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Quantitative analysis of hyperoside and isoquercitrin in methanolic extract of *Stewartia koreana* leaves using HPLC-DAD

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Abstract Since *Stewartia koreana* leaves are registered with the Food and Drug Administration as edible herbal materials, they are used in the development of functional foods, cosmetics, and medicines. In this study, we established an analysis method that can simultaneously analyze two indicators, hyperoside (quercetin 3-*O*-galactoside) and isoquercitrin (quercetin 3-*O*-glucoside) contained in the leaves of *S. koreana* using HPLC-DAD. In accordance with the Ministry of Food and Drug Safety's health functional food guidelines, the analysis method was verified for specificity, accuracy, precision, limit of quantification, and linearity. The analysis method established in this study showed more than 0.9989 of the correlation coefficient values (r^2) for the calibration. The total recovery rates of isoquercitrin and hyperoside were 100.55 and 98.87% with 0.14-0.78 and 0.47-0.67% of the relative standard deviation, respectively. Therefore, it was suggested that the new analytical method would be applied to standardize raw materials and high value-added products originated from the leaves of the *S. koreana* in the future.

Keywords High-performance liquid chromatography with diode-array detection · Hyperoside · Isoquercitrin · Quantitative analysis · *Stewartia koreana*

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Introduction

Stewartia koreana belongs to the family of Theaceae, is designated as an endemic plant in Korea, and is known to grow naturally in some parts of China and Japan. *S. koreana* is a deciduous small arboreous tree native to the southern area of South Korea (Gyeongsangnam-do, Gyeongsangbuk-do, Jeollanam-do, Jeollabuk-do). Many individuals are distributed in the Jirisan(Mt) area, and the height reaches 7-15 m. The bark of *S. koreana* is peeled off thinly, and the texture under the bark is soft. The peeled surface appears mottled with red and gray patterns. Leaves of *S. koreana* are alternated, 4-10 cm long, 2-5 cm wide, and oval shape. Flowers are bisexual, attached to leaves at the base of new branches, and bloom with 5 to 6 petals from late June to early August. The fruit is a pentagon-shaped capsule and matures from late September to mid-October. *S. koreana* is planted as a landscaping tree because of its beautiful flowers, and also used for furniture or decorative materials [1]. In particular, *S. koreana* had been used for the traditional Korean wooden utensils for a long time and the traditional manufacturing technology had been sustained in specific area such as Namwon because of the abundant *S. koreana* resources of Jirisan (Mt.) [2]. Besides the values of *S. koreana* as wooden materials, it would be worthy to investigate and to characterize the biological active compounds in *S. koreana.*

In recent years, not only the interests in personal health due to the pandemic such as COVID-19 but also the interests in strengthening immunity to enhance the resistance to various diseases and stresses have been increased. In this regard, the consumption of health-related functional foods has been increased in these days. It has been reported that the steady intake of health-related functional foods or supplements can directly affect the health promotion and the disease prevention through the strengthening immunity [3] Because of the increasing awareness about the importance of strengthening immunity, the development and researches on biologically active compounds derived from

plants had been drawn the attentions. At the same time, the market sizes of health-related natural compounds have been gradually expanded and the plant originated natural compounds has been emerging as a high-value-added industry [4,5].

In oriental medicine, the stem and root bark of *S. koreana* called as Molan has been used for relieving blood from bruises, for treating quadriplegia and for alleviating pains [6]. Recently, various studies have been reported to reveal and to characterize the physiological activities and components of the *S. koreana*. According to the research, *S. koreana* exhibits antioxidant, anti-inflammatory, and whitening effects, and natural compounds including dihydrochalcones, flavonoids, lignans, and sterols have been reported as substances exerting these activities [7,8,9,10,11]. It had been also reported the extract of *S. koreana* was used for bone resorption, angiogenesis, wound healing, and anti-allergy [12], and syringaresinol isolated from the stems of *S. koreana* showed antioxidant and anti-inflammatory activities [13]. The branch extracts of *S. koreana* showed antioxidant activity, and the main substances responsible for these activities were catechin, proanthocyanindin-A2, fraxin, (2*R*, 3*R*)-taxifolin-3-*β*-D-glucopyranoside, and (2*S*-3*S*)-taxifolin-3-β-glucopyranoside [6]. The main compounds of the leaves of *S. koreana* were quercein, hyperoside, quercetin-3-*O*-(6″-O-galloyl)-*β*-D-galactopyranoside, and kaempferol 3-*O*-[2″,6″-di-*O*-(trans-*p*-coumaroyl)]-*β*-D-glucopyranoside. Among compounds derived from leaves, kaempferol 3-*O*-[2″,6″-di-*O*-(trans-*p*-coumaroyl)]-*β*-D-glucopyranoside showed the best anti-inflammatory activity [11]. In addition, hyperoside and isoquercitrin, which are a group of flavonol, are the main biological active substances in *S. koreana* leaves. Both substances have been reported to show anti-inflammatory, neuroprotective, antiviral, antidepressant, antibacterial, and organ-protective effects. Additionally, increasing interest is being focused on exploring the antitumor effects of both substances. In particular, it has been reported to be effective against lung, cervical, stomach, colon, pancreatic, breast, and ovarian cancer [13,14].

Not only the physiological activities of barks and leaves but also branch and sap of *S. koreana* are registered as edible biological materials by the Ministry of Food and Drug Safety in Korea. So, it can be applied to the development of functional food additives and supplementary materials. In addition, the mixture of different raw materials is often showed synergetic physiological activities during the development of health-related functional materials in plant species. This synergetic effects of mixture of raw materials in the development of health-related functional foods emphasize the need for a certified standard method to analyze marker compounds for the evaluation of each raw material. Nonetheless, there has been no report on the standardized method to validate the marker substances of *S. koreana*.

In this study, we demonstrate an establishment of standard method to analyze two marker compounds, hyperoside (quercetin 3-*O*-galactoside) and isoquercitrin (quercetin 3-*O*-glucoside) in *S. koreana* based on HPLC-DAD and showed the established method was reached to the satisfaction to the health functional food guidelines (specificity, accuracy, precision, limit of quantification, linearity) of the Ministry of Food and Drug Safety. Consequently, we believed that the established method for validating bioactive materials would be invaluable to manage and to analyze bioactive raw materials efficiently for the development of health-related functional compounds from natural resources as well as *S. koreana*.

Materials and Methods

Analytical equipment and reagents

The leaves extract of *S. koreana* were used for analysis using HPLC (Shimadzu M20A series, Kyoto, Japan) equipped with a UV-DAD detector and an Agilent Polaris C18 (4.6×250 mm) column. Authentic compounds, hyperoside (CAS number 482-36-0) and isoquercitrin (CAS number 482-35-9), were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) (Fig. 1). HPLC-grade methanol (99.8%), acetonitrile (99.8%), and 3rd deionized water were purchased from J.T. Baker (Avantor, Radnor, PA, USA), and formic acid (99%) was purchased from Daejung (Korea).

Experimental materials

S. koreana leaves were purchased from Mori Co., Ltd., an agricultural company, and used for validation of HPLC-based analysis method validation. *S. koreana* leaves were completely

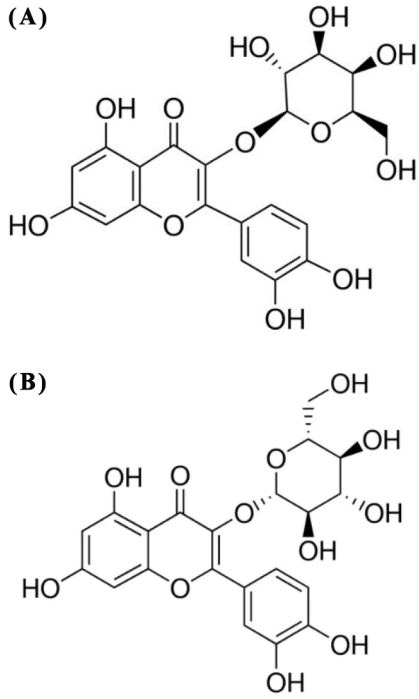


Fig. 1 Chemical structure of hyperoside (A) and isoquercitrin (B)

dried in a dryer at 60 ºC and ground into a fine powder with a blender (Hanil, SHMF-3260S). Methanolic extraction of *S. koreana* leaves were carried out at 100 ºC for 3 h by adding 20 L of 50% methanol (v/v, S1) to 1 kg. The 50% methanolic extract was filtered using filter paper (Whatman, Maidstone, UK) grade 2 and then concentrated using a rotary evaporator (Sunil: N-1300E, Seongnam, Korea). The concentrated sample was completely dried under a freeze dryer (Hanil, HyperCOOL). The powder obtained by lyophilization of *S. koreana* was used for HPLC validation.

Preparation of standard solution and test solution

Standard compounds (10 mg), hyperoside and isoquercitrin were precisely measured and dissolved in 10 mL of 50 % methanol and used as a stock solution. Each standard solution was prepared by diluting the common stock solution with 50% methanol following the Ministry of Food and Drug Safety validation test method. After adding 10 mL of 50 % methanol to 1.0, 2.0, and 3.0 g of the powder of *S. koreana* leaves, respectively, the ultrasonic extraction was performed in a water bath at 40 ºC for 30 min. The extract was filtered (Whatman grade 2), concentrated using a rotary evaporator (Sunil: N-1300E), and finally completely dried with a freeze dryer (Hanil, HyperCOOL). Each sample was again dissolved in 50% methanol at 1 mg/mL, filtered through a 0.45 μm membrane filter, and then used as standard for the index component validation test.

HPLC-DAD analysis

A Shimadzu M20A series HPLC (Shimadzu) equipped with a photodiode array detector was used for the metabolite analysis of *S. koreana* leaves. The flow rate was maintained at 1.0 mL/min, and the gradient concentrations of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) were appropriately adjusted. An Agilent Polaris C18 (4.6×250 mm) was used to separate the metabolites of *S. koreana* leaves. The column temperature was maintained at 40 ºC and the gradient concentration of the solvent for HPLC analysis was as follows; 0-20 min (10-27% B), 20-21 min (27-100% B), 21-29 min (100% B), 29-30 min (90-10% B), 30-35 min (10% B). 20 μL of the sample for analysis was injected, and metabolite monitoring was performed at 340 nm.

Analytical method validation

To validate the analytical method, the standard solution for hyperoside and isoquercitrin and the methanolic extract solution of *S. koreana* leaves were applied to HPLC. To determine the specificity, the retention time, UV spectrum and interferences were analyzed and compared. For the acid hydrolysis of the extract of *S. koreana* leaves, 0.2N HCl and 99.8% MeOH were added at a ratio of 1:1 and heated at 100 ºC for 10 min. After cooling the reactant to room temperature, the same amount of distilled water was added. Then, the same amount of ethyl acetate was added to the reaction mixture, mixed well, and centrifuged at 13,200 rpm for 5 min. The supernatant was transferred to a new tube, dried completely using a speed vac, and dissolved in DMSO for HPLC analysis. Accuracy was evaluated by calculating the concentration difference between the control and experimental groups containing 2.5, 5.0, and 10.0 μg/mL of hyperoside and isoquercitrin. The measurements were repeated more than 3 times to verify the accuracy of analytical method. To evaluate the intraday precision (repeatability), 1, 2, and 3 g of *S. koreana* leaves powder were added to 100 mL of 50% methanol solution and extracted by ultrasonication for 30 min in a 40 ºC water bath. 1.0 mL of each extraction solution was mixed with 9.0 mL of 50% methanol and filtered through a 0.45 μm membrane filter. Then, the solution was applied to HPLC to measure the contents of hyperoside and isoquercitrin. Finally, the hyperoside and isoquercitrin contained in 1.0 g of *S. koreana* leaves powder were converted into μg/g units by considering the dilution factor. The intraday precision was evaluated by repeating the HPLC analysis 5 times. The daily precision (reproducibility) was evaluated by preparing samples in the same way as the intraday precision evaluation. Measurements were repeated 5 times a day, and this process was repeated over five days. To measure the detection limit and quantification limit, the standard solution containing 0 to 100 μg/mL of hyperoside and isoquercitrin were applied to HPLC analysis. The experiments were repeated 3 times and the concentration was plotted against peak area to construct the calibration curves. Then, the limits of detection (LOD) and quantification (LOQ) were obtained using the following formula.

$$\mathrm{LOD} = 3.3\times\frac{\sigma}{S} \qquad \mathrm{LOQ} = 10\times\frac{\sigma}{S}$$

S: Average of the slopes of the calibration curve,
σ: Standard deviation of the y-intercept.

Calibration curve

Standard compounds (10 mg), hyperoside and isoquercitrin were precisely measured and dissolved in 10 mL of 50% methanol and used as a stock solution. Each standard solution was prepared by diluting the common stock solution with 50% methanol following the Ministry of Food and Drug Safety validation test method. For linearity evaluation, standard stock solutions of hyperoside and isoquercitrin with a concentration of 1.0 mg/mL were sequentially diluted with 40% methanol to eight concentrations of 0.1, 0.25, 0.5, 2.5, 10, 25, 50, and 100 μg/mL. The standard solutions of hyperoside and isoquercitrin were analyzed using HPLC, and the area of each concentration was calculated to prepare a calibration curve. The curve was analyzed by a linear regression fit and the the value of the coefficient of determination (r^2) was confirmed.

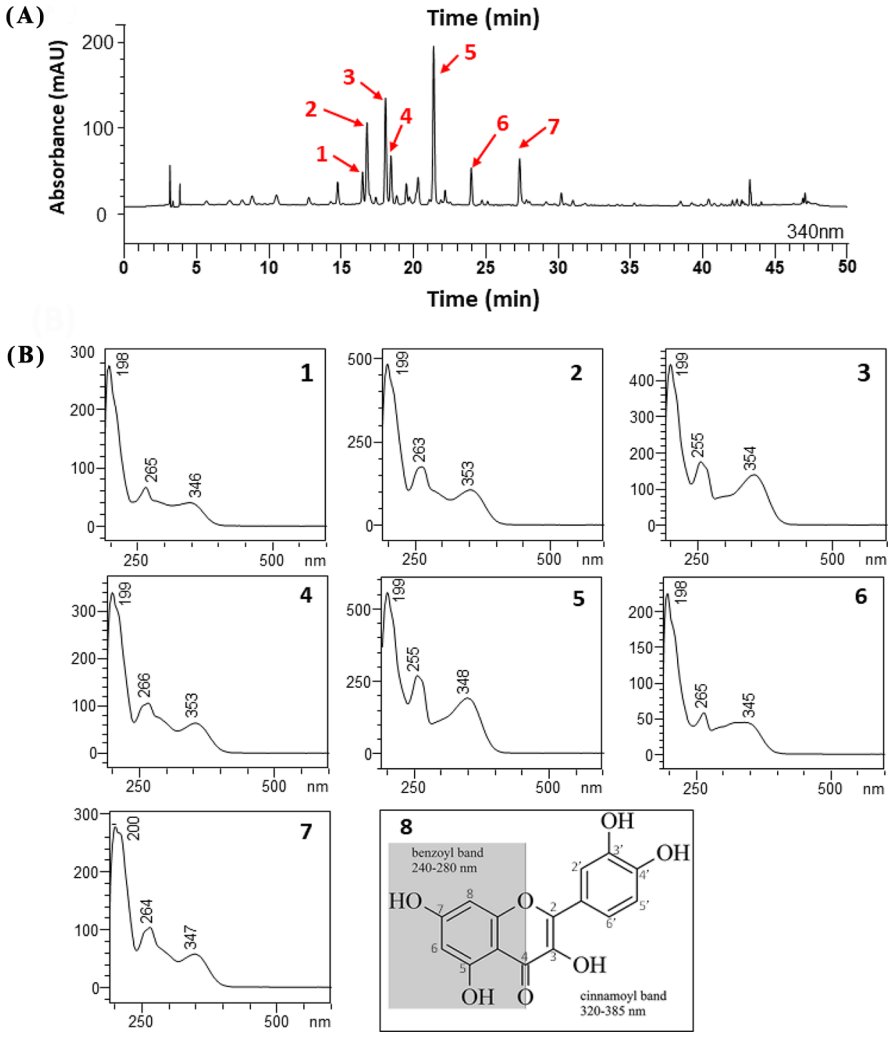


Fig. 2 HPLC chromatogram (A) and UV spectrum (B) of the methanolic extract of *S. koreana* leaves

Results and Discussions

Analysis for marker substances of *S. koreana* leaves

To validate the efficiency of analytical method, the metabolites in *S. koreana* leaves were applied to HPLC-DAD and analyzed the sensitivity, retention time, and resolution. As a result, more than 20 peaks were observed in chromatogram and seven major metabolites were further analyzed (Fig. 2). As shown in Figure 2B, the 7 major peaks showed similar absorbance from HPLC-DAD analysis. This result proposed that the selected major metabolites would share similar backbone structure. In fact, flavonoids are divided into flavanones, flavones, flavonols, and etc. The absorbance of flavonoid is 240-280 nm for the benzoyl band group and 320-285 nm for the cinnamoyl band group (Fig. 2B8). The maximum absorbance of the cinnamoyl bands in flavanone, flavone and flavonol are around at 280 nm, at 330 nm, and at 360 nm, respectively. In Fig. 2B, the major metabolites in *S. koreana* leaves showed the maximum absorbance at about 350 nm. It would be explained that the attachment of sugar group at 3-position of flavonol compound induces a hypochromic shift; thereby the maximum absorbance of the cinnamoyl band shifts to the left [15]. Therefore, it could be known that the major metabolites from the leaves of *S. koreana* would be derivatives of quercetin and kaempferol, which are classified as flavonol compounds. In addition, the assumption was supported by the previous report that revealed the major metabolites in the leaves of *S. koreana* were quercetin, kaempferol, quercitrin, hyperoside, isoquercitrin, quercetin-3-*O*-(6"-*O*-galloyl)-β-D-galactopyranoside, kaempferol 3-*O*-[2", 6"-di-*O*-(trans-p-coumaroyl)]-*β*-D-glucopyranoside [11].

To confirm this assumption, the leaves extract of *S. koreana* was processed through the acid hydrolysis and then analyzed by HPLC-DAD (Fig. 3). The standard solution of quercetin and kaempferol showed at about 29 and 31 min of retention time, respectively, and the UV spectrum were shown in Fig. 3A. Although the leaves extract showed many peaks of metabolites as shown above, 2 major peaks were observed after acid hydrolysis.

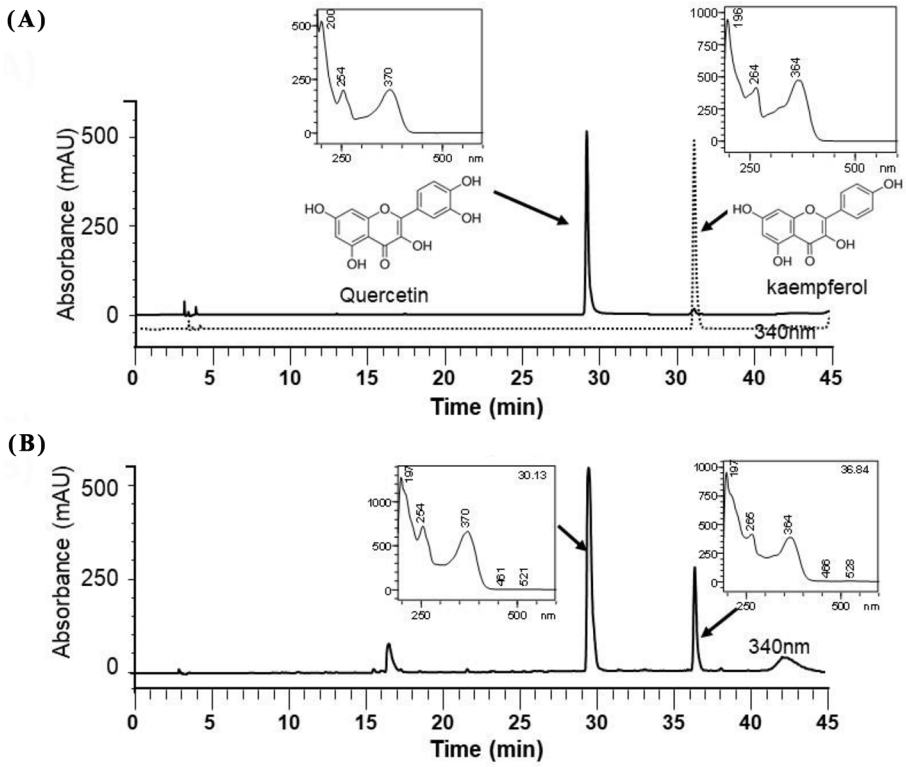


Fig. 3 HPLC chromatogram of HCL hydrolysis of metabolites extracted from *S. koreana* leaves. A: chromatogram of authentic quercetin and kaempferol, B: chromatogram of HCl hydrolyzed metabolites

Moreover, the retention time and the spectrum of UV absorbance were as same as the quercetin and kaempferol (Fig. 3B). Thus, it would be concluded that most of the metabolites in the extract of *S. koreana* leaves were derivatives of quercetin and kaempferol. According to previous studies, it is known that when a glycoside is attached to the carbon at 3-position of quercetin and kaempferol, the maximum absorbance is shifted to around 353 and 348 nm, respectively [14]. In this regard, that the compound No. 1 (maximum absorbance 346 nm), No. 5 (maximum absorbance 348 nm), No. 6 (maximum absorbance 345 nm), and No. 7 (maximum absorbance 347 nm) would be derivatives of kaempferol, while the compound No. 3 (maximum absorbance 354 nm), No. 4 (maximum absorbance 353 nm) would be derivatives of quercetin (Fig. 2). Based on this result, it would be concluded the major compounds of the *S. koreana* leaves were the glycosides of quercetin and kaempferol.

Among the major metabolites observed in HPLC analysis, hyperoside (quercetin 3-*O*-galactoside) and isoquercitrin (quercetin 3-*β*-D-glucoside) are commercially available. Furthermore, their biological activities, such as attenuating fatty liver disease, relieving inflammation in diabetic patients, inducing cell death of breast cancer and gastric cancer, and relieving pulmonary fibrosis, have been reported [5,7,8,11,12,13,14,16]. In these reasons, hyperoside and isoquercitrin were selected for targets as marker substances in *S. koreana* leaves. As shown in Fig. 4, the standard isoquercitrin and hyperoside showed the peaks at 18.08 and 18.45 min that were corresponded to peak No 3 and No 4 from the leave extracts. Since the retention time and UV absorbance were identical, the compound No 3 and No 4 would be isoquercitrin and hyperoside, respectively.

Specificity validation

As described above section, the peaks of isoquercitrin and hyperoside were not interfered by other substances in *S. koreana* under the experimental conditions for established analysis method. The retention time of isoquercitrin (tR =18.07) in the *S. koreana* was coincided with standard isoquercitrin (tR =18.08). In addition, as shown in Fig. 5, the UV absorbance of each substance was measured using HPLC-DAD from 5 different points for standard isoquercitrin and *S. koreana* originating isoquercitrin. The UV absorbance of the authentic isoquercitrin and the isoquercitrin from *S. koreana* leaves extract were similar, and it was confirmed that there was no interference from other substances (Fig. 5). Hyperoside (tR =18.47) from the extract of *S. koreana* leaves analyzed by HPLC was consistent with authentic hyperoside (tR =18.45). Also, as shown in Fig. 6, the UV absorbance of both hyperoside measured by DAD at 5 different points were matched; thereby indicating no interfering substances present (Fig. 6). Consequently, it was suggested that the method established for analyzing the extract of *S. koreana* leaves in this study is capable

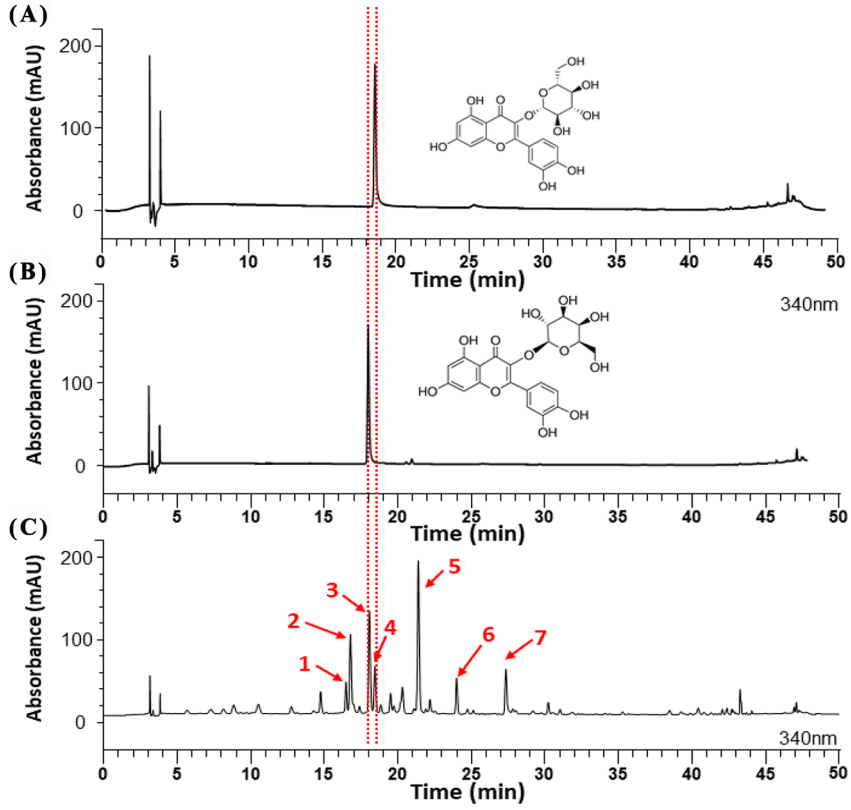


Fig. 4 HPLC chromatogram of authentic isoquercitrin (A), hyperoside (B), and the methanolic extract of *S. koreana* leaves (C)

to analyze isoquercitrin and hyperoside selectively and precisely.

Accuracy validation

The accuracy of the analysis method was evaluated by determining the recovery rate of the added standard isoquercitrin and hyperoside. To test the accuracy, authentic compounds, isoquercitrin, and hyperoside were added to the extracts of *S. Koreana* leaves at the concentrations of 2.5, 5.0, and 10.0 μg/mL, respectively. Based on the fitting curves obtained from calibration curves, the concentration of added compounds was determined. As listed in Table 1, the total recovery rate of isoquercitrin was 100.55%, and the recovery rate ranged from 97.66 to 105.89%. The total recovery rate of hyperoside was 98.87%, and the recovery range was 94.05-106.15%. This result satisfied 90~108% (≥0.1% (1 mg/g)) that is the standard for a recovery rate of marker substances allowed in the Validation Guidelines for Health Functional Food Markers of the Ministry of Food and Drug Safety. It indicates that the analytical method developed in this study has the accuracy to simultaneously analyze and evaluate the two substances, isoquercitrin and hyperoside.

Validation of intra-day precision (repeatability)

To evaluate the intra-day accuracy of the analysis method, the isoquercitrin and hyperoside in 1.0, 2.0, and 3.0 g of the extract of *S. koreana* leaves were analyzed by HPLC with 5 times of repeats. As shown in Table 2, the mean, deviation (SD), and relative standard deviation (RSD) of isoquercitrin and hyperoside were listed. The average content of isiquercitrin was 2714.17 μg/g from 1.0 g, 2,977.80 μg/g at 2.0 g, and 2,930.31 μg/g at 3.0 g of extracts, and there was no significant difference. The RSD of isiquercitrin was 0.14-0.78%. On the other hand, the average content of hyperoside was 961.47 μg/g at 1.0 g, 1,011.11 μg/g at 2.0 g, and 1,114.35 μg/g at 3.0 g. The RSD of hyperoside is 0.47-0.67%. As a result, it was noted that the values lies within the daily precision evaluation criteria (≥0.1%; 1 mg/g) of the Ministry of Food and Drug Safety's Guidelines for Validation of Functional Food Indicator Substances. It means that the analysis method developed in this study meets the daily precision evaluation criteria.

Inter-day precision (reproducibility) validation

For the inter-day precision test, samples corresponding to 1.5 and 2.5 g of the extract of *S. koreana* leaves were applied to HPLC and repeatedly measured five times a day, and this process was carried out for five days. The content of isoquercitrin was 2719.54 μg/g for 1.5 g and 2785.90 μg/g for 2.5 g, and the RSD of isoquercitrin was 0.60% for 1.5 g and 0.64% for 2.5 g. The average of their RSD was calculated as 0.62% (Table 3). The content of hyperoside was measured to be 975.44 μg/g for 1.5 g and 974.71 μg/g for 2.5 g. RSD of hyperoside were determined to

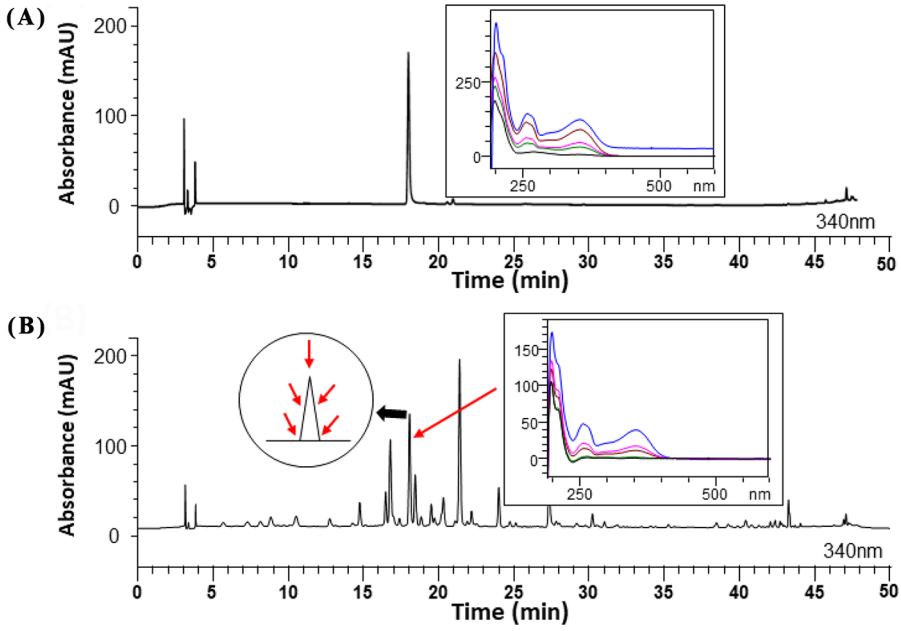


Fig. 5 The HPLC chromatogram of authentic isoquercitrin(A) and the methanolic extract extract(B) of *S. koreana* leaves for the evaluation of specificity

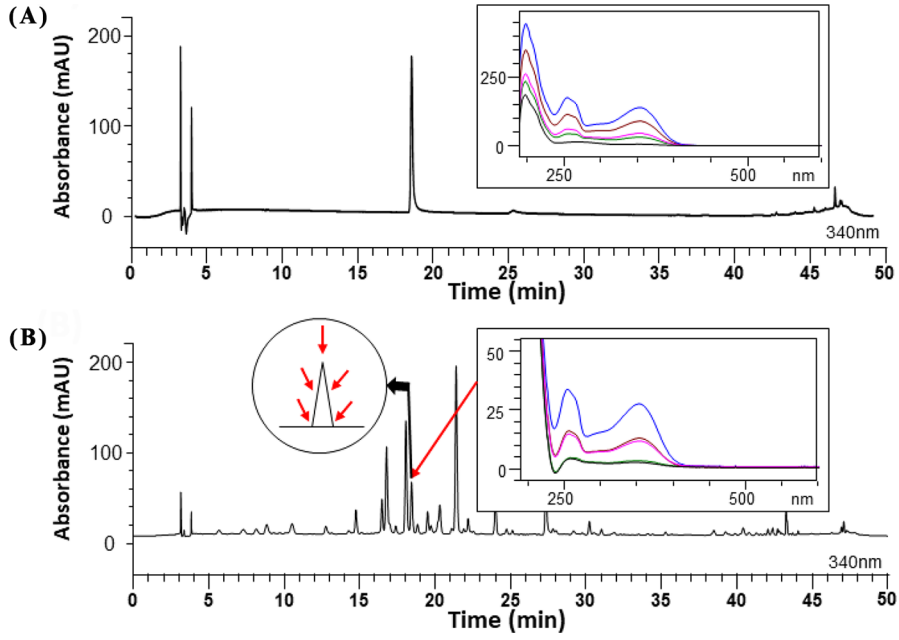


Fig. 6 The HPLC chromatogram of authentic hyperoside(A) and the methanolic extract(B) of *S. koreana* leaves for the evaluation of specificity

be 0.90% for 1.5 g and 1.37% for 2.5 g, and the average was calculated as 1.14%. Thus, the daily precision for analyzing isoquercitrin and hyperoside satisfies the Ministry of Food and Drug Safety guideline within 6% of the reproducibility standard of the concentration range of ≥0.1% (1 mg/g) of the indicator substance.

Determination of limit of detection (LOD) and quantification (LOQ)

A standard solution of isoquercitrin and hyperoside were diluted at 8 different concentrations ranged in 0.1-100.0 μg/mL and analyzed with 3 times of repeats. The slope and y-intercept were obtained from the linear regression fitting model from the

Table 1 Accuracy (recovery test) results of isoquercitrin and hyperoside of *S. koreana* leaves extract

Compounds	Repetition	Added amounts		
		2.5 μg/mL	5.0 μg/mL	10.0 μg/mL
Isoquercitrin	1	101.07	97.66	99.14
	2	104.94	98.13	99.41
	3	105.89	98.19	100.56
	Mean recovery rate (%)	103.97	97.99	99.70
	Net recovery rate (%)		100.55	
	Range of recovery rate (%)		97.66-105.89	
Hyperoside	1	97.48	101.45	94.17
	2	104.24	100.27	94.05
	3	106.15	97.95	94.06
	Mean recovery rate (%)	102.62	99.89	94.09
	Net recovery rate (%)		98.87	
	Range of recovery rate (%)		94.05-106.15	

Table 2 The intra-day precision (repeatability) results of isoquercitrin and hyperoside in the methanolic extract of *S. koreana* leaves

	Amount of mathanolic extract of *S. koreana* leaves		
	1.0 g	2.0 g	3.0 g
Repetition	Isoquercitrin contents (μg/g)	Isoquercitrin contents (μg/g)	Isoquercitrin contents (μg/g)
1	2,705.47	2,966.21	2,927.28
2	2,715.76	2,990.37	2,937.05
3	2,714.22	2,997.12	2,927.63
4	2,727.36	2,993.07	2,928.58
5	2,714.17	2,942.24	2,931.05
Mean	2,715.40	2,977.80	2,930.31
SD	7.82	23.25	4.04
RSD (%)	0.29	0.78	0.14
Range of RSD (%) = 0.14-0.78			
	Amount of mathanolic extract of *S. koreana* leaves		
	1.0 g	2.0 g	3.0 g
Repetition	Hyperoside contents (μg/g)	Hyperoside contents (μg/g)	Hyperoside contents (μg/g)
1	957.25	1007.50	1119.70
2	958.77	1007.93	1101.99
3	957.37	1009.90	1113.14
4	969.56	1010.57	1116.78
5	964.41	1019.10	1120.16
Mean	961.47	1011.00	1114.35
SD	5.38	4.71	7.46
RSD (%)	0.56	0.47	0.67
Range of RSD (%) = 0.47-0.67			

calibration curve constructed by plotting the concentrations against the peak area. The average slope of isoquercitrin was 33968.33, and the standard deviation of the y-intercept was 261.1549, resulting 0.0254 μg/mL of detection limit and 0.0769 μg/mL of the quantification limit. On the other hand, the hyperoside showed 0.0345 and 0.1046 μg/mL of detection limit and quantification limit, respectively (Table 4).

Linearity validation

Linearity was confirmed by selecting five concentrations (0.25, 0.5, 2.5, 10, 50 μg/mL for isoquercitirn; 2.5, 10, 25, 50, 100 μg/mL for hyperoside) that ensure linearity well within the concentration

Table 3 The inter-day precision(reproducibility) results of isoquercitrin and hyperoside in the methanolic extract of *S. koreana* leaves

	Amount of mathanolic extract of S. *koreana* leaves		Amount of mathanolic extract of S. *koreana* leaves	
	1.5 g	2.5 g	1.5 g	2.5 g
Repetition	Isoquercitrin contents (μg/g)	Isoquercitrin contents (μg/g)	Hyperoside contents (μg/g)	Hyperoside contents (μg/g)
1	2708.47	2790.56	957.25	987.52
2	2721.76	2781.23	958.77	982.79
3	2718.22	2796.70	957.37	991.88
4	2729.36	2812.04	969.56	989.55
5	2721.17	2788.16	964.41	993.18
1	2702.08	2767.88	972.71	957.12
2	2702.96	2780.11	975.50	957.54
3	2687.43	2782.67	980.85	959.40
4	2700.86	2786.34	985.26	960.04
5	2679.36	2740.93	991.80	968.14
1	2736.71	2793.84	979.76	979.92
2	2735.28	2812.17	972.77	975.71
3	2737.94	2794.11	973.27	989.62
4	2750.19	2792.23	983.49	988.95
5	2754.07	2773.34	987.39	971.60
1	2725.58	2784.14	978.92	961.98
2	2720.26	2750.47	973.61	965.50
3	2728.06	2773.23	978.06	960.52
4	2713.39	2795.21	976.34	959.92
5	2721.87	2799.70	976.14	954.96
1	2715.01	2780.94	981.05	981.94
2	2714.35	2777.09	980.94	971.28
3	2707.54	2806.47	979.48	991.36
4	2724.69	2792.34	974.63	978.50
5	2731.93	2795.63	976.74	988.95
Mean	2719.54	2785.90	975.44	974.71
SD	17.49	16.60	8.74	13.32
RSD (%)	0.64	0.60	0.90	1.37
	Net RSD (%) = 0.62		Net RSD (%) = 1.14	

range of the standard solutions (0.1-100 μg/mL) used to measure the limit of quantification of isoquercitirn and hyperoside. Measurements were repeated three times to confirm the error range for each indicator substance. As shown in Table 5, it can be seen that the correlation coefficient (r^2) value of the calibration curve in all six test groups shows excellent linearity with a value of 0.9989 or more. The analysis method developed in this study sufficiently provided appropriate precision and accuracy. These results indicate an ability to obtain a linear measurement value for the amount of the analyte contained in the sample.

In this study, isoquercitrin and hyperoside were selected as indicator substances of *S. koreana* leaves. Isoquercitrin and hyperoside, selected as indicators, are known to show various physiological activities but are known to show excellent efficacy in relieving inflammation [13,14]. *S. koreana* leaves are registered with the Food and Drug Administration as edible herbal materials. Therefore, *S. koreana* leaves can be used to develop functional foods, cosmetics, and medicines. To standardize the quality control and manufacturing process of raw materials in the development of products using *S. koreana* leaves, it is essential to select indicator substances and develop methods to analyze them efficiently. As part of this study, we developed an analysis method that meets the criteria for selecting indicator substances for *S. koreana* leaves and the validation criteria for health functional food indicators of the Ministry of Food and Drug Safety. These results are considered helpful for the standardization of raw materials and products when producing high-value-added products targeting the leaves of *S. koreana* in the future.

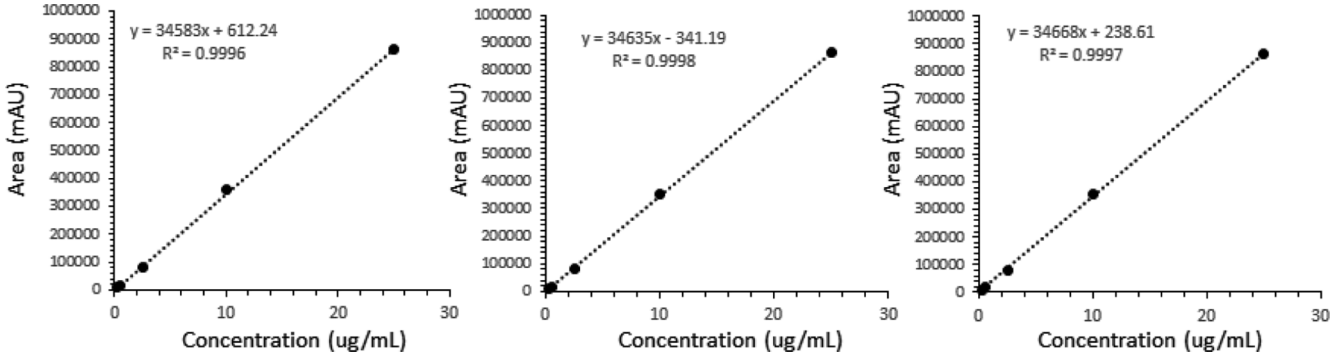
Table 4 The limit of detection (LOD) and quantification (LOQ) for isoquercitrin and hyperoside in the methanolic extract of *S. koreana* leaves

	Repetition	Range (μg/mL)	Regression equation	r^2
Isoquercitrin	1	0.1-100	y=33986x-2573.0	0.9993
	2	0.1-100	y=33940x-2225.7	0.9994
	3	0.1-100	y=33979x-2061.5	0.9994
	Mean of slopes (S)	33968.33	Standard deviation of intrcept (σ)	261.1549
	LOD (3.3×σ/S)		0.0254	
	LOQ (10×σ/S)		0.0769	
	Repetition	Range (μg/mL)	Regression equation	r^2
Hyperoside	1	0.1-100	y=39442x+26968	0.9989
	2	0.1-100	y=39460x+26839	0.9989
	3	0.1-100	y=39669x+26196	0.9989
	Mean of slopes (S)	39523.67	Standard deviation of intrcept (σ)	413.54
	LOD (3.3×σ/S)		0.0345	
	LOQ (10×σ/S)		0.1046	

Table 5 The limit of detection (LOD) and quantification (LOQ) for isoquercitrin (A) and hyperoside (B) in the methanolic extract of *S. koreana* leaves

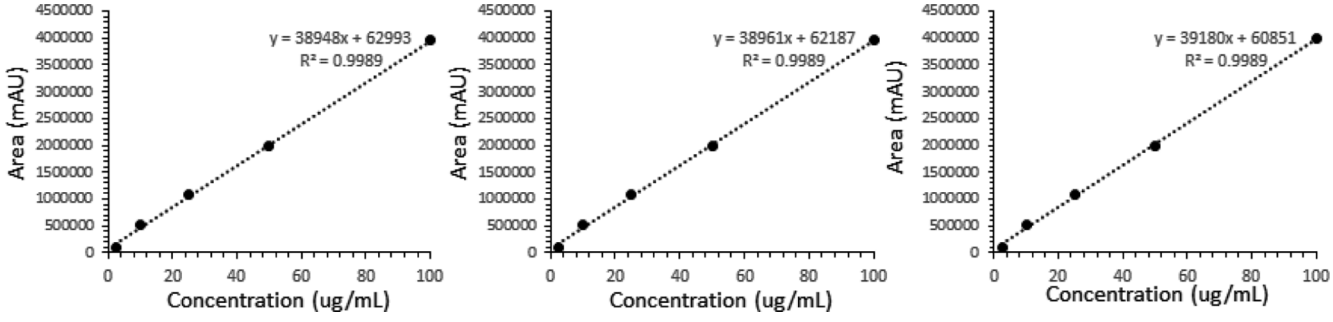
(A)

Repetition	Range (μg/mL)	Regression equation	r^2
1	0.25-25	y=34583x+612.4	0.9996
2	0.25-25	y=34635x+341.2	0.9998
3	0.25-25	y=34668x+238.6	0.9997



(B)

Repetition	Range (μg/mL)	Regression equation	r^2
1	2.5-100	y=38948x+62993	0.9989
2	2.5-100	y=38961x+62187	0.9989
3	2.5-100	y=39180x+60851	0.9989



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