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Quantitative Analysis of Flavonoid Glycosides in *Sophora japonica* and *Sophora flavescens* by HPLC-DAD

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Abstract – Recently, a phytoestrogenic functional food has been developed using the fruits of *Sophora japonica*. Phytochemical investigation of fruits of *S. japonica* led to the isolation of eight flavonoid glycosides using various chromatographic techniques. The isolated compounds were identified as genistin (**1**), sophoricoside (**2**), genistein 7,4'-di-*O*-*β*-D-glucopyransoide (**3**), sophorabioside (**4**), genistein-7-*O*-*β*-D-glucopyranoside-4'-*O*-[(*α*-L-rhamnopyranosyl)-(1→2)-*β*-D-glucopyranoside] (**5**), sophoraflavonoloside (**6**), nicotiflorin (**7**) and kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranoside (**8**), respectively, by comparison of their spectroscopic data with those reported in the literature. In addition, a new HPLC-DAD method for simultaneous determination of the isolated compounds was developed to quantitate the contents of flavonoids in *S. japonica* and *S. flavescens*. The method was validated in terms of limit of detection, limit of quantitation, specificity, linearity, precision and accuracy. The validated method was successfully applied to determine eight flavonoids in two *Sophora* species. The contents of eight flavonoids varied according to the parts and species. Particularly, it was found that only the fruits of *S. japonica* contained sophoricoside, a phytoestrogenic isoflavone.

Keywords – *Sophora japonica*, Flavonoid glycosides, HPLC-DAD, Sophoricoside

Introduction

It is well-established that most middle-aged women in menopausal transition period suffer various symptoms such as hot flushes, insomnia, headache, depression, and cystitis due to estrogen deficiency.[1,2] Since estrogen mediate various physiological functions ranging from regulation of the menstrual cycle and reproduction to the modulation of bone density and cholesterol transport, estrogenic supplement is important for maintaining health-related quality of life of women.[1] For these reasons, hormone replacement therapy (HRT) had been clinically recommended for women to reduce various menopausal symptoms and health risks of low estrogen, including osteoporosis, hypertension, diabetes mellitus, dementia, and so on. However, it may be accompanied by several side effects such as the increased risk of breast and ovarian cancer, stroke, and heart attacks.[3] Therefore, plant-derived estrogens, known as phytoestrogens, have been suggested as an alternative therapy of HRT due to their structural similarity with estrogen.[4] Among them, isoflavonoids have received considerable attention due to their agonistic activity against β-estrogen receptor (ER-β), for selective ER-β agonists can be beneficial for the treatment of menopausal symptoms without estrogen carcinogenesis in breast or uterus.[5]

Recently, health functional food has been getting a great deal of interest from middle-aged women in menopausal transition period. In the Korean health food market, there are several functional food products containing the fruit extract of *Sophora japonica* which belongs to the family of isoflavonoid-rich Leguminosae.[6] The major functional compound in the extract is known to be sophoricoside which has similar biological potency to genistein, a well-known phytoestrogenic isoflavonoid.[7] Daily intake of sophoricoside significantly decreased Kupperman index (KI) score by 3.5 points, representing the improvement of postmenopausal and menopausal

Dedicated to Prof. Jinwoong Kim of the Seoul National University for his pioneering works on Pharmacognosy.

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symptoms.[8] KI is the tool that assesses the 11 common symptoms of menopausal such as hot flashes, insomnia, fatigue and headache, based on the severity and symptoms rated from 0 to 3.[9,10]

As the interest in health supplements increases, the importance of their quality control and standardization of them is increasingly emphasized. Many health supplements, such as the functional foods for menopausal women mentioned above, are produced from certain parts of a particular plant as raw materials. In the case of plants, it should be considered that plants in some genus contain several different species used for various purposes. Plants in the same genus sometimes have a similar appearance. In addition, the drying and cutting or extracting process for market distribution makes it more difficult to authenticate. Therefore, it is necessary to establish a method of distinguishing them.

Several species in the genus *Sophora* (Leguminosae) have been used in traditional medicine in Korea. In particular, the flower and its bud of *S. japonica* and the root of *S. flavescens* are listed in Korean Pharmacopoeia.[11] Among the herbs derived from the genus *Sophora*, these two species are the most distributed on the market. Phytochemical studies have shown that both of them contain flavonoids as an important class of compounds.[12] The first one has been used for the treatment of gastrointestinal disorders, and the later has been used for the treatment of hematuria, hemorrhoids and hypertension.[12]

Thus, in this study, isolation and quantitative analysis of bioactive compounds in the fruits *S. japonica* were conducted using HPLC-DAD. In addition, the contents of bioactive flavonoid glycosides were compared between two *Sophora* species, *S. japonica* and *S. flavescens*.

Experimental

General experimental procedures – Chemical shifts are reported in parts per million (ppm) from TMS. All NMR spectra were recorded on an Agilent 400-MR-NMR spectrometer operated at 400 and 100 MHz for hydrogen and carbon, respectively. Data processing was carried out with the MestReNova ver.6.0.2 program. HR-ESI-MS spectra are obtained using an AGILENT 6550 iFunnel Q-TOF LC/MS system. Preparative HPLC was carried out using an AGILENT 1200 HPLC system. Column chromatography was performed on silica-gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) or YMC RP-18 resins (30 - 50 μm, Fujisilisa Chemical Ltd.). For thin layer chromatography (TLC), a pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck) were used. First grade solvents for extraction, fractionation and isolation were purchased from SK chemical Co. Ltd., Korea. HPLC grade solvents were purchased from Burdick & Jackson (Muskegon, MI) and J.T. baker (Chemical Co., Philipsburg, NJ) and HPLC grade formic acid from Sigma-Aldrich (St. Louis, MO, USA). Other reagents and chemicals are of analytical grade.

Plant materials – The samples of *S. japonica* and *S. flavescens* were purchased at Kyung-dong herbal market, Seoul, Korea in 2015 as shown in Table 1. Since the health supplement for menopausal women uses seed-containing fruits of *S. japonica*, fruits containing seeds were used in this study. These samples were authenticated using DNA barcoding analysis by Professor Tae-Jin Yang in the College of Agriculture and Life Sciences, Seoul National University, Seoul, Korea. A voucher specimen was deposited at the Herbarium of College of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, Incheon, Korea.

Extraction and isolation – The dried fruits of *S. japonica* (4.8 kg) were extracted with 100% methanol (3 × 10 L, room temperature) for 3 days in sonicator and the extract was evaporated in vacuo. The methanol extract (1132.6 g) was suspended in water and fractionated with organic solvent to produce *n*-hexane (3.8 g), $CHCl_3$ (4.2 g), EtOAc (17.6 g) *n*-BuOH (245.7 g), water (357.5 g) fractions. Since the EtOAc and *n*-BuOH fractions showed similar LC-UV chromatograms and TLC band patterns, these two fractions were combined and then chromatographed on a silica gel column and eluting with a gradient of $CHCl_3$/MeOH (20 : 1 → 1 : 1, v/v) to obtain five sub-fractions, SJ-E1, SJ-E2, SJ-E3, SJ-E4 and SJ-E5. The SJ-E5 fraction was chromatographed on a silica gel column eluting with $CHCl_3$/acetone/water (1 : 3 : 0.2, v/v/v) to give three smaller fractions, SJ-E5-1, SJ-E5-2 and SJ-E5-3. SJ-E5-1 fraction was chromatographed on YMC RP-18 column eluting with MeOH/water (1 : 1.5, v/v) to give six

Table 1. Species, geographic origin and plants parts used in this study

Species	Parts	Geographic origin	Code
S. japonica	Fruits	Korea	SJ1
		Korea	SJ2
		China	SJ3
	Flowers	Korea	SJ4
S. flavescens	Roots	Korea	SF1
	Flowers	Korea	SF1-1

sub-fraction, SJ-E5-1A, SJ-E5-1B, SJ-E5-1C, SJ-E5-1D, SJ-E5-1E and SJ-E5-1F. SJ-E5-1A fraction then chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm column, 15% aq. ACN, and a flow rate of 3 mL/min to yield **1** (7.6 mg) and **2** (13.4 mg). In addition, SJ-E5-2B fraction further chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm column, 16% aq. ACN, and a flow rate of 3 mL/min to yield 3 (7.2 mg). The SJ-E5-1D subfraction was chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm column, 20% aq. ACN, and a flow rate of 3 mL/min to yield **4** (80.4 mg) and **5** (2.2 mg) each, while the SJ-E5-1F sub-fraction was chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm column, 22% aq. ACN, and a flow rate of 3 mL/min to yield **6** (4.5 mg), **7** (6.1 mg) and **8** (37.6 mg).

Genistin (1) – Yellow amorphous powder, $C_{21}H_{20}O_{10}$ [ESI-QTOF/MS *m/z* 455.0951 $[M+Na]^+$ (calcd for $C_{21}H_{20}O_{10}Na$, 455.0954)]; ^{1}H-NMR (CD_3OD, 400 MHz) : δ 8.34 (1H, s, H-2), 7.39 (2H, d, *J*=8.7 Hz, H-2', 6'), 6.83 (2H, d, *J* = 8.7 Hz, H-3', 5'), 6.71 (1H, d, *J* = 2.1 Hz, H-8), 6.47 (1H, d, *J* = 2.1 Hz, H-6), 5.05 (1H, d, *J* = 7.2 Hz, H-1"), 3.71 (1H, d, *J* = 10.2 Hz, H-6"b), 3.42 (overlapped, m, H-5", 6"a), 3.32 (1H, t, *J* = 8.6 Hz, H-3"), 3.27 (1H, t, *J* = 8.6 Hz, H-2"), 3.17 (1H, t, *J* = 8.6 Hz, H-4"); ^{13}C NMR (CD_3OD, 100 MHz) data : see Table 2.

Table 2. ^{13}C-NMR data of compounds **1-8** from the fruits of *S. japonica* (δ in ppm, 100 MHz, CD_3OD)

δ*C*	**1**	**2**	**3**	**4**	**5**
2	155.0	154.4	155.1	155.0	155.6
3	123.0	124.2	123.9	126.3	126.1
4	180.9	180.0	180.4	181.9	182.2
5	162.0	162.2	161.6	163.8	163.5
6	100.3	99.1	100.3	100.2	101.1
7	163.4	164.8	163.1	166.2	164.7
8	95.0	93.8	94.6	94.9	95.9
9	161.7	157.6	157.3	158.7	159.2
10	106.5	104.3	106.1	106.2	108.0
1'	121.5	121.9	122.2	124.2	124.5
2'	130.6	130.0	130.1	131.3	131.4
3'	115.5	116.0	116.1	117.3	117.3
4'	157.6	157.3	157.2	159.7	158.8
5'	115.5	116.0	116.1	117.3	117.3
6'	130.6	130.0	130.1	131.3	131.4
1"	100.0	100.3	99.8	100.3	101.6
2"	73.4	73.2	73.2	78.1	73.1
3"	76.6	76.6	76.6	79.0	76.4
4"	69.9	69.7	69.7	72.2	71.1
5"	77.5	77.1	77.2	79.2	79.2
6"	60.9	60.7	60.6	62.5	62.5
1"'			99.6	102.5	100.2
2"'			73.0	72.3	76.4
3"'			76.4	71.5	79.0
4"'			69.6	73.9	69.9
5"'			77.0	69.9	76.9
6"'			60.6	18.0	62.4
1""					102.4
2""					71.5
3""					71.2
4""					72.2
5""					69.9
6""					18.2

Table 2. Continued

δC	6	7	8
2	158.6	158.6	159.2
3	134.9	135.5	134.7
4	179.7	179.3	179.4
5	163.2	163.0	163.0
6	99.9	100.2	100.4
7	165.9	166.6	167.3
8	94.7	95.1	95.2
9	158.9	159.4	158.6
10	105.8	105.6	105.3
1'	122.8	122.7	122.9
2'	132.4	132.4	132.4
3'	116.3	116.1	116.2
4'	161.6	161.5	161.5
5'	116.3	116.1	116.2
6'	132.4	132.4	132.4
1"	101.0	104.6	101.2
2"	82.5	75.7	76.9
3"	77.9	78.1	81.9
4"	71.3	71.4	71.5
5"	78.2	77.2	77.8
6"	62.6	68.5	62.6
1"'	104.7	102.4	104.4
2"'	75.6	72.1	75.5
3"'	77.9	72.3	78.3
4"'	71.1	73.9	71.5
5"'	78.3	69.7	77.9
6"'	62.5	17.9	68.3
1""			102.2
2""			72.4
3""			72.2
4""			74.0
5""			69.8
6""			17.9

Sophoricoside (2) – Yellow amorphous powder, $C_{21}H_{20}O_{10}$ [ESI-QTOF/MS *m/z* 455.0957 $[M+Na]^+$ (calcd for $C_{21}H_{20}O_{10}Na$, 455.0954)]; ^{1}H-NMR (CD_3OD, 400 MHz) : δ 12.90 (1H, s, 5-OH), 8.37 (1H, s, H-2), 7.49 (2H, d, *J* = 8.8 Hz, H-2', 6'), 7.09 (2H, d, *J* = 8.8 Hz, H-3', 5'), 6.39 (1H, d, *J* = 2.0 Hz, H-8), 6.23 (1H, d, *J* = 2.0 Hz, H-6), 4.91 (1H, d, *J* = 7.3 Hz, H-1"); 13C NMR (CD_3OD, 100 MHz) data : see Table 2.

Genistein 7,4'-di-*O*-*β*-D-glucopyransoside (3) – White amorphous powder, $C_{27}H_{30}O_{15}$ [ESI-QTOF/MS *m/z* 617.1481 $[M+Na]^+$ (calcd for $C_{27}H_{30}O_{15}Na$, 617.1482)]; ^{1}H-NMR (CD_3OD, 400 MHz) : δ 12.89 (1H, s, 5-OH), 8.49 (1H, s, H-2), 6.49 (1H, d, *J* = 2 Hz, H-6), 6.74 (1H, d, *J* = 2 Hz, H-8), 7.53 (2H, d, *J* = 9 Hz, H-2', 6'), 7.12 (2H, d, *J* = 9 Hz, H-3', 5'), 5.07 (1H, d, *J* = 7.2 Hz, H-1"), 4.93 (1H, d, *J* = 7 Hz, H-1"'); ^{13}C NMR (CD_3OD, 100 MHz) data : see Table 2.

Sophorabioside (4) – Yellow amorphous powder, $C_{27}H_{30}O_{14}$ [ESI-QTOF/MS m/z 601.1535 $[M+Na]^+$ (calcd for $C_{27}H_{30}O_{14}Na$, 601.1533)]; ^{1}H-NMR (CD_3OD, 400 MHz) : δ 12.89 (1H, s, 5-OH), 8.35 (1H, s, H-2), 7.50 (2H, d, *J* = 8.7 Hz, H-2', 6'), 7.07 (2H, d, *J* = 8.7 Hz, H-3', 5'), 6.41 (1H, d, *J* = 2.0 Hz, H-8), 6.25 (1H, d, *J* = 2.0 Hz, H-6), 5.15 (1H, s, H-1"'), 5.07 (1H, d, *J* = 7.4 Hz, H-1"),

1.23 (3H, d, J = 6.2 Hz, H-6'''); ^{13}C NMR (CD_3OD, 100 MHz) data : see Table 2.

Genistein-7-*O*-*β*-D-glucopyranoside-4'-*O*-[(*α*-L-rhamnopyranosyl)-(1→2)-*β*-D-glucopyranoside] (5) – White amorphous powder, $C_{33}H_{40}O_{19}$ [ESI-QTOF/MS *m/z* 763.2059 $[M+Na]^+$ (calcd for $C_{33}H_{40}O_{19}Na$, 763.2061)]; ^{1}H-NMR (CD_3OD, 400 MHz) : δ 12.92 (1H, s, 5-OH), 8.48 (1H, s, H-2), 7.51 (2H, d, J = 8.6 Hz, H-2', 6'), 7.04 (2H, d, J = 8.6 Hz, H-3', 5'), 6.74 (1H, d, J = 1.8 Hz, H-8), 6.48 (1H, d, J = 1.8 Hz, H-6), 5.36 (1H, s, H-1'''), 5.07 (1H, d, J = 7.2 Hz, H-1''), 4.68 (1H, d, J = 7.6 Hz, H-1''''), 3.54 (overlapped, m, H-2''',6'''', 3'''), 3.20 (overlapped, m, H-4'''', 5''''); ^{13}C NMR (CD_3OD, 100 MHz) data : see Table 2.

Sophoraflavonoloside (6) – Yellow amorphous powder, $C_{27}H_{30}O_{16}$ [ESI-QTOF/MS *m/z* 633.1430 $[M+Na]^+$ (calcd for $C_{27}H_{30}O_{16}Na$, 633.1431)]; ^{1}H-NMR (CD_3OD, 400 MHz) : δ 8.00 (2H, d, J = 8.0 Hz, H-2', 6'), 6.81 (2H, d, J = 8.0 Hz, H-3', 5'), 6.30 (1H, s, H-8), 6.09 (1H, s, H-6), 5.20 (1H, d, J = 7.6 Hz, H-1''), 4.65 (1H, d, J = 7.6 Hz, H-1'''); ^{13}C NMR (CD_3OD, 100 MHz) data : see Table 2.

Nicotiflorin (7) – Yellow needles, $C_{27}H_{30}O_{15}$ [ESI-QTOF/MS *m/z* 617.1484 $[M+Na]^+$ (calcd for $C_{27}H_{30}O_{15}$ Na, 617.1482)]; ^{1}H-NMR (CD_3OD, 400 MHz) : δ 1.11 (3H, d, J = 6.3 Hz, H-6'''), 3.51 (1H, dd, J = 3.3, 9.6 Hz, H-3'''),3.62 (1H, dd, J = 1.5, 3.3 Hz, H-2'''), 4.51 (1H, d, J = 1.5 Hz, H-1'''), 5.13 (1H, d, J = 7.8 Hz, H-1''), 6.21 (1H, d, J = 2.1 Hz, H-6), 6.41 (1H, d, J = 2.1 Hz, H-8), 6.89 (2H, d, J = 9.0 Hz, H-3', 5'), 8.06(2H, d, J = 9.0 Hz, H-2', 6'); ^{13}C NMR (CD_3OD, 100 MHz) data : see Table 2.

Kaempferol-3-*O*-*β*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranoside (8) – Yellow amorphous powder, $C_{33}H_{40}O_{20}$ [ESI-QTOF/MS *m/z* 779.2013 $[M+Na]^+$ (calcd for : $C_{33}H_{40}O_{20}Na$,779.2011)]; ^{1}H-NMR (CD_3OD, 400 MHz) : δ 4.75 (1H, d, J = 7.0 Hz, H-1''') 4.47 (1H, d, J = 1.0 Hz, H-1''''), 5.35 (1H, d, J = 7.5 Hz, H-1''), 6.19 (1H, d, J = 2.0 Hz, H-6), 6.38 (1H, d, J = 2.0 Hz, H-8), 6.89 (2H, d, J = 9.0 Hz, H-3', 5'), 8.02 (2H, d, J = 9.0 Hz, H-2', 6'); ^{13}C NMR (CD_3OD, 100 MHz) data : see Table 2.

HPLC apparatus and chromatographic conditions – The quantitative determination of the isolated compounds was performed on an Agilent 1290 Infinity liquid chromatography system equipped with the G4220A Quad pump solvent delivery system and UV-VIS photodiode array detector G4212A (Agilent Technologies, Inc., CA, USA). Chromatographic analysis was carried out on an Agilent 1260 Series HPLC system (Agilent Technologies, CA, USA) with DAD (Agilent Technologies, CA, USA). Chromatographic separation was performed at 30°C on an YMC Hydrosphere C18 (4.6 × 150 mm, 5 μm; YMC Co., Kyoto, Japan) column. The mobile phase consisted of 5 mM ammonium acetate in water (A) and MeOH (B) using a gradient elution of 36–58% B at 0–15 min. The wavelength was set at 260 nm and the mobile flow rate was at 0.8 mL/min. The injection volume was 5 μL, and the system was controlled by Mass Hunter software (Agilent Corporation, MA, USA).

Preparation of standard solutions – Individual standard stock solutions of the eight compounds were prepared at a concentration of 1 mg/mL in 50% methanol. Working standard solutions were prepared by serial dilution to appropriate concentrations with 50% methanol.

Method validation – The HPLC method was validated in terms of linearity, precision, and accuracy according to the International Conference on Harmonization guidelines (ICH 1995, ICH 1997). To calculate calibration curves of the eight compounds, standard solutions for each compound were prepared and diluted to the appropriate concentrations. Those solutions of six different concentrations of each compound were analyzed in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the signal-to-noise ratio (LOD = 3.3 δ/S, LOQ = 10 δ/S, where δ is the standard deviation of the response and S is the slope of the calibration curve). The precision was validated by the determination of intra- and inter-day variances. The precision of the developed method was expressed by the relative standard deviation (RSD). The accuracy was determined by the method of standard addition. The diluted sample solution of 100% methanol extract of *S. japonica* seeds (1 g/10 mL) was spiked with the mixture of standard compounds in the ratios of 2:1, 1:1, and 1:2. Then, the resultant samples were analyzed by the proposed method, and triplicate experiments were carried out. For comparison, an unspiked sample was concurrently prepared and analyzed simultaneously.

Result and Discussion

The reference standards namely genistin (**1**),[13] sophoricoside (**2**),[14] genistein 7,4'-di-*O*-*β*-D-glucopyransoide (**3**),[15] sophorabioside (**4**),[15] genistein-7-*O*-*β*-D-glucopyranoside-4'-*O*-[(α-L-rhamnopyranosyl)-(1→2)-*β*-D-glucopyranoside] (**5**),[16] sophoraflavonoloside (**6**),[15] nicotiflorin (**7**),[17] kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranoside (**8**)[18] (Fig. 1) were isolated and purified from the fruits of *S. japonica* using various chromatographic techniques. Comparison of the MS and NMR data with reported values in the literature

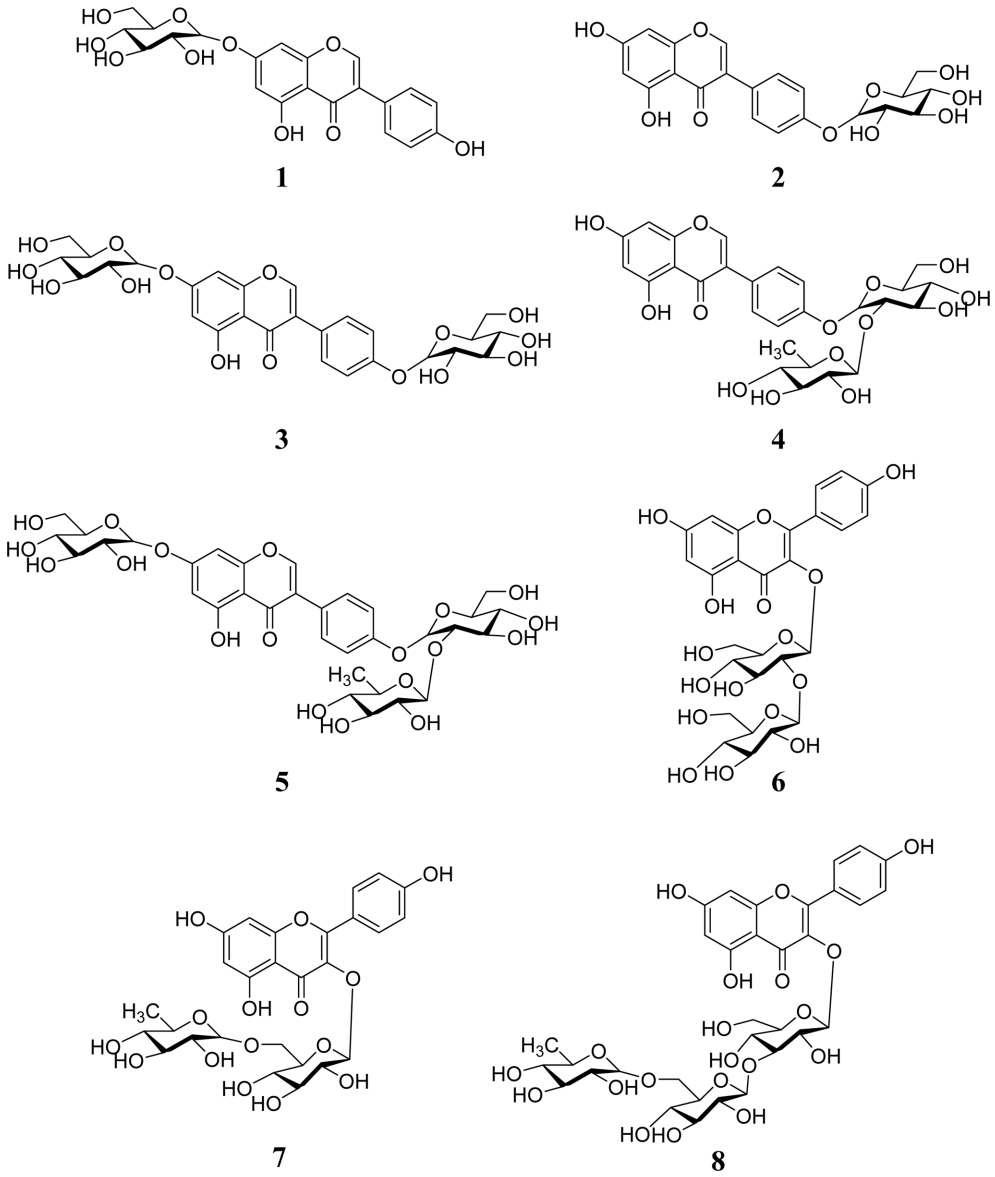

Fig. 1. Structures of the isolated compounds from the fruits of *S. japonica.*

led to the identification of these structures, and purities were determined to be >98% by HPLC-DAD analysis based on a peak area normalization method.

The chromatographic condition was established for simultaneous determination of eight components of *S. japonica* fruits extract. The combination of water containing ammonium acetate and methanol was employed as a mobile phase. This solvent system is known to attain better separation for phenolic compounds by reducing the tailing of the peaks compared with the mixture of acetic acid and methanol. Under the chromatographic condition, the addition of ammonium acetate in water increased the resolution of the peaks, whereas the peaks tailing occurred when acetic acid was added in water. Furthermore, the detection wavelength was set at 260 nm, where all compounds presented the maximum absorption. The presence of eight components in the extract was confirmed by comparing each retention time and UV spectrum with those of standards. As a result, the optimized mobile phase comprising methanol and water including ammonium acetate was successively used for the analysis of *S. japonica* fruits extract resulted in a good resolution and satisfactory peak shape at 260 nm.

The newly developed HPLC-DAD method was validated in terms of precision, selectivity, linearity, sensitivity, and accuracy test. The absorption spectrum of a single com-

Table 3. Regression equation and detection limit for the isolated components

Analyte	Regression equation[a]	Correlation coefficient (R^2)	Linear range (μg)	LOD (μg)	LOQ (μg)
1	y = 1,233.62x – 0.43	0.99996	0.0327 - 0.626	0.0093	0.0282
2	y = 979.78x + 3.51	0.99992	0.0318 - 0.627	0.0118	0.0358
3	y = 2,814.25x + 3.46	0.99995	0.0320 - 0.625	0.0014	0.0041
4	y = 10,291.18x + 18.81	0.99996	0.0312 - 0.625	0.0018	0.0055
5	y = 2,817.51x + 3.85	0.99999	0.0313 - 0.625	0.0080	0.0243
6	y = 3,127.12x + 4.23	0.99997	0.0325 - 0.626	0.0023	0.0070
7	y = 1,043.45x + 2.63	0.99998	0.0317 - 0.626	0.0064	0.0195
8	y = 1,566.13x + 2.18	0.99998	0.0318 - 0.625	0.0043	0.0130

[a]y : peak area, x : amount (μg)

Table 4. Analytical results of precision and accuracy of the eight isolated components

Analyte	Precision RSD (%)				Accuracy RSD (%)				
	Quantity (μg)	Intra-day (n=3)	Inter-day (n=3)	Repeatability RSD (%) (n=5)	Sam.: Std.	Spiked (μg)	Detected (μg)	Recovery (%)	RSD (%)
	0.312	0.669	0.612		1 : 2	0.924	1.008	110.00	0.724
1	0.248	0.692	1.604	1.183	1 : 1	1.230	1.385	112.88	0.265
	0.124	0.346	0.394		2 : 1	1.535	1.739	114.44	1.254
	0.310	0.548	0.748		1 : 2	1.572	1.612	103.87	1.708
2	0.248	0.720	1.798	1.037	1 : 1	2.202	2.321	105.60	0.280
	0.124	0.867	1.024		2 : 1	2.832	2.999	105.28	0.493
	0.314	0.163	0.613		1 : 2	0.753	0.772	102.98	0.451
3	0.250	0.208	1.001	1.349	1 : 1	0.974	1.017	105.23	0.694
	0.124	1.039	1.067		2 : 1	1.194	1.252	105.36	0.409
	0.312	0.212	0.532		1 : 2	0.844	0.879	104.55	0.517
4	0.253	0.146	1.707	0.171	1 : 1	1.110	1.183	106.63	0.242
	0.124	0.027	1.820		2 : 1	1.376	1.465	106.67	0.336
	0.313	0.326	0.697		1 : 2	0.852	0.885	104.26	0.592
5	0.251	0.129	0.685	0.188	1 : 1	1.121	1.189	105.56	0.428
	0.124	0.243	0.895		2 : 1	1.391	1.466	105.45	0.216
	0.311	0.223	0.430		1 : 2	1.333	1.380	103.54	0.041
6	0.250	0.355	0.716	0.703	1 : 1	1.843	1.953	106.01	0.032
	0.124	0.096	1.610		2 : 1	2.353	2.475	105.54	0.316
	0.311	1.317	0.577		1 : 2	0.750	0.774	104.65	2.025
7	0.251	1.082	1.415	1.227	1 : 1	0.968	1.025	106.09	0.386
	0.125	1.671	1.457		2 : 1	1.187	1.248	106.20	0.853
	0.312	1.042	0.656		1 : 2	1.093	1.091	99.95	0.387
8	0.250	0.604	0.542	1.966	1 : 1	1.484	1.483	100.78	0.752
	0.123	0.782	0.711		2 : 1	1.874	1.898	101.37	0.264

ponent remained constant at each retention time in one peak, which supported the specificity of each peak. Calibration curves were obtained linear at a relatively wide range of quantity, and all presented satisfactory linear regressions with high correlation coefficient values ($R^2 > 0.9999$) between peak area (y) and amount of each compound (x, μg) (Table 3). The LOD and the LOQ of eight compounds were between 1.35 and 11.8 ng and between 4.08 and 35.77 ng, respectively. They appeared high sensitivity under this chromatographic condition. In addition, the precision test was carried out by the intraday and interday variability for eight compounds in *S. japonica* fruits extract. The intraday variability was assayed at three concentrations on the same day and interday

Table 5. Contents (%, n = 3) of the isoflavonoids and flavonoids in *S. japonica* and *S. flavescens* samples

Compounds	SF1	SF1-1	SJ1	SJ2	SJ3	SJ4
1	0.03 ± 0.01	n.d.	8.99 ± 0.04	7.08 ± 0.09	7.64 ± 0.11	6.58 ± 0.27
2	0.43 ± 0.47	n.d.	81.13 ± 0.22	87.21 ± 0.09	77.69 ± 0.12	n.d.
3	n.d.	n.d.	4.99 ± 0.68	5.21 ± 0.48	3.28 ± 0.99	n.d.
4	0.19 ± 0.17	n.d.	5.94 ± 0.16	4.83 ± 0.32	4.89 ± 0.38	n.d.
5	n.d.	n.d.	5.88 ± 0.81	3.93 ± 0.22	2.86 ± 0.06	n.d.
6	0.16 ± 0.55	0.08 ± 0.54	10.24 ± 0.23	13.22 ± 0.06	8.84 ± 0.07	n.d.
7	n.d.	2.16 ± 0.53	0.13 ± 2.74	0.09 ± 1.09	0.04 ± 0.76	n.d.
8	n.d.	n.d.	7.99 ± 1.63	7.66 ± 0.95	5.58 ± 1.00	n.d.

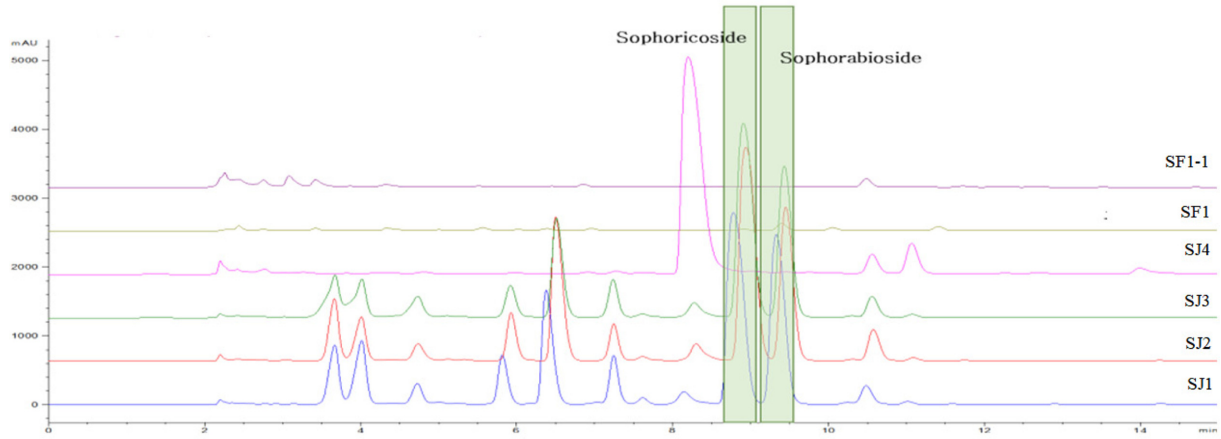

Fig. 2. HPLC chromatograms of *S. japonica* and *S. flavescens* extracts.

variability at three concentrations on three different days (1, 3, 4 days). As listed in Table 4, the RSD of intraday and interday variability was less than 1.82%, which revealed the high precision of this method. The accuracy was determined by the method of standard addition. The diluted sample solution (10 mg/mL) was spiked with the mixture of standard solutions of eight compounds, the isolated compounds **1** – **8**, in the ratios of 2:1, 1:1, and 1:2. The resultant samples were analyzed using the proposed method. For comparison, an unspiked sample was concurrently prepared and analyzed simultaneously. The mean recovery of each compound was 99.95 – 114.44%. (Table 4)

The established method has been applied to the determination of the eight components in the flowers (SJ4) and fruits (SJ1, SJ2, SJ3) of *S. japonica* and the roots (SF1) and flowers (SF1-1) of *S. flavescens* extracts (Fig. 2). As shown in Table 5, the chromatographic separation of the compounds **1** – **8** in the extracts of *S. japonica* and *S. flavescens* was well attained by the developed method. Most of the compounds were detected in SJ1~3, whereas only compound **1** was detected in SJ4. Isoflavonoids such as compounds **2** – **5** were not detected in SJ4. There were significant differences in the contents of compound **2**, a main bioactive component of *S. japonica* for the treatment of menopausal symptoms, among the tested samples. This result can be applied to discriminate the fruits of *S. japonica* from other parts of the same species or other species of the same genus samples. In addition, the contents of flavonoids were much higher in *S. japonica* samples from Korea (SJ1, SJ2) than the sample from China (SJ3) (Table 5). The proposed method could be readily utilized for the quality control of *S. japonica* products as well as comparison with forged samples by offering more information on the constituents of the fruits *S. japonica*.

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