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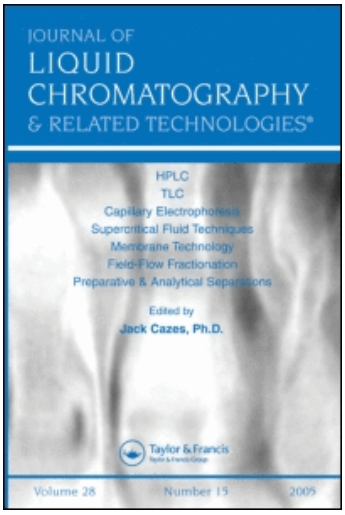

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Simultaneous Determination of Five Active Constituents in the Aerial Parts of *Saururus chinensis* by HPLC-DAD

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Simultaneous Determination of Five Active Constituents in the Aerial Parts of *Saururus chinensis* by HPLC-DAD

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Abstract: The chemical profile of five main bioactive constituents including rutin, quercetin 3-*O*-*β*-D-glucuronopyranoside, quercetin 3-*O*-*β*-D-glucopyranosyl(1→2)-*O*-α-L-rhamnopyranoside, quercitrin and sauchinone in extract of *Saururus chinensis* was studied using high performance liquid chromatography-diode array detector-electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS). The HPLC-DAD conditions were optimized for the simultaneous analysis of these five compounds. This method was validated in terms of specificity, linearity ($R^2 > 0.9987$), precision (<6.4% RSD), and recoveries (93.9 – 103.0%). The limits of detection of these compounds were ranged from 38.7 to 648.4 ng. In addition, the seasonal variation of the chemical composition of these main compounds in *S. chinensis* was investigated using this validated method, which resulted in a significant difference in the contents of these compounds according to the harvest time.

Keywords: HPLC-DAD, *Saururus chinensis*, Seasonal variation, Simultaneous analysis, Validation

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INTRODUCTION

Medicinal plants have played a key role in world health. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. Currently, the major pharmaceutical companies have demonstrated renewed interest in investigating plants as sources for new lead structures and also for the development of standardized phytotherapeutic agents.[1] It is widely accepted that multiple constituents are responsible for the pharmacological and biological effect of phytotherapeutic agents. Thus, it is necessary to quantitatively analyze the multibioactive compounds to achieve proper standardization and quality control of phytotheraputic agents. In addition, the source and quality of raw materials play a pivotal role in guaranteeing the quality and stability of phytotherapeutic agents. Since the chemical constituents of plants are greatly dependent on seasonal variation, the optimization of the harvesting and collecting time should be considered to expect a stable quality of phytotherapeutic agents including the chemical profile and biological effects.

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Saururus chinensis (Saururaceae) is a perennial herbaceous plant used in the treatment of various conditions, such as edema, jaundice, gonorrhea, as an antipyretic, diuretic, and anti-inflammatory agent in Korean traditional medicine.[2] Lignans, flavonoids, and aristolactams have been reported to be constituents of *S. chinensis*.[3–5] Among these compounds, sauchinone, a major lignan, has been reported to have hepatoprotective, immunosuppressive, and anti-inflammatory activities.[6–8] Flavonoids have been also known to be active components of *S. chinensis* due to their hepatoprotective, antioxidative, and anti-inflammatory effects.[4,9,10] Thus, in the present study, we tried to develop a simultaneous analytical method for five major active constituents of the aerial parts of *S. chinensis*, rutin, quercetin 3-*O*-*β*-D-glucuronopyranoside

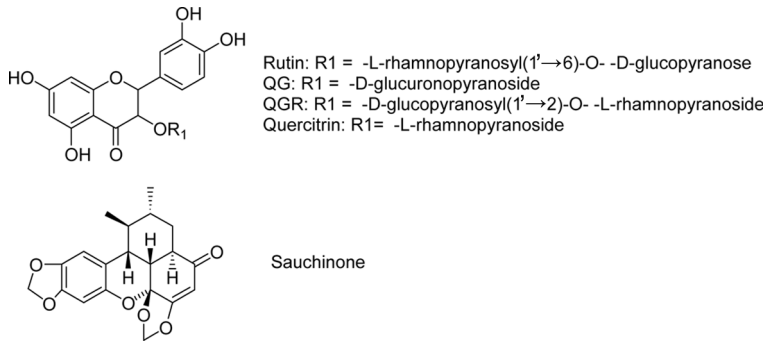

Figure 1. Structures of the active constituents in the aerial parts of *S. chinensis*.

(QG), quercetin 3-*O*-*β*-D-glucopyranosyl(1→2)-*O*-α-L-rhamnopyranoside (QGR), quercitrin, and sauchinone (Figure 1), which were selected based on the previous reports.[4,6–10] In addition, the contents of these active constituents with regard to the seasonal variation were estimated using the simultaneous analysis method developed in this study to optimize the harvesting and collecting time of the aerial parts of *S. chinensis*.

EXPERIMENTAL

Materials

All the five compounds were isolated from the aerial parts of *S. chinensis* and identified by comparison of their spectral data with those reported in the literature.[2–4] The purity of all compounds is more than 95%, as determined by HPLC-UV with two wavelengths (250 and 350 nm). Ten samples of the aerial parts of *S. chinensis* were collected during the summer (May 31, June 9, July 4, July 22, August 2, August 16, August 23, August 30, 2005) and autumn (September 12, October 2, 2005) from the Medicinal Herb Garden, College of Pharmacy, Seoul National University, Gyunggi, Korea. Average temperature and amount of precipitation detected during the months of harvest are presented in Table 1.

HPLC grade solvents (acetonitrile, water, and methanol) and reagents were obtained from BDH chemicals (Poole, UK). Acetic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

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Table 1. Average climate data detected during the periods preceding the harvest

Month	Average temperature (°C)	Amount of precipitation (mm)	Extraction yield (%)
May 31[a]	17.7	85.5	27.2
June 9	21.1	3.0	35.2
July 4	23.3	239.0	34.5
July 22	24.9	76.7	38.7
August 2	26.8	146.2	22.4
August 16	27.0	135.0	25.5
August 23	24.2	19.5	26.6
August 30	22.1	103.5	22.2
September 12	24.2	3.2	25.7
October 2	20.3	324.7	24.6

[a]From May 1 to May 31.

Chromatographic Conditions

The HPLC system consisted of a chromatographic pump (P680, Dionex, Germany) and injector (7725i, Rheodyne, USA) equipped with a photodiode array (UVD 340U, Dionex, Germany). The output signal of the detector was recorded using a Dionex Chromelon^{TM} Chromatography Data System. Chromatographic separation was achieved on a Shiseido Capcell Pak RP18 MG (5 μm, 4.6 mm × 150 mm). The mobile phases were composed of acetonitrile (A) and water with 0.3% formic acid (B) at a flow rate of 1.0 mL/min, and monitored at 250 nm. The gradient profile was as follows: 0–5 min: isocratic 15% of A; 5–20 min: linear 15–25% of A; 20–30 min: linear 25–45% of A; 30–40 min: linear 45–60% of A; 40–48 min: isocratic 60% of A; 48–50 min: linear 60–15% of A; 50–52 min: isocratic 15% of A (v/v).

The high performance liquid chromatography diode array detector electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS) system consisted of Finnigan Surveyor HPLC system with a pump, an autosampler, a PDA plus detector, and Finnigan LCQ advantage MAX with Xcalibur software. Separation was achieved on a Waters XTerra^{TM} RP18 (5 μm, 4.6 mm × 150 mm). A linear gradient elution of acetonitrile (A), 0.03% formic acid (B) and methanol (C) was used at a flow rate of 0.3 mL/min. The gradient profile was as follows: 0–30 min: linear 15–50% of A and 85–50% of B; 30–35 min: linear 50% of A, 50–35% of B and 0–15% of C; 35–65 min: isocratic 50% of A, 35% of B and 15% of C; 65–66 min: linear 50–15% of A, 35–85% of B and 15–0% of C; 66–70 min: isocratic 15% of A and 85% of B (v/v). Ion polarity was negative for flavonoids and positive for sauchinone, respectively.

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Preparation of Standard Solution

Stock standard solution of rutin, QG, QGR, quercitrin, and sauchinone was prepared in methanol at a concentration of 1 mg/mL, respectively. The appropriate amount of every standard solution was mixed and diluted with methanol as indicated.

Sample Preparation for HPLC

The dried and minced leaves and twigs of *S. chinensis* (3.0 g) was weighed accurately and extracted with 70% methanol for 2 h at 90°C, using a reflux. This extract was filtered and evaporated in vacuum, and then suspended with 50% methanol at an appropriate concentration. This sample

solution was filtered through a 0.45 μm membrane filter (Millipore, Nylon, 170 mm) and analyzed with HPLC.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of adjacent peaks. Reversed phase columns have been usefully applied to analyze the components of natural resources. The preferred chromatographic condition was obtained using Shiseido Capcell Pak RP18 MG (5 μm, 4.6 mm × 150 mm). *S. chinensis* contains both polar flavonoids and nonpolar lignans. Thus, a gradient elution system consisting of acetonitrile and 0.3% formic acid was employed for the simultaneous determination of these five active constituents of *S. chinensis*. Various mixtures of water and acetonitrile in combination with formic acid were tested as a mobile phase. Acid is known to achieve better separation for phenolic compounds by depressing the tailing of the peaks.[11,12] In our chromatographic condition, addition of 0.3% formic acid in water increased the resolution of the peaks, whereas the distortion of peaks occurred when formic acid was added less than 0.3%. The wavelength for detection was set at 250 nm, where the five compounds showed the maximum absorption as measured by DAD. The presence of rutin, QG, QGR, quercitrin, and sauchinone in this herb was verified by comparing each retention time and UV spectrum with those of each standard compound and spiking with authentic standards. As a result, the optimal mobile phase consisting of acetonitrile-water with 0.3% formic acid was subsequently employed for the analysis of *S. chinensis*, which led to good resolution and satisfactory peak shape at 250 nm (Figure 2a).

HPLC-ESI-MS Identity Confirmation

A HPLC-ESI-MS experiment was also performed to confirm the identity of five marker constituents (Figure 2b). A different solvent condition was used for this analysis to reduce the flow rate for the MS detector. The molecular weights of flavonoids and sauchinone were obtained in negative and positive modes, respectively. The ESI-MS spectra for the compounds were detected in selected ion monitoring mode. Rutin, QG, QGR, and quercitrin showed their molecular ions $[M\text{-}H]^-$ at m/z 609.1, 467.0, 609.1, and 447.0, respectively. Sauchinone showed a molecular ion $[M]^+$ at m/z 357.0.

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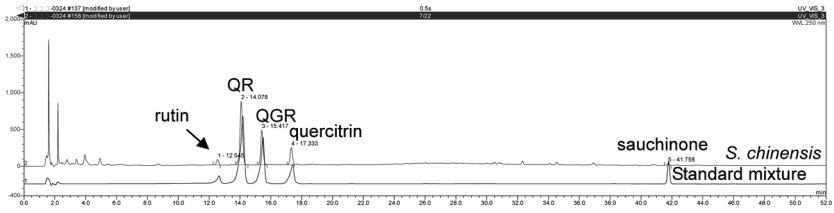
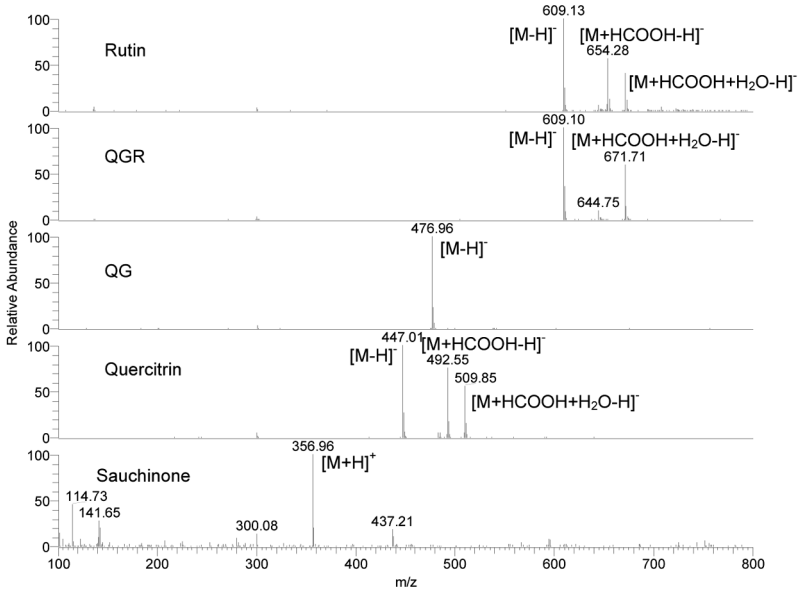


(a)



(b)

Figure 2. HPLC chromatogram of *S. chinensis* and standard mixture at 250 nm (a) and HPLC-DAD-ESI-MS spectrum of rutin, QG, QGR, quercitrin, and sauchinone in *S. chinensis* (b).

Validation of the HPLC Method

Specificity was determined by the calculation of peak purity facilitated by DAD. The peak purity was evaluated using DAD and its corresponding computer software, which confirms the singularity of the peak component. The absorption spectrum of a single component remained invariable at each time point in one peak, which supported specificity of each peak. In HPLC-ESI-MS spectra, the molecular ions and fragmentation patterns of each compound were well matched with each chemical structure. These results clearly showed the specificity of each peak for

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rutin, QG, QGR, quercitrin, and sauchinone, respectively (Figure 2b). According to the International Conference on Harmonization (ICH) guidelines, the linearity of calibration curves was suggested to be evaluated by at least five concentrations of each compound. In our present study, the linearity of rutin, QG, QGR, quercitrin, and sauchinone was calculated by six concentrations of each compound and the regression equation was calculated in the form of $y = ax + b$, where y and x were the values of peak area and amount of each compound, respectively. The results of regression analyses and the correlation coefficients (r^2) were listed in Table 2. Calibration curves were linear in relatively wide ranges of concentrations (0.048–2.4 μg for rutin, 0.256–12.8 μg for QG, 0.224–11.2 μg for QGR, 0.128–6.4 μg for quercitrin and 0.048–2.4 μg for sauchinone) with high correlation coefficient values ($R^2 > 0.9987$) between peak area (y) and amount of each compound (x, μg). The LOD was measured based on the method recommended by ICH ($LOD = 3.3\,\delta/S$, δ = standard deviation of the response, S = slope of the calibration curve). The LOD of rutin, QG, QGR, quercitrin, and sauchinone were 38.7, 108.7, 39.2, 214.0, and 69.7 ng, respectively, which showed a high sensitivity at this chromatographic condition (Table 2). The precision test was carried out by the intra-day and inter-day variability for rutin, QG, QGR, quercitrin, and sauchinone. The intra-day variability was assayed at three concentrations on the same day and inter-day variability was assayed at three concentrations on three sequential days (1, 3, 5 days). As listed in Table 3, the relative standard deviation (RSD) of intra-day and inter-day variability was less than 6.4%, which demonstrated good precision of this method. The accuracy of the method set up in this study was determined by the method of standard addition. The dilute sample solution (10.0 mg/mL) was spiked with the mixture standard samples of rutin (0.024 mg/mL), QG (0.128 mg/mL), QGR (0.112 mg/mL), quercitrin (0.064 mg/mL), and sauchinone (0.024 mg/mL) at the ratio of 2:1, 1:1, and 1:2, respectively. The resultant samples

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Table 2. Regression equation and detection limit for different compound

Compound	Retention time (min)	Linear regression equation[a]	Correlation coefficient (R^2)	LOD (ng)
Rutin	12.6	$y = 26.0727x - 0.5159$	0.9996	38.7
QG	14.1	$y = 42.0423x + 4.0144$	0.9987	108.7
QGR	15.4	$y = 28.9988x + 0.0491$	0.9998	39.2
Quercitrin	17.3	$y = 25.3972x + 0.1173$	0.9989	214.0
Sauchinone	41.8	$y = 40.1232x - 1.0020$	0.9993	69.7

[a]y = peak area, x = concentration (μg/ml).

Table 3. Analytical results of precision and accuracy

		Precision				
		Inter-day	Intra-day	Accuracy		
Compound	Amount (μg)	RSD (%)[a]	RSD (%)[a]	Spiked amount (μg)	Accuracy (%)	RSD (%)[a]
Rutin	1.20	1.94	1.72	0.5790	102.2	2.63
	0.48	1.85	2.12	0.6285	99.0	6.27
	0.24	3.44	1.67	0.6780	98.2	4.08
QG	6.40	1.64	2.44	3.0857	101.6	3.23
	2.56	2.03	3.55	3.3486	93.9	7.47
	1.28	2.38	1.21	3.6115	98.1	3.28
QGR	5.60	1.33	2.07	2.3528	101.8	5.17
	2.24	1.30	0.30	2.4092	102.7	3.55
	1.12	1.21	0.66	2.4656	101.0	0.96
Quercitrin	3.20	5.77	1.69	1.2759	95.3	3.02
	1.28	3.02	3.71	1.2739	95.1	2.90
	0.64	6.36	2.64	1.2719	95.2	0.51
Sauchinone	1.20	2.99	2.44	0.3818	95.4	2.79
	0.48	1.38	1.35	0.3327	103.0	1.81
	0.24	0.47	2.37	0.2836	96.3	3.62

[a]RSD (%) = (SD of amount detected/mean of amount detected) × 100.

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were analyzed by using the proposed method. For comparison, an unspiked sample was concurrently prepared and analyzed simultaneously. As listed in Table 3, the mean recovery of each compound was 93.9–103.0% with RSD values less than 7.5%.

Seasonal Variation of the Active Constituents in *S. chinensis*

In Korean traditional medicine, the aerial parts of *S. chinenesis* are collected in July, August, and September.[13] Since the amount of leaves were not enough during winter and spring, the aerial parts of *S. chinensis* were collected from May to October to study the influence of seasonal variation on the contents of active constituents. The effect of harvest time on the yield of extraction was estimated using the reflux extraction method. To extract both polar and nonpolar compounds, water-methanol mixture, 70% methanol, was used as an extraction solvent. As shown in Table 1, high yields of extraction were obtained in June and July. The contents of five active constituents were distributed unevenly among seasons. Before August 2, rutin

Table 4. Seasonal variation in the active constituents of *S. chinensis* (g/g%)

Month	Rutin	QG	QGR	Quercitrin	Sauchinone
May 31	0.2865	2.158	0.7535	0.7638	0.0440
June 9	0.2491	1.8689	0.7271	0.6864	0.0438
July 4	0.3171	2.9179	1.6722	1.1766	0.1016
July 22	0.2747	2l1127	1.541	0.8687	0.0641
August 2	0.2532	1.4382	1.0471	0.4967	0.0656
August 16	0.295	1.7578	1.2431	0.5747	0.0887
August 23	0.3201	1.8254	1.1169	0.542	0.0897
August 30	0.3416	2.0527	1.3147	0.6646	0.0769
September 12	0.3263	2.1896	1.5355	0.832	0.0440
October 2	0.3807	2.0686	1.2892	0.6339	0.0927

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showed the highest content on July 4 (0.3171%). From August 2 (0.2532%), the content of rutin consistently increased till October 2 (0.3807%). Other quercetin glycosides, QG, QGR, and quercitrin, showed similar trends over all experiment periods. These quercetin glycosides showed the maximum yields on July 4 (Table 4). This is in agreement with the literature reporting that flavonoid quantities of some leafy plants showed the highest levels in summer.[14] Since the formation of flavonoids is light dependent,[15] the decrease in the amount of these compounds during July and August can be explained by monsoon and typhoon rainfall in Korea, at least in part. Taken together, for more efficiency in harvesting, the aerial parts of *S. chinensis* should be collected in July, considering the extraction yield and the contents of active constituents.

CONCLUSIONS

In this paper, a rapid and reliable HPLC method for simultaneous determination of five active constituents of the aerial parts of *S. chinensis*, rutin, QG, QGR, quercitrin, and sauchinone, has been developed and validated. The method fulfilled all the requirements to be identified as a reliable and feasible method, showing good specificity, precision, linearity, and accuracy date. Therefore, this established method is useful for the quality control of *S. chinensis* by simultaneous quantitative analysis of these constituents. In addition, the present study demonstrated that the variations in the extraction yield and the contents of active constituents were associated with seasonal influences.

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