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# Validation of an optimized HPLC/UV method for the quantification of flavonoids in lotus

Ju Sung Lee<sup>1</sup>, Leo Adrienne Paje<sup>1</sup>, Won-Hee Choi<sup>2</sup>, Eun Ju Cho<sup>3</sup>, Hyun Young Kim<sup>4</sup>, Sonia D. Jacinto<sup>5</sup> and Sanghyun Lee<sup>1\*</sup>

## Abstract

Flavonoids present in the leaves of lotus (*Nelumbo nucifera*) grown in different regions of South Korea (Yeongcheon, Haenam, and Seocheon) and at different harvest times (July to September) were determined. Flavonoid contents in lotus extracts were identified and analyzed using high-performance liquid chromatography (HPLC). The HPLC results revealed that the flavonoid contents of the lotus extracts varied at different harvesting times, with the highest content in July. Analysis of the flavonoid content in the leaves from the different regions showed the highest contents of isorhamnetin-3-O-glucoside, quercetin 3-O-glucuronide, and quercetin 3-O-glucoside in Yeongcheon, Korea, and highest contents of rutin, myricetin, kaempferol 3-O-glucoside, and quercetin in Haenam, Korea. The HPLC method was validated and optimized to quantify quercetin 3-O-glucuronide; it showed good linearity (1000–62.5 µg/mL,  $r^2 = 0.9999$ ), accuracy (106%–108%), and precision (RSD ≤ 1.70%). Determination of flavonoid content in lotus is valuable for producing medicinal crops and identifying the optimal sources to increase the quantity of clinically available medicines.

**Keywords:** Flavonoid, HPLC, Method, Validation, *Nelumbo nucifera*

## Introduction

Lotus (*Nelumbo nucifera*) has a wide distribution throughout North America, Australia, and East Asia [1]. The plant is used for various medicinal purposes and folk remedies in China [2] and is considered to be of cultural and historical importance in Egypt, India, and China [3–5] as it symbolizes perfection, purity, and beauty. In recent years, there have been reports on anti-viral [2], anti-obesity [6], anti-oxidant [7], and lipolytic [8] activities with simultaneous determinations of the components of lotus [9].

As oxygen free radicals, reactive oxygen species (ROS) contribute to aging and many related diseases [10]. The oxygen taken up via breathing is used by mitochondria within the body to generate energy, but they produce byproducts such as ROS which interfere with cell

function; their effects are long-lasting and can eventually lead to cell damage. An excess supply of oxygen produces free radicals that oxidize cells, destroying the cell structure, damaging DNA, and attacking the cell membrane. The resulting damaged cells grow and proliferate and eventually become cancer cells [11]. As ROS are highly unstable biochemical substances, they oxidize fat in the cells and blood to yield lipid peroxides, the main cause of aging. A potential solution to prevent cell damage caused by free radicals is the use of anti-oxidants such as flavonoids. Flavonoids are a large class of secondary metabolites that are widely present in plant species. They are important in human diet [12, 13] as they perform different metabolic functions are largely valued because of their anti-oxidant properties [14].

In this study, flavonoids were identified in the extracts of lotus leaves. A simultaneous analysis of the flavonoid content of lotus grown in different regions and at different harvesting times was also performed. Thus, the aim of this study was to evaluate whether lotus could be a

\*Correspondence: slee@cau.ac.kr

<sup>1</sup> Department of Plant Science and Technology, Chung-Ang University, Anseong 17546, Republic of Korea  
Full list of author information is available at the end of the article

rich natural source of flavonoids based on the measured concentrations.

## Materials and methods

### Plant materials

The samples of lotus leaves were collected from different regions of South Korea (Haenam, Seochon, and Yeongcheon) by Teazen Co., Korea. The samples were collected during the months of July, August, and September. A voucher specimen was deposited at Chung-Ang University, Korea.

### Instruments, chemicals, and reagents

Chromatographic analysis was performed using an HPLC system (Agilent technology 1290 Infinity II) equipped with a pump, auto-sampler, and UV detector (Santa Clara, CA, USA). The flavonoids [myricetin (MYR), isorhamnetin 3-*O*-glucoside (I3Glc), quercetin (QUE), kaempferol 3-*O*-glucoside (K3Glc), quercetin 3-*O*-glucoside (Q3Glc), rutin (RUT), and quercetin 3-*O*-glucuronide (Q3Glu)] were prepared by the Natural Product Institute of Science and Technology ([www.nist.re.kr](http://www.nist.re.kr)), Anseong, Korea (Fig. 1). HPLC grade solvents (acetonitrile, methanol, and water) were obtained from J. T. Baker (Avantor, Radnor, PA, USA). Ethanol and acetic acid (99.7%) were obtained from Samchun Pure Chemicals (Pyeongtaek, Korea).

### Sample preparation

Dried lotus leaves (15 g) were extracted in distilled water using the Soxhlet procedure and dried using a freeze

dryer. This extract was dissolved in methanol (MeOH) and filtered with a syringe filter (0.45  $\mu$ m). MeOH was used to dissolve the compounds, and stock solutions (0.125–1000  $\mu$ g/mL) were prepared.

### HPLC conditions

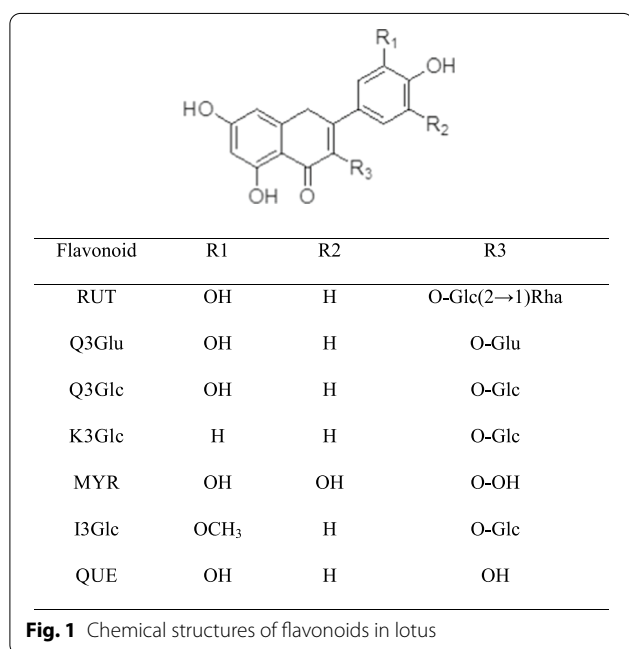
Flavonoids were quantitatively analysed using a reverse-phase HPLC system with a UV system. An INNO C18 column (25 cm  $\times$  4.6 mm, 5  $\mu$ m) was used to separate the compounds. The column temperature and flow rate were maintained at 35  $^{\circ}$ C and 1 mL/min, respectively. The injection volume was 10  $\mu$ L, and the wavelength was detected at 350 nm. The mobile phase with a gradient elution system composed of 0.5% acetic acid in water (A) and acetonitrile (B); 80% A at 0 min, 70% A at 30 min, and 80% A at 35 min.

### Method validation

The validation of the HPLC method for Q3Glu was evaluated in terms of specificity, accuracy, linearity, and precision. To evaluate potential interference with the signals of the analytes, specificity was assessed. Using five concentrations of the standard mixture (1000–0.1  $\mu$ g/mL), linearity was determined with triplicate injections/level. The chromatogram peak areas at 350 nm were plotted against the known concentrations of the standard solutions to construct the calibration curve. The standard curves for Q3Glu were analyzed using linear least-squares regression. For accuracy and precision validation, Q3Glu in the different standard mixtures (1000, 100, and 10  $\mu$ g/mL) was used for intra- and inter-day precision analysis. For intra-day precision, the retention time and peak area of three different concentrations of the standard solutions were calculated in one day and expressed as the relative standard deviations (RSD %). For inter-day precision, the RSD % of the standard solutions was determined on three separate days. The recovery tests that analyzed the extracts were spiked with three concentrations of the standard mixture ( $\mu$ g/mg), and the % recovery was used to determine the accuracy of the HPLC method.

### Calibration curves

Each compound was dissolved in MeOH (1 mg/mL) to prepare standard stock solutions. The selected stock solutions were serially diluted to the desired concentrations to construct calibration curves. All samples were dissolved in MeOH (20 mg/mL). Prior to use, all examined solutions were filtered through a 0.45- $\mu$ m PVDF filter. The flavonoid calibration functions were calculated using the concentration ( $X$ ,  $\mu$ g/10  $\mu$ L), peak area ( $Y$ ), and mean values  $\pm$  standard deviation ( $n = 5$ ).



## Results and discussion

Flavonoids, a group of natural substances with variable phenolic structures, are found in vegetables, fruits, tea, flowers, grains, and wine. Lotus contains biologically effective flavonoids, including I3Glc, K3Glc, MYR, QUE, Q3Glc, Q3Glu, and RUT, which exhibit pharmacological activities [15]. Optimization and validation is important to facilitate the production of plant-derived health products [16–18] and source out the plant containing the most flavonoids where it can help increase its quantity in clinically available medicines.

Content of flavonoids in lotus leaves at different times (July to September) and from different regions (Yeongcheon, Haenam, and Seochon in South Korea) were performed using HPLC. The calibration curve of the compounds was determined by plotting the peak area of the prepared concentrations and was calculated using

linear regression. The coefficient values ( $r^2$ ) of the compounds ranged from 0.9997 to 1.0000 (Table 1). For quantitative analyses, HPLC separation of the compounds was performed using a reverse phase system and gradient elution system (Fig. 2). Table 1 shows the standard calibration curves for the compounds. The HPLC method efficiently separated the compounds, and the retention times ranged from 8.1 to 25.3 min. The effective wavelength for detecting the flavonoids and the main component of Q3Glu in a single run was 350 nm (Fig. 3).

The results suggested that the flavonoid contents in lotus extracts varied depending on the harvesting time (July, August, and September). The highest contents of K3Glc (1.09 mg/g), RUT (1.46 mg/g), I3Glc (1.23 mg/g), QUE (1.55 mg/g), and Q3Glu (63.83 mg/g) were found in July, whereas the highest contents of MYR (1.16 mg/g) and Q3Glc (8.77 mg/g) were found in August. Further, harvesting in July yielded a higher flavonoid content than in August and September. The flavonoid content from different regions was analyzed; it was the highest in Yeongcheon for Q3Glc (10.19 mg/g), I3Glc (1.96 mg/g), and Q3Glu (77.58 mg/g). In contrast, the content of K3Glc (1.07 mg/g), RUT (1.71 mg/g), QUE (1.38 mg/g), and MYR (1.27 mg/g) from Haenam was also high. The overall flavonoid content of lotus leaf extracts obtained from Yeongcheon was higher than from the other regions (Tables 2 and 3).

Our results corroborate previously reported data [19]. A previous study reported similar results with measured concentrations of hyperoside, Q3Glc, and K3Glc at 9.1, 4.6, and 3.0 mg, respectively [19]. Another study showed

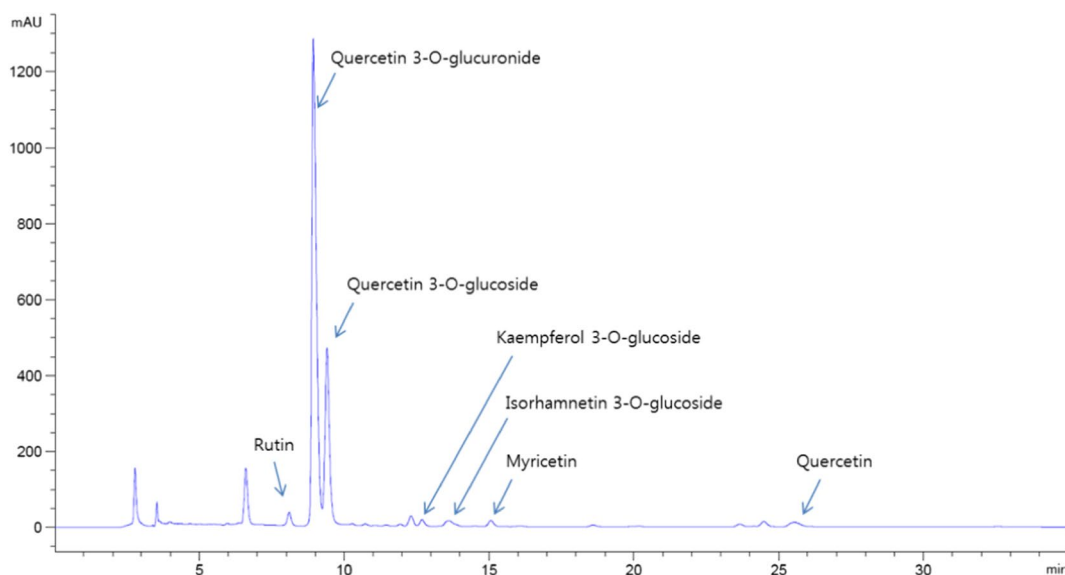
**Table 1** Calibration curves of flavonoids in lotus

Compound	$t_R$	Calibration equation	Correlation factor, $r^2$
RUT	8.1	$Y = 917.46X + 3.4761$	0.9999
Q3Glu	8.9	$Y = 1158.4X + 28.395$	0.9999
Q3Glc	9.2	$Y = 1913.5X + 63.777$	1.0000
K3Glc	12.7	$Y = 1267.8X + 10.943$	0.9998
I3Glc	13.7	$Y = 865.57X + 4.8457$	1.0000
MYR	15.6	$Y = 1058.7X - 14.542$	0.9999
QUE	25.3	$Y = 1849.5X - 36.422$	0.9997

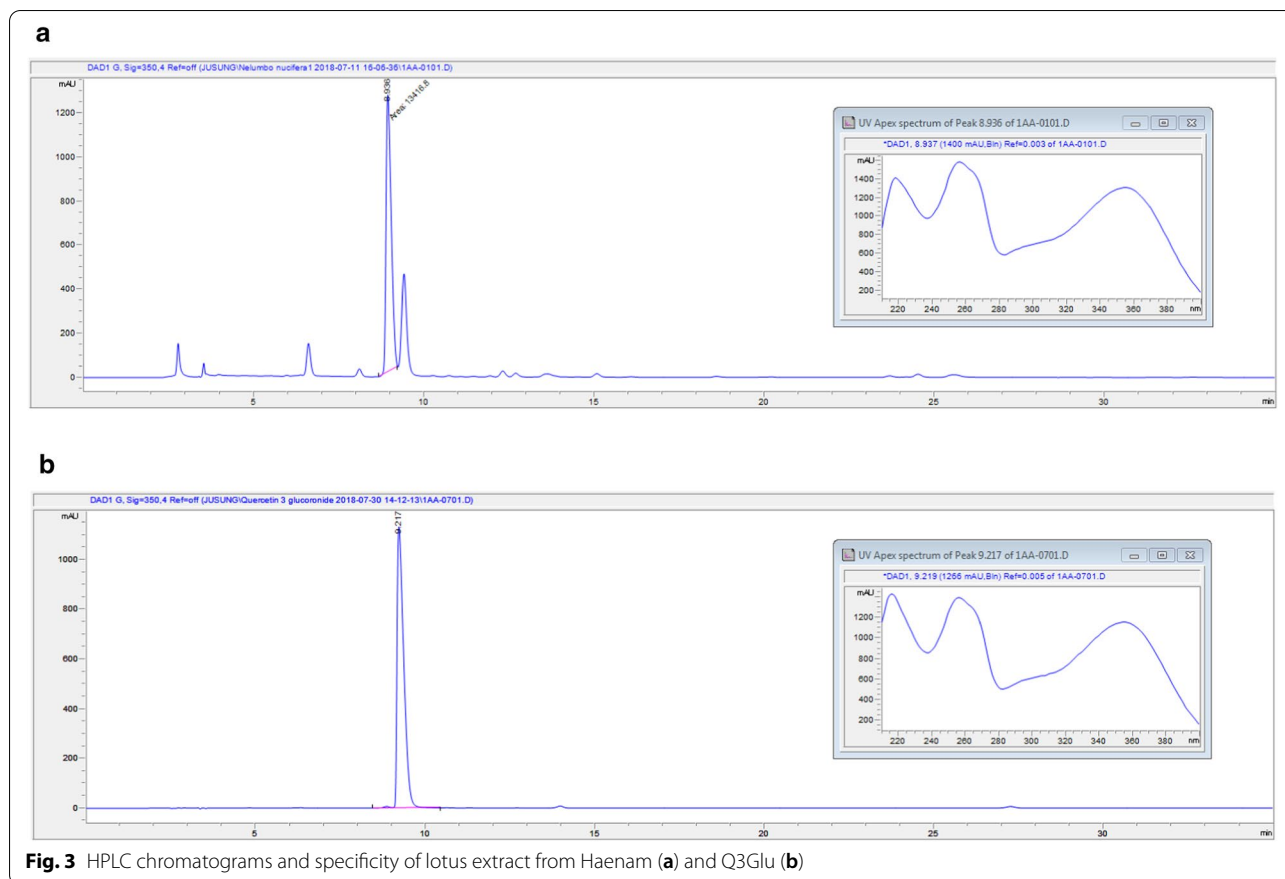
$t_R$  = retention times

Y = peak area, X = concentration of standard ( $\mu\text{g/mL}$ )

$r^2$  = correlation coefficient for five data points in the calibration curve



**Fig. 2** HPLC profiling of the lotus extract



**Fig. 3** HPLC chromatograms and specificity of lotus extract from Haenam (a) and Q3Glu (b)

**Table 2** Contents of flavonoids in lotus at different harvesting times from Haenam region

Harvest	Content (mg/g extract)						
	RUT	Q3Glu	Q3Glc	K3Glc	I3Glc	MYR	QUE
July	1.46 ± 0.15	63.83 ± 0.47	5.67 ± 0.10	1.09 ± 0.07	1.23 ± 0.00	0.52 ± 0.02	1.55 ± 0.16
August	1.19 ± 0.01	61.76 ± 0.16	8.77 ± 0.16	0.71 ± 0.01	1.22 ± 0.28	1.16 ± 0.09	1.27 ± 0.02
September	0.40 ± 0.05	15.41 ± 0.11	1.77 ± 0.07	0.28 ± 0.03	0.53 ± 0.04	0.91 ± 0.05	0.82 ± 0.03

**Table 3** Contents of flavonoids in lotus from different regions harvested in July

Region	Content (mg/g extract)						
	RUT	Q3Glu	Q3Glc	K3Glc	I3Glc	MYR	QUE
Yeongcheon	0.79 ± 0.01	77.58 ± 1.16	10.19 ± 0.18	1.06 ± 0.05	1.96 ± 0.06	0.94 ± 0.09	1.07 ± 0.03
Haenam	1.71 ± 0.13	54.27 ± 0.29	9.67 ± 0.12	1.07 ± 0.01	1.75 ± 0.17	1.27 ± 0.03	1.38 ± 0.01
Seocheon	1.10 ± 0.12	74.02 ± 0.66	8.64 ± 0.57	0.65 ± 0.01	1.14 ± 0.10	0.78 ± 0.05	0.92 ± 0.02

that Q3Glu (6.1 mg), obtained at a purity of 97.0%, was the most dominant flavonoid in the lotus extract [20].

Intra- and inter-day precision levels were used to evaluate the method's precision in analyzing Q3Glu. These values were obtained within one day by triplicate

analysis and over a 5-day period, respectively (Table 4). The coefficient of variation of Q3Glu ranged from 0.14 to 0.91% and 0.89 to 1.70% for intra- and inter-day precision, respectively. The obtained values were lower than 2%, suggesting that the analytical method for quantifying

**Table 4** Intra- and inter-day precision for the determination of Q3Glu in lotus

Compound	Spiked concentration (mg/mL)	Intra-day (n = 5)		Inter-day (n = 5)	
		Found concentration (mg/g)	RSD (%)	Found concentration (mg/g)	RSD (%)
Q3Glu	20.0	34.32	0.14	34.79	1.70
	17.5	34.91	0.91	34.83	0.89
	15.0	35.32	0.73	34.50	1.47

**Table 5** Accuracy for the determination of Q3Glu in lotus

Compound	Concentration (mg/mL)	Found content (mg)					Recovery (%)	RSD (%)
		1st	2nd	3rd	4th	5th		
Q3Glu	0.25	0.28	0.27	0.27	0.27	0.28	108.61	1.22
	0.5	0.53	0.53	0.54	0.54	0.54	106.79	1.14
	1	1.07	1.06	1.07	1.08	1.09	107.41	1.12

Q3Glu from the lotus extract showed good precision (Table 4).

Recoveries for Q3Glu were examined to validate the accuracy of the analytical method. Three different concentrations were analyzed five times. The recovery of Q3Glu ranged from 106.79 to 108.61%, indicating that the analytical method used to quantify Q3Glu exhibited good accuracy (Table 5).

In conclusion, the flavonoids in lotus are important in producing medicinal crops, and analyses to identify optimal sources can increase the quantity of clinically available medicines. Additionally, the majority of the flavonoid content has been obtained from lotus grown domestically, for example, in Yeongcheon. This suggests that lotus grown in Yeongcheon, South Korea, can be an internationally competitive source in developing lotus-derived products as it contained the highest flavonoid contents among the lotus from other places.

#### Authors' contributions

JSL experiments of specificity, accuracy, and precision; LAP HPLC analysis and HPLC profiling; W-HC harvesting and collecting samples and experimental design; EJC and HYK, content analysis and data reading; SDJ, data interpretation and English writing; SL, experimental design and English writing. All authors read and approved the final manuscript.

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#### Availability of data and materials

Not applicable.

#### Competing interests

There is no conflict of interests.

#### Author details

<sup>1</sup> Department of Plant Science and Technology, Chung-Ang University, Anseong 17546, Republic of Korea. <sup>2</sup> R&D Center, Teazen Co, Anyang 14067, Republic of Korea. <sup>3</sup> Department of Food Science and Nutrition, Pusan National University, Busan 46241, Republic of Korea. <sup>4</sup> Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 52725, Republic of Korea. <sup>5</sup> Institute of Biology, University of the Philippines, 1101 Diliman, Quezon, Philippines.

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