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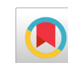

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Validation of an HPLC/UV-based method for *Salicornia herbacea*-derived isorhamnetin-3-*O*-glucoside and quercetin-3-*O*-glucoside quantification

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Abstract *Salicornia herbacea* is a type of salt marsh plant that has been used in traditional medicine to treat several diseases. Isorhamnetin-3-*O*-glucoside (I3G) and quercetin-3-*O*-glucoside (Q3G) are major flavonoids in *S. herbacea* that are known to exert various pharmacological activities. Therefore, our study sought to validate and optimize an HPLC/UV-based analytical method for I3G and Q3G yield quantification, as well as to determine its limit of detection, limit of quantification, linearity, precision, and accuracy. Upon testing a concentration range of 31.5-1.9 μg/mL the results exhibited good linearity (r^2 ≥0.9996 and r^2 ≥0.9999 for I3G and Q3G, respectively), and the procedure was deemed precise (relative standard deviation of ≤3.19 and ≤3.85%, respectively), and accurate (102.6-105.0 and 92.9-95.2%, respectively). The results showed that our proposed method could be used for rapid I3G and Q3G evaluation in *S. herbacea*.

Keywords Isorhamnetin-3-*O*-glucoside · Method validation · Quercetin-3-*O*-glucoside · *Salicornia herbacea*

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Introduction

Salicornia herbacea is a salt marsh plant that commonly grows in coastlines influenced by salinity gradients. This plant is an annual succulent shrub that grows throughout Eurasia, North America, the Middle East and the western coast of Korea [1-3]. *S. herbacea* is considered among the halophyte species with the highest tolerance for salinity. Moreover, it grows to approximately 10-40 cm in height and its stem has a dark green color, which turns to red during fall [4]. This plant has recently begun to be consumed in local cuisine and as a remedy against obesity, constipation and hepatitis. Therefore, in addition to its consumption as a 'sea vegetable', the medicinal qualities of this lesser-known marsh plant (e.g., immunomodulatory, osteoprotective, antilipidemic, anti-proliferative, and hypoglycemic) make it a good target for further phytochemical studies [5]. Parallel to the emergence of new functional and biologically active products, plants have recently been increasingly used as functional food items and herbal medicine [6,7].

S. herbacea is rich in calcium, potassium, magnesium, dietary fibers and essential fatty acids [8,9], and has also been found to possess therapeutic properties against arteriosclerosis, hyperlipidemia, diabetes, and fatty liver *in vivo* [10-12]. Different phytochemicals have been isolated from *S. herbacea* including various flavonoids such as quercetin-3-*O*-glucoside, isoquercitrin 6-*O*-methyloxalate and isorhamnetin-3-*O*-glucoside [13,14]. Among the flavonoids present in *S. herbacea*, most studies have focused on two of its major flavonoid constituents, which are known to possess important biological activities including cytotoxicity and phytotoxicity, as well as anti-microbial, anti-cancer, hepatoprotective, anti-oxidant, anti-inflammatory, and anti-lipogenic properties [14-18].

However, one of the challenges that prevent the widespread implementation of herbal medicines is the lack of standard quality controls in terms of extraction, compound determination and analysis [19]. Therefore, new methods and techniques must be developed to overcome these limitations. Validation of analytical methods with regards to their efficiency and precision is an

important step towards standardizing the effectiveness of bioactive extracts [20]. Despite the known therapeutic qualities of *S. herbacea* extracts, the qualitative and quantitative determination of its phytochemical markers has not been explored in previous studies.

Our study aimed to develop a technique for the detection of *S. herbacea* flavones, isorhamnetin-3-*O*-glucoside (I3G) and quercetin-3-*O*-glucoside (Q3G). Q3G have been already detected using a validated HPLC-DAD method in *Azadirachta indica* [21]. I3G has been detected in many plant extracts but there have been no studies regarding the validation of the HPLC method used in detecting the compound. This study serves as a pre-requisite for the production and registration of the bioactive compounds derived from this medicinal plant.

Material and Methods

Plant materials

S. herbacea was obtained from Suncheonman Byeolryang Yeomjeon (Suncheon, Korea) and identified by Prof. J. S. Choi, Pukyong National University, Korea. A voucher specimen (No. LEE2016-01) was deposited in the Department of Plant Science and Technology herbarium, Chung-Ang University, Korea.

Instrumentation and chemicals

A Waters 1525 system equipped with a binary HPLC pump (Miami, FL, USA), an auto-sampler, and an INNO C18 (4.6×250 mm, 5 µm) column equipped with a Waters 2489 UV/Visible Detector (Milford, MA, USA) was used for HPLC analysis. Flavonoids I3G (99.6% purity) and Q3G (99.7% purity) were isolated in our laboratory (Fig. 1) from *S. herbacea* [10] and purchased from Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea. HPLC-grade solvent (methanol, soil, and acetonitrile) were acquired from J. T. Baker (Radnor, PA, USA). Acetic acid (99.7%) was acquired from Samchun Pure Chemicals, Pyeongtaek, Korea.

Sample preparation

S. herbacea extraction was carried out under reflux using 30% EtOH for 3 h. The resulting extract was then concentrated and lyophilized. For the preparation of a calibration curve, 1 mg/mL of each flavone was dissolved in 70% acetonitrile (ACN) and serially diluted. The individual peak areas of I3G and Q3G were compared with the standard curves prepared from their corresponding standards. The concentration (X, µg/mL), peak area (Y), and mean values (n =5) of the calibration functions of I3G and Q3G were then calculated.

Preparation of standard solutions and chromatographic conditions

I3G and Q3G stock solutions were prepared by dissolving 1 mg of each standard compound in 70% ACN. The *S. herbacea* flavonoids were analyzed using an INNO C_{18} column (4.6 mm× 250 mm, 5 µm). The mobile phase was comprised of two solvents: 0.5% acetic acid in water (A) and ACN (B). Gradient elution system was composed of 0.5% acetic acid in water (A) and acetonitrile (B). The initial condition started at 83:17 (A:B), 70:30 (A:B) after 10 min and maintained until it reached 25 min. The solvent system was changed to 20:80 (A:B) after 30 min and to 0:100 (A:B) at 35 min, and then maintained until 40 min. Afterwards, the solvent was changed to 83:100 (A:B) until 50 min and maintained for another 5 min. The total analysis time was 55 min. The flow rate of the mobile phase was 1 mL/min, the injection volume was 10 µL, and a UV absorbance of 270 nm was set for detection. The column was maintained at 30 °C.

Linearity, limit of detection (LOD), and limit of quantification (LOQ)

The linearity of the analytical method developed herein was evaluated according to the correlation coefficient (r^2) of the calibration curve of each standard compound using six serial concentrations. The procedure was conducted by dissolving together 1 mg of each standard compound in 1 mL of 70% ACN

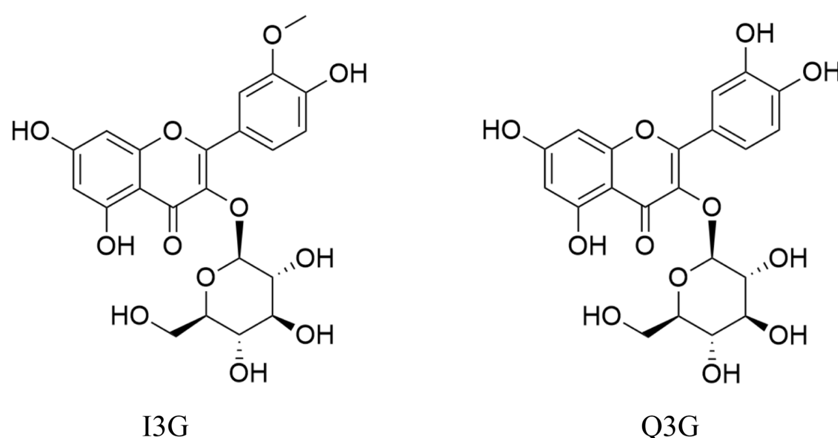

Fig. 1 Chemical structures of I3G and Q3G

and then serially diluting the solution to obtain appropriate concentrations. HPLC analysis was then performed using the developed method. Based on the calibration curve for each standard compound, LOD and LOQ were determined using the values of the standard deviation of the intercept (σ) and the slope (S). LOD is defined as the lowest analyte quantity that renders a measurable signal that is three times greater than the noise level, and LOQ is the lowest analyte amount that can be quantitated with a signal-to-noise ratio of 10. The following equations were used to calculate LOD and LOQ: $LOD = 3.3\ (\sigma/S)$ and $LOQ = 10\ (\sigma/S)$.

Intra- and inter-day precision and accuracy

The intra-day (repeatability) and inter-day (intermediate precision) variability of the developed analytical procedure was assessed to determine its precision. Nine determinations were performed for each assay, covering low, medium, and high concentrations (5.0, 7.5, and 10 g/mL, respectively). The injections were conducted on the same day for the intra-day, and on three different days for the inter-day assay. The relative standard deviation (RSD) for each assay was used to express its precision. Comparing the nominal and measured concentrations of the QC samples under three trials validated the accuracy of the analytical method and was expressed as a percentage of the nominal concentration.

Calibration curves

Different concentrations of I3G and Q3G ranging from 1.9 to 31.5 μg/mL were prepared by serially diluting the standard stock solutions. For each standard compound, the calibration curve was calculated by plotting the peak area (Y) against the concentration (X, mg/mL). Analyte concentrations in the samples were calculated with the calibration equation. All values are reported as their means (n =3) ± standard deviation.

Results and Discussion

Efficient chromatography and high sensitivity were achieved using acetonitrile and a 0.5% acetic acid aqueous solution as the mobile phase with 270 nm as the detection wavelength. Figure 2 depicts the chromatographic separation of I3G and Q3G, indicating the specificity of I3G and Q3G. As demonstrated in Fig. 2, the HPLC method displayed good separation. The retention time of I3G and Q3G was recorded at 12.00 and 10.30 min, respectively. A wavelength of 270 nm was found to be the most effective to detect and measure all impurities and the key components of I3G and Q3G in a single run. No peak was found close to the I3G and Q3G retention times, which highlights the specificity of our proposed method. The chromatogram shown in Fig. 2 depicts an apparent complete separation of I3G and Q3G. This indicates that the HPLC approach was sufficiently specific for the analysis of I3G and Q3G derived from *S. herbacea.*

The quantification parameters for I3G and Q3G were examined using the aforementioned HPLC conditions. Furthermore, linear regression analysis confirmed the linearity of the calibration curves for the isolated compounds established by plotting the peak areas of the prepared concentrations. Linearity was tested using six optimized solutions with concentrations ranging from 1.9 to 31.5 μg/mL (n =3). The regression equation was determined by plotting the peak area (Y) against the I3G and Q3G concentrations (X) expressed in mg/mL. The correlation coefficients (r^2 =0.9996 and r^2 =0.9999, respectively) obtained for the regression line indicated a strong linearity between the peak area (AU) and concentration of I3G and Q3G (Table 1).

The LOD represents the lowest concentration of the analyzed compounds that can be estimated by the HPLC instrument and the analytical method, whereas the LOQ is the lowest concentration of the analyzed compounds that can be quantified by the instrument and the analytical method with acceptable accuracy and precision. In this study, the LOD of I3G and Q3G were 1.49 and 0.8 μg/mL, respectively, and the LOQ were 4.0 and 2.0 μg/mL, respectively, highlighting the good sensitivity of the proposed analytical method for the quantification of both *S. herbacea*-derived compounds (Table 1).

The intra-day and inter-day precision (% RSD) of the chromatographic method was estimated from triplicate experiments conducted within a single day and over a 3-day period, respectively. Table 2 summarizes the coefficient of variation of intra-day and inter-day precision values for I3G and Q3G ranging from 0.93 to 3.19% and 1.75 to 3.85%, respectively (i.e., values were below 5%). These results demonstrated that the proposed analytical method for *S. herbacea*-derived I3G and Q3G quantification had good precision. The analytical method accuracy is determined by how close its results are to the true values. Triplicate analyses were performed on three injections. As shown in Table 3, good recovery rates (92.9-136.6%) were achieved for both I3G and Q3G indicating that the analytical method had good I3G and Q3G quantification accuracy.

Many studies have already suggested that flavonoids such as I3G and Q3G exhibit various biological activities such as anti-microbial, cytotoxicity, and phytotoxicity [13-17]. The anti-inflammatory effect of *S. herbacea* is reported to be due to the presence of I3G. Another study indicated that I3G is a promising drug candidate for cancer therapy because its glycosylation conferred more advantageous pharmacological changes [22]. Q3G also exhibited antidepressant activity and was found to help prevent complications associated with diabetes [23].

In our previous study, we identified I3G and Q3G as marker compounds of *S. herbacea* extract. However, to the best of our knowledge, this is the first study to establish a validated method using HPLC-UV to quantify the mentioned compounds present in *S. herbacea*. The aim of validation was to establish an analytical method suitable for quantitative determination of the compound of interest. As we presented in the results, our HPLC-UV method

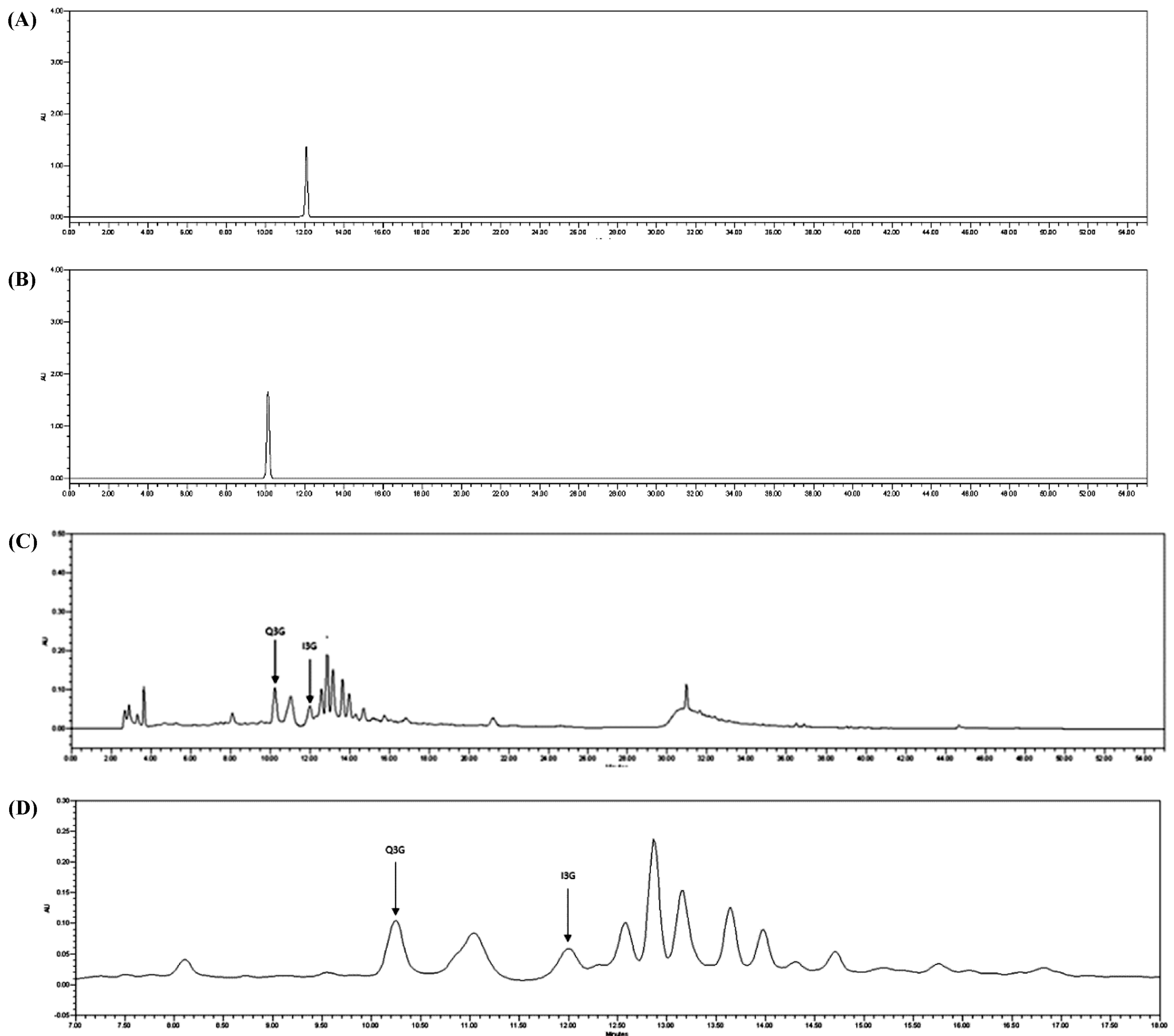

Fig. 2 HPLC chromatograms of I3G (A), Q3G (B), and *S. herbacea* extract (C), and expanded version of *S. herbacea* extract (D) Q3G, quercetin-3-*O*-glucoside; I3G, isorhamnetin-3-*O*-glucoside

Table 1 Linearity, LOD, and LOQ for I3G and Q3G

Compound	t_R	Range (μg/mL)	Calibration equation[a]	$r^{2\ b}$	LOD (μg/mL)	LOQ (μg/mL)
I3G	12.00	31.5-1.9	Y = 16819X + 7.621	0.9996	1.49	4.0
Q3G	10.30	31.5-1.9	Y = 22017X – 5.625	0.9999	0.8	2.0

[a]Y = peak area, X = concentration of standards (mg/mL)
[b]r^2 = correlation coefficient for five calibration data points (n =5)

showed efficiency in the analysis and all values obtained from different parameters used were within an acceptable range to be considered valid.

An HPLC/UV-based method was developed for the simultaneous quantification of I3G and Q3G from *S. herbacea* which can be used in routine analyses, large scale extraction processes, and content determination. The proposed procedure exhibited good accuracy, precision, specificity and quantification parameters. Compared to previous studies in detecting these compounds, our method is more convenient as it uses cheaper solvent system and a simpler chromatographic condition for the quantification of bioactive flavonoids in *S. herbacea.* Moreover, the findings of the

Table 2 Intra- and inter-day precision for the determination of I3G and Q3G

Compound	Spiked concentration (mg/mL)	Intra-day (n =5)		Inter-day (n =5)	
		Measured concentration (mg/g)	RSD (%)	Measured concentration (mg/g)	RSD (%)
I3G	10	0.93	2.150	0.94	3.19
	7.5	1.16	1.724	1.17	2.56
	5.0	1.08	1.851	1.07	0.93
Q3G	10	0.52	3.846	0.52	3.85
	7.5	0.53	1.886	0.53	3.77
	5.0	0.57	1.754	0.57	1.75

Table 3 I3G and Q3G determination accuracy

Compound	Sample	Measured content (mg/g)			Average	Predicted content (mg/g)	Recovery (%)	RSD (%)
		1st	2nd	3rd				
I3G	QC1	0.508	0.508	0.511	0.509	0.378	134.6	1.06
	QC2	0.271	0.261	0.262	0.265	0.194	136.6	1.79
	QC3	0.135	0.135	0.135	0.135	0.101	133.7	0.10
Q3G	QC1	0.449	0.451	0.453	0.453	0.476	95.1	0.45
	QC2	0.235	0.235	0.235	0.234	0.246	95.2	0.19
	QC3	0.115	0.116	0.117	0.116	0.125	92.9	0.53

present study could be applied to the industrialization of marker compounds present in *S. herbacea.*

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