

Validation of an Analytical Methods of 3',4',5-Trihydroxy-3-Methoxy-6,7- Methylenedioxyflavone 4'-Glucuronide for Standardization of *Spinacia oleracea*

Yun Gon Son

Gyeongsang National University

Juyoung Jung

Gyeongsang National University

Dong Kun Lee

Gyeongsang National University

Sang Won Park

Gyeongsang National University

Jeong Yoon Kim

jykim21@gnu.ac.kr

Gyeongsang National University

Hyun Joon Kim

Gyeongsang National University

Research Article

Keywords: Spinach, HPLC method validation, Quantitative analysis, Nutraceuticals, TMG

Posted Date: March 18th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4098773/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Molecules on May 24th, 2024. See the published version at <https://doi.org/10.3390/molecules29112494>.

Abstract

Spinach (*Spinacia oleracea*) is one of the most famous vegetables worldwide, rich in essential metabolites for various health benefits. It is a valuable plant source that has the potential to be a nutraceutical. In the process of developing functional foods, it is crucial to establish a standard compound and quantitative analysis methods to ensure standardization. Thus, the analytical method validation was established using 3',4',5-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide (TMG) as a marker compound. The specificity, accuracy, linearity, precision, repeatability, limit of detection (LOD), and limit of quantification (LOQ) was proved according to validation guideline. The specificity was confirmed from the BPI and UV chromatogram of the standard compound (TMG) as the predominant metabolite in the spinach sample. The calibration curve of TMG (15.625 ~ 500 µg/ml) had reasonable linearity ($R^2 = 0.999$) considering with LOD and LOQ values, respectively. Recovery rate of TMG was 93.31-100.98% for FDS and 90.37–95.04% for SEC. The precision was less than 3 and 6% in the intra-day and inter-day. The spinach samples (FDS and SEC) were validated using TMC as a standard compound to comply with AOAC and KFPA regulations for approving functional ingredients in health functional food.

Introduction

Spinach (*Spinacia oleracea*) is an annual plant belonging to the Amaranthaceae family (Amir et al. 2024). It is widely cultivated as a green leafy versatile vegetable for various cuisines, including fresh ones, soups, sauces, etc (Zhou et al. 2023). Spinach is widely recognized for its nutritional value, containing sugars, organic acids, amino acids, vitamins, minerals, and phytochemicals (Salehi et al. 2019; Waseem et al. 2021; Sharma et al. 2023). Recent studies have highlighted the association between phytochemicals found in spinach and several health benefits (Roberts and Moreau 2016). Spinach has been found to have positive impacts on eye health, as well as anti-inflammatory and antioxidant effects (Metha and Belemkar 2014; Khalid et al. 2017; Thandapani et al. 2023). Moreover, it has also been linked to the prevention and management of chronic diseases such as depression, obesity, and hypolipidemia (Son et al. 2018). With its impressive benefits, spinach has valuable potential for development as a nutraceutical.

The healthy benefits of spinach have been noted to involve its specific phytochemicals, such as carotenoids, flavonoids, terpenoids, polyketides, lignans, saponins, and peptides (Riso et al. 2004; Park et al. 2011; Karasawa et al. 2021). One of the key components responsible for these benefits is flavonoids, a type of phytochemical in spinach (Bergquist et al. 2005; Tao et al. 2023). The types of spinach flavonoids have been known as patuletin derivatives, spinacetin derivatives, spinach flavonoids glucuronide derivatives, and other basic skeleton flavonoids (Mi et al. 2008; Singh et al. 2018; Yuk et al. 2019). One of the patuletin derivatives, patuletin-3-O-(2"-coumaroylglucosyl)-(1→6)-[apiosyl-(1→2)]-β-D-glucopyranoside, has been reported to inhibit the formation of advanced glycation end products (AGEs) and aldose reductase activity (RLAR) (Perez Gutierrez and Velazquez 2020). 3',4',5-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide (TMG) is a specific flavonoid glucuronide derivative found in

spinach leaves (Aritomi and Kawasaki 1984; Ferreres et al. 1997; Koh et al. 2012). TMG has inhibitory activity on granulation in basophilic leukemia RBL-2G3 cells (Yuta Morishita 2015). The results indicate that spinach flavonoids have therapeutic potential and specific skeletons for standard compounds of spinach.

Establishing and verifying standard compounds in bioresources play a crucial role in the development of nutraceuticals (Dillard and Bruce German). These compounds serve as indicators or reference points for quality control and standardization of nutraceutical products (Morris; 2001). The selection of standard compounds is typically based on their presence in the botanical source or their known bioactivity (Moreno et al. 2006; Devi et al. 2024). For the validation, it is imperative to ensure their safety, efficacy, and quality according to Association of Official Analytical Collaboration (AOAC) international guidelines (2023).

In this study, 3',4',5-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide (TMG) was identified as a standard compound to develop nutraceuticals of spinach extract using UPLC-Q-TOF/MS analysis. In addition, the effectiveness of the analytical method validation was verified by confirming specificity, precision, repeatability, accuracy, LOD, LOQ, and recovery rate according to the AOAC international guideline using HPLC.

Materials and Methods

Plant materials

The spinach was cultivated at the local farm in Goseong-gun, Gyeongsangnam-do, Republic of Korea. The seed of spinach was sown around September to October 2022. The aerial parts were harvested between November 2022 and March 2023 when 60 days after sowing to use for experiments. The harvested spinach was washed to remove the contaminants and then dried using a freeze dryer (30 l, IlshinBioBase Ltd., Gyeonggi-do, Republic of Korea) for 48 hours. The freeze-dried spinach (500 kg) was extracted with 70% ethanol to be six times the volume of the plant material. The filtered crude spinach extract was concentrated using an evaporator and dried using freeze-drying to obtain a powder type at a company equipped for extraction facilities (Kuan Industrial Co., Ltd. Factory, Geumsan-gun, Chungcheongnam-do, Republic of Korea). In this experiment, freeze-dried spinach (FDS) and spinach extract concentrate (SEC) were used for analysis.

LC-Q-TOF/MS analysis

The metabolites from the spinach samples were annotated using liquid chromatography quadrupole time of flight mass (LC-Q-TOF/MS, AB SCIEX X500R, Framingham, MA, USA). To prepare the analytical sample, 1 g of freeze-dried spinach was sonicated with 50 ml of distilled water for 1 hour. The spinach extract (1 µl) was injected to the column (Poroshell 120 EC C18, 2.7 µm, 2.1 × 100 mm, Agilent Technologies, Santa Clara, CA, USA). The eluent solvents comprised water (A) and acetonitrile containing 0.1% acetic acid (B). The gradient conditions were conducted for 40 min: 0 min, 0% B; 30 min, 50% B; 40

min, 100% B. The MS ionization was positive mode using electrospray ionization (ESI) with 5500 V of ionspray voltage, 450°C of ion source temperature. The gas condition was kept at 30 psi of curtain gas pressure, 50 psi nebulizer gas pressure and 50 psi of heating gas pressure with 50 V decluttering potential. The MS scan range was 50-1000 m/z with a collision energy ramp of 10–30 eV.

Method validation

The standard compound (TMG) of spinach was validated using a high-performance liquid chromatography-diode array detector (HPLC-DAD) following the guidelines of the Korea Food and Drug Administration (KFDA). The validation method comprised of five criteria including linearity, specificity, accuracy, precision with repeatability and reproducibility, and limit of detection and quantitation (LOD and LOQ). According to these guidelines, verification of the standard compound (TMG) analysis method was performed, along with quantification of TMG in the analysis sample (FDS and SEC). The experiments were validated through appropriate repetitions of each procedure.

Standard solution preparation using TMG

3',4',5-Trihydroxy-3-methoxy-6,7-methylene-dioxyflavone 4'-glucuronide (TMG) as the standard compound of spinach sample was purchased from Molport, Inc. (Beacon, NY, USA). 10 mg of TMG was weighed and dissolved in 10 ml of methanol to make a stock solution. A TMG standard solution was prepared by appropriately diluting the above standard stock solution with methanol to use for validation methods.

Sample solution preparation using FDS and SEC

The 0.5, 1.0, and 1.5 g of each sample (FDS and SEC) were extracted with 20 ml of 80% ethanol using a sonicator for 90 min to prepare the samples with the concentration at 25, 50, and 75 mg/ml. Each solution was filtered through 0.2 μm membrane filter (hydrophilic unit, Advantec MFD, Inc., Dublin, CA, USA) to use in accordance with the validation methods.

HPLC-DAD analysis

The method validation was mainly performed using Agilent Technology Infinity 1260 HPLC equipped with autosampler, binary pump, diode array detector (DAD). The standard and sample solution (injection volume: 10 μl) was chromatographed on the column (XBridge C18, 4.6 $\times$ 150 mm, 5 μm , Waters Corporation, Milford, MA, USA). The elution solvents A and B were 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile. The gradient mode of elution was set as follows: 0–5 min, 90% A; 5–13 min, 85% A; 13–40 min, 70% A; 40–50 min, 50% A; 50–60 min, 0% A. The UV wavelength was 350 nm, which was selected due to the relatively high intensity of TMG.

Specificity

The specificity of the HPLC analysis was determined by comparing the results of the standard solution (TMG, 62.5 $\mu\text{g/ml}$) and sample solution (FDS and SEC, 50 mg/ml). The TMG peak was confirmed to be completely separated from other peaks present in the chromatogram of spinach samples based on its

retention time and UV spectrum. The specificity of the standard and sample solutions was confirmed through triplicate analysis.

Linearity

The TMG stock solution (1.0 mg/ml) was diluted to prepare six concentrations: 15.625, 31.25, 62.5, 125, 250, and 500 µg/ml. Methanol was used as a dilution solvent for the blank. All the diluted TMG solution was measured using HPLC. The peak area of each TMG concentration was derived from the HPLC chromatogram to obtain the calibration curves. The coefficient of determination (R^2) was confirmed for calibration curves. The linearity of the results was demonstrated by performing triplicate measurements.

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

A trend line was generated from the TMG standard curve for linearity verification, which displayed the correlation between TMG concentrations (0, 15.625, 31.25, 62.5, 125, 250, and 500 µg/ml) and peak area. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the average (S) of the slope of the trend line and the standard deviation (σ) of the y-intercept of the trend line. The equation for the calculation was as follows: $LOD = 3.3 \times \sigma/S$, $LOQ = 10 \times \sigma/S$.

Accuracy

The FDS and SEC (each 0.5 g) were extracted with 10 ml of 80% ethanol using a sonicator for 90 min. Subsequently, 100 µl of the blank (80% ethanol) or TMG solution corresponding to 50%, 100%, and 150% was mixed with 900 µl of each FDS and SEC extract. The blank content for FDS and SEC were 17.97 and 41.75 µg/ml respectively. To achieve concentrations of 50%, 100%, and 150%, 8.98, 17.97, and 26.95 µg/ml of TMG samples were added to the FDS, while 20.88, 41.75, and 62.63 µg/ml were added to the SEC. The accuracy was proved by triplicates.

Precision

Intraday precision (repeatability)

The accurately weighing 0.5, 1.0, and 1.5 g of FDS and SEC were sonicated with 20 ml of 80% ethanol for 90 min to obtain the sample solutions. The TMG contents were measured in each sample solution using HPLC analysis. All the units of contents were converted to µg/g according to the dilution factor. Intraday precision was performed by measuring the TMG contents per gram of FDS and SEC. The experiment was conducted five times in total to confirm the repeatability.

Interday precision (intermediate precision)

The accurately weighing 0.5 and 1.5 g of FDS and SEC were sonicated with 20 ml of 80% ethanol for 90 min to obtain the sample solutions. The measurement of TMG contents and unit conversion in sample solution were conducted in the same manner as the intraday precision methods. Interday precision was measured five times per day for a total of five days to confirm the intermediate precision.

Results and Discussions

Metabolite identification of spinach extract using LC-Q-TOF/MS

The identification of metabolites was conducted using LC-Q-TOF/MS analysis with positive mode. The predominant six peaks were annotated from the base peak intensity (BPI) gram of FDS extract (Fig. 1A). Additionally, Fig. 1B displayed a UV chromatogram at 350 nm, which corresponded to the high sensitivity of the six target metabolites selectively. The six peaks were separated with sufficient sensitivity, retention time (t_R), and resolution. The mass spectra for the six peaks were compared with previous data (Koh et al. 2012; Yuk et al. 2019).

Peak 1 (t_R = 11.61 min, Fig. 1C) was observed as the molecular ion peak at m/z 803.2242 and two fragment ion peaks at m/z 671.1843 and 347.0771. The fragment ion at m/z 671.1843 ($[M + H]^+ - 132$ amu) was derived from its apioside moiety cleavage. Furthermore, the two glucose moieties was fragmented to obtain the ion peak at m/z 347.0771 ($[M + H]^+ - 324$ amu). Based on this typical fragment pattern, peak 1 was identified as spinacetin 3-O-glucosyl-(1→6)-[apiosyl(1→2)]-glucoside. Peak 2 (t_R = 12.72 min, Fig. 1D) exhibited the molecular ion peak at m/z 671.1818 ($[M + H]^+$) with the fragment ion observed at m/z 347.0769 ($[M + H]^+ - 324$ amu) corresponded to the loss of two glucose moieties. Based on these mass data, it was tentatively assigned as spinacetin 3-O-glucosyl-(1→6)-glucoside. Peaks 3–6 only showed the molecular ions, which were compared to the theoretical value to presume the metabolite with a reasonable error value (Table 1). Peak 3 (t_R = 14.96 min, Fig. 1E) was assigned as spinatoside, which had the molecular ion peak at m/z 523.1066 with – 4.18 ppm of error value by comparing the theoretical mass at m/z 523.1087. Peak 4 (t_R = 15.87 min, Fig. 1F) displayed the molecular ion peak at m/z 537.1240, corresponding to the theoretical mass of m/z 537.1240 with an error value of -0.81 ppm to identify jaceidin 4'-glucuronide. Peak 5 (t_R = 17.69 min, Fig. 1G) was confirmed as -1.99 ppm of an error value. In accordance, it was annotated as 3',4',5-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide with the chemical formula C₂₃H₂₀O₁₄. Peak 6 (t_R = 18.62 min, Fig. 1H) was observed as a molecular ion peak at m/z 535.1068 with – 5.2 ppm of an error ppm. It was in agreement with 5,4'-dihydroxy-3,3'-dimethoxy-6,7-methylendioxyflavone-4'-glucuronide. Thus, the mass spectral data were elucidated that the six compounds were flavonoid derivatives as spinacetin 3-O-glucosyl-(1→6)-[apiosyl(1→2)]-glucoside (1), spinacetin-3-O-glucosyl-(1→6)-glucoside (2), spinatoside (3), jaceidin 4'-glucuronide (4), 3',4',5-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide (5), and 5,4'-dihydroxy-3,3'-dimethoxy-6,7-methylendioxyflavone-4'-glucuronide (6) through positive ion mode by comparison with relevant references.

Table 1
Characterization of flavonoid derivatives from *S. oleracea* extracts using LC-TOF/MS

Peaks	t_R (min)	Observed ion (m/z)	Theoretical ion (m/z)	Error (ppm)	Formula	Identification
1	11.6	803.2242	803.2246	-0.50	$C_{34}H_{42}O_{22}$	Spinacetin 3-O-glucosyl-(1→6)-[apiosyl(1→2)]-glucoside
2	12.7	671.1818	671.1823	-0.81	$C_{29}H_{34}O_{18}$	Spinacetin-3-O-glucosyl-(1→6)-glucoside
3	15.0	523.1066	523.1087	-4.81	$C_{23}H_{22}O_{14}$	Spinatoside
4	15.9	537.1240	537.1244	-0.81	$C_{24}H_{24}O_{14}$	Jaceidin 4'-glucuronide
5	17.7	521.0921	521.0931	-1.99	$C_{23}H_{20}O_{14}$	3',4',5-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide
6	19.6	535.1060	535.1088	-5.20	$C_{24}H_{22}O_{14}$	5,4'-Dihydroxy-3,3'-dimethoxy-6,7-methylenedioxyflavone-4'-glucuronide

Establishment of TMG as a standard compound of spinach

Based on LC-Q-TOF/MS analysis, it was confirmed that spinach contained a high level of six flavonoid derivatives. Among them, TMG was selected as a standard compound for establishing the quantitative validation method of spinach. TMG was not only commercially available, but also the most sensitivity in UV chromatogram with excellent specificity compared to other compounds.

Development HPLC validation method for spinach sample using standard compound

Specificity

HPLC analysis was conducted at a wavelength of 350 nm, where the identified six flavonoid derivatives were selectively detected with high levels. As shown in Fig. 2B and 2C, HPLC chromatogram of the sample solution (FDS and SEC) confirmed that the metabolites were properly separated. Figure 2A showed that the retention time ($t_R = 31.459$ min) of TMG through established HPLC methods.

Furthermore, the location of TMG in the sample solution (FDS and SEC) was accurately characterized to detect without the influence of interfering substances. Therefore, these findings supported specificity of TMG as a standard compound through the verified HPLC methods.

Linearity

TMG standard solutions ranging from 15.625, 31.25, 62.5, 125, 250, and 500 $\mu\text{g/ml}$ were prepared and analyzed under the established HPLC conditions to obtain peak areas at each concentration. A standard curve was created using TMG concentration on the x-axis and peak area on the y-axis. The TMG-trend

line was drawn using experimental values for the standard curves with more than three repetitions. All three lines had 0.999 of the coefficient values (R^2), representing a significant linearity.

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

LOD and LOQ were determined using the TMG (Total Measurement Uncertainty) trend line of the standard curves that were also used for linear verification. To calculate the LOD and LOQ, we obtained the slope (S) and intercept (σ) values through the regression equation derived from the trend line. The results of three experiments showed the mean slope (S) at 0.1464 and the standard deviation of the intercept (σ) at 0.0074. Using these values in the properly equation, LOD and LOQ of TMG were calculated to be 0.167 $\mu\text{g/ml}$ and 0.505 $\mu\text{g/ml}$, respectively (Table 2).

Table 2
The calibration curve and the responsible variables (equation, coefficient value, LOD, and LOQ) of 3',4',5-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide (TMG) by HPLC analysis

Repetition #	Range ($\mu\text{g/ml}$)	Regression equation	R^2
1	15.625-500	$y = 34.065x - 33.662$	0.999
2	15.625-500	$y = 33.582x - 15.960$	0.999
3	15.625-500	$y = 33.878x - 71.495$	0.999
Mean of slopes (S)	33.842	Standard deviation of intercept (σ)	23.163
LOD ($3.3 \times \sigma/S$)	2.259 $\mu\text{g/ml}$		
LOQ ($10 \times \sigma/S$)	6.845 $\mu\text{g/ml}$		

Accuracy

The recovery rate was confirmed by adding TMG (standard compound) corresponding to the concentrations at 50%, 100%, and 150% to TMG-containing sample solutions (FDS and SEC). In the FDS sample, the concentration of TMG was 17.97 $\mu\text{g/ml}$. Thus, TMG was added to the FDS sample with 8.98, 17.97, and 26.95 $\mu\text{g/ml}$ corresponding to 50%, 100%, and 150% of this content, respectively. The average recovery rate for TMG in FDS samples was 96.81%, with a range of 93.31–100.98% (Table 3). The TMG concentration in the SEC sample solution was 41.75 $\mu\text{g/ml}$. To achieve 50%, 100%, and 150% of the TMG content, the SEC sample was supplemented with 20.88, 41.75, and 62.63 $\mu\text{g/ml}$ of TMG respectively. As a result, the average and range of recovery rate exhibited 92.52% and 90.37 ~ 95.04%, respectively (Table 4). The accuracy of the recovery test complied with the standard ranges of 90–108% at a concentration of 0.1% (1 mg/g) according to AOAC international accuracy and KFDA guidelines for functional ingredient approval in health functional foods (2023; Taverniers et al. 2004).

Table 3
Accuracy (recovery rate) results of 3',4',5-trihydroxy-3-methoxy-6,7-methylene-dioxyflavone 4'-glucuronide (TMG) contents in freeze-dried spinach (FDS) solutions

Repetition #	Spiked amounts of TMG		
	8.98 µg/ml	17.97 µg/ml	26.95 µg/ml
1	93.41	100.98	95.60
2	95.10	100.52	95.29
3	93.31	100.75	96.34
Mean recovery rate (%)	93.94	100.75	95.74
Net recovery rate (%)	96.81		
Range of recovery rate (%)	93.31-100.98		

Table 4
Accuracy (recovery rate) results of 3',4',5-trihydroxy-3-methoxy-6,7-methylene-dioxyflavone 4'-glucuronide (TMG) contents in spinach extract concentrate (SEC) solutions

Repetition #	Spiked amounts of TMG		
	20.88 µg/ml	41.75 µg/ml	62.63 µg/ml
1	91.48	91.78	94.98
2	91.62	90.42	95.04
3	91.99	90.37	95.03
Mean recovery rate (%)	91.70	90.86	95.01
Net recovery rate (%)	92.52		
Range of recovery rate (%)	90.37–95.04		

Precision

Intraday precision (repeatability)

Repeated measurements ($n = 5$) were taken with 0.5, 1.0, and 1.5 g of FDS and SEC to confirm repeatability with respect to the sample amount. For intraday precision for FDS, 0.5, 1.0, and 1.5 g had a mean of TMG contents with 1042.52, 995.56, and 1090.07 µg/g for 5 times repeated experiments. As shown in Table 5, the relative standard deviation (RSD, %) was deviated as 1.87, 2.15, and 0.32 at each FDS amount, respectively. In intraday precision test for SEC, the mean TMG contents for 0.5 g, 1.0 g, and 1.5 g were 2575.84, 2465.55, and 2475.00 µg/g, respectively, based on five repeated experiments. Table 6 showed that the RSD (%) varied 1.26, 0.91, and 1.74 for 0.5, 1.0, and 1.5 g of SEC amounts.

Intraday precision confirmed the appropriate RSD level (< 3%) at the concentration of 0.1% (1 mg/g) based on AOAC international and KFDA guidelines (Choi et al. 2022; 2023).

Table 5
The intra-day precision (repeatability) results of 3',4',5-trihydroxy-3-methoxy-6,7-methylene-dioxyflavone 4'-glucuronide (TMG) contents in freeze-dried spinach (FDS) solution (*n* = 5)

	Amount of FDS		
	0.5 g	1.0 g	1.5 g
Repetition #	TMG contents (µg/g)	TMG contents (µg/g)	TMG contents (µg/g)
1	1025.43	1018.94	1089.93
2	1030.19	993.78	1095.79
3	1027.51	1008.37	1088.94
4	1053.72	1000.63	1090.62
5	1075.75	956.11	1085.07
Mean	1042.52	995.56	1090.07
SD	19.49	21.43	3.44
RSD (%)	1.87	2.15	0.32

Table 6
The intra-day precision (repeatability) results of 3',4',5-trihydroxy-3-methoxy-6,7-methylene-dioxyflavone 4'-glucuronide (TMG) contents in spinach extract concentrate (SEC) solution (*n* = 5)

	Amount of SEC		
	0.5 g	1.0 g	1.5 g
Repetition #	TMG contents (µg/g)	TMG contents (µg/g)	TMG contents (µg/g)
1	2599.19	2504.85	2455.34
2	2587.28	2464.36	2549.44
3	2531.00	2458.55	2418.82
4	2545.29	2465.10	2485.12
5	2616.46	2434.88	2466.26
Mean	2575.84	2465.55	2475.00
SD	32.47	22.52	43.04
RSD (%)	1.26	0.91	1.74

Interday precision (intermediate precision)

To ensure intraday precision, the repeated experiments were conducted five times a day for five consecutive days. The mean of TMG content exhibited 1110.95 and 1091.38 $\mu\text{g/g}$ in 0.5 g and 1.5 g of FDS, respectively. The RSD for both weights was 0.72% and 0.61%, respectively. The Net RSD was approximately 1.11% (Table 7). The mean of TMG content in 0.5 g of SEC was 2603.24 $\mu\text{g/g}$, while in 1.5 g of SEC it was 2587.33 $\mu\text{g/g}$. The RSD for both weights was 1.44% and 1.91% respectively. The Net RSD for both weights was approximately 1.71% (Table 8). According to the guidelines, intermediate precision was achieved as the Net RSD was less than 6% based on a concentration of 0.1% (1 mg/g).

Table 7

The intra-day precision (intermediate precision) results of 3',4',5-trihydroxy-3-methoxy-6,7-methylene-dioxyflavone 4'-glucuronide (TMG) contents in freeze-dried spinach (FDS) solution ($n = 50$)

Amount of FDS				
0.5 g			1.5 g	
Day	Repetition #	TMG contents ($\mu\text{g/g}$)	Repetition #	TMG contents ($\mu\text{g/g}$)
1	1	1092.13	1	1087.15
	2	1104.34	2	1086.06
	3	1107.91	3	1088.74
	4	1108.81	4	1091.02
	5	1110.89	5	1092.81
2	1	1123.99	1	1064.82
	2	1128.46	2	1101.54
	3	1106.43	3	1089.93
	4	1117.15	4	1092.51
	5	1105.53	5	1094.10
3	1	1112.08	1	1093.40
	2	1110.59	2	1093.01
	3	1106.43	3	1096.28
	4	1106.43	4	1093.11
	5	1107.62	5	1094.79
4	1	1121.31	1	1095.79
	2	1121.02	2	1096.88
	3	1119.53	3	1095.88
	4	1117.15	4	1097.37
	5	1117.74	5	1096.88
5	1	1109.40	1	1089.93
	2	1103.15	2	1087.75
	3	1102.85	3	1088.94
	4	1109.40	4	1090.62

Amount of FDS			
0.5 g		1.5 g	
5	1103.45	5	1085.07
Mean	1110.95	Mean	1091.38
SD	7.96	SD	6.69
RSD (%)	0.72	RSD (%)	0.61
Net RSD (%) = 1.11			

Table 8

The intra-day precision (intermediate precision) results of 3',4',5-trihydroxy-3-methoxy-6,7-methylene-dioxyflavone 4'-glucuronide (TMG) contents in spinach extract concentrate (SEC) solution ($n = 50$)

Amount of SEC				
0.5 g			1.5 g	
Day	Repetition #	TMG contents ($\mu\text{g/g}$)	Repetition #	TMG contents ($\mu\text{g/g}$)
1	1	2537.85	1	2514.20
	2	2532.49	2	2516.88
	3	2532.49	3	2521.65
	4	2539.04	4	2570.98
	5	2544.10	5	2535.84
2	1	2588.17	1	2505.57
	2	2602.47	2	2550.04
	3	2587.87	3	2553.71
	4	2573.58	4	2555.60
	5	2601.87	5	2558.87
3	1	2618.25	1	2555.79
	2	2623.01	2	2578.72
	3	2637.60	3	2555.10
	4	2631.05	4	2623.19
	5	2625.39	5	2584.88
4	1	2634.92	1	2631.23
	2	2617.95	2	2632.22
	3	2613.19	3	2632.72
	4	2628.67	4	2636.09
	5	2621.82	5	2636.09
5	1	2648.03	1	2640.96
	2	2645.94	2	2633.32
	3	2627.48	3	2648.60
	4	2637.31	4	2654.95

Amount of SEC			
0.5 g		1.5 g	
5	2630.46	5	2656.05
Mean	2603.24	Mean	2587.33
SD	37.39	SD	49.30
RSD (%)	1.44	RSD (%)	1.91
Net RSD (%) = 1.71			

Conclusions

Six flavonoid glycosides (**1–6**) were identified from spinach extract using LC-Q-TOF/MS. Two sample types were prepared for effective nutraceutical development: freeze-dried spinach (FDS) and spinach extract concentrate (SEC). TMG with high specificity was selected as a standard compound of spinach samples to validate the HPLC analytical method according to AOAC international guidelines for linearity, LOD, LOQ, accuracy, and precision. The linearity of the TMG calibration curve from 15.625 to 500 µg/ml was verified with a high coefficient factor of 0.999. LOD and LOQ values were determined to be 0.167 and 0.505 µg/ml. The accuracy of the validation method was also confirmed to be in an average recovery rate range of 96.81% for FDS and 92.52% for SEC. The precision was also evaluated as appropriate for both intraday and interday RSD levels. The repeatability of the analysis was within the acceptable range of 0.32–2.15% for FDS and 0.91–1.74% for SEC, based on the amount at 0.5, 1.0, and 1.5 g. Additionally, the intermediate precision was determined to be highly reliable, with a Net RSD of 1.11% for FDS and 1.71% for SEC. These results showed that the method validation using TMG as a standard compound for spinach was reliable by proven according to the AOAC international guidelines.

Declarations

CRedit authorship contribution statement

Yun Gon Son: Conceptualization, Formal analysis, Investigation; **Juyoung Jung:** Resources, Investigation; **Dong Kun Lee and Sang Won Park:** Writing-Review & Editing, Resources; **Jeong Yoon Kim:** Writing-Original Draft, supervision; **Hyun Joon Kim:** Project administration, Funding acquisition.

Conflict of Interest

The authors declare no conflict of interest.

Funding

This research was supported by the National Research Foundation of Korea (NRF) funded by Ministry of Science and ICT (MSIT) (NRF-2022R1A2C1005296 and NRF-2021R1A5A8029490), and the Commercializations Promotion Agency for R&D Outcomes (COMPA) (Grant number 1711173796) funded by the MSIT.

Author Contribution

Y.G. Son: Conceptualization, Formal analysis, Investigation; J. Jung: Resources, Investigation; D.K. Lee and S.W. Park: Writing-Review & Editing, Resources; J.Y. Kim: Writing-Original Draft, supervision; H.J. Kim: Project administration, Funding acquisition.

References

1. Amir M, Raheem A, Kumar A et al (2024) Role of phytofabricated gold nanoparticles for enhancing sustainable *Spinacia oleracea* L. production. *South Afr J Bot* 166:386–397. <https://doi.org/10.1016/J.SAJB.2024.01.028>
2. Aritomi M, Kawasaki T (1984) Three highly oxygenated flavone glucuronides in leaves of *Spinacia oleracea*. *Phytochemistry* 23:2043–2047. [https://doi.org/10.1016/S0031-9422\(00\)84967-5](https://doi.org/10.1016/S0031-9422(00)84967-5)
3. Bergquist SÅ, Gertsson UE, Knuthsen P, Olsson ME (2005) Flavonoids in baby spinach (*Spinacia oleracea* L.): Changes during plant growth and storage. *J Agric Food Chem* 53:9459–9464. <https://doi.org/10.1021/JF051430H/ASSET./IMAGES/LARGE/JF051430HF00002.JPEG>
4. Choi S, Il, Han X, Lee SJ et al (2022) Validation of an Analytical Method for the Determination of Thiabendazole in Various Food Matrices. *Sep* 2022, Vol 9, Page 135 9:135. <https://doi.org/10.3390/SEPARATIONS9060135>
5. Devi PV, Islam J, Narzary P et al (2024) Bioactive compounds, nutraceutical values and its application in food product development of oyster mushroom. *J Futur Foods* 4:335–342. <https://doi.org/10.1016/J.JFUTFO.2023.11.005>
6. Dillard CJ, Bruce German J Review Phytochemicals: nutraceuticals and human health. <https://doi.org/10.1002/1097-0010>
7. Ferreres F, Castañer M, Tomás-Barberán FA (1997) Acylated flavonol glycosides from spinach leaves (*Spinacia oleracea*). *Phytochemistry* 45:1701–1705. [https://doi.org/10.1016/S0031-9422\(97\)00244-6](https://doi.org/10.1016/S0031-9422(97)00244-6)
8. Karasawa Y, Miyano K, Fujii H et al (2021) In Vitro Analyses of Spinach-Derived Opioid Peptides, Rubiscolins: Receptor Selectivity and Intracellular Activities through G Protein- and β -Arrestin-Mediated Pathways. *Mol* 2021, Vol 26, Page 6079 26:6079. <https://doi.org/10.3390/MOLECULES26196079>
9. Khalid M, Hassani D, Bilal M et al (2017) Influence of bio-fertilizer containing beneficial fungi and rhizospheric bacteria on health promoting compounds and antioxidant activity of *Spinacia oleracea* L. *Bot Stud* 58:35. <https://doi.org/10.1186/S40529-017-0189-3>

10. Koh E, Charoenprasert S, Mitchell AE (2012) Effect of organic and conventional cropping systems on ascorbic acid, vitamin C, flavonoids, nitrate, and oxalate in 27 varieties of spinach (*Spinacia oleracea* L). *J Agric Food Chem* 60:3144–3150. https://doi.org/10.1021/JF300051F/ASSET/IMAGES/LARGE/JF-2012-00051F_0005.JPEG
11. Metha D, Belemkar S (2014) PHARMACOLOGICAL ACTIVITY OF *SPINACIA OLERACEA* LINN.- A COMPLETE OVERVIEW. *Asian J Pharm Res Dev* 2:83–93
12. Mi JC, Howard LR, Prior RL, Morelock T (2008) Flavonoid content and antioxidant capacity of spinach genotypes determined by high-performance liquid chromatography/mass spectrometry. *J Sci Food Agric* 88:1099–1106. <https://doi.org/10.1002/JSFA.3206>
13. Moreno DA, Carvajal M, López-Berenguer C, García-Viguera C (2006) Chemical and biological characterisation of nutraceutical compounds of broccoli. *J Pharm Biomed Anal* 41:1508–1522. <https://doi.org/10.1016/J.JPBA.2006.04.003>
14. Morris RCT (2001) Striving to Validate Nutraceuticals. 19
15. Park HJ, Cho HJ, Choi SA et al (2011) Dietary Spinach saponin-enriched lipophilic fraction inhibits platelet aggregation and blood coagulation. *J Med Food* 14:784–791. <https://doi.org/10.1089/JMF.2010.1411/ASSET/IMAGES/LARGE/FIGURE4.JPEG>
16. Perez Gutierrez RM, Velazquez EG (2020) Glucopyranoside flavonoids isolated from leaves of *Spinacia oleracea* (spinach) inhibit the formation of advanced glycation end products (AGEs) and aldose reductase activity (RLAR). *Biomed Pharmacother* 128:110299. <https://doi.org/10.1016/J.BIOPHA.2020.110299>
17. Riso P, Brusamolino A, Scalfi L, Porrini M (2004) Bioavailability of carotenoids from spinach and tomatoes. *Nutr Metab Cardiovasc Dis* 14:150–156. [https://doi.org/10.1016/S0939-4753\(04\)80035-8](https://doi.org/10.1016/S0939-4753(04)80035-8)
18. Roberts JL, Moreau R (2016) Functional properties of spinach (*Spinacia oleracea* L.) phytochemicals and bioactives. *Food Funct* 7:3337–3353. <https://doi.org/10.1039/C6FO00051G>
19. Salehi B, Tumer TB, Ozleyen A et al (2019) Plants of the genus *Spinacia*: From bioactive molecules to food and phytopharmacological applications. *Trends Food Sci Technol* 88:260–273. <https://doi.org/10.1016/J.TIFS.2019.03.028>
20. Sharma A, Kumar S, Singh R (2023) Formulation of Zinc oxide/Gum acacia nanocomposite as a novel slow-release fertilizer for enhancing Zn uptake and growth performance of *Spinacia oleracea* L. *Plant Physiol Biochem* 201:107884. <https://doi.org/10.1016/J.PLAPHY.2023.107884>
21. Singh J, Jayaprakasha GK, Patil BS (2018) An optimized solvent extraction and characterization of unidentified flavonoid glucuronide derivatives from spinach by UHPLC-HR-QTOF-MS. *Talanta* 188:763–771. <https://doi.org/10.1016/J.TALANTA.2018.06.025>
22. Son H, Jung S, Shin JH et al (2018) Anti-Stress and Anti-Depressive Effects of Spinach Extracts on a Chronic Stress-Induced Depression Mouse Model through Lowering Blood Corticosterone and Increasing Brain Glutamate and Glutamine Levels. *J Clin Med* 2018 7:406. <https://doi.org/10.3390/JCM7110406>

23. Tao H, Zhao Y, Li L et al (2023) Comparative metabolomics of flavonoids in twenty vegetables reveal their nutritional diversity and potential health benefits. *Food Res Int* 164:112384. <https://doi.org/10.1016/J.FOODRES.2022.112384>
24. Taverniers I, De Loose M, Van Bockstaele E (2004) Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. *TrAC Trends Anal Chem* 23:535–552. <https://doi.org/10.1016/J.TRAC.2004.04.001>
25. Thandapani G, Arthi K, Pazhanisamy P et al (2023) Green synthesis of copper oxide nanoparticles using *Spinacia oleracea* leaf extract and evaluation of biological applications: Antioxidant, antibacterial, larvicidal and biosafety assay. *Mater Today Commun* 34:105248. <https://doi.org/10.1016/J.MTCOMM.2022.105248>
26. Waseem M, Akhtar S, Manzoor MF et al (2021) Nutritional characterization and food value addition properties of dehydrated spinach powder. *Food Sci Nutr* 9:1213–1221. <https://doi.org/10.1002/FSN3.2110>
27. Yuk HJ, Ryu HW, Kim DY et al (2019) Comparison of flavonoid and policosanol profiles in Korean winter-spinach (*Spinacia oleracea* L.) cultivated in different regions. *Food Chem* 279:202–208. <https://doi.org/10.1016/J.FOODCHEM.2018.11.143>
28. Yuta Morishita ESTFYKS (2015) Flavonoid glucuronides isolated from spinach inhibit IgE- mediated degranulation in basophilic leukemia RBL-2H3 cells and passive cutaneous anaphylaxis reaction in mice
29. Zhou Q, Liang W, Wan J, Wang M (2023) Spinach (*Spinacia oleracea*) microgreen prevents the formation of advanced glycation end products in model systems and breads. *Curr Res Food Sci* 6:100490. <https://doi.org/10.1016/J.CRFS.2023.100490> (2023) Guidelines for Dietary Supplements and Botanicals. *Off Methods Anal AOAC Int*. <https://doi.org/10.1093/9780197610145.005.011>

Figures

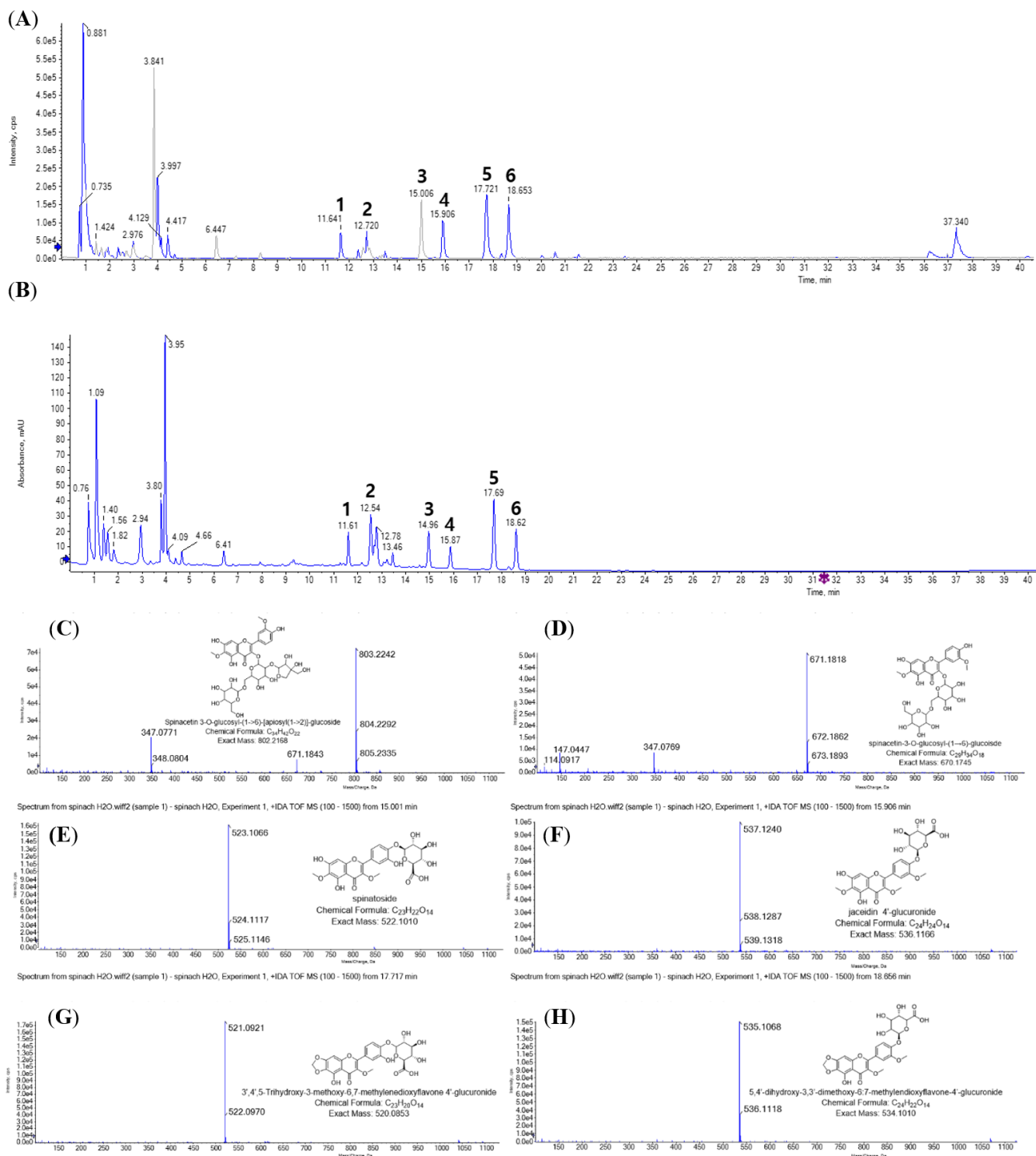


Figure 1

LC-Q-TOF/MS analysis of 80% ethanol extract of freeze-dried spinach (FDS). (A) BPI and (B) UV chromatogram of FDS 80% ethanol extract. Individual Mass gram of (C) peak 1, spinacetin 3-O-glucosyl-(1→6)-[apiosyl(1→2)]-glucoside; (D) peak 2, spinacetin-3-O-glucosyl-(1→6)-glucoside; (E) peak 3, spinatoside; (F) peak 4, jaceidin 4'-glucuronide; (G) peak 5, 3',4',5-trihydroxy-3-methoxy-6,7-

methylenedioxyflavone 4'-glucuronide; (H) peak 6, 5,4'-dihydroxy-3,3'-dimethoxy-6,7-methylenedioxyflavone-4'-glucuronide.

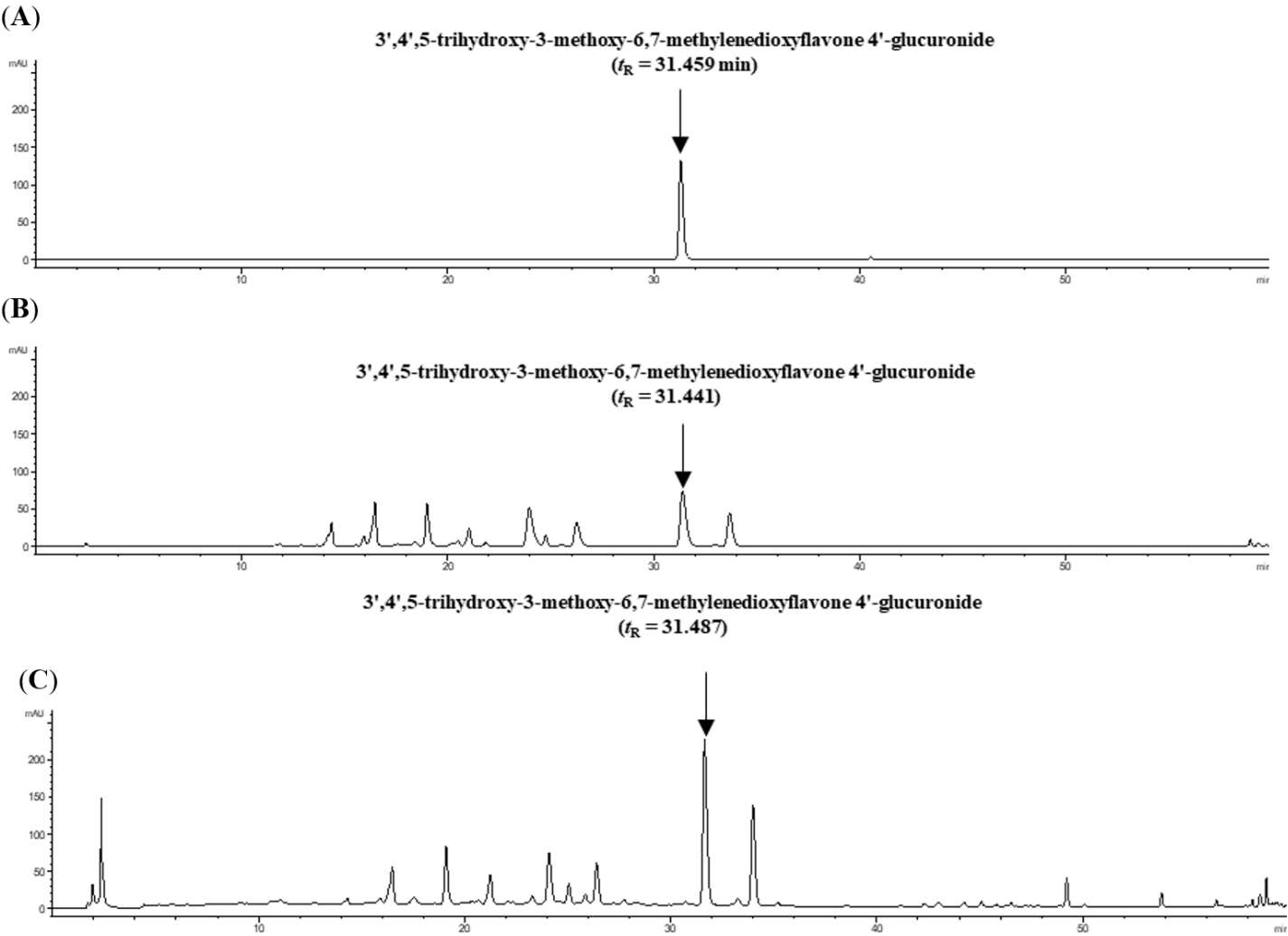


Figure 2

HPLC chromatogram of (A) the standard (TMG) solution at 62.5 µg/ml, (B) freeze-dried spinach (FDS), and (C) spinach extract concentrate (SEC) solutions at 350 nm.