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Method Validation of Analytical Method for 12 Flavonol Glycosides in Foods Using Ultra High-performance Liquid Chromatography Coupled with Photodiode Array Detection

Jong-Chan Kim[1] and You-Shin Shim[1,2,*]

[1]*Food Standard Research Center, Korea Food Research Institute, Seongnam, Gyeonggi 13539, Korea*
[2]*Department of Food Biotechnology, University of Science & Technology, Daejeon 34113, Korea*

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*Corresponding Author
Tel: +82-31-780-9330
Fax: +82-31-780-9280
E-mail: ysshim@kfri.re.kr

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Abstract An analytical method for the simultaneous determination of 12 flavonol glycosides in buckwheat, black tea, and wild parsley using ultra high-performance liquid chromatography (UHPLC) coupled with a simple liquid extraction method using dimethyl sulfoxide (DMSO) was validated in precision, accuracy, and linearity. The UHPLC separation of target compounds was performed on a C18 column using a photodiode array (PDA) detector and the wavelength was fixed at 350 nm. The recovery values for flavonol glycosides ranged from 85.44 to 108.79%. The limits of detection and limits of quantification were less than 0.32 mg/kg and less than 0.97 mg/kg, respectively. The intraday and interday precisions were less than 13.69% for all the test samples. This method coupled with UHPLC-PDA detection could be expected to provide more convenient sample preparation than conventional methods in the tested foods.

Keywords: flavonol glycoside, UHPLC, analytical method, validation

Introduction

Flavonols, the major representative of the flavonoid subclasses, are the most widely distributed flavonoids in nature and are considered the most active compounds within the flavonoid group (1). It is well known that flavonol is a polyphenol containing 3-hydroxyflavone. Flavonols are unstable to light; they undergo photo-oxidation, resulting in fading or formation of darker colors. These characteristics of flavonols explain why onion skins and marigolds, which are rich in flavonols, are not used for commercial dyeing (2).

Generally, the biological activities of flavonols are summarized as four types of activities in human body: antiinflammation (3,4), anticancer (5,6), anticardiovascular disease (7), and antibacterial (8,9). In addition, some are active constituents or characteristic markers in herbal drugs; flavonols such as kaempferol and quercetin show several biological activities. Due to their phenolic nature, flavonoids are quite polar but poorly water soluble. These properties have limited their use in pharmaceuticals (10).

Analytical methods for detecting flavonol glycosides have been continuously performed to date. The most general method for the identification and quantification of flavonol glycosides involves a high-performance liquid chromatography (HPLC) system coupled with MS/MS or hybrid mass spectrometry (11-13) and single mass spectrometry (15-17). The investigation reports that formation of flavonol glycosides using nuclear magnetic resonance (NMR) (17-19) has also become a common research topic in recent years. However, the analytical methods using LC/MS, LC/MS/MS, or NMR have not been easily available in some laboratories because of their high cost. Moreover, these previous studies did not report data with sufficient precision and accuracy in terms of method validation tests. Most papers restrict analytical values to a specific matrix or just one food matrix (11-16).

In this study, we focused on evaluating a fully validating analytical method for the determination of 12 flavonol glycosides in buckwheat, black tea, and wild parsley using ultra high-performance liquid chromatography (UHPLC) and photodiode array (PDA), coupled with a simple liquid extraction method.

Materials and Methods

Chemicals and materials The reference materials of quercetin-3,4-*O*-diglucoside (QUE-3,4-diglucoside), myricetin-3-*O*-galactoside (MYR-3-*O*-galactoside), myricetin-3-*O*-glucoside (MYR-3-*O*-glucoside),

kaempferol-3-*O*-robinoside-7-*O*-rhamnoside (robinin), myricetin-3-*O*-rhamnoside (myricitrin), quercetin-3-*O*-rutinoside(rutin), quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucopyranoside (QUE-3-glucopyranoside), kaempferol-3-*O*-glucoside (KAE-3-glucoside), kaempferol-7-*O*-glucoside (KAE-7-glucoside), isorhamnetin-3-*O*-rutinoside (ISO-3-rutinoside), and isorhamnetin-3-*O*-glucoside (ISO-3-glucoside) were purchased from Extrasynthese (Genay, France). Standard stock solutions of flavonol glycoside reference materials were dissolved in methanol, preparing at concentration ranging from 180.17 to 198.05 mg/kg, respectively. The organic solvents (methanol and acetonitrile) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Buckwheat, black tea, and wild parsley were purchased at a local market in Seoul, Republic of Korea. The samples were stored in 4°C and must be used immediately after grinding to prevent degradation or deformation of target compounds by polyphenol oxidase in the foods matrix.

Preparation of working standard solutions The working flavonol glycoside mixtures were prepared by diluting the stock standard solutions with methanol to five different concentrations, 0.88, 1.75, 3.50, 7.00, and 14.00 mg/kg.

Sample preparation Approximately 1.0 g of buckwheat sample (0.5 g of black tea and wild parsley samples) was massed into 22 mL amber vials (Supelco, Bellefonte, PA, USA) and 5 mL DMSO was added followed by vortex mixing for 5 min. After vortex mixing, 7 mL of 50% methanol was added followed by vortex mixing for another 10 min. Finally, the supernatants were filtered through a 0.2-μm syringe filter (Whatman, Maidstone, UK) and transferred into a small glass vial for UHPLC analysis. The total time for sample preparation for this method was 20 min.

The condition of the UHPLC The UHPLC/PDA detection system (LaChromUltra L-2000 U series; Hitachi High-Technologies Corp., Tokyo, Japan) was used to separate 12 flavonol glycosides. The system comprised a fixed injection volume of 3 μL and EZChrome Elite software from the Hitachi (Version 3.1.8b). The used analytical column was a LaChrom Ultra C18 (2.0 mm i.d., 100 mm length, 2 μm particle size) purchased from Hitachi. The analytical detector was an L-2455U PDA detector, which was set to measure the specific wavelength of 350 nm for flavonol glycosides. To identify each peak correctly, the detector was concurrently set to a wavelength of 200-500 nm. A mobile phase of 15% acetonitrile containing 1% formic acid solution was used in the isocratic elution mode. The flow rate in UHPLC was 0.2 mL/min and the temperature of the analytical column was maintained at 40°C.

Method validation The validation procedures in this study were performed according to the ICH Q2B Validation Methodology (20).

Results and Discussion

Optimization of UHPLC detection The most commonly used analytical column in previous reports for the identification and quantification of flavonol glycoside was C18 column (10,15). Thus, C18 column was used for separating 12 flavonol glycosides, in this study. To evaluate HPLC detection method for flavonol glycosides, Thabitis mobile phase condition was initially considered (15). However, this was not adequate for separation of the 12 flavonol glycosides in UHPLC because the polarities of MYR-3-galactoside and KAE-7-glucoside were very similar to those of MYR-3-glucoside and ISO-3-rutinoside. To separate the 12 flavonol glycosides, it was necessary to retain MYR-3-galactoside and KAE-7-glucoside peaks for

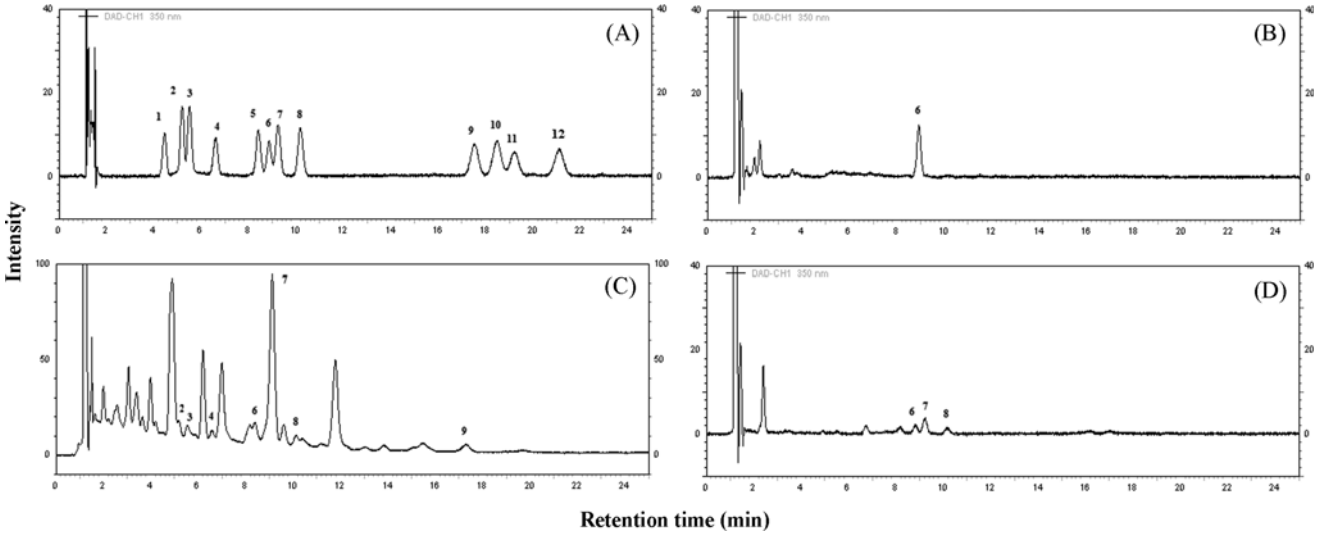

Fig. 1. Typical chromatograms of 12 flavonol glycosides (A) standard solution 7.00 mg/kg, (B) buckwheat, (C) blacktea, and (D) wildparsley. Peaks 1, QUE-3,4-diglucoside; 2, MYR-3-galactoside; 3, MYR-3-glucoside; 4, Robinin; 5, Myricitrin; 6, Rutin; 7, Hyperoside; 8, QUE-3-glucopyranoside; 9, KAE-3-glucoside; 10, KAE-7-glucoside; 11, ISO-3-rutinoside; 12, ISO-3-glucoside

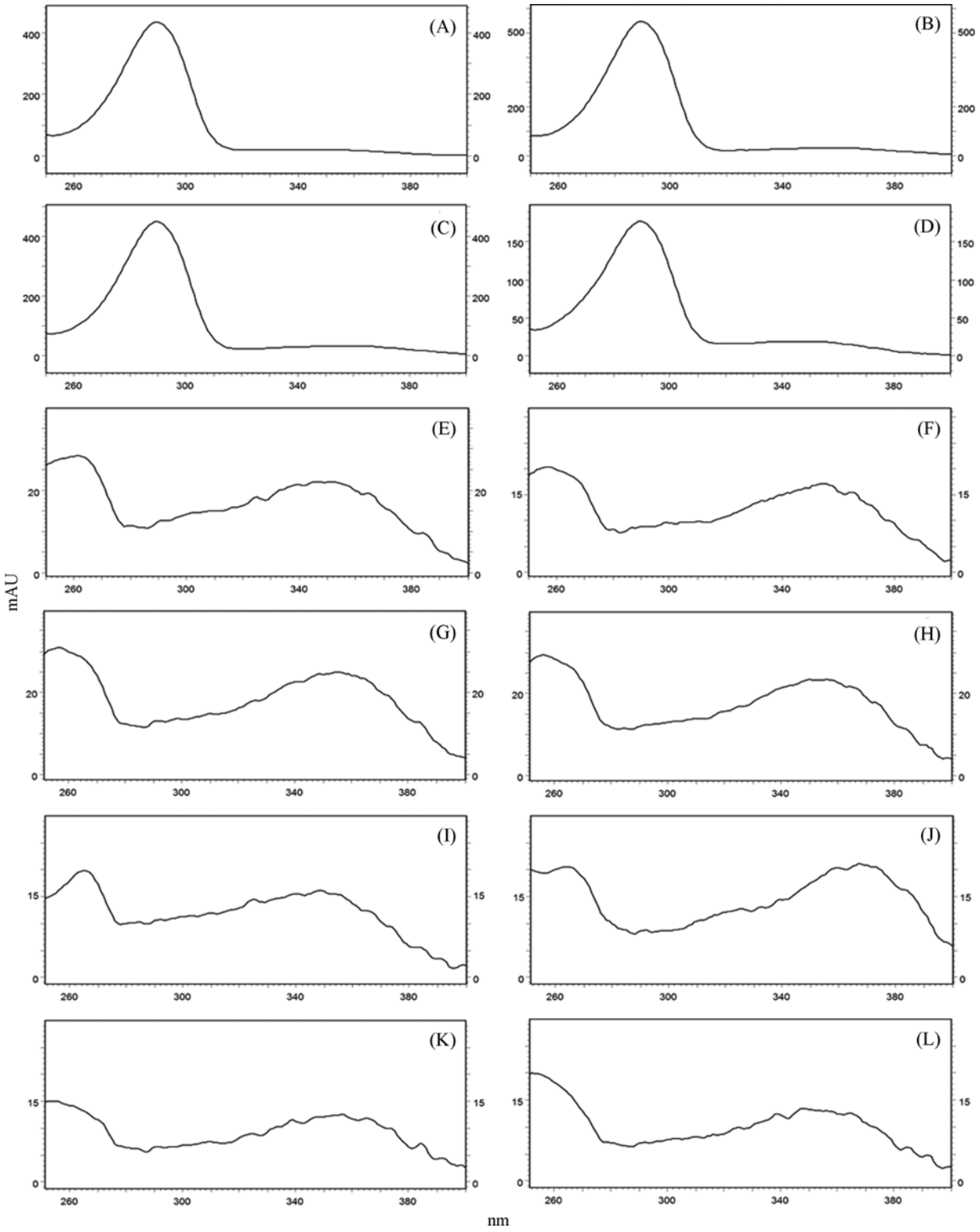

Fig. 2. Individual spectra for flavonol glycosides. QUE-3,4-diglucoside (A), MYR-3-galactoside (B), MYR-3-glucoside (C), Robinin (D), Myricitrin (E), Rutin (F), Hyperoside (G), QUE-3-glucopyranoside (H), KAE-3-glucoside (I), KAE-7-glucoside (J), ISO-3-rutinoside (K), and ISO-3-glucoside (L)

at least 4 and 18 min, respectively, as an isocratic elution mode in order to resolve MYR-3-glucoside and ISO-3-rutinoside, as you can see in Fig. 1A. The individual spectra for the flavonol glycosides were displayed in Fig. 2. The optimized run time was 25 min and the consumption of mobile phase was only 7.5 mL, which is very fast compared with conventional HPLC detection (15).

Table 1. The linearity and sensitivity of 12 flavonol glycosides[1)]

Component	Linear range (mg/kg)	*r*	LOD (mg/kg)	LOQ (mg/kg)
QUE-3,4-diglucoside	0.88-14.00	0.9997	0.27	0.81
MYR-3-galactoside	0.88-14.00	0.9996	0.25	0.76
MYR-3-glucoside	0.88-14.00	0.9993	0.26	0.81
Robinin	0.88-14.00	0.9997	0.32	0.97
Myricitrin	0.88-14.00	0.9998	0.29	0.88
Rutin	0.88-14.00	0.9992	0.22	0.69
Hyperoside	0.88-14.00	0.9998	0.22	0.69
QUE-3-glucopyranoside	0.88-14.00	0.9997	0.27	0.83
KAE-3-glucoside	0.88-14.00	0.9998	0.29	0.88
KAE-7-glucoside	0.88-14.00	0.9994	0.21	0.64
ISO-3-rutinoside	0.88-14.00	0.9992	0.27	0.84
ISO-3-glucoside	0.88-14.00	0.9994	0.31	0.94

[1)]All values were calculated using standard solution, intraday (*n*=10) analyses.

Selection of extraction solvent It is well known that DMSO is an amphipatic molecule with a highly polar domain and two apolar groups. Because of its physical and chemical properties, DMSO could be a very efficient extraction solvent for both of water-soluble and water-insoluble compounds (21). We also found that DMSO acts as an excellent extraction solvent for compounds containing glycoside such as anthocyanin glycosides and isoflavones (22,23). DMSO is well tolerated by humans and presents low or no toxicity (24), which leads us to use DMSO as an extraction solvent for flavonol glycosides.

To evaluate a rapid sample pretreatment method, we considered using DMSO as a pre-extraction solvent and considered 50% methanol as the main extraction solvent according to a previous study (15). Using DMSO allowed for the omission of the reflux extraction step and long cooling time of about 20 min without the loss of target compounds and reduced the pretreatment time two-fold compared with the conventional method (16).

Linearity, limits of detection (LOD), and limits of quantification (LOQ) To determine the linearity of the flavonol glycosides, five different concentrations (0.88, 1.75, 3.50, 7.00, and 14.00 mg/kg) of the flavonol glycoside standards were used. Each solution was injected 10 times, and average concentrations are presented in Table 1. Regression analyses calculated as correlation coefficient (*r*) values were ranged from 0.9992 to 0.9998. Sensitivity values for flavonol glycosides were calculated using five previously described standard concentrations. The LOD and LOQ for the analytical method were estimated as the SD multiplied by 3.3 and 10 over b (SD/b), respectively. SD represents the standard deviation of the intercept, and b represents the average slope of the linear regression. All LOD and LOQ values in this study were less than 1.00 mg/kg.

Precision and accuracy To determine the precision and accuracy of this method, intraday (*n*=6) and interday (*n*=3) tests were performed on the spiked buckwheat, black tea, and wild parsley. The concentrations of target compounds in initial samples (buckwheat, black tea, and wild parsley) were calculated as values described in the footnote of Table 2-4. These values are concentrations of test solutions in vials for HPLC analyses. To calculate the concentration in the real sample, these values should be multiplied by factors of 12 (dilution factor for buckwheat) or 24 (dilution factor for black tea and wild parsley). The concentration of rutin was 120.0 mg/kg in buckwheat samples (groats buckwheat). These results are similar to those of a previous report (25). In our previous report (10), wild parsley contained quercetin, kaempferol, and isorhamnetin and black tea contained myricetin, quercetin, and kaempferol. The analytical results for the initial samples in this study indicated that only quercetin glycoside was found in wild parsley. In black tea, myricetin, quercetin, and kaempferol glycosides were equally found. In case of wild parsley, it could be estimated that kaempferol and isorhamnetin were entirely aglycone forms. The corresponding chromatograms are displayed in Fig. 1B-1D.

Table 2. The precision[1)] and accuracy[2)] for the analyses of 12 flavonol glycosides in spiked buckwheat samples

Component	Spiked amount (mg/kg)	Intraday (RSD, %)	Interday (RSD, %)	Recovery, %
QUE-3,4-diglucoside	1.17	8.20	10.55	94.63±9.99
	2.33	1.98	3.14	91.43±2.87
MYR-3-galactoside	1.17	3.60	4.13	99.07±4.10
	2.33	6.15	5.44	100.26±5.46
MYR-3-glucoside	1.17	2.10	7.04	101.77±7.16
	2.33	5.63	4.89	96.37±4.71
Robinin	1.17	2.93	7.10	103.00±7.31
	2.33	2.95	3.22	106.85±3.44
Myricitrin	1.17	3.62	5.71	102.53±5.85
	2.33	2.81	3.92	108.79±4.27
Rutin	1.17	5.73	12.07	96.27±11.62
	2.33	3.87	3.36	100.84±3.39
Hyperoside	1.17	3.71	5.36	103.15±5.53
	2.33	3.05	7.62	93.46±7.12
QUE-3-glupyranoside	1.17	4.20	4.15	102.33±4.25
	2.33	3.14	2.90	105.02±3.05
KAE-3-glucoside	1.17	5.67	8.28	100.09±8.28
	2.33	2.27	5.67	96.09±5.45
KAE-7-glucoside	1.17	6.03	12.34	97.18±11.99
	2.33	3.04	4.61	101.82±4.70
ISO-3-rutinoside	1.17	7.31	9.67	96.32±9.31
	2.33	2.31	2.24	103.92±2.33
ISO-3-glucoside	1.17	3.09	9.60	100.16±9.61
	2.33	2.93	3.28	105.85±3.47

[1)]Amount of buckwheat sample=1.0 g; values represent the results of the intraday (*n*=6) and interday (*n*=3) analyses; The initial rutin concentration in buckwheat was estimated to be 10.0 mg/kg.
[2)]Values represent the mean±standard deviation of the intraday (*n*=6) and interday (*n*=3) analyses.

The relative standard deviations (RSD) for intraday and interday repeatability with the aforementioned samples (buckwheat, blacktea, and wildparsley) spiked at levels of 1.17 and 2.33 mg/kg are shown in Table 2-4. To determine the recovery values, intraday (*n*=6) and interday (*n*=3) tests were also performed at the same conditions for

Table 3. The precision[1)] and accuracy[2)] for the analyses of 12 flavonol glycosides in spiked blacktea samples

Component	Spiked amount (mg/kg)	Intraday (RSD, %)	Interday (RSD, %)	Recovery, %
QUE-3,4-diglucoside	1.17	5.17	5.04	86.57±4.36
	2.33	0.66	3.50	86.57±3.03
MYR-3-galactoside	1.17	8.57	12.47	96.13±11.98
	2.33	4.02	8.08	99.06±8.00
MYR-3-glucoside	1.17	4.17	5.98	90.76±5.42
	2.33	4.89	5.05	88.17±4.45
Robinin	1.17	5.16	9.24	93.08±8.60
	2.33	3.97	10.19	88.22±8.99
Myricitrin	1.17	7.37	10.31	98.87±10.20
	2.33	5.65	5.38	99.91±5.38
Rutin	1.17	5.58	5.25	86.40±4.54
	2.33	4.06	8.75	95.82±8.39
Hyperoside	1.17	4.19	9.90	85.44±8.45
	2.33	1.72	11.60	93.89±10.89
QUE-3-glupyranoside	1.17	4.49	6.48	106.65±65.00
	2.33	4.39	6.36	105.61±6.71
KAE-3-glucoside	1.17	5.94	9.00	102.81±9.26
	2.33	3.95	9.99	99.10±9.90
KAE-7-glucoside	1.17	4.19	6.59	102.05±6.73
	2.33	6.02	5.89	96.90±5.71
ISO-3-rutinoside	1.17	3.33	9.91	101.83±10.09
	2.33	3.41	10.41	91.96±9.57
ISO-3-glucoside	1.17	6.07	8.95	91.23±8.16
	2.33	5.06	5.38	87.23±4.69

[1)]Amount of blacktea sample=0.5 g; values represent the results of the intraday (n=6) and interday (n=3) analyses; the initial QUE-3,4-diglucoside, MYR-3-galactoside, robinin, myricitrin, rutin, hyperoside, QUE-3-glucopyranoside, and KAE-3-glucoside concentrations in blacktea were estimated to be 2.95, 2.15, 2.07, 5.60, 2.68, 51.11, 2.00, and 3.63 mg/kg, respectively.
[2)]Values represent the mean±standard deviation of the intraday (n=6) and interday (n=3) analyses.

Table 4. The precision[1)] and accuracy[2)] for the analyses of 12 flavonol glycosides in spiked wildparsley samples

Component	Spiked amount (mg/kg)	Intraday (RSD, %)	Interday (RSD, %)	Recovery, %
QUE-3,4-diglucoside	1.17	3.59	9.66	95.31±9.21
	2.33	3.37	8.70	91.61±7.97
MYR-3-galactoside	1.17	3.69	4.32	88.93±3.85
	2.33	3.62	11.05	95.27±10.53
MYR-3-glucoside	1.17	1.93	13.69	104.97±14.37
	2.33	4.58	8.86	100.35±8.89
Robinin	1.17	1.94	9.65	89.27±8.62
	2.33	7.37	11.28	85.08±9.60
Myricitrin	1.17	4.16	13.36	93.41±12.48
	2.33	6.99	10.01	91.10±9.12
Rutin	1.17	4.82	13.09	92.32±12.08
	2.33	1.36	8.62	86.75±7.48
Hyperoside	1.17	2.70	7.40	89.15±6.60
	2.33	1.88	10.02	95.76±9.59
QUE-3-glupyranoside	1.17	5.85	12.27	97.83±12.00
	2.33	3.63	6.06	101.77±6.17
KAE-3-glucoside	1.17	4.08	4.20	108.09±4.54
	2.33	4.84	13.36	99.68±13.32
KAE-7-glucoside	1.17	5.56	11.82	96.13±11.37
	2.33	7.96	7.67	98.04±7.52
ISO-3-rutinoside	1.17	3.42	5.98	89.22±5.33
	2.33	6.22	7.72	98.10±7.58
ISO-3-glucoside	1.17	6.73	9.71	95.72±9.29
	2.33	6.14	9.73	93.69±9.11

[1)]Amount of wildparsley sample=0.5 g; values represent the results of the intraday (n=6) and interday (n=3) analyses; the initial rutin, hyperoside and QUE-3-glucopyranoside concentrations in wildparsley were estimated to be 2.85, 2.50, and 1.11 mg/kg, respectively.
[2)]Values represent the mean±standard deviation of the intraday (n=6) and interday (n=3) analyses.

the precision and accuracy tests. The assay samples were calculated from the following equation:

$$\text{Recovery (\%)}=[(C_t-C_u)/C_a]\times 100$$

where C_t is the total concentration of the analyte; C_u is the concentration of the analyte in the original samples; and C_a is the concentration of the standard spiked to the samples.

The RSD values of the intraday and interday repeatability were below 13.69% for flavonol glycosides in the precision test. The recovery values were 85.44-108.79% for all the analytes. All the values for the validation of this study were in accordance with the FDA guidelines for bioanalytical validation (26). Moreover, previous research (19) reported that the recovery values for flavonol glycosides in sea buckthorn were in the range of 94.5-106.2%, similar to the results obtained from recovery values in this study using UHPLC-PDA detection coupled with a liquid solvent extraction method. The results of the precision and accuracy analysis are shown in Table 2-4.

Consequently, the UHPLC method suggested in this study could be more conveniently applied for the analyses of 12 kinds of flavonol glycosides in buckwheat, blacktea, and wildparsley using UHPLC/PDA coupled with a simple liquid extraction method.

Disclosure The authors declare no conflict of interest.

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