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ARTICLE

Simultaneous Quantification of Flavonoids and Phenolic Acids in Plant Materials by a Newly Developed Isocratic High-Performance Liquid Chromatography Approach

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A simple reversed-phase high-performance liquid chromatographic (RP-HPLC) method based on isocratic elution has been developed and validated for the simultaneous quantitation of flavonols (myricetin, quercetin, kaempferol, and isorhamnetin), flavones (luteolin and apigenin), and phenolic acids (chlorogenic, caffeic, ellagic, and rosmarinic acids) as important constituents in fruits, vegetables, and medicinal plants. Analysis was achieved on a C_{18} column at ambient temperature. The wavelengths used for the detection of flavonols, flavones, and phenolic acids were 370, 350, and 325 nm, respectively. After acid hydrolysis, the flavonoid aglycones were quantified straightforwardly in 20 dry herbal samples. The plants with the highest flavonoids were *Rosa damascena*, *Solidago virgaurea*, *Ginkgo biloba*, and *Camellia sinensis*. The contents of flavonoids aglycons ranged from 0.54 to 11.10 mg/g, from 0.03 to 14.80 mg/g, from 0.19 to 2.76 mg/g, from 0.15 to 2.36 mg/g, from 0.27 to 2.05 mg/g, and from 0.42 to 1.82 mg/g for quercetin, kaempferol, isorhamnetin, luteolin, apigenin, and myricetin in dry plant samples, respectively.

KEYWORDS: HPLC; flavonols; flavones; phenolic acids

INTRODUCTION

Phenolic compounds are commonly found in both edible and nonedible plants, and they have been reported to have multiple biological effects. Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease (*1*). They are one class of the most important phenolic compounds that have widely been distributed in plants and consist mainly of anthocyanidins, flavonols, flavones, catechins, and flavanones. Among them, flavonols and flavones are of particular importance in the human diet as antioxidants and the associated health benefits of a high-flavonoid diet, which has received the most attention in recent literature (*2*,*3*). The occurrence of flavonols and flavones in food plants has been reviewed (*4*, *5*). Epidemiological studies have indicated that their consumption is associated with a reduced risk of cancer and cardiovascular disease (*6*−*8*). The main difference between flavones and flavonols is the presence of a hydroxyl group at C_3 in flavonols (**Figure 1**).

Flavonoids are usually found in plants as glycosides. More than 4000 different naturally occurring flavonoids have been described, and this list is still growing (*9*). Almost 180 different glycosides of quercetin have been described in nature, with rutin (quercetin-3-*O*-rutinoside) being one of the most common (*10*). Quantitative determination of individual flavonoid glycosides in foods is difficult because most reference compounds are not commercially available (*11*). Thus, to reduce the complexity of the analysis, plant flavonoids are identified and quantified as aglycone after hydrolysis. Methods for extraction, hydrolysis, and analysis of flavonoids by HPLC have been published (*11*−*28*). Despite the various reports on the analysis of flavonoids, a few HPLC methods are available for the simultaneous analysis of flavonoid aglycones, which do not contain an adequate separation. It has been found that peaks luteolin/quercetin and isorhamnetin/apigenin eluted at the same retention time or slightly different retention times with marginal separation (*12*, *13*, *15*, *18*). However, the incomplete separation was problematic because these coeluting flavonoids coexist in some plant samples. Another important class of phenolic compounds is hydroxycinnamic acids, which usually exist as esters of organic acid or glycosides. The occurrence of such compounds in foods significantly affects stability, color, flavor, nutritional value, and other food qualities. They also possess some biological activities (*29*). The major representative of this phenolics class is caffeic acid, which normally occurs in foods mainly as an ester with quinic acid (QA), known as chlorogenic acid (**Figure 1**). Chlorogenic acid (5-caffeoylquinic acid or 5-CQA) and caffeic acid are found in almost every plant. Chlorogenic acid has several isomers having molar extinction coefficients that are almost the same (*30*). Chlorogenic acid and caffeic acid are antioxidant in vitro, which protects low-density lipoprotein (LDL) from oxidation and, therefore, is thought to prevent various age-related diseases (*31*). Rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, has been reported to have several biological activities including a potent anti-inflammatory effect resulting from decreased arachidonate formation, inhibition of hemolysis, and suppression of hyaluronidase and β-hexoaminidase, antioxidative, anti-inflammatory, and antidepressive activities, and protective activity on lipopolysaccharide-induced liver injury (*32*,*33*). Ellagic acid, a phenolic lactone compound (dimeric derivative of gallic

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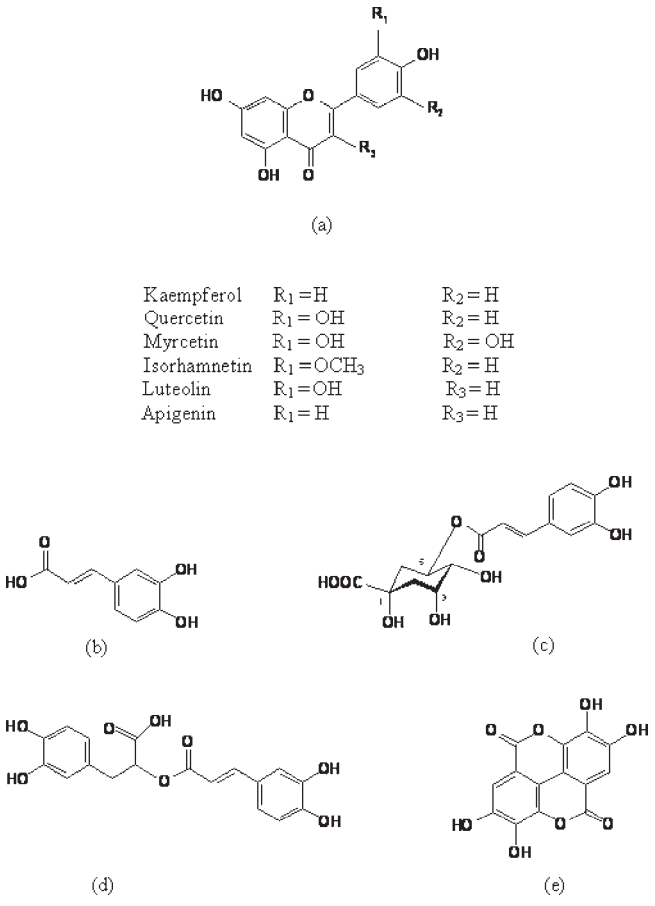

Figure 1. Chemical structures of flavonols and flavones (**a**), caffeic acid (**b**), chlorogenic acid (**c**), rosmarinic acid (**d**), and ellagic acid (**e**).

acid) that exists in plants, is a degradation product of hydrolyzable plant tannins (*34*). It has antibacterial and antioxidative properties (*35*). The aim of this study was the development and validation of a simple and reliable HPLC method for the simultaneous analysis of flavonols (myricetin, quercetin, kaempferol, and isorhamnetin), flavones (luteolin and apigenin), and phenolic acids (chlorogenic, caffeic, ellagic, and rosmarinic acids) because of their wide distribution and important biological activities in materials of plant origin.

MATERIALS AND METHODS

Solvents and Chemicals. Standards including myricetin, luteolin, quercetin dihydrate, apigenin, kaempferol, isorhamnetin, rosmarinic acid, ellagic acid, and chlorogenic acid were purchased from Carl Roth (Karlsruhe, Germany) and morin (standard Fluka, for microscopy) was from Sigma-Aldrich. HPLC grade acetonitrile (ACN), methanol (MeOH), tetrahydrofuran (THF), and caffeic acid were obtained from Merck (Darmstadt, Germany).

Plant Material. All samples were obtained from a marketplace or collected from nature. The dried samples were powdered and immediately used. The botanical names of samples are listed in **Table 1**.

Establishment of Calibration Curves. Stock solutions of the standards were freshly prepared within the range of 200 μg/mL by dissolving 10 authentic compounds in methanol. The concentration of standard solutions was selected according to the level of the analytes expected in the samples. Working standard solutions were made by gradual dilution with methanol to the required concentration, which was based on the sensitivity of detection and the linearity range of the study. The concentration of the standards ranged from 0.5 to 40 μg/mL for flavonoids and from 5 to 100 μg/mL for phenolic acids except caffeic acid, which was 1−50 μg/mL. The calibration curve was constructed for each standard by plotting the concentration of the standard (μg/mL) against peak area.

Extraction and Hydrolysis. Methods for acid hydrolysis of flavonoids have been published (*11*, *14*, *15*, *21*, *22*). The extraction and hydrolysis of samples containing flavonoids were done as previously reported (*21*). Five milliliters of 50% aqueous methanol containing 1.2 M HCl and 0.04% (w/v) ascorbic acid as antioxidant was added to 50 mg of dried sample. The hydrolysis was performed at 80 °C under reflux for 2 h and the extract allowed to cool; the extract was subsequently diluted to 10 mL with methanol. The extract (2 mL) was filtered through a Chromafil syringe filter (0.45 μm pore size) and injected three times for HPLC analysis. Each sample was analyzed three times. The extraction of rosmarinic acid, caffeic acid, and chlorogenic acid was carried out in accordance with the method reported in the literature (*33*). Briefly, 50 mg of dried sample was extracted with 25 mL of ethanol/water (30:70 v/v). The extract was filtered and transferred to a 50 mL volumetric flask. The residual solid was rinsed with 20 mL of the same solvent and added to the filtered extract. The filtrate volume was adjusted with the solvent to the mark. To sample comprising ellagic acid, the extraction and hydrolysis were performed as previously reported elsewhere (*36*).

Columns. The C_{18} analytical columns tested were Novapak (150 × 3.9 mm i.d., 4 μm), Hypersil (150 × 4.6 mm i.d., 5 μm), Eurospher (250 × 4.6 mm i.d., 5 μm), Econosil (250 × 4.6 mm i.d., 5 μm), and Nucleosil (250 × 4.6 mm i.d., 5 μm).

Chromatographic Conditions. The HPLC analysis was performed with Knauer (WellChrom, Germany) equipment including a K-1001 pump, a K-2600 fast scanning UV detector with simultaneous detection at four wavelengths, a K-5004 analytical degasser, and a 2301 injector with a 20 μL loop on an Eurospher C_{18} column (250 × 4.6 mm i.d., 5 μm particle size). The mobile phase was 4% (v/v) THF in ACN and 0.4% (v/v) phosphoric acid in water (35:65) at a flow rate of 1.0 mL/min at ambient temperature. Each sample was analyzed in triplicate to determine the mean contents of analytes in herbal samples. The injection volume of all samples was 20 μL. Column effluent was simultaneously monitored at four wavelengths between 240 and 370 nm and detection affected at 325 nm for hydroxycinnamic acids, at 350 nm for flavones, and at 370 nm for flavonols and ellagic acid. The spectral data of signals from the UV detector were collected during the whole run in the range of 240−400 nm. The analytes were identified by matching the retention times and spectral characteristics against those of standards, and their absorbance ratios in simultaneous detection were determined, spiking samples with standards, with those reported in the literature (*37*). Morin, a flavonol derivative found in a few herbs, is proposed as internal standard. However, quantification was based on an external standard method.

Method Validation. The method was validated for linearity, sensitivity, and repeatability recovery. To construct calibration curves, linearity was tested at five different concentrations of phenolic compounds and injected in triplicate. Limits of detection (LOD) and quantification (LOQ) for each detected analyte were calculated with corresponding standard solution on the basis of a signal-to-noise ratios (S/N) of 3 and 10, respectively. Measurements of intra- and interday variability were utilized to determine the repeatability of the method. The repeatability of the method, as relative standard deviation (RSD), was assessed by analyzing 50 mg of the same samples within one day (four parallel samples) and on three consecutive days (two parallel samples each day). Accuracy was evaluated by the standard addition method. The standard solutions were added at three different contents to samples, and recovery was measured in triplicate. For comparison, an unspiked sample was concurrently prepared and analyzed.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions. The HPLC analysis of flavonoids and phenolic acids using several mobile phases and columns under gradient elution has been previously reported. The earlier methods were unsuitable due to coelution of quercetin/luteolin and apigenin/isorhamnetin peaks (*12*, *13*, *15*). In this study, we developed a simple isocratic RP-HPLC method for the direct determination of flavonoids and phenolic acids in plant materials.

For sample pretreatment, the chromatographic procedure described (*13*) was duplicated for the analysis of six flavonoid aglycones on available columns, but the separation of luteolin/

Table 1. Mean Content of Analytes (Milligrams per Gram ± SD) of Dry Samples in This Study

	flavonoids[a]						phenolic acids[a]			
sample	Q	K	I	L	A	M	CA	EA	CGA	RA
Solidago virgaurea leaf	3.95 ± 0.11	3.53 ± 0.10	0.68 ± 0.02	nd[b]	nd	nd	nd	nd	0.93 ± 0.04	nd
Rosa damascena flower	11.10 ± 0.31	14.80 ± 0.35	0.71 ± 0.02	0.15 ± 0.01	nd	1.71 ± 0.04	nd	nd	1.81 ± 0.05	nd
Calendula officinalis flower	1.12 ± 0.04	0.18 ± 0.01	2.76 ± 0.09	nd	nd	nd	nd	nd	0.14 ± 0.008	nd
Ginkgo biloba leaf	1.36 ± 0.06	2.67 ± 0.07	0.85 ± 0.02	0.12 ± 0.01	nd	0.42 ± 0.02	nd	nd	0.19 ± 0.007	nd
Achillea millefolium aerial parts	1.07 ± 0.03	nd	0.19 ± 0.01	0.66 ± 0.03	0.27 ± 0.01	nd	nd	nd	nd	nd
Apium gravolens aerial parts	nd	nd	nd	0.95 ± 0.04	2.05 ± 0.06	nd	nd	nd	0.98 ± 0.03	nd
lettuce leaf (*Lactuca sativa*)	0.82 ± 0.02	nd	nd	0.22 ± 0.01	nd	nd	nd	nd	nd	nd
red onion bulb (*Allium cepa*)	3.27 ± 0.11	0.36 ± 0.03	nd	nd	nd	nd	nd	nd	nd	nd
yellow onion (*Allium cepa*)	4.81 ± 0.07	0.25 ± 0.01	nd	nd	nd	nd	nd	nd	nd	nd
white onion (*Allium cepa*)	1.16 ± 0.06	nd	nd	nd	nd	nd	nd	nd	nd	nd
olive leaf (*Olea europaea*)	0.54 ± 0.03	nd	nd	1.42 ± 0.04	nd	nd	nd	nd	nd	nd
grape leaf (*Vitis vinifera*)	2.93 ± 0.06	0.19 ± 0.01	nd	nd	nd	1.82 ± 0.05	nd	1.11[c] 15.86[d]	nd	nd
strawberry leaf (*Fragaria vesca*)	2.16 ± 0.05	0.34 ± 0.02	nd	nd	nd	nd	nd	1.72[c] 21.66[d]	nd	nd
Camellia sinensis leaf (green)	2.78 ± 0.07	0.93 ± 0.03	nd	nd	nd	1.31 ± 0.04	nd	nd	1.63 ± 0.06	nd
red apple peel	0.89 ± 0.03	0.03 ± 0.002	nd	nd	nd	nd	nd	nd	nd	nd
Pimpinellia anisum seed	nd	nd	nd	0.29 ± 0.02	nd	nd	nd	nd	1.36 ± 0.05	nd
Salvia officinalis leaf	nd	nd	nd	nd	nd	nd	nd	nd	nd	9.98 ± 0.33
Melissa officinalis leaf	nd	nd	nd	nd	nd	nd	nd	nd	nd	21.78 ± 0.59
Cynara scolymus leaf	nd	nd	nd	2.36 ± 0.08	nd	nd	0.15 ± 0.007	nd	2.88 ± 0.07	nd
Gundelia tournefortii	nd	nd	nd	nd	nd	nd	0.12 ± 0.005	nd	8.14 ± 0.28	nd

[a] Q, quercetin; K, kaempferol; I, isorhamnetin; CA, caffeic acid; EA, ellagic acid; CGA, chlorogenic acid; RA, rosmarinic acid; L, luteolin; A, apigenin; M, myricetin. [b] nd, not detected. [c] Unhydrolyzed. [d] Hydroylzed.

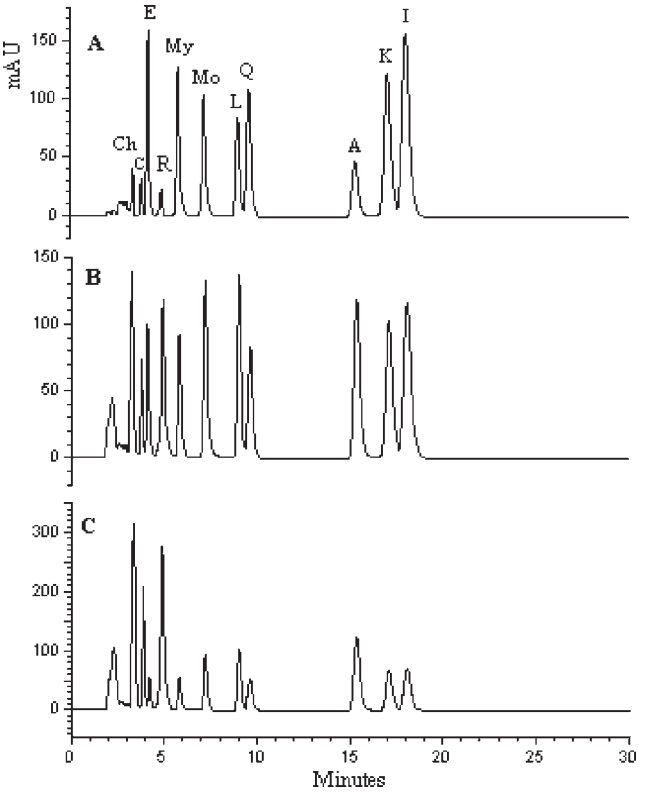

Figure 2. Typical chromatogram of standards on an Eurospher column: (**A**) 370 nm; (**B**) 350 nm; (**C**) 325 nm. Ch, chlorogenic acid; C, caffeic acid; E, ellagic acid; R, rosmarinic acid; My, myricetin; Mo, morin; L, luteolin; Q, quercetin; A, apigenin; K, kaempferol; I, isorhamnetin.

quercetin peaks was without success. To optimize the chromatographic conditions, the mobile phase, column type, and detection wavelength were considered. First, several mobile phases consisting of mixtures of MeOH or ACN as an organic modifier in different ratios with water containing an acidic modifier such as phosphoric acid or trifluoroacetic acid (TFA) were surveyed using authentic compounds. The modifiers enhance the resolution, restrain the ionization, and inhibit the tailing and broadening of the peak. MeOH/water mixtures in different proportions did not enable an acceptable separation for apigenin and isorhamnetin. When different proportions of ACN/H_2O were used as mobile phase instead of MeOH/H_2O, the results showed that luteolin and quercetin peaks coeluted at the same retention time. The addition of THF to the ACN/water mixture improved the separation for the examined analytes. Phosphoric acid in comparison with TFA was found to be the better choice. Eventually, a mobile phase consisting of 4% THF in ACN as eluent A and 0.4% aqueous phosphoric acid as eluent B (A:B, 35:65 v/v) was selected to achieve optimal separation.

Table 2. Column Performance for the Separation of Analytes

	efficiency parameters			
analyte	retention factor (*k*)	selectivity factor (*a*)	resolution (R_S)	theoretical plate (*N*)
chlorogenic acid	0.67			1936
caffeic acid	0.97	1.45	1.76	2704
ellagic acid	1.17	1.21	1.32	3287
rosmarinic acid	1.52	1.30	1.85	3226
myricetin	2.03	1.33	2.48	3624
luteolin	3.59	1.77	6.21	5298
quercetin	3.95	1.10	1.38	5089
apigenin	6.73	1.1	7.85	5856
kaempferol	7.64	1.13	2.54	7312
isorhamnetin	8.24	1.07	1.41	6864

Column Efficiency. For a satisfactory separation and decreased run time, available C_{18} columns were tested using standard compounds. The better separation was observed on the Eurospher column at a run time of < 20 min. **Figure 2** shows a typical chromatogram obtained from standard compounds under the optimized HPLC-UV method. The following parameters have been calculated for the representative chromatogram to check the column efficiency:

Table 3. Linearity and Sensitivity Data for the Proposed Method

analyte	linearity range (μg/mL)	regression equation	LOD (μg/mL)	LOQ (μg/mL)	correlation coefficient (r)
quercetin	0.5−40	$Y = 74489X - 32343$	0.16	0.5	0.9995
myricetin	0.5−40	$Y = 64653X - 98958$	0.18	0.5	0.9992
kaempferol	0.5−40	$Y = 85079X - 12512$	0.15	0.5	0.9998
isorhamnetin	0.5−40	$Y = 69851X - 18123$	0.17	0.5	0.9997
luteolin	0.5−40	$Y = 94737X - 62335$	0.14	0.5	0.9993
apigenin	0.5−40	$Y = 143223\ X - 54123$	0.12	0.5	0.9996
ellagic acid	5−100	$Y = 31312X - 16420$	1.2	3.2	0.9993
caffeic acid	1−50	$Y = 101572X - 4366$	0.14	0.5	0.9998
chlorogenic acid	5−100	$Y = 66446X - 34710$	0.33	1.0	0.9997
rosmarinic acid	5−100	$Y = 52637X - 44664$	0.75	2.1	0.9998

The capacity factor or retention factor ($k = t_R - t_0/t_0$) is a measure of the retention time of a compound in the sample with a given combination of mobile phase and column, in which t_R is the retention time of the compound and t_0 refers to the retention time for an unretained compound. The selectivity factor ($a = t_{R2} - t_0/t_{R1} - t_0$) or ($k_2/k_1$) is a measure of separation of two adjacent peaks in the sample under given conditions. Resolution (R_S) is a measure of the degree of separation between contiguous peaks. It is expressed as $R_S = 2(t_{R2} - t_{R1})/(w_1 + w_2)$, in which t_{R2} and t_{R1} are the retention times and w_1 and w_2 the baseline peak widths of successive peaks. A value of 1.5 for resolution implies a complete separation of two peaks. The number of theoretical plates, $N = 16(t_R/w)^2$, refers to the performance of the stationary phase (*38*). The column performance for the separation of analytes is given in **Table 2**. To get the best response, the standards were scanned between 240 and 400 nm on the HPLC detector. The UV spectra showed that suitable absorbance was approximately at 370 nm for flavonols and ellagic acid, at 350 nm for flavones, and at 325 nm for hydroxycinnamic acids. The detection was set at these wavelengths in this method.

Validation Results. The calibration curves were linear in the ranges of 0.5−40 μg/mL for flavonoids, 5−100 μg/mL for chlorogenic acid, ellagic acid, and rosmarinic acid, and 1−50 μg/mL for caffeic acid. Regression equations and correlation coefficients revealed linear response for the developed method. The LODs and LOQs for the reference compounds were 0.12−1.2 and 0.5−2.1 μg/mL in standard dilutions, respectively. The results are summarized in **Table 3**. The intra- and interday precisions (expressed in terms of %RSD) were obtained in the range of 1.26−1.58 and 1.32−1.79%, respectively. The recovery of flavonoids and phenolic acids was determined by the method of standard addition as above-reported. The mean recoveries were found to be 96.5−103.8% with a mean RSD in the range of 1.86−4.72%. **Table 4** shows the results of precision and recovery in this study.

The presence of analytes in selected herbs was confirmed by comparison of their retention times, spiking the extracts with pure standards and overlaying UV spectra with those of standard compounds and with those reported in the literature (*37*). **Table 1** represents mean contents of the analytes found in samples. The hydrolyzed extracts of *Gingko biloba* leaf and flower of *Rosa damascena* showed the presence of myricetin, luteolin, quercetin, kaempferol, isorhamnetin, and chlorogenic acid. Quercetin was found in all selected plants except *Apium gravolens* and *Cynara scolymus*. Quercetin was found to be a major flavonoid, followed by kaempferol and isorhamnetin. Myricetin was detected in *Camellia sinensis*, *Gingko biloba*, *Vitis vinifera*, and *Rosa damascene* and luteolin a major flavonoid in *Olea europaea*. Apigenin was observed in low amount in *Achillea milleafolium*, *Apium gravolens*, and *Camellia sinensis*. Phenolic acids were detected in *Cynara scolymus*, *Gundelia tournefortii*, *Vitis vinifera*, *Fragaria vesca*, *Rosa damascena*, *Apium gravolens*, *Camellia sinensis*, *Pimpinellia anisum*, and *Calendula officinalis*. The dried onion bulb with colored skins contained 0.11−0.48% quercetin and 0.025−0.036% kaempferol. Kaempferol was not detected in the white onion, and quercetin was lower than in colored bulbs. Most of the samples surveyed in this study contained chlorogenic acid and its isomers. HPLC analysis of chlorogenic acid and its isomers was performed according to our present and previous methods (*39*) and compared together. In the present method, three isomers showed one peak at 3.2 min. Therefore, the content of total isomers in samples was determined, and results were expressed as chlorogenic acid equivalent in samples. The concentration of ellagic acid is generally increased after hydrolysis due to the presence of ellagitannins in the crude extract. The contents of ellagic acid in unhydrolyzed samples of *Vitis vinifera* and *Fragaria vesca* were 1.11 and 1.72 mg/g and in hydrolyzed samples were 15.86 and 21.66 mg/g, respectively.

Table 4. Intra- and Interday Precision and Recovery Data for the Proposed Method

compound	intraday av amount (μg)	RSD (%)	interday av amount (μg)	RSD (%)	recovery (%)	RSD (%)
quercetin	555	1.26	552	1.32	102.7	4.72
myricetin	85.6	1.35	86.4	1.39	103.8	3.47
kaempferol	739	1.31	742	1.36	99.6	2.43
isorhamnetin	35	1.38	33	1.45	100.8	2.12
chlorogenic acid	134	1.42	132	1.53	97.3	1.97
apigenin	102.5	1.52	104	1.67	101.4	1.87
luteolin	118	1.36	114	1.55	102.9	1.86
caffeic acid	6	1.54	6.3	1.68	96.5	2.74
ellagic acid	793	1.58	784	1.79	97.2	2.32
rosmarinic acid	499	1.28	514	1.47	99.4	1.89

In summary, this study presents a developed and validated method for the simultaneous separation and quantification of six flavonoid aglycones and four phenolic acids in 20 herbal samples. This procedure exhibits a satisfactory separation and uses a simple mobile phase for isocratic elution at ambient temperature at a run time of < 20 min.

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