Optimization of a Quantitative HPLC Determination of Potentially Anticarcinogenic Flavonoids in Vegetables and Fruits

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A rapid method based on RP-HPLC with UV detection is presented for the quantitative determination of five major flavonoid aglycons, viz. quercetin, kaempferol, myricetin, luteolin, and apigenin, in freeze-dried vegetables and fruits, after acid hydrolysis of the parent glycosides. Completeness of hydrolysis and extraction have been optimized by testing systematically different conditions such as acid concentration, reaction period, and methanol concentration in the extraction solution using samples containing various types of flavonoid glycosides. Optimum hydrolysis conditions are presented for flavonol glucuronides, flavonol glucosides, and flavone glycosides. Identity of the flavonoids was confirmed with diode array. Repeatability of the method was good, with coefficients of variation of 2.5–3.1% for quercetin, 4.6–5.6% for kaempferol, 4.6% for myricetin, 3.3% for luteolin, and 2.8% for apigenin. CV of the within-laboratory reproducibility was less than 2 times the CV of repeatability. Recoveries of the flavonols quercetin, kaempferol, and myricetin ranged from 77 to 110%, and recoveries of the flavones apigenin and luteolin ranged from 99 to 106%. The method presented allows a fast, quantitative, and reproducible determination of five flavonoids in freeze-dried foods.

INTRODUCTION

Flavonoids are widely distributed in plant foods and therefore important constituents of the human diet. It has been suggested that humans consume several grams of flavonoids a day (Markham, 1989). Although flavonoids generally are considered to be nonnutritive agents, interest in these substances has arisen because of possible effects on human health. Several studies on the mutagenicity of some major flavonoids, e.g., quercetin, kaempferol, and myricetin, have been published (Bjeldanes and Chang, 1977; Stavric, 1984; van der Hoeven et al., 1984; MacGregor, 1984). Some evidence for carcinogenicity of these flavonoids has been found (Pamucku et al., 1980; Erturk et al., 1984), but these results could not be confirmed in other studies (Saito et al., 1980; Hirono et al., 1981; Morino et al., 1982). Current research shows that these flavonoids have antimutagenic and anticarcinogenic properties in experimental animal studies (Kato et al., 1983; Huang et al., 1983; Fujiki et al., 1986; Mukhtar et al., 1988; Verma et al., 1988; Francis et al., 1989; Deschner et al., 1991). Therefore, it is generally unclear whether flavonoids are beneficial or harmful to humans. Epidemiological studies could be very valuable in assessing the importance of flavonoids to human cancer. However, reliable quantitative data on the occurrence of flavonoids in foods needed for these kind of studies are lacking.

Flavonoids share the common skeleton of diphenylpropanes (C_6–C_3–C_6) (Figure 1). Flavonols and flavones occur in plants usually as *O*-glycosides. The main difference between flavones and flavonols is the presence of a hydroxy group at C3 in flavonols. The occurrence of flavonols and flavones in foods has been reviewed by Herrmann (1976, 1988). Flavonols and flavones occur mainly in the leaves and outer parts of the plants, while only trace amounts are found in parts of the plant below the soil surface. In vegetables, quercetin glycosides predominate, but glycosides of kaempferol, luteolin, and apigenin are also present. Fruits almost exclusively

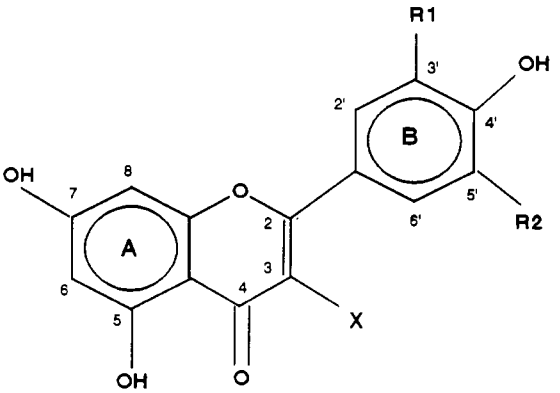

Figure 1. Structure of flavonoids. Flavonols: X = OH; quercetin, R1 = OH, R2 = H; kaempferol, R1 = H, R2 = H; myricetin, R1 = OH, R2 = OH. Flavones: X = H; apigenin, R1 = H, R2 = H; luteolin, R1 = OH, R2 = H.

contain quercetin glycosides, whereas kaempferol and myricetin glycosides are found only in trace quantities (Herrmann, 1988). So far, little attention has been paid to quantitative aspects of the determination of flavonols and flavones in foods, e.g., completeness of hydrolysis and extraction. Furthermore, the quantitative data published were mainly obtained with thin-layer chromatography followed by a spectrophotometric measurement. More recently, Bilyk and co-workers published results of flavonoid analyses in foods based on HPLC and UV detection (Bilyk et al., 1984; Bilyk and Sapers, 1985, 1986). However, hydrolysis conditions were not optimized in these studies.

Quantitative determination of individual flavonoid glycosides in foods is difficult because most reference compounds are not commercially available. Furthermore, more than 50 different glycosides of the most common flavonoids have been described (Herrmann, 1988). Hydrolysis of all glycosides to aglycons offers a practical method for the quantitative determination of flavonoids in foods. Hydrolysis of flavonoids with HCl has been described by Harborne (1965), who performed acid hydrolysis with 2.0 M HCl in boiling 50% aqueous methanol (v/v). Under these conditions flavonol 3-*O*-glucosides are

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Table I. Retention Time t_R, Capacity Factors (k'), and Plate Numbers of Five Flavonoids

		eluent I[a]			eluent II[b]		
peak	compound	t_R, min	k'	plate no.	t_R, min	k'	plate no.
1	myricetin	3.08	2.1	4080	3.45	2.5	1325
2	quercetin	5.78	4.8	5670	6.34	5.3	2080
3	luteolin	5.65	4.7	5330	7.88	6.9	2600
4	apigenin	10.32	9.3	5625	12.9	11.9	3355
5	kaempferol	11.52	10.5	5554	11.53	10.5	3125

[a] Eluent I, 25% acetonitrile in 0.025 M phosphate buffer (pH 2.4). [b] Eluent II, 45% methanol in 0.025 M phosphate buffer (pH 2.4). Detection, 370 nm, 0.01 AUFS. Flow rate, 0.9 mL/min.

hydrolyzed completely within a few minutes, whereas complete hydrolysis of flavonol 3,7- and 4'-*O*-glucuronides takes 60–250 min.

On the basis of data from the literature (Bilyk and Sapers, 1985; Herrmann, 1988) we selected five food samples, viz. lettuce, endive, cranberry, onion, leek, in which the three major flavonols quercetin, myricetin, and kaempferol occur both as the rapidly hydrolyzable glycosides, mainly glucosides, and as the slowly hydrolyzable glucuronides. In lettuce quercetin 3-D-glucuronide predominates and luteolin 3-D-glucuronide is present in trace quantities. In endive kaempferol 3-D-glucuronide predominates. In cranberry high concentrations of quercetin and myricetin 3-D-glucosides are present. Onion has an exceptionally high content of quercetin 4'-D-glucosides, whereas leek contains kaempferol 3-D-glucosides. Celery, containing the flavones apigenin and luteolin 7-apiosyl-D-glucosides, was used as an additional food sample.

In this paper we present a HPLC method for the determination of the above-mentioned flavonoids in freeze-dried vegetables and fruits. Extraction and hydrolysis conditions have been systematically optimized for every food sample by varying acid concentration and reaction period. The effect of flavonoid and glycoside type on hydrolysis conditions could thus be investigated.

MATERIALS AND METHODS

HPLC. Chromatographic separations were performed on a Nova-Pak C_{18} (Waters Associates, Milford, MA) column (3.9 × 150 mm, 4 μm) protected by a Perisorb RP-18 (3.9 × 40 mm, 30–40 μm) guard column. Both columns were placed in a column oven set at 30 °C. The HPLC system consisted of a Kratos (Kratos Analytical Systems, Ramsey, NJ) Spectroflow 400 solvent pump controlled by a Kratos Spectroflow 450 solvent programmer and a Linear (Linear Instrument Corp., Reno, NV) Model 204 UV–vis detector set at 370 nm. A Marathon (Spark Holland, Emmen, The Netherlands) autoinjector was used with a fixed 10-μL loop. Two mobile phases were used. The first mobile phase consisted of 25% acetonitrile in 0.025 M KH_2PO_4 (pH 2.4) with a flow rate of 0.9 mL/min (eluent I). However, since inadequate separation of quercetin and luteolin was achieved, a second eluent consisting of 45% methanol in 0.025 M KH_2PO_4 (pH 2.4) with a flow rate of 0.9 mL/min was used (eluent II). Detector output was sampled using a Nelson (P E Nelson, Cupertino, CA) Series 900 interface and Nelson integrator software (Model 2600, rev. 5.0). Quantification was based on peak area as determined by Nelson. Eluent I was used for quantification of the compounds, whereas eluent II was used for additional peak identification. However, when both quercetin and luteolin were present, eluent II was used for quantification of these flavonoids. A Hewlett-Packard (Palo Alto, CA) Model 1040 A photodiode array UV–vis detector was used to record UV spectra of the flavonoids in samples on-line. Spectra were recorded upslope, apex, and downslope (220–450 nm, 2-nm steps, sampling interval 1280 ms). The spectra of each peak were superimposed after subtraction of the corresponding baseline spectrum. Peaks were considered to be pure when there was exact correspondence among the spectra (peak purity match >990). Similarly, peak identity was confirmed by superimposing the spectrum of each peak with the corresponding standard spectrum (peak identity match >990) and by comparison of retention times (time window 0.5%) in both eluents. Peaks were only quantitated if they matched the above-mentioned criteria.

Sample Preparation. Fresh lettuce (*Lactuca sativa* L), leek (*Allium porrum* L), celery (*Apium graveolens* L), onion (*Allium cepa* L), endive (*Chicorium endivia* L), and cranberries (*Vaccinium macrocarpon* Ait.) were purchased in December at a local supermarket and prepared the same day. The whole foods were cleaned, chopped under liquid nitrogen, and immediately stored at −20 °C until lyophilized (Delta, condenser temperature −45 °C, pressure 0.01 mbar). The outer dry skin of onions was removed before cleaning. After lyophilization, the freeze-dried tissues were ground to pass a 0.5-mm sieve and allowed to equilibrate in open air. Percentage of moisture was measured (80 °C, vacuo). The food samples were stored at −20 °C until analyzed.

Standards. Flavonoid standards were purchased from Fluka (myricetin 70050, quercetin dihydrate 83370, kaempferol 60010, and apigenin 10790) and from Roth (luteolin 5801). The standards were dissolved in methanol to a concentration of 500 μg/mL and stored at 4 °C. Every week stability of the compounds in methanol was checked spectrophotometrically at 375 (flavonols) and 340 nm (flavones) after dilution to 10 μg/mL in methanol. Standard solutions proved to be stable for over 3 months at 4 °C. An exception was myricetin, which deteriorated by approximately 10% after 1 month. Calibration curves of the standards ranging from 0.5 to 25 μg/mL were constructed for both eluents. As peak height, peak shape, and retention time are dependent on the composition of the injection solution, we decided to match standard and sample solution. Standard stock solutions were diluted in 20 mL of 62.5% aqueous methanol to which 2 g/L of antioxidant *tert*-butylhydroquinone (TBHQ) was added. To this solution was added 5 mL of 6 M HCl, and the solution was subsequently made up to 50 mL with methanol. All compounds had linear calibration curves (peak area vs concentration) through the origin. R^2 values exceeded 0.9995. The limit of detection was defined as the amount of flavonoid which resulted in a peak height 3 times the standard deviation of the baseline noise.

Extraction and Hydrolysis. Unless otherwise stated, extracts were prepared as follows: 40 mL of 62.5% aqueous methanol (2 g/L TBHQ) was added to 0.500 g of freeze-dried sample material. To this extract was added 10 mL of 6 M HCl with careful mixing. The extraction solution thus obtained consisted of 1.2 M HCl in 50% aqueous methanol (v/v). After refluxing at 90 °C for 2 h with regular swirling, the extract was allowed to cool and was subsequently made up to 100 mL with methanol and sonicated for 5 min. Approximately 2 mL was filtered through a 0.45-μm filter for organic solvents (Acrodisc CR PTFE, Gelman) prior to injection.

RESULTS

Eluents I and II, described under HPLC, were both tested for separation efficiency. Standard working solutions were made by diluting the five stock solutions to 5 μg/mL as described before. The working solutions were injected onto the column which was previously equilibrated with the eluent for 60 min. As can be seen from Table I, eluent I yielded, with similar capacity factors, much higher plate numbers compared to those with eluent II. Interestingly, the elution order of the pair luteolin and quer-

Table II. Influence of Reaction Period, Acid Type, and Acid Concentration on Quercetin Yield[a] in Lettuce

	H_2SO_4		HCl	
reaction period, h	0.3 M	0.6 M	0.6 M	1.2 M
1	70 mg/kg, 27%	141 mg/kg, 55%	174 mg/kg, 68%	216 mg/kg, 85%
2	108 mg/kg, 42%	198 mg/kg, 78%	185 mg/kg, 73%	230 mg/kg, 90%
4	134 mg/kg, 53%	209 mg/kg, 82%	204 mg/kg, 80%	245 mg/kg, 96%
6	181 mg/kg, 71%	231 mg/kg, 90%	209 mg/kg, 82%	255 mg/kg, 100%

[a] Results are expressed in mg/kg of dry weight and as percentage of maximum yield found in this sample. Mean for duplicate determination.

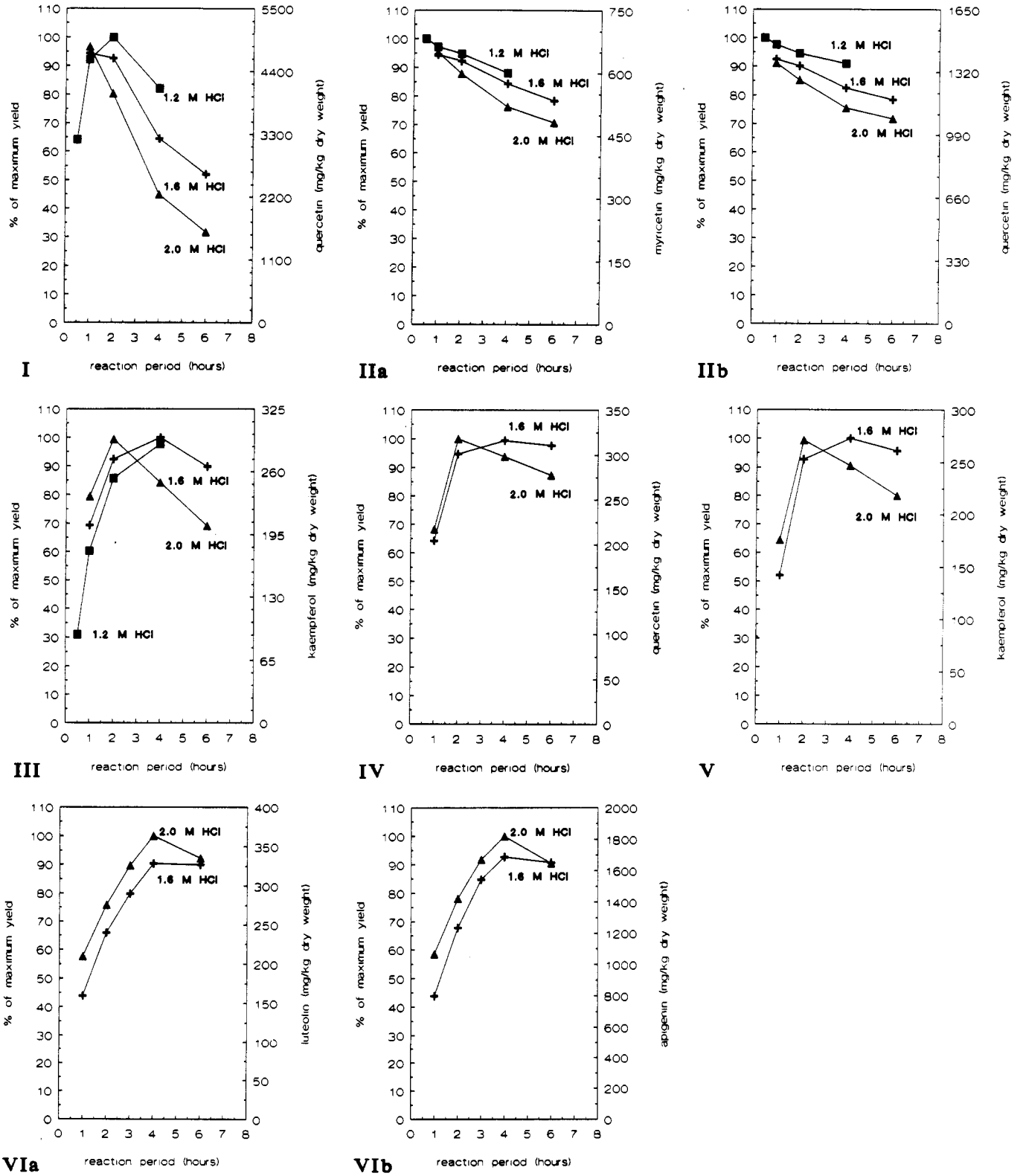


Figure 2. Influence of acid concentration (1.2, 1.6, 2.0 M HCl) and reaction period (0.5, 1, 2, 4, 6 h) on flavonoid yield in onion (I), cranberry (IIa,b), leek (III), lettuce (IV), endive (V), and celery (VIa,b). All graph points are the mean of a duplicate determination. Results are expressed as percentage of the highest yield found in that food sample and as milligrams per kilogram of dry weight (right *y* axis).

Table III. Flavonoid Content[a] and Corresponding Optimum Hydrolysis Conditions in Six Food Samples

sample	conditions	mg/kg of dry wt				
		quercetin	kaempferol	myricetin	luteolin	apigenin
cranberry	1.2 M HCl/0.5 h	1485	<20[c]	662	<10[c]	<40[c]
onion	1.2 M HCl/2 h	5076	<20[c]	<10[c]	<10[c]	<40[c]
leek	1.6 M HCl/4 h	20[b]	295	<10[c]	<10[c]	<40[c]
lettuce	2.0 M HCl/2 h	319	<20[c]	<10[c]	<10[c]	<40[c]
endive	1.6 M HCl/4 h	<10[c]	271	<10[c]	<10[c]	<40[c]
celery	2.0 M HCl/4 h	<10[c]	<20[c]	<10[c]	358	1787

[a] Mean for duplicate determination. [b] Conditions: 1.2 M HCl/2 h. [c] Below limit of detection.

cetin and the pair apigenin and kaempferol is reversed in eluent I compared to that in eluent II, indicating a different selectivity of both modifiers for flavonols and flavones. Analyses of samples revealed that eluent I resulted in less interfering peaks compared to eluent II (Figures 3 and 4). However, with eluent I quercetin and luteolin could not be separated. Therefore, we decided to apply eluent I for quantification and eluent II for additional peak identification and for quantification only when both quercetin and luteolin were present. However, as we did not find both quercetin and luteolin in the same product, this proved not to be necessary for the food samples investigated in this study.

Wildanger and Herrmann (1973) reported the use of 1% sulfuric acid in methanol for hydrolysis of flavonoid glycosides in plant materials. Accordingly, we investigated the effects of sulfuric acid and hydrochloric acid on hydrolysis of quercetin 3-D-glucuronides in lettuce. Lettuce was prepared and analyzed as described before, taking care that the water/methanol ratio was kept equal for both acids. As can be seen from Table II, higher yields were found with HCl compared to H_2SO_4. All further hydrolysis experiments were thus carried out with hydrochloric acid.

Optimization of Extraction and Hydrolysis. The six food samples were prepared and analyzed as described before. Three hydrochloric acid concentrations were tested (1.2, 1.6, and 2.0 M), and the reaction period was varied (0.5, 1, 2, 4, and 6 h) in the procedure described under Extraction and Hydrolysis. The lowest acid concentration and the shortest reaction period were omitted in the analysis of lettuce, endive and celery. Determinations at each HCl concentration and reaction period were carried out in duplicate for each food sample.

Henning (1980) reports that flavonoid glycosides are more soluble in water and flavonoid aglycons more soluble in methanol. Extraction efficiency could thus depend on the water/methanol ratio. In a preliminary experiment, two additional extraction solutions with methanol concentrations of 20 and 80% were tested in the analysis of onion, endive, and celery. Care was taken to keep acid concentration in all extraction solutions equal. It appeared that extraction was most efficient with 50% aqueous methanol. Flavonoid levels were up to 30% higher compared to 20 and 80% aqueous methanol (results not shown). All further experiments were thus carried out with 50% aqueous methanol (v/v).

Stability of the flavonoid standards under hydrolysis conditions was tested. It appeared that all flavonoids (5 μg/mL) were stable in a solution consisting of 2.0 M HCl in 50% aqueous methanol (v/v) (2 g/L TBHQ) with boiling for up to 6 h. Losses were less than 5% (results not shown).

The influence of acid concentration and reaction period on flavonoid yield in the six food samples is presented in Figure 2. Highest amounts found are summarized in Table III together with the corresponding hydrolysis condition.

Cranberry, Onion, Leek: Glucosides of Quercetin, Kaempferol, and Myricetin. In onion, we found a high amount of quercetin (5076 mg/kg of dry weight), but no kaempferol could be detected. The highest yield was found using 1.2 M HCl and a reaction period of 2 h (Figure 2I). Increasing acid concentration and reaction time led to a significant degradation of quercetin. After 6 h, quercetin had deteriorated by approximately 70% using 2.0 M HCl. Chromatograms of the onion sample analyzed with both eluents are shown in Figure 3. A small unknown peak with a retention time corresponding to luteolin (t_R = 8.25 min) was detected in eluent II. However, the spectrum of this peak did not match the spectrum of luteolin (Figure 4).

We found quercetin (1485 mg/kg of dry weight) and myricetin (662 mg/kg of dry weight) in cranberry. As can be seen in Figure 2IIa,b, the highest yields were found with 1.2 M HCl and a reaction period of 30 min. An increased acid concentration and an increased reaction period led to degradation of both quercetin and myricetin. After 6 h, a loss of up to 20% of myricetin and quercetin was observed.

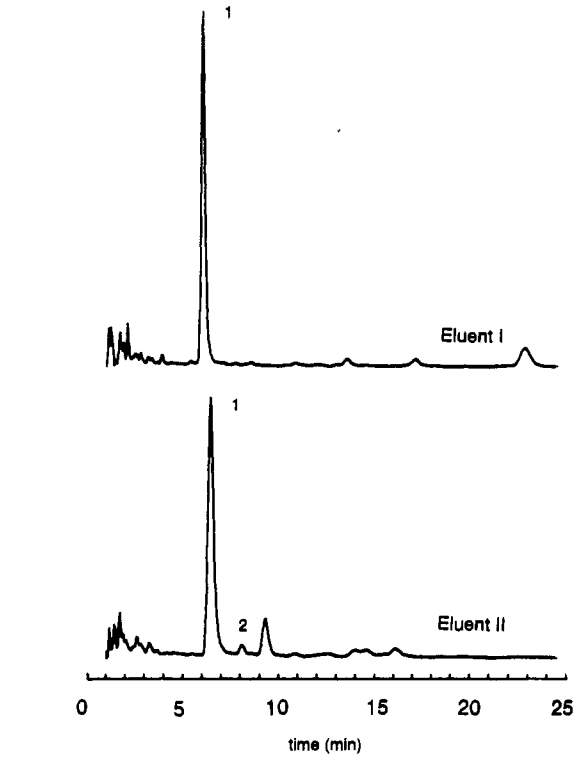
Kaempferol (295 mg/kg of dry weight) was detected in leek. Results are summarized in Figure 2III. Highest levels of kaempferol were found using 1.6 M HCl and a reaction period of 4 h. We also detected trace quantities of quercetin (20 mg/kg of dry weight) when both 1.2 M HCl and a reaction period of 2 h and 1.6 M HCl and a reaction period of 1 h were used. Quercetin was only detected with eluent I, and due to the low level, identification was only tentative. At more severe hydrolysis conditions the quercetin peak disappeared.

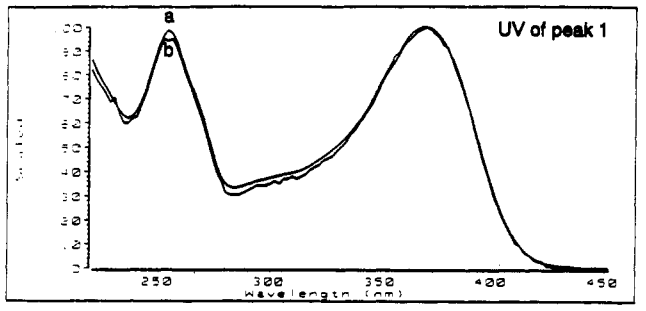
Lettuce, Endive: Glucuronides of Quercetin and Kaempferol. Only quercetin (319 mg/kg of dry weight) was detected in lettuce. Figure 2IV shows that the highest yield was found using 2.0 M HCl and a reaction period of 2 h. Similar yields were found using 1.6 M HCl and a reaction period of 4 h. Increasing the reaction period up to 6 h with 2.0 M HCl led to a degradation of quercetin of approximately 10%. No luteolin was detected in this sample.

In endive, only kaempferol (271 mg/kg of dry weight) was found. As shown in Figure 2V the highest yield was found using 2.0 M HCl and a reaction period of 2 h. Similar yields were found with 1.6 M HCl and a reaction period of 4 h. No significant additional yield could be gained by increasing the reaction period and/or hydrochloric acid concentration (2.5 M HCl, not shown).

Lettuce and endive samples were also treated with a (nonspecific) β-glucuronidase solution (Fluka 49310) using a method described by Kunzeman and Herrmann (1977). The amounts of quercetin and kaempferol liberated by β-glucuronidase in lettuce and endive corresponded well with the amounts obtained after acid hydrolysis. About 69% of the glycosides in onion were liberated with β-glucuronidase (Table IV).

The presence of flavonol glucuronides in both samples was confirmed by a method described by Sagara (1985). Flavonol glucuronides can be separated from other fla-





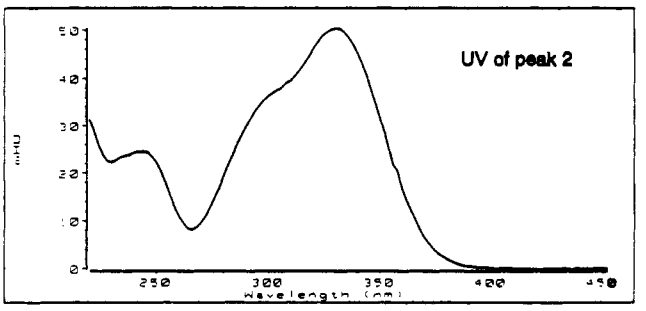
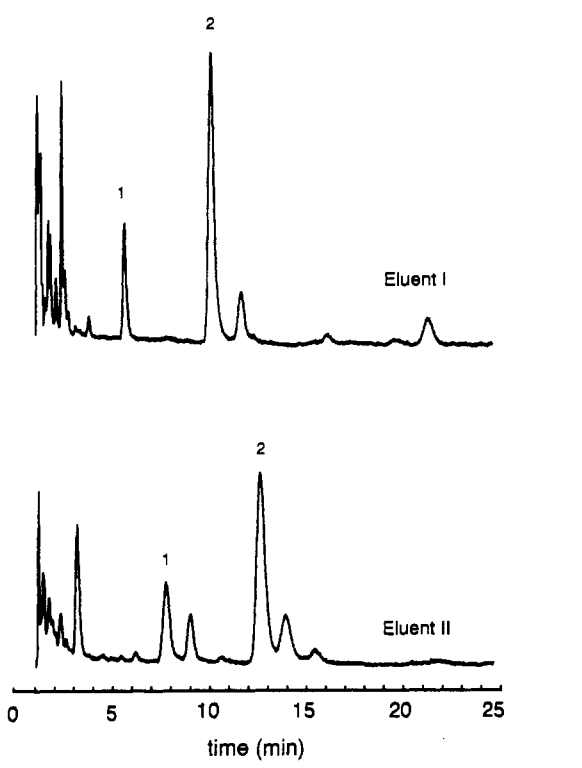


Figure 3. Typical chromatograms of an onion extract in eluents I [25% acetonitrile in phosphate buffer (pH 2.4)] and II [45% methanol in phosphate buffer (pH 2.4)]. Hydrolysis conditions: 1.2 M HCl/2 h (for details see text). Peaks: 1, quercetin; 2, unknown. Detection: 370 nm; 0.01 AUFS. Flow rate: 0.9 mL/min. Also shown are the diode array spectrum of quercetin standard (a) and quercetin in onion (b), both recorded in eluent I, and the spectrum of unknown peak 2 at t_R = 8.25 min, recorded with eluent II.

vonoid compounds by adding an ion-pair reagent [5 mM tetra-*n*-pentylammonium bromide (TPAB)] to the eluent. At pH 4.0 only flavonol glucuronides will be partially ionized. When an ion-pair reagent is added, the retention of flavonol glucuronides will be enhanced, whereas the retention of the other, nonionized glycosides will remain virtually unchanged. After addition of the ion-pair reagent, the retention time of the major quercetin glycoside peak in lettuce changed from 8.8 to 18.2 min and the retention time of the major kaempferol glycoside peak



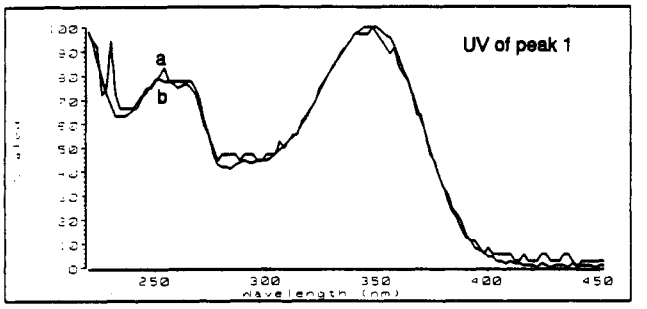


Figure 4. Typical chromatograms of a celery extract in eluents I and II (see Figure 3). Hydrolysis conditions: 2.0 M HCl/4 h (for details see text). Peaks: 1, luteolin; 2, apigenin. Detection: 370 nm; 0.01 AUFS. Flow rate: 0.9 mL/min. Also shown is the diode array spectrum of luteolin standard (a) and luteolin in celery (b), both recorded in eluent I.

in endive changed from 10.2 to 18.6 min. This confirmed that both glycosides are presumably glucuronides (Table IV).

Celery: Glucosides of Apigenin and Luteolin. Celery was prepared and analyzed as described before. An additional reaction period of 3 h was used. We found luteolin (358 mg/kg of dry weight) and apigenin (1787 mg/kg of dry weight). Both gave the highest yields when 2.0 M HCl and a reaction period of 4 h were used. Increasing the reaction period did not increase the flavone yield. Results are shown in Figure 2VIa,b. Typical chromatograms of a celery extract using both eluents are shown in Figure 4 together with the spectrum of the luteolin peak provided by diode array.

Precision. For quality control of future analysis of vegetables and fruits, control samples were composed of the foods investigated in this study containing the various flavonoid glycosides. Control sample A containing flavonol glucosides was made by mixing onion and leek (1:9 w/w). Unfortunately, addition of cranberry for myricetin glucosides to this control sample led to low flavonol levels and interfering peaks. Thus, a separate control sample for myricetin (cranberry, control sample B) was necessary. Lettuce and endive were mixed (1:1 w/w) to a control

Table IV. Influence of Ion-Pair Reagent (5 mM TPAB) on Retention Times[a] of Flavonol Standards, Glucosides, and Glucuronides

sample	component	t_R, min without ion pair	t_R, min with ion pair	mg/kg of dry wt after hydrolysis: enzymatic	mg/kg of dry wt after hydrolysis: acid
standard	quercetin	15.6	17.2		
standard	kaempferol	18.2	19.0		
lettuce[b]	quercetin glucuronide	8.8	18.2	23	27
endive	kaempferol glucuronide	10.4	18.6	282	271
onion[b]	quercetin glucoside	11.8	14.2	1973	2880

[a] Eluent A, 0.025 M phosphate buffer (pH 4.0)/methanol (90/10 v/v). Eluent B, 0.025 M phosphate buffer (pH 4.0)/methanol (20/80 v/v). Gradient, 20–70% B in 16 min. Detection, 370 nm, 0.02 AUFS. Flow rate, 0.9 mL/min. Comparison between flavonol yield with enzymatic hydrolysis and acid hydrolysis (for conditions see text). [b] Samples not identical with samples reported in Table III.

Table V. Mean Content, Coefficients of Variation (CV), and Recoveries of Four Control Samples

sample[a]	conditions	component	mean content $n = 10$, mg/kg of dry wt	CV %[c]	CV %[d]	recovery,[b] % 100% add	recovery,[b] % 50% add
control sample A (onion/leek)	1.2 M HCl/2-h hydrolysis	quercetin	381.4	2.5	4.9	88	98
		kaempferol	235.6	5.6	11.8	102	110
control sample B (cranberry)	1.2 M HCl/2-h hydrolysis	quercetin	1411.4	3.1	4.1	91	91
		myricetin	601.6	4.6	6.1	94	88
control sample C (endive/lettuce)	2.0 M HCl/2-h hydrolysis	quercetin	121.4	3.1	7.6	77	94
		kaempferol	142.4	4.6	8.3	96	102
control sample D (celery)	2.0 M HCl/4-h hydrolysis	apigenin	1783.2	2.8	4.1	99	104
		luteolin	333.4	3.3	4.6	101	106

[a] Composition of control samples is described under heading precision. [b] Mean for duplicate determination. [c] Within-laboratory repeatability ($n = 10$). [d] Within-laboratory reproducibility ($n = 5$).

sample (C) containing flavonol glucuronides. Furthermore, celery was used as a control sample (D) for flavone glucosides. Repeatability of the method within our laboratory was determined by 10 analyses of these four control samples. Hydrolysis and extraction conditions were based on the optimization curves established (Figure 2). However, as optimum conditions differ for all of the foods investigated, we decided to define three "optimum" conditions depending on the following flavonoid and glycoside types: flavonol glucosides, flavonol glucuronides, and flavone glucosides. For flavonol glucosides (control samples A and B) this choice (2-h hydrolysis with 1.2 M HCl) was a compromise leading to underestimations of the myricetin and kaempferol content of less than 5 and 15%, respectively. Recoveries were measured in the control samples by spiking pure standards to the extraction solutions at two levels (50 and 100% of the measured flavonoid content) prior to sample analysis. Within-laboratory reproducibility was established by five duplicate analyses on separate days within a period of 1 month. Results, together with the hydrolysis conditions, are shown in Table V.

DISCUSSION

From our results it clearly appears that completeness of hydrolysis depends on both reaction period and acid concentration. It is noteworthy that suboptimal hydrolysis conditions could therefore lead to an underestimation of up to 50% of the true flavonoid level in foods. In samples with flavonol glucosides, an optimum between flavonol yield and flavonol degradation was observed as prolonged extraction and hydrolysis showed a decrease in the flavonol contents measured (Figure 2I–III). A reaction period of more than 4 h with 1.6 M HCl in general led to loss of flavonols. This effect was enhanced when 2.0 M HCl was used in the extraction medium. However, flavonol degradation could not be observed in standard solutions treated under identical conditions. Therefore, the presence of some unknown compounds in the sample matrix accelerating the degradation of flavonoids should be considered.

Identical optimum hydrolysis conditions for flavonol glucosides in leek, onion, and cranberry could not be achieved. Time required for complete hydrolysis is dependent on the binding site of the sugar on the flavonoid nucleus: C7 > C4′ > C3 (Harborne, 1965). Glucose moieties of flavonol glucosides in cranberry are bound to C3 and in onion to C4′ (Herrmann, 1976). This might explain different optimum conditions found for, e.g., quercetin in onion and quercetin in cranberry. However, long hydrolysis conditions needed for complete hydrolysis of kaempferol 3-glucosides presumably present in leek could not be explained. Other kaempferol glycosides may be present in leek. Possibly, matrix composition is an important factor determining hydrolysis rate.

The following hydrolysis conditions for flavonoid glycosides are proposed. For analysis of foods containing predominantly flavonol glucuronides an extraction medium consisting of 2.0 M HCl in boiling 50% aqueous methanol (v/v) and a reaction period of 2 h will result in complete hydrolysis of flavonols. Similar results are found with 1.6 M HCl and a reaction time of 4 h. Foods containing flavonol glucosides should be hydrolyzed with 1.2 M HCl in boiling 50% aqueous methanol (v/v) with a reaction period not exceeding 2 h. Under these conditions possible degradation of flavonol 3-glucosides is less than 10%. Eventually, a second analysis under identical conditions with a reaction period of 4 h should be applied for quantification of kaempferol glucosides. Flavone glucosides are more resistant to acid hydrolysis. Complete hydrolysis of flavones takes 4 h; the extraction medium consists of 2.0 M HCl in 50% aqueous methanol (v/v).

The method employed to confirm the presence of flavonol glucuronides as the major glycosides in lettuce and endive was shown to be sufficiently specific to distinguish between flavonol glucuronides and glucosides. As the available β-glucuronidase preparations lack specificity (Markham, 1989), an enzymatically based distinction of these glycosides is not possible. Interestingly, amounts liberated by enzymatic hydrolysis corresponded well with the amounts found with the optimized acid hydrolysis.

Table VI. Comparison of Flavonoid Levels in Foods Found in the Present Study and Levels Reported by Herrmann and Co-workers and Bilyk and Co-workers

		mg/kg of fresh wt[a]		
product	compound	present study	Herrmann and co-workers	Bilyk and co-workers
lettuce	quercetin	9	6–273[b]	1–38[c]
leek	kaempferol	31	90–200[d]	20[c]
	quercetin	2	10–25[d]	ND[e]
onion	quercetin	544[f]	104–1260[g]	15–62[h]
	kaempferol	<2.5[f]	21–235[g]	3–7[h]
cranberry	quercetin	172	NA[i]	73–250[j]
	myricetin	77	NA	4–2[j]
endive	kaempferol	18	150[b]	NA
celery	apigenin	108	75[d]	NA
	luteolin	22	14[d]	NA

[a] In the whole foods unless otherwise stated. [b] Outer leaves (Wöldecke and Herrmann, 1974). [c] Green portion (leek) (Bilyk and Sapers, 1985). [d] Herrmann (1976). [e] ND, not detectable. [f] Outer dry skin removed. [g] Open air (Starke and Herrmann, 1976). [h] Edible portions (Bilyk et al., 1984). [i] NA, no data available. [j] Bilyk and Sapers (1986).

In general, recoveries of flavonoids in the samples spiked with 50 and 100% of the original level were good. However, in control sample B recoveries of myricetin spiked with 50% was somewhat inferior (88%). Probably, hydrolysis conditions were too severe. This also resulted in a lower mean level compared to the level found under optimal conditions in the optimization experiments (Table V). In control sample C, recovery of quercetin was rather poor, possibly due to the low level of quercetin in that sample.

Coefficients of variation of the repeatability and within-laboratory reproducibility were good, being less than 5 and 9%, respectively. Repeatability of kaempferol in control sample A is somewhat poor because hydrolysis conditions applied were not optimal. This is also reflected in the CV of within-laboratory reproducibility.

In general, our findings compare well with findings reported earlier by Herrmann and co-workers and Bilyk and co-workers (Table VI). Differences found may be due to varietal or seasonal differences or to the fact that hydrolysis conditions were optimized in our study but not in the studies reported by Herrmann (1976, 1988) and Bilyk et al. (Bilyk and Sapers, 1985, 1986; Bilyk et al., 1984).

The HPLC column in this study, used for over 1 year on an almost daily basis, did not show any significant loss of plate numbers during this period. However, a long preconditioning period was necessary due to a high absorption of the new column. This period could be shortened by repeated injections of a concentrated quercetin solution. Furthermore, it was necessary to wash injection needle and tubings regularly with water to avoid blockings and damage to the autoinjector from the acid.

In conclusion, the method presented here allows a fast, quantitative, and reproducible determination of the five flavonoid aglycons in freeze-dried foods after acid hydrolysis. As a large number of different glycosides are present in foods, the quantitative determination of individual flavonoid glycosides in vegetables and fruits commonly consumed would be complicated. In addition, because of the low levels of individual flavonoid glycosides in foods and the limitations indicated in the Introduction, only analysis of the aglycons after hydrolysis proved to be a practical method for the quantitative determination of flavonoids in foods. Currently, the above-described method is applied in the study of a large group of fruits and vegetables at our laboratory.

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LITERATURE CITED

Bilyk, A.; Sapers, G. M. Distribution of quercetin and kaempferol in lettuce, kale, chive, garlic chive, leek, horseradish, red radish and red cabbage tissues. *J. Agric. Food Chem.* **1985**, *33*, 226–228.

Bilyk, A.; Sapers, G. M. Varietal differences in the quercetin, kaempferol, and myricitin contents of highbush blueberry, cranberry, and thornless blackberry fruits. *J. Agric. Food Chem.* **1986**, *34*, 585–588.

Bilyk, A.; Cooper, P. L.; Sapers, G. M. Varietal differences in distribution of quercetin and kaempferol in onion (*Allium cepa* L.) tissue. *J. Agric. Food Chem.* **1984**, *32*, 274–276.

Bjeldanes, L. F.; Chang, G. W. Mutagenic activity of quercetin and related compounds. *Science (Washington, D.C.)* **1977**, *197*, 577–578.

Deschner, E. E.; Ruperto, J.; Wong, G.; Newmark, H. L. Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. *Carcinogenesis* **1991**, *7*, 1193–1196.

Erturk, E.; Hatcher, J. F.; Nunoya, T.; Pamucku, A. M.; Bryan, G. T. Hepatic tumors in Sprague-Dawley (SD) and Fischer 344 (F) female rats chronically exposed to quercetin (Q) or its glycoside rutin (R). *Proc. Am. Assoc. Cancer Res.* **1984**, *25*, 95–97.

Francis, A. R.; Shetty, T. K.; Bhattacharya, R. K. Modifying role of dietary factors on the mutagenicity of aflatoxin B1: in vitro effect of plant flavonoids. *Mutat. Res.* **1989**, *222* (4), 393–401.

Fujiki, H.; Horiuchi, T.; Yamashita, K.; Hakii, H.; Suganuma, M.; Hishino, H.; Iwashima, A.; Hirata, Y.; Sugimura, T. Inhibition of tumor promotion by flavonoids. In *Plant flavonoids in biology and medicine: biochemical, pharmacological and structure-activity relationships* (Proceedings of a symposium held in Buffalo, NY, July 22–26, 1986); Cody, V., Middleton, E., Harborne, J. F., Eds.; Liss: New York, 1986; Vol. 213, 429–440.

Harborne, J. B. Plant polyphenols XIV. Characterisation of flavonoid glycosides by acidic and enzymic hydrolyses. *Phytochemistry* **1965**, *4*, 107–120.

Henning, W. HPLC determination of flavonol glycosides present in plums, cherries, peaches, and apricots. Ph.D. Dissertation, University of Hannover, Federal Republic of Germany, 1980.

Herrmann, K. Flavonols and flavones in food plants. A review. *J. Food Technol.* **1976**, *11*, 433–448.

Herrmann, K. On the occurrence of flavonol and flavone glycosides in vegetables. *Z. Lebensm. Unters. Forsch.* **1988**, *186*, 1–5.

Hirono, I.; Ueno, I.; Hosaka, S.; Takanashi, H.; Matsushima, T.; Sugimura, T.; Natori, S. Carcinogenicity examination of quercetin and rutin in ACI rats. *Cancer Lett.* **1981**, *13*, 15–21.

Huang, M-T.; Wood, A. W.; Newmark, H. L.; Sayer, J. M.; Yagi, H.; Jerina, D. M.; Conney, A. H. Inhibition of the mutagenicity of bay-region diol-epoxides of polycyclic aromatic hydrocarbons by phenolic plant flavonoids. *Carcinogenesis* **1983**, *4*, 1631–1637.

Kato, R.; Nakadate, T.; Yamamoto, S.; Sugimura, T. Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion and ornithine decarboxylase activity by quercetin: possible involvement of lipoxygenase inhibition. *Carcinogenesis* **1983**, *4*, 1301–1305.

Kunzeman, J.; Herrmann, K. Isolation and Identification of flavon(ol)-O-glycosides in Caraway (*Carrum carvi* L.), Fennel (*Foeniculum vulgare* Mill), Anise (*Pimpinella anisum* L.), and Coriander (*Coriandrum sativum* L.) and of flavon-C-glycosides in Anise. *Z. Lebensm. Unters. Forsch.* **1977**, *164*, 194–200.

MacGregor, J. T. Genetic and carcinogenic effects of plant flavonoids: an overview. *Adv. Exp. Med. Biol.* **1984**, *177*, 497–526.

Markham, K. R. Flavones, Flavonols and their glycosides. *Methods Plant Biochem.* **1989**, *1*, 197–235.

Morino, K.; Matsukura, N.; Ohgaki, H.; Kawachi, T.; Sugimura, T.; Hirono, I. Carcinogenicity test of quercetin and rutin in golden hamsters by oral administration. *Carcinogenesis* **1982**, *3*, 93–97.

Mukhtar, H.; Das, M.; Khan, W. A.; Wang, Z. Y.; Bik, D. P.; Bickers, D. R. Exceptional activity of tannic acid among naturally occurring plant phenols in protecting against 7,12-dimethylbenz(a)anthracene-, benzo(a)pyrene-, 3-methylcholanthrene-, and N-methyl-N-nitrosourea-induced skin tumorgenesis in mice. *Cancer Res.* **1988**, *48*, 2361–2365.

Pamucku, A. M.; Yalçiner, S.; Hatcher, J. F.; Bryan, G. T. Quercetin, a rat intestinal and bladder carcinogen present in bracken fern (*Pteridium aquilinium*). *Cancer Res.* **1980**, *40*, 3468–3472.

Sagara, K.; Ito, Y.; Oshima, T.; Misaki, T.; Murayama, H. Simultaneous determination of baicalein, wogonin, oroxylin-a and their glucuronides in *scuttellariae radix* by ion-pair HPLC. *J. Chromatogr.* **1985**, *328*, 289–297.

Saito, D.; Shirai, A.; Matsushima, T.; Sugimura, T.; Hirono, I. Test of carcinogenicity of quercetin, a widely distributed mutagen in food. *Teratog., Carcinog., Mutagen.* **1980**, *1*, 213–221.

Starke, H.; Herrmann, K. Flavonols and flavones of vegetables. VII. Flavonols of leek, chive, and garlic. *Z. Lebensm. Unters. Forsch.* **1976**, *161*, 25–30.

Stavric, B. Mutagenic food flavonoids. *Fed. Proc.* **1984**, *43* (9), 2454–2458.

van der Hoeven, J. C. M.; Bruggeman, I. M.; Debets, F. M. H. Genotoxicity of quercetin in cultured mammalian cells. *Mutat. Res.* **1984**, *136*, 9–21.

Verma, A. K.; Johnson, J. A.; Gould, M. N.; Tanner, M. A. Inhibition of 7,12-dimethylbenz(a)anthracene and N-nitrosomethylurea induced rat mammary cancer by dietary flavonol quercetin. *Cancer Res.* **1988**, *48*, 5754–5788.

Wildanger, W.; Herrmann, K. Qualitative identification and quantitative determination of flavonols and flavones. *J. Chromatogr.* **1973**, *76*, 433–440.

Woeldecke, M.; Herrmann, K. Isolation and identification of flavon(ol) glycosides in Endive and Lettuce. *Z. Naturforsch.* **1974**, *29C*, 355–359.

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Registry No. Myricetin, 529-44-2; quercetin, 117-39-5; luteolin, 491-70-3; apigenin, 520-36-5; kaempferol, 520-18-3; quercetin 3-D-glucuronide, 22688-79-5; kaempferol 3-D-glucoronide, 22688-78-4.