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Orange and Mandarin Peel Oils Differentiation Using Polymethoxylated Flavone Composition

Emile M. Gaydou,* Jean-Pierre Bianchini,[1] and Robert P. Randriamiharisoa

Six *Citrus* methoxyflavones have been separated and quantitatively determined by normal-phase HPLC using a LiChrosorb Si60 packed column and heptane–isopropyl alcohol eluent system. The response factors were determined with a UV detector at 280 nm. The methoxyflavone composition of peel oils was determined for 15 various samples obtained from mandarins (*Citrus reticulata*) and oranges (*Citrus sinensis*). The major components were tangeretin (0.5–2.8 $g \cdot L^{-1}$), heptamethoxyflavone (0.2–2.7 $g \cdot L^{-1}$), and nobiletin (0.4–2.0 $g \cdot L^{-1}$). The six flavones and the sum of flavones were used in standardized principal-component analysis for the sample classification. A taxonomic significance of the content of these flavones was shown by factorial discriminant analysis for the differentiation of *C. reticulata* and *C. sinensis*.

Polymethoxylated flavones (PMF) are found in high concentration in peel and in low amounts in juice of some *Citrus* species (Veldhuis et al., 1970). Tangeretin (5,6,7,8,4′-pentamethoxyflavone, **1**) was crystallized from tangerine oil by Nelson (1934). Tetra-*O*-methylscutellarein (5,6,7,4′-tetramethoxyflavone, **2**) and heptamethoxyflavone (3,5,6,7,8,3′,4′-heptamethoxyflavone, **3**) were reported and identified by Swift (1967) in the neutral fraction of orange peel oil. Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, **4**) was first isolated from orange peel by Tseng (1938). Thirteen flavonoids were isolated and identified from Valencia orange and Robinson tangerine peel and peel juice (Tatum and Berry, 1972). Among them a minor component (3,5,6,7,3′,4′-hexamethoxyflavone) was characterized by TLC. 5,6,7,3′,4′-Pentamethoxyflavone (**6**), first isolated by Born (1960), was named by Swift (1964) as sinensetin. Because of their important physiological response in higher animals (Kupchan et al., 1965; Robbins, 1977; Mabry and Ulubelen, 1980), the separation and quantitation of these compounds has been done via TLC–spectrophotometric procedure (Veldhuis et al., 1970) and by HPLC procedures (Ting et al., 1979; Bianchini and Gaydou, 1981, 1983). Since the relative flavonoid composition varies with each variety, the composition of PMF in *Citrus* juices has been proposed as a measure of juice authenticity (Kefford and Chandler, 1970). The presence of various PMF from leaf extracts was used to distinguish between nucellar and zygotic seedlings (Tatum et al., 1978). Quantitative as well as qualitative differences in PMF content from the juices of different *Citrus* varieties were found by Ting et al. (1979).

Since PMF are found particularly in the peel oil of two *Citrus* species, orange (*Citrus sinensis*) and mandarin (*Citrus reticulata*), the purpose of this work was to study the range of PMF content in 15 peel oil samples obtained from 14 cultivars. The determination using an internal standard of the main PMF cited above (**1–6**) was achieved by a normal-phase HPLC procedure. Multivariate statistical techniques that have been successfully applied in enological research (Kwan and Kowalski, 1980; Noble et al., 1980) or in lipid research (Gaydou et al., 1984) were used to distinguish peel oils of oranges from those of mandarins.

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EXPERIMENTAL SECTION

Citrus Oil Preparation. The fruits were collected in the Station de Recherches Agronomiques of San Giulano (Corsica, France) in 1981, and the botanical origins of the various cultivars were guaranteed. About 30–60 fruits harvested on three to five trees were used for the preparation of the *Citrus* peel oils. The 15 samples of *Citrus* peel oils were prepared by expression of the oil from the ground peel in a hydraulic press.

Standards. The six flavones used as standards given in Table I were isolated from industrial orange oils as previously described (Bianchini and Gaydou, 1981). The standards were chromatographed separately and in combinations to determine their capacity factors and their identities. At least four to eight injections of various mixtures of PMF were made, and two to four results were obtained for each flavones. The response factor of each flavone was determined, for quantitative estimation of these components in the samples, with 4-methoxyacetophenone (Fluka, Buchs, Switzerland) as internal standard.

Chromatography. All chromatographic solvents were spectrograde quality from Merck (Darmstadt, GFR) and were dried in the usual manner and distilled. The solvents were degassed on a sonicator for 5 min. Water concentration (0.1%) was obtained by addition of a known volume of deionized water to 1 L of anhydrous solvent. The water content was controlled by Karl-Fisher determination. The HPLC equipment consisted of an Orlita (Giessen, GFR) DMP AE 1044 dual-stroke pump and an ISCO (Lincoln, NE) Model UA 5 dual-beam UV–visible detector at 280 nm. Injections were carried out with a Rheodyne 70-10 valve equipped with a 10-μL loop. The response factors (RF) were determined on solutions of flavones (50 $\mu g \cdot mL^{-1}$) and 4-methoxyacetophenone (20 $\mu g \cdot mL^{-1}$).

Quantitation of Flavones in Citrus Peel Oils. A *Citrus* peel oil sample (500 μL) was diluted in methylene chloride (25 mL) containing 500 μL of a solution of 4-methoxyacetophenone (200 $\mu g \cdot mL^{-1}$). For the samples, 10 μL of the prepared solution was used in each injection. In some experiments the *Citrus* peel oil solutions were co-chromatographed with standards to verify the identity of the peaks. The amounts of the flavones were determined from the area given by the integrator using the response factor of the standards.

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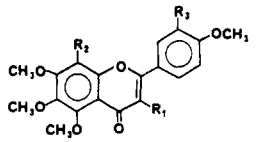
Table I. Formula, Capacity, and Response Factors of Polymethoxylated Flavones Investigated



no.	flavone trivial name	R_1	R_2	R_3	k'^a	RF[b]
1	tangeretin	H	OCH_3	H	0.72 ± 0.02	2.2 ± 0.1
2	tetra-*O*-methylscutellarein	H	H	H	1.05 ± 0.03	3.9 ± 0.2
3	heptamethoxyflavone	OCH_3	OCH_3	OCH_3	1.20 ± 0.05	6.5 ± 0.4
4	nobiletin	H	OCH_3	OCH_3	1.50 ± 0.05	4.4 ± 0.3
5	3,5,6,7,3′,4′-hexamethoxyflavone	OCH_3	H	OCH_3	1.80 ± 0.06	4.0 ± 0.3
6	sinensetin	H	H	OCH_3	2.40 ± 0.08	8.3 ± 0.5

[a] Determined on a LiChrosorb Si60 column; solvent, heptane–isopropyl alcohol (60:40, v/v). [b] Response factors expressed against 4-methoxyacetophenone as internal standard.

Statistical Analyses. Principal-component analysis (PCA) was performed with a data set transformed into centered and reduced variables (standardized PCA). The initial data set was composed of the values taken by seven variables (flavones 1–6 and the sum of flavones) for the 15 *Citrus* peel oils. Factor discriminant analysis (FDA) was performed to classify the oil samples into two categories. Further descriptions of PCA and FDA are provided by Romeder (1973), Lebart et al., (1982), and Foucart (1982). All processing was done on the computer (Hewlett-Packard HP 1000) of the Ecole Supérieure de Chimie of Marseilles (France).

RESULTS AND DISCUSSION

Capacity and Response Factors of PMF. We have shown that PMF are well separated from each other using heptane–isopropyl alcohol (60:40, v/v) as solvent system for a water concentration between 0.02 and 0.05% for normal-phase HPLC (Bianchini and Gaydou, 1981). Retention times and capacity factors were determined for each PMF. Capacity factors were averaged and are given for PMF 1–6 in Table I with the standard deviation. The response factors and precision of PMF 1–6 expressed against 4-methoxyacetophenone used as internal standard are given in Table I. These response factors ranged from 2.2 ± 0.1 in the case of tangeretin to 8.3 ± 0.5 for sinensetin, showing therefore the role of the internal standard for the quantitation of PMF in peel oils.

PMF in Various Citrus Peel Oils. Different classification systems have been proposed by taxonomists who specialized in *Citrus* (Blondel, 1978). The number of species is variable according to the different authors. For example, in the systematics of Swingle (Swingle and Reece, 1943; Swingle, 1967), *Citrus* contains 16 species; in the systematics of Hodgson (1961), this genus contains 36 species; and in the systematics of Tanaka (1961, 1969) a great number of species (157) are described. The differences between the Swingle and Tanaka systems, according to Barrett and Rhodes (1976), may be explained taking into account the concept of speciation: Tanaka produced an excellent descriptive morphology of *Citrus* biotypes, equating morphological diversity with speciation. Table II gives the names of the cultivars of the 15 samples of *Citrus* peel oils investigated and their classification following the Swingle and Tanaka systems.

Figure 1 shows the separation of the PMF in the case of Tangerine and Washington navel peel oils. The quantitative determinations of the six PMF for the various samples are given in Table III. Three flavones are found in higher amounts: tangeretin (0.5–2.8 $g \cdot L^{-1}$), heptamethoxyflavone (0.2–2.7 $g \cdot L^{-1}$), and nobiletin (0.4–2.0 $g \cdot L^{-1}$). Tetra-*O*-methylscutellarein (0.0–0.6 $g \cdot L^{-1}$), 3,5,6,7,3′,4′-hexamethoxyflavone (0.0–0.04 $g \cdot L^{-1}$), and sinensetin (0.07–0.3 $g \cdot L^{-1}$) are found in lower amounts. The sum of flavones ranges from 2.22 to 6.49 $g \cdot L^{-1}$. From these results some characteristic differences are shown: the amount of tangeretin and the sum of PMF are higher in mandarins than in oranges. Tetra-*O*-methylscutellarein content is higher in oranges than in mandarins. Flavone 5 is in minute amount in all samples. These results for orange peel oils are in agreement with those previously obtained from industrial orange oils (Bianchini and Gaydou, 1981). The range of variation of these PMF shows

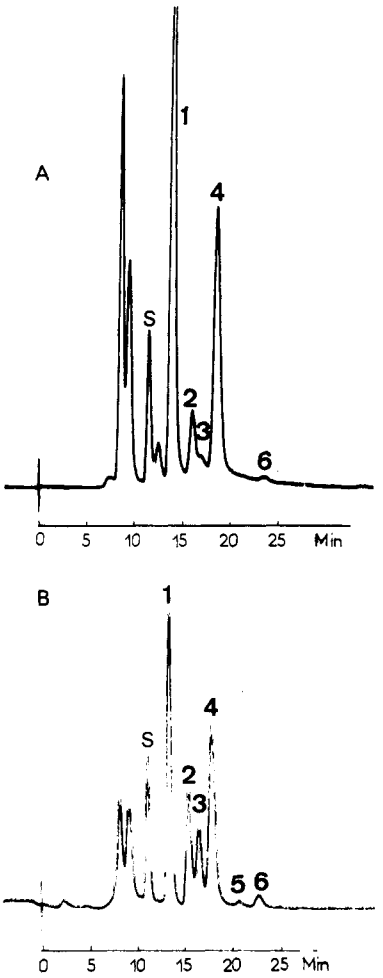


Figure 1. Separation of polymethoxylated flavones of *Citrus* peel oils. Conditions: column, LiChrosorb Si60; solvent, heptane–isopropyl alcohol (60:40, v/v); detector, UV at 280 nm. Key: (A) tangerine; (B) Washington navel; S, standard (4-methoxyacetophenone). For peak identification see Table I.

Table II. Description of the Various *Citrus* Samples Investigated

code	cultivar	classification	
		Tanaka	Swingle
O1	Cadenera orange	*C. sinensis* (L.) Osb.	*C. sinensis* (L.) Osb.
O2	Cadenera orange	*C. sinensis* (L.) Osb.	*C. sinensis* (L.) Osb.
O3	Hamlin orange	*C. sinensis* (L.) Osb.	*C. sinensis* (L.) Osb.
O4	Moro orange	*C. sinensis* (L.) Osb.	*C. sinensis* (L.) Osb.
O5	Valencia late orange *C. sinensis* (L.) Osb.	*C. sinensis* (L.) Osb.	*C. sinensis* (L.) Osb.
O6	Navelate orange	*C. sinensis* (L.) Osb.	*C. sinensis* (L.) Osb.
W	Washington navel	*C. oblonga* Hort. ex Y. Tan	*C. sinensis* (L.) Osb.
M1	Common mandarin	*C. deliciosa* Ten.	*C. reticulata* Bl.
M2	Kara mandarin	*C. reticulata* Bl.	*C. reticulata* Bl.
M3	Murcot mandarin	*C. reticulata* Bl.	*C. reticulata* Bl.
M4	Malvasio mandarin	*C. reticulata* Bl.	*C. reticulata* Bl.
M5	Ortanique mandarin	*C. reticulata* Bl.	*C. reticulata* Bl.
K	King mandarin	*C. nobilis* Lour.	*C. reticulata* Bl.
C	Clementine	*C. clementina* Hort. ex Tan.	*C. reticulata* Bl.
T	Tangerine (Dancy)	*C. tangerina* Hort. ex Tan.	*C. reticulata* Bl.

that it is difficult to perform some classifications among *C. sinensis* and *C. reticulata* species according to Swingle classification or among six species according to Tanaka classification, without the utilization of multidimensional data analysis.

Multivariate Statistical Analysis. In standardized principal-component analysis (PCA), the content of the seven variables indicated in Table III was used for the classification of the 15 *Citrus* peel oils. The correlation coefficient matrix (Table IV) shows a positive coefficient of the sum of flavones with tangeretin ($r = 0.80$) and with heptamethoxyflavone (0.64). A negative coefficient between tetra-*O*-methylscutellarein and sinensetin ($r = -0.60$) was also observed. The factor loadings, the eigenvalues, and the percentage of the total variance are given in Table V. On the first principal component (axis 1, 38.9% of the variance) tangeretin and sinensetin are positively loaded (0.82 and 0.68, respectively) and tetra-*O*-methylscutellarein and flavone 5 are negatively loaded (−0.77 and −0.60, respectively). On the second principal component (axis 2, 29.3% of the variance) nobiletin and the sum of flavones are negatively loaded (−0.79 and −0.75, respectively). Heptamethoxyflavone has a high factor loading (−0.77) with the third component (axis 3, 15.7% of the variance). In Figure 2 the projections of the variables are plotted onto the two first principal components. Figure 3 represents the graphical projection of the peel juice samples for principal components 1 and 2, which together account for 68.2% variation. One can notice that the orange group is well separated from the mandarin group by the first principal component, showing the role of PMF in the differentiation of *C. sinensis* from *C. reticulata*. However, clementine (*Citrus clementina* according to Tanaka) and tangerine (*Citrus tangerina* according to Tanaka) are found in the negative part of the axis 1 and with the orange group. This result seems to show that PMF content follows the variation of morphological *Citrus* biotypes, and therefore an agreement with the Tanaka system is observed. In the case of Washington navel sample (*Citrus oblonga* according to Tanaka), a good agreement between PMF content and the Swingle classification is observed since this sample is found in the orange (*C. sinensis*) category. The second principal component permits the separation of mandarin M1 from the other ones (M2–M5). This sample, which is strongly positively loaded to nobiletin and the sum of flavones, was classified as a different species (*Citrus deliciosa*) by Tanaka. Another sample (King mandarin, K), strongly positively loaded to heptamethoxyflavone and the sum of flavones, was differentiated by the second axis form the mandarins M2–M5, in agreement with the distinction of these species by Tanaka (*Citrus nobilis*) from the *C. reticulata* of Swingle. A classification into two categories according to Swingle systematics (group 1, O1–O6 and W; group 2, M1–M5 and K, C, and T) was obtained by using standardized factorial discriminant analysis (FDA) for the 15 samples. A correct attribution was observed since the samples K, C, and T were classified with the *C. reticulata* group and W with the *C. sinensis* group.

CONCLUSION

The results obtained show the differences in PMF content among various orange and mandarin peel oils. The differentiation observed is in agreement with the classification (Table II) of Swingle and of Tanaka (1961), showing therefore the role of the variations of PMF content in the different morphologic *Citrus* biotypes. Albach and Redman (1969) showed previously that *Citrus* species could be differentiated on the basis of their contents of neohesperidosides or rutinosides. On another hand, PMF composition could be used as a measure of peel oil authenticity, taking into account that, in the case of commercial oils, tangeretin could precipitate upon standing,

Table III. Quantitative Determination of Polymethoxylated Flavones Found in Peel Oils of Various *Citrus* Samples

code[a]	flavone[b] content, $g \cdot L^{-1}$						
	1	2	3	4	5	6	sum
O1	0.5	0.30	0.56	0.75	0.02	0.10	2.22
O2	0.8	0.40	0.40	0.60	0.00	0.20	2.40
O3	0.6	0.45	0.70	1.10	0.00	0.15	3.00
O4	0.5	0.45	1.00	0.90	0.04	0.10	2.99
O5	0.7	0.20	0.30	0.50	0.00	0.30	1.80
O6	0.7	0.50	2.00	0.70	0.00	0.10	4.00
W	1.0	0.60	0.70	1.00	0.03	0.14	3.47
M1	2.8	0.50	1.10	2.00	0.02	0.07	6.49
M2	1.9	0.00	1.60	0.60	0.00	0.20	4.30
M3	2.0	0.20	0.30	0.80	0.00	0.20	3.50
M4	2.1	0.00	0.20	0.90	0.00	0.20	3.40
M5	2.2	0.05	0.70	0.70	0.00	0.30	3.95
K	2.5	0.00	2.70	0.60	0.00	0.20	6.00
C	1.1	0.25	0.75	0.40	0.03	0.07	3.60
T	1.5	0.35	0.22	1.50	0.00	0.07	3.64

[a] For identification of *Citrus* samples see Table II. [b] For identification of flavones see Table I.

Table IV. Correlation Coefficient Matrix for the Flavones Investigated in *Citrus* Peel Oils

flavone[a]	2	3	4	5	6	sum[b]
1	−0.51	0.25	0.34	0.29	0.40	0.80
2		−0.13	0.45	0.48	−0.60	−0.16
3			−0.15	−0.05	0.06	0.64
4				0.13	−0.36	0.42
5					−0.50	−0.07
6						

[a] For flavone identification see Table I. [b] Sum of content of flavones 1–6.

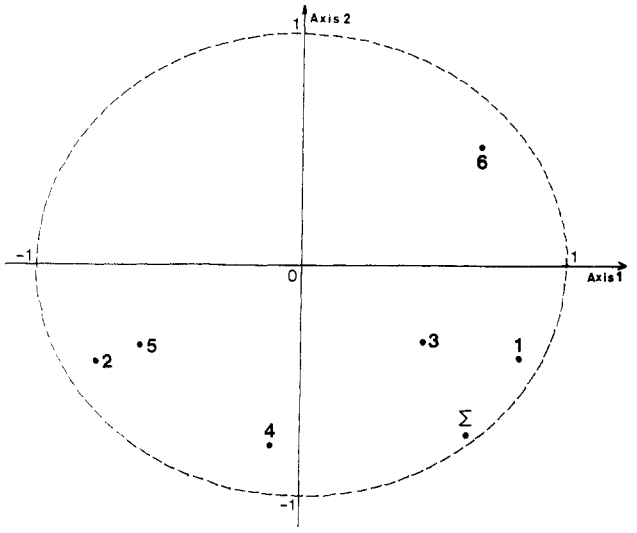


Figure 2. Graphical projection of variables (flavones 1–6 and the sum of flavones (Σ) given in Table I) onto the two first principal components obtained in standardized PCA for the various *Citrus* peel oils investigated.

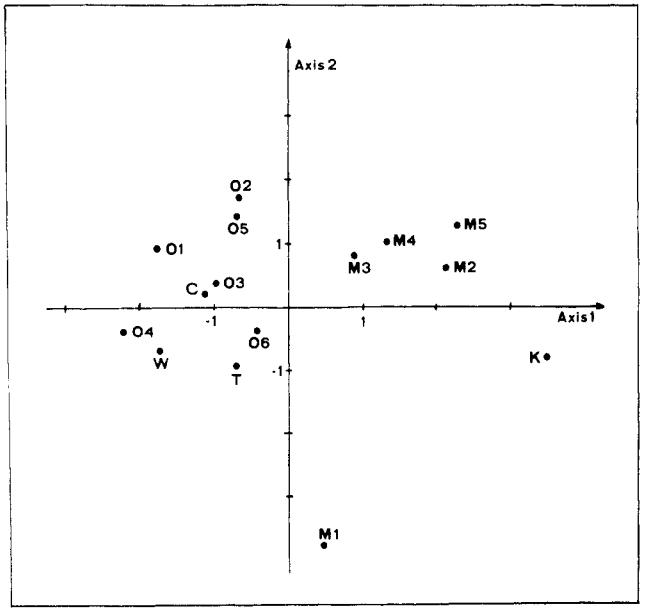


Figure 3. Graphical projection onto the two first principal components of the 15 *Citrus* peel oils used in standardized PCA. For sample identification see Table II.

especially in tangerine oils. However, more results are needed to know the range of PMF for the various species and cultivars since numerous factors such as fruit size, horticultural conditions, age of the trees, and climatic conditions from one year to another would affect the PMF content.

Table V. Factor Loadings, Eigenvalues, and Percentage of Variance Using Standardized PCA for the Various *Citrus* Peel Oils Investigated

	axis		
	1	2	3
factor loading			
1[a]	0.82	−0.42	−0.28
2	−0.77	−0.43	0.00
3	0.46	−0.35	0.77
4	−0.11	−0.79	−0.55
5	−0.60	−0.36	0.30
6	0.68	0.50	−0.14
sum[b]	0.63	−0.75	0.14
eigenvalues	2.72	2.05	1.10
%[c]	38.9	29.3	15.7

[a] For flavone identification see Table I. [b] Sum of content of flavones 1–6. [c] Percentage of total variance.

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Registry No. 1, 481-53-8; 2, 478-01-3; 3, 1178-24-1; 4, 478-01-3; 5, 1251-84-9; 6, 2306-27-6.

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Flavonoid Glycosides of Spartan Apple Peel

Arthur J. Dick,* Peter R. Redden, Alphonse C. DeMarco, Perry D. Lidster, and T. Bruce Grindley

The flavonoid glycosides of Spartan apples were isolated by column chromatography on polyamide and Sephadex resins and by RP-HPLC. They were characterized by ^1H and ^{13}C NMR as phlorizin and the following glycosides of quercetin: α-L-arabinofuranoside, β-D-galactopyranoside, β-D-glucopyranoside, α-L-rhamnopyranoside, β-D-xylopyranoside. The coupling constants in the ^1H NMR spectra were used to establish anomeric configurations of all glycosides.

A flavonoid glycoside mixture isolated from apples has been reported to have inhibitory properties toward a β-galactosidase of apples and toward softening of fresh apples (Dick et al., 1985). This paper describes the characterization of this mixture. The quercetin glycosides of apple peel were described by identification of the sugar moiety after hydrolysis of the separated glycosides (Siegelman, 1955; Walker, 1964) and by isolation of the individual glycosides by paper and cellulose chromatography (Teuber Wuenscher and Herrmann, 1978). Changes in the quercetin glycoside content of apples during storage have been reported by Donchev (1977). This paper describes procedures for HPLC analysis of the flavonoids in extracts of Spartan apples, procedures for the effective preparative fractionation of the flavonoid glycosides from apples, and their detailed characterization, by NMR methods. The occurrence of phlorizin, long known to be present in other apple tissues (deKoninck, 1835) and in apple juice (Lea and Timberlake, 1974), is now described in apple peel.

MATERIALS AND METHODS

Phlorizin, quercitrin, and rutin were obtained from Sigma Chemical Co. After recrystallization from ethanol–water, each substance gave a single peak on HPLC (see below) and the expected UV spectrum (Mabry et al., 1970). Polyamide 6S was obtained from Riedel-deHaen AG, Hannover. The flavonoid glycoside fraction was prepared from a purified ethyl acetate extract of Spartan apples by chromatography on an acrylic ester resin column (Dick et al., 1985).

High-Performance Liquid Chromatography (HPLC). Apple peel samples for HPLC analysis were prepared by blending 5 g of scraped apple peel in 100 mL of methanol, filtration, concentration to 10.0 mL, and injection of a 10-μL sample. Quantitative extraction of flavonoid glycosides was indicated by the quantitative recovery of rutin from spiked peel samples. Column fraction samples (see later) were injected directly after filtration. A Varian liquid chromatograph was used with detection of absorbance at 270 nm. A Waters Radial-Pak reversed-phase column was used with a solvent program of 25–50% tetrahydrofuran in 0.1% aqueous trifluoroacetic acid over 20 min at a flow rate of 2 mL/min. The labeled peaks of the chromatogram (Figure 1) were identified as (1) quercetin glucoside plus quercetin galactoside (isoquercitrin and hyperin), (2) quercetin xyloside (reynoutrin), (3) quercetin rhamnoside (quercitrin), (4) quercetin arabinoside (avicularin), and (5) phlorizin.

Preparative Column Chromatography. The purified flavonoid glycoside mixture (1.44 g) was fractionated by chromatography on polyamide 6S (240-mL settled bed volume) in a glass column of 3-cm diameter. The sample as a 10% solution in methanol was applied to the column equilibrated with 30% methanol in 0.1% aqueous acetic acid. The column was developed at a flow rate of 3 mL/min with 300 mL of the equilibration solvent followed by a linear gradient of 30–70% methanol in 0.1% aqueous acetic acid of volume 700 mL. This was followed by 700 mL of 70% methanol in 0.1% aqueous acetic acid. Elution of flavonoid glycosides was monitored by absorbance at 260 nm which, however, did not reveal any resolution. Examination of column fractions by HPLC as described gave the profile shown in Figure 2. Phlorizin, HPLC peak 5, was eluted first and resolved from the second component which was a mixture of the quercetin hexosides, the glucoside, galactoside, and rhamnoside, HPLC peaks 1 and 3. The third eluent was the partially resolved pentosides, the xyloside and arabinoside, HPLC peaks 2 and 4. The tail of this peak, labeled 4′ in Figure 2, was quercetin arabinoside. Column fractions of Figure 2 that were pooled were those that contained HPLC peaks 5, 1 and 3, 2 and 4, and 4′. Each was evaporated to dryness. After recrystallization of the residues of peaks 5 and 4′ from ethyl acetate–hexane, 22 mg of phlorizin and 17.5 mg quercetin arabinoside, respectively, were obtained.

The combined fractions containing HPLC peaks 1 and 3 from the polyamide column were evaporated to dryness, dissolved in a minimum volume of methanol and applied to a Sephadex G-10 column (40-mL bed volume), which was developed with a linear gradient of water–20% aqueous methanol at a flow rate of 0.1 mL/min (Redden, 1985). Monitoring by HPLC showed a succession of three

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