Characterization of Flavonoids in Petals of *Rosa damascena* by HPLC and Spectral Analysis

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High-performance liquid chromatography (HPLC), performed with a photodiode array detector, was applied to separation of the anthocyanins and other flavonoids in the petals of *Rosa damascena* Mill., used to produce rose petal jam and attar of rose. Over 25 discrete peaks were detected, and separation of these took less than 50 min. Most of the HPLC peaks were collected as separate fractions from the column outlet and reanalyzed by HPLC, paper chromatography, and spectral analysis. The concentration of total anthocyanins was 285 mg/kg of fresh petals. The main anthocyanin was cyanidin 3,5-diglucoside, which accounted for over 95% of the total anthocyanins. Also identified were several kaempferol and quercetin glucosides, galactoside, arabinosides, and rhamnosides.

INTRODUCTION

Rosa damascena Mill. (Damask or Kazanlik rose) is a species of rose grown primarily in Turkey, Bulgaria, and the USSR. The economic products of these roses are attar of rose, a fragrant essential oil extracted from rose petals, and a unique food product called rose petal jam. Because of their perishability and short harvesting period, rose petals require rapid processing, and changes in color of the petals occur during processing and storage. Anthocyanins are the pigments primarily responsible for the color of rose petals and the jam made from them. However, it is well-known that colorless flavonoids aid in the intensification and stabilization of color (Mazza and Brouillard, 1990). This study was, therefore, initiated to characterize both anthocyanins and colorless flavonoids present in petals of *R. damescena* to develop the required information for improving color retention during processing and storage of the rose jam.

MATERIALS AND METHODS

Flower petals of *R. damascena* Mill. were harvested near Isparta, Turkey, in June 1989 and were frozen and stored at -20° C for 1 month prior to analysis. A 20-g sample was blended with 200 mL of 10:1:9 ethanol–acetic acid–water (EAW) for 5 min in a Waring blender at full speed. The homogenate was then suction filtered through a Whatman No. 44 filter paper and the residue washed with 50 mL of extraction solvent. The extract was dried under vacuum at 30 °C. Just prior to high-performance liquid chromatography (HPLC) and paper chromatography (PC), dried pigment extract was redissolved in methanol–acetic acid–water (MAW) and filtered through a 45-μm Millipore filter. Petals of John Cabot roses (*Rosa kordesii* hybrid), harvested near Morden, Canada, in July 1989, were analyzed concurrently with samples of *R. damascena* as the petal colors of both roses are very similar.

Standards. Protocatechic acid, *p*-coumaric acid, ferulic acid, chlorogenic acid, syringic acid, and *p*-hydroxybenzoic acid were purchased from Carl Roth Chemical Co. (Karlsruhe, W. Germany), cinnamic acid, quercetin, rutin, myricetin, apigenin, and chrysin from Sigma Chemical Co. (St. Louis, MO), and D-catechin and naringenin from Nutritional Biochemical Corp. (Cleveland, OH). Gallic acid was purchased from Fisher Scientific (Fairlawn, NJ) and esculin from J. T. Baker Chemical Co. (Phillipsburg, NJ). Cyanidin 3-glucoside, cyanidin 3-galactoside, cyanidin 3-rutinoside, and peonidin chloride were purchased from Extrasynthese (Genay, France). Peonidin 3,5-diglucoside was donated by J. G. Sweeny (The Coca-Cola Co., Atlanta, GA), cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, pelargonidin chloride, and cyanidin chloride were donated by K. Yoshitama (University of Kyoto, Kyoto, Japan), and quercetin 3-arabinoside and kaempferol were donated by T. Fuleki (Ontario Ministry of Agriculture and Food, HRIO, Vineland, ON, Canada).

Purification and HPLC Analysis. Concentrated petal extracts were applied to Whatman No. 3 chromatographic paper and developed with *n*-butanol–acetic acid–water (4:1:5) (BAW). The resulting pigment bands were cut and eluted with MAW prior to further purification by HPLC.

The equipment used for HPLC consisted of an LKB liquid chromatography system (LKB-Produkter, Bromma, Sweden) equipped with two pumps (LKB Model 2150), a controller (LKB Model 2152), a Rheodyne 7125 injector valve with a 20- or 100-μL loop, an UltroPac prepared column (250 × 4.6 mm) of Spheri 10-RP18 (10 μm) (Brownlee Labs, Santa Clara, CA), a photodiode array detector (LKB Model 2140), interfaced with an IBM personal computer and a Canon A-1210 color printer, and a fraction collector (LKB Model 2211 SuperRac).

The following solvent system and elution profiles were used for the separation of flavonoids: solvent A, formic acid–water (5:95 v/v), solvent B, methanol; elution profile 0–10 min, 17–22% B (linear gradient), 10–12 min, 22–27% B, 12–33 min, 27–37% B, 33–39 min, 37–55% B, and 39–49 min, 55–59% B. The solvent flow rate was 1.0 mL/min and the column pressure 34–40 bar. All separations were performed at 22 ± 1 °C, and all solvents were of HPLC grade filtered through a 0.45-μm Millipore filter before use. Detection was performed simultaneously at 190-370 nm. The retention times were calculated with an IBM personal computer equipped with a Wavescan spectral detector program (LKB 2140-202). The capacity factor (k') was calculated by the equation (Kirkland, 1971)

$$k' = (t_r - t_0)/t_0$$

where t_r is the retention time of the compound, t_0 is the time of zero retention measured as the time of the nonretained solvent peak, and k' is the capacity factor of the compound.

The peak area and area percent were calculated with the same IBM computer equipped with Model 2600 chromatography software, revision 3.1 (Nelson Analytical, Inc., Cupertino, CA). The UV maxima were determined from the spectrum of each

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HPLC peak displayed on the IBM personal computer equipped with the Wavescan spectral detector program. Selected HPLC peaks were collected as separate fractions from the column outlet, concentrated on a rotary evaporator at 30 °C, and subjected to further analyses.

Spectral Analysis. Purified anthocyanin pigment was dissolved in methanolic 0.01% HCl; other flavonoids were dissolved in methanol. UV and visible spectra were obtained with a Beckman DU-50 spectrophotometer connected to an Epson RX-80 printer and an IBM personal computer equipped with a "peak pick" program (Beckman Quant 1 Soft-Pak, Beckman Instruments Inc., Scientific Instruments Div., Irvine, CA). Sodium methoxide (NaOMe), aluminum chloride ($AlCl_3$), aluminum chloride plus hydrochloric acid ($AlCl_3$ + HCl), sodium acetate (NaOAc), and sodium acetate plus boric acid (NaOAc + H_3BO_3) shifts were recorded and interpreted as described by Mabry et al. (1970) and Markham (1982).

Hydrolysis of Flavonoids. Purified flavonoids were subjected to acid hydrolysis as described by Francis (1982). One to 2 mg of flavonoid and methanol in 2 mL of 2 N HCl were used. The aglycon and sugar portions of the flavonoid were obtained by heating the pigment–solvent mixture in a water bath at 100 °C for 1 h. After cooling, the aglycon was extracted with amyl alcohol and characterized by HPLC. The aqueous solution containing the sugar was dried under vacuum, and the sugar was dissolved in a few drops of water and chromatographed on Whatman No. 1 paper with reference standards using solvent 4:1:2.2 *n*-butanol–ethanol–water (BEW). For anthocyanins, prior to paper chromatography, aqueous solvent was washed with 10% di-*n*-octylmethylamine in chloroform as described by Francis (1982). Paper chromatograms of sugar were viewed after spraying with aniline hydrogen phthalate reagent (Markham, 1982). The position of the sugar in the flavonoid molecule was determined from spectral shifts. (Markham, 1982; Mabry et al., 1970) and by comparing HPLC retention times of rose flavonoids with those of authentic compounds analyzed under the same conditions.

Total Anthocyanins. Total anthocyanins were determined by the pH differential method of Fuleki and Francis (1968) and calculated by using the extinction coefficient of cyanidin 3-glucoside in methanol containing 0.01% HCl.

RESULTS AND DISCUSSION

Typical HPLC chromatograms of methanolic extracts of petals of *R. damascena* and John Cabot roses are shown in Figure 1. Peaks 1–13 and 15 had generally higher absorbance at 280 nm than at 335 nm. Similarly, peak 14 and peaks 16–26 had higher absorbance at 335 nm than at 280 nm. It is well-known (Harborne, 1967; Markham, 1982; Putman and Butler, 1989) that the principal ultraviolet absorption maximum for anthocyanins, flavan-3-ols, and procyanidins, or condensed tannins, is at 270–280 nm and that for other flavonoids is at 310–370 nm. Thus, from the difference in absorbance of peaks at 280 and 335 nm it can be deduced that peaks 1–13 and 15 were probably anthocyanins, anthocyanidins, flavan-3-ols, or procyanidins. Retention times, UV maxima in methanol–formic acid–water, and capacity factors of 28 phenolic compounds are listed in Table I. These data confirm and extend the findings of Wulf and Nagel (1976), Casteele et al. (1983), Mazza (1986), and others.

A comparison of the retention times and spectral characteristics of the flavonoids of *R. damascena* (Table II) with the retention and spectral data of standard compounds listed in Table I reveals that the identity of only peaks 8 and 18 can be predicted. Peak 8 corresponds to cyanidin 3,5-diglucoside, while peak 18 corresponds to quercetin 3-arabinoside. Nonetheless, the comparison does reveal that, contrary to recent literature reports (Sharma, 1981), there is no cyanidin 3-glucoside or pelargonidin 3,5-diglucoside in the petals of *R. damascena* from the Isparta region of Turkey. The lack of agreement between results is most probably related to the use of a different cultivar by Sharma (1981), who did not give the name of the cultivar used for his study. In addition, the comparison between Tables I and II also reveals that no

Table I. Retention Times (t_r), UV Maxima (λ_{max}), and Capacity Factors (k') of Standard Anthocyanins and Related Substances on Spheri 10 RP18 Using Methanol–Formic Acid–Water as Eluent[a]

compd[b]	t_r, min	λ_{max},[c] nm	k'
gallic acid	3.5	271	0.3
protocatechuic acid	5.3	259, 293	1.0
D(−)-catechin	6.5	239, 277	1.3
esculin	6.5	246, 296 sh, 332	1.4
p-hydroxybenzoic acid	8.3	254	2.0
chlorogenic acid	9.5	244, 301 sh, 321	2.4
caffeic acid	11.4	244, 319	3.2
syringic acid	13.2	273	3.7
Cn 3,5-G	14.1	274	3.8
Pg 3,5-G	15.5	272	4.2
p-coumaric acid	16.3	291 sh, 308	4.9
Pn 3,5-G	17.3	276	5.2
Cn 3-Ga	18.5	243 sh, 278	5.4
ferulic acid	19.4	243, 301 sh, 321	5.7
Cn 3-G	20.2	278	6.1
Cn 3-Ru	24.1	279	7.2
rutin	30.4	256, 354	10.0
myrcetin	31.1	254, 370	10.0
Que 3-A	31.2	256, 301 sh, 351	9.4
cinnamic acid	32.1	276	10.4
Cn chloride	38.3	274	12.4
Pg chloride	42.2	266	13.0
naringenin	40.1	288, 355 sh	14.3
quercetin	40.6	255, 370	13.5
Pn chloride	43.5	272	13.5
kaempferol	43.6	263, 364	13.6
apigenin	44.1	266, 337	14.3
chrysin	45.3	266, 313	14.4

[a] Flow rate = 1 mL/min; t_0 = 2.62 min. [b] Cn = cyanidin; Pg = pelargonidin; Pn = peonidin; G = glucoside; Ga = galactoside; Ru = rutinoside; Que = quercetin. [c] sh = shoulder.

Table II. Retention Times (t_r), UV Maxima (λ_{max}), Capacity Factors (k'), and Area Percents of *R. damascena* Flavonoids on Spheri 10 RP18 Using Methanol–Formic Acid–Water as Eluent[a]

				peak area (×10⁵)		area, %	
peak no.[b]	t_r, min	λ_{max}, nm	k'	280 nm	335 nm	280 nm	335 nm
1	4.2	242 sh, 272	0.4	64.2		3.0	
2	4.9	245 sh, 305	0.6	16.3	9.4	0.8	0.6
3	5.7	252 sh, 270	0.9	36.4	2.5	1.7	0.2
4	7.0	243 sh, 272	1.3	86.5	0.7	4.1	0.1
5	7.8	243 sh, 275	1.6	17.3		0.8	
6	8.7	243 sh, 274	1.8	27.2		1.3	
7	10.3	248 sh, 274	2.4	51.4	1.3	2.4	0.1
8	14.1	274	3.7	171.9	8.0	8.1	0.6
9	15.9	274	4.3	116.8	2.0	5.5	0.1
10	17.2	275	4.7	59.0	4.3	2.8	0.3
11	18.0	245 sh, 275	5.0	191.2	3.8	9.0	0.3
12	18.9	252 sh, 276	5.2	77.0	0.8	3.6	0.1
13	20.0	275	5.6	54.1		2.6	
14	24.7	262, 285 sh, 351	7.2	43.0	16.0	2.0	1.1
15	27.8	275	8.2	46.9		2.2	
16	29.0	255, 301 sh, 352	8.6	87.7	152.1	4.1	10.5
17	29.8	256, 301 sh, 352	8.9	91.4	149.8	4.3	10.3
18	31.6	257, 301 sh, 351	9.4	7.3	8.9	0.3	0.6
19	32.7	261, 301 sh, 354	9.8	26.2	21.9	1.2	1.5
20	34.6	262, 301 sh, 347	10.5	98.4	158.1	4.6	10.9
21	36.8	264, 298 sh, 345	11.2	280.0	450.0	13.3	31.0
22	38.8	265, 293 sh, 345	11.8	63.9	70.5	3.0	4.9
23	40.0	263, 298 sh, 345	12.2	58.3	88.2	2.8	6.0
24	40.4	262, 298 sh, 342	12.4	109.1	146.0	5.2	10.1
25	42.8	263, 298 sh, 341	13.2	41.6	50.1	2.0	3.5
26	43.5	265, 313	13.4	65.0	62.6	3.1	4.3

[a] Flow rate = 1 mL/min; t_0 = 3.02 min. [b] Refer to peak number in Figure 1.

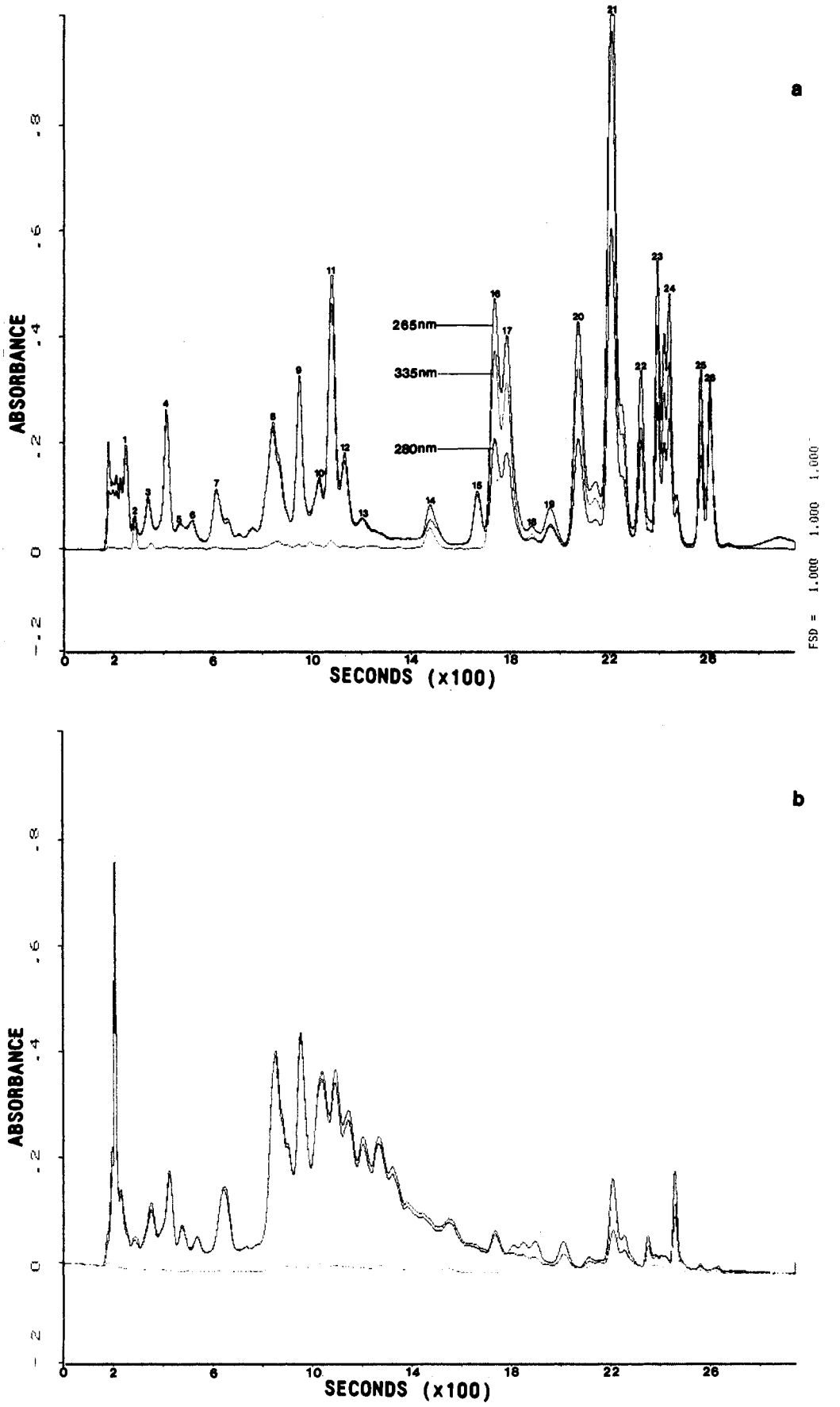

Figure 1. HPLC of rose petal flavonoid on Spheri 10-RP18 column: (a) *R. damascena*; (b) John Cabot rose (*R. kordesii*) hybrid. Determined by photodiode array detection.

phenolic acids or commonly occurring flavonoids such as rutin, naringenin, and apigenin were detected in the petals of *R. damascena*. Asen (1982) also reported that the flavonoids in flowers of White Masterpiece, Bridal Pink, and

Table III. Chromatographic Data for Aglycons and Sugar Moieties of the *R. damascena* Flavonoids

	peak no.									standards[a]							
	8	16	17	20	21	22	24	25	26	G	Ga	Xy	Ar	Rh	Cn	Que	Ka
aglycons																	
retention time, min	38.1	41.1	40.0	42.4	43.1	42.5	43.3	43.3	43.1	–	–	–	–	–	38.3	40.6	43.6
λ_{max} band II	274	255	255	264	263	263	263	263	264	–	–	–	–	–	274	255	263
λ_{max} band I	–	370	370	365	366	364	366	365	367	–	–	–	–	–	–	370	365
sugars																	
RG × 100[b]	100	100	91 98	90 147	100 144	150	96 270	89 275	95 146 265	100	88	176	154	284	–	–	–

[a] G = glucose; Ga = galactose; Xy = xylose; Ar = arabinose; Rh = rhamnose; Cn = cyanidin; Que = quercetin; Ka = kaempferol. [b] Solvent: BEW.

Table IV. Spectral Characteristics of *R. damascena* Flavonal Glycosides in Six Shift Reagents

	λ_{max} in MeOH, nm		$\Delta\lambda H_3BO_3$	$\Delta\lambda AlCl_3$	$\Delta\lambda AlCl_3$/HCl	$\Delta\lambda NaOAc$	$\Delta\lambda H_3BO_3$
peak no.	band II	band I	band I	band I	band I	band II	band I
16	255, 293 sh[a]	357	+48	+74	+45	+17	+18
17	255, 295 sh	357	+50	+68	+46	+17	+18
20	264, 301 sh	352	+46	-3	+41	+9	+7
21	264, 301 sh	345	+52	+5	+41	+8	+9
23	265, 294 sh	346	+46	+45	+47	+8	+4
24	264	341	+46	+5	+53	+7	+2
25	264	338	+47	+52	+55	+7	+4
26	266	313	+55	+25	+78	+8	+0

[a] sh = shoulder.

Table V. Flavonoids of *R. damascena* Mill.

peak no.[a]	compd
1, 3–7	flavan-3-ols
8	cyanidin 3,5-diglucoside
9–13, 15	flavan-3-ols
16	quercetin 3-glucoside
17	quercetin 3-glucosylgalactoside
18	quercetin 3-arabinoside
20	kaempferol 3-galactosylarabinoside
21	kaempferol 3-glucosylarabinoside
23	kaempferol 3-arabinoside
24	kaempferol 3-rhamnosylglucoside
25	kaempferol 3-rhamnosylgalactoside
26	kaempferol 3-rutinosyl-7-arabinoside

[a] Refers to peak number in Figure 1.

Samantha roses are anthocyanins and kaempferol or quercetin 3-glucosides.

From the data in Tables I and II it can further be noted that peaks 1, 3–7, 9–13, and 15 have the spectral characteristics of flavan-3-ols and procyanidins (Putman and Butler, 1989; Table I). The overall identity of these peaks was further confirmed by subjecting the HPLC fractions containing these peaks to the well-known vanillin assay (Butler et al., 1982) and the *n*-butanol–HCl procyanidin test (Porter et al., 1986). The vanillin reaction with peaks 1, 3–7, 9–13, and 15 in glacial acetic acid gave a positive reaction indicated by the appearance of a light pink to deep cherry red coloration and an increase in absorbance at 500 nm. This suggests that the peaks were indeed procyanidins or flavan-3-ols. The *n*-butanol–HCl test for procyanidins, however, yielded no anthocyanidins, thus indicating that these peaks were flavan-3-ols. The exact identity of individual compounds was, however, not determined.

The identity of peak 8 was further confirmed by fluorescence and color reaction, absorbance ratios, spectral shift comparisons, and chromatography of the anthocyanidins and sugars produced on acid hydrolysis (Harborne, 1967; Table IV).

Peaks 16–18, 20, 21, and 23–26 were colorless in visible light, dark purple in ultraviolet light, and yellow-green after treatment with NH_3. Their MeOH spectra consisted of two absorption maxima in the range 255–266 and 313–357 nm (Tables III–IV). These color and spectral characteristics strongly suggest that these peaks were flavonols (Mabry et al., 1970; Markham, 1982). Acid hydrolysis of purified fractions, followed by HPLC, paper chromatography, and spectral analysis of aglycons, gave quercetin for peaks 16–18 and kaempferol for peaks 20, 21, and 23–26. The sugars obtained on hydrolysis of these peaks were glucose, galactose, arabinose, and rhamnose. Measurements of the changes in the UV spectra of each peak in MeOH and in the presence of NaOMe, $NaOAc/H_3BO_3$, $AlCl_3$, and $AlCl_3$/HCl (Table IV) provided the position of attachment of sugars to quercetin and kaempferol (Table IV). Thus, peaks 16–18 were characterized as the 3-glucoside, 3-glucosylgalactoside, and 3-arabinoside of quercetin, respectively, and peaks 20, 21, and 23–26 were characterized as 3-galactosylarabinoside, 3-glucosylarabinoside, 3-arabinoside, 3-rhamnosylglucoside, 3-rhamnosylgalactoside, and 3-rutinosyl-7-arabinoside of kaempferol, respectively. The flavonol glycoside present in largest concentration in petals of *R. damascena* and John Cabot roses was kaempferol 3-glucosylarabinoside. Its concentration, determined by HPLC analysis, was, however, approximately 2.5 times the concentration of cyanidin 3,5-diglucoside in *R. damascena* and less than half the concentration of cyanidin 3,5-diglucoside in John Cabot rose petals.

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Registry No. Cyanidin 3,5-diglucoside, 2611-67-8; quercetin 3-glucoside, 482-35-9; quercetin 3-glucosylgalactoside, 53023-35-1; quercetin 3-arabinoside, 30370-87-7; kaempferol 3-galactosylarabinoside, 131063-21-3; kaempferol 3-glucosylarabinoside, 56778-06-4; kaempferol 3-arabinoside, 5041-67-8; kaempferol 3-rhamnosylglucoside, 27554-19-4; kaempferol 3-rhamnosylgalactoside, 29859-91-4; kaempferol 3-rutinosyl-7-arabinoside, 130984-19-9.