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Characterization of Flavonols in Cranberry (*Vaccinium macrocarpon*) Powder

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Flavonoids were extracted from cranberry powder with acetone and ethyl acetate and subsequently fractionated with Sephadex LH-20 column chromatography. The fraction eluted with a 60% methanol solution was composed primarily of phenolic constituents with maximum absorbance at 340 nm. A high-performance liquid chromatography procedure was developed, which resolved 22 distinct peaks with UV/vis and mass spectra corresponding to flavonol glycoside conjugates. Six new constituents not previously reported in cranberry or in cranberry products were determined through NMR spectroscopy to be myricetin-3-β-xylopyranoside, quercetin-3-β-glucoside, quercetin-3-α-arabinopyranoside, 3′-methoxyquercetin-3-α-xylopyranoside, quercetin-3-*O*-(6″-*p*-coumaroyl)-β-galactoside, and quercetin-3-*O*-(6″-benzoyl)-β-galactoside. Quercetin-3-*O*-(6″-*p*-coumaroyl)-β-galactoside and quercetin-3-*O*-(6″-benzoyl)-β-galactoside represent a new class of cranberry flavonol compounds with three conjugated components consisting of a flavonol, sugar, and carboxylic acid (benzoic or hydroxycinnamic acids). This is also the first report identifying quercetin-3-arabinoside in both furanose and pyranose forms in cranberry. Elucidation of specific flavonol glycosides in cranberry is significant since the specificity of the sugar moiety may play a role in the bioavailability of the flavonol glycosides in vivo.

KEYWORDS: ***Vaccinium macrocarpon*** **Ait.; cranberry; flavonol glycosides; quercetin; myricetin; arabinopyranoside; acylated quercetin-3-glycosides**

INTRODUCTION

The fruit of the American cranberry (*Vaccinium macrocarpon* Ait.) has received considerable attention for its putative human health benefits. Most of the focus is on the flavonoid constituents due to their relatively high biological activity in various assays. In vitro chemical assays have rated cranberries as having some of the highest antioxidant values of over 21 fruits (*1*, *2*), and the overall phenolic content appears to correlate with the level of antioxidant activity. The Folin−Ciocalteu colorimetric test has shown cranberry to have one of the highest phenolic contents of a number of fruit species tested (*1*, *2*). The phenolic classes identified in cranberry include phenolic acids (*3*−*6*), anthocyanins (*7*, *8*), flavonols (*9*, *10*), and flavan-3-ols, which consist of both monomers and the polymer classes of procyanidins and proanthocyanidins (*11*−*14*). Cranberry A type proanthocyanidins have been recognized for their antiadherence activities against uropathogenic P type *Escherichia coli* (*12*, *13*) and may play a role in urinary tract health.

Of fruit species, the cranberry is one of the highest in the concentration of quercetin, ranging from 11 to 25 mg/100 g fresh fruit (*15*, *16*). Quercetin is receiving much attention for potential health benefits and is associated with numerous biological properties (*17*). Quercetin and related compounds exhibit DPPH radical scavenging activity and inhibit a number of the processes associated with inflammation including lipopolysaccharide-induced nitric oxide and cytokine production (*10*, *18*−*21*). Quercetin is predominantly found in a conjugated form with various sugars, and the sugar may significantly influence its bioavailability and absorption (*22*−*24*). This investigation focuses on the characterization of the flavonol glycosides in spray-dried cranberry powder equivalent to those used in dietary supplements.

MATERIALS AND METHODS

Extraction and Fractionation of Phenolic Constituents. Phenolic constituents from 1.0 kg of freeze-dried cranberry powder 90-MX supplied by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA)

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were extracted twice with 10.0 L of 80% acetone/water (1:10; v/v), filtered, and partially evaporated under reduced pressure at 35 °C to remove the acetone. The resultant aqueous phase was defatted by extraction with 0.5 L of hexane (1:1), and the aqueous layer was further extracted with three portions of 0.5 L of ethyl acetate (1:1). After the extract was concentrated in vacuo, the pooled ethyl acetate fraction (24.8 g) was further fractionated using column chromatography. A portion of ethyl acetate extract (8.0 g) was dissolved in 60% methanol and loaded onto a 100 mm × 45 mm column packed with hydrated Sephadex LH-20. Subsequent elution with water (1.5 L), 60% methanol/water (v/v) (2.4 L), 100% methanol (1.5 L), and 70% acetone/water (v/v) (1.5 L) was applied to fractionate phenolic classes and remove all phenolic constituents from the column. Fractions eluted were monitored by analytical high-performance liquid chromatography (HPLC). The 60% methanol fraction, containing mostly constituents with maximum absorbance at 340 nm, was eluted from the column in two sequential 1200 mL volumes yielding 660 (fraction 1) and 600 mg (fraction 2) of phenolics. Fraction 1 was used for the further isolation of individual constituents by preparative HPLC. The final isolation of compound **16a** from the peak **16** obtained by preparative HPLC was achieved on a 40 mm × 20 mm Sephadex LH-20 column using 60% methanol as the eluting solvent.

Reagents. All reagents were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma (St. Louis, MO) and were of analytical or HPLC grade. Dimethyl sulfoxide (DMSO)-d_6 (99.96% D) was obtained from Cambridge Isotope Laboratories (Andover, MA). Standards of quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, quercetin, myricetin, and kaempferol were purchased from Indofine Chemical Company, Inc. (Somerville, NJ). Sephadex LH-20 for column chromatography was obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

HPLC Apparatus and Chromatographic Conditions. *Analytical HPLC.* HPLC analysis was performed on a Waters Millenium HPLC system composed of an in-line degasser, a 600E multisolvent delivery system, a 717 plus autosampler, and a 996 photodiode array detector. A 250 mm × 4.6 mm i.d. Zorbax SB-C18 reversed phase column protected with a Waters Guard-Pak precolumn module was used for analysis. Separations were carried out in a binary solvent system: solvent A, 2% formic acid; solvent B, 2% formic acid in methanol. A linear gradient of 5−25% B from 0 to 5 min; 25−40% B from 5 to 25 min; an isocratic elution with 40% B from 25 to 30 min; a linear gradient of 40−95% B from 30 to 45 min; and an isocratic elution with 95% B from 45 to 50 min at a flow rate of 1 mL/min was used. A PDA detector from 210 to 700 nm was used to monitor elution.

Preparative HPLC. A 250 mm × 21.2 mm i.d. Zorbax SB-C18 column was used with a gradient elution developed for analytical separations (methanol/water/formic acid) at a flow rate of 15 mL/min. The column effluents were monitored from 210 to 400 nm. Fractions were collected using a fraction collector. A gradient elution program afforded eight individual compounds, which were purified by rechromatography under the same conditions.

Mass Spectrometry (MS). Atmospheric pressure chemical ionization (APCI) MS in the negative ion detection mode was obtained on a VG Platform mass spectrometer (Micromass, Manchester, U.K.). A 250 mm × 4.6 mm i.d. Zorbax SB-C18 reversed phase column and a mobile phase of methanol/formic acid/water at a flow rate of 1 mL/min as described above were used. Typical tuning parameters were as follows: corona, 3 kV; high voltage lens, 0.0 kV; cone, 15 V; source temperature, 150 °C; and APCI probe temperature, 450 °C. Spectra were scanned over a mass range of *m*/*z* 150−1100 at 1.0 s per cycle.

Positive ion electrospray ionization (ESI) MS was acquired on a ThermoFinnigan TSQ-Quantum mass spectrometer using standard operating parameters. Separations were carried out in a 150 mm × 4.6 mm i.d. Zorbax Eclipse XDB C18 reversed phase column and a binary solvent gradient of A [water/acetonitrile/trifluoroacetic acid (TFA); 95:5:0.1] and B [water/acetonitrile/TFA; 5:95:0.1]. A linear gradient of 0−100% B from 0 to 10 min and an isocratic elution with 100% B from 10 to 12 min at a flow rate of 1 mL/min were used. Spectra were scanned over a mass range of *m*/*z* 190−800 at 1.0 s per cycle.

High-Resolution Mass Spectrometry (HRMS). HRMS data were acquired on a Finnigan MAT-900ST mass spectrometer operating in the ESI mode. Accurate mass measurement was carried out by linear E-scan peak matching. Resolution was calculated using a $m/\Delta m$ 10% valley definition. The responses from polypropylene glycol at *m*/*z* 447, 505, 563, 621, and 679 were used as references to match vs the ion from the sample. Ten measurements were taken for each structure.

NMR Spectroscopy. The NMR data were obtained on either a Varian INOVA three-channel NMR spectrometer operating at a ^{1}H observation frequency of 599.730 MHz and equipped with a 3 mm Nalorac Z-SPEC MIDTG gradient inverse triple resonance NMR probe or a Varian INOVA three-channel NMR spectrometer operating at a ^{1}H observation frequency of 499.792 MHz and equipped with a 5 mm Varian Chili-probe gradient inverse triple resonance NMR probe operating at a coil temperature of 25 K. The sample temperature was regulated at 20 °C for all samples except **9**, for which data were acquired at 32 °C. Samples were dissolved in ∼150 mL of DMSO-d_6 and transferred to a Wilmad 3 mm NMR tube for analysis. Peak **16a** was dissolved in ∼150 mL of 90:10 DMSO-d_6:benzene-d_6. Chemical shifts were referenced relative to the residual solvent resonances at 2.49 and 39.5 ppm for ^{1}H and ^{13}C, respectively. All ^{1}H NMR data were acquired with a spectral width of 16 ppm. Correlated spectroscopy (COSY) data were acquired as 256 increments with eight transients per increment; squared sinebell apodization was used in both dimensions. Heteronuclear single quantum coherence (HSQC) and HSQC total correlation spectroscopy (HSQCTOCSY) data were acquired as 96 increments with 24 and 96 transients per increment, respectively. Both data sets had an F1 spectral window of 146 ppm and were apodized with a Gaussian weighing function in both dimensions; the HSQCTOCSY mixing time was set to 18 ms. Heteronuclear multiple bond correlation (HMBC) data were acquired as 96 increments with 320 transients per increment. The F1 spectral width was 241 ppm. Squared sinebell apodization was used in both dimensions.

Identification of Flavonol Glycosides and Aglycones. Standard curves for identification of flavonols were prepared using authentic standards dissolved in methanol at a concentration of 1 mg/mL and stored at −20 °C as stock solutions. Identification of quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, myricetin, and quercetin was performed by matching their retention times and spectrometric characteristics measured at 340 nm against those of standards.

RESULTS AND DISCUSSION

Cranberry powder extracted with aqueous acetone (80%) followed by partitioning with ethyl acetate yielded a phenolic fraction, which was further fractionated on Sephadex LH-20 column using serial elutions with organic solvents. HPLC analysis with PDA detection indicated that the fraction eluted with 60% methanol was composed primarily of phenolics with maximum absorbance near 350−360 nm. Further isolation and determination of the components were undertaken.

To optimize resolution of compounds within the main phenolic classes (phenolic acids, anthocyanins, flavonols, and proanthocyanidins), analytical C18 reverse phase columns, eluants, and gradients were used to obtain well-resolved HPLC profiles. The method described herein provided optimal simultaneous resolution for cranberry phenolic acids, anthocyanins, and flavonols.

Figures 1 and **2** show chromatograms of 60% methanol fractions 1 and 2, respectively, eluted from the Sephadex LH-20 column at 340 nm absorbance. Fraction 1 exhibited well-resolved peaks at R_t 29−48 min and included some minor constituents with R_t 15−26 min. Fraction 2 exhibited four peaks, two of which (R_t 39.4 and 44.6) were predominant. All peaks in the fraction 1 chromatogram with retention times between 29 and 48 min numbered from 1 to 22 (**Figure 1**) displayed absorbance profiles corresponding to those of flavonols (*25*, *26*). Three peaks of R_t 34.3, 35.1, and 40.4 min were found to be consistent with retention times and UV/vis spectra of standards quercetin-3-galactoside (**5**), quercetin-3-glucoside (**6**), and quer-

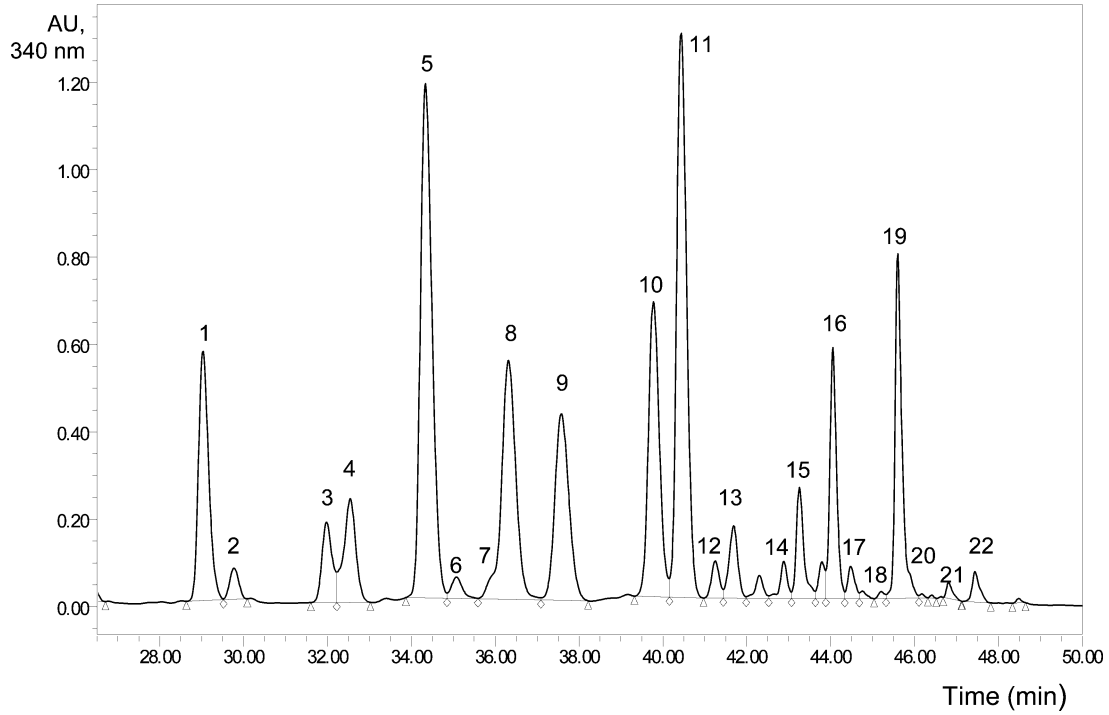

Figure 1. HPLC chromatogram of 60% methanol fraction 1 after Sephadex LH-20 column chromatography. Peaks correspond to numbering of compounds in **Table 1**.

Table 1. Retention Times, Relative Peak Areas, λ_{max}, *m/z* Values of $[M + H]^+$, $[M - H]^-$, and Fragment Ions of Flavonoid Constituents Resolved by HPLC from 60% Methanol Fraction 1[a]

compd	retention time (min)	relative peak area (%)	λ_{max} (nm)	$[M - H]^-$ and fragment ions in APCI MS	$[M + H]^+$ in ESI MS	structures
1	29.0	8.3	261.5, 356.5	479 (25%), 317 (100%)	NA	myricetin-3-β-galactoside[b,d]
2	29.8	0.9	261.5, 356.5	449 (100%), 317 (90%)	451	myricetin-3-α-xylopyranoside[e]
3	32.0	2.2	233.2, 266.2, 299.4, 356.5	ND	NA	ND
4	32.5	3.6	261.5, 356.5	449 (65%), 317 (100%)	NA	myricetin-3-α-arabinofuranoside[b−d]
5	34.3	21.5	256.8, 356.5	463 (55%), 301 (100%)	465	quercetin-3-β-galactoside[b−e]
6	35.1	0.9	256.8, 356.5	463 (60%), 301 (100%)	465	quercetin-3-β-glucoside[f]
7	35.8	1.3	256.8, 356.5	ND	NA	ND
8	36.3	8.2	256.8, 356.5	433 (45%), 301 (100%)	NA	quercetin-3-α-xylopyranoside[b,d]
9	37.6	6.7	256.8, 356.5	433 (35%), 301 (100%)	435	quercetin-3-α-arabinopyranoside[f]
10	39.8	9.7	256.8, 356.5	433 (35%), 301 (100%)	435	quercetin-3-α-arabinofuranoside[b−e]
11	40.4	14.3	256.8, 351.7	447 (55%), 301 (100%)	NA	quercetin-3-rhamnopyranoside[b−d]
12	41.2	1.6	256.8, 356.5	477 (100%), 315 (25%)	NA	3′-methoxyquercetin-3-β-galactoside[b,d]
13	41.7	3.9	256.8, 356.5	507 (100%), 463 (48%), 345 (48%), 331 (46%)	NA	dimethoxymyricetin-hexoside[d], methoxymyricetin-pentoside[d]
14	42.9	0.6	256.8, 356.5	447 (100%), 315 (55%)	NA	methoxyquercetin-pentoside[d]
15	43.3	2.3	256.8, 356.5	447 (100%), 315 (32%)	NA	3′-methoxyquercetin-3-α-xylopyranoside[e]
16–**16a**	44.0	4.6	256.8, 351.7, 256.8, 318.4, 351.7sh	447 (70%), 315 (25%), 609 (35%), 301 (100%)	NA, 611[g]	methoxyquercetin-pentoside[d], quercetin-3-*O*-(6″-*p*-coumaroyl)-β-galactoside[f]
17	44.5	0.8	256.8, 356.5	ND	NA	ND
18	44.8	1.5	256.8, 313.6, 356.5	ND	NA	ND
19	45.6	5.6	256.8, 356.5	567 (100%), 301 (52%)	569	quercetin-3-*O*-(6″-benzoyl)-β-galactoside[f]
20	46.2	0.1	256.8, 356.5	ND	NA	ND
21	46.8	0.3	256.8, 356.5	581 (53%), 431 (100%), 299 (28%)	NA	methoxykaempferol derivatives[d]
22	47.4	1.1	256.8, 290sh, 364.6	593 (50%), 431 (100%), 345 (68%), 299 (75%)	NA	methoxykaempferol derivatives[d]

[a] Note: ND, not determined; NA, not available. [b] According to Yan et al. (*10*). [c] According to Puski and Francis (*9*). [d] Structures based on LC-MS data. [e] Structures based on LC-MS and NMR data. [f] Structures characterized with LC-MS, HRMS, and NMR. [g] $[M + H]^+$ for peak **16a**.

cetin-3-rhamnoside (**11**), respectively (**Figure 1** and **Table 1**). Two major flavonoids eluted in fraction 2 (**Figure 2**) were identified as myricetin (R_t 39.8) and quercetin (R_t 44.6).

APCI LC-MS analysis in the negative ion mode was used to identify the molecular weights of constituents eluting in 60% methanol fraction 1 and to ascertain whether they were sugar conjugates as evidenced by loss of 162/132 mass units from the pseudomolecular ion. The results obtained are summarized in **Table 1** together with spectrometric characteristics, distribution of peaks by area percentages (at 340 nm), and retention

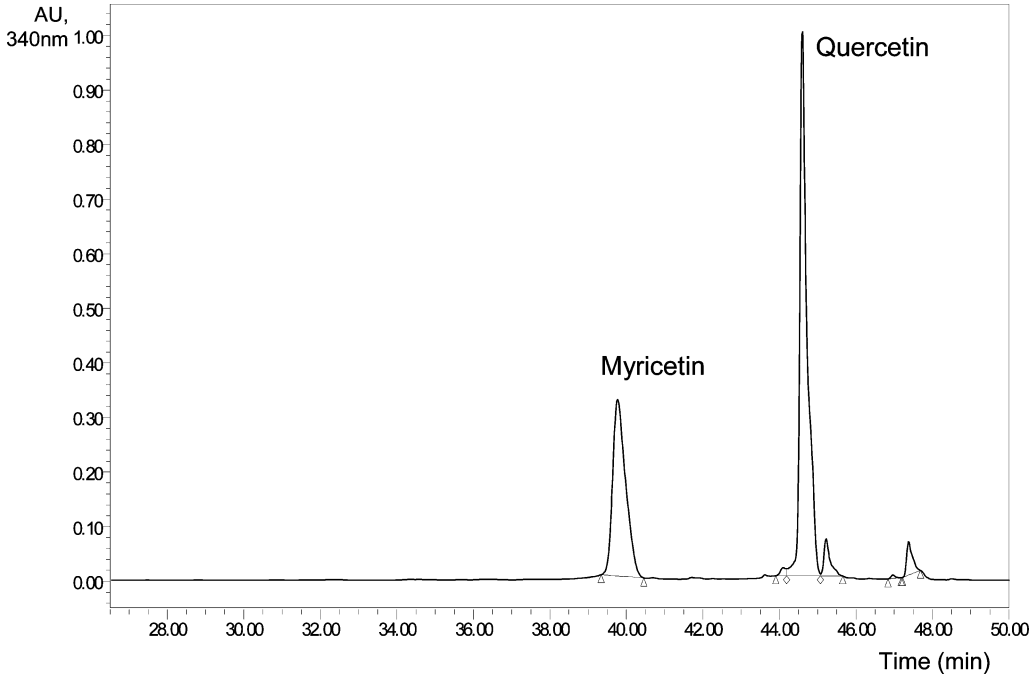

Figure 2. HPLC chromatogram of 60% methanol fraction 2 after Sephadex LH-20 column chromatography.

time under chromatographic conditions employed. APCI LC-MS of phenolics gives intense deprotonated molecular ions $[M - H]^-$ in the negative ion mode.

Peak **1** exhibited an intense $[M - H]^-$ ion peak at *m*/*z* 479 and a fragment ion at *m*/*z* 317 $[M - C_6H_{11}O_5]^-$ corresponding to a myricetin hexoside. The spectrum of the peaks **2** and **4** both gave $[M - H]^-$ ion peaks at *m*/*z* 449 and a fragment ion at *m*/*z* 317 $[M - C_5H_9O_4]^-$ consistent with myricetin pentoside conjugates. Peaks **5** and **6** exhibited in the APCI mass spectra characteristic $[M - H]^-$ ions at *m*/*z* 463 and fragment ions at *m*/*z* 301 $[M - C_6H_{11}O_5]^-$ corresponding to quercetin hexosides. These peaks, when compared with chromatographic behavior and UV/vis spectra of standards, were identified as quercetin-3-*β*-galactoside and quercetin-3-*β*-glucoside, respectively.

Three peaks of R_t 36.3, 37.6, and 39.8 (**8**−**10**) showed intense $[M - H]^-$ ion peaks at *m*/*z* 433 and fragment ions at *m*/*z* 301 $[M - C_5H_9O_4]^-$ consistent with quercetin pentoside structures. The exact nature of the sugar moiety cannot be ascertained by LC-MS. The spectra obtained for peak **11** exhibited a $[M - H]^-$ ion at *m*/*z* 447 and a fragment ion at *m*/*z* 301 $[M - C_6H_{11}O_4]^-$, which corresponds to quercetin-3-α-rhamnoside as determined by comparison with a standard.

Peak **12** gave a $[M - H]^-$ ion peak at *m*/*z* 477 with a fragment ion at *m*/*z* 315 $[M - C_6H_{11}O_5]^-$ consistent with a possible structure containing a monomethoxyquercetin hexoside. The position of the methyl substituent could not be determined. Peaks **14** and **15** exhibited $[M - H]^-$ ions at *m*/*z* 447 and had fragment ions at *m*/*z* 315 $[M - C_5H_9O_4]^-$ corresponding to possible methoxylated quercetin pentosides. The MS spectra of peaks **13**, **16**, **21**, and **22** appeared more complex and suggested the presence of a mixture of components. Possible constituents eluted correspond to monomethoxymyricetin pentoside (*m*/*z* 463, 331) and dimethoxymyricetin hexoside (*m*/*z* 507, 345) for peak **13** and to derivatives of methoxykaempferol (*m*/*z* 299) for peaks **21** and **22**. Deprotonated molecular ions for peak **16** indicate the presence of possible monomethoxyquercetin pentoside (*m*/*z* 447, 315) and acylated derivative of quercetin hexoside (*m*/*z* 609, 301).

One of the latest eluting peaks at R_t 45.6, peak **19**, gave $[M - H]^-$ and fragment ion peaks at *m*/*z* 567 and 301, which we initially speculated to be a quercetin hexose ester with benzoic acid. This conjecture was subsequently confirmed by NMR spectroscopy.

For structural determination of various flavonoid constituents by NMR spectroscopy, fraction 1 of the 60% methanol eluate was further fractionated by preparative HPLC. The procedure applied for the elution of constituents with two sequential volumes of 60% methanol during Sephadex LH-20 column chromatography was useful to prevent a possible coelution of abundant simple flavonols, such as myricetin and quercetin, with peaks of flavonol conjugates (**Figures 1** and **2**).

Sufficient amounts (1−2 mg) of eight pure components (**2**, **5**, **6**, **9**, **10**, **15**, **16**, and **19**) were obtained. Peak **16**, represented by a mixture of coeluting compounds, was subjected to additional chromatography on a Sephadex LH-20 column with 60% methanol as the elution solvent. The compound with $[M - H]^-$ ion peak at *m*/*z* 609 (**16a**) was used for structural determination by NMR spectroscopy. The NMR analysis of purified peaks was supported by ESI LC-MS data performed in the positive ion mode (**Table 1**).

The compounds conclusively identified using NMR spectroscopy include myricetin-3-*β*-xylopyranoside (**2**), quercetin-3-*β*-galactoside (**5**), quercetin-3-*β*-glucoside (**6**), quercetin-3-α-arabinopyranoside (**9**), quercetin-3-α-arabinofuranoside (**10**), 3′-methoxyquercetin-3-α-xylopyranoside (**15**), quercetin-3-*O*-(6″-*p*-coumaroyl)-*β*-galactoside (**16a**), and quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside (**19**) (**Figure 3**).

The assignment of peaks **5** and **6** previously identified as quercetin-3-*β*-galactoside and quercetin-3-*β*-glucoside, respectively, was confirmed by NMR data (**Table 2**). For peak **6**, the anomeric proton appeared as a doublet ($J = 7.6$ Hz) while the 2″, 3″, and 4″ proton resonances were apparent triplets ($J =$ 7.8−8.8 Hz), consistent with a glucose moiety. HRMS determined the mass of the compound in sample peak **6** to be 464.09540 Da with a standard deviation of 0.00059 Da ($N =$ 10) taken at a resolution of 12 500. The experimental mass differs by −0.2 ppm from the theoretical mass of the empirical formula for the proposed structure, $C_{21}H_{20}O_{12}$ (quercetin-3-*β*-glucoside). The flavonoid at R_t 39.8 (peak **10**) was identified as quercetin-3-α-arabinofuranoside, which is consistent with

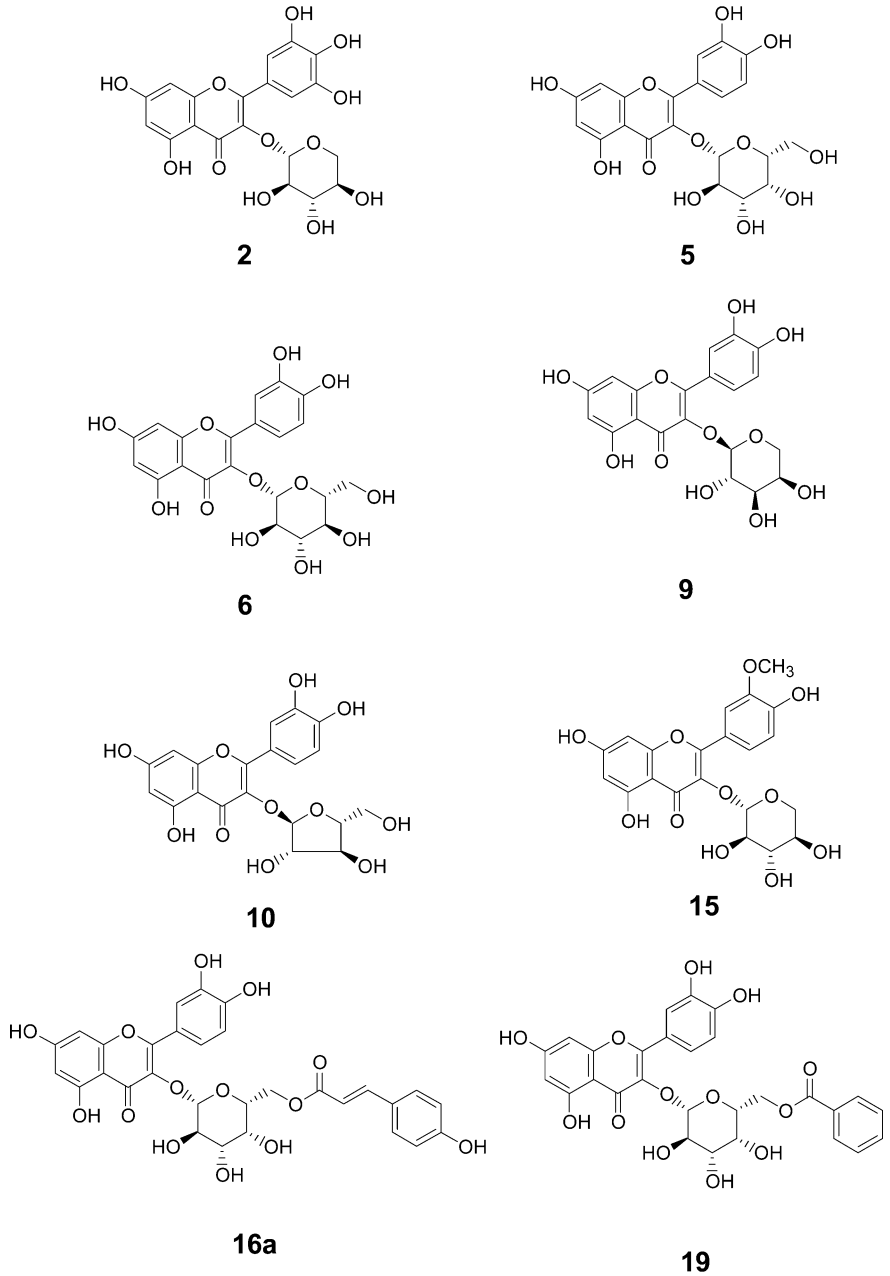

Figure 3. Chemical structures of flavonol glycosides isolated from cranberry powder: myricetin-3-β-xylopyranoside (**2**), quercetin-3-β-galactoside (**5**), quercetin-3-β-glucoside (**6**), quercetin-3-α-arabinopyranoside (**9**), quercetin-3-α-arabinofuranoside (**10**), 3′-methoxyquercetin-3-α-xylopyranoside (**15**), quercetin-3-*O*-(6″-*p*-coumaroyl)-β-galactoside (**16a**), and quercetin-3-*O*-(6″-benzoyl)-β-galactoside (**19**).

previously reported data (*9*, *10*). Quercetin-3-β-glucoside (**6**) and structures **2**, **9**, **15**, **16a**, and **19** represent compounds not previously reported in cranberry or cranberry products.

Myricetin-3-β-xyloside (**2**) in the pyranose form was apparent due to the 4″ carbon chemical shift and the strongly anisochronous 5″ methylene responses, which are consistent with those of a pentose in the pyranose form. The anomeric signal appeared as a $J = 7.43$ Hz doublet, and taken along with the downfield shift of the carbon resonance, indicated a β-configuration. The 3″ proton resonance was an apparent triplet ($J = 8.66$ Hz), establishing a *trans*−diaxial relationship for the 2″−3″ and 3″−4″ pairs.

The pyranose form of quercetin-3-α-arabinoside (**9**) was indicated by the 4″ carbon chemical shift and the anisochronous 5″ methylene group. The anomeric signal appeared as a $J = 5.2$ Hz doublet at 5.22/102.3 ppm, implying the α-configuration. The 2″−3″ coupling constant of $J = 6.7$ Hz indicated a *trans*−diaxial configuration, while a 3″−4″ coupling constant ($J = 3.1$ Hz) implies an axial−equatorial orientation, yielding the assignment of arabinopyranose. HRMS determined the mass of the compound in sample peak **9** to be 434.08459 Da with a standard deviation of 0.00056 Da ($N = 10$) taken at a resolution of 10 300. The experimental mass differs by −0.7 ppm from the theoretical mass of the empirical formula of the proposed structure, $C_{20}H_{18}O_{11}$. Thus, the relatively major flavonol quercetin-3-arabinoside exists in two sugar forms, as a pyranose (**9**) (∼6.7%) and the previously reported (*10*) furanose form (**10**) (∼9.7%) (**Table 1**).

Peak **15** presented NMR spectra indicative of quercetin and a xylopyranose sugar moiety. An aromatic methoxy resonance was also observed (3.71, 51.9 ppm) that yielded a 3-bond HMBC response to the 3′ carbon resonance at ∼144 ppm, allowing assignment of the site of the methoxy substitution.

Table 2. ^{13}C and ^{1}H NMR Shifts of Cranberry Flavonol Glycosides (ppm)

	2		5		6		9		10		15		16a		19	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
6	99.5	6.15	99.4	6.04	100.4	6.06	99.6	6.14	99.5	6.14	94.6	6.14	99.8	6.41	99.4	6.13
8	94.1	6.32	94.8	6.23	95.5	6.27	94.5	6.35	94.3	6.35	89.7	6.39	94.6	6.54	94.0	6.29
2′	109.1	7.12	117.1	7.59	116.8	7.50	116.8	7.47	116.1	7.43	108.6	7.71	117.0	7.84	116.5	7.45
5′			116.1	6.79	116.1	6.81	116.1	6.80	116.2	6.79	111.0	6.82	116.4	7.05	115.8	6.77
6′	109.1	7.12	122.7	7.39	122.7	7.44	122.7	7.60	122.4	7.50	118.2	7.46	122.7	7.85	122.8	7.58
1″	102.5	5.31	103.1	4.97	102.2	5.06	102.3	5.22	108.6	5.53	97.5	5.20	102.9	5.66	101.9	5.45
2″	74.3	3.34	71.8	3.59	74.3	3.26	71.7	3.73	83.0	4.11	69.5	3.17	71.8	3.58	72.1	3.58
3″	77.0	3.14	73.3	3.43	76.2	3.25	72.6	3.48	77.6	3.67	71.3	3.14	73.9	3.41	74.4	3.42
4″	70.2	3.31	68.6	3.69	69.9	3.15	66.9	3.62	86.8	3.51	65.1	3.21	69.1	3.64	69.3	3.68
5″	66.9	2.91/ 3.60	75.8	3.36	77.1	3.06	65.2	3.18/ 3.57	61.6	3.25	61.7	3.54/ 2.89	73.8	3.66	74.2	3.75
6″			60.9	3.34/ 3.44	61.1	3.34/ 3.47							64.3	4.26/ 4.35	64.9	4.23

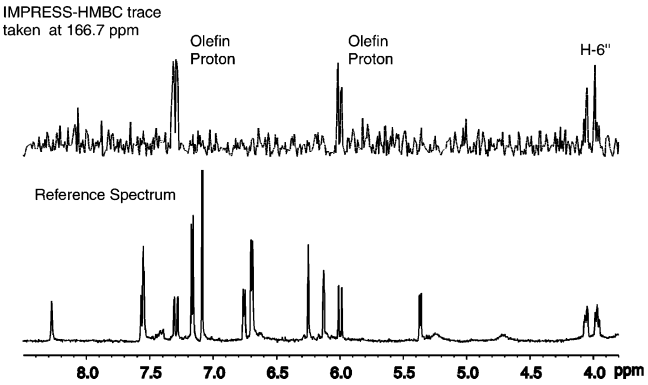

Figure 4. Peak **16a** ^{1}H NMR spectrum (bottom), and the trace extracted from the 6 Hz optimized IMPRESS-HMBC data set at 166.7 ppm (upper) showing 2- and 3-bond coupling responses from the olefin protons of the coumaroyl moiety and the galactose C-6″ protons to the same ester carbonyl resonance.

A quercetin moiety was observed in the isolate of peak **16a** by NMR spectroscopy, and resonances consistent with the presence of a galactopyranose sugar moiety were observed. Two new AB spin systems were observed in the downfield region; one integrating for a total of two protons (7.58/145.5 and 6.29/114.6 ppm), the other integrating for four protons (7.46/130.9 and 6.99/116.7 ppm). The larger set of resonances was readily assigned as a 1,4-substituted aromatic system based on HMBC data, while the smaller system was assigned as a *trans*-olefin due to its 15.9 Hz coupling constant. An IMPRESS-HMBC data set was used to establish an ester carbonyl linkage between the galactopyranose and the olefinic moieties (*27*). HRMS determined the mass of the compound in sample peak **16a** to be 610.13161 Da with a standard deviation of 0.00070 Da ($N = 10$) taken at a resolution of 10 700. The experimental mass differs by −1.1 ppm from the theoretical mass of the empirical formula of the proposed structure, $C_{30}H_{26}O_{14}$. Thus, the NMR data coupled with the molecular mass information yielded the assignment of this isolate as the C-6″ *para*-hydroxycinnamic acid ester of quercetin-3-β-galactopyranose (**Figure 4**).

Interpretation of the NMR spectra obtained on the peak **19** (^{1}H reference, COSY, HSQC, HSQCTOCSY, and HMBC) revealed several structurally significant features. The resonances indicating a quercetin moiety were readily observed and assigned by inspection. Resonances consistent with a sugar moiety were also observed. The sugar resonances were somewhat obscured by the residual water in the sample, but several key assignments were possible. The anomeric methine doublet ($J = 7.6$ Hz) was observed at 5.46/101.9 ppm, indicating an axial proton orientation and therefore a β-linkage to the quercetin backbone. COSY and HSQCTOCSY responses allowed complete, sequential assignment of the sugar ring. A large coupling constant ($J = 8.3$ Hz) was observed for the 2″−3″ interaction, indicative of a *trans*−diaxial interaction, while the 4″ proton resonance was observed as a broad singlet. This result requires axial−equatorial interactions for both 3″−4″ and 4″−5″ and indicated the assignment of the sugar moiety as β-galactose.

Another spin system was identified in the aromatic region of the spectrum. This system consisted of an apparent doublet−triplet−triplet pattern in a 2:2:1 ratio and could be readily assigned as a phenyl group (*ortho*, 7.62/129.4; *meta*, 7.26/129.3; and *para*, 7.49/133.9 ppm). Investigation of the HMBC data set revealed a 3-bond response between the *ortho*-protons of this phenyl ring with a carbonyl resonance at 166.1 ppm. A second 3-bond response to this same carbonyl carbon was observed from the 6″ methylene protons. Taken with the C-6″ chemical shift of 64.9 ppm and the C-9″ chemical shift of 129.5 ppm, these data clearly indicated the presence of a benzyl ester moiety attached at the 6″ position of the galactose ring. HRMS determined the mass of the compound in sample peak **19** to be 568.12212 Da with a standard deviation of 0.00046 Da ($N = 10$) taken at a resolution of 12 200. The experimental mass differs by 0.8 ppm from the theoretical mass of the empirical formula of the proposed structure, $C_{28}H_{24}O_{13}$.

The cumulative data showed the presence of mostly glycosylated forms of myricetin and quercetin in the 60% methanol fraction 1/ethyl acetate powder extract. Together with UV spectra and peak area percentages, these results confirm the identity of peaks **4**, **5**, **10**, and **11** as myricetin-3-α-arabinofuranoside, quercetin-3-β-galactoside, quercetin-3-α-arabinofuranoside, and quercetin-3-α-rhamnoside (*9*, *10*). The peaks labeled **1**, **8**, and **12** correspond to myricetin-3-β-galactoside, quercetin-3-xyloside, and 3′-methoxyquercetin-3-β-galactoside (*iso*-rhamnetin-galactoside), respectively. These components were recently identified in extracts from fresh cranberry by Yan et al. (*10*).

Flavonol constituents previously reported from American cranberry include those already mentioned as well as quercetin and glycosides of kaempferol including kaempferol-3-glucoside (*9*, *28*). In fresh fruit, flavonols were reported to be comprised of mainly quercetin derivatives, with quercetin-3-β-galactoside as the major glycoside (*10*). Similar to fresh fruit, quercetin-3-β-galactoside (**5**) was found to be the major flavonol glycoside (21.5% relative peak area; **Table 1**) in this study. However, quercetin-3-rhamnopyranoside (**12**), with a peak area of 14.3%, was the second highest in concentration in powder, whereas quercetin-3-α-arabinofuranoside was the second major flavonol

in whole cranberries (*10*). It is noteworthy that in addition to the furanose form of quercetin-3-arabinose, the pyranose form was also identified whereas only quercetin-3-arabinofuranoside was found in fresh fruit (*10*). The methods presented in this study have identified quercetin-3-arabinopyranoside in fresh fruit as well (Vvedenskaya and Vorsa, unpublished data).

Although kaempferol hexoside (*28*) and diglycosides of myricetin and quercetin (*9*, *29*) in cranberry extracts have been reported, evidence of these compounds was not found in this study. However, some peaks present in the chromatogram remain unidentified and may account for these structures. Kandil et al. (*29*) reported the presence of a flavonol constituent in cranberry with a molecular ion $[M + Na]^+$ at *m*/*z* 632.9, which was identified as rutin. No evidence for the presence of rutin in either cranberry powder or fresh fruit extracts was found in this study. However, a flavonol conjugate with the same molecular weight (610) was identified as quercetin-3-*O*-(6″-coumaroyl)-*β*-galactoside (**16a**).

The flavonol glycosides profile of cranberry powder, although similar to that of fresh cranberry, does appear to have some distinct differences that may arise from the processing procedure. The most pronounced is the presence of the flavonol aglycons myricetin and quercetin and the constituent quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside. Evaluation of a number of cranberry juice concentrates and powder samples indicates that these components are present in significant quantities only after processing (Vvedenskaya and Vorsa, unpublished data).

Little information exists with regard to the presence of methyl ethers of flavonols in cranberry. To our knowledge, except for *iso*-rhamnetin galactoside (*10*), the presence of other methyl ethers has not been reported. Among various fruit species, 3′-methoxyquercetin derivatives were previously reported for lemon, sweet cherry, grape, and grapefruit (*25*). The flavonols of pear are characterized by the presence of mainly *iso*-rhamnetin glycosides (*30*). Besides the second fully characterized methoxy ether derivative, 3′-methoxyquercetin-3-α-xylopyranoside (**15**), the LC-MS data suggest the presence of a number of other methoxylated flavonols including derivatives of quercetin, myricetin, and kaempferol (**Table 1**).

Thus, the solvent system and elution program for HPLC developed in our study revealed 22 peaks in contrast to the nine reported previously in cranberry (*10*) and were more effective for the separation of individual flavonol glycosides within this class of flavonoids. Although the HPLC analysis time presented here is longer, it provides increased resolution, enabling the identification of additional flavonoids of which six were structurally determined. Two newly identified compounds, **16a** and **19**, are very rare acylated quercetin-galactosides. Quercetin-3-*O*-(6″-coumaroyl)-*β*-galactoside (**16a**) was only reported in *Ledum palustre* L. (*31*). Quercetin-3-*O*-(6″-benzoyl)-*β*-galactoside (**19**) is isolated for the first time from a processed natural source.

Currently, there is little information regarding the bioactivity of individual flavonol glycosides found in cranberry. Reports indicate that DPPH radical scavenging activities of the flavonols myricetin-3-arabinoside, myricetin-3-galactoside, quercetin-3-galactoside, and quercetin-3-rhamnoside are superior to that of vitamin E, and quercetin-3-xyloside prevents LDL oxidation (*10*). Quercetin-3-rhamnoside from the leaves of *Erythrospermum monoticolum* was found to be active against acute inflammation in mice induced by TPA, significantly reducing edema (62%) when compared with the reference drug, indomethacin (*32*). The 60% methanol flavonol fraction described in this study was found to exhibit fairly high activity in a mouse ear TPA-induced edema bioassay (Vvedenskaya and Vorsa, unpublished data).

There has been considerable interest on the bioactivity of the flavonol aglycons, kaempferol, quercetin, and myricetin (*33*, *34*). Quercetin has been found to mediate the lipopolysaccharide (LPS)-induced inflammatory response by inhibiting cytokine production (*21*) and the expression of LPS inducible nitric oxide synthase (*20*). Because flavonols occur predominately as sugar conjugates, their availability in vivo may also be significantly influenced by the specificity of the sugar moiety (*22*, *23*, *35*).

The total flavonol content of American cranberry is one of the highest among fruit and berry crops (*15*, *16*). It is comparable with that of apple peel, pea pods, kale, and onions, which are known to be rich in total flavonols (*36*−*38*). The information obtained in the present study significantly extends our knowledge on flavonol glycosides of cranberry powders, which are widely utilized in cranberry-derived products, and may serve as the basis for future research of cranberry phytochemicals with potential human health benefits.

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