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Identification of flavonol glycosides in American cranberry fruit

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Abstract

Two flavonol glycosides, quercetin galactoside and quercetin arabinoside, have been identified in American cranberry fruit, as a complementary investigation of our previous study. The analysis processes included separation, hydrolysis and structure elucidation of flavonol glycosides. The separation of flavonol glycosides was carried out by solvent extraction, thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). After hydrolysis of the obtained flavonol glycosides, flavonol aglycones and sugars were identified by HPLC and gas chromatography–mass spectrometry (GC–MS), respectively.

Keywords: Identification; Flavonol glycoside; Cranberry; TLC; HPLC; GC–MS

1. Introduction

Flavonoids are one of the most important natural pigments and widely distributed in vegetables, berries and fruits. Interest in separation and determination of flavonoid and other phenolic compounds in plant has been increasing during the past decade (Ayaz, Hayirlioglu-Ayaz, Gruz, Novak, & Strnad, 2005; Chen, Zuo, & Deng, 2001; Daigle & Conkerton, 1988; Endale, Kammerer, Gebre-Mariam, & Schmidt, 2005; Mattila, Astola, & Kumpulainen, 2000; Merken & Beecher, 2000; Nessa, Ismail, Karupiah, & Mohamed, 2005; Robards & Antolovich, 1997; Vvedenskaya et al., 2004; Zhang & Zuo, 2004; Zuo, Chen, & Deng, 2002; Zuo, Wang, & Zhan, 2002) because flavonoids and phenolic acids have definitive anticarcinogenic and cardioprotective effects on human health (Deshner, Ruperto, Wong, & Newmark, 1991; Lee, Krueger, Reed, & Richards, 2006; Pamucku, Yalciner, Hatcher, & Bryan, 1980) as important antioxidants. Flavonoids comprise flavonols, flavones, flavanones, anthocyanidins, catechins and bioflavans. Among these subclasses of flavonoids, flavonols are a major one and their characteristic skeletons are diphenylpropanes (C_6–C_3–C_6) with a hydroxyl group attached on C3. However, flavonols rarely occur in the free state but usually present as O- and C-glycosides (Häkkinen & Auriola, 1998; Swain, 1962).

Cranberry, a good food source of flavonoids (Chen et al., 2001; Chu, Clydesdale, & Francis, 1973; Hong & Wrolstad, 1986; Vvedenskaya et al., 2004; Zhang & Zuo, 2004), has being given much attention as a folk remedy since the 19th century. Native Americans used cranberries as a poultice for wounds. Pilgrims found that eating the berries could help to prevent scurvy (Thomos, 1900). Clinical studies over the course of six months suggested that pyuria could be reduced by nearly 50% in elderly women who drink 300 mL of cranberry juice cocktail each day probably because cranberry is able to block bacterial adherence to urinary epithelial cells (James, 1994). Recent study further indicates that cranberry juice may inhibit the development of human breast cancer (Zuo, Wang, & Wen, 2003). A combination of these unique antibacterial and anticarcinogenic functions of cranberry implies that cranberry may possess a very different antioxidants composition from other kinds of fruits (Avorn et al., 1994; Ofek et al., 1991). Researches focusing on analyses of antioxidants in cranberry have been extensively performed. However, most studies on cranberry in the literature were done

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with respect to the determination of phenolic acids and anthocyanin pigments while flavonoids in cranberry, especially flavonol glycosides, were largely ignored and the reports on the identification of cranberry flavonol glycosides (Chen et al., 2001; Häkkinen and Auriola, 1998; Lees and Francis, 1971; Puski and Francis, 1967; ; Zhang and Zuo, 2004; Zuo, Wang et al., 2002) are still limited so far. Previously we analyzed flavonol aglycones and phenolic acids in cranberry by HPLC and GC–MS (Chen et al., 2001; ; Zuo, Wang et al., 2002). A significant amount of quercetin was found in hydrolyzed cranberry juices while no free quercetin was detected in original cranberry juices, suggesting that flavonols exist in combined forms. Unfortunately, direct identification of flavonol glycosides by HPLC is still difficult due to a general lack of availability of pure flavonol glycoside standards, although some flavonol glycoside standards were recently used in the application of HPLC (Schieber, Keller, & Carle, 2001). In this follow-up study, a new method for the determination of flavonol glycosides in cranberry using TLC, HPLC and GC–MS was developed. It involved the extraction, purification and hydrolyses of flavonol glycosides, followed by individual identifications of flavonol aglycones and sugars. By using this method, we identified two flavonol glycosides, quercetin galactoside and quercetin arabinoside, in the freshly-squeezed cranberry juice and successfully interpreted two previously-unknown peaks in the HPLC chromatogram corresponding to these two glycosides.

2. Methodology

2.1. Experimental apparatus

Backman HPLC was equipped with a photodiode array detector and a reversed phase column RP-C_{18} (15 cm × 4.6 mm, 5 μm). The detection wavelengths were chosen at both 280 nm and 360 nm. Solvent gradients were formed by the dual pumping system by varying the proportion of solvent A (water:acetic acid, 97:3 by volume) to solvent B (methanol). The solvent gradient elution program is presented in Table 1. The chromatographic conditions were the same as those previously reported (Chen et al., 2001) except that the injection volume is 15 μL for qualitative analysis and 100 μL for the purification of flavonol glycosides, respectively.

HP 5890 gas chromatograph was equipped with a J&W Scientific DB-5ms column (30 m × 0.32 mm ID, 0.25 μm film) and a HP 5970 series mass selective detector. Helium gas was used as the carrier gas. The temperature of the injector port was 260 °C. The initial temperature of the column was 100 °C, which was kept for 0.5 min. Then the temperature of column was increased to 280 °C at a rate of 8.0 °C/min where it was kept for 5 min. The delay time for detecting was 2 min and the scanning range was 50–800 *m/z*.

Table 1
HPLC solvent gradient elution program

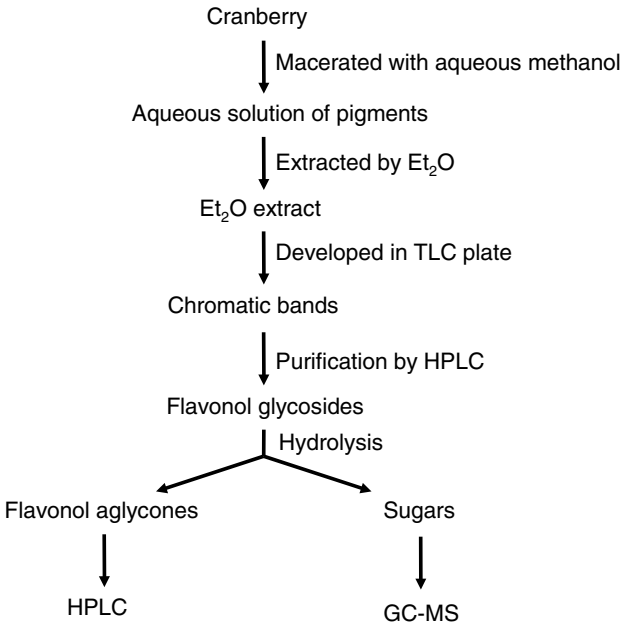
Time (min)	Solvent B (%)	Flow-rate (mL/min)
0	0	0.9
10	10	1.0
40	70	1.0
44	0	0.9
47	0	0.9

2.2. Chemicals

Standards of quercetin, D(−)-arabinose, D(−)-fructose, D(+)-galactose, D(+)-glucose and L(+)-rhamnose were purchased from Acros Organics (Geel Belgium, NJ, USA). The derivatizing reagent, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was obtained from Supelco Company (Bellefonte, PA, USA). Methanol was purchased from Pharmco Products (Brookfield, CT, USA). Acetic acid, ethyl ether, petroleum ether (b.p. 40–65 °C) and pyridine were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.3. Sample preparation and extraction

Natural cranberry fruit (*Vaccinium macrocarpon* Ait., variety Early Black) was obtained from the Cranberry Experiment Station, East Wareham, MA, USA and stored at −20 °C. Cranberry (66.5 g) was macerated in a Waring blendor with 100.0 mL of 70% aqueous methanol and the resultant slurry was then filtered through a Büchner funnel using Whatman No. 1 filter paper. The residue including the filter paper was then remacerated with 100.0 mL of 70% aqueous methanol and filtered as before. The rema-



Scheme 1. Flow chart of the total analysis process.

ceration and filtration procedure was repeated. All filtrate was combined and methanol was removed by a rotary evaporator to leave an aqueous solution of pigments. The aqueous solution was extracted twice with 60.0 mL of petroleum ether. The remaining aqueous solution was then extracted four times with 60.0 mL of ethyl ether. The ethyl ether layers were combined, filtered and then concentrated by a rotary evaporator and stored at 4 °C for TLC separation.

2.4. Preliminary separation by TLC

The concentrated ethyl ether extract was applied on the silica plates (20 cm × 20 cm, Aldrich) using ethyl acetate as the developing solvent. The developing process was stopped when the solvent front was 2 cm from the top edge of the silica plate. Chromatic bands on the silica plate were shown under UV lamp and circled with pencil. Then chromatic bands were scraped out respectively and extracted by

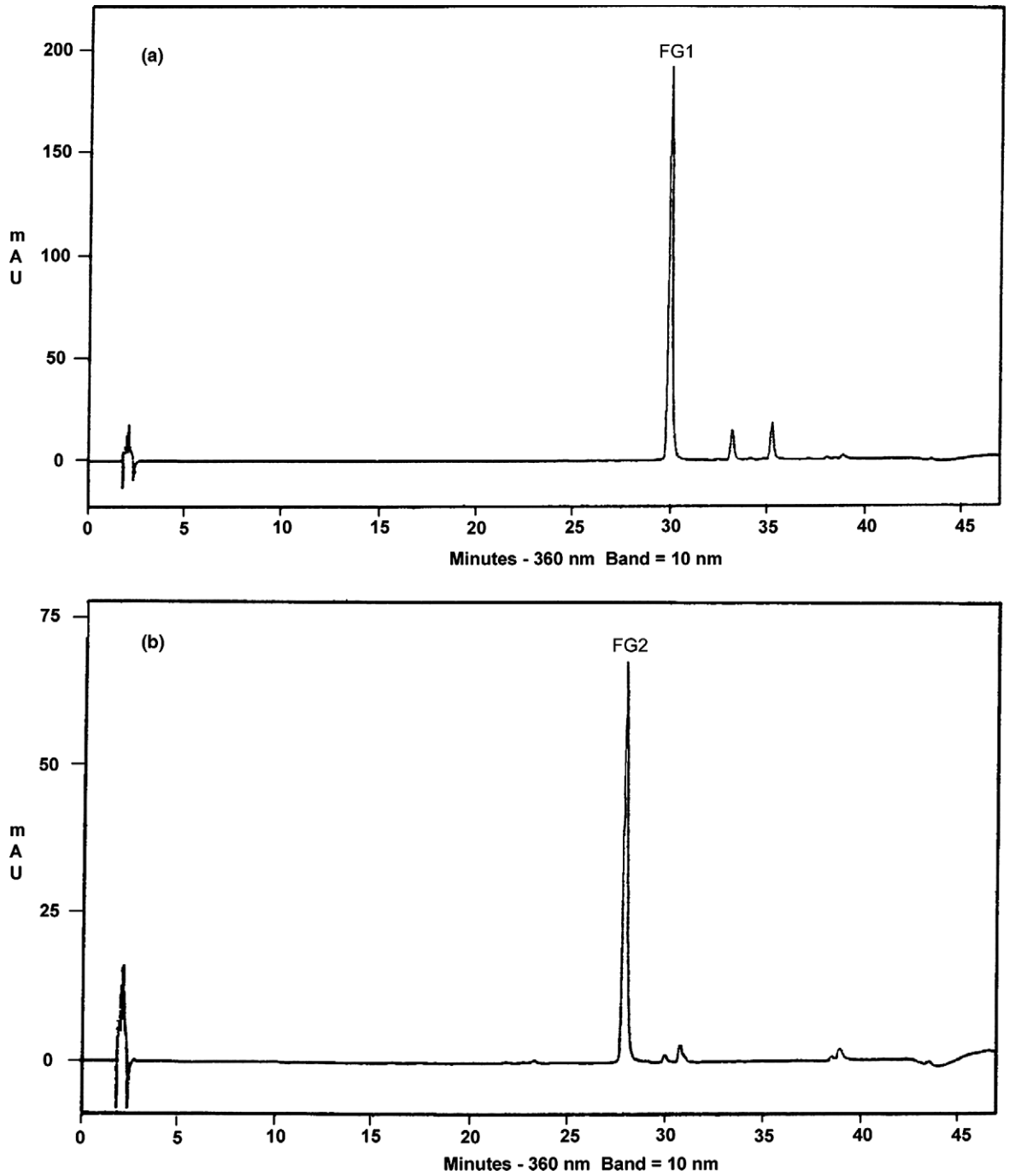


Fig. 1. HPLC chromatograms of the methanolic extracts from two major chromatic bands in TLC detected at 360 nm. The flavonol glycosides present in the extracts, FG1 and FG2, are shown in (a) and (b), respectively.

methanol (2×2.0 mL). The obtained methanol solutions were filtered and subjected to HPLC analysis. If there was any peak detected at 360 nm in the methanol extract, it would undergo the next step purification by HPLC.

2.5. Purification (by HPLC) and hydrolysis of flavonol glycosides

A 100.0 μL aliquot of the methanol extract was injected into the HPLC each time and the eluate was collected in a series of vials as the peak was appearing in the chromatogram detected at 360 nm. The solution in each vial was subjected to HPLC analysis again to check the composition of each fraction. N_2 gas was bubbled through the collected fractions in a water bath at 80 °C. 0.5 mL of solvent (H_2O:CH_3OH:CH_3COOH = 9.5:5.0:0.5, v/v/v) was added to the residue. The solution under N_2 was heated with a water bath at 80 °C for 30 min.

2.6. Identification of flavonol aglycones by HPLC and the conjugated sugars by GC–MS

The hydrolyzed solution was diluted by 0.5 mL of methanol and subjected to HPLC analysis in order to identify the flavonol aglycone. The solution spiked with quercetin standard solution was also subjected to HPLC analysis.

N_2 gas was bubbled through the hydrolyzed solution to remove methanol, followed by extraction by ethyl ether (3×1.0 mL) to remove the flavonol aglycone. N_2 gas was again bubbled through the aqueous phase till dryness at 80 °C. A 50.0 μL of newly distilled pyridine was added to dissolve the residue. The pyridine solution was derivatized with 50.0 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) at 70 °C on a hot plate for 15 min. Then 1.0 μL of the derivatized solution was injected into the GC–MS. 1.0 mg of arabinose, fructose, galactose, glucose and rhamnose standards were dissolved in 1.5 mL of newly distilled pyridine. A 50.0 μL aliquot was subjected to silylation and 1.0 μL of the derivatized solution was injected into the GC–MS.

3. Results and discussion

The selected liquid chromatographic conditions are the same as those previously reported (Chen et al., 2001) since a good separation of 13 flavonoids and phenolic acids was achieved in a short elution time of 47 min under these optimized chromatographic conditions. One of the detection wavelengths was chosen at 360 nm because most flavonols have characteristic UV absorption around this wavelength. In the previous study, only a few limited peaks were identified in the chromatogram of cranberry (Chen et al., 2001). This experiment was designed to identify flavonol glycosides and their corresponding peaks in the chromatogram of cranberry juice, which were not recognized in the previous study.

The flow chart of total analytical processes is shown in Scheme 1. The cranberry was first macerated with aqueous methanol and filtered through 0.45 μm filter membrane. Methanol in solution was subsequently removed using a rotary evaporator and the aqueous solution left was then extracted by petroleum ether to remove the impurities such as chlorophylls and waxy materials (Lees & Francis, 1971). After the obtained aqueous solution was extracted by ethyl ether, most of flavonol glycosides entered into the ethyl ether layer while anthocyanins, the main pigments in cranberry, remained in the aqueous phase (Lees & Francis,

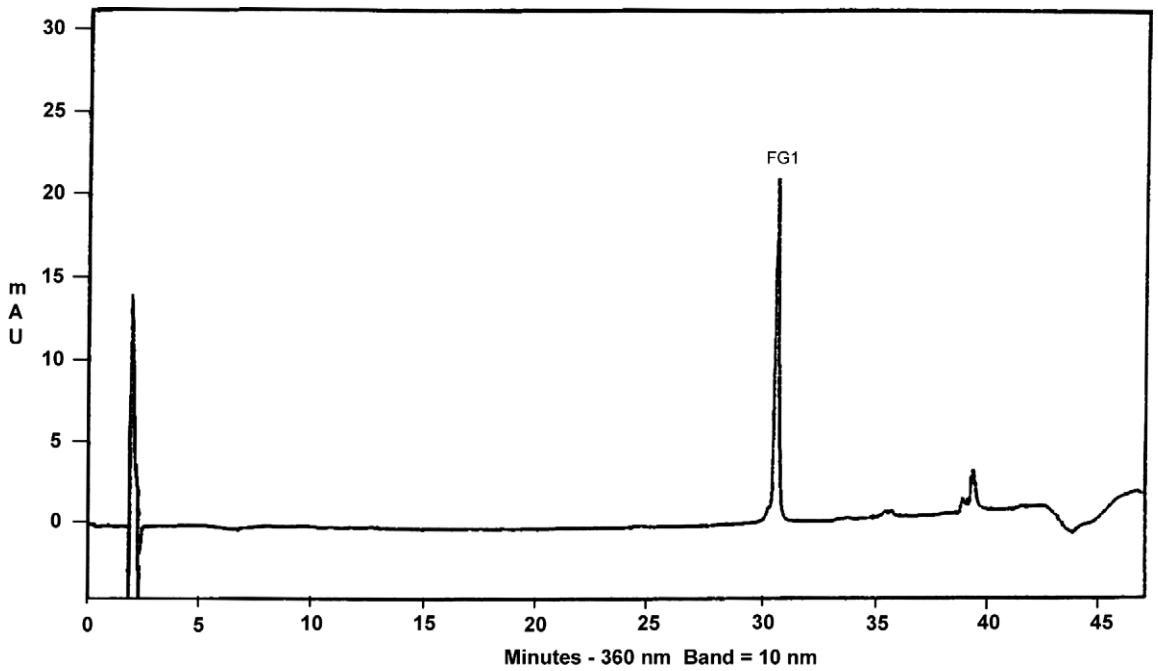


Fig. 2. HPLC chromatogram of the purified FG1 detected at 360 nm.

1971). The ethyl ether extract of cranberry was preliminarily separated by TLC. Two of major chromatic bands obtained from TLC separation were indicated to contain flavonol glycosides FG1 and FG2 by further HPLC analysis, based on the corresponding chromatograms detected at 360 nm shown in Fig. 1. Compounds FG1 and FG2 were further purified by using preparative HPLC separately and Fig. 2 shows the chromatogram of the purified flavonol glycoside FG1.

The flavonol glycosides FG1 and FG2 obtained were hydrolyzed to yield flavonol aglycones and sugars. The flavonol aglycone from the hydrolysis of FG1 was identified as quercetin based on the following information: (1) it has the same retention time as quercetin standard and completely overlaps with quercetin standard if spiked with quercetin (compare Fig. 3a with Fig. 3b); (2) it has the same UV absorption spectrum as the authentic standard with a maximum absorption at 367 nm, which is character-

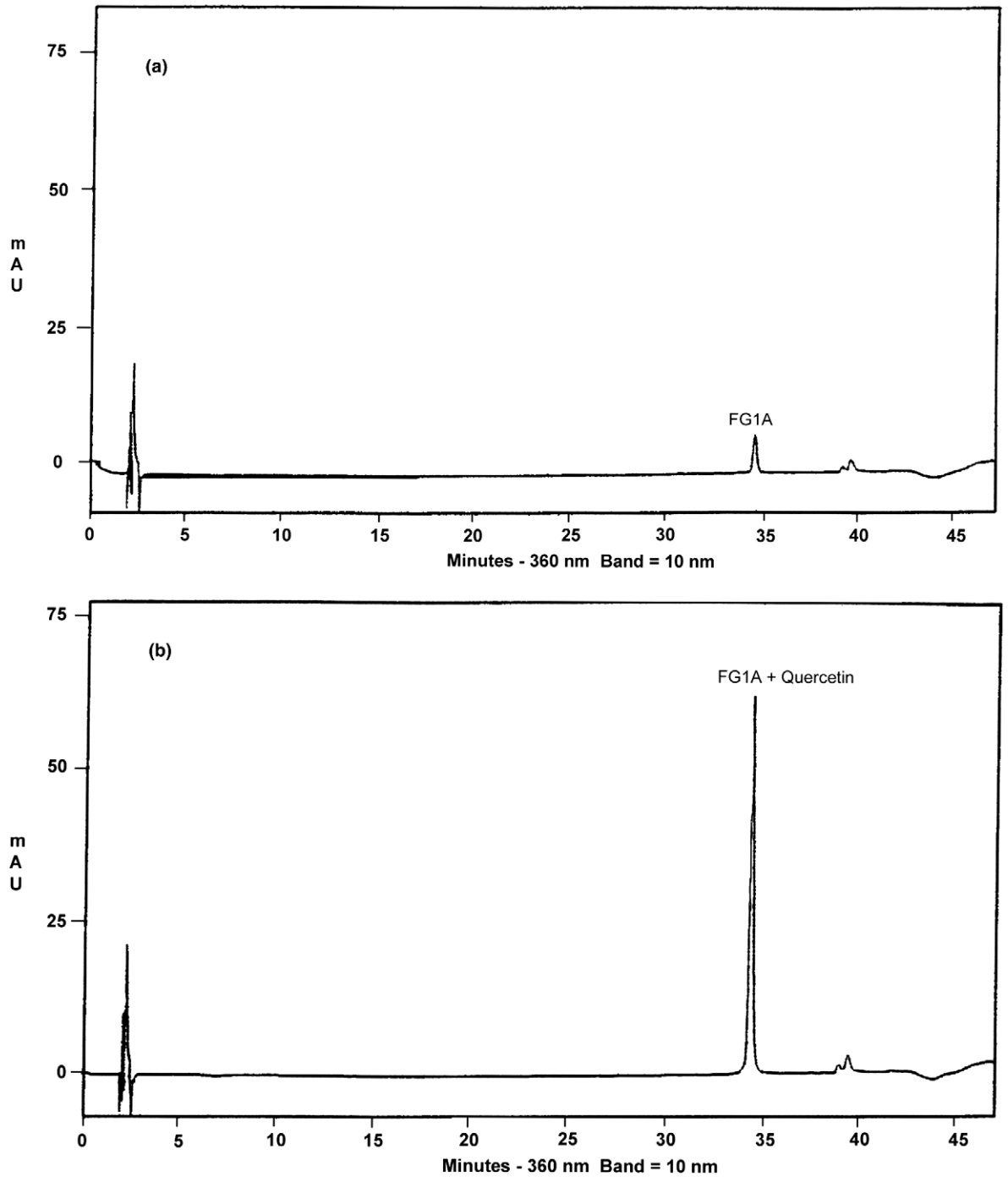


Fig. 3. (a) HPLC chromatogram of the flavonol aglycone of FG1 detected at 360 nm. (b) HPLC chromatogram of the flavonol aglycone of FG1 spiked with quercetin standard compound.

istic for flavonols as mentioned before. The corresponding conjugated sugar moiety of FG1 was derivatized by BSTFA to form the silylated sugar and then analyzed by GC–MS. It was identified as arabinose because the derivatized sugar has three identical diagnostic peaks with the same retention times (9.6, 10.0 and 10.5 min, respectively) in the total ion chromatogram (TIC) as the derivatized arabinose standard and similar corresponding MS spectra (Table 2). Note that there are multiple peaks in TIC for this derivatized sugar. It is due to its well-known different isomeric forms, such as furanose or pyranose. Recently, Vvedenskaya et al. (2004) also reported the findings of both furanose and pyranose forms of quercetin-3-arabinose in cranberry powder by LC–MS. Therefore, FG1 is identified as quercetin arabinoside. In the same way, the other major flavonol glycoside, FG2, is identified as quercetin galactoside in the cranberry juice. The chemical structures of these two identified flavonol glycosides are shown in Fig. 4.

Attempt has also been made to identify other pure flavonol glycosides isolated from TLC. Unfortunately, the identifications failed because of the insufficient quantity. As to the linkage way between the flavonol aglycone and the

Table 2
Retention times in total ion chromatogram (TIC) and m/z of the corresponding mass spectra data for individual derivatized sugar standards and the sugars obtained from the hydrolysis of compounds FG1 and FG2

Compounds	Retention times (min)	m/z of peaks in mass spectra	Identified as
Standard arabinose	9.6	73(100), 147, 191, 217, 259, 305, 393	
	10.0	–	
	10.5	73, 103, 147, 191, 217(100), 291, 305	
Standard fructose	12.3	73(100), 147, 217, 257, 291, 347, 437	
	13.4	73(100), 147, 217, 219, 306, 345, 437	
Standard galactose	12.5	73, 147, 191, 217(100), 319, 332, 435	
	13.0	73, 147, 204(100), 217, 305, 361, 393	
	13.6	73, 147, 191, 217(100), 319, 332, 435	
Standard glucose	12.8	73, 147, 217(100), 218, 318, 345, 435	
	13.5	73, 147, 204(100), 217, 305, 345, 435	
	14.6	73, 147, 204(100), 218, 305, 361, 435	
Standard rhamnose	9.8	73, 147, 191, 204(100), 291, 305, 393	
	10.7	73, 147, 191, 204(100), 217, 291, 347, 393	
Sugar 1[a]	9.6	73(100), 101, 147, 191, 217, 259, 279, 305	Arabinose
	10.0	73(100), 101, 147, 191, 217, 231, 279, 305	
	10.5	73, 79, 103, 147, 149, 189, 217(100), 230	
Sugar 2[b]	12.5	73, 75, 117, 133, 147, 204, 217(100)	Galactose
	13.0	73, 129, 147, 204(100), 285, 291, 332, 393	
	13.6	73, 103, 147, 191, 204(100), 243, 305, 317	

[a] From FG1.
[b] From FG2.

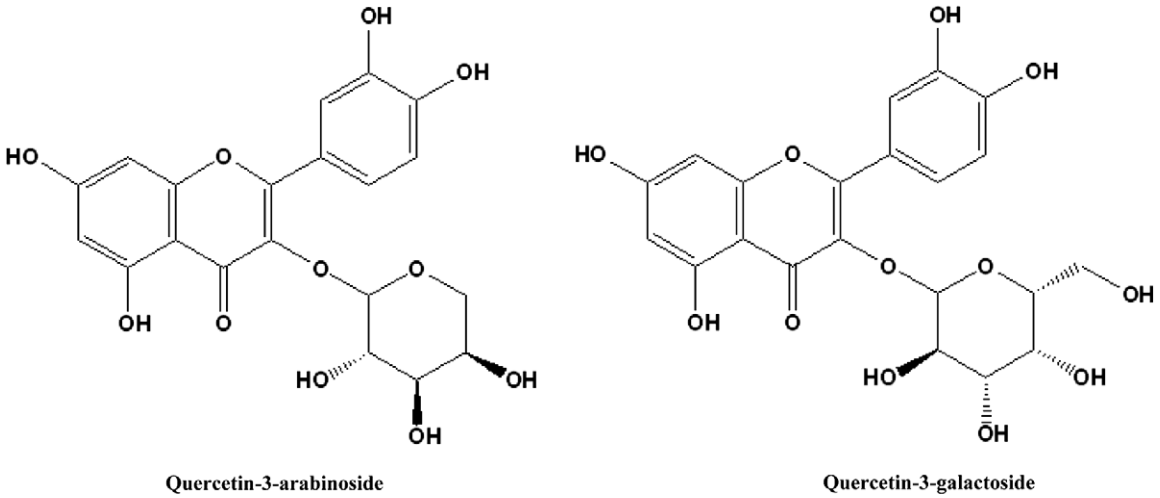


Fig. 4. Chemical structures of two identified flavonol glycosides, quercetin-3-arabinoside and quercetin-3-galactoside.

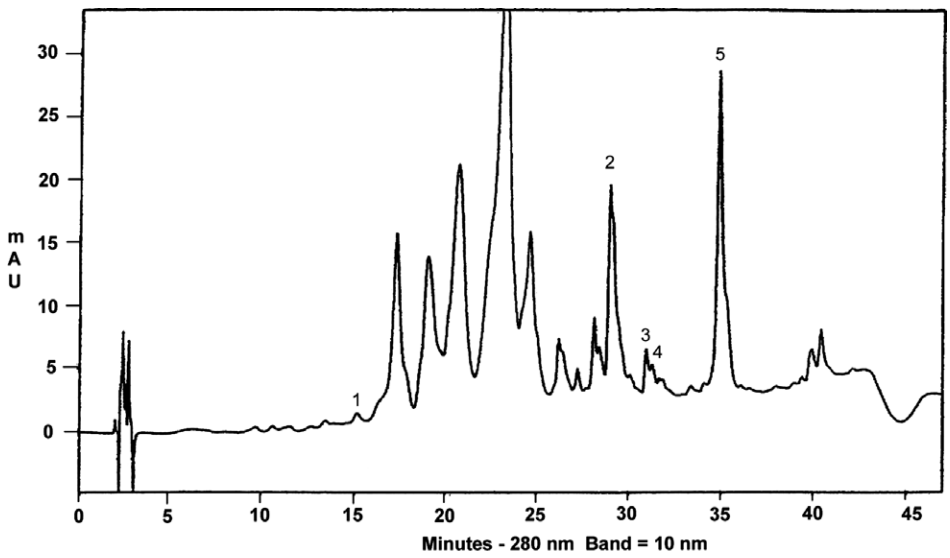


Fig. 5. HPLC chromatogram of the neutral fraction of the freshly-squeezed cranberry juice detected at 280 nm. Peaks: 1 = (+)-catechin; 2 = quercetin galactoside; 3 = quercetin arabinoside; 4 = myricetin; 5 = quercetin (added as an internal standard).

sugar in flavonol glycosides such as the α- or β-configuration and linking position, hydrolysis by a specific enzyme or scanning by NMR will be warranted in the future studies.

With the two identified flavonol glycosides in hand, two more peaks 2 and 3 can be recognized in the chromatogram of cranberry in our previous report as displayed in Fig. 5 (Fig. 4 in Chen et al., 2001). The elution order of both flavonol glycosides, quercetin galactoside and quercetin arabinoside, is in agreement with that reported by Schieber et al. (2001).

4. Conclusions

Two flavonol glycosides, quercetin galactoside and quercetin arabinoside, were separated from cranberry and their structures were then identified by HPLC and GC–MS. Therefore, two more corresponding peaks which were not identified in our previous study have been recognized in the chromatogram of cranberry juice. This work also provides an alternative method for identifying compounds in complex matrix by HPLC and GC–MS when the standard compounds are not available.

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References

Avorn, J., Monane, M., Gurwitz, J. H., Glynm, R. J., Choodnovskiy, I., & Lipsitz, L. A. (1994). Reduction of bacteriuria and pyuria after ingestion of cranberry. *Journal of the American Medical Association, 217*, 751–754.

Ayaz, F. A., Hayirlioglu-Ayaz, S., Gruz, J., Novak, O., & Strnad, M. (2005). Separation, characterization, and quantitation of phenolic acids in a little-known blueberry (*Vaccinium arctostaphylos* L.) fruit by HPLC–MS. *Journal of Agricultural and Food Chemistry, 53*, 8116–8122.

Chen, H., Zuo, Y., & Deng, Y. (2001). Separation of determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. *Journal of Chromatography A, 913*, 387–395.

Chu, N. T., Clydesdale, F. M., & Francis, F. J. (1973). Isolation and identification of some fluorescent phenolic compounds in cranberries. *Journal of Food Science, 38*, 1038–1042.

Daigle, D. J., & Conkerton, E. J. (1988). Analysis of flavonoids by HPLC: an update. *Journal of Liquid Chromatography, 11*, 309–325.

Deshner, E. E., Ruperto, J., Wong, G., & Newmark, H. L. (1991). Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. *Carcinogenisis, 7*, 1193–1196.

Endale, A., Kammerer, B., Gebre-Mariam, T., & Schmidt, P. C. (2005). Quantitative determination of the group of flavonoids and saponins from the extracts of the seeds of *Glinus lotoides* and tablet formulation thereof by high-performance liquid chromatography. *Journal of Chromatography A, 1083*, 32–41.

Häkkinen, S. H., & Auriola, S. (1998). High-performance liquid chromatography with electrospray ionization mass spectrometry and diode array ultraviolet detection in the identification of flavonol aglycone and glycosides in berries. *Journal of Chromatography A, 829*, 91–100.

Hong, V., & Wrolstad, R. E. (1986). Cranberry juice composition. *Journal of the Association of Official Analytical Chemists, 69*, 199–207.

James, C. F. (1994). New support for a folk remedy: cranberry juice reduces bacteriuria and pyuria in elderly women. *Nutrition Reviews, 5*(5), 168–170.

Lee, C.-H., Krueger, C. G., Reed, J. D., & Richards, M. P. (2006). Inhibition of hemoglobin-mediated lipid oxidation in washed fish muscle by cranberry components. *Food Chemistry, 99*(3), 591–599.

Lees, D. H., & Francis, F. J. (1971). Quantitative methods for anthocyanins, flavonols and anthocyanins in cranberries. *Journal of Food Science, 36*, 1056–1060.

Mattila, P., Astola, J., & Kumpulainen, J. (2000). Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. *Journal of Agricultural and Food Chemistry, 48*, 5834–5841.

Merken, H. M., & Beecher, G. R. (2000). Measurement of food flavonoids by high performance liquid chromatography: a review. *Journal of Agricultural and Food Chemistry, 48*, 577–599.

Nessa, F., Ismail, Z., Karupiah, S., & Mohamed, N. (2005). RP-HPLC method for the quantitative analysis of naturally occurring flavonoids in leaves of *Blumea balsamifera* DC. *Journal of Chromatographic Science, 43*, 416–420.

Ofek, I., Goldhar, J., Zafriri, D., Lis, H., Adar, R., & Sharon, N. (1991). Anti-*Escherichia coli* adhesion activity of cranberry and blueberry juices. *New England Journal of Medicine, 324*, 1599.

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

Puski, G., & Francis, F. J. (1967). Flavonol glycosides in cranberries. *Journal of Food Science, 32*, 527–530.

Robards, K., & Antolovich, M. (1997). Analytical chemistry of fruit bioflavonoids. *Analyst, 122*, 11R–34R.

Schieber, A., Keller, P., & Carle, R. (2001). Determination of phenolic acids and flavonoids of apple and pear by high performance liquid chromatography. *Journal of Chromatography A, 910*, 265–273.

Swain, T. (1962). In T. A. Geissman (Ed.), *Economic importance of flavonoid compounds: Foodstuffs, the chemistry of flavonoid compounds* (pp. 513–552). New York: Macmillan Co..

Thomos, J. D. (1900). *Cranberry harvest: A history of cranberry growing in Massachusetts*. New Bedford: Spinner publication.

Vvedenskaya, I. O., Rosen, R. T., Guideo, G. E., Russell, D. J., Mills, K. A., & Vorsa, N. (2004). Characterization of flavonols in cranberry powder. *Journal of Agricultural and Food Chemistry, 52*, 188–195.

Zhang, K., & Zuo, Y. (2004). GC–MS determination of flavonoids and phenolic and benzoic acids in human plasma after consumption of cranberry juice. *Journal of Agricultural and Food Chemistry, 52*, 222–227.

Zuo, Y., Chen, H., & Deng, Y. (2002). Simultaneous determination of catechins, caffeine and gallic acids in green, oolong, black and pu-erh teas using HPLC with a photodiode array detector. *Talanta, 57*, 307–316.

Zuo, Y., Wang, C., & Wen, J. (2003). Antioxidant and antibreast cancer capacity of American cranberry and other fruits. In: *The 225th American Chemical Society annual meeting*, New Orleans, LA, 23–27 March, 2003.

Zuo, Y., Wang, C., & Zhan, J. (2002). Separation, characterization, and quantitation of benzoic and phenolic antioxidants in American cranberry fruits by GC–MS. *Journal of Agricultural and Food Chemistry, 50*, 3789–3794.