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Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography

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Abstract

A HPLC method was developed for the separation and determination of flavonoid and phenolic antioxidants in cranberry juices. Free flavonoid and phenolic compounds were fractionated into neutral and acidic groups by means of a solid-phase extraction method, followed by subsequent HPLC separations. Combined flavonoids and phenolics were hydrolyzed by acid before HPLC analysis. This developed method provides a fast and high resolution of individual flavonoid and phenolic compounds. In cranberry fruit, flavonoids and phenolic acids exist predominantly in combined forms, such as glycosides and esters. A total of 400 mg of total flavonoids and phenolic compounds/l of sample was found in a freshly squeezed cranberry juice, which was distributed as about 44% of phenolic acids and 56% of flavonoids. Benzoic acid was the major phenolic compound. Major flavonoids in the freshly squeezed cranberry juice were quercetin and myricetin. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Fruit juices; Food analysis; Flavonoids; Phenolic acids; Quercetin; Catechin; Myricetin; Benzoic acid; Antioxidants

1. Introduction

Many fruit phenolic compounds are good sources of natural antioxidants and have inhibitory effects on mutagenesis and carcinogenesis [1,2]. During the past decades, extensive analytical research has been carried out on the separation and determination of phenolic constituents in various fresh fruit products and environmental samples. The techniques previously used include UV–Vis spectrophotometry, thin-layer chromatography (TLC) [3,4], gas–liquid chromatography (GLC) [5], high-performance liquid chromatography (HPLC) [6–11] and capillary electrophoresis (CE) [12,13]. Isolation and quantification, however, is difficult because of the great variety of species present and the wide variations in their levels. Thus, it is essential to devise a sample preparation stage, which will ensure reliable identification and quantification. In the literature, little information is available on the content and types of flavonoids and phenolic constituents in cranberry. The unique antibacterial activities of cranberry implicate that cranberry may possess a very different flavonoid and phenolic composition from other kinds of fruits [14,15]. An efficient separation and quantitation method is essential for understanding the components of flavonoid and phenolic antioxidants in cranberry and their health benefits.

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Reversed-phase HPLC is now commonly used for the separation of complex mixtures of phenolic compounds and other natural products in plant extracts [6–10,16]. However, usually only one group of phenolics [11] or anthocyanins [17] or a limited number of flavonoid compounds [18] have been analyzed. Very few suitable methods [10,19] for simultaneous screening of flavonoids and phenolics have been reported. The main difficulty existed in the often-occurred low resolutions between flavonoids and phenolic acids. The purpose of this study was to separate, identify and quantitate flavonoids and phenolic acids in cranberry juice using HPLC with UV–Vis photodiode array detection. A solid-phase extraction (SPE) with a Sep-Pak C_{18} cartridge was used to clean and fractionate free phenolic acids and flavonoids. An acid-catalyzed hydrolysis process was employed to liberate flavonoids and phenolic acids from their bound forms.

2. Experimental

2.1. Sample preparation

Commercially canned cranberry juice cocktail (containing 27% cranberry juice) was purchased at a local supermarket. It was stored at 4°C and filtered before analysis. 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 until crushed to provide a fresh cranberry juice, which is sequentially filtered before HPLC separation.

2.2. Chemicals

Standards of gallic, caffeic, sinapic, *p*-coumaric, chlorogenic, and 3,4,5-trimethoxycinnamic acids, myricetin and quercetin were purchased from Acros Organics (Geel, Belglum, NJ, USA). (−)-Epicatechin was obtained from ICN Biomedicals (OH, USA), benzoic acid from Baker (Phillipsburg, NJ, USA), *p*-anisic acid from Eastman. Gentisic acid and (+)-catechin were from Sigma (St. Louis, MO, USA).

Methanol was purchased from Pharmco Products (Brookfield, CT, USA). Acetic acid was obtained from EM Science (Cherry Hill, NJ, USA). All solution preparations were made using distilled–deionized water. All solutions were filtered through 0.20-μm membranes (Fisher Scientific) before HPLC analysis and the mobile phase solvents were degassed before use.

2.3. Extraction and fractionation of flavonoids and phenolic compounds

a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) was preconditioned for neutral flavonoids and phenolics by sequentially passing 8 ml of methanol and 4 ml of distilled–deionized water adjusted to pH 7.0. For acidic phenolics, cartridges were preconditioned by passing 4 ml of 0.01 *M* HCl instead of distilled–deionized water.

A 0.50-ml volume of 10 g/l ascorbic acid was added to a suitable volume of cranberry juice samples (3.0 ml of commercially canned cranberry juice or 0.80 ml of freshly squeezed cranberry juice) to avoid oxidation of phenolic compounds. This solution was adjusted to pH 7.0 with diluted NaOH solutions, loaded onto the neutral fractionating Sep-Pak C_{18} column and washed with 10 ml of pH 7.0 distilled–deionized water. The effluent portion was adjusted to pH 2.0 with 2.0 *M* HCl, passed through the preconditioned acidic column, and washed with 5 ml of 0.01 *M* HCl. The adsorbed fractions were eluted with 12 ml of methanol, and evaporated using a rotary evaporator until dryness at 35°C with a water bath. The residue was redissolved in 3.0 ml of methanol, filtered and 15 μl was injected onto the HPLC system. The SPE recoveries varied from 68 to 110% except for gallic acid (3%). The low recovery observed for the gallic acid is due to its high polarity. The relative standard deviation is less than 20%.

2.4. Hydrolysis

An 80-mg amount ascorbic acid was dissolved in 15 ml of distilled–deionized water in a 100-ml round bottomed flask. The sample (10 ml of canned cranberry juice or 3.0 ml of freshly squeezed cranberry juice plus 7.0 ml distilled–deionized water) and 25 ml of methanol were added to the flask. To this solution, 10 ml of 6 *M* HCl was added slowly within 5 min. Under an N_2 atmosphere, this solution

was stirred using a magnetic stirrer at 35°C for 16 h. The solution was allowed to cool and filtered. A 15-ml portion of the filtrate was evaporated to dryness using a rotary evaporator and a water bath at 35°C. The residue was redissolved in 3.0 ml of methanol, filtered and 15 μl was injected onto the HPLC system.

2.5. HPLC analysis

HPLC analysis was conducted with Beckman liquid chromatograph equipped with a Model 125 dual solvent pump, a 508 autosampler, a 168 photodiode array detector and Gold Nouveau Software. An Eclipse XDR-C_{18} reversed-phase column (15 cm× 4.6 mm, 5 μm; Waters) was used throughout this study.

The flavonoids and phenolic acids were detected 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, (v/v)] to solvent B (methanol). The solvent gradient elution program is presented in Table 1.

All flavonoids and phenolic compounds were identified by matching the retention time and their spectral characteristics against those of standards. Quantitation was made according to the linear calibration curves of standard compounds. Quercetin and 3,4,5-trimethoxycinnamic acid were chosen as internal standards for the fractionated cranberry juices because their retention times are close to the peaks of interest and there is no large light absorbance near their retention times in the chromatograms. External standard methods were used for the hydrolyzed products of cranberry juices since a suitable internal standard is hard to get.

Table 1
HPLC solvent gradient elution program

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

3. Results and discussion

After multiple preliminary assays, an XDR-C_{18} column and a gradient elution program using methanol–acetic acid–water as solvent was chosen (Table 1). Fig. 1 illustrates the separation of a standard mixture of 13 flavonoids and phenolic acids. A good separation can be achieved in a short separation time of 47 min. In this selected solvent gradient system, it is essential to increase the volume percentage of solvent B by 2.0%/min from 10 min to 40 min for achieving good peak shapes and resolution of flavonoids since round peaks for myricetin and quercetin often occurred [19]. Retention data and UV–Vis maximum absorption wavelengths for some studied flavonoids and phenolic acids are listed in Table 2.

The canned cranberry juice and the freshly squeezed cranberry juice were fractionated into neutral and acidic groups, respectively by means of an SPE method described in the Experimental section, followed by subsequent HPLC separations. Their chromatograms spiked with internal standards are shown in Figs. 2–5. Myricetin, catechin, *p*-coumaric, benzoic and chlorogenic acids in the canned cranberry juice and myricetin, catechin, *p*-anisic and benzoic acids in the freshly squeezed cranberry have been identified according to their retention times and the spectral characteristics of their peaks against those of standards. The presence of myricetin in cranberry juices has been reported previously by Häkkinen and Auriola [20]. However, no quercetin could be detected in either the canned cranberry juice or the freshly squeezed cranberry juice.

The canned and the freshly squeezed cranberry juices have very similar composition of identified flavonoids and phenolic acid such as myricetin, catechin and benzoic acid. The concentration of identified components were calculated by using the obtained calibration curves and listed in Table 3. Benzoic acid is the major phenolic acid detected in both studied cranberry juices. The canned cranberry juice contains about 34 mg/l of benzoic acid, and freshly squeezed juice contains more than 41 mg/l. This amount is in the range that used in the preservation of most perishable foods. The concentrations of myricetin, catechin and benzoic acid in the freshly squeezed cranberry juice are significantly higher than

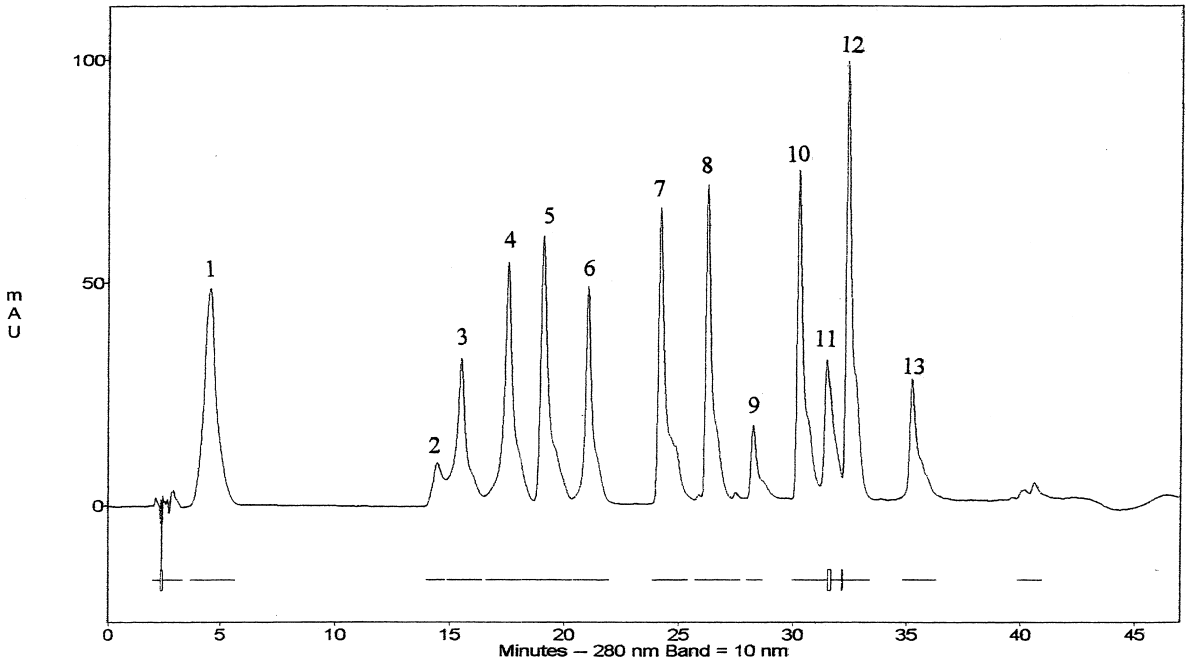

Fig. 1. HPLC chromatogram of the flavonoid and phenolic acid standards at 280 nm. Peaks: 1=gallic acid; 2=gentisic acid; 3=(+)-catechin; 4=chlorogenic acid; 5=caffeic acid; 6=(−)-epicatechin; 7=*p*-coumaric acid; 8=sinapic acid; 9=benzoic acid; 10=*p*-anisic acid; 11=myricetin; 12=3,4,5-trimethoxycinnamic acid; 13=quercetin.

those in the canned cranberry juice. This can be ascribed to the dilution and loss during the manufacture processes. The canned juice studied contains 27% cranberry juice.

It is well known that flavonoids and phenolic acids occur in plants mainly as glycosides or simple ester or are bound to the cell wall. Hydrolysis by acid can release the combined flavonoids and phenolic acids [21]. In our experiment, hydrolysis with HCl was carried out according to Hertog et al. [18]. After

Table 2
Retention times and absorption maxima nm of some flavonoids and phenolic acids

Compound	Retention time (min)	Absorbance maximum wavelength (nm)
Gallic acid	4.57	272
Gentisic acid	14.48	240, 327
(+)-Catechin	15.52	242, 279
Chlorogenic acid	17.57	248, 296, 326
Caffeic acid	19.10	246, 295, 323
(−)-Epicatechin	21.03	242, 279
p-Coumaric acid	24.20	310
Sinapic acid	26.25	244, 279, 323
Benzoic acid	28.27	241, 277
p-Anisic acid	30.27	257
Myricetin	31.52	255, 373
3,4,5-Trimethoxycinnamic acid	32.45	245, 303
Quercetin	35.27	250, 370

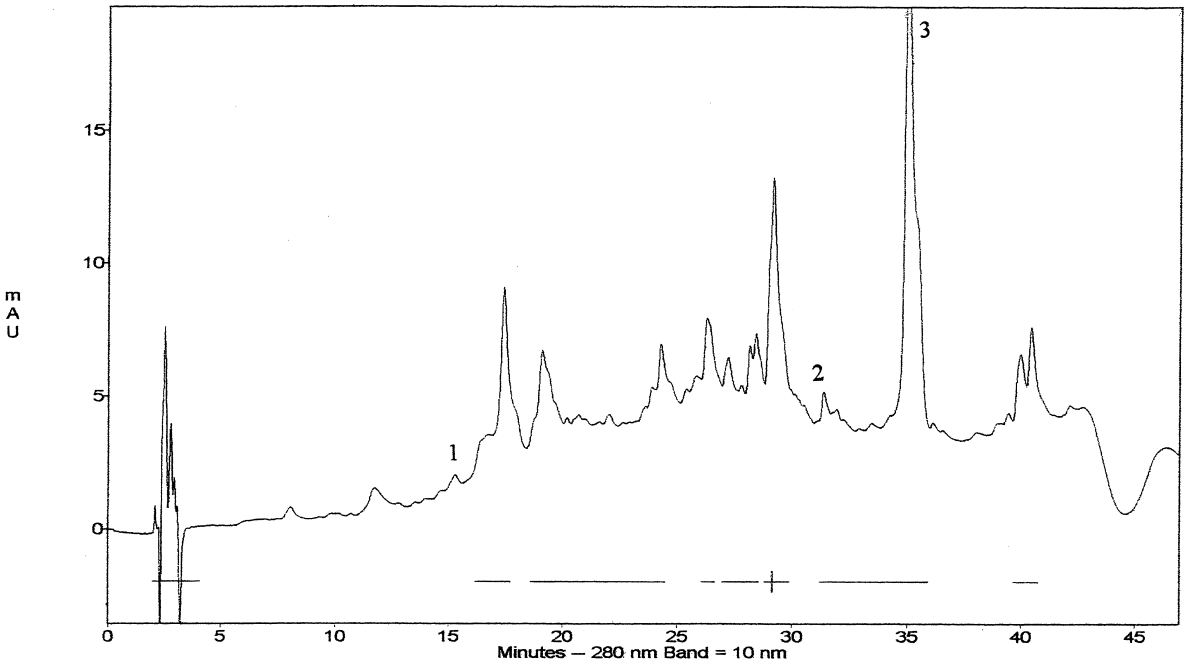

Fig. 2. HPLC chromatogram of the neutral fraction of the commercially canned cranberry juice detected at 280 nm. Peaks: 1=(+)-catechin; 2=myricetin; 3=quercetin (added as an internal standard).

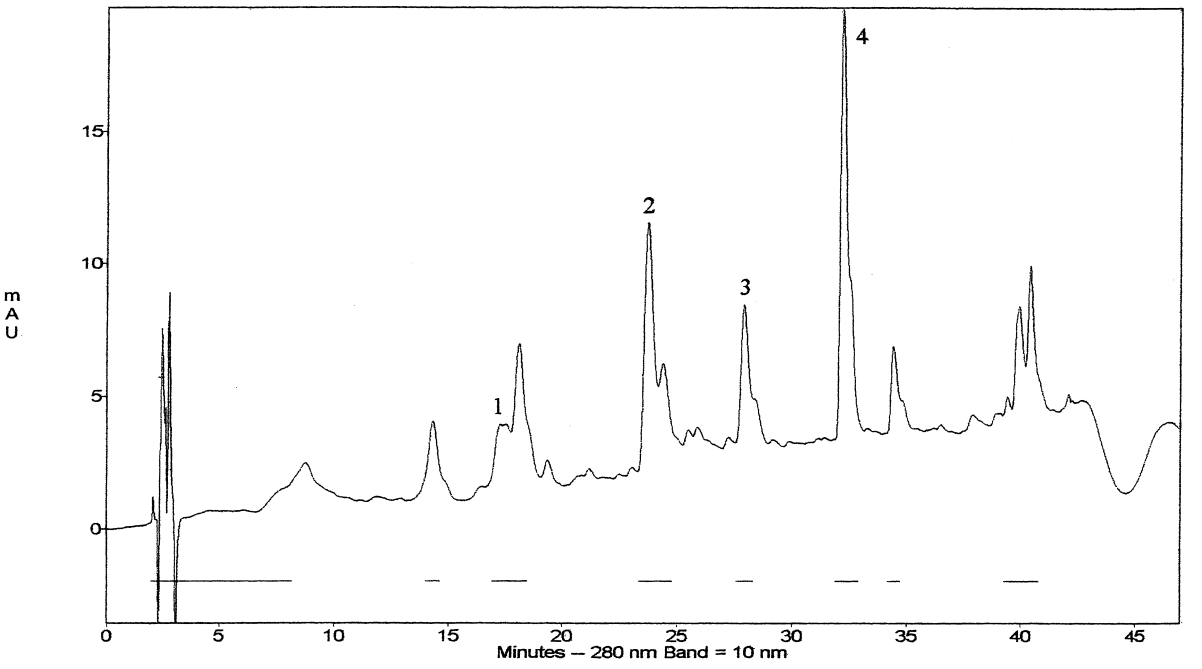

Fig. 3. HPLC chromatogram of the acidic fraction of the canned cranberry juice detected at 280 nm. Peaks: 1=chlorogenic acid; 2=*p*-coumaric acid; 3=benzoic acid; 4=3,4,5-trimethoxycinnamic acid (added as an internal standard).

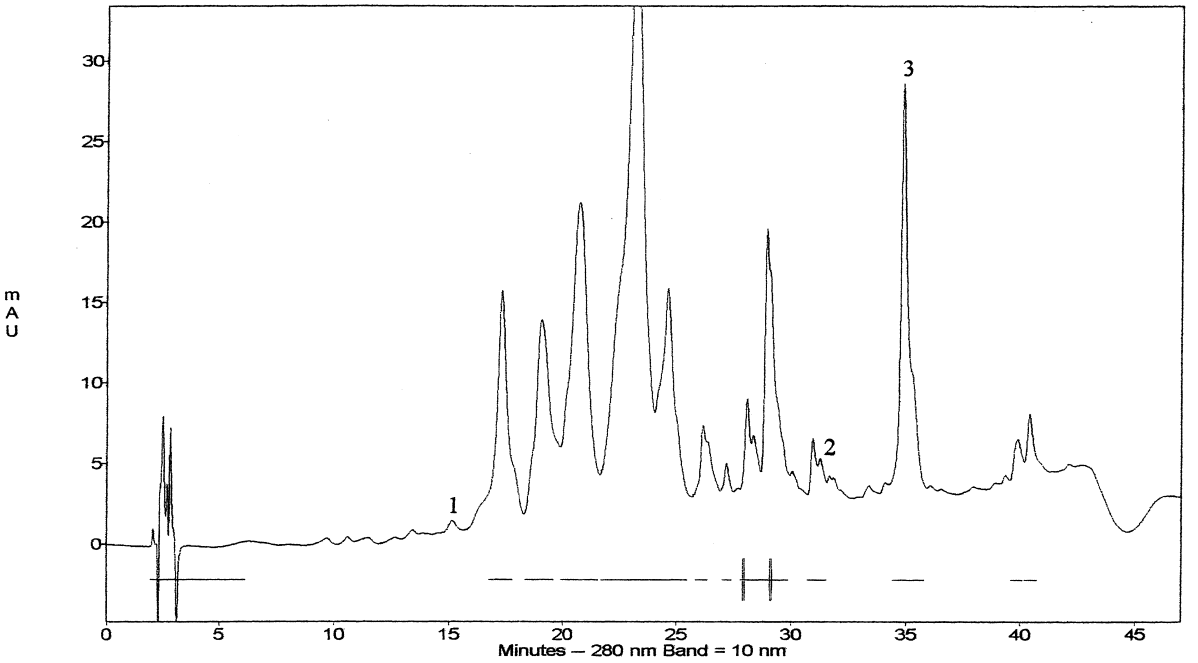

Fig. 4. HPLC chromatogram of the neutral fraction of the freshly squeezed juice detected at 280 nm. Peaks: 1=(+)-catechin; 2=myricetin; 3=quercetin (added as an internal standard).

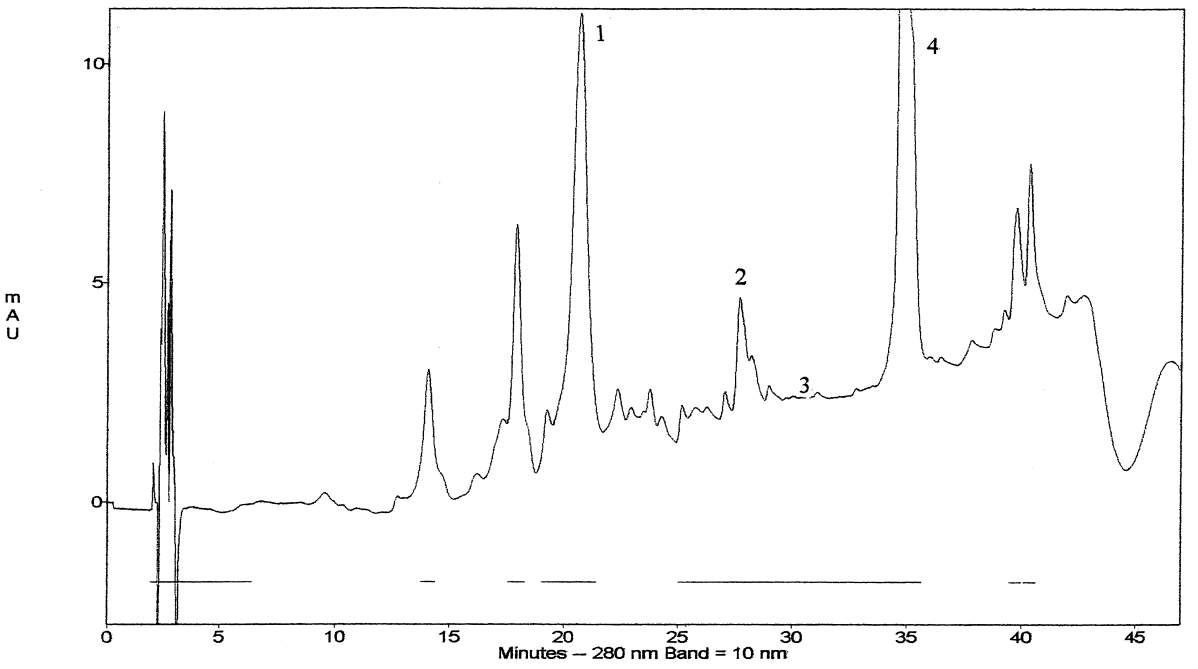

Fig. 5. HPLC chromatogram of the acidic fraction of the freshly squeezed cranberry juice detected at 280 nm. Peaks: 1=anthocyanin derivative I; 2=benzoic acid; 3=*p*-anisic acid; 4=quercetin (added as an internal standard).

Table 3
Concentrations of flavonoids and phenolic acids in cranberry juice samples

Component	Concentration (mg/l)			
	Canned cranberry juice	Hydrolyzed products of the canned juice	Fresh cranberry juice	Hydrolyzed product of freshly squeezed juice
(+)-Catechin	1.1	2.0	8.1	9.8
Chlorogenic acid	5.1	ND[a]	ND	ND
p-Coumaric acid	5.2	6.1	ND	ND
Benzoic acid	34	28	41	178
p-Anisic acid	ND	ND	2.2	3.2
Myricetin	1.7	2.9	8.3	47
Quercetin	ND	12	ND	175

[a] ND: Not detected.

hydrolysis, the products were concentrated and then subjected to HPLC analysis directly. The corresponding chromatograms were shown in Figs. 6 and 7 and the concentrations of detected components were determined by the external standard method.

Quercetin was found as one of the major flavonoids in hydrolyzed products of both studied cranberry juices while it was absence in the unhydrolyzed juices. The concentration of quercetin found in the hydrolyzed freshly squeezed cranberry juice was about 170 mg/l, accounted for about 75% of the total flavonoids and 42% of the total phenolics recovered. The concentration of myricetin was determined at about 50 mg/l, which is about six-times higher than that measured in the unhydrolyzed juice. Also, several of anthocyanin and the unknown phenolic compounds separated from unhydrolyzed phenolic extracts were missing from hydrolyzed

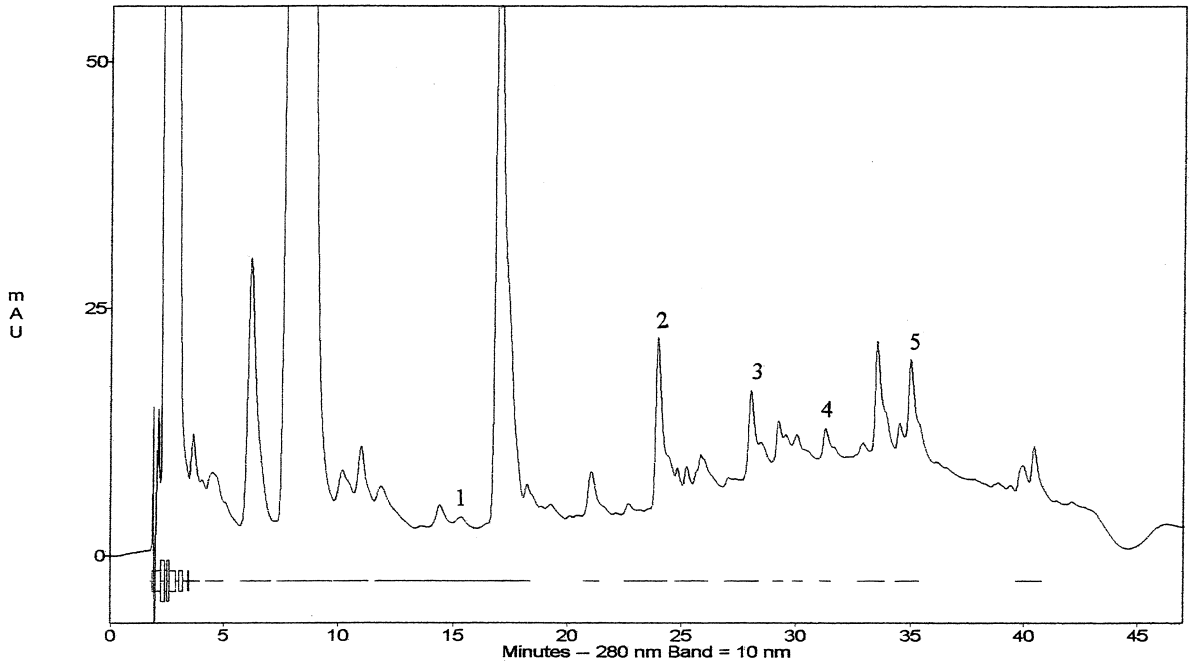

Fig. 6. HPLC chromatogram of the acid-catalyzed hydrolyzed product of the canned cranberry juice detected at 280 nm. Peaks: 1=(+)-catechin; 2=*p*-coumaric acid; 3=benzoic acid; 4=myricetin; 5=quercetin.

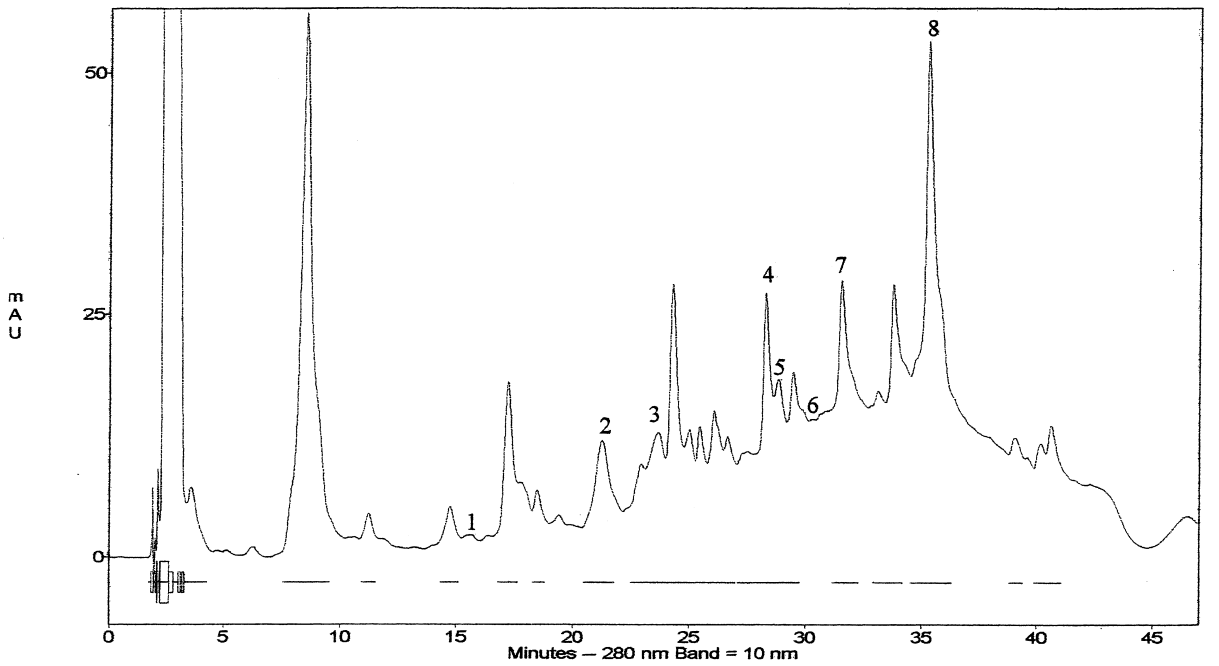

Fig. 7. HPLC chromatogram of the acid-catalyzed hydrolyzed product of the freshly squeezed cranberry juice detected at 280 nm. Peaks: 1=(+)-catechin; 2=anthocyanin derivative I; 3=anthocyanin derivative II; 4=benzoic acid; 5=anthocyanin derivative III; 6=*p*-anisic acid; 7=myricetin; 8=quercetin.

cranberry juice patterns. This simultaneous loss of unknown phenolic components and the increase in quercetin and myricetin contents in cranberry juices following hydrolysis are circumstantial evidence that up to 85% of the flavonoids and phenolic acids exists in cranberry juice as esters or bound phenolic compounds. Further studies on the components of these combined flavonoids, phenolic acids and their health effects are warranted.

4. Conclusions

The described SPE–HPLC method allows easy and simultaneous determinations of flavonoids and other phenolic compounds in cranberry juices. The main free phenolic acid in cranberry has been found to be benzoic acid. Quercetin and myricetin are the major flavonoids detected in the hydrolyzed cranberry juices. This method can also be used in the separation of phenolic antioxidants in other fruit juices.

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