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Hydrophilic Carboxylic Acids and Iridoid Glycosides in the Juice of American and European Cranberries (*Vaccinium macrocarpon* and *V. oxycoccos*), Lingonberries (*V. vitis-idaea*), and Blueberries (*V. myrtillus)*

HEIDI D. JENSEN,[†,‡] KAREN A. KROGFELT,[‡] CLAUS CORNETT,[§] S. HONORÉ HANSEN,[§] AND S. BRØGGER CHRISTENSEN*[,†]

Department of Medicinal Chemistry, Royal Danish School of Pharmacy, Copenhagen, Denmark, Department of Gastro-Intestinal Infections, Statens Serum Institut, Copenhagen, Denmark, and Department of Analytical and Pharmaceutical Chemistry, Royal Danish School of Pharmacy, Copenhagen, Denmark

Analysis of the hydrophilic fraction of cranberry juice by reversed-phase HPLC using an Aqua LUNA column with diode array or MS detection revealed the presence of quinic acid, malic acid, shikimic acid, and citric acid. For the first time, two iridoid glucosides were found in the juice. The two iridoid glucosides were shown to be monotropein and 6,7-dihydromonotropein by MS and NMR spectroscopy. A fast reversed-phase HPLC method for quantification of the hydrophilic carboxylic acids was developed and used for analyses of cranberry, lingonberry, and blueberry juices. The level of hydrophilic carboxylic acids in cranberries was 2.67−3.57% (w/v), in lingonberries 2.27−3.05%, and in blueberries 0.35−0.75%. In lingonberries both iridoid glucosides were present, whereas only monotropein was present in blueberries.

KEYWORDS: HPLC; American cranberry (*Vaccinium macrocarpon*); European cranberry (*Vaccinium oxycoccus*); lingonberry (*Vaccinium vitis-idaea*); blueberry (*Vaccinium myrtillus*); quinic acid; malic acid; shikimic acid; citric acid; iridoid glucosides; monotropein; 6,7-dihydromonotropein

INTRODUCTION

Approximately 25% of women will have at least one urinary tract infection in their lifetime caused by infection by bacteria, especially *Escherichia coli* (*1*). Many of these will have several infections (*2*). Whereas some clinical trials have shown that cranberry juice prevents urinary tract infections in women (*3*, *4*), other reports still question the beneficial effects (*5*, *6*). In vitro experiments have revealed that cranberry fruit juice (*Vaccinium macrocarpon* Aiton, family Ericaceae) possesses an antimicrobial (*7*) and an antiadhesive effect (*8*) on *E. coli*. Since lack of sensitivity toward common antibiotics increasingly complicates treatment of infections, antiadhesion therapy might become an important alternative to the presently used methods. Some previous studies have related the antiadhesion effects to the presence of proanthocyanidins (*9*), fructose, and macromolecules (*8*) in the juice, whereas other studies have correlated the effect to the acidificaton of urine caused by the organic acids (*5*). Despite these hypotheses, only very limited attention has been paid to the hydrophilic fraction, maybe because of difficulties in performing such analyses. Reversed-phase column packing materials applicable for separation of very hydrophilic compounds have enabled development of the fast method for analyses of the hydrophilic carboxylic acids presented in this paper. Previous studies have only elucidated the structures of the very polar compounds by their retention times (*10*, *11*). The present study was undertaken in order to confirm the structures of the polar acids present in cranberry juice and to identify possible unknown constituents which might contribute to the biological activity and to the taste of the juice. Techniques such as HPLC-MS and HPLC-NMR have facilitated unequivocal structural determination of the detected compounds. For comparison the study also includes analyses of the juices of European cranberries (*Vaccinium oxycoccos* L.), lingonberries (*Vaccinium vitis-idaea* L.), and blueberries (*Vaccinium myrtillus* L.).

EXPERIMENTAL PROCEDURES

Chemicals and Materials. All solvents and reagents were of analytical grade. Trifluoroacetic acid 99% was from Aldrich, and acetonitrile and methanol were HPLC-grade. Water was deionized and filtered through a 0.45 μm poresize Millipore filter (Millipore, Ireland) before use. Authentic (−)-quinic acid (1β,3α,4α,5β-tetrahydroxycy-

* Corresponding author. Address: Department of Medicinal Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark. Tel.: +45 35306253. Fax: +45 3530 6040. E-mail: sbc@dfh.dk.

[†] Department of Medicinal Chemistry, Royal Danish School of Pharmacy.

[‡] Department of Gastro-Intestinal Infections, Statens Serum Institut.

[§] Department of Analytical and Pharmaceutical Chemistry, Royal Danish School of Pharmacy.

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Table 1. Sample Number, Origin, and Concentration of Four Organic Acids in the Hydrophilic Fractions of Cranberry, Lingonberry, and Blueberry Juices (% W/V ± RSD %)

sample[a] (**4**)	species	origin	quinic acid (**1**)	malic acid (**2**)	shikimic acid (**3**)	citric acid
1	*V. macrocarpon*	USA[a]	1.14 ± 0.01	0.82 ± 0.01	0.041 ± 0.0003	1.20 ± 0.01
2	*V. macrocarpon*	USA[a]	1.180 ± 0.003	0.80 ± 0.01	0.0293 ± 0.0002	1.14 ± 0.01
3	*V. macrocarpon*	Holland (Thershellingen)	0.892 ± 0.003	0.626 ± 0.004	0.059 ± 0.001	1.10 ± 0.01
4	*V. macrocarpon*	Czech Republic	0.605 ± 0.001	1.052 ± 0.002	0.01092± 0.00004	1.863 ± 0.001
5	*V. oxycoccos*	Denmark	0.55 ± 0.01	0.95 ± 0.01	0.00670 ± 0.00004	1.63± 0.01
6	*V. vitis-idaea*	Sweden	0.88 ± 0.03	tr.[b]	0.0040 ± 0.0002	1.391 ± 0.003
7	*V. vitis-idaea*	Switzerland	1.530 ± 0.001	tr.		1.52 ± 0.01
8	*V. myrtillus*	Germany	tr.	tr.	tr.	0.75 ± 0.01
9	*V. myrtillus*	Argentina	tr.	tr.	tr.	0.350 ± 0.003

[a] The two samples were obtained from different suppliers. [b] tr., trace amounts.

clohexane carboxylic acid) (Aldrich), l-(−)-malic acid (*S*-hydroxysuccinic acid) (Fluka), (−)-shikimic acid (3*R*,4*S*,5*R*)-3,4,5-trihydroxycyclohexene-1-carboxylic acid) (Aldrich), and citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) (Riedel-de Haën) were used as standards. Pectinex Ultra SP-L (Novozymes) was used as pectinase. Solid-phase extraction catridges were 500 mg, 3 mL C-18 BondElut (Varian). RP-18 LiChroprep, 40−63 μm silylated silica gel (Merck), was used for preparative removal of the less hydrophilic constituents of the juices.

NMR Apparatus. The NMR spectra were recorded on a Gemini 2000 spectrometer at 300 and 75 MHz for ^{1}H- and ^{13}C NMR spectra, respectively. The spectra were recorded in deuterium oxide using acetonitrile (2.06 ppm) as an internal standard.

HRMS. The high-resolution mass spectra were recorded on a Q-Tof1 (Micromass) with a 3.6 GHz TDC. Negative ions obtained after electron spray were detected using a mixture of corn syrup and maltose as references.

HPLC Apparatus. The quantitative analyses were performed using a Water Associates pump Model 510, a Rheodyne 7125 injector valve with a 20 μL injection loop, and a Shimadzu SPD-10A UV−vis Detector. Data acquisition and manipulation were performed on a C-R8A Chromatopac, Shimadzu, Japan. Separation was accomplished at room temperature on a 150 × 4.6 mm i.d., 5 μm Aqua LUNA C-18 column (Phenomenex). The preparative isolation of the compounds was performed on a 150 × 21.2 mm i.d., 5 μm Aqua LUNA C-18 column (Phenomenex) and with the same apparatus as for the quantitative analysis except for a 2 mL loop and a preparative UV detector cell. HPLC-MS was performed on an 1100 HPLC system equipped with a diode array and a mass spectrometric detectors (Agilent Technologies, USA), and HPLC NMR was performed on a Bruker spectrometer working at 400 MHz using a flow cell.

Berry Samples. The origins of the berries used in this study are given in **Table 1**. Samples 1, 2, 3, 6, 8, and 9 were purchased in a grocery store. Sample 4 was a generous gift from Rynkeby A/S, Denmark. One of the authors collected sample 5 in the fall 2000 in Bøllemose moor, north of Copenhagen, Denmark, and sample 7 in the fall 2001 in the Alps near Lausanne, Switzerland. The identities of samples 5 and 7 were confirmed by Dr. Per Mølgaard, The Royal Danish School of Pharmacy. All the berries were frozen immediately after collection or purchase and stored at −20 °C.

Sample Preparation. Frozen berries were thawed at 5 °C overnight, and 1 kg of the berries blended with 0.7 L of deionized water for five minutes in a Waring Commercial Blendor. The pulp was centrifuged at 420*g* for 15 min in a SIGMA 3 centrifuge. The supernatant was filtered to give 1 L of juice.

Pectinase Treatment. The large amount of pectin in blueberries (samples 8 and 9) necessitated treatment with a pectinase (Pectinex Ultra) before centrifugation. The pectinase (0.1% v/v) was added to the pulp, and the pulp was stirred for 60 min at 35 °C. Further sample preparation was performed as described above.

Quantitative Analysis of the Hydrophilic Carboxylic Acids. A BondElut cartridge was activated by washing with 10 mL of acetonitrile−water (1:1) and dried by sucking 10 mL of air through the cartridge. Two portions of 5 mL of the fresh juice were sucked through the cartridge. The first 5 mL of eluate was discarded and the following 5 mL collected. After filtration through 0.45 μm poresize Whatman filter, 200 μL of the filtrate was diluted to 5000 μL, and 100 μL of the diluted sample was injected into the HPLC system using water containing 0.06% of trifluoroacetic acid as the mobile phase, flow 1.0 mL/min, detection 214 nm. Quantification was based on linear regression analysis of the ratio between the peak areas in the chromatograms of the juice and the peak areas of the standards.

Linearity. Standard solutions of authentic samples of quinic acid, malic acid, shikimic acid, and citric acid were prepared in mobile phase. The standard curves were based on five or six concentrations each analyzed in triplicate. The final concentrations of the acids in the solutions were as follows: quinic acid, 0.125, 0.167, 0.215, 0.301, 0.378, and 0.501 mg/mL; malic acid, 0.064, 0.128, 0.171, 0.205, and 0.256 mg/mL; shikimic acid, 2.35, 3.10, 4.70, 6.27, and 7.50 μg/mL; and citric acid, 0.124, 0.165, 0.212, 0.247, 0.297, 0.371, and 0.495 mg/mL. Quality control samples were analyzed before an analysis. Both standard solutions and samples were stored at 5 °C. Good linearity for quinic acid and citric acid was achieved between 0.124 and 0.495 mg/mL with correlation coefficients of 0.9992 and 0.9818, respectively, for malic acid between 0.064 and 0.256 mg/mL with a correlation coefficient of 0.9982, and for shikimic acid between 2.4 and 9.4 μg/mL with a correlation coefficient of 0.9991.

Recovery. The extraction efficiency (recovery) was determined by adding authentic standards to the juice and comparing the found amounts of acids with the amounts in unspiked juice. The recovery data for quinic acid, malic acid, shikimic acid, and citric acid were 103%, 91%, 97%, and 95%, respectively, with coefficients of variation between 6 and 8.

Stability Studies. No changes in the contents of the carboxylic acids could be observed after freezing and thawing of the juice, as revealed by comparison of the chromatograms of a freshly prepared juice and a juice which had been frozen at −20 °C.

Preparative Isolation of Iridoid Glycosides and Carboxylic Acids. Frozen berries (1020 g) were thawed at 5 °C, and water (700 mL) was added. The mixture was blended and the pulp centrifuged at 420*g* for 15 min. A 200 mL sample of the supernatant was applicated with a FMI LAB pump model QD (Fluid Metering, Inc. USA) on 20 g of RP-18 silylated silica gel in a 300 × 20 mm column activated with methanol and washed with deionized water. The column was eluted with 300 mL water, and the eluates were pooled and evaporated in vacuo at 40 °C to the volume of the applied juice (200 mL). For the preparative HPLC, the compounds eluted with a flow of 10 mL/min were collected and the fractions lyophilized. Detection and mobile phase were as in the quantitative analysis of the compounds. The ^{1}H and ^{13}C NMR spectra of quinic acid, malic acid, shikimic acid, citric acid, and monotropein were superimposable to those of authentic samples and the expected peaks for (M-1) were found in all the mass spectra.

LC-MS Analysis. An isocratic elution mode with 0.1% (v/v) aqueous formic acid as an eluent was used at a flow rate of 0.5 mL/min. The same HPLC column as for the UV detection was used, and the injection volume was 50 μL. The mass spectrometric detection was performed using API-ES in the negative mode.

Molecular Analysis Data. Quinic acid, shikimic acid, and citric acid were obtained as a colorless solid. Monotropein (16 mg) was

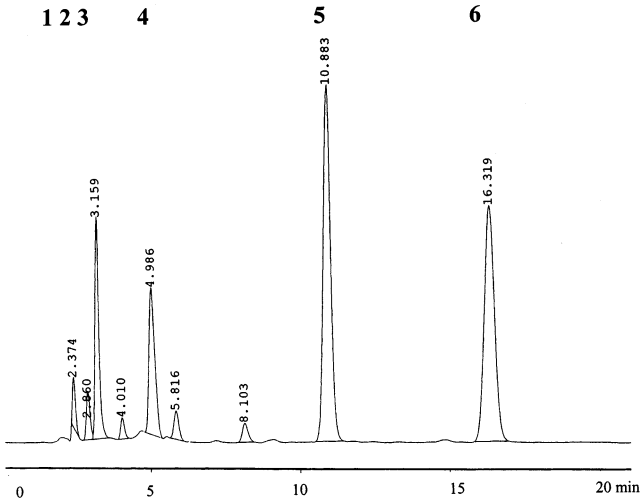

Figure 1. Chromatogram of the hydrophilic fraction of cranberry juice (*Vaccinium macrocarpon*). Peak at 2.4 min quinic acid (**1**), 2.9 min malic acid (**2**), 3.2 min shikimic acid (**3**), 5.0 min citric acid (**4**), 10.9 min monotropein (**5**), and 16.3 min 6,7-dihydromonotropein (**6**). Detection at 240 nm.

isolated from 200 mL juice as an amorphous colorless solid, corresponding to 0.01% w/v in single strength cranberry juice. $[\alpha]^{25}_{D} = -132.1°$ (*c* 0.165, H_2O). λ_{max} (measured with a diode array detector, which prevented measurement of ϵ) (H_2O) 239 nm. ^{1}H NMR (D_2O, 300 MHz) δ 7.47 (1H, d, $J_{3,5} = 1.0$ Hz, H-3), 6.29 (1H, dd, $J_{5,6} = 2.7$ Hz, $J_{6,7} = 6.0$ Hz, H-6), 5.73 (1H, dd, $J_{5,7} = 1.5$, $J_{6,7} = 6.0$ Hz, H-7), 5.67 (1H, d, $J_{1,9} = 1.8$ Hz, H-1), 4.82 (1H, d, $J_{1',2'} = 8.1$ Hz, H-1′), 3.94 (1H, dd, $J_{6'a,6'b} = 12.3$, $J_{6',5'} = 2.4$ Hz, H-6′a), 3.75 (1H, dd, $J_{6'b,6'a} = 12.3$, $J_{6'b,5'} = 5.7$ Hz H-6′b), 3.72 (1H, d, $J_{10a,10b} = 11.4$ Hz, H-10a), 3.66 (1H, d, $J_{10b,10a} = 11.4$, H-10b), 3.52 (1H, ddd, $J_{4',5'} = 9.9$ Hz, $J_{6a',5'} = 2.4$ Hz, $J_{6'b,5'} = 5.7$ Hz, H-5′), 3.57−3.60 (1H, m, H-5), 3.51 (1H, t, $J_{2',3'} = 9.3$ Hz, $J_{3',4'} = 9.0$ Hz, H-3'), 3.40 (1H, t, $J_{3',4'} = 9.0$ Hz, $J_{4',5'} = 9.9$ Hz, H-4'), 3.28 (1H, dd, $J_{1',2'} = 8.1$ Hz, $J_{2',3'} = 9.3$ Hz, H-2'), 2.74 (1H, dd, $J_{1,9} = 1.8$ Hz, $J_{5,9} = 8.7$ Hz, H-9).^{13}C NMR (D_2O, 75.5 MHz) δ 95.38 (C-1), 153.04 (C-3), 111.53 (C-4), 37.73 (C-5), 133.32 (C-6), 138.70 (C-7), 85.81 (C-8), 44.73 (C-9), 67.34 (C-10), 172.33 (C-11), 99.38 (C-1′), 73.61 (C-2′) 76.60 (C-3′), 70.51 (C-4′), 77.29 (C-5′), 61.55 (C-6′). MS API-ES negative $[M - 1]^-$ *m/z* = 389.0. 6,7-Dihydromonotropein (22 mg) was isolated from 200 mL juice as an amorphous colorless solid, corresponding to 0.01% w/v in single strength cranberry juice. $[\alpha]^{25}_{D} = -204.6°$ (*c* 0.100, H_2O). λ_{max} (measured with a diode array detector, which prevented measurement of ϵ) (H_2O) 239 nm. ^{1}H NMR (D_2O, 300 MHz) δ 7.55 (1H, br s, H-3), 5.54 (1H, d, $J_{1,9} = 3.6$ Hz, H-1), 4.82 (1H, d, $J_{1',2'} = 8.4$ Hz, H-1′), 3.92 (1H, br d, $J_{6'a,6'b} = 12.3$, H-6′b), 3.74 (1H, dd, $J_{6'b,6'a} = 12.3$, $J_{6'b,5'} = 5.7$ Hz H-6'b), 3.62 (1H, d, $J_{10a,10b} = 17.5$ Hz, H-10a), 3.56 (1H, d, $J_{10b,10a} = 17.5$, H-10b), 3.54−3.48 (1H, m, H-5′), 3.51 (1H, t, $J_{3',4'} = 9.1$ Hz, H-3′), 3.41 (1H, t, $J_{3',4'} = J_{4',5'} = 9.1$ Hz, H-4′), (1H, d, $J_{1',2'} = 8.1$ Hz, H-1′), 3.29 (1H, t, $J_{2',3'} = 9.1$ Hz, $J_{1',2'} = 8.1$ Hz, H-2′), 2.95 (1H, m, H-5), 2.34 (1H, dd, $J_{1,9} = 3.6$ Hz, $J_{5,9} = 9.3$ Hz, H-9), 2,08−1.63 (4H, m, 2H-6, 2H-7). ^{13}C NMR (D_2O, 75.5 MHz) δ 95.6 (C-1), 153.4 (C-3),112.4 (C-4), 32.5 (C-5), 30.1 (C-6), 35.7 (C-7), 82.8 (C-8), 45.7 (C-9), 68.2 (C-10), 172.0 (C-11), 99.4 (C-1′), 73.3 (C-2′), 76.3 (C-3′), 70.1 (C-4′), 77.0 (C-5′), 61.3 (C-6′). MS API-ES negative $[M - 1]^-$ *m/z* = 391.1 and exact mass ES negative $[M - 1]^-$ *m/z* = 391.1228, cald for $C_{16}H_{23}O_{11}$ 391.1240.

RESULTS AND DISCUSSION

Chromatographic Data. Isocratic elution using aqueous trifluoroacetic acid (0.06%) as an eluent afforded good separation of a series of carboxylic acids in the most hydrophilic fraction of the juice (**Figure 1**).

Structural Elucidation. The carboxylic acids, quinic acid (peak 2), malic acid (peak 3), shikimic acid (peak 4), and citric acid (peak 5), had previously been stated to be present in the juice (*10*, *11*), but no verification of the structures had been given. HPLC-NMR and HPLC-MS was used in this study to establish the structures. The above four carboxylic acids were isolated by preparative HPLC and comparison of their ^{1}H NMR spectra with those of authentic samples unequivocally established their structures. In addition to these four carboxylic acids, two additional peaks at 10.9 and 16.3 min were observed in the chromatogram. The characteristic signal at 7.5 ppm and the presence of a glucopyranosyl moiety as revealed by the signal pattern in the HPLC-^{1}H NMR spectrum indicated that both of these compounds were iridoid glucosides. After isolation by preparative HPLC, the ^{1}H NMR and ^{13}C NMR spectra of the more hydrophilic iridoid glucoside were found to be superimposable to the spectra of monotropein (*12*). The spectra of the less polar iridoid glucoside were very similar to those of monotropein except for the missing signals originating in the nuclei at the 6,7 double bond. Instead, a complex pattern was found at 2.08−1.63 ppm in the ^{1}H NMR spectrum and two signals at 30.1 and 35.7 ppm in the ^{13}C NMR spectrum. MS revealed that the molecular weight of the compound was two units higher than that of monotropein. Consequently the compound was concluded to be 6,7-dihydromonotropein. The methyl ester of 6,7-dihydromonotropein (splendoside) has previously been isolated from *Fouquieria splendens* (*13*), but this is the first report of natural occurrence of the free acid. The structures of the compounds isolated from the cranberry juice are given in **Figure 2**. Monotropein is proven to be present in all the analyzed *Vaccinium* species by LC-MS. 6,7-Dihydromonotropein was proven to be present in cranberries and lingonberries by LC-MS, but absent in blueberries. A compound with the R_f values of monotropein on TLC and PC has previously been demonstrated in the green parts of *V. oxycoccos* L. (European cranberries), in the stem and fruit of *V. myrtillus* L. (blueberry), and in the leaves and stem of *V. vitis-ideae* L. (lingonberry) (*14*).

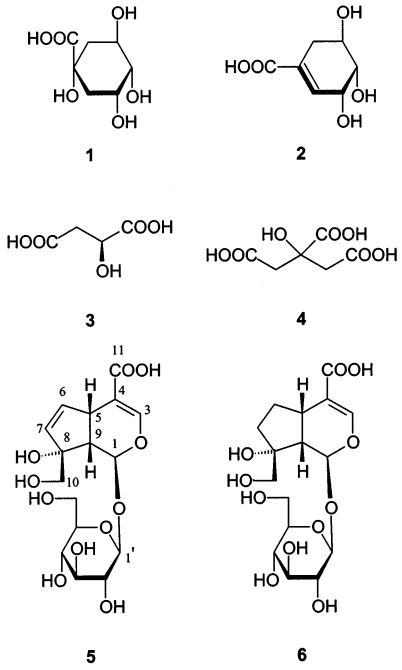

Figure 2. Structures of the isolated acids and iridoid glucosides, (–)-quinic acid (**1**), (–)-shikimic acid (**2**), L-malic acid (**3**), citric acid (**4**), monotropein (**5**), and 6,7-dihydromonotropein (**6**).

Quantification of Quinic, Malic, Shikimic, and Citric Acid. The amounts of the four carboxylic acids (**1**−**4**) in cranberry,

lingonberry, and blueberry juices are given in **Table 1**, which reveals that no major differences in the concentrations of the acids are found in the different samples of juices. The results are in agreement with previous finding in juice from *V. macrocarpon* (*10*, *11*, *14*). The analyses were extended to a series of juices prepared from berries of other *Vaccinium* species such as European cranberries, lingonberries and blueberries (**Table 1**). Preliminary studies revealed that the two iridoid glucosides do not contribute to the antiadhesive effect of the juice, but the presence of acids and iridoid glucosides contributes to the taste of the products. Consequently analyses of the contents of such compounds are important for making products with similar tastes. The new analytical protocol facilitates quantification of the carboxylic acids and has revealed the presence of iridoid glucosides in cranberry, lingonberry, and blueberry juices.

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