Degradation Products of Cyanidin Glycosides from Tart Cherries and Their Bioactivities

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The bioactive anthocyanins present in tart cherries, *Prunus cerasus* L. (Rosaceae) cv. Balaton, are cyanidin 3-glucosylrutinoside (**1**), cyanidin 3-rutinoside (**2**), and cyanidin 3-glucoside (**3**). Cyanidin (**4**) is the major anthocyanidin in tart cherries. In our continued evaluation of the in vivo and in vitro efficacy of these anthocyanins to prevent inflammation and colon cancer, we have added these compounds to McCoy's 5A medium in an effort to identify their degradation products during in vitro cell culture studies. This resulted in the isolation and characterization of protocatechuic acid (**5**), the predominant degradation product. In addition, 2,4-dihydroxybenzoic acid (**6**) and 2,4,6-trihydroxybenzoic acid (**7**) were identified as degradation products. However, these degradation products were not quantified. Compounds **5**−**7** were also identified as degradation products when anthocyanins were subjected to varying pH and thermal conditions. In cyclooxygenase (COX)-I and -II enzyme inhibitory assays, compounds **5**−**7** did not show significant activities when compared to the NSAIDs Naproxen, Celebrex, and Vioxx, or Ibuprofen, at 50 μM concentrations. However, at a test concentration of 50 μM, the antioxidant activity of protocatechuic acid (**5**) was comparable to those of the commercial antioxidants *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA), and superior to that of vitamin E at 10 μM concentrations.

Keywords: *Prunus cerasus; tart cherries; anthocyanins; cyanidin; degradation; antioxidant; cyclooxygenase*

INTRODUCTION

Tart cherry anthocyanins and their aglycon, cyanidin, are reported to have excellent antiinflammatory and antioxidant properties comparable to those of commercial products (*1*−*3*). These reports have resulted in an increased consumer demand for fresh and frozen cherries, cherry concentrate, dried cherries, and various other forms of processed cherry products. Because consumers are including various forms of cherries and cherry products in their diets for relief of arthritis- and gout-related pains, it is important to understand the fate of anthocyanins and cyanidin in order to maintain their integrity in food products. This is essential if there is a desire to retain the biological activities of these natural coloring pigments.

Although anthocyanins occur widely in many fruits, flowers, and other plant materials, they are not stable pigments, and they tend to be decolorized or degraded during processing and storage of the commodity. There are numerous studies into the mechanisms of anthocyanin breakdown (*4*−*6*). Among the most important factors involved in the processing of cherry products are pH and temperature (*4*). Numerous pseudo forms of anthocyanins exist at various pH values (*7*). The major anthocyanidin found in tart cherries, cyanidin, exists as the red flavylium cation at pH < 3 thereby giving the fruit its characteristic color. The flavylium cation also exists as the colorless carbinol pseudobase at pH 3−6 and the blue-purple quinoidal-base at pH 7−8, which eventually converts to the chalcone. The red flavylium cation, stable at low pH, forms ketoquinonoidal bases by deprotonation and an ionized quinonoid base at pH > 7 (*4*, *5*). At pH 3−6, the cation forms a carbinol pseudobase or chalcone pseudobase (*4*, *5*).

Anthocyanins degrade by either of two pathways to form chalcones and coumarin glycosides or aldehydes and benzoic acid derivatives (*4*). A number of physical and environmental factors affect the stability of anthocyanins. The temperature of processing and storage affects degradation, with the rate of degradation increasing with rising temperatures. Also, varying values of pH influence anthocyanin degradation. Low pH stabilizes anthocyanins because the equilibrium between the colored flavylium cation and the colorless pseudobase is shifted toward the more stable flavylium ion. Enzymatic action has also been implicated in the degradation of anthocyanins. Glycosidases (anthocyanases) may hydrolyze the glycosidic bonds of anthocyanins to yield the much more unstable anthocyanidins which undergo spontaneous decolorization and degradation.

In this study we have investigated the stability and degradation products of the cyanidin glycosides isolated from tart cherries under varying pH levels and temper-

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atures, and in McCoy's 5A medium under cell culture conditions at 37 °C for 72 h. The antioxidant and COX-I and -II inhibitory activities of the degradation products of cherry anthocyanins have also been determined.

MATERIALS AND METHODS

General Experimental Procedures. All NMR spectra (^{1}H and ^{13}C) were recorded on a Varian VXR 500 MHz spectrometer. ^{13}C NMR spectra were recorded at 126 MHz. Chemical shifts were recorded in DMSO-d_6, and the values are in δ (ppm) based on residual DMSO at 2.5 ppm for ^{1}H NMR and DMSO at 39.51 ppm for ^{13}C NMR. Coupling constants, J, are in Hz. For preparative HPLC purification (LC-20, Japan Analytical Industry Co., Tokyo), two JAIGEL-ODS, A-343-10, 250 mm × 20 mm i.d., 10 μm (Dychrom, Santa Clara, CA) columns were used in tandem. Peaks were detected at 210 nm, unless otherwise stated, using a UV detector equipped with a model D-2500 Chromato-integrator (Hitachi, Tokyo). All solvents were ACS reagent grade and were purchased from Sigma-Aldrich Chemical Co., Inc. (St, Louis, MO). Standards for the degradation products, protocatechuic acid, 2,4-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid and positive controls used in the antioxidant (TBHQ, BHA, BHT, and α-tocopherol) and COX inhibitory (Ibuprofen and Naproxen) bioassays were also purchased from Sigma-Aldrich. Celebrex capsules and Vioxx tablets were physician's professional samples provided by Dr. Subash Gupta, Sparrow Pain Center, Sparrow Hospital, MI.

Fruits. IQF (Individual quick frozen) tart cherries, *P. cerasus* L. cv. Balaton were obtained through the Cherry Marketing Institute, and originated from commercial growers in Traverse City, MI.

Analytical HPLC Conditions. All samples (20 μL injection volume) were filtered (0.22 μm) and analyzed on an Xterra (Waters Corp., Milford, MA) RP-18 column (250 mm × 4.6 mm i.d., 5 μm) at a column temperature of 35 °C. The mobile phase, 4% aqueous H_3PO_4/CH_3CN (90:10 v/v), was used under isocratic conditions at a flow rate of 0.75 mL/min for detection of anthocyanins and the degradation products. Anthocyanins were detected at 520 nm and degradation products were detected at 210 nm using a PDA detector (Waters). For the detection of cyanidin, the mobile phase of 4% aqueous H_3PO_4/MeOH (70:30 v/v) was used in isocratic mode with a flow of 0.75 mL/min.

Cell Culture Studies. Human colon cancer cell lines, HT-29 (ATCC 38-HTB) and HCT-116 (ATCC 247-CCL), were purchased from American Type Culture Collection, Rockville, MD. Cells were cultured in McCoy's 5A medium (M9720) purchased from Sigma Co., St. Louis, MO and supplemented with 10% fetal bovine serum (Sigma). Sodium bicarbonate (2.2 g) was added for each liter of final volume of medium being prepared, and the pH was adjusted to 7.2 with 1 M HCl. The components of McCoy's 5A medium are calcium chloride, magnesium sulfate, potassium chloride, sodium chloride, sodium phosphate, L-alanine, L-arginine·HCl, l-asparagine·H_2O, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, l-histidine·HCl·H_2O, *trans*-4-hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine·HCl, L-methionine, l-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, ascorbic acid, *p*-aminobenzoic acid, D-biotin, choline chloride, folic acid, myo-inositol, niacinamide, nicotinic acid, D-panthothenic acid (hemicalcium), pyridoxal·HCl, pyridoxine·HCl, riboflavin, thiamine·HCl, vitamin B-12, peptone, D-glucose, and glutathione (reduced).

Isolation of Anthocyanins. Anthocyanins were isolated from Balaton tart cherries according to the previously published method (*3*). The anthocyanin mixture (1.51 g) was dissolved in H_2O (2 mL) and fractionated by medium-pressure liquid chromatography (MPLC) on a C-18 column (500 × 40 mm i.d.), and eluted with a MeOH (0.1% HCl):H_2O solvent system, under gradient conditions, starting with 30% MeOH to 100% MeOH at a flow rate of 2 mL/min. Five fractions were collected: I, 150 mL; II, 300 mL; III, 250 mL; IV, 200 mL; and V, 400 mL. MeOH from the fractions was evaporated in vacuo, and the residue was lyophilized. Fraction IV (493.7 mg) revealed a high content of anthocyanins by analytical HPLC and was dissolved in H_2O (20 mL) then extracted with n-BuOH (3 × 10 mL) to remove undesired flavonoids and other polyphenols. The aqueous portion was then lyophilized to yield anthocyanins (210.3 mg) containing cyanidin 3- glucosylrutinoside (**1**)/cyanidin 3-rutinoside (**2**) in a ratio of 3:2.

Preparation of Cyanidin. The aglycon, cyanidin, was prepared by the acid hydrolysis of anthocyanins isolated from Balaton tart cherries. The anthocyanin mixture (50 mg) was hydrolyzed with 3 M HCl (15 mL) under an atmosphere of nitrogen for 3 h at 80 °C. The dark red precipitate obtained was subjected to MPLC on a C-18 column (350 × 40 mm i.d.), under gradient conditions starting with 70% MeOH/H_2O to 100% MeOH (0.1% HCl). The red band that eluted with 100% acidic MeOH was evaporated in vacuo to yield pure cyanidin (**4**) (10 mg).

Isolation and Identification of Compounds 5−7. An aqueous solution of the anthocyanins or cyanidin (158 mg/5 mL H_2O, pH 3.4) was filtered sterilized separately into McCoy's 5A cell growth medium supplemented with 10% fetal bovine serum. The resulting solutions (pH 7.4) were placed in a 5% CO_2 incubator at 37 °C for 72 h, after which the pH was adjusted to pH 3 with 3 N HCl (1 mL). This was purified on an XAD-16 column (Amberlite resin) by eluting with H_2O (250 mL) followed by MeOH (0.1% HCl) (100 mL). The MeOH eluate (3.03 g) was further purified by MPLC on a C-18 column (300 mm × 22 mm i.d.) under stepwise gradient conditions starting with 10% MeOH/H_2O to 100% MeOH (0.1% HCl). The fractions I (603 mg, 10% MeOH/H_2O, 250 mL), II (12 mg, 50% MeOH/H_2O, 75 mL), and III (22 mg, 70% MeOH/H_2O, 55 mL) were collected and evaporated in vacuo. The purification of fraction II by preparative HPLC utilizing a mobile phase of A: MeOH/H_2O (1:9; v/v); B: MeOH/H_2O (1:1; v/v); gradient 100% A, 0−60 min; 100% B, 61−200 min, and a flow of 3 mL/min, gave compound **5**, protocatechuic acid (1.7 mg, R_t 130 min). The fraction collected at R_t 140 min gave compounds **6** and **7**, which were not isolated in pure form. They were confirmed as 2,4-dihydroxybenzoic and 2,4,6-trihydroxy benzoic acids, respectively, by comparing their retention values (R_t) using authentic samples purchased from Aldrich Chemical Co.

Compound 5. Compound **5** was identified as protocatechuic acid. The ^{1}H and ^{13}C NMR spectral data of compound **5** were identical to the published spectral data (*8*) and also to the data of an authentic sample purchased from Aldrich Chemical Co.

Cyclooxygenase Inhibitory Assay. The COX-I enzyme inhibitory assay was conducted with an enzyme preparation from ram seminal vesicles purchased from Oxford Biomedical Research, Inc., Oxford, MI (ca. 0.46 mg protein/mL in 30 mM Tris buffer, pH 7). COX-II activity was determined using a preparation from insect cell lysate (supplied by Dr. Dave Dewitt, Department of Biochemistry, MSU, MI) diluted with Tris buffer (pH 7) to give an approximate final concentration of 1.5 mg protein/mL. COX assays were measured at 37 °C by observing the initial rate of O_2 consumption using an Instech micro oxygen chamber and electrode (Instech Laboratories, Plymouth Meeting, PA) attached to a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument, Inc., Yellow Springs, OH). Each assay mix contained 12 mL of pH 7, 0.1 M Tris 1 mmol phenol buffer and 340 μg of hemoglobin. For all test samples and controls, activities were determined following a 10-min preincubation of test samples with 5−25 μg of enzyme in a volume of 10−20 μL in the assay chamber (*9*). Reaction was initiated by adding 10 μL of arachidonic acid (0.25 mg/0.5 mL of Tris buffer). Data were recorded using Quicklog for Windows data acquisition and control software (Strawberry Tree Inc., Sunnyvale, CA).

Antioxidant Assay. Bioassays were conducted by analysis of model liposome oxidation using fluorescence spectroscopy. The lipid, 1-stearoyl-2-linoleoyl-sn-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc. Alabaster, AL) and fluorescent probe, 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]-phenylpropionic acid (Molecular Probes Inc., Eugene, OR), were combined in DMF and dried in vacuo at RT. Large unilamellar vesicles (LUVs) were

Table 1. Degradation Products of Tart Cherry Anthocyanins Exposed to Varying pH and Temperature for 1 h Detected by HPLC

conditions		
pH	temperature	degradation products
3	ambient	no degradation (Figure 1a).
7	ambient	protocatechuic acid (**5**), 2,4-dihydroxy benzoic acid (**6**), and 2,4,6-trihydroxybenzoic acid (**7**) (Figure 1e).
10	ambient	protocatechuic acid (**5**), 2,4-dihydroxy benzoic acid (**6**), and 2,4,6-trihydroxybenzoic acid (**7**) (Figure 1f).
3	refluxed	Anthocyanins, protocatechuic acid (**5**), 2,4-dihydroxy benzoic acid (**6**), and 2,4,6-trihydroxybenzoic acid (**7**). Hydrolysis of cyanidin-3-glucosylrutinoside (**1**) to cyanidin-3-glucoside (**3**) and cyanidin (**4**) was monitored by HPLC and quantified at 30 (Figure 1g) and 60 min (Figure 1h).
7	refluxed	protocatechuic acid (**5**), 2,4-dihydroxy benzoic acid (**6**), and 2,4,6-trihydroxybenzoic acid (**7**) (Figure 1i).
10	refluxed	protocatechuic acid (**5**), 2,4-dihydroxy benzoic acid (**6**), and 2,4,6-trihydroxybenzoic acid (**7**) (Figure 1j).
3	microwave heating for 5 min	anthocyanins, protocatechuic acid (**5**), 2,4-dihydroxy benzoic acid (**6**), and 2,4,6-trihydroxybenzoic acid (**7**) (Figure 1k).

produced by resuspension of the lipid−probe mixture (0.15 M NaCl, 0.1 mM EDTA, and 0.01 M MOPS maintained over Chelex resin) followed by ten freeze−thaw cycles in a dry ice−EtOH bath, and extrusion (29 times) through a 100-nm pore size membrane (Avestin, Inc., Ottawa, Canada). The final assay volume was 2 mL consisting of 100 μL of HEPES buffer (50 mM HEPES and 50 mM Tris), 200 μL of 1 M NaCl, 1.64 mL of N_2 sparged water, 20 μL of test sample or DMSO (blank), and a 20-μL aliquot of liposome suspension. Peroxidation was initiated by addition of 20 μL of $FeCl_2 \cdot 4H_2O$ (0.5 mM) for positive controls (BHA, BHT, TBHQ, and α-tocopherol/Vitamin E, all 10 μM) and test samples. Fluorescence was measured at 384 nm and monitored at 0, 1, 3, and every 3 min thereafter up to 21 min using a Turner model 450 Digital Fluorometer (Barnstead Thermolyne, Dubuque, IA). The decrease of relative fluorescence intensity with time indicated the rate of peroxidation. Relative fluorescence (F_t/F_0) was calculated by dividing the fluorescence value at a given point (F_t) by that at $t = 0$ min (F_0).

RESULTS AND DISCUSSION

Anthocyanins are extremely unstable plant pigments known to spontaneously degrade with changes in pH and temperature. In addition to our interest in the stability of anthocyanins in processed cherry products, we are evaluating the efficacy of these compounds in vivo and in vitro for colon cancer prevention in humans (unpublished results). In in vitro cell culture studies, the colon cancer cell lines HT 29 and HCT 116 are cultured in McCoy's 5A medium. Hence, it is important to determine the degradation and/or stability of these compounds in the cell culture medium in the absence of the HT 29 and HCT 116 cell lines. The tart cherry anthocyanins (**1**−**3**) and cyanidin (**4**) were added to McCoy's 5A cell culture medium at pH 7.4 and at 37 °C and incubated under cell culture conditions. Similarly, aqueous solutions of anthocyanins and cyanidin and McCoy's 5A cell medium supplemented with 10% fetal bovine serum as controls were incubated under identical conditions and analyzed by HPLC. Three benzoic acid derivatives, protocatechuic acid (**5**), 2,4-dihydroxybenzoic acid (**6**), and 2,4,6-trihydroxybenzoic acid (**7**), were identified as degradation products. However, these compounds were not detected by HPLC in the control samples studied.

Protocatechuic acid (**5**), an off-white amorphous solid, was isolated in pure form by preparative HPLC. The ^{1}H NMR spectrum revealed signals attributed to three aromatic protons at δ 7.33 (1H, d, $J = 3.5$ Hz), 7.28 (1H, dd, $J = 14.0$, 3.5 Hz), and 6.76 (1H, d, $J = 14.0$ Hz) in an ABX coupling system. Two additional signals at δ 9.61 and 9.23, both singlets exchangeable with D_2O, were attributable to the phenolic protons. The ^{13}C NMR spectrum confirmed the presence of a disubstituted benzoic acid derivative with the carbonyl of the acid resonating at 167.4 ppm. The spectral data of compound **5** were identical to those of a commercially obtained standard of protocatechuic acid and were also similar to the reported values (*8*). Protocatechuic acid and 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran have been reported as degradation products resulting from the oxidation of cyanidin 3-glucoside (**3**) (*8*, *10*). There are two major pathways for the degradation of anthocyanins (*4*). The first pathway proceeds through the carbinol pseudobase to the chalcone and coumarin glycoside. The second route involves hydrolysis of the glycosidic bond as the first step in anthocyanin degradation to form the anthocyanidin. The aglycon, which is more unstable than its glycosides, proceeds through a highly unstable α-diketone intermediate to eventually form aldehydes and benzoic acid derivatives. We have observed the latter route of anthocyanin degradation in our studies.

The fraction collected after protocatechuic acid yielded a mixture of two compounds. These were identified as 2,4-dihydroxybenzoic acid (**6**) and 2,4,6-trihydroxybenzoic acid (**7**) by comparing their retention times to authentic standards. These compounds are plausible degradation fragments of flavylium cation similar to protocatechuic acid. Compounds **5**−**7** were also formed, as detected by HPLC analysis, when anthocyanins were subjected to a combination of high pH and elevated temperatures. The stability and degradation products, detected by HPLC, resulting from anthocyanin degradation under varying experimental conditions are shown in Table 1 , Figure 1a−k, and Figure 2. We have observed that the anthocyanins at pH 3 are very stable at room temperature. However, when the temperature was elevated to 100 °C (boiling or reflux conditions for 1 h) increased levels of cyanidin 3-glucoside (**3**) and cyanidin (**4**) were observed because of the hydrolysis of cyanidin 3-glucosyl rutinoside (**1**). This was confirmed from the time-course experiments and by HPLC analyses of samples monitored at 30 (Figure 1g) and 60 min (Figure 1h). The anthocyanins were quantified from standard curves generated using pure anthocyanins **1**−**3** as previously reported (*3*). When the pH was increased to 7 or 10 by the addition of 1N KOH, anthocyanins rapidly degraded to the benzoic acid derivatives. A combination of high pH and temperature also resulted in rapid anthocyanin degradation (Table 1, Figure 1a−k). We have also studied the degradation of cyanidin glycosides under microwave conditions used for cooking and heating food. After 5 min of heating the anthocyanins under high heat, trace quantities of anthocyanin **1** and substantial quantities of compound **6**,

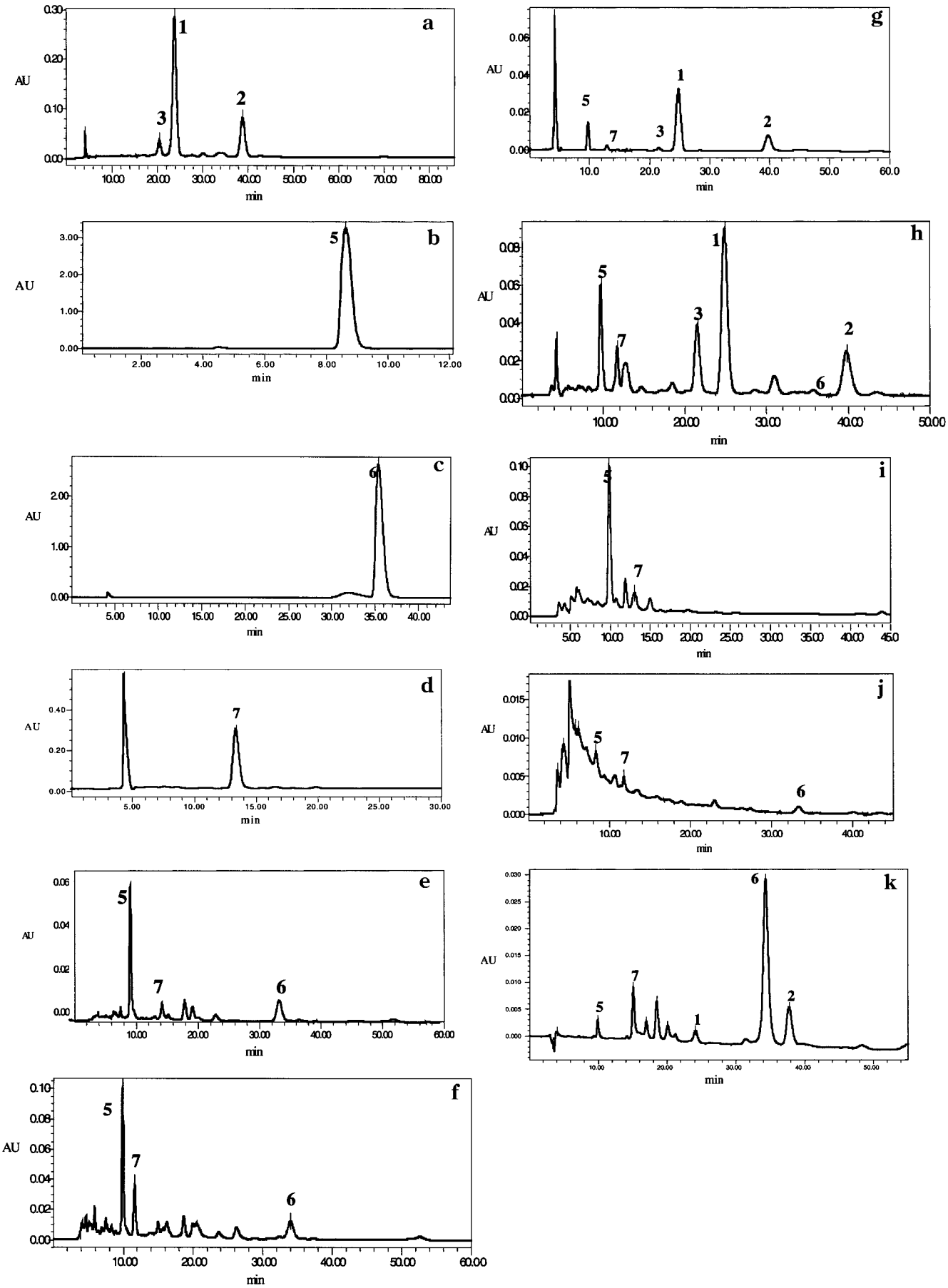

Figure 1. Panels a−k. HPLC chromatograms of degradation products (monitored at 210 nm) formed from anthocyanins (monitored at 520 nm) exposed to varying pH and temperature levels for 1 h. Analyses of authentic samples of **5**, **6**, and **7** are shown in Figures 1b, 1c, and 1d, respectively.

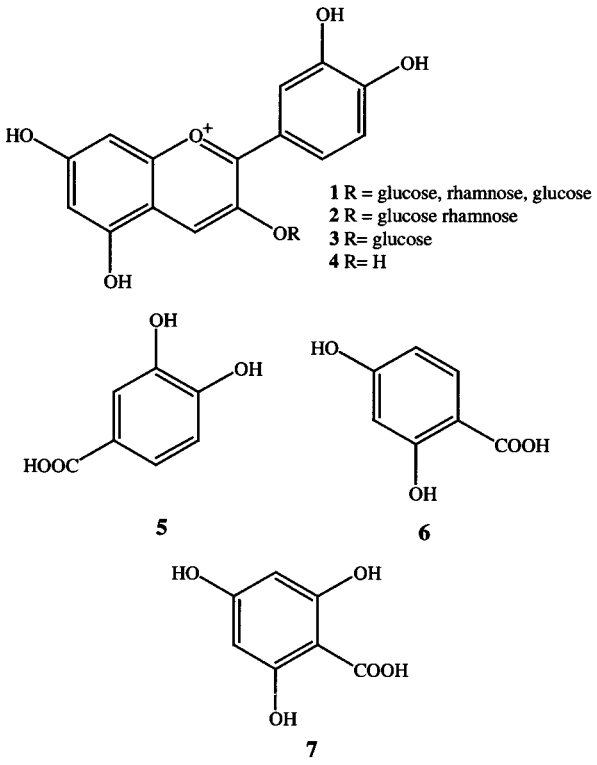

Figure 2. Structures of anthocyanins and their aglycon obtained from tart cherries (**1**−**4**) and degradation products identified under varying conditions of pH and temperature (**5**−**7**).

2,4-dihydroxy benzoic acid, were detected by HPLC (Figure 1k). No attempts were made to quantify these degradation products mainly because of the low levels of degradation products detected. We believe that the cyanidin glycosides underwent rapid degradation and further oxidized to products that are characteristic of typical phenolic oxidation products. However, further studies on the degradation kinetics of these anthocyanins will be helpful to ensure the quality of processed cherry products.

We have previously reported that tart cherry anthocyanins **1**−**3** and their aglycon cyanidin **4** have in vitro antioxidant and cyclooxygenase-I and -II enzyme inhibitory activities comparable to those of commercial products (*3*). Therefore, we have evaluated the biological activities of the degradation products **5**−**7** to determine their potential phytoceutical properties. However, these compounds did not show significant COX-I and -II inhibitory activities at test concentrations of 100 μM when compared to those of commercial antiinflammatory drugs tested at 10 μM. The antioxidant assay was conducted by using an iron-catalyzed liposomal model and fluorescence spectroscopy to monitor the inhibition of lipid peroxidation (*11*). At a test concentration of 50 μM, only protocatechuic acid **5** showed significant antioxidant activity compared to those of the commercial antioxidants BHA, BHT, TBHQ, and vitamin E when tested at 10 μM concentrations (Figure 3).

We have identified only three benzoic acid derivatives from the degradation products of tart cherry anthocyanins and cyanidin under varying pH and temperature conditions. The antioxidative activity of the anthocyanins in tart cherries may be retained by the formation of protocatechuic acid under physiological conditions. The tart cherry anthocyanins are stable at room temperature and pH 3, as is cherry fruit. At high temperatures, such as those in cooking, they will be hydrolyzed to cyanidin aglycon with enhanced antiinflammatory and antioxidant activities (*3*). However, prolonged exposure of cherries to high temperatures during product formulation should be avoided in order to retain the health benefits of anthocyanins, as the aglycon cyanidin undergoes spontaneous degradation to various byproducts with little or no antiinflammatory activity. To yield the full potency of cherry anthocyanins for pain relief, the cherry products should be prepared under mild temperature and low pH conditions. Both temperature

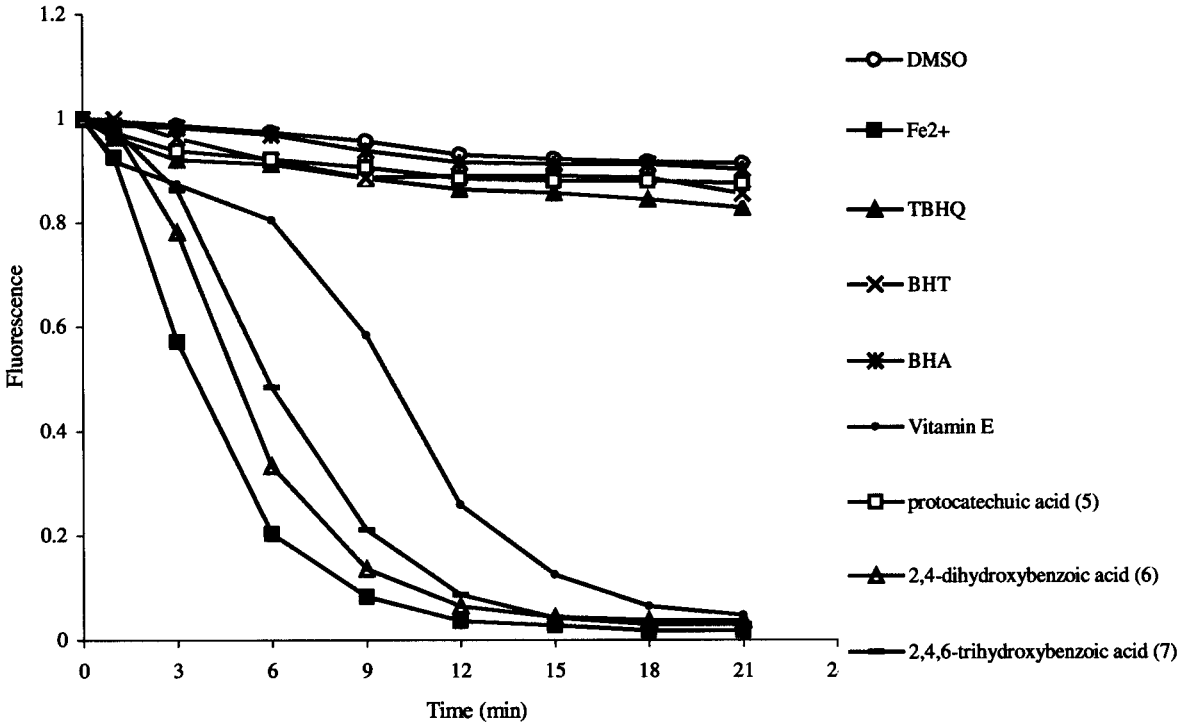

Figure 3. Comparative antioxidant activities of benzoic acid derivatives **5**−**7** obtained from the degradation of cherry anthocyanins in a liposomal model system. Samples were tested at 50 μM and standards were at 10 μM concentrations.

and pH will also determine the shelf life of the finished cherry products as nutraceuticals or phytoceuticals. This suggests that fresh or frozen cherries and cherry products prepared under ideal conditions that maintain the desired levels of anthocyanins would be beneficial to human health when consumed, and they may be included in the regular diet for alleviating arthritis- and gout-related pain.

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