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Stability and Antioxidant Activity of Black Currant Anthocyanins in Solution and Encapsulated in Glucan Gel

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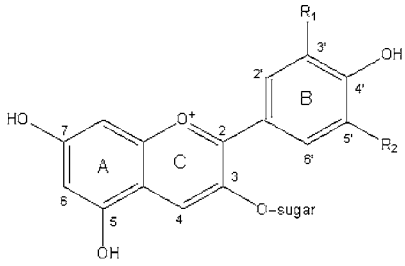
The effects of ferric and ferrous ions, pH, and temperature on the stability and antioxidant activity of black currant anthocyanins (BCA) were studied, and the recovery of BCA from glucan gel [mixed linked (1→3,1→4)-β-D-glucan] after using different encapsulating procedures was determined. The degradation of individual anthocyanins follows first-order kinetics and shows Arrhenius temperature dependence. The activation energies of individual anthocyanins, evaluated over the temperature range 60−100 °C, decrease with an increase in pH. While the antioxidant activity of BCA, measured by the ferric reducing antioxidant power assay, decreased with the degradation of anthocyanins, the completely degraded products still exhibited ∼30% of the initial antioxidant activity. Ferric ions have a detrimental effect on the stability of BCA, especially for delphinidins. Freeze drying of encapsulated BCA gives ∼20% higher recovery of individual anthocyanins than infrared drying.

KEYWORDS: Anthocyanins; black currant; antioxidant activity; FRAP assay; HPLC; degradation kinetics; thermal stability; ferric-anthocyanin complexes; (1→3,1→4)-β-D-glucan; encapsulation

INTRODUCTION

Anthocyanins are responsible for the attractive red, purple, and blue colors in many fruits and flowers. They may be used as natural colorants and as markers of good manufacturing practices in food systems (*1*). Recent studies suggest that anthocyanins are beneficial to human health by reducing the risk of cardiovascular disease and improving vision (*1*). These compounds can be absorbed by the human digestive system and have been detected intact in blood (*2*).

Four anthocyanins account for ∼97% of the total anthocyanin content of black currant extract (*3*). These anthocyanins are delphinidin-3-O-glucoside (Del-3-O-G), delphinidin-3-O-rutinoside (Del-3-O-R), cyanidin-3-O-glucoside (Cyn-3-O-G), and cyanidin 3-O-rutinoside (Cyn-3-O-R) (**1**−**4**) (**Figure 1**). It has been reported (*4*−*6*) that the stability of anthocyanins and their colors is highly dependent on pH, due to changes in the concentration of the four species: flavylium cation, quinonoidal base, pseudobase, or carbinol and chalcone. Conversion of one species to another is typically accompanied by dramatic changes in color and stability. Among the four species, the red flavylium cation present at pH 1.0−2.4 is the most stable (*4*). Other factors that affect the rate of anthocyanin degradation include temperature, oxygen, enzymes, light, acylation, copigments, and metal ions (*1*, *7*). Hoshino and co-workers (*8*) reported that metal ions such as Mg^{2+}, Fe^{3+}, and K^{+} stabilize acylated anthocyanins due to the formation of anthocyanin−metal complexes. In contrast, Mazza and Brouillard (*7*) suggested that metal−anthocyanin complexes did not contribute to the stability of anthocyanins, due to the decomposition of the complexes with time.



	Anthocyanin	R_1	R_2	MW
1	Delphinidin 3-O-glucoside	OH	OH	465
2	Delphinidin 3-O-rutinoside	OH	OH	611
3	Cyanidin 3-O-glucoside	OH	H	449
4	Cyanidin 3-O-rutinoside	OH	H	595
5	Petunidin 3-O-rutinoside	OCH_3	OH	625

Figure 1. Chemical structures and molecular weights of the anthocyanins found in black currant extract.

The stability of anthocyanins from pomace was found to be increased by encapsulation in DE20 maltodextrin (*9*). Likewise, the stability of anthocyanins from *Hibiscus sabdariffa* L. was improved by encapsulation in pullulan (*10*). Consequently, encapsulation of anthocyanins with natural polymers can be

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expected to enhance their stability and potentially provide controlled release of these functional ingredients in the human body for more efficient nutraceutical usage. In the present study, mixed linked (1→3,1→4)-β-D-glucan extracted from barley (*11*) was used as an encapsulating medium for black currant anthocyanins (BCA). The glucan is a thermoreversible gelling agent whose gelling behavior depends on molecular weight and concentration (*12*). During cooling of the glucan solution in water from 80 °C to room temperature, a network structure forms through interacting chain segment association and aggregated junction zones (*11*). It was expected, consequently, that BCAs could be trapped in the network structure.

A number of assays such as the oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox equivalent antioxidative capacity (TEAC), and ferric reducing antioxidant power (FRAP) that are being used to evaluate antioxidant activity depend on different mechanisms (*13*). The FRAP assay determines the ferric reducing ability of a sample, and it can be argued that the antioxidant power measured as the ferric reducing ability has little relationship with the free radical scavenging ability of an antioxidant. However, Nielsen et al. (*14*) reported that the FRAP assay and TEAC correlated well for measurement of antioxidant capacity of black currant juices. Cao and Prior (*15*) also reported that there are significant linear relationships between the ORAC assay and the FRAP assay in assessment of antioxidant capacity of human serum especially when protein was removed in the ORAC assay. The FRAP assay has a good response to black currant extract and is simple and easy to perform for large amounts of sample. Consequently, in our study, the FRAP assay was chosen to investigate the change in antioxidant activity of black currant extracts during heating at different pH values.

The objectives of the present study were to quantify the changes in anthocyanins and antioxidant activity of BCAs in response to a range of physical and chemical stimuli including pH and temperature, and encapsulation with glucan, and to study the effect of ferric and ferrous ions on the stability of the major anthocyanin components of BCAs. We also investigated the recovery of BCAs from dried glucan gel-encapsulated material.

MATERIALS AND METHODS

Materials. Dried black currant extract was supplied by New Zealand Pharmaceuticals Ltd. (Nelson, New Zealand), and glucan was supplied by Gracelink Ltd. (Wellington, New Zealand). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was obtained from Aldrich Chemicals (Milwaukee, WI), and formic acid, ferric chloride ($FeCl_3 \cdot 6H_2O$), and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) were Analar grade materials from BDH Chemicals (Poole,United Kingdom). Acetonitrile [high-performance liquid chromatography (HPLC) grade] was from Scharlau (Barcelona, Spain). All chemicals used for preparation of buffer solutions were from BDH. Ondina oil 68 (white mineral oil) was supplied by The Shell Company (Melbourne, Australia), and soybean oil was purchased in a supermarket.

Identification of BCAs. Identification of the component anthocyanins of BCA was accomplished using an online photodiode array (PDA) together with HPLC/QTOF-MS (quadrupole time-of-flight mass spectrometer) (Thermo Electron Corporation, Framington, MA) and a Phenomenex LUNA C18 column (4.6 mm × 250 mm, i.d. 5 μm) (Torrance, CA) attached to a Finnigan HPLC system (Thermo Electron Corp.). The mobile phase for HPLC/QTOF-MS analysis was 2.2 M formic acid (solvent A) and acetonitrile (solvent B). Elution was performed at a flow rate of 1 mL min^{-1} using a gradient elution starting with 1% B for 0.5 min, to 7% B at 1 min; 7% B, 1.0−7.0 min; to 10% B at 9.5 min; 10% B, 9.5−13 min; to 13% B at 15 min; 13% B, 15−18 min; to 40% at 20 min; to 1% B at 21 min; and 1% B, 21−27 min. After the sample (10 mg mL^{-1}) (5−25 μL) was introduced into the HPLC column, the PDA was monitored at 520 nm. The elution profile for the HPLC/QTOF-MS system was slightly different from that for the HPLC for analysis of individual anthocyanins. The eluent was split after PDA detection to give a flow of 0.2 mL min^{-1} into the MS. The HPLC/QTOF-MS was equipped with an electrospray interface. Xcalibur Version 1.4 (Thermo Electron Corporation) software was used to control the instruments and determine accurate masses. Electrospray ionization was operated in positive ion mode under the following conditions: spray voltage, 4200 V; sheath gas (N_2) pressure, 563 kPa; auxiliary gas (N_2) pressure, 35 kPa; capillary temperature, 230 °C; tube lens offset, 119 V; CID-10 source; quad MS/MS bias, −3.6; and fragmentation conditions, 0.093 kPa argon (99.99%) and 30 V. N_2 was produced with a nitrogen generator (Peak Scientific Instruments, Bedford, MA). Tandem mass spectrometry (MS/MS) was performed in selective reaction monitoring mode. The analyses were carried out in triplicate.

Table 1. Buffer Solution Compositions[a]

pH	composition
1.2	HCl (0.2 M, 40.7 mL) + KCl (0.2 M, 9.3 mL)
2.4	HCl (0.1 M, 42.2 mL) + KH phthalate (0.1 M, 50.0 mL)
3.6	HOAc (0.2 M, 46.3 mL) + NaOAc (0.2 M, 3.7 mL)
4.5	HOAc (0.2 M, 58.5 mL) + NaOAc (0.2 M, 41.5 mL)
5.5	HOAc (0.2 M, 87.5 mL) + NaOAc (0.2 M, 12.5 mL)
6.8	NaH maleate (0.2 M, 25.0 mL) + NaOH (0.1 M, 44.4 mL)

[a] Each was diluted to 100 mL with water.

Buffer Solutions. Buffer solutions with pH values 1.2, 2.4, 3.6, 4.5, 5.5, and 6.8 were prepared as specified in **Table 1** (*16*). The pH of each buffer solution was measured with a pH meter.

Effect of Ferric and Ferrous Ions on the Stability of BCA. BCA (1.0 mg mL^{-1}) was prepared in 2.2 M formic acid solution and then filtered with disposable poly(tetrafluoroethylene) (PTFE) 0.45 μm filters (Phenomenex). Ferric and ferrous ion solutions were added to vials containing the BCA solution (total volume, 1 mL) with or without flushing with N_2, and the vials were sealed. HPLC analysis of individual anthocyanins in this study was performed using an Agilent Technologies HP 1100 instrument (Wilmington, DE) with an autosampler and PDA detector. The same column as the HPLC/QTOF-MS was used and maintained at 25 °C during the analysis. The mobile phase consisted of 2.2 M formic acid (solvent A) and acetonitrile (solvent B). The elution conditions were as follow: isocratic elution 1% B, 0−0.5 min; linear gradient from 1 to 7% B during 0.5−1.0 min; 7% B, 1.0−7.0 min; to 10% B during 7.0−9.5 min; to 13% B during 9.5−15 min; to 50% B during 15−18 min; to 1% B during 18−20 min; 1% B from 20 to 21 min; posttime 5 min with 1% B before next injection. The solvent flow rate was 1.0 mL min^{-1}, and 50 μL of sample solution was injected onto the column. The detection wavelengths were 520, 350, and 290 nm, and spectra from 200 to 700 nm were recorded for all eluted peaks. After each sampling, the vials initially filled with N_2 were refilled with N_2 and resealed for the next analysis in the time course. The control sample used a similar amount of MilliQ water instead of ferric ion solution. The experiment was performed in triplicate.

Stability of BCA at Different pH Values and Temperatures. BCA (0.1 mg mL^{-1}) solutions buffered at pH 2.4, 3.6, 4.5, 5.5, and 6.8 were incubated in a thermostated shaking water bath at 60, 80, and 100 °C. Aliquots (2.00 mL) were transferred to cuvettes cooled in an ice bath at predetermined time intervals over 12 h, and then, samples (1.00 mL) were immediately acidified with the calculated amount of 4 mol L^{-1} HCl to give final diluted samples (2.00 mL) at a pH of approximately 1.7. After the samples were filtered without contact with metal, using disposable PTFE 0.45 μm filters, the samples were analyzed for the individual anthocyanin components using HPLC. The study was performed in duplicate.

Effects of pH and Temperature on Antioxidant Activity of BCA. The antioxidant activity of BCA was assessed using the FRAP assay (*17*). FRAP reagent was made by mixing 10 volumes of acetate buffer (pH 3.6, 0.3 mol L^{-1}), 1 volume of TPTZ (10 mM in 0.04 M HCl), and 1 volume of ferric chloride solution (20 mM) and then incubated at 30 °C for 2 h before use. After the FRAP reagent (1800 μL), water

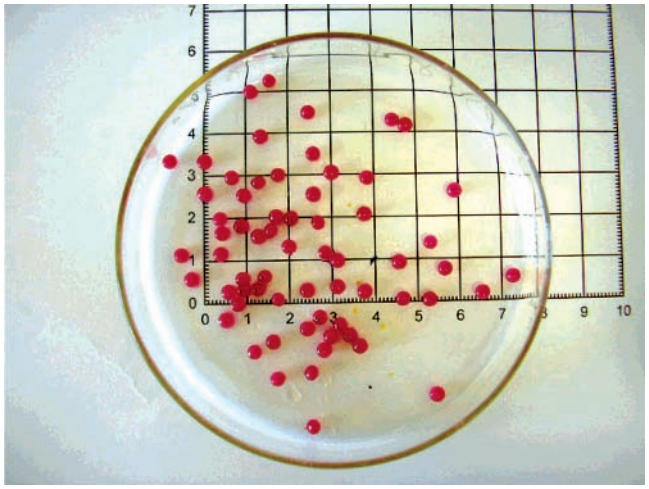

Figure 2. Fresh preparation of β-glucan gel beads encapsulated with BCA. The grid scale is in centimeters.

(180 μL), and samples (60 μL; the samples that were collected for study of stability of BCA at different pH values and temperatures) were mixed, the mixture was left for 50 min, and then, the absorbance of the resulting solutions was measured using a Hewlett-Packard model 8452A spectrophotometer (Wilmington, DE) at 593 nm. The absorbance at 700 nm was selected as background correction, and the corresponding buffers (60 μL) were used as blanks. The molar absorptivities of the four anthocyanins were reported as follows: ϵ_{520nm} of 27800 for Del-3-O-G, ϵ_{517nm} of 27500 for Del-3-O-R, ϵ_{512nm} of 27400 for Cyn-3-O-G, and ϵ_{510nm} of 26300 for Cyn-3-O-R (*18*). The value of the molar absorptivities for the four anthocyanins was similar to each other. Consequently, the relative amounts of anthocyanins were estimated as: $\sum$[peak area (t)/molecular weight]/$\sum$[peak area (t_0)/molecular weight]. The relative antioxidant activity of BCA was estimated as antioxidant activity (A_t)/antioxidant activity (A_0) where the absorbances A_0 and A_t were determined using the FRAP assay initially and at time t.

Encapsulation of BCAs with Glucan. Glucan (2.0 g) was slowly added to 90 °C MilliQ water (20 mL) with continuous stirring until the solid was fully dispersed. The resulting dispersion was held at 80 °C with gentle stirring for 1 h, and then, 0.5 mL of acetic acid was added together with black currant extract (16.0 mg) dissolved in water (3 mL). The mixture was stirred for a further 5 min and then used to make gel cubes and beads using the following procedures.

Gel cubes were made by pouring the warm mixture into a Petri dish and allowing it to cool to ambient temperature. The resulting gel was cut into approximately 5 mm cubes that were held at 4 °C for 4 h and then dried as explained below. In the case of gel beads, the heated mixture was transferred to a syringe heated to 80 °C, which was connected to a 0.15 mm i.d. catheter tube. The mixture in the syringe was added dropwise into soybean oil or Ondina oil 68 and cooled in an ice bath to produce beads with a diameter ~2.5 mm (**Figure 2**). The beads were kept at 4 °C for 4 h before drying.

The gel cubes and beads were dried by four methods: (i) in a conventional laboratory oven (Sanyo Electric, Japan) at 40 °C; (ii) in a vacuum oven (Heraeus Vacutherm, Munich, Germany) at 1 kPa and 40 °C; (iii) by infrared drying (Westinghouse Electric, Philadelphia, PA) at 38.9 °C; and (iv) by freeze drying for 12 h. The dried beads were rinsed with CCl_4 (20 mL) to remove oil and then flushed with N_2 to remove residual CCl_4.

Recovery of Anthocyanins from Encapsulated Samples. The dried gel cubes or beads (0.1 g) were transferred to a pH 1.2 HCl buffer (2.0 mL), crushed, and stirred with a glass rod. The mixture was centrifuged, and the supernatant liquid containing anthocyanins was collected. The centrifugation procedure was repeated 10 times, and all anthocyanin extracts were added together. After the extracts were filtered with a disposable PTFE filter (0.45 μm), the release of anthocyanins from encapsulated samples was measured using HPLC. Samples were measured in duplicate.

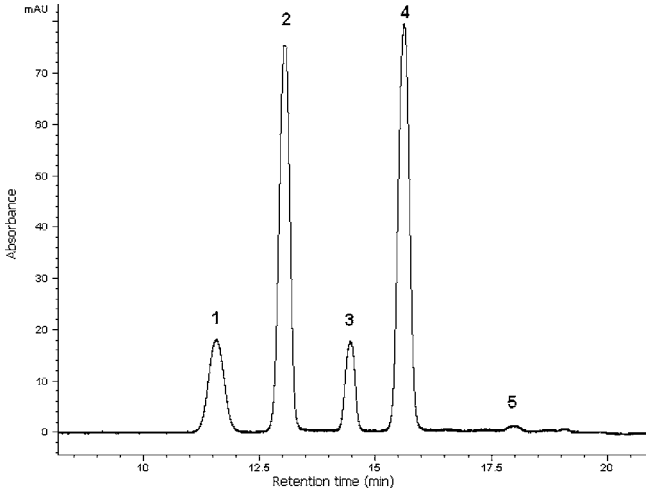

Figure 3. HPLC chromatogram of BCAs with detection at 520 nm. Compounds corresponding to the peaks are **1**, **2**, **3**, **4**, and petunidin 3-O-rutinoside (**5**).

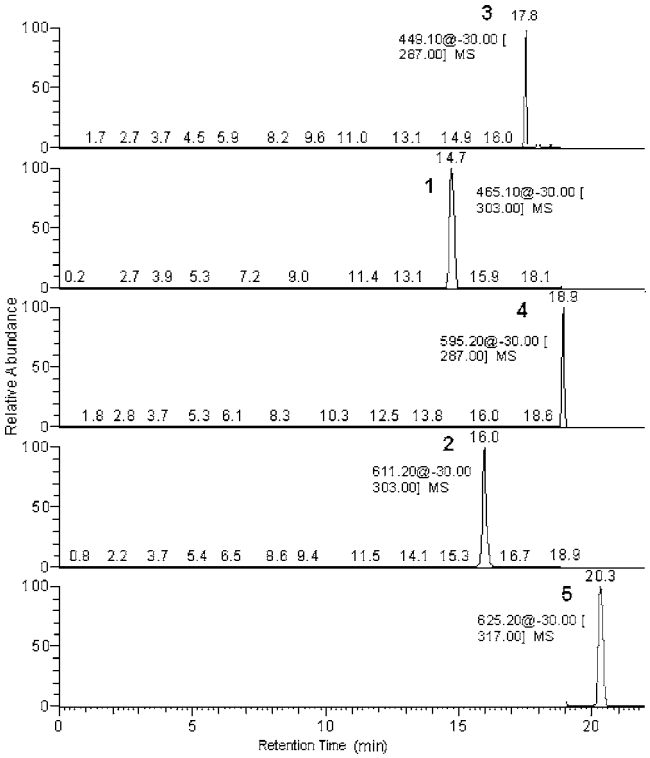

Figure 4. Product ion mass spectra measured using HPLC/QTOF-MS. Compounds corresponding to the peaks are **1**, **2**, **3**, **4**, and petunidin 3-O-rutinoside (**5**).

RESULTS AND DISCUSSION

Identification of Individual Anthocyanins from BCAs. Both the HPLC chromatogram (**Figure 3**) and the liquid chromatography−mass spectrometry (LC-MS) showed that the black currant extract contains four major anthocyanin components and one minor component. Mass spectrometry was used to identify the components from the molecular masses of the parent ions and the measured molecular masses of the reacting fragment ions corresponding to the peaks detected by PDA online LC-MS (**Figure 4**). The slightly different elution profile and void volumes in the HPLC-PDA and HPLC-QTOF systems caused the differences in retention times shown in **Figures 3** and **4**. The fragment ions generated represented corresponding aglycon ions of the anthocyanins after loss of the sugar moiety

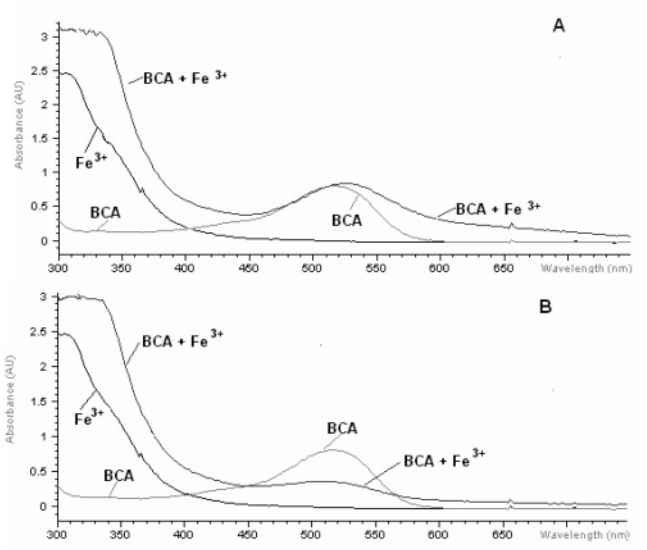

Figure 5. UV–vis spectra in 2.2 M formic acid of (**A**) BCA (1.0 mg mL^{-1}, 0.9 mL) + H_2O (0.1 mL), BCA (1.0 mg mL^{-1}, 0.9 mL) + Fe^{3+} (5×10^{-2} mol L^{-1}, 0.1 mL), and Fe^{3+} (5×10^{-2} mol L^{-1}, 0.1 mL) + 2.2 M formic acid (0.9 mL), measured immediately after mixing; (**B**) BCA (1.0 mg mL^{-1}, 0.9 mL) + H_2O (0.1 mL), BCA (1.0 mg mL^{-1}, 0.9 mL) + Fe^{3+} (5×10^{-2} mol L^{-1}, 0.1 mL), and Fe^{3+} (5×10^{-2} mol L^{-1}, 0.1 mL) + 2.2 M formic acid (0.9 mL), measured 15 min after mixing.

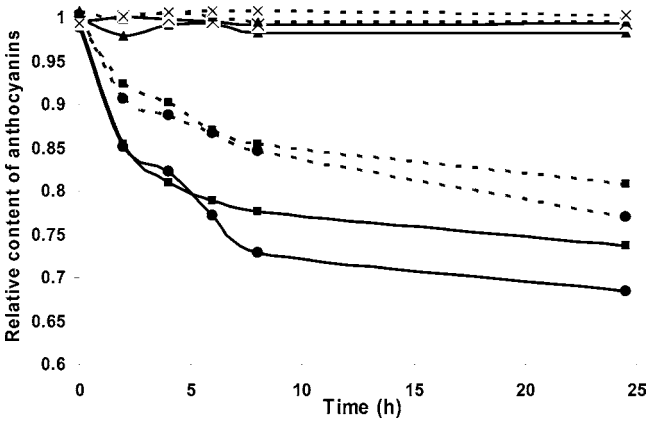

Figure 6. Effect of ferric ion (5×10^{-5} mol L^{-1}) on the stability of BCA. The following four are without a nitrogen purge (represented by solid lines): Del-3-O-G, ●; Del-3-O-R, ■; Cyn-3-O-G, ▲; and Cyn-3-O-R, ×. The following four are with a nitrogen purge (represented by dashed lines): Del-3-O-G, ●; Del-3-O-R, ■; Cyn-3-O-G, ▲; and Cyn-3-O-R, ×. The standard deviation of triplicate determinations was ≤1.5%.

from the parent ions. The data established that the BCA used in this study contains Del-3-O-G, Del-3-O-R, Cyn-3-O-G, and Cyn-3-O-R as major components and petunidin 3-O-rutinoside (**Figure 1**) as a minor component. The results for the four major components of BCA are consistent with previous reports (*3*, *14*, *19*) in terms of order of elution, absorption spectrum, and mass and fragments ions.

Effect of Ferric and Ferrous Ion on Stability of BCA. Jurd and Asen (*20*) proposed that Cyn-3-O-G forms stable complexes with ferric, ferrous, and aluminum ions at pH > 5.5. However, their experimental data were given only for aluminum ions and showed rapid decomposition of the complex. It is generally considered that the change in color of anthocyanins in the presence of metallic ions is due to the formation of metal−anthocyanin complexes, especially when the anthocyanins contain an *ortho*-dihydroxyl in the B ring.

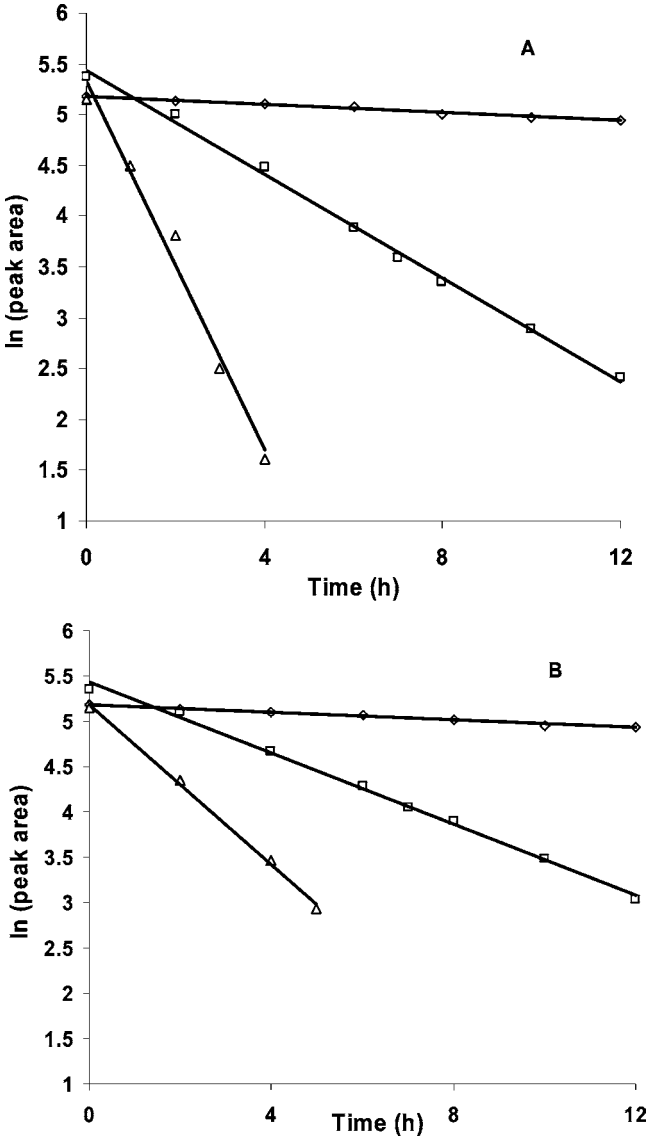

Figure 7. First-order plots for the degradation of Del-3-O-G from black currant extract at 60 (◇), 80 (□), and 100 °C (△) at (**A**) pH 2.4 and (**B**) pH 3.6. The standard deviation of duplicate determinations was ≤1%.

In this study, PTFE filters (0.45 μm) in a stainless steel holder were initially used for filtering anthocyanin solutions prior to HPLC analysis. Irregular changes in peak areas of delphinidins were observed during every HPLC run, presumably due to traces of ferric ions leached from the filter holder into the anthocyanin solutions during filtration. Because traces of ferric and ferrous ions from industrial processing equipment could find their way into anthocyanin solutions, the effects of ferric and ferrous ions on anthocyanins were investigated. The addition of ferric ions (5×10^{-2} M, 0.1 mL) to BCA solution (1 mg mL^{-1}, 0.9 mL) in 2.2 M formic acid caused an immediate color change from red to purple, indicating the formation of either a metal−anthocyanin complex or a transitory oxidation product. Comparison with the spectra of anthocyanins with no added ferric ions, exhibiting an absorption maximum at 518 μm, revealed a small bathochromic shift to λ_{max} of 524 μm for the purple mixture (**Figure 5**). The absorbance markedly decreased within 15 min with λ_{max} shifting progres-

Table 2. Degradation Reaction Rate Constants (k) and Standard Errors of the Slope (s_k), Half-Life Periods ($t_{1/2}$), Activation Energies (E_a), and Correlation Coefficients (in Parentheses) for the Major Anthocyanins from Black Currant Extract Incubated at Different pH Values and Temperatures

		pH 2.4	pH 3.6	pH 4.5	pH 5.5	pH 6.8
			Del-3-O-G			
60 °C	$10^3(k \pm s_k)$ (h^{-1})	20.1 ± 1.2 (0.981)	21.3 ± 1.2 (0.985)	35.2 ± 1.0 (0.996)	324 ± 15 (0.991)	2047 ± 398 (0.930)
	$t_{1/2}$ (h)	34.5	32.5	19.7	2.1	0.34
80 °C	$10^3(k \pm s_k)$ (h^{-1})	255 ± 5 (0.997)	196 ± 4 (0.997)	231 ± 12 (0.983)	877 ± 19 (0.992)	3098 ± 127 (0.998)
	$t_{1/2}$ (h)	2.7	3.5	3.0	0.8	0.22
100 °C	$10^3(k \pm s_k)$ (h^{-1})	910 ± 69 (0.983)	440 ± 16 (0.997)	535 ± 16 (0.997)	1414 ± 91 (0.992)	6160 ± 231 (0.998)
	$t_{1/2}$ (h)	0.76	1.6	1.3	0.5	0.11
	E_a (kJ mol^{-1})	98.9 ± 15.7 (0.943)	78.8 ± 18.3 (0.948)	70.7 ± 13.2 (0.966)	38.3 ± 6.5 (0.972)	28.3 ± 5.0 (0.961)
			Del-3-O-R			
60 °C	$10^3(k \pm s_k)$ (h^{-1})	19.0 ± 1.0 (0.987)	19.2 ± 0.7 (0.993)	31.1 ± 0.9 (0.996)	302 ± 20 (0.983)	1827 ± 247 (0.948)
	$t_{1/2}$ (h)	36.5	36.1	22.3	2.3	0.38
80 °C	$10^3(k \pm s_k)$ (h^{-1})	169 ± 8 (0.987)	143 ± 6 (0.991)	202 ± 7 (0.970)	622 ± 63 (0.993)	3564 ± 62 (0.993)
	$t_{1/2}$ (h)	4.1	4.9	3.4	1.11	0.19
100 °C	$10^3(k \pm s_k)$ (h^{-1})	491 ± 14 (0.991)	284 ± 15 (0.989)	372 ± 11 (0.997)	1051 ± 70 (0.987)	4457 ± 60 (0.999)
	$t_{1/2}$ (h)	1.4	2.5	1.9	0.66	0.16
	E_a (kJ mol^{-1})	84.4 ± 13.8 (0.974)	70.1 ± 17.3 (0.943)	64.6 ± 16.6 (0.938)	32.2 ± 1.9 (0.997)	23.3 ± 5.9 (0.860)
			Cyn-3-O-G			
60 °C	$10^3(k \pm s_k)$ (h^{-1})	4.7 ± 0.7 (0.889)	10.3 ± 0.4 (0.994)	16.9 ± 0.8 (0.990)	66.7 ± 2.2 (0.995)	340 ± 10 (0.992)
	$t_{1/2}$ (h)	147.5	67.3	41.0	10.4	2.0
80 °C	$10^3(k \pm s_k)$ (h^{-1})	45 ± 7 (0.899)	97 ± 5 (0.986)	116 ± 3 (0.995)	367 ± 18 (0.988)	1119 ± 60 (0.991)
	$t_{1/2}$ (h)	15.4	7.1	6.0	1.9	0.62
100 °C	$10^3(k \pm s_k)$ (h^{-1})	259 ± 10 (0.992)	333 ± 13 (0.994)	403 ± 11 (0.998)	593 ± 19 (0.997)	1994 ± 259 (0.967)
	$t_{1/2}$ (h)	2.7	2.1	1.72	1.2	0.35
	E_a (kJ mol^{-1})	125 ± 12 (0.998)	90.1 ± 12.2 (0.982)	82.1 ± 7.4 (0.991)	56.9 ± 12.2 (0.923)	45.9 ± 7.6 (0.973)
			Cyn-3-O-R			
60 °C	$10^3(k \pm s_k)$ (h^{-1})	1.85 ± 0.5 (0.888)	8.2 ± 0.5 (0.980)	14.4 ± 1.2 (0.966)	59.8 ± 1.5 (0.998)	342.1 ± 4.3 (0.997)
	$t_{1/2}$ (h)	374.7	84.5	48.1	11.6	4.3
80 °C	$10^3(k \pm s_k)$ (h^{-1})	32 ± 5 (0.905)	63 ± 4 (0.977)	64 ± 8 (0.934)	179 ± 13 (0.973)	1093 ± 48 (0.986)
	$t_{1/2}$ (h)	21.5	11.0	10.8	3.9	0.64
100 °C	$10^3(k \pm s_k)$ (h^{-1})	236 ± 14 (0.982)	231 ± 11 (0.989)	318 ± 13 (0.993)	494 ± 36 (0.979)	1600 ± 119 (0.984)
	$t_{1/2}$ (h)	2.94	3.0	2.18	1.4	0.43
	E_a (kJ mol^{-1})	125.5 ± 8.9 (0.990)	86.5 ± 8.3 (0.991)	79.8 ± 4.2 (0.993)	54.5 ± 0.5 (0.990)	40.2 ± 10.3 (0.938)

sively from 524 to 510 μm (**Figure 5B**), indicating that the anthocyanin content had rapidly declined in the 15 min time interval.

The anthocyanin content measured by HPLC immediately after mixing of BCA and ferric ion showed that the peaks for delphinidins had vanished and about 82% of the cyanidins remained. The HPLC results confirmed the rapid decrease in absorbance, measured using a diode array spectrometer, in the first 15 min. In the presence of a lower ferric ion concentration (5×10^{-5} mol L^{-1}), the relative content of delphinidins decreased with time whereas little change occurred in the relative content of cyanidins (**Figure 6**). Flushing with nitrogen reduced the rate of degradation of delphinidins (**Figure 6**). In contrast, the spectra and absorbance of BCA in the presence of ferrous ions (5×10^{-3} mol L^{-1}) did not change with time for a period of 12 h (data not shown). These observations suggest that the degradation of BCAs in the presence of ferric ions can be attributed to a redox reaction involving ferric ions and BCA. Ferric ions probably act as an initiator or catalyst in the presence of oxygen. While delphinidins and cyanindins have the same backbone structure, the additional phenolic hydroxy groups in the B ring (**Figure 1**) may facilitate degradation of delphinidins. The electron-donating ability of hydroxy groups confers a stronger antioxidant capability on delphinidins as compared to cyanindins. Other studies have observed that the antioxidant capacity increases with an increase in the number of phenolic hydroxyl groups in the B ring (*21*).

Stability of BCA as a Function of pH and Temperature. The ln(peak area) vs time data (from HPLC) of each individual BCA, at different temperatures, fit first-order reaction kinetics. The data for Del-3-O-G at pH 2.4 and 3.6, incubated at 60, 80, and 100 °C, are shown in **Figure 7**. Rate constants were evaluated as the slope of ln[peak area(t)/peak area(t_0)] vs time plots, where the peak areas at zero time t_0 and time t were obtained using HPLC.

Table 2 summarizes the experimentally determined values of the degradation rate constant (k), the half-life ($t_{1/2}$, calculated as $-\ln 0.5/k$), the activation energy (E_a, estimated using the Arrhenius equation), and the correlation coefficients for the four major anthocyanins from black currant extract incubated at a range of pH values and temperatures. The observation of first-order kinetics for the degradation of anthocyanins is in agreement with other studies on anthocyanins from *H. sabdariffa* L. (*10*) and blood orange (*22*). As far as we are aware, the $t_{1/2}$ values and activation energies for the individual anthocyanins (Del-3-O-G, Del-3-O-R, Cyn-3-O-G, and Cyn-3-O-R) have not been reported previously. Most studies on degradation kinetics of anthocyanins have been carried out on fruit juice rather than an extract. For example, Kirca and Cemeroglu (*22*) reported $t_{1/2} = 3.6$ h at pH 3.44 and 80 °C and $E_a = 73.6$ kJ mol^{-1} (from 70 to 90 °C) for anthocyanins in blood orange juice in which Cyn-3-O-G accounted for 50% of the total anthocyanins.

As expected, anthocyanin degradation increased with temperature and time. **Table 2** also shows that the same aglycon is more stable when linked to rutinoside than to glucoside at the same pH and temperature, which may be attributable to the larger sugar moiety having greater protection against nucleophilic attack by water on C-2 in the C ring. Comparison of rate constants for degradation of aglycons with the same sugar moiety at corresponding pH and temperature indicates that cyanidin O-sugars are considerably more stable than the corresponding delphinidin O-sugars. The results are consistent with other dynamic studies (*7*), indicating that decolorization

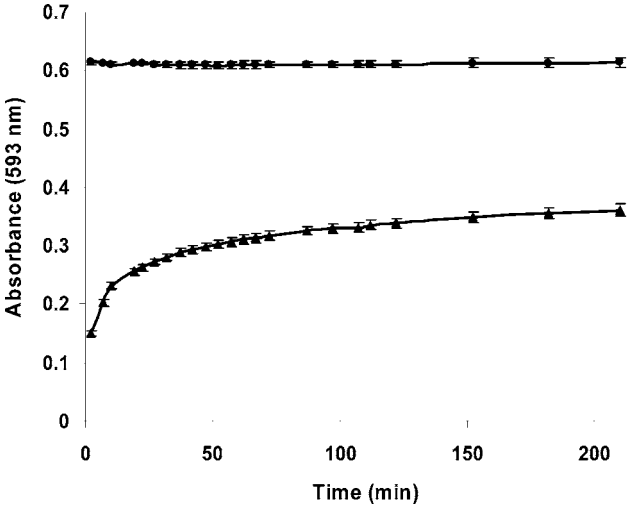

Figure 8. FRAP reaction kinetics of ferrous ions (1.0 mmol L^{-1}) (●) and BCA (1.0 mg mL^{-1}) (▲). The determinations were in triplicate.

of anthocyanins during heating is favored through nucleophilic attack by water on C-2 giving the pseudobase form. The flavonoid structure is then opened to form chalcone, which is degraded further to a brown product in the presence of oxygen. Because C-2 of the delphinidin aglycon has a higher partial charge than for the cyanindins (*23*), nucleophilic attack by water on C-2 of delphinidin aglycon will be faster than for cyanidin aglycon. However, the lower E_a for delphinidin O-sugars than for corresponding cyanidin O-sugars in the pH range 2.4−6.8 shows that the effect of temperature on the stability of cyanidin O-sugars is larger than for the corresponding delphindin O-sugars.

The pH effect on the stability of Cyn-3-O-G and Cyn-3-O-R showed that the rate of decomposition increased slowly with an increase in pH from 2.4 to 5.5 and rapidly with a further increase in pH. For the Del-3-O-G and Del-3-O-R, a similar trend was observed in the pH range 3.6−6.8. However, the decline in Del O-sugar content was slightly higher at pH 2.4 than at pH 3.6, at 80 and 100 °C. It seems that at pH 3.6, 4.5, and 5.5, buffer solution constituents such as acetic acid may react with anthocyanins at elevated temperature, producing acylated anthocyanins, which have a stabilizing effect (*24*). The appearance of a new peak at 1740 cm^{-1} (ester C═O stretching vibration) in the FTIR spectrum was evidence for the formation of acylated anthocyanins.

Antioxidant Activity of Black Currant Berry Extract Determined Using FRAP Assay. The absorbance at 593 nm for mixtures of the FRAP reagent and BCA increased with time, whereas a mixture of the FRAP reagent and ferrous ions gave constant absorbance after 2 min (**Figure 8**). Absorbance was measured in the FRAP assay 50 min after mixing FRAP reagent and BCA samples, because after 50 min the absorbance was changing slowly and had increased to more than 80% of the value at the plateau region (∼200 min). A time-dependent increase in the ferric reducing ability has been reported in a study of antioxidant activity of polyphenols (*25*).

Figure 9 shows that the ferric reducing ability of BCA at pH 2.4−4.5 slowly decreased during heating at 60 and 80 °C for 12 h. The loss of ferric reducing ability was more rapid at higher pH and increased temperature; BCA lost about 40 and 55% of the initial antioxidant activity at pH 6.8 in 2 h at 80 and 100 °C, respectively. Although no anthocyanin peaks were detected by HPLC after heating for 6 h at 80 °C and 4 h at 100 °C at pH 6.8, those solutions had about 30% of the initial antioxidant activity, which clearly indicates that the degradation products of BCA exhibit significant ferric reducing ability.

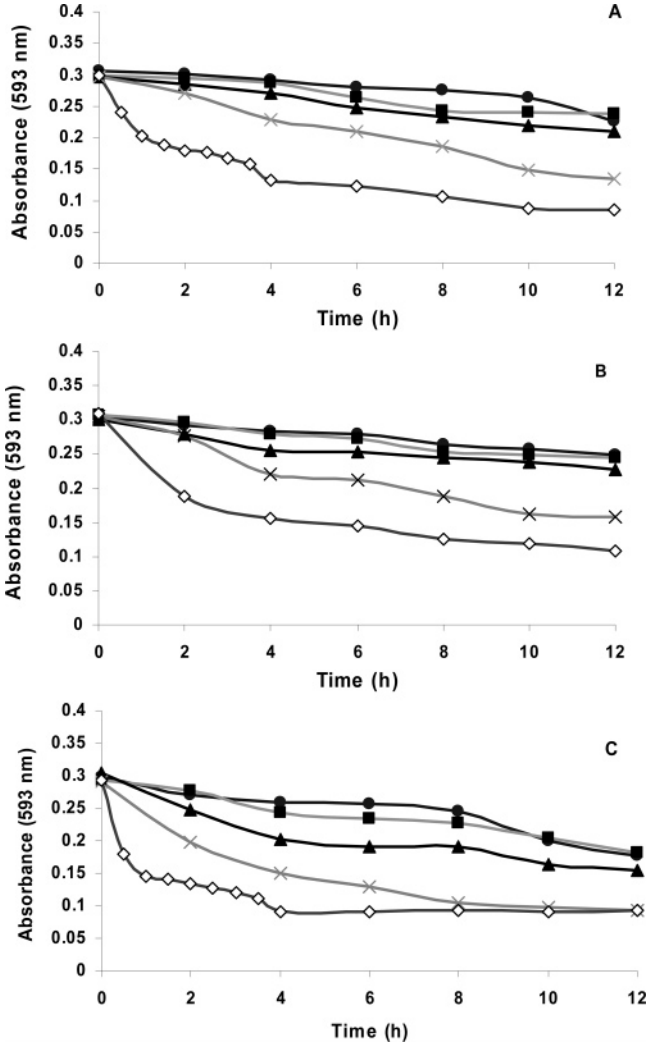

Figure 9. Antioxidant activity of black currant extract anthocyanins at (**A**) 60, (**B**) 80, and (**C**) 100 °C with pH 2.4 (●), 3.6 (■), 4.5 (▲), 5.5 (×), and 6.8 (◇) determined using the FRAP assay. The standard deviation of triplicate determinations was ≤2%.

Table 3 gives the relative amount of anthocyanins and relative antioxidant activity at a range of pH values and temperatures. The change in relative antioxidant activity is more rapid than the decrease in relative amount of anthocyanins at pH 2.4−4.5 at 60 °C. At higher pH and temperature, however, the rate of degradation of anthocyanins is much higher with a relatively slow decrease in antioxidant activity. The results suggest first that more than one breakdown product was produced, at least one of which has antioxidant activity. Second, individual anthocyanins have different contributions to ferric reducing ability in the FRAP assay. During heating, individual anthocyanins degraded with different rates, resulting in the differing changes in the relative amounts of anthocyanins and relative antioxidant activity.

Encapsulation and Release of Anthocyanins. Four drying processes were used to produce BCA encapsulated in glucan cubes and beads. Immediately after drying, the release of anthocyanins was determined using HPLC. **Table 4** shows the extent of release of the anthocyanins from the dried cubes or beads into pH 1.2 HCl/KCl solution. Larger amounts of anthocyanins were released from glucagel-BCA cubes than from

Table 3. Time Dependence of the Relative Amount of Anthocyanins Determined Using HPLC, and Relative Antioxidant Activity by FRAP Assay, as a Function of pH and Temperature

		relative amount of anthocyanins[a]				
temp (°C)	time (h)	pH 2.4	pH 3.6	pH 4.5	pH 5.5	pH 6.8
60	0	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)
	2	0.979 (0.960)	0.976 (0.968)	0.938 (0.928)	0.703 (0.905)	0.305 (0.609)
	4	0.968 (0.928)	0.952 (0.908)	0.902 (0.848)	0.562 (0.726)	0.151 (0.502)
	6	0.942 (0.916)	0.924 (0.889)	0.864 (0.839)	0.451 (0.692)	0.069 (0.467)
	8	0.926 (0.854)	0.895 (0.823)	0.820 (0.814)	0.360 (0.617)	0.033 (0.404)
	10	0.905 (0.847)	0.870 (0.811)	0.799 (0.786)	0.291 (0.530)	0.016 (0.387)
	12	0.878 (0.818)	0.847 (0.795)	0.745 (0.750)	0.234 (0.515)	0.008 (0.354)
80	0	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)
	2	0.933 (0.983)	0.951 (0.983)	0.889 (0.961)	0.684 (0.917)	0.073 (0.594)
	4	0.899 (0.956)	0.806 (0.958)	0.791 (0.914)	0.493 (0.769)	0.005 (0.437)
	6	0.792 (0.927)	0.781 (0.877)	0.699 (0.833)	0.339 (0.710)	0.00 (0.413)
	8	0.658 (0.900)	0.665 (0.810)	0.547 (0.784)	0.141 (0.627)	0.00 (0.353)
	10	0.539 (0.857)	0.524 (0.798)	0.403 (0.736)	0.067 (0.502)	0.00 (0.288)
	12	0.438 (0.736)	0.470 (0.792)	0.316 (0.709)	0.058 (0.452)	0.00 (0.285)
100	0	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)	1.0 (1.0)
	2	0.544 (0.909)	0.628 (0.940)	0.507 (0.814)	0.371 (0.677)	0.017 (0.456)
	4	0.330 (0.870)	0.416 (0.829)	0.280 (0.659)	0.112 (0.512)	0.00 (0.311)
	6	0.164 (0.864)	0.209 (0.799)	0.113 (0.624)	0.026 (0.440)	0.00 (0.313)
	8	0.082 (0.826)	0.083 (0.769)	0.037 (0.623)	0.0 (0.357)	0.00 (0.321)
	10	0.051 (0.672)	0.052 (0.697)	0.018 (0.540)	0.0 (0.338)	0.00 (0.308)
	12	0.024 (0.591)	0.027 (0.620)	0.00 (0.505)	0.0 (0.316)	0.00 (0.319)

[a] The relative antioxidant activity is given in parentheses. Determinations were carried out in duplicate and triplicate with standard deviations ≤1.5 and ≤2.0%, respectively.

Table 4. Recovery of Anthocyanins from BCA Encapsulated in Glucan after Various Drying Procedures[a]

	recovery (%)			
	vacuum drying	oven drying	infrared drying	freeze drying
	cubes			
Del-3-O-G	83.1	72.8	80.7	NP
Del-3-O-R	86.2	77.8	84.7	NP
Cyn3-O-G	83.8	73.7	81.4	NP
Cyn3-O-R	85.9	76.7	84.1	NP
	beads made in soybean oil			
Del-3-O-G	45.7	31.7	NP	NP
Del-3-O-R	47.0	33.3	NP	NP
Cyn3-O-G	39.5	25.4	NP	NP
Cyn3-O-R	40.4	27.3	NP	NP
	beads made in Ondina oil 68			
Del-3-O-G	77.2	75.6	61.3	83.3
Del-3-O-R	78.4	78.7	63.4	82.3
Cyn3-O-G	76.8	72.9	57.6	85.8
Cyn3-O-R	78.4	76.5	61.0	84.4

[a] All determinations were carried out in duplicate and had standard deviations ≤3%; NP, not performed.

glucagel-BCA beads using the same drying process. From 25 to 33% of the anthocyanins were recovered (i.e., released) from the beads produced in soybean oil after infrared drying and from 40 to 47% after vacuum oven drying, indicating that anthocyanins act as primary antioxidants as well as superoxide anion scavengers (*26*). The implication is that anthocyanin was depleted through reaction with lipid, alkoxy, and peroxy radicals as a result of lipid oxidation. Interestingly, the recovery of individual anthocyanins from the beads produced in soybean oil was not significantly different, implying that individual anthocyanins have approximately equal ability as free radical scavengers. By contrast, Ondina oil 68 does not contain such free radicals, and the recovery of anthocyanins from beads produced in Ondina oil 68 was slightly lower than from glucagel-BCA gel cubes. Although the anthocyanins were predominantly present as AH^+ species at low pH, during encapsulation and drying, a proportion of the anthocyanins may be converted to the pseudobase form, which probably became dispersed in the oil phase. Comparison of the experimental data obtained using different drying processes suggests that infrared radiation reduced the stability of BCA. Considering the effects of all processing parameters including temperature, light, and oxygen, we conclude that freeze drying is the best way to preserve anthocyanins, followed by vacuum oven drying and oven drying.

In conclusion, we have observed that in the presence of ferric ions the spectrum of BCA has a bathochromic shift of 6 nm, relative to BCA alone. The formation of ferric−anthocyanins complex accelerates the degradation of the delphinidin components but has little effect on the stability of the cyanidins. We suggest that the degradation of BCA in the presence of ferric ions can be attributed to oxidation−reduction. In regard to thermal stability of BCA at different pH values, the larger the sugar moiety attached to aglycon is, the more thermally stable the anthocyanin is. Moreover, the stability of the anthocyanins on heating also depends on the number of hydroxy groups in the B ring; the cyanidin O-sugars are more stable than the corresponding delphinidin O-sugars at a constant temperature. However, the degradation of cyanidin O-sugars is more sensitive to a change in temperature than the corresponding delphinidin O-sugars.

Recovery of 73−79% of encapsulated anthocyanins was achieved using normal oven drying to dehydrate the gel matrix. A still higher recovery can be achieved with vacuum oven drying and freeze drying. The low recovery rate from encapsulated BCA using soybean oil is likely to be due to the reaction of anthocyanins with radicals as a result of lipid oxidation. However, the similar recovery of individual anthocyanins indicates that the four major anthocyanins in black currant extract exhibit little difference as free radical scavengers. Moreover, BCAs show increased reducing ability with time, and the degradation products retain antioxidant ability. Encapsulation of anthocyanins with natural polymers, followed by appropriate processing, may enhance the stability of anthocyanins for efficient utilization in food systems.

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