

Characterization of Antioxidant Capacity from Fruits with Distinct Anthocyanin Biosynthetic Pathways

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Abstract

The antioxidant capacity of individual anthocyanins is well established. Less information is available however, about the relative contribution to which specific anthocyanins in a complex mixture affect total antioxidant capacity in different soft fruit sources; especially those that share a similar pathway for anthocyanin synthesis. The objectives of this work were to compare the antioxidant capacity of two different soft fruits, blackcurrant and grape, which share similarities in anthocyanin biosynthetic pathways but are composed of distinctly different anthocyanin profiles.

Anthocyanin composition profiles of grape and blackcurrant were characterized by High Performance Liquid Chromatography/Mass Spectrometry (HPLC). ORAC (Oxygen Radical Absorbance Capacity) and ABTS (2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) assays were used for antioxidant activity quantification. An anthocyanin antioxidant capacity index (AACI) was derived from the product of antioxidant (ORAC) activity for each of major anthocyanins present in blackcurrant and grape, and the sum of anthocyanins recovered from purified fruit extracts to determine the extent that the total antioxidant activity derived from different anthocyanin combinations.

Blackcurrant contained four predominant anthocyanins, cyanidin3-glucoside (Cy3G), delphinidin3-glucoside (Dp3G), cyanidin3-rutinoside (Cy3R), and delphinidin3-rutinoside (Dp3R). Major anthocyanins found in grape were malvidin3-glucoside (Mv3G), Dp3G, Cy3G, petunidin3-glucoside (Pt3G), and peonidin3-glucoside (Pn3G). A greater ($p < 0.05$) total antioxidant capacity existed for blackcurrant compared to grape when measured by ORAC and ABTS methods. An antioxidant synergy was confirmed for blackcurrant and wine grape thus indicating that this phenomenon is a factor for characterizing total antioxidant activity in both blackcurrant and wine grape.

Keywords: Blackcurrant; Grape; Anthocyanin; Antioxidant synergy

Introduction

Anthocyanins are a diverse group of over 600 naturally occurring flavonoid compounds conferring hues of blue, purple, or deep red to several plants, including fruits of several commercially important species. The presence and relative abundance of different anthocyanins have been previously determined for several commercially important fruits [1-4]. Anthocyanins possess potent antioxidant activity as scavengers of reactive oxygen (ROS) and nitrogen (RNS) species, such as superoxide, nitric oxide, hydroxyl and peroxy radicals and hydrogen peroxide [5-7]; which in many cases surpasses that of ascorbic acid [8] and the vitamin E analogue, Trolox [5,7,9]. Numerous reports have quantitated the total antioxidant capacities of anthocyanin containing extracts derived from various fruit and plant materials in general [3,4,8-11]. In comparison, there is relatively little information on the contribution that individual anthocyanins in a complex mixture, governed by subtle differences in flavonoid biosynthesis, have on the antioxidant quality of the fruit source. Structure-antioxidant activity relationships, such as the presence of free hydroxyl groups located at the 3' and 4' positions of the B-ring of anthocyanidins have been shown to be important for Trolox equivalent antioxidant capacity (TEAC) and radical scavenging activity in low density lipoprotein oxidation assays [12-14]. This activity may in part be attributed to the enhanced stabilization of the radical state during electron transfer when assayed relative to compounds that lacked the orthodiphenolic structure [2,12-14]. Further, the addition of a glycoside residue (e.g. rutinose, glucose) at position 3 on the C-ring or methylation of the 3' and/or 5' hydroxyl group on the anthocyanidin B-ring has been shown to reduce Trolox

equivalent antioxidant capacity and radical scavenging activity [2,8,12-14].

Interactions between different plant polyphenolics have yielded antioxidant activity owed to antagonism or synergism [15,16]; however, only a very limited number of studies have investigated combinations of purified anthocyanin compounds and potential synergistic effects on antioxidant capacity. For example, an approximate four-fold increase in superoxide radical scavenging activity was reported for an artificial reconstitution of a mix of bilberry (*Vaccinium myrtillus* L.) anthocyanins in comparison to the sum of individually characterized activities of each constituent anthocyanin, indicating synergism [17]. Antioxidant assays in the bilberry study were carried out at neutral pH, which may not have reflected the native structures of the anthocyanins tested.

In this study, we report on the relative antioxidant activities for naturally occurring anthocyanidin-glycosides in two distinct anthocyanin rich fruit sources, namely blackcurrant (*Ribes nigrum*)

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and wine grape (*Vitis vinifera* L.). Blackcurrant is a rich source of delphinidin 3-rutinoside, compared to wine grape which is dominant in malvidin 3-glucoside. The aim of this study was to compare the antioxidant capacity of these two fruit sources of anthocyanins that possess a common anthocyanin biosynthesis pathway, but have distinctly different anthocyanin compositional mixtures. In particular, we have attempted to determine if the *in vitro* antioxidant behaviour for blackcurrant and wine grape could in part be attributed to a synergism between the different mixtures of anthocyanins as observed previously for bilberry [17].

Materials and Methods

Plant materials

Four different cultivars of blackcurrant (*Ribes nigrum*) were obtained from Agriculture Canada (Dr. Chris Nesson, Alberta, Canada). The cultivars included, *Ben Alder*, *Ben Nevis* cultivated in Edmonton Alberta, *Ben Sarek* cultivars derived from Red Deer Alberta and *Lentiyay* which was grown in Brooks, Alberta. Berries from three different grape (*Vitis vinifera* L.) cultivars, *Cabernet Sauvignon*, *Merlot*, and *Pinot Noir*, were obtained from a commercial vineyard near Osoyoos, BC (Canada). For all collections, berries were snap-frozen in liquid nitrogen in the field and then transported on dry ice for storage at UBC Vancouver at -80°C.

Reagents

Methanol, ethyl acetate, hydrochloric acid, formic acid, sodium acetate and sodium carbonate were obtained from Fisher Scientific Canada (Ottawa, ON). Potassium persulphate, potassium chloride, 2, 2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and gallic acid were purchased from Sigma Co. (St. Louis, MO). Fluorescein, Trolox and 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). Folin Ciocalteu reagent was from Fluka (Sigma-Aldrich Canada Ltd., Oakville, ON). HPLC-grade Cy3G standard was purchased from Polyphenols Laboratories AS (Sandnes, Norway). Dp3G and Cy3R, Cy3G, Mv3G, and Pn3G were obtained from Extrasynthese (Genay, France). Dp3R was purchased from Apin Chemicals Ltd. (Abingdon, Oxon, United Kingdom).

Chemical analysis

General chemistry analysis: A number of general chemical composition analyses were made on both fruit sources to establish base-line differences that may exist within individual fruits as well as with the different cultivars from or each fruit source. Juice samples from thawed berries were analyzed for °Brix measurements in triplicate using a digital refract meter (SPER Scientific, Cole-Parmer, Montreal, QC, Canada) to determine soluble solids. Titratable acidity (TA) was measured by titrating diluted fruit juice with 0.1 M NaOH to a final end-point of pH 8.2. Results are given as gram tartaric acid equivalent/L of fresh fruit juice extract. Sample pH was measured using a Fisher Accumet, Model AB 15, pH meter (Fisher Scientific Ltd., Edmonton, AB, Canada) in triplicate, at room temperature. Two point calibrations were accomplished using pH 4.0 and 7.0 buffers.

Determination of total phenolic content: The total phenolic concentration in the blackcurrant and grape extracts determined in this study was performed using the Folin Ciocalteu procedure [13]. Upon transfer of electrons from the phenolic compounds to

phosphomolybdic/phosphotungstic acid complexes in an alkaline medium, the blue complexes formed were detected spectroscopically at 765 nm. Briefly, 20 µl of sample was mixed with 100 µl of 10-times diluted Folin Ciocalteu reagent and 80 µl sodium carbonate (Na₂CO₃) solution (7.5% w/w). After 10 min of incubation at room temperature, the absorbance was measured at 765 nm. (Multiskan Spectrum, Thermo Labsystem). Samples were calibrated against gallic acid and the total phenol content was expressed as µmol gallic acid equivalent (GAE) per gram freeze-dried sample [18,19].

Total anthocyanin analysis: Samples of lyophilized blackcurrant and grape berries were extracted with 200 ml of methanol/water/hydrochloric acid (80:20:0.05% (v/v), MeOH/H₂O/HCl), for 2 h with constant shaking at 4°C. The resulting extracts were centrifuged at room temperature at 1500 rpm for 20 min and the supernatant removed. The extraction process was repeated three times on the dried powder until the sample became colorless. The collected supernatants were combined and transferred to a 200 ml volumetric flask. Pooled supernatants were brought to a final volume of 200 ml with MeOH/H₂O/HCl and stored at -25°C until further analyses were conducted. The total anthocyanin content in both blackcurrant and wine grape berries was determined spectrophotometrically (UV-1700 Pharma Spec spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA)), using the pH differential method [20]. The maximum absorbance wavelength of crude blackcurrant and grape fruit extracts was 520 nm. The concentration of anthocyanins was calculated using the following formula and expressed in mg/L of Cy3G.

Monomeric anthocyanins pigment (mg/L) = $(A * MW * DF * 1000) / (\epsilon * l)$ Where: A = Absorbance of diluted sample, MW = Molecular weight (g), DF = Dilution Factor, ϵ = Extinction factor (absorption of 1% solution measured through a 1 cm path at the λ vis-max, or molar absorption coefficient.) and l = cell path length (cm). MW and ϵ used in this formula correspond to the predominant anthocyanin in the sample. The MW and ϵ of Cy3G used in this experiment was 449.2 and 26,900 respectively [21].

Individual anthocyanin measurement: Major anthocyanins present in both blackcurrant and grape samples were further identified and quantified using High Performance Liquid Chromatography (HPLC) (Agilent 1100 series; Agilent Technologies Canada Inc., Mississauga, ON), equipped with a diode array detector. Sample extracts, re-dissolved in 95% solvent A (aqueous 5% formic acid) and 5% solvent B (100% methanol) were injected (10 µl) onto a Zorbax RX-C18 column, 5 µm (250 x 4.6mm) (Agilent) that had a flow rate of 1 ml/min. The gradient applied for blackcurrant anthocyanin composition was 95 % A and 5% B going to 15% B over 5 min and then to 25% B for 20 min before returning to, initial solvent conditions after an additional 22 min [2,21]. The gradient applied for grape anthocyanin detection was 95% A and 5% B for 20 minutes going to 35% B for 4 minutes, before returning to the original mobile phase composition [7]. Cy3G (100 µg/ml) was used as the external standard for determining Cy3G equivalents for the separated anthocyanins of interest. Liquid chromatography-mass spectrometry of anthocyanin extracts were also performed to enable accurate anthocyanin fingerprinting necessary to conduct the synergy studies.

Purification of anthocyanin extracts: A pre-conditioned solid phase extraction (SPE) C-18 column (500 mg/4cm; Burdick and Jackson, Honeywell, VWR International, Edmonton, AB, Canada)

was used to obtain purified anthocyanin extracts. Cartridges were preconditioned with 0.01% HCl-methanol, ethyl acetate, and 0.01% HCl-water, respectively, and dried under vacuum. Blackcurrant and grape extracts (200 µl) were loaded onto a pre-conditioned SPE cartridge. Sugars and other water-soluble constituents were eluted in acidified water while phenolic compounds were washed from the column using ethyl acetate [6,7]. Finally, 5 ml of 0.01% HCl-methanol was used to recover the anthocyanin extract. Information on the efficiency of recovering purified anthocyanin mixtures as well as obtaining structural information of individual anthocyanins recovered in the purified extracts derived from both blackcurrant and grape was determined by LC/MSD Trap XCT Plus spectrometry equipped with an electrospray ionization (ESI) interface (Agilent) was used for anthocyanin composition analysis. The separation of anthocyanins was performed using a Zorbax RX-C18 (250 x 4.6 mm i.d, particle size = 5 µm, Agilent) column at 45°C with a linear gradient mobile phase, containing solvent A (aqueous 5% formic acid) and solvent B (100% methanol). The flow rate was 1 ml/min, and detection for anthocyanins was set at 520 nm. The chromatographic conditions were as follows: 95% A and 5% B at the time of injection (10 µl), changed at 5 min to 15% B; in 20 min to 25% B, and finally back to the initial condition in 22 min. Positive ion was applied with the following conditions: Capillary voltage, 3.5 kV; dry temperature, 350°C; nebulizer, 60.0 psi; scan range 40-700 m/z. Anthocyanins were identified by mass profile in comparison to authentic standards (Extasynthese, Genav, France) or inferred from other studies [22,23].

ABTS assay: The ABTS radical scavenging activity test for evaluating the free radical scavenging power of blackcurrant and grape crude extracts was used according to our previous study [9]. The discoloration reaction was measured at 734 nm in a 96-well assay plate using a microplate spectrophotometer (Multiskan Spectrum, Thermo Labsystems, Franklin, MA, USA). The antioxidant capacity was expressed as TEAC and is expressed as µmol Trolox/g freeze-dried sample [24].

ORAC assay: The ORAC assay is the only measurement of antioxidant activity that combines both the percentage of inhibition and the length of inhibition of free radical formation by antioxidants compounds into a single identity. Trolox standard (20 µM), fluorescein (200 nM), and 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (60 mM) solutions were prepared for use in phosphate buffer (75 mM, pH 7.0). The plate was read at excitation and emission wavelengths of 485 and 527 nm, respectively, at 37°C and 1 min intervals for 60 min. (Fluoroskan Ascent FL, Fisher Scientific). A Trolox standard curve was obtained by plotting Trolox concentrations against the average net area under the curve (AUC) of at least three measurements for each concentration [25]. Final ORAC values were expressed as µmol Trolox/g freeze-dried sample.

Anthocyanin Antioxidant Capacity Index (AACI): An anthocyanin antioxidant capacity index (AACI) was developed to evaluate the proportion of total antioxidant capacity measured using ORAC that was influenced by individual anthocyanins contained in each of the fruit extracts. AACI were calculated using the following formula and expressed as µmol Trolox equivalent (TE)/ gram freeze-dried sample:

$$AACI (\mu\text{mol TE}/ \text{g dw}) = \sum (\text{Relative Individual Anthocyanin Concentration in purified extract (mg/g)} \times \text{Individual Standard Anthocyanin ORAC value } (\mu\text{mol TE}/ \text{mg std})).$$

Statistical analysis: Analyses were performed on triplicate sub-samples from each cultivar. Results were expressed as mean ± standard deviation. All statistical analyses were done using the Statistical Package for Social Science (SPSS) for Windows v. 10.0 (SPSS Inc., Chicago, IL, USA). Statistical significance of differences between cultivars was evaluated by one-way analysis of variance (ANOVA) and differences between means were determined using Tukey HSD and the Games Howell test ($p < 0.05$) for homogenous and heterogeneous variances, respectively.

Results

Determination of pH, soluble solids content and titratable acidity

Blackcurrant soluble solids (°Brix) ranged between 12.87 to 15.40% (Table 1). A significant difference ($p < 0.05$) in titratable acidity was found among different blackcurrant cultivars, with *Ben Alder* and *Ben Sarek* having significantly higher titratable acidity compared to *Lentiy* and *Ben Nevis* ($p < 0.05$). There were no significant differences between *Ben Alder* and *Ben Sarek* or *Ben Nevis* and *Lentiy*. The pH of juice extracts derived from different blackcurrant cultivars ranged between 2.75 ± 0.01 to 2.79 ± 0.02 showing no significant difference among cultivars. The soluble solids content of grape juice from different grapevine cultivars was also not significantly different (Table 1). Titratable acidity was not significantly different between *Cabernet Sauvignon* and *Merlot* cultivars, but significantly lower ($p < 0.05$) than the *Pinot Noir* cultivar (Table 1). A Brix × pH² coefficient, commonly used as an indicator of fruit ripeness, ranged from 97 to 117 for different blackcurrant cultivars and 250 to 284 for the different grape cultivars (Table 1). A significant difference ($p < 0.05$) in total phenolic content was found between blackcurrant cultivars (Table 2). *Ben Alder* and *Ben Sarek* both contained significantly ($p < 0.05$) lower total phenolics than *Ben Nevis*. *Lentiy* crude extract contained 94 ± 8 µmol of gallic acid per gram of freeze-dried sample, which was the lowest total phenolic content among other blackcurrant cultivars tested ($p < 0.05$). The total phenolic content in the grape cultivars varied between 107 to 121 µmol gallic acid/g freeze-dried powders and was not significantly different between cultivars (Table 2). Total anthocyanin content of the different blackcurrant cultivars as estimated by the pH differential method

Fruits	Brix (Brix%)	pH	Titratable Acidity (g TA /L) ²	(Brix * pH ²)
Blackcurrant				
Ben Alder	15.40±0.00	2.76 ± 0.11	48.20 ± 0.25 ^a	117
Ben Nevis	14.23±0.06	2.79 ± 0.02	38.80 ± 0.82 ^b	111
Ben Sarek	12.87±0.06	2.75 ± 0.01	49.10 ± 0.24 ^a	97
Lentiy	13.50±0.00	2.76 ± 0.04	39.70 ± 0.80 ^b	103
Grape				
Cabernet Sauvignon	18.06 ± 0.97	3.77 ± 0.02	3.73 ± 0.01 ^a	257
Merlot	18.86 ± 0.96	3.64 ± 0.01	3.72 ± 0.05 ^a	250
Pinot Noir	18.88 ± 2.17	3.88 ± 0.01	4.14 ± 0.10 ^b	284

¹Value represents mean ± SD (n=3). Different superscripted letters indicate significant differences ($p < 0.05$).

²Titratable acidity results are expressed as grams of tartaric acid per liter of fresh fruit extract.

Table1: Soluble solids (°Brix), pH and titratable acidity¹ in blackcurrant and grape samples¹.

ranged from 29 ± 1 to 75 ± 6 mg Cy3G per L of extract (Table 2). *Ben Sarek* and *Lentiy* contained a similar total anthocyanin content, but were both significantly less ($p < 0.05$) than that found in *Ben Nevis* and *Ben Alder* cultivars, respectively. *Ben Nevis* had the highest total anthocyanin content among all cultivars. The total anthocyanin content in wine grapes ranged from 18 to 44 mg Cy3G/L sample extract (Table 2). Grape cultivars, *Cabernet Sauvignon* and *Merlot* contained similar anthocyanin content which was significantly greater ($p < 0.05$) than the anthocyanin content *Pinot Noir*.

Quantification and characterization of anthocyanins contained in blackcurrant and grape crude extract

Anthocyanin profiles were obtained for different extracts from each blackcurrant cultivar using HPLC-UV analyses; a representative chromatogram from the *Ben Alder* cultivar in addition to a table contain quantification of major peaks of blackcurrant is shown in (Table 3). The elution order of the four major anthocyanins in each of the blackcurrant cultivars was Dp3G, followed by Dp3R, Cy3G, and Cy3R, respectively. Quantification of each separate anthocyanin was performed relative to a Cy3G standard concentration. Purification of both blackcurrant and grape extracts for anthocyanin content, and related structural information for individual anthocyanins, was confirmed via LC-MS/Trap spectrometry (Figure 1). The major anthocyanin in three of the cultivars was Dp3R (peak 4), with 42.3% total concentration in *Ben Alder*, 43.0% *Ben Nevis* and 49.3% in *Lentiy* (Table 3). The one exception was *Ben Sarek*, wherein Cy3R (peak 6) was highest (Table 3). Peak 5 (Cy3G) was lowest in all cultivars (Table 3). We also identified trace amounts of petunidin 3-O-rutinoside and peonidin 3-O-rutinoside in blackcurrant, however these anthocyanins were only very minor components of the total mixture and not quantitated. A representative anthocyanin profile from the *Cabernet Sauvignon* grape cultivar also determined using HPLC-UV is shown in (Table 4). All grape anthocyanin extracts exhibited seven distinct peaks and had similar retention times for each. The purity of anthocyanins in each extract along with structural information for individual anthocyanins was confirmed via LC-MS/Trap spectrometry (Figure 2). Relative concentrations of individual anthocyanins in all grape cultivars examined indicated that peak numbers 3, 5, and 7 were major anthocyanins present in *Cabernet Sauvignon* and *Merlot*,

Cultivar	Total phenolic content ($\mu\text{mol GA/g}$) ²	Total Anthocyanin Content (mg Cy3G/L) ³
Blackcurrant		
Ben Alder	137 ± 11^a	61 ± 6^a
Ben Nevis	163 ± 2^b	75 ± 6^b
Ben Sarek	132 ± 4^a	30 ± 1^c
Lentiy	94 ± 8^c	29 ± 1^c
Grape		
Cabernet Sauvignon	117 ± 16^a	44 ± 3^a
Merlot	107 ± 2^a	43 ± 1^a
Pinot Noir	121 ± 12^a	18 ± 2^b

¹Values represent mean \pm SD (n=3). Different superscripted letters indicate significant differences ($p < 0.05$).

²Total phenolic results are expressed as micromole gallic acid per gram of freeze-dried sample.

³Total anthocyanin content is expressed as milligram of equivalent (cyanidin 3-glucoside) per liter extract.

Table 2. Total phenolic ($\mu\text{mol GA/g}$) and total anthocyanin in blackcurrant and grape samples ¹.

Cultivar	Peak	HPLC Retention time (min)	Concentration (mg/g) ¹	Percentage ²
Ben Alder	3	11.94	77.53 ± 5.23	15.00
	4	13.57	218.59 ± 13.63	42.28
	5	14.74	34.59 ± 1.90	6.69
	6	16.93	174.00 ± 12.00	33.65
Ben Nevis	3	11.9	87.57 ± 4.50	14.52
	4	13.5	259.53 ± 12.89	43.04
	5	14.7	37.96 ± 2.89	6.30
	6	16.9	205.72 ± 9.95	34.12
Ben Sarek	3	12.0	49.52 ± 3.30	25.16
	4	13.7	50.40 ± 3.23	25.61
	5	14.8	29.84 ± 1.76	15.16
	6	17.1	63.24 ± 3.70	32.13
Lentiy	3	11.9	37.33 ± 2.10	17.14
	4	13.6	107.28 ± 5.90	49.25
	5	14.8	12.72 ± 0.72	5.84
	6	17.0	56.61 ± 2.79	26.00

¹Results are expressed as milligram equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean \pm SD), (n=3).

²Percentage = Relative % of total concentration.

Table 3: HPLC/UV quantification of major anthocyanins in four different blackcurrant cultivars.

Cultivar	Peak	HPLC Retention time (min)	Concentration (mg/g) ¹	Percentage ²	
Cabernet Sauvignon	3	11.4	19.55 ± 0.09	21.21	
	4	13.6	4.10 ± 0.14	4.45	
	5	15.3	10.47 ± 0.26	11.36	
	6	17.3	8.00 ± 0.16	8.69	
	7	18.4	43.26 ± 1.29	46.92	
	Merlot	3	11.4	28.72 ± 0.49	24.25
		4	13.6	12.57 ± 0.10	10.62
5		15.3	17.28 ± 0.46	14.59	
6		17.3	14.47 ± 0.32	12.22	
Pinot Noir	3	11.6	3.33 ± 0.13	6.12	
	4	13.6	1.85 ± 0.03	3.40	
	5	15.3	3.95 ± 0.06	7.26	
	6	17.4	17.28 ± 0.28	31.79	
	7	18.5	23.71 ± 0.85	43.61	

¹Results are expressed as milligram of fraction equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean \pm SD). n=3.

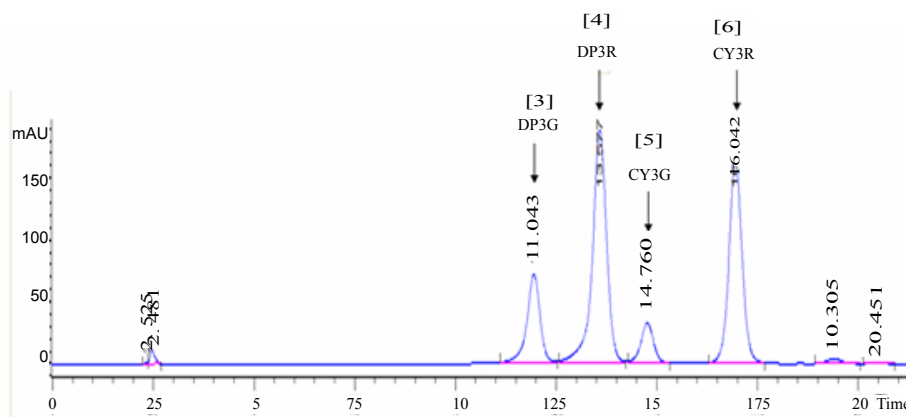
²Percentage = Relative % of total concentration.

Table 4: HPLC/UV quantification of major anthocyanins in three different grape cultivars.

whereas *Pinot Noir* displayed the majority of its anthocyanins in peak numbers 6 and 7 (Table 4). The dominant anthocyanin in all wine grape cultivars was peak 7, which accounted for 46.9, 32.2, and 43.6% of the total anthocyanins analyzed for *Cabernet Sauvignon*, *Merlot*, and *Pinot Noir*, respectively (Table 4). These data indicate that the highest percentage of anthocyanins in each of the grape cultivars was Mv3G, whereas Dp3G was the second most abundant anthocyanin recovered from *Cabernet Sauvignon* and *Merlot*. The exception to this was found with *Pinot Noir*, in which Pn3G was the second most abundant anthocyanin. Other relatively minor anthocyanins present in all grape cultivars were Pn3G and Cy3G.

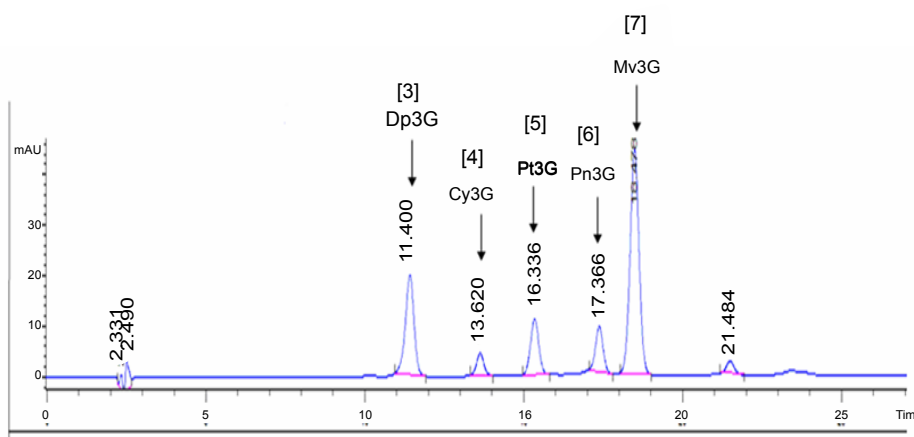
Antioxidant capacity of different fruit crude extracts

ABTS results for blackcurrant crude extracts ranged from 106 \pm



Peak	HPLC-UV (nm)	[M] ⁺ m/z	ESI mode	Fragments, m/z	Retention Time	ID
3	520	465	+	303,223	11.943	Delphinidin 3-glucoside
4	520	611	+	300,283,252	13.577	Delphinidin 3-rutinoside
5	520	449	+	287,162	14.768	Cyanidin 3-glucoside
6	520	595	+	367,344,327	16.942	Cyanidin 3-rutinoside

Figure 1: A typical HPLC/MS chromatogram (520nm) showing anthocyanin separation in blackcurrant (Ben Alder). Dp3G = delphinidin 3-glucoside, Dp3R = delphinidin 3-rutinoside, Cy3G = cyanidin 3-glucoside, Cy3R = cyanidin 3-rutinoside.



Peak	HPLC-UV (nm)	[M] ⁺ m/z	ESI mode	Fragments, m/z	Retention Time	ID
3	520	465	+	303,223	11.409	Delphinidin 3-glucoside
4	520	449	+	358,280	13.620	Cyanidin 3-glucoside
5	520	479	+	358,280,388,342,295,195	15.335	Petunidin 3-glucoside
6	520	463	+	237	17.366	Peonidin 3-glucoside
7	520	493	+	299,372,432	18.478	Malvidin 3-glucoside

Figure 2: A typical HPLC/MS chromatogram (520nm) showing anthocyanin separation in grape (*Cabernet Sauvignon*). Dp3G = delphinidin 3-glucoside, Cy3G = cyanidin 3-glucoside, Pt3G = petunidin 3-glucoside, Pn3G = peonidin 3-glucoside and Mv3G = malvidin 3-glucoside.

6 to 138 ± 8 μmole Trolox per gram of freeze-dried sample (Table 5). The *Ben Nevis* and *Lentiy* cultivars had the highest and lowest ABTS radical quenching activities, respectively, of the four different cultivars

(p<0.05) (Table 5). For grape crude extracts, *Cabernet Sauvignon* produced the greatest ABTS quenching power (p<0.05) compared with both *Merlot* and *Pinot Noir* grape samples (Table 5). Antioxidant

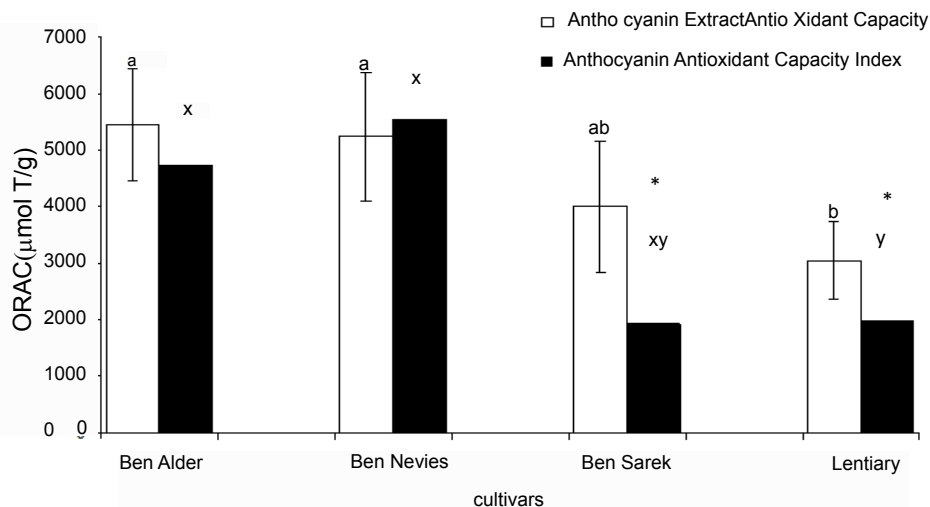


Figure 3: Indication of potential synergy from anthocyanin extract antioxidant capacity and anthocyanin antioxidant capacity index in different cultivars of blackcurrant. Values represent mean \pm SD, (n=3). Different superscripted letters ^{a,b} indicate significant differences in anthocyanin extract antioxidant capacity of different Blackcurrant cultivars while ^{xy} indicate the significant difference in anthocyanin antioxidant capacity index. (p<0.05). * Denotes the significant difference between AAACI and the anthocyanin extract antioxidant capacity.

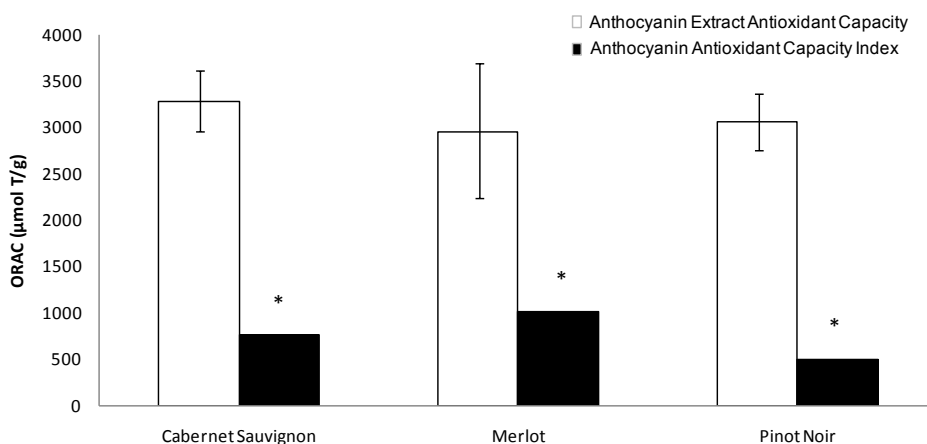


Figure 4: Indication of potential synergy from anthocyanin extract antioxidant capacity and anthocyanin antioxidant capacity index in different cultivars of grape. Values represent mean \pm SD (n=3). *Denotes the significant difference between AAACI and the anthocyanin extract antioxidant capacity.

capacity of blackcurrant crude extracts as measured by ORAC ranged from 620 ± 123 to 828 ± 52 of $\mu\text{mol TE}/\text{gram}$ of freeze-dried blackcurrant (Table 5). Similar to the findings of the ABTS results, the *Ben Nevis* and *Lentiary* crude extracts gave the highest and lowest antioxidant capacities, respectively ($p < 0.05$). In the grape samples, unlike the result obtained with ABTS, no significant difference ($p < 0.05$) was observed among the three grape cultivars tested. ORAC readings for the grape cultivars varied from 520 to 606 $\mu\text{mol Trolox}$ per gram freeze-dried sample (Table 5).

ORAC measurements of antioxidant capacities of purified anthocyanin extracts

The ORAC antioxidant capacity of purified anthocyanin extracts collected from the different blackcurrant cultivars ranged between 3047 ± 690 to 5450 ± 990 $\mu\text{mol TE}$ per gram of freeze-dried sample, with significant differences ($p < 0.05$) found between cultivars. The

antioxidant capacity of the purified anthocyanin extract of *Lentiary* was significantly ($p < 0.05$) lower than other purified anthocyanin extracts collected from *Ben Alder* and *Ben Nevis*. No significant difference was observed in the ORAC activity of *Ben Sarek* and *Lentiary* purified anthocyanin extract antioxidant activity.

For the grape extracts, the three anthocyanin extract antioxidant capacities varied within a narrow range and no significant difference ($p < 0.05$) was found among the purified anthocyanin mixtures from each cultivar (Figures 3 and 4).

ORAC analyses for pure anthocyanin standards

The antioxidant capacities of pure anthocyanin standards representing the specific anthocyanins detected in blackcurrant and wine grape were determined by ORAC analysis. ORAC values ($\mu\text{mol TE}$ per gram of freeze-dried sample), ranged between 4.05 ± 0.27

(Dp3G) to 6.36 ± 0.37 for Cy3G (Table 6). Assays with the cyanidins and peonidin and petunidin 3-glucoside had significantly higher ORAC antioxidant capacities ($p < 0.05$) when compared with delphinidins or malvidin 3-glucoside. No significant difference was observed between same anthocyanins containing different sugar moieties. These data indicate that purified dihydroquercetin-derived anthocyanins (Cy3G, Cy3R, and Pn3G) have higher ORAC antioxidant capacities than dihydromyricetin-derived anthocyanins (Dp3G, Dp3R, and Mv3G, Pt3G).

Results of the anthocyanin antioxidant capacity index (AACI)

The AACI was derived from the antioxidant (ORAC) activity that was determined for each of six anthocyanin standards, and the relative concentrations of corresponding anthocyanins in blackcurrant (Figure 3) or grape, (Figure 4) expressed as Cy3G equivalents. These values were compared to the range of ORAC measures determined from purified anthocyanin extracts in each cultivar as described in Materials and Methods. AACI values fell within the range of ORAC antioxidant measures for purified extracts from the blackcurrant cultivars, *Ben Alder* and *Ben Nevis*, indicating a strong association between the antioxidant capacities of individual anthocyanins in comparison to the sum of individual anthocyanins in the purified extracts. In contrast, the blackcurrant cultivars, *Ben Sarek* and *Lentiy*, gave AACI values that were less than the expected range for the antioxidant capacities of partially purified anthocyanin extracts measured in these cultivars (Figure 3). These data corresponded to a lower percentage of Dp3R in *Ben Sarek* and a lower percentage of Cy3R and Cy3G in *Lentiy*. The AACI values in the grape cultivars showed a significantly lower level than the range of ORAC antioxidant capacity of different anthocyanin (Figure 4).

Potential synergies between anthocyanin components in regards to ORAC antioxidant capacity for each cultivar are also shown in (Figures 3 and 4) for black current and wine grape, respectively. Synergy was predicted by comparing the difference between the AACI and the actual ORAC values for the purified anthocyanin extracts. *Ben Sarek* and *Lentiy* exhibited the greatest potential for synergy of anthocyanin components in purified extracts, compared to *Ben Nevis* and *Ben Alder*, respectively.

Cultivar	Crude Extract	
	ABTS ($\mu\text{mol TE/g}$) ²	ORAC ($\mu\text{mol TE/g}$) ²
Blackcurrant		
Ben Alder	123 ± 2^a	651 ± 27^a
Ben Nevis	138 ± 8^b	828 ± 52^b
Ben Sarek	115 ± 1^{ac}	715 ± 44^c
Lentiy	106 ± 6^c	620 ± 123^{cd}
Grape		
Cabernet Sauvignon	65 ± 2^a	520 ± 78^a
Merlot	53 ± 1^b	535 ± 16^a
Pinot Noir	53 ± 3^b	606 ± 31^a

¹Values represent mean \pm SD (n=3). Different superscripted letters indicate significant differences ($p < 0.05$).

²Results are expressed as micromole of Trolox equivalent per gram of freeze-dried sample.

Table 5: Antioxidant activities of crude extracts from different blackcurrant and grape cultivars¹.

Anthocyanins	ORAC ($\mu\text{mol TE}/\mu\text{mol Std}$) ¹	Relative Capacity ²
Cyanidin 3-glucoside	6.36 ± 0.37^a	1:1
Cyanidin 3-rutinoside	6.06 ± 0.26^a	0.95:1
Delphinidin 3-glucoside	4.05 ± 0.27^b	0.64:1
Delphinidin 3-rutinoside	5.03 ± 0.03^b	0.79:1
Malvidin 3-glucoside	4.52 ± 0.60^b	0.71:1
Petunidin 3-glucoside	5.23 ± 0.78^b	0.82:1
Peonidin 3-glucoside	5.92 ± 0.52^a	0.93:1

¹Values represent mean \pm SD (n=3). Different superscripted letters indicate significant differences ($p < 0.05$). Results are expressed as micromole of Trolox equivalent per micromole of standard sample.

²Relative capacity, given cyanidin 3-glucoside = 1.0.

Table 6: ORAC value for pure anthocyanin standards known to be present in blackcurrant and grape.

Discussion

The antioxidant properties of blackcurrant and wine grape; respectively, are a function of both the total anthocyanin content and the activity associated with individual anthocyanins, that compose the complex mixture, and which also reflect the metabolic pathway for anthocyanin biosynthesis in both fruit berries. In both cases, blackcurrant and wine grape anthocyanin metabolism dominate the dihydromyricetin pathway and yield delphinidin anthocyanins; however, despite this similarity, it is noteworthy that the extended anthocyanin pathway to malvidin in wine grapes, is characteristically different from blackcurrant. Using this difference in anthocyanin metabolism, and notwithstanding the total anthocyanin composition between the two berry sources, the aim of this research was to characterize the antioxidant capacity of two different sources of anthocyanin constituents sharing similar anthocyanin biosynthesis pathways that reflect higher relative amounts of delphinidin (e.g. blackcurrant), or malvidin (e.g. grape). Moreover, we were interested in attempting to compare the antioxidant activity of additional anthocyanin constituents that are also present in both berries from the dihydroquercetin pathway.

The total phenolic content of fruit is an important factor governing total antioxidant capacity, as well as contributing to flavor, particularly, astringency. In this study, the total phenolic content of blackcurrant berries and wine grapes, as determined by Folin Ciocalteu procedure, was higher than the total phenolic content reported by others [2,3, 26,27]. Differences in total phenolic content between studies have been attributed to differences in the maturity of berries, sources of cultivars, or the use of different extraction solvents to recover polyphenolic constituents [28]. We detected and quantified six major anthocyanins, which were common to all blackcurrant cultivars examined in this study, but also represented less than half of the 15 different anthocyanins reported in the blackcurrant anthocyanin profile by other workers [22,29]. However, the four major anthocyanin compounds including Dp3R, Cy3R, Dp3G and Cy3G were responsible for more than 97% of the total anthocyanin content measured in the blackcurrant cultivars tested. Only trace amounts of petunidin and peonidin rutinosides (<1 area %) were also detected which is supported by others [28].

Anthocyanin 3-rutinosides comprised more than 60% of the anthocyanin profile in blackcurrant. Dp3R was the major anthocyanin present in all the blackcurrant cultivars with the one exception of *Ben*

Sarek. The anthocyanin 3-rutinosides were found to be at least 3 times higher than those of anthocyanin 3-glucosides (except for *Ben Sarek*) which agree with the data reported by Malien-Aubert et al. [30]. Again, with the exception of the *Ben Sarek* cultivar, the delphinidin derivatives, in particular rutinoside, was 2.8 times higher than that of the glucoside. A similar finding was also found for cyaniding derivatives.

For the three different wine grape cultivars examined in this study, five similar major anthocyanins were present in all grape cultivars examined, thus indicating a general similarity in the anthocyanin composition between grape cultivars sampled in this study. Our data confirms earlier reports [23,31] that showed anthocyanin 3-glucoside to be the most abundant anthocyanin among all anthocyanin derivatives in grape. Mv3G and Cy3G represented the highest and lowest concentration of anthocyanin pigment in all grape cultivars, respectively. *Cabernet Sauvignon* and *Merlot* had the same relative concentration of different anthocyanins (Mv-3-G > Dp-3-G > Pt-3-G > Pn-3-G > Cy-3-G), whereas *Pinot Noir* had a very different pattern (Mv-3-G > Pn-3-G > Pt-3-G > Dp-3-G > Cy-3-G). The difference between the relative concentrations of different anthocyanins in *Cabernet Sauvignon* and *Merlot* with *Pinot Noir* could simply be the result of differences between cultivars. For example, Mori et al. [32] reported that with the exception of Mv3G, the quantities of other individual anthocyanins decrease during berry development under warmer conditions. Notwithstanding this, the five major anthocyanin compounds identified in grape were responsible for more than 92% of the total anthocyanin content measured in the different grape cultivars, of which Mv3G was responsible for more than 30% of the total anthocyanin content in *Cabernet Sauvignon* and *Merlot*. In all grape cultivars, the percent of 3',4',5'-trihydroxy (Dp, Mv, Pt) was also at least 2 times higher than that of 3',4'-Dihydroxy (Cy and Pn).

The ORAC assay used to measure antioxidant activity of individual purified anthocyanin aglycons provided additional information about related antioxidant capacities of flavonoid products derived from different anthocyanin biosynthesis pathways. The ORAC antioxidant assay tests the affinity of anthocyanins to scavenge the production of peroxy radicals that are generated from AAPH. The data showed that Cy3G, which is the first anthocyanin metabolite produced from the dihydroquercetin pathway, produced the highest ORAC antioxidant capacity, compared to delphinidin, which is the first metabolite of the dihydromyricetin pathway. These two compounds differ only in the hydroxylation pattern of ring B. The position and the degree of hydroxylation pattern of B-ring substitutions is an important aspect of anthocyanins to scavenge free radicals using a variety of different *in vitro* assay methods [8,33,34]. Wang et al. [5] reported from structure-activity data with individual anthocyanins that both antioxidant and prooxidant activities varied depending on the number of hydroxyl groups present on the aglycone moiety. Our results showed delphinidin, a trihydroxy anthocyanin at positions 3',4',5'-B ring had relatively lower ORAC activity than cyanidin which has two hydroxyl groups at positions 3' and 4' on the B ring. Our finding agrees with other studies that have used the ORAC assay [5] and the *in vitro* Cu₂⁺-mediated LDL oxidation assay [14], but is in contrast to studies that have shown delphinidin to possess greater activity at scavenging both superoxide and peroxy radicals than cyanidin [35]. Malvidin, which contains O-methyl group on 4' position on the B-ring, was also lower in activity than cyanidin, which has two hydroxyl groups attached. The apparent importance of extra hydroxyl groups associated

with the B-ring for eliciting high antioxidant activity does not hold true for delphinidin 3-glucoside, which possesses an extra hydroxyl group in C-5' position, but had a relatively lower ORAC value compared to cyanidin 3-glucoside. Methoxylation of cyanidin and delphinidin did not have a significant effect on ORAC antioxidant capacity, but does adversely affect the scavenging activity of superoxide radicals [35]. It was also of particular interest that the attached sugar moiety did not affect the ORAC antioxidant capacity of same aglycon, as 3-glucoside and 3-rutinoside were similar for both cyanidin and delphinidin anthocyanins. Other workers have reported only slight reductions in superoxide radical scavenging in both the cyanidin and delphinidin families that were glycosylated at 3-OH position [36].

The ORAC values for purified anthocyanin extracts derived from blackcurrant contained more than five times the antioxidant capacity compared to the respective crude blackcurrant extracts. We attribute this finding to the use of anthocyanin purification steps that resulted in higher yields of purified anthocyanin mixtures and potential phytochemical antioxidant capacity in blackcurrant. Similar results have been achieved with anthocyanins purified by column chromatography from blackberry, which provided measures of ORAC antioxidant capacity that were five-fold higher than crude extracts [6].

Unlike the blackcurrant, the different grape cultivars examined in this study did not exhibit significantly different ORAC results. Following the analysis on crude grape extracts, and similar to what had been done for blackcurrant sample, subsequent experiments were performed on purified extracts that retained the anthocyanins at the cost of removing other phenolics. This was achieved by using a solid phase extraction (SPE)-C18 column. The purified anthocyanin extracts compared to the respective crude extracts indicated higher antioxidant capacity, again showing that anthocyanin purification was indeed achieved producing a relatively higher yield of antioxidant capacity. It is of particular interest that the *Ben Nevis* cultivar examined in this study, which had the greatest concentration of total anthocyanins and phenolics, also exhibited the highest ORAC antioxidant capacity. Regression analyses showed that ABTS antioxidant activity was highly correlated with total anthocyanin and total phenolic contents in blackcurrant.

A more in-depth evaluation of the different anthocyanins present in mixtures collected from all blackcurrant and grape cultivars was used to explain the subtle differences in anthocyanin composition that responded to the measured antioxidant activity. The ORAC assay was used to test the hypothesis that the relative antioxidant capacities of major, anthocyanins present in wine grape and blackcurrant fruits provide synergistic antioxidant activity. An anthocyanin antioxidant capacity index (AACI) was derived to show the importance of anthocyanin-specific composition, and the potential synergies that may exist between cultivar-specific anthocyanin compositions in regard to total ORAC activity. The standard AACI was consistently lower than the range of ORAC values obtained for the anthocyanin extracts from each blackcurrant and grape cultivar. Others have reported a similar result with a standardized extract resembling bilberry which was 5-times weaker than the actual bilberry extract [35]. Thus, a potential synergy between anthocyanin components in a complex mixture along with the total anthocyanin content together contribute to the total antioxidant capacity of the fruit.

Natural polyphenolic compounds, such as anthocyanins, exhibit antioxidant activity by one or more mechanisms that include, affinity to quench reactive oxygen species, donate hydrogen (reducing activity)

and sequester metal ions [5,6,14]. In theory, therefore synergistic antioxidant activity of anthocyanins could easily exist in fruit that vary in both relative concentration and antioxidant activity specific to each mechanism of action. However, in our study whereby the *in vitro* ORAC method was used to quantify the antioxidant activity, synergy between different anthocyanins would likely be limited to only the relative affinity to scavenge peroxy radicals. One possible explanation for the synergy observed in both studies could be the phase partition that results from the complex anthocyanin mixture of the fruit extract and which influences the affinity of the anthocyanin polyphenols to react with the peroxy radicals generated by AAPH. Unique steric relationships of the dihydroxy groups and possibly ortho arrangement could also represent additional molecular aspects that explain the relative higher affinity of a complex mixture of anthocyanins to interact with peroxy radicals, compared to a standardized extract. For example, with the exception of *Ben Nevis*, the three other cultivars of blackcurrant studied herein all exhibited both distinct anthocyanin compositions and a relatively higher measure of total antioxidant capacity compared to the calculated AACI. On the other hand, *Ben Sarek*, which had approximately a two-fold lower amount of Dp3R compared to other tested blackcurrant cultivars, exhibited the lowest AACI. The anthocyanin profile found in *Lentiy*, which had the second lowest AACI, compared to other three blackcurrant tested cultivars also had a lower Cy3G content. Collectively subtle differences in a lipophilic/hydrophilic balance required to optimize H-donating ability of anthocyanins for interaction with lipid peroxy radicals could be the underlying factor for the synergy observed in complex anthocyanin mixtures. Other workers have observed a similar enhanced antioxidant activity, or synergistic activity, amongst anthocyanins recovered from bilberry towards both superoxide and peroxy radical radicals [35]. Similar to our findings, these workers reported a markedly higher antioxidant capacity for the standardized bilberry extract, compared to a calculated estimate of activity based on the activity of each of the 15 different purified anthocyanin components present in the mixture.

Conclusion

In this study we compared the physicochemical properties of two soft fruits rich in delphinidin (e.g. blackcurrant) and malvidin (e.g. grape) anthocyanins. Chemical characterization of blackcurrant anthocyanins using HPLC/UV-MS disclosed that 3-rutinoside anthocyanins were the major anthocyanins, comprising more than 60% of the anthocyanin profile. Regression analyses showed that ABTS antioxidant activity was highly correlated with total anthocyanin ($r^2=0.88$) contents in blackcurrant. A potential synergy between anthocyanin components in regard to ORAC antioxidant capacity existed for each blackcurrant cultivar. Berries at the midpoint of ripening from three different wine grape cultivars contained Mv3G (>32%) and Cy3G (< 11%), which were the highest and lowest concentration of anthocyanin pigment in all cultivars, respectively. Grape cultivars had comparatively lower ORAC antioxidant capacity than blackcurrant cultivars. ORAC results showed that the dihydroquercetin pathway anthocyanin metabolites, including cyanidin and peonidin gave higher antioxidant capacity than metabolites derived from the dihydromyricetin pathway (e.g. Dp and Mv). The sugar moiety for each aglycon did not affect the ORAC antioxidant capacity to the same extent. An AACI was constructed to evaluate the proportion of antioxidant capacity influenced by the mixture of anthocyanins naturally occurring in both soft fruit sources. Using this index, we confirmed the antioxidant synergy reported for anthocyanins in bilberry earlier, with observed antioxidant synergies was also characteristic for blackcurrant and wine grape berries.

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