Characterization of antioxidant activities from fruits rich in delphinidin or malvidin anthocyanins

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Food Science)

THE UNIVERSITY OF BRITISH COLUMBIA

January 2008

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ABSTRACT

Anthocyanins have been shown to possess specific antioxidant capacities, which may provide an underlying protective effect against many chronic diseases. Although the antioxidant capacity of anthocyanins has been well established, less is known about the extent to which specific anthocyanin composition affects total antioxidant capacity. The aim of the present study was to compare the antioxidant capacity of two different soft fruits, blackcurrant and grape, which have distinctly different anthocyanin profiles.

The anthocyanin profiles of grape and blackcurrant were characterized by HPLC/MS coupled with a diode array detector. Results showed that blackcurrant contained four predominant anthocyanins, cyanidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-rutinoside, and delphinidin 3-rutinoside. In contrast, malvidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, and peonidin 3-glucoside were the major anthocyanins found in grape. The concentration of individual anthocyanins in all berries was quantified with HPLC/UV using cyanidin 3-glucoside as an external standard. Finally, results showed a greater \( p<0.05 \) antioxidant capacity of blackcurrant compared to grape. The total antioxidant capacity of crude extracts from each was measured by Oxygen Radical Absorbance Capacity (ORAC) and ABTS assays.

Anthocyanin antioxidant capacity index (AACI), derived from the product of antioxidant (ORAC) activity for each of major anthocyanin present in blackcurrant and grape, was also used to determine whether the antioxidant capacity of crude anthocyanin fractions represents either the sum total anthocyanin content or, alternatively, a synergy between different anthocyanins components. Our results indicated that a plausible
potential synergy between anthocyanin components in regards to ORAC antioxidant capacity existed in blackcurrant and grape semi-purified anthocyanin extracts.

Furthermore, it could be concluded that both total anthocyanin content as well as the composition of individual anthocyanins in soft fruits is important to assess total antioxidant capacity of different berry sources.
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LIST OF ABBREVIATIONS

AACI  Anthocyanin antioxidant capacity index
AAPH  2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS  2,2’-azinobis 3-ethylbenzothiazoline-6-sulfonic acid
ABTS+  ABTS radical cations
ANS   Anthocyanin synthase
AUC   Area under the curve
BC    British Columbia
BHA   Butylated hydroxyanisol
β-PE  β-Phycoerythrin
CHD   Coronary heart disease
Cy    Cyanidin
Cy3G  Cyanidin 3-glucoside
Cy3R  Cyanidin 3-rutinoside
CVD   Cardiovascular heart disease
DFR   Dihydroflavonol reductase
DNA   Deoxyribonucleic acid
Dp    Delphinidin
DPPH  2,2’- diphenyl-1-picrylhydrazyl
Dp3G  Delphinidin 3-glucoside
Dp3R  Delphinidin 3-rutinoside
EDTA  Ethylenedinitrilotetraacetic acid
ESI   Electrospray ionization
FAO  Food and Agriculture Organization
GA   Gallic acid
GIT  Gastrointestinal tract cell
HAT  Hydrogen atom transfer
HOO' Hydroperoxyl
HPLC High performance liquid chromatography
HL-60 Human promyelocytic leukemia cell line
HT 29 Human colon adenocarcinoma cell line
LC/MS Liquid chromatography/mass spectrometry
LDL  Low density lipoprotein
MCF-7 Human breast adenocarcinoma cell line
Mv   Malvidin
Mv3G Malvidin 3-glucoside
ORAC Oxygen radical absorbance capacity
O2^- Superoxide
^1O2  Singlet Oxygen
OH'  Hydroxyl radical
Pg   Pelargonidin
Pn   Peonidin
Pn3G Peonidin 3-glucoside
Pt   Petunidin
Pt3G Petunidin 3-glucoside
RO'  Alkoxy radicals
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ROO'</td>
<td>Peroxyl radicals</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SET</td>
<td>Single electron transfer</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>TA</td>
<td>Titratable acidity</td>
</tr>
<tr>
<td>TE</td>
<td>Trolox equivalent</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VDT</td>
<td>Video display terminal</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>F3GT</td>
<td>Flavonoid-3-glucosyltransferase</td>
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ACKNOWLEDGMENT

Foremost, I would like to express my sincere gratitude to my supervisor, Dr. David D. Kitts, for his insight, guidance, and wise supervision throughout this research project. Without his kind assistance and patience, its completion would not have been possible. His moral support and leads from my first day in Vancouver were fundamentally formative in helping me pursue my academic goals.

My sincere thanks are also due to Dr. Steven Lund and Dr. Eunice Li-Chan for their constructive suggestions and enormous input. Their critical comments significantly contributed in overcoming the research problems and improving its quality.

I also extend my thanks to Dr. Parastoo Yaghmaee and Dr. Amir Farid Moayer for their valuable assistance in my attempt to start this stage of my life.

I am genuinely grateful to Dr. Charles Hu, Dr. Pedro Aloise, Val Skura, and specially Crystal Cinq-Mars for their technical assistance and the coherent answers they have provided to my endless questions.

Many thanks to all my dear friends and talented colleagues. Your support undoubtedly contributed to the quality of this work. The flaws that remain result from my own shortcomings.
Most important I would like to thank my family. My dad, Mohammad Hashem Hosseini Beheshti, my mum Shahin Nikkar, and my dear brother Alireza Hosseini Beheshti for the unending love and wisdom they have given throughout my education. They have always been a boundless source of strength and inspiration.
To my parents

for their endless love and support

and to the dearest

Alireza and Nasim
CO-AUTHORSHIP STATEMENT

I have conducted all the analysis and experiments in chapter II and chapter III.

Figures and tables that I contributed to these chapters (II, III) are all listed on list of figures an list of tables.
General introduction

A proper balance between antioxidant and oxidant states is important for maintaining an optimal physiological body condition (Halliwell, 2006). Free radicals are produced both endogenously through ordinary metabolic processes (Ou et al., 2002) and exogenously by exposure to different reaction products derived from ultra-violet light, alcohol consumption, cigarette smoking and exercise, to name a few (Hu et al., 2005). Overproduction of reactive oxygen species (ROS) will lead to an imbalance between generation of reacting oxidation products, resulting in oxidative stress. There is also overwhelming evidence to suggest that an excess formation of ROS is involved in the development of different disease states, such as atherosclerosis, cardiovascular disease, stroke, cancer, arthritis, aging and Alzheimer’s disease (Crujeiras et al., 2006; Frank et al., 2002; Halliwell, 1995).

Recently, considerable scientific interest in plant secondary metabolic compounds has developed due to a multitude of reported protective effects of these compounds against cardiovascular heart disease, including coronary heart disease, type II diabetes, hypertension, dyslipidemia (e.g. high total cholesterol, or high level of triglycerides), stroke, and some cancers. Flavonoids including anthocyanins, flavonols, flavones, and flavanols have been reported to have anti-carcinogenic, anti-mutagenic, anti-bacterial (Puupponen-Pimia et al., 2005) and vasodilating effects in animal systems (Steinmetz et al., 2005).

Anthocyanins are polyphenolic pigments that belong to the flavonoid family (Nielsen et al., 2003) and in general are water-soluble, nontoxic pigments (Malien-
that have been previously demonstrated to possess numerous bioactive properties *in vivo and in vitro*. Previous studies have reported many protective effects of anthocyanins on human health preservation, such as anti-inflammatory, anti-mutagenic and anti-genotoxic properties, improvement of night visual acuity and overall reduced risk to heart disease due to antioxidant capacity. Blackcurrant represents one of the most important and richest sources of phenolics (Cacace et al., 2002). In particular, blackcurrant has been reported to contain four predominant anthocyanins, which include cyanidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-rutinoside, and delphinidin 3-rutinoside (Slimestad et al., 2002). In contrast, malvidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside and peonidin 3-glucoside are the major anthocyanins found in grape (Pomar et al., 2005; Monagas et al., 2006). Although the antioxidant capacity of anthocyanins has been well established, less is known about the extent to which specific anthocyanin composition can affect total antioxidant capacity. The aim of the present study was to characterize, identify, quantify, and evaluate the antioxidant capacity of two different sources of anthocyanin constituents that reflect higher relative amount of delphinidin (e.g. blackcurrant) or malvidin (e.g. grape). Related to this was the aim to compare the antioxidant capacity of blackcurrant and grape, which have distinctly different anthocyanin profiles. In addition, it was tested whether the antioxidant capacity of crude anthocyanin fractions represents either the sum of total anthocyanin content or, alternatively, a synergy between different anthocyanin components.
CHAPTER I. Literature review

1.1. Reactive oxygen species and the effects of oxidative stress

1.1.1. Properties of reactive oxygen species

Reactive oxygen species (ROS) are one of the primary causes for the development of many pathological conditions such as cancer, ischemia, atherosclerosis, cardiovascular disease, stroke, diabetes, and Alzheimer's disease (Frank et al., 2002; Kaur et al., 2006; Bryan et al., 1996). ROS is a collective term including both oxygen radical-centered free radicals and non-radical oxidants (Roberfroid et al., 1995). By definition, free radicals are atomic or molecular species with at least one unpaired electron on the outer shell (Roberfroid et al., 1995). ROS include superoxide (O_2^-), hydroxyl (OH^-), hydroperoxyl (HOO'), alkoxy radicals (RO'), and peroxyl radicals (ROO'). Examples of non-radical oxidants, which can shift to be oxidized into radicals relatively easily, are hydrogen peroxide (H_2O_2) and singlet oxygen (^1O_2) (Hu et al., 2005).

1.1.2. Source of production of reactive oxygen species

ROS come into contact with the metabolizing organism as the result of either endogenous or exogenous production. Oxygen metabolism, energy generation in the mitochondria, and detoxification reactions in the liver are some of the main sources of endogenous ROS generation, while exposure to environmental pollutants and cigarette
smoke, ionizing radiation, alcohol consumption, and fungal, or viral infections, are examples of exogenous sources (Roberfroid et al., 1995). A certain amount of ROS is beneficial for controlling antimicrobial activity and regulating cell proliferation in the body; however, when free radicals and other oxidative molecules are generated beyond the capacity of the body defense network, they are not effectively detoxified leading to oxidative stress.

1.1.3. Oxidative stress

As mentioned above, several pathological situations, including atherosclerosis, cardiovascular disease, stroke, cancer, arthritis, Alzheimer’s disease, and age-related disorders are thought to be caused by free radicals and ROS, which generate oxidative stress (Frank et al., 2002). A balance between oxidants and antioxidants in the body is essential to avoid oxidative stress. Oxidative stress is imposed on the cell as a result of decreased levels of antioxidants. This can be caused intrinsically, for example, by DNA mutations that have altered the cellular antioxidant defense system activity, or extrinsically by a deficiency in dietary minerals (cofactors), or by toxins and other factors which deplete the antioxidant defenses. An increased level of oxidants in the cell can also result in oxidative stress.

Oxidative stress may result in adaptation of the cell or organism by triggering up-regulation of the immune defense system; however, this can also result in cell injury and cell death. Cellular interaction with ROS results in damage to DNA molecules, indicating that oxidative stress likely plays an important role in increasing the risk of
cancer through enhanced mutagenesis, carcinogenesis, and aging (Baskin et al., 1997; Halliwell et al., 2004). Oxidative stress can also elicit structural and compositional alterations to enzymes, receptors, and transport proteins that affect their functions. These defective proteins are degraded and removed from the cell (Acworth et al., 1997).

1.1.4. Lipid peroxidation

The reaction of ROS with polyunsaturated lipids has been researched extensively not only for its important role in the development of undesired odours and flavours in food, but also for the initiation of atherosclerosis by oxidizing LDL (Acworth et al., 1997; Pal et al., 2003). Lipid peroxidation, whether occurring in vivo or in vitro, has three distinct steps: Initiation, propagation, and termination. The initiation step involves the reaction between free radicals (R') and polyunsaturated fatty acids or lipid molecules (LH) in which a hydrogen atom is extracted from the fatty acid or lipid molecule to produce an alkyl radical (L') (Equation 1).

\[ R' + LH \rightarrow RH + L' \]  
(Equation 1)

The alkyl radical will be further oxidized during the propagation step to form a peroxyl radical (LOO'), which further extracts a hydrogen atom from another fatty acid or lipid molecule. These products can easily interact with nearby lipid molecules and produce secondary lipid radicals (Equation 2-4).

\[ L' + O_2 \rightarrow LOO' \]  
(Equation 2)
The free radical production in the propagation step will be continued until the termination step takes place. The final step involves the combination of two free radicals, which results in a non-radical product (Bryan et al., 1996; Fennema, 1996).

\[
{\text{LOO}}^\cdot + {\text{LOO}}^\cdot \rightarrow \text{Non-radical products} \quad \text{(Equation 5)}
\]

\[
{\text{L}}^\cdot + {\text{LOO}}^\cdot \rightarrow \text{Non-radical products} \quad \text{(Equation 6)}
\]

\[
{\text{L}}^\cdot + {\text{L}}^\cdot \rightarrow \text{Non-radical products} \quad \text{(Equation 7)}
\]

Of all biological products, lipid oxidation products have one of the longest half-lives \((10^3 \text{ to } 10^6 \text{ times})\) and, therefore, are predisposed to attacking critically important cellular components and increasing the potential of cell damage that causes different disorders, such as atherosclerosis and cardiovascular disease. These situations may be potentially prevented by consuming dietary antioxidants, including anthocyanins, which work by counteracting the imbalance of oxidative agents present in the body (Halliwell and Whiteman, 2004).
1.2. Antioxidants and the measurement of antioxidant capacity

1.2.1. Antioxidant definition

Antioxidants are defined as “any substrate, that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (Tang et al., 2004). In food science, antioxidants are further defined as a “substance present in food that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans” (Huang et al., 2005).

1.2.2. Antioxidant classification

1.2.2.1. Biological antioxidant classification

Biological antioxidant sources can be classified into two major groups. First, enzymatic antioxidants, which are produced endogenously to protect the body from the adverse effects of free radicals, such as superoxide dismutase (in mitochondria and the cellular cytosol), catalase (in peroxisomes) and glutathione peroxidase (in the cell membrane). Secondly, non-enzymatic or dietary antioxidants, which are external antioxidant sources that combat oxidation when internal defense systems fail, due to a decrease in cellular antioxidants or an increase in the production of free radicals. Antioxidant enzyme cofactors (e.g. selenium), oxidative enzyme inhibitors (e.g. aspirin), transition metal chelators (e.g. EDTA), and radical scavengers (e.g. vitamins and phenolics) are different examples of non-enzymatic antioxidants (Kaur et al., 2006; Huang et al., 2005).
1.2.2.2. Antioxidant classification by chemical reaction basis

Antioxidants can be classified on a chemical reaction basis by describing the process of how free radicals are deactivated using either Hydrogen Atom Transfer (HAT) or Single Electron Transfer (SET) (Huang et al., 2005). The end-result of both mechanisms is the same; however, they are different in terms of radical elimination kinetics and the potential for side reactions define (Prior et al., 2005).

**HAT reaction:** This reaction involves the transfer of proton-coupled electrons and demonstrates the affinity of any antioxidant such as a hydrogen donor to quench free radicals. The following equation shows the HAT reaction, where AH is an antioxidant and X is the oxidized substrate.

\[ X^+ + AH \rightarrow XH + A^- \]  \hspace{1cm} \text{(Equation 8)}

HAT reactions are solvent- and pH-independent and usually occur in a few seconds or minutes. The activity of this rapid reaction is based on the bond dissociation energy constant of the antioxidant hydrogen donor (Wright et al., 2001; Mayer, 2004).

**SET reaction:** This reaction indicates the affinity of the antioxidant for transferring an electron to reduce free radicals. The activity of this reaction is usually detected by the ionization potential constant of the reactive group; therefore, the SET reaction is pH-dependent.

\[ X^+ + AH \rightarrow X^- + AH^+ \]  \hspace{1cm} \text{(Equation 9)}

\[ AH^+ \xLeftarrow{H,O} A^- + H_3O^+ \]  \hspace{1cm} \text{(Equation 10)}

\[ X^- + H_3O^+ \rightarrow XH + H_2O \]  \hspace{1cm} \text{(Equation 11)}
By this mechanism, the reaction takes a longer time to complete, so the calculation of antioxidant capacity is based on the percent decrease of products rather than by the kinetics of the reaction (Prior et al., 2005; Mayer, 2004).

1.2.2.2.1. ABTS assay

The ABTS assay was first reported by Miller and Rice-Evans (1993). ABTS is usually classified as a SET reaction. This spectrophotometric method is also known as the Trolox Equivalent Antioxidant Capacity (TEAC). The activity of the antioxidant sample in terminating the ABTS radical is measured spectrophotometrically via the chemical decolorization of the ABTS radical solution (Kaur et al., 2006; Antolovich et al., 2002). The ABTS radical is generated by either chemical reactions (i.e. hydrogen peroxide, sodium persulfate) or enzymatic reactions (i.e. metmyoglobin, hemoglobin, etc), of which the latter reaction is faster and the reaction temperature needed to initiate the reaction is milder (Prior et al., 2005). The ABTS has maximum absorbances at 415, 645, 734 and 815 nm. Among these, a 734 nm wavelength has been adopted by most researchers to decrease interference from other components and minimize sample turbidity (Antolovich et al., 2002).

The ABTS assay is a simple and rapid method that can be used over a wide pH range. ABTS is also soluble in both organic and aqueous solvents, which makes it a common antioxidant capacity test for food and nutraceutical products. On the other hand, ABTS is not a physiological radical source and can, therefore, underestimate the antioxidant capacity of the compound when using an endpoint of short duration (~10
min), since some of the antioxidant components may exhibit a relatively slower reaction mechanism (Prior et al., 2005).

![ABTS reaction](image)

**ABTS** (Dark Blue) → ABTS\(^2\) (Colorless)

**Figure 1.1. ABTS reduction (adapted from Huang et al., 2003)**

1.2.2.2.1. ORAC assay

Among different antioxidant capacity tests, the Oxygen Radical Absorbance Capacity (ORAC) assay has been found to have the broadest applicability for measuring the antioxidant capacity in different food samples (Huang et al., 2005). ORAC is one of the only methods that can detect both general and specific antioxidant activities. This assay was first suggested by Ghiselli et al. (1995) and Glazer (1990), and was developed further by Cao et al. (1993) (Prior et al., 2005). The ORAC assay is a temperature-sensitive method in which peroxyl radicals, which are generated, react with a fluorescent probe and produce a non-fluorescent product. The decreased rate and amount of peroxyl radical produced over time is an index to determine the antioxidant capacity of antioxidants.
Previously, β-Phycoerythrin (β-PE), which is a protein isolated from *Prophyridium cruentum*, was used as the fluorescent probe in the ORAC assay; however, β-PE has large lot-to-lot variability in reactivity with peroxyl radicals, becomes photo-bleached after exposure to excitation light, and also interacts with polyphenols via nonspecific protein binding all of which result in a loss in fluorescence and, therefore, cause an underestimation in the ORAC value (Huang et al., 2005; Prior et al., 2005). To avoid an underestimation of antioxidant capacity, it has been recommended to use one of two alternative fluorescent probes, fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1][3H],9'[9H]-xanthen-3-one), or dichlorofluorescein (2',7'-dichlorofluorescein diacetate) which are more stable and less reactive (Huang et al., 2005).

In the ORAC assay, the radical initiator component of the assay, 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH), a water-soluble free radical initiator, decomposes at 37°C to form two carbon-centered radicals. These radicals quickly combine with oxygen to generate peroxyl radical, which initiates lipid oxidation. Fluorescein will decompose upon exposure to these free radicals (Niki, 1990). The oxidized reaction product of fluorescein, following exposure to the peroxyl radical, has been characterized by LC/MS, and the product pattern is consistent with a classical HAT mechanism (Prior et al., 2005). The following equations show the reaction, where the R-N=N-R is a radical initiator, ROO' is a peroxyl radical and AH is an antioxidant.
R-N=N-R + O₂ → N₂ + 2ROO⁺ (Equation 12)

ROO⁺ + fluorescence probe → ROOH + oxidized fluorescence probe (↓ in fluorescence)

ROO⁺ + AH → ROOH + A⁺ (Equation 13)

ROO⁺ + A⁺ → ROOA (Equation 14)

In the ORAC assay, the antioxidant capacity of the antioxidant is assessed by calculating the net integrated areas under the fluorescence decay curves (\(AUC_{\text{net}} = AUC_{\text{sample}} - AUC_{\text{blank}}\)). Trolox, which is a water-soluble counterpart of vitamin E, is used as a standard in most of the antioxidant capacity tests. The ORAC value of antioxidant samples is always reported in Trolox equivalents (µmol TE/g).

Overall, ORAC is the only assay that combines both the degree of inhibition and inhibition time into a single quantitative parameter (Ou et al., 2001). It is also known for using a peroxyl radical source that is generated at a constant rate during the heating of AAPH. This assay can also be adapted to detect both hydrophilic and hydrophobic antioxidants by changing the extraction solvent or the peroxyl radical source. Nevertheless, in this temperature sensitive assay, strict temperature control throughout the plate is essential. Small changes in temperature within each individual microplate well will cause a decrease in assay reproducibility. Further, this assay has been criticized for its relatively high cost, the need for special equipment (i.e. fluorometer), and the long
analysis time; however, its high-throughput capability and adaptability to different sample types overcomes these limitations.

Figure 1.2. Fluorescence oxidation in the presence of AAPH (Adapted from Ou et al., 2001)
1.3. Anthocyanins

1.3.1. Anthocyanin structure

Anthocyanins are the largest group of water-soluble secondary metabolites produced by plants (Tsuda et al., 2004) and are responsible for most of the red, blue, purple, and other intermediate colors of many plant tissues (Frank et al., 2002).

Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium (flavylium) salts (Wu et al., 2004). Approximately 400 different anthocyanins are found in nature (Kong et al., 2003); the most common anthocyanidin aglycons in plants are cyanidin (Cy), delphinidin (Dp), malvidin (Mv), petunidin (Pt) peonidin (Pn) and pelargonidin (Pg). The chemical structures of these different aglycons vary only in the position of the R group that is located at position C-3’ or C-5’. Aglycons have rarely been found in fresh plant materials (Prior et al., 2004). The flavylium ion structures of these six anthocyanidins are shown in **Figure 1.3.**

![Common anthocyanidin structure](image)
Table 1.1. Different anthocyanidin aglycons (Wu et al., 2004; Prior et al., 2004).

<table>
<thead>
<tr>
<th>Anthocyanidin</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$MW$</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
<td>287</td>
<td>Orange red</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
<td>303</td>
<td>Bluish red</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OCH$_3$</td>
<td>OCH$_3$</td>
<td>331</td>
<td>Bluish red</td>
</tr>
<tr>
<td>Petunidin</td>
<td>OCH$_3$</td>
<td>OH</td>
<td>317</td>
<td>Bluish red</td>
</tr>
<tr>
<td>Peonidin</td>
<td>OCH$_3$</td>
<td>H</td>
<td>301</td>
<td>Red</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
<td>271</td>
<td>Orange</td>
</tr>
</tbody>
</table>

Glycosylation provides stability to anthocyanidins. Glucose, galactose, rhamnose, and arabinose are the most common monosaccharides that bind to anthocyanidins. Disaccharide and trisaccharide glycosylates, which are formed by combinations of these four monosaccharides, can also occur. The first sugar always replaces the hydroxyl group in the 3 position of the anthocyanin aglycon structure, and if a second sugar is present, it generally occurs at position C-5.
In some cases, the sugar residues of anthocyanins are acylated by p-coumaric, caffeic, ferulic, or sinapic acids, and sometimes by p-hydroxybenzoic, malonic, or acetic acids. Acyl substitutions usually occur to the C-3 position of the sugar moiety. Red cabbage, red lettuce, garlic, red-skinned potato, and purple sweet potato are some of the foodstuffs which contain acylated anthocyanins (Prior et al., 2004). A few pigment acyl substitutes are attached to the 6-hydroxyl position; they have also been shown to be attached to the 4-hydroxyl group of the sugar moiety.

Anthocyanins are predominantly found in vacuoles of outer cell layers, such as the epidermis of land plants, but are not found in animals or microorganisms. Water plants also lack anthocyanins, as the light penetration under water is not enough to trigger biosynthesis. Not all land plants contain anthocyanin, however. Fruits, namely berries such as blueberry, blackcurrant, raspberry, saskatoon berry, grape, chokeberry, and
cherry are rich in anthocyanins. Conversely, anthocyanins are found in a much lower concentration in banana, asparagus, pea, fennel, pear, potato (Clifford et al., 2000) and grains (Hu et al., 2007).

Since the spectral properties of anthocyanins are dependent on a number of factors including pH and co-pigmentation with flavonoids, the colour of anthocyanin will not necessarily obey the “Beer-Lambert law” at any given concentration (Degenhardt et al., 2000). Anthocyanins exist in equilibrium among four different structural conformations: The quinoidal base, the flavylium cation, the pseudobase or carbinol, and the chalcone. The red flavylium cation occurs at pH 1 and is the most stable form of anthocyanins. Increasing pH will change the anthocyanin conformation, and with this, the spectral properties. The less stable colorless compounds at higher pH, such as chalcone, have been suggested to be the precursor of anthocyanin degradation (Prior et al., 2004). Anthocyanins are also sensitive to light and elevated temperature (Nielsen et al., 2003). The equilibrium among the four different structural conformation of anthocyanin is shown in Figure 1.5 (Dimitric Makovic et al., 2005).
Figure 1.5. Structural transformation of anthocyanin in strongly acidic to alkaline aqueous media at room temperature (Dimitric Makovic et al., 2005).
1.3.2. **Anthocyanin biosynthesis**

Chalcone is the common precursor for flavonoid biosynthesis in plants. It is synthesized from 4-coumaryl-CoA and three molecules of malonyl Co-A by the chalcone synthase enzyme. This product is then converted to naringenin by the action of chalcone isomerase. By a sequence of hydroxylation reactions, naringenin is further converted to different intermediate products, mainly dihydromyricetin, dihydroquercitin and dihydrokaempferol. As shown in Figure 1.6, dihydroflavonol reductase (DFR), anthocyanin synthase (ANS), and flavonoid 3-glucosyltransferase (F3GT) are responsible for the final conversions to delphinidin 3-glucoside, cyanidin 3-glucoside and pelargonidin 3-glucoside, respectively (Suzuki et al., 1999).

1.3.3. **Anthocyanin antioxidant capacity in vitro and in vivo**

Plants produce an extraordinary diversity of phenolic compounds, including flavonols, anthocyanins, and tannins due to the lower electron reduction potential of phenolic compounds compared to oxygen radicals. Since phenoxy radicals are generally less reactive than oxygen radicals, phenolic compounds are excellent oxygen radical scavengers (Ainsworth et al., 1999); however, berry cultivar and other factors such as weather conditions, geographical location, soil type, and berry ripeness can influence the phenolic content and the antioxidant capacity of berry extracts. For example, there is a positive relationship between total anthocyanin content, total phenolic content, and the relative antioxidant capacities in different grape berry tissues (Monagas et al., 2006; Moyer et al., 2002; Kahkonen et al., 2001). Results with Oregon cranberries (Wada et al., 2002) also suggested a strong linear relationship between ORAC and total phenolic
content \( r^2 = 0.95 \), indicating that the antioxidant capacity of cranberry is highly dependent on the presence of phenolic compounds. Similar relationships have also been found between anthocyanins and ORAC value, yet they are not as strong as the former. This observation is also in agreement with others studies (Prior et al., 1998; Wada et al., 2002).

The mechanism of antioxidant activity of flavonoids, particularly anthocyanins, is well known. The phenolic structure of anthocyanins suggests that the antioxidant property stems from electron donation or hydrogen transfer from hydroxyl or methoxyl groups to free radicals. The difference between aglycon anthocyanin structures occurs in both C-3’ and C-5’ positions. Cyanidin 3-glucoside, which is the most common anthocyanin, occurs in 90% of fruit species and has been found to be twice as effective an antioxidant compared to butylated hydroxyanisol (BHA) and \( \alpha \)-tocopherol (Fukmoto et al., 2000). The structural effect of aglycon moieties on antioxidant capacities has been compared among five anthocyanins having the same glucose moiety; delphinidin aglycon apparently yields a higher intensity than the other four (measured by chemilluminescence in a neutral pH region). Other structural effects of sugar moieties were also compared, indicating that the rutinoside sugar moiety has a higher antioxidant capacity at pH=7 than the corresponding glucose moiety in human plasma (Matsumoto et al., 2002). Finally, acylated anthocyanins, as well as glycosylated anthocyanins, have been reported to have lower antioxidant activities compared to corresponding aglycons (Monagas et al., 2006).

Anthocyanins are the major class of flavonoids in the Western diet, which have received considerable interest by health scientists and nutritionists, due to potential protective effects against disease (Clifford et al., 2000; Santos-Buelga et al., 2000). In
animal studies, consumption of dark-colored berries has been shown to protect against peroxynitrite-induced endothelial dysfunction and chemically induced cancer (Serraino et al., 2003; Casto et al., 2002). Further, Olsson et al. (2004) demonstrated that both fruit and berry extracts inhibited cancer cell proliferation in a dose-dependent manner. In particular, they showed that the proliferation of colon cancer cells in the cell line HT29 was inhibited to a higher degree than the proliferation of breast cancer cells (e.g. MCF-7). Their results also suggested that delphinidin 3-glucoside, cyanidin 3-glucoside, and malvidin 3-glucoside each at a concentration of 200 µg/mL significantly decreased cell proliferation of both HT29 and MCF-7 lines, whereas a non-fractionated mixture of anthocyanins at the same concentration decreased cell proliferation to a lesser extent (Olsson et al., 2004).

The potential anti-cancer activity of anthocyanins has also been evaluated in several studies. Anthocyanins have demonstrated the highest anti-proliferation and apoptosis induction in HL-60 human promyelocytic leukemia cells and HCT-116 colon carcinoma cells (Katsube et al., 2003). In particular, cyanidin and delphinidin were reported to be more effective than malvidin at inhibiting the growth of human tumor cells in vitro (Merers et al., 2001). Hou et al. (2003) found that delphinidin, cyanidin and petunidin induced apoptosis in the HL-60 human promyelocytic leukemia cell line, whereas pelargonidin, peonidin and malvidin showed no similar inductive effect. According to Matsumoto et al. (2002) and Serraino et al. (2003), cranberry and blackberry anthocyanins protected cultured human vascular endothelial cells against both oxidative membrane damage and DNA strand breakage (Matsumoto et al., 2002; Serraino
et al., 2003), as well as inhibited colon cancer cell proliferation \textit{in vitro} (Kang et al., 2003).

Pal et al. (2002) reported a reduction of intracellular cholesterol in cells when in the presence of red wine polyphenolics (dealcoholized wine). They suggested that the reduction of ApoB100 secretion from hepatocytes, which is synthesized mainly as VLDL, is due to a reduction of cholesterol availability when in the presence of red wine polyphenolics (Pal et al. 2003). Heo et al. (2005) suggested that strawberry, among other common fruit such as plum, red grape, banana and orange, had a higher antioxidant capacity \textit{in vitro} when tested in PC-12 cells treated with H$_2$O$_2$. Their result suggested that the consumption of strawberry could have an important role in reducing the oxidative stress-induced risk of neurodegenerative disorders such as Alzheimer’s disease due to the high anthocyanin content. Elderberry extract has also been reported to possess some anti-inflammatory activity and anti-virus activity against influenza viruses \textit{in vitro} (Zakay-Rones et al., 1995).

Feeding grape juice to rats significantly inhibited mammary adenocarcinoma multiplicity and tumor mass, while anthocyanins from camellia, hibiscus, and glutinous red rice increased survival rates in mice inoculated with syngeneic tumor cells (Kamei et al., 1996). Red wine also has been reported to decrease plasma lipids in animals. According to Vinson et al. (2001), LDL plasma concentrations as well as cholesterol and plasma total triglyceride were decreased by 14, 25 and 13%, respectively, in hyperlipidemic hamsters fed dealcoholized red wine compare to a control group (Vinson et al., 2001).
It has also been reported in animal studies that blackcurrant anthocyanins can improve blood circulation due to platelet coagulability reduction and improvement in blood filterability (Matsumoto et al., 2005). Oral intake of a mixture of blackcurrant fruits has been claimed to be beneficial for visual function (Nakajima et al., 2004). Blackcurrant anthocyanin prepared from blackcurrant fruits improved dark adaptation and video display terminal work-induced transient refractive alternation in humans (Nilelsen et al., 2003; Matsumoto et al., 2002). Matsumoto et al. (2005) suggested that blackcurrant anthocyanin intake could improve the shoulder stiffness that occurs during typing by increasing the peripheral circulation.

Many studies, each employing different demographic populations, have shown that diets rich in antioxidants are associated with a lower incidence of atherosclerotic cardiovascular disease (Xia et al., 2003). Specifically, fruits rich in anthocyanins were reported to have free radical scavenging activity and have also been used for the treatment of cardiovascular disorders (Slimestad et al., 2002). The investigation of the ‘French Paradox’, which refers to the observation that people in France suffer low incidences of cardiovascular heart disease although they have a diet rich in saturated fats but frequently consume red wine, has drawn similar attention to the possible health benefits of the consumption of anthocyanin-rich foods. Anthocyanins, once absorbed into the membrane and cytosol of vascular endothelial cell, play a role in preventing the risk of coronary heart disease by protecting the cell against oxidative stress (Degenhardt et al., 2000).
Chalcone

Naringenin

Dihydrokaempferol
Dihydromyricetin  Dihydroquercitin  Pelargonidin-3-glucoside

Figure 1.6. Steps in anthocyanin biosynthesis
1.3.4. Anthocyanin absorption and metabolism

The majority of anthocyanins can be absorbed intact in the small intestine as glycosides (Nilelsen et al., 2003; Sung et al., 2000; Talavera et al., 2004). Even though the mechanism of anthocyanin absorption is still unknown, analyses of biological fluids have revealed several circulating forms of anthocyanins and possible mechanisms defining anthocyanin metabolism (Talavera et al., 2004). A possible metabolic pathway of orally administered Cy3G is shown in Figure 1.7. According to Ichiyanagi et al. (2005), orally administered Cy3G can either be cleaved to its aglycon form by a β-glucosidase, or metabolized to two other aglycons (Pn and 4’-O-methyl Cy) by an O-methyltransferase in the liver.
Figure 1.7. Possible pathway of Cy3G metabolism. Adapted from Ichiyanagi et al., (2005)

Kay et al. (2005) proposed that glucuronidation and methylation are the two major modifications of anthocyanins in humans. Specifically, 59.8% and 57.8% of the total anthocyanin metabolites in blood and urine were found to follow the glucuronidation pathway, while 43.8 and 51.4% of the total anthocyanins detected in serum and urine samples were representative of the methylation metabolic pathway (Kay et al., 2005); however, absorption of anthocyanins appears to be one-tenth that of flavonoids, and at least 13 different anthocyanins from 7 different sources have been detected as unchanged in plasma, urine, blood and feces samples (Prior et al., 2004).
According to Mutsumoto et al. (2001), modified anthocyanins were detected after the oral intake of blackcurrant anthocyanins Dp3R and Cy3R with a maximum concentration being reached after two hours, and Dp3G and Cy3G producing maximum concentrations one hour after ingestion (Matsumoto et al., 2001). Furthermore, Nielsen et al. (2003) reported that other food components could also affect anthocyanin absorption and increase bioavailability (Nilelsen et al., 2003). For example, higher plasma levels of anthocyanins have been detected in both rabbits and human subjects after consumption of juice, compared to consumption of other anthocyanin food sources, such as an aqueous citric acid matrix (in rabbits) and a carbohydrate-rich meal (in humans).

Talavera et al. (2004) studied both the absorption of anthocyanins in the small intestine after in situ intestinal perfusion and the effect of a specific sugar moieties on the anthocyanin absorption. Based on their results, anthocyanin structure was established as an important factor affecting intestinal absorption. For instance, absorption of Cy3G was 50% greater than that for Mv3G. Cyanidin 3-glucoside was also more readily absorbed than cyanidin 3-galactoside or cyanidin 3-rutinoside. Anthocyanin arabinoside appeared to be better absorbed than all other anthocyanins; however, it may have been absorbed or metabolized in a different manner compared to other glycosides. More research is required to confirm the arabinoside metabolic pathway (Wu et al., 2002).

As was mentioned earlier, anthocyanins can be detected rapidly in plasma after feeding (Tsuda et al., 2004; Matsumoto et al., 2001). On the other hand, because of the higher stability of anthocyanins in acidic pH, stomach conditions should be a favourable environment for uptake of anthocyanin molecules (Talavera et al., 2004).
While anthocyanins have not been shown to inhibit breast or lung cancers (Seeram et al., 2003; Carlto et al., 2000), Bernadene et al. (2005) suggested that anthocyanins in the intestines may interact with the gastrointestinal tract cell (GIT) and somehow inhibit GIT cancer, including oral, esophageal, intestinal, and colorectal cancer (Casto et al., 2002; Malik et al., 2003). These results suggested that feces are the major clearance route for ingested anthocyanin. The results also confirmed that the number and type of sugar substitution, and also acylation could be important factors that collectively affect anthocyanin absorption or degradation in the gut.

1.4. Blackcurrant fruit

1.4.1. Introduction

Blackcurrant (Ribes nigrum) is a fruit species with a centre of origin in Central and Northern Europe and the northern part of Asia. It is now widely cultivated in North America, Europe, and New Zealand (Takata et al., 2005). These small shrub plants have long, simple, and broad leaves (4-9), with five-petalled reddish-green to brown flowers. The edible berries, which are a very dark purple in color, are mostly sour in taste. The blackcurrant fruit normally matures from early July to mid-August in the Northern Hemisphere.

1.4.2. Blackcurrant nutritional composition

The nutritional composition of blackcurrant fruit varies depending on the stage of growth. Levels of sugars, anthocyanins, and acid content change during ripening. Environmental conditions can also cause differences in composition within the same
namely bayberry, blackcurrant, blackberry, raspberry, strawberry, blueberry, cowberry, cranberry, and redcurrant (Amakura et al., 2000).

Consumption of blackcurrants has been claimed to be beneficial in the prevention and treatment of many disorders. For example, cardiovascular disease (Slimestad et al., 2002) may be remediated by decreasing platelet coagulability, improving blood filterability and capillary penetration, and by having a relaxing effect on thoracic aorta, as reported in rats (Matsumoto et al., 2005). It has been also reported by Edwards et al. (2000) that dietary intake of anthocyanins can result in a significant reduction of fatigue and sleep disturbance and in the management of primary fibromyalgia. These results suggest that poor peripheral circulation may cause some of these problems related with fibromyalgia. Similar findings were also reported by Matsomoto et al. (2005) who indicated that subjective symptoms of fatigue of the head, neck, arm, eye and lower back during two hours of typing work could be reduced by consuming blackcurrants.

Many studies have further investigated the effect of blackcurrant in the improvement of visual function and dark adaptation (Matsumoto et al., 2005; Nakajima et al., 2004). In addition, the regeneration of the retinal pigment responsible for the perception of light, rhodopsin, may be influenced by blackcurrant consumption (Matsumoto et al., 2003). It has also been suggested that cyanidin can affect the phototransduction cascade, a process that converts light to electrical signals in rod cells, cone cells, and photosensitive ganglion cells of the retina of the eye, by activating or inhibiting phosphodiesterase activity (Matsumoto et al., 2003).
1.5. Grape

1.5.1. Introduction

Grape is a perennial, deciduous woody vine of the genus *Vitis*. Commonly known as grapevine, *Vitis* belongs to the botanical family *Vitaceae* (Burns et al., 2002) and is divided into two subgenera, *Euvitis* and *Muscadinia*, based on various morphological criteria. For example, *Euvitis* berries grow in clusters that are harvested as an intact unit upon ripening. In contrast, muscadine cultivars are harvested as individual berries that ripen over an extended harvest period. Compared to the *Euvitis* genera, Muscadine grapes are a better source of ellagic acid, resveratrol, quercetin, and other flavonols including anthocyanin 3,5-diglucoside (Yi et al., 2005).

According to the Food and Agriculture Organization (FAO) of the United Nations, 71% of total grape berry tonnage in the world is used for wine production, while only 27% and 2% of that are used as fresh and dried fruit, respectively. Most grapes grown and cultivated in the world today come from cultivars of *Vitis vinifera*, the European grapevine native to Mediterranean Central Asia. Some species such as *Vitis labrusca*, *Vitis riparia* and *Vitis rotundifolia* are also cultivated in North America and Asia (Walker et al., 2006). *Vitis vinifera* grows to 35 m in height with alternate, palmately lobed, 5-20 cm long and broad leaves. The grape berries can be vastly different in colour, including red, blue, white/green, and golden. In addition to adding colour, anthocyanins also contribute to the organoleptic and chemical properties of grapes and wines because of the interaction with other phenolic compounds as well as with proteins and polysaccharides (Pomar et al., 2005).
On average, grape berries contain 81% moisture, 94% carbohydrate, 4% protein, and 2% fat. Grape is very low in saturated fat, sodium, and cholesterol, and is also a very good source of Vitamin C and K; however, since a large proportion of its calories come from simple sugars, it is best consumed as part of a diverse diet of fruits, vegetables, and whole grains.

1.5.2. Grape anthocyanin

Grape skin and leaves contain high levels of anthocyanins and flavonols. The anthocyanins in grape skins are predominantly the 3-O-glucosides of malvidin, delphinidin, cyanidin, petunidin, and peonidin (Monagas et al., 2006). In addition to 3-monoglucosides, the 3-O-acylated monoglucosides also occur via acylation at the C-6 position of the glucose molecule by sterification with acetic, p-coumaric, and caffeic acids. Grape seeds are important sources of proanthocyanidins, namely procyanidins (oligomers and polymers of (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate), while skins and stems also contain prodelphinidins (Monagas et al., 2006).

Anthocyanins found in grapes are similar across cultivars; however, the relative amounts of individual compounds differ. For example, Pinot Noir grapes contain no acylated anthocyanins (Monagas et al., 2006). It has also been observed that climate conditions and the physical and chemical characteristics of the soil will influence the distribution and concentration of anthocyanins in grapes. Nevertheless, most references concur that environmental conditions and viticultural practices have a greater effect on
overall anthocyanin concentration rather than on relative distribution (Pomar et al., 2005). As such, the total anthocyanin content of red grapes can range anywhere from 300 to 7500 µg/g of the fresh weight of ripe berries (Monagas et al., 2006).

Relative concentrations of all anthocyanins, however, are not constant throughout maturation. Overall, total anthocyanin levels accumulate gradually in the skin during ripening. During ripening, levels of malvidin 3-glucoside and peonidin 3-glucoside increase, while other anthocyanin monoglucosides decrease due to the activity of flavonoid O-methyltransferase activity. Simultaneously, acylated anthocyanins tend to increase throughout ripening. (Kedage et al., 2007).

1.5.3. Bioactive properties of grape

In addition to the important role for red wine in cardiovascular disease reduction as part of the French Paradox phenomenon, polyphenols found in grape skin, such as resveratrol, have been demonstrated to prevent cancer, heart disease, and degenerative nerve disease. Grape skin polyphenols are also suspected to decrease the susceptibility to vascular damage, as well as decrease the activity of angiotensin, which is a systematic hormone causing blood vessel constriction. Terra et al. (2007) reported that proanthocyanidin extract recovered from grape seed had anti-inflammatory activity. Compared to aspirin (3 mM), indomethacin (20 µM), and dexamethasone (9 nM), the procyanidin extract showed a higher inhibitory effect on NO biosynthesis.

In terms of regenerative support, Zhong et al. (2007) reported on the effects of grape procyanidins in the inhibition of hepatocyte damage induced by ethanol and carbon tetrachloride and the stimulation of normal hepatocyte proliferation. Their results
indicated that the injury level of the grape procyanidin-treated group decreased in a dose dependent manner (5-100mg/L). Furthermore, their results showed that normal cells treated with grape procyanidin also had higher proliferative activities compared to the control group.

Finally, according to Jung et al. (2006), purple grape juice had a protective effect against breast cancer. Their data showed that grape juice consumption significantly inhibited \textit{in vivo} 7,12-dimethylbenzanthracence-DNA adduct formation in rat mammarys (Ju et al., 2007). In a clinical study, Park et al. (2004) also tested the effect of daily grape juice consumption on 67 healthy volunteers (16 women and 51 men). Evidently, consumption of grape juice increased the plasma antioxidant capacity, causing a reduction in DNA damage in peripheral lymphocytes achieved at least partially by a reduced release of ROS (Pask et al., 2003).

The literature summarized herein on the chemical composition of blackcurrant and wine grape berries suggests that respective antioxidant properties of each soft fruit source will be greatly influenced by the anthocyanin composition common to both the blackcurrant and wine grape which dominates the dihydromyricetin pathway and yields delphinidin anthocyanin; however, despite this similarity, it is noteworthy that the anthocyanin pathway is extended to malvidin in the grape, which is characteristically different from blackcurrant. The overall objective of this dissertation is to clarify the importance of the relative difference in anthocyanin composition between blackcurrant and wine grape fruit in terms of antioxidant functional properties.
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CHAPTER II.

Experiment 1. Physicochemical properties of blackcurrant anthocyanins

2.1. Introduction

Recently, considerable interest in plant secondary metabolites present in fruits has arisen due to a number of reported protective effects associated with these compounds against Alzheimer’s disease, cardiovascular heart disease (CVD), coronary heart disease (CHD), type II diabetes, hypertension, dyslipidemia (e.g. high total cholesterol, or high level of triglycerides), stroke and some cancers (Frank et al., 2002; Cacace et al., 2002). Among the large number of recognized polyphenols present in fruits, anthocyanins in particular have been specifically investigated due to the presence in a wide variety of fruits, which have high consumer consumption interest (He et al., 2005). In particular, anthocyanins from blackcurrant have been cited to improve visual function (Matsumoto et al., 2005; Matsumoto et al., 2002). It has been estimated in the United States for example, that the average per capita daily intake of anthocyanins is 200 mg (Wu et al., 2004; Matsumoto et al., 2001).

The major underlying mechanism for the physiological health benefits attributed to anthocyanins can be associated with antioxidant activities that modulate potential cellular metabolic disorders. For example, reports of a fatigue reducing effect and

\[1\] A version of this chapter will be submitted for publication. Hosseini Beheshti E. Kitts DD, Lund ST.
improvement of peripheral circulation due to blackcurrant consumption have been cited. In human studies, oral intake of blackcurrant anthocyanins in a mixture can result in significant improvement for dark adaptation and video display terminal (VDT) work-induced transient refractive alternation due to regeneration of rhodopsin (Matsumoto et al., 2002; Nakaishi et al., 2000; Matsumoto et al., 2001).

Anthocyanins represent a predominant class of water-soluble secondary metabolites present in plants, which are responsible for the red, violet, and blue colours of most berries and fruits (Nielsen et al., 2003). Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of a flavylium (2-phenylbenzopyrylium) salt. The major glycosidic sugars in the anthocyanin structure are glucose, galactose, rhamnose, and arabinose, while the common anthocyanin aglycones in plants are cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg) and malvidin (Mv) (Meskin et al., 2004). Of all soft fruits, berries represent one of the most important sources of dietary phenolic compounds (Cacace et al., 2002). Blackcurrants are the second strongest radical scavenger amongst other berries, and are known to contain approximately 250 mg anthocyanin per 100 g of fresh fruit (Cacace et al., 2002; Slimestad et al., 2002); however, of the 15 different anthocyanins that have been detected in the blackcurrant anthocyanin profile, cyanidin 3-glucoside (Cy3G), delphinidin 3-glucoside (Dp3G), cyanidin 3-rutinoside (Cy3R) and delphinidin 3-rutinoside (Dp3R) are responsible for approximately 98% of the total anthocyanin content (Nielsen et al., 2003).

Although there have been several studies demonstrating the effect of different environmental factors such as temperature, light source, and pH on anthocyanin
concentration, less is known about the extent to which anthocyanin composition influences total antioxidant capacity. This may be particularly important in cases where the chemical pathways for anthocyanin biosynthesis may differ characteristically between various sources of soft fruits. The present study was conducted to characterize, identify, and quantify the anthocyanin content in blackcurrant. In addition, the total phenolic content of blackcurrant was measured so that a comparison between the total phenolic content, total anthocyanin content and composition, and total antioxidant capacity could be made. As such, the main objective of this research was to determine whether the antioxidant capacity of a crude extract of blackcurrant represented either the sum of the individual anthocyanin fraction activities or, alternately, was due to a synergy between different anthocyanins.
2.2. Hypothesis and objectives

Experiment 1. Physiochemical properties of blackcurrant anthocyanin

Null hypothesis ($H_0$)

The content and individual anthocyanin components present in blackcurrants reflects the characteristic antioxidant capacity of the fruit.

Objective 1

To extract anthocyanins from different blackcurrant cultivars and determine total anthocyanin content and anthocyanin composition using semi-quantitative and qualitative methods.

Objective 2

To measure the antioxidant activities of the crude extract in blackcurrant using different antioxidant test methods that represent Single Electron Transfer (SET) and Hydrogen Atom Transfer (HAT).

Objective 3

To determine the relative contribution of each anthocyanin compound in blackcurrant to the total antioxidant capacity.
2.3. Materials and methods

2.3.1. Materials

Four different cultivars of blackcurrants (*Ribes nigrum*) were obtained from Agriculture Canada (Dr. Chris Nesson, Alberta, Canada). *Ben Alder, Ben Nevis, and Ben Sarek* cultivars were derived from a Scottish variety, whereas *Lentiay* is a Russian variety. *Ben Alder* and *Ben Nevis* were cultivated in Edmonton, Alberta, while *Ben Sarek* was grown in Red Deer, Alberta. *Lentiay* was grown in Brooks, Alberta. Methanol (MeOH), ethyl acetate (C₄H₈O₂), hydrochloric acid (HCl), formic acid (CH₂O₂), sodium acetate (CH₃COONa.3H₂O) and sodium carbonate (Na₂CO₃) were obtained from Fisher Scientific (Nepean, ON). Potassium persulphate (K₂S₂O₈), potassium chloride (KCl), 2, 2’- azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and gallic acid (C₇H₆O₅) were purchased from Sigma Co. (St. Louis, MO). Fluorescein, Trolox and 2, 2’-azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma Aldrich Chem Co. (Oakville, ON). Folin-Cioalteu reagent was from Fluka (Sigma-Aldrich Chemie GmbH, Switzerland). HPLC-grade cyanidin 3-glucoside standard was purchased from Polyphenol AS (Sandnes, Norway). Delphinidin 3-glucoside and cyanidin 3-rutinoside standards were obtained from Extrasynthese (Genay, France). Delphinidin 3-rutinoside standard was purchased from Apin Chemical Ltd (Oxfordshire, United Kingdom).
2.3.2. Methods

2.3.2.1. Plant material and sample preparation

The different samples of blackcurrant cultivars were received frozen and stored at -25°C in the Food Science Laboratory at UBC until used for analysis. After cleaning and removing all leaves and other debris, three sub-samples of each cultivar, consisting of 100-150 g of sample, were freeze-dried. The freeze-dried blackcurrant fruits containing skin, pumice and seeds, were ground to a fine powder at 4°C. The powder was kept in a container sealed with Parafilm “M” laboratory film to reduce oxygen contact and with aluminum foil to prevent light exposure while stored at -80 °C.

2.3.2.2. °Brix measurement

The term “Brix degrees” (°Brix) is defined as the mass-to-volume percentage of soluble solids present in a pure solution. Juice samples from thawed berries was analyzed for °Brix by passing the fruit homogenate through a double layer of cheesecloth. °Brix of the blackcurrant juice samples was then measured by a digital refractometer (SPER Scientific) in triplicate and results are given as °Brix. No corrections were made for frozen-thawed berries due to minimal drip loss.
2.3.2.3. Titratable acidity and pH

Titratable acidity was measured by placing 1.0 gram of the fruit juice extract in 100 mL deionized water and titrating the extract with 0.1 M sodium hydroxide (NaOH) to a final endpoint of pH 8.2. Results are given as gram tartaric acid equivalent per liter of fresh fruit juice extract.

Sample pH of fruit juice was measured using a Fisher Accumet, Model AB 15, pH meter (Fisher Scientific, Edmonton, AB) in triplicate at room temperature. Two point calibrations were accomplished using pH 4.0 and 7.0 buffers.

2.3.2.4. Extraction procedure

For anthocyanin extraction from blackcurrant, 1 g of freeze-dried fruit powder was weighed into a 50 mL screw-cap tube. The sample was extracted gradually 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 extract was centrifuged at room temperature at 1500 rpm for 20 min and the supernatant removed. The extraction process was repeated 2-3 times on the dried powder until the sample became colourless. The collected supernatants were combined and transferred to a 200 mL volumetric flask. MeOH/H₂O/HCl was added to the pooled supernatant to adjust the final volume to 200 mL. The final blackcurrant extracts were kept at -25°C until further analyses were conducted.
2.3.2.5. Determination of total anthocyanin content

The pH differential method, as described by Wrolstad and Giusti (2000), was used to measure the total anthocyanin content in blackcurrant. This method is derived from the fact that the absorptivity of anthocyanins is highly dependent on pH. Anthocyanin pigments change hue and intensity with pH due to a change in structure. The maximum wavelength and appropriate dilution factor was determined by preliminary experiments. Blackcurrant extracts were scanned using a Mandel UV-1700 spectrophotometer (Phamaspec) for maximum absorbance wavelength ($\lambda_{\text{vis-max}}$). The maximum absorbance wavelength of blackcurrant crude extract was determined to be 520 nm (Figure 2.1).

Blackcurrant crude extracts were diluted to an appropriate concentration (500-1000 µg/ml) with a potassium chloride buffer (0.025 M, pH 1) and a sodium acetate buffer (CH$_3$CONa.3H$_2$O, 0.4 M, pH 4.5) until the absorbance of the sample was within the linear range of the spectrophotometer readings. The mixture was then allowed to equilibrate for 15 min at room temperature and the absorbance was read at 520 nm and 700 nm, respectively, against a blank cell filled with distilled deionized water to correct for haze.

The absorbance of diluted sample ($A$) was calculated according to the following equation:

$$A = (A_{\lambda_{\text{vis-max}}} - A_{700}) \text{ pH } 1.0 - (A_{\lambda_{\text{vis-max}}} - A_{700}) \text{ pH } 4.5$$

The concentration of anthocyanins was calculated using the following formula and expressed in mg of cyanidin 3-glucoside / L:
Monomeric anthocyanins pigment (mg/L) = \((A \times MW \times DF \times 1000) / (\varepsilon \times l)\)

Where:

\(A\) = Absorbance of diluted sample

\(MW\) = Molecular weight (g/mol)

\(DF\) = Dilution Factor

\(\varepsilon\) = Extinction factor (L/cm×mol)

\(l\) = cell path length (cm)

MW and \(\varepsilon\) used in this formula correspond to the common anthocyanin in both samples. The MW and \(\varepsilon\) of Cy3G used in this experiment was 449.2 and 26,900 (L/cm×mol) respectively (Rubinskiene et al., 2005).

2.3.2.6. Determination of total phenolic content

The total phenolic concentration in the blackcurrant extracts determined in this study was performed using the Folin-Ciocalteu procedure (Hu et al., 2005). 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 (Ainsworth et al., 2007); however, the chemical nature of the Folin-Ciocalteu reaction mixture is unknown and other oxidation substrates may interfere with the color development in an inhibitory, additive, or enhancing manner. Nonetheless,
this method is a rapid, simple, and precise assay with high-throughput capacity, and has been widely used for total phenolic content measurement.

Briefly, 20µL of sample was mixed with 100 µL of 10-times diluted Folin-Ciocalteu reagent and 80 µL sodium carbonate (Na$_2$CO$_3$) 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 (Burns et al., 2001; Landbo and Mayer, 2001).

2.3.2.7. Measurement of antioxidant capacity of crude extract

The antioxidant capacity of the blackcurrant crude extract samples was measured using two antioxidant assays. First, the ABTS radical scavenging activity test represented a single electron transfer (SET) assay that was performed at room temperature. The data were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) with the final unit being µmol Trolox equivalent (TE)/g freeze-dried sample. The Oxygen Absorbance Radical Capacity (ORAC) assay represented a hydrogen atom transfer (HAT) method and was performed using the procedure of Kitts et al. (2005). Results were expressed in terms of micromole of Trolox equivalents (TE)/g freeze-dried sample.
2.3.2.7.1. ABTS method

The ABTS radical scavenging activity test was used to evaluate the free radical scavenging effect of blackcurrant crude extracts. This method was developed by Miller (1996) and is based on a discolouration reaction whereby ABTS radical cations (ABTS\(^+\)) are produced by adding 88 µL of a potassium persulphate solution (140 mM) to 5 mL of a 7 mM ABTS solution, and keeping the mixture overnight in the dark at room temperature (Valls-Belles et al., 2006). The blue-green species of the ABTS\(^+\) produced is best detected at 734 nm. Antioxidants, with scavenging activity inhibit this color production, which is proportional to the antioxidant capacity of the sample. In this method, results are expressed as an equivalent of the micromole concentration of Trolox standard solution per gram freeze-dried sample (Kaur and Geetha, 2006).

After 10 minutes of incubation at room temperature, the discolouration reaction at 734 nm was measured in a 96-well assay plate using a microplate spectrophotometer (Multiskan Spectrum, ThermoLabsystem) (Hu et al., 2005). The antioxidant capacity was expressed as TEAC and is expressed as µmol/g freeze-dried sample.

2.3.2.7.2. Oxygen radical absorbance capacity method

The ORAC assay is the only measurement of antioxidant capacity that combines both the percentage of inhibition and the length of inhibition of free radical formation by antioxidant compounds into a single identity. This method depends on a free radical attack of a fluorescent marker that results in a loss of fluorescence intensity. The extent of
decline in fluorescence intensity is an index of the extent of free radical damage to the probe. Antioxidant capacity is determined by measuring the area under the curve, which reflects the decreased rate and a reduced amount of product formed over time, as a proportion of activity present in a control sample without antioxidant protection (Prior et al., 2005).

Trolox standard (20µM), fluorescein (200 nM), and 2, 2’-azobis (2-amidinopropane) dihydrochloride (AAPH) (60 mM) solutions were prepared prior to use in phosphate buffer (75 mM, pH 7.0). Different volumes of samples (5-60 µL) and different volumes of Trolox (5-60 µL) were placed into the wells of 96-well assay plates and 60 µL of fluorescein was added to each well. Distilled deionized water was used as a blank. The final volume in each well was made up to 200 µL using buffer solution. The plate was incubated for 15 min with slow shaking and, subsequently, 40 µL of AAPH solution was added to each well. 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, Labsystem). A standard curve was obtained by plotting Trolox concentrations against the average net area under the curve (AUC) derived from at least three measurements for each concentration in each plate (Davalos et al., 2004). Final ORAC values were calculated based on the following formula:

\[
\text{ORAC number} = \frac{\text{Slope}_{\text{sample}}}{\text{Slope}_{\text{Trolox}}}
\]

The ORAC value was expressed as µmol Trolox/g freeze-dried sample.
2.3.2.8. **Anthocyanins characterization and quantification**

Anthocyanin profiles of blackcurrant were characterized and quantified using HPLC/UV (Agilent 1100 series), equipped with a diode array detector. A Zorbax RX-C18 (250×4.6 mm i.d, particle size = 5µm, Agilent) column was used at 45 °C with a linear gradient mobile phase containing solvent A (5% formic acid in water v/v) and solvent B (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), change at 5 min to 15% B, in 20 min to 25% B, and finally back to the initial solvent run conditions in 22 min (Wu et al., 2004; Rubinskiene et al., 2005).

Cyanidin 3-glucoside injected at a concentration of 0.10 mg/mL was used as the standard for relative quantitation of eluted unknown peaks.

2.3.2.9. **Anthocyanin separation with SPE column**

A pre-conditioned solid phase extraction C-18 column (500mg/4 cm; Burdick and Jackson, Honeywell) was used to separate phenolic acids from anthocyanins. Cartridges were preconditioned with 0.01% HCl-methanol, ethyl acetate, and 0.01% HCl-water, respectively, and dried under vacuum.
Blackcurrant 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 (Hu et al., 2003; Hu et al., 2007). Finally, 5 mL of 0.01% HCl-methanol was used to recover the anthocyanin extract.

2.2.2.10. Anthocyanins identification by LC/MS

The structural information of individual anthocyanins was obtained by LC/MSD Trap XCT Plus spectrometry equipped with an electrospray ionization interface (ESI). The separation of anthocyanins was performed using a Zorbax RX-C 18 (250 × 4.6 mm i.d, particle size = 5µm, Agilent) column at 45 °C with a linear gradient mobile phase, containing solvent A (5% (v/v) formic acid in water) and solvent B (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), change 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 (Hu et al., 2003).
2.3.2.11. Anthocyanin antioxidant capacity index

An anthocyanin antioxidant capacity index was constructed to evaluate the proportion of antioxidant capacity influenced by individual measured anthocyanins in the fruit extract. The Anthocyanin Antioxidant Capacity Index (AACI) was calculated using the following formula and expressed as μmol Trolox equivalent/ gram freeze-dried sample.

\[
\text{AAIC (µmol TE/ g dw) = } \sum (\text{Relative Individual Anthocyanin Concentration (mg/g)} \times \text{Individual Standard Anthocyanin ORAC value (µmol TE/ mg std)})
\]

2.3.2.12. Data evaluation

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. 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.
2.4. Results

2.4.1. Characterization and quantification of blackcurrant fruit components

2.4.1.1. Determination of pH, soluble solids content and titratable acidity

The pH of juice extract from different blackcurrant cultivars ranged between 2.75±0.01 to 2.79±0.02 showing no significant difference among cultivars. The percent soluble solids, measured using a digital refractometer, varied between 12.87 to 15.40%. The titratable acidity, calculated as grams of tartaric acid per liter fresh fruit juice, was found to range between 39.70±0.80 and 49.10±0.24 in different blackcurrant cultivars. A significant difference \((p<0.05)\) in titratable acidity between different cultivars was found. Ben Alder and Ben Sarek had significantly higher titratable acidity compared to Lentiay and Ben Nevis \((p<0.05)\), while there were no significant differences between Ben Alder and Ben Sarek or Ben Nevis and Lentiay \((p>0.05)\). The values for °Brix \(\times\) pH\(^2\) in different blackcurrant cultivars varied within the range of 97 to 117 (Table 2.1).
Table 2.1. Soluble solids (°Brix), pH and titratable acidity of blackcurrant cultivars.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Brix Reading (Brix%)</th>
<th>pH</th>
<th>Titratable Acidity (g TA/L)²</th>
<th>(Brix × pH²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ben Alder</td>
<td>15.40±0.00</td>
<td>2.76 ± 0.11</td>
<td>48.20 ± 0.25²</td>
<td>117</td>
</tr>
<tr>
<td>Ben Nevis</td>
<td>14.23±0.06</td>
<td>2.79 ± 0.02</td>
<td>38.80 ± 0.82²</td>
<td>111</td>
</tr>
<tr>
<td>Ben Sarek</td>
<td>12.87±0.06</td>
<td>2.75 ± 0.01</td>
<td>49.10 ± 0.24²</td>
<td>97</td>
</tr>
<tr>
<td>Lentiay</td>
<td>13.50±0.00</td>
<td>2.76 ± 0.04</td>
<td>39.70 ± 0.80²</td>
<td>103</td>
</tr>
</tbody>
</table>

¹ Value represents mean± SD (n=3). Different superscripted letters indicate significant differences (p<0.05).
² Titratable acidity results are expressed as grams of tartaric acid per liter of fresh fruit extract.

2.4.1.2. Determination of total phenolic and anthocyanin content in blackcurrant

Total phenolics: The total phenolic and anthocyanin contents of blackcurrant berries is presented in Table 2.2. For different cultivars, total phenolic content ranged from 94±8 to 163±2 µmol of gallic acid per gram of freeze-dried sample. A significant difference (p<0.05) in total phenolic content existed among cultivars. While Ben Alder and Ben Sarek were not significantly different (p>0.05), both cultivars contained significantly (p<0.05) lower total phenolics than Ben Nevis. Lentiay crude extract with 94±8 µmol of gallic acid per gram of freeze-dried sample had the lowest total phenolic content among other blackcurrant cultivars tested (p<0.05).

Total Anthocyanins: The total anthocyanin content of different blackcurrant cultivars, as measured by the pH differential method at maximum wavelength (520 nm)(Figure 2.1), ranged from 29±1 to 75±6 mg Cy3G/L sample extracts (Table 2.2). Ben Sarek and Lentiay were not significantly different in anthocyanin content, but when compared to Ben Nevis and Ben Alder cultivars, each contained significantly less.
anthocyanin \((p<0.05)\). *Ben Nevis* with 75±6 mg Cy3G per liter extract had the highest total anthocyanin content among all cultivars.

**Table 2.2. Total phenolic (µmol GA/g) and total anthocyanin (mg Cy3G/L) contents in blackcurrant cultivars**

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Total phenolic content (µmol GA/ g)(^2)</th>
<th>Total Anthocyanin Content (mg Cy3G/L)(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ben Alder</td>
<td>137 ± 11 (^a)</td>
<td>61 ± 6 (^a)</td>
</tr>
<tr>
<td>Ben Nevis</td>
<td>163 ± 2 (^b)</td>
<td>75 ± 6 (^b)</td>
</tr>
<tr>
<td>Ben Sarek</td>
<td>132 ± 4 (^a)</td>
<td>30 ± 1 (^c)</td>
</tr>
<tr>
<td>Lentiay</td>
<td>94 ± 8 (^c)</td>
<td>29 ± 1 (^c)</td>
</tr>
</tbody>
</table>

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

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

\(^3\) Total anthocyanin content is expressed as milligram of equivalent (cyanidin 3-glucoside) per liter extract.
2.4.2. Characterization of anthocyanins in blackcurrant

2.4.2.1. Quantification and characterization of anthocyanins in blackcurrant crude extract

The HPLC/UV chromatograms of different anthocyanin profiles obtained for each cultivar are illustrated in Figures 2.2, 2.3, 2.4, and 2.5 for Ben Alder, Ben Nevis, Ben Sarek and Lentiay blackcurrant cultivars, respectively. The elution order of the four major anthocyanins in all tested blackcurrant cultivars were delphinidin 3-glucoside (Dp3G), delphinidin 3-rutinoside (Dp3R), cyanidin 3-glucoside (Cy3G) and cyanidin 3-rutinoside (Cy3R), respectively. Quantification of each separate anthocyanin was performed relative to a known cyanidin 3-glucoside concentration. The major anthocyanin in all the cultivars was delphinidin 3-rutinoside (peak 4), with 42.28 mg Cy3G equivalent per gram freeze-dried blackcurrant sample in Ben Alder, 43.04 mg Cy3G equivalent/g (dw) Ben Nevis and 49.25 mg Cy3G equivalent/g (dw) in Lentiay. The one exception was Ben Sarek, which
had the relatively highest amount of cyanidin 3-rutinoside (peak 6) in its anthocyanin profile. Peak 5 (Cy3G) displayed the lowest amount of anthocyanins in all cultivars. Data are shown in Table 2.3 (Ben Alder), Table 2.4 (Ben Nevis), Table 2.5 (Ben Sarek) and Table 2.6 (Lentiay).

Different anthocyanins were identified on the basis of molecular weight and product ion fragments using LC/MS. As previously mentioned, the structural identification of the individual anthocyanins was obtained by liquid chromatography coupled with ion-trap mass spectrometry (LC/MS); the four identified anthocyanin peaks in all different blackcurrant cultivars were delphinidin 3-glucoside (peak 3), delphinidin 3-rutinoside (peak 4), cyanidin 3-glucoside (peak 5) and cyanidin 3-rutinoside (peak 6), respectively (Table 2.7).
Figure 2.2. A typical HPLC/UV chromatogram (520nm) showing anthocyanin separation from blackcurrant *Ben Alder* cultivar. Dp3G = delphinidin 3-glucoside, Dp3R = delphinidin 3-rutinoside, Cy3G = cyanidin 3-glucoside, Cy3R = cyanidin 3-rutinoside.

Figure 2.3. A typical HPLC/UV chromatogram (520nm) showing anthocyanin separation from blackcurrant *Ben Nevis* cultivar. Dp3G = delphinidin 3-glucoside, Dp3R = delphinidin 3-rutinoside, Cy3G = cyanidin 3-glucoside, Cy3R = cyanidin 3-rutinoside.
Figure 2.4. A typical HPLC/UV chromatogram (520nm) showing anthocyanin separation from blackcurrant *Ben Sarek* cultivar. Dp3G = delphinidin 3-glucoside, Dp3R = delphinidin 3-rutinoside, Cy3G = cyanidin 3-glucoside, Cy3R = cyanidin 3-rutinoside.

Figure 2.5. A typical HPLC/UV/UV chromatogram (520nm) showing anthocyanin separation from blackcurrant *Lentiay* cultivar. Dp3G = delphinidin 3-glucoside, Dp3R = delphinidin 3-rutinoside, Cy3G = cyanidin 3-glucoside, Cy3R = cyanidin 3-rutinoside.
Table 2.3. HPLC/UV quantification of major anthocyanins in *Ben Alder* blackcurrant.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Peak</th>
<th>HPLC Retention time (min)</th>
<th>Concentration (mg/g)¹</th>
<th>Percentage²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ben Alder</td>
<td>3</td>
<td>11.94</td>
<td>77.53 ± 5.23</td>
<td>15.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.57</td>
<td>218.59 ± 13.63</td>
<td>42.28</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.74</td>
<td>34.59 ± 1.90</td>
<td>6.69</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>16.93</td>
<td>174.00 ± 12.00</td>
<td>33.65</td>
</tr>
</tbody>
</table>

¹ Results are expressed as milligram equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean ± SD), (n=3).
² Percentage = Relative % of total concentration.

Table 2.4. HPLC/UV quantification of major anthocyanins in *Ben Nevis* blackcurrant.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Peak</th>
<th>HPLC Retention time (min)</th>
<th>Concentration (mg/g)¹</th>
<th>Percentage²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ben Nevis</td>
<td>3</td>
<td>11.9</td>
<td>87.57 ± 4.50</td>
<td>14.52</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.5</td>
<td>259.53 ± 12.89</td>
<td>43.04</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.7</td>
<td>37.96 ± 2.89</td>
<td>6.30</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>16.9</td>
<td>205.72 ± 9.95</td>
<td>34.12</td>
</tr>
</tbody>
</table>

¹ Results are expressed as milligram equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean ± SD), (n=3).
² Percentage = Relative % of total concentration.

Table 2.5. HPLC/UV quantification of major anthocyanins in *Ben Sarek* blackcurrant.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Peak</th>
<th>HPLC Retention time (min)</th>
<th>Concentration (mg/g)¹</th>
<th>Percentage²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ben Sarek</td>
<td>3</td>
<td>12.0</td>
<td>49.52 ± 3.30</td>
<td>25.16</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.7</td>
<td>50.40 ± 3.23</td>
<td>25.61</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.8</td>
<td>29.84 ± 1.76</td>
<td>15.16</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>17.1</td>
<td>63.24 ± 3.70</td>
<td>32.13</td>
</tr>
</tbody>
</table>

¹ Results are expressed as milligram equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean ± SD), (n=3).
² Percentage = Relative % of total concentration.

Table 2.6. HPLC/UV quantification of major anthocyanin in *Lentiay* blackcurrant.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Peak</th>
<th>HPLC Retention time (min)</th>
<th>Concentration (mg/g)¹</th>
<th>Percentage²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lentiay</td>
<td>3</td>
<td>11.9</td>
<td>37.33 ± 2.10</td>
<td>17.14</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.6</td>
<td>107.28 ± 5.90</td>
<td>49.25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.8</td>
<td>12.72 ± 0.72</td>
<td>5.84</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>17.0</td>
<td>56.61 ± 2.79</td>
<td>26.00</td>
</tr>
</tbody>
</table>

¹ Results are expressed as milligram equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean ± SD), (n=3).
² Percentage = Relative % of total concentration.
Table 2.7. Characterization of anthocyanins present in blackcurrant analyzed by LC/MS.

<table>
<thead>
<tr>
<th>Cultivar&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Peak&lt;sup&gt;2&lt;/sup&gt;</th>
<th>[M+H]&lt;sup&gt;+3&lt;/sup&gt;</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, B, C, D</td>
<td>3</td>
<td>465</td>
<td>Delphinidin 3-glucoside</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>611</td>
<td>Delphinidin 3-rutinoside</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>449</td>
<td>Cyanidin 3-glucoside</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>595</td>
<td>Cyanidin 3-rutinoside</td>
</tr>
</tbody>
</table>

<sup>1</sup>Data represent analysis of major anthocyanin in each blackcurrant cultivar. A=Ben Alder, B=Ben Nevis, C=Ben Sarek, D=Lentiay, (n=3).

<sup>2</sup>Peaks 1, 2, 7, 8 on chromatogram were not identified.

<sup>3</sup>[M+H]<sup>+</sup>: Parent ion.
2.4.3. Antioxidant capacity determination

2.4.3.1. Determination of crude extracts antioxidant capacity

*ABTS*: The antioxidant capacity results of blackcurrant crude extracts measured using the ABTS assay are shown in Table 2.8. ABTS results ranged from 106±6 to 138±8 μmole Trolox/g freeze-dried sample. The *Ben Nevis* and *Lentiay* cultivars had the highest and lowest ABTS radical quenching activities, respectively, of the four different cultivars (*p*<0.05).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Crude Extract</th>
<th></th>
<th>ORAC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABTS</td>
<td>(μmol TE/g)²</td>
<td>(μmol TE/g)²</td>
<td></td>
</tr>
<tr>
<td><em>Ben Alder</em></td>
<td>123 ± 2</td>
<td>a</td>
<td>651±27</td>
<td>a</td>
</tr>
<tr>
<td><em>Ben Nevis</em></td>
<td>138 ± 8</td>
<td>b</td>
<td>828±52</td>
<td>b</td>
</tr>
<tr>
<td><em>Ben Sarek</em></td>
<td>115 ± 1</td>
<td>ac</td>
<td>715±44</td>
<td>c</td>
</tr>
<tr>
<td><em>Lentiay</em></td>
<td>106 ± 6</td>
<td>c</td>
<td>620±123</td>
<td>cd</td>
</tr>
</tbody>
</table>

1Values represent mean ± 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.

*ORAC*: Antioxidant capacity of blackcurrant crude extracts as measured by ORAC is also given in Table 2.8. ORAC values ranged from 620±123 to 828±52 of μmol Trolox equivalents (TE)/gram of freeze-dried blackcurrant. Similar to the ABTS results, the *Ben Nevis* and *Lentiay* crude extracts gave the highest and lowest antioxidant capacities, respectively (*p*<0.05).
2.4.3.2. ORAC measurements of antioxidant capacities of semi-purified anthocyanin extracts collected from different blackcurrant cultivars.

![Graph showing ORAC antioxidant capacity of blackcurrant semi-purified anthocyanin extracts](image)

**Figure 2.6. Antioxidant capacity of blackcurrant semi-purified anthocyanin extract**

Values represent mean ± SD, (n=3). Different superscripted letters indicate significant differences (p<0.05).

The ORAC antioxidant capacity of semi-purified anthocyanin extracts collected from different blackcurrant cultivars ranged between 3047±690 to 5450±990 µmol TE per gram of freeze-dried sample with significant differences (p<0.05) found between cultivars (Figure 2.6). The antioxidant capacity of the semi-purified anthocyanin extract of Lentiay was significantly (p<0.05) lower than other semi-purified anthocyanin extracts collected from Ben Alder and Ben Nevis. No significant difference was observed in the ORAC activity of Ben Sarek and Lentiay semi-purified anthocyanin extract antioxidant activity.
2.4.3.3. ORAC value for pure anthocyanin standards

ORAC antioxidant capacity of four major anthocyanin standards determined to be present in blackcurrant extract is presented in Table 2.9. Results for antioxidant capacities of four anthocyanin standards varied between 4.05±0.27 for delphinidin 3-glucoside to 6.36±0.37 for cyanidin 3-glucoside. Results indicated that all cyanidin aglycons produced significantly \( p<0.05 \) higher ORAC antioxidant capacities compare with delphinidin anthocyanin. No significant difference was observed between same anthocyanins containing different sugar moieties.

<table>
<thead>
<tr>
<th>Anthocyanins</th>
<th>ORAC (μmol TE/μmol Std)(^1)</th>
<th>Relative Capacity(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin 3-glucoside</td>
<td>6.36±0.37(^a)</td>
<td>1:1</td>
</tr>
<tr>
<td>Cyanidin 3-rutinoside</td>
<td>6.06±0.26(^a)</td>
<td>0.95:1</td>
</tr>
<tr>
<td>Delphinidin 3-glucoside</td>
<td>4.05±0.27(^b)</td>
<td>0.64:1</td>
</tr>
<tr>
<td>Delphinidin 3-rutinoside</td>
<td>5.03±0.03(^b)</td>
<td>0.79:1</td>
</tr>
</tbody>
</table>

\(^1\)Values represent mean±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.

\(^2\)Relative capacity, given cyanidin 3-glucoside=1.0.
2.4.3.4. Determination of anthocyanin antioxidant capacity index

The anthocyanin antioxidant capacity index (AACI), which was derived from the antioxidant (ORAC) activity for each of four anthocyanin standards (Table 2.9) and the relative concentrations of corresponding anthocyanins (determined as cyanidin 3-glucoside equivalent) (Tables 2.3 to 2.6) is presented in Table 2.10. These values were compared to the range of ORAC measures determined from semi-purified anthocyanin extracts in each cultivar. In the cases of Ben Alder and Ben Nevis, AACI values fell within the range of ORAC antioxidant measures for semi-purified extracts, indicating strong association between antioxidant capacity of individual anthocyanins and the sum of individual anthocyanins in the mixture. In contrast, blackcurrant cultivars Ben Sarek and Lentiay had AACI values that were not in the range of the semi-purified anthocyanin ORAC activity. These two cultivars had AACI values that were less than the expected range for antioxidant capacity of anthocyanin extracts measured in these cultivars. This result corresponded to a lower percentage of delphinidin 3-rutinoside in Ben Sarek and a lower percentage of cyanidin 3-rutinoside and cyaniding-3-glucoside in Lentiay.

Potential synergy between anthocyanin components in regards to ORAC antioxidant capacity for each cultivar is illustrated in Figure 2.7. Synergy is predicted from the difference between AACI and the actual ORAC values for the semi-purified anthocyanin extracts (Table 2.10). Ben Sarek and Lentiay exhibited the greatest potential for synergy of anthocyanin components in semi-purified extracts, compared to Ben Nevis and Ben Alder, respectively.
Table 2.10. Blackcurrant anthocyanin extract antioxidant capacity and anthocyanin antioxidant capacity index.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Anthocyanin Extract Antioxidant Capacity (µmol TE/g)¹,²</th>
<th>Anthocyanin Antioxidant Capacity Index (µmol TE/g)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ben Alder</td>
<td>4217-6254</td>
<td>4737</td>
</tr>
<tr>
<td>Ben Nevis</td>
<td>4278-6494</td>
<td>5532</td>
</tr>
<tr>
<td>Ben Sarek</td>
<td>2861-5341</td>
<td>1913</td>
</tr>
<tr>
<td>Lentiay</td>
<td>2387-3760</td>
<td>1965</td>
</tr>
</tbody>
</table>

¹Results represent the range of anthocyanin extract antioxidant capacity (n=3). ²Results are expressed as micromole of Trolox per gram of freeze-dried sample.

Figure 2.7. Indication of potential synergy from anthocyanin extract antioxidant capacity and anthocyanin antioxidant capacity index in different cultivars of blackcurrants¹.

¹Values represent mean ± SD, (n=3). Different superscripted letters indicate significant differences (p<0.05).
2.5. Discussion

2.5.1. Blackcurrant fruit pH, soluble solids content and titratable acidity

Blackcurrant fruit usually ripens from early July to mid-August in Northern latitudes; however, environmental conditions such as cold weather or a wet season can delay the time of harvesting fruit. Blackcurrant fruit is also very susceptible to mould growth and must be harvested in a sound condition which is both not under- or over-ripe. Over ripening of fruit will decrease fruit yield due to fruit “shivering” and “dropping”. The samples of different blackcurrant cultivars analyzed in this study were harvested in the middle of August, when the fruit was ripe and there was no evidence of mould growth on the samples.

It is well known that with maturation of the berry, or ripening, an increase in sugar content, pH, and berry size occurs, while the total acidity decreases (Anonymous 2007). The data determined for soluble solids content, pH, and titratable acidity of blackcurrant cultivars indicated that all samples were harvested at approximately the same development stage. The range of blackcurrant fruit pH agrees with a pH range reported by Heiberg et al. (1992). The range for titratable acidity also measured in this study agrees with previously reported data for blackcurrant (Zatyný et al., 2005); however, the range of soluble solids measured in this study was slightly lower than that reported by Heiberg et al. (1992). It is very likely that the slight difference noted herein between blackcurrant fruit cultivars and that reported by other workers can be explained by differences in environmental growing conditions, fruit genotype, and maturity.
2.5.2. Blackcurrant fruit total phenolic and anthocyanin

*Total Phenolics:* Total phenolic content provides a crude measurement of the phenolic compounds extracted in acidified methanol-water. The total phenolic content of fruit is an important factor governing total antioxidant capacity, as well as contributing to flavour, particularly, astringency. In this study, the total phenolic content of blackcurrant berries, as determined by Folin-Ciocalteu procedure, was higher than the total phenolic content reported for blackcurrant by Wu et al. (2004). Differences in blackcurrant total phenolic content between studies may be attributed to notable differences in maturity of blackcurrant cultivars or the use of different extraction solvents (Howard et al., 2000). In addition, the analysis used in this study was performed on the whole berry, which included seeds that contribute to the total phenolic content of the fruit extract.

*Total Anthocyanins:* Rubinskiene et al. (2005) showed that biosynthesis of anthocyanins occurs throughout berry maturation and that the most remarkable increase occurs when the skin colour changes to a dark hue, with nearly a two-fold increase in anthocyanin content occurring in over-ripe berries.

In this study the total anthocyanin content, as determined using a pH differential method, ranged almost three-fold which is in agreement with the total anthocyanin content and range of anthocyanin concentration reported for blackcurrant in other studies (Wu et al., 2004; Wada et al., 2002). Again, different growing conditions, stage of ripeness, cultivar differences, and variations expected using different extraction procedures are all considered to be important factors that will yield variability in total anthocyanin concentrations reported between laboratories. In this study, precautions were
taken to ensure that sampling of fruits from different cultivars was performed in a manner that randomized potential differences in maturity stages of sub-samples.

2.5.3. Blackcurrant fruit anthocyanin quantification and identification

A total of at least six anthocyanins was detected across all blackcurrant cultivars, which represent only a fraction of the 15 different anthocyanins reported in the blackcurrant anthocyanin profile by Nielsen et al. (2003). The concentrations of the different anthocyanins identified in the blackcurrant samples studied herein followed a general relative order of delphinidin 3-rutinoside > cyanidin 3-rutinoside > delphinidin 3-glucoside > cyanidin 3-glucoside, which is in agreement with published data (Wada et al., 2002; Kapasakalidis et al., 2006).

Individual anthocyanins identified by LC/MS from blackcurrant fruit were confirmed to be delphinidin 3-glucoside, delphinidin 3-rutinoside, cyanidin 3-glucoside, and cyanidin 3-rutinoside, respectively. These four major anthocyanin compounds were responsible for more than 97% of the total anthocyanin content measured in the blackcurrant cultivars tested. Additionally, in two of the four cultivars, two minor anthocyanin peaks (e.g. peak 7, 8 in figure 2.2 and 2.3) were also detected, but were not identified. On the basis of previous studies by Wu et al. (2004) and retention times obtained in this experiment, these peaks were likely petunidin 3-glucoside and pelargonidin 3-glucoside (Wu et al., 2004); however, Nielsen et al. (2003) determined that pelargonidin 3-glucoside was not present in blackcurrant. Other minor anthocyanins that have been identified in blackcurrant and represent less than 3% of blackcurrant anthocyanin content are petunidin 3-rutinoside, peonidin 3-glucoside, delphinidin 3-
xyloside, pelargonidin 3-rutinoside, peonidin 3-rutinoside, cyanidin 3-xyloside, petunidin 3-(6-coumaryl)-glucoside and cyanidin 3-(6-coumaryl)-glucoside (Wu et al., 2004).

Delphinidin 3-rutinoside was the major anthocyanin present in all the blackcurrant cultivars with the one exception being Ben Sarek. The anthocyanin 3-rutinosides were found to be at least 3.2 times higher than those of anthocyanin 3-glucosides (except for Ben Sarek) which agrees with the data reported by Malien-Aubert et al. (2001). With the exception of the Ben Sarek cultivar, the delphinidin derivatives (in particular rutinoside) was at least 2.8 times higher than that of glucoside. A similar finding was also found for cyanidin derivatives.

2.5.4. Blackcurrant fruit antioxidant capacity

ABTS: Antioxidant capacity of blackcurrant crude extracts determined using the ABTS method, a Single Electron Transfer mechanism (SET-based) assay, produced results that indicated variation in antioxidant capacity among cultivars. The ABTS assay is based on free radical scavenging alone. In other words, the ABTS result should be regarded as a measure of antioxidant recycling activity (e.g. vitamin C regeneration) (Johnston et al., 2006). Kapasakalidis et al. (2006) reported lesser ABTS antioxidant capacity for blackcurrant; however, those data were obtained with a blackcurrant juice extract, whereas our findings relate to the total berry which included both seeds and skin as well as the pulp fractions. The results of this study show that blackcurrant cultivars containing the greatest total phenolic content and total anthocyanin content also exhibit the highest antioxidant capacity. Regression analysis revealed that ABTS antioxidant
capacity data were highly correlated with total anthocyanin ($r^2=0.88$) and total phenolic
($r^2=0.92$) contents.

**ORAC:** Oxygen Radical Absorbance Capacity (ORAC) assay and Total Radical
Trapping Antioxidant Parameter (TRAP) assay are two assays which involve a hydrogen
atom transfer (HAT) reaction with unique differences associated mainly with relative
quantification approaches. The ORAC assay specifically applies the area under a lipid
peroxidation kinetic curve (AUC) and is an assay in which an antioxidant capacity
containing constituent in a sample reduces the oxidation of a probe by a biologically
relevant radical source (e.g. peroxyl radical source), which is generated by thermal
decomposition of AAPH (Huang et al., 2005). The antioxidant capacities of crude
blackcurrant extracts as measured by ORAC varied between cultivars and were
approximately two times higher than those reported by Wu et al. (2004). These
differences may be due, in part, to the differences in cultivars tested, or the presence of
seed phenolics which have ORAC activity. The cultivars tested in this research were Ben
Alder, Ben Nevis, Ben Sarek, and Lentiay, whereas Wu et al. (2004) used four other
cultivars in addition to Ben Alder and Ben Nevis. It was of particular interest that the Ben
Nevis cultivar examined in this study which had the greatest concentration of anthocyanin
and phenolics also exhibited the highest ORAC antioxidant capacity.

In addition to the different pre-harvesting conditions employed, using different
parts of the berry will also produce different results (Wu et al., 2004). According to
Dimitrios (2006), phenolic compounds including flavanols, hydroxycinamnic acids,
hydroxybenzoic acids, and anthocyanins are active antioxidants in berry fruits.
In the present study, a linear relationship was found between ORAC values and total phenolic content \( (r^2=0.78) \), implying that the total antioxidant capacity of blackcurrant crude extract was influenced to a marked amount by the presence of phenolic compounds. It is also noteworthy that the relationship between total anthocyanin content and ORAC was not strong \( (r^2=0.43) \) in comparison to the ABTS findings.

ORAC values obtained for semi-purified anthocyanin extracts derived from blackcurrant contained more than five times the antioxidant capacity compared to the respective crude blackcurrant extracts. This finding indicates that anthocyanin purification steps produce higher yields of antioxidant capacity, which were related to a purified, standardized compositional mixture of anthocyanins in blackcurrant. These results agree with similar anthocyanins purified by column chromatography from blackberry in which ORAC antioxidant capacity gave a 5-fold higher ORAC activity that corresponded to the enriched anthocyanin content was observed (Elisia et al., 2007).

Evaluation of the different anthocyanins in the mixtures from all blackcurrant cultivars supports the hypothesis that antioxidant capacity of blackcurrant can be explained by subtle difference in anthocyanin composition and concentration. To further test this hypothesis, the relative antioxidant capacities of four different major anthocyanin standards present in blackcurrant fruits were assessed using the ORAC assay. The data suggested that some structure-activity relationship (SAR) exists with individual anthocyanin standards tested in blackcurrant by ORAC. Indeed, the ORAC value of the four aglycons studied varied with the difference between the hydroxyl group positions in the B-ring. These results indicate the importance of different hydroxyl group substitutions on antioxidant capacity. Our data agree with published results of Wang et al. (1997), who
found that cyanidin which is a 3',4'-dihydroxy anthocyanin has a relatively higher antioxidant capacity among other aglycons. The exception to this rule is that delphinidin, which is 3',4',5'-trihydroxy anthocyanin, yields lower ORAC activity than cyanidin (Wang et al., 1997). It was also of particular interest that the sugar moiety did not affect the ORAC antioxidant capacity of same aglycon to the same extent. For example, no differences in ORAC activity were found between the 3-glucoside and 3-rutinoside of cyanidin and delphinidin anthocyanins.

Our findings also describe a potential synergistic effect in the antioxidant capacity of the anthocyanin fractions. With the exception of Ben Nevis, the three other cultivars of blackcurrant with unique anthocyanin compositions had a higher total antioxidant capacity compared to a calculated anthocyanin antioxidant capacity index. These data not only suggested that the total anthocyanin composition of each fruit is important for antioxidant capacity, but also the proportion in each anthocyanin in the total mixture could strongly affect antioxidant capacity of blackcurrant fruit. For example, Ben Sarek, which has approximately a two-fold lower amount of delphinidin 3-rutinoside compared to other tested blackcurrant cultivars, exhibited the lowest anthocyanin antioxidant capacity index. The anthocyanin profile found in Lentiay, which had the second lowest anthocyanin antioxidant capacity index compared to other three blackcurrant tested cultivars, corresponded to lower cyanidin 3-glucoside. Taken together, these results show how small differences between the concentration of anthocyanins in different blackcurrant cultivars can affect the absolute total antioxidant capacity of fruits. It is important to recognize the fact that calculations made in this study are an attempt to predict synergies between anthocyanins only involving 4 of the 15 individual
anthocyanins present in blackcurrant. These calculations, however, can be justified on the basis that the four anthocyanins measured in this study represent more than 97% of total anthocyanin in blackcurrant.
2.6. References


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CHAPTER III.

Experiment 2. Physicochemical properties of grape anthocyanins

3.1. Introduction

An imbalance between the overproduction of free radicals and the reduction of biological detoxification factors of Reactive Oxygen Species (ROS) by antioxidants in vivo results in a condition commonly known as oxidative stress (Halliwell et al., 2004). ROS are a highly toxic, mutagenic, and reactive species, which are continuously produced in cells as a result of ordinary energy producing metabolic processes. Many free radicals are highly unstable and can interact non-specifically with cellular macromolecules such as lipids, protein, and DNA, which in turn will result in oxidative damage (Wang et al., 2000). Oxidative damage has been implicated as a contributing factor in at least 50 diseases, such as atherosclerosis, cardiovascular heart disease, cancers, Parkinson’s, and Alzheimer’s diseases (Hu et al., 2005; Frank et al., 2002).

Due to a worldwide movement towards increased consumption of fruit and vegetable food products, dietary antioxidants are of interest to scientists who study the link between soft fruit consumption patterns and protection against free radical-associated disease (Hu et al., 2005). Specifically, investigation into the possible health benefits of red wine consumption in the Mediterranean and French diets has drawn attention to foods

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1 A version of this chapter will be submitted for publication. Hosseini Beheshti E, Lund ST, Kitts DD.
that are rich in anthocyanins (Degenhardt et al., 2000). Some epidemiological data have shown that moderate consumption of red wine can reduce the risk of cardiovascular heart diseases (CVD) and lower the susceptibility of plasma and low-density lipoprotein (LDL) to lipid peroxidation (Yamakoshi et al., 1999). Because of these properties, anthocyanin antioxidative activity could play an important role in reducing the risk of many diseases such as CVD.

Anthocyanins are responsible for the coloring of grapes and red wines (Burns et al., 2002). Different factors, such as the variety, maturity, climate, and the fruit yield affect both the amount and concentration of anthocyanins in grapes. The total anthocyanin content of red grape ranges widely between 300 to 7500 µg/g fresh weight of ripe berries (Burns et al., 2002). Many studies have reported that the predominant anthocyanins in grape skin are the 3-glucosides of cyanidin, delphinidin, petunidin, peonidin, and malvidin (Burns et al., 2002). Less information is available concerning the extent to which anthocyanin composition in grape influences total antioxidant capacity. This may be particularly important in cases where the chemical pathways for anthocyanin biosynthesis may differ characteristically between various sources of soft fruits.

In response to this question, the main objective of this research was to determine whether the antioxidant capacity of crude extracts derived from different grape cultivars either represent the sum of the individual anthocyanin fraction activities or, alternately, is due to a synergy between different anthocyanin components. To achieve this objective, the anthocyanin content of different grape cultivars was characterized. In addition, the
total phenolic content of grape was measured so that a comparison between the total phenolic content, total anthocyanin content, and total antioxidant capacity could be made.
3.2. Hypothesis and objectives

Experiment 2. Physiochemical properties of grape anthocyanin

Null hypothesis (H₀)

The total and sum of the individual anthocyanins in grape equivalently reflect the characteristic antioxidant capacity of the fruit.

Objective 1

To extract anthocyanins from different grape cultivars and determine total anthocyanin content and anthocyanin composition using HPLC/UV and LC/MS analytical methods.

Objective 2

To measure the antioxidant capacities of the grape crude extract using ABTS and ORAC assays.

Objective 3

To determine the relative contribution of each anthocyanin compound in different grape cultivars to total antioxidant capacity.
3.3. Materials and methods

3.3.1. Materials

Berries from three *V. vinifera* cultivars, *Cabernet Sauvignon*, *Merlot*, and *Pinot Noir*, were obtained from a commercial vineyard near Osoyoos, BC (Canada). Methanol (MeOH), ethyl acetate (C₄H₈O₂), hydrochloric acid (HCl), formic acid (CH₂O₂), sodium acetate (CH₃COONa.3H₂O) and sodium carbonate (Na₂CO₃) were obtained from Fisher Scientific (Nepean, ON). Potassium persulphate (K₂S₂O₈), potassium chloride (KCl), 2, 2’- azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and gallic acid (C₇H₆O₅) were purchased from Sigma Co. (St. Louis, MO). Fluorescein, Trolox, and 2, 2’-azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma Aldrich Chem. Co. (Oakville, ON). Folin-Ciocalteu reagent was purchased from Fluka (Sigma-Aldrich Chem. GmbH, Switzerland). HPLC-grade cyanidin 3-glucoside standard was purchased from Polyphenol AS (Sandnes, Norway). Delphinidin 3-glucoside and cyanidin 3-glucoside, malvidin 3-glucoside, and peonidin 3-glucoside standards were obtained from Extrasynthese (France).
3.3.2. Methods

3.3.2.1. Plant material and sample preparation

Grape samples arrived to UBC frozen. Samples were stored frozen at -25°C in the Food Science laboratory until used. After cleaning, 100-150 g of each of the three different grape cultivars were freeze dried. Similar to the processing of blackcurrant samples (2.3.2.1.), the freeze-dried grape samples were ground at 4°C with the skin, pumice, and seeds still intact. Prior to extraction, the powder was kept at -81°C in a sealed container with parafilm and aluminum foil to limit oxidation as well as light exposure.

3.3.2.2. °Brix measurement

Refer to determination of °Brix measurement in 2.3.2.2.

3.3.2.3. pH and titratable acidity

Refer to determination of pH and titratable acidity in 2.3.2.3.

3.3.2.4. Extraction procedure

Extraction of anthocyanins from grape samples was performed using a similar extraction procedure described for the blackcurrant sample (2.3.2.4.). Anthocyanin extraction was performed by weighing approximately 1 g of freeze-dried grape powder from each cultivar in a 50 mL screw-cap tube. The sample was extracted with 100 mL of
methanol/water/hydrochloric acid (80:20:0.05%; v/v, MeOH/H₂O/HCl). The extraction process was repeated 2-3 times on the dried powder until the sample became colorless.

3.3.2.5. Determination of total anthocyanin content

Refer to determination of total anthocyanin content in 2.3.2.5.

3.3.2.6. Determination of total phenolic content

Refer to determination of total phenolic content in 2.3.2.6.

3.3.2.7. Measurement of antioxidant capacity of crude extract

Grape crude extracts from different cultivars were prepared for total antioxidant capacity determination. Antioxidant analysis of grape extracts was performed using the ABTS (2.3.2.7.1) and Oxygen Radical Scavenging Activity (ORAC) assay (2.3.2.7.2.).

3.3.2.8. Anthocyanins characterization and quantification

High Performance Liquid Chromatography (HPLC) (Agilent 1100 series) equipped with a diode array detector was used to identify and quantify grape anthocyanins. A Zorbax RX-C18 column, 5µm; (250×4.6 mm) (Agilent) was used at 40 °C. The mobile phase consisted of aqueous 5% formic acid (A) and 100% methanol
(B). The flow rate was 1 mL/min, and detection for anthocyanin was set at 520 nm. The gradient applied for analysis was: 90 % A and 10% B at the separation time of injection (10 µL), for 20 min which was shifted to 35% B; for 4 minutes and returned to the remainder of the run time. Total run time was 28 min.

Cyanidin 3-glucoside injected at a concentration of 0.10 mg/mL was used as the external standard to quantify the relative eluted unknown peaks.

3.3.2.9. Grape anthocyanin separation with SPE column

Anthocyanins were separated as described in 2.3.2.9.

3.3.2.10. Anthocyanin identification by LC/MS

An Agilent 1100 series LC/MS, consisting of a binary pump and equipped with an electrospray interface operating in the positive ion mode, was employed using the following conditions: Capillary voltage, 3.5 kV; dry temperature, 350 °C; nebulizer 60.0 psi; scan range 40-750 m/z. The separation of anthocyanins was performed on a Zorbax RX-C18 (250×4.6 mm i.d, particle size = 5µm, Agilent) column at 40 °C with a linear gradient mobile phase containing solvent A (5% formic acid in water (v/v)) and solvent B (methanol). The injection volume was 10 µL and UV detection for anthocyanins was set at 520 nm. The gradient elution program was set to deliver 90% A and 10% B for 20 minutes changing to 35% B for 4 minutes and 10% B for the remainder of the retention
time. The flow rate was set for 1 mL/min and total elution time was programmed for 28 minutes.

3.3.2.11. Anthocyanin Antioxidant Capacity Index

Refer to anthocyanin antioxidant capacity index measurement in 2.3.2.11.

3.3.2.12. Data evaluation

Analyses were preformed on three sub-samples. Samples collected from each of these cultivars and results were expressed as mean ± SD (n=3). All statistical analyses were done using SPSS© for Windows v. 10.0. Statistical analysis was performed using one-way analysis of variance (ANOVA). Differences between means were evaluated using Tukey HSD and Games Howell test ($p<0.05$) for homogenous and heterogeneous variance, respectively.
3.4. Results

3.4.1. Characterization and quantification of anthocyanins in grape

3.4.1.1. Determination of pH, soluble solids content, and titratable acidity

The soluble solids content (degrees Brix) results varied within a narrow range of 18.06±0.97 to 18.88±2.17 with no significant difference attributed to cultivar source (Table 3.1). Grape pH ranged within 3.64 ± 0.01 to 3.88 ± 0.01 for all samples. Titratable acidity, which was calculated as gram of tartaric acid per liter fruit juice sample, was found to range between 3.72 to 4.14 (g TA/L) in grape samples, showing no significant difference between the Cabernet Sauvignon and Merlot cultivars, but when compared to the Pinot Noir cultivar, it was significantly \((p<0.05)\) lower. While the basis for the change in pH is complex and not always a good index for berry ripeness, a Brix \(\times\) pH\(^2\) coefficient, often used as an index indicating the ripeness of grape berry, showed that Pinot Noir gave relatively higher values than Cabernet Sauvignon and Merlot, respectively (Table 3.1). The results of Brix \(\times\) pH\(^2\) varied from 250 to 284.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Brix Reading (Brix%)</th>
<th>pH</th>
<th>Titratable Acidity (g TA/L)(^2)</th>
<th>(Brix (\times) pH(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabernet Sauvignon</td>
<td>18.06 ± 0.97</td>
<td>3.77 ± 0.02</td>
<td>3.73 ± 0.01(^a)</td>
<td>257</td>
</tr>
<tr>
<td>Merlot</td>
<td>18.86 ± 0.96</td>
<td>3.64 ± 0.01</td>
<td>3.72 ± 0.05(^a)</td>
<td>250</td>
</tr>
<tr>
<td>Pinot Noir</td>
<td>18.88 ± 2.17</td>
<td>3.88 ± 0.01</td>
<td>4.14 ± 0.10(^b)</td>
<td>284</td>
</tr>
</tbody>
</table>

\(^a\) Value represent mean ± SD \((n=3)\) for sub-sample obtained from each cultivar. Different letters indicate significant differences \((p<0.05)\).

\(^b\) Titratable acidity results are expressed as grams of tartaric acid per liter of fruit extract.
3.4.1.2. Determination of total phenolic and anthocyanin content in grape

*Total phenolics*: Total phenolic content, as measured by Folin-Ciocalteu procedure, varied between 107 to 121 μmol gallic acid/g freeze-dried powder and was not significantly different between cultivars (*Table 3.2*).

*Total anthocyanin*: The total anthocyanin content from different grape cultivars, measured using the pH differential method, is reported in *Table 3.2*. *Cabernet Sauvignon* and *Merlot* were not significantly different from one another, but the *Pinot Noir* cultivar was significantly (*p*<0.05) lower. In general, the total anthocyanin content in wine grapes ranged from 18 to 44 mg cyanidin 3-glucoside/L sample extract.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Total Phenolics (μmol GA/g)</th>
<th>Total Anthocyanin Content (mg C3G/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabernet Sauvignon</td>
<td>117 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Merlot</td>
<td>107 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pinot Noir</td>
<td>121 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Values represent mean± SD. Different letters indicate significant differences (*p*<0.05).
<sup>2</sup> Total phenolic results are expressed as micromole gallic acid per gram of freeze-dried sample.
<sup>3</sup> Total anthocyanins content expressed as milligram of equivalent (cyanidin 3-glucoside) per liter.
3.4.2. Identification of anthocyanins in grape

3.4.2.1. Quantification and identification of anthocyanins in grape crude extract

Typical HPLC/UV chromatograms for anthocyanin composition are shown in Figures 3.2, 3.3, and 3.4 for Cabernet Sauvignon, Merlot, and Pinot Noir, respectively. All grape cultivars exhibited seven distinct peak areas and had similar retention times for each. Quantification of anthocyanins separated from each cultivar of grape is given in Table 3.3 (Cabernet Sauvignon), Table 3.4 (Merlot), Table 3.5 (Pinot Noir). 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, whereas Pinot Noir displayed the majority of its anthocyanins in peak numbers 6 and 7. The dominant anthocyanin in all wine grape cultivars was peak 7, which accounted
for 46.92, 32.21, and 43.61% of the total anthocyanin for Cabernet Sauvignon, Merlot, and Pinot Noir, respectively.

Thus, these results indicate that the highest percentage of anthocyanins in all wine-grape cultivars was in fact malvidin 3-glucoside (Mv3G); whereas delphinidin 3-glucoside (Dp3G) was second most abundant anthocyanin recovered from Cabernet Sauvignon and Merlot. The exception to this was found with Pinot Noir, in which peonidin 3-glucoside (Pn3G) was the second most abundant anthocyanin. Other relatively minor anthocyanins present in all grape cultivars were petunidin 3-glucoside (Pn3G) and cyanidin 3-glucoside (Cy3G).

Using LC/MS, the different anthocyanins recovered from each grape cultivar were identified on the basis of molecular weight and product ion fragments. The five different anthocyanin 3-glucosides identified in grape cultivars included delphinidin 3-glucoside (peak 3), cyanidin 3-glucoside (peak 4), petunidin 3-glucoside (peak 5), peonidin 3-glucoside (peak 6), and malvidin 3-glucoside (peak 7).
Figure 3.2. A typical HPLC/UV chromatogram (520nm) showing anthocyanin separation from the Cabernet Sauvignon cultivar. Dp3G = delphinidin 3-glucoside, Cy3G = cyanidin 3-glucoside, Pt3G = petunidin 3-glucoside, Pn3G = peonidin 3-glucoside and Mv3G = malvidin 3-glucoside.

Figure 3.3. A typical HPLC/UV chromatogram (520nm) showing anthocyanin separation from the Merlot cultivar. Dp3G = delphinidin 3-glucoside, Cy3G = cyanidin 3-glucoside, Pt3G = petunidin 3-glucoside, Pn3G = peonidin 3-glucoside and Mv3G = malvidin 3-glucoside.

Figure 3.4. A typical HPLC/UV chromatogram (520nm) showing anthocyanin separation from the Pinot Noir cultivar. Dp3G = delphinidin 3-glucoside, Cy3G = cyanidin 3-glucoside, Pt3G = petunidin 3-glucoside, Pn3G = peonidin 3-glucoside and Mv3G = malvidin 3-glucoside.
Table 3.3. HPLC/UV quantification of major anthocyanins in *Cabernet Sauvignon*.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Peak</th>
<th>HPLC Retention time (min)</th>
<th>Concentration (mg/g)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Percentage&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabernet Sauvignon</td>
<td>3</td>
<td>11.4</td>
<td>19.55 ± 0.09</td>
<td>21.21</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.6</td>
<td>4.10 ± 0.14</td>
<td>4.45</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15.3</td>
<td>10.47 ± 0.26</td>
<td>11.36</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>17.3</td>
<td>8.00 ± 0.16</td>
<td>8.69</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>18.4</td>
<td>43.26 ± 1.29</td>
<td>46.92</td>
</tr>
</tbody>
</table>

<sup>1</sup> Results are expressed as milligram of fraction equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean ± SD). n=3.

<sup>2</sup> Percentage = Relative % of total concentration.

Table 3.4. HPLC/UV quantification of major anthocyanins in *Merlot*.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Peak</th>
<th>HPLC Retention time (min)</th>
<th>Concentration (mg/g)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Percentage&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merlot</td>
<td>3</td>
<td>11.4</td>
<td>28.72 ± 0.49</td>
<td>24.25</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.6</td>
<td>12.57 ± 0.10</td>
<td>10.62</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15.3</td>
<td>17.28 ± 0.46</td>
<td>14.59</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>17.3</td>
<td>14.47 ± 0.32</td>
<td>12.22</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>18.5</td>
<td>38.14 ± 0.68</td>
<td>32.21</td>
</tr>
</tbody>
</table>

<sup>1</sup> Results are expressed as milligram of fraction equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean ± SD). n=3.

<sup>2</sup> Percentage = Relative % of total concentration.

Table 3.5. HPLC/UV quantification of major anthocyanins in *Pinot Noir*.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Peak</th>
<th>HPLC Retention time (min)</th>
<th>Concentration (mg/g)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Percentage&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinot Noir</td>
<td>3</td>
<td>11.6</td>
<td>3.33 ± 0.13</td>
<td>6.12</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.6</td>
<td>1.85 ± 0.03</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15.3</td>
<td>3.95 ± 0.06</td>
<td>7.26</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>17.4</td>
<td>17.28 ± 0.28</td>
<td>31.79</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>18.5</td>
<td>23.71 ± 0.85</td>
<td>43.61</td>
</tr>
</tbody>
</table>

<sup>1</sup> Results are expressed as milligram of fraction equivalent (cyanidin 3-glucoside) per gram of freeze-dried sample (mean ± SD). n=3.

<sup>2</sup> Percentage = Relative % of total concentration.
Table 3.6. Characterization of anthocyanin present in grape cultivars analyzed by LC/MS

<table>
<thead>
<tr>
<th>Cultivar(^1)</th>
<th>Peak(^2)</th>
<th>[M+H](^3)</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A,B,C</td>
<td>3</td>
<td>465</td>
<td>Delphinidin 3-glucoside</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>449</td>
<td>Cyanidin 3-glucoside</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>479</td>
<td>Petunidin 3-glucoside</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>463</td>
<td>Peonidin 3-glucoside</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>493</td>
<td>Malvidin 3-glucoside</td>
</tr>
</tbody>
</table>

\(^1\)Data represent analysis of major anthocyanins in each grape cultivar. A=Cabernet Sauvignon, B=Merlot, C=Pinot Noir, \((n=3)\).
\(^2\)Peaks 1, 2, 8 on chromatogram were not identified.
\(^3\)[M+H]: Parent ion.

3.4.3. Antioxidant capacity determination

3.4.3.1. Determination of antioxidant capacity of crude extracts

**ABTS:** The antioxidant capacities of the different cultivars measured using the ABTS procedure are given in *Table 3.7*. *Cabernet Sauvignon* produced the greatest \((p<0.05)\) ABTS quenching power compared to both *Merlot* and *Pinot Noir*.

Table 3.7. Antioxidant activities of crude extracts from different grape cultivars\(^1\).

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Crude Extract (µmol TE/g)(^2)</th>
<th>ORAC (µmol TE/g)(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cabernet Sauvignon</em></td>
<td>65 ± 2 (^a)</td>
<td>520 ± 78 (^a)</td>
</tr>
<tr>
<td><em>Merlot</em></td>
<td>53 ± 1 (^b)</td>
<td>535 ± 16 (^a)</td>
</tr>
<tr>
<td><em>Pinot Noir</em></td>
<td>53 ± 3 (^b)</td>
<td>606 ± 31 (^a)</td>
</tr>
</tbody>
</table>

\(^1\)Values represent mean ± SD \((n=3)\). Different letters indicate significantly differences \((p<0.05)\).
\(^2\)Results are expressed as micromole of Trolox equivalent per gram of freeze-dried sample.
ORAC: The antioxidant capacities of the crude extracts derived from different wine-grape cultivars are presented in Table 3.7. Unlike the result obtained with ABTS, no significant difference \((p<0.05)\) was observed between different grape cultivars. In general, ORAC readings for all grape cultivars varied from 520 to 606 micromole Trolox per gram freeze-dried sample.

3.4.3.2. ORAC measurement of antioxidant capacity of semi-purified anthocyanin mixture collected from different grape cultivars

Semi-purified anthocyanin extract antioxidant capacity of different grape cultivars measured using ORAC is shown in Figure 3.5. The three anthocyanin extract antioxidant capacities varied within a narrow range and no significant difference \((p<0.05)\) was found among the semi-purified anthocyanin mixtures from each cultivar.

![Figure 3.5. Antioxidant capacity of grape semi-purified anthocyanin extract.](image)

Figure 3.5. Antioxidant capacity of grape semi-purified anthocyanin extract\(^1\).

\(^1\)Values represent mean ± SD \((n=3)\).
3.4.3.3. ORAC value for pure anthocyanin standards

As shown in the Table 3.8, the antioxidant capacity of the major anthocyanins presented in grape cultivars, excluding petunidin 3-glucoside, was measured using ORAC assay. The data varied between 4.05±0.27 to 6.36±0.37 µmol Trolox per µmol anthocyanin standard. Dihydroquercetin-derived anthocyanins (Cy3G and Pn3G) and dihydromyricetin-derived anthocyanins (Dp3G and Mv3G) showed the highest and lowest relative antioxidant capacities, respectively, of pure anthocyanins tested and known to be present in grape. No significant difference ($p<0.05$) was observed between Cy3G and Pn3G, nor Dp3G and Mv3G, ORAC value.

Table 3.8. ORAC value for pure anthocyanin standards known to be present in grape.

<table>
<thead>
<tr>
<th>Anthocyanin Fraction</th>
<th>ORAC Value (µmol TE/µmol Std)$^1$</th>
<th>Relative Capacity$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin 3-glucoside</td>
<td>6.36±0.37$^a$</td>
<td>1:1</td>
</tr>
<tr>
<td>Delphinidin 3-glucoside</td>
<td>4.05±0.27$^b$</td>
<td>0.63:1</td>
</tr>
<tr>
<td>Malvidin 3-glucoside</td>
<td>4.52±0.60$^b$</td>
<td>0.71:1</td>
</tr>
<tr>
<td>Peonidin 3-glucoside</td>
<td>5.92±0.52$^a$</td>
<td>0.93:1</td>
</tr>
</tbody>
</table>

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

$^2$Relative Capacity, given cyanidin 3-glucoside=1.0.

3.4.3.4. Determination of anthocyanin antioxidant capacity index

Figure 3.6 shows anthocyanin extract antioxidant capacity and the anthocyanin antioxidant capacity index (AACI) of different grape cultivars. The AACI in all grape
fruits tested from different cultivars shows a significantly lower level than the range of antioxidant capacity of different anthocyanin extracts measured using ORAC assay.

Table 3.9. Grape anthocyanin extract antioxidant capacity and anthocyanin antioxidant capacity index.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Anthocyanin Extract Antioxidant Capacity (µmol TE/ g)&lt;sup&gt;1,2&lt;/sup&gt;</th>
<th>Standards Anthocyanin Antioxidant Capacity Index (µmol TE/ g)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabernet Sauvignon</td>
<td>2762-3811</td>
<td>727</td>
</tr>
<tr>
<td>Merlot</td>
<td>2889-3031</td>
<td>963</td>
</tr>
<tr>
<td>Pinot Noir</td>
<td>2744-3369</td>
<td>493</td>
</tr>
</tbody>
</table>

<sup>1</sup>Results represent the range of anthocyanin extract antioxidant capacity (n=3).

<sup>2</sup>Results are expressed as micromoles of Trolox per gram of freeze-dried sample.
Figure 3.6. Indication of potential synergy from anthocyanin extract antioxidant capacity and anthocyanin antioxidant capacity index in different cultivars of grape.

Values represent mean±SD, (n=3).

1Values represent mean±SD, (n=3).
3.5. Discussion

3.5.1. Grape pH, soluble solids content, and titratable acidity

The development of grape berries consists of two growth phases separated by a lag phase. In the first phase, the berry is green and hard and the growth rate is rapid, followed by a period of slowed growth while the seed matures. In the third stage of development, which is known as ripening, the sugar content, pH, colour, and size of the berry increases while the total acidity decreases (Andaur et al., 2004; Castellarin et al., 2007). Factors such as berry temperature, carbon availability (Pereira et al., 2006), and light exposure can affect berry maturation and also chemical composition. Concurrently, sugar content, acidity, pH, colour, and flavour are used to determine grape maturity in different cultivars.

Andaur et al. (2004) reported that the distribution of °Brix within a cluster of grape is a non-random factor and that the outer layer berries consistently show a higher soluble solids content, size, and number of seeds. Since berries from different parts of the cluster were randomly used in this study, the effect of berry position in the cluster was minimized. As was mentioned earlier, °Brix is one of the best indexes used to determine the ripeness of grape berries. Bisson et al. (2001) determined that a °Brix $\times$ pH$^2$ range of 220 to 260 indicated that grape berries had reached an optimal sugar/acidity balance. Results outside of this range are an indicator of a late harvest fruit.

The °Brix, pH, and TA results shown here are comparable to other studies (Andaur et al., 2004; Pereira et al., 2006; Cortell et al., 2007; Mazza et al., 1999; Hulya Orak, 2007), and indicate that all the grape cultivars were harvested in the midpoint
period of ripening. This was an essential step to allow comparisons between antioxidant capacities of different grapes to be made.

3.5.2. Grape total phenolic and anthocyanin

Total Phenolics: The phenolic content of the grape samples, as measured by the Folin-Ciocalteu procedure, was higher than the total phenolic content reported by others for grape (Monagas et al., 2006; Hulya Orak, 2007; Parry et al., 2006). The differences in total phenolic content between studies can be attributed not only to the different part of the berry used or the stage of grape sample maturity, but also the use of different extraction solvents. Baydar et al. (2007) have reported that grape seed extracts have more than 28 times more phenolic content per gram dry weight (mg GA/g), compared to grape skin extract or grape juice.

According to Hulya Orak (2007), grape phenolic composition includes flavonoids, flavan-3-ols such as catechins, anthocyanins, flavanols, and also non-flavonoid compounds such as hydroxycinnamic acids and hydroxybenzoic acids. It is also well known that the antioxidant capacity of grape is largely attributed to the phenolic content; for example, grape cultivars with a higher phenolic content such as Mourvedre have a higher antioxidant capacity (Huly and Orak, 2007). The finding of this experiment agrees with other studies which showed that the antioxidant capacity of grape is highly correlated to its total phenolic content ($r^2 = 0.85$).

Total Anthocyanin: There are many studies indicating that the anthocyanin content of wine grapes changes during ripening, depending largely on seasonal environmental conditions. In this study, the total anthocyanin content, as measured by
the pH differential method, varied from 18±2 to 44±3 mg cyanidin 3-glucoside/L extract samples. These data are in agreement with the total anthocyanin content reported for *Cabernet Sauvignon* by Huly and Orak (2007) and others (Monagas et al., 2006; Parry et al., 2006).

In this study, precautions were taken to ensure that grape berries form the outer and inner layers of different grape clusters were randomly chosen. The difference in total anthocyanin content of *Pinot Noir* with the other two cultivars, for example, is likely attributed to the differences between cultivars, rather than selection of different grape berries.

3.5.3. Grape anthocyanin quantification and identification

Although the anthocyanin profile is a characteristic factor for each grape cultivar, the total of five similar major anthocyanins detected across all grape cultivars examined in this thesis indicates general similarity in the anthocyanin biosynthesis pathway between cultivars. According to other reported data, anthocyanin 3-glucoside represented the most abundant anthocyanin among all anthocyanin derivatives. Malvidin 3-glucoside and cyanidin 3-glucoside represented the highest and lowest concentration of anthocyanin pigment in all cultivars, respectively. These data are in agreement with those reported by Monagas et al. (2006). *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* showed a 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* is simply a result of differences between cultivars.

Results of this study identified peaks 3 to 7 to be delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside, respectively. These five major anthocyanin compounds were responsible for more than 92% of the total anthocyanin content measured in the different grape cultivars. Malvidin 3-glucoside was responsible for more than 30% of the total anthocyanin content in *Cabernet Sauvignon* and *Merlot*. Other workers have reported that the proportion of delphinidin and petunidin was related to canopy light exposure, whereas malvidin 3-glucoside has not been reported to be light or nitrogen sensitive (Mori et al., 2007).

3',4'-dihydroxy (Cy and Pn) and 3',4',5'-trihydroxy (Dp, Mv, Pt) anthocyanin percentages were also determined. In all grape cultivars, the percent of 3',4',5'-trihydroxy (Dp, Mv, Pt) was at least 2 times higher than that of 3',4'-Dihydroxy (Cy and Pn). Many factors have been reported to influence anthocyanin composition. For example, with the exception of malvidin 3-glucoside, the quantities of other individual anthocyanins have been shown to decrease under high temperature during berry development (Mori et al., 2007). Methoxylation, glycosylation, and acylation of anthocyanin derivatives have been reported to increase the thermal stability of the grape anthocyanin, and this could explain the higher amount of malvidin 3-glucoside compared to other anthocyanins at high temperature (Mori et al., 2007). It has also been suggested that black cultivars’ skin compared to red skinned grapes have a stronger ability to adapt anthocyanin biosynthesis to temperature.
Canopy light exposure, vigor zone, nitrogen status, shading, temperature-mediated expression of anthocyanin biosynthetic genes, and anthocyanin accumulation and anthocyanin degradation are some of the factors which are being further studied as determinants of anthocyanin composition (Cortell et al., 2007; Mori et al., 2007; Romero et al., 2000).

3.5.4. Grape antioxidant capacity

*ABTS:* The antioxidant capacity of solvent-derived crude extracts of grape samples was measured using the ABTS method. Results ranged between 53±3 to 65±2 μmol of Trolox equivalent per gram freeze-dried sample. A similar range of ABTS values was reported for red wine by Rivero-Perez et al. (2007). This latter result was achieved without measuring the differences between specific cultivars of grape.

ABTS is a Single Electron Transfer (SET) reaction antioxidant assay, which is based on a redox reaction (Johnston et al., 2006). The assay requires a short time to complete and measures the radical scavenging capacity of antioxidant in neutral pH (Huang et al., 2005), which can be performed in both organic and aqueous solvent systems. This makes ABTS a better radical scavenging assay than DPPH (2,2’-diphenyl-1-picrylhydrazyl), which can only be employed in ethanol (Lee et al., 2003).

The presence of different phytochemical constituents in grape tissues such as pigments, alkaloids, polyamines, ascorbate, phenolic compounds, and anthocyanins are factors that will contribute to the antioxidant capacity of soft fruits such as grape. These compounds are known to be involved in the direct removal of ROS and also with
antioxidant regeneration (Johnston et al., 2006). That is often related to a synergistic activity between different antioxidant constituents (e.g. vitamin C and vitamin E).

**ORAC**: The total antioxidant capacity of grape crude extracts was also measured using the ORAC assay, which represents a HAT-based reaction and involves the mechanism of radical chain breaking capacity specific to the generation of peroxyl radicals. In this study, our ORAC results were higher than those reported for seedless grape samples by Kedage et al. (2007). Differences in total ORAC antioxidant capacity between the two studies could be attributed to the fact that extracts used herein were derived from whole berries and included seeds. It has also been reported that the antioxidant activities of fruits and vegetables are strongly dependent on cultivation conditions and genotype (Ou et al., 2002). The data reported herein, however, were not found to be significantly different between grape cultivars.

Following the analysis on crude grape extracts, subsequent experiments were performed on semi-purified extracts that retained the anthocyanins at the cost of other phenolics. This was achieved by using a solid phase extraction (SPE)-C18 column. The semi-purified anthocyanin extracts compared to the respective crude extracts indicated higher antioxidant capacity, suggesting that anthocyanin purification was indeed achieved with a relatively higher yield of antioxidant capacity. Comparing the three different grape crude and semi-purified anthocyanin extracts showed that the semi-purified anthocyanin extracts had at least a 4.8 times higher antioxidant capacity than the crude extract.

Further interpretation of the data obtained for antioxidant capacity and anthocyanin content from different grape cultivars involved constructing an anthocyanin antioxidant capacity index similar to that reported for blackcurrant in Chapter 2 of this
thesis. This calculation demonstrates the importance of anthocyanin-specific compositions, which may reveal potential synergies between cultivar-specific anthocyanin compositions in regard to total ORAC activity. To this end, it was clear that the standard anthocyanin antioxidant capacity index was consistently lower than the range of ORAC values obtained for the anthocyanin extracts from each grape cultivar; however, it is particularly noteworthy that Pinot Noir produced an anthocyanin antioxidant capacity index value that was markedly lower than that observed for Cabernet Sauvignon and Merlot. To explain this observation, it is important to recognize that this difference in Pinot Noir anthocyanin antioxidant capacity index could not be accounted for by the lower proportion of delphinidin 3-glucoside or, alternately, the higher proportion of peonidin 3-glucoside, regardless of the individual ORAC values for each standard. Thus, it appears that in the grape unlike the blackcurrant, the total anthocyanin content rather than its anthocyanin composition has a greater effect in producing the characteristic degree of antioxidant capacity between cultivars. It is plausible, however, that anthocyanins not identified or examined in the experiment that accounted for approximately 20% of the total anthocyanin content may be involved in the synergy observed herein. In comparison, an anthocyanin synergy could be identified in blackcurrant, which represented 97% of anthocyanins.

The ORAC measurement on individual purified anthocyanin aglycons was also intended to provide additional information about anthocyanin biosynthesis pathways and related antioxidant capacities. The data showed that cyanidin 3-glucoside, which is the first anthocyanin metabolite produced from dihydroquercitin pathway, gave the highest antioxidant capacity, compared to delphinidin, which is the first metabolite of the
dihydromyricetin pathway. Further investigation showed these anthocyanins, which contain one hydroxyl group on the B-ring, such as malvidin, had a relatively lower antioxidant capacity compared to cyanidin, which has two hydroxyl groups attached. This observation is in agreement with the data reported by Wang et al. (1997) that indicated C-3 'and C-4' positions are important for free radical scavenging properties. 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 has a relatively lower ORAC value compared to cyanidin 3-glucoside. Methoxylation of cyanidin and delphinidin does not appear to have a significant effect on antioxidant capacity.
3.6. References


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CHAPTER IV. General conclusion

The focus of this study was to compare the physiochemical properties of two soft fruits rich in delphinidin (e.g. blackcurrant) and malvidin (e.g. grape) anthocyanins. Ripe fruit from four different blackcurrant cultivars were studied and it was determined that blackcurrant is a rich source of delphinidin 3-rutinoside, following by cyanidin 3-rutinoside, delphinidin 3-glucoside and cyanidin 3-glucoside at relatively lower concentrations. Characterization of blackcurrant anthocyanins using HPLC/UV demonstrated that 3-rutinoside anthocyanins were the main anthocyanins, comprising more than 60% of the anthocyanin profile. Blackcurrant crude extract also exhibited considerable antioxidant capacity. Regression analyses showed that ABTS antioxidant activity was highly correlated with total anthocyanin \( (r^2=0.88) \) and total phenolic \( (r^2=0.92) \) contents in blackcurrant. Furthermore, ORAC values obtained for semi-purified anthocyanin extracts derived from blackcurrant gave more than 5 times the antioxidant capacity compared to the respective crude extracts, indicating that anthocyanin purification steps produce higher yields of antioxidant capacity. Our findings supported our hypothesis that a plausible potential synergy between anthocyanin components in regard to ORAC antioxidant capacity existed for each blackcurrant cultivar. It was concluded that total anthocyanin content as well as the composition of individual anthocyanins and the proportion of each anthocyanin in blackcurrant are important factors to assess total antioxidant capacity.

Berries at the midpoint of ripening from three different grape cultivars were also studied in this research. Anthocyanin 3-glucoside represented the most abundant
anthocyanin among all anthocyanin derivatives in all grape samples. Malvidin 3-glucoside (>32%) and cyandindin 3-glucoside (< 11%) represent the highest and lowest concentration of anthocyanin pigment in all cultivars, respectively. Grape crude extract antioxidant capacity was also evaluated using ABTS and ORAC. ORAC results indicated that in both assays, grape had comparatively lower antioxidant capacity than blackcurrant cultivars tested in this study. Similar to the blackcurrant findings, different antioxidant capacities between different grape crude and semi-purified anthocyanin extracts were of the magnitude of 4.8 times more for semi-purified anthocyanin extract.

Further interpretation of the data obtained from semi-purified anthocyanin antioxidant capacity was directed towards constructing an anthocyanin antioxidant capacity index (AACI), which was constructed to evaluate the proportion of antioxidant capacity influenced by individually measured anthocyanins in the fruit extract. AACI was calculated by multiplying the concentration of individual anthocyanin (mg/g) with individual standard anthocyanin ORAC value (µmol TE/ mg std). Using this index, it was calculated that grape exhibited a stronger potential synergistic effect in antioxidant capacity of individual anthocyanin aglycon species compare to blackcurrant berries. Our results also demonstrate that grape antioxidant activity is influenced more by the concentration of anthocyanins than the individual proportion of anthocyanins that comprise the profile. In addition, this research showed that the antioxidant capacity of major individual anthocyanins known to be present in blackcurrant and grape berries (except for petunidin) could be quantitated using ORAC. ORAC results suggest that the dihydroquercetin pathway metabolites including cyanidin and peonidin produce significantly higher antioxidant capacity than metabolites derived from the
dihydromyricetin pathway (e.g. delphinidin and malvidin). Our data also demonstrated that the sugar moiety for each aglycon did not affect the ORAC antioxidant capacity to the same extent.

Further studies will be needed to demonstrate the role of other phytochemicals present in soft fruits that can affect antioxidant capacity. More studies are also required to focus on the antioxidant capacity of the whole anthocyanin biosynthesis pathway, including dihydrokaempferol anthocyanin biosynthesis, and also to study the antioxidant activity of different anthocyanin combinations.