Analysis and Entrapment of Select Antioxidants from Chokecherry and Saskatoon Berry Fruits

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ABSTRACT

The major objectives of this research were to produce a phenolic rich isolate from two locally grown Saskatchewan fruits, chokecherries and saskatoons, develop an encapsulation system for the phenolic isolate, and test this system for the delivery of the phenolic isolate in an animal (rat) model.

Natural phenolic compounds present in plants such as fruits have antioxidant and free radical scavenging activities, which have been proposed to have health benefits. The extraction of these compounds from plants is commonly performed using methanol despite being toxic to both humans and animals. As such, ethanol was investigated for its ability to extract phenolics from plants as a food safe alternative to methanol. Phenolic extraction from chokecherries with ethanol:formic acid:water (EFW) resulted in higher concentrations (9.83 mg gallic acid equivalents (GAE)/g fresh weight) than with methanol:formic acid:water (MFW) (7.97 mg GAE/g fresh weight). Results from saskatoons showed similar phenolic levels of 4.26 and 4.21 mg GAE/g fresh weight with MFW and ethanol (EFW), respectively. These results showed that EFW was a suitable substitute for MFW in phenolic compound isolation from chokecherries and saskatoons, and could be used to produce extracts that were safe for use in foods and feeds.

High performance liquid chromatography with photodiode array detection (HPLC-PDA) was used to determine the phenolic compound composition of the raw fruits and their phenolic rich isolates. Chlorogenic acid was identified in both chokecherry and saskatoon samples, and rutin was also shown to be present in saskatoons. These identifications were based on the relative retention time and ultra violet-visual spectra comparisons to standards. Solid phase extraction (SPE) using Amberlite XAD-16 was employed to produce phenolic isolates from chokecherries and saskatoons. HPLC-PDA results determined that there was a ~2.7x and ~1.6x increase in peak area for chokecherries and saskatoons, respectively when SPE was employed. The antioxidant activity of the extracts and isolates was determined using *in vitro* radical scavenging tests including 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2´-azinobis-3-ethylbenzthiazoline-sulphonic acid (ABTS). The EFW chokecherry extract and isolate had the highest overall free radical scavenging activity. Crude fruit extracts exhibited

lower free radical scavenging values compared to the isolate samples in both of the assays performed.

The fruit phenolic isolates were encapsulated in chitosan (CH) sodium tripolyphosphate (TPP) nanoparticles at a ratio of 4.0:1.0 (CH:TPP). HPLC-PDA was used to determine the entrapment efficiency of phenolic isolates to be 15.9 ± 2.7% and 23.0 ± 7.1% for chokecherries and saskatoons, respectively. Characteristics such as the size, surface potential and phenolic release were determined for the two fruit isolate containing nanoparticles. The size of the nanoparticles were 527.90 ± 74.57 nm and 443.03 ± 15.79 nm for chokecherries and saskatoons, respectively. Both of the nanoparticle systems had positive surface charges at 52.70 ± 2.93 mV and 54.43 ± 1.27 mV for chokecherries and saskatoons, respectively. The release properties of the CH:TPP nanoparticles containing fruit phenolics were examined in enzymatic simulated intestinal fluid and resulted in ~23% and ~28% release of chokecherry and saskatoon phenolics, respectively.

Saskatoon phenolic isolates and isolates encapsulated in CH:TPP were gavage fed to rats (six animals in each of the two groups) at a dosage rate of 276.36 ± 9.74 mg/kg body weight. The saskatoon isolate contained 12.44 ± 0.44 mg/kg body weight anthocyanins (~3.30 mg anthocyanin per rat). These animals were sacrificed after 1 h and all stomach tissue samples in each of the treatment groups contained detectable levels of anthocyanins. In the small intestine tissues all six of the saskatoon isolate and three of the encapsulated isolate groups had detectable amounts of anthocyanins, while in the large intestine tissue, only one sample from the isolate group showed detectable amounts of anthocyanins. Although other tissues were tested (brain, heart, kidney and liver), anthocyanins were not detected. Therefore anthocyanins were detected in the gastrointestinal tract of both of the treatment groups.

The research performed therefore illustrated that phenolic compounds can be extracted from fruit sources using EFW and can be successfully encapsulated in chitosan tripolyphosphate capsules allowing for targeted delivery in an animal model.

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LIST OF ABBREVIATIONS

%EE	percent entrapment efficiency
ABTS	2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AH	antioxidant
bw	body weight
CH	chitosan
CHD	coronary heart disease
CVD	cardiovascular disease
ddH_2O	Milli-Q^{TM} water
DPPH	2,2-Diphenyl-1-picrylhydrazyl
dw	dry weight
EFW	ethanol, formic acid, water
EM	electrophoretic mobility
ESIF	enzymatic simulated intestinal fluid
FAE	ferulic acid equivalents
FC	Folin-Ciocalteau
FCR	Folin-Ciocalteau reagent
fw	fresh weight
GAE	gallic acid equivalents
GC	gas chromatography
GI	gastro intestinal
kg	kilograms
h	hour(s)
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HPLC-PDA	high performance liquid chromatography photodiode array detector
IC_{50}	concentration for 50% inhibition
L	litre
LC	liquid chromatography
LDL	low-density lipoprotein

mAU	milli-absorbance units
mM	millimolar
M	molar
MFW	methanol, formic acid, water
mg	milligrams
min	minute(s)
mL	millilitres
mm	millimetres
MS	mass spectrometry
mV	milli volts
MW	molecular weight
N	normality
nm	nanometre(s)
nM	nanomolar
NaOH	sodium hydroxide
PBS	phosphate buffered saline
PDA	photodiode array detector
POH	phenolic antioxidants
ROS	reactive oxygen species
RP	reverse phase
rpm	revolutions per minute
RRT	relative retention time
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SPE	solid phase extraction
TEAC	trolox equivalent antioxidant capacity
TPC	total phenolic content
TPP	tripolyphosphate
TSS	total soluble solids
µg	microgram(s)
µm	micrometer(s)

USDA	United States Department of Agriculture
UV	ultraviolet
UV-vis	UV-visual
v/v	volume per volume
w:v	weight per volume
w/w	weight per weight
ζ	zeta potential

1.0 INTRODUCTION

Interest in natural antioxidants is increasing in the food, nutraceutical and natural health product industries because of their purported ability to protect the body against reactive oxygen and nitrogen species that are associated with degenerative diseases (Shahidi and Naczk, 2004b). Naturally occurring phenolic compounds in fruits have been shown to have antioxidant and free radical scavenging activities (Rice-Evans et al., 1997; Wang and Jiao, 2000; Ross and Kasum, 2002). In fact, many of the phenolics identified in fruits have been shown to have better free radical scavenging abilities than other natural antioxidants such as vitamin E and C on a molar basis (Rice-Evans et al., 1996). Antioxidants may provide some of the solutions for the effective treatment of degenerative diseases including Alzheimer's, Parkinson's and dementia, and may also reduce the impact of aging (Scalbert et al., 2005). Therefore, the addition of fruit phenolics to our diet through the production and consumption of functional foods, nutraceuticals and nutritional supplements may provide important health benefits. The use of phenolics in foods may be hindered by attributes such as taste, solubility and stability. These limitations may be solved by encapsulation technologies, which can provide both phenolic compound protection and site directed delivery, as well as the ability to mask their off-flavours and improve their miscibility in foods/other formulations. The encapsulation of phenolic compounds is also important because this technology has the possibility of increasing the bioavailability of these compounds.

The overall goal of this research was to produce a food safe phenolic isolate from Saskatchewan fruits (chokecherry and saskatoons) that could then be encapsulated allowing for phenolic compound protection against oxidation coupled with targeted delivery. A number of objectives were established to meet this goal. The first objective of this research was to determine if ethanol was a suitable substitute to methanol for the extraction of phenolics from the aforementioned fruits to produce a phenolic isolate using solid phase extraction (SPE). The second objective was to investigate the mass ratio of chitosan to sodium tripolyphosphate (CH:TPP) required to create an encapsulation delivery system for the phenolic isolate (core material), examining particle size, surface charge, and the entrapment efficiency of the core material. The final objective was to

determine the release of anthocyanins (a component of the phenolic isolate) from the particles in an animal system (rat) to understand their transport and absorption into tissues.

2.0 LITERATURE REVIEW

2.1 Plant phenolics

Phenolics are defined as chemical substances which possess an aromatic ring that contains one or more attached hydroxyl group(s) (Harborne and Simmonds, 1964). Most naturally occurring plant phenolics contain two or more hydroxyl groups and are considered to be bioactive compounds (Ho, 1992). Plant phenolics are secondary metabolites, which are derived from acetate via the cyclization of linear polyketide chains. These secondary metabolites are not required for normal plant growth but rather aid in their protection from herbivores, microbial infection, stress and ultra violet (UV) radiation (Goldberg, 2003; Shetty and McCue, 2003). Phenolics in plants constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with over 8000 structures currently identified (Bravo, 1998). Plant phenolics have been commonly referred to in literature as polyphenols or polyphenolics. These terms originally meant a compound that consisted of many phenolic groups; more recently the term has been used to describe all of the different phenolic compounds present in a plant extract (Wrolstad, 2005). In this thesis the term phenolic(s) will be used to describe all classes of these compounds.

2.1.1 Basic structure

The most basic phenolic is phenol (Figure 2.1). Phenolic structures can range from the simple phenol molecule to highly polymerized complex structures, such as proanthocyanins (Bravo, 1998; Santos-Buelga and Scalbert, 2000). Plant phenolics are primarily present in a conjugated form with carbohydrate residues linked to hydroxyl groups on the aromatic ring(s) (Bravo, 1998). The most common carbohydrate conjugate is glucose although galactose, rhamnose, xylose, arabinose, glucuronic and galacturonic acids have also been identified (Rice-Evans et al., 1996; Bravo, 1998; Karakaya, 2004). Compounds that form other covalent linkages with plant phenolics include organic acids, amines, lipids and other phenolic molecules (Bravo, 1998; Manach et al., 2004).

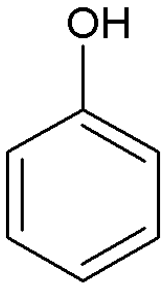


Figure 2.1 Chemical structure of phenol.

2.2 Classes of phenolics

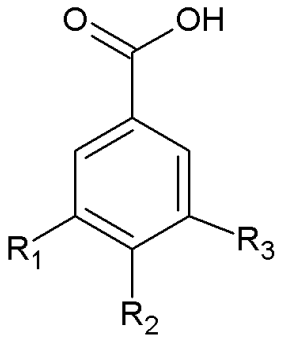
Various structural classes of phenolics exist and can be grouped in a number of ways. Ho (1992) classified them into three groups, simple phenols and phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. In contrast, Harborne and Simmonds (1964) classified plant phenolics based on the number of carbon atoms present in their structure. Based on structure, there are at least ten different phenolic classes (Table 2.1), with the most abundant being the simple phenols and flavonoids (Bravo, 1998).

Table 2.1 Classes of phenolics present in the kingdom Plantae (adapted from Bravo 1998 and Vermerris and Nicholson 2006).

Class	Structure
Simple phenols	C_6
Hydroxybenzoic acids	C_6-C_1
Phenylacetic acids	C_6-C_2
Hydroxycinnamic acids	C_6-C_3
Coumarins, isocoumarins	C_6-C_3
Naftoquinones	C_6-C_4
Xanthones	C_6-C_1-C_6
Stilbenes	C_6-C_2-C_6
Anthraquinones	C_6-C_2-C_6
Flavonoids	C_6-C_3-C_6

Simple phenols have the most basic chemical structures and are also referred to as substituted phenolics (Vermerris and Nicholson, 2006a). These compounds can have multiple hydroxyl groups substituted on the benzene ring. Simple phenols are the monomeric components of more complex compounds which are present in many plant tissues (Brielmann, 1999). Examples of compounds in this class include arbutin, hydroquinone, orcinol and resorcinol (Brielmann, 1999).

In literature, a common group of phenolics found in plants are termed phenolic acids; in this thesis this group will be divided into hydroxybenzoic (C_6-C_1) and hydroxycinnamic acids derivatives (C_6-C_3). Hydroxybenzoic acids are characterized by a substitution of a hydrogen with a carboxyl group on the phenol molecule (Figure 2.2). Some examples of these compounds include gallic acid, p-hydroxybenzoic acid, protocatechuic acid and vanillic acid (Vermerris and Nicholson, 2006a). The level of hydroxybenzoic acids derivatives in edible plants is very low with the exception of certain red fruits, black radish and onions (Manach et al., 2004). Onions contain higher levels of hydroxybenzoic acids at 103 mg/kg fresh weight (fw) compared to other vegetables such as potato and bell pepper which have lower levels at 1-3 mg/kg fw (Herrmann and Nagel, 1989).

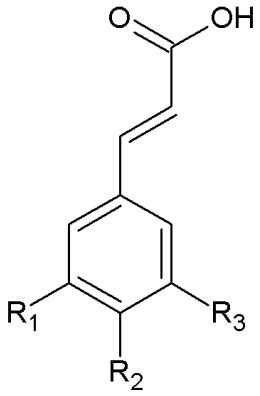


Compound	R_1	R_2	R_3
gallic acid	OH	OH	OH
p-hydroxybenozic acid	H	OH	H
protocatechunic acid	H	OH	OH
vanillic acid	H	OH	OCH_3

Figure 2.2 Examples of chemical structures of the common hydroxybenozic acids found in plants (adapted from Vermerris and Nicholson, 2006b).

Hydroxycinnamic acids are more common to edible plants than are hydroxybenzoic acids (Manach et al., 2004), and are the most widely distributed phenolic class in plant tissues (Wrolstad, 2005). The most common hydroxycinnamic acids are caffeic, cinnamic, p-coumaric, ferulic, 5-hydroxyferulic and sinapic acids (Figure 2.3), with most plants containing at least three of these compounds (Vermerris and Nicholson, 2006a).

The hydroxycinnamic acids that are found in plants are normally glycosylated or esterified, however free forms are found in processed foods that have been heat treated, fermented or frozen (Manach et al., 2004). Caffeic acid in both its free and esterifed forms (e.g. chlorogenic acid) makes up 75-100% of the hydroxycinnamic acids content of most fruits (Manach et al., 2004). Although derivatives of hydroxycinnamic acids can be present in all portions of the fruit, the highest concentrations are found in the outer portion known as the pericarp (Manach et al., 2004).



Compound	**R_1**	**R_2**	**R_3**
caffeic acid	H	OH	OH
cinnamic acid	H	H	H
ferulic acid	H	OH	OCH_3
5-hydroxyferulic acid	OH	OH	OCH_3
p-coumaric acid	H	OH	H
sinapic acid	OCH_3	OH	OCH_3

Figure 2.3 Chemical structures of common hydroxycinnamic acid derivatives (adapted from Manach et al., 2004).

2.2.1 Flavonoids

Flavonoids are a diverse group of phenolics that are widely distributed in plants and more than 5000 have been identified (Ross and Kasum, 2002). Their basic structure consists of two (A, B) benzene rings linked through a three carbon chain. One of these carbon atoms is always linked to a carbon of one of the benzene rings, either directly or through an oxygen bridge. This results in the formation of a third heterocyclic (C) ring, which is either five or six membered (Figure 2.4). There is a common numbering system for flavonoids in order to locate the substituents from the rings. It starts by assigning the ring oxygen with the lowest possible number. Flavonoids can be further divided into sub-classes based upon variations in the heterocyclic C ring (Ross and Kasum, 2002). These sub-classes include anthocyanidins, flavanols, flavanones, flavones, flavonols and isoflavones (Bravo, 1998). Flavonols are commonly found in foods with the main compounds being, kaempferol, myricetin and quercetin (Figure 2.5); (Manach et al., 2004). Plant flavonols are usually glycosylated at the 3-position on the C ring. Flavonols are concentrated in the exocap (skin) of fruits and the leaves of plants as their synthesis is stimulated by light, and they exist in both monomeric and polymeric forms (Manach et al., 2004). The polymethoxylation of flavones results in increased hydrophobicity and they are the most non-polar of the flavonoid class (Manach et al., 2004).

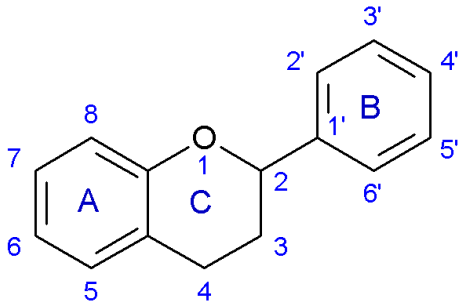
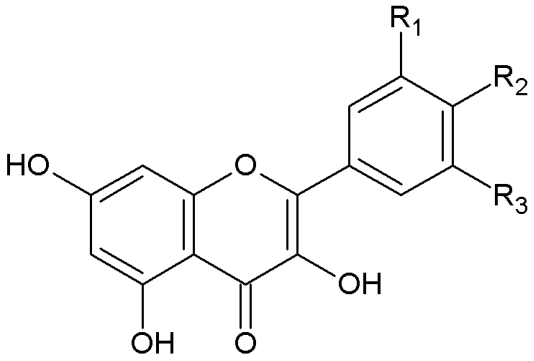


Figure 2.4 Basic chemical structure of the flavonoid molecule, illustrating its three ring structure and numbering.

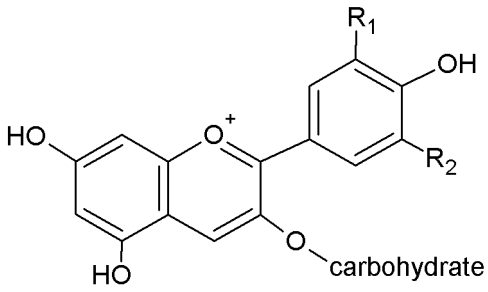


Compound	R_1	R_2	R_3
kaempferol	H	OH	H
quercetin	OH	OH	H
myricetin	OH	OH	OH

Figure 2.5 Chemical structures of common food flavonols (adapted from Manach et al., 2004).

2.2.1.1 Anthocyanins

Anthocyanins are a group of water soluble pigments that are responsible for the red to purple colours present in flowers and fruits. These flavonoids, are considered to be the most important group of phenolics in foods, which have multiple substitution patterns (Figure 2.6). The term anthocyanin refers to the glycoside of the anthocyanidin at the 3- and/or 5-position(s) with carbohydrate(s) such as glucose, galactose, arabinose and di- and trisaccharides (Krenn et al., 2007).



Anthocyanin	R_1	R_2
pelargonidin	H	H
cyanidin	OH	H
peonidin	OCH_3	H
delphinidin	OH	OH
petunidin	OCH_3	OH
malvidin	OCH_3	OCH_3

Figure 2.6 Most abundant plant anthocyanins showing their substitution patterns (adapted from Ozga et al., 2007).

Anthocyanin colour depends on a number of environmental and chemical factors including pH, presence of metal ions and carbohydrate substitution pattern (Vermerris and Nicholson, 2006a). These compounds can exist in a number of chemical forms which results in both coloured and non-coloured compounds based on environmental conditions (Manach et al., 2004). At pH values <3, anthocyanins have a red colour which becomes colourless at pH 4-5 as the flavylium cation is converted to the carbinol base (pseudobase). At pH values >6, a blue/purple colour is formed due to the presence of the quinoidal base (anhydro base) (Manach et al., 2004; Krenn et al., 2007).

2.2.1.2 Proanthocyanidins

Proanthocyanidins, which are also referred to as condensed tannins, are high molecular weight polymers of flavonoid molecules. These compounds consist of oligomeric or polymeric flavonoids such as flavan-3-ol units (Vermerris and Nicholson, 2006a). Individual monomeric units are linked through oxidative condensation at the 4-

position of the C ring and either the 6- or 8-position of the A ring on two adjacent flavonol units.

2.3 Plant sources of phenolics

Plants can provide an abundant natural source of phenolics in the human diet, as they are present in a wide variety of commonly consumed foods including fruits, vegetables, cereals, legumes and beverages such as juices, tea, coffee and wine (Table 2.2) (Scalbert et al., 2005). The average consumption of phenolics in the United States, has been estimated to range between 189 mg/day and 1000 mg/day (Scalbert et al., 2005; Chun et al., 2007). The levels of phenolics present in plants are based on genetic factors (cultivar and variety) and environmental growing conditions such as temperature, moisture, irradiation and disease (Duthie and Crozier, 2000; Howard et al., 2003). Environmental factors can lead to the uneven distribution of phenolics in plant tissues, and food processing can result in either the loss or enrichment of phenolics (Scalbert and Williamson, 2000). A common way of reporting the phenolic levels in plant sources is by gallic acid equivalents (GAE) units determined by the Folin-Ciocalteu method (Kahkonen et al., 1999). This method compares the phenolic material of the plant source to a commercial source of gallic acid.

Cereal products, specifically whole grains, are a source of phenolic acids (Scalbert and Williamson, 2000), with ferulic acid being identified as the most abundant compound in this class (Manach et al., 2004). Phenolic classes such as hydroxybenzoic acids, hydroxycinnamic acids, flavonoids and tannins are also present in whole grains (Salunkhe et al., 1982). During the processing of cereal grains (i.e. roasting) it has been shown that a number of phenolics are formed as by-products of enzymatic or thermal degradation (Naczk and Shahidi, 2006).

Beverages such as coffee, tea, fruit juice and red wine are good sources of phenolics (Table 2.2). For example, red wine is an excellent source of hydroxybenzoic and hydroxycinnamic acids, flavan-3-ols (catechin and epicatechin), flavonols (quercetin and myricetin) and anthocyanins (Bravo, 1998; Minussi et al., 2003; Manach et al., 2004). The phenolic content and composition of wines vary widely due to several factors including the variety of grape, growing conditions, production, maturity and processing

parameters (Balasundram et al., 2006). The levels of phenolics in red wines range from 1018-4177 mg GAE/L (Balasundram et al., 2006); more specifically, red wines contain ~200-350 mg anthocyanins/L (Manach et al., 2004).

Both fruits and vegetables are excellent sources of phenolics (Table 2.2) (Macheix et al., 1990; Shahidi, 2000; Shahidi and Naczk, 2004a). The intake of phenolics from fruit consumption is approximately three times that from vegetables because of the lower phenolic concentrations in the latter (Brat et al., 2006). Fruits have been found to contain the following classes of phenolics: hydroxybenzoic acids, hydroxycinnamic acids, flavonoids (anthocyanins, flavanols and flavonols), proanthocyanins and tannins (Brat et al., 2006). Fruits are a good source of anthocyanins, which are not present in most vegetables (Scalbert and Williamson, 2000). A commonly consumed fruit source of phenolics are berries from the following genera *Vacciuium* spp., *Ribes* spp., *Rubus* spp. and *Fragaria* spp. (Wang et al., 1996; Kahkonen et al., 1999; Kahkonen et al., 2001). Berries are an excellent source of a wide variety of phenolic classess such as anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, proanthocyanidins and flavonoids (Bakowska-Barczak et al., 2007), and contain higher concentrations of these compounds when compared to other fruits (Kalt et al., 2008).

As an example, the American cranberry (*Vaccinium macrocarpon*) is commercially grown in North America and is used to produce juices, juice concentrates, canned products, infused dried fruits and sauces (Shahidi and Naczk, 2004a). The wild variety (European cranberry; *V. oxycoccus*) has a smaller economic value compared to the American variety (Huopalahti et al., 2000). The total phenolic content (TPC) of American cranberries range from 192-527 mg GAE/100 g fw (Sun et al., 2002; Borowska et al., 2009), whereas European cranberries range from 139-288 mg GAE/100 g fw (Taruscio et al., 2004; Borowska et al., 2009). Phenolics that have been identified in both types of cranberries include anthocyanins, flavanols, flavonols, hydroxybenzoic acids, hydroxycinnamic aicds and proanthocyanidins (Kahkonen et al., 2001; Prior et al., 2001; Zuo et al., 2002; Taruscio et al., 2004; Borowska et al., 2009). Anthocyanin levels for five different American cranberry cultivars ranged from 51-77 mg cyanidin-3-glucoside equivalents/100 g fw (Borowska et al., 2009). The main anthocyanins that were identified in American cranberry were, cyanidin-3-arabinoside, cyanidin-3-glucoside,

peonidin-3-arabinoside and peonidin-3-glucoside (Prior et al., 2001). The American cranberry has also been identified as a good source of the flavanol quercetin ranging from 11-25 mg/100 g fw (Häkkinen et al., 1999).

Phenolic extracts from two local berry fruits, chokecherry and saskatoons were prepared, encapsulated and used in animal feeding trials in this study and their chemical compositions are discussed below (Table 2.2).

2.4 Chokecherry

The chokecherry (*Prunus virginiana* L.) is a shrub or small tree that is widespread throughout Canada and the Western part of the United States (Vilkitis, 1974; Looman and Best, 1979). The shrub is found in moist, rich soils that are well drained and ranges from 2-10 meters in height (Vilkitis, 1974; Looman and Best, 1979; St-Pierre, 1993). This plant likes full sun and is commonly found along fence lines, roadsides and along the edges of wooded areas or ravines (St-Pierre, 1993). The fruit of this plant ranges in colour from dark red to purplish black and are approximately 6-8 mm in diameter, each containing a single pit. The fruit maturity is dependent on the latitude of growth and the majority mature from late June to August, while in Saskatchewan the fruit matures slightly later from late August to early September (Looman and Best, 1979; St-Pierre, 1993; Green, 2007). The chokecherry is closely related to domesticated cherries (Zatylny et al., 2005a). Chokecherry shrubs are currently being used in Saskatchewan for multiple row shelterbelts, wildlife habitat improvement, slope stabilization and erosion prevention (St-Pierre, 1993). Historically, Native Americans used this wild fruit in soups, stews and pemmican (St-Pierre, 1993; Kraft et al., 2008). Chokecherries are also used as a base for

Table 2.2 Source, extraction methods and the concentration of total phenolics present in selected foods.

Food Source	Extraction method	Total phenolics	Reference
Fruits			
Apple	Acetone/water (7:3)	179.1 mg GAE/ 100 g[1]	(Brat et al., 2006)
Cherry	Acetone/water (7:3)	94.3 mg GAE/ 100 g[1]	(Brat et al., 2006)
Chokecherry	Methanol/water (8:2) (0.01% formic acid)	1045.4 mg GAE/ 100 g[1]	(Bakowska-Barczak et al., 2007)
Cranberry	Acetone/water (7/3)	212.0 mg GAE/ 100 g[2]	(Kahkonen et al., 1999)
Grape	Acetone/water (7:3)	195.5 mg GAE/ 100 g[1]	(Brat et al., 2006)
Saskatoon	Methanol/water (8:2) (0.01% formic acid)	650.9 mg GAE/ 100 g[1]	(Bakowska-Barczak and Kolodzeijczyk, 2008)
Vegetables			
Broccoli	Acetone/water (7:3)	98.9 mg GAE/ 100 g[1]	(Brat et al., 2006)
Potato	Acetone/water (7:3)	23.1 mg GAE/ 100 g[1]	(Brat et al., 2006)
Tomato	Acetone/water (7:3)	13.7 mg GAE/ 100 g[1]	(Brat et al., 2006)
Carrot	Acetone/water (7:3)	10.1 mg GAE/ 100 g[1]	(Brat et al., 2006)
Pea	Methanol/water (8:2)	160.0 mg GAE/ 100 g[2]	(Kahkonen et al., 1999)
Cereals and Grains			
Oat (grain)	80% Methanol	30.0 mg GAE/ 100 g[2]	(Kahkonen et al., 1999)
Wheat (grain)	80% Methanol	20.0 mg GAE/ 100 g[2]	(Kahkonen et al., 1999)
Barley (grain)	80% Methanol	40.0 mg GAE/ 100 g[2]	(Kahkonen et al., 1999)
Beverages			
Red Wine	No extraction	1018-4177 mg GAE/ L[4]	(Balasundram et al., 2006)
Green Tea	No extraction	80.0 mg GAE/ 100 g[5]	(Seeram et al., 2008)
Coffee	ethyl acetate, n-hexane, dichloromethane 1:1 ratio (v/v)	343.4 mg GAE/ 100 g[5]	(Pulido et al., 2003)

[1] Gallic acid equivalents per 100 grams of fresh edible portion.
[2] Gallic acid equivalents per 100 grams of dry weight.
[3] Ferulic acid equivalents per 100 grams.
[4] Gallic acid equivalents per L of beverage.
[5] Gallic acid equivalents per 100 grams of beverage.

jams, jellies, syrups, wines and fruit juice blends (St-Pierre, 1993). The fruit extract has also been used in the treatment of canker sores, sore throat, sore eye and diarrhea (Stromberg, 2001).

2.4.1 Chemical composition of chokecherry

The fw of ten chokecherries has been reported to range from 6.6-9.2 g, whereas the values for the weight percentage of pit to fruit ranged from 9.4-16.0% (Zatylny et al., 2005a). Green (2007) reported a higher value of pit weight to fruit of 19.2%. The pH of the fruit ranges from 3.9-4.4, with a mean value of 4.2 (Green, 2007). The mean moisture content of chokecherries has been reported as 66.8% (Green, 2007), with a range of 62.0-68.5% (Zatylny et al., 2005a). The crude protein, lipid and ash for chokecherries are 1.15, 0.10 and 0.82% respectively (Hosseinian et al., 2007). Literature values for total and soluble solids range from 35.2-45.8% and 18.3-29.8°Brix, respectively (Zatylny et al., 2005a; Green, 2007).

The TPC of chokecherry has been reported as 131.88 ± 8.06 mg/g ferulic acid equivalents (FAE) (Li et al., 2009). When TPC was calculated using GAE the value ranged from 1045.43 ± 78.57 mg GAE/100 g of fw (Bakowska-Barczak et al., 2007) to 1330 ± 160 mg GAE/100 g fw (Green, 2007). The major phenolics identified included chlorogenic acid, quercetin and rutin (Green, 2007), with caffeic, ferulic, p-coumaric, protocatechuic and sinapic acids also identified (Kraft et al., 2008; Li et al., 2009).

Total reported anthocyanin levels for chokecherry ranged from 105-285 mg cyanidin-3-glucoside equivalents/100 g fw (Bakowska-Barczak et al., 2007; Green, 2007). The two major anthocyanins identified were cyanidin-3-glucoside and cyanidin-3-rutinoside (Green, 2007). Other identified chokecherry anthocyanins include cyanidin-3-(6 acetyl)-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3,5-diglucoside, delphinidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3-rutinoside, pelargonidin-3-rutinoside and peonidin-3-rutinoside (Bakowska-Barczak et al., 2007; Kraft et al., 2008).

2.5 Saskatoons

Saskatoons (*Amelanchier alnifolia* Nutt) are a shrub or small tree perennial from the Rosaceae family that is native to the plains of North America (Zatylny and St-Pierre, 2005). The height of the shrub/tree ranges from 0.3 to over 6.0 meters. Plants are commonly found in coulees, bluffs, hillsides, gulleys, stream banks, and dry rocky soils in full sunlight to moist fertile soils (St-Pierre, 1997). The fruit from this plant are purple to blue-black in colour and range in diameter from 10-15 mm depending on the cultivar (Zatylny et al., 2002). The harvest of mature fruit occurs in July to August (St-Pierre, 1997). Historically, saskatoons were collected from wild stands and were used by aboriginal people and the early settlers of the prairies. The fruit was eaten fresh or added to a protein and dried to produce pemmican. Many portions of the plant were used medicinally including the preparation of concoctions prepared from the inner bark and roots that were used to treat diarrhea, dysentery, painful menstruation and bleeding during pregnancy (St-Pierre, 1997). Mixtures of the fruit were also used for the treatment of eye and stomach ailments (St-Pierre, 1997).

The majority of the orchards today are comprised of a mixture of cultivars including, Honeywood, Martin, Northline, Pembina, Smoky and Thiessen. Saskatoons are eaten fresh or are used in processed products such as jams, jellies, pie fillings, syrups and wine (Zatylny and St-Pierre, 2005). There are more than 1200 hectares of commercial saskatoons plantations across Saskatchewan, Alberta and Manitoba, which account for approximately 6-8 million kg of fruit per year (Bakowska-Barczak et al., 2009). In literature saskatoons are referred to as Saskatoon Berry (Bakowska-Barczak et al., 2009), Saskatoons (Bakowska-Barczak and Kolodziejczyk, 2008) as well as saskatoons (Zatylny et al., 2002; Zatylny et al., 2005b). In this thesis they will be referred to as saskatoons.

2.5.1 Chemical composition of saskatoons

The fresh weight of sixteen different saskatoon cultivars ranged from 0.79-1.66 g per berry with a mean value of 1.14 g, with the Martin variety being the heaviest (Zatylny et al., 2005b). According to literature, fruit pH ranges from 3.65-4.18, with a pH for the Martin cultivar of 3.71 (Zatylny et al., 2005b). The moisture range content of saskatoons

has been reported as 75.3-77.6% (Mazza, 2006; Hosseinian et al., 2007). Crude protein, lipid and ash contents of saskatoons are 1.05, 0.48 and 0.59%, respectively (Hosseinian and Beta, 2007). Literature values for total and soluble solids range from 19.9-25.1% and 14.0-20.1°Brix, respectively (Zatylny et al., 2005b). The specific values for the Martin cultivar were 20.8% total solids and 15.3°Brix (Zatylny et al., 2005b). Each of the saskatoon cultivars has been shown to be a good source of phenolics, although they have some variations in the concentrations of these compounds (Zatylny et al., 2005b; Bakowska-Barczak and Kolodziejczyk, 2008). The TPC of saskatoons has been reported as 37.91 ± 0.61 mg/g FAE (Li et al., 2009), and a range of 554-801 mg GAE/100 g fw (Bakowska-Barczak and Kolodziejczyk, 2008).

Identified phenolics/phenolic classes in saskatoons include hydroxybenzoic acids (ellagic, gallic and p-hydroxybenzoic acids), hydroxycinnamic acids (caffeic acid, chlorogenic, ferulic, neochlorogenic, *p*-coumaric, protocatechin acids) and flavonoids (anthocyanins, catechin, epicatechin, epicatechin gallate, quercetin and rutin) (Bakowska-Barczak et al., 2007; Ozga et al., 2007; Bakowska-Barczak and Kolodziejczyk, 2008; Gazdik et al., 2008; Li et al., 2009). The four major anthocyanins identified in saskatoons are cyanidin-3-arabinoside, cyanidin-3-galactoside, cyanidin-3-glucoside and cyanidin-3-xyloside (Bakowska-Barczak et al., 2007; Ozga et al., 2007; Bakowska-Barczak and Kolodziejczyk, 2008). Minor anthocyanins identified include, cyanidin-3,5-diglucoside, cyanidin-3-xyloside, petunidin-3-galactoside and petunidin-3-glucoside (Kraft et al., 2008). According to literature, the anthocyanin concentration of 17 different saskatoon varieties ranged from 382.1-189.7 mg/100 g fw (Bakowska-Barczak and Kolodziejczyk, 2008).

2.6 Bioavailability of phenolics

One definition of bioavailability is the portion of a compound which is digested, absorbed and metabolized through normal metabolic pathways (McGhie and Walton, 2007). In order to take advantage of the biological properties of phenolics they must first be bioavailable. The behaviour of phenolics in the digestive tract will influence both their nutritional benefits and potential systemic effects. To assess phenolic bioavailability, both the amount consumed and the amount that enters the blood stream

following consumption must be known. Interactions of phenolics with other compounds in foods such as binding to proteins or polysaccharides can significantly reduce their bioavailability (Manach et al., 2004). The chemical structures of phenolics, the type and amount of their metabolites circulating in the blood, also play a role in their bioavailability. Structural factors that affect absorption include conjugation, degree of glycosylation/acylation, degree of polymerization, molecular size and solubility (Manach et al., 2004).

When phenolic-rich foods are consumed, there is an assumption by the general public that they retain their bioavailability. However, the majority of phenolics present in foods are in the form of esters, glycosides or polymers which are not easily absorbed in these chemical forms (Manach et al., 2004; Saura-Calixto et al., 2007). Typically, phenolics must be hydrolyzed by intestinal enzymes or colonic microflora to produce the aglycones and/or cleave the ring structure in order to be absorbed. Therefore, the large intestine is an important region of the gastrointestinal tract for phenolic bioavailability because it is rich in the microflora which produce these enzymes. In the large intestine there are two routes for phenolic uptake: 1) absorption of intact phenolics across the colonic epithelium or 2) via the hydrolysis of the original structure by microbes (Williamson and Manach, 2005). Complicating the structure-function relationship of phenolic bioavailability are the presence of functional groups. Flavonoids such as isoflavones, flavones and anthocyanins are usually glycosylated, which can influence the physical, chemical and biological properties of these molecules.

In order to determine food phenolic bioavailability, detection methods have focused on their presence in plasma and urine after consumption (McGhie and Walton, 2007). Phenolics that have been found to be the most well absorbed in humans are the catechins, flavanones, gallic acid, isoflavones and quercetin glucosides (Manach et al., 2005b). Studies determining the absorption of phenolics from berries (blackberry, chokeberry, raspberry and strawberry) in humans showed significant increases in both plasma levels and urinary excretion. In one study, the total intake of phenolics from berries (bilberries, black currants, chokeberries, lingonberries, raspberries and strawberries) was 837 mg/day for 8 weeks and results showed a significant increase in plasma levels of caffeic acid, p-coumaric acid, homovanillic acid, 3-hydroxyphenylacetic

acid, 3-(3-hydroxyphenyl) propionic acid, protocatechuic acid, quercetin and vanillic acid when compared to the control group. The observed increase in plasma levels for these compounds ranged from 7-81% showing that a portion of the source fruit phenolics were bioavailable (Koli et al., 2010).

Anthocyanin absorption differs from other flavonoids because it has been determined through animal studies that the intact glycosidic form can be absorbed (McGhie and Walton, 2007). Studies in rats have shown that anthocyanins reach the circulatory system within 0.25-2 h after consumption (McGhie and Walton, 2007). An animal (rat) study showed that oral administration of 400 mg/kg of body weight of cyanidin-3-glucoside resulted in plasma concentrations of 2-3 µg/mL of the intact compound after 30 minutes (Tsuda et al., 1999). Felgines et al. (2002) determined that blackberry anthocyanins were excreted in both their intact and methylated forms, and as aglycones/conjugates.

The sites of anthocyanin absorption have been reported in literature (El Mohsen et al., 2006; Hassimotto et al., 2008; He et al., 2009) and have lead to the suggestion that they are partially absorbed from the stomach unlike other phenolic classes that are absorbed through the small intestine (McGhie and Walton, 2007). As an example, in a human study involving the feeding of a black currant concentrate containing 3.58 mg cyanidin-3-glucoside/kg of body weight was delivered to 8 individuals, this compound was shown to be present in the plasma (0.12 nM) and the urine (0.1% of the original dose) of all subjects as the intact glycosylate (Matsumoto et al., 2001). These results show that intact anthocyanins are both absorbed and excreted rapidly within 2 h of ingestion. As human studies have shown phenolic absorption from foods, there is a growing need for more long term human feeding trials so as to better understand the mechanisms of phenolic uptake, and the relationship between phenolic class and bioavailablity (Williamson and Manach, 2005).

2.7 Functional and medicinal properties of phenolics

2.7.1 Functionality in foods

Phenolics are partially responsible for the nutritional and sensory qualities of plant foods (Bravo, 1998). The sensory qualities that phenolics contribute to foods are related to overall aroma and taste (Lule and Wenshui, 2005). In beverages, the astringency and bitterness associated with phenolics depends on their concentration and structure (Bravo, 1998). Another functional aspect of phenolics (i.e. anthocyanins) are the colours they contribute to foods such as fruits and vegetables (Lule and Wenshui, 2005). Although other classes of phenolics are colourless, they can be converted via oxidation, to coloured brown compounds during food handling and processing (Lule and Wenshui, 2005). This process may be undesirable in many products, however in others such as coffee, leaf teas or dates this colour change is desirable (Bravo, 1998; Lule and Wenshui, 2005). Phenolics can also act as antioxidants in foods, which helps to prevent/delay oxidative deleterious effects by reacting with reactive oxygen species or through the stimulation of endogenous enzymes (Zhu et al., 1999; Shahidi and Naczk, 2004b).

2.7.2 Medicinal properties

Phenolics are important natural constituents of edible plants and are increasingly recognized for their possible health-promoting roles through food consumption and nutritional supplements. These compounds have been suggested to have the ability to prevent a number of human diseases based on their ability to interact with reactive oxygen and nitrogen species (Figure 2.7). Reactive oxygen species (ROS) include the, superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and singlet state oxygen ($^1O_2$). Reactive oxygen species are produced within the cells of our bodies during normal cellular activities and provide important physiological functions (Seifried et al., 2007). These include antimicrobial activities, energy production and signal transduction for cellular communication (Noguchi and Niki, 1998). Of the oxygen we consume 1-3% is converted to ROS by our body (Sohal and Weindruch, 1996). When concentrations of ROS are present in excess they can damage cellular lipids, proteins or form DNA adducts that have the potential to

promote carcinogenic activity (Seifried et al., 2007). These processes are kept under control by endogenous cellular antioxidant mechanisms (Seifried et al., 2007). Therefore, the interest in the consumption of natural antioxidants is rising amongst consumers because of their purported ability to protect the body against reactive oxygen and nitrogen species that are associated with degenerative diseases (Shahidi and Naczk, 2004b).

Numerous *in vitro* and *in vivo* studies have shown that when phenolics are added to the diet there is a prevention of cancers (Imai et al., 1997; Wu et al., 2003), cardiovascular diseases (CVD) (Diebolt et al., 2001; Manach et al., 2005a), neurodegenerative diseases (Levites et al., 2001), diabetes (Elliott et al., 2009) and osteoporosis (Scalbert et al., 2005). *In vitro* models have shown the ability of phenolics to inhibit the oxidation of low density lipoproteins (LDL) which are involved in the formation of atheromatous lesions (Zhu et al., 1999). Phenolic classes such as flavonoids have shown positive preventative effects towards cardiovascular diseases. Hertog et al. (1993) showed that a diet high in flavonoids (particularly quercetin) was associated with a decrease in the risk of coronary heart disease mortality in males. In another study, similar preventative results in females were observed as flavonoid intakes increased (Knekt et al., 1996).

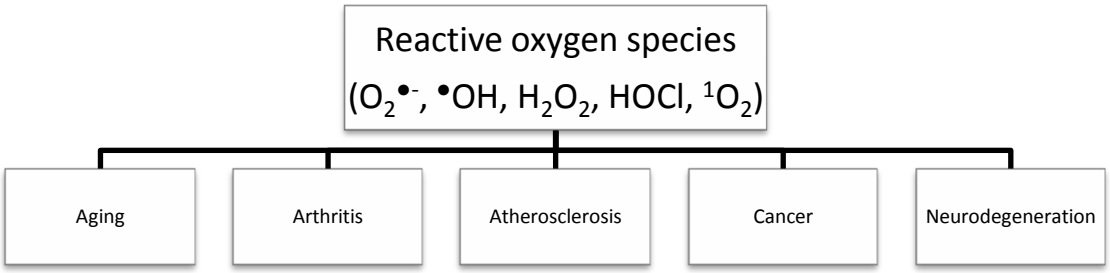


Figure 2.7 Diseases associated with reactive oxygen species (adapted from Shahidi and Naczk 2004a).

Phenolics have also been reported to have anticarcinogenic effects in animal models through the blocking of initiation steps such as the metabolism of procarcinogens by modulating the expression of cytochrome P450 enzymes involved in their activation, increasing the expression of phase II conjugation enzymes and through the stimulation of

DNA repair (Webster et al., 1996; Scalbert et al., 2005). Another anticarcinogenic mechanism of phenolics includes the prevention of tumour formation and growth by inhibiting cell proliferation (Kuntz et al., 1999). Diseases, such as Alzheimer's, Parkinson's and dementia, result through ischemic injury which results in the production of ROS (Scalbert et al., 2005). As these diseases are associated with oxidative stress on brain tissues, antioxidants may help to delay or prevent onset (Halliwell, 2001). Mitochondria have been identified as a main producer of ROS in cells and the presence and concentration of ROS have been correlated with the aging process (Beckman and Ames, 1998; Melov, 2004). Studies on phenolics, such as resveratrol, have shown that increased accumulation in the mitochondria can decrease ROS production due to the negative potential of this organelle, and may delay the negative cellular consequences of aging (Biasutto et al., 2008).

2.7.3 Controversy surrounding *in vitro* versus *in vivo* antioxidant activity results of fruits and vegetables and their prevention of disease(s) in humans

A general perception exists that the consumption of fruits and vegetables in the human diet will lead to the prevention of chronic diseases such as cancer and CVD. As outlined in previous sections of this thesis, both fruits and vegetables are rich sources of phenolic compounds, and these compounds are known to display antioxidant activities *in vitro* (Galluzzo et al., 2009; Androutsopoulos et al., 2011; Nickel et al., 2011). In addition to being rich sources of phenolic compounds, fruits and vegetables have also been shown to contain other chemical compounds such as vitamins, minerals and dietary fibre, which exhibit antioxidant activities (Meester et al., 2008). It is the relationship between phenolic compound structure and their mechanism(s) of antioxidant activity that have led to the widespread belief in their chronic disease prevention function. However, results from human studies (i.e. *in vivo*) on fruit and vegetable consumption and chronic disease prevention have been less positive leading to some scientific controversy regarding experimental design an methodology.

Boffetta et al. (2010) investigated the cancer (all cancer types) prevention properties of fruit and vegetable consumption. In this cohort study, a total of 142 605 men and 335 873 women reported their fruit and vegetable consumption over a 12 month

period, which ranged from 32-1061 g/day. Results were measured as the incidence of any cancer development after the median follow up time of 8.7 years. They found that there was only a modest cancer preventative effect associated with the increased intake of fruits and vegetables from 0-226 g/day to ≥647 g/day. Because this study only looked at the total cancer incidence, any significant preventative results with individual cancer types and/or specific compound/group of compounds were masked by this type of experimental design/data reporting. Another cohort study by George et al. (2009) investigated fruit and vegetable consumption on total and 18 specific cancers (i.e. bladder, brain, breast, colorectal, endometrial, esophagus, head and neck, kidney, liver, lung, myeloid leukemia, myeloma, non-Hodgkin lymphoma, ovarian, pancreas, skin, stomach and thyroid). The study consisted of in 483 338 participants, including 288 109 men and 195 229 women who reported their dietary intake of fruit and vegetables (excluding potatoes) over a 12 month period. Results were gathered after a 7 year follow up time. Data showed that an increase in fruit (from 0-142.2 g/1000 kcal diet up to 450.3-1322.5 g/1000 kcal diet) and vegetable (from 0-132.7 g/1000 kcal diet up to 338.9-1038.1 g/1000 kcal diet) consumption had no significant effect on the risk of total cancer as well as any of the identified types in the female cohort. In the male cohort the increase in fruit (from 0-104.3 g/1000 kcal diet up to 376.8-1215.8 g/1000 kcal diet) consumption had no significant effect on total cancer or any of the individual cancers, whereas the increase in vegetable (from 0-104.3 g/1000 kcal diet up to 260.7-770.3 g/1000 kcal diet) consumption did have a significant effect on reducing the incidence of thyroid cancer. A case control study by Cui et al. (2008) investigated the preventative effects of flavonoids on lung cancer. The study consisted of 1395 participants who reported their dietary intake of fruit, vegetables and beverages for a period of 12 months. The total amount of flavonoids consumed was determined by taking the mean published (USDA, 2003) flavonoid content (mg/100 g) of these fruits, vegetables and beverages. Experimental data was collected after a 5 year follow up time and the results indicated that there was an inverse association between the consumption of black tea and lung cancer in smoking men. When higher levels of specific flavonoids were consumed, such as catechin (4 mg/day), epicatechin (10 mg/day), kaempferol (2 mg/day) and quercetin (9 mg/day); a preventative effect was found in male smokers. These correlative results appear to show

that the consumption of specific flavonoids and or flavonoid ratios were able to act as strong antioxidants against ROS generated by tobacco smoking.

Literature results on the relationship fruit and vegetable consumption and the prevention of CVD are more positive. Hung et al. (2004) investigated the incidence of CVD as related to fruit and vegetable consumption. In this study a cohort of 71 910 female and 37 725 male participants who's diet was followed over a 10 year period with food frequency questionnaires conducted at the start date, and with dietary follow ups at years 2, 6 and 10. Data was collected after a 14 year experimental period and results were based on the incidence of CVD as a function of fruit and vegetable consumption. The authors found that CVD incidence decreased as fruit and vegetable consumption increased (from 1.5 to 5 servings/day). The participants that consumed at least 5 servings of fruits and vegetables daily had a 28% lower risk of CVD than those eating only 1.5 servings. Experimental data also showed that fruits had a greater effect on CVD reduction than did vegetables. The flavonoids present in fruits have been shown to have a preventative effect on CVD (Knekt et al., 2002). Mink et al. (2007) investigated the effects of flavonoids on CVD and coronary heart disease (CHD) in a cohort of 34 489 postmenopausal women. Dietary flavonoid intake was determined by conducting five 24 h food frequency recall interviews over an 11 year period. Data was collected after a 16 experimental time period and results were based on the relationship between subject mortality due to CVD and/or CHD and flavonoid consumption. The authors found that mortality rate decreased as the intake of total flavonoids increased (from 0.6-133.2 mg/day up to >425.3 mg/day). Individual flavonoid consumption was also investigated and it was determined that any increase in anthocyanin consumption (from 0 mg/day) lead to a decrease in subject mortality due to CVD and/or CHD. In addition, the authors noted that when flavanone (a specific class of flavonoids) consumption increased from 0-16.1 mg/day to ≥72.8 mg/day, mortality due to CVD and/or CHD decreased. . These results show that the dietary intake of flavonoids, specifically anthocyanins and flavanones present in fruits and vegetables were associated with mortality reduction due to CVD and/or CHD.

Based on this selection of literature results, the prevention of some cancers (lung and thyroid) has been demonstrated to be linked with the increased consumption of fruits

and vegetables, however, the overall prevention of all cancers is not likely to occur with just fruit and vegetable consumption alone. Also, a reduced risk of CVD/CHD mortality appears to be closely correlated with fruit and vegetable consumption particularly when associated with the presence and concentration of specific phenolic compounds and classes.

2.8 Antioxidant activity and capacity of phenolics

Antioxidant activity describes the rate at which a reaction between a certain antioxidant and oxidant proceeds, whereas antioxidant capacity is a measure of the amount of free radical that can be scavenged (MacDonald-Wicks et al., 2006). The presence of a phenolic group gives phenolics their characteristic antioxidant activity so they can act as oxygen scavengers, free radical terminators, singlet state oxygen quenchers and metal chelators (Shahidi et al., 1992; Bravo, 1998; Fraga, 2007). The predominant antioxidant mechanism of phenolics is the scavenging of free radicals, which limits the oxidation of susceptible compounds in both foods and the human body (Bravo, 1998). As various phenolic classes exist in foods, they vary in their free radical scavenging activity. Many factors influence the antioxidant capacity and activity of phenolics including, their degree of hydroxylation, polarity, solubility, reducing potential, stability through food processing and storage, and radical stability (McClements and Decker, 2008).

2.8.1 General mechanism

Antioxidants can be classified by their mode of action, and phenolics are included in the category of free radical terminators (Shahidi et al., 1992). Phenolic antioxidants (POH) can react with ROS ($ROO^{\bullet}$ or $RO^{\bullet}$) or interfere with the oxidation process through the donation of a hydrogen atom to the radical (Gordon, 1990). After donation, the POH is converted to an oxidized phenoxy radical ($PO^{\bullet}$), which is stabilized through resonance as shown in Figure 2.8 (Gordon, 1990; Shahidi et al., 1992). When free radicals are scavenged there is an antioxidant effect which slows or inhibits the oxidative chain reaction. The reduction of both peroxyl and alkoxy radicals by phenolics is illustrated below:

$$ROO^{\bullet} + POH \rightarrow ROOH + PO^{\bullet}$$

$$RO^{\bullet} + POH \rightarrow ROH + PO^{\bullet}$$

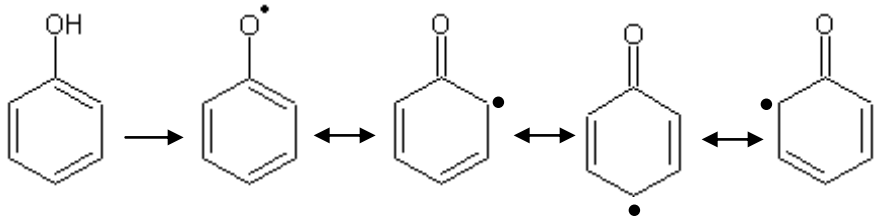


Figure 2.8 Stabilization of the phenoxy free radical through resonance (adapted from Gordon 1990).

The intermediates produced in this reaction are relatively stable so that further free radical reactions (propagation reactions) are not initiated (Shahidi et al., 1992). Phenoxy radicals can also act as terminators of propagation reactions by interacting with other free radicals as shown below (Bravo, 1998):

$$R^{\bullet} + PO^{\bullet} \rightarrow ROP$$

$$RO^{\bullet} + PO^{\bullet} \rightarrow ROOP$$

The activity of a phenolic as an antioxidant depends greatly on its structure (Bravo, 1998). The basic phenol molecule is a very poor antioxidant, however the addition of hydroxyl or n-butyl groups at the ortho and para positions significantly increases antioxidant activity (Bravo, 1998). With respect to flavonoids, the most important structural features are the 4'- and the 3-hydroxyl groups (Duthie and Crozier, 2000). Also, the addition of hydroxyl groups ortho to the 4' position has been shown to increase the antioxidant capacity of the compound (Duthie and Crozier, 2000).

The antioxidant activity of flavonoids has been reported to be greater than those of the natural antioxidant vitamin E (Duthie and Crozier, 2000). Reasons for this greater activity are based on the following structural features: an extended conjugated system to support the unpaired electrons, reduced steric hindrance at the active site(s) and the presence of two or more hydroxyl groups (Duthie and Crozier, 2000).

In order to determine if phenolics have antioxidant activity, *in vitro* assay methods are often used. These methods are quantitative and are typically based on colourimetric reactions. In contrast, *in vivo* methods are used to investigate antioxidant behaviour in a biological system.

2.8.2 Select *in vitro* antioxidant assays

There are a number of different *in vitro* assays available for measuring antioxidant activity based on the free radical scavenging ability of phenolics including 2,2-α-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), aldehyde/carboxylic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), ferric reducing/antioxidant power, ferric thiocyanate, hydrogen peroxide scavenging, hypochlorous acid scavenging, oxygen radical absorbance capacity, peroxyl radical scavenging, superoxide radical scavenging, and thiobarbituric acid assays (Sanchez-Moreno, 2002; Huang et al., 2005). However, as only the ABTS and DPPH assays were employed in this research, they will be the ones discussed.

The ABTS assay has been extensively used in literature to determine the antioxidant activity of compounds in foods (Kao et al., 2008; Vasco et al., 2008; Moon and Shibamoto, 2009; Valavanidis et al., 2009; Hervert-Hernandez et al., 2010; Tagliazucchi et al., 2010). In addition, the DPPH assay has been used for food antioxidant measurements as illustrated by a number of recent literature citations (Kao et al., 2008; Vasco et al., 2008; Moon and Shibamoto, 2009; Rufino et al., 2009; Akillioglu and Karakaya, 2010; Faller and Fialho, 2010; Ramchandani et al., 2010). In the DPPH assay the scavenging potential of the DPPH radical (DPPH•) by an antioxidant (AH) is measured spectrophotometrically. The antioxidant is allowed to react with DPPH• in an alcoholic solution and the reduction of AH is monitored by a decrease in solution absorbance (measured at 515 nm). This assay has a major limitation as the lifetime of DPPH• has little similarity to the reactive free radicals present in biological systems (MacDonald-Wicks et al., 2006). Consequently, an antioxidant may react much slower with DPPH• than free radicals under native conditions, underestimating its free radical scavenging potential.

The ABTS radical cation ($ABTS^{\bullet+}$) assay is also known as the Trolox equivalent antioxidant capacity (TEAC) (Millar, 1996). The reaction is initiated by the addition of hydrogen peroxide or potassium persulfate to ABTS to produce $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ has an absorbance maxima at 734 nm and when an antioxidant is added to the solution its colour is suppressed in proportion to its concentration (Kaur and Geetha, 2006). The TEAC value is normally reported as the ability of the antioxidant to scavenge $ABTS^{\bullet+}$ as compared to Trolox, which is a synthetic analogue of vitamin E. A problem with this *in vitro* assay is that $ABTS^{\bullet+}$ is unlike free radicals present in biological systems (MacDonald-Wicks et al., 2006). However, a favourable aspect of this assay is that it can be used to determine antioxidant capacity in both aqueous and lipid phases (Arnao et al., 1998; MacDonald-Wicks et al., 2006).

In vitro scavenging assays do not necessarily predict how an antioxidant will act in a complex system, such as food. They also fail to take into account the bioavailability of the antioxidant, its storage patterns in tissues, the reactivity of the sample, the effects of the sample matrix or synergies between the presence of other antioxidants (MacDonald-Wicks et al., 2006).

2.8.3 *In vivo* antioxidant methods

Although *in vitro* assays provide an estimate of the antioxidant abilities of compounds they do not mimic biological systems. To better understand the antioxidant activities of phenolics in biological systems *in vivo* studies with animals (rats, mice, rabbits etc.) or humans are conducted, which are followed by the chemical analysis of biological fluids/tissues to determine antioxidant activity and bioavailability post consumption. *In vivo* methods include the indirect detection of oxidative stress that occurs in the body on lipids, proteins and DNA (Wood et al., 2006). The products from oxidative reactions on the aforementioned compounds can be used to monitor oxidative stress and are an indirect method of measuring antioxidant capacity.

The measurement of oxidative damage to polyunsaturated fatty acids is the most commonly studied effect of free radical production in the human body (Kneepkens, 1997). These assays include the monitoring of lipid peroxidation products including, alkanes, ethane, F2-isoprostanes, 4-hydroxy-2-nonenol, isoprene, malondialdehyde and

pentane (Wood et al., 2006). These peroxidation products are present in the biological fluids and tissues of animals/humans. The most common methods for their detection include gas chromatography (GC), GC mass spectroscopy and enzyme immunoassays (Esterbauer et al., 1986; Janero, 1990).

A common *in vivo* assay for LDL involves the monitoring of peroxide damage as measured by antibodies that recognize oxidative modifications of LDL (Tatzber et al., 1997). It has been shown that the major product of ROS attack on proteins are protein carbonyls which result in the modification of amino acid side chains (Levine et al., 2000). The most common method used to measure these chemical changes is HPLC (Levine et al., 2000).

The last major class of compounds that are measured *in vivo* are the DNA oxidation products which can be detected in urine. Radical damage of DNA results in the cross-linking between bases, the coupling of bases to proteins, the chemical modification of bases and strand breaks (Wood et al., 2006). As a result of these reactions, DNA oxidation products such as 8-hydroxy-deoxyguanosine, 8-hydroxy-adenine or 7-methyl-8-hydroxyguanine can be detected in urine (Halliwell and Aruoma, 1997). These oxidation products can be analyzed by GC or HPLC and/or their concentrations relative to controls can be used as a measure of DNA protection by antioxidants (Svoboda and Kasai, 2004).

2.9 Phenolic extraction methods

The main goal of the extraction process is to separate and concentrate the bioactive components (i.e. phenolics) in the raw material from other chemical constituents. The extraction procedure is also one of the initial steps required to purify a bioactive compound for further chemical and physical analysis. The raw material sources containing the compound(s) of interest are normally pre-treated prior to extraction in order to increase yields. These initial treatment steps include crushing, grinding, maceration or milling (Lee, 2004). Pre-treatment increases the surface area of the raw material allowing for better contact time with the extraction solvent. In addition to the role of pre-treatment in the extraction process, the chemical structures of the sample phenolics also play an important role in their extraction efficiency. The final sample

obtained following solvent extraction is usually a mixture of phenolic classes and further purification is required to obtain individual compounds.

2.9.1 Solvent extraction

The majority of phenolics in plants are present in the vacuoles and are commonly extracted using organic solvents (Santos-Buelga and Scalbert, 2000; Robbins, 2003). The most common organic solvents used for the extraction of phenolics include acetone, diethyl ether, ethanol, ethyl acetate and methanol (Kahkonen et al., 2001; Robbins, 2003; Lee, 2004). These solvents are typically used as aqueous solutions ranging from 60-80% (v/v) (Harborne and Williams, 2000). When paired with appropriate extraction conditions, the solvent system destroys the cell membrane and simultaneously dissolves the phenolics (Naczk and Shahidi, 2004). Mechanical force methods can also be employed in conjunction with solvent extraction which enhances raw material exposure to the aqueous organic solvent. These mechanical force methods include centrifugation, continuous rotary action, homogenization, microwave irradiation, stirring, ultrasound or vortexing (Robbins, 2003; Naczk and Shahidi, 2006; Krenn et al., 2007). Other factors which impact phenolic solvent extraction efficiency include solvent to sample ratio and frequency, extraction time, and temperature (Naczk and Shahidi, 2004). Solvent extraction is the most common method reported in literature for the isolation of phenolics from fruits (Kahkonen et al., 2001).

Pinelo et al. (2005) showed that phenolic compound extraction from grape pomace at a solvent:sample ratio of 1:1 (w:v) compared to 5:1 (w:v) resulted in an improved extraction. In literature, samples are usually solvent-extracted multiple times (2-6) (Robbins, 2003), however it has been shown that 2-3 times is sufficient for most raw materials (Shahidi and Naczk, 2004b).

Extraction times for phenolic compound recovery from raw materials range from 1 min (Price and Butler, 1977) to 24 h (Maxson and Rooney, 1972). A major disadvantage of extended extraction times is the risk of compound oxidation, however this can be minimized by the addition of a reducing agent such as ascorbic acid to the extraction solvent (Naczk and Shahidi, 2006). By-products (i.e. skins) from grapes, and black and red currants showed the highest total phenolic compound recovery with an

extraction time of 12 h when compared to those of 1 and 24 hr (Lapornik et al., 2005). The same trend was found for the extraction of anthocyanins from these fruit by-products.

The effect of temperature on phenolic extraction is illustrated by the published work of Pinelo et al. (2005). The authors reported higher phenolic levels in samples that were extracted in either ethanol or methanol at 50°C when compared to those extracted at 25°C. In another study, when grape pomace was extracted with water at temperatures ranging from 45-60°C, the total phenolic concentration increased from 0.97 to 1.46 GAE (mg/L) with the temperature increase (Spigno et al., 2007).

Flavonoids, such as anthocyanins and anthocyanidins, are commonly extracted with acidified methanol, ethanol, or an aqueous solution of these solvents in conjunction with ultrasound (Naczk and Shahidi, 2004). Common acidulants include acetic, citric, formic, hydrochloric or trifluoracetic acids (Durst and Wrolstad, 2005). An acid is added to the extraction solvent because it creates the environmental conditions for the formation of the flavylium cation, and this chemical form has both increased solubility and stability (Durst and Wrolstad, 2005). In order to reduce the potential toxic effects from residual solvent levels in phenolic extracts to be used in feeding trials, ethanol can be used alone or as an aqueous mixture. Phenolic extracts prepared using these solvent systems are considered safe for human consumption (Nawaz et al., 2006).

2.9.2 Solid phase extraction

For phenolics, the solid phase extraction (SPE) method normally employs a hydrophobic (i.e. Amberlite XAD-16) or C_{18} loaded silica gel stationary phase in conjunction with an intermediate polarity liquid phase (i.e. methanol or ethanol). This system has been shown to effectively isolate phenolics from unwanted plant material components such as carbohydrates, organic acids and proteins (Kahkonen et al., 1999; Robbins, 2003; Lee, 2004; Green, 2007; Krenn et al., 2007).

Amberlite XAD-16, a non-ionic polymeric absorbent has been successfully used for phenolic compound recovery from saskatoon extracts (Bakowska-Barczak and Kolodziejczyk, 2008), blackberry (Srivastava et al.), buffaloberry, chokecherry and sea buckthorn (Green, 2007). Whereas, C_{18} SPE has been successfully used for phenolic

isolation from saskatoons (Ozga et al., 2007), blueberry, cranberries, chokeberries and lingonberries (Zheng and Wang, 2003), tart cherry and elderberry (Chandra et al., 2001), and apple (Vrhovsek et al., 2004).

2.10 Total phenolic analysis

2.10.1 Total phenolics by Folin Ciocalteu method

The Folin Ciocalteu (FC) method is known as the total phenolic assay, however the method actually measures the total reducing capacity of the sample (Huang et al., 2005). This method is also referred to as the total phenolic content (TPC), and has been reported frequently in literature for fruits and food ingredients because it is convenient, simple and reproducible (Huang et al., 2005; MacDonald-Wicks et al., 2006). The FC method employs an electron-based transfer reaction to determine the reducing ability of the compounds present in the sample. The reagent is a mixture of tungsten and molybdenum oxides which attracts an electron from the antioxidant compound (i.e. phenolic) which causes a reagent colour change (Huang et al., 2005). This colour change is monitored spectrophotometrically at 765 nm. The FC reagent reacts preferentially with phenolic compounds under basic conditions (pH ~10). Under these basic conditions, hydroxyl group dissociation of phenolic compounds leads to the formation of the phenolate anion which has the potential to reduce the FC reagent (Huang et al., 2005; MacDonald-Wicks et al., 2006).

As the FC reagent is reduced, the colour change is proportional to the concentration of antioxidant (i.e. phenolic) present in the sample. Standards are used to create a linear curve with concentration vs. absorption and samples are usually expressed as GAE. Sample results based on GAE have been determined for numerous foods including, fruits, vegetables, cereals, grains and beverages (Velioglu et al., 1998; Kahkonen et al., 1999; Pulido et al., 2003; Brat et al., 2006; Bakowska-Barczak et al., 2007; Seeram et al., 2008).

2.10.2 Chromatographic analysis

High performance liquid chromatography (HPLC) has been widely employed for the separation, identification and isolation of phenolics in fruits/berries (Merken and Beecher, 2000; Robbins, 2003; Naczk and Shahidi, 2006). The choice of stationary and mobile phases allows for the analysis of numerous phenolic classes (Naczk and Shahidi, 2004). The most commonly used stationary phase is C_{18} reverse phase (RP), which range in column size from 100-300 mm in length and 4.6 mm in diameter (Robbins, 2003). The mobile phase is typically an acidified aqueous phase employing acetic, formic, perchloric or phosphoric acid, and an organic phase such as acetonitrile or methanol (Merken and Beecher, 2000; Valls et al., 2009). The injection volumes for samples range from 1-100 µL (Merken and Beecher, 2000; Robbins, 2003). A selection of review articles on RP analysis of fruit phenolics include Merken and Beecher (2000); Robbins (2003); Tsao and Deng (2004) and Valls et al. (2009).

Sample phenolics are most often detected using a photodiode array detector (PDA) as these compounds absorb UV radiation over a wide wavelength range (254-520 nm) (Merken and Beecher, 2000; Robbins, 2003; Valls et al., 2009). Phenolic classes show characteristic absorbance patterns and this provides a method for their identification (Green, 2007; Valls et al., 2009). Phenolic acids and flavonoids show characteristic absorbancies in the range of 190-380 nm (Merken and Beecher, 2000; Robbins, 2003; Green, 2007), while anthocyanin detection can be determined at wavelengths between 515-540 nm (Green, 2007; Krenn et al., 2007; Valls et al., 2009).

Recently, reverse phase HPLC-PDA has been used for the separation and identification of phenolics in the following food sources: green tea (Komes et al., 2010), herbs (Grevsen et al., 2009), cocoa products (Belscak et al., 2009), and fruit (blueberries, chokecherries, grapes, raspberries, sea buckthorn, saskatoons and strawberries) (Green, 2007; Li et al., 2009; Ratnasooriya et al., 2010).

2.11 Encapsulation

As only a small percentage of the phenolics ingested are bioavailable, improved and targeted delivery systems are required to provide the maximum potential health benefits of these compounds (Chen et al., 2006; Bononi and Tateo, 2007; Ferreira et al.,

2007; Zhang and Kosaraju, 2007). One such method is through bioactive compound encapsulation. Encapsulation is a process whereby a continuous thin coating is formed around solid particles, liquid droplets or gas cells (King, 1995). The capsules are comprised of a core material, containing the bioactive ingredient(s) surrounded by a wall material of somewhat uniform thickness. The wall can be engineered through solvent and/or polymer choice and processing conditions to produce either a single or multi-layered capsule (Madene et al., 2006). In the latter case, greater control of core release (prolonged versus pulse) can be achieved in addition to greater core compound protection and capsule mechanical stability. However, because multi-layer capsule designs are more costly and complex to produce, the more common design is a single layer carrier (Xiong et al., 2006; Lucas-Abellan et al., 2007; Zhang and Kosaraju, 2007; Laine et al., 2008; Wu et al., 2008).

Microencapsulation has been used by the food industry for more than 60 years as a method to protect ingredients from environmental or chemical interactions until the desired core release rates and sites are reached (Barbosa-Canovas et al., 2005; Desai and Park, 2005). Encapsulation technology enables core materials to be incorporated into a wider range of products, ensures that optimal dosages are delivered, and aids in balancing cost effectiveness with health promoting effects (i.e., improved bioavailability of core materials) (Shahidi and Han, 1993; Gibbs et al., 1999; Gouin, 2004). A brief list of the advantages of core material encapsulation is presented in Table 2.3.

Table 2.3 Advantages of core material encapsulation (adapted from Barbosa-Canovas et al., 2005; Desai and Park 2005).

Advantages of Encapsulation
1. Controlled release
2. Improved bioavailability
3. Enhanced stability to environmental factors -temperature, moisture, oxidation and light
4. Protection against nutritional loss
5. Masking of undesirable flavours
6. Reduction of interaction with other compounds
7. Easier handling
8. Addition of insoluble ingredients to a product

Capsule wall materials can be composed of natural or synthetic polymers. Some of the natural polymers used as wall materials in the food industry include polysaccharides (i.e. cyclodextrins, dextrin, maltodextrin, starch, cellulose, agar, carrageenan, gum Arabic and sodium alginate); lipids (i.e. beeswax, diglycerides, monoglycerides and paraffin); and proteins (i.e. albumin, casein, gelatine, pea and whey) (Shahidi and Han, 1993; de Kruif, 2004; Ducel et al., 2004; Barbosa-Canovas et al., 2005; Liu et al., 2010). The composition of the wall material is the main determinant of the functional properties of the capsule. Once the wall and core materials have been selected, there are a number of physical techniques which can be used for capsule formation which include spray drying, chilling, cooling, extrusion, fluidized bed coating, liposome entrapment, coacervation, inclusion complexation and centrifugal extraction (Gibbs et al., 1999). The resulting capsules can be classified into three size categories: macro (>5000 μm), micro (200 nm - 5000 μm) and nano (<200 nm) (King, 1995).

Controlled release is defined as a method where one or more of the core materials are released at a desired site and time, and at a specific rate (Pothakamury and Barbosa-Canovas, 1995). The mechanisms of core release from capsules can be divided into five main categories, fracturation or pressure activated, diffusion, dissolution (melting activated), solvent activated and biodegradation or pH sensitive release (Barbosa-

Canovas et al., 2005; Pegg and Shahidi, 2007). There are three main parameters that affect the release of encapsulated core materials, the first being the core coating properties including the density of the material, solubility and cross-linking. The second encompasses capsule properties including size, wall thickness and the number of coating layers. Lastly, the experimental parameters which include temperature, pH, moisture, solvents and mechanical action (Shahidi and Han, 1993). Capsules are typically designed to be sensitive to various environmental triggers that will allow for the controlled release of their core materials. These triggers include, changes in pH, enzymatic activity, osmotic force, mechanical stress, temperature change, and time (Gouin, 2004). The release of the core material from the capsule can be site-specific, stage-specific, or signalled by one of the aforementioned environmental triggers (Lopez-Rubio et al., 2006).

2.11 Wall material

As listed in the previous section there are numerous wall material choices for the encapsulation process. In literature, phenolics have been encapsulated employing the following wall materials, alginate (Deladino et al., 2008), cellulose (Lauro et al., 2005), chitosan (Kosaraju et al., 2006; Zhang and Kosaraju, 2007; Hu et al., 2008), cyclodextrins (Lucas-Abellan et al., 2007), gelatine (Shutava et al., 2009), glucan (Xiong et al., 2006), maltodextrins (Laine et al., 2008; Robert et al., 2010) and triacylglyerols (Barras et al., 2009). Research conducted and presented in this thesis was based on the use of CH-TPP as the wall material. Information about this wall material will be presented in some detail in the following sections.

2.11.1 Chitosan

Chitosan is a naturally occurring biopolymer derived by the deacetylation of chitin from crustacean shells. Chitosan is mainly an unbranched polymer of repeating β-(1→4)-2-amino-2-deoxy-D-glucopyranose units (Figure 2.9). Capsules containing chitosan as the wall material can be produced by covalent or ionic cross-linking; however, the most common method used is ionic cross-linking because of its mild formation conditions. Also, cross-linking through electrostatic interactions eliminates the

possible toxic effects encountered when glutaraldehyde is used as the chemical agent for covalent cross-linking (Hu et al., 2008).

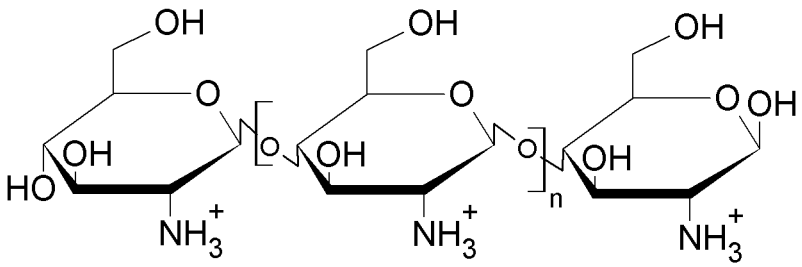


Figure 2.9 The basic structure of chitosan.

2.11.2 Cross-linking of chitosan with tripolyphosphate

Ionic cross-linking of chitosan can be achieved with the multivalent counter ion TPP. Tripolyphosphate consists of three covalently linked phosphate molecules which are negatively charged at pH values below 7.0 (Mi et al., 1999) (Figure 2.10). Ionic interactions of the positively charged amino groups of chitosan with the negatively charged TPP are the driving force for chitosan-tripolyphosphate (CH-TPP) nanoparticle formation (Mi et al., 1999).

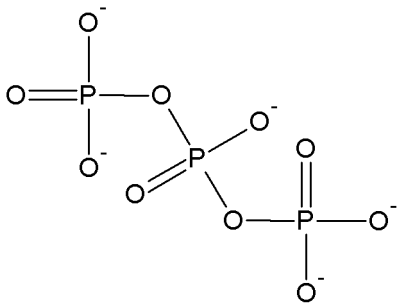


Figure 2.10 The chemical structure of tripolyphosphate.

This nanoparticle formation method using ionic cross-linking of CH-TPP with TPP is also referred to as ionic gelation. Ionic gelation can be accomplished by the addition of an alkaline phase (pH 7-9) containing TPP to an acidic phase (pH 4-6) containing chitosan (Zhang and Kosaraju, 2007). Under these conditions, protonation of the chitosan amino group allows for the interaction with the negatively charged TPP (Suheyla, 1997). These formed junction zones are ionic and form an overall network. The formation of gel nanoparticles occurs because of the intermolecular and intramolecular cross-linking between these oppositely charged molecules (Mi et al.,

1999; Janes et al., 2001). The swelling and breakdown of these nanoparticles can be influenced by the cross-linking time and anion concentration. In general, when both are increased, particle swelling decreases (Shu and Zhu, 2002).

The size of particles produced using the ionic cross-linking method range from 200 nm–1 μm (Zhang and Kosaraju, 2007; Hu et al., 2008; Jang and Lee, 2008; Papadimitriou et al., 2008). The observed variation in particle size is due to the formation conditions, molecular weight of the starting material and the bioactive molecule being entrapped. Particle size distribution can also be affected by the CH to TPP ratio (Papadimitriou et al., 2008). The size of the particle is a significant factor in the uptake into mucosal and epithelial tissues and intracellular trafficking (Hu et al., 2008). Particles that are 500 nm and smaller have shown higher particle uptake which leads to higher active ingredient absorption (Acosta, 2009).

Another important characteristic of the particles is their surface charge or zeta potential. Zeta potential for CH-TPP particles is dependent on a number of factors including the measurement conditions (pH) and the CH concentration. Current published research on CH-TPP particles report positive zeta potential values ranging from 19 - 53 mV (Zhang et al., 2004; Zhang and Kosaraju, 2007; Hu et al., 2008; Jang and Lee, 2008). Particles having a greater positive surface charge or zeta potential would have better abilities to adhere to the lining of the gastrointestinal tract and increasing bioadhesive properties (Bonferoni et al., 2009; Pedro et al., 2009). This occurs because of the gastro-intestinal (GI) tract is lined with a protective mucus layer which contains negatively charged glycoproteins (Bonferoni et al., 2009; Pedro et al., 2009). Higher zeta potential values also prevent particle agglomeration and therefore keep the solution uniform (Jahanshahi and Babaei, 2008)

Many different core materials have been encapsulated using the ionic gelation technique of CH and TPP including protein (bovine serum albumin) (Rayment and Butler, 2008), vitamin C (Jang and Lee, 2008), antimicrobials (i.e. copper) (Qi et al., 2004), dorzolamide and pramipexole (Parkinson disease drugs) (Papadimitriou et al., 2008), and phenolics including tea catechins (Zhang and Kosaraju, 2007; Hu et al., 2008) and quercetin (Zhang et al., 2008).

2.11.3 Bioadhesion

Bioadhesion is the process whereby synthetic and/or natural polymers are able to adhere to mucosal surfaces of the body (Woodley, 2001; Grabovac et al., 2005). Chitosan has been identified in literature as a bioadhesive macromolecule (Woodley, 2001; Hejazi and Amiji, 2003). The cationic nature of CH at physiological pH is ideal for bioadhesion in the human GI tract (Bonferoni et al., 2009). Ionic interactions occur between the positively charged groups of chitosan (pKa of 6.3) and the sialic acid (pKa of 2.6) moieties of mucius interact by strong electrostatic interactions at physiological pH (7.3-7.4) (He et al., 1998; Woodley, 2001; Bowman and Leong, 2006; Bonferoni et al., 2009; Pedro et al., 2009). Other physicochemical processes such as hydrogen bonding, hydrophobic interactions and van der Waals forces may also influence chitosan bioadhesion (Woodley, 2001). Bioadhesion is directly related to the number of free amino groups on CH and its overall charge density, and it is also affected by the degree of cross-linking (Bonferoni et al., 2009; Pedro et al., 2009). It has been shown that microparticles prepared by ionic gelation had greater bioadhesion ability when compared to those produced through other methods such as thermal cross-linking or glutaraldehyde cross-linking. This was most likely due to the observation that particles produced from the latter methods had a lower zeta potential (Dhawan et al., 2004). Therefore, the cross-linking method used for chitosan particle formation will influence bioadhesion ability, with those produced through ionic gelation having the best adhesive properties (Pedro et al., 2009). The advantages of particles having bioadhesion properties include prolonged residence time in the intestine at the site of core material absorption due to mucosa interactions, and that particles may also have an effect on the gating properties of epithelium cell tight junctions and/or that they may increase cellular permeability (Hejazi and Amiji, 2003; Bowman and Leong, 2006; Pedro et al., 2009).

2.12 Animal feeding methods

In order to fully understand the potential health benefits of anthocyanins or other phenolics it is important to know how they are absorbed, metabolised, distributed and excreted (El Mohsen et al., 2006). Literature is abundant in detailing the effects of phenolics on biological systems *in vitro*, however minimal consideration has been

directed towards their bioavailability or metabolism as observed *in vivo* (Williamson and Manach, 2005). Therapeutic effects of anthocyanins are dependent on their absorption into cells and organs from the diet (McGhie and Walton, 2007). When examining compound bioavailability there are a number of advantages of animal models when compared to those for humans. These advantages include the ability to induce dietary deficiencies, use of radioisotopes, tissue collection and cost (Gallaher, 1992; Failla et al., 2008). Although there are ethical guidelines for the use of animals in experimental studies, the rules for human exposure to test compounds are more strict (Gallaher, 1992). When extrapolating experimental results obtained from animal studies to humans, differences such as the physiology between the animal being studied and humans must be taken into account as they can lead to differences in the bioavailability and metabolism of the compounds under investigation (Espín et al., 2007). When using animal models to help explain bioavailability, an understanding of the similarities and differences to humans provides clarity to the results, and validity of the model (Gallaher, 1992).

There are two main techniques used for the delivery of bioactive compounds in a rat model: gavage or dietary feeding. The term gavage means that the compound of interest is administered by passing a tube down the esophagus directly to the stomach of the animal. In dietary feeding the compound is mixed with the feed administered to the animal. The gavage method can be used when the bioactive compounds being investigated are not stable for long periods of time in the feed, or are not palatable to the animal. One of the advantages of the gavage method is that the precise dose of the bioactive can be given to the subject, which can reduce the amount of test compound required (Savenije et al., 2010). The main disadvantage of the gavage technique is that it can be stressful for the animals if they are not properly conditioned to the procedure and if performed improperly can lead to respiratory problems.

The main advantages of the dietary feeding method are; the interactions between the bioactive compounds and the food matrix are taken into account, resulting in a more realistic account of bioavailability; (Ross and Kasum, 2002) it is less time consuming because the active ingredient can be mixed into a large batch of feed (Johnson, 2007; Walton et al., 2009). The bioactive compound studied must be stable in the feed,

palatable to the animal, and must be homogeneously distributed and consumed so that all subjects receive the same dosage (Johnson, 2007).

The analysis of tissues, blood, urine and feces of test animals provides important data on the amount of the administered compound that was absorbed and excreted. Tissues that are commonly examined include the liver, kidney, stomach, small and large intestines, spleen, heart, brain, pancreas, lung and testes.

Animal studies investigating the bioavailability of anthocyanins have shown that these compounds appear in blood and urine as unchanged or in their methylated, glucurono- or sulpho-conjugated forms (Felgines et al., 2009; Sakakibara et al., 2009). Sakakibara et al. (2009) determined that 51.5 % of the anthocyanins (isolated from bilberry) administered to rats at 33.7 mg/kg body weight were localized in the liver, demonstrating that this organ may be the main absorption site for these compounds. Animal studies have determined that the majority of anthocyanins are absorbed in their glycosidic form from the stomach by a process that involves bilitranslocase, and reach the circulatory system in 0.25 to 2 h (McGhie and Walton, 2007; Felgines et al., 2009). Further absorption also appears to take place in the small intestine (McGhie and Walton, 2007; Felgines et al., 2009). From animal studies (mice and rat) through either dietary feeding or gavage, anthocyanins have been found in the liver and kidney (Frank et al., 2002; Talavera et al., 2005; El Mohsen et al., 2006; Vanzo et al., 2008; Sakakibara et al., 2009), digestive system including the stomach, small intestine and large intestine (Frank et al., 2002; Talavera et al., 2005; El Mohsen et al., 2006; Borges et al., 2007; He et al., 2009; Walton et al., 2009), the lungs (El Mohsen et al., 2006; Sakakibara et al., 2009), brain (Talavera et al., 2005; El Mohsen et al., 2006) and testes (Sakakibara et al., 2009).

In order to determine the anthocyanin content in excised animal tissue, an extraction procedure prior to HPLC analysis is employed. Initially, tissue samples of interest are perfused prior to excision by pumping phosphate buffered saline through the animal's circulatory system. The excised tissues are then washed with the same buffer to remove residual blood present on the tissue (Talavera et al., 2005; Ichiyanagi et al., 2006). Tissue samples are then frozen in liquid nitrogen or lyophilized (de Boer et al., 2005; Hassimotto et al., 2008), and anthocyanins are extracted via homogenization with a solvent such as acidified methanol or acetone (Hassimotto et al., 2008). Supernatants

are then collected through centrifugation and dried by rotary evaporation (Hassimotto et al., 2008). Samples are then re-dissolved and filtered before HPLC-PDA and/or HPLC-mass spectrometry analysis (Gee et al., 2004; Ichiyanagi et al., 2006). The majority of studies that have examined (by HPLC-PDA/HPLC-MS) the bioavailability of anthocyanins are based on the levels of the flavylium cation in animal tissues (stomach, small intestines and large intestines) (McGhie and Walton, 2007). It is postulated that the metabolism of anthocyanins occurs through the digestive tract because of the pH differences observed, coupled with the presence of microbial populations (McGhie and Walton, 2007).

Bioactive compound targeting to specific organelles such as the mitochondria may be desired in the control of degenerative diseases (Yao and Vieira, 2007). The mitochondria of cells continuously generate ROS, which play a vital role in cell life and death (Victor et al., 2004) and also play a key role in cellular aging (Sastre et al., 2000). Therefore, the targeting of antioxidants to the mitochondrial respiratory chain may be an important mechanism in cell protection (Victor et al., 2004). One of the biophysical properties of the mitochondria is its high negative internal potential, which has been exploited to deliver lipophilic cations to this organelle (Sheu et al., 2006).

3.0 MATERIALS AND METHODS

3.1 Chemicals

The following chemicals were obtained through Sigma-Aldrich Canada Ltd. (Oakville, ON): Amberlite XAD-16, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), caffeic acid, catechin, chitosan (low molecular weight 92% deacetylated), chlorogenic acid, citric acid, cyanidin-3-galactoside (ideain), cyanidin-3-glucoside (kuromain), cyanidin-3-rutinoside (keracyanin), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin & Ciocalteu's phenol reagent (FCR), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), narigenin, phloridzin, porcine pancreatin, quercetin, rutin hydrate and sodium tripolyphosphate (TPP).

Chemicals from BDH obtained through VWR Canada (Mississauga, ON) included: citric acid, hydrochloric acid (HCl), ortho-phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4) and sodium bicarbonate ($NaHCO_3$).

Chemicals from EMD obtained through VWR Canada (Mississauga, ON) included: acetic acid (glacial), acetonitrile (HPLC grade), formic acid, methanol (HPLC grade), potassium chloride, sodium acetate, sodium chloride (NaCl) and sodium phosphate dibasic.

Sodium hydroxide solution (50% w/w; NaOH) from Fisher Scientific was obtained through VWR Canada (Mississauga, ON). Ethanol (95% (v/v)) from Commercial Alcohols Inc. (Brampton, ON) was obtained through The College of Agriculture and Bioresources stores (Saskatoon, SK). Oxgall dehydrated bile media from Difco was obtained through VWR Canada (Mississauga, ON).

The following anaerobic gases were obtained from Praxair (Saskatoon, SK): ultra high purity nitrogen and a mixed system containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide. Oxygen used in animal experiments was also obtained from Praxair.

Isoflurane was obtained from the Baxter Corporation (Mississauga, ON) through the animal resource centre at the University of Saskatchewan.

3.2 Fruit material

Fruit sources used for this project were saskatoons (*Amelanchier alnifolia* Nutt.) and chokecherries (*Prunus virginiana*). All samples were collected in 2008. The saskatoons (cultivar Martin) were harvested by hand in mid July from J.W.D Market garden in Outlook, SK. The chokecherries were harvested by hand from the Prairie Farm Rehabilitation Administration (PFRA) in Outlook, SK at the end of August. Saskatoons were collected when berries were a purple colour which is present in the last stage of ripeness (Rogiers and Knowles, 1997). Chokecherries were collected when the fruit was dark purple to black in colour. Approximately five kilograms of each fruit were collected. After the fruit was collected it was placed on ice for transportation. Fruit was rinsed with Milli-Q^{TM} water (Millipore Corporation, Billerica, MA) referred to as ddH_2O in this thesis, placed into freezer bags and frozen (-30°C) until used for experiments. The time between fruit harvest and frozen storage was approximately 3 h.

3.3 Total soluble solids

The total soluble solids (TTS) of the two fruits were determined by refractometry (Auto Abbe refractometer; Leica Inc., Buffalo, NY) and were reported as °Brix. Fruit samples ~5 g were thawed overnight at 4°C, and were manually macerated and analyzed. The °Brix of the fruit samples was determined with temperature compensation. Both of the fruit samples were analyzed in triplicate.

3.4 Crude extraction of fruit material

The crude extraction method was adapted from Green (2007) with solvent modification. Twenty grams ± 1.0 g of frozen fruit (saskatoons or chokecherries) were weighed into a 250 mL beaker. Sixty millilitres of solvent consisting of either methanol:formic acid:water (MFW) at a ratio of 70:2:28 (v:v:v) or ethanol:formic acid:water (EFW) at the same ratio was added to the fruit. The molarities of these solutions were 12.6 M and 17.3 M for EFW and MFW, respectively. The solution was then homogenized in a ten speed blender (Sunbeam Canada, Toronto, ON) on setting 6 for 2 min. The homogenate was transferred to a beaker, covered and stored overnight (16-20 h) at 4°C in the dark. The homogenate was vacuum filtered (VWR-413, 12.5 cm)

and washed with 20 mL of solvent. The solid material was re-suspended in 60 mL of solvent and stirred (Ika, Wilmington, NC) for 10 min at 600 rpm. The mixture was then re-filtered and washed with 60 mL of solvent. The final volume of the filtrate was brought to 200 mL in a volumetric flask with solvent.

3.5 Total phenolic content

The Folin-Ciocalteau (FC) method was adapted from Singleton et al. (1999) for total phenolic content. One mL of sample (section 3.4) was added to 5.0 mL of 1:10 (v/v) of FCR solution in glass test tubes (16 x 150 mm). After 5 min, 4.0 mL of 15% (w/v) sodium carbonate solution was added to each tube and the tubes were covered and mixed by inversion. The samples were then left for 2 h at room temperature (22 ± 2°C) before analysis. A blank was prepared which contained all of the reagents plus 1.0 mL of ddH_2O. After the incubation period, the samples were analyzed by UV-visible spectroscopy (Mecasys Co, Daejeon, South Korea) at 765 nm. A standard curve was prepared using gallic acid at concentrations ranging from 5-50 ± 0.2 mg/L in ddH_2O. A standard was run in conjunction with samples. Standard curves had correlation coefficients ≥0.990. All standards were run in triplicate whereas fruit samples were run in duplicate. Duplicate samples were produced from two separate crude fruit extraction batches. Sample results were reported as GAE.

3.5.1 Phenolic estimation for chitosan particles

Concentrations of rutin, chokecherry and saskatoon isolates in supernatants (section 3.11) were determined using the FC method. One mL of the supernatant was passed through a nylon syringe filter (13 mm diameter, 0.2 μm pore size; Chromatographic Specialties Inc., Brockville, ON) and added to 5.0 mL of 10% (v/v) Folin-Ciocalteau reagent. Samples were kept at room temperature for 5 min followed by the addition of 4.0 mL of 15% (v/v) sodium carbonate. The samples stood for 2 h at room temperature and their absorbances were determined spectrophotometrically at 765 nm. Standards of rutin and both fruit extracts were prepared at concentrations of 0.5 mg/mL and 2.5 mg/mL. All standards and samples were run in triplicate.

3.6 Anthocyanin estimation

The estimation of monomeric anthocyanins was determined by the pH differential method (Giusti and Wrolstad, 2001). The method relies on the colour changes of the anthocyanin chromophore at different pH values which can be measured spectrophotometrically. The pH values of 1.0 and 4.5 are used because at the lower pH the compounds are coloured while at 4.5 they are colourless. A pH 1.0 buffer of 0.025 M potassium chloride was prepared in ddH_2O and adjusted using concentrated HCl. A pH 4.5 buffer of 0.4 M sodium acetate was prepared in ddH_2O and adjusted using concentrated HCl. For each sample, 2.0 mL of crude extract (section 3.4) was added to separate 50 mL volumetric flasks and were brought to volume with the appropriate buffer solution. The samples were allowed to equilibrate for 2 h at room temperature in the dark. Sample absorbance was measured at 512 and 700 nm. The measurement at 700 nm was used to correct for solution turbidity. The absorbance change was calculated as follows:

$$\Delta A = (A_{512nm} - A_{700nm})_{pH\ 1.0} - (A_{512nm} - A_{700nm})_{pH\ 4.5} \quad \text{(eq. 3.1)}$$

The anthocyanin content of the sample was expressed as mg cyanidin-3-glucoside/100 g of fresh fruit using the following equation (Giusti and Wrolstad, 2001):

$$\text{Anthocyanin content} = \left(\frac{(\Delta A \times MW) \times DF \times 1000}{\varepsilon \times 1} \right) \quad \text{(eq. 3.2)}$$

Where:

MW = molecular weight of cyanindin-3-glucoside (449.2 g/mole).

DF = the dilution factor of the sample

1000 = conversion of mg to g

ε = molar absorbance coefficient of cyanidin-3-glucoside (26 900 M^{-1} cm^{-1})

1 = path length (cm)

3.7 Phenolic isolate production

Amberlite XAD-16 resin was used to partially purify the crude fruit extracts (section 3.4). The resin was first hydrated in either 50% (v/v) ethanol or methanol for at least 24 h. All isolates were individually evaporated to dryness at 30°C using a vacuum evaporator (Buchi Rotavapor, Flawil, Switzerland). The dried material was rehydrated in 1.0-2.0 mL of ddH_2O and freeze dried (Hetovac, Birkerod, Denmark) at room temperature and stored in light proof containers at -18°C until needed.

Approximately 55 mL of hydrated resin was transferred to a glass column (11.2 cm x 2.5 cm). The resin was pre-conditioned by first washing with 110 mL of ddH_2O, followed by 110 mL of 90% (v/v) of either ethanol or methanol, followed by 110 mL of ddH_2O. Approximately 12 mL of syringe filtered (13 mm diameter, 0.2 μm pore size) crude extract was loaded onto the column bed. Water soluble materials were removed by washing the column with 110 mL of ddH_2O, while the phenolic compounds (phenolic isolate) were eluted by washing the column with 110 mL of 70% (v/v) methanol or ethanol. The flow rate used was 0.5 mL/min. In order to obtain larger amounts of the phenolic isolates for animal trial studies the process was scaled up as follows: the glass column size was to 100 cm x 2.5 cm, the resin bed volume was 165 mL and the solvent volumes used were 330 mL.

3.8 Anthocyanin isolation

Anthocyanins were isolated using a C_{18} Sep-Pak (Waters Corporation, Milford, MA). Solid phase cartridges were pre-conditioned by washing with 5.0 mL of methanol-0.01% HCl followed by 2.0 mL of ddH_2O-0.01% HCl. One mL of crude extract (section 3.4) was added to the cartridge and the sample was washed with 2.0 mL of ddH_2O-0.01% HCl, and sample phenolics were eluted using 2.0 mL of methanol-0.01% HCl. These samples were used to determine the anthocyanin profiles in the crude fruit extracts as outlined in section 3.9.1.

3.9 HPLC-PDA analysis

All chromatographic analyses in this project were performed on an 1100 series HPLC system (Agilent Technologies Canada Incorporated, Mississauga, ON). The

components of the HPLC system were the solvent degasser, quaternary pump, auto sampler, column heater, UV-visible wavelength PDA, with system controlled by Chemstation LC-3D software (Revision B.04.01).

Phenolic separation was achieved using a 250 x 4.6 mm Prodigy ODS-3 5 μm, C_{18} column (Phenomenex, Torrance, CA) in series with a C_{18} guard cartridge (Phenomenex, Torrance, CA). All analyses were conducted at 25 ± 1 °C.

Detection was performed using a PDA detector with monitoring at 254, 280, 360 and 520 nm, with reference at 360, 400, 700 and 700 nm respectively. The mobile phase system used for phenolic compound separation was: 0.05 M KH_2PO_4 adjusted to pH 3.0 ± 0.1 (solvent A), and 70% (v/v) acetonitrile in solvent A (solvent B). The following gradient program was used: initial, 100% A for 3 min, followed by a linear gradient to 4% B at 6 min, followed by a linear gradient to 10% B at 15 min, followed by a linear gradient to 15% B at 30 min, followed by a linear gradient to 20% B at 35 min, followed by a linear gradient to 23% B at 50 min, followed by a linear gradient to 25% B at 60 min, followed by a linear gradient to 30% B at 66 min, followed by a linear gradient to 50% B at 80 min, followed by a linear gradient to 80% B at 85 min, which was held at 80% B for 5 min (method 1). The injection volume was 20 μL and the mobile phase flow rate was 0.8 mL/min. All samples were syringe filtered before analysis.

Standards were run to determine their relative retention times. Standards used were: caffeic acid, catechin, chlorogenic acid, gallic acid, narigenin, phloridzin, quercetin and rutin. All standards were run in triplicate at a concentration of 100.0 ± 0.2 mg/L.

3.9.1 HPLC-PDA analysis of anthocyanins

A second gradient elution system was employed for anthocyanin analysis. Mobile phases consisted of 4.0% (v/v) phosphoric acid at pH 1.4 (solvent A) and acetonitrile (solvent B). The flow rate was 0.8 mL/min and anthocyanins were separated under the following gradient system: initial, 6% B for 12 min, followed by a linear gradient to 20% B at 66 min, which was held at 20% B for 18 min (method 2). Sample injection volume was 20 μL and all samples were syringe filtered before analysis. Detection was performed using a PDA detector with monitoring at 520 nm with reference at 700 nm.

Anthocyanin standards used for compound identification were: cyanidin-3-galactoside (ideain), cyanidin-3-glucoside (kuromain) and cyanidin-3-rutinoside (keracyanin). Standard curves were run at concentrations ranging from 10-100 mg/L, and had correlation coefficients ≥ 0.90.

3.9.1.1 HPLC-PDA analysis of anthocyanins in animal (rat) plasma and tissues

The mobile phases consisted of 1.0% (v/v) phosphoric acid at pH 2.0 (solvent A) and acetonitrile (solvent B). The flow rate was 0.8 mL/min and anthocyanins were separated using the following gradient system: initial, 6% B for 12 min, followed by a linear gradient to 20% B at 66 min, and a hold at 20% B for 18 min. Sample injection volume was 20 μL and all samples were syringe filtered before analysis. Detection was performed using a PDA with monitoring at 520 nm with reference at 700 nm.

3.9.2 HPLC-PDA analysis of chitosan particles

The analysis of phenolics from chitosan particles was analysed using the following HPLC-PDA methods:

(i) Supernatant rutin concentration was determined by HPLC-PDA relative to a single point standard (8.0 mg rutin in 2.0 mL [10% (v/v)] acetic acid:8.0 mL [70% (v/v)] ethanol:6.0 mL [2 mg/mL] TPP). Mobile phases consisted of ddH_2O (solvent A) and acetonitrile (solvent B). The flow rate was 0.8 mL/min and the following gradient system was used: initial, 100% A followed by linear gradient to 55% B at 15 min (method 3). Sample injection volume was 20 μL and all samples were syringe filtered before analysis. Detection was performed using a PDA with monitoring at 254, 280, 360 and 520 nm.

(ii) Supernatant chokecherry and saskatoon isolate concentrations were determined by HPLC-PDA relative to a berry standard (40.0 ± 0.2 mg fruit isolate in 2.0 mL [10% (v/v)] acetic acid:8.0 mL [70% (v/v)] ethanol:6.0 mL [2 mg/mL]:tripolyphosphate (TPP)). Mobile phases consisted of 0.05 M potassium dihydrogen phosphate (pH 3.0) (solvent A) and 70% acetonitrile/30% solvent A (solvent B). The flow rate was 0.8 mL/min and the following gradient system was used: 100% A followed by linear gradient to 100% B at 25 min and a hold at 100% B for 10 min (method 4). Sample injection volume was 20 μL and all samples were syringe-filtered

before analysis. Detection was performed using a PDA with monitoring at 254, 280, 360 and 520 nm.

3.10 *In vitro* antioxidant activities

3.10.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The methods of Brand-Williams (1995) and Green (2007) were used to determine the DPPH free radical scavenging activity of crude extracts (section 3.4) and isolates (section 3.7). A 500 μM DPPH solution was prepared by dissolving 9.8 ± 0.2 mg of DPPH in a 50 mL volumetric flask with 70% (v/v) methanol using a sonicator (Bransonic, Danbury, CT). Fresh DPPH solutions were prepared for each day of analysis.

Freeze dried samples of crude extracts and isolates were prepared at concentrations ranging from 0.1-5.0 mg/mL in 70% (v/v) methanol by dilution. All samples used in the DPPH assays were placed in the sonicator for 20 min to ensure that the material was dissolved. A 0.5 mL aliquot of sample was added to 2.0 mL of DPPH solution, however if this sample amount was not available the same ratio of sample:DPPH solution was used. The control consisted of 0.5 mL of 70% (v/v) methanol in 2.0 mL of DPPH solution. The reagent blank was 70% (v/v) methanol. Sample blanks consisted of 0.5 mL of sample in 2.0 mL 70% (v/v) methanol to account for the sample absorbance. Samples were vortexed (Vortex Genie Scientific Industries, Bohemia, NY) for 10 to 15 s and held at room temperature in the dark for 15 min. Sample, blank and control absorbances were determined at 517 nm, and the percent DPPH radical scavenging activity was calculated as follows:

$$\% \text{ DPPH radical scavenging activity} = \left[1 - \left(\frac{A_{517} \text{ sample}}{A_{517} \text{ control} - A_{517} \text{ sample blank}}\right)\right] \times 100 \quad \text{(eq. 3.3)}$$

Where:

A_{517} sample = sample absorbance at 517 nm.

A_{517} control = control absorbance at 517 nm.

A_{517} sample blank = sample blank absorbance at 517 nm.

Results were plotted as percent DPPH radical scavenging versus sample concentration. Using linear regression the concentration that gave a 50% radical inhibition (IC_{50}) was determined. All regression equations had correlation coefficients ≥ 0.92. The IC_{50} value was expressed as mg solids/mL of DPPH solution and the antioxidant activity was reported as $1/IC_{50}$. All sample analyses was performed in duplicate.

3.10.2 Trolox equivalent antioxidant capacity (TEAC) assay for ABTS radical scavenging activity

Stock solutions were prepared 7.0 mM ABTS, and 7.0 mM potassium persulfate in ddH_2O. The radical form of ABTS was produced by mixing 4.0 mL of the ABTS solution with 2 mL of the potassium persulfate solution. This mixture was held at room temperature for 12 h in the dark to allow for complete ABTS oxidation. The resulting $ABTS^{\bullet+}$ solution was diluted approximately 1 in 50 with 70% (v/v) methanol to give an absorbance reading of 0.75 ± 0.05 at 734 nm. Solutions of both phenolic sources (chokecherry and saskatoons) were prepared in 70% (v/v) methanol from freeze dried samples at the following concentrations; crude extracts (section 3.4) 1.0 to 20.0 mg solids/mL and phenolic isolates (section 3.7) 0.5 to 20.0 mg solids/mL. The Trolox standard was prepared in 70% (v/v) methanol at the following concentrations: 0.1 mg/mL (0.4 mM) to 0.5 mg/mL (2.0 mM).

This radical scavenging assay was conducted by mixing 20 µL of the phenolic sample solution with 2.0 mL of $ABTS^{\bullet+}$ solution. The absorbance was read at 734 nm at 1, 4 and 6 min intervals. A blank solution of 20 µL 70% (v/v) methanol in 2.0 mL

$ABTS^{\bullet+}$ solution was prepared and analyzed at the same wavelength. Values from the 6 min interval were used to calculate the percent radical inhibition as follows:

$$\% \text{ ABTS radical inhibition} = \left[1 - \left(\frac{A_{734} \text{ sample}}{A_{734} \text{ control}}\right)\right] \times 100 \qquad \text{(eq. 3.4)}$$

Where:

A_{734} sample = sample absorbance at 734 nm.

A_{734} control = control absorbance at 734 nm.

Percent $ABTS^{\bullet+}$ inhibition was plotted against sample concentration in order to determine the linear regression equation for the sample. All correlation coefficients for the samples calculated using linear regressions were ≥0.96. The $ABTS^{\bullet+}$ inhibition of 1.0 mM Trolox was calculated from the linear regression equation as 37.8%. The sample concentration that had the same inhibition activity as 1.0 mM Trolox was determined as follows:

$$Y_{TE} = a + (b \times (\% \text{ Trolox activity})) \qquad \text{(eq. 3.5)}$$

Where:

Y_{TE} = concentration of sample (mg/mL) to produce the same % $ABTS^{\bullet+}$ inhibition as 1.0 mM Trolox.

a = intercept of the sample linear regression equation

b = slope of the sample linear regression equation

% Trolox activity= inhibition of $ABTS^{\bullet+}$ by 1.0 mM Trolox

The Trolox equivalent antioxidant capacity (TEAC) was expressed as the equivalent activity of Trolox (mM) in 100.0 mg sample/mL and was determined by:

$$TEAC = 100/Y_{TE}$$

(eq. 3.6)

Where:

100 = conversion factor to standardize all samples to 100 mg/mL

Y_{TE} = concentration of sample (mg/mL) to produce the same % $ABTS^{\bullet+}$ inhibition as 1 mM Trolox.

All sample analyses was performed in duplicate.

3.11 Chitosan nanoparticles

Chitosan nanoparticles were prepared using the ionic gelling techniques of Zhang and Kosaraju (2007) and Jang and Lee (2008) with modification. Particles were prepared at different CH:TPP (w/w) ratios of 2.0:1.0, 2.5:1.0, 3.0:1.0, 4.0:1.0 and 5.0:1.0, with entrapped phenolics. Chitosan solutions were prepared by dissolving chitosan powder (Sigma, Oakville, ON) at the following concentrations (2.4, 3.0, 3.6, 4.8, 6.0 ± 0.02 mg/mL) in a 10 mL solution containing 2.0 mL acetic acid 10% (v/v) and 8.0 mL of 70% (v/v) ethanol. The solution was then mixed at 480 rpm using a magnetic stir plate (Ika, Wilmington, NC) until complete dissolution occurred (~10 min). Solution pH was adjusted to 4.8 using 1.0 M sodium hydroxide (NaOH) (~100uL), and was allowed to stir for an additional 3 h at 250 rpm. Sodium tripolyphosphate (TPP) (0.075 % w/v) was added (6 mL, 2 mg/mL) drop-wise to the mixture, and allowed to stir for 1 h for particle hardening. Chitosan particles were separated by centrifugation (Beckman J2-HC, Mississauga, Ontario) at 9820 x g for 30 min. The supernatant was collected and used for the determination of entrapment efficiencies (section 3.11.1), and the pellet was either used in release studies (section 3.12) or re-suspended in 10 mL of ddH_2O and subsequently used for size (section 3.11.2) and surface charge determinations (section 3.11.3). In the case of animal feeding trials (section 3.12), the pellet was freeze dried and stored (-20°C) for later use.

For phenolic entrapment, solutions containing approximately 1 mg/mL of rutin, or 5 mg/mL of chokecherry isolate (section 3.7) or 5 mg/mL of saskatoon isolate (section

3.7) were prepared by dissolving their respective powders in 70% (v/v) ethanol by sonication for 30 min. This solution was substituted for the ethanol phase in particle formation as described above.

3.11.1 Entrapment efficiency

Entrapment efficiencies for rutin, saskatoon and chokecherry isolates within CH:TPP particles were determined by the analysis of their supernatants relative to their original solution concentrations (Zhang et al., 2004). Analyses were made by HPLC-PDA (section 3.9.2) and the Folin-Ciocalteau method (section 3.5.1). All analyses were performed in triplicate.

3.11.2 Particle size

Particle size was determined for all CH:TPP ratios containing rutin (2.0:1.0, 2.5:1.0, 3.0:1.0, 4.0:1.0, 5.0:1.0) and fruit phenolics using a Zetasizer Nanoseries instrument (Malvern, Westborough, MA). The mode settings used for the determination of size included the measurement angle of 90° and the measurement position was automatically determined by the software to seek the optimum position on the cell. Samples were taken after centrifugation to determine particle size. The recovered pellet was re-suspended in 10.0 mL of ddH_2O and left to stir overnight at 250 rpm. A 0.5 mL aliquot of this solution was added to 9.5 mL of ddH_2O and sonicated for 10 min before particle size determination. The pH of the solution after the above dilution was ~4.5. The cuvette was then filled with the diluted sample for analysis. Sample measurements were run at room temperature and each CH:TPP ratio was measured in triplicate.

3.11.3 Surface charge

The surface charge of all rutin (zeta potential [ζ]) and fruit phenolic particles were determined using a folded capillary cell and the aforementioned Zetasizer Nanoseries instrument. Zeta potential was calculated from the electrophoretic mobility (EM) of the particles by the Henry equation:

$$U_E = \left(\frac{2\varepsilon\,\zeta\,f(\kappa\alpha)}{3\eta}\right)$$

(eq. 3.7)

Where:

U_E= electrophoretic mobility

ε= dielectric constant

ζ= zeta potential

f(κα)= function related particle radius (α) (using the Smoluchowski approximation where *f*(κα) is equal to 1.5)

η= viscosity

The same sample dilution procedures were used as outlined in section 3.11.2. One mL of sample was placed into the capillary cell and the surface charge was determined in triplicate for each CH:TPP ratio.

3.12 Phenolic release studies (PRS)

3.12.1 PRS in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

The methods of Zhang and Kosaraju (2007) were adapted to determine the release of rutin from the 4.0:1.0 ratio (CH:TPP) particles in SGF and SIF. Simulated gastric fluid was made by adding 1.0 g of NaCl and 3.5 mL of concentrated HCl (12.0 M) in a 500 mL volumetric flask which was brought to volume with ddH_2O. The pH of the solution was 1.4 ± 0.05. Simulated intestinal fluid was prepared by mixing 7.7 mL of 0.2 N sodium hydroxide and 680 mg KH_2PO_4 in a 100 mL volumetric flask and brought to volume with ddH_2O. The pH of the solution was adjusted to 6.8 ± 0.05 using 1.0 M NaOH.

Chitosan particles containing rutin, saskatoon and chokecherry isolates (section 3.7) were prepared using a 4.0:1.0 (CH:TPP) ratio. The prepared particles with a wet weight of ~ 0.7-0.8 g were added to either 15 mL of SGF or 15 mL of SIF and incubated at 37°C in a water bath with continuous stirring at ~100 rpm. Aliquots of 250 μL were taken at time 0 and every h for a total of 2 (SGF) or 3 h (SIF). Aliquots were syringe

filtered and analyzed for their phenolic content by HPLC-PDA (section 3.9.2) and FC method (section 3.5.1). All experiments were run in triplicate.

3.12.2 Enzymatic SIF (ESIF)

An ESIF solution was prepared with modification from that of Laird et al. (2007). The ESIF was made by dissolving 6.0 g oxgall dehydrated bile media, 12.5 g $NaHCO_3$ and 0.9 g pancreatin into a 1 L volumetric flask and was brought to volume with ddH_2O. The pancreatin enzyme mixture consisted of amylase (108 USP units/mg), protease (100 USP units/mg), lipase and ribonuclease (at an unknown concentration). Enzymatic SIF solutions were placed in an anaerobic chamber 12 h prior to experiments to remove the oxygen. All ESIF experiments were performed at 37°C in an anaerobic chamber (Forma Sci. Inc., Marietta, GA) with 80% nitrogen, 10% hydrogen and 10% carbon dioxide. The ESIF solution was kept at 4 °C for a maximum of 3 days before use.

Particles (0.7-0.8 g wet weight) prepared with a 4.0:1.0 (CH:TPP) ratios containing (a) rutin, (b) chokecherry isolate or (c) saskatoon isolate (section 3.11) were individually added to 15 mL of ESIF. These suspensions were placed at 37°C with continuous stirring ~100 rpm for the duration of the experiments. Aliquots (250 µL) were taken at time 0, 10, 20, 30, 45, 60, 120, 180, 240 min. Samples were syringe filtered before analysis. All samples were analyzed by HPLC-PDA (section 3.9.2) to determine phenolic release over time. Analysis of samples was performed in triplicate.

3.13 Animal studies

Eighteen male Wistar rats (Charles River, Wilmington, MA) were received at 6 weeks of age and were housed (3 per cage) in a controlled environment at 23 ± 1°C with a 12 h dark/light cycle. The rats were allowed free access to water and a commercial diet (ProLab RMH300) for 7 days prior to experimentation. However, during this period the animals were introduced to the gavage technique with water administered. Animals were handled humanely in compliance with the guidelines of the Canadian Council on Animal Care and with the approval of the University Committee on Animal Care and Supply. Once animals arrived they were acclimatized to the conditions for 7 days before feeding trials were preformed.

The evening before commencing the feeding trials, the animals were deprived of food for at least 12 h. On the day of the feeding trials, animals were assigned to one of three groups consisting of saskatoon isolate (section 3.7; re-suspended in 0.1% citric acid) (n= 6), CH:TPP (4.0:1.0) particles containing saskatoon isolate (section 3.11) (n=6), or the control (n=6).

In order to deliver the CH:TPP particles containing the saskatoon isolate by gavage, 184.8 ± 8.0 mg of freeze dried material was hydrated in 2.5 mL 0.1% citric acid and homogenized (Omni International, Inc., Marietta, GA) at 4000 rpm for 3 min. Once the particles were re-suspended it formed a gel like substance. The dose was administered using the gavage technique with a 16 gage, 3 inch tube length, 3 mm ball diameter gavage needle attached to a syringe (Cadence Science, Lake Success, NY). The control group was fed 3 mL of 0.1% citric acid while the 2 other groups were fed approximately 275 mg/kg body weight of saskatoon isolate or encapsulated isolate in 3 mL of 0.1% citric acid. Following the treatment the rats were deprived of water and sacrificed after 1 h.

3.13.1 Plasma and tissue recovery

After 1 hour the rats were anaesthetized with isoflurane (2% in oxygen delivered at 1 mL/min) and blood (3-4 mL) was collected (heparinized tubes) by cardiac puncture. Plasma was recovered by centrifugation at 10000 x g for 8 min at 4°C and was stored at -30°C until analyzed. Phosphate buffered saline (PBS) was prepared by adding 7.1 g sodium phosphate, 20.0 mL 1.0 M HCl and 8.9 g NaCl to ~700 mL ddH_2O. The pH was adjusted to 7.4 using 1.0 M HCl and the final volume was brought to 1 L with ddH_2O in a volumetric flask. The final concentration of the PBS was 50 mM sodium phosphate and 0.15 M NaCl. The body was exsanguinated with chilled (~4°C) PBS to remove the blood from the tissues at a rate of 52 mL/min (Isomatec, Chicago, IL) for ~2 min. Tissues that were collected included the brain, liver, kidneys, heart, stomach and small and large intestines. After removal, the tissues were immediately flash frozen in liquid nitrogen and stored at -70°C until analyzed.

3.13.2 Tissue extraction and anthocyanin detection

All tissue samples were powdered using a liquid nitrogen cooled pulverizer (Biospec, Bartlesville, OK) before anthocyanin extraction. The extraction of anthocyanins from the resulting powdered tissues was adapted from the protocol of Talavera et al. (2005). Methanol containing 1.0 % (v/v) formic acid was added to powdered tissues at a ratio of 9.0 mL per 1.0 g of tissue. Samples were homogenized (Talboys, Thorofare, NJ) in 55 mL glass tubes with a tight fitting Potter-Elvehjem tissue grinder (Wheaton, Millville, NJ) for 3 passes (each pass was ~1 min in duration). The resulting homogenate was placed in a centrifuge tube (30 mL) and vortexed for 15 seconds on the highest setting and centrifuged (Beckman Coulter, Mississauga, ON) at 4000 x g for 8 min at 4°C. The supernatant was collected and the pellet was then re-extracted using a pellet pestle motor (Kontes, Vineland, NJ) in methanol:1.0 % formic acid at a ratio of 4.0 mL per 1.0 g of tissue followed by vortexing and centrifugation as above. The resulting supernatant was removed and combined with the one collected previously and was rotary evaporated at room temperature. The dried tissue extracts were then re-suspended in 500 µL of 1.0% (v/v) formic acid in methanol and frozen at -30°C until analyzed by HPLC-PDA (section 3.9.1.1). Prior to analysis the samples were centrifuged at 12 000 x g for 5 min at room temperature and were syringe-filtered.

3.13.3 Plasma extraction and anthocyanin detection

The extraction of anthocyanins from plasma was adapted from the protocol of Miniati (2007). Plasma collected (section 3.13.1) was thawed at room temperature and 500 µL was mixed with 150 µL of 1.0 M potassium dihydrogen phosphate and 15 µL of phosphoric acid (85%). To this solution was added 250 µL of acetonitrile followed by vortexing for 1 min. Samples were then centrifuged at 3500 x g for 10 min at 5°C, and the supernatants were recovered. The supernatant solutions were evaporated to dryness by rotary evaporation at room temperature. The dried plasma extracts were then rehydrated with 400 µL of methanol and frozen at -30°C until analyzed. Anthocyanins were detected in plasma using the HPLC-PDA protocol described in section 3.9.1.1. All samples were syringe-filtered prior to analysis.

3.14 Statistical analysis

All statistical analyses were performed using SPSS software (SPSS (17), Chicago, IL). One way analysis of variance (ANOVA) with a post hoc Tukey test was used to determine statistical significance. Analysis confidence intervals used in all work were 95% ($p \leq 0.05$).

4.0 RESULTS AND DISCUSSION

4.1 Study 1- Determination of total phenolic content, antioxidant activity and phenolic class identification of solvent (methanol and ethanol) extracts of chokecherries and saskatoons.

The extraction solvents used in all research studies were ethanol:formic acid:water (70:2:28 (v:v:v)) and methanol:formic acid:water (70:2:28 (v:v:v)), and will be referred to as EFW and MFW in the following sections of this thesis.

4.1.1 Total phenolic content of extracts as determined by the Folin-Ciocalteau method

The TPC of crude extracts prepared using EFW and MFW for the two fruits was determined using the Folin-Ciocalteau (FC) method (Singleton et al., 1999). This spectrophotometric method is commonly used for the determination of TPC in plant materials and foods (Kahkonen et al., 1999; Brat et al., 2006; Bakowska-Barczak et al., 2007). In this study, differences in the TPC for the two fruits (chokecherry and saskatoons) and the effect of the extraction solvent (EFW and MFW) on TPC results were determined.

The mean TPC of chokecherries was determined as 9.83 mg GAE/g fw for EFW and 7.97 mg GAE/g fw for MFW (Table 4.1). These values were lower than those previously reported for chokecherry (MFW extract) in our laboratory with TPC levels of 13.3 ± 1.6 mg GAE/g fw (Green, 2007). These differences could be explained by seasonal changes that impact phenolic levels. In literature, seasonal variations were shown to alter the TPC levels by 28-56% in five different blueberry cultivars (Howard et al., 2003). Seasonal conditions that have been found to influence the production of phenolics in fruit include sunlight, degree temperature days and moisture (Kalt, 2005). The mean TPC values for saskatoons were lower than those for chokecherries at 4.21 mg GAE/g fw for EFW and 4.26 mg GAE/g fw for MFW. In literature, the reported TPC for saskatoons ranged from 4.05 ± 0.21 to 4.98 ± 0.49 mg GAE/g fw for Smokey and Northline varieties, respectively (Mazza, 2006). These values are close to those found in the current work and the small differences observed can be explained by seasonal and/or

varietal differences as the Martin variety was used in this study. Bakowska-Barczak and Kolodziejczyk (2008) determined that there were significant TPC differences (~30%) found in the 17 saskatoon varieties analyzed in their study.

Methanol has been commonly used in the extraction of phenolics from plant tissues (Vermerris and Nicholson, 2006b). Although methanol is efficient for phenolic compound extraction from plant tissues, it is toxic, and residual levels in extracts following solvent evaporation may prevent the use of these extracts for animal and human consumption (Nawaz et al., 2006). Therefore, ethanol was investigated for its phenolic extraction ability so that the resulting extracts could be used in animal feeding studies.

The results shown in Table 4.1 illustrate two major differences in the TPC values for these fruit extracts. First, the mean TPC value for chokecherries was approximately 2x that of saskatoons. The second major difference observed was that the TPC of the chokecherry extract samples differed with the solvent used; with a mean value for EFW of 9.83 GAE/g fw versus 7.97 GAE/g fw for MFW. However, unlike the chokecherry samples, the saskatoon extracts showed similar TPC results for each solvent.

The observed differences in TPC values for the two fruit sources may be explained by a number of factors including, but not limited to: variations in their non-phenolic chemical composition (reducing carbohydrate and ascorbic acid), phenolic classes present and/or their phenolic class concentration and composition, extraction solvent polarity, and sample soluble solids content. Each of these factors will be discussed in some detail.

Table 4.1 Total and mean phenolic contents of chokecherry and saskatoon solvent (EFW and MFW) extracts as determined by the Folin-Ciocalteau method.

Fruit	Gallic acid equivalents [a]	Mean
Chokecherry- EFW[b]		
sample #1	9.57	9.83
sample #2	10.08	
Chokecherry- MFW[b]		
sample #1	7.80	7.97
sample #2	8.14	
Saskatoon- EFW		
sample #1	4.05	4.21
sample #2	4.37	
Saskatoon- MFW		
sample #1	4.20	4.26
sample #2	4.32	

[a] expressed as mg gallic acid equivalents (GAE)/g fresh weight.
[b] chokecherry fresh weight was the flesh weight not including the seeds.
n=2

The two major compounds/class of compounds that have been shown to impact (i.e., are considered to be interferences) TPC results are reducing carbohydrates and ascorbic acid. This observation is due to the fact that the FC method is based on the chemical reduction of metal oxide compounds (FC reagent) which can be induced by these compounds (Singleton et al., 1999; Wrolstad, 2005). Ascorbic acid levels of 1.2 ± 1.2 mg/100 g fw in chokecherry (without seeds) and 3.7 ± 0.7 mg/100 g fw (whole fruit) for saskatoons have been reported (Mazza, 2004; Green, 2007). Previous work done by Green (2007) determined that the ascorbic acid contribution to FC results was a ratio of 0.76 mg GAE:1.00 mg ascorbic acid. Using this ratio and assuming the highest level of ascorbic acid for each solvent extract (2.4 for chokecherry and 4.4 mg/100 g fw for saskatoons) the contribution of ascorbic acid to the total GAE results would be 0.15 and 0.19% for chokecherry, and 0.79 and 0.78% for saskatoons, for EFW and MFW,

respectively. Based on these results, the observed differences in TPC values for the two fruit extracts was not due to their ascorbic acid content.

With respect to the possible interference of reducing carbohydrates, it has been shown that they do not react appreciably with the FC reagent at low concentrations (10 g invert carbohydrate/100 g sample) at room temperature (Singleton et al., 1999). The reducing carbohydrate levels of chokecherries and saskatoons have been reported as ~7 g and ~11 g/100 g of fw, respectively (Mazza, 2006; Green, 2007). According to the values reported by Singleton et al. (1999) the reducing carbohydrates levels listed above would contribute ~3% to the TPC results. Previous work done by Green (2007), determined that the reducing carbohydrate (fructose and glucose) contribution to FC results was 0.28 mg GAE/g reducing carbohydrate. Based on literature reducing carbohydrate concentrations, chokecherry TCP values would be reduced by 0.020 mg GAE/g fw and saskatoons by 0.031 mg GAE/g fw. These results represent 0.20% and 0.25% of the chokecherry EFW and MFW TCP values with saskatoons values slightly higher at 0.73% and 0.72%. Therefore, the contribution of reducing carbohydrates to the TPC results for these two fruits would not explain the large observed differences.

The HPLC-PDA phenolics profiles for each fruit were determined (section 4.1.3; Figures 4.1 and 4.4) and showed 3 major peaks for chokecherries and 4 major peaks for saskatoons. For chokecherries, 2 of the peaks were identified as hydroxycinnamic acids and one as anthocyanins. For saskatoons, 2 peaks were identified as hydroxycinnamic acids, one as anthocyanins and one was tentatively identified as flavonoids. Chromatography results showed that the chokecherry solvent extracts had higher peak areas (~2.8x) for the hydroxycinnamic acids when compared to the saskatoon extracts. The anthocyanin peak (relative retention time (RRT) ~39-40 min) areas for both the solvents were also higher (~1.2x) in the chokecherry samples when compared to the saskatoons. Based on these results it was assumed that the phenolic compound concentration and composition was responsible for the majority of the observed differences in the TPC values between these two fruits. The crude fruit extract samples that were analyzed by HPLC-PDA were initially freeze dried and diluted to achieve similar concentrations. Therefore, sample moisture content was removed as a variable. Finally, literature shows that phenolic compound structure (i.e. phenolic class) influences

TPC values. For example, molar response factors of 1.00, 1.03, 1.26 and 2.02 have been reported for gallic acid (hydroxybenzoic acid class), caffeic and chlorogenic acids (hydroxycinnamic acid class), and rutin (flavonoids), respectively (Slimestad et al., 2009).

The TPC differences observed for chokecherry solvent extracts (Table 4.1) may be explained by the polarity differences of the two solvents. Solvent polarity is commonly classified on the basis of dielectric constant with the lower the value indicating the less polar the solvent. The dielectric constants (at standard temperature and pressure conditions) for water, methanol, ethanol and benzene have been reported as 78.54, 32.63, 24.30 and 2.28, respectively (Weast, 1978). The dielectric constant value for ethanol when compared to methanol would indicate the possibility of improved hydrophobic phenolic compound solubility while maintaining reasonable hydrophilic phenolic compound solubility. This difference in hydrophobicity may account for the observed higher TPC results observed for chokecherries extracted with EFW. Chromatographic (HPLC-PDA) results showed higher (~1.2x) peak areas for phenolic compounds eluting in the 48-67 min range for EFW extracts when compared to those produced by MFW. Therefore, the higher concentrations of these compounds in the EFW extracts may account for the differences seen in the TPC values. The fact that saskatoons showed no appreciable differences in TPC results for the two extraction solvents would indicate that this fruit may contain lower levels of MFW/EFW extractable hydrophobic phenolics. This conclusion is supported by chromatographic results as the HPLC-PDA peak areas for hydroxycinnamic, flavonoid and anthocyanin fractions were similar for both EFW and MFW saskatoon extracts.

The observed differences in TPC values between the two fruit extracts would also be impacted by their soluble solids contents. The total soluble solids (TSS) content was measured using refractometry, °Brix values for chokecherry and saskatoons were 25.28 ± 0.75 and 16.18 ± 0.87, respectively. Therefore in 10 g of fresh fruit there would be 2.53 ± 0.08 g TSS and 1.62 ± 0.09 g TSS in chokecherries and saskatoons, respectively. The TSS value for chokecherries was lower than previously reported of 29.8 ± 6.6 °Brix (Green, 2007), while the value for saskatoons was higher than those previously reported for the Martin variety at 15.3 °Brix (Zatylny et al., 2005b). Based on these results and

assuming similar total phenolic concentrations, the 36% difference in TSS between the two fruits would be a significant contributing factor to the observed difference in their TPC values.

The observed variation in TPC values between the duplicate samples was 4.5% for chokecherry and 7.1% for saskatoons. This variation may be explained by the small original sample size used (20g) and/or the lack of a completely homogeneous starting sample set. Previous work by Green (2007) reported variations in chokecherry TPC values of up to 21% in triplicate measurements.

In conclusion, the observed difference in TPC values between the two fruits, independent of the solvents used for extraction, may be explained by both the difference in phenolic content of the fruits and the TSS of the starting materials. As discussed previously, the TPC of chokecherries was ~2.1x higher than that of saskatoons. The difference in the phenolic content of the fruits was determined to be ~1.7x higher in chokecherries compared to saskatoons and this accounts for the majority of the difference found in the TPC values. The TSS would make up the remaining difference that occurred in the TPC values of the two fruit solvent extracts.

4.1.2 Total anthocyanin content of chokecherry and saskatoon crude extracts by the pH differential method

The pH differential method was used to determine the monomeric anthocyanin content of fruit or fruit extracts. Sample absorbances were determined at pH 1.0 and 4.5 at both 512 and 700 nm. These pH values were used because at pH 1.0 monomeric anthocyanins are coloured while at pH 4.5 they are colourless (Wrolstad, 2005). The maximum absorbance of anthocyanins occurs at 512 nm, and the absorbance at 700 nm was used to account for any turbidity in the samples. The pH differential method was used in this study to determine the differences in monomeric anthocyanin content between the two fruit extracts and also to determine the effect of extraction solvent on anthocyanin extraction.

Experimental results for chokecherry and saskatoon crude fruit extracts employing EFW and MFW as extraction solvents are shown in Table 4.2. These results show that crude solvent extracts for chokecherry had mean anthocyanin contents that

were approximately 57% greater than those of saskatoons. Anthocyanin concentrations for chokecherries were similar (within 5%) for both solvents whereas the saskatoon EFW extract was approximately 7% greater than that of the MFW extract. As only two true replicates were analyzed, no experimental data exists to support any significance to the effect of solvent on the anthocyanin extraction for either fruit employing the pH differential method.

From the HPLC-PDA area results for the crude fruit extracts, the anthocyanin (RRT ~ 39-40 min; Figures 4.1 and 4.4) content of the chokecherry samples were 1.2x higher than saskatoons. Data shown in Table 4.2 shows that the monomeric anthocyanin content of the chokecherry samples was 1.7x higher than those of saskatoons supporting the HPLC-PDA results. As discussed in section 4.1.1 the total soluble solids may also play a role in the anthocyanin content of the fruit. The TSS of chokecherries was higher than that of saskatoons, resulting in more extractable anthocyanins from the original fruit sample.

As discussed in section 4.1.1, ethanol is a less polar solvent than methanol. Therefore, ethanol may be more effective in extracting anthocyanins from saskatoons, which would contribute to the observed pH differential results. If anthocyanidins (i.e. deglycosylated anthocyanins) were present in the sample, these would be better extracted with a more hydrophobic solvent, such as ethanol. The anthocyanidins are more hydrophobic than anthocyanins because they lack carbohydrate moieties (Giusti and Wrolstad, 2001). In addition, the solubility of anthocyanins in protic solvents, such as ethanol and methanol, depends on the number, type and the linkage position of the carbohydrate (Lapornik et al., 2005). Literature pH differential method results have shown that higher values (~19%) were observed for red currants that were extracted with 70% ethanol versus 70% methanol (Lapornik et al., 2005). Black currants were also examined using the pH differential method and showed that 70% methanol versus 70% ethanol extracts produced higher monomeric anthocyanin contents (~8%) (Lapornik et al., 2005). Therefore, the original fruit source and the solvent hydrophobicity influence the extraction of anthocyanins.

Table 4.2 Total and mean anthocyanin contents of chokecherry and saskatoon solvent (EFW and MFW) extracts as determined by the pH differential method.

Sample	Anthocyanin content [a]	Mean
Chokecherry- EFW[b]		
sample #1	240	244
sample #2	247	
Chokecherry- MFW[b]		
sample #1	256	257
sample #2	259	
Saskatoon- EFW		
sample #1	144	147
sample #2	149	
Saskatoon- MFW		
sample #1	132	138
sample #2	143	

[a] reported as the mg cyanidin3-glucoside/100 g fresh weight
[b] chokecherry fresh weight was the flesh weight not including the seeds.
n= 2

The observed variation in monomeric anthocyanin values between the duplicate samples was 2.5% for chokecherry and 8.5% for saskatoons. This variation may be explained by the small original sample size used (20g) and/or the lack of a completely homogeneous starting sample set. Previous work by Green (2007) reported variation in chokecherry MFW produced anthocyanin extracts ranging from 4 to 16% in triplicate measurements.

The monomeric anthocyanin content of saskatoons from this reported in this thesis was 138 to 147 mg cyanidin3-glucoside/100 g fw which is similar to those reported by Rogiers and Knowles (1997), with values of 110 for the Northline and 92 for the Smokey varieties. In literature, the anthocyanin content of the Nelson and Martin varieties have been shown to have the highest anthocyanin levels while Smokey contains the lowest (Bakowska-Barczak and Kolodziejczyk, 2008).

The monomeric anthocyanin content for chokecherries found in this study were higher than those reported by both Bakowska-Barczak et al. (2007) and Green (2007). Bakowska-Barczak et al. (2007) reported anthocyanin values of 228 ± 9 mg cyanidin-3-glucoside/100 g fw and Green (2007) reported a mean value of 234 ± 46 mg cyanidin-3-glucoside/100 g fw. Green (2007) determined monomeric anthocyanin values for chokecherries over a 4-year growing period, which ranged from 169-285 mg cyanidin-3-glucoside/100 g fw. The observed wide range in values was explained by environmental factors such as light, temperature and agronomic practices (Bakowska-Barczak et al., 2007; Angzzas et al., 2009).

All of the aforementioned factors have been shown to affect the anthocyanin concentration in chokecherries and saskatoons. Therefore, the results obtained in this study were well within the ranges reported in the literature.

4.1.3 Characterization of the phenolic profiles of chokecherry and saskatoon crude extracts and isolates as a function of solvent (ethanol and methanol) by HPLC-PDA

In this study, the effect of solvent, specifically EFW and MFW on phenolic compound extraction from chokecherry and saskatoons was examined employing HPLC-PDA. This analytical technique was used to characterize the phenolic profiles of crude extracts and isolates (sections 3.4 and 3.7) so as to classify/identify the major phenolic peaks present in the chromatograms of these fruits. All compounds present in the chromatograms with absorbance peaks at 280 nm were assumed to be phenolics. In the literature, the majority of reports on phenolic extraction from fruits have employed acetone, acetonitrile or methanol (Bakowska-Barczak et al., 2007; Ozga et al., 2007; Bakowska-Barczak and Kolodziejczyk, 2008; Kalt et al., 2008). Recently, phenolic extraction from fruits employing ethanol has been reported (Karvela et al., 2009; Li et al., 2009). A comparison of the phenolic profiles obtained from EFW and MFW extracts and isolates from these two fruits would provide important information on phenolic class and compound extraction as a function of solvent polarity.

Chromatographic results from saskatoon crude extracts (section 3.4) produced employing EFW and MFW, showed virtually identical phenolic profiles as illustrated in

Figure 4.1. Four major (peak area >100 mAU) phenolic classes/specific compounds were identified in solvent extracts from saskatoons as hydroxycinnamic acid(s) (RRT ~27 min), chlorogenic acid (RRT ~37 min), anthocyanins (RRT ~39 min) and rutin (RRT ~67 min). The total peak area for the four identified phenolic classes/compounds was found to be 5.27 x 10^4 mAU/s and 5.48 x 10^4 mAU/s for EFW and MFW respectively, indicating that the solvent employed had a similar impact on TPC extraction. The peak at RRT ~27 min did not match any of the common phenolics previously identified in fruits (Figure 4.2), however through the comparison of UV-visible (UV-vis) spectra to phenolic standards it was determined to be a hydroxycinnamic acid (Figure 4.3; A & H). The second peak with a RRT of ~37 min was tentatively identified as chlorogenic acid based on its similar RRT to that of the standard (37.6 min) and by comparison of the UV-vis spectra of this chromatographic peak to the chlorogenic acid standard (Figure 4.3; B & H). The peak at ~39 min was identified as anthocyanins which were determined by UV-vis spectral analysis based on an absorbance at ~520 nm (Figure 4.3; C & I). Although the highest absorbance for anthocyanins occurs at 520 nm, they also display an appreciable absorbance band at 280 nm (Robards and Antolovich, 1997). The last major peak in the saskatoons profile occurred at a RRT of ~67 min and was tentatively identified as rutin based on its similar RRT to that of the standard (66.2 min) as well as by comparison of the UV-vis spectra of this chromatographic peak to the rutin standard (Figure 4.3; D & J). Although the maximum absorbance of flavonoids, such as rutin, occurs at ~360 nm an absorbance band also occurs at 280 nm (Mabry et al., 1970).

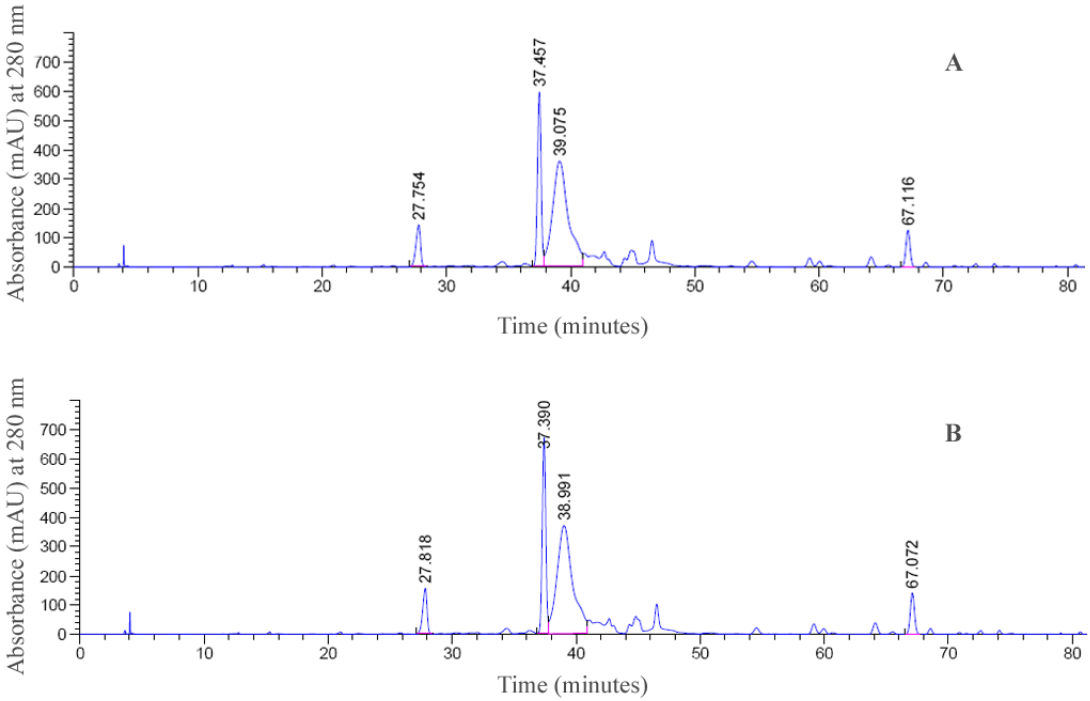


Figure 4.1 **Reverse phase HPLC-PDA chromatograms (280 nm) of the phenolic profiles of saskatoon crude extracts produced with EFW (A) and MFW (B).**

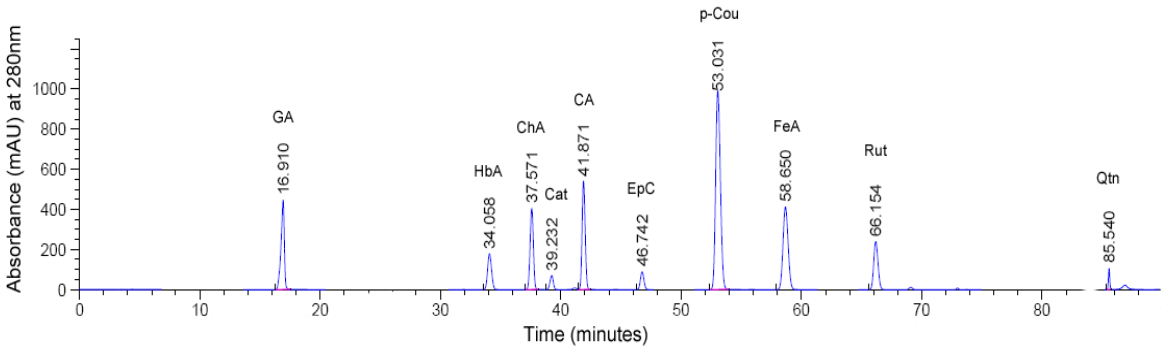


Figure 4.2 **Reverse phase HPLC-PDA chromatogram (280 nm) of the ten most common phenolic compounds present in fruits. Peak identities: GA, gallic acid; HbA, hydroxybenzoic acid; ChA, chlorogenic acid; Cat, catechin; CA, caffeic acid; EpC, epicatechin; p-Cou, p-coumaric acid; FeA, ferulic acid; Rut, rutin; Qtn, quercetin. (standard concentrations ranged from 100 to 150ppm).**

Abundant literature exists on the phenolic composition of saskatoon varieties. Gazdik et al. (2008) reported that saskatoons contained appreciable concentrations of gallic acid, quercetin and rutin. Li et al. (2009) identified seven phenolics in saskatoon extracts including caffeic, p-coumaric, ellagic, ferulic, gallic, hydroxybenzoic and protocatechuic acids. Saskatoons have also been reported to contain chlorogenic acid, catechin, epicatechin, epicatechin gallate, and four anthocyanins (Kraft et al., 2008). Mazza (1986) identified chlorogenic acid and rutin as the main non-anthocyanin phenolics present in saskatoons. Based on the relative retention times of standard compounds (Figure 4.2), two of the major HPLC-PDA peaks could be tentatively identified as chlorogenic acid at ~37 min and rutin at ~67 min. While the other major peaks at ~27 and ~39 min were assigned to the phenolic classes of hydroxycinnamic acids and flavonoids (anthocyanins), respectively. Supporting scientific evidence for compound identification was obtained by comparing the UV-vis spectra of these chromatographic peaks with those of chlorogenic acid (a hydroxycinnamic acid) and cyanidin-3-rutinoside (an anthocyanin) standards.

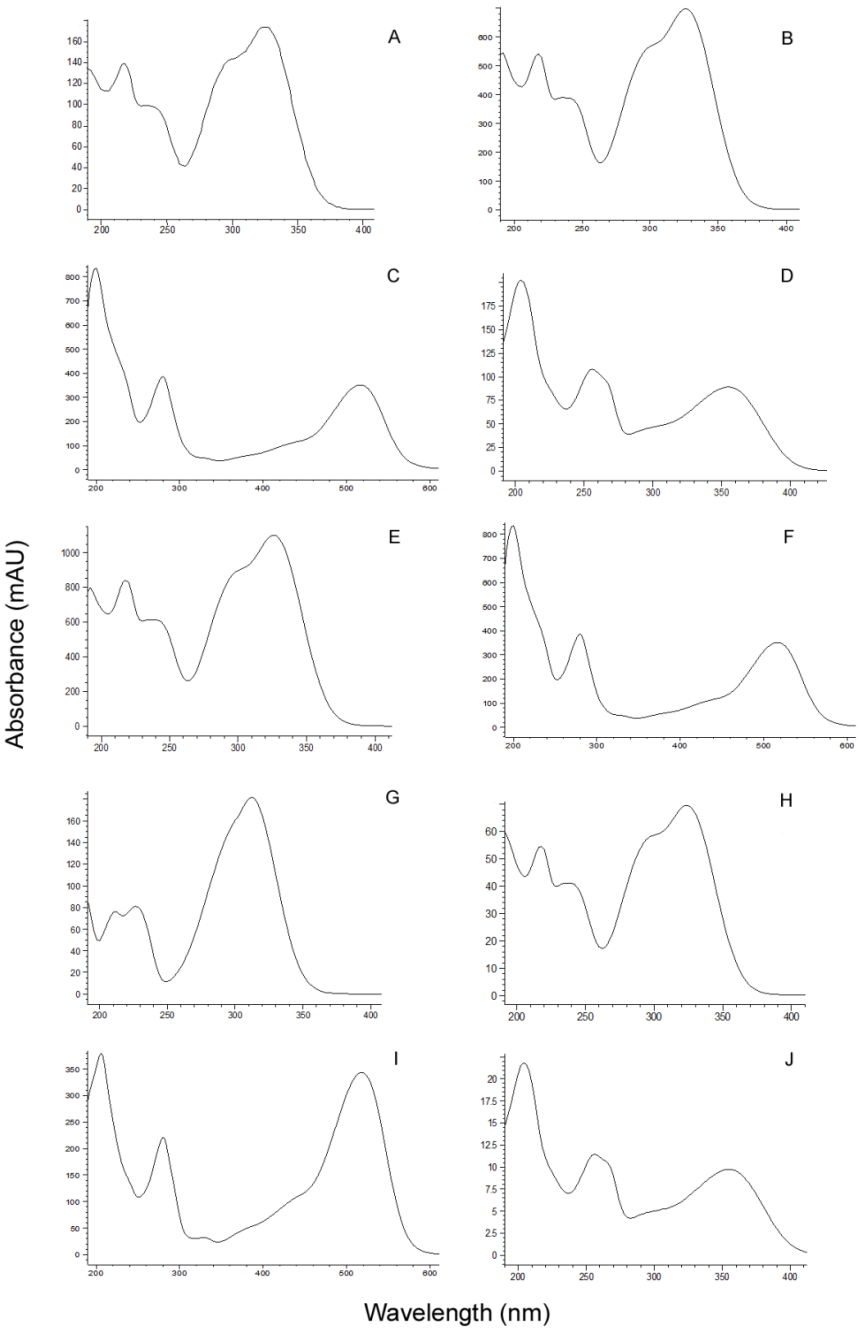


Figure 4.3 **UV-visible spectral profiles of the major phenolic peaks of saskatoon and chokecherry crude solvent (EFW and MFW) extracts and a selection of phenolic standards. (A: saskatoon peak RRT~27 min; B: saskatoon peak RRT~37 min; C: saskatoon peak RRT~39 min; D: saskatoon peak RRT~67 min; E: chokecherry peak RRT~37; F: chokecherry peak RRT~40 min; G: chokecherry peak RRT~44 min; H: chlorogenic acid, a hydroxycinnamic acid; I: cyanidin-3-rutinoside, an anthocyanin; J: rutin, a flavonoid).**

Chromatographic (HPLC-PDA) profile results of chokecherry crude extracts (section 3.4) produced from EFW and MFW are shown in Figure 4.4. Three major (peak area >100 mAU) chromatographic peaks were identified with RRTs of ~37, 40 and 44 minutes, respectively. The total peak area for the major phenolic classes/compounds were combined and were found to be 9.22×10^4 mAU/s and 8.73×10^4 mAU/s for EFW and MFW respectively, indicating that the solvent employed had a minimal impact on total phenolic compound extraction (~5% higher with EFW). The first major peak was identified as the hydroxycinnamic acid, chlorogenic acid (RRT of ~37 min) based on both RRT (Figure 4.2) and UV-vis absorbances (Figure 4.3; E & H). The second peak with a RRT of ~40 min was tentatively identified as anthocyanins based on an absorbance at 520 nm (Figure 4.3; F & I). The last major peak in the chokecherry HPLC-PDA profile had a RRT of ~44 min, and was tentatively identified as hydroxycinnamic acid(s) based on UV-vis spectral data (Figure 4.3; G & H). Although this peak was also identified as a hydroxycinnamic acid, the RRTs of compound within this class can vary based on the number of hydroxyl or methoxy groups attached to the aromatic ring (Wrolstad, 2005). The RRT (~44 min) for this peak did not match any of the phenolic standards commonly present in fruits (Figure 4.2) and therefore no specific compound identification could be made.

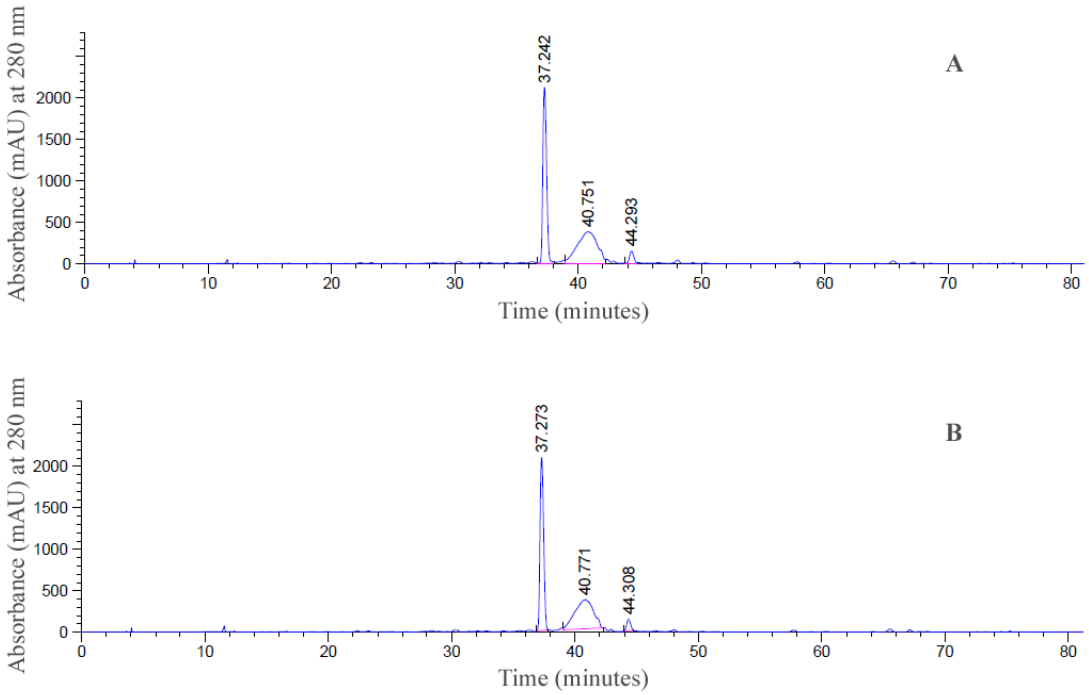


Figure 4.4 Reverse phase HPLC-PDA chromatograms (280 nm) of the phenolic profiles of chokecherry crude extracts produced with EFW (A) and MFW (B).

In the literature, the major phenolics identified in chokecherries by HPLC-PDA were anthocyanins (cyanidin-3-glucoside and cyanidin-3-galactoside), caffeic, chlorogenic, p-coumaric, ferulic, protocatechuic and sinapic acids (Kraft et al., 2008; Li et al., 2009). Previous work done by Green (2007) reported that the three main phenolics present in chokecherry MFW extracts were chlorogenic acid, quercetin and rutin. Therefore, the tentative identification of the phenolics in chokecherry solvent extracts conducted in this research matched those reported in the literature.

When comparing the two fruit sources, the total major phenolics peak areas differed, with the chokecherry crude extract samples having a higher value than the saskatoons. This peak area difference showed that chokecherry samples were 1.7x higher in phenolic peak area than saskatoons. Therefore, the chokecherry samples contained higher amounts of EFW/MFW extractable phenolics.

Solid phase extraction employing Amberlite XAD 16 was used to isolate phenolics from the crude extracts. This process allows for the concentration of phenolics via the removal/reduction in concentration of unwanted plant material components such as carbohydrates, organic acids and proteins (Kahkonen et al., 1999; Robbins, 2003; Lee,

2004; Green, 2007; Krenn et al., 2007). These compounds are solubilised in the water wash once the plant extract is loaded onto the column. The isolates that were produced from SPE with a single protic solvent concentration (70% ethanol or methanol) (section 3.7) and were compared to determine the effect of solvents (EFW vs. MFW). The 70% protic solvent concentration used offered complete elution of all phenolics (Green, 2007). The phenolic isolates from both fruits were then used for encapsulation studies (section 3.11).

For the saskatoon isolates, the four major phenolic HPLC-PDA peak areas were very similar for both solvents (chromatograms/data not shown) showing that the clean-up using SPE with either EFW or MFW led to similar amounts of extracted phenolics. The total (i.e. combination of all four major peaks) phenolics HPLC-PDA peak areas for the SPE isolates were 1.6x higher than those of the crude extracts for both EFW and MFW, respectively. These results indicated that the SPE step removed the majority of water soluble contaminants and concentrated the phenolics.

The chokecherry isolates three major phenolic HPLC-PDA peak areas were similar in major peak area with the EFW and MFW (chromatograms/data not shown). The total phenolic HPLC-PDA peak areas for the SPE isolates were 2.5x and 2.8x higher than those of the crude extracts for EFW and MFW, respectively.

From these results it was clear that all of the SPE produced extracts contained a higher amount of phenolic material than the original crude extracts on a w/w basis. The total phenolic HPLC-PDA peak area values for chokecherry were 2.9x higher than those of saskatoons indicating that the chokecherry isolate samples contained higher amounts of phenolic compounds.

4.1.4 Identification and concentration of anthocyanins in SPE produced chokecherry and saskatoon crude extracts by HPLC-PDA

The determination of total and individual anthocyanin concentrations in fruit extracts produced employing SPE (C_{18} Sep-Pak) were accomplished by RRT and response factor comparison to standards using HPLC-PDA (section 3.9.1). This extract production method was used to compare and contrast EFW and MFW with respect to anthocyanin extraction from chokecherry and saskatoon samples.

The anthocyanin chromatographic profiles of SPE-produced saskatoon crude extracts using EFW and MFW showed four peaks with approximate RRTs of 37.7, 40.7, 42.9 and 50.4 min, respectively (Figure 4.5). Based on comparison of these RRTs with those of standards, cyanidin-3-galactoside (~37.7 min) and cyanidin-3-glucoside (~40.7 min) were identified in saskatoon extracts. The remaining peaks were identified using literature results as cyanidin-3-arbinoside (~43 min) and cyanidin-3-xyloside (~50 min) (Bakowska-Barczak et al., 2007; Ozga et al., 2007; Bakowska-Barczak and Kolodziejczyk, 2008). In the literature, 17 different cultivars of saskatoons were examined for their anthocyanin content, and anthocyanin concentrations were reported as 65-78% cyanidin-3-galactoside, 9-23% cyanidin-3-glucoside, 8-13% cyanidin-3-arabinoside and 0.4-11% cyanidin-3-xyloside (Bakowska-Barczak and Kolodziejczyk, 2008). The average values determined in this research based on HPLC-PDA for the saskatoon EFW samples were, 32.0 mg/100 g fw (49.1%) cyanidin-3-galatoside, 17.8 mg/100 g fw (27.3%) cyanidin-3-glucoside, 9.2 mg/100 g fw (14.1%) cyanidin-3-arabinoside and 6.2 mg/100 g fw (9.5%) cyanidin-3-xyloside (Table 4.3). While the saskatoon MFW extract had average values of 33.2 mg/100 g fw (42.6%) cyanidin-3-galatoside, 23.2 mg/100 g fw (29.8%) cyanidin-3-glucoside, 11.7 mg/100 g fw (15.0%) cyanidin-3-arabinoside and 9.8 mg/100 g fw (12.6%) cyanidin-3-xyloside. The concentrations of cyanidin-3-arabinoside and cyanidin-3-xyloside in these samples were estimated employing the response factor for cyanidin-3-glucoside. The observed concentrations for these four anthocyanins were slightly different than the ranges reported for saskatoons in the literature, as a lower concentration of cyanidin-3-galactoside was found in the current work, as well as higher concentrations of cyanindin-3-glucoside and cyanidin-3-arabinoside.

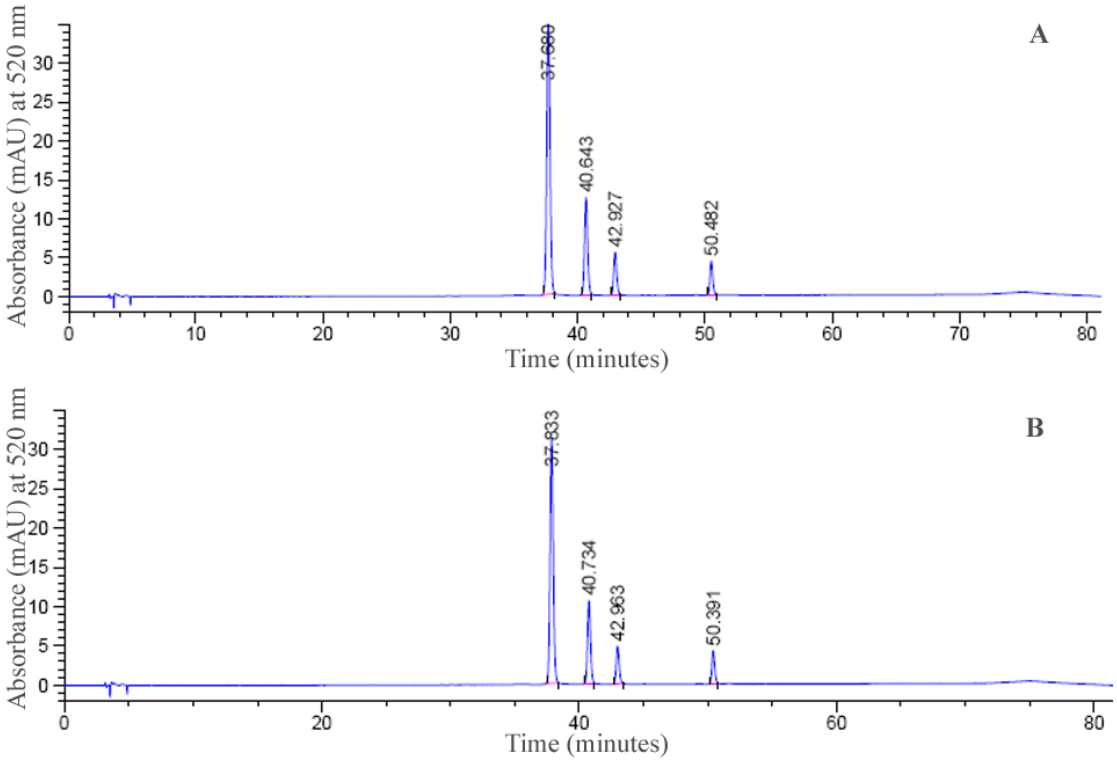


Figure 4.5 Reverse phase HPLC-PDA anthocyanin chromatograms (520 nm) of SPE produced saskatoon crude extracts employing EFW (A) and MFW (B) as solvents.

The anthocyanin chromatographic profile of SPE produced chokecherry crude extracts showed two peaks with approximate RRTs of 41.0 and 43.5 min, respectively (Figure 4.6). Based on comparison of these RRTs with those of standards, cyanidin-3-glucoside (~40.7 min) and cyanidin 3-rutinoside (~43.3 min) were identified. The average concentration of anthocyanins was 61.7 and 61.9 mg/100 g fw for cyanidin-3-rutinoside in EFW and MFW SPE extracts, respectively, while the average concentration of cyanidin-3-glucoside was 49.8 and 51.2 mg/100 g fw in MFW and EFW SPE extracts, respectively.

Table 4.3 Anthocyanin identification and mean concentration in EFW and MFW SPE produced crude chokecherry and saskatoon extracts as determined by reverse phase HPLC-PDA (520 nm).

Anthocyanin	Saskatoon-EFW	Saskatoon-MFW	Chokecherry-EFW[b]	Chokecherry-MFW[b]
Cyanidin-3-galactoside	32.0 [a]	33.2		
Cyanidin-3-glucoside	17.8	23.2	61.7	61.9
Cyanidin-3-arabinoside[c]	9.2	11.7		
Cyanidin-3-xyloside[c]	6.2	9.8		
Cyanidin-3-rutinoside			49.8	51.2
Total	65.2	77.9	111.5	113.1

[a] expressed as the average mg /100 grams fresh fruit.
[b] chokecherry fresh weight was the flesh weight not including the seeds.
[c] Estimated using the response factor of cyanidin-3-glucoside.
n= 2

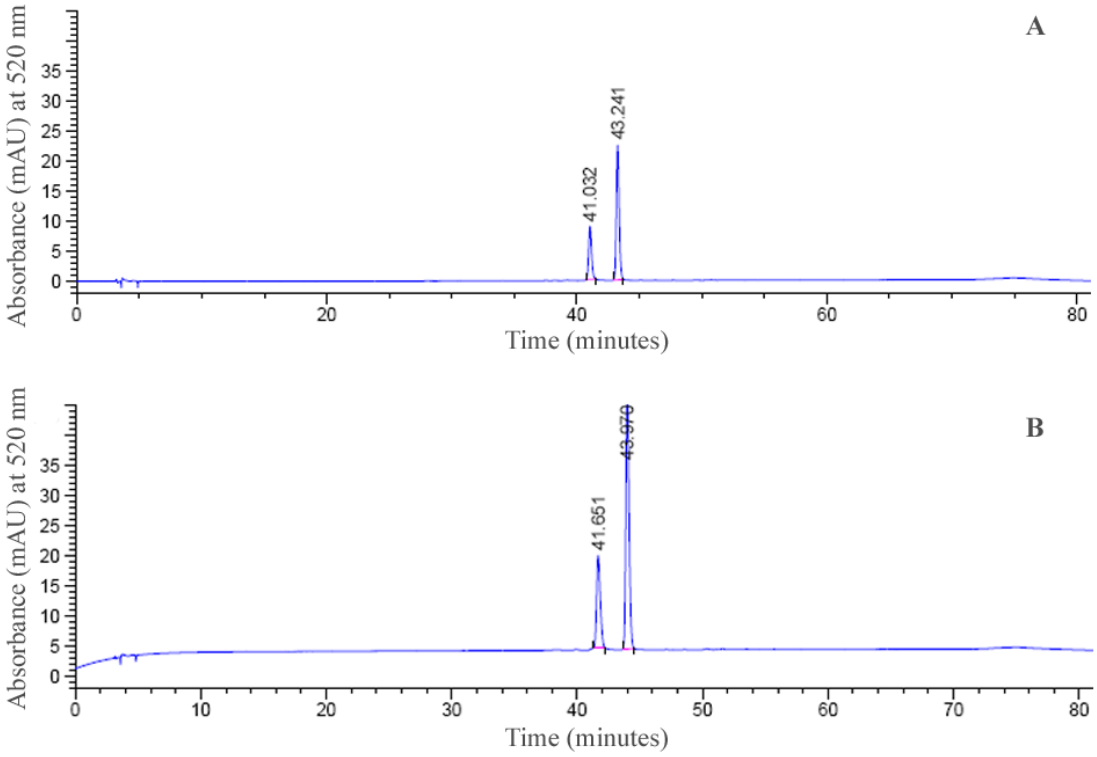


Figure 4.6 Reverse phase HPLC-PDA anthocyanin chromatograms (520 nm) of SPE produced chokecherry crude extracts employing EFW (A) and MFW (B) as solvents.

The total anthocyanin concentrations present in the SPE produced crude extracts as determined by HPLC-PDA are shown in Table 4.3. In general, the concentration of anthocyanins in the SPE produced MFW extracts for saskatoons were higher than those observed in the EFW extracts. This result is most likely due to the polar characteristics of anthocyanins which include a positive charge at the acidic pH of the extraction solvent and the presence of carbohydrate moieties, which are more readily solubilized in MFW. Results for SPE produced crude chokecherry extracts showed similar anthocyanin concentrations (as determined by HPLC-PDA area results) for EFW and MFW.

In the literature, anthocyanins have been extracted from fruits using polar solvents such as acetone, ethanol, methanol and water (Lapornik et al., 2005; Nicoue et al., 2007). Lapornik et al. (2005) determined that the solubility of anthocyanins was generally the highest in methanol, slightly lower in ethanol and the lowest in water. Metivier et al. (1980) determined that methanol was the most effective extraction solvent when compared to ethanol and water for the extraction of anthocyanins from grape pomace. Ju

and Howard (2003) also determined that higher concentrations of anthocyanins were extracted using acidified methanol as compared to acidified ethanol from grape skins. Therefore, data from literature would support the results found in the current study.

Based on the analytical results shown in Table 4.3 the total mean anthocyanin concentration for SPE produced crude chokecherry extracts were 1.4x and 1.7x greater than those for saskatoon EFW and MFW, respectively.

These anthocyanin concentrations for saskatoons, as determined by HPLC-PDA, were ~70 mg/100g fw lower than those previously calculated using the pH differential method (section 4.1.2). Reasons for this decrease may be explained by an underestimation of cyanidin-3-arabinoside and cyanidin-3-xyloside because these concentrations were calculated using the response factor of cyanidin-3-glucoside. Analytical differences may also have resulted from the use of crude extracts of the fruit for the pH differential work, whereas the SPE produced extracts were used for HPLC-PDA analysis. Burdulis et al. (2008) reported that sample purification using SPE can result in the loss of anthocyanins from extracts. In addition, a sample clean-up step employing a C_{18} Sep-Pak was used prior to HPLC-PDA analysis. It was noted that this procedure resulted in colour absorption which was not completely removed upon solvent washing. It was observed that this loss was greater in the saskatoon EFW samples when compared to the MFW samples. This observation was also made by Kraemer-Schafhalter et al. (1998) for SPE produced black chokeberry ethanol extracts. The authors reported that this band of colour was not removed from the solid phase material even when the concentration of the elution solvent was raised to 96%.

The anthocyanin concentrations as determined by HPLC-PDA for chokecherries were ~138 mg/100 g fw lower than those calculated using the pH differential method (section 4.1.2). As discussed above with saskatoons, these differences may be due to the extracts used for these measurements (crude versus SPE). Previous work by Green (2007) reported a decrease (from 255 to 147 mg/ 100 g fw) in the anthocyanin concentration of chokecherry extracts as determined by the pH differential (crude extract) method when compared to HPLC-PDA (SPE extract) method. The use of a single extinction coefficient in the pH differential method may also influence the reported

anthocyanin levels, because it may not accurately represent all anthocyanins present in the sample (Zatylny et al., 2005b).

Literature results for the anthocyanin concentration of saskatoons vary greatly. Ozga et al. (2007) reported values of 131 and 140 mg/100 g fw for Honeywood and Smokey cultivars, respectively. A similar value of 139 mg/100 g fw was reported by Hosseinian and Beta (2007), with no cultivar identified. A value of 55 mg/100 g fw was report for the Martin variety by Zatylny et al. (2005b), which was lower than the results reported in this research of 65.2-77.9 mg/100 g fw. Hu et al. (2005) reported values of 60 mg/100 g fw for the Thiessen cultivar and 40 mg/100 g fw for Smoky. It is difficult to make a direct comparison of the results of this research to literature values because there are many factors that can impact these analytical results including cultivar, environmental conditions during the growing season of the fruit, sample preparation, and HPLC protocols.

Hosseinian and Beta (2007) reported that the major anthocyanins in saskatoons were delphinidin-3glucoside and malvidin-3-glucoside. However, the majority of literature results identified cyanindin-3-galactoside and cyanindin-3-glucoside are identified as the major anthocyanins in saskatoons (Mazza, 1986; Zatylny et al., 2005b; Ozga et al., 2007; Bakowska-Barczak and Kolodziejczyk, 2008). Results from this study agreed with the majority of literature with cyanidin-3-glucoside and cyanidin-3-galactoside being the most prevalent anthocyanins found in saskatoons (Martin cultivar).

Previous work done in our laboratory (Green, 2007) had identified the major anthocyanins in chokecherry extracts to be cyanidin-3-glucoside and cyanidin-3-rutinoside. Green (2007) reported total anthocyanin values in chokecherry extracts of 147 mg/100 g fw as determined by liquid chromatography mass spectrometry, while in this study values of 112 mg/100 g fw and 113 mg/100 g fw were found for SPE produced EFW and MFW extracts, respectively. These values were lower than those reported by Green (2007), with the major decrease due to low concentrations of cyanidin-3-rutinoside. The anthocyanin concentration differences observed in this work from that reported by Green (2007) would be best explained by environmental factors, as the sample preparation and analysis methods used were similar. Environmental factors that have been shown to impact fruit anthocyanin content include light, temperature and

agronomic conditions (Hu et al., 2005; Hosseinian and Beta, 2007). In addition, genetic variance also has been shown to have an impact on the anthocyanin content of fruit (Kalt et al., 1999; Connor et al., 2005; Zadernowski et al., 2005).

4.1.5 *In vitro* antioxidant activity

Phenolics act as antioxidants mainly through the donation of a hydrogen atom to a free radical (Heim et al., 2002). *In vitro* antioxidant assays, such as DPPH and ABTS (TEAC), can be used to provide an estimation of the antioxidant ability of a pure compound or group of compounds in a sample. Both of these assays were used in this research project to determine if there were differences in the antioxidant activities of the crude fruit solvent (EFW and MFW) extracts. In addition, the antioxidant activities of the extracts produced from an Amberlite column were compared to determine the impact of solvent (EFW versus MFW) on the radical scavenging activities of the phenolic rich extracts.

4.1.5.1 DPPH radical scavenging activity

The DPPH radical scavenging activities of the four crude fruit extracts were determined and the results of these experiments are shown in Table 4.4. These results show that chokecherry DPPH values for both extraction solvents were ~1.6x higher than those observed for the saskatoons samples. Based on these results, it can be concluded that the crude chokecherry extracts had a greater DPPH radical scavenging ability than the crude saskatoon extracts, indicating a higher concentration of phenolic compounds. These results were supported by the TPC values (Table 4.1), which showed that chokecherries had approximately twice the concentration of phenolic compounds when compared to saskatoons. Also, as discussed in section 4.1.3, the crude chokecherry extracts had higher (~1.7x) total phenolic HPLC-PDA peak areas than saskatoons for both extraction solvents, which would be expected to result in higher DPPH radical scavenging ability values. When comparing the impact of the solvent used to produce crude fruit extracts on DPPH value, the results showed very similar mean DPPH values for EFW versus MFW for both fruits.

The observed variations in DPPH radical scavenging activity values between the duplicate samples of crude chokecherry extracts were 13% and 9% for EFW and MFW, respectively. While the crude saskatoon extract sample variations were smaller at 8% and 4% for EFW and MFW, respectively. These variations may be explained by the small original fruit sample size used (20 g) and/or the lack of a completely homogeneous starting material. Previous work by Green (2007) reported ABTS radical scavenging value differences of 14% between triplicate crude chokecherry extracts produced with MFW.

Table 4.4 Individual and mean antioxidant capacities of crude chokecherry and saskatoon solvent (EFW and MFW) extracts using DPPH free radical scavenging assay.

Sample	DPPH scavenging value [a]	Mean
Chokecherry- EFW	2.13	2.21
	2.46	
Chokecherry- MFW	1.98	2.08
	2.17	
Saskatoon- EFW	1.31	1.35
	1.40	
Saskatoon- MFW	1.33	1.36
	1.39	

[a] Reported as 1/mg of sample needed to reduce the DPPH radical by 50%.
n= 2

Fruit phenolic extracts produced with Amberlite resin (section 3.7) showed, with the exception of the chokecherry MFW isolates, at least a doubling (w/w) of the DPPH radical scavenging activity values when compared to the crude fruit extracts (Table 4.5). These results are supported by an increase in the total phenolic HPLC-PDA areas observed for these extracts (section 4.1.3) of ~2.6x and ~1.6x over those of the crude extracts for chokecherries and saskatoons, respectively.

Table 4.5 Individual and mean antioxidant capacities of crude chokecherry and saskatoon solvent Amberlite extracts (EFW and MFW) using DPPH free radical scavenging assay.

Sample	DPPH scavenging value [a]	Mean
Chokecherry- EFW	4.50	4.97
	5.43	
Chokecherry- MFW	3.14	3.58
	4.02	
Saskatoon- EFW	3.71	3.86
	4.01	
Saskatoon- MFW	3.18	3.48
	3.79	

[a] Reported as 1/mg of sample needed to reduce the DPPH radical by 50%.
n= 2

The differences in the DPPH values for chokecherry samples may be explained by the lower values of hydrophobic compounds (flavonoids) present in the MFW isolates. Generally, flavonoids have a greater radical scavenging ability when compared to other classes of phenolics such as hydroxycinnamic acids (Rice-Evans et al., 1996; Heim et al., 2002; Villaño et al., 2007). Rutin, which is a flavonoid has a DPPH radical scavenging activity 2.2x higher than caffeic acid, a hydroxycinnamic acid (Villaño et al., 2007). Therefore, any increase in the concentration of these compounds in the EFW extract would result in a higher radical scavenging ability when compared to that produced with MFW.

The observed variations in DPPH radical scavenging activity values between the duplicate samples of the chokecherry isolates were 17% and 22% for EFW and MFW, respectively. While the saskatoon isolate sample variations were smaller at 7% and 16% for EFW and MFW, respectively. These variations may be explained by the small original sample size used (20 g) and/or the lack of a completely homogeneous starting material. Previous work by Green (2007), reported ABTS radical scavenging value differences of 23% between triplicate chokecherry isolates produced with MFW.

4.1.5.2 ABTS radical assay (TEAC value)

The antioxidant activity results of the crude fruit solvent extracts employing the ABTS radical assay (TEAC value) are shown in Table 4.6. These results show that the mean TEAC values for crude chokecherry extracts produced using EFW and MFW were ~2.3x and ~1.6x higher than those of saskatoon for EFW and MFW, respectively. As discussed previously (section 4.1.5.1), these results are supported by higher TPC and DPPH values and by higher total HPLC-PDA phenolic peak areas.

The higher mean TEAC value for the EFW versus the MFW crude chokecherry extracts may be explained by a higher level of extractable flavonoids in the original sample set used to produce these extracts. Research has shown that flavonoids, such as rutin, have greater (~38%) solubility in EFW (less polar solvent) than methanol (more polar solvent) (Zi et al., 2007). In addition, it has been shown that antioxidant activity values are dependent upon the phenolic compound/class/concentration present in the sample (Villaño et al., 2005; Villaño et al., 2007). Therefore, the role of extraction solvent hydrophobicity on sample phenolic compound/class/concentration would have a significant impact on TEAC values. The fact that the observed mean TEAC values were similar for the crude saskatoon EFW and MFW extracts would appear to indicate that no significant differences in phenolic compound, class or concentration existed between these two extracts.

Table 4.6 Individual and mean antioxidant capacities of crude chokecherry and saskatoon solvent (EFW and MFW) extracts using ABTS free radical scavenging assay, reported as TEAC.

Sample	TEAC value [a]	Mean
Chokecherry- EFW	22.66	25.40
	28.14	
Chokecherry- MFW	16.15	17.58
	19.01	
Saskatoon- EFW	10.01	10.93
	11.84	
Saskatoon- MFW	10.94	11.20
	11.45	

[a] Reported as mM Trolox equivalents/100 mg sample.
n= 2

The observed variation in TEAC mean values between the duplicate crude chokecherry extracts were 19% and 15% for EFW and MFW, respectively. While the saskatoon crude extract sample variation were 15% and 4% for EFW and MFW, respectively. These variations may be explained by the small original fruit sample size used (20g) and/or the lack of a completely homogeneous starting material. Previous work by Green (2007) reported variations in chokecherry TEAC values of 12% between triplicate crude chokecherry extracts produced with MFW.

The ABTS radical scavenging activities of the chokecherry and saskatoon Amberlite extracts were found to range from ~1.7 to ~3.0x higher in TEAC value when compared to the crude fruit extracts (Table 4.7). As discussed previously, these results were supported by DPPH radical scavenging activity and HPLC-PDA total phenolic area results. The observed variations in TEAC values for duplicate samples have been discussed previously.

Table 4.7 Individual and mean antioxidant capacities of crude chokecherry and saskatoon solvent Amberlite extracts (EFW and MFW) using DPPH free radical scavenging assay, reported as TEAC.

Sample	TEAC value [a]	Mean
Chokecherry- EFW	42.23	43.17
	44.11	
Chokecherry- MFW	38.28	44.27
	50.26	
Saskatoon- EFW	31.60	34.25
	36.90	
Saskatoon- MFW	33.26	33.33
	33.36	

[a] Reported as mM Trolox equivalents/100 mg sample.
n= 2

The results obtained for the two fruit sources from both antioxidant activity tests showed similar general trends. First, the Amberlite produced fruit extracts generally showed higher antioxidant activities (~2x) than the crude extracts. This was due to a concentrating of the phenolic material in the Amberlite extracts coupled with the bulk removal of non-phenolic compounds from the samples. Similar results have been reported in the literature where extracts tested before the removal of bulk sample carbohydrates were less active than the purified extracts (Re et al., 1999). The research results from this work clearly illustrate that the use of Amberlite resin with EFW or MFW as solvents was a facile way to produce phenolic compound rich chokecherry and saskatoon extracts which showed increased free radical scavenging activities when compared to crude extracts.

4.2 Study 2- Entrapment and release of phenolics from chitosan-based nanoparticles.

4.2.1 Preparation and characterization of CH:TPP nanoparticles for rutin delivery

Chitosan-based nanoparticles were formed by ionotropic cross-linking with TPP at different mass ratios (2.0:1.0 – 5.0:1.0 CH:TPP), and used to entrap rutin. Formed particles were assessed for their entrapment efficiencies, surface charge, size and release properties. Rutin was chosen as a model phenolic for entrapment purposes based on its structural similarities (three conjugated rings and a carbohydrate moiety) to anthocyanins found in chokecherries and saskatoons. However, unlike anthocyanins, rutin lacks the pyrilium cation of the C ring (Vermerris and Nicholson, 2006a). In all cases, nanoparticles were formed after 1 h of curing within the TPP solution. The chitosan material used for particle formation had a 92% deacetylation rate which therefore contains many $-NH_2$ groups. Under the acidic conditions used (pH 4.8), $-NH_2$ groups on the CH backbone become protonated to form $-NH_3^+$ enabling interaction with the anionic TPP so as to form intermolecular and/or intramolecular linkages between CH chains to create a porous gel matrix (Mi et al., 1999). Cross-linking by TPP is attractive for developing controlled delivery systems since it produces reversible interactions and is considered to be less toxic than other alternatives, such as glutaraldehyde (Mi et al., 1999). Furthermore, the resulting CH:TPP cross-linked gel is reversible on pH change, giving the nanoparticles a unique pH-sensitive release mechanism (Hu et al., 2008).

4.2.1.1 Particle size and surface charge

Particle size and surface charge are both important considerations when designing controlled delivery vehicles. Sub-micron particles are essential for mucosal and epithelial tissue uptake, whereas particles of high positive charge aid in suspension stability, mucoadhesiveness and permeation (Hu et al., 2008; Harris et al., 2011). Particle size and distribution is related to CH concentration and size, along with mixing conditions (time, shear, temperature) (Zhang and Kosaraju, 2007). In general, the CH:TPP particles experienced a decline in size with increasing mass ratio (Figure 4.7A) as the geometric mean diameter of the nanoparticles decreased from ~814 nm for the 2.0:1:0 mass ratio to ~528 nm for ratios between 2.5:1.0 and 4.0:1.0, and to ~322 nm for the 5:0:1.0 mass

ratio. Dynamic light scattering describes particle size based on the Stokes-Einstein equation, which estimates the diameter of an imagery sphere surrounding the particle in solution (Alexander and Dalgleish, 2006). In solution, the anionic phosphate groups in TPP interact with an extended CH polymer (under acidic conditions) as follows: first by forming intermolecular and intramolecular cross-links between amino groups along the polymer backbone; and second by screening occupied or unoccupied charged reactive sites to reduce the overall surface charge of the polysaccharide (Figure 4.8) (Mi et al., 1999). This screening effect acts to reduce the polymer's electric double layer and increase its conformational entropy (or chain flexibility) (Mi et al., 1999). Hu et al. (2008) reported a linear decline in particle size with increasing CH:TPP mass ratios from 3.0:1:0 to 7:0:1:0. The authors attributed the larger particles at lower mass ratios (i.e., more TPP present relative to available $-NH_3^+$ groups) to the cross linking of multiple smaller mono-particles to give an overall larger cluster in solution. In contrast, at higher mass ratios (i.e., where TPP is more limiting) fewer inter-particle cross links may be formed. Zhang and Kosaraju (2007) employed transmission electron microscopy to show that aggregation of CH:TPP particles were difficult to avoid, and found clear evidence of nucleation and growth induced by ionic gelation. The authors produced CH:TPP particles ranging in size from 4.27-6.29 μm.

In the current study, surface charge of the formed CH:TPP particles were found to increase from ~+21 mV to ~+59 mV as the mass ratio increased from 2.0:1.0 to 5.0:1.0, indicating that less neutralization of protonated amino groups by TPP was taking place (i.e., TPP was becoming limiting) and that more CH sites were left unbound as polysaccharide levels increased (Figure 4.7B). The positive zeta potential over the entire molar range indicates that even at the low 2.0:1.0 mass ratio, there are unbound $-NH_3^+$ groups present (Harris et al., 2010). The significantly lower zeta potential at a mass ratio of 2.0:1.0 may reflect the electrostatic charge screening effect of TPP (Liu and Gao, 2009) and the larger clustering of the CH particles due to TPP ionic cross linking (Zhang and Kosaraju, 2007). The latter would result in less protonated amino groups at the surface, which are exposed to the solvent (Figure 4.7B). A similar trend was also reported by Hu et al. (2008) for CH:TPP systems at higher mass ratios (3.0:1.0-7.0:1.0). Zhang and Kosaraju (2007) reported that CH:TPP particles only formed at a specific CH

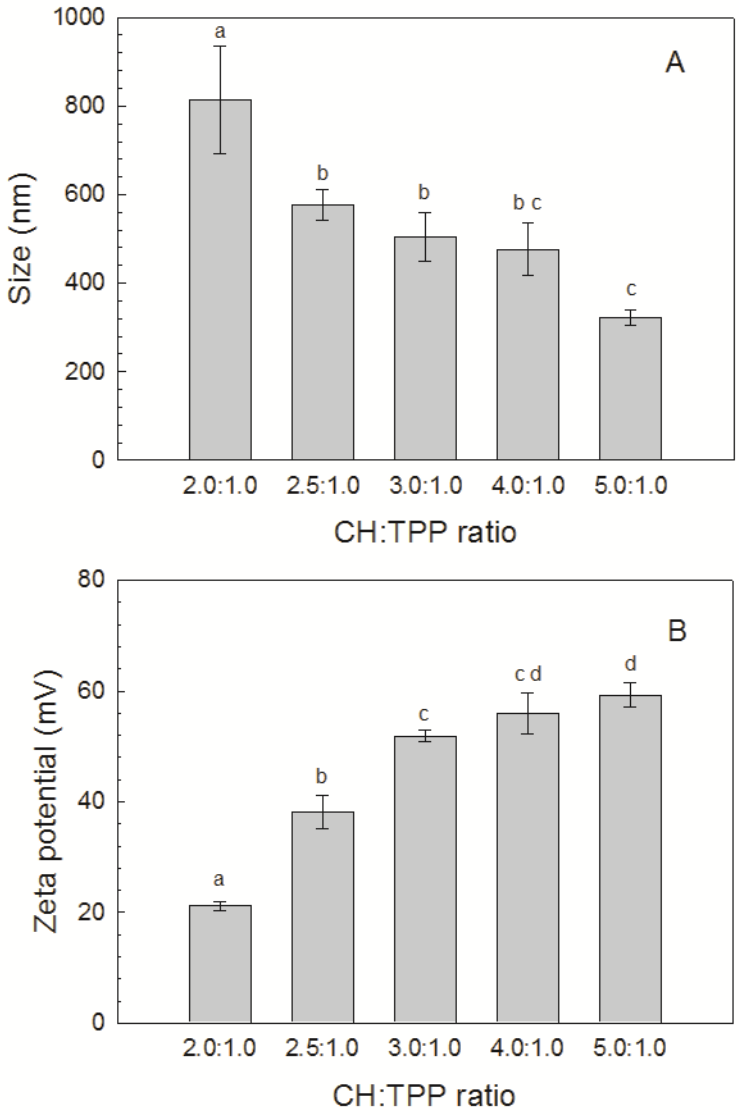


Figure 4.7 **Size (A) and zeta potential (B) of CH:TPP rutin particles as a function of mass ratio at pH 4.8. Data represent the mean ± one standard deviation (n= 3). Means with common letters (a, b, c, d) are not significantly different ($p \leq 0.05$).**

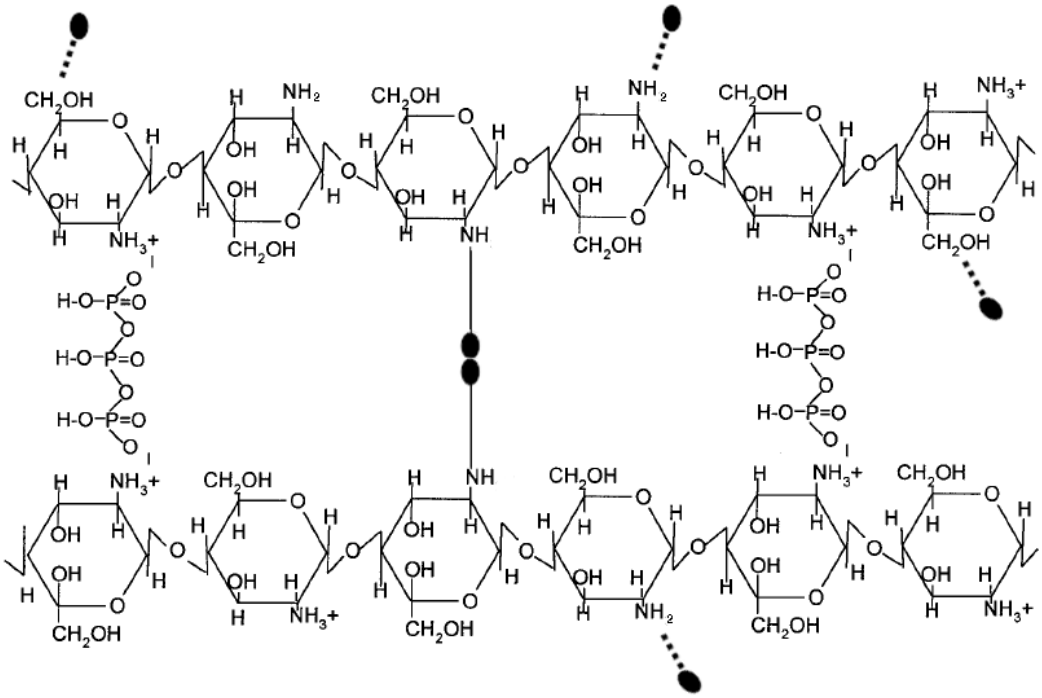


Figure 4.8 **Ionic cross linking of CH-TPP solution in the presence of phenolic compounds. Solid circles represent phenolic compounds, solid lines represent covalent bonding and dotted lines represent hydrogen bonding with CH (adapted from Mi et al., 1999; Zhang and Kosaraju, 2007).**

and TPP concentration range, below and beyond which no particles formed. Once formed, CH:TPP particles are inherently thermodynamically unstable especially to changes in pH, making them excellent bioactive delivery vehicles.

Although not studied in the present research, particle size and charge may be influenced by other factors, such as molecular mass and concentration of CH, and the initial pH of the CH solution. Hu et al. (2008) studied all of these factors for designing CH:TPP nanoparticles loaded with tea catechins. While studying the effect of CH molecular weight (MW) (30-300 kDa) on particle size, the authors reported a trend related to the amount of shear degradation experienced by the CH chains when exposed to magnetic stirring during preparation. Particle size decreased from ~201 nm to ~169 nm as MW increased from 30 to 50 kDa, and then remained constant between 50 and 150 kDa, before increasing up to ~309 nm at 300 kDa. The authors reasoned that the higher particle size observed at 30 kDa, relative to those between 50 and 150 kDa, reflects the shorter nature of the CH fragments making them non-conducive to shear degradation. Constant size between 50 and 150 kDa reflects the streamlining of polysaccharides within the direction of the shear field, where the elongation shear flow was strong enough to disrupt points of entanglement, leading the formation of smaller particles once cross linked by TPP. The rebound to larger particle sizes with the 300 kDa CH chains suggests that the elongation shear flow was not sufficient to disrupt all points of entanglement, leading to larger particles once exposed to TPP.

Hu et al. (2008) also found zeta potential to increase with CH MW due to a greater number of protonated amino groups (at the appropriate pH conditions) present in solution. The authors also reported that particle size and zeta potential increased linearly with increasing CH concentration within their experimental range of 0.5 to 2.5 mg/mL. These results were thought to be associated with fewer un-neutralized amino groups (charged) at the higher molar mass ratios. Furthermore, the authors investigated the effect of the initial pH at which CH was solubilised, in relation to particle size and charge. Chitosan is considered to have reduced solubility at neutral and alkaline pH since the amino groups remain de-protonated ($-NH_2$) on the C-2 position of D-glucosamine. However, under acidic conditions the $-NH_2$ groups are protonated to form NH_3^+, a cationic polyelectrolyte in solution. The authors reported both the smallest particles and

the lowest surface charge at a pH of 4.5. At pH<4.5 both the size and charge of the particles increased (until pH 3.6), whereas at pH>4.5 particle size increased as pH was raised to 5.5, but charged decreased. The possible explanation for these results were as follows: at pH <4.5, CH chains become more positively charged and stretched as a result of decreased conformational entropy (i.e., the polysaccharides become more rigid) leading to greater particle size, while at pH>4.5-5.0 the zeta potential declined as $-NH_3^+$ are groups de-protonated and particle size increased as CH chains became more open due to fewer cross links with TPP. Particles of high surface charge generally have good suspension stability due to repulsive forces between particles to prevent aggregation, and tend to lead to more uniform size distributions (Zhang and Kosaraju, 2007; Jahanshahi and Babaei, 2008).

In general, nanoparticles <500 nm have been shown to have higher uptake through mucosal and epithelial tissues than larger particles, leading to increased levels of bioactive core material absorption (Acosta, 2009). Jung et al. (2000) reported that particles ranging in size from 50-100 nm showed greater absorption (26-33%) through rat intestines than 500 nm particles (10%). There are two main methods in which particles are transported into cells, which are passive and active transport. For passive transport, particles diffuse through epithelial cell tissue whereas active transport requires receptors on the cell surface (Acosta, 2009). In the present study, with the exception of the 2.0:1.0 CH:TPP ratio, all particles were at or below 570 nm in diameter suggesting that the nanoparticles produced were within the range that could facilitate absorption across the mucosal and epithelial tissues in the small intestine. The positive charge on these nanoparticles would aid in their suspension stability, and facilitate their adhesion to the lining of the small intestines, where there are mucosal cells that contain negatively charged glycoproteins (Bonferoni et al., 2009; Pedro et al., 2009).

4.2.1.2 Rutin entrapment

In the current study, the entrapment efficiencies of rutin were indirectly measured by determining rutin content in supernatant so as to estimate levels entrapped within CH:TPP particles. These levels were investigated as a function of CH:TPP mass ratio using rutin peak are determined by HPLC-PDA and the FC assay (Figure 4.9). Percent

entrapment efficiency (%EE) (as measured by HPLC-PDA) was found to increase from 3.7 ± 1.4 to 57.6 ± 5.1% as the CH:TPP ratios increased from 2.0:1:0 to 4.0:1:0, respectively ($p<0.05$), after which a plateau was reached up to the 5.0:1:0 ratio ($p>0.05$) (Figure 4.9A). A similar trend was found using the FC assay where %EE increased from 8.9 ± 6.1% up to 62.1 ± 6.1% as ratios increased from a CH:TPP ratio of 2.0:1.0 to 4.0:1.0 ($p<0.05$), respectively, before leveling off ($p>0.05$) (Figure 4.9B). The rise in %EE with an increasing CH:TPP ratio (up to 4.0:1.0) is thought to be associated with the larger number of charged $–NH_3^+$ groups available for interactions with the rutin through hydrogen bonding (Zhang and Kosaraju, 2007). Once the plateau was reached (4.0:1.0 mass ratio), the rutin concentration was thought to become limiting relative to the amount of un-neutralized $–NH_3^+$ groups present, resulting in no further increases in %EE as a function of mass ratios increase.

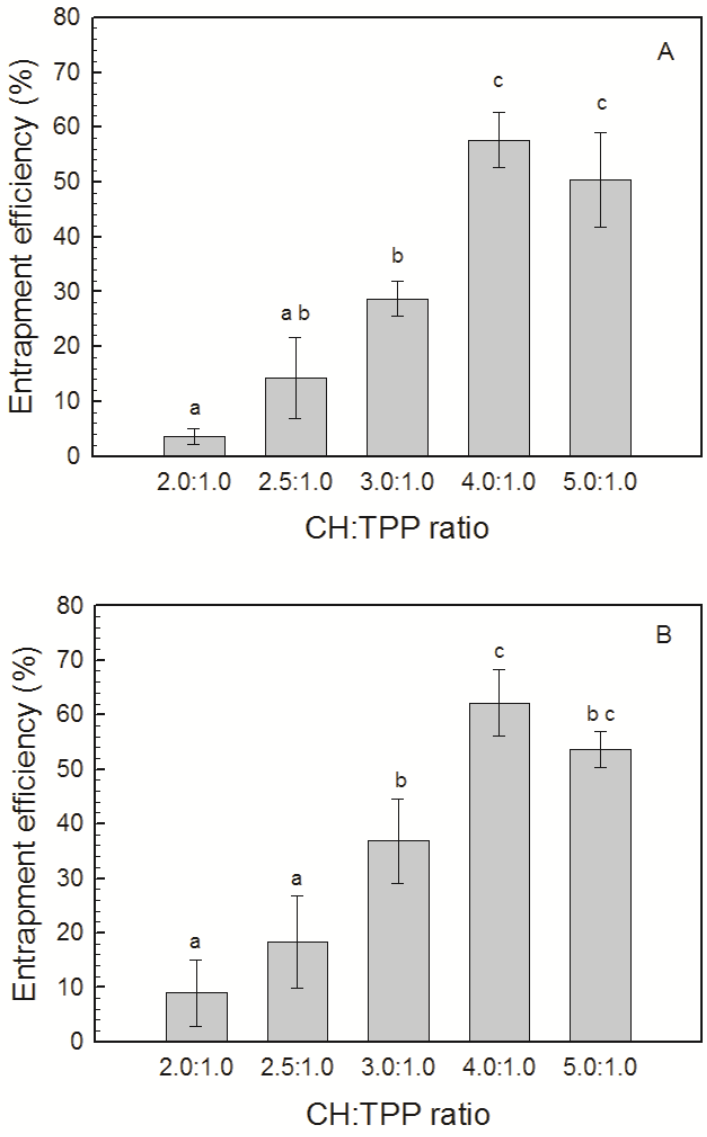


Figure 4.9 **Percent entrapment efficiency of rutin as a function of CH:TPP mixing ratio, as determined by (A) HPLC-PDA and (B) the FC assay. Data represent the mean ± one standard deviation (n= 3). Means with common letters (a, b, c) are not significantly different (p>0.05).**

Rutin interactions within the CH:TPP nanoparticle are proposed to be primarily associated with weak non-covalent associations, such as hydrogen bonding between the hydroxyl groups on the carbohydrate moiety and rutin with the $-NH_3^+$ groups of CH. The covalent linkages are proposed to occur in minor amounts involving the oxidized quinone ring of rutin and the free amino groups of CH. Similar interaction mechanisms were also proposed to occur in CH-tea catechins (Zhang and Kosaraju, 2007) and CH-olive leaf extracted phenols (Kosaraju et al., 2006). Siebert et al. (1996) and Strauss and Gibson (2004) discussed phenolic interactions with proteins to describe a similar mechanism, where both hydrophobic interactions and hydrogen bonding played a role in the weaker non-covalent interactions whereas covalent bonding first involved the oxidation of the aromatic phenolics. Strauss and Gibson (2004) proposed a generic interaction mechanism for phenolic acids and side chain amino groups of peptides (Figure 4.10). In brief, the diphenol structure of the phenolic (**1**) (Note: bolded numbers related to structures in Figure 4.10) can be easily oxidized into an orthoquinone, which can then react with another quinine to form a dimer (**2**) or with the amino side group of a peptide (or in our case, CH). The latter can be oxidized further with a second amino group to form a cross link between neighbouring chains (**3**) or react with a second orthoquinone to form a dimer so as to cross link two chains together (**4**).

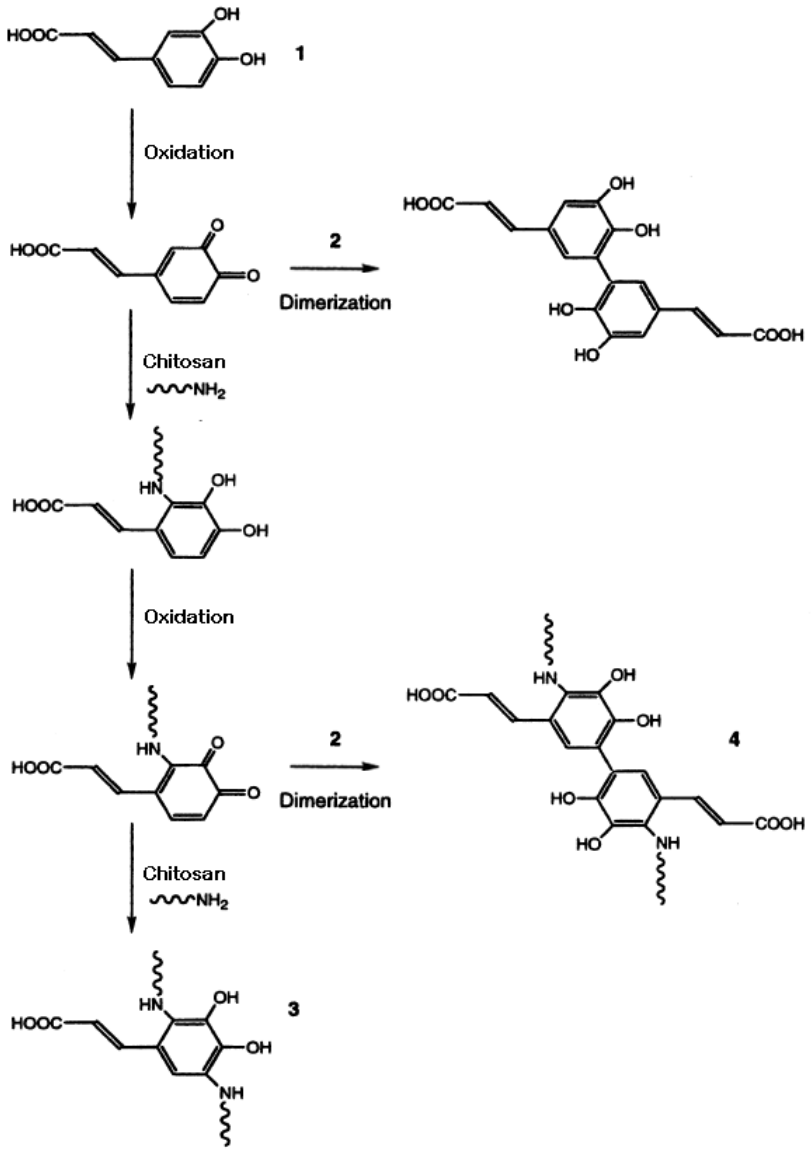
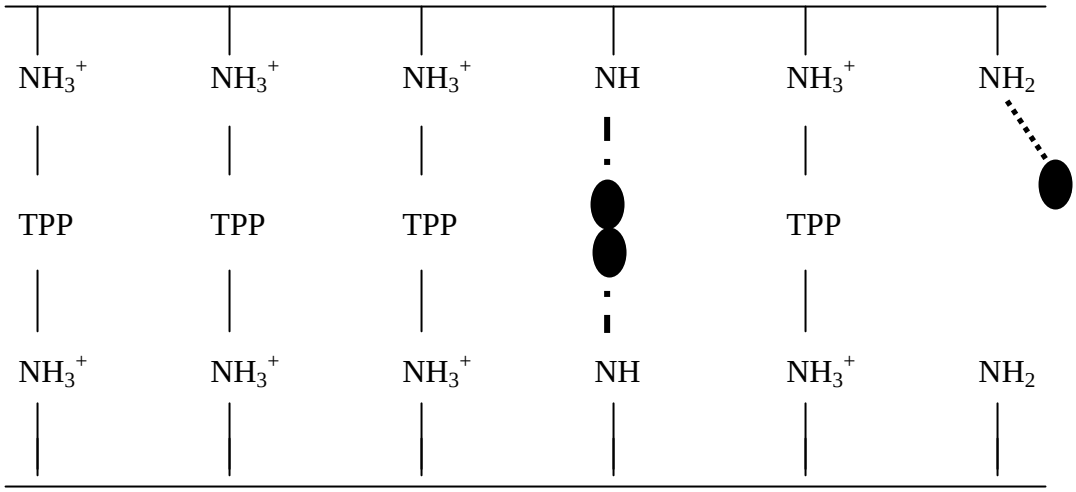


Figure 4.10 Generic reaction scheme of a phenolic acid with an amino group of chitosan (adapted from Strauss and Gibson, 2004). Numbers outline the reactions 1) oxidation, 2) dimerization, 3) cross link between neighbouring chains, 4) dimerization to cross link two chains.

In the present study, TPP acted to form intermolecular and intramolecular electrostatic cross links within or between neighbouring CH chains, and also to screen the electric double layer so as to reduce the electrostatic attractive forces acting towards counterions (or counter-molecules) in solution. At low molar ratios (2.0:1.0 CH:TPP), available $–NH_3^+$ groups were reduced due to higher levels of TPP leading to less hydrogen bonding between CH and rutin (%EE ~3.7%) (Figure 4.11A). However, as the mass ratio increased (i.e., increase the CH content relative to TPP), the amount of available $–NH_3$+ groups increased, allowing for greater hydrogen bonding to occur between CH and rutin (Figure 4.11B). Hu et al. (2008) reported a similar phenomenon while studying the effect of CH molecular weight on the entrapment of catechins within chitosan nanoparticles formed at a 5.0:1.0 CH:TPP mass ratio (pH 4.5). In contrast to the present study in which the levels of TPP influenced the amount of $–NH_3^+$ groups available for hydrogen bonding with the phenolics, Hu and co-workers (2008) discussed the availability of $–NH_3^+$ groups in reference to the size and spatial distribution of CH polymers in solution. Their findings showed that both CH molecular weight and spatial distribution in solution influenced %EE, where for low masses (50-150 kDa), CH chains remained fully extended in solution due to the short nature of the polymer and the electrostatic repulsion between neighbouring $–NH_3^+$ groups. Entrapment of catechins was found to increase linearly over this range, which was attributed to an increased in the amount of hydrogen bonding between CH and the phenolics. However, as CH mass increased to 300 kDa, the chain length reached a critical point in which folding or entanglement ensued. The folding process created steric hindrances and disrupted hydrogen bonding between CH and catechin, leading to lower %EE. The authors also reported entrapment of catechins to be related to contact time with CH polymers, and also molar ratio and CH concentration. Depending on the conditions used, CH:TPP nanoparticles entrapped 24 to 53% catechins. Zhang and Kosaraju (2007) found similar %EE for catechins within CH:TPP nanoparticles with levels ranging from 28 to 40%. Kosaraju et al. (2006) entrapped (27% EE) polyphenols from olive leaf extracts within CH microspheres produced by spray drying using a direct method of analysis, which involved particle degradation in 0.1 N HCl for 24 h.

(a) Low mass ratio (e.g., 2.0:1.0 CH:TPP ratio)



(b) High mass ratio (e.g., 4.0:1.0 CH:TPP ratio)

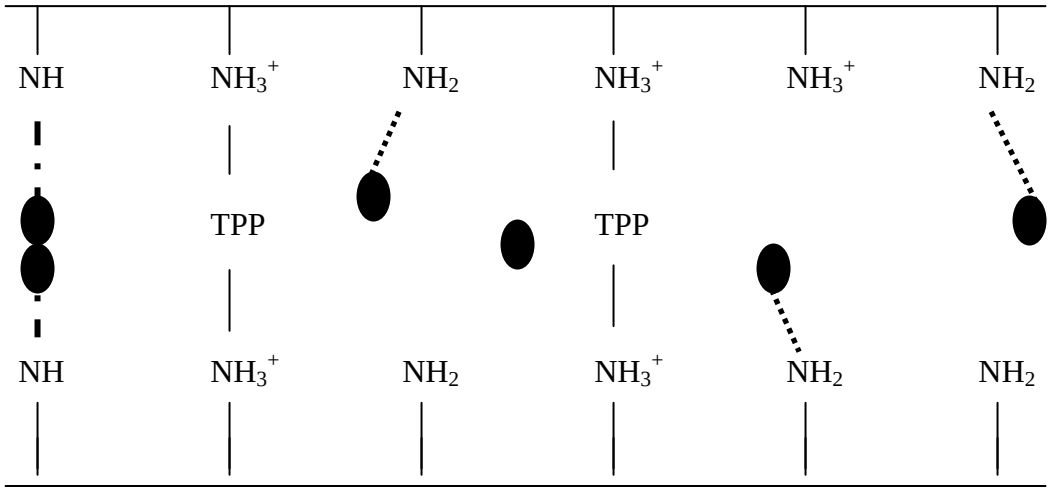


Figure 4.11 Proposed chitosan-TPP and chitosan-phenolic interactions as a function of low (a) and high (b) CH:TPP mass ratios in the presence of phenolic compounds. Solid circles represents phenolic compounds, solid lines represent ionic bonding, dotted lines represent hydrogen bonding and dash dot lines represent covalent bonding (modified from Mi et al., 1999).

4.2.2 Entrapment of fruit phenolics within CH:TPP nanoparticles

The entrapment of phenolic isolates from chokecherries and saskatoons within CH:TPP nanoparticles were investigated at a 4.0:1.0 mass ratio. The carrier system chosen was based on findings from section 4.2.1, where particles showed high entrapment of rutin (%EE = 57.6) and gave characteristic size (<500 nm) and surface charge (~+55 mV) to facilitate good dispersion characteristics, bioadhesion and uptake once delivered to the small intestine (Dhawan et al., 2004; Acosta, 2009). CH:TPP nanoparticles were found to be similar in size to one another at 527.9 ± 74.6 nm and 443.0 ± 15.8 nm for carriers with entrapped chokecherry and saskatoon phenolic isolates, respectively ($p>0.05$), and comparable to those with entrapped rutin (section 4.2.1, $p>0.05$). CH:TPP nanoparticles at the 4.0:1.0 mass ratio prepared without entrapped phenolics was found to have a similar diameter of 453.7 ± 25.5 nm ($p>0.05$), indicating that phenolic:CH interactions was not influencing particle size. In contrast, Zhang and Kosaraju (2007) for CH:TPP micro-spheres prepared at mass ratios between 2.5:1.0 and 7.5:1.0 containing tea catechins resulted in smaller spheres than those without phenolics, which the authors attributed to an increased cross-linking density. Also in the present study, the surface charge on CH:TPP nanoparticles containing chokecherry and saskatoon phenolic isolates were found to be similar at +52.7 ± 2.9 mV and +54.4 ± 1.3 mV, respectively ($p>0.05$), and comparable to those with entrapped rutin (section 4.2.1, $p>0.05$). Nanoparticles prepared without phenolics were found to also display a similar positive charge (+57.5 ± 3.9 mV) ($p>0.05$), suggesting that charge was related solely to the level of CH–TPP interactions, rather than CH-phenolic hydrogen bonding and minor amounts of covalent linkages being formed.

Entrapment efficiency of phenolic isolates from chokecherries and saskatoons within the CH:TPP nanoparticles were found to be similar at 15.9 ± 2.7% and 23.0 ± 7.1%, respectively as measured by HPLC-PDA ($p>0.05$), and 22.8 ± 1.8% and 24.1 ± 4.9%, respectively using the FC assay ($p>0.05$). However, the level of entrapment was found to be significantly lower than that found for rutin (%EE = 57.6 ± 5.1%) ($p<0.05$). Differences in %EE between rutin and the phenolic isolates may be attributed to the level of heterogeneity within the material. Rutin is considered a commercially pure product (95% purity), whereas the saskatoon and chokecherry phenolic isolates had average

purities of 18.9% and 22.9%, respectively (as determined by FC assay), therefore the isolates were not as highly concentrated as rutin. The fruit phenolics are also quite heterogeneous in nature relative to the rutin, possibly leading to slight differences in phenolic-CH interactions. For instance, fruit samples contained multiple classes of phenolics including hydroxycinnamic acids and flavonoids. Hydroxycinnamic acids contain only one cyclic ring and less hydroxyl groups than compounds of the flavonoid class, therefore potentially leading to less interactions with the CH matrix. Fang and Bhandari (2010) determined that the adsorption of phenolics in a CH nanoparticle depends on the characteristics of the phenolic. Compounds that have higher molecular weights and a greater number of hydroxyl groups were found to have higher absorption into particles (Fang and Bhandari, 2010). Since hydroxycinnamic acids make up a large portion of the fruit isolates phenolic content, it was not surprising to find lower %EE than for rutin. The large standard deviations in the %EE data are thought to reflect the heterogeneous nature of the phenolic isolates (as discussed in section 4.1.3).

4.2.3 *In vitro* release studies

The release of rutin and phenolic isolates from chokecherries and saskatoons entrapped within CH:TPP nanoparticles (4.0:1.0 mass ratio) were investigated over time within simulated enzyme-free gastric and intestinal fluids in order to mimic their release. In all cases, no release of rutin or the phenolic isolates was detected after 2 h within simulated gastric fluid (SGF) at pH 1.40 or after 3 h in simulated intestinal fluids (SIF; without enzymes) at pH 6.80 as measured by concentrations present in the supernatant by the FC assay and HPLC-PDA (data not shown). These findings indicated that phenolic-CH interactions remained relatively strong, despite a pH change from pH 1.40 (SGF) versus pH 6.80 (SIF). Zhang and Kosaraju (2007) using an enzyme-free SGF and SIF reported catechin release from the CH:TPP nanoparticles range between 5 and 15% in SGF, and 9 and 25% in SIF depending on the mass ratio (2.5:1.0 – 10.0:1.0 CH:TPP) and level of catechin-CH interactions.

Release of rutin and fruit phenolics from the CH:TPP nanoparticles was also studied within SIF containing enzymes (ESIF) (pancreatin) over time. In contrast to the enzyme-free SIF, this fluid contained a mixture of amylases, lipases, nucleases and

proteases; dehydrated bile and was higher in pH (7.70) (Barrett, 2006). Rutin loaded particles showed an initial burst release over the first 30 min, and then showed no further release for the duration of the experiment (Figure 4.12). Maximum release of rutin was observed at ~20%, indicating that ~80% of the rutin was still trapped within the nanoparticles. In the case of nanoparticles loaded with fruit phenolics, an initial burst of release was evident within the first 30 min, followed by a plateau similar to rutin (Figure 4.12). However, variability in the data was substantially higher with the fruit phenolics than with rutin, possibly due to the heterogeneous nature of the isolates. Their release observed in ESIF in the presence of enzymes but not in the absence is thought to be related to the higher pH (pH 7.70 vs. 6.80) and the presence of bile salts, both of which would alter hydrogen bonding between the phenolic and CH, and electrostatic bonding between CH and TPP. Lin et al. (2008) reported CH:TPP particles to become unstable and begin to break down at pH >7.2 due to the deprotonation of the amino group of CH. In the current study, it was presumed that the presence of enzymes was only a minor reason for release due to the inability to break CH:TPP interactions or covalent bonding between CH and the phenolics. McConnell et al. (2008) reported CH films prepared without TPP were completely degraded when incubated (37°C) with pancreatin enzymes, whereas in their presence, only partial degradation occurred to a degree and it depended upon the level of TPP added. The digestion of chitosan by porcine pancreatic enzyme was attributed to either the lipase present or to enzyme contaminants in the preparation (McConnell et al., 2008).

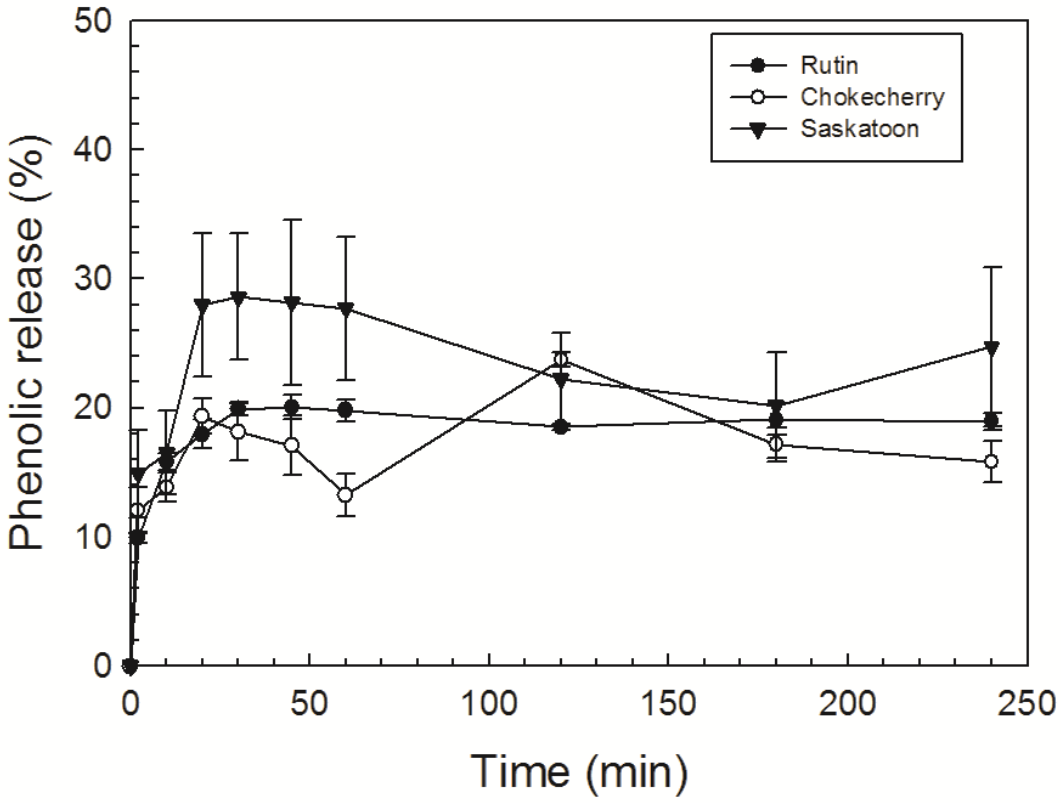


Figure 4.12 Phenolic release percentage from CH:TPP nanoparticles (4.0:1.0 mass ratio) within enzymatic simulated intestinal, as a function of time (min) as measured by reverse phase HPLC-PDA. Data represent the mean ± one standard deviation (n= 3).

Findings from this study were slightly lower at ~20% than others found in the literature, however percent release is related to both material characteristics (e.g., CH molecular weight/molar ratio, TPP levels and bioactive core material), along with the *in vitro* conditions (e.g., pH, duration, enzymes, and salts). Zhang et al. (2002) found that CH:TPP particles released only 20-40% of their entrapped material (bovine serum albumin) after 20 h in SIF with enzymes. Hu et al. (2008) reported ~45-55 % release of entrapped catechins from the CH:TPP nanoparticles depending on the mass ratio after 12 h of emersion in distilled water at 37^{o}C.

4.2.4 Particle choice for *in vivo* delivery studies

Although the total anthocyanin concentration and antioxidant activity (sections 4.1.4, 4.1.5.1 and 4.1.5.2) for chokecherries were shown to be higher than those found in saskatoons, the higher entrapment efficiency and percent phenolic release were the deciding factors in choosing the saskatoon isolate material for the animal feeding trial (section 4.3) studies.

4.3 Study 3- Oral (gavage) administration of an ethanol-Amberlite produced saskatoon isolate in an animal (rat) feeding trial.

As presented in the materials and methods section, the animal feeding trial consisted of 18 laboratory animals (male Wistar rats), which were assigned to three separate groups (control, saskatoon SPE extract, and CH:TPP nanoparticles + saskatoon SPE extract) consisting of six animals per group. The animal trial consisted of delivery: (a) the control group 3 mL of 0.1% citric acid; (b) the second group 276.4 ± 9.7 mg/kg body weight (bw) of the saskatoon extract which contained 12.4 ± 0.4 mg/kg bw of anthocyanins (~3.3 mg anthocyanin per rat) in 3 mL of 0.1% citric acid; and (c) the same amount of saskatoon extract/anthocyanins encapsulated in a CH:TPP matrix in 3 mL of 0.1% citric acid. Following animal feeding, the animals were deprived of food and water and were sacrificed after one hour. The average weight of the rats at the time of sacrifice was 238.7 ± 12.3 g.

4.3.1 Detection of anthocyanins in plasma

Plasma samples from the twelve saskatoon extract treated animals were collected and analyzed by HPLC-PDA (section 3.9.1.1) and showed no detectable levels of anthocyanins following the 1 h oral delivery period (data not shown). These results indicate that either the anthocyanin levels in the plasma were below the employed detection limits (0.25 mg/L; based on cyanidin-3-glucoside) or no anthocyanins were taken up into the blood stream after 1 h. These results could be due to the timing of the experiment (i.e. 1 h after administration) which may have resulted in the orally administered anthocyanins not being taken up into the blood stream, being taken up in the

blood stream earlier, being broken down or conjugated (i.e. metabolite production), and/or taken up by other organs.

In the literature, it has been shown that the highest levels of orally administered anthocyanins appear in the plasma 15-30 min after administration (Matsumoto et al., 2001; Ichiyanagi et al., 2006; Matsumoto et al., 2006; Hassimotto et al., 2008; Sakakibara et al., 2009; Walton et al., 2009). A number of studies have detected anthocyanins in plasma 1 h following oral administration, however these studies employed significantly higher concentrations (100-835 mg anthocyanins/kg bw) of these compounds than were used in this study (Matsumoto et al., 2001; El Mohsen et al., 2006; Ichiyanagi et al., 2006; Borges et al., 2007; Hassimotto et al., 2008; Sakakibara et al., 2009; Walton et al., 2009).

It has been shown that anthocyanin plasma levels in rats are dependent on several factors including their uptake from the gastrointestinal tract, tissue distribution and excretion in urine and bile (Ichiyanagi et al., 2006).

4.3.2 Detection of anthocyanins in tissues

Stomach tissues collected at the time of sacrifice from the extract feed group (b) animals were dark pink in colour compared to that of the control group (a), which were clear to yellowish in colour. These results appeared to indicate that a portion of the saskatoon extract, which was dark red in colour, was present in this organ and had not passed in its entirety into the small intestine. This colour was also observed in the upper portion of the small intestine but was absent in the large intestine. A similar result was observed for the encapsulated extract fed group (c) animals, however the dark pink colour was less intense.

Analytical (HPLC-PDA) results showed no detectable anthocyanins in the stomach tissue of the control group, however in the saskatoon extract treated (free and encapsulated) groups eleven of the twelve animals showed the presence of anthocyanins (Figure 4.13). A slight shift in the RRTs (<1 min) of these compounds was noted and was most likely due to the chromatographic influence of stomach tissue sample matrix. This relatively small shift in anthocyanin RRTs was not believed to be due to metabolite production. Differences in the presence of specific anthocyanins and their concentrations

were noted between the two extract treated groups. The nanoparticle treated group showed lower overall anthocyanin stomach tissue concentrations and only two of the six samples contained all four anthocyanins (data not shown). These chromatographic results did not show the presence (detection limit of 0.25 mg/L based on cyanidin-3-glucoside) of cyanidin (i.e. the aglycone), which has an RRT >60 minutes (Figure 4.14). The absence of cyanidin in the stomach tissue in extract treated animals shows that the saskatoon anthocyanins were not de-glycosylated during the initial stages of digestion. Also, nanoparticle gel like material (section 3.13) were visible in the stomachs of all group (c) treated animals.

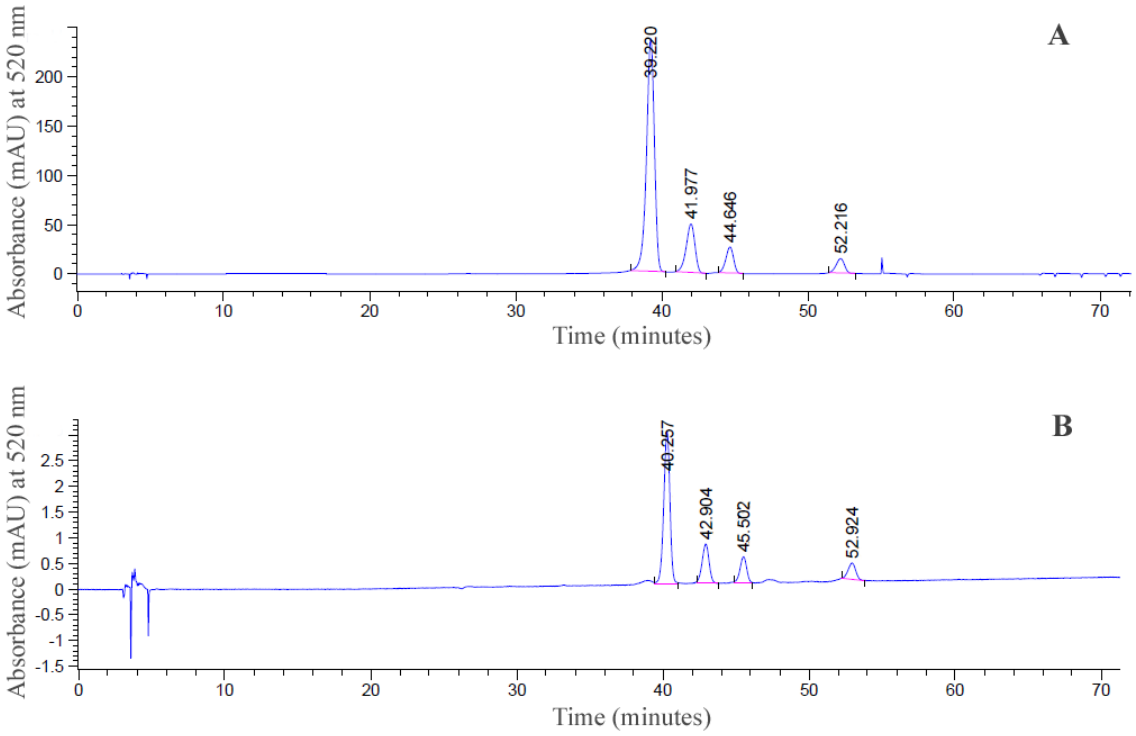


Figure 4.13 Reverse phase HPLC-PDA anthocyanin chromatogram (520 nm) profile of the saskatoon isolate used in the feeding trials (A) and stomach tissue extract from a rat fed the same concentration of saskatoon isolate (B).

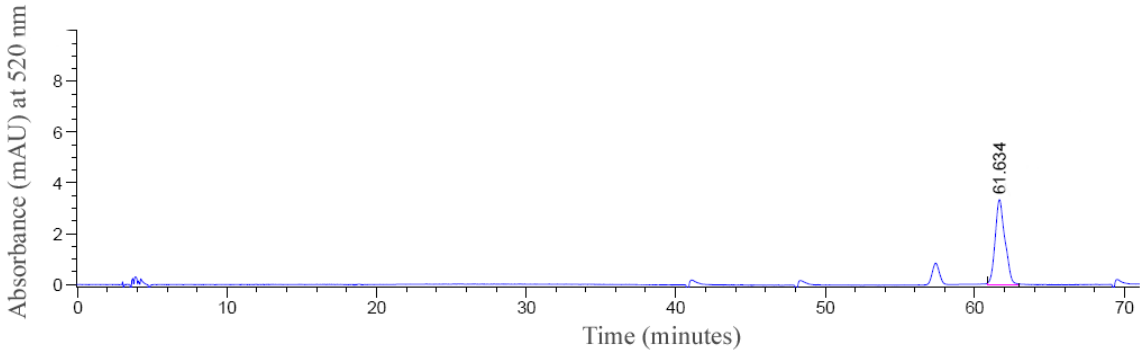


Figure 4.14 Reverse phase HPLC-PDA chromatogram (520 nm) of the aglycone (anthocyanidin) cyanidin standard.

Literature reports suggest that the stomach can be a potential site of absorption of anthocyanins from the diet as they can permeate the gastric wall (Passamonti et al., 2003; Talavera et al., 2003). Unlike the plasma results, the gavage delivered anthocyanins were present in rat stomach tissue up to 180 min after treatment (Walton et al., 2009). Published studies have also shown that the structures of gastric absorbed anthocyanins were unchanged. In addition, black raspberry anthocyanins were shown to bind with the proteins present in stomach tissue, resulting in difficulties in the HPLC-PDA analysis of samples both qualitatively and quantitatively (He et al., 2009).

Analytical (HPLC-PDA) results showed no detectable anthocyanins in the small intestine tissue of the control group, however in the saskatoon extract treated (free and encapsulated) groups, nine of the twelve animals showed the presence of anthocyanins (Figure 4.15). Also, nanoparticle gel like material (section 3.13) was observed in the upper portion of the small intestine of all group (c) treated animals.

As reported previously, a slight (< 1 min) shift in anthocyanin RRTs was observed in these tissues, which can be explained by the influences of small intestine sample matrix. Differences in the presence of specific anthocyanins and their concentrations were noted between the two extract treated groups. In the nanoparticle treated group, three of the six samples showed detectable levels of anthocyanins, although only two of the tissue samples showed the presence of all four compounds. In the other samples, only one (RRT ~39 min) of the four anthocyanins was detected. As observed in stomach tissue, cyanidin was not detected in small intestine tissue samples from any of the animals in either of the saskatoon extract treated groups. A comparison

of the total HPLC-PDA peak areas for the extract versus the encapsulated extract fed intestine tissue samples where all four compounds were present, showed an 8.3x higher level in the former. As nanoparticles were present in the upper portion of the small intestine, it is most likely that full release of anthocyanins from the nanoparticles had not occurred before the time of sacrifice.

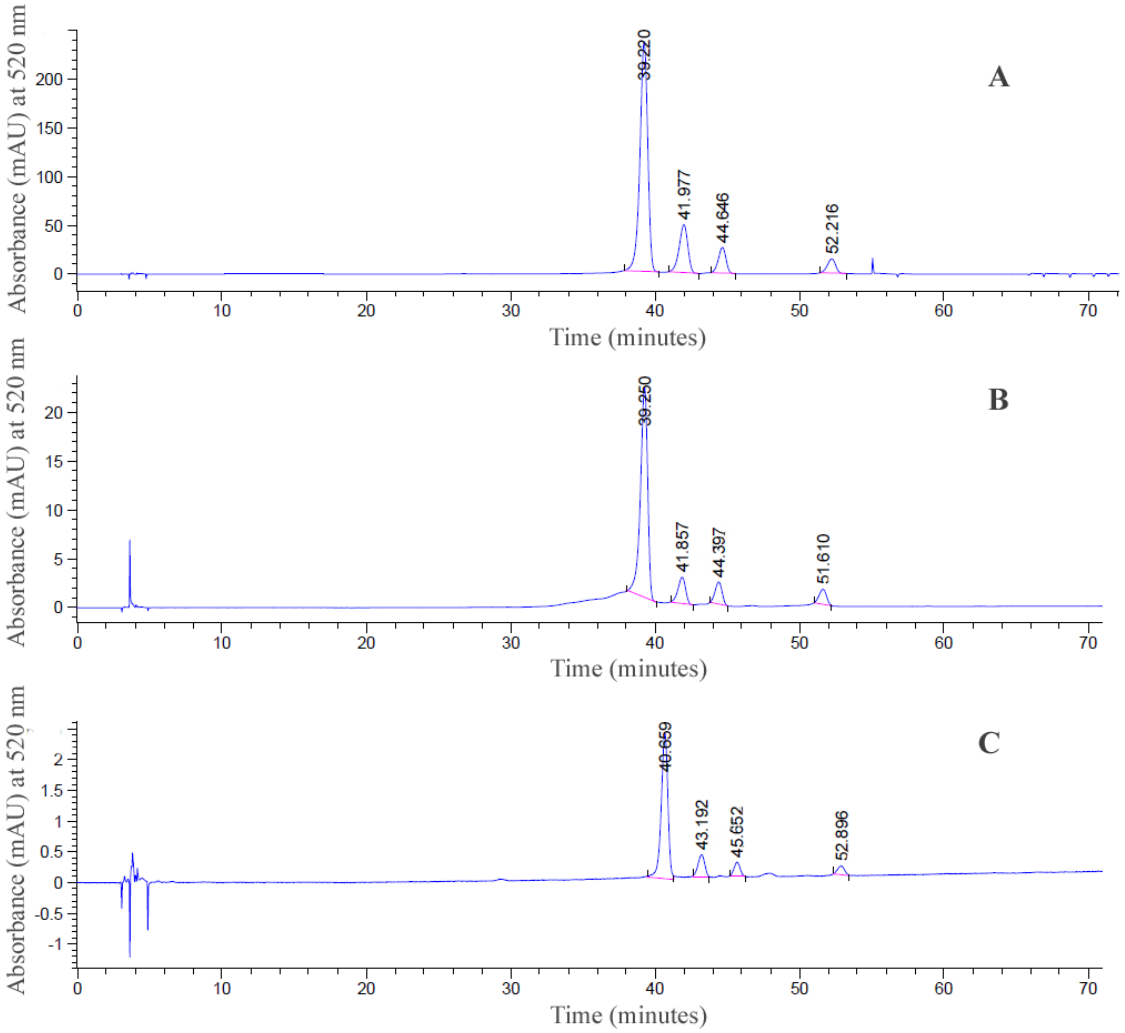


Figure 4.15 Reverse phase HPLC-PDA anthocyanin chromatograms of the saskatoon isolate (A), small intestine tissue extract from a rat fed saskatoon isolate (B) and small intestine tissue from rat fed the encapsulated isolate (C).

In literature, the small intestines have been reported as a key organ for the absorption of anthocyanins in both their original and aglycone forms (Talavera et al., 2005; Hassimotto et al., 2008). The highest anthocyanin levels were detected in the small intestine of rats between 1 and 2 h after oral administration (He et al., 2009; Walton et al., 2009). The uptake of anthocyanins in the gastrointestinal tract is mainly governed by the carbohydrate moiety attached to the anthocyanidin (Ichiyanagi et al., 2006). For example, it has been shown that the galactosides are more effectively absorbed than the arabinosides when attached to the same aglycones (Ichiyanagi et al., 2006).

Analytical (HPLC-PDA) results of large intestine tissues showed the presence of saskatoons anthocyanins in only one sample from the extract fed group, with only cyanidin-3-galactoside (RRT of ~40 min) present at detectable levels. Literature reports have supported the uptake of anthocyanins from the large intestine, however the dosages used in these studies (He et al., 2009; Walton et al., 2009) were significantly higher (150 mg-250 mg anthocyanins/kg of body weight) than that used in the present study. Walton et al. (2009) found that the highest levels of black currant anthocyanins were present in large intestine tissues 2h following oral administration, whereas those of mulberry were detected at 3 h (Hassimotto et al., 2008). Based on these literature results, a possible explanation for the lack of detectable anthocyanins in the large intestine tissues from the saskatoon extract (free and encapsulated) treated animals in this study is most likely due to the timing (i.e. 1 h following oral administration) of tissue harvesting.

Four other animal tissues were examined for the presence of saskatoon anthocyanins, which included the brain, heart, kidney and liver. Experimental results did not show detectable anthocyanin levels in any of the 12 saskatoon extract (free and encapsulated) treated animals. Because saskatoon anthocyanins were not detected in liver tissue samples, analysis of the mitochondria from this organ was not performed.

A major limitation of this study was the use of a single time point (i.e. 1 h after oral administration) for animal tissue harvesting. The fact that only one time point was used did not afford the study of anthocyanin absorption throughout the entire digestive tract of the target animals. However, the results obtained in this study are in agreement with those reported by Borges et al. (2007) who found that within one hour after

ingestion, 60% of the anthocyanins were found in the ileum and 32% remained in the stomach.

Talavera et al. (2005) detected anthocyanins in the brain, kidney, liver, small intestine, and stomach tissues of rats fed a daily (15 day study) diet of a blackberry phenolic extract (10.76 g anthocyanins/kg bw). A similar study found blackberry anthocyanins in adipose tissue, bladder, heart, prostate gland and testes (Felgines et al., 2009). These results suggest that the consumption of anthocyanins on a daily basis results in absorption/up-take not only by digestive organs but also to other tissues of the body.

As examples, an *in vivo* model using an everted sac showed that both cyanidin-3-glucoside and cyanidin-3-rutinoside passed through intestinal mucosa in their glycoside form and were not de-glycosylated (Hassimotto et al., 2008). The study of *in vitro* absorption of blackcurrant anthocyanins by human intestinal epithelial caco-2 cells showed that their transport across the apical membrane occurred to a greater extent than their translocation across the basolateral membrane into the plasma (Steinert et al., 2008). Although these studies showed that anthocyanins can be taken up in to tissues or cells and then transported through the basal membrane this may not equate to the same uptake in the tissues in the body.

5.0 RESULTS SUMMARY

The extraction of phenolics from plants and fruits has been extensively researched in literature, although the production of feed/food safe extracts has not. Replacing traditional extraction solvents (acetone, acetonitrile or methanol) with ethanol allows for the production of feed/food safe extracts. Although, fruit phenolic extracts have high *in vitro* antioxidant potential, little research has investigated their stability in food systems or our body's digestive system. Therefore ways to preserve their activity need to be implemented and encapsulation allows for this protection.

The major findings of this study on the production of phenolic extracts from chokecherries and saskatoons, their encapsulation and *in vivo* delivery of saskatoon isolate nanoparticles are as follows:

1. The mean TPC of chokecherry crude extract was 9.83 mg GAE/g fresh weight and 7.97 mg GAE/g fw for EFW and MFW respectively, while the mean TPC values for saskatoon crude extracts were lower at 4.21 mg GAE/g fw and 4.26 mg GAE/g fw for EFW and MFW respectively. These results showed that the TPC values for chokecherry crude extract were ~2x higher than for saskatoons. Differences in chokecherry TPC values were found for the extraction solvents, with EFW having ~1.2x higher TPC compared to MFW. Unlike chokecherries, saskatoons had similar TPC values with each of the solvents.

2. Based on HPLC-PDA results, chlorogenic acid was identified in chokecherries, while chlorogenic acid and rutin were identified in saskatoons. SPE using Amberlite XAD-16 was employed to produce phenolic isolates from both chokecherries and saskatoons. The SPE isolates had similar HPLC-PDA profiles as the crude EFW and MFW extracts. The HPLC-PDA results determined that there was a ~2.7x and ~1.6x increase in total major peak area for chokecherry and saskatoon isolates, respectively compared to the crude extracts. The increase in peak area illustrates that there is an increase in the concentration of phenolic material in the samples.

3. Anthocyanins were identified in both fruit sources with chokecherries having a concentration ~1.6x greater than saskatoons. HPLC-PDA results identified the two major anthocyanins in chokecherries as cyanidin-3-glucoside and cyanidin-3-rutinoside, while the four major anthocyanins were identified in saskatoons as cyanidin-3-arbinoside, cyanidin-3-galactoside, cyanidin-3-glucoside and cyanidin-3-xyloside. Chokecherry anthocyanin concentrations were similar with both extraction solvents, while saskatoons had a higher concentration (~1.2x) in the MFW extract compared to the EFW.

4. The antioxidant activity using *in vitro* radical scavenging tests showed that the chokecherry EFW extract and isolate had the highest overall free radical scavenging activity of the four samples. Dried crude fruit extracts exhibited lower overall free radical scavenging values compared to the all of the dried isolate samples using both of the *in vitro* radical scavenging tests.

5. The extraction of phenolics from chokecherries and saskatoons with EFW lead to extracts and isolates that had either comparable or greater concentrations of phenolic material compared to MFW extracts. Therefore, ethanol was determined to be a suitable solvent replacement for methanol.

6. Fruit phenolic isolates were successfully encapsulated in CH:TPP nanoparticles at a ratio of 4.0:1.0 (CH:TPP). The entrapment efficiency of the phenolics was 15.9 ± 2.7% and 23.0 ± 7.1% for chokecherry and saskatoons, respectively. The size of the nanoparticles were 527.90 ± 74.57 nm and 443.03 ± 15.79 nm for chokecherry and saskatoons, respectively. Both of the nanoparticle systems had positive surface charges at 52.70 ± 2.93 mV and 54.43 ± 1.27 mV for chokecherries and saskatoons. Both a positive surface charge and size of ≤500 nm have been shown to be important characteristics for absorption and bioadhesion in the digestive tract.

7. *In vitro* release from CH:TPP nanoparticles in enzymatic simulated intestinal fluid resulted in ~23% and ~28% release of chokecherry and saskatoon phenolics, respectively.

8. The animal study showed that anthocyanins from saskatoon isolates were detected in the stomach, small intestine, large intestine tissues 1h after delivery. Encapsulated saskatoon isolate were also delivered and anthocyanins were detected in both the stomach and small intestine tissues at the 1h sacrifice time. Although other tissues were tested (brain, heart, kidney and liver) anthocyanins were not detected in either of the treatment groups 1h after delivery.

6.0 GENERAL CONCLUSION

In the initial study EFW was tested as a substitute to MFW for phenolic extraction from chokecherries and saskatoons so as to produce a safe material for the proposed animal studies. Results from the HPLC-PDA studies clearly showed that the EFW extracts were similar in phenolic compound extraction ability with respect to both profile and concentration. From these results it is clear that EFW can be effectively used as a phenolic extraction solvent for fruits so as to produce materials which are safe for human and animal consumption.

Solid phase extraction of crude chokecherry and saskatoon solutions employing Amberlite XAD-16 produced phenolic rich isolates as determined by HPLC-PDA. This process allowed for the removal of water soluble compounds present in the original crude fruit extract while also concentrating the phenolics.

The fruit isolates produced in the initial study were successfully encapsulated in CH:TPP capsules. This wall material allowed for the creation of small capsules that possessed a positive surface charge. The CH:TPP capsules remained stable in SGF solution over the 2 hour time period illustrating their strong structure. When the capsules were placed in SIF only a small portion of the phenolic isolates were released.

When CH:TPP capsules containing a saskatoon phenolic rich isolate were delivered *in vivo* to rats, anthocyanins were detected as indicated by HPLC-PDA results in both the stomach and small intestinal tissues of the animals. These results illustrate that *in vivo* release and targeted delivery of anthocyanins occurred with the developed CH:TPP capsules.

7.0 FUTURE STUDIES

This research has shown that a fruit isolates can successfully be produced using EFW and can be encapsulated into CH:TPP nanoparticles. Release from the particles was only 23-28% of the encapsulated material therefore changing the nanoparticle characteristics may increase its release. A decrease in the time used for curing (1h) nanoparticles may decrease the cross-linking densities and therefore result in higher release percentages. Changes in the current nanoparticle characteristics may also result in changes of percent entrapment, size or surface charge if there is lower cross-linking. An increase in initial concentration of the fruit phenolics may result in higher loading into the particles.

This study encapsulated fruit phenolic material in CH:TPP, although other potential wall materials such as plant protein could be investigated. The use of plant proteins instead of chitosan would allow for the enzymatic breakdown of protein nanoparticles by animal/human digestion enzymes, potentially leading to a greater percent release from the nanoparticles in the small intestine.

To further investigate the absorption of anthocyanins released from CH:TPP nanoparticles, animal studies incorporating longer time intervals between administration and collection of tissue could been performed. The increased number of sacrifice time points would allow for the investigation of nanoparticle release throughout the entire digestive system and not only the upper portion as done in the current study. Increasing the concentration of anthocyanin material delivered in nanoparticles may also allow for better detection in the collected tissues because only low amounts (~3.3 mg) were delivered to each rat in the current investigation.

Lastly, in order to test the full ability of encapsulated phenolics and more specifically anthocyanins a animal feeding study should be employed. Mixing either the nanoparticles containing fruit isolates or un-encapsulated isolate material directly into the feed would demonstrate if the CH:TPP nanoparticles are able to protect the anthocyanins through feed production, consumption and digestion. This type of animal feeding study would provide scientific results to help understand how specific compounds interact with food and how they are transported and absorbed.

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