PHYSICOCHEMICAL PROPERTIES AND PHENOLIC COMPOSITION OF SELECTED SASKATCHEWAN FRUITS: BUFFALOBERRY, CHOKECHERRY AND SEA BUCKTHORN

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By

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ABSTRACT

There is increasing interest in the commericalization of native fruits for utilization as foods and medicinal extracts. This study was undertaken to determine the physicochemical properties and phenolic composition of selected Saskatchewan native fruits, including buffaloberry (*Shepherdia argentea* Nutt.), chokecherry (*Prunus virginiana* L.) and sea buckthorn (*Hippophae rhamnoides* L.). The physicochemical analyses included carbohydrate content, CIELAB colour values, organic acid composition, phenolic content, % seed, soluble solids, pH, total solids, total titratable acidity and proximate composition. Fruit samples were collected and analyzed over four crop years. The proanthocyanidin content was also determined photometrically after acid depolymerization in acid-butanol. Buffaloberry contained a proanthocyanidin concentration of 505 ± 32 mg/100 g fresh fruit and this level was 10 fold higher than that of chokecherry and sea buckthorn. Chokecherry was found to contain an anthocyanin concentration of 255 ± 35 mg/100 g fresh fruit, as determined using the pH differential method.

Two high performance liquid chromatography (HPLC) methods were developed for simultaneous determination of seven phenolic classes, including anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavanones, flavones and flavonols in aqueous methanol extracts. Based on the semi-quantitative analysis of the total phenolic chromatographic index (TPCI), chokecherry contained the highest levels of phenolic compounds with a concentration of 3,327 ± 469 µg/g fresh fruit followed by buffaloberry (578 ± 73 µg/g fresh fruit) and sea buckthorn (477 ± µg/g fresh fruit).

The antioxdant activity of the fruit extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2´-azinobis-3-ethylbenzthiazoline-sulphonic acid (ABTS) radical scavenging assays. Buffaloberry and chokecherry produced the highest radical scavenging activity and were at least five fold greater than that of sea buckthorn. The major radical scavenging compounds in buffaloberry were ascorbic acid and proanthocyanidins. Radical scavenging activity of chokecherry fruit was largely attributable to its anthocyanins, flavonols and hydroxycinnamic acids. Prominent antioxidants in sea buckthorn included ascorbic acid, proanthocyanidins and flavonols.

Certain individual compounds in the phenolic extracts were identified by HPLC-photodiode array and HPLC-mass spectrometry. Rutin was found in all of the extracts. Other phenolic compounds identified included catechin in sea buckthorn, and chlorogenic acid and quercetin in chokecherry. The chokecherry fruit pigments were comprised of two major anthocyanins and these were identified as cyanidin 3-glucoside and cyanidin 3-rutinoside. A preparative scale purification method for these anthocyanins using centrifugal partition chromatography (CPC) was determined. Under the CPC conditions employed, cyanidin 3-glucoside and cyanidin 3-rutinoside were purified to concentrations of 84 and 90%, respectively.

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LIST OF ABBREVIATIONS AND SYMBOLS IN THE TEXT

Å	angstroms
A_{512nm}	absorbance at 512 nanometers
A_{700nm}	absorbance at 700 nanometers
A_{765nm}	absorbance at 765 nanometers
AA	ascorbic acid
AAPH	2,2′-azobis-(2-amidinopropane) dihydrochloride
ABTS	2,2′-azinobis-3-ethylbenzthiazoline-sulphonic acid
$ABTS^{\bullet+}$	2,2′-azinobis-3-ethylbenzthiazoline-sulphonic acid radical cation
ACC	1-aminocyclo-propane-1-carboxylic acid
ACY1	chokecherry anthocyanin peak that occurred at retention time of approximately 37 minutes on the HPLC chromatogram.
ACY2	chokecherry anthocyanin peak that occurred at retention time of approximately 41 minutes on the HPLC chromatogram.
AH	antioxidant in the non-radical (reduced) state
ANOVA	analysis of variance
ArO•	aromatic hydroxyl (phenoxy) radical
ArOH	aromatic hydroxyl
ATP	adenosine triphosphate
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
ca	approximately
CCC	counter-current chromatography
cm	centimetres
CoA	coenzyme A

$CoAS^-$	Coenzyme A thiol anion
CPC	centrifugal partition chromatography
Cy 3-glu	cyanidin 3-glucoside
Cy 3-rut	cyanidin 3-rutinoside
Da	Daltons
DAD	diode array detector
ddH_2O	deionized, distilled water
DMPD	N,N-dimethyl-p-phenylene-diamine
$DMPD^{\bullet+}$	N,N-dimethyl-p-phenylene-diamine radical cation
DNA	deoxyribo-nucleic acid
DP	degree of polymerization
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH•	2,2-diphenyl-1-picrylhydrazyl radical
DTT	1,2-dithio-DL-threitol
EDTA	ethylenediaminetetraacetic acid
EI	electron ionization
eq.	equation
ESI	electro-spray ionization
FAB	fast atom bombardment
F-C	Folin-Ciocealteu reagent
g	force of gravity (9.8 m/s^2)
g	grams
GC	gas chromatograph or gas chromatography
h	hours
HPLC	high performance liquid chromatograph or high performance liquid chromatography

HX-OX	hypoxanthine-xanthine oxidase superoxide generating system
IC_{50}	concentration for 50% inhibition
IUPAC	International Union of Pure and Applied Chemistry
kg	kilograms
L	litres
LDL	low density lipoprotein
m/z	mass to charge ratio
M	molar
MALDI	matrix-assisted laser desorption ionization
mAU	milli-absorbance units
MFW	methanol/formic acid/water (70/2/28, volume basis)
mg	milligrams
mL	millilitres
mm	millimetres
mM	millimolar
MS	mass spectrometry
MW	gram molecular weight
N	normality
NAD	nicotine adenine dinucleotide
NADPH	nicotine adenine dinucleotide phosphate
NBT	nitro-blue tetrazolium
nm	nanometers
No.	number
$O_2^{-\bullet}$	superoxide anion
$^1O_2$	singlet state oxygen

ODS	octyldecylsilyl
ORAC	oxygen radical antioxidant capacity
P	partition coefficient
PC	paper chromatography
PFRA	Prairie Farm Rehabilitation Administration
PG	propyl gallate
psi	pounds per square inch
r	correlation coefficient
RI	refractive index
RO•	alkoxy radical
ROO•	peroxyl radical
ROOH	alkyl peroxide
ROS	reactive oxygen species
RP	reverse phase
RRT	relative retention time, defined as the retention time of a chromatographic peak compared to a standard peak chromatographed under the same conditions.
s	seconds
SD	standard deviation
sh	peak shoulder on the spectral profile of the phenolic compound
TBA	thiobarbituric acid
TBHQ	tert-butyl hydroxyquinone
TBME	tert-butyl methyl ether
TDF	total dietary fibre
TEAC	trolox equivalent antioxidant capacity
TFA	trifluoroacetic acid

TLC	thin layer chromatography
TPCI	total phenolic chromatographic index, defined as the sum of all the phenolic classes calculated from the chromatogram.
TPI	total phenolic index
t_R	absolute retention time
TTA	total titratable acidity
UDP	uridine diphosphate
USDA	United States Department of Agriculture
UV	ultraviolet
V	volts
v	volume
XAD	Amberlite XAD-16 resin
°C	degrees Celcius
ΔA	difference in absorbance values
ϵ	molar absorbance coefficient
λ_{max}	maximum wavelength
$[M]^+$	molecular cation
$[M-H]^-$	parent molecule less one hydrogen proton
$[M-2H]^-$	parent molecule less two hydrogen protons
#	number
µg	micrograms
µL	microlitres
µM	micromolar
µRIU	micro-refractive index units
µs	microseconds
%	percent

~	approximately
@	at

1.0 INTRODUCTION

Saskatchewan is host to a wide variety of native fruits that are attracting considerable interest for commercial production. Currently there are an estimated 550 fruit growers and 1,800 acres of planted fruit crops in Saskatchewan (Anonymous, 2005). Chokecherry (*Prunus virginiana* L.) and sea buckthorn (*Hippophae rhamnoides* L.*)* are among the four major native fruit crops in the Province while buffaloberry (*Shepherdia agentea* Nutt.) is of recent interest to the fruit processing industry (Anonymous, 2005).

Chokecherry is a shrub that grows throughout the Canadian Prairies and much of the United States (Vilkitis, 1974; Looman and Best, 1979). The shrub is oval rounded to narrow in shape and ranges in height from two to ten metres, depending on the climatic and soil conditions (Vilkitis, 1974; Looman and Best, 1979; St. Pierre, 1993). The fruit are spherical, six to eight millimeters in diameter, dark red to purple in colour and contain a single seed or stone (Looman and Best, 1979; St. Pierre, 1993). Chokecherry has a history of use in soups, stews and pemmican by native North Americans and was utilized as a food and medicinal source by European settlers in North America (St. Pierre, 1993). Although the chokecherry fruit is notably astringent, it is currently valued as a base for jams, jellies, syrups and fruit juice blends (Mazza, 1979; Hetherington and Steck, 1997; Anonymous, 2004). Chokecherry orchards are in production in Saskatchewan, however most of the fruit is collected from wild populations (Anonymous, 2004). Very little chemical composition data has been published in peer-review literature on chokecherry fruit and leaves.

Sea buckthorn is a hardy deciduous shrub currently being domesticated in several parts of the world, including the Canadian Prairies. The bush is described as a spiny shrub or tree with a willow-like appearance reaching two to four metres in height (Bailey and Bailey, 1976). The branches are brown or black with narrow, silver-gray leaves. Sea buckthorn is a drought and cold tolerant bush useful for shelterbelts, enhancement of wildlife habitat and land reclamation (Li and Schroeder, 1996). It originated in Eastern Europe and Asia and has been cultivated in China, Finland, Germany, Mongolia, Russia and other areas of Europe (Li and Schroeder, 1999). The fruit of sea buckthorn is yellow to orange in colour, spherical in shape and ranges from three to eight millimeters in diameter (Harrison and Beveridge, 2002). The fruit, including the seed is highly valued for its nutritional content, containing large quantities of essential fatty acids, vitamin C and flavonoids (Li and Schroeder, 1996). The vitamin C concentration in sea buckthorn fruit varies depending on the species, geographical location and physiological maturity (Bernath and Foldesi, 1992), and has been reported to range from 28 to 2,500 mg/100 g juice (Tang and Tigerstedt, 2001). Sea buckthorn is reputed to have considerable medicinal value as an anti-inflammatory, anti-microbial and anti-cancer agent, and in providing protective action against cardiovascular disease (Li and Tan, 1993; Li and Schroeder, 1996; Eccleston et al., 2002). These medicinal effects are largely attributed to antioxidant compounds in the oil, including β-carotene and tocopherols and in the juice of sea buckthorn, primarily vitamin C and flavonoids. The vitamin C and flavonoid composition of sea buckthorn grown in Saskatchewan has not been reported.

Buffaloberry is a shrub varying in height from one to five metres with silvery-green oval leaves on thorny twigs. The fruit are red in colour, approximately six to nine millimeters in diameter, slightly fleshy and contain a single seed. Buffaloberry is a common native plant of moist areas such as river valleys, around sloughs and in low meadows throughout the Canadian Prairies (Looman and Best, 1979; Anonymous, 2004). The nutritional composition of buffaloberry is not well known, however, these berries have been reported to contain high levels of vitamin C (Remlinger and St. Pierre, 1995). Buffaloberry was originally used in pemmican by native North Americans and presently it is processed into jellies and preserves by Saskatchewan fruit processors (Anonymous, 2005).

Phenolic compounds constitute a large group of secondary metabolites derived from phenylalanine and are widely distributed throughout the Plant Kingdom (Mann, 1987; Harborne, 1994). Although they typically comprise less than 2% of the fresh weight basis of the plant, phenolic compounds serve such diverse functions as imparting colour to leaves and fruits, attracting or repelling insects, antimicrobial action, antiviral activity, protection from harmful ultraviolet radiation and protection from herbivores (Harborne, 1967; Macheix et al., 1990; Harborne and Williams, 2000).

Chemically, phenolic compounds are defined as compounds possessing an aromatic ring bearing one or more hydroxyl groups, including their derivatives (Harborne, 1967; Macheix et al., 1990; Shahidi and Naczk, 1995). More than 8,000 phenolic compounds have been identified in plants (Wrolstad, 2005) and the major

phenolic compounds in fruits include the phenolic acids and flavonoids (Macheix et al., 1990; Robbins, 2003).

Phenolic acids in plants are predominantly substituted derivatives of hydroxybenzoic and hydroxycinnamic acids. These derivatives differ in patterns of hydroxylation and methoxylation of their aromatic rings (Harborne, 1994).

The flavonoids share a common base structure consisting of two phenolic rings connected via an oxygenated heterocyclic pyran ring (Harborne, 1967). They are divided into several groups differing in the oxidation state of the pyran ring and include five major classes: anthocyanins, flavanols, flavanones, flavones and flavonols.

In addition to general nutritional properties, berries and fruits are attracting particular interest for potential medicinal related benefits of fruit consumption and products produced from the fruit. Increasing epidemiological data suggest that a high intake of fruits offers a number of health benefits against degenerative diseases (Rissanen et al., 2003). These disease prevention properties are due in part to the phenolic content of the fruit (Harborne, 1994; Wollgast and Anklam, 2000). Numerous studies have suggested that the antioxidant activity, due to the phenolic composition of a food or natural health product, contributes to their protective effects against chronic and degenerative diseases (Heinonen et al., 1998; Record et al., 2001). Several specific plant phenolic compounds and fruit extracts have been reported to exhibit anti-inflammatory, anti-carcinogenic, vasodilatory and anti-microbial activities (Rice-Evans et al., 1996; Robards and Antolovich, 1997; Harborne and Williams, 2000; Wollgast and Anklam, 2000).

Therefore, in assessing the potential value of a fruit for human consumption it is pertinent to determine the phenolic composition and potential antioxidant activity of such compounds. The phenolic composition of a plant is generally unique to the plant species and can vary with its growth climate (Manach et al., 2004). It has also been shown that the antioxidant activity varies with the types of phenolic compounds present in the fruit and that certain types of phenolic compounds show greater antioxidant activity than others (Rice-Evans et al., 1995). It has also been found that some phenolic compounds act synergistically to increase overall antioxidant activity.

The hypothesis of the present study was as follows: the fruit and leaves of buffaloberry, chokecherry and sea buckthorn contain phenolic compounds that could add commercial value to these crops. Furthermore, the characterization of the chemical composition and phenolic profile of these Saskatchewan fruits would contribute to further development of natural health products and the native fruit industry in Saskatchewan.

The objectives of this research were as follows:

1. Characterize the chemical composition of buffaloberry, chokecherry and sea buckthorn fruit.
2. Identify the major phenolic classes present in the fruit and leaves of buffaloberry, chokecherry and sea buckthorn.
3. Determine the effect of harvest date on the chemical and phenolic composition of the fruit.

4. Determine the phenolic components/fractions in the fruit and leaves that exhibit antioxidant activity.
5. Investigate the use of centrifugal partition chromatography (CPC) for phenolic compound isolation and purification from chokecherry fruit.

2.0 LITERATURE REVIEW

2.1 Buffaloberry

Buffaloberry (*Shepherdia argentea*) is a shrub varying in height from one to five metres with thorny branches, silvery-green oval leaves and small red berries (Looman and Best, 1979; Anonymous, 2004). The buffaloberry is a member of the Oleaster Family (Elaeagnaceae) and is a common native plant of the Western and Central North American Great Plains (Remlinger and St. Pierre, 1995; Anonymous, 2004). It grows along riverbeds and sloughs as well as in moist areas of valleys, plains and meadows. Once established, the buffaloberry bush is considered winter hardy, saline tolerant and drought resistant (Remlinger and St. Pierre, 1995).

Buffaloberry was used by Native Americans and pioneers as an accompaniment to bison meat. The fruit was eaten raw or dried and also used to prepare pemmican, soup and juice (Remlinger and St. Pierre, 1995). In addition to its use as human food, buffaloberry bushes were also used as a source of food and shelter by the bison on the prairies. This traditional identification of bison with the plant accounts for its common name (Remlinger and St. Pierre, 1995).

Buffaloberry fruit are approximately six to nine millimeters in diameter, slightly fleshy and contain a single seed. In addition to the typical red-fruited shrubs, yellow-fruited buffaloberry plants are known to occur (Remlinger and St. Pierre, 1995). Fruit begin to ripen about 107 days from first flowering and are considered ripe when fully red or yellow (Remlinger and St. Pierre, 1995;

Anonymous, 2001). In Saskatchewan, the fruit are typically ripe in August and September, although they stay on the bush and can be harvested after frost (Looman and Best, 1979; Anonymous, 2004). Currently, in Saskatchewan, buffaloberry fruit is processed into jellies and preserves (Anonymous, 2005).

2.1.1 Chemical Composition

The nutritional composition of buffaloberry fruit was reported by Alberta Agriculture as cited by Remlinger and St. Pierre (1995). The fruit contain 75.1% moisture, 1.4% protein, 0.5% lipid, 0.51% ash and 4% dietary fibre. The total carbohydrate content of the fruit was calculated by difference from the proximate analyses and was reported to be 22.3% with glucose and fructose being the only carbohydrates identified at 6.4% and 3.7% fresh weight, respectively. The fruit is high in ascorbic acid content at a concentration of 150 mg/100 g fresh fruit (Remlinger and St. Pierre, 1995).

Buffaloberry fruit has been reported to contain 0.6% tannins (Hetherington and Steck, 1997). The fruit is also assumed to contain considerable levels of saponins due to the high foaming ability of buffaloberry juice, however, the actual saponin concentration of the fruit has not been determined (Looman and Best, 1979; Remlinger and St. Pierre, 1995).

Phenolic acids in the leaves of buffaloberry have been reported to include caffeic, chlorogenic, *p*-coumaric, ellagic, ferulic, gentisinic, *p*-hydroxybenzoic, protocatechuic (3,4-dihydroxybenzoic), sinapic and syringic (3,4,5-trihydroxybenzoic) acids (Bekker and Glushenkova, 2001).

Detailed information on other chemical parameters of buffaloberry fruit and leaf has not been reported in peer reviewed literature.

2.2 Sea buckthorn

Sea buckthorn (Hippophae *rhamnoides* L.) originated in Europe and Asia and has been domesticated in several countries including Britian, China, Finland, France, Germany, India, Nepal, Pakistan, Romania and Russia (Beveridge et al., 1999; Negi et al., 2005). It belongs to the Family Elaeagnaceae and thus, is related to buffaloberry. Consistent with other members of the Elaeagnaceae family, sea buckthorn is also a nitrogen fixer (Schroeder and Yao, 2003).

The sea buckthorn is a hardy deciduous shrub reaching two to four meters in height and develops a tree-like appearance since usually only the upper buds sprout and branch (Bernath and Foldesi, 1992). The leaves are narrow and a silver-brown colour. Sea buckthorn is drought and cold tolerant, useful for land reclamation and farmstead protection. Over one million sea buckthorn seedlings have been planted in the Canadian Prairies since 1982 (Schroeder and Yao, 2003). The shrub is also planted in British Columbia, Newfoundland and Quebec (Beveridge et al., 1999).

The mature fruit of sea buckthorn is usually yellow to orange-red in colour, spherical and contains a brown seed that is enveloped by a membranous seed sac (Harrison and Beveridge, 2002). The fruit are approximately eight millimeters long and seven millimeters in diameter and a natural sea buckthorn habitat can yield 750 to 1500 kg fruit/ha (Li and Schroeder, 1996). Sea buckthorn fruit is classified as nonclimacteric (Zhang et al., 1989b) and will remain on the branches throughout the

winter (Li and Schroeder, 1999).

Sea buckthorn fruit has attracted considerable attention, mainly for its medicinal value and great economic potential (Li and Schroeder, 1996). The fruit and leaves have been used for more than one thousand years in traditional medicines in China, Mongolia and Tibet (Xu et al., 1994). Fruit and seed extracts have been used for medicinal applications in Asia and Russia for the treatment of burns, cancer, cardiovascular disease, gastric ulcers and oral inflammation (Looman and Best, 1979; Mingyu, 1991; Jiang et al., 1993; Li and Tan, 1993; Che et al., 1998).

Sea buckthorn oil is approved for clinical use in hospitals in China and Russia, where, in 1977, it was formally listed in the Pharmacopoeia (Xu et al., 1994; Li and Schroeder, 1996). More than ten different drugs have been developed from sea buckthorn in these countries (Li and Schroeder, 1996).

Johansson and coworkers (2000) showed that sea buckthorn oil inhibited platelet aggregation in humans. A similar inhibitory effect to aspirin on platelet aggregation induced by collagen in mouse femoral artery was reported for a total flavone extract from sea buckthorn (Cheng et al., 2003). This ability to prevent *in vivo* thrombogenesis suggested that sea buckthorn fruit consumption may help prevent cardiac and cerebral thrombosis in humans.

Sea buckthorn has been shown to possess appreciable levels of antioxidant activity. Geetha et al. (2002) found that concentrated (500 μg/mL) alcoholic extracts of fruit and leaves of sea buckthorn could inhibit chromium-induced free radical apoptosis, DNA fragmentation and restored antioxidant status to that of control cells in a lymphocyte *in vitro* model system. They concluded that fruit and

leaf extracts of sea buckthorn have a cytoprotective effect against chromium-induced cytotoxicity as well as immunomodulating activity. Negi et al. (2005) investigated the antioxidant and antibacterial activities of various sea buckthorn seed alcohol extracts and found high levels of these activities. They postulated that these activities were attributed to the high phenolic content of these extracts.

Eccleston et al. (2002) reported that sea buckthorn juice was rich in antioxidants and moderately decreased the susceptibility of low density lipoprotein to oxidation.

2.2.1 Chemical Composition of Sea buckthorn

Sea buckthorn fruit is recognized for its nutritional benefits, being rich in amino acids, carbohydrates, organic acids, protein and vitamins (Bernath and Foldesi, 1992). A number of studies have shown that the chemical composition of the fruit varies greatly according to the species, and climatic and geological conditions of the growth areas of the plant (Yao and Tigerstedt, 1994; Kallio et al., 2000; Tang and Tigerstedt, 2001).

The moisture content of sea buckthorn fruit has been reported to range from 74 to 86.7% (Chen et al., 1991; Tang and Tigerstedt, 2001). Soluble solids content of the juice ranges from 9.3 to 22.7°Brix and glucose and fructose are the major carbohydrates in Chinese sea buckthorn (subspecies *sinensis*) and are present in approximately equal amounts (Tong et al., 1989; Zhang et al., 1989b). The soluble solids content of Canadian sea buckthorn has been reported to range from 8.4 to 10.7°Brix (Beveridge et al., 2002).

The protein content of the fruit varies with the variety and geographical location and is reported to vary from 0.79 to 3.11% on a fresh weight basis (Bekker and Glushenkova, 2001)

Malic acid was the major organic acid reported in Finnish sea buckthorn fruit (subspecies *rhamnoides*) with minor quantities of citric and tartaric acid (Kallio et al., 2000). Sea buckthorn grown in Canada, however, has been reported to contain quinic acid as the major organic acid while malic was next, and citric, oxalic and tartaric acids were minor components (Beveridge et al., 2002).

Sea buckthorn is reputed to be an excellent source of vitamin C, although a wide concentration range has been reported from 2 to 2500 mg/100 g juice (Bernath and Foldesi, 1992; Yao and Tigerstedt, 1994; Eccleston et al., 2002; Kallio et al., 2002; Sabir et al., 2005). Sea buckthorn also contains vitamin E at levels of 13 mg/L juice (Eccleston et al., 2002) and 160 mg/100 g fruit (Bernath and Foldesi, 1992).

Yang and Kallio (2001) determined the oil content of Chinese and Finnish sea buckthorn varieties. The whole berry oil content of Chinese sea buckthorn was 2.1 ± 0.5% with lipid levels of 7.3 ± 1.4% and 1.7 ± 0.5% of the seed and pulp/peel, respectively. The whole berry oil content of Finnish sea buckthorn was reported to be 3.5 ± 0.7% with an oil content of 11.3 ± 2.5% of the seed and 2.8 ± 0.8% of the pulp/peel.

The major fatty acid in sea buckthorn pulp/peel is palmitoleic acid (16:1 n-7) at a concentration of 12.1 to 39.0% and is 8.9 to 31.0% of that found in the whole fruit (Yang and Kallio, 2001). Linoleic (18:2n-6) and α-linolenic (18:3n-3) acid

comprise 70% of the seed oil fatty acids and palmitoleic acid is practically absent in this oil (Yang and Kallio, 2001).

The total sterol content in sea buckthorn oil was found (Yang et al., 2001b) to range from 1200 to 1800, 240 to 400 and 340 to 520 mg/kg in the seeds, fresh pulp/peel and whole berries, respectively. Sitosterol constituted 57 to 76 and 61 to 83% of the seed and pulp/peel sterols, respectively.

The carotenoid content of sea buckthorn fruit varies with the growing conditions but typically ranges from 30 to 40 mg/100 g fruit (Bernath and Foldesi, 1992). Eccleston et al. (2002) reported a total carotenoid content of 73 mg/100 mL for sea buckthorn juice with β-carotene accounting for 45% of this amount.

The total phenolic content of sea buckthorn fruit was reported to range from 114 to 244 mg/100 g fruit expressed as gallic acid equivalents (Gao et al., 2000). These authors reported a strong positive correlation between the antioxidant capacity of the fruit and its total phenolic and ascorbic acid contents.

Zadernowski et al. (2005) found that the phenolic acid composition in sea buckthorn berries ranged from 3570 ± 282 to 4439 ± 405 mg/kg on a dry weight basis. They tentatively identified 17 phenolic acids in the fruit with salicylic acid accounting for 55 to 74% of the total. The phenolic acids in the fruit were mainly in their esterified and glycosylated forms, whereas the maximum free phenolic acids content was 2.3%.

The flavonoid content in the leaves and fruit of seabuckthorn has been reported to range from 310 to 2100 mg/100 g dried leaf and 120 to 1000 mg/100 g fresh fruit, respectively (Glauzunova et al., 1984; Glauzunova et al., 1985; Chen et

al., 1991). Eccleston et al. (2002) reported a flavonoid content of 1182 mg/L sea buckthorn juice and identified isorhamnetin-rutinoside (355 mg/L), isorhamnetin-glycoside (142 mg/L), quercetin-rutinoside (35 mg/L) and quercetin-glycoside (35 mg/L) as the main flavonoids present. Hakkinen et al. (1999b) reported that quercetin was the main flavonoid in European sea buckthorn fruit.

2.3 Chokecherry

Chokecherry (*Prunus virginiana* L.) is a shrub that grows throughout Canada and much of the Western United States (Vilkitis, 1974; Looman and Best, 1979). The shrub is oval rounded to narrow in shape and ranges in height from two to ten metres, depending on the climatic and soil conditions (Vilkitis, 1974; Looman and Best, 1979; St. Pierre, 1993). Chokecherry is commonly found on rich, moist, but well-drained soils. It is intolerant of shade and therefore is usually found in the open along fence lines, roadsides and streambanks, on cleared land, and along the borders of wooded areas and ravines (St. Pierre, 1993).

Chokecherry leaves are broadly oval and pointed in shape with serrated edges. The fruit are spherical, six to eight millimeters in diameter and vary in colour from deep red to purple-black and contain a single seed or stone. The fruit mature from late June to late August, depending on the latitude of growth (Looman and Best, 1979; St. Pierre, 1993). Chokecherry fruit matures from late August to early September in Saskatchewan (Anonymous, 2004). Chokecherry is a true cherry and is a close relative of domesticated apricots, cherries, peaches and plums (USDA, 2004b).

Chokecherry originated in Eurasia from 4,000 to 5,000 B.C., was imported to England in 1629 and was first cultivated in North America as an orchard crop in 1724 (USDA, 2004b). The common name, chokecherry, came from the bitter and astringent taste of the fruit (USDA, 2004b).

Dried or cooked chokecherry was used in soups, stews and pemmican by native North Americans (St. Pierre, 1993; USDA, 2004b). Chokecherry was used for medicinal purposes by the native North Americans and European settlers. The fruit was boiled and eaten to treat internal bleeding of the digestive tract, post-partum hemorrhage, diarrhea and sore throats (USDA, 2004b). Infusions prepared from the leaves and bark of chokecherry were used to treat open sores, and skin ulcers, and a medicinal tea from the leaves and twigs was used to treat colds and rheumatism (USDA, 2004b).

Currently, the chokecherry is becoming more widely used as an ornamental in multiple-row shelterbelts for wildlife habitat improvement, and for reclamation and rehabilitation for slope stabilization, and erosion control (St. Pierre, 1993). The fruit is also valued as a base for jams, jellies, syrups, wines and fruit juice blends (Mazza, 1979; St. Pierre, 1993; Hetherington and Steck, 1997; Anonymous, 2004). There are a number of organizations in Saskatchewan that grow and process products containing chokecherry (Anonymous, 2004).

The chokecherry has not undergone breeding and selection for cultivated environments although a number of selections having large, good quality fruit have been chosen from the wild. It is these selections that are occasionally propagated and cultivated (St. Pierre, 1993).

2.3.1 Chemical Composition of Chokecherry

Chokecherry leaves and seeds are toxic due to the presence of prunasin and amygdalin, respectively, which occurs at significantly higher levels than in other prairie Prunus species. Prunasin and amygdalin can be catabolized by digestive enzymes in the stomach to yield hydrogen cyanide with the possible consequence of cyanide poisoning. Fresh leaves have been reported to contain 143 mg prunasin/100 g (St. Pierre, 1993). This concentration is 100 times greater than the level (1 mg prunasin/100 g fresh weight) suggested for a plant material to be classed as cyanogenic and potentially toxic (Davis, 1991). The highest levels of prunasin occur in the spring and summer and the leaves become non-toxic once the fruits mature in late summer. Drying or boiling the leaves and seeds will denature the prunasin and amygdalin resulting in edible materials.

Very little has been reported on the chemical composition of chokecherries although recently, some physicochemical properties of chokecherry fruit were reported (Zatylny et al., 2005a). Fruit of the chokecherry ranged in their ten-fruit weights from 6.6 to 9.2 grams, percent pit from 9.4 to 16.0%, estimated percent flesh from 17.2 to 23.7% and pH from 3.86 to 4.25. Mean total solids and soluble solids contents were 35.2% and 18.3°Brix, respectively (Zatylny et al., 2005a).

2.4 Types of Phenolic Compounds and Their Biosynthesis

Phenolic compounds can be defined as compounds possessing an aromatic ring bearing one or more hydroxy substituents (Harborne, 1967; Mann, 1987). Their chemical structure may range from quite simple compounds like phenol

(Figure 2.1) with a gram molecular weight of 94 g/mole, to highly polymerized compounds such as proanthocyanidins, which range in gram molecular weight from 500 to greater than 10,000 g/mole (Santos-Buelga and Scalbert, 2000). Phenolic compounds are also commonly referred to as polyphenolics, a term that originally meant "many phenolics" and probably referred to a compound having many phenolic groups (Wrolstad, 2005). The more recently accepted meaning of the term is the multiplicity of different phenolic compounds present in a plant extract (Wrolstad, 2005). The terms polyphenolics and polyphenols are used interchangeably, however, due to the past ambiguity of these terms, they are not employed to describe phenolic compounds in this thesis.

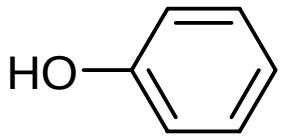


Figure 2.1 Phenol, the simplest phenolic compound.

In plants, phenolic compounds occur primarily in their mono-glycosylated form (Shahidi and Naczk, 1995; Manach et al., 2004). Glucose is the predominant glycosyl moiety, however, arabinose, galactose, rhamnose and xylose are also common (Klick and Herrmann, 1988; Bravo, 1998). Phenolic compounds may also be conjugated with aliphatic organic acids, amines, lipids, oligosaccharides or other substituents (Carotenuto et al., 1997; Bravo, 1998; Norbaek and Kondo, 1999; Lin et al., 2002).

In addition to conjugation, the aromatic ring(s) of phenolic compounds contain varying levels of hydroxylation and methoxylation. Variations in complexity of structure, conjugation, hydroxylation and methoxylation contribute to the wide range of naturally occurring phenolic molecules, and more than 8,000 phenolic compounds have been identified in plants (Harborne and Williams, 2000; Wrolstad, 2005).

Phenolic compounds can be divided into at least 13 different classes depending on their basic carbon skeleton structure (Shahidi and Naczk, 1995; Bravo, 1998; Manach et al., 2004) as shown in Table 2.1. Distinction between these classes is drawn first on the basis of the number of constitutive carbon atoms and then by the structure of the basic skeleton.

The metabolic pathways responsible for phenolic compound biosynthesis are the shikimate and the acetate pathways. Certain phenolic compounds are biosynthesized by each of these pathways alone, however the largest phenolic class, the flavonoids, are products of mixed biosynthetic origin whereby they incorporate within their structures the biogentic sub-units of both the shikimate and acetate pathways (Mann, 1987; Dewick, 2002). An overview of the key intermediates and pathways involved in producing phenolic metabolites is shown in Figure 2.2. Plant metabolism begins with photosynthesis whereby ultraviolet light energy from the sun is absorbed by chlorophyll to synthesize NADPH (nicotinamide adenine diphosphate) and ATP (adenosine triphosphate), which act as reducing and activating reagents, respectively in metabolic reactions. In the subsequent 'dark reaction' of photosynthesis, carbon dioxide is reduced to produce four-, five- six-

Table 2.1 Phenolic classes in plants. (Adapted from Bravo, 1998)

Phenolic Class	Carbon Skeleton
simple phenols	C_6
hydroxybenzoic acids	C_6-C_1
phenylacetic acids	C_6-C_2
hydroxycinnamic acids, coumarins	C_6-C_3
naphthoquinones	C_6-C_4
xanthones	C_6-C_1-C_6
stilbenes, anthraquinones	C_6-C_2-C_6
flavonoids	C_6-C_3-C_6
tannins	$(C_6$-$C_1)_n$, $(C_6$-C_3-$C_6)_n$
biflavonoids	$(C_6$-C_3-$C_6)_2$
lignans	$(C_6$-$C_3)_2$
lignins	$(C_6$-$C_3)_n$

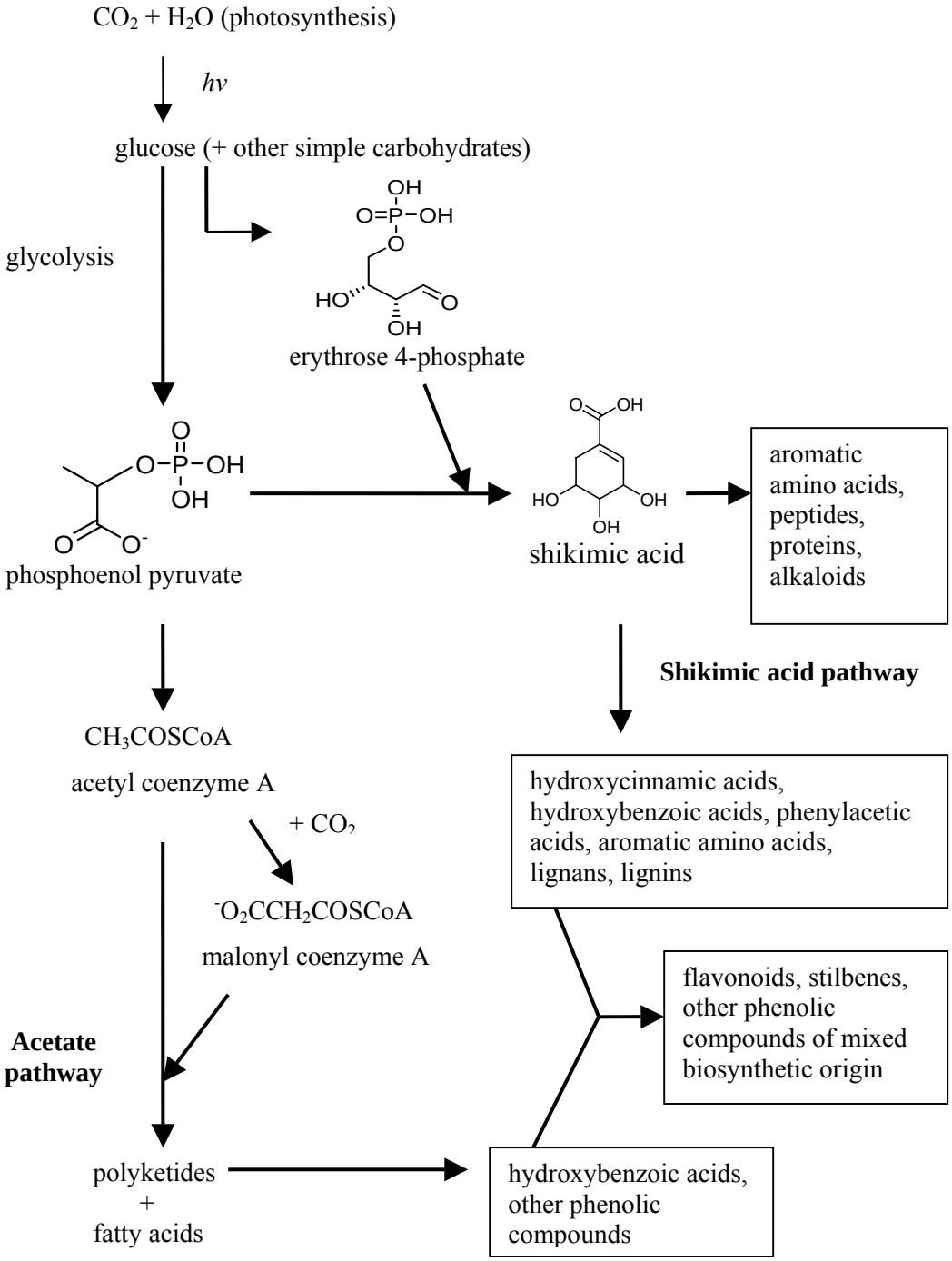


Figure 2.2 Overview of the biosynthesis of phenolic compounds. (Adapted from Mann, 1987)

and seven-carbon carbohydrates, including glucose.

The key precursor intermediates in the biosynthesis of phenolic compounds include shikimic acid, which is a central intermediate in the shikimate pathway, and acetyl coenzyme A (acetyl-CoA) which is the starting unit for the acetate pathway.

Coenzyme A is an important cofactor used by enzymes to activate substrates for subsequent reaction. This coenzyme is an acyl-transfer reagent and functions by forming acyl-thioester species upon reaction with acyl-substrates (Mann, 1987). The thioester group activates the acyl species for nucleophilic attack at the carbonyl carbon atom, resulting in displacement of the $CoAS^-$ and alkylation at the α-carbon atom (Mann, 1987; Dewick, 2002).

Details of the shikimate and acetate biosynthetic pathways have been described by others (Mann, 1987; Dewick, 2002). An overview of the shikimic acid pathway is provided in Figure 2.3. This biosynthetic pathway begins with a coupling of phosphoenol pyruvate and D-erythrose-4-phosphate via a series of transformations to produce shikimic acid and 3-dehydroshikimic acid. Typical hydroxylation patterns of phenolic compounds biosynthesized by the shikimic acid pathway include a single hydroxy para to the side-chain function, dihydroxy groups arranged ortho to each other, usually 3,4- to the side-chain, and trihydroxy groups also ortho to each other and 3,4,5- to the side-chain (Dewick, 2002). The single para-hydroxylation and the ortho- polyhydroxylation patterns contrast with the typical meta-hydroxylation patterns characteristic of phenolic compounds derived via the acetate pathway (Dewick, 2002).

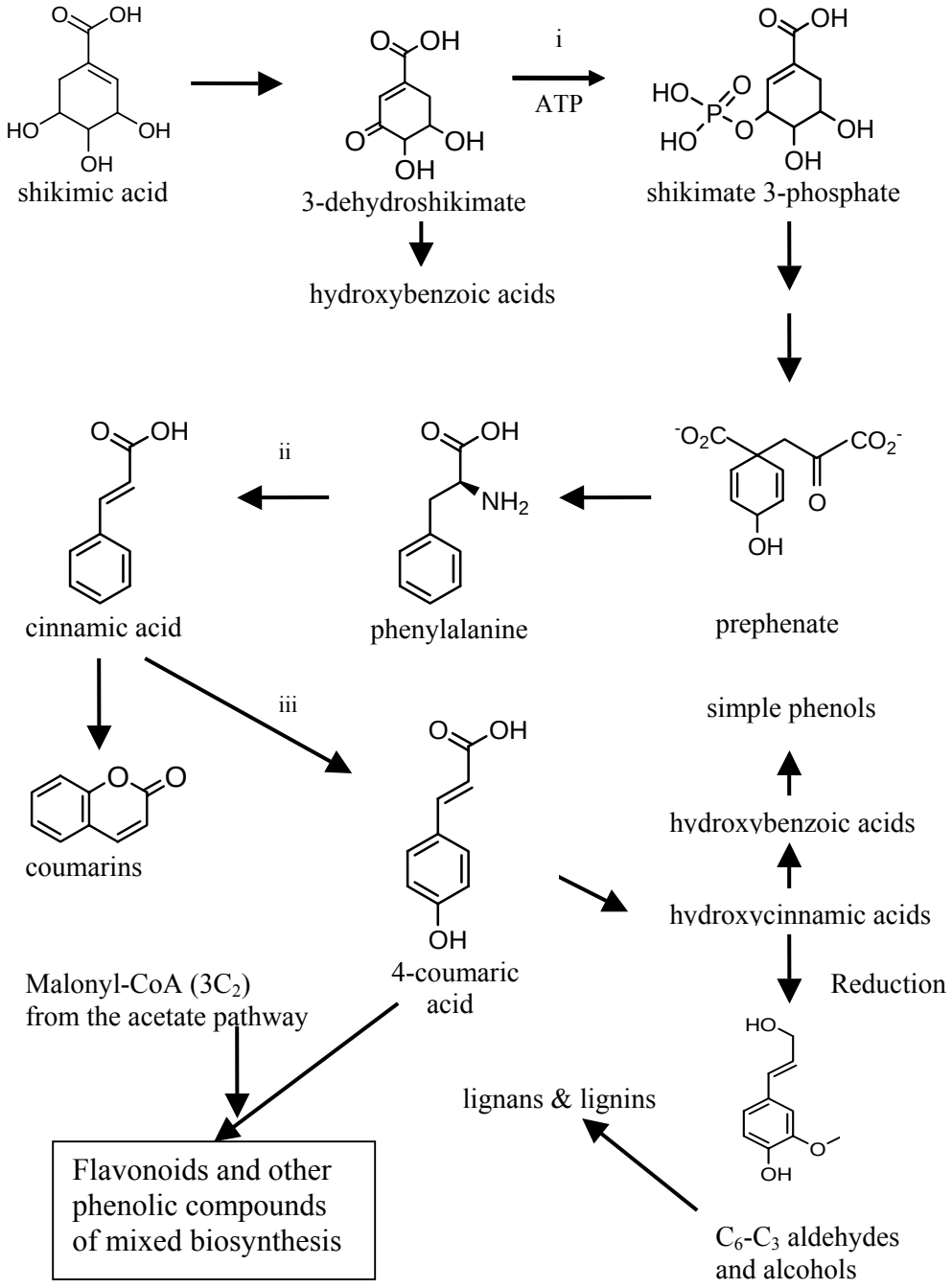


Figure 2.3 Formation of phenolic compounds from the shikimic acid pathway. Enzymes: i) shikimate kinase, ii) phenylalanine ammonia lyase (PAL), iii) cinnamic acid 4-hydroxylase. (Adapted from Mann, 1987 and Dewick, 1993)

Natural products originating via the acetate pathway fall into the biosynthetic class of compounds known as polyketides. Aromatic polyketides are natural products derived from poly-β-keto chains, formed by the coupling of acetate and malonate units via condensation reactions (Mann, 1987). The formation of 6-methylsalicylic acid, shown in Figure 2.4, found in many microorganisms and some higher plants demonstrates the biosythesis of a phenolic compound via the acetate pathway. The acetate and malonate units are assembled by polyketide synthase enzymes which utilize coenzyme A to facilitate the condensation of these sub-units (McDaniel et al., 1995; Dewick, 2002). The conversion of acetyl-CoA into malonyl-CoA increases the acidity of the α-hydrogens and thus provides a better nucleophile for the Claisen condensation reaction (Dewick, 2002). The condensation of acetyl-CoA with malonyl-CoA is shown in Figure 2.5. As the condensation reaction occurs, the carboxyl group of the malonyl-CoA is simultaneously lost by a decarboxylation reaction, thus, the polyketide chain is only extended by two carbon units (Mann, 1987). The coiling of the polyketide chain leads to an intra-molecular condensation to produce the phenolic ring (Dewick, 2002).

2.4.1 Simple Phenols

Simple phenols (C_6) are relatively rare in their natural distribution and are either absent or comprise only a small proportion of the phenolic content in plants (Cowan, 1999). Examples of these compounds include arbutin, catechol, hydroquinone,

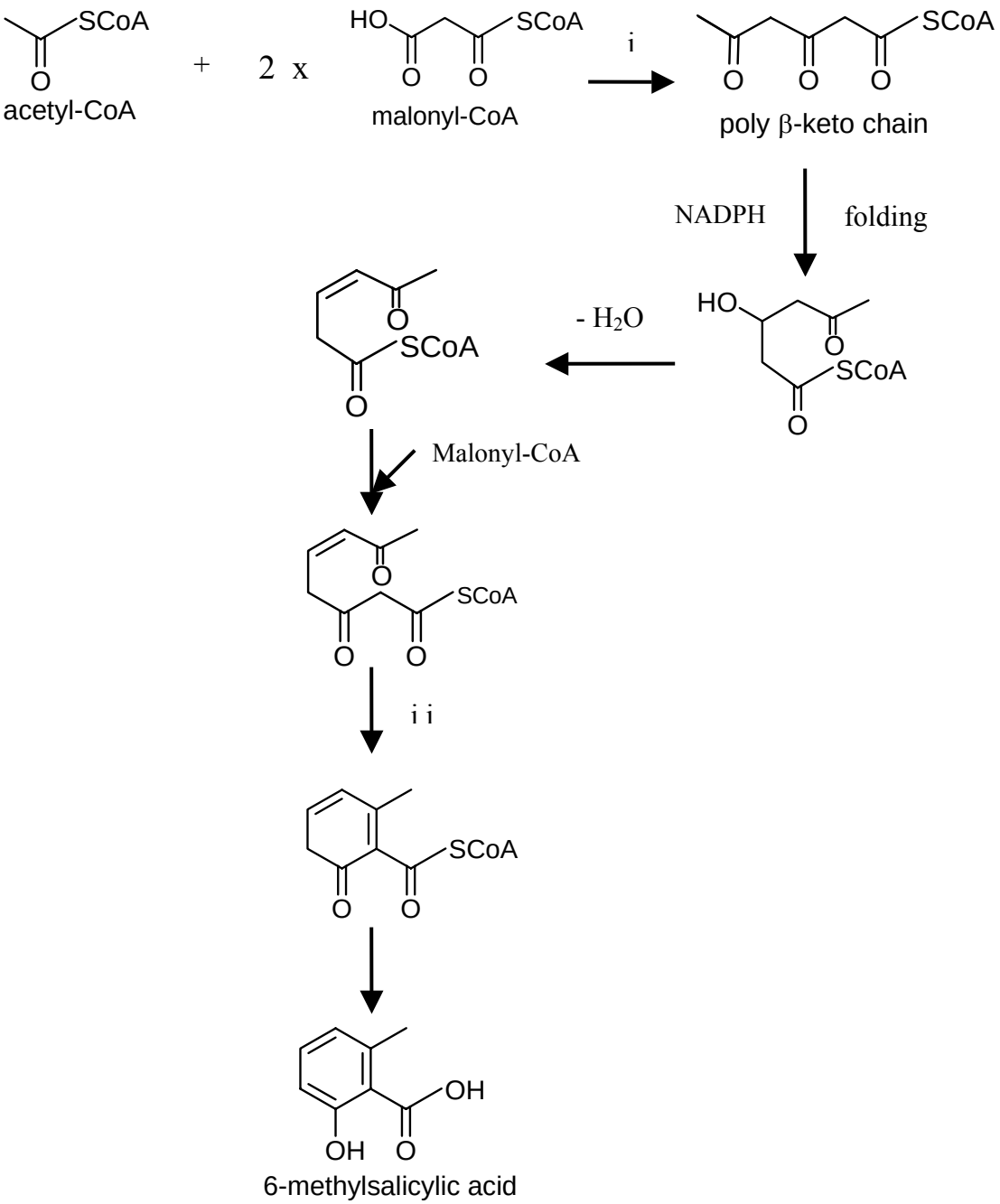


Figure 2.4 Biosynthesis (acetate pathway) of 6-methylsalicylic acid. Enzymes: i) polyketide synthase, ii) polyketide cyclase. (Adapted from Dewick, 1993)

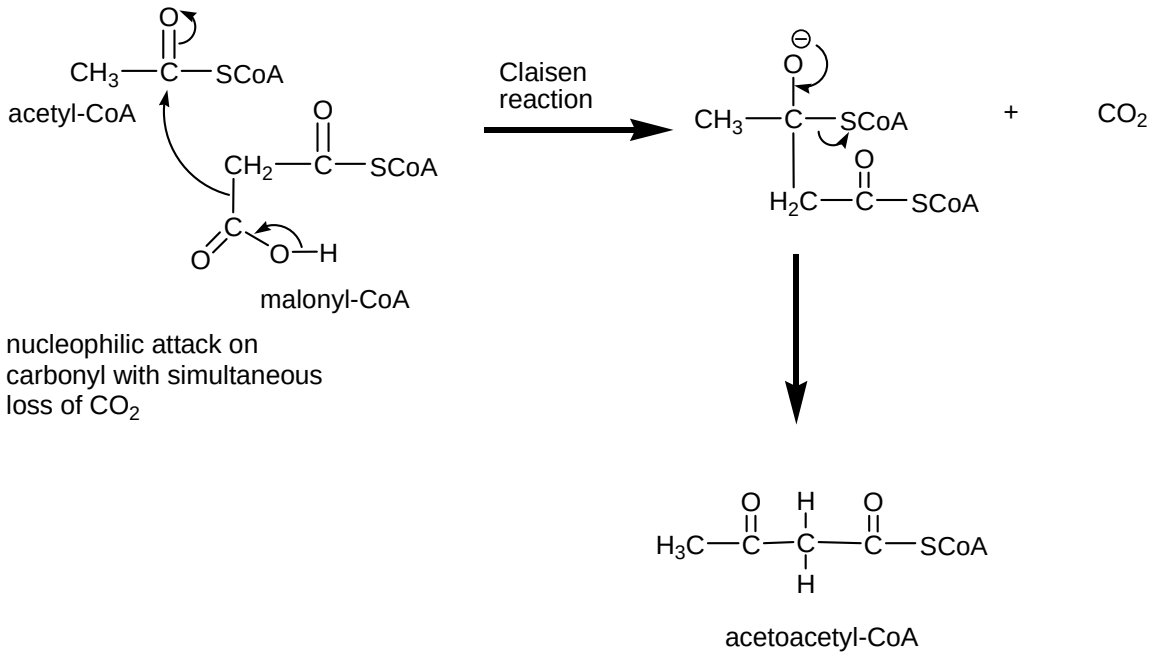


Figure 2.5 Claisen condensation mechanism in the acetate pathway. (Adapted from Mann, 1987)

phloroglucinol and resorcinol (Figure 2.6). Hydroquinone has been shown to be formed by decarboxylation and reduction of *p*-hydroxybenzoic acid, a product of the shikimic acid pathway (Mann, 1987). Arbutin is the β-D-glucoside of hydroquinone and is biosynthesized by substitution of one of the hydroxyls with D-glucose (Mann, 1987). Other simple phenols are reported to occur as a result of decarboxylation of phenolic acids or by thermal degradation of lignin during processing of the food of plant origin (Maga, 1978).

2.4.2 Hydroxybenzoic Acids

The hydroxybenzoic acids (C_6-C_1 structure) are phenolic compounds that possess one carboxylic acid functionality and along with the hydroxycinnamic acids

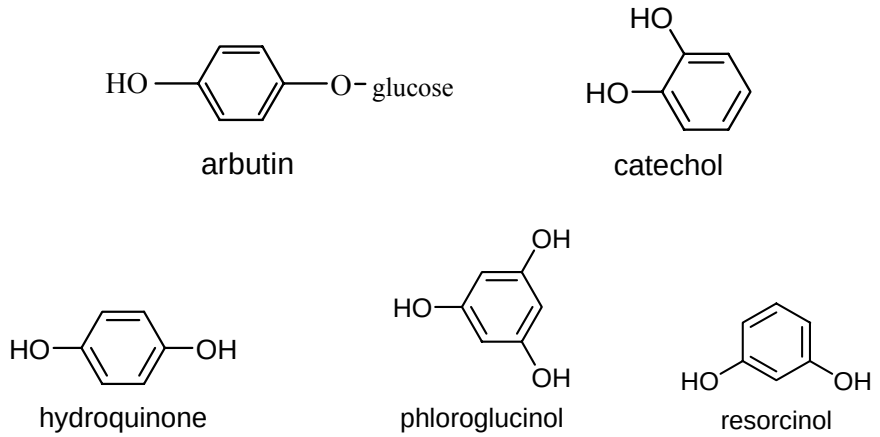
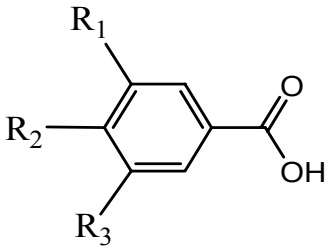


Figure 2.6 Some simple phenols.

(section 2.4.4), constitute the phenolic acids (Robbins, 2003; Shahidi and Naczk, 2004). Variations in the hydroxybenzoic acids occur due to differing patterns of hydroxylation and methoxylation of their aromatic rings (Figure 2.7). The majority occur as glycosides or esters linked with aliphatic organic acids such as maleic and tartaric acids (Shuster and Herrmann, 1985), or larger phenolic compounds such as flavonoids (Winter and Herrmann, 1986; Klick and Herrmann, 1988). Hydroxybenzoic acids such as gallic, *p*-hydroxybenzoic and vanillic acid are present in nearly all plants (Shahidi and Naczk, 1995; Robbins, 2003). In addition to free and conjugated forms with other compounds, gallic acid is the basic phenolic constituent of hydrolysable tannins (Section 2.4.9).

The most widely occurring hydroxybenzoic acids are biosynthesized via the shikimic acid pathway either from 3-dehydroshikimate or from degradation of the side chain of hydroxycinnamic acids (Figure 2.3). Hydroxybenzoic acids with di-



Acid	R_1	R_2	R_3
p-hydroxybenzoic	H	OH	H
3,4-hydroxybenzoic	H	OH	OH
vanillic	OCH_3	OH	H
syringic	OCH_3	OH	OCH_3
gallic	OH	OH	OH

Figure 2.7 Structures of common hydroxybenzoic acids.

hydroxy groups in the meta-position are typically derived from the acetate pathway (Figure 2.4).

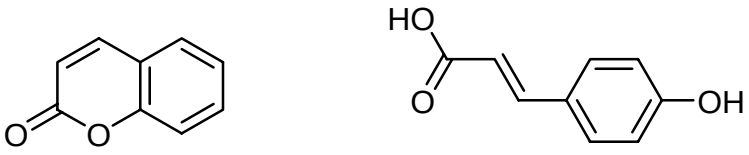
Aldehydes such as vanillin and *p*-hydroxybenzaldehyde are common flavour compounds derived from the reduction of hydroxybenzoic acids (Shahidi and Naczk, 1995).

2.4.3 Phenylacetic Acids

The phenylacetic acids (C_6-C_2) are not widely occurring phenolic compounds (Bravo, 1998) although they are known to play a role in the growth regulation of some plants (Hammad et al., 2003). These compounds are more common in microorganisms, particularly in moulds (Dewick, 1993). Biosynthesis of phenylacetic acids is reported to originate from phenylalanine and proceed via the shikimic acid pathway (Hammad et al., 2003).

2.4.4 Hydroxycinnamic Acids and Other Phenylpropanoids

Phenolic compounds consisting of the C_6-C_3 skeleton are collectively known as phenylpropanoids and largely consist of the hydroxycinnamic acid derivatives and coumarins (Figure 2.8). The hydroxycinnamic acids are the most widely occurring phenylpropanoids and are precursors to their cyclic derivatives, the coumarins.



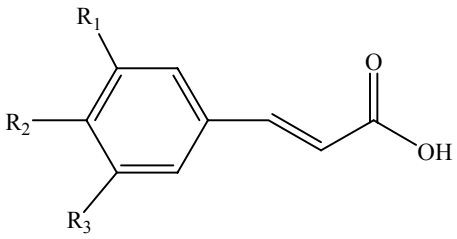
A B

Figure 2.8 Structures of A) coumarin and B) *p*-hydroxycinnamic acid.

Hydroxycinnamic acids are the most widely distributed phenolic acids in plant tissues (Robbins, 2003). They are rarely encountered in the free state in nature and are predominantly found as hydroxyacid esters with quinic, shikimic or tartaric acid (Herrmann, 1989), with larger phenolic compounds such as flavonoids or with structural components of the plant such as cellulose, lignin and protein (Clifford, 1999; Scalbert and Williamson, 2000).

The hydroxycinnamic acids have their biosynthetic origin from phenylalanine and the conversion of phenylalanine to the various hydroxycinnamic acids and coumarins is referred to as general phenylpropanoid metabolism (Mann, 1987). The first step in phenylpropanoid metabolism is the elimination of ammonia

from phenylalanine by the enzyme phenylalanine ammonia lyase (PAL) to produce cinnamic acid (Figure 2.2). Cinnamic acid is then hydroxylated to *p*-coumaric acid via the enzyme cinnamic acid 4-hyroxylase (Dewick, 1993). Other hydroxycinnamic acids are obtained by further hydroxylation and methylation reactions. Some of the common natural hydroxycinnamic acids are caffeic, ferulic, *p*-coumaric and sinapic acid. These structures are shown in Figure 2.9.



Acid	R_1	R_2	R_3
p-coumaric	H	OH	H
caffeic	H	OH	OH
ferulic	OCH_3	OH	H
sinapic	OCH_3	OH	OCH_3

Figure 2.9 Structures of common hydroxycinnamic acids.

Caffeic acid and to a lesser extent, ferulic acid are the most prominent phenolic acids occurring in foods of plant origin such as cereals, coffee, fruits and vegetables (Andreasen et al., 2000; Scalbert and Williamson, 2000; Robbins, 2003). The chlorogenic acids are a family of esters formed between trans-cinnamic acid and quinic acid which has axial hydroxyls on carbon C-1 and C-3 and equatorial hydroxyls on carbons C-4 and C-5. Using the IUPAC numbering, the most widely occurring individual chlorogenic acid is 5-*O*-caffeoyl quinic acid (Clifford, 1999). It is the only one commercially available and is typically referred to as chlorogenic

acid or 5-*O*-caffeoyl in literature (Clifford, 1999). The compound chlorogenic acid as used in this thesis refers to 5-*O*-caffeoyl quinic acid (Chemical Abstract Service number 327-97-9) and the structure of this compound is shown in Figure 2.10.

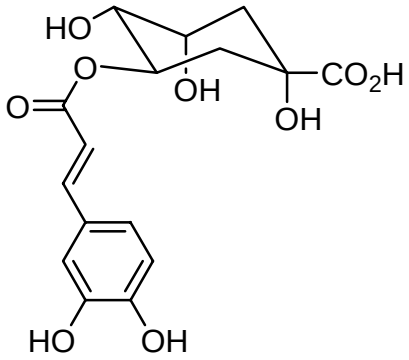


Figure 2.10 Structure of chlorogenic acid.

2.4.5 Naphthoquinones

Naphthoquinone (C_6-C_4) is an oxidized diphenolic compound to form a quinine; an aromatic ring possessing a double ketone functional group (Figure 2.11) (Cowan, 1999). These compounds are produced by the shikimic acid pathway and are usually conjugated with aliphatic isoprenoid units to form phylloquinones. Phylloquinones are thus, produced from mixed biosynthetic pathways: the shikimate acid and mevalonate (a polyketide) pathways. The vitamin K series of compounds are phylloquinones that have blood clotting functionality and are widely found in green vegetables (Shearer, 1995; Dewick, 2002).

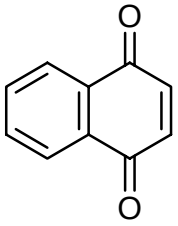


Figure 2.11 Basic structure of naphthoquinones.

2.4.6 Xanthones

The basic structure of xanthones (C_6-C_1-C_6) includes a pyrylium ring possessing a ketone functional group between two aromatic rings (Figure 2.12). Xanthones are synthesized from phenylalanine and three acetate units, thus these compounds are metabolites of mixed biosynthetic pathways. Xanthones are rare constituents of dietary plants, however, mangosteen is a well known source of the xanthone γ-mangostin (Parveen and Khan, 1988; Bennett and Lee, 1989).

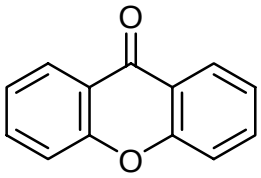


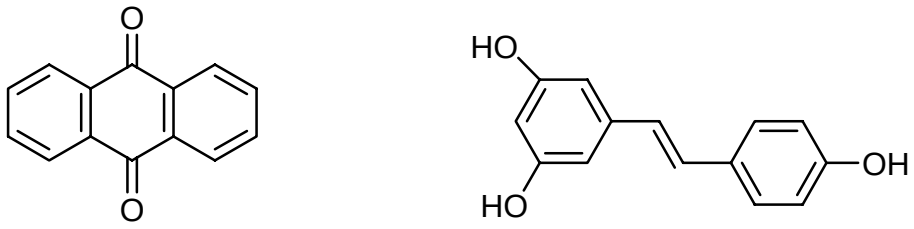
Figure 2.12 Basic structure of xanthones.

2.4.7 Anthraquinones and Stilbenes

Anthraquinones (C_6-C_2-C_6) possess a diketone linkage between the aromatic moieties (Figure 2.13). These compounds are primarily formed from an octaketide that is oxidized to produce the diketone (Dewick, 2002), thus, biosynthesis of anthraquinones is typically via the polyketide pathway. Alternatively, the anthraquinone, alizarin, an orange-red pigment isolated from the tropical madder plant, is derived from the shikimic acid and mevalonate pathways (Mann, 1987). The anthraquinones, however, are not prominent phenolic compounds in plants.

Stilbenes also possess a C_6-C_2-C_6 basic carbon skelton containing 1,2-diphenylethylene as a functional group. Resveratrol (3,5,4′-trihydroxystilbene) is a member of the stilbene family produced in some fruits occurring in both free and

glycoside forms (Nichenametla et al., 2006). This compound is of particular interest in grapes and red wine (Dercks and Creasy, 1989; Celotti et al., 1996) in which it is considered to impart health benefits (Section 2.7.4). The structure of resveratrol is shown in Figure 2.13.



A B

Figure 2.13 Structures of A) an anthraquinone and B) resveratrol.

Stilbenes are phenolic compounds of mixed biosynthetic origin produced from the shikimic acid and acetate pathways. The starter unit is cinnamoyl-CoA from the shikimic acid pathway. Extension of the cinnamoyl side-chain is then accomplished by the enzyme stilbene synthase by coupling the cinnamoyl-CoA unit with three C_2 units supplied by malonyl-CoA to produce the stilbenes. Hydrolysis and decarboxylation take place during this transformation (Dewick, 2002).

2.4.8 Flavonoids

Flavonoids (C_6-C_3-C_6) consist of two phenyl rings linked through three carbons that form an oxygenated heterocycle of three rings commonly labeled as A, B and C. The basic structure of flavonoids is shown in Figure 2.14. These compounds account for 60% of the total dietary phenolic compounds (Harborne and

Williams, 2000; Shahidi and Naczk, 2004; Nichenametla et al., 2006) and there are an estimated 4,000 known flavonoids (Wrolstad, 2005).

Flavonoids occasionally occur in plants as aglycones although they most commonly occur as glycoside derivatives. Each hydroxyl group and certain carbons can be substituted with one or more of a range of different simple carbohydrates which, in turn, may be acylated with a variety of phenolic or aliphatic acids (Harborne and Williams, 2000; Williams and Grayer, 2004). Additional substituents on the flavonoid skeleton structure can include benzyl, cinnamyl, hydroxyl, isoprenyl and methoxyl (Harborne and Williams, 2000).

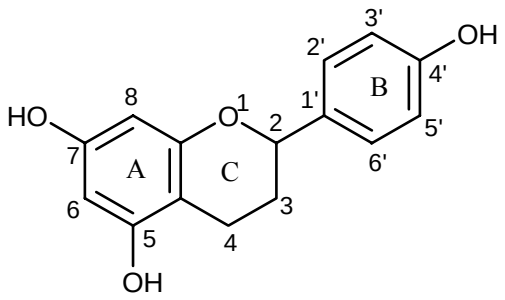


Figure 2.14 Basic structure of the flavonoids.

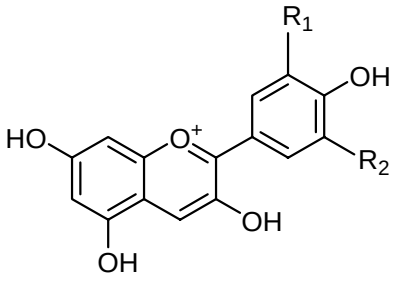
Flavonoids can be classified into six different subclasses that include anthocyanins, flavanols, flavanones, flavones, flavonols and isoflavones. The molecular structures of these subclasses are discussed in Sections 4.2.7.1 to 4.2.7.6. The biosynthesis of flavonoids is presented in Section 4.2.7.8.

Two stereoisomeric forms are possible for flavonoids unsaturated between C-2 and C-3, since C-2 is an asymmetic centre. Consequently, the B-ring can be either in the (2S)- or (2R)-configuration. The majority of the flavonoids isolated

from plants have the (2S)-configuration because the enzymatic reaction catalyzing the formation of these compounds is stereospecific (Grayer and Veitch, 2005).

2.4.8.1 Anthocyanins

Anthocyanins are flavonoids, they are water-soluble pigments and are responsible for providing red, blue and violet colours found in most plant species. These pigments are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrilium (flavylium) salts, characterized by conjugated unsaturation in the C-ring and by glycosylation at the C-3 position (Brouillard, 1982; Springob et al., 2003). The basic aglycone of the anthocyanin is unstable and is referred to as the anthocyanidin. There are six major anthocyanidins and these are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Brouillard, 1982). The flavylium ion structures of these anthocyanidins are shown in Figure 2.15.



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

Figure 2.15 The flavylium ion structures of the six major anthocyanidins.

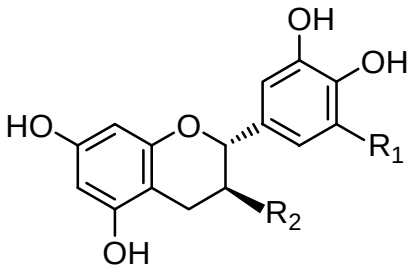
Glycosylation provides stability to the anthocyanins and may also occur at other postions in addition to C-3, often at C-5 (Mazza and Miniati, 1993; Springob et al., 2003). The most common glycosyl groups are monosaccharides, especially glucose, galactose, rhamnose and arabinose. Di- and trisaccharides formed by combinations of these four monosaccharides also occur (Brouillard, 1982). The C-3 glycosylation is a prerequisite for further modifications, such as a second glycosylation, acylation, methylation and prenylation (Springob et al., 2003). As a result of modifications to the basic structures of these pigments, more than 500 types of anthocyanins have been reported in plants (see Mazza and Miniati, 1993).

The most common acyl groups associated with anthocyanins are the hydroxycinnamic acids, particulary caffeic, ferulic and *p*-coumaric acids, and the aliphatic dicarboxylate, malonic acid (Williams and Grayer, 2004). Acylation with aromatic organic acids is presumed to contribute to intra- and/or inter-molecular stacking for the stabilization of the pigment molecules (Williams and Grayer, 2004). Furthermore, acylation increases water solubility and may serve as protection against enzyme-catalyzed glycosidase degradation (Springob et al., 2003).

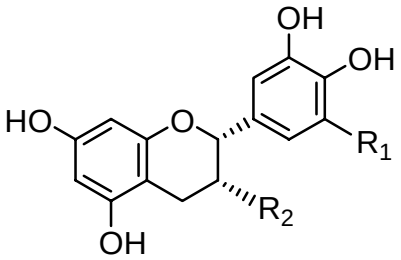
2.4.8.2 Flavanols

Flavanols possess a saturated C-ring with a hydroxyl or galloyl group attached at C-3 (Figure 2.16). The flavanols largely occur in the aglycone form, contrary to the fact that most flavonoids exist in plants as glycosides (Harborne, 1994). The most widely distributed members of this subclass of phenolic compounds in nature are the diastereoisomeric pair (+)-catechin and (-)-epicatechin

and consequently, the flavanols are commonly referred to as the catechins (Shahidi and Naczk, 2004). Flavanols can undergo polymerization to form proanthocyanidins (Section 2.4.8.2).



A

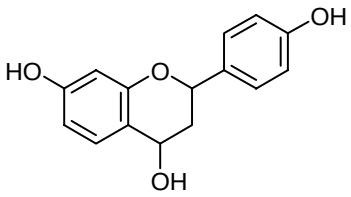


B

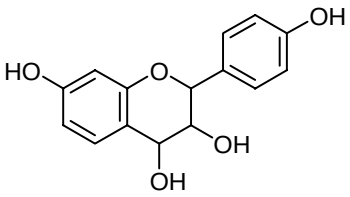
Flavanol (A/B)	R_1	R_2
(+)catechin/(-)epicatechin	H	OH
(+)catechin gallate/(-)epicatechin gallate	H	O-gallate
(+)gallocatechin/(-)epigallocatechin	OH	OH
(+)gallocatechin gallate/(-)epigallocatechin gallate	OH	O-gallate

Figure 2.16 Structures of flavanols.

Monomeric flavanols hydroxylated at position C-4 only or with an additional hydroxyl group at position C-3 of ring C are known as flavan-4-ols and flavan-3,4-diols, respectively (Figure 2.17). These compounds, also known as



A



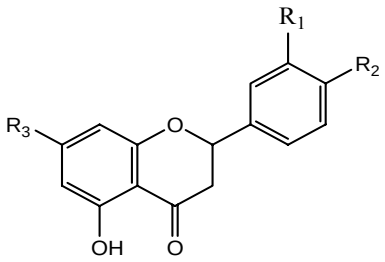
B

Figure 2.17 Structures of A) flavan-4-ol and B) flavan-3,4-diol.

leucoanthocyanidins are converted to anthocyanidins by cleavage of the C-ring hydroxyl group upon heating with acid (Ferreira et al., 2006).

2.4.8.3 Flavanones

Flavanones are characterized by the presence of a carbonyl group at C-4 of ring C. The flavanones are especially abundant in citrus fruits and the most commonly occurring aglycones are hesperedin, hesperetin and naringenin (Figure 2.18). There are numerous structural variations of flavanones found in plants due to acylation, glycoslyation, hydroxylation, methylation and methoxylation of the A- and B-rings of the carbon skeleton (Grayer and Veitch, 2005). Glycosylation of flavanones typically occurs at the C-7 position (ring A).



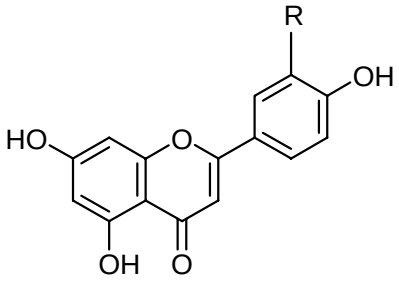
Flavanone	R_1	R_2	R_3
eriodictyol	OH	OH	O-rutinoside
hesperedin	OH	H	OH
hesperetin	OH	OCH_3	OH
naringenin	H	OH	OH

Figure 2.18 Structures of some common flavanones.

2.4.8.4 Flavones

Flavones are similar in structure to flavanones as indicated by the presence of a carbonyl at C-4, however, they are unsaturated between C-2 and C-3 of ring C

(Harborne and Williams, 1976). The structures of two common flavone aglycones, apigenin and luteolin, are shown in Figure 2.19. Nearly 700 flavones (Williams and Grayer, 2004) have been identified in plants with variation in acylation, glycosylation, hydroxylation, methylation and sulfation in the aromatic rings accounting for this large number (Williams and Grayer, 2004). Flavones are less common in plants as compared to other flavonoids but are prominent in citrus fruits (Kim and Lee, 2005a).

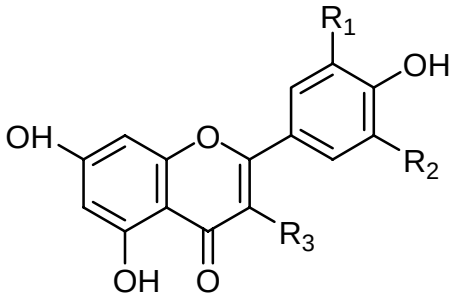


Flavone	R
apigenin	H
luteolin	OH

Figure 2.19 Structures of apigenin and luteolin.

2.4.8.5 Flavonols

Flavonols are the most prevalent flavonoid in the plant kingdom (Harborne and Williams, 2000). The basic aglycone structure of flavonol differs from that of a flavone in that the C-3 position is hydroxylated/glycosylated. Commonly occurring flavonol aglycones include kaempferol, myricetin and quercetin. The structures of these compounds are shown in Figure 2.20.



Flavonol	R_1	R_2	R_3
kaempferol	H	H	OH
quercetin	OH	H	OH
myricetin	OH	OH	OH
quercitrin	OH	H	O-rhamnoside
rutin	OH	H	O-rutinoside

Figure 2.20 Structures of some common flavonols.

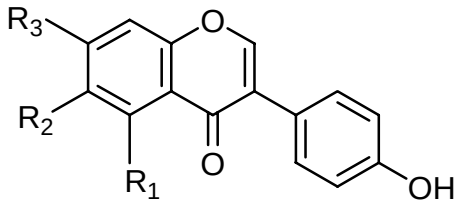
Flavonols are usually found as their glycoside derivatives in plants and as with other flavonoids, various mono- and disaccharides are part of the flavonol structure. Further variations in structure are due to acylation with aliphatic and aromatic acids, methoxylation, and prenylation. More than 1400 glycosylated flavonols have been identified in plants (Harborne and Williams, 2000) and glycosides of quercetin, such as quercitrin and rutin (Figure 2.20), are the most predominant naturally occurring flavonols (Harborne and Williams, 2000; Stefova et al., 2001; Williams and Grayer, 2004). Due to their wide distribution in plants and their purported health potential, there is significant interest in the flavonol content/composition of fruits (Section 2.7).

2.4.8.6 Isoflavones

Isoflavones are structural isomers of flavanones that are often treated

separately from the former five sub-classes of flavonoids because they typically occur only in plants of the family Leguminaceae (Mann, 1987). The isoflavones are characterized by the attachment of the B- to the C-ring at C-3, rather than C-2.

Isoflavones commonly occur as aglycones, glycosides, acetyl-glycosides and malonyl-glycosides. The aglycones diadzein, genistein, glycitein and their glycosides are the major isoflavones found in plants (Figure 2.21).



Isoflavone	R_1	R_2	R_3
diadzein	H	H	OH
diadzin	H	H	O-glucoside
genistein	OH	H	OH
genistin	OH	H	O-glucoside
glycitein	H	OCH_3	OH
glycitin	H	OCH_3	O-glucoside

Figure 2.21 Structures of the major plant isoflavones.

2.4.8.7 Other Minor Flavonoids

In addition to the aforementioned main subclasses of flavonoids there are three minor flavonoid subclasses that include chalcones, dihydrochalcones and aurones. The chalcones are structurally one of the most diverse groups of flavonoids and are found as monomers, dimers, oligomers and conjugates of various types. They are of great significance biosynthetically as the immediate precursors of all other classes of flavonoids (Section 2.4.7.8). The unique feature that distinguishes chalcones and dihydrochalcones from other flavonoids is the open-

chain three-carbon structure linking the A- and B-rings in place of a heterocyclic C-ring (Figure 2.22). This three-carbon linkage between the aromatic rings is unsaturated in chalcones and saturated in dihydrochalcones. Chalcones and dihydrochalcones are unstable and upon acid treatment, they readily isomerise to the corresponding flavanone (Harborne, 1967).

The aurones are based on the 2-benzylidine-coumaranone or 2-benzylidin-3(2H)-benzofuranone structure (Harborne, 1967). Aurones are not common in dietary plants although they provide strong yellow colours in a variety of flowers (Veitch and Grayer, 2005) and have been identified in bark from some ornamental trees (Veitch and Grayer, 2005) such as Golden larch (*Pseudolarix amabilis*) (Li et al., 1999) and leaves from medicinal shrubs such as *Uvaria hamiltonii* from Thailand (Huang et al., 1998).

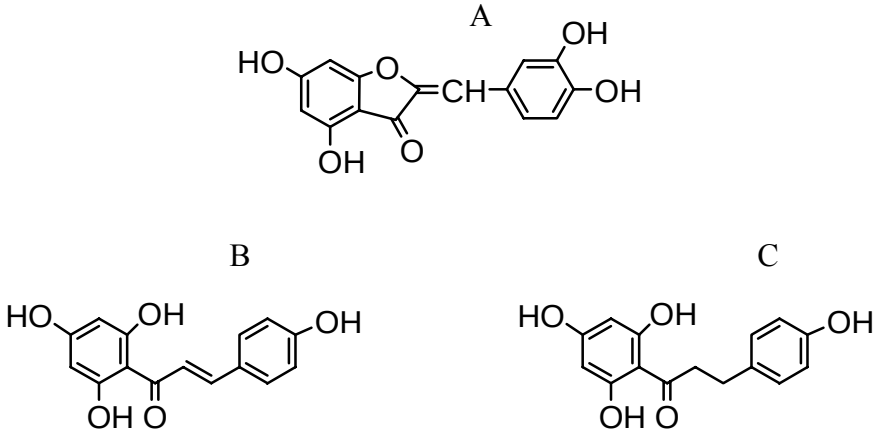


Figure 2.22 Structure of A) aurone, B) chalcone and C) dihydrochalcone.

2.4.8.8 Biosynthesis of the Flavonoids

Biosynthesis of flavonoids is shown in Figure 2.23. Biogenetically, the flavonoids

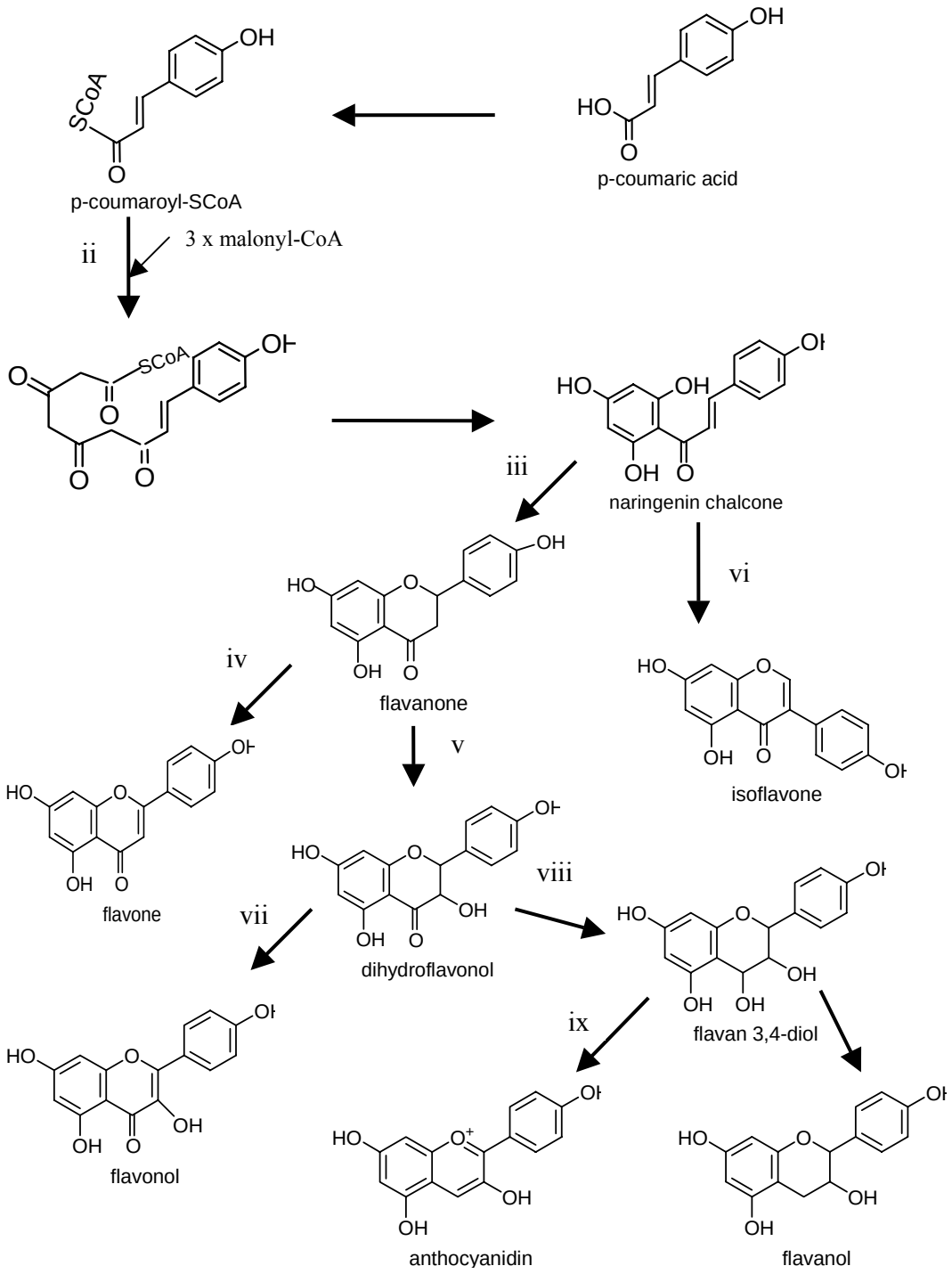


Figure 2.23 Biosynthesis of the flavonoids. Enzymes: i) *p*-coumarate-CoA ligase, ii) chalcone synthase, iii) chalcone isomerase, iv) flavone synthase II, v) flavanone 3-β-hydroxylase, vi) isoflavone synthase, vii) flavonol synthase, viii) dihydroflavonol 4-reductase, ix) anthocyanidin synthase. Adapted from Mann, 1982; Dewick, 1993; and Davies and Schwinn, 2005.

are produced from mixed metabolite synthesis whereby the A ring comes from the acetate pathway and the B-ring is derived from the shikimic acid pathway (Mann, 1987). Initially, *p*-coumaric acid produced from the shikimic acid pathway (Figure 2.4) is activated to *p*-coumaroyl-CoA by the enzyme *p*-coumarate-CoA ligase. The first step in the biosynthesis of flavonoids is then catalyzed by the enzyme chalcone synthase which performs sequential condensations with *p*-coumaroyl-CoA and three acetate units from malonyl-CoA (Springob et al., 2003). The resulting tetraketide intermediate undergoes intramolecular cyclization to naringenin chalcone (2',4,4',6'-tetrahydroxychalcone). Naringenin chalcone is then converted stereospecifically to the flavanone, (2S)-naringenin by the enzyme chalcone isomerase. In the absence of chalcone isomerase this reaction occurs spontaneously yielding racemic (2R/S)-naringenin (Springob et al., 2003). Flavones can be produced directly from flavanones by hydroxylation at C-2 followed by dehydration resulting in unsaturation between C-2 and C-3. In most plants, the conversion to flavones is catalyzed by cytochrome P450 flavone synthase II (Davies and Schwinn, 2005). Flavanones can also be converted to dihydroflavonols by hydroxylation at position C-3 catalyzed by flavanone 3-β-hydroxylase (Knaggs, 2003). The dihydroflavonols may be converted to flavonols via flavonol synthase or to flavan 3,4-diols via the enzyme dihydroflavonol 4-reductase. The resulting flavan 3,4-diols may be reduced at position C-4 to produce a flavanol or may be converted to coloured anthocyanidins by the enzyme anthocyanidin synthase (Springob et al., 2003). Glycosylation of anthocyanidins to anthocyanins occurs via a glycosyltransferase using the UDP-bound carbohydrate as the glycosyl donor

(Davies and Schwinn, 2005).

Due to the numerous defense functions of phenolic compounds in plants, several environmental factors such as light, temperature, humidity and internal factors, including genetic differences, nutrients and hormones contribute to the regulation of their synthesis (Macheix et al., 1990; Wollgast and Anklam, 2000; Kahkonen et al., 2001).

2.4.9 Tannins

In addition to simple monomeric soluble forms of phenolic compounds there are polymerized forms of varying solubility such as the tannins. Tannins are phenolic compounds of intermediate to high molecular weight ranging from 500 to >20,000 Da (Santos-Buelga and Scalbert, 2000). These compounds can form insoluble complexes with carbohydrates and protein through hydrogen bonding of the hydroxyl groups (Salunkhe et al., 1989). The term "tannin" comes from the capacity these compounds have in transforming animal hides into leather by forming stable tannin-protein complexes with skin collagen (Makkar, 1989; Bravo, 1998; Wollgast and Anklam, 2000).

There are two main types of tannins which include the hydrolysable tannins, based on gallic acid polymers, and condensed tannins or proanthocyanidins which are composed of flavanol polymers (Manach et al., 2004).

2.4.9.1 Hydrolyzable Tannins

Hydrolyzable tannins consist of gallic acid polymers and include the

gallotannins and ellagitannins. Gallotannins are composed of gallic acid units esterified to a central core carbohydrate or polyol, which is mainly glucose or sorbitol, respectively. Each single gallic acid monomer in gallotannin is called a galloyl unit and are incorporated onto the central core polyol via galloyltransferase (Dewick, 2002). One of the more common gallotannins is pentagalloylglucose (1,2,3,4,6-penta-O-galloyl-β-D-glucose) which possesses the structure shown in Figure 2.24 (Mueller-Harvey, 2001). Pentagalloylglucose is a simple tannin and is the starting unit for more complex hydrolysable tannins. Additional galloyl subunits can attach to the galloyls on the pentagalloylglucose molecule by oxidative coupling to produce larger gallotannin molecules (Mueller-Harvey, 2001).

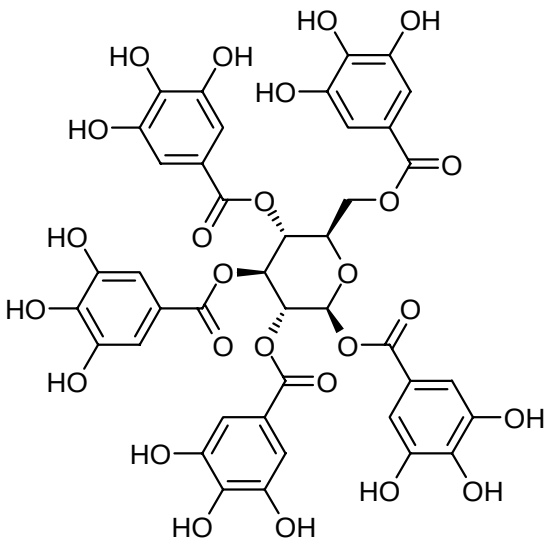
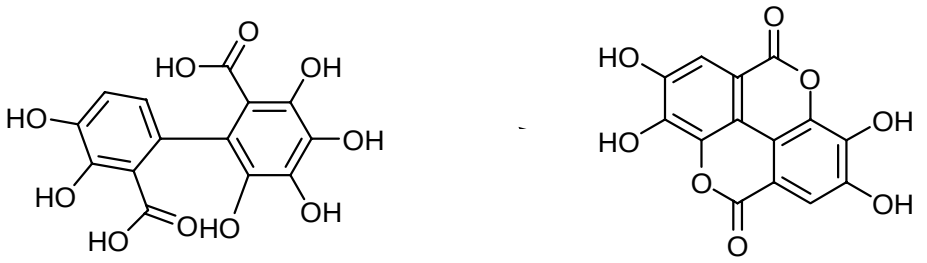


Figure 2.24 Structure of 1,2,3,4,6-penta-O-galloyl-β-D-glucose, a gallotannin.

Hydrolysable tannins are easily hydrolyzed with weak aqueous acid or alkali and by enzymatic action yielding the central core polyol, and the constituent phenolic acid (Bravo, 1998; Mueller-Harvey, 2001). Gallotannins hydrolyze to the

polyol and gallic acid whereas the phenolic acid liberated from hydrolysis of ellagitannins is hexahydroxydiphenic acid that, in the aqueous acidic or basic environment, converts to the lactone form, ellagic acid (Figure 2.25) (Shahidi and Naczk, 1995; Dewick, 2002). The biosynthesis of ellagitannins is not well known, however, they are believed to be derived from pentagalloylglucose by oxidative reactions between the gallic acid units (Nonaka, 1989; Mueller-Harvey, 2001).



A B

Figure 2.25 Structures of A) hexahydroxydiphenic acid and B) ellagic acid.

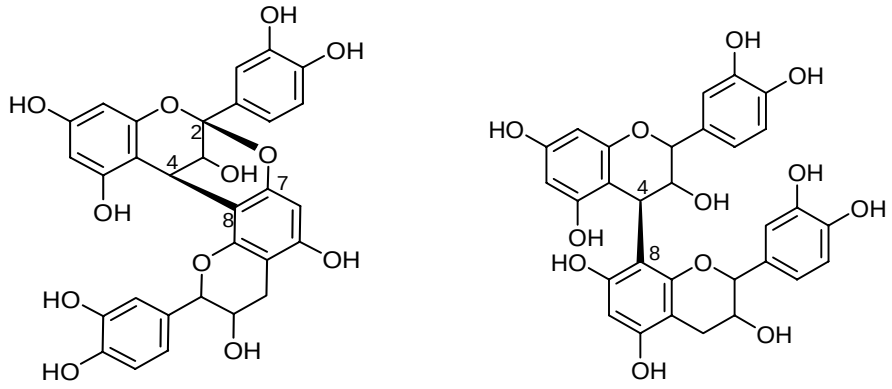
2.4.9.2 Proanthocyanidins

Proanthocyanidins are high molecular weight flavonoid polymers that are also referred to as 'condensed tannins'. The monomeric unit is a flavanol, such as catechin or epicatechin with a flavan-3,4-diol or flavan-4-ol molecule as its precursor (Santos-Buelga and Scalbert, 2000). Oxidative condensation occurs between carbon C-4 of the C-ring and carbons C-6 or C-8 of the A-ring on adjacent flavanol units. Proanthocyanidins are typically described by their degree of polymerization (DP) with DP=1 as monomers and those with DP = 2 to 10 and DP > 10 as oligomers and polymers, respectively (Santos-Buelga and Scalbert, 2000;

Gu et al., 2003; Gu et al., 2004). Most of the proanthocyanidins identified occur as oligomers due to the difficulty in analyzing highly polymerized molecules (USDA, 2004a). However, proanthocyanidins with an average degree of polymerization up to 85 in grape skins (*Vitis vinifera* L.) (Monagas et al., 2003), and 190 in cider apples (*Malus domestica*) (Guyot et al., 2001) have been identified.

Under strongly acidic conditions, the proanthocyanidins produce anthocyanidins by cleavage of a C-C interflavanyl bond. If the sub-units consist only of catechin and epicatechin, cyanidin is the only resulting product of hydrolysis and those proanthocyanidins are called procyanidins. The procyanidins are the most common proanthocyanidins in dietary plants, however prodelphinidins derived from epigallocatechin and propelargonidins derived from the flavanol afzelechin have also been identified (Santos-Buelga and Scalbert, 2000; Gu et al., 2004; USDA, 2004a).

Proanthocyanidins have been classified into two types based on the linkages between the flavanyl units. B-type proanthocyanidins are characterized by singly linked flavanyl units, usually between C-4 of the chain-extension unit and C-6 or C-8 of the chain-terminating moiety (Ferreira et al., 2006). They are classified according to the hydroxylation pattern of the chain-extension units. Proanthocyanidins of the A-type possess a second ether linkage to C-2 of the top or chain-extension unit. The structures of A- and B-type proanthocyanidins are shown in Figure 2.26.



A B

Figure 2.26 Structures of proanthocyanidin dimers: A) A-type and B) B-type.

2.4.10 Biflavonoids and Triflavonoids

Biflavonoids and triflavonoids are dimers and trimers, respectively of flavonoids that do not result in cleavage to anthocyanidins on treatment with strong acid. The dimers and trimers are products of oxidative coupling mainly of flavones, flavanones and/or aurones and thus, they possess a carbonyl group at C-4 (Geiger and Quinn, 1976; Ferreira et al., 2006). As a result of this C-4 carbonyl group, the bi- and tri-flavonoids do not produce anthocyanidins upon hydrolysis in strongly acidic environments (Bennie et al., 2000; Bennie et al., 2001).

The structure of the biflavone, 2,3-dihydroapigeninyl-(I-3′,II-3′)-apigenin is shown in Figure 2.27. This compound has a C-C interflavanyl linkage between the C-3′ carbons on each flavone moiety. Several biflavonoids with C-O-C interflavanyl bonds have recently been identified (Bennie et al., 2000; Bennie et al., 2001; Bennie et al., 2002; Ferreira et al., 2006).

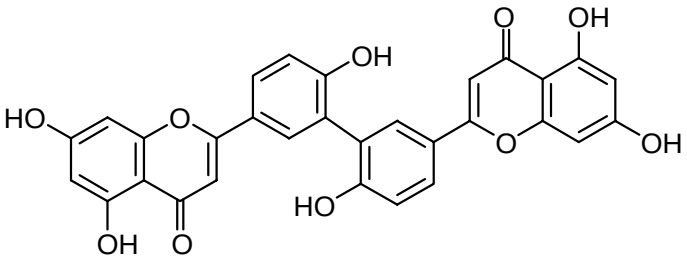


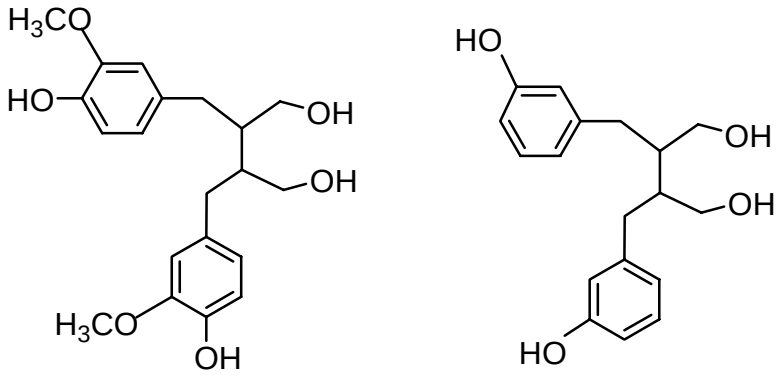
Figure 2.27 Structure of 2,3-dihydroapigeninyl-(I-3′,II-3′)-apigenin, a biflavonoid.

2.4.11 Lignins and Lignans

Lignin is formed by oxidative polymerization of cinnamic acid derivatives, particularly hydroxycinnamyl alcohol monomers (Mann, 1987). The most prominent of cinnamic acid derivatives found in lignins are 4-hydroxycinnamyl alcohol, coniferyl alcohol and sinapyl alcohol, although these monomers vary with the plant type (Mann, 1987; Dewick, 2002). Lignin is held within a cellulose matrix and is utilized to strengthen the cell wall of the plant against external physical and chemical stresses. Formation of lignin increases dramatically when plants come under fungal or bactericidal attack (Mann, 1987). In contrast to most other natural polymeric materials, lignin is devoid of ordered repeating units and thus, the native structure is inconsistent. The mode of polymerization of in the biosynthesis of lignin is not known but has suggested to be a result of a free radical or ionic process rather than a stereospecific, enzyme-mediated one, since lignins are optically inactive (Mann, 1987).

On the contrary, lignans are dimeric compounds also formed from cinnamic acid derivatives coupled in a specific manner, thus forming specific compounds such as secoisolariciresinol (Dewick, 2002) (Figure 2.28). Further cyclization and

other modifications can create a wide range of lignans of very different structural types. Food lignans are only found in considerable quantities in flaxseed and when ingested by humans, they are metabolized by the intestinal microflora into the "mammalian lignans" (Borriello et al., 1985; Meagher et al., 1999). The mammalian lignans enterolactone and enterodiol (Figure 2.28) are considered to be products of colonic bacterial metabolism of the plant-derived precursors mateirresinol and secoisolariciresinol, respectively (Borriello et al., 1985; Heinonen et al., 2001).



A B

Figure 2.28 Structure of A) secoisolariciresinol, a plant lignan and B) enterodiol, a mammalian lignan.

2.5 Role of Phenolic Compounds in Plants

Phenolic compounds are secondary metabolites that play important roles in defense and physiology of the plant. One important protective role of flavonoids is to filter harmful ultraviolet (UV) radiation from damaging the plant (Stafford, 1991; Iwashina, 2003). Flavonoids, especially flavones and flavonols, exhibit high absorption in the UV-B region (280 to 320 nm); these wavelengths have been

shown to damage the nucleic acids and metabolic enzymes of plants (Mann, 1987; Stafford, 1991).

Some phenolic compounds are synthesized and accumulate in plant cells as a defense mechanism after exposure to microorganisms (Afek et al., 1986; Laks and Pruner, 1989; Butler, 1992; Iwashina, 2003), these compounds are called phytoalexins. Examples of phenolic compounds that inhibit the growth of pathogenic fungi include catechin which can inhibit *Aspergillus* species (Weidenborner et al., 1990), and kaempferol and quercetin 3-glucoside which can inhibit *Verticillium* fungi that cause plant wilt (Picman et al., 1995). The mechanisms responsible for phenolic toxicity to microorganisms include enzyme inhibition, complexation with extracellular proteins and cell wall discruption (Mason and Wasserman, 1987; Scalbert, 1991; Chabot et al., 1992).

Other phenolic substances protect plants by acting as feeding deterrents to insects (Beier and Oertli, 1983; Nahrstedt, 1990). For example, flavonoids such as luteolin, naringenin, phloretin, quercetin 3-rhamnoside and myricetin 3-rhamnoside have been shown to act as feeding deterrents to aphids (Dreyer and Jones, 1981). The phenolic compounds chlorogenic acid and rutin are toxic to the larvae of the fruitworm *Heliothis zea* and thus inhibit the growth of the larvae feeding on leaves containing these compounds (Isman and Duffey, 1982).

Phenolic compounds have been shown to be feed inhibitors to mammalian herbivores. For example, proanthocyanidins in blackbrush, a plant in the Rosaceae family have been shown to act as a feed inhibitor to the snowshoe hare (Clausen et al., 1990). High concentrations of tannins in the diet of some mammals have also

been shown to be highly toxic. For example, weaning hamsters treated with a diet containing 4% dry weight of sorghum tannin were reported to suffer weight loss and then perish within three to 21 days (Harborne, 2001).

In contrast to deterring insects and herbivores from plants, certain flavonoids are believed to function as attractants for pollen and seed dispersal. The anthocyanins are the most widespread pigments in the plant kingdom and are responsible for most of the red, blue, purple and intermediate hues of flowers and fruits (Mazza and Miniati, 1993; Springob et al., 2003). The colour imparted by anthocyanins to flowers and fruits is believed to attract specific species of animals, birds and insects for pollen and seed dispersal, respectively (Mazza and Miniati, 1993; Harborne, 2001; Springob et al., 2003). Furthermore, although flavonols are not coloured, they absorb strongly in the UV region and they can be seen by insects. Flavonols such as kaempferol 3-glucoside, and quercetin 3-glucoside have been reported to attract insects for pollen dispersal in the flowers of Leguminaceae (Harborne and Boardley, 1983; Mann, 1987).

Many phenolic compounds are allelopathic that is, they are released by plants to inhibit the growth of other plants and thereby reduce competition (Mann, 1987; Onyilagha and Grotewold, 2004). For example, phlorizin is a chalcone released from the roots of apple trees in order to play an allelopathic role to reduce competition of other plants near the tree (Mann, 1987).

Phenolic compounds such as hydroxycinnamic acids play important roles in lignification and therefore, influence the physical structure of plants (Faulds and Williamson, 1999; Russell et al., 1999).

2.6 Phenolic Compounds in Foods of Plant Origin

2.6.1 Impact on Food Quality

Phenolic compounds are produced by plants and some microorganisms, and the occurrence of these compounds in animal tissues is due plant ingestion. The sensory attributes typically attributed to phenolic compounds are astringency, and bitterness (Shahidi and Naczk, 1995). Astringency is related to the ability of the phenolic compound to precipitate salivary proteins, bringing about a drying sensation over the surface of the tongue (Bate-Smith, 1973). Tannins are the most astringent phenolic compounds although even though less complex phenolic compounds such as catechin and *p*-coumaric acid can impart astringency (Delcour et al., 1984; Huang and Zayas, 1991). Hydroxycinnamic acid derivatives have been found to contribute bitter flavours to berries such as cranberries (Marwan and Nagel, 1982) while catechin and epicatechin have been reported to contribute bitterness to red wine (Kallithraka et al., 1997), tea and cocoa powder (Drewnowski and Gomez-Carneros, 2000). Flavanone glycosides such as hesperidin, naringin and neohesperidin are major contributors to the bitter taste of citrus fruits (USDA, 1998).

Phenolic compounds may contribute other desirable and non-desirable flavours to foods (Macheix et al., 1990; Scalbert and Williamson, 2000; Shahidi and Naczk, 2004; Kyle and Duthie, 2006). The characteristic flavour of vanilla extract is due to the benzoic acid derivative vanillin (*p*-hydroxybenzaldehyde). Strawberry volatiles contain esters of phenolic acids such as methyl cinnamic and ethylbenzoic

acids, and several coumarins contribute to lime and mandarin flavours (Shahidi and Naczk, 1995). Chalcones and dihydrochalcones are sweet in flavour although the onset of sweetness is reportedly slow and leaves a lingering aftertaste (Dubois et al., 1977). Sinapine is a bitter phenolic acid derivative found in rapeseed and is linked to a fishy taint in the eggs of hens fed high levels of rapeseed meal (Sosulski et al., 1977; Butler et al., 1982). Free ferulic acid is a precursor of objectionable flavour in stored orange juice caused by the presence of *p*-vinyl guaiacol which is produced as a decarboxylation product of ferulic acid (Naim et al., 1988).

The colour of many fruits and fruit products is attributable to phenolic compounds. Anthocyanin pigments naturally present in the raw fruits and certain vegetables are also responsible for the desirable colours of foods prepared from these plant products (Mazza and Miniati, 1993). In contrast, undesirable enzymatic browning may occur during the aging or as a result of injury to fruits and vegetables containing phenolic compounds. This browning reaction occurs due to polyphenoloxidases which catalyze the oxidation of phenolic compounds that readily condense to form brown off-colour, off-flavour and off-odour products (Burnette, 1977; Shahidi and Naczk, 1995).

In addition to their contributions to the sensory properties of food, there is significant interest in phenolic compounds due to the antioxidant and free radical scavenging abilities associated with these compounds (section 2.7) for their potential to increase the shelf-life of the food (Shahidi, 2000). Food manufacturers use food-grade phenolic antioxidants to prevent the deterioration of product quality and nutritional value due to oxidation (Shahidi and Naczk, 2004). Synthetic and

natural antioxidants are used routinely in foods especially those containing oils and fats. The common synthetic antioxidants used in foods include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroxyquinone (TBHQ); all phenolic compounds (Papas, 1993). Natural antioxidants used in food product manufacture include ascorbic acid and phenolic compounds extracted from plants (Schuler, 1990; Sakihama et al., 2002; Shahidi and Naczk, 2004) such as tocopherols and rosemary extract (Schuler, 1990).

2.6.2 Occurrence in Foods

The average per capita consumption of phenolic compounds in the U.S. has been estimated to range from 255 mg/day (Vinson et al., 2001) to as high as 1,000 mg/day (Kyle and Duthie, 2006). In particular, anthocyanins, flavonols, flavanols, phenolic acids and tannins are the major phenolic compounds found in foods (Macheix et al., 1990; Shahidi and Naczk, 1995).

Due to the growing awareness of the potential importance of flavonoids in diet; in 2003 the United States Department of Agriculture (USDA) established a database containing the flavonoid content of foods compiled from bibiliographic sources (USDA, 2003). New foods are added to the database periodically and the information is made available on the USDA website (http://www.nal.usda.gov/nutrientdata).

2.6.2.1 Occurrence in Berries

Berry fruits are an important source of potential health-promoting phenolic

compounds, particularly flavonoids and phenolic acids. The berry fruits encompass several distinct botanincal families and thus differ markedly in their phenolic composition. Bilberry (*Vaccinium myrtillus*) is one of the richest sources of phenolic compounds, reported to contain approximately 3,300 mg/100 g dry weight basis as gallic acid equivalents (Kahkonen et al., 2001) and 844 mg/100 g fresh weight basis (Maatta-Riihinen et al., 2004b). This berry is the European blueberry typically found in the wild and has been used for some medicincal applications (Shahidi and Naczk, 2004). Anthocyanins are the major phenolic compounds in bilberry and account for greater than 75% of the phenolic composition in the fruit (Kahkonen et al., 2001; Maatta-Riihinen et al., 2004b). The total content of hydroxycinnamic and hydroxybenzoic acids in bilberry was reported to range from 113 to 231 mg of chlorogenic acid equivalents/100 g dry weight and 3 to 6 mg gallic acid equivalents/100 g dry weight, respectively, and the flavonol content was reported to be 54 to 130 mg of rutin equivalents/100 g dry weight of bilberry (Kahkonen et al., 2001).

The highbush blueberry (*Vaccinium corymbosum*) is a cultivated blueberry used for commercial production in North America (Kalt et al., 2001; Shahidi and Naczk, 2004). The total phenolic content of highbush blueberry has been reported to range from 115 (Taruscio et al., 2004) to 191 mg/100 g fresh weight basis reported as gallic acid equivalents (Kalt et al., 2001). This berry contains moderate amounts of anthocyanins, reported to average 100 mg/100 g fresh weight basis, which is lower than the range for lowbush blueberries (*Vaccinium angustifolium*),

reported to be 150 to 200 mg/100 g fresh weight (Gao and Mazza, 1994a; Kalt et al., 2001).

The lowbush blueberry is a wild blueberry that contains significantly higher levels of total phenolic content (376 mg gallic acid equivalents/100 g fresh weight) in comparison to the highbush blueberry (Kalt et al., 2001). Chlorogenic acid is the major phenolic acid present in both highbush and lowbush blueberry (Schuster and Herrmann, 1985; Gao and Mazza, 1994a).

The American cranberry (*Vaccinium macrocarpon*) is a cultivated berry that is grown commercially in North America and processed into sauce, concentrates and juice. The European cranberry (*Vaccinium oxycoccus*) is a wild variety that is of lesser economic value in comparison to the American cranberry (Huopalahti et al., 2000). Appreciable total phenolic levels, reported at 527 mg gallic acid equivalents/100 g fresh weight (Sun et al., 2002) occur in the American cranberry whereas the European cranberry contains a lower total phenolic content, reported to be 139 mg gallic acid equivalents/100 g fresh weight (Taruscio et al., 2004). Kahkonen et al. (Kahkonen et al., 2001) reported a total phenolic content of 2200 mg/100 g dry weight of European cranberry, expressed as gallic acid equivalents. The phenolic compounds identified in cranberry include anthocyanins (Hong and Wrolstad, 1990b; Kahkonen et al., 2001; Wang and Stretch, 2001), flavanols (Taruscio et al., 2004), flavonols (Yan et al., 2002), phenolic acids (Zuo et al., 2002) and proanthocyanidins (Foo et al., 2000). Anthocyanins are the predominant phenolic compounds in cranberry, reported at levels of 19 to 63 mg cyanidin 3-galactoside/100 g fresh weight in American cranberry (Wang and Stretch, 2001) and

31 mg cyanidin 3-glucoside/100 g fresh weight basis in European cranberry (Taruscio et al., 2004). The major anthocyanins of American cranberry are reported to consist of 3-galactosides and 3-arabinosides of cyanidin and peonidin (Hong and Wrolstad, 1990a; Prior et al., 2001) while European cranberry anthocyanins are mainly the 3-glucosides of cyanidin and peonidin (Mazza and Miniati, 1993). The American cranberry is also a good source of quercetin, ranging from 11 to 25 mg/100 g fresh fruit (Hakkinen et al., 1999b). Major aromatic acids in American cranberry, in the order of decreasing quantity, was reported to be benzoic (470 mg/100 g fresh weight), *p*-coumaric (25 mg/100 g fresh weight), sinapic (21 mg/100 g fresh weight), caffeic (15 mg/100 g fresh weight), ferulic (9 mg/100 g fresh weight) and vanillic (9 mg/100 g fresh weight) acid (Zuo et al., 2002). Flavanols reported in European cranberry consist of catechin and epicatechin at concentrations ranging from 2.4 to 8.2 and from 0.7 to 11.9 mg/100 g fresh weight, respectively (Maatta-Riihinen et al., 2004b; Taruscio et al., 2004). Prior et al. (2001) identified catechin and epicatechin in American cranberry, although the concentration of these flavanols was not reported. The total proanthocyanidin content of American cranberry is approximately 2 mg/100 g fresh weight (Prior et al., 2001).

The total phenolic content of other *Vaccinium* species such as black huckleberry (*V. membranaceum*), evergreen huckleberry (*V. ovatum*), oval-leaf blueberry (*V. ovalifolium*), and red huckleberry (*V. parvifolium*) collected from the northwestern United States have been reported to range from 170 to 335, 284 to 842, 281 to 678, and 81 to 228 mg/100 g fresh weight basis, expressed as gallic acid

equivalents (Moyer et al., 2002; Taruscio et al., 2004), respectively. Kahkonen et al. (1999) reported the total phenolic content of whortleberry (*V. uligonosum*), and cowberry (*V. vitis-idaea*) from Finland to be 2870 mg/100 g and 2490 mg/100 g dry weight expressed as gallic acid equivalents, respectively.

Blackcurrant (*Ribes nigrum* L.) is commercially grown for the production of juices, jams and fruit-based yogurts (Shahidi and Naczk, 2004). The total phenolic content of blackcurrant berry grown in Finland was reported to range from 2230 to 2790 mg/100 g dry weight expressed as gallic acid equivalents (Kahkonen et al., 2001). Moyer et al. (2002) determined the phenolic composition of 32 cultivars of blackcurrant grown in North America and found that the total phenolic content varied considerably among the cultivars, ranging from 498 to 1342 with an overall mean of 799 mg gallic acid equivalents/100 g fresh weight basis. Anthocyanins are the most abundant phenolic compounds in blackcurrant ranging from 156 to 319 mg/100 g fresh fruit expressed as cyanidin 3-glucoside (Moyer et al., 2002). The predominant anthocyanins of the berry have been reported to include cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside and delphinidin 3-rutinoside (Mazza and Miniati, 1993; Matsumoto et al., 2001). Myricetin is the most abundant flavonol in blackcurrant followed by quercetin and kaempferol with their concentrations ranging from 8.9 to 24.5, 5.2 to 12.2, and 0.9 to 2.3 mg/100 g fresh weight, respectively (Mikkonen et al., 2001). The concentration of hydroxycinnamic acid and hydroxybenzoic acid derivatives in blackcurrant has been reported to range from 58 to 93 mg chlorogenic acid/100 g dry weight and from 6 to 12 mg gallic acid/100 g dry weight, respectively (Kahkonen et al., 2001). The

major hydroxycinnamic acids were reported to include caffeic, *p*-coumaric and ferulic and hydroxybenzoic acids were reported include ellagic, gallic, *p*-hydroxybenzoic and vanillic (Hakkinen et al., 1999a; Sellappan et al., 2002; Maatta et al., 2003).

The phenolic content of blackberry (*Rubus sp.*) has been reported to range from 383 to 844 mg gallic acid equivalents/100 g fresh weight (Fukumoto and Mazza, 2000; Moyer et al., 2002; Sellappan et al., 2002; Siriwoharn and Wrolstad, 2004). Anthocyanins are the major phenolic class in this berry with concentrations reported as 75 to 225 mg cyanidin 3-glucoside/100 g fresh weight (Siriwoharn and Wrolstad, 2004; Fan-Chiang and Wrolstad, 2005). Cyanidin 3-glucoside was identified as the major anthocyanin with cyanidin 3-glucoside, cyanidin 3-rutinoside and cyandin 3-xyloside esterified with malonic acid also being present (Fan-Chiang and Wrolstad, 2005). Blackberry fruit also contains flavonols, primarily the glycosides of quercetin, flavanols, mainly epicatechin, procyanidins, ellagic acid derivatives and ellagitannins (Siriwoharn and Wrolstad, 2004).

Red raspberry (*Rubus idaeus*) is also abundant in phenolic compounds with reported levels ranging from 2390 to 2990 mg/100 g dry weight (Kahkonen et al., 1999; Kahkonen et al., 2001) and 193 to 359 mg/100 g fresh weight (Anttonen and Karjalainen, 2005) expressed as gallic acid equivalents. Ellagitannins are the most abundant class of phenolic compounds in red raspberry, reported to range from 103 to 119 mg/100 g fresh weight, expressed as ellagic acid equivalents (Maatta-Riihinen et al., 2004a).

Strawberry fruit (*Fragaria* spp.) is reported to contain a moderate level of total phenolic compounds at 160 mg/100 g fresh weight expressed as gallic acid equivalents with the major phenolic compounds identified as anthocyanins and ellagitannins (Sun et al., 2002). Kahkonen et al. (2001) reported dry weight (mg/100 g) total phenolic compound content of 1600 to 2410, an anthocyanin content of 184 to 232 mg and an ellagitannin content of 81 to 184 as ellagic acid, cyanidin 3-glucoside and ellagic acid equivalents, respectively.

The saskatoon berry (*Amelanchier alnifolia*, Nutt.) is a fruit grown commercially in Western Canada for the fresh and frozen fruit market as well as processed jams, jellies and juice concentrates (Anonymous, 2005). The total phenolic compound content of saskatoon berry was reported to range from 170 to 520 mg tannic acid/100 g fresh weight and varied with the cultivar (Green and Mazza, 1986). Anthocyanins are the main phenolic constituents in saskatoon berry and cyanidin 3-galactoside and 3-glucoside were identified as the predominant anthocyanins in the fruit (Mazza, 1986).

2.6.3 Occurrence in Other Dietary Fruits and Vegetables

The phenolic content of other fruits is typically lower than berries on a fresh fruit basis. For example, Sun et al. (2002) reported the phenolic content (expressed as gallic acid equivalents) of a variety of dietary fruits in the United States. Among the non-berry fruits, apple (296 mg/100 g) contained the highest phenolic content followed by red grape (201 mg/100 g), pineapple (94 mg/100 g), banana (90 mg/100 g), peach (84 mg/100 g), lemon (82 mg/100 g), orange (81 mg/100 g), pear (71

mg/100 g) and grapefruit (50 mg/100 g).

Red wine, tea and pomegranate juice contain relatively high levels of phenolic compounds. Magalhaes et al. (2006) reported phenolic contents of up to 2,526 mg/L of red wine compared to 305 mg/L for white wine and 773 mg/L for green tea. Gil et al. (2000) also determined the total phenolic content of red wine, green tea and pomegranate juice and reported levels of 2,036, 1,029, and 2,566 mg *p*-coumaric acid equivalents/L, respectively.

Vegetables can be an important source of dietary phenolic compounds although less information has been published on the phenolic compound content of vegetables in comparison to fruits. The total phenolic content of carrot is reported to range from 509 to 779 mg chlorogenic acid equivalents/100 g dry weight (Talcott et al., 1999) with phenolic acids and coumarins being the most abundant (Babic et al., 1993). Onions are one of the most widely consumed vegetables and are rich in flavonoids, particularly flavonols (Shahidi and Naczk, 2004). Quercetin conjugates are the main flavonols in onions reported to range from 11 to 29 mg/100 g fresh weight in the bulb of red onion cultivars (Bilyk et al., 1984). The total phenolic content of potato tubers has been reported to range from 500 to 1,200 mg/kg dry weight basis (Griffiths et al., 1995) with chlorogenic acid accounting for up to 90% of this value (Friedman, 1997). Spinach leaf contains 162 to 483 mg chlorogenic acid equivalents/100 g fresh weight (Howard et al., 2002) and is dominated by *p*-coumaroyl-tartaric acid at levels of approximately 20 mg/100 g fresh weight (Tadera and Mitusda, 1971; Winter and Herrmann, 1986).

Leafy herbs in the Lamiaceae, including basil, marjoram, oregano, peppermint, rosemary, sage, spearmint and thyme are characterized by the occurrence of rosmarinic acid, the caffeic acid conjugate of α-hydroxyhydrocaffeic acid at concentrations ranging from 100 to 1700 mg/100 g dry basis (Exarchou et al., 2001; Exarchou et al., 2002).

Various leaf extracts from plants, shrubs and trees are of interest due to their high phenolic content and antioxidant activity. For example, the major compounds identified in phenolic extracts from soybean (*Glycine max*) were reported to be caffeic, ferulic, genistic, 4-hydroxybenzoic, 4-hydroxycinnamic, salicylic, syringic and vanillic acids as well as diadzein, genistein, naringenin and quercetin (Porter et al., 1986b). The phenolic extract from *Xanthosoma violaceum* leaves, a herbaceous plant of tropical American origin, was found to contain 2600 to 33500 mg/100 g extract, expressed as apigenin equivalents (Picerno et al., 2003). Phenolic extracts from *Leea guineense* leaves, a tropical tree used in folk medicine for its anti-inflammatory properties, were found to contain primarily quercetin conjugates as well as gallic acid, ethyl gallate, kaempferol, quercetin and quercitrin (de Beck et al., 2003) although these compounds were not quantified. Amarowicz et al. (2004) determined the antioxidant activity and total phenolic content of ethanolic extracts from the leaves of selected plants from the Canadian Prairies including bearberry (*Arctostaphylos uva-ursi*), horsetail (*Equisetum* spp.), narrow-leaved echinacea root (*Echinacea angustifolia*), senega root (*Polygala senega*) and wild-licorice (*Glycyrrhiza lepidota*). The total phenolic content of the extracts ranged from 5800

mg/100 g for horsetail leaf to 31200 mg/100 g for bearberry leaf, expressed as (+)-catechin equivalents.

2.6.4 Effect of Processing

In general, processed products such as fruit juices have significantly different flavonoid levels and profiles than the original fresh product (Spanos and Wrolstad, 1990a; Spanos and Wrolstad, 1990b; Spanos et al., 1990). Processing and preservation can expose fresh products to increased risk of oxidative damage and the activation of oxidative enzymes such as polyphenol oxidase (Lee et al., 1990; Amiot, 1995), producing brown off-colours. In addition, procedures such as canning, drying, heating, enzymic clarification, fermentation, pasteurization, solvent extraction and sulfur dioxide treatment have been reported to affect phenolic profiles and concentrations in products such as fruit juice (Spanos and Wrolstad, 1990a; Spanos and Wrolstad, 1990b; Spanos et al., 1990; Bengoechea et al., 1997; Lu and Foo, 1997) grapes (Karadeniz et al., 2000), tomatoes and related sauces (Re et al., 2002) and processed berry products prepared from bilberry, black currant, lingonberry, red raspberry and strawberry (Hakkinen et al., 2000).

Domestic preparation procedures such as chopping, shredding, peeling and cooking may decrease the phenolic content in foods (Price et al., 1997; Ewald et al., 1999) as well as inducing glucosidase-mediated formation of phenolic aglycones (Rhodes and Price, 1996). Boiling is reported to lead to reduced flavonol content in vegetables (Price et al., 1997; Ewald et al., 1999), although microwave cooking and frying have less effect presumably due to decreased leaching of phenolic

compounds from foods during these cooking procedures (Crozier et al., 1997; Price et al., 1997; Ewald et al., 1999).

Tannins in meals and flours from some oilseeds, grains and legumes possess antinutritional properties by binding and precipitating carbohydrate and protein, thereby reducing food digestibility (Shahidi and Naczk, 1995). Tannins may also precipitate a wide range of essential minerals, thus lowering their bioavailability (Faithful, 1984). Several processing methods for lowering the potential antinutritional effect of phenolic compounds are available. These include cooking and extraction of the phenolic antinutrients with aqueous or alcohol solvents, dehulling of seeds and supplementation of the diet with tannin-binding materials (Salunkhe et al., 1989).

2.7 Antioxidant and Medicinal Properties of Phenolic Compounds

It is suspected that disease prevention properties of plant and plant extracts are largely attributable to the antioxidant activity of the phenolic compounds naturally present in the plant (Heinonen et al., 1998; Lampe, 1999; Gil et al., 2000; Record et al., 2001). Due to their antioxidant activity, phenolic compounds and fruit extracts have been reported to have positive effects on cancer, cardiovascular disease, immune disorders, microbial infections, neurogenerative disease and viral infections (Macheix et al., 1990; Duarte et al., 1993; Knekt et al., 1997; Papas, 1999; Le-Marchand et al., 2000; Xu et al., 2000; DiSilvestro, 2001). The proposed antioxidant mechanisms, influence of structure and potential medicinal benefits of phenolic compounds are presented in Sections 2.7.1 to 2.7.4.

2.7.1 Reactive Oxygen Species and Free Radical Scavengers

The term reactive oxygen species (ROS) is widely used to denote oxygen-containing molecules that are more reactive than triplet state oxygen in air (Noguchi and Niki, 1998). Many ROS are reactive free radicals, that is, chemical species that contain one or more unpaired electron and are capable of independent existence (Halliwell et al., 1995). The ROS radicals (Darley-Usmar and Halliwell, 1996) include, alkoxyl radical (RO•), hydroperoxyl radical (HOO•), hydroxyl radical (HO•), nitric oxide (NO•), peroxyl radical (ROO•) and superoxide radical (O_2^-•). Certain non-radical compounds that easily form radicals are also classed as ROS and include hydrogen peroxide (HOOH), peroxynitrite ($ONOO^-$) and singlet oxygen ($^1O_2$) (Papas, 1999). The ROS that contain both nitrogen and oxygen are also referred to as reactive nitrogen species (Darley-Usmar and Halliwell, 1996).

Reactive oxygen species can be generated accidentally however they are typically produced deliberately in the human body to carry out important physiological functions. For example, ROS are essential for antimicrobial activity, energy production, and signal transduction for cellular communication (Noguchi and Niki, 1998).

In contrast to the beneficial effects of limited levels of ROS, a large excess of these chemical species in the body can damage some biological targets. The body has mechanisms to remove ROS, however, when these compounds are found in excess the body is said to be in a state of oxidative stress. There is increasing evidence, indicating that oxidative stress may play a causative role in a variety of

diseases including cancer, heart disease, immunomodulatory diseases and other chronic degenerative diseases associated with aging (Noguchi and Niki, 1998, Halliwell and Aruoma, 1997, Darley-Usmar and Halliwell, 1996).

2.7.2 Antioxidant Mechanism of Phenolic Compounds

The antioxidant activity of phenolic compounds is regarded to be related to 1) scavenging free radicals, 2) chelating transition-metals involved in free-radical production and 3) inhibiting the enzymes participating in free-radical generation (Yang et al., 2001a; Aruoma, 2002; Hensley et al., 2004).

Free radical scavengers are compounds that are capable of donating electrons or hydrogen atoms to inhibit a free radical reaction (Halliwell et al., 1995). An antioxidant effect is observed by scavenging free radicals that are involved in slowing or inhibiting the oxidative chain reaction. The free radical scavenging activity of phenolic compounds is generally attributed to their ability to donate a hydrogen atom to reduce ROS radicals (Halliwell et al., 1995). In doing so, the phenolic compounds are converted to oxidized phenoxy radicals (ArO•) that are stabile due to resonance-stabilized delocalization of the unpaired electron over the aromatic ring (Figure 2.29) (Pietta, 2000; Aruoma, 2002). For example, the reduction of peroxyl and hydroxyl radicals by phenolic compounds can be represented as follows:

$$ROO\bullet + ArOH \rightarrow ROOH + ArO\bullet$$

$$HO\bullet + ArOH \rightarrow HOH + ArO\bullet$$

whereby ArOH represents the phenolic compound and ArO• is the phenoxy radical.

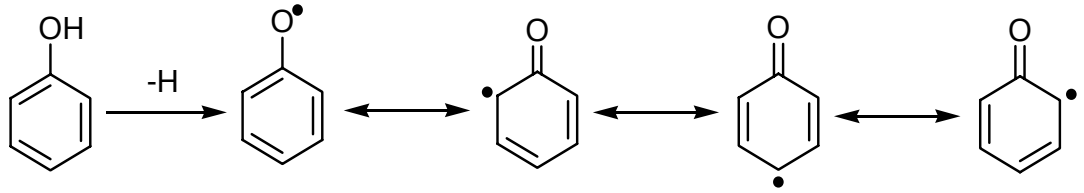


Figure 2.29 Delocalization of the unpaired electron on a phenol radical (Adapted from Gordon, 1990).

The phenoxy radical intermediates are relatively stable and therefore further oxidation reactions are not easily initiated. Non-radical products may also be formed by the coupling of ROS radicals with phenoxy radicals (Halliwell et al., 1995) as follows:

$$ROO\bullet + ArO\bullet \rightarrow ROOArO$$

Phenolic compounds may also enhance the antioxidant activity of non-phenolic antioxidants by regenerating the oxidized forms of these compounds. For example, phenolic compounds have been reported to regenerate dehydroascorbic acid to its reduced form (Cossins et al., 1998). Furthermore, Pedrielli and Skibsted (2002) reported synergistic antioxidant interactions between flavonoids and α-tocopherol *in vitro*. Their studies included the flavonoids (+)-catechin, (-)-epicatechin and quercetin, and found that the combination of any one of these flavonoids with α-tocopherol resulted in longer induction periods and an efficient inhibition of oxidation. All experimental results indicated that α-tocopherol was regenerated by the flavonoid. The suggested mechanism of interaction was by hydrogen atom transfer between flavonoids and α-tocopherol. However, the possibility of more than one type of mechanism being responsible for the

antioxidant interaction, such as formation of complexes between antioxidants or interaction through H-bonding, even though likely not to be the main mechanism, were not ruled out on the basis of the results achieved.

Phenolic compounds may also impart antioxidant properties by functioning as chelators of metal ions that are capable of catalyzing oxidation (Gordon, 1990; Aruoma, 2002). Phenolic acids and flavonoids have been shown to complex with iron (Rice-Evans et al., 1996) and copper ions (Brown et al., 1998) to provide secondary antioxidant effects.

It has been proposed that the phenolic antioxidants that are consumed in foods, medical foods and/or dietary supplements seldom reach levels *in vivo* that are sufficient to function as effective antioxidants. According to Hensley et al. (2004), the level of external phenolic antioxidants can range from concentrations of high nM to low μM concentrations in cells and blood plasma. These authors suggested that the minimum concentration for effective free radical scavenging activity of these antioxidants, however, is in the medium to high μM concentration range (i.e. 50 to greater than 100 μM). In spite of the limited antioxidant levels in foods, they noted that there are still observed physiological benefits of phenolic free radical scavengers in inhibiting diseases associated with oxidative stress. It was proposed that the protective effect of many phenolic antioxidants, however, may be largely be due to inhibition by these compounds of enzyme systems or signal transducers that are responsible for the generation of reactive oxygen species.

In support of this hypothesis for *in vivo* antioxidant activity, others have reported that phenolic compounds show marked inhibition of enzymes responsible

for the generation of ROS such as nitric oxide synthase (Fitzpatrick et al., 1998; Park et al., 2000; Wang and Mazza, 2002), tyrosine kinase (Aikayama et al., 1987; Traxler et al., 1999) and xanthine oxidase (Plessi et al., 1998).

It is important to note that under certain conditions phenolic antioxidants may act as promoters of free radicals and thus, act as pro-oxidants. Such conditions have been reported to include high concentrations of the phenoxy radicals resulting from low concentrations of synergistic antioxidants or a lack of reducing enzymes to regenerate the antioxidant from its phenoxy radical state (Patel et al., 2001).

2.7.3 Influence of Structure

The antioxidant activity of phenolic compounds reportedly varies with the structure and degree of hydroxylation of the aromatic ring (Cuvelier et al., 1992; Fukumoto and Mazza, 2000; Burda and Oleszek, 2001; Aruoma, 2002). For example, it has been found that cinnamic acid derivatives are more active antioxidants than benzoic acid derivatives, indicating that the propenoic acid moiety (-CH=CH-COOH) of the cinnamic acids enables greater antioxidant efficiency than the carboxyl (-COOH) group in benzoic acids (Cuvelier et al., 1992). This result suggested that the double bond of the propenoic acid moiety participates in stabilizing the resulting phenoxy radical by resonance (Cuvelier et al., 1992; Marinova and Yanishleiva, 2003).

It has been proposed that phenolic compounds bearing a substituent in the ortho position to the hydroxyl group form an intramolecular hydrogen bond, which

is energetically favourable, resulting in easier donation of hydrogen atoms to free radicals (De Heer et al., 1999).

Fukumoto and Mazza (2000) reported that for benzoic and cinnamic acid derivatives, flavonols and anthocyanidins, an increase in the number of hydroxyl groups on the aromatic ring lead to higher antioxidant activity *in vitro*. Compounds with three hydroxyl groups on the phenyl ring of phenolic acids or the B-ring of flavonoids had high antioxidant acitivity. The loss of one hydroxyl group decreased activity slightly whereas the loss of two hydroxyl groups significantly decreased activity. These authors also reported that glycosylation of quercetin, cyanidin, pelargonidin and peonidin resulted in lower antioxidant activity and the addition of a second glycosyl moiety decreased activity further. This decrease in antioxidant effect was attributed to steric hindrance by carbohydrate moiety.

Yang et al., (2001a) found that quercetin exhibited the highest antioxidant activity among eight flavonols studied. When the 3-hydroxyl group of quercetin was glycosylated, as in rutin, the result was a significant decrease in antioxidant activity.

Burda and Oleszek (2001) investigated the relationship between the structure of 42 flavonoids and their antioxidant and antiradical activities. They reported that flavonols with a free hydroxyl group at the C-3 position of the flavonoid skeleton showed the highest inhibitory activity to β-carotene oxidation. Antiradical activity depended on the presence of a flavonol structure or free hydroxyl group at the C-4′ position. The effect of the 4′-hydroxyl was strongly modified by other structural

features, such as the presence of free hydroxyls at C-3 and/or C-3′ and a C2-C3 double bond.

Flavonoids are widely recognized as effective antioxidants (Bors et al., 1990; Huang et al., 1992; Rice-Evans et al., 1995; Bors et al., 1997; Pietta, 2000; Shahidi, 2000; DiSilvestro, 2001). The structural arrangements imparting the greatest radical scavenging activity in these compounds have been found to be the presence of an ortho 3′,4′-dihydroxy moiety in the B-ring (e.g. catechin and quercetin), a meta 5,7-dihydroxy arrangement in the A-ring (e.g. kaempferol and apigenin) and a 2,3-double bond in combination with both the 4-keto group and 3-hydroxyl group in the C-ring for effective electron delocalization (e.g. in quercetin), as long as the *o*-dihydroxy structure is also present (Cao et al., 1977; Bors et al., 1990; Joyeux et al., 1995.; Foti et al., 1996; Bors et al., 1997; Burda and Oleszek, 2001; Tsao et al., 2005). Alterations in the arrangement of the hydroxyl groups and substitution of contributing hydroxyl groups by glycosylation decreases the antioxidant activity of these structures (Rice-Evans et al., 1995; Rice-Evans et al., 1997)

For metal ion chelation, the two points of attachment of transition metal ions to the flavonoid molecule are the o-diphenolic groups in the 3′,4′-dihydroxy positions in the B-ring and the structures, 4-keto/3-hydroxy or 4-keto/5-hydroxy in the C-ring of the flavonols (Rice-Evans et al., 1995; Rice-Evans et al., 1997).

2.7.4 Potential Medicinal Benefits of Phenolic Compounds

There are numerous *in vitro* and *in vivo* studies that have indicated the potential medicinal benefits of phenolic compounds. Excellent reviews on this topic have been published (Huang et al., 1992; Hertog et al., 1993; Boris, 1996; Heinecke, 1997; Bravo, 1998; Lampe, 1999; Nichenametla et al., 2006)

The anthocyanins extracted from bilberry (*Vaccinium myrtillus*) juice have been reported to protect the vascular system *in vivo* by increasing the permeability of capillary blood vessels (Azar et al., 1987). Anthocyanins have been shown to have stimulatory effect on the secretion of tumour necrosis factor-α in macrophages *in vitro* (Wang and Mazza, 2002) which are responsible for cytostatic and cytotoxic activities on malignant cells (Camussi et al., 1991).

Tea phenolic compounds have been reported to exhibit a very broad spectrum of medicinal activities. Green tea phenolic components inhibit intestinal uptake of glucose through rabbit intestinal epithelial cells and thus may contribute to the reduction of blood glucose levels (Kobayashi et al., 2000). Epigallocatechin gallate, a phenolic component of green tea, has been found to reduce the incidence of chemically induced tumours in the esophagus, liver, lungs, skin and stomach of experimental animals (Huang et al., 1992; Shahidi and Naczk, 2004). Epigallocatechin gallate was also reported to possess effective antioxidant properties and can provide protection *in vitro* against both peroxyl radical and hydroxyl radical-induced oxidation of DNA (Hu and Kitts, 2001). This radical scavenging activity suggests that phenolic compounds may provide protection against carcinogens. Yen and Chen (1994) showed that aqueous extracts of black,

green, oolong and pouchong tea inhibit > 90% of the mutagenicity of a selected number of chemicals toward *S. typhimurium* TA98 and TA100.

Various *in vitro* studies have reported that flavonols inhibit many pathogenic microorganisms including *Vibrio cholerae* (Boris, 1996), *Streptococcus mutans* (Sakanaka et al., 1992) and *Shigella* (Vijaya et al., 1995).

Proanthocyanidin-rich extracts from grape seeds also display anticataract activity in rats (Yamakoshi et al., 2002). Since oxidative stress is responsible for cataract formation (Taylor et al., 1995) the inhibition of cataract progression was suggested to be due to the antioxidant activity of the grape seed proanthocyanidins (Yamakoshi et al., 2002). In other studies, grape seed extracts were found to possess antiulcer properties in rats (Saito et al., 1998). It was suggested that the antiulcer property was due to the radical scavenging activity of the grape seed proanthocyanidins and their ability to bind proteins.

Oxidation of low density lipoprotein (LDL) is a key event leading to the formation of atherosclerotic plaques (De Vries et al., 1998). It is believed that ROS as well as enzymes, such as lipoxygenase and myeloperoxidase, are involved in the oxidation of LDL (Heinecke, 1997; Morton et al., 2000). Some studies have shown support for the role of dietary phenolics against cardiovascular disease due to their antioxidant effects (Hertog et al., 1993; Reynaud and Lorgeril, 1993; Nardini et al., 1995). Flavonoids have also been reported to exert positive effects on the cardiovascular system through modulation of the nitric oxide synthase system (Fitzpatrick et al., 1998; Visioli et al., 1998; Park et al., 2000). However, not all

epidemiological studies have found a protective effect of dietary phenolic compounds against heart disease (Rimm et al., 1996; Hertog et al., 1997).

Phenolic compounds in red wine, particularly the anthocyanins, hydroxycinnamic acids, other flavonoids and stilbenes, are hypothesized to be the beneficial components responsible for inhibiting the oxidation of low-density lipoprotein (LDL) and thus, providing protection against atherosclerosis (Frankel et al., 1993).

Resveratrol (a stilbene) has been reported to exhibit antioxidant (Soares et al., 2003), cardioprotective (Chen and Pace-Asciak, 1996; Wang et al., 2002) and anti-inflammatory properties (Subbaramaiah et al., 1998; Martin et al., 2004). Other studies have shown that resveratrol can inhibit cellular events associated with carcinogenesis including tumour initiation, promotion and progression (Jian et al., 1999). *In vivo* studies have indicated that resveratrol has anticancer effects against intestinal (Ziegler et al., 2004), colon (Tessitore et al., 2000) and skin cancer (Nichenametla et al., 2006). In contrast, other studies reported no anticancer effect and even carcinogenic properties at high dosage levels of resveratrol (Bove et al., 2002; Sato et al., 2003). Furthermore, Scalbert and Williamson (2000) noted that the very low concentration of resveratrol in red wine (0.3 to 2.0 mg/L) (Frankel et al., 1995) makes the attribution of protective effects to this compound unlikely.

Mammalian lignans are recognized as phytoestrogens due to their estrogen moderating activity (Dewick, 2002). There is evidence that lignans from flaxseed can reduce mammary tumor size and number in rats (Thompson et al., 1996a; 1996b). Hibisami et al. (1996) reported that catechin-rich persimmon extract

induced programmed cell death (apoptosis) in human lymphoid leukemia cells. Citrus flavonoids, in particular, flavanones have been shown to display anticarcinogenic and antiallergenic (Noguchi et al., 1999) activity *in vitro*. Wang et al. (2005) reported that lingonberries contained potent free radical scavenging activities for DPPH, peroxyl, hydroxyl and superoxide radicals. Extracts of the lingonberries also applied to cell cultures induced cancer cell apoptosis and suppressed cell damage from UV radiation. The authors suggested that the antioxidant compounds in lingonberry may make them possible candidates for a role in cancer chemoprevention and treatment.

Correlation between ingesting phenolic compounds and improved health has been reported in epidemiological studies (Knekt et al., 2002; Nichenametla et al., 2006). Knekt et al. (1997) studied 9,959 Finnish men and women and observed an inverse association between the intake of flavonoids and incidence of lung cancer. The association between flavonoid intake and lung cancer incidence was not due to the intake of antioxidant vitamins or other potential confounding factors, as adjustment for factors such as smoking and intakes of energy, vitamin E, vitamin C and β-carotene did not alter the results.

Caffeic acid is known to selectively block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma and allergic reactions (Koshihara et al., 1984). Other studies have suggested that caffeic acid and some of its esters possess antitumour activity against colon carcinogenesis (Rao et al., 1993; Olthof et al., 2001). Caffeic acid derivatives have also been shown to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase

which catalyzes the integration of the viral DNA into the host cell. Caffeic and ferulic acid derivatives have also been suggested to be potential protective agents against photooxidative skin damage (Saija et al., 1999).

Quercetin has been reported to be a potent inhibitor of human immunodeficiency virus (HIV)-1 protease (Xu et al., 2000). This compound has also been shown to decrease the infectivity of herpes simplex virus type I, poliovirus type I and parainfluenza virus *in vitro* (Kaul et al., 1985).

2.8 Determination of Antioxidant Activity

Free radical scavenging is believed to be the predominant mechanism by which phenolic antioxidants limit oxidation in food systems as well as in the human body (Hall and Cuppert, 1997; Nawar, 1985). As a result, evaluation of the potential antioxidant activity of a phenolic compound or extract typically begins with *in vitro* tests for free radical scavenging (Aruoma, 2002; Sanchez-Moreno, 2002). Compared to *in vivo* methods, *in vitro* analysis is generally lower in cost, faster and may provide mechanistic information on the antioxidant by testing various types of free radicals and/or reactive oxygen species. Compounds that have poor antioxidant activity *in vitro* will not be effective *in vivo* or in a food system (Aruoma et al., 1997). Effective antioxidant activity *in vitro*, however, does not necessarily indicate that the same compound will be effective *in vivo* since the *in vitro* test systems cannot effectively simulate the complex metabolism of the human body.

2.8.1 *In Vitro* Assays

In vitro methods to determine free radical scavenging activity often involve the use of chemicals to generate free radicals so that the radical scavenging ability of the test antioxidant can be determined (Aruoma, 2002). Several methods have been developed in which the antioxidant activity is assessed by the scavenging of synthetic radicals in polar organic solvents. Common synthetic radicals used include 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzthiazoline-sulphonic acid (ABTS) radicals, N,N-dimethyl-p-phenylene-diamine (DMPD) and 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH).

One of the most frequently used methods is the DPPH assay (Williams and Grayer, 2004). In this test, the scavenging of the stable DPPH radical (DPPH•) by an antioxidant (AH) is measured colourimetrically. The test antioxidant is allowed to react with DPPH• in methanol solution and the reduction of the radical by the AH is followed by monitoring the decrease in absorbance of the solution at 515 nm. In its radical form, DPPH• absorbs at 515 nm but upon reduction by an antioxidant, the absorption disappears (Brand-Williams et al., 1995). Most research articles in which the DPPH method has been used report the scavenging after 15 or 30 minutes of reaction time. The data is commonly reported as the concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time period (Aruoma, 2002).

Another spectrophotometric technique uses the ABTS radical cation ($ABTS^{\bullet+}$) and is known as the Trolox equivalent antioxidant activity (TEAC) (Re et al., 1999). The method involves generation of the stable $ABTS^{\bullet+}$ by reaction with

ferrylmyoglobin radical or potassium persulphate. An aliquot of this $ABTS^{\bullet+}$ solution is then mixed with the test antioxidant and allowed to react for a specified reaction time. Typically the reaction times reported in literature for this test vary from only one to six minutes, as the $ABTS^{\bullet+}$ scavenging is a fast reaction. The $ABTS^{\bullet+}$ has an absorbance maxima at 734 nm and this absorbance is lost upon reduction of the radical by the antioxidant. The antioxidant suppresses the $ABTS^{\bullet+}$ absorbance to an extent and at a rate dependent on the antioxidant activity. The TEAC value is typically reported as the ability of hydrogen– or electron-donating antioxidant to scavenge the $ABTS^{\bullet+}$ compared to that of Trolox, a synthetic, water-soluble analogue of vitamin E (Re et al., 1999) at the same molar concentration. The TEAC value for Trolox was set at 1.0, thus any antioxidant with a TEAC value greater than 1.0 is considered to be a more effective scavenger of $ABTS^{\bullet+}$ than Trolox, and the greater the TEAC value, the stronger the antioxidant activity.

Scavenging of a radical cation from the hydrophilic compound N,N-dimethyl-p-phenylene-diamine (DMPD) can also be used to test potential antioxidants for the direct scavenging ability of free radicals. In the presence of a suitable oxidant such as ferric chloride at acidic pH, DMPD is converted to a stable and coloured DMPD radical cation ($DMPD^{\bullet+}$). The UV-visible spectrum of this compound shows a maximum absorbance at 505 nm and antioxidant compounds that are able to transfer a hydrogen atom to $DMPD^{\bullet+}$ cause a decolouration of the solution which is proportional to their concentration. The antioxidant activity may also be expressed as Trolox equivalents using this assay. The presence of organic acids in some extracts may interfere with the DMPD assay (Gil et al., 2000).

Methods for determining the scavenging ability of reactive oxygen species, particularly hydroxyl radical, superoxide, hydrogen peroxide, hypochlorous acid and peroxynitrite have been developed and are widely used for *in vitro* assessment of potential antioxidant compounds.

The ORAC (Oxygen Radical Absorbance Capacity) assay measures the scavenging of peroxyl radical induced by 2,2′-azobis- (2-amidinopropane) dihydrochloride (AAPH) at 37°C using fluorescence at a wavelength of 565 nm with excitation at 540 nm (Cao et al., 1993). The determination of peroxyl radical scavenging has also been determined in human plasma and the degree of oxidation was monitored by measuring the oxygen consumed during the reaction (Cao and Prior, 1998). The test antioxidants are usually compared to Trolox .

Hydroxyl radical scavenging ability is often detemined using the deoxyribose assay (Halliwell et al., 1987). This assay uses the Fenton reaction to generate hydroxyl radical:

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + HO^- + HO\bullet$$

Hydroxyl radicals are generated by reaction of an iron-EDTA complex with hydrogen peroxide in the presence of ascorbic acid. The hydroxyl radicals attack deoxyribose to form products that, upon heating with thiobarbituric acid yield a pink chromogen. The hydroxyl radical scavenging activity of test compounds added to the reaction mixture can be measured on the basis of their ability to inhibit the degradation of deoxyribose. Hagerman et al. (1998) modified the deoxyribose

method by omitting ascorbic acid (i.e. the antioxidant radical regeneration mechanism) to evaluate the potential of certain tannins to behave as pro-oxidants.

Saint-Cricq de Gaulejac et al. (1999) evaluated hydroxyl radical scavenging in an *in vitro* system called the 3D assay (damaged DNA detection). In this system the hydroxyl radical, generated by Fenton's reaction, induced some strand breaks, modifications, or loss of bases in plasmid DNA. The protective power of the potential antioxidant on DNA was measured in terms of the ratio of percentage of repair to damaged DNA.

The enzyme xanthine oxidase is present in tissue and is one of the main sources of reactive oxygen species *in vivo*. The enzyme normally operates as a dehydrogenase that transfers electrons to nicotinamide adenine dinucleotide (NAD), as it oxidizes xanthine or hypoxanthine to uric acid (Halliwell and Gutteridge, 1990; Sanchez-Moreno, 2002). Under certain stress conditions (e.g. in a high oxidation environment), the enzyme is converted to an oxidase whereby it reacts with the same electron donors, but reduces oxygen instead of NAD, thus producing superoxide and hydrogen peroxide (Halliwell et al., 1995). An antioxidant assay based on this enzyme involves stressing tissue samples containing xanthine oxidase to produce superoxide in a system called the hypoxanthine-xanthine oxidase superoxide generating system (HX-OX). The superoxide scavenging activity of test antioxidants is determined by the decrease in superoxide. Superoxide reduces nitro-blue tetrazolium (NBT) into formazan, and formazan generation is followed by spectrophotometry at 560 nm. Any added compound that is capable of reacting with

$O_2^{\bullet-}$ inhibits the production of formazan (Robak and Gryglewski, 1988; Constantino et al., 1992).

Hydrogen peroxide-scavenging activity can be measured by using a perxoidase-based assay system. Horseradish peroxidase is often used in the assay which uses H_2O_2 to oxidize scopoletin into a nonfluorescent product. In this test system, the loss of fluorescence is an indication of the lack of antioxidant activity (Halliwell and Gutteridge, 1990).

Scavenging of hypochlorous acid (HOCl) can be determined using a myeloperoxidase/hydrogen peroxide/sodium chloride reaction system. The test antioxidant is added with 1-aminocyclo-propane-1-carboxylic acid (ACC). ACC is oxidized by HOCl to produce ethane which can be monitored by gas chromatography. Thus, the suppression of ethene by the test compound is used to indicate that HOCl scavenging activity is present (Halliwell and Gutteridge, 1990; Sanchez-Moreno, 2002).

Scavenging of peroxynitrite can be determined by the oxidation of dihydro-rhodamine 123 to fluorescent rhodamine 123. A mixture of the dihydro-rhodamine 123 with the test antioxidant is allowed to react and the level of fluorescent rhodamine 123 is measured with a fluorescence spectrophotometer with excitation and emission wavelengths of 485 and 539 nm, respectively (Chung et al., 1998).

2.8.2 *In Vivo* Assays

The first stage of *in vivo* trials usually involve administering the test compound or extract, either in a controlled diet or by other means to test animals.

The results of administering the test compound are usually monitored indirectly by measuring marker compounds that indicate antioxidant effects in, or expelled from, the test animals. Final stage *in vivo* testing can involve human clinical trials that are usually designed to show physiological or clinical benefits of the test antioxidant (Halliwell and Aruoma, 1997; Aruoma, 2002).

Peroxidation of polyunsaturated fatty acids is one of the most intensely investigated effects of free radical production in the human body (Kneepkens, 1997). A common test of the effectiveness of dietary antioxidants is to measure their effects on the peroxidizability of low-density lipoprotein (LDL) isolated from blood plasma. Two detrimental products of lipid oxidation are malondialdehyde and 4-hydroxynonenal as these compounds can cause damage to proteins and DNA (Esterbauer et al., 1992). An HPLC assay has been developed for the detection of these products (Chaudhary et al., 1994)

The hydrocarbon breath test is used to indicate the total level of lipid peroxidation in the body and involves the determination of lipid oxidation products in breath samples (Dillard et al., 1978) using gas chromatography. For example, ethane and pentane are end-products of the peroxidation of n-3 and n-6 polyunsaturated fatty acids (Dumelin and Tappel, 1977) and the concentration of alkanes are determined in breath samples by gas chromatography (Dillard et al., 1978).

The measurement of DNA oxidation in the body can be determined by measuring DNA-base damage products excreted in the urine. Such degradation compounds include 8-hydroxy-deoxyguanosine, 8-hydroxy-adenine, and 7-methyl-

8-hydroxyguanine (Stillwell et al., 1989). After determining normal background levels of these compounds in the subject's urine, the effect of a controlled diet or other means to administer the test antioxidant are measured. The DNA-damage indicator compounds are typically assayed by HPLC or GC (Loft et al., 1992).

It is evident that *in vivo* tests usually only determine indirect marker compounds of oxidation, thus leaving uncertainty of the exact physiological effect or relevance of the test antioxidant (Halliwell and Aruoma, 1997). As a result, there is still a significant requirement for the development of valid *in vivo* methods for determining free radical scavenging and antioxidant activity.

2.9 Extraction of Phenolic Compounds from Plant Material

Most phenolic compounds are stored in vacuoles in the plant and are commonly extracted in alcoholic or other organic solvents (Merken and Beecher, 2000; Santos-Buelga and Scalbert, 2000; Robbins, 2003). The phenolic composition of an extract is influenced by the chemical nature of the phenolic compounds present in the plant, the extraction method employed and the presence of interferring compounds (Naczk and Shahidi, 2004).

Typically, extraction is carried out on dried or frozen plant material since phenolic compounds are susceptible to polymerization and enzymatic degradation during storage of the harvested plant material (Price et al., 1997). The extraction solvent is added to the dried/frozen plant material and made into a slurry through blending or homogenizing (Kim and Lee, 2005b). Ultrasound has also been applied

to aid in the extraction of phenolic compounds from plant materials (Vinatoru et al., 1997). Other extraction techniques such as percolation or Soxhlet may also be used for preparing phenolic extracts (Cimpan and Gocan, 2002).

The solubility of phenolic compounds is dependent on the polarity of the solvent used. Typical solvents for phenolic compound extraction include, aqueous solutions of 60 to 80% (v/v) acetone, ethanol, ethyl acetate or methanol (Harborne and Williams, 2000; Shahidi and Naczk, 2004). These solvent systems when coupled with appropriate extraction conditions destroys cell membranes and simultaneously dissolves the phenolic compounds (Naczk and Shahidi, 2004; Kim and Lee, 2005b). Aqueous methanol solutions, specifically, are one of the most commonly employed solvents for extracting phenolic compounds, particularly phenolic acids and flavonoids, from fruit and vegetable material (Merken and Beecher, 2000) since phenolic compounds are quite stable in these methanol solutions. For example, flavones and flavonols were reported to be stable in methanol for greater than three months at 4°C (Hertog et al., 1992b). Aqueous methanol solutions also result in higher extraction yields of phenolic acids and flavonoids. Metivier et al. (1980) reported that aqueous methanol was 20% more effective than aqueous ethanol solutions of the same concentration and 73% more effective than water in extracting anthocyanins from grape pomace. Julkunen-Tiito (1985) found that higher levels of total phenolic compounds were extracted from leaves of northern willows with aqueous methanol (50% v/v) in comparison to 50% acetone.

Hot or boiling water has also been used to extract flavonoids and phenolic acids from plant materials such as tea (Lin et al., 1993; Dalluge et al., 1998).

The extraction period affects the recovery of phenolic compounds from the plant material. Extraction periods varying from one minute (Price and Butler, 1977) to 24 hours (Maxson and Rooney, 1972) have been reported.

The solvent to plant solid ratio influences the recovery of phenolic compounds from plant material. For instance, Naczk et al. (1992) found that changing the solvent to sample ratio from 5:1 to 10:1 increased the extraction of total phenolics from 773 to 805 mg/100 g of canola meal when using 70% acetone.

Typically, extractions are repeated two to three times and the extracts are combined (Merken and Beecher, 2000; Robbins, 2003). Further extractions (up to six in total) only marginally enhances the yield of phenolic compound extraction (Shahidi and Naczk, 2004).

Anthocyanins are usually extracted with an acidified organic solvent; typical acidulants include, acetic, citric, formic, hydrochloric or trifluoroacetic acid (Metivier et al., 1980; Mazza and Miniati, 1993; Wrolstad, 1993; Durst and Wrolstad, 2005). The acid provides favourable conditions for formation the red flavylium cation structure of anthocyanins which is more readily soluble and stable in the acidified solvent (Brouillard, 1982; Mazza and Miniati, 1993; Wrolstad, 1993)

Due to their low concentration, anthocyanin extracts are typically concentrated under vacuum prior to further analysis or purification (Fuleki and Francis, 1968a). Concentrating phenolic extracts, especially acidified solutions may

cause losses of labile acyl and carbohydrate conjugates from the anthocyanins (Moore et al., 1982a). Degradation of conjugates of phenolic compounds are minimized or avoided by using neutral organic solvents or boiling water or by using volatile, weak organic acids such as formic or acetic acid to acidify the solvents (Moore et al., 1982a; Moore et al., 1982b; Antolovich et al., 2000).

After removal of the extractable phenolic compounds, insoluble phenolic compounds remain in the plant matrix. These insoluble phenolic compounds include those that are bound to insoluble carbohydrates and proteins within the plant matrix, and high molecular weight phenolics such as lignins. Often, saponification is employed prior to extraction in order to cleave ester linkages to the plant insoluble matrix (Robbins, 2003). For example, Kroon et al. (1996) incubated bran cell walls in 1 M sodium hydroxide for 24 hours at 37°C prior to solvent extraction.

2.10 Analysis of Phenolic Compounds

Historically, the determination of the phenolic content of plants was carried out using spectrophotometric methods on sample extracts. A number of spectrophotometric methods for phenolic compound quantification in plant materials have been developed (Swain and Hillis, 1959; Goldstein and Swain, 1963; Price and Butler, 1977; Slinkard and Singleton, 1977; Porter et al., 1986a).

The Folin-Denis assay is a procedure often employed for quantifying the total phenolic content of plant materials and beverages. It is based on the reduction of phosphomolybdic-phosphotungstic acid (Folin-Denis) reagent to a blue-coloured complex in alkaline solution that occurs in the presence of phenolic compounds

(Swain and Hillis, 1959). Colour development and thus, the quantity of phenolic compounds is measured at 725 nm and is determined from a standard curve prepared from a pure phenolic standard.

Singleton and Rossi (1965) improved this assay by using the Folin-Ciocalteu version of the phenol-oxidizing reagent rather than the less sensitive Folin-Denis reagent. In comparison to the Folin-Denis reagent, the Folin-Ciocalteu reagent contains a higher concentration of molybdate in the reactive complex, resulting in a more sensitive reagent to reduction (Singleton and Rossi, 1965). The Folin-Ciocalteu assay is commonly used to determine the total phenolic content of foods (Shahidi and Naczk, 1995; Waterhouse, 2005). This assay is not compound specific and detects all phenolic groups found in extracts including those found in extractable proteins, such as tryptophan and other reducing substances in the extract such as ascorbic acid (Singleton and Rossi, 1965; Slinkard and Singleton, 1977; Waterhouse, 2005).

The Prussian blue method involves the reduction of ferric to ferrous ion by phenolic compounds and the formation of a ferricyanide-ferrous ion complex, also called Prussian blue (Price and Butler, 1977). This assay has been employed for the determination of the phenolic compound content of sorghum grain (Price and Butler, 1977) and mung bean (Deshpande and Cheryan, 1987).

The vanillin method is used to quantify proanthocyanins and is based on the condensation of the vanillin reagent with proanthocyanins in acidic solutions, resulting in a pink to reddish coloured product that is measured at 500 nm (Price et al., 1978). This method is specific for flavan-3-ols, dihydrochalcones and

proanthocyanins and has been employed to detect these compounds in fruits (Goldstein and Swain, 1963), grains (Burns, 1971) and legumes (Deshpande and Cheryan, 1987).

A more ubiquitous common assay for proanthocyanidins is carried out in a solution of butanol-concentrated hydrochloric acid (95:5, v/v). The solution is heated to convert proanthocyanidins to highly coloured anthocyanidins which is measured at 550 nm (Porter et al., 1986a).

Anthocyanins are typically assayed colourimetrically by the pH differential method of Fuleki and Francis (1968a; 1968b). This method involves diluting the anthocycanin extract in pH 1.0 and pH 4.5 buffers and determining the absorbance at the maximum wavelength in the visible range, typically in the range of 510 to 520 nm at both pH's. This method takes advantage of the characteristic behaviour of monomeric anthocyanins existing in their highly coloured oxonium ion form at pH 1.0 and are essentially in their colourless pseudobase form at pH 4.5 (Fuleki and Francis, 1968b; Brouillard, 1982; Mazza and Miniati, 1993). The difference in the absorbance of the same amount of extract diluted in pH 1.0 and 4.5 buffers is measured and this value is divided by the molar absorbance coefficient of the principal anthocyanin of the sample to determine the total anthocyanin content.

2.10.1 Chromatographic Methods for Phenolic Compounds

Various chromatographic techniques have been employed for the separation, purification and identification of phenolic compounds (Merken and Beecher, 2000; Robbins, 2003; Shahidi and Naczk, 2004). These include gas chromatography (GC)

(Dabrowski and Sosulski, 1984; Liggins et al., 1998; Tasioula-Maragari and Okogeri, 2001), high performance liquid chromatography (HPLC) (Merken and Beecher, 2000; Proestos et al., 2005), liquid column chromatography (Salagoity-Auguste and Bertrand, 1984; Fulcrand et al., 1999) paper chromatography (PC) (Haslam, 1965; Jackman et al., 1987) and thin-layer chromatography (TLC) (Mabry et al., 1970; Azar et al., 1987).

The most widely used technique for the isolation and identification of phenolic compounds is HPLC (Merken and Beecher, 2000; Maatta et al., 2003; Robbins, 2003). Phenolic extracts can be filtered and injected directly onto reversed phase HPLC column (Goiffon et al., 1991; Tsao et al., 2003) or pre-fractionated by gel chromatography (Amarowicz and Shahidi, 1996; Fulcrand et al., 1999), liquid-liquid extraction (Donner et al., 1997; Robards et al., 1997) or solid phase extraction (Ozmianski and Lee, 1990; Price and Wrolstad, 1995). Solid phase extraction using a disposable C_{18} cartridge is the preferred method for sample clean-up and the fractionation of phenolic acids and flavonoids (Kim and Lee, 2005b).

Some sample preparation methods of phenolic extracts include acid, alkaline or enzymatic hydrolysis to remove acyl and glycosyl moieties from the phenolic compounds (Hong and Wrolstad, 1990a; Finger et al., 1991; Gao and Mazza, 1994a; Hakkinen et al., 1998). Vigorous acid or alkaline hydrolysis may degrade the most unstable aglycones or some of the glycosides may not be hydrolyzed completely and therefore, erroneous results may be obtained (Tolonen and Uusitalo, 2004). For example, Hertog et al. (1992a) reported that even under optimized hydrolysis

conditions, the true phenolic compound content in foods may be underestimated by up to 50%.

There are numerous reviews published on the application of HPLC methodology for the analysis of phenolic compounds (Daigle and Conkerton, 1983; Robards and Antolovich, 1997; Merken and Beecher, 2000; Robbins, 2003). The HPLC columns are typically reversed phase (RP), ranging from 100 to 300 mm in length and 4.6 mm in diameter. Elution systems are usually binary with an aqueous acidified polar solvent such as aqueous acetic, formic, perchloric or phosphoric acid (solvent A) and a less polar organic solvent such as methanol or acetonitrile (solvent B) (Merken and Beecher, 2000). Thermostatically controlled columns are normally held at ambient or slightly above ambient temperatures and injections generally range from 1 to 100 μL (Daigle and Conkerton, 1983; Merken and Beecher, 2000; Robbins, 2003).

Phenolic compounds absorb ultraviolet radiation and phenolic acids and flavonoids show characteristic absorbance patterns in the ultraviolet (UV) range from 190 to 380 nm (Mabry et al., 1970; Merken and Beecher, 2000; Robbins, 2003). The anthocyanin pigments absorb in the visible range with maximum absorbance at 510 to 525 nm (Harborne, 1967; Williams and Grayer, 2004). As a result of these strong UV-visible absorption properties, phenolic compounds are most commonly detected with a photodiode array (DAD) detector (Merken and Beecher, 2000).

Structural characteristics of the flavonoids can be determined from the UV-visible spectra and two absorption bands, referred to as Band I and Band II are

characteristic of this class of phenolic compounds (Merken and Beecher, 2000; Kim and Lee, 2005a). Band II, with a maximum in the 240 to 285 nm range, is believed to arise from the A-ring of flavonoids while Band I, with a maximum in the 300 to 550 nm range, arises from the B-ring (Mabry et al., 1970). UV spectra of flavones and flavonols have a Band II peak around 240 to 280 nm and a Band I peak around 300 to 380 nm (Mabry et al., 1970). Because there is little or no conjugation between the A- and B-rings, UV spectra of flavanones and isoflavones usually have an intense Band II peak and a small Band I peak (Mabry et al., 1970). Similarily, the lack of conjugation in flavanols results in the absence of Band I peaks in the spectra of these compounds.

Anthocyanins show Band II and Band I absorption maxima in the 265 to 275 and 465 to 560 nm regions, respectively (Robards and Antolovich, 1997). Further important structural properties of the anthocyanins can be obtained from spectral data including the nature of the aglycon (anthocyanidin), the position of attachment of the sugar molecule and acylation by phenolic acids (Mabry et al., 1970; Brouillard, 1982). The presence of glycosidic substituents can be differentiated by reversed phase HPLC retention characteristics. The nature of carbohydrate substitution, however, has no effect on the anthocyanin absorbance spectrum (Harborne, 1967; Williams and Grayer, 2004).

Phenolic acids with the benzoic acid structure have their maximum absorbance wavelength (λ_{max}) in the 200 to 290 nm range. The cinnamic acid derivatives, due to additional conjugation, show an additional broad absorbance band from 270 to 360 nm (Robbins, 2003).

Based on the UV-visible absorbance spectra, the phenolic class and flavonoid sub-class can be identified for each chromatographic peak separated and detected by HPLC-DAD (Merken and Beecher, 2000; Robbins, 2003). After the quantification of individual phenolic peaks separated and detected by HPLC-DAD, Escarpa and Gonzalez (2001a) defined the total extractable phenolic chromatographic index (TPCI) as the sum of all extractable phenolic compounds obtained from the chromatogram and calculated for the anthocyanidins, chalcones, hydroxybenzoic acids, hydroxycinnamic acids, flavanols and flavonols. Tsao and Yang. (2003) also employed this method for determination of the TPCI in apples, although they identified the term as the total polyphenolic index (TPI). Compared to spectrophotometric methods, the advantages of the TPCI method for total phenolic content determination is that it is free from interference from other compounds such as proteins and it provides additional information on the content of phenolic sub-classes in the extract (Escarpa and Gonzalez, 2001a; Escarpa and Gonzalez, 2001b; Tsao and Yang, 2003; Tsao et al., 2005).

Much research has been devoted to the structural elucidation of flavonoids by mass spectroscopy (Stobiecki, 2000; Cuyckens and Claeys, 2004). Several groups have reported flavonoid identification by electron ionization (EI) (Schels et al., 1977; Schels et al., 1978; Stobiecki, 2000; Cuyckens and Claeys, 2004) or fast atom bombardment (FAB) mass spectroscopy (Li et al., 1992; Ma et al., 1997; Ma et al., 2000; Borges et al., 2001; Ma et al., 2001). More recently, electrospray ionization (ESI) (Maatta et al., 2003; Tsao et al., 2003), and matrix-assisted laser desorption ionization (MALDI) (Wang and Sporns, 1999) have been used to analyze

flavonoids. The coupling of MS to HPLC systems (LC-MS, LC-MS-MS, LC-DAD-MS) for phenolic compound separation and structural identification (Merken and Beecher, 2000; Fabre et al., 2001; Maatta et al., 2003; Parejo et al., 2004).

2.10.2 Preparative Counter-Current Chromatography of Phenolic Compounds

The development of techniques to fractionate and isolate phenolic compounds on a preparative scale is a prerequisite to more detailed studies for a better understanding of their properties. As an alternative to the classical gel chromatographic and preparative scale HPLC techniques, countercurrent chromatography (CCC) has been examined and has shown potential benefits in the preparative fractionation of plant phenolic compounds (Berthod et al., 1999; Delaunay et al., 2002; Schwarz et al., 2003).

CCC can be described as a chromatographic separation process in which a liquid phase is retained in a coil or channels by centrifugal force, while a second immiscible liquid phase continuously passes through it (Ito et al., 1966; Foucault and Chevolot, 1998). Because it is a support-free liquid-liquid chromatographic technique, solute retention is determined exclusively by their partition coefficients and equally important, the problem of adsorption of solutes onto the stationary phase is excluded (Conway, 1991; Ito, 1991).

Centrifugal partition chromatography (CPC) is a specific design of CCC whereby the stationery phase is contained in a series of partition channels as opposed to coils or circular tubes in the CCC instruments (Foucault and Chevolot,

1998). Solvent systems employed for CCC are also applicable for CPC (Foucault and Chevolot, 1998).

Salas et al. (2005) used a biphasic solvent system consisting of tert-butyl methyl ether/n-butanol/acetonitrile/water (2/2/1/5, v/v) acidified with 0.1% (v/v) trifluoroacetic acid to separate the anthocyanins in red wine by CCC. Degenhardt et al. (2000b) employed the same solvent system for the fractionation of wine mono-glycosylated anthocyanins and used a more polar biphasic solvent system consisting of ethyl acetate/n-butanol/water (2/3/5, v/v) acidified with 0.1% (v/v) trifluoroacetic acid for the separation of diglycoside anthocyanins. These authors also used a more hydrophobic solvent system of ethyl acetate/n-butanol/water (4/1/5, v/v) acidified with 0.1% (v/v) trifluoroacetic acid to separate acylated anthocyanins.

Du et al. (2004) employed a biphasic solvent system consisting of methyl tert-butyl ether/n-butanol/acetonitrile/water/trifluoroacetic acid (1/4/1/5/0.01, v/v) to isolate the anthocyanins cyanidin-3-xylosylglucoside and delphinidin-3-xylosylglucoside from bilberry (*Vaccinium myrtillus*) fruit using CCC.

Berthod et al. (1999) utilized a solvent system of butanone/water (1/1, v/v) to separate flavonols and a solvent system composed of butanol/ethyl acetate/water/acetic acid (0.3/4.6/4.95/0.05) to separate hydrolysable tannins by CCC.

CPC was employed by Delauney et al. (2002) to fractionate phenolic compounds from grape seed extract. A solvent system consisting of hexane/ethyl acetate/ethanol/water (1/8/2/7, v/v) was used to separate these compounds into two fractions; one containing approximately 75% flavanol monomers (catechin and

epicatechin) corresponding to 18% of the crude extract and another fraction of proanthocyanidins comprising 22% of the crude extract. In the same study, these authors used the same solvent system to separate stilbenoid compounds from flavanols in the phenolic extracts from grape stalks. Trans-resveratrol was then isolated to 90% purity by CPC from the stilbenoid fraction by adjusting the solvent ratio to 4/5/3/3/ (v/v).

3.0 MATERIALS AND METHODS

3.1 Raw Material

Samples of fruit and leaves from buffaloberry (*Shepherdia argentea*), chokecherry (*Prunus virginiana*) and sea buckthorn (*Hippophae rhamnoides* L.) were collected from the Prairie Farm Rehabilitation Administration (PFRA) cultivated shelterbelt at Outlook, SK. Ten bushes of each species were selected and used for sample collection over four consecutive years from 1999 to 2002. Samples were collected when the fruit first ripened in late August and also in mid-September to mid-October. Approximately two to three kilograms of fruit and 500 g of leaves were collected at each harvest. Following collection, the samples were placed in freezer bags and were transferred to a cooler containing ice within two hours after harvesting. Samples were transferred to a -20ºC freezer within five hours of harvesting and stored under these conditions until analyzed. Sub-samples (ca. 50 g) were also freeze dried (Labconco Freezone 6; Labconco Corp., Kansas City, MO, USA) and stored at room temperature (22 ± 2°C).

3.2 Chemicals

The following chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON): apigenin, arbutin, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), caffeic acid, catechin, chlorogenic acid, *p*-coumaric acid, 2-deoxy-D-ribose, 1,1-diphenyl picrylhydrazyl (DPPH), ellagic acid, epicatechin, ferric

chloride, ferulic acid, Folin-Ciocaulteu reagent, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 4-hydroxybenzoic acid, isoquercetin, kaempferol, malic acid, maltose, myricetin, naringenin, phloridzin, resveratrol, rutin, shikimic acid, sucrose, thiobarbituric acid (TBA), trans-cinnamic acid, trichloroacetic acid, and vanillic acid.

Isoflavone standards, diadzein, diadzin, genistein, genistin, glycitein and glycitin were purchased from Indofine Chemical Company (Hillsborough, NJ).

Acetic acid, acetone, acetonitrile, ascorbic acid, *n*-butanol, citric acid, dipotassium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), formic acid, fructose, galactose, glucose, hydrochloric acid, hydrogen peroxide, maleic acid, methanol (HPLC grade), oxalic acid, phosphoric acid, potassium dihydrogen phosphate, quinic acid, sodium carbonate, sodium hydroxide, succinic acid, tartaric acid and tert-butyl methyl ether were obtained from BDH (Edmonton, AB).

Anthocyanin standards, cyanidin 3-galactoside (ideain) chloride, cyanidin 3-glucoside (kuromanin) chloride, cyanidin 3-rutinoside (keracyanin) chloride and pelargonidin 3-glucoside (callistiphen) were purchased from Extrasynthase (Genay, France).

1,2-Dithio-DL-threitol (DTT) was purchased from Fluka Chemie GmbH (Buchs, Switzerland).

The water used in this research, labeled as ddH_2O (distilled and deionized water) was produced from a Millipore Milli-Q^{TM} water system (Millipore Corporation, Milford, MA).

3.3 General Physicochemical Analyses

3.3.1 Total Solids

Total solids content of the fruit was determined gravimetrically using a vacuum oven (Napco Model 5861 Vacuum Oven; Napco Scientific Co., Tulatin, OR, USA). Aluminum weigh dishes (70 mm; Fisher Scientific, Edmonton, AB) were pre-dried in a forced draft oven (Thelco Model 28; GCA/Precision Scientific, Chicago, IL, USA) at 105°C for six hours and cooled to ambient temperature (22 ± 2°C) in a glass dessicator containing Drierite dessicant (Fisher Scientific). Frozen fruit was thawed to 15 ± 5°C for 30 to 45 minutes at room temperature and ground using a mortar and pestle. Seeds of the fruit were left intact and included in the analysis of the buffaloberry and sea buckthorn fruit, and were removed from the chokecherry sample. The ground fruit sample (4 to 7 g) was accurately weighed (± 0.0002 g) into a pre-weighed moisture dish using an analytical balance (Sartorius AC 120 S, Fisher Scientific, Edmonton, AB), spread evenly throughout the dish using a spatula and the spatula was rinsed with ddH_2O (ca. 2 to 4 mL) into the dish. Samples were dried in a vacuum oven at 70°C, 30 mm Hg vacuum for 16 to 20 h. After drying, samples were cooled in a dessicator for 1 hour and weighed. Total solids content was determined using the following calculation:

$$\% \text{ total solids} = \frac{(DS - d)}{(WS - d)} \times 100 \qquad \text{(eq. 3.1)}$$

Where:

DS = weight of dried sample + dish

d = weight of dry dish

WS = weight of sample before drying + dish

All fruit samples were analyzed in triplicate.

Total solids content of the leaves was determined gravimetrically using a forced draft air oven (Thelco Model 28; GCA/Precision Scientific, Chicago, IL, USA). Aluminum weigh dishes (70 mm) were pre-dried in the oven at 105°C for six hours and cooled to ambient temperature (22 ± 2°C) in a glass dessicator containing Drierite dessicant. Frozen, whole leaves (2 to 5 g) were accurately weighed (± 0.0002 g) into a pre-weighed moisture dish using an analytical balance. Samples were dried in the forced air oven at 120 ± 2°C for 2 ± 0.2 hours. After drying, samples were cooled in a dessicator for 1 hour and weighed. Total solids content was calculated using equation 2.1. All samples were analyzed in triplicate.

3.3.2 Fruit Sample Preparation

Aqueous extracts of fruit were prepared for analysis of pH, total titratable acidity and total phenolic content. Twenty-five grams of frozen fruit were macerated with 50 mL ddH_2O in a Waring 700 laboratory blender (Waring Incorporated, Torrington, CT, USA) or a Sunbeam 10 Speed Blender (Sunbeam Canada, Toronto, ON) set to level 6, for three minutes. Seeds of the fruit remained intact under these blending conditions. The macerate was transferred to a 250 mL beaker; the blender jar was rinsed with 25 mL of ddH_2O and this solution was added to the beaker. This solution was placed on a hotplate (Corning Models PC-520 and PC-351, Corning Glass Works, Corning, NY, USA) and brought to boil for 1 hour with intermittent stirring. Water lost to evaporation was replaced at 10 – 15 minute intervals. After boiling, the sample was removed from the hotplate and allowed to

cool at room temperature (22 ± 3°C) for 30 minutes. The cooled mixture was poured into a 250 mL graduated cylinder and diluted to 175 ± 2 mL with ddH_2O. The sample was filtered (Whatman no. 4, 12.5 cm diameter; VWR Scientific, Mississauga, ON) under vacuum and stored at 4 ± 2°C for 2 to 48 hours until analyzed. This sample preparation protocol was a modification of AOAC Method 920.149 (AOAC, 2000). All samples were analyzed in triplicate.

3.3.3 pH

Sample pH was determined in triplicate at room temperature using a Fisher Accumet, Model 15, pH Meter (Fisher Scientific, Edmonton, AB). Three point calibration was accomplished employing pH 7.0, 4.0 and 2.0 buffers (Fischer Scientific).

3.3.4 Total titratable acidity (TTA)

Total titratable acidity was determined by titration with 0.1 N NaOH. Thirty mL of sample (Section 3.3.2) was poured into a 100 mL beaker and placed on a stirplate (Corning Model PC-520 and PC-351). The pH electrode on a Fisher Accumet Model 15 pH meter (Fisher Scientific) or a Radiometer PHM62 Standard pH Meter (Bach-Simpson Limited, London, ON) previously calibrated with pH 7.0 and 4.0 standard buffer solutions was immersed in the test sample contained in the beaker. The solution was moderately stirred and 0.1 N NaOH was added by burette at a constant flow until the solution pH was 6.0 to 6.8. At this point, alkali was added (4 to 8 drops at a time) until the pH reached 7.2 to 7.6. Titration was then

continued to pH 8.1 by adding single drops of alkali with rinsing of the outlet of the buret with minimal water after each drop. The volume of 0.1 N NaOH required to reach pH 8.1 ± 0.2 was determined. Percent total titratable acidity was expressed as malic acid using a conversion factor of 0.067 (AOAC, 2000) and calculated as follows:

$$\% \text{ TTA} = \frac{\text{mL 0.1 N NaOH}}{\text{mL sample}} \text{ x DF x 100 x 0.067} \qquad \text{(eq. 3.2)}$$

Where:

DF = dilution factor of the sample (175 mL/25 g fruit)

All samples were analyzed in triplicate.

3.3.5 Total Phenolic Content

One mL of appropriately diluted sample was mixed with 5 mL of Folin-Ciocalteu reagent (1:10, v/v, diluted with ddH_2O) and 4 mL of 15% (w/v) sodium carbonate in a 20 mL test tube. The mixture was vortexed on an S822 S/P® Deluxe Mixer (Scientific Products, McGaw Park, IL) for 10 to 15 seconds at a mid-range (4 to 6) setting to create a vortex to approximately one cm from the bottom of the test tube. Samples were held for 2 hours at 22 ± 2°C before the absorbance at 765 nm was measured on a Shimadzu 265 UV-visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD).

The total phenolic content was determined from a gallic acid calibration curve prepared and analyzed concurrently with the fruit samples. Standard solutions of gallic acid were prepared in ddH_2O at concentrations ranging from 10 to 50 mg/L. Gallic acid was dissolved in ddH_2O by holding the standard solutions in a

Fisher FS60 ultrasonic bath (Fisher Scientific, Edmonton, AB) for 20 minutes at 15 to 20°C. The calibration curve was constructed by plotting the absorbance at 765 nm (A_{765}) versus standard concentration. Calibration curves had correlation coefficients of 0.990 or greater. This method for total phenolic content analysis was first described by Singleton and Rossi (1965).

All samples were analyzed in triplicate.

3.3.6 Soluble Solids

Soluble solids was expressed as ºBrix and determined by refractometer. The fruit was thawed to 15 ± 5°C and juice was expressed by placing 3 to 5 fruit between two layers of cheesecloth and manually squeezing the sample. The ºBrix was determined on the juice using a Leica Auto Abbe refractometer, Model 10504 (Leica Inc., Buffalo, NY) with temperature compensation. Fruit samples were analyzed in triplicate.

3.3.7 % Seed

Whole frozen fruit (8 to 12 g) were thawed to 15 ± 5°C and accurately (± 0.0002 g) weighed on an analytical balance (Sartorious AC 120 S; Fisher Scientific, Edmonton, AB). The fruit was ground using a mortar and pestle, maintaining the seeds intact. Sample was transferred to a Tyler number 14 mesh screen (850 µm aperture) and the seeds were washed with ddH_2O (22 ± 3°C) to remove fruit pulp and skin. The washed, bare seeds were transferred into pre-dried and pre-weighed (± 0.0002 g) aluminum dish (70 mm; Fisher Scientific, Edmonton, AB) and dried at

105ºC in a forced air oven for 16 hours. The dry seeds were cooled to room temperature (22 ± 3°C), weighed (±0.0002 g) and the % seed calculated as follows:

$$\% \text{ Seed} = \frac{(\text{DSeed} + \text{d}) - \text{d}}{(\text{WF} + \text{d}) - \text{d}} \times 100 \qquad \text{(eq. 3.3)}$$

Where:

DSeed = weight of dried bare seed

d = weight of aluminum dish

WF = weight of whole fruit sample

Samples were analyzed in triplicate.

3.3.8 CIELAB Colour Parameters

CIELAB tristimulus colour coordinates were measured on a Hunterlab Labscan 6000 (Hunter Associates Laboratory, Incorporated, Reston, VA) colour meter using a 2° observer angle. The colour meter was standardized using a white tile (standard no. LS-13903) with colour coordinates L* = 94.65, a* = -0.82 and b* = 0.83. Frozen fruit was thawed to 15 ± 5°C at room temperature. The thawed fruit (25 ± 1 g) was transferred to a glass sample cup (Hunter Associates) and placed over the analysis port on the colour meter. A black cover cup was placed over the sample and the CIELAB L*, a* and b* values were measured. The hue angle (θ*) of the samples was determined by calculating $\tan^{-1}$ b*/a* (Little, 1976). Samples were analyzed in triplicate.

3.3.9 Proximate Analysis of the Fruit

Proximate analysis of the fruit samples harvested on August 30, 2002.

3.3.9.1 Moisture

Moisture content of the fruit was determined gravimetrically using a vacuum oven. Aluminum weigh dishes (70 mm) were pre-dried in a forced draft oven at 105°C for six hours and cooled to ambient temperature (22 ± 2°C) in a glass dessicator containing Drierite dessicant (Fisher Scientific). Frozen fruit was thawed to 15 ± 5°C for 30 to 45 minutes at room temperature and ground using a mortar and pestle. Seeds of the fruit were left intact and included in the analysis of the buffaloberry and sea buckthorn fruit, and were removed from the chokecherry sample. The ground fruit sample (4 to 7 g) was accurately weighed (± 0.0002 g) into a pre-weighed moisture dish using an analytical balance, spread evenly throughout the dish using a spatula and the spatula was rinsed with ddH_2O (ca. 2 to 4 mL) into the dish. Samples were dried in a vacuum oven at 70°C, 30 mm Hg vacuum for 16 to 20 h. After drying, samples were cooled in a dessicator for 1 hour and weighed. Moisture content was determined using the following calculation:

$$\% \text{ moisture} = \frac{WS - DS}{WS - d} \times 100 \qquad \text{(eq. 3.4)}$$

Where:

DS = weight of dried sample + dish

d = weight of dry dish

WS = weight of sample before drying + dish

All fruit samples were analyzed in triplicate.

3.3.9.2 Ash Content

Ash content of the fruit was determined using a muffle furnace. Crucibles

(50 mL; Fisher Scientific, Edmonton, AB) were ignited in a Thermolyne Furnatrol II model F-6020 muffle furnace (Thermolyne Corporation, Dubuque, IA) at 520 ± 5°C for 30 minutes, cooled to ambient temperature (22 ± 3°C) in a glass dessicator containing Drierite dessicant and accurately weighed (± 0.0002 g). Frozen fruit was thawed to 15 ± 5°C for 30 to 45 minutes at room temperature and ground using a mortar and pestle. The ground fruit (5 to 10 g) was accurately weighed (± 0.0002 g) into a pre-weighed crucible using an analytical balance and dried in a forced draft oven at 105 ± 3°C for 18 hours. Samples were transferred to a muffle furnace and ashed at 520 ± 5°C for 2 hours then cooled in a glass dessicator for 1 hour. A few drops of ddH_2O were added to the sample and the crucibles were placed in a forced draft oven at 105 ± 3°C for 2 hours. The crucibles were transferred into the muffle furnace and ashing was continued at 520 ± 5°C for 2 hours. Following ashing, the samples were cooled in a glass dessicator for 1 hour and weighed. The total ash content of the fruit was determined using the following calculation:

$$\% \text{ ash} = \frac{CS - C}{S} \times 100 \qquad \text{(eq. 3.5)}$$

Where:

CS = weight of crucible + sample

C = weight of dry crucible

S = weight of ground sample

3.3.9.3 Lipid Content

Freeze-dried fruit sample was ground in a coffee mill to pass through a No. 35 ASTM mesh screen (500 μm aperture). A 3.0000 ± 0.0005 g sample was

transferred onto a 150 mm filter paper (Whatman No. 2) and enclosed in a second filter paper, folded so as to prevent escape of the sample. The second filter paper was left open at one end to form a thimble shape and glass wool was placed in the top of the thimble. A 50 mL Soxhlet flask was pre-weighed and approximately 25 mL of petroleum ether was transferred into the flask. The wrapped sample was placed in a Butt extraction tube, fitted onto the Soxhlet flask and placed on a hotplate. A water-cooled condenser was fitted with a one-holed rubber stopper and inserted into the top of the Butt extraction tube. Extraction was performed by applying sufficient heat to the Soxhlet flask to cause the petroleum ether to evaporate and drop from the condenser at a rate of at 150 to 200 drops per minute. The extraction was continued for three hours. Following the extraction, the Soxhlet flask cooled to 30 ± 5°C and removed from the extraction apparatus. The flask was placed on a rotary evaporator and the petroleum ether was evaporated at 70 ± 3°C. Following evaporation, the Soxhlet flask was dried in a forced draft oven for 20 minutes and cooled to 22 ± 3°C. The cooled flask was weighed (± 0.0002 g) and the lipid content was calculated as follows:

$$\% \text{ Lipid} = \frac{SFL - SF}{SP} \times 100 \qquad \text{(eq. 3.6)}$$

Where:

SFL = weight of Soxhlet flask + lipid

SF = weight of pre-weighed Soxhlet flask

SP = weight of freeze-dried fruit sample

3.3.9.4 Protein

Protein content of the freeze-dried fruit was determined using a Leco Nitrogen Determinator System FP-428 (Leco Corporation, St. Joseph, MI). Sample cups for the nitrogen analyzer were pre-dried at 105 ± 3°C for 1 hour in forced draft oven then cooled to 22 ± 3°C in a dessicator. The freeze-dried fruit was milled in a coffee mill to pass through a No. 35 ASTM mesh screen. Milled sample (0.1000 ± 0.0005 g) was transferred to a pre-dried foil sample cone and inserted into the sample port of the Leco analyzer. Samples were analyzed using a combustion temperature of 850 ± 5°C. Nitrogen was calculated to protein using the following:

$$\% \text{ protein} = \left(\frac{N}{S} \times 100 \right) \times 6.25 \qquad \text{eq. (3.7)}$$

Where:

N = weight of nitrogen in the sample

S = weight of the dry sample.

3.3.9.5 Total Dietary Fibre

Freeze-dried fruit was milled in a coffee grinder to pass through a No. 35 ASTM mesh screen. Samples were prepared in duplicates and duplicate controls were also analyzed with the samples to measure any contribution from reagents to the residue. Milled freeze-dried fruit (1.0000 ± 0.0002) was transferred into a 400 mL beaker and mixed into 50 mL of phosphate buffer (1.40 g sodium phosphate, 8.42 g sodium dihydrogen phosphate in 1 L of ddH_2O, pH 6.0). A 0.1 ± 0.02 mL aliquot of α-amylase enzyme solution A-0164 (Sigma-Aldrich) was added to the mixture and stirred at medium speed on a stirrer hotplate (Corning). The beaker

was covered with aluminum foil and placed in a boiling water bath for 30 minutes. The sample was gently shaken at five-minute intervals during the incubation. Following the incubation the sample was cooled to 22 ± 3°C and the pH was adjusted to 7.5 ± 0.2 with sodium hydroxide solution (0.275 N). A 0.1 ± 0.02 mL aliquot of protease solution P-3910 (Sigma-Aldrich) solution (50 mg/mL) was added. The beaker was covered with aluminum foil and incubated in a water bath at 60 ± 3°C for 30 minutes. The sample was gently shaken at five-minute intervals during the incubation. Sample was cooled to 21 ± 3°C and the pH of was adjusted to 4.3 ± 0.2 with 0.35 N hydrochloric acid. A 0.3 ± 0.02 mL aliquot of amyloglucosidase solution A-9913 (Sigma-Aldrich) was added and the beaker was covered with aluminum foil followed by incubation in a 60 ± 3°C water bath for 30 minutes. The sample was gently shaken at five-minute intervals during the incubation. Dietary fibre was precipitated out of the enzyme digest by adding 280 mL of 95% (v/v) ethanol preheated to 60 ± 2°C. Precipitate was allowed to form in the sample at 22 ± 3°C for one hour.

Acid washed celite (No. C-8656; Sigma Chemical Company, St. Louis, MO) (0.5 ± 0.05 g) was transferred to a fritted, porosity no. 2 crucible (Pyrex no. 32940, coarse ASTM 40 ~ 60 μm, 60 mL), dried at 130 ± 3°C for one hour, cooled to 22 ± 3°C in a desiccator then weighed (± 0.0002). The crucible was fitted to a 500 mL filter flask and a filter bed was prepared by wetting the celite with 78% ethanol and applying vacuum to the draw the celite onto the fritted glass as an even bed. The ethanol precipitated enzyme digest was vacuum filtered through the celite bed and the resulting residue was washed successively with 3 x 20 mL of 78% ethanol, 2 x

10 mL of 95% ethanol followed by 2 x 10 mL of acetone. The crucible with the residue was dried at 105 ± 3°C in a forced draft air oven for 16 hours, cooled to 22 ± 3°C in a desiccator and weighed (± 0.0002 g). Weight of the dry residue was determined by:

$$WR = WCCR - WCC \qquad \text{eq. (3.8)}$$

Where:

WR = weight of the sample residue

WCCR = weight of crucible + celite + residue

WCC = weight of crucible + celite

The residue from one of each of the duplicates was removed from the celite bed and analyzed for protein content using the method described in Section 3.3.9.5. The second residue sample from each set of duplicates was incinerated in a muffle furnace at 525 ± 5°C for 5 hours. Samples were cooled to 21 ± 3°C in a desiccator and the crucible + celite + ash (WCCA) was weighed (± 0.0002 g). The weight of the ash was determined by:

$$WA = WCCA - WCC \qquad \text{eq. (3.9)}$$

Where:

WA = weight of the ash in the residue

WCCA = crucible + celite + ash

WCC = crucible + celite

Total dietary fibre was calculated from equation 3.10:

$$TDF\ (\%) = ([WR - WP - WA - WB] \times 100) \div WS \qquad \text{eq. (3.10)}$$

Where:

TDF = total dietary fibre

WR = average weight of the duplicate residues

WP = weight of the protein in the residue (N x 6.25)

WA = weight of the ash in the residue

WB = average weight of the duplicate control

WS = average weight of the duplicate sample

Samples were analyzed in triplicate.

3.3.10 Total Anthocyanin Content

3.3.10.1 Extraction of Anthocyanins

Anthocyanins in the chokecherry fruit were extracted in methanol/formic acid/water (MFW; 70/2/28, v/v/v). Twenty grams of frozen chokecherries were macerated with 80 mL of MFW for two minutes in a Waring 700 laboratory blender (Waring Incorporated, Torrington, CT) or a Sunbeam 10 Speed Blender (Sunbeam Canada, Mississauga, ON) set at level 6. The homogenate was transferred to a beaker, covered with parafilm and stored overnight at 4°C. The cooled sample was centrifuged at 2,500 x g for 10 minutes in a Beckman Coulter TJ-25 centrifuge (Beckman Coulter Canada Incorporated, Mississauga, ON) and the supernatant was decanted from the sample. The sediment was transferred to a 100 mL beaker and re-suspended in 40 mL of MFW with stirring. The mixture was centrifuged (2,500 x g, 10 minutes). The supernatant was recovered and the sediment was washed with 40 mL then 60 mL of MFW. Centrifugation (10 minutes at 2,500 x g) was used to recover the supernatant and sediment after each wash. All supernatant recovered from the four washings were pooled and brought to 200 mL with fresh MFW.

3.3.10.2 Determination of Total Anthocyanin Content

Total anthocyanin content of the chokecherry MFW extract was determined by the pH differential method (Fuleki and Francis, 1968a; Wrolstad, 1993). A pH 1.0 buffer solution was prepared by mixing 125 mL of 0.2 N KCl with 385 mL of 0.2 N HCl and 490 mL ddH_2O. The pH of the buffer was adjusted to pH 1.0 with 0.2 N HCl. A pH 4.5 buffer solution was prepared by mixing 440 mL 1.0 M sodium acetate with 200 mL 1.0 M HCl and 360 mL ddH_2O. The pH of the solution was measured and adjusted to pH 4.5 with 1.0 M HCl.

Two mL of anthocyanin extract was diluted to 50 mL in each of the pH 1.0 and 4.5 buffers and allowed to equilibrate in the dark for two hours. The absorbance of the samples at 512 (A_{512nm}) and 700 nm (A_{700nm}) was measured on a Shimadzu 265 UV-visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The difference in absorbance (ΔA) between the anthocyanin extract diluted in pH 1.0 and pH 4.5 buffers was calculated using equation 3.11.

$$\Delta A = (A_{512nm}\ pH\ 1.0 - A_{700nm}\ pH\ 1.0) - (A_{512nm}\ pH\ 4.5 - A_{700nm}\ pH\ 4.5) \quad \text{(eq. 3.11)}$$

The A_{700nm} was employed in the calculation of ΔA to correct for any background absorbance due to turbidity in the samples. The anthocyanin content was expressed as mg cyanidin 3-glucoside/100 g berries using a molar absorbance coefficient (ε) of 26,900 $L^{-1}M^{-1}cm^{-1}$ (Giusti and Wrolstad, 2001). Samples were analyzed in triplicate.

Calculation of the anthocyanin content was as follows:

$$TACY = \frac{((\Delta A \times MW) \times DF \times 1000)}{\varepsilon} \times 0.1 \qquad \text{(eq. 3.12)}$$

Where:

TACY = total anthocyanins expressed as mg cyanidin 3-glucoside/100g fruit.

MW = molecular weight of cyanidin 3-glucoside (449.2 g/L).

DF = dilution factor to express the samples on a per gram of fruit basis.

1000 is the conversion factor for grams to mg.

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

0.1 is the conversion factor for per 1000 grams to 100 grams basis.

3.3.11 Proanthocyanidin Content

The proanthocyanidin content of the fruits and leaves from the August, 2002 crop was determined spectrophotometrically. Phenolic extracts were diluted to provide spectrophotometric readings between 0.10 and 0.80 absorbance units. A 1.0 mL sample aliquot of adequately diluted phenolic extract was added to 9.0 mL of concentrated hydrochloric acid in *n*-butanol (10/90, v/v) in a screw top vial. The resulting solution was mixed by vortexing at a mid-range setting (5 to 6) for 10 to 15 seconds. Samples were heated for 90 minutes in an 85°C water bath (Haake W19; Haake, Berlin, Germany) then cooled to 15 to 25°C in an ice bath. The absorbance at 550 nm was measured on a UV-visible spectrophotometer. A control solution of each extract was prepared to account for background absorbance due to pigments in the extracts. The control solution consisted of the dilute phenolic extract prepared in the hydrochloric acid/*n*-butanol solvent without heating. The proanthocyanidin content was expressed as mg cyanidin/kg of sample and

calculated using the molar absorbance coefficient (ε) of cyanidin of 17,360 $L^{-1}M^{-1}cm^{-1}$ (Rosch et al., 2003).

$$\text{Proanthocyanidin content} = \frac{A_{550}\ \text{sample} - A_{550}\ \text{control}}{\varepsilon \times L} \times MW \times DF \times 1000 \quad \text{(eq. 3.13)}$$

Where:

A_{550} sample = sample absorbance at 550 nm

A_{550} control = control sample absorbance at 550 nm

ε = Molar absorbance coefficient of cyanidin (17,360 $L^{-1}M^{-1}cm^{-1}$)

L = cell path length (1 cm)

MW = molecular weight of cyanidin (287 g/mole)

DF = dilution factor to express as g/L

1000 is the conversion factor for g to mg

Samples were analyzed in triplicate.

3.4 Preparation of Crude Phenolic Extracts

Phenolic compounds of the fruits were extracted with MFW. Twenty grams of frozen fruit were added to 60 mL of MFW and homogenized in a blender for 2 minutes. The mixture was transferred to a 250 mL beaker, covered with parafilm and held for 16 to 20 h at 4°C. The sample was vacuum filtered through a Whatman #41, 12.5 cm diameter filter paper and washed with 20 mL of MFW. The sediment was removed from the filter paper and re-suspended in 60 mL of fresh MFW. After mixing moderately for 10 minutes on a stirrer the mixture was vacuum filtered and the sediment was washed with 60 mL of MFW. The filtrates from each washing

were pooled in a 200 mL volumetric flask and brought to volume with MFW.

Leaf phenolics were extracted from samples of freeze-dried leaves. Samples (20 to 30 g) of freeze-dried leaves were ground in a coffee mill and passed through a No. 35 ASTM mesh screen (500 μm aperture). Five grams of the screened powdered sample was extracted with 50 mL of MFW by blending for two minutes in a laboratory blender. The homogenate was vacuum filtered and the sediment was washed with an additional 25 mL of MFW. The filtrates were pooled in a 100 mL volumetric flask and brought to volume with MFW. A 10 mL aliquot of the resulting extract was transferred into a second 100 mL volumetric flask and brought to volume with MFW prior to analysis for total leaf phenolic content and HPLC analysis.

Further isolation, fractionation and analysis of the fruit and leaf MFW phenolic extracts were performed according to the scheme shown in Figure 3.1.

3.5 Preparation of Phenolic Isolates

Fruit and leaf crude phenolic extracts from the August, 2002 crop were isolated employing Amberlite XAD-16 (Sigma-Aldrich Canada Limited, Oakville, ON) resin. The resin was hydrated in 50% (v/v) methanol for 24 hours then poured into a glass column (11.2 cm x 2.5 cm) to produce a bed volume of about 55 mL. The resin bed was pre-conditioned by washing with 110 mL of ddH_2O followed by 110 mL of 90% (v/v) aqueous methanol and another 110 mL of ddH_2O. Phenolic extracts (10 to 14 mL) in MFW were filtered through a nylon syringe filter (13 mm diameter, 0.2 μm pore size) (Chromatographic Specialties Incorporated, Brockville,

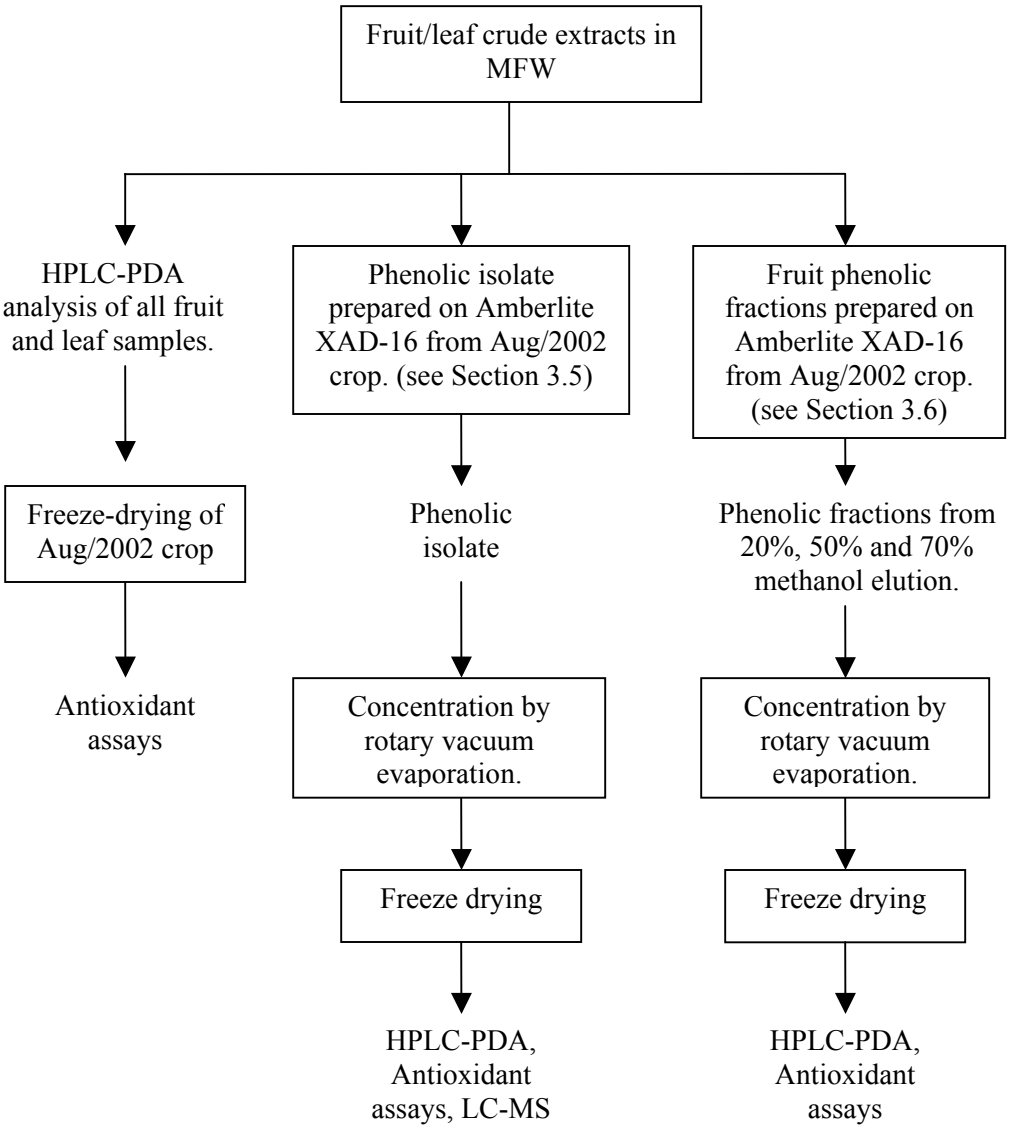


Figure 3.1 Preparation and analysis scheme for fruit/leaf phenolic extracts.

ON, Canada) and loaded onto the resin bed. Water-soluble compounds were eluted with 110 mL of ddH_2O and the phenolic compounds were collected by eluting with 110 mL of 70% (v/v) methanol. Separate Amberlite XAD-16 columns were used for each fruit and leaf phenolic extract.

3.6 Fractionation of the Fruit Crude Extracts

Fruit crude extracts were fractionated on Amberlite XAD-16 resin. The resin was hydrated in 50% (v/v) methanol for 24 hours then poured into a glass column (17 cm x 1 cm) to produce a resin bed of about 13 mL. The resin bed was pre-conditioned by washing with 40 mL of ddH_2O followed by 40 mL of 90% (v/v) methanol and another 40 mL of ddH_2O. A 4 mL sample of phenolic extract was filtered and loaded onto the resin bed. Water-soluble components of the sample were eluted with two bed volumes (27 mL) of ddH_2O (fraction #1). The phenolics were eluted in three fractions (#2 to #4) with methanol-ddH_2O solutions of increasing aqueous methanol content. Fraction #2 was produced by eluting with 27 mL of 20% (v/v) methanol. A 50% (v/v) methanol solution (27 mL) was used to elute fraction #3 and 27 mL of 70% (v/v) methanol was employed to elute fraction #4. The phenolic fractions were concentrated to 3 to 8 mL on a Buchii III Rotavapor vacuum evaporator (Brinkmann Instruments Canada Limited, Rexdale, ON) at 45°C and freeze-dried on a Hetovac VR-1 lyophilizer (Heto-Holten A/S, Allerod, Denmark). All freeze-dried phenolic fractions were stored at ambient temperature in a dessicator in the dark until analyzed by HPLC and/or for their antioxidant activity.

3.7 Acid Hydrolysis of the Fruit and Leaf Crude Extracts

Selected crude phenolic extracts were acid hydrolyzed by mixing 1 mL of extract with 1 mL of 2 N HCl in a screw top vial. The vial was flushed with nitrogen, sealed and incubated at 80°C for 1 hour. The mixtures were cooled in an ice bath and analyzed by HPLC within 2 to 12 hours.

3.8 Base Hydrolysis of the Fruit and Leaf Crude Extracts

Base hydrolysis was performed by mixing 5 mL of the crude phenolic extract with 5 mL of 2 N NaOH in a screw top vial. The vial was sealed and held at room temperature for 30 minutes then neutralized to pH 7.0 ± 0.5 using 2 N HCl. Resulting samples were analyzed by HPLC within 2 – 12 hours of preparation.

3.9 Sample Preparation for Organic Acid and Carbohydrate Analysis

Frozen fruit was thawed to 15 ± 5°C at room temperature. Sea buckthorn, chokecherry and buffaloberry fruit (4.5 ± 0.2 g) was added to 5, 7 and 11 ± 0.5 mL of ddH_2O, respectively and macerated by mortar and pestle. These dilutions resulted in juice samples measuring approximately 5.5°Brix (Leica Incorporated, Buffalo, NY). A 1.5 mL aliquot of the dilute juice was clarified by centrifuging in a Hettich Mikro 20 centrifuge (PRO Scientific Incorporated, Oxford, CT) at 7,500 rpm for five minutes. The supernatant was filtered prior to HPLC analysis. Samples were prepared in triplicate and analyzed within 12 hours of preparation.

3.10 HPLC and LC-MS Systems

All HPLC analyses in this study were performed on an Agilent Technologies 1100 Series HPLC system (Agilent Technologies Canada Incorporated, Mississauga, ON). The HPLC system consisted of a quaternary pump (model G1311A), a solvent degasser (model G1322A), an autosampler (model G1329A), a column heater (model G1316A), a UV-visible wavelength photo-diode array detector (DAD; model G1315A), a refractive index detector (model G1362A) and was controlled by ChemStation™ LC-3D software (Revision A.08.03).

The LC-MS system was a separate Agilent 1100 series HPLC system consisting of a quaternary pump (model G1312A), a solvent degasser (model G1379A) an autosampler (model G1329A) and a photodiode array detector (model G1315B). The mass spectrometer was a QStar® XL Hybrid™ (Applied Biosytems, Foster City, CA) quadrupole time of flight LC/MS/MS interfaced with a Turbo Ionspray electrospray ionizer.

3.10.1 Phenolic Profile by HPLC-DAD

Sample phenolic profiles were determined by HPLC using the aforementioned equipment. Chromatographic separation was carried out on a 250 x 4.6 mm Prodigy ODS-3, C_{18} column (5 μm; Phenomenex, Torrance, CA) in series with a C_{18} guard cartridge at 30 ± 1°C. Phenolic compound detection was achieved employing DAD detector with monitoring at wavelengths of 254, 280, 360 and 520 nm.

Two different mobile phase systems were used to separate the phenolic

compounds. Both mobile phase systems consisted of a binary solvent combination using a polar solvent A and a less polar solvent B. The initial mobile phase system consisted of 0.05 M KH_2PO_4 adjusted to pH 3.0 with 0.05 M phosphoric acid (solvent A) and 70% (v/v) acetonitrile in A (solvent B). HPLC analysis that employed this mobile phase is referred to as Method 1 throughout the remainder of this thesis. Since the phosphate solvent system was not suitable for LC-MS, a second mobile phase solvent system was developed. The second mobile phase system consisted of 10 mM formic acid in ddH_2O, pH 2.4 (solvent A) and 70% acetonitrile in A (solvent B). HPLC analysis that employed this mobile phase is referred to as Method 2 throughout the remainder of this thesis. The same elution profile was used for both mobile phase solvent systems in the following gradient system: initial, 100% A for 3 minutes, followed by a linear gradient to 4% B at 6 minutes, followed by a linear gradient to 10% B at 15 minutes, followed by a linear gradient to 15% B at 30 minutes, followed by a linear gradient to 20% B at 35 minutes, followed by a linear gradient to 23% B at 50 minutes, followed by a linear gradient to 25% B at 60 minutes, followed by a linear gradient to 30% B at 66 minutes, followed by a linear gradient to 50% B at 80 minutes, followed by a linear gradient to 80% B at 85 minutes, and a hold at 80% B for 5 minutes. The mobile phase flow rate was 0.8 mL/minute and the sample (crude extract, phenolic isolate or phenolic fraction [Figure 3.1]) injection volume was 20 µL. Samples were filtered (13 mm diameter, 0.2 µm nylon filter) before HPLC-DAD analysis.

Phenolic standards employed for retention time and spectral (190 to 700 nm) comparison and quantitation (0.4 to 25 µg/mL in MFW) were apigenin, caffeic acid,

catechin, cyanidin 3-glucoside, gallic acid, naringenin and quercetin, representing the flavones, hydroxycinnamic acids, flavanols, anthocyanins, hydroxybenzoic acids, flavanones and flavonols, respectively. All samples and standards were prepared and analyzed in triplicate. Calibration curves of these standards had correlation coefficients of 0.99 or greater.

3.10.2 LC-MS of the Fruit and Leaf Phenolic Isolates

The LC-MS analyses on fruit and leaf phenolic isolates (Section 3.5) were performed using the equipment outlined in Section 3.10. Chromatographic column and elution conditions were using Method 2 as described in Section 3.10.1. The sample injection volume was 5 μL. Mass spectrometer parameters were as follows: capillary voltage, 4000 V; drying gas temperature 400°C; negative ion mode scanning from 100 to 1000 *m/z* at a scan rate of 1.43 s/cycle and pulse duration of 10 μs.

Phenolic standards consisting of gallic acid (8.6 mg/100 mL), chlorogenic acid (17.5 mg/100 mL), phloridzin (9.1 mg/100), quercetin dihydrate (16.9 mg/100 mL) and rutin dihydrate (11.4 mg/100 mL) were prepared in MFW and analyzed by LC-MS. A second standard solution consisting of arbutin (4.8 mg/100 mL), caffeic acid (2.7 mg/100 mL), p-coumaric acid (3.3 mg/100 mL), (+)-catechin (3.2 mg/100 mL), diadzein (3.5 mg/100 mL), diadzin (4.4 mg/100 mL), (-)-epicatechin (5.9 mg/100 mL), ferulic acid (3.8 mg/100 mL), genistin (3.1 mg/100 mL), glycitein (0.4 mg/100 mL), 4-hydroxybenzoic acid (3.4 mg/100 mL), isoquercitrin (5.7 mg/100 mL), kaempferol (4.0 mg/100 mL), myricetin (1.4 mg/100 mL), (±)-naringenin (7.3

mg/mL), vanillic acid (4.5 mg/100 mL) was prepared in MFW and analyzed using the LC-MS system. The mass spectra of the standards was employed in the identification of phenolic compounds in the fruit and leaf samples.

3.10.3 HPLC Analysis of Chokecherry Anthocyanins

3.10.3.1 Pre-treatment

One milliliter of the chokecherry anthocyanin MFW extract (Section 3.3.10.1) was applied to a conditioned C_{18} Sep-Pak (Waters Corporation, Milford, MA) and washed with 2 mL water-0.01% HCl. The anthocyanins were eluted with 2 mL of methanol-0.01% HCl. The solid phase cartridge was pre-conditioned by washing with 5 mL methanol-0.01% HCl followed by 2 mL of ddH_2O-0.01% HCl.

3.10.3.2 HPLC-DAD of Anthocyanins

Anthocyanin analysis was carried out using the equipment and column outlined in Sections 3.10 and 3.10.1, employing a binary elution system described by Hong and Wrolstad (1990a). This elution solvent system consisted of aqueous 4% (v/v) phosphoric acid, pH 1.4 as solvent A and acetonitrile as solvent B. The flow rate was 0.8 mL/minute and anthocyanins were separated in the following gradient system: initial, 6% B for 12 minutes, followed by a linear gradient to 20% B at 66 minutes, and a hold at 20% B for 18 minutes. Sample injection volume was 20 μL. Sample anthocyanins were detected at 520 nm with reference at 700 nm. The spectra at the points of inflection on the increasing and decreasing slopes of each peak were taken as the reference spectra.

3.10.3.3 LC-MS of Anthocyanins

LC-MS of chokecherry anthocyanins was performed using the equipment and column outlined in Sections 3.10 and 3.10.1 and the gradient system described in Section 3.10.3.2 except 4% formic acid, pH 1.7 was employed as solvent A. The sample (Section 3.10.3.1) injection volume was 5 µL. Mass spectrometer parameters were as follows: capillary voltage, 4000 V; drying gas temperature 400°C; negative and positive ion scanning modes from 100 to 1000 *m/z* at a scan rate of 1.43 s/cycle and pulse duration of 10 µs.

A combined anthocyanin standard consisting of cyanidin 3-glucoside chloride (2.2 mg/100 mL), cyanidin 3-galactoside chloride (0.8 mg/100 mL), cyanidin 3-rutinoside chloride (4.8 mg/100 mL) and pelargonidin 3-glucoside chloride (6.2 mg/100 mL) was prepared in MFW. The anthocyanin standard solution was analyzed by LC-MS and the retention times and mass spectra of the standards was employed to identify anthocyanins in the chokecherry phenolic isolate.

3.10.4 Organic Acid Analysis

HPLC analysis of the organic acids in the fruit samples was performed using the equipment outlined in Section 3.10. Organic acid separation was carried out on a Restek Allure organic acids column, 300 x 4.6 mm, particle size 5 µm, pore size 60 Å (Chromatographic Specialties Incorporated, Brockville, ON) with elution afforded using an isocratic mobile phase of 100 mM K_2HPO_4 adjusted to pH 2.5

with 50 mM ortho-phosphoric acid at a flow rate of 0.7 mL/minute. Solute detection was at 226 nm with reference at 360 nm.

Standards consisting of acetic, ascorbic, citric, isocitric, maleic, malic, malonic, oxalic, quinic and succinic acid were prepared and chromatographed individually. Organic acids in the fruit samples were identified by retention time comparison to standards. Quantification of the organic acids was determined from calibration curves of the peak area versus concentration of the standards. The concentration of these standards ranged from 2 to 200 mg/100 mL in ddH_2O and calibration curve correlation coefficients were 0.99 or greater.

1, 2-Dithiothreitol (1.5 mg/2ml) was added to samples and standards for ascorbic acid analysis to ensure that ascorbic acid was in the reduced state.

All samples and standards were analyzed in triplicate.

3.10.5 HPLC of Carbohydrates

HPLC analysis of the carbohydrates in the fruit samples was performed using the equipment outlined in Section 3.10. Carbohydrate separation was carried out on a CapCell Pak 6 μm NH_2 UG-80A column, 250 x 4.6 mm, with a CapCell guard cartridge (Phenomenex, Torrance, CA) with elution afforded using an isocratic mobile phase of 20% (v/v) acetonitrile in ddH_2O at a flow rate of 1.0 mL/minute. Carbohydrate detection was by refractive index maintained at 30 ± 1°C.

Carbohydrates were quantified from calibration curves of standards that included D-fructose, D-glucose, sucrose and maltose. The calibration curves were constructed by plotting peak area versus concentration of the standards. The

concentration of these standards ranged from 0.1 to 6.0 g/100 mL. Calibration curves had correlation coefficients of 0.99 or greater.

All samples and standards were analyzed in triplicate.

3.11 Samples for Antioxidant Assays

Antioxidant assays were performed on phenolic extracts, isolates and fractions prepared from fruits and leaves harvested in August 2002 (Sections 3.4, 3.5 and 3.6). All spectroscopic measurements were performed on a Shimadzu 250 UV/visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD).

3.11.1 DPPH Radical Scavenging Activity

A 500 μM DPPH solution was prepared by adding 9.8 ± 0.2 mg of DPPH to a 50 mL volumetric flask and diluting to volume with 70% (v/v) aqueous methanol. The sample of freeze-dried phenolic crude extract, isolate or fraction was prepared in two to five mL of 70% (v/v) methanol at three to five concentrations ranging from 0.1 to 5.0 mg/mL to produce DPPH radical scavenging levels ranging from approximately 10 to 85%. All sample and DPPH solutions were placed in an FS ultrasonic bath (20 ± 3°C; Fisher Scientific, Edmonton, AB) for 20 minutes to ensure that all soluble components were dissolved. Fresh solutions were prepared each day of analysis.

A 0.5 mL aliquot of the sample solution was added to 2.0 mL of DPPH in a 20 mL test tube. A control solution was prepared by adding 0.5 mL 70% (v/v) methanol to 2 mL DPPH solution. Samples were vortexed on level 5 for 10 to 15

seconds and held at room temperature (22 ± 3°C) in the dark for 15 minutes. The absorbance of the sample and control solutions was determined at 517 nm and the percent DPPH radical scavenging was calculated as follows:

$$\% \text{ DPPH radical scavenging} = [1 - (\frac{A_{517nm} \text{ sample}}{A_{517nm} \text{ control}})] \times 100 \qquad \text{(eq. 3.14)}$$

Where:

A_{517nm} sample = sample absorbance @ 517 nm

A_{517nm} control = control sample absorbance @ 517 nm

A plot of the %DPPH radical scavenging versus concentration of each sample was prepared and the concentration at 50% radical inhibition (IC_{50}) was determined from the linear regression equation. Regression equations had correlation coefficients ≥ 0.91. The IC_{50} was expressed as mg solids/mL DPPH solution and the antioxidant activity of the sample was reported as $1/IC_{50}$. Hence, the higher the $1/IC_{50}$ value, the higher the radical scavenging activity. Samples and standards were analyzed in triplicate.

3.11.2 DPPH Radical Scavenging of HPLC Fractions

DPPH (14 ± 2 mg) was added to a 50 mL volumetric flask and made to volume with 70% (v/v) methanol. The solution was placed in an ultrasonic bath (20 ± 3°C) for 20 minutes to dissolve the DPPH. Fresh DPPH solution was prepared each day of analysis. Aliquots (1.0 mL) of the DPPH solution were dispensed into 85 fraction collector tubes containing 1.0 mL methanol and were placed in a Bio-Rad 2110 fraction collector (Bio-Rad Laboratories, Hercules, CA) connected to the

HPLC detector outlet. HPLC fractionation of the phenolic crude extract sample (Section 3.4: 50 μL injection) was performed using the equipment and conditions as outlined in Sections 3.10 and 3.10.1 with fractions collected at one minute intervals (0.8 mL). Each fraction was mixed by vortexing (setting 5, 10 to 15 seconds) within two minutes after collection. After 15 minutes, the absorbance at 517 nm of each fraction was measured. All fractions were measured against a control sample consisting of 1.0 mL DPPH solution in 1.0 mL methanol and 0.8 mL 70% methanol. The absorbance of the control solution was determined 15 minutes after preparation. The DPPH radical scavenging activity of the mobile phase was determined by taking fractions at 1, 20, 45 and 85 minutes of the HPLC gradient elution program.

Percent DPPH radical scavenging activity of each fraction was calculated employing the following equation:

$$\% \text{ scavenging} = [1 - (\frac{A_{517} \text{ of fraction}}{A_{517} \text{ of control}})] \times 100 \qquad \text{(eq. 3.15)}$$

All samples and standards were analyzed in duplicate.

3.11.3 TEAC Assay for ABTS Radical Scavenging Activity

A 7 mM ABTS stock solution was prepared by adding 9.8 ± 0.2 mg ABTS to a 10 mL volumetric flask and diluting to volume with ddH_2O. ABTS radical cations ($ABTS^{\bullet+}$) were produced by mixing 4 mL of the ABTS stock solution with 2 mL of 7 mM potassium persulfate (18.9 mg/10 mL ddH_2O). In order to ensure complete oxidation of ABTS, the mixture was held at room temperature in the dark for 6 to 12 h prior to analysis. The resulting $ABTS^{\bullet+}$ solution was diluted with 70%

methanol (ca 1 mL $ABTS^{\bullet+}$/50 mL) to give an absorbance reading of 0.75 ± 0.05 at 734 nm. Solutions of the phenolic samples in 70% (v/v) methanol were prepared in the following concentrations: freeze dried fruit phenolic extracts (Section 3.4), 5.0 to 100.0 mg solids/mL; fruit phenolic isolates (Section 3.5) and fractions (Section 3.6), 0.5 to 20.0 mg solids/mL; leaf phenolic extracts (Section 3.4), 0.2 to 25.0 mg solids/mL; and Trolox (Sigma-Aldrich), 0.1 mg/mL (0.47 mM) to 0.5 mg/mL (2.06 mM).

Radical scavenging analysis was performed by mixing 20 µL of the phenolic solution into 2.0 mL of $ABTS^{\bullet+}$ solution and reading the absorbance at 734 nm after 1, 2, 3, 4 and 5 minutes. A blank solution of 20 µL 70% methanol in 2.0 mL of $ABTS^{\bullet+}$ solution was prepared and analyzed. The decolourization reaction was complete after 1 minute, therefore, readings taken at 1 minute were used to calculate percent inhibition values. The percentage inhibition of absorbance of the samples was calculated as follows:

$$\%\ ABTS^{\bullet+}\ \text{inhibition} = \left[1 - \left(\frac{A_{734nm}\ \text{Sample}}{A_{734nm}\ \text{Blank}}\right)\right] \times 100 \qquad \text{(eq. 3.16)}$$

Where:

A_{734nm} Sample = absorbance of the sample @ 734 nm

A_{734nm} Blank = absorbance of the blank @ 734 nm.

The % $ABTS^{\bullet+}$ inhibition was plotted as a function of sample concentration and the linear regression equations were calculated. Correlation coefficients of the linear regression equations were ≥ 0.98. The % $ABTS^{\bullet+}$ inhibition of 1 mM Trolox was determined from the slope of the Trolox linear regression curve (r = 0.993) and was

found to be 39.9%. The sample concentration equivalent to the inhibition activity of 1 mM Trolox was calculated as follows:

$$Y_{TE} = a + (b \times 39.9) \qquad \text{(eq. 3.17)}$$

Where:

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

a = intercept of the sample linear regression equation

b = slope of the sample linear regression equation

39.9 is the % $ABTS^{\bullet+}$ inhibition of 1 mM Trolox under assay conditions.

The Trolox equivalent antioxidant capacity (TEAC) was expressed as the equivalent activity of Trolox (mM) in 100 mg sample/mL and was calculated as follows:

$$TEAC = 100/Y_{TE} \qquad \text{(eq. 3.18)}$$

Where:

TEAC = Trolox equivalent antioxidant capacity (Trolox equivalents in 100 mg sample/mL)

100 is the conversion factor to standardize the sample to 100 mg/mL

Y_{TE} = sample concentration (mg/mL) producing $ABTS^{\bullet+}$ inhibition equivalent to 1 mM Trolox.

The analysis was performed in triplicate for all samples. This antioxidant analysis method employing potassium persulfate to generate the ABTS radical was developed by Re et al. (1999).

3.12 Centrifugal Partition Chromatography (CPC) of Chokecherry Anthocyanins

3.12.1 Extraction of Anthocyanins for CPC

Frozen chokecherries (40 ± 0.2 g) harvested in August, 2002 were added to 160 ± 2 mL MFW in a blender jar. The sample was macerated for 2 minutes on blender level 6 (mid-range) and transferred into 250 mL centrifuge bottles. The macerate was centrifuged at 2,500 x g for 10 minutes and the supernatant (anthocyanin extract) was recovered for anthocyanin isolation.

3.12.2 Anthocyanin Isolation on Amberlite Resin

Amberlite XAD-16 resin (Sigma-Aldrich) was hydrated in 50% (v/v) methanol for 16 hours and poured into a glass column (30 cm x 2.5 cm) to produce a bed volume of 59 mL (12 cm x 2.5 cm). The resin bed was pre-conditioned by sequential washings of 120 mL ddH_2O, 120 mL of 90% methanol and 120 mL ddH_2O. Chokecherry anthocyanin extract (6.5 mL: Section 3.12.1)) was loaded onto the pre-conditioned bed and washed with 55 mL of ddH_2O. Anthocyanins were then eluted from the XAD-16 bed with 55 mL of 70% methanol. The procedure was repeated 11x and the anthocyanin isolates were pooled (572 mL) and concentrated on a vacuum evaporator at 45°C to 3.3 mL. The resulting solution was placed in an amber glass vial and stored at 4 ± 1°C.

3.12.3 Selection of Solvent System

Three potential solvent systems for CPC separation were evaluated by

determining the partition coefficient of the two major chokecherry anthocyanins in the solvents. Solvent system I consisted of ethyl acetate/ethanol/water (4:1:5, v/v/v) with 0.025% formic acid. Solvent system II was *n*-butanol/tert-butyl methyl ether (TBME)/acetonitrile/water (2:2:1:5, v/v/v/v) containing 0.1% formic acid and solvent system III consisted of hexane/ethanol/water (5:4:1, v/v/v). Each solvent system (100 mL) was prepared in a 100 mL volumetric flask and transferred to individual 100 mL separatory funnels. The solvent was shaken vigorously in the separatory funnel and allowed to separate overnight. Upper and lower phases were collected separately. Partition coefficient of the anthocyanins in each solvent system was determined by mixing 2 mL each of upper and lower solvent phase in a 7 mL culture tube. One mL of the concentrated anthocyanin isolate (Section 3.12.2) was added to the culture tube and vortexed at level 6 for 30 seconds. The culture tubes were covered with parafilm and held at room temperature (22 ± 3°C) without agitation for 1 hour to allow the upper and lower solvent phases to separate. Upper and lower phases were collected separately using transfer pipets and the anthocyanin content of each phase was determined by HPLC analysis (section 3.10.3.2). Partition coefficients were ascertained for each of the two anthocyanin peaks by determining the ratio of the peak area in the upper versus the lower solvent phase (Schwarz et al., 2003) and calculated using equation 3.19:

$$P = \frac{\text{ACY upper phase}}{\text{ACY lower phase}} \qquad \text{(eq. 3.19)}$$

Where:

P = partition coefficient

ACY upper phase = chromatographic area of the anthocyanin peak at 520 nm with reference 700 nm of the upper phase solvent.

ACY lower phase = chromatographic area of the anthocyanin peak at 520 nm with reference 700 nm of the lower phase solvent.

3.12.4 Separation by CPC

Separation of the anthocyanins was performed on a model CPC-LLB centrifugal partition chromatograph (Sanki Engineering Limited, Kyoto, Japan). The CPC column consisted of a circular partition disk with 2,136 channels and a total column capacity of 1,360 mL. A Lab Alliance Series III HPLC pump (Rose Scientific, Edmonton, AB) was connected to the CPC for delivery of the stationary and mobile phases onto the column. Solvent system II was employed for CPC separation of the anthocyanins and was prepared by adding *n*-butanol (800 mL), TBME (800 mL), acetonitrile (400 mL), ddH_2O (2,000 mL), and 90% (v/v) formic acid (4 mL) to a 4,000 mL separatory funnel. The solvent mixture was shaken vigorously for 1 minute, held overnight without agitation and the resulting lower and upper phases were collected separately. Upper phase solvent was pumped onto the CPC column at 15.8 mL/minute, ascending mode, at 300 rpm column rotor speed. Equilibration of the stationary and mobile phases on the column was achieved by pumping the lower phase solvent (mobile phase) in descending mode through the column at 7.8 mL/minutes, 900 rpm and 960 psi. The hydrodynamic equilibrium of the two phases was reached when the mobile phase emerged from the

column. Under these conditions, 200 ± 20 mL of stationery phase was displaced from the column. Three mL of anthocyanin concentrate (17% total solids, w/w) was diluted in 2 mL of stationary (upper) phase and injected onto the column through a 6 mL injection loop on the CPC system. Elution was performed in descending mode at a flow rate of 6.5 mL/minute, column rotation of 800 rpm and backpressure of 690 psi. The eluant was monitored with a Waters 490 UV-visible detector (Water Corporation, Milford, MA) at 520 nm. Eluant stream was split so the flow rate to the detector was 0.8 ± 0.1 mL/minute. Fractions were collected in 20 mL screw-top vials. The preparative scale separation the anthocyanins was performed in triplicate.

3.13 Statistical Analysis

Statistical analysis of was performed using JMP statistical analysis software (SAS Institute, Cary, NC). Two-way main effects analysis of variance was used to determine significant differences (5% confidence level) among the crop years and harvest date. The harvest date was partitioned into two dates consisting of the August picking and September/October collection. Means were ranked according to Tukey's HSD (honestly significant difference) multiple comparison test (Steel and Torie, 1980) for responses that showed significance difference in the year and/or date of harvest.

4.0 RESULTS AND DISCUSSION

4.1 Physicochemical Parameters of the Fruit

4.1.1 Introduction

Samples were harvested when the fruit first ripened in mid- to late August. Since buffaloberry and sea buckthorn fruit are often harvested in the fall or winter (Remlinger and St. Pierre, 1995; Li and Schroeder, 1999), a second harvest was collected in September of 1999, 2000 and 2001 and in October of 2002. Except for the 2000 crop, the overnight temperatures during the week before harvest were below 0°C prior to the September/October collection. In general, there was a greater abundance of ripe fruit in August compared to September and October. The chokecherry bushes were especially depleted in fruit in September and October and most of the chokecherry fruit on these harvest dates was considered overripe as the harvest season for this fruit in Saskatachewan is from mid-August to mid-September (Anonymous, 2004).

Proximate analysis was determined on the fruit harvested on August 30, 2002. Further chemical and physical analyses were determined at each harvest date for each of the four crop years, with the exception of the August, 2001 samples. The August, 2001 samples were not analyzed since they thawed and decayed somewhat due to a breakdown during freezer storage.

Monomeric anthocyanins were not detected in the buffaloberry or sea

buckthorn samples using the pH differential method and therefore the total anthocyanin content was not included in the results of these fruits.

4.1.2 Proximate Composition of the Fruit

The proximate composition provides a general overview of the nutritional value of a food and includes analysis of the ash, moisture, lipid and protein content (Kirk, 1993). Analysis for total dietary fibre (TDF) content was also included in this study since TDF is relevant to the nutritional value of fruits (Salunkhe et al., 1991). Proximate composition of the buffaloberry, sea buckthorn and chokecherry fruit harvested on August 30, 2002 is shown in Table 4.1. The chokecherry seed was removed from the fruit prior to proximate analysis since the seed is considered to be inedible due to its high content of the toxin, hydrocyanic acid (St. Pierre, 1993; USDA, 2004b).

There was considerable difference in moisture content among the fruits and sea buckthorn was found to contain the highest moisture level at 81.0 ± 0.5%. This moisture level was lower than the range of 83.4 to 86.7% reported for sea buckthorn grown in Finland (Tang and Tigerstedt, 2001) and higher than the 74% reported in Chinese sea buckthorn by Ma and Cui (1987) as cited by Tang and Tiegerstadt (2001). Chokecherry contained the lowest moisture level at 66.8 ± 0.4%. This moisture content was similar to the range of 62.0 to 68.5% reported for chokecherry fruit cultivars grown in Saskatoon, SK (Zatylny et al., 2005a). Buffaloberry fruit was lower in moisture content (71.5 ± 0.5%) than sea buckthorn and higher than chokecherry fruit. The proximate analysis of buffaloberry fruit has not been

previously reported.

Table 4.1 Proximate Composition of Buffaloberry, Chokecherry and Sea buckthorn Fruit Harvested on August 30, 2002[1]

	Buffaloberry	Chokecherry[2]	Sea buckthorn
Moisture	71.5 ± 0.5	66.8 ± 0.4	81.0 ± 0.5
Protein[3]	2.5 ± 0.1	2.2 ± 0.1	2.5 ± 0.1
Lipid[3]	0.5 ± 0.1	0.2 ± 0.1	2.6 ± 0.1
Ash[3]	0.6 ± 0.1	0.9 ± 0.1	0.4 ± 0.1
TDF[4]	6.0 ± 0.2	8.9 ± 0.3	4.2 ± 0.1

[1] Mean ± standard deviation of triplicate samples.
[2] Fruit – seed. Buffaloberry and sea buckthorn included the seed.
[3] % fresh weight basis.
[4] % total dietary fibre.

The protein contents of buffaloberry and sea buckthorn fruit were similar (2.5 ± 0.1 and 2.5 ± 0.1%, respectively) and marginally higher than chokecherry (2.2 ± 0.1%). Bekkar and Glushenkova (2001) reported that the protein content of sea buckthorn fruit ranged from 0.9 to 3.11% fresh weight basis, depending on the variety and geograpical location. A protein content of 2.6% was reported for wild chokecherry pulp from the northeastern United States, (Wittmer, 1996; Wittmer, 1998).

Chokecherry was found to contain the highest level of ash of 0.9 ± 0.1% in

comparison to buffaloberry of 0.6 ± 0.1% and sea buckthorn of 0.4 ± 0.1%. The ash content of these fruits has not previously been reported.

Chokecherry was shown to contain the highest total dietary fibre content of 8.9 ± 0.3%. All of the fruit analyzed was higher in TDF than the ranges of 2.3 to 3.0 g/100 g fresh weight basis and 1.0 to 2.2 g/100 g fresh weight basis reported for blueberries and strawberries, respectively (Mongeau and Brassard, 1989).

Sea buckthorn contained the highest (2.6 ± 0.1%) lipid content among the fruit samples analyzed. This content was similar to the level reported for sea buckthorn grown in China (subsp. *sinensis*) and lower than sea buckthorn grown in Finland of 2.1 ± 0.5% and 3.5 ± 0.7%, respectively (Yang and Kallio, 2001). Both the seed (7.3 ± 1.4 to 11.3 ± 2.5%) and flesh (1.6 ± 0.5 to 2.8 ± 0.7%) of sea buckthorn have been reported to contain high levels of lipid and greater than 75% of the fruit lipid is contained in the flesh (Yang and Kallio, 2001). Potential medicinal and functional food uses of sea buckthorn lipid include lowering the risk of cardiovascular and cerebrovascular diseases, regulation of immunofunctions and anti-inflammatory action (Jiang et al., 1993; Li and Tan, 1993; Li and Schroeder, 1996; Che et al., 1998; Eccleston et al., 2002).

4.1.3 Physicochemical Composition of Buffaloberry Fruit

4.1.3.1 pH

The mean pH of buffaloberry fruit was found to be 3.1 ± 0.1 and ranged from 3.0 ± 0.1 in the fruit harvested in August of 1999, 2000 and 2002 to pH 3.3 ± 0.1 in the October 14, 2002 sample picked on (Table 4.2). Fruit pH was

Table 4.2 Physicochemical Composition of Buffaloberry Fruit

Year	Harvest date	pH	TTA[1]	°Brix	°Brix / TTA	Total Solids Content[2]	Total Phenolic Content[2]	% Seed/Fruit
1999	August 17	3.0 ± 0.1*a*[3,4]	2.75 ± 0.12*c*	21.4 ± 0.2*ab*	7.8 ± 0.4*a*	25.8 ± 0.4*a*	0.95 ± 0.04*c*	4.5 ± 0.1
	September 18	3.1 ± 0.1*bc*	2.25 ± 0.01*ab*	19.9 ± 0.7*a*	8.9 ± 0.4*ab*	27.9 ± 0.3*b*	0.84 ± 0.03*b*	5.5 ± 0.3
2000	August 25	3.0 ± 0.1*ab*	2.60 ± 0.02*c*	21.7 ± 1.2*ab*	8.4 ± 0.5*a*	28.5 ± 0.4*b*	0.86 ± 0.02*b*	5.1 ± 0.4
	September 17	3.2 ± 0.1*c*	2.36 ± 0.10*b*	20.0 ± 1.0*a*	8.5 ± 0.7*a*	28.0 ± 0.7*b*	0.79 ± 0.03*a*	4.3 ± 0.4
2001	September 30	3.2 ± 0.1*c*	2.33 ± 0.06*b*	23.9 ± 0.7*bc*	10.2 ± 0.5*c*	27.0 ± 0.6*ab*	0.77 ± 0.02*a*	4.5 ± 0.1
2002	August 30	3.0 ± 0.1*ab*	2.32 ± 0.03*b*	23.3 ± 0.1*bc*	10.0 ± 0.1*bc*	27.1 ± 0.6*ab*	0.82 ± 0.01*ab*	4.1 ± 0.4
	October 14	3.3 ± 0.1*c*	2.10 ± 0.03*ab*	24.0 ± 0.5*bc*	11.5 ± 0.4*c*	28.0 ± 0.9*b*	0.83 ± 0.01*ab*	4.5 ± 0.3
Overall Mean ± SD		3.1 ± 0.1	2.38 ± 0.21	22.0 ± 1.7	9.3 ± 1.3	27.6 ± 1.1	0.84 ± 0.06	4.7 ± 0.5
Year/harvest significance[5]		Y, M	Y, M	Y	Y	Y, M	Y, M	NS

[1] % total titratable acidity as malic acid.
[2] % fresh weight basis of whole fruit.
[3] Mean ± standard deviation of three replicates.
[4] Means in the same column followed by a common letter are not statistically different ($P \leq 0.05$) by Tukey's HSD (Honestly Significant Difference) multiple range test.
[5] Significant difference ($P \leq 0.05$) among the years (Y) and/or months (M) of harvest by two-way ANOVA. NS = no significant difference.

significantly higher in samples harvested after August and is attributed to the decrease in total titratable acidity with increasing harvest dates (see Section 4.1.3.2). An increase in fruit pH with later harvesting dates has also been reported for other fruits such as raspberries (Sjulin and Robbins, 1987) and blueberries (Galletta et al., 1971).

4.1.3.2 Total Titratable Acidity (TTA)

Total titratable acidity expressed as citric acid ranged from 2.10 ± 0.03% to 2.75 ± 0.12% with a mean value of 2.38 ± 0.21% (Table 4.2). The TTA did not significantly vary between the years of harvest. Fruit harvested in September and October was 10 to 20% lower in TTA than samples collected in August. The lower levels of TTA in fruit harvested after August may have been due to the exposure of the developing fruit to freezing temperatures that occurred prior to harvest in September and October. Freezing of fruit during its on-bush development has been reported to cause damage to cell structure which leads to metabolic disturbances resulting in changes in fruit acidity (Hendrix and Redd, 1995). The TTA of buffaloberry was higher than that reported for other berry fruits such as blueberry, (Galletta et al., 1971) and saskatoon berry, reported at 1.56 ± 0.38% and 0.2 to 0.5%, respectively (Green and Mazza, 1986; Zatylny et al., 2005b).

4.1.3.3 Total Solids Content

Buffaloberry fruit had a mean total solids content of 27.6 ± 1.1% and ranged from 25.8 ± 0.4% in the August 17, 1999 sample to 28.5 ± 0.4% in the fruit

harvested on August 25, 2000 (Table 4.2). There was no significant difference in total solids content of the fruit among the year or date of harvest. Although overripe, dehydrated berries were present on the bushes in September and October, only ripe fruit were collected for analysis. This result suggested that the total solids content did not change in ripe fruit harvested in summer or fall.

4.1.3.4 Soluble Solids

The mean soluble solids content of the fruit as determined by the °Brix value, was 22.0 ± 1.7 °Brix and ranged from 19.9 ± 0.7 to 24.0 ± 0.5 °Brix (Table 4.2). There was no significant difference in the °Brix between harvest dates within the years of collection. The fruit in 2001 and 2002 crops were approximately 14% higher in °Brix than the 1999 and 2000 crops. Interestingly, data supplied by PFRA, Outlook, indicated a total rainfall in the growing season (i.e. from May to October) was lower in 2001 and 2002 compared to 1999 and 2000 (Figure 4.1). The total rainfall in these growing seasons was 267.4, 240.9, 142.2 and 182.0 mm. in 1999, 2000, 2001 and 2002, respectively. In agreement with this rainfall-soluble solids relationship, others have reported that lower levels of irrigation resulted in higher levels of soluble solids in fruits such as apples (Leib et al., 2006) and peaches (Crisosto et al., 1994). There was no notable trend in either the mean monthly temperature (Figure 4.2) or mean temperature throughout the growing season among the four crop years (range of 12.7 in 1999 to 15.2°C in 2001).

Although buffaloberry fruit is generally regarded as astringent (Remlinger and St. Pierre, 1995) and not recognized for its sweet taste, the determined soluble

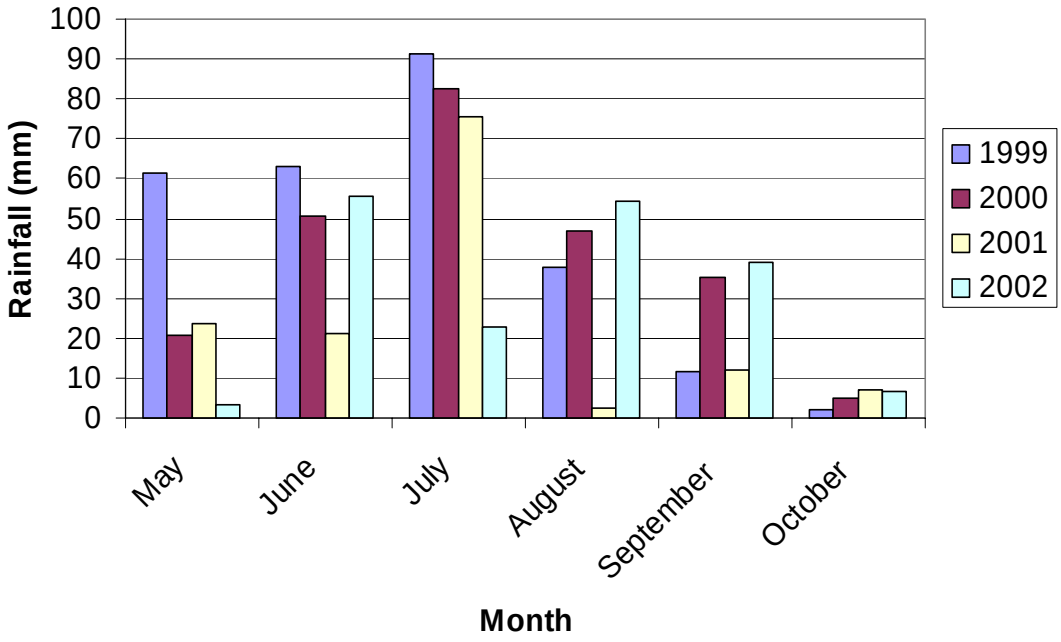


Figure 4.1 Total monthly rainfall measured by tipping bucket rain gauge at PFRA Research Station, Outlook, SK for the growing seasons of 1999 to 2002. Data supplied by PFRA Research Station, Outlook.

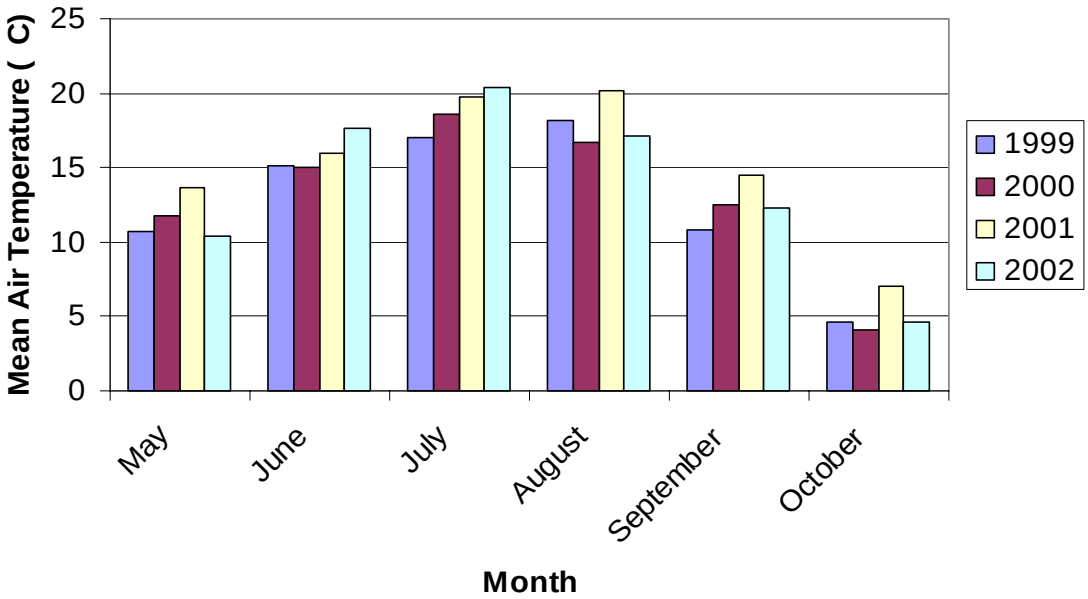


Figure 4.2 Mean monthly air temperature at PFRA Research Station, Outlook, SK for growing seasons of 1999 to 2002. Data supplied by PFRA Research Station, Outlook.

solids level was similar to sweet berries such as blueberry, reported at 10 °Brix (Galletta et al., 1971; Kalt and McDonald, 1996) and saskatoon berry, reported to be 16.1 ± 1.04 °Brix (Zatylny et al., 2005b). Soluble solids of fruits are largely composed of carbohydrates and to a lesser extent, organic acids (Potter, 1978). Thus, despite the high level of °Brix, the astringent character of buffaloberry fruit may be due to its relatively high level of organic acids, as evidenced by the higher TTA level observed for this fruit (Section 4.1.3.2). In addition, the phenolic composition of the fruit may also contribute to its astringency (Section 2.6.1).

4.1.3.5 °Brix/TTA Ratio

The °Brix/TTA ratio is a quality attribute used by the fruit industry to indicate the tartness of fruits and fruit juices (Potter, 1978). The higher the °Brix/TTA ratio the sweeter and less tart is the fruit and/or fruit juice (Potter, 1978). This ratio increases with maturity of the fruit and is used to identify the optimum maturity for harvesting to produce maximum product quality (Rebeck, 1995). Typical levels of °Brix/TTA ratio vary with the type of fruit (Potter, 1978; Rebeck, 1995).

The mean °Brix/TTA ratio was 9.3 ± 1.3 and ranged from 7.8 ± 0.4 in the August, 1999 sample to 11.5 ± 0.4 in the October, 2002 buffaloberry samples (Table 4.2). Fruit harvested in 2001 and 2002 were higher in °Brix/TTA ratio than the 1999 and 2000 crops. The higher values for the 2001 and 2002 crop years was due to higher °Brix values in these berries since the TTA values did not vary significantly among the crop years (Table 4.2).

The °Brix/TTA ratios were similar to those reported for blueberry by Galletta et al. (1971) of 7.14 ± 2.01 and lower than those reported for saskatoon berry of 39.9 ± 4.4 (Zatylny et al., 2005b). There was no significant change in the °Brix/TTA ratio between the harvest dates within the crop years. This result indicated that the °Brix/TTA ratio cannot be used as an indicator of fruit maturity from August to mid-October for buffaloberry fruit. The lack of significant difference among the harvest dates also indicated that ripe buffaloberries can be collected in August through to mid-October.

4.1.3.6 Total Phenolic Content

Total phenolic content was determined using the Folin-Ciocalteu reagent on aqueous extracts of macerated fruit. The extracts were prepared following the AOAC method for extraction of water soluble compounds from fruit (AOAC, 2000). This method involves boiling the sample to enhance extraction and to inactivate enzymes present in the fruit tissue. The total phenolic content determined using the Folin-Ciocalteu reagent provides a crude measurement of the water extractable total phenolic compounds in the fruit. These compounds impact the antioxidant capacity of the fruit and can impart flavour and astringency.

The mean total phenolic content determined using the Folin-Ciocalteu reagent was 0.84 ± 0.06% and ranged from 0.79 ± 0.03% in the buffaloberries harvested in September, 2000 to 0.95 ± 0.04% in the berries harvested in August, 1999 (Table 4.2). There was a significant decrease in phenolic content of fruit harvested later in the season in 1999 and 2000. The decrease in fruit phenolic

content in the fall may be attributed to lower temperatures (Figure 4.1) and rainfall (Figure 4.2) during fruit development in September and October of 2000. Others have reported that phenolic biosynthesis is dependent on the environmental temperature during fruit development (Tang and Tigerstedt, 2001). The phenolic content has been reported to increase at higher temperatures of growth and decrease at lower temperatures of the growing season (Wang and Zheng, 2001).

4.1.3.7 % Seed/Fruit

The mean % seed/fruit content in buffaloberry was 4.7 ± 0.5% and ranged from 4.1 ± 0.4% in the August 30, 2002 sample to 5.5 ± 0.3% in the September 18, 1999 sample (Table 4.2). There was no change in the % seed/fruit content among the harvest dates or among the crop years.

4.1.3.8 Organic Acids

Organic acids identified in the buffaloberry fruit determined by comparison of the relative retention time (RRT) of the HPLC peaks to standards, and by spiking samples of the fruit extract with the following standards, ascorbic, citric, malic, oxalic, quinic and succinic acids (Figure 4.3). The concentration of individual organic acids in selected fruit samples from 1999 to 2002 are shown in Table 4.3. Citric acid was the most abundant organic acid (1499 ± 253 mg/100 g) and succinic (963 ± 159 mg/100 g) and malic acid (924 ± 189 mg/100 g) were next highest in concentration. The mean ascorbic acid content in buffaloberry was 221 ± 32 mg/100 g and ranged from 176 ± 44 to 235 ± 28 mg/100 g of fruit. This level of

ascorbic acid is higher than other common commercial berry fruits such as blueberry and cranberry which have been reported at 7 to 20 mg/100 g (Kalt and Dufour, 1997) and 9 mg/100 g (USDA, 2005), respectively. The ascorbic acid content of buffaloberry is approximately 4x higher than that of oranges (53 mg/100 g; (USDA, 2005). These results suggest that buffaloberry is an excellent source of ascorbic acid and enhances the potential of this fruit for food use.

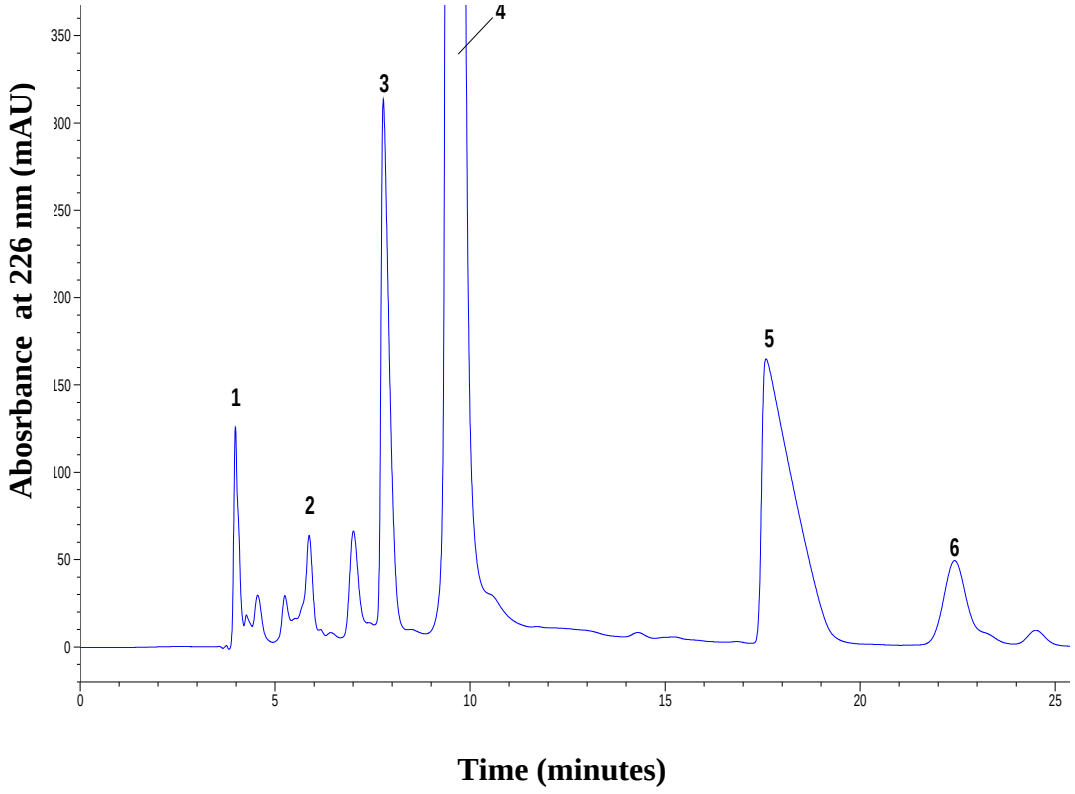


Figure 4.3 HPLC-DAD chromatogram of the organic acid profile of buffaloberry fruit. Peak identities: 1) oxalic acid; 2) quinic acid; 3) malic acid; 4) ascorbic acid; 5) citric acid; 6) succinic acid.

Table 4.3 Organic Acid Composition and Concentration in Buffaloberry Fruit

Year	Harvest date	Ascorbic Acid[1]	Citric Acid[1]	Malic Acid[1]	Oxalic Acid[1]	Quinic Acid[1]	Succinic Acid[1]	Total Acids[1]
1999	August 17	176 ± 44[2,3]	1275 ± 211	869 ± 27	109 ± 18	193 ± 50	808 ± 158	3430
2000	September 18	235 ± 28	1709 ± 33	865 ± 93	86 ± 16	199 ± 83	1091 ± 65	4185
2001	September 30	218 ± 10	1668 ± 29	1149 ± 87	69 ± 20	243 ± 36	1043 ± 40	4390
2002	August 30	216 ± 5	1347 ± 295	812 ± 38	75 ± 11	144 ± 41	908 ± 181	3502
Overall Mean		211 ± 32	1499 ± 253	924 ± 189	84 ± 22	195 ± 60	963 ± 159	3877 ± 483

[1] mg/100 g whole fruit (wet weight basis)
[2] Mean ± standard deviation
[3] Means in the same columns were not statistically different ($P \leq 0.05$) by Tukey's HSD (honestly significant difference) multiple range test.

4.1.3.9 Fructose, Glucose and Sorbitol

The major soluble carbohydrates and polyol in buffaloberry harvested in August 2002 were identified by comparison of the HPLC-RI peaks to those of standards and by spiking samples of the fruit extracts with standards. Glucose, fructose and sorbitol were identified in the expressed juice (Figure 4.4) and the concentration of these compounds was determined from standard curves prepared in ddH_2O. The major carbohydrate was glucose and was 1.3 fold greater than fructose and 14.7 fold greater than the concentration of sorbitol (Table 4.4).

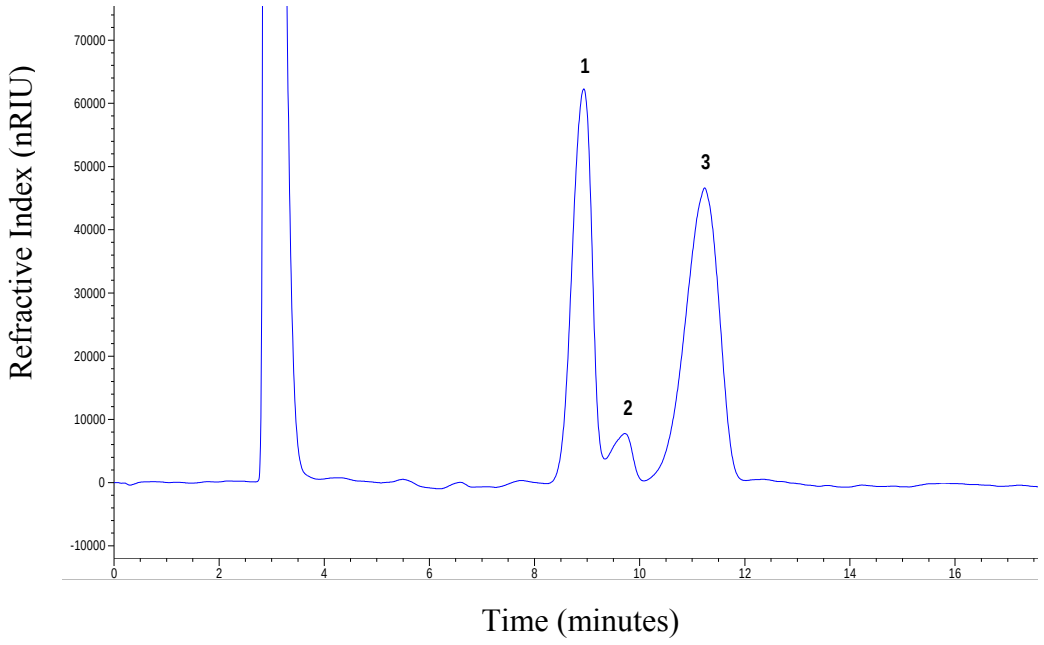


Figure 4.4 HPLC-RI chromatogram of fructose, glucose and sorbitol in buffaloberry fruit. Peak identities: 1) fructose, 2) sorbitol, 3) glucose.

The total soluble carbohydrate and polyol concentration (10.4 g/100 g juice) of buffaloberry juice determined by HPLC-RI was less than one-half of the soluble solids content of the juice (22.0 °Brix; Table 4.2) as determined by refractometry.

This result indicates that the other soluble components in the fruit, primarily organic acids (overall mean = 3.9 g/100 g juice; Table 4.3) and soluble phenolic acids (overall mean = 0.84 g/100 g fruit; Table 4.2), play a major role in the °Brix content of buffaloberry juice.

Table 4.4 Fructose, Glucose and Sorbitol Content of Buffaloberry, Chokecherry and Sea buckthorn Fruit Harvested August 30, 2002

	Buffaloberry	Chokecherry	Sea buckthorn
Glucose[1]	5.9 ± 0.5[2]	3.2 ± 0.4	2.7 ± 0.5
Fructose[1]	4.5 ± 0.1	3.6 ± 0.1	1.3 ± 0.1
Sorbitol[1]	0.4 ± 0.1	2.6 ± 0.2	0.3 ± 0.1
Total[1]	10.4 ± 0.1	6.8 ± 0.1	4.1 ± 0.1
Glucose/Fructose	1.3	0.9	2.1

[1] g/100 g fruit, fresh weight basis.
[2] Mean ± standard deviation of three replicates.

4.1.3.10 Colour Parameters

The CIELAB L* value is a measure of the lightness of the sample, a* value measures redness when positive and greenness when negative, and b* measures yellowness when positive and blueness when negative. Hue angle (θ*) is calculated from the $\tan^{-1}$ b*/a* and identifies the predominant colour of the sample (Little, 1976). Colour parameters for the buffaloberry fruit over the four years of collection

are shown in Table 4.5.

The a* values in the 1999, 2000 and 2001 crops were significantly higher than those of the 2002 fruit. The lower a* value for the 2002 crop may be largely attributed to the fruit picked on October 14, 2002 (a* value of 23.8 ± 2.7) which was harvested at least 14 days later than fruit harvested from the 1999 to 2001 crop years. This result suggests there was a loss of red colour late in the harvest season. Fruit harvested in 1999 had the greatest b* values. The hue angle did not vary among the years of harvest, suggesting the variation in the a* and b* values was not perceived in the overall colour of the fruit.

Table 4.5 CIELAB Colour Values of Buffaloberry Fruit

Year	Harvest date	L*	a*	b*	Hue angle*
1999	August 17	28.7 ± 0.3[1]	33.4 ± 2.4*b*[2]	25.4 ± 2.1*b*	37.3 ± 4.2
	September 18	23.4 ± 2.1	31.9 ± 2.5*b*	21.3 ± 1.6*ab*	33.7 ± 1.9
2000	August 25	28.5 ± 3.8	30.9 ± 1.4*b*	18.0 ± 0.4*a*	30.2 ± 0.7
	September 17	25.8 ± 1.2	31.3 ± 1.5*b*	21.1 ± 1.2*ab*	34.0 ± 1.1
2001	September 30	28.8 ± 3.3	30.7 ± 1.3*b*	20.0 ± 3.4*a*	32.9 ± 3.2
2002	August 30	24.8 ± 0.6	29.0 ± 1.8a*b*	20.6 ± 2.0*ab*	35.4 ± 0.9
	October 14	24.9 ± 0.4	23.8 ± 2.7*a*	17.4 ± 0.7*a*	36.3 ± 2.0
Overall Mean		26.4 ± 2.9	30.1 ± 3.4	21.0 ± 4.0	34.3 ± 3.0
Year, harvest significance[3]		NS	Y	Y	NS

[1] Mean ± standard deviation.
[2] Means in the same column followed by a common letter are not statistically different ($P \leq 0.05$) by Tukey's HSD multiple range test.
[3] Significant difference ($P \leq 0.05$) among the years (Y) and/or months (M) of harvest by two-way ANOVA. NS = no significant difference.

4.1.4 Physicochemical Composition of Chokecherry

4.1.4.1 pH

Chokecherry fruit had a mean pH value of 4.2 ± 0.2 and ranged from 3.9 ± 0.1 to 4.4 ± 0.1 (Table 4.6). A similar mean pH of 4.06 ± 0.08 for chokecherry fruit was recently reported by Zatylny et al. (2005a). Chokecherry fruit harvested in August was lower in pH than samples collected in September and October. This observed increase in pH at later harvest dates can be attributed to the degradation of organic acids in the fruit as ripening progressed (Galletta et al., 1971; Potter, 1978; Terrier et al., 2001). In Saskatchewan, chokecherries are at optimum ripeness in late August to mid-September (St. Pierre, 1993; Anonymous, 2004) and thereafter, degradation of organic acids can be expected.

The pH of chokecherry was considerably higher than that of buffaloberry (3.0 ± 0.1; Table 4.2) fruit. This can be explained by the higher levels of organic acids in buffaloberry (Table 4.3) which was 3.7x higher than those in chokecherry fruit.

4.1.4.2 Total Solids Content

The mean total solids content of whole chokecherry fruit was 45.8 ± 4.1% and ranged from 39.3 ± 0.3 to 53.4 ± 2.7% (Table 4.6). Chokecherry fruit contained significantly higher levels of total solids in comparison to buffaloberry (27.6 ± 1.1%; Table 4.2). The high level of total solids in chokecherry could be attributed to the large seed that is present in the fruit (Section 4.1.4.6). Fruit harvested on

Table 4.6 Physicochemical Composition of Chokecherry Fruit

Year	Harvest date	pH	TTA[1]	°Brix	°Brix / TTA	Total Solids Content[2]	Total Phenolic Content[2]	Total Anthocyanin Content[3]	% Seed/Fruit
1999	August 17	3.9 ± 0.1*a*[4,5]	0.54 ± 0.03*a*	22.7 ± 0.9*a*	42.4 ± 2.2*a*	45.6 ± 0.2*b*	0.48 ± 0.01*a*	195 ± 12*ab*	15.6 ± 0.4*a*
	September 18	4.1 ± 0.1*b*	0.53 ± 0.02*a*	23.3 ± 0.5*ab*	43.8 ± 2.0*a*	46.2 ± 0.5*b*	0.48 ± 0.01*a*	204 ± 5*ab*	19.0 ± 0.6*ab*
2000	August 25	4.1 ± 0.1*b*	0.62 ± 0.03*b*	26.0 ± 0.7*ab*	42.7 ± 2.8*a*	46.6 ± 0.2*b*	0.50 ± 0.03*ab*	233 ± 12*bc*	19.5 ± 0.4*b*
	September 17	4.4 ± 0.1*c*	0.64 ± 0.01*b*	33.0 ± 4.0*c*	51.8 ± 6.1*a*	45.5 ± 2.5*b*	0.69 ± 0.01*c*	272 ± 24*cd*	19.4 ± 2.4*b*
2001	September 30	4.4 ± 0.1*c*	0.63 ± 0.14*b*	33.0 ± 3.2*c*	54.2 ± 10.8*a*	44.4 ± 1.2*b*	0.58 ± 0.07*b*	168 ± 7*a*	16.4 ± 0.4*ab*
2002	August 30	4.2 ± 0.1*b*	0.54 ± 0.01*a*	29.2 ± 0.8*bc*	53.7 ± 2.5*a*	39.3 ± 0.3*a*	0.48 ± 0.05*a*	285 ± 19*cd*	19.1 ± 0.4*b*
	October 14	4.4 ± 0.1*c*	0.54 ± 0.02*a*	41.4 ± 3.0*d*	76.4 ± 8.1*b*	53.4 ± 2.7*c*	0.79 ± 0.04*c*	279 ± 25*cd*	25.2 ± 2.1*c*
Overall Mean		4.2 ± 0.2	0.58 ± 0.05	29.8 ± 6.6	52.2 ± 12.3	45.8 ± 4.1	0.57 ± 0.12	234 ± 46	19.2 ± 3.1
Year, harvest significance		Y[6], M	Y	Y, M	Y, M	M	Y,M	Y	Y, M

[1] % total titratable acidity as malic acid.
[2] % fresh weight basis of whole fruit.
[3] mg cyanidin 3-glucoside/100 g fruit.
[4] Mean ± standard deviation.
[5] Means in the same column followed by a common letter are not statistically different ($P \leq 0.05$) by Tukey's HSD (honestly significant difference) multiple range test.
[6] Significant difference ($P \leq 0.05$) among the years (Y) and/or months (M) of harvest by one-way ANOVA. NS = no significant difference.

October 14 was significantly higher in total solids content than the samples collected on August 30 in 2002. The chokecherry sample harvested in October was collected after the optimal harvest date for the fruit (St. Pierre, 1993) and as a result, the fruit was notably dehydrated.

4.1.4.3 Total Titratable Acidity (TTA)

The mean total titratable acidity of chokecherry was 0.58 ± 0.05% malic acid and ranged from 0.53 ± 0.02% in the berries harvested in September, 1999 to 0.64 ± 0.01% for those harvested on September 17, 2000 (Table 4.6). There was no significant difference in the TTA among the samples harvested at different dates within the same year. This result was somewhat contradictory to the trend of higher pH values in the samples harvested later in the year (Section 4.1.4.1), suggesting the TTA analysis was less sensitive to the change in organic acid composition of the fruit. This lower sensitivity may be due to the buffer system in the fruit. For instance, during the alkaline titration to pH 8.1 in the TTA analysis, the natural buffer system in the fruit has been shown to be resistant to pH change (Galletta et al., 1971).

The chokecherry fruit was considerably lower in TTA levels than buffaloberry (mean = 2.54 ± 0.19%; Table 4.2). Similarily, as mentioned in Section 4.1.4.1, the organic acid content in buffaloberry was considerably higher than that of chokecherry.

4.1.4.4 Soluble Solids Content

The mean soluble solids content of chokecherry fruit was 29.8 ± 6.7 °Brix and ranged from 22.7 ± 0.9 °Brix in the August 17, 1999 sample to 41.4 ± 3.0 °Brix in the fruit harvested October 14, 2002 (Table 4.6). There was a significant increase in °Brix at later harvest dates with the largest increase being 47% in the October 14 versus August 30 samples in 2002. This result may be partly attributed to dehydration of the chokecherry fruit harvested in October, after its optimal harvest date (St. Pierre, 1993).

Soluble solids content of the chokecherries in this study was high in comparison to the values of 18.3 °Brix reported by Zatlyny et al. (2005a).

4.1.4.5 °Brix/TTA

The mean °Brix/TTA ratio of chokecherry fruit was 52.1 ± 12.3 and ranged from 42.4 ± 2.2 in the August, 1999 sample to 76.4 ± 8.1 in the fruit harvested in October, 2002 (Table 4.6). With the exception of the 2002 crop in which the fruit collected in October was overripe, there was no significant difference in the °Brix/TTA ratio at different harvest dates within the year of harvest. The °Brix/TTA ratio for ripe chokecherry fruit ranged from 42.7 ± 2.2 in the August, 1999 sample to 53.7 ± 10.8 in the September 30, 2001 fruit. In comparison, °Brix/TTA ratios for ripe apple, blueberry and saskatoon berry have been reported to range from 10.0 to 37.7 (Fuleki et al., 1995), 11.2 to 38.5 (Galletta et al., 1971) and 19.7 to 66.2 (Green and Mazza, 1986), respectively.

4.1.4.6 % Seed/Fruit Content

The mean seed content of chokecherry fruit was 19.2 ± 3.1% and ranged from 15.6 ± 0.4% in the August, 1999 sample to 25.2 ± 2.1% in the fruit harvested on October 14, 2002 (Table 4.6). These % seed/fruit values were higher than those values reported by Zatlyny et al. (2005a) for chokecherry fruit of 13.2 ± 0.90% and ranged from 9.4 to 16.0%.

4.1.4.7 Total Phenolic Content

Total phenolic content of the chokecherry fruit aqueous extracts determined using the Folin-Ciocalteu reagent ranged from 0.48 ± 0.01 to 0.79 ± 0.04 g/100 g with an overall mean value of 0.57 ± 0.52 g/100 g (Table 4.6). The total phenolic content of chokecherry was 1.5x lower than that of buffaloberry (Table 4.2).

4.1.4.8 Total Anthocyanin Content

The total anthocyanin content was determined using the pH differential method in which the absorbance of anthocyanin extracts at pH 1.0 and 4.5 are determined. Monomeric anthocyanins are highly coloured at pH 1.0 and colourless at pH 4.5 (Wrolstad, 2005). Since the visible spectrum showed the anthocyanin maximum absorbance to be at 512 nm, the difference in absorbance (ΔA) was calculated from (A_{512nm} pH 1.0 – A_{700nm} pH 1.0) – (A_{512nm} pH 4.5 - A_{700nm} pH 4.5). Absorbance at 700 nm was used to account for any turbidity in the samples. The total anthocyanin content was expressed as mg cyanidin 3-glucoside/100 g fruit and

calculated using its molar absorbance coefficient of 26,900 $L^{-1}M^{-1}cm^{-1}$ (Wrolstad, 2005).

The monomeric anthocyanin content of the chokecherry fruit ranged from 168 ± 7 to 285 ± 19 with a mean value of 255 ± 35 mg/100 g fruit (Table 4.6). This anthocyanin concentration can be considered high when compared to those reported for other berries such as saskatoon (*Amelanchier alnifolia*) and cranberry (*Vaccinium oxycoccus*) of 25 to 179 mg/100 g and 78 mg/100 g fresh fruit, respectively (Mazza and Miniati, 1993). In further support of this finding, the anthocyanin content of a saskatoon berry fruit sample collected from a local orchard was determined to be 138 ± 14 mg/100 g fresh fruit, expressed as cyanidin 3-glucoside. This anthocyanin concentration was within the range reported in literature and lower than that of chokecherry fruit. The anthocyanin content of chokecherry is considerably lower, however, than the levels reported for highbush blueberry (*Vaccinium corymbosum*) and bilberry (*Vaccinium myritillus*) of up to 495 mg/100 g (Ballinger, 1972) and 300 to 698 mg/100 g fresh fruit (Mazza and Miniati, 1993), respectively.

4.1.4.9 Organic Acids

Organic acids impart flavour characteristics to fruits and fruit products. For example, succinic acid is reported to be bitter, while malic acid alone or in comibination with citric acid is sour (Rubico and McDaniel, 1992).

Organic acids identified in the chokecherry fruit included ascorbic, citric, malic, oxalic, succinic and quinic acids (Figure 4.5) and their concentrations in

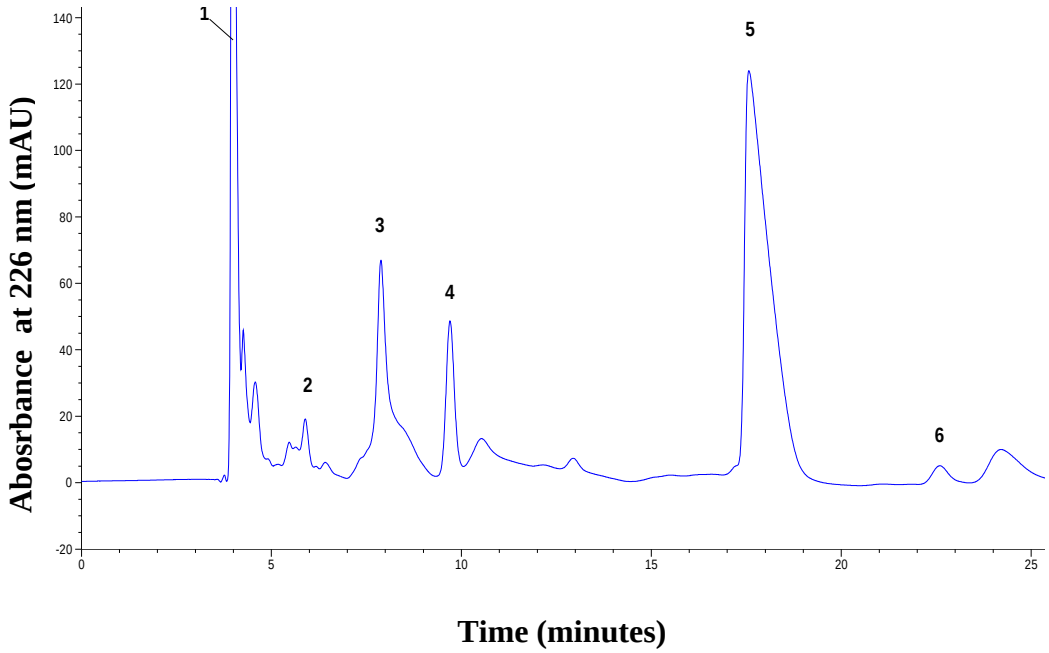


Figure 4.5 HPLC-DAD chromatogram of the organic acid profile of chokecherry fruit. Peak identities: 1) oxalic acid; 2) quinic acid; 3) malic acid; 4) ascorbic acid; 5) citric acid; 6) succinic acid.

samples from select years are shown in Table 4.7. The overall mean total organic acid content (903 ± 182 mg/100 g fruit) determined by HPLC was 55% greater than the TTA value (580 mg/100 g fruit; Table 4.6). Kalt and McDonald (1996) reported the total acid content in lowbush blueberry (*Vaccinium angustifolium* Aiton) cultivars was up to 70% greater when measured by HPLC compared to the TTA value.

The organic acid composition of chokecherry fruit has not been reported in literature. Citric acid was the most abundant organic acid (471 ± 59 mg/100 g fruit), followed by malic (189 ± 54 mg/100 g) and succinic (142 ± 50 mg/100 g) acid. The ascorbic acid content of the chokecherry fruit was low and this fruit would not be

Table 4.7 Organic Acid Composition and Concentration in Chokecherry Fruit

Year	Harvest date	Ascorbic Acid[1]	Citric Acid[1]	Malic Acid[1]	Oxalic Acid[1]	Quinic Acid[1]	Succinic Acid[1]	Total Acids[1]
1999	August 17	2 ± 1	482 ± 47*ab*[2,,3]	183 ± 35*a*	52 ± 2*b*[3]	48 ± 5*a*	109 ± 9*a*	872 ± 37
2001	September 30	1 ± 1	526 ± 24*b*	250 ± 15*b*	42 ± 5*ab*	100 ± 33*b*	205 ± 29*b*	1123 ± 51
2002	August 30	1 ± 1	407 ± 17*a*	135 ± 20*a*	37 ± 2*a*	27 ± 6*a*	111 ± 13*a*	712 ± 19
Overall Mean		1 ± 1	43 ± 7	189 ± 54	43 ± 7	59 ± 37	142 ± 50	903 ± 182

[1] mg/100 g fruit, fresh weight basis.
[2] Mean ± standard deviation.
[3] Means in the same column followed by a common letter are not statistically different ($P \leq 0.05$) by Tukey's HSD (honestly significant difference) multiple range test.

considered a good source of of this organic acid compared to the others analyzed in this study.

When compared to other fruits, the overall mean total organic acid content of the chokecherry fruit was lower than grapefruit (1040 mg/100 g fruit), navel orange (1256 mg/100 g fruit), lemon (3743 mg/100 g fruit) (Nisperos-Carriedo et al., 1992) and lowbush blueberry (2687 mg malic acid/100 g fruit; Kalt and McDonald, 1996

4.1.4.10 Fructose, Glucose and Sorbitol

The main soluble carbohydrates in chokecherry fruit harvested in August 2002 were glucose, fructose and sorbitol as detected by HPLC-RI (Figure 4.6). The glucose content was 3.15 ± 0.4 g/100 g and fructose was 3.64 ± 0.08 g/100 g fruit, resulting in a glucose/fructose ratio of 0.90 (Table 4.4). The content of individual carbohydrates and the glucose/fructose ratio has been utilized as one indicator of fruit juice authenticity (Fry et al., 1995). The glucose/fructose ratio of chokecherry was similar to that of other sweet fruits such as bilberry, blackberry, highbush blueberry and orange which have been reported as 1.0 (Ayaz et al., 2001), 0.7 (Wrolstad et al., 1980) and 1.0 (Fry et al., 1995), respectively, and lower than that of apple, reported at >2.0 (Lea, 1995).

4.1.4.11 CIELAB Values

Hunterlab colourimeter values have been used to determine the change during ripening or between different cultivars of fruits that are coloured by anthocyanins, including black currant (Zatylny et al., 2005a), cranberry

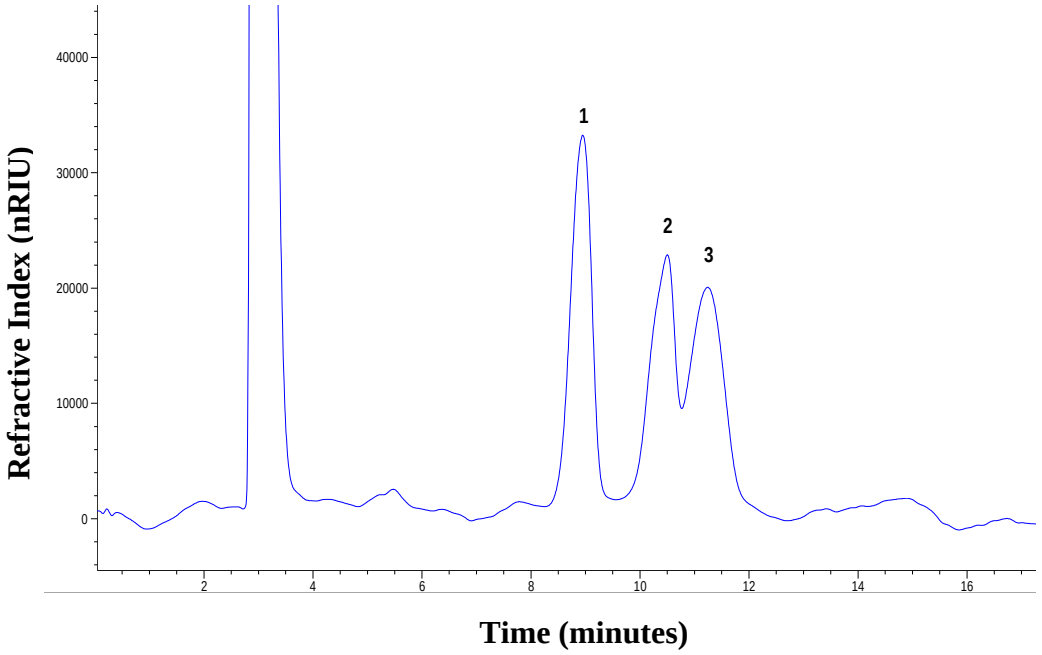


Figure 4.6 HPLC-RI chromatogram of the fructose, glucose and sorbitol of chokecherry fruit. Peak identities; 1) fructose, 2) sorbitol, 3) glucose.

(Sapers et al., 1983) and saskatoon berry (Green and Mazza, 1986; Zatylny et al., 2005b). Zatylny et al. (2005a) also employed Hunterlab colour analysis to determine the colour of chokecherry fruit cultivars. In the present study, the CIELAB values were employed because, according to the equipment manufacturer (Hunter Associates Laboratory, Reston, VA), this colour difference scale generally provides a more accurate approximation to visual evaluation of colour difference for very dark colours.

The L*, a*, b* and hue* angle values of the chokecherry fruit showed no significant difference among the harvest dates or years (Table 4.8). The mean L* value was 12.7 ± 1.7 and ranged from 11.7 ± 1.4 in the August 1999 fruit to 14.7 ±

Table 4.8 CIELAB Colour Values of Chokecherry

Year	Harvest date	L*	a*	b*	Hue angle*
1999	August 17	11.7 ± 1.4[1]	1.7 ± 0.6	1.0 ± 0.3	31.6 ± 13.7
	September 18	12.6 ± 1.1	1.8 ± 0.7	1.2 ± 0.5	34.6 ± 5.7
2000	August 25	14.7 ± 0.6	2.5 ± 0.3	1.3 ± 0.4	28.3 ± 10.1
	September 17	12.0 ± 3.1	1.6 ± 0.9	1.0 ± 0.7	30.4 ± 2.3
2001	September 30	13.4 ± 1.7	1.2 ± 0.2	0.4 ± 0.2	18.7 ± 8.8
2002	August 30	11.8 ± 1.6	0.9 ± 0.2	0.1 ± 0.1	21.2 ± 2.6
	October 14	12.6 ± 0.6	1.2 ± 0.2	0.8 ± 0.4	31.1 ± 9.0
Overall Mean		12.7 ± 1.7	1.6 ± 0.7	0.8 ± 0.5	26.5 ± 10.7
Year/harvest significance[2]		NS	NS	NS	NS

[1] Mean ± standard deviation.
[2] Significant difference ($P \leq 0.05$) among the years (Y) and/or months (M) of harvest by two-way ANOVA. NS = no significant difference.

0.6 for the fruit harvested on August 25, 2000. The a* mean value was 1.6 ± 0.7 and ranged from 0.9 ± 0.2 in the fruit picked on August 30, 2002 to 2.5 ± 0.3 in the fruit harvested on August 25, 2000. Chokecherry fruit had a mean b* value of 0.8 ± 0.5 and ranged in b* value from 0.1 ± 0.1 in the September, 2001 crop to 1.3 ± 0.4 in the August 25, 2000 sample. The mean hue angle* value was 26.2 ± 11.3 and ranged from 8.9 ± 5.8 to 34.6 ± 5.7. None of the colour values changed significantly among the years of harvest or harvest dates within each of the years. This result indicated very that the CIELAB colour measurement scale was not sufficiently sensistive to detect changes in anthocyanin concentration of the fruit (Section 4.1.4.8). Similarly, Kalt and McDonald (1996) reported that Hunterlab

values were not significantly correlated to the anthocyanin content of lowbush blueberry.

4.1.5 Physicochemical Composition of Sea Buckthorn Fruit

4.1.5.1 pH

The mean pH of sea buckthorn fruit was 3.0 ± 0.1 and ranged from 2.9 ± 0.1 in the samples harvested in August 1999 and 2002 to pH 3.2 ± 0.1 for the fruit harvested in September, 1999 and 2001 (Table 4.9). There was a significant increase in pH at later harvest dates in the 1999 and 2002 crops. The pH values were within the range of 3.0 to 3.3, reported for sea buckthorn juice processed from fruit harvested in Southern Saskatchewan (Beveridge et al., 2002). Similar pH values have also been reported for sea buckthorn fruit harvested in Finland, ranging from 2.87 to 3.28 (Tang and Tigerstedt, 2001).

4.1.5.2 Total Titratable Acidity

The mean TTA expressed as malic acid of the sea buckthorn fruit was 1.49 ± 0.19% and ranged from 1.33 ± 0.04% in the September 17, 2000 sample to 1.89 ± 0.01% in the sample harvested on August 30, 2002 (Table 4.9). These TTA values were lower than the minimum range of 1.78 to 2.09% malic acid reported by Beveridge et al. (2002) for sea buckthorn juice processed from Saskatchewan grown fruit. However, the TTA of sea buckthorn grown throughout Europe and Asia has been reported to vary from 1.49 to 4.79% expressed as citric acid (Tang and Tigerstedt, 2001).

Table 4.9 Physicochemical Composition of Sea buckthorn Fruit

Year	Harvest date	pH	TTA[1]	°Brix	Brix / TTA	Total Solids Content[2]	Total Phenolic Content[2]	% Seed/Fruit
1999	August 17	2.9 ± 0.1*a*[3,4]	1.45 ± 0.03*b*	8.9 ± 0.8	6.2 ± 0.6*a*	15.9 ± 0.6*a*	0.44 ± 0.01*d*	4.6 ± 0.1*a*
	September 18	3.2 ± 0.1*b*	1.35 ± 0.03*ab*	11.6 ± 0.5	8.6 ± 0.5*b*	20.6 ± 0.5*cd*	0.31 ± 0.02*ab*	4.6 ± 0.3*a*
2000	August 25	3.0 ± 0.1*a*	1.42 ± 0.03*ab*	12.5 ± 0.7	8.8 ± 0.4*b*	19.5 ± 0.5*c*	0.28 ± 0.03*a*	5.3 ± 0.2*b*
	September 17	3.2 ± 0.1*b*	1.33 ± 0.04*a*	11.4 ± 0.3	8.6 ± 0.4*b*	21.6 ± 0.4*de*	0.29 ± 0.02*a*	6.3 ± 0.2*c*
2001	September 30	3.1 ± 0.2*b*	1.44 ± 0.03*b*	12.2 ± 0.8	8.5 ± 0.4*b*	21.2 ± 0.7*de*	0.35 ± 0.01*bc*	4.7 ± 0.1*a*
2002	August 30	2.9 ± 0.1*a*	1.89 ± 0.01*d*	11.0 ± 1.3	5.8 ± 0.7*a*	18.0 ± 0.5*b*	0.31 ± 0.03*ab*	5.1 ± 0.2*ab*
	October 14	3.1 ± 0.1*b*	1.60 ± 0.05*c*	12.4 ± 0.6	7.8 ± 0.5*b*	22.3 ± 0.5*e*	0.37 ± 0.02*c*	6.1 ± 0.2*c*
Overall Mean		3.0 ± 0.1	1.49 ± 0.19	11.4 ± 1.3	7.8 ± 1.3	19.9 ± 2.2	0.34 ± 0.06	5.2 ± 0.7
Year/harvest significance[5]		M	Y, M	NS	Y, M	Y, M	Y	Y, M

[1] % total titratable acidity.
[2] Percent wet weight of whole fruit.
[3] Mean ± standard deviation.
[4] Means in the same column followed by a common letter are not statistically different ($P \leq 0.05$) by Tukey's HSD (honestly significant difference) multiple range test.
[5] Significant difference ($P \leq 0.05$) among the years (Y) and/or months (M) of harvest by one-way ANOVA. NS = no significant difference.

In the present study it was found that the TTA decreased by ~7% from August to September in 1999 and 2000 and by ~15% from August to October in 2002. This trend of lower TTA at later harvest dates has been previously reported for sea buckthorn (Tang and Tigerstedt, 2001). TTA changes in the samples analyzed in this study were most likely due to the effect of low temperature (frost) on fruit metabolism that occurred before the fruit was harvested from mid-September to mid-October (Hendrix and Redd, 1995).

4.1.5.3 Total Solids Content

Total solids content of the sea buckthorn whole fruit ranged from 15.9 ± 0.6% in the August, 1999 berries to 22.3 ± 0.5% in the October, 2002 crop and had a mean of 19.9 ± 2.2% (Table 4.9). A total solids content of 12.3 to 17.8% has been reported for sea buckthorn fruit harvested in Europe and Asia, respectively (Tang and Tigerstedt, 2001).

The fruit were significantly higher in total solids content at later harvest dates (September and October) compared to the samples harvested in August, increasing approximately 10% in the 2000 crop to greater than 20% in the 1999 and 2002 crop years. This observed increase can be partly attributed to an increase in the % seed/berry (Section 4.1.5.8) and probably to an increase in the insoluble solids of the fruit since the soluble solids content did not change significantly with harvest date (Section 4.1.5.4).

4.1.5.4 Soluble Solids

The mean soluble solids content of the juice expressed from sea buckthorn fruit was 11.4 ± 1.3 °Brix and ranged from 8.9 ± 0.8 to 12.5 ± 0.7 °Brix (Table 4.9). This level of soluble solids is in the range of 8.4 to 10.7 °Brix reported by Beveridge et al. (2002) for sea buckthorn harvested in Saskatchewan. The soluble solids content of Chinese sea buckthorn juice has been reported to range from 9.3 to 22.7 °Brix (Zhang et al., 1989a; Beveridge et al., 1999).

The soluble solids content did not differ significantly among the years of harvest or at different harvest dates within each crop year. Although the °Brix value of the sample harvested on August 17 (8.9 ± 0.8 °Brix) was lower than the value (11.6 ± 0.5 °Brix) of the fruit harvested on September 18 of 1999, the combined data of three years (1999, 2000 and 2002) showed no significant differences in the soluble solids content at different harvest dates within each year.

4.1.5.5 °Brix/TTA Ratio

The °Brix/TTA ratio of the sea buckthorn fruit varied from 5.8 ± 0.7 in the August, 2002 to 8.8 ± 0.4 in the August 2000 samples (Table 4.9). Fruit harvested in August were significantly lower in °Brix/TTA ratio than those harvested in September and October in 1999 and 2002. The mean °Brix/TTA ratio was 7.8 ± 1.3 and was high in comparison to values for sea buckthorn juice, reported to range from 4.7 to 5.8 (Beveridge et al., 1999). Sea buckthorn contained a similar °Brix/TTA ratio to buffaloberry (9.3 ± 1.3; Table 4.2).

4.1.5.6 Organic Acids

Organic acids identified in the sea buckthorn fruit included ascorbic, citric, malic, oxalic, quinic and succinic acids (Figure 4.7). Quinic and malic acids comprised 86% of the total organic acids at 1,466 ± 412 and 1,314 ± 343 mg/100 g fruit, respectively (Table 4.10). Similar organic acid compositions were reported by Kallio et al. (2000) with quinic and malic acids comprising 77 to 97% of the total organic acids in sea buckthorn juice. Beveridge et al. (2002) reported quinic and malic acid was responsible for 91% of the organic acids in sea buckthorn juice.

The mean ascorbic acid content of the fruit analyzed in this study was 96 ±

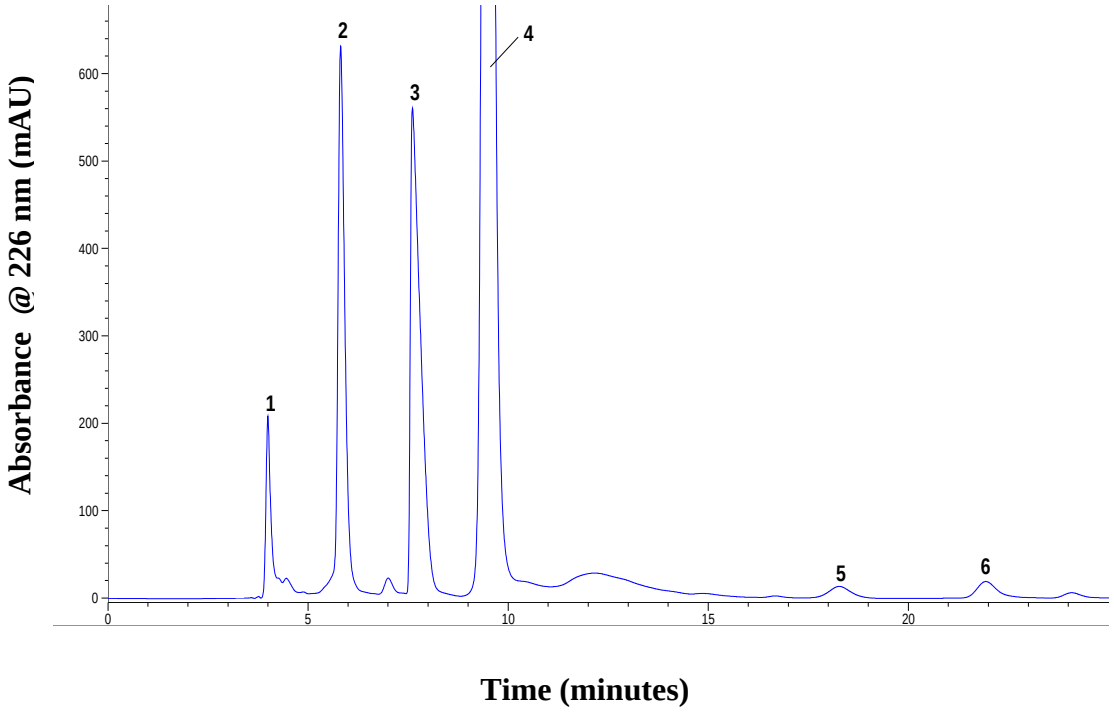


Figure 4.7 HPLC-DAD chromatogram of the organic acid profile of sea buckthorn fruit. Peak identities: 1) oxalic acid; 2) quinic acid; 3) malic acid; 4) ascorbic acid; 5) citric acid; 6) succinic acid.

Table 4.10 Organic Acid Composition and Concentration of Sea buckthorn Fruit

Year	Harvest date	Ascorbic Acid[1]	Citric Acid[1]	Malic Acid[1]	Oxalic Acid[1]	Quinic Acid[1]	Succinic Acid[1]	Total Acids[1]
1999	September 18	98 ± 29[2]	76 ± 3	1162 ± 202	56 ± 3*b*[3]	1552 ± 221	234 ± 33	3179
2000	August 25	91 ± 41	65 ± 26	1145 ± 401	29 ± 9*a*	1101 ± 463	196 ± 86	2628
2002	August 30	98 ± 7	71 ± 9	1635 ± 192	50 ± 13*ab*	1744 ± 295	241 ± 33	3840
Overall Mean		96 ± 26	71 ± 15	1314 ± 343	45 ± 15	1466 ± 412	224 ± 54	3216 ± 607

[1] mg/100 g fruit, fresh weight basis.
[2] Mean ± standard deviation.
[3] Means in the same column followed by a common letter are not statistically different ($P \leq 0.05$) by Tukey's HSD (honestly significant difference) multiple range test.

26 mg/100 g and did not significantly differ among the three crop years. This ascorbic acid content is in the lower range reported for sea buckthorn fruit. Kallio et al. (2002) reported ascorbic acid contents of 2 to 1,300 mg/100 g fruit and Yao and Tigerstedt (1994) reported a range of 28 to 2,500 mg/100 g for sea buckthorn juice. Ascorbic acid content varies widely with the subspecies of sea buckthorn; the subspecies *sinensis* is reported to contain 5 to 10 times more ascorbic acid than subspecies *rhamnoides* which in turn contains higher levels of ascorbic acid than subspecies *mongolica* (Kallio et al., 2002). Since subspecies *mongolica* was the source of sea buckthorn in the present study, the relatively lower range of ascorbic acid in this fruit is most likely due to its genetic composition. The ascorbic acid content of sea buckthorn fruit was approximately one-half of the ascorbic acid content of the buffaloberry fruit found in this study (206 ± 37 mg/100g; Table 4.3).

4.1.5.7 Fructose, Glucose and Sorbitol

The fructose and glucose content of the sea buckthorn fruit was 1.33 ± 0.01 and 2.73 ± 0.05 g/100 g fruit, respectively (Table 4.4). These values are in agreement with carbohydrate levels of 1.05% fructose and 2.98% glucose in sea buckthorn juice reported by Beveridge et al. (2002). Kallio et al. (2000) reported a fructose content ranging from 0.2 to 3.8 g/100 mL and 0.9 to 5.5 g/100 mL glucose in European and Chinese sea buckthorn juice.

A small sorbitol peak was observed at 9.5 minutes on the chromatogram (Figure 4.8), corresponding to a concentration of 0.3 ± 0.1 g/100 g fruit fresh weight basis. The identity of the sorbitol peak and its concentration was confirmed by

HPLC with pulsed amperometric detection (HPLC-PAD), utilizing an internal method and equipment in the Food Chemistry Laboratory, University of Saskatchewan. In contrast, others have reported that sorbitol was not detected in sea buckthorn fruit harvested in Saskatchewan (Beveridge et al., 2002), and in China, Finland and Russia (Kallio et al., 2000; Bekker and Glushenkova, 2001). Makinen and Soderling (1980), however, reported a sorbitol concentration ranging from 13 to 640 µg/g fruit in Finnish sea buckthorn and that the concentration of this polyol increased with maturity of the fruit. The findings of the present study confirmed the presence of sorbitol in sea buckthorn.

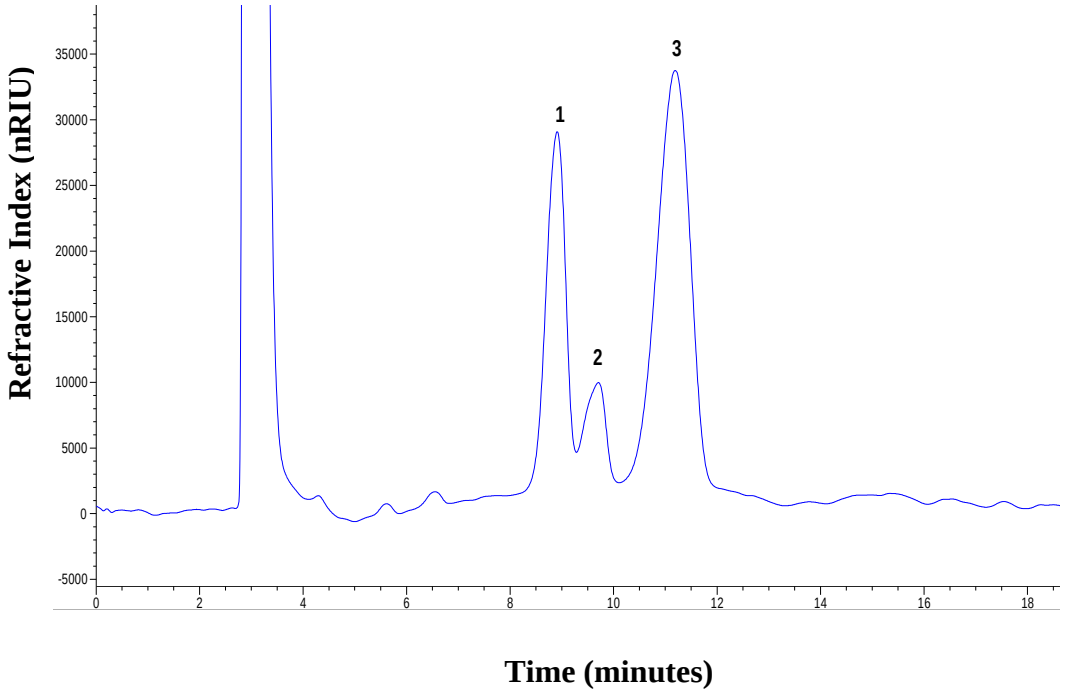


Figure 4.8 HPLC-RI chromatogram of fructose, glucose and sorbitol in sea buckthorn fruit. Peak identities: 1) fructose; 2) sorbitol; 3) glucose.

4.1.5.8 % Seed/Fruit Content

The mean % seed/fruit content of the sea buckthorn was 5.2 ± 0.7% and ranged from 4.6 ± 0.3 to 6.1 ± 0.2%. This % seed/fruit content is in the range reported for sea buckthorn from Finland (3.4 to 6.8%) and Russia (2.6 to 5.9%) and in the lower range reported for Chinese (5.0 to 12.9%) fruit (Kallio et al., 2000). Others have reported a % seed/fruit content of 3.9 to 9.0% in a Chinese species (Yang and Kallio, 2001) and a range of 2.9 to 8.4% in a Finnish species of sea buckthorn (Yang and Kallio, 2002).

The mean % seed/fruit content of sea buckthorn in this study was comparable to that observed for buffaloberry, at 4.7 ± 0.5% (Table 4.2).

4.1.5.9 Total Phenolic Content

The total phenolic content ranged from 0.28 ± 0.03 to 0.44 ± 0.01 g/100 g fruit and the mean was 0.34 ± 0.06 g/100 g fruit. These total phenolic content values were more than two fold higher than the range of 0.11 to 0.24 g/100 g reported for European sea buckthorn berries (*H. rhamnoides*) although the subspecies of the fruit was not identified (Gao et al., 2000). The difference in phenolic content of the sea buckthorn samples analyzed in this study versus the previously published data may be attributable to variation in environmental conditions, subspecies of the fruit and analytical methodology (Tang and Tigerstedt, 2001; Zadernowski et al., 2005).

The total phenolic content of sea buckthorn fruit was 40% of the total phenolic content in buffaloberry fruit (0.84 ± 0.06 g/100 g; Table 4.2)

4.1.5.10 CIELAB Colour

The CIELAB colour values of the sea buckthorn fruit samples are shown in Table 4.11. There was no significant difference in the L* values among the year or month of harvest. The a* and b* values were greater in 1999 and 2000 compared to those of 2001 and 2002. Hue angle values were significantly greater in the August

Table 4.11 CIELAB Colour Values of Sea Buckthorn Fruit

Year	Harvest date	L*	a*	b*	Hue angle*
1999	August 17	46.1[1] ± 4.2	17.3 ± 1.4*a*[2]	49.1 ± 7.0*b*	70.5 ± 2.2*b*
	September 18	46.3 ± 0.8	31.5 ± 2.3*c*	49.8 ± 1.1*b*	57.7 ± 1.9*a*
2000	August 25	48.5 ± 1.2	33.8 ± 2.8*c*	59.1 ± 4.4*b*	60.2 ± 2.2*a*
	September 17	45.9 ± 2.1	31.5 ± 2.3*c*	49.8 ± 1.1*b*	57.7 ± 1.9*a*
2001	September 30	45.7 ± 0.9	24.3 ± 1.5*b*	35.0 ± 2.0*a*	60.5 ± 2.6*a*
2002	August 30	47.4 ± 2.6	18.6 ± 4.9*ab*	37.3 ± 2.6*a*	63.7 ± 4.3*a*
	October 14	46.5 ± 2.4	17.6 ± 0.9*a*	34.3 ± 2.8*a*	62.8 ± 2.3*a*
Overall Mean		46.6 ± 2.2	25.1 ± 7.4	45.9 ± 10.3	62.2 ± 4.5
Year/harvest significance[3]		NS	Y	Y	Y, M

[1] Mean ± standard deviation.
[2] Means in the same column followed by a common letter are not statistically different ($P \leq 0.05$) by Tukey's HSD multiple range test.
[3] Significant difference ($P \leq 0.05$) among the years (Y) and/or months (M) of harvest by one-way ANOVA. NS = no significant difference.

1999 sample compared to all other samples of the fruit. The colour of sea buckthorn berry is due to its carotenoid content (Bernath and Foldesi, 1992; Li and Schroeder, 1996; Zeb, 2004) and thus, higher levels of these compounds can be expected to

result in greater positive a* (i.e. redness) and b* (i.e. yellowness) values. The relationship between the carotenoid content in sea buckthorn fruit and environmental growth conditions, however, has not been reported in the literature.

4.2 Fruit Phenolic Composition

4.2.1 Introduction

The phenolic composition of the fruits used in this study was determined by HPLC-DAD. Chromatographic peaks were assigned to the appropriate phenolic acid and flavonoid class based on their spectral profiles and the concentration of the class was determined from linear calibration equations of phenolic standards. The total phenolic chromatographic index (TPCI) was then determined from the sum of all the phenolic classes calculated from the chromatogram. The provisional identification of chromatographic peaks to phenolic classes and determination of the TPCI value was first defined and employed by Escarpa and Gonzalez (1998; 1999; 2001a; 2001b) for characterizing the phenolic profiles of apples, pears and various vegetables.

4.2.2 Extraction Solvent Selection

Aqueous solutions of acidified methanol, ethanol and acetone are common solvent systems for the extraction of phenolic compounds from plant materials (Merken and Beecher, 2000; Wrolstad, 2005). The extraction solvents evaluated in the present study included 80% (v/v) acetone in ddH_2O, 70% (v/v) ethanol in ddH2O-0.01% (v/v) HCl, 70% methanol in ddH2O-0.01% (v/v) HCl and methanol-

formic acid-ddH2O (MFW; 70:2:28, v/v). All fruit samples (20 g) were extracted in a total of 200 mL solvent using the method described in section 3.4. HPLC analysis of the various solvent extracts produced similar chromatographic profiles, however, larger peaks with better resolution were observed on chromatograms at 280 nm of fruits extracted with MFW.

4.2.3 HPLC Mobile Phases

The HPLC analysis of the fruit phenolic compounds was initially accomplished using a binary solvent system consisting of potassium phosphate buffer at pH 3.0 for solvent A and 70% acetonitrile in the phosphate buffer for solvent B (Method 1; Section 3.10.1). The Method 1 mobile phase was not compatible for LC-MS analysis since the non-volatile potassium phosphate can leave a residue on the ionization probe of the mass spectrometer that can compromise the performance of the instrument (Mosi and Eigendorf, 1998). As a result, the identification of an LC-MS compatible solvent system for effective separation of the phenolic compounds was required.

Initially, the use of ddH_2O for solvent A and 70% acetonitrile in ddH_2O for solvent B was used. HPLC analysis of fruit phenolic crude extracts eluted using this mobile phase, however, produced chromatograms with unresolved peaks (Figure 4.9). A more effective phenolic compound separation was achieved by acidifiying the mobile phase with volatile organic acids, specifically, formic and acetic acid. Acid concentrations ranging from 5, 10, 50 and 100 mM were prepared and used for

HPLC analysis of the fruit extracts. The highest peak resolution was achieved by using 10 mM formic acid in ddH_2O for solvent A and 70% acetonitrile/30% aqueous formic acid (10mM) for solvent B (see Figures 4.15, 4.16 and 4.17). As a

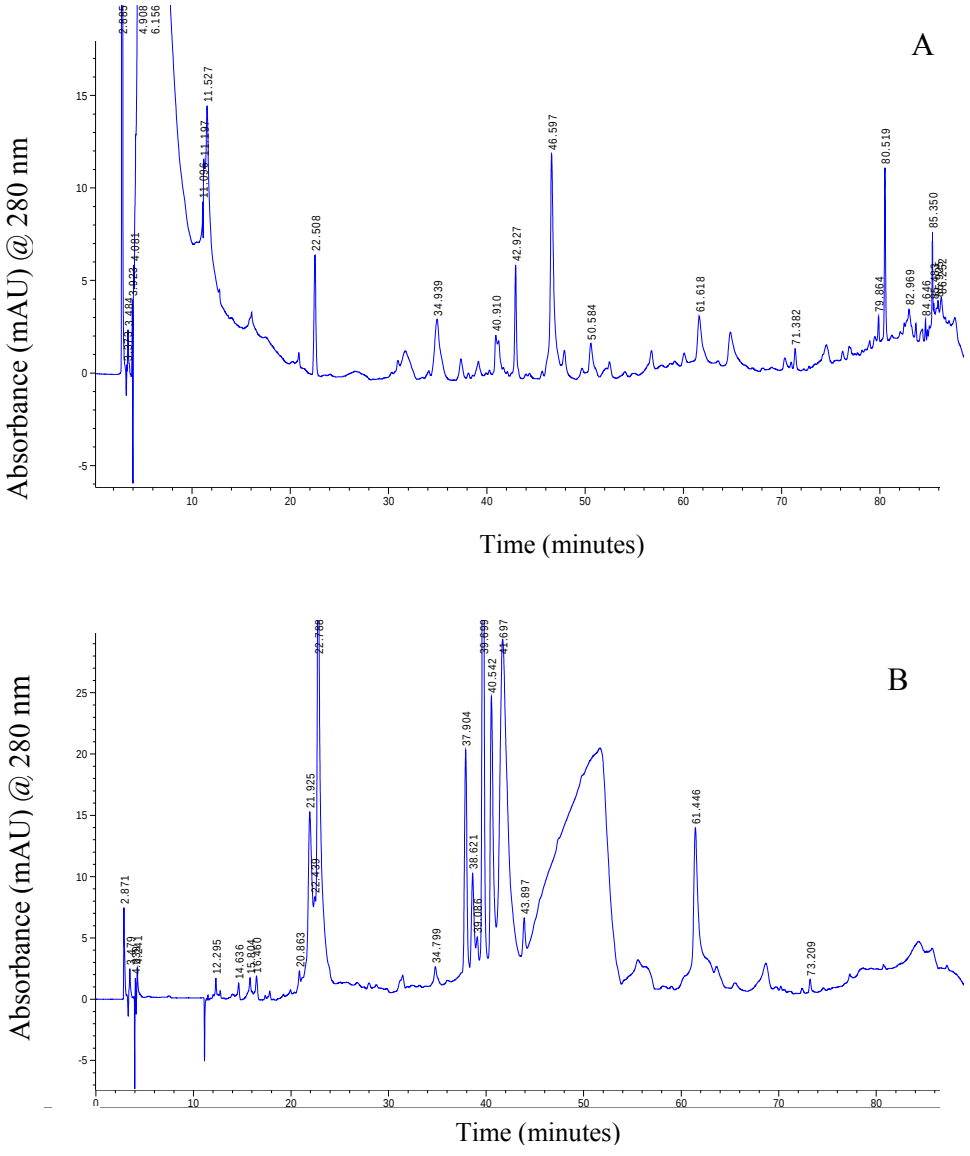


Figure 4.9 HPLC-PDA chromatograms of: A) buffaloberry and B) chokecherry fruit extracts using ddH_2O-acetonitrile as mobile phase.

result, this solvent system was selected as an additional binary mobile phase for HPLC-DAD and LC-MS analyses of the phenolic compounds and is referred to as Method 2 in the remainder of this thesis. The same gradient elution profile was used for both Methods 1 and 2. In addition to analysis using Method 1, HPLC-DAD analyses of the fruit and leaf phenolic crude extracts from the August 2002 samples were acquired employing Method 2.

4.2.4 Phenolic Peak Assignment

Phenolic compounds for which standards were available were identified on chromatograms according to their relative retention times (RRT's), spiking experiments and their UV-visible absorbance spectra. Typical HPLC-DAD chromatograms of the phenolic standards analyzed using Method 1 and Method 2 are shown in Figure 4.10. Elution times increased by approximately 30 seconds to five minutes using Method 2 compared to those in Method 1. The pH of the Method 2 mobile phase was 2.4 versus 3.0 for Method 1. At lower pH values the phenolic compounds exist in a less polar, non-ionized state compared to a more polar state and ionized at higher pH levels. In the less polar state, the phenolic compounds had a greater affinity for the C_{18} stationary phase, resulting in longer RRT's (Kim and Lee, 2005a).

Caffeic acid showed the greatest shift in retention time from 32.5 minutes in the pH 3.0 mobile phase employed for Method 1 to 37.6 minutes in the pH 2.4 mboile phase used in Method 2. This resulted in a change in the elution order of the

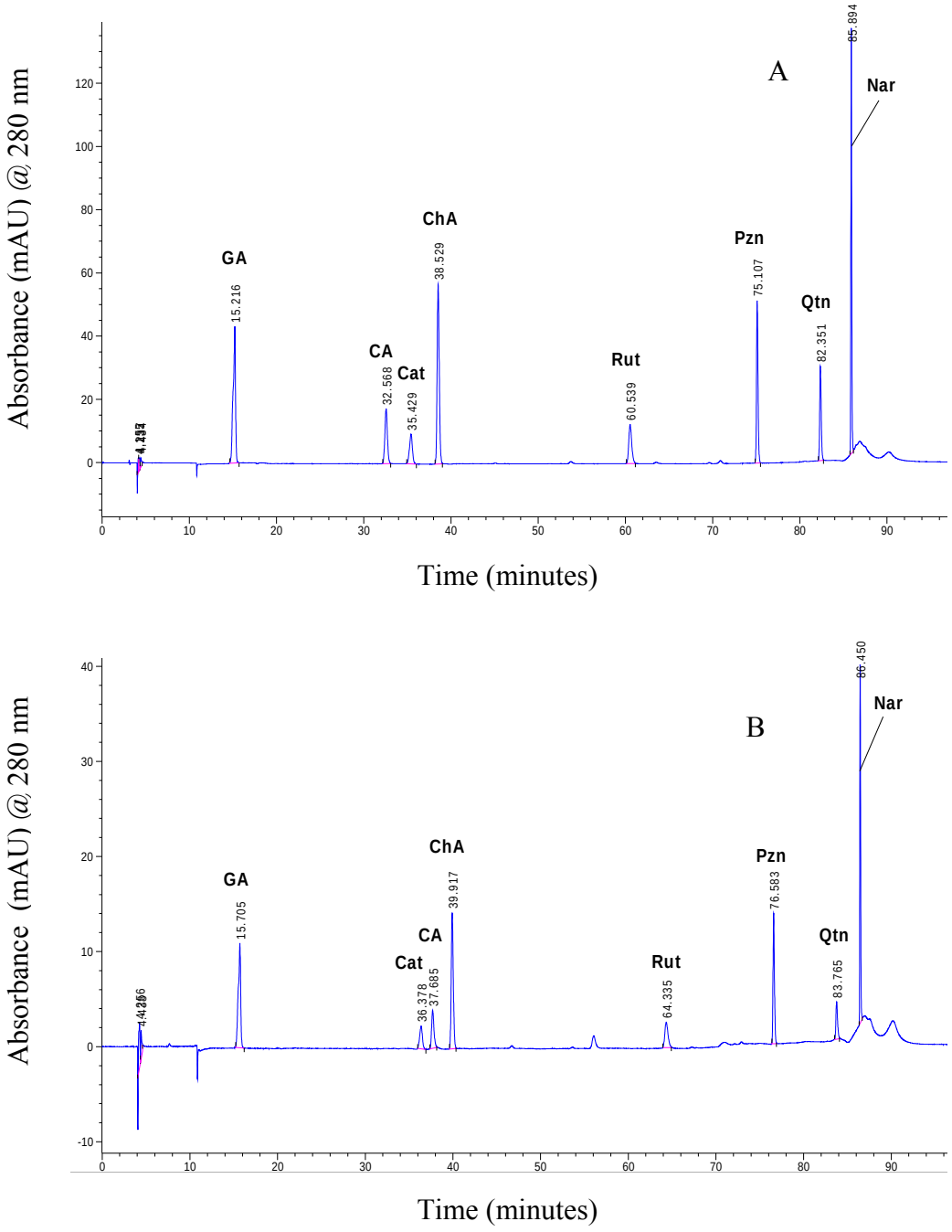


Figure 4.10 HPLC-PDA chromatograms of phenolic standards using: A) Method 1 and B) Method 2. Peak identities: GA, gallic acid; Cat, catechin; CA, caffeic acid; ChA, chlorogenic acid; Rut, rutin; Pzn, phloridzin; Qtn, quercetin; Nar, naringenin.

standards with the caffeic acid peak occurring after the catechin peak on the chromatogram of standards using Method 2 (Figure 4.10).

Chromatographic peaks that did not match the RRT's of external standards were provisionally assigned to phenolic classes based on the comparison of their spectral characteristics with those of the standards. The phenolic classes assigned included hydroxybenzoic acids, hydroxycinnamic acids and the flavonoids: anthocyanins, flavanols, flavanones/dihydrochalcones, flavones and flavonols.

Typical spectral profiles of the phenolic standards are shown in Figure 4.11. The hydroxybenzoic acids exhibited characteristic λ_{max} in the 200 to 240 nm range with other absorbance peaks from 260 to 300 nm (Kim and Lee, 2005a). Hydroxycinnamic acids, due to conjugation in their C_3 moiety (Robbins, 2003), showed an additional absorbance band at 310 to 330 nm with a shoulder on the lower wavelength side of the peak (Figure 4.11).

All flavonoids showed absorbance peaks in the UV range of 190 to 220 nm. Flavanols showed a small absorbance peak in the 275 to 285 nm region and a shoulder at 230 to 240 nm. Assignment of the other flavonoid peaks was based on the occurrence of two absorption bands that are characteristic of these compounds (Harborne, 1967; Mabry, 1970; Merken and Beecher, 2000). Band I, with a maximum in the 300 to 550 nm range, arises from the B-ring of the flavonoid structure while Band II, with a maximum absorbance in the 240 to 295 nm range, arises from the A-ring. Anthocyanins were identified by their Band I absorbance in the visible range of 510 to 530 nm and Band II absorbance in the UV region of

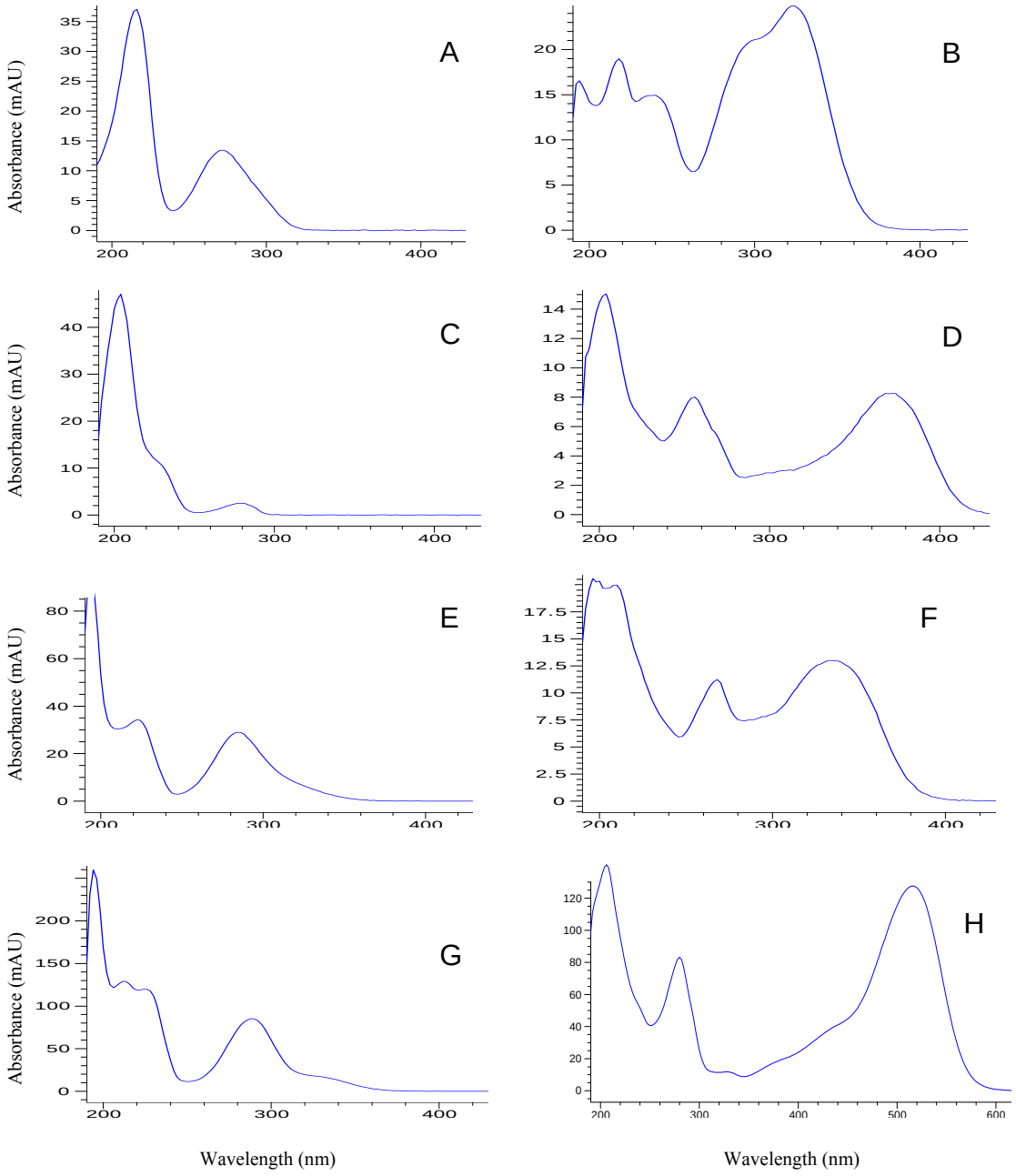


Figure 4.11 Spectral profiles of phenolic standards. A. gallic acid, a hydroxybenzoic acid; B. caffeic acid, a hydroxycinnamic acid; C. catechin, a flavanol, D. quercetin, a flavonol; E. phloridzin, a dihydrochalcone; F. apigenin, a flavone; G. naringenin, a flavanone; H. cyanidin 3-glucoside, an anthocyanin.

275 to 285 nm. Flavanones were identified by an absorbance peak occurring at 278 to 292 nm and a broad shoulder in the range of 320 to 360 nm. Dihydrochalcones such as phloridzin have a similar UV spectral profile as flavanones. The dihydrochalcones are unstable and readily convert to the flavanone form in acidic conditions, as employed in the HPLC-DAD analysis in the present study (Harborne, 1967). As a result, flavanones and dihydrochalcones were assigned to the same phenolic class. Flavones were identified by their Band II absorbance at 250 to 275 nm and Band I at 304 to 350 nm. The flavonols showed a similar Band II absorbance, however, their Band I occurred at a longer wavelength in the range of 352 to 385 nm. Isoflavones, characterized by intense absorbance in the 240 to 265 nm region with a shoulder in the 320 to 340 nm range, were not detected in any of the fruit and leaf extracts analyzed in this study.

The HPLC-DAD spectral profiles of the flavonoid standards showed very little change (± 2 nm) in the Band I and Band II λ_{max} values between Method 1 and Method 2. The observed spectral profiles of the phenolic standards were consistent with spectral data reported in literature for these compounds and their respective phenolic classes (Mabry et al., 1970; Hong and Wrolstad, 1990a; Merken and Beecher, 2000; Maatta et al., 2003; Sakakibara et al., 2003; Tsao and Yang, 2003).

Phenolic peaks occurring in each chromatogram were assigned to the appropriate phenolic class and peak areas for each class were summed. The concentration of each class was determined from linear regression equations of the phenolic standards. Hydroxybenzoic and hydroxycinnamic acids were expressed as gallic acid and caffeic acid, respectively. Anthocyanins, flavanols,

flavanones/dihydrochalcones, flavones and flavonols were expressed as cyanidin 3-glucoside, catechin, naringenin, apigenin and quercetin, respectively. Except for the anthocyanins, the quantification of all phenolic peaks was determined at 280 nm. Although flavones and flavonols show higher absorbance values at 320 to 375 nm compared to 280 nm, the slopes of the representative standard curves (i.e. apigenin and quercetin) were greater at 280 nm versus 360 nm. The greater slope of the observed linear equations indicated greater sensitivity to a change in the analyte concentration and as a result, the flavonols and flavones were quantified at 280 nm. Anthocyanins were quantified at their λ_{max} of 520 nm, as the standard curve showed the highest sensitivity at this wavelength. Following quantification of each phenolic class, the TPCI was determined from the sum of all classes calculated from chromatographic results.

4.2.5 Phenolic Composition Using HPLC-DAD Method 1

Typical HPLC-DAD chromatograms of the fruit crude extracts acquired using Method 1 are shown in Figures 4.12 to 4.14. Generally, the elution order of the phenolic compounds was hydroxybenzoic acids followed by hydroxycinnamic acids followed by the flavonoids, with some overlap between members of the different classes. Similar elution profiles for phenolic acids and flavonoids using reversed phase chromatography have been reported in literature (Hakkinen and Torronen, 2000; Merken and Beecher, 2000; Tsao and Yang, 2003).

The more polar phenolic compounds such as gallic acid and caffeic acid generally elute first under reverse phase chromatographic conditions followed by

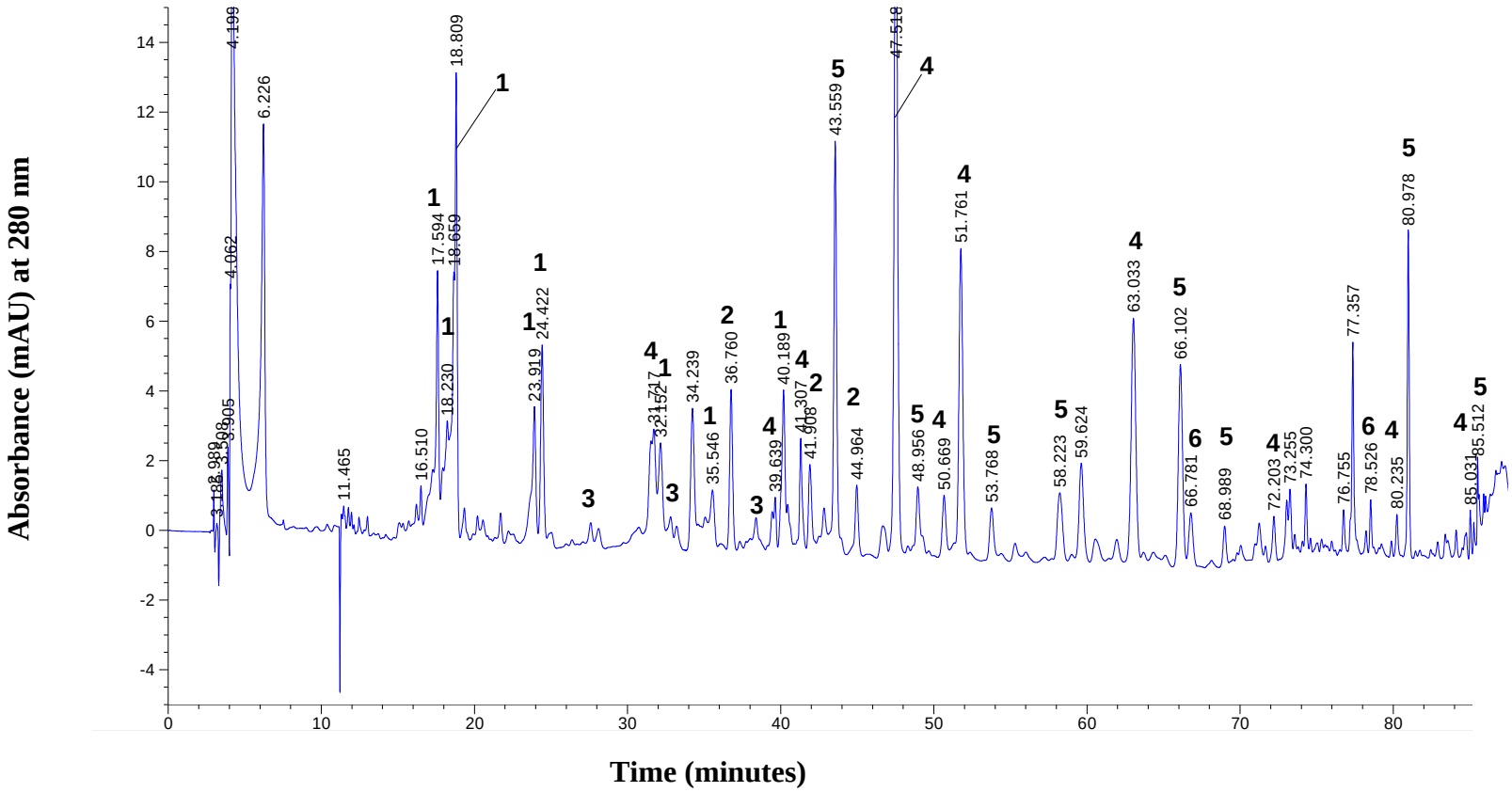


Figure 4.12 HPLC-DAD Method 1 chromatogram of buffaloberry fruit crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavones; 6) flavanones/dihydrochalcones.

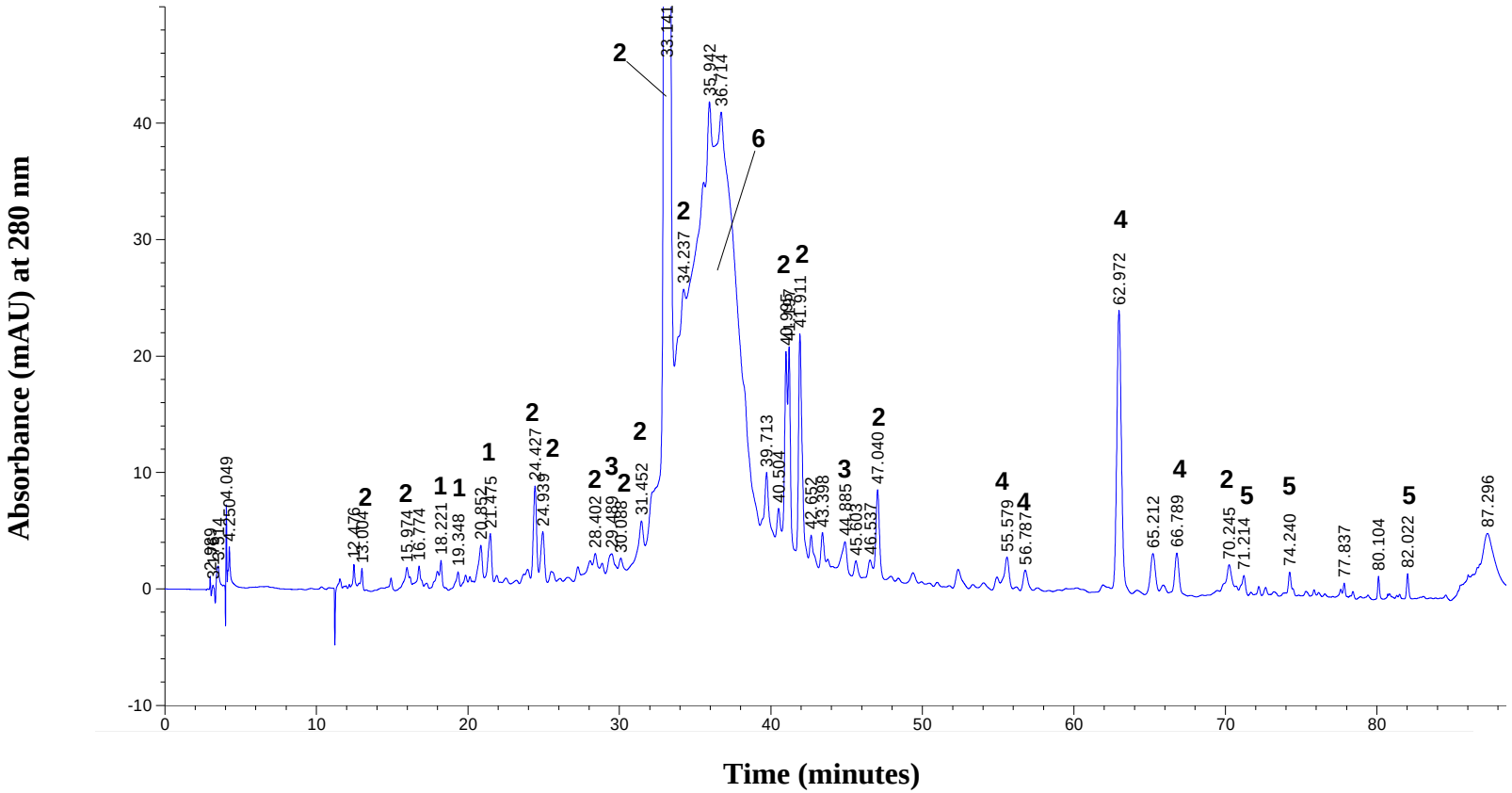


Figure 4.13 HPLC-DAD Method 1 chromatogram of chokecherry fruit crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavanones/dihydrochalcones; 6) anthocyanins.

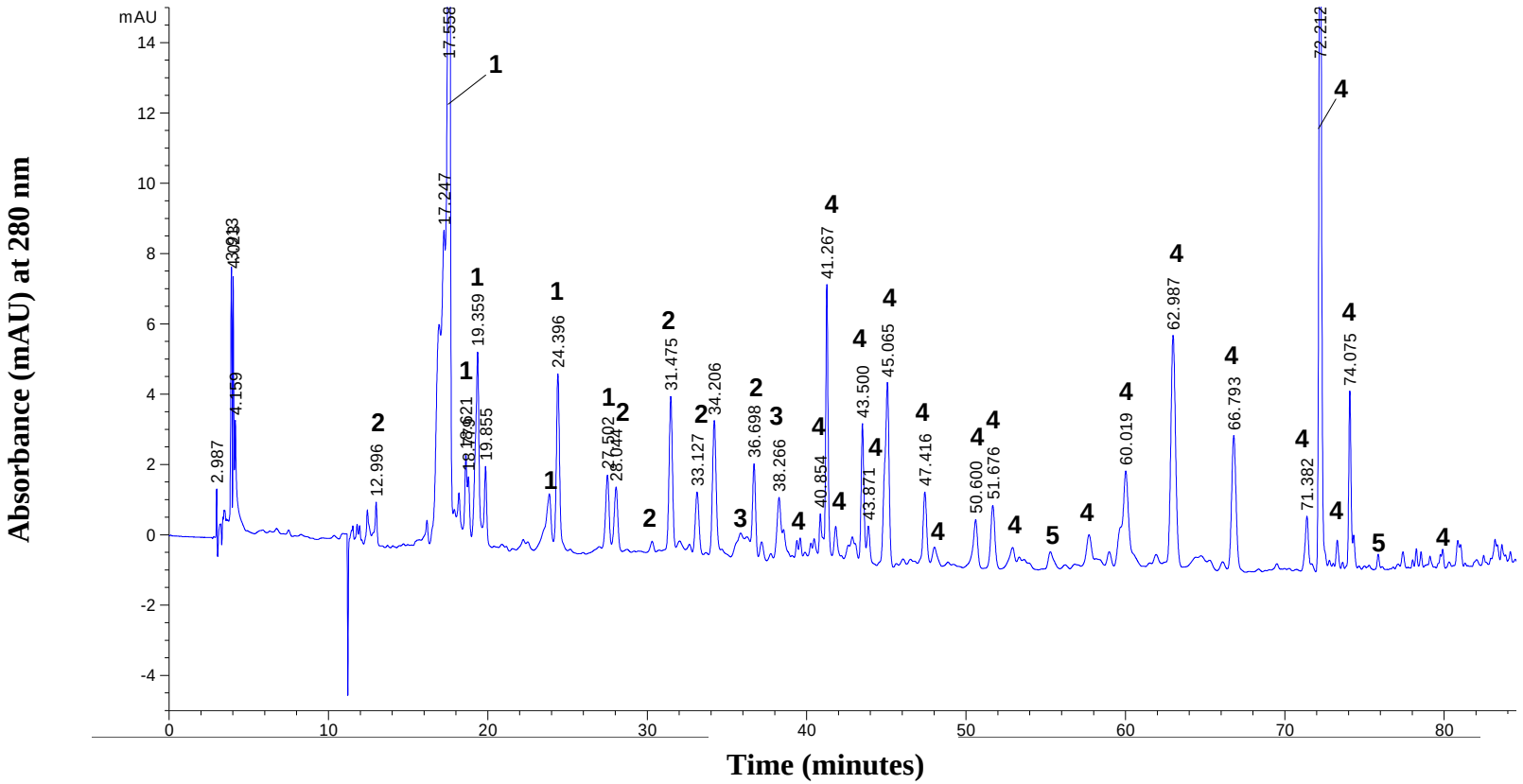


Figure 4.14 HPLC-DAD Method 1 chromatogram of sea buckthorn fruit crude extract. Peak assignment: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavones.

glycosylated flavonoids. Triglycosides (e.g. quercetin 3-*O*-triglucoside) elute before diglycosides (e.g. rutin), followed by monoglycosides (quercitrin), and then aglycones (e.g. quercetin). Monomeric flavanols such as catechin are relatively polar and elute at similar retention times to hydroxycinnamic acids. The elution order within an individual phenolic class is typically dependent on the number of polar hydroxyl groups and hydrophobic methoxyl groups (Kim and Lee, 2005a). For example, the elution order for hydroxycinnamic acids is as follows: caffeic acid (two hydroxyls on the phenyl ring), *p*-coumaric acid (one hydroxyl), ferulic acid (one methoxyl, one hydroxyl) and cinnamic acid (no hydroxyl or methoxyl) (Schieber et al., 2001).

Acylation of flavonoids with phenolic acids reduces their mobility and therefore increases their retention times under reversed phase HPLC conditions. For example, acylated anthocyanins elute later than those that are non-acylated (Durst and Wrolstad, 2005). The spectral properties of flavonoids acylated with phenolic acids may show a shift in the observed λ_{max} in the Band I and/or II regions of their spectral profile (Mabry et al., 1970). However, the overall spectral profile is consistent with the phenolic class of the parent flavonoid and will be identified as that class.

A minimum of eight hydroxybenzoic acid derivatives, three hydroxycinnamic acid derivatives, three flavanols, ten flavonols, eight flavones and two flavanones/dihydrochalcones were detected in buffaloberry fruit (Figure 4.12). Chokcherry fruit contained at least three hydroxybenzoic acids, thirteen hydroxycinnamic acids, two flavanols, four flavonols, three

flavanones/dihydrochalcones and at least two major anthocyanin peaks on the chromatogram (Figure 4.13). Sea buckthorn fruit contained six hydroxybenzoic acids, six hydroxycinnamic acids, two flavanols, twenty-one flavonols, and two flavanones/dihydrochalcones (Figure 4.14).

In addition to the identification of the phenolic class, peaks that matched the retention time and spectral profile of individual standards and co-eluted under spiking experiments were tentatively identified. Based on these parameters, buffaloberry fruit crude extract was found to contain catechin (RRT of 35.5 minutes) and rutin (RRT of 63.0 minutes), chokecherry fruit crude extract contained chlorogenic acid (RRT of 33.1 minutes), epicatechin (RRT of 44.8 minutes) and rutin (RRT of 62.9 minutes), and sea buckthorn fruit crude extract contained catechin (35.5 minutes) and rutin (RRT of 62.9 minutes).

4.2.5.1 Total Phenolic Chromatographic Index of Fruit Crude Extracts Employing HPLC-DAD Method 1

The phenolic composition of the fruit extracts determined by semi-quantitative analysis of the chromatographic (Method 1) peaks is shown in Table 4.12. The concentration of individual phenolic classes in buffaloberry and sea buckthorn fruit was consistent throughout the harvest dates and crop years. Chokecherry samples harvested on October 14, 2002 were at least 40% lower in anthocyanin and hydroxycinnamic acid concentrations than the fruit harvested on August 30, 2002. Since the chokecherry fruit harvested in October was considered to be overripe (Section 4.1.2.5), this data was not included in the calculation of the

Table 4.12 Phenolic Composition of Buffaloberry, Chokecherry and Sea Buckthorn Fruit Determined by HPLC-DAD

Fruit	Harvest Year	Harvest Date	Hydroxy-benzoic acids	Hydroxy-cinnamic acids	Flavanols	Flavonols	Flavones	Flavanones/ dihydrochalcones	Anthocyanins	TPCI[a]
Buffaloberry	1999	Aug. 17	41 ± 2[b]	45 ± 8	60 ± 8	216 ± 13	49 ± 14	45 ± 15	ND[b]	457 ± 23
	1999	Sept. 18	19 ± 6	138 ± 17	54 ± 21	239 ± 23	125 ± 43	47 ± 10	ND	622 ± 24
	2000	Aug. 25	18 ± 7	88 ± 10	60 ± 15	284 ± 36	118 ± 22	24 ± 12	ND	592 ± 68
	2000	Sept. 18	38 ± 16	51 ± 20	48 ± 20	375 ± 25	94 ± 8	20 ± 6	ND	626 ± 76
	2001	Sept. 30	35 ± 9	93 ± 8	63 ± 15	205 ± 26	110 ± 17	7 ± 3	ND	514 ± 19
	2002	Aug. 30	41 ± 8	47 ± 3	5 ± 1	393 ± 24	110 ± 3	17 ± 2	ND	613 ± 24
	2002	Oct. 14	33 ± 4	50 ± 4	4 ± 3	409 ± 7	114 ± 7	15 ± 3	ND	625 ± 9
	Overall Mean		**32 ± 11**	**73 ± 34**	**42 ± 27**	**303 ± 85**	**103 ± 29**	**25 ± 16**	**ND**	**578 ± 73**
Chokecherry	1999	Aug. 17	45 ± 12	2798 ± 59	120 ± 20	365 ± 19	ND	4 ± 2	241 ± 2	3573 ± 60
	1999	Sept. 18	38 ± 16	2702 ± 102	115 ± 24	297 ± 15	ND	2 ± 1	201 ± 21	3355 ± 69
	2000	Aug. 25	29 ± 4	2863 ± 47	87 ± 26	350 ± 29	ND	2 ± 1	194 ± 8	3525 ± 79
	2000	Sept. 18	48 ± 9	2762 ± 69	94 ± 21	256 ± 23	ND	4 ± 1	310 ± 25	3474 ± 43
	2001	Sept. 30	33 ± 10	2467 ± 74	90 ± 6	240 ± 21	ND	3 ± 2	270 ± 21	3102 ± 70
	2002	Aug. 30	31 ± 5	1770 ± 23	39 ± 16	240 ± 25	ND	2 ± 1	252 ± 5	2333 ± 19
	2002	Oct. 14	27 ± 1	990 ± 30	47 ± 15	280 ± 29	ND	2 ± 1	91 ± 6	1436 ± 31
	Overall Mean[d]		**37 ± 8**	**2560 ± 410**	**91 ± 29**	**291 ± 55**	**ND**	**2 ± 2**	**245 ± 43**	**3327 ± 469**
Sea buckthorn	1999	Aug. 17	54 ± 11	67 ± 8	14 ± 3	346 ± 18	ND	4 ± 2	ND	484 ± 17
	1999	Sept. 18	32 ± 4	51 ± 9	13 ± 4	325 ± 16	ND	3 ± 2	ND	425 ± 14
	2000	Aug. 25	50 ± 13	37 ± 9	10 ± 3	282 ± 14	ND	3 ± 1	ND	371 ± 16
	2000	Sept. 18	34 ± 6	64 ± 13	18 ± 6	374 ± 23	ND	2 ± 1	ND	492 ± 45
	2001	Sept. 30	38 ± 5	62 ± 7	16 ± 3	392 ± 16	ND	6 ± 3	ND	514 ± 29
	2002	Aug. 30	53 ± 1	30 ± 6	24 ± 2	419 ± 40	ND	7 ± 4	ND	529 ± 51
	2002	Oct. 14	72 ± 26	31 ± 4	20 ± 3	399 ± 56	ND	6 ± 5	ND	527 ± 75
	Overall Mean		**47 ± 14**	**49 ± 16**	**16 ± 5**	**362 ± 48**	**ND**	**4 ± 2**	**ND**	**477 ± 59**

[a] Total Phenolic Chromatographic Index = sum of all identified and quantified phenolic peaks.
[b] Mean (µg/g fresh weight basis) ± standard deviation of three replications.
[c] Not detected.
[d] Overall mean does not include results from October 14, 2002 sample as this fruit was considered to be overripe.

overall mean.

Chokecherry fruit contained the highest level of TPCI (3,327 ± 469 μg/g) and was >5x higher than the level in buffaloberry (578 ± 73 μg/g) and >6x higher than the TPCI of sea buckthorn fruit (477 ± 59 μg/g). In comparison to the TPCI of other fruits, chokecherry can be considered to be significantly higher than that reported for strawberry (64 μg/g), red grape (133 μg/g), apple (212 μg/g), cherry (958 μg/g), blueberry (1,737 μg/g) and blackberry (2,003 μg/g) (Tsao and Yang, 2003).

The flavonols were major phenolic compounds found in buffaloberry fruit crude extracts, accounting for 52% (303 ± 85 μg/g based on quercetin) of the TPCI. In comparison to other fruits, the flavonol content of buffaloberry was considerably higher than the levels reported for strawberry (8 μg/g), red grape (23 μg/g) and blackberry (49 μg/g) and were 30% lower than the flavonol content reported for apple pomace (423 μg/g) by Tsao and Tang (2003). Other phenolic classes detected in buffaloberry fruit crude extract included flavones, hydroxycinnamic acids, flavanols, hydroxybenzoic acids and flavanones/ dihydrochalcones, comprising 18, 13, 7, 5, and 4% of the TPCI, respectively. Anthocyanins were not detected in buffaloberry crude extracts and this result was consistent with the observed absence of these pigments in this fruit as determined using the pH differential method (Section 4.1.1).

The hydroxycinnamic acids were the most abundant phenolic class in chokecherry at a concentration of 2,560 μg/g fruit based on caffeic acid, contributing 78% of the TPCI. Flavonols accounted for almost 10% (291 μg/g) and

anthocyanins comprised 7% (245 μg/g) of the TPCI in the chokecherry fruit crude extract. The two anthocyanin peaks were not well resolved (Figure 4.13), however, there was good reproducibility of their total concentration in the samples harvested in August and September over the four crop years. Further characterization of the chokecherry anthocyanins is provided in Section 4.4.

Flavonols were the major phenolic class in sea buckthorn fruit crude extract with a concentration of 362 ± 48 μg/g fresh fruit, based on quercetin and comprised 76% of the TPCI. This result was similar to the flavonol concentration of 357 μg/mL sea buckthorn juice reported by Rosch et al. (2003). These authors identified the predominant sea buckthorn juice flavonols to be quercetin 3-*O*-rutinoside (i.e. rutin), quercetin 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside-7-*O*-rhamnoside, isorhamnetin 3-*O*-rutinoside, isorhamnetin 3-*O*-glucoside, isorhamnetin 7-*O*-rhamnoside and isorhamnetin. Hydroxybenzoic and hydroxycinnamic acids each comprised 10% of the TPCI of the sea buckthorn fruit crude extract. The flavanols and flavanones/ dihydrochalcones were detected in the chromatograms, however, these compounds contributed to less than 5% of the TPCI. Anthocyanins were not detected in the sea buckthorn fruit crude extracts and this result was consistent with the observed absence of monomeric anthocyanins in this fruit as determined using the pH differential method (Section 4.1.1).

Phenolic compounds are important components useful for the standardization of fruit extracts. The high precision of the TPCI using the HPLC-DAD conditions employed in Method 1 over the four crop years suggested that this method was effective for characterizing the phenolic composition of the fruit.

Chromatograms of the fruit crude extracts acquired using Method 1 consistently showed good peak resolution for the seven major classes of phenolic compounds separated.

4.2.6 Total Phenolic Content of Fruit Crude Extracts by Folin-Ciocalteu Analysis

Samples of the crude extract from the fruit harvested on August 30, 2002 were analyzed for total phenolic content (TPC) using the Folin-Ciocalteu (F-C) reagent in order to compare the TPC with the TCPI as determined by HPLC-DAD. The TPC (Table 4.13) was higher than the TPCI values (Table 4.12) for these extracts. Buffaloberry fruit had the greatest difference with the TPC being 24x greater than the TPCI (57.8 mg/100 g fresh fruit). The TPC of chokecherry was 4x that of the TPCI (332.7 mg/100 g fresh fruit) and the TPC of sea buckthorn was 9x higher than the TPCI (47.7 mg/100 g fresh fruit). Total phenolic content as

Table 4.13 Total Phenolic Content and Proanthocyanidin Content of Fruit Crude Extracts.

Sample	Total Phenolic Content[a]	Proanthocyanidin Content[b]
Buffaloberry	1420 ± 80[c]	505 ± 32
Chokecherry	1330 ± 160	52 ± 10
Sea buckthorn	440 ± 60	51 ± 17

[a] Determined using the Folin-Ciocalteu reagent and expressed as mg gallic acid/100 g fruit fresh weight basis.
[b] mg cyanidin/100 g fruit, fresh weight basis.
[c] Mean ± standard deviation of three replicates.

determined by the F-C method measures the total reducing activity of the sample and compounds such as ascorbic acid, reducing carbohydrates and proteins contribute to this analytical measurement (Waterhouse, 2005). In spite of these interferences, the F-C reagent is routinely employed for the phenolic content analysis of fruits, juices and other plant-based materials without correction for interfering compounds (Escarpa and Gonzalez, 2001b; Waterhouse, 2005).

Buffaloberry and sea buckthorn fruit both contained appreciable levels of ascorbic acid of 211 ± 32 (Section 4.1.3.7) and 96 ± 26 mg/100 g fruit (Section 4.1.4.6), respectively. The contribution of ascorbic acid (AA) to the TPC of these fruits was determined by preparing standard solutions of AA in MFW solvent ranging from 5 to 25 mg/L. These standard solutions, prepared in duplicate, were assayed using the Folin-Ciocalteu reagent as described in Section 3.3.5. The AA contribution was consistent in a ratio of 0.76:1.00 (w/w), gallic acid versus ascorbic acid. Applying this correction factor of 0.76 using the AA concentrations in the fresh fruit to the TPC's would reduce the total phenolic content values by 11 and 16% for buffaloberry and sea buckthorn, respectively. This correction for ascorbic acid would not fully account for the considerably higher TPC versus the TPCI values for crude extracts from these fruits.

The contribution of fructose and glucose to the TPC was determined by assaying 5% (w/v) solutions of these carbohydrates using the F-C reagent according to the method described in Section 3.3.5. These carbohydrates produced responses of 0.35 mg gallic acid/g fructose and 0.21 mg gallic acid/g glucose. Applying these correction factors using the fruit carbohydrate concentrations (Table 4.4) would

reduce the TPC values insignificantly, by 2.8, 1.9 and 1.0 mg gallic acid/100 g fruit in buffaloberry, chokecherry and sea buckthorn, respectively.

Other components of the fruit crude extracts, such as proanthocyanidins (Section 4.2.7) would have contributed to the observed higher TPC values versus TPCI of the fruit crude extracts.

4.2.7 Proanthocyanidin Content of Fruit Crude Extracts

Proanthocyanidins are polymeric compounds comprised of flavanol subunits and under oxidative and acidic conditions, these compounds are converted to lower molecular weight flavanol subunits and anthocyanidins. The proanthocyanidins can be determined by reversed phase HPLC methods up to a degree of polymerization of four (Schofield et al., 2001), thus, only a small proportion of these compounds would have been separated and detected under the HPLC conditions employed in this study. Because of the lack of standard compounds, the proanthocyanidin content was determined photometrically after acid depolymerization to anthocyanidins using the acid-butanol method described by Nikfardjam (2001) as cited by Rosch et al. (2003) (Section 3.3.11). The proanthocyanidin results are shown in Table 4.13. Buffaloberry fruit extract contained the highest level of proanthocyanidins (505 ± 32 mg/100 g fruit) and was 10x higher than the levels in chokecherry and sea buckthorn fruit. The proanthocyanidin content of sea buckthorn berry was within the range of 35 to 57 mg/100 mL reported for sea buckthorn juice (Rosch et al., 2003). Chokecherry proanthocyanidin concentration has not been reported in literature.

Proanthocyanidins contribute to the TPC value determined using the F-C reagent (Waterhouse, 2005). The high concentration of proanthocyanidins in buffaloberry fruit crude extract suggests these compounds contributed significantly to the TPC of this fruit.

4.2.8 HPLC-DAD Using Method 2

Typical HPLC-DAD chromatograms of the fruit extracts using Method 2 are shown in Figures 4.15 to 4.17. In comparison to the Method 1 chromatogram, the buffaloberry fruit crude extract Method 2 chromatogram had one less hydroxybenzoic acid (i.e. seven in total), nine additional hydroxycinnamic acids (i.e. twelve in total), one additional flavanol (i.e. four in total), one less flavonol (i.e. nine in total), three less flavones (i.e. five in total) and one additional flavanone/dihydrochalcone (i.e. three in total). Individual peaks tentatively identified on the buffaloberry fruit crude extract Method 2 chromatogram, based on RRT's, spectral profiles and spiking experiments with phenolic standards, included gallic acid (RRT of 16.1 minutes), catechin (RRT of 36.4 minutes), chlorogenic acid (RRT of 37.7 minutes) and rutin (RRT of 62.9 minutes). The identification of the rutin peak was confirmed by LC-MS (Section 4.5.1).

The chokecherry fruit crude extract Method 2 chromatogram had two additional hydroxybenzoic acids (i.e. five in total), three less hydroxycinnamic acids (i.e. 10 in total), one additional flavanol (i.e. three in total), three additional flavonol peaks (i.e. seven in total), one additional flavone (i.e. one in total), and one less flavanone/dihydrochalcone peak (i.e. two in total) incomparison to its Method 1

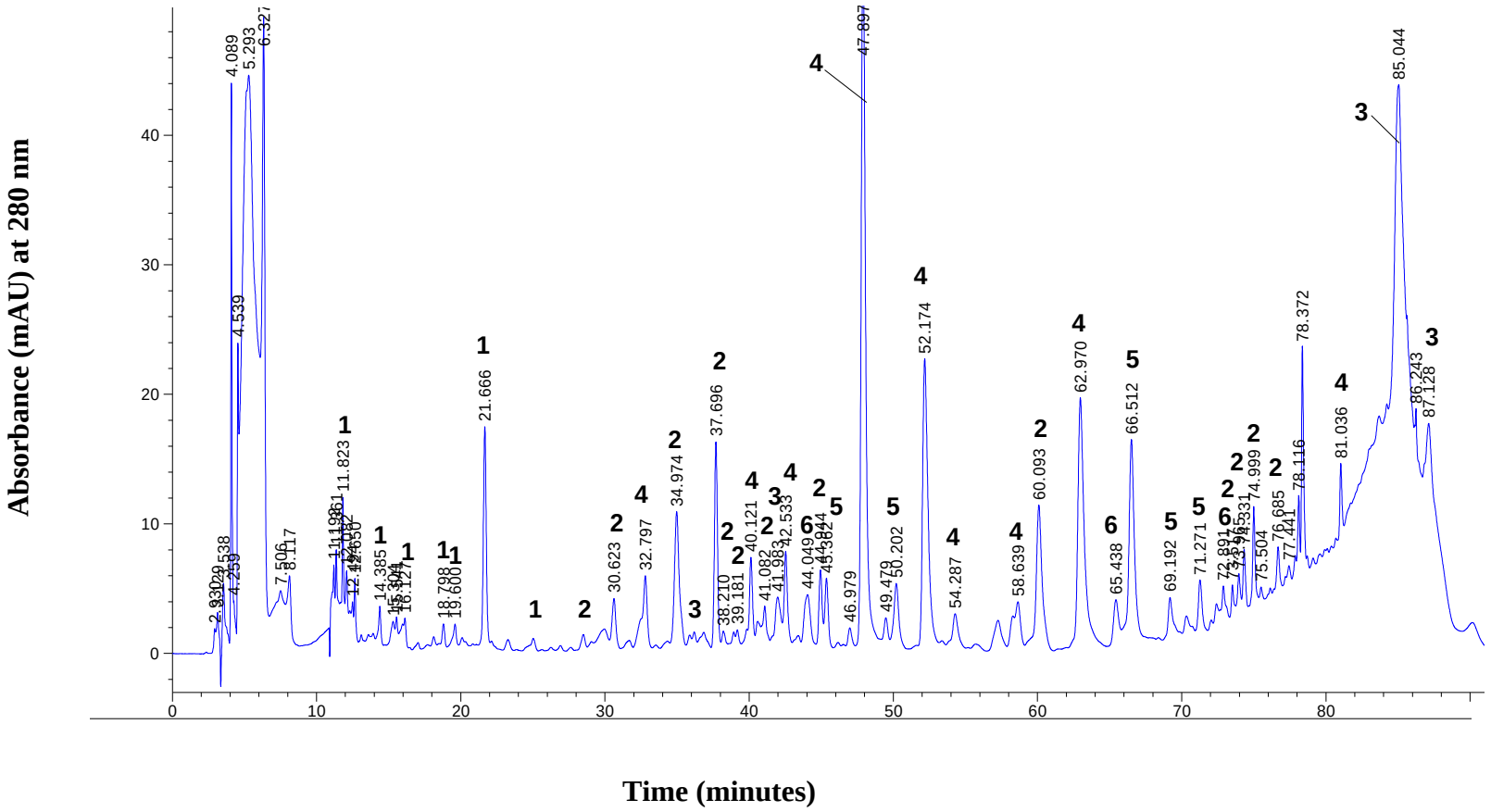


Figure 4.15 HPLC-DAD Method 2 chromatogram of buffaloberry fruit crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavones; 6) flavanones/dihydrochalcones.

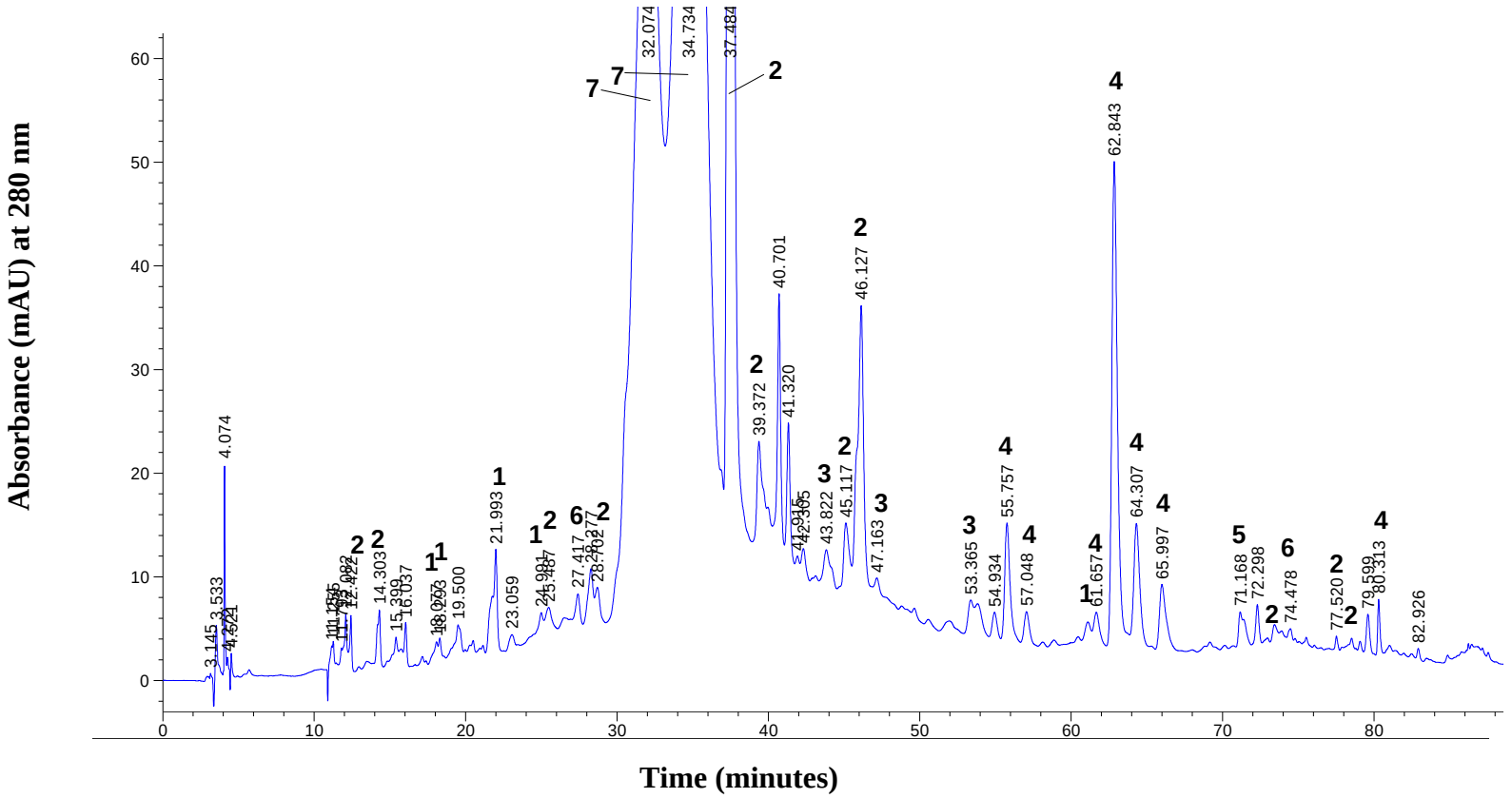


Figure 4.16 HPLC-DAD Method 2 chromatogram of chokecherry fruit crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavones; 6) flavanones/dihydrochalcones; 7) anthocyanins.

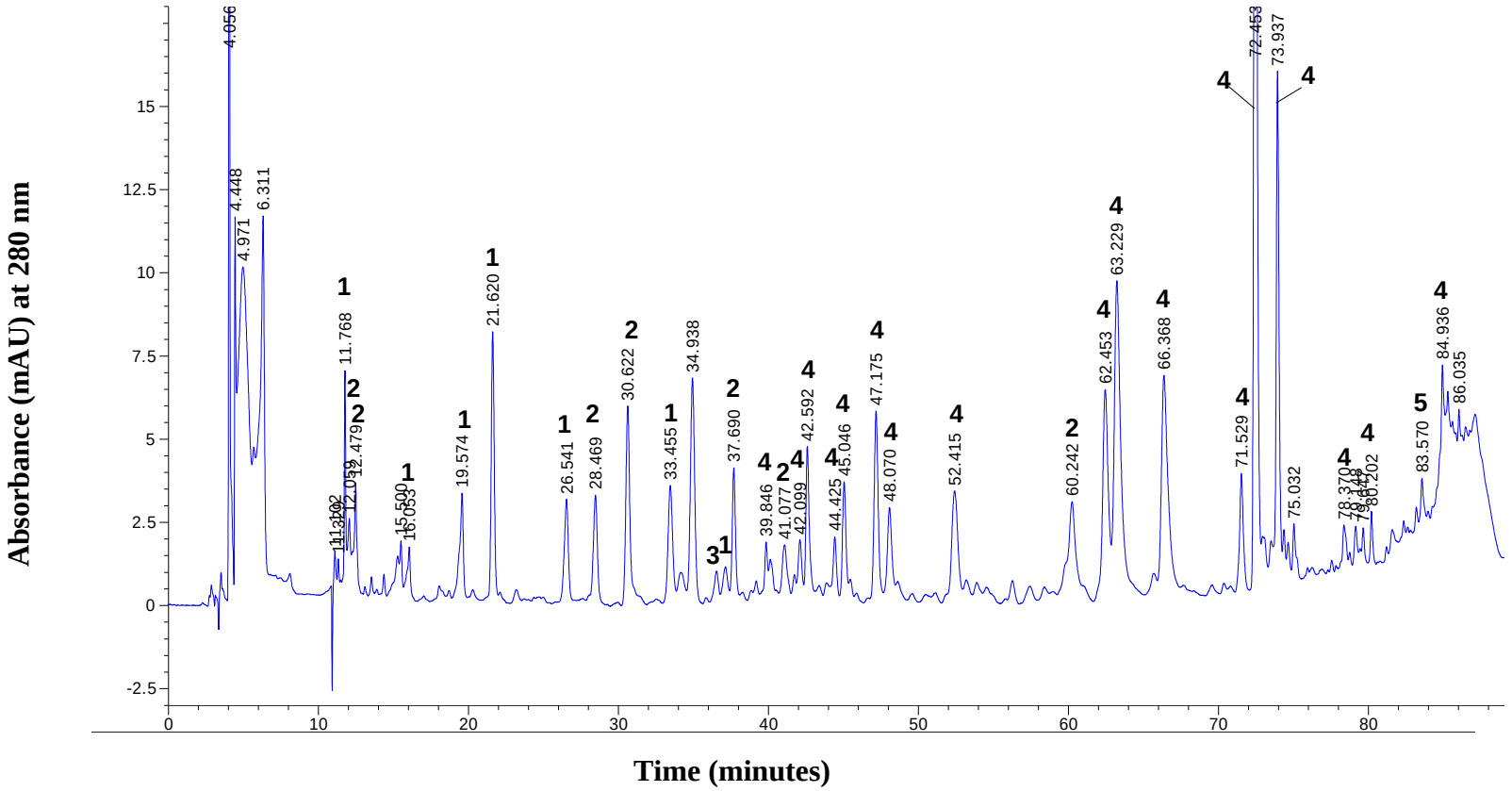


Figure 4.17 HPLC-DAD Method 2 chromatogram of sea buckthorn fruit crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavones.

chromatogram. Individual peaks on the Method 2 chromatogram were tentatively identified chlorogenic acid (RRT of 37.4 minutes), rutin (RRT of 62.7 minutes) and cinnamic acid (RRT of 77.5 minutes). The peak identities of chlorogenic acid and rutin were confirmed by LC-MS (Section 4.5.2). HPLC-DAD of the chokecherry fruit crude extract using Method 2 caused a decrease in the retention time of the two anthocyanin peaks to 32.0 and 34.7 minutes (Figure 4.16) versus 35.9 and 36.7 minutes in Method 1 (Figure 4.13). Anthocyanins are weak acids and their structure and chromatographic mobility respond to changes in mobile phase pH. In the pH range between 1 and 4, these pigments exist in an equilibrium between the oxonium cation and the carbinol forms (Brouillard, 1982). At the lower pH (2.4) condition in Method 2 this equilibrium was shifted towards the cationic form and the anthocyanins would elute faster in the reversed phase system compared to the pH 3.0 mobile phase employed in Method 1.

In comparison to the Method 1 chromatogram, the sea buckthorn fruit crude extract Method 2 chromatogram had one additional peak of both hydroxybenzoic (i.e. seven in total) and cinnamic acids (i.e. seven in total), one less flavanol (i.e. one in total), four less flavonols (i.e. seventeen in total) one additional flavone (i.e. one in total), and no (i.e. two less) flavanone/ dihydrochalcone peaks. Based on their RRT's, spectral properties and spiking experiments with phenolic standards, individual peaks were tentatively assigned as catechin (RRT of 35.3 minutes), chlorogenic acid (RRT of 37.7 minutes) and rutin (RRT of 63.2 minutes). The presence of catechin and rutin in sea buckthorn fruit was confirmed by LC-MS (Section 4.5.3).

Overall, the chromatographic results of the fruit crude extracts employing Method 2 resulted in the detection of nine additional phenolic acid peaks and the concomitant loss of four flavonoid peaks. The increase in phenolic acid peaks may be partly attributable to the lower pH of the Method 2 mobile phase with a pH of 2.4 versus 3.0 for that of Method 1. This lower pH environment may initiate *in situ* hydrolysis of phenolic compounds, particularly acylated and glycosylated flavonoids, resulting in the liberation of free phenolic acids (Herrmann, 1989; Robbins, 2003). For example, a peak matching the retention time (RRT of 37.7 minutes) and spectral properties of chlorogenic acid peak was observed in the buffaloberry fruit crude extract chromatogram using Method 2 whereas this phenolic acid was not detected in the chromatogram of this extract using Method 1.

Generally, larger peaks occurring on the Method 1 chromatograms also occurred on the Method 2 chromatograms. For example, chlorogenic acid, anthocyanins and rutin were prominent peaks on both the chokecherry fruit crude extract Method 1 and Method 2 chromatograms. Both Method 1 and Method 2 were effective in separating the seven phenolic classes of interest in the fruit phenolic extracts in the present study.

4.2.8.1 TPCI of Fruit Crude Extracts Employing the Method 2

The phenolic composition of the fruit crude extracts harvested on August 30, 2002 determined by semi-quantitative analysis of the chromatographic peaks using Method 2 is shown in Table 4.14. Chokecherry fruit extract had the highest TPCI at 6.7x and 5.6x higher than that of buffaloberry and sea buckthorn fruit crude extracts,

Table 4.14 Phenolic Composition of Buffaloberry, Chokecherry and Sea Buckthorn Fruit Crude Extracts Determined by HPLC-PDA Method 2.

Fruit	Total hydroxy-benzoic acids	Total hydroxy-cinnamic acids	Total flavanols	Total flavonols	Total flavones	Total flavanones/ dihydrochalcones	Total anthocyanins	TPCI[a]
Buffaloberry	13 ± 6[b]	155 ± 22	42 ± 6	376 ± 40	24 ±8	21 ± 7	ND[c]	631 ± 23
Chokecherry	54 ± 13	2472 ± 67	52 ± 18	646 ± 56	ND	6 ± 2	1025 ± 78	4255 ± 48
Sea buckthorn	23 ± 10	96 ± 16	6 ± 2	624 ± 58	ND	11 ± 5	ND	760 ± 27

[a] Total Phenolic Chromatographic Index = total of all identified and quantified phenolic peaks.
[b] Mean ± standard deviation of three replications expressed as µg/g whole fruit.
[c] Not detected.

respectively.

The major phenolic classes found in buffaloberry fruit crude extract were flavonols and hydroxycinnamic acids which comprised 59% and 24% of the TPCI, respectively. Flavanols were responsible for approximately 7% of the TPCI. The remaining 10% of the TPCI was attributable to the hydroxybenzoic acids, flavanones/ dihydrochalcones, and flavones with each of these contributing to less than 4% of the phenolic compounds identified in the chromatogram. Anthocyanins were not detected in buffaloberry fruit using Method 2. This result was consistent with the HPLC-DAD results employing Method 1 (Section 4.2.5.1) and by the pH differential method (Section 4.1.1). These results indicated that anthocyanins are not responsible for the red colour of buffaloberry fruit. It is notable that the crude extracts of the fruit were not coloured, indicating that the pigments were not soluble in the MFW solvent. The red pigments in the buffaloberry fruit may be carotenoids, however, the presence of these compounds was not determined in the present study.

The hydroxycinnamic acids, anthocyanins and flavonols were the predominant phenolic classes in chokecherry fruit crude extract, accounting for 58, 24, and 15% of the TPCI, respectively. Hydroxybenzoic acids and flavanones comprised less than 2% of the TPCI and flavones were not detected in the chromatogram.

Phenolic compounds in the sea buckthorn fruit crude extract were largely comprised of flavonols with this phenolic class accounting for 82% of the TPCI. Anthocyanins and flavones were not detected in the crude extract of this fruit and

this result was consistent with the chromatographic analysis employing Method 1 (Section 4.1.1).

The TPCI values obtained using Method 2 (Table 4.14) were 1.1x, 1.4x and 1.8x higher than those determined using Method 1 (Table 4.12) for crude extracts of buffaloberry, chokecherry and sea buckthorn fruit, respectively, harvested on August 30, 2002. Except for the buffaloberry fruit, these TPCI values were significantly different within the limits of standard deviation of the means for the samples. The higher TPCI's in Method 2 may be partly attributed to changes in the molar absorbance coefficients (ε) of the phenolic compounds due to a change in pH and different solvent composition of these two methods. These results indicate that the comparison of the phenolic profile and TPCI of fruits using different HPLC mobile phases must be interpreted with caution due to the variability in peak area/height of the observed chromatographic profiles.

4.2.8.2 HPLC-DAD of Acid and Base Hydrolyzed Fruit Crude Extracts

Flavonoid and phenolic acid content of fruits and vegetables are often determined following acid and/or base hydrolysis of the extracts and quantitated using aglycone standards representing the phenolic classes (Hertog et al., 1992b; Gao and Mazza, 1994b; Hakkinen et al., 1998; Hakkinen et al., 1999a; USDA, 2003). The HPLC-DAD chromatograms of the acid hydrolyzed crude extracts from the August 30, 2002 fruit using Method 2, are shown in Figures 4.18 to 4.20. Acid

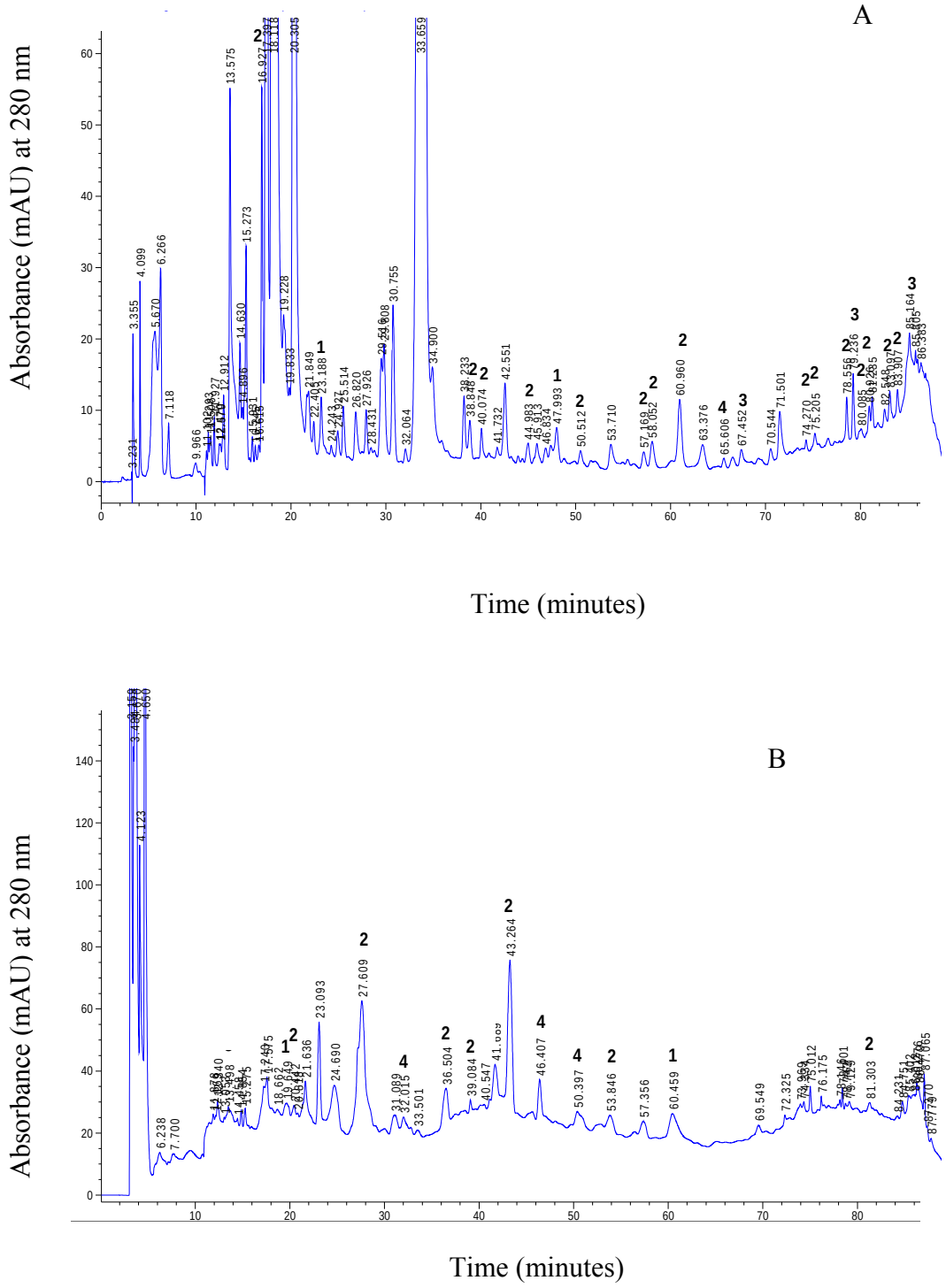


Figure 4.18 HPLC-DAD Method 2 chromatograms of: A) acid and B) base hydrolyzed buffaloberry fruit crude extract using Method 2. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols.

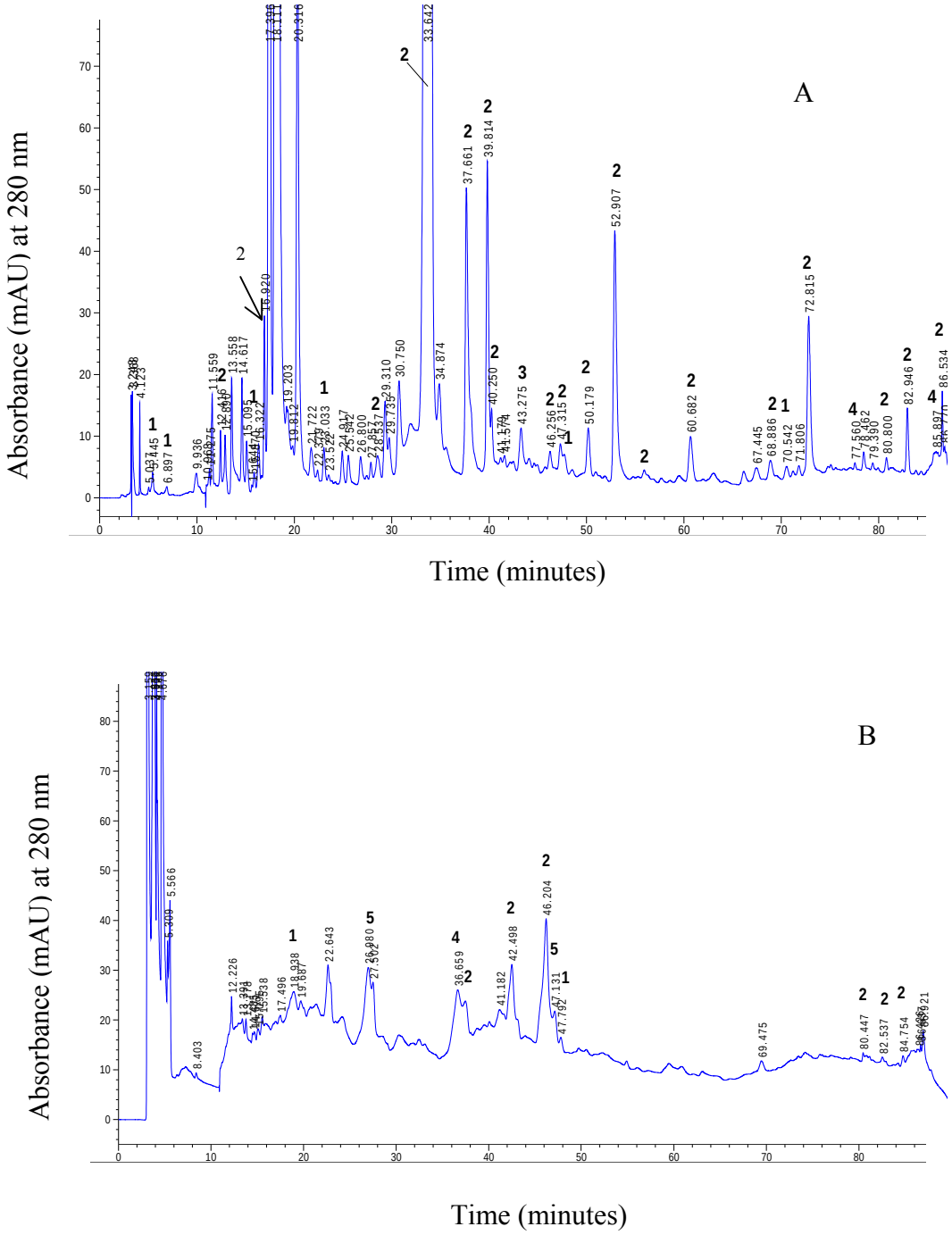


Figure 4.19 HPLC-DAD Method 2 chromatograms of: A) acid and B) base hydrolyzed chokecherry fruit crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavanones/dihydrochalcones.

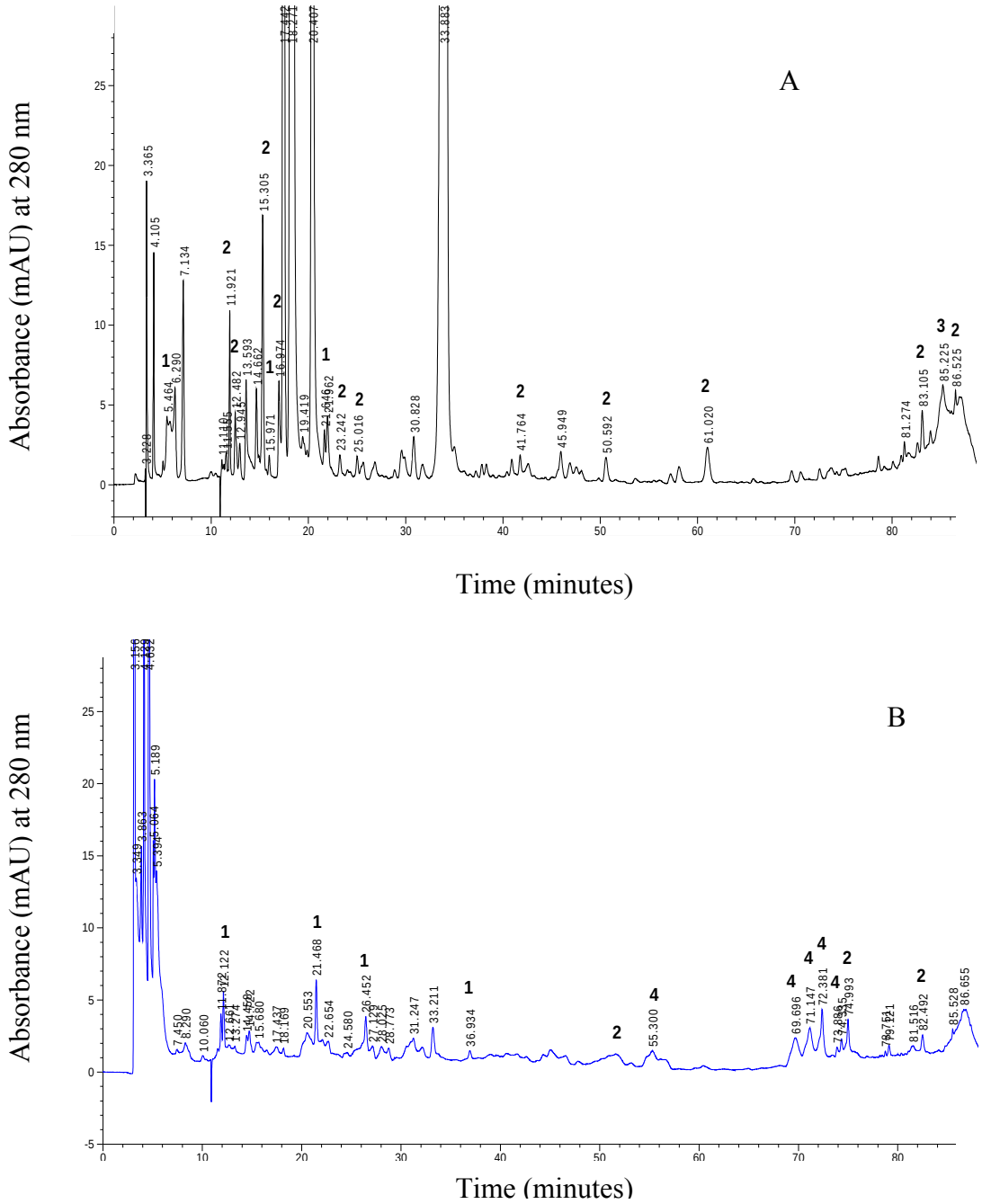


Figure 4.20 HPLC-DAD Method 2 chromatograms of: A) acid and B) base hydrolyzed sea buckthorn fruit crude extract . Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols.

hydrolysis was carried out by mixing (1:1, v/v) the fruit extracts with 2 N HCl, for 1 hour at 85 ± 3°C under nitrogen. Base hydrolysis was afforded by treating the fruit crude extracts (1:1, v/v) with 2 N NaOH, at 21 ± 2°C for 30 minutes, then neutralizing to pH 7.0 ± 0.5 using 2 N HCl.

In comparison to the buffaloberry fruit crude extract (Figure 4.15), the acid hydrolyzed sample (Figure 4.18A) contained five less hydroxybenzoic acids (i.e. two in total), three additional (i.e. fifteen in total) hydroxycinnamic acids, one less (i.e. three in total) flavanol, eight less (i.e. one in total) flavonols, five less (i.e. none) flavones and three less (i.e. none) flavanones/ dihydrochalcones. A similar result was observed in the acid hydrolyzed chokecherry fruit chromatograms (Figure 4.19) with a net increase of one additional (six in total) hydroxybenzoic acid peak, seven additional (i.e. seventeen in total) hydroxycinnamic acids, one less (i.e. one in total) anthocyanin, one less (i.e. two total) flavanol, one less (i.e. none) flavone, seven less (i.e. none) flavonols and two less (i.e. none) flavanones/dihydrochalcones compared to the HPLC-DAD chromatogram (@ 280nm) of the non-hydrolyzed fruit crude extract (Figure 4.16). In comparison to the sea buckthorn fruit crude extract, the chromatogram of the acid hydrolyzed sample had four less (i.e. three in total) hydroxybenzoic acids, five greater (i.e. twelve in total) hydroxycinnamic acids, the same number (i.e. one) of flavanol peaks, seventeen less (i.e. none) flavonols, one less (i.e. none) flavone and the same (i.e. none) flavanones/ dihydrochalcones.

Following acid hydrolysis, the chlorogenic acid peak was lost in the buffaloberry fruit crude extract. A peak occurring at retention time of 50.5 minutes was tentatively identified as *p*-coumaric acid, based on its RRT, spectral properties

and spiking with a phenolic standard. Individual phenolic acid peaks identified in acid hydrolyzed chokecherry fruit crude extracts included gallic acid (RRT of 16.3 minutes), chlorogenic acid (RRT of 37.6 minutes) and caffeic acid (RRT of 40.2 minutes) and those in acid hydrolyzed sea buckthorn fruit crude extract included gallic acid (RRT 15.9 minutes) and *p*-coumaric acid (RRT of 50.6 minutes).

Overall, while some phenolic acid peaks were lost, there was a net increase in the number of hydroxycinnamic acid peaks observed on the chromatograms of all fruit crude extracts following acid hydrolysis. A number of these new hydroxycinnamic acids eluted under less polar gradient elution conditions with retention times of in the range of 57 to 86 minutes. Gao and Mazza (1994b) reported that acid hydrolysis of pure hydroxycinnamic acids in methanol by mixing (1:1, v/v) with 2 N HCl for 1 hour at 100ºC resulted in the formation of less polar hydroxycinnamic acid methyl esters (Gao and Mazza, 1994b). These phenolic acid methyl esters may account for some of the unidentified hydroxycinnamic acid peaks on the chromatograms of the acid hydrolyzed fruit crude extracts. The identities of these newly occurring hydroxycinnamic acids however, was not determined in the present study.

Other new large peaks with retention times of 17.3 ± 0.2, 18.1 ± 0.2, 20.3 ± 0.2 and 33.6 ± 0.2 minutes occurred on the chromatograms of all acid hydrolyzed fruit crude extracts. These new peaks showed λ_{max} values in the range of 276 to 282 nm, with only one narrow absorbance band or with three to four absorbance peaks in the range of 215 to 282 nm. The spectral properties of these new peaks did not match those of the phenolic standards.

The TPCI of the acid hydrolyzed buffaloberry fruit crude extract (Table 4.15) was 1.6x greater than that of the crude extract (Table 4.14), attributable to the liberated hydroxycinnamic acids following hydrolysis. In comparison to the TPCI's of the fruit crude extracts, those of chokecherry and sea buckthorn were 40 and 50% lower following acid hydrolysis, respectively. This result was consistent with the observed loss of phenolic peaks on the chromatograms of these hydrolyzed fruit extracts.

HPLC-DAD chromatograms of all base hydrolyzed fruit crude extracts showed a decrease in the number of phenolic peaks (Figure 4.18B, 4.19B and 4.20B). Concurrently, large new peaks with retention times ranging from 3.2 to 5.5 minutes were observed on these chromatograms. These new peaks showed λ_{max} values less than 270 nm and did not match spectral profiles of any of the phenolic standards included in the present study.

Peak resolution of the phenolic compounds on the base hydrolyzed fruit extract chromatograms was poor and many of the peaks were not pure as indicated by variation in the spectral profiles at the incline and decline of these peaks. As a result, the TPCI's were not determined for the base hydrolyzed fruit crude extracts.

The purpose of the acid and base hydrolysis was to determine the phenolic content of the fruit crude extracts based on the hydrolyzed aglycones of the flavonoids and thereby accurately quantitate the phenolic classes from standard curves of the pure phenolic standards. Both the acid and base hydrolysis conditions employed in the present study were similar to methods in literature (Hertog et al., 1992b; Gao and Mazza, 1994b; Hakkinen et al, 1998). However, due to

Table 4.15 Phenolic Composition of Acid and Base Hydrolyzed Buffaloberry, Chokecherry and Sea Buckthorn Fruit Crude Extracts Determined by HPLC-PDA Method 2

Fruit	Sample	Total hydroxy-benzoic acids	Total hydroxy-cinnamic acids	Total flavanols	Total flavonols	Total flavones	Total flavanones	Total anthocyanins	TPCI[a]
Buffaloberry	Acid hydrolyzed	88 ± 9	834 ± 46	68 ± 12	14 ± 5	ND	ND	ND	1004 ± 24
	Base hydrolyzed	22 ± 8	274 ± 23	67 ± 14	129 ± 38	ND	ND	ND	492 ± 52
Chokecherry	Acid hydrolyzed	23 ± 11	2423 ± 82	122 ± 46	ND	ND	ND	211 ± 8	2719 ± 92
	Base hydrolyzed	54 ± 13	2340 ± 48	773 ± 59	ND	ND	142 ± 37	ND	3309 ± 74
Sea buckthorn	Acid hydrolyzed	12 ± 7	311 ± 26	83 ± 12	ND	ND	ND	ND	406 ± 37
	Base hydrolyzed	13 ± 4	37 ± 9	ND	66 ± 17	ND	ND	ND	116 ± 12

[a] Total Phenolic Chromatographic Index = total of all identified and quantified phenolic peaks.
[b] Mean ± standard deviation of three replications expressed as µg/g whole fruit.
[c] Not detected.

loss of flavonoid peaks on the chromatograms of the acid hydrolyzed fruit crude extracts, and general loss of all phenolic peaks on those of the base hydrolyzed samples, the hydrolysis methods were not reliable for the identification and quantification of the phenolic compounds in the fruits included in the present study.

The optimum hydrolysis conditions are reported to vary with the reaction period, acid concentration, temperature and type of fruit or vegetable (Hertog et al., 1992a; Hakkinen et al., 1998; Merken and Beecher, 2000).

4.3 Antioxidant Analysis of Fruit Crude Extracts, Phenolic Isolates and Phenolic Fractions

4.3.1 Introduction

Phenolic compounds behave as antioxidants due to the reactivity of the hydroxyl substituent on the aromatic ring. Although several mechanisms have been proposed; the predominant mode of antioxidant activity is believed to be radical scavenging via hydrogen atom donation from the phenolic hydroxyl moiety (Robbins, 2003; Rosch et al., 2003). For this reason, radical scavenging assays were selected for evaluation of the antioxidant activity of the phenolic extracts, isolates and fractions from fruit harvested on August 30, 2002. The assays included the DPPH radical scavenging assay and the Trolox equivalent antioxidant capacity (TEAC) assay. These assays, are widely employed as *in vitro* screening assays for antioxidant activity (Rice-Evans et al., 1995; Aruoma, 2002). In the DPPH test, the scavenging of DPPH radicals is followed by monitoring the decrease in absorbance

at 515 nm, which occurs due to reduction by the sample antioxidants (Aruoma, 2002). The TEAC test involves generation of the stable ABTS radical by reaction with potassium persulphate. This assay reflects the ability of hydrogen or electron-donating sample antioxidants to scavenge the ABTS radical compared to that of Trolox, a synthetic, water-soluble analogue of vitamin E. The antioxidant suppresses ABTS radical absorption at 734 nm to an extent and on a time scale dependent on the antioxidant activity (Re et al., 1999).

In addition to the established antioxidant assays, the DPPH radical scavenging ability of one-minute fractions collected from HPLC-DAD analysis of the phenolic extracts using Method 2 was determined. This method enabled the measurement of antioxidant activity of chromatographically separated phenolic crude extracts.

4.3.2 Radical Scavenging Activity of the Fruit Crude Extracts

The radical scavenging activities of the fruit crude extracts can be largely attributed to the ascorbic acid content, proanthocyanidin content, and the phenolic acid and flavonoid content as determined by the TPCI. Concentrations of these compounds in each of the fruit crude extracts was determined in the present study and are shown in Table 4.13 and 4.12 for the proanthocyanidin content and TPCI, and in Tables 4.3, 4.7, and 4.10 for the ascorbic acid contents of buffaloberry, chokecherry and sea buckthorn fruit, respectively. The total content of these antioxidant compounds in buffaloberry fruit crude extract (782 mg/100 g fruit) was 2.7x greater than the levels in chokecherry (290 mg/100 g fruit) and 3.9x greater

than the levels in sea buckthorn (200 mg/100 g fruit). The TPCI comprised 8%, 80% and 26% of the total radical scavenging substances in buffaloberry, chokecherry and sea buckthorn, respectively.

The radical scavenging assays of freeze-dried crude extracts from fruit samples harvested on August 30, 2002, are shown in Table 4.16. Buffaloberry crude extract showed the highest DPPH• scavenging activity and was 5.5x and 15.4x greater than those of chokecherry and sea buckthorn, respectively. The observed higher level of DPPH radical scavenging in the buffaloberry crude extract could be largely attributable to its high levels of ascorbic acid and proanthocyanidins. Both ascorbic acid (Brand-Williams et al., 1995) and proanthocyanidins (Maldonado, et al., 2005; Es-Safi et al., 2006) have been reported to have strong DPPH radical scavenging activities.

Table 4.16 Radical Scavenging Activity of Freeze-Dried Crude Extracts of Buffaloberry, Chokecherry and Sea Buckthorn Fruit.

Sample	DPPH Radical Scavenging[a]	TEAC Value[b]
Buffaloberry crude extract	7.7 ± 0.5	4.8 ± 0.7
Chokecherry crude extract	1.4 ± 0.1	5.1 ± 0.3
Seabuckthorn crude extract	0.5 ± 0.1	0.9 ± 0.1

[a/] Expressed as $1/IC_{50}$ (1/mg sample for 50% DPPH radical inhibition).
[b/] Expressed as mM Trolox equivalents/100 mg sample.

The TEAC values of chokecherry and buffaloberry fruit crude extracts were equivalent within the determined experimental error (i.e. standard deviation of three replicates) at 5.1 ± 0.3 and 4.8 ± 0.7 mM Trolox/100 mg sample, respectively, and these values were 5x higher than those of sea buckthorn. Chokecherry fruit contained lower levels of ascorbic acid and proanthocyanidins, however, its TPCI was 6.7x greater than that of buffaloberry and 5.6x higher than that of sea buckthorn fruit. Flavonoids and many phenolic acids have been shown to have TEAC values that are 1.5 to 3.0x higher than that of ascorbic acid (Rice Evans et al., 1997; Re et al., 1999). Buffaloberry fruit extract was rich in proanthocyanidins, however, these compounds did not result in a significantly higher TEAC value compared to that of chokecherry fruit. It has been reported in literature that radical scavenging compounds may have different scavenging activities for different radicals. For example, Schleisier et al. (2002) performed six *in vitro* antioxidant assays, including the TEAC and DPPH assays, for analyzing the antioxidant activity of ten beverages, and found the ranking based on their radical scavenging activity differed with the assay method.

Samples of commerical blueberry and cranberry fruit concentrates were assayed for radical scavenging activity for comparison to the fruit crude extracts included in the present study. The fruit concentrates were adjusted to 12ºBrix and assayed for DPPH radical scavenging and TEAC. The DPPH radical scavenging activities were 0.6 ± 0.2 and 0.8 ± 0.1, expressed as 1/mg solids for 50% DPPH inhibition, for blueberry and cranberry, respectively. These radical scavenging activities were similar to the corresponding value for sea buckthorn and lower than

those of buffaloberry and chokecherry. Blueberry concentrate produced a TEAC value of 4.2 ± 0.2 which was comparable to the TEAC values of buffaloberry and chokecherry, and 4.7x higher than that of sea buckthorn. The TEAC value of the cranberry concentrate was 8.2 ± 1.7 which was approxiamtely 1.6x higher than those of buffaloberry and chokecherry and 9.1x higher than the TEAC of sea buckthorn fruit crude extract. Overall, the radical scavenging activities of the fruit crude extracts included in this study compared favourably to those of blueberry and cranberry concentrates, two commerical fruit products reputed as functional foods.

4.3.3 Radical Scavenging Activity of Fruit Freeze Dried Phenolic Isolates

Phenolic isolates were prepared using Amberlite XAD-16 as described in section 3.5. The fruit crude extracts were loaded onto the XAD-16 resin bed and polar, water-soluble compounds were eluted with ddH_2O. The phenolic isolate was then collected by elution with 70% methanol, concentrated by rotary vacuum evaporator at 45°C and freeze-dried. Antioxidant assays were performed on the freeze dried phenolic isolates.

Chokecherry had a DPPH radical scavenging activity that was 13 and 6.5x greater than the radical scavenging activities of buffaloberry and sea buckthorn, respectively (Table 4.17). This result can be attributed to chokecherry's high TPCI, which was 40x and 11x greater than those of buffaloberry and sea buckthorn, respectively (Table 4.18). The chokecherry phenolic isolate was comprised largely of hydroxycinnamic acids, flavonols and anthocyanins, which represented 72, 10 and 8% of the TPCI.

Table 4.17 Radical Scavenging Activity of Dried Phenolic Isolates of Buffaloberry, Chokecherry and Sea Buckthorn Fruit.

Sample	DPPH Radical Scavenging[a]	TEAC Value[b]
Buffaloberry phenolic isolate	1.8 ± 0.2	15.2 ± 1.2
Chokecherry phenolic isolate	23.4 ± 3.1	122.8 ± 34.4
Sea buckthorn phenolic isolate	3.6 ± 0.2	7.1 ± 0.9

[a/] Expressed as $1/IC_{50}$ (1/mg sample for 50% DPPH radical inhibition).
[b/] Expressed as mM Trolox equivalents/100 mg sample.

The TEAC value of the chokecherry phenolic isolate was 8x and 17x greater than those of buffaloberry and sea buckthorn, respectively. Sea buckthorn produced a TEAC value that was 53% lower although its TPCI was 3.6x greater in comparison to buffaloberry phenolic isolate. The buffaloberry phenolic isolate contained a flavanol concentration that was 4.1x greater than that of the sea buckthorn isolate and this may have contributed to the higher TEAC value of the buffaloberry sample. Flavanols have been reported to produce TEAC values approximately 1.1 to 6x higher than other flavonoids and approximately 1.6 to 122x higher than phenolic acids (Rice-Evans et al., 1996). Furthermore, phenolic compounds possessing ortho-dihydroxy groups and/or tri-hydroxy groups on aromatic ring(s) are reported to result in TEAC values that are greater than the corresponding phenolic acid or flavonoid containing a mono-hydroxy group (Rice-Evans et al., 1995; Rice-Evans et al., 1996; Rosch et al., 2003). It is possible that the buffaloberry phenolic isolate contained higher quantities of ortho-dihydroxy and tri-hydroxy phenolic compounds compared that of sea buckthorn. Information on

Table 4.18 Phenolic Composition of Buffaloberry, Chokecherry and Sea Buckthorn Fruit Phenolic Isolates as Determined by HPLC-PDA Method 2

Fruit	Total hydroxy-benzoic acids	Total hydroxy-cinnamic acids	Total flavanols	Total flavonols	Total flavones	Total flavanones /dihydrochalcones	Total anthocyanins	TPCI[a]
Buffaloberry	212 ± 19[b]	1487 ± 87	2614 ± 141	4106 ± 281	733 ± 54	938 ± 46	ND[b]	10090 ± 167
Chokecherry	1414 ± 66	299410 ± 9926	31086 ± 1273	42287 ± 2165	1437 ± 139	4625 ± 244	32104 ± 2612	412363 ± 7634
Sea buckthorn	308 ± 22	4984 ± 280	635 ± 46	28917 ± 1905	ND	2342 ± 185	ND	37186 ± 792

[a] Total Phenolic Chromatographic Index = sum of all identified and quantified phenolic peaks.
[b] Mean ± standard deviation of two replications reported as µg/g dry sample.
[c] Not detected.

the individual structures of the flavonoids and phenolic acids present in the fruit samples could not be completely elucidated under the HPLC-DAD and limited LC-MS analyses employed in this study.

4.3.4 Radical Scavenging Activity of XAD Fractions

The DPPH radical scavenging activity and TEAC values of XAD fractions from the 20%, 50% and 70% methanol elutions from the Amberlite XAD-16 (XAD) column are shown in Table 4.19. Typical chromatograms at 280 nm of the 20% and 50% fractions, and the 70% methanol fraction acquired from buffaloberry are shown in Figures 4.21 and 4.22, respectively. The retention times and spectral properties

Table 4.19 Radical Scavenging Activity of Freeze-Dried XAD Fractions from Fruit Crude Extract.

XAD Fraction	DPPH Radical Scavenging[a]	TEAC Value[b]
Buffaloberry		
20% methanol	0.7 ± 0.1[c]	4.6 ± 0.5
50% methanol	8.1 ± 0.3	8.8 ± 0.2
70% methanol	23.1 ± 5.9	26.3 ± 0.8
Chokecherry		
20% methanol	1.0 ± 0.1	5.4 ± 0.2
50% methanol	22.6 ± 5.9	206.1 ± 1.4
70% methanol	13.9 ± 0.5	163.9 ± 5.7
Seabuckthorn		
20% methanol	3.2 ± 0.3	7.7 ± 0.0
50% methanol	10.2 ± 1.4	14.8 ± 0.5
70% methanol	7.5 ± 3.0	22.1 ± 1.0

[a/] Expressed as $1/IC_{50}$ (1/mg sample for 50% DPPH radical inhibition).
[b/] Expressed as mM Trolox equivalents/100 mg sample solids.
[c/] Mean ± standard deviation of three replicates.

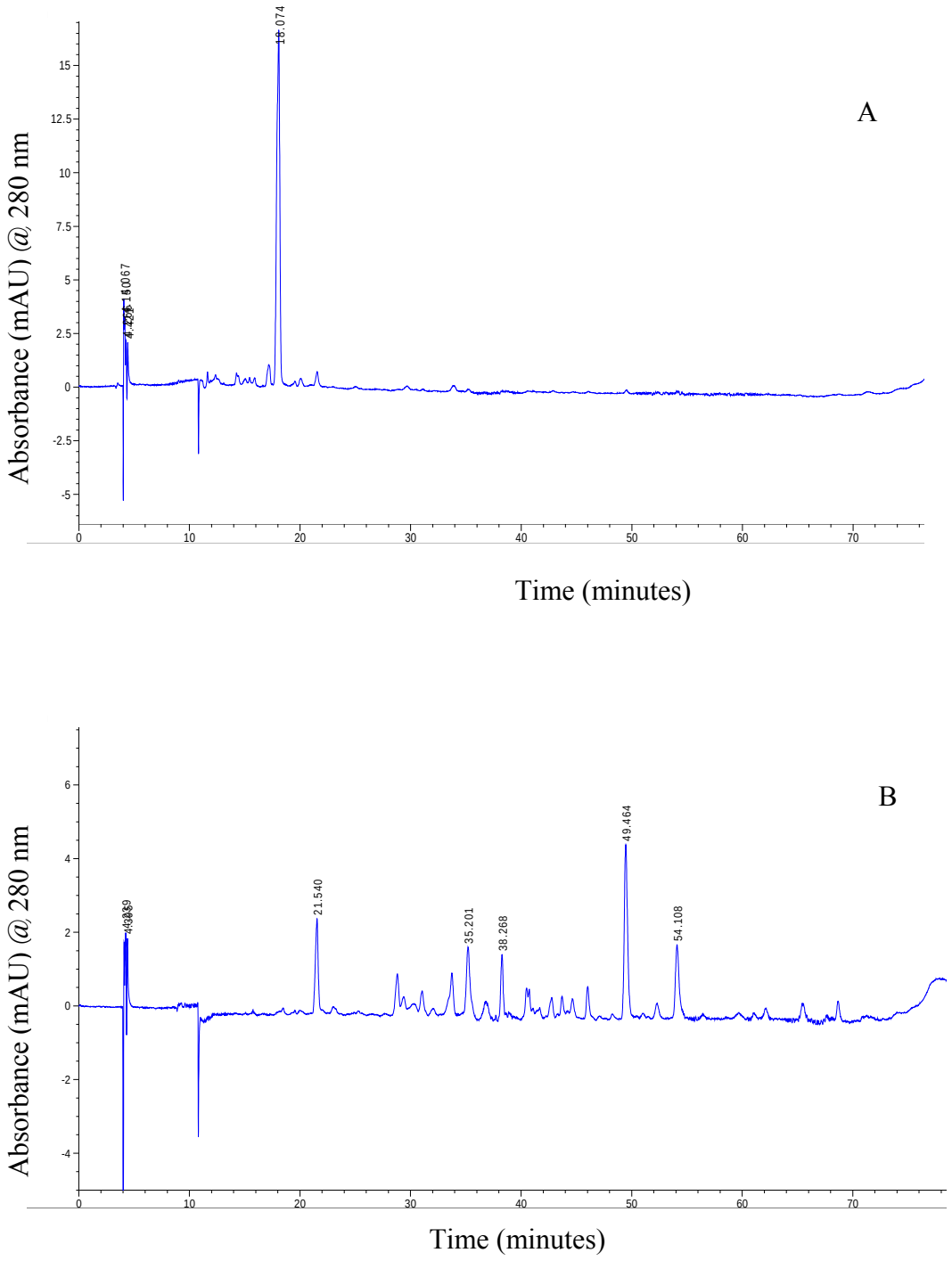


Figure 4.21 HPLC-DAD Method 2 chromatogram of XAD-16 fractions from buffaloberry fruit extract: A) 20% methanol fraction; B) 50% methanol fraction.

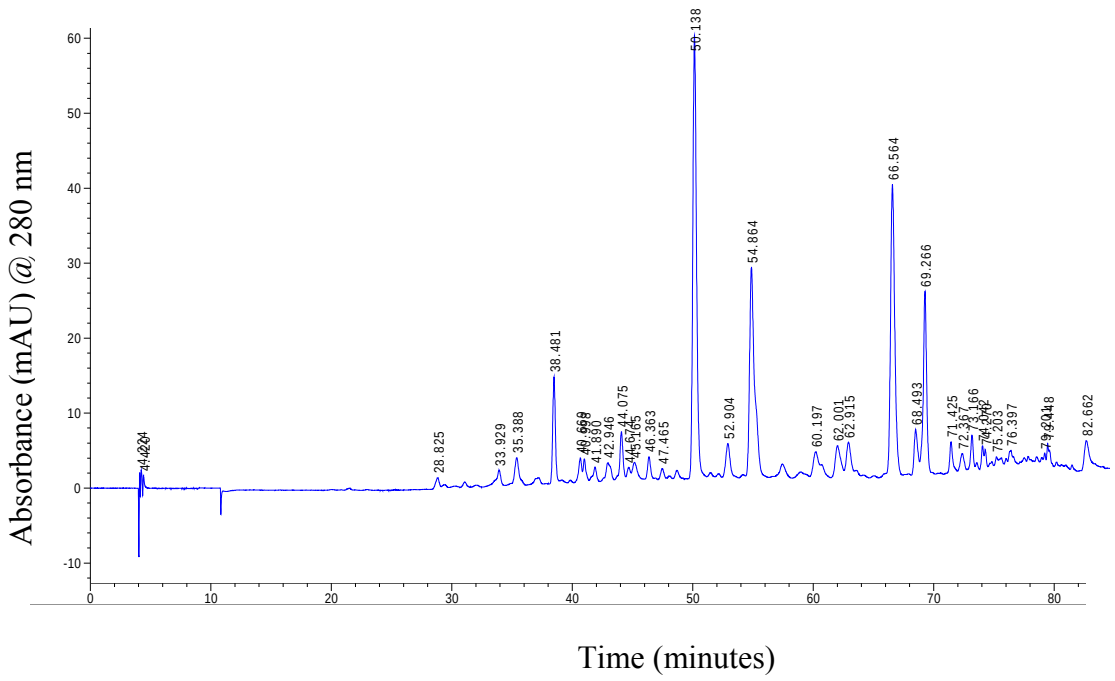


Figure 4.22 HPLC-DAD Method 2 chromatogram of 70% methanol XAD fraction from buffaloberry fruit extract.

of the peaks present in the chromatograms indicated that polar phenolic compounds, primarily phenolic acids eluted in the 20% methanol fraction and flavonoids eluted in the 50 and 70% methanol fractions. The phenolic compostion and TPCI's of the fruit XAD fractions is provided in Table 4.20.

Overall, the highest radical scavenging activity was observed in the 70% methanol XAD fractions for buffaloberry and sea buckthorn (Table 4.19). These fractions had the highest TPCI's, and flavonols were the most abundant phenolic class (Table 4.20). The highest radical scavenging activity in the chokecherry fruit was found in the 50% methanol XAD fraction. This fraction was 54% lower in TPCI, however 1.8x, 6.5x, 2.4x and 2.9x greater in flavanols, hydroxybenzoic

Table 4.20 Phenolic Composition of Buffaloberry, Chokecherry and Sea Buckthorn Fruit Fractions Determined by HPLC-PDA Method 2.

Fruit	XAD Fraction	Total hydroxy-benzoic acids	Total hydroxy-cinnamic acids	Total flavanols	Total flavonols	Total flavones	Total flavanones/ dihydro-chalcones	Total anthocyanins	TPCI[a]
Buffaloberry	20% methanol	149 ± 12	606 ± 52	212 ± 23	ND	ND	ND	ND	967 ± 48
	50% methanol	731 ± 85	4262 ± 338	1002 ± 136	8044 ± 792	796 ± 77	204 ± 26	ND	15039 ± 437
	70% methanol	218 ± 13	2866 ± 192	443 ± 42	34849 ± 2934	8767 ± 1087	1002 ± 114	ND	48145 ± 1827
Chokecherry	20% methanol	357 ± 33	3739 ± 334	ND	ND	ND	ND	ND	4096 ± 279
	50% methanol	1722 ± 241	203010 ± 15469	21054 ± 1675	3614 ± 354	ND	9944 ± 1277	31536 ± 2193	270880 ± 1526
	70% methanol	266 ± 36	441637 ± 17911	11869 ± 1583	111793 ± 4735	2743 ± 236	4108 ± 388	10708 ± 599	583124 ± 14875
Sea buckthorn	20% methanol	527 ± 71	787 ± 91	ND	ND	ND	18 ± 7	ND	1332 ± 82
	50% methanol	1409 ± 191	26093 ± 2412	2042 ± 194	13553 ± 1118	ND	279 ± 31	ND	43376 ± 1468
	70% methanol	142 ± 23	7380 ± 693	1541 ± 208	143987 ± 8970	1381 ± 123	1861 ± 53	ND	156229 ± 746

[a] Total Phenolic Chromatographic Index = sum of all identified and quantified phenolic peaks.
[b] Mean ± standard deviation of two replications. Results reported as µg/g dry sample.
[c] Not detected.

acids, flavanones and anthocyanins, respectively, in comparison to the chokecherry 70% methanol fraction (Table 4.20). The flavanols and anthocyanins have been shown to possess high radical scavenging activity (Rice-Evans et al., 1995; Rice-Evans et al., 1996; Rosch et al., 2003), and this may explain the observed higher radical scavenging activity of the chokecherry 50% methanol fraction. Flavanols such as catechin and epicatechin are effective radical scavengers due to their ortho-dihydroxyl moieties on the B-ring. Anthocyanins, flavonols and flavones have high radical scavenging activities due to unsaturation in their C-rings, allowing for electron delocalization across the molecule for stabilization of the phenoxy radical (Rice-Evans et al., 1996).

The observed trend of high antioxidant activities concomittant with XAD fractions containing higher concentrations of flavanols, flavonols and anthocyanins is consistent with those of pure phenolic acids and flavonoids (Rice-Evans et al., 1996). The authors reported high TEAC values for flavanols, flavonols, anthocyanidins and anthocyanins ranging from 1.8 to 4.9 mM Trolox equivalents when compared to flavanones, flavones and most phenolic acids with values less than 1.74 mM Trolox. Epicatechin gallate possessed the highest TEAC value of the flavonoids studied at 4.9 mM Trolox followed by epigallocatechin gallate (4.8), quercetin (4.7), delphinidin (4.7) and cyanidin (4.4). Glycosylated flavonols and anthocyanins were also shown to have high antioxidant activities with TEAC values for rutin and cyanidin 3-rutinoside of 2.4 and 3.25. Glycosylated flavones and flavanones had TEAC values ranging from 0.76 to 1.74, and phenolic acids ranged from 0.04 for salicylic acid to 2.19 for caffeic acid.

4.3.5 DPPH Radical Scavenging Activities of Fruit Crude Extract HPLC Fractions

In order to determine the specific compounds responsible for the radical scavenging activity in the fruit crude extracts, a modified DPPH assay was developed to measure the %DPPH radical (DPPH•) inhibition of one-minute fractions eluting from the HPLC column (Section 3.11) employing Method 2. The %DPPH• inhibition of the HPLC mobile phase (i.e. 10 mM formic acid-acetonitrile) was also measured and found to be consistent throughout the gradient elution at levels of 8 to 12%DPPH• inhibition. Therefore, DPPH• scavenging activity values >12% were considered to be due to the compounds present in the fractions.

The highest level of radical scavenging activity at 55% DPPH• inhibition was observed in the four to five minute HPLC fraction of the buffaloberry fruit crude extract (Figure 4.23). The corresponding chromatographic peak at 4.3 minutes had tangent peaks at 4.0, 4.2 and 4.6 minutes, and contained compounds eluted in the MFW solvent front. Chromatographic peaks at 4.2, 4.3 and 4.6 minutes showed UV spectra that matched that of ascorbic acid (Figure 4.24). Ascorbic acid standard (210 mg/ 100 mL MFW) chromatographed using HPLC-DAD Method 2 eluted as a wide band with a small peak with a retention time 4.2 ± 0.3 minutes and a larger, asymmetrical peak at 6.3 ± 0.3 minutes. These results suggest that ascorbic acid, or a derivative of this compound, was a component of the four to five minute HPLC fraction. Additional, unidentified compounds also contributed to the DPPH• scavenging activity of this fraction as at least three tangent peaks eluted with the solvent front peak at 4.3 minutes (Figure 4.23). The

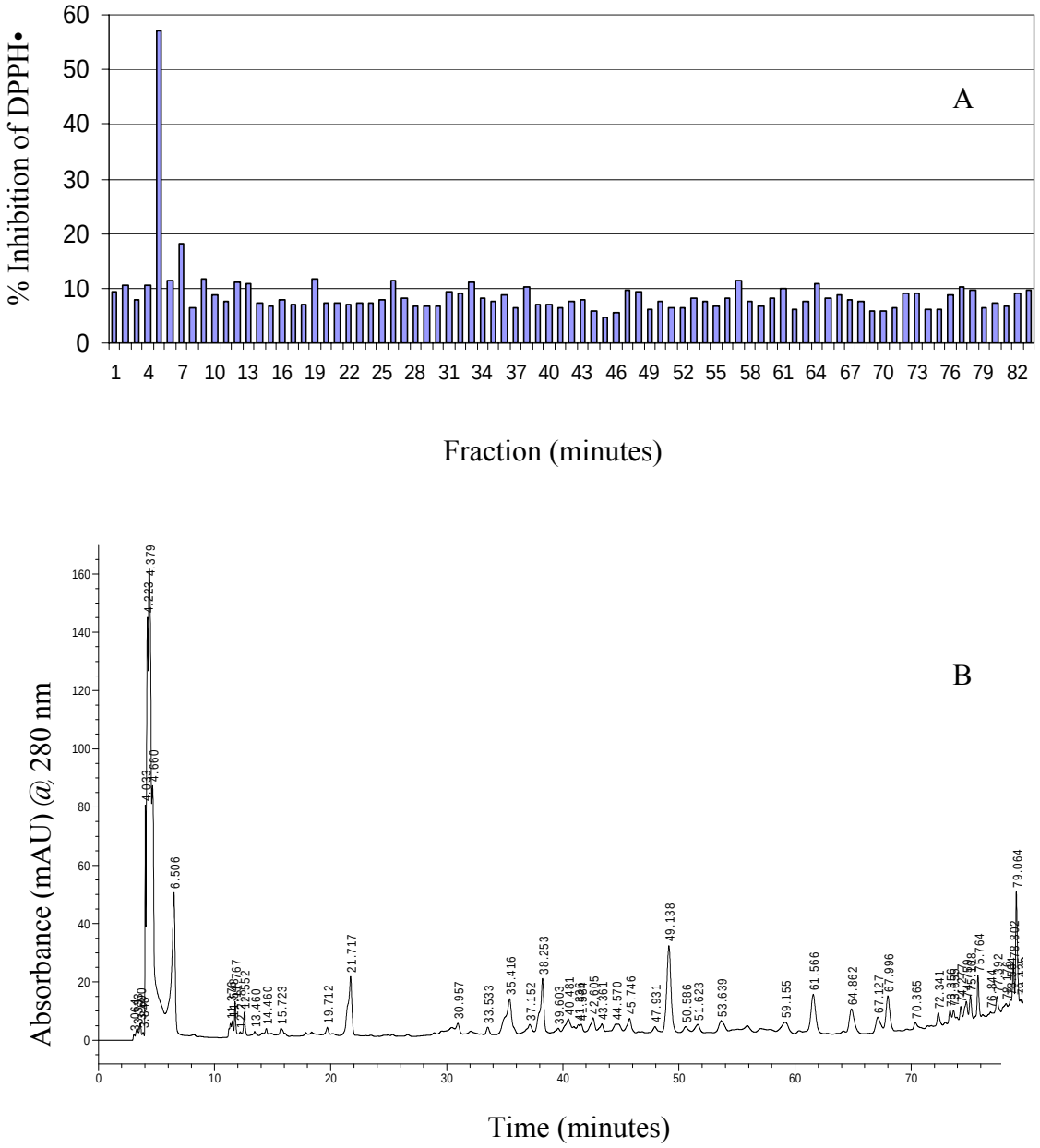


Figure 4.23 Buffaloberry fruit crude extract: A) DPPH scavenging activity of HPLC one-minute fractions; B) HPLC-DAD Method 2 chromatogram.

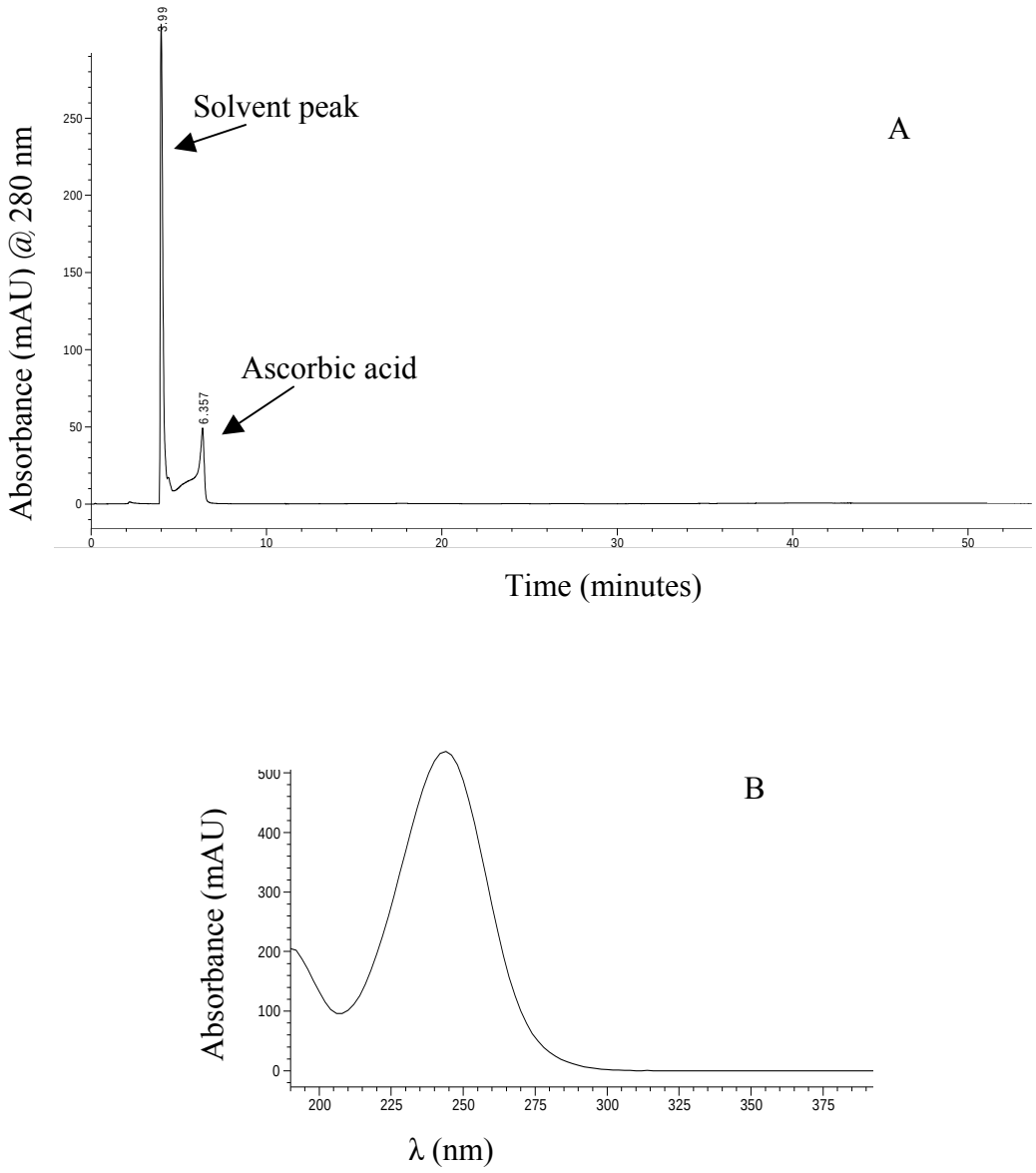


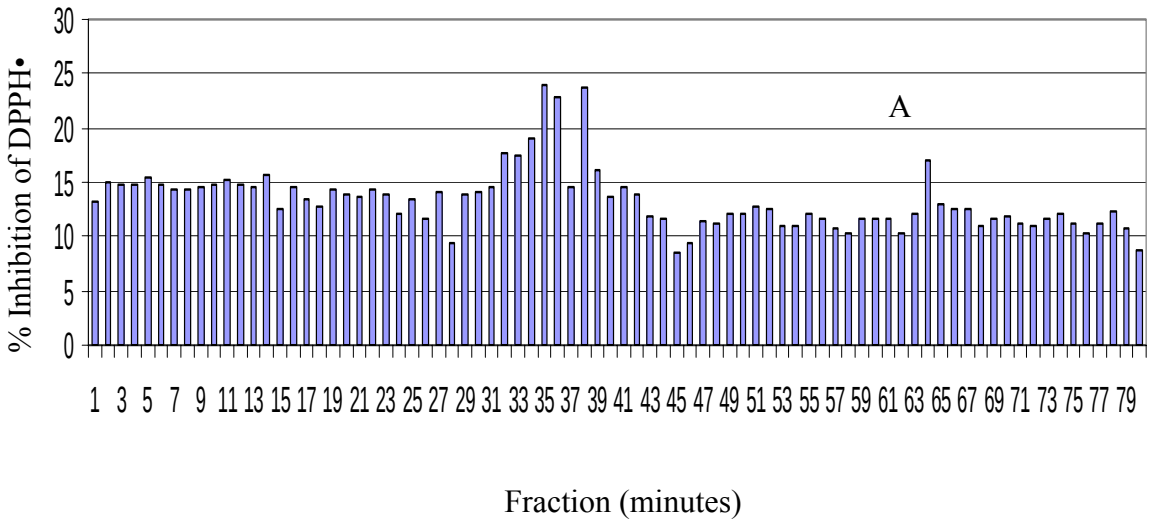
Figure 4.24 Ascorbic acid: A) Standard HPLC-DAD chromatogram (@280 nm); B) absorbance spectrum of the ascorbic acid peak (λ_{max} = 244 nm).

spectral profile of the tangent peak at 4.0 minutes showed a λ_{max} at 210 nm and a smaller absorbance peak at 266 nm. This spectral profile did not match any of the chemical standards employed in this study. Furthermore, the lack of absorbance at 280 nm indicated that phenolic compounds were not present in this tangent peak.

A relatively high level of DPPH radical scavenging (18% DPPH• inhibition) was also observed in the six to seven minute HPLC fraction. This fraction corresponded to the ascorbic acid peak occurring at a retention time of 6.5 minutes and matched the spectral profile of this compound. This result further supports the finding that ascorbic acid was an important radical scavenging component of buffaloberry fruit (Section 4.3.2).

The buffaloberry fruit HPLC fractions collected after seven minutes showed no notable DPPH• inhibition, suggesting that the compounds contained in these fractions were of insufficient concentration to provide radical scavenging activity under these assay conditions.

Chokecherry HPLC fractions having RRT's ranging from 31 to 38 minutes and from 63 to 64 minutes showed >16% DPPH• inhibition (Figure 4.25). The identities of the phenolic peaks in these fractions were of interest, since the chokecherry fruit phenolic isolate was found to possess higher radical scavenging activities than those of the buffaloberry and sea buckthorn fruits (Seaction 4.3.3).



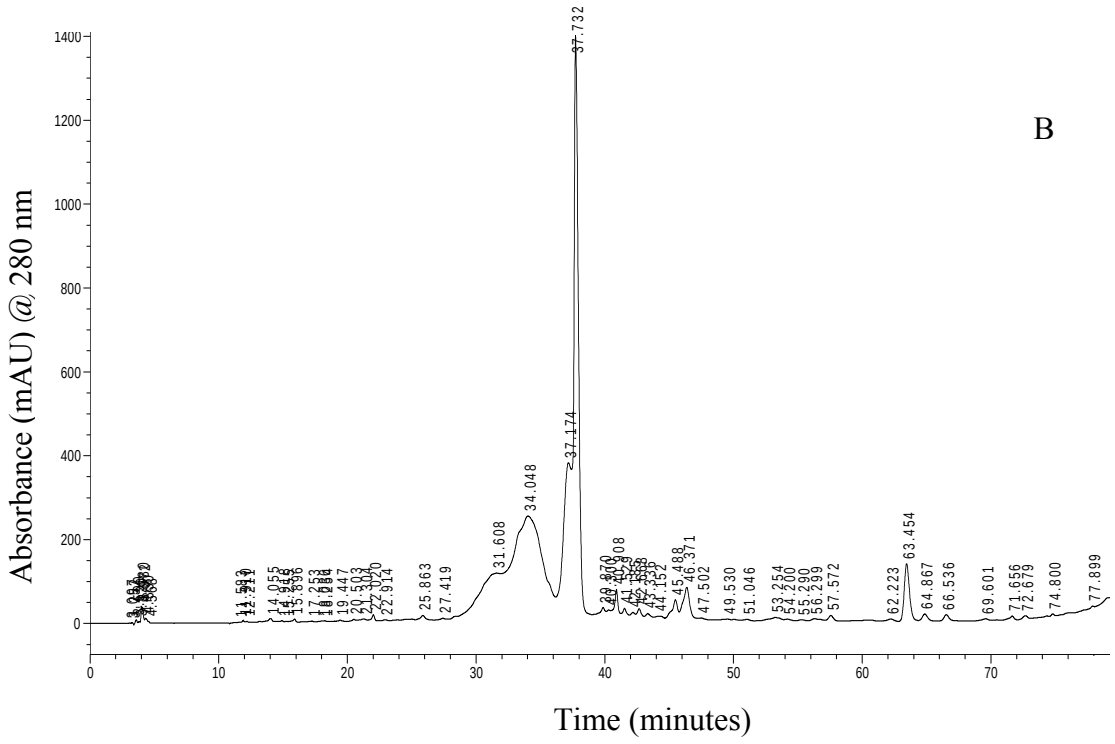


Figure 4.25 Chokecherry fruit crude extract: A) DPPH scavenging activity of HPLC one-minute fractions; B) HPLC-DAD chromatogram.

The largest peak on the chokecherry crude extract chromatogram occurred at a RRT of 37.7 minutes and the corresponding HPLC fraction (37 to 38 minutes) showed a DPPH• inhibition of 24%. This peak was tentatively identified as chlorogenic acid, based on its RRT and spectral properties when compared to those of a pure standard under the same chromatographic conditions. Confirmation of the peak identity was done by LC-MS and is presented in Section 4.5.2.

Two broad peaks, identified to be anthocyanins (Sections 4.2.8) with RRT's at 31.6 and 34.0 minutes also corresponded to high levels (17 to 24%, respectively) of DPPH• inhibition. Confirmation of the identities of these anthocyanins was done by further HPLC-DAD and LC-MS analyses, and these are provided in Section 4.4. Anthocyanins were also found to be important contributors to the radical scavenging activity in chokecherry extract, isolate and XAD fractions (Sections 4.3.2, 4.3.3 and 4.3.4, respectively).

The phenolic peak with a RRT of 63.4 minutes corresponded to the HPLC fraction showing 16% DPPH• inhibition. This peak was identified as rutin, based on its matching spectral profile and RRT to a rutin standard under the same chromatographic conditions. Confirmation of peak identity was done by LC-MS analyses and is presented in Section 4.5.2.

In addition to the prescence of anthocyanins, chlorogenic acid and rutin in the chromatogram of chokecherry fruit crude extract, the observed high DPPH• inhibition of these compounds is in agreement with radical scavenging data reported in literature. For example, Fukomoto and Mazza (2000) determined the DPPH radical scavenging activity of numerous pure phenolic compounds and found that

chlorogenic acid and rutin were among the most effective of those studied. De Heer et al. (1999) proposed that phenolic compounds bearing a substituent in the ortho position to the hydroxyl group typically enables easier donation of hydrogen atoms to free radicals due to intramolecular hydrogen bonding that stabilizes the phenoxy radical. Anthocyanidins/anthocyanins were also identified as effective DPPH radical scavengers, especially those that possess ortho-dihydroxy groups on the B-ring of the molecular structure (Fukomoto and Mazza, 2000).

Notable DPPH radical scavenging activity (>25% DPPH• inhibition) was observed in the HPLC fractions collected from 4 to 15 minutes from the sea buckthorn fruit crude extract (Figure 4.26). An ascorbic peak was identified at a retention time of 6.4 minutes on the fruit extract chromatogram, based on matching RRT, spectral profile and sample spiked with an ascorbic acid standard. Other than the ascorbic acid peak with an RRT of 6.4 minutes, there were few significant chromatographic peaks that occurred in these HPLC fractions. The spectral profiles selected at one-minute intervals with RRT's of 8 to 11 minutes showed UV absorbances less than 240 nm. This result suggested that other, non-phenolic compounds were largely responsible for the observed DPPH• scavenging activity in these fractions, as all phenolic compounds show UV absorbance at 280 nm (Mabry et al., 1970).

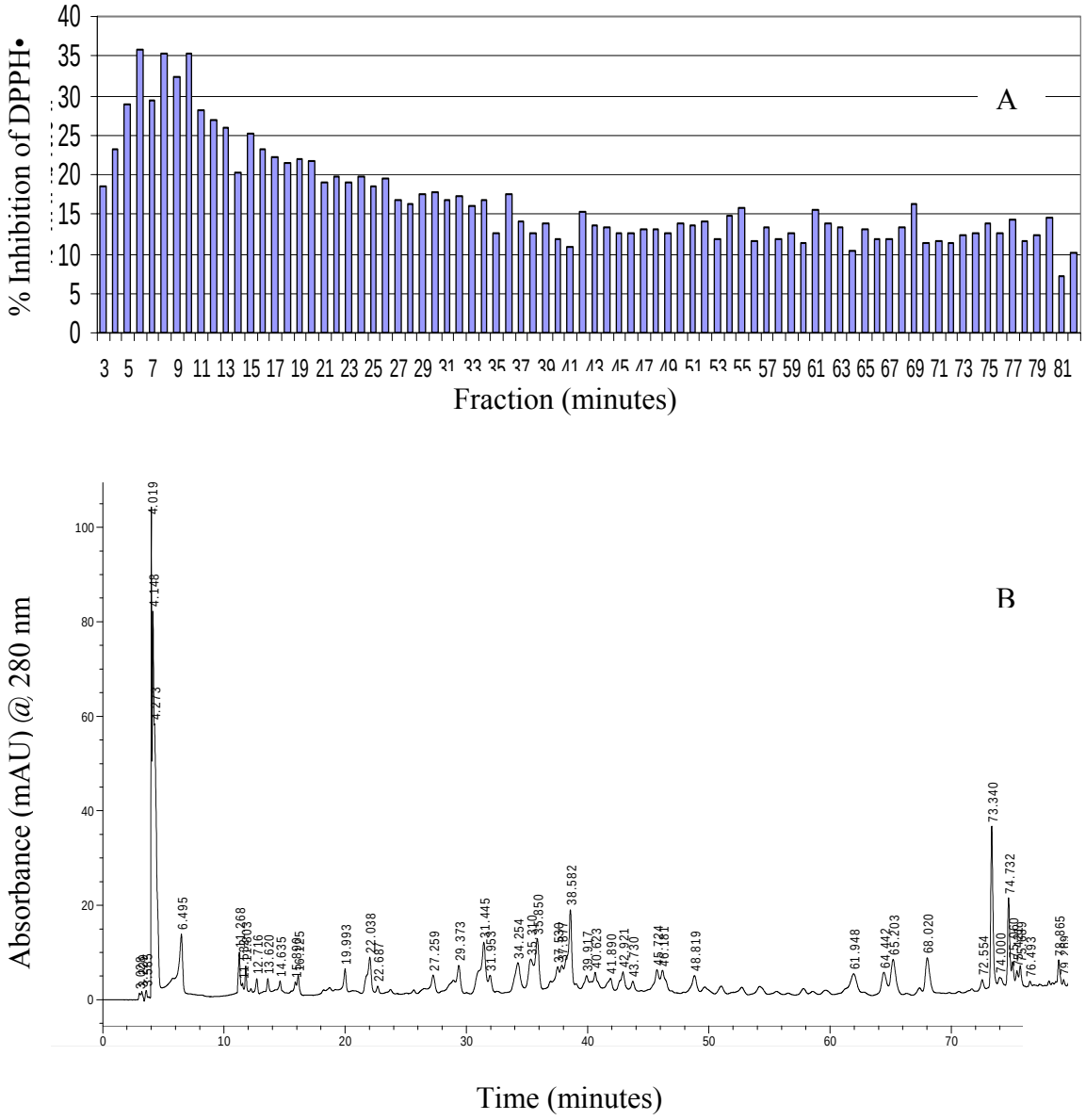


Figure 4.26 Sea buckthorn fruit crude extract: A) DPPH scavenging activity of HPLC one-minute fractions; B) HPLC-DAD chromatogram.

4.4 Identification of Chokecherry Anthocyanins

Radical scavenging activity assays and HPLC-DAD analysis of the phenolic isolates and fractions from chokecherry fruit indicated that the anthocyanins were important contributors to its radical scavenging activity. Furthermore, the anthocyanin content of 255 ± 35 mg/100 g chokecherry fruit was found to be high in comparison to other native fruits, such as the saskatoon berry (179 mg/100 g fruit) and some commercial fruits such as cranberry (78 mg/100 g fruit; Section 4.1.5.8), noted for their high antioxidant activity due to their anthocyanin content (Mazza and Miniati, 1993). As a result of these findings and due to the lack of published information on chokecherry pigments, the identification of the major anthocyanins in this fruit was of interest in the present research.

Chokecherry anthocyanins were pre-isolated on a C_{18} Sep-Pak cartridge (Waters Corporation) prior to HPLC analysis (section 3.10.3.1). Reversed phase HPLC-DAD analysis was carried out using a mobile phase of 4% (v/v) phosphoric acid-acetonitrile mobile phase elution with detection at 520 nm (section 3.10.3.2). The chokecherry fruit was found to contain thirteen peaks in total that absorbed in the visible range, however, two major peaks accounted for greater than 98% of the total chromatographic peak area. These two anthocyanin peaks with retention times of 43.9 and 46.0 minutes (Figure 4.27) were designated ACY1 and ACY2, respectively. These pigments were most likely to be anthocyanins, as anthocyanidins are unstable and typically occur in the glycosylated form (i.e. anthocyanins) in plants (Mazza and Miniati, 1993; Springob et al., 2003).

A series of anthocyanin standards including, cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-rutinoside and pelargonidin 3-glucoside were chromatographed using the 4% phosphoric acid-acetonitrile mobile phase. These anthocyanin compounds were selected as they are widely distributed throughout the Plant Kingdom (Brouillard, 1982; Mazza and Miniati, 1993) and were available commercially. Based on similar RRT's, ACY1 and ACY2 were tentatively assigned as cyanidin 3-glucoside (RRT of 43.9 minutes) and cyanidin 3-rutinoside (RRT of 46.0 minutes), respectively. Spiked samples of these anthocyanin isolates with the anthocyanin standards supported these tentative assignments.

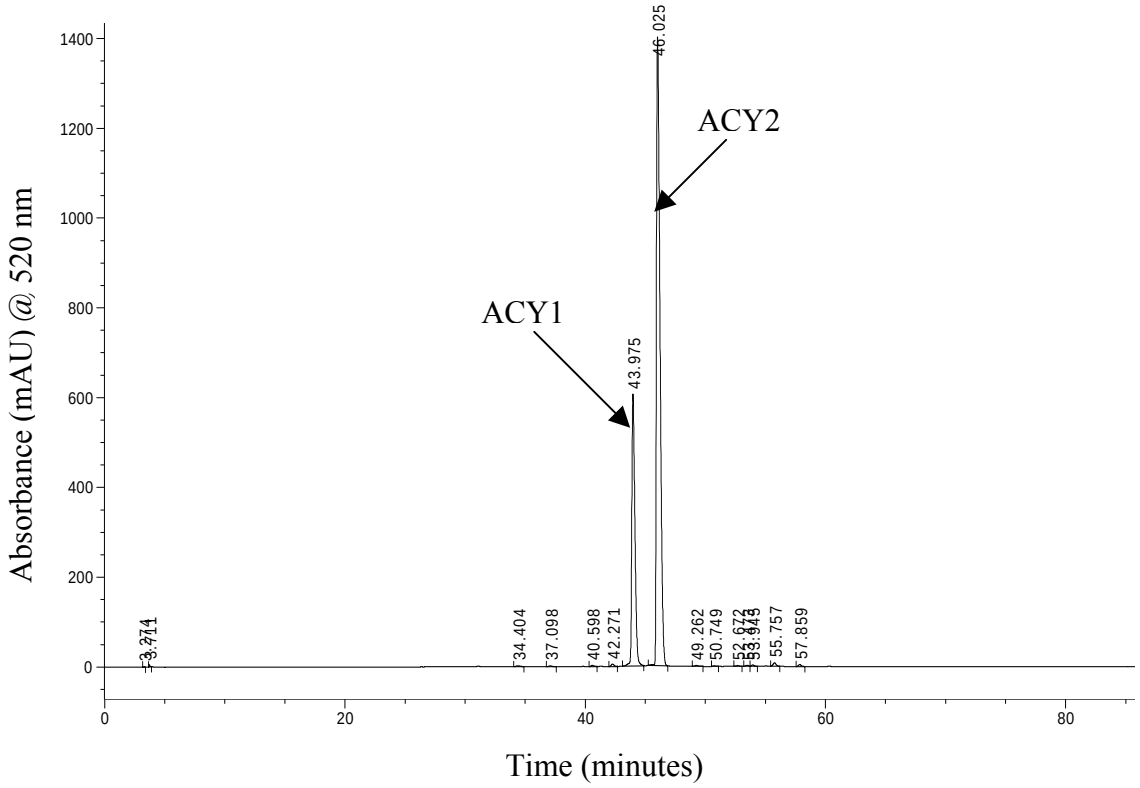


Figure 4.27 HPLC-DAD chromatogram of chokecherry fruit anthocyanins. The two major anthocyanin peaks ACY1 and ACY2 are cyanidin glycosides

Further identification of the chokecherry anthocyanins by HPLC-DAD was carried out by pigment hydrolysis. The two anthocyanin peaks were collected separately from the reversed phase HPLC column and hydrolyzed using 2 N HCl as described in section 3.10.3.2. Both hydrolyzed anthocyanins showed an anthocyanidin peak with the same RRT of 60.7 ± 0.2 minutes and spectral profile as cyanidin (Figure 4.28).

This result further supported the tentative identification of both major chokecherry anthocyanins to be cyanidin glycosides. Glycosylation was assigned at the 3-position of cyanidin based on the matching RRT's of ACY1 and ACY2 with the cyanidin 3-glucoside and 3-rutinoside standards, respectively. The assignment of the glycosylation at C-3 was also consistent with literature information on anthocyanin biosynthesis, where it is reported that anthocyanidins are initially stabilized by glycosylation at the 3-position (Springob et al., 2003). The 3-O-glycosylation is a pre-requisite for further modifications, such as a second glycosylation, acylation, methylation and prenylation (Springob et al., 2003).

Confirmation of the structures of the two major anthocyanins in chokecherry fruit was achieved by LC-MS using a 4% formic acid-acetonitrile mobile phase. The anthocyanin standards, cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-rutinoside and pelargonidin 3-glucoside, and ACY1 and ACY2 were analyzed by LC-MS in the positive and negative ion modes. Selected mass spectroscopic results are shown in Table 4.21 and the complete spectral information for each cyanidin 3-glucoside, cyanidin 3-rutinoside, ACY1 and ACY2, is presented in the Appendix A.1 to A.4, respectively.

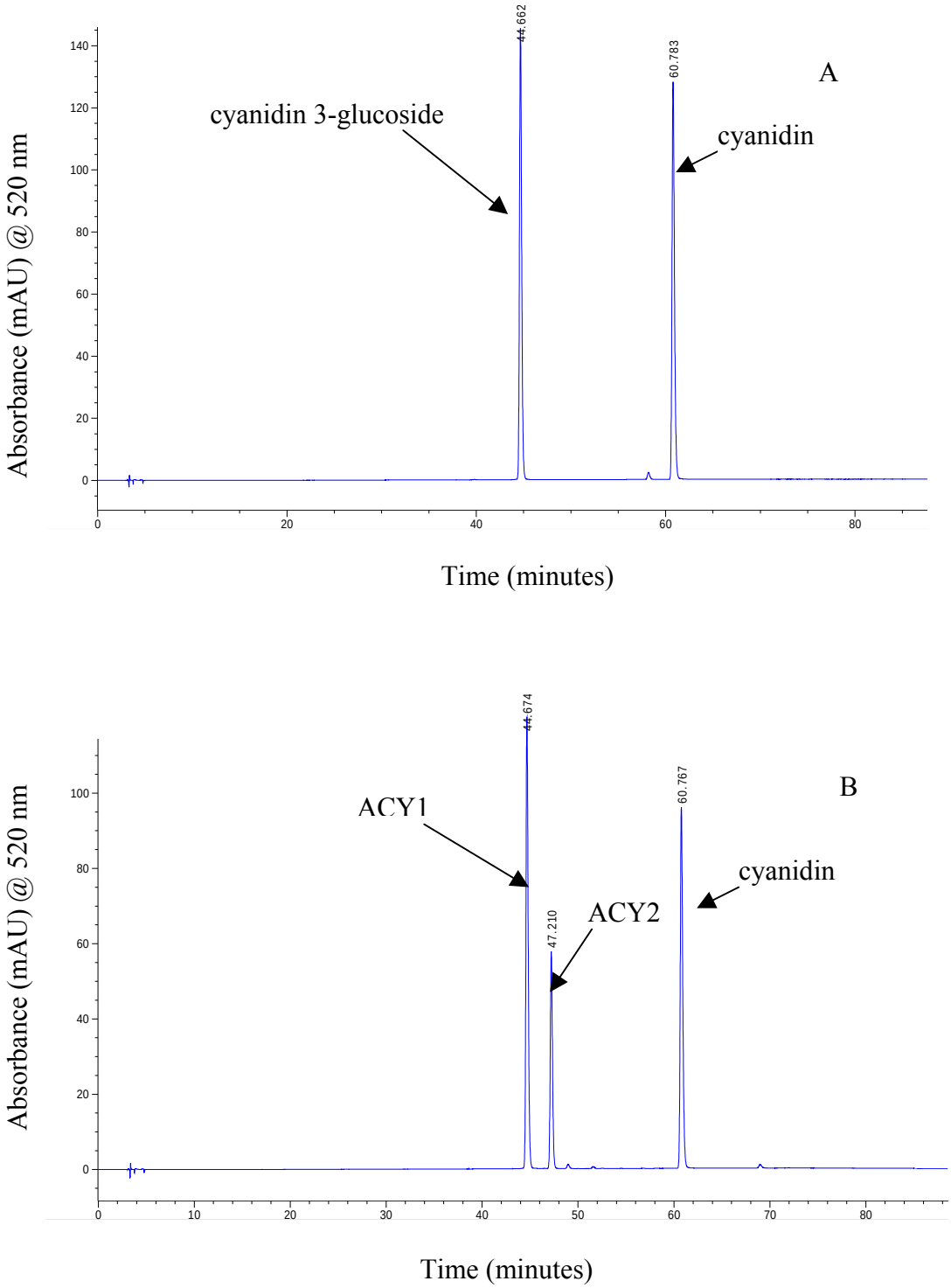


Figure 4.28 HPLC-DAD chromatogram of A) acid-hydrolyzed cyanidin 3-glucoside and B) acid hydrolyzed chokecherry fruit anthocyanins. The two major chokecherry anthocyanin peaks ACY1 and ACY2 are cyanidin glycosides.

Table 4.21 HPLC-DAD RRT and Spectral Characteristics and LC-MS Positive and Negative Ion Mode Data of Anthocyanin Standards and Chokecherry Fruit ACY1 and ACY2.

Anthocyanin	RRT (minutes)	Absorbance peaks and sh (nm)	MW	MS (*m/z*) positive ion	MS (*m/z*) negative ion
Cyanidin 3-galactoside	34.3	516, (sh 434), 280, 206	449	449, 287	447, 285
Cyanidin 3-glucoside	37.4	516, (sh 434), 280, 206	449	449, 287	447, 285
Cyanidin 3-rutinoside	40.9	518, (sh 438), 280,206	595	595, 287	593, 285
Pelargonidin 3-glucoside	42.2	502, (sh 430), 276, 206	433	433, 271	431, 269
ACY1	37.4	516, (sh 434), 280, 206	449	449, 287	447, 285
ACY2	40.9	518, (sh 438), 280, 206	595	595, 287	593, 285

Anthocyanins and their corresponding anthocyanidins exist in the cation form in the 4% formic acid mobile phase (pH = 1.7) employed in the LC-MS analysis and thus, the mass of the molecular ion in the positive ion mode is the molecular weight of the pigment molecule (Tian et al., 2005). The LC-MS spectra of ACY1 showed an $[M]^+$ peak at *m/z* 449 matching that of cyanidin 3-glucoside (Wu and Prior, 2005). The fragmentation pattern showed a diagnostic fragment at *m/z* 287 corresponding to cyanidin and was consistent with the LC-MS of the cyanidin 3-glucoside standard (Table 4.21). These fragmentation patterns matched the LC-MS fragmentation patterns for cyanidin 3-glucoside recently reported by others (Tian et al., 2005; Wu and Prior, 2005). The cyanidin 3-galactoside standard also showed an LC-MS spectral profile comparable to that of ACY1, however, the RRT of the galactoside was 34.5 minutes versus 37.4 minutes for ACY1. Based on

HPLC-DAD and LC-MS results, ACY1 was identified as cyanidin 3-glucoside and had an approximate concentration of 37 mg/100 g chokecherry whole fruit on a fresh weight basis.

The LC-MS positive ion mode analysis of ACY2 produced a parent ion of *m/z* 595 and a fragment ion at *m/z* 287, corresponding to cyanidin. This MS profile was similar to that of the cyanidin 3-rutinoside standard and mass spectra data reported by others for this compound (Giusti et al., 1999; Tian et al., 2005; Wu and Prior, 2005). Based on the chromatographic and LC-MS results, ACY2 was identified as cyanidin 3-rutinoside and had an approximate concentration of 110 mg/100 g chokecherry whole fruit on a fresh weight basis.

For comparison, negative mode LC-MS was also conducted on the chokecherry anthocyanins and standards. Loss of two protons from the parent ion $[M-2H]^-$ corresponding to each anthocyanin was detected rather than the commonly observed deprotonated molecular $[M-H]^-$. The lack of deprotonated molecular ion using the negative ion mode is attributed to the fact that anthocyanins and their corresponding anthocyanidins carry a positive charge at the low pH (1.7) conditions employed in this analysis, and the deprotonated molecule could not be detected due to the neutralization of the charge (Tian et al., 2005). Parent ions for cyanidin 3-glucoside and cyanidin 3-rutinoside were consistent with those obtained for ACY1 and ACY2 of *m/z* 447 and *m/z* 593, respectively.

Results of the LC-MS in the negative ion mode confirmed the identity of chokecherry anthocyanins ACY1 and ACY2 to be cyanidin 3-glucoside and cyanidin 3-rutinoside, respectively (Table 4.21).

4.5 Confirmation of Individual Fruit Phenolic Compounds

In addition to peak identification using HPLC-DAD data, the identities of selected phenolic peaks were confirmed based on their LC-MS spectral properties compared to those of phenolic standards. Peaks that showed high DPPH radical scavenging activity in the HPLC one-minute fractions were of particular interest.

Literature reports that acidic hydroxybenzoic and hydroxycinnamic acids deprotonate readily in the LC-MS negative ion mode (Gioacchini et al., 1996; Perez-Magarino et al., 1999) whereas in the LC-MS positive ion mode they can form adducts with cations in the sample or mobile phase (Swatsitang et al., 2000; Baderschneider and Winterhalter, 2001; Johnsson et al., 2002). Monomeric flavanols favour both protonation to positive ions (Lin et al., 1993) and deprotonation to negative ions (Poon, 1998; Perez-Magarino et al., 1999). Flavonols and flavonol glycosides also show response in both positive and negative LC ion modes (Hakkinen and Auriola, 1998; Andlauer et al., 1999; Schieber et al., 2001). In the present research, non-anthocyanin compounds in the phenolic isolates were determined using LC-MS in the negative ion mode because the positive one produced few spectral fragments.

Parent and fragment ions of the standard compounds used in this research are shown in Table 4.22. Parent ions of $[M-H]^-$ for each standard were apparent from their LC-MS chromatograms and fragment ions were observed for arbutin, caffeic acid, chlorogenic acid, *p*-coumaric acid, kaempferol, phloridzin and vanillic acid.

Rutin is a glycosylated flavonol consisting of quercetin 3-*O*-glucosyl-rhamnoside (quercetin 3-rutinoside). The molecular weights of the glucose and rhamnose moieties are 162 and 146, respectively. The aglycone fragment, quercetin (*m/z* 301) was not observed on the LC-MS spectrum of rutin. This result indicated

Table 4.22 HPLC-DAD RRT and Spectral Characteristics, and LC-MS Negative Ion Mode Data of Phenolic Standards.

Phenolic Standard	RRT (minutes)	UV absorbance peaks/(sh) (nm)	MW	MS (*m/z*) negative ion
arbutin	12.9	284, 220, 192	318	317, 271
gallic acid	16.2	272, 216	170	169
vanillic acid	26.7	292, 260, 218, 204	358	357, 167
4-hydroxy benzoic acid	31.9	256, (sh 208), 196	138	137
catechin	36.7	278, (sh 228), 204	290	289
chlorogenic acid	37.9	324, (sh 296), 240, 218, 194	354	353, 191
caffeic acid	40.2	326, (sh 300), 242, 218, 196	180	179, 135
epicatechin	43.6	278, (sh 228), 204	290	289
p-coumaric acid	50.4	312, (sh 296), 226, (sh 212)	164	163, 119
ferulic acid	56.0	322, (sh 298), 236, 218, 192	194	193
rutin	63.5	356, 256, 204	610	609
isoquercitrin	67.5	354, 256, 204	464	463
phloridzin	75.1	284, 222, 194	436	435, 273
quercetin	83.6	372, 256, 204	302	301
naringenin	86.3	288, 226, 212, 194	272	271
apigenin	86.7	336, 268, 210	270	269
kaempferol	86.9	366, 266, 224, 196	286	285, 265

that the glycosidic bond between quercetin and the glucosyl moiety of rutinose was not readily fragmented under the LC-MS conditions employed in this study.

Identification of phenolic compounds present in chokecherry, buffaloberry and sea buckthorn fruit extracts that matched the LC-MS spectral data of the standard phenolic compounds are shown in Table 4.23. Phenolic isolates prepared on the XAD-16 resin were used for the LC-MS analysis in order to remove possible interferences of polar, non-phenolic compounds such as organic acids, and soluble carbohydrates and proteins. Relatively few LC-MS peaks were assigned since the phenolic acids and flavonoids mainly exist in the glycosylated and conjugated state whereas the standard phenolic compounds were predominantly aglycones and monomers.

4.5.1 Buffaloberry Fruit Isolate

Rutin was identified in buffaloberry fruit as determined by LC-MS spectral data, RRT and spectral comparisons to phenolic standards. Rutin was also identified in buffaloberry by HPLC-DAD RRT comparison to a standard employing both HPLC Method 1 and Method 2 mobile phases with RRT's of 62.9 and 63.5 minutes, respectively. The HPLC fraction containing rutin did not show notable DPPH radical scavenging activity in comparison to other fractions (Figure 4.25), indicating this compound was not present in sufficient concentration in the fraction to provide considerable antioxidant activity in the extract. The sensitivity of the DPPH radical scavenging assay could be increased by such adjustments as increasing the injection volume and/or concentration of the extract, and/or

decreasing the concentration of DPPH radical in the assay solution.

Table 4.23 Identification of Phenolic Compounds in Buffaloberry, Chokecherry and Sea Buckthorn Fruit Determined by HPLC-DAD and LC-MS Negative Ion Mode.

Fruit	RRT (minutes)	Spectral characteristics (nm)	MS (*m/z*) negative ion	Peak Tentative Identification
Buffaloberry	63.7	356, 256, 204	609	rutin
Chokecherry	37.7	324, (sh 296), 240, 218, 194	353, 191	chlorogenic acid
	63.4	356, 256, 204	609	rutin
	83.7	372, 256, 204	301	quercetin
Sea buckthorn	36.8	280, (sh 228), 204	289	catechin
	63.7	356, 256, 204	609	rutin

4.5.2 Chokecherry Fruit Phenolic Isolate

The largest phenolic peak on the HPLC-DAD chromatogram (280 nm detection) had a RRT of 37.7 minutes and also showed high DPPH radical scavenging activity (Figure 4.25). This peak matched the retention time and UV spectral data of chlorogenic acid with an absorbance maximum at 324 nm and a shoulder at 296 nm. The LC-MS negative ion mode of this peak matched that of the chlorogenic acid standard (Table 4.22) with the parent ion $[M-H]^-$ at *m/z* 353 corresponding to the parent ion and a fragment at *m/z* 191 corresponding to the

quinic acid moiety. The concentration of chlorogenic acid in the chokecherry was 47 mg/100 g fresh fruit as determined by HPLC-DAD (Section 3.10.1). In addition to chlorogenic acid, other compounds identified by LC-MS spectral data compared to standards in the chokecherry fruit phenolic isolate included rutin and quercetin (Table 4.23).

4.5.3 Sea buckthorn Fruit Phenolic Compounds

Phenolic compounds identified by HPLC-DAD and LC-MS in sea buckthorn included catechin and rutin (Table 4.23). The HPLC fractions containing catechin and rutin produced antioxidant activities of 17% and 13% DPPH• inhibition, respectively. These levels of antioxidant activity were lower than fractions eluted in the first 25 minutes on the chromatogram (Figure 4.26).

4.6 Phenolic Compounds in the Leaves

4.6.1 HPLC-DAD of the Leaf Phenolic Extracts

The HPLC-DAD chromatograms using Method 2 of the phenolic compounds are shown in Figures 4.29 to 4.31 for buffaloberry, chokecherry and sea buckthorn leaf crude extracts, respectively. A total of forty peaks were identified in the chromatogram of buffaloberry leaf (Figure 4.29) consisting of nineteen flavonols, eight hydroxycinnamic acids, five hydroxybenzoic acids, five flavones, two flavanones/dihydrochalcones and one flavanol. Twenty-four peaks were identified on the chokecherry leaf chromatogram (Figure 4.30), consisting of four

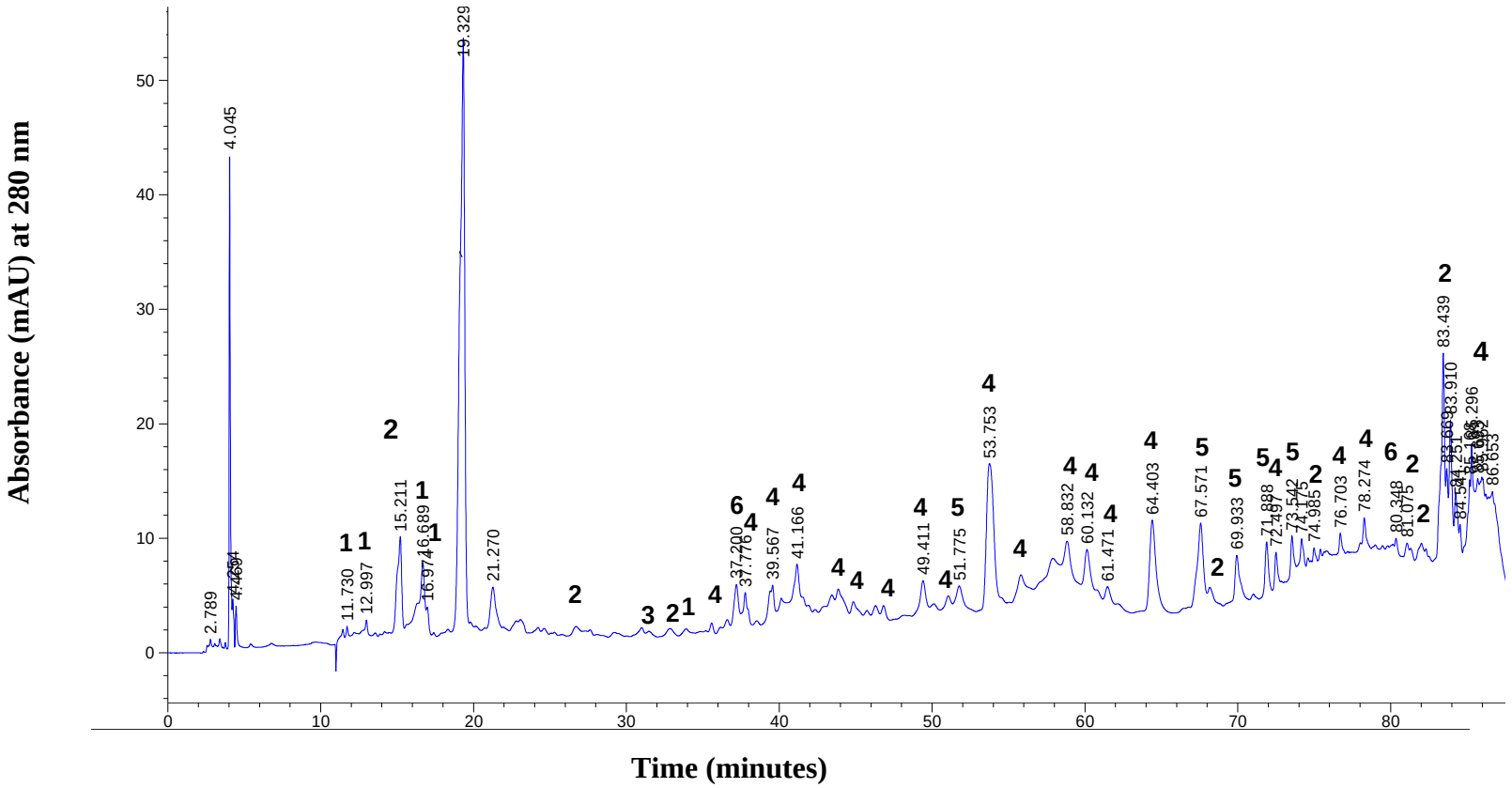


Figure 4.29 HPLC-DAD Method 2 chromatogram of buffaloberry leaf crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavones; 6) flavanones/dihydrochalcones.

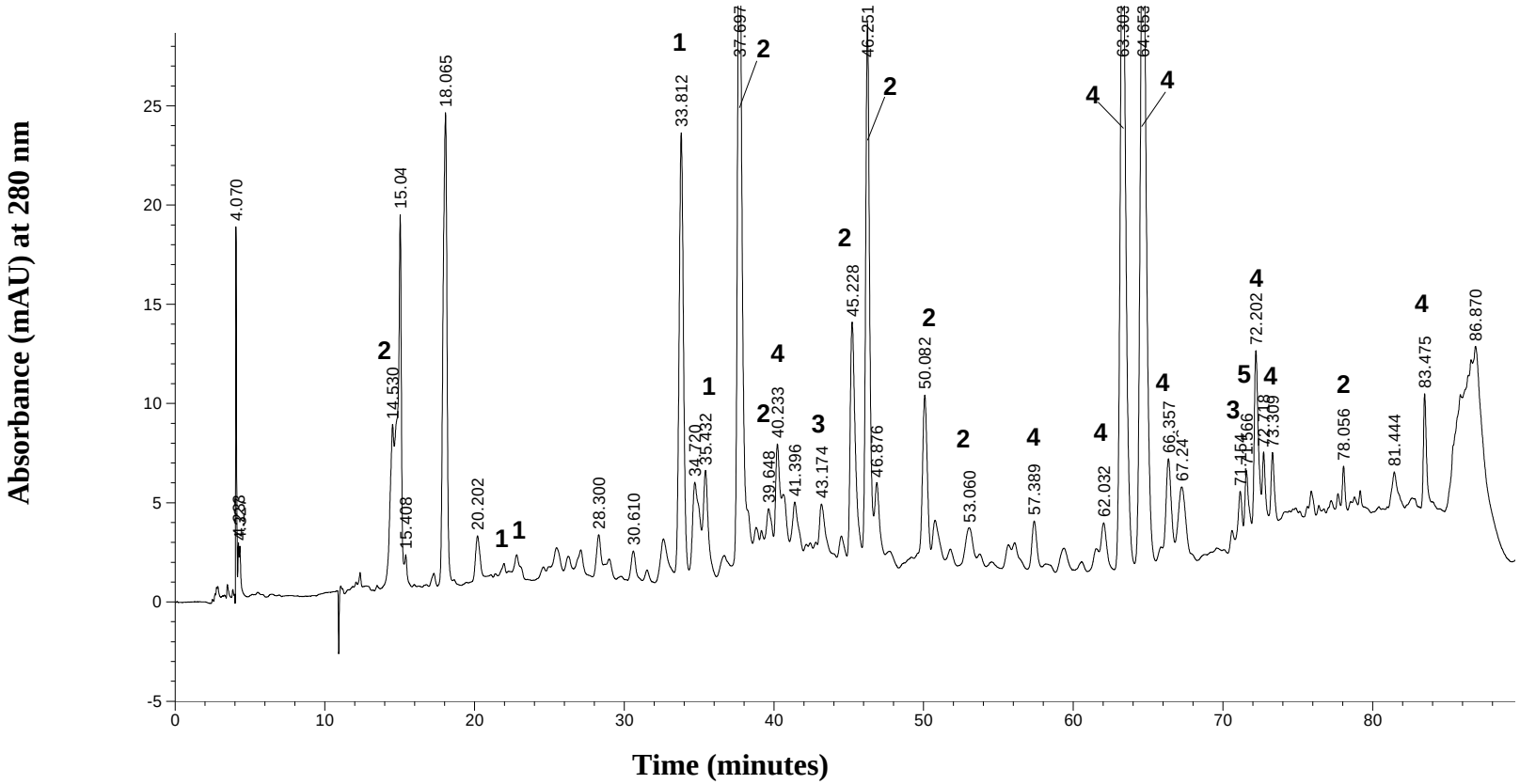


Figure 4.30 HPLC-DAD Method 2 chromatogram of chokecherry leaf crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavones; 6) flavanones/dihydrochalcones.

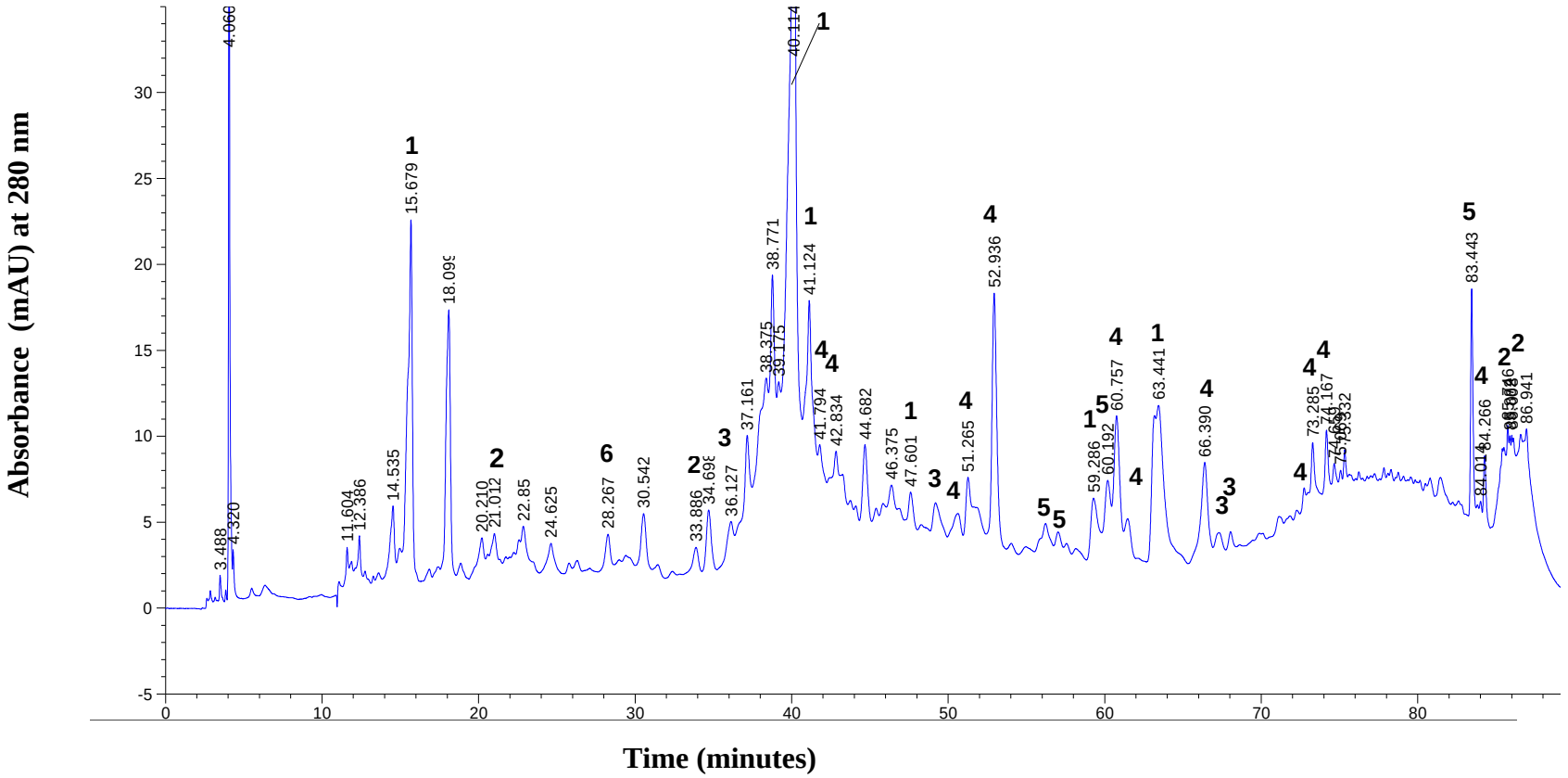


Figure 4.31 HPLC-DAD Method 2 chromatogram of sea buckthorn leaf crude extract. Peak assignments: 1) hydroxybenzoic acids; 2) hydroxycinnamic acids; 3) flavanols; 4) flavonols; 5) flavones; 6) flavanones/dihydrochalcones.

hydroxybenzoic acids, eight hydroxycinnamic acids, two flavanols, nine flavonols, one flavone and no flavanones/dihydrochalcones. Thirty-one peaks were identified on the sea buckthorn leaf chromatogram (Figure 4.31), consisting of twelve flavonols, five hydroxycinnamic acids, six hydroxybenzoic acids, four flavanols, three flavones and one flavanone/dihydrochalcone. Rutin and quercetin were identified in each of the leaf chromatograms. In addition, gallic acid (RRT of 16.6 minutes) was identified in buffaloberry leaf; chlorogenic acid (RRT of 37.7 minutes) in chokecherry leaf; and gallic acid (RRT of 15.7 minutes), and catechin (RRT of 36.1 minutes) was identified in sea buckthorn leaf. These phenolic compounds were identified by matching the RRT's and spectral properties of the peaks with those of pure phenolic standards.

The TPCI's of the freeze-dried leaf crude extracts are shown in Table 4.24. The highest TPCI's were observed in chokecherry (19,179 ± 2,481 μg/g) and sea buckthorn (19,870 ± 1,819 μg/g) and these were 1.6x higher than the TPCI of buffaloberry leaf.

The flavonols were the major phenolic class found in buffaloberry leaf crude extract, accounting for 61% of the TPCI. Flavones comprised 20% of the buffaloberry leaf TPCI and hydroxycinnamic acids contributed 16%. Hydroxybenzoic acids accounted for 1.5%, and the flavanols and flavanones/dihydrochalcones comprised less than 1% each of the TPCI. The flavonols and hydroxycinnamic acids were the predominant phenolic classes in chokecherry leaf contributing to 46 and 44% of the TPCI, respectively. The

Table 4.24 Phenolic Composition of Buffaloberry, Chokecherry and Sea Buckthorn Leaf Crude Extracts Determined by HPLC-PDA Method 2[a]

Leaf Extract	Total hydroxy-benzoic acids	Total hydroxy-cinnamic acids	Total flavanols	Total flavonols	Total flavones	Total flavanones / dihydro-chalcones	TPCI[b]
Buffaloberry	180 ± 29[c]	1958 ± 195	45 ± 6	7440 ± 954	2474 ± 684	63 ± 44	12160 ± 1458
Chokecherry	618 ± 23	8406 ± 1137	940 ± 180	8744 ± 1487	433 ± 118	38 ± 15	19179 ± 2481
Sea buckthorn	697 ± 211	1506 ± 248	3144 ±681	13384 ± 1584	1138 ± 427	11 ± 5	19870 ± 1818

[a] Results expressed as µg/g dry weight basis.
[b] Total Phenolic Chromatographic Index = total of all identified and quantified phenolic peaks.
[c] Mean ± standard deviation of three replications.
[d] Not detected.

remaining 10% of the TPCI was comprised of flavanols, hydroxybenzoic acids and flavones.

Flavonols were the most abundant phenolic compounds in sea buckthorn leaf extract with a concentration of 13,384 ± 1,584 μg/g dry leaf and comprised 67% of the TPCI. The flavanols contributed to 16% of the TPCI followed by hydroxycinnamic acids and flavones at 7 and 6%, respectively.

The flavonoid content of sea buckthorn leaf has been reported to range from 3,100 to 21,000 μg/g dry leaf (Glazunova et al., 1985 as reported in Li and Schroeder, 1996). By summation of the flavonoid classes determined by HPLC-DAD Method 2 in the present study (Table 4.24) the flavonoid content of sea buckthorn leaf was determined to be 17,667 μg/g dry leaf and within the range reported in literature. In comparison, the flavonoid content of buffaloberry and chokecherry leaf was 10,772 and 10,155 μg/g dry leaf, respectively.

4.6.2 Total Phenolic Content of Leaf Crude Extracts by Folin-Ciocalteu Analysis

Samples of the leaf crude extracts harvested on August 30, 2002 were analyzed for total phenolic content (TPC) using the F-C reagent. Sea buckthorn leaf had the highest TPC value (4,065 ± 137 mg/100 g dry leaf, expressed as gallic acid) and was 1.2x and 1.4x higher than the TPC's of buffaloberry and chokecherry leaf, respectively (Table 4.25).

The higher TPC could be attributed to the influence of proanthocyanidins (Waterhouse, 2005) and other non-phenolic reducing compounds such as proteins

(Section 2.10). Ascorbic acid was not detected by HPLC-DAD Method 2 (detection limit of 8 mg/100 g dry leaf; 3 x signal/noise) in the leaves of buffaloberry, chokecherry or sea buckthorn and therefore did not contribute significantly to the TPC value.

The proanthocyanidin content of buffaloberry and sea buckthorn leaf was highest at 269 ± 27 and 257 ± 96 mg cyanidin/100 g dry leaf, respectively (Table 4.25). These levels were 1.8x higher than the proanthocyanidin content of chokecherry leaf (137 ± 20).

Table 4.25 Total Phenolic, Proanthocyanidin and Solids Contents of Leaf Crude Extracts.

Sample	Total Phenolic Content[a]	Proanthocyanidin Content[b]	Total Solids Content[c]
Buffaloberry leaf	3419 ± 437[d]	269 ± 27	43.8 ± 1.5
Chokecherry leaf	2932 ± 48	137 ± 20	49.4 ± 1.2
Sea buckthorn leaf	4067 ± 137	257 ± 96	37.7 ± 0.8

[a] Determined using the Folin-Ciocalteu reagent. Expressed as mg gallic acid/100 g dry weight basis.
[b] Expressed as mg cyanidin/100 g dry weight basis.
[c] g/100 g leaf fresh weight basis.
[d] Mean ± standard deviation of three replicates.

4.6.2.1 HPLC-DAD of Acid and Base Hydrolyzed Leaf Crude Extracts

The phenolic composition of the acid and base hydrolyzed leaf crude extracts determined by HPLC-DAD Method 2 is shown in Table 4.26. The flavonols in all leaf extracts were completely degraded by the acid hydrolysis, resulting in a decrease in TPCI's ranging from 44% in buffaloberry to a 66% loss in sea buckthorn leaf. While flavonols were lost during acid hydrolysis, the flavanol concentration of the buffaloberry leaf crude extract increased significantly from 45 ± 6 μg/g in the crude extract to 1,929 ± 6 μg/g dry leaf following acid hydrolysis. This result suggested that proanthocyanidins in buffaloberry leaf crude extract were hydrolyzed to flavanol subunits that were separated and detected under the HPLC-DAD Method 2 conditions employed (Schofield et al., 2001). The presence of anthocyanidin peaks that would have been released from complete hydrolysis of proanthocyanidins was not observed on the acid hydrolzyed buffaloberry leaf extract chromatogram.

Under the base hydrolysis conditions employed, the flavonol concentration decreased by greater than 80% in buffaloberry leaf, greater than 70% in sea buckthorn leaf, and by 100% in chokecherry leaf (Table 4.26). A similar loss in the concentration of flavones occurred with a 70% decrease in the flavone concentrations of both buffaloberry and sea buckthorn leaf and complete loss of the flavone chromatographic peaks in chokecherry leaf crude extract following base hydrolysis. The hydroxycinnamic acid content of sea buckthorn leaf increased by 4x that of the crude extract following base hydrolysis. This result suggests there was a considerable level of conjugated hydroxycinnamic acids in sea buckthorn leaf

Table 4.26 Phenolic Composition of Acid and Base Hydrolyzed Buffaloberry, Chokecherry and Sea Buckthorn Leaf Extracts Determined by HPLC-PDA Method 2[a].

Leaf	Extract	Total hydroxy-benzoic acids	Total hydroxy-cinnamic acids	Total flavanols	Total flavonols	Total flavones	Total flavanones	TPCI[b]
Buffaloberry	Acid hydrolyzed	1225 ± 56	2333 ± 247	1929 ± 123	ND[d]	ND	156 ± 34	5643 ± 522
	Base hydrolyzed	107 ± 18	2372 ± 318	ND	1414 ± 359	763 ± 187	ND	4643 ± 582
Chokecherry	Acid hydrolyzed	832 ± 59	5367 ± 735	1241 ± 246	ND	ND	71 ± 31	7511 ± 637
	Base hydrolyzed	26 ± 16	7613 ± 748	ND	ND	ND	97 ± 46	7736 ± 735
Sea buckthorn	Acid hydrolyzed	1526 ± 234	2445 ± 281	2652 ± 218	ND	ND	ND	6623 ± 761
	Base hydrolyzed	84 ± 27	6015 ± 743	ND	3636 ± 392	795 ± 94	142 ± 52	10672 ± 1323

[a] Results expressed as µg/g dry weight basis.
[b] Total Phenolic Chromatographic Index = total of all identified and quantified phenolic peaks.
[c] Mean ± standard deviation of three replications.
[d] Not detected.

extract.

4.6.3 Radical Scavenging Activity of Leaf Phenolic Extracts

The antioxidant activity of freeze dried leaf extracts using DPPH radical scavenging and TEAC assays is shown in Table 4.27. Sea buckthorn leaf possessed the highest scavenging activity of both DPPH (1.7 ± 0.3) and the ABTS radicals (TEAC = 53 ± 1). The TPCI of sea buckthorn leaf (19870 ± 1818) was similar to that of chokecherry leaf (19179 ± 2481) and approximately 30% greater than the level of buffaloberry leaf (12160 ± 1458 µg/g dwb) (Table 4.24). Although chokecherry leaf contained a similar TPCI level as sea buckthorn leaf, the phenolic profile of sea buckthorn showed 3.3x more flavanols, 2.6x more flavones and 1.5x more flavonols than chokecherry leaf. These classes of flavonoids generally show higher TEAC values than the hydroxycinnamic acids (Rice-Evans et al., 1996) which were of higher concentration in chokecherry leaf. As a result, radical scavenging activity was notably higher in the sea buckthorn leaf extract in comparison to the leaves of buffaloberry and chokecherry.

Buffaloberry leaf showed higher levels of radical scavenging activity than chokecherry leaf, although the TPCI of chokecherry leaf was 1.2x greater than that of buffaloberry leaf (Table 4.24). Both samples contained comparable levels of flavonoids, however, chokecherry was also 59% greater in hydroxycinnamic acids concentration. The observed higher radical scavenging activity of buffaloberry over chokecherry leaf may be attributed a 2x higher proanthocyanidin content in the buffaloberry (Table 4.25). Additional antioxidants such as ascorbic acid were not

apparent in the chromatogram of the leaf extracts.

Table 4.27 DPPH and ABTS Radical Scavenging Activity of Leaf Crude Extracts.

Sample	DPPH• Scavenging (1/IC50)	TEAC (mM Trolox/100 mg)
Buffaloberry leaf	1.2 ± 0.1	22 ± 1
Chokecherry leaf	0.3 ± 0.0	5 ± 0.5
Sea buckthorn leaf	1.7 ± 0.3	53 ± 1

4.6.4 DPPH Radical Scavenging Activity of Leaf HPLC Fractions

DPPH radical scavenging profiles of the HPLC fractions and the chromatograms of the leaf phenolic extracts are shown in Figures 4.32 to 4.34 for the buffaloberry, sea buckthorn and chokecherry leaf samples, respectively. Generally, the prominent compounds on the chromatogram at 280 nm also showed relatively higher inhibition of DPPH radical. In addition to identification of the phenolic peaks occurring on the HPLC-DAD chromatogram, LC-MS was performed to determine the identity of the prominent antioxidant compounds.

4.6.4.1 Buffaloberry Leaf

Buffaloberry leaf exhibited high radical scavenging activity in HPLC fractions eluting from three to five minutes (Figure 4.32). The observed radical scavenging activity was consistent with a large peak at four minutes on the chromatogram at 280 nm. The UV-Visible spectral profile of the peaks did not

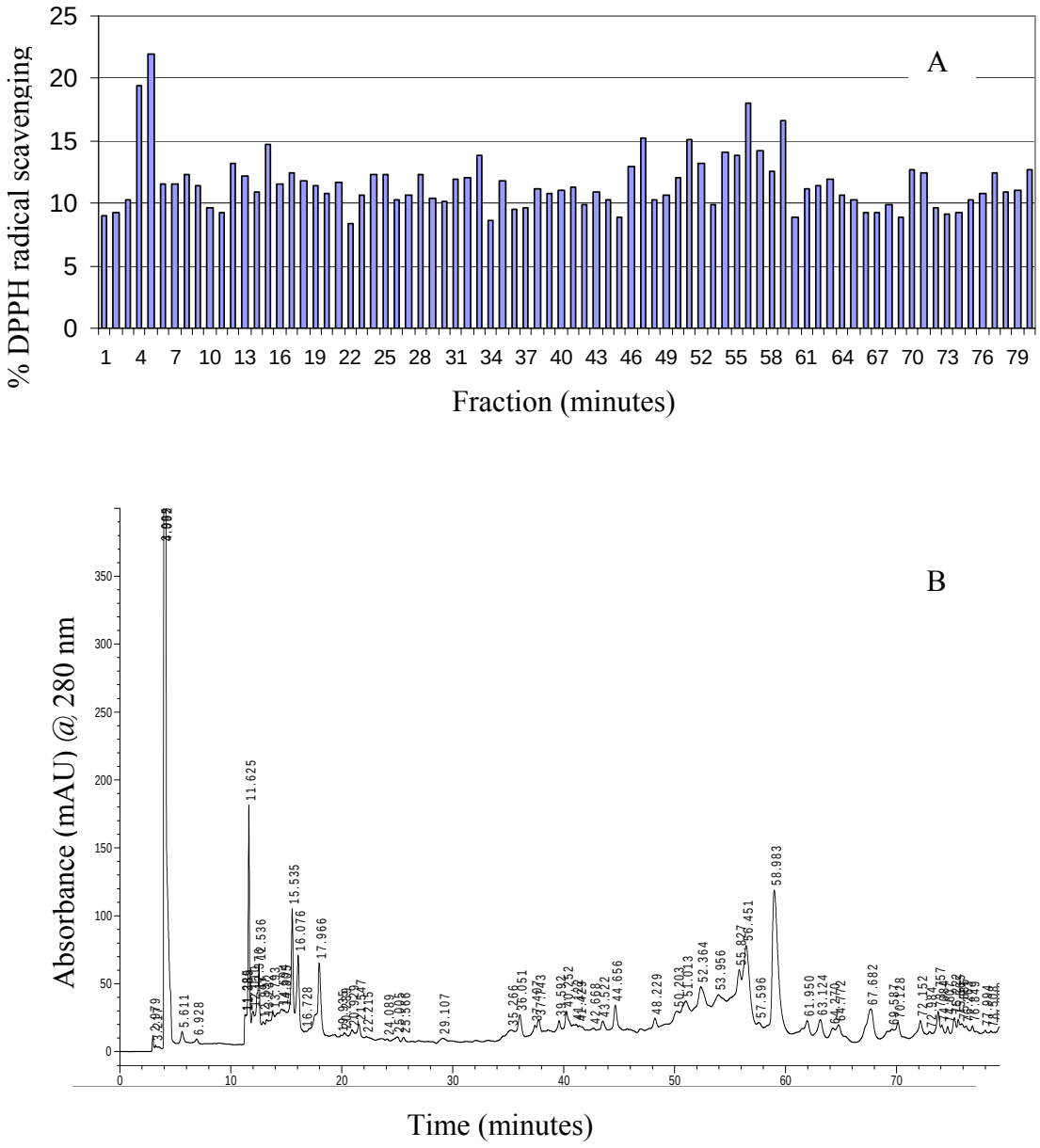


Figure 4.32 Buffaloberry leaf extract: A) DPPH• scavenging activity of HPLC one-minute fractions and B) HPLC-DAD chromatogram (@ 280 nm).

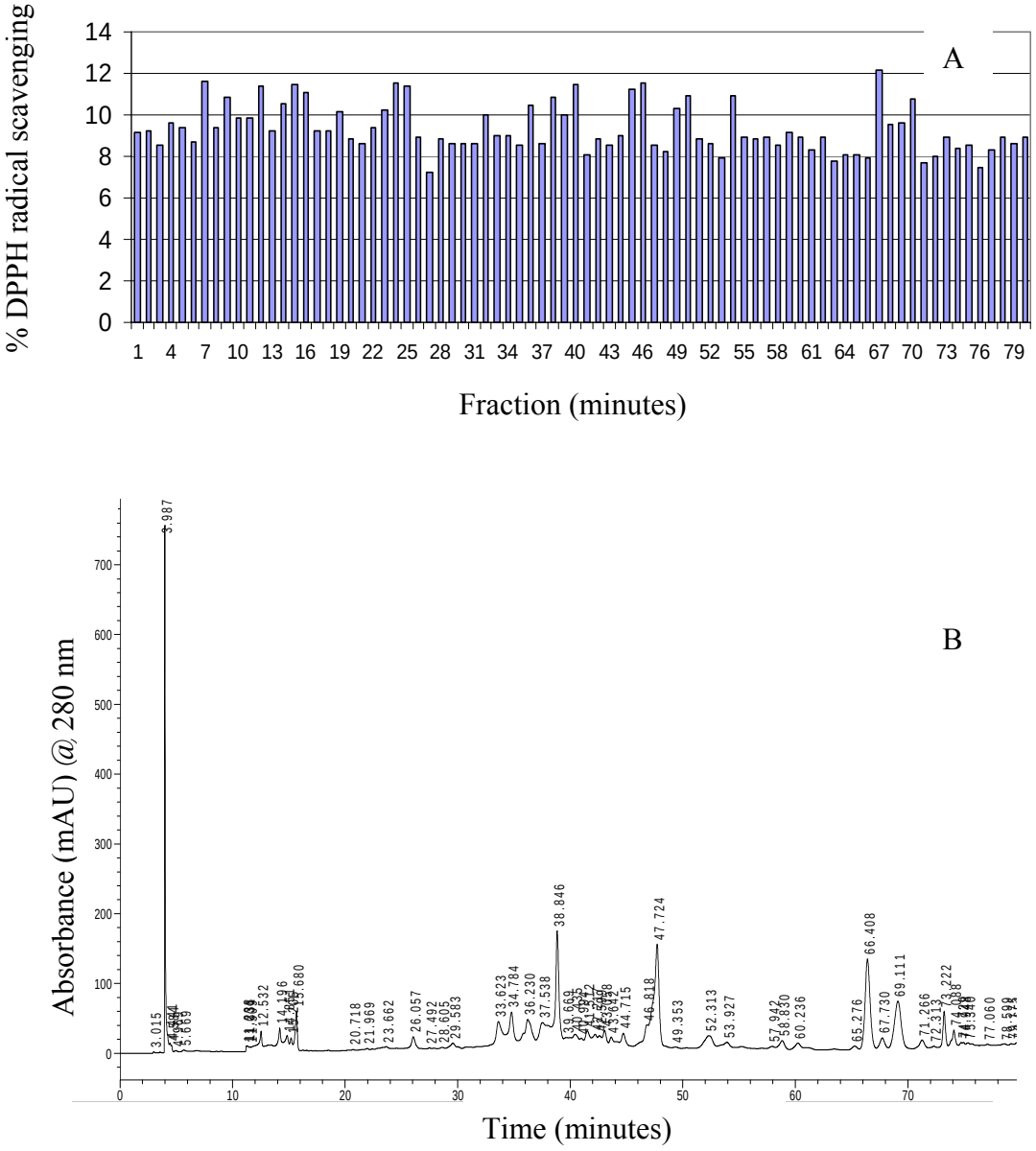


Figure 4.33 Chokechery leaf extract: A) DPPH• scavenging activity of HPLC one-minute fractions and B) HPLC-DAD chromatogram (@ 280 nm).

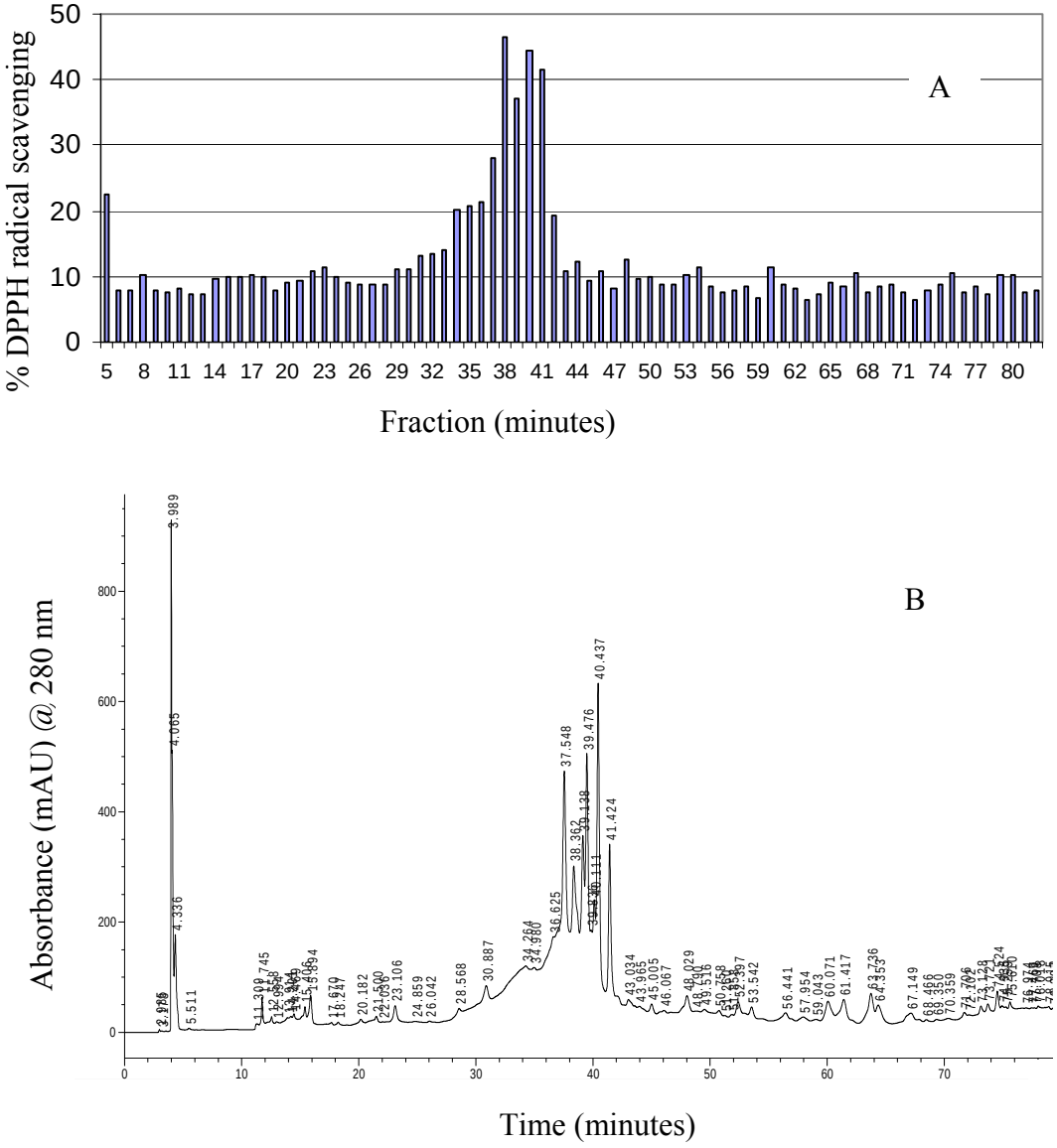


Figure 4.34 Sea buckthorn leaf extract: A) DPPH• scavenging activity of HPLC one-minute fractions and B) HPLC-DAD chromatogram (@ 280 nm).

match any of the phenolic standard compounds and were not identified in the present study.

Other notable buffaloberry leaf fractions showing DPPH radical scavenging activity greater than 12% inhibition occurred at 55 to 56 minutes and 58 to 59 minutes. A flavonol peak occurred at 55.8 minutes on the chromatogram with UV absorbance peaks at 356, 256 and 204 nm. The LC-MS negative ion mode of this peak showed one ion with *m/z* 433 which did not match the phenolic standards. A relatively large peak at 58.9 minutes occurred in the 58 to 59 minute fraction and the spectral properties of this peak showed a λ_{max} at 216 nm and a shoulder at 262 nm. These spectral properties did not coincide with any phenolic compounds. LC-MS analysis of the peak at 58.9 minutes showed a negative ion *m/z* 739 and fragment at *m/z* 567, however, these ions did not match any of the phenolic standards.

The identity of specific phenolic compounds in buffaloberry leaf matching the HPLC-DAD and LC-MS spectral profiles and retention time of standard compounds are shown in Table 4.28. Gallic acid and rutin were identified at 16.7 and 64.4 minutes on the buffaloberry leaf LC-MS chromatogram.

4.6.4.2 Chokecherry Leaf

Chokecherry leaf fractions were generally low in radical scavenging activity, showing DPPH• inhibition levels less than 12% (Figure 4.33). The low antioxidant activity was consistent with the low DPPH scavenging and TEAC assays of the chokecherry leaf extract (Table 4.27).

LC-MS spectra of the chokecherry leaf peaks confirmed the presence of

chlorogenic acid as a major peak on the chromatogram at 37.7 minutes (see Figure 4.30). The retention time and spectral properties of other peaks at 43.2, 64.6 and 83.5 minutes were identified as epicatechin, rutin and quercetin, respectively.

Table 4.28 Spectral HPLC-DAD Characteristics and LC-MS Negative Ion Mode of Buffaloberry, Chokecherry and Sea buckthornLeaf Crude Extracts

Leaf	t_R (minutes)	UV absorbance peaks/shoulders (nm)	MS (*m/z*) negative ion	Peak Tentative Identification
Buffaloberry	16.6	272, 216	169	Gallic acid
	64.4	356, 256, 204	609	Rutin
Chokecherry	37.7	324, (sh 296), 240, 218, 194	353, 191	Chlorogenic acid
	43.2	280, (sh 228), 204	289	Epicatechin
	64.6	356, 256, 204	609	Rutin
	83.5	356, 256, 204	301	Quercetin
Sea buckthorn	15.7	272, 216	169	Gallic acid
	36.1	280, (sh 228), 204	289	Catechin
	83.4	372, 256, 204	301	Quercetin

4.6.4.3 Sea buckthorn Leaf

Higher levels of DPPH radical scavenging activity of the sea buckthorn leaf occurred in HPLC fractions at 37 to 41 minutes eluting from the HPLC-DAD system (Figure 4.34). These phenolic fractions were comprised of a wide band of eluants with peaks from 37.5 to 40.4 minutes on the chromatogram at 280 nm. The

UV and LC-MS spectral properties of the individual peaks in this retention time range did not match spectral properties of phenolic compounds.

The identities of phenolic peaks of sea buckthorn leaf that were confirmed by matching LC-MS retention time and spectra of phenolic standards included gallic acid, catechin and quercetin (Table 4.28). These compounds were also identified in sea buckthorn leaf by HPLC-DAD analysis.

4.7 Fractionation of Chokecherry Fruit Anthocyanins by Centrifugal Partition Chromatography

The chokecherry fruit anthocyanins, cyanidin 3-glucoside and cyanidin 3-rutinoside were identified as prominent compounds that exhibited high levels of radical scavenging activity (Section 4.3.5). The antioxidant properties of anthocyanins are well documented (Kong et al., 2003) and there is consumer interest in the consumption of foods containing these compounds and a demand for their presence in medicinal extracts (Clifford and Brown, 2005). Recoveries of high purity anthocyanin standards prepared by preparative scale HPLC are limited due to adherence of these compounds to the hydrophobic stationary phase (Durst and Wrolstad, 2005). In addition, the strong pH required for effective HPLC separation of anthocyanins in the present study was 1.7 and at this level, the reversed phase column packing material was unstable. This fact was observed during the initial preparation of the anthocyanins on a semi-preparative HPLC column (Phenomenex) using a scale-up factor of four from the analytical column (Section 3.10.3). The

cyanidin 3-glucoside and 3-rutinoside fractions were collected, concentrated and freeze-dried. These freeze-dried pigment fractions were found to contain appreciable levels of the stripped HPLC stationary phase that was evident by the presence of a white, gel-like precipitate. In order to alleviate these stationary phase problems, the isolation of purified chokecherry anthocyanins was attempted using centrifugal partition chromatography (CPC).

CPC is a liquid-liquid chromatographic separation involving the partitioning of a solute between two immiscible solvents (section 2.10.3), the relative proportions of the solute passing into each of the two phases being determined by their respective partition coefficients (*P*). The solvent combination is typically adjusted to ensure the distribution ratio of the analyte is within the *P* value range of 0.2 to 10.0 (Glinski and Caviness, 1995). *P* values lower than 0.2 indicate that the analyte has low solublity in the stationary phase and will elute closer to the solvent front with lower peak resolution, while *P* values larger than 10.0 result in better resolution but broader, more dilute peaks due to a longer elution time (Glinski and Caviness, 1995).

Partition coefficients of the concentrated chokecherry anthocyanin isolate were determined in three solvent systems. Solvent system I consisted of ethyl acetate/ethanol/water (4:1:5, v/v/v) with 0.1% formic acid. Solvent system II was *n*-butanol/tert-butyl methyl ether (TBME)/acetonitrile/water (2:2:1:5, v/v/v/v) containing 0.1% formic acid and solvent system III consisted of hexane/ethanol/water (5:4:1, v/v/v). Solvent system I was selected from a list of CPC eluants recommended for the separation of compounds of mid-polarity as

supplied by the manufacturer of the CPC used in this research (Sanki Engineering Limited, Kyoto, Japan). Solvent system II was chosen because it was successfully used by Degenhardt et al. (2000b) for the separation of red wine anthocyanins. These authors employed 0.1% trifluoroacetic acid (TFA) to acidify the solvent system, whereas formic acid was used in the present study so as to be consistent with the extraction solvent (Section 3.3.10.1). Solvent system III was developed to determine the separation ability of a highly nonpolar stationary phase (hexane) with a polar mobile phase (75% v/v, aqueous ethanol) in which the anthocyanins are highly soluble.

The partition coefficients for the aforementioned chokecherry anthocyanins were determined as described in Section 3.12.3. Briefly, a 1 mL aliquot of the concentrated anthocyanin isolate was mixed with 2 mL of the test solvent system. The upper and lower solvent phases were allowed to separate and the anthocyanin content in each phase was determined by HPLC-DAD (Section 3.10.3.2). Experimentally, determined *P* values were ascertained for each of the anthocyanins (cyanidin 3-glucoside and cyanidin 3-rutinoside) by determining the ratio of their concentration in upper and lower solvent phase (Schwarz et al., 2003).

Under these separation conditions, the *P*'s for cyanidin 3-glucoside and cyanidin 3-rutinoside were 0.73 and 0.22, respectively, in solvent system II. These partition coefficients were within the range acceptable for CPC (Glinski and Caviness, 1995) separation of solutes with a *P* difference of 0.51. The anthocyanins were not detected in the upper phases in solvent systems I and III ($P = 0$). Based on these results, system II was selected for the preparative CPC.

The anthocyanin crude extract used for the CPC experiments is outlined in Section 3.12.2 and the CPC separation was conducted as described in Section 3.12.4. Briefly, the anthocyanins were loaded onto a pre-conditioned column of Amberlite XAD-16 resin and polar compounds were eluted with ddH_2O. The anthocyanins were then eluted from the XAD-16 bed with 70% methanol and concentrated on a vacuum evaporator at 45ºC. The resulting anthocyanin isolate (3.0 mL at 17% total solids) was injected onto the CPC.

The CPC chromatogram (@ 522 nm) of the chokecherry anthocyanin extract is shown in Figure 4.35. A large cyanidin 3-rutinoside peak eluted from 50 to 100 minutes, followed by cyanidin 3-glucoside at 110 to 150 minutes. This pigment elution order has been reported by Schwarz et al. (2003) for counter-current chromatography of blackberry fruit extract. In contrast to RP-HPLC, the CPC elution order was cyanidin 3-rutinoside then cyanidin 3-glucoside under these analysis conditions. According to Durst and Wrolstad (2005), the 3-rutinoside has a longer RP-HPLC retention time due to the nonpolarity imparted by its C-6 methyl group on the rhamnosyl moiety.

Up to 500 mg of the chokecherry anthocyanin crude extract was separated in a single CPC run and fractions of the separated anthocyanins were collected. The CPC purification was repeated three times with the same chromatogram reproduced for each replicate. In addition, the stationary phase was not eluted under the CPC conditions employed in this study.

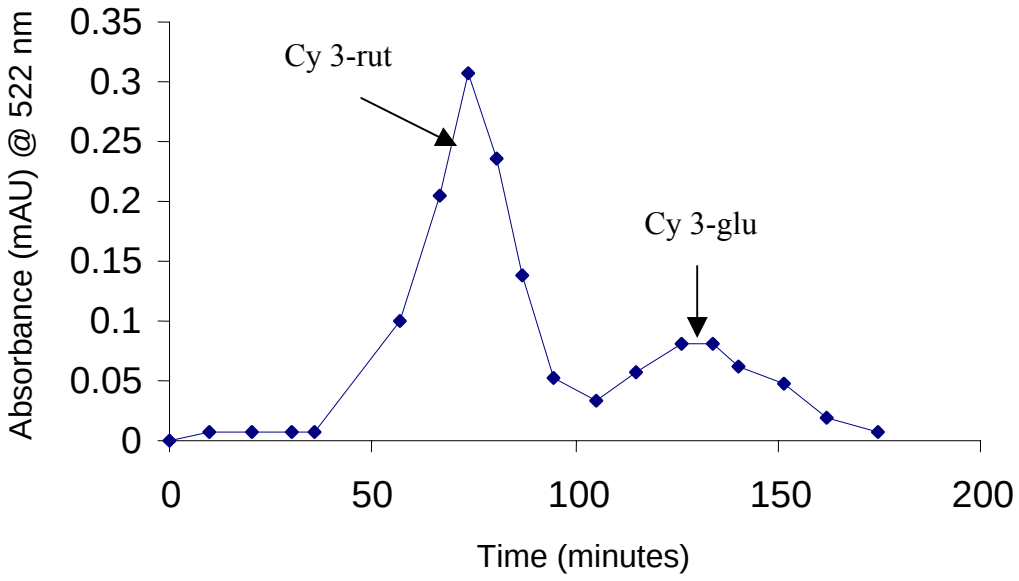


Figure 4.35 CPC chromatogram of chokecherry anthocyanin extract: cyanidin 3-rutinoside (Cy 3-rut) and cyanidin 3-glucoside (Cy 3-glu).

HPLC chromatograms of the recovered anthocyanin fractions are shown in Figures 4.36 and 4.37 for cyanidin 3-rutinoside and cyanidin 3-glucoside, respectively. Other non-anthocyanin peaks with RRT's of approximately 4.3, 14.2 and 19.4 minutes appeared on the chromatogram with detection @ 280 nm, however their UV spectral profiles did not match any of the phenolic standards employed in this study. The fractions collected from 63 to 78 minutes had a concentration of 90% cyanidin 3-rutinoside, dry weight basis. The fractions collected from 126 to 138 minutes had a concentration of 84% cyanidin 3-glucoside, dry weight basis. In

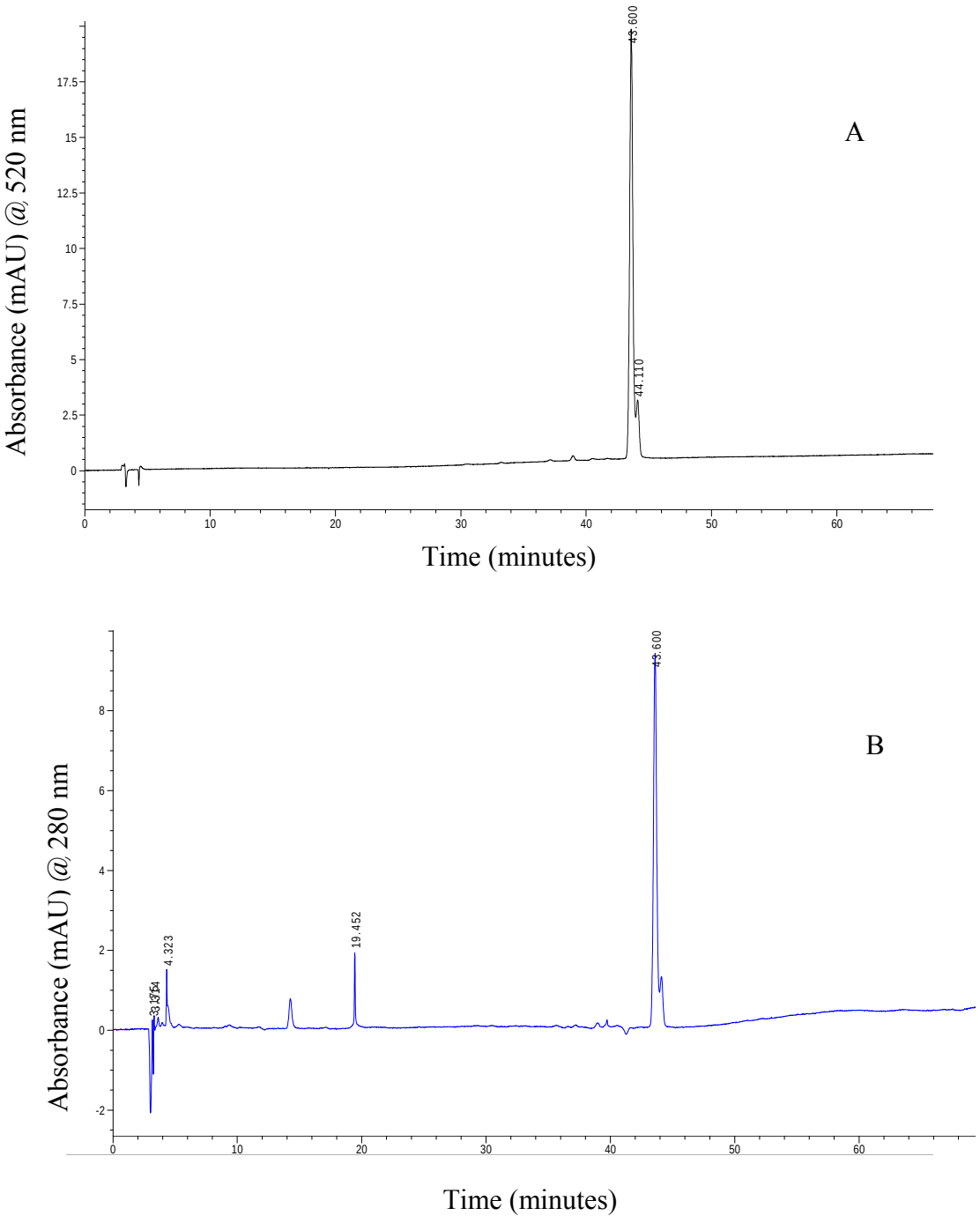


Figure 4.36 HPLC-DAD chromatograms of chokecherry anthocyanin CPC fractions from 63 to 78 minutes (cyanidin 3-rutinoside) at: A) 520 nm and B) 280 nm.

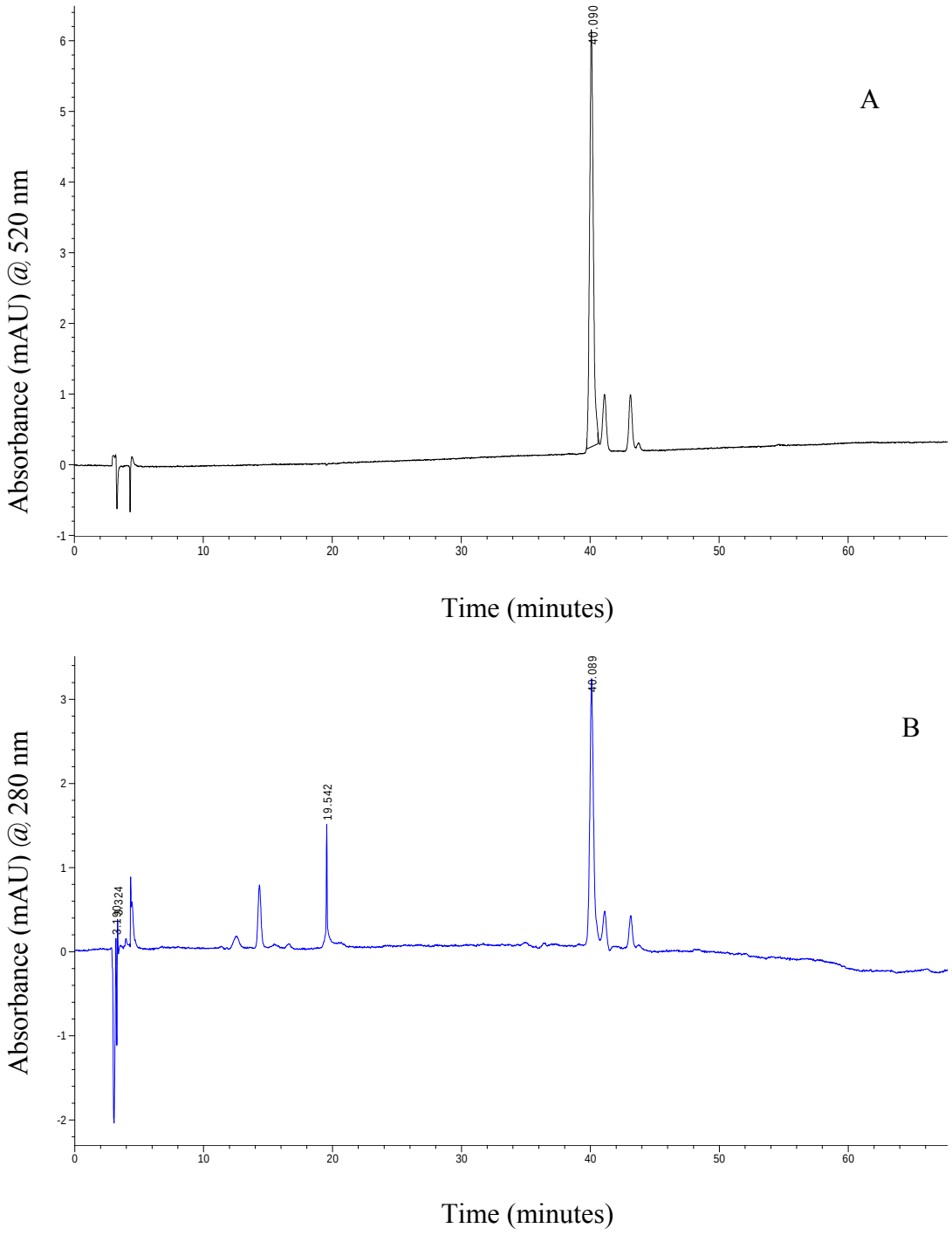


Figure 4.37 HPLC-DAD chromatograms of chokecherry extract CPC fractions from 126 to 138 minutes (cyanidin 3-glucoside) at: A) 520 nm and B) 280 nm.

comparison, the commercial anthocyanin standards showed purities of 95 and 96% for cyanidin 3-rutinoside and cyanidin 3-glucoside, respectively.

Based on the cyanidin 3-rutinoside and cyanidin 3-glucoside concentrations of 110 and 37 mg /100 g fresh fruit (Section 4.4), respectively, 20 g of chokecherry fruit contained 22 mg and 7.4 mg of cyanidin 3-rutinoside and 3-glucoside, respectively. The recoveries from 20 g of fruit using CPC was 3.1 mg of cyandin 3-rutinoside at 90% purity and 0.35 mg cyanidin 3-glucoside at 84% purity. These recoveries corresponded to 12.7% for cyanidin 3-rutinoside and 4.0% for cyanidin 3-glucoside. Additional recovery of the chokcherry anthocyanins could be achieved by re-chromatographing the lower purity pigment fractions. Scale-up of this CPC procedure is possible and sample loads in the gram range can be applied. These results suggest that the CPC conditions employed in this study represent an effective preparative scale method for chokecherry anthocyanin purification.

5. CONCLUSIONS

Although buffaloberry and chokecherry fruit have historically been used as a food source in Saskatchewan, their physicochemical properties have not been reported in literature. Sea buckthorn has also been utilized for its nutitional and health benefits throughout Asia and Europe and its physicochemical composition has been reported to vary considerably with environmental conditions and geographical location. In addition to their general nutritional properties, fruits and leaves are attracting interest for potential medicinal related benefits due to their antioxidant activity. The phenolic compounds present in these fruits and leaves are of particular interest for their radical scavenging activity and other health benefits.

The major findings from this study on the physicochemical properties and phenolic content of a selection of fruits native to Saskatchewan are as follows:

1. Proximate composition provides a general overview of the nutritional value of a food. The proximate composition of buffaloberry fruit has been determined. Results showed that this fruit has a moisture content of 71.5%, ash content of 0.6%, lipid content of 0.5%, protein content of 2.5% and total dietary fibre content of 6.0%, fresh weight basis.

2. The proximate composition of chokecherry fruit has been determined. Results showed this fruit has a moisture content of 66.8%, ash content of 0.9%, lipid content of 0.2%, protein content of 2.2% and total dietary fibre content of 8.9%, fresh weight basis of the fruit less the seed.

3. The proximate composition of sea buckthorn fruit has been determined. Results showed this fruit has a moisture content of 81.0%, ash content of 0.4%, lipid content of 2.6%, protein content of 2.5% and total dietary fibre content of 4.2%, fresh weight basis.

4. In conjunction with an endcapped, C_{18} (5µm and 100 Å pore size) stationary phase, a mobile phase system consisting of 0.05 M dipotassium phosphate with 0.05 M phosphoric acid, pH 3.0 (solvent A) and 70% (v/v) acetonitrile in A (solvent B) was developed for the effective separation of seven classes of phenolic compounds, including anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavanones/dihydrochalcones, flavones and flavonols. The stationary/mobile phase system was also found to be effective in phenolic compound separation within each class.

5. In conjunction with an endcapped, C_{18} (5µm and 100 Å pore size) stationary phase, a second mobile phase consisting of aqueous 10 mM formic acid (solvent A) and 30% 10 mM formic acid in acetonitrile (solvent B) was developed for the effective separation of seven classes of phenolic compounds, including

anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavanones/ dihydrochalcones, flavones and flavonols. This mobile phase was applicable for LC-MS as well as HPLC-DAD.

6. The phenolic acid and flavonoid composition of aqueous methanol extracts of fruits of buffaloberry, chokecherry and sea buckthorn has been determined by HPLC-DAD. Based on the total phenolic chromatographic index (TPCI), chokecherry contained the highest concentration of these phenolic compounds at 3,327 μg/g fruit, followed by buffaloberry at 578 μg/g fruit, followed by sea buckthorn at 477 μg/g fruit, fresh weight basis.

7. Phenolic compounds identified by LC-MS of the fruit extracts included rutin in buffaloberry, chlorogenic acid, rutin and quercetin in chokecherry, and catechin and rutin in sea buckthorn. Phenolic compounds identified by LC-MS of the leaf extracts included gallic acid and rutin in buffaloberry, chlorogenic acid, epicatechin, rutin and quercetin in chokecherry, and gallic acid, catechin and quercetin in sea buckthorn.

8. Based on HPLC-DAD analysis, the major flavonoid compounds in buffaloberry and sea buckthorn were flavonols at concentrations of 303 and 362 μg/g fruit, fresh weight basis, respectively. Anthocyanins and flavonols were major flavonoid compounds in chokecherry with concentrations of 245 and 291 μg/g fruit on a fresh

weight basis, respectively. Chokecherry fruit also contained a high concentration of hydroxycinnamic acids of 2,560 μg/g fruit, fresh weight basis.

9. The major chokecherry anthocyanins have been determined by HPLC-DAD and by LC-MS. Results showed there are two major anthocyanins in chokecherry fruit and these are cyanidin 3-glucoside and cyanidin 3-rutinoside.

10. A method to separate and purify chokecherry anthocyanins by centrifugal partition chromatography was identified. Based on this method, cyanidin 3-glucoside and cyanidin 3-rutinoside can be purified to concentrations of 84 and 90%, respectively.

11. Antioxidant activity testing using *in vitro* radical scavenging assays has been determined on extracts of the buffaloberry, chokecherry and sea buckthorn fruit. Based on these assays, buffaloberry and chokecherry showed the highest radical scavenging activity. The radical scavenging activity of buffaloberry fruit was largely attributed to its high concentrations of ascorbic acid and proanthocyanidins. The radical scavenging activity of chokecherry was attributed to its high concentrations of anthocyanins, hydroxycinnamic acids and flavonols.

12. Antioxidant activity testing using *in vitro* assays has been determined on leaf extracts of buffaloberry, chokecherry and sea buckthorn. Based on these assays, sea buckthorn leaves showed the highest radical scavenging activity, followed by

buffaloberry, followed by chokecherry. Radical scavenging of sea buckthorn leaf was attributed to its high flavonol content.

13. Based on results of the TPCI, *in vitro* radical scavenging assays and physicochemical composition, the native Saskatchewan fruits included in this study compare favourably with commercial fruit concentrates from blueberry and cranberry, reputed to be functional foods.

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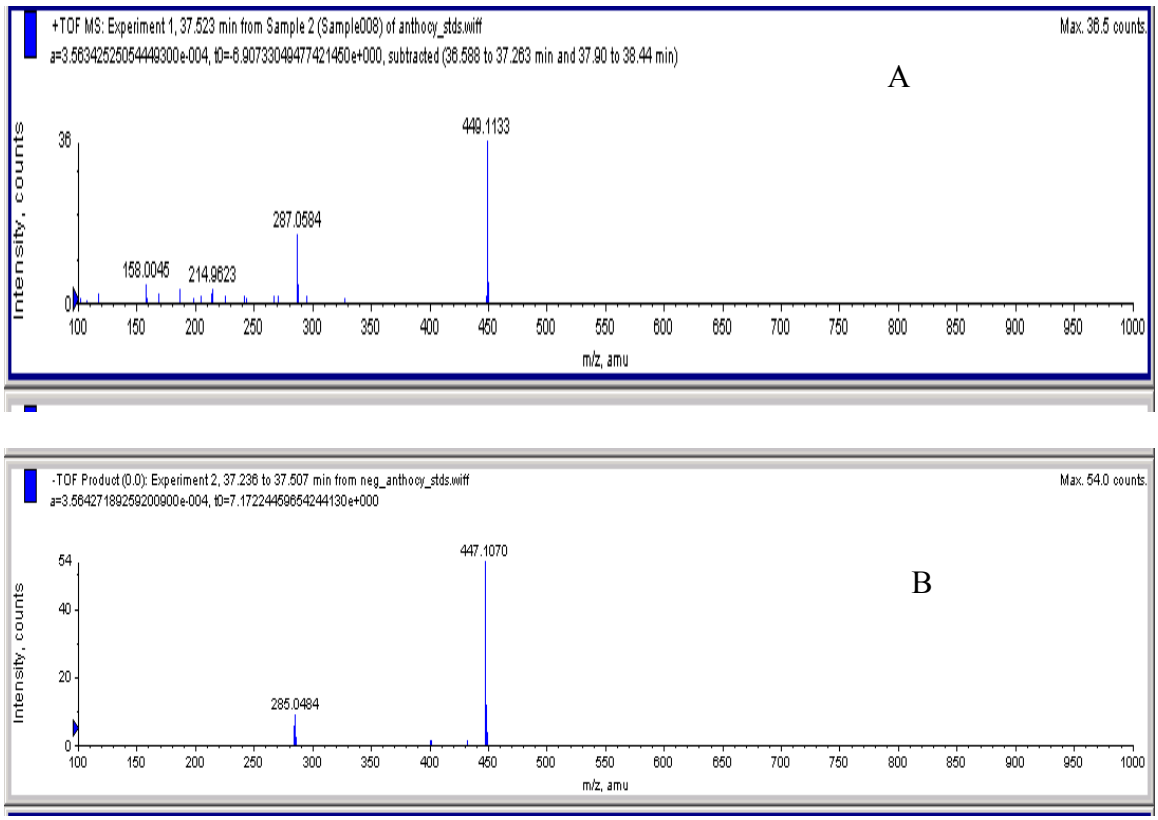
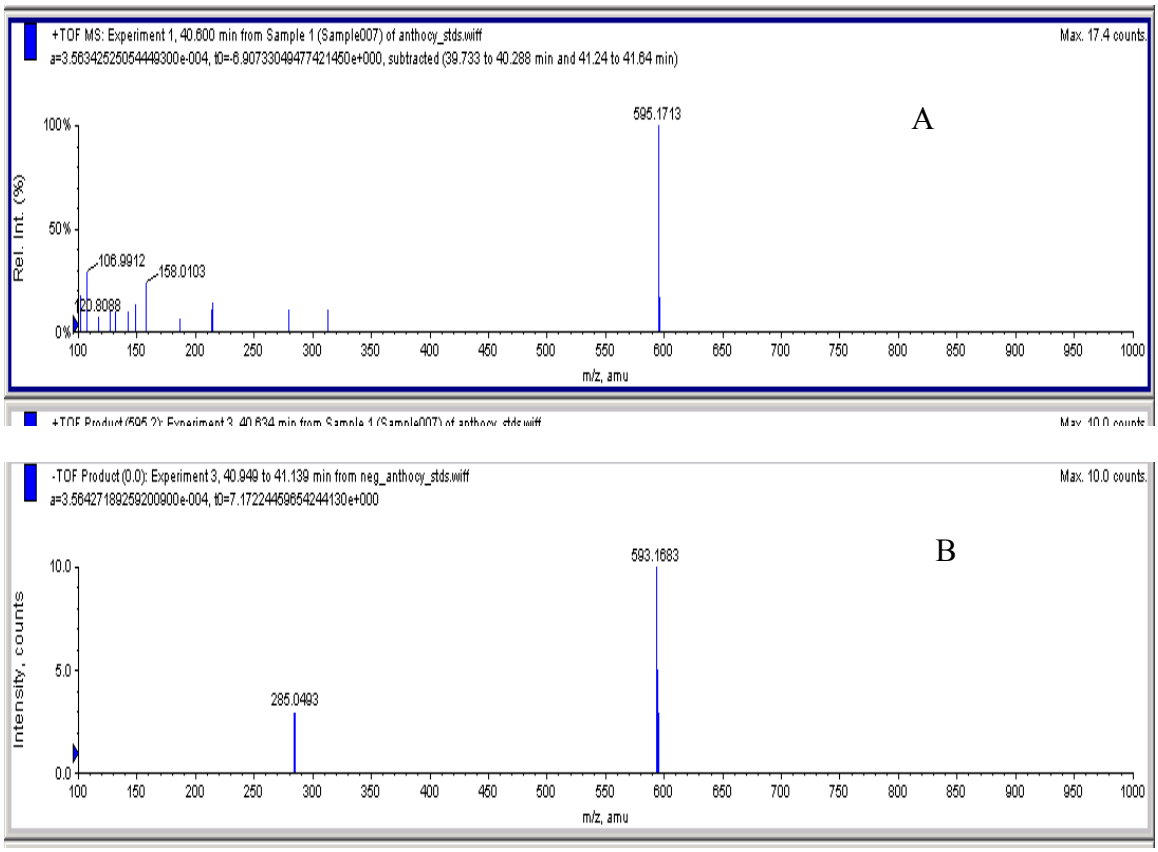
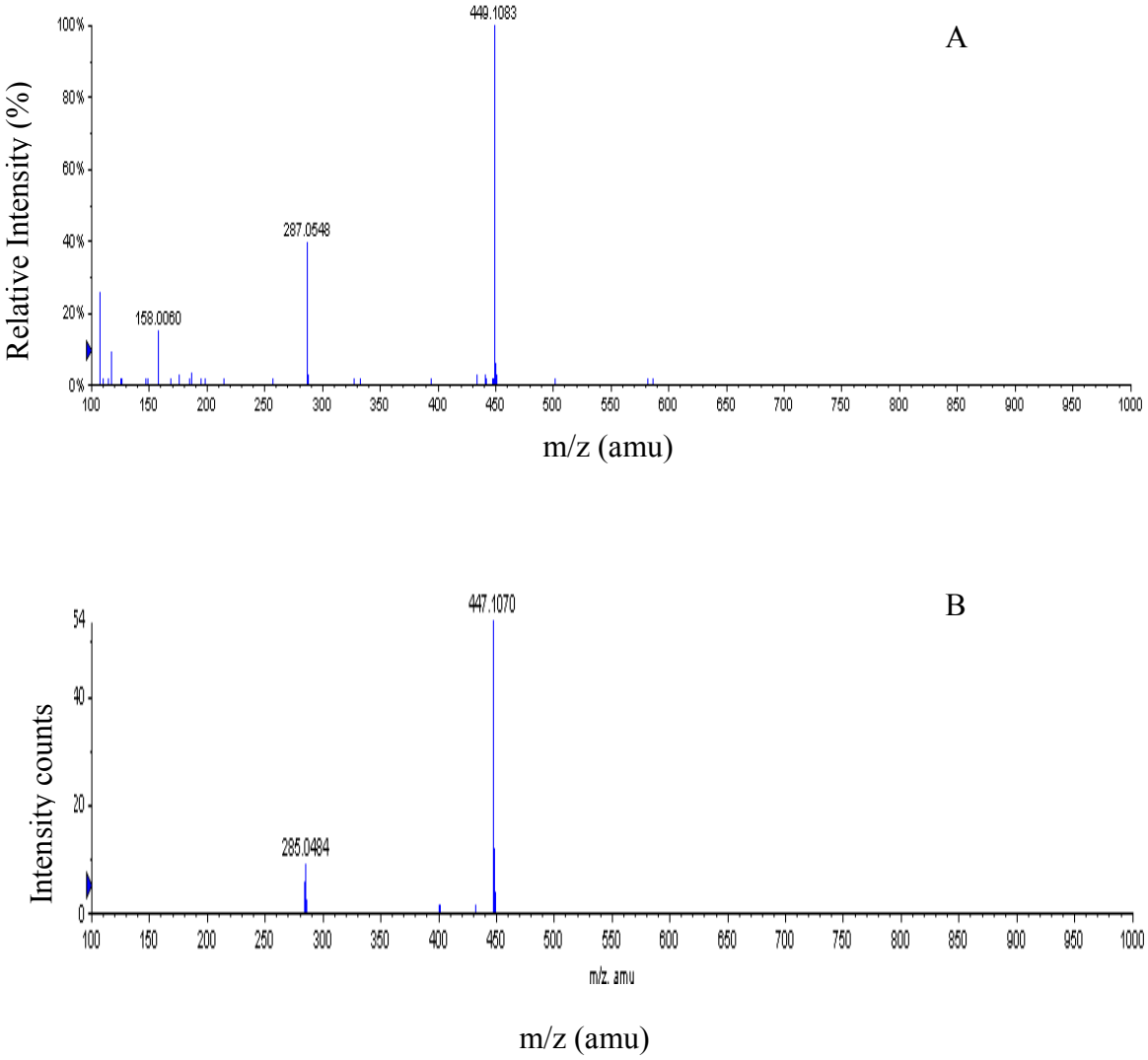
9.0 APPENDICES



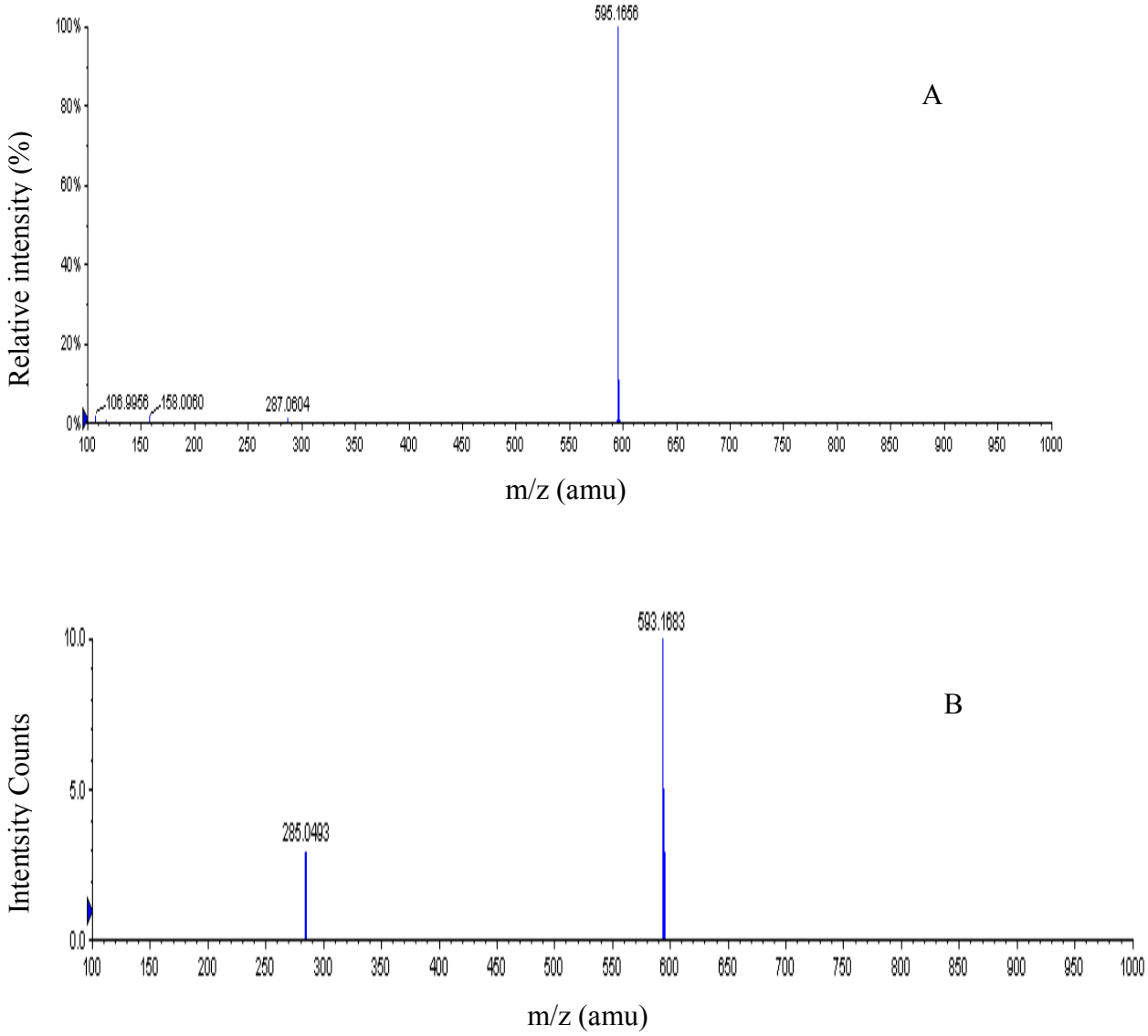
A.1 LC-MS spectra of cyanidin 3-glucoside: A) positive ion mode scan; B) negative ion mode scan.



A.2 LC-MS spectra of cyanidin 3-rutinoside: A) positive ion mode scan; B) negative ion mode scan.



A.3 LC-MS spectra of chokecherry fruit anthocyanin peak ACY1: A) positive ion mode scan; B) negative ion scan mode scan.



A.4. LC-MS spectra of chokecherry fruit anthocyanin peak ACY2: A) positive ion mode scan ; B) negative ion mode scan.