

**ANALYSIS OF THE BIOACTIVITY, METABOLISM, AND
PHARMACOKINETICS OF ANTHOCYANINS IN HUMANS**

A Thesis

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ABSTRACT

ANALYSIS OF THE BIOACTIVITY, METABOLISM, AND PHARMACOKINETICS OF ANTHOCYANINS IN HUMANS

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Recent interest in the health-promoting properties of berry anthocyanins has been based on studies reporting their significant *in vitro* antioxidant activities. However, information regarding the bioavailability, metabolism, and antioxidant activity of anthocyanins in humans is largely unexplored. The overall objective of this thesis research was to examine the antioxidant bioactivity, metabolism, and pharmacokinetics of anthocyanins in humans through a series of three exploratory investigations. The objective of the first investigation was to determine if the consumption of blueberries resulted in the absorption of anthocyanins and if the appearance of anthocyanins corresponded with changes in the antioxidant capacity of the blood. The aim of the second investigation was to identify metabolites of anthocyanins (specifically cyanidin 3-glycosides) post-consumption of chokeberries. Lastly, the goal of the third investigation was to establish the pharmacokinetics of parent and metabolized anthocyanins. In the first investigation, the concentration of anthocyanins in human serum positively correlated with an increase in antioxidant capacity of the serum; however, the concentration of

parent anthocyanins appeared insufficient to account for the magnitude of antioxidant effect. It was hypothesised that unidentified anthocyanin metabolites likely contributed to the observed antioxidant effect. In the second investigation, anthocyanin metabolites were identified in the serum and urine as glucuronide and methyl derivatives of the parent cyanidin 3-glycosides. The third study evaluated the pharmacokinetic parameters of both parent and metabolized anthocyanins. The total cumulative concentration of anthocyanins (parent and metabolites) detected in the serum over a 7h sampling regime was $172.96 \pm 7.44 \mu\text{g}\cdot\text{h}/\text{mL}$ with a maximum concentration of $44.86 \pm 2.82 \mu\text{g}/\text{mL}$ occurring within 2.8h. Additionally, the total urinary excretion of metabolites and parent compounds over 24h was $1071.54 \pm 375.46 \mu\text{g}$, reaching a maximal rate of excretion of $202.74 \pm 85.06 \mu\text{g}/\text{h}$ at $3.72 \pm 0.83\text{h}$ and having an elimination half-life of $4.12 \pm 0.4\text{h}$. Only 33% of the total anthocyanins detected in the serum were identified as the parent cyanidin 3-glycosides with 67% occurring as conjugated metabolites. This evidence suggests that anthocyanins are absorbed, metabolized, and readily excreted in humans, and their metabolites likely contribute significantly to their overall antioxidant bioactivity.

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LIST OF ABBREVIATIONS

AAPH	2,2-azobis (2-amidinopropane) dihydrochloride
ABTS	2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate)
ANOVA	analysis of variance
AUC	area under plasma concentration time curve
β -gal	β -galactosidase
BMI	body mass index
BP	blood pressure
β -PE	beta-phycoerythrin
C-3-ara	cyanidin 3-arabinoside
C-3-gal	cyanidin 3-galactoside
C-3-glu	cyanidin 3-glucoside
C-3-xyl	cyanidin 3-xyloside
CBG	cytosolic beta-glucosidase
C-gluc	cyanidin glucuronide
C _{max}	maximum concentration
COMT	catechol-O-methyltransferase
CVD	cardiovascular disease
cyt-P450	cytochromes-P450
DAD	diode array detector
E ₄₄₀ /E _{max}	ratio of the absorbance intensity at 440nm versus the maximum absorbance intensity

EHC	enterohepatic circulation
ESI-MS	electrospray ionisation mass spectrometry
FID	flame ionization detector
GC	gas chromatography
Gluc	glucuronic acid
GST	glutathione-S-transferase
HCL	hydrochloric acid
HDL	high density lipoprotein
HPLC	high pressure liquid chromatography
LC	liquid chromatography
LDL	low density lipoprotein
LPH	lactase-phlorizin hydrolase
m	molecular mass
m/z	mass to charge ratio
M-3-gal	malvidin 3-galactoside
MeOH	methanol
M-gluc	malvidin glucuronide
MS	mass spectroscopy
NMR	nuclear magnetic resonance
NOS	nitric oxide synthase
ORAC	oxygen radical absorbance capacity
P	peak
P-3-gal	peonidin 3-galactoside

PAPS	phosphoadenosine-5'-phosphosulfate
PCA	perchloric acid
P-gluc	peonidin glucuronide
Prep-HPLC	preparative high pressure liquid chromatography
Rf	reference value
Rmax	maximum rate of urinary excretion
RP-HPLC	reverse phase high pressure liquid chromatography
Rt	retention time
SAM	S-adenosyl methionine
SAS	statistical analysis systems
SGULT	sodium-glucose co-transporter
SOD	superoxide dismutase
SPE	solid phase extraction
SULT	sulfotransferases
t	time
$t_{1/2}$	elimination half-life
$t_{1/2a}$	absorption half-life
TAG	triacylglycerol
TAS	total antioxidant status
TFA	trifluoroacetic acid
tmax	time point where maximal serum concentration occurs
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UDP-GA	UDP-glucuronic acid

UDP-GT	UDP-glucuronosyltransferase
UV	ultraviolet
UV-vis	ultraviolet-visible

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LIST OF ORIGINAL PUBLICATIONS

Work completed during the progress of this thesis dissertation was utilized for the publication of the following manuscripts.

Refereed Papers Published in Scientific Journals:

Mazza G, Kay CD, Cottrell T & Holub BJ. Absorption of Anthocyanins from Blueberries and Serum Antioxidant Status in Humans. *Journal of Agricultural and Food Chemistry*. 2002;50:7731-7737.

Kay CD, Mazza G, Holub BJ & Wang J. Anthocyanin Metabolites in Human Urine and Serum. *British Journal of Nutrition*. 2004;91:933-942.

Kay CD, Mazza G & Holub BJ. The Metabolism and Pharmacokinetics of Cyanidin-3-glycosides in Humans. Manuscript in progress.

Critical Reviews/Book Chapters:

Kay CD & Holub BJ. The Postprandial Effects of Dietary Antioxidants in Humans. *Current Atherosclerosis Reports*. 2003;5:452-458.

Mazza G, Cacace JE & Kay CD. Methods of Analysis for Anthocyanins in Plants and Biological Fluids. *Journal of AOAC International*. 2004;87(1):129-145.

Kay CD & Holub BJ. Anthocyanins and Cancer Prevention. In: *Nutrition and Cancer Prevention*. Marcel Dekker, Inc. 2005.

CHAPTER 1.

BIOLOGICAL ACTIVITY OF ANTHOCYANINS

NATURAL OCCURRENCE AND ABUNDANCE OF ANTHOCYANINS

Flavonoids are a class of polyphenols comprising more than 4000 identified structures (Hollman et al., 1997a). Flavonoids share a common C6-C3-C6 configuration consisting of 2 aromatic rings linked by 3 carbons (Figure 1.1). Anthocyanins are a class of flavonoid which are prominent in many coloured plants. The intense absorption of anthocyanins at visible wavelengths of light impart colour (most commonly orange, red, and blue) to plant tissues including flowers, vegetables, and fruits. As a result of their intense colours, they have a history of use as dyeing agents and food additives. There are 18 common base anthocyanidin (aglycone) species which differ in their patterns of hydroxylation and methylation (Figure 1.2). In addition, there are well over 300 glycosides and a rapidly expanding list of identified acylated derivatives (Swain, 1976; Mazza & Miniati, 1993). In plants, anthocyanins occur in glycosylated forms, generally linked with glucose, galactose, arabinose, rhamnose, xylose, or fructose. The sugar moiety is most often found on the 3 or 5 position, but can also occur at the 7, 3', or 5' positions (for an extensive list of glycosides refer to Mazza & Miniati, 1993). Cyanidin, delphinidin, and pelargonidin are the most common anthocyanins in nature (Swain, 1976) with cyanidin glycosides reportedly present in nearly 90% of all fruits (Prior, 2003). Anthocyanins are found in very high concentrations in berry fruits and can range from 10 to 600mg/100g fresh weight. It is estimated that the average daily consumption

of anthocyanins is anywhere between 2-215mg/d (Kuhnau, 1976). A list of berry fruits containing high concentrations of anthocyanins is given in Table 1.1.

Figure 1.1 Basic Flavonoid Structure

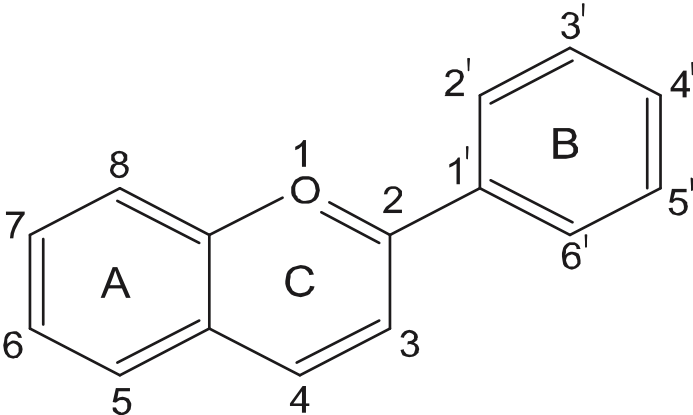
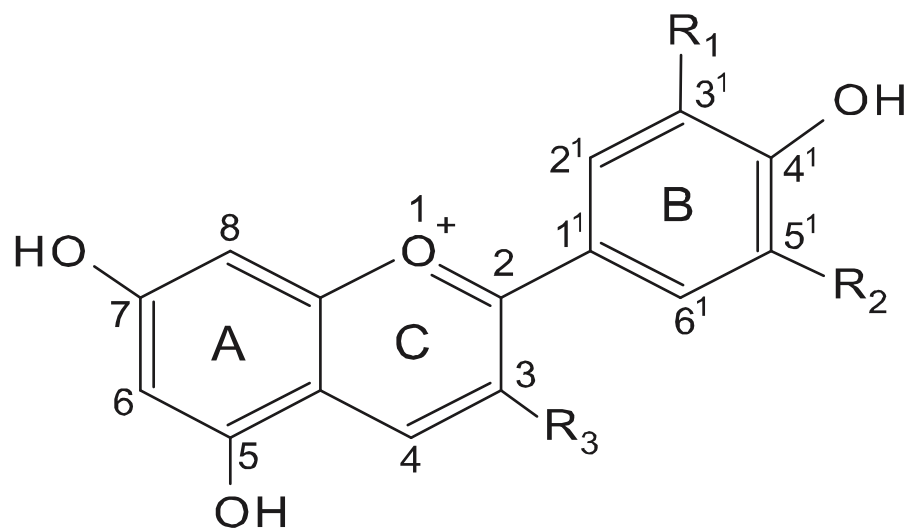


Figure 1.2 Common Anthocyanidin Structures



Anthocyanidin			
Aglycone	R ₁	R ₂	R ₃
Delphinidin	OH	OH	OH
Cyanidin	OH	H	OH
Petunidin	OCH ₃	OH	OH
Peonidin	OCH ₃	H	OH
Malvidin	OCH ₃	OCH ₃	OH
Pelargonidin	H	H	OH
Tricetinidin	OH	OH	H
Luteolinidin	OH	H	H
Apigenidin	H	H	H

Table 1.1 Berry Fruits Containing High Concentrations of Anthocyanins

Berry Fruit	*Anthocyanins (mg/100g Fresh Weight)
Bilberry	300-370
Black currant	110-430
Black Raspberry	197-428
Blackberry	133-172
Chokeberry	305-631
Cranberry	78
†Grapes	0-603
Highbush Blueberry	63-484
Lingonberry	174
Lowbush (Wild) Blueberry	91-255
Strawberry	8-79

Adapted from Skrede & Wrolstad (2002).

*Concentrations represent data from specific investigations. Anthocyanin variability will depend on genus tested, maturity of fruit, size of fruit, and environmental conditions.

†The anthocyanin concentration is given for *Vitis vinifera L.* grapes. The high variability results from including both white and red varieties.

REPORTED BIOLOGICAL ACTIVITY

In recent years, numerous studies have suggested that anthocyanins are protective against many chronic degenerative diseases (Kamei et al., 1995; Laplaud et al., 1997; Andriambelason et al., 1998; Trevithick & Mitton, 1999; Mazza, 2000; Parthasarathy et al., 2001). For the most part, the beneficial qualities of anthocyanins have been primarily attributed to their antioxidant capacities (Bohm et al., 1998; Kong et al., 2003; Galvano et al., 2004). Similar associations have been made for antioxidant phytochemicals such as lycopene (Rao & Agarwal 2000). Cancer is generally believed to be initiated in part by oxidative mechanisms acting upon genetic materials such as DNA and RNA (Scandalios 1992; Ross 1993). As well, the oxidation of macromolecules such as lipids and proteins may result in altered cellular processes

associated with cancer development and progression (Kaplan & Aviram, 1999; Vendemiale et al., 1999). Anthocyanins have been observed to prevent oxidation of these structures *in vitro* (Fuhrman et al., 1995; Tsuda et al., 1996; Rossetto et al., 2002; Hou, 2003) and a growing body of evidence now suggests a possible association between anthocyanins and cancer prevention (Middleton et al., 2000; Hou, 2003). Furthermore, an increased level of oxidation in the blood is assumed to be associated with cardiovascular disease (CVD) (Maggi et al., 1994; Vogel 1997; Staprans et al., 1999; Vendemiale et al., 1999). Elevated oxidation in the serum/plasma results in a variety of reactions including the oxidative modification of LDL particles (Morel et al., 1986; Vendemiale et al., 1999), initiation of inflammation, activation of nitric oxide synthase (NOS), increased platelet aggregation, and increased foam cell production (Parthasarathy et al., 2001). Recently, anthocyanins have been observed to reduce serum oxidation in the postprandial hyperlipidemic state (Kay & Holub, 2002; Mazza et al., 2002) and to enhance the resistance of LDL to oxidative modification (Natella et al., 2002). The antioxidant characteristics of anthocyanins have been associated with a variety of properties including free radical scavenging, chelation of trace metals, and inhibition of lipid peroxidation and DNA oxidation. Additionally, anthocyanin consumption has been observed to directly increase the hydrophilic and lipophilic antioxidant capacity of the blood serum/plasma in many investigations (Maxwell et al., 1994; Fuhrman et al., 1995; Whitehead et al., 1995; Tsuda et al., 1996; Sarma et al., 1997; Cao et al., 1998; Serafini et al., 1998; Miyazawa et al., 1999; Pool-Zobel et al., 1999; Young et al., 1999; Wang & Jiao, 2000; Kay & Holub, 2002; Mazza et al., 2002; Rossetto et al., 2002; Seeram & Nair, 2002; Hou 2003).

ANTIOXIDANT ACTIVITY

Anthocyanins are highly reactive radical scavengers in various *in vitro* environments. Anthocyanins not only scavenge radicals, but through their ability to bind heavy metals such as iron, zinc, and copper, also prevent the formation of radicals (Havsteen, 1983). Anthocyanins may also exert antioxidant abilities through the protection or enhancement of endogenous antioxidants (i.e., sparing effect), or through the induction of antioxidant enzymes such as glutathione-S-transferase (GST) and superoxide dismutase (SOD) (Fiander & Schneider, 2000; Ross & Kasum, 2002). Also, there appears to be a synergism between anthocyanins, vitamin C, and other flavonoids which is similar to the reported recycling effect of vitamin E by vitamin C. This outcome was observed in an investigation by Rossetto et al. (2002) where the flavonoid catechin was observed to regenerate malvidin 3-glucosides therefore increasing their antioxidant capacity in a micellar system of induced linoleic acid peroxidation.

Structural Characteristics Effecting Antioxidant Activity

The structural characteristics responsible for the antioxidant effect of anthocyanins are generally associated with the number of free hydroxyls around the pyrone ring (greater number of hydroxyls = greater antioxidant capacity; refer to Figure 1.2) although this is an oversimplification. The antioxidant capacity of a polyphenolic is dictated not only by the number of free hydroxyls, but also by the basic structural orientation of the compound. The ring orientation will determine the ease by which a hydrogen atom from a hydroxyl group can be donated to a free radical and the ability of the compound to support an unpaired electron. The conjugation of the anthocyanin ring structure is also important. The C2-C3 double bond of the C-ring is consistently

associated with a higher antioxidant capacity, reportedly having a stabilizing effect on the phenoxy radical (Middleton et al., 2000; Zheng & Wang, 2003). The positioning of hydroxyls in relation to one another is also a very important determinant of the antioxidant capacity of anthocyanins. Hydroxyl groups in close proximity, such as the ortho-hydroxyls of the B-ring, appear to greatly enhance the antioxidant capacity of the anthocyanin (Lien et al., 1999; Zheng & Wang, 2003) in experimental (*in vitro*) models; however, the availability of the highly reactive ortho-hydroxyls in a biological system (*in vivo*) has yet to be established. Conceptually, this site on the B-ring could form bonds with many compounds within biological fluids thus inhibiting the ability of this reactive site to participate in oxidation, metal chelation, or protein binding *in vivo*.

Glycosylation and Antioxidant Capacity

Anthocyanins are found in plants in glycosylated forms. Glycosylation is reported to influence the antioxidant capacity of anthocyanins/ flavonoids (Ioku et al., 1995; Noroozi et al., 1998; Seeram & Nair, 2002). It is generally stated in the literature that glycosylation decreases the antioxidant capacity of anthocyanins by reducing free hydroxyls and metal chelation sites; however, contradictory results have been reported (Wang et al., 1997; Pool-Zobel et al., 1999; Kähkönen & Heinonen, 2003). It is important to note that the effect of glycosylation on antioxidant capacity will depend upon the environment in which oxidation is being assessed; i.e., aqueous-soluble or lipid-soluble phases.

Glycosylation diminishes the antioxidant capacity of the anthocyanin in an artificial membrane system by decreasing the number of free hydroxyls and metal chelation sites. More importantly, glycosylation will decrease the accessibility of the

flavonoids to membranes as a result of the increased polarity (i.e., increased water-solubility) associated with the sugar moiety. The physiological relevance of this effect has, however, not been sufficiently established *in vivo*. Aglycones are less water-soluble and therefore have an increased partitioning into the lipid-soluble phase of the artificial membrane system. One would assume that the increased antioxidant capacity of anthocyanidins (aglycones) in this environment would therefore be partly a result of the increased lipid solubility of the aglycones over the glycosides. Conversely, other assay systems such as the oxygen radical absorbance capacity (ORAC) assay (Wang et al., 1997), the ferric reducing assay (Pool-Zobel et al., 1999), and certain lipid oxidation models (Kähkönen & Heinonen, 2003) have found some glycosides to have higher antioxidant capacities than their respective aglycones. Therefore, the *in vitro* effect of glycosylation on antioxidant capacity will depend upon the environment in which oxidation is being assessed (aqueous-soluble or lipid-soluble phase). Additionally, since anthocyanin aglycones have not been identified in the blood or urine, the physiological relevance of the antioxidant capacity of aglycones in the circulation is questionable. This being said, as anthocyanin glycosides are generally believed to be cleaved by colonic microflora, the aglycones could have physiological relevance within the colon with the glycosides having more systemic relevance. It is clear that the respective *in vivo* antioxidant capabilities of the anthocyanin aglycones versus their glycoside derivatives require further investigation.

Effect of pH on Antioxidant Activity

Anthocyanins exist in equilibrium in a variety of protonated, deprotonated, and hydrated forms. These range from coloured quinonoid forms, to the flavylium ion, and

to colourless hemi-acetal forms (Kähkönen & Heinonen, 2003). The expression of the predominant form is generally pH dependent. There is little evidence regarding the effect of pH on the biological activity of these compounds. Although in spite of the loss of colour of anthocyanins at physiological pH (7), evidence presented by Narayan et al. (1999) suggests that anthocyanin glycosides retain their antioxidant activity.

Compartmentalization & Antioxidant Activity

Results of trials aimed at determining the link between antioxidant consumption, antioxidant status, and oxidative-associated disease have been inconsistent (Willett, 2000). Although anthocyanins have shown promise in many *in vitro* antioxidant models, it has yet to be established if these compounds can reach their target of suspected action and if high enough concentrations are attained to elicit a biologically significant response. Youdim et al. (2000) were one of the first groups to show evidence of the incorporation of anthocyanins into cells and cell membranes. In a cell culture experiment, using human aortic endothelial cells, cyanidin glycosides from the elderberry were observed to be incorporated into both the plasma membrane and cytosol. The cells containing anthocyanins were determined to have significant protection against oxidation induced by reactive oxygen species. Subsequently, Bagchi et al. (2004) recently reported the cellular uptake of berry anthocyanins by endothelial cells. Although uptake was indicated in these studies, the mechanism by which anthocyanins enter intracellular compartments has yet to be determined.

CHAPTER 2.

ABSORPTION, METABOLISM & PHARMACOKINETICS OF ANTHOCYANINS/FLAVONOIDS

IDEOLOGY

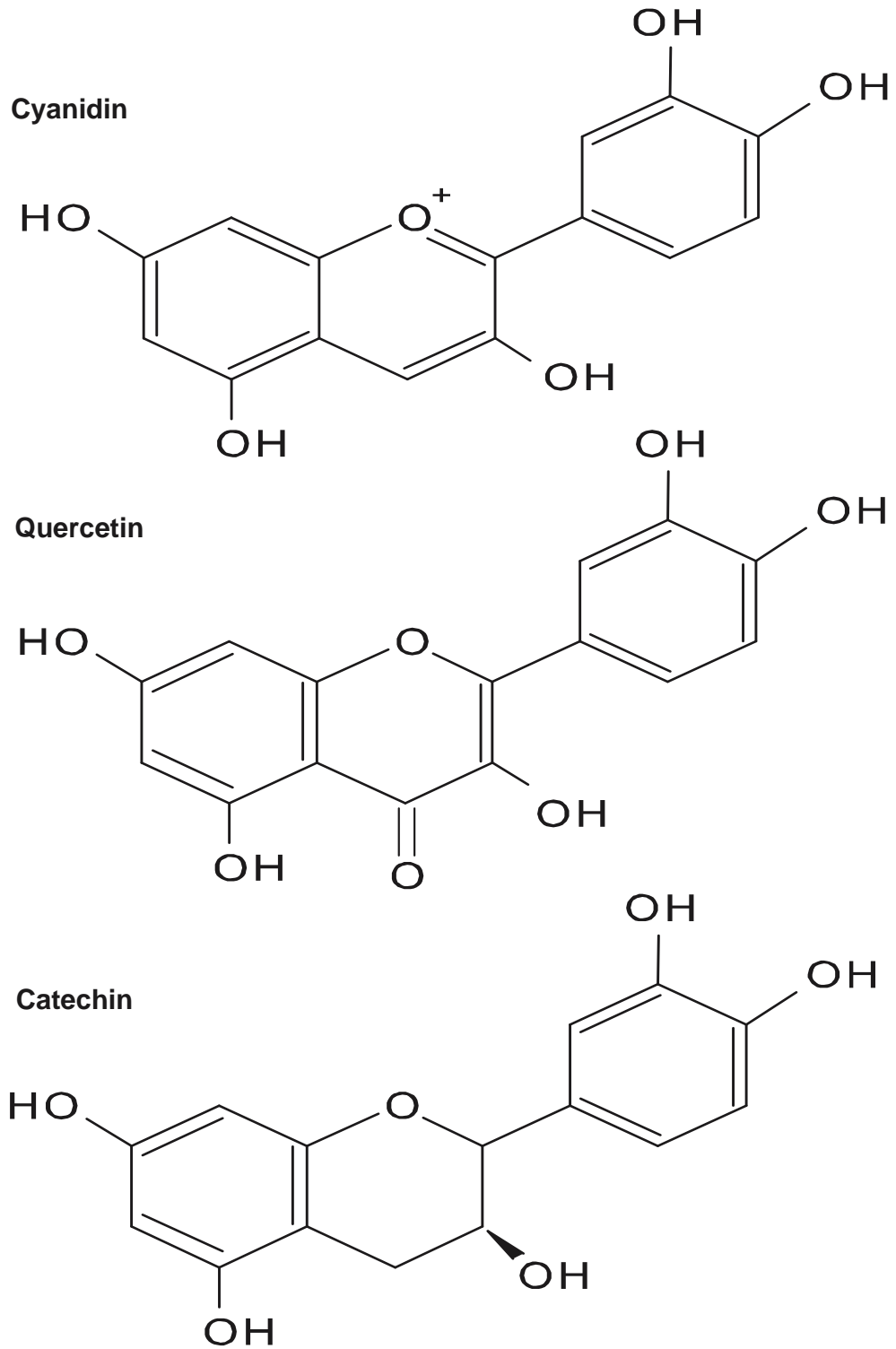
In the first investigation detailed in this thesis dissertation (“Absorption of Anthocyanins from Blueberries and Serum Antioxidant Status in Human Subjects”; Chapter 3), the total concentration of anthocyanins identified in human blood serum post-consumption of blueberries correlated with an increase in serum antioxidant capacity (as determined using the ORAC and TEAC assays). However, it was questionable if the concentration of anthocyanins in the circulation was sufficient to explain the magnitude of the observed increase in antioxidant capacity. One possible explanation was that unidentified anthocyanin metabolites were contributing to the observed antioxidant effect. Researchers have reported that the antioxidant activities of anthocyanins are maintained long after their clearance from the blood, suggesting that their metabolic by-products are responsible for the continued antioxidant effect (Tsuda et al., 1999a; Ross & Kasum, 2002). As well, researchers administering quercetin and (–)-catechin to rats have observed an increased antioxidant capacity of the plasma/serum, even though the compounds were identified in the biological fluids as glucuronide and sulfate derivatives (Terao, 1999); therefore suggesting that, although anthocyanins are reported to have a low bioavailability, the biological activity of their metabolites may be significant.

At the beginning of this thesis research, it was generally believed that anthocyanins were absorbed intact and circulated in the blood in their parent forms. No study had identified anthocyanin metabolites in human biological fluids. It was the quantification and identification of anthocyanin metabolites that would become the major focus of the remainder of this thesis investigation. Since that time, ourselves and others have reported anthocyanin metabolites in human biological fluids. In the succeeding review, the limited available literature will be assessed in an attempt to describe the potential mechanisms involved in the absorption and metabolism of anthocyanins. Additionally, subsequent chapters will detail the identification and pharmacokinetics of anthocyanin metabolites in human serum and urine.

INTRODUCTION

Within the last decade, many studies have focused on the potential biological activities or health effects of anthocyanins in humans (Reviews: Bohm et al., 1998; Kong et al., 2003; Galvano et al., 2004). Although there is a great deal of evidence indicating the bioactivity of anthocyanins, very little progress has been made establishing the mechanisms associated with their absorption, metabolism, and pharmacokinetics. Since there is limited data on the metabolism and bioavailability of anthocyanins to date, the following chapter will consider evidence of the flavonoid quercetin primarily as its structure is particularly comparable to anthocyanins (Figure 2.1). Reviewing quercetin metabolism will provide a foundation of evidence for the elucidation of mechanisms involved in anthocyanin absorption and metabolism.

Figure 2.1 Structural Similarities Between Cyanidin, Quercetin, and Catechin



ABSORPTION

Flavonoids are mostly present in foods as glycosides (Hollman & Katan, 1998a). Glycosylation influences their chemical and biological properties, having the greatest effect on partitioning coefficients. Partition coefficients relate to the solubility or polarity of a compound and are a measure of the relative affinity of the compound to exist in the aqueous or organic phase. This is important in determining mechanisms of absorption; i.e., whether a compound will passively diffuse across a biological membrane and how it might partition within internal cell phases. Aglycones are primarily hydrophobic and can passively diffuse through biological membranes. The linkage with sugars increases the water-solubility of the flavonoids and limits passive diffusion. Diffusion of a hydrophilic flavonoid glycoside across biological membranes is not likely to occur. Therefore, the absorption of a flavonoid glycoside likely requires either a specific active transport mechanism or hydrolysis of the beta-glycoside prior to absorption (Brown et al., 1998).

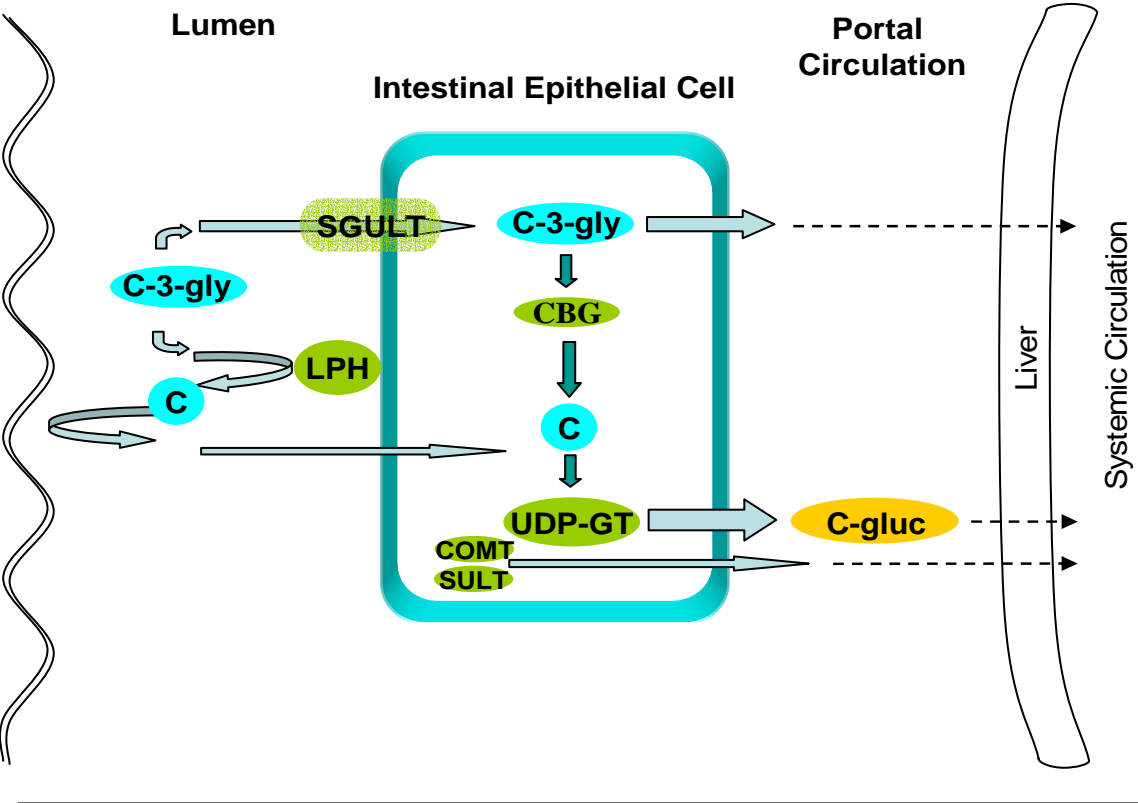
Absorption of Flavonoids

In the past, flavonoid glycosides were not considered capable of crossing the gut wall (Griffiths & Borrow, 1972) and hydrolysis of the glycoside was believed to be necessary for absorption. As no enzymes capable of cleaving glycosidic bonds were known to be secreted in the gut, the absorption of flavonoid glycosides was thought to only occur in the colon prior to hydrolysis via colonic microorganisms (Griffiths & Borrow, 1972; Kühnau, 1976). Human studies within the last decade have proven this theory to be incorrect as the absorption of flavonoids from the small intestine has been

documented. Hollman et al. (1995) conducted a study in ileostomized patients (patients lacking a colon with bacterial flora) to examine the extent of the absorption of quercetin. They found that the absorption of orally administered quercetin aglycone was approximately 24%; however, the absorption of quercetin glycosides was 52% suggesting that the glycoside moiety enhanced absorption. Additional studies in healthy individuals confirmed this finding (Williamson et al., 2000). Further investigations identified glycosidases capable of hydrolyzing glycosidic bonds within the cells of the gastrointestinal mucosa, thus establishing the small intestine as a major site of flavonoid absorption (Hollman et al., 1995; Hollman et al., 1996; Scalbert & Williamson, 2000).

The exact mechanisms involved in flavonoid absorption are a matter of much debate. It is speculated that the absorption of non-glycosylated flavonoids occurs in the small intestine via passive diffusion (Hollman et al., 1997a; Donovan et al., 2001). However, it has yet to be definitively established how flavonoid glycosides enter the enterocyte. The question is whether flavonoid glycosides enter the enterocyte as intact structures and are cleaved prior to passage across the basolateral membrane or if they are cleaved at the cell interface and move passively across the luminal membrane. Two potential transport mechanisms for flavonoid glycosides have been proposed (Figure 2.2), either the transport of the intact glucosides by a sodium-glucose co-transporter (SGULT), or the extracellular hydrolysis of the glycoside via lactate phlorizin hydrolase (LPH) at the brush border, followed by passive diffusion of the aglycone (Hollman et al., 1999a; Gee et al., 2000). In addition, a fraction of flavonoids will escape absorption in the upper small intestine and undergo bacterial metabolism in the lower intestine where the compounds will be deglycosylated and the aglycones will be subjected to transport or further metabolism (Scalbert & Williamson, 2000; Williamson et al., 2000).

Figure 2.2 Potential Mechanisms of Anthocyanin Absorption



C, cyanidin; CBG, cytosolic beta-glucosidase; C-3-gly, cyanidin 3-glycoside ; C-gluc, cyanidin glucuronide; COMT, catechol-O-methyltransferase; LPH, lactate phlorizin hydrolase; SGULT, sodium-glucose co-transporter; SULT, sulfotransferase; UDP-GT, UDP-glucuronosyltransferase.

Adapted from Williamson et al., 2002

Evidence suggests that SGLT1 is involved in the transport of quercetin glycosides across the luminal membrane surface of enterocytes in the small intestine. Quercetin absorption has been documented extensively in humans (Mizuma et al., 1994; Hollman et al., 1995; Wolfram et al., 1995; Paganga & Rice-Evens, 1997; Aziz et al., 1998; Gee et al., 1998; Hollman et al., 1999; Walgren et al., 2000) and its absorption is inhibited by the absence of Na⁺ (Hollman et al., 1995) and presence of sugars (Wollfram et al., 2002) suggesting the importance of SGLT in the transport of flavonoid glycosides. If the glycosides do enter the enterocyte in this manner, the broad specificity cytosolic beta-glucosidase (CBG) will likely be responsible for hydrolysis of the glycoside within the enterocyte (Day et al., 2000a; Walle et al., 2000) as CBG is known to catalyze the hydrolysis of a wide variety of xenobiotic glycosides (Lamarco and Glew, 1986; Gopalan et al., 1992) (Figure 2.2).

An alternative mechanism for the absorption of flavonoids may involve the hydrolysis of the glycoside by LPH prior to absorption. LPH an enzyme located outside the luminal boundary of the brush border membrane (Figure 2.2). The resulting aglycone would be subsequently absorbed by passive diffusion (Day et al., 2000a; Williamson et al., 2000). LPH is a family of beta-glucosidases used primarily for the hydrolysis of lactose from milk in newborns and persists into adulthood in the human intestine (Leese & Semenza, 1973). An *in vitro* investigation has revealed LPH is capable of cleaving quercetin 3-glucoside, quercetin 4'-glucoside, quercetin 3,4'-glucoside, and 3' methylquercetin-3-glucoside. Because LPH has been shown to catalyze the hydrolysis of quercetin-3-O-glucosides, which are not substrates for CBG (Day et al., 2000a), it is believed that this pathway plays a significant role in the absorption and metabolism of flavonoid glycosides. Although this has yet to be

adequately established in humans, experimental evidence presented in Chapters 5 & 6 suggests that both of these pathways may be involved in anthocyanin glycoside absorption.

Absorption of Anthocyanins

The mechanisms involved in the absorption and metabolism of anthocyanins are likely similar to those of other hydrophilic flavonoid glycosides, and may occur by one or both of the above suggested routes (Figure 2.2). First, anthocyanin glycosides may be hydrolyzed at the mucosal brush border membrane by LPH (Gee et al., 2000; Williamson et al., 2000). Once at the brush border, the aglycone may diffuse into the enterocyte where it could either enter the portal circulation as an aglycone, or be conjugated before transference across the basolateral membrane into the serosal fluid. (Laitinen & Walkins, 1986; Hollman, 2001). If anthocyanin glycosides are hydrolyzed at the mucosal brush border membrane by LPH prior to absorption and conjugation with glucuronic acid, one would not expect to find parent anthocyanin glycosides in biological fluids. A second pathway must exist as many investigators have identified anthocyanin glycosides in human blood and urine (Bub et al., 2001; Cao et al., 2001; Felgines et al., 2003; Mazza et al., 2002; Müllleder et al., 2002; Wu et al., 2002; Galvano et al., 2004; Kay et al., 2004). The second possible route of absorption is likely to occur via transport of the intact anthocyanin glycoside into the enterocyte, possibly by SGULT (Hollman et al., 1995; Wolffram et al., 1995; Hollman & Katan, 1998a; Williamson et al., 2000; Müllleder et al., 2002). Supplementation of anthocyanins with various sugars has been observed to reduce the excretion of anthocyanins (Müllleder et al., 2002) suggesting the potential role of SGULT in the transport of anthocyanin glycosides. Once

inside the cell, the intact glycoside could either directly cross the basolateral membrane into the portal circulation, or be hydrolyzed by CBG (Day et al., 1998a; Walle et al., 2000; Williamson et al., 2000; Mülleder et al., 2002) prior to intestinal metabolism and transport (Hollman, 2001). Saturation of the above detailed pathways could account for the large variation in results presented in the literature. The theory of a saturatable transport mechanism involving a sodium-dependent transporter has been previously proposed for the absorption of quercetin glycosides (Mizuma et al., 1994; Hollman et al., 1995; Wolffram et al., 1995; Hollman & Katan, 1998a). As both anthocyanin glycosides and glucuronide derivatives have been identified in human plasma/serum (Mazza et al., 2002; Wu et al., 2002; Felgines et al., 2003; Galvano et al., 2004; Kay et al., 2004) the current evidence suggests that both pathways are involved in the absorption of anthocyanins.

METABOLISM & PHARMACOKINETICS

Experimental Variability

Flavonoid metabolism has yet to be adequately characterized partly due to the large variation in findings in the literature. This is likely the result of variations in dosages used in clinical studies. Since metabolic pathways that rely on co-factors are saturatable, large doses of flavonoids (pharmacological doses) will likely result in the saturation of metabolic pathways and consequently large amounts of un-conjugated parent compounds will enter the circulation. Alternatively, flavonoids fed in lower doses, more similar to the levels found in the diet, would not be expected to saturate

metabolic pathways and the circulating species would most likely be largely conjugated. For this reason, studies feeding a single dose of flavonoids will not establish a complete representation of their metabolism. More advanced multiple-dosing studies are required to establish a true understanding of the metabolism of these compounds.

Metabolic Variability

The inter-individual variability in response to any pharmacokinetic parameter is extensive. It is not uncommon for inter-individual variations to be 5–60% (Fuhr & Kummert, 1995). This makes it difficult to compare findings among populations, age groups, genders, and ethnicities. Therefore, findings in pharmacological investigations must not be conceptualized in absolute terms but rather as representations of the subset of individuals' investigated. Numerous factors can contribute to the extreme variation in metabolism between subjects. Factors affecting pharmacokinetics include: age, gender, genetic ethnicity, hormone levels, gut flora and intestinal integrity, diet, environment, incomplete absorption, enterohepatic cycling with incomplete reabsorption, or metabolism in the gut, gut wall, portal blood, or liver prior to entry into the systemic circulation (Benet, 1984). In addition, conditions where liver function is directly affected, such as cirrhosis, reduced blood flow, alcohol and drug use (acute and chronic), malnutrition, and liver pathology, compound this variability. Also, the variation in the age of subjects often has a sizeable impact on variability. Alterations in intestinal integrity, gastrointestinal pH, gastric emptying time, intestinal transit time, intestinal and hepatic metabolic activity, and splanchnic blood flow are known to occur with aging. Even slight variation in fasting time between individuals can affect pharmacokinetic data. Fasting causes changes in the activity of several enzymes

responsible for biotransformation including UDP-glucuronosyltransferase, beta-glucosidase, and NADPH Cyt-P450 reductase (Rozman, 1986). As a result of the above listed dynamics, large variations in pharmacokinetic parameters between studies are often described.

Metabolism and Pharmacokinetics of Flavonoids

Basic concepts in flavonoid metabolism. Many flavonoids are extensively metabolised in humans with less than 5-10% of the ingested parent compounds (following a low to moderate oral dose) excreted in the urine (Bravo et al., 1994). It has been reported that as much as 52% of an oral dose of radiolabelled quercetin was exhaled as $^{14}\text{CO}_2$ (Walle et al., 2001) in humans indicating that significant absorption and metabolism had occurred. Even though many flavonoids are reported to have low bioavailabilities as a result of their extensive metabolism, their metabolites may persist in the circulation for long periods of time and consequently the bioactivity of the metabolites may have significant effects. In order to form a working hypothesis of the metabolic fate(s) that anthocyanins may have in the body, it is necessary to first review the basic processes associated with flavonoid/polyphenol metabolism.

Conjugation. Conjugation is generally regarded as a detoxification reaction conferring a progressive increase in the polarity of a compound leading to its increased solubility and consequent excretion in the bile or urine (Dutton, 1980). Conjugation requires a variety of enzymatic reactions collectively referred to as Phase I and Phase II detoxification. Phase I reactions are primarily responsible for adding or exposing polar groups, such as

hydroxyl, carboxyl, or amino, for further phase II conjugation. Phase I reactions consist of oxidations, reductions, and hydroxylations. The enzyme system responsible for these reactions is the cytochrome P-450 monooxygenase system (Andrews, 1978). Phase II reactions involve glucuronidation, sulfation, methylation, acylation, and glutathione and amino acid conjugation. Once conjugated, the foreign compound can access existing transport systems utilized by endogenous conjugates, thus facilitating their excretion. The products of Phase II reactions are usually less reactive as a result of their increased water-solubility; conversely, products of phase I reactions are not always less reactive (Andrews, 1978; Dutton, 1980). Research within the last decade suggests that the majority of flavonoids are found in the circulation and urine as methylated, sulfated, glucuronidated and glycosylated conjugates (Aziz et al., 1998; Hollman & Katan, 1998a,b; Shimoi et al., 1998; Donovan et al., 2001), with only 0.1-1.5% of ingested dietary quercetin reported to be excreted unmetabolized (Balant et al., 1979; Hollman et al., 1995; Hollman et al., 1997b; Aziz et al., 1998; Hollman et al., 1999).

Glucuronidation. Glucuronide conjugation is regarded as the major conjugation reaction involved in flavonoid metabolism (Shimoi et al., 1998; Spencer et al., 1999; Kuhnle et al., 2000; Donovan et al., 2001; Oliveria et al., 2002). There are two main reasons for the widespread utilization of the glucuronidation pathway; one, glucuronic acid is derived directly from glucose, and its store, glycogen, and is therefore readily available; and two, glucuronic acid has the capacity to be conjugated with a wide range of compounds. O-glucuronides (linkage through an oxygen atom) are the most common form of glucuronide conjugation (Dutton, 1980), making the highly hydroxylated flavonoids prime targets for glucuronidation. The glucuronidation reaction is catalyzed

by UDP-glucuronosyltransferases (UDPGT) which is found in high concentrations in the liver, intestine and kidneys. Of all the tissues, the liver has the greatest capacity for glucuronidation (Mojarrabi & MacKenzie, 1998; Strassburg et al., 1998) although more and more evidence points toward the intestine as being the initial and primary site for flavonoid glucuronidation in humans (Cheng et al., 1999; Crespy et al., 1999; Donovan et al., 2001).

Methylation. Methylation appears to be the second most significant conjugation reaction involving flavonoids (Kuhnle et al., 2000; Williamson et al., 2000). Methylation is driven by a group of enzymes referred to as methyltransferases. These non-specific enzymes are found in many tissues including the liver and intestine (Laitinen & Watkins, 1986). The most common methylation reaction associated with flavonoid metabolism is O-methylation. O-methylation is catalyzed by catechol-O-methyltransferase (COMT) utilizing S-adenosyl methionine (SAM) as a cofactor. The liver has the highest COMT activity and is the main organ responsible for methylation (Piskula & Terao, 1998). The hydroxylation pattern of a flavonoids ring structure will determine the primary site of methylation. Studies reveal that quercetin is extensively methylated following low oral doses in humans and in animals (Manach et al., 1998; Williamson et al., 2000).

Sulfation. Sulfation or glycylation are common conjugation reactions which predominate when low doses of phenolic drugs are administered. Sulfation reactions are catalyzed by sulfotransferases (SULT) which are a small group of cytosolic enzymes widely distributed throughout the body. They utilize phosphoadenosine-5'-

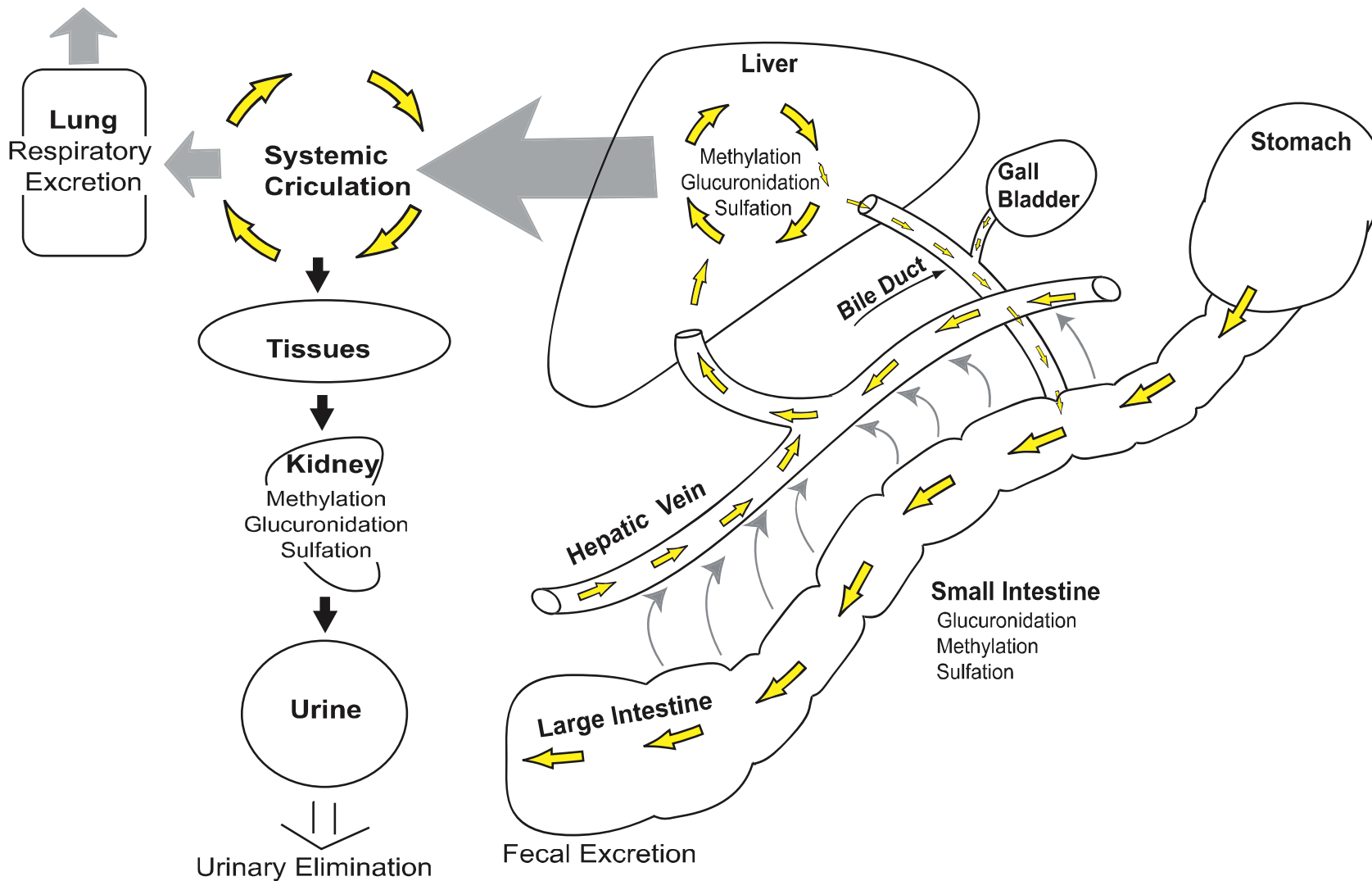
phosphosulfate (PAPS) as a cofactor and their known substrates include phenols and polyphenols (i.e., flavonoids), iodothyronines, 4-nitrophenol, and hydroxyarylamines (Coughtrie et al., 1998). Additionally, sulfation, as a conjugation reaction, is relatively costly in ATP and sulphate and is more likely to be rapidly limited by aglycone loading than is glucuronidation. Therefore, sulfate conjugation is regarded as a highly saturatable pathway making it difficult to identify flavonoid sulfides as a result of their extremely low concentrations in the blood and urine. The sulfation of quercetin has been documented (Williamson et al., 2000; Donovan et al., 2001) and one recent study has identified sulfoconjugates of anthocyanin glycosides (Felgines et al., 2003).

Pharmacokinetics of flavonoids in plasma/serum. Studies have shown that the concentrations of intact flavonoids in human serum/plasma rarely exceed 1 mM although low levels can be maintained for significant periods of time (24-48h) (Scalbert & Williamson, 2000). Quercetin glucosides have been detected in the plasma of non-supplemented individuals (basal levels) at levels of 0.5-1.6 μ M (Paganga & Rice-Evens, 1997). For most flavonoids, the plasma clearance is usually rapid with an absorption half-life of 1–2h and elimination half-life of 4-6h (Lee et al., 1995; Hollman et al., 1996; Hollman et al., 1997ab; Aziz et al., 1998). Glycosides, such as rutin, are an exception to this as their hydrolysis only occurs in the colon, thereby, delaying the appearance of quercetin rutoside in the serum (maximum plasma concentration = 9h; Hollman et al., 1997a,b). In addition to the type of glycosidic bond, subtle changes in the structure of individual flavonoids, as well as the food matrix in which they exist, will have significant effects on their pharmacokinetics. For example, peak quercetin plasma levels

have been observed to be highly variable ranging from 0.7-10h depending on their form and source. After the ingestion of onions, quercetin reached its maximum concentration (C_{max}) at 0.7h; however, it peaked at 2.5h after the consumption of apples and 9h after the consumption of quercetin rutinoid (Hollman et al., 1997b). Once in the systemic circulation the flavonoid distributions will be similar, with compounds distributed to tissues and eventually eliminated through urinary excretion. Conversely, compounds entering the bile may undergo further metabolism in the small or large intestine prior to excretion or reabsorption.

Elimination. Once conjugated, the main routes of excretion for xenobiotics are urinary, biliary, and expiratory (Figure 2.3). Highly polar compounds, when taken orally or those formed in the intestine as a result of conjugation will enter the circulation and be filtered by the kidneys. Water-soluble substances of molecular weight less than 60 000 are generally filtered by the renal glomeruli, unless bound to proteins, while highly polar compounds formed in the liver are readily available for biliary excretion and may be reabsorbed from the intestine subsequent to biliary excretion (process known as enterohepatic circulation; EHC). The various routes of elimination and pharmacokinetics of flavonoids are discussed below (Rozman, 1986; Gregus & Klaassen, 1986).

Figure 2.3 Potential Route for Anthocyanin Metabolism and Excretion



Urinary excretion and pharmacokinetics of flavonoids. Urinary excretion is the primary route of elimination for highly polar flavonoids; however, the urinary excretion of total quercetin is low, and generally reported between 0.1-1.5% of the ingested dose (Hollman et al., 1995; Hollman et al., 1997a,b; Aziz et al., 1998; Hollman et al., 1999). Additionally, more than 98% of the excreted quercetin occurs as conjugates in the urine (Balant et al., 1979; Hollman et al., 1997b; Aziz et al., 1998; Hollman et al., 1999). The half-lives of elimination for quercetin have been reported between 8-28h (Hollman et al., 1997a,b; Graefe et al., 1999) revealing a large variation in elimination kinetics between quercetin forms and sources. The delayed urinary elimination of quercetin has been attributed to its high affinity for plasma proteins, mainly albumin (Scalbert & Williamson, 2000).

Biliary excretion. Studies have shown that many products of flavonoid metabolism that still have considerable polarity and molecular mass (>300-400) are readily excreted into the bile (Barrow & Griffiths, 1974; Hackett & Griffiths, 1981; Brown & Griffiths, 1983; Ueno et al., 1983). The substitution pattern of a flavonoid is influential in determining the extent of biliary excretion with the highest excretion rate observed for mono- and di-hydroxylated compounds and lowest for fully hydroxylated flavonoids (Barrow & Griffiths, 1974). Flavonoids excreted in the bile come into contact with the vast absorptive surface of the intestine where they may be reabsorbed into the portal circulation and returned to the liver (EHC) or they may be excreted through the large intestine. EHC prolongs the residence of a chemical/xenobiotic in the body by reducing its fecal excretion. If a compound undergoing EHC is also available to the systemic

circulation, this will result in a longer plasma elimination half-life (Gregus and Klaassen, 1986). EHC is likely to be a major factor in the metabolism of many flavonoids and is responsible for their low but consistent levels in the circulation long after their time to reach maximum concentration (t_{max}) is achieved (e.g., quercetin C_{max} 1-2h, $t_{1/2}$ 8-28h). Although it appears that various forms of quercetin may undergo EHC, this is likely not a major route for the elimination for anthocyanins as their elimination half-life is relatively short ($t_{1/2} = 3-4h$) (Cao et al., 2001; Matsumoto et al., 2001; Mülleder et al., 2002; Table 2.1)

The route of elimination of flavonoids will ultimately depend on the type of conjugates produced and their site of production. Glucuronides formed in the intestine tend to enter the systemic circulation directly and are not readily available for biliary excretion unlike those newly formed in the liver which are predominantly excreted into the bile. This implies that glucuronidation in the intestinal cells facilitates the loss of chemicals from EHC towards the systemic circulation. Compounds undergoing EHC may also undergo further sulfation in the intestine or hepatocyte which would divert them from biliary excretion to eventual urinary elimination as enteral reabsorption of strongly polar compounds is negligible (Gregus & Klaassen, 1986).

Table 2.1 Pharmacokinetics of Anthocyanins Following Oral Consumption in Humans

Anthocyanin	Source	Study Period (h)	Dose	% Urinary Recoverys	C _{max}	t _{max} (h)	t _{1/2} (h)	Reference
Mixed (M-3-G)	red wine	6	68mg	0.03	^p 1.4 nmol/L	0.3		Bub et al., 2001
Mixed	de-alc red wine	6	56mg	0.03	^{l,p} 1.7 nmol/L	1.5		Bub et al., 2001
Mixed	red grape	6	117mg	0.03	^{l,p} 2.8 nmol/L	3		Bub et al., 2001
Mixed (C-3-gly)	elderberry	24	720mg	0.05	^p 97.4 nmol/L	1.2	2.2	Cao et al., 2001
C-3-G	black currant	8	² 11mg	0.6	^p 5.0 nmol/L	1.2	1.3	Matsumoto et al., 2001
C-3-R	black currant	8	² 82mg	0.1	^p 46.3 nmol/L	1.5	3.4	Matsumoto et al., 2001
D-3-G	black currant	8	² 32mg	0.7	^p 22.7 nmol/L	1.5	4.1	Matsumoto et al., 2001
D-3-R	black currant	8	² 111mg	0.1	^p 73.4 nmol/L	1.7	3.2	Matsumoto et al., 2001
Mixed	blueberry	4	1200mg	0.003	^p 13.0 ug/L	4.0		Mazza et al., 2002
Mixed	elderberry	6	1900mg	0.003	^u 21.7 ug/urine	1.5	4	Mülleder et al., 2002
Mixed	elderberry	24	720mg	0.07	^p 97.4 nmol/L			Wu et al., 2002
Mixed	strawberries	24	179umol	1.8	^{l,u} 1.2 μmol/L	2-4		Felgines et al., 2003
Mixed	black currant	4	1239mg	0.07	^{l,p} 52ng/L	0.7		Nielsen et al., 2003
Mixed	black currant	4	716mg	0.05	^{l,p} 16 ng/L	0.7		Nielsen et al., 2003

C-3-G, cyanidin 3-glucoside; C-3-gly, cyanidin 3-glycosides; C-3-R, cyanidin 3-rutanoside; C-3-S, cyanidin 3-sambabioside; C_{max}, maximum concentration; D-3-G, delphinidin 3-glucoside; D-3-R, delphinidin 3-rutanoside; M-3-G, malvidin 3-glucoside; t_{max}, time to reach maximum concentration; t_{1/2}, half-life of elimination.

¹Data interpreted visually from a concentration vs time graph as exact values were not given in the manuscript.

²Dose derived from conversion of mg/kg to mg, based on reported average subject weight of 66Kg (median of range 60-72).

^pC_{max} derived from plasma data.

^uC_{max} derived from urine data.

Blank cells represent missing data from original manuscripts.

Colonic metabolism and re-absorption. Colonic bacteria play a major role in the metabolism of many flavonoids (Das & Sothy, 1971; Shaw & Griffiths, 1980; Hackett, 1986). Colonic bacteria cleave glucosides from their parent compounds for catabolism leaving aglycones for re-absorption through the intestinal wall or further degradation (Boulton et al., 1999; Schneider et al., 1999; Skibola & Smith, 2000). The degradation of flavonoids occurs through pyrone ring cleavage producing phenylacetic and phenylpropionic acids as well as lower molecular weight byproducts (Piskula & Terao, 1998). Reabsorbed phenolic acids are found in the circulation and eventually excreted in the urine (Hollman & Katan, 1998b). In a study conducted by Das & Sothy (1971) utilizing radiolabelled catechin, ¹⁴C-phenolic acid metabolites were found to be reabsorbed and excreted in the urine of rats post bacterial degradation. Conversely, in a study by Schneider et al. (1999) utilizing colonic bacteria isolated from human feces, quercetin-3-glucoside was observed to be degraded to quercetin; however, the majority of quercetin aglycone was left intact. Additionally, varying proportions of xenobiotics present in food will also pass through the GIT unabsorbed and be eliminated in the feces (Rozman, 1986). Studies feeding oral doses or radiolabelled quercetin (Ueno et al., 1983) and catechin (Matsumoto et al., 2001) to rats reported 30-32% of the radiolabelled compounds to be excreted in the feces unmetabolized. The extent to which flavonoids are eliminated in the feces of humans is undetermined for many flavonoids including anthocyanins.

Respiratory excretion. The lung is responsible for the elimination of volatile substances from the body and is a major site of excretion for many xenobiotics. In a study by

Petrakis et al. (1959), 44% of the radioactivity of ^{14}C labelled quercetin was detected in the GI of rats and 11% was detected in the lungs (Petrakis et al., 1959). More recently, it has been reported that as much as 52% of an oral dose of radiolabelled quercetin in humans was exhaled as $^{14}\text{CO}_2$ (Walle et al., 2001). Unfortunately, the relative respiratory excretion of many flavonoids, including anthocyanins, is still unknown.

Metabolism and Pharmacokinetics of Anthocyanins

Pharmacokinetics. The limited pharmacokinetic data available suggests that maximum plasma concentrations of anthocyanins in humans are anywhere between 1-100 nmol/L, and are generally reported to occur between 0.7-4h post-consumption of doses ranging between 0.05-1.5g. Additionally, the average urinary excretion of parent glycosides is generally reported between 0.03-1% of the ingested dose with a maximum rate of urinary excretion occurring between 1-4h (Murkovic et al., 2001; Milbury et al., 2002) and having an elimination half-life of 2-4h (Table 2.1) (Bub et al., 2001; Cao et al., 2001; Matsumoto et al., 2001; Mazza et al., 2002; Mülleder et al., 2002; Wu et al., 2002; Felgines et al., 2003; Nielsen et al., 2003).

Metabolism. The presence of unmetabolized (parent) anthocyanin glycosides in human blood and urine has been extensively documented (Miyazawa et al., 1999; Bub et al., 2001; Cao et al., 2001; Felgines et al., 2002; Mazza et al., 2002; Mülleder et al., 2002; Wu et al., 2002; Galvano et al., 2004; Kay et al., 2004). Even though many investigators suggest that anthocyanins are not metabolized prior to release into the systemic circulation, recent evidence indicates otherwise. The detection of glucuronide

(Wu et al., 2002; Wu et al. 2002; Felgines et al., 2003; Kay et al., 2004), methyl (Wu et al., 2002; Wu et al., 2002; Kay et al., 2004), and sulfoconjugates (Felgines et al., 2003) have been documented with one investigation (Felgines et al., 2003) reporting as much as 80% of anthocyanins in the urine existing as conjugated metabolites.

Low/dietary levels of anthocyanins are likely to be primarily metabolized in the intestinal mucosa while larger pharmacological doses are likely to undergo significant metabolism in the liver (Figure 2.3) (Scalbert & Williamson, 2000). The primary conjugation reaction for anthocyanins appears to be glucuronidation, and the site of the majority of glucuronidation is likely the small intestine (Cheng et al., 1999; Crespy et al., 1999; Donovan et al., 2001); however, portions of anthocyanin glycosides may also be glucuronidated in the liver or to a lesser extent in the kidney (Mojarrabi & MacKenzie, 1998; Strassburg et al., 1998). The next most significant conjugation reaction is methylation and is likely to occur primarily in the liver (Piskula & Terao, 1998), followed by sulfation in the liver, small intestine, or kidney (Chapman et al., 2004).

Elimination. The rapid urinary excretion rate reported for anthocyanins suggests that anthocyanin glycosides are primarily absorbed from the small intestine (Hollman et al., 1999; Scalbert & Williamson, 2000). Conjugates produced in the intestine will likely enter the systemic circulation directly while conjugates formed in the liver are more likely to be excreted in the bile (Griffiths, 1982). Conjugates entering the systemic circulation will be distributed to tissues and eventually eliminated through urinary excretion while compounds entering the bile may undergo further metabolism in the

small or large intestine. An illustration of the pathway that anthocyanins may follow in the body is given in Figure 2.3. Colonic bacteria are also likely to play a major role in the metabolism of anthocyanins. Many flavonoids are extensively metabolized by colonic bacteria leaving a range of phenolic acids which may be reabsorbed. (Das & Sothy, 1971; Shaw & Griffiths, 1980; Hackett, 1986; Hollman, 2001). Tsuda et al. (1999a) reported a significant increase in plasma concentrations of protocatechuic acid following the administration of cyanidin glycosides to rats. The researchers suggested that the protocatechuic acid may be derived from the breakdown of anthocyanins. The concentration of protocatechuic acid in the plasma was reported to be 8-fold higher than the parent anthocyanins. A recent *in vitro* fermentation study has confirmed that protocatechuic acid is a major metabolic by-product of anthocyanins by human fecal bacteria (Aura et al., 2004); however, radiolabelling studies are required to establish the extent to which this occurs in humans *in vivo*. Although the limited data suggests that anthocyanins are not metabolized by bacteria to the extent seen with other flavonoids (Hollman & Katan, 1998b) this possibility must not be overlooked. If anthocyanins are readily absorbed and less than 2% are identified in the urine, the remainder of the anthocyanins are likely to exist as colonic metabolites. Additionally, if a large conversion of anthocyanins to phenolic acids and phenolic acid residues does occur, these compounds would not be easily identified and would likely go undetected. In addition to colonic metabolism, varying proportions of anthocyanins/flavonoids present in food would pass through the GIT unabsorbed and be eliminated in the feces (Rozman, 1986). Empirical evidence for the excretion of anthocyanins in human feces is currently not available.

The lung is also a major site for the excretion of xenobiotics. Recently, it has been reported that as much as 52% of an oral dose of radiolabelled quercetin in humans was exhaled as $^{14}\text{CO}_2$ (Walle et al., 2001). Unfortunately, the relative respiratory excretion of anthocyanins is unknown. From the above discussion, it is quite apparent that a great deal of research is required before the absorption and metabolism of anthocyanins is fully understood.

CHAPTER 3.

EXTRACTION & ANALYSIS OF ANTHOCYANINS IN SERUM AND URINE

Anthocyanins have been identified and characterized for more than two decades. The identification of anthocyanins has historically been accomplished by one of two chromatographic techniques- silica gel column chromatography or reverse-phase high pressure liquid chromatography (RP-HPLC). The latter is definitely the most utilized today. The major advantage of a reverse-phase system is that the aqueous environment of the mobile phase is highly compatible with biological samples (Hostettmann & Hostettmann, 1982; Markham, 1982). Due to the vast diversity of HPLC systems, column types, and solvent mixtures, no single method can be applied to all situations/applications. Some specific methods, as cited in the literature, are summarized in Table 3.1.

Various complementary techniques may be utilized to aid in the identification of flavonoids. Shift reagents may be used to determine sites of conjugation (Markham, 1982) and acid-hydrolysis may be utilized to determine the nature of the base aglycone when comparable standards are available. Enzymatic methods have also been utilized to determine the basic structure of flavonoid glycosides (Matsumoto et al., 2001; Liu & Hu, 2002; Mazza et al., 2004). By using specific glycosidases, one can determine the nature of the conjugating sugar. Additionally, the liberated aglycone can also be compared to available standards; however, these methods cannot be easily applied to the analysis of anthocyanin conjugates for the following reasons. Enzymatic hydrolysis is

problematic as a consequence of the instability of anthocyanins in the higher pH ranges needed for optimal enzymatic activity (pH 4-5). Also, once an aglycone has been liberated, its spontaneous degradation is rapid. Therefore, the use of traditional techniques for the identification of anthocyanin conjugates is problematic (Mazza et al., 2004).

To date, very few researchers have adapted methods to extract and quantify anthocyanin metabolites from human biological fluids or tissues. Concentrations of anthocyanins in the blood and urine are at levels approaching the detection limit of current methodologies such as HPLC, making the identification of metabolic by-products difficult. Additionally, glycosides and glucuronides can have almost identical retention times and absorption spectra when using HPLC analysis (Day & Williamson, 2001). Also, pure standards of anthocyanin metabolites are not available and have to be manufactured in-house. This can be accomplished enzymatically and chemically; however, few laboratories have the expertise to do so. More advanced techniques, such as mass spectroscopy (MS), are often coupled with HPLC to identify novel structures in biological fluids; however, many compounds can have near identical molecular weights making MS analysis difficult. For example, an anthocyanin glucuronide can have the same mass, to one decimal place, as its methylated hexose glycoside derivative. Ideally, identification using NMR should be utilized. The difficulty lies in the amount of sample required for proper structural elucidation. NMR requires large quantities of pure compounds and many labs do not have the resources to conduct analyses of this magnitude (Mazza et al., 2004). Regardless, the structural conformation of anthocyanin metabolites requires significant chemical/experimental evidence. Sufficient evidence

will require isolation and extraction of mg quantities of metabolites through large scale, bulk extractions.

The extraction of anthocyanins in biological fluids can be a complex task and the following should be considered. Anthocyanins are moderately polar structures and should be extracted in polar solvents such as water, methanol (MeOH), or acetonitrile (Takeoka & Dao 2002). Anthocyanins are generally unstable structures in aglycone form and should therefore be extracted at temperatures below 40°C. Furthermore, due to their stability in the low pH ranges, it is suggested that extraction solvents be of a pH between 1-3. At higher pH's (> pH 4.5), decolourisation of the anthocyanins in solution can occur as a result of the hydroxylation of the flavylum salt (Fuleki & Francis, 1968; Wrolstad, 1976; Hostettmann & Hostettmann, 1982; Brouillard et al., 1997; Takeoka & Dao 2002).

When extracting anthocyanins from biological materials for HPLC analysis, sample-cleanup is generally required. Basic sample-cleanup is best achieved using commercially available disposable solid-phase extraction C₈ or C₁₈ cartridges (Takeoka & Dao 2002). Specifics of common SPE procedures, as found in the literature, are presented in Table 3.2. Although this technique is sufficient when standards are available, the identification of novel metabolites requires larger scale extractions. This can be accomplished using open column chromatography or preparative HPCL or TLC (Takeoka & Dao 2002). Once sufficient quantities of the metabolites are obtained, identification will likely require multiple techniques and will not be achieved simply by HPLC methods alone (Mazza et al., 2004).

The techniques utilized in the ensuing thesis dissertation are the result of ongoing and progressive method development and are by no means definitive. It is important to

point out that the identification of anthocyanin metabolites is an extremely new avenue in anthocyanin research and much work needs to be done before an adequate model of anthocyanin metabolism in humans is established.

Table 3.1 HPLC Analysis of Anthocyanins in Biological Fluids using Reverse Phase Chromatography

Column	Mobile Phase	Elution	Reference
Nova-Pack C ₁₈	0.05 M dihydrogen ammonium phosphate adjusted to pH 2.6 using orthophosphoric acid B) 20% A / 80% ethanol C) 0.2 M orthophosphoric acid pH 1.6	Linear gradient for 80 min	Lapidot et al., 1998.
Capcell Pac C ₁₈	86:14 H ₂ O (1.5 mol/L formic acid) / MeOH	Not cited	Miyazawa et al., 1999.
Develosil ODS	85:15 H ₂ O (50 mM sodium phosphate) / acetonitrile	Not cited	Tsuda et al., 1999a.
Nova-Pack C ₁₈	A ₁) Plasma - 84:16 H ₂ O (1% orthophosphoric acid) / acetonitrile A ₂) Urine - 92:4:4 1% orthophosphoric acid/ MeOH / acetonitrile B) 100% acetonitrile	Isocratic run at 92%A, 8%B for 3-min; with progression to a linear gradient with 10% B at 35min, 25% B at 45min, and 5 min at 100% B	Bub et al., 2001.
Zorbex C ₁₈	A) 0.5% phosphoric acid B) MeOH	Linear gradient from 20%B to 23% B at 15 min, hold at 23% for 8 min	Matsumoto et al., 2001.
Symmetry	79:10:11 H ₂ O / formic acid / acetonitrile	Isocratic run	Mülleder et al., 2002.
Wakosil II-AR 4	85:15 H ₂ O / acetonitrile	Isocratic run	Suda et al., 2002.
Zorbax C ₁₈	A) 4.5% formic acid in water B) 100% MeOH	Linear gradient starting at 10% B, 0-30 min from 10-25% B, 30-50 min from 25-45% B, 50-55 min from 45-100% B, 55-60min at 100% B, 60-65min from 100-10% B	Mazza et al., 2002.

Table 3.2 Extraction of Anthocyanins from Biological Fluids

Acidification	SPE Cartridge	Wash	Elute	Re-dissolve	Reference
Serum/Plasma					
Equal volume of 10mM oxalic acid	Sep-Pack C ₁₈ Waters	10 mM oxalic acid	MeOH containing 1.0% TFA	100 µl 15% acetonitrile in water containing 50 mM sodium phosphate (pH 1.7)	Tsuda et al., 1999a.
200 µl 0.44 M TFA/mL	Sep-Pack C ₁₈ Waters	10 mL 0.44 mol/L TFA, 10 mL dichloromethane, 10 mL benzene	5 mL 0.44 mol/L TFA in MeOH	200 µl 0.44 mol/L aqueous TFA	Cao & Prior 1999.
100 µl (85%) ortho-phosphoric acid/mL	Sep-Pack C ₁₈ Waters	10 mL orthophosphoric acid in H ₂ O	10 mL 1% orthophosphoric acid in MeOH	200 µl HPLC mobile phase	Bub et al., 2001.
200 µl 0.44 M TFA/mL	Sep-Pack C ₁₈ Waters	10 mL 25 M Na acetate (pH 1.5), 10 mL dichloromethane, 10 mL benzene	2 mL 25 mmol Na acetate/L of MeOH	MeOH containing 25 M Na acetate	Cao et al., 2001.
200 µl 5% TFA/mL	Sep-Pack C ₁₈ Waters	10 mL of 7% formic acid, 15mL dichloromethane, 15 mL benzene	5 mL 5% TFA in MeOH	200 µl 15% acetonitrile in water containing 1.5% phosphoric acid	Suda et al., 2002.
25 µl of 6N HCL/mL	Supelclean ENVI-18 Sigma	7 mL 10 mM oxalic acid	7 mL MeOH containing 0.1% TFA	200 µl 4.5% formic acid in water and MeOH 90:10	Mazza et al., 2002.
Urine					
25 µl 6 N HCL/mL	Sep-Pack C ₁₈ Waters	10 mmol/L oxalic acid in water	5% TFA in MeOH	200 µl 3% phosphoric acid	Matsumoto et al., 2001.
200 µl 0.44 M TFA/mL	Sep-Pack C ₁₈ Waters	10 mL 1.5 M formic acid, 10 mL dichloromethane, 10 mL benzene	5 mL 0.44 mol/L TFA in MeOH	200 µl 0.44 mol/L TFA in MeOH	Miyazawa et al., 1999.
25 µl 6 N HCL/mL	Sep-Pack C ₁₈ Waters	10 mmol/L oxalic acid in water	5% TFA in MeOH	200 µl 3% phosphoric acid	Matsumoto et al., 2001.
100 µl ortho-phosphoric acid/mL	Sep-Pack C ₁₈ Waters	10 mL orthophosphoric acid in H ₂ O	10 mL 1% H ₃ PP ₄ in MeOH	200 µl HPLC mobile phase	Bub et al., 2001.
200 µl 0.44 mol/L TFA/mL	Sep-Pack C ₁₈ Waters	15 mL 25 M Na acetate (pH 1.5)	2 mL 25 mmol Na acetate/L of MeOH	Mobile phase	Cao et al., 2001.

CHAPTER 4.

ABSORPTION OF ANTHOCYANINS FROM BLUEBERRIES AND SERUM ANTIOXIDANT STATUS IN HUMAN SUBJECTS

ABSTRACT

In recent years, numerous studies have revealed that polyphenolics present in fruit and vegetable products possess anticarcinogenic, anti-inflammatory, antihepatotoxic, antibacterial, antiviral, antiallergic, antithrombotic, and antioxidant effects. However, there is little reliable information on the absorption of anthocyanins, especially glycosylated and acylated conjugates, in humans. In the present study, 5 middle-aged, male subjects were fed a high-fat meal and a control supplement followed one week later by the same high-fat meal supplemented with 100g of freeze-dried lowbush blueberries (*Vaccinium angustifolium*) containing 1.20g of total anthocyanins (25 individual anthocyanins, including six acylated structures). Initial and postprandial blood samples were taken sequentially (1, 2, 3, 4h) and analyzed for anthocyanins and for antioxidant status. Nineteen of the 25 anthocyanins present in the lowbush blueberries were detected in the human blood serum, and eleven anthocyanins could be analyzed quantitatively. The serum concentrations of total anthocyanins varied from 5.43 to 16.9 ng/mL. Maximum levels of anthocyanins were observed at 4 hours post-consumption of the blueberry treatment and the anthocyanins identified in highest abundance were malvidin-3-galactoside, malvidin-3-glucoside, and delphinidin-3-glucoside. Likewise, the blueberry treatment was associated with a maximal increase in

serum antioxidant capacity above the control at 4h post-consumption of the high-fat meal (ORAC_{acetone}, P = 0.04). The blueberry treatment prevented the reduction in serum antioxidant capacity associated with the consumption of a high-fat meal as observed using the TEAC assay (treatment effect, P = 0.001). Furthermore, the appearance of total anthocyanins in the serum was directly correlated with the serum antioxidant capacity (ORAC_{acetone}, P < 0.01). These results indicate that anthocyanins can be absorbed in their intact forms, and that the consumption of wild blueberries, a food source with high *in vitro* antioxidant properties, is associated with a diet-induced increase in *ex vivo* serum antioxidant status.

INTRODUCTION

Anthocyanins are widely distributed in the human diet through fruits, vegetables, and grain products. They are best known for their ability to impart intense colours to the plants or plant products in which they occur. Lowbush 'wild' blueberries (*Vaccinium angustifolium*) are one of the highest fruit sources of anthocyanins (Francis 1989; Gao & Mazza, 1995) and have exhibited one of the highest recorded *in vitro* antioxidant capacities of various fruits and vegetables tested (Wang et al., 1996; Camire 2000). Wild blueberries are relatively low in antioxidant vitamins and minerals (Bushway et al., 1983), and their *in vitro* antioxidant capacity has been attributed to their high concentration of anthocyanins (Prior et al., 1998; Kalt et al., 1999).

Diet and oxidative stress have been implicated in the development of cardiovascular diseases (Castelli 1998; Kaplan & Aviram, 1999), diabetes (Georgopoulos, 1999; Vendemiale et al., 1999), and cancer (Ames et al., 1995; Willett

2001). Increasing the blood antioxidant status has also been proposed as a preventative means to reduce the development of these diseases (Kaplan & Aviram, 1999; Vendemiale et al., 1999; Willett, 2001). As anthocyanins have displayed strong antioxidant activity *in vitro* (Tsuda et al., 1994; Mazza, 1997; Wang et al., 1997; Narayan et al., 1999), they may have potentially beneficial effects in humans with respect to the prevention and/or treatment of oxidative associated disease. Unfortunately, information regarding their absorption in humans is scarce (Cook & Samman, 1996). The purpose of the present *in vivo* study was to evaluate the absorption of anthocyanins, and to determine if their consumption correlated with their effect on serum antioxidant status, as measured in healthy human subjects.

MATERIALS AND METHODS

Clinical Procedures

Subjects. Five male subjects (46.9 ± 1.9 years, BMI of 23.8 ± 0.8 kg/m²) were recruited from the Guelph area (ON, Canada). Baseline (fasting) values are given in Table 4.1. The principal criteria for eligibility were: 1) absence of clinical disease; 2) no history of renal or gastrointestinal disorders; 3) no alcoholism; and 4) no smoking. Subjects taking lipid-altering or blood pressure medications were excluded. Subjects also refrained from taking aspirin or anti-inflammatory medications prior to, or during the study, and discontinued all forms of antioxidant supplementation one month prior to the investigation. Furthermore, subjects were instructed to maintain a consistent diet throughout the study period. This study conformed with the ethical guidelines of the

University of Guelph (ON, Canada) and was approved by the Human Subjects Committee. All subjects gave written consent in advance.

TABLE 4.1 Fasting Baseline Characteristics of Study Subjects Before Initiation of Treatment^a

Measurement	Treatment	
	Control Mean \pm SD (n=5)	Blueberry Mean \pm SD (n=5)
TEAC ($\mu\text{mol Trolox equivalents/L}$) ^b	1370 \pm 40	1270 \pm 60
ORAC _{total} ($\mu\text{mol Trolox equivalents/L}$)	2479 \pm 360	2497 \pm 263
ORAC _{PCA} ($\mu\text{mol Trolox equivalents/L}$)	658 \pm 69	672 \pm 56
ORAC _{acetone} ($\mu\text{mol Trolox equivalents/L}$)	759 \pm 145	735 \pm 96
Triacylglycerols (mmol/L)	1.33 \pm 0.33	1.22 \pm 0.40

Abbreviation; TEAC, Trolox equivalent antioxidant capacity. ORAC, oxygen radical absorbance capacity.

^a $\bar{x} \pm \text{SD}$ (n = 5).

^b Significant difference between treatment groups at baseline (t = 0), (paired t test): P < 0.05.

Supplementation . The freeze-dried wild blueberry powder used in this trial was produced at the University of Guelph (Department of Human Biology and Nutritional Sciences) in conjunction with the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA, Guelph, ON, Canada), (Table 4.2). The powder was produced from lowbush blueberries (*Vaccinium angustifolium*) obtained from the Sudbury area of Northern Ontario. The wild blueberries were carefully hand-picked and frozen at $-30\text{ }^{\circ}\text{C}$. The berries were then freeze-dried (whole) and ground into a fine powder using a household food processor. The blueberry supplement (100g) contained 1.20g of total anthocyanins (42% of the total phenolics) and had an ORAC value of 147 $\mu\text{mol Trolox}$

equivalents/g (as determined by Brunswick Laboratories, Wareham, MA, USA). The control supplement was matched for digestible carbohydrate and contained 76.4g (305 calories) of glucose (Alantic Sugar Ltd., Toronto, ON, Canada), 0.5g of sugar-free Kool Aid (Kraft Canada, Don Mills, ON, Canada), and had an ORAC value of 5 μ mol Trolox equivalents/g (Table 4.2).

Experimental design. This study followed a singly-blinded crossover design. Subjects were admitted to the Human Testing Lab (Department of Human Biology and Nutritional Sciences, University of Guelph) in the morning of the study dates in a fasted state (12-14 h, 24h no alcohol). Subjects were cannulated with an Intima 20 gage intravenous catheter (Becton Dickinson, Rutherford, NJ, USA) in a brachial vein and overnight fasting blood samples were drawn. Subjects received the control supplement (dissolved in 500 mL water) with a high-fat meal (853 calories, 46.7g fat with 15.5g saturated fat, as determined by Maxxam Analytics Inc, Mississauga, ON, Canada) consisting of 1 Egg McMuffin, 1 Sausage McMuffin and 2 hash brown patties (McDonald's Corporation), (Table 4.3). Time $t = 0$ was obtained upon initiation of the high-fat meal; subsequent blood samples were performed at 1, 2, 3, and 4h. Procedures were repeated (on the same subjects) 7d later using the same high-fat meal, with 100g of the freeze-dried wild blueberry powder (dispersed in 500 mL of water) in place of the control supplement. All subjects consumed both the blueberry and control supplements and were fully compliant with the study regime. No adverse effects were reported.

TABLE 4.2 Composition of Treatment Supplements (100g)

Component	Concentration	
	Blueberry	Control
ORAC ^a (µmol TE/g)	147	5
Total phenolics (g/100g)	2.79	0.00
Anthocyanins (g/100g)	1.16	0.00
Vitamin C (g/100g)	0.01	0.00
Carbohydrate ^b (digestible), (g/100g)	76.4	76.4
Total dietary fibre ^b (g/100g)	17.8	0.00
Protein ^b (g/100g)	2.7	0.00
Fat ^b (g/100g)	2.4	0.00
Ash ^b (mineral content), (g/100g)	1.0	0.00
Calories ^b (/100g)	338	305
KJ ^b (energy), (/100g)	1410	1272

^a ORAC, oxygen radical absorbance capacity (TE/g, Trolox equivalents per gram), conducted by Brunswick Laboratories.

^b Chemical analyses of freeze-dried blueberry powder conducted by Maxxam Analytics Inc.

TABLE 4.3 Composition of High-Fat Meal

Component	Amount	% Calories
Carbohydrate ^a (digestible)	75.2g	35.4 %
Protein ^a	32.4g	15.3 %
Fat ^a	46.7g	49.3 %
Total dietary fibre ^a	4.5g	
Ash (mineral content) ^a	9.1g	
Vitamin C	6.0mg	
Vitamin E	2.1mg	
Calories ^a	853	
KJ (energy) ^a	3564	

^a Chemical analyses of the high-fat meal conducted by Maxxam Analytics Inc.

Sampling procedures. Twenty mL of blood (per time point) was drawn from a brachial vein catheter into evacuated glass tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, USA). Samples were allowed to clot at room temperature for 25 min. Samples were then immediately centrifuged (3000 rpm, 1000 x g) for 15 min to recover serum which was extracted and aliquoted into 2 mL vials (Cryovial, Fisher Scientific Ltd., Nepean, ON, Canada) over an ice bath (3-4°C) using disposable glass pipettes. The serum was then snap-frozen in liquid nitrogen and stored at -80°C.

Anthocyanins Extraction

The below method was modified from Tsuda et al., (1999a). Anthocyanins in human serum were extracted by solid phase extraction using a SPE C₁₈ cartridge (Supelclean ENVI-18, 6 mL tube, 0.5g loading, Sigma-Aldrich/Supelco, Bellefonte, PA). The cartridge was preconditioned using 7 mL of methanol containing 0.1 % trifluoroacetic acid (TFA) and then equilibrated with 7 mL of 10 mM oxalic acid before use. Human serum (1.5 mL) was acidified with a 1/40 volume of 6 N HCl. The acidified serum was diluted with 1.5 mL of 10 mM oxalic acid. The mixture was then loaded on the cartridge. The cartridge was washed with 7 mL of 10 mM oxalic acid. Before eluting anthocyanins, the remained aqueous was drained off using vacuum. Finally, anthocyanins were eluted with 7 mL of methanol containing 0.1 % TFA. The eluate was carefully evaporated using Speed-Vac Plus SC 110A and Universal vacuum system UVS 400 (Savant Instruments, Inc. Holbrook, NY) at room temperature and finally taken to dryness under a stream of nitrogen. The dry residue was redissolved in 200 µL of 4.5 % formic acid in water and methanol in a ratio of 90:10, and filtered through a 13mm 0.45µ GHP Acrodisc filter (Pall-Gelman) into a 300µl vial insert for HPLC.

HPLC Analysis

The HPLC system used was an Agilent 1100 Series (Agilent Technologies Inc., Palo Alto, CA) with a photo diode array detector. The following procedure was modified from previously published methods (Mazza et al., 2002). Anthocyanins were separated on a Zorbax SB C₁₈, 250 × 4.6 mm (5µm) reverse phase column (Agilent Technologies, Inc Palo Alto, CA) with an Inertsil 30 × 4.6 mm (5µm) guard column (Phenomenex, Torrance, CA). The solvents used were 4.5% (V/V) aqueous formic acid (solvent A) and 100% HPLC grade methanol (solvent B). The flow rate was 1 mL/min with a gradient profile of solvent A with following proportions of solvent B: 0 min, 10%; 0-30 min, 10-25%; 30-50 min, 25-45%; 50-55 min, 45-100%; 55-60 min, 100%; 60-65 min, 100-10%. Total run time was 70 min. The column temperature was maintained at 35°C, the injection volume was 50 µL, the detector signal was 525nm (16nm bandwidth) and the reference was 700nm (100nm bandwidth). Anthocyanins in the blueberry diet and in blood serum extracts were identified as described by Gao and Mazza (1994, 1995) by reversed-phase high-performance liquid chromatography (RP-HPLC) and using retention times and UV-vis absorption spectra. The aliphatic acylating acids and the sugars were characterized by capillary gas-liquid chromatography (GLC) analysis of trimethylsilyl derivatives of sugars and aliphatic acids and methyl esters of aliphatic acids.

Serum Antioxidant Capacity

Serum antioxidant capacity was measured using the TEAC and ORAC (ORAC_{total}, ORAC_{PCA}, and ORAC_{acetone} fractions) assays. TEAC was measured on an automated Hitachi 911 Biochemical Analyzer using the Randox-TEAC assay (Randox Laboratories, Mississauga, ON, Canada). Briefly, the TEAC assay is based on the inhibition by antioxidants to absorb free radicals. Plasma samples were added to a 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate), (ABTS^{•+}) radical solution. The change in radical concentration over a 10 min incubation period was monitored spectrophotometrically (600 nm) and compared to the decrease of a known standard (Trolox). Plasma ORAC was determined by Genox Laboratories, Baltimore, MD, USA, using the ORAC assay as outlined by Cao et al. (1993). Modifications to the ORAC assay allowed for the isolation of lipid- and water-soluble phases of the plasma sample. The ORAC_{PCA} fraction (serum treated with perchloric acid) is a nonprotein fraction that preserves the water-soluble antioxidants within the sample. ORAC_{acetone} (plasma treated with acetone) is a nonprotein fraction containing both water-soluble and lipid-soluble antioxidants (Cao et al., 1993). Briefly, the ORAC assay was conducted using the automated COBAS FARA II spectrofluorometric analyzer (Roche Diagnostics, Basel, Switzerland) with fluorescent filters at an excitation wavelength of 546 nm and an emission wavelength of 565 nm. *R*-phycoerythrin (*R*-PE) was used as the target molecule for free radical attack, with 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) as the peroxy radical generator. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) an aqueous-soluble vitamin E analogue was used as a control standard. Fluorescence of *R*-PE was recorded every 55 sec and expressed

relative to the initial reading. Final results were calculated by using the differences of the areas under the *R*-PE decay curves between the blank and the sample. ORAC readings are expressed as μmol Trolox equivalents, where 1 Trolox equivalent equals the net protection area under the curve provided by 1 μmol Trolox. Specifics of the ORAC and comparisons between the ORAC and TEAC assays have been outlined by Cao and Prior (1998).

Statistical Analysis

Results are expressed as means \pm SD. Baseline characteristics of the treatment groups were compared using independent paired *t* tests. The effects of diet, treatment and postprandial times as well as interactions between them were determined by repeated measures analysis of variance (ANOVA) using the Statistical Analysis Systems statistical software package version 6.1 (SAS Institute, Cary, NC, USA). Linear regression analysis was also conducted using SAS.

RESULTS AND DISCUSSION

Anthocyanins in Human Serum

Figure 4.1 shows typical HPLC chromatograms of human serum extracts before ($t=0$) (A), and 1 (B) and 3h (C) after consumption of 100g of freeze-dried blueberries that contained 1.20g of total anthocyanins. No anthocyanins were detected before consumption of the blueberry anthocyanins. Blood samples collected after consumption

of the blueberries, contained most of the 25 anthocyanins present in lowbush blueberries, and eleven anthocyanins, namely, delphinidin-3-galactoside, delphinidin-3-glucoside, cyanidin-3-galactoside, delphinidin-3-arabinoside, cyanidin-3-glucoside, petunidin-3-galactoside, cyanidin-3-arabinoside, petunidin-3-glucoside, peonidin-3-arabinoside, malvidin-3-galactoside, and malvidin-3-glucoside could be analysed quantitatively in blood serum. The contents of total and major anthocyanins in the serum extracts 1, 2, 3 and 4h after consumption of the freeze-dried berries are presented in Table 4.4. The serum concentration of total anthocyanins in the five subjects expressed as cyanidin-3-glucoside varied from 5.43 to 16.9 ng/mL. Maximum levels were observed 4h after consumption of blueberries and the anthocyanins present most abundantly were malvidin-3-galactoside, malvidin-3-glucoside, and delphinidin-3-glucoside accounting for 27, 20, and 10% of the total, respectively.

Figure 4.1 Typical Chromatograms of Blood Serum Extracts

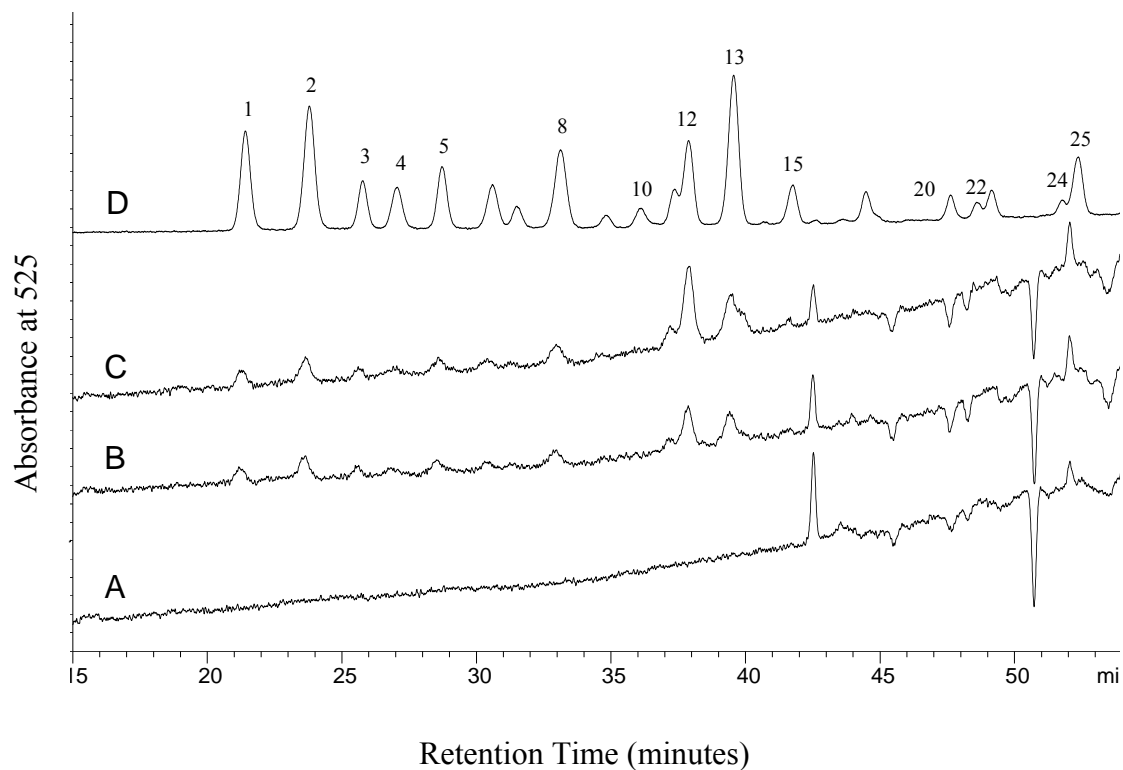


Figure 4.1 Typical chromatograms of blood serum extracts for a subject before (A), and 1 hour (B) and 3 hours (C) after diet ingestion, and blood serum spiked with blueberry diet extract (D). Peak identities : 1 = del-3-gal; 2 = del-3-glu; 3 = cy-3-gal; 4 = del-3-ara; 5 = cy-3-glu; 6 = pet-3-gal; 7 = cy-3-ara; 8 = pet-3-glu; 9 = pn-3-gal; 10 = pet-3-ara; 11 = pn-3-glu; 12 = mal-3-gal; 13 = mal-3-glu; 14 = del-3-gal-ac; 15 = mal-3-ara; 16 = cy-3-gal-ac; 17 = cy-3-ara-ac; 18 = del-3-glu-ac; 19 = pet-3-gal-ac; 20 = pn-3-gal-ac; 21 = cy-3-glu-ac; 22 = mal-3-gal-ac; 23 = pet-3-glu-ac; 24 = pn-3-glu-ac; 25 = mal-3-glu-ac.

Table 4.4 Mean Concentration^a and Standard Deviation of Anthocyanins in Blood Serum

Anthocyanin ^b	Time After Ingestion			
	1hr	2hrs	3hrs	4hrs
del-3-gal	0.49± 0.12	0.60± 0.09	0.68± 0.15	0.65± 0.12
del-3-glu	0.74± 0.21	0.98± 0.17	1.17± 0.41	1.25± 0.41
cy-3-gal	0.35± 0.00	0.39± 0.07	0.45± 0.13	0.39± 0.08
del-3-ara	0.38± 0.11	0.48± 0.22	0.64± 0.31	0.84± 0.25
cy-3-glu	0.52± 0.09	0.67± 0.16	0.91± 0.49	0.82± 0.43
pet-3-gal	0.31± 0.02	0.36± 0.08	0.42± 0.08	0.46± 0.09
cy-3-ara	0.27± 0.03	0.23± 0.03	0.29± 0.08	0.32± 0.06
pet-3-glu	0.67± 0.12	0.82± 0.12	0.98± 0.11	1.06± 0.19
pn-3-ara	0.44± 0.06	0.64± 0.13	0.83± 0.15	0.93± 0.19
mal-3-gal	1.59± 0.27	2.65± 0.73	3.30± 0.45	3.68± 0.73
mal-3-glu	1.09± 0.35	1.81± 0.51	2.44± 0.66	2.70± 0.59
Total	6.63± 1.35	9.58± 2.05	12.10± 2.82	13.09± 2.74

^a Serum concentration (ng/mL) expressed as cyanidin-3-glucosied chloride. All are means of 5 subjects, analysed in duplicate.

^b Anthocyanin identities : del = delphinidin; cy = cyanidin; pet = petunidin; pn = peonidin; mal = malvidin; glu = glucoside; gal = galactoside; ara = arabinoside.

Figure 4.2 shows the time course of changes in the concentrations of total anthocyanins (A), malvidin-3-galactoside (B), malvidin-3-glucoside (C), and delphinidin-3-glucoside (D) in human serum after consumption of lowbush blueberries. The mean concentration of total anthocyanins increased from 6.6 ng/mL at 1h post-consumption to 9.6, 12.1, and 13.1 ng/mL at 2, 3, and 4h post-intake, respectively. Considering that the average weight of the subjects used in this study was about 80kg, and that each subject consumed 1.20g of anthocyanins, the average intake of anthocyanins was 15mg/kg of body weight. Serum total anthocyanins (as cyanidin-3-

glucoside equivalents) in the subjects 3h after consumption was $12.1 \pm 2.8 \mu\text{g/L}$, or approximately $0.454 \mu\text{g/kg}$ of body weight (assuming 5 L of blood per person and 40-60% of the blood being serum). Thus, these results indicate that only 0.002-0.003 % (or 20-30 ppm) of the ingested amount of anthocyanins was present in the human serum 3h post-ingestion. This level of absorption appears to be relatively low but it is consistent to values recently reported by Matsumoto et al. (2001). Similarly, the serum anthocyanin concentrations, as reported in this study, are comparable to the plasma total concentrations of anthocyanins reported by Miyazawa et al. (1999) and Matsumoto et al. (2001). Although the time to reach maximum plasma concentration was longer than that reported by Miyazawa et al. (1999) and Matsumoto et al. (2001), this is likely a result of different dietary treatments. In this study, subjects received the anthocyanin-rich blueberry powder with a high-fat meal while in the study reported by Miyazawa et al. (1999) the subjects received a typical Japanese diet low in fat. HPLC chromatograms of the control serum samples spiked with the blueberry supplement extract (Figure 4.1D) revealed the presence of 25 anthocyanins which were identified as described by Gao and Mazza (1994, 1995). The results presented here appear to indicate the presence of the non-acylated and acetylated forms of galactosides, glucosides, and arabinosides of delphinidin, cyanidin, petunidin and malvidin. These results are in full agreement with earlier findings (Gao & Mazza, 1995). The major non-acylated anthocyanins appear to be 3-galactosides and 3-glucosides of delphinidin and malvidin (Figure 4.1D), peaks # 1, 2, 12 and 13) and the major acetylated anthocyanins were consistent with 3-acetylglucosides of delphinidin, cyanidin, petunidin and malvidin (peaks # 16, 21, 23 and 25).

Figure 4.2 Changes in Serum Concentration of Total Anthocyanins over Time

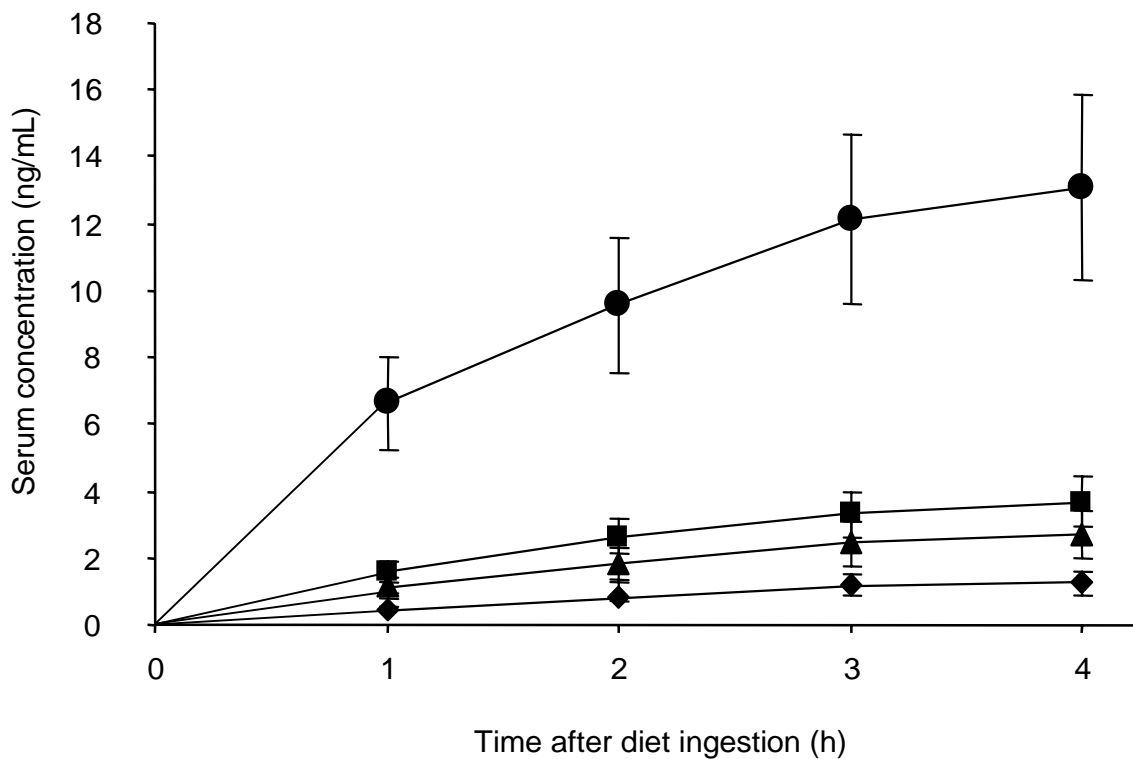


Figure 4.2 Changes in serum concentration of total anthocyanins (●), del-3-glu (■), mal-3-gal (▲) and mal-3-glu (◆). Values are the mean \pm SD of 5 subjects, and are expressed as cyanidin-3-glucoside chloride.

Table 4.5 Recovery^a of Anthocyanins from Clean-up Process

Anthocyanin ^b	RT ^c	Peak Areas		% Recovery
		Diet extract	Serum spike	
del-3-gal	21.40	21.82	17.07	78.2
del-3-glu	23.78	30.12	23.37	77.6
cy-3-gal	25.78	9.81	7.77	79.2
del-3-ara	27.05	11.05	7.93	71.7
cy-3-glu	28.73	13.17	10.54	80.1
pet-3-gal	30.61	10.37	8.34	80.4
cy-3-ara	31.52	5.10	3.91	76.7
pet-3-glu	33.14	20.27	16.38	80.8
pn-3-gal	34.85	2.39	1.98	83.1
pet-3-ara	36.13	4.90	3.70	75.5
pn-3-ara	37.37	6.67	5.36	80.3
mal-3-gal	37.89	18.33	15.10	82.4
mal-3-glu	39.56	32.89	26.70	81.2
mal-3-ara	41.75	8.13	6.29	77.4
del-3-glu-ac	44.49	7.47	4.92	65.9
cy-3-glu-ac	47.64	5.17	3.43	66.2
mal-3-gal-ac	48.64	3.37	2.35	69.9
pet-3-glu-ac	49.16	6.20	4.34	70.0
mal-3-glu-ac	52.37	14.47	12.21	84.4
Mean recovery				76.9

^aComparison of anthocyanin peak areas from diet extract before and after spiking into blood serum.

^bAnthocyanin identity as in Table 4.1; ac = acetyl.

^cComponent retention time (minutes).

A comparison of the concentration of the 19 major anthocyanins present in the methanolic extract of the freeze-dried blueberry supplement and in control serum samples spiked with the blueberry supplement extract (Table 4.5) shows that the recovery of anthocyanins from the serum sample clean-up process averaged 76.9%. Lower recoveries were obtained for acylated anthocyanins (peaks 15-19, Figure. 4.1D)

than non-acylated structures. These results indicate that, during sample preparation, some anthocyanins may be more or less effectively separated from the other blood serum components though there is a possibility that some acetylated anthocyanins may be hydrolyzed to anthocyanin glucosides and acetic acid by hydrolase in the control blood serum samples. This, however, remains to be demonstrated. Additionally, the metabolism and functions of these circulating anthocyanins as reported here in human subjects requires further investigation.

Serum Antioxidant Status

The decision to feed subjects a high-fat meal was based on the evidence that the consumption of a high-fat meal is associated with postprandial lipemia, increased oxidative stress, increased oxidation of low-density lipoprotein cholesterol, decreased nitric oxide bioactivity, and decreased endothelial response (Vogel, 1997; Starprans et al., 1999; West 2001; Kay & Holub, 2003). We therefore aimed to create a dietary stress similar to that encountered when eating a typical ‘fast-food’ meal (Vogel, 1997) in order to test the antioxidant capacity of the blueberry under such a postprandial stress.

The blueberry treatment appeared to prevent a mean decrease in serum antioxidant capacity as experienced in the control group following the consumption of the high-fat meal (as determined by TEAC analysis, treatment effect $P = 0.001$) (Table 4.6). The consumption of both of the treatment meals was associated with a significant time effect which was a result of a progressive increase in serum ORAC_{total} ($P < 0.001$) and ORAC_{acetone} ($P < 0.001$) over time (Table 4.6). Furthermore, the blueberry treatment was associated with a significant increase in serum lipid-soluble antioxidant status (ORAC_{acetone}, $P = 0.04$) above the control at the 4h time points (Table 4.6).

Table 4.6 Percent Change in Serum Antioxidant Capacity and Triacylglycerol Over Time

Measure	Time	Treatment				P value
		Control		Blueberry		
		Mean	SD	Mean	SD	
TEAC ^{a,e}	0	0.0	0.0	0.0	0.0	
	1	-6.1	8.2	0.6	4.3	0.07
	2	-4.0	3.9	-1.1	6.8	0.55
	3	-3.5 ^d	2.6	-2.7	5.4	0.84
	4	-4.3	4.0	-1.7	5.2	0.49
ORAC _{total} ^{b,f}	0	0.0	0.0	0.0	0.0	
	1	1.9	12.7	0.2	14.8	0.87
	2	15.2	17.6	11.3	7.7	0.68
	3	29.0	19.0	39.1 ^d	10.0	0.16
	4	43.5 ^d	14.1	50.5 ^d	8.9	0.06
ORAC _{pca}	0	0.0	0.0	0.0	0.0	
	1	0.2	3.8	6.5	10.7	0.24
	2	1.4	12.4	-1.3	8.5	0.79
	3	6.2	17.2	-4.1	4.4	0.23
	4	-1.5	7.1	1.9	11.2	0.36
ORAC _{acetone} ^{b,f}	0	0.0	0.0	0.0	0.0	
	1	2.1	17.9	0.6	12.8	0.88
	2	17.3	21.0	17.4	11.3	0.99
	3	40.3 ^d	13.1	48.5 ^d	11.4	0.10
	4	50.7 ^d	14.2	58.9 ^d	10.1	0.04 ^c
Triacylglycerol ^{b,f}	0	0.0	0.0	0.0	0.0	
	1	13.1 ^d	2.5	4.4	5.7	0.46
	2	26.3 ^d	15.8	21.6	11.7	0.63
	3	40.3 ^d	13.2	40.5 ^d	6.4	0.97
	4	50.6 ^d	10.8	55.2 ^d	5.3	0.44

^a Treatment effect as determined by repeated measures ANOVA (P < 0.05).

^b Time effect as determined by repeated measures ANOVA (P < 0.01).

^c Significant difference from control group as determined by paired t-test (P < 0.05).

^d Significant difference from baseline (t = 0) as determined by paired t-test (P < 0.05).

^e Significant difference at baseline (t = 0) between treatment groups as determined by paired t-test (P = 0.05).

^f Analyses sharing the same f-subscript are positively correlated (P < 0.001).

An increase from baseline in the serum antioxidant capacity using the ORAC assay ($ORAC_{total}$ and $ORAC_{acetone}$) following consumption of the high-fat meal occurred in the control group (as well as the blueberry treatment group) regardless of an essentially antioxidant-free control supplement. No such increase was found in the control group when using the TEAC assay. This dissimilarity in findings between the two assays is likely a result of mechanistic differences between the assays. The TEAC assay measures the inhibition of free radical action, which is similar to the basis of the ORAC assay; however, the TEAC assay measures the inhibition at a fixed time (10 min) whereas the ORAC assay measures the time it takes for this reaction to reach completion (≥ 60 min). Measuring the reaction to completion may allow the oxidation of fatty acids within absorbed triacylglycerol particles (TAG) (as a result of the high-fat meal) to retard the decay of the target molecule (R -PE) under these experimental conditions. The results of correlation analysis in fact reveal that the appearance of TAG in blood is correlated with increases in $ORAC_{total}$ and $ORAC_{acetone}$ fractions ($P < 0.01$). Although a correlation exists, further research is needed to determine the effect of TAG on the antioxidant assays used in this trial.

Correlation between Serum Anthocyanin Content and Postprandial Antioxidant Status

Figure 4.3 shows that there was a significant positive correlation between serum anthocyanin content and postprandial antioxidant status ($P < 0.01$). These results suggest that the compounds within the blueberry responsible for the increase in serum antioxidant status above the control group are most likely the anthocyanins.

Figure 4.3 Correlation between Serum Antioxidant Capacity and Concentration of Serum Total Anthocyanins

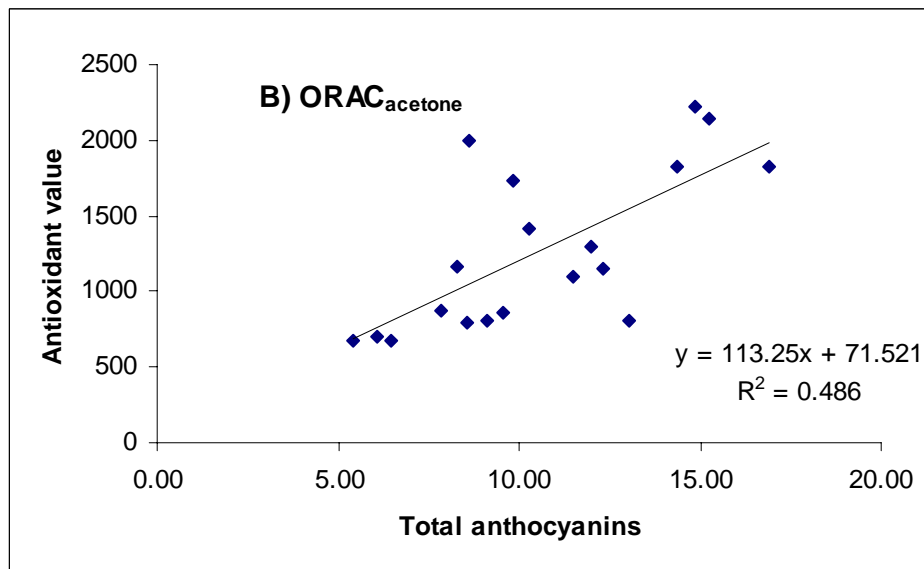
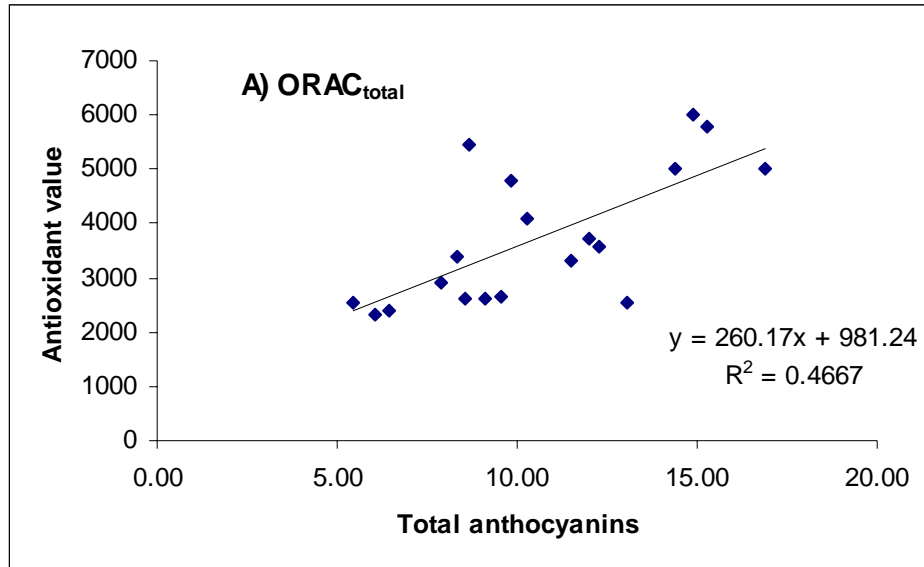


Figure 4.3 Correlation between serum antioxidant capacity and concentration of serum total anthocyanins. Antioxidant value expressed as μm Trolox equivalents/L, and anthocyanins expressed (ng/mL of serum) as cyanidin-3-glucoside chloride.

Anthocyanins comprise the highest concentration of all phenolic subgroups in the blueberry (Mazza & Miniati, 1993; Camire, 2000). Previous research has determined that the ORAC value of the blueberry correlates stronger with anthocyanins than with other phenolics in the ripe berry (Wang & Mazza, 2002). Furthermore, the antioxidant properties of anthocyanins have been validated using other systems of oxidation such as their ability to prevent LDL oxidation *in vitro* (Laplaud et al., 1997). Although we cannot exclude other phenolics within the blueberries as contributing to the observed effects in the blueberry treatment, correlational evidence indicates that the *in vitro* antioxidant properties of wild blueberries are mainly a result of their high concentration of anthocyanins and not their concentrations of antioxidant vitamins, minerals, or fibres (Bushway et al., 1983; Prior et al., 1998; Kalt et al., 1999).

We have demonstrated that supplementation with a freeze-dried wild blueberry powder prevented the decrease in serum antioxidant capacity in the postprandial state, although the magnitude of the observed response cannot be arbitrarily translated into a decreased risk of chronic degenerative disease. Studies have indicated an increased risk of chronic disorders in individuals with low levels of fasting antioxidants (Lachance 1998). However, there is insufficient data at the present time in the literature to determine the magnitude of chronic degenerative disease risk reduction with given increases in serum/plasma antioxidant status.

CONCLUSIONS

The results of this study show that anthocyanins can be absorbed in their intact glycosylated and possibly acylated forms in middle-aged men soon after the consumption of blueberries, and that the presence of anthocyanins in the serum correlates with a diet-induced increase in *ex vivo* serum antioxidant status.

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CHAPTER 5.

ANTHOCYANIN METABOLITES IN HUMAN URINE AND SERUM

ABSTRACT

In this study we have investigated the metabolic conversion of cyanidin glycosides in humans using solid phase extraction, HPLC-DAD, MS, GC, and enzymatic techniques. Volunteers consumed approximately 20g of chokeberry extract containing 1.3g of cyanidin 3-glycosides (899mg cyanidin 3-galactoside, 321mg cyanidin 3-arabinoside, 51mg cyanidin 3-xyloside, and 50mg cyanidin 3-glucoside). Blood samples were drawn at 0, 0.5, 1, 2h post-consumption of the extract. Urine samples were also collected at 0, 4-5, and 22-24h. We have confirmed that humans have the capacity to metabolize cyanidin 3-glycosides, as observed by at least 10 individual anthocyanin metabolites in the urine and serum. Average concentrations of anthocyanins and anthocyanin metabolites in the urine reached levels of 8 µg/mL within 5h post-consumption and persisted in 24h urine samples at levels of 5-6 ng/mL. In addition, average total levels of anthocyanins and anthocyanin metabolites detected in the serum were observed at 312 ng/mL within 2h post-consumption. Cyanidin 3-galactoside accounted for 56.4% (4µg) and 65.0% (202ng) of the detected anthocyanins, in the urine and serum samples, respectively. The metabolites were identified as glucuronide conjugates, as well as methylated and oxidized derivatives of cyanidin 3-galactoside and cyanidin glucuronide. Conjugation likely affects the biological activity of anthocyanins and these metabolic products are likely in

part responsible for the reported health benefits associated with the consumption of anthocyanins.

INTRODUCTION

In recent years, numerous studies have suggested that anthocyanins, present in fruit and vegetable products, are protective against many chronic degenerative diseases (Kamei et al., 1995; Laplaud et al., 1997; Andriambeloson et al., 1998; Trevithick & Mitton, 1999; Mazza, 2000; Parthasarathy et al., 2001). However, there is little reliable information on their absorption and metabolism in humans. Several investigators report that anthocyanins are transported in biological fluids exclusively as intact glycosides (Miyazawa et al., 1999; Murkovic et al., 2000; Mazza et al., 2002; Müllleder et al., 2002; Suda et al., 2002), while few have identified glucuronide or sulphide derivatives (Wu et al., 2002; Felgines et al., 2003). Adequate identification of metabolized anthocyanins must be established prior to the elucidation of their health effects. Since conjugation and derivatization likely alter the bioactive properties of anthocyanins, future *in vitro* studies should be conducted using anthocyanins in their metabolized forms, as they appear in the human body.

The aim of this study was to investigate the metabolic fate of cyanidin 3-glycosides through the identification of intact or conjugated structures in human urine and serum.

MATERIALS & METHODS

Materials/Reagents

The chokeberry extract (no. 74190000, lot L18010) was purchased from Artemis International, Inc., Fort Wayne, IN, USA. The β -glucuronidase (type-3), β -galactosidase (Aspergillus), β -glucosidase (from almonds) and sulfatase (aryl sulfatase) were purchased from Sigma (Oakville, ON, Canada). The anthocyanin standards, cyanidin 3-glucoside chloride, cyanidin 3-galactoside chloride (ideain chloride), malvidin 3-glucoside chloride (oenin chloride), peonidin 3-glucoside chloride, and pelargonidin 3-glucoside chloride (callistephin), were purchased from Extrasynthese (ZI Lyon Nord, Genay, France). The phenolic acid standards, syringic acid, vanillic acid, p-hydroxybenzoic acid, protocatechuic acid, caffeic acid, and gallic acid, were purchased from Sigma (Oakville, ON, Canada). Ferulic acid was acquired from K&K Rare & Fine Chemicals (Costa Mesa, CA, USA) and p-hydroxycinnamic acid (or trans-p-hydroxycinnamic acid) was obtained from Baker Chemical Co. (Phillipsburg, NJ, USA). The Tri-Sil Z (Pierce; Rockford, IL., USA), methanol (MeOH), chloroform (Caledon; Toronto, ON, Canada), formic acid (Fisher Scientific; Springfield, NJ, USA), oxalic acid (Baker Chemical Co.; Phillipsburg, NJ, USA), amyl alcohol, hydrochloric acid (HCL), and trifluoroacetic acid (TFA) (DH Ltd., Toronto, ON, Canada) were all reagent grade. All solvents used for HPLC analysis were HPLC grade.

Clinical Procedures

Two healthy, male volunteers, aged 27 and 54y, participated in the chokeberry consumption trial. Subjects had an average BMI of 27.5 kg/m² and were absent of clinical disease as determined using a medical history questionnaire. Subjects were instructed to consume an essentially anthocyanin-free diet for 2 days prior to the study and to avoid taking aspirin or anti-inflammatory medications, and antioxidant or herbal supplements for 2 weeks prior to the investigation. The major constituents of the anthocyanin-free wash-out diet were milk, tuna, white bread, chicken, and white rice. No caloric restrictions were imposed. The 2-day wash-out diet was performed before baseline sampling. After the overnight fast (12-14h), the volunteers consumed approximately 20g of chokeberry extract (containing 1.3g cyanidin glycosides) dissolved in 250 mL water. The extract contained 4 cyanidin 3-glycosides: 899mg cyanidin 3-galactoside, 321mg cyanidin 3-arabinoside, 51mg cyanidin 3-xyloside, and 50mg cyanidin 3-glucoside (as determined by HPLC-DAD). The chromatogram of the chokeberry extract is given in Figure 5.1B. The extract was consumed directly following a baseline blood sample (t = 0). Subsequent blood samples were taken at t = 0.5, 1, and 2h post-consumption of the extract. Blood samples were drawn by venipuncture from a brachial vein into 10 mL evacuated glass tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA). The blood samples were allowed to clot at room temperature for 25 min. Samples were then immediately centrifuged (2500 rpm, 1000 g) for 15 min at 5°C to recover the serum. Urine voids were collected in the morning of the study date (first void, t = 0) along with 4-5 and 22-24h samples. The serum and urine were stored at -70°C upon removal/collection.

Anthocyanin Extraction

Methods of extraction are modified from Tsuda et al., (1999a). Anthocyanins were extracted from biological fluids using disposable solid phase extraction (SPE) C₁₈ cartridges (Supelclean ENVI-18 6 mL 500mg; Sigma, Oakville, ON, Canada). Cartridges were pre-conditioned using 7 mL acidified MeOH (0.1% TFA, pH 2.1), followed by 7 mL acidified H₂O (10mM oxalic acid, pH 2.2). 1.5 mL of unfiltered blood serum or 1 mL of unfiltered urine was acidified (40µl 6N HCl), diluted with oxalic acid (equal volume 10mM), vortexed, and loaded directly onto the SPE cartridge. The sample was drained under gravity (~1drop/sec) and washed with 2 volumes of acidified water (10mM oxalic acid, ≈ 12mL). The remaining anthocyanin extract was eluted with 6 mL acidified MeOH (0.1% TFA). The extract was evaporated at ambient temperature in a SpeedVac Plus-SC110A condenser (Savant Instruments Inc.; Farmingdale, NY, USA) to 0.1 - 0.5 mL. The residual elute was brought to dryness under nitrogen at room temperature and the residue was redissolved in 200µl of HPLC mobile phase solution and filtered through a 13 mm 0.45 µm GHB Acrodisc syringe filter (Pall-Gelman; Ann Arbor, MI, USA; Lot # A10310523). Anthocyanins were quantified by comparison to a standard curve obtained using known concentrations of cyanidin 3-galactoside.

HPLC Analysis

HPLC analysis was performed on an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA) using a Zorbax SB C₁₈ (5µ, 4.6 x 250mm; Agilent Technologies, Palo Alto, CA, USA) reverse-phase column with an Inertsil Spherisorb guard column (C₁₈ 5µ, 4.6 x 30mm; Phenomenex, Torrance, CA, USA). The following

procedure was modified from previously published methods (Mazza et al., 2002). The column temperature was set at 35°C and injector temperature at 15°C, with an injection volume of 50 µl. The mobile phase consisted of 4.5% v/v formic acid in water (solvent A) and 100% MeOH (solvent B). The flow rate was 1.0 mL/min and the solvent gradient program used as follows; 10% B at 0 min, 25% B at 30 min, 45% B at 50 min, 100% B at 55 min maintaining 100% for 10 min. A diode array detector (DAD) (G1315B; Agilent Technologies, Palo Alto, CA, USA) monitored absorbance at 280, 360, and 525 nm. Peaks on the chromatogram corresponding to anthocyanins as identified by spectral analysis (peaks detected at 525 nm with λ_{max} 250-300, 500-550) were collected from the analytical column (Foxy 200 X-Y fraction collector, ISCO, Inc.; Lincoln, NE, USA) and concentrated at ambient temperature in a vacuum condenser (SpeedVac; Savant, Farmingdale, NY, USA) for further analysis. All water used for HPLC was 18 MΩ·cm Milli-Q water and degassed using an inline Agilent 1100 micro vacuum degasser (Agilent Technologies, Palo Alto, CA, USA).

MS Analysis

Mass Spectrometry (MS) identification of individual compounds was conducted post-separation via HPLC (as described above). Individual peaks were collected on a Foxy 200 X-Y fraction collector (ISCO, Inc.; Lincoln, NE, USA), concentrated, and analysed by electrospray ionisation mass spectrometry (ESI-MS). Micromass ZQ single quadrupole mass spectrometer with electrospray interface and MassLynx 3.5 software (Micromass UK Ltd.; Manchester, U.K.) was used for data acquisition. Mass spectrometer parameters were set as follows: Ionization mode: electrospray positive ion

mode; capillary voltage: 3.25 kV; source temperature: 130°C; desolvation temperature: 280°C; nebulizer nitrogen flow rate: 95 l/hr; desolvation nitrogen gas flow rate: 610 l/hr; LM resolution: 15; HM resolution: 15; ion energy: 0.8 V; Multiplier voltage: 650 V; Cone voltage: 20 V; RF lens: 0.5 V; extractor: 6 V. The flow rate of a built-in syringe pump was set at 20 μ l/min. For the flow injection, samples (HPLC fractions) and chokeberry extracts (2.4mg) were redissolved in 2 mL of a mixture of acetonitrile and water (50:50) containing 0.1% formic acid. Spectra were recorded by scanning a mass range from m/z 100 to 1000 with scan time of 1s, inter-scan time of 0.02s, and run duration of 0.5 min. The MS parameters were loosely based on methods previously published by Felgines et al. (2003) and García-Beneytez et al. (2003).

Enzymatic Hydrolysis

The dried anthocyanin extract obtained using the above outlined SPE procedure was subjected to enzymatic hydrolysis using β -galactosidase, β -glucuronidase, β -glucosidase, and sulfatase. The enzymatic methods were based on principles previously described by Matsumoto et al. 2001, Liu & Hu, 2002, and Mazza et al. 2004. The dried extract was mixed with 1 mL of 0.1M sodium acetate buffer containing activated enzyme. The four individual enzyme buffer solutions (700 U β -galactosidase, 500 U β -glucuronidase, 500 U β -glucosidase, 75 U sulfatase) were created by adding 1 mL of preincubated (37°C) sodium acetate buffer (pH 3.8) to the pre-weighed enzymes. The enzyme buffer solutions were then vortexed and added to the dried anthocyanin extract. The mixtures were further vortexed and incubated at 37°C for 1h. The anthocyanin enzyme extracts were then diluted with 1 mL acidified MeOH (4.5% formic acid in

methanol, pH 2.1) and centrifuged for 10 min at 14000 rpm. The supernatants were removed, evaporated under N₂ and redissolved in 150 µl mobile phase (as outlined above). If precipitate persisted, the sample was re-centrifuged (10 min at 14000 rpm) before filtration. The enzymatically hydrolyzed aglycone rich extracts were injected into the HPLC column and analyzed using the above outlined HPLC methodology.

GC Analysis, Acid Hydrolysis and Derivatization of Sugars

Chemical characterization of the glycosylating compounds was conducted using capillary gas-liquid chromatography post-acid hydrolysis. This was accomplished using methods as published by Gao & Mazza (1994), with slight modifications. Samples were silylated after concentration by adding 50 µl Tri-Sil Z, and incubated at 65°C for 1h. GC analysis of the derivatised sugars was conducted on a Hewlett Packard 5890A Gas Chromatograph with a FID detector (Hewlett Packard (now Agilent), PA, USA) using a fused silica capillary column (J&W DB-1701, 30m x 0.32 mm x 1 µm; J&W Scientific Inc.; Folsom, CA, USA) . The injector and detector temperatures were 250°C. The carrier gas was helium at a head pressure of 80 kpa, flow of 1.8 mL/min, and a linear velocity of 35 cm/sec. The sample (1µl) was injected into the column via a split/splitless injector in split mode using a split ratio of 11:1. The initial column temperature was increased from 120°C to 180°C at a rate of 20°C/min, then increased from 180°C to 200°C at a rate of 5°C/min and held for 8 min.

RESULTS

The consumption of chokeberry extract containing 4 cyanidin glycosides (899mg cyanidin 3-galactoside, 321mg cyanidin 3-arabinoside, 51mg cyanidin 3-xyloside, and 50mg cyanidin 3-glucoside) resulted in the appearance of at least 10 anthocyanin metabolites (as separated by RP-HPLC-DAD) in the human serum and urine (Figures 5.1 & 5.2). Concentrations of individual anthocyanins in the urine and serum extracts are given in Tables 5.1 and 5.2 respectively. HPLC-DAD analysis of anthocyanin standards together with their molecular weights are given in Table 5.3. The UV-vis spectral analysis of all potential metabolites is given in Figure 5.2. Initial attempts to fully characterize anthocyanin metabolites in the urine by enzymatic and chromatographic methods were unsuccessful and results of both enzymatic hydrolysis (detection of aglycone via HPLC-DAD) and acid hydrolysis (detection of derivatized sugars and glucuronic acid via capillary GC) experiments were deemed inconclusive. MS was necessary for adequate identification of urinary metabolites. Post MS analysis, the identification of anthocyanins (Table 5.1) was based on the matching of molecular weights for parent (anthocyanin) and daughter (anthocyanidin or aglycone; when obtainable) fragments, along with HPLC-DAD data (retention time, $\lambda_{\max_{\text{vis}}}$ and E_{440}/E_{\max}) with that of available standards (Table 5.3). Identification was based on metabolites isolated from pooled urine samples, while relative anthocyanin concentrations in individual subject samples were determined by HPLC-DAD analysis in relation to a cyanidin 3-galactoside standard. Results indicated that cyanidin 3-galactoside was the primary anthocyanin in the chokeberry extract accounting for 68.0% of its total anthocyanins. Cyanidin 3-galactoside was also the primary anthocyanin

identified in the urine and serum samples accounting for 54.8% (4.5 μ g) and 64.7.0% (202.2ng) of the identified anthocyanins respectively. No anthocyanins were detected in the baseline serum or urine samples. Results indicated the presence of both cyanidin 3-galactosides (m/z = 449, P2 Figure 5.1) and cyanidin glucuronides (m/z = 463, P3 Figure 5.1), along with mono- and dimethylated cyanidin galactosides (m+14, P 4&5; m+28, P9 Figure 5.1) and glucuronides (m+14, P 6&7; m+28, P10 Figure 5.1). Oxidative modification was also indicated in one peak (m/z = 493/331, P 11 Figure 5.1) by an increased mass (m+16) above the di-methyl derivative of cyanidin 3-galactoside (represented by P9 Figure 5.1, m/z = 477).

Figure 5.1 Anthocyanins in Chokeberry Extract, and Human Urine and Serum Post Consumption of Extract.

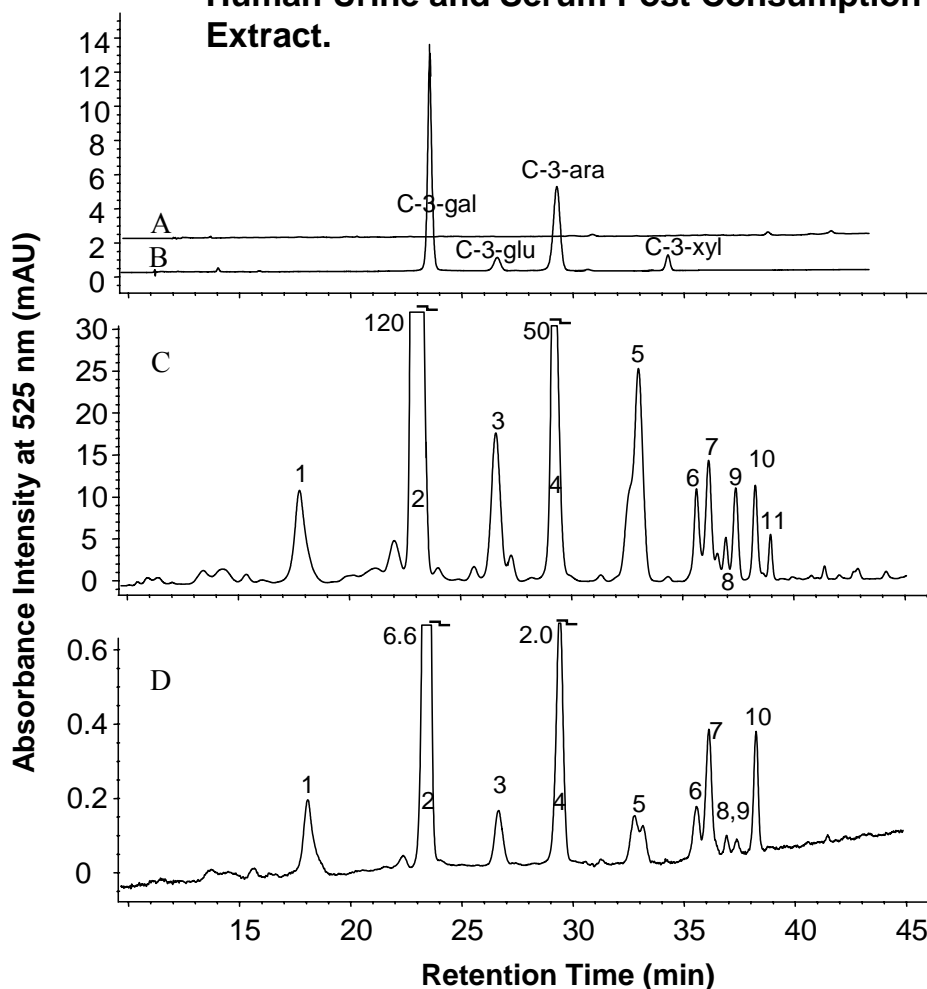


Figure 5.1 Anthocyanins in chokeberry extract, and human urine and serum post consumption of extract. (A) Chromatogram of baseline urine sample. (B) Chromatogram of chokeberry extract. (C) Chromatogram of 5h urine sample. (D) Chromatogram of 2h serum sample. Cyanidin 3-galactoside (C-3-gal), cyanidin 3-glucoside (C-3-glu), cyanidin 3-arabinoside (C-3-ara), cyanidin 3-xyloside (C-3-xyl). HPLC analysis as outlined in the methods. Identification of chokeberry anthocyanins was based on that of known standards. When standards were not available identification was based on retention times and spectral data from the literature (Chandra et al. 2001). Urine and serum concentrations of each compound represented by peaks 1 through 11 are given in Table 5.1 and Table 5.2, respectively. Urine and serum concentrations of anthocyanins were determined using UV-vis HPLC and quantified in relation to known concentrations of a cyanidin 3-galactoside standard.

Table 5.1 Identification^a and Concentration^b of Anthocyanins and Anthocyanin Metabolites in Human Urine (Mean Values with their Standard Deviations for Two Subjects)

Peak	Rt ^c (min)	λ_{\max} ^c (nm)	E ₄₄₀ / E _{max} ^c (as %)	m/z ^d (total/ aglycone)	Conjugation/ derivatization ^a	Concentration ($\mu\text{g/mL}$) ^b	
						t=5 (h) Mean	SD
1	18.1	510	39	449	Unknown	0.27	0.56
2	23.4	515	31	449	Intact C-3-gal	4.46	1.71
3	26.5	516	24	463	Gluc	0.36	0.55
4	29.3	517	37	463/301	CH ₃	1.26	0.35
5	32.9	515	36	463/301	CH ₃	0.85	0.18
6	35.5	516	33	447	Gluc + CH ₃	0.15	0.07
7	36.0	514	40	447	Gluc + CH ₃	0.16	0.19
8	36.8	516	38	Unknown	Unknown	0.12	0.00
9	37.7	517	30	447	2 x CH ₃	0.22	0.04
10	38.5	520	30	491	Gluc + 2 x CH ₃	0.21	0.22
11	39.0	518	31	493/331	2 x CH ₃ + OH	0.07	0.01
Total						8.14	1.28

Glucuronic acid (Gluc), cyanidin 3-galactoside (C-3-gal).

^aIdentification based on relative polarity (retention time), MS, and spectral data (as outlined in the methods) using standards of known composition when available. For spectral characteristics of anthocyanin standards, see Table 5.3.

^bConcentrations ($\mu\text{g/mL}$) are means of two subjects determined using UV-vis HPLC and quantified in relation to known concentrations of a cyanidin 3-galactoside standard. No anthocyanins were detected in baseline (t=0) samples.

^cRetention times (Rt) and spectral data obtained by HPLC as outlined in the methods. For HPLC chromatographic plot of individual peaks, see Figure 5.1

^dMolecular weights obtained by MS (as outlined in the methods).

Table 5.2 Concentrations^a of Identifiable^b Anthocyanins in Human Serum (Mean Values with their Standard Deviations for Two Subjects)

Peak ^b	Rt ^c (min)	Concentration (ng/mL) ^a								Total/ peak (Mean)
		t=0 (h)		t=0.5 (h)		t=1 (h)		t=2 (h)		
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
1	18.1	0.00	0.00	0.00	0.89	1.27	4.56	0.53	5.46	
2	23.4	0.00	17.84	16.93	58.02	63.07	126.38	54.23	202.24	
3	26.5	0.00	1.87	0.85	4.50	2.38	7.61	1.44	13.98	
4	29.3	0.00	6.45	6.23	19.04	20.77	38.67	16.33	64.16	
5	32.9	0.00	0.00	0.00	2.66	2.62	5.64	4.84	8.29	
6	35.5	0.00	0.00	0.00	0.79	1.11	0.90	1.27	1.69	
7	36.0	0.00	0.00	0.00	1.86	2.64	1.95	1.30	3.82	
8	36.8	0.00	0.00	0.00	0.00	0.00	1.08	0.24	1.08	
9	37.7	0.00	0.00	0.00	0.00	0.00	1.06	0.14	1.06	
10	38.5	0.00	0.99	1.40	3.27	3.11	6.38	2.50	10.64	
Total		0.00	27.15	6.92	91.03	23.31	194.23	40.36	312.41	

^aConcentrations (ng/mL) are means of two subjects determined using UV-vis HPLC and quantified in relation to known concentrations of a cyanidin 3-galactoside standard.

^bIndividual peaks identified as anthocyanins by analysis of UV-vis spectrum having maxima in the 250-300 and 500-550 nm range.

^cRetention times (Rt) obtained by HPLC as outlined in the methods.

Table 5.3 UV-vis HPLC Characteristics of Anthocyanin Standards

Peak	Rt ^a (min)	Absorption Spectra		Molecular Weight (m/z)	
		λ_{\max} (vis) (nm)	E ₄₄₀ /E _{max} (as %)	Glycoside	Aglycone
Cyanidin-3-galactoside	24.1	518	31.7	449	287
Cyanidin-3-glucoside	26.6	517	32.5	449	287
Cyanidin-3-arabinoside ^b	29.2	517	30.7	419	287
Pelargonidin-3-glucoside	32.3	502	44.0	433	271
Peonidin-3-glucoside	36.0	518	32.6	463	301
Malvidin-3-glucoside	38.5	528	27.5	493	331
Cyanidin-3-xyloside ^b	38.9	517	43.6	419	287
Cyanidin	43.0	526	23.5		287

^aRetention times (Rt) and spectral data obtained by HPLC (as outlined in the methods) using standards of known composition when available.

^bIdentification of cyanidin 3-arabinoside and cyanidin 3-xyloside in the chokeberry extract was based on retention times and spectral data (as standards were not available) and was confirmed in the literature (Chandra et al., 2001).

Figure 5.2 UV-Absorption Spectra of Anthocyanins in Human Urine and Serum

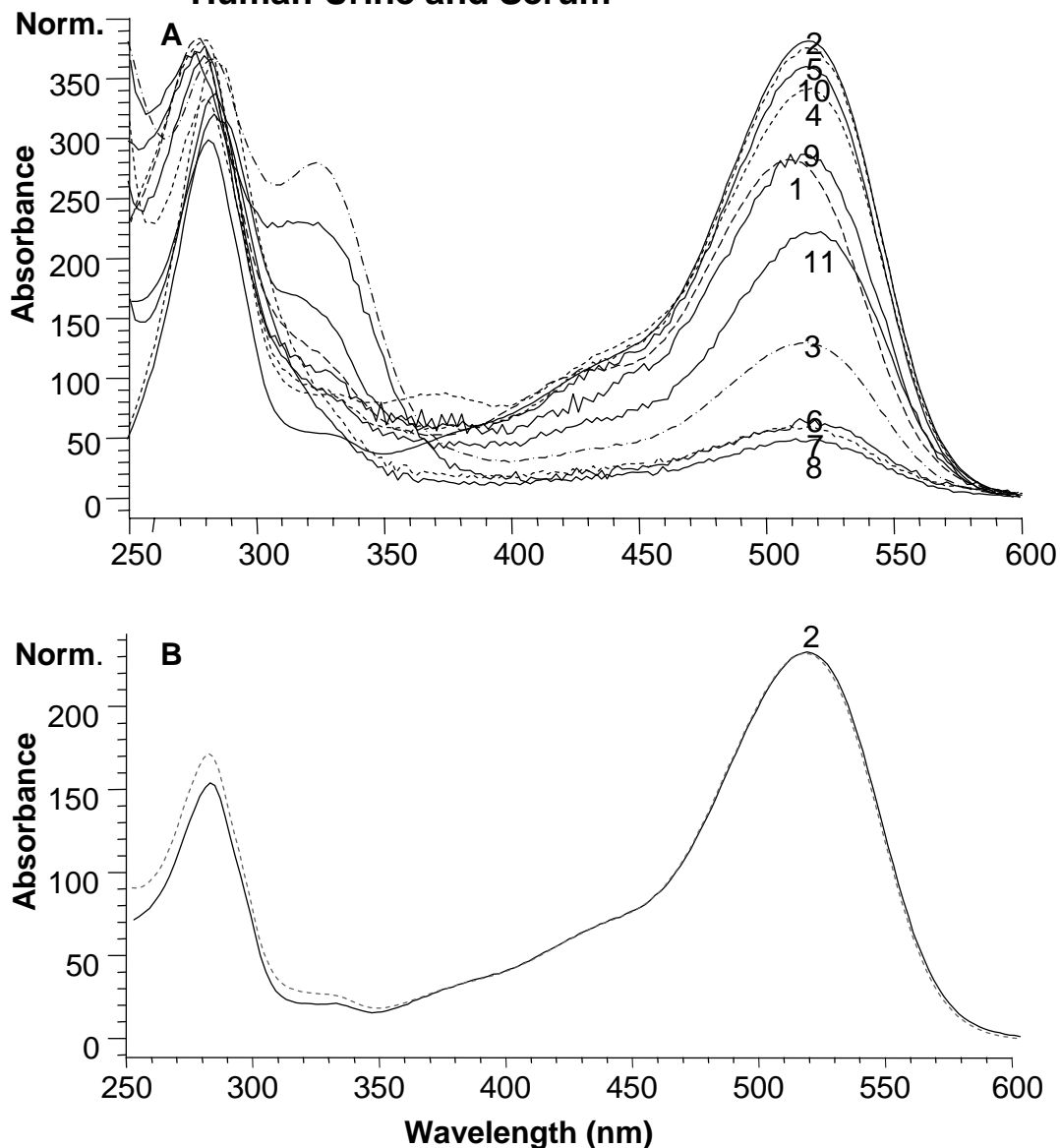


Figure 5.2 UV-absorption spectra of anthocyanins in human urine and serum samples post consumption of chokeberry extract monitored at 525nm by HPLC-DAD. (A) Typical UV-absorption spectra of individual peaks in human urine and serum samples post consumption of the extract. (B) UV-absorption spectra of peak 2 (—) and cyanidin 3-galactoside standard (---). HPLC chromatogram of urine and serum samples given in Figure 5.1. Urine and serum concentrations of each compound represented by peaks 1 through 11 are given in Table 5.1 and Table 5.2, respectively. Urine and serum concentrations of anthocyanins were determined using HPLC-DAD and quantified in relation to known concentrations of a cyanidin 3-galactoside standard.

The HPLC quantitative analysis of the individual anthocyanin peaks in the urine (pooled samples) revealed that cyanidin 3-galactoside and derivatives of cyanidin 3-galactoside accounted for an average of 84.3% (6.9 μ g) of the identified metabolites while cyanidin glucuronide and glucuronide derivatives accounted for 10.8% (0.88 μ g); with the remaining 4.8% or 0.39 μ g uncharacterized (peaks 1&8; Figure 5.1 C&D). In addition to the urinary analysis of metabolites, comparisons were made between urinary and serum metabolites using HPLC-DAD data (Figures 5.1 & 5.2, and Tables 5.1 & 5.2). Results indicated that the serum peaks matched (both retention time and UV-vis spectrum) the peaks identified in the urine with the exception of one compound (P11) that was observed in the urine but did not appear in the serum (Figure 5.1 C&D). Cyanidin 3-galactoside and derivatives of cyanidin galactoside identified in the serum accounted for 88.3% (275.7ng) of the metabolites while glucuronide and glucuronide derivatives accounted for 9.6% (31.2ng); with the remaining 2.1% or 6.5ng uncharacterized (peaks 1&8; Figure 5.1 C&D).

DISCUSSION

The purpose of this work was to identify potential metabolites of cyanidin 3-glycosides in human urine and serum. To date there is little reliable information on the absorption and metabolism of anthocyanins in humans, and the studies available have reported contradictory results. Results of this investigation indicate that anthocyanins are metabolized prior to entry into the systemic circulation. It is therefore likely that their

metabolites will be responsible for many of the reported health effects associated with anthocyanin consumption.

The chokeberry extract utilized in this trial was chosen because it contained only one anthocyanidin species, cyanidin. Most other fruit extracts contain two or more anthocyanidins making it difficult to identify the origin of any one metabolic by-product. Unfortunately, the highly concentrated chokeberry extract contained a high percentage of phenolics/polyphenolics. As a result, it was very astringent and its palatability (when dispersed in water) was low. Consequently, incomplete subject compliance made it difficult to establish the exact concentration of the supplement consumed by the 2 subjects. For this reason, no attempt has been made to establish the percentage of anthocyanins appearing in the urine and serum relative to the initial dose (bioavailability). Future studies will establish bioavailability using an encapsulated extract or a product more similar in composition to the intact fruit. The aim of this study was simply to consume a high enough concentration of cyanidin glycosides to identify their possible metabolites in biological fluids and to elicit a potential metabolic pathway for anthocyanin biotransformation in humans. It is important to note that the structural derivatives of anthocyanins proposed in this study result from the consumption of a high dose of anthocyanins (1.3g). The metabolic route under these circumstances may differ from the route following the ingestion of a more 'typical' dose of anthocyanins, as would be encountered with high fruit and vegetable consumption or with moderate to high wine consumption.

The consumption of 4 cyanidin glycosides (cyanidin 3-galactoside, cyanidin 3-arabinoside, cyanidin 3-xyloside, and cyanidin 3-glucoside) resulted in the appearance

of at least 10 individual anthocyanin metabolites in the human urine and serum (Figures 5.1 & 5.2). To our knowledge, no other study has identified this number of anthocyanin metabolites in the urine or serum to date. Findings from studies using similar concentrations of total anthocyanins but reporting fewer metabolites are likely the result of individual metabolite concentrations being below the detection limit of the methodologies used. In our study, only cyanidin was consumed, with the majority (68%) attributed to one cyanidin glycoside (cyanidin 3-galactoside), therefore, resulting in fewer numbers and higher concentrations of individual metabolites. In the present investigation, the total concentration of identifiable anthocyanins in the urine reached an average of 8.1 $\mu\text{g/mL}$ within 5h post-consumption of the chokeberry extract (Table 5.1). 22-24h urine samples showed cyanidin 3-galactoside and metabolized derivatives of cyanidin 3-galactoside to persist in the urine at levels of 5-6 ng/mL. The identification of anthocyanin metabolites in 24h urine samples has also recently been noted by Felgines et al. (2003), and may signify the potential for minor tissue accumulation. Additionally, the concentrations of identifiable anthocyanins and anthocyanin metabolites in the serum (2h sample) were observed at a level of 194.2 ng/mL within 2h post-consumption, with a cumulative total (0-2h) serum concentration reaching 312.4 ng/mL over the 2h sampling period (Table 5.2). Magnification of the chromatograms revealed the appearance of many small peaks which were at concentrations too low to adequately identify but were well above any baseline noise. Adequate structural identification was not possible at this concentration but many peaks had spectral characteristics representative of anthocyanins ($\lambda_{\text{max}_{\text{vis}}}$ in the 500⁺ nm range). Analysis of the total peak areas at 525 nm (all integratable peaks with $\lambda_{\text{max}_{\text{vis}}} > 500$ not observed in baseline samples) revealed that

the urine total concentration of anthocyanins and/or 'anthocyanin-like' compounds reached levels as high as 10.2 $\mu\text{g/mL}$ within 5h post-consumption, while total (0-2h) serum levels reached 447.9 ng/mL within 2h post-consumption of the extract (concentration of unknown compounds based on cyanidin 3-galactoside molar equivalents). These results indicate that the body may have the capacity to transform anthocyanins into numerous metabolites, many of which likely go undetected as a result of their substantial numbers and subsequently low concentrations.

Efforts to identify the main anthocyanin metabolites through enzymatic hydrolysis experiments were unsuccessful, as the enzymes β -galactosidase, β -glucuronidase, and β -glucosidase had affinities for all anthocyanin metabolite peaks to varying extents. This led us to suspect the presence of both glycoside and glucuronide derivatives of cyanidin in our samples. GC analysis, post-acid hydrolysis and derivatization of sugars, was also utilized to identify the possible glycosylating structures (galactose, glucose, arabinose, xylose, glucuronic acid). These results suggested both glycosides and glucuronides were present. However, as a result of the low concentrations and large numbers of derivatized sugars produced, adequate chromatographic identification was not possible. Further analysis using ESI-MS was necessary for sufficient identification of the potential metabolites.

Identification of urinary metabolites (Table 5.1) was based on the matching of molecular weights for parent and daughter fragments, along with the retention time, $\lambda_{\text{max}_{\text{vis}}}$ and E_{440}/E_{max} , to that of available standards (Table 5.3 & Figure 5.2). The data obtained indicated the presence of both cyanidin 3-galactosides ($m/e = 449$, P2 Figure 5.1) and cyanidin glucuronides ($m/e = 463$, P3 Figure 5.1). Although there is little data

regarding the identification of anthocyanin glucuronides to date (Wu et al., 2002; Felgines et al., 2003), the urinary excretion of other flavonoid and isoflavonoid glucuronides such as catechin, quercetin, & genisten has been well documented (Wermeille et al., 1983; Piskula & Terao, 1998; Hollman & Katan, 1998a,b; Holder et al., 1999; Okushio et al., 1999; Walle et al., 2000; Williamson et al., 2000; Oliveria et al., 2002).

Mono- and dimethylated cyanidin 3-galactoside-derivatives (m+14, P 4&5; m+28, P9 Figure 5.1) and glucuronide derivatives (m+14, P 6&7; m+28, P10 Figure 5.1) were also indicated in this investigation (Table 5.1). Monomethylated derivatives had similar retention times and E_{440}/E_{max} to the peonidin 3-glucoside standard. While only a few researchers have reported methylated anthocyanins in the urine and blood of humans and animals (Miyazawa et al., 1999; Tsuda et al., 1999a), methylated derivatives of quercetin and catechin have been documented extensively (Harborne, 1958; Wermeille et al., 1983; Hollman & Katan, 1998a,b; Miyazawa et al., 1999; Okushio et al., 1999; Day & Williamson, 2001; Donovan et al., 2001). In addition, oxidative modification was also indicated in one metabolite (peak 11; m/z 493) by an increased mass (m+16) above the di-methyl derivative of cyanidin 3-galactoside (represented by P9 Figure 5.1, m/z = 477). This structure (m/z 493/331) matched the molecular weights for parent and daughter fragments of malvidin 3-galactoside, had a similar retention time to that of the malvidin 3-glucoside standard but differed in spectral characteristics (Tables 5.1 & 5.3). No other oxidized derivatives of anthocyanins have been previously identified to our knowledge. Comparisons were made between anthocyanin metabolites in the serum and those in the urine; unfortunately, as a result of low concentrations of individual

anthocyanin metabolites in the serum, collection and concentration of a sufficient quantity of sample was not possible. Consequently, MS analysis using the above methods could not be utilized. However, retention times and UV-vis spectra were obtainable and the data indicated that the serum peaks matched the peaks identified in the urine (Table 5.2 & Figure 5.1). When comparisons were made between urinary and serum metabolites, it was apparent that one anthocyanin metabolite (P11) occurred in the urine but not in the serum (Figure 5.1C&D). The identification of an anthocyanin metabolite exclusively in the urine may indicate that this metabolic product is either, formed exclusively in the kidney, accumulates in the kidney, or the concentration of this metabolite in the serum may have been below the detection limit of our methodology. MS analysis (of the urinary metabolite) revealed this compound (P11) to be a highly metabolized cyanidin 3-galactoside (indicated by 2 methylations and 1 hydroxylation) having the same mass and daughter fragment as malvidin 3-galactoside (m/z 449/331). Tsuda et al. (1999a) has previously reported the methylation of cyanidin 3-glucoside at both the 3' and 4' positions in the liver of rats. Also, the addition of hydroxyl groups to flavones has been characterized in animal models, where these metabolites were identified in the urine (Buset & Scheline, 1980; Hollman & Katan, 1998a). In theory, dimethylation of the B-ring, resulting in reduced polarity, could merit further phase I cyt-P450 oxidation, as observed with other flavonoids (Griffiths, 1982), in an attempt to increase the water-solubility of the structure for elimination in the urine. This may explain the appearance of this compound in the urine but not in the serum. The parent aglycone cyanidin was not identified in any urine or serum samples. This result is consistent with other studies on anthocyanin metabolism as anthocyanin aglycones are

generally regarded as unstable at physiological pH (Tsuda et al., 1999a; Wu et al., 2002; Felgines et al., 2003).

Compounds having identical molecular weights but different retention times and absorption spectra were observed and are likely the result of methylation of the ortho hydroxyls of cyanidins B-ring (i.e., 3' versus 4' methylation; peaks 4&5, 6&7 Figure 5.1). Methylation at different sites of the B-ring may cause a slight shift in retention time, producing 2 distinct peaks on a HPLC chromatogram. It should be noted that the methylation pattern we have proposed, only accounts for derivatization of the B-ring, which has been documented as a likely site for methylation and oxidation of flavonoids (Griffiths, 1982; Hollman & Katan, 1998a; Tsuda et al., 1999a; Doostdar et al., 2000). However, methylation and hydroxylation at other sites of cyanidin may be possible and cannot be excluded using the above methodologies. In addition, the sites of anthocyanin glucuronide conjugation in humans are unknown. The glucuronidation of quercetin, a flavonoid of similar structure to anthocyanins, has been documented at the 4', 3', 7, and 3 positions of the polyphenol ring (Day et al., 2000b). The absence of available standards for anthocyanin metabolites, along with the problems associated with compound identification when sample impurities exist prevents the undeniable identification of the metabolites. NMR is required to elicit the actual positioning of the glucuronide, hydroxyl, or methyl subgroups.

Total urinary analysis of identified peaks revealed that cyanidin galactosides (cyanidin 3-galactoside and derivatives of cyanidin 3-galactoside) accounted for 84.3% (6.7 μ g) of the identified anthocyanins, with 54.8% (4.5 μ g) being the parent compound C-3-gal (10.8% or 0.88 μ g glucuronides; Table 5.1). Accordingly, cyanidin galactosides

accounted for 88.3% (275.7ng) of the anthocyanins (9.6% or 30.1ng glucuronides; Table 5.1) in the serum, with 64.7% (202.2ng) being the parent compound C-3-gal. The high percentage of anthocyanin glycosides in relation to glucuronides as observed in this trial has also been reported by Wu et al., (2002) (90% anthocyanin glycosides) but is not consistent with other flavonoids as reported in the literature. Most studies of flavonoid glycoside consumption indicate that the major metabolites in the urine are the glucuronide derivatives of the parent compound (Piskula & Terao, 1998; Holder et al., 1999; Kuhnle et al., 2000; Donovan et al., 2001 Oliveria et al., 2002; Felgines et al., 2003). Felgines et al., (2003) recently reported that approximately 80% of excreted anthocyanins were monoglucuronides. The high percentage of glycoside observed in this trial may be the result of the consumption of a high dose of anthocyanins and may not occur under more normal physiological concentrations.

For a HPLC chromatographic plot of individual peaks, refer to Figure 5.1; and for MS and for HPLC identification of individual peaks, see Table 5.1. No anthocyanins were detected in baseline (t =0) samples. Total analysis of identified peaks revealed that methyl derivatives of cyanidin galactosides and glucuronides accounted for 37% and 33% of the identified metabolites in the urine and serum, respectively. Although a high percentage of metabolites were methylated in this investigation, the level of methylation, as well as oxidation, observed may have been, as suggested above, the result of the high dose of anthocyanins consumed.

We found no evidence of sulfation in this study; however, studies indicate this pathway can be easily saturated (Dutton, 1980; Laitinen & Watkins, 1986; Williamson et al., 2000; Oliveria et al., 2002). As the sulfation pathway is substrate limited, it is

therefore possible that this pathway was overwhelmed by the high concentration of anthocyanin glycosides in this investigation, and any sulfate derivatives appearing in the urine or blood may have been at a concentration below the detection limit of our methodology. It is also possible that metabolites of lower concentration had similar retention times (overlapping peaks on chromatogram) to those of more concentrated metabolites and were not identified and collected. This illustration could also be used to explain why no arabinosides, glucosides, or xylosides were identified in the urine and serum. Alternatively, the route of absorption for anthocyanin glycosides may have specificity towards galactosides over arabinosides or xylosides (Williamson et al., 2000).

Researchers have reported that many flavonoid glycosides are cleaved before entry into intestinal cells where they are either transported to the liver as aglycones, or metabolized to glucuronide and/or sulfide conjugates before transport (Hollman & Katan, 1998a,b; Spencer et al., 1999; Kuhnle et al., 2000; Williamson et al., 2000; Donovan et al., 2001; Oliveria et al., 2002). In this investigation, both glycoside and glucuronides were identified in the urine and serum. This evidence leads us to believe that there may be more than one route of absorption. The combination of our results and others in the literature seems to indicate that a dose dependent or saturatable metabolic pathway may predominate (Mizuma et al., 1994; Hollman et al., 1995; Wolfram et al., 1995). This could explain the inconsistency in findings reported between studies using varying doses of anthocyanins/flavonoids.

The metabolism of anthocyanins (particularly by colonic microflora) may also result in the formation of phenolic acids, phenolic acid residues, H₂, or CO₂ (Rozman, 1986; Hollman & Katan, 1998a). Tsuda et al., (1999a) reported that cyanidin 3-

glycosides administered to rats were metabolized to protocatechuic acid. In our investigation, protocatechuic acid was observed in trace amounts in both the urine (μg concentrations) and serum (ng concentrations) samples; however, the source of the protocatechuic acid could not be determined and results have therefore been excluded from this report. In addition, colonic microflora have significant potential to transform flavonoids into lower molecular weight compounds, which may also have protective biological activities. Future research in this area is essential to establish a more complete understanding of flavonoid metabolism.

CONCLUSION

The above results indicate that orally administered cyanidin 3-glycosides are absorbed and are transported in human serum and urine as glycosides and glucuronides, both of which appear to undergo further methylation and oxidation. The contribution of each individual structure to the reported bioactivity (health effects) of anthocyanins should be the focus of future research.

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CHAPTER 6.

THE METABOLISM & PHARMACOKINETICS OF CYANIDIN 3- GLYCOSIDES IN HUMANS

ABSTRACT

Despite numerous reports of the biological activities of anthocyanins, their absorption and metabolism in humans is still poorly understood. Evidence presented in this manuscript details the pharmacokinetic parameters of anthocyanins after the administration of a 721mg oral dose of cyanidin 3-glycosides to human subjects. Solid-phase extraction (SPE), preparative high pressure liquid chromatography (prep-HPLC), preparative thin layer chromatography (prep-TLC), HPLC-diode array detection (HPLC-DAD), HPLC-mass spectroscopy (HPLC-MS), and nuclear magnetic resonance (NMR) were utilized to isolate, identify, and quantify anthocyanins in 0-7h serum and 0-24h urine samples. As well as parent cyanidin 3-glycosides, glucuronidated and methylated derivatives were identified in the serum and urine samples. The total cumulative concentration of anthocyanins (parent and metabolites) detected in the serum over the 7h sampling regime was $172.96 \pm 7.44 \mu\text{g}\cdot\text{h}/\text{mL}$, with a maximum concentration of $44.86 \pm 2.82 \mu\text{g}/\text{mL}$ (C_{max}) occurring within 2.8h (t_{max}). Only 32.7% ($52.54 \mu\text{g}\cdot\text{h}/\text{mL}$) of the total anthocyanins detected in the serum were the parent cyanidin 3-glycosides with an average of 67.3% ($120.42 \mu\text{g}\cdot\text{h}/\text{mL}$) identified as conjugated metabolites. Additionally, the total urinary excretion of metabolites and parent compounds over 24h was $1071.54 \pm 375.46 \mu\text{g}$, reaching a maximal rate of excretion (R_{max}) of $202.74 \pm 85.06 \mu\text{g}/\text{h}$ at 3.72

± 0.83 h (t_{max}) and having an elimination half-life ($t_{1/2}$) of 4.12 ± 0.4 h. Correspondingly, only 32.5% (347.85 μ g) of the anthocyanins excreted in the urine were the parent compounds with an average of 67.5% (723.69 μ g) occurring as conjugated metabolites. Although, the absorption and elimination of parent anthocyanins appears relatively low compared to the initial dose (0.048%; total urinary recovery over 24h), glucuronidated and methylated anthocyanin metabolites were observed in the present investigation at levels more than twice that of the parent (intact) compounds (0.10% of initial dose).

INTRODUCTION

Within the last decade, many studies have focused on the potential biological activities or health effects of anthocyanins in humans (Bohm et al., 1998; Kong et al., 2003; Galvano et al., 2004). Although there is a great deal of evidence indicating the bioactivity of anthocyanins, very little progress has been made in establishing the pharmacokinetics of these compounds, with aspects such as absorption and metabolism left essentially unstudied. Previously it was assumed that anthocyanins were poorly absorbed and circulated in the blood exclusively as un-metabolized parent glycosides (Miyazawa et al., 1999; Cao et al., 2001; Matsumoto et al., 2001). It is only recently that researchers have begun to suggest that anthocyanins are metabolized; however, the identification of derived metabolites has been limited as a result of their diversity and low concentrations in the blood. The aim of the present investigation was to determine the pharmacokinetics of parent anthocyanins in humans as well as to establish the extent of their metabolic fate.

MATERIALS & METHODS

Materials/Reagents

The chokeberry extract (no. 74190, lot L18010) was purchased from Artemis International, Inc., Fort Wayne, IN. The anthocyanin standards, cyanidin 3-glucoside chloride, cyanidin 3-galactoside chloride (ideain chloride), peonidin 3-glucoside chloride, cyanidin chloride and peonidin chloride were purchased from Extrasynthese (Genay, France). Formic acid (Fisher Scientific; NJ, USA), hydrochloric acid (HCL), trifluoroacetic acid (TFA), and glacial acetic acid (DH Ltd., Toronto, ON, Canada) were all reagent grade and all solvents used for HPLC analysis were HPLC grade.

Clinical Procedures

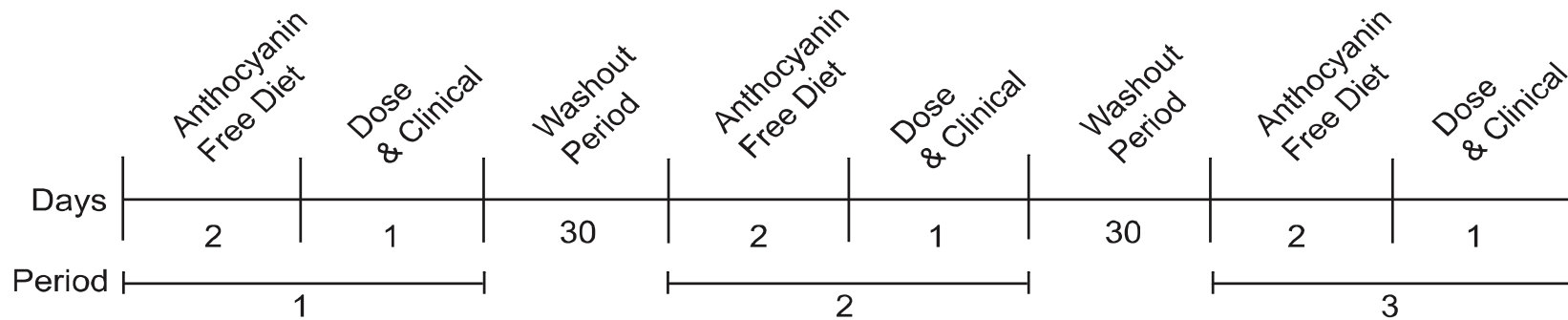
Three healthy male volunteers aged 40 ± 14.2 years participated in the cyanidin 3-glycoside consumption intervention. All subjects gave informed written consent prior to the commencement of the investigation. Subjects had an average BMI of 28.3 ± 1.6 kg/m² and were absent of clinical disease as determined using a medical history questionnaire. Subjects were instructed to consume an essentially anthocyanin-free diet (no fruit or vegetables, including foods coloured with red or blue dyes) for two days prior to the study and to avoid taking aspirin or anti-inflammatory medications, and antioxidant or herbal supplements for two weeks prior to the investigation. The major constituents of the anthocyanin-free wash-out diet were milk, tuna, white bread, chicken, and white rice. Compliance with the anthocyanin-free diet was monitored using food diaries and confirmed in baseline samples via RP-HPLC with diode array detection (RP-

HPLC-DAD). The diets met recommended dietary allowances for macro nutrients and no caloric restrictions were imposed.

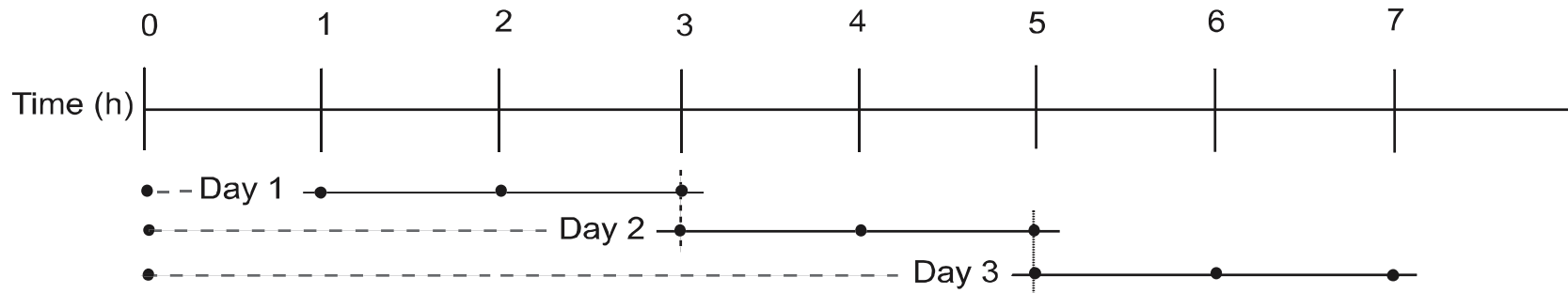
Subjects were admitted to the clinic (Okanagan Clinical Laboratory; Penticton, BC) on the morning of the study dates in a fasted state (12h, 24h no alcohol). Baseline urine samples (first void, $t = 0$) were taken in the morning of each study date along with individual urine voids over the next 24h. Immediately following baseline ($t = 0$) blood samples the volunteers consumed 7.1g of encapsulated (gel caps) chokeberry extract with 250 mL of water. The extract contained 4 cyanidin 3-glycosides (721.4mg): 491.0mg cyanidin 3-galactoside (C-3-gal), 175.3mg cyanidin 3-arabinoside (C-3-ara), 27.8mg cyanidin 3-xyloside (C-3-xyl), and 27.3mg cyanidin 3-glucoside (C-3-glu), as determined by HPLC-DAD. Subsequent blood samples were taken at $t = 1, 2, 3$ h post-consumption of the extract. The experiment was repeated at a later date (30d wash-out) following the protocol outlined above with one variation. On the second day, blood samples were taken at $t = 0, 3, 4, 5$ h and on the third day at $t = 0, 5, 6, 7$ h. Diagrams of the dosing schedule and extraction profile are given in Figures 6.1A and B respectively. The sampling regime was necessary to acquire the volume of blood needed for analysis. Following the consumption of the extract, subjects were instructed to consume 250 mL of water every h for 5h with subsequent adlibitum consumption. An anthocyanin-free lunch and dinner was provided for the subjects at 4h and 8h post-consumption of the extract.

Figure 6.1 Clinical Procedures

A. Study repetition profile



B. Blood sample regime



• Blood Sample.

All study dates included a baseline (t=0) blood and urine sample.

Individual urine voids for 24h were collected for each subject on each repetition.

The blood samples (approx. 20 mL) were drawn by venipuncture from a brachial vein into 10 mL evacuated glass tubes (2 tubes per time point) (Vacutainer; Becton Dickinson, NJ, USA). The blood samples were allowed to clot at room temperature for 25-30 min. Samples were then immediately centrifuged (2500 rpm, 1000 g) for 15 min at 5°C to recover the serum. Urine samples were acidified with 20 ul/mL 12N HCL upon collection. The serum and urine were stored at –80°C upon removal/collection.

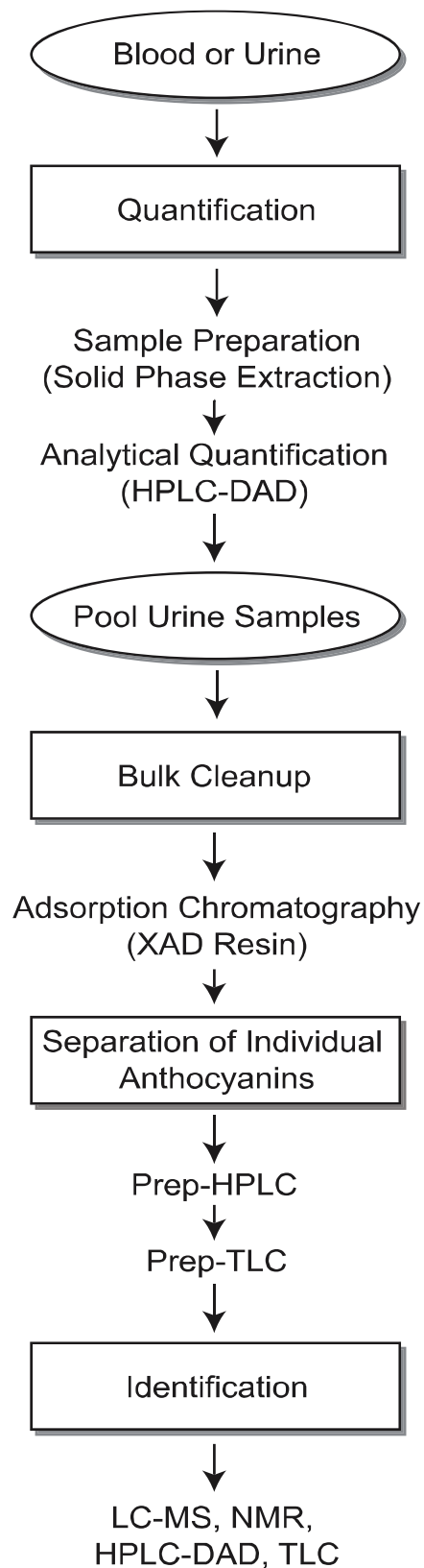
Anthocyanin Extraction

A flow chart of the analytical methods used is illustrated in Figure 6.2.

C₁₈ solid phase extraction. Methods of extraction are modified from Tsuda et al., (1999a). Anthocyanins were extracted from biological fluids prior to HPLC analysis using disposable solid phase extraction C₁₈ cartridges (Supelclean ENVI-18 6 mL 2000mg; Sigma, Oakville, ON, Canada; lot # SP2419C). Cartridges were pre-conditioned using 12 mL acidified MeOH (0.1% TFA, pH 2.1), followed by 12 mL acidified H₂O (0.1% TFA, pH 2.2). Four mL of unfiltered blood serum or 2-10 mL of unfiltered acidified urine (2 mL t = 0-9h or 10 mL t = 10-24h), were diluted with 0.1% TFA in H₂O (v/v), vortexed, and loaded directly onto the SPE cartridge. The sample was drained under gravity (~1 drop/sec) and washed with 20 mL acidified water (0.1% TFA). The remaining anthocyanin extract was eluted with 10 mL of acidified methanol (MeOH) (0.1% TFA). The extract was evaporated at ambient temperature in a SpeedVac Plus-SC110A condenser (Savant, NY, USA) to 0.1 - 0.5 mL. The residual elute was brought to dryness under nitrogen gas at room temperature and the residue was

redissolved in 100-200 μ l (100 μ l for serum 200 μ l for urine) of the HPLC mobile phase solution (90% HCOOH : 10% MeOH) for HPLC analysis. Each blood or urine sample (non-pooled) was extracted in duplicate, and each extract was injected into the HPLC column in duplicate (total of 4 injections per sample) for quantitative HPLC analysis.

Figure 6.2 Analytical Procedures



Amberlite XAD-7 adsorption chromatography. Purification of anthocyanins in pooled human urine samples (post quantitative HPLC analysis) was performed using Amberlite XAD-7 nonionic polymeric adsorbent (Sigma, MO, USA; Lot # 77H0157) prior to isolation of individual anthocyanin/metabolite peaks using Prep-HPLC. The procedure was based on general methods as described by Markham, (1982). The column (50 x 3.0 cm) was filled with pre-soaked (24h EtOH:H₂O v/v) XAD-7 resin to a volume of 212 cm³. The column was conditioned by washing with 500 mL of 100% acetone followed by 500 mL H₂O. The column was then back flushed with H₂O for 60 min (2 mL/min) and allowed to settle to ensure a consistent sorbent bed void of air bubbles. One L of unfiltered acidified urine (pH ≈ 2.5) was then loaded onto the column using a separatory funnel. The column was drained under gravity using a flow rate of 5 mL/min. The column was then washed with 500 mL acidified H₂O (0.1% TFA) at a rate of 10 mL/min. The column was next washed with 500 mL MeOH:H₂O (30:70) containing 0.1% TFA at a rate of 10 mL/min. Finally, the anthocyanin extract was eluted with 500 mL of MeOH:H₂O (75:25) containing 0.1% TFA (flow rate 10 mL/min). The MeOH was removed from the remaining elute using a rotary evaporator and the elute was freeze-dried to remove any remaining H₂O. The anthocyanin-rich urine extract was then further purified via prep-LC.

HPLC Analysis

Analytical HPLC. HPLC analysis was performed on an Agilent 1100 series HPLC (Agilent Technologies, CA, USA) using a Zorbax SB C₁₈ (5 μ, 4.6 x 250 mm; Agilent Technologies, CA, USA) reverse-phase column with an Supelguard LC-18 guard column (C₁₈ 5μm, 4.6 x 20mm; Supelco, Sigma-Aldrich; Oakville, ON, Canada). The

following procedure was modified from previously published methods (Mazza et al., 2002). The column temperature was set at 35°C and injector temperature at 15°C, with an injection volume of 50 µl. The mobile phase consisted of 4.5% v/v formic acid in water (solvent A) and 100% MeOH (solvent B). The flow rate was 1.0 mL/min and the solvent gradient program used as follows; 10% B at 0 min, 25% B at 30 min, 45% B at 50 min, 100% B at 55 min maintaining 100% for 10 min. A diode array detector (DAD) (G1315B; Agilent Technologies, CA, USA) monitored absorbance at 280, 360, and 525 nm. All samples were filtered through a 13 mm 0.45 µm GHB Acrodisc syringe filter (Pall-Gelman, MI, USA; Lot # A10310523) into a 200 µl vile insert prior to analysis. Anthocyanins were quantified by comparison to a standard curve obtained using known concentrations of cyanidin 3-galactoside. All water used for HPLC was 18 MΩ·cm Milli-Q water and degassed using an inline Agilent 1100 micro vacuum degasser (Agilent Technologies, CA, USA).

Prep-HPLC. Separation of individual anthocyanins from pooled urine samples was performed on a Waters Chromatographic system (Waters, MA, USA) comprised of two Model 510 pumps, and a Model 490 programmable multi wavelength detector set at 525nm. The preparative column system (Waters PrepPak) consisted of two Nova-Pak HR C₁₈ radial compression cartridges (25 x 100 mm; 6 µm, 60Å; PrepPak Cartridge; Waters, MA, USA) with a Nova-Pak HR C₁₈ guard insert (Waters, MA, USA). Injections were carried out on a manual injection port (Rheodyne, Cotati, CA, USA) equipped with a 500 µl injection loop. The column and injector were kept at ambient temperature, with an injection volume of 250-500 µl. The mobile phase consisted of 0.1% TFA in water (solvent A) and 100% MeOH (solvent B). The flow rate was 15

mL/min with an isocratic run of 80% A and 20% B. Peaks on the chromatogram corresponding to anthocyanins, as identified by spectral analysis (peaks detected at 525 nm with λ_{max} 250-300, 500-550), were collected manually from the prep-HPLC column and concentrated using a rotary evaporator. The remaining H₂O was removed in a freeze-drier and samples were sealed under nitrogen gas and stored at -80°C until further analysis.

TLC

TLC procedures were based on methods described by Wagner & Blatt, (1996). Normal phase prep-TLC was utilized for final purification of individual anthocyanins separated from the pooled urine samples. The 20 x 20 250 μm silica gel Redi/plates (Analtech Inc, Newark, DE, USA) were preconditioned prior to use. For preconditioning, the plates were let run for 2.5h in 100% MeOH, and then dried for 1h in a fume hood under nitrogen at ambient temperature prior to baking for 2h at 120°C. Once the plates had cooled to ambient temperature, the anthocyanin extracts were applied in a horizontal 1 cm band running the length of the plate. The solvent system consisted of ethyl acetate, glacial acetic acid, formic acid, and H₂O (100:11:11:26). After the plates were developed, the anthocyanin bands were removed from the plates and dissolved in 5 mL of MeOH containing 0.1% formic acid. The solution was stirred for 10 min and vacuum filtered through both a Watman disposable glass microfibre GA filter, followed by #2 Watman filter paper (Watman; Saukkola, Finland). The cake was recovered and redissolved, and the above procedure was repeated twice more. The final solution was filtered through a 0.45 PVDF syringe filter, evaporated in a rotary

evaporator, and brought to complete dryness in a freeze-dryer. The remaining extract was sealed under nitrogen gas and stored at -80 °C until further analysis.

0.20 mm silica gel 60 analytical TLC plates (Macherey-Nagel GmbH & Co, Postfach Düren, Germany; Batch 901/021) containing a fluorescent indicator (UV 254) were utilized post-acid hydrolysis of anthocyanins for verification of aglycones. Acid hydrolysis of the anthocyanin glycosides was achieved by dissolving a portion of the dry anthocyanin extracts in 200 µl of 2N HCl. The solution was then sealed under nitrogen gas and heated to 100°C for 1.5h. The samples were then cooled immediately in an ice bath and plated using the above solvent system.

MS Analysis

Mass Spectrometry (MS) identification of individual compounds was conducted post-separation via prep-LC and prep-TLC (as outlined above). The analysis was carried out on a Waters Alliance 2695 HPLC coupled serially with a Waters 2996 photodiode array detector and a Waters ZQ 2000 quadrupole analyzer utilizing the electrospray ionisation interface (ESI-MS) (Waters, MA, USA). The chromatographic separation was performed on a 250 x 2.0 mm Synergi 4µ Max-RP 80Å column (Phenomenex; Torrance, CA, USA) with a 4 x 2 mm Phenomenex Max RP guard cartridge (Phenomenex; Torrance, CA, USA). The column temperature was set at 35°C with an ambient injector temperature and an injection volume of 2 µl. The mobile phase consisted of an acidified (0.18% v/v acetic acid) water:acetonitrile mixture (95:5) (solvent A) and 100% acetonitrile (solvent B). The flow rate was 130 µl/min and the solvent gradient program

used 100% A at 0-2 min, and ramped to 100% B at 60 min. Micromass ZQ single quadrupole mass spectrometer with electrospray interface and MassLynx 4.0 software (Micromass, Manchester, U.K.) was used for data acquisition. Mass spectrometer parameters were set as follow: capillary voltage, 3400 V; cone voltage, 30 V (with ramping 20-40 V); source temperature, 120°C; desolvation temperature, 300°C; cone gas flow rate, 51 L/hr; desolvation gas flow rate, 400 L/hr; LM resolution, 15; HM resolution, 15; ion energy, 1 V; and multiplier voltage, 500 V. The instrument was operated in electrospray positive ion mode (ES+) scanning from 100 to 900 at a rate of 1.2 s/cycle. The MS parameters were loosely based on methods previously published by Felgines et al., (2003) and García-Beneytez et al., (2003).

NMR Analysis

NMR spectra were obtained on a Bruker Avance DRX 500 MHz spectrometer (Bruker Biospin Ltd., Milton, ON, Canada), equipped with a cryoprobe, at 300K. For ^1H (500 MHz) NMR, a solvent mixture of $\text{CD}_3\text{OD}:\text{CF}_3\text{COOD}$ (98:2, v/v, 200 μl) was used and δ values were referenced to CD_3OD (CHD_2OD at 3.30 ppm). Analysis of the ^1H NMR spectra was based on the comparison of the chemical shift and relative intensity of the signals with those of standard compounds.

Statistical Analysis

Results are expressed as means \pm SDs. Between subject variance, as well as the effect of time and period, were determined by repeated-measures ANOVA (analysis of variance) using the Statistical Analysis Systems statistical software package version 8.2

(SAS Institute, Cary, NC, USA). Least significant difference tests (LSD) were conducted using SAS and were utilized to determine differences between individual subject means. Independent paired t-tests were also utilized to determine differences between two individual subjects' serum data. Statistical analysis was conducted with $P < 0.05$ considered statistically significant. Non-compartmental pharmacokinetic evaluation was performed according to standard methods (Rowland & Tozer, 1989). Calculation of area under the plasma concentration time curve (AUC) was based on the average serum concentrations of individual subjects using the trapezoidal rule. Absorption half-lives were determined graphically (SPSS SigmaPlot, IL) using the method of residuals. The geometric means of the elimination half-lives were determined graphically from the renal excretion rates of individual subjects.

RESULTS

The consumed chokeberry extract contained 721.4mg of cyanidin glycosides (491.0mg C-3-gal, 175.3mg C-3-ara, 27.8mg C-3-xyl, and 27.3mg C-3-glu; Figure 6.3B). Serum and urine samples collected prior to the administration of the chokeberry extract (baseline, $t=0$) contained no detectable anthocyanins (Figure 6.3A). Data obtained by HPLC-DAD, HPLC-MS, and TLC analysis of the anthocyanin standards, isolated chokeberry anthocyanins, and serum and urinary anthocyanins are given in Tables 6.1, 6.2 and 6.3, respectively; the chromatographic representations are illustrated in Figure 6.3, and the MS spectral analysis is given in Figure 6.4. Standard curves were linear in all matrices, having an average $r^2 \geq 0.99$.

Figure 6.3 Anthocyanins in Chokeberry Extract, Serum, and Urine

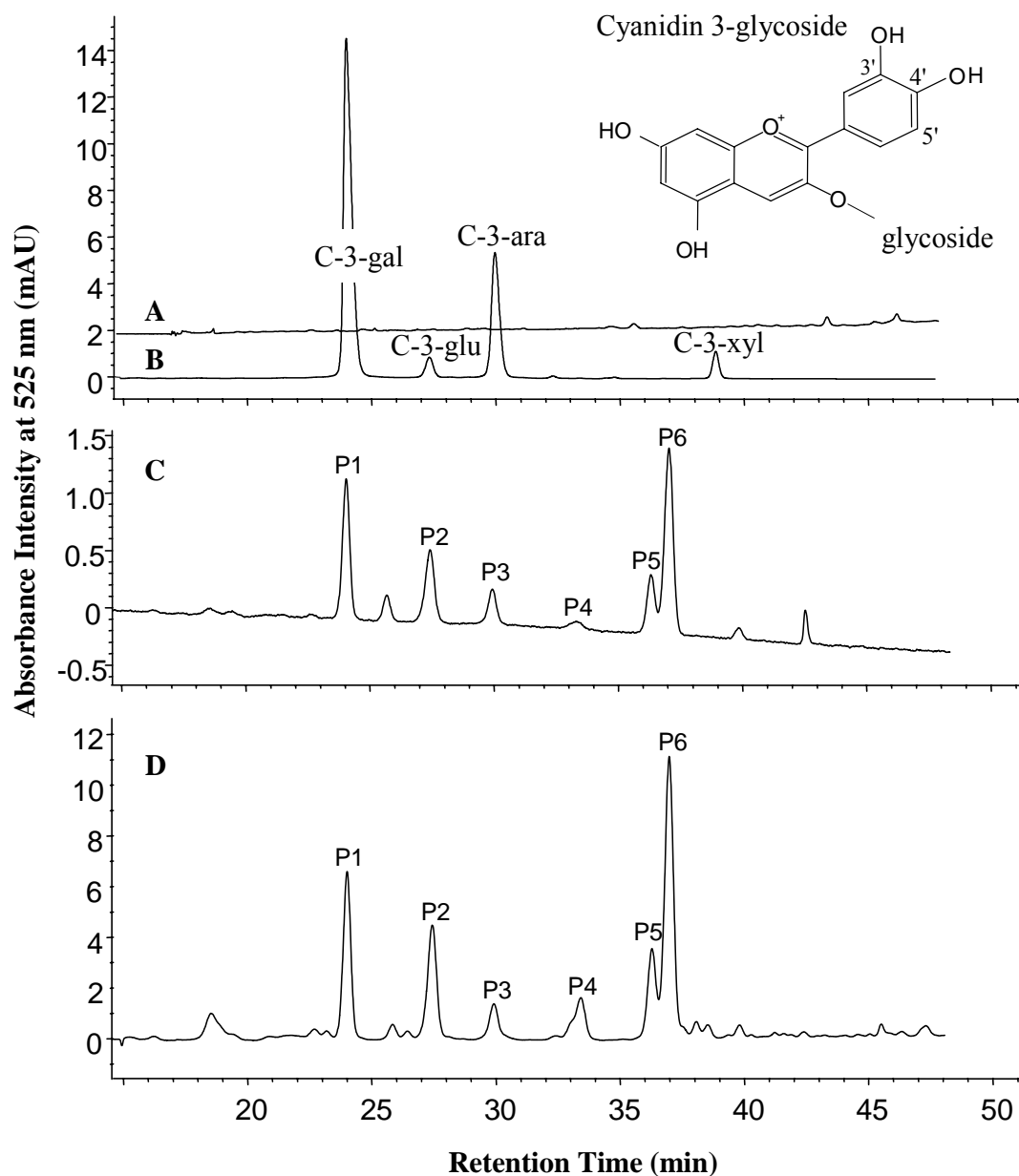


Figure 6.3 Chromatograms of anthocyanins in chokeberry extract, serum, and urine. (A) Chromatogram of baseline urine sample. (B) Chromatogram of chokeberry extract. (C) Chromatogram of serum sample. (D) Chromatogram of urine sample. Cyanidin 3-galactoside (C-3-gal), cyanidin 3-glucoside (C-3-glu), cyanidin 3-arabinoside (C-3-ara), cyanidin 3-xyloside (C-3-xyl). HPLC analysis as outlined in the methods. Identification of each peak represented above (peaks 1 through 6) is given in Table 6.1. Serum and Urine pharmacokinetic data of each compound represented by peaks 1 through 6 is given in Table 6.2 and 6.3, respectively.

Table 6.1 Identification of Anthocyanins and Anthocyanin Metabolites in Human Urine

Identification of anthocyanins in urine and serum samples						
Peak	Anthocyanin/Identity	¹ Rt (min)	² Molecular weight (m/z) Parent/Daughter Fragment	¹ Absorption spectra		TLC (Rf)
				λ_{\max}	E ₄₄₀ /E _{max} (as %)	
1	Cyanidin 3-galactoside	23.8	449/287	280, 517	31	0.36
2	Cyanidin glucuronide	27.6	463/287	280, 517	31	0.38
3	Cyanidin 3-arabinoside	29.6	419/287	280, 517	31	0.45
4	Peonidin 3-galactoside	33.4	463/301	280, 517	31	0.42
5	Methylated cyanidin glucuronide	36.3	477/301	280, 514	37	0.39
6	Methylated cyanidin glucuronide	37.0	477/301	280, 515	32	0.44
Characteristics of anthocyanins standards						
^{3,4}	Cyanidin 3-galactoside	23.8	449/287	280, 517	31	0.36
³	Cyanidin 3-arabioside	29.6	419/287	280, 517	31	0.45
³	Cyanidin 3-xyloside	39.0	419/287	280, 517	29	0.48
^{3,4}	Cyanidin 3-glucoside	27.3	449/287	280, 517	37	0.40
⁴	Cyanidin	43.6	287	280, 526	23	0.80
⁴	Peonidin	50.8	301	280, 528	26	0.84

¹Identification (retention time and absorption spectra) based on Agilent HPLC-DAD.

²Identification (molecular weight) based on Waters micro capillary HPLC-MS.

³Data based on analysis of purified anthocyanin from chokeberry extract.

⁴Data based on analysis of purchased standard (Extrasynthese; Genay, France).

Table 6.2 Pharmacokinetic Parameters of Cyanidin 3-Glycosides and Corresponding Metabolites in Human Serum¹

Peak	Anthocyanin/Identity	Cmax		tmax		AUC		t _{1/2a}
		(ng/mL)	SD	(h)	Range (h) ²	(ng·h/mL)	SD	(h)
1	Cyanidin 3-galactoside	10.94	1.09	2.5	(2-3)	29.92	1.47	<1.35
2	Cyanidin glucuronide	6.72	1.87	2.0	(2)	42.10	1.89	
3	Cyanidin 3-arabinoside	3.71	0.21	3.5	(3-4)	22.62	1.02	<1.67
4	Peonidin 3-galactoside	1.74	0.36	4.0	(4)	14.20	0.52	
5	Methylated cyanidin glucuronide	6.11	0.20	2.5	(2-3)	16.42	1.32	
6	Methylated cyanidin glucuronide	15.64	5.19	2.5	(2-3)	47.70	6.13	
Total	Parent and metabolite	44.86	2.82	2.8	(2-4)	172.96	7.44	
	Parent	14.65	0.65	3.00	(2-4)	52.54	1.24	1.51 ± 0.24
	Metabolites	30.21	1.97	2.75	(2-4)	120.42	2.46	

¹Average of serum concentration data for 3 subjects (n=3; t=0-7h).

²Median (range).

Cmax, maximum concentration of anthocyanins observed in the serum.

tmax, time point where the maximum serum concentration is observed.

AUC, total area under the plasma concentration-time curve (ng·h/mL).

t_{1/2a}, absorption half-life determined using the method of residuals (Rowland & Tozer, 1989). Precise absorption half-lives for subjects 2 and 3 cannot be determined as there was only one serum sample (t=1h) during the absorption phase. Therefore, as a result of the rapid absorption phase (< 1h) for two of the three subjects, SD's cannot be specified. The absorption half-life values above are for subject one only and it can be assumed that the true absorption half-life of anthocyanins is less than 1h for the remaining subjects. For a more accurate estimate of absorption half-life samples are required prior to 1h.

Table 6.3 Pharmacokinetic Parameters of Cyanidin 3-Glycosides and Corresponding Metabolites in Human Urine¹

Peak	Anthocyanin/Identity	Quantity		Rmax		tmax _R		t _{1/2}	
		(µg)	SD	(µg/h)	SD	(h)	SD	(h)	SD
1	Cyanidin 3-galactoside	267.55	90.82	48.74	14.80	3.39	1.14	3.72	0.42
2	Cyanidin glucuronide	173.25	86.46	39.47	20.77	3.17	0.86	3.63	0.56
3	Cyanidin 3-arabinoside	80.30	31.30	13.17	4.68	2.94	1.39	4.05	0.82
4	Peonidin 3-galactoside	103.88	35.63	19.27	7.74	3.39	1.14	4.28	0.59
5	Methylated cyanidin glucuronide	111.06	60.61	19.61	10.51	4.05	1.21	4.53	0.92
6	Methylated cyanidin glucuronide	335.50	187.66	66.63	37.13	4.05	1.21	4.39	0.83
Total	Parent and metabolite	1071.54	375.46	202.74	85.06	3.72	0.83	4.12	0.40
	Parent	347.85	60.61	61.91	9.74	3.16	1.26	3.88	0.62
	Metabolites	723.69	92.59	144.98	19.04	3.66	1.05	4.02	0.72

¹Average of urine concentration data for all subject urine samples (n=9; t=0-24h).

Rmax, maximal rate of excretion (µg/h).

tmax_R, time point where maximal rate of excretion occurs.

t_{1/2}, elimination half-life or the time to reach half-maximal excretion.

Figure 6.4 Mass Spectral Analysis of Isolated Anthocyanins from Pooled Urine Samples

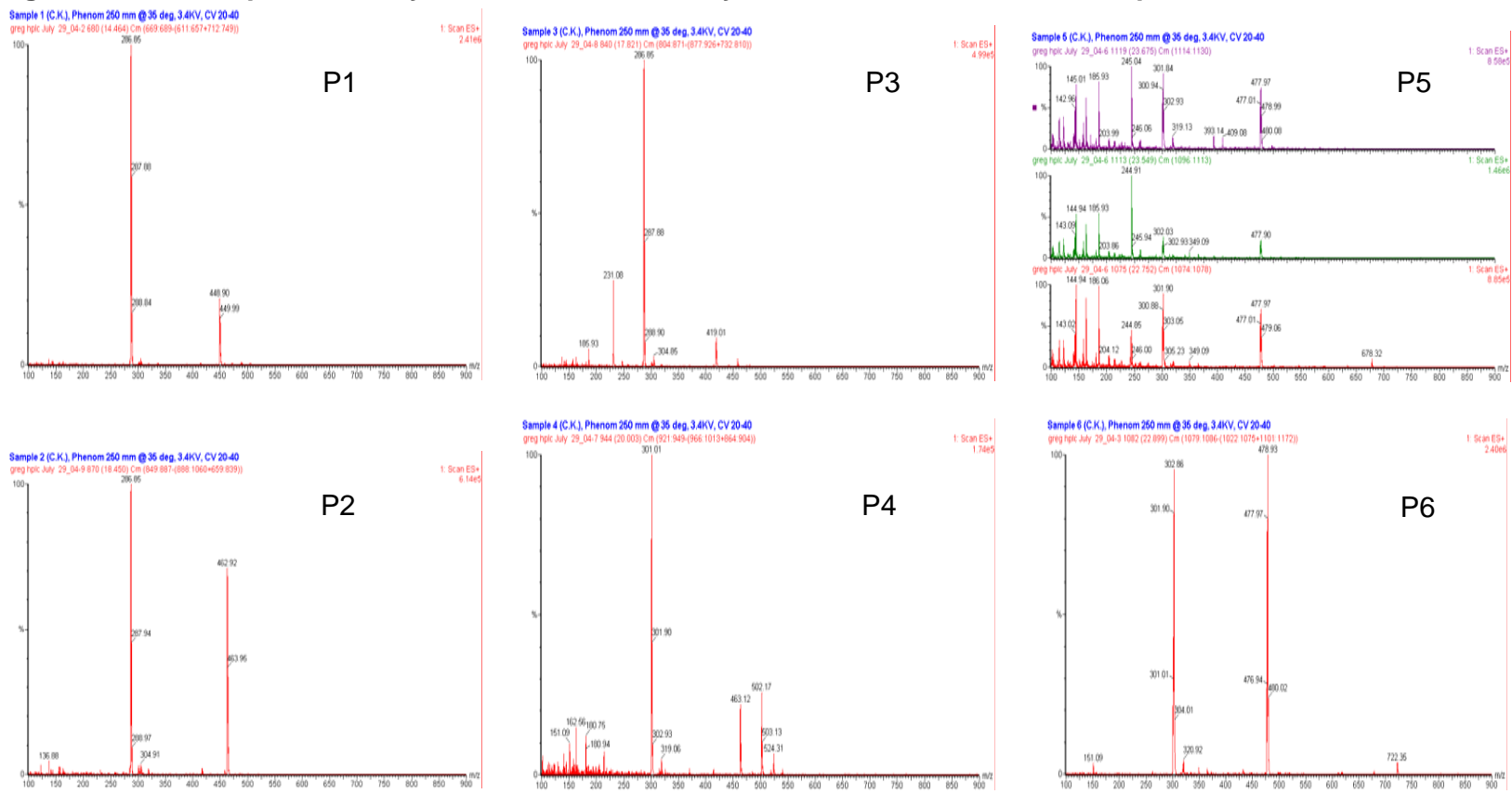


Figure 6.4 Mass spectral analysis of isolated anthocyanins from pooled urine samples. Mass spectra represent original profiles. Identification was based on the matching of compounds to standards following the subtraction of extraneous peaks from the solvent blank. The MS spectra of all samples as well as standards and blanks can be found in Appendix 1. Peak 5 appeared to have a distinctive shoulder and individual analyses of the front, apex, and tail were obtained to establish identification. Identification of all peaks listed above is given in Table 6.1.

Identification of Anthocyanins

Identification of cyanidin 3-glycosides. Identification was based on the analysis of isolated anthocyanins from pooled urine samples subsequent to HPLC-DAD quantification of individual samples. Comparisons were made between serum and urinary samples using HPLC-DAD data (Figure 6.3C,D; Table 6.1) and the results indicated that the serum peaks matched both retention time (Rt) and UV-vis spectrum with the peaks identified as anthocyanins in the urine, and were therefore regarded as the same compounds. The identification of anthocyanins was based on the comparisons of molecular weights for parent (anthocyanin) and daughter (aglycone) fragments, along with HPLC-DAD data (Rt, $\lambda_{\max_{\text{vis}}}$ and E_{440}/E_{\max}), to that of available standards (Table 6.1; Figure 6.4). When necessary, further identification was based on TLC evidence. Results indicated the presence of both cyanidin 3-galactoside (m/z 449/287, Table 6.1; P1, Figure 6.3) and cyanidin 3-arabinoside (m/z 419/287, Table 6.1; P3, Figure 6.3) which were the two major anthocyanins in the chokeberry extract (92.4% of total). Additionally, the proportions of the compounds corresponded with their relative concentration in the extract. The compounds also matched HPLC Rt, UV-vis characteristics (λ_{\max} and E_{440}/E_{\max} ; Table 6.1) and Rf values (as determined by normal phase TLC) as with that of the corresponding purchased standards and isolated chokeberry anthocyanins (C-3-gal and C-3-ara; Table 6.1).

Identification of conjugated cyanidin derivatives. The identification of anthocyanin metabolites was based on MS, HPLC-DAD, and TLC data as described above (Table 6.1). Results indicated the presence of both glucuronidated (m-176) and methylated (m+14) derivatives of cyanidin. In total, 4 derivatives were isolated from the urine in

high enough quantities for structural identification. One metabolite (Figure 6.3D, P2) was identified as a cyanidin glucuronide, as indicated by its molecular ion at m/z 463 and fragment at m/z 287 (Table 6.1). The second identified metabolite (Figure 6.3D, P4) had the chemical characteristics of a methylated derivative of C-3-gal, having a parent ion of m/z 463 and daughter fragment of m/z 301 (Table 6.1); including matching HPLC R_t and having an aglycone with spectral characteristics and a R_f value consistent with peonidin. Two other metabolites (P3,4; Figure 6.3) were identified as methylated derivatives of cyanidin glucuronide, having parent ions of m/z 477 and daughter fragments of m/z 301 (Table 6.1). Further confirmation (HPLC-DAD and TLC data) of the aforementioned compounds is detailed in Table 6.1 as well as in the following discussion.

Pharmacokinetic Evaluation

The calculated pharmacokinetic parameters of individual anthocyanins in the serum and urine are summarized in Tables 6.2 and 6.3, respectively. There were no period effect or significant difference between subjects for total serum or urine anthocyanin pharmacokinetics ($P>0.1$). There was also no significant difference between overlapping serum samples (Figure 6.1B) (within subject variability). There was a significant difference in anthocyanin concentration (total and individual) over time ($P<0.0001$), as anthocyanins were absorbed and eliminated from the blood and urine. There was also a significant difference between grouped total (P1-6), parent (P1,3), and metabolized anthocyanins (P2,4,5,6, Figure 6.3) in blood and urine samples ($P<0.001$). Figure 6.5 illustrates the inter-individual variability in the serum concentration and renal excretion of anthocyanins.

Figure 6.5 Serum and Urine Elimination of Total Anthocyanins Following Ingestion of 721 mg Cyanidin 3-glycosides

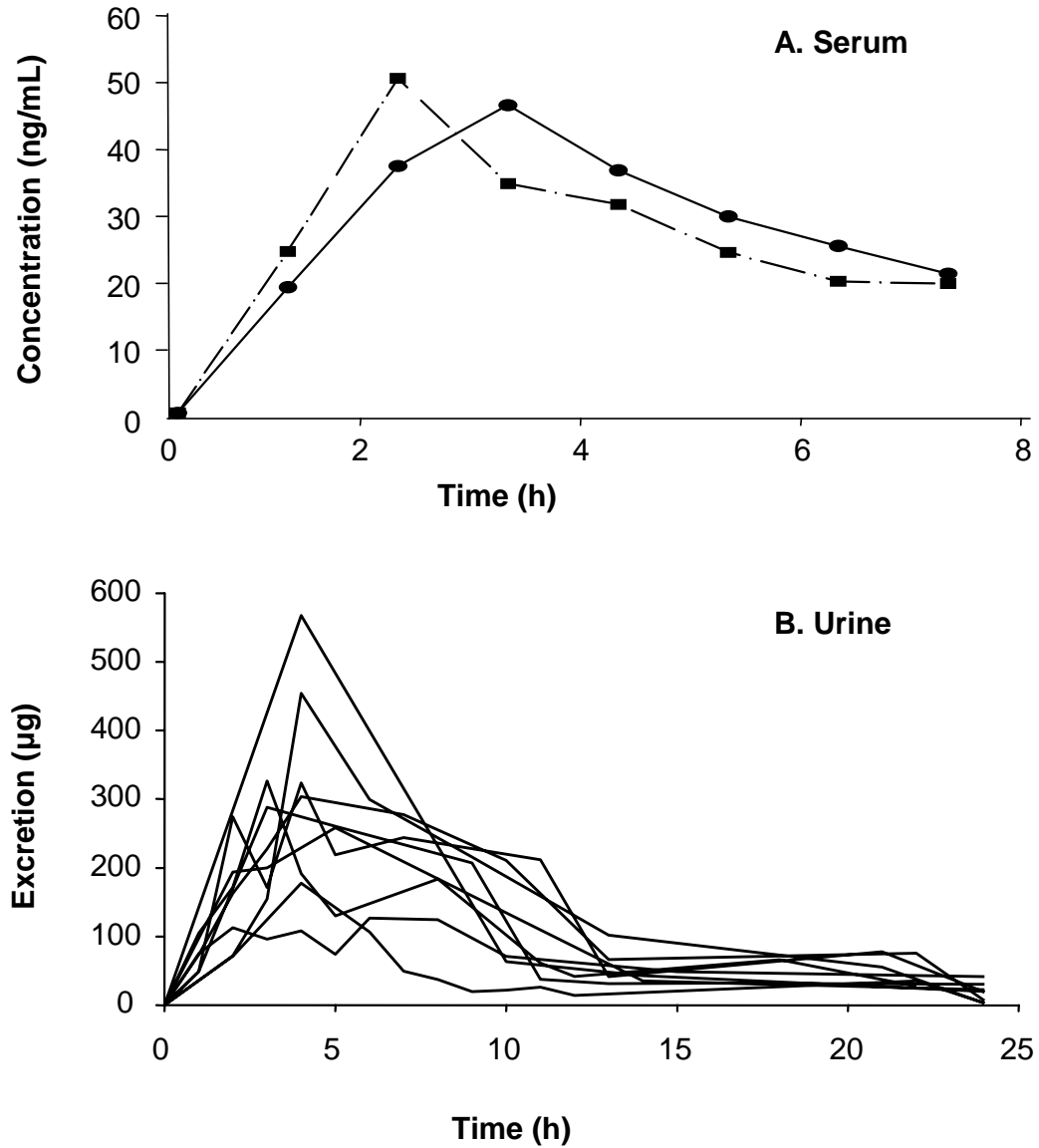


Figure 6.5 Time course of total anthocyanins in human serum (A) and urine (B) of individual subjects following the consumption of 721 mg of cyanidin 3-glycosides. The serum analysis (A) represents two subjects elimination data as the third subjects data was incomplete (refer to Figure 6.1B for sampling regime). The urine analysis (B) represents 9 individual 24h urine collections as each subject (n=3) participated in 3 repetitions of the study protocol (refer to Figure 6.1A for repetition profile).

Pharmacokinetic evaluation of parent cyanidin 3-glycosides. The average relative concentrations, as well as cumulative levels of anthocyanins in the serum and urine, are graphically represented in Figure 6.6 and 6.7. The AUC (Table 6.2) of the parent anthocyanins (C-3-gal and C-3-are) over the 7h serum sampling regime totaled 52.54 ± 1.24 ng·h/mL. The parent anthocyanin 3-glycosides appeared rapidly in the blood, having an average absorption half-life ($t_{1/2a}$) of 1.51 ± 0.24 h and a maximum serum concentration (C_{max}) of 14.65 ± 0.65 ng/mL occurring within 3h (t_{max} ; range 2-4h) (Table 6.2; Figure 6.6A). Additionally, the total amount of parent anthocyanins excreted in the urine was 347.85 ± 61.61 µg, having an average elimination half-life ($t_{1/2}$) of 3.88 ± 0.62 h (Table 6.2). The average maximum observed excretion rate (R_{max}) of the total parent anthocyanins was 61.91 ± 9.74 µg/h and occurred rapidly (Figure 6.6B), as indicated by an average time to reach maximum excretion rate (t_{maxR}) of 3.16 ± 1.26 h (Table 6.3). The excretion of parent anthocyanins in the urine represented 0.048% of the ingested dose (% recovery).

Pharmacokinetics of total and conjugated cyanidin metabolites. The total cumulative concentration of anthocyanins (parent and metabolites) detected in the serum over the 7h sampling regime was 172.96 ± 7.44 µg·h/mL (AUC; Table 6.1). The C_{max} for total anthocyanins (parent and metabolites) in the serum was 44.86 ± 2.82 µg/mL with a t_{max} of 2.8h (range 2-4h) post-consumption; however, only 32.7 % (52.54 µg·h/mL) of the total anthocyanins detected in the serum were the parent compounds (P1,3) with an average of 67.3% (120.42 µg·h/mL) identified as conjugated metabolites (P2,4,5,6, Table 6.2; Figure 6.3C). Similarly, 32.5 % (347.85 µg) of the anthocyanins excreted in the urine were the parent compounds (P1,3) with an average of 67.5% (723.69 µg)

occurring as conjugated metabolites (P2,4,5,6, Table 6.3; Figure 6.3C). The total urinary excretion of metabolites and parent compounds over 24h was $1071.54 \pm 375.46\mu\text{g}$, reaching a R_{max} of $202.74 \pm 85.06 \mu\text{g/h}$, a t_{max_R} of $3.72 \pm 0.83\text{h}$ and $t_{1/2}$ of $4.12 \pm 0.40\text{h}$ (Table 6.3).

The total cumulative concentration of anthocyanin metabolites detected in the serum over the 7h sampling regime was $120.42 \pm 2.46 \mu\text{g}\cdot\text{h/mL}$ (AUC; Table 6.1), with a C_{max} of $30.21 \pm 1.97 \mu\text{g/mL}$ and t_{max} of 2.75h (range 2-4h) post-consumption. Similarly, the total urinary excretion of metabolites over 24h was $723.69 \pm 92.59\mu\text{g}$, reaching a R_{max} of $144.98 \pm 19.04 \mu\text{g/h}$, a t_{max_R} of $3.66 \pm 1.05\text{h}$, and $t_{1/2}$ of $4.02 \pm 0.72\text{h}$ (Table 6.3).

Figure 6.6 Serum and Urine Elimination of Total, Parent, and Metabolized Anthocyanins Following Ingestion of 721 mg Cyanidin 3-glycosides

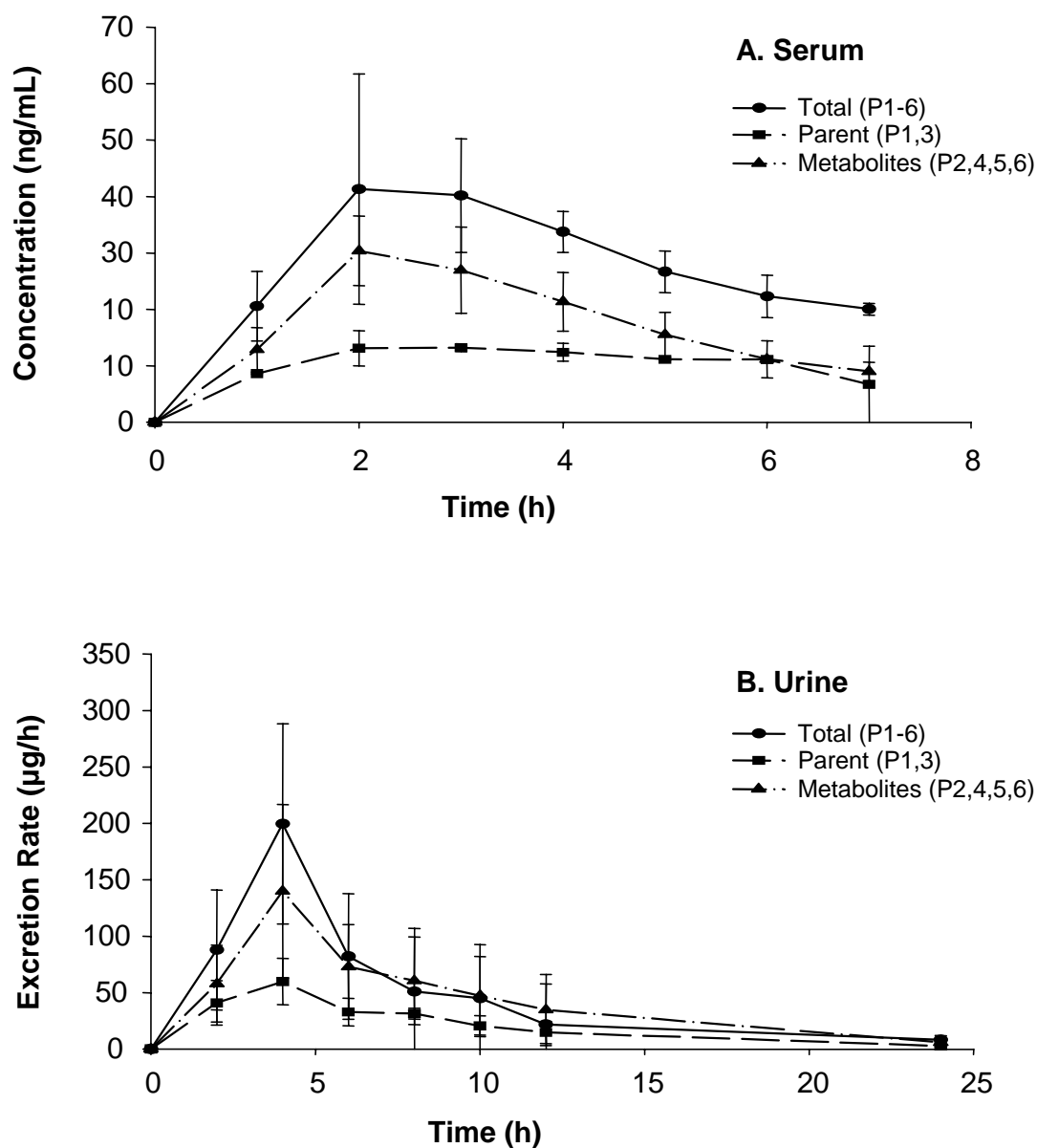


Figure 6.6 Time course of total, parent, and metabolized anthocyanins in human serum (A) and urine (B) of individual subjects following the consumption of 721 mg of cyanidin 3-glycosides. The serum analysis (A) represents two subjects elimination data as the third subjects data was incomplete (refer to Figure 6.1B for sampling regime). The urine analysis (B) represents 9 individual 24h urine collections as each subject (n=3) participated in 3 repetitions of the study protocol (refer to Figure 6.1A for repetition profile).

Figure 6.7 Cumulative Serum and Urine Elimination of Individual Anthocyanins Following Ingestion of 721 mg Cyanidin 3-glycosides

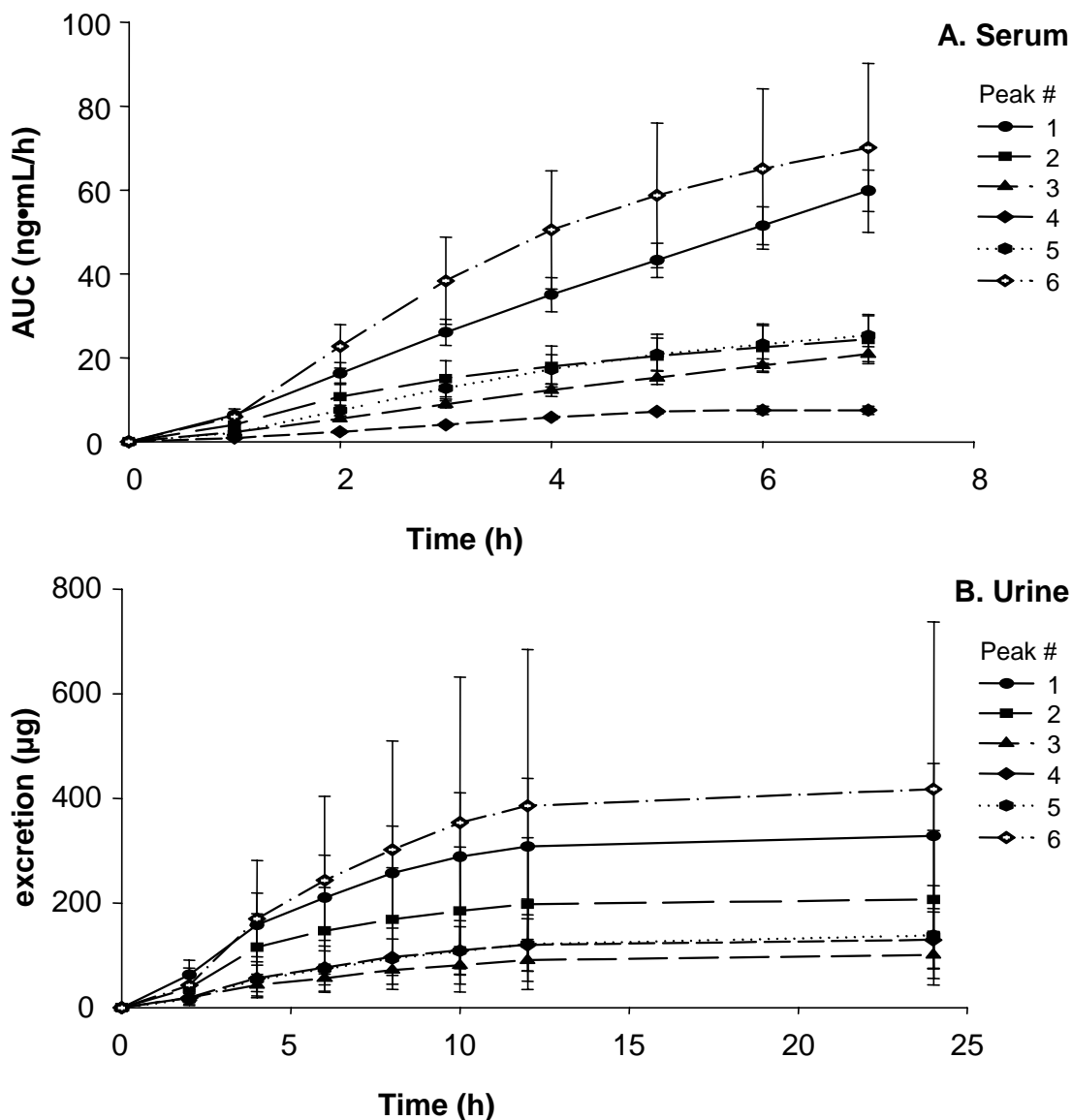


Figure 6.7 Cumulative time course of individual anthocyanins in human serum (A) and urine (B) following the consumption of 721 mg of cyanidin 3-glycosides. The serum analysis (A) represents three subjects elimination data as the third subjects data was incomplete (refer to Figure 6.1B for sampling regime). The urine analysis (B) represents 9 individual 24h urine collections as each subject (n=3) participated in 3 repetitions of the study protocol (refer to Figure 6.1A for repetition profile). For peak identities refer to Table 6.1. AUC, area under plasma concentration time curve

DISCUSSION

The absorption and metabolism of anthocyanins in humans is poorly understood. Evidence presented in this manuscript details the identification and pharmacokinetics of both parent anthocyanin glycosides and metabolized derivatives in the serum and urine.

Identification

In the present investigation, three subjects consumed approximately 721.4mg of cyanidin glycosides (as contained in the chokeberry extract) on three separate occasions. The chokeberry extract as utilized in the present investigation was chosen for its simplistic anthocyanin profile, consisting of only cyanidin 3-glycosides (491.0mg C-3-gal, 175.3mg C-3-ara, 27.8mg C-3-xyl, and 27.3mg C-3-glu). The use of a fruit extract containing only one anthocyanin species (cyanidin) was crucial for establishing the origin of methylated cyanidin derivatives.

Individual urine (0-24h) and blood (0-7h) samples were collected on each date following the protocol as outlined in Figure 6.1B. Individual serum (0-7h) and urine (0-24h) samples were analyzed via HPLC-DAD for the quantification of anthocyanins. Subsequent to quantification, urine samples were pooled for purification (XAD adsorption chromatography), isolation (Prep-HPLC and prep-TLC), and identification (HPLC-MS; HPLC-DAD; TLC; NMR) (Figure 6.2). Identification of urinary metabolites (Table 6.1) was based on the matching of molecular weights (parent and daughter fragments), R_t , $\lambda_{\max_{\text{vis}}}$ and E_{440}/E_{\max} , to that of available standards. TLC data was also utilized for additional confirmation when considered necessary. The results indicate the presence of intact anthocyanin glycosides in the serum and urine samples. It has previously been suggested that deglycosylation of flavonoid glycosides is a pre-

requisite for their absorption (Németh et al., 2003); however, the presence of intact cyanidin glycosides in the urine and serum detailed in this investigation, as well as others (Tsuda et al., 1999a,b; Cao et al., 2001; Felgines et al., 2002; Mazza et al., 2002; Mülleder et al., 2002; Wu et al., 2002; Galvano et al., 2004; Kay et al., 2004), demonstrates that intact anthocyanin glycosides can be absorbed.

Identification of parent anthocyanin glycosides. Peaks 1 and 3 of the chromatogram, as illustrated in Figure 6.3, were identified as the parent chokeberry anthocyanins C-3-gal and C-3-ara, respectively. These compounds had identical mass spectrum (parent ion, daughter fragment, and loss of sugar fragment), HPLC Rt & UV-vis spectrum, as well as TLC Rf values (both glycoside and hydrolyzed aglycone) with that of purchased standards and respective isolated chokeberry anthocyanins (Table 6.1). The presence of cyanidin glycosides in human serum/plasma and urine has previously been documented (Cao et al., 2001; Felgines et al., 2002; Mazza et al., 2002; Mülleder et al., 2002; Wu et al., 2002; Galvano et al., 2004; Kay et al., 2004). Although, parent cyanidin glycosides were identified in the serum and urine samples, metabolized derivatives accounted for the majority of the anthocyanins detected in the serum and urine (67.3% and 67.5% respectively).

Identification of anthocyanin metabolites. The evidence presented in this investigation confirms peak 2 (Figure 6.3) to be a cyanidin glucuronide as indicated by its molecular ion at m/z 463 and fragment at m/z 287. This reveals a loss of m/z 176 which is indicative of the loss of a glucuronide residue as opposed to a hexose sugar residue which has a mass of 162. Additionally, the fragment ion m/z 287 has a mass equivalent

to cyanidin. Likewise, the spectral data (λ_{\max} and E_{440}/E_{\max}) of the intact compound was similar to C-3-gal having a negligible maxima shift (0-2 units) which is consistent with the negligible shift observed between flavonoid glucosides and glucuronides reported in the literature (Day & Williamson, 2001). This evidence is further reinforced by the fact that the hydrolysis of this compound produced an aglycone with the same HPLC Rt, UV-vis spectral data and TLC Rf value as the purchased cyanidin standard. Unfortunately, we do not have enough evidence to determine the position of the glucuronide residue since we were unable to obtain adequate NMR spectra (result of insufficient quantity and poor solubility). One metabolite, Peak 4 (Figure 6.3), had structural/chemical characteristics equivalent to P-3-gal, the methylated derivative of C-3-gal. Identification of this compound is based on the following evidence. The parent ion had a mass of m/z 463 with a daughter fragment of m/z 301. The increased mass of 14 ($m/z +14$) above that of cyanidin (287) is consistent with methylation ($287+14 = 301$), and a daughter fragment of m/z 301 is consistent with peonidin. The compound was also observed to have an increased HPLC Rt over C-3-gal which is consistent with the reduced polarity resulting from methylation of a hydroxyl group. Additionally, the hydrolysis of the compound resulted in an aglycone with similar HPLC Rt, UV-vis spectral data, and TLC Rf value, as the purchased peonidin standard. The identification of this compound as a peonidin galactoside is reinforced by its loss of m/z 162 upon fragmentation, which is indicative of the fragmentation of a hexose sugar. Since the galactoside was the hexose sugar conjugate (C-3-gal) consumed in the highest concentration, the compound is most likely a methylated derivative of the parent C-3-gal. Also, since there is no major detoxification pathway involving glycosidation with hexose sugars other than with glucuronic acid (Dutton 1980), it can be concluded that

the most likely hexose conjugate in this case is the parent 3-galactoside. The possible metabolic transformation of cyanidin 3-glycosides to peonidin 3-glycosides has previously been documented (Felgines et al., 2002; Wu et al., 2002; Kay et al., 2004). Although, a great deal of evidence suggests this compound is P-3-gal, we cannot prove indubitably that the methylation occurred at the 3' position and the glycosylation occurred at the 3 position as we did not obtain sufficient NMR data for structural elucidation. Two other metabolites, P5 and P6, m/z 477/301 (Table 6.1; Figure 6.3) were identified as methylated derivatives of cyanidin glucuronide. They had daughter fragments consistent with peonidin (m/z 301) and a loss of m/z 176 upon fragmentation which is consistent with the loss of a glucuronic acid residue. These derivatives were dissimilar to peonidin (having different HPLC & TLC characteristics) and are therefore likely methylated at positions other than the 3' position of the B-ring. The differences were observed as the aglycone yielded post-acid hydrolysis did not match the Rt (HPLC) or Rf (TLC) of the peonidin standard. An additional point to note is that the HPLC-MS data indicated a prominent shoulder on the chromatogram of one metabolite (P4, Figure 6.3) that could not be sufficiently resolved but had the same mass spectra; this may indicate a third methyl derivative. It appears from the above presented results that methylation and glucuronidation are the primary conjugation reactions involved in anthocyanin metabolism.

The presence of anthocyanin mono-glucuronides (P2,5,6, Figure 6.3) in the serum and urine, as observed in this investigation (Tables 6.2 & 6.3), is not surprising. Glucuronidation is the most common pathway utilized to increase the water-solubility of phenolic xenobiotics prior to elimination from the body (Dutton, 1980). Furthermore, in recent years, a few researchers have reported the detection of anthocyanin glucuronides

in biological fluids (Wu et al., 2002; Felgines et al., 2003; Kay et al., 2004).

Glucuronidation was the major metabolic pathway for anthocyanin metabolism in the present investigation representing 61.4% and 57.8% of the total anthocyanins (parent and metabolites) detected in the blood and urine, respectively. Felgines et al., (2003) recently identified as much as 80% of excreted anthocyanins as monoglucuronides. The relative differences in glucuronidation between these two investigations are likely the result of the different forms and doses of anthocyanins utilized. Mono-methylated cyanidin derivatives ($m+14$; P4,5,6, Figure 6.3) were also identified in this investigation (Tables 6.2 & 6.3). Methylation was the second most observed metabolic transformation for anthocyanin metabolism representing 45.3% and 51.4% of the total anthocyanins detected in the serum and urine, respectively. Methylated derivatives of anthocyanins in the blood and urine have also previously been documented (Miyazawa et al., 1999; Tsuda et al., 1999a; Kay et al., 2004) and the high percentage of methylated metabolites observed in this investigation is consistent with the particularly high catechol-O-methyltransferase concentration of the liver (Kopin, 1985). Even though only a few researchers have reported methylated anthocyanins in the urine and blood of humans and animals, methylated derivatives of the flavonoids quercetin and catechin are well documented (Harborne, 1958; Wermeille et al., 1983; Hollman & Katan, 1998a,b; Miyazawa et al., 1999; Okushio et al., 1999; Donovan et al., 2001).

We did not detect sulfoconjugates in the present investigation; however, sulfoconjugates of anthocyanin glycosides have recently been identified (Felgines et al., 2003). Magnification of the chromatograms in the present investigation revealed the appearance of many small peaks (at 525 nm) which were at concentrations too low to adequately identify but were well above any baseline noise. Many of these peaks had

spectral characteristics representative of anthocyanins ($\lambda_{\text{max}_{\text{vis}}}$ in 280 and 500 nm range). Some of these peaks may have been sulfoconjugates. The presence of many trace metabolites indicates that the body may have a high capacity to metabolize anthocyanins. These metabolites likely go undetected in many investigations as a result of their vast numbers and subsequent low concentrations. The presence of many trace metabolites is supported by the findings of the study discussed in Chapter 5 where the consumption of a much higher dose of anthocyanins (1.3g) resulted in the appearance of at least 10 individual anthocyanin metabolites. Notably, in the present investigation, we isolated a significant concentration of an unidentified compound in the urine which was highly non-polar. This compound had an intense absorption in the visible spectrum with maximas at 539, 360, and 290. This compound also had a parent and daughter fragment of 453/263 and did not match UV-vis or TLC data for any run standard. The low polarity of the compound (as determined by its long R_t under reverse-phase conditions) indicates that this compound was likely a non-glycosylated structure. This compound may be missed by other investigators using reverse phase HPLC as the compound would elute in the wash-out phase of most column elution programs. Unfortunately, at this time, there is insufficient evidence to present a potential structure for this compound or elucidate its apparent origin.

Pharmacokinetics

Pharmacokinetics of parent cyanidin 3-glycosides. In the present investigation no anthocyanins were identified in the fasting serum or urine samples suggesting that the wash-out phase and pre-study dietary exclusion of anthocyanins was sufficient. The AUC of the parent anthocyanins (C-3-gal and C-3-are) over the 7h serum sampling

regime totalled 52.54 ± 1.24 ng·h/mL. The anthocyanins appeared rapidly in the blood having an average $t_{1/2a}$ of 1.51 ± 0.24 h, a C_{max} of 14.65 ± 0.65 ng/mL, and t_{max} of 2.8h (range 2-4h) (Table 6.2; Figure 6.6A). The data suggests that the elimination of anthocyanins from the body follows a first-order model. This elimination kinetic model has also been reported by Cao et al. (2001) following the consumption of an anthocyanin mixture derived from elderberries. The total amount of parent anthocyanins excreted in the urine was 347.85 ± 61.61 μ g, with an average $t_{1/2}$ of 3.88 ± 0.62 h, R_{max} of 61.91 ± 9.74 μ g/h, and t_{maxR} at 3.16 ± 1.26 h. (Table 6.3; Figure 6.6B). Furthermore the percentage of parent anthocyanins excreted in the urine was approximately 0.048% of the total ingested dose which is consistent with the literature. Many studies reporting the consumption of anthocyanins ranging from doses of 50 to 550mg have expressed urinary recoveries of parent anthocyanins generally between 0.02 and 0.1% (Lapidot et al., 1998; Miyazawa et al., 1999; Bub et al., 2001; Cao et al., 2001; Wu et al., 2002). The low plasma and urinary concentrations of anthocyanins observed suggests either a low absorption or a rapid and efficient metabolism.

Pharmacokinetics of total anthocyanins (parent & metabolites). The absorption and elimination of parent anthocyanins appears relatively low compared to the initial dose; however, glucuronidated and methylated anthocyanin metabolites were observed in the present investigation at levels more than twice that of the parent (intact) compounds. In the present study, the cumulative concentration of total anthocyanins (parent and metabolites) detected in the serum over the 7h sampling regime was 172.96 ± 7.44 ng·h/mL (Table 6.2), having a C_{max} of 44.86 ± 2.82 ng/mL and t_{max} of 2.8h (range, 2-4h); however, only 32.7 % (52.54 ng·h/mL) of the total anthocyanins detected in the

serum were the parent compounds (P1,3) with an average of 67.3% (120.42 ng·h/mL) identified as conjugated metabolites (P2,4,5,6, Figure 6.3; Table 6.2). Similarly, only 32.5 % (347.85µg) of the anthocyanins excreted in the urine were the parent compounds (P1,3, Figure 6.3; Table 6.2) with an average of 67.5% (723.69µg) occurring as conjugated metabolites (P2,4,5,6, Figure 6.3).

The total urinary excretion of metabolites and parent compounds over 24h was $1071.54 \pm 375.46\mu\text{g}$, accounting for 0.15% of the initial dose. The R_{max} ($202.74 \pm 85.06 \mu\text{g/h}$) was observed at $3.72 \pm 0.83\text{h}$ (Table 6.3), with a $t_{1/2}$ of $4.12 \pm 0.4\text{h}$ (Table 6.3). Similar elimination kinetics were reported by Murkovic et al., (2001) and Milbury et al., (2002) who observed maximum urinary excretion at 3-4h. Additionally, Mülleder et al, (2002) reported a maximum excretion rate at 1-3h and elimination half-life of 4h following oral doses of elderberry anthocyanins. Although some recent investigations have described the identification of anthocyanin metabolites in human urine this is the first study to give detailed pharmacokinetic parameters for anthocyanin metabolites.

In this investigation the total cumulative concentration of anthocyanin metabolites detected in the serum over the 7h sampling regime was $120.42 \pm 2.46 \mu\text{g}\cdot\text{h/mL}$ (AUC; Table 6.1), with a C_{max} of $30.21 \pm 1.97 \mu\text{g/mL}$ and t_{max} of 2.75h (range 2-4h). Additionally, the total urinary excretion of metabolites over 24h was $723.69 \pm 92.59\mu\text{g}$, reaching a R_{max} of $144.98 \pm 19.04 \mu\text{g/h}$, a t_{max_R} of $3.66 \pm 1.05\text{h}$, and $t_{1/2}$ of $4.02 \pm 0.72\text{h}$ (Table 6.3). The elimination of the metabolites in this investigation mirrored that of the parent compounds having similar t_{max} , t_{max_R} , and $t_{1/2}$ suggesting the metabolites and parent compounds follow the same metabolic pathway in the body.

Many studies have reported the excretion of anthocyanins to account for as little as 0.03% of the initial dose. However, many of these studies have been unable to identify

anthocyanin metabolites in biological fluids. In this investigation, both parent glycoside and metabolized derivatives were identified in the urine and serum, with the excretion of metabolites being greater than 2 times that of the parent glycosides. Similarly, Felgines et al. (2003) has recently reported the urinary excretion of anthocyanin metabolites to be greater than 1.8% of the ingested dose. Studies unable to identify metabolites of anthocyanins in biological fluids therefore likely underestimate their absorption. Furthermore, the metabolism of anthocyanins may also result in the formation of phenolic acids, phenolic acid residues, H₂, or CO₂ (Rozman, 1986; Hollman & Katan, 1998a) which augments their underestimation in biological fluids. Recently, it has been reported that as much as 52% of an oral dose of radiolabelled quercetin in humans was exhaled as ¹⁴CO₂ (Walle et al., 2001) indicating significant absorption and metabolism had occurred. It is therefore clear that further research is required to establish a more comprehensive understanding of anthocyanin absorption and metabolism in humans.

CONCLUSION

To conclude, the above results indicate that orally administered cyanidin 3-glycosides are absorbed and transported in human serum and urine primarily as glucuronide and methy-glucuronide derivatives. The contribution of each individual structure to the reported bioactivity (health effects) of anthocyanins should be the focus of future research.

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CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

The overall aim of this thesis dissertation was: to identify the significance of the reported antioxidant effect of anthocyanins in human subjects; to develop effective methods for the extraction, separation, and quantification of anthocyanins in human biological fluids; and to investigate the metabolism and pharmacokinetics of anthocyanins in humans.

In the first investigation detailed in this thesis (“Absorption of Anthocyanins from Blueberries and Serum Antioxidant Status in Human Subjects”; Chapter 4), the total concentration of anthocyanins identified in human blood serum post-consumption of blueberries (1.2g mixed anthocyanins) correlated with an increase in serum antioxidant capacity ($P < 0.01$; as determined using the ORAC and TEAC assays). However, it was questionable if the concentration of anthocyanins in the circulation (5.43-16.9 ng/mL) was sufficient to account for the magnitude of antioxidant activity observed. It was hypothesised that unidentified anthocyanin metabolites may contribute significantly to the increased antioxidant capacity of the serum. Even though the anthocyanins were observed to have a low bioavailability, the biological antioxidant activity of their metabolites may be significant. The quantification and identification of anthocyanin metabolites is the focus of the remainder of this thesis dissertation.

Currently, the mechanisms of anthocyanin absorption and metabolism are unknown. The most commonly cited conjugation reactions for flavonoids and isoflavonoids of similar structure to anthocyanins include glucuronidation, methylation, and sulfation (Manach et al., 1998; Shimoi et al., 1998; Spencer et al., 1999; Kuhnle et

al., 2000; Williamson et al., 2000; Donovan et al., 2001; Oliveria et al., 2002).

Therefore, it was hypothesised that anthocyanins would be metabolized in much the same manner. However, at the initiation of this research, there was little evidence to suggest that anthocyanins were metabolized to any significant extent in humans as studies reported only intact parent anthocyanin glycosides in biological fluids (Miyazawa et al., 1999; Cao et al., 2001; Mazza et al., 2002; Mülleder et al., 2002).

The act of investigating the metabolism of flavonoids is difficult as there are generally no available standards upon which to base their identification. In addition, the levels of metabolites observed frequently approach the detection limit of current methodologies. Consequently, the identification of metabolites following the consumption of a complex mixture of anthocyanins, such as that of the blueberry, required a significant increase in dose above the 1.2g of anthocyanins as fed in the previous investigation. It was therefore necessary to feed a fruit/fruit-product containing a more simplified anthocyanin profile in order to provide sufficient quantities of any one (or more) metabolized derivative(s) for identification; such was the rationale behind the design of the second study as detailed in this thesis dissertation (“The Identification of Cyanidin Metabolites in Human Urine and Serum”; Chapter 5).

In the second investigation, human subjects were fed cyanidin 3-glycosides as contained in the chokeberry. The chokeberry extract was utilized for its simplistic anthocyanin profile, consisting of only cyanidin 3-glycosides. Cyanidin is the most abundant anthocyanins in nature and was one of the 5 anthocyanin species in the blueberry. The blueberry extract fed in the first investigation contained 5 individual anthocyanidin species comprising 23 glycoside and acetyl-glycoside derivatives, while the chokeberry contains only 4 glycoside derivatives of cyanidin. The use of a fruit

extract containing only one parent anthocyanin species (cyanidin) is crucial for establishing the origin of any one metabolite in addition to maintaining the dose of total anthocyanins as utilized in the first investigation.

In the second investigation, subjects were fed 1.3g cyanidin 3-glycosides which resulted in the appearance of at least 10 individual anthocyanin metabolites in the urine. The metabolites were identified as glucuronide conjugates, as well as methylated derivatives of cyanidin 3-galactoside and cyanidin glucuronide. The average concentrations of anthocyanins and anthocyanin metabolites in the serum were observed at 312 ng/mL within 2h post-consumption, and reached levels of 8 µg/mL in 5h urine samples. This number and concentration of metabolites was unexpected as evidence in the literature indicated that only the parent anthocyanidin glycosides were absorbed and identified in the blood or urine. The results of this study therefore indicated that orally administered cyanidin 3-glycosides are absorbed and transported in human serum and urine as glycosides and glucuronides, both of which undergo further methylation. The design of this study was intended for the characterization of metabolites alone. The aim was to feed a sufficient amount of the parent compounds to produce adequate quantities of metabolites for their detection via HPLC-DAD. Although this investigation produced important findings, i.e., the identification of novel metabolites, the design did not allow for the determination of relevant pharmacokinetic data. In order to form a more complete model of anthocyanin metabolism, further time course studies were required using a more realistic anthocyanin dose. Additionally, the development of more involved extraction procedures and sensitive detection methods was necessary to characterize pharmacokinetic parameters following a lower oral dose.

In the third and final investigation (“The Metabolism & Pharmacokinetics of Cyanidin 3-Glycosides in Humans”; Chapter 6), the pharmacokinetic parameters of anthocyanins were established following the administration of a 721mg oral dose of cyanidin 3-glycosides to human subjects. Solid-phase extraction (SPE), prep-HPLC, prep-TLC, HPLC-DAD, HPLC-MS, and NMR were utilized to isolate, quantify, and identify anthocyanins in 0-7h serum and 0-24h urine samples. The results indicated the presence of the parent cyanidin 3-glycosides as well as glucuronidated and methylated derivatives. The excretion of metabolites in this investigation was 2 times greater than that of the parent cyanidin 3-glycosides. Felgines et al. (2003) had also recently reported the urinary excretion of anthocyanin metabolites to be much greater than that of the parent compounds. In the second investigation (Chapter 5), 66% of the identified anthocyanins in the serum and urine were as the parent cyanidin 3-glycosides; however, in the third low-dose investigation (Chapter 6), only 32.5% were as the parent compounds. As the dosage in the third study was half of the dosage in the second, it appears that the metabolic pathways involved in the metabolism of anthocyanins may approach a plateau or saturation at higher doses. Therefore, the appearance of high concentrations of parent anthocyanins in the serum and urine, as reported in the literature (Miyazawa et al., 1999; Cao et al., 2001; Mazza et al., 2002; Müllleder et al., 2002), may be an abnormal occurrence which is only prevalent following an oral pharmacological dose of anthocyanins.

The maximum concentration of anthocyanins (parent and metabolites) detected in the serum over the 7h sampling regime in the third investigation (Chapter 6) was $44.86 \pm 2.82 \mu\text{g/mL}$ and occurred within 2.8h, displaying an absorption half-life of $1.38 \pm 0.24\text{h}$. Additionally, the maximal rate of urinary excretion was $202.74 \pm 85.06 \mu\text{g/h}$ at

3.72 ± 0.83 h, with an elimination half-life of 4.12 ± 0.4 h. This pharmacokinetic profile indicates rapid absorption and elimination of anthocyanins. If anthocyanins and their respective metabolites are determined to have significant biological activity at these concentrations, the use of anthocyanins for chronic disease prevention would require repeated daily consumption as anthocyanins and their identified metabolites are rapidly eliminated from the body.

The pharmacokinetic parameters as described in the above experiment are based on the metabolism of cyanidin 3-glycosides following the consumption of a berry extract containing a very unique anthocyanin profile. The impact of one anthocyanidin species in isolation versus a complex mixture of many individual anthocyanins on metabolism is unknown. Additionally, the absorption and metabolism of anthocyanins following the consumption of various fruit matrices (i.e, whole fruit, dried extracts, fruit juice concentrates, and synthetic pure compounds) is also likely highly variable. It is important to note that the performance of cyanidin 3-glycosies as described above is the result of the consumption of a chokeberry fruit extract and cannot be directly applied to all fruits or fruit products which contain variable and highly complex anthocyanin profiles.

The total urinary excretion of anthocyanins (parent & metabolites) over 24h was 1071.54 ± 375.46 µg, representing 0.15% of the ingested dose. Several studies have reported the excretion of anthocyanins to account for as little as 0.05% of the initial dose (Bub et al., 2001; Cao et al., 2001; Mülleder et al., 2002; Nielsen et al., 2003); however, many of these studies were unable to identify anthocyanin metabolites in biological fluids and, as a result, underestimated the extent of anthocyanin absorption. The level of anthocyanins in the blood (serum/plasma) and urine reported in this investigation, as

well as in others, raises many questions about the extent of anthocyanin absorption and metabolism. For example, if less than 1% of anthocyanins are identified in biological fluids, what proportion of absorbed and metabolized anthocyanins escape detection? Additionally, what is the fraction of anthocyanins and/or anthocyanin metabolites that are eliminated through fecal or respiratory excretion? It is evident that a great deal of additional research is necessary before a complete understanding of anthocyanin metabolism and biological activity is realized.

The following is a list of future research that is required for the complete determination of anthocyanin absorption, metabolism, and biological activity. Cell suspension/culture studies are required to characterize the mechanisms of absorption, including transport, enzymology, and saturation levels. Animal studies involving the collection of portal blood and bile are required to determine the extent of intestinal and liver metabolism, as well as EHC. Animal studies are also required to fully characterize the sites of metabolism, tissue distribution, accumulation, and toxicity of anthocyanins and anthocyanin metabolites (including phenolic acid derivatives). Complete metabolic studies involving compound labelling and the collection of blood, urine, CO₂, and feces are required for the characterization of anthocyanin absorption and metabolism. Furthermore, multi-dose interventions involving extravascular and intravascular administration of anthocyanins are required for the determination of complete pharmacokinetic profiles of all major anthocyanins. Finally, *in vitro* and *in vivo* investigations are required to determine the biological activities of all major anthocyanins and anthocyanin metabolites in relation to their probable circulating concentrations.

In conclusion, the above results indicate that cyanidin 3-glycosides, following a low oral dose of chokeberry extract, are absorbed and transported in human serum and urine primarily as glucuronide and methyl-glucuronide derivatives. As the anthocyanin metabolites retain many of their free hydroxyls, it is likely that all of the above identified metabolites contribute, to some degree, to the antioxidant capacity of the serum. The contribution of each individual metabolite to the reported biological activity of anthocyanins should be the focus of future research.

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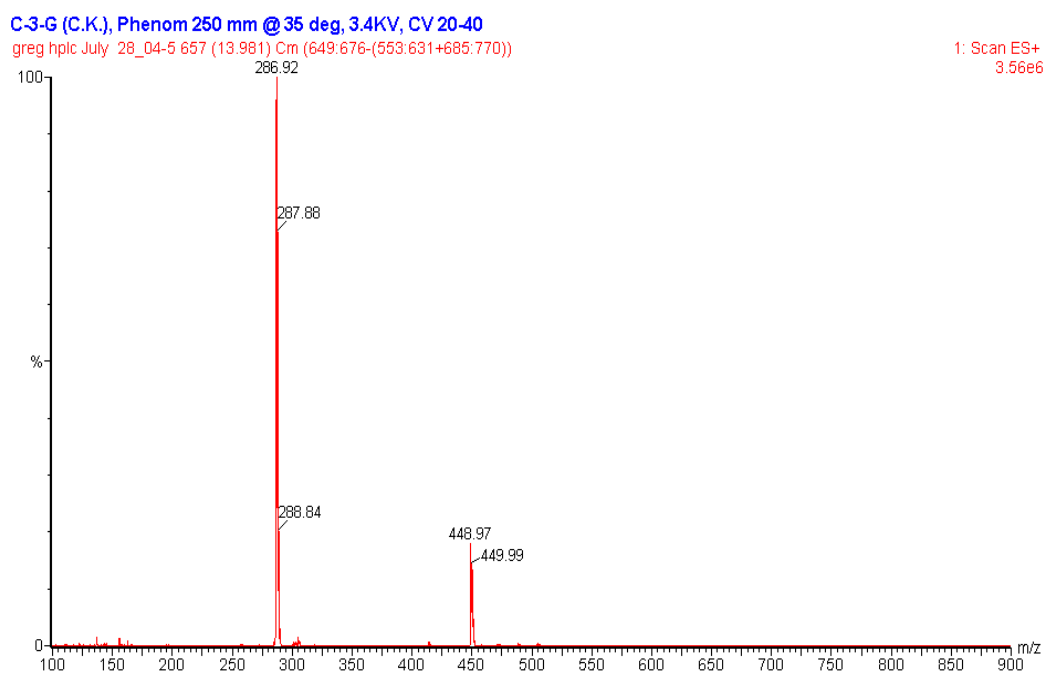
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APPENDIX 1.

MASS SPECTRAL ANALYSIS OF ISOLATED ANTHOCYANINS FROM POOLED URINE SAMPLES AS DETAILED IN CHAPTER 6

The following appendix contains the mass spectral analysis of isolated anthocyanins from pooled urine samples as detailed in Chapter 6 (“The Metabolism & Pharmacokinetics of Cyanidin 3-Glycosides in Humans). Mass spectra represent original profiles. Identification was based on the matching of compounds to standards following the subtraction of extraneous peaks from the solvent blank. Identification of peaks 1-6 is given in Chapter 6 (Table 6.1). M+1 peaks on the mass spectra are the result of using deuterated solvents.

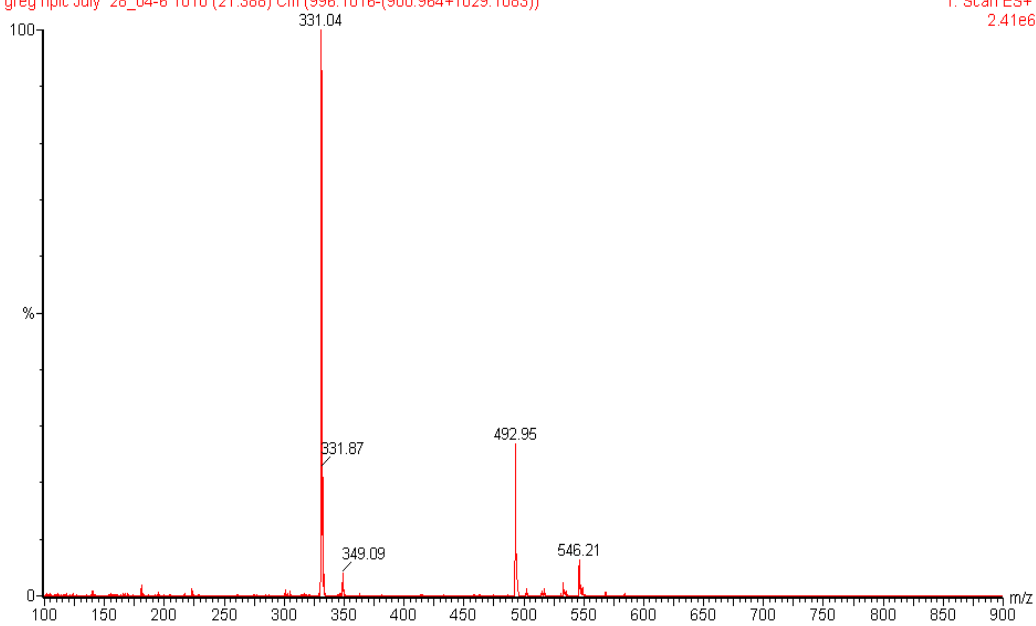
MASS SPECTRA of ANTHOCYANIN STANDARDS



M-3-Glu (C.K.), Phenom 250 mm @ 35 deg, 3.4KV, CV 20-40

greg hplc July 28_04-6 1010 (21.388) Cm (996:1016-(900:964+1029:1083))

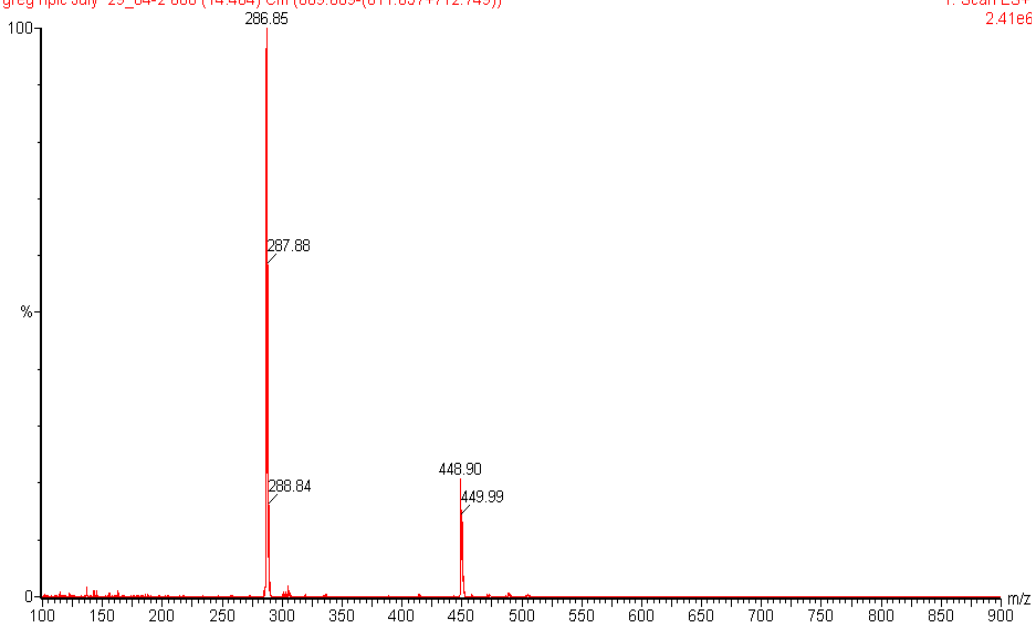
1: Scan ES+
2.41e6



**MASS SPECTRA of SAMPLES/PEAKS 1-6 as REPRESENTED
in CHAPTER 6 (TABLE 6.1)**

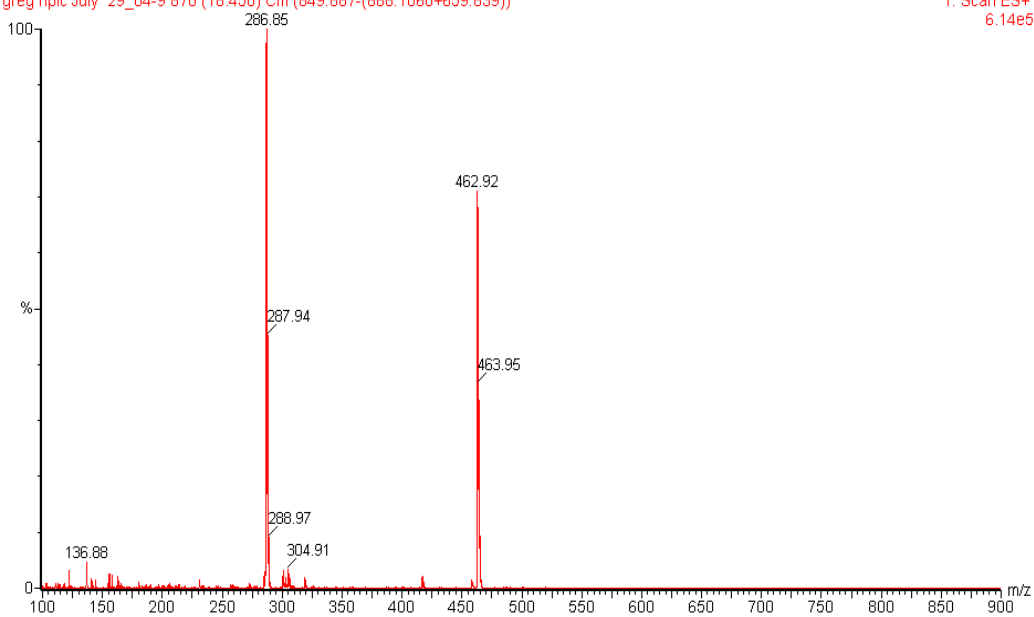
Sample 1 (C.K.), Phenom 250 mm @ 35 deg, 3.4KV, CV 20-40
greg hplc July 29_04-2 680 (14.464) Cm (669:689-(611:657+712:749))

1: Scan ES+
2.41e6



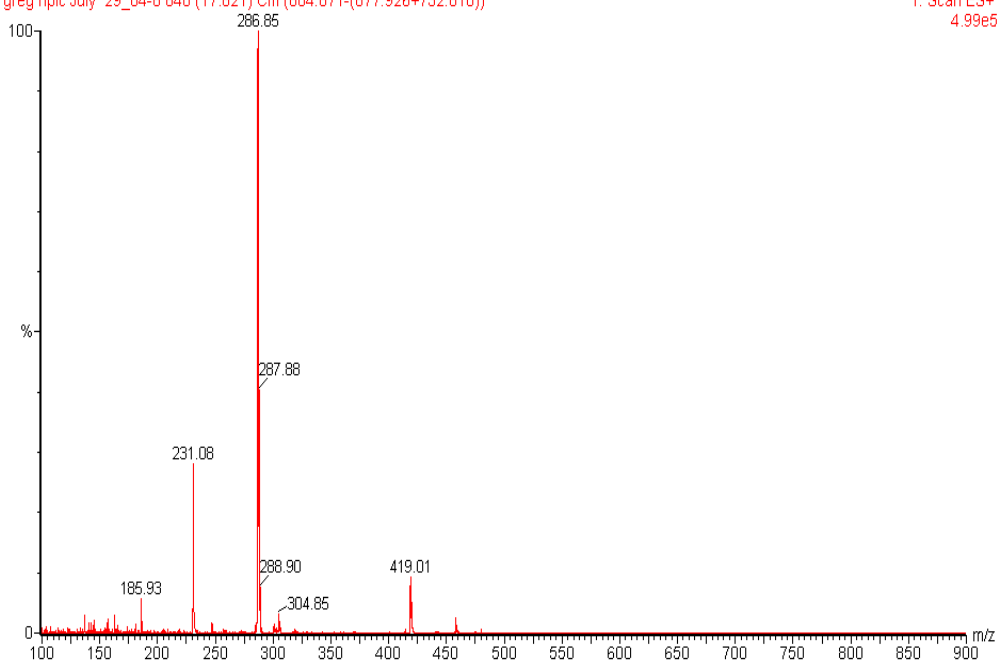
Sample 2 (C.K.), Phenom 250 mm @ 35 deg, 3.4KV, CV 20-40
greg hpic July 29_04-9 870 (18.450) Cm (849:887-(888:1060+659:839))

1: Scan ES+
6.14e5



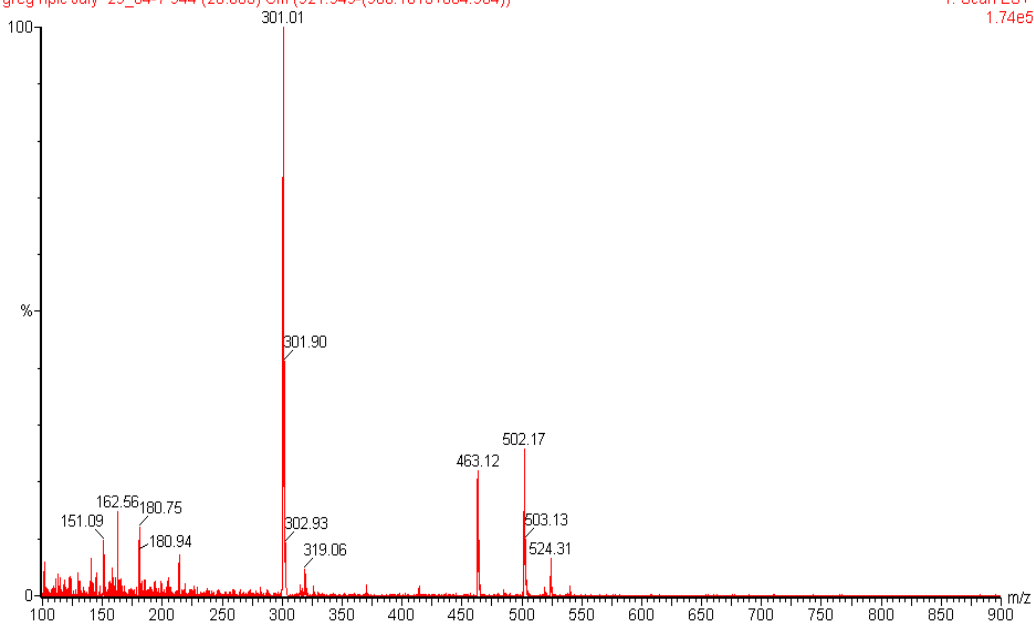
Sample 3 (C.K.), Phenom 250 mm @ 35 deg, 3.4KV, CV 20-40
greg hplc July 29_04-8 840 (17.821) Cm (804:871-(877:926+732:810))

1: Scan ES+
4.99e5

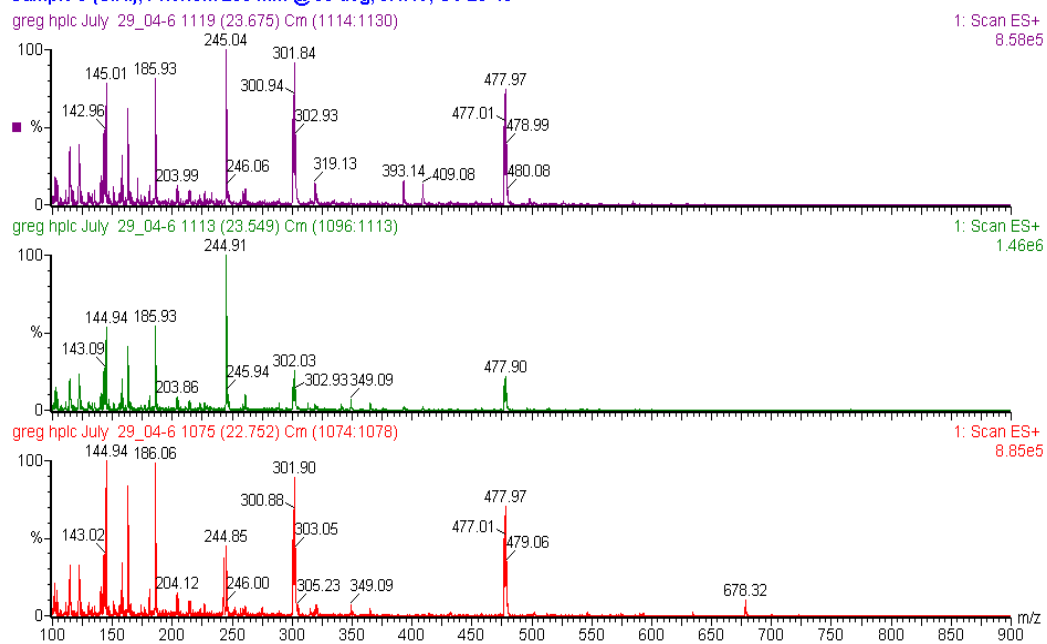


Sample 4 (C.K.), Phenom 250 mm @ 35 deg, 3.4KV, CV 20-40
greg hplc July 29_04-7 944 (20.003) Cm (921:949-(966:1013+864:904))

1: Scan ES+
1.74e5

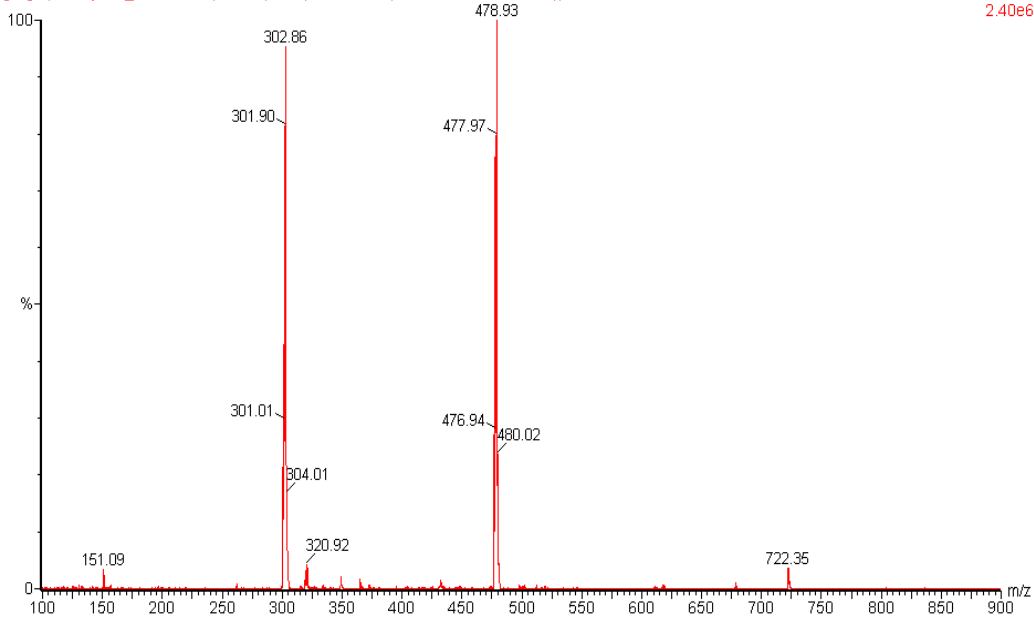


Sample 5 (C.K.), Phenom 250 mm @ 35 deg, 3.4KV, CV 20-40



Sample 6 (C.K.), Phenom 250 mm @ 35 deg, 3.4KV, CV 20-40
greg hplc July 29_04-3 1082 (22.899) Cm (1079:1086-(1022:1075+1101:1172))

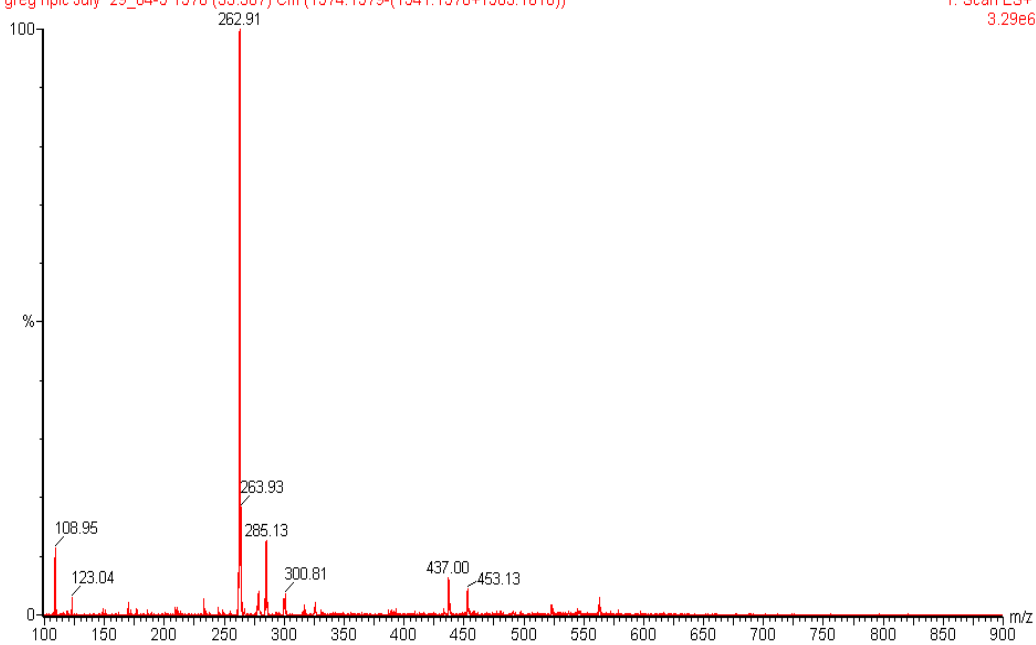
1: Scan ES+
2.40e6



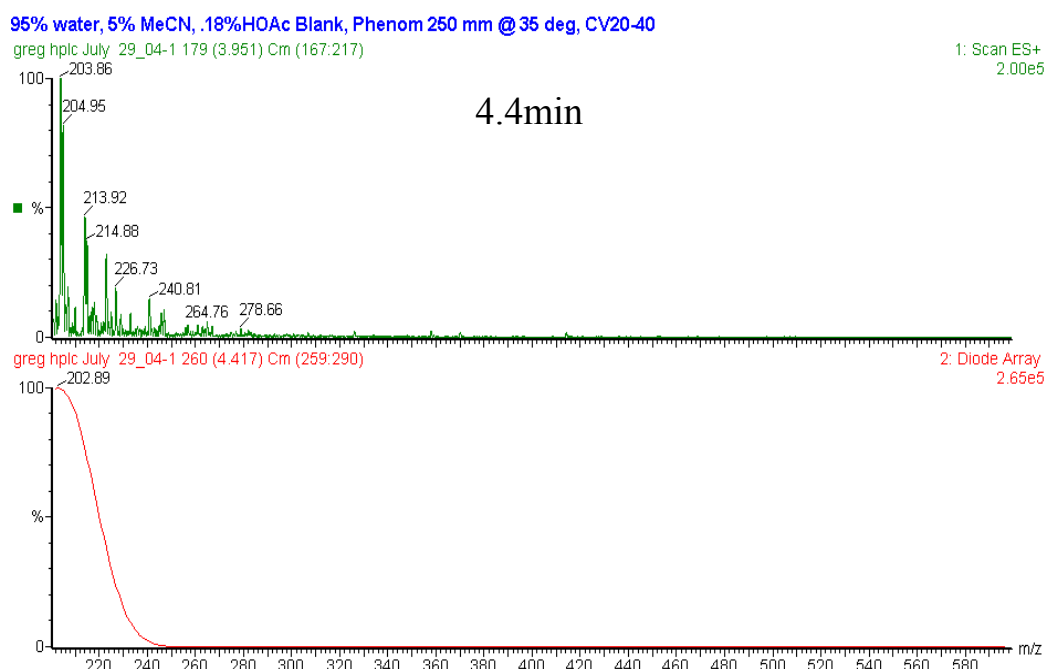
Sample 7 (C.K.), Phenom 250 mm @ 35 deg, 3.4KV, CV 20-40

greg hplc July 29_04-5 1578 (33.307) Cm (1574:1579-(1541:1570+1583:1610))

1: Scan ES+
3.29e6



MASS SPECTRA of SOLVENT BLANK OVER TIME

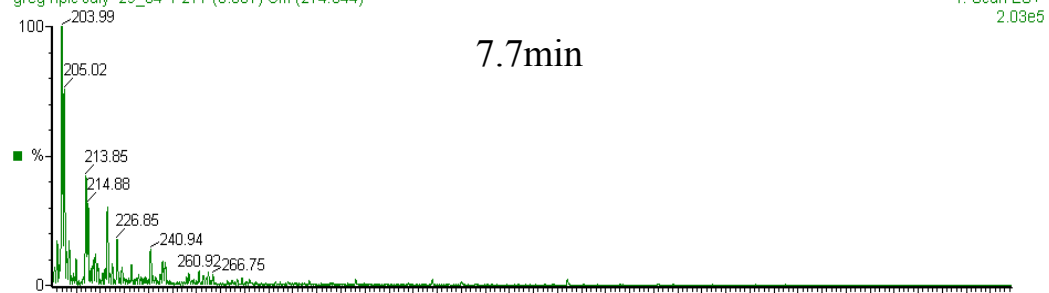


95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

greg hplc July 29_04-1 277 (6.007) Cm (274:344)

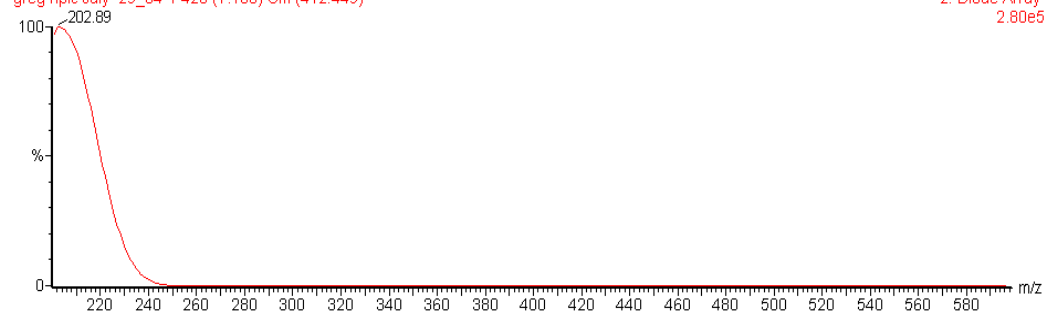
1: Scan ES+
2.03e5

7.7min



greg hplc July 29_04-1 426 (7.183) Cm (412:449)

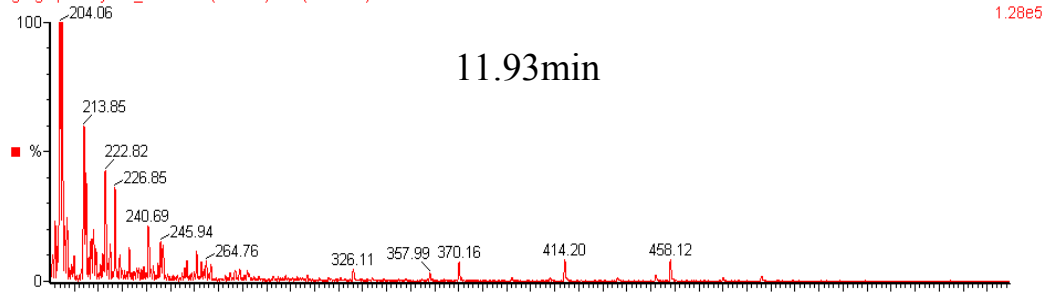
2: Diode Array
2.80e5



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

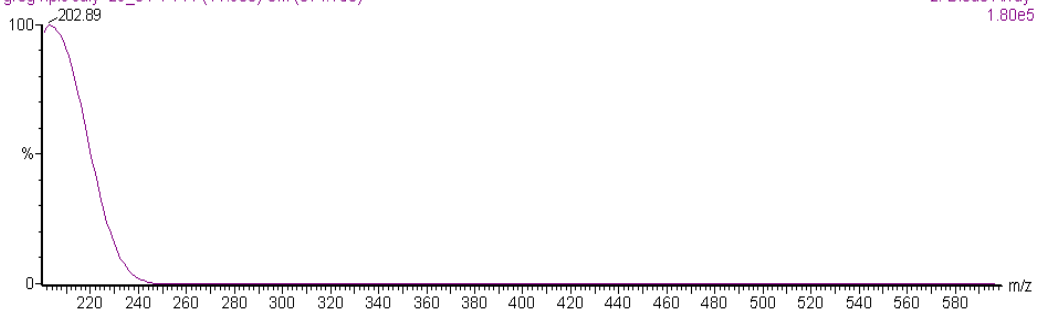
greg hplc July 29_04-1 653 (13.897) Cm (540.661)

1: Scan ES+
1.28e5



greg hplc July 29_04-1 711 (11.933) Cm (674.753)

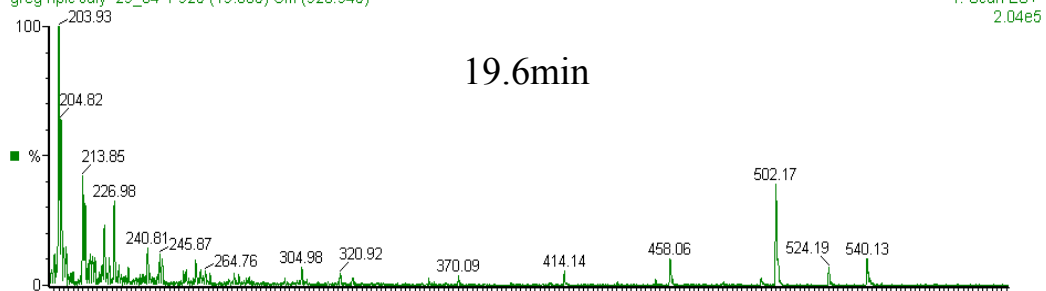
2: Diode Array
1.80e5



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

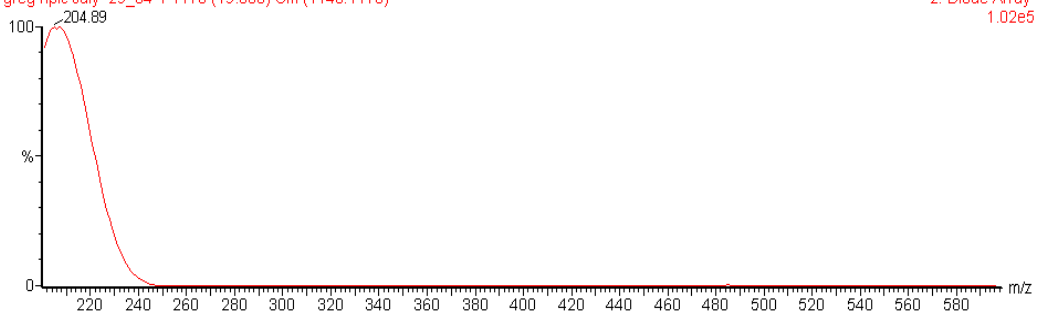
greg hplc July 29_04-1 925 (19.605) Cm (923:945)

1: Scan ES+
2.04e5



greg hplc July 29_04-1 1176 (19.683) Cm (1140:1176)

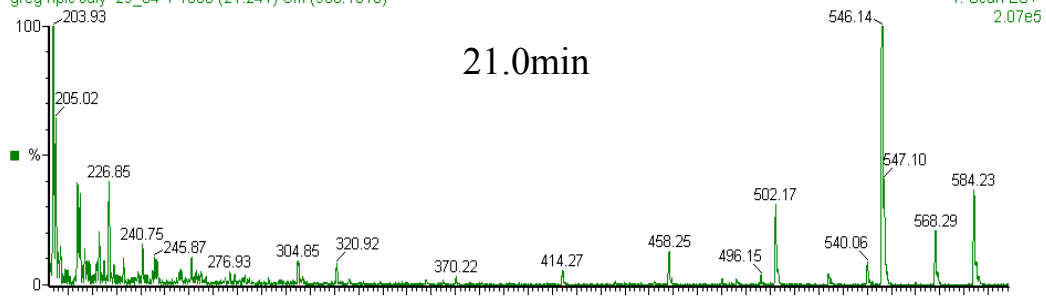
2: Diode Array
1.02e5



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

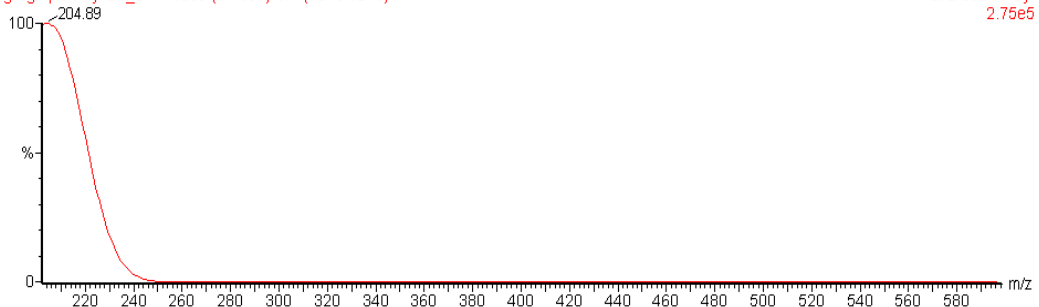
greg hplc July 29_04-1 1003 (21.241) Cm (988:1010)

1: Scan ES+
2.07e5



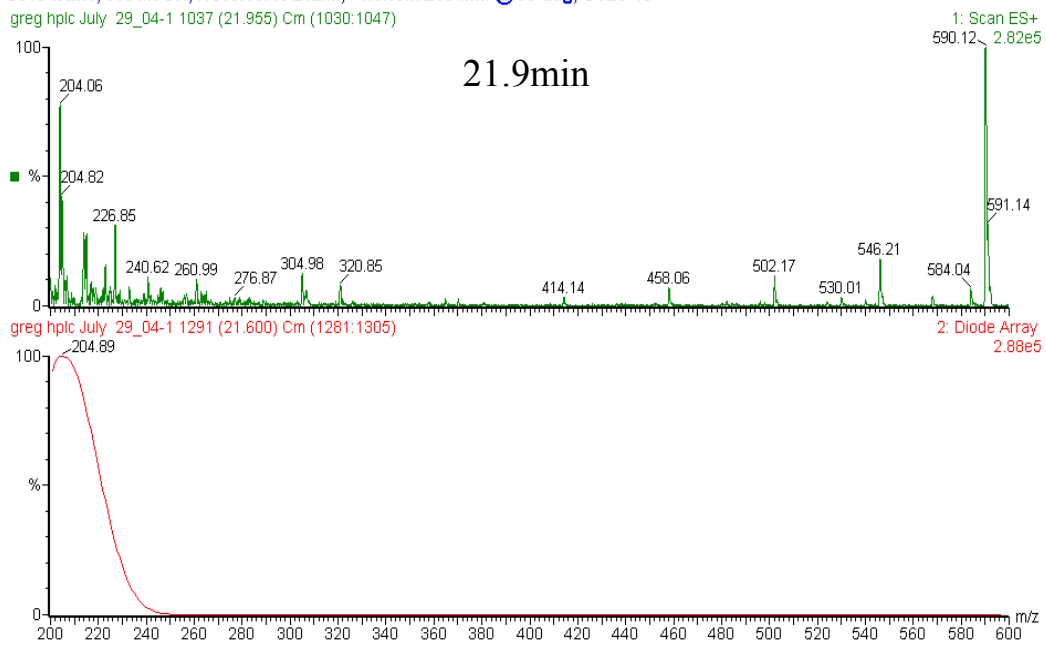
greg hplc July 29_04-1 1255 (21.000) Cm (1248:1270)

2: Diode Array
2.75e5



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

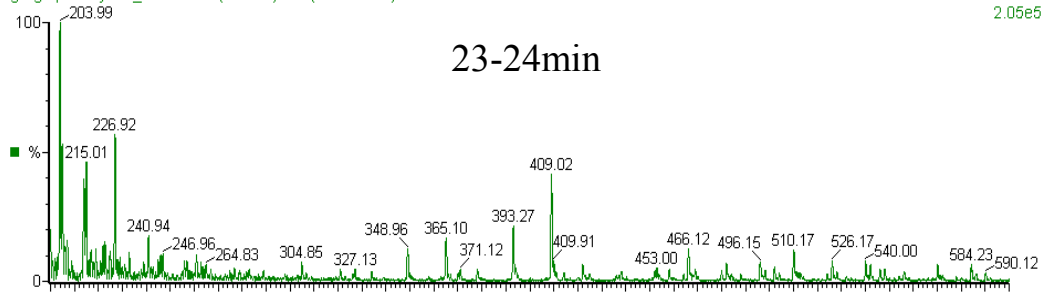
greg hplc July 29_04-1 1037 (21.955) Cm (1030:1047)



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

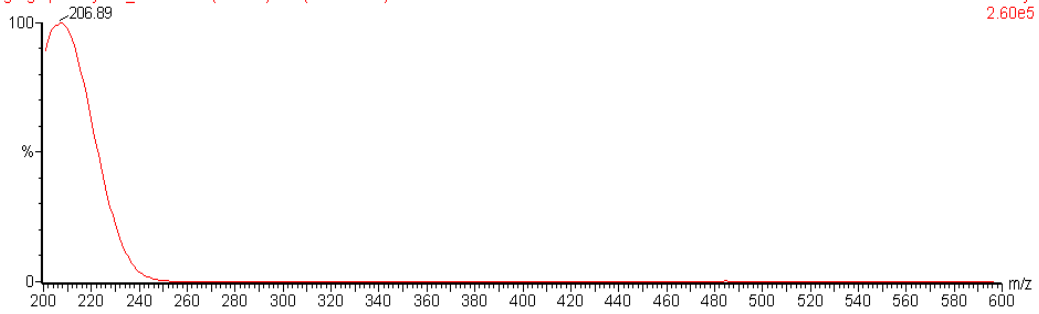
greg hplc July 29_04-1 1148 (24.284) Cm (1092:1148)

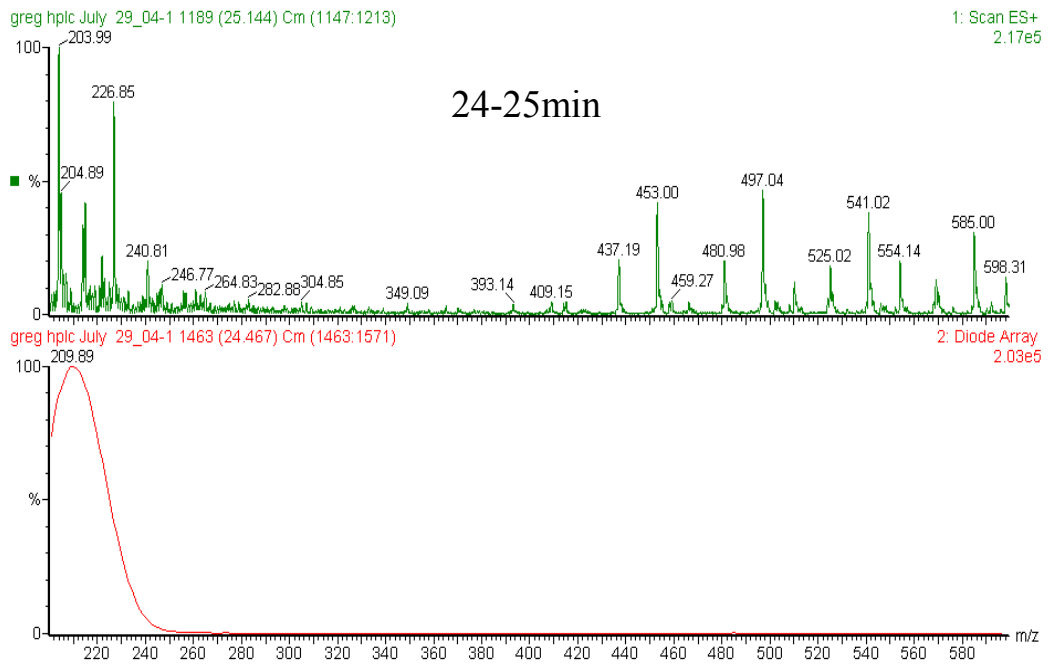
1: Scan ES+
2.05e5



greg hplc July 29_04-1 1376 (23.017) Cm (1349:1438)

2: Diode Array
2.60e5



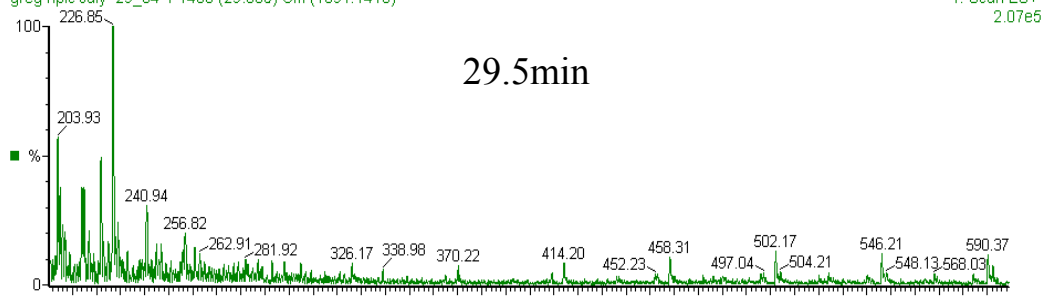


95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

greg hplc July 29_04-1 1403 (29.635) Cm (1391:1418)

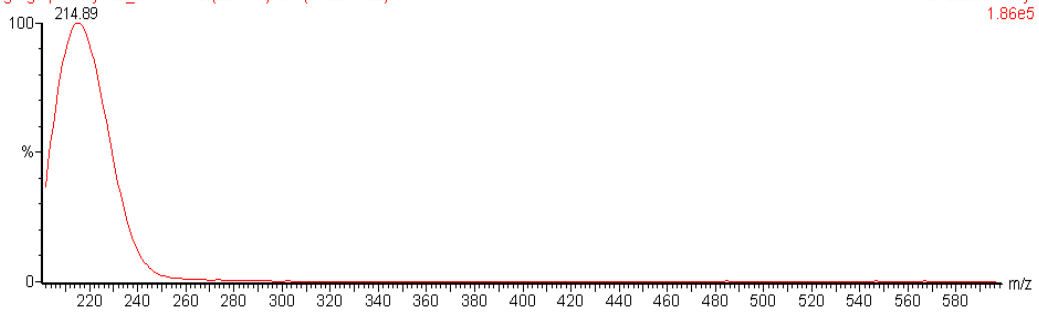
1: Scan ES+
2.07e5

29.5min



greg hplc July 29_04-1 1778 (29.717) Cm (1738:1782)

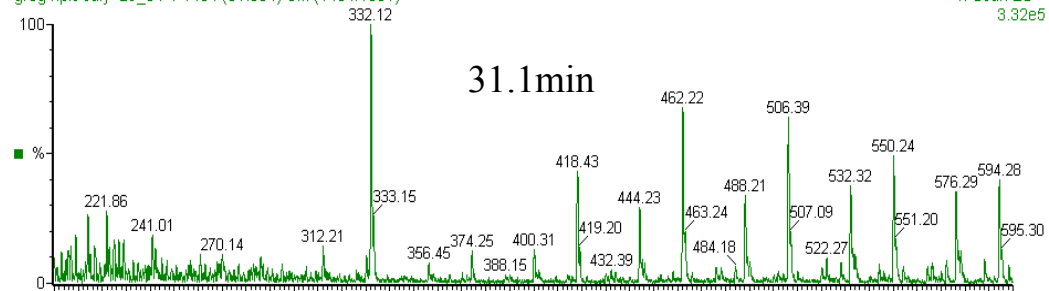
2: Diode Array
1.86e5



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

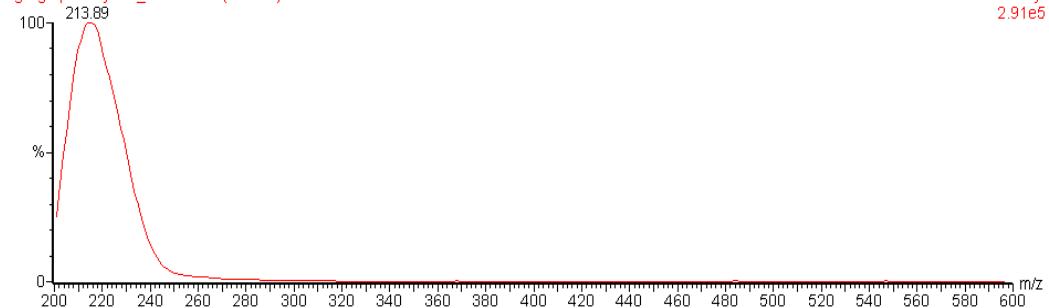
greg hplc July 29_04-1 1484 (31.334) Cm (1484:1501)

1: Scan ES+
3.32e5



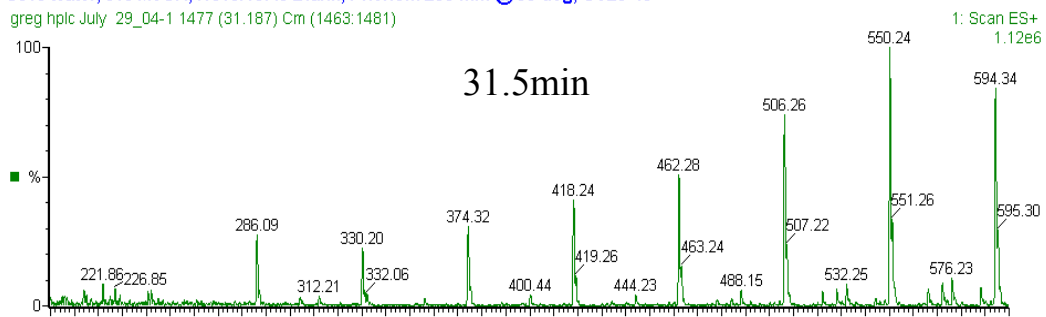
greg hplc July 29_04-1 1895 (31.667)

2: Diode Array
2.91e5



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

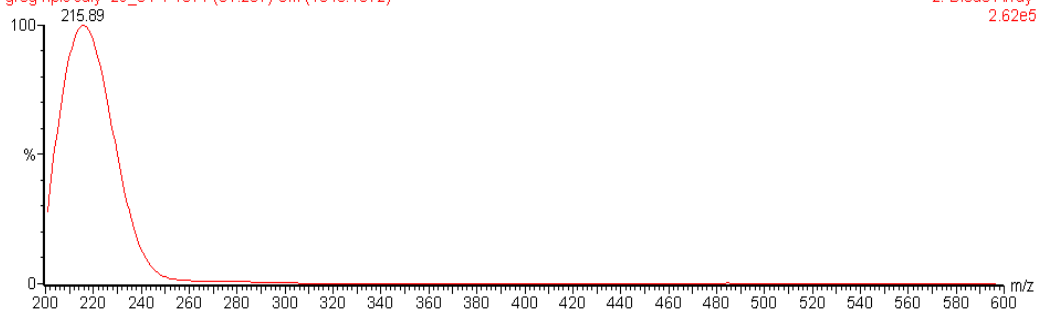
greg hplc July 29_04-1 1477 (31.187) Cm (1463:1481)



1: Scan ES+
1.12e6

greg hplc July 29_04-1 1871 (31.267) Cm (1840:1872)

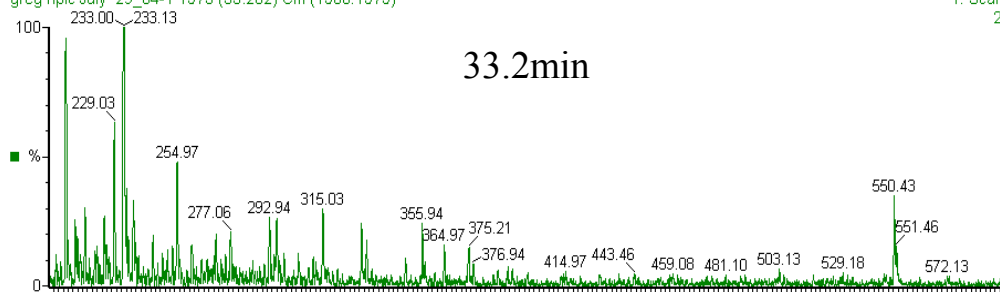
2: Diode Array
2.62e5



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @ 35 deg, CV20-40

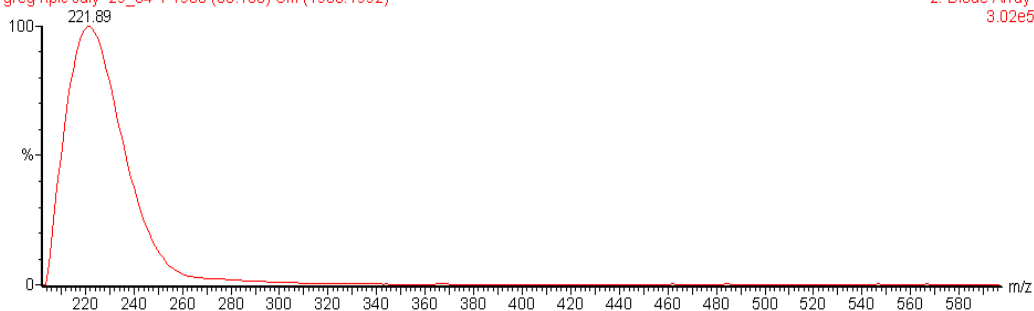
greg hplc July 29_04-1 1573 (33.202) Cm (1568:1575)

1: Scan ES+
2.25e5



greg hplc July 29_04-1 1986 (33.183) Cm (1980:1992)

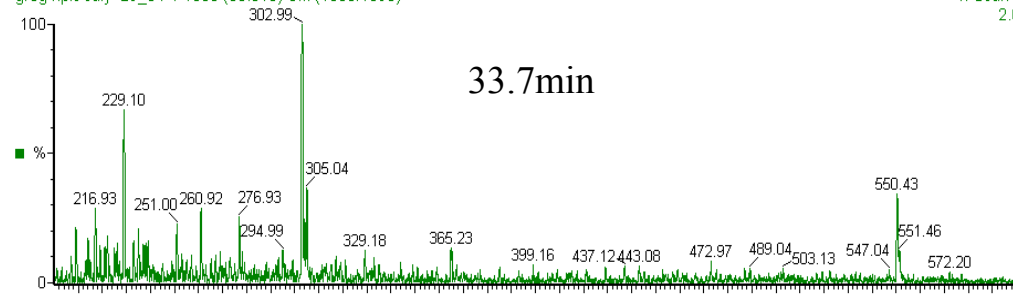
2: Diode Array
3.02e5



95% water, 5% MeCN, .18%HOAc Blank, Phenom 250 mm @35 deg, CV20-40

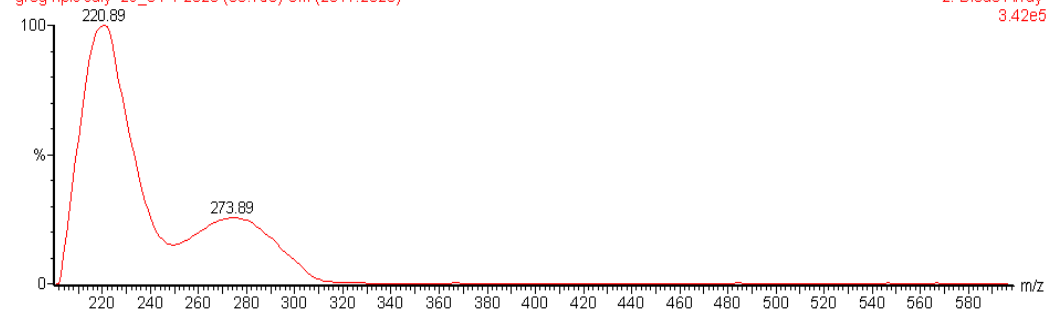
greg hplc July 29_04-1 1588 (33.516) Cm (1588:1596)

1: Scan ES+
2.00e5



greg hplc July 29_04-1 2020 (33.750) Cm (2017:2023)

2: Diode Array
3.42e5



APPENDIX 2.

SUPERVISORY/EXAMINATION COMMITTEE MEMBERS

Supervisory Committee

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Chair:
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