

ABSTRACT

Title of Dissertation / Thesis: **ABSORPTION, EXCRETION, AND TRANSFORMATION OF INDIVIDUAL ANTHOCYANINS IN RATS**

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Anthocyanins are polyphenolics responsible for most red to purple colors in plants. Human consumption is increasing because of their potential health benefits and use as natural colorants. However, their absorption and metabolism are not well characterized. We compared anthocyanin absorption and excretion in rats receiving chokeberry, bilberry or grape enriched diet (4g anthocyanin/kg) for 13 weeks. Traces of anthocyanins and metabolites were detected in plasma. In urine, intact anthocyanins and methylated derivatives (~ 24, 8, 15 mg cy-3-gla equivalent/L urine for chokeberry, bilberry, grape) were found. High metabolite concentration suggested accumulation of methylated anthocyanins in tissue. Fecal anthocyanin extraction was maximized with aqueous methanol (60%). Anthocyanin concentration in feces ranged from 0.7 to 2g anthocyanin/kg, similar to cecal content. In the gut, anthocyanin degradation was high for glucosides, moderate for galactosides and negligible for arabinosides and xylosides. Both, glycosylation and acylation seemed to affect the bioavailability of anthocyanins *in vivo*.

ABSORPTION, EXCRETION, AND TRANSFORMATION OF INDIVIDUAL
ANTHOCYANINS IN RATS

By

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Dedication

The thesis is dedicated to my beloved father Shide He and mother Zonghui Hu, for supporting my education, and to my dear wife Weishu Xue, from whom the time devoted to this thesis has been withdrawn.

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I would like to thank my advisor Dr M. Monica Giusti for her support all the time. Her advice and encouragement throughout the research, even after her leaving from University of Maryland, guided me to achieve every success. Her valuable experience and outstanding intelligence opened my vision and nourished my thought. Her coordination among different research groups made this project possible. I want to thank Dr Bernadene A. Magnuson, my co-advisor, for her contribution on the rats feeding trial, and her devoted guidance to me. I would also thank another member of my advisory committee, Dr Liangli Yu for her invaluable opinions to my research.

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List of Tables

Table 1 Anthocyanin content in selected common fruits, vegetables, beverages and wines	13
Table 2 The composition of experimental diets.....	36
Table 3 Individual anthocyanins recovered from feces spiked chokeberry ARE.....	50
Table 4 Individual anthocyanins recovered from feces spiked bilberry ARE	52
Table 5 Individual anthocyanins recovered from feces spiked grape ARE.....	54
Table 6 Fecal and urinary anthocyanin percentage peak areas in chokeberry treatment	62
Table 7 Fecal and urinary anthocyanin percentage peak areas in bilberry treatment.	63
Table 8 Fecal and urinary anthocyanin percentage peak areas in grape treatment.....	64

List of Figures

Figure 1. Basic structure of anthocyanins.....	7
Figure 2. Structure of common anthocyanidin glucoside	8
Figure 3. Chemical structure of acylated pelargonidin derivatives	9
Figure 4. Common cinnamic acids acylated with sugar moieties on anthocyanins...	10
Figure 5. Common aliphatic acids acylated with sugar moieties on anthocyanins ...	10
Figure 6. Conformations of anthocyanins in aqueous solution under varying pH	23
Figure 7. The generalized effect of pH value on non-acylated mono glucoside anthocyanin equilibria.....	24
Figure 8. Formation of colorless anthocyanin-sulfonic acid adducts	25
Figure 9. Structures of vitisins from <i>Vitis vinifera</i>	25
Figure 10. Co-pigmentation of wine anthocyanins.....	28
Figure 11. Suggested mechanism of anthocyanin-metal-ascorbic acid complexation	29
Figure 12. Molecular complex between an anthocyanidin (apigenidin) and a polyhydroxy-flavone sulfonate	30
Figure 13. Scheme of the copigmentation reaction	31
Figure 14. Relative efficiency of four solvents on fecal anthocyanin extraction	46
Figure 15. Typical UV-HPLC chromatogram of fecal anthocyanins recovered from chokeberry anthocyanin rich extract spiked feces	49
Figure 16. Typical UV-HPLC chromatogram of fecal anthocyanins recovered from bilberry anthocyanin rich extract spiked feces.....	50
Figure 17. Typical UV-HPLC chromatogram of fecal anthocyanins recovered from grape anthocyanin rich extract spiked feces	51
Figure 18. Recovery of spiked anthocyanins in feces.....	56
Figure 19. Total anthocyanins recovered from rat urine.....	60
Figure 20. Typical UV-HPLC chromatograms of urinary anthocyanins detected in chokeberry, bilberry, and grape diet groups	69
Figure 21. Total anthocyanins recovered from rat feces.....	72
Figure 22. Comparison of anthocyanins in the chokeberry anthocyanin rich extract to the cecal and fecal content from a rat fed chokeberry diet	77
Figure 23. Comparison of anthocyanins in the bilberry anthocyanin rich extract to the cecal and fecal content from a rat fed bilberry diet	78
Figure 24. Comparison of anthocyanins in the grape anthocyanin rich extract to the cecal and fecal content from a rat fed grape diet	79
Figure 25. Anthocyanins and anthocyanin-like compounds detected in rat plasma in control, chokeberry, bilberry, and grape diet groups	81
Figure 26. Spectrum of the two compounds of interest in plasma.....	83
Figure 27. Typical UV-HPLC chromatograms of SPE purified plasma at 320 nm in control, chokeberry, bilberry, and grape diet groups	84
Figure 28. Proposed three possible structures of compound 1 in plasma.....	85

List of Abbreviations

Ac	Acetate
ACF	Aberrant Crypt Foci
ADI	Acceptable Daily Intake
Arab	Arabinoside
ARE	Anthocyanin Rich Extract
Coum	p-coumarate
CRD	Completely Randomized Design
Cya (Cy)	Cyanidin
Del (Dp)	Delphinidin
Gal	Galactoside
GIT	Gastrointestinal Tract
Glu	Glucoside
HPLC	High Performance Liquid Chromatography
IACUC	Institutional Animal Care and Use Committee
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LPH	Lactase Phlorizin Hydrolase
Mal (Mv)	Malvidin
MS	Mass Spectrum
NOEL	No Observed Effect Level
PC	Protocatechuic acid
Pel (Pg)	Pelargonidin
Peo (Pn)	Peonidin
Pet (Pt)	Petunidin
PPO	Polyphenol Oxidase
SGLT1	Sodium Dependant Glucose Transporter
SPE	Solid Phase Extraction
UMCP	University of Maryland at College Park
UV	Ultraviolet
Xyl	Xyloside

Table of Contents

Dedication	ii
List of Figures	vi
List of Abbreviations	vii
Table of Contents	viii
Chapter 1: Introduction	1
Chapter 2: Literature Review	5
2.1. Chemical Structures of Anthocyanins	5
2.1.1. Basic Structure	5
2.1.2. Glycosylation	7
2.1.3. Acylation	9
2.2. Distribution, Daily Intake and Safety	11
2.2.1. Distribution	11
2.2.2. Daily Intake	14
2.2.3. Toxicity and Safety	15
2.3. Different Bioactivities Among Individual Anthocyanins	16
2.3.1. Differences Associated With Aglycones	16
2.3.2. Differences Associated With Sugar Moieties	18
2.3.3. Differences Associated With Organic Acid Acylation	21
2.4. The Biochemistry and Stability of Anthocyanins	21
2.4.1. The Major Factors Influencing Stability	21
2.4.2. The Influence of pH	22
2.4.3. Substitution	24
2.4.4. Acylation	25
2.4.5. Copigmentation	27
2.4.6. Condensation	31
2.4.7. Enzymatic System	32
2.4.8. Other Influencing Factors	33
Chapter 3: Materials and Methods	35
3.1. Chemicals and Materials	35
3.2. Animals and Experimental Design	35
3.2.1. Diets and Treatments	35
3.2.2. Animal Care and Feeding Trial	37
3.3. Sample Collection	38
3.3.1. Urine and Feces Collection	38
3.3.2. Plasma and Cecal Content Collection	38
3.4. Feces Anthocyanin Extraction Method Development	39
3.4.1. Method Development	39
3.4.2. Recovery Tests	40
3.5. Determination of Anthocyanins	41
3.5.1. Semi-purification of Anthocyanins in the Plasma and Urine	41
3.5.2. Extraction and Semi-purification of Anthocyanins in the Feces and Cecal Content	41

3.5.3.	High Performance Liquid Chromatography (HPLC) Analysis	42
3.5.4.	Standards and Calibration Curves.....	43
3.5.5.	Statistical Analysis.....	43
Chapter 4: Results and Discussion.....		44
4.1.	Fecal Anthocyanin Extraction Method Development.....	44
4.1.1.	Solvents Comparison	45
4.1.2.	Stability and Recovery	48
4.1.2.1.	Stability of Fecal Anthocyanins	48
4.1.2.2.	Recovery of the Extraction Method.....	55
4.2.	Anthocyanin Absorption, Excretion and Transformation in Rats	58
4.2.1.	Anthocyanin in Urine.....	59
4.2.1.1.	Urinary Anthocyanin Concentration.....	59
4.2.1.2.	Effect of Sugar Moiety on Urinary Anthocyanin Content.....	61
4.2.1.3.	Influence of Acylation on Absorption	65
4.2.1.4.	Anthocyanin Metabolites in Urine.....	66
4.2.2.	Anthocyanin in Gut Content	70
4.2.2.1.	Gut Content Anthocyanin	70
4.2.2.2.	Effects of Sugar Moieties on Gut Content Anthocyanins.....	73
4.2.2.3.	Proposed Mechanisms of Anthocyanin Degradation in Gut.....	75
4.2.3.	Anthocyanins and Possible Anthocyanin Metabolites in Plasma	79
4.2.3.1.	Determination of Anthocyanins in Plasma	79
4.2.3.2.	Determination of Possible Anthocyanin Metabolites in Plasma.....	82
Chapter 5: Summary and Future Research		86
Appendices.....		88
References.....		89

Chapter 1: Introduction

As the most abundant antioxidants in our diets, polyphenols are receiving increasing interest from consumers and food manufacturers (Scalbert and Williamson 2000). Among all the recognized polyphenols, anthocyanins are especially important because of their unparalleled consumption, which was estimated to be 100+ mg/day (Hertog *et al.* 1993; Galvano 2004; Prior 2004). They are very widespread in fruits, vegetables and processed foods or beverages like juices and wines (Francis 1989; Clifford 2000), and have been consumed by humans for generations after generations. Recently as the worldwide concern about synthetic food dyes rises, demands and interests in anthocyanins as a potential natural colorant alternative are steadily increasing (Francis 1989; Giusti and Wrolstad 2003).

The explosive interest in anthocyanins was aroused by the recognition of their potential health benefits (Giusti and Wrolstad 2003). Epidemiological studies have suggested associations between the consumption of anthocyanin-rich wines and the prevention of coronary heart disease, which was known as the “French Paradox” (Renaud and De Lorgeril 1992). Later on, a vast number of studies have been carried out on the potential benefits of anthocyanins on human health. To date, the potential health benefits of anthocyanins include radical scavenging (Tsuda *et al.* 1994; Pool - Zobel *et al.* 1999), inhibition of lipoprotein oxidation and platelet aggregation (Whitehead *et al.* 1995; Tsuda *et al.* 1996; Ramirez -tortosa *et al.* 2001; Kay and Holub 2002), anti-inflammatory activity (Wang *et al.* 1999), reduction of capillary

permeability and fragility (Cohen-Boulakia *et al.* 2000), prevention of obesity (Tsuda *et al.* 2003), protecting on liver ischemia (Tsuda *et al.* 1999), anti-neoplastic and anti-tumor activity (Koide *et al.* 1997; Kamei *et al.* 1998; Hagiwaraa *et al.* 2001; Harris *et al.* 2001; Malik *et al.* 2003; Singletary *et al.* 2003), improvement of eye vision (Nakaishi *et al.* 2000; Canter and Ernst 2004), control of diabetes (Jankowski *et al.* 2000), and others. All the effects listed above are more or less related to the antioxidant mechanisms (Kong *et al.* 2003).

However, there are two critical issues that may impair the merit of anthocyanins. First, although anthocyanins can have antioxidant effects in cell culture and other in vitro systems at relatively high concentrations, it is not clear whether concentrations can be reached in vivo at the tissue level to produce antioxidant effects (Prior 2004). Numerous studies have suggested the low bioavailability of anthocyanins as indicated by the very low recovery in the plasma and urine after ingestion (Lapidot *et al.* 1998; Cao and Prior 1999; Miyazawa *et al.* 1999; Bub *et al.* 2001; Murkovic *et al.* 2001; Felgines *et al.* 2002; Mazza *et al.* 2002; Milbury *et al.* 2002; Wu *et al.* 2002; Frank *et al.* 2003; Mcghie *et al.* 2003; Nielsen *et al.* 2003; Felgines *et al.* 2003). Second, anthocyanins may not have sufficient stability to survive the physiological conditions during absorption and distribution. In any cell or tissue culture study using anthocyanins, one must be aware that at neutral pH, the anthocyanins may degrade (Prior 2004). In addition, in the gastrointestinal tract and body tissues, enzymes such as β -glucosidase may also accelerate the degradation of anthocyanins (Tsuda *et al.* 1999; Tsuda *et al.* 2000). It is not clear whether anthocyanins remain intact in tissues long enough to act as antioxidants.

This study was designed to contribute information for answering the above two uncertainties. Anthocyanins in the urine, plasma, cecal content and feces of rats fed anthocyanin rich extracts (AREs) as well as control diet were analyzed to evaluate the absorption, metabolism, and excretion of anthocyanins in vivo. Three AREs, namely chokeberry, bilberry, and grape extracts, were chosen as the additives in diets. They were all reported to be good sources of antioxidants (Camire 2002; Skrede and Wrolstad 2002; Shahidi and Naczsk 2004a), yet contained a wide variety of individual anthocyanins, which might provide valuable information on the difference of individual anthocyanins. Unlike many other animal studies, in which relatively high intakes (up to 400 mg/kg BW) of anthocyanins were used to observe health-related responses in a short time (Tsuda *et al.* 1999; Prior 2004), relatively low dose diet and long term feeding were employed in our study to simulate the normal intake of anthocyanins by humans.

A focus of our study is the comparison of individual anthocyanin compounds. It's not surprising that the chemical structure of anthocyanins will affect their biological properties. The number of hydroxyl groups, type of sugar moieties, as well as the acylated groups obviously can influence the polarity, size, and spatial conformations of individual compounds, and consequently have a certain impact on the bioavailability. Studies on the bioavailability of anthocyanins have been widely carried out (Cao and Prior 1999; Tsuda *et al.* 1999; Miyazawa *et al.* 1999; Tsuda *et al.* 2000; Cao *et al.* 2001; Matsumoto *et al.* 2001; Felgines *et al.* 2002; Milbury *et al.* 2002; Mazza *et al.* 2002; Wu *et al.* 2002; Nielsen *et al.* 2003; Felgines *et al.* 2003). Differences among individual anthocyanins have been noticed (Felgines *et al.* 2002;

Wu *et al.* 2002), but generally the studies didn't focus on the comparison of individual anthocyanins, thus available information in this field is scarce. Our objective was to provide information for the screening of more bioavailable anthocyanins in foods.

Chapter 2: Literature Review

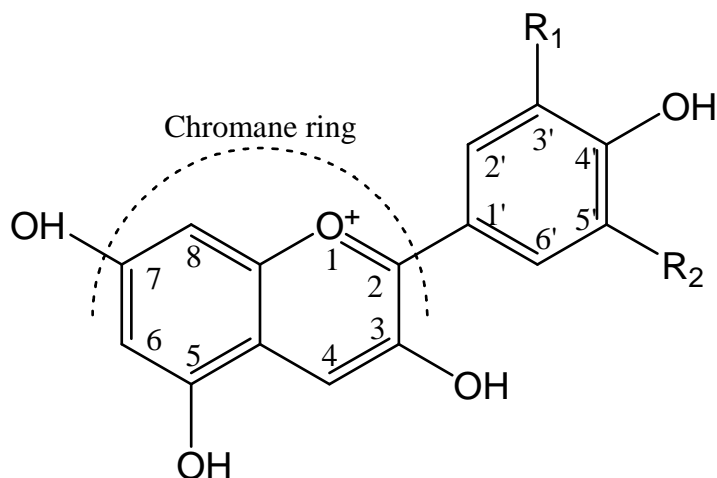
Anthocyanins are generally accepted as the largest and most important group of water-soluble pigments in nature (Harborne 1998; Clifford *et al.* 2000; Eder 2000; Takeoka and Dao 2002). The word anthocyanin derived from two Greek words anthos, which means flower, and kyanos, which means dark blue, reveals its important characteristic as a natural colorant (Eder 2000; Delgado-Vargas *et al.* 2000; Delgado-Vargas and Paredes-López 2003). In plant tissues the anthocyanins are responsible for the attractive red, purple, violet and blue colors (Brouillard 1982; Takeoka and Dao 2002). Contributing to the colorful appearance of fruits, vegetables, and flowers, anthocyanins help them to attract animals, leading to seed dispersal and pollination. Mazza and Miniati (1993) reported that anthocyanins might be important in protecting plants against ultraviolet-induced damage.

2.1. Chemical Structures of Anthocyanins

2.1.1. Basic Structure

The anthocyanins are hydroxylated and methoxylated derivatives of phenyl-2-benzopyrylium or flavylium salts, regarded as flavonoid compounds (Eder 2000). Their basic structure of the aglycone shown in Figure 1 is a C-6 (A-ring)-C-3 (C ring)-C-6 (B ring) carbon skeleton, figuring a chromane ring bearing a second aromatic ring B in position 2 (Harborne 1998). Until 2003, there were 17 known

naturally occurring anthocyanidins or aglycones, but only six are common in higher plants - cyanidin (cy), peonidin (pn), pelargonidin (pg), malvidin (mv), delphinidin (dp), and petunidin (pt) (Eder 2000; Kong *et al.* 2003), with cyanidin being the most common (Harborne 1998). Other 11 anthocyanidins including apigeninidin, aurantinidin, capensinidin, europinidin, hirsutidin, 6-hydroxycyanidin, luteolinidin, 5-methylcyanidin, pulchellidin, rosinidin, and tricetinidin have also been detected in plants (Hou 2003). The differences in chemical structure of these six common anthocyanidins occur at the 3' and 5' positions (Figure 1). Due to the long chromophore of eight conjugated double bonds carrying a positive charge (Figure 1), anthocyanins are intensely colored under acidic conditions. The maximum absorption in the visible region is usually between 465 and 550 nm, while the other maximum absorption band falls in the UV range between 270 and 280 nm (Eder 2000). Interestingly, the color is affected by the number of hydroxyl and methoxyl groups (Figure 1): if more hydroxyl groups, then the color goes toward a more bluish shade; if more methoxyl groups, then redness is increased (Heredia *et al.* 1998; Delgado-Vargas and Paredes-López 2003).



Name	Substitution		Visible color	Visible max. (nm) in MeOH-HCl
	R ₁	R ₂		
Cyanidin (Cy)	OH	H	} magenta	535
Peonidin (Pn)	OCH ₃	H		532
Pelargonidin (Pg)	H	H	red	520
Malvidin (Mv)	OCH ₃	OCH ₃	} purple	542
Delphinidin (Dp)	OH	OH		546
Petunidin (Pt)	OCH ₃	OH		543

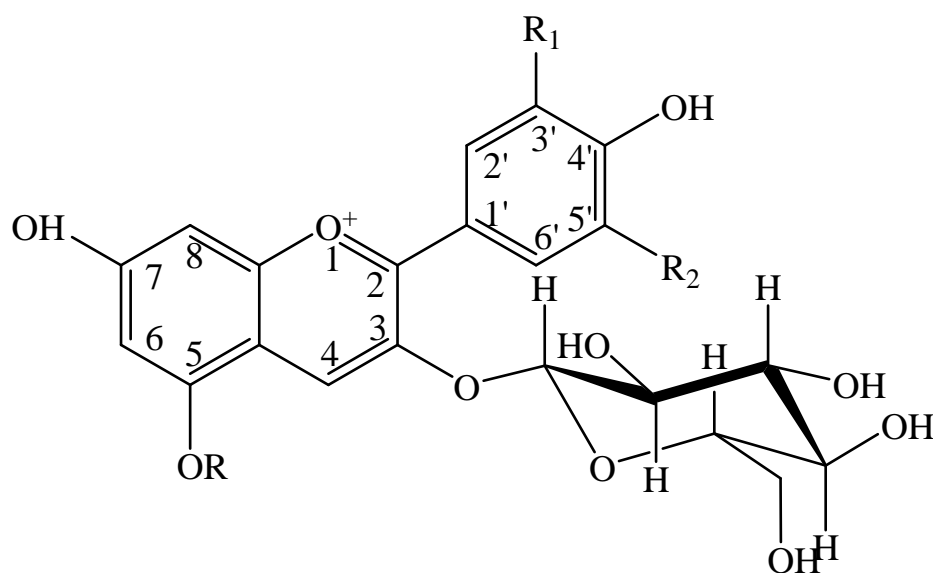
Adapted from Harborne (1998).

Figure 1 Basic structure of anthocyanins (flavylium cation).

2.1.2. Glycosylation

The aglycones are rarely found in fresh plant material (Clifford 2000; Prior 2004) or commercial products such as wine (Waterhouse 2002), except in trace quantities, because they are quite unstable. The glucosides of anthocyanidins are called anthocyanins (Willstätter 1920). The stability is enhanced by one or more

sugar molecules bonded at different hydroxyl positions. With a few exceptions anthocyanins are always glycosylated at C-3 (Takeoka and Dao 2002). Besides the C-3 position, other sugars can also be attached at any one of the hydroxyls at C-5, C-7, C-3', C-5', and even C-4' (Brouillard 1988; Mazza and Miniati 1993; Eder 2000). Usually anthocyanidin glycosides are 3-monosides and 3, 5-diglycosides as shown in Figure 2. Sometimes 3, 7-diglycosides or 3-triosides are also known to occur (Clifford 2000). The most common sugar is glucose, but rhamnose, xylose, galactose, arabinose, and fructose as well as rutinose (6-O- α -L-rhamnosyl-D-glucose), sophorose (2-O- β -D-xylosyl-D-glucose), gentobiose (6-O- β -D-glucosyl-D-glucose), sambubiose (2-O- β -D-xylosyl-D-glucose), xylosylrutinose and glycosylrutinose may also be present (Clifford 2000; Takeoka and Dao 2002; Delgado-Vargas and Paredes-López 2003; Shahidi and Naczki 2004b).



Anthocyanidin 3-glucoside, R = H
 Anthocyanidin 3,5-diglucoside, R = glucose

Figure 2 Structure of common anthocyanidin glucoside.

2.1.3. Acylation

The sugar residues may be further acylated with organic acids (Mazza 1993; Giusti *et al.* 1998; Eder 2000) (Figure 3). Common acylating agents include cinnamic acids such as caffeic, p-coumaric, ferulic and sinapic acid (Figure 4), as well as a range of aliphatic acids such as acetic, malic, malonic, oxalic, and succinic acid (Figure 5). Cinnamic acids may themselves bear glycosidic sugars. Aromatic and aliphatic acylation may occur in the same molecule, and from zero to at least three acylating residues may be present (Harborne 1998; Clifford 2000; Eder 2000; Takeoka and Dao 2002).

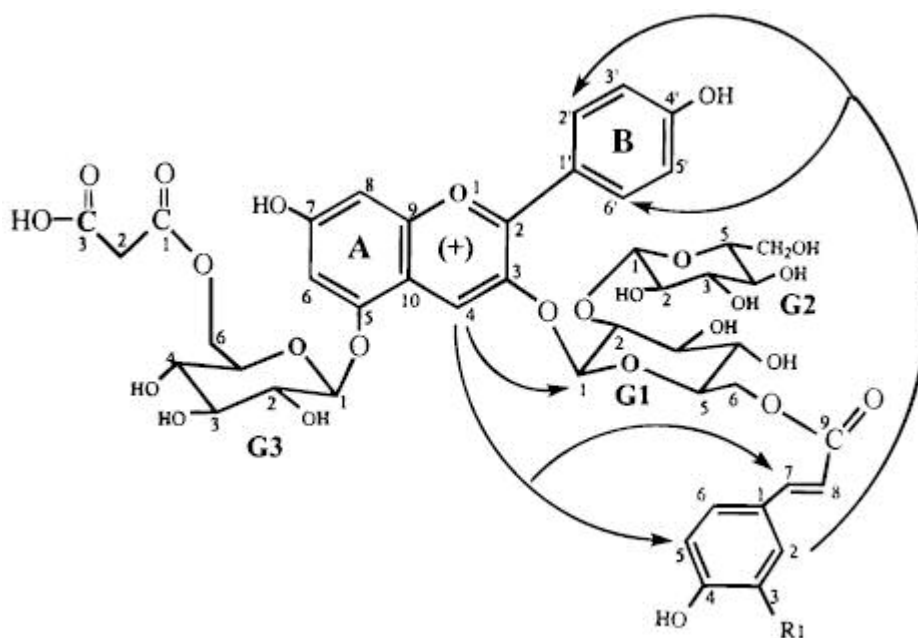


Figure 3 Chemical structure of acylated pelargonidin derivatives encountered in red radish. Arrows indicate hydrogens close in space. (Source: Giusti *et al.* 1998)

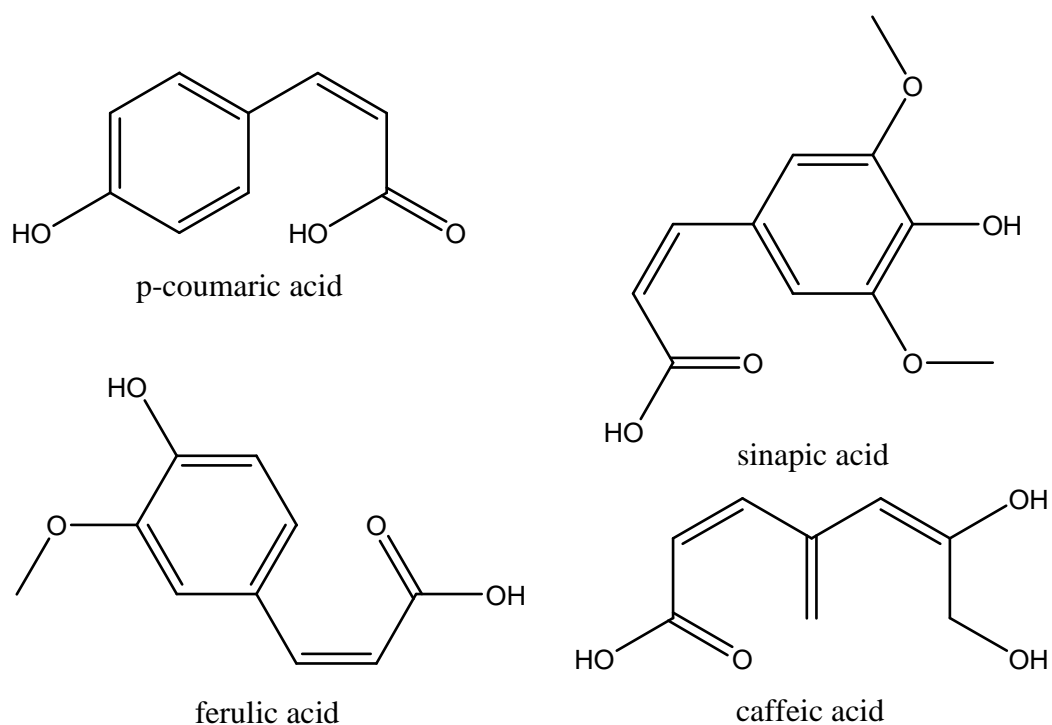


Figure 4 Common cinnamic acids acylated with sugar moieties on anthocyanins.

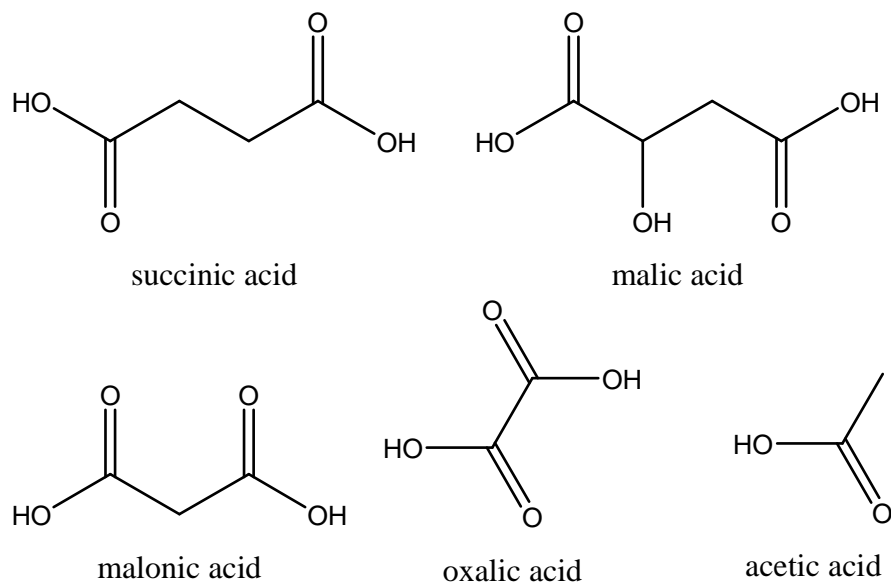


Figure 5 Common aliphatic acids acylated with sugar moieties on anthocyanins.

Since each anthocyanidin may be glycosylated and acylated by various sugars and acids at different positions, a great number of chemical combinations exist (Harborne 1998; Delgado-Vargas and Paredes-López 2003). Based on several reviews, it was estimated that more than 600 anthocyanins had been found in nature (Andersen 2002). Such tremendous variations together with the pH dependent and chelating metal ion dependent color change (Hou 2003) reasonably elucidated the amazing gamut of natural colors.

2.2. Distribution, Daily Intake and Safety

2.2.1. Distribution

Anthocyanins are water-soluble and vacuolar pigments found in most species in the plant kingdom (Harborne 1998; Shahidi and Naczk 2004b). They are produced in most higher plants such as blackberry, red and black raspberries, nectarines, peaches, blueberries, bilberries, cherries, currants, pomegranates, ripe gooseberries, onion, red radish, red soybeans, purple corn, basil, blood orange, elderberries, red cabbage, fennel, red lettuce, grapes, red-skinned potato and purple sweet potato (Eder 2000; Prior 2004; Shahidi and Naczk 2004b). Anthocyanins can be found in all parts of the plants. Though aggregate mostly in flowers and fruits (Brouillard 1988), they also present in leaves, stems and storage organs (Delgado-Vargas and Paredes-López 2003). Among different plants or even cultivars in the same plant, the total anthocyanin content varies considerably, affected by genetic make-up, light, temperature, and agronomic factors (Shahidi and Naczk 2004b). Available data show very wide range of anthocyanin content, though more or less due to the lack of robust

quantification method for anthocyanins (Clifford 2000). Table 1 is a summary based on the information from several researchers. Although content varies widely, anthocyanins profiles are used as a fingerprint for different commodities given that the anthocyanins profile is very typical for each commodity (Giusti *et al.* 1999b; Revilla *et al.* 2001; Chaovanalikit *et al.* 2004; Núñez *et al.* 2004).

The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) (Kong 2003). The three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature. Some commodities like peach skin contain a limited number of anthocyanin pigments, whereas others like hybrid red grapes may contain a mixture of more than 20 (Clifford 2000). Overall, cyanidin aglycone occurs in about 90% of fruits and is the most frequently appearing aglycone (Prior 2004).

In plants the following three classes of anthocyanidin glycosides are common: 3-monoglycosides, 3-biosides, and 3,5-diglycosides. 3-glycosides occur about two and half times more frequently than 3,5-diglycosides (Kong 2003). Considering that glucoside form is the most abundant comparing to other glycosides (Prior 2004), cyanidin 3-glucoside is not surprisingly the most widespread anthocyanin in nature (Kong 2003).

Table 1 Anthocyanin content in selected common fruits, vegetables, beverages and wines.

Commodity	Total anthocyanin concentration (mg/kg)	Sources
Apple (peel)	100-21,600	5
Bilberry	4600	5
Blackberry	820-1800	5, 6
Blueberry	825-5030	1, 5, 6, 9
Boysenberry	1609	6
Cherry (sweet)	3500-4500	5
Cherry (tart)	288	5
Chokeberry	5060-10000	6
Cranberry	460-2000	1, 5, 9
Elderberry	2000-15,600	5, 6
Grape (red)	300-7500	1, 6
Grape (blue)	80-3880	5
Loganberry	774	6
Marion berry	237	9
Orange, Blood (juice)	2000	6
Plum	19-250	1, 5
Raspberry (red)	100-600	1, 5, 6
Raspberry (black)	763-4277	1, 6, 9
Strawberry	127-360	1, 5, 9
Cabbage (red)	250	1
Current (black)	1300-4000	1, 5
Currant (red)	119-186	5
Eggplant	7500	6
Radish (red)	110-600	3
Potato (red)	150-450	4
Purple corn	16420	8
Onion	Up to 250	1
Rhubarb	Up to 2000	1
Wines (red)	90-400	7
Wines (Port)	140-1100	7

Source: ¹Timberlake 1988; ²Mazza and Miniati 1993; ³Giusti and Wrolstad 1998; ⁴Rodríguez-Saona *et al.* 1998; ⁵Eder 2000; ⁶Clifford 2000; ⁷Waterhouse 2002; ⁸Cevallos-Casals *et al.* 2003 and ⁹Prior 2004.

2.2.2. Daily Intake

Anthocyanins are widely ingested by humans, mainly deriving from fruits and red wines (Galvano 2004). The world wide annual consumption has been estimated as 10,000 tons of anthocyanins from black grapes alone (Clifford 2000). Of the various classes of flavonoids, the potential dietary intake of anthocyanins is perhaps the greatest (100+ mg/day per person) (Prior 2004), which is much higher than the intake (23 mg/day per person) estimated for other flavonoids, including quercetin, kaempferol, myricetin, apigenin, and luteolin (Hertog *et al.* 1993; Cao and Prior 1999; Galvano 2004). Depending on the nutritional habits, the daily intake of anthocyanins for individuals has been estimated to range from several milligrams to hundreds of milligrams per person. In the USA, average daily intakes were estimated at 215 mg per person during the summer and 180 mg per person during the winter (Kühnau 1976). However, as pointed out by Timberlake (1988), regular consumers of red wine are likely to have significantly higher intakes.

Intake of anthocyanin is increasing because extracts with high anthocyanin contents from fruits and vegetables like bilberry or elderberry are commercially available. Due to the antioxidant and other potential beneficial properties, grape, cabbage and other natural anthocyanin colorants are becoming more popular. In the future, the use of value added anthocyanins as natural food colorants is expected to steadily increase, following the current trend away from synthetic colors (Gao and Mazza 1994).

2.2.3. Toxicity and Safety

Anthocyanins are generally regarded safe, since they have been consumed by animals and human for countless generations without apparent adverse effects to health (Brouillard 1982). In 1996, a highly purified extract from *V. myrtillus* berries containing 36% anthocyanins was used to test the tolerability and safety of anthocyanins in animals (Morazzoni *et al.* 1996; Hou 2003). In mouse and rats the LD₅₀ values were over 2000 mg/kg, without any toxic symptoms. In dogs, single doses of 3000 mg/kg did not induce any sign of adverse effects. The oral administration of the extract to rats and dogs at doses of 125-150 mg/kg or 80-320 mg/kg daily respectively for 6 months did not induce mortality or any toxic effects. Post marketing surveillance study confirmed the clinical safety (Morazzoni *et al.* 1996). Most of persons took 160 mg twice daily for 1-2 months. Generally the extract was well or very well tolerated, only 4% persons complained side effects, mainly gastrointestinal or concerning skin and cutaneous annexes, and nervous system.

The use of natural anthocyanins for food and beverages is widely permitted within the European Community (E163), Japan, and the United States (Eder 2000). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that anthocyanin-containing extracts are of a very low order of toxicity, based on limited toxicological studies including mutagenicity, reproductive toxicity and teratogenicity. In a two-generation reproduction study the no-observed-effect-level (NOEL) for young rats was determined to be 225 mg/kg body weight (diet containing 7.5% grape skin extract, or 3% anthocyanin pigments, equivalent to 7500 mg diet per kg body

weight). Based on the above result, in 1982 the estimated acceptable daily intake (ADI) for man was calculated to be 2.5 mg/kg body weight per day, using the equation of $ADI = NOEL/100$ (WHO Food Additives Series 17; Clifford 2000).

2.3. Different Bioactivities Among Individual Anthocyanins

Anthocyanins represent a large group of chemically related substances and the effect observed with one specific anthocyanin may not be applicable to another. For instance, Matsumoto *et al.* (2002) reported that in the neutral pH region in human plasma, comparison of the antioxidant activities among the 3-glucosides with five different aglycones and the 3-glycosides with three different sugar moieties at C-3 showed that their strongest activities were given by the delphinidin aglycone and 3-rutinosyl moiety, respectively.

2.3.1. Differences Associated With Aglycones

The structural variations in the B-ring with the presence of hydrogen, hydroxyl or methoxyl substitutions give the six most common anthocyanidins. The number and position of hydroxyl groups and methoxyl groups may significantly vary the aglycones' chemical reactivity.

Wang *et al.* (1997) reported the oxygen radical absorbance capacities (ORAC) of five of the anthocyanidins as following in decreasing order: cyanidin (2.239 ± 0.029) > malvidin (2.009 ± 0.167) > delphinidin (1.809 ± 0.068) > peonidin (1.693 ± 0.035) > pelargonidin (1.540 ± 0.033). Similarly, the trolox equivalent antioxidant

capacity (TEAC) was reported as the decreasing order: cyanidin (4.4 ± 0.01) = delphinidin (4.4 ± 0.01) > peonidin (2.2 ± 0.02) > malvidin (2.1 ± 0.1) > pelargonidin (1.3 ± 0.1) (Rice-Evans *et al.* 1996).

For other health related functions, structure also has important impact. Andriambeloson *et al.* (1998) reported that among the anthocyanidins, delphinidin, but not malvidin or cyanidin, showed endothelium-dependent vasorelaxation. An *in vitro* study showed that the cyanidin and delphinidin were potent inhibitors of the epidermal growth-factor receptor (EGFR) and inhibited the growth of the human tumor cell lines tested (Meiers *et al.* 2001). However, malvidin was completely inactive up to 100 μ M. In a human study involving both elderberry extract and low bush blueberry extract diet, only the cyanidin glycosides were found to exist as methylated or glucuronidated forms in urine (Wu *et al.* 2002).

Anthocyanins with their 3', 4'-dihydroxy groups can rapidly chelate metal ions to form stable anthocyanin-metal complexes (Sarma *et al.* 1997). As a result, anthocyanins with the ortho-dihydroxyl groups have the potential to scavenge hydroxyl radical through the inhibition of \cdot OH generation by chelating iron (Noda *et al.* 1998; Noda *et al.* 2000), and to prevent iron -induced lipid peroxidation (Wang *et al.* 1999). The ortho-dihydroxyl group also helps to form anthocyanin-metal-copigment complexes at physiological pH ranges with various organic compounds such as ascorbic acid (Sarma *et al.* 1997) and partially through this mechanism to spare Vitamin C.

2.3.2. Differences Associated With Sugar Moieties

The sugars combined with the anthocyanidins play an important role in the bioactivities. However, glycosylation may modulate the antioxidant capacity depending on the aglycones (Wang *et al.* 1997). Glycosylation either increased (cyanidin-3-glucoside vs. cyanidin), or decreased (malvadin-3-glucoside vs. malvidin), or did not have a significant effect (pelargonidin-3-glucoside vs. pelargonidin) on the ORAC activity of aglycones. In another study, the light emission (chemiluminescence; CL) were observed in the reaction of anthocyanins with tertbutyl hydroperoxide (t-BuOOH) in the presence of acetaldehyde, and the CL intensity of anthocyanins indicated that glucosylation at 3 and 5 positions of the anthocyanin skeleton enhances the antioxidant effect of anthocyanins on hydroperoxidation (Yoshiki *et al.* 1995).

The type of sugar attached to the aglycone may also have different effects. For example, 3-glycosylation in the C ring of cyanidin increased its antioxidant activity for glucose and rhamnoglucose, but decreased activity for galactose and rutinose. The effects are particularly intriguing when comparing the glucoside and galactosides, because the only difference is the orientation of one hydroxyl group at their pyran ring (Rice-Evans *et al.* 1996; Wang *et al.* 1997). In another study with flavonoids including anthocyanins on the suppression of tumor growth *in vitro*, glucose attachment at the A phenol caused suppression of tumor cell growth, but other sugars such as rhamnose and rutinose at that position did not suppress the growth (Kamei *et al.* 1996).

It has to be emphasized that among all these sugars, glucose seems to be associated with some unique properties of anthocyanins, including the stability, absorption and metabolism. In the alkaline region it has been found that certain anthocyanidin 3-glucosides showed a relatively high stability (Delgado-Vargas and Paredes-López 2003). Bearing in mind that once the anthocyanins are inside the cell and in plasma, the circumstance pH will be above 7, the anthocyanidin 3-glucosides are expected to have better availability if only consider the stability factor. However, the absorption pathway might not function in the same way for all the different glycosides. In the *in vitro* studies there are already evidences that the absorption of some flavonol glucosides includes the interaction with the intestinal sodium dependent glucose transporter (SGLT1) (Gee *et al.* 1998; Gee *et al.* 2000). But there are currently two contradictory explanations. Some researchers suggested that it is possible for the similarly structured anthocyanidin glucosides to be absorbed partially through this pathway (Clifford 2000). The study done by Mülleder *et al.* (2002) indicated such a mechanism also applied to anthocyanins. They observed that the addition of sucrose to the elderberry juice resulted in a delay and lower amount of the anthocyanin excretion in human. There were also other researchers believed that the interaction with SGLT1 did not necessarily indicate that transport occurred, as phenolic glucosides could interact by inhibiting sugar transport but not themselves be transported across the membrane, because of the bulky size of aglycones attached to the glucose (Day *et al.* 2000). Despite the proved direct absorption of anthocyanin glycosides (Cao *et al.* 1999; Miyazawa *et al.* 1999; Matsumoto *et al.* 2001; Wu *et al.* 2002; Mcghee *et al.* 2003), there is another suggested pathway of anthocyanin

absorption. Tsuda *et al.* (1999) speculated that cyanidin-3-glucoside was absorbed in the small intestine as aglycone after being hydrolyzed by β -glucosidase. On the other hand, results from Day *et al.* (1998) demonstrated that the cell-free extracts from human small intestine and liver had the ability to deglycosylate various flavonoid glucosides. In particular, lactase phlorizin hydrolase (LPH), the only mammalian β -glucosidase having an activity within the gut lumen, may be primarily responsible for the hydrolysis (Day *et al.* 2000).

Aside from the sugar type, the position and number of glycosylation may also affect the bioactivity of anthocyanins. As shown by Wang *et al.* (1997), 5-glucosylation significantly decreased the ORAC activity of cyanidin-3-glucoside and pelargonidin-3-glucoside, yet slightly increased the activity of malvidin-3-glucoside. The effect of different sugar molecules on the radical scavenging efficiency was believed to be associated with their ability to enhance or diminish the parent compounds' capacity to form stable radicals. In the *in vivo* studies, Mülleder *et al.* found a greater urinary excretion of cyanidin 3-sambutbioside than cyanidin 3-glucoside. Prior cited his unpublished data to show that the complexity of the glycosidic pattern does not seem to noticeably affect the absorption. However, the reduced excretion of cyanidin-3-glucoside may be the result of increased degradation relative to cyanidin-3-sambubioside in the gastrointestinal tract (Wu and Prior, unpublished data).

2.3.3. Differences Associated With Organic Acid Acylation

Sugar acylation of anthocyanins with cinnamic acids and a range of aliphatic acids induces resistance to other factors such as heat, light, SO₂, high pH, improving the color quality and stability (Delgado-Vargas and Paredes-López 2003). However, the acylation also impairs the bioavailability of anthocyanins (Mazza *et al.* 2002 ; Prior 2004).

2.4. The Biochemistry and Stability of Anthocyanins

2.4.1. The Major Factors Influencing Stability

The color of anthocyanins is based on the fully conjugated 10-electron A-C ring π -system, with some contribution by the B ring as well. If that is disrupted, the color is lost as when anthocyanins are in high pH medium or bleached by bisulfite (Waterhouse 2002). However, the structure in resonance is the cause of their instability, and consequently the groups attached to the structure (namely hydroxyl, methoxyl, glycosyl, and acyl) influence the stability substantially (Delgado-Vargas and Paredes-López 2003).

Other factors like the pH, temperature, light, presence of other phenolic compounds, enzymes, metal ions, sugars, ascorbic acid, and oxygen etc. also have significant impact on the stability of anthocyanins (Shahidi and Naczk 2004b). Generally the anthocyanin degradation follows first-order reaction kinetics (Giusti and Wrolstad 1996a; Eder 2000).

2.4.2. The Influence of pH

In aqueous solution, anthocyanins undergo structural transformations that are pH-dependent (Figure 6), which had been studied and summarized by Brouillard *et al.* (1982). It has been found that four major anthocyanin forms exist in equilibria: the red flavylium cation, the blue quinonoidal base, the colorless carbinol pseudobase, and the colorless chalcone. At pH below 2, anthocyanins exist primarily in the form of the red flavylium cation. Solvation of a flavylium salt in a slightly acidic or neutral aqueous solution results in the immediate formation of neutral and/or ionized quinonoidal bases (Takeoka and Dao 2002). The flavylium cation by virtue of its positive charge is resistant to attack by electrophiles such as aldehyde and quinone (Clifford 2000). Rapid and almost complete hydration of the flavylium cation occurs almost exclusively at the C-2 position to give the colorless carbinol pseudobase at pH values ranging from 3 to 6. This can further equilibrate to an open form, the colorless chalcone pseudobase, at a slower rate. The generalized effect of pH on these equilibria for a non-acylated mono glucoside is illustrated in Figure 7. Interestingly, under the same conditions the 3, 5-diglycosides have a smaller concentration of the cationic form at any given pH value whereas acylated forms have noticeably more cation especially above pH 5 (Dangles *et al.* 1993). As pointed out by Brouillard in 1977, the hemiacetal form is most likely to be the target for attacking at slightly acidic pH. This was evidenced by later discovered anthocyanin-quinone adducts in the hemiacetal form detected by LC-MS (Sarni-Manchado *et al.* 1997).

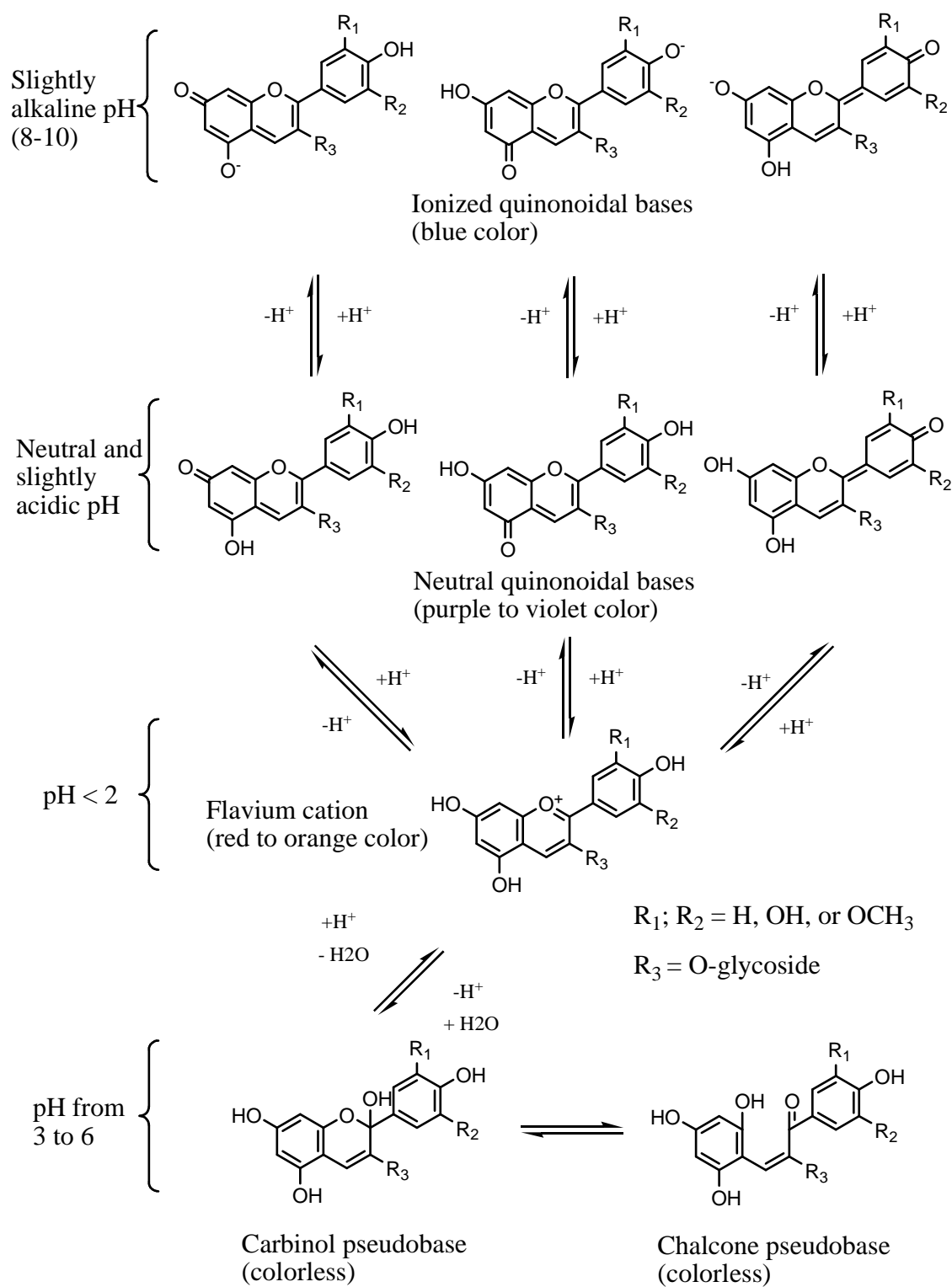


Figure 6 Conformations of anthocyanins in aqueous solution under varying pH. (Adapted from Brouillard 1988)

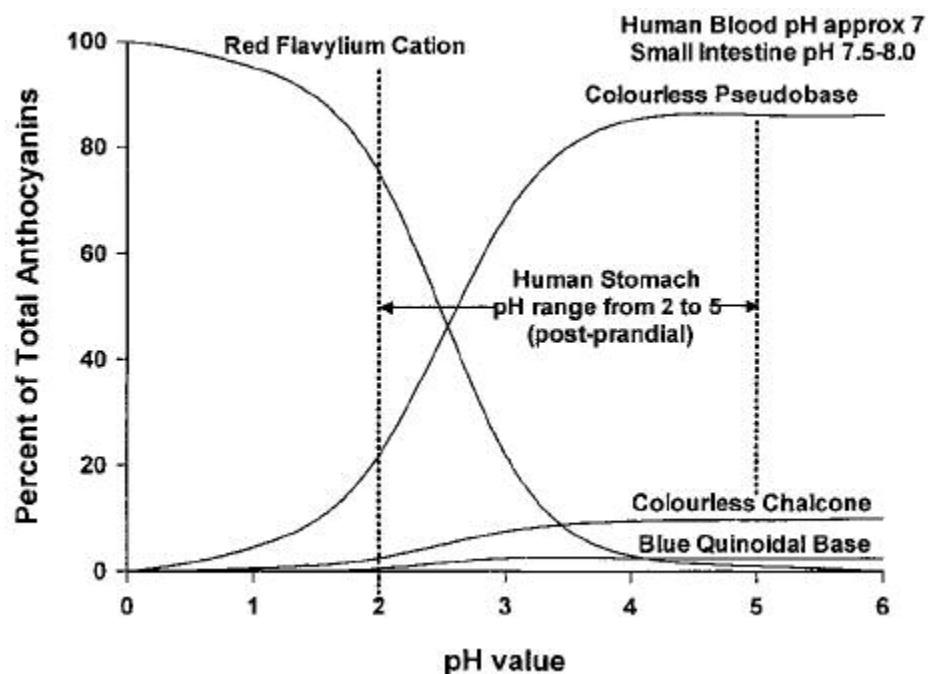


Figure 7 The generalized effect of pH value on non-acylated mono glucoside anthocyanin equilibria. (Source: Brouillard 1982)

2.4.3. Substitution

Anthocyanins are stabilized by the substitution at the C-4 position. This inhibits the addition of water and subsequent formation of colorless species (Mazza and Miniati 1993). Monomeric anthocyanin pigments will combine with bisulfite to form a colorless adduct, the sulfonic acid substitution occurring at the C-4 position (Figure 8). Polymeric anthocyanins and some wine pigments will not be bleached since the C-4 position is blocked. For instance, Vitisins isolated from *Vitis vinifera* contain a link between C-4 and the 5-hydroxyl group of the molecule, improving characteristic of color and stability (Figure 9). Their resistance to sulfur dioxide and high pH values has been observed (Bakker and Timberlake 1997). The absorbance at

420 nm of the bisulfite-treated sample is an index for browning (Wrolstad *et al.* 2002).

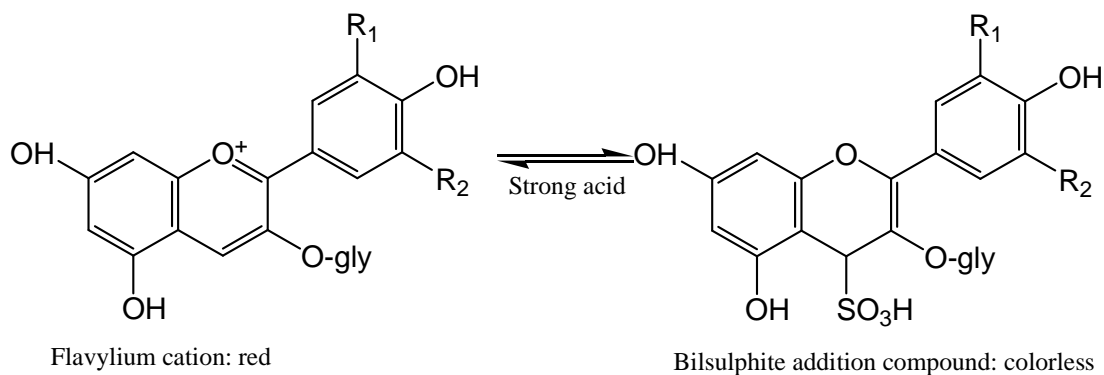


Figure 8 Formation of colorless anthocyanin-sulfonic acid adducts. (Source: Giusti *et al.* 2001)

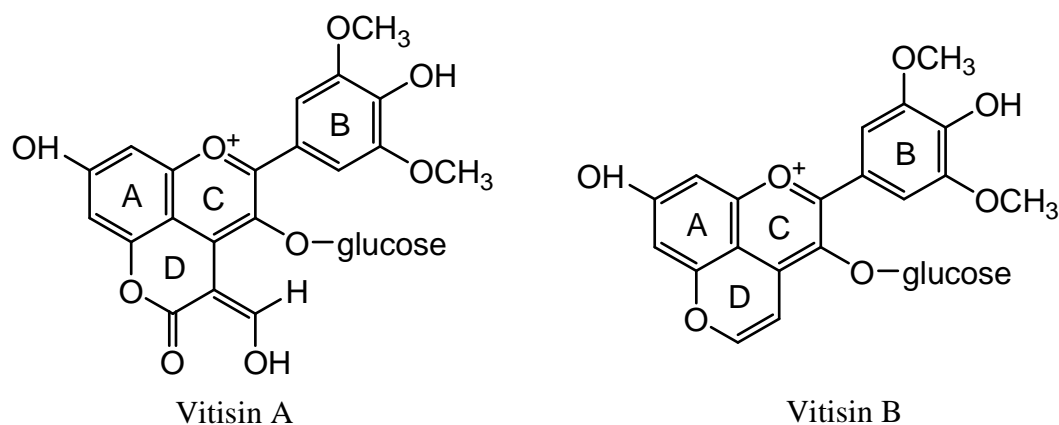


Figure 9 Structures of vitisins from *Vitis vinifera*. (Source: Bakker and Timberlake 1997)

2.4.4. Acylation

It is well established that acylation, particularly with cinnamic acids, will give much greater stability to the pigment. The improved stability is believed to be due to the intramolecular copigmentation, later described. Acylation usually occurs with the

sugar at the C-3 position. Presence of two or more acyl groups also increases the color stability of anthocyanins in aqueous solution (Brouillard, 1982). It is suggested that acyl groups interact with the basic anthocyanin structure avoiding formation of the hydrated species (Giusti *et al.* 1998; Delgado-Vargas and Paredes-López 2003). It is believed that the acids will fold over the main anthocyanidin, and there is evidence of proximity between hydrogens from acids and hydrogens at position C-4 (Mazza and Miniati 1993; Giusti *et al.* 1998). Radishes, purple carrots, red cabbage are being used by the food industry due to their increased stability attributed to the presence of anthocyanins with two or more acylations. Maraschino cherries colored with radish anthocyanins have a shelf life of at least 6 months at 25 °C, and their good stability has been associated with the presence of two acylating groups on the pelargonidin derivatives (Giusti and Wrolstad 1996b; Rodríguez-Saona *et al.* 2001).

However, acylation with aliphatic acids through ester bond often goes undetected since these esters are quite labile to acid hydrolysis (Wrolstad *et al.* 2002). When malonated anthocyanins are subjected to standard extraction procedures using methanol acidified with 0.1 to 1.0% HCl, the malonyl group is lost in a short time (Harborne 1988). Data from the grape anthocyanins extraction suggested that the use of solvents containing up to 1% of 12 N HCl could produce partial hydrolysis of some acetylated anthocyanins during extraction (Revilla *et al.* 1998). Comparing to HCl, TFA (boiling point 72.4°C) has the advantage that it is easily removed by evaporation under reduced pressure. Aqueous TFA of 3% had been employed to isolate malonated pigments from *Monarda didyma* (Takeoka and Dao 2002).

2.4.5. Copigmentation

Stability of anthocyanins can be enhanced through intramolecular or intermolecular copigmentation. Acylated anthocyanins containing two or more aromatic acyl groups may affect the color through a mechanism called intramolecular copigmentation (Mazza and Miniati 1993; Harborne and Williams 2001).

Anthocyanins also interact with other flavonoids and related compounds to produce an increase in color intensity (hyperchromic effect) and a shift in the wavelength of maximum absorbance toward higher wavelengths (bathochromic effect). Such a phenomenon is called intermolecular copigmentation, which can take place in acidic, neutral and even slightly alkaline aqueous solution (Mazza and Miniati 1993; Brouillard and Dangles 1994).

The occurrence of copigmentation relies on at least two effects (Waterhouse 2002). First, the formation of the π - π complex causes changes in the spectral properties of the molecules in the flavylum form, resulting in hyperchromic shift and bathochromic shift (Giusti *et al.* 1999a). Secondly, the stabilization of the flavylum form by the π complex shifts the equilibrium to better favor the flavylum, thus boosting the proportion of anthocyanin molecules in the red-colored form (Figure 10).

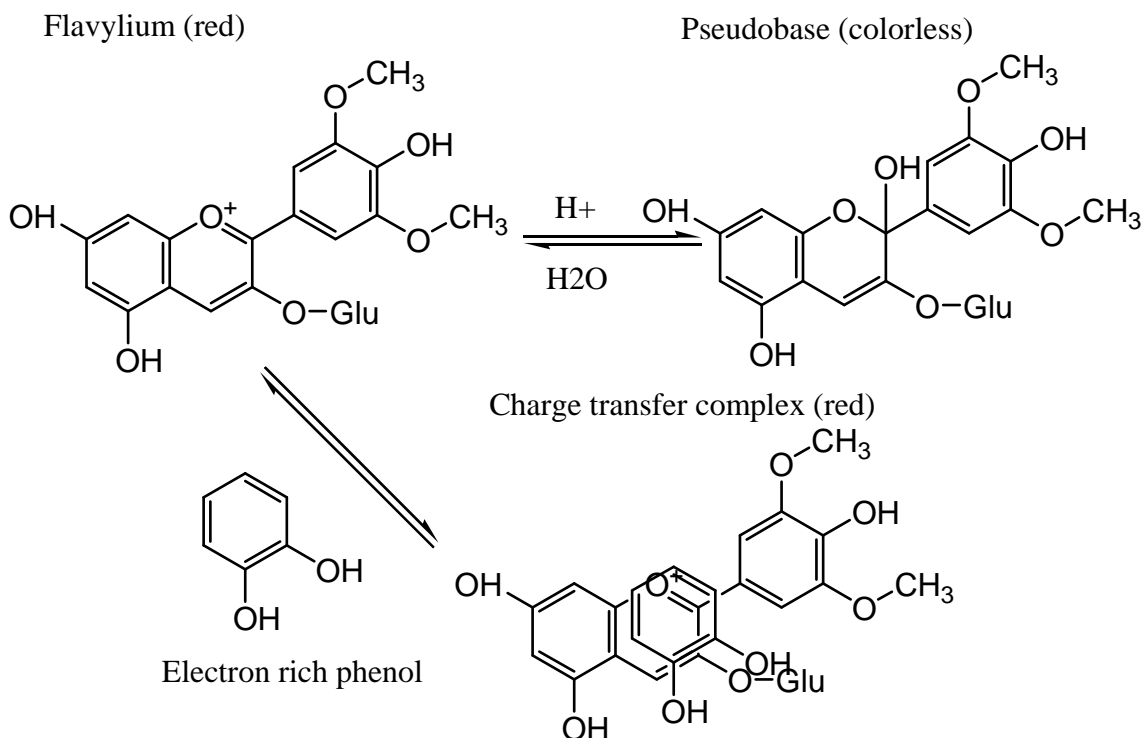


Figure 10 Co-pigmentation of wine anthocyanins. (Source: Waterhouse 2002)

Several types of chemical groups were observed to induce anthocyanin copigmentation. Among those, flavonones, aurones, and flavonols show the most significant color modifications, including chroma and lightness (Delgado-Vargas and Paredes-López 2003). Other phenomena contributing to the copigmentation involve the anthocyanin self-association (Clifford 2000) and metal complexation as illustrated in Figure 11 (Somaatmadja *et al.* 1964). Because of the self-association, anthocyanin absorbance in the solution doesn't follow Beer's law, especially at high concentration.

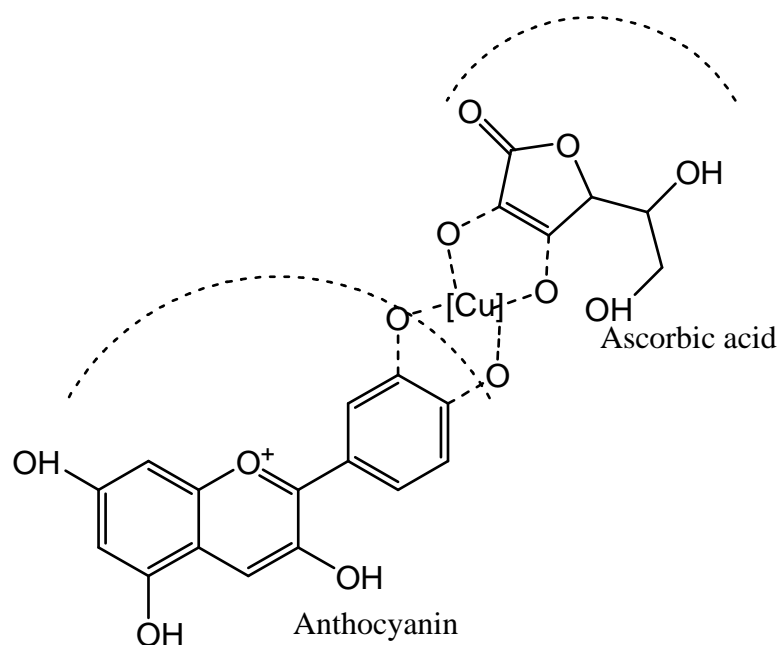


Figure 11 Suggested mechanism of anthocyanin-metal-ascorbic acid complexation. (Source: Delgado-Vargas *et al.* 2000)

Copigmentation of anthocyanins usually results in improved stability, by protecting the colored flavylium cation from the nucleophilic attack of the water molecule (Mazza and Miniati 1993; Delgado-Vargas and Paredes-López 2003). In terms of the intramolecular copigmentation, a sandwich type stacking of the aromatic residue of acyl groups with the pyrylium ring of the flavylium cation decreases hydration at C-2 and C-4 positions (Mazza and Miniati 1993). Intermolecular copigmentation also enhances the stability through intermolecular stacking (Clifford 2000), which was evidenced by the observations from independent researchers. In an anthocyanin stability study, Madhavi and coworkers attributed the better *in vitro* stability of the pigment to the production of co pigmenting agents such as flavonols, phenolic acids, and tannins (Madhavi *et al.* 1996). Reaction between anthocyanins

and tannins produces a “stabilized ” anthocyanin or pigmented tannin, which persists much longer in wine than the initial form, and it is this stabilized color that persists in most red wines more than a few years old (Waterhouse 2002). Sweeney and co-workers reported that polyhydroxylated flavone, isoflavone, and aurone sulphonates increased the photo stability of anthocyanins (Sweeney *et al.*1981). They attributed this effect to extensive H-bonding and ionic bonding between the negatively charged sulfonate and the electron deficient flavylum structure. As shown in Figure 12, the addition of quercetin-5'-sulfonate made the product more stable to light.

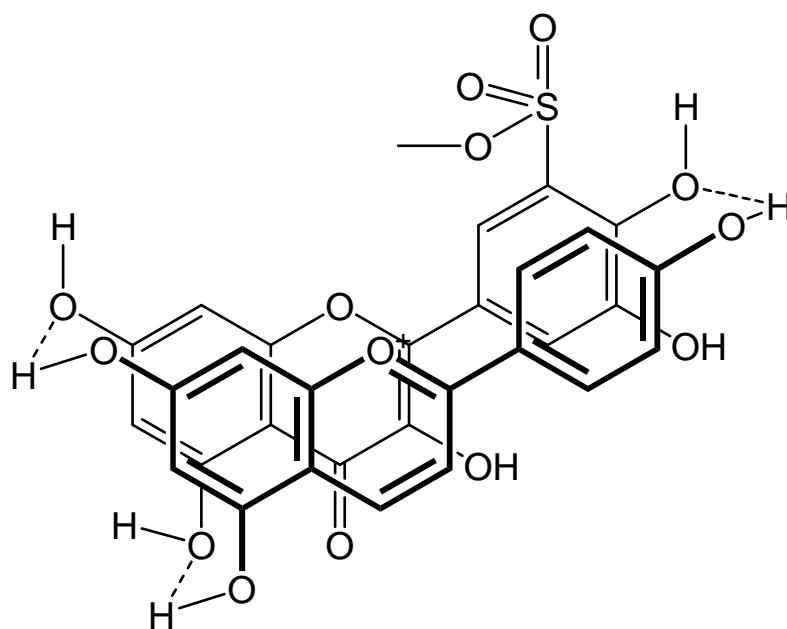


Figure 12 Molecular complex between an anthocyanidin (apigenidin) and a polyhydroxy-flavone sulfonate. (Source: Sweeny *et al.*1981)

The copigmentation is an extremely fast dynamic process and the lifetime of a given complex is probably less than one microsecond. But for a covalent bond to establish between anthocyanin and copigment a minimum period of time, much

longer than a complex mean lifetime, is necessary. It is only from time to time that a complex can escape from the copigmentation equilibrium to give a new pigment molecule (Brouillard and Dangles 1994). The reaction is simplified in Figure 13. This theory shades a light on explaining the slow process of red wine aging.

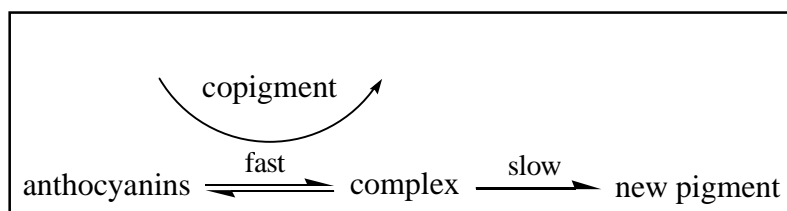


Figure 13 Scheme of the copigmentation reaction. (Adapted from Brouillard and Dangles 1994)

2.4.6. Condensation

The difference between copigmentation and condensation is vague, but it is generally considered condensation when covalent bonds are formed (Francis 1989; Fossen *et al.* 2000). Many flavylium salts condense easily with amino acids, phloroglucinol, catechin, and other compounds to yield colorless flavones. This type of reaction may lead to condensation products, which can proceed to produce brown polymers which precipitate out and cause turbidity but also contribute to polymeric color. However, in some cases the condensation dramatically increases color density (Timberlake and Bridle 1977). When anthocyanins and flavan-3-ols such as catechins and procyanidins interact with acetaldehyde, the increase in color can be up to seven times. It is believed to be due to a linking of the anthocyanin and flavan-3-ol with a CH₃-CH bridge.

2.4.7. Enzymatic System

Fresh blueberry fruits develop an intense browning along with a color loss after crushing (Kader *et al.* 1998; Lee *et al.* 2002). Studies indicate that native enzyme polyphenol oxidase (PPO, which is mainly located in the cytoplasm), anthocyanins, and polyphenolics (predominantly located in the vacuole) undergo significant degradation when the fruit is processed (Lee *et al.* 2002). PPO oxidizes polyphenolics to produce quinines (Jiménez *et al.* 1999). However anthocyanins are not substrates for PPO. It is the secondary quinones that induce the pigment degradation (Kader *et al.* 1998). The quinones subsequently react with anthocyanins by coupled oxidation mechanisms with a ratio of 2:1 (Kader *et al.* 1998) or forming adducts with a stoichiometry of 1:1 (Sarni-Manchado *et al.* 1997; Kader *et al.* 1999) to produce brown pigments.

In addition to the PPO, glycosidases are very important in anthocyanin stability because of a degradation effect producing very unstable anthocyanidins and glycosides (Skrede *et al.* 2000; Lee *et al.* 2002; Delgado-Vargas and Paredes-López 2003). But their source is unlikely to be the fruits themselves (Skrede and Wrolstad 2002). Moldy fruit is a potential source to have glycosidase side activities (Wrolstad *et al.* 1994). Juice processing enzymes, such as pectinases and cellulases, may also affect the stability of anthocyanins due to glucosidase side activity of some enzyme preparations, and therefore extreme caution must be used when choosing an enzyme preparation for use by the food industry (Wightman and Wrolstad 1996). In the *in vivo* studies, cyanidin 3-glucoside (Tsuda *et al.* 1999) and other flavonoid glycosides

(Andrea *et al.* 1998; Andrea *et al.* 2000; Gee *et al.* 2000) were detected in the small intestine as deglycosylated form after administration to the rats or human. β -glucosidase was hypothesized to be responsible for this chemical conversion.

Native peroxidase enzymes may be a cause of anthocyanin destruction in some berry commodities (Skrede and Wrolstad 2002). Active peroxidase may be a major contributor to anthocyanin pigment destruction in many processed strawberry products (Zabetakis *et al.* 2000).

2.4.8. Other Influencing Factors

Processing and storage under low temperature can improve the stability of anthocyanins (Delgado-Vargas and Paredes-López 2003). Temperature has been reported to induce a logarithmic destruction of pigment with time of heating at a constant temperature (Delgado-Vargas *et al.* 2000). When temperature is increased, the unstable formation of chalcone (Figure 6) is favored, and the chalcone is further degraded to brown products.

Light is usually deleterious to anthocyanin components. Early in 1936 Tressler and Pederson had reported the adverse effect of light on the color of Concord grape juice in bottles. Palamidis and Markakis (1975) reported that grape colorants in beverage had the half-life of 416 days in dark against 197 days in daylight at 20°C. Again, acylated anthocyanins are less affected by light, with only slight difference on pigment stability when exposed to light as compared to stored in dark (Giusti and Wrolstad 1996b).

Oxygen and hydrogen peroxide can easily oxidize anthocyanins (Delgado-Vargas and Paredes-López 2003), but this mechanism is often accelerated by the presence of ascorbic acid. The mutual destruction between ascorbic acid and anthocyanin has been of great concern because of the universal of ascorbic acid in fruit juice (Francis 1989). The interaction of ascorbic acid and oxygen may be mediated by H_2O_2 because one of the mechanisms of oxidation of ascorbic acid produces peroxide, and peroxide is known to bleach anthocyanins.

Increased sugar level may affect the rate of anthocyanin destruction (Delgado-Vargas and Paredes-López 2003). It was reported that sucrose addition improved the color characteristics of frozen strawberries (Wrolstad *et al.* 1990). The mechanism could be associated with the inhibition of degradative enzymatic activities of PPO and peroxidase (Delgado-Vargas and Paredes-López 2003).

Anthocyanins are very reactive toward metals, and they form stable complexes with tin, copper, and iron (Figure 11) (Francis 1989). For instance, cyanidin 3-glucoside forms a stable colored complex in the presence of aluminum ions at pH 5.5. Actually the addition of $AlCl_3$ is an analytical test for anthocyanidins which have two adjacent OH groups (Cy, Pt, Dp) and those which do not (Pg, Pn, Mv). It has been proposed that metal complexes could be used as colorants (Delgado-Vargas *et al.* 2000).

Chapter 3: Materials and Methods

3.1. Chemicals and Materials

Commercially available Anthocyanin-Rich Extracts (AREs) of bilberry (*Vaccinium myrtillus* L.) and chokeberry (*Aronia melanocarpa* E.) extracts were supplied by Artemis International, Inc. (Fort Wayne, IN). Grape extract (*Vitis vinifera*) was supplied by Polyphenolics, Inc. (Madera, CA). Cyanidin 3-galactoside standard for HPLC analysis was purchased from Polyphenols Laboratories (Sandnes, Norway). Acetonitrile, acetic acid, methanol, acetone, and ethyl acetate were HPLC grade reagents from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) and AOM (lot no.111k1502), a potent carcinogen, were obtained from Sigma chemical (St. Louis, MO). Tissue homogenizer was from Biospec Products, Inc. (Bartlesville, OK). Sep Pak Vac (6cc, 1g; 12cc 2g) C₁₈ Cartridges for solid-phase extraction (SPE) were purchased from Waters (Milford, MA).

3.2. Animals and Experimental Design

3.2.1. Diets and Treatments

Biological samples used in the current study were obtained from rats exposed to AOM, a carcinogen, as a part of an experiment to assay chemopreventive activity of the AREs (Magnuson, Univ. of Maryland, unpublished). Diets containing 4g/kg monomeric anthocyanin from chokeberry, bilberry or grape AREs were prepared by

supplementing AIN-93 powdered diet (Dyets Inc. PA). All the three anthocyanin extracts were added in at the expense of cornstarch, based on their monomeric anthocyanin content. The composition of experimental diets is summarized in Table 2.1.1. All diets were prepared fresh on a weekly basis and stored at 4°C until use. After feeding for one week, all animals randomly received one dose of a subcutaneous injection of AOM in saline at 20mg/kg body weight.

Table 2 The composition of experimental diets.

Ingredient (g/kg)	Diets			
	AIN-93*	AIN-93 with 5% Chokeberry ARE	AIN-93 with 3.5% Bilberry ARE	AIN-93 with 2.6% Grape ARE
Casein	200	200	200	200
L-Cystine	3.0	3.0	3.0	3.0
Sucrose	100	100	100	100
Corn starch	397.5	347.5	362.5	371.0
Soybean oil	70	70	70	70
Fiber (cellulose)	50	50	50	50
Mineral Mix	35	35	35	35
Vitamin mix	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
AREs	—	50	35	26

* AIN-93 is a standard rat diet.

The experiment was performed following a completely randomized design (CRD). Rats were randomly assigned to four groups of ten animals each. Within each group, either an anthocyanin enriched diet or control diet was given (Table 2). Each

rat was considered an experimental unit. It was assumed that data from different experimental unit was independent.

3.2.2. Animal Care and Feeding Trial

Forty 3-4 week old male specific pathogen free male Fischer 344 rats (Matsunaga *et al.*2002) were obtained from Harlan (Indianapolis Indiana) and maintained under the University of Maryland at College Park (UMCP) Institutional Animal Care and Use Committee (IACUC) approved protocols. In the first week of acclimatization rats were slowly weaned from pelleted rat chow to powdered AIN – 93 diets. Chewing bones (Bio-Serv, NJ) were also provided for overgrowing teeth due to feeding of powdered diet for long time. The animals were housed in pairs in suspended stainless steel cages with wire mesh floor and front. Powdered diet was provided in standard feeding cups. Diet and tap water was available *adlibitum*. Artificial light was supplied from fluorescent tubes, in a 12h light –12h dark cycle. The number of air changes was ~10 per hour. Relative humidity was maintained at 25% - 60%. Clinical signs for all the animals were recorded regularly. Body weight was recorded twice in a week and 3-days food intake was measured two times for all the animals in 14 weeks study. These data are not included in the current report.

3.3. Sample Collection

3.3.1. Urine and Feces Collection

Urine samples and feces were collected from eight animals randomly selected from each group one week before the final (14 week) sacrifice. Each animal was placed individually in Nalgene metabolic cages (Mini Mitter Inc., Bend, Oregon) over a period of 12 hours of dark cycle. Because there were only 16 metabolic cages available, each day only 4 rats from each treatment group were used for urine collection, for 2 consecutive days. The powdered diet was not provided during this time to reduce any contamination of urine. Urine was collected from 6 am to 12 pm, and 20% TFA was added right after the collection (Miyazawa *et al.* 1999; Cao *et al.* 2001; Wu *et al.* 2002; Milbury *et al.* 2002). Feces were collected twice at 12 pm and 6 pm and sealed in polypropylene bags. All the samples were immediately stored at 80°C until analyzed.

3.3.2. Plasma and Cecal Content Collection

At the end of 14 weeks, all rats were anesthetized in the carbon dioxide chamber in early morning without feeding. Rats were immediately decapitated to collect blood using heparinized tubes. Blood was mixed by inversion at least 3 times and afterwards placed on ice. Plasma samples were immediately prepared according to the method of Tsuda *et al.* (1999) with slight modification. Collected blood was centrifuged at 3000 rpm for 15 min at room temperature. The separation was finished within 30 min. Then plasma samples were quickly removed and immediately treated

with an aqueous solution of 0.44 mol/L TFA (1:0.2; v/v) (Wu *et al.* 2002). Proteins in the plasma were precipitated by centrifuging for 5 min at 3000 rpm at 4°C (Felgines *et al.* 2002). The cecal contents were wrapped in foil and rapidly frozen in liquid nitrogen. All treated samples were stored at -80°C prior to anthocyanin analysis.

3.4. Feces Anthocyanin Extraction Method Development

3.4.1. Method Development

To date, a procedure for anthocyanin analysis in feces had not been reported in the literature. Our extraction method was designed based on reported procedures for cecal content anthocyanins extraction (Felgines *et al.* 2002) as well as the fecal quercetin extraction (Aura *et al.* 2002). An aliquot of 0.100g was taken from each frozen fecal sample, and placed in a 40 mL centrifuge tube. After breaking the sample into smaller pieces with a spatula, 20 mL of extracting solvent was added. Tissue homogenizer was used to homogenize the feces. Then the suspension was sonicated for 3 min and centrifuged for 10 min at 4000 rpm 4°C to precipitate the protein and other water insoluble content. The supernatant was taken and the deposit was re-extracted with 10mL of extracting solvent twice in the same way. The combination of supernatants was carefully evaporated in a Büchi rotovapor at 40°C. During the evaporation, pressure was carefully controlled to avoid boiling. After evaporation to almost dry, the solution was diluted with small amount of acidified water, and then applied to a Sep-Pak Vac C₁₈ (12cc, 2g) cartridge pre-conditioned with one volume of methanol containing 1% TFA followed by one volume of water containing 1% TFA. After washing with one volume of water containing 1% TFA, anthocyanins were

eluted with one volume of methanol containing 1% TFA. The eluate was carefully evaporated in a Büchi rotovapor at 40°C, and then redissolved in water containing 1% TFA to 5mL.

Four solvents were studied for the extraction efficiency using the fecal samples in ARE treated groups including methanol, acetone, methanol water mixture (60:40; v/v), and water, all acidified with 1% TFA. The experiment was done by duplicate. For each replication, one sample from each group was randomly selected and divided to 4 equal weighted aliquots of 0.100g.

3.4.2. Recovery Tests

In day 1, four fecal samples from the control group were randomly selected. A portion of each sample was taken out and pooled. After mixing the pooled feces with a spatula, 18 equal weighted aliquots were put into 18 capped tubes and randomly assigned to 3 groups. Within each group, known amount of chokeberry, bilberry, and grape AREs were spiked into different tubes with 2 replications. Samples in group 3 were pasteurized in 95°C water bath for 2 minutes before spiking (Lee *et al.* 2002). After brief mixing, all the tubes were stored at -18°C prior to anthocyanin extraction. In day 2, samples in group 1 and group 3 were extracted with 60% methanol following the procedures described in 3.4.1. Immediately after the semi-purification, anthocyanin content was analyzed by HPLC. In day 4, samples in group 3 were analyzed in the same way.

3.5. Determination of Anthocyanins

3.5.1. Semi-purification of Anthocyanins in the Plasma and Urine

Plasma semi-purification method was adapted from Matsumoto *et al.*(2001). All the available plasma (around 4 mL) was applied to a Sep PakVac C18 (6cc, 1g) cartridge pre-conditioned with one volume of methanol containing 1% TFA followed by one volume of water containing 1% TFA. After washing with one volume of water containing 1% TFA, anthocyanins were eluted with one volume of methanol containing 1% TFA. The eluate was carefully evaporated in a Büchii rotovapor at 40°C, and then redissolved in water containing 1% TFA to 1mL.

Urine samples were semi-purified following the same procedure used for plasma, except that the sample volume was carefully controlled for quantification purpose. Only 0.75 mL was used for each sample.

3.5.2. Extraction and Semi-purification of Anthocyanins in the Feces and Cecal Content

Fecal samples were defrosted in polypropylene bags at room temperature. An aliquot of 0.100 g was carefully weighted out, and then extracted and semi-purified as described in 3.4.1.

Cecal content was obtained from the defrosted cecum and then extracted and semi-purified in exactly the same way as feces.

3.5.3. High Performance Liquid Chromatography (HPLC) Analysis

All the samples were filtered through 0.45µm Whatman polypropylene filter to the special vials before injection into HPLC. Analyses were conducted on a HPLC (Waters Delta 600) system equipped with a photodiode array detector (Waters 996), Millennium³² software (Waters Corp., Milford, MA), and autosampler (Waters 717 plus). The separation of anthocyanins was accomplished on a Symmetry C18 column (5µm; 4.6×150mm). Mobile phases and gradients were modified from Rodríguez-Saona *et al.*(1998) and Durst and Wrolstad (2001). Mobile phases were A, 10% acetic acid and 1% phosphoric acid in deionized water and B, acetonitrile. The gradient condition was 0-5 min, 100% A isocratically; 5-40 min linearly decrease to 65% A; 40-45 min linearly increase to 100% A. Spectral data (260-650nm) was collected for all samples. Elution of compounds of interest was monitored at wavelength 520 nm for anthocyanins, 280nm for phenolics, and 320nm for hydroxycinnamic acids. Other chromatographic conditions were as follows: flow rate, 1 mL/min; injection volume, 200 µL for urine and plasma samples, 300 µL for cecal and fecal samples; and post run time, 10 min.

Peak identification of bilberry and chokeberry anthocyanins was based on comparison of relative retention times, percentage peak area, and spectral data with available anthocyanin profiles of chokeberry and bilberry ARE obtained from Artemis International, Inc. (Fort Wayne, IN), as well as information from literature (Camire 2002; Skrede and Wrolstad 2002; Shahidi and Naczki 2004a). For grape anthocyanins, peaks were compared to unknown anthocyanins as well as profile from

literature (Mazza and Miniati 1993). HPLC-MS was also used to assist on peak identification (Tian *et al.* unpublished).

3.5.4. Standards and Calibration Curves

Commercially available cyanidin 3-galactoside standard was dissolved in DD water containing 1% TFA to 0.100 g/L, and thereafter diluted to 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128 of the initial concentration to make standard solutions. Standard solutions were injected separately into HPLC under the conditions described in 3.5.3 to generate calibration curve for all interested anthocyanin compounds (Andriambeloson *et al.* 2004). Calibration curve was linear, with $R^2 \geq 0.99$. All of the anthocyanin peaks being analyzed fell within the range of the standard curve. All the individual anthocyanins were expressed as cyanidin 3-galactoside equivalent in weight.

3.5.5. Statistical Analysis

One way ANOVA was conducted by SPSS (version 10, 1999, SPSS Inc., Chicago, IL), and values were given as means \pm SEM. When appropriate, significance of differences between values was determined by LSD. Differences of $P < 0.05$ were considered significant.

Chapter 4: Results and Discussion

4.1. Fecal Anthocyanin Extraction Method Development

Through the years, anthocyanin biological activity has been overlooked given their poor absorption in the gastrointestinal tract (GIT). It has been only in the last decade that methods have become available that allow for detection and quantitation of anthocyanins in biological samples. Unlike many other flavonoids, anthocyanins are not well degraded by gut microflora, judging by the visual color of feces from animals fed anthocyanin rich diets (Brouillard 1982). Because of the close contact between gut content and colon epithelium cells, direct influence of anthocyanins to gut health including colon cancer is plausible. Actually, our hypothesis is supported by the fact that to date all of the cancers that may be inhibited by anthocyanins are related to the GIT. Breast cancer, on the other hand, was found not effectively inhibited by anthocyanins. Felgines *et al.*(2002) were probably among the first to report anthocyanin availability in the cecal contents. However to date there is no established method available for analysis of fecal anthocyanins. In our study we developed such a method based on reported procedures for cecal content anthocyanin extraction (Felgines *et al.* 2002) as well as the fecal quercetin extraction (Aura *et al.* 2002) in order to systematically study anthocyanins in the gut contents.

4.1.1. Solvents Comparison

Methanol (Frøytlog *et al.* 1998; Torskangerpoll *et al.* 2001), acetone (Giusti *et al.* 1996a, Rodriguez-Saona and Wrolstad 2002), water, and organic-inorganic mixture solvents (Ju and Howard 2003a; Ju and Howard 2003b) have been widely used for anthocyanin extraction. In our study, we compared the extraction efficiency of four solvent systems: aqueous methanol (60:40, v/v), water, methanol, and acetone. All solvents were acidified with 1% of TFA (Torskangerpoll *et al.* 2001) to preserve anthocyanins. Fecal anthocyanins were extracted and semi-purified following procedures described in 3.4.1. HPLC calibration curve (described in 3.5.4) was used to calibrate the concentration. Total anthocyanins recovered from fecal samples were calculated by adding the area under curve of individual peaks together and then calibration. Anthocyanin recovery by water extraction was arbitrarily considered 100%, and recoveries by all the other solvents were calculated based on the relative efficiency to water extraction. Results were summarized in Figure 14.

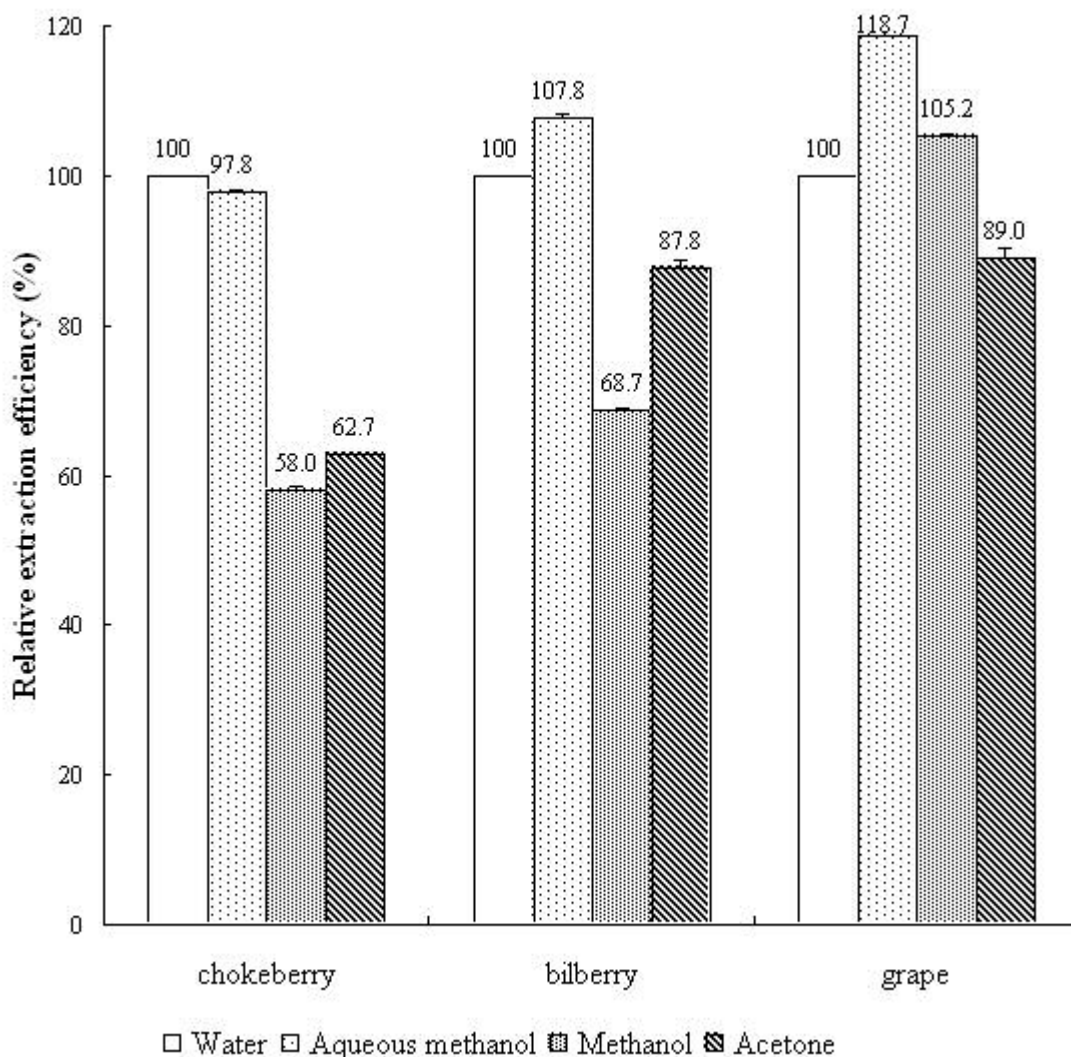


Figure 14 Relative efficiency of four solvents on fecal anthocyanin extraction. Efficiency of acidified water was arbitrarily considered 100%. Efficiency of other solvents was calculated based on their relative efficiency to acidified water. Values are the mean \pm SEM of two replications.

Figure 14 clearly shows that the extraction efficiency with organic solvents (methanol and acetone) or organic-inorganic solvent mixture (aqueous methanol) was dependant of the hydrophilicity of the anthocyanins present in the extract, increasing in the order of chokeberry-bilberry-grape. This was reasonable since the distribution of more or less hydrophilic anthocyanins in those three extracts was different. In the chokeberry ARE only the hydrophilic cyanidin mono glycosides were present, yet in

the bilberry ARE there were large amount of less hydrophilic anthocyanins such as peonidin 3-glycosides and malvidin 3-glycosides. In the grape ARE, however, significant amount of acylated anthocyanins, which were even more hydrophobic, were present, resulting in a higher affinity for organic solvents. Overall, aqueous methanol (60% methanol) gave the best extraction efficiency. This mixture of inorganic and organic solvents seemed to have good efficiency for a wide range of individual anthocyanins. In addition, methanol helped to precipitate enzymes (proteins) during centrifugation. Traditionally organic solvents were preferred for anthocyanin extraction from fruits and vegetables (Giusti *et al.* 1996a; Harborne 1998; Eder 2000; Takeoka and Dao 2002), partly because organic solvents have the advantage of easily penetrating the cell membranes and consequently extracting anthocyanins, the vacuolar pigments (Delgado-Vargas and Paredes-López 2003). However, fecal anthocyanins were not as compartmentalized as in fruits and vegetables, since the consumed foods containing anthocyanins had been digested in gastrointestinal tract (GIT) before excretion. Anthocyanins present in feces were not trapped in cell membranes, and therefore the advantage of organic solvents was not apparent.

Water is a good alternative for aqueous methanol, not only because it is an environmental friendly solvent, but also because water extraction can be directly applied to the C₁₈ cartridge, and therefore save a step of evaporation. Generally the vacuum evaporation is conducted at around 35-40°C for anthocyanin solutions, yet this temperature range is so close to the body temperature, and is very likely to accelerate the degradation of anthocyanins in the presence of fecal microflora. Using

acidified water may diminish such a concern and give more control to the reproducibility. In our study, we found water extraction gave the lowest SD/Mean ratio in chokeberry and bilberry treatment, and the second lowest SD/Mean ratio (next to aqueous methanol treatment) in grape treatment.

In our study, we chose acidified aqueous methanol (60:40, v/v) to extract all the fecal samples and cecal content samples because of the higher extraction efficiency obtained.

4.1.2. Stability and Recovery

In preliminary studies we observed that at room temperature, crude extracts of fecal chokeberry anthocyanins in acidified water degraded very quickly. After one day, the majority of anthocyanins had degraded, judging by the disappearance of red color. Even when stored at -18°C, fecal chokeberry anthocyanin extracts in acidified water also lost some color after a few week . We suspected that the fecal microflora were responsible for the quick degradation. Therefore the stability of fecal anthocyanins at the time of collection (after excretion but before collection) as well as during storage and extraction was a huge concern for accurate analysis. To acquire the efficiency of our method, meanwhile to evaluate the role of fecal microflora, we designed a study of the stability and recovery of fecal anthocyanins, described below.

4.1.2.1. Stability of Fecal Anthocyanins

Profiles of recovered anthocyanins in unpasteurized fecal samples were shown in Figure 15, Figure 16, and Figure 17, with the profiles of anthocyanins recovered

from pasteurized samples as references. Pasteurized samples are assumed to have no microflora and therefore no enzymatic activity on anthocyanins. This assumption was supported by the fact that pasteurized samples had identical shapes of anthocyanin profiles with the spiked AREs. Thus the difference of total anthocyanins between the spiked AREs and that recovered from pasteurized samples was attributed only to the loss during extraction and semi-purification. The overlapped profiles clearly showed that the unpasteurized fecal samples underwent significant anthocyanin degradation when stored at -18°C for three days. More interestingly, the severe degradation occurred for the anthocyanidin galactosides, followed by anthocyanidin glucosides, and not much significant degradation for anthocyanidin arabinosides and anthocyanidin xylosides was observed. This trend was better illustrated in Table 3, Table 4, Table 5, and Figure 18.

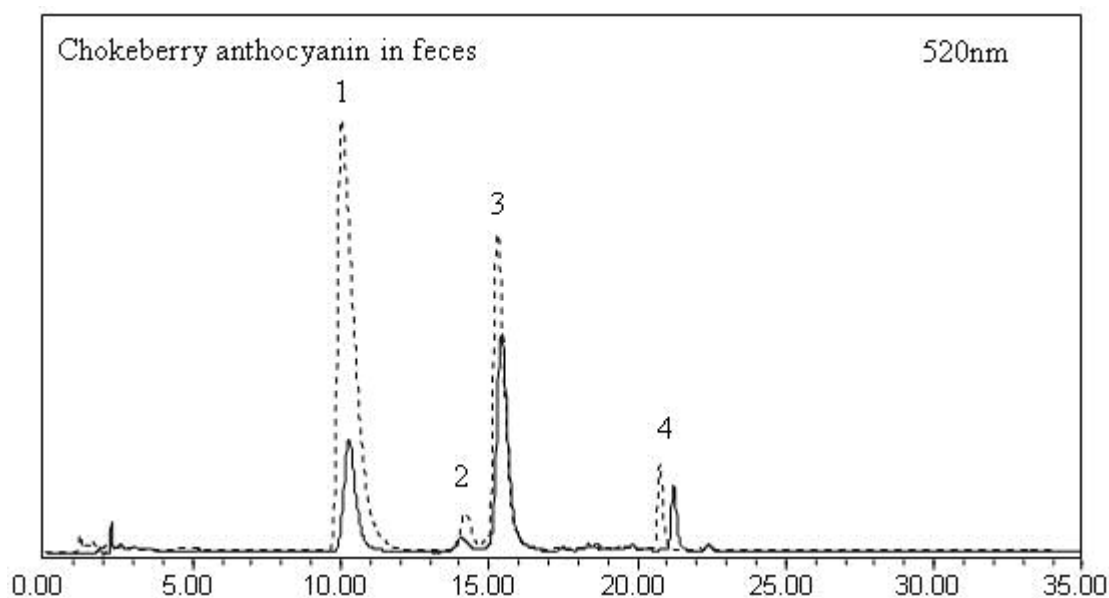


Figure 15 Typical UV-HPLC chromatogram of fecal anthocyanins recovered from chokeberry anthocyanin rich extract spiked feces. Solid line: Unpasteurized samples stored for 3 days; Dot line: pasteurized samples. Peak identities: 1, cya 3-gal; 2, cya 3-glu; 3, cya 3-arab; 4, cya 3-xyl.

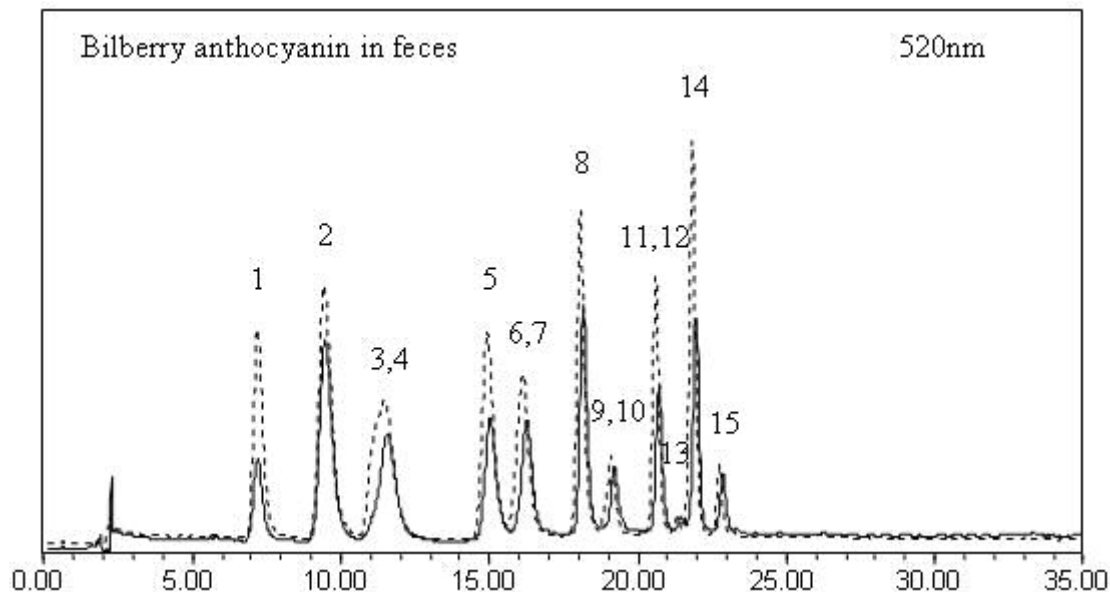


Figure 16 Typical UV-HPLC chromatogram of fecal anthocyanins recovered from bilberry anthocyanin rich extract spiked feces. Solid line: Unpasteurized samples stored for 3 days; Dot line: pasteurized samples. Peak identities: 1, del 3-gal; 2, del 3-glu; 3, cya 3-gal; 4, del 3-arab; 5, cya 3-glu; 6, pet 3-gal; 7, cya 3-arab; 8, peo 3-glu; 9, peo 3-glu; 10, pet 3-arab; 11, peo 3-glu; 12, mal 3-gal; 13, peo 3-arab; 14, mal 3-glu; 15, mal 3-arab.

Table 3 Individual anthocyanins recovered from feces spiked chokeberry ARE¹.

Anthocyanins	Spiked	Recovered		
		Pasteurized	Unpasteurized	
			After 1 day	After 3 days
Cya 3-gal	105a ²	90.1b	61.1c	20.8d
Cya 3-glu	6.2a	5.3b	5.6b	3.6c
Cya 3-arab	49.2a	45.5a	45.6a	36.8b
Cya 3-xyl	7.0a	6.2a	6.7a	6.2a
unknown	3.1a	0.6c	1.2bc	2.1ab
Total	170.5a	147.7b	120.2c	69.5d

¹ Fecal anthocyanin concentration (mg cya 3-gal equivalent / 100g feces) expressed as mean of seven replications. ² Means within a row with similar letters are not significantly different ($P < 0.05$).

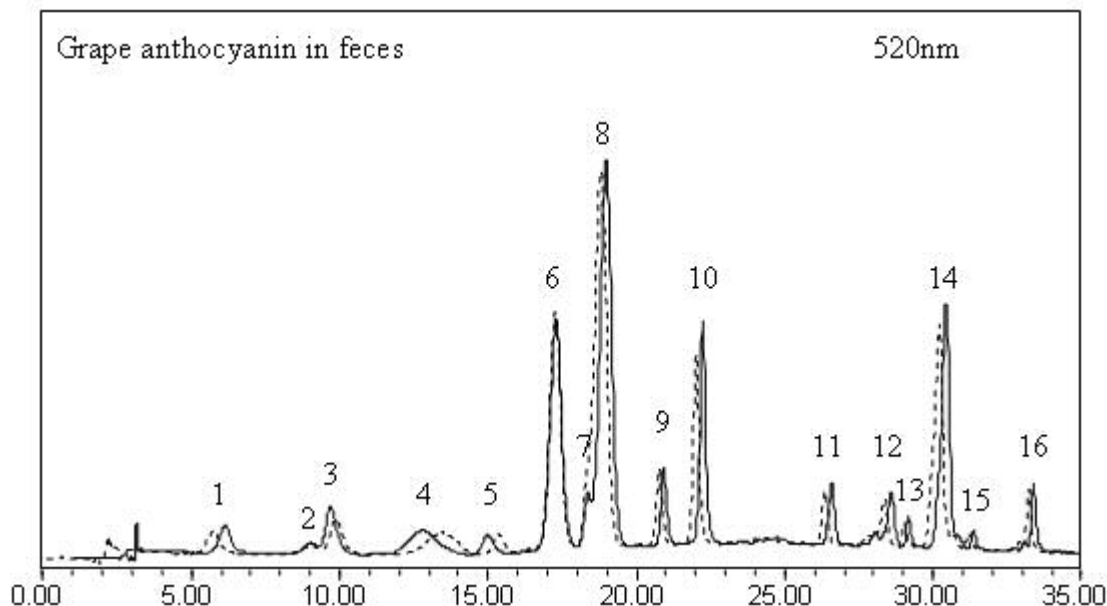


Figure 17 Typical UV-HPLC chromatogram of fecal anthocyanins recovered from grape anthocyanin rich extract spiked feces. Solid line: Unpasteurized samples stored for 3 days; Dot line: pasteurized samples. Peak identities: 1, del 3,5-diglu; 2, cya 3,5-diglu; 3, del 3-glu; 4, pet 3,5-diglu; 5, cya 3-glu; 6, peo 3,5-diglu; 7, pet 3-glu; 8, mal 3,5-diglu; 9, peo 3-glu; 10, mal 3-glu; 11, cya 3-glu-ac; 12, del 3-glu-*p*-coum; 13, cya 3-glu-*p*-coum; 14, pet 3-glu-*p*-coum; 15, pel 3-glu-*p*-coum; 16, mal 3-glu-*p*-coum.

Table 3 shows the individual anthocyanins recovered from chokeberry ARE spiked feces. Among all the four cyanidin glycosides present in chokeberry, cyanidin 3-galactoside underwent the most severe degradation, from 90.1 mg/100g pasteurized feces (without exposure to the enzymatic activity) to 61.1 mg/100g feces with one day exposure to fecal microflora under -18 °C, and then to 21.8 mg/100g feces with three days exposure. The recovery of other three cyanidin 3-glycosides all showed no significant difference between without fecal microflora treatment and with one day exposure to the fecal microflora treatment. After three days exposure to the fecal microflora, only cyanidin 3-xyloside showed no significant degradation, but cyanidin 3-arabinoside was also very resistant to degradation as compared to the others.

Table 4 Individual anthocyanins recovered from feces spiked bilberry ARE¹.

Anthocyanins	Spiked	Recovered		
		Pasteurized	Unpasteurized	
			After 1 day	After 3 days
Del 3-gal	17.5a ²	15.6ab	13.9b	7.7c
Del 3-glu	26.9a	25.2b	25.8ab	21.0c
Cya 3-gal + Del3- arab	26.6a	23.2b	21.0b	15.8c
Cya 3-glu	20.8a	18.7b	18.2b	11.9c
Pet 3-gal + Cya 3-arab	17.7a	16.2b	15.5b	11.3c
Pet 3-glu	19.6a	18.6a	18.8a	14.2b
Peo 3-gal + Pet 3-arab	6.8a	05.9b	5.8b	5.3c
Peo 3-glu + Mal 3-gal	15.4a	13.4b	13.3b	8.5c
Peo 3-arab	1.4b	1.3c	1.4b	1.8a
Mal 3-glu	20.9a	18.3b	18.3b	10.9c
Mal 3-arab	4.4a	3.9c	4.3ab	4.1bc
Total	178.0a	160.3b	156.3b	112.5c

¹ Fecal anthocyanin concentration (mg cya 3-gal equivalent / 100g feces) expressed as mean of seven replications. ² Means within a row with similar letters are not significantly different ($P < 0.05$).

Table 4 shows the individual anthocyanins recovered from bilberry ARE spiked feces. Bilberry anthocyanin profile was much more complexed than that of chokeberry, but the data demonstrated similar trend of degradation. Although some of the anthocyanins were not completely separated by the HPLC conditions, the other peaks still delivered enough information to compare the degradation of anthocyanins with different types of glycosylation. After three days of exposure to the fecal microflora, delphinidin 3-galactoside degraded most severely, by more than 55%. Delphinidin 3-glucoside, cyanidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-

glucoside degraded from ~ 22% to ~48%. While peonidin 3-arabinoside and malvidin 3-arabinoside showed no significant degradation. Co-eluting peaks seemed to have degraded to an extent half way between the contained individual compounds.

Grape ARE only contained monomeric anthocyanidin glucosides and acylated anthocyanidin glucosides. This fact is widely accepted by researchers (Mazza and Miniati 1993; Skrede and Wrolstad 2002), and supported by our HPLC results. Table 5 summarizes the recovery of individual anthocyanins from grape ARE spiked feces. Since all the compounds were glycosylated with only β -glucose, they showed very uniform characteristic in terms of resistance to the fecal microflora. For most compounds there was no significant degradation detected after one day or three days of exposure to the fecal microflora. However, two major compounds, namely peonidin 3,5-diglucoside and malvidin 3,5-diglucoside degraded significantly after three days of storage. Their corresponding 3 mono glucoside, namely peonidin 3-glucoside and malvidin 3-glucoside, both increased to levels even higher than the spiked amount. What's more, the decreases of 3,5-diglucosides closely correlated to the increases of 3 mono glucosides. This phenomenon strongly indicated the partial hydrolysis of malvidin 3,5-diglucoside to malvidin 3-glucoside as well as peonidin 3,5-diglucoside to peonidin 3-glucoside. Some very small peaks betrayed the trend described above, probably because of the base line variation. Comparing the fecal anthocyanidin glucosides in grape treatment to that in bilberry treatment, we can see that even the same anthocyanin glucoside like cyanidin 3-glucoside displayed different resistance to degradation in different samples. Probably the derivatives of anthocyanidin 3-galactosides degradation in bilberry treatment accelerated the

degradation of anthocyanidin 3-glucosides. Similar mechanism had been suggested when PPO was involved in anthocyanin degradation (Kader *et al.* 1998) .

Anthocyanins themselves are not substrates for PPO, yet the secondary quinones induce the pigment degradation.

Table 5 Individual anthocyanins recovered from feces spiked grape ARE ¹.

Anthocyanins	Spiked	Recovered		
		Pasteurized	Unpasteurized	
			After 1 day	After 3 days
Del 3,5-glu	3.9a ²	2.6d	2.9c	3.3b
Cya 3,5-glu	2.1b	1.8c	1.8c	2.3a
Del 3-glu	5.4a	4.7a	4.8a	5.2a
Pet 3,5-glu	7.3a	5.7c	6.1bc	6.3b
Cya 3-glu	3.1a	2.6b	2.9ab	2.8b
Peo 3,5-glu	32.2a	26.2b	26.9b	22.3c
Mal 3,5-glu + pet 3-glu ³	52.4a	44.9b	45.1b	39.7c
Peo 3-glu	4.1a	3.3b	4.1a	4.6a
Mal 3-glu	7.8b	6.5b	8.1b	10.7a
Del 3-glu-ac	2.4a	2.0b	1.9b	1.9b
Cya 3-glu-ac	3.3ab	3.2ab	3.1b	3.9a
Del 3-glu-coum	4.1a	4.1a	4.0a	4.8a
Cya 3-glu-coum	1.8ab	1.7ab	1.4b	2.2a
Pet 3-glu-coum	18.4a	19.5a	18.6a	17.7a
Pel 3-glu-coum	1.1b	1.2b	1.0b	1.9a
Mal 3-glu-coum	2.3a	0.030a	0.023a	0.040a
Total	151.7a	130.0b	132.7b	129.6b

¹ Fecal anthocyanin concentration (mg cya 3-gal equivalent / 100g feces) expressed as mean of seven replications. ² Means within a row with similar letters are not significantly different (P < 0.05). ³ Pet 3-glu is a co-eluted minor peak.

4.1.2.2. Recovery of the Extraction Method

The recoveries of total anthocyanins from all the fecal samples are presented in Figure 18. Clearly, the chokeberry and bilberry treatments, where significant proportion of anthocyanidin galactosides was present, presented considerable anthocyanin degradation by fecal microflora. In the grape treatment, anthocyanins were highly resistant to the fecal microflora. Consequently, it is reasonable to infer that at the time of fecal sample collection, since there was a gap between rat excretion and sample collection, some anthocyanins might have experienced further degradation. In our results, fecal anthocyanin level (chokeberry treatment in particular) may have been underestimated.

Figure 18 indirectly tells us it is very likely that fecal microflora didn't exhibit significant effect during extraction procedure. Although our results didn't show directly how much influence of fecal microflora came from the extraction period alone, and how much came from the storage period. Shown in Figure 18 (chokeberry treatment), the recovered total anthocyanin decreased by ~16% (86.4 to 70.3%) with one day exposure to fecal microflora, but decreased by ~46% (86.4 to 40.7%) with three days exposure. Apparently the extent of degradation (46% vs. 16%, ratio ≈ 3) was highly correlated to the time of exposure (3 days vs. 1 day, ratio ≈ 3). If we suppose that during extraction procedure (about one hour) fecal microflora caused great loss, and equal amount of such a loss had been incorporated into all the unpasteurized samples, then the ratio of degradation between three days exposure and one day exposure should have been less than three. Therefore the data indirectly

shows us that fecal microflora didn't exhibit high activity during extraction period. This is not surprising because our method has at least three advantages in preventing enzymatic degradation. First, centrifugation (4000 rpm, 10 min) precipitated enzymes (proteins), which was evidenced by the fact that centrifugation treated samples were much more stable than crude extracts. Second, controlled low temperature (4°C) during extraction reduced enzyme activity. Third, short processing time (1h) comparing to traditional long time extraction (6h, Frøytlog *et al.* 1998) safeguarded the degradation.

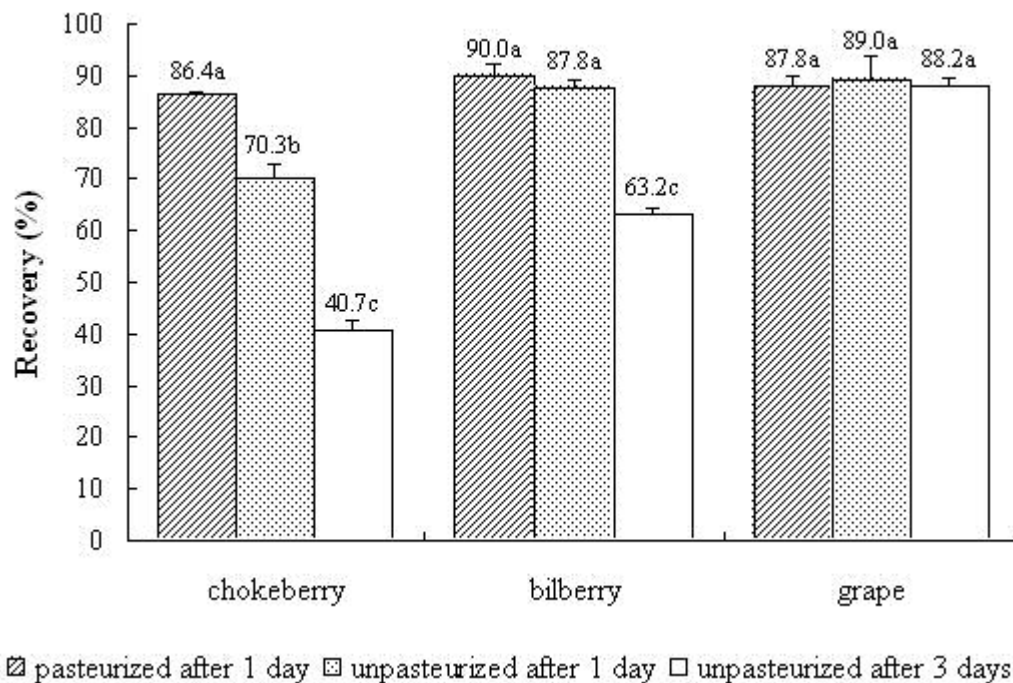


Figure 18 Recovery of spiked anthocyanins in feces. Anthocyanin rich extracts were spiked into pasteurized or unpasteurized fecal samples and then left under -18°C for storage until further analysis. Extraction and HPLC analysis were done after one day or three days of storage. Recovery rate is calculated by dividing the recovered amount by the spiked amount. Values are the mean \pm SEM of two replications. Means with different letters are significantly different at $P < 0.05$.

The recovery rate of fecal anthocyanins was calculated based on the data from pasteurized samples and spiked amounts. The average recovery rate for chokeberry ARE, bilberry ARE, and grape ARE was $88.1 \pm 2.3 \%$ ($n=6$). The loss was resulted from extraction step as well as semi-purification step. For the purification step, Wu *et al.* (2002) reported that the recovery of cyanidin 3-glucoside on Sep Pak C₁₈ SPE cartridge was $90.0 \pm 1.5 \%$ from urine and $91.4 \pm 0.9 \%$ from plasma. Excluding the loss on C₁₈ cartridge, our extraction procedure alone indeed recovered over 95% of the fecal anthocyanins. Certainly recovery of spiked samples may not represent exactly how much could be recovered from real samples, since binding of anthocyanins to other components was possible during digestion. But when extracting the real fecal samples from rats, hardly any red color could be seen in the precipitate by naked eyes after the extraction. This also is an indication of the good extraction efficiency of our method.

A limitation of this experiment was the potential interference of trace oxygen. Oxygen is a factor that can accelerate anthocyanin degradation. Since oxygen was not expelled from the capped tubes containing fecal samples, the role of free oxygen was not clearly determined. But during the extraction procedure, contact between fecal samples and oxygen was always inevitable.

In summary, storage of fecal samples at -18°C does not protect the anthocyanins from degradation, most likely due to the enzymatic activity of microflora. Under these conditions we found the most degradation for anthocyanin galactosides, smaller but significant degradation for anthocyanin glucosides, and little

degradation for anthocyanin arabinosides and xylosides. All the observations strongly suggested that fecal microflora had strong activity on fecal anthocyanins, and the storage of fecal anthocyanins must be under a much lower temperature than -18°C. Despite that centrifugation may reduce enzyme activity by precipitating enzymes, the extraction and semi-purification procedure to evaluate anthocyanin from fecal samples must be done as quickly as possible under controlled low temperature. Our extraction method, in which the aqueous methanol solvent was utilized, allowed the samples to be extracted and semi-purified within one hour (procedures described in 3.4.1). During this period we kept the temperature under 4°C at most of the time, and achieved an excellent recovery rate.

4.2. Anthocyanin Absorption, Excretion and Transformation in Rats

Anthocyanin absorption and excretion in the rat animal model were assessed by analyzing anthocyanin content in plasma, urine, cecal content and feces in order to better understand the bioavailability of anthocyanins in diet. Because the current experiment was part of a project designed to study the chemopreventive properties of anthocyanins, rats were treated with carcinogen. However at the very early stages of lesion development rats were unlikely to have abnormal physiological reactions. This was evidenced by the normal food and drink consumption of all groups. Although exact amount of anthocyanins consumed was not determined, food and drink consumption was approximately equal for all groups (control and treatments).

4.2.1. Anthocyanin in Urine

4.2.1.1. Urinary Anthocyanin Concentration

At the time of collection urine samples showed no red coloration, but immediately turned red after acidification with 20% volume(v/v) of 0.44 mol/L TFA. This color change was attributed to the re-equilibration of the chemical forms of anthocyanin in acidic pH. As shown in Figure 7, under neutral pH condition anthocyanins were predominantly in colorless pseudobase form, but under acidic pH most anthocyanins converted to the red colored flavylium form. Concentrations of total anthocyanins in rat urines are reported in Figure 19. Significant differences among the three treatments were observed. The highest concentration of anthocyanin was detected in the urine from rats in the chokeberry treatment, while the lowest level was detected in the bilberry treatment. Considering the approximate volume of urines (~1-1.5mL), and the average anthocyanin daily intake of rats (64-72mg), less than 0.05% was excreted within 6 hours after ingestion. These results agree with numerous previous reports that anthocyanins were poorly recovered in rat urine (Tsuda *et al.* 1999; Miyazawa *et al.* 1999; Matsumoto *et al.* 2001; Felgines *et al.* 2002).

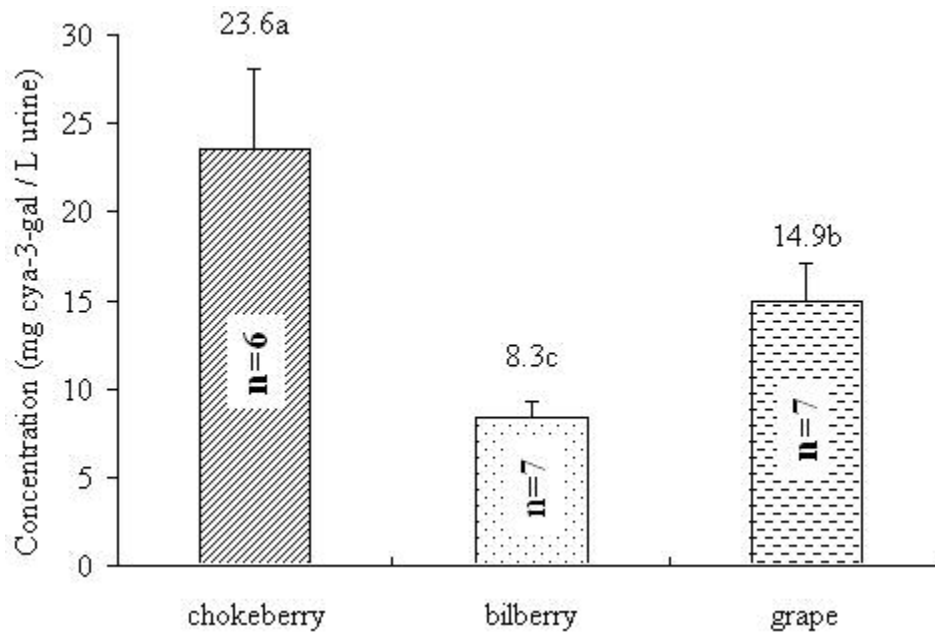


Figure 19 Total anthocyanins recovered from rat urine. Values are the mean \pm SEM of six replications for chokeberry, seven replications for bilberry and grape treatments. Means with different letters are significantly different at $P < 0.05$.

The concentration of urinary anthocyanins was not only related to the amount of anthocyanin excretion, but also related to the urine volume. In urine samples from the chokeberry treatment, the high anthocyanin concentration was at least partially due to the small urinary volume. It had been noticed that among all the 31 urine samples collected, 8 samples had less than 1 mL volume, and 5 of those were from chokeberry treatment. One rat didn't produce any urine excretion at all, representing the only missing sample in this study, and that rat was also from the chokeberry treatment. One rat in each treatment gave greater than 2 mL urine. It seemed to be abnormal because all the rest samples had similar volume of less than 1.5 mL. These data points represented outlier and were discarded during statistical analysis. Apparently, chokeberry ARE enriched diet reduced the urine excretion in rats. However, consumption of water in each group was not significantly different. The reason was not clear, but a possible explanation may be increased fecal moisture

(unpublished data). Since different ARE diets might have varied ability to seize water in the gut, varied urine volume was not surprising.

4.2.1.2. Effect of Sugar Moiety on Urinary Anthocyanin Content

Urine volume was not the only reason for the significantly different urinary anthocyanin concentrations among diet groups. The sugar moiety seemed to play an important role on anthocyanin during absorption. The percentage of individual anthocyanins excreted in the urine was compared with the ARE consumed in the diet, and results obtained are presented in Tables 6 through 8. As shown in Tables 6 and 7, the proportion of anthocyanin mono-glucosides generally decreased as compared to their proportions in AREs. A possible reason is the degradation of anthocyanin glucosides in the small intestine, which will be discussed later in this paper. A more interesting observation stands in Table 8. All anthocyanin 3,5-diglucosides in grape were excreted in the urine consistently in higher proportion than their corresponding 3-glucosides, as evidence by a comparison to their proportion in the AREs. The second glucose moiety seemed to have improved the absorption of anthocyanidin diglucosides. In grape ARE, around 65% of anthocyanins were 3,5-diglucosides; in bilberry ARE, around 50% were 3-glucosides. This may partially explain why in bilberry and grape treatments the urinary total anthocyanin concentrations differed by one fold (exact consumption not known), yet the urine volumes were not so different.

Table 6 Fecal and urinary anthocyanin percentage peak areas in chokeberry treatment¹.

Anthocyanin components	Percentage area			Proportion to ARE	
	ARE	Urine	feces	urine	feces
cya 3-gal	61.0±0.10	37.9±1.6 ²	23.2±3.8	0.62	0.38
cya 3-glu	3.40±0.003	0.95±0.18 ²	0.84±0.33	0.28	0.25
cya 3-arab	28.0±0.06	16.2±0.56 ²	63.7±4.5	0.58	2.27
peo 3-gal	ND ³	20.3±0.53	ND	ND	ND
peo 3-glu	ND	4.8±0.63	ND	ND	ND
peo 3-arab	ND	8.9±0.56	ND	ND	ND
cya 3-xyl	3.9±0.01	ND	5.93±0.94	ND	1.52
Unknown	3.6±0.17	ND	1.57±0.30	ND	0.44

¹ Percentage areas are expressed as Mean ± SEM. ² Newly generated anthocyanins result in decreased percentage of native anthocyanins. ³ ND means not detectable.

Generally diglucosides are more stable than monoglucosides (Delgado-Vargas and Paredes-López 2003). In the grape treatment group (Table 8), only four major peaks were recovered from fecal samples: peonidin 3,5-diglucoside, malvidin 3,5-diglucoside, peonidin 3-glucosides, and malvidin 3-glucoside. Yet the ratio of peonidin 3,5-diglucoside to peonidin 3-glucoside as well as the ratio of malvidin 3,5-diglucoside to malvidin 3-glucoside both increased, in agreement with the hypothesis that diglucosides were less degraded in the GIT. Prior (2004) suggested that cyanidin 3-glucoside might be more susceptible to degradation than cyanidin 3-sambubioside in GIT and thus resulting in reduced excretion. This explanation is in agreement with our findings. However, we also need to consider the potential interaction between glucosides and the intestinal glucose transport (SGLT1) pathway (Williamson *et al.* 2000).

Table 7 Fecal and urinary anthocyanin percentage peak areas in bilberry treatment¹.

Anthocyanin components	Percentage area			Proportion to ARE	
	ARE	Urine	feces	urine	feces
Del 3-gal	10.13±0.05	4.97±0.38	19.35±1.25	0.49	1.91
Del 3-glu	15.75±0.11	5.57±0.22	2.98±0.54	0.35	0.19
Cya 3-gal Del 3-arab	15.48±0.10 ²	10.34±0.43	31.61±1.23	0.67	2.04
Cya 3-glu	11.18±0.00	12.17±0.49	0.71±0.14	1.09	0.06
Pet 3-gal Cya 3-arab	8.90±0.28 ²	13.06±0.30	17.81±0.80	1.47	2.00
Pet 3-glu	11.35±0.05	9.42±0.15	1.99±0.21	0.83	0.18
Peo 3-gal Pet 3-arab	4.06±0.03 ²	7.40±0.19	9.53±0.59	1.82	2.35
Peo 3-glu Mal 3-gal	8.96±0.17 ²	16.95±0.28	6.87±0.84	1.89	0.77
Peo 3-arab	0.23±0.00	2.56±0.17	1.02±0.20	11.34	4.52
Mal 3-glu	11.61±0.00	14.92±0.40	1.09±0.15	1.28	0.09
Mal 3-arab	1.44±0.08	2.23±0.15	7.03±0.83	1.55	4.88

¹ Percentage areas are expressed as Mean ± SEM. ² One row of data with two rows of names means peaks with these two names co-eluted.

Table 8 Fecal and urinary anthocyanin percentage peak areas in grape treatment¹.

Anthocyanin components	Percentage area			Proportion to ARE	
	ARE	Urine	feces	urine	feces
Del 3,5-glu	2.91±0.02	2.00±0.06	ND ²	0.69	ND
Cya 3,5-glu	0.85±0.23	1.18±0.06	ND	1.39	ND
Del 3-glu	3.03±0.30	0.75±0.10	ND	0.25	ND
Pet 3,5-glu	4.83±0.11	5.24±0.13	ND	1.08	ND
Cya 3-glu	1.71±0.16	0.43±0.04	ND	0.25	ND
Peo 3,5-glu	21.2±0.15	32.2±0.24	22.1±0.51	1.52	1.04 ³
Mal 3,5-glu + pet 3-glu ⁴	35.5±0.44	46.2±0.25	47.8±1.52	1.30	1.35 ³
Peo 3-glu	2.38±0.03	2.97±0.09	1.80±0.07	1.25	0.76
Mal 3-Glu	4.79±0.01	5.31±0.12	4.31±0.18	1.11	0.90
Del 3-glu-ac	0.55±0.01	0.15±0.02	ND	0.27	ND
Cya 3-glu-ac	1.83±0.02	0.22±0.02	1.00±0.07	0.12	0.55
Del 3-glu-coum	2.61±0.05	ND	1.63±0.43	ND	0.62
Pet 3-glu-coum	14.1±0.18	2.2±0.2	19.5±1.59	0.16	1.38 ³
Pel 3-glu-coum	0.45±0.04	0.11±0.03	ND	0.24	ND
Mal 3-glu-coum	1.50±0.03	ND	0.63±0.08	ND	0.42

¹ Percentage areas are expressed as Mean ± SEM. ² ND means not detectable. ³ Major peaks such as peo 3,5-glu, mal 3,5-glu, and pet 3-glu-coum in feces increased in terms of percentage area. This was because some other anthocyanins were almost completely not detectable. ⁴ Pet 3-glu is a co-eluted minor peak.

Recently it had been suggested that quercetin glucosides, a group of compounds very similar to anthocyanidin glucosides in structure, interact with SGLT1 pathway (Gee *et al.*1998; Gee *et al.*2000). More recently, Bub *et al.* (2001) and Mülleder *et al.*(2002) reported that the ingestion of glucose or sucrose led to a delayed excretion of anthocyanins. A very interesting phenomenon was shown in Mülleder's data. From the chromatograms in their paper we can see that in the

administered diet cyanidin 3-glucoside and cyanidin 3-sambubioside were at very close molar concentration levels. But they determined that without addition of sugar, the average maximum concentration was 13.6 µg/ urinary sample for cyanidin 3-glucoside, and 29.8µg/ urinary sample for cyanidin 3-sambubioside; when sucrose was added to the diet, the maximum was 11.3 µg/ urinary sample for cyanidin 3-glucoside and 30.8µg/ urinary sample for cyanidin 3-sambubioside. Considering the difference of molecular weight (cyanidin 3-glucoside 449, cyanidin 3-sambubioside 581), the molar excretion of cyanidin 3-sambubioside was one fold higher than cyanidin 3-glucoside, and cyanidin 3-sambubioside was more competitive to sucrose for the transporter. This was possibly due to the presence of an extra glucose moiety of cyanidin 3-sambubioside. Similar mechanism may also apply to our case to explain the apparently superior absorption of diglucosides than monoglucosides.

4.2.1.3. Influence of Acylation on Absorption

Acylated anthocyanins were found in urine samples of rats in the grape treatment. As of 2004, Prior reported that only one study, by Mazza *et al.* (2002) had detected the *in vivo* absorption of intact acylated anthocyanins in serum, though in a lower extent as compared to other monomeric anthocyanins. No other literature to date had reported the detection of acylated anthocyanins in either plasma or urine. Most likely it was because the acylated anthocyanins were present in low concentrations in the foods and or feeding treatments used (most studies have been done choosing isolated anthocyanins or berries that contain no acylated anthocyanins), and current methods were not sensitive enough to detect them. In the grape ARE we

used, acylated anthocyanins accounted for ~22% of the total anthocyanin, and petunidin 3-glucoside-*p*-coumarate in particular accounted for ~14% of total. Therefore our instrument successfully detected clear peaks of acylated anthocyanins in urine samples, though the excretion of acylated anthocyanins was significantly lower than non acylated anthocyanins. For example, the percentage of petunidin 3-glucoside-*p*-coumarate dropped from ~14% in the ARE to ~2% in the urine. The decreasing may not be attributed to the hydrolysis of acylated anthocyanins in the stomach and gut, as traditionally thought. In our study we only found slight decreases of acylated anthocyanins in cecal content as well as fecal samples (Figure 24). This observation supported an alternative explanation that the acyl group on anthocyanins greatly impaired its ability to transport through the mucosal cells on the intestine inner wall.

4.2.1.4. Anthocyanin Metabolites in Urine

Three anthocyanin-like metabolites were detected in rat urine from the chokeberry treatment (Figure 20 A, Table 6). By comparing the retention time and spectrum to bilberry ARE, those three metabolites were tentatively identified to be peonidin 3-galactoside, peonidin 3-glucoside, and peonidin 3-arabinoside. Interestingly, the proportion of cyanidin 3-glucoside to peonidin 3-glucoside (1.87) in urine samples was almost identical with the proportion of cyanidin 3-arabinoside to peonidin 3-arabinoside (1.83). The proportion of cyanidin 3-glucoside to peonidin 3-glucoside (0.20) in urine samples was much lower, indicating the trace amount of cyanidin 3-glucoside was more efficiently transformed to peonidin 3-glucoside, or in

another way, the trace amount of peonidin 3-glucoside was well accumulated in tissues as described shortly after.

In bilberry treatment (Figure 20 B, Table 7), since peonidin glycosides were present in bilberry ARE, we couldn't conclude that peonidin glycosides were produced by metabolism. However, dramatic percentage increase of all the three peonidin glycosides in urine clearly suggests the transformation of cyanidin glycosides to peonidin glycosides. Percentage area of peonidin 3-arabinoside surprisingly increased to 11.34 times. Percentage area of peaks of peonidin 3-galactoside and peonidin 3-glucoside increased to 1.82 and 1.89 times respectively. Because these two peaks were both co-eluting peaks, the actual increase of peonidin 3-galactoside and peonidin 3-glucoside could be greater than 1.82 and 1.89. As cyanidin 3-arabinoside accounted for about 5% of total anthocyanin in bilberry ARE, which is ~20 times higher than peonidin 3-arabinoside (0.23%). The transformation of cyanidin 3-arabinoside to peonidin 3-arabinoside may reasonably explain the rocketing percentage of peonidin 3-arabinoside in urine.

Methylation of cyanidin 3-glucoside was first reported by Tsuda *et al.*(1999). Felgines *et al.*(2002) and Wu *et al.*(2002) also identified trace amounts of peonidin 3-glucoside and peonidin 3-sambubioside as methylated metabolites when diets containing cyanidin 3-glucoside and cyanidin 3-sambubioside were administered. The studies performed by Wu *et al.* (2002) and Felgines *et al.*(2002) involved only short term adaptation, and only trace amount of peonidin glucosides were found. In many other short term studies about urinary anthocyanins the methylated metabolites were

not reported at all. In contrast, our study was a long term study. Samples were collected after 13 weeks of ARE feeding. It has to be noticed that peonidin glycosides accounted for about one third of the total anthocyanins recovered from urine samples (Table 6). This high percentage was unmatched by reported studies indicating the possible accumulation and later release of methylated cyanidin glycosides, i.e. peonidin glycosides, in tissues. We didn't measure the anthocyanin concentration in tissues, but according to Tsuda *et al.* (1999) methylated cyanidin 3-glucoside was found high in liver as well as in kidney, though cyanidin 3-glucoside was not even detected in liver.

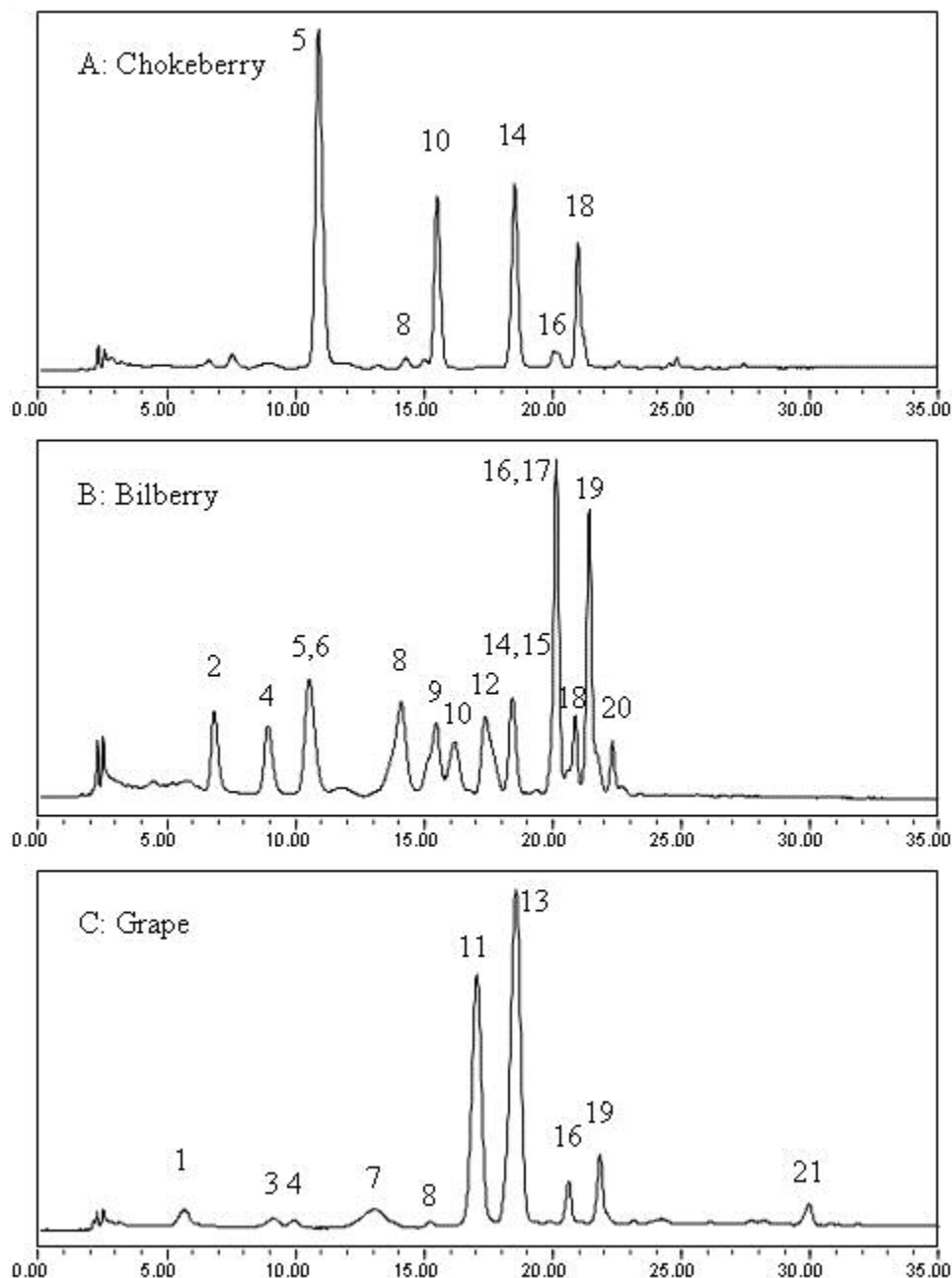


Figure 20 Typical UV-HPLC chromatograms of urinary anthocyanins detected in chokeberry (A), bilberry (B), and grape (C) diet groups. Detection wavelength: 520 nm. Peak identities: 1, del 3,5-diglu; 2, del 3-gal; 3, cya 3,5-diglu; 4, del 3-glu; 5, cya 3-gal; 6, del 3-arab; 7, peo 3,5-diglu; 8, cya 3-glu; 9, pet 3-gal; 10, cya 3-arab; 11, peo 3,5-diglu; 12, pet 3-glu; 13, mal 3,5-diglu; 14, peo 3-gal; 15, pet 3-arab; 16, peo 3-glu; 17, mal 3-gal; 18, peo 3-arab; 19, mal 3-glu; 20, mal 3-arab; 21, pet 3-glu-*p*-coum.

4.2.2. Anthocyanin in Gut Content

4.2.2.1. Gut Content Anthocyanin

Visual appearance of fecal samples suggested the presence of anthocyanin compounds. At the time of collection, feces from anthocyanin treated rats appeared dark purple to black, and red color was clearly seen in the solution as soon as acidified solvent was used for extraction. Similar to the urinary anthocyanins, this color change was attributed to the equilibrium of anthocyanin structure conversion too. However, the intense coloration of the samples suggested that in feces anthocyanins existed in higher concentration and at least partly in a different chemical form than in urine, given the fact that urine was almost colorless. As shown in Figure 6, under neutral or slightly acidic conditions anthocyanins may exist in colorless pseudobase form or purple to violet colored quinonoidal base form. Feces might not have sufficient water to hydrate flavylium anthocyanins to form pseudobases, consequently quinonoidal bases were dominant in the feces. Therefore, the concentrated purple color resulted in very dark feces.

Anthocyanin concentrations found in fecal samples from different treatments are summarized in Tables 6- 8. Also, three cecal samples collected from each diet group were analyzed to compare the anthocyanin in cecal content to that in the colon. As cecum is the startingpoint of large intestine, and feces are collected at the end of large intestine, the comparison of cecal anthocyanin to fecal anthocyanin was conducted to better understand if any change in anthocyanin occurred in the large intestine, where almost 90% of microflora in the entire body resides. Anthocyanin

profiles in the cecal content were very similar to that in the fecal samples, except that the total anthocyanin concentrations were generally lower in the cecal content. This is reasonable because when the cecal content pass through the large intestine, moisture is reabsorbed producing a concentrating effect.

In 4.1.1.2 the recovery efficiency of the extraction method has been discussed. Nearly 90% of the fecal anthocyanins were extracted with acidified 60% methanol, and the extraction efficiency was not significantly different for each treatment. The total anthocyanin level in feces (Figure 21) was in the same order with that in diets (4g/kgdiet).

Researchers have assumed that most anthocyanins would be excreted in the feces (Brouillard 1982), but no one had really studied it. To date our study was the first systematic study of anthocyanins in feces. Our observation supported the traditional thought that fecal anthocyanin was a major excretion of ingested anthocyanins, and meanwhile it unveiled a fact that not all anthocyanins behaved in the same way in gut. Anthocyanin concentration in gut samples from the chokeberry and bilberry treatments were higher than those from the grape treatment, with no significant difference between chokeberry and bilberry treatments. This suggested that grape ARE experienced more degradation in the gut. Consequently grape ARE was expected to produce more derivatives (anthocyanin aglycones as an example, and further derivatives were also possible), which were potentially absorbable to have health benefits or toxic effects as well. Interestingly, we do found the highest level of derivatives in plasma from rats fed grape diet as discussed later in this thesis.

Anthocyanins in gut content may have several benefits. In a parallel study conducted with the same rats used in this study, Magnuson *et al.* (unpublished data) found more inhibition of aberrant crypt foci (ACF), the early stage of colon cancer, in the rats fed chokeberry and bilberry diets than in the rats fed grape diet. The inhibition positively correlated to the anthocyanin concentration in feces, suggesting that the anticarcinogenic effect was related to the concentration of anthocyanins, rather than their derivatives. Fecal moisture analysis done by Magnuson *et al.* (unpublished data) revealed that anthocyanin diets led to moister feces. This water absorbing capability suggested potentially more excretion of water soluble compounds, and consequently more excretion of water soluble toxins including bile acids.

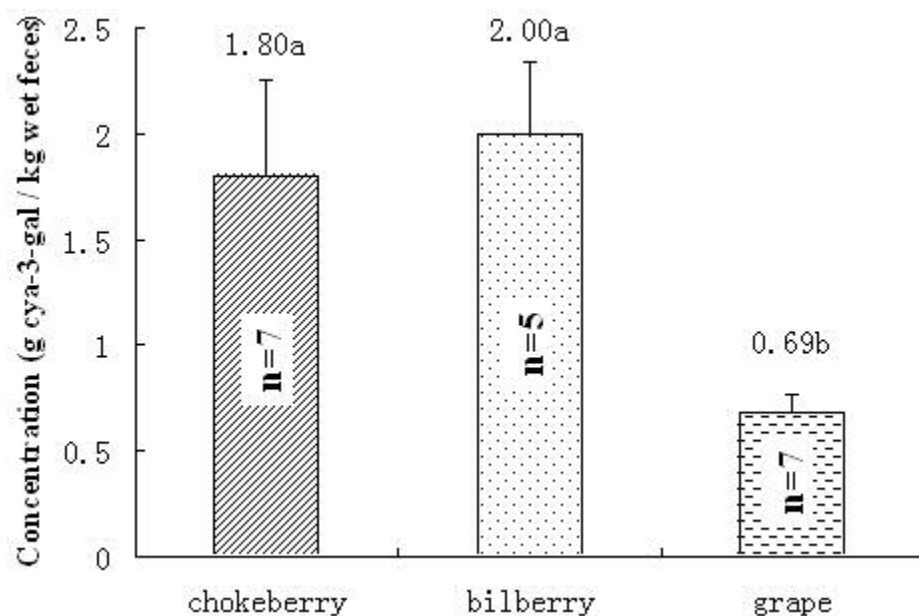


Figure 21 Total anthocyanins recovered from rat feces. Values are the mean \pm SEM of seven replications for chokeberry and grape treatment, five replications for bilberry treatment. Means with different letters are significantly different at $P < 0.05$.

4.2.2.2. Effects of Sugar Moieties on Gut Content Anthocyanins

In addition to the differences in total anthocyanin concentrations in the gut contents from rats fed different diets, we also found differences in terms of individual anthocyanins. Very low amount of anthocyanin glucosides was detected in cecal content and feces (Figure 22 and 23), suggesting they were almost completely metabolized before entering large intestine. Shown in Tables 6 and 7, every anthocyanin glucoside peak except peonidin 3-glucoside decreased to 6-25% of its percentage in ARE. Indeed, the co-elution of peonidin 3-glucoside with another anthocyanin prevented that particular peak from dramatic dropping

All the mono anthocyanins in grape were 3-glucosides or 3,5-diglucosides . Therefore there was not much difference resulted from sugar moieties, and the profile of grape anthocyanins in cecal content and feces kept similar in shape as compared to the grape ARE (Figure 24 and Table 8). Moreover, the overwhelming anthocyanin glucosides in grape ARE reasonably explained the low concentration of total grape anthocyanins found in gut content, since glucosides were easily degraded. About fifty percent of anthocyanins in the bilberry were 3-glucosides. As nearly all those 3-glucosides were reduced, the relative percentage of the next dominant anthocyanins, the 3-galactosides almost doubled.

Considerable degradation of galactosides was also observed. Shown in Table 6, fecal cyanidin 3-galactoside decreased to 38% of its percentage in chokeberry ARE. It was very unfortunate that the HPLC separation of the complex profile from bilberry anthocyanins did not achieve complete resolution of all anthocyanins, with four of the

3-galactosides co-eluting with other components. Out of this four, three peaks co-eluted with anthocyanin arabinosides, and had greater proportion to ARE than the single peak of delphinidin 3-galactoside; one peak co-eluted with peonidin 3-glucoside, and had a much lower proportion to ARE than delphinidin 3-galactoside. Some rats might have much stronger ability to break down galactosides. Those rats brought large variation to the total anthocyanin level in chokeberry treatment, because in chokeberry ARE 70% was cyanidin 3-galactosides. When those experimental units (2 units) were excluded, the mean of fecal total anthocyanin concentration in chokeberry treatment became the highest.

In bilberry treatment two of the well separated 3-arabinosides (peonidin-3-arabinoside and malvidin-3-arabinoside) increased to greater than 4.5 times in feces as compared to their original percentage in ARE. This again evidenced that anthocyanin arabinosides was very stable under physiological conditions, which has also been discussed in 4.1.2.1.

If we consider the different changes in proportion of the individual anthocyanin from bilberry in gut content versus in ARE, we can see that the proportion of arabinosides to galactosides increased by more than 2 fold. Even if we assume no degradation of anthocyanin 3-arabinosides, the anthocyanin galactosides must have degraded at least by half through the GIT.

Summing up, all the observations suggest high glucosidase activity and moderate galactosidase activity in the overall GIT, with low or no arabinosidase activity.

4.2.2.3. Proposed Mechanisms of Anthocyanin Degradation in Gut

In our study, three AREs containing a wide range of individual anthocyanins strongly suggest that anthocyanins in rat gut content were subject to at least two degradation mechanisms, with the influence of glucosidase or galactosidase.

In section 4.1 the distinct influence of fecal microflora on galactosides has been discussed. Our stability study analyzed the change under -18°C. In gut the temperature was around 37°C and the influence of microflora logically would have been magnified. This trend was clearly illustrated in Figure 22, where cyanidin 3-galactoside decreased in the cecal content as compared to the chokeberry ARE, and further decreased in the feces. This suggests that galactosidase exists in the cecum as well as in the colon.

Comparing the anthocyanin profiles in the cecal content and fecal samples (Figures 23-25), it is clear that the degradation of 3-glucosides occurred, and nearly completed before or in the cecum, the connection point of small intestine and large intestine. This observation challenges the traditional thought that flavonoids aglycones were released by colonic microflora (Kühnau 1976; Day *et al.* 2000), indicating the possible role of cytosolic β -glucosidases present in the cell-free extracts of small intestine and liver (Day *et al.* 1998; Williamson *et al.* 2000). Of course if we had studied the small intestine content, the conclusion would have been clearer. A series of studies had evidenced the effects of β -glucosidases on dietary flavonoids and isoflavonoids in the small intestine (Day *et al.* 1998; Day *et al.* 2000; Gee *et al.* 2000; Aura *et al.* 2002). The broad-specificity liver β -glucosidase as well

as substrate specific β -glucosidase such as lactase phlorizin hydrolase (LPH), which is present on the luminal side of the brush border in the small intestine, are potential factors responsible for the flavonoids deglycosylation. Information on the deglycosylation of anthocyanins is scarce. It has only been mentioned by Tsuda *et al.* in several papers (Tsuda *et al.* 1999a; Tsuda *et al.* 1999b; Tsuda *et al.* 2000). However, it's reasonable to speculate that anthocyanins undergo likewise deglycosylation procedures as the similarly structured flavonoids like quercetin glucosides. Of course, direct absorption of anthocyanins also undergo in the meantime.

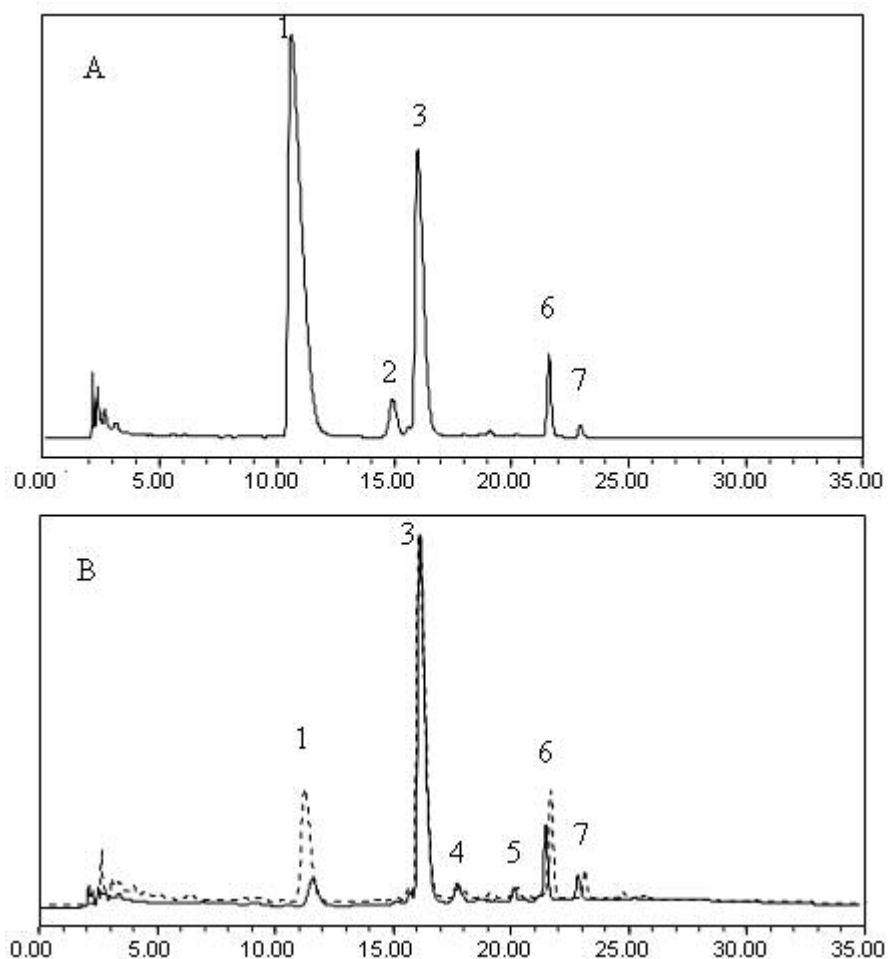


Figure 22 Comparison of anthocyanins in the chokeberry anthocyanin rich extract (A) to the cecal (B dot line) and fecal content (B solid line) from a rat fed chokeberry diet. Detection wavelength: 520 nm. Peak identities: 1, cya 3-gal; 2, cya 3-glu; 3, cya 3-arab; 4, 5, unknown; 6, cya 3-xyl; 7, unknown (probably cyanidin glycoside acylated with an aliphatic acid).

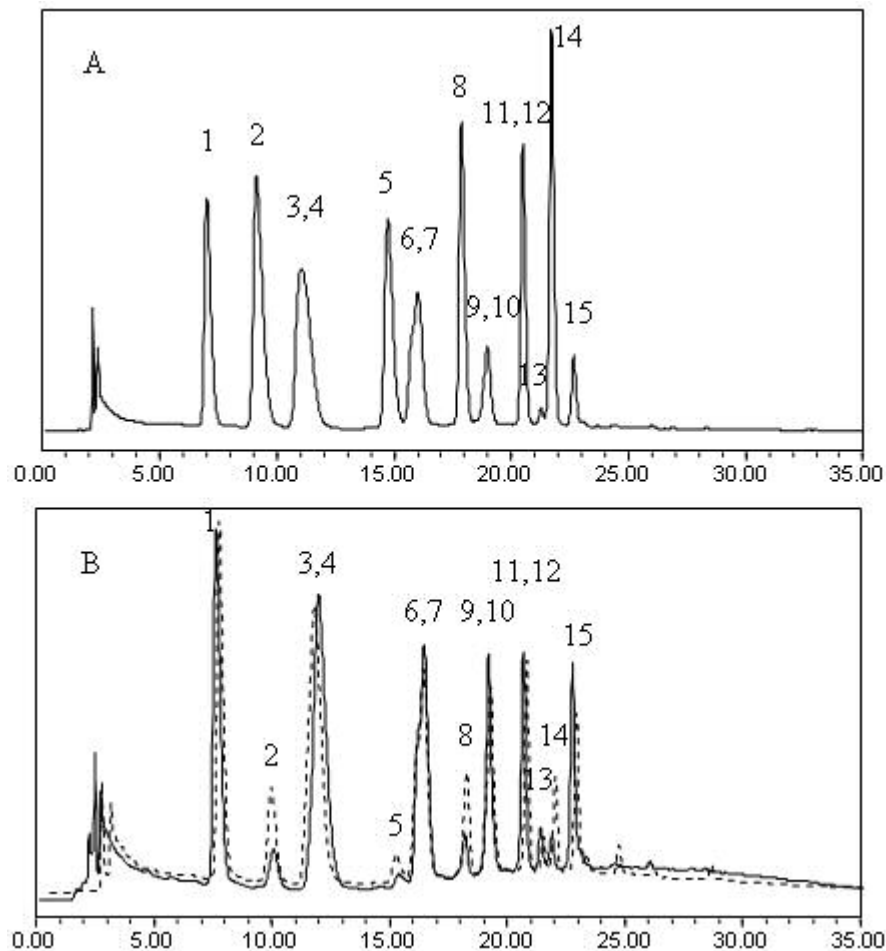


Figure 23 Comparison of anthocyanins in the bilberry anthocyanin rich extract (A) to the cecal (B dot line) and fecal content (B solid line) from a rat fed bilberry diet. Detection wavelength: 520 nm. Peak identities: 1, del 3-gal; 2, del 3-glu; 3, cya 3-gal; 4, del 3-arab; 5, cya 3-glu; 6, pet 3-gal; 7, cya 3-arab; 8, peo 3-glu; 9, peo 3-glu; 10, pet 3-arab; 11, peo 3-glu; 12, mal 3-gal; 13, peo 3-arab; 14, mal 3-glu; 15, mal 3-arab.

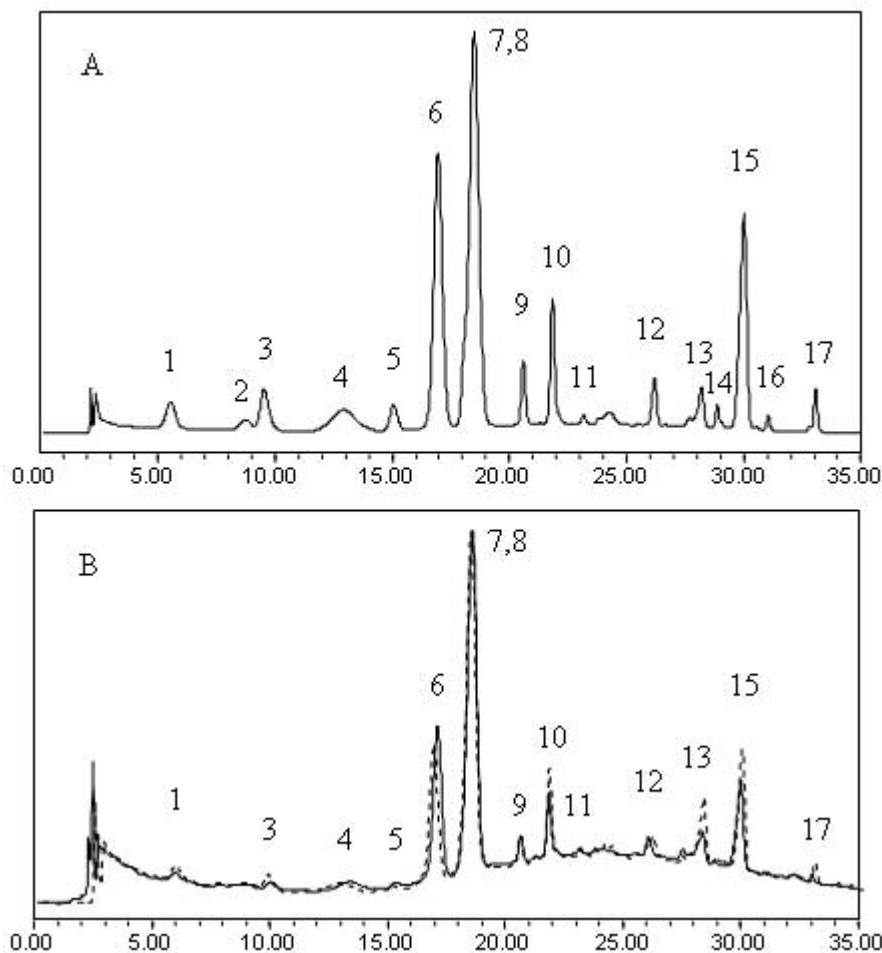


Figure 24 Comparison of anthocyanins in the grape anthocyanin rich extract (A) to the cecal (Bdot line) and fecal content (B solid line) from a rat fed grape diet. Detection wavelength: 520 nm. Peak identities: 1, del 3,5-diglu; 2, cya 3,5-diglu; 3, del 3-glu; 4, pet 3,5-diglu; 5, cya 3-glu; 6, peo 3,5-diglu; 7, pet 3-glu; 8, mal 3,5-diglu; 9, peo 3-glu; 10, mal 3-glu; 11, del 3-glu; 12, cya 3-glu-ac; 13, del 3-glu-*p*-coum; 14, cya 3-glu-*p*-coum; 15, pet 3-glu-*p*-coum; 16, pel 3-glu-*p*-coum; 17, mal 3-glu-*p*-coum.

4.2.3. Anthocyanins and Possible Anthocyanin Metabolites in Plasma

4.2.3.1. Determination of Anthocyanins in Plasma

Most anthocyanins were detected in plasma as intact glycosylated forms (Figure 25). The concentration was too low for accurate quantification with our analytical methodology. However, total anthocyanin level in the plasmas was estimated based on the under curve area to be in the range of 0.1 to 1 $\mu\text{g/mL}$ plasma.

Cyanidin 3-galactoside, cyanidin 3-glucoside and cyanidin 3-arabinoside were the dominant anthocyanins in both the chokeberry and bilberry treatments (Figure 25 B and C). A minor peak of delphinidin 3-galactoside was also found in bilberry treatment. The major anthocyanin peaks in grape ARE (peonidin 3,5-diglucoside and malvidin 3,5-diglucoside) were observed in the plasma as well. However, in grape treatment, cyanidin 3-glucoside also seemed to be preferably absorbed in terms of its percentage area. An interesting finding here was that plasma seemed to favor anthocyanins with cyanidin aglycone. In all the three treatments, six anthocyanin-like compounds with max absorption close to 520nm were unable to be identified because their retention times didn't match any known anthocyanin in corresponding AREs. Therefore they were suspected to be metabolites of anthocyanins administered.

Acylated anthocyanin (malvidin 3-glucoside-acetate) was found in plasma samples from the grape treatment (Figure 25 D), confirming the report by Mazza *et al.* (2002), that anthocyanins can possibly be absorbed in acylated forms. As expected, no anthocyanidins were detected in any of the samples evaluated, likely due to their extremely low stability.

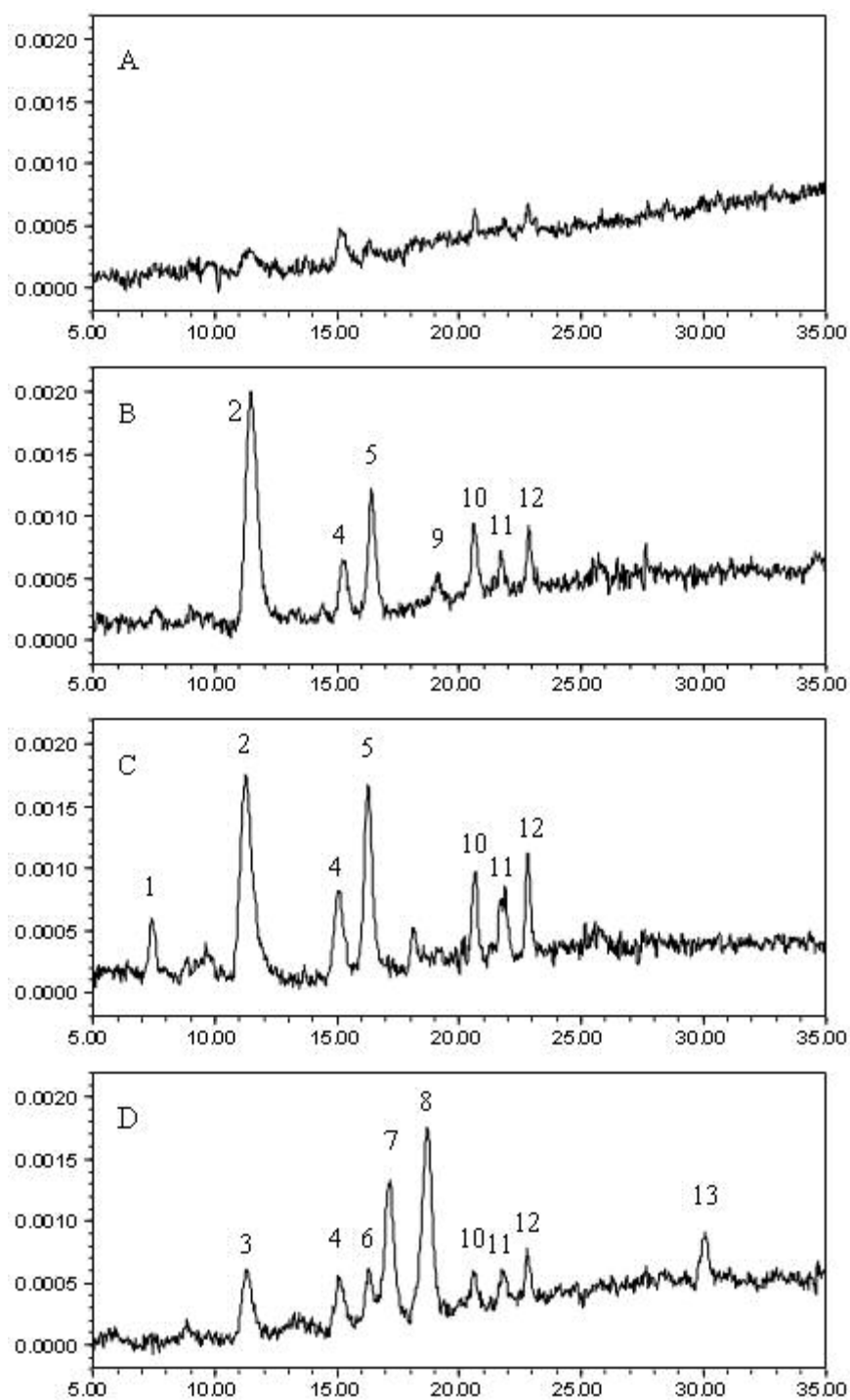


Figure 25 Anthocyanins and anthocyanin-like compounds detected in rat plasma in control (A), chokeberry (B), bilberry (C), and grape (D) diet groups. Detection wavelength: 520 nm. Peak identities: 1, del 3-galactoside; 2, cya 3-arab; 3, unknown; 4, cya 3-glu; 5, cya 3-arab; 6, unknown; 7, peo 3,5-diglu; 8, mal 3,5-diglu; 9, 10, 11, 12, unknown; 13, pet 3-glu-coum.

4.2.3.2. Determination of Possible Anthocyanin Metabolites in Plasma

Derivatives of anthocyanins as protocatechuic acid (PC) or other phenolics *in vitro* and *in vivo* have been reported in several studies (Tsuda *et al.* 1999a; Tsuda *et al.* 1999b; Tsuda *et al.* 2000; Seeram *et al.* 2001). In our study, two major non-anthocyanin peaks (compounds 1 and 2) with high absorption at 320nm were found in every plasma samples from ARE fed groups (three plasma samples in each treatment were analyzed) (Figure 26, 27). These peaks were not present in plasma samples from the control group. Their peak areas were several folds larger than plasma anthocyanins, yet they were not found in the feces or cecal content, neither in any ARE. These facts suggested that they were anthocyanin metabolites, and that the conversion of anthocyanins to such compounds occurred in the blood after absorption. Based on spectrum information (Figure 26), we suspected that compound 2 in plasma was a certain flavonoid.

The concentration of compound 1 found in the plasma was much higher in grape treatment than in bilberry and chokeberry treatments. Combining this observation together with the evidence of extensive deglycosylation of grape ARE in gut described in 4.2.2.2., we hypothesized that it was the deglycosylated anthocyanins (aglycones) that further degraded to compound 1 in plasma.

Chokeberry, bilberry and grape AREs had distinctly different anthocyanin aglycones, but surprisingly the same compound 1 was observed in plasma samples from all treatments. As introduced in 2.1.1, the only difference among the 6 common anthocyanin aglycones is the B ring. Therefore we hypothesized that compound 1 is a

derivative from A or C ring of anthocyanidins, probably under the peroxide attack at the C-2 position at physiological pH. Based on information from anthocyanin degradation studies (Wong 1989; Seeram *et al.*2001) , we proposed three possible structures of compound 1 in plasma (Figure 28).

Neither, anthocyanin aglycones nor compounds 1, 2 were found in feces or cecal content. However this fact does not disputethe proposed scheme because : (1) the aglycones may only produce such derivatives in the plasma; (2) it has been known that anthocyanin aglycones are very unstable under neutral pH condition; (3) many phenolics are susceptible to the gut microflora, so even if such derivatives had been produced in the gut, very likely they would rapidly convert into other metabolites.

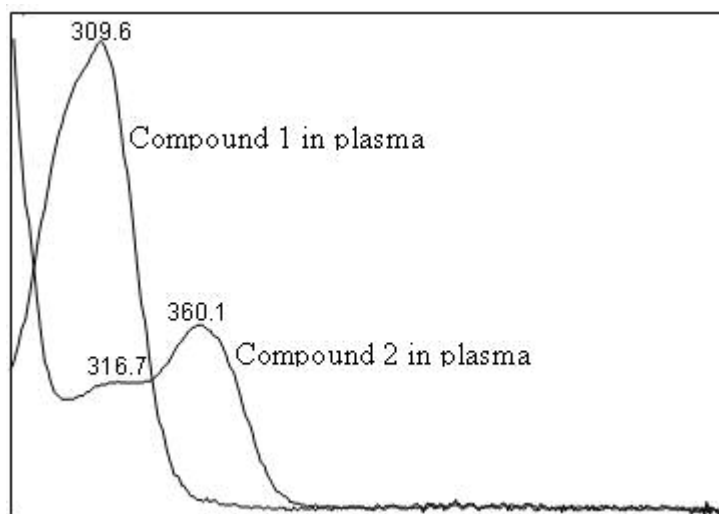


Figure 26 Spectrum of the two compounds of interest in plasma.

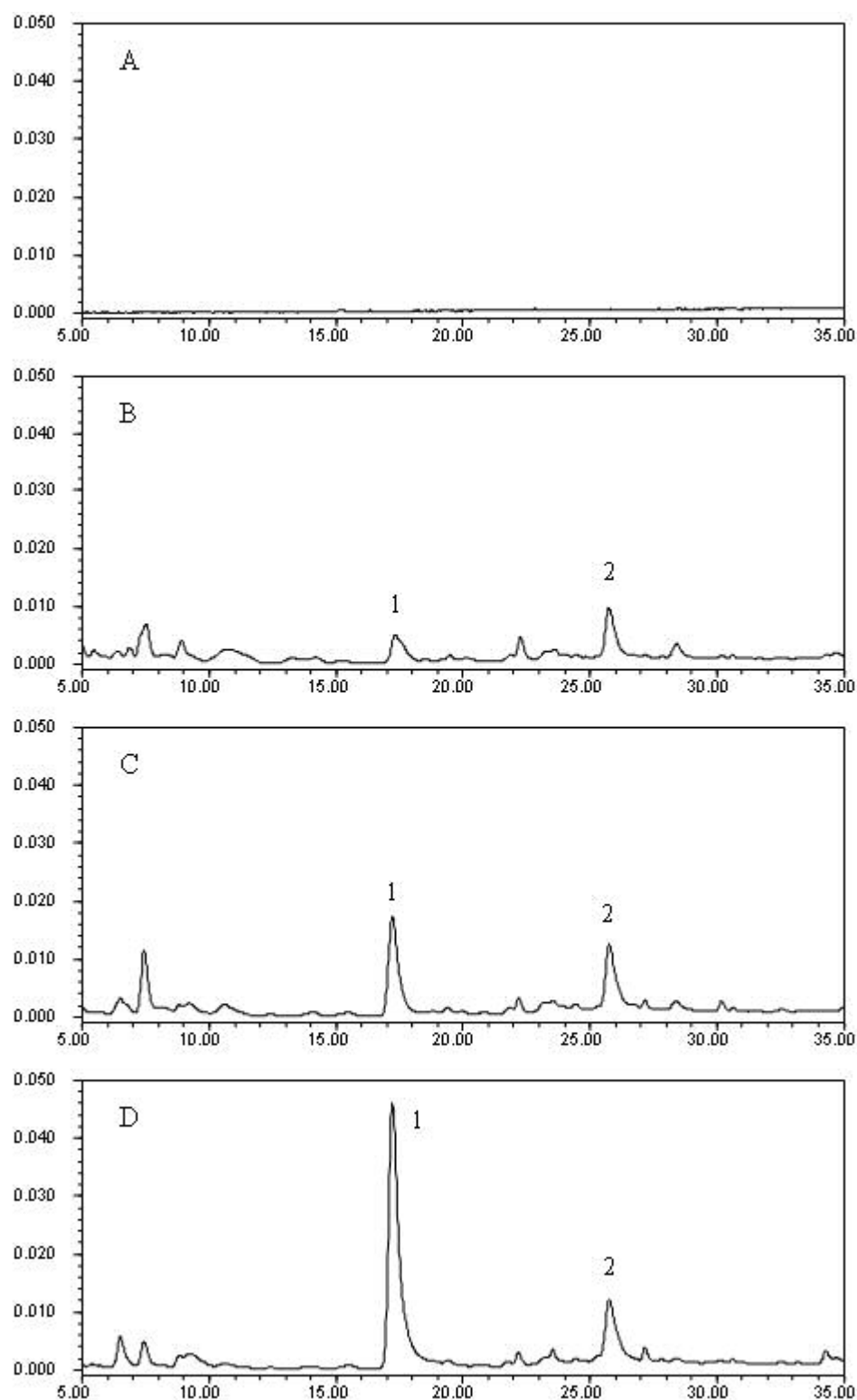


Figure 27 Typical UV-HPLC chromatograms of SPE purified plasma at 320 nm in control (A), chokeberry (B), bilberry (C), and grape diet groups (D).

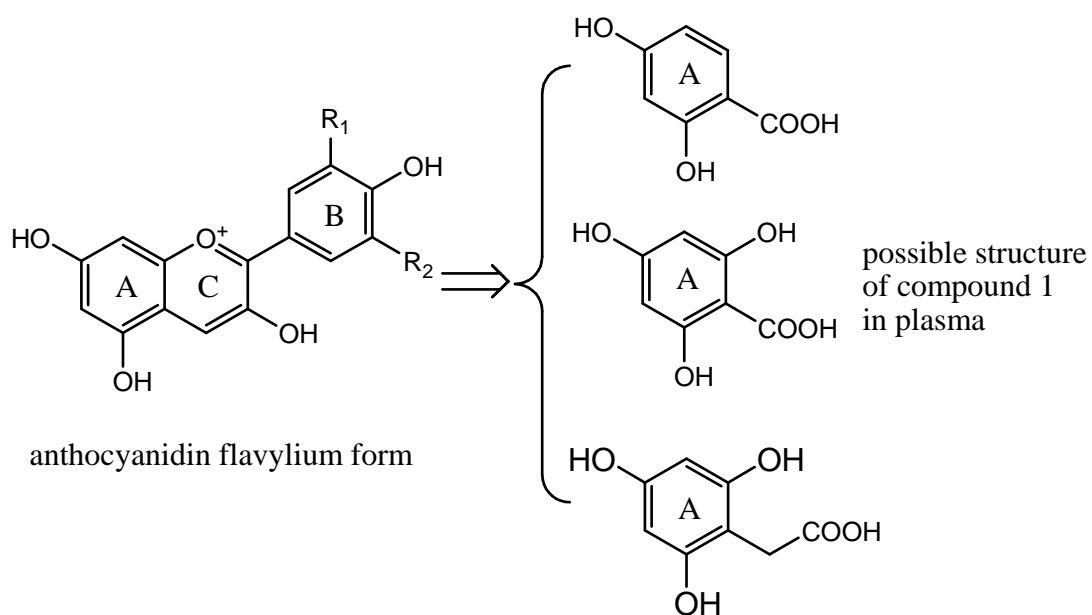


Figure 28 Three proposed possible structures of compound 1 in plasma that may be formed as a result of anthocyanin degradation .

Chapter 5: Summary and Future Research

Using rats as an animal model, we evaluated the impact of chemical structure on bioavailability of anthocyanins. In a long term feeding trial we observed that sugar moiety had significant impact on the apparent absorption, metabolism, and excretion of individual anthocyanins in rats. The method we developed for fecal anthocyanin analysis allowed us to accurately measure anthocyanins in cecal content and feces, therefore made it possible to depict the pattern of anthocyanin degradation in the large intestine. Overall in the gut anthocyanin glucosides were the least stable, probably due to the β -glucosidase present in the small intestine. Anthocyanin galactosides were fairly unstable under the influence of large intestine microflora. Anthocyanin arabinosides and xylosides seemed to be quite resistant to the gut microflora. These findings provide information for the screening of more stable anthocyanins *in vivo*. As found in a parallel study, higher availability of anthocyanins in the colon seemed to be correlated with higher inhibition of colon cancer.

In the future *in vitro* experiments can be developed using the crude extract of gut content to evaluate the metabolism of anthocyanins. This approach gives less interference and makes it easier to monitor many factors of concern, such as the production of anthocyanin aglycones, and the kinetics of degradation. An anthocyanin source with simple aglycone and various sugars is recommended for such purpose.

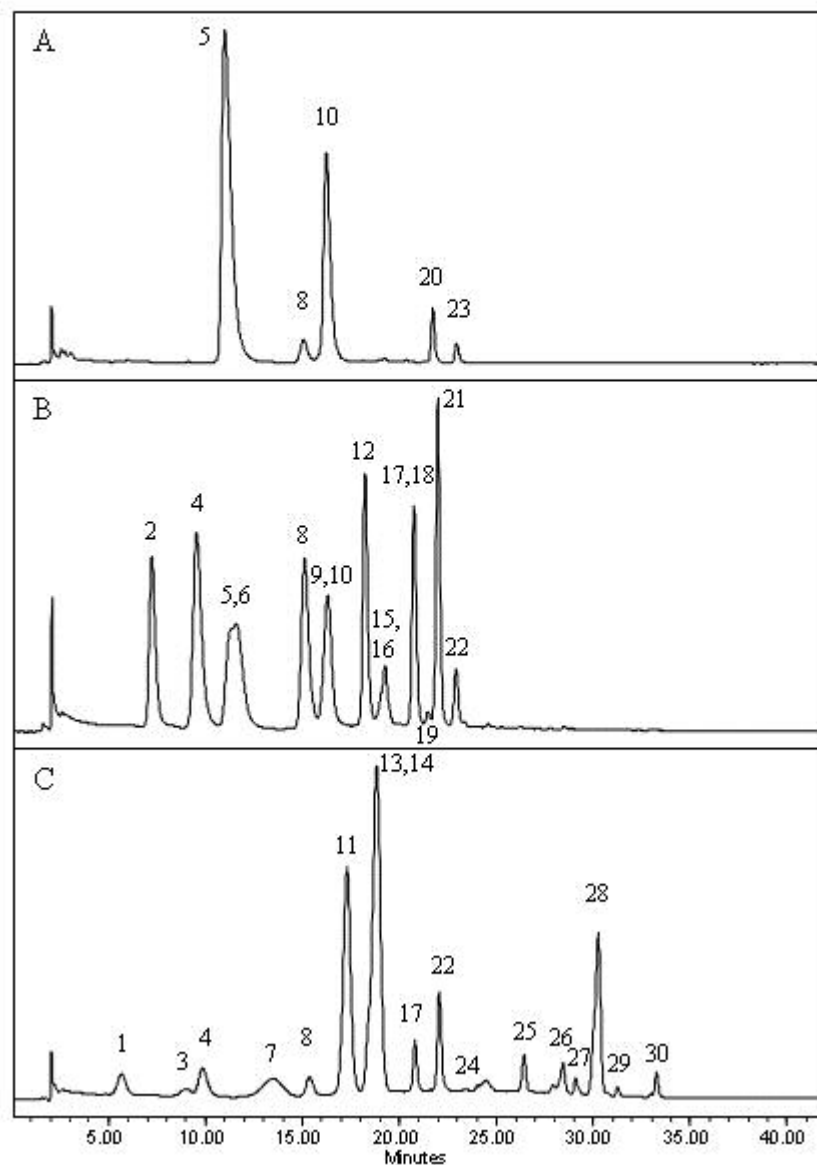
Our study of rat urine and plasma supported the finding by numerous researchers that anthocyanins have very low absorption. This, on the other hand, also

supports our hypothesis that anthocyanins in gut may have health benefits even without being absorbed. We also noticed in plasma the presence of large amount of two possible anthocyanin derivatives. They were found in all the three treatments but neither in the control nor the anthocyanin diets. One of them had the concentration positively correlated to the degradation extent of total anthocyanins in gut. Mass spectrum (MS) may provide more information on the identity of this compound, but judging by its spectrum this compound seemed to have a double bond conjugated to a benzene ring. Since anthocyanins themselves were proved to have low bioavailability, the health benefit of their *in vivo* derivatives may worth our notice in the future.

In urine we found anthocyanin metabolites having maximum absorbance close to 520nm. They were tentatively identified as methylated anthocyanins (cyanidin glycosides methylated to peonidin glycosides) based on retention time and spectrum. MS will provide the molecular weight of these compounds, as well as the molecular weight on the parent compound (anthocyanidin), allowing for definite identification of the aglycone. Anthocyanins were generally regarded as not being accumulated in the plasma, but people rarely measure the anthocyanin in tissues. In this long term study we observed surprisingly larger urine excretion of methylated anthocyanins than in two short term studies reported, suggesting the possible accumulation of anthocyanins in tissues (liver in particular). Measuring tissue bound anthocyanin is a promising way to explain the contradiction of widely observed health benefit and low plasma availability of anthocyanins. Also, we should test in the future if the same anthocyanin metabolites are present in human plasma and urine.

Appendices

Appendices



Appendix 1 UV-HPLC chromatograms of anthocyanins from chokeberry (A), bilberry (B), and grape (C) extracts. Detection wavelength: 520 nm. Peak identities: 1, del 3,5-diglu; 2, del 3-gal; 3, cya 3,5-diglu; 4, del 3-glu; 5, cya 3-gal; 6, del 3-arab; 7, peo 3,5-diglu; 8, cya 3-glu; 9, pet 3-gal; 10, cya 3-arab; 11, peo 3,5-diglu; 12, pet 3-glu; 13, pet 3-glu; 14, mal 3,5-diglu; 15, peo 3-gal; 16, pet 3-arab; 17, peo 3-glu; 18, mal 3-gal; 19, peo 3-arab; 20, cya 3-xyl; 21, mal 3-glu; 22, mal 3-arab; 23, unknown (tentative: cya glycoside acylated with aliphatic acids); 24, del 3-glu-ac; 25, cya 3-glu-ac; 26, del 3-glu-*p*-cumarate; 27, cya 3-glu-*p*-coum; 28, pet 3-glu-*p*-coum; 29, pel 3-glu-*p*-coum; 30, mal 3-glu-*p*-coum.

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