ISOLATION OF ANTHOCYANIN MIXTURES FROM FRUITS AND VEGETABLES AND EVALUATION OF THEIR STABILITY, AVAILABILITY AND BIOTRANSFORMATION IN THE GASTROINTESTINAL TRACT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

The Ohio State University
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ABSTRACT

Anthocyanins are among the most abundant polyphenols in fruits and vegetables and exhibit potent antioxidant activity. Our previous studies suggested that anthocyanins present in the gastrointestinal tract (GIT) of rats exert chemopreventive effect on colon cancer. Since such effect was dose dependent, it is important to examine the stability and transformation of anthocyanins in the GIT. Based on some in vitro studies and limited in vivo evidence, we hypothesized that anthocyanins were moderately stable under the influence of digestive enzymes and physiological conditions in the GIT, and their chemical structures determine the stability, accessibility, biotransformation, and health-promoting effects.

To validate our hypothesis a series of studies were conducted. We first examined the transit, stability, transformation, and uptake of black raspberry anthocyanins in rat GIT. Our results suggest that ingested anthocyanin glycosides remained relatively stable in the GIT lumen and were efficiently taken up into the GIT tissues with limited transfer to the plasma compartment. We also observed selective degradation of cyanidin-3-glucoside in the small intestine lumen, which was tentatively attributed to β-glucosidase activity in the small intestine.
In order to eliminate interferencing non-anthocyanin compounds in bioassays, a novel mixed mode cation-exchange/reversed-phase (MCX) solid-phase extraction (SPE) technique was developed for high purity isolation of anthocyanins. The new methodology drastically increased purity and efficiency as compared to three widely used SPE techniques – C\textsubscript{18}, HLB, and LH-20 columns. For the majority of plant materials evaluated, the new technique achieved above 99% anthocyanin purity while maintaining excellent yield (93.6 ± 0.55%) and low cost. The resulted high purity anthocyanins were later used in the enzymatic assays.

To elucidate the effect of intestinal enzymes on anthocyanin digestion, an \textit{in vitro} model was employed. Cell-free extract of pig small intestinal mucosa and crude extract of lactase containing foods/supplement were examined with respect to their \(\beta\)-glycosidase activities on highly purified anthocyanins. Selective degradation of anthocyanin glucosides and galactosides was observed in the presence of small intestinal mucosa cell-free extract and lactase supplement extract, respectively. The type of aglycone also substantially affected the resistance of anthocyanins to enzymatic degradation.

The outcome of this dissertation demonstrates that chemical structure of anthocyanins greatly affects their stability in the GIT. Such information will contribute to the exploration of anthocyanin’s health benefits \textit{in vivo}, and may eventually facilitate the development of value added foods/nutraceuticals that promote the healthiness of people.
The dissertation is dedicated to my mother Zonghui Hu, my father Shide He, and my dear wife Weishu Xue for their love and ultimate support to me.
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**FIELDS OF STUDY**

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>ii</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGMENTS</strong></td>
<td>v</td>
</tr>
<tr>
<td><strong>VITA</strong></td>
<td>vii</td>
</tr>
<tr>
<td><strong>TABLE OF CONTENTS</strong></td>
<td>ix</td>
</tr>
<tr>
<td><strong>LIST OF TABLES</strong></td>
<td>xvi</td>
</tr>
<tr>
<td><strong>LIST OF FIGURES</strong></td>
<td>xvii</td>
</tr>
<tr>
<td><strong>LIST OF ABBREVIATIONS</strong></td>
<td>xx</td>
</tr>
<tr>
<td><strong>1. INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>2. LITERATURE REVIEW</strong></td>
<td>3</td>
</tr>
<tr>
<td>2.1 ANTHOCYANINS AS NATURAL PIGMENTS</td>
<td>3</td>
</tr>
<tr>
<td>2.2 CHEMICAL STRUCTURE OF ANTHOCYANINS</td>
<td>4</td>
</tr>
<tr>
<td>2.2.1 Chemical structure of flavonoids</td>
<td>4</td>
</tr>
<tr>
<td>2.2.2 Anthocyanin aglycones</td>
<td>5</td>
</tr>
<tr>
<td>2.2.3 Glycosylation and acylation</td>
<td>7</td>
</tr>
</tbody>
</table>
2.5.4  Anti-carcinogenic activity ................................................................. 27
2.5.5  Prevention of obesity ................................................................. 32
2.5.6  Control of diabetes ................................................................. 33
2.5.7  Improvement of eye vision .................................................... 34
2.5.8  Antimicrobial activity ............................................................. 34

2.6  BIOAVAILABILITY AND METABOLISM OF ANTHOCYANINS …… 35

2.6.1  Overview .......................................................... 36
2.6.2  Gastric absorption ............................................................ 37
2.6.3  Direct absorption in the small intestine ................................... 40
2.6.4  Deconjugation of carbohydrate moieties .................................. 43
2.6.5  The influence of colonic microflora ......................................... 46
2.6.6  Metabolism in intestinal mucosa and tissues .......................... 47
2.6.7  Tissue distribution .............................................................. 48
2.6.8  Excretion ......................................................................... 49

3.  BLACK RASPBERRY ANTHOCYANINS ARE STABLE IN THE DIGESTIVE TRACT LUMEN AND EFFICIENTLY TRANSPORTED INTO GASTRIC AND SMALL INTESTINAL TISSUES IN RAT ................................................................. 52

3.1.  ABSTRACT ................................................................. 52
3.2. INTRODUCTION .......................................................................................................... 53

3.3. MATERIALS AND METHODS .................................................................................... 55
   3.3.1 Chemicals and materials ...................................................................................... 55
   3.3.2 Black raspberry extract ....................................................................................... 55
   3.3.3 Animals and experimental design ......................................................................... 56
   3.3.4 Sample preparation .............................................................................................. 57
   3.3.5 Recovery ............................................................................................................... 59
   3.3.6 HPLC-MS analysis of anthocyanins and anthocyanin metabolites ...................... 59
   3.3.7 Calibration curve .................................................................................................. 60
   3.3.8 Statistical analysis .............................................................................................. 61

3.4. RESULTS AND DISCUSSION .................................................................................... 61
   3.4.1 Flux of anthocyanins in GIT lumen ...................................................................... 61
   3.4.2 Anthocyanins in GIT tissues ................................................................................ 67
   3.4.3 Anthocyanin transformation in the GIT ............................................................... 69
   3.4.4 Metabolism and excretion of absorbed anthocyanins ......................................... 75

3.5. CONCLUSIONS .......................................................................................................... 77

3.6. ACKNOWLEDGEMENTS ........................................................................................... 77
4. HIGH-PURITY ISOLATION OF ANTHOCYANIN MIXTURES FROM FRUITS AND VEGETABLES – A NOVEL SOLID-PHASE EXTRACTION METHOD …….. 79

4.1. ABSTRACT ……………………………………………………………………………………………… 79

4.2. INTRODUCTION ……………………………………………………………………………………… 80

4.3. MATERIALS AND METHODS …………………………………………………………………………. 83

4.3.1 Reagents and standards ……………………………………………………………………………… 83

4.3.2 Anthocyanin sources and sample preparation ………………………………………………….. 84

4.3.3 Fractionation methods comparison ……………………………………………………………… 84

4.3.4 Extended evaluation on a variety of anthocyanin sources ……………………………………… 89

4.3.5 Optimization for food application ……………………………………………………………… 89

4.3.6 Statistical analysis ………………………………………………………………………………… 89

4.4. RESULTS AND DISCUSSION ……………………………………………………………………… 90

4.4.1 MCX method development ……………………………………………………………………… 90

4.4.2 Fractionation methods comparison ……………………………………………………………… 91

4.4.3 Extended evaluation on a variety of anthocyanin sources ……………………………………… 102

4.4.4 Optimization for food application ……………………………………………………………… 104

4.5. CONCLUSIONS …………………………………………………………………………………………………… 104

4.6. ACKNOWLEDGEMENTS ………………………………………………………………………………… 105
5. IMPACT OF SMALL INTESTINAL $\beta$-GLUCOSIDASE AND DIETARY LACTASE ON ANTHOCYANIN DIGESTION .................................................. 106

5.1. ABSTRACT ........................................................................................................... 106

5.2. INTRODUCTION ................................................................................................... 107

5.3. MATERIALS AND METHODS .......................................................................... 108

5.3.1 Chemicals and materials ............................................................................... 109

5.3.2 Purification of anthocyanins ......................................................................... 109

5.3.3 $\beta$-glucosidase activity in porcine small intestinal mucosa ...................... 111

5.3.4 Lactase from foods ......................................................................................... 114

5.3.5 HPLC-MS analysis of anthocyanins ............................................................ 118

5.3.6 Calibration curve ............................................................................................ 119

5.3.7 Statistical analysis ........................................................................................ 119

5.4. RESULTS AND DISCUSSION ......................................................................... 120

5.4.1 Lactase activity of pig small intestinal mucosa extract ............................... 120

5.4.2 $\beta$-glucosidase activity on anthocyanins .................................................... 120

5.4.3 Calculation of apparent $K_m$ and $V_{max}$ for intestinal $\beta$-glucosidase .... 124

5.4.4 Substrate specificity of small intestinal $\beta$-glucosidase ......................... 127

5.4.5 Activity of lactase supplement on anthocyanins ........................................ 131
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1 Differences on chemical structure, color and $\lambda_{\text{max}}$ of anthocyanidins most commonly found in nature</td>
<td>6</td>
</tr>
<tr>
<td>Table 2.2 $K_m$ of quercetin 4'-glu and genistein 7-glu by $\beta$-glucosidase from human and animal intestine and liver</td>
<td>45</td>
</tr>
<tr>
<td>Table 3.1 Peak assignments of anthocyanins extracted from the gastric and intestinal contents of rats administered black raspberry extract</td>
<td>63</td>
</tr>
<tr>
<td>Table 3.2 Black raspberry anthocyanins in urine collected from rat bladder</td>
<td>76</td>
</tr>
<tr>
<td>Table 4.1 Mobile phases used to elute compounds of interest from the SPE cartridges</td>
<td>85</td>
</tr>
<tr>
<td>Table 4.2 Visually observed anthocyanin color loss during purification of anthocyanin mixtures by the different SPE procedures</td>
<td>94</td>
</tr>
<tr>
<td>Table 5.1 Composition of mixtures used to test the effect of pig small intestinal enzymes on black raspberry anthocyanins</td>
<td>113</td>
</tr>
<tr>
<td>Table 5.2 Compositions of buffered solutions used to investigate the effect of lactase supplement on blueberry anthocyanins</td>
<td>116</td>
</tr>
<tr>
<td>Table 5.3 Kinetic constants for the hydrolysis of LPH substrates</td>
<td>124</td>
</tr>
<tr>
<td>Table 5.4 Profile change of grape anthocyanins after incubation with LPH for 30 min</td>
<td>129</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 2.1 Representative aglycone structures of the common flavonoid sub-classes</td>
<td>5</td>
</tr>
<tr>
<td>Figure 2.2 Basic chemical structure of a common anthocyanidin</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2.3 Chemical structure of an acylated anthocyanin (Mv-3-(p-coumaryl)glu) in grape skin</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2.4 Scheme of the pH-dependent structural interconversion between dominant forms of mono-glycosylated anthocyanins in aqueous phase</td>
<td>9</td>
</tr>
<tr>
<td>Figure 2.5 A representative chemical structure of pyranoanthocyanins</td>
<td>12</td>
</tr>
<tr>
<td>Figure 2.6 Effect of feeding anthocyanin rich diets on total anthocyanin concentration in rat urine, colonic cell proliferation index, large ACF multiplicity, total anthocyanin concentration in feces, fecal moisture content, and fecal bile acids concentration</td>
<td>31</td>
</tr>
<tr>
<td>Figure 2.7 Mv-3-glu concentration in plasma sampled from either the portal vein or the heart of rats administered with grape anthocyanins</td>
<td>39</td>
</tr>
<tr>
<td>Figure 2.8 The ratio of mean 24h cumulative urinary excretion: intake of quercetin</td>
<td>42</td>
</tr>
<tr>
<td>Figure 2.9 Intergrated putative pathways of dietary flavonoids absorption, metabolism, distribution, and excretion</td>
<td>51</td>
</tr>
</tbody>
</table>
Figure 3.1 Chromatographic profiles of Cy standard, black raspberry anthocyanins in the administered crude extract, and representative samples of luminal contents from stomach and small intestine from a rat killed 120 min after gavage................................................................. 64

Figure 3.2 Black raspberry anthocyanins in the gastric and small intestinal contents and in the small intestinal tissue .................................................................................................. 65

Figure 3.3 UV-Vis spectra of the anthocyanin-protein complex extracted from the stomach tissue ................................................................................................................... 68

Figure 3.4 Relative abundance of black raspberry anthocyanins in the gastric and small intestinal lumens ............................................................................................................... 71

Figure 3.5 Relative abundance of Cy-3-glu is decreased in the small intestinal content . 73

Figure 4.1 The chemical structure of MCX sorbent and an anthocyanin molecule at varied pH conditions.................................................................................................................... 83

Figure 4.2 Percentage of individual chokeberry anthocyanin peaks after purification .... 94

Figure 4.3 HPLC-PDA chromatograms of chokeberry crude extract and purified anthocyanin fractions ........................................................................................................ 97

Figure 4.4 The purity and recovery of anthocyanins and other-phenols based on the AUC of HPLC-PDA chromatograms ............................................................................................ 98

Figure 4.5 Impurities in chokeberry anthocyanin fractions as indicated by the appearance of noise peaks in the MS chromatogram................................................................. 100

Figure 4.6 HCA dendrograms of chokeberry and purple corn extracts....................... 101

Figure 4.7 The purity of anthocyanins purified from 10 additional commodities....... 103
Figure 5.1 Degradation of purified black raspberry anthocyanins during incubation with pig small intestinal cell-free extract in the presence and absence of β-glucosidase inhibitors ................................................................. 123

Figure 5.2 Saturation curve for the small intestinal mucosal β-glucosidase showing the relationship between Cy-3-glu substrate concentration and rate of Cy-3-glu hydrolysis 125

Figure 5.3 HPLC chromatograms of purified grape anthocyanins incubated with LPH for 0 min and 30 min in the presence or absence of D-gluconolactone ......................... 128

Figure 5.4 Chemical structures of the common anthocyanidins glycosylated on the C-3 position .......................................................................................................................... 130

Figure 5.5 HPLC chromatograms of purified blueberry anthocyanins incubated with either heat-inactivated lactase from supplement, active lactase from supplement extract, or lactase from supplement in the presence of 584 mM lactose for 15 min .......... 133

Figure 5.6 Profile change of anthocyanin galactosides in blueberry after incubation with the lactase supplement extract for 15 min ........................................................................ 134

Figure 5.7 Lactase supplement extract substrate specificity on the 5 anthocyanin galactosides in blueberry ................................................................. 136

Figure 5.8 Saturation curve for the lactase supplement extract showing the relationship between Cy-3-gal substrate concentration and rate of Cy-3-gal hydrolysis. ................. 137

Figure 5.9 Intact chokeberry anthocyanins remaining after incubation with buffer or dietary lactase extract for 45 min .......................................................................................... 138

Figure 5.10 Growth inhibition (48 h) of human colon cancer cell line HT-29 by purified chokeberry anthocyanins and lactase treated chokeberry anthocyanins ..................... 140
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACF</td>
<td>Aberrant crypt foci</td>
</tr>
<tr>
<td>ADI</td>
<td>Acceptable daily intake</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethane</td>
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<tr>
<td>Ara</td>
<td>Arabinoside</td>
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<tr>
<td>CBG</td>
<td>Cytosolic β-glucosidase</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenases</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>DD</td>
<td>Double distilled</td>
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<tr>
<td>Gal</td>
<td>Galactoside</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
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<tr>
<td>Glc</td>
<td>Glucuronide</td>
</tr>
<tr>
<td>Glu</td>
<td>Glucoside</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycoside</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<tr>
<td>HPLC</td>
<td>High-pressure liquid chromatography</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>JECFA</td>
<td>Joint FAO/WHO Expert Committee on Food Additives</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>LPH</td>
<td>Lactase-phlorizin hydrolase</td>
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<tr>
<td>MPLC</td>
<td>Medium-pressure liquid chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectroscopy</td>
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<tr>
<td>Mv</td>
<td>Malvidin</td>
</tr>
<tr>
<td>NOEL</td>
<td>No-observed-effect-level</td>
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<tr>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
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<tr>
<td>PC</td>
<td>Protocatechuic acid</td>
</tr>
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<td>Pg</td>
<td>Pelargonidin</td>
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<tr>
<td>POD</td>
<td>Polyphenol proxidase</td>
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<tr>
<td>PPO</td>
<td>Polyphenol oxidase</td>
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<td>Pn</td>
<td>Peonidin</td>
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<td>Pt</td>
<td>Petunidin</td>
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<tr>
<td>Rha</td>
<td>Rhamnose</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>Rut</td>
<td>Rutinoside</td>
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<tr>
<td>Sam</td>
<td>Sambubioside</td>
</tr>
<tr>
<td>SAOC</td>
<td>Serum antioxidant capacity</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SGLT1</td>
<td>Sodium-dependent glucose transporter</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>SULT</td>
<td>Phenol sulfotransferases</td>
</tr>
<tr>
<td>TAC</td>
<td>Total antioxidant capacity</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>UDPGD</td>
<td>Uridine diphosphoglucose glucose dehydrogenase</td>
</tr>
<tr>
<td>UDPGT</td>
<td>Uridine diphosphoglucose glucuronosyl transferase</td>
</tr>
<tr>
<td>Xyl</td>
<td>Xyloside</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Anthocyanins are a group of polyphenols widely distributed in fruits, vegetables, and fruit based beverages as natural pigments. Among all the recognized polyphenols, anthocyanins are especially important because of their potent antioxidant property and high consumption, probably the highest among all flavonoids (1-3). Although cell culture studies, animal studies and epidemiological studies have suggested association between anthocyanin intake and disease prevention, the bioactive form of anthocyanins or anthocyanin metabolites is still controversial. Studies have repeatedly shown that only very limited amount of intact anthocyanins are absorbed (4). Amounts of glucuronidated, sulfoconjugated, and methylated anthocyanin metabolites also appear to be limited (4-7).

Our research group has suggested that the unabsorbed intact anthocyanins remaining in the gastrointestinal tract (GIT) may exert chemopreventive activity through direct contact with epithelial cells, in that a positive correlation between anthocyanins concentration in the colon content and the reduced incidence of colon lesion was observed in our previous study (8). Thus anthocyanin stability in the GIT appears to be a determination factor of anthocyanin’s health benefit on GIT related organs. In addition,
the way that anthocyanins are present in the GIT absorption sites likely determines the absorption of anthocyanins or their metabolites into the blood stream and consequently being exposed to other organs (5, 6, 9).

Above all, understanding the stability and transformation of anthocyanins in the GIT is a prerequisite for the evaluation of their putative health benefits. Based on some in vitro studies about anthocyanin’s stability during simulated digestion (10-12) and limited in vivo evidence (7, 13, 14), we hypothesized that anthocyanins were moderately stable under the influence of digestive enzymes and neutral pH in the GIT and their chemical structures determine the stability, accessibility, biotransformation, and health-promoting effects. The overall objective of this dissertation research was to increase understanding of the relationship between anthocyanin chemical structure and their existance in the GIT, which potentially affects the health-promoting value of anthocyanin-rich diet. First we investigated the in vivo stability, transformation and uptake of semi-purified black raspberry anthocyanins in the GIT using a rat model (Chapter 3). Then in order to better understand the effect of the GIT enzymes on anthocyanins we developed a simple and efficient methodology to remove co-extracted interference plant components from anthocyanin mixtures (chapter 4). The highly purified anthocyanins were subsequently tested in vitro with extracted β-glydosidases (chapter 5). Two types of β-glycosidases, the endogenous intestinal brush border β-glucosidase and the exogenous lactase, were evaluated with respect to selective degradation of various anthocyanins.
2.1 ANTHOCYANINS AS NATURAL PIGMENTS

Anthocyanins constitute the largest and probably the most important group of water soluble natural pigments (15). To date there has been at least 539 anthocyanins identified in nature and such a versatile group is responsible for the vivid blue, purple, and red color of many fruits, vegetables and flowers (16). In fact the word anthocyanin was derived from two Greek words: anthos and kyanos, meaning flower and dark blue respectively (17). Anthocyanins are believed to be important to plants as their color attracts animals, leading to seed dispersal and pollination. Due to strong absorption of light, they may also be important in protecting plants from ultraviolet-induced damage (18).

Anthocyanins are primarily used as colorants in beverages in the food industry. As public concern about synthetic food dyes has increased recently, consumers and food manufacturers desire colorants from natural sources. The commonly used synthetic dyes in the food industry have been suspected to cause adverse behavioral and neurological
effects in the past. A recent trial involving 153 3-year-old and 144 8/9-year-old children concluded that artificial colorants combined with sodium benzoate preservative in the diet resulted in statistically significant increase of hyperactivity in children (19). As promising alternatives to the most widely used synthetic food dye FD&C Red #40 (called Allura red in Europe), anthocyanins have recently attracted great interest.

2.2 CHEMICAL STRUCTURE OF ANTHOCYANINS

2.2.1 Chemical structure of flavonoids

Anthocyanins belong to a large group of polyphenolics named flavonoids, which are secondary metabolites synthesized by higher plants. Based on the characteristic of aglycones, flavonoids are divided into sub-classes (Figure 2.1). Their aglycones share a C-6 (A-ring)-C-3 (C-ring)-C-6 (B-ring) carbon skeleton (20). The presence and absence of the double bond and carbonyl group on the C ring are the major differences among sub-classes, whereas a shift of B ring substitution from C-2 to C-3 position separates isoflavones from others. Because quercetin, catechin, and isoflavones have similar structure to anthocyanins, their extensively studied bioactivities can provide implication for the evaluation of anthocyanins.
2.2.2 Anthocyanin aglycones

Due to the long chromophore of eight conjugated double bonds carrying a positive charge (Figure 2.2), anthocyanins are intensely colored under acidic conditions. The maximum absorption in the visible range is usually between 465 and 550 nm, while the other maximum absorption band falls in the UV range between 270 and 280 nm (21). Differing in the number of hydroxyl and methoxyl groups, 6 common anthocyanidins (aglycones) exist in nature - cyanidin (Cy), peonidin (Pn), pelargonidin (Pg), malvidin (Mv), delphinidin (Dp), and petunidin (Pt) (21, 22). The color varies among aglycones (Table 2.1) with the B ring possessing more hydroxyl groups being more blue and more methoxyl groups being more red (17, 23).
**Figure 2.2** Basic chemical structure of a common anthocyanidin.

![Chemical Structure](image)

**Table 2.1** Differences on chemical structure, color and $\lambda_{\text{max}}$ of anthocyanidins most commonly found in nature.

<table>
<thead>
<tr>
<th>Name</th>
<th>Substitution</th>
<th>Color</th>
<th>$\lambda_{\text{max}}$ (nm) in HCl acidified MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy</td>
<td>OH</td>
<td>H</td>
<td>magenta</td>
</tr>
<tr>
<td>Pn</td>
<td>OCH$_3$</td>
<td>H</td>
<td>magenta</td>
</tr>
<tr>
<td>Pg</td>
<td>H</td>
<td>H</td>
<td>red</td>
</tr>
<tr>
<td>Mv</td>
<td>OCH$_3$</td>
<td>OCH$_3$</td>
<td>purple</td>
</tr>
<tr>
<td>Dp</td>
<td>OH</td>
<td>OH</td>
<td>purple</td>
</tr>
<tr>
<td>Pt</td>
<td>OCH$_3$</td>
<td>OH</td>
<td>purple</td>
</tr>
</tbody>
</table>

$\lambda_{\text{max}}$ represents the maximum wavelength of light absorbed by the compound.
2.2.3 Glycosylation and acylation

The hydroxyl groups on the aglycone may be substituted by carbohydrate moieties and the carbohydrate moieties may further be linked to other sugars through glycosidic bond or acylated with organic acids (cinnamic acid, melonic acid, and acetic acid, to name a few) through ester bond (Figure 2.3). Both glycosylation and acylation affect the physical-chemical properties of anthocyanins in that they modify the molecular size and polarity. Glycosylation increases water solubility and acylation decreases water solubility. Perhaps due to increased molecular size, acylated anthocyanins are much less efficiently absorbed than their counterparts without the acylation (24, 25). The aglycone form of anthocyanins is rarely found in nature due to the poor stability. Glycosylation improves anthocyanin stability by forming intramolecular H-bonding network within the anthocyanin molecule (26). Glucose (glu) and rhamnose (rha) are common sugar moieties attached to the aglycone, but galactose (gal), arabinose (ara), xylose (xyl), rutinose (rut), sambubiose (sam) and other sugars are also frequently found. Acylated organic acids comprise a broad range of compounds as well, which are normally classified into two categorizes, aliphatic acids and cinnamic acids. Due to the various types of substitutions, many varieties of anthocyanin derivatives exist.
2.2.4 The influence of pH on anthocyanin chemical structure

Anthocyanins are unique among flavonoids as their structures reversibly undergo pH-dependent transformation in aqueous solution (Figure 2.4). Four major anthocyanin forms exist in equilibria: the red flavylium cation; the blue quinonoidal base; the colorless carbinol pseudobase; and, the colorless chalcone (27). At pH below 2, anthocyanins exist predominantly in the red flavylium cation form. Rapid hydration of the flavylium cation occurs at the C-2 position to generate the colorless carbinol pseudobase at pH values ranging from 3 to 6. As red color is bleached out in this transformation, the mechanism of reaction has been extensively investigated.
Figure 2.4 Scheme of the pH-dependent structural interconversion between dominant forms of mono-glycosylated anthocyanins in aqueous phase. (Source: Houbiers, Lima et al. 1998)
The fundamental work conducted by Brouillard and Dubois (28) demonstrated that the hydration process is fairly rapid and, depending on the extent of pH change, can take between 30 and \(\sim 10^3\) s to reach equilibrium, whereas the reverse transition from carbinol pseudobase to flavylium cation is almost instant upon acidification. The \(pK_a\) for the hydration reaction has been well studied with Mv-3-glu, a major anthocyanin in grape and wines using different methodologies (27, 29, 30). The reported \(pK_a\) was 2.60, 2.80, or 1.76 using UV/Vis spectroscopy, \(^1\)H NMR spectroscopy, or electrophoresis respectively. It is noteworthy that under the same conditions the 3, 5-di-glu has less proportion in cation form than the corresponding 3-mono-glu, while acylation leads to noticeably increased cations especially at above pH 4 (31). For example, larger number of acylated cinnamic acids results in higher \(pK_a\), and thereby more red color is retained at low acidic conditions. This characteristic of acylated anthocyanins makes them preferable food colorant in moderately acidic foods like yogurt.

The carbinol form can further equilibrate to an open ring form, the colorless chalcone pseudobase, at a slow rate, and the reaction is favored by increased temperature (27). However, at any pH condition the chalcone form exists in a much smaller proportion as compared to the carbinol form. Unlike the carbinol form, reconversion of chalcone to flavylium cation is a very slow process taking hours to reach completion (32). Deprotonation of flavylium cation to generate quinonoidal base occurs at slightly acidic to neutral condition and the reaction is extremely fast (28). At such a condition, kinetic competition between the deprotonation and hydration reactions predominantly favors
deprotonation. As the pH increase to above 8, the quinonoidal base can be ionized to carry one or two negative charges (30, 33).

2.2.5 Pyrananthocyanins

Pyrananthocyanins are a group of color-stable anthocyanins first identified in aged red wines (34-36). The characteristic structure of pyrananthocyanins is an additional pyran ring linking C-4 position and C-5 OH group on the backbone (Figure 2.5). This pyran structure stabilizes the parent anthocyanin’s color markedly in that the flavylium cation form become more resistant to pH increase and SO₂ bleaching (34). It has been proposed that the new ring protects pyrananthocyanins from nucleophilic attack by water, delaying the hydrolysis of flavylium cations at low acid condition. The resistance to SO₂, a compound commonly used in winery as antioxidant and antimicrobial agent, is attributed to the occupied C-4 position. A free C-4 position can rapidly form covalent bond with hydrogen sulfite anion and result in rupture of the conjugated system in the chromophore.
Figure 2.5 A representative chemical structure of pyranoanthocyanins. R3 is usually a hydrogen, a carbonyl group, a phenol, or a flavonoid molecule (Source: Vivar-Quintana, Santos-Buelga et al. 2002; Alcalde-Eon, Escribano-Bailon et al. 2004).

It is known that anthocyanins react with pyruvic acid, acetaldehyde, acetone, 4-vinylphenol, vinylflavanols, or cinnamic acids to generate different classes of pyranoanthocyanins (35, 37, 38). Nucleophilic attack on the C-4 position of anthocyanin aglycone is the initial step of reaction, and subsequent cyclic reaction requires a free OH group on the C-5 position. Therefore 3, 5-diglycosylated anthocyanins are not substrate in this type of reaction. The generation of pyranoanthocyanins requires weeks for completion during wine aging, but can be significantly accelerated by increased temperature. Schwarz and Winterhalter (37) found that the reaction between Mv-3-glu and p-dimethylamino cinnamic acid required 6 wk to complete at 15°C, but only 3 days at 35°C. However, high temperature may also cause more browning via condensation.
reaction. Appropriate percentage of ethanol in the solvent also accelerates pyranoanthocyanin generation as ethanol improves solubility of organic substrates. However, high concentrations of ethanol deter covalent binding, leading to slower reactions (39). Fermentation during wine making was previously thought to be necessary for the generation of pyranoanthocyanins, but later it was discovered that juices naturally containing appropriate precursors could also produce pyranoanthocyanins during storage (40). For the purpose of enhanced color stability, artificially added cinnamic acids had been evaluated in some berry juices and pyranoanthocyanins were successfully produced (41). Synthesis of novel pyranoanthocyanins with orange to blue hues has also been investigated in the attempt to provide stable and novel pigments for application in food industry (37, 42).

2.3 ANTHOCYANINS IN HUMAN DIET

2.3.1 Occurrence of anthocyanins in plant materials

Anthocyanins are water-soluble vacuolar pigments found in many plant tissues (43). Edible anthocyanin sources in nature include colored fruits such as berries, cherries, peaches, grapes, pomegranates and plums as well as many dark colored vegetables such as black currant, red onion, red radish, black bean, eggplant, purple corn, red cabbage, and purple sweet potato (21, 44). Although most commonly accumulated in flowers and fruits, they are also present in leaves, stems and storage organs (17). Total anthocyanin content varies substantially across plant species or even cultivars (44). Available data show very wide range of anthocyanin content in plant material with berries usually
providing the most anthocyanins per serving. Environmental factors such as light, temperature, and altitude also affect anthocyanin concentration considerably (43).

Abundance of the six common anthocyanidins in the edible parts of plants varies considerably. Some commodities like strawberry contain a limited number of anthocyanin pigments, whereas others like low bush blueberry may contain a complex mixture. In a previous review Kong et al. (22) estimated the following abundance order: Cy (50%), Pg (12%), Pn (12%), Dp (12%), pt (7%), and Mv (7%). In a later published summary an updated evaluation was probably more accurate as more anthocyanins were included (16). The abundance order was estimated to be Cy (30%), Dp (22%), Pg (18%), Pn (7.5%), Mv (7.5%), and Pt (5%). In both reports the three non-methylated anthocyanidins (Cy, Dp and Pg) were shown to be more wide spread than the three methylated anthocyanins (Pn, Mv and Pt). Considering that glucoside sugar moiety occurs in over 90% of the identified anthocyanins (16), it is not surprising that Cy-3-glu is the most widespread anthocyanin in nature (22).

2.3.2 Anthocyanins in foods and beverages

Dietary anthocyanin sources include many colored fruits and vegetables, as well as fruit based processed foods and beverages like jelly, juices, and red wine. The global anthocyanin consumption from black grapes alone was estimated to be 10,000 tons annually (45). With regard to mass consumed, anthocyanins constitute perhaps the most important sub-class of flavonoids. Daily intake of anthocyanins had previously been overestimated to be 185-215 mg/day/person in the US, and according to a recent report by USDA evaluating more than 100 common foods the more accurate estimation is 12.5
mg/day/person (44). Still this is a significant number comparing to other phytochemicals with known or proposed health-promoting benefits. It has to be noted that dietary habits and choices have great impact on anthocyanin consumption. For example, one serving of blueberry increases anthocyanin consumption to greater than 500 mg. Likewise one serving of Concord grape provides about 200 mg, and one serving of elderberry can supply 2000 mg anthocyanins. Regular red wine drinkers or juice drinkers can also benefit more from anthocyanins as one bottle can readily provide more than 200 mg of anthocyanins (45). Nowadays as the consumers are increasingly concerned about the adverse health effect of synthetic food dyes, more and more food manufacturers are attempting to use anthocyanins as substitutes for FD&C red #40, the most widely used synthetic colorant. For instance, in fruit yogurt and many types of fruit flavored dry mix anthocyanins are gradually gaining application. Indeed synthetic dyes are not allowed in the rapidly growing whole foods market, where anthocyanins become highly important. Acylated anthocyanins are usually used as food colorants due to superior stability than non-acylated anthocyanins (46). However, certain commodities such as elderberry, barberry, and purple corn can provide exceedingly high level of non-acylated anthocyanins at relatively low cost, thus they also have potential use in the food industry (47, 48).

2.3.3 Toxicity of anthocyanins

Animals and human have consumed anthocyanins since ancient time. No adverse impact on health has been reported with oral consumption of anthocyanins in foods (49). The use of natural anthocyanins as additives in foods and beverages is widely permitted
within Europe (E163), Japan, the United States, and many other countries (21). Based on early toxicological studies including mutagenicity, reproductive toxicity, teratogenicity, as well as acute and short-term toxicity evaluations, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that anthocyanin-containing extracts had a very low toxicity (50). The no-observed-effect-level (NOEL) for young rats was determined to be approximately 225 mg/kg body weight in a two-generation reproduction study. Based on the above result, the estimated acceptable daily intake (ADI) for human was estimated to be 2.5 mg/kg body weight per day in 1982, using the equation of ADI = NOEL/100 (45).

2.4 STABILITY OF ANTHOCYANINS

2.4.1 The effect of pH

At pH lower than 3, anthocyanins exist primarily in the red colored flavylium cation form, which is the most stable form of anthocyanins in the presence of oxygen (32). As pH increases above the pK\textsubscript{a}, the majority of anthocyanin molecules exist in the hemiacetal (chalcone) form. As pointed out by Brouillard, the hemiacetal form is most likely the target for attacking at slightly acidic pH (28). This speculation was supported by a later discovery of anthocyanin-quinone adducts in the hemiacetal form using LC-MS (51). A systematic study of the two anthocyanins, Cy-3-glu and Pt-3-(p-coumaryl)rha,5-glu, revealed that pH is a critical factor for anthocyanin stability (52). Cy-3-glu stability slightly decreased from pH 1 to 3, and plummeted at pH above 4 at 23°C in the dark, whereas Pt-3-(p-coumaryl)rha,5-glu stability dramatically decreased above pH 5. At
alkaline pH anthocyanins exist in the quinonoidal bases form, which participate more readily in chemical reactions than the flavylium cations. The o-quinones are also known for their tendency to polymerize (53).

2.4.2 The effect of temperature

A logarithmic relationship has been established between temperature and anthocyanin degradation rate (53). Possible degradation mechanism is that higher temperature favors transition to the unstable chalcone form (Figure 2.4), while the opened C ring of chalcone is further degraded to brown products (32). Therefore, refrigeration was recommended as an efficient means of preserving anthocyanins. When stored at 4°C for 9 wk, approximately 40% of anthocyanins in blackcurrant juice (pH 2.0) were lost, while no detectable level was found at 37°C (53). The reported half life for grape pomace extract color in carbonated beverage was 1536 days at 3.5°C as compared to 769 days at 10°C, 416 days at 20°C, and 80 days at 38°C (54). Thermal processing procedures like canning and hot fill adversely affect anthocyanin color. Strawberry pigment at 100°C only has a half-life of 1 h. But a short time high temperature treatment may improve anthocyanin stability by facilitating inactivation of native enzymes that are detrimental to anthocyanins. Heat aided drying processes decompose anthocyanins considerably, not only because of the elevated temperature, but also because the drying process increases the concentration of reactants in the solution. In contrast, the freeze drying method commonly employed in high quality food preparation has the advantage that thermal sensitive phytochemicals, including anthocyanins, can be well preserved during the drying process.
2.4.3 The effect of light

Light is another deteriorating factor with respect to anthocyanin stability. Due to the intrinsic light absorption property, anthocyanin pigments absorb energy from photons efficiently. In daylight versus dark storage, the half-life of grape pomace color extract at 20°C can be shortened by more than 50% (54). Therefore, it is recommended that anthocyanin colored foods and beverages are stored in the dark. Opaque package material helps to preserve anthocyanins owing to blocked incoming light. Similarly, acylated anthocyanins are more stable than non-acylated anthocyanins when exposed to light under low acid and high temperature conditions (32). Other factors such as the second sugar moiety on the C-5 position and co-existence of other polyhydroxylated flavonoids in the solution also stabilize anthocyanin molecules. Plausible mechanisms of such stabilizing effects include the established H-bonding network and intramolecular/intermolecular co-pigmentation.

2.4.4 The effect of enzymes

Native plant enzymes, particularly polyphenol oxidase (PPO) and proxidase (POD), are usually released from cell compartments during anthocyanin extraction, accelerating anthocyanin degradation. Enzymatic browning along with color loss can occur very fast after crushing. Studies have shown that PPO does not metabolize anthocyanins directly, but instead generate quinones from phenolic acids that subsequently react with anthocyanins through condensation or coupled oxidation (51, 55). In the presence of H₂O₂, POD oxidize phenolic acids to their corresponding quinones,
which polymerize with anthocyanins to increase color degradation and browning (56). Both the flavylium cation form and the hydrated forms can form adduct with quinones, although the hydrated forms are more reactive. Prompt inactivation of PPO by means of increased extraction temperature and addition of SO₂ efficiently preserves anthocyanins during extraction (57).

Glycosidases are another group of enzymes that affect anthocyanin stability. Fungal enzymes are widely used to facilitate macerating and pressing of fruits to increase juice yield by breaking cell structure. However crude enzyme extract usually contains β-glycosidases, which are capable of hydrolyzing the glycosidic linkage between anthocyanidins and sugar moieties (58-60). As mentioned above, anthocyanidins lacking the sugar moiety are extremely unstable and decompose spontaneously. Therefore, caution must be taken when choosing an enzyme preparation for use in the food industry (60). In addition to the fungal β-glycosidases, endogenous β-glycosidases in the animal digestive tract may also have impact on anthocyanin stability and consequently affect the availability of anthocyanins in vivo.

2.4.5 The influence of oxygen, peroxide and ascorbic acid

Oxygen has been well known to accelerate anthocyanin decomposition. As the ultimate electron accepter, oxygen participates directly or indirectly in multiple mechanisms of anthocyanin degradation, including H₂O₂ bleaching. Bottled cranberry juice without head space oxygen can have four times higher anthocyanin concentration remained than that with 2 mL of headspace oxygen after 32 wk of storage (32). In an
ascorbic acid-anthocyanin-flavanol model system, bottled samples of purified Pg-3-glu stored in a nitrogen atmosphere was preserved as compared to samples stored in an oxygen atmosphere (61). H₂O₂, the most commonly used packaging sterilant in the food industry, is a particularly reactive form of oxygen causing anthocyanin degradation. Residual amount of H₂O₂ is likely to be present in anthocyanin rich product. Millimolar levels of H₂O₂ still accelerate sour cherry and pomegranate anthocyanin degradation in a concentration dependent manner (62).

The effect of ascorbic acid is complex in that it is matrix dependent. In the presence of H₂O₂, ascorbic acid at both 60 and 80 mg/L concentrations protected anthocyanins in pomegranate juice, whereas 80 mg/L of ascorbic acid dramatically accelerated anthocyanin degradation in sour cherry juice (62). In the ascorbic acid-anthocyanin-flavanol model system mentioned above, addition of approximately 0.42 mg/mL of ascorbic acid adversely affected anthocyanin stability compared to addition of 0.64 mg/mL of catechin or sparging the head space with oxygen (61). Two mechanisms have been proposed for the adverse effect of ascorbic acid, i.e. 1) oxidation of ascorbic acid generates H₂O₂ that oxidizes anthocyanins and 2) ascorbic acid directly reacts with anthocyanins causing condensation. The investigation of dehydroascorbic acid (the oxidized form of ascorbic acid) level throughout the storage period suggested that direct condensation was likely to be the predominant mechanism.
2.4.6 Self-association, acylation and co-pigmentation

It has been established that anthocyanins at high concentration do not follow Beer’s Law due to self-association effect (32). For example, a 100-fold increase of Cy-3,5-di-glu concentration from 0.1 mM to 10 mM at pH 3.16 could result in a 300-fold increase in absorbance. The reason lies in that when the anthocyanin molecules are close to each other at high concentration, they associate with each other creating a stacking effect that enhances absorbance. Generally anthocyanin color is expected to undergo a second order increase in response to greater concentration (39). The stacking effect was believed to protect the anthocyanidins from the nucleophilic attack of the water molecule or other reactants.

Another two types of stacking effect that occur with anthocyanins are intramolecular co-pigmentation and intermolecular co-pigmentation. Intramolecular co-pigmentation occurs when the acyl group in acylated anthocyanins interacts with the aglycone. Acylation, particularly with cinnamic acids, will render a bathochromic shift (from more reddish hue to more bluish) and greater stability to the parent anthocyanins. Intramolecular copigmentation has been proposed to be responsible for this stabilization effect (46). It is suggested that acids will fold over the anthocyanidin aglycone avoiding formation of the hydrated species (17), and there is evidence of proximity between hydrogens from acids and the C-4 position (18, 63). Presence of two or more acyl groups further increases the color stability of anthocyanins in aqueous solution (64). Radishes, purple carrots, red cabbage, and purple sweet potato extracts are being commercialized as colorants in the food industry due to their increased stability attributed to the presence of
two or more acyl groups. Maraschino cherries colored with radish anthocyanins have a shelf life of at least 6 months at 25 °C, and the excellent stability of the major pigments has been associated with the presence of two acyl groups on the pelargonidin derivatives (65, 66).

Anthocyanins also interact with other phenols and flavonoids to produce a hyperchromic effect (increase in color intensity) and a bathochromic effect. Such a phenomenon is called intermolecular co-pigmentation, which can occur in acidic, neutral and even slightly alkaline aqueous solution (18, 67). The occurrence of intermolecular co-pigmentation relies on at least two effects (68). First, the formation of the π-π complex causes changes in the spectral properties of the molecules in the flavylium form, resulting in hyperchromic shift and bathochromic shift (69). Second, the stabilization of the flavylium form by the π complex shifts the equilibrium to better favor the flavylium, thus boosting the proportion of anthocyanin molecules in the red-colored form.

2.5 PUTATIVE HEALTH-PROMOTING EFFECTS OF ANTHOCYANINS

Interests in dietary polyphenols including anthocyanins drastically intensified after the recognition of their potential health benefits (70). Epidemiological studies have suggested a reverse association between high consumption of polyphenols and incidence of some chronic diseases. For example, drinking red wine regularly has been associated with the relatively low incidence of coronary heart disease in French people despite a high fat diet, the well known “French Paradox” (71). Since then a vast number of studies have been carried out on the biological effects of polyphenols using in vitro and in vivo
models. Anthocyanins are among the most abundant polyphenols in fruits and vegetables and possess potent antioxidant activity. In vitro models have the merits of low cost and high efficiency, thus they have been widely employed. Animal and human clinical studies on health benefits of anthocyanins are still in the early stage. To date, the suggested health benefits of anthocyanins are more or less related to their antioxidant activity (22). It has to be noted that not a single class of compounds may explain most of the health promoting effects of consuming fruits and vegetables. Apparently the phytochemicals contained in fruits and vegetables work collaboratively to benefit our body (72, 73).

2.5.1 Relief of oxidative stress

Reactive oxygen species (ROS) including free radicals, singlet oxygen and peroxides are generated in the body. They are important to the immune system, cell signaling, and many other normal body functions. However, if ROS are overly produced they can elicit cellular damage, leading to degenerative diseases such as inflammation, cardiovascular disease, cancer, and aging (74).

Anthocyanins are potent antioxidants in vitro. They effectively quench free radicals and terminate the chain reaction that is responsible for the oxidative damage. Because pH in the human body is generally neutral except in the stomach, the antioxidant activity of anthocyanins at neutral pH is of particular importance. Using a widely accepted antioxidant assessment method, the oxygen radical absorbance capacity (ORAC) assay, antioxidant activity of 14 anthocyanins including Dp, Cy, Pg, Mv, Pn and their glycosylated derivatives was determined in aqueous phase at neutral pH (75). Among
these anthocyanins, Cy-3-glu had the highest ORAC value, 3.5 times as potent as Trolox (water soluble vitamin E analogue). Pg had the lowest ORAC value of tested anthocyanins, but still as potent as Trolox. As compared to the natural form of vitamin E (α-tocopherol), Cy-3-glu and its aglycone Cy were shown to have similar antioxidant potency in linoleic acid autoxidation, liposome, rabbit erythrocyte membrane, and rat liver microsomal systems (76). Such potent antioxidant activity from anthocyanins may have protective effects in the biological environment. An in vitro study using human erythrocytes treated with H$_2$O$_2$ as an oxidative model revealed that red wine fractions rich in anthocyanins significantly lowered ROS in human red blood cells (77).

Protective effect of anthocyanins on oxidative stress induced damage is promising as shown using in vivo models. In a rat study utilizing hepatic ischemia-reperfusion as an oxidative stress model, Cy-3-glu efficiently attenuated changes of biomarkers in liver injury (78). In another rat study the animals were fed vitamin E deficient diets for 12 wk followed by supplementation with purified anthocyanin-rich extracts. The anthocyanin diet significantly improved plasma antioxidant capacity and lowered the level of hydroperoxides and 8-Oxo-deoxyguanosine, indicating significant reductions of the vitamin E deficiency induced lipid peroxidation and DNA damage, respectively (79).

2.5.2 Prevention of cardiovascular diseases

Oxidation of low-density lipoprotein (LDL) triggers accumulation of macrophage white blood cells in the artery wall. Rapture of the plaque deposits oxidized cholesterol into the artery wall, leading to atherosclerosis and eventually cardiovascular diseases (80,
Dietary antioxidants including anthocyanins have the potential to increase serum antioxidant capacity and thereby protect against LDL oxidation and prevent cardiovascular diseases. Research has initially focused on anthocyanin-rich red wine because of the famous “French paradox” (71). Using a chemiluminescent assay of serum antioxidant capacity (SAOC), the effects in normal human subjects ingesting 300 mL of red wine, white wine, or high dose (1000 mg) of vitamin C were studied. In subjects who ingested red wine the mean SAOC was increased by 18% and 11% after 1 and 2 h, both higher than that in the white wine group although not as high as that in the vitamin C group (82).

Following the pioneering studies on red wines, more and more attention had been given to anthocyanins and other polyphenols present in red wines. A trial involving 7 human subjects demonstrated that daily consumption of 125 mL of concentrated red grape juice markedly raised serum total antioxidant capacity (TAC) as compared to the baseline. The susceptibility of LDL to oxidation was also reduced. Therefore the nonalcoholic red grape extract was suggested to have similar beneficial effects to red wine (83). Other anthocyanin-rich foods were also extensively studied. Monitoring of the chemiluminescent emission intensity of human blood plasma for 8 h following oral administration of black currant anthocyanins demonstrated a rapid increase of plasma antioxidant capacity until 2 h (84). Spray-dried elderberry juice containing high anthocyanin content was investigated with respect to the protective effect on human LDL in vitro (85). A concentration-dependent prolongation of the lag phase was observed in copper-induced oxidation. Meanwhile a similar prolongation effect was also observed in
peroxyl-radical-driven LDL oxidation together with a reduction of maximum oxidation rate. It is important to note that anthocyanins may not explain all of the protective effects observed in these foods, but likely contributed to some extent. In a UV light radiation induced lipid peroxidation model, three purified anthocyanins (Pg-3-glu, Cy-3-glu, and Dp-3-glu), as well as their aglycones all demonstrated strong inhibition of lipid peroxidation and acted as active oxygen radical scavenging agents (86).

2.5.3 Anti-inflammatory activity

Inflammation is a complex biological response in response to tissue injury. Many cancers occur at sites of inflammation because inflammatory cells provide microenvironment favorable for tumor development (87). Anti-inflammatory therapy has the potential to prevent early neoplastic progression and malignant conversion. Because cyclooxygenases (COX) convert arachidonic acid to prostaglandins that stimulate inflammation, inhibitory effect on COX enzymes, especially selective inhibition on one of the isoenzymes COX-2, is highly desirable. Cy aglycone was reported to possess better anti-inflammatory activity than the positive control aspirin in the COX activities assays (88). Purified anthocyanin fractions from tart cherries, sweet cherries, bilberries, blackberries, blueberries, cranberries, elderberries, raspberries, and strawberries were evaluated using cyclooxygenase-inhibitory assays (89). All the anthocyanin fractions demonstrated inhibitory effect on COX-1 and COX-2 enzymes, while strawberry, blackberry and raspberry showed the highest activity, comparable to that of the positive controls ibuprofen and naproxen at 10 μM concentrations. In an in vivo study the therapeutic efficacy of blackberry anthocyanins (Cy-3-glu accounts for 80%) was
investigated in rats with carrageenan induced lung inflammation (90). All parameters of inflammation were effectively reduced in a dose-dependent manner by anthocyanins.

2.5.4 Anti-carcinogenic activity

Anti-cancer activity of anthocyanins has been established largely based on in vitro evidence. Anthocyanins extracted from flower petals were found to be more potent than combined non-anthocyanin flavonoids regarding cell growth inhibition in a human malignant intestinal carcinoma derived HCT-15 cell line (91). Dose required for 50% inhibition ranged from 0.5 to 5 µg/mL for representative individual anthocyanins and anthocyanidins, whereas higher concentrations of other flavonoid evaluated in this study were required to exhibit the same effect. Similarly, anthocyanin fraction isolated from red wine was also discovered to be significantly more effective than non-anthocyanin flavonoids in red wine or white wine using HCT-15 cell line and AGS cell line, which was derived from human gastric cancer (92). The anti-proliferative effect of anthocyanin fraction from 4 cultivars of muscadine grapes were evaluated using two human colon cancer derived cell lines, HT-29 and Caco-2 (93). In all cultivars and both cell lines, greater inhibitory activity was observed from the anthocyanin fraction than from the phenolic acids fraction or the crude extract. Zhao et al. (94) demonstrated that anthocyanin fractions from commercially available bilberry, chokeberry, and grape extracts all exerted anti-proliferative effect in the HT-29 cell line. Dose of 25 µg/mL chokeberry anthocyanins provided 50% inhibition but not affecting the growth of normal colonic NCM460 cells. More in-depth investigation revealed that the chokeberry anthocyanins arrested the cell cycle of HT-29 cells by blocking at the G1/G0 and G2/M
phases (95). Highly purified anthocyanins had also been evaluated. Four anthocyanins isolated from strawberry by means of medium-pressure liquid chromatography (MPLC) were all shown to reduced cell viability of human oral (CAL-27, KB), colon (HT29, HCT-116), and prostate (LNCaP, DU145) cancer cells at 100 μg/mL dose level, although different sensitivity was recorded for each individual compound (72).

Anthocyanins are shown to be promising phytochemicals responsible for, at least part of, the anti-cancer property of many fruits and vegetables, but it is more than likely that anthocyanins work collaboratively with other phytochemicals to help the body defense. Seeram et al. (73) evaluated the antiproliferative effects of total cranberry extract versus its flavonol glycosides (gly), anthocyanins, proanthocyanidins, and organic acids fractions using human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620), and prostate (RWPE-1, RWPE-2, 22Rv1) cancer cell lines. Both the anthocyanin fraction and the proanthocyanidin fraction exhibited substantial inhibitory effect on all but the SW480 cell lines. However, the combination of these two fractions was the most active against all cell lines.

In animal studies the growing body of data has demonstrated chemopreventive effect of anthocyanins in multiple types of cancers. Nevertheless, the observed preventive effects were primarily limited to the GIT related organs including the oral cavity, the esophagus (96, 97), and the colon (8, 98-100). In the GIT lumen, anthocyanins are largely available and can contact directly with the epithelial layer (13). In contrast, strawberry anthocyanins failed to inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-and benzo[a]pyrene-induced lung cancer in a mice model (101).
Anti-cancer activity of anthocyanins may be attributed to the collaborative effect of multiple mechanisms \((8, 102, 103)\). Possible mechanisms that have been suggested include antimutagenic activity \((104-106)\), inhibition of oxidative DNA damage \((107)\), inhibition of carcinogen activation and induction of phase II enzymes for detoxification \((108, 109)\), cell cycle arrest \((110)\), inhibition of COX-2 enzymes, induction of apoptosis \((93, 111)\), and anti-angiogenesis \((112)\). In the particular case of GIT related cancer, the influence of anthocyanins on the GIT luminal condition is of great importance too. Bruce et al. \((113)\) suggested two mechanisms that initiate colon cancer development, one involving a local irritation that produces a local inflammatory response, and the other relating to an electrolyte imbalance. Both mechanisms result from a defect in the epithelial barrier and both lead to elevated ROS and COX-2 levels in epithelial cells. Therefore, agents that can improve colon luminal condition hence reduce epithelial barrier damage, can inhibit expression of COX-2 and inflammation, or can quench ROS in local cells have the potential to prevent colon cancer. Anthocyanins have been shown to be powerful antioxidants and selective COX-2 inhibitor, as discussed previously in this section. However, Lala et al. \((8)\) suggested that the inhibitory effect of dietary anthocyanins in a colon carcinogen azoxymethane (AOM) induced rat colon cancer model was primarily attributed to the direct effect on improving colon luminal condition. The patterns of inhibition on colonic cell proliferation and large aberrant crypt foci (ACF) multiplicity \((Figure 2.6)\) were not correlated with the total antioxidant capacity in the diet, with anthocyanin absorption as accessed by urinary anthocyanin concentration, or with the colonic mucosa COX-2 mRNA levels. The highest correlation was between colon cancer growth and the total anthocyanin content in the colonic lumen as represented by
Fecal anthocyanin concentration. Luminal anthocyanins appeared to promote fecal moisture content and excretion of bile acids, a group of endogenous tumor-promoting compounds (Figure 2.6). In addition, luminal anthocyanins may benefit the colon health by protecting the epithelial cells against oxidative damage and microbial infection.
Figure 2.6 Effect of feeding anthocyanin rich diets on total anthocyanin concentration in rat urine, colonic cell proliferation index, large ACF multiplicity, total anthocyanin concentration in feces, fecal moisture content, and fecal bile acids concentration (Source: Lala, Malik et al. 2006). Data are means ± SE. Values with a different letter differ significantly (P<0.05) within a same category.
2.5.5 Prevention of obesity

Obesity is the result of accumulated excessive adipose tissue caused by the imbalance of energy intake and expenditure. It is usually associated with various metabolic disorders. Consumption of anthocyanins can possibly ameliorate the function of adipocytes, thus may prevent metabolic syndrome and obesity (114). In a fundamental study conducted by Tsuda et al. (115), 24 male mice were fed control, purple corn color, high-fat, or high-fat plus purple corn extract diet for 12 wk. Supplementation with purple corn color suppressed the high-fat diet induced gain of body weight and white/brown adipose tissue weights. Down-regulation of the mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis was suggested to contribute to this anti-obesity effect. Two additional in vivo studies supported anthocyanin’s anti-obesity effect on high-fat diet. In one of the studies black soybean anthocyanins were found to effectively reverse the weight gain of high-fat diet group rats to the same as that in the control group (116). Serum lipids composition was also improved by the addition of black soybean anthocyanins into high-fat diet. Serum triglyceride and cholesterol levels were significantly reduced, whereas the high-density lipoprotein (HDL)-cholesterol concentration markedly increased. In the second study male mice were fed high-fat diet for 8 wk with or without supplementation of blueberry anthocyanins in drinking water (117). Both the whole blueberry and the purified blueberry anthocyanins were evaluated. The purified anthocyanins resulted in lower body weight gains and body fat than the controls while the whole blueberry with the same level of anthocyanins actually increased obesity, probably due to added calorie intake from sugar. A further study of
anthocyanin’s effect on gene expression of adipocytes employed an *in vitro* model using isolated rat adipocytes (118). The total RNA isolated from the adipocytes was analyzed using GeneChip microarray. After the treatment of adipocytes with 100 µM of Cy-3-glu or Cy, 633 and 427 genes, respectively, were up-regulated by greater than 1.5-fold. Based on the gene expression profile, the up-regulation of hormone sensitive lipase and enhancement of the lipolytic activity were suggested to be the result of anthocyanin treatment on adipocytes.

2.5.6 Control of diabetes

Type-2 diabetes is a metabolic disorder associated with insulin resistance. Insulin secreted from the β-cells of the pancreas is responsible for stimulation of blood glucose transportation into skeletal muscle and adipose tissue as well as suppression of hepatic glucose production (119). Obesity and excessive intake of high fat or high glycemic index foods are possible reasons for the relative inadequacy of insulin in type-2 diabetes. Anthocyanins have the potential to control obesity, and consequently may contribute to the prevention of type-2 diabetes. Furthermore, the antioxidant activity of anthocyanins may protect β-cells from glucose induced oxidative stress (120). Sugimoto et al. (121) examined the protective effects of boysenberry anthocyanins against oxidative stress in streptozotocin induced diabetic rats. Increased plasma and liver biomarkers oxidation was observed in diabetic rats as compared to control rats. Administration of diet with boysenberry anthocyanins restored or tended to restore the biomarkers to the level of the control rats. The results indicated that anthocyanins are effective in preventing the development of *in vivo* oxidation that may lead to diabetes. More details about the role of
anthocyanins in diabetes prevention can be found in the comprehensive review by Ghosh and Konishi (2007).

2.5.7 Improvement of eye vision

Anecdotal evidence suggests that consumption of anthocyanins can improve eye vision (122). In a double-blind, placebo-controlled, crossover study with healthy human subjects, feeding black currant anthocyanin concentrate at 12.5, 20, and 50 mg/subject resulted in dose-dependent lowering of the dark adaptation threshold (123). The effect with the highest dose (50 mg/subject) was statistically significant effect (P = 0.011). However, a systematic review of placebo-controlled trials revealed conflicting evidence in the use of anthocyanins to improve night vision (124). The negative outcomes reported may be associated with low doses tested in some trials, the different methodologies used for evaluation, the variation of subjects, and the source of anthocyanins (119). Recently, a study on blueberry anthocyanin distribution in pig tissues confirmed that anthocyanins accumulated in pig eyes after feeding blueberry diet for 4 wk (125). Although the detected concentrations in eye tissue were extremely low (pmol/g), the concentrations were comparable to that in other evaluated tissues including liver.

2.5.8 Antimicrobial activity

Plant phenolics are well known to play an important role in defence against pathogens. Thus, their effects on human intestinal bacteria, both beneficial and pathogenic, have been extensively investigated (126). In a study of the phenolic compounds in 8 common Finnish berries, the berry extracts, as well as the representative
individual phenolic compounds contained in the berries, were evaluated against human intestinal bacteria (127). All four anthocyanins tested including Pg chloride, Cy chloride, Dp chloride, and Cy-3-glu were found to be effective inhibitor of Gram-negative *Escherichia coli* strain CM 871, a DNA repair-deficient strain, but not inhibiting regular *E. coli* and the beneficial Gram-positive probiotic bacteria. Therefore the antimicrobial activity of anthocyanins was speculated to involve reactions related to DNA. In another study evaluating berry phenolics against severe human pathogens, anthocyanin fraction was the most potent phenolic fraction in berries for reducing viability of *Salmonella enterica* serovar Typhimurium (126). Such an effect was attributed to the ability of anthocyanins to induce the release of lipopolysaccharide molecules from the outer membrane of the Gram-negative bacteria.

### 2.6 BIOAVAILABILITY AND METABOLISM OF ANTHOCYANINS

To validate the prominent health-promoting effects revealed in many *in vitro* models it is important to consider the bioavailability *in vivo*. The doses reported in some *in vitro* studies might be inappropriate since the level of intact anthocyanins exposed to tissues (except GIT luminal side tissues) could be very limited due to the observed low concentration in blood (128). The other important issue is the form of metabolites that are generated in the tissues. Some metabolites of flavonoids have comparable to or even more potent bioactivity than the precursors (129). Therefore, to truly evaluate the bioactivity of anthocyanins, it is critical to understand their bioavailability and metabolism.
Regarding absorption and metabolic pathways, anthocyanins had been thought to differ from the common flavonoids, since only intact anthocyanin glycosides were detected in urine and plasma (130). However, improved analytical techniques have revealed that anthocyanins are also methylated, sulfated and glucuronidated (4, 131-134). It is now believed that the absorption, metabolism and excretion of anthocyanins share many similarities with structurally similar flavonoids. In this section both anthocyanins and several well-documented flavonoids are discussed together as a whole.

2.6.1 Overview

After consumption of flavonoid containing foods, the flavonoids are released from the food matrix by chewing. Absorption could start at the stomach. Flavonoids absorption by the stomach would appear in the blood extremely rapidly after ingestion (135). Small intestine is the major site for flavonoid absorption. Endogenic β-glucosidases are involved at this stage to release aglycones from primarily flavonoid- glu and to a lesser extent gal, xyl, and ara. Free aglycones are more hydrophobic and have smaller size than the glycosides, thus are more likely to penetrate the epithelial layer passively. In contrast, intact glycosides are also absorbed by the small intestine, either by inefficient passive diffusion or by the sodium-dependent glucose transporter (SGLT1). Acylated flavonoids are generally recognized as non-absorbable in the small intestine due to their larger molecular size and lack of a free sugar moiety for transporter binding. However, recent evidence suggests that acylated anthocyanins are slightly bioavailable as the intact form (13, 136). Unabsorbed flavonoids travel down to the colon, where tremendous amount of microorganisms (~10^{12}/ cm^3) reside to provide catalytic and hydrolytic potential (137).
Glycosidic and ester bonds are cleaved by colonic microflora \((138)\). Aglycones then undergo spontaneous ring fission to some extent to generate simple compounds such as phenolic acids. The released aglycones and generated phenolic acids could be absorbed by the colon, yet only marginal absorption is expected since the colon is much less efficient than the small intestine with respect to absorption. For this reason it is anticipated that the sugar moiety of flavonoids glycosides governs the absorption and bioavailability of the aglycones of many flavonoids. But so far little is known about the effect of enzymatic deglycosylation on anthocyanin absorption. Within the same subclass, the aglycones have not been reported to be a determining factor for bioavailability \((139)\).

Flavonoid aglycones taken up from GIT lumen are subsequently metabolized by phase II drug-metabolizing enzymes to glucuronides, sulfates and methylates in the intestine epithelium, liver and kidney \((4, 128)\). The conjugated metabolites may be excreted into the jejunum via bile, and later recycled in the intestine/colon by the process referred to as the enterohepatic circulation pathway.

2.6.2 Gastric absorption

In a study involving 32 Wistar male rats \((135)\), isoflavone aglycones (daidzein and genistein) and glucosides (daidzin and genistin) were administered to rats at 7.9 \(\mu \text{mol/kg body weight}\). Analysis of plasma samples collected following administration showed that isoflavone aglycones were quickly absorbed (in <3 min) while glycosides were not detected within 5 minutes. This indicated that isoflavone glycosides were not
absorbed until reaching the small intestine. Further experiment conducted on absorption site restricted (pylorus ligated and abdominal wall sutured) rats revealed that only aglycones but not glycosides were absorbed in the stomach.

Nevertheless, the rule does not apply to all the flavonoids. Two research groups used similar approaches to demonstrate that anthocyanidin glycosides were efficiently absorbed in the stomach (140, 141). Passamonti et al. (140) injected grape anthocyanins into the stomach of 19 Wistar male rats, which had surgically blocked cardias, and collected blood from both the portal vein and the heart at 6 min intervals. Quantification of the anthocyanins by HPLC-MS using single ion monitoring revealed that Mv-3-glu was present in both portal and systemic plasma (0.789 ± 0.491 µM and 0.098 ± 0.078 µM, respectively; n = 19). Importantly, Mv 3-glu appeared in the plasma even within 6 min (Figure 2.7), presenting an evidence of stomach absorption. Pn-3-glu, Pt-3-glu and Mv-3-glu-acetic were inconsistently detected, perhaps due to animal variability. Neither anthocyanin aglycones nor conjugated derivatives were detected in the plasma.
Figure 2.7 Mv-3-glu concentration in plasma sampled from either the portal vein or the heart of rats administered with grape anthocyanins. n = 5, 5, 4, 5 for the time points 6, 12, 24, 36 min respectively (Source: Passamonti, Vrhovsek et al. 2003).

Talavera et al. (141) infused anthocyanin standards as well as bilberry and blackberry extract into the stomach of pylorus and sphincter ligated rats. Gastric contents and blood were collected from the gastric vein and abdominal aorta 30 min after the administration. HPLC analysis utilizing an internal standard PEG revealed that a high proportion (~25%) of anthocyanin mono-glycosides, including glucoside and galactoside, was absorbed from the stomach, whereas the rutinoside was poorly absorbed. It was suggested that gastric absorption of anthocyanins involves bilitranslocase (TC 2.A.65.1.1), an organic anion membrane carrier in the gastric mucosa (142).
2.6.3 Direct absorption in the small intestine

Small intestine is generally regarded as the most important site for absorption of nutrients. Absorption of anthocyanins in rat small intestine has been evaluated using an in situ perfusion method (143). Intestinal perfusion of anthocyanin supplemented in physiological buffers was conducted on anesthetized rats. Anthocyanin amount remaining in the effluent was used to estimate the rate of anthocyanin absorption in the small intestine. Depending on structure, the absorption rate of supplemented anthocyanins ranged from 22.4 ± 2.0% (Cy-3-glu) to 10.7 ± 1.1% (Mv-3-glu). Such high absorption rates seemed to be contradict the very low levels of anthocyanins observed in the blood (3). However, it has to be noted that these absorption rates were calculated based on the disappeared amount in the effluent, thus they could indicate the portion of anthocyanins being taken up into the small intestine tissue but not necessarily transferred into the blood. Interestingly, the disappearance of Cy-3-glu was significantly higher than Cy-3-gal and Cy-3-rut. Two mechanisms are likely responsible for such difference: preferable uptake or prominent degradation of anthocyanin glucoside in the small intestine lumen. The former is supported by evidence with quercetin glycosides, and the latter is probably due to the β-glucosidase activity in the small intestine, which will be discussed in the next section.

An early human pharmacokinetic study of quercetin rich foods indicated that absorption of quercetin was affected by the type of glycosides (144). Five women and four men received either quercetin-glu rich, quercetin-gal rich, or quercetin-rut rich diet for breakfast in three separated periods. Blood was collected every 6 h over 36 h, and
urine was collected continuously for 24 h. Total quercetin concentration was measured after being hydrolyzed to the aglycone form. Urinary quercetin equivalents indicated that the proportion excreted was significantly higher for the quercetin-glu rich diet than for the quercetin-gal rich and quercetin-rut rich diets (Figure 2.8), suggesting preferable absorption of quercetin-glu in the small intestine. The urinary data were also in agreement with the kinetics analysis of plasma quercetin. Plasma quercetin reached peak level 0.7 h after ingestion of quercetin-glu rich diet, 2.5 h after ingestion of quercetin-gal rich diet, and 9 h after ingestion of quercetin-rut rich diet. Almost no quercetin was detected in the plasma for 4 h with the rut diet. The authors suggested that quercetin-glu was absorbed efficiently in the stomach and small intestine, while quercetin-rut must be transported to the colon to be hydrolyzed by the colonic microflora before the aglycone could be absorbed.
Figure 2.8 The ratio of mean 24h cumulative urinary excretion: intake of quercetin (Source: Hollman, van Trijp et al. 1997). Data are means ± SD. Results with a different letter differ significantly (P<0.001).

The hypothesis was supported by Gee et al. (145) with an in situ stimulated efflux model. Jejunal sacs were obtained from male Wister rats, and then everted, ligatured at one end and tied on to syringes to load Krebs bicarbonate buffer. Subsequently, sacs were pre-loaded with $^{14}$C radio labeled galactose solution by incubation. Next, rinsed tissue was submerged into buffer bath with or without test compounds (quercetin mono- or di-glu and rut). In the presence or absence of sodium ions, the initial and cumulative efflux rates of labeled galactose were measured. The results showed potent counterflow stimulation by quercetin mono- and di-glu, as opposed to no flux of quercetin-gal and rha. Furthermore, the presence of sodium ions stimulated the rate of efflux of quercetin...
glycosides. The data suggested that quercetin mono- and di-glu interacted with the sodium-dependent glucose/galactose transport pathway of the small intestine brush-border mucosal cells. A follow up study carried out by the same group of researchers strengthened the conclusion by demonstrating that the presence of quercetin-3-glu stimulated the efflux of preloaded galactose and competitively suppressed the uptake of labeled galactose via rat jejunum (146).

2.6.4 Deconjugation of carbohydrate moieties

Glycosylated flavonoids are more hydrophilic than the corresponding aglycones. For instance, quercetin has a partition coefficient (log value of concentrations in octanol/water) of 1.2 ± 0.1, while quercetin-3-rut has only 0.37 ± 0.06 (70). With smaller molecular size and better lipid solubility, aglycones are anticipated to penetrate the lipid bi-layer of cell membranes, possibly leading to passive diffusion across the small intestine brush border. This pathway necessitates deglycosylation of ingested anthocyanins. Nonenzymatic deglycosylation is unlikely to play an important role, despite the acidic condition in the stomach. Deglycosylation of quercetin glycosides (145) or anthocyanidins glycosides (10) was not noted after pepsin-HCl digestion at pH 2.0 and 37°C for 2h. Therefore the question is left to the possibility of enzymatic deglycosylation in vivo.

Recent advances in the study of small intestinal β-glucosidases support the hypothesis that they deglycosylate some flavonoids, and play an important role in the digestion of dietary flavonoids. Day et al. (147) were the first to investigate the effect of
human β-glucosidases on flavonoids. Crude enzyme extract was obtained from homogenized intestine or liver specimens surgically removed from human patients and incubated with a broad range of flavonoids at 37°C for up to 90 min. The $K_m$ for hydrolysis of each compound was estimated. Most of the mono-glucosides, except for those glu at the 3-position on B-ring, were successfully deglycosylated by both small intestine and liver β-glucosidases, regardless of the type of aglycone (quercetin, kaempferol, naringenin, apigenin, genistein, and daidzein). In contrast, rutinosides remained intact. The results agreed with a number of previous in situ or in vivo studies specifying the absorption site difference between mono-glu and rut ($144, 145, 148$). The authors accentuated the importance of the glycosylation position. The glucosylation at C-3 position clearly hampered catalytic activity. Although the small intestinal β-glucosidase slowly hydrolyzed quercetin-3-glu, hepatic β-glucosidase did not. Whether this inhibition was likely due to steric effect or other reasons was not discussed. Cytosolic β-glucosidase (CBG) was purified from pig liver ($149$) and the lactase-phlorizin hydrolase (LPH; EC 3.2.1.62) from lamb small intestine brush border ($150$) using chromatographic and gel separation techniques. Hydrolytic activity with flavonoids confirmed the previous human tissue study. Most of the mono-glu, except 3-glu, were substrates for the pig hepatic CBG while all mono-glu were substrates for the lamb LPH. Rutinosides were not a substrate for either enzymes. Notably, the $K_m$ values of human and animals originated enzymes were different but comparable for both enzymes (Table 2.2). These studies suggested the possibility of using animal models for future research. In situ or ex vivo rat jejunum perfusion model, two research groups independently demonstrated that LPH was capable of hydrolyzing quercetin-glu efficiently, and influencing the transport of quercetin across...
the epithelial membrane \((151, 152)\). Both studies employed N-butyldodeoxygalactonojirimycin, a LPH inhibitor, to control the activity of LPH, in the attempt to establish the association between LPH activity and the degree of quercetin deglycosylation and absorption.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (µM)</th>
<th>Human</th>
<th>Pig</th>
<th>Lamb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Small intestine</td>
<td>Liver</td>
</tr>
<tr>
<td>Quercetin 4’-glu</td>
<td>27 ± 13</td>
<td>37 ± 12</td>
<td>65</td>
<td>44 ± 7</td>
</tr>
<tr>
<td>Genistein 7-glu</td>
<td>13 ± 1</td>
<td>14 ± 5</td>
<td>35</td>
<td>85 ± 11</td>
</tr>
</tbody>
</table>

**Table 2.2** K_m of quercetin 4’-glu and genistein 7-glu by β-glucosidase from human and animal intestine and liver (Source: Day, DuPont et al. 1998; Lambert, Kroon et al. 1999; Day, Canada et al. 2000).

Unlike the well-documented flavonoid glucosides and rutinosides, other glycosides such as gal, ara, and xyl are not well studied. It is unclear if their absorption may partly depend on β-glucosidase but there is a lack of evidence. Limited information from our recent research suggested that anthocyanin- xyl and ara were better retained in the cecal content and feces as opposed to anthocyanin- gal and glu \((13)\). Further research is needed to elucidate the fate of such glycosides.
Evidence of enzymatic deglycosylation of anthocyanins is still very limited. Examination of pig and rat GIT content indicated selective degradation of anthocyanin glucoside in the small intestine (5, 7, 13), but further characterization of anthocyanin deglycosylation pattern under the effect of isolated small intestinal β-glucosidases is needed.

2.6.5 The influence of colonic microflora

The enzymes present in the small intestine, including β-glucosidase, cannot account for hydrolysis of all glycosidic bonds, and hence flavonoid- rha and rut can survive through the small intestine and reach the colon (70). There are no endogenous esterases in human to release phenolic acids either. Thus, the esterase activity of colonic microflora is required for the metabolism of acylated flavonoids (153).

Using in vitro anaerobic fecal fermentation model, Aura et al. (154) demonstrated that human fecal flora readily deconjugates quercetin- rut, glu, and glucuronide (glc). The deglycosylated quercetin undergoes ring fission to generate simple phenolics such as 3,4-dihydroxyphenylacetic acid and its derivatives. One of the microorganisms responsible for the degradation of flavonoids may be Eubacterium ramulus, as addressed by Schneider and Blaut (155). Anaerobic incubation with a broad range of flavonoids was performed after inoculating the media with exponentially growing culture of Eubacterium ramulus, which had been previously isolated. The fermentation end products included hydroxyphenylacetic acids and hydroxyphenylpropionic acids. These degradation
products, as well as the deglycosylated aglycones, may be absorbed by the colon, and consequently contribute to the bioactivity of ingested flavonoids.

Fermentation of Cy-3-rut and Cy-3-glu in the presence of human fecal slurry revealed that anthocyanins could also be converted by gut microflora (9). Hydrolysis of Cy-3-glu was almost complete after 2 h of incubation, and less than 1/3 of the Cy-3-rut remained. Protocatechuic acid (PC), a ring fission product of Cy aglycone, was the major metabolite. In another study Cy-3,5-di-glu was incubated with human fecal suspension (6). Over 90% of the Cy-3,5-glu was degraded after 2 h and partial hydrolysis generated Cy-mono-glu as a degradation intermediate, which also underwent degradation in the mean time. Corresponding generation and accumulation of PC was again observed. Further examination of di-acylated anthocyanins from red radish revealed that the acyl group could be cleaved by fecal microflora and the released acids were relatively stable. Deacylated anthocyanins would then follow the same pathway of degradation as discussed above.

2.6.6  Metabolism in intestinal mucosa and tissues

Several phase II drug detoxification enzymes involved in xenobiotic conjugation appear to be the key enzymes for flavonoid metabolism after absorption. Catechol-O-methyltransferase (COMT; EC 2.1.1.6), which occurs in various tissues may transfer a methyl group to the flavonoid aglycone (156, 157). Uridine diphosphoglucose glucuronosyl transferase (UDPGT; EC 2.4.1.17) and uridine diphosphoglucose glucose dehydrogenase (UDPGD; EC 1.1.11.22), both abundant in liver and intestine, were
proposed to catalyze the glucuronidation of flavonoid aglycones \((158)\). Cytosolic enzymes phenol sulfotransferases (SULT; EC 2.8.2.1) are widely distributed throughout the body. They are likely to sulfate flavonoids \((70)\).

Some of the metabolites contribute to the bioactivity of flavonoids. For instance, methylated Cy-3-glu is converted to Pn-3-glu \((131)\). Benzoic acid generated by the metabolism of quercetin-3-rut may provide antioxidant activity or even anticancer effect \((159)\). Equol as a colonic metabolite of daidzein is more estrogenic than daidzein and the other metabolites of isoflavones. Hence the production of this metabolite has attracted great interest \((129)\).

2.6.7 Tissues distribution

The protective effects of flavonoids have been associated with diseases occurring in various tissues, but such claims are mainly based on \textit{in vitro} evidence using different types of cell lines. Knowledge about their availability to target tissues is quite limited. Quercetin is one of the well investigated flavonoids regarding distribution into tissues. For example, 2 groups of rats fed either 0.1% or 1% quercetin diet for 11 wk demonstrated the same pattern of tissue distribution \((160)\). The combined concentration of quercetin and its metabolites was high in lung, testes and kidney, moderate in thymus, heart and liver, low in brown fat, muscle and bone, and extremely low in white fat, brain and spleen. The highest tissue concentrations were 3.98 and 15.3 nmol/g in lung for diets with 0.1 and 1% quercetin, respectively. The authors also reported that liver (5.87 nmol/g tissue) and kidney (2.51 nmol/g tissue) contained high concentrations of quercetin in pigs.
fed 3 d of 500 mg quercetin/kg body wt diet, while brain, heart, and spleen had much lower concentrations (160).

Anthocyanin distribution in tissues has recently been evaluated in rat and pig models. Male Wistar rats were fed blackberry extract (370 nmol anthocyanin/d) for 15 d and killed at 3 h after the beginning of the last meal. Total anthocyanins averaged 605 nmol/g in jejunum, 68.6 nmol/g in stomach, 3.27 nmol/g in kidney, 0.38 nmol/g in liver, and 0.25 nmol/g in brain (161). In pigs fed diets supplemented with 0, 1, 2, or 4% w/w blueberries for 4 wk and fasted for 18-21 h before euthanasia, 1.30 pmol/g of anthocyanins were identified in the liver, 1.58 pmol/g in eyes, 0.878 pmol/g in cortex, and 0.664 pmol/g in cerebellum (125). The results suggested that anthocyanins crossed the blood-brain barrier and the blood-retinal barrier to potentially provide protection for brain and eye tissues.

2.6.8 Excretion

Unabsorbed flavonoids are excreted through feces (13, 162, 163). The absorbed intact anthocyanins and flavonoid aglycones are largely excreted in urine (130, 164). Conjugated flavonoid metabolites are likely excreted in urine as well (131), but alternatively a portion of them may re-enter the jejunum with the bile, and later either being absorbed by the colon entering the enterohepatic circulation (157, 165), or being excreted with feces. Lung has been reported as a major excretion site for many phytochemicals including quercetin (166). Over 50% of the orally ingested $^{14}$C-labeled
quercetin were found exhaled as $^{14}\text{CO}_2$ in humans. There are no data regarding the respiratory excretion of anthocyanins.

Understanding the bioavailability and metabolism pathway is important to the health benefits evaluation of anthocyanins. Such knowledge is also necessary for the screening of suitable anthocyanins from numerous sources to facilitate development of functional foods/supplement that promote human health. In the last decade our knowledge of the bioavailability and metabolism of anthocyanins has steadily increased. The pathways reviewed in this section are summarized in Figure 2.9.
**Figure 2.9** Integrated putative pathways of dietary flavonoids absorption, metabolism, distribution, and excretion.
CHAPTER 3

BLACK RASPBERRY ANTHOCYANINS ARE STABLE IN THE DIGESTIVE TRACT LUMEN AND EFFICIENTLY TRANSPORTED INTO GASTRIC AND SMALL INTESTINAL TISSUES IN RAT

3.1. ABSTRACT

Anthocyanins are abundantly consumed natural pigments with reported health promoting activities. However information regarding their fate in the gastrointestinal tract (GIT) is scarce. Kinetic flux and absorption of black raspberry anthocyanins throughout rat stomach and small intestine were evaluated. Fasted male rats were administered either vehicle only (n = 6) and killed at 30 min (control) or 12 ± 3 mg of anthocyanins (n = 24) and euthanatized at 30, 60, 120, and 180 min to collect bladder urine, GIT contents, stomach and small intestine. HPLC-MS analysis showed that anthocyanin content in the gastric lumen decreased linearly over time with t½ = 30 min. Anthocyanin content in small intestinal tissue and lumen peaked at 120 min. Uptake by the small intestine tissue reached 7.5% of the administered dose, a much higher percentage than the previously reported absorption of these pigments (normally <0.1%). The relatively high recovery (75-79%) of administered anthocyanins from GIT contents demonstrated their overall

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stability at physiological conditions. Selective decrease of the concentration of cyanidin-3-glucoside in the small intestinal content resulted from β-glucosidase activity. Urine anthocyanin analysis indicated that profile of absorbed anthocyanins reflected that present in the GIT at the time of absorption. Our results suggest that ingested anthocyanin glycosides remain relatively stable in the GIT lumen and are efficiently taken up into the GIT tissues with limited transfer to the plasma compartment.

Key words: Anthocyanin; black raspberry; digestion; gastrointestinal tract; tissue uptake; absorption; β-glucosidase; stability; metabolism

3.2. INTRODUCTION

Anthocyanins are a group of natural pigments that belong to the large family of flavonoids and are responsible for the red, purple and blue colors of many plant materials. Consumption of anthocyanins is high compared to other flavonoids (70) with the daily intake estimated to be 12.5 mg/day/person in the USA (44). Berries are generally high in anthocyanin content and contribute a major portion of anthocyanin consumed (167).

Increasing evidence shows that anthocyanins are potent antioxidants and are associated with protective effects against many chronic diseases such as cancer, cardiovascular diseases, and even obesity (3). However, such postulated health-promoting effects are largely based on in vitro evidence, while data related to the in vivo absorption and metabolism of anthocyanins remains limited (168). Recent animal and clinical studies have focused on the bioavailability of anthocyanins by monitoring the
concentration in plasma and urine, as well as distribution in organs (133, 161, 169). In contrast, information about the fate of anthocyanins in the GIT is scarce. The lack of such information impairs our understanding of their dietary value. First, since stomach and small intestine have been considered as potential absorption sites of anthocyanins (140, 141, 143), the availability of anthocyanins in these segments would likely be a major factor influencing absorption. Existing data suggest rapid absorption and elimination of a small percentage of ingested anthocyanins (169). Therefore, the emptying time in the stomach and transit rate in the small intestine is expected to determine the duration of mucosal cell uptake and circulation in the blood. Second, intact anthocyanins in the GIT lumen may directly interact with GIT tissues, where these antioxidants may protect GIT against acute distress and chronic diseases associated with oxidative stress (8, 13, 113, 170). Third, metabolism of anthocyanin glycosides has been proposed to be possible before absorption (171). It was suggested that less hydrophilic aglycones could be produced in the small intestine by the action of β-glucosidase with the resulting aglycone being transported across the mucosal epithelium via passive diffusion in a manner analogous to some other flavonoids (168). Further derivatives from the ring fission of aglycones may also be absorbed and contribute to the health-promoting effects (6, 171). Direct evidence of anthocyanin deglycosylation in the GIT is required to support such speculations.

Black raspberry (*Rubus occidentalis*) can provide 845 mg of anthocyanins in a single serving and was selected as a representative anthocyanin-rich source for the present study (44). Its phenolic content, primarily anthocyanins, has been shown to
effectively inhibit proliferation of human oral, breast, prostate, and colon cancer cells in vitro (172). Here we investigated the kinetics of black raspberry anthocyanin flux in stomach and intestine of fasted rats, focusing on the stability, rate of emptying, uptake into GIT tissues, and selective deglycosylation by intestinal β-glucosidase activity.

3.3. MATERIALS AND METHODS

3.3.1 Chemicals and materials

Reagent grade tri-fluoroacetic acid (TFA), high performance liquid chromatography (HPLC) grade acetone and methanol for extraction, and HPLC- mass spectrometer (MS) grade water and acetonitrile for chromatography were purchased from Fisher Scientific (Fair Lawn, NJ). Formic acid (99%) was from Acros Organics (Morris Plains, NJ). Cyanidin (Cy) aglycone was prepared from Cy-3-glu standard (ChromaDex Inc., Irvine, CA) by acid hydrolysis (173). Protocatechuic acid standard was from MP Biomedical (Solon, OH).

3.3.2 Black raspberry extract

Lyophilized black raspberry powder was a generous gift from Dr. Laura Kresty, OSU Comprehensive Cancer Center. The powder was extracted following a modified procedure by Jing et al. (174). One part of the dry powder was mixed with three parts of acetone/water (70:30, v/v) containing 0.1% formic acid, briefly homogenized by a handheld tissuemiser (Fisher Scientific, Fair Lawn, NJ), and sonicated in a Fisher FS30
ultrasonic bath for 15 min. The slurry was then centrifuged at 1800×g for 10 min and the supernatant was collected. The pellet was re-extracted twice for further removal of pigmentation from the matrix. The pooled supernatant was mixed (1:1, v/v) with chloroform and centrifuged at 1800×g for 15 min. The red aqueous fraction was transferred to a round-bottomed flask and evaporated with a Büchii rotovaporator at 35 °C to remove organic solvents. The extract was lyophilized to powder form and stored at -80°C under N₂.

3.3.3 Animals and experimental design

Male Fischer 344 rats (8 wk of age; ~200 g) were purchased (Taconic Farms, Germantown, NY) and maintained in the University Laboratory Animal Resources facility on the Ohio State University campus in accordance with NIH Laboratory Animal Use Guidelines. The Institutional Animal Care and Use Committee at the Ohio State University approved the experimental protocol (OSU Protocol #2004A0139). Animals were group housed with two animals per cage and maintained under standard conditions (22 ± 2 °C, 50 ± 10% relative humidity, 12 h light/dark cycle). Commercial diet and water were available ad libitum prior to the experiment.

Rats (n=30) at 11 wk of age were randomly assigned to one of five groups. After being fasted overnight, animals were lightly anesthetized for the delivery of 1.2 ± 0.3 mL water either containing 0.1% citric acid alone (control) or including black raspberry extract (treatment) by stomach tube. The precise volume delivered was determined by weighing the syringe and intubation tube before and after gavage. The administered
extract contained 10 mg Cy-3-glu equivalent/mL as measured by pH differential method (175). Treatment groups were asphyxiated with CO₂ after 30 (n = 6), 60 (n = 6), 120 (n = 6), and 180 min (n = 6). Control group (n = 6) was asphyxiated at 30 min after intubation. Contents from stomach and small intestine were separately collected by perfusing the lumen with 25 mL of ice-cold 20% formic acid in phosphate buffered saline (PBS). Urine samples (when present) were collected from bladder and acidified immediately with 20% (v/v) formic acid. Stomach and small intestine were then removed and immediately frozen by liquid nitrogen. All samples were stored at -80°C until analysis.

3.3.4 Sample preparation.

Urine samples were thawed and weighted with the container. The weight of container was later recorded and subtracted from the total weight. Urine volume was calculated based on weight assuming a density of 1.0 g/mL. A 50 μL aliquot was added to 550 μL of 0.1% TFA and centrifuged at 16000×g for 15 min prior to HPLC analysis of the supernatant (143). The remaining samples were pooled and semi-purified by a 1cc Sep-Pak® C₁₈ cartridge (Waters, Milford, MA) for HPLC-MS analysis to confirm peak identities.

Gastric and small intestinal contents were thawed, sonicated for 15 min in ice water bath, and centrifuged at 1800×g for 10 min. An aliquot of the supernatant was filtered (0.45 μm Whatman® polypropylene filter) for HPLC analysis. A second aliquot of the remaining sample was pooled by group and semi-purified by a 6cc Sep-Pak® C₁₈ cartridge for HPLC-MS analysis to confirm peak identities. The C₁₈ semi-purification
method was modified from our previous studies (13, 25). Briefly, sample was loaded onto a pre-conditioned C_{18} cartridge and washed with 2 vol of 0.1% TFA acidified water before eluting anthocyanins with 1 vol of 0.1% TFA acidified methanol. The methanolic eluate of urine was dried under N_{2} and redissolved in an aliquot of acidified water for HPLC analyses. In order to preserve the labile anthocyanidin aglycone in gastrointestinal samples, the methanolic eluate was not evaporated but instead diluted in acidified water (1:9, v/v) to maintain adsorption of anthocyanin peaks on the reverse-phase HPLC column.

Extraction of anthocyanins from the stomach (1.43 ± 0.032 g, n = 30) and small intestine tissues (7.1 ± 0.12 g, n = 30) were carried out separately. The samples and solutions were kept on ice during the entire process to prevent enzymatic and/or heat degradation. Mucus and epithelial layer were removed from each tissue because dark food particles adhered to the surface. The remaining muscular tissue was homogenized at high speed for approximately 3 min in 10 mL (stomach) or 15 mL (intestine) of methanol (with 0.1% formic acid) using a hand-hold tissuemiser followed by sonication in iced water-bath for 20 s. The methanolic extract was then centrifuged at 1800×g for 10 min. Supernatants were collected, pellets were re-extracted and centrifuged at 1800×g for 10 min, and the pooled supernatants were diluted 6 fold using water containing 0.1% formic acid. Diluted supernatants were then passed through a Sep-Pak® C_{18} (5 g, 20 cc) cartridge and recovered with 10mL of 0.1% formic acid in methanol which was evaporated using a Büchii rotary evaporator. The remaining anthocyanins were redissolved to 5mL using
10% formic acid prior to centrifugation at 16000×g for 10 min, filtration with 0.45 μL polypropylene syringe filters, and HPLC-MS analysis.

3.3.5 Recovery

Black raspberry extract was spiked into effluents of gastric and small intestinal contents from control animals (n = 4) to simulate the concentration found in the treatment groups. The slurry was sonicated in ice water bath for 15 min to facilitate anthocyanin penetration into particulate materials. Spiked samples were frozen and analyzed on the following day according to the procedures described above. Recovery was calculated by total amount recovered/total amount added × 100%. Profiles of recovered anthocyanins from the spiked samples were compared to that from the gastrointestinal contents of treatment groups to identify specific changes not attributable to non-specific binding.

3.3.6 HPLC-MS analysis of anthocyanins and anthocyanin metabolites

Samples were analyzed using a Shimadzu LCMS-2010 EV HPLC-MS (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with an SPD-M20A photodiode array (PDA) detector and a single quadrupole electron spray ionization (ESI) MS detector. Separation was accomplished on a Symmetry® C18 column (3.5 μm, 4.6×150 mm; Waters Corp., Milford, MA) with a flow rate of 0.8 mL/min. Conditions for urine samples were as follows: injection volume: 200 μL; mobile phase: A, 4.5% formic acid in HPLC-MS grade water; B, HPLC-MS grade acetonitrile; gradient: 0-5 min, 2-7% B; 5-20 min, 7-12% B; 20-25 min, 12-17% B; 25-40 min, 17-25% B. Conditions for gastric and small intestinal contents/tissues samples were the following: injection volume: 20 μL; mobile
phase: A, 10% formic acid in HPLC-MS grade water; B, HPLC-MS grade acetonitrile; gradient: 0-6 min, 5-8% B; 6-15 min, 8-10% B; 15-20 min, 10-25% B; 20-25 min, 25% B. After each run, the column was equilibrated for 5 min under the initial condition. Spectral data (250-700 nm) were collected during the entire separation procedure. When MS was coupled to the HPLC, spectra were obtained under positive ion condition using SCAN (from m/z 200 to 1200) and Selective Ion Monitoring (SIM) modes. Six channels including m/z 287 for Cy and m/z ratios for other common anthocyanin aglycones were monitored in the SIM mode. Anthocyanin concentrations in the urine, perfusate of gastrointestinal contents and tissues were calculated using areas under curve (AUC) of HPLC chromatograms at 520 nm and a standard calibration curve. Anthocyanin amount was calculated by multiplying the concentration in the aliquot with the corresponding volume.

3.3.7 Calibration curve

Commercially available Cy-3-glu standard (10 mg) was dissolved in 10 mL of double distilled water containing 0.1% TFA, and a series of dilutions were prepared to generate a standard curve ($R^2 > 0.99$). All anthocyanins analyzed fell within the range of the standard curve and were expressed as Cy-3-glu equivalents by weight. The total amount of recovered anthocyanins was calculated by summing up the AUC of individual anthocyanin peaks at 520 nm and using this calibration curve.
3.3.8 Statistical analysis

One-way ANOVA or its non-parametric analogue Kruskal-Wallis Test (equal variance not assumed) was conducted using SPSS (version 13, 2004, SPSS Inc., Chicago, IL) to compare amounts of total and individual anthocyanins in different treatment groups, and data are given as means ± SE. When appropriate, significance of differences between means was determined by Tukey's HSD or Dunnett’s T3 (equal variance not assumed). Differences of P<0.05 were considered significant.

3.4. RESULTS AND DISCUSSION

3.4.1 Flux of anthocyanins in GIT lumen

Kinetic flux of anthocyanins in the GIT is critical for understanding anthocyanin absorption. Recent studies have suggested that the stomach is a potential site for the absorption of anthocyanins (140, 141), in addition to the small intestine which is a known common absorption site for other structurally related flavonoids (143). Therefore, absorption may begin immediately after ingested anthocyanins reach the stomach. The rate of gastric emptying, transit through the small intestine, as well as anthocyanin stability, will determine the duration and extent of anthocyanin accessibility to the mucosal epithelia for uptake and possible transfer to circulation. Combining kinetic flux through the GIT with pharmacokinetic data is necessary to determine the primary site(s) of anthocyanin absorption.
A preliminary examination using spiked perfusate samples from fasted rats demonstrated recovery rates of 93 ± 2% (n = 4) and 84 ± 10% (n = 4) for black raspberry anthocyanins added to gastric and small intestinal contents, respectively. We observed that contents from the small intestine had a gel-like property causing difficulty with passage through the 0.45 μm polypropylene filter. The extent of pigmentation on filters was greater for samples with more viscous or gel-like consistency. The viscosity variability of luminal contents from the small intestine samples contributed to the relatively large variation in recovery of anthocyanins from the small intestinal contents.

Anthocyanins in the black raspberry extract and collected biological materials were identified by comparing retention times and UV-visible spectra to known standards, and by comparing m/z ratios of whole molecular ions and fragments to established values (176). The four major anthocyanins identified in the crude extract of black raspberry, as well as gastric and intestinal contents (Table 3.1, Figure 3.1) included Cy-3-sambubioside (Cy-3-sam), Cy-3-glu, Cy-3-xylosylrutinoside (Cy-3-(xyl)-rut), and Cy-3-rutinoside (Cy-3-rut). Anthocyanin total concentrations in the gastric and small intestinal contents were adjusted for the efficiency of extraction and are presented in Figure 3.2. Approximately 52% of the total amount of black raspberry anthocyanins delivered to the stomach (12 mg) was present in gastric luminal contents 30 min after administration of the bolus. Anthocyanin content in the gastric lumen decreased linearly (R² = 0.67) during the 180 min study. The estimated time to deplete one half of the anthocyanin content in the gastric lumen (t½) of the fasted rat was approximately 120 min, suggesting that minimal amounts of anthocyanins would be present in the stomach after 4 h. Our result
agrees with a previous study by Borges et al. (14) who reported almost no raspberry anthocyanins remained in the gastric lumen of fasted rats 4 h after gavaging raspberry juice. However, these investigators observed a non-linear decrease of the anthocyanin content in the stomach, with a more rapid decrease during the first hour. This difference in the two studies may be due to the administration of a much larger dose of anthocyanins than that in our study (920 nmol vs. 26.7 nmol, respectively).

<table>
<thead>
<tr>
<th>Peak number</th>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>(m/z)</th>
<th>Peak identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intact molecule</td>
<td>Aglycone fragment</td>
</tr>
<tr>
<td>1</td>
<td>520</td>
<td>581</td>
<td>287</td>
</tr>
<tr>
<td>2</td>
<td>516</td>
<td>449</td>
<td>287</td>
</tr>
<tr>
<td>3</td>
<td>522</td>
<td>727</td>
<td>287</td>
</tr>
<tr>
<td>4</td>
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<td>287</td>
</tr>
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<td>5</td>
<td>520</td>
<td>287</td>
<td>287</td>
</tr>
</tbody>
</table>

Table 3.1 Peak assignments of anthocyanins extracted from the gastric and intestinal contents of rats administered black raspberry extract.
Figure 3.1 Chromatographic profiles of Cy standard (A), black raspberry anthocyanins in the administered crude extract (B), and representative samples of luminal contents from stomach (C) and small intestine (D) from a rat killed 120 min after gavage. Chromatograms are monitored at 520 nm for detection of anthocyanins. Peak identities are presented in Table 1.
Figure 3.2 Black raspberry anthocyanins in the gastric and small intestinal contents (A) and in the small intestinal tissue (B). Rats were administered approximately 12 mg (Cy-3-glu equivalent) of anthocyanins in acidic solution by stomach tube. Data are means ± SE for 6 rats. Within each line, means with different letters are significantly different (P < 0.05).
The amount of anthocyanins in the small intestinal lumen increased between 30 and 120 min after administering the black raspberry extract, and then decreased by 180 min (Figure 3.2). This suggests that the amount entering the small intestine prior to 120 min exceeded the total amount of small intestinal mucosa uptake, exiting to the lower intestine, and perhaps degradation. Combining such information with the fact that plasma anthocyanin concentration usually is maximum within 0.25-0.5 h in rats after gastric intubation of anthocyanin extract (169, 177, 178), it is reasonable to speculate that the stomach has a central role in anthocyanin absorption. Otherwise, plasma concentration of anthocyanins would be maintained at the same or a higher concentration after 0.5 h as anthocyanin content in the small intestine continues to increase from 30-120 min.

The total amount of administered anthocyanins recovered from gastric and small intestinal contents was 75-79% (∼9 mg Cy-3-glu equivalents) at 30-120 min after delivery (Figure 3.2), demonstrating that the anthocyanins delivered in the absence of a food/meal are stable within the lumen of the upper GIT of fasted rats. In contrast, losses of greater than 50% of anthocyanins have been reported during in vitro digestion (6, 10, 11). Anthocyanin are not stable at neutral pH, forming pseudobases, quinoidal bases, and chalcones that subject to nucleophilic attack by water (11). However, we hypothesize that binding of anthocyanins to mucus, secretions and food residues in vivo may increase stability (47). Knowing the stability of anthocyanins in the GIT lumen is important not only because the accessibility affects absorption but also because some suspected health benefits depend on continuous exposure to anthocyanins. For example, Malik et al. reported that exposure of colon cancer cells to anthocyanins caused cell cycle arrest at
G1/G0 and G2/M phases, yet after the anthocyanins were removed the cells could recover from both the G1/G0 and G2/M blocks (95). By 180 min the anthocyanin content in the combined gastric and small intestinal contents decreased significantly as anthocyanins presumably entered the large intestine (Figure 3.2A).

3.4.2 Anthocyanins in GIT tissues

Intense red color was present in the acidic extract of all gastric and small intestine tissue samples. The intensity of the red color in the gastric tissue extract steadily decreased over time, coinciding with the linear decrease of anthocyanins in the gastric lumen. However, anthocyanins appeared to bind to unidentified protein in the stomach tissue thus could not be quantified as free anthocyanins by HPLC. The presence of anthocyanins in the gastric tissue extract was confirmed by observing spectral change at the pH of 1.0, 4.5, and 10.0 (Figure 3.3). Such binding may be attributed to non-specific binding or perhaps specific binding to transporter protein in the stomach (142). There was a parallel trend of anthocyanin concentrations in the small intestinal tissue and luminal contents (Figures 3.1 A and B). This correlation suggests that some portion of the newly acquired anthocyanins within GIT mucosa was quickly degraded and/or effluxed across the apical or basolateral membranes. Otherwise, anthocyanins would be expected to continue to accumulate in the tissues with increasing time.
Figure 3.3 UV-Vis spectra of the anthocyanin-protein complex extracted from the stomach tissue. Reversible decrease of absorbance near 520nm is unique for anthocyanins when pH changes from 1 to 4.5.

The total amount of anthocyanins in the small intestine tissue reached 7.5% of the administered dose (120 min), a much higher percentage than the reported anthocyanin absorption (normally <0.1%), which was based on plasma and urine anthocyanin concentrations (3). Considering the fact that anthocyanins would degrade or exit GIT tissue rapidly, the actual uptake by the small intestine tissue may be much larger than 7.5%. In the current study when 26.7 nmol of black raspberry anthocyanins was administered as a bolus, the highest concentration in the small intestine tissue (282 pmol Cy-3-glu equivalent/g, n = 6) was observed at 120 min. This observation agrees with a recent report by Talavera et al. in which 605 pmol Cy-3-glu equivalent/g jejunum tissue
was found in rats (n = 6) fed ~370 nmol of blackberry anthocyanins for 15 days (161). The exceptionally high anthocyanin concentration in GIT tissues as compared to the much lower concentrations in blood or other tissues (125, 161) suggests that intact anthocyanins may be taken up by GIT tissues efficiently but not effectively transported into circulation. Our speculation is supported by a recent study evaluating absorption of black currant anthocyanins by monolayers of human intestinal epithelial Caco-2 cells (179). This in vitro model revealed that transport across the apical membrane occurs to a much larger extent than further translocation across the basolateral membrane. Despite inefficient transfer across the basolateral membrane into plasma, anthocyanins in GIT tissues may have protective effects in situ. Bruce et al. (113) suggested that colon cancer development might be inhibited by agents that prevent epithelial barrier damage, inhibit inflammation, or quench reactive oxygen species in local epithelial cells. Anthocyanins have been shown to be potent antioxidant and anti-inflammatory agents in vitro (89). In vivo studies examining the ability of anthocyanins to protect GIT against oxidative stress are merited.

3.4.3 Anthocyanin transformation in the GIT

Besides the 4 major anthocyanins identified in the black raspberry diet, a minor peak of Cy (anthocyanidin) was observed in both stomach and intestinal contents (Peak 5 in Figure 3.1 C and D). Anthocyanidins are degradation intermediate of glycosylated anthocyanins when deglycosylation occurs. As anthocyanidins degrade quickly at room temperature, in order to preserve them we shortened sample preparation to avoid using rotary evaporation or a stream of N₂ to remove organic solvents. Slight change of the
anthocyanin profile over time occurred in the gastric content, probably due to acid hydrolysis. Anthocyanidins are usually produced from glycosylated anthocyanins via acid hydrolysis in the laboratory (173). Boiling and 2N HCl ensure nearly complete cleavage of glycosidic bonds within 30 min. The pH in the gastric lumen after fasting can be as low as 1 and this acidic environment may cause limited hydrolysis. It has to be noted that the vehicle for delivery of the black raspberry extract to rat stomach was slightly acidic and thus expected to stabilize anthocyanins. In contrast, introduction of food matrix into the gastric lumen leads to rapid elevation of gastric pH (180), resulting in perhaps no acid hydrolysis and decreased overall stability of anthocyanins. The presence of Cy in the gastric content (Figure 3.1C) suggested cleavage of glycosidic bond between anthocyanidin and sugar moieties. As cleavage may also occur at glycosidic bond within a multi-glycoside moiety, hydrolysis of tri-glycoside (e.g. between rutinose and xylose) or di-glycoside (e.g. between glucose and rhamnose) to the simpler glycoside was also observed (Figure 3.4). Such hydrolysis explained the slight but steady decrease of Cy-3-(xyl)-rut with proportional increases in Cy-3-rut and Cy-3-glu in the stomach over time. The small intestinal contents are neutral so further acid hydrolysis is not expected. Changes in the anthocyanin profile in the GIT are likely to influence the profile observed in plasma, tissues and urine (7). Failure to consider this factor may overestimate the absorption of mono-glycosides and underestimate the absorption of multi-glycosides present in foods, beverages and formulations.
Figure 3.4 Relative abundance of black raspberry anthocyanins in the gastric and small intestinal lumens. ANOVA for linear regression model indicated significant (P < 0.05) decrease in the relative amount of Cy-3-(xyl)-rut and a significant increase in the relative amounts of Cy-3-rut and Cy-3-glu in gastric lumen. Pattern of anthocyanin profile in the small intestine is similar to that in the stomach, indicating no further metabolism with the exception of the decrease in Cy-3-glu.
The anthocyanin profile of small intestinal contents was similar to that in the stomach (Figure 3.4) with one exception. The relative amount of Cy-3-glu in the small intestinal contents was significantly (p<0.001) decreased in comparison to that in the administered extract and gastric contents (Figure 3.5). Such selective degradation of this anthocyanin glucoside was reported in our previous animal study using rats (13) and by Wu et al. using a pig model (5, 7). We observed additional supporting evidence for the hydrolysis of anthocyanins as Cy appeared in the intestinal contents (Figure 3.1D). But interpretation of Cy generation must be cautious, as it is possible that the observed Cy was carried over from the stomach. LPH, which is located in the brush border membrane of mature small intestine enterocytes (181), and/or glycosidase activity in microorganisms residing in the small intestine are likely the basis for this hydrolysis.
Figure 3.5 Relative abundance of Cy-3-glu is decreased in the small intestinal content. Due to negligible change with time, all data from the 4 treatment groups are pooled. Means with different letters are significantly different (P < 0.05) as determined by Dunnett’s T3 test following Kruskal-Wallis Test.

A preliminary test using the small intestine mucosal layer of 8-month old pigs has also provided supporting evidence of LPH activity by showing the selective hydrolysis of Cy-3-glu in black raspberry anthocyanins after incubation (unpublished data). An alternative explanation to the selective decrease of Cy-3-glu in the intestinal lumen is high uptake. However, the Cy-3-glu recovered from small intestine tissue represented 7.5 ± 0.16% (n = 23) of the total anthocyanins extracted, which was significantly (P<0.001) lower than that in the black raspberry extract and in the spiked control samples. Such evidence confirmed that the selective decrease was due to degradation rather than uptake.
In a previous study using *in situ* intestine perfusion to evaluate anthocyanins uptake, Talavera et al. stated that Cy-3-glu was preferably absorbed as compared to other Cy glycosides based on the evidence that Cy-3-glu disappeared in a large extent in the intestinal efflux (143). We now suggest that their observation might instead be attributed to degradation of Cy-3-glu during intestine perfusion. In the present study, degradation of anthocyanin glucoside did not appear to be a major factor for overall anthocyanin stability. However, it is noteworthy that >90% anthocyanins identified in nature contain glucoside moiety (16), and in weaning mammals (150) or some adults (182) LPH activity remains remarkably high, potentially causing extensive biotransformation of ingested anthocyanins.

Hydrolysis of the glycosidic bond is considered critical for flavonoids digestion since their aglycones rather than the ingested glycosides are readily absorbed (147, 151, 152, 183). Anthocyanins differ from other flavonoids in that anthocyanidins (aglycones) are considered to be relatively unstable and rarely detected in plasma or urine in many studies (169). However, even if only present for a relatively short time, anthocyanidins may have the potential to be taken up by epithelial cells lining the gastric and small intestinal mucosa where they are metabolized to become conjugated metabolites with some being absorbed (184). The anthocyanidins may also undergo spontaneous ring fission to generate smaller phenolic compounds that may be absorbed to have health promoting benefits (6, 171). In the case of the Cy aglycone, protocatechuic acid (PC) is expected to be one of the ring fission products. Although we have observed the presence of PC when genuine Cy standard was degraded in buffer, PC was not detected in either
the gastric or small intestinal contents. In another study Cy and PC were particularly monitored but not found in rat plasma after gavage feeding anthocyanin-rich extract from wild mulberry (185). Perhaps Cy and PC were promptly degraded during digestion in gastric and small intestinal lumens, or only primarily produced in the large intestine where absorption is much less efficient than the small intestine (6, 9).

3.4.4 Metabolism and excretion of absorbed anthocyanins

Black raspberry anthocyanins appeared in the urine within 30 min after stomach intubation (Table 3.2). At 120 min total anthocyanins in the urine collected directly from bladder accounted for 0.045% of that administered. Although urine collection was incomplete, this low amount agrees with other studies showing the low bioavailability of anthocyanins (186). Anthocyanin content in the urine collected from bladder at 180 min post-administration declined suggesting excretion and further metabolism. Methylated anthocyanin metabolites, primarily from the most abundant black raspberry anthocyanin Cy-3-rut, were identified in urine and accounted for 18.2% (n = 15) of total anthocyanins recovered. Nevertheless, such methylated metabolites were not detected in the GIT contents. Enterohepatic cycle extends the plasma elimination half-life of many flavonoids due to re-absorption (168), but with such low concentrations, if any, of anthocyanin metabolites found in the GIT lumen, enterohepatic cycle (184) appeared not capable of influencing anthocyanin excretion half-life (161, 187).
Table 3.2 Black raspberry anthocyanins in urine collected from rat bladder.\(^a\)

Relative abundance of individual anthocyanins in urine was similar to that in the GIT. Wu et al. also reported a positive relationship between the anthocyanin profile in the GIT and that in the urine of weaned pigs (7). Similarly, we found that hydrolysis of Cy-3-glu in the small intestine (~2.5% of the total anthocyanins, \textbf{Figure 3.5}) was associated with a relative decrease of Cy-3-glu in urine at 180 min (~2.9% drop comparing to 30 min, \textbf{Table 3.2}). At 180 min the stomach was almost empty, thus gastric absorption was negligible and the small intestinal content profile determined the urine profile. At 30 min gastric absorption appeared to be predominant as the urinary profile of anthocyanins was quite similar to that in the gastric lumen (\textbf{Table 3.2}).

<table>
<thead>
<tr>
<th>Recovery of administered anthocyanins (%)</th>
<th>30 min (n=3)</th>
<th>60 min (n=5)</th>
<th>120 min (n=5)</th>
<th>180 min (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.007 (0.003-0.010)(^b)</td>
<td>0.024 (0.007-0.042)</td>
<td>0.045 (0.011-0.010)</td>
<td>0.013 (0.010-0.017)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative abundance of co-eluted Cy-3-glu and Cy-3-sam (%)</th>
<th>30 min (n=3)</th>
<th>60 min (n=5)</th>
<th>120 min (n=5)</th>
<th>180 min (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9.3 (8.9-9.7)</td>
<td>8.6 (7.3-9.7)</td>
<td>7.4 (6.7-8.1)</td>
<td>6.4 (6.3-6.5)</td>
</tr>
</tbody>
</table>

\(^a\): Data presented as mean values of all available samples in each group.

\(^b\): Numbers in parentheses indicate the range for each group.
3.5. CONCLUSIONS

In conclusion, black raspberry anthocyanins were relatively stable in the gastric and small intestinal lumens of fasted rats in contrast to reports from some in vitro studies. Total anthocyanins in gastric lumen and tissue steadily decreased during the 180 min period following administration, whereas anthocyanin contents in the small intestinal lumen and tissue were maximum at 120 min before decreasing. A significant portion of administrated anthocyanins was taken up into the GIT tissues, but neither extensively delivered into the blood nor cumulatively retained. The profile of anthocyanins changed in the GIT with some hydrolysis of glycosidic linkages occurring in the acidic gastric lumen, as well as selective hydrolysis of Cy-3-glu in the small intestinal content likely due to endogenous β-glucosidase activity. Unlike some other flavonoids, the stomach is an important site for absorption of anthocyanins. As anthocyanins transit the GIT lumen, the predominant absorption site changed gradually from the stomach to the small intestine. The present study provides novel information about the kinetics of the gastrointestinal flux of anthocyanins in vivo and is expected to contribute to a better understanding of the metabolism, absorption, tissue uptake, and health-promoting activities of these water-soluble plant pigments.

3.6. ACKNOWLEDGEMENTS

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supporting this study. We also thank Dr Laura Kresty for providing lyophilized black raspberry powder.
CHAPTER 4

HIGH-PURITY ISOLATION OF ANTHOCYANIN MIXTURES FROM FRUITS AND VEGETABLES – A NOVEL SOLID-PHASE EXTRACTION METHOD $^{a,b}$

4.1. ABSTRACT

Research on biological activity of anthocyanins requires the availability of high purity materials. However, current methods to isolate anthocyanins or anthocyanin mixtures are insufficient for complete removal of chemically related compounds. We employed a novel cation-exchange/reversed-phase combination technique, the Oasis® MCX solid-phase extraction (SPE) cartridge, and optimized the use of water/organic buffer mobile phases to selectively separate anthocyanins. Crude extracts of various representative anthocyanin sources were purified with this technique and compared to 3 commonly used SPE sorbents: C$_{18}$, HLB, and LH-20. Purified anthocyanin fractions were analyzed with high performance liquid chromatography (HPLC) coupled to photodiode array (PDA) and mass spectrometry (MS) detectors and by Fourier transform infrared (FT-IR) spectroscopy. The UV-visible chromatograms quantitatively demonstrated that

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$^b$ Patent pending.
our novel technique achieved significantly higher (P<0.05) anthocyanin purity than the C$_{18}$ cartridge, the next best method, for 11 of the 12 anthocyanin sources tested. Among the 12 sources, eight were purified to greater than 99% purity (based on UV-Visible chromatograms). The new method efficiently removed non-anthocyanin phenolics. MS and FT-IR semi-quantitatively confirmed extensive reduction of impurities. Due to strong ionic interaction, the MCX sorbent capacity was superior to others, resulting in the highest throughput and least use of organic solvents. This new methodology for isolation of anthocyanin mixtures drastically increased purity and efficiency while maintaining excellent yield (93.6 ± 0.55%) and low cost. The availability of high purity anthocyanins facilitates expansion of scientific studies, as well as applications in the food and nutraceutical industries.

Key words: cation-exchange; anthocyanin; flavylium cation; purification; solid-phase extraction; polyphenols

4.2. INTRODUCTION

Anthocyanins, a class of polyphenols, are responsible for the red, purple and blue color of most fruits and vegetables. Increasing evidence shows that anthocyanins are potent antioxidants and anthocyanin rich fruits and vegetables, as well as anthocyanin rich extracts, have been associated with protective effects against many chronic diseases such as cancer, cardiovascular diseases, and even obesity (3). Interest in the use of anthocyanins as alternatives to synthetic food colorants has increased, and many
researchers are investigating their potential health benefits. Obtaining high-purity anthocyanins is essential for such research (188). Many bioassays on anthocyanin-rich commodities are not feasible without eliminating bioactive impurities that confound interpretation of results. To date there have been 637 naturally occurring anthocyanins identified (189). Unfortunately, there are only a limited number of pure standards commercially available and the cost is high. Therefore, many biological studies are performed using crude anthocyanin extracts from fruits and vegetables.

Isolation methods range from simple solvent extraction to various forms of chromatography (15). SPE methods have gained popularity recently, due to a balance of efficiency and cost (190, 191). However, SPE sorbents normally adsorb analytes via non-selective interactions, which would inevitably allow for a broad spectrum of plant constituents to contaminate the anthocyanin fraction. The impurities, mostly phenolics, are likely to have biological effects and therefore become confounding factors in bioassays. Thus, explanation of anthocyanin bioactivity could be vague (73), and results from different labs are difficult to compare given the different isolation methods employed. Removal of undesirable compounds from anthocyanins is also of great importance in the food colorant and nutraceutical industries. Sugars, phenolic compounds, amino acids, and metals accelerate degradation of anthocyanins and therefore high purity is necessary for improved stability (192). Some potential low-cost sources of anthocyanins are not being commercialized because of co-extracted adverse flavor/aroma or even natural toxins (65). Removal of undesirable odors or flavors can only be achieved with multiple purification steps and tedious manipulations, increasing cost (15, 193).
In this study we explored a low-cost and high-throughput isolation technique for anthocyanin based on a unique property of anthocyanins. Anthocyanin molecules have the unique property that they acquire a positive charge to become flavylium cations at highly acidic pH while losing the positive charge or even obtaining one or two negative charges at basic pH to become ionized quinonoidal bases (Figure 4.1) (30, 33). Such transformation does not occur with most other plant compounds. We hypothesized that the positively charged anthocyanin molecules (pK$_h$ ~2.8) (29) would interact with strong cation-exchange sorbent. Meanwhile most other compounds without positive charges could be easily removed. Dissociation of anthocyanins from the solid phase was carried out by increasing the elution solvent pH. A recently introduced sorbent combining both cation-exchange and reversed-phase interaction mechanisms was employed to develop such a method (194), and evaluated against three established common fractionation methods of anthocyanins.
Figure 4.1 The chemical structure of MCX sorbent and an anthocyanin molecule at varied pH conditions.

4.3. MATERIALS AND METHODS

4.3.1 Reagents and standards

Reagent grade tri-fluoroacetic acid (TFA), certified ACS grade HCl (12 N), HPLC grade methanol, ethyl acetate and acetone for extraction, and HPLC-MS grade water and acetonitrile for chromatography were purchased from Fisher Scientific (Fair Lawn, NJ). Formic acid (99%) was from Acros Organics (Morris Plains, NJ). Cyanidin-3-glucoside standard was from ChromaDex Inc. (Irvine, CA). All other chemicals were certified ACS grade from Fisher Scientific.
4.3.2 Anthocyanin sources and sample preparation

Different anthocyanin-rich fruit and vegetable sources were tested. Fresh blueberry and radish were purchased from a local whole food grocery store. Whole blueberry and peeled radish skin were quickly frozen and fine powdered in a stainless blender in the presence of liquid nitrogen (195). The powder was extracted three times by acidified methanol and centrifuged. The combined supernatant was evaporated in a Büchii rotovaporator at 35 °C and redissolved in 0.1% trifluoroacetic acid (TFA) acidified water. Black raspberry and strawberry as freeze-dried powders were extracted in the same manner. Commercially available crude extracts of red cabbage extract (Food Ingredient Solutions, Llc., Blauvelt, NY) in liquid, as well as bilberry, black currant, chokeberry, elderberry (Artemis International, Inc., Fort Wayne, IN), grape, and purple carrot (Polyphenols, Lab., Sandnes, Norway) in spray-dried powder containing ~10% anthocyanins, were diluted in acidified water. All aqueous solutions were prepared to a concentration of ~1 mM anthocyanin.

4.3.3 Fractionation methods comparison

4.3.3.1 Cation-exchange SPE

Two vol of chokeberry and purple corn crude extracts in aqueous solution were applied to a strong cation-exchange Oasis® MCX SPE cartridge (6cc, 1g sorbent; Waters Corp., Milford, MA). After washing with 2 vol of 0.1% TFA, the other-phenols fraction was collected by elution with 2 vol of methanol (0.1% TFA). Subsequently anthocyanins were eluted with 1 vol of methanol and 1 vol of water/methanol (40:60, v/v), both
containing 1% NH₄OH. The combined alkaline eluate was immediately mixed with an aliquot (250 µL) of formic acid (99%) to lower the pH to < 2. Table 4.1 summarizes the purification conditions used in the present study. For each step, 2 vol of specified eluting solvent was used.

<table>
<thead>
<tr>
<th>Cartridges</th>
<th>Oasis® MCX</th>
<th>Sep-pak® C₁₈</th>
<th>Sephadex® LH-20</th>
<th>Oasis® HLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washing</td>
<td>H₂O</td>
<td>H₂O</td>
<td>20% MeOH</td>
<td>15% MeOH</td>
</tr>
<tr>
<td>Other-phenols fraction</td>
<td>MeOH</td>
<td>EtOAc</td>
<td>N/A b</td>
<td>EtOAc</td>
</tr>
<tr>
<td>Anthocyanin fraction</td>
<td>60%-100% MeOH with 1% NH₄OH c</td>
<td>MeOH</td>
<td>70% MeOH</td>
<td>MeOH</td>
</tr>
<tr>
<td>Other-phenols fraction</td>
<td>N/A</td>
<td>N/A</td>
<td>MeOH and 70% acetone</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 4.1 Mobile phasesa used to elute compounds of interest from the SPE cartridges.

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a: 0.1% TFA was added to all acidified solvents except in the original C₁₈ method where 0.01% HCl was used as described in the literature. TFA as a volatile acid effectively reduced the accumulation of acid after evaporation. In each step, 2 vol of mobile phase was passed through the cartridges.

b: N/A, not available. This fraction was not designed to be collected.

c: This alkaline eluate was immediately mixed with an aliquot of formic acid to bring the pH to <2. Acidification must be prompt to prevent anthocyanin degradation.
4.3.3.2 C\textsubscript{18} and HLB SPE

Two vol of chokeberry and purple corn crude extracts in aqueous solution were loaded onto Sep-pak\textsuperscript{®} C\textsubscript{18} (6cc, 1g sorbent; Waters Corp., Milford, MA) and Oasis\textsuperscript{®} HLB (3cc, 60mg sorbent; Waters Corp., Milford, MA) SPE cartridges. Purification conditions were adapted from established procedures (195-198) and presented in Table 4.1. Purified anthocyanin and other-phenol fractions were obtained for HPLC analysis.

4.3.3.3 LH-20 SPE

The Sephadex\textsuperscript{®} LH-20 (Sigma-Aldrich Corp., St. Louis, MO) sorbent is widely used in open column chromatography, but SPE cartridge with LH-20 sorbent was not commercially available. LH-20 cartridges were made by inserting 1g of LH-20 material in between two polyethylene frits in a polypropylene cartridge (6cc) and then soaking in 20% methanol overnight for activation. The conditions for purification are presented in Table 4.1. Both other-phenols and anthocyanin fractions were obtained for HPLC analysis.

4.3.3.4 HPLC-PDA-MS analysis

All the fractions with 8 duplicates were briefly evaporated in a Büchii rotovaporator at 35°C to remove organic solvent, redissolved in 0.1% TFA acidified water, filtered through 0.45 μm Whatman polypropylene filter, and then analyzed using a Shimadzu LCMS-2010 EV Liquid Chromatograph Mass Spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with a SPD-M20A PDA detector.
and a single quadrupole electron spray ionization (ESI) MS detector. Separation was accomplished on a Symmetry® C18 column (3.5 um, 4.6×150 mm; Waters Corp., Milford, MA). Other chromatographic conditions were as follows: flow rate: 0.8 mL/min; mobile phase: A, 4.5% formic acid in LCMS grade water; B, LCMS grade acetonitrile; gradient: 0-5 min, 2-7% B; 5-20 min, 7-12% B; 20-25 min, 12-17% B; 25-40 min, 17-25% B; 40-45 min, 25-30%; 45-55 min, 30% (the gradient stopped at 40 min if all anthocyanins in the sample were non-acylated); and after each run 5 min was given to equilibrate the column to initial condition; injection vol: 20 μL. Spectral data (250-700 nm) was collected throughout the run. Elution of anthocyanins was monitored at wavelength 510-530 nm. Mass spectra were obtained under positive ion condition using SCAN (from m/z 200 to 1000) and Selective Ion Monitoring (SIM) modes. Six channels including m/z 271 (Pelargonidin), m/z 287 (Cyanidin), m/z 301 (Peonidin), m/z 303 (Delphinidin), m/z 317 (Petunidin) and m/z 331 (Malvidin) were monitored in the SIM mode to detect the 6 common anthocyanin aglycone fragments.

4.3.3.5 Purity and recovery evaluation

Concentrations of anthocyanins and total phenols were represented by area under the curve (AUC) in the 510-530 nm and the 250-700 nm max-plots respectively. Polyphenols usually have absorbance within 260-400 nm range whereas most anthocyanins, including all anthocyanins tested in this study, have max absorbance in the 510-530 nm range (red color). Since the emphasis of the present study was to compare the extent of purity improvement by different methods, converting AUC to absolute concentration is not necessary, and perhaps not possible for all the detectable peaks.
Anthocyanin purity was calculated by dividing the AUC of anthocyanin peaks by the AUC of all peaks in the 250-700 nm max-plot. The current method for computing anthocyanin purity based upon UV-visible chromatogram was adapted from a previous report in which \( \text{AUC}_{280\text{ nm}} \) was used to represent total phenolics (69). It has to be noted that the purities calculated in this study are not the true purities based on weight. Recovery was calculated by ratio of eluted total anthocyanin/initially loaded total anthocyanin.

4.3.3.6 FT-IR

FT-IR spectrometer analysis of anthocyanin fractions for composition profiling were conducted following a protocol previously developed in our lab (199). The acid-base neutralization product ammonium formate interfered with anthocyanin IR signal, and therefore a desalting procedure was necessary. Aqueous MCX anthocyanin fractions were loaded onto a \( C_{18} \) cartridge, washed with water to remove the salt, and recovered with acidified methanol.

4.3.3.7 Sorbent capacity

Sorbent capacity was determined by 2 means, a visual observation of breaking-through volume and instrumental measurement of total anthocyanins (175) recovered from saturated cartridges. Chokeberry extract was used as representative source of anthocyanins to determine sorbent capacity. The mass of total anthocyanins recovered was calculated based on a Cy-3-glu standard calibration curve (\( R^2 > 0.99 \)).
4.3.4 Extended evaluation on a variety of anthocyanin sources

Aqueous solution of bilberry, black currant, blueberry, black raspberry, elderberry, grape, purple carrot, radish, red cabbage and strawberry crude extracts were loaded onto the MCX cartridge or the C<sub>18</sub> cartridge (0.1% TFA) and purified as described in Table 4.1 (4 replications). Purity and recovery were calculated with AUC of HPLC-PDA chromatograms as described above.

4.3.5 Optimization for food application

Crude extract of radish skin was purified by the MCX and C<sub>18</sub> methods (Table 4.1) in the attempt to evaluate the removal of adverse aroma. Purified anthocyanin fractions were rotoevaporated prior to redissolving in water with 0.01% HCl. Due to the adverse aroma of residual ammonium, the MCX method was conducted with 1% Na<sub>2</sub>CO<sub>3</sub> to substitute NH<sub>4</sub>OH in eluting solvents. Anthocyanin fractions along with the initial crude extract were filled into separated glass vials with caps and blindly evaluated for adverse aroma in random order.

4.3.6 Statistical analysis

One-way ANOVA or its non-parametric analogue Kruskal-Wallis Test (equal variance not assumed) was conducted using SPSS (version 13, 2004, SPSS Inc., Chicago, IL) to compare amounts of total and individual anthocyanins in different treatment groups, and data are given as means ± SE. When appropriate, significance of differences between
means was determined by Tukey’s HSD or Dunnett’s T3 (equal variance not assumed). Differences of $P<0.05$ were considered significant.

4.4. RESULTS AND DISCUSSION

4.4.1 MCX method development

A novel means for anthocyanin separation based on cation-exchange mechanism was developed and optimized. Both our preliminary study with an Alltech® SCX cation-exchange SPE cartridge (Fisher Scientific, Fair Lawn, NJ) and reported literature revealed that sorbents relying on solely strong cation-exchange mechanism tend to retain anthocyanins irreversibly and the column capacity was very low ($190, 200$). Here, we employed the MCX cartridge, which was packed with a mixed-mode resin combining both strong cation-exchange mechanism and reversed-phase interaction, a mechanism commonly employed for isolation of anthocyanins (Figure 4.1). This sorbent is a modified divinylbenzene-vinylpyrrolidone copolymer with a hydrogen atom on benzene substituted by a sulfonic group ($201$). The divinylbenzene units provide retention via hydrophobic interaction. The benzene structure forms strong $\pi-\pi$ interaction with the ring structure of anthocyanins as well as other phenolic compounds. To elute phenolics other than anthocyanins, acidified methanol was used. Due to the additional ionic interaction with sulfonic group, positively charged anthocyanin flavylium cations remain adsorbed to the sorbent. To subsequently elute anthocyanins from the sorbent ($194, 201$), $\text{NH}_4\text{OH}$ was added to the mobile phase to raise the pH to above 9.5 and deprotonate anthocyanins.
to quinonoidal base anion or di-anion (30), which repels the negatively charged sulfonic group on the sorbent to dissociate anthocyanins. According to Brouillard and Dubois (28), this pH-dependent structural conversion occurs without delay, therefore equilibration time is not necessary to ensure complete recovery of anthocyanins from the sorbent. Excessive NH4⁺ could also compete with cation binding sites on the resin to facilitate dissociation of flavylium cations remaining in the equilibrium. Eluted alkaline anthocyanin solution was immediately acidified to prevent degradation, as anthocyanins are unstable at high pH (52). Due to the neutralization of alkaline and acid, a trace amount of salt was formed. The residual salt is generally not a concern in chemical and biological studies. However, in situation that salts need to be removed, an additional SPE technique such as C_{18} can be easily employed. We also investigated neutral pH mobile phase for eluting anthocyanins, but elution was much slower and incomplete because the hydration from flavylium cation form to hemiacetal form (Figure 4.1) can take up to 15 min to reach equilibrium (28). It is noteworthy that when acylated anthocyanins are eluted, the alkaline pH (9.5-10) may cause slight saponification, although the eluted solution is immediately acidified. In this situation neutral or slightly alkaline pH is recommended to preserve anthocyanin profile.

4.4.2 Fractionation methods comparison

4.4.2.1 SPE sorbent selection and condition optimization

Sixteen SPE materials were explored by Karemer-Schafhalter et al. (191) for anthocyanin purification, and two sorbents (Amberlite XAD-7 and RP C_{18}) based on reversed-phase interaction mechanism achieved the best separation and recovery.
Currently the often employed SPE purification method for anthocyanins is based on C_{18}, an octadecyl group bonded silica gel (202). Anthocyanins and other-phenols are adsorbed onto the gel by reversed-phase interaction. Water was used to elute hydrophilic compounds including sugar, salt, and simple acids. Acidified methanol was used to elute anthocyanins and other polyphenolic compounds. Oszmianski et al. (196) improved this method by adding an additional cleanup step to remove less polar polyphenolics using ethyl acetate elution. Giusti et al. (195) followed this method and used HCl at low concentration (0.01%) in eluting solvents to prevent acid hydrolysis of anthocyanin, which might occur later when methanol was removed by vacuum evaporation leaving more concentrated acid residue. However, considering the pK_{h} of ~2.8 (29), 0.01% 12N HCl (pH=3) was not sufficient to maintain the majority of anthocyanins as flavylum cations, the most stable form for anthocyanins. Insufficient acidity may not ensure protonation of some low pK_{a} phenolic acids, resulting in incomplete elution of such interfering compounds with non-polar ethyl acetate. We used 0.1% TFA instead of 0.01% HCl to optimize the C_{18} method. The 0.1% TFA aqueous solution provided an acidic environment approximately 1 pH unit below 0.01% HCl. Yet volatility (bp 72.4°C) of TFA prevented increase in concentration during vacuum evaporation (15).

The HLB cartridge explored in this study was packed with a recently developed porous polymeric sorbent sharing the same divinylbenzene-vinylpyrrolidone structure with the MCX, but without the sulfonic group. Such polymers provide stronger reversed-phase interaction and they maintain water wettability better than the C_{18} gel due to the hydrophilic N-vinylpyrrolidone group (191). Also, the polymer structure is more durable
and sustains wider pH range than silica based gel. Therefore HLB was promoted as a replacement of the C\textsubscript{18} gel for polyphenolics purification.

The hydroxypropylated dextran based LH-20 material is a beaded and cross-linked gel often used in open column chromatography (15). It is a size exclusion gel also with polar-polar interaction to adsorb analytes (203). LH-20 has been successfully applied to fractionate polymeric proanthocyanins (197, 204, 205); these compounds are difficult to separate with reversed-phase SPE methods. Aqueous methanol was used to elute anthocyanins and aqueous acetone with its carbonyl oxygen as a strong acceptor for hydrogen bonding was used to subsequently remove polymers (203).

4.4.2.2 SPE methods comparison

The MCX and C\textsubscript{18} cartridges appeared to have unbiased selection on all anthocyanins (Figure 4.2), whereas the HLB and LH-20 cartridges recovered the hydrophilic anthocyanin (cyanidin-3-galactoside and cyaniding-3-glucoside) less efficiently. Visual observation (Table 4.2) revealed leaching of color into the aqueous washing solution in the case of HLB and LH-20 cartridges, explaining the loss of hydrophilic anthocyanins.
**Figure 4.2** Percentage of individual chokeberry anthocyanin peaks after purification.

<table>
<thead>
<tr>
<th>Cartridges</th>
<th>Oasis&lt;sup&gt;®&lt;/sup&gt; MCX</th>
<th>Sep-Pak&lt;sup&gt;®&lt;/sup&gt; C&lt;sub&gt;18&lt;/sub&gt;</th>
<th>Sephadex&lt;sup&gt;®&lt;/sup&gt; LH-20</th>
<th>Oasis&lt;sup&gt;®&lt;/sup&gt; HLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading &amp; washing</td>
<td>–</td>
<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Phenol/tannin fraction</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Residue on sorbent</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

<sup>a</sup>: –, not observable; +, slightly observed; ++, obvious.

**Table 4.2** Visually observed anthocyanin color loss during purification of anthocyanin mixtures by the different SPE procedures.
Visual observation revealed strong retention of anthocyanins (high break-through volume) by the MCX and C\textsubscript{18} sorbents (Table 4.2). Therefore, capacity was further determined for these two sorbents quantitatively (n=3). Presumably due to the additional ionic interaction, MCX had stronger retention of anthocyanins (45.8 mg cyanidin-3-glucoside equivalent/g sorbent) than C\textsubscript{18} (19.0 mg cyanidin-3-glucoside equivalent/g sorbent). This high capacity of MCX resulted in less organic waste per gram of product. Another advantage of this strong retention is that even methanol extracts could be directly loaded and anthocyanins would bind to the MCX sorbent as long as the environment is acidic. All other methods required evaporation of organic solvents, an extra step causing delays and possibly resulting in degradation. Enhanced dissociation of anthocyanins from MCX was observed as compared to the HLB method (Table 4.2). This was probably attributed to the repelling between negatively charged anthocyanin molecule and sulfonic group on sorbent (Figure 4.1).

HPLC-PDA chromatograms clearly show that chokeberry anthocyanins (Figure 4.3 A) purified by all methods (Figure 4.3 B-F) contained less impurities than the crude extract (Figure 4.3 G). The MCX method produced almost pure anthocyanins as in the 250-700 nm max-plot besides the 4 anthocyanin peaks the baseline was flat. C\textsubscript{18} methods were apparently the next best with respect to purification. Quantitative analysis of chokeberry and purple corn purity and recovery data was conducted by Kruskal-Wallis test, a non-parametric analogue of ANOVA, using SPSS (version 13). The unequal variances indicated varied reproducibility of different cartridges (Figure 4.4). The MCX and C\textsubscript{18} cartridges notably generated highly reproducible results. Post-hoc Dunnett’s T3
multiple mean comparisons determined that the MCX method resulted in significantly higher purity (P<0.05) of the anthocyanin fraction (Figure 4.4 A) and significantly lower residue amount anthocyanin (P<0.05) in the other-phenols fraction (Figure 4.4 B) than all other methods. The recovery rates of the MCX method for both anthocyanins (Figure 4.4 C) and other-phenols (Figure 4.4 C) were also the highest or in par with the highest. The other-phenols represent an important class of compounds of interests for research and food applications. Removal of anthocyanins is favored in certain situations, such as to remove color. With the MCX method, the phenolics fraction was recovered essentially free of anthocyanins.
Figure 4.3 HPLC-PDA chromatograms of chokeberry crude extract and purified anthocyanin fractions. Anthocyanins exclusively are observed in the 510-530 nm max-plots, and due to high similarity only one chromatogram is shown here (A). Overall compositions are observed in the 250-700 nm max-plots (B, C, D, E, F and G). Peak identities: 1, cyanidin-3-galactoside; 2, cyanidin-3-glucoside; 3, cyanidin-3-arabinoside; 4, cyanidin-3-xyloside.
Figure 4.4 The purity and recovery of anthocyanins and other-phenols based on the AUC of HPLC-PDA chromatograms. Values are the mean ± SE (n=8). Within the same category means with different letters are significantly different at P<0.05.
Our optimized C\textsubscript{18} method achieved significantly (P<0.05) higher anthocyanin purity from chokeberry and significantly (P<0.05) higher anthocyanin recovery from purple corn than the original C\textsubscript{18} method. As shown in Figure 4.3 C and D, at least one major impurity peak was not completely removed by the original procedure but cleared by the optimized procedure. LH-20 and HLB methods lacked adequate capability to separate anthocyanins from other-phenols (Figure 4.3 A), and the recovery of anthocyanins were also not satisfactory (Figure 4.3 C). In the next experiment testing extended anthocyanin sources, only the MCX and optimized C\textsubscript{18} methods were evaluated.

4.4.2.3 Confirmation of purity improvement with supplementary analytical methods

Purity was quantified based on absorbance in the 250-700 nm range. Phenolic compounds and many other organic compounds belong to this class. However, this method has a limitation in that compounds without absorbance in this UV-visible range are not detectable (16). Therefore, two additional analytical methods were employed to confirm the purity. Total ion concentration recorded by a MS detector showed only anthocyanin peaks for the MCX anthocyanin fraction (Figure 4.5), indicating absence of impurities; yet, multiple noise peaks were present with other isolation methods. Hierarchical cluster analysis (HCA), an unsupervised clustering analysis was carried out on the infrared spectra to determine compositional similarity (199). Anthocyanin fractions (de-salted) with similar compositions were grouped close together. The decreasing similarity from the crude extract to the HLB, LH-20, C\textsubscript{18}, and MCX eluents indicated increasing purity (Figure 4.6).
Figure 4.5 Impurities in chokeberry anthocyanin fractions as indicated by the appearance of noise peaks in the MS chromatogram. The MS was operated at positive mode.
Figure 4.6 HCA dendrograms of chokeberry and purple corn extracts. Compositionally similar samples are clustered together. Similarity of 1 indicates identical composition and 0 indicates the largest difference in the sample set.
4.4.3 Extended evaluation on a variety of anthocyanin sources

Besides the chokeberry and purple corn being elaborated above, we further tested (n=4) other common anthocyanin-rich fruits and vegetables. We successfully purified bilberry, black currant, blueberry, elderberry, purple carrot and red cabbage anthocyanins to greater than 99% purity (Figure 4.7). Black raspberry, strawberry, grape and radish anthocyanin purities were also significantly (P<0.05) improved as compared to the optimized C18 method using Mann-Whitney U test (non-parametric analogue of Student’s t-test). It is reasonable to speculate that our method may also be applicable for many other anthocyanin-rich fruits and vegetables as well. Overall recovery of anthocyanins from all 12 commodities (n=56) tested in this study was compared between the MCX and optimized C18 methods using Student’s t-test. The recovery by the MCX method (93.6±0.55%) was not significantly different (P>0.05) from that by the C18 method (93.8±0.36%), and both were exceptionally satisfactory.

Sequential coupling of more than one type of resin was explored. As mentioned above, anthocyanins present in organic solvents can be directly loaded onto the MCX resin, and this makes it easy to directly load organic eluents from other resins. When black raspberry extract was sequentially purified by C18 and MCX columns, the anthocyanin purity was successfully increased to almost 100%.
Figure 4.7 The purity (based on the AUC of HPLC-PDA chromatograms) of anthocyanins purified from 10 additional commodities. Values are the mean ± SE (n=4). MCX achieved significantly higher (P<0.05) purity in all commodities except purple carrot (P>0.05).
4.4.4 Optimization for food application

Radish extract, a mixture providing stable color and representative hue to synthetic food colorant Red #40, was particularly studied with respect to removal of its strong adverse aroma (173). Our preliminary test determined that the crude extract had high intensity odor whereas the C\textsubscript{18} and MCX purified fractions only had moderate and slight odor, respectively. Higher radish anthocyanin purity (85.6\%) obtained by the MCX method (Figure 4.7) than the C\textsubscript{18} method (47.0\%) correlated well with the removal of odor. We propose that by optimizing the pH of eluting solvents anthocyanins could be even further separated from positively charged interfering compounds. For instance, nitro-containing odorants have positive charge at acidic condition but their pK\textsubscript{a} are higher than the pK\textsubscript{b} anthocyanin flavylium cations. Adjusting the polarity of eluting solvents, for example, using ethyl acetate, acetone and hexane, may also help to remove more non-polar aromatic compounds extracted from radish.

For the purpose of food application, less toxic solvents and chemicals are proposed. We found ethanol to work similarly to methanol. Na\textsubscript{2}CO\textsubscript{3} will be neutralized by citric acid instead of formic acid. The resulted residual amount of sodium citrate is a common ingredient found in foods.

4.5 CONCLUSIONS

An innovative mixed-mode cation-exchange anthocyanin isolation method was successfully developed. This method was superior to the commonly used SPE methods
regarding purity, recovery, sorbent capacity, low cost, simplicity of manipulations, and organic waste generated per gram of product. Therefore it could become a rapid, low cost, and high throughput method to provide high-purity anthocyanins in research labs for minimized interference from other bioactive compounds. A scale-up production based on this new technique may provide the food colorant industry and nutraceutical industry a practical way to separate high quality anthocyanins from fruits and vegetables, and even from industry by-products.

4.6. ACKNOWLEDGEMENTS

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CHAPTER 5

IMPACT OF SMALL INTESTINAL β-GLUCOSIDASE AND DIETARY LACTASE ON ANTHOCYANIN DIGESTION

5.1. ABSTRACT

Anthocyanins are natural pigments with high antioxidant capacity and potential health benefits. Studies suggest that their stability on the GIT may greatly impact their bioactivity. Previous studies have shown selectively degradation of anthocyanin glucosides in animal small intestinal lumen. Such evidence points to the possibility of enzymatic deglycosylation by digestive enzymes. In this study we attempted to evaluate the impact of β-glycosidase that can be present in the small intestine epithelium on anthocyanin stability. Cell-free extract of pig small intestinal mucosa and crude extract of lactase containing foods/supplement were incubated with highly purified anthocyanins at physiological temperature and pH. Selective degradation of anthocyanin glucosides was observed in the presence of small intestinal mucosa cell-free extract, whereas selective degradation of anthocyanin galactosides occurred in the presence of lactase supplement.

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extract. In both cases cyanidin glycosides were hydrolyzed the fastest among all the five common anthocyanidins examined while malvidin glycosides were the most resistant to enzymatic degradation. Kinetic parameters $V_{\text{max}}$ and apparent $K_m$ were determined for both enzyme extracts using their highest affinity substrates, respectively. Our results demonstrate that small intestinal mucosal enzyme and lactase supplement affect anthocyanin stability. Bioactivity and bioavailability studies of anthocyanins should not overlook the influence of enzymatic deglycosylation, which may considerably alter anthocyanin profiles in the GIT.

Key words: anthocyanin; deglycosylation; $\beta$-glucosidase; $\beta$-galactosidase; lactase; stability; biotransformation; small intestine

5.2. INTRODUCTION

Cellular, animal and epidemiological studies have suggested association between anthocyanin intake and chronic diseases prevention. However, the bioactive forms of anthocyanins or anthocyanin metabolites is still in dispute. Studies have repeatedly shown that intact anthocyanins undergo metabolism after absorption (4), but limited information is available on anthocyanin transformation in the GIT lumen before uptake into epithelial cells. It is critical to understand the stability of anthocyanins and possible transformation in the GIT for several reasons. First, the concentration of accessible anthocyanins in the GIT lumen is positively associated with absorption (7). Second, metabolism of anthocyanins in the GIT may produce bioactive phenolic derivatives that
have health benefits (4-7). Third, stable anthocyanins remaining intact in the GIT may exert antioxidant, anti-inflammatory, and chemopreventive activity through direct contact with GIT epithelial cells (8, 13).

Our previous studies on anthocyanins in rat GIT content (13), as well as extensive literature on the metabolism of flavonoids (150), revealed that LPH, a β-glucosidase residing on the brush boarder membrane of small intestine, may hydrolyze anthocyanins containing glucose sugar moiety and result in loss of anthocyanins in the GIT. Further investigation using isolated small intestinal enzyme extract and highly purified anthocyanin substrate is merited to better explain the proposed enzymatic activity towards anthocyanins. In the present study, pig small intestinal mucosa extract was used to examine β-glycosidase activity with various anthocyanins.

Exogeneous enzymes responsible for hydrolysis of β-glycosidic linkage are also likely to affect anthocyanin stability. As the deficiency of LPH causes lactose intolerance (206), many individuals with low LPH activity rely on exogeneous lactases to hydrolyze lactose. Fungal lactase in supplement form is widely used. Dairy foods rich in lactose can be fermented or treated with fungal lactase to substantially reduce lactose content. Here we also investigated the effect of both, the lactase supplement and the residual dietary lactase on cleavage of anthocyanin 3-O-β-glycosidic bonds in the digestive tract.

5.3. MATERIALS AND METHODS
5.3.1 Chemicals and materials

Molecular biology grade dithiothreitol (DTT) was purchased from Promega Corporation (Madison, WI). Protease inhibitor cocktail was from Sigma (St Louis, MI). Reagent grade tri-fluoroacetic acid (TFA), high performance liquid chromatography (HPLC) grade acetone and methanol for extraction, and HPLC-mass spectrometer (MS) grade water and acetonitrile for chromatography were purchased from Fisher Scientific (Fair Lawn, NJ). HEPES buffer (>99%), D-gluconolactone (>99%) and formic acid (99%) were from Acros Organics (Morris Plains, NJ). Cyanidin (Cy) aglycone was prepared from Cy-3-glu standard (ChromaDex Inc., Irvine, CA) by acid hydrolysis (19). Protocatechuic acid standard was from MP Biomedicals (Solon, OH). α-D-lactose monohydrate and all other chemicals were ACS grade from Fisher Scientific. The human colon cancer cell line HT-29 was purchased from the American Type Culture Collection (Rockville, MD).

5.3.2 Purification of anthocyanins

Blueberry and grape anthocyanins were selected as substrates in the present study because of their representativeness. Blueberry contains 5 common anthocyanidins each linked to 3 common glycosides. Grape contains 5 common anthocyanidins each linked to a glucoside. Highbush blueberry and crimson seedless red grape were purchased from a local whole food grocery store. Blueberry or red grape (28 g, equivalent to 1/5 serving) was crushed by a spatula and then mixed with 20 mL of methanol (0.1% TFA). The mixture was then briefly homogenized using a handheld tissuemiser (Fisher Scientific,
Fair Lawn, NJ) briefly followed by sonication in a Fisher FS30 ultrasonic bath for 20 min. The slurry was then centrifuged at 1800×g for 10 min and the supernatant was collected. The pellet was re-extracted three times with 25 mL of methanol (0.1% TFA) for further removal of pigmentation from the matrix. The pooled supernatant was directly applied onto a strong cation-exchange Oasis® MCX SPE cartridge (6cc, 1g sorbent; Waters Corp., Milford, MA) and subsequently purified according to the procedure described in Chapter 4. Briefly, the cartridge was washed with 2 vol of DD water and methanol, both containing 0.1% TFA, and anthocyanins were eluted by 2 vol of aqueous methanol containing 1% NH₄OH. The alkaline eluent was immediately mixed with an aliquot of formic acid to decrease pH to acidic region. This aqueous methanol mixture containing highly purified anthocyanins, salts and residual formic acid was then diluted with water to decrease methanol concentration to <10%, and applied to a Sep-pak® C₁₈ cartridge (20cc, 5g sorbent; Waters Corp., Milford, MA) for further removal of salts. After washing with 2 vol of DD water (0.01% HCl), the anthocyanins were eluted with 1 vol of methanol (0.01% HCl), evaporated in a Büchii rotovaporator at 35°C to almost dryness, and then diluted to 20 mL with DD water. As such, 1mL of the extract contained anthocyanins equivalent to 1/100 serving of the corresponding fruit.

Black raspberry was studied to compare with the in vivo results obtained in Chapter 3. Lyophilized black raspberry powder was a gift from Dr. Laura Kresty, OSU Comprehensive Cancer Center. Acetone/water extraction of the powder has been described in Chapter 3. An aliquot of the extract containing 25 mg of total anthocyanins as determined by pH differential method (175) was further purified using MCX and C₁₈
cartridges with the same procedure described above. Eventually the black raspberry anthocyanins were dissolved in 25mL of DD water.

Chokeberry extract was selected for lactase treatment and further evaluation in cell culture because of its high Cy-3-gal content, a discovered substrate for dietary lactase. Commercially available chokeberry extract powder (containing 10% anthocyanin) was donated by Artemis International, Inc. (Fort Wayne, IN). Following the same MCX/C<sub>18</sub> purification procedure above, 540 mg of chokeberry powder dissolved in acidified water was purified and eventually redissolved in 10 mL of DD water.

5.3.3 β-glucosidase activity in porcine small intestinal mucosa

5.3.3.1 Preparation of cell-free extract

Fresh mid-jejunum samples from two 8-month old pigs were generous gifts from Dr Henry N. Zerby, OSU Department of Animal Sciences. The samples were opened longitudinally on an ice-cold glass plate and washed with ice-cold Phosphate Buffered Saline (PBS, 50mM, pH 7.0). The mucosa layer was scraped using glass slides and transferred to a tube containing 10 mL of ice-cold HEPES buffer (25 mM HEPES buffer, pH 7.0, containing 154 mM NaCl, 1 mM EDTA, 5 mM DTT, and a 400 fold diluted protease inhibitor cocktail). Mucosal cells were disrupted using a handheld glass homogenizer (15 mL, up and down 15 ×) followed by further homogenization with a tissuemizer for 10 s, and then diluted to 25mL with ice-cold HEPES buffer. Aliquots (1 mL) of the crude extract was transferred and distributed into 5 micro-centrifuge tubes. The remainder was centrifuged at 10,000×g, 4°C for 40 min, and the resultant as cell-free
supernatant was aliquoted into 12 micro- centrifuge tubes (1 mL each), pellets saved. Samples were frozen at –80°C until enzymatic assays. Frozen samples were thawed in cold water bath and inverted a few times to mix before use.

5.3.3.2 Protein assay

The protein contents of crude enzyme extract and cell-free extract were measured in triplicate using a Bio-Rad protein assay kit (Hercules, CA) following the recommended procedures. Bovine serum albumin (BSA) was used as protein standard.

5.3.3.3 Reference velocity

One hundred µL of the cell-free supernatant prepared from homogenized mucosal epithelium was diluted in 1 mL of PBS (50 mM, pH 6.5) containing 584 mM lactose (close to saturation concentration). Initial rate of lactase hydrolysis was measured based on the velocity of glucose released. Samples in micro-centrifuge tubes were incubated at 37°C in water bath. After 45min, the tubes were immerged in boiling water bath for 2.5 min to stop the reaction, followed by centrifugation at 16,000×g for 5 min (4°C). Aliquots (0.5 mL) of the supernatant were analyzed using a GAGO-20 glucose kit (Sigma, St Louis, MI) following instructed procedures. The reactions were conducted in triplicate.

5.3.3.4 Confirmation of enzymatic activity

One micro-centrifuge tube of thawed cell-free supernatant was heated in boiling water for 2.5 min to inactivate enzymes prior to centrifugation (16,000×g for 5 min at 4°C) for removing protein precipitate. Heat-inactivated and non-heated enzyme extracts
(300 µL) were added to polystyrene tubes (15 mL size) containing 10 µL of purified black raspberry anthocyanins (2.10 mM) and 2.19 mL of PBS (50mM, pH 6.5) alone or PBS with LPH inhibitors (584 mM α-D-lactose or 0.1M D-gluconolactone) (Table 5.1). These compounds were used to confirmed that hydrolysis of anthocyanins to the aglycone was due to LPH activity. D-gluconolactone is a known potent inhibitor of the lactase active site of LPH (147), whereas α-D-lactose is a substrate of LPH.

<table>
<thead>
<tr>
<th></th>
<th>Black raspberry anthocyanins (µL)</th>
<th>Heat-inactivated extract (µL)</th>
<th>Active enzyme extract (µL)</th>
<th>Buffer pH 6.5 (2.190 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>300</td>
<td>—</td>
<td>PBS</td>
</tr>
<tr>
<td>Active enzyme</td>
<td>10</td>
<td>—</td>
<td>300</td>
<td>PBS</td>
</tr>
<tr>
<td>Competitive inhibition</td>
<td>10</td>
<td>—</td>
<td>300</td>
<td>200 g/L α-D-lactose in PBS</td>
</tr>
<tr>
<td>Complete inhibition</td>
<td>10</td>
<td>—</td>
<td>300</td>
<td>0.1 M D-gluconolactone in PBS</td>
</tr>
</tbody>
</table>

Table 5.1 Composition of mixtures used to test the effect of pig small intestinal enzymes on black raspberry anthocyanins.

Triplicate samples of each mixture were incubated in a water bath at 37°C avoiding light. After 0, 15, 30, 60, and 90 min, the tubes were gently mixed and an aliquot (0.5 mL) was removed from each tube and immediately mixed with 1 mL of ice-cold 10% formic acid to terminate enzymatic reaction. Samples were then centrifuged at 16,000×g for 5 min (4°C) and the supernatants were directly analyzed by HPLC.
5.3.3.5 Determination of apparent $K_m$ and $V_{max}$

Tubes containing 100 µL of cell-free supernatant and 390 µL of PBS (50 mM pH 6.5) were pre-equilibrated in water bath to 37°C. Various concentrations (50-2000 mg/L) of Cy-3-glu (10 µL) were added into each tube, mixed, and placed in 37°C water bath. After designated times, 1 mL of 10% formic acid was added to terminate the reaction and samples were centrifuged at 16,000×g for 5 min (4°C). Supernatants were directly analyzed by HPLC. The experiment was repeated three times.

5.3.3.6 Substrate specificity

An assay was carried out on purified grape anthocyanins to determine substrate specificity. Five µL of the purified grape anthocyanins (1.37 mM) were mixed with 0.5 mL of the cell-free supernatant and 0.995 mL of PBS (50mM, pH 6.5) and incubated in water bathed at 37°C avoiding light. The assay was also conducted in parallel with 0.1M D-gluconolactone in PBS. Aliquots (0.5 mL) were removed from each tube after 0, 30, and 60 min, and mixed with 1mL of ice-cold 10% formic acid to terminate enzymatic activity. Samples were centrifuged at 16,000×g for 5 min (4°C) and the supernatants were directly analyzed by HPLC. The assay was conducted with duplicate samples.

5.3.4 Lactase from foods

5.3.4.1 Extraction of crude enzyme

An aliquot (1/10 serving) of either a lactase supplement containing 9000 FCC lactase units/serving, yogurt with live-culture (22.5 g), or lactase treated skim milk (24
mL) was mixed with PBS (50mM, pH 6.5) to ~70 mL followed by hand-shaking the containers for 1 min. The mixtures were centrifuged at 10,000×g for 40 min (4°C) and the supernatants were diluted to 100 mL with PBS. As such, 1 mL aliquots represented 1/1000 serving of the lactase supplement, yogurt or milk. All samples were either used fresh or frozen in separate tubes at –18°C. Prior to enzymatic assay, aliquots of the frozen extract were thawed in cold water bath and inverted several times to mix.

5.3.4.2 Confirmation of enzymatic activity

One micro-centrifuge tube of thawed lactase supplement extract was heated in boiling water for 2.5 min to inactivate the enzymes prior to centrifugation at 16,000×g for 5 min (4°C) to remove protein precipitate. The heat-inactivated and non-heated extracts (1 mL) were subsequently mixed in polystyrene tubes (15 mL size) with 100 µL of purified blueberry anthocyanins (2.52 mM) and 1.1 mL of PBS (50mM, pH 6.5) with or without α-D-lactose, a known substrate of lastase (Table 5.2). The mixtures were incubated in a water bath at 37°C avoiding light in triplicate. An aliquot (0.5 mL) was removed from each tube at 15 min and added to 1 mL of ice-cold 10% formic acid to terminate enzymatic activity. Samples were centrifuged (16,000×g for 5 min at 4°C) and the supernatants were analyzed by HPLC.
Table 5.2 Compositions of buffered solutions used to investigate the effect of lactase supplement on blueberry anthocyanins.

<table>
<thead>
<tr>
<th></th>
<th>Blueberry anthocyanins (µL)</th>
<th>Heat-inactivated extract (mL)</th>
<th>Active enzyme extract (mL)</th>
<th>Buffer pH 6.5 (1.1 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>1</td>
<td>—</td>
<td>PBS</td>
</tr>
<tr>
<td>Active enzyme</td>
<td>100</td>
<td>—</td>
<td>1</td>
<td>PBS</td>
</tr>
<tr>
<td>Competitive inhibition</td>
<td>100</td>
<td>—</td>
<td>1</td>
<td>200 g/L α-D-lactose in PBS</td>
</tr>
</tbody>
</table>

5.3.4.3 Substrate specificity assays

Five hundred µL of the purified blueberry anthocyanins (equivalent to 1/200 serving) were mixed with 5 mL (equivalent to 1/200 serving) of the thawed lactase supplement extract or freshly prepared yogurt and milk extract and incubated at 37°C avoiding light. An aliquot (0.5 mL) was removed from each tube after 1, 5, 10, 20, 30, 60, 90, and 150 min, and immediately mixed with 1 mL of ice-cold 10% formic acid to terminate enzymatic activity. Samples were then centrifuged (16,000×g for 5 min at 4°C) and the supernatants were directly analyzed by HPLC. The assays were repeated in triplicate.

5.3.4.4 Determination of apparent $K_m$ and $V_{max}$

Six micro-centrifuge tubes each containing 10 µL of freshly prepared lactase supplement extract and 390 µL of PBS (50 mM pH 6.5) were pre-equilibrated in water
bath to 37°C. A series dilution of purified chokeberry anthocyanins (100 µL) were pipetted into each tube to give a range of 38.4 – 570.3 mg/L of cy-3-gal concentrations as determined by HPLC. Samples were incubated in 37°C water bath for 3 min prior to addition of 1 mL of 10% formic acid to terminate enzymatic reaction. Samples were centrifuged (16,000×g for 5 min at 4°C) and the supernatants were directly analyzed by HPLC. The experiment was repeated in triplicate.

5.3.4.5 Anti-proliferative effect on colon cancer cell line

Two mL of purified chokeberry extract (~10 mg anthocyanins) were mixed with 10mL of freshly prepared lactase supplement extract (treatment) or 50mM, pH 6.5 PBS (control). The mixed solutions were incubated in water bath at 37°C avoiding light for 45 min. An aliquot (0.5 mL) was removed from each solution and immediately mixed with 1mL of ice-cold 10% formic acid to be analyzed by HPLC. The rest of the solutions were cooled in ice-water bath and frozen at -80°C until being used for cell culture.

HT-29 cells were cultured in McCoy’s 5A medium (GIBCO Laboratories, Gran Island, NY) supplemented with 10% fetal bovine serum (FBS) and incubated in humidified atmosphere (5% CO₂, 95% O₂) at 37°C. Purified chokeberry was dissolved in medium to give a final concentration of 100 µg/mL and the enzyme treated chokeberry extract was used at equivalent volume. Two fold dilutions were made. Negative control wells contained cells in medium without anthocyanins while blank wells contained only media.
HT-29 cells were plated in 24-well cell microtiter plates at a density of 3×10^4 cells/mL. Cells were incubated for 24 h in culture medium. Growth medium was aspirated and cells were treated for 48 h with the different test media. After incubation, growth inhibition was measured using a modified Sulforhodamine B (SRB) assay. Briefly, test media was removed and cells were fixed by adding 500μL of 10% trichloroacetic acid (TCA) and plates were incubated for 1 h at 4°C. After that, plates were washed with water and air-dried. 500μL of 0.4% SRB solution was applied to each well and plates were incubated for 30 min. Plates were washed using 1% acetic acid and allowed to dry. Bound SRB was solubilized by adding 1mL of 10mM Tris Base Solution. Optical density (OD) was read at 565nm. The cell culture experiment was repeated in duplicate.

Growth inhibition was calculated as follow:

$$\text{Cell Growth Inhibition}\% = 100 - \left( \frac{OD_{\text{sample}} - OD_{\text{day 0}}}{OD_{\text{negative control}} - OD_{\text{day 0}}} \times 100 \right)$$

5.3.5 HPLC-MS analysis of anthocyanins

Samples were analyzed using a Shimadzu LCMS-2010 EV HPLC-MS (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with an SPD-M20A photodiode array (PDA) detector and a single quadrupole electron spray ionization (ESI) MS detector. Separation was accomplished on a Symmetry® C18 column (3.5 μm, 4.6×150 mm; Waters Corp., Milford, MA) with a flow rate of 0.8 mL/min. Mobile phase conditions were as follows: A, 4.5% formic acid in HPLC-MS grade water; B, HPLC-MS grade acetonitrile; gradient: 0-5 min, 2-7% B; 5-20 min, 7-12% B; 20-25 min, 12-17% B; 25-40 min, 17-
25% B. After each run, the column was equilibrated for 5 min under the initial condition. Spectral data (250-700 nm) were collected during the entire separation procedure. When MS was coupled to the HPLC for identification, spectra were obtained under positive ion condition using total ion scan (from m/z 200 to 1200) and selective ion monitoring (SIM) modes. Six channels including m/z 287 for Cy and m/z ratios for other common anthocyanin aglycones were monitored in the SIM mode. Anthocyanin concentrations were calculated using areas under curve (AUC) of HPLC chromatograms at 520 nm and a standard calibration curve.

5.3.6 Calibration curve

Commercially available Cy-3-glu standard (10 mg) was dissolved in 10 mL of double distilled water containing 0.1% TFA, and a series of dilutions were prepared to generate a standard curve ($R^2 > 0.99$). All anthocyanins analyzed fell within the range of the standard curve and were expressed as Cy-3-glu equivalents by weight. The amount of anthocyanins was calculated using the AUC of individual anthocyanin peaks at 520 nm and this calibration curve.

5.3.7 Statistical analysis

One-way ANOVA was conducted using SPSS (version 13, 2004) to compare amounts of individual anthocyanins at different time points, and data are given as means ± SE. When appropriate, significance of differences between means was determined by Tukey's HSD. Differences of $P<0.05$ were considered significant. Kinetic parameters $K_m$ and $V_{max}$ were calculated using SigmaPlot (version 10, 2006).
5.4. RESULTS AND DISCUSSION

5.4.1 Lactase activity of pig small intestinal mucosa extract

Homogenate prepared from porcine small intestinal mucosa contained 4.97 ± 0.06 g protein/L, while the cell-free extract contained 4.53 ± 0.12 g protein/L. Therefore, the majority of the protein was present in the supernatant but not in the pellet. This is not surprising because centrifugation of homogenized cells at 10,000×g for 40 min was expected to remove nucleus and mitochondria, leaving most of the cytosolic and membrane bound proteins in the aqueous supernatant. Similarly, enzymatic assay using lactose as substrate revealed 22.7 ± 0.15 nmol/min·mg protein lactase activity in the crude extract and 24.0 ± 0.08 nmol/min·mL activity in the cell-free extract. Thus, the supernatant maintained the majority of enzymatic activity in the crude extract. We observed that repeated freezing and thawing significantly lower enzymatic activity. Thus, each thawed aliquot of enzyme extract was discarded after single use. It is noteworthy that the β-glucosidase activity measured in the present study may be underestimated because samples were analyzed after thawing.

5.4.2 β-glucosidase activity on anthocyanins

Stability of black raspberry anthocyanins in rat small intestine was examined and selective degradation of Cy-3-glu was observed (Chapter 3). LPH residing on the brush boarder of small intestine was proposed to be responsible for the selective hydrolysis of Cy-3-glu. In the present study, cell-free extract of isolated pig small intestinal mucosa layer was investigated with highly purified black raspberry anthocyanins as substrates.
Purified anthocyanins eliminate the possibility of interference from numerous other compounds present in the plant extracts, and therefore improved the reliability of the enzymatic assay. For the four major anthocyanins present in black raspberry (Cy-3-sam, Cy-3-glu, Cy-3-(xyl)-rut, and Cy-3-rut), only Cy-3-glu was significantly (P<0.05) degraded (by 55.3% in 90 min) by the cell-free extract (Figure 5.1 A). This degradation was attributed to enzymatic activity rather than chemical instability of Cy-3-glu, as the amount of Cy-3-glu was not affected during incubation with heat-inactivated cell-free extract (Figure 5.1 B). To confirm that the enzymatic activity was due to β-glucosidase, a membrane bound β-glucosidase inhibitor D-gluconolactone was used. Addition of 0.1 M D-gluconolactone in the PBS buffer completely ceased selective degradation (P>0.05) of Cy-3-glu (Figure 5.1 C), confirming that β-glucosidase was the catalytic entity for the hydrolysis.

Similarly, some flavonoid glucosides could be substrates for β-glucosidases present in the small intestine cell-free extract (150), while the other three types of glycosides were not substrates for these enzymes. It is noteworthy that Cy-3-sam, Cy-3-(xyl)-rut and Cy-3-rut all have a glucose moiety on the C-3 position of aglycone, but other molecules linked to the glucose moiety likely prevente approximate binding to the active site of β-glucosidase. Nemeth et al. (181) purified human small intestinal β-glucosidases and determined that in the epithelial cell the membrane bound LPH was responsible for the observed β-glucosidase activity towards flavonoid glucosides. Although no attempt was made to identify the specific enzyme responsible for anthocyanin hydrolysis in the current study, we speculated that LPH was responsible for...
deglucosylation of Cy-3-glu. Lactose, a common substrate for LPH, was investigated at nearly saturated concentration for competitive inhibition on Cy-3-glu hydrolysis. Slight but significant (P<0.05) decrease (16.3%) of Cy-3-glu was observed in 90 min (Figure 5.1 D). Clearly lactose competed with Cy-3-glu for the active site of β-glucosidase. However, caution must be taken in that part of the β-glucosidase activity might come from luminal microbial β-glucosidase adhering to the mucosal layer and cytosolic β-glucosidase present in mucosal cells.

In all assays, anthocyanin deglycosylation was accompanied by the appearance of anthocyanidins. However, the concentrations of the anthocyanidins were dependent on the delay between incubation and HPLC analysis due to the labile characteristic of anthocyanidins. As a result quantification of the anthocyanidins was not attempted.
Figure 5.1 Degradation of purified black raspberry anthocyanins during incubation with pig small intestinal cell-free extract in the presence and absence of β-glucosidase inhibitors (n = 3). The initial amount of each anthocyanin was considered as 100%.
5.4.3 Calculation of apparent $K_m$ and $V_{\text{max}}$ for intestinal $\beta$-glucosidase

$\beta$-glucosidase mediated degradation of Cy-3-glu with Michaelis-Menten kinetics (Figure 5.2). Apparent $K_m$ and maximum velocity ($V_{\text{max}}$) were calculated from the substrate saturation curve for comparison with values reported in literature (Table 5.3). $K_m$ is equivalent to the substrate concentration that reaches half velocity of $V_{\text{max}}$, and smaller $K_m$ represents higher affinity of enzyme towards its substrate. As summarized in Table 5.3, Cy-3-glu has comparable apparent $K_m$ with structurally similar flavonoid glucosides, yet much lower than lactose. Therefore, Cy-3-glu is expected to be the preferred substrate for the small intestinal epithelial $\beta$-glucosidases in the presence of lactose.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$ (µmol/min·g protein)</th>
<th>Apparent $K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy-3-glu</td>
<td>1.21 ± 0.14</td>
<td>29.3 ± 9.0</td>
</tr>
<tr>
<td>Quercetin-4’-glu (147)</td>
<td>—</td>
<td>37 ± 12</td>
</tr>
<tr>
<td>Genistein-7-glu (147)</td>
<td>—</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>Lactose (206)</td>
<td>23.96</td>
<td>8800 ± 300</td>
</tr>
</tbody>
</table>

Table 5.3 Kinetic constants for the hydrolysis of LPH substrates.
Figure 5.2 Saturation curve for the small intestinal mucosal β-glucosidase showing the relationship between Cy-3-glu substrate concentration and rate of Cy-3-glu hydrolysis. Markers with error bars represent means ± SE (n = 3).
The concentration of Cy-3-glu required in the reaction to reach half of \( V_{\text{max}} \) was 29.3 \( \mu \text{M} \), which can be easily exceeded by a single serving of red grapes (44). Based on the \( V_{\text{max}} \) and the total mucosal protein extracted from the segment (~20 cm in length) of weaned pig small intestine, we estimate that in 30 min approximately 0.5-1 mg of Cy-3-glu could be hydrolyzed within this segment. Although the estimated efficiency of anthocyanin glucoside hydrolysis may appear to be insignificant considering an average daily consumption of 12.5 mg anthocyanins, the impact of this enzymatic reaction should not be underestimated. As discussed above, the cell-free extract was thawed and had lost a significant part of its enzymatic activity. Therefore we probably underestimated the maximum rate of degradation. Also, tissue was obtained from 8-month old pigs. Pigs at this age are not expected to express LPH at high level. Pre-weaned animals and humans express high level of LPH to digest lactose in milk (150). Hence, the degradation of anthocyanins is expected to be much greater for newly weaned populations. Some populations, particularly individuals of Northern European origin, maintain high level of LPH throughout adult life (182). In those people perhaps metabolism of anthocyanin glucosides differs greatly with normal subjects. High LPH activity may be induced by diet (182). For example, milk-drinking population have higher LPH activity than non-milk-drinking population, and high \( \alpha \)-saccharides diet effectively elevates LPH in rats (207). In our previous study, rats after weaning were fed starch-rich diet, thus little anthocyanin glucosides remained in the GIT segments below the small intestine, causing over 50% loss of total anthocyanins (13).
5.4.4 Substrate specificity of small intestinal β-glucosidase

The impact of anthocyanin structure on the extent of enzymatic hydrolysis was evaluated. A substrate specificity assay was performed with purified grape anthocyanins (Figure 5.3 A). Grape contains 5 of the most common anthocyanidins conjugated to glucose at C-3 position (Table 5.4). The identities of grape anthocyanins were determined by comparing retention times and UV-visible spectra to known standards, and by comparing m/z ratios of whole molecular ions and fragments to established values (208). The profile of grape anthocyanins was not altered when incubated with cell free extract in the presence of D-gluconolactone (Figure 5.3 B). Incubation with LPH alone for 30 min resulted in loss of 38.1% total anthocyanins (Figure 5.3 C, Table 5.4) and 20-50% loss of Cy-3-glu, Pn-3-glu and Dp-3-glu, while Pt-3-glu and Mv-3-glu were quite stable. Further incubation to 60 min resulted in 19% loss of Pt-3-glu, while Mv-3-glu remained almost unchanged. Such substrate specificity may be explained by the B ring structure of the anthocyanidins (Figure 5.4). Mv is the only anthocyanidin that has a methoxyl group on the 5’ position of B ring. Due to the proximity between the 5’ position and the sugar moiety on the C-3 position, the bulk sized hydrophobic methoxyl group may limit binding to the enzyme and consequently prevent hydrolysis of Mv-3-glu.
Figure 5.3 HPLC chromatograms of purified grape anthocyanins incubated with LPH for 0 min (A) and 30 min in the presence (B) or absence of D-gluconolactone (C). Peak identities are listed in Table 5.4.
<table>
<thead>
<tr>
<th>Peak#</th>
<th>Peak identity</th>
<th>m/z</th>
<th>AUC&lt;sub&gt;treatment/AUC&lt;sub&gt;control% after 30 min incubation (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Intact molecule</td>
<td>Aglycone fragment</td>
</tr>
<tr>
<td>1</td>
<td>Dp-3-glu</td>
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<td>303</td>
</tr>
<tr>
<td>2</td>
<td>Cy-3-glu</td>
<td>449</td>
<td>287</td>
</tr>
<tr>
<td>3</td>
<td>Pt-3-glu</td>
<td>479</td>
<td>317</td>
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<tr>
<td>4</td>
<td>Pn-3-glu</td>
<td>463</td>
<td>301</td>
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<tr>
<td>5</td>
<td>Mv-3-glu</td>
<td>493</td>
<td>331</td>
</tr>
<tr>
<td>6</td>
<td>Pn-3-((p-coumaryl)-glu</td>
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<td>301</td>
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<tr>
<td>7</td>
<td>Mv-3-((p-coumaryl)-glu</td>
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<td>331</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
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</tr>
</tbody>
</table>

**Table 5.4** Profile change of grape anthocyanins after incubation with LPH for 30 min. D-gluconolactone was added to the buffer as negative control.
Figure 5.4 Chemical structures of the common anthocyanidins glycosylated on the C-3 position.
In this assay Cy-3-glu and Pn-3-glu were hydrolyzed at the highest velocity. The absence of a bulky group on the 5’ position of the B ring (Figure 5.4) is speculated to give advantage to Cy-3-glu and Pn-3-glu for accessing the binding site of β-glucosidase. Our hypothesis is also supported by the data published by Nemeth et al. (181): purified sheep LPH hydrolyzed kaempferol-3-glu 16% faster than quercetin-3-glu. The only difference between these two substrates is that kaempferol-3-glu has one less hydroxyl group on the B ring.

5.4.5 Activity of lactase supplement on anthocyanins

The effect of lactase from the supplement on blueberry anthocyanins was investigated. Because lactose is a disaccharide composed of glucose and galactose, both β-glucosidase and β-galactosidase may hydrolyze lactose. Unlike LPH, which is a β-glucosidase, fungal lactase selectively hydrolyzed anthocyanin galactosides (Figure 5.5), whereas other glycosides were intact. In the presence of lactose, hydrolysis of anthocyanin galactosides was reduced due to competitive inhibition (Figure 5.5 and 5.6).

5.4.6 The influence of lactase residual in foods on anthocyanins

Because of the concern about anthocyanin degradation under the effect of food source lactase, yogurt with live-culture (as an example of fermented foods) and lactase treated skim milk were also examined for lactase activity towards blueberry anthocyanins. However, neither of the crude enzyme extracts (at consumable levels) demonstrated significant enzymatic activity on purified blueberry anthocyanins after 30 min of incubation (data not shown). Lactase activity assay using a GAGO-20 glucose kit
revealed that yogurt with live-culture (one serving) had about 1/40 of the activity as lactase supplement (one serving) using lactose as substrate. We speculate that due to the low enzymatic activity and rich sugar content co-extracted with lactase, anthocyanin hydrolysis was competitively inhibited thus no significant degradation was observed. Enzymatic activity of the milk extract towards lactose was not detected. This suggested that the processing of lactase treated milk might not leave active lactase residual that could affect anthocyanin stability when ingested together.
Figure 5.5 HPLC chromatograms of purified blueberry anthocyanins incubated with either heat-inactivated lactase from supplement (A), active lactase from supplement extract (B), or lactase from supplement in the presence of 584 mM lactose (C) for 15 min.
Figure 5.6 Profile change of anthocyanin galactosides in blueberry after incubation with the lactase supplement extract for 15 min (n = 3).
5.4.7 Substrate specificity of lactase from supplement

Lactase substrate specificity assay was carried out on purified blueberry anthocyanins. Similar to the observations with intestinal \( \beta \)-glucosidase, Mv-3-gal was the most resistant to hydrolysis among all the five anthocyanin-3-gly (Figure 5.7). Such an effect was again attributed to the steric effect of the methoxyl group on 5’ position of B ring.

5.4.8 Calculation of apparent \( K_m \) and \( V_{\text{max}} \) for dietary lactase

For kinetic parameters determination, lactase supplement (9000 FCC lactase units/serving) was extracted prior to assay without being frozen. Purified chokeberry extract containing 70% Cy-3-gal served as source of substrate. The reaction exhibited Michaelis-Menten kinetics (Figure 5.8). The \( V_{\text{max}} \) was 2.38 ± 0.14 mmol/min·serving and the apparent \( K_m \) was 414 ± 58 µM. It is noteworthy that the apparent \( K_m \) is about 14 times of that for intestinal \( \beta \)-glucosidase. Thus, fungal lactase from the supplement has much lower affinity towards Cy-3-gal as compared to the affinity between \( \beta \)-glucosidase and Cy-3-glu. However, owing to the high level of lactase contained in one serving of the supplement, hydrolysis of anthocyanin galactosides was extremely rapid. Based on the calculated \( V_{\text{max}} \) we estimate that 1 g of Cy-3-gal can be hydrolyzed in 2 min giving the half maximum velocity. Therefore it appears that any person who takes lactase supplement regularly may benefit less from the intact anthocyanins that he/she consumes.
Figure 5.7 Lactase supplement extract substrate specificity on the 5 anthocyanin galactosides in blueberry.
Figure 5.8 Saturation curve for the lactase supplement extract showing the relationship between Cy-3-gal substrate concentration and rate of Cy-3-gal hydrolysis. Markers with error bars represent means ± SE (n = 3).

5.4.9 Anti-proliferative effect on colon cancer cell line

After incubation with dietary lactase for 45 min most Cy-3-gal in purified chokeberry extract disappeared, Cy-3-arab was lost by 46%, while Cy-3-glu and Cy-3-xyl were in comparable levels with that in the control (incubated with PBS buffer). It appears that dietary lactase had slight enzymatic activity on Cy-3-arab but such activity
was suppressed when Cy-3-gal was abundant. Overall about 80% of the chokeberry anthocyanins were hydrolyzed at the end of incubation (Figure 5.9). Large amount of Cy aglycone was produced as degradation intermediate but a significant portion of it rapidly disappeared.

Figure 5.9 Intact chokeberry anthocyanins remaining after incubation with buffer or dietary lactase extract for 45 min (n=3).
The chokeberry anthocyanins (control) inhibited HT-29 colon cancer cell growth by 17% at 100µg/mL concentration in medium, whereas at equivalent concentration (equal initial anthocyanin concentration before lactase treatment) the dietary lactase treated chokeberry anthocyanins only inhibited cell growth by 10% (Figure 5.10). At diluted concentrations the lactase treated anthocyanins also demonstrated lower anti-proliferative effect. Obviously the exposure to lactase decreased the total amount of chokeberry anthocyanins and as a result reduced their potency of colon cancer prevention. The mechanism of anthocyanin’s chemoprevention mechanism in HT-29 cells had been researched before (95). The cells could be blocked at G1/G0 and G2/M phases of the cell cycle when exposed to anthocyanins in the medium, but upon removal of anthocyanins from the medium the cells could recover from both the G1/G0 and G2/M blocks. Therefore both, the concentration of anthocyanins remaining in the colon and the duration of exposure to the colon epithelial cells could be important factors in anthocyanin’s health benefits. According to our present study, anthocyanins with specific chemical structures such as Mv aglyone and non-glucoside/galactoside sugar moieties may be more resistant to enzymatic hydrolysis in the GIT than other anthocyanins and consequently have a better chance to protect the GIT tissues from chronic diseases including colon cancer.
Figure 5.10 Growth inhibition (48 h) of human colon cancer cell line HT-29 by purified chokeberry anthocyanins and lactase treated chokeberry anthocyanins. Markers represent means of 4 replicates. The experiment was repeated twice independently and data from one experiment is shown.
5.5. CONCLUSIONS

Deglycosylation of various anthocyanins by pig small intestinal mucosal extract and lactase supplement extract was observed in vitro. The intestinal mucosal extract specifically hydrolyzed anthocyanin glucosides while the lactase supplement extract specifically decomposed anthocyanin galactosides. The type of aglycone also influenced the velocity of enzymatic hydrolysis with Mv being the slowest and Cy being the fastest. Deglycosylation of anthocyanins under the effect of endogenous and exogenous β-glycosidases may significantly affect the accessibility and health-promoting effects of intact anthocyanin in the digestive tract. The large variations of β-glucosidase activity among individuals, as well as the ingestion of exogenous lactase are likely to influence the ability of individuals to metabolize anthocyanins. Further investigation of the effect of β-glycosidases on digestion, metabolism and health benefit of anthocyanins warrants attention.

5.6. ACKNOWLEDGEMENTS

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147


