Transport and Uptake of Anthocyanins in Gastric Tissue and Their Effect on the Gastric Inflammatory Response: Developing an *in vitro* Model Using the NCI-N87 Gastric Cell Line

DISSERTATION

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

By

Allison Atnip, M.S.

Graduate Program in Food Science and Nutrition

The Ohio State University

2014

Dissertation Committee:

Dr. M. Monica Giusti, Advisor

Dr. Joshua Bomser

Dr. Luis Rodriguez-Saona

Dr. Christopher Simons
Copyrighted by
Allison A. Atnip
2014
ABSTRACT

Anthocyanins are polyphenolic pigment compounds that are ubiquitous in the plant kingdom, imparting color to many fruits commonly consumed by humans. Anthocyanins have been researched for their many potential health benefits, including antioxidant, anti-inflammatory, anti-cancer, and cardioprotective activities. In order to maximize the benefits of anthocyanin intake for health (including for the development of functional foods), the uptake, metabolism, and bioavailability of anthocyanins throughout the gastrointestinal tract must be elucidated. Animal and human studies have shown that anthocyanin glycosides appear in plasma very rapidly after oral ingestion, suggesting a possible role of the stomach on the in vivo fate of anthocyanins. The effects of the gastric environment and chemical structure on the transport and uptake of anthocyanins in the stomach have not been well characterized. Cell culture models can be useful tools for investigating these behaviors; however the environment must be kept at physiological pH (7.4), not ideal for the study of the acidic environment of the stomach. The NCI-N87 cell line is an acid-stable model of the gastric epithelium, and has successfully been used to study drug metabolism in the stomach. We investigated the NCI-N87 cell line, seeded on 0.4 μm pore inserts, as a model to study the uptake and metabolism of anthocyanins in the stomach.
The NCI-N87 cell line formed a coherent monolayer that was stable over a period of 18-32 days post-confluence. The pH levels of apical chambers were adjusted to pH 3.0, 5.0, or 7.4, with a basolateral pH of 7.4, with minimal effects on monolayer integrity. A slight initial drop in monolayer integrity at pH 3.0 was quickly recovered by 120 min treatment at 37°C. The mucus layer generated by the cells trapped a small amount of anthocyanins, but was easily removed by washing and did not appear to have significant effects on the analysis of cellular uptake.

Transport and uptake of anthocyanins from chokeberry extract by NCI-N87 cells was dependent on treatment time, initial anthocyanin concentration, and apical pH. Overall anthocyanin recovery was significantly higher at apical pH 3.0 as compared with pH 5.0 and 7.4. As expected, over time and with increasing initial concentrations, the basolateral concentration of anthocyanins increased. The rate of anthocyanin transport increased over time, suggesting that the transport was not by passive diffusion alone. Generally, an apical pH of 3.0 showed the highest amount of transport, and pH 5.0 showed the lowest amount. There was also an observed effect of anthocyanin structure on transport, both for anthocyanins from chokeberry (significantly higher transport of cy-3-arabinoside and lower transport of cy-3-galactoside) and from red grape (higher transport of cyanidin-3-glu and lower transport of malvidin-3-glu). Finally, anthocyanins from chokeberry were shown to significantly inhibit increased IL-8 production in response to the IL-1β induced inflammation of NCI-N87 cells. This suggests a potential
anti-inflammatory activity of anthocyanins in the stomach, at least with respect to this acute inflammation model.

We have shown with this work that the NCI-N87 cell line was a useful model to study the behavior of anthocyanins in the stomach. The transport and uptake of anthocyanins in this model were dependent upon initial concentration, time, apical pH, and sometimes, chemical structure. This work is further evidence that the stomach may play a significant role in the \emph{in vivo} fate of anthocyanins.
This dissertation is dedicated to my Mom- thank you for being a woman I am proud to be slowly but surely turning into; my Dad, who is my inspiration for any and all academic achievement; and my brother Richard, who is by far the coolest thing about me.

and

In memory of Nelson Tyler Gunterman, who always stubbornly believed I could do this, even when I wasn’t so sure.
ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Monica Giusti, for her guidance and support. Your genuine care and concern for you students is obvious, and I have felt so lucky to be in your lab. Thank you for teaching me how to tell a story, how to trust the data, and how to believe I am capable of more. To my Giusti labmates, both past and present- thank you for your support, both in and out of the lab. Specifically, thank you to Neda, Greg, Fei, Andrew, and Alex for the help, and the laughs. I would like to especially thank Dr. Kom Kamonpatana for his guidance and work before me.

I would also like to thank Dr. Joshua Bomser for all his training, help, and insights with regard to the cell culture portion of this work. Thank you to Bomser lab members Daniel Straiton, Colin McGuinness, and Patrick Talbott for all your help in the cell culture lab. Thank you to my other committee members for your insights and feedback on my project- Dr. Simons, and Dr. Luis Rodriguez-Saona, who provided me with many teaching opportunities during my time at Ohio State. I would also like to acknowledge the Ohio Agricultural Research and Development Center for financial support of this project.

A sincere thank-you to Dr. David Min, for bringing me to Ohio State in the first place.
I would also like to sincerely thank my friends and colleagues from the Food Science Department at Ohio State. Jess, for being a most consistent and reliable friend, and for pushing me to be a better scientist. Mike, for taking on the extrovert role for me whenever I needed it, and for always listening to my ridiculous problems. Andrew W, for always being up for a beer (or an Ice) or some really bad golf. Ashley, for the countless lunches, coffees, chats, and for being the same kind of weirdo as me about so many things. Thank you Gabe, Greg, Matt, and Erin for keeping me sane so many afternoons. To my IFT friends- Jon, Jay, Matt, Anna, - I look forward to seeing you guys in the real world! My other wonderful food science friends- Taylor, Jen, and Alex M, the Kentucky kids; Paige, Liz, Alex S, Matt T., Hilary, Rachel, Andrew B, Mei-Ling, Morgan, Christian, Sarah, Sarrah, and so many others who made my time here unforgettable. Thank you!!!
VITA

1983................................................. Born, Louisville Kentucky

2001..................................................... New Albany Senior High School, New

Albany Indiana

2006..................................................... B.S. Nutrition Science, Indiana University,

Bloomington Indiana

2006-2008 ............................................. Quality Assurance, Paradise Tomato

Kitchens, Louisville Kentucky

2008-2010 ............................................. M.S. Food Science & Technology, The Ohio

State University, Columbus Ohio

2010 to present ......................................... Graduate Research Associate, Department

of Food Science & Technology, The Ohio

State University, Columbus Ohio

Publications


   Stability of Docosahexaenoic Acid Enriched Algal Oil. Available online:
   http://ole1.ohiolink.edu.proxy.lib.ohio-state.edu/record=b28895016~S0


   Authentication of Fruit Juices by Infrared Spectroscopic Techniques. In: Progress

Fields of Study

Major Field: Food Science and Nutrition
TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii

ACKNOWLEDGEMENTS ..................................................................................................... vi

VITA...................................................................................................................................... viii

LIST OF TABLES ................................................................................................................ xv

LIST OF FIGURES ............................................................................................................... xvi

INTRODUCTION .................................................................................................................. 1

CHAPTER 1: REVIEW OF LITERATURE AND SPECIFIC AIMS ................................. 5

1.1 Properties of anthocyanins ............................................................................................. 5

1.1.1 Chemical structure ...................................................................................................... 5

1.1.2 Stability ....................................................................................................................... 9

1.2 Uptake and bioavailability of anthocyanins ................................................................. 10

1.2.1 Bioavailability of anthocyanins ................................................................................ 10

1.2.2 Anthocyanin metabolism .......................................................................................... 12

1.2.3 Antioxidant activity .................................................................................................... 13

1.2.4 Anti-inflammatory activity ........................................................................................ 15
1.2.5 Anti-cancer activity ................................................................. 17
1.2.6 Other possible health benefits .............................................. 18
1.3 Anthocyanin activity in the stomach ........................................... 18
  1.3.2 Anthocyanin transport in the stomach ................................... 21
1.3.3 Gastric inflammation ............................................................. 24
1.3.4 Effect of anthocyanins on gastric inflammation and cancer ............ 26
1.3.5 The NCI-N87 cell line ............................................................ 26
1.4 Dissertation Research ............................................................... 27
  1.4.1 Overview and main research objectives ................................... 27
  1.4.2 Central hypothesis and overall research goal ............................ 29
  1.4.3 Overview of specific aims .................................................... 29

CHAPTER 2: THE NCI-N87 CELL LINE AS A MODEL OF THE GASTRIC
EPITHELIUM TO STUDY ANTHOCYANIN TRANSPORT AND UPTAKE IN THE
STOMACH ............................................................................................ 32

Abstract ............................................................................................. 32

2.1 Introduction .................................................................................. 33

2.2 Materials and Methods ................................................................ 34
  2.2.1 Standards and reagents ......................................................... 34
2.2.2 Cell culture conditions

2.2.3 Extraction and purification of chokeberry anthocyanins

2.2.4 Effects of anthocyanins on cell density

2.2.5 pH stability of treatment media

2.2.6 Monolayer integrity of NCI-N87 cells on inserts under treatment conditions

2.2.7 Effects of the mucus layer on the analysis of cellular uptake of anthocyanins by NCI-N87 cells

2.3 Results and Discussion

2.3.1 Extraction, characterization, and stability of anthocyanin-rich extracts

2.3.2 Effect of anthocyanins on NCI-N87 cell density

2.3.3 pH stability of treatment media

2.3.4: NCI-N87 monolayer integrity

2.4.5: Effects of the NCI-N87 mucus layer on the analysis of anthocyanin uptake by NCI-N87 cells

Conclusions

CHAPTER 3: EFFECTS OF pH, TIME, AND ANTHOCYANIN CHEMICAL STRUCTURE ON THE TRANSPORT AND UPTAKE OF ANTHOCYANINS THROUGH THE NCI-N87 GASTRIC EPITHELIAL MONOLAYER

Abstract

3.1 Introduction
3.2 Materials and Methods ........................................................................................................ 55
  3.2.1 Standards and reagents ................................................................................................ 55
  3.2.3 Cell culture conditions ............................................................................................ 56
  3.2.4 HPLC analyses of apical and basolateral samples .................................................... 57
  3.2.5 Anthocyanin extraction from NCI-N87 cells ............................................................. 58
  3.2.6 Effects of concentration and time on the transport of anthocyanins through the
      NCI-N87 monolayer ........................................................................................................ 59
  3.2.7 Effects of pH on the transport of anthocyanins through the NCI-N87 monolayer
       ................................................................................................................................. 59
  3.2.8 Effects of anthocyanin chemical structure on the transport of anthocyanins
       through the NCI-N87 monolayer .................................................................................. 60
  3.2.9 Statistical Analysis .................................................................................................. 60

3.3 Results and Discussion ..................................................................................................... 61
  3.3.1 Effects of time and initial concentration on the transport and uptake of
       anthocyanins by NCI-N87 cells .................................................................................... 61
  3.3.2 Effects of pH on the total recovery, and transport and uptake of anthocyanins
       by NCI-N87 cells ......................................................................................................... 72
  3.3.2 Effects of anthocyanin chemical structure on the transport of anthocyanins
       through the NCI-N87 monolayer .................................................................................. 82

Conclusions .......................................................................................................................... 100
CHAPTER 4: EFFECTS OF ANTHOCYANINS ON THE IL-1β INDUCED
INFLAMMATION OF NCI-N87 GASTRIC CELLS.................................................. 102

Abstract ....................................................................................................................... 102

4.1 Introduction .......................................................................................................... 103

4.2 Materials and Methods ...................................................................................... 105

  4.2.1 Standards and reagents .................................................................................. 105

  4.2.2 Cell culture conditions .................................................................................. 106

  4.2.4 Anti-inflammatory activity of chokeberry anthocyanins................................. 107

  4.2.5 Cytokine ELISA assay.................................................................................... 108

4.3 Results and Discussion ....................................................................................... 108

  4.3.1 Anti-inflammatory activities of chokeberry anthocyanins ............................. 108

Conclusions ................................................................................................................. 112

CONCLUDING REMARKS AND FUTURE WORK .............................................. 113

REFERENCES ............................................................................................................. 115
LIST OF TABLES

Table 1: Types of cells found in human gastric glands (Campbell and Reese 2006) ........ 21
Table 2: Anthocyanin recovery (%), from initial anthocyanin addition, from apical, basolateral, and cell layers at varying apical pH values after 3 h treatment at 37°C and an initial anthocyanin concentration 1500µM. ......................................................... 74
Table 3: Individual anthocyanin concentration (mg/L) and percent transported from the apical to basolateral compartments, at apical pH 3.0 and basolateral pH 7.4, after 180 min treatment and initial anthocyanin concentration of 1500 µM (727 mg/L)................. 83
Table 4: Individual anthocyanin amounts (mg/L) and percent uptake from the apical compartment to the cellular layer, after 180 min at apical pH 3.0 and basolateral pH 7.4 and initial anthocyanin concentration of 1500 µM (727 mg/L) from chokeberry extract. ................................................................. 86
Table 5: Individual HPLC peak area % values for anthocyanins from chokeberry extract, from the basolateral, apical, and cellular layers after 3 h treatment at apical pH 3.0 and basolateral pH 7.4, at 37°C... ................................................................. 89
LIST OF FIGURES

Figure 1: Anthocyanin aglycone structures found commonly in the diet, including B-ring substitutions. Frequently the R₃ position is occupied by sugar moieties. .................................. 6

Figure 2: Structure of cyanidin-3-glucoside in its flavylum cation form, found predominantly at pH 1-4. .................................................................................................................. 7

Figure 3: Effects of pH on the chemical structure of anthocyanins in solution. ................. 8

Figure 4: Basic anatomy of the human stomach (Encyclopedia Britannica 2014). ........... 19

Figure 5: Histology of the gastric epithelium (Betts and others 2014).............................. 25

Figure 6: HPLC-PDA profile of predominant anthocyanins in chokeberry juice extract. 41

Figure 7: Effect of anthocyanins on cell density of the NCI-N87 cell line ..................... 42

Figure 8: Fluctuations in apical pH observed over 3 h at 37°C. ...................................... 44

Figure 9: Phenol red diffusion from apical to basolateral chamber of NCI-N87 cells at 37°C over time. .............................................................................................................. 46

Figure 10: Anthocyanin recovery from media, mucus, and cell layers at 37°C vs 0°C at pH 7.4 ........................................................................................................................................ 48
Figure 11: Effect of time on the concentration (µM) of anthocyanins through the NCI-N87 monolayer (apical to basolateral chambers) at apical pH 3.0, with varying initial concentrations over 3 h at 37°C. .......................................................... 62

Figure 12: Effect of initial anthocyanin concentration on the transport efficiency (%) of anthocyanins through the NCI-N87 monolayer over 3 h at 37°C at apical pH 3.0 and basolateral pH 7.4. .......................................................... 65

Figure 13: Effect of initial anthocyanin concentration (µM) on the total anthocyanin amount (µg) extracted from the NCI-N87 cell layer after 3 h treatment at 37°C at apical pH 3.0 and basolateral pH 7.4. .......................... 68

Figure 14: Efficiency (%) of anthocyanin uptake by NCI-N87 cells at varying initial apical concentrations, at apical pH 3.0 and basolateral pH 7.4, after 180 min treatment at 37°C. .......................................................... 71

Figure 15: Effect of changing apical pH on the transport of chokeberry anthocyanins through the NCI-N87 cell monolayer after 3 h at 37°C and basolateral pH 7.4 as determined by anthocyanin concentration in basolateral chamber. ...................... 75

Figure 16: Effect of apical pH on the transport efficiency of chokeberry anthocyanins through the NCI-N87 monolayer over 3 h at 37°C at basolateral pH 7.4. ................. 78

Figure 17: Effects of apical pH on the cellular uptake efficiency (%) of NCI-N87 cells after 3 h treatment at 37°C and basolateral pH 7.4. .......................................................... 80

Figure 18: Anti-inflammatory activity of chokeberry anthocyanins in NCI-N87 cells as measured by IL-8 secretion induced by IL-1β. ......................................................... 110
Figure 19: Dose response of the anti-inflammatory activity of chokeberry anthocyanins on the IL-1β induced inflammation of NCI-N87 cells. ................................................................. 111
INTRODUCTION

Anthocyanins are polyphenolic pigment compounds found in many plants, and are a subgroup of the large family of chemical compounds known as flavonoids. Anthocyanins impart a range of colors to plants including blue, purple, and red. These color properties have lead to high interest in the food industry, as potential natural replacements for synthetic food dyes, which have been shown by some studies to cause hyperactivity in children. Additionally, consumer backlash against synthetic dyes have created a higher demand for natural alternatives for the coloring of food products (Aberoumand 2011). In addition to their potential as natural colorants, dietary intake of anthocyanins has long been known to have a multitude of health benefits, including antioxidant and anti-inflammatory activities. This is most likely due to their phenolic structure (Figure 1) and thus their free-radical scavenging capabilities (Rice-Evans and others 1997). Although intake of anthocyanins is estimated to be quite high (approximately 200 mg/day) as compared to other flavonoids (estimated to be approximately 20 mg/day), the bioavailability of these compounds has been shown in many studies to be quite low, and the metabolism of action within the body has not been well-characterized (Kuhnau 1976, Hertog and others 1993, Prior and Wu 2006). Still, epidemiological evidence consistently shows that regular intake of fruits and vegetables
(Steevens and others 2011, Hui and others 2013, Jennings and others 2014) and in one case, specifically anthocyanins (Zamora-Ros and others 2011), leads to reduced instances of many cancers and chronic diseases. Structurally, anthocyanins found in the diet are composed mainly of six aglycone compounds (known alone as anthocyanidins), attached to various sugar and/or acid attachments. The stability of anthocyanins is highly susceptible to the environment’s pH, with the most stable form (known as the flavylium cation) occurring as the predominant structure under acidic (pH 1-3) conditions.

In humans, most nutritional absorption occurs in the small intestine, and the stomach has been widely ignored as an absorptive organ. Several studies, however, suggest that significant amounts of some compounds, including anthocyanins, are in fact absorbed in the stomach (Fernandes and others 2012; Passamonti and others 2002, 2003, 2005; Talavera and others 2003). The rate and mechanism of absorption are not yet fully characterized, but the most significant body of evidence points to uptake via the transporter protein bilitranslocase. The involvement of an active transport mechanism of some kind is likely, as the molecular weight of intact anthocyanins is considered too high for passive diffusion through the tight junctions of the gastric epithelial layer. So far, the effects of chemical structure on the absorption of anthocyanins in the stomach have not been fully elucidated, partially due to the lack of an in vitro model of the gastric epithelium.

Established from human gastric carcinoma, the NCI-N87 cell line forms a well-differentiated monolayer which expresses epithelial-type junctions (E-cadherin and ZO-1) and, at 18 days post-confluence, secretes gastric mucin-6 glycoprotein. At this stage
the cell line is also acid-resistant at least to pH 3. These factors make this cell line a physiologically relevant model of the gastric epithelium, and indeed it has been used to investigate drug metabolism in the stomach (Park and others 1990, Chaille and Menard 2005, Lemieux and others 2011). We hypothesize that this cell line can be used to investigate the uptake of anthocyanins in the stomach, including the varying pH environments to which the stomach is exposed. We also hypothesize that different anthocyanin chemical structures found in various fruit extracts may be differentially taken up by the cells.

Gastritis, or the inflammation of the stomach lining, can have several different causes, including overuse of alcohol or non-steroidal anti-inflammatory drugs (NSAIDs), and, most significantly, infection with the bacteria *Helicobacter pylori*. Chronic stomach inflammation is the most well-known risk factor in the development of gastric cancers, the 4th most common type of cancer worldwide (Brenner and others 2009). That anthocyanins can change the inflammatory response of a tissue has been well-documented. We further hypothesize, therefore, that anthocyanin-rich extracts will act as anti-inflammatory agents in the induced inflammatory response of the NCI-N87 gastric cell line.

Based on the hypotheses outlined here, we have three main research objectives:

I: Determine the feasibility of the NCI-N87 cell line as a model for anthocyanin uptake in gastric tissue.
II: Evaluate the transport and uptake of anthocyanins in the stomach using the NCI-N87 cell line.

a. Effects of time, initial concentration, and apical pH on the transport and uptake of chokeberry anthocyanins by NCI-N87 cells

b. Effects of anthocyanin chemical structure on the transport and uptake of anthocyanins from chokeberry (effects of sugar substitution) and red grape (effects of B-ring substitution) by NCI-N87 cells

III: Determine the effects of anthocyanins on the induced inflammation of NCI-N87 gastric cells.
CHAPTER 1: REVIEW OF LITERATURE AND SPECIFIC AIMS

1.1 Properties of anthocyanins

1.1.1 Chemical structure

Anthocyanins (from the Greek *anthos* = flower, and *kianos* = blue) commonly found in the diet are comprised mainly of six different aglycones, with many possible combinations of sugar and acid attachments. The basic structure of the aglycone is that of an aromatic ring (the A ring) bound to a heterocyclic, oxygen-containing ring (the C ring), which is bound by C-C bond to another aromatic ring (the B ring). Substitutions of –OH and -CH₃ groups at the 3’ and 5’ position on the B-ring account for the different anthocyanidin structures, as seen in Figure 1. Adding further diversity to this group of compounds, attachment of sugar and acid moieties is common, mostly on the C-3 and C-5 positions of the aglycone on the A and C rings. Some of the more common sugar attachments include glucose, galactose, xylose, and arabinose, and rhamnose. These sugar attachments may also be acylated, mostly by aromatic and/or aliphatic acids, such as cinnamic acid. Owing to these many combinations of substitution variations, more than 700 different anthocyanins have been identified in nature, with a wide range of molecular weights (approximately 400-1200 g/mol) (Prior and Wu 2006, Andersen and
Jordheim 2006, He and Giusti 2010). The structure in Figure 2 is an example of an anthocyanin monoglycoside commonly found in fruits, cyanidin-3-glucoside (C3G), widely believed to be the most prevalent anthocyanin, up to 50% of those found in nature (Castaneda-Ovando and others 2009).

![Anthocyanin structure](image)

<table>
<thead>
<tr>
<th>Aglycone</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Petunidin</td>
<td>OCH₃</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Peonidin</td>
<td>OCH₃</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OH</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
<td>OH</td>
</tr>
</tbody>
</table>

Figure 1: Anthocyanin aglycone structures found commonly in the diet, including B-ring substitutions. Frequently the R₃ position is occupied by sugar moieties.
This particular structure shows cyanidin-3-glucoside in its flavylum cation form (note the positive charge on the oxygen of the heterocyclic C ring), the most stable form of an anthocyanin. This is also the predominant structure found at a low environmental pH. Anthocyanins exist in a dynamic equilibrium in solution that is dependent on the pH: under acidic conditions (pH 1-3) the flavylum cation is the most dominant form and the color is stable. Approaching a more neutral pH (5-6), the chalcone or hemiketal form is predominant, and the compound loses its color. Under basic conditions (7 and higher), the quinoidal base is dominant and an unstable blue color is formed (Castaneda-Ovando and others 2009, He and Giusti 2010). This property is one that differentiates anthocyanins from the rest of the flavonoid family, and has consequences in terms of the
uptake and metabolism of anthocyanins throughout the GI tract (Prior and Wu 2006). The effects of pH on the chemical structure of anthocyanins in solution are shown in Figure 3.

Figure 3: Effects of pH on the chemical structure of anthocyanins in solution.

Additionally, this behavior creates technical challenges when using anthocyanins as natural colorants in food systems, or utilizing the health benefits of anthocyanins in the formulation of functional foods. The stability of anthocyanins is dependent on a host of other factors as well, making them an intriguing but challenging tool for food product development.
1.1.2 Stability

Aside from stability issues arising from environmental pH, anthocyanins are also susceptible to degradation from other sources. Heat, light, enzymes, and chemical reactions with other compounds can all cause degradation of the anthocyanin molecule. These of course are issues that must be addressed when working with anthocyanins in food systems: processing temperature, storage time and temperature, ingredient interactions, and light exposure must all be taken into account for product quality.

The structure of anthocyanins can also have a significant effect on their stability. As mentioned previously, the aglycone anthocyanidin is considerably less stable in solution than the glycosylated anthocyanin (Woodward and others 2009). Further structural factors that contribute to anthocyanin stability include acylating groups: more acylation tends to provide more stability, possibly due to the ability of these groups to contribute to anthocyanins’ ability to stack with each other, thereby protecting the reactive groups from outside influences such as oxygen. Similarly, anthocyanins with more hydroxyl groups on the B-ring tend to be more reactive, therefore less stable. Use of anthocyanins with acylating groups can have an effect on the color of a solution or product: generally, more substitutions on an anthocyanin will give a deeper hue (a bathochromic shift) (Castaneda-Ovando and others 2009).

Anthocyanins are also capable of interacting with other molecules, such as phenolic acids (ex: ferulic, caffeic) resulting in increased stability- this is known as copigmentation. Salts of metal ions such as magnesium and copper can also react this way with anthocyanins and increase their stability by interacting with the B-rings of
multiple anthocyanins. Co-pigments themselves are generally electron rich molecules, allowing them to readily react with the flavylium cation, providing protection from nucleophilic attack at the 2-C position, and peroxide degradation at the 4-C position (Castaneda-Ovando and others 2009). Copigmentation likely has a profound effect on the stabilization of color in plants and food systems.

1.2 Uptake and bioavailability of anthocyanins

Due to the interest in the health benefits associated with high anthocyanin intake, many studies of the pharmacokinetics and bioavailability of anthocyanins have been published, with varied results. Intake of anthocyanins is already generally higher than other flavonols- depending on an individual’s diet, the daily intake of anthocyanins can be as high as 200 mg/day (Prior and Wu 2006). The complex chemistry of anthocyanins makes the study of their metabolism more difficult. Given their different structures at various pH levels and their susceptibility to breakdown by pH, microbes and enzymes, studies of the rates of uptake, metabolism, and cellular distributions of anthocyanins remain difficult. Still, in order to investigate the role of these compounds in disease prevention, determining their metabolic fate is a key step.

1.2.1 Bioavailability of anthocyanins

In addition to different in vitro chemical characteristics, anthocyanins differ from other flavonoids in their in vivo behavior. For example, studies have repeatedly shown that anthocyanins are taken up by some tissues in their intact, glycoside form, as opposed
to their aglycone form. Most other flavonoids, such as quercetin, are predominantly taken up as aglycones. Many studies have shown that anthocyanins are poorly absorbed when compared to other flavonols: absorption of less than 1% of ingested anthocyanins has been shown by various animal studies (Manach and others 2005, Stoner and others 2005, McGhie and Walton 2007). Additionally, the majority of ingested anthocyanin has been shown to disappear from the GI tract within 4 hours (Prior and Wu 2006). Taken together, these results suggest that there may be extensive breakdown and metabolism of anthocyanins in the GI tract before tissue uptake. These metabolism and breakdown products may have very different biological activity than the intact anthocyanins. Several researchers have suggested that these metabolites of anthocyanins are in fact the compounds responsible for the observed health benefits of anthocyanin intake (Prior and Wu 2006, Forester and Waterhouse 2010). In fact, recent results discussed below show that it is possible that health effects are due to a combination of the intact ingested anthocyanin, chemical breakdown products, and microbial and cellular metabolic products.

Very recently, a $^{13}$C labeled anthocyanin study was published in which the bioavailability of anthocyanins was estimated to be as high as 12-15%, substantially higher than the <1% previously reported in many studies (Czank and others 2013). This study suggests that traditional bioavailability studies which, look only at urine excretion, are most likely underestimating bioavailability. This may especially be true for compounds which have not yet had their metabolism fully characterized, as in the case
with anthocyanins. Without having an understanding of which metabolites are formed, true bioavailability is difficult to estimate (Rein and others 2013).

1.2.2 Anthocyanin metabolism

Given the large amount of structural variation within the anthocyanin family found in nature, the metabolism of these compounds is quite complex, and is not yet fully understood. Evidence for the effects of structure on metabolism of anthocyanins is widely varied, and seems to depend on both the anthocyanin source and environmental influences (enzymatic, pH, and microbial). In a human study by Wu and others (2002), different participants were fed an oral dose of whole blueberries. Results showed the proportions of anthocyanins found in the urine as compared to the proportion in the original blueberry sample were quite similar, except in the case of the anthocyanin-arabinosides (delphinidin, petunidin, and malvidin). This result may indicate that the arabinoside forms of these anthocyanins are absorbed and/or metabolized differently, which begs the question: what effect(s) do substitutions and the different aglycones themselves have on the uptake, metabolism, distribution, and elimination of anthocyanins? Recent research by Kamonpatana and others (2012) began to address this question in the oral cavity. Using commodities with distinct anthocyanin profiles (blueberry, chokeberry, black raspberry, red grape, and strawberry), the authors showed that all anthocyanins were partially broken down in the oral cavity. Additionally, the observed degradation was structure-dependent, and was mediated by microbiota in the mouth. Glycosides of delphinidin and petunidin were more susceptible than those of
cyanidin, malvidin, pelargonidin and peonidin, suggesting that the aglycone structure itself may affect metabolism in the mouth. Anthocyanins with 2 or 3 sugar attachments were also shown to be slightly more stable than those with attached monosaccharides in the oral cavity.

1.2.3 Antioxidant activity

Many of the observed health benefits from increased anthocyanin intake are attributed to the antioxidant capabilities of these compounds. In the body, compounds known as reactive oxygen species (ROS) are naturally generated and have many important functions, including involvement in cell signaling and immune responses. If ROS compounds start to become over-produced, however, they can start to cause damage within the body at the cellular level. High levels of ROS can lead to many disease states, including chronic inflammation, heart disease, and cancer (Allen and Tresini 2000, He and Giusti 2010). Examples of ROS compounds include free radicals, singlet oxygen, and peroxides, all of which contribute to the so-called chain reaction of the oxidation process. These ROS can interact not only with each other, but with other electron-rich compounds, to propagate this chain reaction. Compounds which can react with ROS to stop this chain reaction (or, “quench” the ROS) make potent antioxidants. Anthocyanins can not only quench ROS, but form relatively stable free radicals in the reaction, making them very effective antioxidants (Prior and Wu 2006, He and Giusti 2010). This is supported both by numerous in vitro studies and by several animal and human studies. Chiou and others (2014) were able to show ROS scavenging (by 2,2-diphenyl-1-
picrylhydrazyl scavenging capacity) and reducing power (by ferric ion reduction) of anthocyanins from currants in vitro. There are many studies that show the overall in vitro antioxidant activity from a wide variety of anthocyanin sources, such as: black carrot (Algarra and others 2014), blueberry (Reque and others 2014), cherries (Ou and others 2012), pomegranate (Kar and others 2011), purple corn (Yang and others 2010, Lopez-Martinez and others 2011), mulberry (Liang and others 2012), and many other fruits and plants.

In vivo activity of anthocyanins can get more complicated; however many studies tend to agree with the in vitro data. An in vivo animal study looked at the blood antioxidant capacity of sheep, and found that after consumption of anthocyanins from purple corn, oxidation was significantly suppressed as compared to control sheep (Hosoda and others 2011). Another study exposed rats to a daily oral diet which included purified red grape skin anthocyanin extract for 10 days, both with and without the co-administration of an oxidative agent (CCl₄). Results showed that the anthocyanin intake was able to completely reverse the increase in oxidative stress induced by CCl₄ administration. Additionally, the antioxidant capacity of the serum in the normal non CCl₄ treated rats was also significantly higher. The researchers also found that there was no toxicity or cellular damage associated with increased anthocyanin intake (Lionetto and others 2011). In human trials, there are positive results as well. Mazza and others (2002) measured antioxidant activity in human serum after ingestion of blueberry powder, and found high correlation between increased anthocyanin consumption and increased
antioxidant activity. These findings agree with a later study by McGhie and others (2007), which utilized boysenberry and black currant as sources of anthocyanins.

1.2.4 Anti-inflammatory activity

The *in vitro* and *in vivo* anti-inflammatory activities of anthocyanins are fairly well-documented. Chinese medicine has long utilized the anti-inflammatory properties of flavonoids in practice, usually in the form of crude plant extracts (Kim and others 2004). The *in vitro* anti-inflammatory properties of anthocyanins have been shown to be in the same potency realm as non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and naproxen. One theory of the *in vitro* mechanism of anti-inflammatory action of anthocyanins is thus as cyclooxygenase (COX) I & II inhibitors, similar to NSAIDs (Seeram and others 2001, Wang and others 1999). Anthocyanins have also been shown to have *in vivo* anti-inflammatory activity, both in rats and humans. Tall and others (2004) showed that anthocyanins from tart cherry extract were able to reduce inflammation-induced hyperalgesia in rats as well as indomethacin (in terms of onset, duration, and magnitude), a potent NSAID. The authors also suggested that the mechanism of action was by suppression of COX-mediated synthesis of prostaglandins.

Another study by Wang and Mazza (2002) showed the *in vitro* inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide (NO) production by macrophages when induced by lipopolysaccharide (LPS) and interferon-γ (IFN-γ). This study suggests an alternative or additional anti-inflammatory mechanism: NO is an important modulator of the host inflammatory response- if it is generated in excess, it can
contribute to oxidative stress and tissue injury, and possibly cancer (Maeda and Akaike 1998). The inhibition by anthocyanins of NO production in LPS-IFN-γ activated macrophages was dose-dependent and also structure dependent: cyanidins showed generally less inhibition as other anthocyanins. Glycosylation in general reduced the inhibitory effect as well, however in the case of malvidin-3-glucoside and 3,5 diglucoside, the effect was larger than other glycosylated anthocyanidins. The authors speculated that this may be due to higher absorption by the cells, or by higher free radical scavenging activity of these particular compounds (Wang and Mazza 2002).

Additionally, crude extracts and purified concentrated extracts of blackberry, black currant, Saskatoon berries and blueberries were tested. The crude extracts of blackberries and black currant showed moderate inhibition of NO production, but no effect was seen with the Saskatoon and blueberries. When the purified concentrates were tested, however, all berries showed significantly more NO production inhibition, and these inhibitory effects could be correlated with the differing phenolic contents. The results showed that total phenolics and total anthocyanins may have more impact on the inhibition of NO production than other flavonols (Wang and Mazza 2002).

A study on overweight adults showed that consumption of strawberry anthocyanins with a high carbohydrate, moderate fat meal showed that the anthocyanin consumption significantly reduced postprandial inflammation as measured by C-reactive protein and by IL-6 levels (Edirisinghe and others 2011).
1.2.5 Anti-cancer activity

Dietary interventions for the prevention of cancer are widely researched, and there is epidemiological data to support it. The World Cancer Research Fund, along with the American Institute for Cancer Research, summarizes its authoritative findings on this topic in its publication *Food, Nutrition, Physical Activity and the Prevention of Cancer: A Global Perspective* (2007). The report clearly shows changes in patterns of not only dietary habits and cancer rates in industrialized nations, but also changes in cancer rates of certain populations due to migration and changes in diets. It is evident that environmental factors have a huge impact on the development of cancer, and many of these factors can be altered (such as tobacco use, diet, and body composition). One important finding of the report was a reduced risk for many types of cancer associated with a high intake of fruits and vegetables.

Anthocyanins have been extensively researched for their observed chemopreventative activities. Anthocyanins from bilberries and grapes have been shown by Teller and others (2009) to inhibit the activity of receptor tyrosine kinases, compounds which promote tumor growth, in rats. Also in rats, Stoner and colleagues (2006) showed that a diet high in anthocyanins (5% from black raspberry, strawberry, and blackberry) was associated with a 24-56% decrease in esophageal tumors. In cell culture, anticancer activity of anthocyanins has also been studied. Inhibition of cell proliferation has been extensively observed (oral, colon and prostate by Zhang and others 2008; liver by Liu and others 2002; oral, breast, colon, and prostate by Seeram and others 2006; leukemia and colon carcinoma cells by Katsube and others 2003).
1.2.6 Other possible health benefits

Anthocyanins have been shown to have a wide variety of other health benefits, likely based on their anti-inflammatory and/or antioxidant capacity. Suppressed weight gain was observed in mice over several studies (Tsuda and others 2003, Rojo and others 2012); and anthocyanins have even been shown to increase the production of insulin in mice (Jayaprakasam and others 2005). Cardiovascular health is another major potential benefit of anthocyanin intake. Animal models (Bell and Gochenaur 2006, Toufe/tsian 2008, Youdim and others 2000), cell culture models (Hidalgo and others 2012) and even randomized controlled human clinical trials (Basu and others 2010) have all shown that anthocyanin intake can improve cardiovascular health. The anti-aging benefits of anthocyanins have also been shown in both animal models and at least one human clinical trial, specifically their effects on the slowing of age-related cognitive decline (In rats-Galli and others 2002; in humans- Krikorian 2010a, 2010b) and Alzheimer’s disease (In rats- Joseph and others 1998, 1999).

1.3 Anthocyanin activity in the stomach

1.3.1 Stomach physiology

The primary roles of the stomach are to store and mix food, with some preliminary digestive function. Figure 4 shows the basic anatomy of the human stomach.
Food enters the stomach from the esophagus, where it is exposed to proteases and hydrochloric acid (providing a low pH for protease function and to kill harmful bacteria) secreted by the stomach. Contractions of the stomach wall (known as peristalsis) churn the food and aid in the conversion of the food bolus to chyme, which enters the duodenum of the small intestine via the pyloric sphincter.

Figure 4 shows the basic anatomy of the human stomach. Food enters the stomach from the esophagus, where it is exposed to proteases and hydrochloric acid (providing a low pH for protease function) secreted by the stomach. Contractions of the stomach wall (known as peristalsis) churn the food and aid in the conversion of the food bolus to chyme, which enters the small intestine via the pyloric sphincter.

Figure 4: Basic anatomy of the human stomach (Encyclopedia Britannica 2014).
Inside the stomach, the inner surface has many folds, known as rugae, which give way to openings into the lumen known as gastric pits. These gastric pits serve as the openings to the gastric glands, which consist of several different types of cells, descriptions of which can be found in Table 1 below.

Intrinsic factor, secreted by parietal cells, is critical to the absorption of vitamin B$_{12}$, and therefore critical to human survival, as B$_{12}$ is required for the maturation of red blood cells. Pepsinogen secreted by chief cells is activated to pepsin in the presence of HCl secreted by parietal cells, and serves to break down proteins into shorter polypeptide chains. Pepsin has an optimal active pH of approximately 2.0. At this pH, the tertiary structure of many proteins will also be denatured, making them more digestible. Other macronutrients are not significantly digested in the stomach (Fox 2006).
Table 1: Types of cells found in human gastric glands (Campbell and Reese 2006).

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goblet cells</td>
<td>Secretion of mucus</td>
</tr>
<tr>
<td>Parietal cells</td>
<td>Secretion of hydrochloric acid and intrinsic factor</td>
</tr>
<tr>
<td>Chief (also known as zymogen) cells</td>
<td>Secretion of pepsinogen (the inactive form of pepsin) and gastric lipase</td>
</tr>
<tr>
<td>Enterochromaffin-like (ECL) cells</td>
<td>Secretion of histamine and serotonin, regulating hormones in the GI tract</td>
</tr>
<tr>
<td>G cells</td>
<td>Secretion of gastrin</td>
</tr>
<tr>
<td>D cells</td>
<td>Secretion of somatostatin</td>
</tr>
<tr>
<td>Ghrelin cells</td>
<td>Secretion of ghrelin</td>
</tr>
</tbody>
</table>

1.3.2 Anthocyanin transport in the stomach

Though the stomach is mostly regarded as a storage organ where some mixing and chemical digestion takes place, in the case of anthocyanins, it may actually serve as a site of significant absorption. As anthocyanins are known to be stable in the acidic environment of the stomach, this absorption is most likely the intact, native form of the
compounds (Fang 2014). Animal models have shown intact anthocyanins appearing very quickly in the plasma of rats, along with high concentrations of anthocyanin metabolic products such as protocatechuic acid (PCA) and 4-hydroxybenzoic acid (Tsuda and others 1999). In human studies, intact anthocyanins have been detected in the plasma and urine after an oral dose. In the plasma, peaks corresponding to anthocyanins have been visible after just 10 minutes (Milbury and others 2002). The absorption of anthocyanins specifically by the stomach has been previously investigated with animal models. A 2003 study by Talavera and colleagues looked at the uptake and metabolism of anthocyanins in rat stomachs. The stomachs of anesthetized rats were directly exposed to either a standard mixture of anthocyanins, blackberry extract, or bilberry extract. Overall results showed that about 25% of administered anthocyanin monoglycosides were rapidly absorbed, intact, regardless of the aglycone structure. Cyanidin-3-rutinoside (an anthocyanin disaccharide) absorption was significantly lower than the cyanidin monosaccharide glucose or galactose attachments. After infusion with bilberry anthocyanins, total absorption from the gastric lumen was approximately 27%, however there were marked differences in the proportions of each individual anthocyanins, ranging from 18.7% for peonidin-3-galactoside, to 36.6% of delphinidin-3-arabinoside. In fact, anthocyanin arabinosides were shown to be better absorbed than other anthocyanin monoglycosides, corresponding to the findings by Wu and others (2002) that suggested arabinosides were metabolized differently in human subjects. In the Talavera rat study, it was also shown that cyanidin-3-glucoside absorption from blackberry extract tended to be higher than from the standard mixture, and that a higher dosage of total
anthocyanins from blackberry yielded a lower absorption rate than a lower dosage (approximately 30% from a dose of 14 µM and approximately 7% from a dose of 750 µM), suggesting that the mechanism of absorption may involve a carrier of some kind which is capable of being saturated.

Several published works by Passamonti and colleagues have also shown rapid appearance of anthocyanins in plasma upon ingestion (in both humans and rats) suggesting significant gastric uptake. The researchers’ working hypothesis is that the uptake in the stomach is by bilitranslocase, a transport protein capable of interacting with many substrates (Passamonti and others 2000, 2002, 2003, 2005). Bilitranslocase is present in both the liver and gastric epithelium, in similar forms. In 2002, Passamonti and others showed (via a bilitranslocase transport activity assay in rat plasma membrane vesicles) that 17 out of 20 tested anthocyanins acted as competitive inhibitors of bilitranslocase transport activity ($K_I = 1.4-22$ µM). Additionally, they were able to show structural differences in this inhibition: anthocyanin mono and diglycosides were better inhibitors than aglycones (Passamonti and others 2000, 2002). In another study (2003) which also used anaesthetized rats (similar to the Talavera study design), the group used a standardized mixture of anthocyanins to show that several anthocyanin monoglycosides were detected in plasma after direct injection into the stomach. No aglycones or conjugated derivatives were detected.

An alternate theory to the bilitranslocase transport, supported by Fernandes and colleagues (2012), is that glucose transporters in the stomach may recognize the sugar moieties found attached to many dietary anthocyanins. A similar theory involves the
interaction of quercetin glucosides with intestinal glucose transporters (quercetin and
anthocyanins are similar in structure) (Gee and others 1998). Given their relative size
and the fact that at lower pH the anthocyanins are in their charged cationic form, passive
diffusion through the gastric cells is unlikely.

1.3.3 Gastric inflammation

Gastric inflammation is an extremely common disease state worldwide, and is
known to be the most predictive factor in the development of gastric cancer. The
bacterium Helicobacter pylori is thought to be present in over half the US population
over 60 years old. In developing countries, the problem is even more widespread and
affects a higher percentage of children. Infection with this bacterium causes gastric
inflammation, which can lead to peptic ulcer disease, gastric adenocarcinoma, and gastric
lymphoma (Brawley 2009). H. pylori, unlike other species of bacteria, have evolved over
time to withstand the acidity and peristalsis found in the stomach’s environment (Israel
and Peek 2001). The incidence of gastric inflammation causes varying degrees of
deterioration of the epithelial layer and secretion of inflammatory cells (lymphocytes,
neutrophils, plasma cells). An illustration of the epithelial layer of the stomach can be
seen below in Figure 5.
While the mechanism of inflammation previously included secretion of these inflammatory cells into the mucosal layer, *in vivo* evidence has not supported that hypothesis (Bodger and Crabtree 1998, Israel and Peek 2001). The detailed mechanism is not well understood, but one current hypothesis is that *H. pylori* may secrete compounds that inflame the mucosal layer either from afar, or through direct contact with gastric epithelial cells, triggering the release of pro-inflammatory cytokines such as interleukin-1 β (IL-1β), IL-2, IL-6, IL-8, and TNF-α. Regardless of the mechanism, *H. pylori* is known to be a significant cause of both acute and chronic stomach inflammation (Israel and Peek 2001).

Chronic gastric inflammation caused by *H. pylori* is thought to be the most well-established risk factor in developing stomach cancer, the fourth most common cancer worldwide. In 1994, it was listed as a “definite carcinogen” for stomach cancer by the
International Agency for Research on Cancer. While the incidence of stomach cancer has declined in some countries, there are some areas of the world where it is quite prevalent—East Asia, Central and South America, and Eastern Europe. Additionally, the cardia subtype of gastric cancer has increased in several countries, while the non-cardia subtype has decreased. Diet, lifestyle, and environment may be important factors in the development of risk for gastric cancer (Zamora-Ros and others 2012). There is also a risk difference based on sex; males are nearly twice as likely as females to develop stomach cancer. Once a patient is diagnosed, prognosis is poor: the 5 year relative survival rate is less than 30% in most parts of the world (Brenner and others 2009).

1.3.4 Effect of anthocyanins on gastric inflammation and cancer

Intake of flavonoids, and specifically anthocyanins, was shown in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study to significantly reduce the risk of gastric cancer in women, but was not significant in the male group. Several case-control studies have also shown this association, however others did not find the relationship between flavonol intake and decreased incidence of gastric cancer to be significant (Zamora-Ros and others 2012). More research is needed to determine the nature of the effect of flavonoid intake on the risk of developing gastric cancer.

1.3.5 The NCI-N87 cell line

While cell culture can be an exceedingly useful tool to study the behavior of compounds in various tissues, many limitations exist and must be considered when
interpreting data. One hurdle in developing an *in vitro* model of the gastric epithelium is that most cell lines must be maintained at physiological pH, approximately 7.4, at all times, or the cell monolayer integrity will be lost, cells will detach from their surface, or even die (Paul 1970, Phelan 2007). The NCI-N87 cell line has been shown to offer more flexibility in terms of its environmental pH, however. Established from human gastric carcinoma, the NCI-N87 cell line forms a well-differentiated monolayer, which expresses epithelial-type junctions (E-cadherin and ZO-1 expression) and, at 18 days post-confluence, secretes gastric mucin-6 glycoprotein. At this stage the cell line is also acid-resistant at least to pH 3, and up to at least pH 7.4. These factors make this cell line a physiologically relevant model of the gastric epithelium, and indeed it has been used to investigate drug metabolism in the stomach (Park and others 1990, Chailler and Menard 2005, Lemieux and others 2011). This cell line has not yet been used to investigate the uptake and metabolism of dietary flavonoids in the stomach.

1.4 Dissertation Research

1.4.1 Overview and main research objectives

Despite the low bioavailability of anthocyanins, their health benefits are repeatedly shown to be significant. Our limited knowledge about the *in vivo* fate of these compounds hinders our ability to make recommendations about the intake of different anthocyanins for specific beneficial properties. This also hinders progress in developing and marketing anthocyanin-rich functional foods targeted to benefit different tissues. As
we move towards a more thorough understanding about what happens to anthocyanins at each stage of digestion and absorption, we can move towards such recommendations and product innovations.

The fate of anthocyanins in the small intestine and the colon have been extensively studied, and recently published work has carefully looked at what happens to these compounds in the oral cavity, showing that different anthocyanin structures are broken down differently, and that the oral microbiota have a significant effect on metabolism of these compounds (Kamonpatana and others 2013, 2014). Literature also convincingly points towards the stomach as an important site for the absorption of anthocyanins. However, limited information is currently available about the effects of changing pH environments of the stomach, or of anthocyanin structure on the transport of anthocyanins in the stomach, and the potential health effects that may occur in the gastric tissue with exposure to anthocyanins. This is partially due to the lack of an accepted in vitro model of the gastric epithelium- a complicated environment due to its wide fluctuations in pH in vivo, and the lack of stability of most cell culture lines at pH other than approximately 7.4. The NCI-N87 cell line has been shown to maintain monolayer integrity at least as low as pH 3, expresses tight junction proteins, and secretes gastric mucin. These attributes make this cell line a promising potential model for the study of anthocyanin uptake in the stomach.

Although the mechanism has not been well characterized, the evidence for the gastric absorption of anthocyanins is convincing, and must be further investigated so that
it may be utilized for the improvement of gastric health and potentially the development of functional foods.

1.4.2 Central hypothesis and overall research goal

We hypothesize that anthocyanins in foods have beneficial health effects for gastric tissue, and that functional foods rich in anthocyanins can be developed for targeted therapy in the stomach. In order to approach these over-arching goals, we have outlined three main research objectives, to study a potential *in vitro* gastric model using the NCI-N87 cell line, with specific aims outlined below.

The overall goal of this research is to investigate the use of the NCI-N87 cell line as an *in vitro* model to study the gastric transport and uptake of anthocyanins - an important first step towards understanding the effects of anthocyanins on the gastric tissue, and the effects of different gastric environments on the anthocyanins.

1.4.3 Overview of specific aims

Based on our central hypothesis, we have outlined three main research objectives, in italics, and specific aims below each objective:

*I: Determine the feasibility of the NCI-N87 cell line as a model for anthocyanin uptake in gastric tissue.*

  a) Evaluate the stability of chokeberry extract in treatment media over incubation time, at various pH levels.
b) Determine possible cytotoxic effects of chokeberry extract on the NCI-N87 cell line.

c) Ensure the stability of pH differentials at apical pH 3.0 and 5.0, and basolateral pH of 7.4, to mimic various *in vivo* gastric environments over treatment time.

d) Determine the monolayer integrity of the cell line over time post-confluence, and determine monolayer integrity of the cell line at pH 3.0 and pH 5.0, as compared to pH 7.4.

e) Evaluate the effects of the mucus layer on anthocyanin transport across the epithelium.

*II: Evaluate the uptake of anthocyanins in the stomach using the NCI-N87 cell line.*

a) Evaluate the transport of anthocyanins across the cell monolayer over 3h at incubation temperature.

b) Evaluate the differences in transport of anthocyanins from the apical to basolateral chambers with varied apical pH levels (pH 3.0 and 5.0).

c) Investigate the effects of structural differences of anthocyanins on the transport by utilizing chokeberry and red grape extracts: chokeberry to analyze effects of different monosaccharide attachments, and red grape to determine possible differences due to different anthocyanin aglycone structures.
III: Determine the effects of anthocyanins on the induced inflammation of NCI-N87 gastric cells.

a) Using the enzyme-linked immunosorbent assay technique, we will determine the effects of chokeberry extract on the inflammatory response of the cell line.
CHAPTER 2: THE NCI-N87 CELL LINE AS A MODEL OF THE GASTRIC EPITHELIUM TO STUDY ANTHOCYANIN TRANSPORT AND UPTAKE IN THE STOMACH

Abstract

Anthocyanins are flavonoid pigment compounds, which are widely distributed in nature, and are common in the human diet in the form of fruits and vegetables. Health benefits such as anti-inflammatory, antioxidant, and anti-cancer activities have been associated with anthocyanins, but the uptake, bioavailability, and metabolism of these compounds is not yet well characterized. Studies have shown that the stomach may be a site of significant absorption of anthocyanins, but the lack of a physiologically relevant in vitro model has limited research progress. The NCI-N87 cell line may provide a useful model to study anthocyanin transport and uptake in the stomach, as it has been shown to be stable at a pH of as low as 3.0. This research has shown that anthocyanins from chokeberry were not cytotoxic to confluent NCI-N87 cells. Adjustment of the apical pH to 3.0 resulted in an increase of the apical pH over 180 minutes. To maintain an apical pH of 3.0, small increments of HCl were added during treatments, which did not affect the basolateral pH of 7.4. The NCI-N87 cell line formed a tightly coherent monolayer that was stable at pH 7.4 over a period of 18-32 days, and that the monolayer integrity...
was maintained at apical pH 3.0 over a period of 180 minutes. To test the model and
determine if anthocyanins were trapped in the mucus layer secreted by NCI-N87 cells, a
parallel uptake experiment at 0°C and 37°C was performed. Results showed that a small
amount of anthocyanins were trapped in the mucus layer, and that the mucus was
removed by washing. Significantly more cellular uptake was observed at 37°C as
compared to 0°C, suggesting an uptake mechanism involving cellular activity which is
slowed or halted at freezing temperatures, such as active transport. The NCI-N87 cell
line provided a stable in vitro model of the gastric epithelium to study the behavior of
anthocyanins in the stomach.

2.1 Introduction

The lack of a relevant in vitro model of the gastric epithelium has prevented
cellular-level analysis of the uptake of anthocyanins by the stomach. Before attempting
to analyze the differences due to environmental pH or anthocyanin structure on the
uptake in the gastric environment, the NCI-N87 cell line must be confirmed as a
functional model. For this purpose, NCI-N87 cells were grown on porous inserts,
creating an apical chamber and a basolateral chamber, to better represent the in vivo
gastric environment. On inserts, treatments of the cells at a basolateral pH of 7.4 and a
variable apical pH (of 3.0 or 5.0) can better mimic the in vivo environment of the stomach
for future studies, as compared to growing the cells on a non-porous plastic surface. In
addition to information already published on the expression of E-cadherin, ZO-1, and
Mucin-6 by NCI-N87 cells, we must answer several more questions to better characterize this model. The pH of each compartment must be confirmed to remain stable over the time of treatment, and the cell monolayer integrity must be maintained at the desired pH differentials. The effects of the environment in the absence of cells on the stability of the anthocyanin compounds used for treatment must also be investigated. Additionally, the effects of the mucus layer formed by NCI-N87 cells after 18 days post confluence on the uptake of anthocyanins must be determined. Previous work with this cell line has shown that caffeine, a weak base known to be absorbed in the stomach by passive transport, is highly permeable to this cell line in both directions (apical » basolateral; basolateral » apical) and at varying pH from 3.0-7.4 (Eteng 1997, Lemieux and others 2011). Thus, caffeine was used as a positive control to monitor transport across the monolayer. The objective of this study was to show that the NCI-N87 cell line, in this environment, will allow for the study the transport of anthocyanins across the gastric epithelium.

2.2 Materials and Methods

2.2.1 Standards and reagents

Unless otherwise indicated, all supplies and chemicals were purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA).
2.2.2 Cell culture conditions

The human gastric epithelial cell line NCI-N87 is derived from human gastric carcinoma tissue and was purchased from the American Type Culture Collection (Manassas, VA). Cells were initially seeded in 75-cm\(^2\) culture flasks (Corning, Corning NY) and maintained with RPMI-1640 complete medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin. Cells were grown at 37\(^\circ\)C under 5% CO\(_2\). Seeding on plastic and on inserts (0.4 µM pore size) was at a density of 2.5 x 10\(^5\). All experiments were performed on passages 4-30 of the NCI-N87 cell line.

2.2.3 Extraction and purification of chokeberry anthocyanins

Chokeberry juice concentrate was obtained courtesy of Artemis International (Fort Wayne, IN) and stored under dark refrigeration (4\(^\circ\)C) conditions. The anthocyanin-rich chokeberry extract was prepared by the methods of Rodriguez-Saona and Wrolstad (2005) and He and Giusti (2011). Briefly, the juice concentrate was extracted with an equal volume of acetone and filtered (Whatman #1). The residual material was extracted 2x more with 70% acetone to further remove the organic phase. Remaining hydrophobic compounds were removed by extraction with chloroform 2:1 v/v overnight at 4\(^\circ\)C. Excess chloroform in the aqueous phase was then removed by rotary evaporation. The resulting material was then purified by cation exchange (MCX SPE cartridge, Waters Corp.). The column was conditioned with methanol and 0.1% v/v trifluoroacetic acid (TFA) prior to sample loading. Neutral and anionic phenolics were removed with 0.1%
TFA and 0.1% TFA in methanol. Anthocyanins were then eluted with water:methanol 40:60 v/v with 1% NH$_4$OH, followed by methanol with 1% NH$_4$OH into a flask containing 500 µL 88% formic acid to drop the final pH to <2. Total monomeric anthocyanins were determined by the pH differential spectrophotometric method (Giusti and Wrolstad 2005). Chromatographic and mass analysis were performed using an HPLC (Shimadzu; Columbia, Md., U.S.A.) equipped with LC-20AD pumps, SIL-20AC auto sampler, CTA-20A column oven, coupled to an LCMS-2010, SPD-M20A Photodiode Array (Shimadzu), RF-10AXL Fluorescence (Shimadzu), and Mass Spectrometer (Shimadzu) detectors, and LCMS Solution Software (Version 3.0, Shimadzu). Mass spectrometry was conducted on a quadrupole ion-tunnel mass spectrometer equipped with electrospray ionization (ESI) interface (Shimadzu). Anthocyanin separation was achieved on a Symmetry C18 reverse-phase column (4.6 x 75 mm, 3.5 µm; Waters Corp., Milford, MA) fitted with an analytical guard column (NovaPak®, 4 x 2.0mm C18; Waters Corp.). The mobile phases were (A) 4.5% formic acid and (B) acetonitrile. All solvents were filtered through 13-mm 0.45µm polypropylene filters. Solvent gradient for chokeberry anthocyanin analysis follows: 0-3 min, 7-10%B; 3-10 min, 10—20%B.

Samples were quantified by external standard via construction of a 5-point standard curve with cyanidin-3-glucoside and area under the curve (AUC). Statistical analysis was performed with Minitab software (v 16) and Microsoft Excel (2011).
2.2.4 Effects of anthocyanins on cell density

To determine any possible cytotoxic effects of anthocyanins from chokeberry extract on the NCI-N87 cell line, the Sulforhodamine B *in vitro* Toxicology Assay Kit (Sigma Aldrich, St. Louis, MO) was used. This assay is a spectrophotometric measure of protein content, and is used to determine cell density. The protocol provided by the manufacturer was followed, including reading results at the recommended sub-optimal wavelength of 490nm, to reduce interference from possible anthocyanin absorption near 595nm. For the assay, NCI-N87 cells were grown on 96 well plates at a density of $1.0 \times 10^5$ and tested 48h after seeding. Anthocyanins from chokeberry were mixed with HBSS to the desired concentration and added to wells prior to incubation for 180 min at 37°C and 5% CO$_2$.

2.2.5 pH stability of treatment media

For transport experiments, Hank’s Balanced Salt Solution (HBSS) was used as the treatment medium to eliminate anthocyanin/protein interactions. Acidification of HBSS to pH 3.0 and 5.0 was achieved by addition of 6 M HCl and 0.1 M NaOH, not to exceed 0.5% of the total treatment solution. These solutions were stable in the absence of cells at incubation temperature (37°C) over 8 h (data not shown). In order to ensure that the apical and basolateral pH levels remain consistent throughout treatment time, the pH levels of the treatment solution were monitored in the presence of cells, but the absence of anthocyanins, before any treatments were attempted.
2.2.6 Monolayer integrity of NCI-N87 cells on inserts under treatment conditions

One important aspect of determining the validity of the NCI-N87 cell line as a model to study gastric permeability is the formation of tight junctions and the maintenance of monolayer integrity at treatment pH levels. The phenol red assay is a noninvasive, nondestructive measure of confluence and tight junction formation (Jovov and others 1991). Briefly, cells were plated on inserts and grown to 18-27 days post-confluency. The media was removed and both chambers washed with PBS. The basolateral layer was replaced with phenol red-free RPMI, and the apical layer was replaced with high phenol red RPMI (500µM phenol red). At desired timepoints, 150 µL aliquots of the basolateral media were collected and replaced with phenol red-free RPMI. A standard curve was prepared using serial dilutions of high phenol red media. Samples and standards were added to duplicate wells of a 96-well plate. 20 µL of 0.1 N NaOH was added to each well, and the plate shook at medium-intensity for 15 s. The plate was then read at 560 nm, and the phenol red concentration calculated using Excel.

2.2.7 Effects of the mucus layer on the analysis of cellular uptake of anthocyanins by NCI-N87 cells

The NCI-N87 cell line is a mucus-secreting line, which aids in its physiological relevance as a stomach model. However, the thick layer of mucus could trap anthocyanins, and during collection of the cell layer, the mucus must be washed away in order to ensure that any anthocyanin analysis of the cell layer did not overestimate cellular uptake. Washing 3x with cold phosphate buffered saline was shown to be an
effective method of mucus removal. To further investigate the validity of the model and confirm that anthocyanins were being actively taken up by the cells and not just caught in the mucus, a parallel uptake experiment was performed at 0°C versus 37°C. Cellular function is significantly reduced or stopped at 0°C, so if there is a difference in uptake at the different treatment temperatures, it can be reasonably concluded that the uptake is by some kind of active transport mechanism. If, however, there is no difference in uptake between 0°C and 37°C, it is possible that any anthocyanins measured in the cellular layer could simply be accumulating in the mucus layer, and that the washing technique used was not sufficient to separate the mucus from the cells. Anthocyanins were mixed with HBSS to the desired concentrations and added to wells for treatment—either at 37°C or 0°C for 180 min. After treatment, spent media was collected and immediately mixed 1:1 (v/v) with acidified (1% HCl) methanol. Mucus layers were collected by quick-washing x2 with cold PBS, collected and mixed immediately with acidified (1% HCl) methanol. These samples were then vortexed, centrifuged, and filtered with 0.45 µm syringe filters for HPLC analysis. If storage was required, samples were stored in a dark freezer until analysis. Cell samples were collected via cell lifter in PBS, centrifuged, and PBS removed. 0.75 mL of new PBS was added to the cells, which were then lysed and homogenized by sonication. 0.75 mL of extraction solution (70:25:5 acetone: water: formic acid v/v) was then be added and the samples frozen (-20°C) overnight to ensure complete extraction. After thawing, samples were again sonicated, vortexed and centrifuged (14000rpm, 5 min). Supernatant was collected into pre-weighed glass tubes. This extraction was then repeated (except for overnight freezing). Supernatants were
partially dried under N\textsubscript{2} (30 min, 37°C), weighted, and filtered (0.2 µm) for HPLC analysis. Density of samples was approximated to be 1 mg/mL as determined by careful weighing and pipet measurement.

2.3 Results and Discussion

2.3.1 Extraction, characterization, and stability of anthocyanin-rich extracts

The anthocyanin-rich chokeberry extract was made from chokeberry juice concentrate as described, and the anthocyanin profile is shown in the HPLC-PDA chromatogram below (Figure 6).
In agreement with published literature, the predominant anthocyanins in chokeberry were shown to be cyanidin-3-galactoside (68.4%), cyanidin-3-glucoside (3.6%), cyanidin-3-arabinoside (23.3%), and cyanidin-3-xyloside (4.7%). This profile is consistent with published data on the anthocyanin makeup of chokeberries, and confirmed by the mass spectrometry analysis (data not shown) (Wu and others 2004). Chokeberry extract was stable over a period of 24 h at pH 3 and 37°C. No changes in the qualitative or quantitative anthocyanin profile were observed under these conditions.
2.3.2 Effect of anthocyanins on NCI-N87 cell density

Figure 7 below shows the results of cytotoxicity effects of chokeberry anthocyanins on NCI-N87 cells by the Sulforhodamine B assay, which measures cellular protein content to determine cell density.

![Graph showing effect of anthocyanins on cell density](image)

Figure 7: Effect of anthocyanins on cell density of the NCI-N87 cell line as determined by the SRB assay from 50-1500 µM anthocyanin concentration after treatment for 3 h at 37°C.

Figure 7 shows that no significant effect on cell density was shown at an anthocyanin concentration of up to 1500 µM, the amount used for treatments. Based on these results, we can conclude that anthocyanins from chokeberry extract in the
concentration range of 0-1500 µM were not toxic to this cell line under these conditions (37°C, 5% CO₂ for 180 min).

2.3.3 pH stability of treatment media

The pH levels of the apical and basolateral chambers were monitored in the absence of any anthocyanin treatment to ensure pH stability. The pH level of the apical treatment media (HBSS) was found to be prone to change when acidified to pH 3.0. Figure 8 shows the upward trend of the apical pH, and also demonstrates the relative stability of the HBSS in the basolateral chamber. This is most likely due to the high buffering capacity of the treatment media at a pH of approximately 7.4. Apical pH levels of 5.0 and 7.4 were found to be stable and consistent under treatment conditions (data not shown).

In order to combat this rise in pH and maintain a consistent pH over the time of treatment and still mimic the in vitro gastric environment, small aliquots of 6N HCl were added at 30 min and 120 min time points throughout treatment. This method was used successfully by Fernandes and colleagues (2012) on the MKN-28 gastric cell line. The periodical addition of 0.5 µL HCl over the treatment time of 180 min allowed for the maintenance of pH 3 in the apical chamber, while maintaining a basolateral pH of 7.4. A total addition of 1.5 µL 6N HCl during treatment gave a final HCl percentage of no more than 0.05% in the treatment media.
Figure 8: Top panel: Fluctuations in apical pH observed over 3 h at 37°C at beginning apical pH of 3.0 and basolateral pH of 7.4. Bottom panel: Apical and basolateral pH levels during incubation for 3 h at 37°C with NCI-N87 cells at 18 days post-confluence. Apical (no HCl) line shows changing apical pH with no intervention over time. Apical (HCl additions) shows the apical pH achieved by addition of 0.5 µL at time points of 30, 60, and 120 m.
Coupled with the pre-treatment HCl addition, the total HCl content of the treatment media was not more than 0.3% HCl. Other pH levels of interest (apical pH 5.0 and 7.4) were maintained over the time of treatment without the need for this intervention.

2.3.4: NCI-N87 monolayer integrity

To confirm the stability of the NCI-N87 monolayer under treatment conditions, the phenol red diffusion assay was used.

As the top panel of figure 9 shows, the diffusion of phenol red through the NCI-N87 monolayer decreased over time, expressed as days post-confluence (dpc), from 23.87% at 2 days post confluence to 3.17% at 18 days post confluence, and remained stable for at least 32 days post confluence. The trend of these data is consistent with patterns of published transepithelial electrical resistance (TEER) values of this cell line plated on inserts and maintained with RPMI-1640 media, and indicated the formation and maintenance of tight junctions over time (Lemieux and others 2011). Indeed, the work by Lemieux and colleagues showed the expression of tight junction proteins E-cadherin and ZO-1, as previously discussed. The stability of phenol red diffusion from the apical to basolateral layers at 18-32 days post confluence indicated monolayer integrity and stability during this time period. Therefore, future experiments were performed during this window.
Figure 9: Phenol red diffusion from apical to basolateral chamber of NCI-N87 cells at 37°C over time. Top panel is monolayer development over time in days post-confluence at apical and basolateral pH 7.4. Bottom panel is monolayer integrity at apical treatment pH 3.0 over 240 min.
In addition to measuring monolayer integrity over time, it must be determined that the monolayer stays intact at the apical pH values used for treatment. The phenol red assay was performed in parallel to each treatment experiment to ensure any transport of anthocyanins was not strictly due to passive transport through a compromised monolayer. Previous work on this topic showed that the monolayer is able to recover an initial loss of monolayer integrity at pH 3.0, and that the monolayer is stable at an apical pH of 5.0, over treatment time of 3 h (Lemieux and others 2011). The bottom panel of figure 9 shows a similar trend: a slight initial loss of monolayer integrity was quickly recovered at apical pH 3.0. After 180 minutes, the phenol red diffusion was not significantly different than the starting time point, indicating that the monolayer integrity remained intact under these treatment conditions. At apical pH 5.0, no loss of monolayer integrity was observed (data not shown). The data collected here agree with the findings of Lemieux and colleagues, suggesting that the NCI-N87 cell line is likely a viable model for the in vivo environment of the stomach than most cell lines, at least at an apical pH range of 3.0-7.4.

2.4.5: Effects of the NCI-N87 mucus layer on the analysis of anthocyanin uptake by NCI-N87 cells

The results of a parallel uptake study at 0°C and 37°C are shown in Figure 10. NCI-N87 cells were treated with 100 or 200 µM anthocyanins from chokeberry for 180 minutes at either 0 or 37°C. Treatment media, mucus layers, and cell layers were collected after treatment and analyzed by HPLC.
Figure 10: Anthocyanin recovery from media, mucus, and cell layers at 37°C vs 0°C at pH 7.4 on plastic wells.
Results from the parallel uptake experiment to determine the effects of the mucus layer on the analysis of anthocyanin uptake are summarized in Figure 10. At 37°C, anthocyanin uptake was significantly higher in the cell layer than at 0°C in the samples containing 200 µg/mL anthocyanin content. Anthocyanin levels were too low in the cellular layer in the samples treated with 100 µg/mL to measure using these methods. Anthocyanins were detected in the mucus layer in small amounts, suggesting that some anthocyanins may be “trapped” by the mucus, but that the mucus layer can in fact be removed by washing 3x with cold phosphate buffered saline. For future experiments, the mucus layer was removed in this manner before collection and analysis of the cell layer. Additionally, these results showed that the cellular uptake appeared to be halted at 0°C, suggesting an active transport mechanism, which is severely slowed or stopped at freezing temperatures. This agrees with some theories of anthocyanin uptake in the stomach already published, and suggests further that the NCI-N87 cell line is a functional model of the gastric epithelium (Passamonti and others 2002, 2003, Fernandes and others 2013).

Conclusions

This work set out to develop a robust in vitro model using the NCI-N87 cell line, which had previously been shown to maintain monolayer integrity at pH as low as 3.0, and express several proteins associated with gastric tissue. We have shown here methods for developing and utilizing this model, including pH stability of treatment solutions, monolayer integrity of the NCI-N87 cell line, potential effects of the mucus layer on the
analysis of anthocyanin uptake by the cells, and that cellular uptake is significantly slowed at freezing conditions. We have not investigated what effects the mucus layer itself may have on anthocyanin breakdown. Based on this work, the NCI-N87 cell line, plated on 0.4 μm pore inserts and treated at 18-32 days post confluence, is a physiologically relevant and useful model for *in vitro* studies of the transport and uptake of anthocyanins in the stomach at an apical pH range of 3.0-7.4.
CHAPTER 3: EFFECTS OF pH, TIME, AND ANTHOCYANIN CHEMICAL STRUCTURE ON THE TRANSPORT AND UPTAKE OF ANTHOCYANINS THROUGH THE NCI-N87 GASTRIC EPITHELIAL MONOLAYER

Abstract

Anthocyanins are ubiquitous pigments in the human diet. Despite their association with a wide variety of health benefits, their metabolic fate of is not well understood. Studies suggest that anthocyanins absorption may occur in the stomach, where the acidic pH favors anthocyanin stability. We have shown that NCI-N87 cell line can be used to study the behavior of anthocyanins in the gastric environment at a pH range of 3.0-7.4. This work examines the effects of time, concentration, pH, and chemical structure on the transport and uptake of anthocyanins by NCI-N87 cells. Anthocyanins were transported from the apical to the basolateral chamber in a time-and dose dependent manner. Over the treatment time of 3 h, it appeared that the rate of transport increased, especially at higher treatment concentrations. This may suggest an active transport mechanism for the transport of anthocyanins across the NCIN87 monolayer. Effects of pH were examined by adjusting apical pH to 3.0, 5.0, or 7.4. At an apical pH 3.0, more transport was observed compared to pH 5.0 and 7.4. Apical pH 5.0 showed the lowest transport of the tested pH levels. To study the effects of anthocyanin structure on the transport and
uptake by NCI-N87 cells, chokeberry and red grape extracts were used. Chokeberry extract showed that there was a small but significant impact of the monoglycoside attachment in the cases of cyanidin-3-glucoside (significantly less transport) and cyanidin-3-arabinoside (significantly more transport). Additionally, there were significant differences in cellular uptake possibly due to the different monoglycoside attachments. From red grape extract, it was clear that B-ring substitution plays a role in the transport and uptake of anthocyanins by NCI-N87 cells. Each anthocyanin from red grape was differentially transported and taken up by the cells, save for cellular uptake of pelargonidin-3-glucoside.

3.1 Introduction

Due to the interest in the health benefits associated with high anthocyanin intake, many studies of the pharmacokinetics and bioavailability of anthocyanins have been published, with varied results depending on the type of study, the anthocyanin source, the tissue in question, and many other factors (He and others 2005, Talavera and others 2005, Kay and others 2005, Kay 2006, Jing and others 2008, Nurmi and others 2009, Novotny and others 2012). The complex chemistry, relative instability, and behavior variations of anthocyanins in vitro makes the study of their uptake and bioavailability quite difficult. Given their different structures at various pH levels and their susceptibility to breakdown by pH, microbes and enzymes, studies of the rates of uptake, metabolism, and cellular distributions of anthocyanins often give differing results. Still, in order to investigate the
role of these compounds in disease prevention, determining the details of their metabolic fate is a key step. Therefore, the effects of time and initial concentration on the transport and uptake of chokeberry anthocyanins by NCI-N87 cells were investigated here. Chokeberry extract was mixed with HBSS to the desired concentration (ranging from 50-1500 µM) and added to the apical side of the NCI-N87 monolayer. Aliquots from the basolateral side were collected at the desired timepoints (15-180 min) to study how time effects anthocyanin transport.

Anthocyanins exist in a dynamic equilibrium in solution that is profoundly dependent on the environmental pH: under acidic conditions (pH 1.0 to approximately 3.5) the flavylium cation is the most dominant form, and the color is relatively more stable. Approaching a more neutral pH (5-6), the open-ring chalcone or hemiketal form is predominant, and the compound loses its color. Under basic conditions (7 and higher), the quinoidal base is dominant and an unstable blue color is formed (Castaneda-Ovando and others 2009). To investigate the role of pH on the transport and uptake of chokeberry anthocyanins by NCI-N87 cells, the pH of the apical chamber was adjusted to either pH 3.0, 5.0, or 7.4 prior to 180 min incubation in the presence of anthocyanins.

Previous studies have shown that in some tissues, chemical structure can affect the rate of uptake of anthocyanins. Wu and others (2004) compared the metabolism of pelargonidin-3-glucoside and cyanidin-3-glucoside from blackberries in pigs, and found a significantly higher (8 fold) apparent absorption of pelargonidin as compared to cyanidin, as measured by urinary output. In a later study by the same researchers (2005), weanling pigs were fed black currant powder, which contained the 3-O-glucosides and rutinosides
of cyanidin and delphinidin. Analyzing the urinary excretion for these anthocyanin parent compounds and some known metabolites, results showed that cyanidins overall were found in higher quantities over delphinidins. Additionally, when comparing within the same aglycone, total excretion of anthocyanin rutosides was found to be higher than that of anthocyanin glucosides. These data show that both B-ring substitutions on the anthocyanin aglycone, and the sugar moieties, may play a role in the metabolism of anthocyanins. In the oral cavity, chemical structure was shown to have a significant effect on the degradation of anthocyanins by the oral microbiota. Delphinidin and petunidin glycosides were more susceptible to degradation than those of cyanidin, pelargonidin, peonidin and malvidin, suggesting that B-ring substitutions play a significant role in anthocyanin behavior in the mouth (Kamonpatana and others 2012). Passamonti and others (2002) have proposed that anthocyanins are absorbed through the gastric epithelium by the transport protein bilitranslocase. Using rat liver plasma membrane vesicles, they showed that the anthocyanin structure effected the interaction of anthocyanins with the transporter: anthocyanin mono and di-glycosides were better ligands for bilitranslocase than the corresponding anthocyanin aglycones. Additionally, the B-ring substitutions played a role when comparing various anthocyanin monoglycosides: increasing degrees of methylation increased the anthocyanin’s affinity for bilitranslocase. Results of this work consistently found that decreasing polarity of the anthocyanin was associated with higher affinity for the transporter. Interestingly, this work also found that 3-O-monogalactoside attachments resulted in entirely different interaction with the transporter compared with other monoglycosides. While most
anthocyanins were shown to be competitive inhibitors of bilitranslocase, cyanidin-3-galactoside acted as a non-competitive inhibitor with a significantly lower affinity for the carrier.

It is clear from these studies that both the aglycone structure and various common sugar attachments can play roles in the in vivo activity of anthocyanins. To investigate the role of chemical structure on the transport and uptake of anthocyanins by NCI-N87 cells, chokeberry and red grape extracts were used. The anthocyanin profile of chokeberry includes four major cyanidin-3-O-monoglycosides, while that of red grape extract includes the six dietary anthocyanin aglycones, all with 3-O-glucoside attachments.

The objective of this work was to determine the effects of time, initial concentration, apical pH, and chemical structure on the transport and uptake of anthocyanins by NCI-N87 gastric cells.

3.2 Materials and Methods

3.2.1 Standards and reagents

Unless otherwise indicated, all supplies and chemicals were purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Anthocyanin extracts were prepared from chokeberry juice concentrate, donated by Artemis International (Fort Wayne, IN), and red grapes obtained from a local market (Kroger, Columbus OH). Extracts were stored under dark freezing (-20°C) conditions.
3.2.2 Extraction and purification of anthocyanins from chokeberry and red grape

Chokeberry juice concentrate was obtained courtesy of Artemis International (Fort Wayne, IN) and stored under dark refrigeration (4°C) conditions. Red grapes were purchased from a local market (Kroger Co., Columbus, OH). The anthocyanin-rich red grape and chokeberry extracts were prepared by the methods of Rodriguez-Saona and Wrolstad (2005) and He and Giusti (2011). Briefly, the juice concentrate was extracted with an equal volume of acetone and filtered (Whatman #1). Red grape skins were frozen with liquid nitrogen and blended to a fine powder, then extracted with an equal volume of acetone and filtered (Whatman #1). The residual materials were extracted 2x more with 70% acetone to further remove the organic phase. Remaining hydrophobic compounds were removed by extraction with chloroform 2:1 v/v overnight at 4°C. Excess chloroform in the aqueous phase was then removed by rotary evaporation. The resulting material was then purified by cation exchange (MCX SPE cartridge, Waters Corp.). The column was conditioned with methanol and 0.1% v/v trifluoroacetic acid (TFA) prior to sample loading. Neutral and anionic phenolics were removed with 0.1% TFA and 0.1% TFA in methanol. Anthocyanins were then eluted with water:methanol 40:60 v/v with 1% NH₄OH, followed by methanol with 1% NH₄OH into a flask containing 500 µL 88% formic acid to drop the final pH to <2.

3.2.3 Cell culture conditions

The human gastric epithelial cell line NCI-N87 is derived from human gastric carcinoma tissue and was purchased from the American Type Culture Collection.
(Manassas VA). Cells were initially seeded in 75-cm² culture flasks (Corning, Corning NY) and maintained with RPMI-1640 complete medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin. Cells were grown at 37°C under 5% CO2. Seeding on plastic and on inserts was at a density of 2.5 x 10⁵. All experiments were performed on passages 4-30.

3.2.4 HPLC analyses of apical and basolateral samples

After treatment, spent media was collected and immediately mixed 1:1 (v/v) with acidified (1% HCl) methanol. Mucus layers were removed by quick-washing 3x with cold PBS. Apical and basolateral samples were vortexed, centrifuged, and filtered with 0.2 µm nylon syringe filters for HPLC analysis. Samples were stored in a dark -20°C freezer until analysis; however when possible analysis was conducted immediately after collection. Chromatographic and mass analysis were performed using an HPLC (Shimadzu; Columbia, Md., U.S.A.) equipped with LC-20AD pumps, SIL-20AC auto sampler, CTA-20A column oven, coupled to an LCMS-2010, SPD-M20A Photodiode Array (Shimadzu), RF-10AXL Fluorescence (Shimadzu), and Mass Spectrometer (Shimadzu) detectors, and LCMS Solution Software (Version 3.0, Shimadzu). Mass spectrometry was conducted on a quadrupole ion-tunnel mass spectrometer equipped with electrospray ionization (ESI) interface (Shimadzu). Anthocyanin separation was achieved on a Symmetry C18 reverse-phase column (4.6 x 75 mm, 3.5 µm; Waters Corp.) fitted with an analytical guard column (NovaPak®, 4 x 2.0mm C18). The mobile phases were (A) 4.5% formic acid and (B) acetonitrile. All solvents were filtered through
13-mm 0.45µm polypropylene filters. Solvent gradient for chokeberry anthocyanin analysis follows: 0-3 min, 5-10% B; 3-10 min, 10—20% B; 10-15 min, 20-35% B; 15-20 min, 35% B. For red grape, the solvent gradient was as follows: 0-3 min, 7% B; 3-6 min, 7-12% B; 6-12 min, 12% B; 12-15 min, 12-35% B; 15-20 min, 35% B.

Samples were quantified by external standard via construction of a 5-point standard curve with cyanidin-3-glucoside (0-1500 µM) and area under the curve (AUC). Statistical analysis was performed with and Microsoft Excel (2011). Unless otherwise noted, all experiments were n=6 and each sample was performed in duplicate.

3.2.5 Anthocyanin extraction from NCI-N87 cells

Cell samples were collected via cell lifter in PBS, centrifuged, and PBS removed. 0.75 mL of new PBS was added to the cells, which were then lysed and homogenized by sonication. 0.75 mL of extraction solution (70:25:5 acetone: water: formic acid v/v) was added and the samples frozen overnight. After thawing, samples were again sonicated, vortexed and centrifuged (14000rpm, 5 min, 0°C). Supernatant was then collected into carefully pre-weighed glass tubes. This extraction was repeated 2x (except for overnight freezing). Supernatants were partially dried under N2 (30-45 min, 37°C), weighted, and filtered (0.2 µm) for HPLC analysis. Density of samples was approximated to be 1 mg/mL by careful weighing and pipet measurement. Samples were analyzed by HPLC under the same conditions as apical and basolateral samples.
3.2.6 Effects of concentration and time on the transport of anthocyanins through the NCI-N87 monolayer

For treatments, chokeberry extract was mixed with HBSS to obtain the desired concentrations (50-1500 µM) and adjusted to pH 3.0 with 6.0 N HCl. Media was removed from apical and basolateral chambers, and all compartments washed with HBSS, then replaced with pH 7.4 HBSS (basolateral chambers) and chokeberry extract-containing pH 3.0-treatment solution (apical chambers). Aliquots of the basolateral chambers were removed at timepoints of 15m, 30m, 60m, 120m, and 180m, and replaced with pH 7.4 HBSS. After 180m, apical and basolateral samples were collected. Samples were immediately mixed 1:1 with 1% HCl in MeOH, vortexed and centrifuged. Cell layers were also collected at 180 min as described above. Samples were collected in triplicate and analyzed by HPLC as described above.

3.2.7 Effects of pH on the transport of anthocyanins through the NCI-N87 monolayer

For experiments, HBSS was adjusted to the desired pH (3.0, 5.0, 7.4) by 6.0 N HCl and 1.0 N NaOH after mixing with purified, concentrated chokeberry extract to obtain the desired treatment concentration. Media was removed and all chambers were washed with HBSS prior to treatment. pH 7.4 HBSS was added to basolateral chambers, and treatment solution of the desired pH and chokeberry anthocyanin concentration was added to apical chambers. Apical pH was monitored throughout the 180 min treatment to ensure stability and consistency. After 180 min treatment at 37°C and 5% CO2 apical,
basolateral, and cell layers were collected, processed, and analyzed by HPLC-MS as described above.

3.2.8 Effects of anthocyanin chemical structure on the transport of anthocyanins through the NCI-N87 monolayer

To investigate the effects of the sugar moiety on transport and uptake of NCI-N87 cells, chokeberry extract was used. The anthocyanin profile of chokeberry includes four predominant anthocyanins, all cyanidin-3-monoglycosides. In addition to the use of chokeberry extract, red grape extract was used to investigate possible structural effects of B-ring substitutions on the uptake of anthocyanins in the stomach. Red grape extract contains each of the six main dietary anthocyanin aglycones, all attached to a 3-O-monoglucoside sugar moiety. Anthocyanin treatments were mixed with HBSS to the desired concentration and added to the apical side of the NCI-N87 monolayer prior to incubation for 180 min at 37°C and 5% CO₂.

3.2.9 Statistical Analysis

Statistical analysis was performed in Microsoft Excel (2011). Paired t-tests were used to obtain p-values to determine statistical significance. P values of 0.05 or less were considered significant. All samples were either duplicate or triplicate, with an n ≥ 6.
3.3 Results and Discussion

3.3.1 Effects of time and initial concentration on the transport and uptake of anthocyanins by NCI-N87 cells

In order to investigate the effects of initial anthocyanin concentration and time on the transport and uptake of anthocyanins by NCI-N87 cells, chokeberry extract was added to the apical side of the cell monolayer at varying concentrations (50-1500 µM). Aliquots of the basolateral layer were removed at the desired timepoints (15-180 min), and the apical, basolateral, and cell layers were collected after 180 mins for HPLC analysis. Figure 11 shows the effects of time and initial concentration on the transport of anthocyanins from chokeberry extract from the apical to the basolateral chamber of NCI-N87 cells, in terms of the basolateral anthocyanin concentration (µM). Treatment was at an apical pH of 3.0 and basolateral pH 7.4, over 180 min at 37°C.
Figure 11: Effect of time on the concentration (µM) of anthocyanins through the NCI-N87 monolayer (apical to basolateral chambers) at apical pH 3.0, with varying initial concentrations over 3 h at 37°C.
At all initial apical concentrations, the concentration of anthocyanin transported from the apical to basolateral chamber increased over time. Anthocyanins were detected in the basolateral chamber after just 15 min at initial concentration of 200 µM (1.0 µM), 500 µM (1.2 µM), 1000 µM (1.2 µM), and 1500 µM (1.4 µM). At the initial concentration of 50 µM, anthocyanins were not detected at 15 min. At 120 min, significant differences in the anthocyanin concentrations in the basolateral chamber were observed for some of the initial concentrations: 1500 µM treatment concentration had a significantly higher concentration (88.7 µM) than 1000 µM treatment (19.2 µM), which was higher than the 500 µM treatment (6.4 µM) (p < 0.05). At 180 min, significant differences were observed between all initial concentrations: 3.7, 6.2, 16.2, 52.6, and 196.2 µM in the basolateral chamber at 50, 200, 500, 1000, and 1500 µM initial concentration, respectively (p < 0.05).

Another observation of figure 11 is that, especially at the higher concentrations of 1000 and 1500 µM initial apical concentration, the rate of transport over time seems to increase in a non-linear way. This could suggest increased activity by a possible active transport mechanism. If the anthocyanins were moving from the apical to the basolateral side by passive diffusion alone, the relationship between time and the basolateral concentration would be approximately linear. While this speculation would agree with some of the existing literature, more information about the mechanism of transport of anthocyanins through the NCI-N87 monolayer is needed (Passamonti and others 2002, 2003, 2005; Fernandes and others 2013). An additional observation from the data in figure 11 is that at the 1500 µM concentration at 180 min, the transport does
not appear to be at a plateau, suggesting that if a transport protein is in fact involved, these conditions have not saturated it. More research, perhaps with much higher concentrations of anthocyanins, or a longer incubation period, is needed to determine if and when the transport of anthocyanins through the NCI-N87 monolayer does plateau—possibly at a higher initial concentration than 1500 µM, or for a longer treatment time than 180 min, or both.

The data presented in figure 11 support existing literature showing the rapid appearance of anthocyanins in the bloodstream after ingestion in animal models and human studies, all suggesting that gastric uptake is likely a significant step in the metabolism of anthocyanins. The time-dependent transport of anthocyanins in the stomach is an important factor when discussing the use of anthocyanins for gastric health, as time spent in the stomach by consumed food can vary greatly from person-to person, and even within individuals, depending on their fed/fasted state. These factors are out of the scope of this work, but will be important for future possible recommendations on the intake of anthocyanins, or for the development of anthocyanin-rich functional foods, for gastric health.
Figure 12: Effect of initial anthocyanin concentration on the transport efficiency (%) of anthocyanins through the NCI-N87 monolayer over 3 h at 37°C at apical pH 3.0 and basolateral pH 7.4. Initial caffeine concentration was 1500 µM. Transport efficiency was determined by (Anthocyanin concentration in basolateral chamber/initial apical concentration) x 100.
Figure 12 details the effects of the initial apical anthocyanin concentration on the transport efficiency (%) from the apical (pH 3.0) to basolateral (pH 7.4) chambers, through the NCI-N87 monolayer over 3 h at 37°C. Transport efficiency was calculated as (concentration in the basolateral chamber/ initial anthocyanin concentration) x 100. Though the concentrations in the basolateral side increased as expected with time and concentration (figure 11), some interesting behavior emerged when the transport efficiencies were calculated. At an initial concentration of just 50 µM showed significantly higher transport efficiency than 200 and 500 µM initial concentrations (p<0.05). An initial concentration of 1000 µM was not significantly different in transport efficiency % than 50 µM, while an initial concentration of 1500 µM showed significantly higher transport efficiency % than all other initial concentrations. As expected, the caffeine control had significantly higher transport efficiency than all anthocyanin concentrations, as caffeine is passively diffused through the gastric monolayer. Notably, the transport efficiency of phenol red was not significantly different from that of the 1000 µM sample, and was higher than that of the 200 and 500 µM samples. Only the initial concentrations of 50 and 1500 µM were significantly higher than phenol red at apical pH 3.0.

The pattern of the transport efficiency raises several questions about the behavior of anthocyanins in this model, and about the relationship between the initial anthocyanin concentration and the mechanism of transport through the NCI-N87 monolayer. Although the actual concentrations in the basolateral compartment follow the expected dose-response trend, the transport efficiencies could suggest that the mechanism is more
complicated than a simple protein transporter, or that there may be a combination of diffusion and active transport taking place.

In addition to monitoring the transport of anthocyanins through the NCI-N87 cell monolayer, the amount of anthocyanins taken up by the cells was analyzed by collecting the cell layer after treatment and extracting the anthocyanins for HPLC analysis. The anthocyanin amount extracted significantly increased with each initial anthocyanin concentration (p < 0.05) (Figure 13).
Figure 13: Effect of initial anthocyanin concentration (µM) on the total anthocyanin amount (µg) extracted from the NCI-N87 cell layer after 3 h treatment at 37°C at apical pH 3.0 and basolateral pH 7.4. Initial caffeine concentration was 1500 µM.
The effects of initial anthocyanin concentration in the apical chamber (pH 3.0) on the total anthocyanin amount (µg) extracted from the NCI-N87 cell monolayer showed a dose dependent relationship, as more anthocyanins were able to be extracted from the cells at each increasing initial concentration of 50, 200, 500, 1000, and 1500 µM. The initial concentration of caffeine was 1500 µM, though the amount of caffeine extracted from the cells was relatively low, only statistically similar to the initial anthocyanin concentration of 50 µM. Caffeine is passively diffused through the gastric monolayer, and not necessarily taken up or metabolized by gastric cells (Liguori and others 1997). At 50 µM initial concentration, some replicates showed anthocyanin peaks, and others were not detected under the analytical conditions presented here. The large standard deviation associated with this data set indicates that consistent results were not obtained under these conditions, though anthocyanins were sometimes detected. The data presented in figure 13 show that NCI-N87 cells do take up anthocyanins from chokeberry extract, and that the amount of uptake is dependent on concentration at least between 50-1500 µM.

Though evidence of anthocyanin transport through the gastric epithelium is convincing, existing data on the uptake or accumulation of anthocyanins by stomach tissue is limited. Talavera and others (2005) fed rats a high anthocyanin diet (14.8 mmol/kg for 15 days) from blueberry and found that just .0005% of the ingested anthocyanin accumulated in the stomach tissue. He and others (2009) observed a red color of gastric tissue samples of rats after administration of black raspberry anthocyanins. The presence of anthocyanins in the gastric tissue was confirmed by
observing spectroscopic changes at pH 1.0, 4.5, and 10, but the anthocyanins appeared to be bound irreversibly to some sort of gastric protein. For this reason, the anthocyanins in the gastric tissue could not be quantified as free anthocyanins, and the researchers hypothesized that the protein bound to the anthocyanins may in fact be the transporter protein responsible for anthocyanin uptake in the stomach. This theory corresponds to an observation from the current study: even after multiple extractions, red/purple color was still observable with the naked eye in some cell samples. This suggests that, while the extraction method used here has been shown to have efficiency of approximately 85% (Kamonpatana 2012), some anthocyanins may still be attached to the cells, possibly bound to the transporter, or some other undetermined protein.
Figure 14: Efficiency (%) of anthocyanin uptake by NCI-N87 cells at varying initial apical concentrations, at apical pH 3.0 and basolateral pH 7.4, after 180 min treatment at 37°C.
Figure 14 shows the efficiency (%) of anthocyanin uptake by NCI-N87 cells, as calculated by \((\text{concentration of anthocyanins extracted from cells} / \text{initial anthocyanin concentration}) \times 100\). The cells were collected after 180 min treatment at 37°C at an apical pH of 3.0 and basolateral pH of 7.4. As with the transport efficiency data, the uptake efficiency was not a dose-response effect with respect to initial anthocyanin concentration, but the patterns did show consistency. At 50 µM initial concentration, the uptake efficiency was higher than that of initial concentrations of 200, 500, and 1000 µM. 1500 µM initial concentration showed the highest uptake efficiency, as was the case with transport efficiency \((p < 0.05)\). Despite the large standard deviation associated with the 50 µM uptake efficiency due to extremely low anthocyanin amounts and the limits of detection of the analytical methods used here, it was still calculated to be significantly higher \((0.41\%)\) than the uptake efficiencies for 200, 500, and 1000 µM \((0.17, 0.14, \text{and } 0.14\%, \text{respectively})\). Additionally, the uptake efficiency at 1500 µM \((0.25\%)\) was not significantly different than that of 50 µM.

3.3.2 Effects of pH on the total recovery, and transport and uptake of anthocyanins by NCI-N87 cells

The apical pH was adjusted to pH 3.0, pH 5.0, or pH 7.4, while the basolateral pH was maintained at pH 7.4, to mimic some of the \textit{in vivo} gastric conditions that may be encountered by ingested anthocyanins. Lemieux and others (2011) showed that the NCI-N87 cell monolayer maintains its integrity at apical pH of 3.0, but below that level, the
monolayer began to lose its transepithelial electrical resistance, another measure of monolayer integrity.

Table 2 below shows anthocyanin recovery at pH 3.0, 5.0, and 7.4 after 3 h treatment at 37°C from each of the basolateral, apical, and cellular layers. Apical pH of 3.0 provided the highest total anthocyanin recovery (91.688% +/- 3.243), as the intact flavylium cation form of the anthocyanin is predominant at this lower pH. At pH 5.0, some anthocyanin loss was expected, as the chalcone form of the anthocyanin was most likely predominant, leading to increased breakdown. As expected, total anthocyanin recovery at pH 5.0 was 81.607% +/- 4.434, lower than at pH 3.0. Furthermore, anthocyanin loss at pH 7.4 was also greater than both pH 3.0 and pH 5.0. Recovery at pH 7.4 was measured as 75.385% +/- 4.112. There was over a 10% difference in recovery from the apical chamber between treatment pH of 3.0 and 7.4, showing that pH did have an important effect on the anthocyanin structural equilibrium in solution. There was also a much higher recovery rate from the basolateral chamber at pH 3.0 (12.212% +/- 0.897) than at pH 5.0 (2.789% +/- 0.441) or pH 7.4 (6.303 +/- 0.076).
Table 2: Anthocyanin recovery (%), from initial anthocyanin addition, from apical, basolateral, and cell layers at varying apical pH values after 3 h treatment at 37°C and an initial anthocyanin concentration 1500µM. Values are averaged triplicate samples +/- standard deviation, n = 6.

<table>
<thead>
<tr>
<th></th>
<th>pH 3.0 (SD)</th>
<th>pH 5.0 (SD)</th>
<th>pH 7.4 (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apical</strong></td>
<td>78.6 (2.6)</td>
<td>77.6 (4.3)</td>
<td>68.5 (2.9)</td>
</tr>
<tr>
<td><strong>Basolateral</strong></td>
<td>12.2 (0.89)</td>
<td>2.8 (0.44)</td>
<td>6.3 (0.08)</td>
</tr>
<tr>
<td><strong>Cell</strong></td>
<td>0.86 (0.04)</td>
<td>1.3 (0.06)</td>
<td>0.59 (0.01)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>91.7 (3.2)</td>
<td>81.6 (4.4)</td>
<td>75.4 (4.1)</td>
</tr>
</tbody>
</table>
Figure 15: Effect of changing apical pH on the transport of chokeberry anthocyanins through the NCI-N87 cell monolayer after 3 h at 37°C and basolateral pH 7.4 as determined by anthocyanin concentration in basolateral chamber. Values are averages of triplicate samples presented as averages, with error bars of standard deviation; n = 6.
The findings presented in Table 2 begin to suggest that pH may play a significant role in the stability of anthocyanins in the various possible gastric environments, but also in the transport of anthocyanins from the apical to basolateral chambers. Since at pH 3 the flavylium cation is likely a predominant anthocyanin structure, these results suggest that this form may be preferentially transported through the NCI-N87 cell monolayer. Table 2 also shows the percent recovery of anthocyanins from the cell layers. At pH 3.0, recovery from the cells was 0.861% +/- 0.043, significantly lower than at pH 5.0, which showed the highest cellular recovery at 1.252% +/- 0.059. Recovery from the cell layer at pH 7.4 was also significantly lower than at pH 5.0 (0.591% +/- 0.009).

Table 2 also shows the percent recovery of anthocyanins from the cell layers. At pH 3.0, recovery from the cells was 0.861% +/- 0.043, significantly lower than at pH 5.0, which showed the highest cellular recovery at 1.252% +/- 0.059. Recovery from the cell layer at pH 7.4 was also significantly lower than at pH 5.0 (0.591% +/- 0.009).

Figure 15 shows the effect of apical pH on the transport of chokeberry anthocyanins through the NCI-N87 monolayer as measured by the anthocyanin concentration in the basolateral chamber after 180 min treatment at 37°C. Each pH level showed an overall dose dependent response, but the pH effects were not uniform over all pH and concentration levels. At pH 3.0, anthocyanin concentration in the basolateral chamber was significantly higher at initial apical concentrations of 50 and 1500 µM (3.65µM +/- 0.77 and 196.25µM +/- 3.25, respectively) as compared to the other pH levels at the same concentrations (pH 5.0: 2.38µM +/- 0.098 and 41.83µM +/- 2.15 for 50 and 1500 µM, and for pH 7.4: 2.03µM +/- 0.15 and 94.55µM +/- 2.33 for 50 and 1500 µM, respectively). At initial concentration of 500 µM, pH 3.0 and 7.4 were not significantly different from each other (16.22µM +/- 3.78 and 24.17µM +/- 5.72, respectively), but were both significantly higher than pH 5.0 (7.83µM +/- 1.48) (p<0.05). At 50 µM, pH 5.0 and pH 7.4 were not significantly different. At 1500 µM, pH 3.0 was
significantly higher than both other pH levels, followed by pH 7.4, and pH 5.0 was the lowest transport at the higher concentration (p<0.05). These results suggest a significant effect overall of apical pH, with the most obvious difference being significantly higher transport at pH 3.0 at the higher 1500 µM treatment concentration. The pH 5.0 treatment also, overall, showed lower transport than the other pH levels at all treatment concentrations. The exception to this trend is that at initial concentration of 50 µM, pH 5.0 and pH 7.4 treatments did not show significantly different transport. One possible explanation for this trend is that the chalcone form of the anthocyanins present in chokeberry extract, most prevalent at pH 5.0, may be transported at a lower rate due to its open-ring formation. As the transport of anthocyanins across the monolayer is most likely via active transport as previously discussed, the interaction between the anthocyanin and the transport protein would likely be affected by this change in structure.
Figure 16: Effect of apical pH on the transport efficiency of chokeberry anthocyanins through the NCI-N87 monolayer over 3 h at 37°C at basolateral pH 7.4. Transport efficiency % was calculated as (Measured anthocyanin concentration in basolateral chamber/ Initial anthocyanin concentration in apical chamber) x 100. Values are with error bars of standard deviation; n=6 with 3 repeated measurements.
These findings are mostly supported by transport efficiency [(concentration in basolateral chamber/ initial concentration) x 100] data. Figure 16 shows the effects of varying apical pH (3.0, 5.0, 7.4) on the transport of chokeberry anthocyanins through the NCI-N87 monolayer at a constant basolateral pH of 7.4. At apical pH of 3.0, treatments of 50 and 1500 µM anthocyanins at the apical side resulted in the highest transport efficiency of all experiments (7.31% +/- 0.21 and 13.08% +/-0.73, respectively). Apical pH 5.0 resulted in significantly lower transport efficiency at all treatment concentrations (4.75% +/- 0.48, 1.57% +/- 0.055, 2.79% +/- 0.098 for initial concentrations of 50, 500 and 1500 µM respectively; p<0.05). Interestingly, at initial concentration of 500 µM, the pH 3.0 and pH 7.4 were not statistically different from each other (3.24% +/- 0.021 and 4.83% +/- 1.14 respectively), nor were the 50 and 500 µM treatments at pH 7.4 (4.05% +/- 0.305 and 4.83% +/- 1.14, respectively) different from each other. At pH 7.4, the most predominant anthocyanin structure in solution is the quinonoidal base, a closed-ring flavan-3-ol similar in structure to the flavylium cation, but without the positive charge associated with the more stable cation (Houbiers and others 1998).
Figure 17: Effects of apical pH on the cellular uptake efficiency (%) of NCI-N87 cells after 3 h treatment at 37°C and basolateral pH 7.4. Uptake efficiency was calculated as (Concentration extracted from cell layer/Initial treatment concentration) x 100. Values are presented with standard deviation as error bars; n = 6 with 3 repeated measurements.
Figure 17 shows the cellular uptake efficiency as affected by changing apical pH over 3 h at 37°C, with a constant basolateral pH 7.4, at initial anthocyanin concentrations of 500 and 1500 µM. At apical concentration of 50 µM, the anthocyanin level in the cell layer was difficult if not impossible to detect under the analytical conditions used here. Apical pH again appeared to play some role in anthocyanin uptake. At 500 µM, pH 3.0 had significantly higher uptake efficiency (0.485% +/- 0.083) as compared to both pH 5.0 and pH 7.4 (0.277% +/-0.006 and 0.183% +/- 0.024, respectively). This suggests that the cell may preferentially take up the flavilyum cation form of the anthocyanin. However, at the higher initial anthocyanin concentration of 1500 µM, the uptake at pH 3.0 (0.861 +/- 0.012) was not significantly different than that at pH 5.0 (0.868% +/- 0.048). Both of these pH levels were significantly higher than uptake at pH 7.4 (0.591% +/-0.032) at 1500 µM initial concentration. This may suggest that the cells take up both the flavilyum cation and the chalcone form of the anthocyanin with equal affinity at higher concentrations, but not at the lower 500 µM treatment concentration. Another possibility is a shift in the equilibrium of the structures, possibly with more anthocyanins in the 1500 µM pH 5.0 sample forming flavilyum cations when exposed to the cells. The lower uptake at pH 7.4 may indicate that the NCI-N87 cells take up the quinoidal base form of the anthocyanin, or it may be a result of the greater overall anthocyanin loss observed at the pH 7.4 treatment (Table 2), as at this pH level, anthocyanins are generally less stable and a lower proportion of the anthocyanins in solution are in the flavilyum cation form.

These results also indicate that the mechanism for cellular uptake appears to differ from that of the mechanism for transport at the higher concentration level of 1500 µM, as
the pattern for transport efficiency (Figure 16) does not follow the same pattern as cellular uptake (Figure 17). The pattern is similar, however, at the treatment concentration of 500 µM. The increased cellular uptake at pH 5.0 at the higher concentration may again suggest a shift in the structural equilibrium of the anthocyanins, or a change in the uptake mechanism by the cells themselves, possibly triggered by the higher anthocyanin concentration at 1500 µM.

3.3.2 Effects of anthocyanin chemical structure on the transport of anthocyanins through the NCI-N87 monolayer

The use of chokeberry extract, with 4 different cyanidin-monoglycosides, and red grape, with 6 different anthocyanin-monoglucosides, allows us to begin an investigation into the effects of chemical structure on the transport and uptake of anthocyanins in this model system of the stomach. Given the wide variety of anthocyanin structures found in nature, and the various effects that the variable pH of the stomach has on the structural equilibrium of anthocyanins in solution, these studies are only the beginning of the investigation into how structure of anthocyanins can affect their reactions with gastric cells. Additionally, the effects of chemical structure on the potential health benefits of anthocyanins for the stomach will require more investigation, outside of the scope of this work.
Table 3: Individual anthocyanin concentration (mg/L) and percent transported from the apical to basolateral compartments, at apical pH 3.0 and basolateral pH 7.4, after 180 min treatment and initial anthocyanin concentration of 1500 µM (727 mg/L). Values are averages +/- standard deviation; n = 6 with 3 repeated measurements.

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Apical mg/L (SD)</th>
<th>Basolateral mg/L (SD)</th>
<th>Transported (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin-3-galactoside</td>
<td>349.7 (5.8)</td>
<td>45.5 (2.78)</td>
<td>13.0</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>24.2 (0.3)</td>
<td>3.0 (0.21)</td>
<td>12.4</td>
</tr>
<tr>
<td>Cyanidin-3-arabinoside</td>
<td>155.4 (3.2)</td>
<td>23.1 (1.07)</td>
<td>14.9</td>
</tr>
<tr>
<td>Cyanidin-3-xyloside</td>
<td>28.1 (0.59)</td>
<td>3.9 (0.03)</td>
<td>14.2</td>
</tr>
</tbody>
</table>
Table 3 displays anthocyanin amounts as quantified by individual peak area in mg/L (cyanidin-3-glucose equivalents) in the apical (pH 3.0) and basolateral chambers, and also includes calculated percent transported of each anthocyanin from apical to the basolateral chamber. The data presented in Table 3 are averages of samples with an anthocyanin concentration of 1500 µM, corresponding to 727 mg/L anthocyanin added initially. These numbers show that only cyanidin-3-glucoside and cyanidin-3-arabinoside were differentially transported through the NCI-N87 monolayer. Cyanidin-3-glucoside (12.43%) was transported at a lower proportion as compared with the other cyanidin-monoglycosides, while cyanidin-3-arabinoside (14.889%) was transported at a significantly higher proportion. Previous work has also suggested that anthocyanin arabinosides may be differently transported and metabolized, as discussed previously (Wu and others 2002, Talavera and others 2004).

The data in table 3 suggest a potential trend regarding the six-carbon versus five-carbon sugar attachments. The six-carbon glucose attachment had the least percent transport, and the five-carbon arabinose attachment had the highest percent, but the six-carbon galactose and the five-carbon xylose were not differentially transported. Glucose and galactose are stereoisomers, meaning the transport differences between the two may either be due to the different initial relative concentrations of each anthocyanin, or due to the slight difference in stereochemistry between the two. Passamonti and others (2002) also observed differing behaviors of anthocyanin-galactosides versus anthocyanin-glucosides in their interactions with bilitranslocase. They observed that cyanidin-3-glucoside acted as a competitive inhibitor of bilitranslocase transport activity, while
cyanidin-3-galactoside seemed to act as a non-competitive inhibitor, despite their very similar structures. The five-carbon sugar attachments of xylose and arabinose showed slightly higher transport, with cyandin-3-arabinoside significantly transported at a higher percentage to the basolateral chamber (p < 0.05). Looking back at the initial concentration of each anthocyanin in the original chokeberry extract, cyandin-3-arabinoside, the highest percent transported, constituted approximately 23.3%, the second-highest relative concentration. Cyandin-3-glucoside, the lowest percent transported, also represents the lowest percent anthocyanin in the original extract, at 3.6%. Cyanidin-3-galactoside is the predominant anthocyanin from the original extract at 68.4%, and cyanidin-3-xyloside was originally 4.7%. It is possible that five-carbon sugar attachments are preferentially transported, and the effect was significant only in cyanidin-3-arabinoside because it was more predominant in the treatment extract. More data, with varying initial concentrations of anthocyanins attached to five and six-carbon sugars, are needed to determine if the initial concentration, or the different stereochemistries, are more significant in determining transport rates.
Table 4: Individual anthocyanin amounts (mg/L) and percent uptake from the apical compartment to the cellular layer, after 180 min at apical pH 3.0 and basolateral pH 7.4 and initial anthocyanin concentration of 1500 µM (727 mg/L) from chokeberry extract. Values are averages +/- standard deviation; n = 6 with 3 repeated measurements.

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Apical mg/L (SD)</th>
<th>Cells mg/L (SD)</th>
<th>Uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin-3-galactoside</td>
<td>3497 (5.8)</td>
<td>6.9 (0.30)</td>
<td>1.97</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>24.1 (0.3)</td>
<td>1.5 (0.04)</td>
<td>5.99</td>
</tr>
<tr>
<td>Cyanidin-3-arabinoside</td>
<td>155.4 (3.2)</td>
<td>4.1 (0.15)</td>
<td>2.64</td>
</tr>
<tr>
<td>Cyanidin-3-xyloside</td>
<td>28.1 (3.6)</td>
<td>1.0 (0.02)</td>
<td>3.45</td>
</tr>
</tbody>
</table>
Table 4 shows individual anthocyanin amounts (mg/L) in the apical chamber as compared with anthocyanin amounts extracted from the cellular layer, and the percent change from the apical to the cellular layer, for each individual anthocyanin. The initial anthocyanin concentration was 1500 µM, or 1.454 mg anthocyanin per well. Cyanidin-3-glucoside has the highest relative uptake (5.99%), followed by cyanidin-3-xyloside (3.445%), cyanidin-3-arabinoside (2.64%), and cyanidin-3-galactoside (1.971%). Although cyanidin-3-galactoside was present in the highest amount (6.894 mg/L) in the cell layer, proportionally it was taken up at a lower percentage as compared to the other anthocyanins present in chokeberry. The different pattern of cellular uptake as opposed to apical to basolateral transport may suggest that the NCI-N87 cells have different mechanisms of distinguishing anthocyanins for uptake versus those that are transported through the monolayer. Cyanidin-3-glucoside, for example, was the anthocyanin taken up by the cells at the highest relative amount, but was transported through to the basolateral chamber at the lowest relative amount. One commonality with the transport data was the lack of pattern concerning the six-carbon versus five-carbon sugar attachments. In the case of cellular uptake, cyanidin with the six-carbon glucose attachment was preferentially taken up, but the five-carbon xylose attachment was second. Cyanidin with the other six-carbon sugar attachment, galactose, was taken up at the lowest percentage, despite having the highest initial concentration in the chokeberry extract. Table 4 does show that in terms of mg/L amounts taken up by the cells, cyanidin-3-galactoside and cyanidin-3-arabinoside were the highest, corresponding to the two highest initial anthocyanins in the extract (68.4% and 23.3%, relative peak areas,
respectively). Cyanidin-3-glucoside was actually present in a lower initial amount than cyanidin-3-xyloside, but still had a higher amount present in the cell layer, suggesting that the structure may influence the uptake, not just initial concentration or amount present.
Table 5: Individual HPLC peak area % values for anthocyanins from chokeberry extract, from the basolateral, apical, and cellular layers after 3 h treatment at apical pH 3.0 and basolateral pH 7.4, at 37°C. Initial anthocyanin concentrations range from 200-1500 µM. Values are average +/- standard deviation with standard errors of means; n = 6 with repeated measurements. The * denotes statistical significance (p < 0.05) of the difference between the apical chamber and the sample marked.

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Apical (SD)</th>
<th>SEM</th>
<th>Basolateral (SD)</th>
<th>SEM</th>
<th>Cellular (SD)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin-3-galactoside</td>
<td>62.3 (0.30)</td>
<td>0.10</td>
<td>61.6 (1.1)</td>
<td>0.34</td>
<td>51.4 (3.6)*</td>
<td>1.1</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>3.4 (0.26)</td>
<td>0.08</td>
<td>3.0 (0.25)*</td>
<td>0.08</td>
<td>2.9 (0.35)*</td>
<td>0.11</td>
</tr>
<tr>
<td>Cyanidin-3-arabinoside</td>
<td>27.9 (0.23)</td>
<td>0.07</td>
<td>29.7 (0.71)*</td>
<td>0.22</td>
<td>30.8 (1.9)*</td>
<td>0.56</td>
</tr>
<tr>
<td>Cyanidin-3-xyloside</td>
<td>4.3 (0.25)</td>
<td>0.08</td>
<td>4.3 (0.42)</td>
<td>0.13</td>
<td>5.7 (0.52)*</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Table 5 contains comparisons of individual HPLC peak areas for each anthocyanin from chokeberry extract (200-1500 µM), after treatment at apical pH 3.0 and basolateral pH 7.4, for 3 h at 37°C. Analyzing percent peak areas in this manner allows for quantification of relative changes of each individual anthocyanin across the apical, basolateral, and cellular layers of treatments. The apical peak area % for each anthocyanin was not significantly different than that of the original chokeberry extract, and the peak areas for the varying concentrations were also not significantly different, thus data from several concentrations were compiled. Asterisks in table 5 denote p-values of 0.05 or less for the differences in peak area % for each anthocyanin from the apical to basolateral, or from the apical to cellular layers. The p-values determined by two-tailed t-test (Microsoft Excel 2011); results are considered significant at p < 0.05.

The HPLC percent peak areas in Table 5 agree with previously the discussed data that cyanidin-3-glucoside and cyanidin-3-arabinoside were differentially transported through the NCI-N87 monolayer: cyanidin-3-glucoside was transported at a significantly lower rate, while cyanidin-3-arabinoside at a significantly higher rate, as compared to their respective original peak area percent (cyanidin-3-glucoside from 3.39 to 2.99% apical to basolateral, and cyanidin-3-arabinoside from 27.89 to 29.70% apical to basolateral). Cyanidin-3-galactoside and cyanidin-3-xyloside were present in similar relative amounts in the apical and basolateral compartments after treatment, suggesting that although they were transported through the monolayer,
their transport did not appear to be significantly favored or un-favored by the transport mechanism of NCI-N87 cells.

Table 5 also shows that there was a significant difference in the peak area percent for each anthocyanin from the apical to the cellular layer, suggesting that there were differences in the uptake of each anthocyanin. Cyanidin-3-galactoside, the most prevalent anthocyanin from chokeberry extract, was present in the cell layer at a relatively lower percent than in the apical chamber. Cyanidin-3-glucoside, the other anthocyanin-six carbon sugar attachment, was also present in the cellular layer at a lower relative amount as compared to its initial amount. Given that these two anthocyanins are present in very different amounts in the original extract, this may suggest that it is not concentration, but the sugar attachment itself, causing differential cellular uptake. This is supported by data from the two anthocyanins with five-carbon sugar attachments, cyanidin-3-arabinoside and xyloside, which were both present in higher relative amounts in the cell layer than in the apical chamber. This pattern of behavior relating to the five and six-carbon attachments was not immediately clear when analyzing the mg/L data from Table 4, but does suggest a preferential uptake of anthocyanins with five-carbon sugar attachments by NCI-N87 cells.
Table 6: Individual anthocyanin concentrations (mg/L) and percent transport from the apical to the basolateral compartment, at apical pH 3.0 and basolateral pH 7.4 and initial anthocyanin concentration of 200 µM (96.95 mg/L) from red grape extract. Values are averages +/- standard deviation; n = 6 with 3 repeated measurements.

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Apical mg/L (SD)</th>
<th>Basolateral mg/L (SD)</th>
<th>Transport (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin-3-glucoside</td>
<td>6.0 (0.002)</td>
<td>0.52 (0.01)</td>
<td>8.69</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>5.2 (0.003)</td>
<td>0.53 (0.01)</td>
<td>10.13</td>
</tr>
<tr>
<td>Malvidin-3-glucoside</td>
<td>54.3 (0.03)</td>
<td>3.3 (0.05)</td>
<td>6.03</td>
</tr>
<tr>
<td>Petunidin-3-glucoside</td>
<td>9.5 (0.004)</td>
<td>0.31 (0.01)</td>
<td>3.23</td>
</tr>
<tr>
<td>Pelargonidin-3-glucoside</td>
<td>5.1 (0.004)</td>
<td>0.33 (0.01)</td>
<td>6.56</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>6.2 (0.04)</td>
<td>0.59 (0.19)</td>
<td>9.51</td>
</tr>
</tbody>
</table>
Table 6 shows individual anthocyanin amounts (mg/L) from apical (pH 3.0) and basolateral (pH 7.4) compartments after 3 h treatment with red grape extract (200 µM, or 96.95 mg/L) at 37°C. Also included in Table 6 are the calculated percent transports of each anthocyanin from the apical to the basolateral chamber. The immediate observation is that the percent differences between these anthocyanins from red grape very much more widely than those from chokeberry, suggesting that B-ring substitutions on the anthocyanin may play a role in the transport of anthocyanins from the apical to basolateral side of NCI-N87 cells. Looking more closely, as was the case with chokeberry, the percent of each anthocyanin transported through the monolayer was low- less than 11% for each anthocyanin from red grape. Cyanidin-3-glucoside showed the highest transport percentage, and was originally present at just 5.214 +/- 0.003 mg/L. Malvidin-3-glucoside, the most predominant anthocyanin from the red grape extract, was transported at 6.026%, at a similar percent of pelargonidin-3-glucoside (6.56%), which was present in the lowest amount in the apical side. Though this still resulted in a higher concentration of malvidin-3-glucoside in the basolateral chamber (as the initial concentration was much higher), it suggests that concentration alone may not be responsible for transport of anthocyanins across the NCI-N87 monolayer.

When the transport percentages are ordered from greatest to least, there is not a clear effect of specific B-ring substitutions. Cyanidin (2 –OH groups), peonidin (1 –OH and 1 –OMe), delphinidin (3 –OH), pelargonidin (1 –OH group), malvidin (1 –OH group and 2 –OMe groups), and petunidin (2 –OH and 1 –OMe)-3-glucosides all had varying transport percentages, seemingly unrelated to their initial concentration. This suggests
that structure may in fact be playing a role in the transport of anthocyanins through the NCI-N87 monolayer.

Table 7 shows individual anthocyanin amounts in the apical compartment and the cellular layer after 3 h treatment with 200 µM (96.95 mg/L) red grape extract at 37°C for 3 h, and the percent uptake from the apical compartment to the cellular layer for each anthocyanin. These percentages seem to show differences in the cellular uptake of each anthocyanin from red grape. Malvidin-3-glucoside, the most abundant anthocyanin in the original extract, was taken up by the cells at the lowest percentage: 0.183%. Pelargonidin-3-glucoside on the other hand, present in the apical side at the lowest concentration, was taken up at 4.57% of its original concentration.
Table 7 Individual anthocyanin amounts (mg/L) and percent uptake from the apical compartment to the cellular layer, at apical pH 3.0 and basolateral pH 7.4 and initial anthocyanin concentration of 200 µM (96.95 mg/L) from red grape extract. Values are averages +/- standard deviation; n = 6 with 3 repeated measurements.

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Apical mg/L (SD)</th>
<th>Cell mg/L (SD)</th>
<th>Uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin-3-glucoside</td>
<td>6.0 (0.002)</td>
<td>0.1 (0.001)</td>
<td>1.21</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>5.2 (0.003)</td>
<td>0.02 (0.002)</td>
<td>3.30</td>
</tr>
<tr>
<td>Malvidin-3-glucoside</td>
<td>54.3 (0.03)</td>
<td>0.011 (0.003)</td>
<td>0.18</td>
</tr>
<tr>
<td>Petunidin-3-glucoside</td>
<td>9.5 (0.004)</td>
<td>0.01 (0.002)</td>
<td>0.99</td>
</tr>
<tr>
<td>Pelargonidin-3-glucoside</td>
<td>5.1 (0.004)</td>
<td>0.08 (0.032)</td>
<td>1.60</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>6.2 (0.4)</td>
<td>0.02 (0.001)</td>
<td>2.83</td>
</tr>
</tbody>
</table>
Taken in order of ascending apical-to-cell percentages (malvidin, petunidin, delphinidin, pelargonidin, peonidin, cyanidin), structural influences are not immediately clear. Malvidin and petunidin, the lower percentages, both are heavily substituted on their respective B-rings. Malvidin is substituted with one –OH group and 2 –OMe groups, while petunidin has 2 –OH groups and one –OMe group. These results suggest that these higher molecular weight/more substituted anthocyanins may be less favorably taken up by NCI-N87 cells. Peonidin and cyandidin-3-glucosides, on the other hand, were found in higher percentages in the cellular layer. Both of these anthocyanins have two groups attached to their B-rings: cyanidin has 2 –OH groups, and peonidin has one –OH and one –OMe group.

These results do suggest some effect of B-ring substitution on the cellular uptake of anthocyanins by NCI-N87 cells; however much more research is needed to determine exactly how these substitutions affect interactions between anthocyanins and the cells.
Table 8: HPLC peak area % for anthocyanins from red grape extract, from the basolateral, apical, and cellular layers after 3 h treatment at apical pH 3.0 and basolateral pH 7.4, at 37°C. Values are average +/- standard deviation, with standard errors of means; n = 6 with 3 repeated measurements. The * denotes significance (p < 0.05) of the difference between the apical chamber and the sample marked.

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Apical (SD)</th>
<th>SEM</th>
<th>Basolateral (SD)</th>
<th>SEM</th>
<th>Cellular (SD)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin-3-glucoside</td>
<td>5.2 (0.3)</td>
<td>0.13</td>
<td>6.0 (0.1)*</td>
<td>0.05</td>
<td>2.8 (0.5)*</td>
<td>0.21</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>4.5 (0.2)</td>
<td>0.09</td>
<td>6.3 (0.2)*</td>
<td>0.08</td>
<td>22.6 (2.1)*</td>
<td>0.94</td>
</tr>
<tr>
<td>Malvidin-3-glucoside</td>
<td>49.1 (4.2)</td>
<td>1.64</td>
<td>75.4 (2.5)*</td>
<td>1.11</td>
<td>22.0 (3.1)*</td>
<td>1.33</td>
</tr>
<tr>
<td>Petunidin-3-glucoside</td>
<td>7.9 (0.6)</td>
<td>0.23</td>
<td>1.3 (0.6)*</td>
<td>0.24</td>
<td>4.5 (0.6)*</td>
<td>0.35</td>
</tr>
<tr>
<td>Pelargonidin-3-glucoside</td>
<td>3.5 (0.6)</td>
<td>0.26</td>
<td>1.5 (0.4)*</td>
<td>0.18</td>
<td>3.3 (0.6)</td>
<td>0.31</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>4.7 (0.5)</td>
<td>0.19</td>
<td>7.7 (0.2)*</td>
<td>0.09</td>
<td>13.5 (1.5)*</td>
<td>0.77</td>
</tr>
</tbody>
</table>
Tables 8 describes the changes in peak area percent for individual anthocyanins from red grape at apical pH 3.0 and basolateral pH 7.4 after treatment for 3 h at 37 °C at an initial anthocyanin concentration of 200 µM. Asterisks in table 8 represent p-values of 0.05 or below for the differences between the percent peak area between the apical and basolateral chambers, and between the apical chamber and cellular layer for each anthocyanin. As the data show, the peak area for each anthocyanin from red grape was significantly different in the basolateral and cell compartments as compared with the initial makeup of the extract, which was not significantly different than the apical makeup after treatment. The one exception was the cellular uptake of pelargonidin-3-glucoside, with a p-value 0.06. This suggests that there was some differential transport and uptake of red grape anthocyanins, either from varying initial concentrations, or from structural differences—specifically, B-ring substitutions.

Delphinidin, cyanidin, malvidin, and peonidin-3-glucosides were all present in the basolateral compartment at higher relative peak area percent than in the apical side, suggesting a higher rate of transport for these compounds. Malvidin-3-glucoside in particular showed a much higher relative concentration in the basolateral side: 49.09% +/- 4.022 on the apical side to 75.41% +/- 2.49 on the basolateral side. Malvidin’s 2 O-Me groups and one –OH group on its B-ring may have an effect on its transport as compared to other anthocyanins in red grape. Petunidin and pelargonidin-3-glucosides showed the opposite trend, a lower relative amount in the basolateral compartment than in the apical side. Structurally, petunidin and pelargonidin have different B-ring substitutions: petunidin has 2 –OH groups on its B-ring, and also an –OMe group- one of
the more highly substituted anthocyanins. Pelargonidin, on the other hand, is substituted with just one –OH group on its B-ring. These two anthocyanins are present in the red grape extract at mid-level relative amounts, so concentration is likely not a factor in their lower transport. For example, Peonidin-3-glucoside is present in the original extract at 4.66% +/- 0.47, lower than both petunidin and pelargonidin-3-glucosides, but is found at a higher percentage than both in the basolateral chamber (7.68% +/- 0.22). More research is certainly needed to determine the effects of anthocyanin B-ring substitution on the transport of anthocyanins through the NCI-N87 cell monolayer.

Table 8 also describes differences between the relative peak areas between the apical and cellular compartments for each anthocyanin from red grape. Again, each anthocyanin except for pelargonidin-3-glucoside appeared in the cell layer at a different relative amount than initially added for treatment. Delphinidin, malvidin, and petunidin-3-glucosides were all found in lower relative amounts in the cell layer than in the apical chamber. Each of these anthocyanins have 3 substitutions on their B-ring: 3 –OH groups, one –OH group and two –OMe groups, and 2 –OH and one –OMe groups, respectively. This could suggest that molecular weight or size of the molecule may play a role in the cellular uptake of anthocyanins by NCI-N87 cells. Malvidin-3-glucoside in particular was found at a much lower relative level in the cellular compartment: 49.095% +/- 4.022 in the apical compartment and just 21.965% +/- 3.131 in the cells. This may suggest that either malvidin-3-glucoside is not well taken up by NCI-N87 cells, or that it may be especially susceptible to degradation or metabolism of some kind by the cells, and thus not detected by the analytical methods used here. Taken together with the apparent
higher transport of Malvidin-3-glucoside from the apical to basolateral chamber, perhaps malvidin-3-glucoside is simply preferentially transported across the monolayer, without being trapped or taken up by the cells. Cyanidin and peonidin-3-glucosides, on the other hand, showed a high relative percent peak area in the cell layer (22.621% +/- 2.085 and 15.144% +/- 1.541, respectively) compared with the apical chamber (4.503% +/- 0.207 and 4.66% +/- 0.47, respectively). Peonidin and cyanidin both have –OH groups on their B-rings: peonidin has one and cyanidin has two, but peonidin additionally has one O-Me group on its B-ring.

Conclusions

This work showed evidence of the effects of time, concentration, pH, and chemical structure in an in vitro model of the gastric epithelium. Anthocyanins were transported through the NCI-N87 cell monolayer, and taken up by the cells, in a time and concentration-dependent manner. The human stomach is subject to a wide range of pH levels, depending on an individual’s fed/fasted state, and can vary between individuals as well. Thus, any discussion of uptake or transport of compounds by the stomach must take into account its ever-changing environment. Additionally, the environmental pH has profound effects on the chemistry of anthocyanins in solution. Though these changes are reversible, at these higher pH levels, anthocyanins may experience significantly more breakdown, an irreversible process that may affect the uptake of anthocyanins. The data presented in this work showed that pH did have a significant impact on the transport and uptake of anthocyanins by NCI-N87 cells. This may suggest that the chalcone form of the
anthocyanin, with its open-ring structure, is less favorably transported by the cells. However, at 1500 µM initial anthocyanin concentration, similar amounts of cellular uptake were observed at pH 5.0 as pH 3.0. Whether or not the chalcone is favorably taken up by the cells (or at least as favorably as the flavilyum cation) remains to be elucidated, but the data here suggest it is a possibility, at least at higher initial anthocyanin concentrations. Results here also showed that some aspects of anthocyanin structure likely play a role in transport and uptake, however, given the vast amount of structural differences amongst anthocyanins in nature, much more work is needed in this area. This work did being to shed light on the basic question of possible impacts of structure on the transport and uptake of anthocyanins in the stomach. More work is needed with more commodities (with differing anthocyanin profiles) to elucidate patterns of B-ring substitution and various sugar and acid moieties with respect to transport and uptake in this model.
CHAPTER 4: EFFECTS OF ANTHOCYANINS ON THE IL-1β INDUCED INFLAMMATION OF NCI-N87 GASTRIC CELLS

Abstract

Anthocyanins, the red, blue, and purple pigments widely found in dietary fruits and vegetables, have frequently been linked with anti-inflammatory capabilities. This activity has not been widely studied in the gastric environment, though studies have shown significant amount of anthocyanin uptake in the stomach. This study aimed to characterize potential anti-inflammatory activity of chokeberry anthocyanins on the IL-1β induced inflammation of NCI-N87 cells. The enzyme-linked immunosorbent assay (ELISA) was used to measure IL-8 secretion of the NCI-N87 cell line in response to IL-1β induced inflammation. Anthocyanins were added to cells either before, at the same time as, or after the inflammatory agent. Secretion of IL-8 was shown to markedly increase in the presence of IL-1β (9 pg/mL +/- 3 in control versus 81 pg/mL +/- 12 with IL-1β treatment). Treatment with anthocyanins 1 h before inflammation was induced, treatment at the same time as inflammation, and treatment 15 min after the introduction of the inflammatory agent, all showed significant inhibition of increased IL-8 secretion by the cells (7 pg/mL IL-8 +/- 2, 10 pg/mL +/- 3, 17 pg/mL +/- 4, respectively). Concentrations of 50, 500, and 1500 µM were used to investigate a possible dose-
response of the IL-8 inhibition. Results showed that each treatment significantly
decreased IL-8 secretion in response to IL-1β inflammation, and that each increasing
concentration significantly reduced secretion from the last (17 pg/mL +/- 5, 8 pg/mL +/-
3, and 5 pg/ml +/- 2, respectively). These findings show that chokeberry anthocyanins
had a powerful inhibitory effect on the increased IL-8 secretion of NCI-N87 cells in
response to IL-1β induced inflammation. More research is warranted on the anti-
inflammatory effect on gastric cells in this acute inflammation model.

4.1 Introduction

Gastric cancer is considered to be a significant global health problem, being the
fourth most common form of cancer, and the second leading cause of death from cancer
worldwide. The exact mechanism by which gastric cancer develops is not clearly
understood, as it most likely involves many factors, including genetics, geographical
environment (developing countries are especially vulnerable to developing gastric
cancer), socioeconomic status, immunity, and individual gut microflora (Serban 2014).
Still, chronic gastric inflammation is considered to be one of, if not the most, potent
predictors of the development of gastric cancer. Infection with Helicobacter pylori has
been shown to be the most common cause of gastric inflammation. As with gastric
cancer, the prevalence of H. pylori varies by region, with developing nations carrying the
heaviest burden. In some counties, up to 80% of the adult population can be infected,
while the worldwide average is closer to 58%. Most of those infected with H. pylori will
remain asymptomatic, though chronic inflammation will develop. Approximately 10% of
infected individuals will develop peptic ulcer disease, and 1-3% will develop gastric cancer (Noto and Peek 2012, Wang and others 2014).

The mechanism of gastric inflammation is not well understood, but likely, infection with *H. pylori* secretes compounds that inflame the mucosal layer either from afar, or through direct contact with gastric epithelial cells, triggering the release of pro-inflammatory cytokines such as interleukin-1 β (IL-1β), IL-2, IL-6, IL-8, and TNF-α (Israel and Peek 2001).

The effects of anthocyanins on *H. pylori* induced gastric inflammation have been previously studied by Kim and colleagues (2013) using AGS (human gastric) cells. This study found that anthocyanins had significant effects on several markers of *H. pylori* infection: decreased ROS production, inhibition of phosphorylation of MAPKs and NF-κB translocation, and a 45.8% inhibition of IL-8 secretion. While these results are promising with regard to anti-inflammatory effects of anthocyanins, Conlin and others (2004) previously found that the AGS cell line may not be a reliable model for the study of *H. pylori* pathogenesis. They found that as compared to primary human antral epithelial cells, the AGS line was significantly different with respect to cell-cell and cell-matrix adhesion, and cell migratory behavior. Conversely, the NCI-N87 cell line was not significantly different from the primary cultured human cells, and therefore may serve as a better model. Previously, Chatterjee and others (2004) observed that extracts from raspberry, strawberry, cranberry, elderberry, blueberry, and bilberry all significantly inhibited *H. pylori* colony growth, and also increased the susceptibility of *H. pylori* to clarithromycin, a commonly used antibiotic treatment for *H. pylori* infection.
The NCI-N87 cell line has previously been used to study the effects of *H. pylori* infection on monolayer permeability and cytokine secretion (Florentino and others 2013). Results from this study showed severe disruption of tight junction proteins and decreased transepithelial electrical resistance (TEER) values when infected with live bacteria. The cell viability was not affected, showing that the *H. pylori* infection mostly targeted tight junction proteins. Additionally, the release of pro-inflammatory cytokines was increased in a polarized manner, mostly basolaterally, when co-cultured with either live or heat-killed bacteria. The researchers concluded that the NCI-N87 cell line provided an excellent model for the study of *H. pylori* induced inflammation of the gastric epithelium (Florentino and others 2013).

The objective of this study was to investigate the effects of anthocyanins on the IL-1β induced inflammation of NCI-N87 cells by monitoring IL-8 secretion with the enzyme-linked immunosorbent assay (ELISA).

### 4.2 Materials and Methods

#### 4.2.1 Standards and reagents

Unless otherwise noted, all chemicals and reagents were purchased from Fisher Scientific (Fair Lawn, NJ), Sigma Aldrich Chemical (St. Louis, MO), and Pierce Biotechnology (Rockford, IL). Anthocyanin extracts were prepared from chokeberry juice concentrate, obtained courtesy of Artemis International (Fort Wayne, IN) and stored under dark refrigeration (4°C) conditions.
4.2.2 Cell culture conditions

The human gastric epithelial cell line NCI-N87 is derived from human gastric carcinoma tissue and was purchased from the American Type Culture Collection (Manassas VA). Cells were initially seeded in 75-cm² culture flasks (Corning, Corning NY) and maintained with RPMI-1640 complete medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin. Cells were grown at 37°C under 5% CO₂.

For experiments, cells were seeded on plastic in 48 well plates at a density of 2.5 x 10⁵ and maintained in complete RPMI media with 10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin and stored at 37°C under 5% CO₂. Medium was changed every 48 hours until cells were 100% confluent, at which point the medium was changed every 24 hours until 18 days post confluency was reached, at which point the cells were treated as described below. All experiments were performed on passages 2-30.

4.2.3 Extraction of anthocyanins from chokeberry juice concentrate

The anthocyanin-rich chokeberry extract was prepared by the methods of Rodriguez-Saona and Wrolstad (2005) and He and Giusti (2011). Briefly, the juice concentrate was extracted with an equal volume of acetone and filtered (Whatman #1). The residual material was extracted 2x more with 70% acetone to further remove the organic phase. Remaining hydrophobic compounds were removed by extraction with chloroform 2:1 v/v overnight at 4°C. Excess chloroform in the aqueous phase was then
removed by rotary evaporation. The resulting material was then purified by cation exchange (MCX SPE cartridge, Waters Corp.). The column was conditioned with methanol and 0.1% v/v trifluoroacetic acid (TFA) prior to sample loading. Neutral and anionic phenolics were removed with 0.1% TFA and 0.1% TFA in methanol. Anthocyanins were then eluted with water:methanol 40:60 v/v with 1% NH₄OH, followed by methanol with 1% NH₄OH into a flask containing 500 µL 88% formic acid to drop the final pH to <2.

Chokeberry extract was quantified by external standard via construction of a 5-point standard curve with cyanidin-3-glucoside (0-1500 µM) and area under the curve (AUC) by HPLC. Anthocyanin separation was achieved on a Symmetry C18 reverse-phase column (4.6 x 75 mm, 3.5 µm; Waters Corp.) fitted with an analytical guard column (NovaPak®, 4 x 2.0mm C18). The mobile phases were (A) 4.5% formic acid and (B) acetonitrile. All solvents were filtered through 13-mm 0.45µm polypropylene filters. Solvent gradient for chokeberry anthocyanin analysis follows: 0-3 min, 5-10% B; 3-10 min, 10—20%B, 10-15 min, 20-35% B; 15-20 min, 35% B. Statistical analysis was performed with and Microsoft Excel (2011).

4.2.4 Anti-inflammatory activity of chokeberry anthocyanins

NCI-N87 cells at 18 days post-confluence were either: pre-treated, co-treated, or post-treated with 0.3 mL pH 3 or pH 7.4 HBSS containing 1500 µM anthocyanins from chokeberry. In the pre-treated samples, cells were washed with warm HBSS and then treated with 50, 500, or 1500 µM pH 3.0 anthocyanin extract. After 1 h of this treatment
at 37°C, 30 µL containing 20 ng/mL recombinant human IL-1β was added to the wells, and incubated for an additional 4 h. This method also allowed for the investigation of a possible dose effect on the anti-inflammatory activities of anthocyanins under these conditions. For co-treatment, the anthocyanins and inflammatory agent were added at the same time and incubated for 4 h. Finally, post-treatment cells were treated first with IL-1β for 15 min, at which time 1500 µM anthocyanin treatment was added, and incubated for an additional 3.75 h. After treatment, media from each sample was collected for IL-8 quantification by ELISA analysis.

4.2.5 Cytokine ELISA assay

Media collected after treatment was analyzed for IL-8 content by the enzyme-linked immunosorbent assay (ELISA) kit (Sigma RPN2764, St. Louis, MO) according to the manufacturer’s instructions. IL-8 was quantified by a 5-point standard curve with materials provided by the manufacturer. Analysis was performed on a Synergy H1 Hybrid Multi-Mode Microplate reader equipped with Gen 5 data analysis software (BioTek, Winooski VT).

4.3 Results and Discussion

4.3.1 Anti-inflammatory activities of chokeberry anthocyanins

Treatment of NCI-N87 cells with IL-1β increased the secretion of IL-8 by the cells significantly, as shown below in figure 18. Treatment of anthocyanins for 1 h prior
to the induction of inflammation significantly decreased the increase of IL-8 secretion as compared to no anthocyanin treatment, and the effect followed a dose-response pattern. IL-8 secretion was significantly increased with IL-1β treatment (9 pg/mL +/- 3 in control versus 81 pg/mL +/- 12 with IL-1β treatment). Figure 18 shows an anti-inflammatory effect of pre-treatment with anthocyanins 1 h before the induction of inflammation by IL-1β in NCI-N87 cells (7 pg/mL IL-8 +/- 2). Co-treatment with anthocyanins (added at the same time as the addition of IL-1β) also significantly blocked the increased secretion of IL-8 by NCI-N87 cells (10 pg/mL +/- 3). Interestingly, treatment with anthocyanins 15 min after the addition of IL-1β also significantly blocked increased IL-8 secretion (17 pg/mL +/- 4). This may suggest that the anti-inflammatory mechanism is likely not by active site binding on the IL-1β molecule itself. It may even suggest that the mediation is not by interaction with the IL-1β receptor in the cells. However, this experiment alone does not offer enough information to deduce the anti-inflammatory mechanism of anthocyanins on NCI-N87 cells.

Figure 19 shows the anti-inflammatory effects of different concentrations of chokeberry anthocyanins on the IL-1β induced inflammation of NCI-N87 cells as measured by IL-8 secretion. Treatment with IL-1β significantly increased IL-8 secretion by the cells (9 pg/mL +/- 3 in control versus 81 pg/mL +/- 12 with IL-1β treatment). Cells were treated with either 50, 500 or 1500 µM for 1 h before the addition of the IL-1β inflammatory agent, at pH 3.0 and 37°C.
Figure 18: Anti-inflammatory activity of chokeberry anthocyanins in NCI-N87 cells as measured by IL-8 secretion induced by IL-1β. Control sample was cells with no treatment; IL-1β sample was no anthocyanin treatment. Pre-treated sample was treated for 1 h with 1500 µM anthocyanin extract before introduction of IL-1β. Co-treated samples were introduced to anthocyanins and IL-1β at the same time, and post-treated samples were treated with IL-1β for 15 min before the introduction of 1500 µM anthocyanins. All treatments were pH 3.0, at 37°C. Asterisks denote significant difference in the mean from no anthocyanin treatment.
Figure 19: Dose response of the anti-inflammatory activity of chokeberry anthocyanins on the IL-1β induced inflammation of NCI-N87 cells. Cells were treated for 1 h at each anthocyanin concentration (pH 3.0) before introduction of 20 ng/mL IL-1β, followed by incubation for 4 h at 37°C. Asterisks denote significant difference in the mean from no anthocyanin treatment.
After the addition of 20 ng/mL IL-1β, cells were incubated for an additional 4 h before removing media for ELISA determination of IL-8 secretion. Each treatment concentration significantly reduced IL-8 secretion by NCI-N87 cells. There was also a dose-response trend in the reduction of IL-8 observed at 50, 500 and 1500 µM treatment (17 pg/mL +/- 5, 8 pg/mL +/- 3, and 5 pg/ml +/- 2, respectively).

Collectively, the data presented in figures 19 and 20 suggest a significant anti-inflammatory effect of anthocyanins on NCI-N87 gastric cells under these conditions. Alterations of environmental pH, anthocyanin structure, and the presence of enzymes which may degrade the anthocyanins, all may have an effect on this response- thus, more research is needed to confirm these promising results in the anti-inflammatory effects of anthocyanins on gastric cells.

**Conclusions**

Anthocyanins from chokeberry showed a significant anti-inflammatory effect on the induced inflammation of NCI-N87 gastric cells, suggesting the potential for anti-inflammatory benefits in the stomach. More research is needed on this topic, as the inflammatory process is complicated, and effects of varying pH and anthocyanin structure have yet to be investigated, but these results suggest that further studies are merited.
CONCLUDING REMARKS AND FUTURE WORK

We have shown here that the NCI-N87 cell line can be used to study the effects of anthocyanins on gastric health. The data presented show that anthocyanin transport through the monolayer and uptake by the cells is dependent on time, initial concentration, and apical pH. There is also likely an effect of anthocyanin structure on these processes, both with respect to the sugar attachment and B-ring substitution on the anthocyanin aglycone. Anthocyanins were also shown to have a significant inhibitory effect on the IL-1β induced inflammation of NCI-N87 cells, further suggesting that anthocyanins may have beneficial biological effects on the stomach.

Many questions remain about the behavior of anthocyanins in the stomach. Studies of different commodities, with different anthocyanin profiles, are needed to further investigate how anthocyanins are transported and taken up by gastric cells. Aside from the monoglycoside attachments and B-ring substitutions studied here, many common dietary anthocyanins contain other monosaccharides, di and tri-saccharide attachments, and acid moieties. The behavior of these complex anthocyanins may differ in their transport over time, and at various apical pH levels.

Research into the potential anti-inflammatory effects of anthocyanins in the stomach is in early stages. Promising results shown in this work are indicative that it is a topic worthy of more investigation. IL-8 is just one pro-inflammatory cytokine
associated with the inflammation process. Measurement of more upstream and downstream compounds could provide more information on the effects of anthocyanins on the overall process of inflammation. Additionally, the effect of different anthocyanin structures on the inflammation state of gastric tissue has not yet been investigated. The use of different commodities could provide more information on this topic as well. Effects of breakdown products of anthocyanins may also provide another piece to this puzzle, as the changing gastric pH may lead to the formation of some of these compounds. These products may even show similar or higher anti-inflammatory benefits when compared directly to intact anthocyanins, but much more research is needed.
REFERENCES


Vergara C, Mardones C, Hermosin-Gutierrez I, von Baer D. Comparison of high-performance liquid chromatography separation of red wine anthocyanins on a mixed-


