Metabolism and Anti-inflammatory Activity of Anthocyanins in Human Oral Cavity

DISSertation

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

By

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2012

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ABSTRACT

Anthocyanins (ACN), natural pigment flavonoids, exhibit chemopreventive, chemotherapeutic, and anti-inflammatory activities in the oral cavity. ACN have recently been included into products for the promotion of oral health. However, it is unclear which ACN-rich foods could be most efficacious. Since fruits and vegetables contain unique mixtures of ACNs, an understanding of effect of ACN structure on metabolism, bioavailability, and bioactivity is needed for providing sound dietary recommendations and development of products containing ACN for promotion of oral health.

ACN extracts from blueberry, chokeberry, black raspberry, red grape, and strawberry were incubated ex vivo for 60 min in human saliva (Chapter 2). Degradation of chokeberry ACN was primarily enzymatic and mediated by bacteria. Glycosides of delphinidin (Dp) and petunidin (Pt) were more susceptible to chemical degradation than those of cyanidin (Cy), pelargonidin (Pg), peonidin (Pn) and malvidin (Mv) in both intact and artificial saliva. Stability of di- and tri-saccharide conjugates of anthocyanidins was slightly greater than that of mono-saccharide derivatives. The ex vivo degradation was significantly decreased in saliva obtained after volunteers rinsed their mouths with anti-bacterial chlorhexidine.

I next examined whether the ex vivo finds could be recapitulated in vivo by having subject retain for 5 minutes either chokeberry or red grape juice in their mouths (Chapter 3). Retained juice, oral washings and buccal scrapings were collected and analyzed for stability of ACN in the oral cavity, binding to buccal mucus and uptake into buccal cells. Cy-3-glucoside preferentially accumulated with the buccal cells compared to other Cy glycosides. Anti-bacterial treatment resulted in greater stability of ACN in retained juice.
Loss of Dp glucosides exceeded that of Pt, Cy, Pn and Mv glucosides during oral retention of red grape juice. Lesser relative amounts of Dp and Pt glucosides were associated with mucus and buccal cells than Cy, Pn and Mv glucosides, suggesting their instability and limited accessibility to the oral epithelium.

I investigated inhibitory activity of ACN mixtures on pro-inflammatory cytokine secretion by oral epithelial cells. ACN extracts from black raspberry, blueberry, chokeberry, red grape, strawberry, and hibiscus were incubated with cells prior to, simultaneously or after addition of IL-1β followed by measurement of IL-8 secretion (Chapter 4). Chokeberry extract reduced IL-1β induced IL-8 secretion by SCC-25 in a dose response manner. The efficacy of inhibitory activity depended on both the ACN composition of the extract (Pg-rich (strawberry) > five anthocyanidins (red grape) > Cy-rich (chokeberry and black raspberry) ≈ Cy-Dp disaccharide (hibiscus) extracts) and the type of cell (effect on SCC-25 cancer cells > OKF-6/TERT-2 non-cancer cells). Pre-conditioning medium, by incubating with extracts for 24 h to allow degradation of approximately 90% of ACN, minimally affected inhibitory activity in SSC-25 cultures, demonstrating that degradation products contributed to the bioactivity.

These data demonstrated that the natural ACN composition determines stability, bioaccessibility to the buccal epithelium, and anti-inflammatory activity with oral epithelium. The effects of ACN structure and their degradation products merit further consideration for the development of sound dietary recommendations and/or formulation of ACN-rich products for the promotion of oral health.
In dedication to
my supporting family,
my ancestors,
and
scientists before me.
ACKNOWLEDGMENTS

First and foremost, I would like to extend my sincere gratitude to Dr. Mark L. Failla for providing great opportunity to work with him and his collaborators. His visionary leadership, friendship, criticisms, patience and positive challenges throughout my doctoral endeavor will not be forgotten. I am thankful to Dr. Chureeporn Chitchumroonchokchokchai for introducing me to nutrition science, her patience and continued supports. I sincerely thank to members of Failla’s laboratory, i.e., Dr. Sagar Thakkar; Ms. Fabiola Gutierrez Orozco, Ms. Emily Carson, and Mr. Emiliano Melgar-Bermudez who always provided their hands in time of need.

I am in debt to Dr. M. Mónica Giusti for her willingness to teach me sound conceptual and technical skills required for the analysis of anthocyanins. I am thankful to members of her lab including Ms. M. Fernanda Polit Arcos, Dr. Taylor C. Wallace, Ms. Neda Ahmadiani, and Ms. Allison Atnip who provided initial training and subsequent assistance with the purification and analysis of HPLC-MS.

I am also in debt to Dr. Purnima S. Kumar and members of her research team, i.e., Ms. Maria MorenoCruz, Dr. Samir Shah, Mr. Matthew Mason, Ms. Megan Fellows, and Ms. Monica Bonnin who literally opened the door for clinical studies and provided training with the culture of oral epithelial cells. Their spirit of ‘get things done’ will be remembered.

I am grateful to Dr. Steven J. Schwartz and his colleagues Dr. Ken Reidl and Dr. Rachel Kopec for their support for the screening of degradation products of chokeberry anthocyanins by HPLC-TOF-MS in the Nutrient and Phytochemical Analytic Shared Resource Core of the OSU Comprehensive Cancer Center (NPASR).

Assistance from Dr. Joseph Scheerens of the OARDC in providing black raspberries from Maurer’s farm will be remembered. I also thank Artemis International...
and Enzyme Development Corp. for the gift of chokeberry samples and food-grade pectinase, respectively.

I would like to thank all participants of clinical studies who show willingness and provide time to take part in 3 years of studies. I thank colleagues in the Ohio State University Nutrition Program (OSUN), i.e., Ms. Kara Kliewer, Dr. Amber Simmons, Ms. Jia Yu Ke, Dr. Rumana Yasmeen, my friends in Food Science & Technology department, and the Thai Student Association of the Ohio State University who were always willing to provide their thoughts about for my data, stimulating intellectual discussion, laughter and friendship.

I am deeply thankful to financial supports and awards for these studies: The College of Education and Human Ecology (EHE) dissertation year fellowship; The Ohio Agricultural Research and Development Center (OARDC); the OARDC Graduate Research grant, OSUN; the Leta Gigax Duhamel Scholarship; the Food Innovation Center (FIC) of OSU; and research competition awards from Institute of Food Technologist (IFT).

Finally and most importantly, I thank my family for their love, support and encouragement throughout my graduate endeavor. My father Mr. Angkorn and mother Prof. Maneewan Kamonpatana who have provided continual inspirations and supports; my wife Dr. Pitiya and daughter Sirin Kamonpatana for their supports, encouragements and being the most important reason for completion of the degree; my in-laws Mr. Mangkorn and Ms Ranee Namwonk who came to welcome Sirin to family; and my siblings, Mr. Kor and Ms. Kamonrat Kamonpatana, who have provided unconditioned support to our family in Thailand in my absence.
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PUBLICATION

Research Publication


Abstracts

2. Kamonpatana, K; Moreno Cruz, M; Kumar, P; Giusti, MM; Failla, ML. (2012). Metabolism and Uptake of Anthocyanins in Human Oral Cavity. #084-28. 2012 IFT annual meeting, Las Vegas, Nevada, U.S.A. (Second place, Nutrition division, graduate student research competition).
3. Kamonpatana, K; Moreno Cruz, M; Kumar, P; Giusti, MM; Failla, ML. (2012). Metabolism and epithelial cell uptake of chokeberry and grape anthocyanins in human oral cavity. Russell Klein Memorial Nutrition Research Symposium, The Ohio State University, Columbus, Ohio, U.S.A.
4. Kamonpatana, K; Moreno Cruz, M; Kumar, P; Giusti, MM; Failla, ML. (2011). Metabolism and uptake of berry and grape anthocyanins in human oral cavity. 2011 EHE Student Research Forum, Columbus, Ohio, U.S.A.
6. Kamonpatana, K; Moreno Cruz, M; Kumar, P; Giusti, MM; Failla, ML. (2011). Differential susceptibility of anthocyanins to degradation in human saliva. #098-18. 2011 IFT annual meeting, New Orleans, Louisiana, U.S.A. (First place, graduate student research competition).


8. Kamonpatana, K; Giusti, MM; Moreno Cruz, M; Kumar, P; Failla, ML. (2011). Effect of anthocyanin structure to the ex vivo degradation in human saliva. 2011 Russell Klein Memorial Nutrition Research Symposium, The Ohio State University, Columbus, Ohio, U.S.A.

9. Kamonpatana, K; Giusti, MM; Moreno Cruz, M; Kumar, P; Failla, ML. (2011). Anthocyanin Structure Affects Extent of Degradation in Human Saliva. 2011 OARDC annual conference, Columbus, Ohio, U.S.A.


11. Kamonpatana, K; Chitchumroonchokchai, C; Giusti, MM; Failla, ML. (2010). Anthocyanins from chokeberry and strawberry are metabolized in saliva. 2010 Russell Klein Memorial Nutrition Research Symposium, The Ohio State University, Columbus, Ohio, U.S.A.

12. Kamonpatana, K; Chitchumroonchokchai, C; Giusti, MM; Failla, ML. (2010). Anthocyanins from chokeberry and strawberry are metabolized in saliva. 2010 EHE Student Research Forum, Columbus, Ohio, U.S.A.


15. Kamonpatana, K; Chitchumroonchokchai, C; Harrison, EH; Failla, ML. (2009). Unsaturated fatty acids promote carotenoid bioavailability in vitro. 2009 Russell Klein Memorial Nutrition Research Symposium, The Ohio State University, Columbus, Ohio, U.S.A.


FIELDS OF STUDY

Major Field: Ohio State University Nutrition Program
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CHAPTER 1

REVIEW OF LITERATURE AND SPECIFIC AIMS

1.1 Introduction

Advances in medical diagnoses and public health practices have altered the profile of morbidity and mortality during the past 50-60 years (Figure 1.1; (Kung and others 2008). While mortality due to cardiovascular and cerebrovascular diseases has continually declined, the number of cancer-related deaths has remained persistently steady. Although cancer primarily involves mutations of the genome, only 5-10% of cancers are inherited (World Cancer Research Fund / American Institute for Cancer Research 2007). Environmental risk factors that include exposure to carcinogens and lifestyle play crucial roles in risk of cancer. However, prevention remains difficult without complete identification of all risk factors. Elimination of some risk factors often requires difficult modification of lifestyle. Drs. Richard Doll and Richard Peto, authors of a landmark cancer prevention study in 1980’s, concluded “It is highly likely that the United States will eventually have the option of adopting a diet that reduces its incidence of cancer by approximately one third” (Doll and Peto 1981). This information emphasizes the importance of identifying dietary factors related to prevention, incidence, age of onset and severity of chronic diseases including malignant neoplasms.
Figure 1.1. Age-adjusted death rates for select leading causes of death in the United State, 1958-2005. (adopted from (Kung and others 2008))
Among malignant neoplasms in the upper GI tract, oral cancers affect 34,000 Americans each year causing 8,000 deaths or approximately one person every hour (Oral Cancer Foundation 2010). Without effective intervention, 40% of those with newly diagnosed oral cancer will not survive longer than 5 years (American Cancer Society 2009). Many epidemiological studies have indicated that consumption of a diet rich in fruits and vegetables is associated with lower prevalence of cancer (Block, Patterson, Subar 1992; Sakagami, Oi, Satoh 1999; Surh 2003). This suggests a potential role for phytochemicals in the prevention of cancer. For example, a recent meta-analysis indicates a strong correlation (relative risk 0.52) between consumption of fruit and vegetable and the risk of oral and esophageal cancer (Lucenteforte and others 2009). In particular, consumption of fruits and vegetables appear to be the dietary factor best associated with low occurrence of oral and pharyngeal cancer (Block, Patterson, Subar 1992). In developed countries, flavonols and anthocyanins represent the major classes of dietary flavonoids present in consumed fruits and beverages (Scalbert and Williamson 2000). These associations have provided impetus to study the chemopreventive properties of these phytochemicals in commonly ingested fruits and vegetables.

Anthocyanins (ACN) are a major class of flavonoids that contribute bright orange, red and blue colors to fruits such as berries and many vegetables. These compounds are of interest for use as natural colorants instead of synthetic food coloring agents such as Red dye no. 40 (Giusti and Wrolstad 2003; Stintzing and Carle 2004). Typical diets contain in the range of 12.5 – 215 mg ACN/day (Kuhnau 1976; Wu and others 2006), which is greater than that of many other flavonoids. This suggests that ACN may play a role in the chemopreventive activity of fruits and vegetables. Studies during the past decade have shown that ACN possess antioxidant, anti-inflammatory and anticarcinogenic activities (Wang and Stoner 2008; Zafra-Stone and others 2007). For example, feeding several types of freeze-dried berries (e.g., black and red raspberries, strawberries, and blueberries) all prevented N-nitrosomethylbenzylamine (NMBA)-induced esophageal cancer in rats (Stoner and others 2010). Interestingly, ACN-rich black raspberry extract show equivalent preventive activity to freeze-dried whole backraspberry for prevention of NMBA-induced tumorigenesis in rat esophagus.
suggesting that ACN are bioactive compounds for such activity (Wang and others 2009). ACN-rich extract from black raspberry also suppressed proliferation of human oral squamous cell carcinoma cells (Rodrigo and others 2006) and reduced the number of chemically-induced tumors in the hamster cheek pouch model (Casto and others 2002). As a result, several strategies to prolong retention of ACN in the oral cavity have been investigated. These include gummy-jelly food products, hard confectioneries, chewing gums, and oral gels (Amoian and others 2010; Mallery and others 2007). Because ACN have the potential to serve as natural colorants for the $119 billion confectionery industry, (USDA Foreign Agricultural Service 2005), these compounds appear to represent economically feasible and accessible vehicles for the promotion of oral health.

There are two evident questions that merit consideration for the strategic use of ACN for promotion of oral health. First, which specific ACN-rich fruits and vegetables are best suited for the formulation of products for the promotion of oral health? There are more than 700 distinct ACN structures (Andersen and Jordheim 2006). Selection of fruit for consumption significantly influences the profile of ACN because of the unique qualitative and quantitative combinations of ACN structures in each fruit (Szajdek and Borowska 2008). It remains unknown if ACN structure is a primary determinant of health promoting activity in the oral cavity. Second, are ACN subject to structure-specific metabolism and tissue delivery in the oral cavity? Gastro-intestinal (GI) digestion and transport are important processes governing the delivery of bioactive compounds to target sites. It is interesting that ACN are among the least bioavailable family of flavonoids. Less than 1% of ingested ACN are absorbed by animals and humans (McGhie and Walton 2007; Stoner and others 2005) and 60-90% disappear from the gastrointestinal (GI) tract within several hours after a meal (Prior and Wu 2006). These data suggest that the observed bioactivities in lower GI tract may be due to the production of bioactive metabolites (Kay, Kroon, Cassidy 2009). It is possible that ACN may be beneficial to oral or esophageal tissues, where the intact molecular structures are likely to be more abundant than in the lower GI tract (Wang and others 2009). However, data on oral metabolism and delivery of these compounds to oral tissues remain largely unknown. Hence, in this dissertation, the effect of ACN structure on a) oral metabolism,
b) tissue delivery, and c) relative activities in oral epithelial cells were investigated to provide novel insights about the reported bioactivities of ACN-rich fruits and extracts in the oral cavity.

1.2 General properties of ACN

ACN are a major class of flavonoids contributing to orange, red, magenta, violet, purple and blue colors of many flowers, vegetables, and fruits such as berries. The word ‘anthocyanin’ originates from Greek ‘anthos’ (flower) and kyanos (dark blue) (Delgado-Vargas and Paredes-López 2003). The functions of ACN in plants include photoprotection from UV irradiation, protection against oxidative stress, antiviral and antimicrobial activities, and the promotion of pollination and seed distribution by attracting animals (Gould 2004). ACN are the largest and generally accepted as the most important group of natural water soluble pigments (Clifford 2000). These compounds have been used as coloring agent as early as 1879 in Italy in the form of ‘enocyanin’, or ACN extract from winery grape residue (Francis 1989). The compounds have received increased attention for identification of fruit components in food products, natural food colorants, and for their proposed health promoting properties (Cooke and others 2005; Giusti and Wrolstad 2003; Stintzing and Carle 2004).

1.2.1 Chemistry of ACN

Natural ACN are glycosides of poly-hydroxyl and/or poly-methoxyl derivatives of 2-phenylenzopyrylum (flavylium salts) that combine two benzene rings (A and B) covalently linked by the oxygen containing pyrane or pyrylium ring (C) (Figure 1.2). The unique structure of ACN is the positive oxonium ion which acts as ‘chromophore’ in that its electrons are excited at visible wavelengths, resulting in selective wavelength absorption (ca. 520 nm) and are responsible for colors of the compounds. Without its glycosylated moieties, these compounds are usually referred as aglycones or ‘anthocyanidins’, whereas ‘anthocyanin (ACN)’ designates the glycosylated anthocyanidin aglycone. Of 31 natural occurring anthocyanidins, there are 6 basic
Figure 1.2. Chemical structure of ACN representing 6 most common anthocyanidins in human diet.
structures that vary in the extent of hydroxylation and/or O-methylation at the 3’- and 5’-
positions and account for the majority (90%) of these compounds present in the human
diet (Andersen and Jordheim 2006). These include cyanidin (Cy), delphinidin (Dp),
pelargonidin (Pg), peonidin (Pn), petunidin (Pt), and malvidin (Mv). Cyanidin is the most
widespread anthocyanidin in the plant kingdom (50%), followed by pelargonidin (12%),
peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) (Kong and others
2003).

With the diversity of 1) degree of hydroxylation / methylation of aglycone, 2)
type, number and location of covalently linked sugars, and 3) type and number of
aliphatic or aromatic acids attached to the sugar, ACN constitute a large family of
compounds with greater than 700 unique structures (Andersen and Jordheim 2006;
Andersen and Markham 2006). In nature, ACN are usually glycosylated in the form of β-
3-O-glycosidic or β-3,5-O-diglycosidic bonds. Most ACN contain mono-, di-, tri-
saccharides covalently glycosylated through O-linkage at 3-, 5-, 7-, 3’,- or 5’ position.
Common glycosylated saccharides include glucose (glu), rhamnose (ram), galactose
(gal), arabinose (arab), xylose (xyl), rutinose (rut), and sambubiose (samb). The
predominant monosaccharide is glucose (90%), whereas sophorose, rutinose and
sambubiose are most common disaccharides (Andersen and Jordheim 2006). Organic
acids are usually covalently acylated to sugar moiety at C-3 (Brouillard 1982). Greater
than 65% of identified ACN are found acylated with organic acids, i.e., a) aliphatic acids
(such as malonic, acetic, malic, oxalic, succinic, and tartaric acids), b) hydroxycinnamic
acid (such as p-coumaric, caffeic, ferulic, sinapic, and 3,5-dihydroxycinnamic acids), and
c) hydroxybenzoic acids (p-hydroxybenzoic or gallic acid) (Andersen and Jordheim
2006). Molecular weight of common ACN range from 400 to 1200 daltons (Prior and Wu
2006).

1.2.2 Stability of ACN

ACN are destabilized by light, heat, high pH, enzymes, and other compounds
(Markakis 1982). The flavylium structure of ACN is electron deficient and readily reacts
with nucleophilic compounds such as water, peroxide, and sulfur dioxide (Jurd 1972). As
a result, ACN can be degraded during processing, storage, digestion and absorption of
foods and beverages. ACN stability in food product has been extensively investigated and reviewed (Francis 1989; Markakis 1982; Schwartz, von Elbe, Giusti 2008).

1.2.2.1 Effect of ACN structure

The stability of ACN is dependent on several molecular features. In general for food products, stability of ACN is decreased with increasing degree of hydroxylation, but increased by methylation of anthocyanidin (Schwartz, von Elbe, Giusti 2008). As a result, Pt and Mv are more stable than Pg, Cy, and Dp. ACN are more stable than their aglycones (Francis 1989) which are rare in nature. Glycosylated di-glucosides have a tendency to be more stable to heat and light compared to mono-glucosides (Markakis 1982). Glycosylation, especially at C 3, is necessary for stability and solubility of ACN at pH 3-7 (Kuhnau 1976). Lacking glycosylation, the 3-hydroxyl group destabilizes the chromophore (Schwartz, von Elbe, Giusti 2008), resulting in loss of the color. Lastly, acylation enhances stability to pH, heat, light, and SO₂ (Delgado-Vargas, Jimenez, Paredes-López 2000). Hence, acylated ACN have the potential to be useful natural colorants in food products (Giusti and Wrolstad 2003).

1.2.2.2 pH

Similar to pH indicators, the color of ACN reversibly shifts from red to blue with increasing pH. ACN are stable at acidic pH at room temperature, but less so at alkaline pH (Brouillard 1982). Within plant tissues and food, ACN exist in equilibrium among 4 structural species, i.e., quinonoidal base (A), flavylium cation (AH⁺), the pseudobase or carbinol (B), and the chalcone (C) and the proportion is dependent on pH (Brouillard 1982). It is noteworthy that each of the four molecular structures is also in equilibrium with a number of their tautomeric derivatives (Brouillard 1982; Clifford 2000). At pH lower than 3 (Figure 1.3), the oxygen in C ring is protonated and becomes the deeply colored flavylium cation (i.e. oxonium form) which is the most stable form (Francis 1989). In slightly acidic aqueous solution (pH 4-5), C-2 of flavylium cation is hydroxylated yielding the colorless carbinol pseudobase (i.e. hemiketal form). Given that fruits and vegetables have pH range between 2 to 5, ACN exists in equilibrium between colorless carbinol and reddish flavylium cation. Further increases in pH result in
Figure 1.3. Structural transformation of ACN at different pH using cyanidin as a representative structure. (adapted from (Fleschhut and others 2006; McGhie and Walton 2007))
formation of quinoide form (or an anhydro base) by loss of a water. At pH 6-8, the compounds are purple to blue color (Kuhnau 1976; Wrolstad 2004). In alkaline medium, the emerging pale yellow color is due to chalcone structure resulting from cleavage of the pyrylium ring. Flavylium cation can be regenerated from the chalcone upon re-acidification. Moreover, the chalcone is unstable and degrades rapidly (Brouillard 1982). An α-diketone was identified as a degradation product of pelargonidin at alkaline pH (Harper 1968). α-diketones are known to be easily hydrolyzed and degraded to acids (Markakis 1974). Depending on pH and structure of ACN, the relative amounts of these four forms varies considerably (Brouillard 1982). Incubation of glucosides of all 6 common anthocyanidins at pH 1-12 revealed that Pt-3-glu and Dp-3-glu are not stable in alkaline solutions, whereas Mv-3-glu exhibits greater stability (Cabrita, Fossen, Andersen 2000).

1.2.2.3 Temperature

ACN degrade extensively with increasing temperature during food processing and storage. In general, hydroxylation of anthocyanidin decreases thermal stability. In contrast, methylation, glycosylation, and acylation stabilize ACN (Schwartz, von Elbe, Giusti 2008). Since opening the heterocyclic pyrylium ring is endothermic, heating shifts equilibria towards the readily degradable chalcone (Brouillard 1982). The reversion from chalcone to flavylium is very slow, e.g., 12 and 6 hours is needed at 25°C to reach equilibrium from 3,5-diglycosides and 3-glycosides, respectively (Markakis 1982). High temperature also promotes hydrolysis of the glycosidic linkage. For example, cyanidin-3-glucosyl rutinoside is hydrolyzed to cyanidin-3-glucoside at 100°C (Seeram, Bourquin, Nair 2001).

1.2.2.4 Sugars

ACN are more stable in solutions containing high concentration of simple sugars. This may be due to a decrease of water activity ($a_w$) required for chemical reaction. However, at low concentrations of sugar, water molecules induce nucleophilic attack at the C-2 position of ACN to generate the colorless carbinol base (Schwartz, von Elbe,
ACN also degrade more extensively in the presence of fructose, arabinose, lactose, and sorbose compared to of glucose, sucrose and maltose (Schwartz, von Elbe, Giusti 2008).

1.2.2.5 **Metals**

In plants, metals form stable complexes with ACN to affect flower color (Schwartz, von Elbe, Giusti 2008). Vicinal phenolic hydroxyls (VPH), *i.e.*, adjacent hydroxyl groups, react with $\text{Al}^{3+}$ to induce a bathochromic shift that differentiates Cy, Dp, and Pt from non VPH-containing ACN (*i.e.*, Pg, Pn, and Mv) (Markakis 1982; Schwartz, von Elbe, Giusti 2008). Stability of ACN in cranberry juice is increased by complexation with Ca, Fe, Al, and Sn (Francis 1977).

1.2.2.6 **Co-pigmentation**

Co-pigmentation has been defined as condensation of ACN with one another and/or other organic compounds (*e.g.* proteins, tannins, other flavonoids, and polysaccharides) (Brouillard 1982; Malien-Aubert, Dangles, Amiot 2001; Osawa 1982; Schwartz, von Elbe, Giusti 2008). This reaction was discovered in 1916 by Willstätter and Zollinger who observed that tannin or gallic acid can make oenin, a grape pigment, more bluish-red (Osawa 1982). In general, co-pigmentation increases stability of ACN in foods during storage (Darias-Martín and others 2001; Malien-Aubert, Dangles, Amiot 2001). Two types of co-pigmentation have been identified, *i.e.*, intra- and inter-molecular co-pigmentation. Many naturally colorless compounds show intermolecular co-pigmentation with ACN resulting in increased stability in food products (Eiro and Heinonen 2002; Malien-Aubert, Dangles, Amiot 2001). For example, Eiro and Heinonen reported strong intermolecular co-pigmentation of ferulic and rosmarinic acids with ACN that stabilized the pigment during storage (Eiro and Heinonen 2002). Degree of methoxylation, acylation and glycosylation of ACN also affect the stabilizing effect of co-pigmented complexes (Davies and Mazza 1993; Mazza and Brouillard 1990). Co-pigmentation commonly occurs in fruit-derived products such as juices and wines (Mazza and Brouillard 1987).
1.3 Dietary intake of ACN

ACN are present in most plant materials, *i.e.*, flowers, fruits and leaves and sometimes in root, tuber, and stem (*Table 1.1*). Conversely, ACN seem scarce in lower plants such as liverworts or algae and some has been reported in mosses and ferns (Swain and Bate-Smith 1962). These compounds are synthesized in the cytoplasm from phenylalanine (Tanaka, Sasaki, Ohmiya 2008) and ultimately localized in vacuolar solutions of epidermal cells (Andersen and Jordheim 2006). During synthesis, the pigments are found in bounded bodies call ‘anthocyanoplast’ prior to dispersal within vacuoles (Delgado-Vargas, Jimenez, Paredes-López 2000). ACN are present in numerous plant foods (*Table 1.1*) (Timberlake and Bridle 1982).
Table 1.1. Examples of common fruits and vegetables containing ACN.

<table>
<thead>
<tr>
<th>Family names</th>
<th>Common names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anacardiaceae</td>
<td>mango</td>
</tr>
<tr>
<td>Araceae</td>
<td>taro</td>
</tr>
<tr>
<td>Brassica oleraceae</td>
<td>red cabbage</td>
</tr>
<tr>
<td>Caprifoliaceae</td>
<td>elderberry</td>
</tr>
<tr>
<td>Compositae</td>
<td>chicory, artichoke</td>
</tr>
<tr>
<td>Cydonia</td>
<td>quince</td>
</tr>
<tr>
<td>Ericaceae</td>
<td>blueberry, bilberry and cranberry</td>
</tr>
<tr>
<td>Fragaria</td>
<td>strawberry</td>
</tr>
<tr>
<td>Garcinia mangostana</td>
<td>mangosteen</td>
</tr>
<tr>
<td>Gramineae</td>
<td>sorghum, sugar cane, rice</td>
</tr>
<tr>
<td>Ipomoea batatas</td>
<td>sweet potatoes</td>
</tr>
<tr>
<td>Leguminosae</td>
<td>legumes</td>
</tr>
<tr>
<td>Liliaceae</td>
<td>onion, garlic, and asparagus</td>
</tr>
<tr>
<td>Litchi chinensis</td>
<td>lychee</td>
</tr>
<tr>
<td>Malus</td>
<td>apple</td>
</tr>
<tr>
<td>Musaceae</td>
<td>bananas</td>
</tr>
<tr>
<td>Oleaceae</td>
<td>olive</td>
</tr>
<tr>
<td>Passifloraceae</td>
<td>passion fruit and pomegranate</td>
</tr>
<tr>
<td>Prunus</td>
<td>apricot, cherry, plum, peach and sloe</td>
</tr>
<tr>
<td>Pyrus</td>
<td>pear</td>
</tr>
<tr>
<td>Raphanus sativus</td>
<td>red radish</td>
</tr>
<tr>
<td>Rubus</td>
<td>blackberry, raspberry, and loganberry</td>
</tr>
<tr>
<td>Rutaceae</td>
<td>blood orange</td>
</tr>
<tr>
<td>Saxifragaceae</td>
<td>gooseberry, black and red currants</td>
</tr>
<tr>
<td>Solanaceae</td>
<td>egg plant</td>
</tr>
<tr>
<td>Umbelliferae</td>
<td>carrot and parsnip</td>
</tr>
<tr>
<td>Vitis</td>
<td>grape</td>
</tr>
</tbody>
</table>

Modified from (Timberlake and Bridle 1982).
In light of the wide distribution of ACN in plant foods, it is reasonable to assume human consumption is relatively high. Consumption of 10,000 tons of ACN per annum has been estimated from black grape alone (Clifford 2000). Concentration in fruits and vegetables ranges from 0.1 – 1.0 % dry weight (Swain and Bate-Smith 1962; Wu and others 2006). ACN represent approximately 50% of total phenolic content in fruits such as blueberry, blackberry, raspberry, chokeberry, bilberry, and blackcurrant (Szajdek and Borowska 2008). Typical consumption is in the range of 3 – 215 mg ACN/day in the US (3 mg/day (Chun, Chung, Song 2007), 12.5 mg/day/person (Wu and others 2006) and 180 to 215 mg/day (Kuhnau 1976) ), which is significantly greater than the estimated daily intake (20-25 mg/day) of many other dietary flavonoids including apigenin, genistein, quercetin, kaempferol, myricetin and luteolin (Cooke and others 2005; Hertog and others 1993). Single servings of common fruits provide considerable ACN intake, e.g., blackberry (353-433 mg ACN/serving), blueberry (579-705 mg ACN/serving), or blackcurrant (553 mg ACN/serving) (Wu and others 2006). Dietary sources determine type of ACN structure ingested (Szajdek and Borowska 2008). ACN profiles of selected dietary source are listed in Table 1.2.
Table 1.2. Profile of ACN in selected dietary sources.

<table>
<thead>
<tr>
<th>Species</th>
<th>ACN dominant</th>
<th>ACN profile</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilberry <em>Vaccinium myrtillus</em></td>
<td>Cy-3-glu, Cy-3-gal, Mv-3-glu, Dp-3-glu</td>
<td>Dp-3-gal, Dp-3-glu, Cy-3-gal, Dp-3-arab, Cy-3-glu, Cy-3-arab, Pt-3-glu, Pt-3-gal, Pn-3-gal, Pt-3-arab, Pn-3-glu, Mv-3-gal, Mv-3-glu, Mv-3-arab, Dp-3-samb, Cy-3-samb</td>
<td>(Borges and others 2010; Dugo and others 2001; Szajdek and Borowska 2008)</td>
</tr>
<tr>
<td>Blackcurrant <em>Ribes nigrum</em></td>
<td>Dp-3-rut, Cy-3-rut</td>
<td>Cy-3-glu, Cy-3-rut, Dp-3-glu, Dp-3-rut, Pn-3-rut, Mv-3-rut</td>
<td>(Szajdek and Borowska 2008; Zheng and others 2012)</td>
</tr>
<tr>
<td>Blackberry <em>Rubus fruticosus</em></td>
<td>Cy-3-glu</td>
<td>Cy-3-glu, Cy-3-arab, Pn-3-glu, Cy-3-xyl, Mv-3-glu</td>
<td>(Dugo and others 2001)</td>
</tr>
<tr>
<td>Black raspberry <em>Rubus occidentalis</em></td>
<td>Cy-3-(2^x)-xyl-rut, Cy-3-rut</td>
<td>Cy-3-glu, Cy-3-samb, Cy-3-(2^x)-xyl-rut, Cy-3-rut, Pg-3-rut</td>
<td>(Tian and others 2005)</td>
</tr>
<tr>
<td>Blueberry <em>Vaccinium corymbosum</em></td>
<td>Mv-3-gal, Mv-3-arab, Dp-3-gal, Cy-3-gal, Pt-3-gal</td>
<td>Dp-3-gal, Dp-3-glu, Cy-3-gal, Dp-3-arab, Cy-3-glu, Pt-3-gal, Pt-3-glu, Pn-3-gal, Pt-3-arab, Pn-3-glu, Mv-3-gal, Mv-3-glu, Mv-3-arab, Mv-3-glu, Mv-3-arab</td>
<td>(Kamonpatana and others 2012; Seeram and others 2006; Tian and others 2005)</td>
</tr>
<tr>
<td>Chokeberry <em>Aronia melanocarpa</em></td>
<td>Cy-3-gal, Cy-3-arab</td>
<td>Cy-3-gal, Cy-3-glu, Cy-3-arab, Cy-3-xyl</td>
<td>(Kamonpatana and others 2012; Wu and others 2004; Zheng and Wang 2003)</td>
</tr>
<tr>
<td>Cranberry <em>Vaccinium oxyccoccus</em></td>
<td>Cy-3-gal, Cy-3-arab, Pn-3-gal, Pn-3-arab</td>
<td>Cy-3-glu, Cy-3-gal, Cy-3-arab, Pn-3-glu, Pn-3-arab, Dp-3-glu, Pt-3-glu, Mv-3-glu, Mv-3-arab</td>
<td>(Borges and others 2010; Szajdek and Borowska 2008)</td>
</tr>
<tr>
<td>Red grape <em>Vitis vinifera</em></td>
<td>Pn-3-glu, Mv-3-glu</td>
<td>Dp-3-glu, Cy-3-glu, Pt-3-glu, Pn-3-glu, Mv-3-glu</td>
<td>(Cai and others 2010; Castillo-Munoz and others 2009; Frank and others 2003)</td>
</tr>
<tr>
<td>Red raspberry <em>Rubus idaeus</em></td>
<td>Cy-3-soph</td>
<td>Cy-3-samb, Cy-3-soph, Cy-3-glu, Cy-3-glu-rut, Cy-3-rut, Pg-3-soph, Pg-3-glu</td>
<td>(Borges and others 2010; Szajdek and Borowska 2008)</td>
</tr>
<tr>
<td>Strawberry <em>Fragaria x ananassa</em></td>
<td>Pg-3-glu</td>
<td>Cy-3-glu, Pg-3-glu, Pg-3-rut, Pg-3-arab, Pg-3-sukcynloglu, Cy-3-sukcynloglu</td>
<td>(Buendia and others 2010; Carkeet, Clevidence, Novotny 2008; Felgines and others 2007; Szajdek and Borowska 2008)</td>
</tr>
</tbody>
</table>
1.4 Digestion, bioavailability, and metabolism of ACN

To understand bioactivities of ACN, an understanding of digestion, metabolism and tissue disposition is needed. Among plant polyphenols, ACN are among the most poorly absorbed (Manach and others 2005). Less than 1% of ingested ACN are absorbed by animals (McGhie and Walton 2007; Stoner and others 2005) and 60-90% disappears from gastrointestinal (GI) tract within 4 hours after a meal (Prior and Wu 2006). This suggests extensive degradation/metabolism within GI tract prior to absorption. However, until the past decade, most of bioefficacy studies of ACN have been explored using anthocyanidin or ACN with limited attention to their metabolites. More recently, it has been proposed that metabolites of ACN are likely responsible for their observed health-promoting activities (Forester and Waterhouse 2010; Prior and Wu 2006; Vitaglione and others 2007). Given that the bioactivities have been reported largely for natural ACN, health promoting activities of these compounds are more likely in the upper GI tract (oral cavity, esophagus and stomach) where the compounds are most abundant (Wang and others 2009). Since ACN are relatively unstable flavonoids, the bioavailability and efficacy of ACN may reflect the combined activities of the consumed compound, chemical degradation, and metabolites generated during digestion, cellular and gut microflora metabolism.

1.4.1 Degradation in physiological environments

Anthocyanidins (aglycone) are significantly less stable than their glycosylated ACN (glycosylated) in physiological solutions (Fleschhut and others 2006; Keppler and Humpf 2005; Markakis 1974; Seeram, Bourquin, Nair 2001; Woodward and others 2009). Woodward (2009) reported instantaneous degradation of anthocyanidins (Pg, Cy, Dp) in water (pH 7.0) and physiological buffer (10mM Na/K phosphate buffer, pH 7.4). The recovery of phenolic aldehydes/acids accounted for 30-40% and 8-18% of original amount of ACN in water and buffer, respectively (Woodward and others 2009). The kinetics of degradation product formation suggests production of intermediary structures prior to final phenolic aldehydes/acids. Some ACN (Pg-3-glu, Cy-3-glu, and Dp-3-glu)
are more susceptible to chemical degradation in physiological buffer than water (Woodward and others 2009), especially when incubated at 37°C, and is accompanied by polymerization of monomeric ACN (Nalliah and others 2009).

Using sterilized inoculum of swine feces, ACN (Cy-3-glu, Mv-3-glu, Cy-3,5-diglu, Mv-3,5-diglu, Cy-3-rut, and Pn-3-glu) were degraded with limited detection of phenolic products (Table 1.3) after 24 hr incubation (Keppler and Humpf 2005). Increased hydroxyla
tion of the B ring was associated with decreased stability in preincubated physiological buffer (10mM Na/K phosphate, pH 7.4) (Woodward and others 2009). Fleschhut (2006) incubated pure ACN (Cy-3-glu, Mv-3-glu, Pn-3-glu, Cy-3,5-diglu, Mv-3,5-diglu) in phosphate buffer (pH 7.4) (Fleschhut and others 2006). Resulting metabolites were detected by MS with m/z at 589, 605, and 587 and were hypothesized to be products of dimerization of Cy via quinoidal structure.

As expected, ACN are not stable in cell culture medium with its standard neutral pH. Kay et al. found that 57% and 96% of Cy-3-glu and Cy spontaneously degraded by 4 hours in cell-free culture medium (DMEM) (Kay, Kroon, Cassidy 2009). Similarly, ACN-rich extracts (ARE) from chokeberry, bilberry and elderberry had half lives less than 5 hr (Wu and others 2006). Dp was least stable (half life 0.43 hr) followed by Pt (2.34 hr), Mv (4.04 hr), Pn (4.31 hr), and Cy (4.45 hr) in cell culture medium. Cy-3-samb-5-glu was more stable than Cy-3-glu. Cyanidin glycosides (i.e. Cy-3-glu-rut, Cy-3-rut, Cy-3-glu, and Cy) from tart cherry degraded in cell-free McCoy’s 5A medium generating PCA as a major product along with 2,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid during incubation (Seeram, Bourquin, Nair 2001). Such observations challenge reported activities attributed to intact ACN in cultures of mammalian cells.

1.4.2 Microbial degradation in gastrointestinal tract

GI microflora have been shown to degrade flavonoids by ring fission and hydrolytic activities. Several bacteria have been shown to metabolize AC, and include Bacteroides, Clostridium, Eubacterium, Ruminococcus, and Eggertheilla (Blaut and Clavel 2007). ACN have been shown to be degraded to phenolic compounds by large intestinal microflora (Keppler and Humpf 2005). Glucoside and di-glucoside derivatives
Table 1.3. Identified metabolites of ACN during incubation with colonic microflora.

<table>
<thead>
<tr>
<th>Anthocyanidin</th>
<th>Degradation product of A ring</th>
<th>Degradation product of B ring</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pg</td>
<td>Phloroglucinol aldehyde (PGA)</td>
<td>4-hydroxy benzoic acid</td>
<td>(Fleschhut and others 2006; Woodward and others 2009)</td>
</tr>
<tr>
<td>Cy</td>
<td>Phloroglucinol aldehyde (PGA)</td>
<td>Protocatechuic acid (PCA)</td>
<td>(Fleschhut and others 2006; Keppler and Humpf 2005; Woodward and others 2009)</td>
</tr>
<tr>
<td>Dp</td>
<td>Phloroglucinol aldehyde (PGA)</td>
<td>Gallic acid</td>
<td>(Woodward and others 2009)</td>
</tr>
<tr>
<td>Pn</td>
<td>Phloroglucinol aldehyde (PGA)</td>
<td>Vanillic acid</td>
<td>(Fleschhut and others 2006)</td>
</tr>
<tr>
<td>Pt</td>
<td>Phloroglucinol aldehyde (PGA)</td>
<td>3-O-methylgallic acid</td>
<td>(Forester and Waterhouse 2008)</td>
</tr>
<tr>
<td>Mv</td>
<td>Phloroglucinol aldehyde (PGA)</td>
<td>Syringic acid, 3-O-methylgallic acid, Vanillic acid</td>
<td>(Fleschhut and others 2006; Keppler and Humpf 2005) (Forester and Waterhouse 2008) (Keppler and Humpf 2005)</td>
</tr>
</tbody>
</table>
of Cy, Pn, and Mv were hydrolyzed by pig gut microflora within 2 h; di-saccharides were hydrolyzed more slowly than mono-saccharides (Keppler and Humpf 2005). ACN (Dp-3-glu, Pt-3-glu, Pn-3-glu, and Mv-3-glu) from Cabernet Sauvignon grapes were rapidly metabolized (two thirds of initial content lost within 0.5 h) after addition to pig large intestinal contents (Forester and Waterhouse 2008). Non-enzymatic degradation or binding was speculated to cause this rapid loss because there was no significant difference when ACN was added to heat-inactivated large intestinal contents comparing to respective control. Respective metabolites, i.e., 3-O-methylgallic acid, syringic acid and PGA (2,4,6-trihydroxybenzaldehyde), were detected after 6 h incubation and though to be generated by chemical degradation.

Aura et al. incubated Cy-3-glu and Cy-3-rut with human fecal microbiota (Aura and others 2005). Small amounts of aglycone, intermediary PCA, and other two unknown products (named Cy-3 and Cy-4) were found as major metabolites for both ACN. However, these products only accounted for 5% of initial amount of ACN inoculated. Bacterial activities such as α,L-rhamnosidase, β,D-glucosidase, catechol-O-methyltransferase and aryl-sulfotransferase are present in the gut and may participate in the generation of PCA. Unknown product ‘Cy-3’ eluded before ACN with absorbance maxima at 290nm and did not have the same retention time and spectra as PGA, 4-hydroxybenzolic acid, PGacid, 4-hydroxyphenylacetic acid or 3,4-dihydroxyphenylacetic acid. ‘Cy-4’ eluded after ACN and had an additional 85 m/z mass to its corresponding ACN. The investigators speculated that Cy-4 formation involved deglycosylation of ACN and re-conjugation of the aglycone with unknown residue (M=85) which may contain nitrogen or sulfur. Incubation (2 hr) of ACN (Cy-3-glu, Mv-3-glu, Pn-3-glu, Cy-3,5-diglu, Mv-3,5-diglu, Cy, Mv, Pn, Pg, Dp) in human fecal suspension resulted in > 90% loss of glucosides of Mv, Pn, and Cy (Fleschhut and others 2006). Approximately 62% of parent ACN was detected as phenolic products of the B ring. Breakdown of di-glucoside initially generated mono-glucoside followed by phenolic acids via unstable quinoid base and conversion to the chalcone. Reported metabolites during incubation with colonic microflora are summarized in Table 1.3.
1.4.3 Absorption of ACN

Like other phenolic compounds, ACN are released from food matrix by mastication, peristalsis and digestion. Polyphenols transiently bind to components in food matrices during digestion, which likely protects more labile ACN from degradation (McDougall and others 2005b). Phytic acid is one such dietary component that was found to enhance the absorption of ACN in rat (Matsumoto and others 2007).

**Fate of ACN in GI tract:** Disappearance of ACN in GI tract was evaluated to better understand absorption. In pigs, approximate 60% of oral dose disappeared during flux of chyme in duodenum, ileum, cecum, and colon (Wu, Pittman III, Prior 2006). In *vitro* digestion models have been used to investigate such disappearance. Loss of 50-90% of ACN occurred during *in vitro* digestion (Bermudez-Soto, Tomas-Barberan, Garcia-Conesa 2007; Pérez-Vicente, Gil-Izquierdo, Garcia-Viguera 2002). ACN are stable during simulated gastric digestion (Talavera and others 2005), but significant losses (43%) occur during small intestinal digestion suggesting instability in the neutral to mild alkaline conditions in this compartment (Bermudez-Soto, Tomas-Barberan, Garcia-Conesa 2007). He et al. found that the ACN from black raspberry were stable in gastric and small intestinal lumen of rat after gavage (He and others 2009) and are not deglycosylated at the low pH of the stomach or in small intestine (He, Magnuson, Giusti 2005). Among 3-*O*-glucosides of Cy, Dp, Pn, Pt, and Mv, only Mv and Pn derivatives were detected after *in vitro* digestion suggesting greater stability of these aglycone structures (McDougall and others 2005a). Glycosylation appeared to have a major impact on stability and absorption. Wu et al. reported that cyanidin linked to samb-rut and rut were more stable than glucosylated ACN in pig gut (Wu, Pittman III, Prior 2006). Glycosides of Dp, but not those of Pt, Cy, Pn, or Mv, in concord grape juice was preferentially lost from mouth to ileum of ileostomy human subjects (Stalmach and others 2012). Type of monosaccharide conjugated to an anthocyanidin aglycone (i.e. Cy, Dp, Pt, Pn and Mv) resulted in preferential presence in rats’ plasma 15 min after
ingestion in the following order: galactoside > glucoside > arabinoside (Ichiyanagi and others 2006).

**Mechanism of absorption:** In general, very low concentrations (nmol/L) of ACN are present in plasma after ingestion. Since other flavonoids need to be hydrolyzed to their aglycones prior to be taken up by intestinal cells, it was assumed that only anthocyanidins are absorbed after deglycosylation (Manach and others 2005). The hydrolysis of glycosylated moiety has been reported to be mediated by brush-border membrane β-glucosidase activity (e.g. lactase phlorizin hydrolase (LPH)) or bacterial β-glucosidase in the lower small intestine and colon (Day and others 2000; Lampe and Chang 2007). However, several research teams have reported the low amounts of intact ACN (Galvano and others 2004; Matsumoto and others 2001; Miyazawa and others 1999; Tsuda, Horio, Osawa 1999). For example, Cy-3-glu, Cy-3,5-di-glu, Cy-3-rut, and Dp-3rut all were detected in plasma and peripheral organs of rats and human (Matsumoto and others 2001; Miyazawa and others 1999). As trans-cellular transport of flavonoids and xenobiotics across the gut epithelium is generally required for absorption, specific transporters that have been suggested include the sodium-dependent glucose co-transporter (SGLT1) (Gee and others 2000; Hollman and others 1999), bili-translocase (Passamonti, Vrhovsek, Mattivi 2002), and GLUT2 (Faria and others 2009). Trans-epithelial transport occurs to a much lower extent than uptake across the apical membrane (Steinert and others 2008). In Caco-2 model, glycosylated Cy and Pt glucosides were transported with greater efficiency than the respective galactosides (Yi and others 2006). This suggests participation of the sodium-dependent glucose transporter in the absorption of ACN (Wolffram, Block, Ader 2002). Interestingly, mouse trans-jejunum transport of Cy-3-glu is not significantly inhibited by phloridzin, a potent inhibitor of SGLT1, but significantly inhibited by addition of quercetin-3-glucoside (Walton and others 2006) suggesting unidentified transporter of can that has greater affinity for the flavonol. Acylated ACN also have been reported to be absorbed intact in rats (Suda and others 2002), although to a significantly lesser extent than non-acylated ACN (Charron and others 2009).
**Absorption site:** Intact ACN have been reported to be absorbed in the upper GI tract including mouth, esophagus, stomach and small intestine. After ingestion, ACN levels in plasma are very low (1-100 nmol/L) with relatively short time required to reach \( C_{\text{max}} \) (0.5-2 hr for plasma and 2.5-4 hr for urine) in animal and human studies (Passamonti and others 2003; Prior and Wu 2006; Rechner and others 2002; Stoner and others 2005; Talavera and others 2003). Additional evidence supporting rapid absorption of ACN in stomach (Passamonti and others 2003; Passamonti and others 2005; Talavera and others 2003) and small intestine (Talavera and others 2004) are available.

**Absorption of metabolites:** The fact that anthocyanidin aglycone is not commonly detected in urine (McGhie and others 2003) and the well established instability of anthocyanidins in physiologic solutions and by gut microbiota (section 1.4.1 and 1.4.2) suggest that anthocyanidins undergo further degradation prior to absorption. PCA was increased after ingestion of Cy-3-glu in plasma of rats (Tsuda, Horio, Osawa 1999) and humans (Vitaglione and others 2007). Phenolic acids were transferred from the apical to basolateral compartment in Caco-2 cell cultures by both paracellular pathway and via monocarboxylic acid transporter (MCT) (Konishi, Kobayashi, Shimizu 2003; Konishi, Kubo, Shimizu 2003; Konishi and Kobayashi 2004a; Konishi and Kobayashi 2004b).

1.4.4 **Metabolism of ACN**

Biotransformation of flavonoids generally occurs in the small intestine and liver as a result of phase-I metabolisms (oxidation of phenolic hydroxyl groups, hydroxylation), hydrolysis (or deglycosylation), and phase-II conjugation reactions (with glucuronic acid, sulphate or \( O \)-methylation) (Rice-Evans and others 2000). Once flavonoids are taken up into gut epithelium, flavonoids are conjugated with glutathione, glucuronic acid (by uridine-5’-diphosphate glucuronsyl-transferase (UGTs)), sulfate (by sulfotransferase (SULT), or methyl groups (by catechol-\( O \)-methyl-transferases (COMTs))). These more hydrophilic derivatives are generally retro-transported to the gut lumen or excreted in bile and urine. Flavonoid glycosides are also deglycosylated by cytosolic glucosidase from pig liver (Lambert and others 1999). It is noteworthy that
human hepatocytes and enterocytes contain cytosolic β-glucosidase, suggesting possible deglycosylation of intact ACN (Daniels and others 1981).

Determination of phase I and phase II metabolism of ACN is complex. ACN-rich extracts used in previous studies contain complex profiles potentially generating diverse metabolites (Prior and Wu 2006). Intestinal and hepatic cells possess catechol-O-methyltransferase activity (Rice-Evans and others 2000) capable of converting Cy to Pn and Dp to Pt (Wu, Cao, Prior 2002). For example, Cy was proposed to be methylated to Pn in rat liver (Felgines and others 2002). Intravenous injection of Cy-3-glu to rats resulted in the presence of Pn-3-glu in plasma, kidney and liver (Vanzo and others 2011). Urinary and biliary recovery of ACN in rat is only 30 and 13.4 %, respectively, during first 4 h after intravenously injection (Ichiyanagi and others 2006) suggesting extensive systemic metabolism. Kidney and liver are believed to play major roles in the methylation and glucuronidation of absorbed ACN in vivo (Talavera and others 2005; Tsuda, Horio, Osawa 1999; Vitaglione and others 2007). Several studies reveal that methylation, sulfation and glucuronidation of ACN are dependent on anthocyanidin aglycone structure and linked moiety (Felgines and others 2003; Felgines and others 2005; Wu and others 2005). For example, mono-glucuronidated anthocyanidins were detected as major metabolites along with methylated glycosides and glucuronidated ACN form in human subjects after consuming a meal with blackberries (rich in Cy-3-glu) (Felgines and others 2005). Similarly, glucuronide conjugates, methylated derivatives of Cy-3-gal and Cy-glucuronides, and methylated-Cy were present in urine of humans and pigs after ingesting a meal with chokeberry ACN (Kay and others 2004; Kay, Mazza, Holub 2005; Wiczkowski, Romaszko, Piskula 2010; Wu and others 2005). Felgines et al. found Pg conjugated primarily (>80%) to glucuronic acid with some of sulfo-conjugate in urine of subjects fed strawberry (Felgines and others 2003). Mullen et al. reported Pg-O-glucuronide as a major metabolite in plasma and urine after strawberry consumption (containing Pg-3-glu, Pg-3-rut, Cy-3-glu) by human subjects, although the amount of Pg-O-glucuronide excreted in urine by 24 h was only 0.75 % of Pg-3-glu ingested (Mullen and others 2008). Interestingly, Dp-3-rut is metabolized differently than Cy-3-glu and Pg-
3-glu in rat as Dp-3-rut was methylated, but not glucuronidated or sulfated, in bile, whereas the intact ingested compound in urine (Matsumoto and others 2006).

As metabolites are often generated prior to absorption (section 1.4.1 and 1.4.2), further metabolism of such metabolites also needs to be considered for bioactivity following intake of ACN. For example, Cy-3-glu was metabolized to protocatechuic acid and phloroglucinol aldehyde during incubation with fecal samples (Kay, Kroon, Cassidy 2009). These compounds can be conjugated with glucuronic acid and sulfate and effluxed across the apical and basolateral membranes by Caco-2 intestinal cells (Kay, Kroon, Cassidy 2009). Tsuda et al. found 8-fold more PCA than Cy-3-glu in plasma after feeding rats Cy-3-glu. Neither Cy-3-glu nor PCA were conjugated to glucuronic acid or sulfate (Tsuda, Horio, Osawa 1999). Vitaglione et al. identified PCA as a major metabolite in human urine accounting for 73% of ingested ACN from blood-orange juice (rich in Cy-3-glu) (Vitaglione and others 2007). Nurmi et al. reported methylated phenolic acids as the most abundant urinary metabolites after human subjects ingested ACN in bilberry-lingonberry (Nurmi and others 2009). The possibility that intact ACN, microbial metabolites and their metabolized products (glucuronidated, sulfated, and methylated) of each contribute to the health promoting activities of ingested ACN merits additional attention.

1.4.5 Excretion of ACN

Clearance of ACN from plasma is relatively rapid with only small amounts detected in plasma at 6 hours after ingestion (Bub and others 2001; Prior and Wu 2006). In humans and animals, minimal absorption and urinary excretion (0.004 – 0.2% of dosage), as well as low fecal excretion, of ACN-glycosides has been reported by numerous investigators (Bub and others 2001; Felgines and others 2005; Manach and others 2005; Matsumoto and others 2001; McGhie and others 2003; McGhie and Walton 2007; Netzel and others 2001; Nielsen and others 2003; Wu and others 2005). Some studies reported slightly greater urinary recovery 1-2 % of ingested amount of ACN from strawberry (Carkeet, Clevidence, Novotny 2008; Felgines and others 2003) or blood orange (Vitaglione and others 2007). In rats, glycosylated ACN was recovered in urine as methylated derivatives. Blackberry ACN were excreted in urine as intact and
methylated-glucosylated ACN (Felgines and others 2002). ACN with more complex structures (Cy-3-xyl-rut) were the most abundant ACN in urine of human subjects despite mono- glycosylated Cy compounds being predominant in the consumed food ingested (Tian and others 2006).

He et al. reported that ACN were partially degraded in rat feces stored at -18°C (He, Magnuson, Giusti 2005), suggesting that quantification in feces can be underestimated due to improper handling. Also, loss of Cy-hexoses (glucoside, galactoside) was significantly greater than that of Cy-pentoses (arabinoside, xyloside) in rats fed diets fortified with chokeberry ACN-rich extract (He, Magnuson, Giusti 2005). Considerable amounts of metabolites of ACN may be retained in the GI tract and excreted in feces. One study indicated poor absorption of Cy-3-glu in mice. Three hr after oral administration of 14C-labeled B-ring of Cy-3-glu, majority of radioactivity (87.9%) was recovered in GI tract. After 24 hr, 44.5% of radioactivity was present in the feces (Felgines and others 2010). The structures of the radio-labeled compounds were not determined. Collectively, the above data confirm the poor absorption of intact ACN (section 4.3.3 and 4.3.4) and suggest an appreciable amount of ingested ACN may exert their activity in the gut with metabolites produced by microflora and mammalian cells active in both the gut and peripheral tissues.

1.4.6 Tissue distribution of ACN

The uptake of ingested ACN into small intestinal tissue may be relatively high. He et al. reported 7.5 % of administered dose of 27 nmol of Cy-3-glu rat was associated with small intestinal tissue (He and others 2009). Glucuronidated and methylated metabolites and intact ACN have been found in rat intestine and urine (Talavera and others 2005), whereas methylated form were present in liver and kidney (Tsuda, Horio, Osawa 1999). Talavera et al. (Talavera and others 2005) found methylated and glucuronide conjugates in peripheral organs (liver and kidney) and ACN-glycosides in stomach of rat fed diet containing blackberry (Cy-3-glu, Cy-3-pentose) for 15 days. Also, ACN was present in the brain at a higher concentration (0.21 nmol/g tissue) than plasma (0.15 nmol/mL), suggesting that the ACN localized in this tissue was not due to contamination with blood. These data suggest that ACN are able to transfer across blood-
brain barrier in agreement with a previous report of uptake of Cy-3-rut and Pg-3-glu by brain endothelial cells (Youdim and others 2003). ACN have been reported to be present in various rat brain regions that are important for learning and memory after feeding blueberry supplemented diet to rats (Andres-Lacueva and others 2005). After pigs were fed diet supplemented with blueberry (0, 1, 2, 4% w/w) for 4 weeks, ACN were present in liver, eye, and brain, but not plasma. The concentration ranged from 300 (cerebellum) to 700 (eye) pg ACN/g tissue (Kalt and others 2008). Milbury and Kalt also detected small amounts of ACN in several regions of the brain after feeding pigs 2% freeze-dried whole blueberry for 8 weeks (Milbury and Kalt 2010).

1.5 Biological activities of ACN

The emerging hypothesis of biological activities of flavonoids, including ACN, has stemmed from their diverse effects on mammalian enzymes (including kinases, lipooxygenases, cyclooxygenases), and anti-inflammatory and other cellular responses (Middleton, Kanadaswami, Theoharides 2000). Antioxidant and anti-inflammatory properties of ingested compounds is often associated with prevention of age-related cognitive decline, cancer and cardiovascular disease. Since ACN are extensively metabolized to phenolics and uncharacterized products, it is possible that bioactivity of ACN can be contributed by their parent structure and/or degradation products. For example, the bioactivities of PCA, a product generated during colonic fermentation of cyanidin glycosides, have been extensively reviewed (Masella and others 2012).

1.5.1 Antioxidant activity

Antioxidant activities of plant flavonoids, including ACN, have been extensively reviewed by Rice-Evans (Rice-Evans, Miller, Paganga 1996). However, exposure to amounts of flavonoids exceeding normal dietary levels was shown to have pro-oxidative effects leading to formation of radicals and DNA damage (Skibola and Smith 2000). Among the numerous bioactive compounds in berries, ACN and ellagitannins are major contributors to antioxidant activity (Borges and others 2010; Cerda, Tomas-Barberan, Espin 2005; Wada and Ou 2002). Various in vitro and in vivo studies have reported
antioxidant capacity of ACN. Content of ACN in berries was directly correlated to antioxidant capacity (Prior and others 1998; Sariburun and others 2010). Among 10 compounds extracted from strawberry, ACN (Cy-3-glu, Pg-3-glu, Pg-3-rut) were the most potent antioxidants as measured by Trolox equivalent antioxidant capacity (TEAC) (Zhang and others 2008). Purified ACN scavenge reactive oxygen and nitrogen up to four times more efficiently than ascorbate and α-tocopherol in aqueous models of oxidation (Bors, Michel, Saran 1994; Rice-Evans, Miller, Paganga 1997; Wang, Cao, Prior 1997). Also, ACN possess antioxidant capacity as effective as strong antioxidants such as ascorbic acid, α-tocopherol, and Trolox in emulsions and the LDL test models (Kahkonen and Heinonen 2003). Data from studies using animal models and human are in line with results from such in vitro studies. When feeding a diet rich in ACN, increased serum ACN content was highly correlated with increased antioxidant capacity in serum from rat (Suda and others 2002) and human (Mazza and others 2002; McGhie and others 2007). Using ACI-rat model with oxidative DNA damage induced by 17β-estradiol (E2), diet enriched with berries and aqueous ACN rich extracts inhibited oxidative DNA damage (Aiyer, Kichambare, Gupta 2008). Feeding mixed red fruit juice rich in ACN reduced oxidative damage to DNA, peroxidation of protein and lipid, and NF-κB binding activity in hemodialysis human patients at high risk for oxidative damage (Spormann and others 2008). Also, supplementation with ACN-rich blackcurrant reduced exercise-induced oxidative stress (e.g. plasma carbonyls) in human subjects (Lyall and others 2009).

Studies of how ACN exert such anti-oxidative activity have been reported. The electron deficiency of ACN with four or more hydroxyl groups makes them highly reactive and confers free radical scavenging and metal chelating activities. Complexed (co-pigmented) ACN with metal such as Cu$^{2+}$ prevents metal induced oxidative loss of ascorbic acid (Sarma, Sreelakshmi, Sharma 1997). Structure of ACN also has been shown to affect antioxidant capacity. Among six anthocyanidin structures conjugated with mono- and di- saccharides, Cy-3-glu possessed greatest antioxidant activity, i.e., 3.5 times greater activity than Trolox, as monitored by oxygen radical absorbance capacity (ORAC) method (Wang, Cao, Prior 1997). These data suggest a key role for the hydroxyl group at 3’- and 4’- of B ring for radical scavenging. Cyanidin derivatives showed strong
antioxidant activity in both in vitro and in vivo studies (Galvano and others 2004; Galvano and others 2007). Increased numbers of hydroxyl group on the B ring in ACN, but not aglycone, is associated with greater antioxidant activity (Tsuda and others 1996). Glycosylation at the 3 position reduces antioxidant capacity (Rice-Evans, Miller, Paganga 1996). Reduction of number of sugar conjugated to anthocyanidin also increased antioxidant activities of cyanidin derivatives (Seeram and others 2001).

Anti-oxidant activity of anthocyanidin aglycone is more efficacious than glycosides (Seeram and others 2001; Wang and others 1999). Since the ACN and resulting anthocyanidin are unstable in physiological conditions, these data suggest a potential role of metabolites. For example, gallic acid, a potential O-demethylated metabolite of syringic acid, possesses three-fold greater anti-antioxidant capacity than syringic acid (Rice-Evans, Miller, Paganga 1996). These data suggest that pattern of metabolism of ACN likely affects anti-oxidative activity.

1.5.2 Anti-inflammatory activity

Chronic inflammation increases the risk of cancer, neurodegenerative disorders, cardiovascular disease, and metabolic disorders (Balkwill and Mantovani 2001; Balkwill and Mantovani 2010; Clevers 2004; Coussens and Werb 2002; O'Byrne and Dalgleish 2001). Chronic increases in production of pro-inflammatory cytokines from inflamed cells can damage DNA and induce tumorogenesis (Mantovani and others 2008). Several in vitro and in vivo models have been used to study the anti-inflammatory activities of ACN.

ACN have been reported to modulate activities of multiple enzymes associated with inflammation. In vitro, cyanidin derivatives from tart cherries inhibited cyclooxygenase-1 and -2 (COX-1 and -2) activities are responsible for converting arachidonate to prostaglandins (PGs) (Seeram and others 2001; Wang and others 1999). Cy-aglycone was more inhibitory than that of the glycosides suggesting greater anti-inflammatory activity of the metabolite. Pre-treatment of monocyte-like THP-1 cells with ACN-rich extract from blackcurrant reduced LPS-stimulated cytokine production and NF-κB activation (Lyall and others 2009). Incubation of murine bone marrow-derived dendritic cells with ARE from blackberry suppressed secretion of Lipid-A induced interleukin-12
(Dai, Patel, Mumper 2007). ARE from wild blackberry inhibited LPS-induced pro-inflammatory responses in RAW 264.7 macrophage-like cells including production of nitric oxide (NO), expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2) (Cuevas-Rodríguez and others 2010). Similarly, pre-treatment of LPS/IFN-γ activated RAW 264.7 model with ACN, anthocyanidins, and blueberry extract inhibited production of NO (Wang and Mazza 2002b) but increased TNF-α production (Wang and Mazza 2002a). Cy, Dp, and Pg anthocyanidins inhibited expression of TPA-induced expression of COX-2 mRNA in HT-29 cells (Kim and others 2008). Production of NO by LPS-activated J774 murine macrophage-like cells was suppressed via inhibition of activation of NF-κB by Pg anthocyanidin (Hamalainen and others 2007) and blackberry extract (rich in Cy-3-glu) (Pergola and others 2006). Dp inhibited TNF-α induced expression of COX-2 in JB6 + mouse epidermal cells (Hwang and others 2009). The same authors provided evidence that Dp inhibited TNF-α mediated phosphorylation of key signaling proteins (i.e., JNK, p38 MAP kinase, Akt, p90RSK, MSK1, and ERK) resulting in blockade of transcription factors (AP-1 and NFκB). These data collectively suggest that the anti-inflammatory activities of ACN involve inhibition of inflammatory pathways in mammalian cells.

*In vivo* studies also suggest anti-inflammatory activity of ACN. C-reactive protein (an inflammatory indicative protein) was reduced in rats fed diet with Norton grape pomace extract (156.9 mg/g as Cy-3-glu equiv) compared to that in animals fed standard diet (Hogan and others 2010). Also, colonic COX-2 mRNA and plasma prostaglandin E2 (PGE2) were suppressed in rats fed diet supplemented with seed coat of black soybean (Kim and others 2008). ACN extract, as well as whole bilberries, were reported to reduce intestinal inflammation and cytokine secretion in acute and chronic DSS-colitis in Balb/c mice (Piberger and others 2011).

1.5.3 **Chemoprevention of cancer**

*In vitro* and animal studies of ACN and cancer prevention were recently reviewed by Wang and Stoner (Wang and Stoner 2008). ACN rich mixtures from bilberries and grapes inhibited the receptor tyrosine kinases (RTKs) EGFR, ErbB2, ErbB3, VEGFR-2 and VEGFR-3 which are play a major role in carcinogenesis and tumor progression
(Teller and others 2009). Diets with 5% freeze-dried black raspberry (Rubus occidentalis), blackberry (Rubus fruticosus), and strawberry (Fragaria ananasia) fed to Fischer 344 rats before, during and after exposure carcinogen induction with N-nitrosomethylbenzylamine (NMBA) decreased numbers of esophageal tumor by 24-56% (Stoner and others 2006). ACN extract from black raspberry was as effective as whole fruit (5% in diet) in prevention NMBA-induced esophageal tumors of F344 rats (Wang and others 2009) suggesting that ACN are bioactive components of the activity.

Crude extract (250 µg/mL) and pure ACN (100µg/mL) from strawberry inhibited proliferation of human oral (CAL-27, KB), colon (HT29, HCT-116), and prostate (LNCaP, DU145) cancer cells (Zhang and others 2008). Anti-proliferative activity of ACN rich extract of raspberry was reported for hepatic human liver cells (HepG2) (Liu and others 2002). ACN rich extracts (25-200 µg/mL) from blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry inhibited proliferation of human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT116), and prostate (LNCaP) tumor cells in a dose dependent manner (Seeram and others 2006). Katsube et al. reported that ACN-rich bilberry extract inhibited growth of HL60 human leukemia cells and HCT116 human colon carcinoma cells (Katsube and others 2003). These authors also reported that Dp and Mv ACN inhibited growth of HL60 by activation of the apoptotic pathway. Possible molecular mechanisms of chemopreventive properties of ACN have been reviewed (Hou and others 2004). In mouse epidermal JB6 Cl 42 cells model, ACN (Cy-3-glu, Cy-3-2G-xyl rut, and Cy-3-rut - rich fractions of black raspberries inhibited benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) induction of NF-κB activity (Hecht and others 2006). These data suggest a crucial role of ACN in the observed chemopreventive property of berries.

Structure of ACN has a profound effect on the mechanism of such bioactivities. Non-acylated mono-glycosylated ACN was associated with greater inhibition of growth of human colorectal adenocarcinoma HT-29 cells than ACN containing triglycosidic, acylated cinnamic acid, and Pg anthocyanidin (Jing and others 2008). Anthocyanidin Cy and Dp are potent inhibitors of epidermal growth factor (EGF) while Mv inhibited cAMP-specific phosphodiesterases (PDEs) in HT-29 cells (Marko and others 2004).
Anti-proliferative activities of metabolites of ACN also have been reported. For example, metabolites of ACN generated by gut microflora (*i.e.* gallic acid, 3-O-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde) were more potent inhibitors than ACN of proliferation of CaCo-2 cells (Forester and Waterhouse 2010). Similarly, gallic acid, degradation product of Dp anthocyanidin, inhibited proliferation of HT-29 cells (Kern and others 2007). These data suggest that metabolism of ACN on the large intestine and colon may contribute to the chemopreventive activities of ACN.

1.5.4 Oral health

Oral diseases such as dental caries, gingivitis, and periodontal diseases are global problems that profoundly affect general health and quality of life for millions of individuals (Petersen and others 2005; Sischo and Broder 2011). For example, most recent NHANES study has estimated that 47% of the adult American population (64.7 million individuals) has periodontitis (Eke and others 2012). Moreover, the WHO lists oral cancers as one of the most common cancers in humans (Petersen and others 2005). In the US, more than 36,000 new cases were diagnosed in 2010 and only 65% of these individuals are expected to survive longer than 5 years after diagnosis (Jemal and others 2010). The estimated expense of treating oral diseases ranks fourth among all chronic diseases in developed countries; representing 5-10% of public health expenditures (Petersen and others 2005). Interestingly, epidemiological studies have repeatedly shown a relationship between fruit and vegetable intake and prevention of oral cancers (Block, Patterson, Subar 1992; Sakagami, Oi, Satoh 1999). Epidemiological evidence also suggested that high polyphenol intake was associated with prevention of oral, esophageal and pharyngeal cancer (Petti and Scully 2009). Thus, plant bioactive compounds have received attention for their preventive and therapeutic use for periodontal diseases and oral cancer (Bodet and others 2008) and other oral disease such as caries, gingivitis, and periodontitis (Varoni and others 2012). The oral mucosa has the greatest exposure to dietary compounds in their intact forms (Halliwell, Zhao, Whiteman 2000). As a result, investigators have reported inclusion of bioactive compounds in confectioneries (Amoian
and others 2010) and muco-adhesive gels (Mallery and others 2007) as a means of prolonging exposure of oral tissues to such compounds.

ACN are also of interest as chemopreventive agents for oral cancers. Treatment of human oral squamous cell carcinoma cells (SCC 4, 9, 15, 25 and 2095) with black raspberry extract resulted in ACN uptake coupled with inhibition of proliferation, VEGF expression and translation, and NOS activity suppression (Rodrigo and others 2006). ACN rich extracts from strawberry (Zhang and others 2008), blackberry, black raspberry, blueberry, cranberry, and red raspberry (Seeram and others 2006) all exhibited antiproliferative activity on human oral cancer KB cells and CAL-27 cells. Topical gel treatment containing 10% (w/w) freezed-dried black raspberry of patients with oral intraepithelial neoplasia resulted in histological improvement and reduced loss of heterozygosity of tumor suppressor gene (Shumway and others 2008), as well as decreased COX-2 protein levels (Mallery and others 2008).

Chronic inflammation and periodontal disease increase the risk of oral cancer. Liu et al. proposed that the progression from Oral Lichen Planus (OLP), a chronic oral inflammatory disease, to oral squamous cell carcinoma (OSCC) involves an alteration in proteins mediated by cytokines produced from infiltrating of T-lymphocytes (Liu and others 2010). In OSCC, Gasche et al. reported that tumorigenesis is promoted by IL-6 induced inflammation via alteration of global LINE-1 hypermethylation (Gasche and others 2010). On the other hand, periodontal diseases derived from complex interaction between bacterial commensals and host cells results in chronic infections and induction of pro-inflammatory cytokines secretion. Increased levels of pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNF-α) are correlated with periodontitis (Hou, Liu, Rossomando 1995; Irwin and Myrillas 1998). Expression of IL-8 is up regulated in oral and gingival epithelial cell after challenge with Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Eikenella corrodens, and Prevotella intermedia (Han and others 2000; Sfakianakis, Barr, Kreutzer 2002; Yumoto and others 1999). These cytokines, as well as prostaglandins, induce expression of TNF-α. Saliva of oral cancer patients contains higher concentration of pro-inflammatory IL-1β and IL-6 cytokines than normal individuals (Brailo and others 2012). A strong association (P < 0.01) between IL-
1β concentration in saliva and severity of periodontal disease has been reported (Yoon and others 2012). Therefore, interventions of chronic and early inflammation of oral tissues are likely to decrease the risk of oral cancer.

Evidence during the last decade has suggested a role for ACN as oral anti-inflammatory agents. Zdarilova et al. found that the phenolic fraction of blue honeysuckle (containing 77% ACN with Cy-3-glu predominating (Chaovanalikit, Thompson, Wrolstad 2004)) suppressed LPS-induced production of IL-1β, IL-6 and TNF-α, as well as expression of COX-2 protein in human gingival fibroblast cells (Zdarilová and others 2010). Black currant extract and Cy-3-glu reduced nicotine-induced cytotoxicity of human oral epithelial cells and gingival fibroblast (Desjardins and others 2012). Interestingly, the authors demonstrated that black currant extract, as well as Cy-3-glu, also reduces LPS-induced expression of IL-6 by macrophages. Black currant extract, as well as standard ACN, inhibited activity of human and bacterial derived proteanase such as MMP-1, MMP-9, and elastase (Santos and others 2011).

Grape phenolic extracts inhibit glucosyltransfrerases, an extracellular polysaccharide producing enzyme, of Streptococcus mutans, a dental pathogen (Thimothe and others 2007). Cranberry polyphenolic compounds also prevent formation of biofilms by pathogenic bacteria (Yamanaka and others 2007). However, antibacterial activity of polyphenols in cranberry may be more contributed by proanthocyanidins than ACN (Bodet and others 2008).

Little is known about the type and activities of metabolic product(s) generated in the oral cavity. Walle and associates showed that saliva possesses activity to hydrolyze some, but not all, tested flavonoid glycosides to their aglycone (Walle and others 2005). They did not report salivary metabolism of ACN. Methyl gallate and gallic acid, colonic metabolites of ACN, inhibit IL-6 and IL-8 production from F. nucleatum-activated KB oral epithelial cells (Kang and others 2009).
1.5.5 Other health promoting benefits

ACN possess health promoting benefits based on aforementioned antioxidative, anti-inflammatory and chemopreventive activities. Additional benefits include prevention of cognitive decline in aging, cardiovascular disease, anti-microbial activities, and possibly even weight management. Examples are listed in Table 1.4.
Table 1.4. Additional health promoting benefits of ACN.

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment</th>
<th>Key observations</th>
<th>Reference</th>
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<tr>
<td><strong>Cognitive health in aging</strong></td>
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<tr>
<td>Aged rats</td>
<td>Blueberry extract</td>
<td>Reduce oxidative stress and improved behavioral cognitive measures</td>
<td>(Galli and others 2002)</td>
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<tr>
<td>Alzheimer Disease model APP + PS1 transgenic mice</td>
<td>Blueberry</td>
<td>Enhanced memory-related neuro-signaling and prevention of behavioral deficit, changed spatial working memory in aged rats involving ERK-CREB-BDNF pathway</td>
<td>(Joseph and others 2003)</td>
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<tr>
<td></td>
<td>Extracts of strawberry and blueberry</td>
<td>Reduced neuronal and cognitive declines and reversed age-related neuronal and behavioral deficits</td>
<td>(Joseph and others 1998)</td>
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<td>(Joseph and others 1999)</td>
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<tr>
<td>Rats</td>
<td>Extracts of strawberry and blueberry</td>
<td>Improved paired associate learning and word list recall</td>
<td>(Krikorian and others 2010a; Krikorian and others 2010b)</td>
</tr>
<tr>
<td>Older adults with early memory changes</td>
<td>Blueberry juice, concord grape juice</td>
<td>Prevention of oxidative damage from H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>(Heo and Lee 2005)</td>
</tr>
<tr>
<td>Rat pheochromocytoma PC12 cells line</td>
<td>strawberry extract</td>
<td></td>
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<tr>
<td><strong>Cardiovascular health</strong></td>
<td></td>
<td></td>
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<tr>
<td>8 weeks randomized controlled human trial</td>
<td>freeze-dried strawberry</td>
<td>Decreased atherosclerotic biomarkers, <em>i.e.</em> total and low-density lipoprotein cholesterol, and circulating vascular cell adhesion molecule-1</td>
<td>(Basu and others 2010)</td>
</tr>
<tr>
<td>Male Wistar rat</td>
<td>ACN-rich diet</td>
<td>Greater cardiac resistance ex vivo to ischaemia and reperfusion insult (ex vivo) and reduced coronary infarction <em>in vivo</em></td>
<td>(Toufektsian and others 2008)</td>
</tr>
<tr>
<td>Isolated pig coronary arterial ring</td>
<td>ARE from chokeberry, bilberry, and elderberry</td>
<td>Vaso-relaxation and prevention of loss of relaxation following exposure to ROS</td>
<td>(Bell and Gochenaur 2006)</td>
</tr>
<tr>
<td><strong>Cell culture of bovine and human aortic endothelial cells</strong></td>
<td>Elderberry ACN extract</td>
<td>ACN were incorporated into membrane and cytosol of endothelial cells after 4 h incubation and provide protection against $\text{H}_2\text{O}_2$, AAPH, and $\text{Fe}_2\text{SO}_4$ induced oxidative cytotoxicity</td>
<td>(Youdim, Martin, Joseph 2000)</td>
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<tr>
<td><strong>Macrophage and human umbilical vein endothelial cell cultures</strong></td>
<td>Standard ACN and phenolic metabolites</td>
<td>PCA inhibit NO production and TNF-$\alpha$ secretion by LPS-IFN-$\gamma$ activated macrophage. Gallic acid reduced secretion of MCP-1, ICAM-1, and VCAM-1 in endothelial cells. ACN exhibit an ACE-inhibitory activity. Dp-3-glu, Pg-3-glu, and gallic acid have affinity for ER$\beta$ and Pg and Pn-3-glu for ER$\alpha$.</td>
<td>(Hidalgo and others 2012)</td>
</tr>
</tbody>
</table>

### Antimicrobial activities

<table>
<thead>
<tr>
<th><strong>Review</strong></th>
<th>Berries phenolics inhibit growth of pathogenic bacteria in GI tract such as <em>Salmonella</em> and <em>Staphylococcus</em></th>
<th>(Puupponen-Pimiä and others 2005)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incubation with human pathogenic microbes</strong></td>
<td>phenolic extracts of 12 Nordic berries</td>
<td>Antimicrobial activities in various human pathogenic microorganisms especially for <em>Helicobacter pylori</em> and <em>Bacillus cereus</em></td>
</tr>
</tbody>
</table>

### Weight management and glucose regulation

<table>
<thead>
<tr>
<th><strong>Mice</strong></th>
<th>Cy 3-glu-rich purple corn color (PCC)</th>
<th>PCC suppressed weight gain, white and brown adipose weight induced by high fat diet</th>
<th>(Tsuda and others 2003)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rodent pancreatic $\beta$-cells (INS-1 832/13)</strong></td>
<td>ACN and anthocyanidins</td>
<td>Cy3-glu, Dp-3-glu, Pg-3-glu increase secretion of insulin.</td>
<td>(Jayaprakasam and others 2005)</td>
</tr>
<tr>
<td><strong>Hyperglycaemic obese C57BL/6J mice</strong></td>
<td>ACN extract from Maqui Berry.</td>
<td>ACN improve fasting blood glucose level and glucose tolerance in mice fed with high fat diet.</td>
<td>(Rojo and others 2012)</td>
</tr>
<tr>
<td><strong>Follow up survey</strong></td>
<td>Nurses’ Health Study (NHS), NHS II, Health Professionals Follow-Up Study</td>
<td>Higher intakes of ACN were significantly associated with a lower risk of type 2 diabetes</td>
<td>(Wedick and others 2012)</td>
</tr>
</tbody>
</table>

### Ocular health

| **C57BL/6 mice** | Bilberry extract (containing about 39% anthocyanins) | Oral administration of the extract prevented endotoxin-induced retinal inflammation by suppression of IL-6 and prevent STAT-3 activation. | (Miyake and others 2012) |

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1.6 Dissertation research

1.6.1 Overview and main research objective

Information regarding the metabolism, epithelial cell uptake and bioefficacy of ACN and their metabolites in oral cavity remains limited. Emerging literature (section 1.4 and 1.5) supports the likelihood that metabolites of ACN are likely generated and also possess health-promoting activities in vivo (Forester and Waterhouse 2010; Vitaglione and others 2007). Moreover, little is known about how structure of ACN (i.e., type of aglycone or type and number of conjugated sugars) affects metabolism of ACN in the mouth. As dietary sources contain unique profiles of ACN structures (section 1.3) and structure appears to affect bioactivities of ACN (section 1.5), it is possible that plant source can influence both delivery to and efficacy of ACN in the oral cavity. An understanding of effect of ACN structure on oral metabolism, tissue delivery, and health promoting activity is expected to provide useful data towards the development of dietary recommendations and formulation of ACN-rich oral care products for optimizing oral health.

Specific questions of interest that will be addressed in my dissertation research projects follow.

1) Are ACN metabolized in the oral cavity?
2) How does ACN structure affect their oral metabolism?
3) What are the specific degradation products of ACN generated in the oral cavity?
4) What are the relative contributions of bacteria, epithelial cells, and secreted saliva to metabolism of ACN in the oral cavity?
5) Does the mucus layer lining the oral cavity block apical uptake of ACN and their metabolites by the oral epithelium?
6) Does the pattern of structure-specific degradation affect uptake into oral epithelial cells?
7) Are dietary ACN and/or their metabolites responsible for the anti-inflammatory activities of ACN?
1.6.2 Central hypothesis

My central hypothesis is that ACN structure affects susceptibility to degradation (chemical, microbial, and oral epithelial) and that both intact ACN and their degradation products of contribute to the anti-inflammatory activity of the ingested pigments in the oral cavity.

1.6.3 Overview of specific aims

In this dissertation project, there were three specific aims.

Aim 1: To investigate the *ex vivo* metabolism of ACN in human saliva. The relative susceptibility of structurally distinct ACN present in several common fruits (black raspberry, blueberry, chokeberry, red grape and strawberry), and possible metabolic products were investigated. I investigated the extent of spontaneous vs. enzyme mediated degradation, the role of oral microbiota in degradation, and the variation in extent of degradation among a small group of adult participants with healthy oral cavities (chapter 2).

Aim 2: To examine metabolism and “uptake” of ACN into oral mucosal/epithelial cells of human subjects. I investigated whether the observed effect of ACN structure also occurs in the oral cavity of human subjects. The effect of ACN structure on extent of ACN association with buccal mucus and epithelium was also investigated (chapter 3).

Aim 3: To assess the anti-inflammatory activity of ACN-rich extracts in oral epithelial cell lines. Anti-inflammatory activity of ACN and metabolites generated *in vitro* was tested using SCC-25 and OKF-6/TERT-2 oral epithelial cell lines. I investigated the effect of ACN structure on the inhibition of secretion of pro-inflammatory cytokines and extent of degradation in cell culture media (chapter 4).
CHAPTER 2

INFLUENCE OF ANTHOCYANIN STRUCTURE ON DEGRADATION
IN HUMAN SALIVA EX VIVO*

* CITATION - Kamonpatana, K; Giusti, MM; Chitchumroonchokchai, C; Moreno Cruz, M; Riedl, KM; Kumar, P; Failla, ML. 2012. Susceptibility of anthocyanins to ex vivo degradation in human saliva. *Food Chem.* 135: 738-747. DOI: 10.1016/j.foodchem.2012.04.110.
2.1 Abstract

Some fruits and their anthocyanin-rich extracts have been reported to exhibit chemopreventive activity in the oral cavity. Insights regarding oral metabolism of anthocyanins remain limited. Anthocyanin-rich extracts from blueberry, chokeberry, black raspberry, red grape, and strawberry were incubated \textit{ex vivo} with human saliva from 14 healthy subjects. All anthocyanins were partially degraded in saliva. Degradation of chokeberry anthocyanins in saliva was temperature dependent and decreased by heating saliva to 80°C and after removal of cells. Glycosides of delphinidin and petunidin were more susceptible to degradation than those of cyanidin, pelargonidin, peonidin and malvidin in both intact and artificial saliva. Stability of di- and tri-saccharide conjugates of anthocyanidins slightly, but significantly, exceeded that of mono-saccharide compounds. \textit{Ex vivo} degradation of anthocyanins in saliva was significantly decreased after oral rinsing with antibacterial chlorhexidine. These results suggest that anthocyanin degradation in the mouth is structure-dependent and largely mediated by oral microbiota.

2.2 Introduction

Anthocyanins (ACN) are a major class of flavonoids that contribute bright orange, red and blue colors to many vegetables and fruits. These compounds are of interest for use as natural colorants for foods and beverages instead of synthetic food coloring agents such as the dye FD&C Red No. 40 (Giusti and Wrolstad 2003). Typical U.S. diets contain a range of 12.5 – 215 mg ACN/day (Kuhnau 1976; Wu and others 2006), which is greater than that of many other flavonoids in foods and beverages (Cooke and others 2005; Hertog and others 1993). \textit{In vitro} and \textit{in vivo} studies during the past decade have shown that ACN possess antioxidant, anti-inflammatory and chemopreventive activities (Wang and Stoner 2008; Zafra-Stone and others 2007). Chemopreventive activity has been reported in the gastrointestinal tract (Casto and others 2002; Rodrigo and others 2006). For example, ACN-rich extract from black raspberry suppressed proliferation of human oral squamous cell carcinoma cells (Rodrigo and others 2006) and reduced the
number of chemically-induced tumors in the hamster cheek pouch (Casto and others 2002) and the esophagus and colon in laboratory rodents (Wang and Stoner 2008). Also, topical application of ACN-rich berry gel reduced heterozyosity indicies (Shumway and others 2008) and COX-2 (Mallery and others 2008) in premalignant oral lesions in human subjects. Such observations have provided impetus to the development of delivery vehicles for ACN such as muco-adhesive gel (Mallery and others 2007) to increase exposure of oral tissues to ACN.

Numerous studies with animals and humans have demonstrated that ingested ACN are poorly absorbed (< 1%) (McGhie and Walton 2007; Stoner and others 2005) and largely disappear from the GI tract within several hours after consuming a meal (Prior and Wu 2006). Bioconversion of ACN to phenolic acids and aldehydes by intestinal microbiota has been demonstrated (Keppler and Humpf 2005). Moreover, cyanidin-3-glucoside was degraded to protocatechuic acid (PCA) and phloroglucinol aldehyde (PGA) in cultures of Caco-2 human intestinal epithelial cells (Kay, Kroon, Cassidy 2009). Collectively, these data suggest that metabolites of ACN may be responsible for observed health-promoting activities of ACN in vivo (Forester and Waterhouse 2010; Vitaglione and others 2007).

At present, the extent of ACN uptake and metabolism in the oral cavity is unknown. An understanding of the influence of ACN structure on susceptibility to and patterns of metabolism in oral cavity is expected to facilitate the strategic development of formulations of ACN-rich products for the promotion of oral health. Walle et al. (Walle and others 2005) reported flavonoids such as quercetin-4’-glucoside and genistein-7-glucoside, but not quercetin-3-rhamnoside and naringenin-7-rhamnoglucoside, were hydrolyzed to their respective aglycones in human saliva ex vivo. They also found that the extent of hydrolysis of genistin in saliva collected from 17 human subjects varied more than 20 fold and hydrolytic activity was inhibited by antibacterial agents, suggesting a key role for oral microbiota in flavonoid metabolism in the oral cavity. We hypothesized that ACN will be metabolized in saliva as a result of microbial activity to unstable ACN aglycones that subsequently degrade to phenolic acids. We present results from studies characterizing the degradation of ACN in two aims. The first aim
characterized the *ex vivo* degradation of ACN extracted from chokeberry in saliva collected at various times from a single participant. The second aim investigated the effect of ACN structure on *ex vivo* susceptibility to degradation in saliva using anthocyanin-rich extracts from blueberry, chokeberry, black raspberry, red grape and strawberry, as well as the role of oral bacteria in such degradation.

2.3 Materials and methods

2.3.1 Standards and reagents

Unless otherwise indicated, all supplies and chemicals were purchased from Sigma-Aldrich and Fisher Scientific. Standard cyanidin chloride was purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ). Blueberry (BluB) (*Vaccinium corymbosum*), red grape (RdGrp) (*Vitis vinifera*), and strawberry (StwB) (*Fragaria x ananassa*) were purchased from a local supermarket. Black raspberries (BlkRB) (*Rubus occidentalis*) were donated by Maurer’s farm (Wooster, Ohio). Concentrated juice and powder made from chokeberry (CkB) (*Aronia meloncarpa E.*) were donated from Artemis International (Fort Wayne, Indiana). All fruits except CkB concentrate were frozen (\(-18^\circ\text{C}\)) upon arrival until use.

2.3.2 Subject recruitment

Approval for the human study protocol was received from the Institutional Review Board for Human Research of the Ohio State University (IRB#20090058). Subjects (*n = 14*; 21-55 yr of age) who were periodontally healthy (as evidenced by all sites with attachment levels \(\leq 2\text{mm}\) and probing depths \(\leq 3\text{ mm}\)) and caries-free as evidenced by a low DMF (decayed, missing, filled teeth) Index were recruited. Subjects who had had antibiotic therapy or professional cleaning within the previous 3 months, use immunosuppressant medications, bisphosphonates or steroids, or report pregnancy; a history of diabetes or HIV were excluded from this study (Kumar and others 2011), since these conditions have been previously shown to affect salivary secretion and oral bacterial profiles (Dawes 1987; Socransky and Haffajee 2005).
2.3.3 Aim 1.1: Characterization of ACN degradation using chokeberry extract in saliva from single human subject

2.3.3.1 Preparation of chokeberry ACN-rich extracts

CkB extract powder was solubilized in 0.01% HCl in water prior to solid phase extraction (Sep-Pak, C-18, SPE Waters®) (Rodriguez-Saona and Wrolstad 2005). Monomeric ACN content was estimated by the pH differential method (Giusti and Wrolstad 2005) as cyanidin-3-glucoside (Cy-3-glu) equivalents using the extinction coefficient of 26,900 L cm⁻¹ mg⁻¹. Profile of ACN species in CkB ACN-rich extract was determined by high pressure liquid chromatography-photodiode array detection-electrospray ionization-mass spectrometry (HPLC-PDA-ESI-MS) as described in section 2.4.3. Purity of ACN in each extract was estimated by dividing collective HPLC-PDA area under curves (AUC) of identified ACN (510-530nm) by total AUC monitored from 250-600 nm. Stock ACN rich extracts were stored at -20 °C until use.

2.3.3.2 Ex vivo assessment of ACN degradation in saliva.

The method was adapted from Walle et al. (Walle and others 2005). Unstimulated whole human saliva was collected from a subject (49 yr) in the morning (7-8 AM) after brushing teeth and eating breakfast. Saliva was stored on ice until use within 2 h. Saliva was diluted with an equal volume of de-ionized water and shaken to reduce viscosity. Aliquots of diluted saliva (0.6 mL) were incubated with CkB extract containing 15 nmol of Cy-3-glu equivalents. At least 3 separate reaction tubes were incubated at 37°C in a shaking water bath at 85 rpm for each characteristic being assessed. Reactions were terminated by addition of 0.6 mL acidified methanol to pH of 2.3 after indicated times. Tubes were centrifuged (27,000 × g, 10 min, 4°C) and supernatant was filtered (0.2 µm syringe filter) prior to analysis. Degradation was calculated as [(nmole ACN added to saliva – nmole ACN remaining after incubation) / nmole ACN added to saliva] × 100 %.

To estimate the extent of ACN loss due to association with ACN precipitated proteins, ACN in the pellet were re-extracted with acetonitrile. Acetonitrile has been used as solvent to separate polyphenols such as catechin and theaflavins from proteins in...
plasma and saliva (Laurent, Besançon, Caporiccio 2007; Lee and others 2000).
Acetonitrile (200 µL) and acidified water (4.5% formic acid, 200µL) were added to
pellets, mixtures were vortexed for 30 sec and sonicated for 1 min. Acidified water (500
µL) was added to the mixture, vortexed and centrifuged (27,000 × g, 10 min, 4°C).
Supernatant was filtered (0.45 µm) for analysis.

2.3.3.3 Evaluation of chemical, enzymatic and cell-mediated degradation of
ACN

Extent of ACN degradation during incubation (60 min) in intact saliva was tested
for the following treatments: intact saliva at 37°C; 4°C; artificial saliva at 37°C; saliva
pre-heated to 80°C for 15 min to destroy enzyme activity and cooled to 37°C; and, cell-
free saliva at 37°C prepared by centrifugation (800 × g, 5 min, 4°C) of diluted saliva
followed by filtration of the supernatant through sterile Millex® 0.22µm (Millipore
Express® PES membrane) to remove microorganisms and shed epithelial cells. Artificial
saliva was prepared according to Oomen et al. (Oomen and others 2003) excluding
enzymes to evaluate chemical (non-enzymatic) degradation of ACN in saliva.

2.3.3.4 β-glycosidase activity in saliva

β-D-glycosidases activity in saliva was examined by incubating (at 37°C) 25
nmol/mL of either p-nitrophenyl galactopyranoside (pNP-gal) or glucopyranoside (pNP-
glu) with intact saliva or centrifuged (800 × g, 5 min, 4°C) and filtered (0.2 µm) to
remove bacterial cells. Activity was quenched after incubation for 30, 45, and 60 min by
placing in ice and centrifugation to remove cells. pNP in supernatant was quantified by
monitoring absorbance at 405 nm compared to a five point standard curve prepared with
pure pNP.

2.3.3.5 Stability of predicted phenolic metabolites of cyanidin-3-glycosides in
saliva

The stabilities of protocatechuic acid (PCA) and phloroglucinol aldehyde (PGA),
two predicted products of cyanidin ring fission (Kay, Kroon, Cassidy 2009; Keppler and
Humpf 2005), were examined in diluted saliva. PCA and PGA (25 nmol/mL) were added
to diluted saliva with and without CkB ACN-rich extract. Tubes were incubated at 37°C for 60 min before analysis. To investigate the production of PCA and PGA from anthocyanidin aglycone, cyanidin chloride (CyCl) was incubated (37°C, 60 min) in saliva (25 nmol/mL diluted saliva) before HPLC analysis.

2.3.3.6 **HPLC-PDA analysis**

Compounds were analyzed by HPLC according to Bonerz et al. (Bonerz, Nikfardjam, Creasy 2008). The HPLC system consists of a Waters 2695 separation module and a Waters 2996 photodiode array detector (PDA). Separation was achieved using a Symmetry C-18 reverse-phase column (4.6 x 75 mm; particle size 3.5 μm) with Phenomenex® security guard™ column (C-18 packing; 4 x 3.0 mm). The mobile phase consisted of (A) water/phosphoric acid (99.5:0.5; v/v) and (B) acetonitrile/water/phosphoric acid (50:49.5:0.5; v/v/v) as follows: 0-2 min, 0 %B; 2-7 min, 0-20 %B; 7-25 min, 20-40 %B; 25-31 min, 40 %B; 31-35 min, 40-80 %B; 35-40 min, 80-100 %B; 40-42 min, 100-0 %B, 42-45 min, 0 %B. Flow rate of mobile phase was 0.4 mL/min. ACN, phloroglucinol aldehyde (PGA), and protocatechuic acid (PCA) were monitored at 520, 292, and 259 nm, respectively. Identification of ACN was accomplished by comparing order of elution and spectral characteristics of ACN to previous literature and later confirmed by HPLC-PDA-ESI-MS data (obtained from method described in section 2.3.4.4). Quantification was achieved by comparison with five-point calibration curves obtained with pure cyanidin chloride, PCA and PGA.

**2.3.4 Aim 1.2: Effects of ACN structure and oral microbiota on extent of salivary degradation in human subjects**

2.3.4.1 **Preparation of ACN-rich extracts**

Five fruits (BlkRB, BluB, CkB, RdGrp, and StwB) were selected for investigation based on their distinct ACN profiles to facilitate comparison of the susceptibility of all 6 anthocyanidins and their derivatives conjugated to either mono-, di- or tri-saccharides (Table 2.1). Frozen fruits were thawed at 4°C overnight and homogenized in a
commercial blender at < 10 °C. ACN were extracted from homogenized fruits and CkB juice with an equal volume of acetone and filtered (Whatman #1). After removal of the organic phase, the residual material was extracted two additional times with an equal volume of acetone : water mixture (70:30 v/v). Hydrophobic compounds in the extract were extracted into chloroform (2:1 v/v) (Rodriguez-Saona and Wrolstad 2005). Anthocyanins in the crude extracts were further enriched using cation exchange columns: Oasis® MCX SPE cartridge (Waters Corp., Milford, MA) for CkB, and STRATA™ X-C cartridge (Phenomenex, Torrance, CA), for BlkRB, BluB, RdGrp, and StwB according to He and Giusti (He and Giusti 2011). The columns were conditioned with 10 mL methanol and 10 mL 0.1% (v/v) tri-fluoroacetic acid (TFA) prior to addition of extracts. Neutral or anionic phenolics were eluted with 10 mL water (0.1% TFA) and 10 mL methanol (0.1% TFA). ACN were then eluted with 5 mL water : methanol (40:60 v/v) containing 1% NH₄OH followed by methanol with 1%NH₄OH into a flask containing 500 µL formic acid (88%) to achieve a final pH < 2. Remaining salts were removed by C18 solid phase extraction as described in section 2.3.1. Monomeric ACN content was estimated by the differential pH method (Giusti and Wrolstad 2005) as Cy-3-glu equivalents (extinction coefficient of 26900 L cm⁻¹ mg⁻¹) for BlkRB, BluB, CkB, and RdGrp, and Pg-3-glu equivalents (extinction coefficient of 15600 L cm⁻¹ mg⁻¹) for StwB. Profile of ACN species in final extract was determined by HPLC-PDA-ESI-MS as described in section 2.3.4.4. Stock ACN rich extracts were stored at -20 °C until use.

2.3.4.2 Ex vivo assessment of ACN degradation in saliva.

Since salivary composition and flow rate are affected by circadian rhythm (Dawes 1974), brushing teeth (Hoek and others 2002), and consumption of food and beverage (Harthoorn and others 2009), unstimulated saliva was collected from human subjects (N=14) at 7-8 AM before brushing their teeth and consuming foods or beverages. Samples were placed in ice and delivered to the laboratory in < 2 h for treatment and analyses. Ex vivo degradation was evaluated as described in section 2.3.2. In a subsequent experiment, saliva was collected before and after subjects rinsed the mouth with 30 mL of antibacterial chlorhexidine (Periogard® chlorhexidine gluconate oral
rinse, 0.12%) for 5 min to determine the contribution of oral bacteria to the observed degradation of ACN in saliva. Aliquots of diluted saliva were incubated with individual ACN rich extract (50 nmol of either Cy-3-glu equivalents for BlkB, BluB, CkB, and RdGrp or Pg-3-glu equivalents for StwB) per mL saliva as outlined in section 2.3.3.2.

2.3.4.3 Chemical degradation of ACN

Three different formulations of enzyme-free artificial saliva (AS) were prepared. These included 1) AS1, as described by Oomen et al. (Oomen and others 2003) but excluding enzymes; 2) AS1-org, i.e., AS1 lacking mucin, urea, and uric acid; and 3) AS2 according to Wong and Sissons (Wong and Sissons 2001) with a mucin concentration at 0.3 g/L (Rayment and others 2000). The compositions of the three formulations of artificial saliva are shown in Table 2.2). Each ACN rich extract was incubated (37°C, 60 min, 25 nmol/mL saliva) in each of the three preparations of artificial saliva to determine extent of degradation.

2.3.4.4 HPLC-PDA-ESI-MS analysis of ACN.

Separation, identification and quantification of compounds were performed using a Shimadzu LCMS-2010 EV HPLC-MS (Shimadzu Scientific Instruments, Inc., Columbia, MD) connected to an SPD-M20A photodiode array (PDA) detector and single quadrupole electro-spray ionization (ESI) – Mass Spectrometer (MS) detector. Separation was achieved on a Symmetry C-18 reverse-phase column (4.6 x 75 mm; 3.5 μm; Waters Corp., Milford, MA) with a NovaPak® (C-18; 4 x 2.0 mm) guard column. The mobile phase consisted of (A) 4.5% formic acid and (B) acetonitrile and the following gradients were used for the indicated ACN-rich extracts: BlkB: 0-3 min, 7-8 %B; 3-10 min, 8-10 %B; BluB: 0-5 min, 7 %B; 5-7 min, 7-8 %B; 7-18 min, 8-10 %B; 18-23 min, 10-15 %B; CkB: 0-3 min, 7-10 %B; 3-10 min, 10-20 %B; RdGrp: 0-3 min, 7 %B; 3-4 min, 7-12 %B; 4-6 min, 12 %B; 6-13 min, 12-35 %B; and, StwB: 0-3 min, 7-10 %B; 3-10 min, 10-15 %B. Flow rate was 0.5 mL/min for all separations and the column was equilibrated for 5 min between runs. Spectroscopic data (200-700 nm) were collected during the whole run. Flow was partially split to MS to monitor mass of parent and daughter (aglycone) fragments. MS conditions were as follows: nebulizing nitrogen gas flow, 1.5 L/min;
interface, +4.50 kV; heat block temperature, 200 °C; focus lens, -2.5 V; entrance lens, -50.0 V; pre-rod bias, -3.6 V; main-rod bias, -3.5 V; detector, +1.50 kV. MS spectra were obtained using positive ionization with full scan (from m/z 100-1200 with scan speed 2000 amu/sec) and selective ion monitoring (SIM) modes (6 aglycones Pg, Cy, Pn, Dp, Pt, and Mv at m/z 271, 287, 301, 303, 317, and 331, respectively).

2.3.5 Data analysis

A minimum of three independent incubations of each ACN rich extract in saliva was performed for each subject. Data were analyzed for statistically significant differences using SPSS Release 17.0 for Windows (SPSS Inc., Chicago, IL). Mixed model analysis of ACN structure (fixed factor) and subject (random factor) with Bonferroni’ adjustment of mean comparison was used for data with BluB, CkB, and RdGrp. Paired-t test was used for data of BlkRB and StwB. Differences were considered significant at $P < 0.05$.

2.4 Results and Discussion

2.4.1 Characterization of ACN rich extracts

ACN in the five fruit extracts were identified by comparison of elution order, peaks spectra, and molecular weight of parent and daughter ions obtained with published literature [BlkRB (Tian and others 2005), BluB (Seeram and others 2006; Tian and others 2005), CkB (Wu and others 2004; Zheng and Wang 2003), RdGrp (Cai and others 2010; Castillo-Munoz and others 2009; Frank and others 2003), and StwB (Buendia and others 2010; Carkeet, Clevidence, Novotny 2008; Felgines and others 2007)] (Table 2.1 and Figure 2.1). HPLC-PDA-ESI-MS analysis of the 5 ACN-rich extracts confirmed the collective presence of 6 anthocyanidin classes and mono-, di-, and tri-saccharide conjugates. Estimated purity of CkB ACN-rich extract used for Aim 1 was $81.1 \pm 0.4 \%$ based on HPLC AUC. Estimated purity of ACN-rich extracts used in Aim 2 for BlkRB, BluB, CkB, RdGrp, and StwB was $95.4 \pm 1.9$, $98.9 \pm 1.4$, $98.2 \pm 0.8$, $71.2 \pm 6.5$, and $85.5 \pm 0.7 \%$ based on AUC, respectively.
Table 2.1 Structure and identification of ACN in ACN-rich extracts. Numbers correspond to HPLC peak assignments shown in chromatograms presented in Figure 2.1.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Identification</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>Absorbance maxima (nm)</th>
<th>Most abundance mass in SCAN, SIM (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyanidin-3-O-sambubioside</td>
<td>OH</td>
<td>H</td>
<td>D-glucose-D-xylose</td>
<td>514, 277</td>
<td>518, 287</td>
</tr>
<tr>
<td>2</td>
<td>Cyanidin-3-O-glucoside</td>
<td>OH</td>
<td>H</td>
<td>D-glucose</td>
<td>514, 278</td>
<td>449, 287</td>
</tr>
<tr>
<td>3</td>
<td>Cyanidin-3-O-(2'-xylosyl rutinoside)</td>
<td>OH</td>
<td>H</td>
<td>D-glucose-L-rhamnoxylose</td>
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<td>727, 287</td>
</tr>
<tr>
<td>4</td>
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<td>H</td>
<td>D-glucose-L-rhamose</td>
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<td>595, 287</td>
</tr>
<tr>
<td>5</td>
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<td>OH</td>
<td>galactose</td>
<td>524, 275</td>
<td>465, 303</td>
</tr>
<tr>
<td>6</td>
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<td>OH</td>
<td>D-glucose</td>
<td>524, 244</td>
<td>465, 303</td>
</tr>
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<td>7</td>
<td>Cyanidin-3-O-galactoside</td>
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<td>H</td>
<td>galactose</td>
<td>517, 278</td>
<td>449, 287</td>
</tr>
<tr>
<td>8</td>
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<td>OH</td>
<td>arabinose</td>
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<td>435, 303</td>
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<td>9</td>
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<td>11</td>
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<td>galactose</td>
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<td>D-glucose</td>
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<td>433, 271</td>
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<td>H</td>
<td>D-glucose-L-rhamose</td>
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<td>579, 271</td>
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Figure 2.1. All 21 ACN were degraded to different extents during incubation with human saliva. Representative HPLC-PDA chromatograms of saliva-extract mixtures before and after incubation (60 min, 37°C) with indicated extracts. Labels above peaks correspond to specific ACN presented in Table 2.1.
2.4.2 Aim 1.1: Characterization of ex vivo degradation of ACN using chokeberry (CkB) extract

2.4.2.1 Ex vivo assessment of ACN degradation in saliva

We first examined the extent of degradation of ACN in CkB extract in saliva from a single subject. The extent of degradation of the four cyanidin glycosides increased in a time-dependent manner during the 60 min incubation at 37°C (Figure 2.2A). Approximately 50% of each of the four cyanidin glycosides was degraded after 60 min incubation, suggesting limited impact of type of monosaccharide on extent of ex vivo degradation (Figure 2.2B). Variability of extent of CkB ACN degradation in saliva collected on three different days was limited (45.9 – 52.1 % degradation) during 60 min incubation (45.9 – 52.1 % degradation). In all subsequent experiments ACN were incubated in saliva for 60 min to allow more precise assessment of the extent of degradation. Indeed, confectionaries and oral adhesive gels containing bioactive compounds are being developed to prolong oral retention of bioactive compounds (Amoian and others 2010; Mallery and others 2007).

Proctor et al. reported that proline-rich proteins, a major class of proteins in saliva, can bind ACN (Laurent, Besancon, Caporiccio 2007; Proctor and others 2005). This suggested that the loss of ACN following addition to saliva may reflect association of ACN with proline-rich proteins precipitated by addition of acidified methanol at 60 min to terminate reactions. Acetonitrile was used previously to separate polyphenols from more hydrophilic compounds such as sugars and proteins in plasma and saliva (Laurent, Besancon, Caporiccio 2007; Lee and others 2000). The precipitate resulting from addition of acidic methanol to salivary samples containing ACN lacked evident color. Mixing the precipitate with acetonitrile yielded an additional 1-3% of initial amount of ACN added to saliva, suggesting that the loss of ACN during incubation was not primarily due to association with precipitated salivary proteins.

ACN have been reported to spontaneously degrade at neutral and alkaline pH in aqueous solutions (Brouillard 1982) and cell culture media (Kay, Kroon, Cassidy 2009), and are enzymatically degraded during in vitro digestion (McDougall and others 2005b).
Figure 2.2. *Ex vivo* degradation of chokeberry ACN-rich extract in human saliva is primarily enzyme-mediated. Panel A: Time-dependent degradation of total ACN from chokeberry during 60 min incubation. Data are mean ± SD, n=3. Panel B: Degradation (%) of cyanidin glycosides in chokeberry after incubation (60 min, 37°C, 85rpm) with intact saliva from a human subject. Data are mean ± SD, n=8. There was no significant difference in extent of degradation of the four cyanidin glycosides (*P* ≥ 0.05). Panel C: Degradation (%) of cyanidin glycosides in chokeberry incubated with saliva from a human subject after indicated treatments. Data are mean ± SD, n= 6. Means not sharing common superscript letters differ significantly (*P* < 0.05).
and incubation with colonic microflora (Keppler and Humpf 2005) and Caco-2 human intestinal epithelial cells (Kay, Kroon, Cassidy 2009). To examine the relative extent of spontaneous vs. enzymatic degradation of CkB ACN, aliquots of saliva were either incubated with CkB ACN at 0°C and 37°C, heated to 80°C to inactivate enzymes before incubating with CkB ACN at 37 °C, or centrifuged and filtered to remove cells before incubation with CkB ACN extract at 37 °C. ACN degradation in saliva during incubation at 0°C was approximately 90% less than that during incubation at 37 °C (Figure 2.2C). Similarly, loss of cyanidin glycosides during incubation at 37°C in heat inactivated saliva, or in cell-free saliva or in enzyme-free artificial saliva was significantly less than that in intact saliva. Collectively, these data suggest that loss of CkB ACN in saliva was primarily enzymatic and dependent on cellular activity rather than secretions from the salivary glands or binding of CkB ACN to salivary proteins.

2.4.2.2 Degradation products of chokeberry cyanidin glycosides in saliva

Protocatechuic acid (PCA) and phloroglucinol aldehyde (PGA) have been identified as microbial metabolites accounting for as much as 20 % of the observed loss of cyanidin glycosides during incubation with human and pig feces (Aura and others 2005; Keppler and Humpf 2005). It has been proposed that microbial β-D-glycosidase activity generated the anthocyanidin aglycone that was spontaneously cleaved to phenolics PCA and PGA (Kay, Kroon, Cassidy 2009). We investigated whether the loss of CkB cyanidin glycosides in saliva was associated with generation of cyanidin aglycone and its phenolic products. Neither cyanidin aglycone, PCA or PGA were detected after incubation of CkB ACN in intact saliva at 37°C (detection limit = 3.6, 2.0 and 1.8 ng/mL, respectively), despite the presence of β-D-galactosidase and β-D-glucosidase activities in intact saliva (data not shown). Cyanidin aglycone (25 nmol/L CyCl) was extensively degraded (> 90%) upon addition to intact saliva with further loss during the next 60 min (Figure 2.3A). PCA was detected in response to the loss of CyCl, although it only accounted for 15.3 % and 9.6 % of the degraded CyCl at 0 and 60 min, respectively (Figure 2.3B). Greater than 86 % of CyCl was recovered immediately after addition to acidified water, although greater than 88 % was lost after incubation for 60 min (Figure 2A). PCA in acidified water accounted for much of the degraded CyCl at 0 min, but only
Figure 2.3. Protocatechuic acid (PCA) and phloroglucinol aldehyde (PGA) are not primary degradation products of cyanidin glycosides in saliva. Panel A: Degradation of cyanidin chloride upon addition to and incubation in acidified water and human saliva. Panel B: Minimal PCA accumulates in acidified water and saliva during degradation of cyanidin chloride. Samples were incubated in saliva at 37°C for 0 and 60 min. Data are mean ± SD, n ≥ 3. The presence of different superscript above error bars denotes significant (P < 0.05) differences. Panel C: Recovery of exogenous PCA and PGA after incubation in human saliva at 37°C for 60 min. Data are mean ± SD, n = 6.
14.5% of that degraded after 60 min (Figure 2.3B). Mallery et al. reported that a very low level of cyanidin aglycone was detected 5 min after a 3 min oral rinse with 10% black raspberry water and was undetectable at 60 min post rinse (Mallery and others 2011). Our detection of some cyanidin aglycone and PCA during ex vivo incubation of cyanidin aglycone, but not cyanidin glycosides, suggests that aglycone formation was not a primary intermediate in the degradation of cyanidin glycosides in saliva. To test the possibility that generated PCA and PGA might be further metabolized in saliva, PCA and PGA were added to intact saliva followed by incubation at 37°C for 60 min. Recoveries of PCA and PGA were 90% and 43%, respectively (Figure 2.3C), which is similar to the observed stability of PCA during in vitro incubation with human fecal microflora (Fleschhut and others 2006) suggesting that PCA should be readily detected if generated in saliva. Mallery et al. reported the presence of PCA and its glucuronidated metabolites in saliva from some subjects for as long as several hours after rinsing their mouths with black raspberry preparation suspended in water for 3 min (Mallery and others 2011). These authors suggested that PCA and its glucuronidated metabolites may be generated after uptake and metabolism of BlkRB ACN by oral epithelium.

Preliminary analysis of metabolic products using high resolution quadrupole Time-Of-Flight mass spectrometry suggest that chalcone-glucosides of Cy may account for as much as 30% of the original amount of standard Cy-3-glu incubated in saliva ex vivo. This observation is in line with an alternative degradation pathway proposed by Markakis (Markakis 1974) that the heterocyclic C-ring of carbinol glycosides can be opened to yield colorless chalcone prior to hydrolysis of the glycosidic bond. It is interesting that several chalcone flavonoids structurally resembling Cy chalcone glycosides have reported bioactivity. For example, butein (3,4,2’,4’-tetrahydroxychalcone) was recently shown to suppress the activity of transcription factor NF-κB in several cancer cell lines (Chua and others 2010; Moon and others 2010) and prevent cytokine-induced nitric oxide production in rat pancreatic β cells (Jeong and others 2011). As a result, it is possible that unidentified metabolites generated in the oral cavity are bioactive, warranting further examination of the degradation products of CkB.
2.4.3 Aim 1.2: Effect of ACN structure and oral microbiota on extent of ex vivo salivary degradation

2.4.3.1 Effect of structure of ACN and inter-subject variation

ACN-rich extracts from BluB, CkB, BlkRB, RdGrp, and StwB were incubated in saliva collected from 14 healthy human subjects. The extracts from these fruits include 6 anthocyanidin aglycones covalently linked to mono-, di-, and tri-saccharides. All 21 identified ACN in the extracts were partially or completely degraded during incubation in saliva from all subjects (Figure 2.1).

The extent of degradation of total ACN incubated in saliva from the different subjects varied considerably, i.e., 45-75% for BluB, 10-90% for CkB, 10-80% for BlkRB, 8-60% for RdGrp, and 13-60% for StwB. The pattern of degradation of CkB ACN in saliva for individual subjects is presented in Figure 2.4A. At least 50% CkB ACN were degraded in saliva from the majority of the subjects. Although mean degradation of the four different cyanidin glycosides was similar in saliva from 13 subjects ($P = 0.560$; Figure 2.4B), the extent of degradation of the galactoside and glucoside conjugates exceeded that of arabinoside and xyloside conjugates in saliva from subjects 3, 7 and 11. These observations suggest inter-subject variability in both extent and pattern of cyanidin glycoside degradation in saliva.

RdGrp was selected to compare the influence of the structures of anthocyanidins on the extent of ACN degradation in saliva. The five anthocyanidins (i.e., Dp, Cy, Pt, Pn, and Mv) in RdGrp are all conjugated with glucose at the 3’ position (Figure 2.1). Degradation of Dp and Pt glucosides exceeded that of Cy, Pn and Mv ($P < 0.001$; Figure 2.4C). This pattern of degradation was consistent in all 14 subjects. The extent of degradation among subjects for each ACN in RdGrp ranged from 60-100% (Dp), 7-60% (Cy), 65-100% (Pt), 5-60% (Pn), and 5-55% (Mv) (data not shown).

ACN-rich extract from StwB was investigated to assess the effect of linkage of a mono-saccharide (Pg-3-glu) versus a di-saccharide (Pg-3-rut) to the anthocyanidin on susceptibility to degradation in saliva. Degradation of Pg-3-rut was significantly ($P < 0.001$) less than that of Pg-3-glu (Figure 2.4D). Slighty, but significantly ($P < 0.001$),
Figure 2.4. **Ex vivo degradation of ACN in saliva is variable among donors and affected by ACN structure.** Panel A: Inter-subject variability in extent of degradation of chokeberry ACN after incubation (60 min, 37°C) in saliva (n = 13). Data are mean ± SE of 4 replicate samples. Salivary degradation (%) of ACN from chokeberry (Panel B, n = 13), red grape (Panel C, n = 14), strawberry (Panel D, n = 14), black raspberry (Panel E, n = 10), and blueberry (Panel F, n = 11). Data are mean ± SE. Means not sharing common letters above bars within a panel differ significantly (P < 0.05).
less degradation of Pg-3-glu compared to Cy-3-glu also was observed, further suggesting differential susceptibility associated with anthocyanidin structure (Figure 2.4D).

To further examine the possible influence of more complex carbohydrates moieties on susceptibility to degradation in saliva, ACN-rich extract from BlkB was tested. Although the presence of Cy-3-samb and Cy-3-glu was confirmed by mass spectrometry (Table 2.1), these compounds were not adequately separated by HPLC (Figure 2.1) and therefore excluded from this comparison. Mean degradation of the tri-saccharide (xylosyl-rotinoside) conjugate of cyanidin for all subjects was significantly ($P < 0.001$) lower than degradation of the di-saccharide rutinose Cy conjugate (Figure 2.4E). Similarly, quercetin di- and tri-saccharides and cyanidin di- and tri-saccharides have been reported to be less susceptible to degradation than their respective mono-saccharide conjugates (Keppler and Humpf 2005; Walle and others 2005).

BlkB extract was tested to compare the influence of structure for five anthocyanidins linked to three different monosaccharides on the extent of degradation in saliva (Figure 2.4F). A mixed model statistical analysis revealed that degradation was significantly affected by type of anthocyanidin ($P < 0.001$), but not by type of monosaccharide ($P = 0.063$) or the interaction between ACN and monosaccharide ($P = 0.495$). As observed with RdGp (Figure 2.4C), degradation of Dp- and Pt-3-glucosides was significantly ($P < 0.001$) greater than that of the 3-glucose conjugates of Cy, Pn and Mv in saliva from all subjects (data not shown). Also, as observed with cyanidin glycosides in CkB extract (Figure 2.4A), the extent of degradation of the anthocyanidin in BlkB extract was not significantly altered by type of monosaccharide for all but three subjects (numbers 3, 7 and 11). Degradation of the hexose conjugates exceeded that of the pentose conjugates in saliva from these three subjects.

The extent of degradation of a specific ACN in saliva appeared to differ across the different extracts. For example, mean degradation of Cy-3-glu in CkB was 63% as compared to 54% degradation in BlkB and only 42% degradation, in RdGrp and StwB (Figure 2.4). We suspect that the presence of multiple ACN and possibly contaminating phenolics may affect the stability of ACN via co-pigmentation (Eiro and Heinonen 2002;
Glucuronidated derivatives of PCA have been detected in saliva collected 5 min after an oral rinse with 10% BlkRB slurry suggesting that phenolic metabolites of anthocyanins were conjugated within and effluxed from the oral epithelium (Mallery and others 2011). We did not detect either PCA or PGA after incubation of ACN extracts in saliva from any subject. Similarly, treatment of post-incubation mixtures in which ACN degradation was high (saliva from subjects 1 and 5 for BluB, subjects 1, 5 and 9 for CkB, subjects 1 and 9 for BlkRB, subjects 1and 5 for RdGrp, and subjects 5 and 9 for StwB) with _H. pomatia_ enzyme preparation containing glucuronidase and sulfatase activities for 3 hr at 37°C also failed to generate detectable PCA and PGA. As the limit of detection of our analytical system was an order of magnitude less than that of the Mallery team (Mallery et al., 2011), it is possible that low concentrations are these metabolites may have been present. Interestingly, a peak corresponding to 4-hydroxybenzoic acid (retention time and absorbance spectrum) was observed following enzyme treatment of saliva incubated with CkB, BlkRB, BluB and StwB extracts.

### 2.4.3.2 Non-enzymatic degradation of Dp and Pt glycosides

To address the basis for the greater susceptibility of Dp and Pt glycosides to degradation in saliva compared to that of the other anthocyanins, ACN-rich extract from RdGrp was first incubated in water and enzyme-free artificial saliva (Figure 2.5A). RdGrp ACN degraded to a significantly (_P_ < 0.001) greater extent in artificial saliva (pH 6.4) than in DI water (pH 7.3). This observation is similar to a recent report that Dp-3-glu, Pg-3-glu, and Cy-3-glu were less stable in Na/K phosphate buffer than in water (Woodward and others 2009). Moreover, degradation of Dp- and Pt-3-glucosides in enzyme-free artificial saliva was significantly (_P_ < 0.001) greater than that of Cy-, Pn- and Mv-3-glucosides and further confirms that the losses during incubation were not due to protein binding. This increased susceptibility of Dp- and Pt-glycosides to non-enzymatic degradation in artificial saliva was also observed with ACN-rich extract from BluB (data not shown) and was aligned with their preferential degradation in intact saliva (Figure 2.4C and 2.4F). Removal of organic compounds from the enzyme-free artificial
Figure 2.5. Dp and Pt glucosides are preferentially degraded in artificial saliva. Panel A: Degradation of Dp and Pt is greater than Cy, Pt and Mv in enzyme-free artificial saliva. Data are mean ± SD, n = 6. Presence of different letter above error bars for ACN in enzyme-free artificial saliva are significantly different (P < 0.05). Panel B: The electrolyte composition of artificial saliva affects the stability of Dp and Pt glucosides. Data are mean ± SD, n = 3. The asterisks above error bars denote mean for AS2 differs significantly from AS1 and AS1-org (P < 0.05).
saliva did not further affect \((P > 0.05)\) the extent of degradation of RdGrp ACN suggesting that mucin, urea, and uric acid do not contribute to the relative instability of Dp- and Pt- glycosides (Figure 2.5B). In contrast, differences in the inorganic contents of artificial saliva preparations AS1 (\(\text{Na}_2\text{SO}_4, \text{NaOH}, \text{KSCN}, \text{and} \ \text{NaH}_2\text{PO}_4\)) and AS2 (\(\text{MgCl}_2, \text{CaCl}_2, \text{NH}_4\text{Cl}, \text{KH}_2\text{PO}_4, \text{and} \ \text{K}_2\text{HPO}_4\)) significantly \((P < 0.001)\) affected the extent of degradation of Dp and Pt in RdGrp extracts (Figure 2.5B), suggesting that one or more electrolytes (Table 2.2) in saliva may affect the stability of the Dp and Pt glucosides. Cabrita et al. reported that Dp and Pt glucosides are less stable than Cy-, Mv-, and Pn- glucosides in aqueous buffer at pH > 7 (Cabrita, Fossen, Andersen 2000). Dp-3-glu and Dp-3-rut were also reported to degrade more rapidly than Cy-3-glu and Cy-3-rut in Dulbecco’s modified Eagle’s medium (Steinert and others 2008). Interestingly, susceptibility to degradation appears to be related to superoxide radical scavenging potential of ACN glycosides as degradation of Dp > Pt > Cy≈Mv > Pn > Pg (Rahman and others 2006). Dp-3-glu also possesses greaterer antioxidant activity than Cy-3-glu in the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Kahkonen and Heinonen 2003). Such results suggest that oxidative degradation of ACN, and especially Dp-glycosides, may contribute to the observed losses in saliva. As Dp and Pt, but not Cy, Pn and Mv, are hydroxylated at the C5’ position of the B-ring, the possible influence of this substitution on stability in saliva merits investigation.
Table 2.2. Compositions of enzyme-free artificial saliva. Artificial saliva modified from Oomen et.al. (Oomen and others 2003) with (AS1), without organic constituents (AS1-org), and artificial saliva modified from Wong and Sissons (Wong and Sissons 2001) with adjusted mucin concentration (Rayment and others 2000) (AS2).

<table>
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2.4.3.3 **Effect of oral microbiota on salivary degradation of ACN**

To investigate the possible role of oral microbiota in the *ex vivo* degradation of ACN, saliva was collected before and after oral rinse with antibacterial chlorhexidine. Antibacterial treatment significantly (*P* < 0.001) decreased the degradation of ACN extracted from RdGrp, StwB, and CkB, in saliva by 49%, 60% and 80%, respectively ([**Figure 2.6**](#)). The degree of inhibition was not affected (*P* > 0.05) by type of conjugated monosaccharides ([**Figure 2.6A**](#)) or number of saccharide units of Pg ([**Figure 2.6C**](#)). In contrast, type of aglycone in RdGrp ACN significantly (*P* < 0.001) affected the impact of treatment with the antibacterial chlorhexidine on the extent of degradation ([**Figure 2.6B**](#)). Degradation of Pt-3-glu in saliva after anti-bacterial treatment decreased by 10% which was significantly (*P* < 0.05) less than that of Dp (30%), Cy (40%), Pn, and Mv (50%). Thus, degradation of glucosides of Dp, Cy, Pn, and Mv was more closely associated with microbial activity than was that of Pt glucosides. Our observation in saliva contrasts with the report of Forester *et al.* who did not observe preferential degradation of Dp, Pt, Pn, Mv glucosides during incubation with large intestinal contents from swine (Forester and Waterhouse 2008). Given the unique profile of microbiota residing in the various sections of GI tract (Kerckhoffs and others 2006) , further characterization of oral microbial activity on ACN metabolism by the oral microbial community merits future investigation.
Figure 2.6. Chlorhexidine oral rinse suppresses ex vivo degradation of ACN in saliva. Data are mean ± SE for 8 human subjects with 4 replicate samples per ACN extract. Means not sharing common letters above bars within same extract are significantly different at $P < 0.05$. 
2.5 Conclusions

To the best of our knowledge, this is the first examination of the effect of ACN structure on susceptibility to degradation in saliva. ACN from 5 dietary sources containing a total of 6 anthocyanidins conjugated to mono-, di- and tri-saccharides were partially degraded during incubation in human saliva at 37°C. Degradation was in part due to microbial activity, although non-enzymatic degradation of Dp and Pt exceeded that of Cy, Pn and Mv. Disappearance of Cy-glycosides or Cy aglycone was not coupled with generation of detectable amounts of PCA or PGA. Preliminary detection of chalcone glycosides of Cy suggests a degradation pathway of ACN in oral cavity that may differ from that in the large intestine. Moderate inter-subject variation in the extent of degradation was observed, but the general patterns of degradation were rather consistent among individuals. While types of monosaccharides did not affect the extent of degradation, anthocyanidins with di- and tri-saccharide conjugates were slightly, but significantly, less susceptible to degradation in saliva. These data collectively suggest that ACN structure affects susceptibility to degradation and perhaps the relative bioaccessibility and efficacy of these compounds in the oral cavity. Studies to assess this possibility have been initiated.
CHAPTER 3

STABILITY AND BIOAVAILABILITY OF ANTHOCYANINS FROM GRAPE AND
CHOKEBERRY JUICE IN HUMAN ORAL CAVITY
3.1 Abstract

Anthocyanins (ACN) are a family of flavonoids (>700 distinct structures) that have been reported to have chemopreventive and chemotherapeutic activities in the oral cavity of hamsters, rats, and human subjects. However, there is very limited information about the oral stability, metabolism and delivery of ACN and their degradation products to tissues in the mouth. Because the relative stability of ACN when incubated ex vivo in saliva has been shown to be dependent on their structure, we hypothesized that structure is likely to affect their availability and perhaps efficacy in oral tissues in vivo. In the present study, either red grape juice or chokeberry juice was retained in the oral cavity of 12 healthy human participants for 5 minutes in a randomized crossover design. Retained juice, oral washings and buccal scrapings were collected and buccal epithelial cells were separated from mucus. ACN in samples were analyzed by HPLC-PDA-ESI-MS to evaluate stability of ACN in oral cavity, their binding to the mucus layer of buccal scrapings and their uptake into buccal epithelial cells. Results showed that loss of delphinidin (Dp) glucosides in red grape juice significantly (P < 0.05) exceeded that of petunidin (Pt), cyanidin (Cy), peonidin (Pn) and malvidin (Mv) glucosides. Relatively lesser amounts of Dp and Pt glucosides were associated with buccal mucus (P < 0.05) and buccal epithelial cells (P = 0.09) than Cy, Pn and Mv glucosides, suggesting that loss of Dp and Pt in oral cavity was likely due to degradation. In chokeberry juice, loss of Cy-3-xyloside exceeded that of Cy-3-glucoside and Cy-3-arabinoside. Less Cy-3-xyloside was associated with mucus than Cy-3-galactoside and Cy-3-arabinoside. Cy-3-glucoside preferentially accumulated in the buccal epithelium (P < 0.05) compared to the other Cy glycosides in chokeberry juice. These results suggest that ACN structure affects stability and buccal cell uptake in the oral cavity and, therefore, the potential efficacy of various ACN-rich products for the promotion of oral health.

3.2 Introduction

Oral diseases such as dental caries, gingivitis, and periodontal diseases are global problems that profoundly affect general health and quality of life for millions of
individuals (Petersen and others 2005; Sischo and Broder 2011). For example, most recent NHANES study has estimated that 47% of the adult American population (64.7 million individuals) has periodontitis (Eke and others 2012). Moreover, the WHO lists oral cancers as one of the most common cancers in humans (Petersen and others 2005). In the US, more than 36,000 new cases were diagnosed in 2010 and only 65% of these individuals are expected to survive longer than 5 years after diagnosis (Jemal and others 2010). The estimated expense of treating oral diseases ranks fourth among all chronic diseases in developed countries; representing 5-10% of public health expenditures (Petersen and others 2005). Interestingly, epidemiological studies have shown an inverse relationship between intake of fruits and vegetables and the prevention of oral cancer (Block, Patterson, Subar 1992; Petti and Scully 2009; Sakagami, Oi, Satoh 1999; Saman 2012). It also has been proposed that the polyphenols in fruits and vegetables contribute to the decreased risk of developing caries, gingivitis and periodontitis as they possess anti-bacterial, anti-mycotic and anti-inflammatory activities (Bodet and others 2008; Varoni and others 2012). These data support the need for cost-effective and efficacious strategies to promote oral health of which dietary chemoprevention appears to represent one such economically sustainable option (Tomar and Cohen 2010).

Anthocyanins (ACN) are a class of flavonoids that are responsible for the orange, red, and blue colors of many fruits and vegetables. Typical daily consumption of ACN ranges from of in the US has been estimated as high as 215 mg (Chun, Chung, Song 2007; Kuhnau 1976; Wu and others 2006) which is greater than that of many other dietary flavonoids (20-25 mg/day) including apigenin, genistein, quercetin, kaempferol, myricetin and luteolin (Cooke and others 2005; Hertog and others 1993). ACN are of particular interest as natural food colorants (Markakis 1974) and for their health-promoting activities (He and Giusti 2010; Mazza 2007; Prior and Wu 2006; Zafra-Stone and others 2007). An emerging body of literature suggests that ACN promote oral health. For example, an ACN-rich extract from black raspberry inhibited proliferation and expression of angiogenic vascular-endothelial-growth-factor (VEGF), and suppressed nitric oxide synthase activity in oral squamous cell carcinoma cell lines (Rodrigo and others 2006). ACN rich extracts from strawberry (Zhang and others 2008), black berry,
black raspberry, blueberry, cranberry and red raspberry (Seeram and others 2006) each inhibited proliferation of KB and CAL-27 human oral cancer cell lines. Inclusion of 5% freeze-dried black raspberry in the diet fed to hamsters reduced the number of chemically-induced tumors in buccal mucosa (Casto and others 2002). Similarly, diets containing either freeze-dried black raspberry or ACN extract from black raspberry reduced chemically-induced esophageal tumorigenesis in rats (Wang and others 2009). Topical application of a gel treatment containing 10% (w/w) freeze-dried black raspberry of human subjects with oral intraepithelial neoplasia was associated with decreased histological basilar hyperplasia and the nuclear to cytoplasmic ratio, decreased loss of heterozygosity for the tumor suppressor gene, and decreased COX-2 protein in lesional oral tissues (Mallery and others 2008; Shumway and others 2008). It also has been demonstrated that the anti-bacterial, anti-mycotic and anti-inflammatory activities of polyphenols in fruits and vegetables contribute to the decreased risk of developing caries, gingivitis and periodontitis (Bodet and others 2008; Varoni and others 2012). Such results have provided impetus to incorporate ACN in products such as bio-adhesive gels and confectioneries (Mallery and others 2008; Song and others 2011) as a mean of increasing interactions of these compounds with oral tissues.

ACN are susceptible to extensive degradation in the small and large intestines (McGhie and Walton 2007; Prior and Wu 2006; Stoner and others 2005) and the intestinal microbiota can convert as much as 20% ingested ACN to phenolic metabolites (Forester and Waterhouse 2008; Keppler and Humpf 2005). Moreover, it has been suggested these products of metabolic degradation may be responsible for the reported bioactivities of ACN (Williamson and Clifford 2010). Also, with estimates of more than 700 distinct ACN in food (Andersen and Markham 2006), the relationship of ACN structure with the extent of metabolism and delivery of the ACN or its products to oral tissues is of particular interest for developing dietary recommendations and food formulations aimed at promoting oral health. We recently reported that salivary microbiota degrade ACN ex vivo and that the extent of degradation in saliva was affected by ACN structure (Kamonpatana and others 2012). The purpose of the present study, therefore, was to examine the in vivo stability and extent of binding of ACN derived from
chokeberry and red grape juices to buccal mucus and epithelial cells. A secondary aim was to examine the role of oral microbiota in degradation of ACN in vivo.

3.3 Materials and methods

3.3.1 Standards and reagents

Unless otherwise indicated, all supplies and chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO) and Fisher Scientific Company (Pittsburgh, PA).

3.3.2 Preparation of juices

Red grapes were purchased from a local supermarket and frozen (-18°C) until preparation of juice. Red grapes were thawed (4°C, overnight) and ground in a commercial blender for 10 minute to produce a cold (<10°C) slurry. Solid particles were removed by passing through nylon mesh (250 µm pore size, NITEX®, Sefar Inc., Ontario, CA). Pectin was degraded by incubation (0.6mL/L juice, 3 h, room temp) with food-grade pectinase (Enzeco® Pectinase DV-2, Enzyme Development Corp., New York, NY). Remaining solids were removed by centrifugation (3000 rpm, 5 min). Concentrated chokeberry juice was a gift from Artemis International (Fort Wayne, IN). Concentration of ACN in chokeberry juice was normalized to 60 nmol Cy-3-glu equivalents /mL by diluting with DI water prior to storage in 20 mL aliquots at -20°C until chemical analysis and use in the human trial. The qualitative and quantitative profile of ACN was not altered during storage at -20°C for 1 month.

3.3.3 Characterization of juices

Content of monomeric anthocyanins of each juice was measured by pH differential method (Giusti and Wrolstad 2005). The total polyphenolic content of each juice was analyzed by the Folin-Ciocalteu assay and reported as gallic acid equivalents (Waterhouse 2005). Soluble sugar and pH were measured by portable refractometer (Fisher Scientific, 13-946-21, 0—32°Brix) and by pH meter (Accument® XL15, Fisher
Scientific), respectively. ACN profile of each juice was analyzed by HPLC as described below.

3.3.4 Experimental design

The human study protocol was approved by Institutional Review Board of the Ohio State University (IRB#20090058). Thirteen systemically and dentally healthy subjects (as evidenced by clinical attachment levels ≤ 2 mm and probing depths ≤ 3 mm and a low DMF (decayed, missing, filled teeth) Index) over the age of 20 were recruited to the study. Subjects who had antibiotic therapy or professional cleaning within the previous 3 months, used immunosuppressant medications, bisphosphonates or steroids, reported pregnancy, or have a history of diabetes or HIV were excluded from this study, in order to minimize variations in salivary secretion and oral bacterial profiles (Dawes 1987; Socransky and Haffajee 2005).

There were two clinical studies. To determine the effect of ACN structure on stability and delivery to buccal tissue, juices from chokeberry and grape were assigned to subjects in randomized cross over design with 1 week wash-out period between juice treatments. Human subjects (n = 12) were instructed to abstain from consuming ACN containing foods and beverages after 10 PM on the day before testing. On the day of study, subjects brushed their teeth and consumed breakfast before arrival at clinic. Each subject served as their own internal control. Control buccal cells were collected upon arrival in the clinic (9-11 AM) by brushing the left buccal mucosal surface 10 times with a tooth brush followed by rinsing with saline (0.9% NaCl, 20 mL). Control cells were analyzed to determine baseline ACN. Subsequently, juice (20 mL in pre-weighed polypropylene tube) was retained and swished around in the mouth for 5 min. Juice remaining in the tube was measured gravimetrically. Retentate was expectorated and collected along with subsequent oral rinse (20 mL DI water) in pre-weighed tubes to determine total amount of juice and saliva expelled. A new toothbrush was used to collect buccal cells from the right buccal mucosal surface followed by saline rinse as previously described. Tubes were re-weighed to estimate the amount of saliva in retained juice and oral rinse. Data from one subject who reported accidental swallowing of a portion of the chokeberry juice were excluded.
To investigate the role of oral microbiota on the loss of ACN in the oral cavity, human subjects (n = 10) were instructed to refrain from consumption of ACN containing foods, coffee, and tea 24 h prior to study. On the day of study, subjects brushed their teeth after awakening, avoided use of antibacterial mouth wash, and did not consume breakfast until arrival to clinic (7-9 AM). Control buccal cells were collected prior to retention of chokeberry juice, followed by post-rinse collection of buccal cells as described above. After a 1 week wash-out period, the same subjects were asked to follow the same procedures prior to arrival at clinic. Human subjects received professional oral cleaning to remove the oral bacterial biofilms, before rinsing their mouths with 15 mL 0.12% chlorhexidine gluconate (Periogard®, Colgate-Palmolive Company, New York, NY) for 1 min, and followed by a 5 min wash-out period. Pre-rinse buccal scrapings, retained chokeberry juice, and post-rinse buccal scrapings were collected and processed using the same procedures as described above.

3.3.5 Sample processing

Retained juices and aqueous oral rinse were separately weighed, acidified with 88% formic acid (final concentration 5% by adding 1.2:20 v/w) within 30 min after sample collection. Both solutions were combined, filtered (0.22 µ pore size), and stored at -20°C until analysis. Buccal scrapings were centrifuged (130 × g, 5 min, 4°C) to separate soluble mucus from buccal cells. Cell pellets were washed 3 times with 10 mL cold sodium phosphate buffer (0.1 M, pH 6.0) and centrifuged to separate extra-cellular ACN and mucin from buccal cells (Sakanaka and others 1996). Washed cells pellets were resuspended in sodium phosphate buffer and stored at -20°C. Supernatant containing mucus also was stored at -20°C, lyophilized to dryness, resolubilized in 15 mL 1:1 (v/v) methanol:water for quantification of mucin, and filtered (0.22 µ pore size) for HPLC analysis of ACN content.

3.3.6 ACN extraction from buccal epithelial cells

Preliminary studies were performed to determine the efficiency of extraction of ACN from buccal cell pellets. Extraction solvents tested were methanol, acetronitrile,
acetone and 70% acetone. All solvents were acidified with formic acid (5% final concentration) to stabilize ACN. ACN-rich extracts (15 nmol of Cy-3-glu equivalents in 25 µL 0.01% HCl solution) from chokeberry and red grape were added to 0.25 mL suspensions of control washed buccal cells isolated from two subjects. Suspensions were sonicated (30 sec, ice-water bath) before addition of 0.75 mL of test solvents and frozen (-20°C) overnight. Samples were thawed, sonicated for 20 min in an ice-water bath, and centrifuged (14000 rpm, 5 min). Supernatant was collected and the pellet was re-extracted twice. Supernatants for the three extractions were transferred to a pre-weighed tube, concentrated by evaporation under N₂ (30 min, 37°C), weighed, and filtered (0.22 µm pore size) prior to HPLC analysis. Recovery was calculated as (amount ACN extracted/amount ACN added) × 100%. Recovery using 70% acetone solvent exceeded 84% for all ACN in chokeberry and red grape juices (data not shown) which was considerably greater than recovery after extraction into the other solvents tested (i.e., 36-51%). The extent of recovery of each ACN was not significantly affected by type of monosaccharide. Therefore, ACN in all test samples were extracted into acidified 70% acetone for analysis.

3.3.7 HPLC analysis

Content of ACN in juices, reentrate, mucus and cell extracts was analyzed by HPLC-PDA-ESI-MS according to previously published methods (Kamonpatana and others 2012) and reported as Cy-3-glu equivalents. Identification of ACN was based on retention time (Rt) and λmax-vis and matched molecular weights of parent compounds and daughter fragments with those of available anthocyanin standards, isolated chokeberry anthocyanins, and reported elution profiles for chokeberry (Wu and others 2004; Zheng and Wang 2003) and red grape (Cai and others 2010; Castillo-Munoz and others 2009; Frank and others 2003). The cellular content of ACN was normalized by DNA content in isolated cell pellets (section 2.8). The quantity of ACN in the mucus layer was normalized per mg of mucin (section 2.9). Separation, identification, and quantification of reported phenolic metabolites were performed using the same HPLC-PDA-ESI-MS system (Kamonpatana and others 2012) using mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1%
formic acid in acetronitrile. The gradient for the mobile phase was 0–30 min, 0–30% B; 30–31 min, 30–100% B; 31–32 min 100–0% B; 32–37 min, 0% B. Partial flow after PDA detector was negatively ionized by the mass spectrometer with previously published parameters (Kamonpatana and others 2012). Mass spectra were obtained using scan mode (from m/z- 50 – 800 with scan speed 1000 amu/s) and selective ion monitoring (SIM) mode for PCA, PGA, gallic acid, syringic acid, vanillic acid, and 4-hydroxy benzoic acid at m/z- 153, 153, 169, 197, 167 , and 137, respectively.

3.3.8 Quantification of DNA in buccal cells

DNA in buccal cell aliquots was determined by diphenylamine (DPA) assay as described by Natarajan et al. (33). Briefly, one volume of cold acetaldehyde (0.16% in DI water) was mixed with five volumes of perchloric acid (20% v/v in DI water) to prepare the reagent. Cell suspensions were sonicated (20 min on ice bath), aliquoted (40 µL) into a 96-well plate before addition of 60 µL of acetaldehyde solution. After mixing, 100µL of DPA (4% in glacial acetic acid) was added to wells and the plate was incubated at 37°C for 24 h. Absorbance was measured at 595 nm (corrected by subtraction of absorbance at a reference wavelength of 700 nm). Corrected absorbance was compared to a five point curve using DNA from herring sperm as standard.

3.3.9 Quantification of mucin

Mucin concentrations in buccal washings were determined by modification of previously described (Sarosiek and others 1994) Alcian Blue assay (Hall and others 1980). Aliquots of supernatant were incubated for 30 min in a 1:1 (v/v) of 1% solution of Alcian Blue in 50 mM sodium acetate buffer containing 25 mM MgCl₂, pH 5.8, under constant agitation at room temperature. Samples were centrifuged (705 × g, 20 min) and supernatant was discarded. The pellets were washed twice within 1 mL 40 % (v/v) ethanol in 50 mM sodium acetate buffer containing 25 mM MgCl₂, pH 5.8, briefly vortexed, incubated for 5 min before centrifugation (705 × g, 20 min) and discard of supernatant. Mucin–dye complexes were dissociated by the addition of 1 mL di-octyl sulfosuccinate solution (1:90 w/v in DI water) followed by brief mixing and ultrasonication at 40 W for 10 s with a Vibra CellTM (Sonics & Materials, Danbury, CT).
Samples were centrifuged (705 × g, 1 min) to eliminate the foam generated during sonication. The absorbance of the dye was determined spectrophotometrically at 605 nm and compared to a five point standard curve using known concentrations of mucin from bovine submaxillary glands as standard. This method does not distinguish larger (MG1) and smaller (MG2) mucins.

3.3.10 Statistical analysis

Each samples (retained juices, oral washes and washed buccal cells) collected and prepared from each subject were mixed and aliquoted for a minimum of three independent analyses. Statistical analysis was performed using SPSS Release 19.0 for Windows (SPSS Inc., Chicago, IL). Mixed model analysis of ACN structure (fixed factor) and subject (random factor) with Bonferoni’s adjustment of mean comparison was used. Pearson's Product Moment Correlation Analysis was used to determine correlation between salivary secretion and extent of degradation of ACN. Two-tailed paired-samples t test was used to evaluate the effect of inhibition of oral microbiota on reduction of ACN loss in the oral cavity. Independent-samples t-test was used to compare 1) extent of ACN association to mucus and buccal cells between chokeberry and red grape juices and 2) abundance (%) of each ACN between samples, i.e., juice, retained juice, mucus-bound, and buccal cell-associated fractions. Differences are considered significant at $P < 0.05$ unless indicated otherwise.

3.4 Results

3.4.1 Characterization of chokeberry and red grape juices.

HPLC-PDA-ESI-MS analysis confirmed that the qualitative and quantitative profiles of ACN in juices administered to subjects in this study was quite similar to the respective ACN extracts used in the previous ex vivo study (Kamonpatana and others 2012). Characteristics of each juice are listed in Table 3.1. Chokeberry juice contained four ACN differing in the types of monosaccharides conjugated to the C3 position of Cy aglycone. Red grape juice contained five different anthocyanidins each conjugated at the
Table 3.1. Characteristic of chokeberry and red grape juices. Data are mean ± SD of 3 independent analyses.

<table>
<thead>
<tr>
<th></th>
<th>Chokeberry juice</th>
<th>Red grape juice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monomeric ACN</strong> (nmol Cy-3-glu equivalent/mL)</td>
<td>64.1 ± 1.4</td>
<td>52.9 ± 2.2</td>
</tr>
<tr>
<td><strong>ACN composition</strong> (% of total ACN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy-3-gal</td>
<td>58.1 ± 1.1</td>
<td>Dp-3-glu</td>
</tr>
<tr>
<td>Cy-3-glu</td>
<td>3.8 ± 0.2</td>
<td>Cy-3-glu</td>
</tr>
<tr>
<td>Cy-3-arab</td>
<td>26.7 ± 0.2</td>
<td>Pt-3-glu</td>
</tr>
<tr>
<td>Cy-3-xyl</td>
<td>11.3 ± 1.0</td>
<td>Pn-3-glu</td>
</tr>
<tr>
<td><strong>Phenolic compounds</strong> (mg gallic acid equivalent/mL)</td>
<td>272.4 ± 17.4</td>
<td>279.5 ± 7.8</td>
</tr>
<tr>
<td>pH</td>
<td>3.75</td>
<td>3.89</td>
</tr>
<tr>
<td>Sugar (ºBx)</td>
<td>0.4 ± 0.2</td>
<td>19.6 ± 0.2</td>
</tr>
</tbody>
</table>
C3 position to glucose. ACN concentration in red grape juice was slightly less than that in chokeberry. While the content of phenolic compounds and pH for the two juices are comparable, red grape juice contained higher sugar content than chokeberry juice.

3.4.2 Loss of ACN in juice after retention in human oral cavity

Subjects (n = 13, 9 females, 4 males; age 32.8 ± 9.5 years, range 24-50 yr; and BMI 22.3 ± 3.1) were recruited to retain CkB and RdGrp juice. Mean loss of total ACN during the 5 minute retention in the mouth was 12.5 ± 1.6 % (range 6.1 – 25.2%) and 15.3 ± 1.8 % (range 7.9 – 30.5%) for chokeberry and red grape juices, respectively. The loss of Cy-3-xyl was slightly, but significantly (P < 0.05), greater than that of Cy-3-gal and Cy-3-arab in chokeberry juice (Figure 3.1A). Losses of Dp and Pt glucosides exceeded that of Cy-, Pn- and Mv-glucosides during oral retention of red grape juice as previously reported for ACN degradation in saliva ex vivo (Kamonpatana and others 2012). However, only the loss of Dp-3-glu in vivo was significantly greater than that of the other anthocyanidins (P < 0.05) (Figure 3.1B).
Figure 3.1. Loss of ACN during 5 min retention of chokeberry (A) and red grape (B) juices in oral cavity. Data are mean ± SEM for 12 participants for chokeberry juice and 13 participants for red grape juice.
Estimated secretion of saliva during 5 min retention of red grape juice (7.86 ± 1.43 g) was significantly ($P < 0.05$) greater than that for chokeberry juice (2.84 ± 0.62 g). The estimated mass of saliva was positively correlated with loss of Dp-3-glu ($r = 0.631$, $P = 0.021$) and Pt-3-glu ($r = 0.613$, $P = 0.026$), but not of the other anthocyanidin glucosides in red grape juice (Figure 3.2). Estimated amount of secreted saliva also was positively correlated with the extent of losses of Cy-3-gal ($r = 0.903$, $P < 0.001$) and Cy-3-arab ($r = 0.778$, $P = 0.002$), but not with loss of Cy-3-glu in chokeberry juice (data not shown).
Figure 3.2. Losses of Dp-3-glu and Pt-3-glu during 5 min retention of red grape juice in the oral cavity were significantly ($P < 0.05$) correlated with amount of secreted saliva. (n = 13).
3.4.3 Microbial-mediated loss of ACN

We recently reported that the majority of ACN degradation in saliva at 37°C ex vivo was due to microbial activity (Kamonpatana and others 2012). To evaluate whether oral microbiota contribute to the in vivo loss of ACN in the oral cavity during 5 min retention of chokeberry juice, the extent of loss of ACN was compared in individual subjects before and after extensive cleaning of the teeth and rinse with antibacterial mouthwash (section 2.4). The extent of loss of chokeberry ACN during the 5 min period of retention in the oral cavity was significantly decreased for 8 out of 10 subjects after treatment to reduce microbial activity (Figure 3.3). The anti-microbial treatment also significantly decreased the mean loss of total ACN in chokeberry juice from 9.57 ± 0.64 to 8.11 ± 0.77 % ($P = 0.036$), supporting the likelihood that the observed loss was in part contributed by microbial activity during the 5 min retention. Loss of Cy-3-glu (10.36 ± 0.54 to 7.04 ± 1.17 %, $P = 0.008$), but not for Cy-3-gal (9.76 ± 0.74 to 7.60 ± 1.13 %, $P = 0.055$) or Cy-3-arab (9.17 ± 0.74 to 8.09 ± 0.76 %, $P = 0.16$), during oral retention was lessened by anti-microbial treatment. Loss of Cy-3-xyl was variable and appeared to increase from 8.97 ± 2.77 to 14.21 ± 3.81 % after reduction of the oral microbiota, although this change was not significant ($P = 0.401$). Collectively, these data further support the hypothesis that the oral microbiota are capable of metabolizing ACN in the oral cavity.
Figure 3.3. Extent of loss of total ACN in chokeberry juice during retention in oral cavity was significantly decreased after removal of plaque and rinsing with anti-bacterial mouthwash. Matched-pair analysis and lines represent change in total chokeberry ACN in the oral cavity before and after removal bacterial biofilms. Asterisk (*) denotes significant ($P < 0.05$) difference between control and clinically cleaned oral cavities of 10 subjects.
3.4.4 Delivery of ACN to mucus and buccal epithelium

Buccal scrapings were fractionated into mucus and epithelial cells to determine the relative distribution of ACN delivered to the surface of this oral tissue. Greater than 97% of total chokeberry ACN in the scrapings were associated with mucus. The relative amounts of Cy-3-glu and Cy-3-arab in the mucus layer were significantly ($P < 0.01$) greater than Cy-3-xyl (Figure 3.4A) after oral retention of chokeberry juice for 5 min. Total ACN detected in washed buccal epithelial cells accounted for 2.2 ± 0.51 % ($n = 8$) of total ACN collected in buccal scrapings. The relative amount of Cy-3-glu in washed buccal epithelial cells was significantly ($P < 0.05$) greater than the other Cy-3-monosaccharides (Figure 3.4B).
Figure 3.4. Adherence of chokeberry cyanidins to buccal surface mucus (panel A) and delivery to buccal epithelial cells (panel B). Data are mean ± SEM for 8 subjects (panel A) and 12 subjects (panel B), respectively. Presence of different letters above bars indicates that means differ significantly ($P < 0.05$).
The amount of red grape ACN associated with buccal mucus fraction was greater than 98% of total scraping ACN after oral retention of the juice for 5 minutes. The ACN-mucus association was significantly ($P < 0.001$) affected by anthocyanidin structure. Dp-3-glu was not detected in the mucus fraction and the relative amount of Pt-3-glu was ($P < 0.05$) less than Cy-, Pn- and Mv-3-glucosides (Figure 3.5A). Total ACN in washed buccal epithelium accounted for $1.38 \pm 0.52\%$ (mean $\pm$ SEM, n = 8) of total ACN collected in buccal scrapings. Dp- and Pt-3-glu were detected in the washed buccal cell fraction, although the relative amounts were less than Cy-, Pn- and Mv-3-glucosides ($P = 0.09$) (Figure 3.5B).

The amount of mucus-associated ACN from red grape juice (Figure 3.5) was significantly less than that from chokeberry (Figure 3.4) ($50.6 \pm 11.1$ vs $139.4 \pm 18.5$ µg ACN / [mg mucin • mg ACN intake], respectively; $P = 0.001$). Similarly, total amount of ACN from red grape juice that was present in washed buccal cells was less than that after retention of chokeberry juice in the mouth for 5 min ($2.16 \pm 0.64$ vs $13.5 \pm 2.3$ µg ACN / [mg DNA • mg ACN intake], respectively; $P = 0.001$). Moreover, all comparison within same subjects showed lesser relative amounts of red grape ACN in both mucus-associated and cellular fractions. (n = 5 and 9, respectively).
Figure 3.5. Adherence of ACN to buccal mucus (panel A) and delivery to epithelium (panel B) during retention of red grape juice in oral cavity. Data are means ± SEM for 8 subjects (panel A) and 12 subjects (panel B). Presence of different letters above error bars indicates that means are significantly different ($P < 0.05$, panel A; $P = 0.09$, panel B). ND, not detected.
To assess changes in the ACN profile of chokeberry and red grape juices during oral retention and delivery to the buccal surface, relative abundances of each ACN (i.e., % area under curve at 520 nm) in juice, after retention in mouth and associated with buccal mucus and epithelium are compared in Figure 3.6. Notably increases of relative abundance of Cy-3-arab (by 70%, $P < 0.001$) and Cy-3-glu (by 330%, $P < 0.001$) as well as decrease of Cy-3-gal (by 30%, $P < 0.001$) and Cy-3-xyl (by 50%, $P < 0.005$) were observed in buccal cells comparing to the chokeberry juice (Figure 3.6A). Decrease of Dp-3-glu (by 80%, $P < 0.001$) and Pt-3-glu (by 63%, $P < 0.005$) were coupled with increase of Pn-3-glu (by 30%, $P < 0.001$) as noted in buccal cells comparing to red grape juice (Figure 3.6B).
Figure 3.6. Change of ACN profile of ACN in chokeberry (A) and red grape (B) juices during oral retention and delivery to buccal surface. Data are mean ± SD for ≥ 8 subjects. Asterisks denote statistical difference comparing to juice before retention at $P < 0.05$ (*), $< 0.01$ (**), $< 0.005$ (***) and $< 0.001$ (***)
Since protocatechuic acid (PCA) and cyanidin aglycone were recently reported to be present in saliva of several subjects after oral retention of black raspberry juice for 3 min (Mallery and others 2011), we examined the juice and cell extracts for the presence of possible metabolites before and after oral retention. HPLC chromatograms (250 - 600 nm) of juices and cell extract before and after retention were compared. Phloroglucinal aldehyde (PGA) was not found in all samples, which may be due to instability of aldehyde. PCA could not be quantified in retained chokeberry juice due to co-elution with other compounds. We did not detect cyanidin aglycone or PCA, in buccal cell extracts collected from 11 subjects after retention of chokeberry juice rinse (limits of detection for cyanidin aglycone and PCA are 2.0 and 3.6 ng/mL, respectively). Similarly, there were no detectable levels of gallic acid, PCA, vanillic acid, and syringic acid, compounds that have been reported as products of cleaved B-ring of Dp, Cy, Pn, and Mv, respectively (Fleschhut and others 2006; Forester and Waterhouse 2008; Keppler and Humpf 2005; Woodward and others 2009), in either post-rinse red grape juice and buccal cell extracts (Limits of detection for gallic acid, vanillic acid and syringic acid were 2.0, 2.5, and 3.3 ng/mL, respectively).

3.5 Discussion

ACN-rich fruits and extracts have been shown to possess chemopreventive and chemotherapeutic activities in oral cell lines (Seeram and others 2006; Zdarilová and others 2010), hamster buccal tissue (Casto and others 2002), rat esophagus (Stoner and others 2010), and the human oral cavity (Mallery and others 2008; Shumway and others 2008). This efficacy of ACN has been reported to be dependent on chemical structure (Jing and others 2008; Marko and others 2004). It is also known that ACN are degraded in the GI tract (He, Magnuson, Giusti 2005; He and others 2009; Keppler and Humpf 2005) and the oral cavity (Kamonpatana and others 2012; Mallery and others 2011). An understanding on the effect of ACN structure on its relative stability, types of metabolites produced in the oral cavity and availability to oral tissues will provide insights for dietary recommendations and the strategic formulation of ACN-containing foods, beverages,
confectionaries and muco-adhesive gels for the promotion of oral health. We recently showed that the extent of biological and chemical degradation of ACN in saliva is dependent in part on their chemical structure (Kamonpatana and others 2012). More specifically, Dp- and Pt-glycosides were degraded to a greater extent than Cy-, Pn-, Mv-glycosides during ex vivo incubation in saliva. In the present study, we investigated in vivo stability of ACN in the oral cavity and the levels of these compounds in buccal mucus and epithelial cells in the same human subjects who participated in the earlier ex vivo study (Kamonpatana and others 2012). Chokeberry and red grape juices were retained in the oral cavity for 5 min to investigate the influence of ACN structure on their stability, the contribution of the oral microbiota to ACN degradation, the extent of adherence of various ACN to the buccal mucosa, and the amounts of ACN delivered to buccal epithelial cells during the brief period of exposure. The extent of in vivo loss of ACN in chokeberry and red grape juice during the brief retention in the mouth was affected by the structure of ACN (Figure 3.1B). The relative amount of Cy-3-xyl in chokeberry juice decreased during oral retention (Figure 3.1A) and this loss was not associated with selective binding to mucus (Figure 3.4A) or uptake by buccal epithelium (Figure 3.4B). Since this preferential loss was not observed during ex vivo incubation of ACN-rich extract from chokeberry in saliva (Kamonpatana and others 2012), it is possible that other components in the juice may have contributed to this outcome in vivo. It is interesting that Bermudez-Soto et al. reported that Cy-3-xyloside was less stable than that the other Cy-3-glycosides during in vitro digestion of chokeberry juice (Bermudez-Soto, Tomas-Barberan, Garcia-Conesa 2007). The preferential loss of Dp-3-glu from red grape juice during oral retention was similar to that during 60 min incubation in saliva (Kamonpatana and others 2012). Our result here is in agreement to the report that glycosides of Dp but not those of Pt, Cy, Pn, or Mv, in concord grape juice was preferentially lost from mouth to ileum of ileostomy human subjects (Stalmach and others 2012). However, the loss of Pt-3-glu in vivo did not achieve statistical significance as occurred ex vivo. This difference may be due to brief period of oral retention and greater variability between subjects in the present study comparing to our ex vivo study (Kamonpatana and others 2012). Various factors are likely responsible for the variability
in the extent of degradation among the participants. First, the amount of saliva secreted during oral retention of red grape juice significantly exceeded that from subjects during retention of chokeberry juice. We suspect that this was due to the higher concentration of simple sugars in the red grape juice (Table 3.1). Enhanced salivary flow is associated with increased concentrations of sodium and bicarbonate ions in saliva (Dawes 1974). We previously showed that the presence of inorganic constituents, including bicarbonate, in artificial saliva resulted in the preferential degradation of Dp and Pt (Kamonpatana and others 2012). Second, loss of ACN during retention of the juices in the oral cavity also reflects one or more of the following: degradation by oral microbiota (Kamonpatana and others 2012); adsorption to teeth and mucus (Proctor and others 2005); and, uptake by epithelial tissues (Mallery and others 2011). To investigate the possible impact of microbial metabolism on loss of ACN in the oral cavity, the extent of total ACN loss was compared in subjects before and after vigorous removal of oral plaque and rinsing with anti-bacterial solution. The intervention resulted in a limited, but notable, inhibition (ca. 15%) of ACN loss during the brief period of retention of juice in the oral cavity (Figure 3.3).

We investigated whether there was preferential delivery of specific ACNs to buccal cells, since squamous cell carcinomas originate in this anatomical location (Casiglia and Woo 2001), and since ACN from black raspberries have been shown to inhibit oral cancer in hamsters (Casto and others 2002) and human buccal lesions (Mallery and others 2008; Shumway and others 2008). Buccal scrapings were fractioned into mucus and epithelial layer after retention of chokeberry juice to assess the inter-play between these compartments. Chokeberry juice was used to investigate effect of the type of conjugated monosaccharide on delivery of ACN to buccal epithelium. Strikingly, there was preferential presence of Cy-3-glu (Figure 3.4B and Figure 3.6A) which suggested preferential binding or uptake to the buccal epithelium. When suspensions of buccal epithelial cells from 2 subjects were spiked with ACN-rich extract from chokeberry and incubated at 0°C for 5 min the relative presence of the four Cy-glycosides was not significantly different. This suggests that the increased bioavailability of Cy-3-glu than that of the other Cy-3-glycosides in chokeberry juice was temperature dependent and
likely to be enzymatic mediated. Presence of glucoside moiety on Cy and Pn was associated with greater absorption to human plasma than galactoside or arabinoside after acute consumption of cranberry juice (Milbury, Vita, Blumberg 2010). Quercetin glucoside was transported greater than quercetin galactoside through murine everted jejunal sacs (Gee and others 1998). Also, transport of Cy-3-glu by Caco-2 human intestinal cells exceeded that of Cy-3-gal (Yi and others 2006). The authors suggested this difference was mediated by the specificity of the sodium-dependent glucose co-transporter-1 (SGLT1) (Wolfram, Block, Ader 2002). SGLT1 has also been detected in primary human buccal epithelial cells (Mallery and others 2011; Oyama and others 1999). These data support the possibility that Cy-3-glu may have been preferentially bound or taken up into buccal epithelium during the brief retention of chokeberry juice in the oral cavity by SGLT1 and/or other unidentified transporters (Walton and others 2006).

Red grape juice allowed us to assess effect of anthocyanidin structure on bioavailability of ACN to buccal epithelium as it contains five distinct anthocyanidin-3-glucose compounds. Preferential loss of Dp-3-glu and Pt-3-glu in expectorated juice (Figure 3.1B) was coupled with lesser amounts of these anthocyanins associated with both buccal mucus and buccal cells (Figure 3.5). These data suggest that the instability of Dp and Pt in the oral cavity lessens their accessibility to the buccal epithelial cells. Similarly, Stalmach et al. reported that loss of Dp glycosides in Concord grape juice was greater than that of Pt-, Cy-, Pn- and Mv-glycosides during passage from the mouth to the ileum of human ileostomists (Stalmach and others 2012). Delivery of ACN to the washed buccal cell fraction during retention of red grape juice in the oral cavity was less than that for chokeberry juice (Figures 3.4 and 3.5). This difference appears to be due to the lower amount of ACN in the red grape juice (Table 3.1) and the greater instability of Dp-3-glu and Pt-3-glu.

Genistin (genistein-7-O-glucoside) is taken up by the SSC-9 human oral epithelial cell line and hydrolyzed to genistein aglycone (Browning, Walle, Walle 2005). It is possible that once ACN are taken up by buccal epithelial cells, they are metabolized to anthocyanin aglycones that spontaneously degrade to phenolic compounds. The
degradation product PCA was detected in saliva from 2 of 10 subjects 4 h after oral rinse with black raspberry for 3 min (Mallery and others 2011). We did not detect ACN aglycone, PCA, PGA or other phenolic products of ACN B-ring in the buccal cell extracts from 11 subjects after the 5 min retention of chokeberry or red grape juices in the oral cavity. It appears that generation of bioactive metabolites of ACN may require increased period of ACN retention in the oral cavity.

In summary, the present study provides novel insights on the effect of ACN structure on oral stability and delivery to buccal mucosa. The results demonstrate that ACN can be degraded in the human oral cavity and that the loss is partially due to microbial activity. These results also suggest that the structure of ACN can affect oral stability and delivery to the buccal surface. Cy-3-glu appeared to be preferentially transferred to the buccal epithelium compared to other Cy-3-glycosides. Instability of Dp-3-glu and Pt-3-glu in oral cavity resulted in association of lesser amounts of the ACN with buccal epithelium. The instability appeared to be associated with amount of salivary secretion. These in vivo results with ACN in a food matrix generally largely agree with the ex vivo stability and metabolism of ACN in saliva. The source of ACN merits consideration for development of ACN-containing products for promoting oral health. The relationship between oral metabolism of ACN and efficacy, e.g., anti-inflammatory activity, as well as the role of metabolites of ACN, warrants further investigation.
CHAPTER 4

ANTHOCYANIN EXTRACTS INHIBIT IL-1β INDUCED SECRETION OF IL-8 BY
SCC-25 AND OKF-6/TERT-2 ORAL EPITHELIAL CELLS
4.1 Abstract

Anthocyanins (ACN) are natural pigments in fruits and vegetables that exhibit chemopreventive and chemotherapeutic activity in the oral cavity of animal and human. Data are limited on the relationship between the structure of ACN and anti-inflammatory activity, thus limiting the development of sound dietary strategies for the promotion of oral health. Here, ACN extracts from black raspberry (BlkRB), blueberry (BluB), chokeberry (CkB), red grape (RdGrp), strawberry (StwB), and Hibiscus (Hbc) were studied for their ability to suppress the increased secretion of IL-8 and IL-6 by IL-1β activated cultures of SCC-25 squamous carcinoma cells and OKF-6/TERT-2 immortalized non-cancer human oral epithelial cells. Results show that pretreatment of SCC-25 cells with CkB extract for 1 h reduced IL-8 secretion in response to both IL-1β and cell-free human saliva. The activity in SCC-25 cells was observed when CkB extract was either pre-incubated, co-incubated, or added after cells were activated by IL-1β. The inhibitory activity was dependent on both source of ACN-rich extract (i.e. Pg-rich StwB > RdGrp extract containing five anthocyanidin aglycone classes > Cy-rich CkB, BlkRB, Cy-Dp disaccharide rich Hbc extracts) and cell type as extracts were more efficacious with SCC-25 cells than OKF-6/TERT-2 cells. Degradation of Dp- and Pt-glycosides in media without cells was greater than that of Cy-, Pn-, and Mv-glycosides in extracts of RdGrp, BluB, and Hbc. Pg-3-glu was more stable in media than Cy-3-glu in StwB. While ca. 90% of Cy-glycosides in ACN extracts were degraded during 24 h incubation, PCA accounted for only 10 % of the initial content of these ACN. Although, presence of cells did not affect the general pattern of degradation of ACN, the identifiable degradation products were significantly less than those in cell-free medium. Pre-conditioning of media containing extracts during 24 h incubation in absence of cells resulted in disappearance of the majority of ACN, but not the inhibitory activity of CkB, RdGrp, StwB, and BlkRB extracts on IL-1β activated cultures of SCC-25 cells. In contrast, loss of ACN from RdGrp during pre-conditioning of medium resulted in decreased suppression of IL-1β induced secretion of IL-8 by OKF-6/TERT-2 cells. These results suggest that ACN and their degradation products possess greater anti-inflammatory
activity in cancer SCC-25 cells than non-cancer OKF-6/TERT-2 cells and that the extent of such activity was dependent on the source of extract.

4.2 Introduction

The global burden of oral diseases significantly impairs the quality of life (Petersen and others 2005; Sischo and Broder 2011). The high cost of oral care and the limited accessibility of oral health service for many (Petersen and others 2005; Sischo and Broder 2011) emphasize the importance of preventing oral diseases (Tomar and Cohen 2010). Many oral diseases (e.g., gingivitis, candidiasis, oral lichen planus, and drug-related oral mucositis) are associated with exacerbated and chronic inflammation (Varoni and others 2012). Chronic inflammation results in increased secretion of pro-inflammatory cytokines and generation of reactive oxygen and nitrogen species in microenvironment that can damage DNA, leading to more serious conditions such as neoplasias, neuro-degenerative and cardiovascular diseases, and metabolic disorders (Balkwill and Mantovani 2001; Balkwill and Mantovani 2010; Clevers 2004; Coussens and Werb 2002; Mantovani and others 2008; O'Byrne and Dalgleish 2001).

The oral epithelium is the first line of defense by serving as a physical barrier and secreting a variety of cytokines, chemokines and other inflammatory mediators in response to injury or infection (Stadnyk 1994). For example, bacterial virulence molecules (e.g., lipo-polysaccharides (LPS), secreted heat-shock proteins (HSP), or bacterial adhesion via fimbriae) activate epithelial cells to secrete pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-8 (Madianos, Bobetsis, Kinane 2005). Under chronic inflammatory conditions, elevation of concentrations of the pro-inflammatory cytokines of IL-1β, IL-6, and IL-8 have been clinically found in saliva from oral cancer patients (Arellano-Garcia and others 2008; Brailo and others 2012; Nagler 2009; St John and others 2004) and individuals with Oral Lichen planus (OLP) (Rhodus and others 2005). The concentration of IL-1β in human saliva is significantly ($P < 0.01$) correlated with severity of periodontal disease (Yoon and others 2012) and IL-1β also induces
expression of macrophage inflammatory protein-1 alpha (MIP-1α) by primary human gingival epithelial cells (Ryu and others 2007).

IL-8 is a pro-inflammatory chemokine that stimulates angiogenesis, cancer cell growth and survival, tumor cell invasion and metastasis, neutrophil chemotaxis and degranulation, and modification of immune responses (Choi and Myers 2008; Watanabe and others 2002; Waugh and Wilson 2008). Expression of IL-8 in oral squamous cell carcinoma (OSCC-9, 15, and 25) cells of tongue is greater than in normal human oral keratinocytes and corresponded with its elevated expression in oral tumors (Rao and others 2010), suggesting an important function of IL-8 in the tumor microenvironment. Infection of oral epithelial cells by Candida albicans induces secretion of both IL-8 and IL-1α (Dongari-Bagtzoglou, Kashleva, Villar 2004; Dongari-Bagtzoglou and Kashleva 2003). IL-1β mediates secretion of IL-6 and IL-8 in primary gingival epithelial cells challenged with P. gingivalis (Eskan and others 2008) or nicotine (Kashiwagi and others 2012). Interestingly, expression of IL-8 in oral epithelium and fibroblast is largely regulated by NF-κB, ERK, JNK and p38 MAPK pathways which can be induced by IL-1 (Hoffmann and others 2002; Watanabe and others 2002). Constitutive secretion of IL-1β by tongue-derived SCC-25 cells can induce expression of IL-6 and COX-2 by co-cultured fibroblasts (Dudás and others 2011). Also, IL-1α induces secretion of IL-6 and IL-8 by KB oral epithelial cells (Sakoda and others 2006). As IL-1β mediates prolonged secretion of pro-inflammatory cytokines by oral epithelium in absence of bacterial or chemical insults, IL-1β induced secretion of IL-8 by oral epithelial cells appears to provide a useful model for identifying potential dietary compounds and extracts capable of preventing chronic inflammation and possibly tumorigenesis in the oral cavity.

Prevention of chronic inflammation by dietary compounds as a strategy for promoting oral health has received attention (Varoni and others 2012). Fruit and vegetable intake has been related to reduced risk of oral cancers (Block, Patterson, Subar 1992; Sakagami, Oi, Satoh 1999) and suggest that polyphenolic compounds have a role in the prevention of oral, esophageal and pharyngeal cancers (Petti and Scully 2009). Thus, the possible use of bioactive compounds for the prevention and treatment of oral inflammation, e.g., periodontal diseases, is of interest (Bodet and others 2008).
There is some evidence that ACN may have anti-inflammatory activity in the mouth (Varoni and others 2012). Zdarilova et al. found that the phenolic fraction of blue honeysuckle (containing 77% ACN with Cy-3-glu most abundant) suppressed LPS-induced production of IL-1β, IL-6 and TNF-α, as well as expression of COX-2 protein, in human gingival fibroblasts (Zdarilová and others 2010). Black currant extract and Cy-3-glu both decreased LPS-induced expression of IL-6 by macrophages (Desjardins and others 2012) and inhibited activity of matrix metallo-proteinases-1 and -9 that appear to be responsible for progression of periodontitis (Santos and others 2011). ACN-rich ethanol extract of black raspberry inhibited proliferation, synthesis of angiogenic vascular-endothelial-growth-factor, and the activity of nitric oxide synthase activity in oral squamous cell carcinomas (SCC) (Rodrigo and others 2006). Consequently, investigators are interested in prolonging retention of ACN in the oral cavity using delivery vehicles such as confectioneries (Amoian and others 2010) and muco-adhesive gels (Mallery and others 2007) to promote oral health.

There are more than 700 unique structures that have been identified for ACN (Andersen and Jordheim 2006) and the profile of those in the diet depends on the food source (Szajdek and Borowska 2008). ACN structure influences the extent of bioactivities including superoxide radical- and peroxynitrite-scavenging activity (Rahman and others 2006), anti-proliferative activity (Jing and others 2008; Kim and others 2008), anti-angiogenic activity (Lamy and others 2006), and inhibition of cell migration (Lamy and others 2007). Therefore, it is possible that the dietary selection of ACN-rich fruits and vegetables may affect anti-inflammatory activity because of the distinct mixture of endogenous ACN. Moreover, ACN are poorly absorbed (< 1%) and subject to extensive degradation within the gastrointestinal (GI) tract (Prior and Wu 2006). It is possible that ACN exert their bioactivities in upper GI tract where the concentration of the ingested compounds is greatest (Wang and Stoner 2008) or that degradation products and metabolites are responsible for such activities (Forester and Waterhouse 2010; Williamson and Clifford 2010).

Little is known about influence of ACN structure on anti-inflammatory activity in oral epithelial cells, specific degradation of ACN products generated in the oral cavity, or
the possible anti-inflammatory activities of such products. Our recent data (Kamonpatana and others 2012) suggest that ACN are subjected to partial degradation during prolonged retention in saliva due to both microbial metabolism and chemical instability. In particular, Dp- and Pt-glycosides were less stable in both saliva and within the oral cavity than Cy-, Pn-, and Mv-glycosides (Chapters 2 and 3). These results suggest that the accessibility of ACN to the oral tissues will likely vary which in turn is expected to affect anti-inflammatory activity. In the present study, two distinct oral epithelial cell lines were treated with several natural mixtures of ACN to examine their effect on the response to pro-inflammatory insult. We hypothesized that ACN and their degradation products would exhibit anti-inflammatory activity and that potency of the inhibitory effect would depend on structure of ACN in the extracts.

4.3 Materials and methods

4.3.1 Chemicals and ACN extracts

Unless indicated otherwise, all chemicals and reagents were purchased from Fisher Scientific (Fair Lawn, NJ), Gibco (Grand Island, NY), Pierce Biotechnology, (Rockford, IL), and Sigma Aldrich Chemical (St. Louis, MO).

Fruits were obtained from various sources as previously described (Kamonpatana and others 2012). Hibiscus (Hibiscus sabdariffa, Hbc) tea powder was purchased from a local market in Bangkok, Thailand. ACN in black raspberry (BlkRB), blueberry (BluB), chokeberry (CkB), Hbc, red grape (RdGrp), and strawberry (StwB) were extracted and semi-purified as described previously (Kamonpatana and others 2012) and extracts were sterilized by filtration (pore size 0.22 µm). Monomeric ACN content in extracts was quantified by pH differential method (Giusti and Wrolstad 2005). Amounts of specific ACN in the mixtures were estimated by HPLC-MS as Cy-3-glu equivalent (Table 4.1). Purity of extracts was estimated by HPLC-PDA as (area under curve (AUC) at 520 nm × 100 / AUC 200-700 nm). ACN extracts were diluted with 0.01% HCl in sterile deionized water to prepare stock solutions and stored at -20°C.
Table 4.1. ACN profile in natural extracts.

<table>
<thead>
<tr>
<th>Source</th>
<th>ACN profile (% of total ACN)</th>
<th>Estimated purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BlkRB</td>
<td>Cy-3-glu (9.8%, co-elude)</td>
<td>95.4 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Cy-3-samb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cy-3-rut (49.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cy-3-(2'-xyl-rut) (40.3%)</td>
<td></td>
</tr>
<tr>
<td>BluB</td>
<td>Cy-3-gal (9.1%)</td>
<td>98.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Dp-3-gal (9.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pt-3-gal (10.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pn-3-gal (4.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mv-3-gal (27.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cy-3-glu (2.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dp-3-glu (1.3%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pt-3-glu (2.3%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pn-3-glu (2.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mv-3-glu (7.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cy-3-arab (3.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dp-3-arab (3.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pt-3-arab (4.1%)</td>
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<tr>
<td></td>
<td>Pn-3-arab (1.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mv-3-arab (12.2%)</td>
<td></td>
</tr>
<tr>
<td>CkB</td>
<td>Cy-3-gal (65.5%)</td>
<td>98.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Cy-3-glu (4.1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cy-3-arab (26.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cy-3-xyl (3.6%)</td>
<td></td>
</tr>
<tr>
<td>Hbc</td>
<td>Cy-3-samb (35.1%)</td>
<td>82.7 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>Dp-3-samb (64.9%)</td>
<td></td>
</tr>
<tr>
<td>RdGrp</td>
<td>Cy-3-glu (16.0%)</td>
<td>71.2 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>Dp-3-glu (5.2%)</td>
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</tr>
<tr>
<td></td>
<td>Pt-3-glu (6.4%)</td>
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</tr>
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<td></td>
<td>Pn-3-glu (46.0%)</td>
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<tr>
<td></td>
<td>Mv-3-glu (26.4%)</td>
<td></td>
</tr>
<tr>
<td>StwB</td>
<td>Pg-3-glu (84.6%)</td>
<td>85.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Cy-3-glu (6.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pg-3-rut (8.9%)</td>
<td></td>
</tr>
</tbody>
</table>
### 4.3.2 Cell culture

SCC-25 cells were obtained from American Type Culture Collection (ATCC) and grown in Dulbecco’s modified Eagle’s medium (DMEM) and F12 (1:1 v/v) supplemented with 10% heat-inactivated fetal bovine serum, 1 % sodium pyruvate (100 mM), 1% hydrocortisone (40 µg/mL), 1% penicillin/streptomycin (10,000 units/10,000 µg per mL, respectively), and 0.2% fungizone (250 µg amphotericin B / mL water). OKF6-TERT2 cells were obtained from Dr. James Rheinwald, Department of Dermatology at Harvard Medical School, and grown in K-sfm medium supplemented with final concentrations of 25 µg/mL bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF), 0.3 mM CaCl₂, 1 % penicillin/streptomycin, and 0.2 % fungizone. Cell cultures were maintained in T-75 flasks in humidified atmosphere of 95% air: 5% CO₂ at 37°C. Media were replaced every second day. Cells were detached from flask when monolayer was ca. 70% confluent by 6-10 min incubation with 0.25% trypsin-EDTA.

For experiments, SCC-25 cells (passage 4 – 24) were seeded into 48 well culture plates (1 × 10⁴ cells/well) in complete DMEM medium. Medium was changed every other day until the monolayer was 100% confluent. OKF-6/TERT-2 cells (passage 5 – 12) were seeded into 48-well plates (1 × 10⁴ cells/well) and grown in supplemented K-sfm medium. At 30% confluency, medium was replaced with ‘high-density’ medium, i.e., 1:1 (v/v) mixture of complete K-sfm medium and complete DF-K medium. The latter consisted of 1:1 (v/v) calcium-free, glutamine-free DMEM (Gibco #21068-028) and Ham’s F-12 medium (Gibco #11765-054) supplemented with 0.2 ng/mL epidermal growth factor (EGF), 25 µg/mL bovine pituitary extract (BPE), 2 mM L-glutamine, 1% penicillin/streptomycin, and 0.2% fungizone. The high density medium was changed every other day until the monolayer was 100% confluence.

### 4.3.3 Stability of ACN in cell culture media

ACN extracts from CkB, BlkRB, RdGrp, StwB, and Hbc were separately added to either complete DMEM/F-12 (for SCC-25 cells) or high-density medium (for OKF6-TERT-2 cells) to listed concentrations, mixed briefly, and incubated (37°C, 5% CO₂).
After 1, 4 and 24 hr, aliquots were collected and acidified by addition of an equal volume of 0.3% HCl in methanol. Samples were centrifuged (20,000 × g, 5 min) and supernatant was filtered (0.22 µm pore size) prior to HPLC analysis.

4.3.4 HPLC-PDA-ESI-MS

ACN and other phenolic compounds were analyzed by HPLC-PDA-ESI-MS as previously described (Kamonpatana and others 2012). Identification of phenolic degradation products was performed as previously reported method (Chapter 3).

4.3.5 Cytotoxicity

Potential cytotoxicity of ACN extracts was qualitatively evaluated by phase contrast microscopy and quantified by reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bicinchoninic acid (BCA) protein assay after 4 h incubation with ACN extracts. SCC-25 cells were seeded in 96 well plates and medium was changed every other day until the monolayer was 100% confluent. To initiate the experiment, spent medium was aspirated and monolayer was washed once with warm 200 µL basal DMEM medium (200 µL). Basal DMEM/F12 without or with ACN extract was added and cultures were incubated for 4 h. Medium was removed and monolayer was washed once with 200 µL warm phosphate buffered saline (PBS). PBS (200µL) containing 0.5 mg MTT/mL to each well and dishes were incubated (37°C, 5% CO₂) for 3 h to allow uptake and mitochondrial reduction to the insoluble formazan dye. The solution was removed from wells and 100 µL of DMSO added before shaking plates at room temperature for 15 min to solubilize the dye. Absorbance was measured at 570 nm and background at 650 nm (Bio-Tek absorbance plate reader, Winooski, VT). Cell viability was reported relative to absorbance in control cultures not exposed to ACN.

BCA protein assay was used to quantify extent of cytotoxicity after treatments with combination between ACN extracts, IL-1β, and saliva comparing to no treatment control. Post-treatment cells were washed once with 200 µL warm PBS and adherent cells were lysed by addition of 60 µL/well of RIPA Cell Lysis Buffer 2 (Enzolifesciences 80-1284) and incubated with constant agitation for 1 h at 37°C. Aliquots (30 µL) were
added to 200 µL BCA reagent and dishes incubated for 30 min before reading absorbance at 562 nm and comparing with a 5 point curve using bovine serum albumin as the standard.

4.3.6 Anti-inflammatory activity of ACN extracts

Cultures of 1-2 d post-confluent monolayers were washed with 0.3 mL of either warm basal DMEM/F12 medium (for SCC-25 cells) or high-density medium for (OKF-6/TERT-2 cells). To evaluate anti-inflammatory activity of ACN, SCC-25 cells were “pre-treated” with 0.3 mL of medium containing either 30 µL of delivery vehicle only or vehicle containing CkB extract. After 1 h, 30 µL containing 20 ng/mL recombinant human IL-1β was added and cultures were incubated for an additional 4 h. In replicates dishes, both CkB ACN and IL-1β were added simultaneously (“co-treatment”) and incubated for 4 h. Also, IL-1β was added to cultures in a third replicate plate 15 min prior to addition of CkB ACN (“pre-activation”) and incubated for 3.75 h. Media were collected and 1% protease inhibitor cocktail (Sigma P2714, 1:100 v/v) added prior to storage at -80°C. After removing medium, monolayers were washed once with warm PBS and lysed to quantify cellular protein by BCA assay (section 2.5) to assess potential cytotoxicity of combined treatment with IL-1β and ACN extracts.

For studies introducing saliva into cell cultures to induce the inflammatory state, saliva from a healthy human subject was collected upon awakening prior to brushing teeth and stored on ice for delivery to laboratory. Saliva was diluted 1:1 v/v with DI water, shaken to reduce viscosity, and filter sterilized (0.22 µm pore size) to remove bacteria. Cultures of SCC-25 cells were prepared as above and pre-incubated 1 h with CkB ACN. Filtered saliva (30 µL) was added to wells, and cultures were incubated for 4 h before collecting and processing medium as above.

To understand the effect of structure of ACN on anti-inflammatory activity, cultures were incubated with aliquots of either CkB, RdGrp, StwB, BlkRB, or Hbc for 1 h prior to exposure to IL-1β for 4 h. To investigate the possible role of degradation products of ACN on cell responses to IL-1β, medium without or with ACN extract was incubated for 24 h. Conditioned media were added to washed cell cultures for 1 h before addition of IL-1β. After 4 h, medium was collected and processed as above.
4.3.7 Cytokine assay

Interleukin (IL)-8 in supernatant collected after centrifugation of medium was quantified by enzyme-linked immunosorbent assay (ELISA) (Thermo Scientific (EH2IL8, Rockford, IL, USA) or R&D Systems (Duo Set® ELISA, Minneapolis, MN, USA) according to the manufacturers’ instructions. Selected samples were analyzed for IL-2, -4, -6, -8, -10, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α by commercial multiplex immunobead-based assay (Bio-Plex Pro™, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instruction.

4.3.8 Statistical analysis of data

Unless otherwise indicated, there were three cell cultures for each analysis and each experiment was repeated at least once to provide a minimum of six independent observations. Statistical analysis was performed using SPSS Release 19.0 for Windows (SPSS Inc., Chicago, IL). One way analysis of variance (ANOVA) followed by mean comparison with Tukey’s adjustment was used for data about the stability of ACN in media and anti-inflammatory activity of each treatment to controls. Differences are considered significant at $P < 0.05$.

4.4 Results

4.4.1 Cytotoxicity of ACN extracts

Cultures of SCC-25 and OKF-6/TERT-2 cells (2 dpc) were incubated initially with varying concentrations of ACN extracts (25-100 µM Cy-3-glu equivalents) for 4 h and observed by phase contrast microscopy to assess possible toxicity, i.e., gross morphological changes including loss of cells from confluent monolayer and rounding of cells that results in decreased interaction with adjacent cells. For monolayers of SCC-25, no morphological changes were observed at concentrations of extracts as great as 100 µM Cy-3-glu equivalents. However, MTT activity in cultures of SCC-25 cells containing 100 µM BlkB, CkB, RdGrp, StwB, and Hbc were $73.1 \pm 6.1, 103.7 \pm 10.7, 79.1 \pm 5.9, 77.6$
± 4.5, and 77.4 ± 8.2 % that in control cultures, respectively. General appearance of the monolayer of OKF-6/TERT-2 was not affected by exposure to mixtures containing as high as 100 µM Cy-3-glu equivalents. Reduction of MTT by OKF-6/TERT-2 cells increased in proportion to concentration of all ACN extracts added to medium (data not shown), suggesting a chemical interference by ACN that was specific to OKF-6/TERT-2 cell cultures. Cell protein content per well in cultures of both cell lines were also quantified after treatment with IL-1β alone, ACN + IL-1β, ACN + saliva, and conditioned media (see below) and did not differ from control cultures (data not shown). ACN concentrations used in all subsequent experiments were ≤ 50 µM Cy-3-glu equivalents unless otherwise noted so that results were not due to cytotoxicity of extracts, IL-1β, saliva, or their combination.

4.4.2 Anti-inflammatory activity of ACN extracts

Incubation of SCC-25 cells with IL-1β (20 ng/mL) for 4 h increased IL-8 secretion 7-fold (Figure 4.1A, P < 0.001). Pre-treatment of the cells with 25 µM CkB ACN for 1 h prior to addition of IL-1β blocked the increased secretion of IL-8 in response to IL-1β. Similarly, simultaneous addition of CkB ACN and IL-1β or delayed addition of CkB ACN until 15 min after IL-1β also prevented cytokine induced increases in IL-8 secretion. The inhibitory activity of CkB ACN on IL-1β induced IL-8 secretion was dose-dependent at concentrations ranging from 5 to 50 µM (Figure 4.1B, P < 0.001). Increased IL-8 secretion also occurred when SCC-25 cells were exposed to diluted saliva (Figure 4.1C, P < 0.001). Pre-incubation of SCC-25 cells with CkB extract (25-100 µM Cy-3-glu equivalents) for 1 h before addition of diluted saliva also inhibited IL-8 secretion in a dose-dependent manner.
Figure 4.1. Anti-inflammatory activity of CkB ACN in SSC-25 cell cultures. **Panel A:** Effect of CkB ACN on IL-1β induced IL-8 secretion. CkB extract (25 µM) was added to medium in SCC-25 cultures 1 h before IL-1β (20 ng/mL) (i.e. pre-treated), at the same time as IL-1β (i.e. co-treated) and 15 min after IL-1β (i.e. post-activated). Media were collected 4 h after addition of IL-1β and analyzed for IL-8. Data are mean ± SD of n = 3. **Panel B:** Effect of dose of CkB ACN on response to IL-1β induced secretion of IL-8. Cultures were incubated with indicated concentrations of CkB ACN for 1 h before 4 h incubation with IL-1β. Data are mean ± SEM, n = 6. **Panel C:** Pre-treatment with CkB extract dose-dependently decreased saliva-induced IL-8 secretion. Cells were pre-treated for 1 h with indicated concentrations of CkB extract prior to 4 h incubation with filtered saliva. Data are mean ± SEM of n = 6. Asterisks (*) denote that mean is significantly differs from induced IL-8 in absence of ACN (P < 0.05).
To determine if composition of ACN in natural mixtures affected anti-inflammatory activity, SCC-25 cells were pre-treated with extracts from RdGrp, StwB, BlkRB, and Hbc for 1 h prior to 4 h induction with IL-1β. All extracts inhibited IL-8 secretion in a dose-response manner (Figure 4.2A, \( P < 0.001 \)). Interestingly, ACN extracts of StwB (IC\(_{50}\) 0.8 \( \mu \)M) and RdGrp (IC\(_{50}\) 2.5 \( \mu \)M) exhibited greater anti-inflammatory activity than those from CkB (IC\(_{50}\) 4.5 \( \mu \)M), Hbc (IC\(_{50}\) 12.6 \( \mu \)M), and BlkRB (IC\(_{50}\) 16.5 \( \mu \)M) in cultures of SCC-25 cells. Similarly, StwB (IC\(_{50}\) 10.4 \( \mu \)M) and RdGrp (IC\(_{50}\) 17.8 \( \mu \)M) extracts also inhibited (\( P < 0.001 \)) IL-1β induced secretion of IL-8 in cultures of OKF-6/TERT-2 cells, although potency was considerably less than for SSC-25 cells (Figure 4.2B). In contrast, 25 \( \mu \)M cyanidin-rich extracts (i.e., CkB, \( P = 0.771 \); BlkRB, \( P = 0.936 \)) or cyanidin-delphinidin rich extract (i.e. Hbc, \( P = 0.961 \)) did not exhibit anti-inflammatory activity in cultures of OKF-6/TERT-2 cells. Interestingly, 50 \( \mu \)M CkB ACN inhibited IL-8 secretion by OKF-6/TERT-2 by ca. 60% (\( P < 0.001 \), data not shown).
**Figure 4.2.** Effect of different ACN extracts on IL-8 secretion by SCC-25 (Panel A) and OKF-6/TERT-2 cells (Panel B) treated with IL-1β. Data are means ± SEM of ≥ 2 independent experiments with triplicate wells per test. Asterisks (*) denote significant differences from IL-8 treated cultures in absence of ACN at $P < 0.05$. 
To determine if the inhibitory effect of natural mixtures of ACN on IL-1β induced secretion of IL-8 might reflect a more general suppressive effect on the inflammatory response of the oral epithelial cell lines, concentrations of other inflammatory cytokines in medium for select samples also were analyzed. Using the Bio-Plex assay system, the effects of treatment with ACN-rich extracts on IL-8 secretion aligned well with the simple ELISA assay (Figure 4.2). For SCC-25 cells, addition of CkB (5 µM), StwB (1 µM), and BlkRB (5 µM), but not RdGrp (1 µM), extracts to SCC-25 cell cultures decreased IL-6 and, to a lesser extent, TNF-α content in medium (Table 4.2). Neither the addition of IL-1β or ACN extracts altered secretion of IL-10 by SCC-25 cells (data not shown). IL-2, IL-4, GM-CSF, and IFN-γ were not detected in control or IL-1β treated cultures.

As for SCC-25 cells, treatment of cultures of OKF-6/TERT-2 cells with StwB ACN extract prior to IL-1β activation inhibited IL-1β induced secretion of IL-8, IL-6, and TNF-α (Table 4.2, Figure 4.2). In contrast, TNF-α secretion was greater in cultures containing RdGrp ACN and IL-6 secretion was similar that in activated cultures without the extract (Table 4.2). Similarly, CkB ACN increased secretion of IL-6 and TNF-α by OKF-6/TERT-2 cells treated with IL-1β. Although BlkRB extract did not inhibit IL-1β induced secretion of IL-8, secretion of IL-6 and TNF-α was less than that in cultures not treated with ACN. GM-CSF was detected in cultures of cells treated with CkB, RdGrp, and StwB with activation of IL-1β (data not shown).
Table 4.2. Secretion cytokines by cultures of cells pre-treated with ACN extracts prior to activation with IL-1β as compared to cultures in absence of ACN. IL-2, IL-4, and IFN-γ were not detected in medium of either cell line. Data are mean ± SEM of 4 analyses for medium in a selected culture compared to IL-1β activated culture in absence of ACN (100 %).

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<tr>
<th>Cytokine secretion by activated in ACN treated cultures compared to cultures without ACN (100 %)</th>
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<tr>
<td><strong>SCC-25 cells</strong></td>
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<td>StwB (1 µM)</td>
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<th><strong>OKF-6/TERT-2 cells</strong></th>
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<td>StwB (25 µM)</td>
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<td>TNF-α</td>
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<td>IL-10</td>
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4.4.3 Degradation of ACN in media and cell cultures

ACN lack stability in saliva (Kamonpatana and others 2012), the oral cavity (Chapter 3), and cell cultures (Forester and Waterhouse 2010; Kay, Kroon, Cassidy 2009; Kern and others 2007). I next examined stability of ACN in media used in these studies to obtain some insights as to whether the observed anti-inflammatory activities were mediated by the intact ACN or perhaps their degradation products. Recovery of CkB ACN from basal DMEM/F12 and high-density media in acidified methanol was 103.5 ± 1.0 and 95.2 ± 1.6 %, respectively, suggesting minimal irreversible binding of ACN to serum and supplemented proteins. Thus, decreases in the concentrations of ACN during incubation in media were due to degradation. The concentration of CkB ACN in both media decreased with increasing time of incubation in the absence of cells (Figure 4.3A). The extent of degradation in high density medium used with OKF-6/TERT-2 cells exceeded that in basal DMEM/F12 used with SCC-25 cells throughout the incubation period (P < 0.001). Extent of ACN degradation during incubation was independent of structure of mono-saccharide conjugated to cyanidin aglycone in both media (P > 0.05, data not shown).

To investigate the effect of ACN structure on the extent of degradation in medium without cells, ACN extracts from BlkRB, BluB, RdGrp, StwB, and Hbc were separately incubated in high-density medium which exhibited higher degradation (Figure 4.3A). Recovery of added ACN (100 µM Cy-3-glu equivalents) from all extracts exceeded 85% (data not shown). Degradation of ACN from all extracts was approximately 50% and greater than 85% after 4 and 24 h incubation, respectively. Interestingly, degradation of Dp- and Pt-glycosides was greater than that of Cy-, Pn-, and Mv-glycosides in medium containing RdGrp extract (Figure 4.3B, P < 0.001 at each time). Such preferential loss of Dp- and Pt-glycosides was also observed using BluB extract (data not shown). Stability of Dp-3-sambubioside (Dp-3-samb) was less than that of Cy-3-samb in Hbc extract (Figure 4.3C, P < 0.001 at each time) and Cy-3-glu was less stable than Pg-3-glu in medium containing StwB extract (Figure 4.3D, P < 0.05 at each time). Differences also were noted for the glycoside conjugates as Pg-3-rut was slightly, but significantly, more stable than Pg-3-glu during incubation of medium containing StwB extract (Figure 4.3D,
$P < 0.05$ at 4 and 24 h), and Cy-3-2G-xyl-rut was more stable than Cy-3-rut in medium containing BlkRB extract (Figure 4.3E, $P < 0.05$ at each time).
Figure 4.3. ACN degradation during incubation of media without cells is dependent on time, concentration and chemical structure. Panel A: Stability of CkB ACN (100 µM at zero hour) during 24 h incubation in basal DMEM/F12 and high-density media. Panel B-E: Stability of RdGrp (Panel B), Hbc (Panel C), StwB (Panel D), and BlkRB (Panel E) ACN in high-density medium during 24 h incubation without cells. Data are means ± SD for n = 3 independent tests.
Products of degraded ACN in medium also were considered. Protocatechuic acid (PCA, $\lambda_{\text{max}}$ 259 nm, m/z- 153) was detected after 24 h incubation of CkB extract in DMEM/F-12 medium and high-density medium (7.9 ± 0.4 and 11.8 ± 1.4 % of added Cy-glycosides, respectively; Figure 4.4A). Degradation products of other extracts during incubation in high-density medium (Figure 4.4B-D) which exhibited higher ACN degradation and phenolic generation. PCA generation during 24 h incubation of high density medium containing Cy-rich BlkRB extract (11.7 ± 1.4 % of added Cy-glycosides; Figure 4B) was similar to that for CkB extract in DMEM/F12 medium (Figure 4A). PCA accounted for 20.0 ± 1.1% of added Cy-3-samb in medium with Hbc extract (Figure 4.4B). PCA content in stock solutions of CkB, BlkRB, and Hbc extracts were 1.15, 0.82, and 0.10 % of total ACN, indicating that spontaneous degradation of Cy-glycosides in medium.

Generation of PCA and syringic acid during 24 h incubation of high density medium containing RdGrp ACN accounted for 30.8 ± 3.9 and 24.5 ± 2.3 % of added Cy-3-glu and Mv-3-glu, respectively (Figure 4.4C). Interestingly, 24 h incubation of high density medium containing StwB extract was associated with a time-dependent increase in 4-hydroxybenzoic acid that accounted for 6.4 ± 0.8 % of the starting concentration of Pg-glycosides after 24 h (Figure 4.4D). An unknown compound eluting at 11.2 min with 366 nm $\lambda_{\text{max}}$ accounted for as much as 81.6 ± 12.9 % PCA equivalents of original amount of StwB ACN. Due to technical difficulty experienced with MS, the molecular weight of this product was not determined.
Figure 4.4. Phenolic products generated during 24 h incubation of ACN extracts in DMEM/F12 and high-density media in absence of cells. Panel A: PCA content in DMEM/F12 medium containing 100 µM CkB ACN extract. Panel B: PCA in high density media with 100 µM cyanidin-rich BlkRB, CkB, and Hbc extracts. Panel C: Generation of PCA and syringic acid from Cy-3-glu and Mv-3-glu, respectively, in RdGrp extract (100 µM Cy-3-glu equivalents) added to high-density medium. Panel D: Generation of 4-hydroxy benzoic acid and unknown degradation product (as PCA equivalents) during incubation of high-density medium containing StwB ACN extract, respectively. Data are means ± SD of n = 3 independent tests.
When medium containing 100 µM CkB ACN was added to cultures of SCC-25 cells for 4 h, recovery of ACN (80.5 ± 2.9 % of added CkB ACN) was marginally (ca. 7%), but significantly ($P < 0.05$), greater than during incubation in absence of cells (87.4 ± 1.3 %, **Figure 4.3A**). This suggested that some ACN may have been taken up by the cells. As observed in cell-free conditions, PCA were detected after incubation of medium containing CkB for 4 h with SCC-25 cells. PCA content in medium in wells containing SCC-25 cells (2.69 ± 0.22 % of original CkB ACN) were 30 % less ($P < 0.05$) than in cell-free wells (3.81 ± 0.20 %, **Figure 4.4A**).

Approximately 50% of the initial concentration of each of the four ACN in CkB extract was recovered in medium after 4 h incubation in cultures of OKF-6/TERT-2 cells which was similar to that during cell-free incubation (**Figure 4.3A**). As observed during incubation of high-density medium containing RdGrp extract in the absence of cells (**Figure 4.3B**), Dp- and Pt-glucosides were preferentially degraded in OKF-6/TERT-2 cell cultures (data not shown). Similarly, the pattern and extent of recovery of StwB ACN in OKF-6/TERT-2 cell cultures were comparable (data not shown) to that during incubation without cells (**Figure 4.3D**). In contrast to cell free incubation (**Figure 4.4**), phenolic acid products were not detected after 4 h incubation of medium containing CkB, RdGrp, or StwB extracts with OKF-6/TERT-2 cells. Collectively, these data suggest that the presence of both cell lines did not considerably alter the extent of loss of ACN during 4 h incubation.

**4.4.4 Comparison of anti-inflammatory activity between ACN and degradation products**

Since ACN are not stable in media and the extent of degradation was affected by their structure (**Figure 4.3**), it was unclear if the observed anti-inflammatory activity was contributed by the intact ACN, their degradation products or both. I next incubated media containing ACN extracts for 24 h to obtain conditioned media with relatively low and high concentrations of ACN and degradation products, respectively. Conditioned basal DMEM/F-12 medium (24 h) to which either CkB, BlkRB, RdGrp or StwB extract had been added contained only 13.5, 12.9, 25.0, and 18.3 % of the initial amount of ACN,
respectively. Cultures of SCC-25 or OKF-6/TERT-2 cells containing either conditioned medium or fresh medium with ACN extract were incubated for 1 h before activated with IL-1β to compare IL-8 content in medium after 4 h. IL-1β induced secretion of IL-8 was similarly suppressed in SSC-25 cell cultures containing fresh and conditioned media for all four tested extracts (P < 0.001) (Figure 4.5A). This suggested that the degradation products of distinct ACN mixtures possess anti-inflammatory activity in SCC-25 cell cultures.

Residual RdGrp and StwB ACN concentrations were only 13.0 and 10.9 %, respectively, of the amounts added to high density medium after cell free incubation for 24 h. In contrast to SCC-25 cells, there was no significant inhibitory effect of conditioned media containing RdGrp ACN degradation products on IL-1β induced secretion of IL-8 by OKF-6/TERT-2 (Figure 4.5B). Inhibition of IL-8 secretion by activated OKF-6/TERT-2 cells was less in cultures with conditioned medium containing StwB degradation products than in cultures with fresh medium with StwB (P < 0.05). However, IL-8 secretion was significantly less in activated cultures with conditioned medium with StwB than in fresh and conditioned medium without StwB extract (P < 0.05).
Table 4.5. Anti-inflammatory activity of ACN extracts and their degradation products. Cultures were incubated for 1 h with either fresh medium containing indicated ACN extracts or medium with CAN extracts for 24 h in absence of cells (“conditioned” medium) followed by 4 h activation by IL-1β. IL-8 in media were analyzed and compared to IL-1β induced secretion without extracts (100%). Data are means ± SEM of n ≥ 6 from 2 experiments. Asterisks (*) denote IL-8 content in medium was significantly less than that in cultures of IL-1β activated cells in absence of either ACN or their degradation products (P < 0.05). Double asterisks indicates that IL-8 secretion in activated cultures with fresh and conditioned media containing ACN extracts differ significantly (P < 0.05).
Select samples of media also were assayed for IL-6, TNF-α, IL-10, IL-2, IL-4, GM-CSF, and IFN-γ to determine if ACN degradation products affected their secretion by activated cells (Table 4.3). Conditioned media for the four extracts inhibited secretion of IL-6, and TNF-α by SCC-25 cells. Media containing StwB and RdGrp extracts and degradation products had greater inhibitory activity than media containing CkB and BlkB extract, as previously observed with fresh medium containing ACN extracts (Figure 4.2). In contrast, conditioned medium to which RdGrp and StwB extracts had been added increased IL-6 and TNF-α concentrations in IL-1β treated cultures of OKF-6/TERT-2 cells.
Table 4.3. Relative secretion (%) of cytokines by IL-1β treated cells incubated in conditioned medium with ACN and degradation products compared to medium lacking ACN and degradation products. Medium with and without ACN extracts were pre-incubated for 24 h in cell-free wells. Conditioned media were added to cell cultures 1 h prior to activation with IL-1β. Cytokine concentrations in media with residual ACN and degradation products are compared with those in cultures containing conditioned media without ACN and their degradation (100 %). IL-2, IL-4, and IFN-γ were not detected in any media. Data are mean ± SEM of 4 readings of a representative sample.

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<tr>
<th>Cytokine secretion by activated cells cultured on conditioned media with residual ACN and degradation products compared to cultures without ACN (100 %)</th>
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<td><strong>SCC-25 cells</strong></td>
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<td>IL-6</td>
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<td>54.8 ± 0.6</td>
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<td>TNF-α</td>
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<th><strong>OKF-6/TERT-2 cells</strong></th>
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<tr>
<td>IL-6</td>
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<tr>
<td>181.4 ± 3.6</td>
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<td>TNF-α</td>
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<td>IL-10</td>
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4.5 Discussion

To explore the effects of ACN on oral epithelial cells, the SCC-25 squamous carcinoma cell line derived from the tongue and immortalized OKF-6/TERT-2 oral mucosal keratinocyte cell line derived from the floor of a healthy mouth (Dickson and others 2000; Rheinwald and others 2002) were used in this study. Genistein has been shown previously to inhibit constitutive expression of COX-2 and PGE2 synthesis by SCC-25 cells (Ye and others 2004), suggesting an anti-inflammatory response of the cells to flavonoids. Similarly, OKF6/TERT-2 cells have been reported to secrete IL-8 in response to exposure to Candida albicans (Dongari-Bagtzoglou and Kashleva 2003).

We found that ACN extract from CkB inhibited IL-1β-induced secretion of IL-8 by SCC-25 cells when ACN was added to medium either 1h before or at the same time as IL-1β. The inhibitory effect of CkB ACN extract was also observed when added to cultures 15 min after IL-1 β. Thus, CkB ACN do not appear to be mediating the inhibitory activity by binding to IL-1β or its receptor. The extent of the inhibitory effect on IL-8 secretion by IL-1β treated cells was dependent on origin of the extract with StwB > RdGrp > CkB > Hbc ≈ BlkRB (Figure 4.2). As extracts from StwB (Pg rich extract) and RdGrp (Dp, Cy, Pt, Pn, Mv monoglycosides) contain aglycone structures differing from the Cy-glycoside rich extracts from CkB and BlkRB, these data suggest that aglycone structure may affect efficacy. Hbc extract (Cy and Dp di-glycosides) inhibited IL-8 secretion by activated cells to a similar extent as to BlkRB (Cy mono-, di-, tri-glycosides), suggesting that Dp may not contribute to the activity. Differential activities of ACN mixtures from various fruits have been reported previously. Jing et al. reported that Cy-3-glu had greater anti-proliferative activity than Pg-3-glu in cultures of HT-29 colon cancer cells (Jing and others 2008). Similarly, Dp and Cy aglycones were more inhibitory to the proliferation of HT-29 cells than Pg (Kim and others 2008). Also, the anti-proliferative activity of the extract from radish and elderberry containing 3,5-diglycosylated-ACN had less anti-proliferative activity for HT-29 cells than the monoglycosylated rich mixtures of ACN (Jing and others 2008). Interestingly, all ACN extracts inhibit secretion of only two of the assayed inflammatory mediators (i.e., IL-8 and IL-6) by activated SCC-25 cells. Elevated levels of the same group of cytokines (i.e.,
IL-1β, IL-8 and IL-6) are also found in saliva from oral cancer patients (Arellano-Garcia and others 2008; Brailo and others 2012; Nagler 2009; St John and others 2004).

The effect of ACN on the IL-1β induced secretion of IL-8 by non-cancerous OKF-6/TERT-2 cells was less robust than that by SCC-25 cells. StwB extract was the only ACN source that inhibited secretion of IL-8, IL-6, and TNF-α by both cancerous SCC-25 and non-cancerous OKF-6/TERT-2 cells. BlkRB ACN reduced secretion of IL-6 and TNF-α, but not IL-8, by the cells. Although RdGrp ACN inhibited secretion of IL-8 by the activated OKF-6/TERT-2 cells, secretion of TNF-α was increased suggesting the possibility that the some mixtures of ACN may have a hormetic effect on non-tumor cells. Similarly, CkB ACN increased secretion of IL-6 and TNF-α by the activated OKF-6/TERT-2 cells. These results suggest that some mixtures of ACN may have a hormetic effect on non-cancerous OKF-6/TERT-2 cells.

Since the anti-inflammatory activity of ACN was dependent on both type of extract and cell type, I considered the possible effect of medium composition and cell metabolism on degradation of ACN as contributing factors in the different anti-inflammatory activities. CkB ACN were more stable in basal DMEM/F12 medium used for experiments with SCC-25 cells than DMEM/F12 medium containing supplements (high-density medium) used for experiments with OKF-6/TERT-2 cells. This suggested that one or more supplement(s) in high density medium may have promoted auto-degradation of ACN and decreased exposure of OKF-6/TERT-2 to intact ACN compared to cultures of SCC-25 cells. All ACN in the extracts were partially degraded during incubation in media (Figure 4.3). Aglycone structure affected extent of degradation and this was slightly modified by number, but not the structure, of saccharides linked to the aglycone. The lower stability of catecholic (Dp, Cy) compared to methoxylated (Pn and Mv) anthocyanidins agrees with previous studies comparing aglycone structure (Kern and others 2007) and anthocyanin glycosides (Steinert and others 2008) in DMEM medium. I also observed preferential degradation of Dp- and Pt-glycosides during ex vivo incubation of RdGrp and BluB extracts in saliva (Kamonpatana and others 2012), and retention of RdGrp juice in the oral cavity (Chapter 3). Phenolic products generated during cell-free incubation of medium containing ACN extracts were similar to those reported after
incubation of ACN extracts in artificial saliva (Kamonpatana and others 2012) and medium for CaCO-2 cells (Kay, Kroon, Cassidy 2009). However, the amount of detected PCA accounted for only ca. 12 % of added Cy-glycosides in CkB and BlkRB ACN-rich extracts. Similarly, generation of PCA accounted for ca. 20% of the Cy-3-samb in Hbc extract. This is similar to reports that PCA accounted for approximately 20 % of added Cy-3-glu in colonic innoculum (Keppler and Humpf 2005). Collectively, these data suggest that PCA is not the primary product generated during degradation of Cy-rich mixtures of ACN in cell culture media, saliva, and colonic fermentation. Further characterization of unknown degradation products merits consideration. It is noteworthy that an unknown product was estimated for approximately 80 % (as PCA equivalent) of added StwB ACN (or 87 % of degraded StwB ACN) after 24 h incubation of StwB ACN in high-density medium (Figure 4.4). The characteristics of the unknown compound, viz., molecular weight (302 Da), elution time and UV-Vis spectra matched ellagic acid. This was confirmed by analysis of pure ellagic acid. Ellagic acid and ellagitannin are present in strawberry (Aaby, Ekeberg, Skrede 2007). Ellagic acid has been reported to be a degradation product of ellagitannin in MEM medium (Larrosa, Tomás-Barberán, Espín 2006), and may contribute to the observed inhibitory effect of StwB ACN extract on IL-8 and IL-6 secretion in response to the presence of IL-1β in cultures of SSC-25 cells.

Incubation of medium with ACN extracts with SCC-25 and OKF-6 cells did not increase the extent of loss of ACN during 4 h incubation that occurred in medium without cells. This suggests limited metabolism of ACN by both cell types. However, PCA content in medium added to Scc-25 and OKF-6 cell cultures was only 70% and 0%, respectively of that in medium incubated in absence of cells for after 4 h. Moreover, PCA was not detected after incubation (3 h, 37°C) of the CkB spent medium with glucuronidase and sulfatase from H. pomatia (data not shown). This also indicates limited phase 2 metabolism of the degradation product by these cell lines. One of most interesting findings was that despite the loss of the majority of added ACN in CkB, RdGrp, StwB, and BlkRB extracts during 24 h incubation of medium in absence of cells, conditioned medium decreased secretion of IL-8, IL-6, and TNF-α by SSC-25 cells activated with IL-1β. As phenolics were generated during cell-free incubation, it is
possible that PCA and unidentified metabolites may exert anti-inflammatory activity. Min et al. reported that PCA was more potent than ACN extract from black rice or Cy-3-glu at inhibiting secretion of TNF-α, IL-1β, NO, and PGE2 from lipopolysaccharide (LPS)-induced RAW264.7 murine macrophage (Min, Ryu, Kim 2010). The anti-inflammatory activity of conditioned medium that had contained RdGrp was associated with the presence of PCA and syringic acid. Syringic acid was reported to inhibit the activity of angiotensin-converting enzyme (Hidalgo and others 2012) that produces angiotensin II, a known proinflammatory mediator (Naruszewicz and others 2007). Intraperitoneal injection of syringic acid also reduced serum TNF-α, IFN-γ, and IL-6 after concanavalin A-induced liver injury in mice (Itoh and others 2009). Moreover, it is important to recognize the bioactivity of compounds other than ACN may contribute to the inhibitory effects of StwB and RdGrp extracts as these extracts were estimated to be only 86 % and 72 % pure ACN, respectively (Table 4.1). Extracts from StwB (Aaby, Ekeberg, Skrede 2007) and RdGrp (Nicoletti and others 2008) contain compounds that may possess inhibitory activity on IL-8 secretion such as ellagic acid (Romier and others 2008) and kaempferol-3-glu (Kou and others 2008), respectively. There may also be synergy among these contaminants and ACN (de Kok, van Breda, Manson 2008; Liu 2004).

In contrast to consistent anti-inflammatory activity of both ACN and the products in conditioned medium with SCC-25 cells, conditioned medium had a pro-inflammatory effect on the non-cancer OKF-6/TERT-2 cell line. Although StwB ACN in fresh medium inhibited secretion of IL-8 and IL-6 (Figure 4.2 and Table 2), conditioned medium that had contained StwB has less of an inhibitory effect on IL-1β-induced secretion of IL-8 (Figure 4.5) and increased secretion of IL-6 and TNF-α (Table 4.3). Also, conditioned medium with degradation products of RdGrp ACN lacked effect on IL-8 secretion by activated OKF-6/TERT-2 cells (Figure 4.5), and increased secretion of IL-6 and TNF-α (Table 4.3). The basis for the different responses of the two cell types is unknown. The obvious response would be that SCC-25 is derived from a cancerous tissue and OKF-6/TERT-2 is an immortalized non-cancer oral cell line. However, it is also important to consider the fact that they were derived from distinct tissues in the mouth.
In summary, natural mixtures of ACN inhibited IL-1β induced secretion of IL-8 and IL-6 by SCC-25 squamous cell carcinoma cell line and by the non-cancerous OKF-6/TERT-2 cells, although to a lesser extent. The extent of inhibition varied among the extracts tested, suggesting that ACN composition in the extract determines relative activity. ACN aglycone structure appeared to have a major influence on activity with lesser influence of the number of saccharides conjugated to the aglycone. The anti-inflammatory activity of extracts in SCC-25 cell cultures was associated with greater stability of ACN in medium and generation of bioactive degradation products. Lesser anti-inflammatory activity of ACN extracts in cultures of OKF-6/TERT-2 cells corresponded with lower stability of ACN in the medium. Stability of ACN in media was dependent on structure in a manner similar to that observed in saliva (Chapter 2) and in the oral cavity (Chapter 3). Collectively, the cellular studies support the hypothesis that ACN source and therefore composition affects the extent of anti-inflammatory activity and that ACN degradation products may possess anti- or pro-inflammatory effects depending on the type of oral epithelial cells. Investigation of the anti-inflammatory effects of natural mixtures of ACN in the oral cavity and identification and the bioactivity of ACN degradation products is expected to provide insights that will facilitate strategic development of products for the promotion of oral health.
Anthocyanins (ACN) as a major class of flavonoids that have dual roles as natural colorants and for the promotion of health (chapter 1). My central hypothesis stemmed from the following three facts: 1) greater than 700 structures of ACN have been characterized in nature; 2) the mixtures of ACN structures are qualitatively and quantitatively unique in different fruits and vegetables; and, 3) structure determines extent and mechanism of bioactivities. Experiments were conducted to explore and challenge the hypothesis that “recommendation or formulation of ACN-rich foods can influence a) metabolism, b) bioaccessibility, and c) bioactivities of ACN in oral tissue”. My data consistently show a strong influence of ACN structure on the extent of metabolism in all models tested, i.e., ex vivo degradation of ACN extracts in saliva (chapter 2), in vivo degradation of fruit juices during 5 min retention in the mouth (chapter 3), and in vitro degradation in cell culture media (chapter 4). Type of ACN aglycone appeared to provide greater influence on metabolism and anti-inflammatory activity than that of glycosylation. Dp and Pt structures were preferentially unstable in oral cavity and consequently correlated with limited bioaccessibility in vivo (chapter 2 and 3). Oral microbiota also exhibited a central role in ACN metabolism in both the ex vivo and in vivo models (chapter 2 and 3). ACN accessibility to the buccal epithelium
was dependent on structure (chapter 3). Anti-inflammatory activity was also dependent on ACN composition/source and type of cells (chapter 4). These data collectively support the consideration of ACN structure for the development of recommendations of specific ACN-rich foods and for the development of delivery devices for the promotion of oral health.

This dissertation aimed to laterally link ACN structure, metabolism, bioaccessibility, and bioactivity in the oral cavity. The results provide proof of hypothesis, as well as a map of specific areas that require further consideration. The data also generated a number of questions that merit further in-depth investigations. Questions I recommend for inquiry follow.

1) **How do other structures of ACN affect the metabolism, bioaccessibility, and bioactivity?** Although my dissertation considered all 6 types of anthocyanidins commonly presented in human diets, glycosylation was only limited to only linkage at the C3 position with mono-, di- and tri- saccharides. Acylation of ACN enhances pigment stability that is desirable for use as natural colorants (Giusti and Wrolstad 2003). Effect of type, position and degree of glycosylation and acylation can be further investigated to further advance our understanding of ACN as health promoting natural colorants.

2) **Does ACN structure influence ‘specific’ anti-inflammatory efficacy for other tissues?** Interestingly, ACN structures/sources provide specific inhibition for only IL-8 and IL-6 for SCC-25 tongue carcinoma cells, but complex and somewhat ‘balanced’ inhibition or induction of cytokines for the immortalized normal epithelium OKF-6/TERT-2 from the floor of the mouth (chapter 4). Such specific anti-inflammatory activity of limited cytokines for the cancer cell line suggests potential tissue-targeted strategy with limited side effects which is valuable for food
and pharmaceutical application. Whether this observation is consistent with other tissues in the mouth requires further investigations. ACN in food matrices containing other compounds that may influence the anti-inflammatory activity merits consideration. Effect of ACN structure on anti-inflammatory activity in animal models is essential to provide a bridge towards future clinical studies.

3) **What are the anti-inflammatory metabolites of ACN?** Efficacy of natural ACN mixtures and their degradation products was characterized ([chapter 4](#)) to provide a strategy for future identification of unknown metabolites ([chapter 2 and 3](#)). Characterization of efficacious degradation products from StwB and RdGrp ACN merits further consideration. Characterization of *in vitro* degradation products that provide anti-inflammatory activity will provide an initial guide for *ex vivo* and *in vivo* analyses. Interestingly, the presence of cells seemed to affect degradation products, but not necessarily ACN. Might the cells take up these products that may be responsible for mediating reported bioactivities of ACN?

4) **How do oral microbiota affect the metabolism, bioaccessibility, and anti-inflammatory activity?** My data provide strong evidence that microbial activity substantially contributes to metabolism of ACN both in brief (5 min) *in vivo* retention as well as prolonged (60 min) *ex vivo* incubation in saliva. These data induce questions about the role of the oral microbiota on the effects of various ACN and their uncharacterized metabolites on anti-inflammatory activity. Are metabolites generated by oral microbiota? If so, what are the products? What commensal and pathogenic bacteria participate in metabolism of ACN? Do metabolites possess both anti- and pro-inflammatory activities?
Finally, this dissertation suggests that the selection of food make a difference in terms of metabolism, bioaccessibility and bioactivity of ACN in the oral cavity. While the effect of ACN structure on metabolism was characterized *ex vivo, in vivo*, and *in vitro*, much work is still needed understand anti-inflammatory activity of ACN beyond cellular models. Regarding dietary recommendations for foods or the development of novel formulations, one should be aware of the complexity of 1) variations of many bioactive compounds other than ACN in any food, 2) interactions of compounds in different foods that are part of a meal, 3) likely differences in effects in various organs, 4) inter-subject variability, and 5) inflammatory pathway systems. Therefore, direct translation of these data to recommendation or formulation of ACN-rich foods seems promising yet premature.
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APPENDIX A

IRB APPROVED RESEARCH PROTOCOL

Protocol Number: 2009H0058
Protocol Title: Metabolism of berry anthocyanins in human oral cavity
Version 5 (Fourth amended protocol)
Feb 23, 2012 (Submission date)

Research Protocol

I. Objectives
1. To conduct preliminary experiments examining the ex vivo metabolism of anthocyanins from commonly consumed fruits in unstimulated saliva from human subjects.

2. To examine the relative contributions of secreted saliva, oral bacteria and oral epithelial cells in the metabolism of different types of fruit anthocyanins.

3. To examine the in vivo stability and epithelial uptake of anthocyanins in natural fruit juices in the oral cavity of human subjects.

4. To evaluate the anti-inflammatory activity of anthocyanins and their metabolites using oral cell lines

II. Background and rationale
Numerous epidemiological studies suggest that diets rich in fruits and vegetables decrease the risk of chronic diseases such as diabetes and cancer. Flavonoids represent a large family of plant polyphenolic compounds that likely contribute to the health promoting effects of such foods. Anthocyanins are one class of flavonoids that provide many fruits and vegetables with brilliant red, purple and blue colors. There is increased interest in the use of these non-toxic compounds as natural colorants for foods, beverages and confectionaries, as well in examining their health promoting activities. Anthocyanins are potent antioxidants and have been shown to inhibit the proliferation and induce programmed cell death of cancer cells, act as vasodilators, and exhibit anti-obesity activity. The amounts of anthocyanins in berry fruits is particularly high and a number of...
laboratories have demonstrated that anthocyanin-rich fruits and extracts from several types of berries possess chemopreventive activities against cancer cell lines originating from the oral cavity and colon, as well as in these organs in animal models and human subjects. Of particular interest for the proposed study is that feeding diets containing 5 and 10% lyophilized black raspberry inhibited the number and volume of chemically induced tumors in the hamster cheek pouch model.

It is noteworthy that anthocyanins are poorly absorbed by rats, pigs and human subjects. Moreover, the small fraction of ingested anthocyanins that is absorbed appears in plasma soon after ingestion, suggesting that absorption likely occurs in the oral, esophageal and gastric compartments. Thus, it is unclear if the chemopreventive action of the anthocyanins are mediated by the ingested compounds themselves or their bioactive metabolites, and whether the observed chemopreventive effect is mediated by the absorbed compounds or by direct uptake from the oral cavity and gut lumen by the epithelial cells lining these organs. We are not aware of any research examining the metabolism, uptake and absorption of anthocyanins in the oral cavity. Such information is needed to elucidate mechanisms by which anthocyanin-rich fruits and formulations protect subjects from carcinogenic insults in the oral cavity, as well as for the strategic development of novel confectionaries and functional foods that deliver the most bioactive anthocyanins to oral tissues. Therefore, we plan to initiate a systematic investigation of the ex vivo and in vivo metabolism of anthocyanins in five commonly consumed fruits in the mouth of human subjects. These data are needed to develop competitive proposals for funding from extramural agencies that are interested in the health-promoting activities of anthocyanins in the oral cavity. It is expected that the metabolism of anthocyanins from the various berries in the oral cavity will be dependent on the structures of these compounds. That is, the chemical fingerprint of anthocyanins in the fruits selected for testing differ in regards to the specific types of anthocyanin aglycones and the types, numbers and positions of the covalently linked sugars. Structure has been reported to be an important determinant for the types and extent of metabolism and absorption of other classes of flavonoids, as well as microbial metabolism in the gut and the apparent absorption of some anthocyanins.

Also, it is expected that there will be marked variability in the extent of metabolism among the human subjects. This likely is related to the different microbial communities in the mouth and perhaps polymorphisms in the transporters and enzymes involved in the metabolism of the ingested anthocyanins in the oral cavity.

III. Procedures

A. Research design. The experimental design is primarily based on a publication by Walle and Associates who investigated the metabolism of flavonoid glucosides in the oral cavity of humans. Anthocyanins were not included in their investigation. The design involves three components. The first component is an ex vivo study in which unstimulated saliva will be collected from as many as 30 subjects upon awakening to screen the extent to which anthocyanins extracted from as many as five fruits are degraded. Our preliminary data and related literature suggest that subjects will be categorized as either “high” or “low” metabolizers of the anthocyanins. As many as 10 subjects who exhibit “high” activity and 10 subjects exhibiting “low” will be selected to participate in subsequent
studies. Subjects that agree to continue to participate will first collect unstimulated saliva before and after rinsing their mouths with 1 ounce of antibacterial mouthwash (PerioGard) in order to assess the contribution of oral bacteria in the ex vivo degradation of saliva. One week later, each subject also will brush their teeth before gently scraping their cheeks, gums and tongue with a new toothbrush to release loosely adhered cells from these surfaces. Saliva will then be collected to determine the extent of ex vivo degradation of anthocyanins by the epithelial cells.

We also plan to evaluate the in vivo stability and epithelial cell uptake of anthocyanins in the oral cavity of as many as 10 high and 10 low metabolizers. On three separate occasions Subjects will ingest 20mL of three distinct anthocyanin-rich fruit juices retained in the mouth for 5 minutes. One juice will be administered each week. Saliva and cell scrapings from cheeks will be collected for anthocyanin analysis. The in vivo component of the study facilitates assessment of both the metabolism and uptake of anthocyanins and their metabolites by the cells lining the oral tissues.

To evaluate the contribution of microbial degradation to the observed loss of juice anthocyanins in the oral cavity, we will request that subjects avoid beverages, food and juices that are rich in anthocyanins or contain compounds that may affect the oral metabolism of anthocyanins for one day prior to visiting the office/clinic. On the day of the study, participants will arrive at assigned times, two hours after brushing their teeth. They will brush their inner cheek and the saliva and suspended cells will be collected in a tube. After rinsing their mouth with water, the washing will be collected. Then participants will be given less than one ounce (20 mL) of an anthocyanin-rich juice, i.e., chokeberry juice, for retention in their mouth for 5 minutes. Retained juice and two washings will be collected in a tube. Participants will then brush the other inner cheek and the scrapings and a wash will be collected. The entire procedure will be repeated one week later except that the participants will have a standard dental cleaning in Dr. Kumar’s dental clinic after the initial scapping of the inner cheek and before retaining 20 mL the same juice in their mouth. Retained juice followed by two rinses of the mouth with water will be collected. Then, buccal scrapings will be collected from the opposite cheek to determine the impact of bacteria on extent of anthocyanin degradation in the mouth.

The final series of experiments involve the use of established oral cell lines. Cultures of oral cell lines will be exposed to the proinflammatory cytokine IL1-α according to Sakoda et.al.\textsuperscript{12}. Anthocyanin-rich extracts from chokeberry, red grape and strawberry will be evaluated for their comparative anti-inflammatory activity. Similarly, these extracts will be incubated ex vivo with saliva from 6 subjects as in completed studies (Page 1, I. Objective 2) to obtain a mixture of anthocyanins and their metabolites. Cultures will be pre-treated and co-treated with either a) anthocyanin extracts, b) mixtures of anthocyanins and their metabolites generated during ex vivo incubation with saliva, or c) appropriate controls to determine anti-inflammatory activity. Expression of pro-inflammatory cytokines, chemokines and enzymes producing inflammatory products will be examined. Anthocyanin profiles in starting materials and following incubation with oral cell cultures will be analyzed by HPLC-PDA-ESI-MS.
B. **Sampling.** We will obtain novel empirical data from as many as 30 subjects. We plan to inform biomedical researchers (research staff and students in our laboratories as well as faculty colleagues and their research groups) that we are investigating the metabolism and bioactivities of health promoting pigments in common fruits. As the procedures are not invasive, a high degree of compliance is expected. We seek approval to recruit as many as 10 more subjects than will be needed after the initial screening to ensure participation of an adequate number of “high” metabolizers and in case some participants withdraw before completion of the ex vivo and in vivo components of the study. We expect a fair degree of variability in the extent of anthocyanin degradation among subjects as a previous study revealed marked variation in extent and rate of hydrolysis of dietary flavonoids other than anthocyanins (i.e., conversion from the ingested flavonoid glucosides to their sugar-free aglycone nuclear structures)\(^\text{11}\). Metabolism varied for class of flavonoid compounds and among individuals, and appeared to be independent of gender, age and chronic use of oral antibacterial solutions, although the number of subjects was small. In the limited number of subjects (n=4) we have examined to date, there was one subject whose saliva exhibited a “high” extent of anthocyanin degradation, two subjects whose saliva had a “low” extent of anthocyanin degradation, and a fourth subject in whose saliva anthocyanin degradation was intermediate.

We plan to orally communicate the planned study of anthocyanin metabolism in the oral cavity to members of the Failla, Giusti and Kumar research teams as well as to faculty colleagues at OSU who have overlapping research interest with request they distribute to their teams. Those with interest in the possibility of volunteering for the study will be asked to contact Mr. Kamonpatana or Dr. Failla by email or in person to schedule an appointment. Potential subjects must be 18-55 years of age. For those expressing interest in participating, we will request completion of the form requesting information about oral and general health, sex, race, ethnicity and level of education. Participation requires that volunteers have not had their teeth cleaned during past three months, taken antibiotics during the past month, have open sores in their mouths at present or require antibiotics prior to visiting the dentist. This will ensure that the microflora and oral epithelium of subjects have not changed recently as a result of possible infection or inflammation with the oral cavity. Information on general health status will be sought in the questionnaire in order to exclude individuals with conditions that can affect salivary secretion. Subjects also will be examined their oral health by oral hygienist to excluded individual with inflamed oral tissues. If the individual meets inclusion criteria, we will next discuss the purpose of the study, collection procedures, and answer all their questions about the study. At completion of the session, we will hand individuals the subject approval form and ask them to read the information once again, sign if they decide to participate, and return the form to Mr. Kamonpatana or Dr. Failla within several days. A copy of the signed form will be retained and second copy given to each subject for their records.

C. **Measurements.** We will identify and quantify the concentrations of anthocyanins and their metabolites after incubation with saliva and collected cells. These data will provide information about the extent of metabolism of the abundant anthocyanins present
in each test material in “intact” saliva and the relative contributions of salivary secretions, oral bacteria and epithelial cells lining the oral cavity of the subject to such metabolism. Also, as the chemical fingerprint of the test anthocyanin extracts to be tested differs, the results are expected to provide information about the relationship between anthocyanin structure and extent of metabolism of mixed anthocyanins from a particular fruit or vegetable. For the in vivo study, the difference between the amount of anthocyanins introduced to the oral cavity and the quantity recovered after 5 min retention in the mouth will likewise yield novel information about the relationship between chemical structure, stability, and uptake of anthocyanin or metabolites by oral epithelial cells. Determination of the profiles of anthocyanins and their metabolites in juice retained in the mouths of participants for 5 minutes and in buccal scrapings prior to and after clinical cleaning of teeth and killing bacteria adhered to tissue surfaces will provide novel insights about the role of bacteria in oral degradation of anthocyanins in vivo.

D. Detailed study procedures.

i. Collection of unstimulated saliva for in vitro experiments. All subjects (N=30) will be asked to brush their teeth with toothpaste as usual before sleeping on the night prior to collections. Subjects will also be asked to refrain from consumption of beverages and foods until after collection of saliva for the ex vivo studies, i.e., when saliva is collected for incubation with anthocyanin-rich extracts from test fruits. For the initial screening study (week #1), subjects will be asked to collect approximately 10mL saliva into a screw cap polypropylene tube upon waking in the morning, cap the tube, store on ice and deliver to the Failla lab within 2 hours. Subjects (maximum of 10 “high” and 10 “low” metabolizers of anthocyanins ex vivo) who are invited and agree to participate will collect saliva again the following week. They will collect approximately 10 mL unstimulated saliva upon awakening before brushing their teeth with toothpaste and rinsing. This second collection will be approximately one week after the initial screening. Subjects will then rinse their mouth with 1 ounce of PerioGard, an anti-bacterial mouthwash, for 1 min and rinse with water. Five (5) minutes later, subjects will transfer 10mL saliva to a second tube, cap the tube, store on ice and deliver both tubes to the Failla lab. The following week (week #3), subjects will brush their teeth with toothpaste and rinse their mouth with water. Subjects next will rinse their mouths with 1 ounce of PerioGard for 1 minute followed by rinse with water. Five (5) minutes later, subjects will use a new toothbrush to lightly scrape the surface of their gums, cheeks and tongue to collect loosely adhered epithelial cells. Saliva (approx, 10 mL) containing these scrapings will be collected in a polypropylene tube and sealed. All tubes will be placed on ice after sample collection for transfer to the Failla lab in Campbell Hall.

The collected saliva is diluted with artificial saliva and incubated with three to five of the anthocyanin extracts from test fruits. These include the following: red grape (Vitis labrusca); chokeberry (Aronia melanocarpa); black raspberry (Rubus occidentalis); blueberry (Vaccinium cyanococcus); and strawberry (Fragaria ananassa). These sources have been selected for investigation because their anthocyanin profile is well established and differ markedly, thus facilitating evaluation of the relationship between chemical structure and metabolism. Results from the ex vivo studies will be important for selection of the primary fruit juices that will be tested in vivo.
Saliva also will be collected after retention of anthocyanin-rich juices in oral cavity. Subjects (maximum of 10 “high” and 10 “low” metabolizers of anthocyanins as assessed during ex vivo studies with saliva in week 1) will brush their teeth after breakfast (without berry or grape fruit juices or foods). They will visit the Failla office in Campbell Hall (343E) within 2h after breakfast. Buccal cells from left cheek will be collected by 10 up-down-strokes using a soft toothbrush according to standard procedure to establish a baseline of anthocyanin in the cells. Subjects will be given less than one ounce (20 mL) of a natural juice prepared from one of the five fruits listed above (week #4). Subjects will retain the juice in their mouths for 5 min. Then they will expel saliva into a large polypropylene tube, rinse their mouth well with 20 mL of water and expel the wash into the flask twice. Subjects will then scrape loosely adhered epithelial cells from gums, cheeks and tongue with a new toothbrush we will provide and collect saliva in a separate polypropylene screw cap tube. Mouth will be rinsed with 20 mL of 0.9% salt (NaCl) solution twice which also will be collected in the tube. The tube will be capped and placed in ice for processing. This procedure will be repeated each of the following two weeks using juice from different fruits on weeks #5 and #6. Anthocyanins will be extracted from retained juice containing saliva and collected cells to quantify amounts of starting materials in these two compartments and evaluate their chemical profiles. We plan to collect saliva from 2 subjects working in the Failla lab on as many as four different occasions to test and modify our analytical protocols prior to undertaking the investigation (Objective 3) with the other subjects.

To evaluate the contribution of oral bacteria in degradation within the mouth, a maximum of 10 subjects will be asked to repeat the same procedure on weeks #7 (control) and #8 (treatment) using chokeberry juice. During the 24 h prior to experiments in week #7 and 8, subjects will be asked to refrain from consumption of red wine, coffee, tea, or food and juices that contain anthocyanins, e.g. grape, berries (such as strawberry, blueberry, raspberry), blue corn, blue cauliflower, and compounds that may inhibit oral metabolism of the anthocyanins (compounds in coffee, tea and red wine). On the morning of the experiment, subjects will be asked to brush their teeth and avoid using antibacterial mouth wash and may consume only water prior to arrival to clinic 2 h after brushing their teeth. Upon arrival, participants will brush one inner cheek and removed cells and a washing will be collected in a tube. Participants will then be given 20 mL juice for retention in their mouth for 5 min. Retnetate and two washings will be collected in tubes. Then participants will brush the other cheek and scrapings and a wash will be collected in tubes. In week #8, the oral cavity of the subjects will be cleaned by a dental hygienist to remove plaque on teeth, and bacteria adherent on the tongue and gingiva. This procedure requires approximately 30 min. After oral cleaning, 15 mL of antibacterial chlorhexidine (PerioGard) will be retained in the mouth for 1 min followed before being expelled without mouth rinsing with water according to standard procedure. Five minutes later, 20 mL chokeberry juice will be retained in mouth as detailed above.

Every effort will be made to recruit the same subjects who participated in the earlier studies for experiments 7 and 8. However, if this is not possible, we will recruit new subjects using the same recruitment techniques described above. All subjects will sign a
new consent form describing the specific processes for this experiment. Breakfast will be offered along with $25 compensation upon completion for both week #7 and week #8.

Laboratory manipulations involve dilution of biological materials, centrifugation, spectrophotometric analyses and high performance liquid chromatography.

iii. **Collection of saliva for evaluation of anti-inflammatory activity of salivary metabolites from anthocyanins (week #9).** Saliva (10 mL) will be obtained from 6 subjects upon awakening and before brushing teeth as described above (See protocol i). Saliva will be collected in sterile screw cap polypropylene tubes that will be capped and placed on ice for transfer to the Failla lab within 2 hours. The collected saliva will be pooled and incubated with anthocyanin-rich extracts from fruits to generate metabolites. The mixture of anthocyanins and their metabolites will be resolubilized in cell culture media and added to cultures of oral epithelial cell lines to evaluate anti-inflammatory activity. A maximum of 4 collections of saliva from 2 subjects is needed prior to indicated experiment to optimize experimental conditions.

iv. **Risks for Subjects.** Brushing the teeth and the use of an antibacterial such as PerioGard is a standard daily activity and not associated with known risk. Collections will be made from individuals only once per week. That is, subjects will collect saliva or loosely adhered epithelial cells only one day per week. Sufficient saliva will be collected to examine ex vivo metabolism of anthocyanin extracted from the five different fruits with the same sample. The number of times any volunteer participating in the in vivo component of this study will brush the surfaces of cheeks is six (6) – once for initial collection of the cells to assess enzymatic cleavage ex vivo, three times at retention of juice in oral cavity for 5 min (week # 4-6), and two times at retention of juice before and after oral cleaning (week # 7-8). The collection of loosely adhered cells from the surface of cheeks is a standard procedure used for investigations related to activities in the oral cavity. The procedure has a minor risk of inducing discomfort if the individual uses excessive force for prolonged period. Dr. Failla and Mr. Kamonpatana will receive training in this routine procedure from Dr. P. Kumar or a designated experienced member of her research team in the OSU Dental School prior to initiating this portion of the study. Failla and Kamonpatana will instruct test subjects in proper procedure to minimize stress to tissues. We are not aware of any possibly adverse long term effects.

Each individual will be assigned a number from 1-30 after enrolling in the study known only to Dr. Failla and Mr. Kamonpatana. The assigned number will be recorded on the top of the signed consent form that will be maintained in a binder in a locked file cabinet in Dr. Failla’s office. Other than the master key in the possession of the Dean’s office and housekeeping, Dr. Failla is the only individual with the key to his administrative office, 150 Arps Halls. Collection tubes and flasks will indicate the assigned number rather than subject name. Although highly unlikely, it is possible that Dr. Failla’s office will be broken into, locked file cabinet forced open, and files of subjects read. The only information that would be available is subject participation in the research study as the
consent form does not contain subject employee number or social security number, thus minimizing the possibility of invasion of privacy and identity theft.

iv. Internal Validity. There is a single recent paper published in a peer-reviewed journal regarding the oral metabolism of anthocyanins. These investigators limited their investigation to a solution containing a suspension of lyophilized black raspberry\textsuperscript{14}. Thus, we seek empirical information regarding the stability and epithelial cell uptake of anthocyanins and their metabolites from various sources of fruits as the structure of anthocyanins in juices from different fruits vary and thus metabolism and potential efficacy may differ. Our results are expected to begin to develop a strategy for the development of efficacious confectionaries and functional food products that will have extended resident time on the oral cavity. Such retention is likely required for maximal chemopreventive activity against oral cancers and other pathologies within the oral cavity. In the only related study, the metabolism of other types of anthocyanins in the oral cavity was highly variable among subjects and seemingly independent of race, gender and regular use of mouthwash.\textsuperscript{11} Thus, our selection of human subjects is imply limited to age (18-55 years) and general oral health. We also will exclude women with knowledge of pregnancy as this condition is associated with mild compromise of immune status. Thus, we wish to avoid the minimal risk that brushing teeth and tissues for the experimental protocol will create wounds that provide oral microorganisms with access to peripheral tissues.

v. Data analysis. The biochemical nature of the inquiry only requires routine statistical comparison of chemical profiles of test anthocyanins in sample before and after incubation of collected samples with saliva and shed epithelial cells. Similarly, changes in amount and profile of anthocyanins during retention in oral cavity are evaluated by standard procedures. We routinely analyze data using ANOVA with Tukey’s post hoc analyses to evaluate extent of metabolism of compound in different extracts and foods.
IV. Bibliography

The Ohio State University Consent to Participate in Research

Study Title: Metabolism of berry anthocyanins in human oral cavity
Principal Investigator: Mark Failla, PhD
Sponsor: Ohio Agricultural Research and Development Center

• This is a consent form for research participation. It contains important information about this study and what to expect if you decide to participate. Please consider the information carefully. Feel free to discuss the study with your friends and family and to ask questions before making your decision whether or not to participate.

• Your participation is voluntary. You may refuse to participate in this study. If you decide to take part in the study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your usual benefits. Your decision will not affect your future relationship with The Ohio State University. If you are a student or employee at Ohio State, your decision will not affect your grades or employment status.

• You may or may not benefit as a result of participating in this study. Although most unlikely your participation may result in unintended minor irritation of tissues in your mouth.

• You will be provided with any new information that develops during the study that may affect your decision whether or not to continue to participate. If you decide to participate, you will be asked to sign this form and will receive a copy of the form. You are being asked to consider participating in this study for the reasons explained below.

1. Why is this study being done? We plan to examine the metabolism of a family of water soluble natural pigments called anthocyanins presents in fruits such as berries in the oral cavity. You likely have enjoyed a dish of strawberries or blueberries and noted that afterwards your teeth, gums and tongue are temporarily colored red and blue color,
respectively. The food industry is especially interested in using anthocyanins as natural
dyes in place of synthetic dyes for coloration of foods, beverages and confectionaries.
Anthocyanins also exhibit some health promoting effects including anti-inflammatory
and anti-cancer activity in cells and in the mouth and colon of experimental animals.
Incorporation of anthocyanins in confectionaries such as chewing gum has been proposed
as a strategy for increasing their residence time in the oral cavity. Presently, it is
unknown if these compounds are a) stable in the mouth, b) anthocyanins or their
metabolites are taken up by cells lining the gums, cheeks and tongue, c) anthocyanins or
their metabolites are transferred from cells in the oral cavity to the blood, and d) the
metabolic mixture is responsible for anti-inflammatory activity. In addition, if
metabolism occurs in the oral cavity, it is unclear if bacteria and/or human cells are
responsible for such activity and how the oral bacterial population in different individuals
affects the metabolism and activities of these pigments. Finally, the specific types of
anthocyanins in different fruits and vegetables differ. Recent studies have suggested that
the structural differences likely affect the metabolism and the health-promoting activities
of anthocyanins from various plant foods.

The planned study represents the first systematic effort to address such questions. We expect that the results obtained from this study will provide novel information about how anthocyanins in foods and commercial beverages and confectionaries affect oral health.

2. How many people will take part in this study?
We seek 30 healthy adults to participate in this study.

3. What will happen if I take part in this study
We request that you first complete the one page questionnaire about your general oral
health and background. The questions include information about whether you have open
sores in your mouth at this time or regularly, if you have had your teeth recently cleaned,
or if you have taken antibiotics in the past 3 months, as well as your sex, race, ethnicity
and education level. Once you complete the questionnaire, we will inform you if you meet the criteria required for participation in the study. If so, we will begin discussion of the details for the study so you can determine if you remain interested in being a subject. (Individuals will be given the questionnaire and allowed to complete in private).

The first 6 sessions (listed below) of clinical study have been completed. If you agree
to participate, you will collect saliva at least once and up to a maximum of three
times (during weeks # 7, 8, and 9). Please begin reading from week # 7 onwards.
You will be instructed to brush your teeth with toothpaste the night before each test.

- Saliva collection #1 (week 1). Upon awakening in the morning, you will collect
  a small amount of saliva (approximately 10 ml....or less than one half ounce) in a
test tube before brushing your teeth with toothpaste or consuming any beverage or
food. The collection tube will then be capped and placed in container with ice for
delivery to the Failla research lab in Campbell Hall within 2 hours. The saliva
will be used to determine the extent of degradation of anthocyanins from fruits
such as strawberry, blueberry and red grape. The tests will allow us to arbitrarily categorize your saliva as having “high” vs. “low” anthocyanin degradation activity. Some individuals from these two categories will be selected for additional studies.

- **Saliva collection #2 (week 2).** If selected to continue in the study and you agree to continue to participate in the study, you will be asked to collect saliva once again upon awakening and before brushing your teeth or consuming beverage or food. You will then brush your teeth with toothpaste before rinsing your mouth with 1 ounce of Periogard, an antibacterial mouthwash (Periogard and cup will be provided), for 1 min to kill oral bacteria. You will then rinse your mouth again with water and empty contents in sink. Five minutes later, you will collect approximately 10 mL saliva in the test tube, cap, and place on ice for delivery to the Failla lab within 2 hours.

- **Saliva collection #3 (week 3).** You will also be asked to provide us with saliva collected after gently scraping your gums, cheeks and tongue to remove loosely adhered cells from the surface. After awakening, you will brush your teeth with toothpaste, rinse your mouth with water and then scrape the indicated tissues with a special toothbrush we will provide and show you how to use. You will refrain from beverage and food until after collecting saliva for this test. Saliva containing the scraped cells will be collected in the test tube that will be capped and placed on ice for delivery to the Failla lab.

- **Saliva collections #4 (week 4).** If you agree to participate in the remainder of the study, you will visit the Failla office on campus in the morning. You may consume beverages and foods other than berries or grapes before the visit. You will brush your teeth with toothpaste before visiting the office. You will be given less than one ounce (20 mL) of a natural, anthocyanin-rich fruit juice. The fruit juice will be from one of the following fruits: black raspberry, strawberry, blueberry, red grape and chokeberry. We will ask you to retain in your mouth for 5 min before emptying contents into a large tube. You will then rinse your mouth with less than 1 ounce (20 mL) of water to collect residual juice and saliva. This will help us to determine the stability of anthocyanins in the fruit juice in your mouth. You also will gently brush your cheeks with a new toothbrush to release loosely adhered cells and collect 10 mL saliva in polypropylene tubes.

- **Saliva collection #5 (week 5).** You will visit the Failla office the following week and receive 2 ounces of a different fruit juice than the one you were given the previous week. As above, you may consume beverages and food other than from berries or grapes before visiting. You will brush your teeth with toothpaste before visiting. You will retain the juice in your mouth for 5 minutes, empty contents into a large tube, and rinse your mouth with less than 1 ounce (20 mL) of water with transfer of these rinses into the large tube. Loosely adhered cells will also be removed by gently brushing cheeks as for week #4.
• Saliva collection #6 (week 6). You will visit the Failla office. As above, you may consume beverages and foods other than berries or grapes before visiting the office. You also will brush your teeth with toothpaste before visiting. A juice from a different fruit source than the previous two weeks will be given to you for retention in your mouth for 5 minutes. The contents in your mouth and from two rinses with water will be transferred into a large tube. Loosely adhered cells will also be removed by gently brushing cheeks as for weeks #4 and #5.

• Saliva collection #7 (week 7). You will visit the Failla office (315C Campbell Hall). During 24 h prior to the study, you may not consume wine, coffee, tea, or food and juices containing anthocyanins, e.g. grape, berries (such as strawberry, blueberry, raspberry) and blue corn/chips. On the day of your visit, you will brush your teeth and avoid use of antibacterial mouth wash. You may consume only water prior to arrival in Failla office 2 h after brushing your teeth. A sample of juice that is less than 1 ounce will be given to you for retention in your mouth for 5 minutes. The contents in your mouth and from two rinses with water will be expelled into tubes. Loosely adhered cells will also be collected by gently brushing one of your inner cheeks before transfer of the juice to your mouth. After expelling the juice retained in your mouth for 5 minutes and rinsing with water, the other inner cheek will be brushed to collect the loosely adhered cells. You will receive $25 for your participation.

• Saliva collection #8 (week 8). The dietary and beverage restrictions for this study are the same as for week 7. You will visit the Kumar dentistry clinic at Postle Hall at the assigned time to receive professional oral cleaning by a dental hygienist. This procedure will remove plaque on teeth, and adherent bacteria on the tongue and gingiva which will take 30 min. After the cleaning, 15 mL of antibacterial chlorhexidine (PerioGard) will be retained for 1 min follow by wash out period of 5 min. One inner cheek will be scrapped with a toothbrush for collection of loosely adhered cells. The same juice as in Week #7 then will be retained in the mouth for 5 minutes. The liquid will be expelled into a collection tube and the mouth rinsed twice with water. The other inner cheek will then be brushed and removed cells collected in a tube as for Week #7. You will receive $25 for your participation.

• Saliva collection #9 (week 9). This is the last session of saliva collection. Upon awakening in the morning, you will collect a small amount of saliva (approximately 10 ml…or less than one half ounce) in a test tube before brushing your teeth with toothpaste or consuming any beverage or food. The collection tube will then be capped and placed in container with ice for delivery to the Failla research lab in Campbell Hall within 2 hours. The saliva will be used to generate metabolites of anthocyanins from fruits such as strawberry, chokeberry and red grape. The metabolites will be investigated for their anti-inflammatory activity.
using several oral cell lines. The tests will allow us to understand the linkage between metabolism and bioactivity of anthocyanins.

4. How long will I be in the study?
Participation in the entire study will require a maximum of 3 weeks with one collection per week. The estimated time for collection and visits to the campus laboratory/office is one hour per week. If you only participate in the first collection, you will be volunteering for a maximum of 1 hour of time and effort.

5. Can I stop being in the study?
You may leave the study at any time. If you decide to stop participating in the study, there will be no penalty for you. Your decision will not affect your future relationship with the research team or The Ohio State University.

6. What risks, side effects or discomforts can I expect from being in the study?
Brushing your teeth is a routine daily activity, as is rinsing with an antibacterial mouthwash such as Periogard. You may be asked and agree to provide us with cells from the oral cavity. This involves brushing the surfaces of your gums, cheeks and tongue, an activity you may or may not do on occasion. This is a standard procedure for collecting loosely adhered cells from the mouth and has a very limited risk of causing temporary discomfort.

7. What benefits can I expect from being in the study?
Your participation will assist us to generate information that is needed for the development of novel confectionaries and functional foods for the prevention or complementary treatment of oral diseases.

8. What other choices do I have if I do not take part in the study?
You may choose not to participate without penalty or loss of benefits to which you are otherwise entitled.

9. Will my study-related information be kept confidential?
Every effort will be made to keep your study-related information confidential. The questionnaire on oral health history will not have your name and will be destroyed after completion. Your name will not appear on any sample as you will be assigned an arbitrary number upon consenting to participate in the study. Also, your consent form will be retained in a locked file to which only the PI has access. However, there may be circumstances where this information must be released. For example, personal information regarding your participation in this study may be disclosed if required by state law. Also, your records may be reviewed by the following groups (as applicable to the research):
- Office for Human Research Protections or other federal, state, or international regulatory agencies;
- U.S. Food and Drug Administration; and
10. What are the costs of taking part in this study?
There is no financial cost for your participation.

11. Will I be paid for taking part in this study?
There is generally no financial compensation for participation in this study except 2 sessions i.e. Week # 7 and # 8, which will be $25 upon completion of each week.

12. What happens if I am injured because I took part in this study?
In the most unlikely case that you suffer an injury from participating in this study, you should notify the researcher immediately, who will determine if you should obtain medical treatment at The Ohio State University Medical Center.
The cost for this treatment will be billed to you or your medical or hospital insurance. The Ohio State University has no funds set aside for the payment of health care expenses for this study.

13. What are my rights if I take part in this study?
If you choose to participate in the study, you may discontinue participation at any time without penalty or loss of benefits. By signing this form, you do not give up any personal legal rights you may have as a participant in this study.
You will be provided with any new information that develops during the course of the research that may affect your decision whether or not to continue participation in the study.
You may refuse to participate in this study without penalty or loss of benefits to which you are otherwise entitled.
An Institutional Review Board responsible for human subjects research at The Ohio State University reviewed this research project and found it to be acceptable, according to applicable state and federal regulations and University policies designed to protect the rights and welfare of participants in research.

14. Who can answer my questions about the study?
For questions, concerns, or complaints about the study you may contact either Dr. Mark Failla at 614-247-2412, Dr. Purnima Kumar at 614-247-4532, or Dr. Monica Giusti at 614-247-8016.

For questions about your rights as a participant in this study or to discuss other study-related concerns or complaints with someone who is not part of the research team, you may contact Ms. Sandra Meadows in the Office of Responsible Research Practices at 1-800-678-6251.

If you are injured as a result of participating in this study or for questions about a study-related injury, you may contact Dr. Mark Failla (office, 614-247-2412; cell, 614,560-9401).
**Signing the consent form**

I have read (or someone has read to me) this form and I am aware that I am being asked to participate in a research study. I have had the opportunity to ask questions and have had them answered to my satisfaction. I voluntarily agree to participate in this study.

I am not giving up any legal rights by signing this form. I will be given a copy of this form.

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**Investigator/Research Staff**

I have explained the research to the participant or his/her representative before requesting the signature(s) above. There are no blanks in this document. A copy of this form has been given to the participant or his/her representative.

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