PURPLE CORN ANTHOCYANINS: CHEMICAL STRUCTURE, CHEMOPROTECTIVE ACTIVITY AND STRUCTURE/FUNCTION RELATIONSHIPS

DISSERTATION

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

By

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ABSTRACT

Interest in purple corn (*Zea mays* L.) as a natural colorant has increased because of their potential health benefits. This study evaluated an anthocyanin-based purple corn extract as a natural colorant and chemoprotective source as compared to other anthocyanin sources. The structure/function relationship between anthocyanins and relative biological activity were investigated.

Purple corncob contained high monomeric anthocyanins concentration (290 to 1323 mg/100g DW) and acylated anthocyanins (35 to 54%). Obtaining a colorant from purple corn produces large amounts of a highly colored purple corn waste (PCW) with limited solubility. The limited solubility was associated with the complexation of anthocyanins with macromolecules (tannins and proteins) abundant in PCW. The purple corn pigment extraction procedure was modified to minimize waste production. Deionized water at 50 °C yielded high anthocyanin concentration with relatively low tannin and protein content. Application of a neutral protease during processing might decrease the level of the major protein (29KD) in purple corn and further reduce PCW.

PCW was soluble in neutral environment and tested as a natural colorant for milk. PCW provided an attractive purple color (hue: 324-347°) to milk. This color was

more stable in milk than in a pH6.8 buffer, suggesting that milk components protected anthocyanins and color in an accelerated model (70 ºC).

Purple corn colorant showed higher inhibition of human colon carcinoma HT29 cell proliferation (GI_{50}=~14µg/ml) than other six (chokeberry, bilberry, grape, purple carrot, radish, and elderberry) anthocyanin-rich extracts (ARE) (GI_{50} =31~130µg/ml). Anthocyanin-rich (2.29g/100g) PCW showed high antiproliferation (GI_{50} =21µg/ml), but lower than the purple corn colorant, suggesting that macromolecular complexes might trap monomeric anthocyanins reducing their bioavailability. An anthocyanin fraction separated from other phenolics in ARE played a major role on ARE's chemoprotection. The interaction between anthocyanin and other ARE phenolics on chemoprotection was additive.

Anthocyanin chemical structure affected chemoprotection: cyanidin 3-glucoside had higher inhibitory effect than pelargonidin 3-glucoside. Anthocyanin monoglucosylation showed a higher inhibitory activity than the corresponding 3,5-triglucoside. Effect of acylation on chemoprotection was dependant on the type of aglycone and acylating acid. More research is needed to better understand the impact of anthocyanin structure on chemoprotection.

Dedicated to my mom and Yan

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PUBLICATIONS

Research Publication

1. Wyzgoski, F.J., Rinaldi, P.L., Reese, R.N., Scheerens, J.C., Miller, A.R., Bishop, B.L., Giusti, M.M., Bomser, J.L., Ozgen, M., Tulio Jr., A.Z. and Jing, P. (2006). Using high field cryoprobe NMR experiments and multivariate analysis to

identify bioactive components in black raspberries. American Chemical Society National Meeting.

2. Jing, P., and Giusti, M. M. (2005). Characterization of Anthocyanin-Rich Waste from Purple Corncobs (Zea mays L.) and Its Application to Color Milk. J Agric Food Chem 53, 8775-8781.

3. Jing, P., Ding, X.W., and Su, Y. (2000). Scavenging effect of extract of citrus peel on •OH. Journal of Southwest Agricultural University. 22, 416-418.

4. Jing, P. and Su, Y. (2000). Development of sports drinks. Beverage Industry, China, 3, 7-9.

5. Jing, P. (1999). Development of functional food in China. Chinese Animal Product and Food, 6, 233-234.

6. Xiang Y.F. Jing, P., and Chen, M. (1996). Improvement of Bennett Test. Studies in Higher Education, 7, 61-62.

7. Xiang, Y.F., Jing, P., and Liu, D.Y. 1996. Study on microencapsulation process of Zanthoxylum oleoresin. Journal of Sichuan University (Natural Science Edition), 33, 760-764.

FIELD OF STUDY

Major Field: Food Science and Nutrition

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACN	Anthocyanin fraction
AP	Reconstitution of anthocyanin fraction and other phenolic fraction
ARE	Anthocyanin-rich extract
ARW	Anthocyanin-rich waste
BSA	Bovine serum albumin
C*	Chroma
Cy	Cyanidin
Dp	Delphinidin
h*	Hue
HPLC	High performance liquid chromatograph
GI_{50}	Concentration of treatment to inhibit 50% of cell growth
GI tract	Gastrointestinal tract
L*	Lightness
LC	Liquid chromatograph
MS	Mass spectrometer
Mv	Malvidin
OPF	Other phenolic fraction
PCC	Purple corn colorant
PCE	Purple corn extract
PCW	Purple corn waste

Pg	Pelargonidin
Pn	Peonidin
Pt	Petunidin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

CHAPTER 1

INTRODUCTION

Anthocyanins are a class of flavonoid compounds responsible for the bright attractive orange, red, purple, and blue colors of most fruits and vegetables. There is considerable demand in the world for natural food colorants as a result of both legislative action and consumer concerns over the use of synthetic additives in their foods. Anthocyanins are a potentially alternative to FD&C Red No. 40 which is the synthetic dye with the highest consumption in the United States. However, the use of colorants from natural sources can be challenging: anthocyanins usually exert low tinctorial power and large doses are needed to reproduce similar results to those obtained with small amounts of synthetic dyes, making them an expensive alternative. In addition, anthocyanins are sensitive to light, pH, and neucleophilic agents, so they have limited stability in food matrices as compared to synthetic dyes. Identification of

low cost anthocyanin-rich sources with increased stability is very important to expand the markets of anthocyanin-rich agricultural commodities as natural food colorants and as value-added ingredients for functional foods.

Purple corn (*Zea mays* L.), rich in anthocyanins, has been extensively cultivated in South America, mainly in Peru and Bolivia, and used to prepare drinks and desserts for centuries due to its high pigment content. Purple corn contains high concentration of pigments, very rich in anthocyanins (~1640 mg/100g FW) much higher than other anthocyanin-rich sources, such as berries (20~1500 mg/100g FW), radishes (*Raphanus sativus* L.) (11~60 mg/100g FW), and cabbages (*Brassica oleracea* L.) (322 mg/100g FW). Anthocyanin-rich extracts from purple corn are comparably less expensive than those from others such as berries. Interest in purple corn as a source of anthocyanins as colors and phytonutrients has increased over the last years. Many health benefits have been associated with purple corn, including reduction of oxidation stress, prevention of obesity and diabetics, and chemoprevention of colon cancer. However application of purple corn as a natural food colorant and as a source of phytonutrient is challenged by limited solubility of anthocyanin-rich extracts from purple corn, large amount of waste during extract preparation, and limited information of how anthocyanin profiles may affect bioactivity.

Large amount of purple corn waste is generated during the colorant processing. The limited solubility of the purple corn waste was found to be associated with high

levels of macromolecules (tannins and proteins). We used three approaches to improve utilization of purple corn anthocyanins: to purify anthocyanins from the waste material, to optimize the processing of purple corn colorant; or to find a new way to apply the waste to food matrices.

In this study, we will evaluate an anthocyanin-based color from purple corn (*Zea mays* L.) as a natural food colorant, compare their potential health benefits as a chemopretective agent with other anthocyanin sources, and determine the effect of processing on their chemoprotection of purple corn anthocyanin-rich colorants. The structure/function relationship between anthocyanins and relative biological activity is investigated.

Chapters 3 through 5 will work on anthocyanin concentration and profiles in purple corncobs, growth conditions that might affect purple corncob anthocyanins, the optimization of extraction methods to minimize the solubility problems during colorant processing, and a potentially practical approach to utilize this anthocyanin-rich waste in skim milk and whole milk.

Chapters 6 through 8 will evaluate the purple corn colorant compared with six other anthocyanin-rich sources (chokeberry, bilberry, elderberry, grape, purple carrot, and radish) with respect to their chemoprotective activity by use of an *in vitro* biological model with a human colorectal adenocarcinoma cell model (HT29). The interaction of anthocyanins and other phenolics will be estimated on the growth inhibition of HT29 cells. Chemoprotective activity will be evaluated among purple

corn waste, purple corn extract, and purple corn colorant, which were generated during purple colorant processing. Finally, structural properties (aglycone, glycosylation, and acylation) of anthocyanins are studied the relationship with their anthocyanin biological activity.

CHAPTER 2

LITERATURE REVIEW

2.1 PURPLE CORN (*ZEA MAYS* L.)

Purple corn (*Zea mays* L.) rich in anthocyanins, is an Andean crop, cultivated in low valleys in South America mainly in Peru and Bolivia. It is known as "maiz morado" and has long been used to color desserts and beverages. A colorant from purple corn is widely used in Asia, South America and Europe at present.

The average anthocyanin content of whole purple corn from Peru was 1640 mg/100g fresh weight (Jones, 2005), higher than fresh blueberries (73-430 mg/100g) (Moyer et al., 2002). Anthocyanin from kernel pericarp contributes to the most percentage of total kernel anthocyanin content. The level of anthocyanin ranged from 504.0 to 1473.0 mg/ 100g in kernel pericarp while in degermed kernels ranged from

54.4 to 115.0 mg/ 100g among cultivars including Arrocillo, Conico, Peruano and Purepecha (Moreno et al., 2005).

Cyanidin-3-glucoside is the major anthocyanin in purple corncob (Nakatani et al., 1979), although pelargonidin, and peonidin glucosides have also been found in maize plants (Styles and Ceska, 1972) as well as their respective malonyl derivatives (Pascual-Teresa-S-de et al., 2002). They were identified as cyanidin 3-malonylglucoside, pelargonidin 3-malonylglucoside, and peonidin 3-malonylglucoside, where the malonyl acylation was at different position instead of the C-6" position of glucoside. In addition, Pascucal-Teresa and co-workers found thant cyanidin/ pelargonidin/ peonidin 3-(6"-ethylmalonyglucoside) in the commercial purple corn anthocyanin-rich colorant (Pascual-Teresa-S-de et al., 2002). Aoki and coworkers found dimalonyl acylation of cyanidin in purple corn kernels (Aoki et al., 2002).

2.2 ANTHOCYANIN DISTRIBUTION IN EDIBLE PLANTS

Anthocyanins are water-soluble and vacuolar pigments found in most species in the plant kingdom. They are produced in fruits and vegetables such as berries, cherries, cherries, red onion, red radish, red soybeans, purple corn, red cabbage, red potato and purple carrot. Some common foods containing anthocyanins were listed in **Table 2.1**. The average anthocyanin content of whole purple corn from Peru was 1640 mg/100g fresh weight (Cevallos-Casals and Cisneros-Zevallos, 2003; Jones, 2005). The levels of anthocyanins in berries vary among different species or even only among

Source	Scientific name	Anthocyanin	Reference
Fruits			
Apple	*Malus pumila* P. Mill.	1 ~ 17	(Wu et al., 2006)
Bilberry	*Vaccinium myrtillus* L.	300 ~ 808	(Prior et al., 1998; Maatta-Riihinen et al., 2004)
Blackberry	*Rubus spp.*	72 ~ 1221	(Clark et al., 2002)
Black chokeberry	*Aronia melanocarpa* (Michx.) Elliot	307 ~ 1480	(Strigl et al., 1995; Wu et al., 2004)
Blackcurrant	*Ribes nigrum* L.	96 ~ 452	(Kampuse et al., 2002; Wu et al., 2004)
Blackberry	*Rubus spp.*	72 ~ 1221	(Clark et al., 2002)
Black raspberry	*Rubus occidentalis* L.	145 ~ 607	(McGhie et al., 2002; Moyer et al., 2002)
Blueberry	*Vaccinium corymbosum* L.	63 ~ 430	(Prior et al., 1998; Moyer et al., 2002)
Cranberry	*Vaccinium macrocarpon* Aiton.	20 ~ 360	(Prior et al., 2001; Wang and Stretch, 2001)
Elderberry	*Sambucus nigra* L.	332 ~ 1374	(Maatta-Riihinen et al., 2004; Wu et al., 2004)
Grape	*Vitis vinifera*	27 ~ 120	(Wu et al., 2006)
Lingonberry	*Vaccinium vitis-idaea* L.	31 ~ 92	(Wang et al., 2005)
Marion berry	*Rubus ursinus*	62 ~ 155	(Deighton et al., 2000; Wada and Ou, 2002)
Strawberry	*Fragaria* × *ananassa* D.	13 ~ 55	(Cordenunsi et al., 2002)

Continued

Table 2.1: Anthocyanin content (mg/ 100g FW) in selected common edible plants

Table 2.1 continued

Vegetables			
Black bean	*Phaseolus vulgaris* L.	45	(Wu et al., 2006)
Eggplant	*Solanum melongena* L.	86	(Wu et al., 2006)
Purple carrot	*Daucus carota*	38 ~ 98	(Lazcano et al., 2001)
Purple corn	*Zea mays* L.	1640	(Cevallos-Casals and Cisneros-Zevallos, 2003)
Radish	*Raphanus sativus* L.	11 ~ 60	(Giusti et al., 1998)
Red cabbage	*Brassica oleracea* L.	322	(Wu et al., 2006)
Red potato		14 ~ 45	(Rodríguez-Saona et al., 1998)
Red onion	*Allium cepa* L.	49	(Wu et al., 2006)

different cultivars. Anthocyanins concentration in black choke berries (*Aronia melanocarpa* (Michx.) Elliot), for example, reached up to 1458 mg/100g (FW) whereas anthocyanins weren't detectable in some berries, such as red currant (*Ribes* × *Pallidum* cv. White Duch) and gooseberry (*Ribes uva-crispa* cv. Careless) (Maatta-Riihinen et al., 2004) (Wu et al., 2004). For the same species and cultivars, the levels of anthocyanins in berries may vary according to different technical assays and culture condition. The black currant (*Ribes nigrum* cv. Ojebyn) was tested by different researchers by different evaluation assays at different locations. The anthocyanins

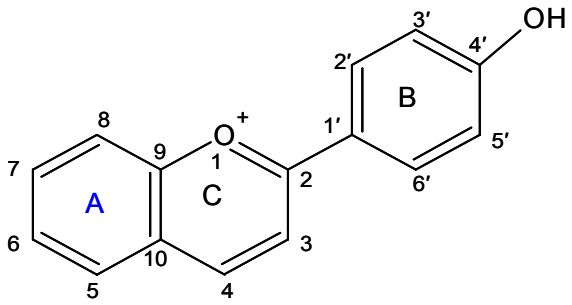
concentration ranged from 165 to 412 mg/g (FW) determined by different extracting methods, evaluation assays (spectrophotometric method or High-performance liquid chromatography (HPLC) analysis), different anthocyanin equivalents or standards for quantification (Kampuse et al., 2002; Moyer et al., 2002; Kähkönen et al., 2003; Maatta et al., 2003; Maatta-Riihinen et al., 2004).

2.3 ANTHOCYANIN CHEMISTRY

2.3.1 Structure and Chemistry

Anthocyanins are nearly universal, water-soluble plant pigments, responsible for the red, purple, and blue colors of many fruits, vegetables, cereal grains, and flowers (Giusti and Wrolstad, 2003). They have long been the subject of investigation by botanists and plant physiologists because of their roles as pollination attractants and phytoprotective agents. Today, interest in anthocyanin pigments has intensified because of their possible health benefits as dietary nutraceuticals.

Anthocyanins belong to the class of flavonoid compounds, which are widely distributed plant polyphenols. The basic structure of aglycones or anthocyanidins was a C6-C3-C6 carbon skeleton. There are more than 19 known naturally occurring anthocyanidins or aglycones (**Table 2.2**) based on the skeleton (Strack and Wray, 1993; Nygaard et al., 1997; Pale et al., 1997; Kong et al., 2003). Among them, the six most common anthocyanidins in nature are cyanidin, pelargonidin, peonidin, delphinidin, petunidin, and malvidin. Anthocyanins as glycosides of anthocyanidin



Name	Abbrev	Substitution						Color
		3	5	6	7	3’	5’	
Common basic structures								
Pelargonidin	Pg	OH	OH	H	OH	H	H	Orange
Cyanidin	Cy	OH	OH	H	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	Bluish-red
Common methylated structures								
Peonidin	Pn	OH	OH	H	OH	OMe	H	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	Bluish-red
Malvidin	Mv	OH	OH	H	OH	OMe	OMe	Bluish-red
3-deoxy structures								
Apigeninidin	Ap	H	OH	H	OH	H	H	Orange
Luteolinidin	Lt	H	OH	H	OH	OH	H	Orange

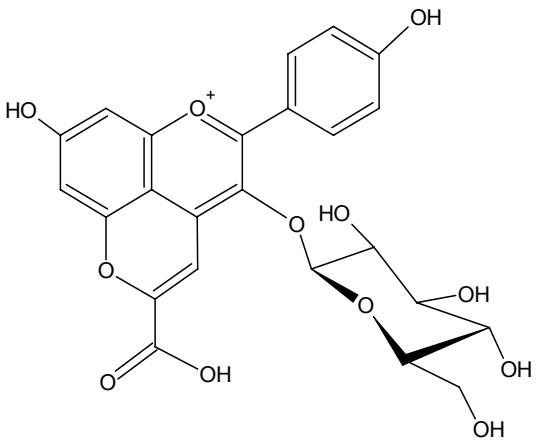
Continued

Table 2.2: Naturally occurring anthocyanidins. Updated from Strack, D. and Wray, V., 1992, Annotation [a] come from difference source: Pale et al, 1997)

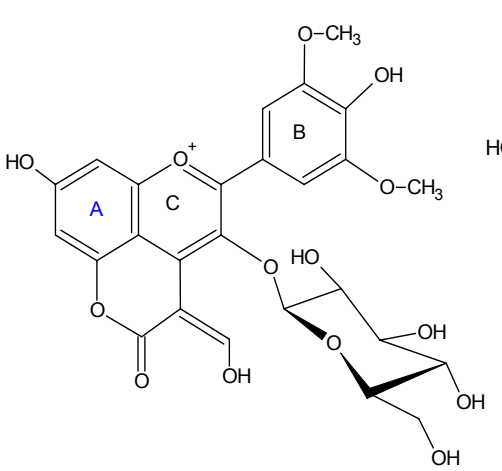
Table 2.2 continued

Tricetinidin	Tr	H	OH	H	OH	OH	OH	Red
Rare hydroxylated structures								
Aurantinidin	Au	OH	OH	OH	OH	H	H	Orange
6-Hydroxycyanidin	6OHCy	OH	OH	OH	OH	OH	H	Red
6-Hydroxydephinidin	6OHDp	OH	OH	OH	OH	OH	OH	Bluish-red
Rare methylated structures								
Capensinidin	Cp	OH	OMe	H	OH	OMe	OMe	Bluish-red
Europinidin	Eu	OH	OMe	H	OH	OMe	OH	Bluish-red
Hirsutidin	Hs	OH	OH	H	OMe	OMe	OMe	Bluish-red
7-O-methylapipeninidin[a]	7-MAp	H	OH	H	OMe	H	H	Orange-red
5-Methylcyanidin	5-MCy	OH	OMe	H	OH	OH	H	Orange-red
Pulchellidin	Pl	OH	OMe	H	OH	OH	OH	Bluish-red
Rosinidin	Rs	OH	OH	H	OMe	OMe	H	Red

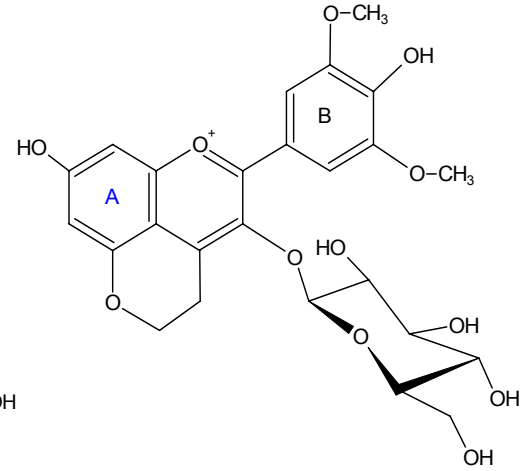
chromophores can be polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium (flavylium) salts linked with aromatic, aliphatic acids, ethyl ester derivatives (Brouillard, 1982). Except the above anthocyanidins, 4-substituted aglycone has been found in nature(**Figure 2.1**), such as 5-carboxypryanopelargonidin 3- *O*- β-glucopyranoside in strawberry (*Fragaria x ananassa* Duch.)



5-carboxypryanopelargonidin
3- *O*- β-glucopyranoside



Vitisin A

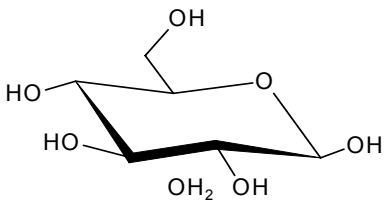


Vitisin B

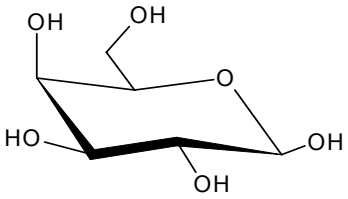
Figure 2.1: Structures of 5-carboxypryanopelargonidin 3- *O*- b-glucopyranoside and vitisin A/B (Source: Andersen et al., 2004b; Bakker and Timberlake 1997)

(Andersen et al., 2004) and, vitisin A and B in red wine (Bakker and Timberlake, 1997). Except for deoxy anthocyandins that are stable, anthocyanidins are very unstable, rarely found in fresh plant material and therefore occur mainly in glycosylated forms, where sugar substitution enhances the stability and solubility of anthocyanin molecule (Harborne, 1979; Clifford, 2000; Giusti and Wrolstad, 2003).

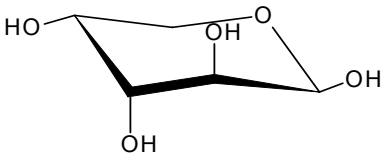
The most common sugar moieties glycosylating aglycones are glucose, galactose, rhamnose, xylose, arabinose, as mono-, di-, and tri-glycosides (**Figure 2.2**). The most widespread anthocyanin is cyanidin 3-glucoside. Besides the C-3 position, other sugars can also be attached at any one of the hydroxyls at C-5, C-7, C-3', C-5', and even C-4' (Brouillard, 1988; Mazza and Miniati, 1993c). These sugars may be acylated with aromatic acids, such as *p*-coumaric, caffeic, ferulic, sinapic, gallic or *p*-hydroxybenzoic acids or aliphatic acids, such as malonic, acetic, malic, succinic or oxalic acids (**Figure 2.3.**) (Robbins, 2003). Acyl substituents are commonly bound to the C-3 sugar, esterified to the 6-OH or less frequently to the 4-OH group of the sugars (Jackman and Smith, 1996.). However, anthocyanins containing rather complicated acylation patterns attached on different sugar moieties have been reported (Andersen and Fossen, 1995; Andersen et al., 1995; Giusti et al., 1998; Torskangerpoll et al., 2005).



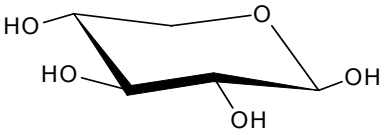
β-D-Glucose



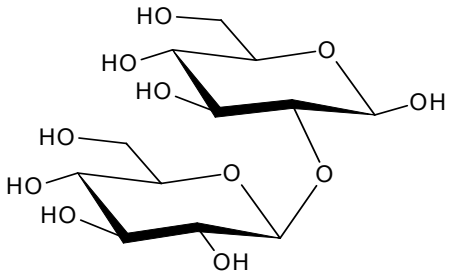
β-D-Galactose



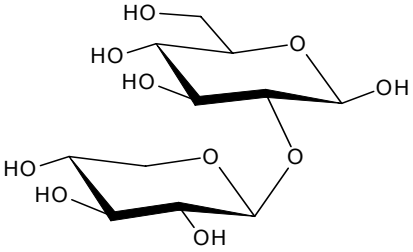
β-D-Arabinose



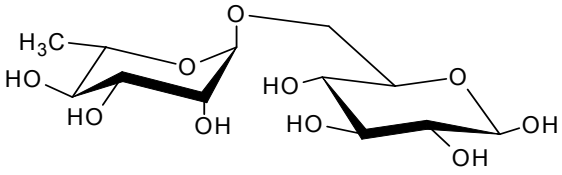
β-D-Xylose



Sophorose
(2-*O*-β-D-glucosyl-D-glucoside)



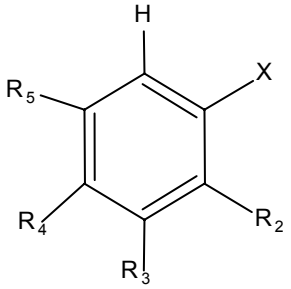
Sambubiose
(2-*O*-β-D-xylosyl-D-glucoside)



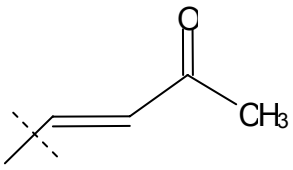
Rutinose
(6-*O*-L-rhamnosyl-D-glucoside)

Figure 2.2: Typical glycosylations in anthocyanins

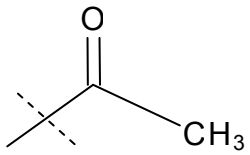
Aromatic acids



Catalogue	Common Name	R_2	R_3	R_4	R_5
	p-coumaric acid	H	H	-OH	H
hydroxycinnamic	ferulic acid	H	$-OCH_3$	-OH	H
acids(Xa)	sinapic acid	H	$-OCH_3$	-OH	-OCH
	caffeic acid	H	-OH	-OH	H
hydroxybenzoic	p-hydroxybenzoic acid	H	H	-OH	H
acids(Xb)	gallic acid	-OH	-OH	-OH	-OH

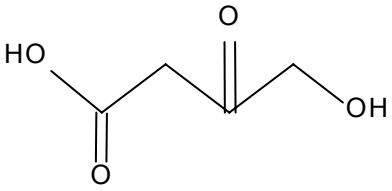


Xa

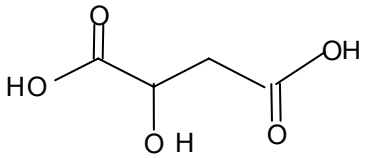


Xb

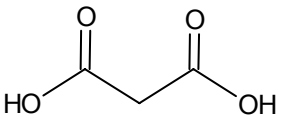
Aliphatic acids



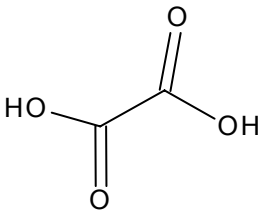
Succinic acid



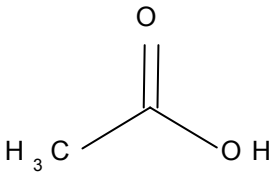
Malic acid



Malonic acid



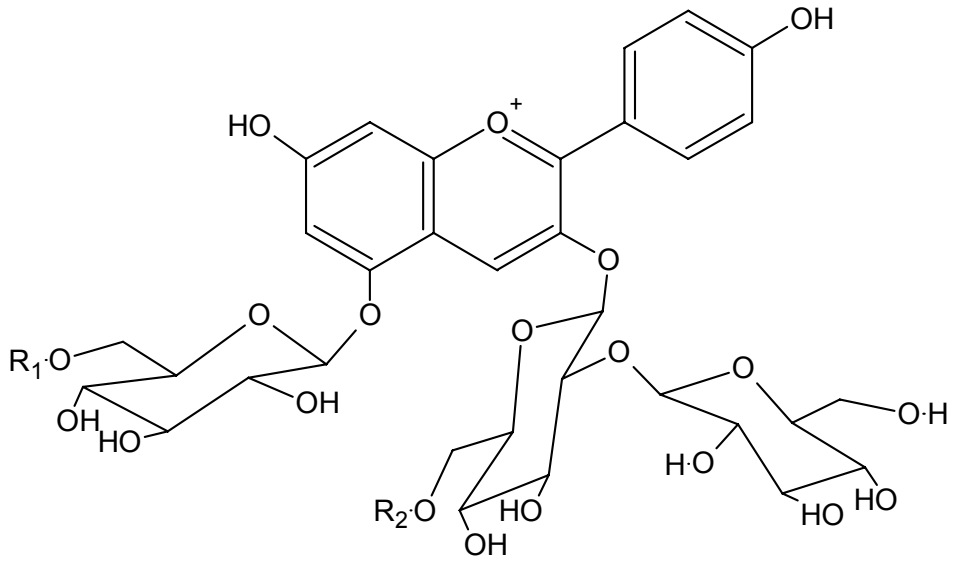
Oxalic acid



Acetic acid

Figure 2.3: Common organic acids acylated with sugar moieties on anthocyanins. (Sources: Robbins, 2003)

There is a wide variety of chemical structures among anthocyanins: different glycosylating patterns, different acylating groups, many different hydroxyl groups available for esterification of the acylating groups and finally presence of cinnamic acids in different stereo isomeric forms (Brouillard, 1988; Giusti et al., 1998) (**Figure 2.4**).



R1: Malonic acid

R2: Ferulic acid

Figure 2.4: Chemical structure of acylated pelargonidin derivatives encountered in red radish (*Raphanus sativus*): pelargonidin-3-sophoroside-5-glucoside acylated with malonic acid and ferulic acid. Arrows indicate hydrogens close in space. (Source: Giusti *et al.* 1998)

As a result, about 600 structurally distinct anthocyanins have been identified in nature (Andersen and Jordheim, 2004).

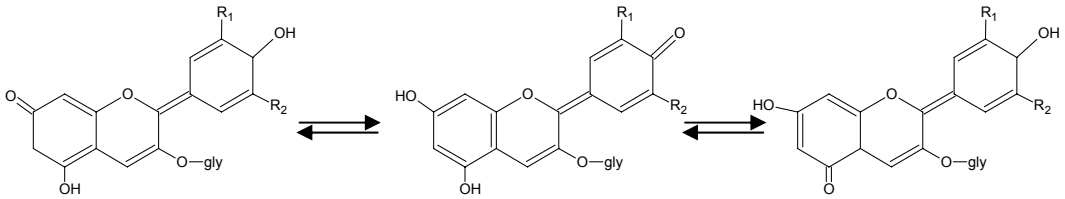
2.3.2 Anthocyanin Stability

The main challenge for the use of anthocyanins as natural food colorant is the relatively low stability. Chemical structure of anthocyanins plays an important role in stability. Anthocyanins owe their color to the high resonance of the fully conjugated 10-electron A-C ring-system, with some contribution by the B ring as well. This structure in resonance is the cause of their light reactivity, and consequently the groups attached to the structure (namely hydroxyl, methoxyl, glycosyl, and acyl) influence the stability substantially (Delgado-Vargas and Paredes-López, 2003). The resonance structure is disrupted and the color is lost, for example, when anthocyanins are in high pH medium or bleached by bisulfite (Brouillard, 1982). Pelargonidin, cyanidin and delphinidin are less stable than peonidin, petunidin and malvidin due to the blocking reactive hydroxyl group by methylation. The anthocyanins with 4-substitution were found more stable than others, such as 5-carboxypryanopelargonidin 3- *O*- b-glucopyranoside in strawberry (*Fragaria x ananassa* Duch.) (Andersen et al., 2004) and vitisin A and B developing during aging process of red wine (Bakker and Timberlake, 1997; Mateus and de Freitas, 2001). The stability of anthocyanins not only depends on their structure but also on the combination of environmental factors, including temperature, light, presence of other phenolic compounds, enzymes, metal

ions, sugars, ascorbic acid, and oxygen etc. have significant impact on the stability of anthocyanins (Delgado-Vargas and Paredes-López, 2003; Shahidi and Naczk, 2004).

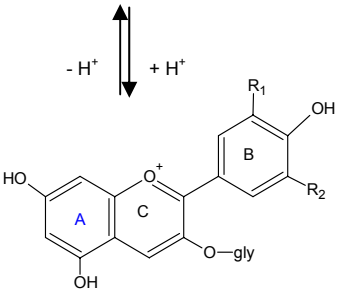
2.3.3 The Influence of pH

Anthocyanins undergo reversible structure transformation in aqueous environment with a change in pH, accompanied by dramatic changes in color (**Figure 2.5.**) (Wrolstad et al., 2002). At pH value around 1, anthocyanins are primarily in the form of flavylium cations. When the pH increases to 4.5, colorless hemiketal form is predominated..Flavylium cation can be hydrolyzed rapidly at the 2-position by nucleophilc attack of water to give the colorless hemiketal form, which can equilibrate to the colorless chalocone pseudobase, an open form. At this point, the conjugated C-ring is destroyed and so lost the color. The blue quinonoidal base exists primarily in neutral aqueous solution (Giusti and Wrolstad, 2001). When pH value continually increases to 8 or above, the quinonoidal base was ionized. Anthocyanins in the form of flavylium cation are much more stable than in the colorless form and quinonoidal base form.



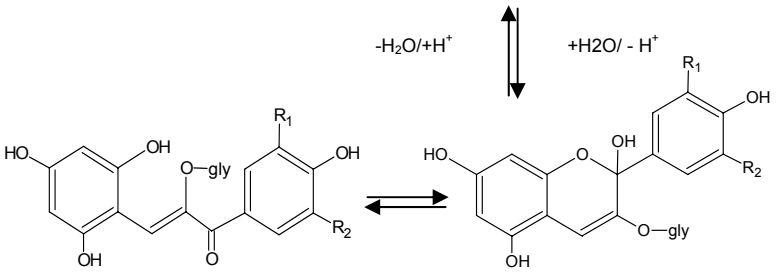
Quinonoidal base: blue

pH = 7



Flavilium cation (oxonium form): orange to purple

pH = 1



Chalcone: colorless

pH = 4.5

Carbinol pseudo-base (hemketal form): colorless

pH = 4.5

Figure 2.5: Predominant structural forms of anthocyanins present at different pH levels. (Source: Wrolstad et al, 2002)

2.3.4 Acylation

Acylation of sugar substitution with aromatic or aliphatic organic acids contribute to an important stabilizing effect on anthocyanins via intermolecular interaction. Acylation with aromatic acid has shown to be more stable than nonacylated form (Bassa and Francis, 1987; Malien-Aubert et al., 2001; Giusti and Wrolstad, 2003). Research found that anthocyanins with a complex pattern of glycosylation and acylation that have greater stability to unfavorable conditions (Francis, 1992; Dangles et al., 1993; Fossen et al., 1998). Acylation with cinnamic acids and even aliphatic acids leads to a bathochromic shift and a hyperchromic effect as electron-donating groups (von Elbe and Schwartz, 1996; Giusti et al., 1999). Acylation of anthocyanins not only has a great impact on the color and tinctorial strength, but also stabilizes the anthocyanins to unfavorable environment, such as in the pH 4-5 range, even in the neutral or slightly alkaline region as compared to nonacylated anthocyanins (Fossen et al., 1998; Torskangerpoll and Andersen, 2005).

A mechanism called intramolecular/intermolecular copigmentation was introduced to illustrate the increased stability of acylated anthocyanins (Dangles et al., 1993; Mazza and Miniati, 1993; Harborne and Williams, 2000; Boulton, 2001). The charged C ring in anthocyanidins can react with nucleophiles as an electrophilic center. Those acyl may not only affect the color but also protect the chromophores from the nucleophilic attack through an intermolecular stacking of chromophore planar or a sandwich spatial structure for anthocyanins containing two or more aromatic acyl

groups which are illustrated in **Figure 2.6** (Dangles et al., 1993; Giusti and Wrolstad, 2003). The spatial proximity between the hydrogens from aglycone and acyl groups in radish was proved very close to each other in two-dimensional NMR spectroscopy analyses (Giusti et al., 1998).

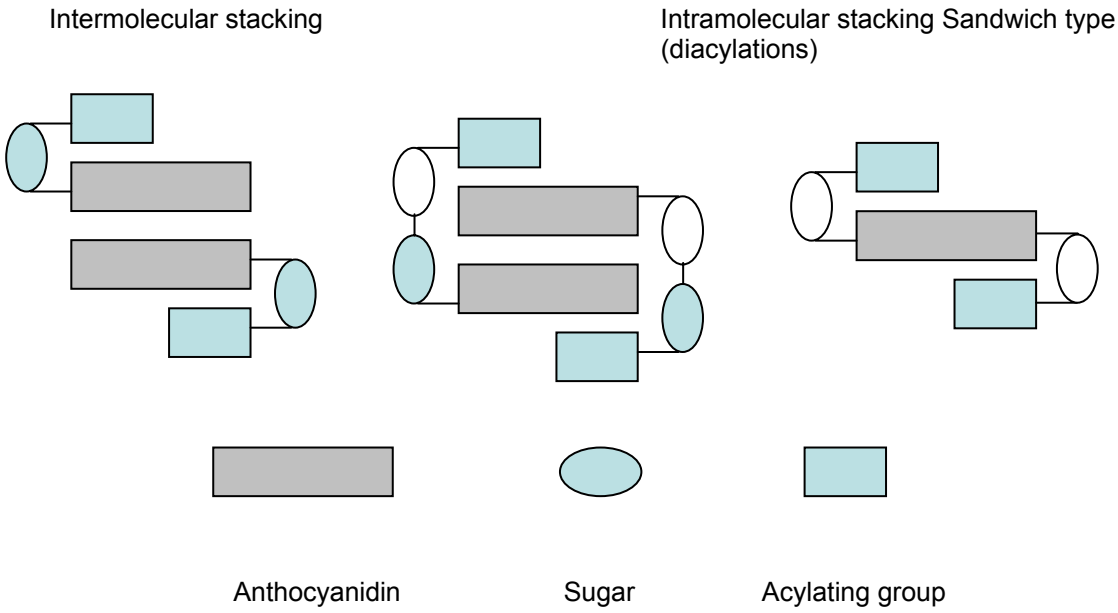


Figure 2.6: Stabilization mechanism of acylated anthocyanins-proposed spatial configuration and stacking of acylated anthocyanins. (source: Giusti and Wrolstad, 2003)

2.3.5 Copigmentation

Anthocyanins are more stable in the aquatic system via intermolrecular copigmentation with other compounds, mostly other phenolic compunds. This formation was forced either by non-covalently interaction (Mazza and Brouillard, 1990), or by covalent bonds with some simple molecules (Mazza and Brouillard, 1987; McDougall et al., 2005). Anthocyanin copigments were found either to exist as a nature formation such as anthocyanin-favanol condensation products detected from black currant (*Ribes nigrum* L.) (McDougall et al., 2005), or develop during extraction, processing or storage (Thorngate and Singleton, 1994; Mateus and de Freitas, 2001). The copigmentation of anthocyanin can be simple phenols such as catechin (Mazza and Brouillard, 1987; Dangles and Brouillard, 1992), flavanol (McDougall et al., 2005), chlorogenic acid, caffeic acid, rutin (Davies and Mazza, 1993), or polyphenols, such as tannins (Thorngate and Singleton, 1994; Remy et al., 2000; Salas et al., 2003). Except the phenolics, metal complexing and condensation of anthocyanins with acetaldehyde also contribute the greater stability (Mazza and Brouillard, 1987; Mirabel et al., 1999).

Most non-covalent interaction involving anthocyanin copigments are instant π-π overlap, dipole-dipole interactions, and possible hydrogen bonding (Dangles and Brouillard, 1992). Copigmentation of malvin with colourless organic molecules was driven enthalpically, an exothermic process with unfavourable entropy change with chlorogenic acids and (+)-catechin or favourable entropy change with caffeine and

tryptophan (Mazza and Brouillard, 1990; Dangles and Brouillard, 1992). Anthocyanin copigmentation with rutin and bacalin was forced by the enthalpy and entropy changes (Oszmianski et al., 2004). The formation of the π-π-complex improves the copigmentation of anthocyanins with tannins, developing during wine aging (Waterhouse, 2002).

2.3.6 Anthocyanins Degradation Kinetics

The degradation of tradescancia anthocyanins at room temperature and exposed to light showed linearity (zero-order reaction) (Baubli et al., 1994). However, most studies on degradation kinetics of anthocyanins have indicated a first-order reaction for red radish anthocyanins (Giusti and Wrolstad, 1996), sour cherries anthocyanins (Cemeroglu et al., 1994), blackberry (Daravingas and Cain, 1968), concord grape, red cabbage, and ajuga anthocyanins (Baubli et al., 1994). Labuza and Riboh pointed out that most quality-related reaction rates are either zero- or first-order reactions and statistical differences between the two types may be insignificant (Labuza and Riboh, 1982).

2.4 EXTRACTION OF ANTHOCYANINS

A good procedure should maximize anthocyanins recovery and minimize degradation or alteration of the nature state. Acids are very important to stablize anthocyanins in aqueous environment necessary for obtaining the flavylium cation form, which is the most stable form (**Figure 2.4**). The acids, however, may also

change the native form of anthocyanins in the tissue during extraction. Hydrochloric acid is the most common mineral acid employed in anthocyanins extraction.

High concentration of acids could cause the hydrolysis of anthocyanins. A concentration of 1% hydrochloric acid in methanol was found to cause partial hydrolysis of acyl moieties in acylated anthocyanins although it is helpful to increase the extraction rate of total monomeric anthocyanins (Revilla et al., 1998). Extraction with solvents containing HCl may cause pigment degradation during concentration. That is why acylations with aliphatic acids had been usually underestimated (Strack and Wray, 1989). To minimize the degradation of anthocyanins, weaker and volatile organic acids such as trifluoroacetic acid about 0.5%-3%, are used in the solvents.

The residue of acid in extracts would affect the cell culture test. Some acids were found to be toxic to cells. Formic acid was very toxic to cells, such as ocular injury, and caused cell death by the disruption of mitochondrial electron transport and energy production (Treichel et al., 2004). Toxicity of acetic acid was evaluated as a NR50 value of 6.4 and 7.4 mM respectively in different fish cells in neutral red assay (Mori and Wakabayashi, 2000).

Many solvents can be used in extraction such as methanol, acetone, ethanol and water due to the polar character of anthocyanin structure. Methanol is the solvent most commonly employed in combination with water, or acetone and water mixture. Most anthocyanins occur naturally as glycosides. Anthocyanin glycosides have higher solubility in water than the corresponding aglycones. Acetone, a universal solvent, has been used to extract anthocyanins due to its more hydrophobic than methanol and

easier penetrating cell membrane (Garcia-Viguera et al., 1998; Rodriguez-Saona and Wrolstad, 2001). Acetone as extracting solvent normally followed by partition with chloroform is more effective compared with the methanol-HCl extraction procedure (Timberlake and Bridle, 1971). Giusti and coworker have developed a method to extract anthocyanins, which involves powdering sample with liquid nitrogen, extraction with aqueous acetone (30:70. v/v) followed by partition with chloroform (Giusti and Wrolstad, 1996). A wide range of anthocyanin-containing plant materials and a high recovery with minimal pigment degradation can be obtained with the procedure. The higher recoveries were found out in acetone extraction for 19 of 20 samples as comparing with methanol extraction (Wrolstad et al., 2002).

2.5 POTENTIAL HEALTH BENEFITS OF ANTHOCYANINS AND BIOAVAILABILITY

2.5.1 Epidemiologic Study

Numerous epidemiologic studies have shown that high consumption of fruits and vegetables are associated with the lower risk of chronic diseases, such as cancers, cardiovascular diseases, cataracts, and hypertension. A report commissioned by the World Cancer Research Fund and the American Institute for Cancer Research mentioned that about 20% or more of all cases of cancers are preventable by high consumption (about 400-800 g/day) of various vegetables and fruits (Glade, 1999). In a review, there are nine of ten ecological studies, two of three case-control studies, and six of sixteen cohort studies that reported a protective effect of fruits and vegetables against cardiovascular diseases (Ness and Powles, 1997). More recently in two

prospective cohort studies, total fruit and vegetable consumption was inversely associated with risk of cardiovascular disease but not with overall cancer incidence (Hung et al., 2004). There is another strong support for the recommendations to consume more than five servings of fruit and vegetables per day likely to cause a major reduction in strokes in a meta-analysis of cohort studies (He et al., 2006). Other epidemiological studies also showed the high intake of fruits and vegetables are associated with low risk of cataracts (Moeller et al., 2004; Christen et al., 2005), obesity (He et al., 2004), and hypertensions (Burt et al., 1995). More recently, a cohort study in Finnish men aged 42-60 y in 1984 -1989 during a follow-up time of 12.8 y indicates that a high consumption of fruits, berries and vegetables is associated with a reduced risk of cardiovascular, noncardiovascular and all-cause mortality. During this study, the protective effect of dietary phytochemicals was investigated against mortality, including ß-carotene and lycopene (Rissanen et al., 2003). Unfortunately anthocyanins, the major dietary phytochemicals in berries didn't include in this study.

Although epidemiological data about anthocyanins or berry food are few, anthocyanin is most aboundant flavonoids in dietary with a consumption of about 200 mg/person estimated in a 1970's report (Kühnan, 1976), much higher than the average daily intake of other flavonoids including quercetin, kaempferol, myricetin, apigenin, and luteolin was 23 mg/person totally (Hertog et al., 1993). Therefore anthocyanins receive more and more attention not only because they are the major dietary phytochemicals in fruits and vegetables potential to provide extra health benefits to our bodies but also because of the French Paradox. Many *in vitro* studies and *in vivo*

studies related to biological activities of anthocyanins or anthocyanin-rich berries have been done or are undergoing around the world at present.

2.5.2 Antioxidants

Living organisms have a redox system trying to keep human life to be at a healthy balance. Free radicals are necessary for the living state of cells and organisms (Nagy, 2001; Droge, 2002). Free radicals protect our bodies' normal function well at a normal level. Some free radicals such as nitric oxide, superoxide anion, and related reactive oxygen species mediate cells in signaling processes (Droge, 2002). However the redox homeostasis could be off balance when in stress situation and extreme environments, and generate radicals exceeding the need of bodies. The overwhelming free radicals could lead aging and many degenerative diseases of aging such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts, et al (Harman, 1956; Ames and Gold, 1991; Harman, 1992; Ames et al., 1993; Davies, 1995; Allen and Tresini, 2000).

Berry extracts or juices were shown a high antioxidant capacity in numerous studies. Fresh strawberry extract was reported to have 5 times higher total antioxidant capacity than Trolox in oxygen radical absorbance capacity (ORAC) assay (Wang et al., 1997). Phenolic extracts of blackberries, black and red currants, blueberries, and black and red raspberries showed a high scavenging activity toward superoxide radicals (Costantino et al., 1992). Extracts of blackberries, red raspberries, blueberries,

and strawberries were shown to inhibit human low-density lipoprotein (LDL) and liposome oxidation (Heinonen et al., 1998).

Different genus, species or even cultivars of berries, as well as the maturity status were shown different antioxidant capacity. The total antioxidant capacity of different berries was found to be ranged 5 times difference as equivalents of Trolox in different species and cultivars of Vaccinium species, including highbush blueberry (*Vaccinium corymbosum* L.), lowbush blueberry (*Vaccinium angustifolium* L.), rabbiteye (*Vaccinium ashei* Reade), and bilberry (*Vaccinium myrtillus* L.) (Prior et al., 1998). Maturity at harvest had an impact on the ORAC, the anthocyanin, and the total phenolic content (Prior et al., 1998). Genus *Vaccinium* (bilberry, bog-whortleberry, cranberry and cowberries), genus *Ribes* (currants and gooseberry), genus *Sorbus* (rowanberries), genus *Aronia* (chokeberry), genus *Rubus* (cloudberry and red raspberry), and genus *Fragaria* (strawberry) were shown different antioxidant capacity with very different phenolic profiles (Kähkönen et al., 2001). Phenolic extracts from bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.) were shown the best antioxidant activities, followed by red raspberry (*Rubus idaeus* L.) and black currant (*Ribes nigrum* L.) in a lactalbumin-liposome system (Viljanen et al., 2004). Blackberries (*Rubus sp*.) and strawberries (*Fragaria* × *ananassa* D.) had the highest ORAC values during the green stages, whereas red raspberries (*Rubus idaeus* L.) had the highest ORAC activity at the ripe stage. Strawberries (*Fragaria* × *ananassa* Duch.) had the highest ORAC activity followed by black raspberry (*Rubus occidentalis* L.), blackberries (*Rubus sp*.), and red raspberries

(*Rubus idaeus* L.) (Wang and Lin, 2000). Blueberry (*Vaccinium corymbosum* cv. Sierra), cranberry (*Vaccinium macrocarpon* cv. Ben Lear), chokeberry (*Aronia melanocarpa*), and lingonberry (*Vaccinium vitis-idaea* cv. Amberland) possessed high antioxidant capacity in terms of ORAC values *in vitro* (Zheng and Wang, 2003). California hackberry (*Vaccinium ovatum*) had greater total anthocyanins, total phenolics, ORAC value, and ferric reducing antioxidant potential (FRAP) than did thinleaf huckberry (*Vaccinium membranaceu*) (Lee et al., 2004).

Berry fruits contain a wide range of flavonoids and phenolic acids that show antioxidant activity, while anthocyanins are major constituents in most berries. Many other phytonutrients in berries such as proanthocyanidin, phenolic acid, ellagitannin, flavanols, et al also play an important role to the total antioxidant activities of berries (Kähkönen et al., 2001; Viljanen et al., 2004). Statistical analysis could not well explain the relaltionship of phenolic compositions and their antioxidant activities in 26 berry samples evaluated by autoxidazing methyl linoleate and predominant phenolic components by HPLC (Kähkönen et al., 2001). On the other side, a linear relationship has been found also between oxygen radical absorbance capability (ORAC) and anthocyanin content among different cultivars of four Vaccinium species (Prior et al., 1998). For ripe berries, a linear relationship existed between ORAC values and anthocyanin content (Wang and Lin, 2000). Total phenolics content was more highly correlated to antioxidant capacity than anthocyanin content (Moyer et al., 2002).

The antioxidant activity of berry phenolics depends not only on the phenolic composition but also on the oxidation model system of choice as well as the oxidation

products monitored. Berry extracts inhibited hexanal formation in LDL in the order: blackberries > red raspberries > blueberries > strawberries, while in lecithin liposomes, the extracts inhibited hexanal formation in the order: blueberries > red raspberries > blackberries > strawberries (Heinonen et al., 1998). That was explained that anthocyanins contributed predominantly to antioxidant activity of berry extract in the LDL oxidation while the amount of hydroxycinnamates was more important and correlated with the activity in the liposome oxidation (Heinonen et al., 1998). The phenolic extracts of lingonberry and bilberry showed best antioxidant protection toward lipid oxidation, followed by black currant and raspberry while bilberry and raspberry performed the best antioxidant activity toward protein oxidation (Viljanen et al., 2004). Six common anthocyanidins and their glycosidic forms were evaluated in three lipid-containing models. [human low-density lipoprotein (LDL) and bulk and emulsified methyl linoleate]. Most anthocyanins and their anthocyanidins acted as strong antioxidants in emulsion methyl linoleate and LDL, comparable to α-tocopherol, Trolox, catechin, and quercetin. In bulk methyl linoleate, anthocyanins and anthocyanidins possessed only a weak antioxidant activity or even oxidation-promoting activity (Kahkonen and Heinonen, 2003).

The relationship between anthocyanin structure and antioxidant capacity has been explored. Glycosylation of the anthocyanidins may be involved in the total antioxidant capacity (Yoshiki et al., 1995; Rice-Evans et al., 1996; Wang et al., 1997). Rice-Evans and co-workers (1996) have shown a general trend of decreasing TEAC by glycosylation (Rice-Evans et al., 1996). However, glucosylation at C-3 and C-5 of

the anthocyanin skeleton have shown an enhancing effect in the chemiluminescence intensity (lipid peroxidation) (Yoshiki et al., 1995). Different sugars may have different effects on the antioxidant activity of an anthocyanin (Wang et al., 1997). In this study, cyanidin-3-glucoside (kuromanin) had the highest ORAC activity, 3.5 times stronger than Trolox (vitamin E analogue), among anthocyanins including the delphinidin, cyanindin, pelargonidin, malvidin, peonidin, and their derivatives with different sugar linkages. Meanwhile, the pelargonin had the lowest antioxidant activity (Wang et al., 1997). Depending on the anthocyanidin, different glycosylation patterns either enhanced or reduced the antioxidant power (Kahkonen and Heinonen, 2003). Although for flavonoids excluding anthocyanins, increasing the number of hydroxyl groups increases the antioxidant capacity (Cao et al., 1997), this extra hydroxylation in delphinidin didn't increase the Trolox equivalent antioxidant capacity in ORAC assay (Wang et al., 1997) but increased the chemiluminescence intensity in the chemiluminescence assay (Yoshiki et al., 1995) compared to cyanidin. Anthocyanins have been shown to chelate metal ions at moderate pH with their ionized hydroxyl groups of the B ring (Satué-Gracia et al., 1997). Cyanidin, with 3',4'-dihydroxy substituents in the B ring and conjugation between the A and B rings, had highly effective radical scavenging structures (Zheng and Wang, 2003). That is the possible mechanism to explain why cyanin showed much better antioxidant activity than pelargonin (Wang et al., 1997).

2.5.3 Cancer Chemoprotective Properties

Carcinogenesis is a multistage process that involves three steps: initiation, promotion and progression (Hong and Sporn, 1997). Phytochemicals in fruits and vegetables are shown to play an important role during the anti-carcinogenesis in numerous studies (Glade, 1999), suppressing any steps of the process (Hou, 2003). Berry fruits have a wild range of phytochemicals, anthocyanins as the major components among them in most berries (Kähkönen et al., 2001). Berry fruits are potential foods to reduce the risk of many cancers not only because of containing many active constituents at a high concentration but also due to the research data from recent cell culture experiments, animal studies, or human intervention studies. The passsible mechanisms related to anticarcinogenese of berries involve in antioxidant activity, detoxification activity, antiproliferation, induction of apoptosis, and anti-angiogenic activity.

2.5.3.1 Antioxidative Activity

When an endogenous and exogenous antioxidant system could not balance off the free radicals generated in human cells, oxidative stress occurs. Oxidative stress can cause oxidative damage to lipids, proteins and DNA. Oxidative modification at oncogenes, tumor-suppressing genes and DNA-repair genes may lead to cancer development (Poulsen et al., 1998). Anthocyanins, the most abundant in berries, possess high antioxidant activity. Rats fed with a diet containing cyanidin 3-glucoside for two weeks were found to significantly decrease the serum the concentration of

thiobarbituric acid reactive substances (TBARS) and be more resistant to the further lipid peroxidation than the control (Tsuda et al., 1998) and also suppressed hepatic ischemia–reperfusion oxidative damage (Tsuda et al., 1999). Delphinidin, cyanidin, and their glycoside and rutinoside derivatives, showed effective against the formation of DNA single strand break and lipid peroxidation in cell cultures (Lazze et al., 2003). Anthocynin-rich extracts from black chokeberry (*Aronia melanocarpa* Elliot), elderberry (*Sambucus nigra* L.) and macqui (*Aristotelia chilensis* (Mol.) Stuntz) were shown to effectively inhibit H_2O_2-induced DNA strand breaks determined in human colon tumor HT-29 cells, but didn't alter the intracellular steady state of oxidized DNA bases (Pool-Zobel et al., 1999). Anthocyanin-rich extract could decrease the hepatic level of 8-Oxo-deoxyguanosine (8-oxodG) and TBARS in Vitamin E deficient rats after 10 weeks of Vitamin E deficient diets followed by 2 weeks supplementation of either this extract or Vitamin E while no effect was observed in Vitamin E sufficient rats (Ramirez-Tortosa et al., 2001). Intake of large amounts of blackcurrant *(Ribes nigrum* L.) juice did not decrease the low steady state levels of oxidative DNA damage in mononuclear blood cells in healthy men and women (Moller et al., 2004). In another study of healthy men, plasma malondialdehyde decreased with time during juice consumption fruit juice abundant in anthocyanins provided by blueberries and boysenberries but no effect on single strand breaks and significantly reduced the oxidative DNA damage in lymphocytes (Bub et al., 2003).

2.5.3.2 Detoxification Activity

Carcinogens are activated by phase I enzymes and inactivated by phase II enzymes, a whole process called as detoxification. The products from a typical phase I reaction are usually more reactive molecules than the parent molecules. If these reactive molecules are not further metabolized by phase II enzymes, they may react with DNA and develop DNA adducts, inducing carcinogenesis. Phase II enzymes include glutathione-S-transferases, UDP-glucuronosyl-transferase, Quinone reductase and others.

The crude extracts, anthocyanin and proanthocyanidin fractions of four Vaccinium species (lowbush blueberry, bilberry, cranberry, and lingonberry) weren't found to be highly active to induce the Phase II detoxification enzyme quinone reductase (QR) while the ethyl acetate extracts were active QR inducers *in vitro* (Bomser et al., 1996). Concord grape juice, rich in anthocyanins, significantly inhibited *in vivo* mammary (7,12-dimethylbenz[a]anthracene) DMBA-DNA adduct formation by 34 and 56%, partially explained by highly increased liver activity of the phase II metabolizing enzyme (glutathione S-transferase) and also inhibited DMBA-induced rat mammary tumorigenesis (Jung et al., 2006).

2.5.3.3 Antiproliferation and Apoptosis Induction

In the healthy state, cell proliferation is balanced by apoptosis. One of the hallmarks of cancer is acquired ability of over-proliferation while resistance to apoptosis is another important hallmark of cancer cells. Therefore proliferation and

apoptosis of cells are critical points to target for chemoprevention of fruits and vegetables (Evan and Vousden, 2001).

Commercially prepared anthocyanin-rich extracts (AREs) from grape (*Vitis vinifera*), bilberry (*Vaccinium myrtillus* L.), and chokeberry (*Aronia meloncarpa* E.) were shown to inhibit the 50% proliferation of human colon tumor cell line (HT-29) at 25-75 μg/mL (equivalents as cyanidin 3-glucoside) after 48 h of treatment, with chokeberry ARE being the most potent inhibitor, whereas they didn't have toxic effect to normal colon cells (NCM460) at AREs concentration range (Zhao et al., 2004b). From this study the varying compositions and degrees of growth inhibition suggest that the anthocyanin chemical structure may play an important role in the growth inhibitory activity of commercially available AREs. The effects of berry fruits to inhibit the growth of neoplastic cells are shown in **Table 2.3**.

Different species and cultivars of berries show different potent biological activities. The ethanol extract from bilberry (*Vaccinium myrtillus*) extract was found to be more effective than lowbush blueberry (*Vaccinium angustifolium*), highbush blueberry (*Vaccinium corymbosum*), cranberry (*Vaccinium oxycoccos*), raspberry (*Rubus idaeus*), and strawberry (*Fragaria* x *ananassa*), black currant (*Ribes nigrum*), red currant (*Ribes sativum*), berry (*Rubus mesogaeus*), cowberry (*Vaccinium vitis-idaea*). The bilberry extract inhibited the growth of HL60 cells and HCT116 cells by 84% and 97% respectively at 4 mg dry wt/mL and induced DNA fragmentation typical of apoptosis in these cells in a dose-dependent manner after 24 h of treatment in HL-60 cells (Katsube et al., 2003). Different cultivars of blueberries was tested for their

Scientific name	Common name	Cell lines	% Inhibition	GI $_{50}$	Reference
Arecaceae					
Serenoa repens (Bartr.) Small	Saw palmetto	Normal prostate cells		>400 nL/mL [d]	(Goldmann et al., 2001)
		267B-1		~20 nL/mL [d]	(Goldmann et al., 2001)
		BRFF-41T		~20 nL/mL [d]	(Goldmann et al., 2001)
		HT-29		200 nL/mL [d]	(Goldmann et al., 2001)
		Jurkat		200 nL/mL [d]	(Goldmann et al., 2001)
		LNCap		200 nL/mL [d]	(Goldmann et al., 2001)
Ericaceae					
Vaccinium corymbosum L.	Blueberry**	HT-29	105 (350 µg/L [a])		(Olsson et al., 2004)
		MCF-7	75 (350 µg/L [a])		(Olsson et al., 2004)
Vaccinium macrocarpon Ait.	Cranberry	$HepG_2$		14.5 mg/mL [b]	(Sun et al., 2002)

Continued

Table 2.3: Reported antiproliferative effects of berries

Table 2.3 Continued

Vaccinium macrocarpon Ait.	Cranberry	KB	60 (200 µg/mL [c])		(Seeram et al., 2004)
		CAL27	8 (200 µg/mL [c])		(Seeram et al., 2004)
Vaccinium myrtillus L.	Bilberry	HT-29		75 µg/mL [a]	(Zhao et al., 2004b)
		HCT116	97 (4 mg /mL [c])		(Katsube et al., 2003)
		HL-60	84 (4 mg /mL [c])		(Katsube et al., 2003)
Vaccinium vitis-idae L.	Lingonberry**	HT-29	85 (64 µg/L [a])		(Olsson et al., 2004)
		MCF-7	102 (64 µg/L [a])		(Olsson et al., 2004)
Grossulariaceae					
Ribes nigrum L.	Black currant**	HT-29	107 (320 µg/L [a])		(Olsson et al., 2004)
		MCF-7	56 (320 µg/L [a])		(Olsson et al., 2004)
Rosaceae					
Aronia meloncarpa E.	Black chokeberry	HT-29		25 µg/mL [a]	(Zhao et al., 2004b)

Continued

Table 2.3 Continued

Aronia meloncarpa E.	Black chokeberry**	HT-29	63 (180 µg/L [a])		(Olsson et al., 2004)
		MCF-7	81 (180 µg/L [a])		(Olsson et al., 2004)
Fragaria × *ananassa* Duch. cv. (Allstar; Annapolis; Earliglow; Evangeline; Jewel; Mesabi; Sable; Sparkle)	Strawberry	$HepG_2$		56 mg/mL [b]	(Sun et al., 2002)
				27; 32; 15; 19; 25; 28; 22; 23 mg/mL [c]	(Meyers et al., 2003)
Rubus idaeus L.	Raspberry**	HT-29	92 (110 µg/L [a])		(Olsson et al., 2004)
		MCF-7	102 (110 µg/L [a])		(Olsson et al., 2004)
Rubus idaeus cv. (Anne; Goldie; Heritage; Kiwigold)	Raspberry	$HepG_2$		33; 13; 15; 14 mg/mL [c]	(Liu et al., 2002)

[a] , concentration of monomeric anthocyanins in media; [b], concentration of total phenolics in media; [c], concentration of mass weight of extracts in media; [d], concentration of extract volume in media; *: oil extract; **: Anthocyanin fraction, Others are phenolic extracts

GI50: the concentration of sample to inhibit 50% of cell growth. CAL27: tongue epithelial carcinoma cells; HepG2: human hepatocellular carcinoma cells; HT-29: human colon adenocarcinoma cells; K562: human chronic myelogenous leukemia cells; KB: mouth epidermal carcinoma cells; MCF-7: human mammary cancer cell; 267B-1 and BRFF-41T: human prostate cancer cell lines; Jurkat and LNCap: human lymphoma cell lines

inhibitory effects on two colon cancer cell lines, HT-29 and CaCo-2, with GI_{50} in the crude extract ranged from 1000 to 3000 μg/mL. The cultivar Briteblue showed the highest potential inhibition than did Tifblue and Powderblue (Yi et al., 2005). Anthocyanins may be most responsible to growth inhibitory effect of anthocyanin-rich berries. Anthocyanin fractions from bilberry were found to decrease the viable cell number by 94% in HL60 cells and 99% in HCT116 cells at a concentration of 400 μg dry wt/mL, more effective than other fractions (Katsube et al., 2003). Anthocyanin fractions were found to show the greatest antiproliferation (> 50% at concentrations of 15-50 μg/mL) than phenolic acids, tannins, flavonols fractions from blueberries and induced the apoptosis in HT-29 and CaCo-2 cells resulted by increment of 2-7 times in DNA fragmentation at 40-80 μg/mL (gallic acid equivalents) compared with no treatment control (Yi et al., 2005).

Anthocyanidins also were studied for effects on cell proliferation. Pure anthocyanidins showed different inhibitory effects in the growth of HL60 and HCT116 cell lines. Their antiproliferation effects were followed in an order: malvidin > delphinidin > peonidin > cyanidin > pelargonidin at 50 ~ 200 μM in HL60 cells, whereas delphinidin > cyanidin > peonidin > pelargonidin > malvidin in HCT116 cells (Katsube et al., 2003). Malvidin showed selective inhibition of different cell lines.

Carcinogenesis is a multistage process that is involved in three steps: initiation, promotion and progression, where signaling transduction pathways were involved in the process development (Hong and Sporn, 1997). In cancer cells, their growth factors and receptors are up-regulated and activated. Besides targeting cell cycles arrest,

inhibition of cancer cells growth also can be evaluated by blocking other signal transduction pathways such as epidermal growth factor receptor (EGFR) and the mitogen activation protein kinases (MAPK). The transcription factor activator protein 1 (AP-1) plays an important role in carcinogenesis by activating transcription of genes involved in cell proliferation (Angel and Karin, 1991). AP-1 activity can be stimulated by growth factor, oxidative stress, or activating mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase or stress-activated protein kinase (JNK/SAPK) and p38 kinase (Huang et al., 1998). NF-κB is an important regulator involving in programmed cell death and proliferation control during carcinogenesis (Baldwin, 1996).

The human HT-29 colon cancer cells treated with semipurified anthocyanin-rich extract from chokeberries (*Aronia meloncarpa* E.) showed a blockage at G1/G0 and G2/M phases of the cell cycle through an increased expression of the p21WAF1 and p27KIP1 genes and decreased expression of cyclin A and B genes (Malik et al., 2003).

Lingonberry (*Vaccinium vitis-idaea* L.) was effective to target signaling transduction pathways, by blocking phosphorylation of ERK1, ERK2, p38, and MEK1/2 induced by either 12-O-tetradecanoylphorbol-13-acetate (TPA) or ultraviolet-B (UVB) in JB6 mouse epidermal cells, and suppressed the activation of AP-1 and nuclear factor-κB (NF-κB) induced by either 12-O-tetradecanoylphorbol-13-acetate (TPA) or ultraviolet-B (UVB), and induced apoptosis in human leukemia HL-60 cells (Wang et al., 2005).

Anthocyanidins showed greater antiproliferation or apoptosis than corresponding glycosidation anthocyanins, the difference among anthocyanidins, however, may attribute to different cell lines applied and principle mechanisms explored in their studies.

Delphinidin inhibited cell growth in uterine carcinoma (HeLa S3 cells) and colon adenocarcinoma cells (CaCo-2 cells) by a reduction of cells in G1 phase, inducing apoptosis with the appearance (Lazze et al., 2004). Three hydroxyl groups on the B ring in the molecular structure of delphinidin may be critical structure to be different from cyanidin in terms of biological activities.

Malvidin was found to be most effective component according chemopreventive properties among five aglycons (cyanidin, delphinidin, malvidin, pelargonidin, and peonidin) and four glycosylated anthocyanins (cyanidin-3-glucoside, malvidin-3-glucoside, pelargonidin-3-glucoside and peonidin-3-glucoside). Malvidin showed the most potent anti-proliferation effect on gastric adenocarcinoma (AGS) cells with cell cycle arrest of AGS cells at the G0/G1 phase and induced apoptosis (Shih et al., 2005). In this study, malvidin increased Bax/Bcl-2 ratio for 1.6-fold of control at 100 μM and significantly induced up-regulation of p38 kinase expression and down-regulation of the ERK activity. However in another interesting study, cyanidin and delphinidin, not malvidin, exerted great inhibitory effect on the growth of human vulva carcinoma cell line A431 *in vitro* by suppressing the epidermal growth-factor receptor (EGFR) and blocking downstream signaling cascades.

Malvidin and the cyanidin 3-galactoside and Malvidin 3-glucoside were inactive up to 100 μmol/L (Meiers et al., 2001).

In another study, six anthocyanidins were also evaluated on inhibition of TPA-induced JB6 mouse epidermal cell transformation and AP-1 transcription activity in an order: dephinidin > petunidin > cyanidin >> pelargonidin > malvidin > peonidin (Hou et al., 2004). Consequently a conclusion could be drawn that an ortho-dihydroxyphenyl structure on the B-ring was the critical structure to suppress this cell transformation and AP-1 activity. Delphinidin blocked the phosphorylation of protein kinases in the ERK pathway and the JNK signaling pathway in a dose-dependent manner.

2.5.3.4 Anti-angiogenic Activity

Angiogenesis is another key regulator for cancer progression, which is defined as new blood vessel formation. Vescular endothelial growth factor (VEGF) is a key stimulator of angiogenesis. VEGF is another target to prevent or threat many cancers (Padhani et al., 2005; Sandler, 2005; Fox, 2006; Yance and Sagar, 2006). Wild blueberry, bilberry, raspberry seeds, and strawberry suppressed significantly both H2O2- and TNF-alpha-induced VEGF expression by human keratinocytes in orders: wild blueberry > raspberry seed > strawberry > bilberry and wild blueberry > bilberry > raspberry seed > strawberry, respectively (Roy et al., 2002). Delphinidin reduced both serum- and vascular VEGF-induced endothelial cell proliferation in bovine aortic endothelial cells by inducing G1 phase arrest of cell cycle through up-regulation of

caveolin-1 and $p21^{WAF1/Cip1}$ and down-regulation of Ras and cyclin D1 (Martin et al., 2003).

2.5.3.5 Animal Study

Anthocyanin-rich extracts showed more inhibitory effect on tumorigenesis in gastrointestinal tract than other sites from animal studies, suggesting low bioavailability caused the difference. Freeze-dried black raspberries at 5 and 10% in the diets were fed to Male Syrian Golden hamsters for 2 weeks prior to treatment with 0.2% 7,12-dimethylbenz(a) anthracene in dimethylsulfoxide reduced significantly the tumor formation in the oral cavity (Casto et al., 2002).

Anthocyanin-rich colorant from purple corn exerted significant inhibitory effect on colorectal carcinogenesis in male F344/DuCrj rats, initially treated with 1, 2-dimethylhydrazine (DMH) and then promoting by 2-amino-1-methyl-6-phenylimidazo pyridine (PhIP) in the diet (Hagiwara et al., 2001).

Freeze-dried strawberries or freeze-dried blueberries, freeze-dried black raspberries were fed on F-344 rats at 5 and 10% freeze-dried strawberries in the diets for 2 weeks before N-nitrosomethylbenzylamine (NMBA) treatmentat. At 25 weeks, 5 and 10% strawberries and black raspberries, not blueberries, were found to significantly inhibit NMBA-induced esophageal tumor multiplicity by 24 and 56% for strawberry and 39 and 49% for black raspberries in the rat esophagus (Aziz et al., 2002; Carlton et al., 2001; Kresty et al., 2001). In post-initiation study, animals fed by 5 and 10% freeze-dried strawberries in the diet only following NMBA treatment

significantly reduced tumor multiplicity by 38 and 31% for strawberries, 62 and 43 % for black raspberries (Carlton et al., 2001; Kresty et al., 2001). A significant decrease in O^6-methylguanine adducts was observed in the esophageal DNA of animals fed with strawberries or black raspberries, but with not blueberries (Aziz et al., 2002; Carlton et al., 2001). Both freeze-dried strawberries and black raspberries were suggested to potentially inhibit both initiation and promotion/progression during NMBA-induced esophageal tumorigenesis.

However, 10% freeze-dried strawberries in the diet fed to the A/J mice didn't suppress lung tumorigenesis induced by the 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) in the A/J mouse (Carlton et al., 2000).

2.5.4 Cardiovasular Diseases

Elevation the level of low density lipoprotein (LDL) and decrease of high density lipoprotein (HDL) level in plasma are associated with atherosclerosis. The lipids in LDL are easily modified by oxidization or aggregation. Oxidized LDL stimulates inflammatory signaling, releasing chemokines and cytokines such as M-CSF and MCP-1 and recruiting monocytes into the arterial wall (Silva et al., 2002). The oxidized LDLs are recognized and internalized by monocytes and macrophages in the vascular endothelium, a key event to develop atherogenic plaque (Esterbauer et al., 1997; Kaul, 2001). A growing body of research demonstrated phenolics and related polyphenolic compounds inhibit the *in vitro* and *in vivo* oxidation of LDL by donation

of a hydrogen to free radicals with the formation of stable intermediates (Shahidi and Naczk, 2004).

Blackberries (*Rubus fructicosus* cv Chester), highbush blueberries (*Vaccinium corymbosum* cv Jersey), red raspberries (*Rubus idaeus* cv Tulameen) and strawberries (*Fragaria ananassa* cv Chandler) showed great inhibition of hexanal formation in human LDL and lecithin liposomes in orders respectively: blackberries > red raspberries > blueberries > strawberries and blueberries > red raspberries > blackberries > strawberries at 10 mol/L of gallic acid equivalents (Heinonen et al., 1998). The aqueous extract of bilberries (*Vaccinium myrtillus*) showed potent protection of LDL against copper-meidated oxidation *in vitro* in many approaches: significant prolongation of the lag-phase of conjugated diene production; reduction in the formation of lipoperoxides and of thiobarbituric acid-reactive substances (Laplaud et al., 1997). Juice of Maquei (*Aristotelia chilensis* (Molina) Stuntz), rich in anthocyanins, was effective in inhibiting copper-induced LDL oxidation and protected from hydrogen peroxide-induced oxidative stress in human endothelial cell cultures in dose-dependant manner (Miranda-Rottmann et al., 2002).

In human intervention studies, intake of berries didn't shown significant reduction of LDL at all. Consumption of 100 g of blackcurrants, bilberries, and lingonberries in healthy 60-year-old men for 8 weeks couldn't show significant inhibition of LDL compared to groups which supplied daily 100 mg of α-tocopherol and 500 mg of ascorbic acid or 500 mg of calcium gluconate instead of berries

(Marniemi et al., 2000). Sea buckthorn (*Hippophaë rhamnoides* L.) juice and placebo randomly given to twenty healthy men showed no significant changes according to plasma lipids, LDL oxidation, platelet aggregation and plasma soluble cell adhesion protein concentration (Eccleston et al., 2002). In a more recent study, twenty healthy female aged 18-40 y supplemented with cranberries juice by about 750 ml/day or placebo for 14 days didn't significantly change the levels of plasma total cholesterol, triglycerides, HDL and LDL and activities of the glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) (Duthie et al., 2006). Anthocyanins or catechins weren't detectable in plasma or urine but plasma vitamin C increased significantly. However, intake of chokeberry for two months in pregnant women was found significantly to reduce the level of oxidized LDL compared to that before treatment (Pawlowicz et al., 2000).

Endothelin-1 (ET-1), a 21-amino acid, plays an important role in the atherosclerotic lesion formation. Nitric oxide, a vasodilating factor, is synthesized and released by endothelium to regulating vascular homeostasis (Taddei et al., 2001). Delphinidin showed the great potential to inhibit the atherosclerotic lesion formation by elevating the level of inducible nitric oxide synthase and decreasing both protein and mRNA level of ET-1 in a dose-dependent manner compared with cyanidin. Dephinidin was also found to suppress the growth of human umbilical vein endothelial cells (HUVECs). Three hydroxyl groups on the B ring suggest to be functional structure that delphinidin exerted greater antioxidant activities than did cyanidin

(Lazze et al., 2006). Delphinidin reduced both serum- and vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation in bovine aortic endothelial cells, which may be important in preventing plaque development and stability in atherosclerosis (Martin et al., 2003).

Peroxynitrite, a toxic oxidant formed from the reaction of NO and superoxide (Koppenol et al., 1992; Squadrito and Pryor, 1995), has been demonstrated in various forms of shock, and may exert cytotoxic effects to the endothelial cells, cause cellular injury and smooth muscle cells, and involve DNA single strand breakage, the activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS) (Packer and Murphy, 1995) (Crow and Beckman, 1995; Elliott, 1996; Szabo et al., 1996). Blackberry (*Rubus sp*) juice and cyanidin-3-O-glucoside (as chloride) at level of 14.5-80 ppm and 0.0085 - 0.085 μM of cyanidin-3-O-glucoside significantly reduced the peroxynitrite-induced suppression of mitochondrial respiration, DNA single strand breakage and PARS activation in HUVECs and also prevented both the peroxynitrite-mediated endothelial dysfunction and suppression of vascular contractility induced by peroxynitrite in endothelium-intact vascular rings (Serraino et al., 2003).

2.5.5 Anti-inflammation

Some berries were found to be able to be good nonsteroidal anti-inflammatory drug to inhibit cyclooxygenase-II (COX II), which catalyze arachidonic acid in to prostaglandin, prostacylin and thromboxane A2. COX-II is inducible enzyme by mitogens, cytokines and bacterial lipopolysacchride *in vitro* and *in vivo* (Mitchell et al.,

1994). Bilberries (*Vaccinum myrtillus* L.), blackberries (*Rubus sp*.), blueberries (*Vaccinium corymbosum* L. cv Jersey), cranberries (*Vaccinium macrocarpon* Ait. cv Early Black), elderberries (*Sambucus canadensis*), raspberries (*Rubus idaeus* L.) and strawberries (*Fragaria* × *ananassa* Duch. cv Honeoye) were shown to inhibit cyclooxygenase about 10% to 45% of COX-I and 5% to 45% of COX-II at 125 μg/ml of anthocyanins, some of them comparable to positive controls: naproxen and ibuprofen, which possessed 47.5% and 54.3% of COX-I and 39.8% and 41.3% of COX-II at 10 μm (Seeram et al., 2001). Blackberries (*Rubus sp*.), strawberries (*Fragaria x ananassa* Duch. cv Honeoye) and elderberries (*Sambucus canadensis*) showed more COX-II inhibitory effect than to COX-I, suggesting they could be potential anti-inflammatory agents as pain-reliever of arthritis and gout-related pain due to the selectively inhibition of COX-II activity.

2.5.6 Antimutation

The mechanism of chemoprevention involves that blocking carcinogenesis and stopping the initial step in the formation of cancer when mutation happens to oncogenes, a tumor suppresser gene, or DNA repair genes. Fresh juices from the fruits of strawberry, blueberry, and raspberry were found to inhibit mutagenesis caused by direct-acting mutagen methyl methanesulfonate and metabolically activated carcinogen benzo[α]pyrene with S. typhimurium strain TA100 as the test organism (Smith et al., 2004).

2.5.7 Obesity

A range of berry phenolics such as flavonols, anthocyanidins, ellagitannins and proanthocyanidins after ingestion of berries such as raspberries and strawberries, inhibited digestive enzymes (α-glucosidase, α-amylase, protease, and lipase), which is a clinical therapic target for controlling type II diabetes and obesity. Among these phenolics, anthocyanins inhibited α-glucosidase activity, therefore reduced blood glucose levels. Meanwhile ellagitannins showed the potential for synergistic effects with anthocyanins to inhibit α-amylase activity. Proanthocyanidins may contribute primarily to inhibit gastrointestinal lipase activity and limit fat digestion after meals (McDougall and Stewart, 2005).

In contrast, consumption of elderberry spray-dried extracts with a high fat meal showed no significant reduction in serum lipids and improvement in antioxidative capacity compared with the group consumption of a high fat meal without elderberry extracts. A higher dose was suggested to improve reduce postprandial serum lipids for future study (Murkovic et al., 2004). Grape anthocyanins were found to inhibit the development of obesity by decreased lipid accumulation in mature adipocytes *in vitro* (Lefevre et al., 2006).

2.5.8 Immune System

Berry foods also report to improve immune system in clinical studies. Juice rich in anthocyanins provided by berries like blueberries and boysenberries, supplemented to healthy men, was found out to increase lymphocyte proliferative

responsiveness. Moreover cytokines (interleukin-2) secretion by activated lymphocytes and the lytic activity of natural killer (NK) cells were significantly enhanced (Bub et al., 2003).

2.5.9 Eye Health

In a systematic review of placebo-controlled trials related to effect of bilberry on night vision, 8 of 12 placebo-control trials reported bilberry (*Vaccinium myrtillum*) positive impact to night vision (Canter and Ernst, 2004). In contrast, bilberry supplements of 160 mg bilberry extract (25% anthocyanins) weren't found to improve night vision in young male at a dose of 3 pills /day for 21 days (Muth et al., 2000). Many factors could affect on the outcome of trails, such as methodology, dose level and extracts geographically variable in anthocyanin composition (Canter and Ernst, 2004). Bilberries have benefits to eye health not only by improving night vision but by treatment of glaucoma and retinopathy (Cluzel et al., 1969; Mian et al., 1977).

Chokeberries *(Aronia melanocarpa)* showed inhibitory effect to ocular inflammation by suppressing of the expression of the inducible nitric oxide synthase and COX-2 enzymes and consequently reducing of the production of lipopolysaccharide-induced nitric oxide (NO), prostaglandin (PG)-E2, and TNF-α in rats (Ohgami et al., 2005).

2.5.10 Neurological Function

High intake of fruits and vegetables may reduce the risk of degenerative disorders, such as Alzheimer's disease (Cantuti-Castelvetri et al., 2000). Blueberry was reported to be effective to improve age-related deficits in neuronal signaling and behavioral parameters after 8-week 2% blueberry in diets in F344 rats. Several anthocyanins (cyaniding 3-galactoside, cyaniding 3-glucoside, cyaniding 3-arabinose, malvidin 3-galactoside, malvidin 3-glucoside, malvidin 3-arabinose, peonidin-3-arabinose and delphinidin 3-galactoside) were found to be able to cross the blood brain barrier and localize in various brain regions in these rats after dietary blueberry for 8-10 weeks in these rats (Andres-Lacueva et al., 2005). In another study, freeze-dried blueberries and bilberries were shown to improve short-term memory of rats at a dose of 3.2 mg/kg per day for 30 days (Ramirez et al., 2005).

2.6 BIOAVAILABILITY

As mentioned earlier in chapter 2 section 2.5.2-(5) , anthocyanin-rich berries more inhibitory effect on tumorigenesis in the gastrointestinal tract than in other sites according to animal studies, suggesting low bioavailability may cause the difference of biological activities (Carlton et al., 2000; Carlton et al., 2001; Kresty et al., 2001; Casto et al., 2002; Aziz et al., 2002). Anthocyanins occurred in plasma and urine in quite small amount after intervention of a large amount of anthocyanin-rich berries.

Bioavailability of anthocyanin pattern with diverse molecular structure and a variety of glycosylation from different dietary sources (blueberry, boysenberry, black

raspberry, and blackcurrant) was confirmed by detection of intact and unmetabolized anthocyanins in urine of male Sprague-Dawley rats and males. The total amount of anthocyanin excreted as a percentage of the amount consumed is less than 0.1% for all anthocyanins (McGhie et al., 2003). The urinary excretion of strawberry anthocyanins from strawberries corresponded to 1.8% of pelargonidin-3-glucoside consumed by humans (Felgines et al., 2003), while total urinary excretion of blackberry anthocyanin was 0.16% of the amount of anthocyanins ingested (Felgines et al., 2005). Four major native anthocyanidin glycosides of blackcurrant juice were detected in human plasma and urine and total anthocyanins excreted was less than 0.133% of total anthocyanins ingested (Rechner et al., 2002). Average concentrations of anthocyanins and anthocyanin metabolites in human urine reached to 17.9 μmol/L within 5 h and detected in the serum were observed at 5917 nmol/ within 2h after consumption of 20g chokeberry extracts (1.3g cyanidin-3-glycosides) (Kay et al., 2004). Anthocyanins were detected in glycosylated form in plasma and urine after a consumption of 720 mg anthocyanins by elderly women, the concentration can reach up to 168 nmol/L in plasma (Cao et al., 2001).

Individual anthocyanin concentration in urine appeared to be different from in berry extracts, suggesting that absorption and excretion of anthocyanins are influenced by structure of the conjugated sugar, especially for single glucose moiety (McGhie et al., 2003). Anthocyanins with either a di- or trisaccharide moiety were excreted in the urine primarily as the intact form in higher percentage of ingested doses than intact forms of monoglycoside (Wu et al., 2005), that can be partially explained by a larger

proportion of the anthocyanin rutinosides than of the glucosides absorbed into blood stream (Nielsen et al., 2003).

Anthocyanins can be metabolized via methylation, glucuronidation, and sulfoconjugation after absorption in weanling pigs which had a single meal with a freeze-dried powder of chokeberry, black currant, or elderberry. Anthocyanins with different aglycons were found to be metabolized differently in bodies. Metabolites of of delphinidin anthocyanins were not detected, whereas anthocyanins of cyanidin were metabolized by methylation, glucuronidation or both (Wu et al., 2005). In a human study, the metabolites were identified as glucuronide conjugates, as well as methylated and oxidised derivatives of cyanidin 3-galactoside and cyanidin glucuronide in urine after consumption of chokeberry extracts (Kay et al., 2004).In the urinary excretion of strawberry consumed, except the pelargonidin-3-glucoside itself, five metabolites were found in urine samples, including three monoglucuronides of pelargonidin, one sulfoconjugate of pelargonidin and pelargonidin, among which monoglucuronide was more than 80% (Felgines et al., 2003). Besides native cyanidin 3-glucoside, several other anthocyanin metabolites were detected in the human urine: methylated glycosides, glucuronides of anthocyanidins and anthocyanins, a sulfoconjugate of cyanidin, and anthocyanidins after consumption of blackberries, among which monoglucuronides of anthocyanidins were major metabolite in urine (>60% of excretion) (Felgines et al., 2005). Malvidin-3-glucoside was detected in plasma and urine after ingestion of red wine, dealcoholized red wine, and red grape juice while its

metabolites including the aglycon, sulfate or glucuronate conjugates were not detected in plasma and urine (Bub et al., 2001).

Metabolism by intestinal microflora and transformation of pure anthocyanins, anthocyanins extracts from radish, and the assumed degradation products, were evaluated in models to mimic *in vivo* conditions (Fleschhut et al., 2006). Glycosylated and acylated anthocyanins were rapidly degraded by the intestinal microflora after anaerobic incubation with a human fecal suspension with an increase of degradation product protocatechuic acid. Anthocyanins were glucuronidated, but no hydroxylation and demethylation, by cytochrome P450 enzymes from rat liver microsomes in the presence of the cofactor NADPH (Fleschhut et al., 2006). The hydroxycinnamates, conjugated and free ferulic, isoferulic, p-coumaric, sinapic and vanillic acids were identified in plasma and urine after consumption of blackcurrant juice (Rechner et al., 2002).

The reasons that anthocyanins have such a low bioavailability are not clear yet. A possible reason is that anthocyanins could not be hydrolyzed by β-glucosidase in the GI tract, resulting in a low absorption into the blood stream (Nemeth et al., 2003). Another possible reason is the degradation of anthocyanins due to neutral and mild alkaline condition in intestines. However, one study found that anthocyanins after metabolism in intestinal microflora after 14-day intake of anthocyanin-rich extracts from chokeberries, bilberries or grapes were abundant in rat feces (0.7/1.8/2.0 g/kg wet feces for chokeberry/ bilberry/ grape, respectively) (He et al., 2005). Food matrix may not exert a negative impact on anthocyanin stability after injection. In humans the

absorption and urinary excretion of anthocyanins from blackcurrant juice were found to be proportional with dose and not influenced by the ingestion of a rice cake (Nielsen et al., 2003). Food ingredients such as protein may enhance anthocyanin stability *in vivo*.

2.7 ANTHOCYANIN-RICH COMMODITIES AND COLORECTAL CANCER

Anthocyanin is most abundant flavonoids in dietary with a daily consumption of about 200 mg/person (Kühnan, 1976), much higher than the average intake of other flavonoids was 23 mg/person totally (Hertog et al., 1993). Anthocyanins are aboundant in many berries, purple carrot, purple corn, red radish, red cabbage, and other red, purple or blue plant foods. They may contribute to cancer preventive potentials of fruits and vegetables which are associated with cancer risk from epidemiological studies (Glade, 1999).

From recent *in vitro* and *in vivo* studies anthocyanin-rich foods and anthocyanin pigments have been suggested as potential foods or food ingredients to reduce the risk of colon cancer. Anthocyanin-rich foods showed great antiproliferation of human colon cancer cells *in vitro*. Anthocyanin-rich berries including blueberries, black currant, black chokeberries, lingonberries, cherries, and raspberries suppressed the proliferation of HT29 in a dose-dependant manner (Olsson et al., 2004). Anthocyanins or anthocyanin-rich extracts from black chokeberry (*Aronia melanocarpa* Elliot) and elderberry (*Sambucus nigra* L.) were shown to suppress DNA single strand break in human tumor HT29 clone 19A cells (Pool-Zobel et al.,

1999). Anthocyanin-rich extracts from grape (*Vitis vinifera*), bilberry (*Vaccinium myrtillus* L.), and chokeberry (*Aronia meloncarpa* E.) were shown to inhibit the 50% proliferation of human colon tumor cell line (HT-29) at 25-75 μg/mL (equivalents as cyanidin 3-glucoside) after 48 h of treatment, with chokeberry ARE being the most potent inhibitor, whereas they didn't have toxic effect to normal colon cells (NCM460) at the same concentration range (Zhao et al., 2004b). From this study the varying compositions and degrees of growth inhibition suggest that the anthocyanin chemical structure may play an important role in the growth inhibitory activity of commercially available anthocyanin-extracts.

Anthocyanins may play an important role in the growth inhibitory effect of anthocyanin-rich berries or extracts. Anthocyanin fractions from bilberry were found to decrease the viable cell number by 94% in HL60 cells and 99% in HCT116 cells at a concentration of 400 μg dry wt/mL, more effective than in other fractions. Pure glycosidation of delphinidin and malvidin isolated from the bilberry extract, inhibited HL60 cells via the induction of apoptosis and while only delphinidin glycoside inhibited the growth of HCT116 cells (Katsube et al., 2003). Anthocyanin fractions were found to show the greater antiproliferation (> 50% at concentrations of 15-50 μg/mL) than any of phenolic acids, tannins, flavonols fractions from blueberries and induced the apoptosis in HT-29 and CaCo-2 cells resulted by increment of 2-7 times in DNA fragmentation at 40-80 μg/mL (gallic acid equivalents) compared with no treatment control (Yi et al., 2005). Anthocyanin fraction from red wine also showed

much higher antiproliferation of HCT15 cells than other flavonoid fractions (Kamei et al., 1998b).

Several *in vivo* studies also have shown that anthocyanin-rich foods and anthocyanin pigment from food are potent inhibitor of carcenogense in the colon (Hagiwara et al., 2001a; Harris et al., 2001; Kang et al., 2003a; Aoki et al., 2004a). Purple corn color was found to modify the mutagenicity induced by the food mutagen 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) in Salmonella typhimurium TA 98 and inhibited the reverse mutation induced by PhIP in the presence of rat liver microsomal activation systems in a dose dependent manner (Aoki et al., 2004). Cyanidin 3-glucoside was demonstrated to reduce the genotoxicity of PhIP, indicating that this anthocyanin pigment is the active components (Aoki et al., 2004). The lyophilized black raspberries inhibited on azoxymethane-induced aberrant crypt foci, colon tumors, and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in male Fischer 344 rats (Harris et al., 2001). The potential of purple corn color showed the potential to reduce colorectal carcinogenesis in male F344/DuCrj rats, initially treated with 1, 2-dimethylhydrazine (DMH) and then promoting by 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) in the diet (Hagiwara et al., 2001). Anthocyanins from tart cherry and cyanidin were found to reduce cecal adenomas significantly in Apc (Min) mice relative to the control group without any anthocyanin administration. The IC_{50} values of anthocyanins and cyanidin were 780 and 63 μmoL/L for HT-29 cells, 285 and 85 μmoL/L for HCT116 cells respectively (Kang et al., 2003).

Total extracts from cranberries (*Vaccinium macrocarpon* Ait.) showed greatest inhibitory effect on HT-29 and HCT116 cells relative to fractions, suggesting an additive or synergistic interaction of anthocyanins, proanthocyanidins, and flavonols (Seeram et al., 2004).

However, the relationship of anthocyanin structure to their chemoprevention has not been well established. Varying composition of anthocyanins results in a different extent of growth inhibition of HT29 cells, suggesting that anthocyanin chemical structure may play an important role in the growth inhibitory activity (Zhao et al., 2004). Different sources of anthocyanins and the presence of glycosylations might affect the inhibition of HCT-15 cancer cell growth (Koide et al., 1997; Kamei et al., 1998; Zhang et al., 2005). Anthocyanidins showed more inhibitory effect than glycosylated anthocyanins. Cyanidin-3-glucoside, cyanidin-3-galactoside, delphinidin-3-galactoside and pelargonidin-3-galactoside anthocyanins did not inhibit cell proliferation of HCT-116 cell lines tested at 200 μg/mL, while malvidin and pelargonidin inhibited HCT-116 cell growth by about 76 %, 63%, respectively, at 200 μg/mL (Zhang et al., 2005). Anthocyanin-rich extracts from red soybeans where the major anthocyanins were cyanidin of 3-glucoside and 3- rhamnoside), not extracts from red beans where cyanidin 3- rhamnoside was the main anthocyanin, showed great inhibition on the growth of HCT-15 cells, suggesting cyanidin conjugated with glucose may show much better inhibition than cyanidin conjugated with rhamnose, although cyanidin showed growth inhibitory (Koide et al., 1997). Also, chemical structure has been correlated with antimutagenic activity, with acylation of

anthocyanin with organic acids markedly increasing antimutagenecity of anthocyanin pigments as compared with the deacylated counterparts (Yoshimoto et al., 2001). The methods of preparation of anthocyanin extracts affected greatly the biological activity (He et al., 2005).

2.8 HEALTH BENEFITS OF ANTHOCYANIN-RICH COLORANT OF PURPLE CORN

Recent studies have found that purple corn has higher antioxidant capacity and antiradical kinetics than blueberry which is well known to possess a high antioxidant capacity (Cevallos-Casals and Cisneros-Zevallos, 2003), reduce the systolic blood pressure of spontaneously hypertensive rats (Toyoshi and Kohda, 2004), and contribute to the prevention of obesity and diabetes (Tsuda et al., 2003). Purple corn color was found to modify the mutagenicity induced by the food mutagen 2-amino-1-methyl-6-phenylimidazo pyridine (PhIP) in Salmonella typhimurium TA 98 and inhibited the reverse mutation induced by PhIP in the presence of rat liver microsomal activation systems in a dose dependent manner (Aoki et al., 2004). Cyanidin 3-glucoside that is the major anthocyanin purified from purple corn color was demonstrated to reduce the genotoxicity of PhIP, indicating that this anthocyanin pigment is the active components (Aoki et al., 2004). The potential of purple corn color showed the potential to reduce colorectal carcinogenesis in male F344/DuCrj rats, initially treated with 1, 2-dimethylhydrazine (DMH) and then promoting by 2-amino-1-methyl-6-phenylimidazo (Aoki et al., 2004) pyridine (PhIP) in the diet (Hagiwara et al., 2001). There is a patent in Japan for the use of anthocyanins with the

aglycone of cyanidin and purple corn aqueous extracts to prevent and even treat cancers, especially colon cancer (Shirai et al., 2002).

Different sources of anthocyanins and the presence of glycosylations might affect the inhibition of HCT-15 cancer cell growth (Koide et al., 1997; Kamei et al., 1998). The methods of preparation of anthocyanin extracts greatly affected the biological activity (He et al., 2005). Also, chemical structure has been correlated with antimutagenic activity, with acylation of anthocyanin with organic acids markedly increasing antimutagenecity of anthocyanin pigments as compared with the deacylated counterparts (Yoshimoto et al., 2001).

2.9 TOXICOLOGY

Anthocyanins have been consumed by humans for many years without any apparent adverse effects. The oral toxicity of anthocyanins was greater than 20 mg/kg/day for rats and no significant toxic effects were found in dogs with a diet containing 15% anthocyanins (Francis, 1987). Cyanidin chloride was not mutagenic when examined in the Ames assay using Salmonella typhimurium strain TA-98 (MacGregor and Jurd, 1978). Cyanidin and delphinidin were inactive in the Ames assay system using 5 different strains of Salmonella typhimurium (TA-1535, TA-100, TA-1537, TA-1538 and TA-98) (Brown and Dietrich, 1979). Anthocyanins were not found to induce any response in both the Ames test using Salmonella typhimurium TA-1538 for mutagenicity and in another *in vitro* test employing E. coli Wf2 for induction of DNA damage (Haveland-Smith, 1981).

2.10 POTENTIAL APPLICATION

2.10.1 Natural Colorants

Europe takes nearly 50% of global natural colorant sales. The American market accounts for about 30% of world sales, followed by Japan at about 20%.(Business-Communications Company, 1 April 2003) In the United States, FD&C Red No. 40, a certified dye, has the highest per capita consumption. With consumers more interested on healthier lifestyles, the food industry is searching for natural alternatives to the use of synthetic dyes. Although grape is the only berry source listed among the food colors exempt from certification (regulated by FDA in 21CFR 73.170 and 21CFR 73.169), many berries such as cranberry, elderberry, chokeberry, bilberry, blueberry, and red raspberry can be used to provide color in the form of a juice concentrates to provide color and to contribute pleasant flavor. The raw materials most suitable for pigment production are the residue or pomace that remains after juice extraction. Different countries will have different restrictions on terms of the regulatory aspects of colorants for foods. In the Europen Union anthocyanins are allowed as colorants, and listed by their E-number (E163). Berry concentrates would be considered as ingredients and not listed as colorants. In the USA, fruit and vegetable juice concentrates are among the approved colorants exempt from certification. However, only physical means of extraction and concentration of the pigments and water as extracting solvent would be allowed in order to fit into the juice

concentrate category. Therefore, a berry concentrate used as a colorant for foods would be listed in the label as a colorant.

The primary requirement for the successful commercial use of anthocyanins-based colorants on food matrixes is low pH. With increases in pH and a consequent decrease in acidity, the protonation of the anthocyanin molecule is lost, and chemical transformations occur, with a resulting loss in color and stability. In addition to pH sensitivity, anthocyanins are susceptible to degradation by light, heat, oxygen, hydrogen peroxide, iron, copper, and ascorbic acid.(Delgado-Vargas and Paredes-Lopez, 2003)

Anthocyanin colorants have been suggested for beverages which represent the biggest market of commercial applications in the United States. Berry powders or concentrates are recommended for jellies, jams, preserves, ice cream, yogurt, gelatin desserts, fruit sauces, candy and confections, chews, bakery fillings, toppings, and pastries.(Decas-Botanical-Synergies, 2005; ARTEMIS-International, 2006; Brownwood-Acres-Foods, 2006)

2.10.2 Value-added Functional Food

There is an increased market for functional foods or nutraceutical and consumers are interested in foods that may help to prevent or reduce the incidence of illness. The market value of anthocyanin containing commodities is presently valued at several billion dollars. The market value of strawberries alone for the year 2000 was $1,013,537,000, while the market value of blueberries was $220,883,000.(USDA,

2000) Optimizing health and performance through the diet is believed to be one of the largest and most lucrative markets in the US. A number of studies point out a relationship between consumption of anthocyanin-rich berries and improved health. Berries have been proven to have high antioxidant power(Prior et al., 1998; Wang and Lin, 2000a; Moyer et al., 2002; Lee et al., 2004) and may prevent or delay the onset of major degenerative diseases of aging including cancer (Stoner et al., 1999; Carlton et al., 2001; Kresty et al., 2001; Casto et al., 2002; Zhao et al., 2004), cardiovascular diseases (Heinonen et al., 1998; Pawlowicz et al., 2000; Miranda-Rottmann et al., 2002), obesity and diabetes (McDougall and Stewart, 2005), cognitive dysfunction (Andres-Lacueva et al., 2005; Ramirez et al., 2005), immune system (Bub et al., 2003) and night blindness (Canter and Ernst, 2004). Thus anthocyanin-rich berry extracts or concentrates are suggested for value-added functional food in a variety of food applications, such as chews and beverages (Decas-Botanical-Synergies, 2005; ARTEMIS-International, 2006). Berry extracts or concentrates are value-added ingredients useful for functional beverages, bars, candies or other foods which claim to contain high ORAC capacity, enhance immune system, and reduce physical effects of stress derived from exercise or extreme work conditions (ARTEMIS-International, 2006). Anthocyanin-rich berry extracts or concentrates are also being commercialized as dietary supplements. Bilberry dietary supplement is intended to provide nutritive support for normal, healthy eyes and circulation (Solaray-company, 2006). Blueberry dietary supplement is a product to maintain a healthy urinary tract and enhance brain function (Brownwood-Acres-Foods, 2006). Cranberry supplements contains

concentrated cranberry juice in a capsule form, is commonly recommended to prevent urinary tract infections (Cran-Max, 2006; Solaray-company, 2006). Elderberry capsules are suggested to promote immune-system function (Solaray-company, 2006), which helps the body fight and recover from colds and flu. Therefore berry extracts or concentrates, rich in anthocyanins can be value-added ingredients for in a variety of functional food or nutraceuticals to meet the increasing demand of consumers for better and healthy life style.

A 100% organic cranberry powder made from the whole cranberry without carriers that is certified by the United States Department of Agriculture (USDA) and National Organic Program (NOP) was introduced to market in 2005. The applications of cranberry powder include nutritional supplements, cranberry-enriched sports drinks, fortified nutrition bars, dental hygiene products, healthy snacks and weight management products and cosmetics, but also be used as a natural colorant or as a natural flavor (Decas-Botanical-Synergies, 2005)

Therefore purple corn anthocyanin-rich extract is potent to be natural food colorant as well as value-added ingredients for a variety of functional foods and nutraceuticals to meet the increasing demand of consumers for a healthy life style.

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CHAPTER 3

ANTHOCYANINS IN PURPLE CORNCOB (*ZEA MAYS* L) [a] [b]

3.1 ABSTRACT

Purple corn (*Zea mays* L.) has been used for centuries as a natural food colorant in South America, and more recently in Asia and Europe. However, limited information is available on factors affecting anthocyanin profiles and concentration in this material. In this study, purple corns grown under different conditions in Peru were evaluated for quantitative and qualitative anthocyanin composition as well as for the level of total phenolics and the ratio of anthocyanins to total phenolics. The

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monomeric anthocyanins in purple corncobs ranged from 290 to 1323 mg/100g of dry matter while the total phenolics ranged from 950 to 3516 mg/100g of dry matter, among the 18 samples evaluated. Based on these numbers, 30.5 to 47.1% of the total phenolics were anthocyanins. The major anthocyanins present were cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-(6”-maloylglucoside), pelargonidin-3-(6”-maloylglucoside), peonidin-3-(6”-maloylglucoside) and the percent of acylated anthocyanins ranged from 35.59 to 53.98% of total monomeric anthocyanins. Neither potassium sources or concentrations treatments on the soil nor seedling density had a significantly effect on the anthocyanin composition. Growing location had a significant impact on the anthocyanins levels and the percentage of anthocyanins to total phenolics (*P<0.0001*), and should be taken into account when choosing a material for color production.

KEYWORDS: purple corncob; anthocyanins; solubility; location; processing; waste

3.2 INTRODUCTION

Anthocyanins are a class of flavonoid compounds responsible for the bright attractive orange, red, purple, and blue colors of most fruits and vegetables. There is considerable demand in the world for natural food colorants as a result of both legislative action and consumer concerns over the use of synthetic additives in their foods. Anthocyanins are a potential alternative to the use of FD&C Red No. 40, which is the synthetic dye with the highest consumption in the United States (Giusti and

Wrolstad, 2003). However, the use of natural colorants such as anthocyanins from natural sources is challenging. Anthocyanins usually exert low tinctorial power and therefore large doses are needed to reproduce similar results to those obtained with small amounts of synthetic dyes, making them an expensive alternative. In addition, anthocyanins are sensitive to light, pH, and neucleophilic agents, therefore they have limited stability in food matrices as compared to synthetic dyes. Identification of low cost anthocyanin-rich sources with increased stability is very important to their practical applications in food industries.

Purple corn (*Zea mays* L.) is rich in anthocyanins. For centuries, purple corn has been cultivated in South America, mainly in Peru and Bolivia, and used to prepare drinks and desserts traditionally. Interest in purple corn as a source of anthocyanins as colors and phytonutrients has increased over the past years. Recently, anthocyanins from purple corn have been associated with possible health benefits. They have been found to have high antioxidant activity (Cevallos-Casals and Cisneros-Zevallos, 2003), reduce the systolic blood pressure of spontaneously hypertensive rats (Toyoshi and Kohda, 2004), and prevent obesity and diabetes in rats (Tsuda et al., 2003). A purple corn colorant was found to inhibit cell mutation induced by 2-amino-1-methyl-6-phenylimidazo pyridine (PhIP) (Aoki et al., 2004) and to reduce chemically induced colorectal carcinogenesis (Hagiwara et al., 2001).

However, knowledge about functional compounds of purple corn as natural food colorants and phytonutrients is limited and application of purple corn is facing the challenges of poor solubility of anthocyanin-rich extracts from purple corn, large

amount of waste produced during extract preparation, lack of information of how anthocyanin profiles may affect stability, and effects of cultivation conditions on anthocyanin contents and profiles in purple corns.

In this research, anthocyanins and other phenolics in purple corncobs were quantified and identified. Our research should provide information to better understand anthocyanins in purple corncobs and provide fundamental knowledge for cultivation and utilization of purple corncobs as anthocyanin-rich sources for natural food colorant and value-added ingredients.

3.3 MATERIALS AND METHODS

3.3.1 Materials and Reagents

A total of 18 different samples of powdered purple corncob (*Zea mays* L.) were provided by the Universidad Nacional Agraria (La Molina, Peru). There were 18 purple corncob samples that were grown under different conditions. Samples with ID from 1 to 10 grew at 5 different locations and 2 seeding density. Samples with ID from 11 to 18 grew with 4 different potassium concentration and 2 different potassium sources as fertilizers.

Folin & Ciocalteau phenol reagent and the standard of gallic acid (crystalline gallic acid, 98% purity) were purchased from Sigma (St. Louis, MO, USA). All high-performance liquid chromatography (HPLC) grade solvents and other chemicals (analytic grade) were from Fisher Scientific (Fair Lawn, NJ, USA).

3.3.2 Anthocyanin Extraction

About 2 g of each purple corncob powder was added to a flask containing 25 ml of 70% aqueous acetone acidified by 0.01% HCl and mixed well. All flasks were shaken on a platform shaker (LabScientific, Inc., New Jersey, USA) at 80 rpm at 10 ºC for one hour. Samples were filtered through a Whatman No.1 filter paper using a Büchner funnel and the slurry was washed by 10 ml acidified 70% acetone. The filtrate was transferred to centrifuge tubes mixing well with 15 ml of chloroform, centrifuged at 2000 rpm for 10 min at 4 °C and the upper aqueous layer, containing the acetone / water mixture was collected while the chloroform/acetone layer was carefully discarded. Residual acetone and chloroform were removed from the anthocyanin extract by using a rotary evaporator at 40 ºC under vacuum condition. Extracts were taken to 25 ml in volumetric flask by 0.01%-HCl-acidified-water. Every treatment had three replicates.

3.3.3 Monomeric Anthocyanins

The total monomeric anthocyanin content was measured by the pH-differential method (Giusti and Wrolstad, 2001). A Shimadzu UV-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan) was used to measure absorbance at 420 nm, 510 nm, and 700 nm. Monomeric anthocyanins were calculated as cyanidin-3-glucoside, using the extinction coefficient of 26,900 L $cm^{-1}mg^{-1}$, and a molecular weight of 449.2 g/L. Disposable cuvettes of 1 cm path length were used.

3.3.4 Total Phenolics

Total phenolics were measured using a modified Folin-Ciocalteu method (Singleton and Rossi, 1965). The absorbance of the samples and standards was measured at 755 nm. Total phenolics were calculated as gallic acid equivalents based on a gallic acid standard curve.

3.3.5 Analytical Chromatography

A high performance liquid chromatograph (HPLC) system (Waters Delta 600 systems, Milford, MA, USA) equipped with a photodiode array detector (Water 996, Milford, MA, USA.), autosampler (Waters 717 plus, Milford, MA, USA), and Millennium^{32} software (Waters Corp., Milford, MA, USA) was used.

Columns and mobile phase: The reversed-phase 5 µm Symmetry C18 column (4.6×150mm, Waters Corp., Milford, MA, USA) fitted with a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corp., Milford, MA, USA) was used. The solvents used were A: 1% phosphoric acid / 10% acetic acid / 5% acetonitrile in water and B: 100% acetonitrile. Solvents and samples were filtered though 0.45 µm poly(tetrafluorothylene) membrane filters (Pall Life Sciences, MI, USA) and 0.45 µm polypropylene filters (Whatman Inc., Clifton, NJ), respectively. Separation was achieved by using a linear gradient from 0 to 30% A in 35 min. An injection volume of 50 µL with a 1 ml/min flow rate was used. Spectral information over the wavelength range of 260-600 nm was collected.

3.3.6 LC/MS/MS

Mass spectrometry was conducted on a triple quadrupole ion-tunnel mass spectrometer (Quattro Ultima, Micromass Limited, Manchester, UK) equipped with a Z-spray ESI source. Calibration of the mass spectrometer was performed using sodium iodide and caesium iodide. Instrument control and data analysis were accomplished using Masslynx V3.5 software. Approximately 1/10 of the HPLC eluate separated by a micro-splitter valve (Upchurch Scientific, Oak Harbor, WA) were delivered to the ESI source. Precursor-ion analysis by scanning the precursors of all six anthocyanidins, including cyanidin (MW 287), delphinidin (MW 303), malvidin (MW 331), peonidin (MW 301), pelargonidin (MW 271), and petunidin (MW 317) and product ion analyses were conducted to screen all the anthocyanins present in the sample. Typical settings of the quadrupole analyzer were as follows: capillary voltage, 3.0 kV; cone voltage, 35 V; RF lense 1, 50 V; desolvation gas temperature, 400 °C at a flow of 17 L/min; source temperature, 105 °C; collision gas (argon) pressure, 7 psi; collision energy was in the range of 25 ~ 35 eV.

3.3.7. Statistical Design and Analysis

The comparison of the 18 purple corncobs was carried out as a Randomized Complete Block Design (RCBD). Each block was performed as a batch which contained duplicates of 18 samples. The least significance tests (LSD) was conducted to evaluated mean differences in a general linear univariate model. All analyses were

performed by SPSS (version 14.0, SPSS Inc., Illinois, USA) software. For all statistics, $P<0.05$ was considered to be statistically significant.

3.4 RESULTS AND DISCUSSION

3.4.1 Anthocyanins and Total Phenolics in Purple Corncobs

There were 18 purple corncob samples that were grown under different conditions. Samples with ID from 1 to 10 grew at 5 different locations and 2 seeding density (**Table 3.1)**. Samples with ID from 11 to 18 grew with 4 different potassium concentration and 2 different potassium sources as fertilizers (**Table 3.2**). The monomeric anthocyanins content ranged from 290 to 1323 mg cy-3-glucoside equivalents / 100g of dry matter while total phenolic content ranged from 950 to 3516 mg/ 100g of dry matter as gallic acid equivalents among the 18 samples. Based on these numbers, 30.5 to 47.1% of the total phenolics were anthocyanins.

Therefore a high level of anthocyanin and percentage of anthocyanins in total phenolics would be ideal for purple corn colorant processing and application. Compared with literature reports, the levels of monomeric anthocyanins in purple corn cobs in this experiment was close to 504 to 1473 mg/ 100g in kernel pericarp (Moreno et al., 2005) but was lower than the literature report which was about 1640 mg/ 100g in purple corn (Cevallos-Casals and Cisneros-Zevallos, 2003).

Purple corncobs which ID number was from 1 to 10 were cultivated at different places and different density of seeding (**Table 3.1**). The seeding density did

not show significant effect on the anthocyanin contents in purple corncobs ($p=0.905$). The growth places (Canta, Cajamarca, Arequipa, La Molina and Pacaran – all in the coast of Peru), however, had significant impact on anthocyanins levels ($p<0.0001$). The purple corncobs grown in Arequipa contained the lowest level of anthocyanins ($P<0.05$). The purple corncobs which cultured in Cajamarca had higher level of anthocyanins and percentage of anthocyanins to total phenolics than in other growing locations ($p<0.05$). Anthocyanin levels in purple corncobs cultivated in Canta, La Molina and Pacaran were not significantly different. There were no significant differences in the percentages of anthocyanins to total phenolics among purple corncobs that were cultivated in Canta, Arequipa, La Molina and Pacaran.

Purple corncobs whose identification number was from 11 to 18 were cultivated at different levels of potassium (0 to 120 kg/ha) or different potassium salts (potassium sulfate or potassium chloride) (**Table 3.2**).

ID	Place	Seeding density	Monomeric anthocyanins (mg/100g)	Total phenolics (mg/100g)	Monomeric anthocyanins of total phenolics (%)	Acylated anthocyanins (%)
1	Canta	D2	355 (9.3)	1105 (36)	32.32 (1.76)	37.11
2	Canta	D1	506 (25)	1360 (149)	37.21 (3.99)	42.29
3	Cajamarca	D2	515 (2.6)	1202 (35)	47.09 (5.36)	53.98
4	Cajamarca	D1	584 (25)	1373 (90)	42.85 (4.39)	49.26
5	La Molina	D2	513 (25)	1420 (57)	36.21 (1.87)	35.59
6	La Molina	D1	519 (18)	1431 (87)	36.41 (1.28)	36.90
7	Arequipa	D2	290 (24)	950 (162)	30.31 (2.99)	36.20
8	Arequipa	D1	384 (22)	998 (19)	38.63 (3.02)	40.24
9	Pacaran	D1	376 (80)	1014 (29)	37.28 (1.49)	39.08
10	Pacaran	D2	519 (9)	1375 (47)	37.87 (1.27)	42.71

Table 3.1: The properties of 18 samples of purple corncob with monomeric anthocyanins, and total phenolics. Values are represented as mean (standard error) (n=4)

ID	Samples	Monomeric anthocyanins (mg/100g)	Total phenolics (mg/100g)	Monomeric anthocyanins of total phenolics (%)	Acylated anthocyanins (%)
11	O-K	1092 (63)	3223 (215)	33.89 (2.04)	38.11
12	60-K	1114 (108)	3240 (247)	34.40 (1.43)	40.18
13	90-K	1002 (163)	2860 (160)	35.11 (1.95)	39.73
14	120-K	1011 (156)	2910 (240)	34.55 (3.77)	41.38
15	SK-60	1193 (113)	3330 (296)	35.86 (1.39)	39.71
16	SK-120	1333 (124)	3445 (260)	38.58 (3.03)	37.40
17	CK-60	1323 (57)	3516 (236)	37.58 (3.49)	38.56
18	CK-120	1153 (94)	3237 (244)	35.49 (2.10)	39.86

Table 3.2: The effect of potassium on levels of anthocyanins and total phenolics in purple corncobs. The 0, 60, 90, and 120 were the potassium treatment concentration from 0, 60, and 120 kg/ha. SK and CK were potassium sulfate and potassium chloride at two concentrations. Values are represented as mean (standard error) (n=4)

Potassium phosphate has been reported to enhance anthocyanin synthesis in 'Fuji' apple callus at 10 mmol/L but decrease anthocyanin concentration when treatment concentration was increased (Li et al., 2004). And the combination of fertilizer (nitrogen, phosphorous, and potassium) and growth regulator was found to increase the anthocyanin content on roselle plants (Al-Badawy et al., 1996). However, in our study, we did not find a significant difference on the anthocyanin concentration of purple corncopbs receiving different concentration or forms of potassium salt applied.

3.4.2 Anthocyanin Profiles in Purple Corns

The major anthocyanins in purple corncobs were identified and showed in **Figure 3.1** and **Table 3.3**. With published literature (Pascual-Teresa et al., 2002; Aoki et al., 2002), they were including cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-(6”-maloylglucoside), pelargonidin-3- (6”-maloylglucoside), peonidin-3- (6”-maloylglucoside). Except these six anthocyanins, there were other three peaks (peak 3, peak 7, and peak 8) appearing the maximum absorbance wavelength around 490 ~ 530 nm (**Figure 3.1**)**.** The results of MS analysis for purple corncobs were showed in **Table 3.3**. They were identified as cyanidin-3-malonylglucoside, pelargonidin-3-malonylglucoside, and peonidin-3-malonylglucoside, where the malonyl acylation was at different position instead of the C-6” position of glucoside. Cyanidin/ pelargonidin/ peonidin-3-(6”-ethylmalonyglucoside) were found in a commercial purple corn colorant by Pascual-

Teresa and coworkers (2002). However, these pigments were not found in the samples we analyzed. Similarly, a dimalonyl derivative of cyanidin reported in purple corn kernels by Aoki and coworkers (2002) was not found in our samples.

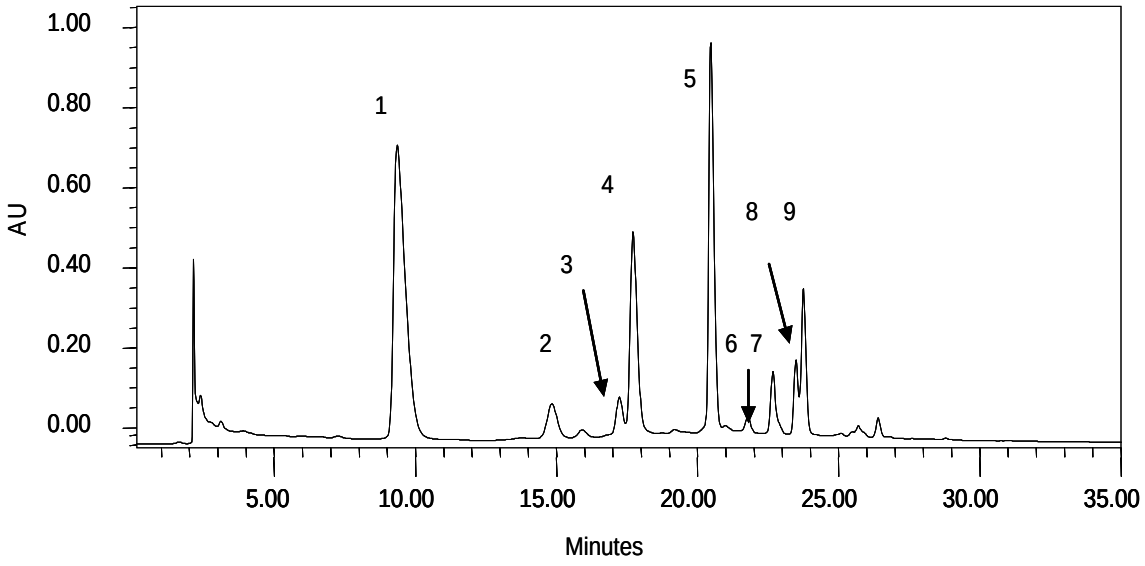

Figure 3.1: Anthocyanins profiles of purple corncob (*Zea mays* L.)

Peak	Compound	m/z	
		[M + H]+ *(amu)*	Fragments [M + H]+ *(amu)*
1	Cyanidin-3-glucoside	449	287
2	Pelargonidin-3-glucoside	433	271
3	Cyanidin-3-malonylglucoside	535	287
4	Peonidin-3-glucoside	463	301
5	Cyanidin-3-malonylglucoside	535	287
6	Pelargonidin-3-malonylglucoside	519	271
7	Pelargonidin-3-malonylglucoside	519	271
8	Peonidin-3-malonylglucoside	549	301
9	Peonidin-3-malonylglucoside	549	301

Table 3.3: MS spectral data for the purple corncob anthocyanins

The proportion of acylated anthocyanins in purple corncobs varied from 35.59 to 53.98% of total monomeric anthocyanins (**Table 3.1** and **Table 3.2**). Acylation of sugar substitution with aliphatic acids donates electrons to chromophores and leads to a bathochromic shift and a hyperchromic effect (von Elbe and Schwartz, 1996; Giusti et al., 1999) and also contributes to an important stabilizing effect on anthocyanins via

intermolecular interaction (Giusti and Wrolstad, 2003). Consequently, acylated anthocyanins with increased stability may impart desirable color and stability for commercial food products (Giusti and Wrolstad, 2003). Hence not only the total monomeric anthocyanins content and the ratio of anthocyanins and total phenolics, but also the percentage of acylated anthocyanins should be considered for purple corn colorant which can provide more desirable color and stability for food products.

3.5 CONCLUSIONS

Purple corncobs are rich in anthocyanins, the level of which may be affected by the growth location but not by seeding density, potassium concentration and sources in this study. Meanwhile, the content of acylated anthocyanins in purple corn should be considered because the range of acylated anthocyanins in purple corn varied greatly and the level of acylated anthocyanins may affect the color property and stability of purple corn colorant when applied in food matrices.

3.6 ACKNOWLEDGMENTS

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CHAPTER 4

EFFECT OF EXTRACTION METHODS ON ANTHOCYANIN AND MACROMOLECULE COMPLEXATION IN ANTHOCYANIN-RICH EXTRACTS FROM PURPLE CORN (*ZEA MAYS* L.) [a] [b]

4.1 ABSTRACT

Purple corn (*Zea mays* L.) rich in anthocyanins is an economic source for anthocyanin colorants or functional ingredients. However, high levels of waste in purple corn are generated during the processing purple corn colorant. Our objective was to optimize the anthocyanin extraction method to reduce purple corn waste, which has limited solubility that is associated with high level of macromolecules. Different

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extraction solvents (deionized water, 0.01%-HCl-acidified water, 0.01%-HCl-acidified-ethanol, and 70% aqueous acetone), and temperatures (room temperature, 50, 75, and 100 °C) were investigated while levels of anthocyanins, tannins, and proteins were evaluated in the final extracts. In addition, the effects of initial extraction duration (20 min and 60 min) and consecutive re-extraction procedures (up to 5 times) were determined by use of 70 % acetone. Deionized water at 50 °C achieved a high yield of anthocyanins with relative low levels of tannins and proteins, comparable to the anthocyanin yield obtained by 70% acetone. The pH (1 to 7) of water had impact on the amount of monomeric anthocyanins, tannins, and proteins in anthocyanin-rich extraction and the lower pH lead to a lower yield of protein (R= 0.704, $p<0.001$). Inactivation of polyphenol oxidases by heat did not improve anthocyanin yield. Consecutive re-extraction procedures with 70% acetone increased total phenolics with little increase in anthocyanins yield. Increasing the extraction time from 20 to 60 minutes with 70% acetone had little effect on the extraction of anthocyanins but caused a significantly higher extraction of other phenolics. A neutral protease was effective to decompose the major protein (29KD) from the extract determined by SDS-PAGE, suggesting an application of neutral proteases might decrease the level of proteins and therefore reduce waste.

Our study suggests that use of water at mild temperatures (50 ºC) for pigment extraction from purple corn could contribute to obtain high concentrations of anthocyanins with lower amounts of other compounds (phenolics and proteins) correlated with precipitated waste production. Extraction time and consecutive re-

extraction procedures should be considered in aspect of high efficiency and purity of anthocyanins for specific samples. Application of neutral protease during extraction may alleviate the formation of complexes between anthocyanins and purple corn proteins.

KEYWORDS: purple corncob; anthocyanins; extraction solvent; tannin; protein

4.2 INTRODUCTION

Anthocyanins are the largest and most important group of water-soluble pigments in nature, contributing to the attractive orange, red, purple, violet and blue colors of fruits, vegetables, and flowers. Anthocyanins, which have been consumed for many years without any apparent adverse effects, have bright pH dependent colors (Mazza and Miniati, 1993). Interest in anthocyanins has increased due to its color properties, and healthy benefits as a promising alternative as food additives (Giusti and Wrolstad, 2003). Close to 25% of the population perceives foods without artificial ingredients as desirable, being this very important in their food and beverage purchase decisions in United States (Sloan, 2005). Recently, anthocyanins have been reported to have various potential health benefits such as antioxidant capacity (Ghiselli et al., 1998; Noda et al., 2000; Prior, 2003; Wang and Lin, 2000), anti-mutagenic activity (Gasiorowski et al., 1997; Peterson and Dwyer, 1998), and chemopreventive activity (Koide et al., 1997; Zhao et al., 2004), contributing to a reduced incidence of chronic diseases. Researchers have shown that an anthocyanin-based food colorant from

purple corn was able to inhibit cell mutation (Aoki et al., 2004), reduce chemically induced colorectal carcinogenesis (Hagiwara et al., 2001), and prevent of obesity and diabetes (Tsuda et al., 2003).

Purple corn (*Zea mays* L.), rich in anthocyanins, has been cultivated in South America, mainly in Peru and Bolivia, and used to prepare drinks and desserts for centuries. A colorant from purple corn is widely used in Asia, South America and Europe. Cyanidin 3-glucoside is the major anthocyanins in purple corncob (Nakatani et al., 1979; Styles and Ceska, 1972). Glucoside of cyanindin, pelargonidin, and peonidin were found in maize plants and their respective malonyl derivatives (Aoki et al., 2002; Pascual-Teresa et al., 2002).

Large quantities of anthocyanin-rich waste (ARW) are generated during the preparation of an anthocyanin colorant, which are obtained by an easy and economic processing of hot-acidified-water extraction from purple corncob. This waste has limited application in foods due to its low solubility in acidified aqueous system. Protein and other complex molecules such as tannins exist in purple corncobs, which may form complexes with anthocyanins resulting in a loss of solubility under typical conditions of anthocyanin food applications. In this study, we will study optimization of the extraction conditions to decrease the yield of tannins and protein and to maximize the yield of anthocyanins, therefore reducing the generation of purple corn waste during the processing of purple corn colorant.

4.3 MATERIALS AND METHODS

Purple corncobs (*Zea mays* L.) were donated by Globenatural International S.A. (Chorrillos-LIMA, Peru). The dry purple corncob was crashed into coarse particles and milled into powder by PERTEN laboratory mill 3600 (Perten Instruments Inc., IL, USA).

4.3.1 Effect of Solvent and Temperature on Anthocyanin Extraction

One gram of each sample powder was added to 25 ml of deionized water, 0.01%-HCl-acidified water, 0.01%-HCl-acidified ethanol, or 70% aqueous acetone. The mixtures in water and 0.01%-HCl-acidified-water were shaken in a NBS C76 Water Bath Shaker (New Brunswick Scientific, NJ, USA) at 80 rpm at different temperatures (room temperature, 50 ºC, 75 ºC and 100 ºC) for 1 hour, while other two solvents were used only at room temperature. The resulting extracts were filtered through a Whatman No.1 filter paper using a Büchner funnel. The filtrates were transferred into a centrifuge tube, mixed with 10 ml of chloroform, and centrifuged at 2000 rpm for 10 min at 4 °C. The chloroform layer was discarded carefully. Residual chloroform with acetone/ ethanol was removed using a rotary evaporator at 40 ºC under vacuum, and the solution was taken back to 25 ml with 0.01%-HCl-acidified-water. Every treatment was done in duplicates.

4.3.2 Effect of pH on Anthocyanin Extraction

One gram of each sample was added to 25 ml of deionized water at pH levels of 1, 2.5, 4, 5.5, and 7 and shaken in a NBS C76 Water Bath Shaker at 80 rpm and 50 ºC for 1 hour. The anthocyanin extracts were filtered through a Whatman No.1 filter paper using a Büchner funnel. The filtrate was then transferred to centrifuge tubes mixing well with 10 ml of chloroform and centrifuged at 2000 rpm and 4 °C for 10 min. Then, the chloroform layer was carefully discarded. Anthocyanin extracts were added up to 25 ml by 0.01%-HCl-acidified-water. Every treatment had three replicates.

4.3.3 Effects of Polyphenol Oxidase Activity on Anthocyanins Stability

Anthocyanin extracts of purple corncob (*Zea mays* L.) obtained with 70% aqueous acetone at room temperature (step 4.3.1) were distributed into 15 ml centrifuge tubes and put in boiling water for 5 min and then cooled down in ice immediately which was recommended to inhibit PPO (Rodriguez-Saona and Wrolstad, 2001). Then anthocyanin extracts with/without the 5-min heat treatment were incubated in water bath at 37 °C and collected about 0.5 ml at time intervals (0, 8, 19, 29, 43, and 67 hr) and put in -18 °C to store for the monomeric anthocyanin analysis. Every treatment had four replicates.

4.3.4 Optimal Time and Consecutive Re-extraction Procedures

One gram of purple corncob powder was added to 25 ml of 70% (v/v) aqueous acetone in a flask. The mixture was stirred at room temperature for 20 min (**Procedure 1**) or 60 min (**Procedure 2**) and filtered by the Whatman No. 1 filter paper using a Büchner funnel under vacuum condition. The cake after filtration was re-extracted using 15 ml of 70% aqueous acetone solvent for 10 min until no more color could be obtained (a total of 4 times).. The acetone was removed using a rotary evaporator at 40 ºC under vacuum. Every treatment was done in duplicate.

4.3.5 Monomeric Anthocyanins

The total monomeric anthocyanins content was measured by the pH-differential method (Giusti and Wrolstad, 2001). A Shimadzu UV-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan) was used to measure absorbance at 510 nm and 700 nm. The total monomeric anthocyanins were calculated as cyanidin-3-glucoside, using the extinction coefficient of 26,900 L $cm^{-1}mg^{-1}$, a molecular weight of 449.2gL^{-1}. Disposable cuvettes of 1 cm path length were used.

4.3.6 Total Phenolics

Total phenolics were measured using a modified Folin-Ciocalteu method (Singleton, 1965). The absorbance of the samples and standards was measured at 755 nm. Total phenolics were calculated as gallic acid equivalents.

4.3.7 Protein Analysis

Total protein concentration was measured by PIERCE BCA (bicinchoninic acids) protein assay kit purchased from Fisher Scientific (Fair Lawn, NJ). The absorbance of the samples, standards of bovine serum albumin (BSA), and controls (samples in deionized water instead of BCA reagent B) were measured at 562 nm. Anthocyanin color background was deducted from the final sample color. Total protein was quantified as BSA equivalents, based on a standard curve of 0, 25, 125, 250, 500, 750 and 1000 μg/ml of BSA.

4.3.8 Tannins Analysis

Tannins were determined by the protein precipitation method (Hagerman and Bulter, 1978). Briefly, 1 ml of BSA was mixed well with 0.5 ml sample at pH 4.9 and sat for 16 hours at room temperature, then centrifuged for 8 min at 1.3 x 10^4 rpm. The pellet was reconstituted into 2 ml SDS/ triethanolamine (TEA) (1 % w/v SDS, 5 % v/v TEA). And then 0.5 ml of 0.01 mol/L $FeCl_3$ solution was added to form the colored ion-phenolate complex. After 15 min standing, absorbance of samples was measured at 510 nm.

4.3.9 Enzyme Hydrolysis and SDS Analysis

Multifect neutral enzyme (Genencor International, Inc. Rochester, NY), a food grade enzyme derived from bacteria, has optimal conditions at pH 7 and 40-60 ºC. Enzeco fungal acid protease (Enzyme Development Corporation, New York, NY), has optimal conditions at pH 3 and 50-60 ºC. Every 100 mg of purple corn powder with

Multifect neutral enzyme or Enzeco fungal acid protease was added up to 2 ml in microcentrifuge tubes by pH 7 deionized water or pH 3 water acidified by HCl, incubating in a shaking water bath at 50 °C for 2 hr and then centrifuged at 1.3 x 10^4 rpm for 5 min at 4 °C. The supernatant was transferred from the tubes to volumetric flask, added up to 10 ml by deionized water for SDS-PAGE analysis.

Extracts or multicolored protein markers (6.5-205 kD) (PerkinElmer Life And Analytical Sciences, Inc. Boston, MA, USA) were prepared by mixing samples well with the same volume of SDS-PAGE sample buffer (3 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 62.5 mmol/L Tris/HCl) to dilute one fold, and then heated at 95 ºC for 3 min and cooled down to room temperature. Denatured extracts (50 μL) or multicolored protein markers (5 μL) were loaded in 10 % to 20 % gradient gel (BioWhittaker Inc., MD, USA) and run at 150 v for 75 min. Proteins that were not decomposed by proteases above were visualized by double-staining with Coomassie blue.

4.3.10 Analytical Chromatography

HPLC system (Waters Delta 600 systems) equipped with a photodiode array detector (Water 996), autosampler (Waters 717 plus), and Millennium^{32} software (Waters Corp.) was used. Columns and mobile phase: A reversed phase 5 μm Symmetry C18 column (4.6 mm × 150 mm, Waters Corp.) fitted with a 4.6 mm × 22 mm Symmetry 2 microguard column (Waters Corp.) was used. The solvents used were A, 1% phosphoric acid/10% acetic acid/5% acetonitrile in water, and B, 100%

acetonitrile. Solvents and samples were filtered through 0.45 μm poly(tetrafluorothylene) membrane filters (Pall Life Sciences, MI, USA) and 0.45 μm polypropylene filters (Whatman Inc., NJ, USA), respectively. Separation was achieved by using a linear gradient from 0 to 30% A in 30 min. An injection volume of 50 μL with a 1 ml/min of flow rate was used. Spectral information over the wavelength range of 260-600 nm was collected.

4.3.11 Statistical analysis

A least significance test (LSD) was performed in general linear univariate model to identify differences among means. Pearson correlation was conducted to evaluate if there were any linear correlation between the pH value in anthocyanin extraction and any of monomeric anthocyanins, tannins, and proteins. All analyses were performed by SPSS (version 14.0, SPSS Inc., Illinois, USA) software. For all statistics, $P<0.05$ was considered to be statistically significant.

4.4 RESULTS AND DISCUSSION

4.4.1 Effect of Extraction Solvent and Temperature on Yields of Anthocyanins, Tannins and Proteins

Samples extracted from different solvents (deionized water, 0.01%-HCl-acidified water, 0.01%-HCl-acidified ethanol, and 70% aqueous acetone) at different temperatures (room temperature, 50 ºC, 75 ºC and 100 ºC) for 1 hr were analyzed for monomeric anthocyanins, tannins, and proteins (**Figure 4.1**). The concentration of monomeric anthocyanins extracted at 50 ºC in deionized water and acidified water was

0.94 and 0.84 mg/100 mg (dry mass), respectively, significantly higher than those at other temperature conditions (room temperature, 75 ºC and 100 ºC) with same exaction solvents (all $p<0.05$, except the comparison of conditions of 50 ºC with 75 ºC with acidified water). High temperature renders the plant cell wall permeable and increases solubility and diffusion coefficient of compounds and decrease the viscosity of solvents, thereby a resulting improvement of the efficiency of the extraction. However, anthocyanins are sensitive to heat and easily convert to the colorless chalcone form during the heating (Wrolstad et al., 2002). The yields of monomeric anthocyanins extracted by 0.01%-HCl-acidified water (pH≈3) were lower than those by water (pH≈7), but not significantly ($p>0.05$) at room temperature, 50 ºC, 75 ºC and 100 ºC. The concentration of tannins and proteins in anthocyanin extracts obtained with deionized water were higher than the corresponding by acidified water, specifically tannins content in extracts obtained by deionized water significantly higher than the corresponding obtained by acidified water ($p<0.05$).

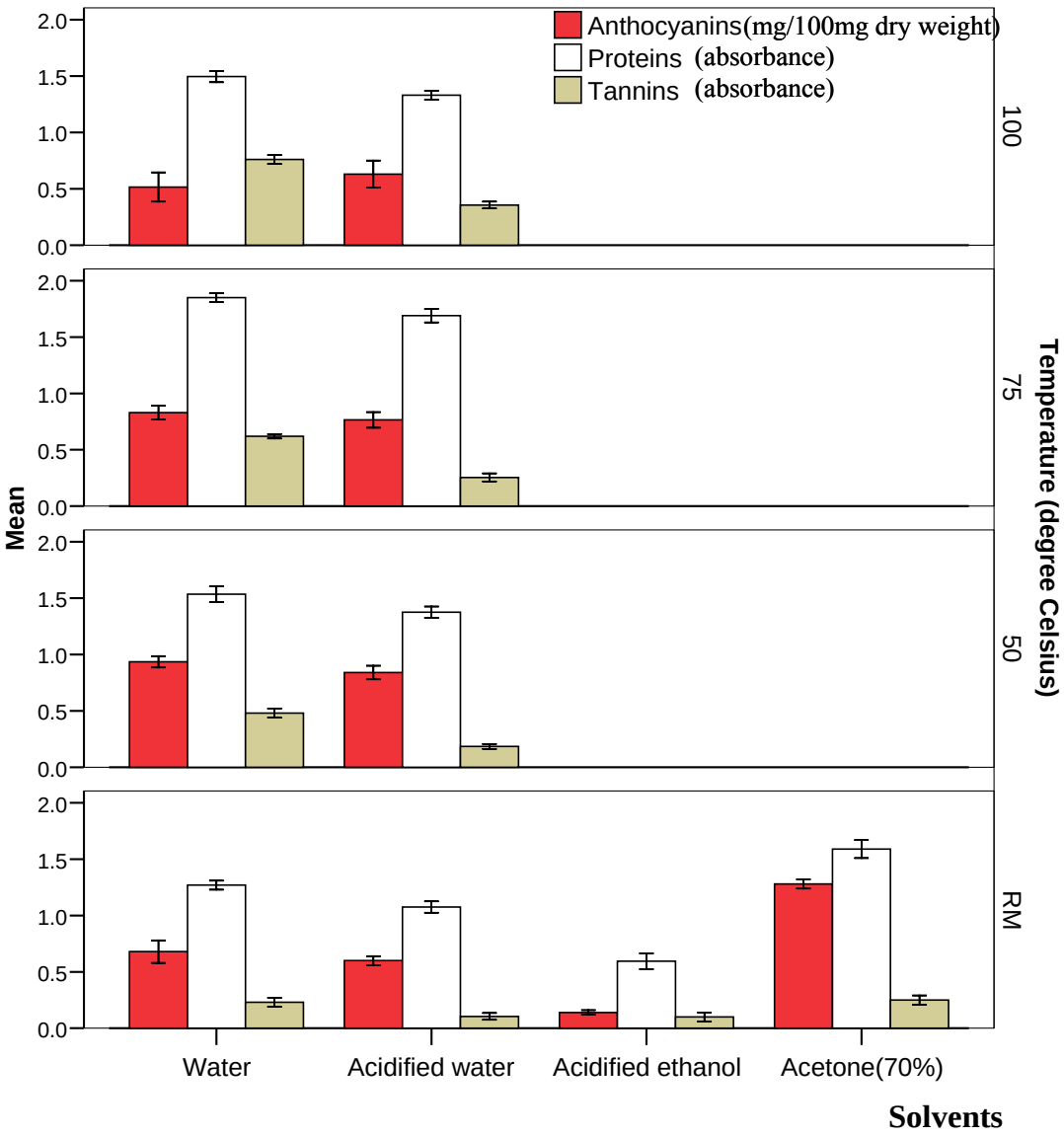

Figure 4.1: Concentrations of monomeric anthocyanins, tannins and protein of the extracts by different extraction methods. Extracting solvents are deionized water, 0.01%-HCl-acidified water, 0.01%-HCl-acidified ethanol, and 70% aqueous acetone at room temperature (RM), 50, 75 and 100 °C for 1 hr. Extracts were analysis for monomeric anthocyanins, tannins, and protein after one hour extracting. Values are represented as Mean ± Standard error (n=2).

Acidified ethanol was not the ideal solvent due to the yield of monomeric anthocyanins significantly lower than others ($p<0.05$) although the yields of proteins and tannins were significantly lower than others too ($p<0.01$). Acetone (70%), a typical solvent to be applied in anthocyanin extraction in laboratories, achieved a significantly highest yield of anthocyanins (0.98 ± 0.08 mg/100 mg corncob, $p<0.01$) with relatively low tannin and protein content. Deionized water was a good and economic solvent with a high yield of monomeric anthocyanins (about 0.94 ± 0.03 mg/100 mg corncob) at 50 °C, however, produced a little higher tannins and proteins than 70% aqueous acetone ($p<0.01$).

Hence water without acidification would be the ideal solvent to achieve a higher yield of anthocyanin extract comparing to other solvents at 50 °C. However, considering the level of tannins and proteins, water with acidification would be good alternative to achieve lower yields of proteins and tannins in price of a little lower yield of anthocyanins.

4.4.2 Effect of pH environment on Anthocyanin-Rich Extraction

Whether water was acidified or not as an extraction solvent had impact on the yields of monomeric anthocyanins, tannins, and proteins. Effect of pH condition on anthocyanin extraction was further evaluated for the levels of monomeric anthocyanins, tannins, and proteins in anthocyanin-rich extracts (**Figure 4.2**).

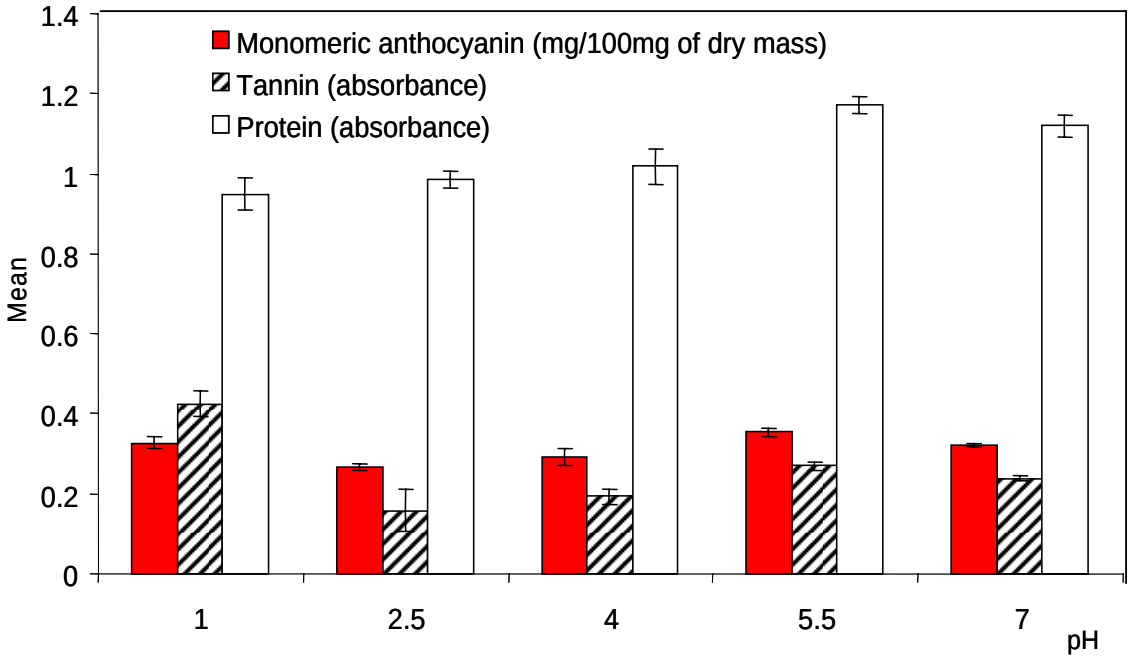

Figure 4.2: Concentration of monomeric anthocyanins, tannin and protein in extracts obtained by water acidified to different pH values. *, $p < 0.05$. Values are represented as Mean ± Standard error (n=3).

The pH during extraction did not show any linear correlation with the levels of monomeric anthocyanins and tannins while showed linear correlation to protein content (R= 0.704, $P<0.001$). The amount of monomeric anthocyanins in the anthocyanin-rich extract obtained by pH 5.5 water showed the highest, significantly higher than in those obtained by pH 2.5 and pH 4 water ($P<0.01$). However, acids are usually used in anthocyanin extraction because the flavylium form of anthocyanins is more stable and the positive charge increases polarity (Wrolstad et al., 2002). Except

the anthocyanin-rich extract obtained by pH 1 contained significantly higher amount of tannins than others ($P<0.01$), others extracts obtained by the pH levels of 1, 2.5, 4, 5.5, and 7 water did not show significant difference. The tannin content in anthocyanin-rich extracts obtained from water at pH 5.5 and 7 was significantly higher than others ($P<0.05$). Anthocyanins are more electrophilic at acid environment due to the charged C-ring and could easily co-pigment in a π-π interaction with nucleophilices such as tannins (Waterhouse, 2002), which can easily precipitate with protein, so developing the anthocyanin complexes with tannins and proteins at acid condition. The acid may contribute to the development of the precipitable complex of anthocyanins, tannins and proteins, consequently decreasing levels of anthocyanin, tannins, and proteins present in final extracts after filtration and centrifugation. However, in **Figure 4.2**, the levels of tannins and monomeric anthocyanins did not show any linear correlation with pH conditions.

4.4.3 Effects of Polyphenol Oxidase Activity on Anthocyanins Stability

Polyphenol oxidase (PPO) may cause the anthocyanins degradation in fruits or fruit products such as strawberries (Wesche-Ebeling and Montgomery, 1990) and blueberry juice (Skrede et al., 2000). There were no literatures to report the effect of purple corn PPO on anthocyanin stability so far. However in this study, the inactivity of PPO did not show improvement of anthocyanin stability but accelerated the anthocyanin degradation (**Figure 4.3**). The treatment group with heat inactivation of PPO in purple corn anthocyanin-rich extracts and the control group which did not

receive the heat treatment were not significantly different during the storage at 37 ºC from 0 to 29 hr. However, when storage time was up to 43 and 67 hr, the treatment group even showed significantly lower anthocyanins than the control group. The reasons were still unexplainable.

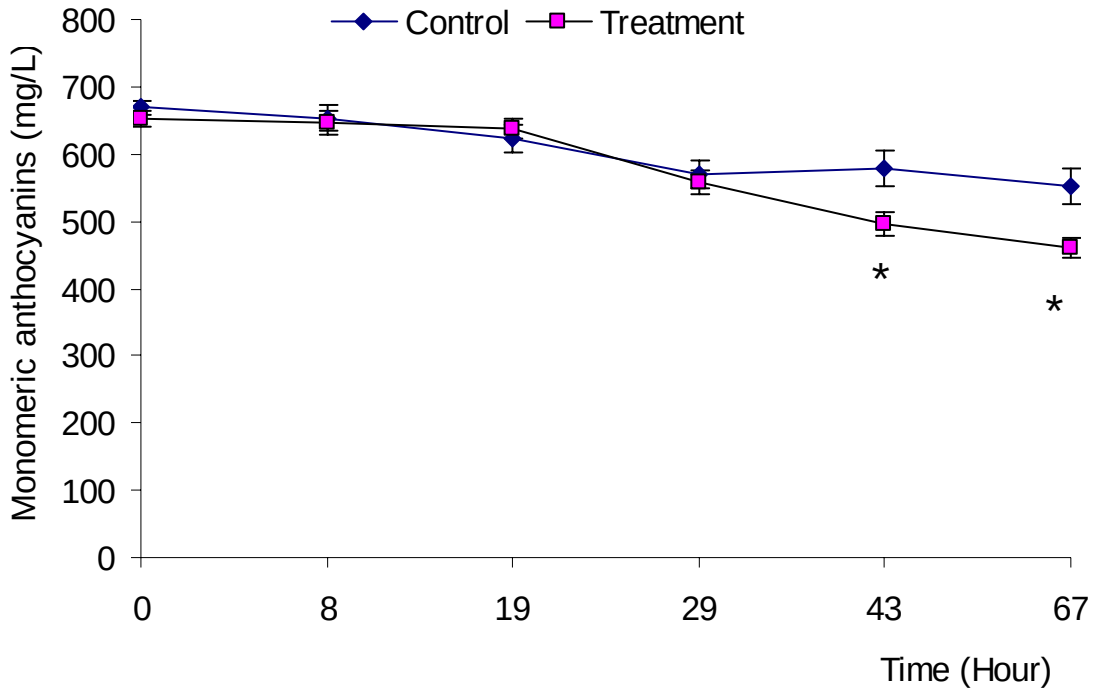

Figure 4.3: Effect of heat treatment on stability of anthocyanins. Student T test was performed to compare the mean differences between the control and treatment group at every time interval. *, $p<0.05$. Values are represented as Mean ± Standard error (n=4).

4.4.4 Optimal Time and Consecutive Re-extraction Procedures

Consecutive re-extraction had effect on the anthocyanin purity of total phenolics. Anthocyanins and phenolics were extracted with 70% aqueous acetone for 60 or 20 min (**Fraction 1**) followed by four re-extraction procedures of 10 min each with acidified 70% aqueous acetone (**Fractions 2-4**) in **Figure 4.4**. Monomeric anthocyanins and total phenolics were measured after each extraction. The forth and fifth extractions did not contribute much to the total monomeric anthocyanins and total phenolics. Extraction with 70% aqueous acetone for 60 min followed by re-extraction (4 times x 10 min) yielded around 18% of total phenolics more than 20 min extraction in a significant degree ($p<0.05$). However, there were no significant difference between in this first extraction for 60 min than that for 20 min in terms of yields of anthocyanins ($p>0.05$). Therefore, extraction with 70% aqueous acetone for 20 min followed by re-extraction (4 times x 10 min) (**procedure 1**) could obtain more pure monomeric anthocyanins than 60 minute extraction (**procedure 2**) at the similar level of anthocyanins and with lower concentration of total phenolics, which not only include the monomeric anthocyanins but also contain other phenolics, such as tannins. The frequency of re-extraction could be reduced to two times since the re-extraction on the third, the fourth or the fifth did not contribute to the total amount of anthocyanins significantly.

Anthocyanin normally occur in flower petals, fruits, stems, roots, leaves accumulating in vacuoles of epidermal and subepidermal cells (Strack and Wray,

1994). Anthocyanins should be easier to be extracted out than other phenolics. Therefore a short time and multiple extractions would be recommended to yield a high amount of monomeric anthocyanins and a low level of total phenolics.

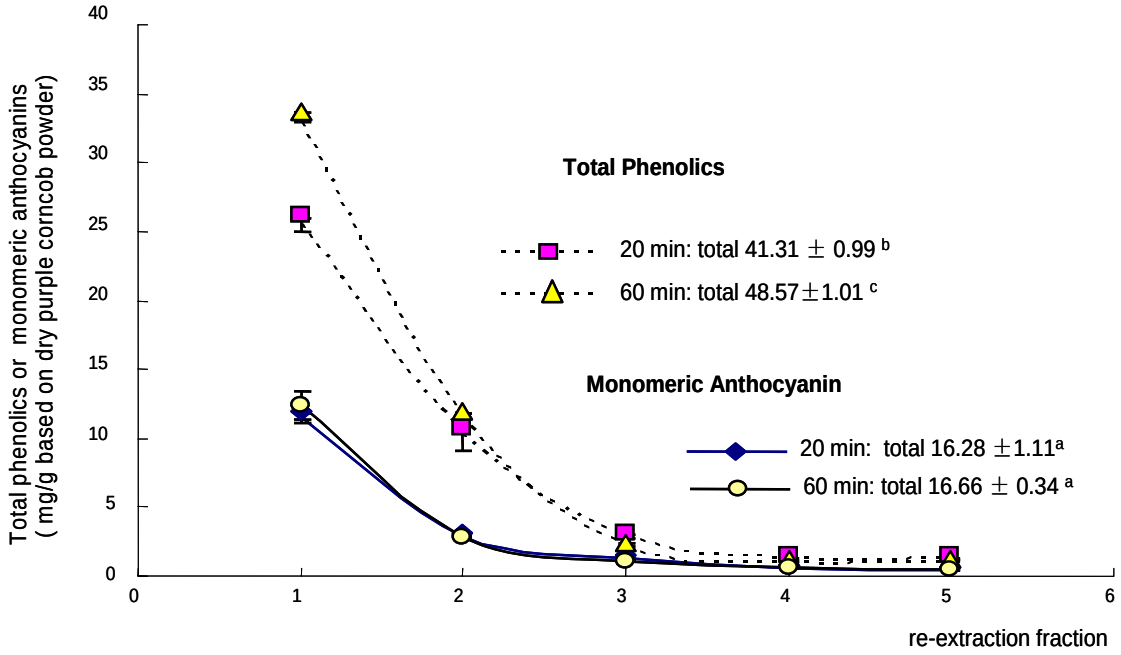

Figure 4.4: Yield of Monomeric anthocyanins and total phenolics during different consecutive re-extraction procedures. Anthocyanins and phenolics were extracted with 70% aqueous acetone for 60 or 20 min (fraction 1) followed by four re-extraction procedures of 10 min each with acidified 70% aqueous acetone (fractions 2-4). Different superscript letters (a, b, c) was that the mean differences were significant at the 0.05 level (n=4). Values are represented as Mean ± Standard error (n=4).

4.4.5 Enzyme Hydrolysis and SDS-PAGE

Large amount of protein was found to play an important role in the complex developing of anthocyanins in our previous study (Chapter 5). There was one band in control and the group treated with acid protease in **Figure 4.5**, while it disappeared in the group treated with neutral protease suggesting that the protein (MW ≈ 29 KD) identified by Multicolored protein markers with a wide range (6.5 –205 KD could be hydrolyzed by the neutral protease but not by the acid protease. The major storage proteins in corn (*Zea mays* L.) endosperm are zeins which are alcohol-soluble proteins. Six classes of zein proteins have been identified so far: zein-A (21-26 KD), zein-B (18-24 KD), zein-C (17 KD), zein-D (14 KD), zein-E (27-31 KD), and zein-F (18 KD) by SDS-PAGE and HPLC (Wilson, 1991). Proteins in purple corncob (*Zea Mays* L.) may be one of them, such as zein-A or zein-E according to the molecular weight. Proteins in purple corncob, a part of the complex of anthocyanins with macromolecules, provided the carboxyl group to form hydrogen-bond with hydroxyl group of tannins, developing complexes which were trapping monomeric anthocyanins. The enzyme hydrolysis of this protein suggested that it was a possible way to reduce the level of anthocyanin complexes and release more monomeric anthocyanins.

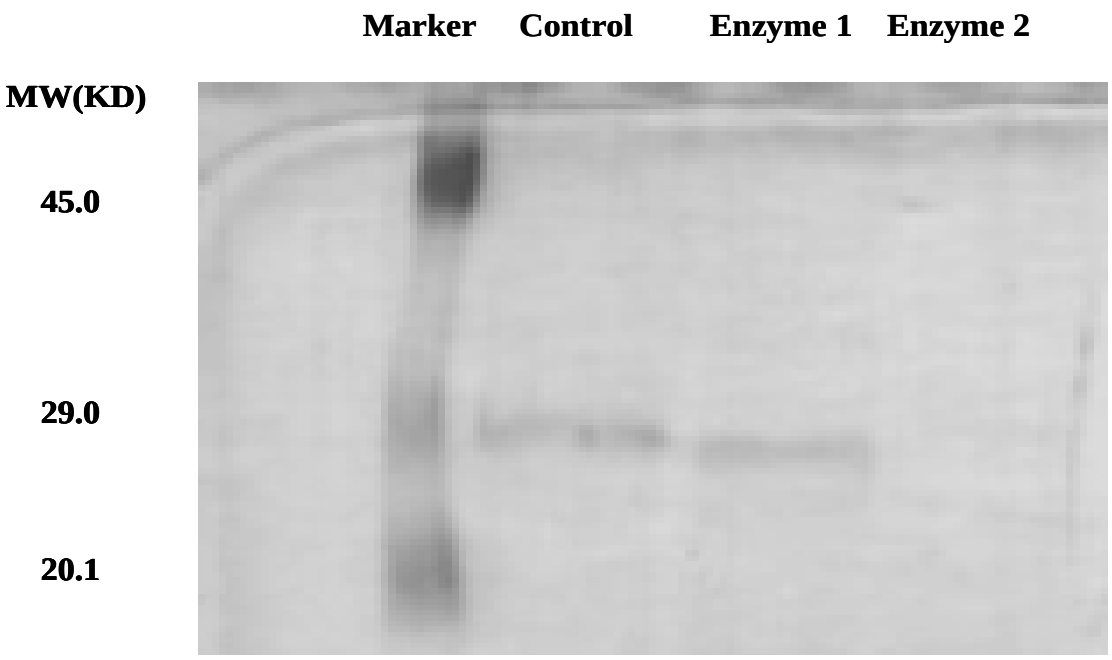

Figure 4.5: SDS-PAGE graph of protein after protein hydrolysis. Enzyme 1: Enzeco fungal acid protease; Enzyme 2: Multifect neutral enzyme

4.4.6 Effect of Extraction Methods on Purple Corncob Anthocyanins Profile

There were nine anthocyanins found in anthocyanin-rich extracts from purple corncobs prepared freshly either by 70% acetone or by boiling water (**Figure 4.6A and B**). They was previously identified to be cyanidin-3-glucoside, pelargonidin-3-glucoside, cyanidin-3-malonylglucoside, peonidin-3-glucoside, cyanidin-3-(6”-maloylglucoside), pelargonidin-3- (6”-maloylglucoside), pelargonidin-3-

malonylglucoside, peonidin-3-malonylglucoside, peonidin-3- (6”-maloylglucoside) in our previous study (Jing et al., 2006). The anthocyanin profile in purple corn extracts by boiling water was similar to those obtained by 70% acetone except the peak 8 (Peonidin-3-malonylglucoside), which was relatively low in purple corncob extractd obtained by boiling water.

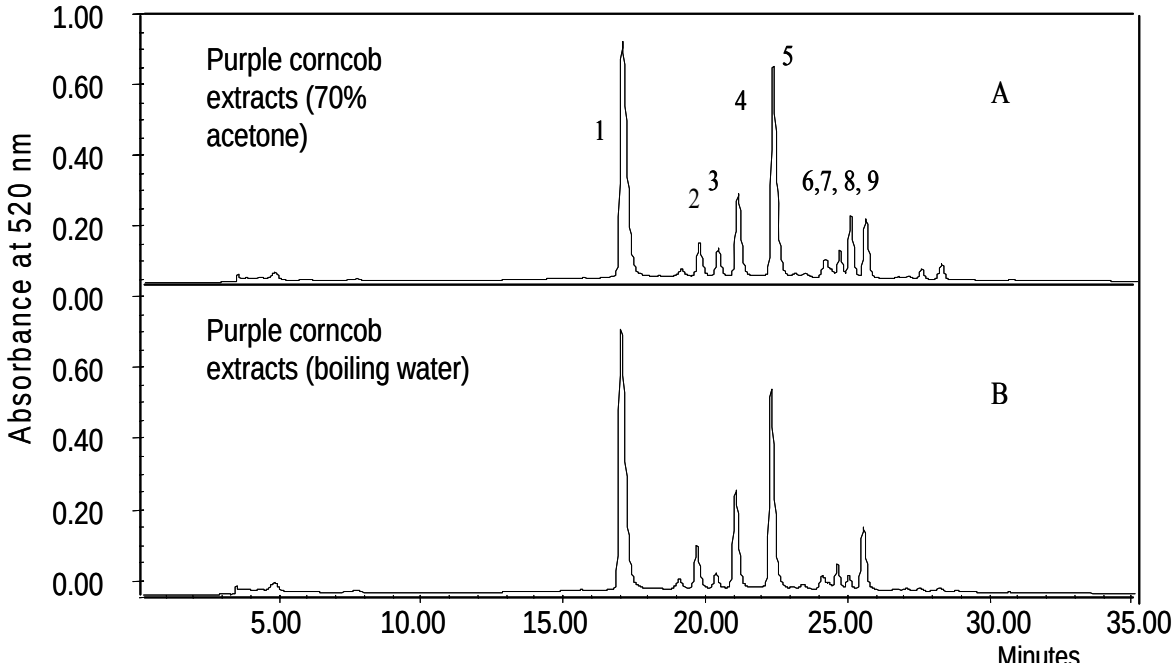

Figure 4.6: HPLC profiles of anthocyanins extracted from purple corncob by 70% acetone and boiling water. 1. cyanidin-3-glucoside; 2. pelargonidin-3-glucoside; 3. cyanidin-3-malonylglucoside; 4. peonidin-3-glucoside; 5. cyanidin-3-(6”-maloylglucoside); 6. pelargonidin-3- (6”-maloylglucoside); 7. pelargonidin-3-malonylglucoside; 8. peonidin-3-malonylglucoside; 9. peonidin-3- (6”-maloylglucoside)

4.5 CONCLUSIONS

Deionized water was a good and economic solvent with a high yield of monomeric anthocyanins (about 0.94 ± 0.03 mg/100 mg corncob) at a relatively mild temperature (50 °C) and compared to other operation temperatures, they produced lower levels of tannins and proteins, which may have unfavorable effect on the application of purple corn colorants in foods, causing pigment precipitation and higher waste. The pH of water had impact on the amount of monomeric anthocyanins, tannins, and proteins in anthocyanin-rich extraction. A short time and multiple extractions resulted in a high yield of monomeric anthocyanins and a low yield of total phenolics. Neutral proteases are potent to be applied to decrease the level of proteins and reduce purple corn waste.

4.6 ACKNOWLEDGMENTS

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CHAPTER 5

CHARACTERIZATION OF ANTHOCYANIN-RICH WASTE FROM PURPLE CORNCOBS (*ZEA MAYS* L.) AND ITS APPLICATION TO COLOR MILK [a] [b]

5.1 ABSTRACT

Pigment production from anthocyanin-rich purple corncobs generates a deeply colored waste precipitate. Our objectives were to characterize this anthocyanin-rich waste (ARW) and to find a suitable application in a food matrix. Composition and

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solubility characteristics of ARW were evaluated. Color (CIELAB) and pigment (monomeric anthocyanin and HPLC profiles) stability of ARW in milk (35 mg /100 ml) was evaluated using an accelerated test at 70 °C, and phosphate buffer as a control. ARW provided milk an attractive purple hue (324-347°). Monomeric anthocyanin degradation followed zero-order kinetics in skim and whole milk and second-order kinetic in the control, with half-lives of 173, 223 and 44 min at 70 °C, respectively. ARW shows potential as natural colorant for a pH range unusual for anthocyanin applications. A protective effect of matrix constituents on the stability of anthocyanins was evident. Anthocyanins may interact with different compounds in biological systems when the pH values are close to neutral.

KEYWORDS: solubility; anthocyanins; macromolecule; phenolics; kinetic

5.2 INTRODUCTION

Anthocyanins are the largest and most important group of water-soluble pigments in nature, contributing to the attractive orange, red, purple, violet and blue colors of fruits, vegetables, and flowers. Anthocyanins, which have been consumed for many years without any apparent adverse effects, have bright pH dependent color (Mazza and Miniati, 1993). Interest in anthocyanins has increased due to its color characteristics and health benefits as a promising alternative as food colorant (Giusti and Wrolstad, 2003). Close to 25% of the population perceives foods without artificial ingredients as desirable, being this very important in their food and beverage purchase decisions (Sloan, 2005). Recently, anthocyanins have been reported to have various

biological activities such as antioxidant (Ghiselli et al., 1998; Noda et al., 2000; Prior, 2003; Wang and Lin, 2000), anti-mutagenic (Gasiorowski et al., 1997; Peterson and Dwyer, 1998), and chemopreventive (Koide et al., 1997; Zhao et al., 2004), contributing to a reduced incidence of chronic diseases. Researchers have shown that an anthocyanin-based food colorant from purple corn inhibited induced cell mutation (Aoki et al., 2004), reduced chemically induced colorectal carcinogenesis (Hagiwara et al., 2001), and contributed to the prevention of obesity and diabetes (Tsuda et al., 2003).

For centuries, anthocyanin rich purple corn (*Zea mays* L.) has been cultivated in South America, mainly in Peru and Bolivia, and used to prepare drinks and desserts. A colorant from purple corn is widely used in Asia, South America and Europe. Cyanidin-3-glucoside is the major anthocyanins in purple corncob (Nakatani et al., 1979; Zhao et al., 2004)), although pelargonidin, and peonidin glucosides have also been found in maize plants (Styles and Ceska, 1972) as well as their respective malonyl derivatives (Pascual-Teresa et al., 2002).

Large quantities of anthocyanin-rich waste (ARW) are generated during the preparation of commercial purple corn colorant, which are obtained by an easy and economic hot-acidified-water extraction procedure. This waste has very limited application in foods due to low solubility in acidified aqueous system.

Anthocyanin pigments have been traditionally used to provide color to acidic food systems (usually pH below 3) such as fruit beverages and jams. However, our preliminary studies suggested that ARW would be water-soluble at pH near to neutral

suggesting that it could find a potential application in foods with a pH level close to neutral such as milk. Nontraditional milk product such as chocolate milk (27%), strawberry (60%), and other flavored milk lead consumption of school vended milk whose market potential is estimated at 130 million of 16-ounces (0.47 L) plastic single servings of milk per year (Sloan, 2005). Therefore, there is a market for natural alternatives to the use of artificial dyes that could provide desired color, stability and value added for these applications. This alternative must be cost effective, and the use of a waste material, such as ARW would be ideal.

Our objectives were to characterize the purple corncob anthocyanin-rich waste (ARW) generated during purple corn color production and to find a suitable food application. In this study we evaluate pigment and solubility characteristics of ARW, tested its potential as natural colorant, and explored the potential interactions between anthocyanins and matrix constituents.

5.3 MATERIALS AND METHODS

5.3.1 Materials and Reagents

Anthocyanin-rich waste (ARW) powder from purple corncob (*Zea mays* L.) was donated by Globenatural International S.A. (Chorrillos-LIMA, Peru). This waste was obtained by sedimentation and spray drying of the water insoluble portion from anthocyanin extracts of purple corncob.

An ARW stock suspension was prepared by mixing 0.5 g ARW powder with 50 ml 0.01% HCl aqueous solution. An ARW stock solution was prepared by mixing 0.5 g ARW powder with 50 ml pH 8 phosphate buffer.

Anthocyanidin standards were prepared by acid hydrolysis (Durst and Wrolstad, 2001) of concentrated anthocyanin extracts from red radishes (pelargonidin) and concord grapes (delphinidin, cyanidin, petunidin, peonidin, and malvidin).

Skim milk (8 g protein, 0 g fat, and 12 g carbohydrate per 240 ml serving) and whole milk (8 g protein, 8 g fat, and 11 g carbohydrate per 240 ml serving) (Richfood, Inc., Richmond, VA, USA) were purchased from a local market.

Folin & Ciocalteau phenol reagent and standard of gallic acid (crystalline gallic acid, 98% purity) were purchased from Sigma (St. Louis, MO, USA). PIERCE BCA (bicinchoninic acids) protein assay kit purchased from Fisher Scientific (Fair Lawn, NJ, USA). All high-performance liquid chromatography (HPLC) grade solvents and other chemicals (analytic grade) were from Fisher Scientific (Fair Lawn, NJ, USA).

5.3.2 Solubility of ARW

Solubility of ARW was evaluated in different solutions. ARW stock suspension was mixed thoroughly with different solutions. Precipitated material was an indicator of lack of solubility and haze was used as a measure of insoluble material suspended in the liquid. Haze was determined immediately after 30-second homogenization as described below.

5.3.2.1 Solubility at different ethanol concentration

ARW stock suspension (0.5 ml) was added into 9.5 ml of different aqueous ethanol solutions with 0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5, and 100% ethanol (v/v) to a final concentration of 0.5 mg /ml based on ARW dry powder. Haze was then measured as described below.

5.3.2.2 Solubility at different pH aqueous system

A series of buffers were prepared: pH 1 potassium chloride buffer (0.025 mol/L), pH 3 sodium citrate-chloride buffer (0.1 mol/L), pH 3.5 sodium citrate-chloride buffer (0.4 mol/L), pH 4 sodium acetate buffer (0.1 mol/L), pH 4.5 sodium acetate buffer (0.4 mol/L), pH 6 sodium-citrate buffer (0.1 mol/L), pH 7 phosphate buffer (1/15 mol/L), and pH 8 phosphate buffer (1/15 mol/L). Anthocyanin-rich waste stock suspension (0.5 ml) was added to tubes containing 5 ml of buffer and mixed reaching a final concentration of 0.91 mg ARW/ml buffer. Haze was determined as described below. Samples were centrifuged at 2000 rpm and 4 ºC for 10 min in a Beckman J2-21M centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). Monomeric anthocyanin content and polymeric color were monitored.

5.3.3 Monomeric Anthocyanins and Polymeric Color

The total monomeric anthocyanin content was measured by the pH-differential method (Giusti and Wrolstad, 2001). A Shimadzu UV-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan) was used at 420 nm, 510 nm, and 700 nm. Monomeric anthocyanins were calculated as cyanidin-3-glucoside, using the

extinction coefficient of 26,900 L $cm^{-1}mg^{-1}$, and a molecular weight of 449.2 g/L. Disposable cuvettes of 1 cm path length were used.

Color density and polymeric color were calculated using absorption at 420 nm, 510 nm, and 700 nm before and after bisulfite treatment, and used as an index of browning (Giusti and Wrolstad, 2001).

5.3.4 Total Phenolics

Total phenolics were measured using a modification of the Folin-Ciocalteu method for total phenols (Singleton and Rossi, 1965). The absorbance of the samples and standards was measured at 755 nm. Total phenols were calculated as gallic acid equivalents based on a gallic acid standard curve.

5.3.5 Protein Analysis

Total protein concentration was measured on a Shimadzu UV-visible spectrophotometer using PIERCE BCA (bicinchoninic acids) protein assay kit. Absorbance at 562 nm of samples, standards of bovine serum albumin (BSA), and controls (samples with deionized distilled water instead of BCA reagent B) were measured. Total protein was quantified as BSA equivalents.

5.3.6 Alkaline and Acid Hydrolysis of Anthocyanins

Anthocyanin-rich waste suspension (1 ml) was saponified in a screw-cap test tube with 10 ml of 10% aqueous KOH for 8 min at room temperature in the dark. (Durst and Wrolstad, 2001; Giusti et al., 1999).The solution was neutralized and

acidified by HCl (2 mol/L), and hydrolysate was purified using a C-18 Sep-Pak cartridge (1 g, Waters Corp., Milford, MA, USA) as described by Giusti et al (Giusti et al., 1999).

Anthocyanidins from ARW were obtained by mixing 1 ml of ARW suspension with 10 ml HCl (2 mol/L) in a 20 ml screw-cap test tube. Tubes were placed in boiling water for 30 min and then cooled down in an ice bath. The hydrolysate was purified with a C-18 Sep-Pak cartridge as previously described (Durst and Wrolstad, 2001; Giusti et al., 1999).

5.3.7 Analytical Chromatography

A high performance liquid chromatograph (HPLC) system (Waters Delta 600 systems, Milford, MA, USA) equipped with a photodiode array detector (Water 996, Milford, MA, USA.), autosampler (Waters 717 plus, Milford, MA, USA), and Millennium^{32} software (Waters Corp., Milford, MA, USA) was used.

Columns and mobile phase: The reversed-phase 5 µm Symmetry C18 column (4.6×150mm, Waters Corp., Milford, MA, USA) fitted with a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corp., Milford, MA, USA) was used. The solvents used were A: 1% phosphoric acid / 10% acetic acid / 5% acetonitrile in water and B: 100% acetonitrile. Solvents and samples were filtered though 0.45 µm poly(tetrafluorothylene) membrane filters (Pall Life Sciences, Ann Arbor, MI) and 0.45 µm polypropylene filters (Whatman Inc., Clifton, NJ, USA), respectively. Separation was achieved by using a linear gradient from 0 to 30% solvent A in 35 min.

An injection volume of 50 µL with a 1 ml/min flow rate was used. Spectral information over the wavelength range of 260-600 nm was collected.

5.3.8 Monomeric Anthocyanins, Total Phenolics, and Proteins from ARW in pH 3 Aqueous Environment

Anthocyanin-rich waste stock suspension (10 mg/ml) was diluted by 10 times with pH 3 sodium citrate-chloride buffer. Diluted suspensions (5 ml) were centrifuged at 2000 rpm and 4 °C for 10 min. The pellet was re-dissolved and taken to 5 ml with pH 8 phosphate buffer. The supernatant was taken to 5 ml with pH 3 sodium citrate-chloride buffer. Monomeric anthocyanins, total phenolics, and proteins were measured on both the supernatant and re-dissolved precipitate portions.

5.3.9 Heat Stability in Dairy Products

Purple corn ARW was used to color skim milk and whole milk. Also, pH 6.8 phosphate buffer colored with ARW was used as a control, to match the pH of the milk samples (pH determined with an Accumet pH-meter 25, from Fisher Scientific). Color and pigment stability were evaluated using an accelerated stability test at a temperature close to typical pasteurization temperature (70 °C). Anthocyanin-rich waste stock solution was mixed with matrices to reach a final concentration of 35 mg ARW /100 ml (containing 3.7 mg monomeric anthocyanins or 44.2 mg total phenolics per 100 ml matrix). A series of 45 test tubes were filled with 20 ml of either pH 6.8 buffer, skim milk, or whole milk (15 test tubes for each matrix), closed with screw caps and covered with aluminum foil. The tubes were then immersed in a water bath at

70 ± 0.1 ºC for 120 minutes with a shake speed of 80 rpm on a reciprocal shaking bath (Precision, Winchester, VA, USA). Nine tubes (3 replicates for each matrix) were collected at regular time intervals (0, 30, 60, 90, and 120 min) and rapidly cooled down to room temperature for color analysis.

5.3.10 Color and Haze Analyses

Hue angle, chroma, L* (CIELch) and haze were measured with a Hunter ColorQuest XE colorimeter (HunterLab, Hunter Associates Laboratories Inc., Reston, USA) using illuminant C and 10 degree observer angle. For color measurements of milk, samples were placed in 1 cm path-length disposable cuvettes and read using the reflectance specular included mode, and covered with light trap while controls were put in the transmittance compartment and measured using total transmittance. For haze measurements, samples were homogenized for 30-second on a Vortex, placed in 2 mm path-length disposable cuvettes and measured using the transmission mode and haze (total transmission and relative transmission).

5.3.11 Anthocyanin Recovery and Purification from Milk

Anthocyanins were recovered from milk by using acidified methanol. Each colored milk (5 ml) was transferred into a Waring Laboratory Blender (New Hartford, CT, USA), mixed with 10 ml of 0.1% acidified methanol for 3 min at low speed and then 2 min at high speed. The mixture was transferred to a 50 ml chloroform-resistant centrifuge tube. Then 15 ml of 0.1% HCL acidified methanol was used to wash the blender and combined with the previous extract. Chloroform (20 ml) was added into

the tubes, mixed well, and centrifuged at 27,200×g and 4 ºC for 10 min in Beckman J20-MI (Beckman Coulter, Inc., Fullerton, CA. USA). The top aqueous phase containing anthocyanins was transferred to a round bottom flask. Residual methanol /chloroform was removed in a rotary evaporator at 40 ºC under vacuum. The remaining aqueous extract was made up to 5 ml with acidified deionized distilled water and centrifuged at 14,000 rpm and 4 ºC for 10 min in an eppendorf centrifuge 5415C (Brinkmann Instruments, Inc., Westbury, NY, USA). The supernatant was refrigerated at 4 ºC until analyzed.

5.3.12 Statistical Analysis

The accelerated stability test was carried out according to a completely randomized design (CRD) with 3 replicates for treatment groups and control. Statistical analysis was done by using the SAS/STAT package (version 8.1, 1999, SAS Institute Inc., Cary, NC). ANOVA and PROC MIXED procedure were conducted. Values of $p<0.05$ were considered significant. Rates for anthocyanin degradation were obtained from linear regression analysis (95% confidence interval).

5.4 RESULTS AND DISCUSSION

5.4.1 Solubility Analysis

Solubility characteristics of colorants are very important since they determine the potential application in food matrices. Anthocyanins, water-soluble pigments, can be usually used to color acidic foods such as drinks, jams, and jellies. ARW, a by-

product of anthocyanin extraction from purple corncob showed poor solubility in acidic aqueous systems, maybe due to other compounds bound to anthocyanins. Therefore evaluation of its solubility characteristics was necessary to evaluate potential applications in food matrices.

The solubility of ARW was different in aqueous solutions of different pH levels (**Figure 5.1**). Anthocyanin-rich waste showed very limited solubility at low pH value while it was water-soluble at pH close to neutral or above. The solubility characteristics of the ARW were very similar to those reported for complexes of protein and tannins.

Hagerman and Butler (1978) reported the formation of tannin-protein complexes that precipitated in a pH-dependant manner. The lowest solubility of the complex was observed within one pH unit of the isoelectric point of the protein (Hagerman and Bulter, 1978), contradicting an earlier statement by Loomis and Battaile (1966) that the ability of tannins to precipitate proteins was pH-independant below pH 8 (Loomis and Battaile, 1966). These interactions are essentially a dynamic surface phenomenon, generally reversible, which involves hydrophobic effects and hydrogen bonds (Charlton et al., 1996; Luck et al., 1994). The tannin's hydroxyl group is an excellent hydrogen donor that can form strong hydrogen bonds with the protein's carboxyl group. Phenolic groups of tannins are ionized and are unavailable for hydrogen bonding at high pH (usually more than 8). In addition, proteins have net negative charges so that precipitates don't occur because protein exhibit repulsive

force (Loomis and Battaile, 1966). Conformation and charge of proteins have an effect on the formation of tannin-protein complexes. Conformationally open proteins which have a high ratio of proline readily form complexes with tannins (Hagerman and Bulter, 1981). The strength of the interactions depends both on the nature of protein and tannin molecule. Tannins have been associated with antinutritional factors in the diet, by decreasing the digestion rate of dietary proteins (Shahidi and Naczk, 2004). However, recent studies have found that tannins may counteract these adverse effects by inducing levels of some digestive proteases, lipases, and billary acid (Horigome et al., 1988; Majumdar and Moudgal, 1994). Further studies would be needed to elucidate the effects of these interactions on the bioavailability of ARW constituents.

Anthocyanins are highly unstable at pH levels of 5-6, and the color fades rapidly at those pH ranges. However, some acylated anthocyanins have shown high stability and color intensity at pH 7-8 (Fossen et al., 1998), suggesting that it may be possible to use them as colorants in food applications in those pH ranges. The high solubility of purple corn ARW at pH close to neutral or above suggested that it was possible to use these waste materials as colorants for food systems around neutral pH.

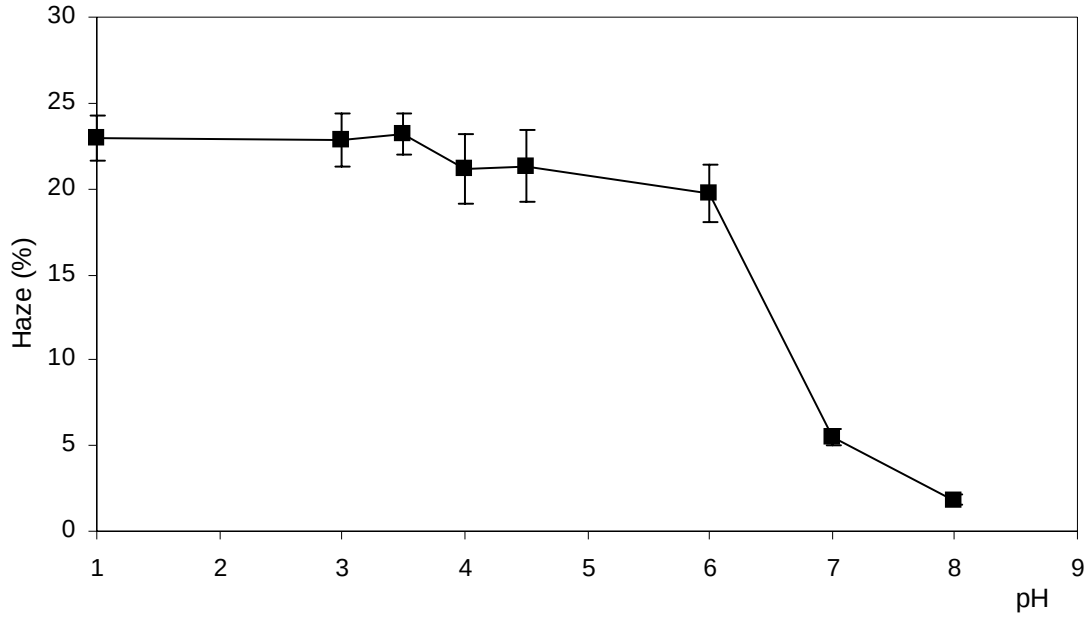

Figure 5.1: Solubility of ARW at different pH levels (0.91 mg ARW/ml aqueous solution)

The solubility characteristics of ARW changed but not proportionally to the ethanol concentration (**Figure 5.2**). ARW solubility was higher in a 50%~62.5% (v/v) ethanol solution with a final concentration of 0.5 mg ARW/ml in aqueous. The total monomeric anthocyanins concentration was highest (about 66.2 mg/g based on ARW dry powder), while haze was lowest (about 1.9%) when concentration of ethanol is

62.5% (**Figure 5.2**), suggesting that anthocyanins had formed complexes with macromolecules that were soluble in a 50% aqueous ethanol environment.

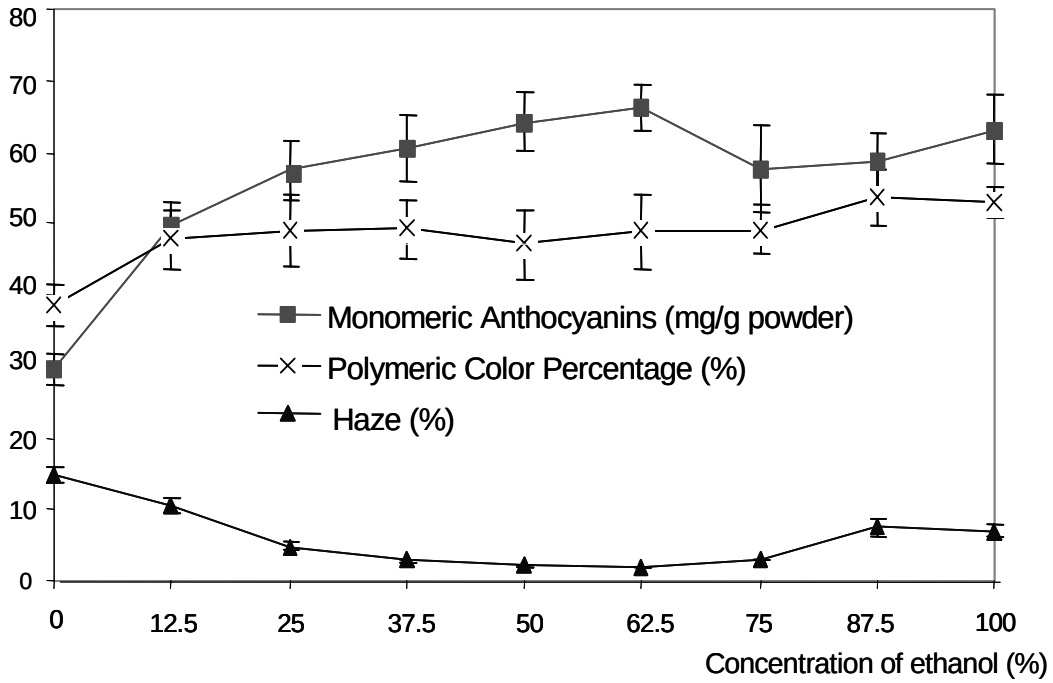

Figure 5.2: Monomeric anthocyanins, polymeric color and haze of ARW at different concentration of aqueous ethanol (0.5mg ARW/ml)

A degradation index of pigments was determined by the ratio between polymerized colored anthocyanin-tannin complexes and color density (Giusti and Wrolstad, 2001). It was very interesting the way that polymeric anthocyanin concentration changed with the increase of ethanol concentration in the solvent

(**Figure 5.2**). Solubility of polymerized colored anthocyanin-tannin complexes may have increased with higher ethanol concentration. When the ethanol concentration in solution increased from 0 to 12.5%, the percent polymeric color increased sharply. However, when ethanol concentration continued to increase from 12.5% to 37.5%, both monomeric anthocyanin and polymerized colored anthocyanin-tannin complexes increased, keeping the percent polymeric color unchanged. Ethanol concentration of 50% exhibited the lowest haze level (1.9%). At this concentration, large quantities of monomeric anthocyanin were released from the complexes so that percentage of polymeric color slightly decreased. When ethanol concentration changed from 50% to 100%, the solubility of tannin-proteins decreased trapping more monomeric anthocyanins and resulting on an increased percentage of polymeric color.

5.4.2 Monomeric Anthocyanins, Total Phenolics, and Proteins from ARW in pH 3 Aqueous Environment

The limited solubility of ARW in water was hypothesized to be associated with complexation reactions between anthocyanins and macromolecules present in purple corncob. Therefore, the distribution of monomeric anthocyanins, total phenolics, and proteins in the supernatant and precipitate portions at pH 3 aqueous environment was investigated.

The monomeric anthocyanins, total phenolics, and protein were distributed in the supernatant portion by 45%, 25%, and 20%, while 55%, 75%, and 80% in the precipitate portion (**Figure 5.3**). Combined with the above information on solubility of

ARW at different pH, it suggested that the precipitates in the ARW are rich in the anthocyanin complexes with macromolecules such as protein and polyphenolics. Hagerman and Butler (1978) pointed out that the low molecular weight phenolics, such as catechin, could bind nonspecifically to the protein-tannin complex (Hagerman and Bulter, 1978). Anthocyanins, with similar structure to catechin, possibly contributed to the nonspecific binding to the protein-tannin complex.

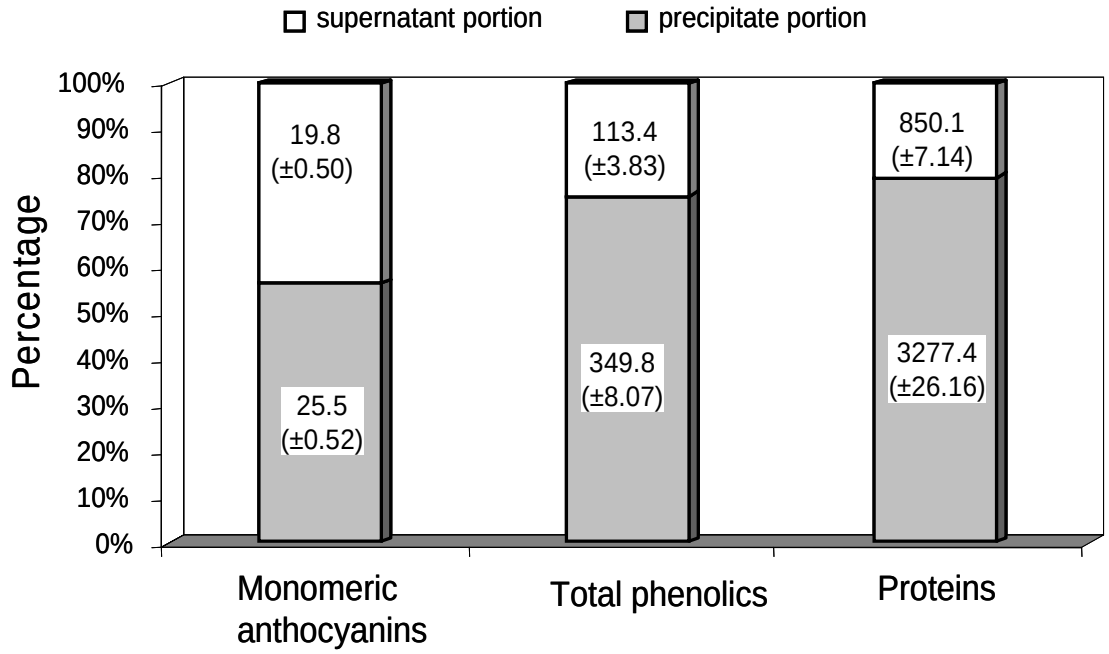

Figure 5.3: Percentage of monomeric anthocyanins, total phenolics, and proteins in the supernatant and precipitate portions of ARW suspension in pH 3. Monomeric anthocyanins, total phenolics and proteins were measured as the equivalents of cyanidin, gallic acid, and BSA (mg/L) respectively and numbers are means of 3 replications. In parenthesis are standard deviations.

5.4.3 HPLC Profiles

The HPLC profiles of ARW were analyzed before and after acid and alkaline hydrolysis (saponification). With published literature (Nakatani et al., 1979; Pascual-Teresa et al., 2002; Styles and Ceska, 1972), six major anthocyanins were identified and showed in **Figure 5.4**. Three different anthocyanidins (cyanidin, pelargonidin, and peonidin) were found in purple corn, based on the comparison of the anthocyanidins profiles from purple corn and the ones from red radish (Giusti and Wrolstad, 1996) and concord grape (Durst and Wrolstad, 2001). Cyanidin, pelargonidin, and peonidin in ARW were 69.9%, 8.3%, and 21.8% of the total peak area at 510 nm, respectively, all glucosylated and present in the acylated and non-acylated forms. The acylating group was reported as malonic acid (Nakatani et al., 1979; Pascual-Teresa et al., 2002; Styles and Ceska, 1972), consistent with our data of long retention times, of peaks 4-6 but no absorption peak in the 320-360 nm range, typical of cinnamic acid acylation.

5.4.4 Application in Milk

Milk samples colored with ARW presented an attractive purple hue while the whole milk exhibited a lighter purple color than skim milk. The white color background of milk changed the color appearance of anthocyanins in milk. We chose an accelerated stability test, with a temperature close to that used for typical pasteurization of milk. Application of ARW in dairy products and interaction of anthocyanins with food components were studied with heat treatments at 70 ± 0.1 °C for different time (0, 30, 60, 90, and 120 min). The skim milk and whole milk were

studied as matrices in the experiment. The control was pH 6.8 buffer phosphate solution.

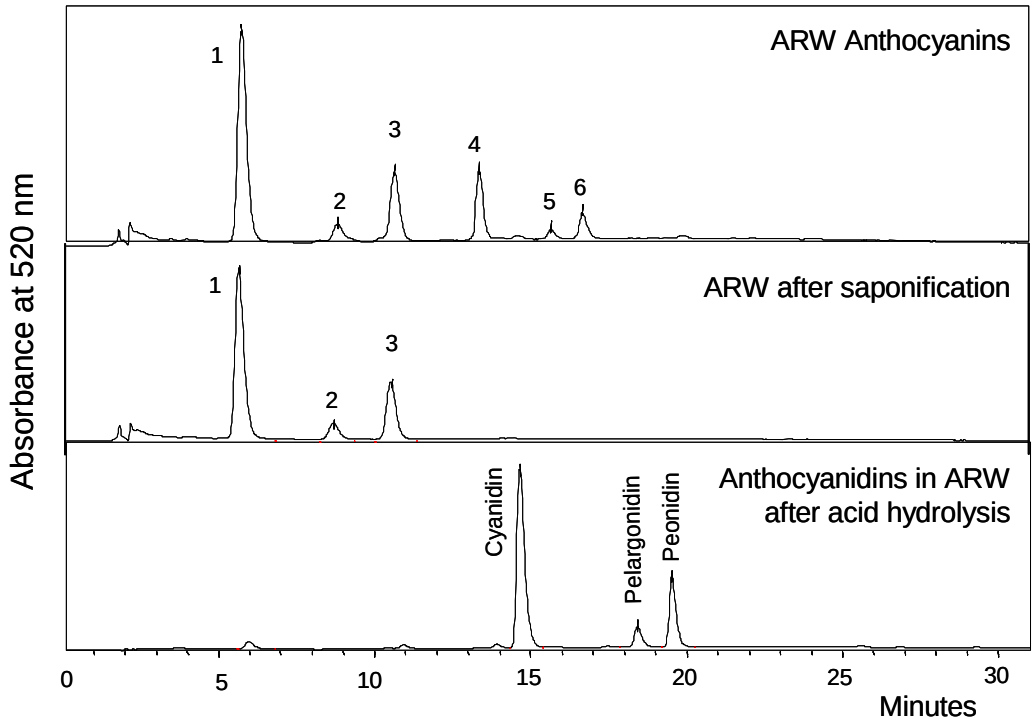

Figure 5.4: HPLC profiles of anthocyanins in ARW before and after saponification and acid hydrolysis. 1: cyanidin-3-glucoside; 2: pelargonidin-3-glucoside; 3: peonidin-3-glucoside; 4: cyanidin-3-(6”-malonylglucoside); 5: pelargonidin-3- (6”-malonylglucoside); 6: peonidin-3- (6”-malonylglucoside)

Changes in color of the samples revealed that anthocyanins were more stable in the milk matrices than in the control (**Table 5.1**). Lightness in skim milk or whole

milk was higher than the corresponding L* in the control at time zero. That may be due to the covering of the nature color of milk. Lightness (L*) values increased from L* = 38.29 (time zero) to L* = 51.56 (after 120 min) in the pH 6.8 buffer, faster than in skim milk and whole milk during the heat treatment, showing that the color in pH 6.8 buffer faded faster than in either of other two matrices. Chroma (C*) was monitored during storage as indicator of changes in color saturation during heat treatment. The chroma of the pH 6.8 buffer was highest among three matrices mainly because the white color contribution of milk components counteracted the intensity of purple color. The chroma in skim and whole milk changed slightly with a 20% and 19% increase, respectively, compared to the 45% increase in pH 6.8 buffer after treatment. The increase of hue angle (h*) in the control indicating a shift in color from a red (h^*_{0min}=29.37˚) to an orange/brown red (h^*_{120min}=52.61˚) was possibly due to the occurrence of browning reactions with anthocyanins degradation during heat treatment. The h* value of skim and whole milk shifted from purple (h^*_{0min}=-35.65˚/-12.01˚)to red (h^*_{120min}=12.65˚/27.58˚), a more commercially desirable color over the treatment period.

Matrix	Time(min)	L*	C*	h*	a*	b*
Ph6.8 buffer	0	38.3	31.5	29.4	27.5	15.5
	30	48.2	41.2	46.9	28.1	30.1
	60	50.1	43.5	45.6	30.4	31.0
	90	51.0	44.4	51.1	27.9	34.6
	120	51.6	45.7	52.6	27.8	36.3
Skim milk	0	56.2	4.0	324.4	3.2	-2.3
	30	59.9	4.2	4.9	4.2	0.4
	60	59.9	4.1	8.4	4.1	0.6
	90	61.2	4.7	10.7	4.7	0.9
	120	61.1	4.5	12.7	4.4	1.0
Whole milk	0	65.1	4.1	348.0	4.0	-0.9
	30	67.8	4.7	25.5	4.3	2.0
	60	68.4	4.9	26.7	4.4	2.2
	90	68.1	4.9	28.8	4.3	2.3
	120	68.7	4.9	27.6	4.3	2.3

L*: lightness; C*: chroma or saturation (a*2+b*2)1/2; h*: hue angle, tan-1(b*/a*)

Table 5.1: Color properties of matrices colored with anthocyanin-rich waste heated at 70 ˚C for different time

In **Figure 5.5**, monomeric anthocyanin concentration in pH 6.8 buffer decreased sharply with time under heating conditions so that only 52.1% was remaining after heating at 70 °C for 30min and decreased down to 30.0% after heat

treatment for a total time of 120 min. The remaining anthocyanins in the skim milk were 90.3% after heating at 70 °C for 30 min and decreased to 63.4% after 120 min heating. The whole milk seemed to protect anthocyanins very well. When heated at 70 °C for 30, 60, 90, and 120 min, the residual anthocyanins in the whole milk was 95.5, 92.0, 85.7, and 76.6%. All above suggested that fat in milk and other components such as proteins might protect anthocyanins from degradation during heat treatment. Anthocyanins intermolecular copigmentation with polyphenolics may increase the stability of anthocyanins (Boulton, 2001; Talcott et al., 2003). Anthocyanins in the milk system might form complexes with macromolecule that protect anthocyanins from degradation.

The statistical analysis showed that the anthocyanin retention greatly depended on treatment time ($p<0.01$). Therefore, linear regression analysis was used to determine the rate of degradation of anthocyanins in each of the milk matrices as well as in the buffer solution. The equations and R-square are presented below. All equations had a R-square of 0.94 or higher, showing that most of the variability was explained by the model. Mean comparisons of the different anthocyanins colored matrices after 120 min of heat treatment revealed that retention of monomeric anthocyanins was significantly different between control (pH 6.8 buffer) and milk matrices at the 0.05 level by Least Significant Difference Test (LSD).

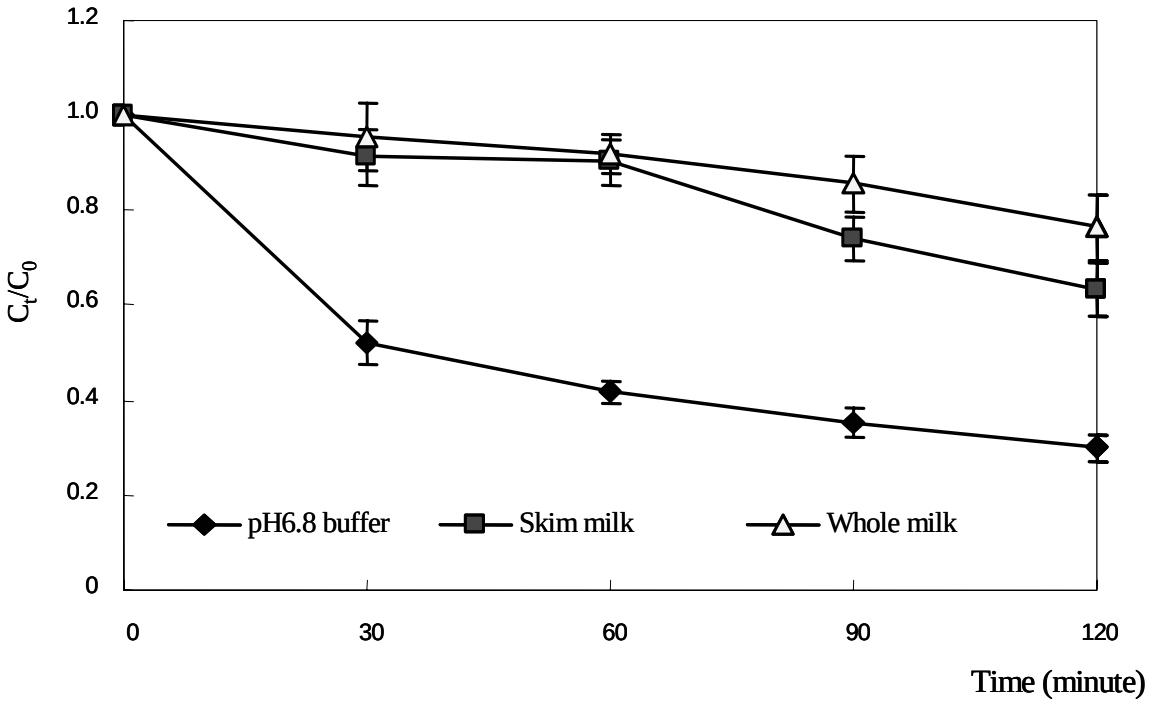

Figure 5.5: Stability of ARW in different matrices heated at 70°C for different time. C_0 is the initial anthocyanins content; Ct is the anthocyanin content after t minute heating at 70 °C; C_0 and C_t are expressed as cyanidin-3-glucoside equivalents (mg/L)

The monomeric anthocyanins content decreased during heating treatment at 70 °C (**Figure 5.5**) and change followed the zero-order kinetics (Equation 1 and 2) in skim milk and whole milk and second-order kinetic (Equation 3) in the control. This indicated that anthocyanins degradation rate in skim milk and whole milk (Equation 1 and 2) didn't depend on substrate concentration and as the concentration of

anthocyanins decreased, the rate of degradation didn't decrease. However the anthocyanin degradation rate in pH 6.8 control (**Equation 3**) depended on the concentration of anthocyanins. When the concentration of monomeric anthocyanins decreased, the rate of degradation decreased sharply. The equations that described the monomeric anthocyanins degradation in skim milk, whole milk, and pH 6.8 control with time during heating treatment are following:

Zero-order kinetics

Skim milk: $C_t/C_0 = -0.003t + 1.019$ $R^2 = 0.94$ (1)

Whole milk: $C_t/C_0 = -0.0021t + 0.969$ $R^2 = 0.96$ (2)

Second-order kinetic

pH 6.8 buffer: $C_0/C_t = 0.019t + 1.180$ $R^2 = 0.97$ (3)

Where C_0 is the initial anthocyanins content (mg/L of cyanidin-3-glucoside equivalents) and Ct is the anthocyanin content (mg/L of cyanidin-3-glucoside equivalents) after t minute heating at 70 °C. The half-lives of monomeric anthocyanins (C_t/ C_0=0.5) at 70 °C were 44, 173 and 223 minute for pH 6.8 buffer, skim milk, and whole milk, respectively. Baublis and coworkers (1994) found that the degradation of tradescancia anthocyanins at room temperature and exposed to light, showed linearity (zero-order reaction) (*34*). However most studies on degradation kinetics of anthocyanins have indicated a first-order reaction for red radish anthocyanins (Giusti and Wrolstad, 1996), sour cherries anthocyanins (Cemeroglu et al., 1994), blackberry (Daravingas and Cain, 1968), concord grape, red cabbage, and ajuga anthocyanins (Baubli et al., 1994), Labuza and Riboh pointed out that most quality related reaction

rates are either zero or first-order reactions and statistical differences between the two types may be insignificant (Labuza and Riboh, 1982). The degradation rate of monomeric anthocyanins in close to neutral environment (pH 6.8) followed a second-rate reaction, which means degradation rate of monomeric anthocyanins change sharply compared to the first-rate reaction as the concentration of anthocyanins decreased. However, the degradation rate of monomeric anthocyanins.

Further analysis was done with HPLC. The percentage of anthocyanins remaining in different matrices after heat treatment at 70 ℃ for 120 min is shown in **Table 5.2**. The concentration of every individual anthocyanin in pH 6.8 buffer decreased significantly faster than in the milk matrices during heat treatment ($p<0.05$). The concentration of individual derivatives of cyanidin, pelargonidin, and peonidin were not significantly different between the skim milk and whole milk except the cyanidin-3-glucoside and penonidin-3-glucoside ($p<0.05$). Cyanidin-3-glucoside was most sensitive to the heat treatment with 20.9%, 64.8%, and 78.4% remaining after heat treatment in pH 6.8 buffer, skim milk, and whole milk, respectively. Generally, acylated anthocyanins were more resistant than the nonacylated for the heat treatment. Peonidin-3- (6′′-malonylglucoside) was most resistant for heat treatment with minimal change in skim milk and whole milk. The higher concentration of anthocyanins present in skim milk and whole milk after heating treatment compared to control, suggested that food components such as protein and fat might exert a protective effect over anthocyanins from heat degradation in milk with the same neutral environment

(pH 6.8) followed the zero-order kinetic, a slower reaction, suggesting that milk components protected anthocyanins from heat damage.

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Anthocyanin	Matrices		
	pH 6.8 buffer	Skim Milk	Whole milk
Cy-3-glucoside	20.9 ± 0.26 [a]	64.8 ± 0.48 [b]	78.4 ± 0.60 [c]
Pg-3-glucoside	24.5 ± 0.20 [a]	72.3 ± 0.15 [b]	79.3 ± 1.3 [b]
Pn-3-glucoside	28.4 ± 0.38 [a]	71.5 ± 0.67 [b]	82.7 ± 0.31 [c]
Cy-3-(6"- malonylglucoside)	33.4 ± 1.2 [a]	84.3 ± 1.1 [b]	91.4 ± 0.62 [b]
Pg-3-(6"- malonylglucoside)	29.1 ± 1.9 [a]	84.8 ± 1.3 [b]	80.7 ± 3.3 [b]
Pn-3-(6"- malonylglucoside)	32.9 ± 0.52 [a]	106.3 ± 10.2 [b]	103.0 ± 4.70 [b]

Cy: cyanidin; Pg: pelargonidin; Pn: peonidin
Different superscript letters in the same row is that the mean difference is significant at the 0.05 level by Least Significant Difference Test (LSD) (mean ±SE, n=3).

Table 5.2: Percentage of anthocyanins remaining in different matrices after heat treatment at 70 ˚C for 120 min

5.5 CONCLUSIONS

The limited solubility of ARW from purple corncob in acidified water was attributed to the formation of anthocyanin complexes with macromolecules such as protein and phenolics. However, ARW was easily solubilized at pH close to neutral or above, suggesting the use of these waste materials as natural colorants for foods with pH close to neutral.

Purple corn ARW was effective providing color to milk matrices. Milk components such as proteins and fats seemed to protect anthocyanins from degradation when exposed to heat. Acylated anthocyanins such as peonidin-3- (6”-malonylglucoside) were more resistant to heat treatment. The results obtained from this study suggest that it may be possible to use a by-product of anthocyanin extraction from purple corncob to provide color to products with a pH range unusual for anthocyanin applications and to develop novelty products with new flavors, colors and health benefits.

5.6 ACKNOWLEDGMENTS

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CHAPTER 6

EFFECTS OF COMPOSITION OF ANTHOCYANIN-RICH EXTRACTS ON THEIR CHEMOPROTECTIVE PROPERTIES [a] [b]

6.1 ABSTRACT

Anthocyanins are potent antioxidants and may be chemoprotective. However, the structure/function relationships are not well understood. Our objectives were to compare the chemoprotective properties of anthocyanin-rich extracts with variable anthocyanin profiles to increase the understanding on the role of anthocyanin structures on their chemoprotection. In addition, we also investigated the role of other

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phenolics present on chemoprotective effects. Anthocyanin-rich extracts (AREs) with different anthocyanin profiles from purple corn (*Zea mays* L.), chokeberry (*Aronia meloncarpa* E.), bilberry (*Vaccinium myrtillus* L.), purple carrot (*Daucus carota* L.), grape (*Vitis vinifera* L.), radish (*Raphanus sativus* L.), and elderberry (*Sambucus nigra* L.) were tested to determine the concentration needed to inhibit growth of human colorectal adenocarcinoma (HT29) by 50% (GI_{50}). Extracts were fractionated into an anthocyanin fraction (ACN) and other phenolics fraction (OPF) using a C18-cartridge and tested for synergistic, additive or antagonistic effect. All AREs inhibited colon cancer cell proliferation to varying degrees. Purple corn ARE showed the highest growth inhibition (GI_{50}~14μg/ml), followed by chokeberry and bilberry. Elderberry and radish showed the lowest inhibition (GI_{50}:~130μg/ml; ~108μg/ml). This variable inhibitory effect may be attributed to the presence of type of aglycone, anthocyanin glycosidation, and /or acylations. ACN, rather than other OPF, played an important role in the chemoprotective effects of AREs although generally they exerted additive interactions towards the total inhibitory effects. Anthocyanin-based colorants may be chemoprotective and therefore valuable ingredients for functional foods. Our results should provide light on what anthocyanin structures to choose for further application in functional foods (increased chemoprotection) and crop and cultivar selection.

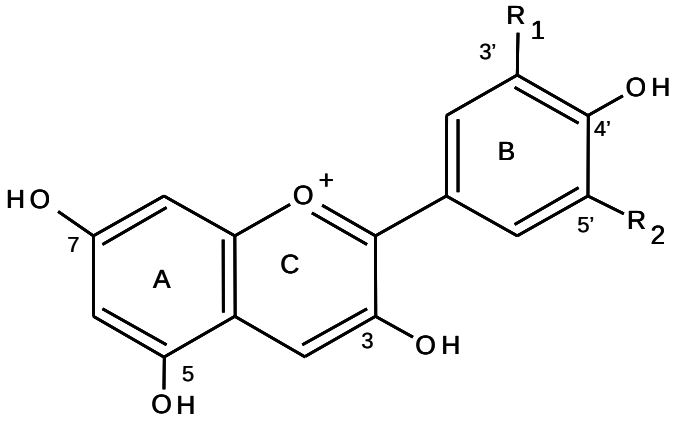
KEYWORDS: anthocyanins; colon cancer; HT29; cell proliferation; anthocyanin-rich extracts

6.2 INTRODUCTION

Anthocyanins are a class of flavonoid compounds responsible for the bright attractive red, orange, purple, and blue colors of most fruits and vegetables. Interest in anthocyanins has increased due to their color characteristics as colorants exempt from certification and potential health benefits as value-added ingredients (Giusti and Wrolstad, 2003). Compared to the average intake of other flavonoids that is 23 mg/person totally (Hertog et al., 1993), anthocyanins are the most abundant dietary flavonoids with a daily consumption of about 200 mg/person (Kühnan, 1976) but it was reported about 12.5 mg/day/person in the United States from a more recent publication {Wu, 2006 #35}. Anthocyanins are rich in many daily foods: berries, purple carrot, purple corn, red radish, red cabbage, et al. Structures of common anthocyanins in fruits and vegetables are showed in **Figure 6.1**. Anthocyanins may contribute to protective effects of fruits and vegetables again chromic disease, including cancer, revealed by epidemiological studies (Glade, 1999).

Colon cancer is the 3rd most common cancer and the 3rd leading cause of cancer death for both men and women in the United States (American-cancer-society, 2006). Anthocyanin-rich foods and anthocyanin pigments have been suggested as potential foods or food ingredients to reduce the risk of colon cancer from recent studies. Anthocyanin-rich foods showed great antiproliferation of human colon cancer cells *in vitro*. Anthocyanin-rich berries including blueberries, black currant, black chokeberries, lingonberries, cherries, and raspberries suppressed the proliferation of

HT29 in a dose-dependant manner (Olsson et al., 2004). AREs from grape, bilberry and chokeberry suppressed 50% proliferation of HT29 cells at 25~75 μg/ml (equivalents as cyanidin 3-glucoside) with chokeberry being the most potent inhibitor, with little effect on non-carcinogenic colon cells (NCM460) at the same concentration range (Zhao et al., 2004).



Anthocyanidins	Substitutes		λ_{max} (nm) visible spectra	Molecular Weight
	R_1	R_2		
Pelargonidin	H	H	494 (orange)	271
Cyanidin	OH	H	506 (orange-red)	287
Delphinidin	OH	OH	508 (red)	303
Peonidin	OCH_3	H	506 (orange-red)	301
Petunidin	OCH_3	OH	508 (red)	317
Malvidin	OCH_3	OCH_3	510 (bluish-red)	331

Figure 6.1: Structures of common anthocyanidins in fruits and vegetables

Anthocyanins were suggested to play an important role in the growth inhibitory effect of anthocyanin-rich berries. Anthocyanin fractions from bilberries (Katsube et al., 2003), blueberries (Yi et al., 2005), and red wine (Kamei et al., 1998), rather than other fractions, were found to effectively inhibit the growth of many human colon cancer cell lines, including CaCo-2 (Yi et al., 2005), HCT116 (Katsube et al., 2003), HCT15 (Kamei et al., 1998), HL60 (Katsube et al., 2003), and HT29 (Yi et al., 2005). Different *in vivo* studies have shown that anthocyanin-rich foods and anthocyanin pigments from food were potent to inhibit carcinogenesis in colon (Aoki et al., 2004; Hagiwara et al., 2001; Harris et al., 2001; Kang et al., 2003).

However so far methods to assess antiproliferation of anthocyanin-rich sources vary due to approaches to prepare samples prior to treatment on cells and different assays applied (Kamei et al., 1998; Kang et al., 2003; Katsube et al., 2003; Olsson et al., 2004; Yi et al., 2005; Zhao et al., 2004). It is difficult to evaluate anthocyanin-rich sources systematically and consistently. Thus our aims of the present research were to understand the relationship of anthocyanin structures and corresponding biological activities and also to develop methodology for *in vitro* screening cancer-suppressive effect of food components and to evaluate the chemoprotective activity of several anthocyanin extracts with different anthocyanin profiles prepared for the food industries as natural colorants or value-added food ingredients. Synergistic or antagonistic effect between anthocyanins with other phenolics was also studied in this study.

6.3 METHODS AND MATERIALS

6.3.1 Anthocyanin Sources

A total of seven anthocyanin extracts were screened to cover different possible main characteristics in pigment profiles (**Table 6.1**): a single aglycone with different sugars substitutions: chokeberry (*Aronia meloncarpa* E.), elderberry (*Sambucus nigra* L.); different aglycones with different simple sugars substituents: bilberry (*Vaccinium myrtillus* L.); different aglycone groups, with only glucose as sugar substitute: grape (*Vitis vinifera* L.); a single aglycone with high number of substitution, including different sugars and acylating groups, representing acylated food colorants: purple carrot (*Daucus dacota* L), and red radish (*Raphanus sativus* L.). In addition, we evaluated the effect of processing on purple corn by evaluating different purple corn extracts (purple corn waste, purple corn extract, and purple corn colorant. All extracts were commercially available and were kindly donated as described in **Table 6.1**.

6.3.2 HT29 Cell Line

The HT29 cell line derived from a colorectal adenocarcinoma (HTB 38; American Type Culture Collection, VA, USA) was grown in McCoy's 5A medium (Fisher Scientific, Fair Lawn, NJ, USA), which was supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA, USA) at 37 ˚C and 5 % CO_2 atmosphere.

Source	Aglycone	Glycosylation	Acylation	Ref.	Supplier
Purple corn (*Zea may* L.)	Cy, Pt, Pn	C3: mono-glycoside	One aliphatic acid	(Jing and Giusti, 2005)	1
Chokeberry (*Aronia meloncarpa* E.)	Cy	C3: mono-glycoside	None	(Wu et al., 2004)	2
Bilberry (*Vaccinium myrtillus* L.)	Dp, Cy, Pt, Pn, Mv	C3: mono-glycoside	None	(Kähkönen et al., 2003)	2
Purple carrot (*Daucus carota* L.)	Cy	C3: di-, tri-glycoside	One cinnamic acid	(Kammerer et al., 2003)	3
Grape (*Vitis inifera* L.)	Dp, Cy, Pt, Pn, Mv	C3: mono-glycoside	One cinnamic acid	(He et al., 2005)	4
Elderberry (*Sambucus nigra* L.)	Cy	C3: mono-; di-glycoside C3 and C5: di-, tri-glycoside	None	(Wu et al., 2004)	2
Radish (*Raphanus sativus* L.)	Pg	C3 and C5: tri-glycoside	More than one cinnamic acid	(Otsuki et al., 2002)	5

Abbreviation: Cyanidin (Cy); Delphinidin (Dp); Pelargonidin (Pg); Peonidin (Pn); Petunidin (Pt); Malvidin (Mv)

1. Globenatural International S.A. (Chorrillos-LIMA, Peru); 2. Artemis International, Inc. (Fort Wayne, IN, USA); 3. Overseal Foods Ltd (Derbyshire, UK); 4. Polyphenolics, Inc. (Madera, CA, USA); 5. RFI Ingredients (Blauvelt, NY, USA)

Table 6.1: Major anthocyanin profiles in seven commercial anthocyanin-rich sources

6.3.3 Reagents and Solvents

All reagents and solvents for HPLC, sample fractionation, and SRB assay were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Folin & Ciocalteau phenol reagent and standard of gallic acid (crystalline gallic acid, 98% purity) were purchased from Sigma (St. Louis, MO, USA).

6.3.4 Preparation of Anthocyanin Extracts

Commercial AREs were semi-purified using the method described by Giusti and Wrolstad (1996), modified for use of the extracts on cell cultures. Briefly, about 0.5 g of each commercial ARE was dissolved in 25 ml of deionized water, sonicated for 15 minute in FS28H Ultrasonic Cleaner from Fisher Scientific and filtered through a Whatman No. 4. About of a 3~5 ml of filtrate was passed through the C18 Sep-Pak solid cartridge (5 g) (Waters Corp., Milford, MA, USA). Anthocyanins and other phenolics were bound to the C18 cartridge, while sugars and other polar compounds were removed with 30 ml of 1%-acetic-acid-acidified water, followed by 10 ml of hexane. Anthocyanins and other phenolics were recovered with 30 ml of methanol containing 1% acetic acid and 5 ml of deionized water. The methanol was removed by rotary evaporation at 40 °C in a Büchi Rotavapor (Brinkmann Instruments, Inc., Westbury, NY, USA), and the residue was taken up to about 10 ml with deionized water in a volumetric flask, then deeply frozen at -70 ºC and lyophilized in a Labconco freeze dry system (Labconco Corp. Kansas, MO, USA).

6.3.5 Anthocyanin Fraction and Other-phenolics Fraction

Anthocyanin fraction (ACN) and other-phenolics fraction (OPF) were prepared by C18 Sep-Pak solid cartridge. About 0.5 g of each commercial ARE was dissolved in 25 ml of deionized water, sonicated for 15 minute, filtered with Whatman No. 1. About 5 ml of the filtrate was passed through a C18 Sep-Pak solid cartridge (5 g). Anthocyanins and other phenolics were bound to the C18 cartridge, while sugars and other polar compounds were removed with 30 ml of 1 %-acetic-acid-acidified water, followed by 10 ml of hexane to remove the water-dead-column- volume. The non-anthocyanin phenolics (OPF) were eluted with 20 ml of diethyl ether followed by 20 ml of ethyl acetate. The anthocyanin fraction was eluted with 30 ml of methanol containing 1% acetic acid followed by 5 ml of deionized water. The other phenolics fraction pooled was mixed with 5 ml of deionized water before rotary evaporation in case other phenolics are too dry to re-dissolve in water and also in order to keep the operation same to anthocyanin fraction. When most of methanol was removed by rotary evaporation at 40 °C, and each residue was added up to about 10 ml with deionized water, then froze and lyophilized.

6.3.6 Cell Growth Inhibition

The HT29 cells were plated at 1.3×10^4 cells/well in 24-well plates (Falcon) using McCoy's 5A medium containing 10% fetal bovine serum. Cells were allowed to grow 24 hours to attain log phase growth at the time of sample addition (time 0). Semi-purified AREs or others were compared for their inhibitory effects at

concentrations normally ranging from 0 to 200 μg/ml of growth media. HT29 cell growth was determined after additional 48 hr of incubation with different levels of anthocyanins by using the sulforhodamine B assay (SRB assay) at 565 nm. Every treatment had 4 replicates every time, and whole experiments were repeated three or four times. The percentage growth inhibition is calculated as:

$$\% \text{ Growth inhibition} = 100 - (T_{trt} - T_0) \times 100 / (T_{ctr} - T_0)$$

T_{trt} is the absorbance of sample with treatment of anthocyanins at 565 nm.

T_0 is the absorbance of sample after the first 24 incubation (time 0) prior to the treatment of anthocyanins.

T_{ctr} is the absorbance of sample without treatment of anthocyanins after the total incubation.

6.3.7 Sulforhodamine B Assay

The detailed methodology for SRB assay was described by Skehan et al., (1990). Briefly, cells were fixed by addition of 250 uL of 50 % trichloroacetic acid (TCA) at 4 °C for 1 hour. TCA and media were removed and wells were washed with water 5 times and dried at room temperature. SRB (0.4 % in acetic water) of 500 μL was added to each well to stain cells at room temperature for 20 min. Wells were washed using 1% acetic acid 5 times and dried at room temperature. The incorporated dye was then solubilized with 1 ml of 10 mM Tris for 5 min at room temperature on a shaker. The absorbance at wavelength of 565 nm was measured using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA)

6.3.8 Monomeric Anthocyanins

The total monomeric anthocyanin content was measured by the pH differential method (Giusti and Wrolstad, 2001). A HP UV-visible spectrophotometer (Agilent Technologies, Inc. Palo Alto, CA, USA) was used to read absorbance at the maximum visible wavelength of absorption of each extract (ranging from 500 -525nm) and at 700 nm. Monomeric anthocyanins were calculated as equivalents of cyanidin-3-glucoside, using the extinction coefficient of 26900 L cm^{-1} mg^{-1} and a molecular weight of 449.2 g/L (Giusti and Wrolstad, 2001), or as equivalents of pelargonidin-3-glucoside, using the extinction coefficient of 31600 L cm^{-1} mg^{-1} and a molecular weight of 433.2 g/L (Giusti and Wrolstad, 1996), in the case of radish which contains only pelargonidin derivatives. Disposable cuvettes of 1-cm-path-length were used.

6.3.9 Total Phenolics

Total phenolics were measured using a modified method of microscale protocol for Folin-Ciocalteu colorimetry (Waterhouse, 2001). The absorbance of the samples and standards was measured at 765 nm. Total phenolics were calculated as gallic acid equivalents based on a gallic acid standard curve. Instead of using cuvettes, 24-well plates were applied in a Multi-Detection Microplate Reader. Briefly, samples and a series of concentration (0 ~500 mg/L) of gallic acid calibration standard were put in 2 ml of vials with caps. Deionized water was added up to 1.6 ml in these vials, followed by 100 μL of Folin-Ciocalteu reagent. They were mixed well by inverting and incubated about 1 to 8 min. Then 300 μL of 20% sodium carbonate solution was

added and mixed well. The final volume was 2 ml. Samples were incubated in 40.0 ± 0.1 ˚C for 20 min and cooled down to room temperature immediately in ice. Each sample (1 ml) was transferred to 24-well plate and absorbance was measured at 765 nm.

6.3.10 HPLC Analysis

A high performance liquid chromatograph (HPLC) system (Waters Delta 600 systems, Milford, MA, USA) equipped with a photodiode array detector (Water 996, Milford, MA, USA.), autosampler (Waters 717 plus, Milford, MA, USA), and Millennium^{32} software (Waters Corp., Milford, MA, USA) was used.

Columns and mobile phase: The reversed-phase 5 µm Symmetry C18 column (4.6×150mm, Waters Corp., Milford, MA, USA) fitted with a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corp., Milford, MA, USA) was used. The solvents used were A: 1% phosphoric acid / 10% acetic acid / 5% acetonitrile in water and B: 100% acetonitrile. Solvents and samples were filtered though 0.45 µm poly(tetrafluorothylene) membrane filters (Pall Life Sciences, MI, USA) and 0.45 µm polypropylene filters (Whatman Inc., Clifton, NJ), respectively. Separation was achieved by using a linear gradient from 0 to 30% A in 35 min. An injection volume of 50 µL with a 1 ml/min flow rate was used. Spectral information over the wavelength range of 260-600 nm was collected.

6.3.11 Statistical Analysis

Regression analysis was used to model the growth inhibition of cells with the different ARE treatments. Tukey HSD test was used to evaluate mean differences among values of GI_{50} values or growth inhibition (%) when at same concentration in one-way ANOVA model. Student t-test was used to determine differences in the mean CI values comparing with a null hypothesized CI = 1 ($p<0.05$). The effects of anthocyanin chemical structure on the properties of the growth inhibition in colon cancer cells were evaluated by classifying anthocyanins according to the type of aglycone (6 aglycones evaluated), number of glycosidic substitutions, and acylation (no acylation versus aliphatic or cinnamic acid substitutes). The multiple linear regression analysis in a general linear univariate model was conducted using a stepwise procedure to remove variables which weren't significantly related to anthocyanin inhibitory activity.

All analyses were performed using SPSS (14.0) software. For all statistical calculations, $p<0.05$ was considered to be statistically significant.

6.4. RESULTS AND DISCUSSION

6.4.1 Methodology Development

The SBR assay was applied through this study to determine the cell growth. This method is recommended for *in vitro* screening for colorful anthocyanins which showed the interference with final reading in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide] assay from our previous study. Adherent cell cultures were fixed in situ by 50% cold TCA in SRB test and the residue of anthocyanins was washed out by 1%-acetate-acid water. The concentration of cell seeding had to be decided by experiment for sure to be the linear regression with the final reading after 72 hr incubation in 24-well plate. Different levels of cell concentrations (0, 1×10^3, 5×10^3, 1×10^4, 5×10^4, and 1×10^5 cells/ml) were tested by SRB assay after 72 hr of growth in McCoy's 5A medium supplemented with 10% fetal bovine serum at 37 ˚C and 5% CO_2 atmosphere. The linear range was located in about 0~1×10^4 cells/well (**Figure 6.2**). The seeding concentration of HT29 was 1.3×10^4 in 24-well plate where the absorbance at 565 nm is about 1, which was determined by the regression curve and equation ($R^2 = 0.9985$) in another experiment.

Acids were added to stabilize anthocyanins during semi-purification and fractionation. However, the residue of acids in extracts would affect the cell culture test due to their cytotoxicity (Mori and Wakabayashi, 2000; Treichel et al., 2004). The ARE from chokeberry were prepared by solution acidified either by 0.01% hydrochloride acid (HCl) or 1% acetic acid via rotary evaporation or rotary evaporation plus lyophilization to remove solvents and acids. The sample prepared in a manner with roto-evaporation and 0.01%-HCL-acidifed solvents showed significantly higher inhibition than other two combinations ($p<0.05$) and the sample prepared with rotary evaporation plus lyophilization and 0.01%-HCl-acidifed solvents still showed higher inhibition than rotary evaporation plus lyophilization and 1%-acetic-acid-acidifed solvents although not significantly different (**Figure 6.3**),

suggesting the amount of HCl residue was more toxic to cells than acetic acids residue, and the operation of rotary evaporation aided with lyophilization could remove the acids more effectively than one of only rotary evaporation.

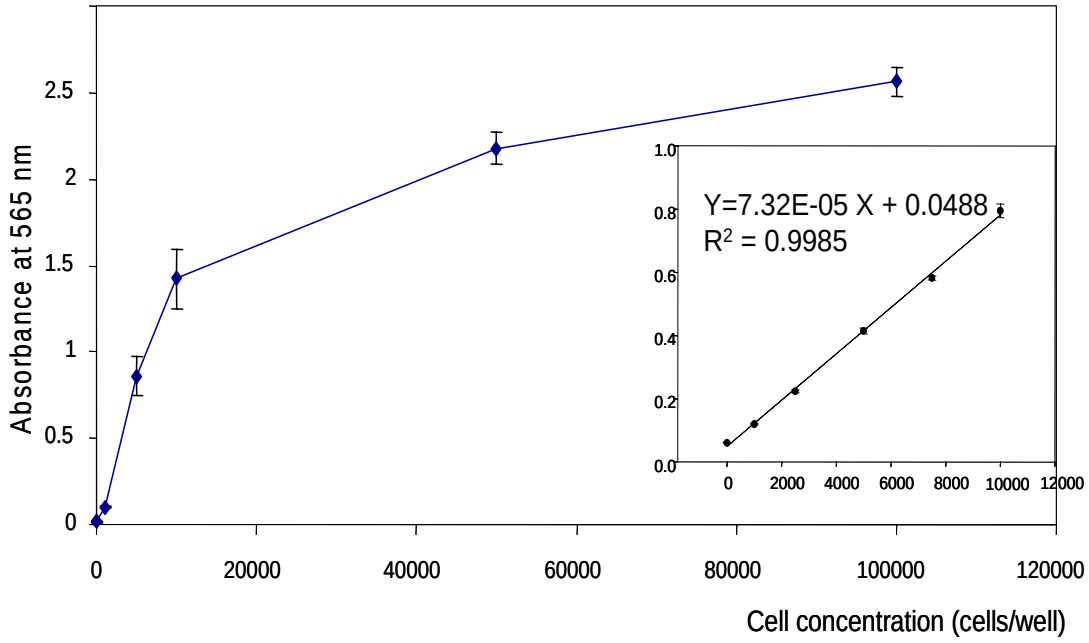


Figure 6.2: Method development of seeding concentration of HT-29 cell line in 24-well plate via SRB test. Values are represented as mean ± standard error (n=4).

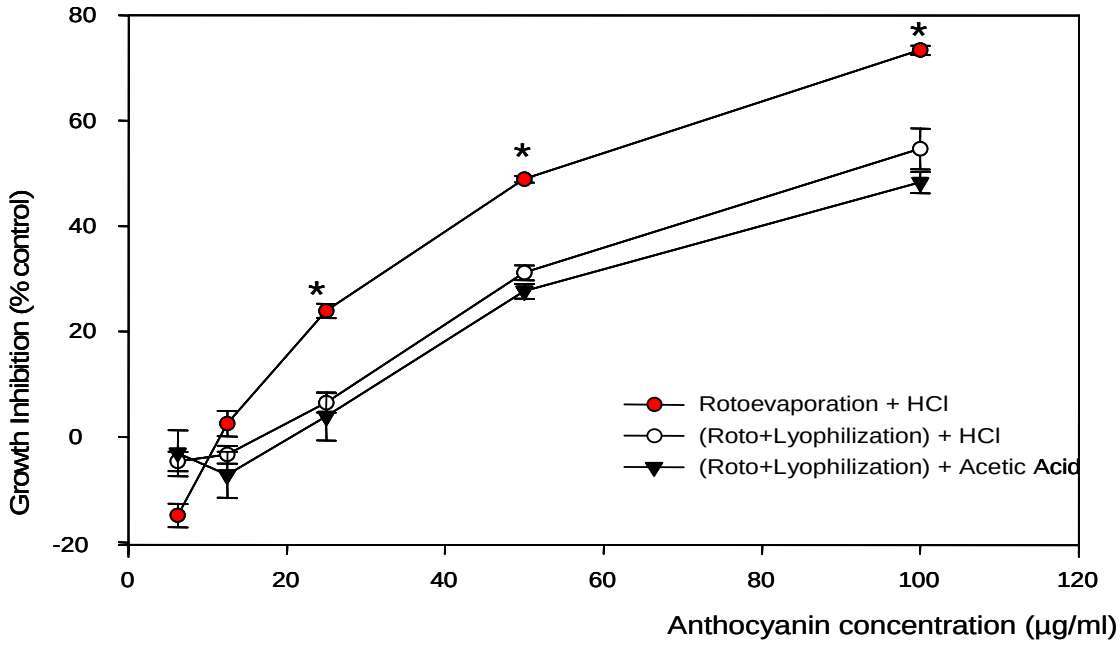


Figure 6.3: Effect of sample preparation on the growth of HT29 cells. Rotoevaporator + HCl: sample was prepared by 0.01%-HCl-acidified solvent which was removed by rotoevaperator; (Roto+Lyophilization) + HCl: sample was prepared by 0.01%-HCl-acidified solvent which was removed by rotary evaporation plus lyophilization; (Roto+Lyomphilization) + acetic acid: sample was preprared by 1%-acetic-acid-acified solvent which was removed by rotary evaporation plus lyophilization. Values are represented at mean ± standard error (n=4).

6.4.2 Growth Inhibition on HT29 Cell Line

All anthocyanin-rich extracts (AREs) exihibited great inhibitory effect on the growth of HT29 cells (**Figure 6.4**). The GI_{50} value, which was the concentration to inhibition 50% of cell proliferation, was calculated and statistically divided into three homogenous groups by Tukey HSD test at the level of $p<0.05$ according to their power to inhibit HT29 growth.

The AREs from purple corn, chokeberry and bilberry showed the greatest growth inhibition in this first group with GI_{50} values of 13.8, 31.2 and 32.2 μg/ml, respectively. The second group was AREs from purple carrot and grape (GI_{50}=68.5 and 71.2 μg/ml respectively). Radish and elderberry (GI_{50}=107.7 and 130.3 μg/ml respectively) were the third group showing relatively lower effectiveness to suppress the HT29 proliferation compared with other groups. The GI_{50} values obtained were much lower than the level of anthocyanins (700~2000 μg/ g wet weight) reported in rat faeces (He et al., 2005), suggesting that it is possible to achieve concentrations I the GIT high enough for any of these AREs to have activity *in vivo*. However, the GI_{50} values obtained with all ARE's were higher than the reported concentrations of anthocyanins in plasma (0.1~1.8%) after consumption of 200 mg anthocyanins/ person daily (Kay et al., 2004), suggesting that these anthocyanins could be less effective against cancers which need delivery of phytochemicals through the blood stream.

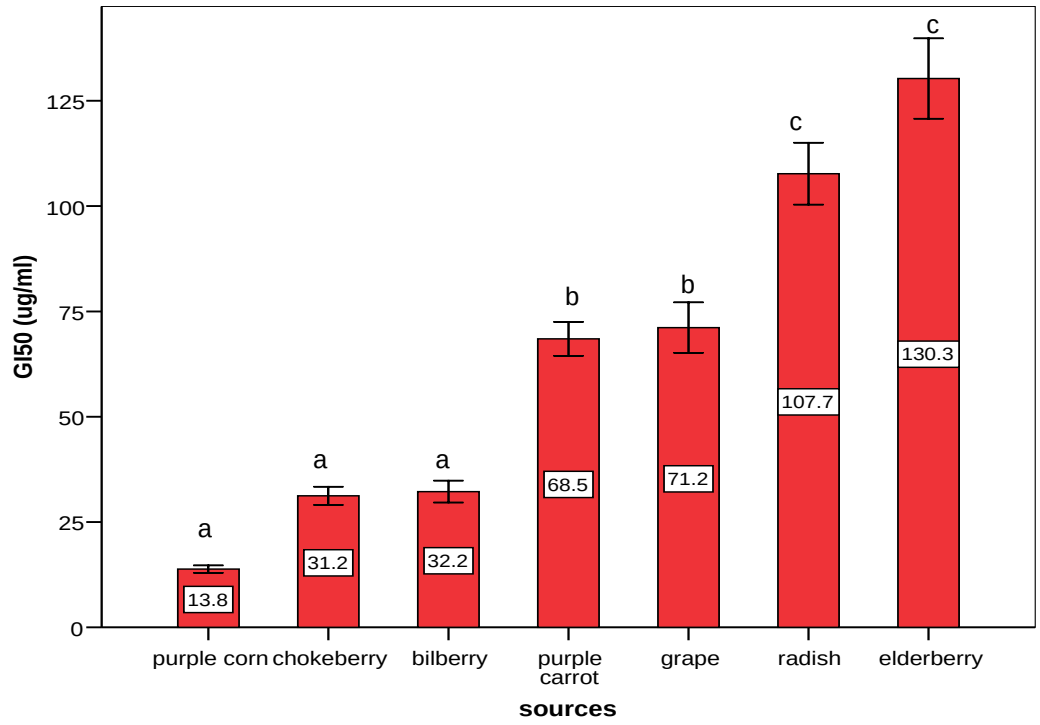


Figure 6.4: The GI_{50} of anthocyanin-rich extracts (based on the monomeric anthocyanins) from 7 natural sources on the growth inhibition of HT-29 cell line. Groups of a, b, and c were divided into homogenous subsets of GI_{50} means of anthocyanin-rich extract from different sources. Anthocyanin-rich extracts were significantly different among groups while anthocyanins-rich extracts within same group were not significantly different at the 0.05 level by least significant difference test (Tukey HSD). Values are represented as equivalents of cyanidin 3-glucoside (µg/ mL) and pg 3-glucoside (µg/mL) for radish.

Anthocyanins might play a major role in the chemoprotective effects of AREs from these different sources, based on our results obtained with the chokeberry

(**Figure 6.5**) and other published studies. Anthocyanin fractions from bilberry, rather than other fractions, suppressed the cell of HL60 cells (Katsube et al., 2003). In another study, the anthocyanin fraction showed the greatest antiproliferation than phenolic acid, tannin, and flavonols fractions from blueberries and induced apoptosis of HT29 cells (Yi et al., 2005). Anthocyanin fraction from red wine also showed much higher antiproliferation of HCT15 cells than other flavonoid fractions (Kamei et al., 1998).

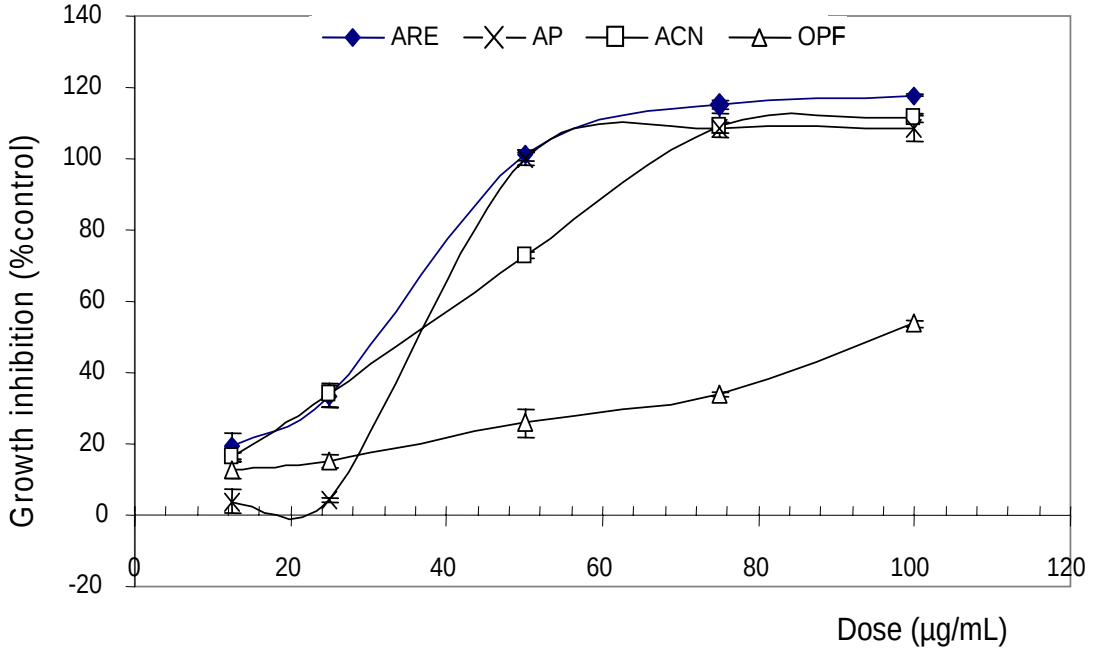


Figure 6.5: Growth inhibition of anthocyanin-rich extracts from chokeberry on HT-29 cell line. ARE: anthocyanin-rich extract; AP: reconstitution of anthocyanin fraction and other phenolic fraction; ACN: anthocyanin fraction; OPF: other phenolic fraction. Values are represented as mean absorbance ± standard error (n=4).

6.4.3 Additive, Synergistic or Antagonistic Interaction

In **Figure 6.5**, we illustrate how the anthocyanin fraction, rather than the other phenolic fraction, played a major role in the chemoprotective effects of AREs from chokeberry. The AP (anthocyanins plus other phenolics) reconstituted from anthocyanin and other phenolic fractions according to their original ratio in chokeberry ARE, showed inhibitory effect which was very similar to the AREs, suggesting our methodology can separate anthocyanins successfully from other phenolics without changing biological activities. The synergistic or antagonistic interaction of anthocyanins with other phenolics was determined by using the combination index (CI) reported by Chou and Talalay (1984) (Chou and Talalay, 1984). Specify what CI correspond to each type of interaction, and how (nule hypothesis) you made your conclusions. An additive effect of anthocyanin fraction and other phenolic fraction from chokeberry was observed in HT29 cell line (CI = 1.222/1.441/1.221 at the each level of GI_{25}, GI_{50} and GI_{100}) (**Table 6.2**).

An additive or synergistic interaction of anthocyanins, proanthocyanidins, and flavonols from cranberries was suggested for the growth inhibition of HT29 and HCT116 cells (Seeram et al., 2004). There were different mechanisms by which phytochemicals could act to exert their anticancer activities *in vivo*. Anthocyanins and other phenolics in chokeberry might have chemoprotective effects on a similar mechanism, which caused the additive effects on the growth inhibition of HT29 cells.

Source	Combination Index		
	GI_{25}	GI_{50}	GI_{100}
Anthocyanins + Other Phenolics	1.222	1.144	1.221
Combination Effect	Additive	Additive	Additive

Table 6.2: Combination index (CI) values of the interaction between anthocyanin fraction and other phenolics. The combination index values were calculated on the multiple drug effect equation. CI<1, =1, and >1 indicate synergistic, additive and antagonistic effects, respectively. Each value represents the mean of three independent experiments carried out in 4 replicates. Student t-test were computed to evaluate if significant differences in the mean CI values comparing with a null hypothesized CI of 1 at the level of 0.05. Values= Means ± Standard error (n=4).

6.4.4 Effect of Anthocyanin Chemical Structure on Chemoprotective Activity

Chemoprotective activities of anthocyanins were affected by the type of aglycones, sugars, and acylated acids as well as the position and degree of glycosylation and acylation (Koide et al., 1997; Yoshimoto et al., 2001; Zhang et al., 2005). Anthocyanin profiles of these seven sources were listed in **Table 6.1**. Chokeberry contains major anthocyanins including cyanidin 3- of galactoside, arabinoside, xyloside, while elderberry has major anthocyanins including cyanidin of 3-glucoside, 3-sambubioside, 3-samubioside-5-glucoside, 3,5-diglucoside (Wu et al., 2004). Chokeberry and elderberry both have the cyanidin as aglycone and are non-acylated anthocyanins. The only differences between them were the type and position

of sugar moieties to cyanidin. Purple carrot is another source which only has the cyanidin as aglycone with acylated or non-acylated complex sugar substitute pattern: cyanidin of 3-xylosyl-galactoside, 3-xylosyl-glucosyl-galactoside and cyanidin of 3-xylosyl-glucosyl-galactoside acylated with one cinnamic acid (Stintzing et al., 2002). The GI_{50} of purple carrot ARE was between the chokeberry and elderberry. And again, anthocyanins in grape are a mixture of 5 different aglycones with glucose moiety acylated and non acylated with one cinnamic acid. Both the purple carrot and grape have the cinnamic acid and they were in same contains pelargonidin 3-sophoroside-5-glucoside acylated with one or two cinnamic homogenous subset of GI_{50} means. However, radish (*Raphanus sativus*) which acids showed relatively low inhibitory effect that is similar to elderberry. This difference might be due to the presence of cinnamic acids acylated with sugar moieties, or to the type of aglycone. Among the sources assayed, only radish and elderberry contained anthocyanins with glycosylation at 3,5-diglycosides. The 3,5-glycosylation on anthocyanidins might have impact on their biological activities.

To evaluate the structure/function relationship of anthocyanins, individual anthocyanins were classified according to the type of aglycone, glycosylation, and acylation in quantity by analytical HPLC (**Table 6.3**) and then anthocyanin structural properties were analyzed statistically with GI_{50}. There were six variables related to aglycone, including cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. Three variables regarding glycosylation: mono-, di-, and tri-glycoside. Anthocyanins were also classified into non-acylated anthocyanins, anthocyanins

acylated with aliphatic acid, and anthocyanins acylated with cinnamic acid. Multiple linear regression analysis (R^2= 0.977, *p<0.001*) determined that if the presence of acylating groups, type of aglycone, and number of sugars substitutions on the anthocyanin molecule affect their chemoprotective properties by using SPSS (14.0) software (**Equation1**).

$$y = - 4.473 + 0.07 \times x_1 + 0.046 \times x_2 + 0.014 \times x_3 – 0.01 \times x_4 + 0.005 \times x_5$$

$R^2 = 0.977$ **Equation 1**

y: $Lg10(GI_{50})$

x_1: percentage of nonacylated anthocyanins

x_2: percentage of anthocyanins acylated with cinnamic acids

x_3: percentage of anthocyanins with pelargonidin as aglycone

x_4: percentage of anthocyanins glycosylated with mono-glycoside

x_5: percentage of anthocyanins glycosylated with tri-glycoside

Pelargonidin (R=0.537, *p=0.006*), tri-glycoside (R=0.609, *p=0.001*), and acylation with cinnamic acid (R=0.591, *p=0.002*) were significantly correlated to the base-10 logarithm of GI_{50} values, suggesting that anthocyanin with pelargonidin, tri-glycoside, or acylation with cinnamic acid might decrease the inhibitory effect of anthocyanins on HT29 cells. Mono-glycoside (R= -0.724, *p<0.0001*) and non-acylation (R= -0.568, *p=0.003*) was negatively correlated with Lg10 (GI_{50}) values, indicating anthocyanins with mono-glycoside or non-acylation might enhance anthocyanin inhibitory effects. Future work need to confirm if these structural properties have impact on their biological activities.

Source	GI_{50} (μg/ml)	Aglycone (%)						Glycosylation (%)			Acylation (%)		
		Cy	Pg	Pn	Dp	Pt	Mv	Mono	Di	Tri	N.A.	Ali	Cinn
Purple corn	13.8	73.4	6.5	20.1	0	0	0	100	0	0	93.6	6.4	0
Chokeberry	31.2	100	0	0	0	0	0	100	0	0	100	0	0
Bilberry	32.2	23.4	0	8.1	27.0	16.1	25.5	100	0	0	100	0	0
Purple carrot	68.5	100	0	0	0	0	0	0	46.1	53.9	58.0	0	42.0
Grape	71.2	3.0	0	28.6	6.5	15.0	44.3	21.3	76.2	0	85.3	0	12.2
Radish	107.7	0	100	0	0	0	0	0	0	100	0	0	100
Elderberry	130.3	100	0	0	0	0	0	45.5	44.7	9.8	100	0	0

Table 6.3: Structural contribution of anthocyanins in different anthocyanin-rich extracts and their GI_{50} on the growth of HT29 cell line. Abbreviation: Cyanidin (Cy); Delphinidin (Dp); Pelargonidin (Pg); Peonidin (Pn); Petunidin (Pt); Malvidin (Mv); Mono: mono-glycoside; Di: diglycoside; Tri: tri-glycoside; N.A.: none; Ali: aliphatic acid; Cinn: cinnamic acid

6.5 CONCLUSIONS

The AREs from purple corn, chokeberry, bilberry, purple carrot, elderberry, and radish may provide chemoprotective benefits to the colon and become value-added food ingredients for functional foods. Additive interaction between anthocyanins and other phenolics suggests that further purification would not be required for their whole chemoprotective activities as functional ingredients. The chemical structures of anthocyanins do make a difference on their biological activities, and more research is being carried out to better understand the partial contribution of the different portions of the molecule and their relationships.

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CHAPTER 7

IMPACT OF ANTHOCYANIN CHEMICAL STRUCTURE ON THEIR CHEMOPROTECTIVE ACTIVITY [a] [b]

7.1 ABSTRACT

Anthocyanins are the pigments responsible for most of the orange/red to purple/blue colors in fruits and vegetables. Anthocyanins have been found to possess chemoprotective activity. However, different chemical structures seem to differ in their bioactivity. In this study, our objective was to study the effects of acylation with aliphatic acids or cinnamic acids, glycosylation pattern as 3-diglycoside-5-glycoside,

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and aglycone on chemoprotection of anthocyanins. Anthocyanins were isolated (anthocyanin fractions) from purple corn (*Zea mays* L.), radish (*Raphanus sativus* L.) and red cabbage (*Brassica oleracea* L.) and compared to their corresponding de-acylated pigments, obtained by saponification followed by purification on a C18-cartridge. Anthocyanin fractions, deacylated anthocyanin fractions, and the pure standards of cyanidin 3-glucoside, and pelargonidin 3-glucoside were evaluated for growth inhibition of human colorectal adenocarcinoma (HT29). Saponification of purple corn anthocyanins resulted in an increased inhibitory effect of HT29 cell proliferation, suggesting that non-acylated purple corn anthocyanins are more effective chemoprotective agents than their counterpart acylated with an aliphatic acid. Acylation with cinnamic acids was found to have different impact on the chemoprotective actions of anthocyanins. Deacylation of cyanidin 3-sophoroside-5-glucoside significantly increased growth inhibition of HT29 cells while deacylation of pelargonidin 3-sophoroside-5-glucoside was on the contrary. The cyanidin 3-glucoside and pelargonidin 3-glucoside both showed a higher inhibitory activity than cyanidin 3-sophoroside-5-glucoside and pelargonidin 3-sophoroside-5-glucoside respectively. Pelargonidin 3-glucoside has been shown to have lower biological effect than cyanidin 3-glucoside in terms of the inhibitory effect on the growth of HT29 cells

KEYWORDS: anthocyaninin chemical structure; HT29; radish; red cabbage; aglycone; glycosylation; acylation

7.2 INTRODUCTION

Interest in anthocyanins has increased due to their appealing color characteristics as certification-exempt colorants and health benefits as value-added ingredients (Giusti and Wrolstad, 2003). Numerous studies have shown that anthocyanin-rich foods and anthocyanin pigment from food possessed high antioxidant power (Lee et al., 2004; Moyer et al., 2002; Prior et al., 1998; Wang and Lin, 2000) and prevented or delayed carcinogenesis in oral cavity (Casto et al., 2002), esophagus (Aziz et al., 2002; Carlton et al., 2001; Kresty et al., 2001; Stoner et al., 1999), and colon (Aoki et al., 2004; Hagiwara et al., 2001; Harris et al., 2001; Kang et al., 2003) in *in vivo* studies.

Commercially prepared anthocyanin-rich extracts (AREs) from purple corn (*Zea mays* L.), chokeberry (*Aronia meloncarpa* E.), bilberry (*Vaccinium myrtillus* L.), purple carrot (*Daucus carota* L.), grape (*Vitis vinifera* L.), radish (*Raphanus sativus* L.), and elderberry (*Sambucus nigra* L.) were shown to inhibit the proliferation of about 50% of the control in human colon tumor cell line HT29 at 14~130 μg/mL (Jing et al., 2006). AREs from chokeberry, bilberry, and grape used at similar concentrations had little effect on NCM460 non-carcinogenic colon cells (Zhao et al., 2004). Anthocyanins have been shown to play a major role in the growth inhibitory effect of anthocyanin-rich berries and anthocyanin chemical structure may affect this inhibitory activity of AREs.

There are a few studies on the relationship of anthocyanin structural effect on their chemopreventive actions. Most of these studies have been focused on different anthocyandins. Delphinidin showed greater inhibitory effect than cyanidin on cell growth in uterine carcinoma HeLa S3 cells and colon adenocarcinoma CaCo-2 cells (Lazze et al., 2004). Six most common anthocyanidins (cyanidin, pelargonidin, peonidin, delphinidin, petunidin, and malvidin) were evaluated on inhibition of TPA-induced JB6 mouse epidermal cell transformation and AP-1 transcription activity in an order: dephinidin > petunidin > cyanidin >> pelargonidin > malvidin > peonidin (Hou et al., 2004). Only pure delphinidin glycosylation isolated from the bilberry extract, but not malvidin glycosylation, inhibited the growth of HCT116 human colon carcinoma cells (Katsube et al., 2003). Cyanidin and delphinidin exerted much greater inhibitory effect than cyanidin 3-galacotoside, malvidin and malvidin 3-glucoside on the growth of human vulva carcinoma cell line A431 by suppressing the epidermal growth-factor receptor (EGFR) and blocking downstream signaling cascades (Meiers et al., 2001). Consequently, an ortho-dihydroxyphenyl structure on the B-ring seems to be a critical structure for anthocyanin chemopreventive activities.

However, very limited information is available on how the glycosylation or acylation of structural properties may affect anthocyanin chemoprotective actions. Although anthocyanin-rich extracts from foods with specific anthocyanin profiles showed variable chemoprotective activities in aspects of the antiproliferative effect on human colon adenocarcinoma HT29 cells, the relationship of anthocyanin structures and relative biological activities should be further clarified. An increased

understanding of the effects of chemical structure on the functionality of anthocyanins should provide light on what anthocyanin structures to choose for food application when looking for value-added ingredients. In addition, this study should provide valuable information regarding structure/function relationships that could be used for future studies on the molecular mechanisms of chemoprotective activities from anthocyanins.

7.3 MATERIALS AND METHODS

7.3.1 Materials

Radish (*Raphanus sativus* L.) and red cabbage (*Brassica oleracea* L.) extracts were provided by RFI Ingredients (Blauvelt, NY, USA). Pelargonidin 3-glucoside and cyanidin 3-glucoside were purchased from Polyphenols Laboratories AS (Sandnes, Norway). Purple corn (*Zea mays* L.) colorant was provided by Globenatural International S.A. (Chorrillos-LIMA, Peru).

7.3.2 HT29 cell line

The HT29 cell line derived from a colorectal adenocarcinoma (HTB 38; American Type Culture Collection, VA) was grown in McCoy's 5A medium (Fisher Scientific, Fair Lawn, NJ, USA), which was supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA) at 37 ˚C and 5 % CO_2 atmosphere.

7.3.3 Reagents and Solvents

All reagents and solvents for HPLC, sample fractionation, and SRB assay were purchased from Fisher Scientific (Fair Lawn, NJ).

7.3.4 Anthocyanin Fractionation

Anthocyanin fractions (ACN) were obtained by purification with a C18 Sep-Pak solid cartridge as described by Jing et al (2006). About 0.5 g of each commercial ARE was dissolved in 25 ml of deionized water, sonicated for 15 minute, filtered with Whatman No. 1. About 5 ml of the filtrate was passed through the C18 Sep-Pak solid cartridge (5 g) (Waters Corp., Milford, MA). Anthocyanins and other phenolics were bound to the C18 cartridge, while sugars and other polar compounds were removed with 30 ml of 1 %-acetic-acid-acidified water, followed by 10 ml of hexane to remove the water-dead-column- volume. The non-anthocyanin phenolics fractions were eluted by 20 ml of diethyl ether and 20 ml of ethyl acetate. The anthocyanin fraction was eluted with 30 ml of methanol containing 1% acetic acid and 5 ml of deionized water. The methanol was removed by rotary evaporation at 40 °C and the residue was added up to 10 ml with deionized water, frozen and lyophilized.

7.3.5 Alkaline Hydrolysis (Saponification) of Anthocyanins

The anthocyanin fraction from anthocyanin-rich extract (1mL) was saponified in a screw-cap test tube with 10 mL of 10% aqueous KOH for 8 min at room temperature in the dark (Durst and Wrolstad, 2001). The solution was neutralized and

acidified by HCl (2 mol/L), and hydrolysate was further purified to remove the acids using the C-18 Sep-Pak cartridge procedure described in step 7.3.4.

7.3.6 Cell Growth Inhibition

The HT29 cells were plated at 1.3×10^4 cells/well in 24-well plates (Falcon) in McCoy's 5A medium containing 10% fetal bovine serum. Cells were allowed to grow 24 hours to attain log phase growth at the time of sample addition (time 0). Anthocyanin-rich extracts, purified anthocyanins or standards were compared for their inhibitory effects at concentrations ranging from 0 to 200 μg/ml of growth media. HT29 cell growth was determined after additional 48 hr of incubation with different levels of anthocyanins by using the sulforhodamine B assay (SRB assay) at 565 nm. Every treatment was in quadruplicates. Percentage growth inhibition was calculated as:

$$\% \text{ Growth inhibition} = 100 - (T_{trt} - T_0) \times 100 / (T_{ctr} - T_0)$$

T_{trt} is the absorbance of sample with treatment of anthocyanins at 565 nm.

T_0 is the absorbance of sample after the first 24 incubation (time 0) prior to the treatment of anthocyanins.

T_{ctr} is the absorbance of sample without treatment of anthocyanins after the total incubation.

7.3.7 Sulforhodamine B assay

The methodology for SRB assay (Skehan et al., 1990) was modified for HT29 cells which grow adherent to the bottom. Briefly, media was aspirated from each well

and 1 ml of Dulbecco's Phosphate Buffered Saline was added in each well. Cells were then fixed by an addition of 250 uL of 50% trichloroacetic acid (TCA) at 4 °C for 1 hour. TCA and other liquid were removed and wells were washed with water 5 times and dried at ambient temperature. SRB (0.4 % in acetic water, 500 µL) was added to each well at room temperature for 20 min to stain cells. Wells were washed using 1% acetic acid 5 times and dried at room temperature. The incorporated dye was then solubilized with 1 ml of 10 mM Tris for 5 min at room temperature on a shaker. The absorbance at wavelength of 565 nm was measured using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., VT, USA).

7.3.8 Monomeric Anthocyanins

The total monomeric anthocyanin content was measured by the pH differential method (Giusti and Wrolstad, 2001). A UV-visible spectrophotometer (Agilent Technologies, Inc. CA, USA) was used at their maximum visible wavelength of absorption (between 510-535nm) and 700 nm. Monomeric anthocyanins were calculated as equivalents of cyanidin-3-glucoside (purple corn and cabbage pigments), using the extinction coefficient of 26900 L cm^{-1} mg^{-1} and a molecular weight of 449.2 g/L (Giusti and Wrolstad, 2001), or as equivalents of pelargonidin-3-glucoside (for radish pigments), using the extinction coefficient of 31600 L cm^{-1} mg^{-1} and a molecular weight of 433.2 g/L (Giusti and Wrolstad, 1996). Disposable cuvettes of 1-cm-path-length were used.

7.3.9 Statistical Analysis

Regression analysis was used to model the growth inhibition of cells with the different ARE treatments. Tukey HSD test was used to evaluate mean differences among GI_{50} values or growth inhibition (%) when at the same concentration in one-way ANOVA model. All analyses were performed using SPSS (14.0) software. For all statistical calculations, $p<0.05$ was considered to be statistically significant.

7.4 RESULTS AND DISCUSSION

7.4.1 Acylation

7.4.1.1 Acylation with aliphatic acid

Purple corn colorant was reported to suppress the carcinogen-induced mutation (Aoki et al., 2004) and reduced colorectal carcinogenesis in rats (Hagiwara et al., 2001). In addition, we have found that purple corn colorant showed the greater inhibition on the growth of HT29 cells with a lower GI_{50} value (13.8 μg/ml) than chokeberry, bilberry, purple carrot, grape, radish and elderberry (Jing et al, 2006). Purple corn contains six major anthocyanins, cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, adding up to 76.9% of the total pigments, and their corresponding malonylated derivatives, that add up to ~ 23.1%. Aliphatic acids (malonic acid) were removed from purple corn anthocyanins by saponification, resulting on only three anthocyanins left: cyanidin-3-glucoside, pelargonidin-3-

glucoside, peonidin-3-glucoside. Deacylated anthocyanins and the counterpart exerted a dose-depend inhibitory effect on the growth of HT29 cells (**Figure 7.1**).

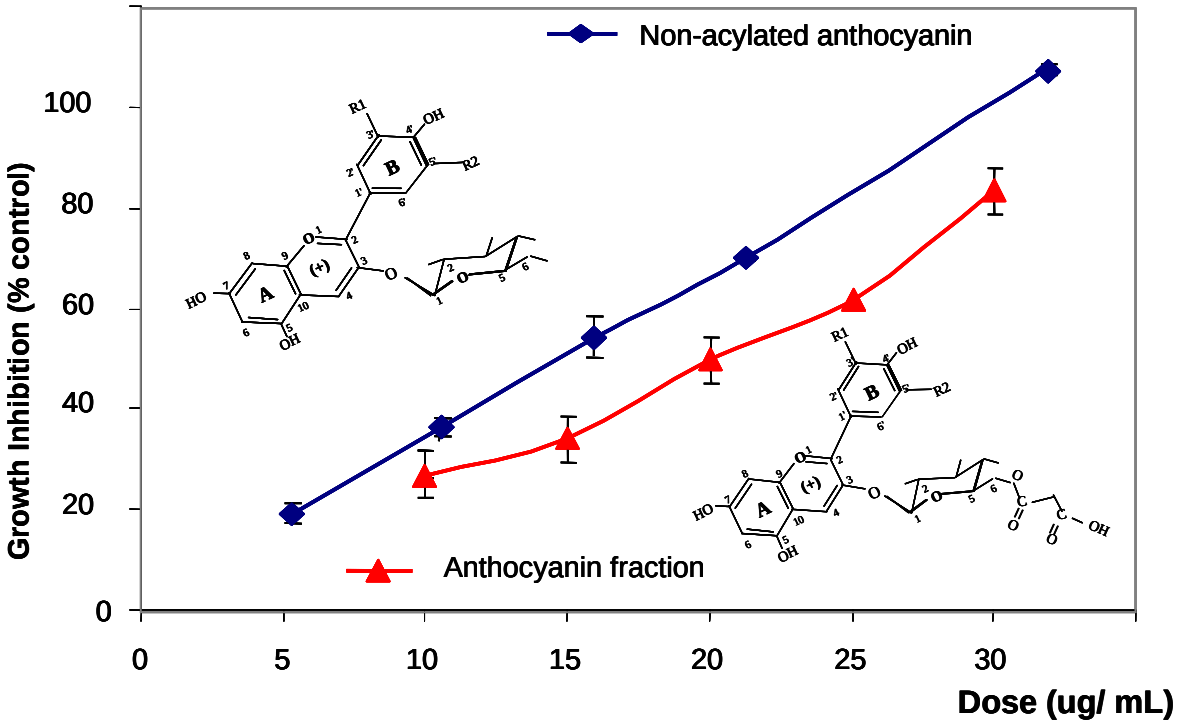

Figure 7.1: Effect of acylated vs deacylated anthocyanins from purple corn on growth inhibition of HT29 cells. Anthocyanins were quantified by the monomeric anthocyanins. Values are represented as Means ± Standard error (n=3).

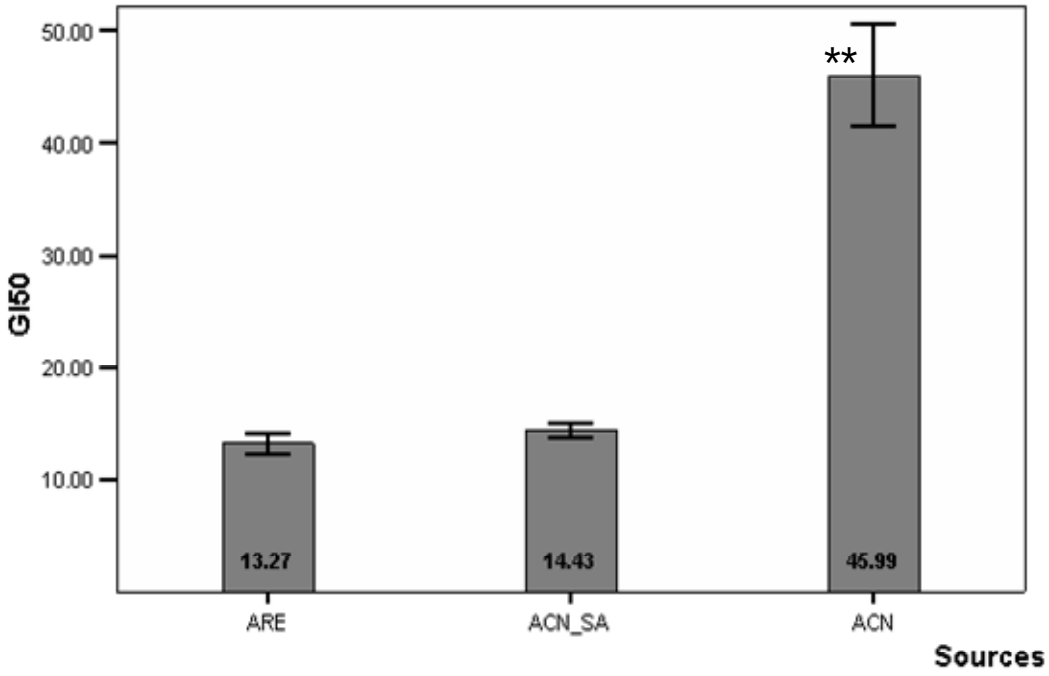

Figure 7.2: The GI_{50} of anthocyanin-rich extract, deacylated anthocyanin fraction and anthocyanin fraction from purple corn on the growth inhibition of HT-29 colon cell line. ARE, anthocyanin-rich extract; ACN_SA, deacylated anthocyanin-fraction; ACN, anthocyanin fraction. The mean of three experiments were carried out in quadruplicates. Anthocyanins were quantified by the monomeric anthocyanins. **, $p<0.01$. Values are represented as Means ± Standard error (n=3).

However deacylated anthocyanins showed higher growth inhibitory effect on HT29 cells than their acylated counterparts at doses ranging from 10 to 30 µg/ml. The

GI_{50} values of ARE, deacyalted anthocyanins, and ACN were 13.27, 14.43, and 44.99 µg/ml (**Figure 7.2**), suggesting that removal of the aliphatic acid increased the inhibitory effect of the pigments to levels comparable to the starting ARE material. Cyanidin 3-glucoside, the major anthocyanin in purple corn was reported to be the active component in purple corn, which reduced the genotoxicity of carcinogens (Aoki et al., 2004). The increased level of cyanidin 3-glucoside in purple corn anthocyanins after saponification might be contributing or playing an important role in the increased inhibitory effect.

7.4.1.2 Acylation with Cinnamic Acids

On a previous study testing different anthocyanin sources on human colon adenocarcinoma HT29 cells we determined by multiple linear regression analysis that anthocyanin acylation with cinnamic acid may be negatively correlated to the growth inhibitory effects (Jing et al, 2006). Red radish (*Raphanus sativus* L.) and red cabbage (*Brassica oleracea* L.) were used in this study to evaluate this structure/function relationship under controlled conditions. The anthocyanin-rich extract from red radish, containing mostly acylated pelargonidin derivatives was previously found to show relatively low inhibition of the growth of human colon cancer HT29 cells as compared to sources rich in cyanidin derivatives. The major anthocyanins in red radish were pelargonidin-3-sophoride-5-glucoside acylated with one or two aromatic acids (ferulic, caffeic and *p*-coumaric acids) to the diglucoside in **Figure 7.3** (Otsuki et al., 2002). The major acylated anthocyanins in red cabbage were cyanidin 3- sophoroside-5-

glucoside derivatives acylated with 1 or 2 aromatic acids (ferulic, sinapic, and *p*-coumaric acids) to the sophorose, as shown in **Figure 7.4** (Shimizu et al., 1997; Wu and Prior, 2005).

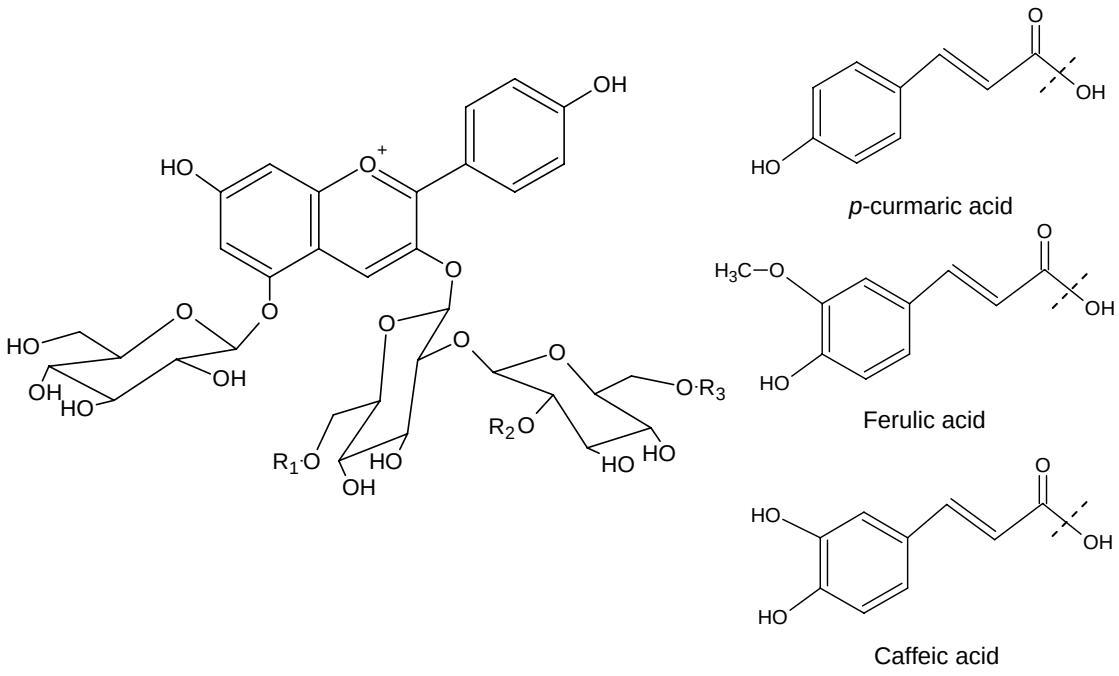

Figure 7.3: Structures of anthocyanins in radish (*Raphanus sativus* L.). R_1, R_2 and R_3 are sites for possible substitution with sinapic acid, ferulic acid, and *p*-coumaric acid. (Source: Otsuki et al., 2002)

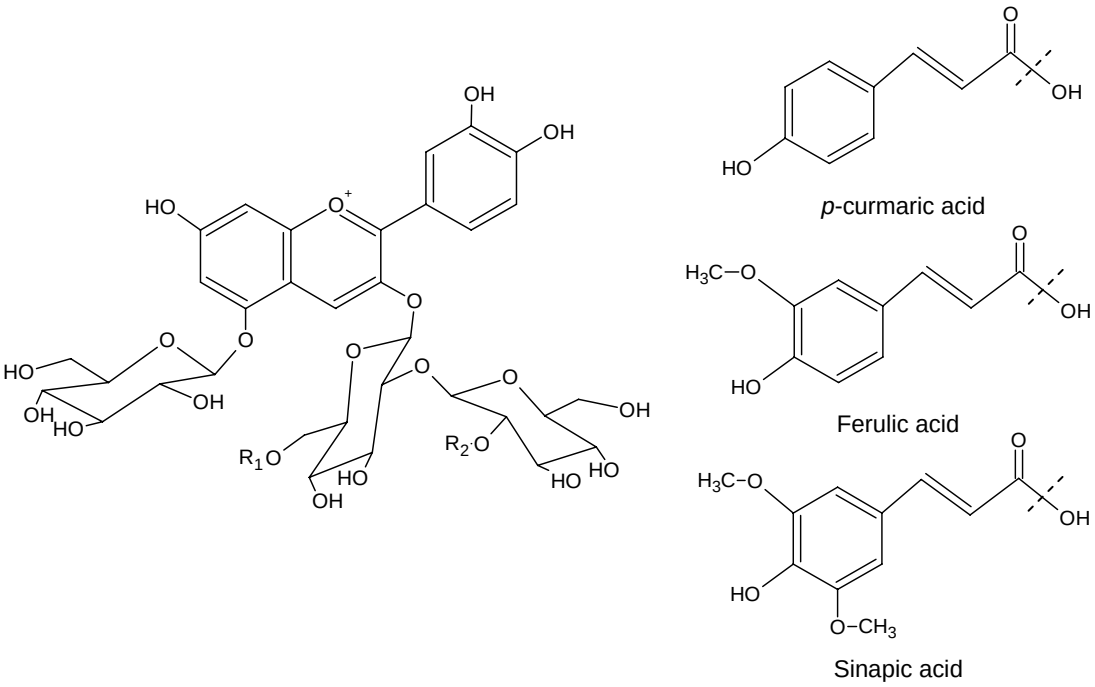

Figure 7.4: Structures of anthocyanins in red cabbage (*Brassica oleracea* L.). R_1 and R_2 are sites for possible substitution with sinapic acid, ferulic acid, and *p*-coumaric acid. (Source: Shimizu et al., 1997; Wu and Prior, 2005)

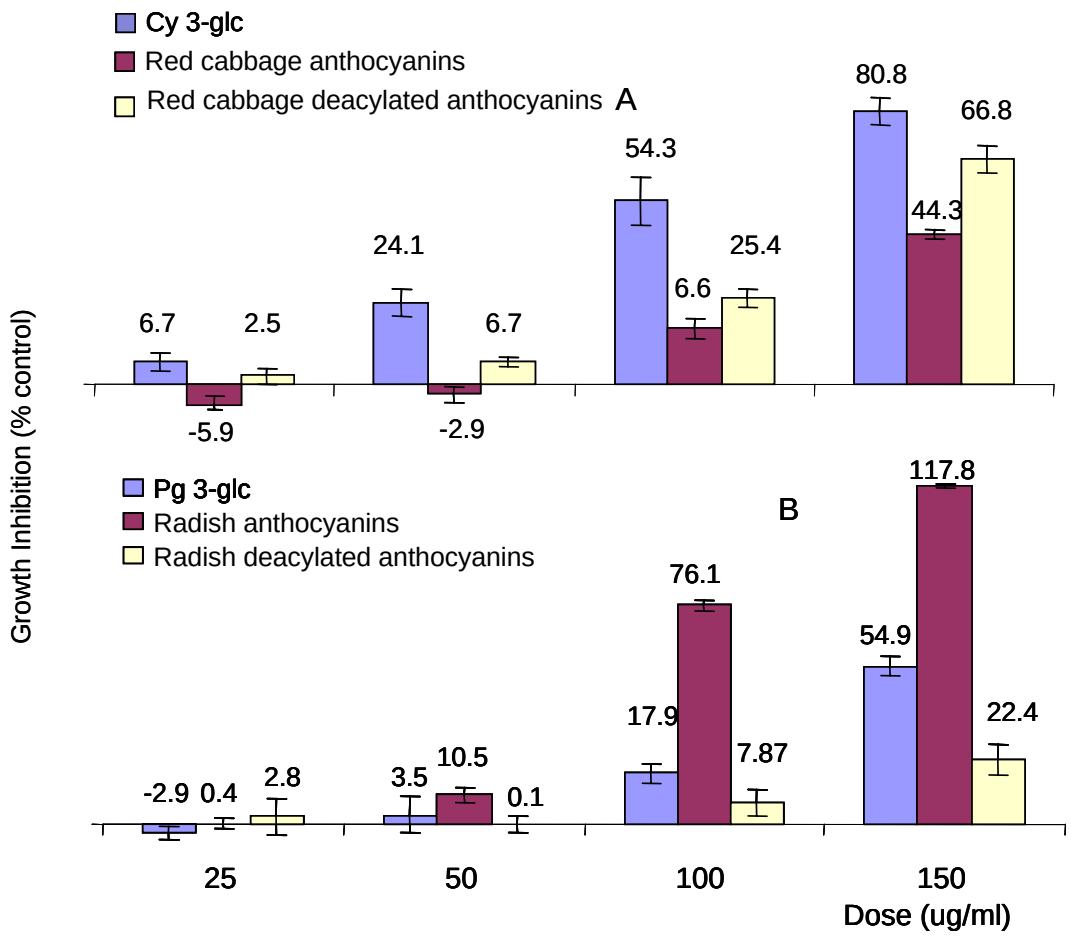

Figure 7.5: Acylation effects of anthocyanins on their chemoprotection. A: red cabbage (*Brassica oleracea* L.); B, radish (*Raphanus sativus* L.). Cy 3-glc: cyanidin 3-glucoside; Pg 3-glc: pelargonidin 3-glucoside

. In **Figure 7.5A**, removal of the cinnamic acid acylation from red cabbage anthocyanins increased the inhibition activity significantly compared with their acylated anthocyanins at a treatment dose of 150 µg/ml ($p<0.01$). The cyanidin-3-

glucoside standard exerted a higher inhibitory effect than either the acylated anthocyanins or deacylated anthocyanins in red cabbage at treatment doses of 50, 100 and 150 µg/ml. The major anthocyanin in the saponified cabbage pigments was the cyanidin 3- sophoroside-5-glucoside obtained by the saponification of red cabbage anthocyanins followed by C_{18} cartridge purification. Cyanidin 3-sophoroside-5-glucoside showed a higher inhibition activity than its acylated counterparts with one or two aromatic acids, where cyanidin 3-(*p*-coumaroyl)sophoroside-5-glucoside and cyanidin 3-(sinapoyl)sophoroside-5-glucoside were dominant anthocyanins. Acylation had an important impact on the chemoprotective effect of anthocyanins, but it was dependant on the type of acid and aglycone. More research is needed to better understand the impact of anthocyanin acylation on their chemoprotective properties.

However, deacylated anthocyanins of radish showed significantly lower inhibition activity than their acylated anthocyanins at the treatment doses of 100 and 150 µg/ml of ($p<0.01$), even lower than the pelargonidin 3-glucoside at a treatment dose of 150 µg/ml ($p<0.01$) (**Figure 7.5B**). The pelargonidin 3-sophoroside-5-glucoside, the major deacylated anthocyanins after saponification of radish anthocyanins, decreased the inhibitory effect compared to its acylated with one or two of aromatic acids (ferulic, caffeic and *p*-coumaric acids), where two of aromatic acids were dominant (Otsuki et al., 2002). Two of ferulic, caffeic and *p*-coumaric acids in a combination with pelargonidin as the aglycone suggested to enhance their inhibitory effect on HT29 cells.

Acylation had an important impact on the chemoprotective effect of anthocyanins, but it was dependant on the type of acid and aglycone. More research is needed to better understand the impact of anthocyanin acylation on their chemoprotective properties. Yoshimoto et al. (2001) found that deacylation of peonidin 3-(caffeylferuly)sophoroside-5-glucoside, markedly reduced antimutagenecity compared to the acylated counterpart while deacylation of cyanidin 3-(caffeylferuly)sophoroside-5-glucoside did not. Hence the biological activities of anthocyanin acylation might depend on their aglycones.

7.4.2 Glycosylation Pattern

Anthocyanins glycosylated with tri-glycoside were compared with anthocyanins with mono-glucoside in terms of the growth inhibition of HT29 cells because in a multiple linear regression model, anthocyanins glycosylated with tri-glycoside was found significantly to be negatively correlated to the inhibitory effect on HT29 cells while mono-glycoside (R= -0.724, $p<0.0001$) was positively correlated to the inhibitory effect (Jing et al, 2006). Anthocyanin-rich extracts from radish (*Raphanus sativus* L.) containing high levels of anthocyanin attached with tri-glycoside about 100%, showed relatively low inhibitory effect (GI_{50}=107.7 μg/ml) (Jing et al., 2006). Therefore, three-sugar substitution might reduce their biological activities. In this study, both of red radish (*Raphanus sativus* L.) and red cabbage (*Brassica oleracea* L.) that are rich in 3,5-position triglycoside, were compared with standards: pelargondin 3-glucoside and cyanidin 3-glucoside (**Figure 7.5**). The

cyanidin 3-glucoside and pelargonidin 3-glucoside both showed a higher inhibitory activity at a dose range from 50 to 150 μg/ml than did the deacylated 3,5-triglycoside anthocyanins, which were cyanidin 3-sophoroside-5-glucoside ($p< 0.05$) (**Figure 7.5A**) and pelargonidin 3-sophoroside-5-glucoside ($p< 0.05$) (**Figure 7.5B**), respectively. That results that anthocyanins glycosylated with tri-glycoside at 3-sophoroside-5-glucoside showed a lower inhibitory effect than the counterpart with mono-glucoside, which was consistent with our statistical results.

7.4.3 Type of Aglycone: Cyanidin versus Pelargonidin

In **Figure 7.5**, pelargonidin 3-glucoside and pelargonidin 3-sophoroside-5-glucoside both exerted a lower inhibitory effect than did the cyanidin pigments with the same glycosylation patterns ($p<0.01$). Anthocyanidins with an ortho-dihydroxyphenyl structure on the B-ring, including delphinidin, cyanidin, and petunidin have been shown to suppress 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell transformation and activator protein-1 transcription activity in mouse epidermal cell line JB6, which was much greater than did anthocyanidins without this structure (pelargonidin, peonidin, and malvidin) (Hou et al., 2004). Delphinidin and cyanidin showed anti-inflammatory properties through the inhibition of mitogen-activated-protein-kinase (MAPK)-mediated cyclooxygenase-2 (COX-2) expression, but pelargonidin, peonidin and malvidin did not (Hou et al., 2005). The structure-activity relationship suggested that the ortho-dihydroxyphenyl structure of anthocyanidins on the B-ring may be the critical structure which was related with the

chemopreventive effect of anthocyanidins. Hence the ortho-dihydroxyphenyl structure of anthocyanidins 3-glucoside on the B-ring might be the critical structure to exert their chemoprotective actions.

7.5 CONCLUSIONS

Aliphatic acid attached to glycoside of anthocyanins in purple corn reduced their chemoprotection. Acylation with cinnamic acids showed different effect on the chemoprotective actions of anthocyanin with different aglycone. Mono-glycoside of cyanidin and pelargonidin might have more biological activities than their 3-diglycoside-5-glycoside pattern. Pelargonidin 3-glucoside has been shown to have lower biological effect than cyanidin 3-glucoside in terms of the inhibitory effect on the growth of HT29 cells, suggesting that the ortho-dihydroxyphenyl structure of anthocyanidins 3-glucoside on the B-ring might be the critical structure to exert their chemoprotective actions.

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CHAPTER 8

EFFECTS OF PROCESSING ON THE CHEMOPROTECTIVE ACTIVITY OF PURPLE CORN COLORANTS [a] [b]

8.1 ABSTRACT

A purple corn colorant was shown to possess high chemoprotective activity in a colon carcinoma cell line. In this study we evaluate the effect of processing on the chemical, physical and chemoprotective activity of purple corn extracts (PCE), colorants (PCC) and waste material (PCW). Materials (PCE, PCC and PCW) were characterized for solubility, qualitative and quantitative anthocyanin composition,

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polymeric anthocyanins and total phenolics. PCE, PCW and PCC were also tested for their inhibitory effect on the growth of human colorectal adenocarcinoma (HT29). Sample solubility was evaluated by haze in 0.1% HCl water with a ColorQuest XE. A decrease in haze (increasing solubility) was negatively correlated with the percentage of anthocyanins to total phenolics in PCW (20.31%, 5.38%), PCE (15.81%, 18.03%), and PCC (2.44%, 25.09%). Anthocyanin profiles were not changed among them. Therefore, a high proportion of non-anthocyanin phenolics was not only associated with lower solubility but also reduced chemoprotective activity of anthocyanins. The purple corn waste, although containing high levels of no-anthocyanin phenolics, was found to be rich in monomeric anthocyanins (2.29 g/ 100g) and have a high chemoprotective (GI_{50}=21.4 μg/ml, R^2= 0.9851), although significantly lower that that of PCE or PCC. Therefore, PCW, a waste material from corn colorant production, could become a source of a chemoprotective ingredient for functional foods. Our study showed that colorant processing had an impact to anthocyanin chemoprotection, and that other phenolics also affected chemoprotection. Elucidating these mechanisms may provide light into understanding how anthocyanins may interact with macromolecules from food or bodies affecting their availability in the GI tract.

KEYWORDS: purple corn colorant; anthocyanin; total phenolics; chemoprotection; HT29; processing, purple corn waste

8.2 INTRODUCTION

Purple corn (*Zea mays* L.) is an Andean crop in South America mainly in Peru and Bolivia. It is known as "maiz morado" and has long been used to color desserts and beverages. A colorant from purple corn is widely used in Asia, South America and Europe currently.

Recent studies have found that purple corns have high antioxidant activity (Cevallos-Casals and Cisneros-Zevallos, 2003), reduce the systolic blood pressure of spontaneously hypertensive rats (Toyoshi and Kohda, 2004), and prevent obesity and diabetes in rats (Tsuda et al., 2003). A purple corn colorant was found to inhibit cell mutation induced by 2-amino-1-methyl-6-phenylimidazo pyridine (PhIP) (Aoki et al., 2004) and to reduce chemically induced colorectal carcinogenesis (Hagiwara et al., 2001). Cyanidin 3-glucoside is the major anthocyanin in purple corn color and has been shown to reduce the genotoxicity of PhIP, indicating that this anthocyanin pigment is an active components (Aoki et al., 2004).

However, large quantities of purple corn waste are generated during the preparation of an anthocyanin-rich colorant, which are obtained by an easy and economic processing of hot-acidified-water extraction from purple corncobs. This waste has limited application in foods due to its low solubility in acidified aqueous system. In a previous study, we showed that a purple corn colorant exerted a greater chemoprotection by inhibition of the growth of human colon adenocarcinoma (HT29) than chokeberry (*Aronia meloncarpa* E.), bilberry (*Vaccinium myrtillus* L.), purple

carrot (*Daucus carota* L.), grape (*Vitis inifera* L.), radish (*Raphanus sativus* L.) and elderberry (*Sambucus nigra* L.).

In this study, our objectives were to study the effect of processing on their chemoprotection of purple corn colorants and to evaluate purple corn waste in terms of its chemoprotective effect.

8.3 MATERIALS AND METHODS

8.3.1 Materials and Reagents

Purple corn extract (PCE), purple corn waste (PCW), and purple corn colorant (PCC) were donated by Globenatural International S.A. (Chorrillos-LIMA, Peru). The process used to obtain these extracts has been illustrated in **Figure 8.1**. PCE is obtained after extraction of pigments with hot water, followed by drying. This first extract is then separated into a water insoluble waste (PCW) and a high quality purple corn colorant.

Folin & Ciocalteau phenol reagent and the standard of gallic acid (crystalline gallic acid, 98% purity) were purchased from Sigma (St. Louis, MO, USA).All reagents and solvents for high-performance liquid chromatography (HPLC), sample fractionation, and SRB assay were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

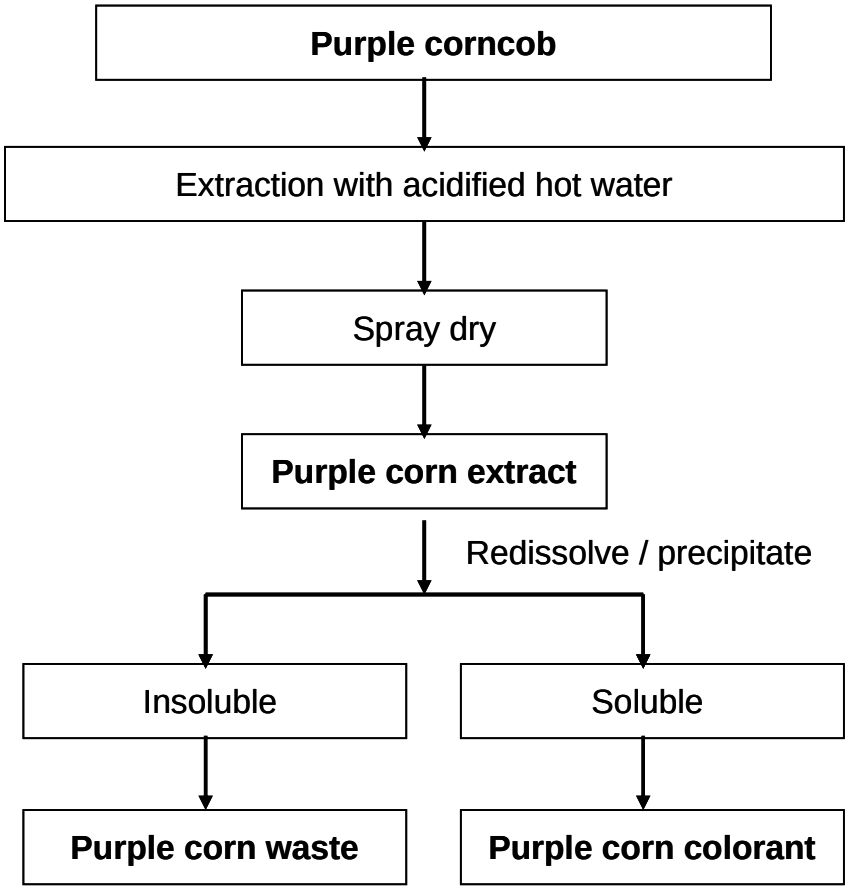

Figure 8.1: Flow chart of processing of purple corn colorant

8.3.2 HT29 Cell Line

The HT29 cell line derived from a colorectal adenocarcinoma (HTB 38; American Type Culture Collection, VA, USA) was grown in McCoy's 5A medium

(Fisher Scientific, Fair Lawn, NJ, USA), which was supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA, USA) at 37 ˚C and 5 % CO_2 atmosphere.

8.3.3 Solubility Test

A sample (100 mg) of each, PCE, PCW and PCC was mixed with 100 ml 0.1%-HCl-water and stirred for 10 min. Haze measured by a Hunter ColorQuest XE spectrophotometer (HunterLab, Hunter Associates Laboratories Inc., Reston, USA) was used as an indirect measure of solubility. The instrument was set up for illuminant C and 10 degree observer angle. Immediately after stirring, samples were placed in a 2 mm path-length disposable cuvette and measured using the transmission mode and haze (total transmission and relative transmission).

8.3.4 Monomeric and Polymeric Anthocyanins

The total monomeric anthocyanin content was measured by the pH differential method (Giusti and Wrolstad, 2001). A HP UV-visible spectrophotometer (Agilent Technologies, Inc. Palo Alto, CA, USA) was used to read absorbance at the maximum visible wavelength of absorption of each extract (ranging from 500 -525nm) and at 700 nm. Monomeric anthocyanins were calculated as equivalents of cyanidin-3-glucoside, using the extinction coefficient of 26900 L cm^{-1} mg^{-1} and a molecular weight of 449.2 g/L (Giusti and Wrolstad, 2001), or as equivalents of pelargonidin-3-glucoside, using the extinction coefficient of 31600 L cm^{-1} mg^{-1}. Disposable cuvettes of 1-cm-path-length were used.

Color density and polymeric color were calculated using absorption at 420 nm, 510 nm, and 700 nm before and after bisulfite treatment, and used as an index of browning (Giusti and Wrolstad, 2001).

8.3.5 Total Phenolics

Total phenolics were measured using a modified Folin-Ciocalteu method (Singleton and Rossi, 1965). Absorbance of samples and standards at 755nm using 1-cm-path-length cuvettes was measured in the UV-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan). Total phenolics were calculated as gallic acid equivalents based on a gallic acid standard curve.

8.3.6 Analytical Chromatography

A high performance liquid chromatograph (HPLC) system (Waters Delta 600 systems, Milford, MA, USA) equipped with a photodiode array detector (Water 996, Milford, MA, USA.), autosampler (Waters 717 plus, Milford, MA, USA), and Millennium^{32} software (Waters Corp., Milford, MA, USA) was used.

Columns and mobile phase: The reversed-phase 5 µm Symmetry C18 column (4.6×150mm, Waters Corp., Milford, MA, USA) fitted with a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corp., Milford, MA, USA) was used. The solvents used were A: 1% phosphoric acid / 10% acetic acid / 5% acetonitrile in water and B: 100% acetonitrile. Solvents and samples were filtered though 0.45 µm poly(tetrafluorothylene) membrane filters (Pall Life Sciences, MI, USA) and 0.45 µm polypropylene filters (Whatman Inc., Clifton, NJ), respectively. Separation was

achieved by using a linear gradient from 0 to 30% A in 35 min. An injection volume of 50 μL with a 1 ml/min flow rate was used. Spectral information over the wavelength range of 260-600 nm was collected.

8.3.7 Cell Growth Inhibition

The HT29 cells were plated at 1.3×10^4 cells/well in 24-well plates (Falcon) using McCoy's 5A medium containing 10% fetal bovine serum. Cells were allowed to grow 24 hours to attain log phase growth at the time of sample addition (time 0). Semi-purified AREs or others were compared for their inhibitory effects at concentrations normally ranging from 0 to 200 μg/ml of growth media. HT29 cell growth was determined after additional 48 hr of incubation with different levels of anthocyanins by using the sulforhodamine B assay (SRB assay) at 565 nm. Every treatment had 4 replicates every time, and whole experiments were repeated three or four times. The percentage growth inhibition is calculated as:

$$\% \text{ Growth inhibition} = 100 - (T_{trt} - T_0) \times 100 / (T_{ctr} - T_0)$$

T_{trt} is the absorbance of sample with treatment of anthocyanins at 565 nm.

T_0 is the absorbance of sample after the first 24 incubation (time 0) prior to the treatment of anthocyanins.

T_{ctr} is the absorbance of sample without treatment of anthocyanins after the total incubation.

8.3.8 Sulforhodamine B Assay

The detailed methodology for SRB assay was described by Skehan et al., (1990). Briefly, cells were fixed by addition of 250 uL of 50 % trichloroacetic acid (TCA) at 4 °C for 1 hour. TCA and media were removed and wells were washed with water 5 times and dried at room temperature. SRB (0.4 % in acetic water) of 500 µL was added to each well to stain cells at room temperature for 20 min. Wells were washed using 1% acetic acid 5 times and dried at room temperature. The incorporated dye was then solubilized with 1 ml of 10 mM Tris for 5 min at room temperature on a shaker. The absorbance at wavelength of 565 nm was measured using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA)

8.3.9 Statistical Analysis

Tukey HSD test was used to evaluate mean differences among growth inhibition (%) when at the same concentration in one-way ANOVA model. All analyses were performed using SPSS (14.0) software. For all statistical calculations, $p<0.05$ was considered to be statistically significant.

8.4 RESULTS AND DISCUSSION

8.4.1 Anthocyanins, Polymeric Anthocyanins and Total Phenolics in Purple Corn Colorant during Processing

The PCC contained high content of anthocyanins (6.69 g/100 g), which was similar to the anthocyanin level in the PCE (6.11 g/ 100g) and was about three times

higher than in waste (2.29 g/ 100g). A high concentration of non-anthocyanin phenolics were accumulated in PCW with an increase for both polymeric color (49.59 %) and total phenolics (42.61 g/ 100g) while a lowest percentage of anthocyanins of total phenolics (5.38 %) compared with either PCE or PCC. The purple corn colorant (PCC) after purification showed great solubility with a low haze about 2.44% in 1 mg powder/ ml acidified deionized H_2O, much lower than purple corn extract (PCE, 15.81%), and purple corn waste (PCW, 20.31%) in **Table 8.1**. The accumulation of phenolics in PCW, especially those with high molecular weight such as polymeric anthocyanins and tannins, as well as proteins that were found to be a high level in purple corn waste (Jing and Giusti, 2005) may explain the poor solubility and high haze of the waste material.

However, PCW was still rich in monomeric anthocyanins (2.29 g/ 100g), much higher than anthocyanin levels in chokeberry (*Aronia melanocarpa* E.) (1.48 g/ 100g) (Wu et al., 2004), in blueberry (*Vaccinium corymbosum* L.) (0.07 ~ 0.43 g/ 100g) or in black raspberry (*Rubus occidentalis* L.) (0.607 g/ 100g) (Moyer et al., 2002).

Sample	Water Solubility (observations)	Haze* (%)	Monomeric Anthocyanins (g/100g)	Polymeric Color (%)	Total Phenolics (g/100g)	Monomeric Anthocyanins of Total phenolics (%)
Purple corn extract	Not very water soluble	15.81 (0.13)	6.11 (0.32)	46.48 (3.16)	33.90 (0.69)	18.03 (1.07)
Purple corn Waste	Some water soluble	20.31 (0.21)	2.29 (0.13)	49.59 (2.54)	42.61 (1.03)	5.38 (0.75)
Purple corn colorant	Very water soluble	2.44 (0.05)	6.69 (0.30)	37.50 (1.78)	26.67 (2.28)	25.09 (1.24)

Table 8.1: Solubility, monomeric anthocyanins, polymeric anthocyanins, and total phenolics during processing. Haze*: 1 mg powder/ml of 0.1%-HCL- H_2O. Total phenolics, monomeric anthocyanins, polymeric Color (%): samples in 70% methanol of purple corn colorant. Values are represented as mean (standard error) (n=3)

8.4.2 Anthocyanin Profiles in Purple Corn Colorants

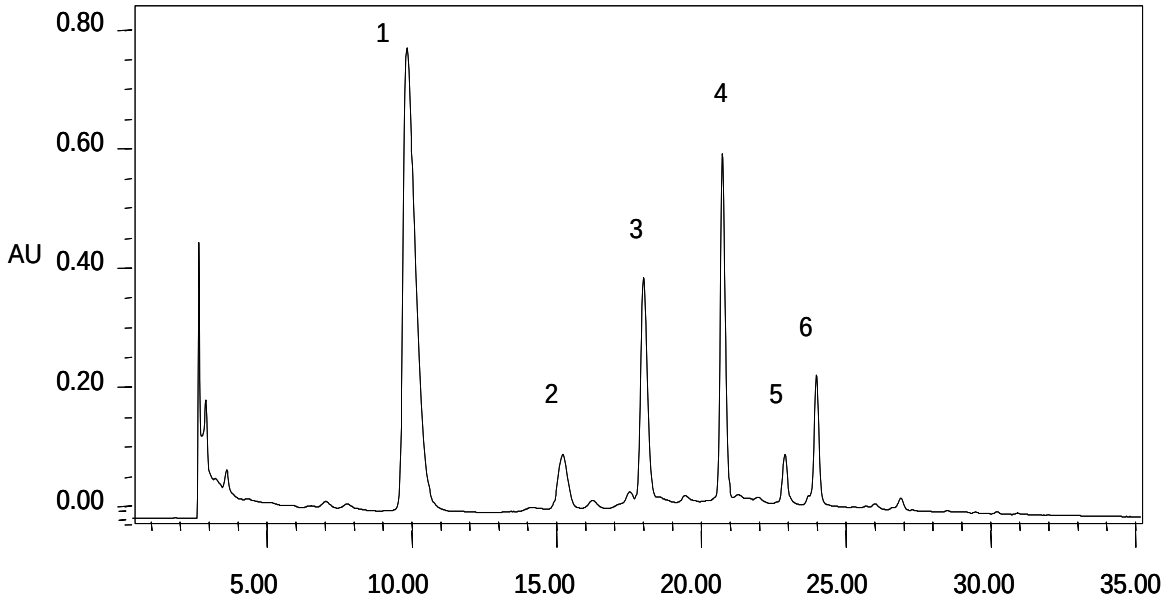

Figure 8.2: Anthocyanin profile in purple corn colorant. 1: cyanidin-3-glucoside; 2: pelargonidin-3-glucoside; 3: peonidin-3-glucoside; 4: cyanidin-3-(6”-malonylglucoside); 5: pelargonidin-3- (6”-malonylglucoside); 6: peonidin-3- (6”-malonylglucoside)

The anthocyanin profiles in PCE, PCW and PCC, were very similar. The chromatogram of PCC was showed in **Figure 8.2.** There were six major anthocyanins: cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-(6”-malonylglucoside), pelargonidin-3-(6”-malonylglucoside), peonidin-3- (6”-

malonylglucoside) as shown in **Figure 8.2**, which were also major anthocyanins in purple corncob anthocyanin-rich extract obtained by 70% acetone (Jing et al., 2006a). Other three minor peaks (peonidin-3-malonylglucoside, cyanidin-3-malonylglucoside, and pelargonidin-3-malonylglucoside) identified in purple corncobs (Jing et al., 2006a) disappeared in PCE, PCC, and PCW, suggesting extraction with hot water was more likely causing sensitive anthocyanin degradation than did 70% acetone extraction.

8.4.3 Effects of Processing on the Chemoprotective Activity of Purple Corn Colorants

A purple corn waste (PCW), purple corn extract (PCE) and purple corn colorant (PCC) obtained during purple corn colorant processing were evaluated in terms of inhibitory effect on the growth of HT29 cells. The purple corn waste exerted a great inhibitory effect with a GI_{50} value of 21.4 μg/ml (R^2= 0.9851), which was lower than GI_{50} values of chokeberry (31.2 μg/ml), bilberry (32.2 μg/ml), purple carrot (68.5 μg/ml), grape (71.2 μg/ml), radish (107.7 μg/ml, and elderberry (130.3 μg/ml) but higher than PCC (13.8 μg/ml) (Jing et al., 2006b). Hence, the purple corn waste is not only potent to be applied as natural food colorants to color neutral foods in our previous study (Jing and Giusti, 2005) but also could be utilized as chemoprotective functional ingredients. In **Figure 8.3**, both PCE and PCC showed better growth inhibition than did purple corn waste, significantly greater at dose range from 20 to 40 μg/ml at the level of 0.05 (Tukey HSD). A 30 μg/ml of monomeric anthocyanins from PCW could exert an inhibitory effect comparable to 20 μg/ml

monomeric anthocyanins from PCE. Antiproliferative effect of 20 μg/ml monomeric anthocyanins from PCW needed lower amount of monomeric anthocyanins (10 μg/ml) from PCC. Tannins and proteins were previously found in a high level in purple corn waste generated in the processing of purple corn colorant (Jing and Giusti, 2005). They could trap free monomeric anthocyanins by π-π interaction (Waterhouse, 2002) and form complexes which might decrease exposure of anthocyanins to HT29 cells. Our results suggest that these complexation reactions may reduce anthocyanin availability to HT29 cells and therefore reduce the inhibitory action of purple corn anthocyanins.

8.5 CONCLUSIONS

The processing of anthocyanin colorant had impact on their chemoprotection. Protein and other complex molecules such as tannins exist in purple corncobs, which may form complexes with anthocyanins resulting in a low availability in GI tract. However, the purple corn waste which was generated during the colorant processing could be alternatively utilized as chemoprotective ingredients for function foods due to their higher biological activity than other anthocyanin-rich sources (chokeberry, bilberry, purple carrot, grape, radish, and elderberry).

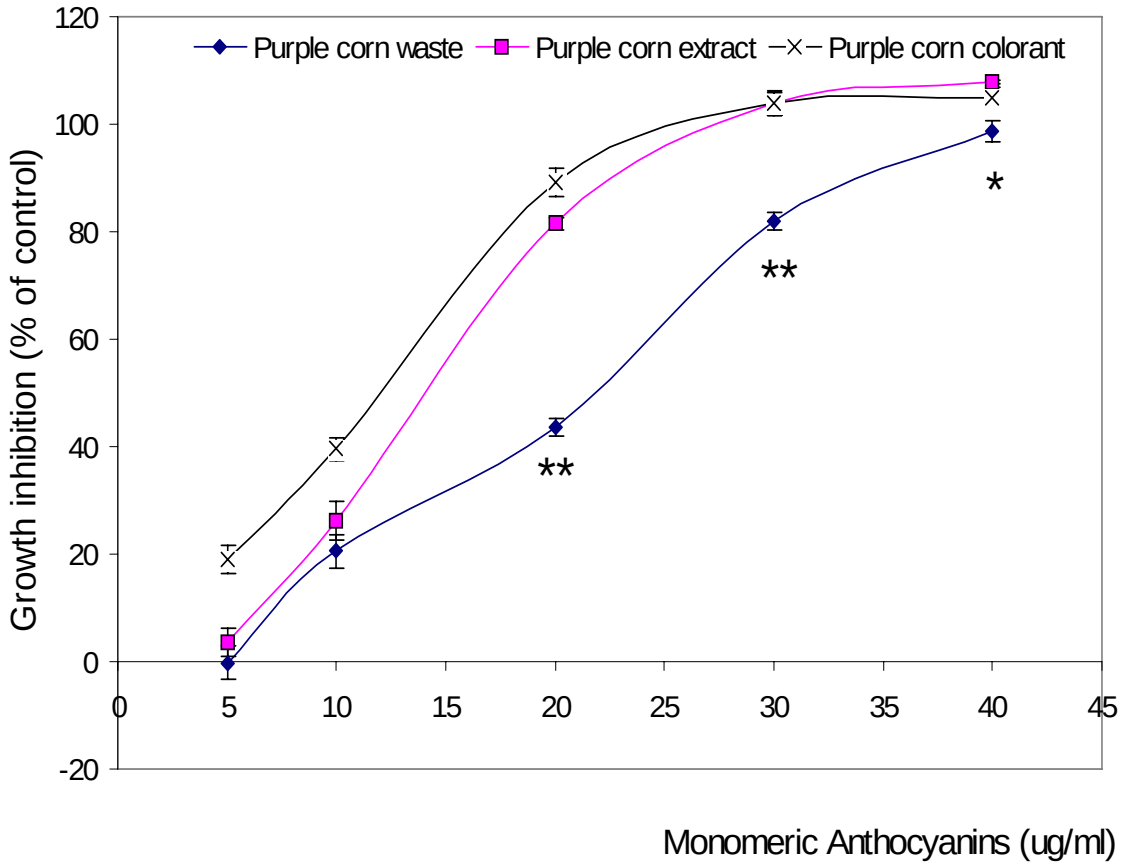

Figure 8.3: Effect of purple corn waste, purple corn extract, and purple corn colorant on the growth of HT29 cell line. Monomeric anthocyanins were calculated as equivalents of cyanidin 3-glucoside. *, $p< 0.05$; **, $p<0.01$ (Tukey HSD). Values are represented as Mean ± Standard error (n=4).

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