Analysis of Procyanidins and Anthocyanins in Food Products using Chromatographic and Spectroscopic Techniques

DISSERTATION

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

By

Taylor Clyde Wallace

Graduate Program in Food Science and Technology

The Ohio State University

2010

Dissertation Committee:

M. Monica Giusti, Advisor, Sheryl Barringer, Joshua Bomser, and John Litchfield

Copyright by Taylor Clyde Wallace 2010

Abstract

Procyanidins and anthocyanins are polyphenol compounds widely distributed throughout nature and are important because of their significant presence in the human diet and potential health benefits post consumption. Even though many food scientists, nutritionists, epidemiologists, and medical practitioners have related a decrease in many age and obesity related chronic diseases to the high consumption of fruits and vegetables containing these potent compounds / antioxidants, scientists have still yet to develop standardized protocols for quantification of procyanidins in food because of their structural complexity, polymerization, and stereochemistry.

Procyanidins and anthocyanins are considered value added food ingredients in a variety of matrices. Depending on the food matrix, procyanidins, anthocyanins, and other polyphenols may or may not be desirable to the consumers. Monomer forms of procyanidins (flavan-3-ols such as catechin and epicatechin) provide a bitter flavor to food products, but become more astringent / less bitter as polymerization increases. Anthocyanins have a distinct influence on the consumer acceptance of a product because of their ability to produce a natural orange-red to blue-violet color. Consumer demand for healthier high quality food products has driven the industry to create food products with higher concentrations of procyanidins / anthocyanins and the need to develop better techniques for analysis of these phytonutrients. There is a need for increased understanding of the chemistry of these compounds in a variety of food matrices, as well as, the development of more reproducible methods for characterization, quantification, and authentication of these powerful phytonutrients for many purposes including validation of labeling claims.

The long-term goal is to increase the knowledge and understanding of the role of

ii

procyanidins and anthocyanins in the diet and the many positive health benefits attributed to these compounds upon consumption by the consumer. The **specific goal** of this research was to develop better analytical methods for identification and quantification of procyanidins and anthocyanins in foods and to apply those methods to understand their chemistry in various food matrices, while monitoring the viability, stability, and integrity of these compounds to select processing conditions.

To validate our hypothesis it was critical to evaluate parameters that affected the 4dimethylaminocinnimaldehyde (DMAC) UV-visible spectrophotometric assay to help the industry develop a more reproducible and standardized protocol for the rapid quantification of proanthocyanidins. To better reproduce and assess total proanthocyanidins in a sample, it is our recommendation that when using catechin as a standard that the following parameters be applied: the use of 2% DMAC (w/v) and 6N H₂SO₄ to prepare the DMAC reagent, a reaction time of >15 min at constant room temperature between 21 and 25°C, and the use of absolute methanol (<1% water in sample). Because there has been significant research conducted with various monomeric and oligomeric standards, our study focused on other parameters that influence the accuracy and reproducibility of the assay. The use of different standards in the assay has a significant effect on the color produced by the DMAC-proanthocyanidin complex. Catechin and epicatechin have been reported to have greater reactivity as compared to proanthocyanidin oligomers. Samples containing high concentrations of oligomeric proanthocyanidins may require a reaction time of up to 20 min as reported in other studies. Our studies as well as previous literature support that the color produced by the DMACproanthocyanidin complex is stable between 20 and 30 min for both monomers and oligomeric proanthocyanidins. The use of absolute methanol is necessary and involves removal of water until samples are dry and re-consituting the proanthocyanidins in methanol prior to analysis. The results from the parameters explored which effect the DMAC assay will aid the food industry in devoping a more reproducible and standard method for quantification and comparison proanthocyanidin contents in foods.

ii

Further evaluation of analytical techniques led us to explore chromatographic techniques to separate and identify procyanidins in cranberry extracts. Cranberries provide a unique opportunity to study both anthocyanins and procyanidins because they are prevalent in the American diet and contain a very diverse matrix of polyphenols, both A- and B-type procyanidins, high concentrations of the more oligomeric and polymeric procyanidins, and 6 non-acylated anthocyanins. Fluorescence and MS detection were used to confirm the identity and quantify procyanidins by their degree of polymerization up to nonamers. Both A- and B-type procyanidins were separated and quantified up to tetramers. Posing an additional barrier to this study, certain cranberry extracts were prepared with an insoluble inert resin, which actively binds polyphenols and aids in their concentration. As the industry has begun to utilize this compound to produce more concentrated phenolic extracts, development of better extraction techniques were explored to be able to better assess the procyanidin and anthocyanin contents of these products. Cranberry extracts containing the inert resin showed higher concentrations of total phenolics, monomeric anthocyanins, and procyanidins as compared to non-resin containing extracts. Commercial cranberry extracts contained between 0.73 and 22.45 mg/g procyanidins as quantified using HPLC - fluorescence detection. The use of Sephadex LH-20 was effective in eliminating interfering compounds prior to DMAC and HPLC analyses. The use of HPLC – fluorescence and MS detection proved to be the most accurate means of quantifying procyanidins in cranberries as compared to the DMAC assay. It was also shown that an acid / alkaline technique of removing the polyphenols from the inert resin degraded compounds, thus a 6X acetone / water extraction technique was developed in order to better assess the the quantity and profile of procyanidins present. Analytical advances in chromatography for anthocyanins were also achieved using HPLC – PDA and MS detection.

Our final study utilized the analytical techniques prepared in the previous studies to solve an emerging industry / quality concern of unfermented cocoa powder extracts. Unfermented cocoa powder is produced with the intention of creating procyanidin-rich extracts used to increase the general public's consumption of these healthy compounds. Because of the lack of fermentation, anthocyanins naturally present in cocoa beans remain viable producing an unwanted violet color in the final extract. Our goal was to decolorize unfermented cocoa powder extracts while monitoring the stability, integrity, and viability of the procyanidins. The use of pH fluctuations, glycosidic enzymes, and potassium meta-bisulfite was explored to see which treatment best removed the violet color and preserved the other polyphenols present in the unfermented cocoa powder extracts. The use of bisulfite to bleach the anthocyanins present eliminated the violet color and preserved the composition and chemistry of the procyanidins and other phenolics present. Higher levels of bisulfite lead to an increased retention of anthocyanins in the final procyanidin-rich extract. The effective use of bisulfite to bleach the violet color present did not have a detrimental / significant effect on the stability of anthocyanins, procyanidins, or other polyphenols present in the procyanidin-rich unfermented cocoa powder extracts as compared to the control throughout a 60 day shelf life study.

Our studies and this dissertation increase the understanding of the chemistry of anthocyanins / procyanidins and help to progress their many applications as value added ingredients. Indirectly, this research may also aid in the advancement of nutritional studies related to the health benefits of these powerful phytonutrients.

Dedication

Dedicated to my father Clyde F. Wallace Jr., to whom I love and owe so much more than this dissertation. Thank you for the many fights we had in high school over doing homework immediately after swim practice, calling me 20 times a day during finals week in college to make sure that I was studying, and being a solid foundation of support to me throughout graduate school. You have sacrificed, encouraged, and opened the door for me to so many opportunities that I never imagined possible. You are a role model to other single parents. I love you and Mamawl so much, and I hope that one day my son can see me as at least half of the person that you are to me.

Acknowledgements

I wish to thank my advisor Dr. M. Monica Giusti, as well as, my dissertation committee Drs. Sheryl Barringer, Joshua Bomser, John Litchfield, and David Min for their guidance and intellectual support which made this dissertation possible. Monica you have crafted me into the scientist I am today and taught me the wonderful skill patience. I would also like to recognize Dr. Rodriguez-Saona for his extensive advice and guidance while conducting research and helping to prepare me for my candidacy examination.

I wish to thank my outstanding colleagues and lab mates. You all make coming to work easy and are the best life-long friends a guy could ask for. Starting with my 2006 trip to Lima, Peru when I first met Dante and Claudia to the many great nights out with Daniel, Carolina, Fer, and Diego you guys left your families to come here to the US, but what you didn't know is that you became part of mine. Fer, I can't thank you enough for being my graduate school mother, supporting and encouraging me during my candidacy exam, and helping review my various presentations. I love all you all so much!

Jen, we've been through so much together. You keep me sane, listen to my deepest thoughts, and have always been there for me through thick and thin. I really don't know where my life would be without you. Thanks for being my best friend.

To the many mentors and friends I have had the opportunity to be acquainted with through IFT, specifically Sheri Schellhaass, Mary Wagner, Barbara Byrd Keenan, and the other IFTSA Presidents, thank you for taking the time to take me under your wing. I have learned so much from you and had so many good times that words can not even express my gratitude. I am forever grateful to my father, to whom this dissertation is dedicated to, for continuously pushing me to advance my education. For all of the money you have spent on my education, I hope you enjoy reading this long and scientifically dynamic document. To Ms. Kaler, my high school advanced placement chemistry teacher, and Dr. Benjy Mikel, my undergraduate advisor, thank you for challenging me in science and for making me believe that I could one day become a PhD scientist. I wish to thank the USDA NRI Competitive Grants Program, the Ohio Agriculture Research and Development Center, CliffStar Incorporated, and Mars Botanical for their support of this research. Thank you to The Ohio State University Department of Food Science and Technology for financially supporting my tuition and allowing me to study at the greatest University both on the field and off. OH-IO!

Vita

1984	 Born – Hopkinsville, KY USA
2002 – 2005	 B.S. Food Science and Technology University of Kentucky Lexington, KY USA
2006 – 2007	 M.S. Food Science and Technology The Ohio State University Columbus, OH USA
2006 – 2009	 Graduate Teaching Assistant The Ohio State University Columbus, OH USA
2006 – 2007	 President, IFT Student Association
2007 – 2010	 PhD Food Science and Technology The Ohio State University Columbus, OH USA
2008	 Campbell's Excellence in Leadership Award Recipient

Publications

 Wallace TC & Giusti MM. 2008. Determination of color, pigment, and phenolic stability in yogurt systems colored with non-acylated anthocyanins from Berberis boliviana L. as compared to other natural/synthetic colorants. J. Food Sci. 73(4): C241-8.

- Giusti MM and Wallace TC. 2009. Flavonoids as natural pigments. In: Mussak AM.
 Handbook of natural colorants 1st ed. New York, NY: John Wiley & Sons. p. 257-75.
- He J, Wallace TC, Keatley K, Failla M, and Giusti MM. 2009. Stability of Black Raspberry Anthocyanins in the Digestive Tract Lumen and Transport Efficiency into Gastric and Small Intestinal Tissues in the Rat. J. Agric. Food Chem. 57(8): 3141-48.
- Wallace TC, Wagner M, Leveille G, Keen CL, Woteki CE, Manley C, Rizk SW, Heber D, and Shrikhande AJ. 2009. Unlocking the benefits of cocoa flavanols. Food Technol. 63(10): 34-41.
- Wallace TC and Giusti MM. 2010. Evaluation of parameters which affect the 4dimethylaminocinnamaldehyde assay for proanthocyanidins. J. Food Sci. (in preparation).
- Wallace TC and Giusti MM. 2010. Assessment of normal and reverse phase HPLC-MS and fluorescence/photodiode array detection methods for characterization and quantification of procyanidins and anthocyanins in cranberry extracts. Food Chem. (in preparation)
- Wallace TC and Giusti MM. 2010. Selective removal of the violet color produced by anthocyanins in procyanidin-rich unfermented cocoa extracts. J. Food Sci. (in preparation).

Field of Study

Major Field: Food Science and Technology

Table of Contents

Abstract			ii
Acknowled	dgement	S	vi
Vita			viii
List of Tab	les		xiv
List of Figu	ures		xv
Chapter 1:	Introdu	ction	1
Chapter 2:	: Literatu	ire Review	3
2.1	Chemi	cal Structure and Properties of Flavonoids	3
	2.1.1 2.1.2 2.1.3 2.1.4 2.1.5 2.1.6 2.1.7	Chemical Structure of Flavonoids Role of Localized Flavonoids in the Plant Biosynthesis of Flavonoids Presence in the Human Diet Separation and Quantification of Flavonoids Affinity of Polyvinyl Pyrrolidone for Flavonoids and Phenolics Health Promoting Effects of Flavonoids.	3 4 6 8 11 11
2.2	Anthoo	yanins	14
	2.2.1 2.2.2 2.2.3 2.2.4	Anthocyanins in Foods and Beverages Anthocyanin Aglycons (Anthocyanidins) and Structures Environmental Factors which Influence Stability of Anthocyanins Bioavailability of Anthocyanins.	14 14 17 26
2.3	Proant	hocyanidins	27
	2.3.1 2.3.2 2.3.3 2.3.4	Proanthocyanidin Chemical Structure and Stereochemistry Separation and Quantification of Proanthocyanidins Proanthocyanidins in Foods and Beverages Bioavailability of Proanthocyanidins.	27 33 38 38
2.4	Cranbe	erries and Cranberry Extracts	39
	2.4.1 2.4.2	Botanic Review Production and Processing of Cranberries	39 40

	2.4.3	Extraction and Concentration of Cranberry Phytonutrients	41
2.5	Cacao	Beans and their Derived Products	41
	2.5.1 2.5.2	Botanic Review Processing of Fermented and Unfermented Cocoa Powder	41 42
Chapter 3	Evalua Assay	ation of Parameters which affect the 4-dimethylaminocinnimaldehyde / for Proanthocyanidins	44
3.1	Abstra	ct	44
3.2	Introdu	uction	45
3.3	Materi	als	48
	3.3.1	Chemicals and Reagents	48
3.4	Metho	ds	48
	3.4.1 3.4.2 3.4.3 3.4.4	Preparation of Catechin Standards. Preparation of DMAC Reagents. DMAC Assay Parameters Tested. Statistical Analysis.	48 49 49 50
3.5	Result	s and Discussion	51
	3.5.1 3.5.2 3.5.3 3.5.4 3.5.5 3.5.6	Acid Nature and Concentration. DMAC Concentration. Reaction Time. Temperature. Water Content of Sample. Interfering Substances.	51 52 53 53 54 55
3.6	Conclu	usion	64
3.7	Acknow	wledgements	64
Chapter 4:	Asses Chara Crant	essment of Normal and Reverse Phase HPLC-MS Methods for the acterization and Quantification of Procyanidins and Anthocyanins in perry Extracts	65
4.1	Abstra	ct	65
4.2	Introdu	uction	66
4.3	Materi	als	68
	4.3.1	Chemicals and Reagents	68
4.4	Metho	ds	69

	4.4.1	Extraction of Cranberry Polyphenols from Extracts Containing an
	4.4.2 4.4.3	Spectrophotometric Analyses HPLC – Fluorescence – PDA – MS Analyses
4.5	Results	s and Discussion
	4.5.1 4.5.2	Method Development Quantification of Anthocyanins using HPLC – PDA – MS and Spectrophotometric Analyses
	4.5.3	Quantification of Procyanidins using HPLC – Fluorescence – MS and Spectrophotometric Analyses.
4.6	Conclu	ision
4.7	Acknow	wledgements
Chapter 5	Select Procya	ive Removal of the Violet Color Produced by Anthocyanins in anidin-rich Unfermented Cocoa Extracts
5.1	Abstra	ct
5.2	Introdu	ction
5.3	Materia	als
	5.3.1	Chemicals and Reagents
5.4	Method	ds
	5.4.1	Preliminary Test: Evaluation of pH, Enzyme, and Bisulfite Bleaching Methods
	5.4.2	Water Extraction of Polyphenols / Bisulfite Bleaching of Anthocyanins from Unfermented Cocca Powder
	5.4.3	Spray Drying of Polyphenol-rich Cocoa Extracts
	5.4.4 5.4.5 5.4.7	Shelf Life Study Extraction of Polyphenols from Cocoa Powders and Extracts Colorimetric Analysis
	5.4.8	Spectrophotometric Analyses
	5.4.9 5.4.10	Sultur Dioxide Equivalent HPLC –Fluorescence – PDA – MS Analyses
5.5	Results	s and Discussion
	5.5.1	Characterization of Anthocyanins and Procyanidins Present in
	5.5.2	Cocoa Powders Preliminary Test: Removing the Violet Color from Unfermented
	5.5.3	Scale Up – Testing the Effects of Bisulfite on Unfermented Cocoa Extracts
	5.5.4 5.5.5	Color, Pigment, and Phenolic Stability
5.6	Conclu	ision

5.7	Acknowledgements	114
Refe	rences	115

List of Tables

Table 2.1	Anthocyanidins more commonly found in nature, their B-ring conjugations, and maximum absorbance.	15
Table 2.2	Structures of common proanthocyanidins	31
Table 4.1	Quantitative comparison of the acid/alkaline and acetone/water extraction procedures	77
Table 4.2	Comparison of HPLC and pH differential analyses of cranberry extract anthocyanin concentrations	82
Table 4.3	Quantitative comparison of cranberry extract anthocyanins	83
Table 4.4	Proportions of anthocyanins in cranberry extracts	84
Table 4.5	Comparison of the HPLC and DMAC analyses of cranberry extract procyanidin concentration	85
Table 4.6	Quantification of cranberry extract procyanidins	88
Table 4.7	Proportions of cranberry extract procyanidins	89
Table 5.1	Color values using reflectance specular included for pH – enzyme – bisulfite feasibility tests	106

List of Figures

Figure 2.1	General base structures of flavonoids	5
Figure 2.2	Schematic of biosynthetic pathway of flavonoids	7
Figure 2.3	Separation of anthocyanins using a semi-preparative C-18 cartridge	10
Figure 2.4	Structure of polyvinyl polypyrrolidone	11
Figure 2.5	Bathochromic and hyperchromic shifts produced by acylation of purple carrot anthocyanins at 520 nm.	16
Figure 2.6	Anthocyanin structural transformations with change in pH	18
Figure 2.7	Degradation of oxonium ion at pH 3.7 accelerated by heat	19
Figure 2.8	Colorless anthocyanin-sulfate complex	23
Figure 2.9	Anthocyanin intermolecular and intramolecular interactions	24
Figure 2.10	Self association of anthocyanins at the C4 position	25
Figure 2.11	Procyanidin configurations	29
Figure 2.12	Proanthocyanidin (dimers) common modes of coupling	30
Figure 2.13	Oxidative cleavage of procyanidin to yield 2 cyanidin and 1 catechin	32
Figure 2.14	Vanillin reaction with flavan-3-ol monomers	35
Figure 2.15	DMAC reaction with flavan-3-ol monomers	36
Figure 2.16	American cranberries	39
Figure 2.17	Cocoa pod and beans	41
Figure 3.1	Products of terminal unit proanthocyanidin reaction with vanillin and DMAC.	47
Figure 3.2	Standard curves for DMAC assay prepared using 6N HCl and 6N $H_2 SO_4$ in the DMAC reagent.	57
Figure 3.3	Standard curves for the DMAC assay prepared using 6N HCI at varying concentrations	58

Figure 3.4	Standard curves for the DMAC assay prepared using 6N H_2SO_4 at varying concentrations.	59
Figure 3.5	Effect of DMAC concentration on color development	60
Figure 3.6	Color produced as the result of reaction of DMAC with catechin over time	61
Figure 3.7	Effect of temperature on the DMAC-catechin reaction	62
Figure 3.8	The bleaching effect of water on the DMAC-catechin reaction	63
Figure 4.1	Structures of procyanidin A- and B-type dimers	67
Figure 4.2	Fluorescence comparison of the acid/alkaline and traditional 3X acetone/water extraction using the non-resin containing CB3 sample.	78
Figure 4.3	Fluorescence comparison of the acid/alkaline and 6X acetone/water extraction using CBR1 sample	79
Figure 4.4	Chromatogram of cranberry anthocyanins	81
Figure 5.1	Chromatogram of cocoa procyanidins	102
Figure 5.2	Anthocyanins present in cocoa	103
Figure 5.3	Unfermented cocoa powder bisulfite treatments	104
Figure 5.4	Procyanidin-rich unfermented cocoa powder extracts	107
Figure 5.5	Total monomeric anthocyanins over storage period monitored by the pH differential method	110
Figure 5.6	Total phenolics over storage period monitored by the Folin-Ciocaltaeu method.	111
Figure 5.7	Total procyanidins over storage period monitored by the DMAC method.	112
Figure 5.8	Total procyanidins (monomers and trimers) over storage period monitored by normal phase HPLC with fluorescence detection	113

Chapter 1

Introduction

Flavonoids are a family of ~6000 polyphenolic compounds which are secondary plant metabolites and widely distributed in nature (Wätjen and others 2005). These compounds are particularly important in foods and beverages because of their significant presence in the human diet and postulated health benefits upon consumption. All flavonoids contain a $C_6 - C_3 - C_6$ monomeric structure, and can be minimally or complexly glycosilated or acylated in nature. A large portion of the flavonoids consumed in the diet are flavan-3-ols, their polymeric condensation products procyanidins, and anthocyanins. These powerful phytonutrients have gained increased awareness among both scientists and consumers not only for their postulated health benefits beyond basic nutritional needs, but also their strong influence on the sensory acceptance of a product (i.e. color, taste, and etc). Even though many food scientists, nutritionists, epidemiologists, and medical practitioners have related a decrease in many age and obesity related chronic diseases to the high consumption of fruits and vegetables containing these value added food components, scientists have still yet to develop procedures for reproducible separation and accurate quantification of anthocyanins and procyanidins in food because of their structural complexities, stereochemistry, and degrees of polymerization (proanthocyanidins).

Consumer demand for healthier food products, particularly those containing polyphenols, has driven the industry to develop food products with higher concentrations of anthocyanins and procyanidins. This has lead to a need for increased understanding of the chemistry of these compounds in a variety of food matrices, as well as, the development of more accurate methods for characterization, quantification, and authentication of these powerful phytonutrients for many

purposes including validation of labeling claims. The goal of this specific research was to develop more reproducible analytical methods for identification and accurate quantification of anthocyanins and procyanidins in foods and to apply those methods to better understand their chemistry in various food matrices, while monitoring the viability, stability, and integrity of these compounds to select processing conditions. The initial hypothesis was that by developing better / improved chromatographic and spectroscopic techniques, we would be able to better identify, quantify, and understand the stability of anthocyanins and procyanidins in food matrices with a high concentration of polyphenolic compounds. To achieve this hypothesis and the overall objective, the following specific objectives of this research were tested:

- Evaluate parameters which affect the reproducibility of the 4dimethylaminocinnamaldehyde (DMAC) UV-Visible spectrophotometric assay for analysis of proanthocyanidins in foods.
- 2. Develop and apply a protocol for the quantification of total polyphenols, procyanidins, and monomeric anthocyanins present in cranberry extracts.
- 3. Enhance an unfermented cocoa powder extract, which contains a high concentration of procyanidins, by making the product more visually acceptable, while maintaining the nutritional components of other polyphenols (most importantly procyanidins) present in the matrix.

Chapter 2

Literature Review

2.1 Chemical Structure and Properties of Flavonoids:

2.1.1 Chemical Structure of Flavonoids:

Flavonoids are a chemically diverse group of secondary plant metabolites which are widely distributed in plants. The chemical nature of a flavonoid depends on its structural class, degree of hydroxylation/methoxylation, sugar/acid conjugations and/or polymerization. Flavonoids contain a C_6 - C_3 - C_6 phenylpropanoid carbon structure, which varies around the characteristic heterocyclic oxygen ring (Figure 2.1). All flavonoid compounds are derivatives of a 2-phenylchromone structure composed of three phenolic rings known as the A, B, and C rings (Clifford and Cuppett 2000). There are six major classes of flavonoids, those being flavanones, flavones, flavans/flavanols, flavonols, isoflavones, and anthocyanins. The presence or absence of the double bond and carbonyl group on the C-ring represents the major difference in the various classes of flavonoids except in isoflavones where a shift in the B-ring substitution from the C₂ to C₃ position occurs (Harborne 1998). Most flavonoids absorb light in the UV-Visible spectrum between 250 to 285 nm (A ring) and 320 to 385 nm (B ring) and are thus colorless or pale yellow to the human eye (Yao and others 2004). Flavonoids that absorb light in the visible spectrum between 400 and 800 nm are known as pigments. Anthocyanins produce the most intense flavonoid pigment color, absorbing light between 490 and 550 nm (Giusti and Wrolstad 2005). Pigments such as anthocyanins contain un-bound or "loosely bound" electrons, which require less energy to become excited and allow the compounds to absorb light in the visible

region (Yao and others 2004). The color produced is not only characteristic of the absorbed light, but that which is reflected or scattered as well.

2.1.2 Role of Localized Flavonoids in the Plant:

Plants are localized and thus are increasingly vulnerable to a variety of harmful environmental stress factors including frost hardiness, drought, fungal/microbial symbiosis, metal composition of soil, and most notably exposure to UV radiation. Production of flavonoids is a mechanism that aids in the plant's protection by providing photo-protective (Ryan and others 2002), antioxidant (Tattini and others 2004), antifungal (Grayer and Harborne 1994), and antimicrobial (Chou 1999) functions. Evidence of photo protection in plants was demonstrated by Rozema and others (1997) who found that epidermal flavonoids absorb UV radiation in order to protect the internal tissues of leaves and stems. Flavonoids may also help plants grow in soils which are rich in toxic metals such as aluminum (Barcelo and Poschenrieder 2002). Several species of herbaceous insects are deterred or are sensitive to flavonoids (Thoison and others 2004). However, the most obvious role of many color producing flavonoids is serving as pollination attractants and encouraging seed dispersal in most angiosperms by providing an attractive visual appeal to leaves, flowers, fruits, and vegetables. Flavonoids accumulate in the epithelial cells of a mature plant's leaves, pollen, stigmata, and/or floral primordia (Peer and others 2001). Build up of these compounds in a young plant's stem is also common. Although more commonly present in the plant cell vacuole, anthocyanins can accumulate in vesticles eventually coalescing into a structure known as an anthocyanoplast in a number of species (Dixon and Steel 1999). Similar vesticle formation can be found with proanthocyanidin phytoalexins in the cytoplasm of sorghum cells near a fungal infection site (Synder and Nicholson 1990). Once attached to a fungal infection site, the phytoalexins are released near the pathogen to aid in the protection and maintenance of the cell (Synder and Nicholson 1990). These antimicrobial effects of flavonoids have been described, but the roles and mode of action are not yet fully understood.



Figure 2.1: General base structures of flavonoids.

Biosynthesis of Flavonoids:

Flavonoids are synthesized via the phenylpropanoid pathway, which is also responsible for producing many plant secondary metabolites including lignans, lignins, stilbenes, and hydroxycinnamic acids (Schwinn and Davies 2004). All flavonoids are synthesized via the shikimate/chorismate pathways from the amino acid phenylalanine and the citric acid cycle derivative malonyl-CoA (Figure 2.2) (Schwinn and Davies 2004). These compounds are first produced in the cytosol of the plant cell and later transferred to the vacuole (Winkel-Shirely 2002). Final conjugation of the flavonoid aglycon with a sugar glucoside is thought to be involved in the transfer of these compounds into the vacuole (Barz and Mackenbrock 1994). The sequences of biological intermediates which lead to the synthesis of anthocyanins are also responsible for the formation of proanthocyanidins (Schwinn and Davies 2004). Polymerization of the anthocyanidin molecule occurs inside the vacuole after transportation of the anthocyanidin/flavanol and synthetic intermediates across the vacuolar membrane.

2.1.3 Presence in the Human Diet:

The flavonoid family comprises the most widely distributed group of polyphenols consumed by humans. Polyphenols (including anthocyanins and procyanidins) are typically consumed in substantial quantities as compared to vitamins and other bioactive substances; it has been estimated that the average adult consumes about 1000 mg of polyphenols per day, whereas only 12 mg of Vitamin E, 5 mg of carotenoids, and 90 mg of Vitamin C are ingested (Heber 2009). Total flavonoid concentration in foods is dependant upon genetic factors of the plant species and environmental conditions such as light, ripeness, and post-harvest processing preparation (Chu and others 2000).

Aside from their potential health benefits to the consumer, flavonoids are responsible for a wide variety of flavors and colors in many fruit and vegetable food products. Catechins and proanthocyanidins significantly contribute to the preferred color and taste of dark chocolate, aged wines, cranberries, and teas.



Figure 2.2: Schematic of the biosynthetic pathway of flavonoids. PAL: phenylalanine ammonialyase; C4H: cinnamate 4-hydroxylase; 4-coumarate CoA ligase; ACC: acetyl CoA carboxylase; CHS: chalcone synthase; CHI: Chalcone isomerase; F3H: flavanone 3β-hydroxylase; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; AUS: aureusidin synthase; FNS: flavone synthase; FLS: flavonol synthase; FNR: flavanones-4-reductase; UF5GT: anthocyanin 5-O-glucosyltransferases (From Schwinn and Davies 2004. *Plant Pigments and their Manipulation* pp 92.)

Limonoids contribute significantly to the citrus flavor in citrus fruit and citrus fruit products. Anthocyanins from fruit and vegetable extracts have traditionally been used to color acidic foods and beverages as a natural alternative to the use of synthetic red FD&C dyes/lakes. Foods which contain high amounts of flavonoids include: apples, black raspberries, blueberries, broccoli, coffee, cranberries, dark chocolate, grapes, nuts, onions, soy beans, and tea. Of all the flavonoids present in the diet, quercetin and kaempferol predominate in fruits and vegetables (Peterson and Dwyer 1998); however, fruits and vegetables typically contain a mixture of flavonoid compounds.

Processing conditions, especially those involving heat and pH changes (IE: canning and hot-fill processes) may drastically affect the amount of flavonoids present in a food/beverage product. Because flavonoids are typically water soluble, they are easily leached from foods during processes such as washing, steaming, and blanching. In fruits and vegetables, flavonoids are the primary substrate for the natural enzyme polyphenol oxidase which causes enzymatic browning and degradation of the compounds. In apple juice and apple cider this enzymatic reaction preferred and utilized. Citrus fruit juice processing may increase the content of flavonoids because extraction processes also help to release these phytonutrients from the rind (Pierpoint 1986).

Flavonoids are not known to be toxic to humans through the consumption of food and beverage products.

2.1.4 Separation and Quantification of Flavonoids:

Essential to the study of flavonoids, isolation and quantification of these compounds helps us to better understand structural characteristics, functionality/concentration/interaction in foods, as well as, the chemistry behind their health promoting effects to the consumer. The majority of current research focuses upon the utilization of High Pressure Liquid Chromatography (HPLC) for separation/identification of flavonoids. HPLC systems coupled to fluorescence, photodiode array (PDA), ultraviolet (UV), and/or nuclear magnetic resonance (NMR) detectors are popular ways of gaining knowledge an individual compound's structural identity, chemical properties, and spectra.

Mass spectroscopy is also commonly used in determination of the molecular weight of the fragment ion (aglycon) and adjacent sugar/acid conjugations present. UV-Vis spectrophotometers are often used in the rapid determination of flavonoid content of a sample. The Folin-Ciocalteu method is commonly used UV-Vis spectrophotometric assay used to assess the total phenolics present in a sample (Wallace and Giusti 2008).

Prior to the separation and/or quantification of flavonoids, extraction techniques and semi-preparative isolation methods are generally utilized. Because most flavonoids are degraded by natural enzymes such as polyphenol oxidase (PPO) in fruits and vegetables, plant materials are typically dried, frozen in liquid nitrogen, and/or lyophilized prior to grinding/pulverizing into a powder form for use in solvent extraction (Marston and Hostettmann 2006). The greater surface area of a fine powder facilitates the extraction of flavonoids from food. Polarity is important in solvent selection when extracting flavonoids. Less polar flavonoids (isoflavones, flavonols, etc.) are typically extracted using acetone, chloroform, dichloromethane, diether ether, or ethyl acetate, while more polar flavonoids are extracted with alcohol-water mixtures (Marston and Hostettmann 2006). A triple extraction with 70/30 acetone and water solution has shown to be most optimum for the extraction of anthocyanins and proanthocyanidins. It should be noted that slight acidification (0.01 to 0.1%) for quantification of anthocyanins is needed for aglycon stability.

There is no single isolation strategy for the semi-preparation of flavonoids. Semipurification of flavonoids is generally achieved by the use of preparatory cartridges containing polyamide substances (eg. C-18), Sephadex LH-20, and/or ion exchange resin (Marston and Hostettmann 2006). In this system, solvent partition methods may be utilized to exclude or isolate certain sub-groups of flavonoids. For example, anthocyanins are generally isolated in a C-18 semi-preparative cartridge, while other phenolics are eluted using ethyl acetate (Figure 2.3). Anthocyanins can then be recovered for further analysis using acidified methanol. Many studies have focused upon semi-preparation/isolation of proanthocyanidins using Sephadex LH-20 cartridges. Thin layer chromatography (TLC) has also been traditionally been utilized for the separation of flavonoids.



Figure 2.3: Separation of anthocyanins using a semi-preparative C-18 cartridge

2.1.6 Affinity of Polyvinyl pyrrolidone for Flavonoids and Phenolics:

The use of soluble polyvinyl pyrrolidone (PVP) and its insoluble polymer form polyvinyl polypyrrolidone (PVPP) have often been used to precipitate or selectively bind phenolic compounds. This is typical in the processing of beer to prevent "chilling haze" and in the production of concentrated phenolic rich extracts. PVPP is utilized by the wine industry as a fining agent for white wines. PVP and PVPP are Generally Recognized as Safe (GRAS) substances in the United States. In fact, during the early 20th century, PVPP was used as a blood plasma expander for trauma victims. PVPP is also utilized by the pharmaceutical industry as a binding agent for many tablets. Recently, the dietary supplement industry has begun to utilize the binding affects of PVP and PVPP to concentrate phenolic compounds for use in capsules and marketable plant extracts. The chemical structure PVPP is exhibited in Figure 2.4.



Figure 2.4: Structure of polyvinyl polypyrrolidone (PVPP).

2.1.5 Health Promoting Effects of Flavonoids:

As early as 1966, researchers have been studying the health promoting effects of flavonoids, most specifically their ability to act as strong antioxidants in foods and in *vitro* studies (Clemetson and Andersen 1966). Quercetin and rutin are the two most studied flavonoids, however each of the 6 groups of flavonoids have been extensively researched for their health promoting properties (Peterson and Dwyer 1998). Flavonoids are thought to play an essential

role in human health by not only demonstrating strong antioxidant activities (Cook and Samman, 1996) but also their potential role in the inhibition of many undesirable transcription factors such as NF-kB, which controls numerous genes involving inflammatory response (Karlsan and others 2008). It is thought that flavonoids are absorbed by passive diffusion after the cleavage of glycosidic groups by the gut micro-flora (Hollman and others 1995). The compounds in general have been noted to help aid in the protection and prevention of many age and obesity related chronic diseases including: cardiovascular diseases (Bagchi and others 2000), cancer (Juranic and Zizak 2005), capillary fragility (Mian and others 1977), atherosclerosis (Kadar and others 1979), and stroke (Zand and others 2002). Recent attention has been given to the ability flavonoids to effectively inhibit the cell cycle, cell proliferation, oxidative stress, and to induce detoxification enzymes, apoptosis, and thoroughly activate the human immune system (Yao and others 2004). This is in addition to the capability of flavonoids to positively influence visual acuity, anti-inflammation, and cognitive functions (Zafra-Stone and others 2007).

Flavonoids are absorbed in the gastrointestinal tract of humans or animals and excreted in-tact or as metabolites in the urine and feces (Cook and Samman, 1996). Bioavailability of the flavonoid aglycon absorbed can range between 0.1% for anthocyanins and tea catechins to 20% for quercetin and isoflavones. Bioavailability may also depend on the food source. For example, absorption of quercetin has been known to be about four times greater when consumed from onions as opposed to apples (Hollman and others 1995). Colonic bacteria are largely responsible for the breakdown of flavonoids into phenyl acids before absorption (Hollman and others 1995). Measurement of flavonoids in the blood stream and plasma after ingestion shows rapid absorption and utilization of these compounds *in vivo* (Benzie and others 1999).

Interest in anthocyanin pigments has increased because of their beautiful color and functionality in food products. There are numerous anecdotal accounts for the bioactive properties of anthocyanins. British RAF pilots were fed bilberries to enhance their night vision during World War II, while Chernobyl victims were directed to consume chokeberry products to alleviate the effects of high dosage radiation (Wrolstad 2004). Numerous studies have found that

anthocyanin absorption into the blood and serum is minimal, however methylated, glucoronidated, and sulphated metabolites have been detected. It is because of the low bioavailability of anthocyanins that recent research has focused upon their effect positive in the gastrointestinal tract, particularly in chemoprotection in the colon (He and others 2009).

It is now recognized that chronic inflammation in tissues can promote the development of vascular diseases. Consumption of procyanidin-rich foods such as cocoa powder may provide vascular protection through modulation of nitric oxide levels in the bloodstream and other mechanisms including positive effects on cell/membrane function, the immune system, and endothelial reactivity. High flavanol cocoa beverages have shown to have similar effects in the vascular system as the known platelet inhibitor aspirin (Ding and others 2006). The bioavailability and bioactivity of flavanols, and their oligomers (procyanidins) differ depending on their chemical structure and chain length, their interactions with other components present in the food matrix, and their breakdown products following interaction with the gut environment and microflora. The most common procyanidin monomers in cocoa, catechin and epicatechin, show a peak presence in the blood stream 1 to 2 hours after ingestion (Holt and others 2002). Procyanidin concentrations in the blood largely return to baseline values within 6 hours (Holt and others 2002). Proanthocyanidin polymers do not seem to cross the intestinal wall in humans but can be degraded by the gut microflora to low-molecular weight monomers, dimers, and phenolic acids prior to absorption (Scalbert and others 2000). Proanthocyanidins have been noted to strongly bind dietary proteins and decrease their uptake into the body (Rios and others 2003). Recently, A-type procyanidin dimers and trimers present in cranberries have been linked to decreased bacterial adhesion in the urinary tract (Foo and others 2000) further implicating their use as value added ingredients in foods and beverages.

2.2 Anthocyanins:

2.2.1 Anthocyanins in Foods and Beverages:

Consumers first assess the freshness and quality of food by its appearance, which makes color a vital characteristic for the ultimate initial acceptance of a product. Many flowers, fruits and vegetables owe their attractive colors to the presence of anthocyanin pigments that range from red to purple to blue. Anthocyanins represent the most common group of natural flavonoid colorants on the market. In processed foods, natural flavonoid pigmentation can be lost easily during manufacturing and enhancement of the product is obtained by the addition of synthetic dyes/lakes or other natural color extracts. Recently, food manufacturers have begun to re-introduce many natural flavonoid colors to foods as opposed to synthetic dyes because of their functional health properties and findings of new colorant extract sources which exhibit an improved performance. The interest in anthocyanin colorants has drastically increased with current research findings and increasing consumer awareness of the potential functional benefits of natural plant ingredients. Aside from the native colors present in fruits and vegetables, several anthocyanin extracts have had commercial success on the market, including those extracted from grape skins, bilberries, and black raspberries.

2.2.2 Anthocyanin Aglycons (Anthocyanidins) and Structures:

Currently, over 539 anthocyanins and 31 anthocyanidins (aglycons) have been identified in nature (Andersen and Jordheim 2007). Six commonly occurring anthocyanidins, those being: Pelargonidin, Cyanidin, Peonidin, Delphinidin, Petunidin, and Malvidin, predominate in nature accounting for over 90% of the anthocyanins currently identified (Andersen and Jordheim 2007). Anthocyanins absorb light in the visible region because they are protonated at the heterocyclic oxygen ring under acidic conditions. Substitutions on the R-groups of the B-ring exert a significant effect on the stability of the pigments and the color produced (Table 2.1). At the primary level the degree of hydroxylation/methoxylation of the anthocyanidin B-ring (Table 2.1) and the nature of sugar and/or acid conjugations have the greatest effect on the color produced by these pigments. An increased number of hydroxyl and/or methoxyl groups on the B-ring of an anthocyanidin results in a bathochromic shift of the visible absorption maximum which has a bluing effect on the color produced (Table 2.1).

Hydroxylation of the B-ring has been reported to decrease the stability of the anthocyanin, while methoxylation increases stability (Mazza and Miniati 1993). Foods containing the aglycon Malvidin and Petunidin seem to show increased color and pigment stability as compared to other hydroxylated anthocyanins.



Table 2.1: Anthocyanidins more commonly found in nature, their B-ring conjugations (R1 and
R2), and maximum absorbance (Giusti and Wrolstad, 2003).

Small differences in chemical structure have a clear impact on color and tinctorial strength of anthocyanins (Dangles and others 1993). Sugar substitution of the anthocyanidin may decrease the visible absorption maximum of the pigment (hypsochromic shift), producing a more red-orange color (Giusti and Wrolstad 2003). Among the 537 anthocyanins reported, 97% are reported to be glycosilated (Andersen and Jordheim 2007).



Figure 2.5: Bathochromic and hyperchromic shifts produced by acylation of purple carrot anthocyanins at 520 nm. Cy-3-gal-xyl-glu (---) Cy-3-gal-xyl-glu + p-coumaric acid (-).
 Bathochromic shift indicates an increase in the visible absorption maximum (nm). Hyperchromic shift indicates an increase in the peak intensity (mAU). From Giusti and Wallace 2009.

Acylation of the sugar substitutions and/or individual anthocyanidins may also produce bathochromic (increased wavelength) and/or hyperchromic (increased absorption) shifts altering the spectra of a compound (Figure 2.5). Acylation of the anthocyanin, mainly with aromatic acids, can drastically improve the color and pigment stability possibly through intermolecular / intramolecular co-pigmentation, and self-association reactions (Giusti and Wrolstad 2003). This effect is more noticeable when there are two or more acylating cinnamic acids attached to the anthocyanin molecule. Acylated anthocyanins may also interact with components of the food matrix including proteins and fat. This results in increased stability of anthocyanins in food matrices (Wallace and Giusti 2008). Because of such interactions, anthocyanins may exhibit increased color and pigment stability to pH changes, heat treatment, and light exposure (Giusti and Wrolstad 2003).

2.2.3 Environmental Factors Which Influence the Stability of Anthocyanins: Structural Transformation and pH:

At any given pH environment anthocyanins exist in equilibrium of different chemical forms. Anthocyanins typically exhibit an absorption maximum at a pH of 1.0 when the anthocyanidin is in its most stable form known as oxonium or the flavylium cation (Figure 2.7). In this form, the pigment produces a bright orange-red to violet color attractive for many applications. However, at a pH of 4.5, anthocyanins become colorless in its chalcone and/or hemiketal forms.

A cyanidin 3-glucoside molecule will only retain 50% of the orange-red color producing oxonium ion at a pH of 3.0 (Wrolstad 2004). When the anthocyanin is in a pH environment of 7.0 the pigment produces a dull blue to green color when the predominant chemical structure is in the form of a quinonoidal base (Figure 2.6).

The food matrix may enhance or decrease the stability of anthocyanins to pH changes. Wallace and Giusti (2008) showed increased anthocyanin color and pigment stability in fatcontaining yogurt matrices (pH ~ 4.3) because of anthocyanin polymerization and an interaction between the fat matrix of the yogurt and the acylating components of anthocyanins. Similar findings were also reported in milk matrices containing high fat content (Jing and Giusti 2005). Such interactions may also be present between acylated anthocyanins and other food, drug, and cosmetic matrices whose environmental conditions (pH, temperature, etc.) are not suitable to anthocyanin color and pigment stability. Kinetic degradation of anthocyanins due to pH changes have been reported to largely follow a first order reaction. Zero order degradation of anthocyanins has been reported; however, statistical differences between zero and first-order kinetic reactions in quality related systems have shown to be potentially insignificant (Labuza and Riboh 1982).



Figure 2.6: Anthocyanin structural transformations of with change in pH.
Temperature:

Heat treatment is one of the most important processes in food manufacturing and has a detrimental influence on the stability of anthocyanin compounds. A logarithmic rise in anthocyanin degradation has been reported by an arithmetic increase in temperature (Francis 1989; Havlikova and Mikova 1985; Rhim 2002). Anthocyanins first undergo hydrolysis of the glycosidic bond when exposed to heat treatment which leads to loss of color since anthocyanidins are much less stable than their glycosylated forms. It is postulated that the first step in anthocyanidin degradation is conversion of the molecule to chalcone which eventually produces an α -diketone (Wrolstad 2004). The result is the production of a dull dark brown to yellow pigment that is unsuitable as a natural colorant. In general, structural characteristics that lead to increased stability to pH changes also lead to increased thermo-stability. Thermo-degradation of anthocyanins has been largely reported to follow first-order kinetics (Ahamed and others 2004) (Figure 2.7). Highly acylated anthocyanins seem to be more stable in the food matrix (Wallace and Giusti 2008). For example, di-acylated anthocyanins are typically more thermo-stable as compared to mono and non-acylated anthocyanins. Complex sugar residues of red cabbage anthocyanins were also proposed to be protective against thermal degradation (Dyrby and others 2001).



Figure 2.7: Degradation of oxonium Ion at pH 3.7 accelerated by heat. From Giusti and Wallace 2009.

Oxygen and Ascorbic Acid:

Oxygen amplifies the impact of other anthocyanin degradation processes. The unsaturation of the anthocyanin structure makes it increasingly susceptible to degradation by oxygen and ascorbic acid. The presence of oxygen accompanied with elevated temperature was the most detrimental combination of many factors tested against color deterioration of different berry juices and isolated anthocyanins in a study conducted by Nebesky and others (1949). It has long been known in the food industry that packaging anthocyanin containing products such as juices under a vacuum or in a nitrogen atmosphere can increase the shelf life of the pigments significantly. Reaction of anthocyanins with oxygen compounds typically yields a dull yellowbrown oxidized color. Damaging effects of oxygen on the anthocyanin pigments can take place through a direct oxidative mechanism and/or through indirect oxidation, where the oxidized components of the media further react with anthocyanins giving rise to colorless or yellow-brown products (Jackman and others 1987).

Fortification of food with ascorbic acid is a common practice to inhibit oxidation while nutritionally enhancing the product. When anthocyanins are in the presence of ascorbic acid both compounds have been known to simultaneously disappear. This is most likely due to the formation of hydrogen peroxide and complete degradation of the anthocyanin during ascorbic acid oxidation (Francis 1989). This reaction can be catalyzed by the presence of copper (Francis 1989). Other theories suggest the direct condensation of ascorbic acid with anthocyanins destroy both molecules (Poei-Langston 1981). Contrary to thermal degradation, galactosides were more stable as compared to arabinosides of cranberry anthocyanins against degradation induced by both oxygen and ascorbic acid (Starr and Francis 1968). In some cases, anthocyanins have been shown to be protected by ascorbic acid against enzymatic degradation (Talcott and others 2003).

Light:

Light exerts two contrasting effects on anthocyanins by favoring their biosynthesis in the plant and accelerating their degradation, particularly after extraction and incorporation into a food matrix as a colorant. Light induced degradation is dependant on the concentration of molecular oxygen present (Attoe and von Elbe 1981). The most vigorous anthocyanin loss can be experienced when the pigments are exposed to florescent light (Palamidis and Markakis 1978). As with most natural pigments that lack the structural stability and tinctorial strength, anthocyanins are predisposed to photo-oxidation by light. Furtado and others (1993) found the end products of light induced degradation of anthocyanins to be the same as the ones produced by thermal degradation. However, the kinetic pathways of the two reactions are different (Furtado and others 1993).

Acylated anthocyanins, once again, seem to exhibit increased stability to light exposure conditions. The stability of acylated pelargonidin derivatives from radish in a maraschino cherry application was compared when the product was stored in the dark vs the same product exposed to light. This comparison showed only a slight increase in the rate of pigment degradation when they were exposed to light (Giusti and Wrolstad 1996). This was attributed to the presence of cinnamic acid acylations attached to the anthocyanin molecules.

Enzymes and Sugars:

Sugars and enzymes are naturally present in fruits, and during processing are often added to enhance a food product. Pectolytic enzymes are often used during juice processing to increase the juice yield and color extraction. Under certain conditions enzyme preparations may degrade anthocyanins and other pigments present in fruit by hydrolyzing glycoside substituents (Wightman and Wrolstad 1996). Hydrolyzed anthocyanins in their pure aglycon form are extremely unstable and degrade quickly loosing their coloring properties. Inactivation of enzymes generally improves an anthocyanin's stability in the food matrix (Garcia-Palazon and others 2004). The most common anthocyanin degrading enzymes are glycosidases which break the glycosidic bond between an anthocyanin and its residual sugar resulting in the more unstable anthocyanidin (Huang 1956). This is of great importance because β -glucosides are the most prevalent pigments in anthocyanin-colored fruits (Macheix and others 1990). Similar findings have also been found in anthocyanin-galactoside rich products containing β -galactosidase enzymes (Wightman and Wrolstad 1996). Mold is a common contributor of these enzymes to fruit products (Wightman and Wrolstad 1996).

Other enzymes present in fruits such as peroxidases and phenolases also commonly degrade anthocyanins (Kader and others 1997). Interestingly, cyanidin was observed to react directly with polyphenol oxidase, but pelargonidin did not react at all (Wesche-Ebeling and Montgomery 1990). Generally, enzymes degrade other phenolic compounds present in the media, after which, their corresponding quinones react with anthocyanins leading to brown condensation products. This has been noted in many studies with pure anthocyanins including cyanidin 3-glucoside (Kader and others 1999), pelargonidin 3-glucoside (Kader and others 2001), and in wine model solutions (Sarni and others 1995).

Sugars as well as their degradation products at lower concentrations are known to decrease the stability of anthocyanins. In a study by Daravingas and Cain (1968), all of the tested sugars (fructose, sucrose, glucose, and xylose) increased anthocyanin degradation in the same way. The reaction of anthocyanins with the degradation products of sugar generally yields a brown polymerized complex. Sugars at high concentrations have also been known to protect anthocyanins from degradation because of their ability to lower a product's water activity (a_w). Sugar solutions also help to stabilize anthocyanins during frozen storage by the inhibition of enzymatic reactions (Wrolstad and others 1990).

Sulfur Dioxide:

Sulfur dioxide has been used extensively in the fruit and vegetable industry, chiefly as an inhibitor of microbial growth and of enzymatic and non-enzymatic browning. The presence of

sulfur dioxide can also lead to the color loss of anthocyanins by a reversible bleaching mechanism that generally occurs when fruits are treated with 500 to 3000 ppm of SO₂. Sulfite bleaching of anthocyanins has been attributed to a nucleophilic attack of the oxonium ion's (flavylium cation) 4 position by the negatively charged bisulfate ion (Jackman and others 1987). This reaction is thought to disrupt the conjugated double bond system resulting in loss of color (Figure 2.8).



Figure 2.8: Colorless anthocyanin-sulfate complex.

The extraction efficiency of anthocyanins from sources such as grape skins can be greatly increased by the addition of sulfur dioxide or its equivalent in bisulfite or meta-bisulfite (Francis 1999). Anthocyanin color loss from sulfite bleaching can be restored by washing before further processing. Color loss in anthocyanin containing fruits and vegetables is a result of a structural complex formation between SO₂ and the C₄ position (Figure 7) of the anthocyanidin (Timberlake and Bridal 1966). This in turn, shifts the maximum absorbance of the complex outside of the visible spectra. Anthocyanins that are resistant to the sulfur bleaching effect generally have a conjugated C₄ position (Timberlake and Bridal 1968).

Co-pigmentation and Metal Complexation:

Co-pigmentation is a valuable and natural tool for enhancing and stabilizing the color of anthocyanin rich products. Co-pigmentation can take place through several interactions: intermolecular complex formations, intramolecular complex formations, self association mechanisms, and metal complexation (Figure 2.9). Co-pigmentation is observed as a bathochromic shift in the visible range towards higher wavelength, which is also called the bluing effect, since the color of an anthocyanin changes from red to more blue hue (Asen and others 1972) or as a hyperchromic shift in which the intensity of the anthocyanin color increases (Giusti and Wrolstad 2005). Anthocyanin co-pigmentation reactions are strongly affected by pH, temperature, concentration, and molecular structure. Co-pigmentation reactions are much weaker at very low pH ranges as compared to pH values between 2 and 5 (Williams and Hrazdina 1979).

Intermolecular interaction is a more prominent means of co-pigmentation in fruits, which contain non-acylated anthocyanins. Intermolecular co-pigmentation is defined as the interaction between a colored anthocyanin to a colorless co-pigment which is not bound covalently to the anthocyanin molecule (Brouillard 1983). Hydrogen bonding, hydrophobic interactions, and electrostatic interactions have been speculated as the main driving force for intermolecular co-pigmentation, resulting in a 1:1 complex formation (Cai and others 1990) (Figure 2.9).



Figure 2.9: Anthocyanin intermolecular and intramolecular interactions. Anthocyanidin (≡); Copigment (#); sugar conjugate (■)

Intermolecular co-pigmentation is loosely defined such that the co-pigment is part of the anthocyanin molecule. The covalent bond between the acylation of the anthocyanin molecule and co-pigment stabilizes the complex (Francis 1989). Intramolecular co-pigmentation is thought to be stronger and more effective in stabilizing anthocyanin color, probably due to the strength of the covalent bonds present (Brouillard 1982). This type of co-pigmentation is mostly associated with anthocyanins derived from flowers and vegetables, which generally contain acylation.

The mechanism of self association has been described as stacking like interactions (Hoshino and others 1981) (Figure 2.10). Self associations of anthocyanins have been observed to take place during wine aging and it is assumed that they may partially contribute to the color of aged wines.

Unlike polyphenolic copigments, metal ions seem to be rarely involved in color stabilization. However, some highly charged metal ions such as Al⁺³ and Mg⁺² have been reported to possibly strengthen the pigment-copigment interaction leading to hyperchromic and/or bathochromic shifts (Elhabiri and others 1997).



Figure 2.10: Self association of anthocyanins at the C-4 position. From Giusti and Wallace 2009.

Goto and Kondo (1991) have utilized x-ray technology to show a natural six pigment-six copigment complex around two magnesium ions in a crystalline state, producing a hyperchromic and bathochromic shift in flower petals. The most common metals and anthocyanin complexes are with tin (Sn), copper (Cu), iron (Fe), aluminum (Al), magnesium (Mg), and potassium (K) (Markakis 1982). Only cyanidin, delphinidin, and petunidin based anthocyanins, which have more than one free hydroxyl group in the B-ring are capable of metal chelation on the aglycon (Osawa 1982).

2.2.4 Bioavailability of Anthocyanins:

Absorption of anthocyanins begins in the stomach and continues on into the colon. Anthocyanins absorbed in the stomach are expected to appear rapidly in the blood stream. He, Wallace and others 2009 have proposed an active transporter protein in the stomach which facilitates the absorption of anthocyanins. This was hypothesized after centrifugation of anthocyanin extracts from stomach tissues of rats showed precipitation of the anthocyanins present. The precipitate was quantifiable using HPLC-MS, but showed the spectra of anthocyanins at a buffered pH of 1.0 and 4.5 (He, Wallace and others 2009). This study noted that the stomach was a major absorption site for anthocyanins.

Anthocyanins are largely absorbed in the intestine, particularly the small intestine which has a pH (7.0) far higher than that needed to achieve maximum stability of anthocyanins (~1.0 – 3.0). Endogenic β -glucosidases are involved in the process of cleaving the sugar moiety from the aglycon, which is in turn smaller and more hydrophobic. This enables the anthocyanidin to be more prone to passive diffusion. About 75 to 78% of absorbed anthocyanins are up-taken in the intestinal tissue (He, Wallace and others 2009). Non-absorbed anthocyanins travel through the intestine where gut microflora are responsible for cleaving glycosidic linkages and breaking down a large amount of the anthocyanins into smaller phenolic acids. The large intestine is much less efficient than the small intestine with respect to absorption (He, Wallace and others 2009).

Anthocyanins may be absorbed in tact (glycosilated and/or acylated) or as free aglycons. They are often subject to glucuronidation, sulfation, and methylation in the intestine enterocyte, liver, and kidney (Felgines and others 2003; Kroon and others 2004). Absorption of anthocyanins has been reported to be very low in *in vivo* studies and ranges between 0.02 to 0.2%. Many factors affect the absorption of anthocyanins including type of sugar moieties, acylation, and the food matrix.

2.3 **Proanthocyanidins:**

2.3.1 Proanthocyanidin Chemical Structure and Stereochemistry:

Proanthocyanidins (condensed tannins) are oligomeric and/or polymeric flavans and flavan-3-ols which play an important role in the organoleptic properties of many food products derived from woody and/or herbaceous plants. Hydroxylation of the A-ring strongly influences the stereochemistry at the C₄ position of the terminal unit (Aron and Kennedy 2008). Procyanidins and prodelphinidins with the 2,3-*cis* stereochemistry frequently adopt the 3,4-*trans* stereochemistry during the interflavanoid bond formation between C₄ \rightarrow C₈ or C₄ \rightarrow C₆ occurring in relative proportion of 3:1 (B-type linkages). The most important chemical aspects of B-type proanthocyanidins are the nucleophilicity of the A-ring and the aptitude of the heterocyclic C-ring to cleavage and subsequent rearrangements (Ferreira and others 2006). Another class of proanthocyanidin dimers (A-type) are similar to B-type conformation, but are different due to the possession of 2 interflavan linkages (C – C and C – O) (Aron and Kennedy 2008). Trimers or C-type proanthocyanidins consist of 3 flavan-3-ol units linked by 2 C₄ \rightarrow C₈ interflavan bonds. The structures of A, B, and C-type proanthocyanidin linkages are presented in Figure 2.11. The best characterized proanthocyanidins are linked via a C – C bond between the C₈ position of the terminal unit and the C₄ position of the extender (B-type).

The four common modes of proanthocyanidin coupling are illustrated in Figure 2.12 (Haslam 1998). In addition to the demonstrated dimeric structures listed in Figure 2.12, other proanthocyanidins linked by the C_6 position of the terminal unit and the C_4 of the extender have

been isolated in nature. B-type linkages of proanthocyanidins are very common in cocoa beans and grape seeds, while A-type linkages are common in cranberries and blueberries.

Table 2.2 shows common proanthocyanidin types. Proanthocyanidin oligomers and polymers typically terminate with catechin subunits and generally range from 500 to 6000 in molecular weight, although proanthocyanidins with molecular weights as high as 20,000 have been reported (Haslam 1998). Although the term "condensed tannin" is used to describe flavonoid based polyphenolic compounds, the chemical term "proanthocyanidin" is gaining increased acceptance. The oxidative cleavage of proanthocyanidins to yield anthocyanins is shown in Figure 2.13. Catechin and epicatechin based oligomers/polymers are known as procyanidins because of their ability to produce the anthocyanin aglycon cyanidin upon acid hydrolysis. Accordingly, gallocatechin and epigallocatechin based polymers are known as prodelphinidins because of their ability to yield the anthocyanin aglycon delphinidin upon oxidation. Rare mono-substituted flavan-3-ol polymers can yield the aglycon pelargonidin and are thus known as propelargonidins.







A-type Dimer (A1)



C-type Trimer (C1)

Figure 2.11: Proanthocyanidin configurations.





B-1 Epicatechin-(4B->8)-catechin







B-3 Cpicatechin-(4a->8)-catechin

B-4 Cpicatechin-(4a->8)-epicatechin

Figure 2.12: Proanthocyanidin (dimers) common modes of coupling.

HO 78 65 R_2	.0 1 2 3 4	2 1 R ₁	R ₄ 3 4 6	OH R5	
Proanthocyanidin Type	\mathbf{R}_{1}	R_2	R_3	R_4	R_5
Procassinidin	Н	Н	Н	Н	Н
Probutinidin	Н	Н	Н	OH	Н
Proapigeninidin	Н	OH	Н	Н	Н
Proluteolinidin	Н	OH	Н	OH	Н
Protricetinidin	Н	OH	Н	OH	OH
Propelargonidin	OH	OH	Н	Н	Н
Procyanidin	OH	OH	Н	OH	Н
Prodelphinidin	OH	OH	Н	OH	OH
Proguibourtinidin	OH	Н	Н	Н	Н
Profisetinidin	OH	Н	Н	OH	Н
Prorobinetinidin	OH	Н	Н	OH	OH
Proteracacinidin	OH	Н	OH	Н	Н
Promelacacinidin	OH	Н	OH	OH	H

 Table 2.2:
 Structures of common proanthocyanidins.



Figure 2.13: Oxidative cleavage of procyanidin to yield 2 cyanidin and 1 catechin.

2.3.2 Separation and Quantification of Proanthocyanidins:

Many attempts have been made to successfully extract, separate, and quantify proanthocyanidins in foods. The major research setback is the inability to adequately separate proanthocyanidins from other phenolic compounds in food prior to analysis. The wide range of chemical properties and molecular weights of these compounds make this group of plant metabolites difficult to purify. Partial purification has been obtained on Sephadex LH-20 columns by introducing phenolics to the column in an ethanol solution and then recovering proanthocyanidins using varying levels of organic solvent (typically aqueous mixtures of ethanol, methanol, and/or acetone). Attempts to isolate proanthocyanidins using semi-preparative C-18 cartridges show little progress. Sun and others (2006) used a series of C-18, Toyopearl 40 and Lichrospher 100 RP18 solid phase extraction cartridges to isolate monomeric, polymeric, and oligomeric proanthocyanidins in red wine. For this reason, fluorescence detection has been utilized in liquid chromatography during the last decade for its increased selectivity for these specific compounds. Because fluorescence excites a molecule, which then emits a photon of energy at a set excitation/emission wavelength, it offers increased accuracy to quantify these compounds such as procyanidins as compared to traditional UV detection at 280 nm. UV detection is highly dependent on a compound's extinction coefficient as stated by Beer's Law. These extinction coefficients vary significantly for different procyanidin oligomers and polymers causing most of the larger compounds to be underestimated when using UV detection. This loss is eliminated with the use of fluorescence detection because chemically similar compounds fluoresce at the same excitation/emission wavelengths.

Even though chromatographic techniques seem to better eliminate interference and be more reproducible for quantification of procyanidins, several UV-Vis spectrophotometric techniques have been developed because of their simplicity, rapid assessment, and cost efficiency. Three spectrophotometric methods have been widely used to describe the flavan-3-ol and proanthocyanidin content of food products, those being the *n*-BuOH – HCl assay, the Vanillin assay, and the dimethylaminocinnamaldehyde (DMAC) assay.

Acid Butanol Assay:

The Acid Butanol Assay uses the characteristic reaction that gave proanthocyanidins their name. In dilute mineral acids (typically HCl), proanthocyanidins are cleaved into the carbocation unit (extension units) and the terminal flavan-3-ol unit. The carbocations produced then experience autoxidation and are rapidly converted to anthocyanidins. The produced color from the anthocyanidin has a visual absorbance maximum (λ_{max}) at ~ 550 nm. While the n-BuOH assay is more specific to proanthocyanidins, it has the disadvantage of depending on the oxidative cleavage of proanthocyanidins into anthocyanidin chromophores (Hageman and others 1997). Furthermore, the transformation of proanthocyanidins to monomeric anthocyanidins is not complete and the color produced is dependant on the anthocyanidin structure, degree of polymerization of the proanthocyanidins present in the sample, and side reactions which may form red-brown polymers (Scalbert 1992). Procyanidins with higher degrees of polymerization produce more anthocyanidins than dimers because of the existence of more extension units, thus allowing for over-estimation of the compounds present (Hummer and Schreier 2008). Additionally, the proanthocyanidin structure influences the yield and kinetics of reaction (Hummer and Schreier 2008). For example, prodelphinidins typically yield higher anthocyanidin concentrations upon hydrolysis as compared to procyanidins. Differences in proanthocyanidin linkages have been known to influence the amount of anthocyanidin produced during the reaction. To remedy this, Porter and others (1986) suggested the use of ferric ions to accelerate autoxidation. Results of guantification in the Acid Butanol Assay often depend on the use of a standard calibration plot. If cyanidin is used as the standard, the total amount of proanthocyanidins will be under-estimated because only a particular percentage of cyanidin is produced (Hummer and Schreier 2008).

Acetone will also interfere with the Acid Butanol Assay, and consequently the most widely used technique of extracting proanthocyanidins (70/30 acetone in water) cannot be used unless the extract is completely dried and the residue re-dissolved in a solvent (such as methanol) which is compatible with the assay (Gessner and Steiner 2005).

Vanillin Assay:

Vanillin assay utilizes the reagent vanillin, which reacts with flavan-3-ols or the terminal unit of the proanthocyanidins (Figure 2.14). It is specific to a narrow range of procyanidins (monomers through polymers) and dihydrochalcones that have *meta* oriented hydroxyl groups and a single bond on the 2, 3 position and on the A-ring (Hageman and others 1997). Subsequently, other compounds with any appropriately substituted phenolic ring could potentially react with the vanillin reagent. Anthocyanins because of their λ_{max} of ~ 520 nm commonly interfere with the vanillin complex which as a λ_{max} at 510 nm (Figure 2.14).



Vanillin - Epicatechin Complex

Figure 2.14: Vanillin reaction with flavan-3-ol monomers.

4-Dimethylaminocinnamaldehyde (DMAC) Assay:

Currently as an alternative to the vanillin and acid butanol assays, the DMAC assay shows less interference and seems to be a more reproducible method for the UV-Vis quantification of proanthocyanidins. Like the vanillin reagent, the aldehyde DMAC is protonated in the presence of a strong acid solution at it's carbonyl oxygen forming an electrophilic carbocation (Figure 2.15). This carbocation reacts with *meta* oriented di- or tri hydroxyl phenols to produce a colored carbonium ion which has a λ_{max} at 640 nm (Scalbert 1992) (Figure 2.15). The most common standard for the DMAC method is catechin, however oligomeric proanthocyanidin standards have been used (Li and others 1996). This method shows increased accuracy because anthocyanins do not interfere with the λ_{max} of the reaction. The DMAC assay has also been known to be approximately 5 higher in its sensitivity as compared to the Vanillin Assay (Marles and others 2003).



Figure 2.15: DMAC reaction with flavan-3-ol monomers.

Because the DMAC and Vanillin reagents may fail to react with some proanthocyanidin sub-units, both of the assays are known to under-estimate the amount of total oligomeric or polymeric proanthocyanidins (Nitao and others 2001). Color yield is lower for monomers as opposed to polymers in the vanillin assay, while monomers tend to give more color than polymers after reaction with DMAC (Nitao and others 2001). The presence of even small amounts of water has the ability to quench/bleach color yield in both the vanillin and DMAC assays. HPLC – MS Characterization of Proanthocyanidins

Because of the variable polarity and wide range of molecular weights exhibited by proanthocyanidins, identification and quantification using HPLC-MS analysis can be quite difficult. Monomeric and polymeric molecular weight proanthocyanidins (monomers through tetramers) can be separated using reverse phase chromatography and a C-18 column. Larger molecular weight oligomeric compounds (hexamers through decamers) exhibit co-elution in reverse phase chromatography, but may be separated by using normal phase (Hammerstone and others 1999; Kelm and others 2006; Robbins and others 2009). Separation of very high oligomeric

Kelm and others 2006; Robbins and others 2009). Separation of very high oligomeric proanthocyanidins has not been achieved to date using HPLC-MS analysis. Recently the traditional normal phase method (Hammerstone and others 1999) utilizing a silica based column and methylene chloride/methanol/water/acetic acid mobile phase has been replaced by a more efficient method utilizing a diol stationary phase column and acetonitrile/methanol/water/acetic acid mobile phase (Kelm and others 2006; Robbins and others 2009) to separate B-type proanthocyanidins in cocoa. As previously stated, fluorescence detection has widely been used as a more selective detection method for accurately quantifying individual procyanidin oligomers and polymers with varying extinction coefficients. Nuclear magnetic resonance (NMR) is a commonly used method in identifying the difference between structural isomers and the monomeric enantiomers of catechin and epicatechin.

2.3.3 Proanthocyanidins in Foods and Beverages:

Proanthocyanidins are commonly found in foods, but are drastically affected by varying environmental conditions similar to that of anthocyanins. De-polymerization is quite common in response to thermal processing, acidic pH environments, and the presence of oxygen. Transformation of B-type to A-type dimers has been shown in processed blueberries (Burger and others 1990). Proanthocyanidin content in foods is largely determined by the geographical origin of the plant species, variety and ripeness / maturity. Proanthocyanidins have the ability to precipitate protein. This ability is a defining characteristic of "tannins.' In dairy products, catechins have proven to delay oxidative rancidity and been useful in the isolation of whey and cream. Quantitative data on the proanthocyanidin determination of herbs and dietary supplements is not as abundant as that concerning other food sources (Aron and Kennedy 2008). Much attention has been focused upon the proanthocyanidin content of apples, chocolate, cranberries, grapes, red wine, and tea because of their high consumption in the United States (Gu and others 2004). De Pascual-Teresa and others (2000) characterized the flavan-3-ol content of 56 Spanish food items ranging from fruits, legumes, vegetables, chocolate, and beverages. Proanthocyanidins are often responsible for astringent flavors in food products, which may be preferable in products such as dark chocolate or undesirable in products such as light beer.

2.3.4 Bioavailability of Proanthocyanidins:

Of all of the classes of flavonoids proanthocyanidins seem to be the least bioavailable in tact due to their high degree of polymerization. The polymerized compounds are 10 to 100 times less bioavailable than their monomeric constituents (Tsang and others 2005). Proanthocyanidins exist in the plasma mainly in their conjugated form but also as sulfated, glucuronidated, and methylated metabolites (Aron and Kennedy 2008). This was confirmed once again by a study of 69 human subjects given 1000 mg/day grape seed extracts (Ward and others 2004). These metabolites have also been detected in the urine of rats (Tsang and others 2005). Metabolism of

proanthocyanidins may occur in the intestine by gut microflora, giving rise to smaller more bioactive compounds (Gonthier and others 2003). A recent discovery by Youdim and others (2004) showed that cocoa proanthocyanidins and their breakdown products may interact favorably at the blood brain barrier. It should be noted that the biological properties the breakdown products of procyanidins may be very significant to the postulated health benefits associated upon consumption of these compounds. In this regard, it has been reported when pre-diabetic rats were fed 30 mg/kg/day of cocoa procyanidins, there was a marked inhibition of NADPH oxidase in the aorta (Ihm and others 2009). This is significant because NADPH oxidase generates the superoxide free radicals which can lead to inflammation and the development of many chronic diseases. Studies of individuals who consumed procyanidin-rich cocoa beverages showed a 30% improvement in measured blood vessel function (Balzer and others 2008).

2.4 Cranberries and Cranberry Extracts:

2.4.1 Botanic Review:

American cranberries are produced by the woody vining evergreen dwarf shrub *Vaccinium macrocarpon*. To date, cranberries have become one of the three commercially important fruits native to North America (the others being blueberries and concord grapes) (McKay and Blumberg 2007) (Figure 2.16).



Figure 2.16: American cranberries.

The plants grow from eastern Canada to the US state of North Carolina. Approximately 500-700 million pounds of cranberries are commercially produced worldwide (85% in the US and 15% in Canada) (McKay and Blumberg 2007). Chile also produces a very small amount of cranberries for export. According to the Economic Research Service of the United States Department of Agriculture (USDA) (2009), Wisconsin is the leading producer of cranberries with over 50% of the US production. The shrubs grow 5 to 30 centimeters in height and approximately 2 meters long with slender wiry stems and small evergreen leaves 5-10 millimeters in size. Small pink flowers eventually produce a white colored fruit, which upon maturity will become bright red because of the presence of anthocyanins in the berry. Cranberries are closely related to blueberries, bilberries, and huckleberries, all in the subspecies *Vaccinium*.

2.4.2 Production and Processing of Cranberries:

Cranberries are gown slightly acidic soil (pH 4.0 - 5.5) with a large supply of fresh water and coarse sand nearby. Good drainage is essential to the healthiness and maximum production of the plant. In the winter months, cranberry shrubs are flooded with water. The ice from the water insulates the vines from severe winter injury.

Cranberries take between 75 and 100 days to fully mature and are typically harvested in mid to late October. 96 % of all cranberries produced are processed (Kundson 2008). The primary products produced from cranberries are cranberry juice blends and cranberry sauce. While the juice industry saw a loss of 2.6% from 2005 to 2006, sales of cranberry juice increased 9.9% in the US and 18% internationally (Kundson 2008). Recently, because of the high biological activities and antioxidant capabilities exhibited by cranberries, concentrated extracts have been introduced into the market as dietary supplements. Of the fruit species, cranberries contain one of the highest amounts of quercetin, a bioavailable phytonutrient present in many fruits and vegetables. Cranberries are also noted for their naturally high concentration of proanthocyanidins and anthocyanins which have numerous biological activities as previously stated.

2.4.3 Extraction and Concentration of Cranberry Phytonutrients:

As dietary supplements become of increasing demand to the consumer, cranberry concentrates have a special appeal because of their known health promoting benefits and high levels of phenolic compounds. Traditional methods of concentration include extraction in ethanol followed by evaporation. Recently the soluble compound polyvinylpyrrolidone (PVP) and its polymeric insoluble form polyvinylpolypyrrolidone (PVPP) have been utilized to precipitate and concentrate the phenolic compounds present in cranberry extracts.

2.5 Cacao Beans (*Theobroma cacao*) and their Derived Products:

2.5.1 Botanic Review:

To be called chocolate, a product must contain cocoa powder derived from the cacao tree (*Theobroma cacao* L.). The cacao tree originates in South and Central America, but is currently grown commercially in humid rainy environments less than 700 meters above sea level and between 20° north and 20° south of the equator (Beckett 2008) (Figure 2.17).



Figure 2.17: Cocoa pod and beans.

Each tree stands approximately 12 to 15 meters in height and will begin to bear pods in 2-3 years. There are four types of cocoa: Nacional originating in the Amazonian region of Ecuador, Criollo, Trinitario, and the most frequently grown variety, Forastero. Three major regions in the world are responsible for the growing of cacao trees and production of cocoa, those being West Africa, Southeastern Asia, and South America. Small green pods known as "cherelles" sprout from the trunk/branches of the tree, and will soon develop into mature pods within 5-6 months. These pods can weigh up to 1 kilogram and contain 30-45 beans each. Pods are harvested several times over a period of a few months (Beckett 2008).

2.5.2 Processing of Fermented and Unfermented Cocoa Powder:

Processing of cocoa powder has a detrimental impact on the amount and quality of the polyphenolic compounds (including anthocyanins and proanthocyanidins) present in the final product. After harvesting of cocoa beans the fermentation process is naturally initiated. There are 2 types of traditional fermentation, those being heap fermentation and box fermentation. Correct fermentation is essential to produce the distinct flavors present in the final chocolate product. During fermentation, the heat generated is sufficient to kill the bean, while ethanol produced activates acetic and lactic acid bacteria. It is at this time that polyphenols such as proanthocyanidins react with proteins and peptides to produce the characteristic brown color of cocoa powder (Maillard browning). Naturally occurring anthocyanins are also degraded during this process. Following fermentation, the cocoa beans are dried prior to transportation. Drying protects the beans from mold growth and rancid flavor development. Beans are typically sun dried on large mats. The fermentation and drying processes seem to result in a significant loss of polyphenolic compounds. Roasting temperatures also affect the stability and chemistry of flavonoids present in the cocoa powder product.

Color has long been recognized as a key attribute when consumers assess the quality and integrity of a food product. Violet color present in cocoa powder has been noted to be unappealing to the consumer. Cocoa beans naturally contain cyanidin-3-galactoside and

cyanidin-3-arabinoside anthocyanins derived from the phenylpropanoid and shikimic pathways during ripening (Niemenak and others 2006). The amount of anthocyanins present in the cocoa bean is dependant on the plant's cultivar, geographical origin, and environmental conditions (Niemenak and others 2006). The traditional process of producing cocoa powder involves the fermentation of cocoa beans, which plays a vital role in the profile of polyphenols present in the final product. Recent studies show that anthocyanins and other polyphenols are rapidly degraded by the traditional fermentation process of producing cocoa powder (Wollgast and Anklam 2000). The traditional fermentation process is also responsible for generating polymerization of polyphenols, resulting in lower bioavailability of the compounds to the consumer. Anthocyanins present in the cocoa beans are rapidly degraded after during the fermentation process by means of hydrolysis of the anthocyanin's galactosidic or arabinosidic linkages. The hydrolyzed anthocyanidin (cyanidin) is very unstable and subject to rapid degradation at fermentation temperatures and pH environment, thus eliminating violet color production in the finalized product.

Unfermented cocoa powder alternatively avoids a large portion of flavonoid degradation. Unfermented cocoa beans are oven dried to a moisture content of ~ 5% (Tomas-Barberan and others 2007). The process of producing unfermented cocoa powder helps to protect the integrity of polyphenolics in the matrix and thus significantly maintain monomer stability. These compounds also show increased bioavailability due to the decrease in polymerization and environmental stress which occurs during the traditional fermentation process. Accordingly, anthocyanins in the unfermented cocoa powder matrix retain their coloring properties post processing (IE. willowing and pressing) (Misnawi and others 2002). Unfermented cocoa powder can be used by the industry to increase the population's consumption of healthy polyphenolic compounds and promote the prevention of certain chronic diseases among consumers. This type of cocoa powder production minimizes the degradation of polyphenolic compounds present in the cocoa bean matrix.

Chapter 3

Evaluation of Parameters which affect the 4-dimethylaminocinnimaldehyde Assay for Proanthocyanidins

3.1 Abstract

Proanthocyanidins (including the procyanidins) are widely distributed in nature and represent the most abundant flavonoids consumed in the diet. Recent attention has been given to these compounds because of their health promoting properties towards chronic disease. Because of their large degrees of chemical variation and stereochemistry, isolation/guantification, particularly is difficult. The 4-dimethylaminocinnamaldehyde (DMAC) spectrophotometric assay has become increasingly popular as a rapid technique to quantify the amount of procyanidins present in foods and beverages; however, there is no current industry standard. In this study, several parameters affecting the DMAC reaction with catechin were examined. Effects of acid nature (HCl and H_2SO_4) and concentration (2-10N), temperature (5-45°C), reaction time (2-35 min), sample water content (1-100%), DMAC concentration (1-3%), and presence of 8 interfering substances were evaluated. A mixture of 2% DMAC in methanol (w/v) in 6N H₂SO₄ (50:50 v/v) showed the highest slope in the standard curve when allowed to react for >15 min prior to analysis. The reaction of catechin with DMAC at constant room temperature (21-25°C), with a sample water content ≤ 1% was found increase reproducibility and better assess in the amount of catechin present. Sample water contents higher than 1% showed a significant bleaching effect on the color produced when catechin concentrations were high (lower levels of catechin did not show any significant differences). None of the 8 tested substances naturally occurring in plants or used as processing aids interfere with the DMAC assay.

Key Words: 4-dimethylaminocinnamaldehyde; DMAC; flavan-3-ol; proanthocyanidin

3.2 Introduction

Flavanols and their polymeric condensation products, proanthocyanidins (condensed tannins) are a group of plant polyphenols responsible for many bitter and astringent flavors in food products such as wine, chocolate, beer, and cranberries (Peleg and others 1999). As the most popular flavonoids consumed in the diet (Aron and Kennedy 2008), proanthocyanidins (including the procyanidins) are considered functional ingredients in many foods and beverages because of their positive effects on human health. Recent studies indicate that dietary intervention with procyanidin-rich foods and beverages shows beneficial effects on platelet aggregation (Holt and others 2002), endothelial function (Heiss and others 2003), blood pressure (Taubert and others 2007), and low-density lipoprotein oxidation (Kondo and others 1996). Increased consumption of procyanidins has been found to decrease the instance of cardiovascular diseases (Porter and others 2001), and cancer (Santos-Buelga and Scalbert 2000) in vivo. Previous studies on the intake of procyanidin-rich cocoa show positive effects on flow mediated dilation and the cardiovascular system. Consumption of cocoa procyanidins has shown similar effects as the known platelet inhibitor aspirin (Ding and others 2006). Recently, Atype dimeric and trimeric procyanidins in cranberries have received considerable attention because of their role in the prevention of bacterial adhesion in the bladder and urinary tract (Foo and others 2000).

Because of the wide variation in stereochemistry and polymerization amongst many proanthocyanidins, isolation and quantification have traditionally been cumbersome and often inaccurate. The most traditional spectrophotometric analysis for the quantification of these compounds is the acid butanol assay. This method uses the characteristic reaction which gave proanthocyanidins their name. By using acid and heat the acid butanol method first hydrolyzes the proanthocyanidins into their carbocation unit (extention unit) and the flavan-3-ol unit (terminal unit). The carbiocation unit quickly converts to an anthocyanidin by autoxidation (Hummer and Schreier 2008). Proanthocyanidins with higher degrees of polymerization produce more anthocyanidins than do smaller compounds such as dimers. Prodelphinidins have been noted to yield higher amounts of anthocyanidins as compared to procyanidins (Bate-Smith 1975). This is just one example of how the chemical structure of each individual proanthocyanidin may influence the yield and kinetics of the reaction. The transformation of proanthocyanidins to monomeric anthocyanidins is not always complete and the color produced may be dependent on the anthocyanidin structure, degree of polymerization of the proanthocyanidins present in the sample, and side reactions which may form red-brown polymers (Scalbert 1992). Because acetone has been known to interfere with the acid butanol assay, the most widely used extraction solvent used for proanthocyanidins (70/30 acetone in water) cannot be used unless the extract is completely dried and re-dissolved in a solvent (such as methanol) which is compatible with the assay (Gessner and Steiner 2005).

Accordingly, the vanillin assay was developed to better account for this predicament. The vanillin assay utilizes the aldehyde vanillin which reacts with *meta* oriented hydroxyl groups on the flavanol A-ring to produce a colored product, which absorbs light at 510 nm (Hageman and others 1997) (Figure 3.1). However, in many fruit and vegetable based products anthocyanins which absorb light in the same region of the visible spectrum interfere with the assay and thus must be subtracted prior to analysis to resolve a potential overestimation of the proanthocyanidins. Chlorophyll may also affect the color development (Walton and others 1983). Higher absorbance values generally occur when using the vanillin assay for the more polymeric procyanidins versus monomers because the reaction does not take place at all extension units or solely at the terminal units (Haggerman and Butler 1994).

The 4-dimethylaminocinnamaldehyde (DMAC) assay is similar to the vanillin assay in its chemistry but can be up to 5-fold higher in its sensitivity as compared to the vanillin assay (Hummer and Schreier 2008) (Figure 3.1).



Figure 3.1: Products of proanthocyanidin terminal unit (flavan-3-ol) reaction with vanillin and DMAC.

The DMAC reaction prefers the C₈ position of the A-ring and reacts only with the terminal units of a proanthocyanidin (Ozmianski and Bourzeix 1996). The DMAC assay has a λ_{max} of ~ 640 nm which is outside of the spectra of anthocyanins, eliminating any interference. Many authors including Treutter and others (1994) and Pascual-Teresa and others (1998) have suggested that use of the DMAC UV-Vis spectrophotometric assay may be more selective and show increased sensitivity as compared to other spectrophotometric protocols. Several DMAC protocols have been developed for quantification of proanthocyanidins in plant materials and food products, most notably those prepared by Li and others (1996) and Broeckling and Vivanco (2008), however wide variation between protocols exist, as well as, a reproducible industry standard.

The objective of this research was to develop a more reproducible DMAC assay for use in food and beverage products containing procyanidins. To accomplish this, a number of experimental parameters of the assay were tested including: acid nature and concentration of the prepared reagent, reaction time, reaction temperature, sample water concentration, concentration of DMAC in reagent preparation, and possible interfering substances in food products.

3.3 Materials

3.3.1 Chemicals and Reagents:

Catechin hydrate, rutin hydrate, chlorogenic acid, sinapic acid, L-ascorbic acid, polyvinyl polypyrrolidone (PVPP) and 4-dimethylaminocinnamaldehyde were purchased from Sigma Aldrich (St. Louis, MO). LCMS grade 99.9% methanol and certified ACS grade sodium acetate, potassium chloride, citric acid monohydrate, caffeine, 36N glacial acetic acid, 17N sulfuric acid, and 10N hydrochloric acid was purchased from Fisher Scientific (Fairlawn, NJ). Reagent grade gallic acid and Folin-Ciocalteu reagent were purchased from MP Biomedical (Aurora, OH). Black raspberry extracts donated from Artemis International Inc. (Fort Wayne, IN).

3.4 Methods

3.4.1 Preparation of Catechin Standards:

Standard curves for the spectrophotometric analysis were prepared by first dissolving 0.5 g catechin in methanol in a 100 mL volumetric flask. The volumetric flask was then sonicated for 30 seconds, inverted, and filled to its 100 mL capacity to ensure uniform distribution. Subsequent dilutions of 50, 100, 200, 300, 400, and 500 mg/L catechin in methanol were prepared by adding 1, 2, 4, 6, 8, and 10 mL of the catechin solution to methanol in order to achieve a final volume of 100 mL solution. Standard curves were prepared by adding 20 μ L of each catechin standard to 2380 μ L of methanol and 100 μ L of the DMAC reagent in 3 mL disposable plastic cuvettes (path length = 1 cm). After reaction at room temperature (~20 °C) for 15 min under minimal light conditions, each standard was subjected to spectrophotometric analysis using a UV-Vis Spectrophotometer 2450 (Shimadzu, Columbia, MD). Standards were replicated and analyzed 3 times at the reaction's visible absorption maximum (λ_{max}) of 640 nm in order to ensure accuracy of the standard curve. Standards were plotted concentration (x-axis) vs. absorbance at 640 nm and subject to a regression analysis. All standard curves were required to have an r² value of > 0.98 in order to be used in the analysis. Spectrophotometric readings were reported as catechin equivalents.

3.4.2 Preparation of DMAC Reagents:

The DMAC reagent was prepared immediately before use by dissolving 1.0, 1.5, 2.0, 2.5, or 3.0% DMAC powder (w/v) in a cold 1:1 mixture of methanol and 2, 4, 6, 8, or 10N HCl or H2SO4 (v/v). Reagents were kept in the freezer (-18°C) under dark conditions between analysis. New reagents were prepared daily because of the relative instability of the DMAC over long periods of time and sensitivity to light. Powder form DMAC was stored in the freezer under dark conditions as recommended to prevent degradation over time.

3.4.3 DMAC Assay Parameters Tested:

Using the prepared standards and reagents, the visible absorption maximum of the reaction (λ_{max}) was monitored at 640 nm. All reactions unless specified below were uniform to the following parameters obtained by a micro-scale study where 2.0% DMAC in 6N H₂SO₄ was allowed to react with the prepared catechin standard curves for 15 min at room temperature (~21 °C). The following variations in the assay were studied (n=3):

Acid Nature and Concentration: Acid concentrations of 2, 4, 6, 8, and 10N HCl or H_2SO_4 were used in the preparation of the DMAC reagent.

DMAC Concentration: Concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0% DMAC powder on a (w/v) basis were used during the preparation of the DMAC reagent. Only the 100, 200, and 300 mg/L catechin standards were used in this study.

Reaction Temperature: The reaction was monitored at 5, 15, 20, 25, 35, and $45 \,^{\circ}\text{C}$ (± 0.5 $^{\circ}\text{C}$) in order to determine the influence of temperature on the color produced. In this study, cuvettes containing the catechin standards (100, 200, and 300 mg/L) and DMAC reagent were covered with parafilm to prevent methanol evaporation during the analysis. The absence of parafilm allowed for evaporation of methanol at temperatures higher than 25 $^{\circ}$ C.

Reaction Time: The absorbance at λ_{max} of 100, 200, and 300 mg/L catechin standards were recorded at 2, 5, 10, 12, 15, 17, 20, 25, 30, and 35 min.

Water Content of the Sample: The absorbance at λ_{max} was measured when catechin standards (100, 200, and 300 mg/L) were prepared with initial water contents of 1, 2, 3, 4, 5, 10, 25, 50, 75, and 100% in the standard. This was accomplished by substituting methanol for water during the preparation of the standards.

Interfering Substances: The interference of the following substances were tested by first solublizing 0.1 g of each compound to 100 mL with methanol in a volumetric flask. The prepared substances were then substituted for the 20 μL of standard. Interfering substances tested included: polyvinyl pyrrolidone (PVP), gallic acid, caffeine, chlorogenic acid, rutin, ascorbic acid, citric acid, sinapic acid, and black raspberry semi-purified anthocyanins. Black raspberry anthocyanins were prepared by solublizing 0.1 g of black raspberry extract in 20 mL of water. The solublized extract was filtered using Whatman #4 filter paper and loaded onto a SepPak semi-preparative C-18 SPE cartridge (Waters Assoc., Milford, MA). Other phenolics were eluted from the SPE cartridge using 20 mL of ethyl acetate. Black raspberry anthocyanins (cy-3-sam, cy-3-glu, cy-3-xyl-rut, and cy-3-rut) were recovered with 10 mL of 0.01% acidified methanol as previously described by Hong and Wrolstad (1990).

3.4.4 Statistical Analysis:

The results for the reaction of catechin standards with the prepared DMAC reagent(s) were subjected to a regression analysis and an analysis of variance (ANOVA) using a Tukey's family error rate comparison test at an $\alpha < 0.05$ acceptance level. All statistical analyses were conducted using MiniTab 15.0 software (State College, PA., U.S.A.).

3.5 Results and Discussion

Individual procyanidins contain very different extinction coefficients and reactivity with the DMAC reagent (Pascual-Teresa and others 1998). Catechin and epicatechin have been reported to have a greater reactivity with the DMAC reagent as compared to oligomeric standards (Li and others 1996), and are the most commercially available standards. Other standards including procyanidin B2 and plant extracts of oligomeric standards quantified by HPLC have been used and are known to more accurately account for the actual amount of proanthocyanidins present in a sample. Plant extracts of proanthocyanidins isolated and quantified by HPLC are appropriate standards because they properly account for the differences in polymerization, stereochemistry, and reactivity among mixtures of proanthocyanidins present in a sample with the DMAC reagent. Isolation of these standards however is quite costly, time intensive, and is often un-achievable depending on the plant matrix. Because current knowledge of how monomer and oligomeric standards effect the DMAC analysis exist, other parameters effecting the color development of the reaction were explored in this study in order to maximize the accuracy of the assay.

3.5.1 Acid Nature and Concentration:

The DMAC assay should be carried out in an acidic medium because of the catalytic role of the acid in the reaction of procyanidins and DMAC (Sun and others 1998). Previous methods have utilized HCl in preparation of the DMAC reagent for UV-Vis spectrophotometric assays and the use of H₂SO₄ has not been widely explored. Sun and others (1998) found that certain parameters such as acid nature and concentration had a significant effect on the reactivity of catechin with the vanillin reagent. Because the DMAC assay is similar in its chemistry to the vanillin assay, further exploration of acid nature and concentration was conducted.

Acid normality had a significant effect on the color produced from the reaction of catechin with the DMAC reagent. The 2N and 4N concentrations of both acids used showed significantly lower absorbencies as compared to the standard curves prepared with 6N, 8N or 10N acid concentrations for both HCL (Figure 3.2) and H_2SO_4 (Figure 3.3) (p < 0.05). The standard curves

using 6N acid concentrations produced absorbencies and slopes which were not significantly different from the higher concentrations (p > 0.1) (Figures 3.2 and 3.3). Increased color development and slope of the standard curve may increase the accuracy of the assay by allowing for better detection between smaller changes in concentration. Figures 3.2 and 3.3 show the standard curves for the DMAC assay established at varying HCl and H₂SO₄ concentrations.

While both hydrochloric and sulfuric acids showed good linearity with the assay ($r^2 > 0.98$) at all catechin concentrations, the standard curves containing H₂SO₄ showed increased slopes and absorbencies as compared to those containing HCl at the same normality (Figure 3.4). Similar results have been obtained in the vanillin assay where it has been noted that the use of H₂SO₄ maximized the reactivity between catechin and vanillin, thus increasing the color intensity of the reaction (Sun and others 1998; Scalbert and others 1989). Figure 3.4 compares the slopes and color intensities of the 6N hydrochloric and 6N sulfuric acid treatments. Because of the data obtained with this portion of the study, the use of 6N H₂SO₄ is recommended for increased reproducibility and in obtaining actual concentrations of proanthocyanidins.

3.5.2 DMAC Concentration:

The DMAC reagent should be used in excess so that the reaction is complete and color development represents the total concentration of procyanidins present in the sample/standard. Previous studies have reported the use of 1 to 3% DMAC (w/v) in preparation of the reagent. The influence of DMAC concentration on a weight to volume basis in preparation of the DMAC reagent is shown in Figure 3.5. As shown in Figure 3.5, the absorbance at λ_{max} 640 nm showed no significant difference when 100, 200, and 300 mg/L catechin eq. standards were allowed to react with \geq 2.0% DMAC (p < 0.05). Therefore, a DMAC concentration of \geq 2.0% DMAC on a weight to volume basis is recommended in preparation of the DMAC reagent. Using a lower concentration of DMAC could result in an underestimation of proanthocyanidins.

3.5.3 Reaction Time:

The influence of reaction time is shown in Figure 3.6. At all of the concentrations tested (100, 200, and 300 mg/L catechin eq.), the reaction time should be >15 min to achieve maximum color development and the lowest standard deviation. Previous studies by Larkin and others (1996) suggest that 20 min is sufficient for the reaction of all proanthocyanidins present in a sample. However, the study by Larkin and others (1996) utilized a different oligomeric proanthocyanidin standard for the DMAC reaction. Differences in reactivity between catechin and proanthocyanidin oligomers should be expected as the oligomeric proanthocyanidins have been known to react more slowly with the DMAC reagent. In our study with catechin, a reaction time of 15 min was enough to achieve maximum color development. To better account for the reactivity of larger proanthocyanidin oligomers, extracts from sainfonin (Onobrychis viciifolia) were purified using HPLC by Tanner and others (1994) who also suggested a reaction time of 20 min. The composition proanthocyanidins present in a matrix should be known prior to determining if 15 min is sufficient for the reaction. Furthermore, the results from our study with catechin (shown in Figure 3.6) and standardized oligomeric proanthocyanidins (Li and others 1996) suggest that a stable colored complex is formed between 20 and 30 min (color loss may occur after 30 min). Reaction times lower than 15 min in our study resulted in an increase in the standard deviations reported and lower absorbencies at λ_{max} , indicating that the entire sample/standard had not fully reacted with the DMAC reagent. Sun and others (1998) found that catechin (monomer) reacted significantly faster with vanillin as compared to a proanthocyanidin oligomeric extract isolated from grape seeds. For this reason we recommend a fixed sample reaction time of > 15 minutes for estimation of monomers such as catechin and > 20 min for samples containing higher amounts of oligomeric/polymeric proanthocyanidins.

3.5.4 Temperature of the Reaction:

The influence of reaction temperature on the DMAC reaction with catechin is shown in Figure 3.7. A 2-way analysis of variance test showed that the reaction was influenced by both the

concentration of the catechin standard, as well as, the reaction temperature (p<0.05). There was a significant difference in all concentrations (100, 200, and 300 mg/L catechin eq.) in which the samples were allowed to react for 15 min at a temperature of 5°C as compared to the other reaction temperatures of 15, 21, 25, 35, and 45°C (p<0.05). Even though there were no significant differences (p>0.05) in the λ_{max} at 640 nm among the other temperatures, it is recommended that the assay be conducted at constant room temperature between 21 and 25°C. The samples measured after reaction at 5 and 15 °C showed consistently higher standard deviations as compared to the samples reacting at higher temperatures. The samples measured after reaction at 45°C showed slightly lower maximum absorbencies at 640 nm. This could be in part because of the relative instability of the DMAC reagent to environmental factors (light, temperature, etc). Interestingly, Sun and others (1998) and Dalby and Shuman (1978), found that the reaction between vanillin and catechin at temperatures of 5 and 35°C was reversible. Because of the similarity in chemistry of the two reactions, this data further suggests that the temperature should be controlled to maximize reproduction and minimize methanol evaporation from the cuvette (this occured at temperatures >25 °C). Evaporation of methanol from the cuvette may cause the blue color complex to concentrate and thus over-estimation the actual proanthocyanidin concentration may occur. From our studies, methanol evaporation did not occur to any significant quantity when the reaction was conducted at a consistent room temperature between 20 and 25 °C and a reaction time of less than 30 min.

3.5.5 Water Content of Sample:

Water content of the sample has a significant influence on the development of color for the DMAC – catechin reaction only if catechin concentrations are high (Figure 3.8). The λ_{max} of the reaction significantly decreased when the sample water content increased greater than 1 % (p > 0.1) in the 300 mg/L catechin standard. This was noticeable but not significantly different (p<0.05) in the 100 and 200 mg/L catechin standards. The bleaching of the color produced by the DMAC-catechin reaction could be partially explained by the theory of Lavassaur, which
suggests that the real pH value of a sample in an organic solution is highly affected by the water content present (Cabannes 1953). Because the DMAC assay is very dependent on the acid concentration to catalyze the reaction, small increases in water content could have a significant effect on the pH of the catechin standard and thus on the final color produced. For this reason absolute methanol should be utilized in the DMAC assay. Li and others (1996) and Vivas and others (1994) reported that methanol was preferable for the assay as compared to ethanol or 1butanol because DMAC is more soluble in methanol. Methanol has also been found to give better results as compared to ethanol or butanol because it accelerates the reaction more efficiently (Treutter, 1994) and produces a more stable condensation product / color (Vivas and others, 1994). Similar bleaching effects of water have also been found in the vanillin assay (Sun and others 1998), however, when compared with our study, the DMAC assay seems to be less susceptible to small changes in sample water content as compared to the vanillin assay. Because of these findings and our studies on the effect of water content of the sample, we recommend the use of absolute methanol in the assay. If water in the sample is unavoidable, it is recommended that the standard used contain the same concentration of water to avoid underestimation of the compounds.

3.5.6 Interfering Substances:

Products containing proanthocyanidins may also contain similar compounds which may interfere with the DMAC assay. Of the interfering substances tested (polyvinyl pyrrolidone (PVP), gallic acid, caffeine, chlorogenic acid, rutin, ascorbic acid, citric acid, sinapic acid, and black raspberry purified anthocyanins) none of the above was effective in producing color at 640 nm in the presence of the prepared DMAC reagent. These findings support previous studies and evidence that the DMAC reagent is very specific to proanthocyanidins. Treutter and others (1994) reported that indole may interfere with the assay, but can be easily eliminated by evaporation because of its high volatility. Additionally, it has been suggested that the sensitivity of DMAC for catechin is so high that one hundred to one thousand fold concentration of an interfering substance would be needed to produce a similar absorbance maximum (Treutter and others 1994; Treutter 1989). It should be further noted that other interfering compounds may be present in food products, specifically those containing di or tri hydroxy phenolic ring structures.



Figure 3.2: Standard curves for the DMAC assay prepared using 6N HCl at varying concentrations.



Figure 3.3: Standard curves for the DMAC assay prepared using 6N H₂SO₄ at varying concentrations.



Figure 3.4: Standard curves for the DMAC assay prepared using 6N HCl and 6N H2SO4 in the DMAC reagent.



Figure 3.5: Effect of DMAC concentration (w/v) on color development. * represents the comparison of the reaction between the same catechin standard concentration and varying DMAC concentrations.



Figure 3.6: Color produced as a result of reaction of DMAC with catechin over time. Bars represent the standard deviation for each time point.



Figure 3.7: Effect of temperature on the DMAC – catechin reaction after 15 min. * represents the comparison of the reaction between the same catechin standard concentration and varying temperatures.



Figure 3.8: The bleaching effect of water on the DMAC – catechin reaction after 15 min. * represents the comparison of the reaction between the same catechin standard concentration and varying water contents.

3.6 Conclusion

The evaluation of the parameters which affect DMAC assay led to advances in the quantification of proanthocyanidins using UV-Visible spectrophotometry. In order to increase reproducibility of the DMAC assay, recommended parameters when using catechin as a standard include: the use of 2% DMAC (w/v) and 6N H₂SO₄ to prepare the DMAC reagent, a reaction time of >15 min (20 min if oligomeric proanthocyanidin concentrations are high) at constant room temperature between 21 and 25°C, and the use absolute methanol (<1% water in sample). It should be noted that even though none of the compounds tested in the study interfere with the DMAC assay, many interfering substances may exist depending on the matrix being tested. It should be further noted that the use of different standards (IE oligomeric/polymeric) may have a significant effect on the color produced by the DMAC-proanthocyanidin complex. While standard curves produced by purifying proanthocyanidins from the individual food being tested preferred, the developed DMAC protocol represents a standard method which can be used universally by the food industry to better quantify, reproduce, and compare proanthocyanidin contents when those purified standards are not available.

3.7 Acknowledgements

The authors would like to thank the USDA NRI Competitive Grants Program, the Ohio Agriculture Research and Development Center, CliffStar Incorporated, and Mars Botanical (a division of Mars Inc.) for their support of this research.

Chapter 4

Assessment of Normal and Reverse Phase HPLC-MS and Fluorescence / Photodiode Array Detection Methods for Characterization and Quantification of Procaynidins and Anthocyanins in Cranberry Extracts

4.1 Abstract

Anthocyanins and procyanidins represent two bioactive and abundant classes of polyphenols found in nature. Cranberries are a major source of these potent phytonutrients, are prevalent among the fruits and vegetables consumed in the American diet. Cranberries have been shown to contain a wide variety of postulated positive health effects in the vascular system and towards many other age / obesity related disorders. Dimeric and trimeric procyanidins have been noted for their prevention of bacterial adhesion in the urinary tract of humans. Anthocyanins in cranberries may be of particular interest to the food / supplement industry because of their attractive coloring properties to many products as an alternative to synthetic FD&C dyes and lakes. The objective of this study was to develop and apply a protocol for the quantification of total monomeric anthocyanins and proanthocyanidins present in 8 cranberry extracts.

Quantification of all cranberry extracts, including those containing inert binding agent were best extracted using an acetone / water technique versus an acid / alkaline extraction. Cranberry anthocyanins ranged from 1.24 to 6.38 mg/g as calculated by reverse phase HPLC – PDA – MS analysis using a cy-3-glu standard curve and a C-18 stationary phase column. The use of Sephadex LH-20 was effective in removing interfering compounds prior to DMAC and HPLC analysis of procyanidins. The use of a 6X acetone / water extraction was more efficient and less harmful to the original composition of procyanidins present in the cranberry extracts containing the inert binding agent. Better resolution and quantification of A- and B-type procyanidins present in cranberries was achieved to tetramers using HPLC – fluorescence and MS detection with a Develosil Diol stationary phase column. Separation and quantification of procyanidins by their degree of polymerization to nonamers was achieved. Total procyanidins ranged from 0.78 to 22.45 mg/g. The samples containing the inert binding agent showed the highest levels of procyanidins. This research advances the understanding of the chromatography and chemistry of these powerful phytonutrients.

Key Words: procyanidin, anthocyanin, cranberry, inert resin.

4.2 Introduction

Interest in flavan-3-ols, their polymeric condensation products procyanidins, and anthocyanins have increased due to the many postulated health benefits of these compounds to humans post consumption. Procyanidins and anthocyanins are polyphenols and members of the flavonoid family, possessing a C_6 - C_3 - C_6 carbon skeleton centered around the characteristic heterocyclic oxygen ring.

The procyanidins, a specific class of proanthocyanidins found naturally in foods such as cranberries, blueberries, grape seeds, cocoa, and wine contain only catechin and epicatechin units. Under acid hydrolysis these compounds produce the cyanidin anthocyanidin / aglycon. Food products such as cocoa contain only the B-type procyanidins, while cranberries contain both A-and B-type oligomers and polymers (Figure 4.1). A-type procyanidins in foods such as cranberries have been found to be of increased significance in the human diet because of their positive benefits in inhibiting bacterial adhesion in the bladder and urinary tract (Foo and others 2000). Procyanidins are typically quantified using normal phase chromatography, where

separation occurs by degree of polymerization and not individual isomers (Santos-Buelga and others 2003).

Compared with lower molecular weight phenolic compounds, procyanidins seem to be difficult to quantify due to difficulties in isolating the higher oligomers/polymers and the lack of appropriate analytical methods (Gu and others 2002). Significant improvements have been made in the past decade in the separation and resolution of cocoa B-type procyanidins, most notably those by Kelm and others (2006) and Robbins and others (2009) who introduced the use of a Diol stationary phase and use of an acidic acetonitrile and acidic aqueous methanol mobile phase in replacement of the traditionally used silica column and methylene chloride, methanol, and water/acetic acid mobile phase.



A-type Dimer (A1)

Figure 4.1: Structures of procyanidin A- and B-type dimers.

However, mixtures of A and B-type procyanidins seem to be more cumbersome in their separation. In 2001, Prior and others showed significant advances in the separation of blueberry and cranberry procyanidins using a silica based column and a ternary mobile phase as described by Hammerstone and others (1999). Recently, Brownmiller and others (2009) further employed

the use of a diol based column with adapted conditions based on the Kelm and others (2006) method for blueberry procyanidins.

Cranberries also contain a significant amount of anthocyanins naturally present in the matrix. These anthocyanins are responsible for imparting the attractive red color to cranberries and their derived food products. In contrast to procyanidins, considerable work has been published on anthocyanins in fruits and vegetables, however quantification of anthocyanins remains difficult because of the lack of appropriate standards (Prior and others, 2001).

The United States is the world's largest producer of cranberries, and these fruits are prevalent in the North American diet as a primary source of procyanidins and anthocyanins which have shown many positive effects towards decreases in many age and obesity related chronic disorders. More health conscious consumers have driven the massive expansion of the functional food market to produce supplements and great tasting foods with value added ingredients derived from fruits such as cranberries. Several industrial scale methods for extraction and concentration have been utilized to increase the quantity of flavonoids in the diet, including the use of inert binding agents.

The overall goal of this research was to evaluate the phytonutrient content and value of cranberry extracts. Quantification of the total phenolic, monomeric anthocyanin, and procyanidin content, as well as, characterization of anthocyanins and procyanidins was achieved by HPLC-MS.

4.3 Materials

4.3.1 Chemicals and Reagents:

Optima LCMS grade acetonitrile, methanol, water, and 88% formic acid, as well as ACS grade acetone, glacial acetic acid, hydrochloric acid, 0.1N sodium hydroxide, potassium chloride, sodium acetate, and Celite 560 fine diatomaceous earth were obtained from Fisher Scientific (Fairlawn, NJ). Reagent grade gallic acid and Folin-Ciocalteu reagent were purchased from MP Biomedical (Aurora, OH). Catechin (98%), cy-3-glu (98%) and reagent grade 4-

dimethylaminocinnamaldehyde (DMAC) were purchased from Sigma Aldrich (St. Louis, MO). Empty 10 mL solid phase extraction cartridges were purchased from Phenomenex (Torrence, CA). Cranberry extracts containing the inert resin (CBR1, CBR2, CBR3 and CBR4) and those with no inert resin added (CB1, CB2, CB3, and CB4) were provided by CliffStar Incorporated (Dunkirk, NY).

4.4 Methods

4.4.1 Extraction of Cranberry Polyphenols from Extracts Containing an Inert Resin:

The inert binding agent in this study is a common insoluble GRAS substance used in the food industry to irreversibly bind and precipitate polyphenolic compounds. It is often used to precipitate tannins in beer to prevent "chilling haze" however; the dietary supplement industry has adopted this method to further concentrate polyphenols for use in extracts and supplements. Because the four cranberry extracts contain the inert binding agent, methods of extraction of polyphenols from the cranberry – inert binding agent matrix were compared in order to maximize recovery of the compounds.

Extraction of Polyphenols using an Acid / Alkaline Technique to Remove the Inert Resin:

All cranberry powders containing the inert binding agent (CBR1, CBR2, CBR3, and CBR4) were subjected to extraction by an acid / alkaline extraction procedure because of the high affinity of the resin to irreversibly bind phenolic compounds. Diatomaceous earth (300 mg) was added to an empty 5 mL semi-prep cartridge and rinsed with 3 mL of 1N HCl followed by 3 mL of LCMS water. The diatomaceous earth is then removed from the semi-prep cartridge and mixed with 100 mg of the cranberry extract powder, homogenized, and placed back into the semi-prep cartridge. LCMS water (1 mL) was added to compact and push through to set the diatomaceous earth/cranberry powder mixture. Concurrently, a 100 mL volumetric flask was filled with 75 mL of 1N HCl and place inside the vacuum kit under the semi-prep cartridge. Following preparation of the volumetric flask and the semi-prep cartridge, three 5 mL aliquots of 0.1N NaOH were slowly

added to the semi-prep cartridge and collected into the volumetric flask. The first two aliquots were allowed to remain in the cartridge for 25 minutes prior to pushing through (rate of 1 drop every 2 seconds). The final aliquot was allowed to remain in the column for 45 minutes before pushing through. This was followed by the addition of 3 mL of LCMS water and 5 mL of 1N HCl. The final solution in the volumetric flask was diluted to its 100 mL volume with 1N HCl for further analysis.

Samples being used for method evaluation / development using HPLC-MS analysis were concentrated and semi-purified using a 5 g SepPak C-18 SPE Cartridge (Waters Corp.; Milford, MA) to remove the acid and base present from the extraction. The loaded samples were washed with 25 mL of water to remove the 1N HCI / 0.1N NaOH and other sugars / aliphatic acids present, prior to elution of the compounds with 0.01% HCl acidified methanol. The eluted samples were rotary evaporated to dryness using a Buchi Rotary Evaporator (Buchi Laboratories; Postfach, Switzerland). Samples used for HPLC-MS anthocyanin analysis were brought to 10 mL in volume in 0.01% HCl acidified water prior to injection. Those samples being used for HPLC-MS procyanidin analysis were brought to volume using a 30% methanol in water solution. Samples were loaded onto a 20 mL solid phase extraction cartridge packed with Sephadex LH-20, which had been allowed to equilibrate over night in 30% methanol in water solution as described by Brownmiller and others (2009). The loaded samples were washed with 40 mL of the 30% methanol in water solution and eluted with 60 mL of 70:29.0:0.1 acetone / water / acetic acid solution. The samples were once again evaporated to dryness and brought to a 5 mL volume in 70:29.5:0.5 acetone / water / acetic acid prior to injection. Each sample was extracted in triplicate.

Extraction of Polyphenols using a 6X Acetone / Water to Remove the Inert Resin:

Polyphenols from the CBR cranberry powders were extracted by first measuring ~0.1 g of powder into a 15 mL centrifuge tube, followed by the edition of 10 mL of a 70:29.9:0.1 acetone/water/HCl solution. The tubes were then vortexed for 2 minutes and centrifuged for 1

min at 3500 x g. After centrifugation, the liquid supernatant fraction was filtered through Whatman #1 filter paper (Whatman Inc.; Florham, NJ) using a Buchner funnel (Fisher Scientific; Farilawn, NJ). This process was replicated 6 times until the color of the supernatant was clear. The acidic aqueous acetone solution was evaporated using a Buchi Rotary Evaporator (Fisher Scientific; Fairlawn, NJ) to remove the acetone portion. The final solution was brought to a volume of 5 mL with the injection solvent (70:29.5:0.5 acetone / water / acetic acid for procyanidin analysis and LCMS grade water for anthocyanin analysis) in a volumetric flask. Samples were subjected to semi-purification using a 3 g Sephadex LH-20 SPE cartridge under the conditions described previously. Each sample was extracted in triplicate.

Extraction of Polyphenols from Non-Inert Resin Containing Extracts:

Anthocyanins from CB3 and CB4 were extracted by first measuring ~0.1 g of powder into a 15 mL centrifuge tube, followed by the edition of 10 mL of a 70:29.99:0.01 acetone/water/HCl solution. The tubes were then vortexed for 2 minutes and centrifuged for 1 min at 3500 x g. After centrifugation, the liquid supernatant fraction was filtered through Whatman #1 filter paper (Whatman Inc.; Florham, NJ) using a Buchner funnel (Fisher Scientific; Farilawn, NJ). This process was replicated 3 times until all of the compounds and pigments had been extracted from the powder matrix (Rodriguez-Saona and Wrolstad 2005). The acidic aqueous acetone solution was evaporated using a Buchi Rotary Evaporator (Fisher Scientific; Fairlawn, NJ) to remove the acetone portion. The final solution was brought to a volume of 5 mL with LCMS grade water for anthocyanin analysis in a volumetric flask. Procyanidin samples brought to 10 mL with a 30% methanol in water solution and subjected to semi-purification using a 3 g Sephadex LH-20 SPE cartridge under the conditions described previously prior to analysis.

CB2 and CB1 cranberry extracts, which are fully soluble in water, were extracted by measuring ~0.1 g of powder into a 15 mL centrifuge tube and adding 10 mL of water prior to 1 min of vortexing. Because these samples contain tricalcium phosphate which precipitates in the presence of organic solvents and magnesium hydroxide which may harm/corrode the LCMS

equipment, a solid phase extraction clean up of these samples was conducted. Aqueous samples were loaded onto a 1 g / 6 cc SepPak C-18 semi-preparative cartridge (Waters Inc.; Milford, MA), washed with 15 mL of 0.01% HCl in water, and eluted with 0.01% HCl in methanol. The methanoic solution was then evaporated using the Buchi Rotary Evaporator, and brought to a volume of 5 mL with LCMS grade water for anthocyanin analysis. Procyanidin samples were brought to 10 mL volume with a 30% methanol in water solution and subjected to semi-purification using a 3 g Sephadex LH-20 SPE cartridge under the conditions described previously prior to injection. Each sample was extracted in triplicate.

4.4.2 Spectrophotometric Analyses:

Monomeric anthocyanin contents were determined using the pH differential method (Giusti and Wrolstad 2005). A UV-Visible Spectrophotometer 2450 (Shimadzu; Columbia, MD) was used to collect spectral data at 520 and 700 nm with 1 cm path length disposable cells. Pigment content was calculated as cy-3-glu equivalents, using a molecular weight of 449.3 and an extinction coefficient of 26,900 L cm-1 mg-1 (Giusti and Wrolstad 2005).

Total phenolics were measured using the microscale Folin-Ciocalteu method (Waterhouse 2005). Cuvettes were prepared with 20 μ L of sample/standard, 1.58 mL water and 100 μ L Folin-Ciocalteu reagent. The gallic acid dilutions standards, water blanks, and samples were let to stand at room temperature for 8 minutes. A 20% Na2CO3 solution (200 μ L) was added and samples were allowed to stand at room temperature for 2 hr. The absorbance of samples and standards was measured at 765 nm. Total phenolics were calculated as gallic acid equivalents based on a gallic acid standard curve.

Total procyanidins were measured using the 4-dimethylaminocinnamaldehyde (DMAC) assay (Wallace and Giusti 2009). Cuvettes were prepared with 20 μ L of sample/standard, 2380 mL of methanol, and 100 μ L of the prepared DMAC reagent (1:1 (v/v) 6N H₂SO₄ and 2% DMAC (w/v) in methanol). Cuvettes were allowed to equilibrate for 20 min when the absorbance of the colored complex was measured at 640 nm. Total procyanidins were calculated as catechin

equivalents based on a catechin standard curve. Each sample extraction was replicated in triplicate for all spectrophotometric assays.

4.4.3 HPLC – Fluorescence – PDA – MS Analysis:

Samples were analyzed using a high performance liquid chromatograph (HPLC) (Shimadzu; Columbia, MD) equipped with LC-20AD pumps, SIL-20AC auto sampler, and a CTA-20A Column Oven coupled to a LCMS-2010, SPD-M20A Photodiode Array (Shimadzu; Columbia, MD), RF-10AXL Fluorescence (Shimadzu; Columbia, MD), and Mass Spectrometer (Shimadzu; Columbia, MD) detectors, and LCMS Solution Software (Version 3, Shimadzu; Columbia, MD). Mass spectrometry was conducted on a quadrupole ion-tunnel mass spectrometer equipped with electrospray ionization (ESI) interface (Shimadzu; Columbia, MD). All samples were filtered through 13 mm 0.45µm polypropylene filters (Fisher Scientific; Fair Lawn, NJ) prior to HPLC analysis.

Anthocyanins:

A reverse phase 4.6 x 150 mm Symmetry C-18 column with a 3.5 micron size (Waters Corp; MA, USA) was connected to a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corp; MA, USA) for the analysis of anthocyanins. Separation of cranberry extract anthocyanins was achieved using a linear gradient from 10% to 14% B, in 4 min; 14% to 16% B, in 10 min; 16% to 40% B, in 12 min; and 40% to 10% B, in 15 min. Solvent A was 4.5% (v/v) formic acid in water and B was 100% Acetonitrile. The flow rate was 0.8 mL / min and an injection volume of 30µL was used. Spectral data was collected using the PDA detector from 250-700 nm. Absorbance of anthocyanins was monitored at 280 and 520 nm. Total anthocyanins were calculated based on PDA detection at 520 nm and a cy-3-glu standard curve.

A 0.25 mL / min flow was diverted to the mass spectrometer. Mass Spectrometric analysis was preformed under positive ion mode with the following settings: nebulizing gas flow, 1.5 L / min; interface bias, +4.50 kV; block temperature, 200 °C; focus lens, -2.5 V; entrance lens,

-50 V; pre-rod bias, -3.6 V; main-rod bias, -3.5 V; detector voltage, 1.5 kV; scan speed, 1000 amu / sec. Full scan Total Ion Monitoring (TIC) was preformed with a mass range from 200-1000 m/z with an interval of 0.1 m/z. Selective Ion Monitoring (SIM) was used to search for the molecular ion of 287 m/z (cyanidin) and 301 m/z (peonidin), as well as, the parent ions 449 m/z (cy-3-gal and cy-3-glu) and 419 m/z (cy-3-arab), 463 m/z (peo-3-gal and peo-3-glu) and 433 m/z (peo-3-arab) in cranberry extracts. Each sample extraction was injected in duplicate for accuracy.

Commercially available cy-3-glu (10 mg) was dissolved in 10 mL of double distilled water containing 0.1% TFA, and a series of dilutions were prepared to generate a standard curve (r^2 >0.999). All samples tested contained anthocyanin concentrations which fell within the standard curve. Total anthocyanins from cranberry extracts were expressed as cy-3-glu equivalents by weight. The total amount of recovered anthocyanins was calculated by summing the total peak area for each anthocyanin identified at 520 nm using this calibration curve.

Procyanidins:

Procyanidin analysis was performed according to an adapted method from Brownmiller and others 2009. A normal phase 4.6 x 250 mm Develosil Diol column with a 5 micron size (Phenomenex; Torrance, CA) was connected to a 4 x 3 mm Cyano SecurityGuard column (Phenomenex; Torrence, CA) for the analysis. Solvents and samples were filtered through 13 mm 0.45µm polypropylene filters (Fisher Scientific, Fair Lawn, NJ). Separation of cranberry extract procyanidins was achieved using a linear gradient from 0% to 40% B, in 35 min; 40% to 100% B, in 40 min; 100% isocratic B, in 45 min; and 100% to 0% B, in 50 min. The column was re-equilibrated for 5 min between samples. The flow rate was set at 0.8 mL/min.

Solvent A was 2% acetic acid in acetonitrile and B was 95:3:2 methanol / water / acetic acid. The injection volume was 5µL, and column temperature was kept at 35°C. Fluorescence of the procyanidins was monitored at excitation and emission wavelengths of 230 and 321 nm with the fluorescence detector. The fluorescence detector was set to low sensitivity with a gain of 4X for the entire run. Absorbance of procyanidins was also monitored at 280 nm with the PDA

detector. Total procyanidins were calculated as catechin equivalents based upon the fluorescence detection and a catechin standard curve.

A 0.25 mL / min flow was diverted to the mass spectrometer. Mass Spectrometric analysis was performed under negative ion mode with the following settings: nebulizing gas flow, 1.5 L / min; interface bias, +4.50 kV; block temperature, 200 °C; focus lens, -2.5 V; entrance lens, -50 V; pre-rod bias, -3.6 V; main-rod bias, -3.5 V; detector voltage, 1.5 kV; scan speed, 2000 amu / sec. Full scan Total Ion Monitoring (TIC) was preformed with a mass range from 200-2000 m/z with an interval of 0.1 m/z. Selective Ion Monitoring (SIM) was used to search for the procyanidin A and B-type monomers-pentamers (m/z 289, 291, 577, 579, 863, 865, 1151, 1153, 1439, and 1441. Each sample extraction was replicated in duplicate for accuracy.

Commercially available catechin (0.1 g) was dissolved in 100 mL of 70:29.5:0.5 acetone / water / acetic acid, and a series of dilutions were prepared to generate a standard curve $(r^2>0.99)$. Each individual procyanidin peak in all cranberry extract samples tested contained a peak area which was within the catechin standard curve. Total procyanidins from all cranberry extracts were expressed as catechin equivalents by weight. Calculation of total procyanidins, as well as, individual procyanidins grouped by their degree of polymerization was reported based on the calibration curve.

4.5 Results and Discussion

4.5.1 Method Development:

For the quantification of both anthocyanins and procyanidins in cranberry extracts containing the inert resin, the acetone / water extraction procedure better preserved the profile of the compounds present and yielded a higher recovery as compared to the acid / alkaline extraction procedure. Because high acid and alkaline conditions have been known to have some degradation effects on procyanidins and other polyphenols, a non-inert resin containing sample (CB3) was subjected to the acid / alkaline process for further analysis by HPLC-MS. The individual profiles of CB3 when subjected to both an acid / alkaline and a traditional 3X acetone /

water extraction are shown in figure 4.2. The profile of CB3 was drastically affected by the acid / alkaline extraction procedure as higher oligomers (DP>4) and polymers were not detected because of their degradation. This is most likely in part because of the extreme acid conditions (use of 1N HCl) which may hydrolyze the oligomers / polymers present in the original sample. Furthermore, the use of 0.1N sodium hydroxide to break the affinity between the procyanidins and the inert binding agent may have had a decretory effect on the compounds. Polyphenols degrade rapidly in alkaline environments due to the auto-oxidation and opening of the phenolic ring (Cilliars and Singleton 1990). Figure 4.3 shows the fluorescence chromatograms of CBR1 cranberry extract (containing the inert resin) subjected to the two different extraction procedures. Once again, the acetone / water extraction seemed to preserve the profile of procyanidins present in the samples, as well as, the quantity of dimers, trimers, and tetramers (note: the acid / alkaline extraction has an mV (x 100) and the acetone water extraction contains an mV (x 1000)). Similar findings occurred with anthocyanins present in the samples; however anthocyanins seemed to be relatively more stable to the acid / alkaline procedure as compared to the procyanidins (data not shown). The anthocyanin's increased stability in its protonated flavylium cation form at a low pH and ability to reversibly change from the flavylium cation to/from a quinonoidal base during pH fluctuations (Giusti and Wallace 2009) may play an important role in the increased stability of the compound to the acid / alkaline extraction.

Procyanidins and other tannins are thought to bind the inert resin in a similar manor as proteins, through the H bonds between the CO-N linkages in the inert resin and phenol groups of the procyanidin (Pierpoint 2004). Stronger interactions of larger molecular weight procyanidins because of their increased number of phenol groups present (Gunilla and Henrik 2001) could also help to explain the lower abundance of these compounds when using the acetone / water extraction procedure.

	Acid/Alkal	ine Extraction	Acetone/Water Extraction			
	CBR1	CB3	CBR1	CB3		
Monomers	3.39	0.60	3.44	0.76		
Dimers	0.65	0.18	2.50	0.95		
Trimers	1.46	0.99	4.22	3.25		
Tetramers	0.56	0.48	1.71	2.28		
Pentamers	ND	ND	ND	0.96		
Hexamers	ND	ND	ND	0.58		
Heptamers	ND	ND	ND	0.26		
Octamers	ND	ND	ND	0.22		
Polymers	ND	ND	ND	5.06		
Total (mg/g)	6.06	2.25	11.87	14.32		

 Table 4.1:
 Quantitative comparison of the acid / alkaline and acetone / water extraction procedures.

 HPLC results are expressed in mg/g catechin equivalents.



Figure 4.2: Fluorescence comparison of the acid/alkaline and traditional 3X acetone/water extraction using the non-inert resin containing CB4 sample.



Figure 4.3: Fluorescence comparison of the acid/alkaline and 6X acetone/water extraction using CBR1 sample.

4.5.2 Quantification of Anthocyanins using HPLC – PDA – MS and Spectrophotometric Analyses:

For the quantification of anthocyanins and procyanidins in cranberry extracts calibration curves were constructed from the purchased catechin standard, which was injected before and after sample analysis (n=2). The area of each anthocyanin or procyanidin peak was recorded and a quadratic relationship was determined when plotted against concentration. All calibration coefficients for both anthocyanin and procyanidin standard curves exceeded an r² value of 0.999. Using the calibration curves the anthocyanin and procyanidin content of the cranberry extracts were calculated and converted to a total weight basis (mg/g). Individual percent areas for each anthocyanin / procyanidin compound were also reported as related to the total anthocyanin or total procyanidin content of each sample.

Peo-3-gal was the most abundant anthocyanin present except in the CBR cranberry extracts where cy-3-arab was the most abundant anthocyanin present. Cy-3-glu was the least abundant anthocyanin present in all cranberry extracts tested (Figure 4.4). This is consistent with the anthocyanin composition of cranberries as reported by Prior and others (2001). Viskelis and others (2009) also reported similar findings among 4 cranberry cultivars, however, peo-3-glu was the least abundant anthocyanin in only the "Stevens" cultivar while cy-3-glu was the least abundant anthocyanin among the 3 other cultivars (Pilgrim, Ben Lear, and Black Veil) tested (Viskelis and others 2009). A representative (CB3) chromatogram of the cranberry extract anthocyanins is presented in Figure 4.4.

HPLC and UV-visible spectrophotometric analyses of anthocyanins show similar quantitative data (Table 4.2). HPLC-PDA analysis is helpful in isolating individual compounds for quantification while eliminating interferences which may be present in the pH differential method (such as compounds which produce a brown color depending on the pH of the solution). Still the pH differential method is a strong tool in rapidly identifying the total amount of monomeric anthocyanins present in a sample. Both assays utilize cy-3-glu as a standard, which may offer

come variability in the quantification of the compounds because of differences in other anthocyanin's extinction coefficients and absorption in the visible region.



Figure 4.4: Chromatogram of cranberry anthocyanins. ¹Cy-3-gal, ²cy-3-glu, ³pet-3-gal, ⁴cy-3-arab, ⁵peo-3-gal, and ⁶peo-3-arab.

	HPLC Analysis Anthocyanins	pH Differential Anthocyanins	Total Phenolics
CB1	1.24	1.38	17.05
	0.05	0.05	1.04
CB2	1.36	1.47	16.26
	0.11	0.05	1.06
CB3	5.30	5.76	152.45
	0.12	0.02	9.22
CB4	2.35	2.32	45.88
	0.17	0.04	1.02
CBR1	6.38	5.49	225.93
	0.05	0.88	14.07
CBR2	6.14	5.27	204.54
	0.33	0.35	14.21
CBR3	6.00	4.94	216.76
	0.21	0.42	5.79
CBR4	5.61	3.61	176.65
	0.25	0.25	9.17

Table 4.2: Comparison of the HPLC and pH differential analyses of cranberry extract anthocyanin concentrations. Results are reported as mg/g.

•

	Non-Inert Resin containing Cranberry Extracts				Inert Resin containing Cranberry Extracts				
	CB1	CB2	CB3	CB4	CBR1	CBR2	CBR3	CBR4	
Cy-3-gal	0.31	0.43	1.43	0.28	1.64	1.53	1.33	1.29	
	<i>0.02</i>	<i>0.03</i>	<i>0.02</i>	<i>0.02</i>	<i>0.01</i>	<i>0.12</i>	<i>0.05</i>	<i>0.07</i>	
Cy-3-glu	0.02	0.03	0.04	0.03	0.13	0.12	0.15	0.13	
	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.00</i>	<i>0.01</i>	<i>0.00</i>	<i>0.00</i>	
Cy-3-arab	0.24	0.25	0.97	0.58	1.91	1.82	1.87	1.58	
	<i>0.01</i>	<i>0.02</i>	<i>0.02</i>	<i>0.04</i>	<i>0.01</i>	<i>0.06</i>	<i>0.06</i>	<i>0.08</i>	
Peo-3-gal	0.46	0.48	1.99	0.71	1.75	1.73	1.57	1.65	
	<i>0.01</i>	<i>0.04</i>	<i>0.04</i>	<i>0.05</i>	<i>0.01</i>	<i>0.09</i>	<i>0.05</i>	<i>0.06</i>	
Peo-3-glu	0.06	0.06	0.19	0.18	0.27	0.26	0.32	0.29	
	<i>0.00</i>	<i>0.00</i>	<i>0.02</i>	<i>0.01</i>	<i>0.00</i>	<i>0.03</i>	<i>0.01</i>	<i>0.00</i>	
Peo-3-arab	0.21	0.17	0.73	0.64	1.01	1.02	1.09	1.01	
	<i>0.01</i>	<i>0.02</i>	<i>0.02</i>	<i>0.05</i>	<i>0.01</i>	<i>0.02</i>	<i>0.04</i>	<i>0.04</i>	
Total (mg/g)	1.31	1.43	5.36	2.42	6.71	6.47	6.33	5.94	

Table 4.3: Quantitation of cranberry extract anthocyanins expressed as mg/g cy-3-glu equivalents by HPLC – PDA – MS analysis.

	Non-Inert Resin containing Cranberry Extracts				Inert Resin containing Cranberry Extracts				
	CB1	CB2	CB3	CB4	CBR1	CBR2	CBR3	CBR4	
Cy-3-gal	24.46	31.08	26.94	11.42	24.89	24.00	21.28	21.85	
	<i>0.33</i>	<i>0.32</i>	<i>0.19</i>	<i>0.09</i>	<i>0.02</i>	<i>0.67</i>	<i>0.11</i>	<i>0.38</i>	
Cy-3-glu	0.89	1.00	0.58	0.82	1.02	0.90	1.38	1.08	
	<i>0.02</i>	<i>0.01</i>	<i>0.07</i>	<i>0.01</i>	<i>0.01</i>	<i>0.16</i>	<i>0.01</i>	<i>0.01</i>	
Cy-3-arab	18.66	17.78	18.10	23.97	29.20	28.93	30.40	27.19	
	<i>0.27</i>	<i>0.09</i>	<i>0.04</i>	<i>0.07</i>	<i>0.02</i>	<i>0.60</i>	<i>0.03</i>	<i>0.39</i>	
Peo-3-gal	36.59	34.95	37.54	29.83	26.67	27.33	25.35	28.34	
	<i>0.54</i>	<i>0.08</i>	<i>0.20</i>	<i>0.07</i>	<i>0.04</i>	<i>0.07</i>	<i>0.07</i>	<i>0.21</i>	
Peo-3-glu	3.67	3.53	3.34	6.91	3.28	3.15	4.32	3.95	
	<i>0.05</i>	<i>0.07</i>	<i>0.26</i>	<i>0.02</i>	<i>0.02</i>	<i>0.32</i>	<i>0.05</i>	<i>0.07</i>	
Peo-3-arab	15.61	11.66	13.49	26.87	14.93	15.69	17.27	16.94	
	<i>0.13</i>	<i>0.25</i>	<i>0.09</i>	<i>0.15</i>	<i>0.07</i>	<i>0.58</i>	<i>0.05</i>	<i>0.13</i>	
Total (%)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	

Table 4.4: Proportions of anthocyanins in cranberry extracts expressed as % area by HPLC – PDA – MS analysis.

4.5.3 Quantification of Procyanidins using HPLC – Fluorescence – MS and Spectrophotometric Analyses:

The total amount of procyanidins present among the 8 cranberry extracts tested varied depending on the extraction / processing methods and use of other additives to produce the extracts. Horticultural variations in the cranberries among individual batches and products produced at different locations may also influence procyanidin concentrations. Of all the samples tested, the CBR samples proved to be more concentrated as compared to the CB1, CB2, CB4, and CB3 samples in both HPLC and UV-visible spectrophotometric (DMAC) analyses (Table 4.5).

	HPLC Analysis	DMAC Analysis	DMAC Analysis
	(Acetone / Water Ex)	(Acetone / Water Ex)	(Acid / Alkaline Ex)
	(Post Sephadex)	(Post Sephadex)	(No Sephadex)
CB1	0.73	2.09	2.38
001	0.05	0.08	0.15
CB2	0.77	1.85	2.15
UDZ	0.05	0.08	0.24
CB3	16.09	22.55	118.49
	0.37	0.83	2.80
CB4	5.40	33.17	22.44
004	0.28	1.60	0.28
CBR1	18.45	17.05	47.25
CBRI	1.11	1.04	1.61
CBR2	22.45	22.05	44.46
ODINZ	1.58	1.21	2.72
CBR3	22.28	22.26	48.19
OBIG	1.93	1.98	2.18
CBPA	16.09	16.05	29.56
	1.33	0.93	1.59

Table 4.5: Comparison of the HPLC and DMAC analysis of cranberry extract procyanidin concentration. Results are reported as mg/g.

Throughout the study, the results of the DMAC assay were consistently higher than those of the HPLC analysis, before and after purification using Sephadex LH-20. Table 4.5 shows the quantification of cranberry procyanidins using HPLC and DMAC methodologies after semi-purification of the compounds with Sephadex LH-20. The semi-purification of the cranberry procyanidins using Sephadex LH-20 showed a ~20 to 40% reduction in the DMAC values of the purified extract due to the elimination of a portion of interfering compounds which may react with the DMAC reagent (data not shown).

Variability among cranberry products including extracts has been reported by Prior and others (2001) because of the high levels of structural variation, due to oxidation or thermal degradation from particular processing techniques. It has also been reported that the A-type linkages and heterogeneous structures among cranberry procyanidins make quantification by UV-visible spectrophotometric methods difficult because of the lack of appropriate standards. Catechin has been universally used in the DMAC assay; however other less commercially available standards such as procyanidin A1, A2, B1, and B2 have been utilized. These standards all show differences in molar extinction coefficients and reaction kinetics with the DMAC reagent (Howell 2007). Currently, there is no universal method for the quantification of procyanidins in cranberries, however the most reproducible methodologies (HPLC and DMAC) were utilized in this study.

Higher values (~ 2 fold) were achieved using the DMAC assay and acid / alkaline extraction of procyanidins from the CBR powders versus the DMAC assay using the 6X acetone / water extraction procedure (this was opposite with HPLC analysis). It has been widely reported that the more complex cranberry procyanidins may be underestimated using the spectrophotometric assays such as the DMAC assay due to the incomplete cleavage of the molecules by the reagent (Hammerstone and others 1999; Howell 2007). However, in this study the acid / alkaline extraction caused an over-estimation of procyanidins by the DMAC assay when compared with the HPLC data. The strong acid and alkaline conditions present during the acid / alkaline extraction seem to have a degrading effect on these complex procyanidins causing them

to hydrolyze (acidic conditions) and the ring structure to open (basic conditions). This may have resulted in an increase in reactive sites contributed by the degradation products (monomers and phenolic acids) causing over-estimation of the compounds by the DMAC reaction during the acid / alkaline extraction.

Normal phase HPLC separations seem to have good resolution for homogeneous B-type procyanidins such as those in cocoa and grapes, however co-elution and low resolution is experienced in cranberries which contain both heterogeneous A-type and homogeneous B-type procyanidins (Hammerstone and others 1999). This makes the cranberry procyanidins increasingly difficult to accurately quantify using HPLC analyses. Even with this dilemma, HPLC-MS analysis eliminates interference and is a better estimate of the actual concentration of procyanidins present in cranberries. Tables 4.6 and 4.7 show the relative concentrations and percent areas of the monomers to polymers present in each cranberry extract.

	Non-Inert Resin containing Cranberry Extracts			Inert Resin containing Cranberry Extracts				
	CB1	CB2	CB3	CB4	CBR1	CBR2	CBR3	CBR4
Monomers	0.06	0.12	1.17	0.57	2.78	4.70	5.15	2.04
	<i>0.01</i>	<i>0.01</i>	<i>0.14</i>	<i>0.01</i>	<i>0.59</i>	<i>0.69</i>	<i>0.98</i>	<i>0.43</i>
A-Type Dimers	0.17	0.25	2.50	1.17	7.19	8.49	8.93	8.36
	<i>0.01</i>	<i>0.01</i>	<i>0.04</i>	<i>0.10</i>	<i>0.52</i>	<i>0.21</i>	<i>0.27</i>	<i>0.36</i>
B-Type Dimers	0.06	0.07	0.84	0.33	1.85	3.33	3.24	1.63
	<i>0.01</i>	<i>0.01</i>	<i>0.04</i>	<i>0.02</i>	<i>0.53</i>	<i>0.62</i>	<i>0.56</i>	<i>0.28</i>
A-Type Trimers	0.13	0.14	2.37	0.83	2.88	3.23	2.72	2.14
	<i>0.01</i>	<i>0.02</i>	<i>0.07</i>	<i>0.05</i>	<i>0.34</i>	<i>0.18</i>	0.17	<i>0.15</i>
B-Type Trimers	0.03	0.03	0.53	0.20	0.92	1.08	0.93	0.72
	<i>0.00</i>	<i>0.01</i>	<i>0.03</i>	<i>0.01</i>	<i>0.07</i>	<i>0.03</i>	<i>0.03</i>	<i>0.06</i>
A-Type Tetramers	0.06	0.06	1.48	0.48	1.12	1.13	0.89	0.77
	<i>0.01</i>	<i>0.01</i>	<i>0.05</i>	<i>0.03</i>	<i>0.05</i>	<i>0.04</i>	<i>0.03</i>	<i>0.04</i>
B-Type Tetramers	0.03	0.02	0.63	0.22	0.55	0.51	0.43	0.45
	<i>0.00</i>	<i>0.00</i>	<i>0.01</i>	<i>0.01</i>	<i>0.05</i>	<i>0.07</i>	<i>0.04</i>	<i>0.03</i>
Pentamers	0.04	0.04	0.57	0.20	ND	ND	ND	ND
	<i>0.01</i>	<i>0.01</i>	<i>0.03</i>	<i>0.01</i>	ND	ND	ND	ND
Hexamers	0.03	0.03	0.47	0.13	ND	ND	ND	ND
	<i>0.01</i>	<i>0.00</i>	<i>0.04</i>	<i>0.01</i>	ND	ND	ND	ND
Heptamers	0.02	ND	0.25	0.09	ND	ND	ND	ND
	<i>0.00</i>	ND	<i>0.02</i>	<i>0.00</i>	ND	ND	ND	ND
Octamers	ND	ND	0.09	0.04	ND	ND	ND	ND
	ND	ND	<i>0.01</i>	<i>0.00</i>	ND	ND	ND	ND
Polymers (DP>8)	0.09	0.02	5.18	1.15	ND	ND	ND	ND
	<i>0.02</i>	<i>0.00</i>	<i>0.55</i>	<i>0.16</i>	ND	ND	ND	ND
Total (mg/g)	0.73	0.77	16.09	5.41	17.28	22.45	22.29	16.10

 Table 4.6:
 Quantification of cranberry extract procyanidins expressed as mg/g catechin equivalents by HPLC – Fluorescence – MS analysis.

	Non-Inert Resin containing Cranberry Extracts			tracts	Inert Resin containing Cranberry Extracts			
	CB1	CB2	CB3	CB4	CBR1	CBR2	CBR3	CBR4
Monomers	8.23	16.31	7.29	10.55	15.91	20.85	22.94	12.54
	<i>0.70</i>	<i>2.40</i>	<i>0.93</i>	<i>0.45</i>	<i>1.62</i>	<i>1.75</i>	<i>2.55</i>	<i>1.56</i>
A-Type Dimers	23.85	32.97	15.55	21.75	41.85	37.93	40.25	52.10
	<i>1.77</i>	1.48	<i>0.33</i>	<i>1.11</i>	<i>2.10</i>	<i>1.85</i>	<i>2.43</i>	<i>2.03</i>
B-Type Dimers	8.29	8.32	5.23	6.01	10.54	14.72	14.44	10.05
	<i>0.96</i>	<i>0.86</i>	<i>0.27</i>	<i>0.38</i>	<i>1.87</i>	<i>1.79</i>	<i>1.40</i>	<i>0.85</i>
A-Type Trimers	18.29	18.06	14.77	15.41	16.64	14.39	12.21	13.28
	<i>0.73</i>	<i>1.32</i>	<i>0.54</i>	<i>0.35</i>	<i>0.28</i>	<i>0.25</i>	<i>0.38</i>	<i>0.22</i>
B-Type Trimers	4.08	4.17	3.31	3.57	5.32	4.80	4.19	4.48
	<i>0.51</i>	<i>0.85</i>	<i>0.18</i>	<i>0.11</i>	<i>0.36</i>	<i>0.21</i>	<i>0.26</i>	<i>0.04</i>
A-Type Tetramers	8.88	7.61	9.20	8.90	6.52	5.04	4.01	4.75
	<i>0.67</i>	<i>0.43</i>	<i>0.40</i>	<i>0.29</i>	<i>0.64</i>	<i>0.54</i>	<i>0.45</i>	<i>0.19</i>
B-Type Tetramers	3.49	2.66	3.90	4.00	3.22	2.27	1.95	2.80
	<i>0.37</i>	<i>0.20</i>	<i>0.12</i>	<i>0.15</i>	<i>0.61</i>	<i>0.46</i>	<i>0.32</i>	<i>0.09</i>
Pentamers	5.37	4.42	3.52	3.63	ND	ND	ND	ND
	<i>0.61</i>	<i>0.59</i>	<i>0.15</i>	<i>0.14</i>	ND	ND	ND	ND
Hexamers	3.77	3.22	2.91	2.42	ND	ND	ND	ND
	<i>0.77</i>	0.51	<i>0.24</i>	0.09	ND	ND	ND	ND
Heptamers	2.65	ND	1.54	1.70	ND	ND	ND	ND
	<i>0.60</i>	ND	<i>0.14</i>	<i>0.10</i>	ND	ND	ND	ND
Octamers	ND	ND	0.53	0.71	ND	ND	ND	ND
	ND	ND	<i>0.05</i>	<i>0.05</i>	ND	ND	ND	ND
Polymers (DP>8)	13.11	2.27	32.21	21.35	ND	ND	ND	ND
	<i>2.17</i>	<i>0.20</i>	<i>3.00</i>	<i>2.42</i>	ND	ND	ND	ND
Total (%)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 4.7: Proportions of cranberry extract procyanidins expressed as % area by HPLC – Fluorescence – MS analysis.

4.6 Conclusion

Advances in chromatography in the separation and quantification of both A- and B-type procyanidins were made. Separation of monomers through nonamers was achieved with good resolution. A- and B- type procyanidins were separated up to tetramer. The 6X Acetone / Water technique to extract polyphenols from the extracts containing an inert resin was found to be more efficient as compared to the Acid / Alkaline technique. CBR extracts showed higher concentrations of total phenolics, monomeric anthocyanins, and procyanidins as compared to the CB extracts. Total monomeric anthocyanins as calculated by HPLC-MS ranged from 1.31 (CB1) to 6.71 mg/g (CBR1). Similar results and accuracy was obtained using the pH differential method. Total phenolics as calculated by the Folin-Ciocalteu method 16.26 (CB2) to 225.93 mg/g (CBR1). The use of Sephadex LH-20 was effective in eliminating interfering compounds prior to HPLC and DMAC analyses. DMAC values for procyanidins as calculated by HPLC – fluorescence and MS detection ranged from 0.78 (CB1) to 22.45 mg/g (CBR2). This study will provide the food industry with advanced analytical techniques needed to identify and evaluate the composition and content of procyanidins present in foods.

4.7 Acknowledgements

The authors would like to thank CliffStar Incorporated for their support of this research project.
Chapter 5

Selective Removal of the Violet Color Produced by Anthocyanins in Procyanidin-rich Unfermented Cocoa Extracts

5.1 Abstract

Consumer demand has driven the food industry to produce cocoa and procyanidin-rich products that provide health benefits beyond the needs of basic human nutrition. To accommodate this trend, the cocoa industry has begun to produce cocoa powders which are unfermented. These unfermented cocoa powders contain higher levels of procyanidins and violet color – producing anthocyanins. The objective of this study was to selectively remove the violet color present in unfermented cocoa extracts while maintaining the stability and composition of procyanidins present in the matrix.

Several processing parameters including pH fluctuations, enzymatic treatments, and the addition of potassium meta-bisulfite were explored to see which treatment best influenced the color characteristics of the extract throughout a 60 day shelf life study. The use of bisulfite at 500 ppm was found to be the most effective means of removing the violet color present and maintaining procyanidin stability and composition. Procyanidins (monomers to pentamers) were quantified and did not show any significant differences among bisulfite treatments as compared to the control. Bisulfite did not have any detrimental effect on the procyanidins present in the final extract. This research will enable the food industry to produce procyanidin-rich extracts to be used in a wider variety of food products without concerns of creating unwanted colors.

91

Key Words: procyanidin, anthocyanin, cocoa.

5.2 Introduction

The diet is a major lifestyle factor that can strongly influence the development of chronic diseases such as type-2-diabetes, cancer, and cardiovascular disorders among humans (Eyre and others 2004). Interest in flavonoid compounds including anthocyanins and procyanidins has increased as consumer demand for functional foods/ingredients that influence human health beyond basic nutrition has expanded. Both the World Health Organization (WHO) and Food and Agriculture Organization (FAO) support accumulating evidence which suggests flavonoids such as anthocyanins and procyanidins derived from fruits and vegetables are important contributors to improved human health associated with fruit and vegetable consumption (Wallace and others 2009). The many postulated health benefits of these compounds may be significant to consumers because of their vast consumption as compared to other phytonutrients. It has been estimated that the average adult consumes ~1000 mg of flavonoids per day as compared to only 12 mg of Vitamin E, 5 mg of carotenoids, and 90 mg of Vitamin C (Heber 2009).

Anthocyanins are a widely studied group of flavonoids which have a unique application in the food industry not only because of their health promoting properties such as noted increases in visual acuity and cognitive functions (Zafra-Stone and others 2007) but their ability to act as a natural alternative to synthetic FD&C dyes and lakes. Anthocyanins are present in many angiosperms and are responsible for the many attractive orange-red to blue-violet colors produced in fruits, vegetables, tubers, and flowers.

Procyanidins otherwise known as a subgroup of proanthocyanidins, flavanols, and / or condensed tannins are of particular interest to scientists and consumers because of recent epidemiological investigations which link these phytonutrients to the decreased risk of mortality from cardiovascular disease (Hertog and others 1997) and development of diabetes (Knekt and others 2002). Ingestion of cocoa procyanidins at levels as low as 0.5 - 2.3 g/day was associated

with reduced systolic and diastolic blood pressure and reduced deaths from cardiovascular diseases (Buijsse and others 2006).

Consumers first assess the freshness and quality of a food by its appearance, which makes color a critical characteristic in the eventual acceptance of a product (Giusti and Wallace 2009). Cacao beans naturally contain the anthocyanins cy-3-gal and cy-3-arab and are rich in Btype procyanidins. However, a large portion of anthocyanins and procyanidins are degraded during the traditional fermentation process. This has lead the industry to develop more functional food ingredients such as unfermented cocoa powders and unfermented cocoa powder extracts, with the goal of increasing the general population's consumption of these healthy compounds. Anthocyanins may be problematic in these types of functional ingredients because their vibrant violet color may be unattractive in certain types of food products.

The objective of this research was to selectively eliminate the violet color produced by anthocyanins present in unfermented cocoa powder extracts, while maintaining the chemical stability and integrity of other polyphenols present in order to produce a more visually acceptable functional ingredient for use in a wide variety of food products.

5.3 Materials

5.3.1 Chemicals and Reagents:

Optima LCMS grade acetonitrile, methanol, water, as well as ACS grade acetone, glacial acetic acid, hydrochloric acid, potassium chloride, and sodium acetate were obtained from Fisher Scientific (Fairlawn, NJ). Reagent grade gallic acid, Folin-Ciocalteu reagent, and potassium meta-bisulfite were purchased from MP Biomedical (Aurora, OH). Catechin (98%) and reagent grade dimethylaminocinnamaldehyde (DMAC) were purchased from Sigma Aldrich (St. Louis, MO). Galactozyme and arabizyme enzymes were provided by Bio Cat Inc. Cocoa powders (regular fermented, unfermented, and unfermented polyphenol extract) were donated by Mars Botanical (Rockville, MD).

5.4 Methods

5.4.1 Preliminary Test: Evaluation of pH, Enzyme, and Bisulfite Bleaching Methods:

Several parameters effecting the chemical structure of the anthocyanins present and physical environment of the unfermented cocoa powder extract including pH fluctuation, the use of glycosidic enzymes, and potassium meta-bisulfite were explored in order to determine the most effective and practical method to remove the violet color present in the product. In order to assess the feasibility of each method 1 g of unfermented cocoa powder was subjected to dilution in 10 mL of luke warm water and the following treatments: 1) control (no treatment added), 2) pH adjusted to 5.75 using sodium hydroxide, 3) galactozyme A (0.1 g), 4) galactozyme B (0.1 g), 5) arabizyme (0.1 g), 6) galactozyme A (0.1 g) and arabizyme (0.1 g) mixture, 7) alactozyme B (0.1 g) and arabizyme (0.1 g) mixture, 8) potassium meta-bisulfite (500 ppm), and 9) potassium meta-bisulfite (1000 ppm).

The pH of the first sample was altered to 5.75 because anthocyanins are known to be most unstable between 5.5 and 6.0 (Cabrita and others 1999). Galactozyme A, B, and Arabizyme enzymes were utilized solely and in mixtures to cleave the glycosidic linkages between the cy-3-gal and cy-3-arab anthocyanins present in cocoa. It was originally hypothesized that this hydrolysis reaction would yield the cyanidin aglycon, which has been known to rapidly degrade at low acid conditions, such as those present in aqueous cocoa powder (~pH 4.8). Finally, the use of bisulfite at 500 and 1000 ppm was explored to bleach the color imparted by the anthocyanins present in unfermented cocoa powder. Color characteristics (L*, a*, b*, chroma, hue angle, and ΔE) of the samples were monitored in this preliminary study using a Hunter ColorQuest XE (Hunter Associates Laboratory; Reston, VA) for 72 hrs to evaluate which method was more impactful and feasible for the study.

Because potassium meta-bisulfite was found to be the most effective way to eliminate the violet color present in the unfermented cocoa powder extracts, a series of samples treated with varying levels of bisulfite (0, 100, 200, 500, 1000, 2000, 3000, and 4000 ppm) were tested to determine the optimum concentration needed to effectively bleach the pigments during the

extraction processes. Color characteristics of the samples were once again monitored both visually and instrumentally using the same conditions as previously described.

5.4.2 Water Extraction of Polyphenols / Bisulfite Bleaching of Anthocyanins from Unfermented Cocoa Powder:

Extraction of procyanidins from unfermented cocoa powder was conducted using a water based extraction technique to mimic that of the industry. Approximately 100 g of unfermented cocoa powder was mixed with 1L of boiling hot water (temperature 98.5-100 °C) containing varying concentrations of potassium meta-bisulfite (0, 200, 500, or 1000 ppm) over a stirring plate for 10 min in a 2L bottle. Samples were then sonicated on high for 5 min in an ultrasonic dismembrator (Fisher Scientific, Fairlawn, NJ), re-mixed on the stirring plate for 5 additional min, and centrifuged using a Sorvall RC5C Plus centrifuge (Kendro Laboratory Products; Newtown, CT) at 15,200 g using a Sorvall SLA-1500 rotor (Kendro Laboratory Products; Newtown, CT) for 5 min. The supernatant was collected and concentrated to ~20% solids using a Buchi rotary evaporator (Buchi Laboratories; Postfach, Switzerland) prior to spray drying. Samples were flushed with N₂ and stored in the refrigerator at 4 °C in between the extraction and spray drying processes.

5.4.3 Spray Drying of Polyphenol-rich Extracts:

Procyanidin-rich extracts (~20% solids (w/v)) were spray dried using a Buchi Mini Spray Drier B-191 (Buchi Laboratories; Postfach, Switzerland). The spray drier conditions were set to an inlet temperature of 170 °C, aspirator 85%, and a pump speed 20%. Samples were spray dried under vacuum at a pressure of 30 mbar. The outlet temperature of the spray drier was monitored and kept between 95 and 105 °C. Parts of the spray drier were disassembled and cleaned in between bisulfite treatment groups so that no cross contamination occurred.

5.4.4 Shelf Life Study:

Unfermented cocoa powder procyanidin-rich extracts were stored in plastic containers at room temperature (21°C) under dark conditions throughout the shelf life study. Samples were collected on each day of the analysis and monitored for color characteristics (L*, a*, b*, chroma, and hue), total monomeric anthocyanins, procyanidins, and phenolics on days 5, 10, 20, 32, 43, 52, and 60.

5.4.5 Extraction of Polyphenols from Cocoa Powders and Extracts:

To extract the polyphenols (including procyanidins) 0.1 g of the cocoa powders and bisulfite-treated procyanidin-rich extracts were placed in a 15 mL disposable centrifuge tube. 7 mL of 70:29.5:0.5 Acetone / Water / Acetic Acid was added to the centrifuge tube, which was then vortexed for 2 min to facilitate dispersion of the sample. The centrifuge tubes were then submerged in water and subjected to sonication in an Ultra Sonic Cleaner SF530 (Fisher Scientific; Fairlawn, NJ) for 5 min. The centrifuge tubes were once again vortexed for 2 min prior to centrifugation at 3250 g for 5 min in a Centrific 225 Centrifuge (Fisher Scientific; Fairlawn, NJ) equipped with a 225 rotor (Fisher Scientific; Fairlawn, NJ). The supernatant was used for HPLC and UV-Vis spectrophotometric analyses (total phenolics and monomeric anthocyanins). For UV-Vis spectrophotometric analysis of procyanidins, 1 mL of the supernatant was collected in a microcentrifuge tube and brought to dryness (approximately 1 hr) using a Savant DNA120 SpeedVac Concentrator (Savant SpeedVac Systems; Ramsey, MN) (medium heat setting). The dry samples were then brought back to their original 1 mL volume with methanol using sonication and vortexing until all compounds were once again dispersed into solution.

5.4.6 Colorimetric Analysis:

Color (L, a, b, chroma, and hue angle) of spray dried procyanidin-rich cocoa extracts were monitored using a Minolta Chroma Meter CR-300 (Konika Minolta Holdings Inc.; Ramsey, NJ) over the shelf life period.

5.4.8 Spectrophotometric Analyses:

Monomeric anthocyanin contents were determined using the pH differential and method (Giusti & Wrolstad 2005). A UV-Visible Spectrophotometer 2450 (Shimadzu; Columbia, MD) was used to collect spectral data at 520 and 700 nm with 1 cm path length disposable cells. Pigment content was calculated as cy-3-glu equivalents, using a molecular weight of 449.3 and an extinction coefficient of 26,900 L cm-1 mg-1 (Giusti and Wrolstad 2005). Each sample extraction was replicated in duplicate.

Total phenolics were measured using the microscale Folin-Ciocalteu method (Waterhouse 2005). Cuvettes were prepared with 20 μ L of sample/standard, 1.58 mL water and 100 μ L Folin-Ciocalteu reagent. The gallic acid dilutions standards, water blanks, and samples were let to stand at room temperature for 8 minutes. A 20% Na2CO3 solution (200 μ L) was added and samples were allowed to stand at room temperature for 2 hr. The absorbance of samples and standards was measured at 765 nm. Total phenolics were calculated as gallic acid equivalents based on a gallic acid standard curve. Each sample extraction was replicated in triplicate.

Total procyanidins were measured using the 4-dimethylaminocinnamaldehyde (DMAC) assay (Wallace and Giusti 2009). Cuvettes were prepared with 20 μ L of sample/standard, 2380 mL of methanol, and 100 μ L of the prepared DMAC reagent (1:1 (v/v) 6N H₂SO₄ and 2% DMAC (w/v) in methanol). Cuvettes were allowed to equilibrate for 15 min when the absorbance of the colored complex was measured at 640 nm. Total proanthocyanidins were calculated as catechin equivalents based on a catechin standard curve. Each sample extraction was replicated in triplicate.

5.4.9 Sulfur Dioxide Equivalent:

The sulfur dioxide (SO₂) level was determined by an iodine titration method (Payne and others 1969) using a standard iodine solution of 0.156 N. Briefly, an iodine stock solution (1.56 N) was prepared by dissolving 198.4 and 310.0 g of resublimed iodine and potassium iodide in a

1 L volumetric flask. 100 mL of the prepared iodine stock solution was further diluted in a 1 L volumetric flask with distilled water to produce a 0.156 N solution. The normality of the 0.156 N iodine solution was confirmed by a direct titration with a 0.1 N solution of sodium thiosulfate (Payne and others 1969).

Sulfur dioxide equivalents were calculated by placing 0.1 g of the unfermented cocoa extract in a 250 mL Erlenmeyer flask containing 100 mL of distilled water. 1 mL of a soluble starch solution (1 g starch / 100 mL water) and 5 drops of 20% concentrated sulfuric acid was added to the flask which was then titrated with the previously prepared 0.156 N iodine solution until a blue color persisted for 30 sec. The mL of iodine required was multiplied by 1,000 to obtain parts per million SO₂ (Payne and others 1969). Levels of SO₂ were measured in all of the unfermented cocoa extracts after completion of the 60 day shelf life study.

5.4.10 HPLC – Fluorescence – PDA – MS Analyses:

Samples were analyzed using a high performance liquid chromatograph (HPLC) (Shimadzu; Columbia, MD) equipped with LC-20AD pumps, SIL-20AC auto sampler, and a CTA-20A Column Oven coupled to a LCMS-2010, SPD-M20A Photodiode Array (Shimadzu; Columbia, MD), RF-10AXL Fluorescence (Shimadzu; Columbia, MD), and Mass Spectrometer (Shimadzu; Columbia, MD) detectors, and LCMS Solution Software (Version 3, Shimadzu; Columbia, MD). Mass spectrometry was conducted on a quadrupole ion-tunnel mass spectrometer equipped with electrospray ionization (ESI) interface (Shimadzu; Columbia, MD). All samples were filtered through 13 mm 0.45µm polypropylene filters (Fisher Scientific; Fair Lawn, NJ) prior to HPLC analysis.

Procyanidins in Cocoa Powders and Extracts:

Procyanidin analysis was performed according to an adapted method from Kelm and others (2006). A normal phase 4.6 x 250 mm Develosil Diol column with a 5 micron size

(Phenomenex; Torrance, CA) was connected to a 4 x 3 mm Cyano SecurityGuard column (Phenomenex; Torrence, CA) for the analysis.

Separation of cocoa powders and cocoa powder extract procyanidins was achieved using a linear gradient from 7% to 37.6% B, in 57 min; 37.6% to 100% B, in 60 min; 100% isocratic B, 65 min; and 100% to 7% B, in 70 min. The column was re-equilibrated 5 min between samples. The flow rate was set at 1.0 mL/min. Solvent A was 2% acetic acid in acetonitrile and B was 95:3:2 methanol, water, and acetic acid. The injection volume was 5µL, and column temperature was kept at 35°C. Fluorescence of the procyanidins was monitored at excitation and emission wavelengths of 230 and 321 nm with the fluorescence detector. Absorbance of procyanidins was monitored at 280 nm with the PDA detector. A 0.25 mL / min flow was diverted to the mass spectrometer. Mass spectrometric analysis was preformed under negative ion mode with the following settings: nebulizing gas flow, 1.5 L / min; interface bias, +4.50 kV; block temperature, 200 °C; focus lens, -2.5 V; entrance lens, -50 V; pre-rod bias, -3.6 V; main-rod bias, -3.5 V; detector voltage, 1.5 kV; scan speed, 2000 amu / sec. Full scan Total Ion Monitoring (TIC) was preformed with a mass range from 200-2000 m/z with an interval of 0.1 m/z. Selective ion monitoring (SIM) was used to search for the proanthocyanidin B-type monomers-pentamers (m/z 289, 577, 865, 1153, and 1441. Each of the three sample extractions were replicated / injected in duplicate for accuracy throughout the shelf life study.

Commercially available catechin (10 mg) was dissolved in 10 mL of 70:29.5:0.5 acetone / water / acetic acid, and a series of dilutions were prepared to generate a standard curve $(r^2>0.999)$. All samples tested contained individual procyanidin concentrations which fell within the standard curve. Total procyanidins from cranberry extracts were expressed as catechin equivalents by weight. The total amount of recovered procyanidins was calculated by summing the total peak area for each procyanidin (grouped by degree of polymerization) identified using fluorescence detection.

99

Anthocyanins in Cocoa Powders:

A reverse phase 4.6 x 150 mm Symmetry C-18 column with a 3.5 micron size (Waters Corp; MA, USA) was connected to a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corp; MA, USA) for the analysis of anthocyanins. Separation of fermented and unfermented cocoa powder anthocyanins was achieved using a linear gradient from 10% to 15% B, in 9 min; 15% to 40% B, in 11 min; and 40% to 10% B, in 15 min. Solvent A was 4.5% (v/v) formic acid in water and B was 100% acetonitrile. The flow rate was 0.8 mL / min and an injection volume of 30µL was used. Spectral data was collected using the PDA detector from 250-700 nm. Absorbance of anthocyanins was monitored at 280 and 520 nm. Total anthocyanins were calculated based on PDA detection at 520 nm and a cy-3-glu standard curve.

A 0.25 mL / min flow was diverted to the mass spectrometer. Mass spectrometric analysis was preformed under positive ion mode with the following settings: nebulizing gas flow, 1.5 L / min; interface bias, +4.50 kV; block temperature, 200 °C; focus lens, -2.5 V; entrance lens, -50 V; pre-rod bias, -3.6 V; main-rod bias, -3.5 V; detector voltage, 1.5 kV; scan speed, 1000 amu / sec. Full scan Total Ion Monitoring (TIC) was preformed with a mass range from 200-1000 m/z with an interval of 0.1 m/z. Selective Ion Monitoring (SIM) was used to search for the molecular ion of 287 m/z (cyanidin), as well as, the parent ions 449 m/z (cy-3-gal) and 419 m/z (cy-3-arab), in cocoa powders. Each sample extraction was injected in duplicate for accuracy.

Commercially available cy-3-glu (10 mg) was dissolved in 10 mL of double distilled water containing 0.1% TFA, and a series of dilutions were prepared to generate a standard curve (r^2 >0.999). All samples tested contained anthocyanin concentrations which fell within the standard curve. Total anthocyanins from cranberry extracts were expressed as cy-3-glu equivalents by weight. The total amount of recovered anthocyanins was calculated by summing the total peak area for each anthocyanin identified at 520 nm using this calibration curve.

100

5.5 Results and Discussion

5.5.1 Characterization of Anthocyanins and Procyanidins Present in Cocoa Powders:

As previously described by Kelm and others (2006), the use of a Develosil Diol normal phase column and fluorescence detection at excitation and emission wavelengths 230 and 321 proved to be the most optimum HPLC-MS method for accurately assessing the amount of procyanidins present in both fermented and unfermented cocoa powders. The Develosil Diol column recovered more procyanidins as compared to a standard Silica based column in our study and one conducted by Robbins and others (2009). Procyanidins elute by their degree of polymerization (Figure 5.1) and decrease in concentrations. Degrees of polymerization as high as DP9 were found in the cocoa powders identified by their fluorescence, order of elution, spectra, comparison to previous literature, and / or mass spectrophotometric data.

Two anthocyanins were identified by reverse phase HPLC-MS, those being cy-3-gal and cy-3-arab (Figure 5.2) in both fermented and unfermented cocoa powders. The two anthocyanins were present in the fermented cocoa powder, but only slightly detectable using the PDA and MS detectors.



Figure 5.1: Normal phase HPLC – fluorescence chromatogram of cocoa procyanidins at ex/em 230/321.



Figure 5.2: Anthocyanins present in cocoa monitored using PDA detection at 520 nm.

5.5.2 Preliminary Test: Removing Violet Color from Unfermented Cocoa Extracts:

The initial feasibility study indicated that changes in pH and the use of glycosidic enzymes were not sufficient to reduce or eliminate the color produced by anthocyanins present in the unfermented cocoa powder samples. Neither of these treatments had an effect on the color values as compared to the control (Table 5.1). While the pH and enzyme treatments did slightly decrease a* value of the samples, a lower L* value and a higher chroma value were also noted (Table 5.1). The samples containing 500 and 1000 ppm potassium meta-bisulfite showed a higher L* value and lower a*, b*, and chroma values (Table 5.1). The hue angle and ΔE of the bisulfite treated samples increased showing a large change in the total color of the samples as compared to the control and other pH/enzyme treatments. This indicated that the bisulfite bleaching method was a more practical approach for eliminating the violet color present (Table 5.1).

Because of the preliminary results obtained in the study described above, an assessment of the effects of different levels of bisulfite was conducted to determine the range of bisulfite needed to effectively bleach the violet color present in the unfermented cocoa powder samples. Bisulfite concentrations tested are shown in Figure 5.3. As shown, the treatment containing 500 ppm of bisulfite effectively decreased the violet color produced by anthocyanins.



Figure 5.3: Unfermented cocoa powder bisulfite treatments.

5.5.3 Scale Up – Testing the Effects of Bisulfite on Unfermented Cocoa Extracts:

Because of these findings, procyanidin-rich extracts were produced from unfermented cocoa powders containing 0, 200, 500, and 1000 ppm of potassium meta-bisulfite to compare the stability of the procyanidins to potassium meta-bisulfite as a potential additive. 200 ppm bisulfite was explored to see if lower levels of the additive could achieve similar results as the 500 ppm treatment. 1000 ppm was also tested to ensure that the bleaching was not reversible on an industrial scale and to see if higher levels of the compound had any additional effect on the procyanidins / other polyphenols present in the matrix. Bisulfite treatment has been utilized by the food industry (mainly the wine industry) since the 1960's to bleach the color produced by anthocyanins, inhibit polyphenols oxidase, and as antimicrobial agents (Ayed and others 1999). Bisulfite has been proven to significantly improve the extraction of anthocyanins in grape pomace (Kammerer and others 2005) as compared to other conventional techniques. Previous studies have not shown that bisulfite significantly effects the extraction of non-anthocyanin polyphenols. Kammerer and others (2005) noted slightly higher levels of phenolic acids during a sulfite assisted extraction; however these findings were not significantly different from the other extraction techniques. Bisulfite bleaching of anthocyanins has been attributed to the nucleophillic attack of the oxonium ion's C_4 position by the negatively charged bisulfite ion, which is thought to disrupt the conjugated double bond system, resulting in color loss (Jackman and others 1987). The sulfur dioxide analysis showed that the final unfermented cocoa extracts contained an average of 0 ± 0.0 (control), 191.6 \pm 14.4 (200 ppm treatments), 458.3 \pm 14.4 (500 ppm treatments), and 975 ± 0.0 (1000 ppm treatments) ppm sulfur dioxide equivalents (n=3). Because the bisulfite-anthocyanin complex is a reversible reaction (Giusti and Wallace 2009), a hot water extraction technique was chosen to produce the unfermented cocoa powder extracts. Use of organic solvents during the production of procyanidin-rich cocoa extracts causes the bisulfite to precipitate. With the bisulfite no longer in solution, anthocyanins reappear in the nonaqueous environment retaining their coloring properties. This also occurred when samples were extracted using a part-aqueous solution of 70/30 acetone and water.

4 Hours After Treatment	L*	a*	b*	Chroma	Hue Angle	ΔE
Control (pH 6.25)	54.26	29.32	24.15	37.98	39.48	
Control pH 5.75	55.37	23.94	33.57	41.23	54.50	2.80
Galactozyme A	43.75	27.54	33.80	43.60	50.83	4.46
Galactozyme B	53.32	21.27	32.57	38.90	56.86	0.23
Arabizyme	45.33	27.15	29.78	40.30	47.65	5.58
Galactozyme A - Arabizyme	50.30	21.25	30.60	37.25	55.22	3.64
Galactozyme B - Arabizyme	38.24	26.58	35.91	44.67	53.49	7.43
500 ppm Bisulfite	73.00	11.84	23.98	26.75	63.72	11.52
1000 ppm Bisulfite	75.89	9.83	22.99	25.00	66.84	13.67
24 Hours After Treatment	L*	а*	b*	Chroma	Hue Angle	ΔE
Control (pH 6.25)	53.32	29.19	28.97	41.12	44.78	1.11
Control pH 5.75	54.53	25.70	46.33	52.98	60.99	9.80
Galactozyme A	50.16	24.95	48.91	54.91	62.97	8.14
Galactozyme B	42.31	25.19	36.24	44.14	55.20	5.09
Arabizyme	44.45	24.08	33.54	41.29	54.32	5.56
Galactozyme A - Arabizyme	51.01	20.59	40.87	45.76	63.26	2.30
Galactozyme B - Arabizyme	35.93	24.58	36.91	44.34	56.34	9.16
500 ppm Bisulfite	76.97	9.95	24.18	26.15	69.10	16.04
1000 ppm Bisulfite	78.57	8.70	22.78	24.39	67.63	15.06
72 Hours After Treatment	L*	a*	b*	Chroma	Hue Angle	ΔE
Control (pH 6.25)	52.97	28.97	36.20	46.36	51.33	4.16
Control pH 5.75	54.07	27.84	54.16	60.89	62.79	15.20
Galactozyme A	50.23	26.73	55.96	62.01	64.47	13.57
Galactozyme B	42.95	24.27	39.26	46.15	58.28	3.19
Arabizyme	44.68	23.42	38.22	44.83	58.50	2.94
Galactozyme A - Arabizyme	48.59	21.43	45.36	50.17	64.72	3.61
Galactozyme B - Arabizyme	36.45	23.95	38.95	45.73	58.41	7.75
500 ppm Bisulfite	79.13	8.94	24.44	26.02	69.90	17.07
1000 ppm Bisulfite	79.88	7.62	22.53	23.78	71.32	17.12

Table 5.1: Color values using reflectance specular included for $pH - enzyme - bisulfite feasibility tests. L* = lightness, a* = red-green value, b* = yellow-blue value, chroma = intensity of color, <math>\Delta E$ = distance between 2 colors (the difference in color of each sample as compared to the control at 4 hours).

5.5.4 Color, Pigment, and Phenolic Stability:

The color of the procyanidin-rich extracts at all treatment levels (including the control) did not change over the shelf life period. The control and 200 ppm treatments showed no significant color differences throughout the shelf life study (data not shown). The samples containing 500 and 1000 ppm bisulfite showed a significantly lighter and acceptable color as compared to the control and 200 ppm treatment groups (data not shown) (Figure 5.4).



Figure 5.4: Procyanidin-rich unfermented cocoa powder extracts. Control – left (L* = 52.84, a* = 11.08, b* = 2.24, chroma = 11.28, and hue = 11.4°) and 500 ppm treatment – right (L* = 71.39, a* = 8.44, b* = 9.61, chroma = 12.79, and hue = 48.8°).

Procyanidin-rich extracts produced with higher levels of bisulfite yielded more anthocyanins as a result of the hot water extraction. The 500 and 1000 ppm treatments showed the highest levels of anthocyanins present in the final extracts. Better extraction efficiency has been widely reported by many authors including Ayed and others (2000). Most recently Kammerer and others (2005) reported higher extraction efficiencies of anthocyanins with the use of bisulfite in grape pomace.

Anthocyanins remained stable throughout the shelf life study as monitored by the pH differential method. There were no significant differences in the monomeric anthocyanin

concentrations of the procyanidin-rich extracts throughout the shelf life study at any treatment level, indicating that the use of bisulfite had no negative effects towards the stability of anthocyanins over time as compared to the control (Figure 5.5). During the extraction of polyphenols from the procyanidin-rich extracts, the anthocyanins reappeared as the added bisulfite precipitated in the presence of organic solvent (70/30 acetone and water), making them easy to spectrophotometrically quantify.

Total phenolics as measured by the Folin-Ciocalteu method seemed to slightly increase throughout the shelf life study; however this increase was not significant at an alpha level of 0.05 (Figure 5.6). The slight increase was also uniform for all treatments, including the control, which suggests that the addition of potassium meta-bisulfite had no significant effect on the stability/degradation of phenolics present in the procyanidin-rich extracts. The use of higher levels of potassium meta-bisulfite did not increase the amount of non-anthocyanin phenolics present in the procyanidin-rich extracts (Figure 5.6).

5.5.5 Procyanidin Stability:

Procyanidins did not show any significant degradation or loss as a result of the addition of potassium meta-bisulfite. Both the DMAC assay and HPLC analysis show no significant differences between bisulfite treatments and the control (Figures 5.7 and 5.8). Furthermore, the DMAC assay results show no significant differences in procyanidin concentrations over time or between treatment groups (p>0.1). The HPLC data indicates significantly higher concentrations of monomers at days 43, 52, and 60 (p > 0.05) as compared to the other data points during the shelf life study. Even though the monomer concentration slightly increased, the concentrations of other oligomers (dimers – pentamers) present in the samples remained stable (cocoa powder and extract chromatograms shown in Figure 5.1). This trend was also noted in the control samples, indicating that the increase in monomers over time was independent of the addition of potassium meta-bisulfite (Figure 5.8). The slight and statistically insignificant decreases in individual

oligomers together may have resulted in the significantly higher monomer values as products of degradation over time for all treatment groups.



Figure 5.5: Total monomeric anthocyanins over the storage period as measured by the pH Differential method expressed as cy-3-glu equivalents. The 1000 ppm bisulfite treatment did not significantly different from 500 ppm treatment (data not shown).



Figure 5.6: Total phenolics over the storage period as measured by the Folin-Ciocaltaeu method expressed as gallic acid equivalents. The 1000 ppm bisulfite treatment did not significantly different from 500 ppm treatment (data not shown).

111



Figure 5.7: Total procyanidins over the storage period as measured by the DMAC method expressed as catechin equivalents. The 1000 ppm bisulfite treatment did not significantly different from 500 ppm treatment (data not shown).



Figure 5.8: Total procyanidins (monomers and trimers) stability over the storage period as measured by the normal phase HPLC with fluorescence detection. The 1000 ppm bisulfite treatment did not significantly different from 500 ppm treatment (data not shown).

5.5 Conclusion

Violet color was effectively removed from the unfermented cocoa powder extracts with the use of 500 ppm potassium meta-bisulfite with no sign of detrimental effects on the procyanidins and other polyphenols present in the matrix. The use of bisulfite was the most efficient means of removing the violet color provided by anthocyanins in unfermented cocoa powder extracts as compared to pH fluctuations and enzymatic treatments. The treatments containing bisulfite at concentrations of 200 ppm did not effectively bleach the violet color present. The total phenolics, procyanidins, and anthocyanins remained stable in the extracts over the shelf life study with the addition of bisulfite. As treatment concentrations of potassium meta-bisulfite increased from 0, 200, 500, and 1000 ppm, the amount of anthocyanins extracted from the unfermented cocoa powder increased. This increase in extraction efficiency was not noted for other non-anthocyanin phenolics including the procyanidins. The use of potassium meta-bisulfite may also have other positive effects such as the inhibition of residual polyphenol oxidase.

5.6 Acknowledgements

The authors would like to thank Mars Botanical (a division of Mars Inc.) for their support of this research.

References

- Adherne AS and Obrien NM. 2002. Dietary flavonols: chemistry, food content, and metabolism, Nutr. 18: 75-81.
- Ahamed J, Shivhare US, and Raghavan GSV. 2004. Thermal degradation kinetics of anthocyanin and visual colour of plum puree. Eur. Food Res. Tech. 218: 525-28.
- Andersen OM and Markham KR. 2006. Flavonoids, chemistry, biochemistry, and applications. Taylor & Francis Group. New York, NY.
- Andersen OM and Jordheim M. 2006. The anthocyanins. Chap. 10. In Flavonoids: chemistry, biochemistry, and applications. OM Andersen and Markham KR. (ed). Taylor and Francis Group. New York, NY. Pp. 471-551.
- Andersen OM. Anthocyanin occurrences and analyses. 2002. Arterielle. 5(4): 187-205.
- Aron PM and Kennedy JA. 2008. Flavan-3-ols: nature, occurrence, and biological activity. Mol. Nutr. Food Res. 52: 79-104.
- Asen S, Stewart RN, and Norris KH. 1972. Copigmentation of anthocyanins in plant tissues and its effect on color. Phytochem. 11: 1139-44.
- Attoe EL and Von Elbe JH. 1981. Photochemical degradation of betanine and selected anthocyanins. J. Food Sci. 46: 1934-37.
- Ayed N, Yu HL, and Lacroix M. 2000. Using gamma irradiation for the recovery of anthocyanins from grape pomace. Radiat. Phys. Chem. 57: 277-79.
- Ayed N, Yu HL, Lacroix M. 1999. Improvement of anthocyanin yield and shelf-life extension of grape pomace by gamma irradiation. Food Res. Int. 32: 539–43.
- Bagchi D, Bagchi M, Stohs SJ, and Das DK. 2000. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology. 148: 187-97.
- Balzer J, Rassaf T, Heiss C, Kleinbongard P, Lauer T, Merx M, Heussen N, Gross HB, Keen CL, Schroeter H, and Kelm M. 2008. Sustained benefits in vascular function through flavanolcontaining cocoa in medicated diabetic patients. J. Am. Coll. Cardiol. 51: 2141-49.
- Barcelo J and Poschenrieder C. 2002. Fast root growth responses. Root exudates. And internal detoxification as clues to the mechanism of aluminum toxicity and resistance: a review. Environ. and Exper. Bot. 48: 75-92.
- Barz W and Mackenbrock U. 1994. Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chickpea (Cicer arietinum) cell suspension cultures. Plant Cell Tissue Organ. Cult. 38: 199-211.
- Bate-Smith EC. 1975. Phytochemistry of proanthocyanidins. Phytochemistry. 14: 1107-13.

- Payne CH, Beavers DV, and Cain RF. 1969. Oregon Agric. Exp. Stn. Circular of Information. Pp. 1-9.
- Beckett S. 2008. The science of chocolate. 2nd ed. Cambridge, United Kingdom: The Royal Society of Chemistry.
- Benzie IFF, Szeto YT, Strain JJ, and Tomlinson B. 1999. Consumption of green tea causes rapid increase in plasma antioxidant power in humans. Nutr. Cancer. 34: 83-87.
- Broeckling CD and Vivanco JM. 2008. A selective, sensitive, and rapid infield assay for soil catechin, and allelochemical of Centaurea naculosa. Soil Biol. Biochem. 40: 1189-96.
- Brouillard R. 1982. Chemical structure of anthocyanins. In: Anthocyanins as Food Colors. Pericles Markakis (eds). Academic Press Inc. New York.
- Brouillard R. 1983. The in vivo expression of anthocyanin color in plants. Phytochem. 22: 1311-23.
- Brownmiller C, Howard LR, and Prior RL. 2009. Processing and storage effects on procyanidin composition and concentration of processed blueberry products. J. Agric.Food Chem. 57(5): 1896-1902.
- Buijsse B, Feskens EJM, Kok FJ, and Kromhout D. 2006. Cocoa intake, blood pressure, and cardiovascular mortality. Arch. Intern. Med. 166: 411-17.
- Cabannes JA. 1953. Le pH pondere et ses possibilities (weighed pH and its possibilities). Chim. Anal. 35: 28-30.
- Cabrita L, Fossen T, and Andersen OM. 1999. Color and stability of the six common anthocyanidin -3 glucosides in aqueous solutions. Food Chem. 68: 101-07.
- Cai Y, Lilley TH, and Haslam E. 1990. Polyphenol-anthocyanin copigmentation. J. Chem. Soc. Chem. Com. 380-83.
- Chou CH. 1999. Roles of allelopathy in plant biodiversity and sustainable agriculture. Crit. Rev. in Plant Sci. 18: 609-36.
- Chu YH, Chang CL, and Hsu HF. 2000. Flavonoid content of several vegetables and their antioxidant acitivity. J. Sci. Food Agric. 80: 561-66.
- Cilliars JJL and Singleton VL. 1990. Autoxidative phenolic ring opening under alkaline conditions as a model for natural polyphenols in food. J. Agric. Food Chem. 38(9): 1797-98.
- Clarkson TB. 2002. Soy phytoestrogens and cardiovascular disease. J. Nutr. 132: 566S-69S.
- Clemetson CAB and Andersen L. 1966. Plant polyphenols as antioxidants for ascorbic acid. Ann. NY Acad. Sci. 136:339-78.
- Clifford AH and Cuppett SL. 2000. Review: anthocyanins-nature, occurrence, and dietary burden, J. Sci. Food Agric. 80: 1063-72.
- Cook NC and Samman S. 1996. Review: Flavonoids-chemistry, metabolism, cardio protective effects, and dietary sources. J. Nutr. Biochem. 7: 66-76.

- Cos P, de Bruyne T, Hermans S, Apers S, and others. 2003. Proanthocyanidins in health care: current and new trends. Curr. Med. Chem. 10: 1345-59.
- Dalby A and Shuman AC. 1978. Temperature induced errors in the colorimetric determination of tannins. Anal. Biochem. 85: 325-27.
- Dangles O, Saito N, and Brouillard R. 1993. Anthocyanin intramolecular copigment effect. Phytochem. 34: 119-24.
- Daravingas G and Cain RF. 1968. Thermal degradation of black raspberry anthocyanin pigments in model systems. J. Food Sci. 33: 138-42.
- De Pascual-Teresa S, Santos-Buelga C, Rivas-Gonzalo JC. 2000. Quantitative analysis of flavan-3-ols in Spanish food-stuffs and beverages. J. Agric. Food Chem. 48: 5331-37.
- Dell Agli M, Busciala A, and Bosisio E. 2004. Vascular effects of wine polyphenols. Cardiovascular Res. 63(4): 593-02.
- Ding EL, Hutfless SM, Ding X, and Girotra S. 2006. Chocolate prevention of cardiovascular disease: A systematic review. Nutr. Metab. 3: 1-12.
- Dixon RA and Steel CL. 1999. Flavonoids and isoflavonoids a gold mine for metabolic engineering. Trends in Plant Sci. 4: 394-400.
- Duffy R, Wiseman H, and File S. 2003. Improved cognitive function in postmenopausal women after 12 weeks of consumption of a soy extract containing isoflavones. Pharmacol. Biochem. Behav. 75: 721-22.
- Dyrby M, Westergaard N, and Stapelfeldt H. 2001. Light and heat sensitivity of red cabbage extract in soft drink model systems. Food Chem. 72: 431–37.
- Elhabiri M, Figueiredo P, Toki K, Saito N, and Brouillard R. 1997. Anthocyanin-aluminum and gallium complexes in aqueous solution. J. Chem. Soc. Perkin Trans. 2: 355-62.
- Eyre H, Kahn R, Robertson RM, and others. 2004. Preventing cancer, cardiovascular disease, and diabetes: a common agenda for the American Cancer Society, the American Diabetes Association and the American Heart Association. Stroke. 35: 1999-2010.
- Felgines C and Talavera S. 2003. Strawberry anthocyanins are recovered in urine as glucuroand sulfoconjugates in humans. J Nutr. 133 (5): 1296-1301.
- Feng Q, Torii Y, Uchida K, Nakamura Y, Hara Y, and Osawa T. 2002. Black tea polyphenols, theaflavins, prevent cellular DNA damage by inhibiting oxidative stress and suppressing cytochrome P450 1A1 in cell cultures. J. Agric. Food Chem. 50: 213-20.
- Ferreira D, Slade D, and Marais JPJ. 2006. Flavans and proanthocyanidins. In: Anderson OM and Markham. Flavonoids chemistry, biochemistry, and applications. New York: Taylor & Francis. Pp. 553-616.
- File SE, Jarrett N, and Fluck E. 2001. Eating soy improves human memory. Psychopharmacology. 157: 430-36.

- Foo LY, Lu Y, Howell AB, and Vorsa N. 2000. A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic p-fimbriated escherichia coli. J. Nat. Prod. 63: 1225-28.
- Francis FJ. 1989. Food colorants: anthocyanins. Crit. Rev. Food Sci. Nutr. 28: 273-14.
- Francis FJ. 1999. Colorants. Eagan Press Handbook. St. Paul.
- Furtado P, Figueiredo P, Chaves das Neves H, and Pina F. 1993. Photochemical and thermal degradation of anthocyanidins. J. Photochem. Photobiol. A. 75: 113-18.
- Garcia-Palazon A, Suthanthangjai W, Kajda P, and Zabetakis I. 2004. The effects of high hydrostatic pressure on b-glucosidase, peroxidase and polyphenoloxidase in red raspberry (*Rubus idaeus*) and strawberry (*Fragaria ananassa*), Food Chem. 88: 7-10.
- Gessner MO and Steiner D. 2005. Acid butanol assay for proanthocyanidins (condensed tannins). In Graca MAS, Barlocher F, and Gessner MO. Methods to study litter decomposition: a practical guide. Springer Inc. Amsterdam, Netherlands. Pp. 107-114.
- Giusti MM and Wallace TC. 2009. Flavonoids as natural pigments. Chap. 16. In Handbook of Natural Colorants. T. Bechtold and R Mussak (ed). John Wiley & Sons Inc. New York, NY. Pp. 257-77.
- Giusti MM and Wrolstad RE. 1996. Radish anthocyanin extract as a natural red colorant for maraschino cherries. J. Food Sci. 61(4): 688-94.
- Giusti MM and Wrolstad RE. 2003. Acylated anthocyanins from edible sources and their applications in food systems. Biochem. Eng. J. 14: 217-25.
- Giusti MM and Wrolstad RE. 2005. Characterization and measurement of anthocyanins by UVvisible spectroscopy. In: Handbook of Food Analytical Chemistry. RE Wrolstad, SJ Schwartz (eds). John Wiley & Sons Inc. New York.
- Giusti MM, Rodriguez-Saona LE, and Wrolstad RE. 2005. Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. J. Agr. Food Chem. 47: 4631-37.
- Gonthier MP and Donovan JL. 2003. Metabolism of dietary procyanidins in rats. Free Rad. Biol. Med. 35: 837-44.
- Goto T and Kondo T. 1991. Angew. Chem. Int. (ed). Engl. 30: 17.
- Grayer RJ and Harborne JB. 1994. A survey of antifungal compounds from higher plants 1982-1993. Phytochem. 37: 19-42.
- Gu L, Kelm M, Hammerstone JF, and Prior RL. 2002. Monomeric, oligomeric, and polymeric proanthocyanidins (PAC) in selected foods: quality and quantity. FASEB J. 16: A1011.
- Gu L, Kelm MA, Hammerstone JF, Beecher G and others. 2004. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. J. Nutr. 134: 613-17.

- Gu L, Kelm MA, Hammerstone JF, Zhang Z, Beecher G, Holden J, Haytowitz D, and Prior RL. 2003. Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. J. Mass Spectrom. 38(12): 1272-80.
- Gunilla BT and Henrik P. 2001. Removal of dissolved brown algal phlorotannins using insoluble polyvinylpolypyrrolidone (PVPP). J. Chem. Ecol. 27(9): 1899-1910.
- Hageman AE, Zhao Y, and Johnson S. 1997. Methods for determinination of condensed and hydrolysable tannins. In: Antinutrients and Phytochemicals in Foods. Fereidoon S.(ed). American Chemical Society. Washington DC. 24: 170-81.
- Haggerman AE and Butler LG. 1994. Assay of condensed tannins or flavonoid oligomers and related flavonoids in plants. Methods Enzymol. 237: 429-37.
- Hammerstone JF, Lazarus SA, Mitchell AE, Rucker R, and Schmitz HH. 1999. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectroscopy. J. Agric. Food Chem. 47: 490-96.
- Harborne JB. 1998. Phenolic Compounds. Phytochemical methods a guide to modern techniques of plant analysis. New York, Chapman & Hall: Pp. 66-74.
- Haslam E. 1998. Plant polyphenols. Practical polyphenolics: from structure to molecular recognition and physiological function. Cambridge University Press: Cambridge UK.
- Havlikova L and Mikova K. 1985. Heat stability of anthocyanins. Z. Lebensm. Unters Frosch. 181: 427–32.
- He J, Magnuson BA, Tian GLQ, Schwartz SJ, and Giusti MM. 2006. Intact anthocyanins and metabolites in rat urine and plasma after 3 months of anthocyanin supplementation. Nutr. and Cancer. 54(1): 3-12.
- He J, Wallace TC, Keatley K, Failla M, and Giusti MM. 2009. Stability of Black Raspberry Anthocyanins in the Digestive Tract Lumen and Transport Efficiency into Gastric and Small Intestinal Tissues in the Rat. J Agric. Food Chem. 57(8): 3141-48.
- Heber D. 2009. Putting out the fire within: pomegranates and plant flavanols effects beyond antioxidation. Presentation at the 2009 Annual Meeting of the Institute of Food Technologists. Session 101.
- Heiss C, Finis D, Kleinbongard P, Hoffman A, Rassaf T, Kelm M, and Sies H. 2007. Sustained increase in flow-mediated dialation after daily intake of high-flavanol cocoa drink over 1 week. J. Cardiovasc Pharmacol. 49(2): 74-80.
- Hertog MG, Feskens EJ, and Kromhout D. 1997. Antioxidant flavanols and coronary heart disease risk. Lancet. 349-699.
- Hollman PCH, De Vries JHM, Van Leeuwen SDD, Mengelers MJB, and Datan MB. 1995. Absorption of dietary quercetin in healthy ileostomy volunteers. Am. J. Clin. Nutr. 62: 1276-82.
- Holt RR, Lazarus SA, Sullards MC, Zhu QY, Schramm DD, Hammerstone JF, Fraga CG, Schmitz HH, and Keen CL. 2002. Procyanidin dimer B2 [Epicatechin-(4β-8)-epicatechin] in human plasma after consumption of a flavanol-rich cocoa. Am. J. Clin. Nutr. 76(4): 798-804.

- Hong V and Wrolstad RE. 1990. Use of HPLC separation / photodiode array detection for characterization of anthocyanins. J. Agric. Food Chem. 38: 708-15.
- Hoshino T, Matsumoto U, Harada N, and Goto T. 1981. Chiral excitation coupled stacking of anthocyanins: interpretation of the origin of anomalous CD induced by anthocyanin association. Tetrahedron Lett. 22: 3621.
- Howell AB. 2007. Review Bioactive compounds in cranberries and their role in prevention of urinary tract infections Mol. Nutr. Food Res. 51: 732 737
- Huang HT. 1956. The kinetics of the decolorization of anthocyanins by fungal anthocyanase. J. Am. Chem. Soc. 78: 2390-93.
- Hummer W and Schreier P. 2008. Analysis of proanthocyanidins. Mol Nutr. Food Res. 52: 1381-98.
- IFT. 2005. Functional Foods: Opportunities and Challenges. An expert report of the Institute of Food Technologists, Chicago, IL.
- Jackman RL, Yada RY, Tung MA, and Speers RA. 1987. Anthocyanins as food colorants–a review. J. Food Biochem. 11: 201–47.
- Jing P, Giusti MM. 2005. Characterization of anthocyanin-rich waste from purple corncobs (Zea mays L.) and its application to color milk. J. Agric. Food Chem. 53: 8775-81.
- Juranic Z and Zizak Z. 2005. Biological activities of berries: from antioxidant capacity to anticancer effects. Biofactors. 23: 207-11.
- Kadar A, Robert L, Miskulin M, Tixier JM, Brechemier D, and Robert AM. 1979. Influence of anthocyanoside treatment on the cholesterol-induced atherosclerosis in the rabbit. Paroi Arterielle. 5(4): 187-205.
- Kader F, Irmouli M, Nicolas JP, and Metche M. 2001. Proposed mechanism for the degradation of pelargonidin 3-glucoside by caffeic acid o-quinone. Food Chem. 75: 139-44.
- Kader F, Irmouli M, Zitouni N, Nicolas J, and Metche M. 1999. Degradation of cyanidin 3glucoside by caffeic acid o-quinone. Determination of the stoichiometry and characterization of the degradation products. J. Agric. Food Chem. 47: 4625-30.
- Kader F, Rovel B, Girardin M, and Metche M. 1997. Mechanism of browning in fresh highbush blueberry fruit (Vaccinium corymbosum L). Partial purification and characterization of blueberry polyphenol oxidase, J. Sci. Food Agric. 73: 513-16.
- Kammerer D, Claus A, Schieber A, and Carle R. 2005. A novel process for the recovery of polyphenols from grape (Vitis vinifera L.) pomace. J. Food Sci. 70(2): 157-63.
- Karlsen A, Retterstol L, Laake P, Paur I, Kjolsrud-Bohn S, Sandvik L, and Blomhoff R. 2008. Anthocyanins inhibit nuclear factor-κB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults. J. Nutr. 137(8): 1951-54.
- Kelm MA, Johnson JC, Robbins RJ, Hammerstone JF, and Schmitz HH. 2006. High-performance liquid chromatography separation and purification of cacao (*Theobroma cacao*)

procyanidins according to degree of polymerization using a diol stationary phase. J. Agric. Food Chem. 54(5): 1571-6.

- Knekt P, Kumpulainen J, Jarvinen and others. 2002. Flavonoid intake and risk of chronic diseases. Am. J. Clin. Nutr. 76: 560-8.
- Kondo K, Hirano R, Matsumoto A, Igarashi O, and Itakura H. 1996. Inhibition of LDL oxidation by cocoa. Lancet 30: 1514.
- Kroon SH, Ahn IS and others. 2004. How should we assess the effects of exposure to dietary polyphenols in vitro? Am. J. Clin. Nutr. 80(1): 15-21.
- Kundson WA. 2008. Economic impact of expanded cranberryproduction. The Strategic Marketing Institute. http://www.aec.msu.edu/Product/documents/cranberries.pdf
- Labuza TP and Riboh D. 1982. Theory and application of arrhenius kinetics to the prediction of nutrient losses in foods. Food Technol. 36: 66-74.
- Leclerq G and Heuson JC. 1979. Physiological and pharmacological effects of estrogens in breast cancer. Biochem. Biophys. Acta. 560: 427-55.
- Li YG, Tanner G, and Larkin P. 1996. The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. J. Sci. Food Agric. 70: 89-101.
- Macheix JJ, Fleuriet A, and Billot J. 1990. Fruit Phenolics. CRC Press. Boca Raton.
- Markakis P. 1982. Stability of anthocyanins in foods. In: Anthocyanins as Food Colors. CRC Press. Boca Raton.
- Marles MA, Ray H, and Gruber MY. 2003. New perspectives on proanthocyanidin biochemistry and molecular regulation. Phytochemistry. 64: 367-83.
- Marston A and Hostettmann K. 2006. Separation and quantification of flavonoids. In: Anderson OM and Markham. Flavonoids chemistry, biochemistry, and applications. New York: Taylor & Francis. Pp. 1-36.
- Mazza G and Miniati E. 1993. Anthocyanins in fruits, vegetables, and grains. CRC Press. Boca Raton.
- McKay DL and Blumberg JB. 2007. Cranberries (Vaccinium macrocarpon) and cardiovascular disease risk factors. Nutr. Rev. 65(11): 490-502.
- Mian E, Curri SB, Lietti A, and Bombardelli E. 1977. Anthocyanosides and the walls of the microvessels: future aspects of the mechanism of action of their protective effect in syndromes due to abnormal capillary fragility. Minerva Med. 68 (52): 3565-81.
- Miles CO and Main L. 1985. Kinetics and mechanism of the cyclisation of 2,6-dihydroxy-4, 4dimethoxychalcone; influence of the 6-hydroxyl group on the rate of cyclisation under neutral conditions. J. Chem. Soc. Perkin Trans. 2: 1639-42.

- Misnawi JS, Jamilah B, and Nazamid S. 2002. Oxidation of polyphenols in unfermented and partially fermented cocoa beans by polyphenol oxidase and tyrosinase. J. Sci. Food Agric. 82: 559-566.
- Murkies AL, Lombard C, Strauss BJG, Wilcox G, Burger HG, and Morton MS. 1995 Dietary flour supplementation decreases postmenopausal hot flushes-effect of soy and wheat. Maturitas. 21: 189-95.
- Murphy AS. 2001. Flavonoid accumulation patterns of transparent testamutants of Arabidopsis, Plant Physiol. 126: 536-548.
- Nebesky EA, Esselen WB, McConnell JEW, and Fellers CR. 1949. Stability of color in fruit juices, Food Research. 14: 261-74.
- Niemenak N, Rohsius C, Elwers S, Ndoumou DO, Lieberei R. 2006. Comparative study of different cocoa (*Theobroma cacao* L.) clones in terms of their phenolics and anthocyanin contents. J. Comp. and Anal. 19: 612-19.
- Nitao JK, Birr BA, Nair MG, Herms DA, and Mattson WJ. 2001. Rapid quantification of proanthocyanidins (condensed tannins) with a continuous flow analyzer. J. Agric. Food Chem. 49: 2207-14.
- Osawa Y. 1982. Copigmentation of anthocyanins. In: Anthocyanins as Food Colors. P Markakis (ed). Academic Press Inc. New York, NY.
- Ozmainski J and Bourzeix M. 1996. Comparison of methods for determining the content and polymerization of proanthocyanidins and catechins. Pol. J. Food Nutr. Sci. 5: 43-50.
- Palamidis N and Markakis. 1978. Stability of grape anthocyanin in carbonated beverages. Semana Vitivinicola. 33: 2633-39.
- Pascual-Teresa S, Treutter D, Rivas-Gonzalo JC, and Santos-Buelga C. 1998. Analysis of flavanols in beverages by high-performance liquid chromatography with chemical reaction detection. J. Agric. Food Chem. 46:4209-13.
- Peer WA, Brown DE, Tague BW, Muday GK, Taiz L. and A.S. Murphy. 2001. Flavonoid accumulation patterns of transparent testa mutants of Arabidopsis. Plant Physiol. 126. 536-548.
- Peleg H, Gacon K, Schlich P, and Noble AC. 1999. Bitterness and astringency of flavan-3-ol monomers, dimers, and trimers. J Sci. Food Agric. 79: 1123-28.
- Peterson J & Dwyer J. 1998. Flavonoids: dietary occurrence and biochemical activity. Nutr. Res. 18(12): 1995-2018.
- Pierpoint WS. 1986. Flavonoids in the human diet. Prog. Clin. Biol. Res. 213: 125-40.
- Pierpoint WS. 1990. Flavonoids in human food and animal feed –stuffs: amounts and consequences. In: Flavonoids in biology and medicine III. Current issues in flavonoids research. NP Das (ed). National University of Singapore. Singapore.

- Pierpoint WS. 2004. The Extraction of Enzymes from Plant Tissues Rich in Phenolic compounds. In Methods in Molecular Biology; Doonan, S., Ed.; Humana Press Inc. Totowa. 244: 65-74.
- Poei-Langston MS. 1981. Color degradation in an ascorbic acid-anthocyanin-flavanol model system. J. of Food Sci. 46(4): 1218.
- Porter LJ. Hrstich LN, and Chan BG. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry. 25: 223-30.
- Porter ML, Krueger CG, Weibe DA, Cunningham DG, and Reed JD. 2001. Cranberry proanthocyanidins associate with low-density lipoprotein and inhibit in vitro Cu+2 induced oxidation. J. Sci. Food Agric. 81: 1306-13.
- Prior, R., Lazarus, S., Cao, G., Muccitelli, H. 2001. Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium spp.) using HPLC/MS, J. Agric. Food Chem. 49:1270-76.
- Ren W, Qian Z, Wang H, Zhu L, and Zhang L. 2003. Flavonoids: promising anticancer agents. Medicinal Res. Rev. 23(4): 519-34.
- Rhim J. 2002. Kinetics of thermal degradation of anthocyanin pigment solutions driven from red flower cabbage. Food Sci. Biotechnol. 11: 361–64.
- Rios LY, Gonthier MP, Remesy C, Mila I, and others. 2003. Chocolate intake increases urinary excretion of polyphenol derived phenolic acids in healthy human subjects. Am. J. Clin. Nutr. 77: 912-18.
- Robbins RJ, Jadwiga LJ, Johnson JC, Li J, Kwik-Uribe C, and Prior RL. 2009. Method performance and multi-laboratory assessment of a normal phase high pressure liquid chromatography–fluorescence detection method for the quantitation of flavanols and procyanidins in cocoa and chocolate containing samples. J. Chrom. A 1216(24): 4831-4840.
- Rodriguez-Saona LE and Wrolstad RE, 2005. Extraction, isolation, and purification of anthocyanins. Unit F1.1. In Handbook of Food Analytical Chemistry. Wrolstad RE and SJ Schwartz (Ed.). John Wiley & Sons, Inc. New York, NY. Pp. 7-18.
- Rozema J, VanDeStaaij J, Bjorn LO, and Caldwell M. 1997. UV-B as an environmental factor in plant life: stress and regulation. Tree. 12: 22-28.
- Ryan KG, Swinny EE, Markham KR, and Winefield C. 2002. Flavonoid gene expression and UV photoprotection in transgenic and mutant Petunia leaves. Phytochem. 59: 23-32.
- Salunkhe DK, Chavan JK, and Kadam SS. 1990. Dietary tannins: consequences and remedies. CRC Press Inc. Boca Raton, FL. Pp. 200.
- Santos-Buelga C and Scalbert A. 2000. Proanthocyanidins and tannin-like compounds nature, occurrence, dietary intake and effects on nutrition and health. J. Sci. Food Agric. 80: 1094-117.
- Sarni P, Fulcrand H, Souillol V, Souquet J, and Cheynier V. 1995. Mechanisms of anthocyanin degradation in grape must-like model solutions. J. Sci. Food Agric. 69: 385-91.

- Scalbert A, Deprez S, Mila I, Albrecht AM, and others. 2000. Proanthocyanidins in human gut health: systematic effects and local effects in the gut. Biofactors. 13: 115-20.
- Scalbert A, Monties B, and Janin G. 1989. Tannins in wood: comparision of different estimation methods. J. Agric. Food Chem. 37: 1324-29.
- Scalbert A. 1992. Quantitative methods for the estimation of tannins in plant tissues. In: Plant polyphenols: synthesis, properties, and significance. Hemmingway RW and Laks PS. (ed). Plenum Press. New York, NY. Pp. 259-80.
- Schwinn KE and Davies KM. 2004. Flavonoids, in Plant Pigments and their Manipulation. KM Davies (ed). Blackwell Publishing. Oxford.
- Setchell KRD and Cassidy A. 1999. Dietary isoflavones: biological effects and relevance to human health. J. of Nutr. 129: 758S-67S.
- Snyder BA and Nicholson RL. 1990. Synthesis of phytoalexins in sorghum as a site specific response to fungal ingress. Science. 248: 1637-39.
- Starr MS and Francis FJ. 1968. Oxygen and ascorbic acid effect on the relative stability of four anthocyanin pigments in cranberry juice. Food Technol. 22: 1293-95.
- Sun B, Liandro MC, Freitas V, and Spranger MI. 2006. Fractionation of red wine polyphenols by solid-phase extraction and liquid chromatography. J. Chrom. A. 1128: 27-38.
- Sun B, Ricardo-da-Silva JM, and Spranger I. 1998. Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agric. Food Chem. 46: 4267-74.
- Talbert D, Roesen R, Lehnann C, Jung N, and Schomig E. 2007. Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: a randomized controlled trial. JAMA. 298: 49-60.
- Talcott ST, Brenes CH, Pires DM, and del Pozo-Insfran D. 2003. Phytochemical stability and color retention of copigmented and processed muscadine grape juice. J. Agric. Food Chem. 51: 957-63.
- Tanner GJ, Moore AE, and Larkin PJ. 1994. Proanthocyanidins inhibit hydrolysis of leaf proteins by rumen microflora in vitro. Br. J. Nutr. 71: 947-58.
- Tattini M, Galardi C, Pinelli P, Massai R, Remorini D, and Agati G. 2004. Deferential accumulation of flavonoids and hydroxycinnamates in leaves of Ligustrum vulgare under excess light and drought stress. New Phytologist. 163: 547-61.
- Teede HJ, Dalais FS, and Kotsopulos D. 2001. Dietary soy has both beneficial and potentially adverse cardiovascular effects: a placebo-controlled study in men and postmenopausal women. J. Clin. Endocrinol. Metab. 86: 3053-60.
- Thoison O, Sevenet T, Niemeyer HM, and Russell GB. 2004. Insect antifeedant compounds from Nothofagus dombeyi and N. pumilio. Phytochem. 65: 2173-76.
- Timberlake CF and Bridal P. 1966. Effect of substituents on the ionization of flavylium salts and anthocyanins and their reactions with sulfur dioxide. Chem. Ind. (2): 1965-66.

- Timberlake CF and Bridal P. 1968. Flavylium salts resistant to sulfur dioxide. Chem. Ind. (1): 1489.
- Tomas-Barberan FA, Cienfuegos-Jovellanos E, Marin A, Muguerza B, Gil-Izquierdo A, Cerda B, Zafrillia P, Morillas J, Mulero J, Ibarra A, Pasamar MA, Ramon D, and Espin JC. 2007. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. J. Agric. Food Chem. 55: 3926-35.
- Treutter D. 1989. Chemical reaction detection of catechins and proanthocyanidins with 4dimethylaminocinnamaldehyde. J. Chromatogr. 467: 185-93.
- Treutter W, Feucht W, and Santos-Buelga C. 1994. Determination of catechins and procyanidins in plant extracts a comparison of methods. Acta Hort. 381: 789-96.
- Tsang C, Auger C, Mullen W, Bornet A, and others. 2005. The absorption, metabolism, and excretion of flavan-3-ols and procyanidins following the ingestion of grape seed extract by rats. Brit. J. Nutr. 94: 170-81.
- United States Code of Federal Regulations. 2007. Title 21.
- United States Department of Agriculture (Internet). Washington, DC. [Accessed January 29th, 2009]. Available from: www.usda.gov.
- Viskelis P, Rubinskiene M, Jasutiene I, Sarkinas A, Daubaras R, and Cesoniene L. 2009. Anthocyanins, antioxidative, and antimicrobial properties of the American cranberry (Vaccinium macrocarpon Ait.) and their press cakes. J. Food Sci. 74(2): C157-61.
- Vivas N, Glories Y, Lagune L, Saucier C, and Augustin M. 1994. Estimation of the degree of polymerization of procyanidins in grapes by a p-dimethylaminocinnimaldehyde method. J. Int. Sci. Vigne Vin. 28: 319-36.
- Wallace TC and Giusti MM. 2008. Determination of color. pigment. and phenolic stability in yogurt systems colored with non-acylated anthocyanins from Berberis boliviana L. as compared to other natural/synthetic colorants. J. Food Sci. 73(4): C241-8
- Wallace TC and Giusti MM. 2009. Optimization of the 4-dimethylaminocinnimaldehyde assay for flavanols/proanthocyanidins. Poster presented at the 238th national meeting of the American Chemical Society. Section D. 158.
- Wallace TC, Wagner M, Leveille G, Keen CL, Woteki CE, Manley C, Rizk SW, Heber D, and Shrikhande AJ. 2009 Unlocking the benefits of cocoa flavanols. Food Technol. October ed. Pp. 34-41.
- Walton MF, Haskins FA, and Gorz HJ. 1983. False positive results in the vanillin-hydrochloric acid assay of tannins in sorgum forage. Crop Sci. 23: 197-200.
- Wang H, Cao G, and Prior RL. 1997. Oxygen radical absorbing capacity of anthocyanins. J. Agric. Food Chem. 45: 304-09.
- Ward NC, Croft KD, Puddey IB, and Hodgson JM. 2004. Supplimentation with grape seed polyphenols results in increased urinary excretion of 3-hydroxyphylpropionic acid, an important metabolite of proanthocyanidins in humans. J. Agric. Food Chem. 52: 5545-49.

- Waterhouse AL. 2005. Determination of total phenolics. Unit I1.1. In Handbook of Food Analytical Chemistry. RE Wrolstad, SJ Schwartz (Ed.). John Wiley & Sons, Inc. New York, NY. Pp. 463-470.
- Watjen W, Michels G, Steffan B, Niering P, Chovolou Y, Kampkotter A, Tran-Thi Q, Proksch P, and Kahl R. 2005. Low concentrations of flavonoids are protective in rat H411E cells whereas high concentrations cause DNA damage and apoptosis. J. Nutr. 135: 525-31.
- Wesche-Ebeling P and Montgomery MW. 1990. Strawberry polyphenol oxidase: its role in anthocyanin degradation. J. Food Sci. 55: 731-34.
- Wightman JD and Wrolstad RE. 1996. β-glucosidase Activity in Juice-processing enzymes based on anthocyanin analysis. J. Food Sci. 61(3): 544-48.
- Williams M and Hrazdina G. 1979. Anthocyanins as food colorants: effect of pH on the formation of anthocyanin-rutin complexes. J. Food Sci. 44: 66-68.
- Winkle-Shirley B. 2002. Biosynthesis of flavonoids and effects of stress. Curr. Opin. Plant Biol. 5: 218-23.
- Wollgast J and Anklam E. 2000. Polyphenols in chocolate: is there a contribution to human health? Food Res. Int. 33: 449-59.
- Wollgast J and Anklam E. 2000. Review on polyphenols in Theobroma cacao: changes in composition during manufacture of chocolate and methodology for identification and quantification. Food Res. Int. 33: 423-47.
- Wrolstad RE, Skrede G, Lea P, and Enersen G. 1990. Influence of sugar on anthocyanin pigment stability in frozen strawberries. J. Food Sci. 55: 1064-65.
- Wrolstad RE. 2004. Anthocyanin pigments-bioactivity and coloring properties. J. Food Sci. 69(5): 419-21.
- Yao LH, Jiang YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R, and Chen SS. Flavonoids in food and their health benefits. Plant Foods Human Nutr. 59: 113-22.
- Youdim KA, Shukitt-Hale B, and Joseph JA. 2004. Flavonoids and the brain: Interactions at the blood-brain barrier and their physiological effects on the central nervous system. Free Rad. Biol. Med. 37: 1683-93.
- Yukawa C, Ichi T, Onishi K, Sato H, United States Patent # 6936292, 2005.
- Zafra-Stone S, Yasmin T, Bagchi M, Chatterjee A, Vinson JA, and Bagchi D. 2007. Berry anthocyanins as novel antioxidants in human health and disease prevention. Mol. Nutr. Food Res. 51: 675-83.
- Zand RSR, Jenkins DJA, and Diamandis EP. 2002. Flavonoids and steroid hormone-dependant cancers. J. Chromatogr. B. 777: 219-32.