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Analysis of Antiviral and Chemoprotective Effects of Strawberry Anthocyanins

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ANALYSIS OF ANTIVIRAL AND CHEMPROTECTIVE EFFECTS OF STRAWBERRY ANTHOCYANINS

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DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the College of Agriculture
at the University of Kentucky

By

Jennifer Anne Willig

Lexington, Kentucky

Director: Melissa Newman, Ph.D., Associate Professor of Food Science

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2013

ABSTRACT OF DISSERTATION

ANALYSIS OF ANTIVIRAL AND CHEMPROTECTIVE EFFECTS OF STRAWBERRY ANTHOCYANINS

This study investigated the antiviral, chemoprotective and proliferative effects of strawberry anthocyanins on herpes simplex virus type-1, cancerous cell lines HT-29 and AGS, and normal cell lines Hs 738.St/Int and CCD-18Co. Antiviral properties were measured by infecting vero cells from adult grivet (*Cercopithecus aethiops*) with herpes simplex virus type-1 (HSV-1) and treating with a concentration of 1.25-20 µg/mL of strawberry anthocyanins. Infectivity and replication were quantified for herpes simplex virus type-1 using the direct plaque assay and reporting PFU/mL. Strawberry anthocyanins (>20 µg/mL) inhibited the herpes simplex virus infectivity in vero cells by 100% ($p<0.05$). Strawberry anthocyanins at concentrations of 5, 10 and 20 µg/mL were reduced to 75.36, 57.98, and 31.46 percent of the control (100%) ($p<0.05$).

Chemoprotective and proliferative effects of strawberry anthocyanins were analyzed for the human cell lines AGS, Hs 738.St/Int, HT-29, and CCD-18Co at a concentration of 25-200 µg/mL and quantified using the sulforhodamine-B assay. Growth inhibition occurred at a level of ≥87% for treatment concentrations 100 and 200 µg/mL for the cancerous AGS and HT-29 cell lines ($p<0.0001$). Proliferation rates for the normal Hs 738.St/Int and CCD-18Co cell lines increased at all treatment concentrations of 25-200 µg/mL ($p<0.0001$); suggesting that the observed proliferative activity may be associated with anthocyanin treatment. Strawberry anthocyanin treatment concentration worked in a dose dependent manner for the HSV-1 and the cancerous AGS and HT-29 cells. The caspase-3 assay was performed to demonstrate potential mechanism of action and confirmed that anthocyanin treatments play a role in apoptosis by the up regulation of caspase-3. Significant differences were seen between the growth characteristics of cancerous cell lines compared to their equivalent normal cell lines ($p<0.0001$).

In summary, the antiviral findings suggest that strawberry anthocyanin extracts could be an effective topical treatment and/or prophylactic agent for oral herpetic infections (HSV-1). Also, the *in vitro* chemoprotective effect of

strawberry anthocyanins found may be relevant to *in vivo* work in the future because when anthocyanins are consumed in the diet they come in direct contact with the gastrointestinal tract and may provide chemoprotection upon contact with the stomach and gastrointestinal tract's epithelial cell layer.

KEYWORDS: strawberries, anthocyanins, HSV-1, colon cancer, stomach cancer

Jennifer Anne Willig

November 21, 2013

ANALYSIS OF ANTIVIRAL AND CHEMPROTECTIVE EFFECTS OF STRAWBERRY ANTHOCYANINS

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Dedicated to Mom and Dad, Debbie and Ray Willig, without whom this dissertation would not have been possible. Thank you for always supporting me, financially and emotionally through this long journey as a student. It is because of your continued generosity and support that I have made it to this point.

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CHAPTER 1

INTRODUCTION

Anthocyanins are a subclass of a group of compounds known as flavonoids and are responsible for the brightly colored and attractive red, purple and blue pigments of many fruits, vegetables and flowers. The basic anthocyanin structure consists of the carbon skeleton with conjugated bonds on the C-ring and two positions, R-1 and R-2, which are substituted with hydroxyl, oxymethyl and hydryl groups. Different combinations of these side groups results in the six anthocyanidin chromophores most commonly found in nature. These six common anthocyanidin aglycones include cyanidin (Cy), pelargonidin (Pg), delphinidin (Dp), petunidin (Pt), malvidin (Mv) and peonidin (Pn) (Fleschhut et al., 2006; Hou et al., 2005; McGhie et al., 2007). Anthocyanins constitute the largest and probably the most important group of water soluble natural pigments (Takeoka and Dao, 2002); to date, there have been more than 635 anthocyanins identified in nature. Epidemiological studies have suggested a reverse association between diets high in polyphenols and incidence of some chronic disease. For example, drinking red wine regularly has been associated with the relatively low incidence of coronary heart disease in French people despite a high fat diet, this phenomenon is well known as the French Paradox (Renaud and de Lorgeril, 1992). Many anthocyanin-rich extracts from berries and vegetables have been reported to slow down the growth of HT-29 colon cancer

cells *in vitro*, each of them to a different extent (Jing et al., 2006; Zhao et al., 2004). Anthocyanins are shown to be promising phytochemicals responsible for at least part of the anticancer property of many fruits and vegetables, but it is more than likely that anthocyanins work collaboratively with other phytochemicals to help the body's defense (He et al., 2009).

Anthocyanins are gaining interest as functional compounds for coloring foods, and as potential antioxidants, phytoalexins and antibacterial agents (Kang et al., 2003). Anthocyanin rich extracts are value added food ingredients which are stable in a variety of food and beverage matrices, including those food and beverages that contain high amounts of protein, fat and low pH. Consumer demand for healthier and non-synthetic food ingredients, particularly those containing polyphenols, has driven the industry to develop food products with higher concentrations of flavanols and anthocyanins. This consumer trend toward non-synthetic food ingredients 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 determining biological function. Research suggests that smaller anthocyanin molecules are more easily taken up and absorbed into the surrounding gastrointestinal tissue than the larger glycosylated and acylated anthocyanins. This indicates that the larger glycosylated and acylated anthocyanins have lower bioavailability than smaller anthocyanins and in turn lower bioactivity (Jing 2006). Anticancer activity of anthocyanins has been

established largely based on *in vitro* evidence. However, there is a growing body of data from *in vivo* studies that has demonstrated chemopreventive and antiviral effects of anthocyanins in multiple types of cancers. Nevertheless, the observed preventive effects were primarily related to gastrointestinal tract (Appendix A) related organs including the oral cavity, the esophagus (Reen et al., 2006), and the colon (Hagiwara et al., 2001,; Harris et al., 2001; Lala et al., 2006).

The herpes simplex virus (HSV) is an epitheliotropic virus that infects children and the majority of adults (Bader et al., 1978). There are two types of herpes simplex virus: herpes simplex virus type 1 refers to the oral herpes virus and herpes simplex virus type 2 refers to the genital herpes virus. The infection of epithelial cells (i.e. mucosa and skin) by the herpes simplex virus results in de-envelopment of the cell membrane, rapid replication, and spread to adjacent cells and nerve cell endings. Due to the uptake of virions by nerve endings the virus can travel retroaxonally to the nerve cell bodies within the trigeminal ganglion, upon which a dormant state is established (Stevens and Cook, 1971; Stevens 1975). The herpes simplex virus DNA resides long-term within the neuronal nuclei in a state that allows for periodic reactivation and recrudescence of progeny virus (Kaufman et al., 1967 and Lachmann 2003). The ability of the virus to lay dormant in immune-privileaged sites makes eradication of the virus difficult.

More than 20% of death in the United States is caused by cancer (Smith, Marks and Lieberman, 2005). Stomach cancer also known as gastric cancer is a disease in which malignant (cancer) cells form in the lining of the stomach (Bruce

et al, 2000). Stomach cancers tend to develop slowly over many years. The stomach is part of the gastrointestinal system, which processes nutrients (vitamins, minerals, carbohydrates, fats, proteins, and water) in foods that are eaten and helps pass waste materials out of the body. The wall of the stomach is made up of three layers of tissue: the mucosal (innermost) layer, the muscularis (middle) layer, and the serosal (outermost) layer. Before true cancer develops, pre-cancerous changes often occur in the lining of the stomach. In many cases, these early changes rarely cause symptoms and therefore often go undetected. Stomach cancer begins in the cells lining the mucosal layer and spreads through the outer layers as it continues to grow and spread. Stomach cancer typically affects the older population. Almost two thirds of people with stomach cancer are 65 years of age or older and the average risk of developing stomach cancer is about 1 in 116 (Bruce et al., 2000). Stomach cancer was the leading cause of cancer related death in the United States until the late 1930s. This is believed to be due to refrigeration and the opportunity for longer food storage. The invention of the refrigerator made it possible for more people to have access to fruits and vegetables year round and in turn decreased the amount of salted and smoked foods consumed. Additionally, stomach related cancers are thought to be less prevalent in first world countries because of the use of antibiotics that kill bacteria that cause infections in the stomach, such as, *Helicobacter pylori* (*H. pylori*). Because of limited resources, stomach cancer is much more prevalent in other parts of the world, particularly in underdeveloped

nations. According the American Cancer Society, stomach cancer is the leading cause of cancer-related deaths in the world today (Bruce et al., 2000).

Of cancers that affect both men and women, colorectal cancer is the second leading cause of cancer related deaths in the United States and the third most common cancer in men and women (Center for Disease Control, 2010). Colorectal cancer is the cancer of the colon or rectum. Colon cancer is cancer that forms in the tissue of the colon, the longest section of the large intestine. The majority of colon cancer arises from preexisting adenomas (Toribara and Sleisenger, 1995). Adenocarcinoma cells are cells that produce and excrete mucus and other fluids. Rectal cancer is cancer that forms in the tissue of the rectum, the last several inches of the large intestine closest to the anus. It was reported that more than 145,083 people were diagnosed with colorectal cancer and 53,580 people died from colorectal cancer in 2004. Colorectal cancer affects both men and women and all races, however, men, African Americans and Alaskan Natives have an exceptionally high colorectal cancer incidence and mortality rate . The treatment options for colorectal cancer depend on the stage of the cancer. According to the Food and Drug Administration (FDA) there are five stages of colorectal cancer: stage 0 (carcinoma in situ), stage I colon cancer, stage II colon cancer, stage III colon cancer and stage IV colon and recurrent colon cancer (Bruce et al., 2000).

The goal of this specific research was to increase the understanding of the role of the anthocyanin chemical structure on their chemoprotective and antiviral properties to better understand anthocyanin chemistry in various *in vitro*

environments. The initial hypothesis was that by testing the chemoprotective and antiviral properties of an anthocyanin source that contains a simple anthocyanin profile that we may be able to more accurately identify and understand the role that anthocyanins have in chemoprotection and antiviral properties in the human gastrointestinal tract. By selecting an anthocyanin source with fewer anthocyanins naturally present, semi-purification and isolation of a single anthocyanin can be more easily achieved. Strawberries have two major anthocyanins present in the fruit tissue; pelargonidin-3-glucoside and cyanidin-3-glucoside, which together account for over 83% of their anthocyanin pigments, making strawberries uniquely simple anthocyanin source. To achieve this hypothesis and the overall objective, the following specific objectives of this research were tested:

1. Use strawberry anthocyanins to test for antiviral properties against Herpes Simplex Virus Type-1.

2. Use strawberry anthocyanins to test for antiproliferation and proliferation rates with the AGS and 738.St/Int human stomach and small intestine cell lines.

3. Use strawberry anthocyanins to test for antiproliferation and proliferation rates with HT-29 and CCD-18Co human colon cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Flavonoids:

2.1.1 Chemical Structure:

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 C_2 to C_3 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 et al., 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 et al., 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 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 et al., 2002), antioxidant (Tattini et al., 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 et al., 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 et al., 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 vesicle formation can be found with proanthocyanidin phytoalexins in the cytoplasm of sorghum cells near a fungal infection site (Snyder 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 (Snyder and Nicholson, 1990). These antimicrobial effects of flavonoids have been described, but the roles and mode of action are not yet fully understood.

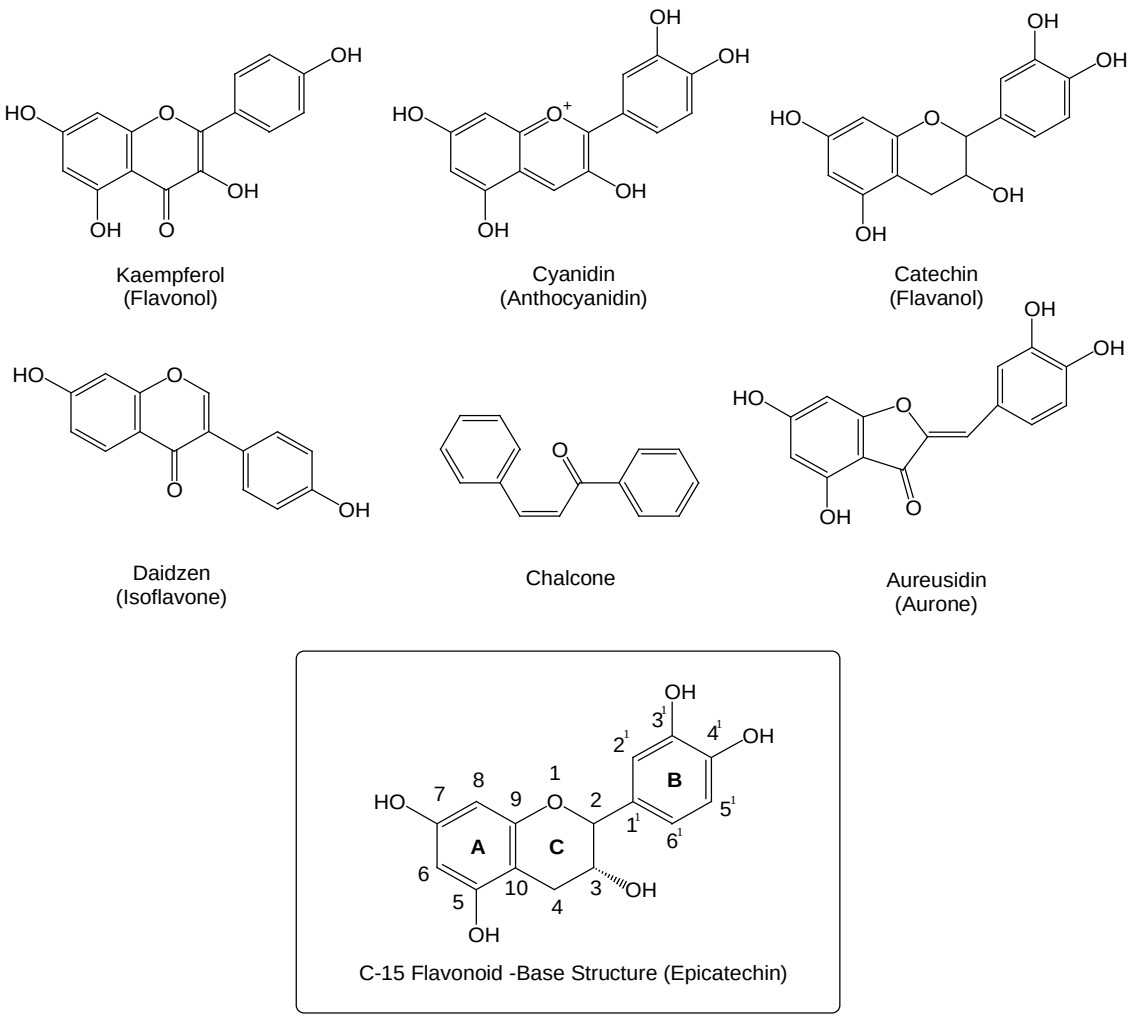


Figure 2.1: General base structures of flavonoids.

2.1.3 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 proanthocyanidin molecule occurs inside the vacuole after transportation of the anthocyanidin/flavanol and synthetic intermediates across the vacuolar membrane.

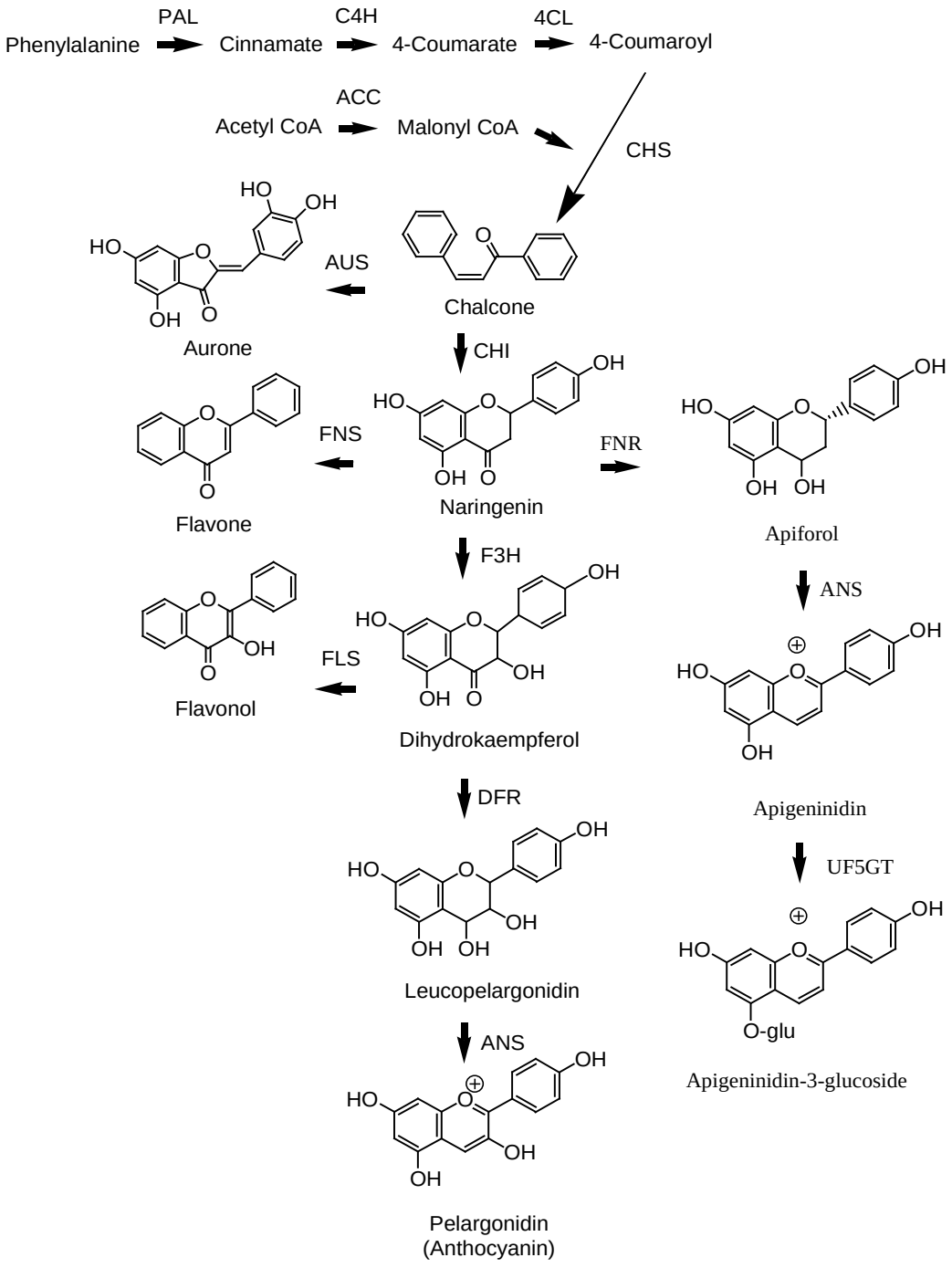


Figure 2.2: Schematic of the biosynthetic pathway of flavonoids. PAL: phenylalanine ammonia-lyase; C4H: cinnamate 4-hydroxylase; 4-coumarate CoA ligase; ACC: acetyl CoA carboxylase; CHS: chalcone synthase; CHI: Chalcone isomerase; F3H: flavanone 3β-hydroxylase; DFR: dihydroflavonol 4-reductase; ANS: 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.)

2.1.4 Incidence 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 dependent upon genetic factors of the plant species and environmental conditions such as light, ripeness, and post-harvest processing preparation (Chu et al., 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. 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.

2.1.5 Fractionation 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. Semi-purification 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.

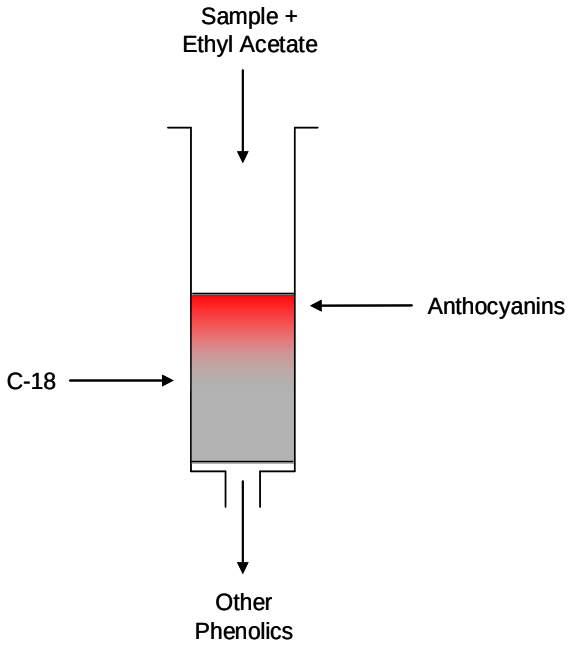


Figure 2.3: Separation of anthocyanins using a semi-preparative C-18 cartridge

2.1.6 Health Benefits 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 (Karlsen et al., 2008). It is thought that flavonoids are absorbed by passive diffusion after the cleavage of glycosidic groups by the gut micro-flora (Hollman et al., 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 et al., 2000), cancer (Juranic and Zizak, 2005), capillary fragility (Mian et al., 1977), atherosclerosis (Kadar et al., 1979), and stroke (Zand et al., 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 et al., 2004). This is in addition to the capability of flavonoids to positively influence visual acuity, anti-inflammation, and cognitive functions (Zafra-Stone et al., 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 et al., 1995). Colonic bacteria are largely responsible for the breakdown of flavonoids into phenyl acids before absorption (Hollman et al., 1995). Measurement of flavonoids in the blood stream and plasma after ingestion shows rapid absorption and utilization of these compounds *in vivo* (Benzie et al., 1999).

Interest in anthocyanin pigments has increased because of their beautiful color and functionality in food products. There are numerous subjective 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 et al., 2009).

2.2 Anthocyanins:

2.2.1 Presence in Foods and Beverages:

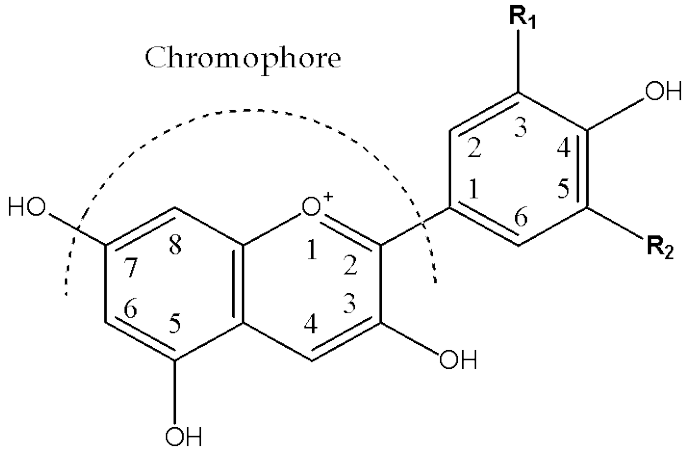
Consumers first assess the freshness and quality of food by its appearance, which makes color a vital characteristic for the ultimate initial acceptance of a product. Many flowers, fruits and vegetables owe their attractive colors to the presence of anthocyanin pigments that range from red to purple 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 Anthocyanidins and Structures:

Currently, over 635 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 (Figure 2.4).

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.



Anthocyanidins	R_1	R_2	Λ_{maz} (nm)
Pelargonidin	H	H	494 nm (orange)
Cyanidin	OH	H	506 nm (orange-red)
Peonidin	OMe	H	506 nm (orange-red)
Delphinidin	OH	OH	508 nm (red)
Petunidin	OMe	OH	508 nm (red)
Malvidin	OMe	OMe	510 nm (bluish-red)

Table 2.1: Anthocyanidins more commonly found in nature, their B-ring conjugations (R_1 and R_2), and maximum absorbance (Giusti and Wrolstad, 2003).

Small differences in chemical structure have a clear impact on color and tinctorial strength of anthocyanins (Dangles et al., 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 635 anthocyanins reported, 97% are reported to be glycosilated (Andersen and Jordheim, 2007).

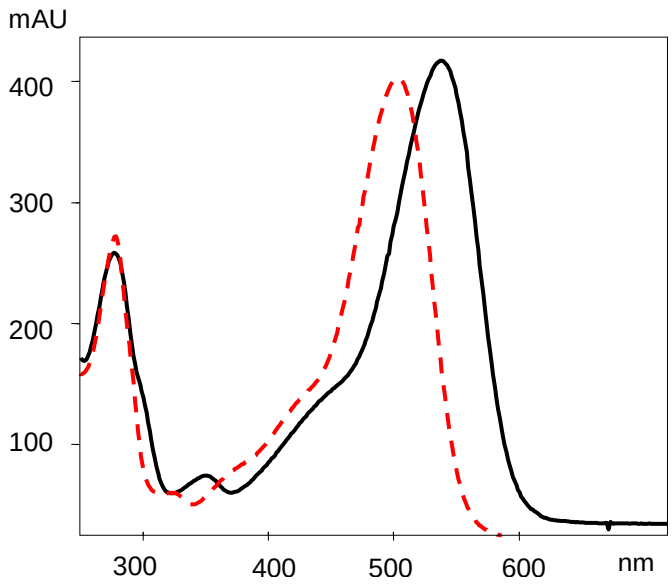


Figure 2.4: Bathochromic and hyperchromic shifts produced by acylation of purple carrot anthocyanins at 520 nm. Cy-3-gal-xyl-glu (---) Cy-3-gal-xyl-glu + p-coumaric acid (—). Bathochromic shift indicates an increase in the visible absorption maximum (nm). Hyperchromic shift indicates an increase in the 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 2.4). 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).

2.3 Stability of Anthocyanin Structure:

2.3.1 Influence of pH on Structure Stability:

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 2.5). 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.

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 fat-containing 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).

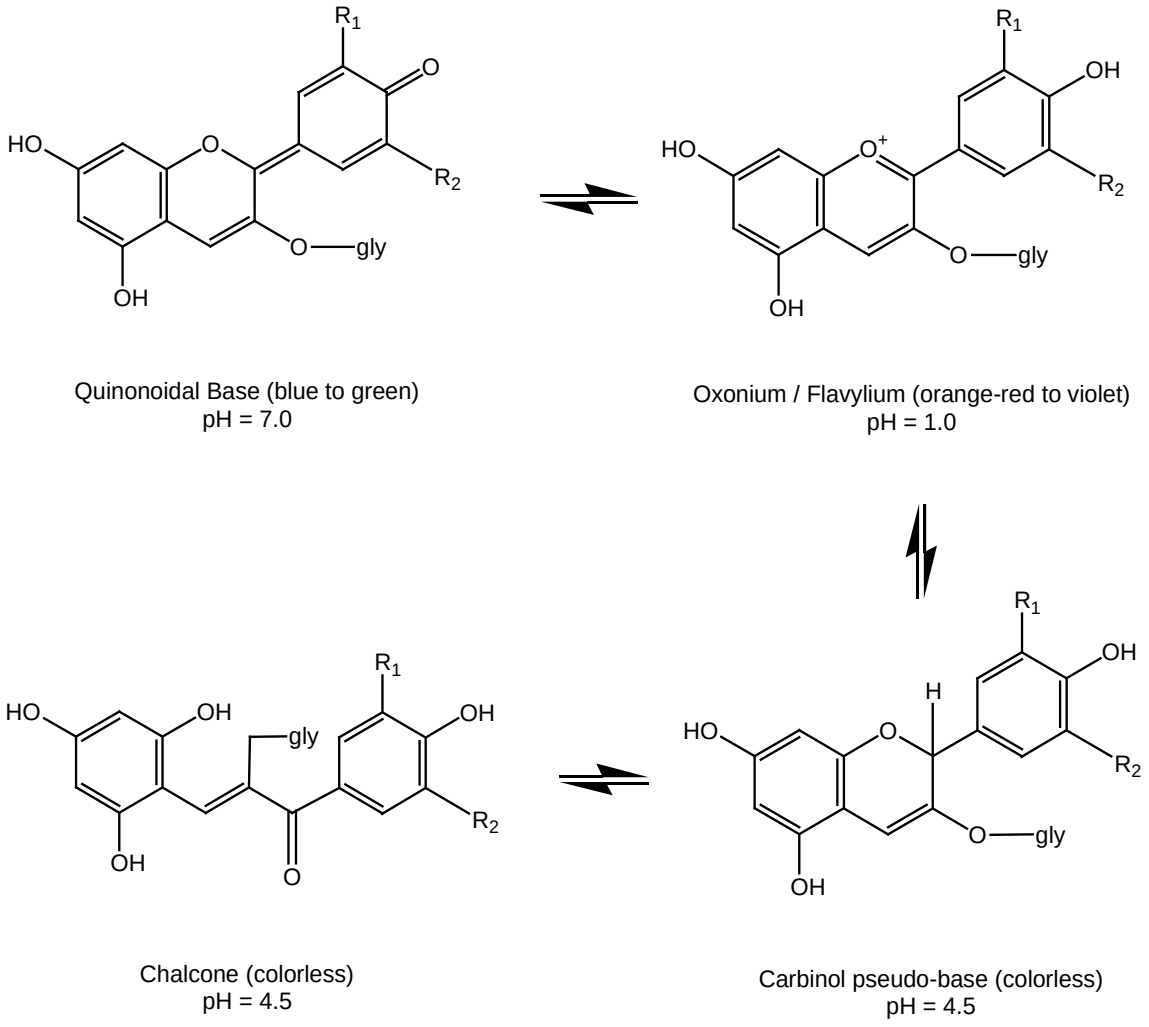
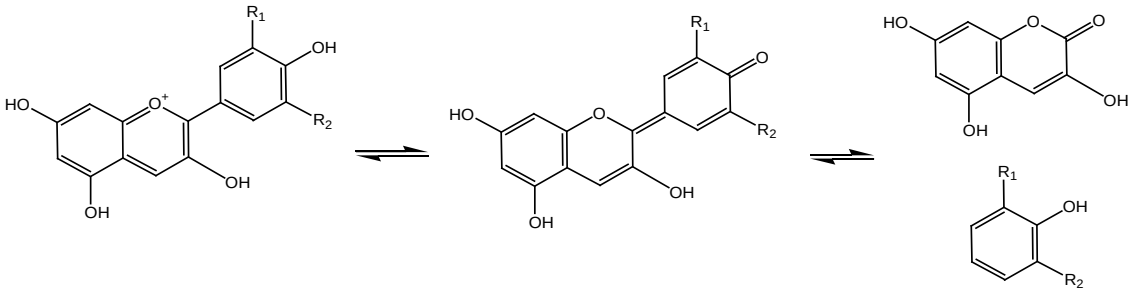


Figure 2.5: Anthocyanin structural transformations with change in pH.

2.3.2 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 a ά-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 et al., 2004) (Figure 2.6). 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 et al., 2001).



Oxonium Ion Quinonoidal Base Benzoic Acid Derivatives

Figure 2.6: Degradation of Oxonium Ion at pH 3.7 accelerated by heat. From Giusti and Wallace 2009.

2.3.3 Oxygen and Ascorbic Acid:

Oxygen amplifies the impact of other anthocyanin degradation processes. The un-saturation 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 yellow-brown 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 et al., 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 et al., 2003).

2.3.4 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 dependent 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 et al., 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.

2.3.5 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 losing their coloring properties. Inactivation of enzymes generally improves an anthocyanin's stability in the food matrix (Garcia-Palazon et al., 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 et al., 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 et al., 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 et al., 1999), pelargonidin 3-glucoside (Kader et al., 2001), and in wine model solutions (Sarni et al., 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 et al., 1990).

2.3.6 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 SO_2. 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 et al., 1987). This reaction is thought to disrupt the conjugated double bond system resulting in loss of color (Figure 2.7).

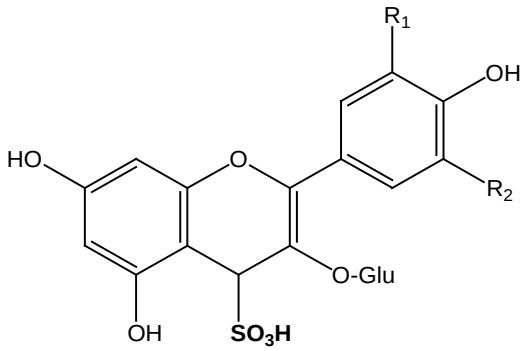


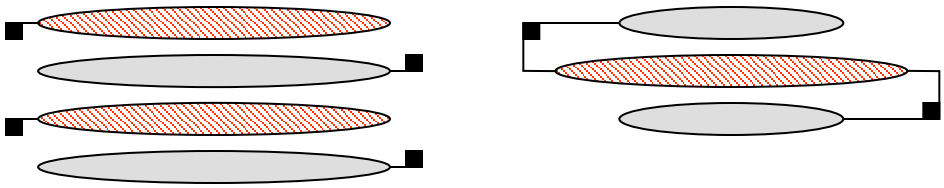
Figure 2.7: Colorless anthocyanin-sulfate complex.

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 SO_2 and the C_4 position 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 C_4 position (Timberlake and Bridal, 1968).

2.3.7 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 2.8). 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 et al., 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 et al., 1990).



Intermolecular Co-pigmentation Intramolecular Co-pigmentation

Figure 2.8: Anthocyanin intermolecular and intramolecular interactions. Anthocyanidin (≡); Co-pigment (#); 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 et al., 1981) (Figure 2.9). 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 et al., 1997).

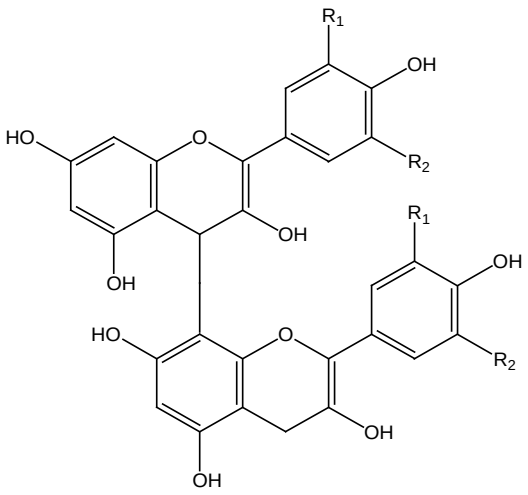


Figure 2.9: Self association of anthocyanins at the C-4 position. From Giusti and Wallace 2009.

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).

2.4 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 and Wallace et al., 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 and Wallace et al., 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 and Wallace et al., 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 et al., 2003; Kroon et al., 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.

2.5 Strawberries and Strawberry Extracts:

2.5.1 Botanic Review:

Strawberries are a hybrid plant species cultivated worldwide for their fruit. The scientific name for strawberries is *Fragaria x ananassa,* meaning garden strawberry. There are many *Fragaria* subspecies today grown worldwide (Figure 2.10).

Figure 2.10: *Fragaria ananassa*

According to the Economic Research Service of the United States Department of Agriculture (USDA) (2008), over 1.3 million metric tons of strawberries are grown annually in the United States alone, making the United States the number one producer and exporter of strawberries in the world. California, Florida and Oregon are the top 3 strawberry-producing states. The strawberry plant is a perennial that grows low to the ground and has three-palmate leaves and toothed

leaflets. The Individual plants range from 4-6 inches (10-15 cm) in height and send out numerous stolons (above ground runners) which root and start new plants. The flowers are white or pink with five rounded petals and are usually produced over an extended period. The strawberry "fruit" is actually a fleshy receptacle with many dry, thin walled achenes imbedded in its surface. Each achene (the true fruit) contains a single seed.

2.5.2 Production and Processing of Strawberries:

Strawberries prefer a slightly acidic soil (pH of 5.8 to 6.2) and grow best in a deep, sandy soil, rich in organic matter. The soil must be well drained and kept away from areas that remain wet late into the spring. The site should receive full sunlight and have a gradual slope. The gradual slope helps to prevent frost injury by allowing cold air to drain away from the plants.

The strawberry plant is considered a perennial but fruit production greatly degrades after the third year. Strawberry plants begin to bloom by early summer and harvesting can start by midsummer. Strawberries are the most popular berry fruit in the world. The majority of strawberries are consumed fresh in the United States. Fresh per capita consumption has increased from 4.21 pounds in 2001, to 7.34 pounds in 2011 (Wu 2012). Continued increases in strawberry imports have helped meet the growing consumption demand for fresh strawberries in the United States. Most processed strawberries are frozen whole (individually flash frozen) or sliced, with less than 10 percent of United States strawberry crops used for juice or puree (USDA). One serving, about 8 strawberries, provides

more vitamin C than an orange. Strawberries contain particularly high levels of polyphenols which act as antioxidants in the body and may contribute to an overall healthy cardiovascular and immune system, as well as, provide anticancer activity.

2.6 Gastric Cancer:

Stomach cancer also known as gastric cancer is a disease in which malignant (cancer) cells form in the lining of the stomach (Eyre 2004). Stomach cancers tend to develop slowly over many years. The stomach is part of the gastrointestinal system, which processes nutrients (vitamins, minerals, carbohydrates, fats, proteins, and water) in foods that are eaten and helps pass waste materials out of the body. The wall of the stomach is made up of three layers of tissue: the mucosal (innermost) layer, the muscularis (middle) layer, and the serosal (outermost) layer. Before true cancer develops, pre-cancerous changes often occur in the lining of the stomach. In many cases, these early changes rarely cause symptoms and therefore often go undetected (Bruce et al., 2000). Stomach cancer begins in the cells lining the mucosal layer and spreads through the outer layers as it continues to grow and spread. Stomach cancer typically affects the older population. Almost two thirds of people with stomach cancer are 65 years of age or older and the average risk of developing stomach cancer is about 1 in 116 (Eyre 2004). Stomach cancer was the leading cause of cancer related death in the United States until the late 1930s. This is believed to be due to refrigeration and the opportunity for longer food storage. The invention

of the refrigerator made it possible for more people to have access to fruits and vegetables year round and in turn decreased the amount of salted and smoked foods consumed. Additionally, stomach related cancers are thought to be less prevalent in first world countries because of the use of antibiotics that kill bacteria that cause infections in the stomach, such as, *Helicobacter pylori* (*H. pylori*). Because of limited resources, stomach cancer is much more prevalent in other parts of the world, particularly in underdeveloped nations. According the American Cancer Society, stomach cancer is the leading cause of cancer-related deaths in the world today (Eyre 2004).

2.7 Colon Cancer:

2.7.1 The Prevalence of Colon Cancer

Of cancers that affect both men and women, colorectal cancer is the second leading cause of cancer related deaths in the United States and the third most common cancer in men and women (Eyre 2004). Colorectal cancer is the cancer of the colon or rectum. Colon cancer is cancer that forms in the tissue of the colon, the longest section of the large intestine. The majority of colon cancer arises from preexisting adenomas (Toribara and Sleisenger, 1995). Adenocarcinoma cells are cells that produce and excrete mucus and other fluids. Rectal cancer is cancer that forms in the tissue of the rectum, the last several inches of the large intestine closest to the anus. It was reported that more than 145,083 people were diagnosed with colorectal cancer and 53,580 people died from colorectal cancer in 2004 (Eyre 2004). Colorectal cancer affects both men

and women and all races, however, men, African Americans and Alaskan Natives have an exceptionally high colorectal cancer incidence and mortality rate (Eyre 2004).

The treatment options for colorectal cancer depend on the stage of the cancer. According to the Food and Drug Administration (FDA) there are five stages of colorectal cancer: stage 0 (carcinoma in situ), stage I colon cancer, stage II colon cancer, stage III colon cancer and stage IV colon and recurrent colon cancer. Pictured below in Figure 2.11 are the different stages of colon cancer.

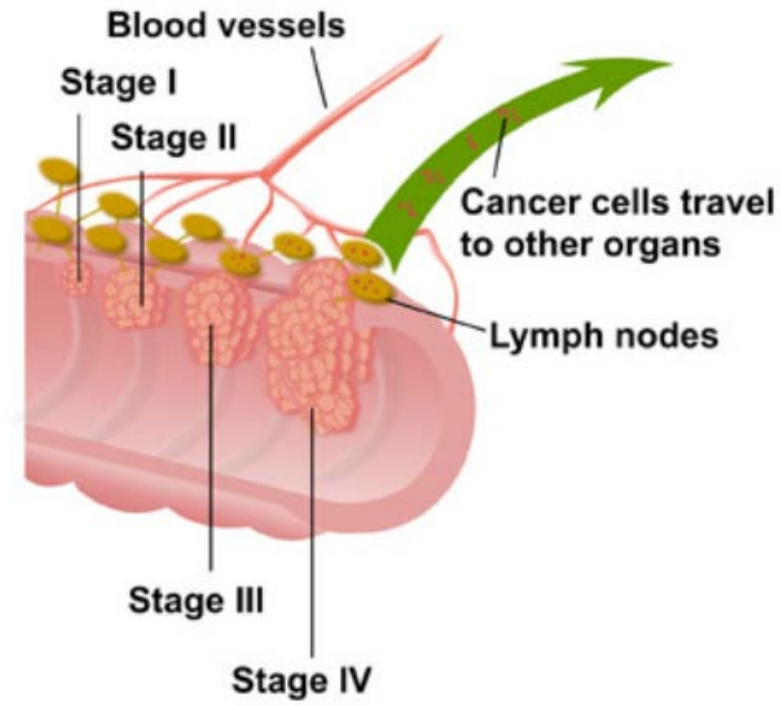


Figure 2.11: Five stages of Colon Cancer (Cedar-Sinai Medical Center 2009). Cedar-Sinai [Internet]. Los Angeles, California: Cedar-Sinai Medical Center; c2000-2009 Los Angeles, California [Accessed 2013 November 8th]. Available from: http://cancersign.us/stage-4-colon-cancer-prognosis/

2.7.2 Five Stages of Colon Cancer

According to the American Cancer Society there are five stages of colorectal cancer: Stage 0 (carcinoma in situ) refers to abnormal cells that have been found in the inner most tissue of the colon or rectum and have the potential to spread to other areas of the colon or rectum. This stage of colon or rectum cancer is also known as Dukes A Colorectal Cancer. Stage 0 is usually treated by localized surgery (Bruce et al., 2000).

Stage I colon cancer indicates that cancer cells have spread beyond the first layer of the colon or rectum into the second and third layers, but has not yet spread to the outside wall of the colon or rectum. Treatment for stage I colon cancer is a surgical procedure known as a resection or anastomosis which removes the diseased section of the colon or rectum and connects healthy sections together.

Stage II colon cancer indicates that cancer cells have spread outside the colon or rectum to the close by tissue but has not yet spread to the lymph nodes. This type of colon or rectum cancer is also known as Dukes B Colorectal Cancer. Stage II colon cancer is also treated with a resection or anastomosis to remove the diseased section of the colon or rectum and connect healthy sections together.

Stage III colon cancer is a more severe stage of colon cancer. It indicates that tumor cells have moved to lymph nodes and organs near the colon or rectum. This stage of colon cancer is also known as Dukes C Colorectal Cancer. Treatment for stage III colon cancer includes a resection/anastomosis with

chemotherapy and/or radiation from x-rays, gamma rays, neutrons, protons and other sources to kill cancer cells and shrink tumors.

The final stage of colorectal cancer is stage IV and recurrent colon cancer. Stage IV colon cancer indicates that the cancer has spread to nearby lymph nodes, as well as, to other organs in the body such as the liver. Recurrent colon cancer means that the colon or rectum cancer has returned from previous treatment. The cancer may or may not return to its previously occurring location. Stage IV and recurrent colon cancer treatments include: resection/anastomosis surgery, chemotherapy, radiation therapy, surgery to remove parts of the other organs where cancer might have spread, clinical trials, biological therapy and some patients may even be offered palliative therapy to improve their quality of life and relieve any discomfort they may be experiencing.

2.8 Cellular Models

2.8.1 Cell Models as a Mechanism to Study Gastrointestinal Health

The use of human cell lines to study the inhibitory effects of anthocyanins against carcinogenesis is advantageous because they allow for complex system to be studied in a rather simplistic model. It is well accepted that any model cancer system is an inadequate representation for the actual human disease; however, the degree of which the system mimics the real disease is an important determinant of its usefulness (Brattain et al., 1981). It is important to understand that different cell models reflect the specific area in which they came from to a different degree. The following four cell lines will be used in this study:

2.8.2 AGS

AGS human cell line originates from the stomach of a *homo sapien*. The cell line is considered to have a epithelial morphology and its culture growth properties are adherent. The AGS human stomach cell lines are gastric adenocarcinoma and therefore cancerous. The base medium for this cell line is ATCC-formulated F-12K Medium. To make the complete growth medium fetal bovine serum to a final concentration of 10 percent must be added.

2.8.3 Hs 738.St/Int

The Hs 738.St/Int human cell line originates from a mix of the stomach and intestine of a male *homo sapien*. The cell line is considered to have a fibroblast morphology and its culture growth properties are adherent. The Hs 738.St/Int human stomach and intestine cell line is considered non-diseased. The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium. To make the complete growth medium fetal bovine serum to a final concentration of 10 percent must be added.

2.8.4 HT-29

The HT-29 human cell line originates from the colon of a female *homo sapien*. The cell line is considered to have an epithelial morphology and its culture growth properties are adherent. The HT-29 human cell line is diseased with colorectal adenocarcinoma and therefore cancerous. The base medium for this cell line is ATCC-formulated McCoy's 5a Medium Modified. To make the

complete growth medium fetal bovine serum to the final concentration of 10 percent must be added.

2.8.5 CCD-18Co

The CCD-18Co human cell line originates from the colon of a female *homo sapien.* The cell line is considered to have a fibroblast morphology and its culture growth properties are adherent. The CCD-18Co human cell line is non-diseased. The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium (MEM). To make the complete growth medium fetal bovine serum to the final concentration of 10 percent must be added.

CHAPTER 3

ANTIVIRAL EFFECTS OF STRAWBERRY ANTHOCYANINS AGAINST HERPES SIMPLEX VIRUS TYPE 1.

3.1 SUMMARY

Anthocyanins are widely distributed in nature and represent an abundant source of flavonoids consumed in the diet. Recent attention has been given to these compounds because of their health promoting antioxidant properties which protect against viral infections. The herpes simplex virus type-1 (HSV-1) has the ability to lay dormant and periodically reactivate causing an infection. Because of its tendency to lay dormant the herpes simplex virus type-1 is difficult to eliminate once the body becomes infected. The virus plaque assay has become increasingly popular as a rapid technique to quantify *in vitro* virus yield (infectivity) and replication of the herpes simplex virus type-1. In this study, the main objective was to evaluate the antiviral properties of strawberry against herpes simplex virus type-1 *in vitro.* Vero cells from the kidney of an adult grivet (*Cercopithecus aethiops*) were used and infected with herpes simplex virus type-1 and treated with strawberry anthocyanins (1.25, 2.5, 5, 10 and 20 µg/mL), and the virus yield (infectivity) and replication were quantified by direct plaque assay. Strawberry anthocyanins (>20 µg/mL) inhibited the herpes simplex virus infectivity in vero cells by 100% ($p<0.05$). Strawberry anthocyanins at concentrations of 5, 10 and 20 µg/mL were reduced to 75.36, 57.98, and 31.46 percent of the control (100%) ($p<0.05$). The virucidal effects were not due to pH changes at concentrations up to 20 µg/mL. Strawberry anthocyanins inhibited

early stages of herpes simplex virus type-1 (strain 17+) replication and had potent virucidal activity. These findings suggest that anthocyanins from strawberries may provide an advantage as a topical prophylactic/therapeutic agent for herpes simplex virus infections.

3.2 INTRODUCTION

Flavonoids have been shown to impart protection from oxidizing species, such as the superoxide anion, hydroxyl radical and peroxy radicals; they have also been proven to be quenchers of singlet oxygen (Harborne et al 2000). The antioxidant activity of flavonoids is thought to present many of the health benefits associated with consumption of fruits and vegetables to humans. Due to the increased interest in the health benefits of flavonoids, there have been an increasing number of biological studies in hopes to better understand these powerful antioxidants. In fact, the United States Department of Agriculture (USDA) recently began documenting the flavonoid content of commonly consumed fruits and vegetables (USDA 2.1, 2007). Anthocyanins represent a subclass of flavonoids and are of great importance to the food industry because of their coloring properties and accordingly, their impact on the visual appeal of a product. Because anthocyanins are responsible for the red to violet hues in many fruits and vegetables, their use in the food industry has rapidly expanded as a value added ingredient and alternatives to synthetic red dyes. The basic anthocyanin structure consists of the carbon skeleton with conjugated bonds on the C-ring and two positions, R-1 and R-2, which are substituted with hydroxyl, oxymethyl and hydryl groups. Different combinations of these side groups results in the six anthocyanidin chromophores most commonly found in nature. These anthocyanidin aglycones include cyanidin (Cy), pelargonidin (Pg), delphinidin (Dp), petunidin (Pt), malvidin (Mv) and peonidin (Pn) (Fleschhut et al., 2006; Hou et al., 2005; McGhie et al., 2007). Anthocyanin sources by nature

contain multiple polyphenolic compounds; because of this the characterization and quantification of anthocyanins can be laborious and expensive. By selecting an anthocyanin source with fewer anthocyanins naturally present, semi-purification and isolation of a single anthocyanin can be more easily achieved. Strawberries have two major anthocyanins present in the fruit tissue; pelargonidin-3-glucoside and cyanidin-3-glucoside (Figure 3.1), which together account for over 83% of their anthocyanin pigments, making strawberries uniquely simple anthocyanin source. Because of this strawberries were selected for this research study.

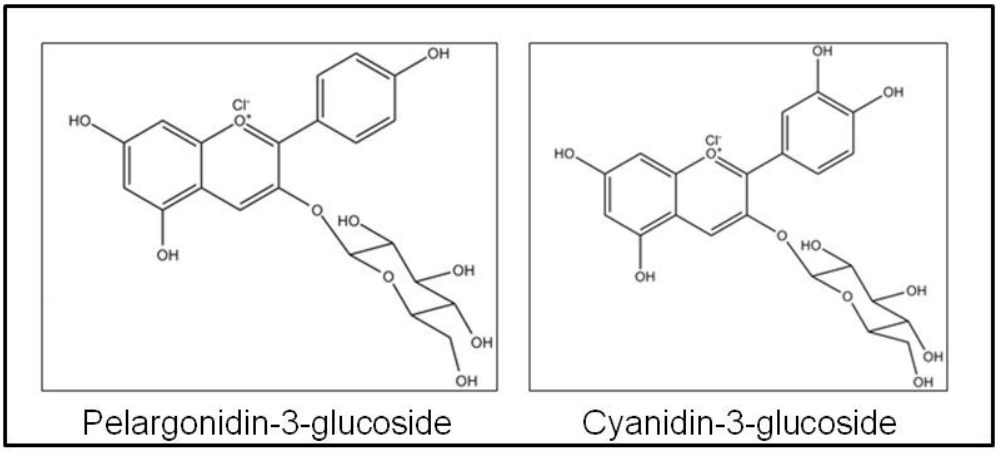


Figure 3.1: The two main strawberry anthocyanins, pelargonidin-3-glucoside and cyanidin-3-glucoside.

The herpes simplex virus (HSV) is an epitheliotropic virus that infects children and the majority of adults (Bader et al., 1978). There are two types of herpes simplex virus: herpes simplex virus type 1 refers to the oral herpes virus and herpes simplex virus type 2 refers to the genital herpes virus. The infection of epithelial cells (i.e. mucosa and skin) by the herpes simplex virus results in de-

envelopment of the cell membrane, rapid replication, and spread to adjacent cells and nerve cell endings. Due to the uptake of virions by nerve endings the virus can travel retroaxonally to the nerve cell bodies within the trigeminal ganglion, upon which a dormant state is established (Stevens and Cook 1971, Stevens 1975). The herpes simplex virus DNA resides long-term within the neuronal nuclei in a state that allows for periodic reactivation and recrudescence of progeny virus (Kaufman et al., 1967 and Lachmann 2003). The ability of the virus to lay dormant in immune-privileaged sites makes eradication of the virus difficult.

The objective of this research was to determine the antiviral effects of strawberry anthocyanins against the herpes simplex virus type-1 (strain 17+). To accomplish this, vero cells from the kidney of an adult grivet (*Cercopithecus aethiops*) were used to infect the herpes simplex virus type-1 (strain 17+) with strawberry anthocyanins (12.5-200 µg/mL), and the virus yield and infectivity were quantified by direct plaque assay.

Although the objective of this study was to determine the antiviral effects of strawberry anthocyanins against herpes simplex virus type-1 and not to determine the exact mechanism of action the strawberry anthocyanin may have on HSV-1 it is important to consider what potential mechanisms of actions for anthocyanins have been proposed. The first and most obvious mechanism of action is that anthocyanins work as an antioxidant and actively scavenge highly reactive oxygen species and prevent them from doing further harm to the body (Nijveldt et al., 2001). The next proposed mechanism of action of anthocyanins

against HSV-1 is blocking. Essentially, anthocyanins provide protection from HSV-1 by blocking factors on the cell surface (for the purpose of this experiment vero cells) that the virus needs for successful infection (Danaher et al., 2011). Lastly, a mechanism of action is recognized for the possibility of the anthocyanin to cause direct inactivation of HSV-1 by anthocyanin binding to the viral envelope (Danaher et al., 2011). Future research in elucidating the specific activity of the strawberry anthocyanin extract will allow a better understanding of it mechanism of action and therefore of its potential applications.

3.3 MATERIALS AND METHODOLOGY

3.3.1 Chemicals and Reagents:

Organic Driscoll's brand strawberries were purchased from a local grocery store in Lexington, Kentucky. Certified ACS grade HCL (12N) and ethyl acetate, LCMS grade 99.9% methanol and 99.9% acetonitrile, and HPLC grade 88% formic acid, 99.9% acetone and 99.9% chloroform were obtained from Fisher Scientific (Fair Lawn, NJ). Reagent grade gallic acid and Folin-Ciocaltaeu reagent were obtained from MP Biomedical (Aurora, OH). Vero cells were obtained from American Type Culture Collection (Rockville, MD). Herpes Simplex Virus (HSV) Type-1 17+ was obtained from N. Fraser of the Wistar Institute, Philadelphia, PA. Sterile Eagle's Minimum Essential Medium and Immunoglobulin IgG (Thermo Fisher Scientific, Waltham, MA), fetal bovine serum (FBS) (Invitrogen Corp. Carlsbad, CA) Delbucco's Phosphate Buffered Saline Solution (DPBS) (Lonza, Walkersville, MD), and 2.5% Trypsin (Lonza, Walkerville, MD) were obtained from their manufacturers. All other chemicals were certified ACS grade from Thermo Fisher Scientific, Waltham, MA.

3.3.2 Extraction of Anthocyanins and other Phenolics from Plant Materials:

The red flesh of strawberries were hand selected using a paring knife and quickly frozen using liquid nitrogen. The frozen strawberry red flesh where then fine powdered using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT) with a 500 mL stainless steel container in the

presence of liquid nitrogen (Giusti and Rodriguez-Soana 1999). Strawberry flesh powder was stored at -20˚C until used for sample extraction.

Strawberry flesh anthocyanins and other phenolics were extracted by blending the powders with 30mL of 70% acetone and 30% 0.1% HCl acidified water (v/v) for 5 minutes using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT). The liquid was subsequently passed through Whatman No. 4 and No. 1 filter paper (Whatman Inc., Florham, NJ) using a Buchner Funnel (Thermo Fisher Scientific, Waltham, MA). This process was repeated until the filtrate was free of haze and all of the pigment had been extracted from the powdered strawberry flesh particles (Rodriguez-Soana and Wrolstad 2001). The strawberry anthocyanin and other phenolics filtrate was then placed into a separatory funnel (Thermo Fisher Scientific, Waltham, MA) and partitioned with 2 volumes of chloroform. The solution was mixed and left to separate for 24 hours at 4˚C in the dark to ensure adequate separation. The top acetone/water layer containing anthocyanins and other phenolics was collected and the bottom chloroform/fat layer was discarded properly in hazardous waste containers. The residual acetone in the sample was evaporated using a Buchi Rotavapor R-215 rotary evaporator (Thermo Fisher Scientific, Waltham, MA) at 40˚C. The solution was brought to 25 mL in a volumetric flask using LCMS grade water.

3.3.3 Fractionation and Concentration of Anthocyanin Sample:

Anthocyanin phenolics from strawberries were concentrated by using Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corporation, Milford, MA). The anthocyanin filtrate was passed through the Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corporation, Milford, MA) 10 mL at a time. Anthocyanins and other phenolics were bound to the C_{18} cartridge, while sugars and other polar compounds were removed with 30 mL of 0.01% HCl acidified water, followed by 10 mL of hexane to remove the residual water. The non-anthocyanin phenolics were eluted with 20 mL of diethyl ether followed by 20 mL of ethyl acetate. The anthocyanin phenolic fraction was eluted with 30 mL of 0.01% HCl acidified methanol followed by 10 mL of 0.01% HCl acidified HPLC grade water. Excess methanol in the anthocyanin phenolic filtrate was evaporated in the Buchi Rotavapor R-215 rotovapor (Thermo Fisher Scientific, Waltham, MA) at 40˚C and the anthocyanin fraction was re-dissolved in 0.01% HCl acidified HPLC grade water. The highly concentrated anthocyanin extract was then quickly frozen using liquid nitrogen and lyophilized using a Labconco Freezone[6] lyophilizer into a fine dry powder (Labconco, Kansas City, MO).

3.3.4 Monomeric and Polymeric Analysis:

Monomeric anthocyanin content was determined using the pH differential method (Giusti and Wrolstad, 2005). This method uses the spectral difference that anthocyanins exhibit at different pH values. Dilution factor was determined by diluting the strawberry anthocyanin extract in 0.025 M potassium chloride

buffer, pH 1.0, until the absorbance was within the appropriate spectrophotometer range (for most spectrophotometers absorbance should be less than 1.2 nm). The strawberry anthocyanin extract was then appropriately diluted using pH 1.0 and pH 4.5 buffer. Solutions were allowed to sit at room temperature for at least 15 minutes in the dark. 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 cyanidin-3-glucoside equivalents, using a molecular weight of 449.3 and an extinction coefficient of 26,900 L cm^{-1} mg^{-1} (Giusti and Wrolstad, 2005). Absorbance was determined in replicates of three.

Polymeric anthocyanin content was determined using the polymeric method (Wrolstad 1999). Appropriate dilutions were determined using pH 1.0 buffer and 1-cm cuvettes were prepared with 2.8 mL of diluted sample in water to each of two cuvettes. 0.2 mL of water was added to one of the cuvettes and 0.2 mL of bisulfate solution to the other and allowed to equilibrate for 15 minutes before reading at 420 nm, 520 nm, and 700 nm. Polymeric anthocyanin content was calculated using the following equations:

Color Density = $[(A_{420nm} - A_{700nm}) + (A_{\lambda\ vis\ max} - A_{700nm})] \times DF$

Polymeric Color = $[(A_{420nm} - A_{700nm}) + (A_{\lambda\ vis\ max} - A_{700nm})] \times DF$

Percent Polymeric Color = (Polymeric Color/Color Density) x 100

Where DF is the dilution factor and absorbance was determined in replicates of three.

3.3.5 Total Phenolics:

Total phenolics were measured using a microscale protocol for Folin-Ciocalteu Colorimetry (Waterhouse 2005). Cuvettes were prepared with 20 µL of sample/standard, 1.58 mL water and 100 µL 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 µL) was added and samples were allowed to stand at room temperature for 2 hours. 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.

3.3.6 LC/MS Analysis:

Strawberry anthocyanin samples were analyzed using a Thermo Finnigan LTQ high performance liquid chromatograph (HPLC) (ThermoFinnigan; Waltham, MA). Positive ion electrospray ionization (ESI) mass spectra were obtained on the Thermo Finnigan LTQ coupled to a Dionex Ultimate 3000 capillary LC system. The column was 20cm x 150um packed with Kromasil C18 (3.5um particle size) (AkzoNobel, Separation Products, Bohus, Sweden). Proteome Discoverer Software 1.1.0 was used on this system (Thermo Fisher Scientific; Waltham, MA). All samples were filtered through 13 mm 0.45µm polypropylene filters (Thermo Fisher Scientific, Waltham, MA) prior to HPLC/MS analysis.

Separation of strawberry extract anthocyanins was achieved using a linear gradient elution from 5% to 60%. Solvent A was 0.1% (v/v) formic acid in water and B was 0.1% (v/v) formic acid in acetonitrile. The flow rate was 0.8 mL / min and an injection volume of 50 µL was used. Spectral data was collected using the Dionex Ultimate 3000 capillary LC system from 250-700 nm. Absorbance of anthocyanins was monitored at 280 and 520 nm.

A 1.5 uL / 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 ºC; 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 and Selective Ion Monitoring (SIM) was used to search for the molecular ions of the common anthocyanins throughout the analysis.

3.3.7 Herpes Simplex Virus Type 1:

The herpes simplex virus (HSV) is an epitheliotropic virus that infects children and the majority of adults (Bader and others 1978). There are two types of herpes simplex virus: herpes simplex virus type-1 refers to the oral herpes virus and herpes simplex virus type-2 refers to the genital herpes virus. The infection of epithelial cells (i.e. mucosa and skin) by the herpes simplex virus results in de-envelopment of the cell membrane, rapid replication, and spread to

adjacent cells and nerve cell endings. Due to the uptake of virions by nerve endings the virus can travel retroaxonally to the nerve cell bodies within the trigeminal ganglion, upon which a dormant state is established (Stevens and Cook, 1971; Stevens 1975). The herpes simplex virus DNA resides long-term within the neuronal nuclei in a state that allows for periodic reactivation and recrudescence of progeny virus (Kaufman et al., 1967 and Lachmann 2003). The ability of the virus to lay dormant in immune-privileged sites makes eradication of the virus difficult.

3.3.8 Vero Cell Line:

The vero cell line derived from kidney of adult *Cercopithecus aethiops* (American Type Culture Collection, Rockville, MA) were grown at 37˚C in a Nuuaire™ IR Autoflow CO_2 Water-Jacketed Incubator at a modified atmosphere (5% CO_2, 95% O_2) in Eagle's Minimum Essential Medium (Thermo Fisher Scientific, Waltham, MA)and supplemented with 10% Fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA). Cultures were split 1:5 when monolayers were 70-80% confluent using Delbucco's Phosphate Buffered Saline solution (DPBS) and 2.5% trypsin (Lonza, Walkersville, MD). Cultures were fed fresh media every 3 days. Growth of vero cell line was determined by microscopic examination. Cell cultures were maintained in 150 cm^2 Flasks with canted necks (Thermo Fisher Scientific, Waltham, MA). All cell culture experiments were performed with the initial 20 passages.

3.3.9 Determining Vero Cell Concentration using a Hemocytometer:

Vero cells were plated in 6-well tissue culture dishes at a volume of 2.3 x 10^5 cells/well. Suspended cells were counted using a Nikon Eclipse 50i Brightfield Microscope and a Reichert Bright-Line hemocytometer (Hausser Scientific; Horsham, PA) to estimate total viable cell concentration. 10 µl of cells were added to 90 µl of Trypan Blue Stain (Thermo Fisher Scientific, Waltham, MA) and subsequently loaded on the hemocytometer. Using the 10x lens of the microscope, cells were counted on 5 primary squares of the hemocytometer using a hand counter. To calculate the vero cell concentration the following formula was used:

Cells/mL = (Total # cells counted)/(Number of primary squares counted) x $1x10^4$

Vero cells were seeded at 2.3 x 10^5 cells/well and allowed to grow and form monolayers for 24 hours. At 24 hours the vero cells were infected with herpes simplex virus type-1 (strain 17+) and treated with strawberry anthocyanin extract and tested for antiviral effects.

3.3.10 Anthocyanin Treatments:

Monomeric anthocyanin content was determined for the strawberry anthocyanin extract using the detailed method explained by Giusti and Wrolstad, 2001. According to the monomeric anthocyanin content of strawberries, 1.25,

2.5, 5, 10 and 20 µg/mL of strawberry anthocyanin was determined and a corresponding treatment value was given in grams of sample weight.

3.3.11 Virus Plaque Assay:

The plaque assay was used to determine the herpes simplex type-1 (strain 17+) as plaque-forming units per milliliter (PFU/mL). In this assay, vero cell monolayers were infected with a low ratio of virus: $7.5x10^4$ PFU/mL for infectivity experiments and 1.875 $x10^4$ PFU/mL for replication experiments such that sporadic cells become infected. In this assay the vero cells required Dulbecco's Modified Eagle Medium (DMEM) with the addition of 0.5% Immunoglobulin G (IgG) to keep the vero cells stable and to limit the spread of the herpes simplex virus type-1. Because of the addition of the immunoglobulin G (IgG) to the Dulbecco's Modified Eagle Medium (DMEM) when each infected cell produced a virus and lysed, only the immediately adjacent cells become infected. Each group of infected cells was referred to as a plaque. Uninfected cells surround the plaques. After several infection cycles, the infected cells in the center of the plaques begin to lyse and the peripheral infected cells remained surrounded by uninfected cells. Because of this, light was able to pass through the infected cells and refract differently than the surrounding uninfected cells, and the plaques could be visualized either by the naked eye or by light microscopy. Each plaque represented a single virus. Therefore, clonal virus populations could be purified by isolating individual plaques. Individual plaques obtained from varying dilutions of a viral stock were counted to determine the viral titer

(PFU/mL) of a given transfection or virus stock. The condition of the vero cells and their even distribution over the surface of the tissue culture plate was very important to the success of a plaque assay. Vero cells were healthy, > 95% viable, and in log-phase growth at the time of the assay. Clumpy cells, cells that were not evenly distributed at the correct density (>70%) over the plate, and cells that did not adhere to the tissue culture dishes within 30 min after plating were detrimental to the assay and were not used.

3.3.12 Viral Infectivity:

A direct plaque technique (Roizman and Roane, 1961) was used for observation of viral cytopathic effect, from which *in vitro* infectivity was quantified. Vero cells were plated in 6-well tissue culture dishes (Corning Inc., Tewksbury, MA) at $2.3x10^5$ cells/well in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37˚C in a humidified incubator with 5% CO_2. The Vero cells were allowed to grow and form monolayers for 24 hours. Following the 24 hour incubation confluent cell monolayers in the 6-well plates were aspirated of the old Dulbecco's Modified Eagle Medium (DMEM) and inoculated with 20 uL of strawberry anthocyanin extract at a levels of 0, 1.25, 2.5, 5, 10 and 20 µg/mL in addition to180 uL of herpes simplex virus type-1 (strain 17+) at a level of $7.5x10^4$ PFU/mL suspended in complete Basal Medium Eagle (BME) with 10% fetal bovine serum (FBS). Immediately following the inoculation of the herpes simplex virus type-1 the virus was adsorbed by intermittent mechanical agitation for one hour at room temperature using an orbital shaker (Thermo Fisher Scientific;

Waltham, MA). Following the one hour agitation 2 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and containing 0.5% human immunoglobulin G (IgG) was added to each well. Infected cultures were incubated at 37˚C in a humidified incubator with 5% CO_2 for 72 hours. 72 hours after inoculation the 6-well plates were aspirated of their media and a 1% crystal violet stain was added to each well at a volume that adequately covered the bottom and was allowed to stand for 20 minutes. After 20 minutes the 1% crystal violet stain was aspirated and the 6-well plates were allowed to air dry. Plaques (1 to 3 mm in diameter), which are clear areas of dead cells, were magnified ten-fold and counted with the aid of a Nikon profile projector. The number of plaques on replicate plates was averaged, and the concentration of virus in plaque-forming units (PFU) per milliliter (PFU/mL) was calculated.

An additional virucidal assay was performed, using a cell-free virus suspension and incubating with the strawberry anthocyanin at room temperature. For this assay, vero cells were plated in 6-well tissue culture dishes (Corning Inc., Tewksbury, MA) at $2.3x10^5$ cells/well in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37˚C in a humidified incubator with 5% CO_2. The Vero cells were allowed to grow and form monolayers for 24 hours. The next day cell-free virus suspensions were prepared with a viral load of $7.5x10^4$ PFU/mL using aliquots of 180 uL of virus and 20 uL of strawberry anthocyanins at concentrations of 0, 1.25, 2.5, 5, 10 and 20 µg/mL the mixture was allowed to stand at room temperature for 15 and 60 minute time intervals. At the end of each time interval 10 uL of the virus/strawberry anthocyanin mixture was added

to 990 uL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Next, following the 24 hour incubation confluent cell monolayers in the 6-well plates were aspirated of the old Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and inoculated with 200 uL of virus and strawberry anthocyanin mixture. Immediately following the inoculation of the herpes simplex virus type-1 the virus was adsorbed by intermittent mechanical agitation for one hour at room temperature using an orbital shaker (Thermo Fisher Scientific; Waltham, MA). Following the one hour agitation 2 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and containing 0.5% human immunoglobulin G (IgG). Infected cultures were incubated at 37˚C in a humidified incubator with 5% CO_2 for 72 hours. 72 hours after inoculation the 6-well plates were aspirated of their media and a 1% crystal violet stain was added to each well at a volume that adequately covered the bottom and was allowed to stand for 20 minutes. After 20 minutes the 1% crystal violet stain was aspirated and the 6-well plates were allowed to air dry. Plaques (1 to 3 mm in diameter), which are clear areas of dead cells, were magnified ten-fold and counted with the aid of a Nikon profile projector. The number of plaques on replicate plates was averaged, and the concentration of virus in plaque-forming units (PFU) per milliliter (PFU/mL) was calculated.

3.3.13 Viral Replication:

In the viral replication inhibitory assay a tittering technique was used for observation of *in vitro* viral replication. The first step to this experiment includes

performing viral replication on a 6-well plate. Vero cells were plated in 6-well tissue culture dishes (Corning Inc., Tewksbury, MA) at $2.3x10^5$ cells/well in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37˚C in a humidified incubator with 5% CO_2. The Vero cells were allowed to grow and form monolayers for 24 hours. Following the 24 hour incubation the 6-well plate was aspirated of the old Dulbecco's Modified Eagle Medium (DMEM) and inoculated with 180 uL of Herpes Simplex virus Type-1 (strain 17+) at a level of $1.875 x10^4$ PFU/ml and 20 ul of strawberry anthocyanin at concentrations of 0, 1.25, 2.5, 5, 10 and 20 µg/mL were systematically diluted by 10 fold per well across the 6-well plate. The 6-well plate was then incubated at 37˚C in a humidified incubator with 5% CO_2 for 1 hour. Following the 1 hour incubation the 6-well plate was aspirated and rinsed with phosphate buffering solution (PBS) two times. Following the rinse the PBS was aspirated and 1.5 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS was added to each well and allowed to incubate at 37˚C in a humidified incubator with 5% CO_2 for 24 hours. After the 24 hour incubation the plate was placed in a -85˚C freezer for 24 hours. The next day the virus infected cells were quickly thawed by placing the plate in a 37˚C humidified incubator with 5% CO_2 for 1 minute and then dislodged with a sterile rubber policeman and harvested and stored in 6 tubes at -85˚C until used in the pilot titer.

The next step to the viral replication inhibitory assay was to perform a pilot titer. To perform the pilot titer three 12-well plates were used and vero cells were seeded at a volume of $2.3x10^5$ cells/well and allowed to grow for 24 hours at

37˚C in a humidified incubator with 5% CO_2. After the 24 hour incubation the 6 tubes generated in the replication experiment above were quickly thawed in a 37˚C water bath and added each in 10 fold dilutions to six different wells starting from the stock and ending with a 10^5 dilution. Directly following the inoculation the three 12-well plates were agitated for one hour at room temperature using an orbital shaker (Thermo Fisher Scientific; Waltham, MA). Next 1 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and immunoglobulin G (IgG) was added to each well and the three 12-well plates were incubated for 72 hours at 37˚C in a humidified incubator with 5% CO_2. 72 hours after inoculation the 6-well plates were aspirated of their media and a 1% crystal violet stain was added to each well at a volume that adequately covered the bottom and was allowed to stand for 20 minutes. After 20 minutes the 1% crystal violet stain was aspirated and the 6-well plates were allowed to air dry. Plaques (1 to 3 mm in diameter), which are clear areas of dead cells, were magnified ten-fold and counted with the aid of a Nikon profile projector. The concentration of virus in plaque-forming units (PFU) per milliliter (PFU/mL) was calculated.

The last step of the viral replication assay was to perform the final titer. To perform the final titer 18 6-well plates were used and vero cells were seeded at a volume of $2.3x10^5$ cells/well and allowed to grow for 24 hours at 37˚C in a humidified incubator with 5% CO_2. Upon 24 hours of incubation the plates were aspirated and each anthocyanin concentration (0, 1.25, 2.5, 5, 10 and 20 µg/mL) that was generated in the initial viral replication experiment was used to perform

the final titer. The 6 tubes generated in the initial replication experiment above were quickly thawed in a 37˚C water bath. Each tube representing a different concentration (0, 1.25, 2.5, 5, 10 and 20 µg/mL) was tittered by adding a volume of 200 uL of the appropriate dilution (as calculated from the pilot titer) to its' own 6-well plate and replicated three times with each plate containing a replicate, for a total of 18 6-well plates. Then the plates were agitated for one hour at room temperature using an orbital shaker (Thermo Fisher Scientific; Waltham, MA). Next 2 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and immunoglobulin G (IgG) was added to each well and the 18 6-well plates were incubated for 72 hours at 37˚C in a humidified incubator with 5% CO_2. 72 hours after inoculation the 6-well plates were aspirated of their media and a 1% crystal violet stain was added to each well at a volume that adequately covered the bottom and was allowed to stand for 20 minutes. After 20 minutes the 1% crystal violet stain was aspirated and the 6-well plates were allowed to air dry. Plaques (1 to 3 mm in diameter), which are clear areas of dead cells, were magnified ten-fold and counted with the aid of a Nikon profile projector. The concentration of virus in plaque-forming units (PFU) per milliliter (PFU/mL) was then calculated.

3.3.14 Statistical Analysis:

The results for the infectivity and replication of herpes simplex virus type-1 (strain 17+) after treatment with strawberry anthocyanin extract was subjected to Analysis of Variance (ANOVA) with a randomized complete block design (SAS 9.3 Software) and an alpha level of $p<0.05$ acceptance of the null hypothesis was used to determine significant variables.

3.4 RESULTS AND DISCUSSION

3.4.1 Characterization of Strawberry Anthocyanin Sample:

Strawberry anthocyanins were identified by comparing retention times, order of elution and UV-visible spectra to previous literature (Lopes-da-Silva and others 2001). The five major anthocyanins identified in strawberry (Figure 3.2 and Table 3.1) include: peak 1 cyanidin-3-glucoside (~23.3%), peak 2 cyanidin-3-rutinoside (~4.7%), peak 3 pelargonidin-3-glucoside (~60.6%), peak 4 pelargonidin-3-rutinoside (~7.1%), and peak 5 cyanidin-3-malonylglucose-5-glocoside (~4.3%). The strawberry anthocyanin profile contained only cyanidin and pelargonidin derivatives. Additionally, the majority of the strawberry anthocyanin profile is made up of cyanidin-3-glucoside (peak 1) and pelargonidin-3-glucoside (peak 3) which combined comprises over 83% of the anthocyanin profile. Below is a representation of the main anthocyanins in strawberry (Figure 3.3). The figure shows the varying glycosylation patterns found in strawberry anthocyanins.

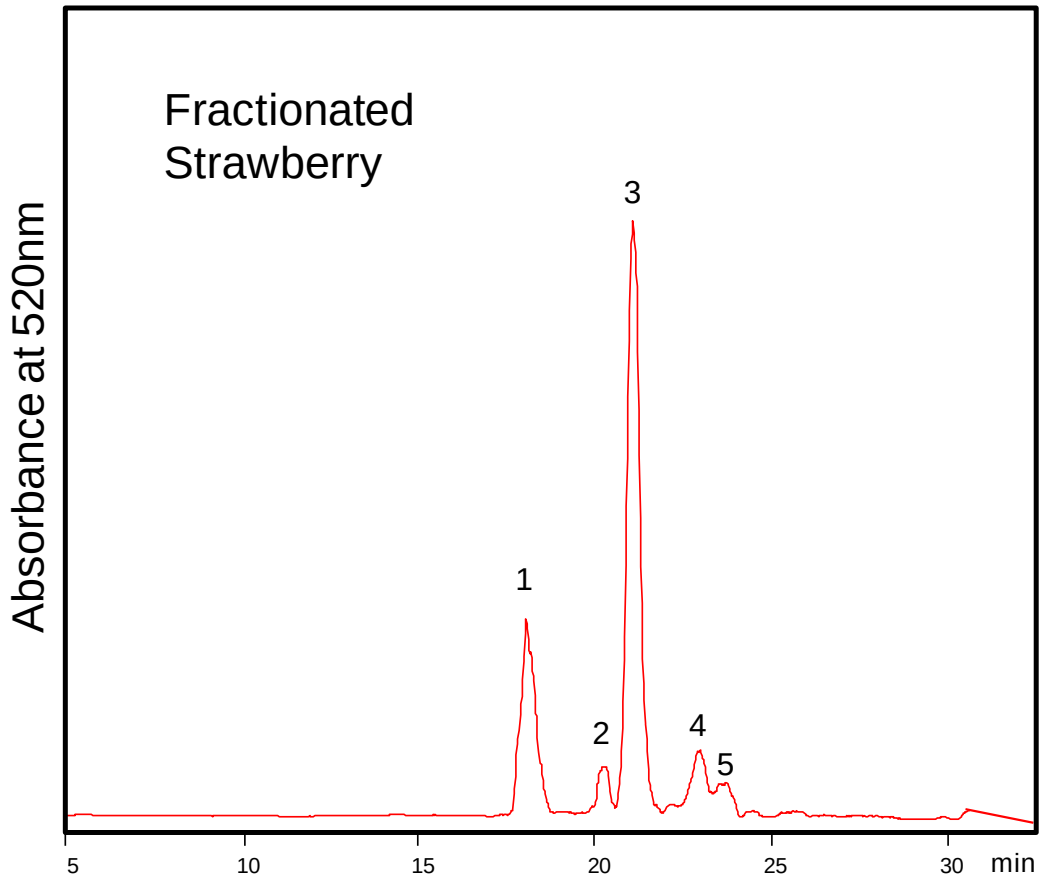


Figure 3.2: Chromatograph of fractionated strawberry extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-glucoside, peak 2 cyanidin-3-rutinoside, peak 3 pelargonidin-3-glucoside, peak 4 pelargonidin-3-rutinoside, and peak 5 cyanidin-3-malonylglucose-5-glocoside.

Fractionated Strawberry			
Peak	Anthocyanin	Retention Time (Min)	%Area@520nm
1	cyanidin-3-glucoside	17.7	23.3
2	cyanidin-3-rutinoside	19.8	4.7
3	pelargonidin-3-glucoside	20.6	60.6
4	pelargonidin-3-rutinoside	22.5	7.1
5	cyanidin-3-malonylglucose-5-glucoside	23.3	4.3

Table 3.1: Fractionated Strawberry Anthocyanins.

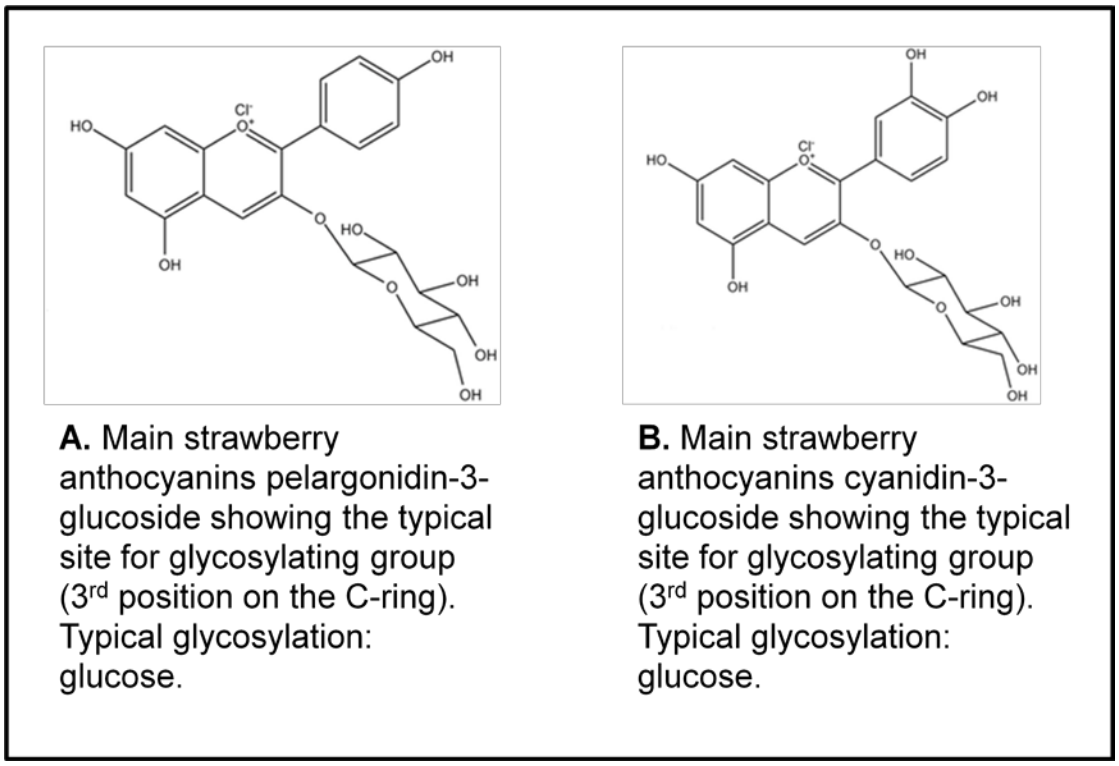


Figure 3.3: Representative chemical structures of main anthocyanins in strawberry and their glycosylating groups.

3.4.2 Herpes Simplex Virus Type-1 Infectivity:

The effects of infectivity on herpes simplex virus type-1(strain 17+) were found to be dose dependent. The plaque assay was used throughout this study to determine the effects of strawberry anthocyanin treatments on the virility of herpes simplex virus type-1 (strain 17+). In this assay, vero cell monolayers were infected with a low ratio of virus: $7.5x10^4$ PFU/mL for infectivity experiments such that sporadic cells became infected. This assay was ultimately used to determine the number of PFU/mL of herpes simplex virus type-1 (strain 17+) was present after strawberry anthocyanin treatment.

Herpes simplex virus type-1 (strain 17+) infected vero cells with the addition of strawberry anthocyanin rich extract at concentrations of 0, 1.25, 2.5, 5, 10 and 20 µg/mL were incubated at 37˚C in a humidified incubator with 5% CO_2 for 72 hours. Strawberry anthocyanin extract appeared to have antiviral properties and as indicated in Figure 3.4, the amount of herpes simplex virus type-1 (strain 17+) plaques remaining after 1 hour of incubation at 37˚C is dose dependently reduced as compared to the control cultures with PFU/mL of 38.85, 19.35, 3.65 and 0.3 at a treatment level of 0, 5, 10, and 20 µg/mL strawberry anthocyanin rich extract respectively. Additionally, Figure 3.5 illustrates the amount of infectious herpes simplex virus type-1 remaining after treatment with strawberry anthocyanin extract, which also shows a dose dependent reduction as compared to the control (no treatment) illustrated at percent growth with 100, 49.80, 9.39 and 0.77 percent at a treatment level of 0, 5, 10, and 20 µg/mL strawberry anthocyanin rich extract respectively. These results support the

findings of Meyer et al., 1997, Amoros et al., 1992, Debiaggi et al., 1990., Shahat et al., 2002 and Danaher et al., 2011 who's research have found that plant polyphenols, specifically from flavonoids, galangin, quercetin, procyanidin and pelargonidin (anthocyanidin) as well as procyanidin C-1 were all found to be virucidal against HSV-1. The fact that strawberry extract at all concentrations reduced virus yield when present throughout the inoculation and replication period indicates that strawberry anthocyanin extract may either inactivate the virus directly and/or block the virus's entry into the cell by providing a protective layer around the cells or by not allowing the viruses to locate or recognize cell membrane attachment sites.

To determine how the strawberry extract inactivates the herpes simplex virus type-1 (strain 17+) the extract was added to cell-free virus suspensions. Our results support the findings of Danaher et al., 2011 whose previous research analyzed blackberry extract in a short-term cell free incubation at room temperature to test herpes simplex virus type-1 inactivation, finding concentrations of ≥1500ug/mL blackberry extract to reduce >90% of infectious virus. In our experiment, strawberry anthocyanin rich extract at concentrations of 0,1.25, 2.5, 5, 10 and 20 µg/mL were added to cell-free virus suspensions and incubated at room temperature for 15 and 60 minutes to mimic the adsorption and entry phase. Strawberry anthocyanin extract-treated virus suspensions were diluted beyond the antiviral concentration (1:100) with culture medium, and incubated for 72 hours. Virus infectivity was quantified by direct plaque assay. Strawberry anthocyanin extract appeared to have antiviral properties. As

indicated in Figure 3.6 and Figure 3.7, the amount of herpes simplex virus type-1 (strain 17+) remaining after 15 and 60 minutes of room temperature incubation is 100 percent eliminated as compared to the control cultures. These findings suggest that strawberry anthocyanins inactivate the herpes simplex virus type-1 (strain 17+) by directly inhibiting the virus and by blocking. Because antiviral effects were most noticeable when free virus was directly exposed to the strawberry anthocyanin extract, and less effective once HSV-1 had adsorbed and entered epithelial cells these findings suggest that the strawberry anthocyanin extract has strong antiviral effects that interfere with adsorption or entry into the host cells and some intracellular activity.

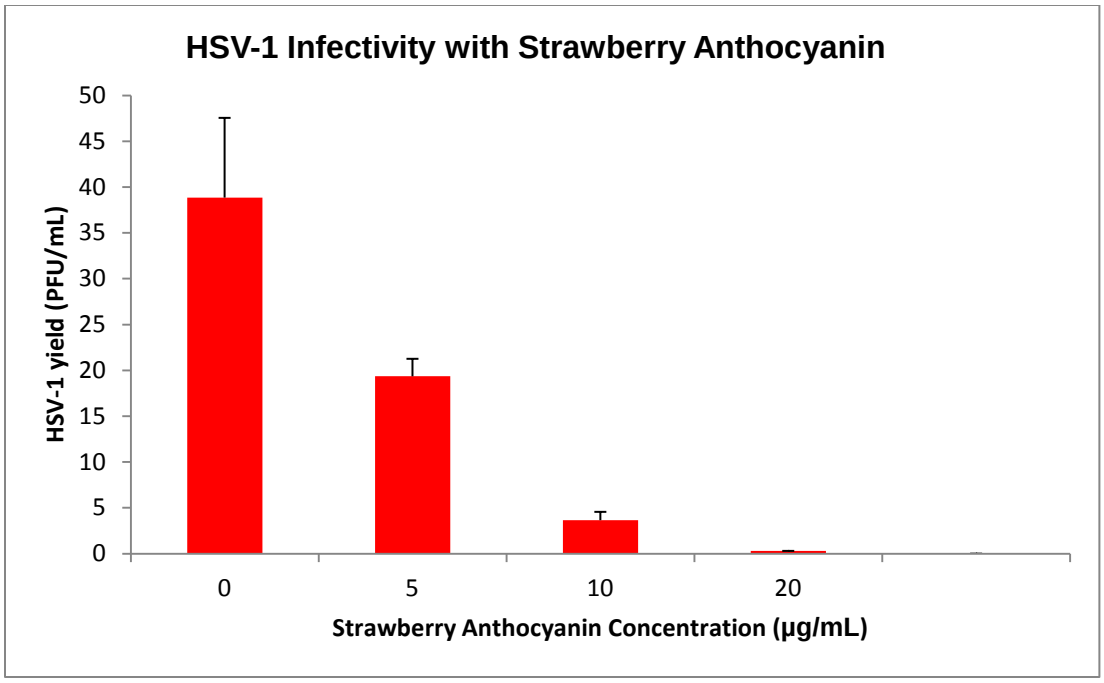


Figure 3.4: Strawberry anthocyanin extract inactivates HSV-1 in a dose dependant manor. Infectivity of the virus treated with strawberry anthocyanins was quantified by the direct plaque assay in triplicate. Results are the average of 2 independent experiments and are presented relative to untreated control samples.

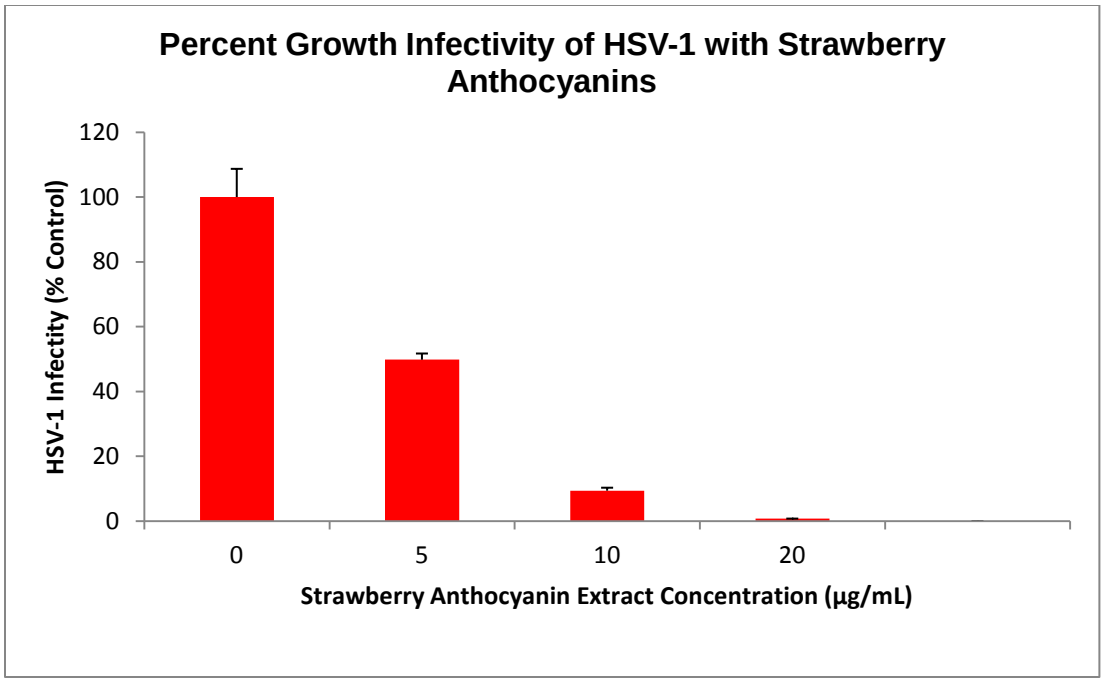


Figure 3.5: Strawberry anthocyanin extract inactivates HSV-1 in a dose dependent manor. Infectivity of the virus treated with strawberry anthocyanins was quantified by the direct plaque assay in triplicate. Results are the average of 2 independent experiments and are presented relative to untreated control samples (100%).

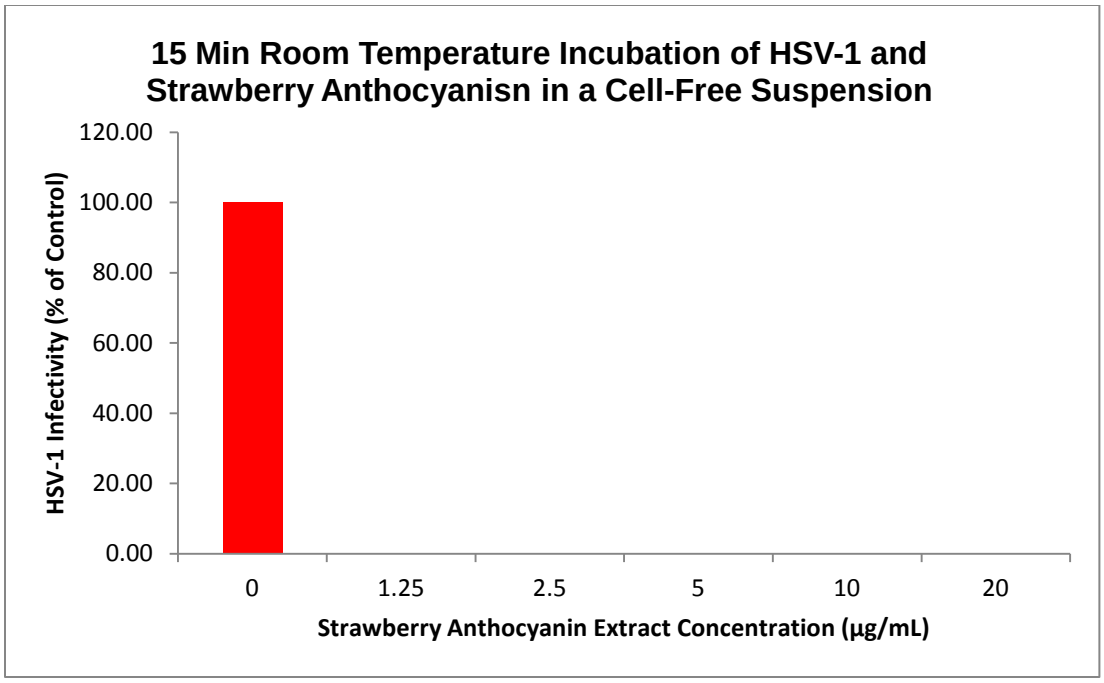


Figure 3.6: Strawberry extract inactivates HSV-1. Cell-free suspensions of HSV-1 (Strain 17+) were incubated for 15 minutes at room temperature with indicated concentrations of strawberry anthocyanin extract. Infectious virus was quantified by the direct plaque assay in triplicate. Results are the average of 2 independent experiments in duplicate and are presented relative to untreated control samples (100%).

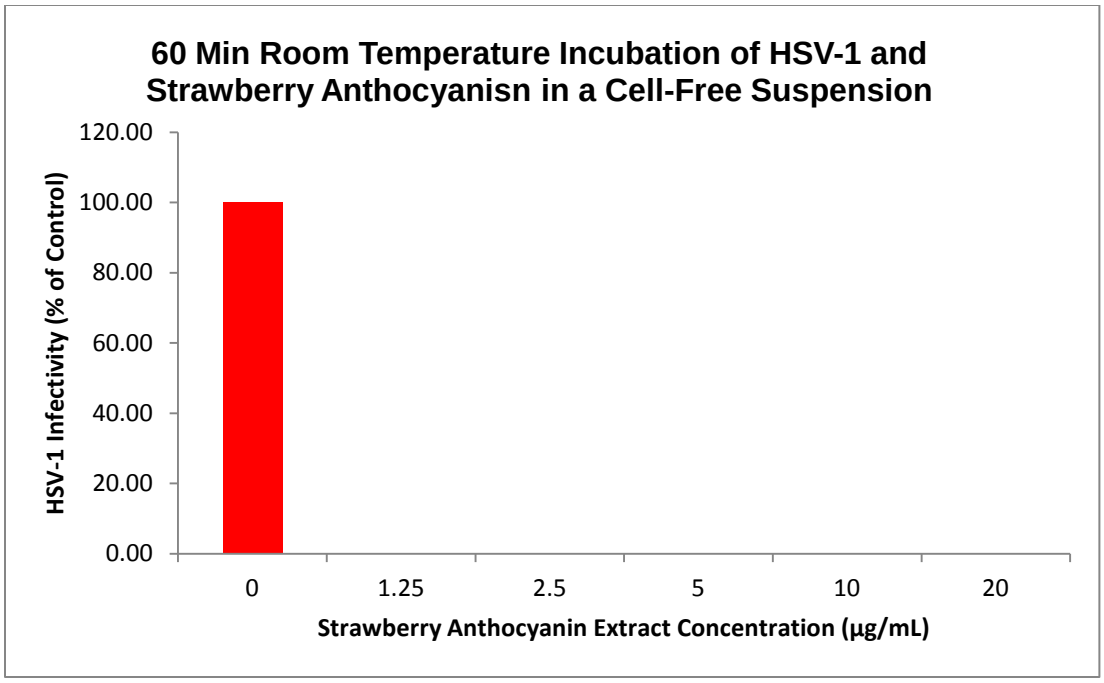


Figure 3.7: Strawberry extract inactivates HSV-1. Cell-free suspensions of HSV-1 (Strain 17+) were incubated for 60 minutes at room temperature with indicated concentrations of strawberry anthocyanin extract. Infectious virus was quantified by the direct plaque assay in triplicate. Results are the average of 2 independent experiments in duplicate and are presented relative to untreated control samples (100%).

3.4.3 Herpes Simplex Virus Type-1 Replication:

In the viral replication inhibitory assay a tittering technique was used for observation of *in vitro* viral replication. Anthocyanin concentrations used for this experiment were determined from the results found for the previous infectivity study. The effects of replication on herpes simplex virus type-1 (strain 17+) were found to be dose dependent. The plaque assay was used throughout this study to determine the effects of strawberry anthocyanin extract treatments on the replication of herpes simplex virus type-1. In this assay, vero cell monolayers were infected with a low ratio of virus: $1.875x10^4$ PFU/mL for replication experiments, such that sporadic cells become infected. This assay was ultimately used to determine the number of PFU/mL of herpes simplex type-1 (strain 17+) present after strawberry anthocyanin treatment.

In our study, strawberry anthocyanins have a significant ($p< 0.005$) antiviral effect on the herpes simplex virus type-1 (strain 17+) at concentrations ≥50ppm. Cultures were maintained in the presence of increasing concentrations of strawberry anthocyanin extract (0, 1.25, 2.5, 5, 10, and 20 µg/mL) throughout the entire replication cycle (i.e., adsorption, entry and production of progeny). As shown in figure 3.8, strawberry anthocyanin extract at 25 µg/mL and higher reduced HSV-1 yield. Strawberry anthocyanins at concentrations of 5, 10 and 20 µg/mL were reduced to 75.36, 57.98, and 31.46 percent of the control (100%) illustrated in figure 3.9. Notably, in all experiments, even at the highest concentrations, the extract was not toxic to the vero cells. The results of our study again support the findings of Danaher et al., 2011, who tested blackberry

extracts on HSV-1 virus yields in vero cells and found dramatically reduced virus yield when present throughout the entire replication cycle, but significant reductions in virus yield when provided after the 1 hour adsorption and entry stage required higher amount of blackberry extract at concentrations of > 280 µg/mL. It is important to note that the research from this project used a highly purified and concentrated strawberry anthocyanin extract, which contributes to the notable difference in treatment concentration (µg/mL) used in this experiment as compared to the work of Danaher et al., 2011.

These results suggest that strawberry anthocyanins provide viral protection both by directly blocking the vero cell from the herpes simplex virus-1 and by potentially binding the virus and enabling it from entering the cell and replicating.

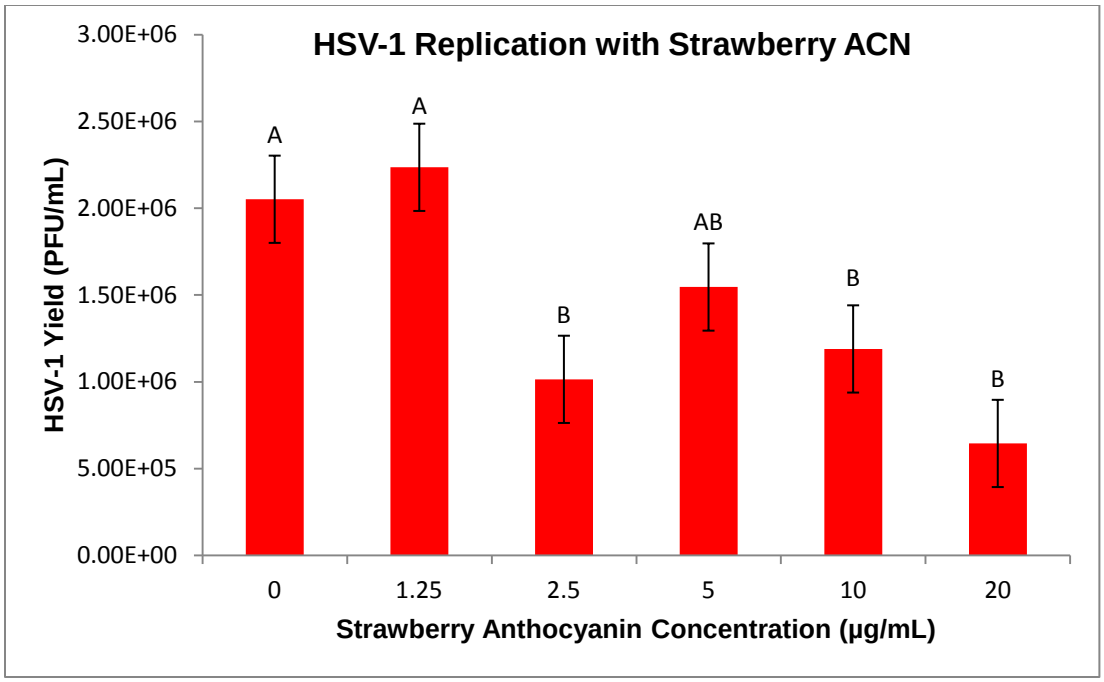


Figure 3.8: Strawberry anthocyanin extract has antiviral properties. Vero cells were inoculated with HSV-1 (strain 17+) (multiplicity of infection 0.05) in the presence of the indicated concentrations of strawberry anthocyanin extract. After cell adsorption at 37˚C and 2 rinses with phosphate-buffered saline solution, fresh cell culture medium containing the indicated concentration of strawberry anthocyanin extract was added and incubation was continued overnight at 37˚C. Twenty-four hours after inoculation, cells were freeze-thawed and virus yield quantified on vero cell monolayers by the direct plaque assay ($P<0.05$).

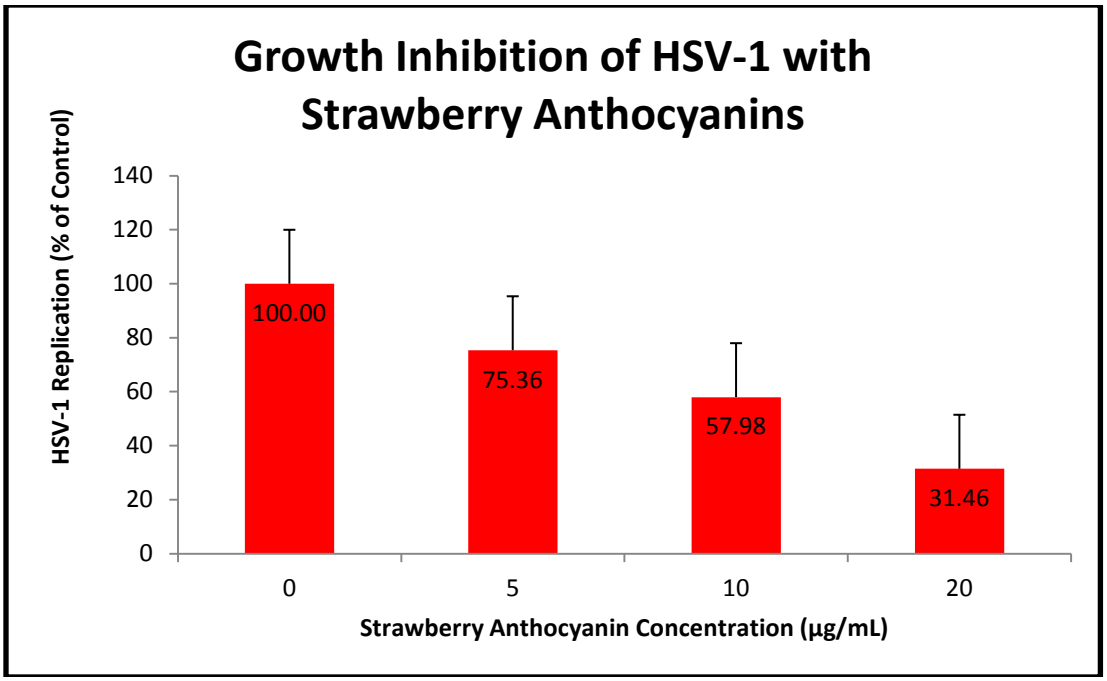


Figure 3.9: Percent growth inhibition as compared to the control of HSV-1 infected vero cells with the 72 hour treatment of fractionated strawberry anthocyanin extract shows significant antiviral properties ($p<0.05$).

3.5 CONCLUSION

Strawberries are a natural and abundant fruit that grow in the wild and as cultivars. Strawberries are a rich source of polyphenols, which have anti-inflammatory, antiviral, and antiproliferative properties (Aaby, Skrede, and Wrolstad, 2005). In the present study we further extended the information known about fruit extracts, which in the past have tested an array of phenolic compounds, not specifically anthocyanins. The results of this research add to the information known about strawberry anthocyanins, specifically pelargonidin-3-glucoside and cyanidin-3-glucoside, by investigating their antiviral properties and mechanism of action. Strawberry anthocyanins were found to have powerful viral replication inhibitory and rapid virucidal activities by inactivating the virus directly and blocking the virus's entry into the cell. These findings indicate that anthocyanin extract may either inactivate the virus directly or by blocking the virus's entry into the cell by providing a protective layer around the cells or by not allowing the viruses to locate or recognize cell membrane attachment sites. The strawberry anthocyanin treatments containing 20 µg/mL significantly inhibited herpes simplex virus (HSV-1) by greater than 68% as compared to the control ($p<0.005$). These findings suggest that strawberry anthocyanin extracts could be an effective topical treatment and/or prophylactic agent for oral herpetic infections. Additionally, because antiviral effects were most noticeable when free virus was directly exposed to the strawberry anthocyanin extract, and less effective once HSV-1 had adsorbed and entered epithelial cells these findings suggest that the strawberry anthocyanin extract has strong antiviral effects that

interfere with adsorption or entry into the host cells and some intracellular activity. The results of this experiment are important for future studies that could isolate and identify whether one anthocyanin from strawberry anthocyanin extracts works more effectively as a bioactive anti-HSV-1 agent and also to determine if the bioactive principles in strawberry anthocyanin extract exhibit antiviral effects against additional HSV strains and serotypes (i.e. HSV-2).

3.6 SPECIAL ACKNOWLEDGEMENTS

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CHAPTER 4

THE EFFECT OF STRAWBERRY ANTHOCYANINS ON THE INHIBITION OF AGS STOMACH CANCER CELLS AND 738.St/Int STOMACH AND INTESTINE CELL PROLIFERATION

4.1 SUMMARY

Anthocyanins are widely distributed in nature and are the red, purple and blue pigments responsible for the beautiful color of many fruits, vegetables and leaves. Previous research suggests a high correlation between diets rich in brightly colored fruits and vegetables and a decreased risk of cardiovascular disease, obesity and the formation of certain cancers, such as gastrointestinal cancers. Due to many fruits and vegetables containing numerous amounts of anthocyanin pigments present in their anthocyanin profile, identifying specific compounds that are responsible for these health promoting properties can become very difficult. In this study, strawberry anthocyanin extracts were chosen because of their very simple anthocyanin profile. In this unique anthocyanin profile, pelargonidin-3-glucoside and cyanidin-3-glucoside together comprise over 83% of the total anthocyanin content. The remaining 17% is comprised of other pelargonidin and cyanidin glycosylates. Pelargonidin-3-glucoside is the most abundant anthocyanin in the strawberry anthocyanin profile and is responsible for the attractive, bright red color of fresh strawberries. The strawberry anthocyanins were extracted and fractionated using acetone, chloroform, methanol, ethyl acetate, hexane and water. The extracts were used in an *in vitro* experiment to treat human gastric adenocarcinoma cells (AGS stomach cancer cells) and human non-diseased gastric cells (Hs 738.St/Int

mixture of stomach and intestine cells). Proliferation rates were measured using the sulfurhodamine B assay. Chemopreventative properties of the strawberry anthocyanin extracts were measured on the AGS and Hs 738.St/Int cell lines. The results of the experiment showed that strawberry anthocyanins are chemoprotective at a concentration of 25-200 µg/mL against human stomach cancer cells line, having antiproliferative effects of ≥90% at treatment levels 100 and 200 µg/mL on the AGS cells lines ($p<0.0001$). The results of this research also showed that strawberry anthocyanins increased the proliferation rates at concentrations of 25, 50, 100, and 200 µg/mL for human gastric cells Hs 738.St/Int ($p<0.0001$). The finding of this study were performed *in vitro* and suggest that the consumption of strawberry anthocyanins may impact overall gastrointestinal health.

4.2 INTRODUCTION

Flavonoids have been shown to impart protection from oxidizing species, such as the superoxide anion, hydroxyl radical and peroxy radicals; they have also been proven to be quenchers of singlet oxygen (Harborne et al 2000). The antioxidant activity of flavonoids is thought to present many of the health benefits associated with consumption of fruits and vegetables to humans. Anthocyanins are the most abundant dietary flavonoids (Jing et al., 2008). Due to the increased interest in the health benefits of anthocyanins, there have been an increasing number of biological studies in hopes to better understand these powerful antioxidants. Anthocyanin consumption has been estimated to be as high as ~200 mg/day/person (Kuhnan 1976), although a more recent study (Wu et al., 2006) reported anthocyanin consumption at about 12.5 mg/day/person in the United States, as compared to the average daily intake of other flavonoids (23 mg/person) (Hertog et al., 1993). Anthocyanins represent a subclass of flavonoids and are of great importance to the food industry because of their coloring properties and accordingly, their impact on the visual appeal of a product. Because anthocyanins are responsible for the red to violet hues in many fruits and vegetables, their use in the food industry has rapidly expanded as a value added ingredient and alternatives to synthetic red dyes. The basic anthocyanin structure consists of the carbon skeleton with conjugated bonds on the C-ring and two positions, R-1 and R-2, which are substituted with hydroxyl, oxymethyl and hydryl groups. Different combinations of these side groups results in the six anthocyanidin chromophores most commonly found in nature.

These anthocyanidin aglycones include cyanidin (Cy), pelargonidin (Pg), delphinidin (Dp), petunidin (Pt), malvidin (Mv) and peonidin (Pn) (Fleschhut et al., 2006; Hou et al., 2005; McGhie et al., 2007). Anthocyanin sources by nature contain multiple polyphenolic compounds; because of this the characterization and quantification of anthocyanins can be laborious and expensive. By selecting an anthocyanin source with fewer anthocyanins naturally present, semi-purification and isolation of a single anthocyanin can be more easily achieved. Strawberries have two major anthocyanins present in the fruit tissue; pelargonidin-3-glucoside and cyanidin-3-glucoside (Figure 4.1), which together account for over 83% of their anthocyanin pigments, making strawberries uniquely simple anthocyanin source. Because of this strawberries were selected for this research study.

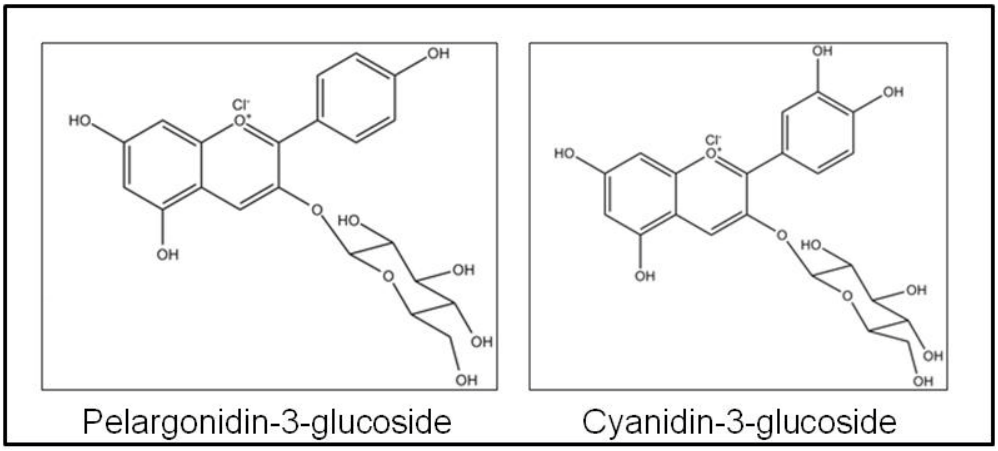


Figure 4.1: The two main strawberry anthocyanins, pelargonidin-3-glucoside and cyanidin-3-glucoside.

More than 20% of death in the United States is caused by cancer (Smith, Marks and Lieberman, 2005). Stomach cancer also known as gastric cancer is a disease in which malignant (cancer) cells form in the lining of the stomach (Eyre

2004). Stomach cancers tend to develop slowly over many years. The stomach is part of the gastrointestinal system, which processes nutrients (vitamins, minerals, carbohydrates, fats, proteins, and water) in foods that are eaten and helps pass waste materials out of the body. The wall of the stomach is made up of three layers of tissue: the mucosal (innermost) layer, the muscularis (middle) layer, and the serosal (outermost) layer. Before true cancer develops, pre-cancerous changes often occur in the lining of the stomach. In many cases, these early changes rarely cause symptoms and therefore often go undetected (Eyre 2004). Stomach cancer begins in the cells lining the mucosal layer and spreads through the outer layers as it continues to grow and spread. Stomach cancer typically affects the older population. Almost two thirds of people with stomach cancer are 65 years of age or older and the average risk of developing stomach cancer is about 1 in 116 (Eyre 2004). Stomach cancer was the leading cause of cancer related death in the United States until the late 1930s. This is believed to be due to refrigeration and the opportunity for longer food storage. The invention of the refrigerator made it possible for more people to have access to fruits and vegetables year round and in turn decreased the amount of salted and smoked foods consumed. Additionally, stomach related cancers are thought to be less prevalent in first world countries because of the use of antibiotics that kill bacteria that cause infections in the stomach, such as, *Helicobacter pylori* (*H. pylori*). Because of limited resources, stomach cancer is much more prevalent in other parts of the world, particularly in underdeveloped nations. According the

American Cancer Society, stomach cancer is the leading cause of cancer-related deaths in the world today (Eyre 2004).

The objective of this research was to determine the chemoprotective effects of strawberry anthocyanins against the human stomach cancer cell line AGS and to determine the proliferative effects of strawberry anthocyanins on the human stomach and small intestine mixed cell line Hs 738.St/Int. To accomplish this, AGS and Hs 738.St/Int cell lines were grown up and treated with fractionated strawberry anthocyanin extract (12.5-200 µg/mL), and the proliferation growth rates were determined using the sulforhodamine-B colorimetric assay.

4.3 MATERIALS AND METHODOLOGY

4.3.1 Chemicals and Reagents:

Organic Driscoll's brand strawberries were purchased from a local grocery store in Lexington, Kentucky. Certified ACS grade HCL (12N) and ethyl acetate, LCMS grade 99.9% methanol and 99.9% acetonitrile, and HPLC grade 88% formic acid, 99.9% acetone and 99.9% chloroform were obtained from Thermo Fisher Scientific (Waltham, MA). Reagent grade gallic acid and Folin-Ciocaltaeu reagent were obtained from MP Biomedical (Aurora, OH). AGS (ATCC® CRL1739™) and Hs 738.St/Int (ATCC® CRL7869™) were obtained from American Type Culture Collection (Rockville, MD). Sterile Dulbecco's Modified Eagle's Medium and F-12K Medium (Thermo Fisher Scientific, Waltham, MA), fetal bovine serum (FBS) (Invitrogen Corp. Carlsbad, CA) Delbucco's Phosphate Buffered Saline Solution (DPBS) (Lonza, Walkersville, MD), and 2.5% Trypsin (Lonza, Walkerville, MD) were obtained from their manufacturers. All other chemicals were certified ACS grade from Thermo Fisher Scientific, Waltham, MA.

4.3.2 Extraction of Anthocyanins and other Phenolics from Plant Materials:

The red flesh of strawberries were hand selected using a paring knife and quickly frozen using liquid nitrogen. The frozen strawberry red flesh where then fine powdered using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT) with a 500 mL stainless steel container in the

presence of liquid nitrogen (Giusti and Rodriguez-Soana, 1999). Strawberry flesh powder was stored at -20˚C until used for sample extraction.

Strawberry flesh anthocyanins and other phenolics were extracted by blending the powders with 30mL of 70% acetone and 30% 0.1% HCl acidified water (v/v) for 5 minutes using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT). The liquid was subsequently passed through Whatman No. 4 and No. 1 filter paper (Whatman Inc., Florham, NJ) using a Buchner Funnel (Thermo Fisher Scientific, Waltham, MA). This process was repeated until the filtrate was free of haze and all of the pigment had been extracted from the powdered strawberry flesh particles (Rodriguez-Soana and Wrolstad, 2001). The strawberry anthocyanin and other phenolics filtrate was then placed into a separatory funnel (Thermo Fisher Scientific, Waltham, MA) and partitioned with 2 volumes of chloroform. The solution was mixed and left to separate for 24 hours at 4˚C in the dark to ensure adequate separation. The top acetone/water layer containing anthocyanins and other phenolics was collected and the bottom chloroform/fat layer was discarded properly in hazardous waste containers. The residual acetone in the sample was evaporated using a Buchi Rotavapor R-215 rotary evaporator (Thermo Fisher Scientific, Waltham, MA) at 40˚C. The solution was brought to 25 mL in a volumetric flask using LCMS grade water.

4.3.3 Fractionation and Concentration of Anthocyanin Sample:

Anthocyanin phenolics from strawberries were concentrated by using Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corporation, Milford, MA). The anthocyanin filtrate was passed through the Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corporation, Milford, MA) 10 mL at a time. Anthocyanins and other phenolics were bound to the C_{18} cartridge, while sugars and other polar compounds were removed with 30 mL of 0.01% HCl acidified water, followed by 10 mL of hexane to remove the residual water. The non-anthocyanin phenolics were eluted with 20 mL of diethyl ether followed by 20 mL of ethyl acetate. The anthocyanin phenolic fraction was eluted with 30 mL of 0.01% HCl acidified methanol followed by 10 mL of 0.01% HCl acidified HPLC grade water. Excess methanol in the anthocyanin phenolic filtrate was evaporated in the Buchi Rotavapor R-215 rotovapor (Thermo Fisher Scientific, Waltham, MA) at 40˚C and the anthocyanin fraction was re-dissolved in 0.01% HCl acidified HPLC grade water. The highly concentrated anthocyanin extract was then quickly frozen using liquid nitrogen and lyophilized using a Labconco Freezone[6] lyophilizer into a fine dry powder (Labconco, Kansas City, MO).

4.3.4 Monomeric and Polymeric Analysis:

Monomeric anthocyanin content was determined using the pH differential method (Giusti and Wrolstad, 2005). This method uses the spectral difference that anthocyanins exhibit at different pH values. Dilution factor was determined by diluting the strawberry anthocyanin extract in 0.025 M potassium chloride

buffer, pH 1.0, until the absorbance was within the appropriate spectrophotometer range (for most spectrophotometers absorbance should be less than 1.2 nm). The strawberry anthocyanin extract was then appropriately diluted using pH 1.0 and pH 4.5 buffer. Solutions were allowed to sit at room temperature for at least 15 minutes in the dark. 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 cyanidin-3-glucoside equivalents, using a molecular weight of 449.3 and an extinction coefficient of 26,900 L cm^{-1} mg^{-1} (Giusti and Wrolstad, 2005). Absorbance was determined in replicates of three.

Polymeric anthocyanin content was determined using the polymeric method (Wrolstad 1999). Appropriate dilutions were determined using pH 1.0 buffer and 1-cm cuvettes were prepared with 2.8 mL of diluted sample in water to each of two cuvettes. 0.2 mL of water was added to one of the cuvettes and 0.2 mL of bisulfate solution to the other and allowed to equilibrate for 15 minutes before reading at 420 nm, 520 nm, and 700 nm. Polymeric anthocyanin content was calculated using the following equations:

Color Density = $[(A_{420nm} - A_{700nm}) + (A_{\lambda\ vis\ max} - A_{700nm})] \times DF$

Polymeric Color = $[(A_{420nm} - A_{700nm}) + (A_{\lambda\ vis\ max} - A_{700nm})] \times DF$

Percent Polymeric Color = (Polymeric Color/Color Density) x 100

Where DF is the dilution factor and absorbance was determined in replicates of three.

4.3.5 Total Phenolics:

Total phenolics were measured using a microscale protocol for Folin-Ciocalteu Colorimetry (Waterhouse 2005). Cuvettes were prepared with 20 µL of sample/standard, 1.58 mL water and 100 µL 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 µL) was added and samples were allowed to stand at room temperature for 2 hours. 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.

4.3.6 LC/MS Analysis:

Strawberry anthocyanin samples were analyzed using a Thermo Finnigan LTQ high performance liquid chromatograph (HPLC) (ThermoFinnigan; Waltham, MA). Positive ion electrospray ionization (ESI) mass spectra were obtained on the Thermo Finnigan LTQ coupled to a Dionex Ultimate 3000 capillary LC system. The column was 20cm x 150um packed with Kromasil C18 (3.5um particle size) (AkzoNobel, Separation Products, Bohus, Sweden). Proteome Discoverer Software 1.1.0 was used on this system (Thermo Fisher Scientific; Waltham, MA). All samples were filtered through 13 mm 0.45µm polypropylene filters (Thermo Fisher Scientific, Waltham, MA) prior to HPLC/MS analysis.

Separation of strawberry extract anthocyanins was achieved using a linear gradient elution from 5% to 60%. Solvent A was 0.1% (v/v) formic acid in water and B was 0.1% (v/v) formic acid in acetonitrile. The flow rate was 0.8 mL / min and an injection volume of 50 µL was used. Spectral data was collected using the Dionex Ultimate 3000 capillary LC system from 250-700 nm. Absorbance of anthocyanins was monitored at 280 and 520 nm.

A 1.5 uL / 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 ºC; 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 and Selective Ion Monitoring (SIM) was used to search for the molecular ions of the common anthocyanins throughout the analysis.

4.3.7 AGS Cell Line:

AGS human cell line originates from the stomach of a *homo sapien*. The cell line is considered to have an epithelial morphology and its culture growth properties are adherent. The AGS human stomach cell lines are gastric adenocarcinoma and therefore cancerous. The AGS cell lines were grown at 37˚C in a Nuuaire™ IR Autoflow CO_2 Water-Jacketed Incubator at a modified atmosphere (5% CO_2, 95% O_2) in ATCC-formulated F-12K Medium. To make the

complete growth medium fetal bovine serum to a final concentration of 10 percent must be added. Cultures were split 1:5 when monolayers were 70-80% confluent using Delbucco's Phosphate Buffered Saline solution (DPBS) and 2.5% trypsin (Lonza, Walkersville, MD). Cultures were fed fresh media every 3 days. Growth of AGS cell line was determined by microscopic examination. Cell cultures were maintained in 75 cm^2 Flasks with canted necks (Thermo Fisher Scientific, Waltham, MA). All cell culture experiments were performed with the initial 20 passages.

4.3.8 Hs 738.St/Int Cell Line:

Hs 738.St/Int cell line originates from a mix of the stomach and intestine of a male *homo sapien*. The cell line is considered to have a fibroblast morphology and its culture growth properties are adherent. The 738.St/Int human stomach and intestine cell line is considered non-diseased. The Hs 738.St/Int cell line was grown at 37˚C in a Nuuaire™ IR Autoflow CO_2 Water-Jacketed Incubator at a modified atmosphere (5% CO_2, 95% O_2) in ATCC-formulated Dulbecco's Modified Eagle's Medium. To make the complete growth medium fetal bovine serum to a final concentration of 10 percent must be added. Cultures were split 1:5 when monolayers were 70-80% confluent using Delbucco's Phosphate Buffered Saline solution (DPBS) and 2.5% trypsin (Lonza, Walkersville, MD). Cultures were fed fresh media every 3 days. Growth of Hs 738.St/Int cell line was determined by microscopic examination. Cell cultures were maintained in

75 cm^2 Flasks with canted necks (Thermo Fisher Scientific, Waltham, MA). All cell culture experiments were performed with the initial 20 passages.

4.3.9 Determining AGS and Hs 738.St/Int Cell Concentration using a Hemocytometer:

Cells were plated in 96-well tissue culture dishes at a volume of 10,000 cells/well. Suspended cells were counted using a Nikon Eclipse 50i Brightfield Microscope and a Reichert Bright-Line hemocytometer (Hausser Scientific; Horsham, PA) to estimate total viable cell concentration. 10 µL of cells were added to 90 µL of Trypan Blue Stain (Thermo Fisher Scientific, Waltham, MA) and subsequently loaded on the hemocytometer. Using the 10x lens of the microscope, cells were counted on 5 primary squares of the hemocytometer using a hand counter. To calculate the AGS and Hs 738.St/Int cell concentration the following formula was used:

Cells/mL = (Total # cells counted)/(Number of primary squares counted) x $1x10^4$

AGS and Hs 738.St/Int cells were seeded at 10,000 cells/well and allowed to grow and form monolayers for 24 hours. At 24 hours the AGS and Hs 738.St/Int cells were treated with strawberry anthocyanin extract and tested for chemopreventative effects.

4.3.10 Anthocyanin Treatments:

Monomeric anthocyanin content was determined for the strawberry anthocyanin extract using the detailed method explained by Giusti and Wrolstad, 2001. According to the monomeric anthocyanin content of strawberries, 25, 50, 100 and 200 µg/mL of sample was determined and a corresponding treatment value was given in grams of sample weight.

4.3.11 Sulforhodamine-B Colorimetric Assay:

The Sulforhodamine-B colorimetric assay is used for cell proliferation and chemosensitivity testing. For this study, AGS stomach cancer cell and Hs 738.St/Int stomach and small intestine cell media was removed and cells were fixed by addition of 150 µL of 50% trichloroacetic acid (TCA) at 4°C for 1 hour. TCA was removed and wells were washed with tap water 5 times and allowed to air dry at room temperature. 75 µL of Sulforhodamine-B (0.4% in water containing 0.01% acetic acid) was added to each well and allowed to stain cells at room temperature for 30 minutes. Each well was washed with 1% acetic acid 5 times to remove any residual unbound dye and dried at room temperature. The protein bound dye was solubilized with 150 µL of 10 mM Tris base for 5 minutes at room temperature on a plate shaker. The absorbance of each plate was then read at a wavelength of 490 nm using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Intruments, Inc., Winooski, VT). Gen5 1.10 Bio-Tek® software was used to analyze the data.

4.3.12 AGS Cell line proliferation with Strawberry Anthocyanins:

The sulforhodamine-B colorimetric assay was used for the *in vitro* observation of chemoprotection against AGS cell lines. First, AGS human stomach cancer cells were plated in 96-well tissue culture plates (Corning Inc., Tewksbury, MA) at a level of 10,000 cells/well in ATCC-formulated F-12K Medium supplemented with 10% FBS and a volume of 150 uL/well and incubated at 37˚C in a humidified incubator with 5% CO_2. The AGS cells were allowed to grow and form monolayers for 24 hours. Following the 24 hour incubation confluent cell monolayers in the 96-well plates were treated in serial dilution with strawberry anthocyanin extract at concentrations of 12.5, 25, 50, 100 and 200 µg/mL. Immediately following the treatment of the AGS cells the plates were incubated at 37˚C in a humidified incubator with 5% CO_2 for 48 hours. 48 hours after strawberry anthocyanin treatment was added the 96-well plates were aspirated of their media and the sulforhodamine-B assay was performed and the absorbance of each plate was then read at a wavelength of 490 nm using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Intruments, Inc., Winooski, VT). Gen5 1.10 Bio-Tek® software was used to analyze the data.

4.3.13 Proliferative Effects of Strawberry Anthocyanin on Hs 738.St/Int Cell Lines:

The sulforhodamine-B colorimetric assay was used for the *in vitro* observation of the proliferative effects of strawberry anthocyanin extracts on the 738.St/Int human stomach and intestine cell line. First, Hs 738.St/Int human stomach and intestine cells were plated in 96-well tissue culture plates (Corning

Inc., Tewksbury, MA) at a level of 10,000 cells/well in ATCC-formulated Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS and a volume of 150 uL/well and incubated at 37˚C in a humidified incubator with 5% CO_2. The Hs 738.St/Int cells were allowed to grow and form monolayers for 24 hours. Following the 24 hour incubation confluent cell monolayers in the 96-well plates were treated in serial dilution with strawberry anthocyanin extract at concentrations of 12.5, 25, 50, 100 and 200 µg/mL. Immediately following the treatment of the Hs 738.St/Int cells the plates were incubated at 37˚C in a humidified incubator with 5% CO_2 for 48 hours. 48 hours after strawberry anthocyanin treatment was added the 96-well plates were aspirated of their media and the sulforhodamine-B assay was performed and the absorbance of each plate was then read at a wavelength of 490 nm using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Intruments, Inc., Winooski, VT). Gen5 1.10 Bio-Tek® software was used to analyze the data.

4.3.14 AGS and Hs 738.St/Int Cell Growth Inhibition Calculation:

AGS and Hs 738.St/Int cells were plated at 10,000 cells/well in 96-well cell culture plates with their appropriate media. Cells were allowed to grow for 24 hours to attain log phase growth at the time of sample addition (time 0). Strawberry anthocyanin samples were compared for their proliferative and/or chemoprotective effects at concentrations of 12.5, 25, 50, 100 and 200 µg/mL. AGS and Hs 738.St/Int cell growth inhibition was determined after 48 hours of incubation at the different anthocyanin concentrations by using the

sulfurhodamine-B assay (SRB) at 490 nm. Each treatment had 6 replicates and every treatment was repeated at least 4 times. The percentage growth inhibition is calculated as:

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

Where T_0: Time zero: The absorbance of sample at 490 nm after first 24-hour incubation period prior to anthocyanin treatment. **T_{trt}:** Absorbance of sample at 490 nm after anthocyanin treatment. **T_{ctr}:** Absorbance of sample at 490 nm after total incubation (72 hours) without anthocyanin treatment.

4.3.15 Caspase-3 Colorimetric Assay:

The caspase-3 colorimetric assay provides for a quick and efficient detection of caspase 3 activity in cell lysates and in purified preparations of caspase 3. The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405nm. The concentration of the pNA released from the substrate is calculated from the absorbance values at 405nm or from a calibration curve prepared with defined pNA solutions. For this study the assay was performed in 100 µL volume on the AGS cell line in 96-well plates using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Intruments, Inc., Winooski, VT). Gen5 1.10 Bio-Tek® software was used to analyze the data.

4.3.16 Statistical Analysis:

The results for the chemoprotective and proliferative analysis of AGS and Hs 738.St/Int cell lines following treatment with strawberry anthocyanin extract was subjected to Analysis of Variance (ANOVA) with an extended randomized complete block design (SAS 9.3 Software) and an alpha level of $p<0.05$ acceptance of the null hypothesis was used to determine significant variables.

4.4 RESULTS AND DISCUSSION

4.4.1 Characterization of Strawberry Anthocyanin Sample:

Strawberry anthocyanins were identified by comparing retention times, order of elution and UV-visible spectra to previous literature (Lopes-da-Silva and others 2001). The five major anthocyanins identified in strawberry (Figure 4.2 and Table 4.1) include: peak 1 cyanidin-3-glucoside (~23.3%), peak 2 cyanidin-3-rutinoside (~4.7%), peak 3 pelargonidin-3-glucoside (~60.6%), peak 4 pelargonidin-3-rutinoside (~7.1%), and peak 5 cyanidin-3-malonylglucose-5-glocoside (~4.3%). The strawberry anthocyanin profile contained only cyanidin and pelargonidin derivatives. Additionally, the majority of the strawberry anthocyanin profile is made up of cyanidin-3-glucoside (peak 1) and pelargonidin-3-glucoside (peak 3) which combined comprises over 83% of the anthocyanin profile. Below is a representation of the main anthocyanins in strawberry (Figure 4.3). The figure shows the varying glycosylation patterns found in strawberry anthocyanins.

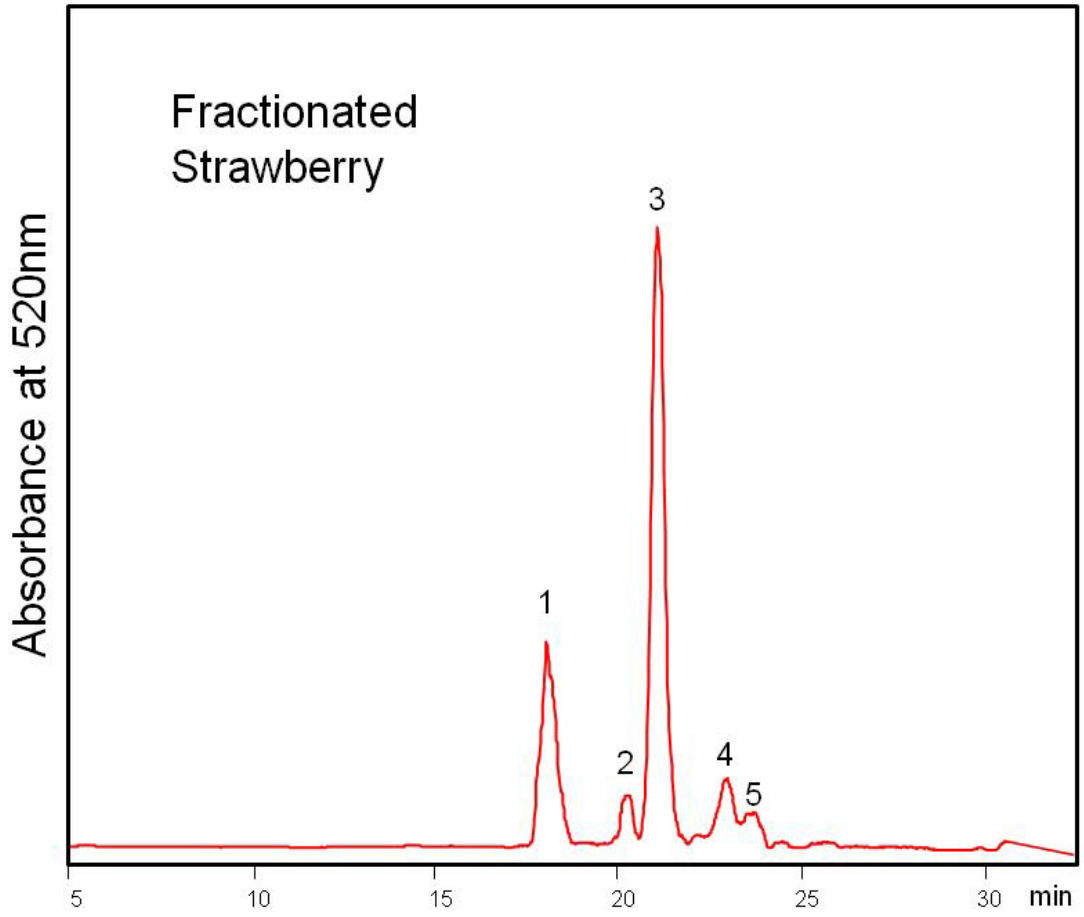


Figure 4.2: Chromatograph of fractionated strawberry extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-glucoside, peak 2 cyanidin-3-rutinoside, peak 3 pelargonidin-3-glucoside, peak 4 pelargonidin-3-rutinoside, and peak 5 cyanidin-3-malonylglucose-5-glocoside.

Fractionated Strawberry			
Peak	Anthocyanin	Retention Time (Min)	%Area@520nm
1	cyanidin-3-glucoside	17.7	23.3
2	cyanidin-3-rutinoside	19.8	4.7
3	pelargonidin-3-glucoside	20.6	60.6
4	pelargonidin-3-rutinoside	22.5	7.1
5	cyanidin-3-malonylglucose-5-glucoside	23.3	4.3

Table 4.1: Fractionated Strawberry Anthocyanins.

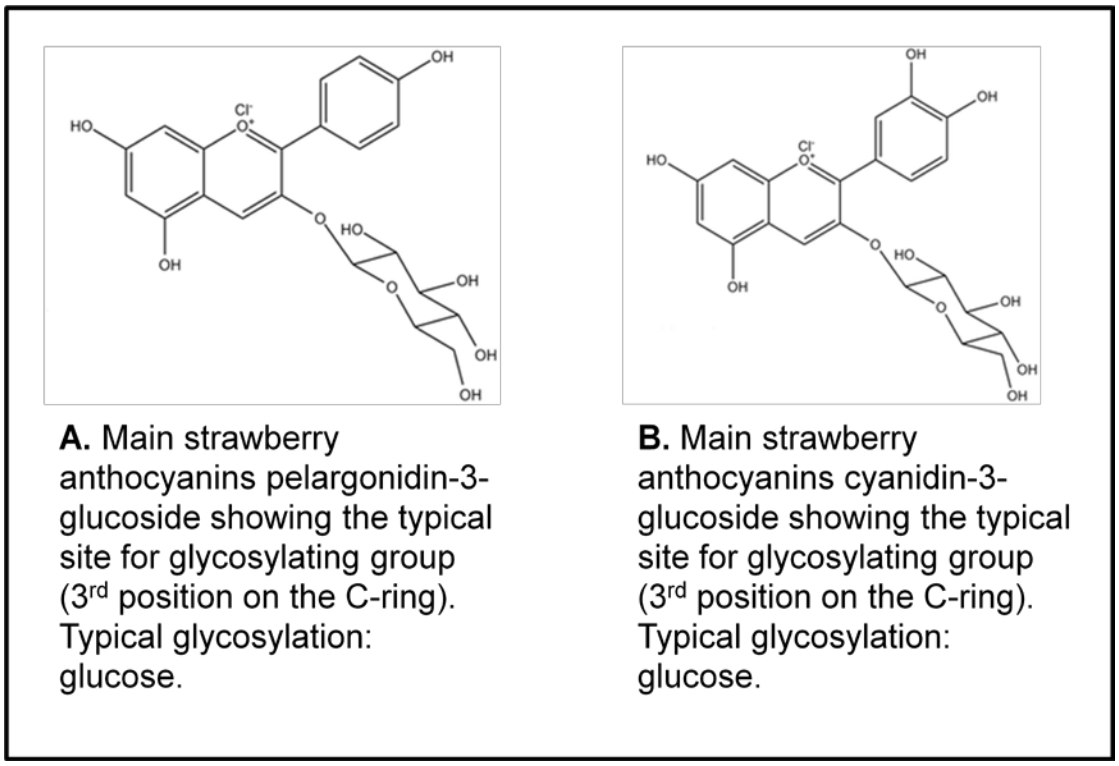


Figure 4.3: Representative chemical structures of main anthocyanins in strawberry and their glycosylating groups.

4.4.2 Growth Characteristics of AGS and Hs 738.St/Int Cell Lines:

Before treating the AGS stomach cancer and the 738.St/Int stomach and intestine mixed cell lines with strawberry anthocyanin extract, their growth characteristics were examined. Proliferation rates for AGS and Hs 738.St/Int cell lines seeded at 10,000 cells/mL in a 24-well plate were determined by manually counting cells using a hemocytometer at 24, 48, and 72 hours. The 72 hour growth pattern for AGS and Hs 738.St/Int is illustrated below (Figure 4.4 and Figure 4.5). AGS and Hs 738.St/Int cell lines exhibited lag phase growth between0-24 hours and log phase (exponential) growth between 24-72 hours. Cells also achieved 80-90% confluence at the end of 72 hours, which is an appropriate confluence for both AGS and Hs 738.St/Int cell treatment according to the American type culture collection product sheet for each cell line.

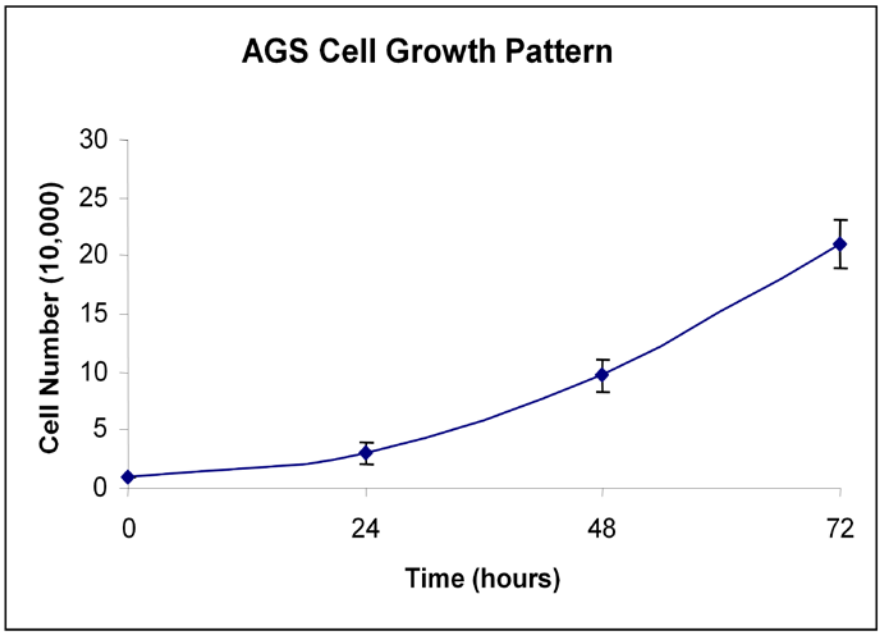


Figure 4.4: AGS human stomach cancer cell culture 72 hour growth pattern.

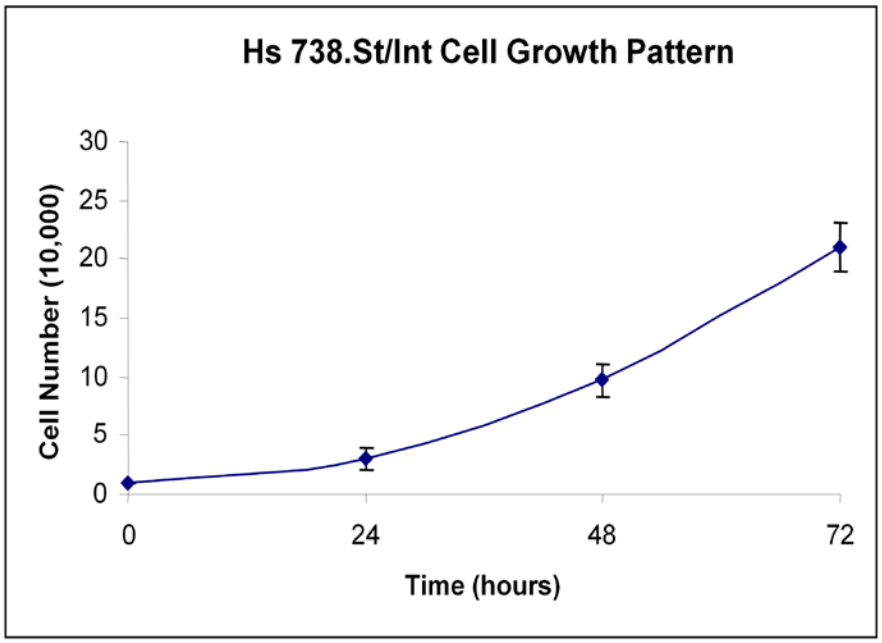


Figure 4.5: Hs 738.St/Int human stomach and small intestine mix cell culture 72 hour growth pattern.

4.4.3 AGS Stomach Cancer Cell Line Growth Inhibition:

The effects of growth inhibition of AGS stomach cancer cells varied according to sample treatment dose. The sulforhodamine-B assay was used throughout the study to determine the effects of strawberry anthocyanin treatments on the proliferation of AGS stomach cancer cells. The sulforhodamine-B colorimetric assay's dye binds to the amino acids of the cellular proteins and through colorimetric evaluation an estimation of the cell proliferation was made as it relates to total protein mass.

AGS stomach cancer cells were incubated in F-12K Medium supplemented with 10% FBS with strawberry anthocyanin rich extract from fractionated strawberries at concentrations of 25, 50, 100 and 200 µg/mL for 48 hours. Fractionated strawberry anthocyanins extracts appeared to significantly reduce the AGS proliferation rates in a dose dependent manner. The findings of Shih et al., 2005, which investigated five anthocyanin aglycone (cyanidin, delphinidin, malvidin, pelargonidin, and peonidin) and four glycosylated (cyanidin-3-glucoside, malvidin-3-glucoside, pelargonidin-3-glucoside and peonidin-3-glucoside) anthocyanins on the induction of cell apoptosis in human gastric adenocarcinoma AGS cells found similar results to our study; showing antiproliferative effects of AGS by anthocyanidins and anthocyanins in a time and dose dependent manner. The results of our study had an overall observed growth inhibition of AGS cells when treated with fractionated strawberry extract at concentrations of 25, 50, 100 and 200 µg/mL of 34.7, 76.1, 92.2, and 87.4 percent respectively and can be seen in figure 4.6 below ($p<0.0001$).

Hs 738.St/Int stomach and small intestine mixed cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS with strawberry anthocyanin rich extract from fractionated strawberries at concentrations of 25, 50, 100 and 200 µg/mL for 48 hours. Fractionated strawberry anthocyanins extracts appeared to increase the proliferation of the Hs 738.St/Int stomach and small intestine mixed cell line. The overall observed growth inhibition rates of the Hs 738.St/Int cells when treated with fractionated strawberry extract at concentrations of 25, 50, 100 and 200 µg/mL were -4.78, -23.9, -27.2, and -24.1 percent respectively and can be seen in figure 4.7 below ($p<.0001$). Because growth inhibition was measured, a negative percent growth inhibition indicates cell growth and not inhibition. Our results further support the work of Tan et al., 2011 who analyzed how fruit polyphenols inhibit cell viability and induce apoptosis in human cancer cell lines. Tan and his lab group's study show significant differences in cell viability between the normal Hs 738.St/Int cells lines and cancerous AGS cell lines ($p<0.05$).

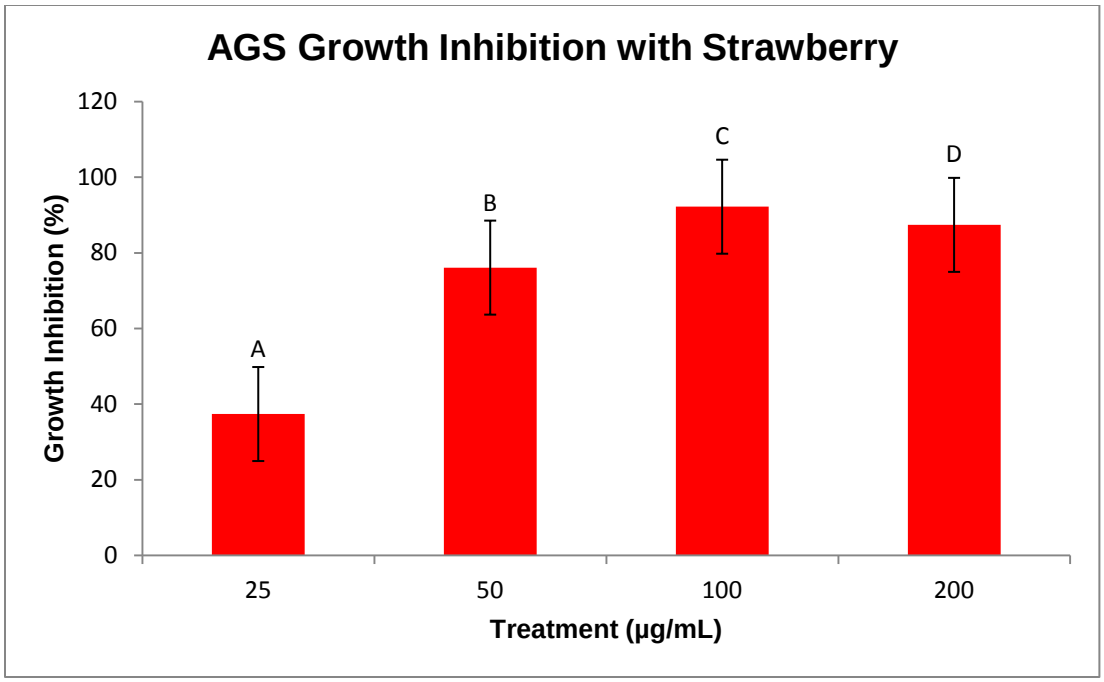


Figure 4.6: Dose dependent growth inhibition of AGS stomach cancer cells when treated with fractionated strawberry anthocyanins at 25, 50, 100 and 200 µg/mL for 48 hours determined by the SRB assay ($p<0.0001$).

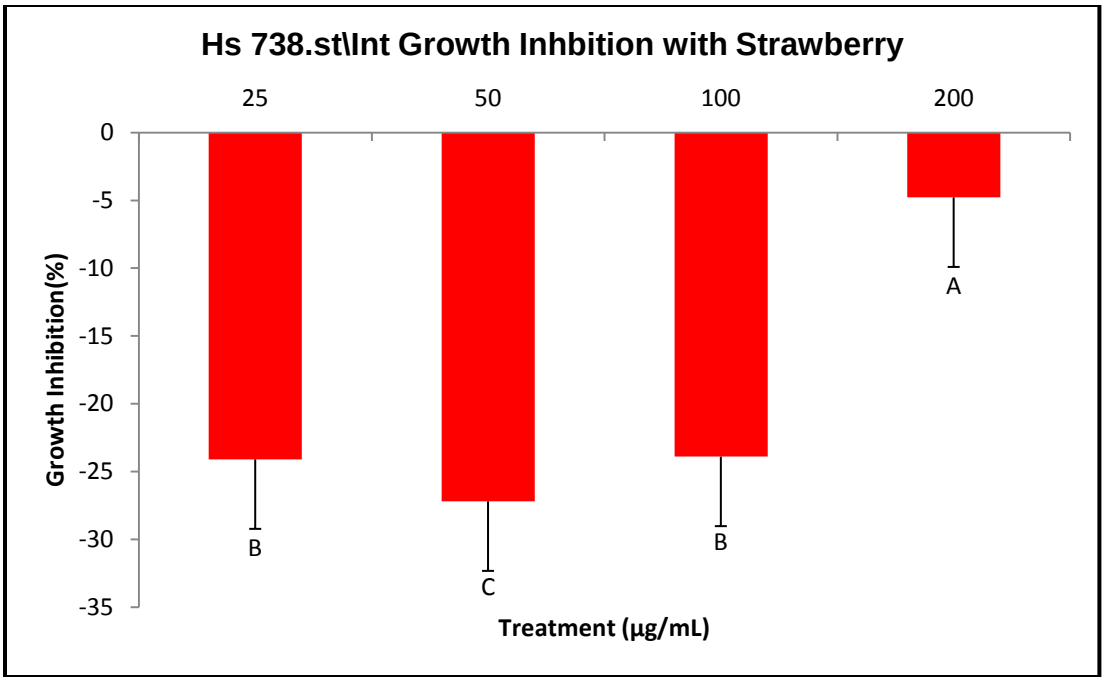


Figure 4.7: Growth inhibition of Hs 738.St/Int stomach and small intestine cells when treated with fractionated strawberry anthocyanins at 25, 50, 100 and 200 µg/mL for 48 hours determined by the SRB assay ($p<0.0001$).

4.4.4 Caspase-3 Colorimetric Assay:

In order to understand the mechanism of action strawberry anthocyanins had on the growth inhibition of the AGS cell line the caspase-3 colorimetric assay was performed. Caspases are cysteine-requiring Aspartate proteases that mediate cell death and are important in the process of apoptosis. Caspase-3 is one of the critical enzymes of apoptosis and is also one of the most studied mammalian caspases. The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405nm. The concentration of the pNA released from the substrate is calculated from the absorbance values at 405nm or from a calibration curve prepared with defined pNA solutions. A pNA calibration curve (figure 4.8) was carried out to compare caspase-3 activity in µmol of pNA released per minute per mL of cell lysate.

The results of the caspase-3 colorimetric assay showed that strawberry anthocyanins at a concentration of 200µg/mL increased the caspase-3 activity as compared to the control by more than 1.8 fold (figure 4.9). This increase in caspase-3 activity can be directly related to the apoptosis seen in the AGS cells when treated with strawberry anthocyanins and may indicate that anthocyanins play a role in the initiation of cell mediated death in human stomach cancer.

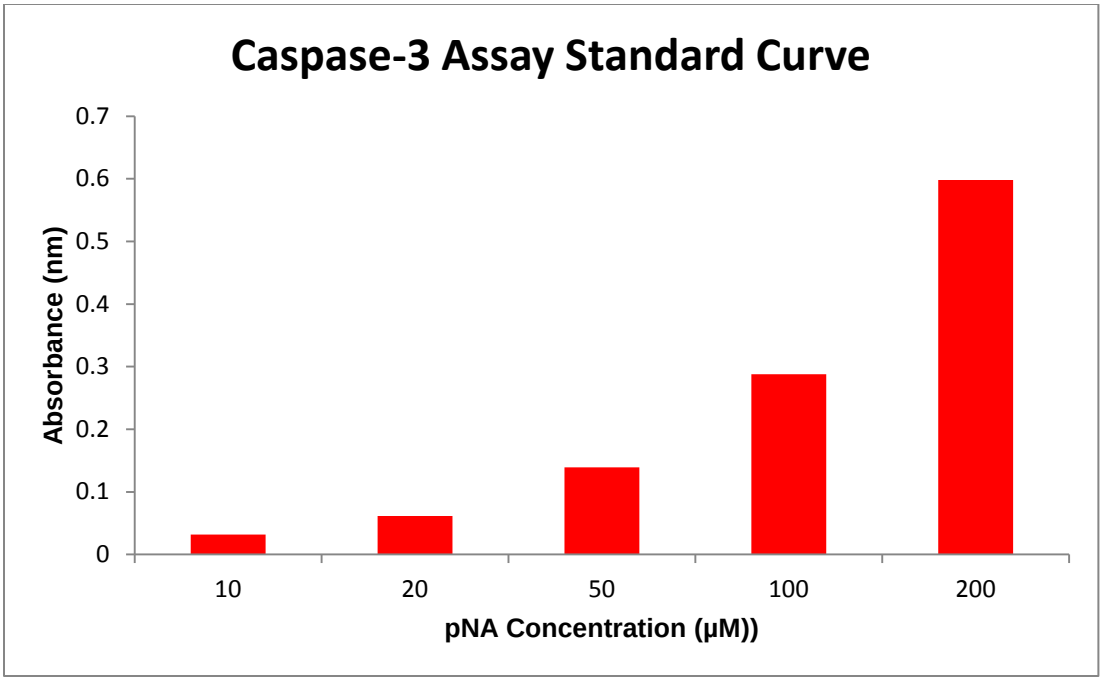


Figure 4.8: p-Nitroaniline (pNA) Calibration Curve.

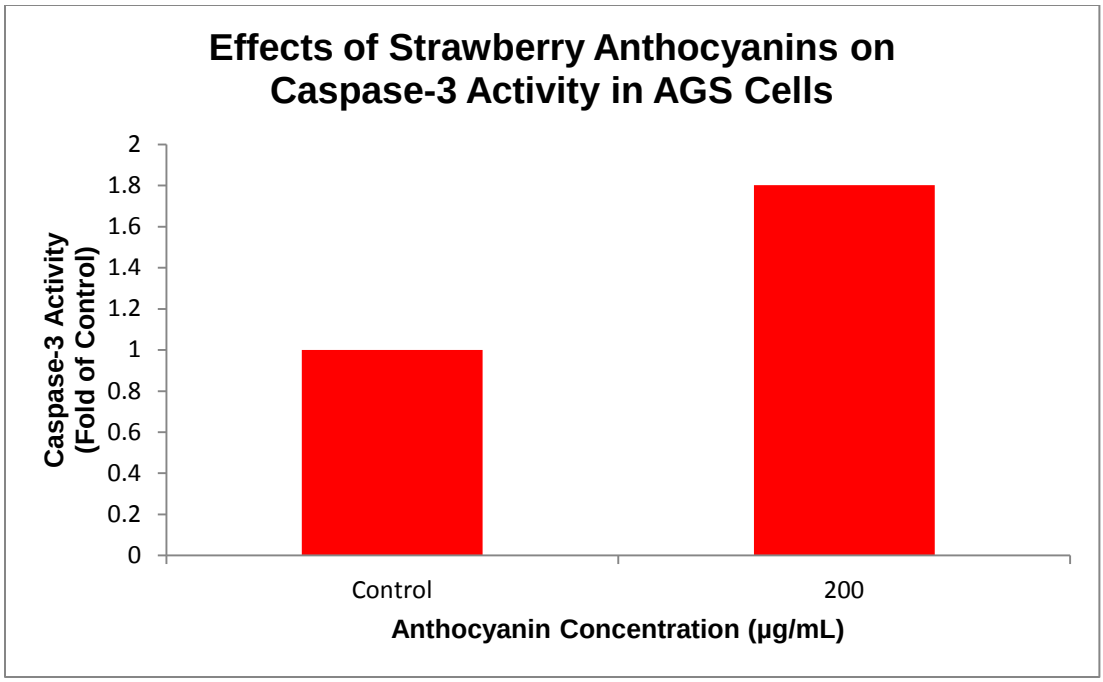


Figure 4.9: Effects of strawberry anthocyanins on caspase-3 activity in AGS cells indicating greater than a 1.8 fold increase in caspase-3 activity.

4.5 CONCLUSION

The results of this study show that anthocyanin extracts from strawberry provide inhibitory effects to the proliferation of AGS cells when treated with fractionated strawberry extract at concentrations of 25, 50, 100 and 200 µg/mL of 34.7, 76.1, 92.2, and 87.4 percent respectively ($p<0.0001$). Furthermore, the results of this study show that anthocyanin extracts from strawberry provide proliferative affects of Hs 738.St/Int stomach and small intestine mixed cell line at a concentration of 25-200 µg/mL. Significant differences were seen between gastric and colorectal cancer cell lines as compared to their equivalent normal cell lines. Again, this data suggests that the observed proliferative activity may be associated with anthocyanin treatment, since the treatment of anthocyanin rich extracts disrupted the cancerous AGS cell line's growth pattern and enhanced the growth pattern of the Hs 738.St/Int stomach and small intestine mixed cell line. The addition of the caspase-3 assay provides information as to what mechanistic role the strawberry anthocyanin extract has in decreasing AGS proliferation rates, suggesting strawberry anthocyanins play a role in the up-regulation of caspase-3 and therefore and important role in apoptosis. The *in vitro* chemotherapeutic effect of strawberry anthocyanins may be relevant to future *in vivo* work because when anthocyanins are ingested in the diet they are in direct contact with the epithelial cell layer of the gastrointestinal tract and may provide chemoprotection upon contact with the stomach and gastrointestinal tract.

CHAPTER 5

THE EFFECT OF STRAWBERRY ANTHOCYANINS ON THE INHIBITION OF HT-29 COLON CANCER CELL AND CCD-18CO COLON CELL PROLIFERATION

5.1 SUMMARY

Anthocyanins are shown to be promising phytochemicals responsible for at least part of the anticancer property of many fruits and vegetables, but it is more than likely that anthocyanins work collaboratively with other phytochemicals to help the body's defense (He and Giusti, 2010). Anthocyanins constitute the largest and probably the most important group of water soluble natural pigments (Takeoka and Dao, 2002). It has been noted that dietary habits and choices have great impact on anthocyanin consumption. For example, one serving of blueberry increases anthocyanin consumption to greater than 500 mg. Additionally, one serving of Concord grapes provides approximately 200 mg, and one serving of elderberry can supply 2000 mg anthocyanin (He and Giusti, 2010). Epidemiological studies have suggested a reverse association between diets high in polyphenols and incidence of some chronic disease. For example, drinking red wine regularly has been associated with the relatively low incidence of coronary heart disease in French people despite a high fat diet, this phenomenon is well known as the French Paradox (Renaud and de Lorgeril, 1992). In this study, strawberry anthocyanin extracts were chosen because of their very simple anthocyanin profile. In this unique anthocyanin profile, pelargonidin-3-glucoside and cyanidin-3-glucoside together comprise over 83% of the total anthocyanin content. The remaining 17% is comprised of other

pelargonidin and cyanidin glycosylates. Pelargonidin-3-glucoside is the most abundant anthocyanin in the strawberry anthocyanin profile and is responsible for the attractive, bright red color of fresh strawberries. The strawberry anthocyanins were extracted and fractionated using acetone, chloroform, methanol, ethyl acetate, hexane and water. The extracts were used in an *in vitro* experiment to treat human colon adenocarcinoma cells (HT-29 colon cancer cells) and human nondiseased colon cells (CCD-18Co colon cells). Proliferation rates were measured using the sulfurhodamine B assay. Chemopreventative properties of the strawberry anthocyanin extracts were measured on the HT-29 and CCD-18Co cell lines. The results of the experiment showed that strawberry anthocyanins are chemoprotective against the human colon cancer cells, having antiproliferative effects on the HT-29 cell line ($p<0.0001$). The results of this research also showed that strawberry anthocyanins increased the proliferation rates of human colon cells by increasing the growth rates of CCD-18Co ($p<0.0001$). The findings of this study were performed *in vitro* and suggest that the consumption of strawberry anthocyanins may impact overall gastrointestinal health.

5.2 INTRODUCTION

Anthocyanins are shown to be promising phytochemicals responsible for at least part of the anticancer property of many fruits and vegetables, but it is more than likely that anthocyanins work collaboratively with other phytochemicals to help the body's defense (He and Giusti, 2010). Anthocyanins constitute the largest and probably the most important group of water soluble natural pigments (Takeoka and Dao, 2002). It has been noted that dietary habits and choices have great impact on anthocyanin consumption. For example, one serving of blueberry increases anthocyanin consumption to greater than 500 mg. Additionally, one serving of Concord grapes provides approximately 200 mg, and one serving of elderberry can supply 2000 mg anthocyanin (He and Giusti, 2010). Epidemiological studies have suggested a reverse association between diets high in polyphenols and incidence of some chronic disease. For example, drinking red wine regularly has been associated with the relatively low incidence of coronary heart disease in French people despite a high fat diet, this phenomenon is well known as the French Paradox (Renaud and de Lorgeril, 1992). Many anthocyanin-rich extracts from berries and vegetables have been reported to slow down the growth of HT-29 colon cancer cells *in vitro*, each of them to a different extent (Jing et al., 2006; Zhao et al., 2004). The United States Department of Agriculture (USDA) recently began documenting the flavonoid content of commonly consumed fruits and vegetables (USDA 2.1, 2007). Dietary anthocyanin sources included many colored fruits and begetables as well as fruit based processed foods and beverages such as jelly, juices and red wine (He and

Giusti, 2010). Anthocyanins represent a subclass of flavonoids and are of great importance to the food industry because of their coloring properties and accordingly, their impact on the visual appeal of a product. This has become very important to the food industry in recent years as many consumers have grown concerned about buying food or beverage products with synthetic food dyes due to possible adverse health effects. Because anthocyanins are responsible for the red to violet hues in many fruits and vegetables, their use in the food industry has rapidly expanded as a value added ingredient and alternatives to synthetic red dyes. The basic anthocyanin structure consists of the carbon skeleton with conjugated bonds on the C-ring and two positions, R-1 and R-2, which are substituted with hydroxyl, oxymethyl and hydryl groups. Different combinations of these side groups results in the six anthocyanidin chromophores most commonly found in nature. These anthocyanidin aglycones include cyanidin (Cy), pelargonidin (Pg), delphinidin (Dp), petunidin (Pt), malvidin (Mv) and peonidin (Pn) (Fleschhut et al., 2006; Hou et al., 2005; McGhie et al., 2007). Anthocyanin sources by nature contain multiple polyphenolic compounds; because of this the characterization and quantification of anthocyanins can be laborious and expensive. By selecting an anthocyanin source with fewer anthocyanins naturally present, semi-purification and isolation of a single anthocyanin can be more easily achieved. Strawberries have two major anthocyanins present in the fruit tissue; pelargonidin-3-glucoside and cyanidin-3-glucoside (Figure 5.1), which together account for over 83% of their anthocyanin

pigments, making strawberries uniquely simple anthocyanin source. Because of this strawberries were selected for this research study.

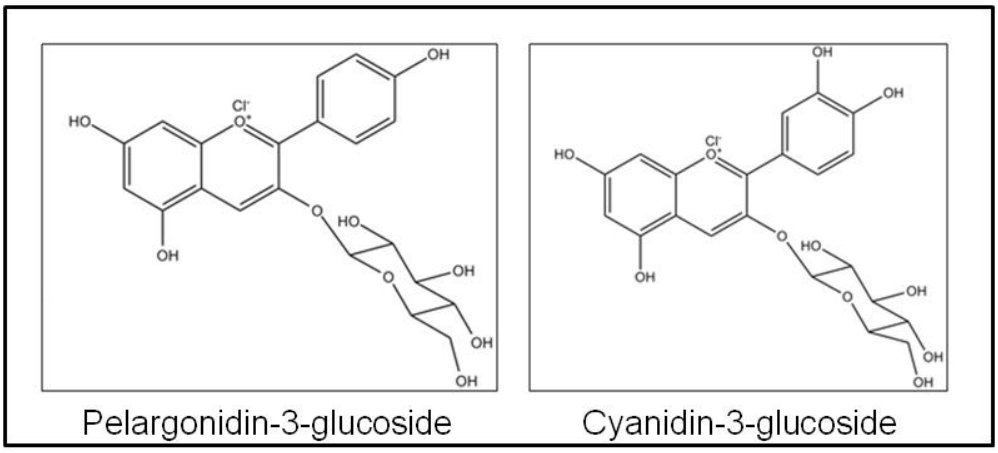


Figure 5.1: The two main strawberry anthocyanins, pelargonidin-3-glucoside and cyanidin-3-glucoside.

Of cancers that affect both men and women, colorectal cancer is the second leading cause of cancer related deaths in the United States and the third most common cancer in men and women (Eyre 2004). Colorectal cancer is the cancer of the colon or rectum. Colon cancer is cancer that forms in the tissue of the colon, the longest section of the large intestine. The majority of colon cancer arises from preexisting adenomas (Toribara and Sleisenger, 1995). Adenocarcinoma cells are cells that produce and excrete mucus and other fluids. Rectal cancer is cancer that forms in the tissue of the rectum, the last several inches of the large intestine closest to the anus. It was reported that more than 145,083 people were diagnosed with colorectal cancer and 53,580 people died from colorectal cancer in 2004 (Eyre 2004). Colorectal cancer affects both men and women and all races, however, men, African Americans and Alaskan Natives have an exceptionally high colorectal cancer incidence and mortality rate

(Eyre 2004). The treatment options for colorectal cancer depend on the stage of the cancer. According to the Food and Drug Administration (FDA) there are five stages of colorectal cancer: stage 0 (carcinoma in situ), stage I colon cancer, stage II colon cancer, stage III colon cancer and stage IV colon and recurrent colon cancer.

The objective of this research was to determine the chemopreventative effects of strawberry anthocyanins against the human colon cancer cell line HT-29 and to determine the proliferative effects of strawberry anthocyanins on the human colon cell line CCD-18Co. To accomplish this, HT-29 and CCD-18Co cell lines were grown up and treated with strawberry anthocyanin extract (25-200 µg/mL), and the proliferation growth rates were determined using the sulforhodamine-B colorimetric assay.

5.3 MATERIALS AND METHODOLOGY

5.3.1 Chemicals and Reagents:

Organic Driscoll's brand strawberries were purchased from a local grocery store in Lexington, Kentucky. Certified ACS grade HCL (12N) and ethyl acetate, LCMS grade 99.9% methanol and 99.9% acetonitrile, and HPLC grade 88% formic acid, 99.9% acetone and 99.9% chloroform were obtained from Fisher Scientific (Fair Lawn, NJ). Reagent grade gallic acid and Folin-Ciocaltaeu reagent were obtained from MP Biomedical (Aurora, OH). HT-29 (ATCC® HTB38™) and CCD-18Co (ATCC® CRL-1459™) were obtained from American Type Culture Collection (Rockville, MD). Sterile McCoy's 5a Medium Modified and Eagle's Minimum Essential Medium (MEM) (Thermo Fisher Scientific, Waltham, MA), fetal bovine serum (FBS) (Invitrogen Corp. Carlsbad, CA) Delbucco's Phosphate Buffered Saline Solution (DPBS) (Lonza, Walkersville, MD), and 2.5% Trypsin (Lonza, Walkerville, MD) were obtained from their manufacturers. All other chemicals were certified ACS grade from Thermo Fisher Scientific, Waltham, MA.

5.3.2 Extraction of Anthocyanins and other Phenolics from Plant Materials:

The red flesh of strawberries were hand selected using a paring knife and quickly frozen using liquid nitrogen. The frozen strawberry red flesh where then fine powdered using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT) with a 500 mL stainless steel container in the

presence of liquid nitrogen (Giusti and Rodriguez-Soana, 1999). Strawberry flesh powder was stored at -20˚C until used for sample extraction.

Strawberry flesh anthocyanins and other phenolics were extracted by blending the powders with 30mL of 70% acetone and 30% 0.1% HCl acidified water (v/v) for 5 minutes using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT). The liquid was subsequently passed through Whatman No. 4 and No. 1 filter paper (Whatman Inc., Florham, NJ) using a Buchner Funnel (Thermo Fisher Scientific, Waltham, MA). This process was repeated until the filtrate was free of haze and all of the pigment had been extracted from the powdered strawberry flesh particles (Rodriguez-Soana and Wrolstad, 2001). The strawberry anthocyanin and other phenolics filtrate was then placed into a separatory funnel (Thermo Fisher Scientific, Waltham, MA) and partitioned with 2 volumes of chloroform. The solution was mixed and left to separate for 24 hours at 4˚C in the dark to ensure adequate separation. The top acetone/water layer containing anthocyanins and other phenolics was collected and the bottom chloroform/fat layer was discarded properly in hazardous waste containers. The residual acetone in the sample was evaporated using a Buchi Rotavapor R-215 rotary evaporator (Thermo Fisher Scientific, Waltham, MA) at 40˚C. The solution was brought to 25 mL in a volumetric flask using LCMS grade water.

5.3.3 Fractionation and Concentration of Anthocyanin Sample:

Anthocyanin phenolics from strawberries were concentrated by using Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corporation, Milford, MA). The anthocyanin filtrate was passed through the Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corporation, Milford, MA) 10 mL at a time. Anthocyanins and other phenolics were bound to the C_{18} cartridge, while sugars and other polar compounds were removed with 30 mL of 0.01% HCl acidified water, followed by 10 mL of hexane to remove the residual water. The non-anthocyanin phenolics were eluted with 20 mL of diethyl ether followed by 20 mL of ethyl acetate. The anthocyanin phenolic fraction was eluted with 30 mL of 0.01% HCl acidified methanol followed by 10 mL of 0.01% HCl acidified HPLC grade water. Excess methanol in the anthocyanin phenolic filtrate was evaporated in the Buchi Rotavapor R-215 rotovapor (Thermo Fisher Scientific, Waltham, MA) at 40˚C and the anthocyanin fraction was re-dissolved in 0.01% HCl acidified HPLC grade water. The highly concentrated anthocyanin extract was then quickly frozen using liquid nitrogen and lyophilized using a Labconco Freezone[6] lyophilizer into a fine dry powder (Labconco, Kansas City, MO).

5.3.4 Monomeric and Polymeric Analysis:

Monomeric anthocyanin content was determined using the pH differential method (Giusti and Wrolstad, 2005). This method uses the spectral difference that anthocyanins exhibit at different pH values. Dilution factor was determined by diluting the strawberry anthocyanin extract in 0.025 M potassium chloride

buffer, pH 1.0, until the absorbance was within the appropriate spectrophotometer range (for most spectrophotometers absorbance should be less than 1.2 nm). The strawberry anthocyanin extract was then appropriately diluted using pH 1.0 and pH 4.5 buffer. Solutions were allowed to sit at room temperature for at least 15 minutes in the dark. 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 cyanidin-3-glucoside equivalents, using a molecular weight of 449.3 and an extinction coefficient of 26,900 L cm^{-1} mg^{-1} (Giusti and Wrolstad, 2005). Absorbance was determined in replicates of three.

Polymeric anthocyanin content was determined using the polymeric method (Wrolstad 1999). Appropriate dilutions were determined using pH 1.0 buffer and 1-cm cuvettes were prepared with 2.8 mL of diluted sample in water to each of two cuvettes. 0.2 mL of water was added to one of the cuvettes and 0.2 mL of bisulfate solution to the other and allowed to equilibrate for 15 minutes before reading at 420 nm, 520 nm, and 700 nm. Polymeric anthocyanin content was calculated using the following equations:

$$\text{Color Density} = [(A_{420nm} - A_{700nm}) + (A_{\lambda\ vis\ max} - A_{700nm})] \times DF$$

$$\text{Polymeric Color} = [(A_{420nm} - A_{700nm}) + (A_{\lambda\ vis\ max} - A_{700nm})] \times DF$$

$$\text{Percent Polymeric Color} = (\text{Polymeric Color}/\text{Color Density}) \times 100$$

Where DF is the dilution factor and absorbance was determined in replicates of three.

5.3.5 Total Phenolics:

Total phenolics were measured using a microscale protocol for Folin-Ciocalteu Colorimetry (Waterhouse 2005). Cuvettes were prepared with 20 µL of sample/standard, 1.58 mL water and 100 µL 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 µL) was added and samples were allowed to stand at room temperature for 2 hours. 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.

5.3.6 LC/MS Analysis:

Strawberry anthocyanin samples were analyzed using a Thermo Finnigan LTQ high performance liquid chromatograph (HPLC) (ThermoFinnigan; Waltham, MA). Positive ion electrospray ionization (ESI) mass spectra were obtained on the Thermo Finnigan LTQ coupled to a Dionex Ultimate 3000 capillary LC system. The column was 20cm x 150um packed with Kromasil C18 (3.5um particle size) (AkzoNobel, Separation Products, Bohus, Sweden). Proteome Discoverer Software 1.1.0 was used on this system (Thermo Fisher Scientific; Waltham, MA). All samples were filtered through 13 mm 0.45µm polypropylene filters (Thermo Fisher Scientific, Waltham, MA)prior to HPLC/MS analysis.

Separation of strawberry extract anthocyanins was achieved using a linear gradient elution from 5% to 60%. Solvent A was 0.1% (v/v) formic acid in water and B was 0.1% (v/v) formic acid in acetonitrile. The flow rate was 0.8 mL / min and an injection volume of 50 µL was used. Spectral data was collected using the Dionex Ultimate 3000 capillary LC system from 250-700 nm. Absorbance of anthocyanins was monitored at 280 and 520 nm.

A 1.5 uL / 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 ºC; 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 and Selective Ion Monitoring (SIM) was used to search for the molecular ions of the common anthocyanins throughout the analysis.

5.3.7 HT-29 Cell Line:

The HT-29 human cell line originates from the colon of a female *homo sapien*. The cell line is considered to have an epithelial morphology and its culture growth properties are adherent. The HT-29 human cell line is diseased with colorectal adenocarcinoma and therefore cancerous. The HT-29 cell line was grown at 37˚C in a Nuuaire™ IR Autoflow CO_2 Water-Jacketed Incubator at a modified atmosphere (5% CO_2, 95% O_2) in ATCC-formulated McCoy's 5a

Medium Modified. To make the complete growth medium fetal bovine serum to a final concentration of 10 percent must be added. Cultures were split 1:5 when monolayers were 70-80% confluent using Delbucco's Phosphate Buffered Saline solution (DPBS) and 2.5% trypsin (Lonza, Walkersville, MD). Cultures were fed fresh media every 3 days. Growth of HT-29 cell line was determined by microscopic examination. Cell cultures were maintained in 75 cm^2 Flasks with canted necks (Thermo Fisher Scientific, Waltham, MA). All cell culture experiments were performed with the initial 20 passages.

5.3.8 CCD-18Co Cell Line:

The CCD-18Co human cell line originates from the colon of a female *homo sapien.* The cell line is considered to have a fibroblast morphology and its culture growth properties are adherent. The CCD-18Co human cell line is non-diseased.The CCD-18Co cell line was grown at 37˚C in a Nuuaire™ IR Autoflow CO_2 Water-Jacketed Incubator at a modified atmosphere (5% CO_2, 95% O_2) in ATCC-formulated Eagle's Minimum Essential Medium (MEM). To make the complete growth medium fetal bovine serum to a final concentration of 10 percent must be added. Cultures were split 1:5 when monolayers were 70-80% confluent using Delbucco's Phosphate Buffered Saline solution (DPBS) and 2.5% trypsin (Lonza, Walkersville, MD). Cultures were fed fresh media every 3 days. Growth of CCD-18Co cell line was determined by microscopic examination. Cell cultures were maintained in 75 cm^2 Flasks with canted necks (Thermo Fisher

Scientific, Waltham, MA). All cell culture experiments were performed with the initial 20 passages.

5.3.9 Determining HT-29 and CCD-18Co Cell Concentration using a Hemocytometer:

Cells were plated in 96-well tissue culture dishes at a volume of 10,000 cells/well. Suspended cells were counted using a Nikon Eclipse 50i Brightfield Microscope and a Reichert Bright-Line hemocytometer (Hausser Scientific; Horsham, PA) to estimate total viable cell concentration. 10 µL of cells were added to 90 µL of Trypan Blue Stain (Thermo Fisher Scientific, Waltham, MA) and subsequently loaded on the hemocytometer. Using the 10x lens of the microscope, cells were counted on 5 primary squares of the hemocytometer using a hand counter. To calculate the HT-29 and CCD-18Co cell concentration the following formula was used:

Cells/mL = (Total # cells counted)/(Number of primary squares counted) x $1x10^4$

HT-29 and CCD-18Co cells were seeded at 10,000 cells/well and allowed to grow and form monolayers for 24 hours. At 24 hours the HT-29 and CCD-18Co cells were treated with strawberry anthocyanin extract and tested for chemoprotective effects.

5.3.10 Anthocyanin Treatments:

Monomeric anthocyanin content was determined for the strawberry anthocyanin extract using the detailed method explained by Giusti and Wrolstad, 2001. According to the monomeric anthocyanin content of strawberries, 25, 50, 100 and 200 µg/mL of sample was determined and a corresponding treatment value was given in grams of sample weight.

5.3.11 Sulforhodamine-B Colorimetric Assay:

The Sulforhodamine-B colorimetric assay is used for cell proliferation and chemosensitivity testing. For this study, HT-29 colon cancer cells and CCD-18Co colon cell media was removed and cells were fixed by addition of 150 µL of 50% trichloroacetic acid (TCA) at 4°C for 1 hour. TCA was removed and wells were washed with tap water 5 times and allowed to air dry at room temperature. 75 µL of Sulforhodamine-B (0.4% in water containing 0.01% acetic acid) was added to each well and allowed to stain cells at room temperature for 30 minutes. Each well was washed with 1% acetic acid 5 times to remove any residual unbound dye and dried at room temperature. The protein bound dye was solubilized with 150 µL of 10 mM Tris base for 5 minutes at room temperature on a plate shaker. The absorbance of each plate was then read at a wavelength of 490 nm using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Intruments, Inc., Winooski, VT). Gen5 1.10 Bio-Tek® software was used to analyze the data.

5.3.12 HT-29 Cell line proliferation with Strawberry Anthocyanins:

The sulforhodamine-B colorimetric assay was used for the *in vitro* observation of chemoprotection against HT-29 cell lines. First, HT-29 human colon cancer cells were plated in 96-well tissue culture plates (Corning Inc., Tewksbury, MA) at a level of 10,000 cells/well in ATCC-formulated McCoy's 5a Medium Modified supplemented with 10% FBS and a volume of 150 uL/well and incubated at 37˚C in a humidified incubator with 5% CO_2. The HT-29 cells were allowed to grow and form monolayers for 24 hours. Following the 24 hour incubation confluent cell monolayers in the 96-well plates were treated in serial dilution with strawberry anthocyanin extract at concentrations of 25, 50, 100 and 200 µg/mL. Immediately following the treatment of the HT-29 cells the plates were incubated at 37˚C in a humidified incubator with 5% CO_2 for 48 hours. 48 hours after strawberry anthocyanin treatment was added the 96-well plates were aspirated of their media and the sulforhodamine-B assay was performed and the absorbance of each plate was then read at a wavelength of 490 nm using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Intruments, Inc., Winooski, VT). Gen5 1.10 Bio-Tek® software was used to analyze the data.

5.3.13 Proliferative Effects of Strawberry Anthocyanins on CCD-18Co Cell Lines:

The sulforhodamine-B colorimetric assay was used for the *in vitro* observation of the proliferative effects of strawberry anthocyanin extracts on the CCD-18Co human colon cell line. First, the CCD-18Co cells were plated in 96-well tissue culture plates (Corning Inc., Tewksbury, MA) at a level of 10,000

cells/well in ATCC-formulated Dulbecco's Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS and a volume of 150 uL/well and incubated at 37˚C in a humidified incubator with 5% CO_2. The CCD-18Co cells were allowed to grow and form monolayers for 24 hours. Following the 24 hour incubation confluent cell monolayers in the 96-well plates were treated in serial dilution with strawberry anthocyanin extract at concentrations of 25, 50, 100 and 200 µg/mL. Immediately following the treatment of the CCD-18Co cells the plates were incubated at 37˚C in a humidified incubator with 5% CO_2 for 48 hours. 48 hours after strawberry anthocyanin treatment was added the 96-well plates were aspirated of their media and the sulforhodamine-B assay was performed and the absorbance of each plate was then read at a wavelength of 490 nm using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Intruments, Inc., Winooski, VT). Gen5 1.10 Bio-Tek® software was used to analyze the data.

5.3.14 HT-29 and CCD-18Co Cell Growth Inhibition Calculation:

HT-29 and CCD-18Co cells were plated at 10,000 cells/well in 96-well cell culture plates with their appropriate media. Cells were allowed to grow for 24 hours to attain log phase growth at the time of sample addition (time 0). Strawberry anthocyanin samples were compared for their proliferative and/or chemoprotective effects at concentrations of 25, 50, 100 and 200 µg/mL. HT-29 and CCD-18Co cell growth inhibition was determined after 48 hours of incubation at the different anthocyanin concentrations by using the sulfurhodamine-B assay

(SRB) at 490 nm. Each treatment had 6 replicates and every treatment was repeated at least 4 times. The percentage growth inhibition is calculated as:

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

Where T_0: Time zero: The absorbance of sample at 490 nm after first 24-hour incubation period prior to anthocyanin treatment. **T_{trt}:** Absorbance of sample at 490 nm after anthocyanin treatment. **T_{ctr}:** Absorbance of sample at 490 nm after total incubation (72 hours) without anthocyanin treatment.

5.3.15 Caspase-3 Colorimetric Assay:

The caspase-3 colorimetric assay provides for a quick and efficient detection of caspase 3 activity in cell lysates and in purified preparations of caspase 3. The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405nm. The concentration of the pNA released from the substrate is calculated from the absorbance values at 405nm or from a calibration curve prepared with defined pNA solutions. For this study the assay was performed in 100 µL volume on the HT-29 cell line in 96-well plates using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Intruments, Inc., Winooski, VT). Gen5 1.10 Bio-Tek® software was used to analyze the data.

5.3.16 Statistical Analysis:

The results for the chemoprotective and proliferative analysis of HT-29 and CCD-18Co cell lines following treatment with strawberry anthocyanin extract was subjected to Analysis of Variance (ANOVA) with an extended randomized complete block design (SAS 9.3 Software) and an alpha level of $p<0.05$ acceptance of the null hypothesis was used to determine significant variables.

5.4 RESULTS AND DISCUSSION

5.4.1 Characterization of Strawberry Anthocyanin Sample:

Strawberry anthocyanins were identified by comparing retention times, order of elution and UV-visible spectra to previous literature (Lopes-da-Silva and others 2001). The five major anthocyanins identified in strawberry (Figure 5.2 and Table 5.1) include: peak 1 cyanidin-3-glucoside (~23.3%), peak 2 cyanidin-3-rutinoside (~4.7%), peak 3 pelargonidin-3-glucoside (~60.6%), peak 4 pelargonidin-3-rutinoside (~7.1%), and peak 5 cyanidin-3-malonylglucose-5-glocoside (~4.3%). The strawberry anthocyanin profile contained only cyanidin and pelargonidin derivatives. Additionally, the majority of the strawberry anthocyanin profile is made up of cyanidin-3-glucoside (peak 1) and pelargonidin-3-glucoside (peak 3) which combined comprises over 83% of the anthocyanin profile. Below is a representation of the main anthocyanins in strawberry (Figure 5.3). The figure shows the varying glycosylation patterns found in strawberry anthocyanins.

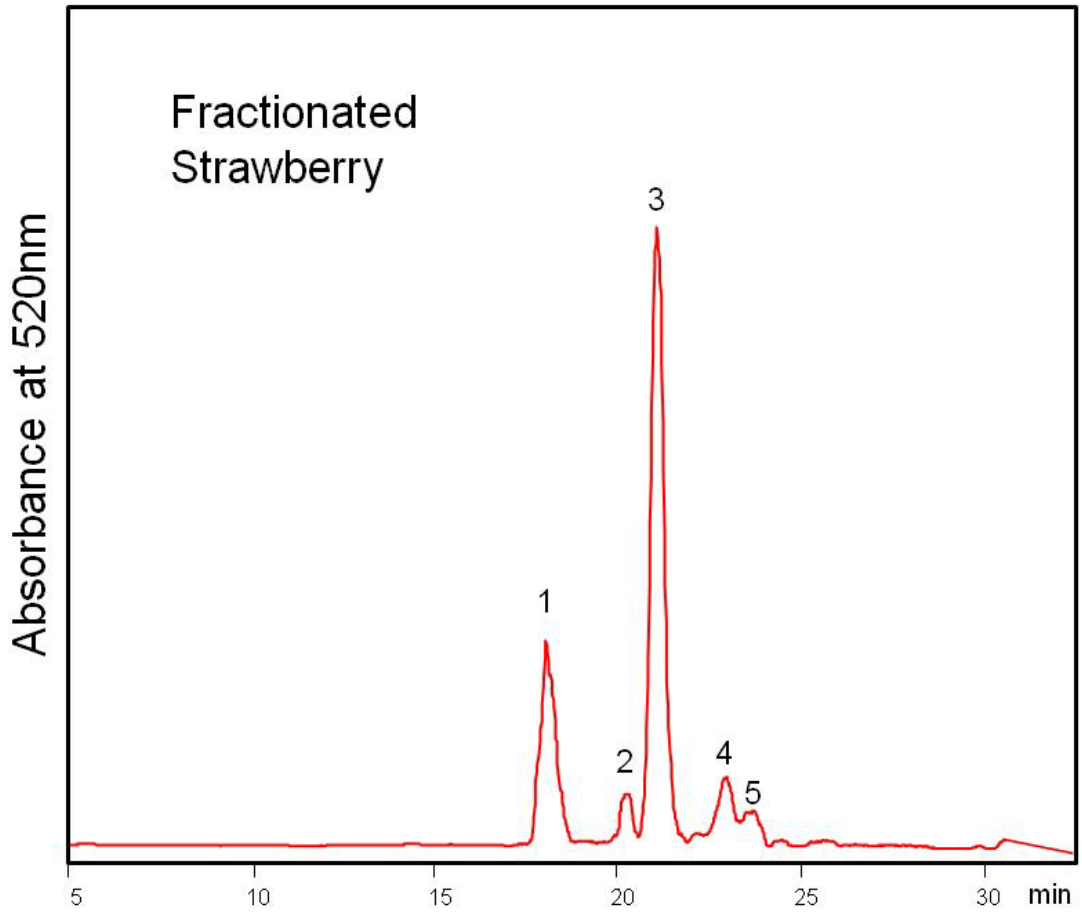
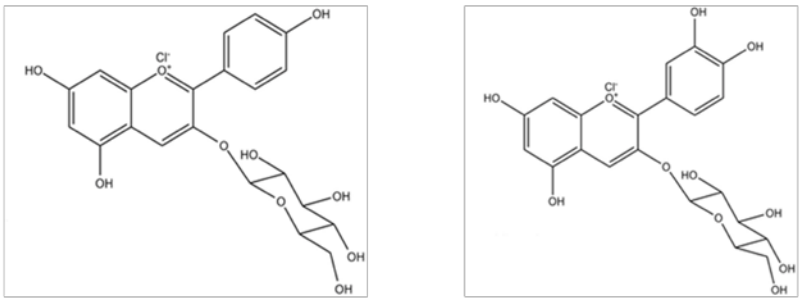


Figure 5.2: Chromatograph of fractionated strawberry extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-glucoside, peak 2 cyanidin-3-rutinoside, peak 3 pelargonidin-3-glucoside, peak 4 pelargonidin-3-rutinoside, and peak 5 cyanidin-3-malonylglucose-5-glocoside.

Fractionated Strawberry			
Peak	Anthocyanin	Retention Time (Min)	%Area 520nm
1	cyanidin-3-glucoside	17.7	23.3
2	cyanidin-3-rutinoside	19.8	4.7
3	pelargonidin-3-glucoside	20.6	60.6
4	pelargonidin-3-rutinoside	22.5	7.1
5	cyanidin-3-malonylglucose-5-glucoside	23.3	4.3

Table 5.1: Fractionated Strawberry Anthocyanins.



A. Main strawberry anthocyanins pelargonidin-3-glucoside showing the typical site for glycosylating group (3rd position on the C-ring). Typical glycosylation: glucose.

B. Main strawberry anthocyanins cyanidin-3-glucoside showing the typical site for glycosylating group (3rd position on the C-ring). Typical glycosylation: glucose.

Figure 5.3: Representative chemical structures of main anthocyanins in strawberry and their glycosylating groups.

5.4.2 Growth Characteristics of HT-29 and CCD-18Co Cell Lines:

Before treating the HT-29 colon cancer and the CCD-18Co cell lines with strawberry anthocyanin extract, their growth characteristics were examined. Proliferation rates for HT-29 and CCD-18Co cell lines seeded at 10,000 cells/mL in a 24-well plate were determined by manually counting cells using a hemocytometer at 24, 48, and 72 hours. The 72 hour growth pattern for HT-29 and CCD-18Co is illustrated below (Figure 5.4 and Figure 5.5). HT-29 and CCD-18Co cell lines exhibited lag phase growth between0-24 hours and log phase (exponential) growth between 24-72 hours. Cells also achieved 80-90% confluence at the end of 72 hours, which is an appropriate confluence for both HT-29 and CCD-18Co cell treatment according to the American type culture collection product sheet for each cell line.

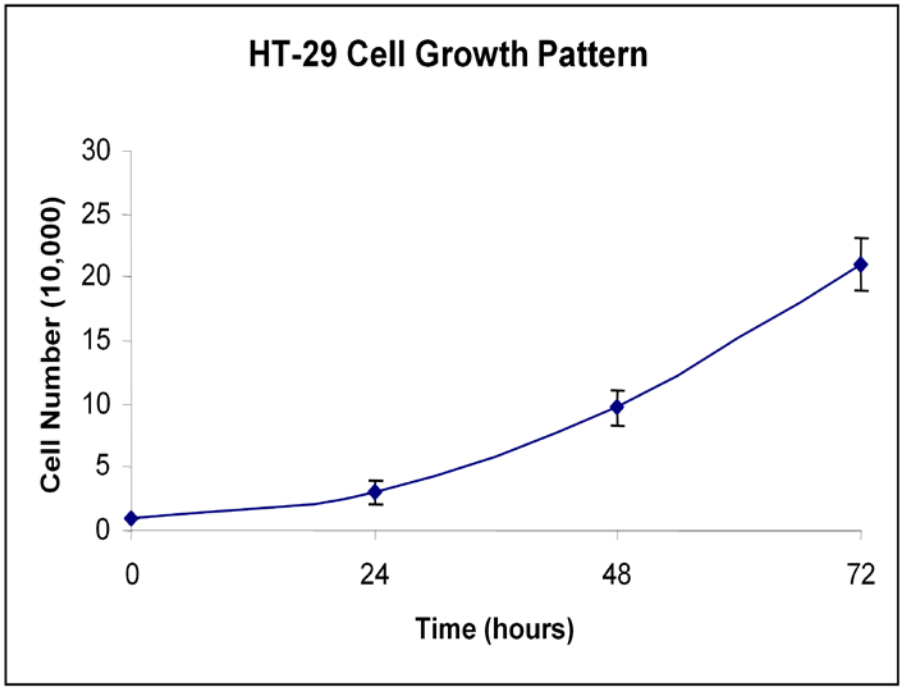


Figure 5.4: HT-29 human colon cancer cell culture 72 hour growth pattern.

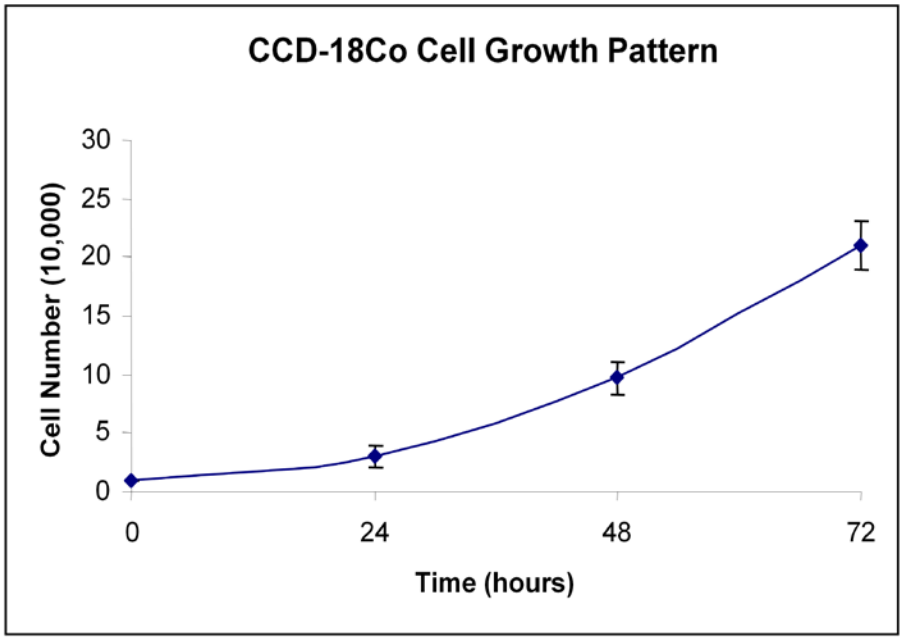


Figure 5.5: CCD-18Co cell culture 72 hour growth pattern.

5.4.3 HT-29 Colon Cancer Cell Line Growth Inhibition:

The effects of growth inhibition of HT-29 colon cancer cells varied according to sample treatment concentration in a dose dependent manner. The sulforhodamine-B assay was used throughout the study to determine the effects of strawberry anthocyanin treatments on the proliferation of HT-29 colon cancer cells. The sulforhodamine-B colorimetric assay's dye binds to the amino acids of the cellular proteins and through colorimetric evaluation an estimation of the cell proliferation was made as it relates to total protein mass.

HT-29 colon cancer cells were incubated in McCoy's 5a Medium Modified supplemented with 10% FBS with strawberry anthocyanin rich extract from fractionated strawberries at concentrations of 25, 50, 100 and 200 µg/mL for 48 hours. Fractionated strawberry anthocyanins extracts appeared to significantly reduce the HT-29 proliferation rates ($p<0.0001$). The results of Jing et al., 2008 who analyzed the chemoprotective effects of purple corn, chokeberry, bilberry, purple carrot, grape, radish, and elderberry on the growth inhibition of human colorectal adenocarcinoma HT-29 cells showed that the anthocyanin rich extracts suppressed the HT-29 cell growth in a dose dependent manner. The findings of Jing et al., 2008 also show that anthocyanin rich extracts exert an additive chemoprotective interaction with other phenolics present. For the present study, growth inhibition of HT-29 cells when treated with fractionated strawberry extract at concentrations of 25, 50, 100 and 200 µg/mL were 34.7, 65.8, 85.1, and 89.8 percent respectively and can be seen in figure 5.6 below ($p<0.0001$).

CCD-18Co colon cells were incubated in Dulbecco's Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS with strawberry anthocyanin rich extract from fractionated strawberries at concentrations of 25, 50, 100 and 200 µg/mL for 48 hours. Fractionated strawberry anthocyanins extracts appeared to increase the proliferation of the CCD-18Co cell line. The overall observed growth inhibition rates of the CCD-18Co cells when treated with fractionated strawberry extract at concentrations of 25, 50, 100 and 200 µg/mL were -1200.6, -1403, -1272.4, and -826.1 percent respectively and can be seen in figure 5.7 below. Because growth inhibition was measure, a negative percent growth inhibition indicates cell growth and not inhibition. Our results further support the work of Tan et al., 2011 who analyzed how fruit polyphenols inhibit cell viability and induce apoptosis in human cancer cell lines and included comparing the HT-29 and CCD-18Co cell lines. Tan and his lab group's study show significant differences in cell viability between the normal CCD-18Co colon cells and cancerous HT-29 colon cells ($p<0.05$).

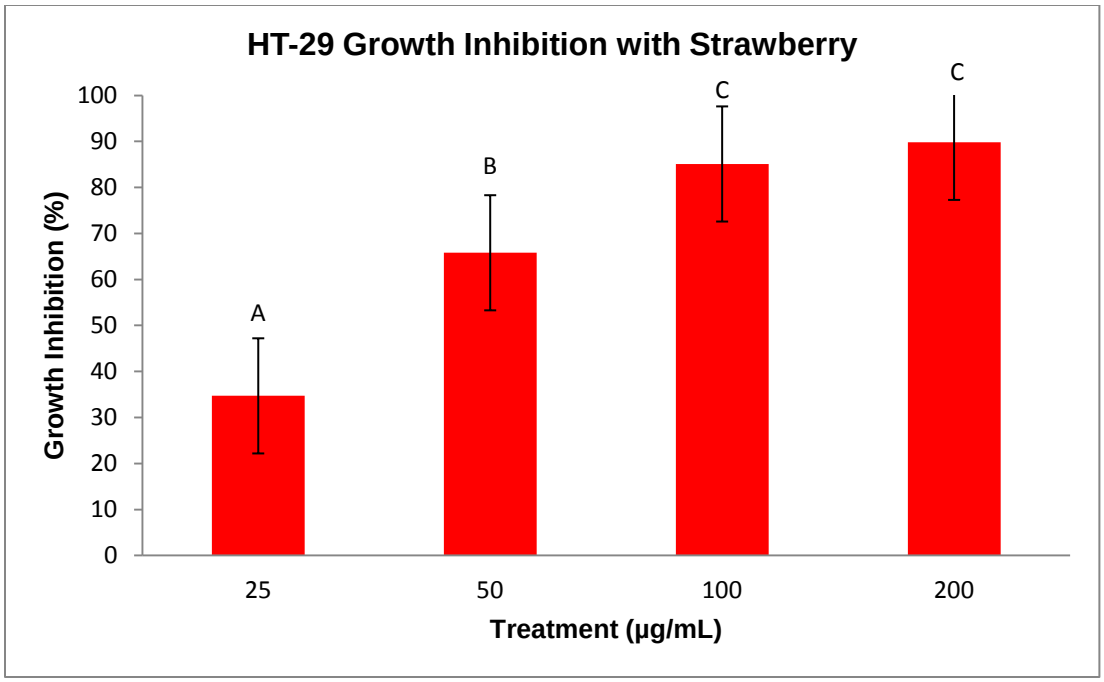


Figure 5.6: Dose dependent growth inhibition of HT-29 colon cancer cells when treated with fractionated strawberry anthocyanins at 25, 50, 100 and 200 µg/mL for 48 hours determined by the SRB assay ($p<0.001$).

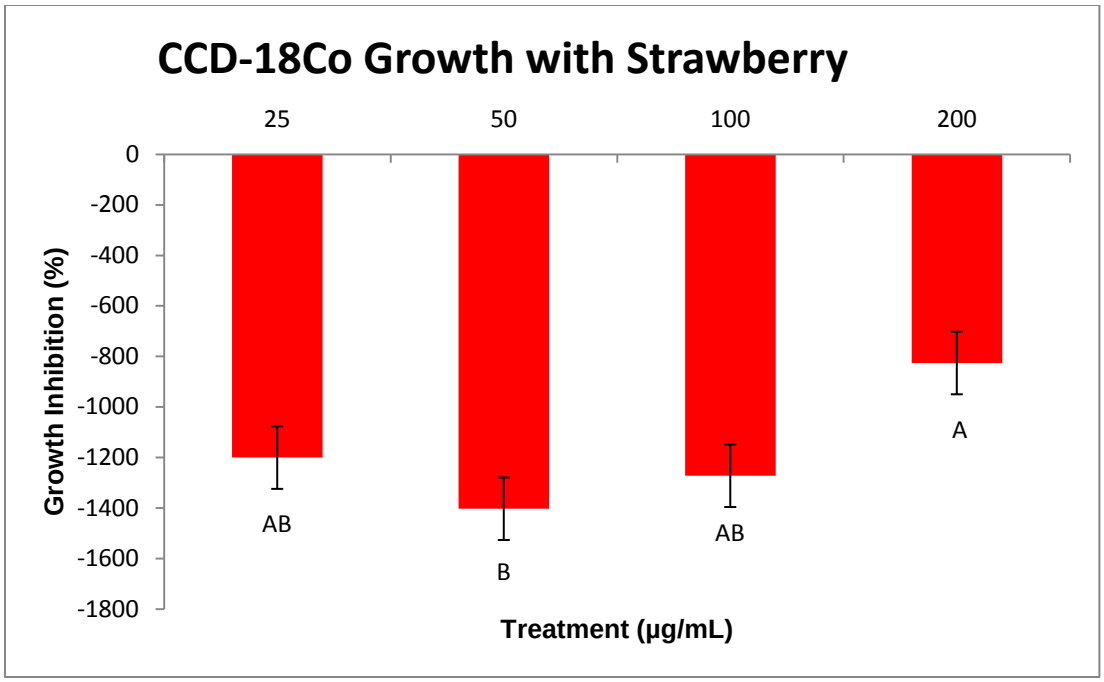


Figure 5.7: Dose dependent growth inhibition of CCD-18Co colon cells when treated with fractionated strawberry anthocyanins at 25, 50, 100 and 200 µg/mL for 48 hours determined by the SRB assay ($P<0.05$).

The results from this study show that anthocyanin extracts from strawberry provide inhibitory effect to the proliferation of HT-29 colon cancer cell line at a concentration of 25-200 µg/mL. Our data suggests that the observed anti-proliferative activity may be associated with anthocyanin treatment, since the treatment of anthocyanin rich extracts disrupted the normal HT-29 cell growth pattern. The proliferation of HT-29 cells was inhibited by the strawberry anthocyanin extracts in a dose dependent manner.

Additionally, the results of this study show that anthocyanin extracts from strawberry provide proliferative affects of CCD-18Co cell line at a concentration of 200 µg/mL or lower. Again, this data suggests that the observed proliferative activity may be associated with anthocyanin treatment, since the treatment of anthocyanin rich extracts disrupted the normal CCD-18Co cell growth pattern.

5.4.4 Caspase-3 Colorimetric Assay:

In order to understand the mechanism of action strawberry anthocyanins had on the growth inhibition of the HT-29 cell line the caspase-3 colorimetric assay was performed. Caspases are cysteine-requiring Aspartate proteases that mediate cell death and are important in the process of apoptosis. Caspase-3 is one of the critical enzymes of apoptosis and is also one of the most studied mammalian caspases. The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405nm. The concentration of the pNA

released from the substrate is calculated from the absorbance values at 405nm or from a calibration curve prepared with defined pNA solutions. A pNA calibration curve (figure 5.8) was carried out to compare caspase-3 activity in µmol of pNA released per minute per mL of cell lysate.

The results of the caspase-3 colorimetric assay showed that strawberry anthocyanins at a concentration of 200µg/mL increased the caspase-3 activity as compared to the control in HT-29 cell lysates by more than 2 fold (figure 5.9). This increase in caspase-3 activity can be directly related to the apoptosis seen in the HT-29 cells when treated with strawberry anthocyanins and may indicate that anthocyanins play a role in the initiation of cell mediated death in human colon cancer.

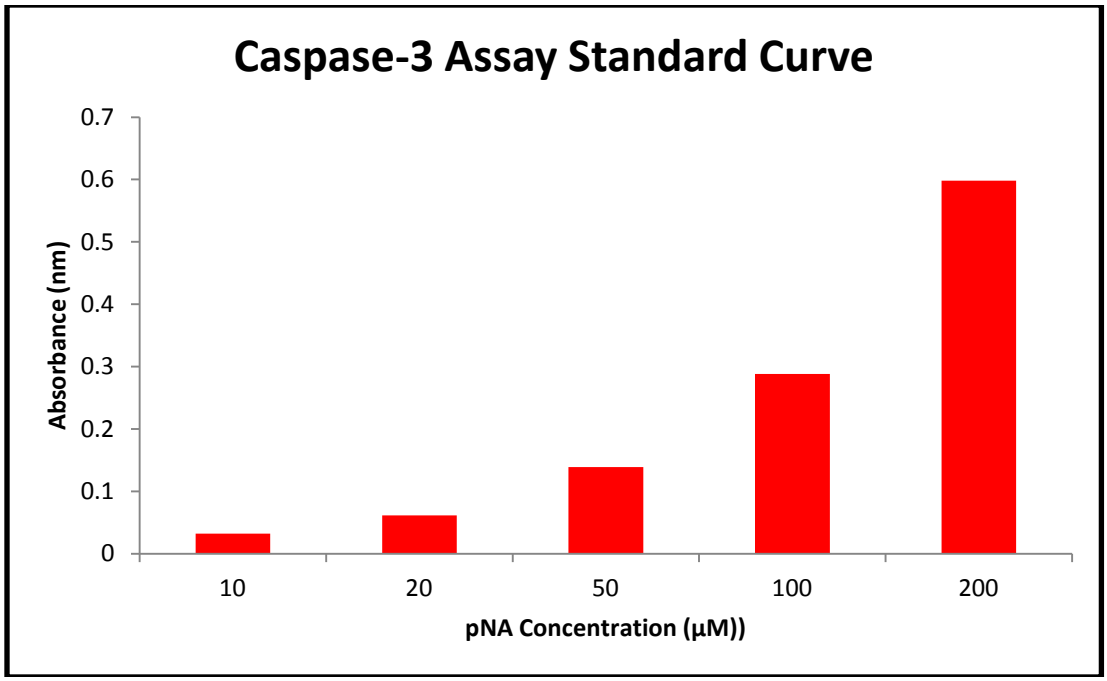


Figure 5.8: p-Nitroaniline (pNA) Calibration Curve

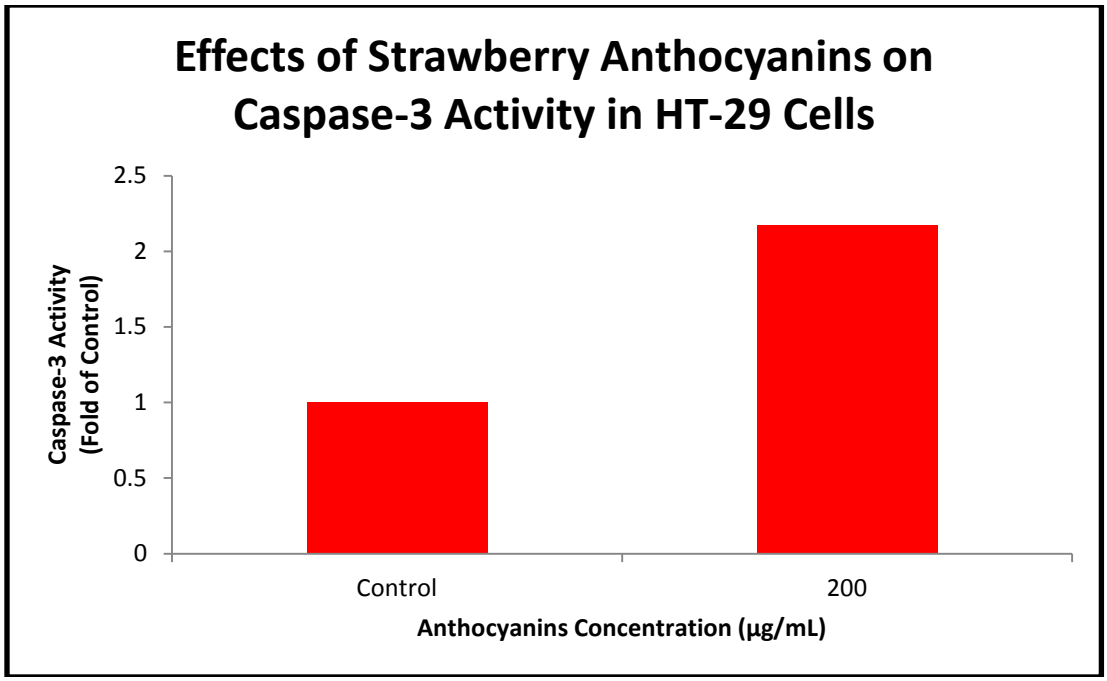


Figure 5.9: Effects of strawberry anthocyanins on caspase-3 activity in HT-29 cells indicating greater than a 2 fold increase in caspase-3 activity.

5.5 CONCLUSION

The results of this study show that anthocyanin extracts from strawberry provide inhibitory effects to the proliferation of HT-29 colon cancer cells when treated with fractionated strawberry extract at concentrations of 25, 50, 100 and 200 µg/mL of 34.7, 65.8, 85.1, and 89.8 percent respectively ($p<0.0001$). Furthermore, the results of this study show that anthocyanin extracts from strawberry provide proliferative affects of the normal colon cell line CCD-18Co at a concentration of 25-200 µg/mL ($p<0.05$). Significant differences were seen between the growth characteristics of colon cancer cell lines as compared to their equivalent normal cell lines ($p<0.0001$). Again, this data suggests that the observed proliferative activity may be associated with anthocyanin treatment, since the treatment of anthocyanin rich extracts disrupted the cancerous HT-29 cell line's growth pattern and enhanced the growth pattern of the CCD-18Co normal colon cell line. The addition of the caspase-3 assay provides information as to what mechanistic role the strawberry anthocyanin extract has in decreasing HT-29 proliferation rates, suggesting strawberry anthocyanins play a role in the up-regulation of caspase-3 and therefore and important role in apoptosis. The *in vitro* chemotherapeutic effect of strawberry anthocyanins may be relevant to future *in vivo* work because when anthocyanins are ingested in the diet they are in direct contact with the epithelial cell layer of the colon during the body's natural digestive cycle and may provide chemoprotection upon contact with the colon.

CHAPTER 6

OVERALL CONCLUSIONS

Strawberries are a natural and abundant fruit that grow in the wild and as cultivars. Strawberries are a rich source of polyphenols, which have anti-inflammatory, antiviral, and antiproliferative properties (Aaby, Skrede, and Wrolstad, 2005). In the present study we further extended the information known about fruit extracts, which in the past have tested an array of phenolic compounds, not specifically anthocyanins. The results of this research add to the information known about strawberry anthocyanins, specifically pelargonidin-3-glucoside and cyanidin-3-glucoside, by investigating their antiviral properties and mechanism of action. Strawberry anthocyanins were found to have powerful viral replication inhibitory and rapid virucidal activities by inactivating the virus directly and blocking the virus's entry into the cell. The strawberry anthocyanin treatments containing 20 µg/mL significantly inhibited herpes simplex virus (HSV-1) by greater than 68% as compared to the control ($p<0.005$). These findings suggest that strawberry anthocyanin extracts could be an effective topical treatment and/or prophylactic agent for oral herpetic infections. The findings also suggest that strawberry anthocyanins inactivate the herpes simplex virus type-1 (strain 17+) by directly inhibiting the virus and by blocking. Because antiviral effects were most noticeable when free virus was directly exposed to the strawberry anthocyanin extract, and less effective once HSV-1 had adsorbed and entered epithelial cells these findings suggest that the strawberry anthocyanin extract has strong antiviral effects that interfere with adsorption or entry into the

host cells and some intracellular activity. The results of this experiment are very useful for future studies that could isolate and identify whether one anthocyanin from strawberry anthocyanin extracts works more effectively as a bioactive anti-HSV-1 agent and also to determine if the bioactive principles in strawberry anthocyanin extract exhibit antiviral effects against additional HSV strains and serotypes (i.e. HSV-2).

The results of this research also found that anthocyanin extracts from strawberry provide inhibitory effects to the proliferation of AGS cells when treated with fractionated strawberry extract at concentrations of 25, 50, 100 and 200 µg/mL of 34.7, 76.1, 92.2, and 87.4 percent respectively ($p<0.0001$). Extracts from strawberry provide proliferative affects of Hs 738.St/Int stomach and small intestine mixed cell line at a concentration of 25-200 µg/mL. Significant differences were seen between gastric and colorectal cancer cell lines as compared to their equivalent normal cell lines. Again, this data suggests that the observed proliferative activity may be associated with anthocyanin treatment, since the treatment of anthocyanin rich extracts disrupted the cancerous AGS cell line's growth pattern and enhanced the growth pattern of the Hs 738.St/Int stomach and small intestine mixed cell line. This research also found that strawberry anthocyanins played a mechanistic role in the up-regulation of caspase-3 activity in AGS cell lysates and therefore contributed to apoptosis in this cancer cell line via mediated cell death. The *in vitro* chemotherapeutic effect of strawberry anthocyanins may be relevant to future *in vivo* work because when anthocyanins are ingested in the diet they are in direct contact with the epithelial

cell layer of the gastrointestinal tract and may provide chemoprotection upon contact with the stomach and gastrointestinal tract.

Lastly, the results of this study found that anthocyanin extracts from strawberry provide inhibitory effects to the proliferation of HT-29 colon cancer cells when treated with fractionated strawberry extract at concentrations of 25, 50, 100 and 200 µg/mL of 34.7, 65.8, 85.1, and 89.8 percent respectively ($p<0.0001$). Extracts from strawberry provide proliferative affects of the normal colon cell line CCD-18Co at a concentration of 25-200 µg/mL ($p<0.05$). Significant differences were seen between the growth characteristics of colon cancer cell lines as compared to their equivalent normal cell lines ($p<0.0001$). Again, this data suggests that the observed proliferative activity may be associated with anthocyanin treatment, since the treatment of anthocyanin rich extracts disrupted the cancerous HT-29 cell line's growth pattern and enhanced the growth pattern of the CCD-18Co normal colon cell line. This research also found that strawberry anthocyanins played a mechanistic role in the up-regulation of caspase-3 activity in HT-29 cell lysates and therefore contributed to apoptosis in this cancer cell line via mediated cell death. The *in vitro* chemotherapeutic effect of strawberry anthocyanins may be relevant to future *in vivo* work because when anthocyanins are ingested in the diet they are in direct contact with the epithelial cell layer of the colon during the body's natural digestive cycle and may provide chemoprotection upon contact with the colon. However, future research is needed to better understand the mechanisms of action regarding strawberry anthocyanin chemoprotection in the body.

The overall conclusion of this research suggests that anthocyanins play an important role in the overall health of the human gastrointestinal tract by providing viral protection from herpes simplex virus type 1 and by playing an important role in cell mediated death or apoptosis of cancerous cells. This is important because it suggests that eating a well balanced diet rich in polyphenols, specifically anthocyanins, may decrease the risk of developing gastrointestinal related infections and cancers.

APPENDIX A

1. RESEARCH OBJECTIVE

Apply the strawberry anthocyanin sample to an in vitro gastrointestinal system that models swine microflora and characterize the resulting metabolites.

2. APPROACH

Gastrointestinal microflora in some animals provides essential nutrients, such as amino acids and certain vitamins, especially in ruminants (Torrallardona et al., 2003). However, for nonruminant omnivores and carnivores the nutritional benefits of the gastrointestinal microflora are not well understood (Torrallardona et al., 2003). It is important to understand the role that the gut microflora plays in the digestion of anthocyanins and their metabolites. Gastrointestinal microflora will be sampled from swine and used to carry out the *in vitro* gastrointestinal system. I propose to characterize the anthocyanins and their breakdown metabolites once exposed to the gastrointestinal microflora of swine. Characterization of the anthocyanins and their metabolites will be done using reverse phase HPLC-MS.

3. MATERIALS AND METHODOLOGY

3.1 Chemicals and Reagents:

Organic Driscoll's brand strawberries were purchased from a local grocery store in Lexington, Kentucky. Certified ACS grade HCL (12N) and ethyl acetate, LCMS grade 99.9% methanol and 99.9% acetonitrile, and HPLC grade 88% formic acid, 99.9% acetone and 99.9% chloroform were obtained from Fisher

Scientific (Fair Lawn, NJ). Reagent grade gallic acid and Folin-Ciocaltaeu reagent were obtained from MP Biomedical (Aurora, OH). All other chemicals were certified ACS grade from Thermo Fisher Scientific, Waltham, MA.

3.2 Extraction of Anthocyanins and other Phenolics from Plant Materials:

The red flesh of strawberries were hand selected using a paring knife and quickly frozen using liquid nitrogen. The frozen strawberry red flesh where then fine powdered using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT) with a 500 mL stainless steel container in the presence of liquid nitrogen (Giusti and Rodriguez-Soana 1999). Strawberry flesh powder was stored at -20˚C until used for sample extraction.

Strawberry flesh anthocyanins and other phenolics were extracted by blending the powders with 30mL of 70% acetone and 30% 0.1% HCl acidified water (v/v) for 5 minutes using a Waring Commercial Laboratory blender (Waring Laboratories, Torrington, CT). The liquid was subsequently passed through Whatman No. 4 and No. 1 filter paper (Whatman Inc., Florham, NJ) using a Buchner Funnel (Thermo Fisher Scientific, Waltham, MA). This process was repeated until the filtrate was free of haze and all of the pigment had been extracted from the powdered strawberry flesh particles (Rodriguez-Soana and Wrolstad, 2001). The strawberry anthocyanin and other phenolics filtrate was then placed into a separatory funnel (Thermo Fisher Scientific, Waltham, MA) and partitioned with 2 volumes of chloroform. The solution was mixed and left to separate for 24 hours at 4˚C in the dark to ensure adequate separation. The top

acetone/water layer containing anthocyanins and other phenolics was collected and the bottom chloroform/fat layer was discarded properly in hazardous waste containers. The residual acetone in the sample was evaporated using a Buchi Rotavapor R-215 rotary evaporator (Thermo Fisher Scientific, Waltham, MA) at 40˚C. The solution was brought to 25 mL in a volumetric flask using LCMS grade water.

3.3 Fractionation and Concentration of Anthocyanin Sample:

Anthocyanin phenolics from strawberries were concentrated by using Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corporation, Milford, MA). The anthocyanin filtrate was passed through the Sep-Pak® C_{18} Vac solid cartridge (20cc, 5g sorbent; Waters Corporation, Milford, MA) 10 mL at a time. Anthocyanins and other phenolics were bound to the C_{18} cartridge, while sugars and other polar compounds were removed with 30 mL of 0.01% HCl acidified water, followed by 10 mL of hexane to remove the residual water. The non-anthocyanin phenolics were eluted with 20 mL of diethyl ether followed by 20 mL of ethyl acetate. The anthocyanin phenolic fraction was eluted with 30 mL of 0.01% HCl acidified methanol followed by 10 mL of 0.01% HCl acidified HPLC grade water. Excess methanol in the anthocyanin phenolic filtrate was evaporated in the Buchi Rotavapor R-215 rotovapor (Thermo Fisher Scientific, Waltham, MA) at 40˚C and the anthocyanin fraction was re-dissolved in 0.01% HCl acidified HPLC grade water. The highly concentrated anthocyanin extract

was then quickly frozen using liquid nitrogen and lyophilized using a Labconco Freezone[6] lyophilizer into a fine dry powder (Labconco, Kansas City, MO).

3.4 Monomeric and Polymeric Analysis:

Monomeric anthocyanin content was determined using the pH differential method (Giusti and Wrolstad, 2005). This method uses the spectral difference that anthocyanins exhibit at different pH values. Dilution factor was determined by diluting the strawberry anthocyanin extract in 0.025 M potassium chloride buffer, pH 1.0, until the absorbance was within the appropriate spectrophotometer range (for most spectrophotometers absorbance should be less than 1.2 nm). The strawberry anthocyanin extract was then appropriately diluted using pH 1.0 and pH 4.5 buffer. Solutions were allowed to sit at room temperature for at least 15 minutes in the dark. 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 cyanidin-3-glucoside equivalents, using a molecular weight of 449.3 and an extinction coefficient of 26,900 L cm^{-1} mg^{-1} (Giusti and Wrolstad, 2005). Absorbance was determined in replicates of three.

Polymeric anthocyanin content was determined using the polymeric method (Wrolstad 1999). Appropriate dilutions were determined using pH 1.0 buffer and 1-cm cuvettes were prepared with 2.8 mL of diluted sample in water to each of two cuvettes. 0.2 mL of water was added to one of the cuvettes and 0.2 mL of bisulfate solution to the other and allowed to equilibrate for 15 minutes

before reading at 420 nm, 520 nm, and 700 nm. Polymeric anthocyanin content was calculated using the following equations:

$$\text{Color Density} = [(A_{420nm} - A_{700nm}) + (A_{\lambda\ vis\ max} - A_{700nm})] \times DF$$

$$\text{Polymeric Color} = [(A_{420nm} - A_{700nm}) + (A_{\lambda\ vis\ max} - A_{700nm})] \times DF$$

$$\text{Percent Polymeric Color} = (\text{Polymeric Color}/\text{Color Density}) \times 100$$

Where DF is the dilution factor and absorbance was determined in replicates of three.

3.5 Total Phenolics:

Total phenolics were measured using a microscale protocol for Folin-Ciocalteu Colorimetry (Waterhouse 2005). Cuvettes were prepared with 20 µL of sample/standard, 1.58 mL water and 100 µL 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 µL) was added and samples were allowed to stand at room temperature for 2 hours. 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.

3.6 LC/MS Analysis:

Strawberry anthocyanin samples were analyzed using a Thermo Finnigan LTQ high performance liquid chromatograph (HPLC) (ThermoFinnigan; Waltham,

MA). Positive ion electrospray ionization (ESI) mass spectra were obtained on the Thermo Finnigan LTQ coupled to a Dionex Ultimate 3000 capillary LC system. The column was 20cm x 150um packed with Kromasil C18 (3.5um particle size) (AkzoNobel, Separation Products, Bohus, Sweden). Proteome Discoverer Software 1.1.0 was used on this system (Thermo Fisher Scientific; Waltham, MA). All samples were filtered through 13 mm 0.45µm polypropylene filters (Thermo Fisher Scientific, Waltham, MA) prior to HPLC/MS analysis. Separation of strawberry extract anthocyanins was achieved using a linear gradient elution from 5% to 60%. Solvent A was 0.1% (v/v) formic acid in water and B was 0.1% (v/v) formic acid in acetonitrile. The flow rate was 0.8 mL / min and an injection volume of 50 µL was used. Spectral data was collected using the Dionex Ultimate 3000 capillary LC system from 250-700 nm. Absorbance of anthocyanins was monitored at 280 and 520 nm.

A 1.5 uL / 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 ºC; 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 and Selective Ion Monitoring (SIM) was used to search for the molecular ions of the common anthocyanins throughout the analysis.

4. PRELIMINARY DATA

4.1 Characterization of Strawberry Anthocyanin Sample:

Strawberry anthocyanins were identified by comparing retention times, order of elution and UV-visible spectra to previous literature (Lopes-da-Silva and others 2001). The five major anthocyanins identified in strawberry (Figure A.1 and Table A.1) include: peak 1 cyanidin-3-glucoside (~23.3%), peak 2 cyanidin-3-rutinoside (~4.7%), peak 3 pelargonidin-3-glucoside (~60.6%), peak 4 pelargonidin-3-rutinoside (~7.1%), and peak 5 cyanidin-3-malonylglucose-5-glocoside (~4.3%). The strawberry anthocyanin profile contained only cyanidin and pelargonidin derivatives. Additionally, the majority of the strawberry anthocyanin profile is made up of cyanidin-3-glucoside (peak 1) and pelargonidin-3-glucoside (peak 3) which combined comprises over 83% of the anthocyanin profile. Mass spectra data was also gathered as shown in figure A.2 below.

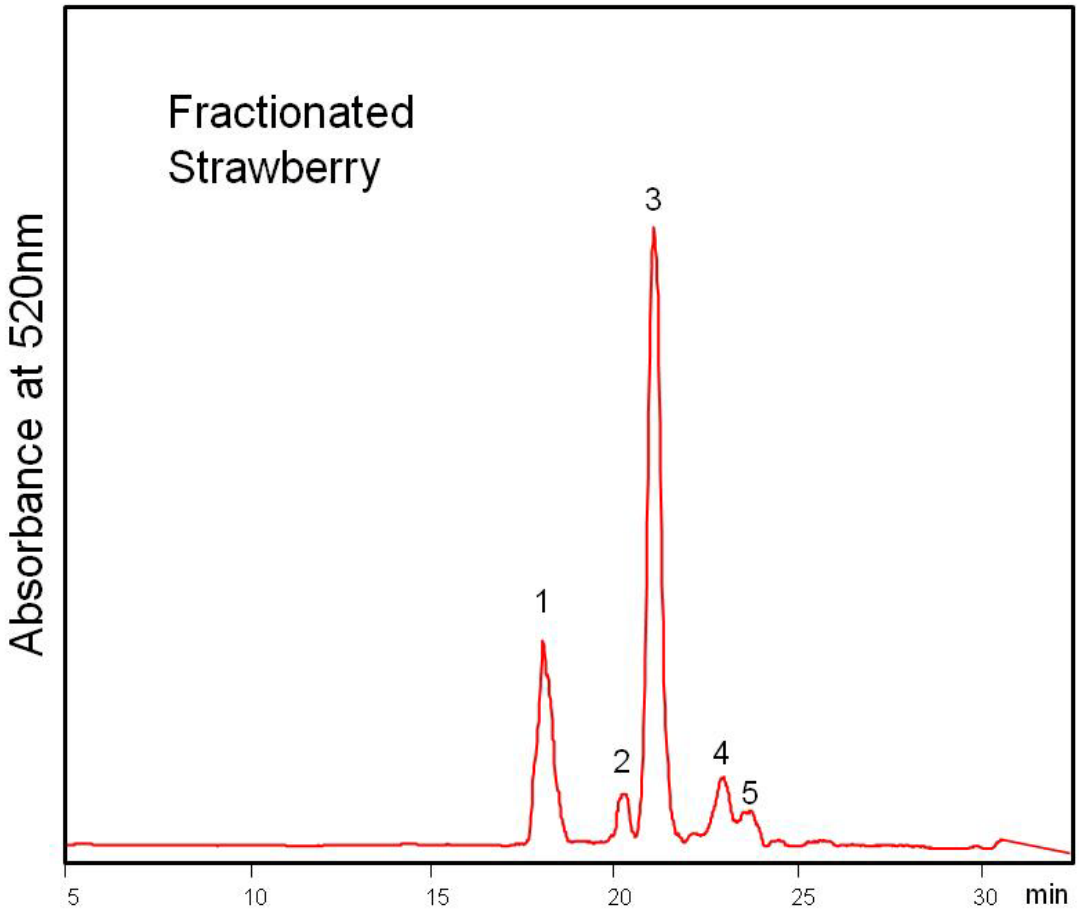


Figure A.1: Chromatograph of fractionated strawberry extract. Anthocyanin profile observed at 520 nm. Peak 1 cyanidin-3-glucoside, peak 2 cyanidin-3-rutinoside, peak 3 pelargonidin-3-glucoside, peak 4 pelargonidin-3-rutinoside, and peak 5 cyanidin-3-malonylglucose-5-glocoside.

Fractionated Strawberry			
Peak	Anthocyanin	Retention Time (Min)	%Area 520nm
1	cyanidin-3-glucoside	17.7	23.3
2	cyanidin-3-rutinoside	19.8	4.7
3	pelargonidin-3-glucoside	20.6	60.6
4	pelargonidin-3-rutinoside	22.5	7.1
5	cyanidin-3-malonylglucose-5-glucoside	23.3	4.3

Table A.1: Fractionated Strawberry Anthocyanins.

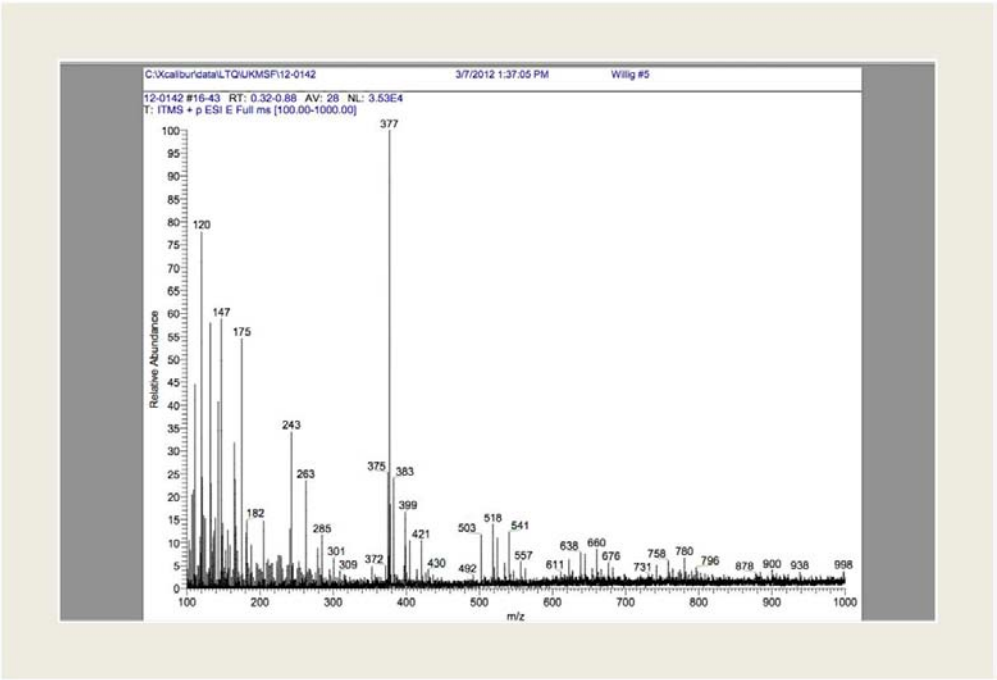


Figure A.2: Mass spectral data on strawberry anthocyanin metabolites after swine microflora exposure.

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Summary of Qualifications and Education

Self-starter, disciplined, confident, and goal oriented. Extensive leadership background with ability to drive bottom-line performance. Strong research background with emphasis in the area of functional foods and phytochemicals. Excellent written and oral communication skills. Ability to motivate members of a team toward common strategic goals.

Master of Science in Food Science and Nutrition
The Effect of Anthocyanin Acylation on the Inhibition of HT-29 Colon Cancer Cell Proliferation
The Ohio State University
March - 2009

Bachelor of Science in Food Science and Technology
University of Kentucky
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Research Experience

University of Kentucky
Lexington, KY
2009-present
Graduate Research Assistant

D.D.Williamson & Co.
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May 2009-August 2009
Natural Colorants Intern

The Ohio State University
Columbus, OH
2006-2009
Graduate Research Assistant

Continental Mills
Hopkinsville, KY
May 2005-August 2005
Quality Assurance Intern

Oral/Poster Presentations

Institute of Food Technologists 2010 Annual Meeting: Chicago, Illinois. Determination of the Antimicrobial Effect of Pungent Spices and Herbs when applied in Combination with the Antimicrobial agent Allicin. The University of Kentucky Department of Animal and Food Science. Jennifer A. Willig, Joe O'Leary, Melissa C. Newman.

Institute of Food Technologists 2009 Annual Meeting: Anaheim, California. "The Effect of Anthocyanin Acylation on the Inhibition of HT-29 Colon Cancer Cell Proliferation" The Ohio State University Department's of Food Science and Human Nutrition. Jennifer A. Willig, Joshua A. Bomser, M. Monica Giusti.

The Ohio Agricultural Research and Development Center poster competition, 2009: Columbus, Ohio. "The Effect of Acylation on the Inhibition of HT-29 Colon Cancer by Anthocyanin Pigments" The Ohio State University Department of Food Science and Human Nutrition. Jennifer A. Willig, Joshua A. Bomser, M. Monica Giusti.

Honors, Awards, and Activities

- Institute of Food Technologist Student Association (IFTSA) President 2010-2013
- Ohio State University Food Product Development Team IFTSA Finalist 2008
- Stolle Daughters Scholarship Recipient 2008
- University of Kentucky Lancaster Aquatic Center's Employee of the Year Award 2006
- Kentucky Soil Conservation Scholarship Recipient 2005
- University of Kentucky College of Agriculture Scholarship Recipient 2005

Professional Affiliations

- Institute of Food Technologists