Effect of Black Raspberry Extracts on Colon Cancer Cell Proliferation

Thesis

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By

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Abstract

Diets rich in colorful fruits and vegetables have been found to correlate with lower incidence of certain cancers. In particular, black raspberries have been shown to inhibit multiple stages of oral, esophageal and colon cancer. At The Ohio State University, the mechanisms by which black raspberry compounds are chemoprotective are under exploration. The objective for this study was to evaluate how compositional variability impacts the antiproliferative activity of 75 black raspberry samples using an *in vitro* colon cancer cell model.

Polyphenolics from black raspberries were extracted, semi-purified and lyophilized to obtain black raspberry extract powders. Human HT-29 colon cancer cells grown in 96-well plates were treated with freeze-dried black raspberry extracts at concentrations of 0.6 and 1.2 mg extract/mL media. Percent growth inhibition for each concentration of the extracts was determined using sulforhodamine B assay. Chemical composition of the extracts was also monitored by spectrophotometric and chromatographic techniques.

All samples tested significantly inhibited the growth of HT-29 colon cancer cells in a dose-dependant manner. Inhibition of cancer cell proliferation obtained with different extracts ranged from 33-118%, with values higher than 100% inhibition indicating cytotoxic effects of the extracts.

Cultivar, growing location and maturity all affected compositional characteristics of black raspberries, and these changes affected their ability to inhibit cancer cell proliferation. Data suggests that interaction of different berry components, rather than one class of compounds may be responsible for the inhibitory action of black raspberries on HT-29 colon cancer cells. These data may aid basic scientists, horticulturists, as well as the general population in the identification of fruits with the greatest potential to prevent and/or reduce the development of chronic disease.

Dedication

Dedicated to my mom. You will always be my hero!

Acknowledgments

This research reflects the dedication and support of many people for whom I am truly thankful. I would like to thank my advisor Dr. Monica Giusti for allowing me the opportunity to combine my two passions of nutrition and research. I would also like to thank her for her advice, patience and support throughout the project. I would like to thank Dr. Joshua Bomser for allowing me to do the *in vitro* research for this project in his lab and for his encouragement throughout the project. To both Dr. Giusti and Dr. Bomser, I would like to thank them for giving me the opportunity to be involved in cancer research and helping me discover a new interest. I would also like to thank Dr. Jeff Culbertson for being a member of my thesis committee. I would like to acknowledge Dr. Joseph Scheerens for sharing his horticultural knowledge with me and answering any questions I might have had about this project. I would like to thank Nam Hee Kim for helping me with the statistical analysis of the data. Additionally, I would like to thank my lab colleagues for their valuable feedback and advice. Especially, Taylor Wallace, Fer Polit, Fabiola Gutierrez and Ray Lombardi for taking the time to answer any questions I might have had and training me on lab techniques and equipment.

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Field of Study

Major Field: Food Science and Nutrition

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Chapter 1: Introduction

Colon cancer is the third most common cancer and the second leading cause of cancer death among both men and women in the United States (Zeller, 2008). The American Cancer Society estimated that in 2008 there were about 108,000 new cases of colon cancer. Among men and women in the United States 1 in 19 will be diagnosed with colon cancer during their lifetime, with this risk being slightly higher for men (National Cancer Institute Website, 2009). The main risk factors that contribute to a person developing colon cancer are heredity, age, a high fat diet, excessive alcohol consumption, obesity, diabetes and smoking (Zeller, 2008). Recently, food-based approaches for treatment of cancer have been explored. Berry extracts rich in anthocyanins have been suggested as potential agents to reduce the risk of colon cancer by inhibiting proliferation of human colon cancer cells *in vitro*, while having little effect on the growth of non-transformed colon epithelial cells (Malik et al., 2003). At The Ohio State University the mechanisms by which black raspberry compounds are chemoprotective are under exploration.

Anthocyanins, which are a class of flavonoid compounds, are responsible for the bright attractive red, orange, purple and blue colors of the leaves, stems, roots, flowers and fruits in plants. In the plant, anthocyanins act as a defensive shield against UV rays,

bacteria, viruses, fungi and oxidative reactions (Kalt, 2005; Kong et al., 2003). In humans, anthocyanins have been shown to have a wide range of bioactivities including being potent antioxidants, and possessing anticancer and anti-inflammatory properties (Seeram, 2008). Berries are one of the richest dietary sources of anthocyanins for humans and black raspberries have one of the highest anthocyanin contents among the berries (Tian et al., 2006). Studies indicate that black raspberry phenolic antioxidants, especially the anthocyanins, may play an important chemoprotective role (Stoner et al., 2007).

Environmental and horticultural (production and post-harvest) factors are known to have an impact on the chemoprotective properties of fruits (Ozgen et al, 2008). In this study, the effects of growing location, cultivar and fruit maturity on black raspberry composition and its ability to inhibit cell proliferation were examined.

This research is part of a large multidisciplinary effort to develop an investigative system to evaluate the interaction of plant-derived compounds as potential bioactive dietary intervention agents. The objective for this study was to evaluate the polyphenolic variability of 75 different black raspberry samples and test their bioactivity by measuring their inhibition of cell growth proliferation on a colon cancer *in vitro* cell system. Samples included berries from 3 different cultivars, grown in 7 different locations and harvested at 3 different maturity stages. The hypothesis for this study was that changes in composition caused by the differences due to cultivar, location and/or maturity would result in different bioactivity of the fruit.

Chapter 2: Literature Review

2.1 Colon Cancer:

2.1.1 The Prevalence of Colon Cancer:

Colon cancer is the third most common cancer and the second leading cause of cancer death among both men and women in the United States (Zeller, 2008). The colon, located in the lower region of the gastrointestinal tract is about 5 feet long and is where some nutrients from food are absorbed and waste is stored until it moves to the rectum for removal. Colon cancer begins in the innermost layer of the colon and can extend through to the other layers. The most common type of colon cancer, causing over 95% of this disease, is adenocarcinoma and it can form in adenoma polyps on the lining of the colon (American Cancer Society, 2009). In 2008, the American Cancer Society estimated that there were about 108,000 new cases of colon cancer. Of the people diagnosed with this disease, more than 90% are over the age of 50, with the average age of diagnosis being 72 (National Cancer Institute Website, 2009).

2.1.2 Development of Colon Cancer and its Five Stages:

As with all cancers the problem first occurs in the cells. Normal cells grow, divide and die as the body needs them, but when abnormal cells start to form this orderly

process no longer works correctly. These abnormal cells form when they are not needed and do not die when they should, causing a mass of tissue to form out of the excess cells present called a tumor (National Cancer Institute, 2009).

In most cases, colon cancer begins as a polyp, which is a growth of tissue on the lining of the colon that extends towards its center. There are five stages of colon cancer used to describe the spread of this disease through the layers of the colon wall (**Figure 2.1**). At stage 0, abnormal cells are found only in the innermost lining of the colon. At stage I, the abnormal cells have formed a tumor that has grown into the inner wall of the colon. At stage II, the tumor extends more deeply into or through the wall of the colon. At this point it may have also invaded nearby tissue, but it has not spread to the lymph nodes yet. At stage III, the cancer has spread to nearby lymph nodes, but not to other organs. At Stage IV, the cancer has spread to other organs (National Cancer Institute, 2009). The survival rate for colon cancer greatly depends on the stage of the cancer. At stage I there is a 93% survival rate, but at stage IV there is only an 8% survival rate.

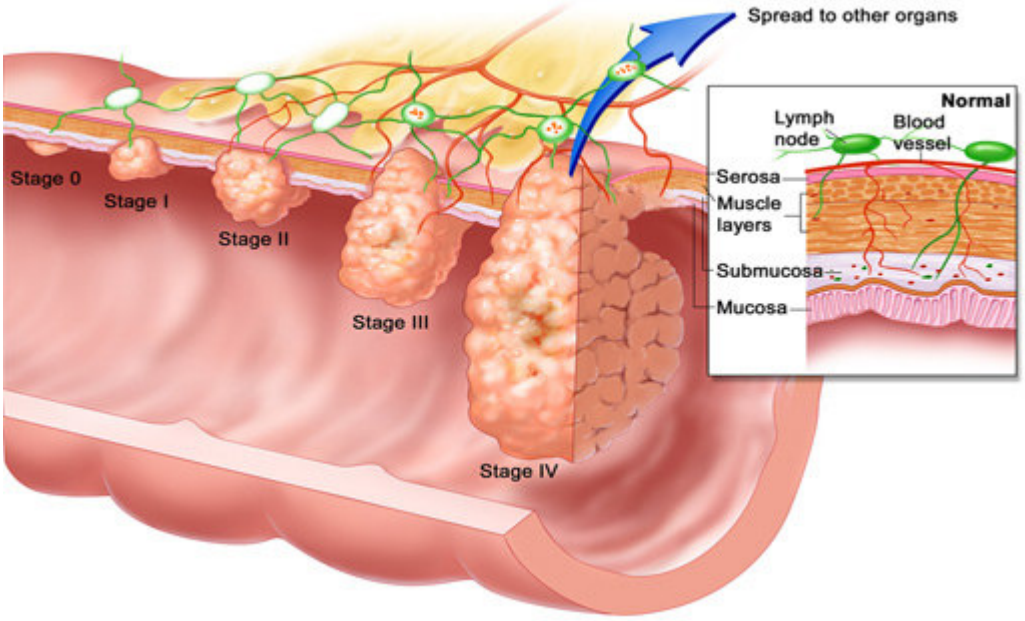

Figure 2.1: The five stages of colon cancer (Source: Nation Cancer Institute Website, 2009).

2.1.3 Common Colon Cancer Risk Factors:

There are several common risk factors that can contribute to a person developing colon cancer. These factors can be divided into two categories: those that can not be controlled (uncontrollable) and those that can be controlled (controllable). Uncontrollable risk factors include heredity, age, sex and race. Controllable risk factors that have been linked to colon cancer include a high fat diet, excessive alcohol consumption, obesity, physical inactivity, Type 2 diabetes and smoking (Stoner et al, 2007). Of these controllable risks factors evidence has shown that about 30-40% of all cancers are linked to poor diets and lack of physical activity (Insel et al., 2002).

2.1.4 Food-based Approaches for Treating Colon Cancer:

Every year about $8.4 billion is spent on colon cancer treatment in the United States. The three main treatment options for patients with colon cancer are surgery, chemotherapy and radiation therapy. The best treatment option for the patient depends on their stage of cancer, whether the cancer has recurred, and the patient's general health (National Cancer Institute Website, 2009).

There are two diet-related strategies involved in cancer research; they are cancer chemoprevention and dietary cancer prevention. Cancer chemoprevention has been defined as the pharmacologic intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis, or prevent the development of invasive cancer (Schatzkin and Kelloff, 1995; Singletary, 2000). Traditionally, beneficial effects of food-derived chemoprotective agents had been assessed either individually or as a few putative active constituents and often at pharmacological doses. Recently, whole food-based approaches for treatment and prevention of cancer have been explored due to the strong correlation between diets rich in colorful fruits and vegetables and lower incidence of certain cancers (Kresty et al., 2001). This type of food-based approach is defined as dietary cancer prevention, which involves modifications in food consumption patterns necessary to decrease cancer development (Schatzkin and Kelloff, 1995; Singletary, 2000).

Many scientists are currently investigating the phytonutrients present in different fruits, as well as the interaction among plant constituents. A study done by Harris and colleagues (2001) compared the effects of freeze-dried whole black raspberries to the effects of single compounds on the inhibition of colon cancer in rats. In the study, a 10%

freeze-dried black raspberry diet that contained 167, 200, 72 and 21 mg/kg of calcium, ellagic acid, β-sitosterol and ferulic acid, respectively was found to inhibit tumor multiplicity in the rats by 71%. Similar studies had to use individual doses of 500, 8,000, 2,000 and 250 mg/kg, respectively of these compounds in order to be as effective as the 10% freeze-dried black raspberry diet at inhibiting the colon cancer. These finding are very positive because they suggest that fruits that contain low doses of multiple compounds could be used in place of large doses of single compounds to provide chemoprotective properties, thereby reducing the possibility of reaching toxic levels of large doses of single compounds.

2.1.5 Cellular Models of Colon Cell Carcinoma:

Cancer cell lines are the most commonly used among the various possible experimental models of cancers because they allow complex biological systems to be studied in a simplified way. The use of human cancer cell lines to study the inhibitory effects of high phytonutrient containing fruits against carcinogenesis is desirable because the cells are pure, easily propagated and genetically manipulated, and they provide reproducible results when used with the same protocol and at the same stage. Cancer cell lines might also be one of the closest equivalents to tumor initiating cells due to the fact that they have the ability to retain the oncogenic mutation that initiated the cancer. It has been found that colon cancer cell lines are able to retain as much as 99% of the sequence mutations from the original tumors (Staveren et al., 2009).

Colon cancer is one of the most extensively studied epithelial tumor types. This type of cancer is usually studied based on the analysis of a primary tumor because it

provides a full spectrum of genetic abnormalities (Gerdes et al., 2000). There are several primary human colon cancer cells lines including Caco-2, HCC2998, HCT 116 and HT-29 (Flatmark et al., 2003).

For this study, the well established HT-29 cell line derived from a human Caucasian colon adenocarcinoma, grade II, was used. The HT-29 cell line was the first human colon carcinoma cell line and it was established in 1975 by Fogh and Temp (Huet et al., 1987). HT-29 cancer cells have the ability to undergo differentiation and the level of differentiation of the cancer cells determines their tumor grade. How different or similar the tumor cells are from the normal cells of the same tissue type determines their level of differentiation. Cells can range from well-differentiated cells that resemble normal cells (low grade) to undifferentiated cells that do not look like normal cells (high grade) (National Cancer Institute Website, 2009).

2.2 Black Raspberries:

Raspberries are thorny bramble plants that belong to the genus *Rubus* in the Rosaceae family (Crandall, 1995; Jennings et al., 1991). They are also considered caneberries because they grow on woody stems called canes. Raspberries may be yellow, red, purple or black. Typically, red raspberries ripen first, followed by black raspberries, and then the purple and yellow raspberries are the last to ripen and they usually do so around the same time. Raspberries, along with blackberries, are not considered true berries. They are actually classified as aggregate fruits because they are clusters of many individual sections each containing one seed (The North American Bramble Growers

Association Website, 2009). Raspberries are rich in vitamins A, C and E, folic acid, iron and potassium, as well as anthocyanins and other phytochemicals.

Of the raspberries, the most talked about type in recent years has been the black raspberry due to its substantial health benefits (Tulio et al., 2008). Black raspberries (*Rubus occidentalis* L.) are native to eastern North America. Traditionally, black raspberry roots were used to make tea to treat stomach aches, while the leaves were boiled in water and used to treat sores. Today, black raspberries are being widely studied for their chemoprotective properties.

2.2.1 Composition of Black Raspberries:

Black raspberries are known for their extremely high phytonutrient content (Stoner et al., 2007). Early black raspberry studies focused on ellagic acid as the primary bioactive compound in this fruit (Ozgen et al., 2008). Ellagic acid ($C_{14}H_6O_8$), a phenolic compound found mainly in a fruit's leaves and seeds, exists in berry fruits in free, glycosylated and acylated forms (Zhao, 2007; Scheerens et al, 2003; Mass et al, 1991). This compound became of interest in the last decade due to its ability to act as a chemoprotective agent (Maas et al., 1991). More recently, research has focused not only on the ellagic acid found in black raspberries but also its other potentially protective compounds (**Table 2.1**) including its vitamins, minerals, phenolics and sterols. There has been an emphasis on the polyphenolic anthocyanins in black raspberries due to them being found in the greatest quantities (1770 mg/100g dry weight) when compared to the other chemoprotective compounds present (Harris et al., 2001).

Dietary Components	Lyophilized Black Raspberry Analysis	Units
Vitamins/provitamins:		
Vitamin E	10.80	IU/100g
Folic Acid	0.13	mg/100g
β-Carotene	0.12	mg/100g
Vitamin C	<0.10	mg/100g
α-Carotene	<0.02	mg/100g
Minerals:		
Calcium	167.00	mg/100g
Magnesium	147.00	mg/100g
Selenium	<0.01	mg/100g
Total Phenolics	5938.00	mg/100g
Others:		
Anthocyanins	1770.00	mg/100g
Ellagic acid	200.00	mg/100g
β-Sitosterol	72.40	mg/100g
Ferulic acid	21.00	mg/100g
p-Coumaric acid	6.72	mg/100g
Campesterol	4.60	mg/100g
Stigmasterol	<3.00	mg/100g
Zeaxanthin	<0.04	mg/100g
Lutein	<0.03	mg/100g

Table 2.1: Levels of nutrients and potential chemoprotective components in lyophilized whole black raspberries (Source: Harris et al., 2001; Kresty et al., 2006).

2.2.2 Black Raspberry Chemoprotective Studies:

Black raspberries have been shown to inhibit multiple stages of oral, esophageal and colon cancer both *in vitro* and *in vivo* (animal studies and clinical trials). In an *in vitro* study done by Rodrigo and others (2006) freeze-dried black raspberry ethanol extracts were used to determine their antiproliferative effects on human oral squamous cell carcinoma tumors. The cells were treated with 10, 50, or 100 µg black raspberry ethanol extract/mL of media and at 24, 48 and 72 hr cells were harvested and counted. The results showed that the freeze-dried black raspberry ethanol extract treatments inhibited the growth of the oral cancer cells in a dose-dependent manner.

An *in vivo* study by Kresty and colleagues (2001) was done to determine if the consumption of freeze-dried black raspberries had an effect on esophageal cancer in rats. In the study, male rats were fed either 5 or 10% freeze-dried black raspberry diets after esophageal tumorigenesis was induced for 25 weeks. At 25 weeks the animals were sacrificed and their tissues were harvested for analysis. The study concluded that both the 5 and 10% freeze-dried black raspberry diets significantly reduced tumor multiplicity in the rats by 62 and 43%, respectively.

A similar experiment was done by Stoner and coworkers (2007) to see if the consumption of black raspberries would have an effect on colon cancer in rats. In this study, rats were induced with the colon cancer and then fed a freeze-dried black raspberry diet of 0, 2.5, 5 or 10%. The freeze-dried black raspberry diets of 2.5, 5 and 10% significantly reduced tumor multiplicity by 42, 45 and 71%, respectively.

In a six month pilot study 10 female and male patients with Barrett's esophagus (a premalignant esophageal condition) consumed 32 or 45 g, respectively, of freeze-dried

black raspberries daily as a slurry in water (Kresty et al., 2006; Stoner et al., 2007). The purpose of the study was to determine if the addition of the freeze-dried black raspberries would help reduce the levels of urinary markers of oxidative stress in these patients. The results concluded that daily consumption of the freeze-dried black raspberry slurry did in fact lead to a reduction in the urinary excretion of two markers of oxidative stress, 8-epi-prostaglandin F2α and 8-hydroxy-2′ -deoxyguanosine, among patients with Barrett's Esophagus. Within the 10 patients, 60% had a significant reduction in 8-epi-prostaglandin F2α levels, while 50% had significant reductions in 8-hydroxy-2′ -deoxyguanosine levels. These findings are important because higher levels of urinary markers significantly increase the risk of patients with Barrett's esophagus developing esophageal adenocarcinoma.

There are many therapies out there that have showed the ability to reduce the growth of cancers, but they are often accompanied by significant toxicities (Rodrigo et al., 2006). It is because of this that it is so vital to find nontoxic, effective treatments to reduce the growth of different types of cancers. Black raspberries are a great option due to their extremely high phytonutrient content and their high tolerability of 45 g per day in humans (Stoner et al., 2005).

2.2.3 Molecular Mechanisms Involved in the Chemoprotective Properties of Black Raspberries:

Transcription factors, activator protein 1 (AP-1) and nuclear factor kappa B (NFκB), are well-known for their roles in cancer formation (**Figure 2.2**). Overexpression of AP-1 and/or NFκB are harmful due to the fact that they regulate the transactivation of

the target genes cyclooxygenase-2 (COX-2) and Vascular Endothelial Growth Factors (VEGF), which in turn regulate cellular proliferation, apoptosis, angiogenesis, and tumor invasion and metastasis**.** Black raspberry extracts have been shown to inhibit both AP-1 and NFκB *in vitro* and *in vivo* by impairing the signal transduction pathways involved in their activations (Lu et al., 2006).

The pathways involved in AP-1 activation are the mitogen-activation protein kinase (MAPK) pathways. The MAPK pathways are important for regulating proteins associated with cell cycle and cellular differentiation. Within the MAPK family there are three signal transduction pathways including extracellular signal-regulated kinase (ERK), Jun-N-terminal kinase (JNK) and p38 kinase. One of these three kinases is activated in the cytosol of a cell when different growth factors (also called signaling molecules) including epidermal growth factors (EGF), platelet-derived growth factors (PDGF), fibroblast growth factors (FGF), insulin or insulin-like growth factor 1 (IGF-1) bind to a receptor tyrosine kinase (RTK) on the surface of the cell. Activation of the kinase causes it to translocate into the nucleus where it in turn activates many transcription factors including AP-1. In the MAPK cascade a mutation in the receptor or a protein due to carcinogens or oncoproteins can lead to the overexpression of AP-1 (Lodish et al., 2007). Black raspberry extracts have been shown to impair the activity of ERK, JNK and p38 kinase pathways leading to the activation of AP-1 and therefore leading to the down-regulation of COX-2 and VEGFs (Huang et al., 2002).

The pathway involved in the activation of NFκB is the IκB kinase (IKK) pathway. This pathway is activated in the cytosol by different signaling molecules including tumor necrosis factor α (TNF-α), bacteria or other pathogens that bind to either a TNF-α,

interleukin 1 (IL-1) or toll-like receptor on the surface of the cell. Activation of IKK causes it to phosphorylate IκBα, targeting it for degradation. Degradation of IκBα leads to the release of NFκB, which translocates into the nucleus where it activates the transcription of many anti-apoptotic genes (Lodish et al., 2007). Mutations in the IKK pathway can lead to the overexpression of NFκB causing it to act as a tumor promoter by suppressing apopotosis (Lin and Karin, 2003). Black raspberry extracts have been shown to inhibit the phosphorylation of IκBα, therefore leading to the down-regulation of NFκB (Lu et al., 2006; Hecht, 2006).

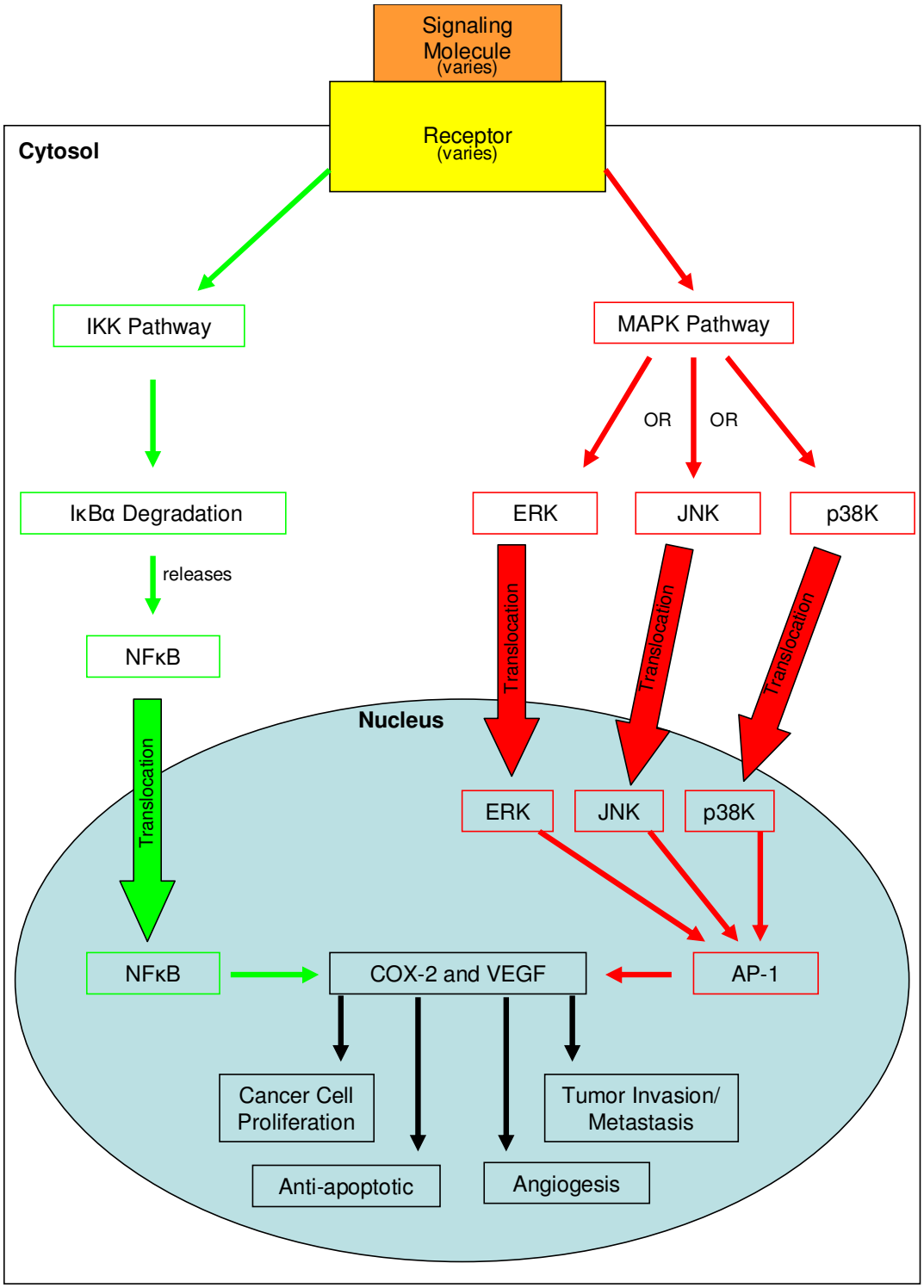

Figure 2.2: Basic model of the two main molecular mechanisms involved in the formation of cancer (Source: Lodish et al., 2007).

2.3 Anthocyanins:

Anthocyanins are the class of polyphenol flavonoid compounds responsible for the bright attractive red, orange, purple and blue colors in many fruits and vegetables. In plants, an anthocyanin's ability to impart color plays an important role in attracting animals for pollination and seed dispersal. They also serve as antioxidants and antibacterial agents for plants (Kong et al., 2003). In humans, anthocyanins have been shown to have a wide range of bioactivities including being potent antioxidants, and possessing anticancer and anti-inflammatory properties (Seeram, 2008).

2.3.1 Anthocyanin Chemical Structure:

Anthocyanins are one of the most widely distributed plant pigments with over 600 structures reported in literature (Andersen, 2002). These water soluble pigments are classified as flavonoids because of their $C_6C_3C_6$ carbon skeleton. When there are no sugar moieties attached to this structure it is known as an anthocyanidin or anthocyanin aglycone. The six most common anthocyanidin structures are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (**Table 2.2**). These anthocyanidins get their names according to what R_1 and R_2 substitutions are on the B-ring (**Figure 2.3**). Due to the fact that anthocyanidins are less water soluble and therefore less stable than anthocyanins they are rarely found in nature. Anthocyanidins can be glycosylated with sugars moieties or glycosylated and acylated with acids in order to make their structures more stable (Zhao, 2007).

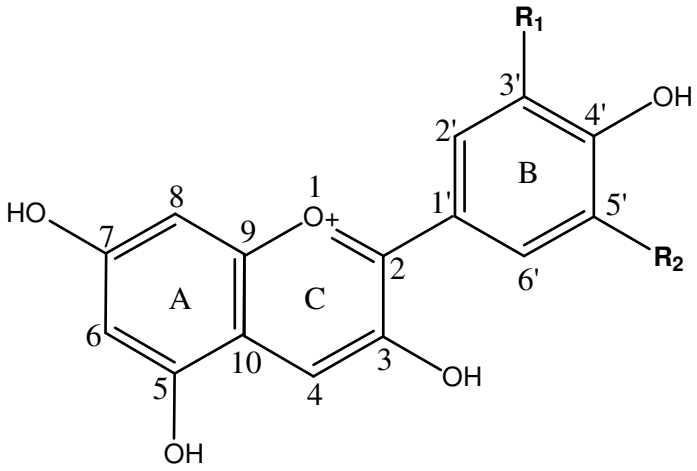

Figure 2.3: Basic chemical structure of an anthocyanidin.

Anthocyanidin	Abbreviation	R_1	R_2	Color	λ_{max} (nm) Visible Color
Pelargonidin	Pg	H	H	Orange	494
Cyanidin	Cy	OH	H	Orange-red	506
Peonidin	Pn	OCH3	H	Orange-red	506
Delphinidin	Dp	OH	OH	Red	508
Petunidin	Pt	OCH3	OH	Red	508
Malvidin	Mv	OCH3	OCH3	Bluish-red	510

Table 2.2: Differences in chemical structure, color and λ_{max} of the six common anthocyanidins (Source: Giusti and Wrolstad, 2003).

2.3.2 Anthocyanin Color and Stability:

The color of an anthocyanin depends greatly on the amount of hydroxyl and methoxyl derivatives that are attached to the anthocyanidin molecule (**Table 2.2**). As more substitutions are added to the anthocyanidin structure the more intense the color becomes resulting from a bathochromic shift in the light absorption band in the visible spectrum (Giusti et al., 1999). A bathochromic shift is the movement of a spectral band position in the absorption of a molecule to a longer wavelength, while a hypsochromic shift has the opposite effect causing the absorption of a molecule to move to a shorter wavelength. The more methoxyl groups attached to the structure the more red the color, while the more hydroxyl groups attached to the structure the more blue the color; both of these substitutions result in a bathochromic shift into the visible region of the spectra (Giusti and Wrolstad, 2003).

The two structural factors that contribute the most to the stability of an anthocyanin are the attachments of sugar moieties (glycosylation) and acids (acylation). Common anthocyanin glycosylations are glucose, galactose, arabinose, xylose, rhamnose, sambubiose, and di- and tri-saccharides formed by a combination of these sugars. If an anthocyanin is glycosylated it always occurs on at least the C-3 position on the C- ring, but other glycosylations can also occur at C-5, C-7, C-3′, C-4′ and C-5′. The more sugar moieties attached to the structure the more stable the molecule (Schwartz et al., 2007).

Acylated anthocyanins are the most stable types anthocyanins found in nature. In order for an anthocyanin molecule to be acylated with an acid it must first be glycosylated with at least one sugar molecule. Common acids used for anthocyanin acylation are caffeic, *p*-coumaric, sinapic, *p*-hydroxybenzoic, ferulic, malonic, malic,

succinic and acetic acids. Acylation occurs when an acid forms an ester bond with a sugar molecule that is attached to an anthocyanin. The increased stability of acylated anthocyanins is due to the intremolecular/intramolecular stacking of molecules, which protects them from nucleophilic attack (Anderson and Jordheim, 2006).

The three environmental factors that affect the stability of anthocyanins the most are pH, oxygen and temperature. In an aqueous environment, anthocyanins undergo reversible structural transformations, which in turn cause color changes due to changes in pH (**Figure 2.4**). At an acidic pH of 1, anthocyanins are present in the red colored oxonium form. A high acid pH is the most desired pH for anthocyanins because it allows them to be in their most stable form. At a less acidic pH of 4.5, anthocyanins are present predominately in the colorless hemiketal form. At this pH the hemiketal form can also equilibrate into the chalcone form, which is colorless as well. As the pH rises further to pH 7, the anthocyanins transform into the quinonoidal base form and give off a blue color (Giusti and Wrolstad, 2005). The quinonoidal base form is the least stable form for anthocyanins.

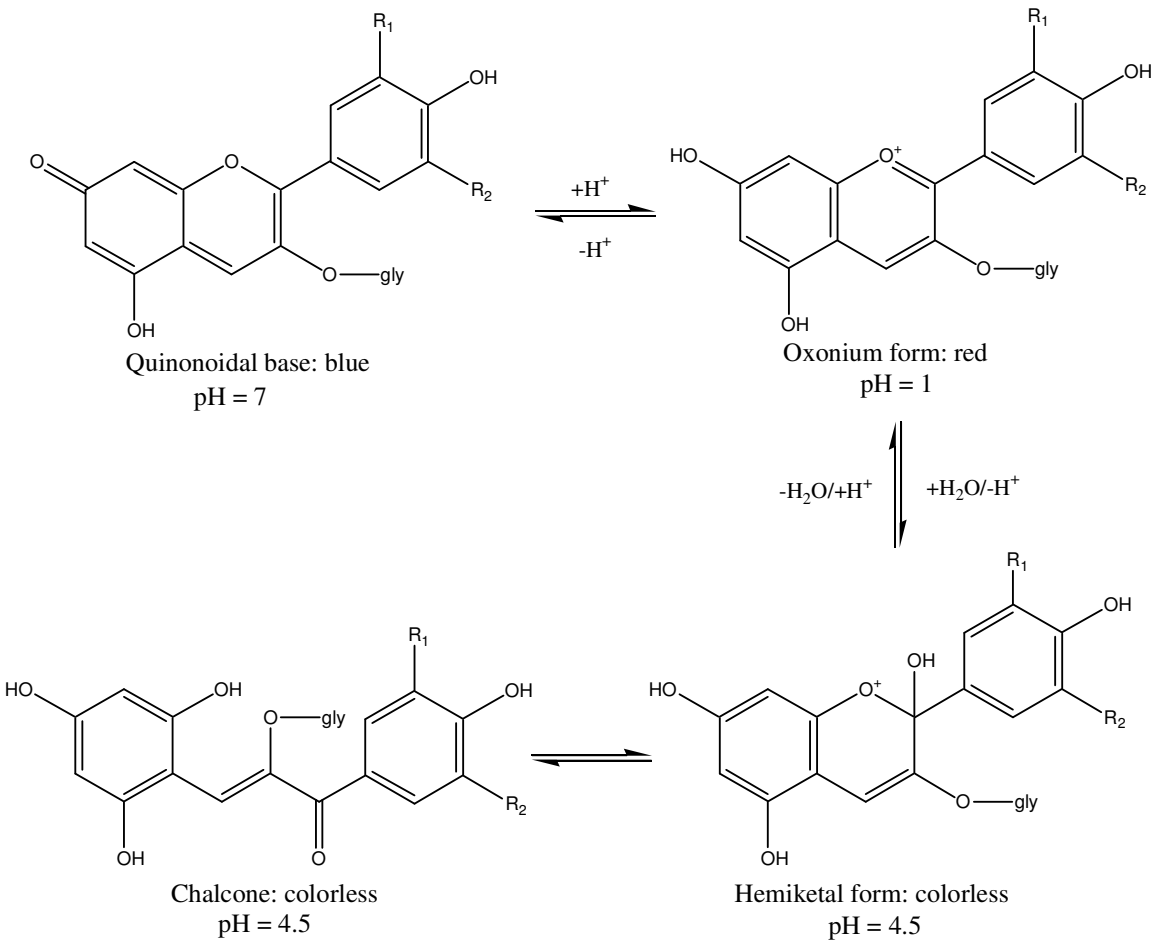

Figure 2.4: Predominant structural forms of anthocyanins present at different pH levels (Source: Giusti and Wrolstad, 2005).

Oxygen is a concern for anthocyanins because it catalyzes the reaction polyphenol oxidase. Polyphenol oxidase is an enzymatic browning reaction that is undesirable in most fruits and vegetables that have been minimally processed and is associated with color quality loss. This reaction occurs when a fruit or vegetable is cut, peeled, bitten or bruised causing the cells beneath the surface of the skin to be damaged and exposed to oxygen. With help from the enzyme polyphenol oxidase, oxygen reacts with anthocyanins, as well as other phenolics compounds, causing browning to occur. Like oxygen, high temperatures can also lead to the degradation of anthocyanin pigments and cause browning to occur (Schwartz et al., 2007).

2.3.3 Anthocyanin Metabolism, Bioavailability and Limitations:

Interest in anthocyanin biochemistry has increased in the last decade due to the newly discovered health benefits of these compounds. Anthocyanin metabolism begins in the gastrointestinal tract (GIT), which contains three distinct environments: the stomach, the small intestine and the large intestine (colon). These regions are distinguished by their different pH and microbial populations. Since the pH of the stomach is the lowest of the three regions (pH 1-2) this is where the anthocyanin compounds are the most stable. The stomach is also where intact anthocyanins are rapidly absorbed into the circulatory system between 15 min to 2 hr after ingestion. The pH of both the small and large intestines is neutral, causing the anthocyanins to be much less stable. Once the remaining anthocyanins in the GIT, about 60% of the ingested amount, reach the colon they are rapidly degraded by its microflora (McGhie and Walton, 2007; Wu et al., 2009). The intestinal microflora has been shown in an *in vitro* model to

hydrolyze the anthocyanidin glycosides and this can take 20 min to 2 hr, depending on the type of sugar moiety that was attached to the anthocyanidin. The neutral pH in the large intestine then causes the anthocyanin aglycones to degrade to phenolic acids and other degradative products before being absorbed into the circulatory system(Keppler and Humpf, 2005; Wu et al., 2009).

The bioavailability of anthocyanins is of great importance because this measures how much of the ingested anthocyanins are absorbed and have the ability to reach other organs and tissues in the body besides those in the GIT. Anthocyanin bioavailability is usually measured as maximum blood plasma concentration or percentage of anthocyanins excreted in the urine (McGhie and Walton, 2007). Anthocyanins can be absorbed into the plasma in their intact forms or as their glucuronide and/or methylated derivatives. The amounts of individual intact anthocyanin compounds that are excreted in the urine vary, indicating that absorption depends on the type of anthocyanin aglycone and its attached sugar moieties (Wu et al., 2005).

A major limitation for anthocyanins is that their bioavailability is very low, often less than 0.1% of the amount ingested is excreted in urine. Complete understanding for why anthocyanins have such low bioavailability is not fully known at this time. A study done by Tsuda and colleagues (1999) found that after oral administration of cyanidin-3-glucoside to rats that the potential degradation product of cyanidin based anthocyanins, protocatechuic acid, was found in the plasma at a concentration eight times that of cyanidin-3-glucoside. This study supports the theory that most of the ingested anthocyanins either remain in the GIT or are transformed into different molecular structures by the colon microflora before they are absorbed into the plasma. These

findings, along with other studies, have found that anthocyanins have a higher inhibitory impact on cancers in the GIT than cancers in other parts of the body due to intact anthocyanins not being able to reach the other organs/tissues in sufficient enough concentrations to be protective (Carlton et al., 2000).

2.3.4 Health Benefits of Anthocyanins:

Consumption of anthocyanin-rich products have several health benefits associated with them including being potent antioxidants, and possessing anti-inflammatory, antiatherogenic and anticarcinogenic properties (Anderson and Jordheim, 2006; Wrolstad, 2000). One of their most important roles is that anthocyanins act as antioxidants by blocking oxidative processes and free radicals. By acting as antioxidants, anthocyanins help delay or even prevent degenerative disease such as cancer, cardiovascular diseases, cataracts and Alzheimer's disease (Wrolstad, 2000). Anthocyanin-rich fruits have been studied for their anti-inflammatory properties. These anti-inflammatory properties are possibly due to an anthocyanin's ability to inhibit signal transduction pathways involved in the activation of NFκB (Wang and Stoner, 2008). Anthocyanins have also been shown to possess antiatherogenic properties due to their ability to promote higher levels of high-density lipoprotein and inhibit oxidation of low-density lipoprotein (Wrolstad, 2000). Anthocyanins have several anticarcinogenic properties as well including their ability to inhibit proliferation of cancer cells, induce apoptosis, induce cellular differentiation and suppress angiogenesis (Wang and Stoner, 2008).

2.3.5 Berry Fruits as Sources of Anthocyanins:

Berries are some of the richest dietary sources of anthocyanins for humans having as high as 1480 mg of anthocyanins/100g fresh weight (Wu et al., 2004). Among the different types of berries the anthocyanin content varies greatly, ranging from 13 to 1480 mg/100g (**Table 2.3**). Black raspberries have been shown to have the highest anthocyanin content (~607 mg/100g) among the caneberries, which include red raspberries, boysenberries, evergreen blackberries and marionberries. Studies indicate that black raspberry polyphenolics, especially the anthocyanins, may play an important chemoprotective role (Stoner et al., 2007). Black raspberry anthocyanins have been identified (**Figure 2.5**) as cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-xylosylrutinoside, cyanidin-3-rutinoside and pelargonidin-3-rutinoside (Tian et al., 2006).

Berry Type	Scientific Name	Anthocyanin Content (mg/100g)	Reference
Bilberry	*Vaccinium myrillus* L.	300 ~ 808	(Prior et al., 1998; Maatta-Riihinen et al., 2004)
Black chokeberry	*Aronia melanocarpa* (Michx.) Elliot	307 ~ 1480	(Strigl et al., 1995; Wu et al., 2004)
Black raspberry*	*Rubus occidentalis* L.	145 ~ 607	(McGhie et al, 2002; Moyer et al., 2002)
Blueberry	*Vaccinium corymosum* L.	63 ~ 430	(Prior et al., 1998; Moyer et al., 2002)
Boysenberry*	*Rubus ursinus x idaeus*	~131	(Wada and Ou, 2002)
Cranberry	*Vaccinium macrocarpon* Aiton.	20 ~ 360	(Prior et al., 1998; Moyer et al., 2002)
Elderberry	*Sambucus nigra* L.	332 ~ 1374	(Maatta-Riihinen et al., 2004; Wu et al., 2004)
Evergreen blackberry*	*Rubus laciniatus*	91 ~ 164	(Wada and Ou, 2002; Siriwoharn et al., 2004)
Lingonberry	*Vaccinium vitis-idaea* L.	31 ~ 92	(Wang et al., 2005)
Marionberry*	*Rubus ursinus*	62 ~ 155	(Deighton et al., 2000; Wada and Ou, 2002)
Red raspberry*	*Rubus idaeus* L.	45 ~ 100	(Wang and Ou, 2002)
Strawberry	*Fragaria x ananassa* D.	13 ~ 55	(Cordenunsi et al., 2002)

*Caneberries

Table 2.3: Anthocyanin content (mg/100g fresh weight) in selected berries (adapted from Jing, 2006).

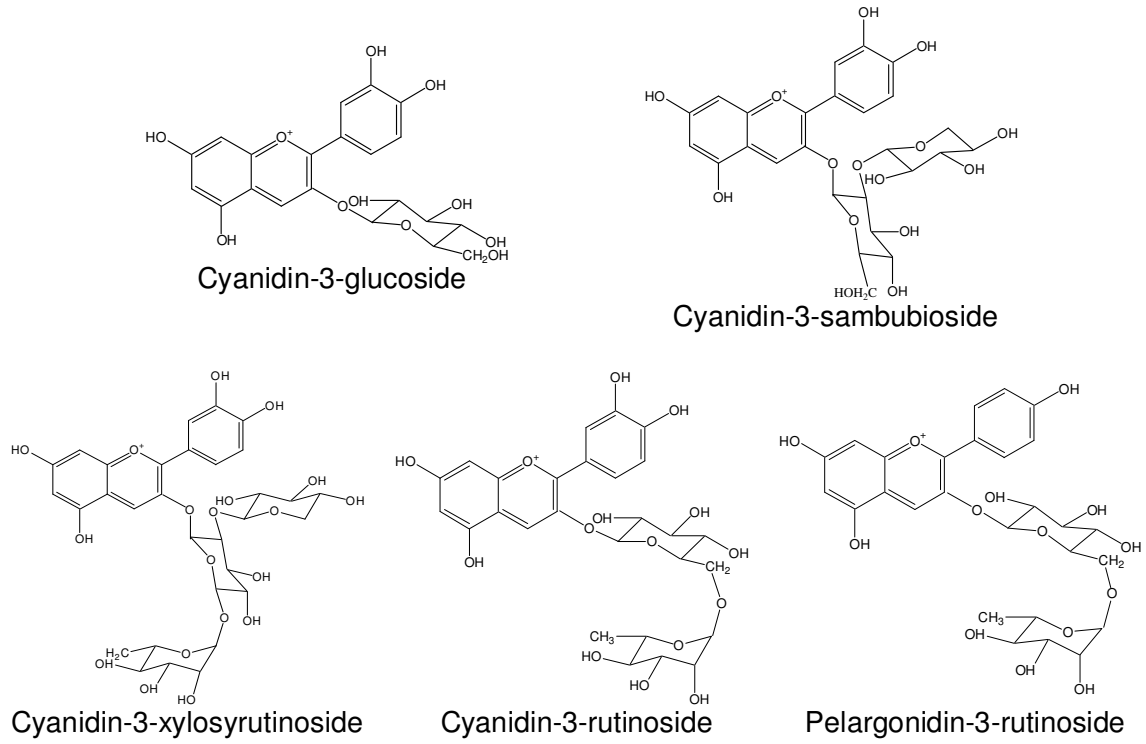

Figure 2.5: The chemical structures of the five anthocyanins found in black raspberries.

2.3.6 Chemoprotective Properties of Black Raspberry Anthocyanins:

A study done by Hecht and colleagues (2006) found that the anthocyanins that contribute the most to a black raspberry's chemoprotective properties are cyanidin-3-glucoside, cyanidin-3 xylosylrutinoside and cyanidin-3-rutinoside. In this study, subfractions from ethanol extracts of freeze-dried black raspberries were used to test their inhibitory effects on induced NFκB and AP-1 activities in rats. Of these subfractions, the ones that contained cyanidin-3-glucoside, cyanidin-3 xylosylrutinoside and cyanidin-3-rutinoside as its major constituents were found to be the most active at inhibiting NFkB and AP-1 activities in rats.

2.4 Horticultural Factors Affecting Fruit Composition:

There is evidence that consistently shows that a high dietary intake of plant-based foods is associated with reduced incidence of many degenerative diseases (Duthie, 2007; Lui, 2004). With specific information about the compounds in plant foods, researchers may be able to identify a variety of bioactive fruit materials or suggest a diet of complementary foods that would protect against a variety of these diseases. Furthermore, horticulturalists will be able to develop genotypes and cultural procedures that optimize their production in fruits.

2.4.1 Effects of Growing Location on Phytonutrient Content:

The growing location can have a major impact on the compositional properties of a fruit due to sunlight (quality, intensity and duration), cultural conditions and climate conditions. While light exposure/UV radiation have been found to have little or no effect

on the ascorbic acid or carotenoid contents in fruits, it has been shown that polyphenolics can greatly increase in order to protect the fruit's tissues from UV damage (Kalt, 2005).

Cultural conditions including soil conditions, nutrient availability, salinity, temperature and water availability have all been shown to impact the phytonutrient quality of fruits. Studies have found that an increase in nitrogen fertilizer can lead to an increase in carotenoid content, but it also leads to a decrease in ascorbic acid content (Kalt, 2005). The addition of compost to soil can be beneficial for fruits because it causes changes in the chemical and physical characteristics of soil, increases beneficial microorganisms and increases nutrient availability (Wang, 2006). For some fruits, saline conditions can lead to an increase in ascorbic acid content, but these conditions may also lead to lower productivity. It has also been found that higher total phenolic and anthocyanin contents are accumulated in fruits that are irrigated than those that are not (Kalt, 2005).

Climate, including total available heat and the extent of high and low temperatures, has a big impact on the growth rate and chemical composition of fruits (Lee and Kader, 2000). Studies have found that higher temperatures lead to a significant increase in total phenolic and anthocyanin contents, while it is the cooler temperatures that help develop higher levels of vitamin C in fruits (Wang, 2006; Lee and Kader, 2000).

2.4.2 Effects of Cultivar on Phytonutrient Content:

The use of different cultivars may also cause compositional differences that can affect the nutritional properties of a fruit. How much of an impact using different cultivars has on the nutritional properties of a fruit really depends on the genetic diversity

among the cultivars within the species. Studies done by Anttonen and Karjalainen (2005) and de Ancos et al. (1999) found that total phenolics, anthocyanins, ellagic acid, quercetin, vitamin C and organic acid concentrations greatly varied among different raspberry cultivars. The highest anthocyanin contents were found in the cultivars that ripened late in the season (de Ancos et al., 1999).

In a more recent study done by Ozgen and colleagues (2008), total phenolic and anthocyanin contents were determined for 19 black raspberry samples from four common Midwestern cultivars (Bristol, Haut, Jewel and MacBlack) grown in 8 Ohio locations to demonstrate the compositional variability among these samples. The Bristol cultivar is the leading black raspberry cultivar in Ohio. It is used for fresh market, freezing and canning, and it ripens between June 23rd and July 4th. The Jewel cultivar, which is considered an improvement of its maternal parent Bristol, is larger in size and more resistant to disease. This cultivar can be used fresh or processed and it ripens early in July. The Haut cultivar is recommended for pick-your-own operations; it is harvested early in the season, but ripens over a long period of time and it is currently in trial use. The MacBlack cultivar produces somewhat soft fruit, while the others produce firm fruit, and this cultivar ripens 7-10 days later than most cultivars (Funt et al., 2009). Due to this cultivar's later ripening season it is the main type used for the fresh market (Finn et al., 2008).

In this study it was determined that the mean total phenolic values among the cultivars Bristol, Jewel and MacBlack were similar, while the Haut cultivar's mean was lower than the others. The 8 Ohio growing locations varied substantially in total monomeric anthocyanin content and to a lesser extent in total phenolics within each of

the cultivars (**Figure 2.6**). The overall results from this study demonstrated that there were not only compositional differences among the four cultivars, but there were also differences within the same cultivar grown in different locations.

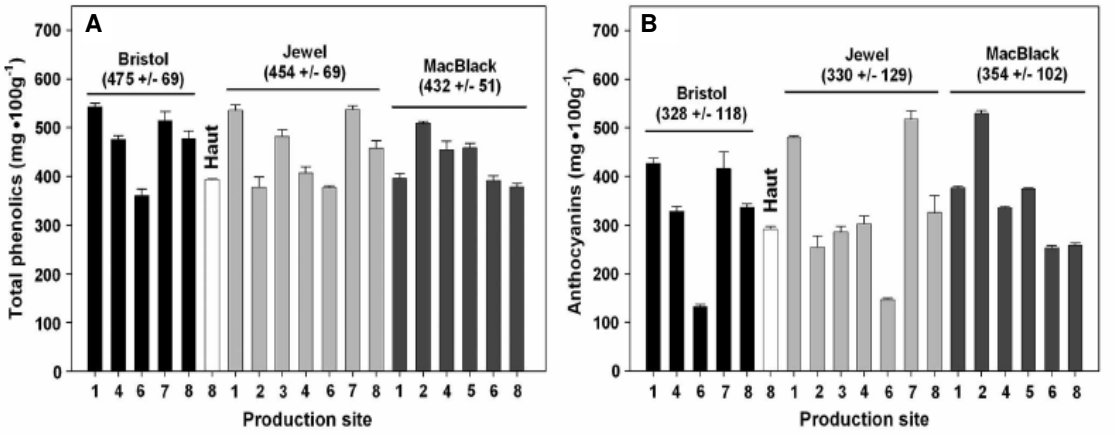

Figure 2.6: Comparison of total phenolic and monomeric anthocyanin contents of 4 black raspberry cultivars grown in 8 Ohio locations (Source: Ozgen et al., 2008).

2.4.3 Effects of Fruit Maturity on Phytonutrient Content:

The stages of maturity are of great interest when studying the effects of phytonutrient content in fruits because as the fruit moves through the different stages of maturity the concentration of some compounds may decrease and/or disappear as other compounds are synthesized and/or accumulated (Ozgen et al., 2008). Many compounds including vitamin C, carotenoids, ellagic acid, total phenolics, anthocyanins, phenolic acids, flavan-3-ols and flavonols have been found to vary greatly among different stages of maturity (Gonalves et al., 2004; Kalt, 2005; Anttonen and Karjalainen, 2005). Most of

these compounds tend to increase as the fruit ripeness, but some compounds like vitamin C and ellagic acid tend to decrease (Kalt, 2005; Anttonen and Karjalainen, 2005).

A study done by Wang and Lin (2000) showed that total phenolic and anthocyanin contents varied among different berries at three stages of maturity (green, pink and ripe) (**Table 2.4**). Among the different stages of maturity, which stage had the highest level of total phenolics greatly depended on the type of berry, while the anthocyanin content was found to increase in all berries as they became ripe.

Species	Maturity	Total Phenolics (mg GAE /100g dry matter)	Anthocyanins (mg/100g dry matter)[a]
Blackberry	Green	2166	6.7
	Pink	1550	57.7
	Ripe	1347	909.3
Black Raspberry	Green	1625	27.9
	Pink	783	93.9
	Ripe	1530	952.4
Red Raspberry	Green	517	2.9
	Pink	400	25.5
	Ripe	1346	391.8
Strawberry	Green	1834	2.2
	Pink	1083	46.2
	Ripe	1033	315.2

[a] Data expressed as milligrams of cy-3-glucoside (blackberry and raspberry) or pg-3-glucoside (strawberry) per 100g dry matter.

Table 2.4: Comparison of total phenolic and anthocyanin contents in fruit juice of different maturities in various berry species (Source: Wang and Lin, 2000).

2.5 Present Study:

The present study was designed to examine the effects of 75 different black raspberry extracts on colon cancer cell proliferation. Among these 75 samples, 3 different cultivars (Bristol, Jewel and MacBlack) and 3 different stages of maturity (underripe, ripe and overripe) grown in 7 different Ohio locations, were used. We hypothesized that changes in composition caused by the differences due to cultivar, growing location and/or maturity would result in different bioactivities of the black raspberry samples.

Aim 1: Quantify total phenolic and total monomeric anthocyanin contents for each of the 75 black raspberry samples.

Aim 2: Test the bioactivity of the 75 black raspberry samples by measuring their inhibition of cell growth proliferation on a colon cancer *in vitro* cell system.

Aim 3: Characterize black raspberry anthocyanins.

Chapter 3: Materials and Methods

3.1 Materials:

3.1.1 Plant Material:

For this project 75 different black raspberry samples were evaluated. Three different cultivars: MacBlack, Jewel and Bristol and three different stages of maturity: underripe, ripe and overripe were harvested from seven different locations. Three samples of the same cultivar were harvested from the same field, at the same maturity stage, designated A, B or C. Harvest locations were in the northern, southern and western regions of Ohio, and to protect the identities of the growers the locations were given a number designation 1-7. In order to make the samples easier to follow a code was developed as seen in **Table 3.1**.

Ohio Location	Cultivar	Maturity	Code	Sample
1	Bristol (B)	ripe (R)	1BR(A-C)*	1, 2, 3
		overripe (O)	1BO(A-C)	4, 5, 6
	Jewel (J)	ripe (R)	1JR(A-C)	7, 8, 9
		overripe (O)	1JO(A-C)	10, 11, 12
	MacBlack (M)	ripe (R)	1MR(A-C)	13, 14, 15
		overripe (O)	1MO(A-C)	16, 17, 18
2	MacBlack (M)	underripe (U)	2MU(A-C)	19, 20, 21
		ripe (R)	2MR(A-C)	22, 23, 24
		overripe (O)	2MO(A-C)	25, 26, 27
3	Jewel (J)	ripe (R)	3JR(A-C)	28, 29 30
		overripe (O)	3JO(A-C)	31, 32, 33
	MacBlack (M)	ripe (R)	3MR(A-C)	34, 35, 36
		overripe (O)	3MO(A-C)	37, 38, 39
4	Bristol (B)	ripe (R)	4BR(A-C)	40, 41, 42
	Jewel (J)	ripe (R)	4JR(A-C)	43, 44, 45
5	Jewel (J)	underripe (U)	5JU(A-C)	46, 47, 48
		ripe (R)	5JR(A-C)	49, 50, 51
		overripe (O)	5JO(A-C)	52, 53, 54
	MacBlack (M)	underripe (U)	5MU(A-C)	55, 56, 57
		ripe (R)	5MR(A-C)	58, 59, 60
		overripe (O)	5MO(A-C)	61, 62, 63
6	Jewel (J)	ripe (R)	6JR(A-C)	64, 65, 66
		overripe (O)	6JO(A-C)	67, 68, 69
7	Jewel (J)	underripe (U)	7JU(A-C)	70, 71, 72
	MacBlack (M)	underripe (U)	7MU(A-C)	73, 74, 75

*A-C: 3 different samples of the same cultivar, harvested from the same field and at the same maturity stage.

Table 3.1: Black raspberry sample code.

3.1.2 HT-29 Colon Cancer Cell Line:

The HT-29 colon cancer cell line derived from human Caucasian colon adenocarcinoma grade II cells was purchased from American Type Culture Collection (Rockville, MD). The cells were grown in sterile McCoy's 5A medium with L-glutamine, which was supplemented with 10% fetal bovine serum (Fisher Scientific, Fair Lawn, NJ).

3.1.3 Reagents and Solvents:

High performance liquid chromatography-mass spectrometer (HPLC-MS) grade 99.9% methanol, 99.9% acetonitrile and water, as well as certified American Chemical Society (ACS) grade potassium chloride, sodium carbonate, sodium acetate and HPLC grade 88% formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Reagent grade gallic acid and Folin-Ciocalteau reagent were purchased from MP Biomedical (Aurora, OH). Delbucco's Phosphate Buffered Saline solution and 2.5% trypsin were purchased from Fisher Scientific (Fair Lawn, NJ). Sulforhodamine B dye, 99% Trichloroacetic acid and 99% Tris(hydroxymethyl) aminomethane were purchased from Sigma-Aldrich (St. Louis, MO).

3.2 Methodology:

3.2.1 Extraction of Anthocyanins and Other Phenolics from Black Raspberries:

Freeze-dried black raspberry extracts were provided by Dr. Joseph Scheerens, from the Horticulture and Crop Science Department at The Ohio State University, Wooster, OH.

Freeze-dried black raspberry extracts were obtained with the following procedure. Black raspberries (200 g) were ground with 100 mL of water in a blender. The material was then filtered through cheesecloth followed by centrifugation to remove the residual pulp. The juice obtained was divided into aliquots and frozen at -20°C for subsequent extraction. Phenolics were extracted on a Phenomenex SPE column (Phenomenex Strata C18-E, 5 g/20 mL Giga Tubes) activated with acidic methanol (0.1% Trifluoroacetic acid added to HPLC grade methanol), followed by 0.1% Trifluoroacetic acid in deionized and distilled (DD) water. Samples (50mL) were loaded onto the column, where phenolics/anthocyanins adhered to the column. The cartridges were washed with 60-100 mL of DD water to remove sugars, organic acids and other polar compounds. The phenolics/anthocyanins were eluted using ~30 mL of acidic methanol. Samples were taken to a final volume of 50 mL with methanol and partitioned into screw-capped test tubes. The tubes were covered with aluminum foil to reduce light exposure and the solvent was removed from each tube under nitrogen stream in an N-Evap system (water bath at 35°C). Solvent free tubes were stored at -80°C and freeze-dried using a Labconco freeze-dry system. Lastly, samples were put into a desiccator, weighed and then stored at -80°C.

3.2.2 Total Phenolic Content Analysis:

Black raspberry extract powders that were used for determination of total phenolic and total monomeric anthocyanin contents for each of the black raspberry samples were weighed (averaging between 1-2 mg of black raspberry extract powder) and then diluted to 2 ml using 0.01% hydrochloric acid acidified water.

Total phenolic content for each black raspberry sample was measured using the modified microscale protocol for Folin-Ciocalteu colorimetry (Singleton and Rossi, 1965). Total phenolic contents were calculated as gallic acid equivalents based on a gallic acid standard curve. Briefly, 20 µL of a sample, a gallic acid calibration standard or a blank (distilled water) was put in a 2 mL cuvette that had 1 cm pathlength. Deionized water (1.58 mL) was added, followed by 100 µL of Folin-Ciocalteu reagent. Cuvettes were mixed well by inverting and standing at room temperature for 8 min. Then, 300 µL of sodium carbonate solution was added and thoroughly mixed. The final volume was 2 mL. Samples were incubated at room temperature for 2 hr in the dark. A Shimadzu UV-Visible Spectrophotometer 2450 (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used for spectral measurements at 765 nm. Finally, total phenolic contents were calculated as gallic acid equivalents based on the created gallic acid standard curve that ranged from 100 to 900 mg/L.

3.2.3 Total Monomeric Anthocyanin Content Analysis:

Monomeric anthocyanin content was determined using the pH differential method (Giusti and Wrolstad, 2005). This method relies on structural transformations of anthocyanins as a function of pH to obtain quantitative information. A dilution factor of 10 was used to ensure that when all black raspberry extracts were diluted in 0.025 M potassium chloride buffer (pH 1.0) their absorbencies would be within the linear range of the spectrophotometer (less than 1.2). Black raspberry extracts (150 µL) were diluted in 1350 µL of both the pH 1.0 and pH 4.5 buffers, separately, in 2 mL cuvettes that had 1 cm pathlengths. Solutions sat in the dark at room temperature for at least 15 min before

they were measured on the spectrophotometer. A Shimadzu UV-Visible Spectrophotometer 2450 (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used for spectral measurements at 520 nm (maximum absorption of anthocyanins) and 700 nm to correct for haze. From these readings the absorbencies of the diluted samples were calculated as shown in **Equation 1**. The calculated absorbencies were then used to calculate total monomeric anthocyanin content (**Equation 2**). Measurements were done twice for each sample.

(Equation 1) $\mathbf{Absorbance = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}}$

(Equation 2) $\mathbf{Monomeric\ anthocyanin\ (mg/L) = (A \times MW \times DF \times 1000) / (\varepsilon \times 1)}$

Where A is the absorbance, MW is the molecular weight. DF is the dilution factor, and ε is the molar absorptivity coefficient. Pigment content was calculated as cyanidin-3-glucoside, where MW = 449.2 and ε = 26,900 cm^{-1} mg^{-1}.

3.2.4 HT-29 Colon Cancer Cell Culture:

The HT-29 colon cancer cells were grown at 37°C in a Nuaire™ IR Autoflow CO_2 Water-Jacketed Incubator at a modified atmosphere (5% CO_2, 95% O_2) in McCoy's 5A medium with L-glutamine and supplemented with 10% fetal bovine serum (Fisher Scientific, Fair Lawn, NJ). Cell cultures were spilt 1:5 when monolayers were 80-90% confluent using Delbucco's Phosphate Buffered Saline solution and 2.5% trypsin (Fisher Scientific, Fair Lawn, NJ). Cultures were supplied with fresh media every 3 days. Growth of the HT-29 cells was determined by microscope examination. Stock cultures

were maintained in 75 cm^2 flasks with canted necks (Fisher Scientific, Fair Lawn, NJ). All cell culture experiments were performed within the initial 20 passages.

3.2.5 Determination of HT-29 Cell Concentration using a Hemocytometer:

After the cells grown in a 75 cm^2 flask were ~80% confluent the media was removed and the cells were washed using 7 mL Delbucco's Phosphate Buffered Saline solution. Next, the cells were detached from the flask using 3 mL 2.5% trypsin and 7 mL of media was added to quench the trypsin, bringing the total volume to 10 mL before the suspension was transported to a 50 mL conical tube. The conical tube was then centrifuged for 5 min at 25°C at 2500RPM and the cell pellet was resuspended in the appropriate amount of media in order to be able to count the cells. Suspended cells were counted on a Reichert Bright-Line hemocytometer using a Nikon Eclipse 50i Brightfield Microscope (Hausser Scientific, Horsham, PA). Using the 10x lens of the microscope and a hand counter, cells were counted in the 5 primary squares of the hemocytometer. The following formula was used to calculate the HT-29 cell concentration:

(Equation 3) $$\frac{\text{Total number of cells counted}}{\text{Number of primary squares counted}} \times 10{,}000 = \text{Cells/mL}$$

The number of cells/mL was used to determine the appropriate dilution needed to get a concentration of 1.0×10^4 cells/well and then the cells were seeded in 96-well plates at this concentration.

3.2.6 Black Raspberry Extract Treatments:

The HT-29 colon cancer cells were seeded in 96-well plates at a concentration of 1.0×10^4 cells/well and incubated for 24 hr in order to achieve log phase growth at the time of treatment with the black raspberry extracts. After the initial 24 hr, the media was removed from the wells and the black raspberry treatment concentrations of 0.6 and 1.2 mg extract/mL media were added to three wells each for every sample. Once the 96-well plates were treated with the extracts they were incubated for an additional 48 hr before sulforhodamine B assay was performed.

On one 96-well plate eight black raspberry extracts could be used at both concentrations. For every sample 3-5 replications were done at both concentrations.

3.2.7 Sulforhodamine B Assay:

In the sulforhodamine B (SRB) assay (Vichai et al., 2006) cells were fixed to the bottom of the wells by the addition of 150 µL of 10% Trichloroacetic acid (TCA) at 4°C for 1 hr. TCA and media were then removed from the wells. The wells were washed with tap water 5 times to remove TCA, growth media and serum proteins, and then dried using a blow drier set to cool. SRB (0.4% dissolved in 1% acetic acid) dye (75 µL) was added to each well to stain cells at room temperature for 30 min. Wells were washed using 1% acetic acid 5 times to remove any unbound dye and dried using a blow drier set to cool. The incorporated dye was then solubilized using 150 µL of 10 mM Tris(hydroxymethyl) aminomethane for 5 min at room temperature on a shaker. The optical density of each well was measured at 500 nm using a Synergy™ HT Multi-

Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). KC4 version 3.03 PowerReport™ Bio-Tek® software was used to analyze the data.

3.2.8 Inhibition of Cell Proliferation:

HT-29 cells plated at 1.0×10^4 cells/well in 96-well plates were allowed to incubate for 24 hr to achieve log phase growth at the time of treatment (time 0). Black raspberry extracts were dissolved in growth media to obtain the concentrations of 0.6 and 1.2 mg extract/mL media for each sample and then tested on the HT-29 cells for their antiproliferative effects. Percent growth inhibition of the HT-29 cells was determined by using the sulforhodamine B assay, as described above, after an additional 48 hr of incubation. Each treatment concentration for a black raspberry sample was applied to 3 wells in a 96-well plate, while the experiments were repeated three to five times. The percent growth inhibition was calculated as:

(Equation 4) $$\% \text{ Growth inhibition} = 100 - \frac{(T_{trt} - T_0) \times 100}{(T_{ctr} - T_0)}$$

$\mathbf{T_0}$ is the absorbance of cellular protein content at 500 nm after the first 24 hr of the incubation period prior to black raspberry extract treatment.

$\mathbf{T_{trt}}$ is the absorbance of cellular protein content at 500 nm after black raspberry extract treatment and incubation for 48 hr.

$\mathbf{T_{ctr}}$ is the absorbance cellular protein content at 500 nm without treatment of black raspberry extracts after the total incubation period of 72 hr.

3.2.9 HPLC-MS Analysis of Black Raspberry Extracts:

Samples were analyzed using a Shimadzu high performance liquid chromatography system (Shimadzu Scientific Instruments, Inc., Columbia, MD) on a 2695 Separation Module equipped with an SIL-20AC autosampler, a 996 photodiode array detector, an LCMS-2010EV quadrupole ion-tunnel mass spectrometer with an electrospray interphase and LCMS solutions software (Version 3, Shimadzu, Columbia, MD) was used to monitor the phenolic composition of the black raspberry extracts. Separation was accomplished on a reversed-phase 3.5 µm Symmetry C18 column (4.6×150 mm; Waters Corp., Milford, MA) fitted with a 4.6 x 22 mm Symmetry 2 micro guard column. The solvents were A: 10% formic acid in HPLC-MS grade water and B: 99.9% HPLC-MS grade acetonitrile. Solvents and samples were filtered though 0.45 µm polypropylene filters. Separation of compounds was achieved by using a linear gradient from 0-6 min, 5-8% B; 6-15 min, 8-10% B; 15-20 min, 10-25% B; 20-25 min, 25% B. The flow rate was set at 0.8 mL/min and an injection volume of 30 µL was used. Spectral information over the wavelength range of 250-700 nm was collected.

A 0.25 mL/min flow was diverted to the mass spectrometer. Mass spectrometry was conducted on a LCMS 2010 quadrupole ion-tunnel mass spectrometer equipped with an electronspray ionization interface (Shimadzu Scientific Instruments, Inc., Columbia, MD). MS analysis was performed under positive mode. A full scan (total ion count, TIC) was performed with a mass range from 200-1500 *m/z*. A selected ion monitoring (SIM) scan was applied for the two black raspberry anthocyanin aglycones: cyanidin (*m/z* 287) and pelargonidin (*m/z* 271). MS analysis was used for confirmation of the five black raspberry anthocyanins.

3.2.10 Statistical Analysis:

The total phenolic, total monomeric anthocyanin and percent inhibition results were all subjected to Analysis of Variance (ANOVA) with Tukey's post-hoc comparisons (SAS 9.1 software). For all statistical calculations, a $p<0.05$ was considered to be statistically significant. The results were split up into four data sets before they were analyzed: data set 1 = total phenolic results, data set 2 = total monomeric anthocyanin results, data set 3 = percent inhibition results of the black raspberry treatment concentration of 0.6 mg extract/mL media and data set 4 = percent inhibition of the black raspberry treatment concentration of 1.2 mg extract/mL media. Among these data sets the results were analyzed by location, by cultivar and by maturity to determine how the responses changed by fixing one of the variables. Interactions between two variables were also analyzed to see if one had an effect on the other. Pearson correlation tests were then used to see if there were any correlations between the bioactivity results (% inhibition) and the analytical chemistry results (total phenolics and total monomeric anthocyanins).

Chapter 4: Results and Discussion

4.1 Methodology Optimization:

4.1.1 Determination of HT-29 Colon Cancer Cell Seeding Concentration:

The appropriate seeding concentration was determined by seeding each column of a 96-well plate with one of the following cell concentrations: 2.5×10^3, 5.0×10^3, 1.0×10^4 and 2.0×10^4 cells/well. The 96-well plate was incubated for 72 hr and then microscopically analyzed to determine which seeding concentration was ~80% confluent. The cell concentration of 2.5×10^3 cells/well exhibited too few of cells after 72 hr of incubation. The cell concentration of 5.0×10^3 was about 50% confluent, while the 2.0×10^4 cells/mL concentration exceeded 100% confluence. It was determined that a concentration of 1.0×10^4 cells/well was the appropriate seeding concentration because after 72 hr of incubation the wells were 80-90% confluent.

4.1.2 Determination of Optical Density Measurement Wavelength:

It has been found that curves of optical density versus SRB dye concentration are generally linear below 1.5-2.0 optical density units. While measurement of optical density at 564 nm provides maximum sensitivity during the readings, measurement at this

wavelength is usually out of the linear range. It has been shown that measuring at suboptimal wavelengths, generally 490-530 nm, allows for samples to remain within the linear range (Skehan et al., 1990). For this experiment a Synergy™ HT Multi-Detection Microplate Reader and KC4 version 3.03 with PowerReports™ Bio-Tek® software were used for the determination of the appropriate wavelength to measure optical density of 96-well plates that were seeded at concentrations of 1.0×10^4 cells/well.

A 96-well plate was seeded and allowed to incubate for 72 hr. After the cells were allowed to grow for 72 hr SRB assay was performed and the plate was used to measure optical density on the microplate reader at wavelengths ranging from 480nm to 640 nm (**Figure 4.1**). By measuring the 96-well plate at the wavelength range of 480-640 nm, it was able to be determined that at a concentration of 1.0×10^4 cells/well wavelengths 530-570 nm were out of the linear range. The wavelengths of 510 and 520 nm were also not appropriate wavelengths to use due to the possible interference of the absorbance of anthocyanins at 520 nm (maximum absorbance of anthocyanins). The wavelength of 500 nm was considered to be the best option for this experiment. Using this wavelength to measure optical density of wells seeded at 1.0×10^4 cells/well was still within the linear range and interference of anthocyanin absorption was not a concern at this wavelength.

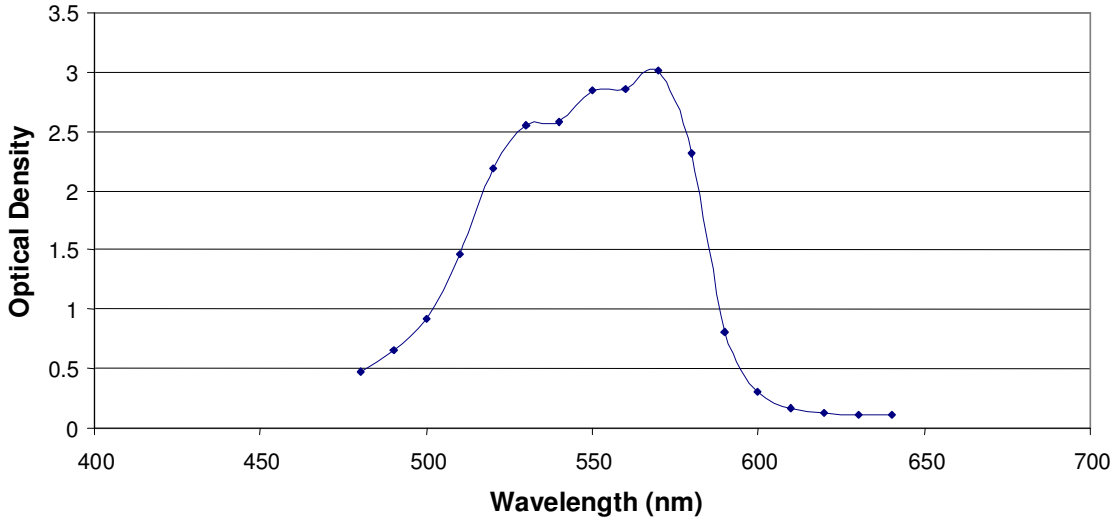

Figure 4.1: Measurement of optical density values at a seeding concentration of 1.0 x 10^4 cells/well taken over the wavelength range of 480-640 nm.

Before treating the HT-29 colon cancer cells with the different black raspberry extracts, the characteristics of the growth of the cells were examined. Proliferation rates for the HT-29 cells seeded at a concentration of 1.0 x 10^4 cells/well in 96-well plates were determined by performing SRB assay and measuring optical density on the microplate reader at 24, 48 and 72 hr. **Figure 4.2** represents the growth behavior of the HT-29 colon cancer cells. The optical density for the cells was not measured when first seeded because the first 24 hr is considered the lag phase and during this time the cells are only attaching to the bottom of the wells and not dividing. Between 24-72 hr the cells exhibited a log growth phase (or exponential growth phase). At the end of the 72 hr period the cells were between 80-90% confluent.

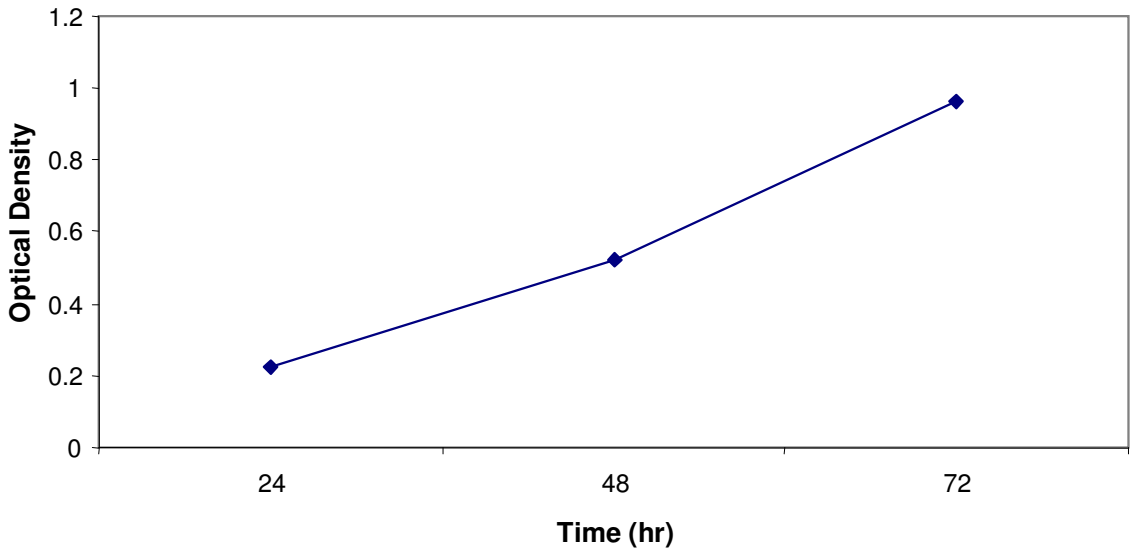

Figure 4.2: Growth rate pattern of HT-29 colon cancer cells.

4.1.3 Determination of Black Raspberry Extract Cell Treatment Concentrations:

A simple test was performed to determine the appropriate black raspberry extract concentrations to be used to evaluate their antiproliferative effects on HT-29 colon cancer cells. Cells seeded at 1.0 x 10^4 cells/well were treated with black raspberry extract concentrations of 0.19, 0.38, 0.75, 1.5 and 3.0 mg of extract/mL growth media. Cell growth inhibition was measured after 48 hr (**Figure 4.3**). Treatment concentrations of 0.6 and 1.2 mg black raspberry extract/mL media were determined to be the appropriate concentrations to show the dose-dependent effects of the black raspberry samples. These two concentrations were chosen because we wanted to use both high and low concentrations that were still within the exponential range of HT-29 cell growth. It was anticipated that for extracts with similar activity as well as extracts with slightly higher or lower bioactivity, the extract concentrations selected would show effects.

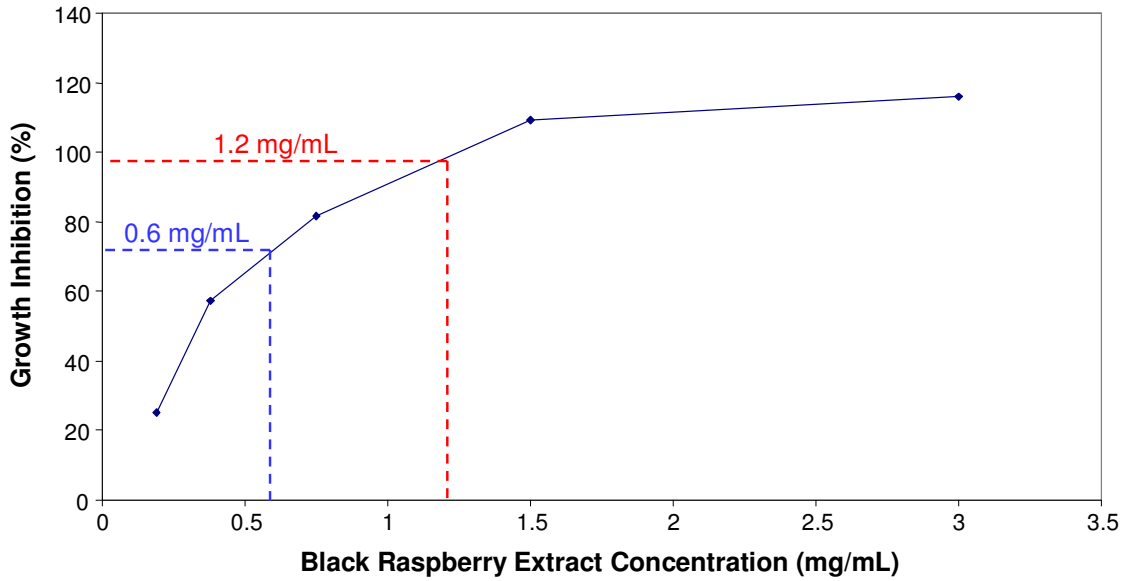

Figure 4.3: Percent growth inhibition of HT-29 colon cancer cells using different black raspberry extract concentrations.

4.2 Black Raspberry Samples:

A total of 25 black raspberry sample combinations were used for this studied. The samples varied in the three factors of maturity, growing location and cultivar. The black raspberry samples were organized into 8 developmental stages of maturity (**Figure 4.4**) by Dr. Joseph Scheerens (Horticulture and Crop Science Department, The Ohio State University, Wooster, OH). For this study, the development stages that were used were stages 4, 7 and 8. Stage 4 berries were picked approximately 4-5 days before being ripe, they were violet in color, and they were firmly attached to their receptacles at the time of harvest. Stage 7 berries were completely black, slightly dull, and could be removed from their receptacles with very minimal to no finger force. Stage 8 berries were the overripe berries that were developed in the lab by widely separating individual ripe berries on Styrofoam trays and allowing them to over-ripen at room temperature for 24 hr.

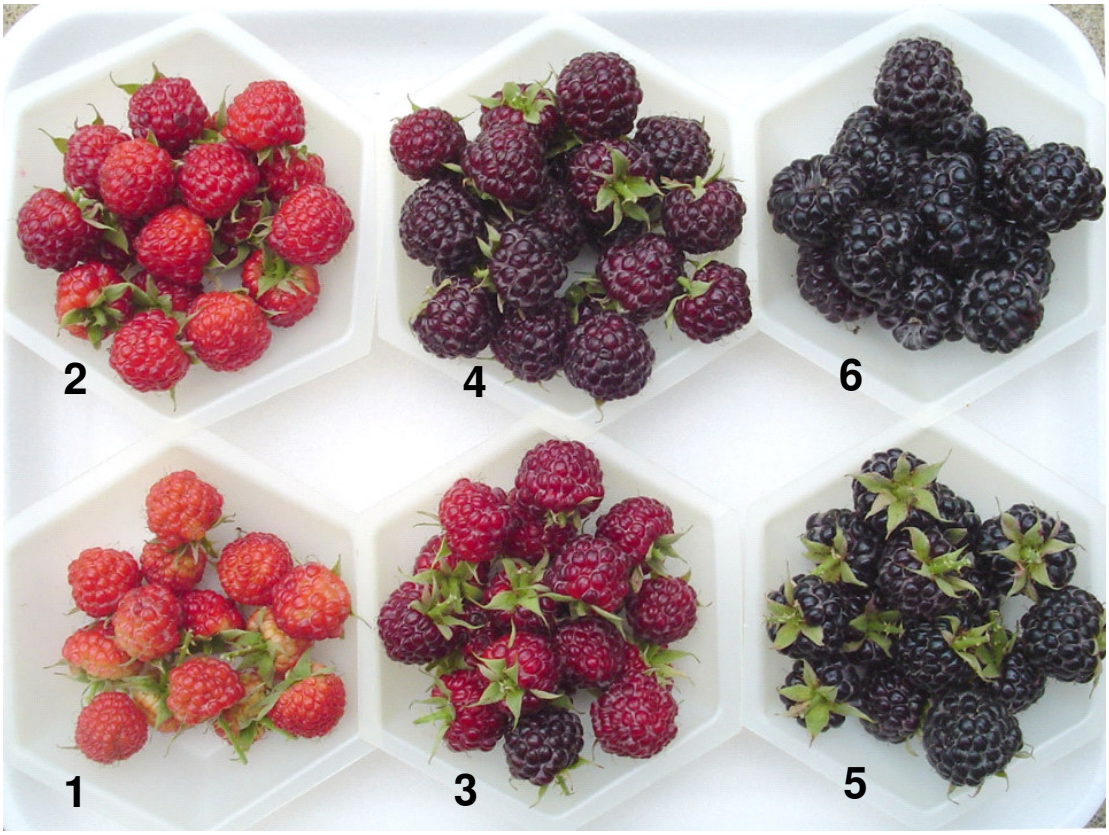

Figure 4.4: Black raspberry fruit developmental stages: Stage 1 – blush; Stage 2 – red; Stage 3 – red violet; Stage 4 – violet; Stage 5 – black, torus firmly attached; Stage 6 – black, torus removed with gentle force. Stages 7 and 8 (not pictured because of similar appearance to Stage 6) represent ripe (black) fruit with torus easily removed and fruit over-ripened for 24 hr at room temperature.

All 7 growing locations were in Ohio and members from the Horticulture and Crop Science Department worked with local growers that already grew black raspberries. However, the three cultivars and the three maturity stages were not able to be picked from each of the 7 locations. The three cultivars were not able to be picked from every location because it was up to the growers in each location to determine what cultivar choices would be grown there and this was determined by them deciding what cultivars would be best for their individual businesses. Underripe samples were unable to be collected from locations 1, 3 and 6 due to not being able to be there in time to collect to them. Only underripe samples were collected from location 7 due to the fact that the growers from there were experiencing bird problems with the ripe berries. At location 4 the berries were picked when ripe by the growers and then frozen on site, which eliminated the chances of being able to over-ripen them. Although the types of black raspberry samples may have been unbalanced it still allowed the main objective of this study to be met, which was to create at least 25 samples that varied significantly in phenolic constituents in order to run a cell modeling study.

Originally for this study the plan was to have 25 black raspberry samples that were harvested in field replications of 3 (A, B and C), but due to the concentration process described in section 3.2.1 every sample had to be treated as individuals, for a total of 75 different samples. The reason that the samples became 75 individual samples was because of their behavior in the SPE columns. Because such large sample volumes were being pushed through the SPE columns, the recovery rates were quite variable causing differences in the field replications.

4.3 Total Phenolic and Total Monomeric Anthocyanin Contents in the Black Raspberry Samples:

The total phenolic (TP) and total monomeric anthocyanin (TMA) contents were determined for the 75 black raspberry extracts, as well as their percent cell growth inhibitions at both treatment concentrations of 0.6 and 1.2 mg extract/mL media (**Table 4.1**). While there was variability among the actual quantities of TP and TMA among the field replications of the same type of black raspberry sample, their proportions of TMA to TP were usually quite similar. There were also similarities in the percent cell growth inhibition for the field replications of the same type of black raspberry sample.

Code Name	TP (mg GAE/100g)	TMA (mg cy-3-glu/100g)	TMA/TP (%)	% Inhibition at 0.6 mg extract/mL media	% Inhibition at 1.2 mg extract/mL media
1BR-A	19303	6794	35	75	108
1BR-B	29504	16493	56	73	96
1BR-C	32988	17341	53	70	101
1BO-A	20845	9147	44	66	101
1BO-B	28542	12593	44	71	98
1BO-C	18373	5922	32	64	94
1JR-A	29658	15041	51	75	113
1JR-B	29681	13504	45	72	106
1JR-C	37255	20611	55	73	97
1JO-A	26235	12785	49	66	96
1JO-B	15311	6216	41	68	95
1JO-C	28809	12200	42	71	96
1MR-A	13006	10158	78	58	86
1MR-B	28875	15641	54	53	85
1MR-C	39723	18619	47	55	88
1MO-A	21673	9114	42	33	71
1MO-B	25212	9477	38	51	84
1MO-C	21450	12075	56	56	84
2MU-A	17581	4053	23	56	83
2MU-B	19019	6502	34	62	96
2MU-C	41030	15941	39	69	109
2MR-A	35177	13645	39	61	89
2MR-B	33540	21569	64	65	88
2MR-C	26403	15196	58	62	93
2MO-A	25786	14053	54	63	87
2MO-B	28280	13380	47	64	87
2MO-C	27905	13311	48	46	82
3JR-A	10177	5272	52	58	90
3JR-B	33017	16411	50	70	101
3JR-C	30795	15790	51	75	110
3JO-A	31566	15802	50	71	101
3JO-B	27404	14871	54	67	92
3JO-C	29796	13939	47	70	102

Table 4.1: Total phenolic content, total monomeric anthocyanin content, and % inhibition at the treatment concentrations of 0.6 and 1.2 mg black raspberry extract/mL media for the 75 black raspberry samples. TP = total phenolics, TMA = total monomeric anthocyanin, Black raspberry code = See table 3.1

Table 4.1 continued

Code Name	TP (mg GAE/100g)	TMA (mg cy-3-glu/100g)	TMA/TP (%)	% Inhibition at 0.6 mg extract/mL media	% Inhibition at 1.2 mg extract/mL media
3MR-A	21866	8218	38	63	87
3MR-B	20662	12302	60	65	91
3MR-C	32469	16554	51	64	90
3MO-A	30946	16356	53	57	75
3MO-B	35951	18327	51	62	88
3MO-C	40383	16198	40	62	82
4BR-A	29814	17983	60	60	96
4BR-B	28814	16592	58	61	89
4BR-C	30553	15206	50	65	103
4JR-A	23408	9300	40	68	92
4JR-B	23560	9652	41	67	89
4JR-C	26135	11484	44	62	93
5JU-A	12219	2042	17	76	104
5JU-B	32631	16156	50	72	103
5JU-C	24772	10765	43	65	92
5JR-A	22158	9936	45	65	93
5JR-B	21216	10363	49	56	84
5JR-C	37044	20116	54	68	101
5JO-A	30705	13060	43	67	98
5JO-B	28280	12775	45	65	90
5JO-C	40780	16688	41	69	93
5MU-A	16121	4582	28	62	91
5MU-B	37674	15312	41	65	95
5MU-C	24481	10585	43	63	92
5MR-A	20651	5488	27	53	86
5MR-B	30892	15387	50	46	76
5MR-C	33768	15934	47	57	80
5MO-A	30098	10896	36	61	84
5MO-B	27954	11552	41	55	78
5MO-C	31087	12691	41	50	81
6JR-A	33010	19844	60	57	91
6JR-B	15729	9228	59	43	82
6JR-C	32810	16330	50	56	84
6JO-A	26292	16490	63	51	84
6JO-B	38471	18387	48	58	83
6JO-C	36176	21405	59	60	84

Continued

Table 4.1 continued

Code Name	TP (mg GAE/100g)	TMA (mg cy-3-glu/100g)	TMA/TP (%)	% Inhibition at 0.6 mg extract/mL media	% Inhibition at 1.2 mg extract/mL media
7JU-A	14027	7459	53	62	88
7JU-B	31450	15620	50	82	118
7JU-C	32187	12350	38	70	98
7MU-A	23843	13178	55	75	108
7MU-B	22598	12921	57	71	101
7MU-C	22255	15446	69	74	106

High variability was obtained in the TP contents of the black raspberry samples evaluated, ranging from 10,177 to 41,030 mg/100g of dry matter as gallic acid equivalents (GAE). There was also high variability in the TMA contents, ranging from 2,042 to 21,569 mg cyanidin-3-glucoside (cy-3-glu) equivalents/100g of dry matter. Based on these numbers, the TMA contents of the black raspberry extracts comprised between 17 to 78% of the TP contents. These results demonstrate that TP and TMA contents in black raspberries greatly vary due to growing conditions and horticultural practices.

The levels of TP and TMA in the black raspberry extracts used in this experiment were much higher than the reported literature values of 5,938 mg GAE/100g and 1,770 mg cy-3-glu/100g of dry matter that came from freeze-dried whole black raspberries (Kresty et al., 2006; Harris et al., 2001). These findings suggest that the extraction and concentration procedures used in this study effectively concentrated the compounds of interest.

For this study, all 75 black raspberry extracts' TP and TMA contents were statistically analyzed by comparing them separately using the three factors: fruit

maturity (underripe, ripe and overripe), cultivar (Bristol, Jewel and MacBlack) and growing location (Ohio locations 1-7). Among these factors, the black raspberries' stage of maturity (**Figure 4.5**) had a significant effect on both the TP content ($p = 0.0425$) and TMA content ($p < 0.01$). It was found that among the three stages of maturity the overripe samples on average (28,678 mg GAE/100g) had the highest TP content, followed by the ripe samples (27,687 mg GAE/100g). The underripe samples, with an average TP content of 24,793 mg GAE/100g, was found to be significantly lower than the overripe samples' TP content.

When comparing TMA contents among the three stages of maturity the ripe samples on average (14,000 mg cy-3-glu/100g) had the highest content, followed by the overripe samples (13,323 mg cy-3-glu/100g). Both of these stages of maturity were significantly higher than the underripe samples' TMA content (10,861 mg cy-3-glu/100g). These results demonstrate that fruit maturity has a major impact on TP and TMA contents present in fruits.

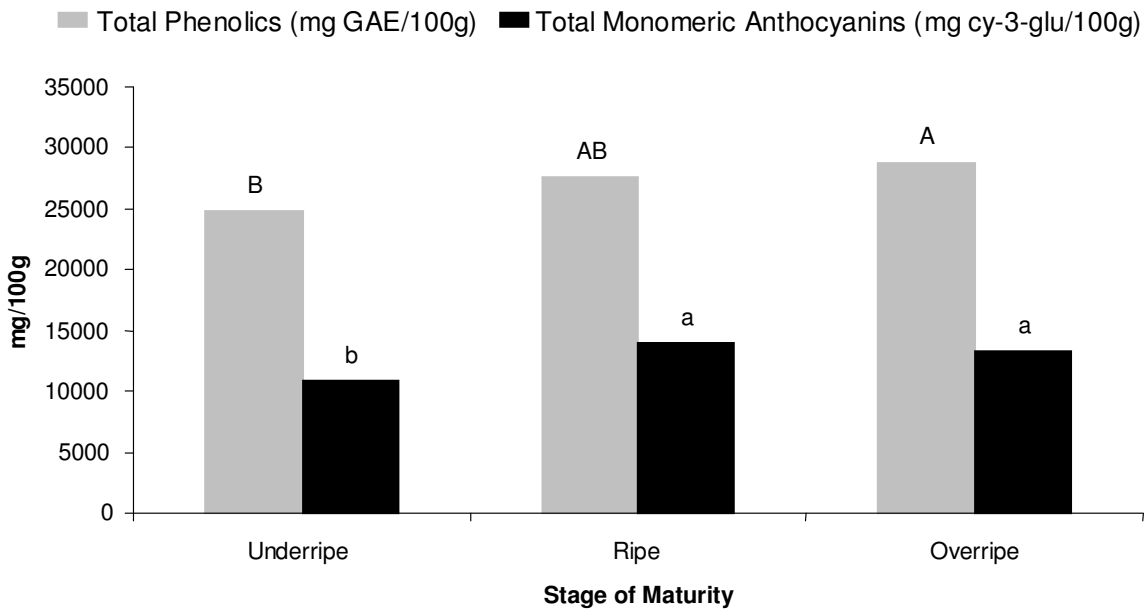

Figure 4.5: Comparison of the TP and TMA contents of the black raspberry samples at the different stages of maturity.

When comparing the black raspberry extracts' TP and TMA contents, growing location (**Figure 4.6**) was found to significantly affect only the TMA contents ($p < 0.01$). Among the 7 growing locations, location 6 grew the berries that had on average the highest TMA content (16,947 mg cy-3-glu/100g). Black raspberry samples grown in location 6 were found to have significantly higher TMA contents than both locations 1 (12,429 mg cy-3-glu/100g) and 5 (11,907 mg cy-3-glu/100g). Berries grown in location 6 also had the highest TP content on average (30,415 mg GAE/100g), while black raspberries grown in location 7 had on average the lowest TP content (24,394 mg GAE/100g). These finding suggest that growing location can have a big impact on the TP and TMA contents of berries due to its sunlight, cultural conditions and climate conditions.

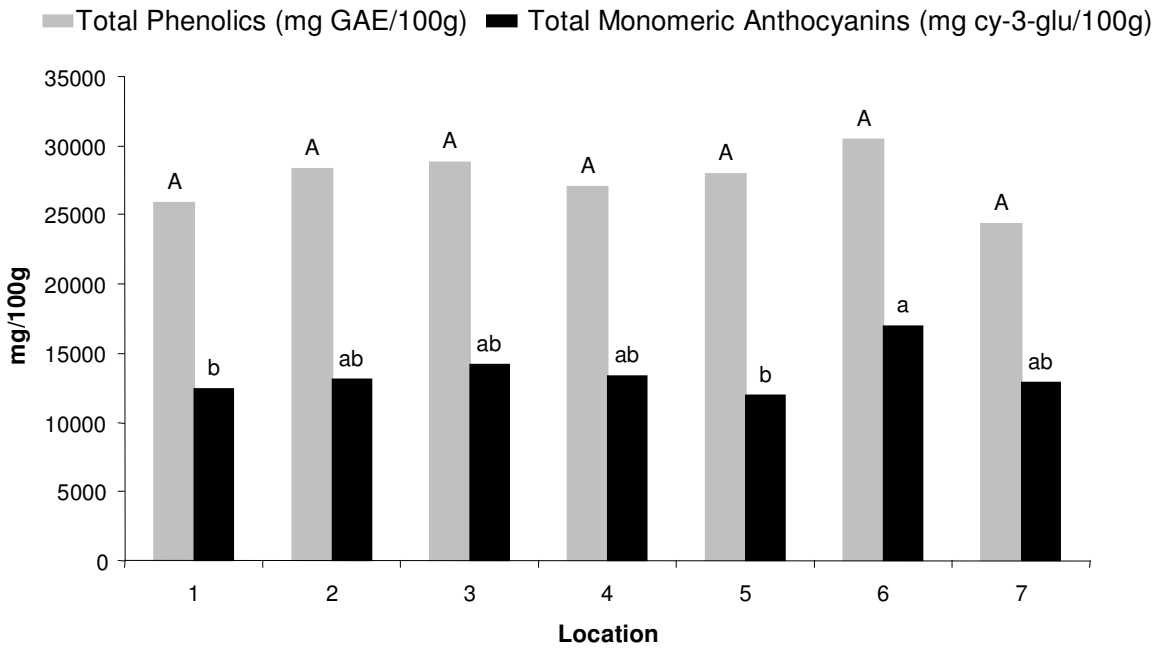

Figure 4.6: Comparison of TP and TMA contents of black raspberry samples grown in the 7 Ohio locations.

4.4 HT-29 Colon Cancer Cell Growth Inhibition:

All 75 black raspberry samples were used to treat HT-29 colon cancer cells at two concentrations of 0.6 and 1.2 mg black raspberry extract/mL media. After the black raspberry extract treatments the cells were incubated for an additional 48 hr before SRB assay was performed to determine percent cell growth inhibition caused by each of the treatments. Percent cell growth inhibition ranged from 33% (sample 1MO-A) to 82% (sample 7JU-B) for the black raspberry concentration of 0.6 mg extract/mL media, while the treatment concentration of 1.2 mg extract/mL media caused inhibition that ranged from 71% (sample 1MO-A) to118% (sample 7JU-B) (**Table 4.1**). Percent inhibition values over 100% indicated that the black raspberry sample was having cytotoxic effects on the HT-29 colon cancer cells.

All black raspberry samples inhibited the growth of HT-29 colon cancer cells in a dose-dependent manner (**Figures 4.7, 4.8 & 4.9).** The three factors evaluated: fruit maturity, cultivar and growing location, all significantly effected ($p < 0.01$) the black raspberry samples' ability to inhibit cell proliferation at both concentrations. **Figure 4.7** summarizes the effect of stage of berry maturity (underripe, ripe and overripe) on growth inhibition (% inhibition) of HT-29 cells. It was found that the underripe black raspberry samples had significantly higher inhibitory effects on HT-29 cell growth than both the ripe and overripe samples, with its inhibitory effects averaging 69% and 99 % for the treatment concentrations of 0.6 and 1.2 mg extract/mL media, respectively. These results show that even though the underripe samples are the black raspberry samples that contained the lowest amounts TP and TMA, they may contain other compounds that are

decreasing and/or disappearing as the berries ripen that are contributing to their high inhibitory properties.

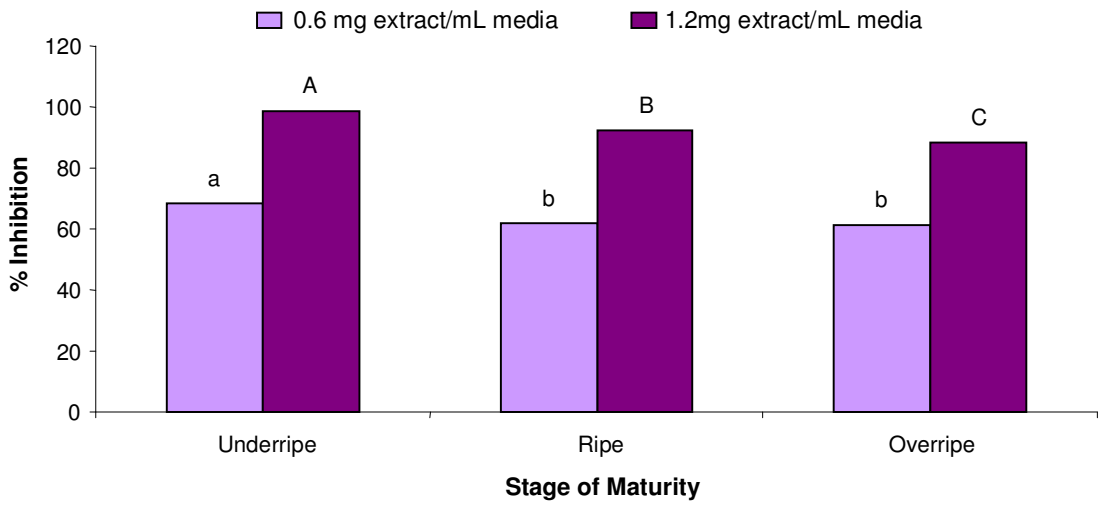

Figure 4.7: Comparison of inhibitory effects of black raspberry samples at the three different stages of maturity.

In **Figure 4.8**, the cultivar shown to contain the black raspberry samples that had the overall highest inhibitory effects on cell growth was Bristol (67 % for 0.6 mg extract/mL media and 98% for 1.2 mg extract/mL media), followed by Jewel (65 and 94%, respectively), and then MacBlack (60 and 88%, respectively). Genetic composition is typically a strong determinant of cultivar performance (Ozgen et al., 2008). In this study, the antiproliferative effects of berries of Jewel cultivar and its maternal parent Bristol did not differ statistically from each other at the low treatment concentration, however they were found to be significantly different from each other at the high concentration. The samples from the MacBlack cultivar on average showed consistently lower antiproliferative effects than berries from the other two cultivars, likely due to the fact that it is unrelated to either of them.

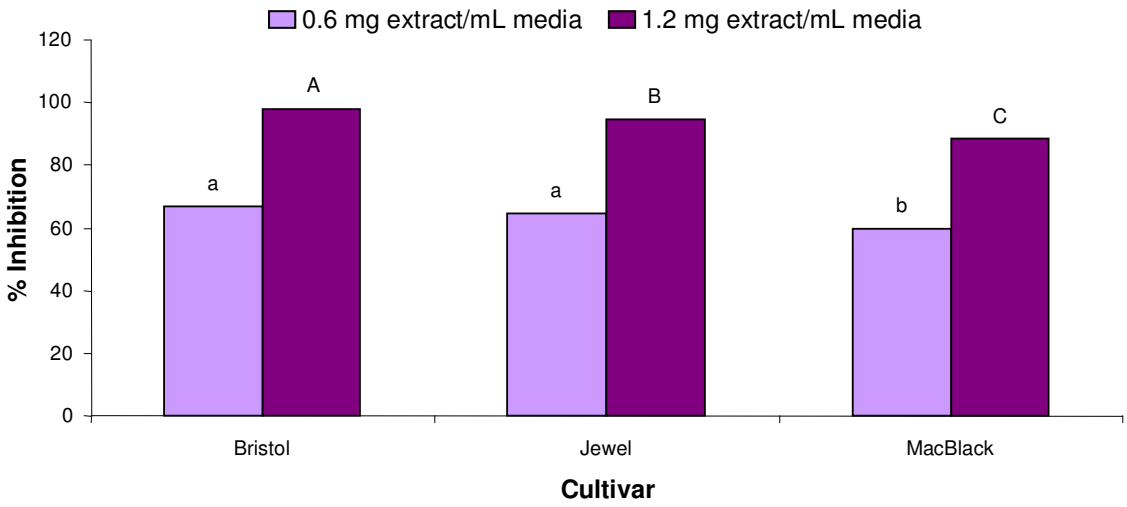

Figure 4.8: Comparison of inhibitory effects of the three different black raspberry cultivars.

Black raspberry samples grown in location 7 (**Figure 4.9**) had the highest inhibitory effects with an average inhibition of 72% for the treatment concentration of 0.6 mg extract/mL media and 103% for the treatment concentration of 1.2 mg extract/mL media. Black raspberry samples grown in location 6, despite having the highest TP and TMA contents, had average the lowest inhibitory effects at both concentrations (54 and 85%, respectively).

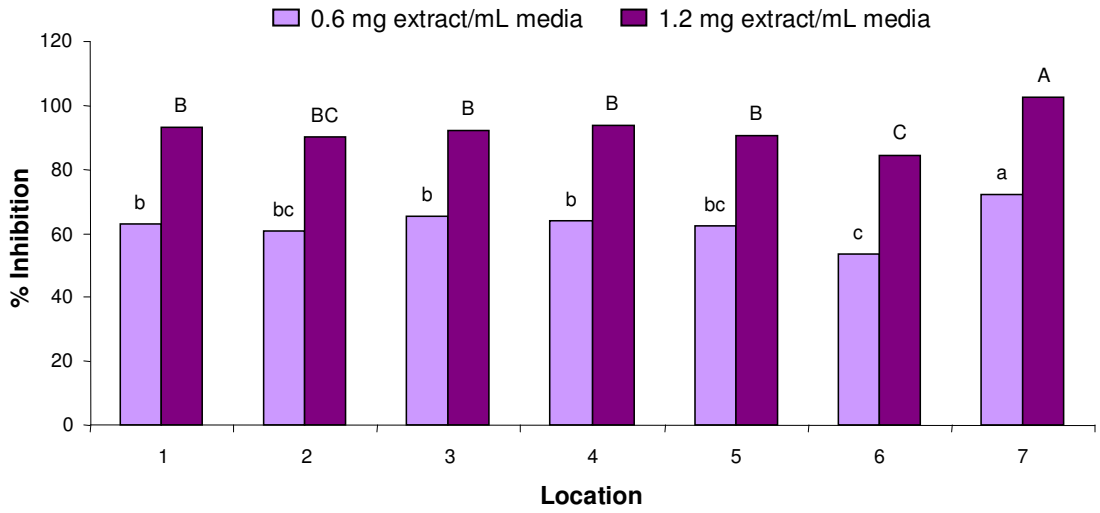

Figure 4.9: Comparison of the black raspberry samples grown in the 7 different Ohio locations.

Different interactions were evaluated using two of the variables at a time to see if one had an effect on the other. When comparing growing locations versus stages of maturity it was found that not all maturity stages (underripe, ripe and overripe) acted the same in every location ($p < 0.01$) for the low black raspberry treatment concentration (**Figure 4.10**). However, the stages of maturity did tend to act the same in the different locations for the high black raspberry treatment concentration ($p > 0.1$), meaning that the underripe samples consistently had the highest inhibitory effects in all the locations they were grown in followed by ripe and then overripe black raspberry samples.

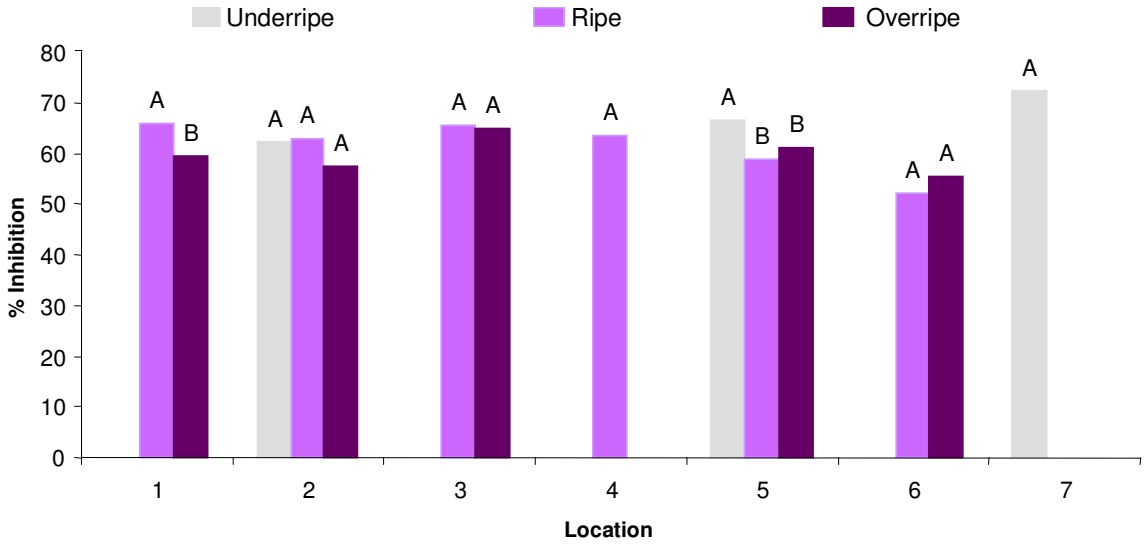

Figure 4.10: Comparison of the three stages of maturity within the 7 Ohio growing locations at the black raspberry treatment concentration of 0.6 mg extract/mL media.

Figures 4.11 and 4.12 compare the 7 growing locations versus the three black raspberry cultivars (Bristol, Jewel and MacBlack) at the low and high black raspberry treatment concentrations. In this comparison it was found that for both black raspberry treatment concentrations of 0.6 and 1.2 mg extract/mL media that the cultivars did not act the same in every location ($p < 0.01$ for the low concentration and $p = 0.0485$ for the high concentration). It was interesting to find that the MacBlack black raspberry samples grown in location 7 had on average the highest inhibitory effects out of the three cultivars and the 7 growing locations for both the low (73%) and high (105%) black raspberry treatment concentrations. This is especially interesting because the results from **Figure 4.8** showed that black raspberry samples of MacBlack cultivar had the overall lowest inhibitory effects out of the three cultivars at both treatment concentrations. The likely explanation for these finding is that some cultivars are known to grow better in some locations than others (Ozgen et al., 2008).

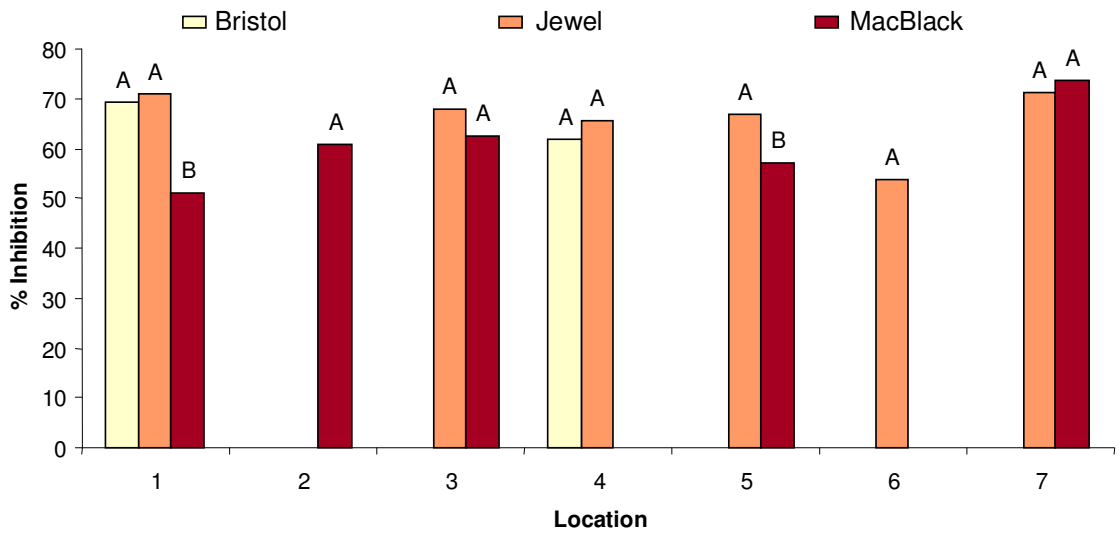

Figure 4.11: Comparison of the three cultivars within the 7 Ohio growing locations at the black raspberry treatment concentration of 0.6 mg extract/mL media.

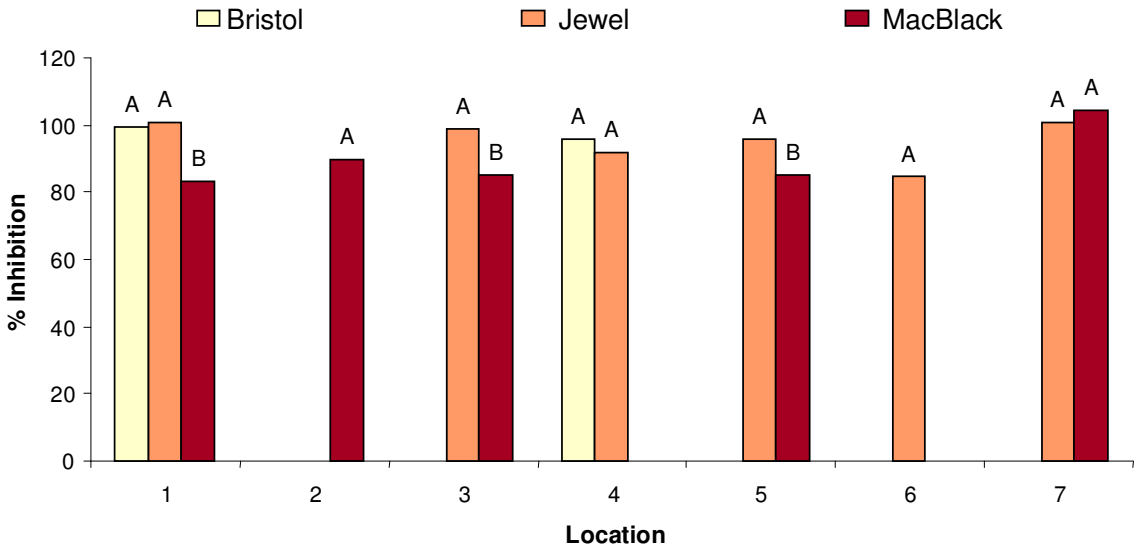

Figure 4.12: Comparison of the three cultivars within the 7 Ohio growing locations at the black raspberry treatment concentration of 1.2 mg extract/mL media.

Overall out of all 75 black raspberry samples the highest inhibition of 118% was obtained with an underripe sample of Jewel cultivar grown in location 7 (sample 7JU-B); while the lowest inhibition of 33% was obtained with an overripe sample of MacBlack cultivar grown in location 1 (sample 1MOA). These findings, along with the statistical analysis conducted on the % inhibition results from this study, suggest that all three factors maturity, cultivar and growing location did have an impact black raspberry antiproliferative activity in an *in vitro* cell model.

4.5 Determination of Possible Correlations between Bioactivity and Analytical Chemistry Results:

Once the bioactivity (% inhibition) results and the analytical chemistry (TP and TMA contents) results were statistically analyzed separately Pearson correlation tests were run to see if there were any correlations between compositional data on polyphenolics and the antiproliferative effects of the samples on HT-29 cells. The correlations that were compared were: % inhibition at the black raspberry treatment concentration of 0.6 mg/mL versus TP content, % inhibition at the black raspberry concentration of 1.2 mg/mL versus TP content, % inhibition (0.6 mg/mL) versus TMA content, and % inhibition (1.2 mg/mL) versus TMA content (**Figure 4.13**). The results showed that there were no correlations found between the bioactivity and analytical chemistry results. These finding suggest that it is other compounds and interactions in conjunction with the TP and TMA contents that are causing the inhibitory effects of the black raspberry samples, not just the TP and TMA contents alone.

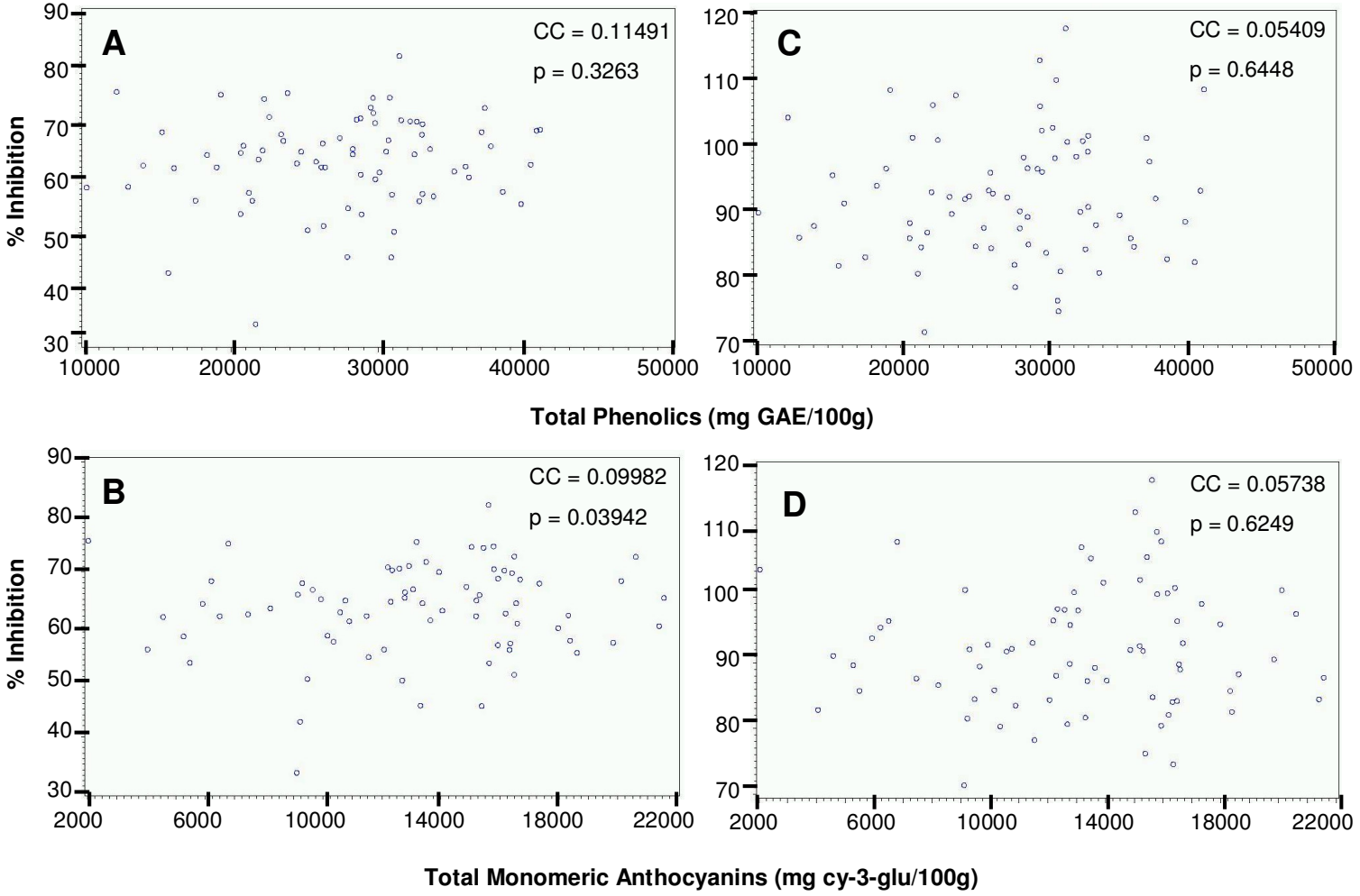

Figure 4.13: Pearson correlations between the bioactivity and polyphenolic results. Figures A & B represent the treatment concentration of 0.6 mg extract/mL media. Figures C & D represent the treatment concentration of 1.2 mg extract/mL media.

4.6 Characterization of Black Raspberry Anthocyanins:

The major anthocyanins in the black raspberry samples were identified in **Table 4.2**. Based on published literature (Tian et al., 2006; He, 2008), order of elution, spectral characteristics and the molecular weights of the compounds, the black raspberry anthocyanins were identified as cyanidin-3-sambubioside, cyanidin-3-glucoside, cyanidin-3-xylosylrutinoside, cyanidin-3-rutinoside and pelargonidin-3-rutinoside.

		m/z		
Peak number	**λ_{max} (nm)**	**$[M^+]$ Intact molecule**	**Aglycone fragment**	**Anthocyanin**
1	520	581	287	cy-3-sambubioside
2	516	449	287	cy-3-glucoside
3	522	727	287	cy-3-xylosylrutinoside
4	522	595	287	cy-3-rutinoside
5	500	579	271	pg-3-rutinoside

Table 4.2: The mass spectra data of the black raspberry anthocyanins (Source: Tian et al., 2006; He, 2008).

The two chromatograms in **Figure 4.14** are representative of the anthocyanin and phenolic profiles of the 75 black raspberry samples used in this study. Cy-3-xylosylrutinoside (peak 3) and cy-3-rutinoside (peak 4) together comprised over 80% of the total anthocyanins found in each of the black raspberry samples, while pg-3-rutinoside usually comprised less than 1% of the total anthocyanins found in each of the samples.

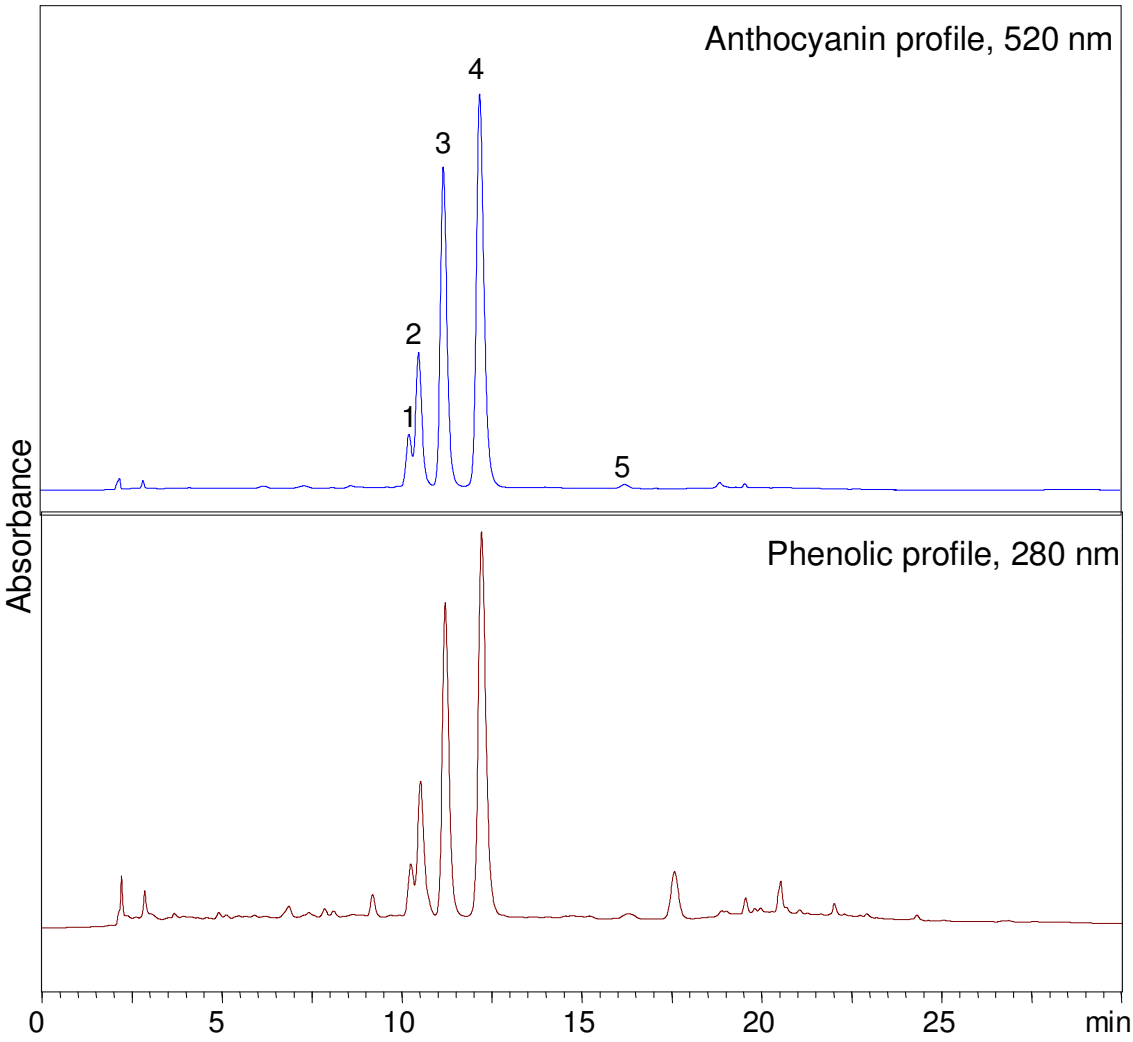

Figure 4.14: Representative chromatograms of the 75 black raspberry samples. Shown is the anthocyanin and phenolic profiles sample 2MR-B.

Peak number	Anthocyanin	% Area at 520 nm for Sample 2MR-B	% Area at 520 nm for Sample 1BR-B	% Area at 520 nm for Sample 7JU-B
1	cy-3-sambubioside	4.4	4.1	5
2	cy-3-glucoside	13.4	10.8	8.9
3	cy-3-xylosylrutinoside	33.4	35	45.5
4	cy-3-rutinoside	47.1	48.6	40.4
5	pg-3-rutinoside	0.4	0.6	0.3

Table 4.3: Characterization of black raspberry anthocyanins from the representative samples 2MR-B, 1BR-B and 7JU-B.

In order to try to identify some of the other phenolics present in the black raspberry samples, besides anthocyanins, two standard cocktails were run along the same elution gradient as the samples. The first cocktail contained caffeic, sinapic, gallic and protocatechuic acids. The second cocktail contained chlorogenic, caffeic, *p*-coumaric and ferulic acids. Retention time, molecular weight and peak absorbencies of the compounds in these standard cocktails were compared with the phenolic compounds' peaks in the black raspberry samples. It was determined that none of the standard compounds were found in the black raspberry samples.

When comparing the five anthocyanin % areas (**Table 4.3**) among the 75 black raspberry samples a noticeable difference was found among the three cultivars. Specially, the difference was found among the three cultivars when comparing peaks 3 (cy-3-xylosylrutinoside) and 4 (cy-3-rutinoside). For black raspberry samples of MacBlack cultivar, representative sample 2MR-B, and of Bristol cultivar, representative sample 1BR-B, peak 4 was usually higher than peak 3. However, for black raspberry samples of Jewel cultivar, representative sample 7JU-B, peak 3 was usually higher than peak 4. Although these finding are interesting, they are not the cause of the differences in antiproliferative activity among the three cultivars. As shown in **Figure 4.8** the antiproliferative activity order was black raspberry samples of the Bristol cultivar with the highest effects, followed by Jewel and then MacBlack.

Previous research has shown that the anthocyanins that contribute the most to a black raspberry's chemoprotective properties are cyanidin-3-glucoside, cyanidin-3 xylosylrutinoside and cyanidin-3-rutinoside (Stoner et al., 2007). These compounds in conjunction with the many other compounds including ellagic acid, ferulic acid, vitamins

C and E, folic acid, calcium, magnesium, carotenoids and β-sitosterol present in black raspberries are what contribute to their overall chemoprotective properties (Stoner et al, 2007, Tulio et al., 2008). In this study, all three factors used: stage of maturity, type of cultivar and growing location significantly impacted the black raspberry samples' ability to inhibit HT-29 cancer cell proliferation; thus further research needs to be conducted to determine what specific impacts these factors are having on the various chemoprotective compounds found in black raspberries.

Chapter 5: Conclusions

The results of this project demonstrated that the black raspberry extracts effectively slowed down the growth of HT-29 colon cancer cells in a dose-dependent manner. Among the black raspberry samples growing location, cultivar and stage of maturity all impacted the composition (TP and TMA contents) and the inhibitory effects of these samples. Black raspberries grown in location 7 on average had the highest inhibitory effects on cell proliferation, but some of the lowest TP and TMA contents. Samples grown in location 6 on average had the highest TP and TMA contents, but some of the lowest inhibitory effects. Of the three cultivars, black raspberry samples of Bristol cultivar were found to have the overall highest inhibitory effects. Out of the three stages of maturity, underripe black raspberry samples had on average the highest inhibitory effects, but the lowest TP and TMA contents. There was no significant correlation between the bioactivity results and polyphenolic results, suggesting that there are other compounds and interactions that are contributing to the chemoprotective properties of the black raspberry samples. Further research is needed to determine what exactly these other compounds and interactions are, besides the TP and TMA contents, that are contributing to the black raspberry samples' antiproliferative properties.

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