EFFECT OF STORAGE CONDITIONS ON PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF BLUEBERRY EXRACT AND THE EFFECT OF ANTHOCYANINS FROM SELECTED CULTIVARS OF GEORGIA-GROWN BLUEBERRIES ON APOPTOSIS AND PHASE-II ENZYMES

By

ANITA SRIVASTAVA

(Under the direction of CASIMIR C. AKOH)

ABSTRACT

Blueberry (*Vaccinium corymbosum*) of the family Ericuceae is reported to have high antioxidant activity compared to other fruits and vegetables. This is highly correlated with the anthocyanins and total polyphenolic content. Blueberries are often converted to extracts such as juice or juice concentrate for subsequent use in beverages, syrups and other food products. Phenolic compounds are highly unstable and may be lost during processing, particularly when heat treatment is involved. Blueberry extract was prepared and stored at different temperatures (-20±1, 6±1, 23±1, and 35±1 ^{o}C) in glass bottles. Changes were observed in total polyphenols (TPP), total anthocyanin (TACY), Trolox-equivalent antioxidant capacity (TEAC), phenolic acids, individual anthocyanins, and cell proliferation during storage. Two Georgia-grown cultivars, Tifblue and Powderblue were chosen for the study. Recovery of TPP, TACY and TEAC in blueberry extract after pressing and heating were ~25, ~29, and ~69%, respectively, for both cultivars. Recovery of gallic acid, catechin and quercetin was ~25% in final extract. Ferulic acid was not

detected in the final extract in both Tifblue and Powderblue cultivars. Recovery of peonidin, malvidin and cyanidin was ~20% in final extract in both the cultivars. These results suggest that most of the phenolic compounds were lost during removal of residue and during heating. Losses due to storage were less when compared with initial loss due to processing. There was no statistically significant loss ($P < 0.05$) of TPP, TACY and TEAC observed up to 30 days at -20±1 ^{o}C. At 6 ^{o}C storage, a significant loss of TPP, TACY and TEAC was observed from 15 to 30 days. Similar results were obtained at 23 ^{o}C and 35 ^{o}C ($P < 0.05$). A linear relationship was observed between TEAC values and total polyphenols and total anthocyanins. There was retention of more than 40% of ellagic acid and quercetin after 60 days at 35±1 ^{o}C. Anthocyanins were not detected after 60 days of storage at 35±1 ^{o}C. Significant retention ($P < 0.05$) was obtained for malvidin (42.8 and 25.8%) and peonidin (74.0 and 79.5%) after 60 days storage at 23±1^{o}C in glass bottles for Tifblue and Powderblue, respectively. Cell viability assay was performed using HT-29 cancer cell line and anthocyanins extracted from 30, 60, and 90 days stored extract at 6±1 and 23±1 ^{o}C. Significant cell proliferation inhibition percentage was observed in 30 days, although this was reduced significantly after 30-90 days. These results suggest that initial preparatory steps like washing, removal of residue mainly skin, heating and storage conditions were significantly affecting the phenolic compounds and their biological activity.

Anthocyanin fractions from four cultivars of Georgia-grown blueberries namely Tifblue, Powderblue, Brightblue, and Brightwell were used for apoptosis study. Apoptosis was confirmed using two different methods: DNA fragmentation and caspase-3 activity. The effect of anthocyanins on the activity of detoxifying enzymes glutathione-

S-transferase (GST) and quinone reductase (QR) were also determined. Cells were treated with 50, 100, and 150 μg/mL of anthocyanin fraction. Low concentration of anthocyanin from all cultivars showed DNA fragmentation. There was a significant difference in the caspase-3 activity ($P < 0.05$) between the control cells and the cells treated with anthocyanins from all the cultivars**.** A positive dose-response relationship was found in all the cultivars. Highest activity (1.4 fold increase over control) was observed in cells treated with 150 μg/mL anthocyanin fraction from the Brightwell cultivar. QR activity was lower in all treated cells than in control cells (0.25 μM/mg protein); A positive dose-response relationship was found in all the cultivars except Brightblue, where activity was the same for all three concentrations. GST activity was statistically higher ($P < 0.05$) in control cells than in cells treated with anthocyanin fractions from all the cultivars and at all levels of concentration. These results indicated that anthocyanins were not highly active in induction of detoxifying enzymes; however, apoptosis was confirmed in HT-29 cancer cells when treated with anthocyanins consisting predominantly of malvidin.

INDEX WORDS: Anthocyanins, blueberries, blueberry extract, caspase-3, cell proliferation, cultivars, detoxifying enzymes, DNA fragmentation, DNA ladder, flavonoids, glutathione-S-transferase, phase-II enzymes, phenolic compounds, quinone reductase,storage,TEAC.

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by

ANITA SRIVASTAVA

B.Sc., University of Delhi, India, 1987

M.Sc., G. B. Pant University of Agriculture and Technology, India, 1990

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2006

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BY

ANITA SRIVASTAVA

Major Professor:	Casimir C. Akoh
Committee:	Ronald R. Eitenmiller Louise Wicker

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2006

DEDICATION

Dad, Mom

Anupam, Rahul, and

Sanjay

ACKNOWLEDGEMENTS

I am deeply thankful to all people who contributed to the completion of this thesis. All help was fully appreciated because no assistance was little when goodwill was involved.

I want to especially thank my major professor, Dr. Casimir C. Akoh, not only for supporting me in the accomplishment of this masters program but also for showing me such great empathy and sensibility to understand my personal goals and needs.

I express also my deep appreciation to each professor that served on my advisory committee, Dr. Ronald R. Eitenmiller, and Dr. Louise Wicker.

I wish to thank Dr. Joan Fischer for providing me valuable guidance all through my research work.

I want to thank Dr. Mout Michael, Dr. Weiguang Yi, Ms. Brenda Jennings, and Ms. Rachel Dulebohn for their technical assistance, my lab collegues, Byung, Stephen, Jeung Hee and Jung-Ah, and my sisters Sunita, Asha, Meenu for their cooperation and moral support all through my work.

Last but not least, thanks to the faculty and staff of the Department of Food Science & Technology, UGA, for being part of my graduate student life and for their contributions to my personal and professional development.

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CHAPTER 1

INTRODUCTION

Blueberries (*Vaccinium corymbosum)* commonly called highbush cultivated blueberries are native to North America and have been commercially produced for many years**.** Blueberries members of the family Ericaceae gained attention in recent years due to its high antioxidant activity compared to other fruits and vegetables (1-2). This is highly correlated to their anthocyanins and total polyphenolic content (3).

Blueberries are often converted to water soluble extracts such as juice or juice concentrate which are subsequently used in beverages, syrups and other food products. Polyphenolic compounds including anthocyanins are not completely stable (4). They are readily oxidized because of their antioxidant properties and thus prone to degradation. Native enzyme, polyphenol oxidase (PPO) present in blueberry is responsible for oxidation of polyphenolics to quinones, which produce brown pigments (5-7), thus affecting the color of the extracts, and similar products like juice or concentrates. Heating was shown to inhibit PPO activity (5). The significant deterioration of phenolic compounds in highbush blueberries when converted to juice has already been discussed in several reports (8-9). However reports describing changes in antioxidant capacity, antiproliferation activity due to storage are rarely found.

Anthocyanins belong to a widespread class of phenolic compounds collectively named flavonoids, and are present in high concentrations in blueberries (10). The difference between individual anthocyanins is related to the number of hydroxyl groups,

the nature and number of sugars, and the position of these attachments (11).

In recent years considerable attention has been paid to anthocyanins due to their abilities to inhibit oxidative stress, cell carcinogenesis, and to induce apoptosis in malignant cells (11-16). Apoptosis is a major form of cell death, characterized chromatin condensation, cytoplasmic blebbing, and DNA fragmentation (17-19). This plays a significant role in the elimination of seriously damaged cells or tumor cells by chemopreventive agents (20-21). Caspases, a family of cystein proteases are said to be involved in this process of apoptosis (22). Activation of caspases during apoptosis results in the cleavage of critical cellular substrates, including poly (ADP-ribose) polymerase and lamins, so precipitating the dramatic morphological changes in apoptosis (23).

Several studies have shown a link between intake of specific flavonoids, including anthocyanins, and a reduction in colon cancer risk (24-26). There are several mechanisms that could contribute to this association. A well characterized defense mechanism involves the induction of detoxification enzymes. Phase-I enzymes, members of cytochrome P450 superfamily, metabolically oxidize many xenochemicals thereby forming electrophilic intermediates (27-29). These electrophilic intermediates have ability to induce DNA damage and mutations, and are responsible for carcinogenic activity of many chemicals (30). Phase-II detoxification enzymes are responsible for metabolizing products of Phase-I metabolic reactions, degrade these reactive intermediates by conjugation or reduction reactions, thereby protecting cells from oxidative DNA damage. The most common conjugation reactions are catalyzed by glutathione-S-transferase (GST) (31), whereas reduction reactions are catalyzed by quinone reductase (QR) (32). GSTs detoxify carcinogens and promote their excretion by

promoting the conjugation of electrophilic compounds with glutathione. QR, another Phase II enzyme, works by catalyzing two-electron reductions on free radicals and toxic oxygen metabolites, which deactivates them and protects the surrounding tissues from mutagenesis and carcinogenesis. Many studies show that flavonoids such as anthocyanins can stimulate GST and QR (33-34).

The present thesis includes five chapters. The first chapter is introduction. The second chapter presents a literature review of topics related to blueberry, phenolic acids, anthocyanins and their health effects, apoptosis, role of caspase-3, detoxifying enzymes, glutathione-S-transferase and quinone reductase.

Third chapter presents effect of storage conditions on biological activity of phenolic compounds of blueberry extract packed in glass bottles. Extract was stored at different temperatures (-20±1, 6±1, 23±1, and 35±1 ^{o}C). Two cultivars, Tifblue and Powderblue were chosen for the study. These are the most commonly used berries by food industries, and Tifblue (Rabbiteye) is gaining attention due to its high anthocyanin content. Anthocyanin fraction was separated from extracts stored at 6±1, 23±1 ^{o}C and their effect on cell proliferation activity using HT-29 colon cancer cell line was evaluated.

The forth chapter presents effect of anthocyanin fractions from Georgia grown cultivars of blueberries (Tifblue, Powderblue, Brightblue, and Brightwell) on apoptosis and phase II enzymes: glutathione-S-transferase and quinone reductase as a measure of chemopreventive properties and mechanisms. Two different methods were chosen to confirm the apoptosis, namely DNA fragmentation and caspase-3 activity.

Chapter 5 summarizes chapters 3 and 4 and presents general conclusions.

The objectives of the present study are: (1) to study the effect of storage on phenolic compounds and their antioxidant activity in blueberry extract, (2) to separate anthocyanin fractions from blueberry extract stored under different temperature conditions and evaluate their effect on cell proliferation activity using HT-29 cancer cell line, and 3) to study the effect of anthocyanins from selected cultivars grown in Georgia on apoptosis and phase-II enzymes.

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CHAPTER 2

LITERATURE REVIEW

BLUEBERRY

Blueberries (*Vaccinium corymbosum)* commonly called highbush cultivated blueberries are native to North America. Blueberries are members of the *Ericaceae* family, which also includes cranberry, huckleberry, and genus *Vaccinium.* The genus *Vaccinium* comes from the Latin "vacca" for cow since cows love them, a fact first noted by Captain James Cook in the late 1700s (1). In the 1930s, horticulturist Stanley Johnston established the first successful cultivated blueberry plantings in the world (2). Although indigenous to North America, blueberries are among the most recently cultivated fruit crops. Domestication began in the late 19th century when selections of wild blueberries were transplanted to gardens and fields in New Jersey and Michigan.

Blueberries are classified as: (1) highbush, the major type cultivated in North America, used in commercial planting in cooler climate, (2) rabbiteye, cultivated in the southeastern United States, and (3) lowbush, harvested from managed wild stands. Highbush and rabbiteye are cultivated in rows. In contrast, lowbush grow naturally as transition vegetation between the open field and forest (3). Blueberries are shallow-rooted plants and require l-2 inches of rainfall per week during the growing season (4). Most northern highbush varieties need at least 30-40 days of temperatures below 45°F, temperatures below -15°F to -20°F damage flower buds and reduce yields. The mature cultivated highbush are less than 10 feet (3-6). Rabbiteye blueberry cultivars have

tolerance of wide range of temperatures, drought resistance, and require low chilling time. Those native to Georgia and northern Florida, require only one-third to one-half as many chilling hours as highbush blueberry cultivars. Georgia is currently the largest producer of rabbiteye with 6000 acres in the southeastern part of the state (7). They have a fibrous root system that penetrates more deeply than does the highbush root system. Rabbiteye can reach heights of 33 feet, but are pruned to manageable heights in commercial plantings (3-4, 8). Lowbush blueberry, as the name suggests, are creeping shrubs, about 1 ft tall or less, and fruits are smaller and lighter blue than other species (9).

The harvest of blueberries usually begins in mid-April in Florida, early May in North Carolina, early June in New Jersey, and early July in Michigan, Oregon, and Washington. Maine's harvest begins in August. Harvest usually ends last in Washington and Michigan. Early maturing blueberries are generally handpicked for the fresh market whereas later berries are mechanically harvested and used for processing. Sugar content of fruit will increase during maturation to about 15 percent when fruit is ripe. Fruit size continues to increase after fruit turns blue, due mainly to water uptake. Accumulation of sugars during ripening increases sweetness. Sugar content does not increase after harvest, but acids are broken down during ripening, thus decreasing tartness. Fruit flavor, much of it associated with the skin, increases during ripening, but not after harvest. Postharvest shelf life of blueberry fruit is increased by rapidly cooling fruit after harvest (7).

Most blueberries are processed, only 30 percent is used fresh. Generally blueberries are used in jams, jellies, syrups, muffin mixes, pies, yogurt bases, canned fruit fillings, bakery products, preserves, juice concentrates, and juice drinks (2). In addition,

individually quick frozen (IQF) blueberries are a retail product. The largest quantity of processed (frozen) blueberries is used by food service establishments and bakeries (4).

PHENOLIC COMPOUNDS

Phenolic compounds are secondary metabolites produced in the plants via shikimate or phenylpropanoid pathway. The biosynthesis of phenylpropanoid compounds is activated in response to environmental stresses such as by wounding, pathogen infection, and/or UV irradiation. Phenolic compounds, as lignin form an integral part of cell-wall structure, and are the second most abundant organic structures on the earth after cellulose (10, 11). Phenylpropanoids play a significant role in the structure and protection of the plant, affects plant qualities such as texture, flavor, color, and processing characteristics (12), and also plays a significant role in pollination and seed dispersal.

Phenylalanine is a common precursor for most phenolic compounds in higher plants (11, 13). All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine ammonia-lyase (PAL), the branch point enzyme between primary (14) and secondary (phenylpropanoid) metabolism (15-18). Several simple phenylpropanoids (with the basic C6-C3 carbon skeleton of phenylalanine) are produced from cinnamate via a series of hydroxylation, methylation, and dehydration reactions; these include p-coumaric, caffeic, ferulic, and sinapic acids and simple coumarins.

Most of the major classes of plant polyphenol are listed in **Table 2.1** according to the number of carbon atoms of the basic skeleton. The structure of natural polyphenols varies from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins (15). Phenolic compounds contain at least one phenol group.

Flavonoids usually have at least two rings while tannins are polymers of flavonoid units. Flavonoids are perhaps the most common in plant-based foods such as fruits, vegetables, nuts and cocoa. They can be subdivided into five classes: flavones, flavonones, isoflavones, flavonols (kaempferol, quercetin), and anthocyanins (19).

Flavonoids represent the most common and widely distributed group of plant phenolics and consist of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle. Their common structure is that of diphenylpropanes (C6-C3-C6) (15, 20-21). **Figure 2.1** shows the basic structure and the system used for the carbon numbering of the flavonoid nucleus. Structural variations within the rings subdivide the flavonoids into several families: flavonols, flavones, flavanols, isoflavones, anthocyanidins and others. These flavonoids often occur as glycosides, rendering the molecule more water-soluble and less reactive toward free radicals. The flavonoid variants are all related by a common biosynthetic pathway, incorporating precursors from both the shikimate and the acetate-malonate pathways shown in **Figure 2.2.** Chalcone synthase (CHS) is the first step in the branch of the pathway that produces the flavonoids including isoflavones, flavones, flavonols and anthocyanins (22).

Anthocyanins are the largest group of flavonoids. They are water soluble, glycosylated and/or acylated flavonoid derivatives that are the source of most red, pink, purple, and blue colors in plant parts. The nonglycosylated form or aglycone is called anthocyanidin. There are 6 commonly occurring anthocyanidins in higher plants: pelargonidin, cyanidin, peonidin, delphinidin, malvidin and petunidin (**Figure 2.3**) (23). Among them, cyanidin is most abundant and malvidin is least (24). Sugars are present most commonly at the C-3 position, second at C-5 position, and very rarely at C-7

position. Sugars provide additional sites for modification as they may be acylated with acids such as *p*-coumaric, caffeic, ferulic, sinapic, acetic, malonic or *p*-hydroxybenzoic acid. Because of the diversity of glycosylation and acylation, there are at least 240 naturally occurring anthocyanins (23).

Stability of anthocyanin is important for bioactive functions and color for food products. This is dependent on many factors, including structure, pH, temperature, light intensity and quality, presence of co-pigments, metallic ions, enzymes, oxygen, ascorbic acid, sugars and their degradation products (25-27). The pH is one of the key factors affecting stability of anthocyanin structure. In acidic media four anthocyanins structures exist in equilibrium: flavilium cation, quinoidal base, carbinol pseudobase and chalcone. The relative amount of these structures at equilibrium varies with pH and anthocyanin structure, the red flavylium cation and the colorless carbinol base (25). The flavylium cation has a positive charge associated with it, while the carbinol base is a hydrated form of the anthocyanin (**Figure 2.4)** (28). Anthocyanins are most stable and highly colored at low pH values but as the pH is increased they gradually lose the color. At pH 4 to 5, the anthocyanins are almost colorless. This color loss is reversible, and the red hue will return upon acidification (29-31). In aqueous media, anthocyanins appear as flavylium cation (red) at acidic pH 0.0-2.0, and as a colourless pseudobase with a small amount of colourless or slightly yellow chalcone structures between pH values 2–6 (30). Thus pH is one of the key factors affecting anthocyanin structure. This behaviour of anthocyanins limits its applications as colorant in food industries. Heat and light treatment also degrades anthocyanins and increase the polymerization of monomeric anthocyanins (25).

Tannins are polymerized flavonoids, with ability to precipitate proteins at neutral pH. There are two major classes of tannins: condensed and hydrolyzable. The hydrolyzable tannins are esters of gallic or ellagic acid and the condensed tannins (proanthocyanidins) are polymers of flavonoids. Hydrolyzable tannins are generally considered antinutritional (32).

Phenolic compounds in blueberries

Blueberry fruit ranks high in antioxidant activity among fresh fruits (33). This is highly correlated with polyphenol content (34). Phenolic acids generally reported in different cultivars of blueberry are gallic acid, p-hydroxybenzoic acid, caffeic acid, p-coumaric acid, ferulic acid, and ellagic acid (35). Major flavonoids are catechin, myricetin (36) kaempferol, and quercetin (37). **Figure 2.5** shows the structures of phenolic compounds present in blueberries. Major anthocyanidins in blueberries are delphinidin, cyanidin, petunidin, peonidin, and malvidin (38). Malvidin and delphinidin are major anthocyanidins present in blueberries (37, 39). There are various factors that affect the antioxidant activity which includes maturity at harvest, season at maturity, genetic differences, preharvest environmental conditions, and processing (40). Late harvest of tifblue and brightwell were reported to possess high antioxidant activity than early harvest (36). Storage at 20 ºC increased anthocyanin content by 20% in *V. corymbosum L* (41).

Absorption and metabolism of phenolic compounds

Many phenolic aglycons are hydrophobic and can passively diffuse through biological membrane (42). Sugar attachment increases the water solubility and limits the passive diffusion (42). Most flavonoids are present in the diet as β-glycosides except

catechin. The first stage of metabolism is deglycosylation. The β-glycosidase present in human small intestine and liver can hydrolyze various phenolic glycosides (43). Once absorbed, phenolics may be metabolized by phase-I enzymes responsible for hydroxylation and demethylation, and phase-II enzymes responsible for the removal of xenobiotics by conjugation of phenolics with glucuronide or sulphate. Conjugation is a common detoxification reaction which increases solubility and molecular mass (44).

Phenolics glycosides that are not absorbed in small intestine pass into the colon. Microorganisms present in colon hydrolyze phenolic sugar glycosidic or ester linkages by the action of β-glycosidase or esterases (45-46). The metabolic pathway of quercetin is summarized in **Figure 2.6** (42).

Stability of phenolic compounds during processing of blueberries

Polyphenolic compounds including anthocyanins are not completely stable (47). After harvest these compounds undergo change on processing and storage (48-49), which may alter their biological activity. Anthocyanins and polyphenolics are readily oxidized because of their antioxidant properties and thus prone to degradation. Primary steps of processing (thawing, crushing, depectinization, and pressing) results in considerable losses of anthocyanins (50). Native enzyme, polyphenol oxidase (PPO) present in blueberry is responsible for oxidation of polyphenolics to quinones, which produce brown pigments (50-52), thus affecting the color of the extract. Heating was shown to inhibit PPO activity (51). The significant deterioration of phenolic compounds in highbush blueberries when converted to juice has already been discussed in several reports (53-54). Oxygen, pH, and various storage conditions are shown to have marked effects on anthocyanins stability (52).

Blueberries and cancer

Lowbush blueberry fruit extract was found active against the initiation and promotion stages of carcinogenesis (55). Highbush blueberry extract reduced cancer proliferation in cervical and breast cancel cell lines (56). The physiological and pharmacological functions of blueberry originate from their antioxidant properties. The antioxidant properties are related to the structures of phenolic compounds (19). Anthocyanins extracted from bilberry such as delphinidin glycosides and cyanidin glycosides induce apoptosis in HL-60, HCT-116 cells (57). Anthocyanins protect against DNA damage by oxidative agents (58). Flavonoids have been classified as "blocking agents" because they are believed to act before the mutagenic step of carcinogenesis, preventing the initiation of cancer. Other dietary compounds, such as retinoid, indoles, and carotenoids, are referred to as "suppressing agents," which act after the mutation occurs to prevent further progression of cancer (59-60).

Carcinogenesis has three stages: intiation, the first stage, which begins when a reactive intermediate alters the genetic make-up of the cell and produces a mutation by modifying oncogenes, tumor suppressor genes, and DNA-repair genes. In the absence of successful repair, the mutated cell enters the stage of promotion, which is characterized by rapid proliferation to convert the initiated cells into a population of cancer cells. This stage is reversible by the same mechanisms as the initiation stage: cell repair or death.

Carcinogenesis initiation is a complicated process, which includes altered genetic changes, damage to DNA, activation of intracellular signaling agents, abnormal cell growth by evading apoptosis and sustained angiogenesis (61). One of the main characteristics of cancer cells is uncontrolled cell proliferation. There are several different

mechanisms to inhibit the cell growth. Activator protein-1(AP-1) is a transcriptional factor that promotes carcinogenesis (62-63). Delphinidin, cyanidin, and petunidin are reported to inhibit TPA-induced AP-1 transcriptional activity and cell transformation in JB6 cells (64). Kamei et al. (65) reported blocking of S, G2, and M phases of the cell cycle in the cells treated with anthocyanins.

Recent studies have suggested that the transcription factor AP-1 plays an important role in promoting carcinogenesis (62-63). AP-1 is a dimeric protein typically composed of the products of the jun and fos oncogene families (66). AP-1 dimers bind to the promoter regions on DNA that contain 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE) to activate the transcription of genes involved in cell proliferation (63), transformation (66-67), and apoptosis (68). A variety of stimuli, such as phorbol esters (66-67, 69), UV radiation (70), growth factors (71) and oxidative agents (72), can stimulate AP-1 activity by activating mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) or stress-activated protein kinase (SAPK) and p38 kinase. Increased AP-1 activity has been shown to be involved in the tumor promotion and progression of various types of cancers, such as skin (70-71), lung (72) and breast cancer (73). In vivo mouse data also demonstrate that AP-1 activity is required for tumor promotion (74-75). Anthocyanidins with ortho-dihydroxyphenyl structure on the B-ring of aglycon suppressed TPA-induced cell transformation and AP-1 transactivation. The potent anthocyanidins, having an ortho-dihydroxy phenyl structure on B-ring of aglycon may block TPA-induced ERK and JNK signaling cascades leading to activation of AP-1 (76).

Oxidative reactions produce large amounts of reactive oxygen radicals, peroxy

and other free radicals. In normal physiological conditions antioxidant defense system composed of enzymatic (superoxide dismutase, catalase, glutathione peroxidase and peroxyredoxins) and low molecular-mass scavengers (such as glutathione) (77), in body keeps the balance between oxidation and oxidation scavenging. When an imbalance between free radical generation and body defense mechanisms occurs, oxidative damage will spread over all the cell targets (DNA, lipids, and proteins). Free radicals are constantly produced in the body firstly by environmental agents such as the ultraviolet (UV) component of sunlight, ionizing radiation, products of normal cellular metabolism, secondly by products of normal cellular metabolism that include reactive oxygen species (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation (78). The oxidative damage is widely accepted as one of the major causes of carcinogenesis (79).

The radical-scavenging activity of flavonoids depends on the molecular structure and the substitution pattern of hydroxyl groups, *i.e.*, on the availability of phenolic hydrogens and on the possibility of stabilization of the resulting phenoxyl radicals *via* hydrogen bonding or by expanded electron delocalization (80-81). Structural requirement considered essential for the effective scavenging activity are: a) the presence of a 3',4'-dihydroxy, *i.e.*, a *o*-dihydroxy group (catechol structure) in the B ring, possessing electron donating properties and being a radical target, b) 3-OH moiety of the C ring (82), c) C2-C3 double bond conjugated with a 4-keto group, which is responsible for electron delocalization from the B ring, and d) presence of both 3-OH and 5-OH groups in combination with a 4-carbonyl function and C2-C3 double bond (80-87). **Figure 2.7** summarizes the structural criteria that modulate the free radical scavenging activity of

flavonoids (88-89).

Several investigators have reported that flavonoids including anthocyanins can protect the DNA damage via protecting DNA from oxidation (90-91). Delphinidin and cyanidin are shown to inhibit the protein tyrosine kinase (PTK) activity of the epidermal growth-factor receptor (EGFR) (92).

Apoptosis

Apoptosis is one of the major mechanisms of cancer suppression (92-93). Apoptosis or programmed cell death is a highly regulated process of selective cell deletion for anticancer drug-induced cell death (94). It is characterized by distinct morphological changes such as membrane blebbing, nuclear condensation and disorganization, and DNA fragmentation (95). These cells are easily recognized by macrophages before cell lysis, and then can be removed without inducing inflammation (96-97). Therefore apoptosis inducing agents are expected to be ideal anticancer drugs.

Cytochrome C is an electron transporting protein that resides within the intermembrane space of the mitochondria, where it plays a critical role in the process of oxidative phosphorylation and production of cellular ATP. Following exposure to apoptotic stimuli, the mitochondrial membrane potential is changed. Then, cytochrome C is rapidly released from the mitochondria into the cytosol (98) and activates caspase 9 (99). Caspase 9 can initiate the caspase cascade involving the downstream executioner caspases, such as caspase 3, 6 and 7 (100). Upon activation caspase 3, cleaves poly (ADP-ribose) polymerase (PARP) and D4-GDI proteins and generates apoptotic fragments in coordination with DNA fragmentation (101). Induction of apoptosis in tumor cells has been shown to be the most common anti-cancer mechanism conjoint by

many cancer therapies, and thus finding potential therapeutic anti-tumor drugs with potent and selective apoptotic effects would be valuable (102).

Delphinidin, cyanidin, and petunidin induced apoptosis of HL-60 cells detected by morphological changes and by DNA fragmentation, whereas pelargonidin, peonidin, and malvidin showed no induction of apoptosis (103). The anthocyanidin glycosides (anthocyanins) extracted from bilberry such as delphinidin glycosides and cyanidin glycosides also induced apoptosis in HL-60 cells (57).

Structure-activity studies indicated that the potency of apoptosis induction of anthocyanidins is associated with the number of hydroxyl groups at the B-ring, and the ortho-dihydroxyphenyl structure at the B-ring appears essential for apoptosis actions (103). It is noteworthy that anthocyanidins increased the levels of hydrogen peroxide in HL-60 cells with a structure-activity relationship that depends on the number of hydroxyl groups at the B-ring (103) and appears in the order of delphinidin > cyanidin, petunidin > pelargonidin, peonidin, and malvidin.

The mechanistic analysis indicates that the apoptosis induction by delphinidin may involve an oxidation/JNKmediated caspase pathway. Delphinidin treatment increased the levels of intracellular reactive oxygen species (ROS), which may be a sensor to activate JNK. Concomitant with the apoptosis, JNK pathway activation such as JNK phosphorylation, *c-jun* gene expression, and caspase-3 activation was observed in delphinidin-treated cells (103). Thus, delphinidin may trigger an apoptotic death program in HL-60 cells through an oxidative stress mediated JNK signaling cascades **(Figure 2.8).**

Accumulated results on structure-activity studies have shown that the biological activities of anthocyanins appear to increase with a decreasing number of sugar units

and/or with an increasing number of hydroxyl groups at their aglycons (104). The ortho dihydroxyphenyl structure on the B ring appears to be essential for anticarcinogenesis, anti-inflammation, and apoptosis induction and the activities of aglycons such as delphinidin and cyanidin are stronger than that of their glycosides (78, 103).

Detoxification enzymes

Xenobiotics are molecules which are introduced into the body from the environment and subsequently metabolized by the body. These can be man-made (drugs, industrial chemicals) or natural (alkaloids, toxins from plants and animals). Continuous exposure to these cytotoxic chemicals may lead to many diseases including cancer (105). Since most xenobiotics occur in a hydrophobic form in nature, they have to transform into hydrophilic compounds which are readily excreted via bile and urine (106). This process is called ***biotransformation*** and is catalyzed by enzymes mainly in the liver of higher organisms but a number of other organs have ability to process xenobiotics such as kidneys, gut and lungs.

Biotransformation reactions are usually classified as Phase I and Phase II reactions, and enzymes involved in the process are called phase-I and phase-II enzymes. Phase I reactions modify the chemical by adding a functional group thereby forming electrophilic intermediates (107-109). A xenobiotic that has undergone a Phase I reaction is now a new intermediate metabolite that contains a reactive chemical group, e.g., hydroxyl (-OH), amino (-NH2), and carboxyl (-COOH). These reactive molecules are more toxic than parent molecule. If they are not further metabolized by Phase II conjugation, they may cause damage to proteins, RNA, and DNA within the cell (110). Cytochrome P450 superfamily enzymes are members of Phase-I enzymes.

Phase-II detoxification enzymes are responsible for metabolizing the products of Phase-I metabolic reactions, degrade these reactive intermediates by conjugations or reductions reactions and thus preventing oxidative DNA damage. The conjugated products are larger molecules than the substrate and generally polar in nature (water-soluble). Thus, they can be readily excreted from the body. Conjugated compounds also have poor ability to cross cell membranes. The most common conjugation reactions are catalyzed by glutathione-S-transferase (GST) (111), uridine 5'diphosphoglucuronosyl transferases (112) and reduction reactions catalyzed by epoxide hydrolase (113) and quinone reductase (QR) (114). Induction of the detoxification enzymes QR and GST is a well-characterized defense mechanism against carcinogens (115-116).

Glutathione S-transferases (EC 2.5.1.18)

GST plays a physiological role in initiating the detoxification of potential alkylating agents (117-118) including pharmacologically active compounds. These enzymes catalyze the reaction of such compounds with the -SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble. Glutathione (GSH), a tripeptide thiol of glutamate, cysteine, and glycine (GluCysGly), is a strong antioxidant found in almost all cells (120). GSH contains a thiol group, which is strongly nucleophilic and forms a stable covalent compound with electrophilic compounds. Glutathione conjugates are thought to be metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, to produce the final product, a mercapturic acid (118-119). The mercapturic acids, i.e. S-alkylated derivatives of N-acetylcysteine, are then excreted. The GST also functions as antioxidants, which is another possible

mechanism for their chemopreventive properties (121).

Research has shown that polyphenols found in red wine and black tea decrease GST expression in colon tumors; this suggests a possible role for polyphenols in preventing drug resistance in these tissues. Therefore, dietary components may have both chemopreventive and chemotherapeutic roles in the development of colon cancer through the regulation of GST expression and activity (122). Moskaug et al. (123) showed that flavonoid increase γ-glutamylcysteine synthetase, which is a rate limiting enzyme in the synthesis of glutathione. The three flavonoids induced a concentration-dependent decrease of both the nuclear GSH content and GST activity. Myricetin, which has the maximum number of hydroxyl groups, was the most active (124). The glycosides rutin and quercetin gave dose-dependent increases in GST activity using HepG2 cells, whereas the fraction containing caffeic acid derivatives were inhibitory (125). Flavonoid administration in vivo, however, induced this activity (126). Rat liver GST was effectively inhibited in vitro by several other flavonoids. This activity was again closely related to the pattern of hydroxylation and presence of a C2-C3 double bond (127).

Quinone reductase enzyme

Quinone reductase (QR) is one of the several enzymes that inactivate electrophilic forms of carcinogens thus providing mechanism of preventing carcinogenesis (128). NAD(P)H:quinone oxidoreductase, also known as DT-diaphorase, is a phase II enzyme. The QR is a single, multi-functional enzyme that exerts its effects in different body tissues, including the liver, lung, colon, and breast (129). It catalyzes two-electron reductions on free radicals and toxic oxygen metabolites, which deactivates them and protects the surrounding tissues from mutagenesis and carcinogenesis. It has been

reported that ethyl acetate extract of anthocyanin and proanthocyanidin fractions were active QR inducers (55). Crude extract of anthocyanins were not highly active in QR induction (55).

Human colon carcinoma cell lines

Human colon carcinoma cell lines are an appropriate experimental model for preliminary research studies, such as the effect of anthocyanins on phase I and phase II enzymes. Cell lines are simpler than a complete organism. Isolating the effects of specific chemicals or compounds on certain tissues can be accomplished easily in cell models, as opposed to complete animal or human systems that have too many variables to control. The disadvantage of cell models is that compounds may not behave the same as they would in a complete living system, in the absence of hormones, buffers, and other regulating factors, so results from cell studies cannot be applied directly to humans.

Several human colon carcinoma cell lines exist, including SW480, HT-29, and Caco-2 cells. Among the different cell lines that exist, SW480 cells are the least differentiated and HT-29 cells used in the present study represent advanced stage of tumor development (130).

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Table 2.1 Most of the major classes of plant polyphenol (15)

The most important class of phenolic compounds in plants		
Number of carbon atom	**Basic skeleton**	**Class**
6	C_6	simple phenols, benzoquinones
7	$C_6 - C_1$	phenolic acids
8	$C_6 - C_2$	acetophenone, phenylacetic acid hydroxycinnamic acid,
9	$C_6 - C_3$	polypropene, coumarin, isocoumarin
10	$C_6 - C_4$	naphtoquinone
13	$C_6 - C_1 - C_6$	xanthone
14	$C_6 - C_2 - C_6$	stilbene, anthrachinone
15	$C_6 - C_3 - C_6$	flavonoids, isoflavonoids
18	$(C_6 - C_3)_2$	lignans, neolignans
30	$(C_6 - C_3 - C_6)_2$	biflavonoids
n	$(C_6 - C_3)_n$	lignins
n	$(C_6)_n$	catecholmelanine
n	$(C_6 - C_3 - C_6)_n$	(condensed tannins)

Figure 2.1 Basic structure and system used for the carbon numbering of the flavonoid nucleus (15)

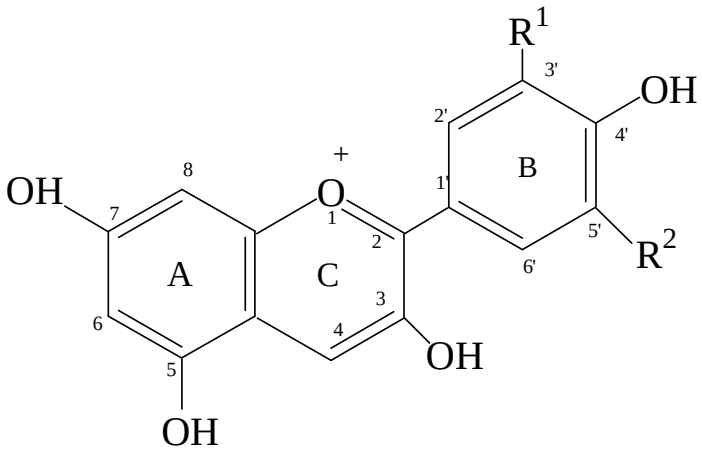
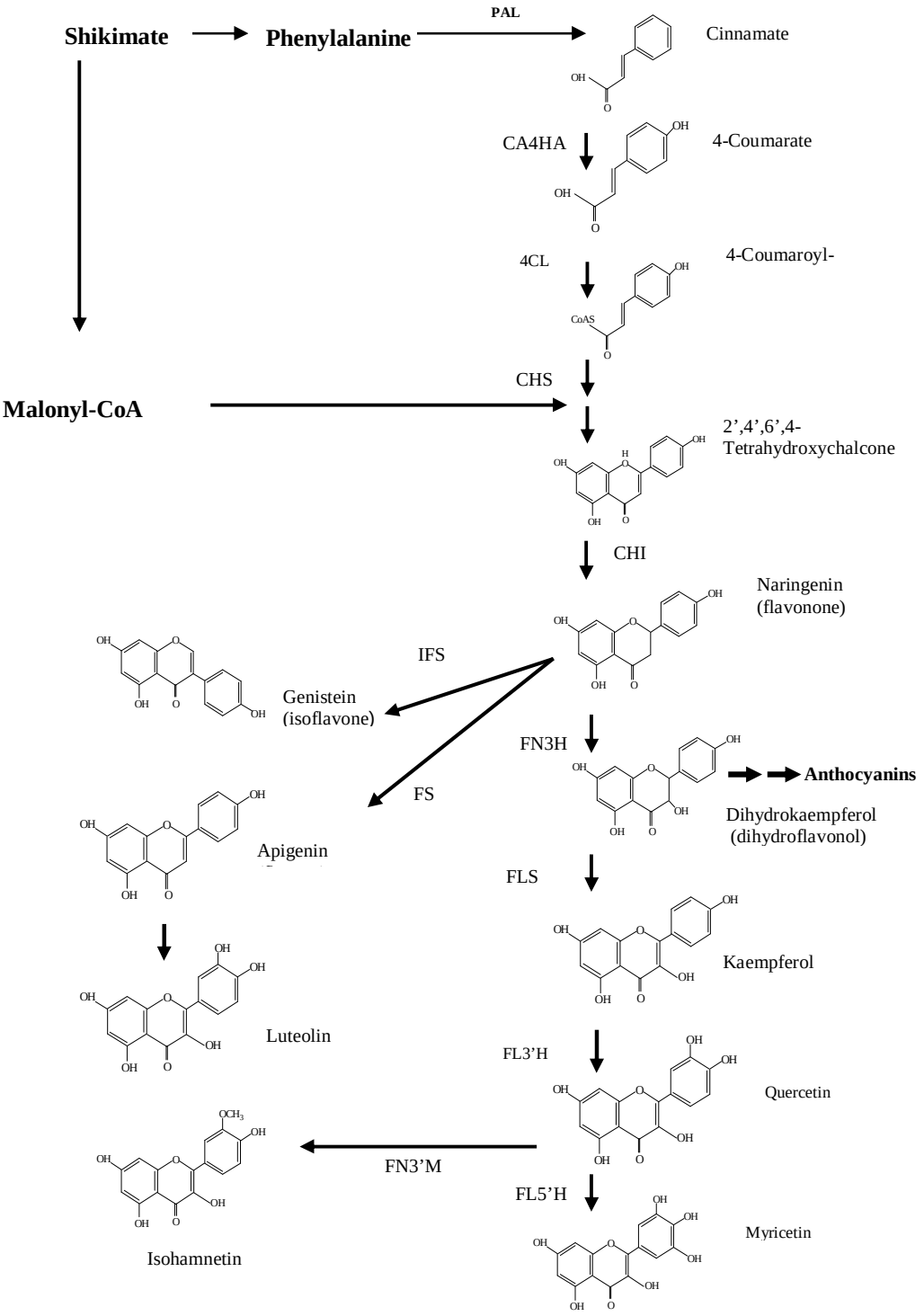
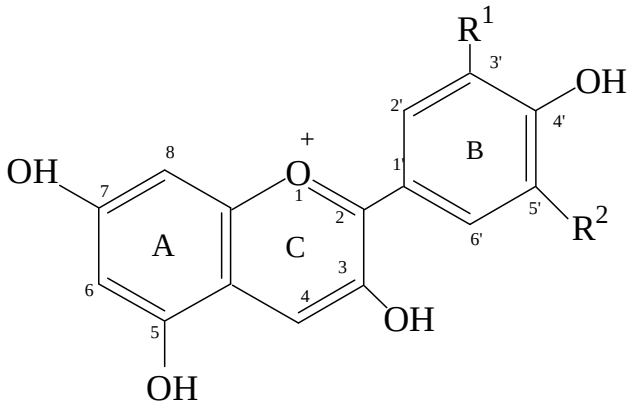


Figure 2.2 The phenylropanoid pathway (Shikimate and acetate pathway) for biosynthesis of flavonoid variants (22)



Abbreviations: PAL, phenylalanine amminia lyase; CA4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; FN3H, flavanone 3-hydroxylase; FS, flavone synthase; F3'H, flavone 3'-hydroxylase; FLS, flavonol synthase; FL3'H, flavonol 3'-hydroxylase; FL3'M, flavonol 3'-methylase; FL5'H, flavonol 5'-hydroxylase.

Figure 2.3 Commonly occurring anthocyanidins and their substitution pattern (23)



Anthocyanidin	**R_1 group**	**R_2 group**
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OMe	H
Petunidin	OMe	OH
Malvidin	OMe	OMe

Figure 2.4 Effect of pH on malvidin-3-glucoside (28)

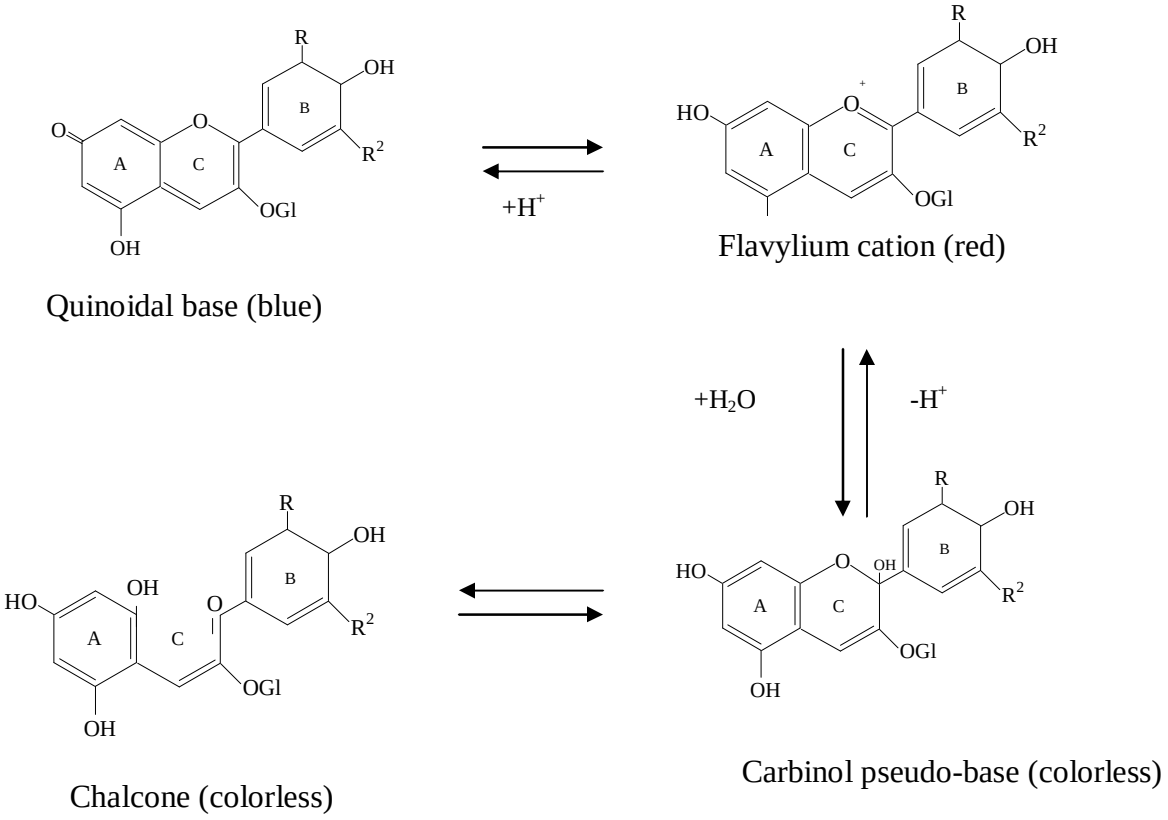
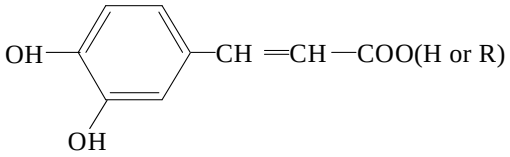
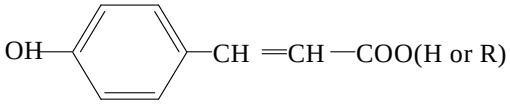


Figure 2.5 Structures of phenolic compounds commonly present in blueberries (21, 23)

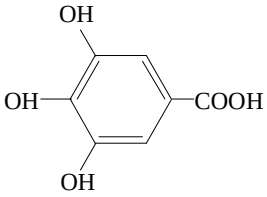
(A) Phenolic acids

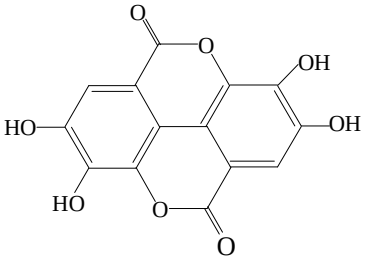




a) Caffeic acid (number of OH-2)

b) *p*-Coumaric acid (number of OH-1)

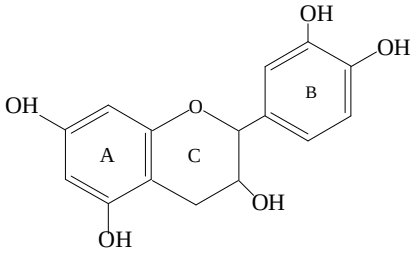


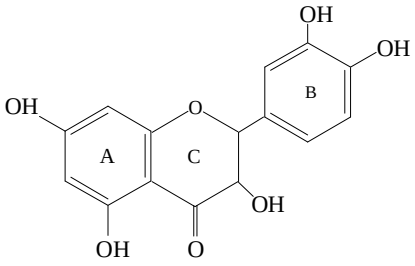


c) Gallic acid (number of OH=3)

d) Ellagic acid (number of OH=4).

(B) Flavonols



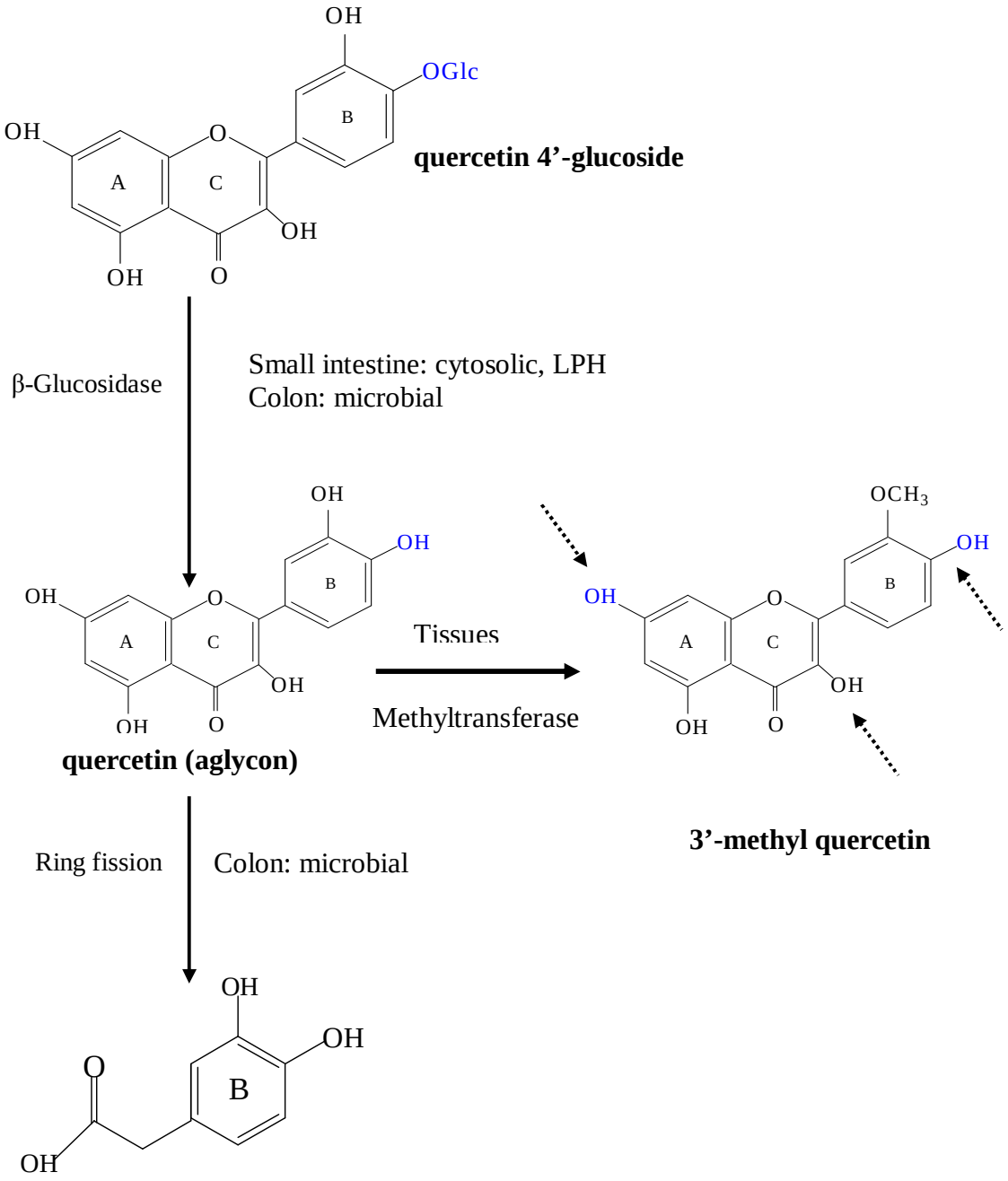


a) (+)-Catechin (number of OH=5)

b) Quercetin (number of OH=5)

(C) Anthocyanins see figure 3.

Figure 2.6 Pathway of quercetin metabolism (42).
Dotted arrows indicate position of sulphate and/or glucuronide conjugation.



Abbreviation: LPH lactase phlorizin hydrolase

Figure 2.7 Structural criteria that modulates the free radical scavenging activity of flavonoids (88-89)

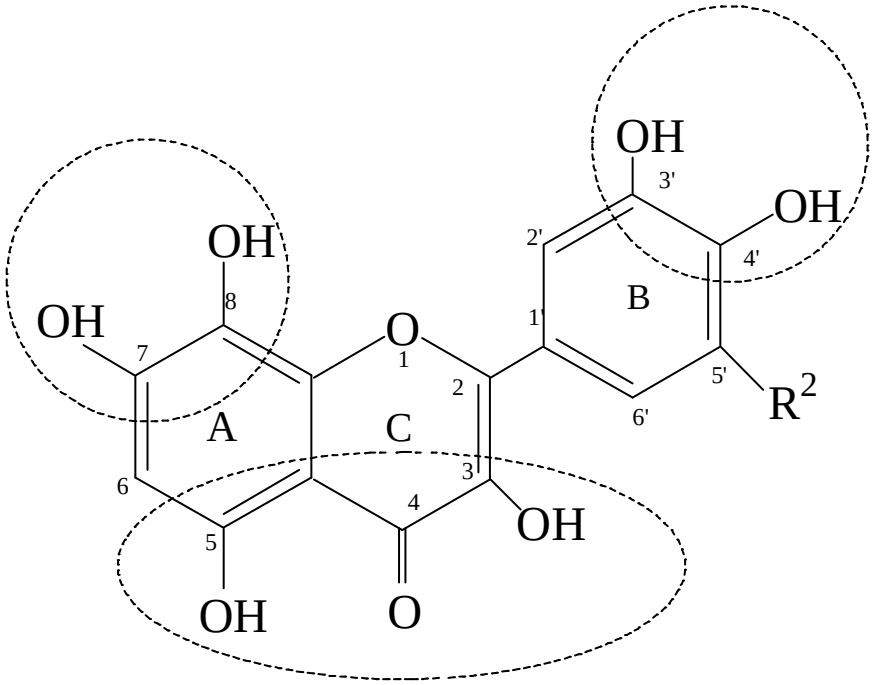
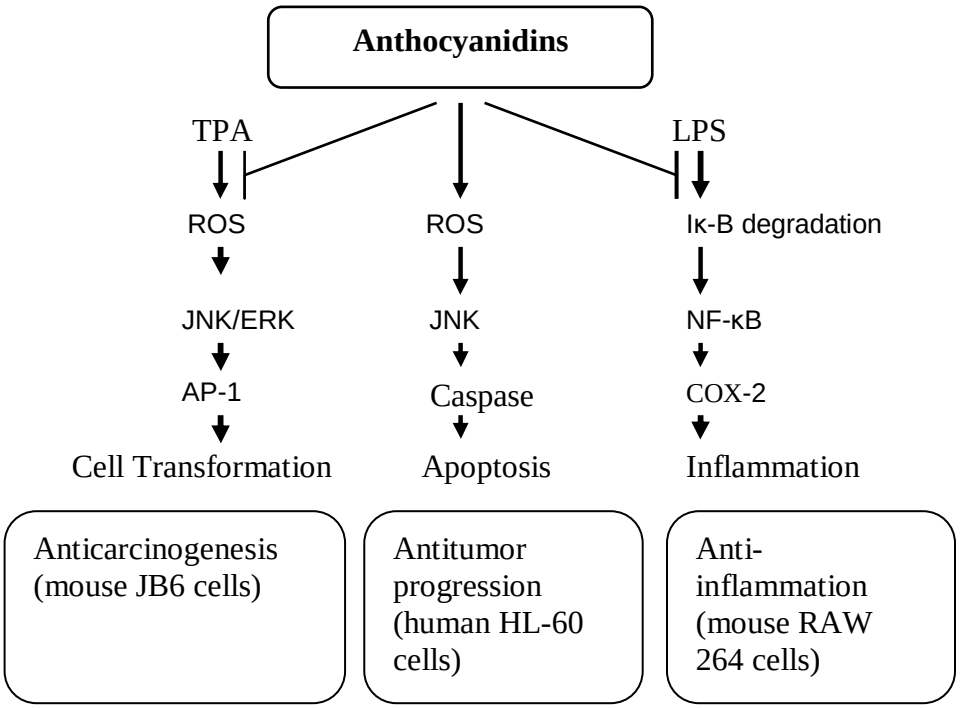


Figure 2.8 A schematic molecular view of cancer chemoprevention by anthocyanidins



Abbreviations: AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; COX-2, cyclooxygenase 2; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B ; ROS, reactive oxygen species; TPA, 12-*O*-tetradecanoylphorbol-13-acetate (103).

CHAPTER 3

EFFECT OF STORAGE CONDITIONS ON BIOLOGICAL ACTIVITY OF PHENOLIC COMPOUNDS OF BLUEBERRY EXTRACT PACKED IN GLASS BOTTLES

Anita Srivastava, Casimir C. Akoh, Weiguang Yi, Joan Fischer, and Gerard Krewer
To be submitted to the Journal of Agricultural and Food Chemistry.

ABSTRACT

Recent research suggests that blueberries are rich in total polyphenols and total anthocyanins. Phenolic compounds are highly unstable and may be lost during processing, particularly when heat treatment is involved. There is no systematic study available providing information on the fate of phenolic compounds during storage and how that affects their biological activity. We provide a systematic evaluation of the changes observed in total polyphenols (TPP), total anthocyanin (TACY), Trolox-equivalent antioxidant activity (TEAC), phenolic acids, individual anthocyanins and cell proliferation from blueberry extract stored in glass bottles. Extract was stored at different temperatures (-20±1, 6±1, 23±1, and 35±1 ^{o}C). Two cultivars, Tifblue and Powderblue were chosen for the study. Recovery of TPP, TACY and TEAC in blueberry extract after pressing and heating were ~25, ~29, and ~69%, respectively for both cultivars. Recovery of gallic acid, catechin and quercetin was ~25% in final extract. Ferulic acid was not detected in the final extract in both Tifblue and Powderblue cultivars. Recovery of peonidin, malvidin and cyanidin was ~20 % in final extract in both cultivars. Losses due to processing were less when compared with initial loss due to processing. At -20 ºC, no statistically significant loss of TPP, TACY and TEAC was observed up to 30 days (P < 0.05). At 6 ºC storage, there was a significant loss observed from 15 to 30 days. Similar results were obtained at 23 ºC and 35 ºC (P < 0.05). There was retention of more than 40% of ellagic and quercetin after 60 days at 35±1 ^{o}C. Anthocyanins were not detected after 60 days of storage at 35±1 ^{o}C. Significant retention (P < 0.05) was obtained for

malvidin (42.8 and 25.8%) and peonidin (74.0 and 79.5%) after 60 days storage at 23±1^{o}C in glass bottles for Tifblue and Powderblue, respectively , when compared with other individual anthocyanins. A linear relationship was observed between TEAC values and total polyphenols and total anthocyanins. Cell viability assay was performed using HT-29 cancer cell lines and anthocyanins extracted from 30, 60, and 90 days stored extract at 6±1 and 23±1 ^{o}C. Significant cell proliferation inhibition percentage was observed in 30 days, although this was reduced significantly after 30-90 days. These results suggest that heating and storage conditions were significantly affecting the phenolic compounds and their biological activity. Frozen and low temperature storage is suggested for blueberry extract in order to retain the bioactive components.

KEYWORDS: anthocyanins; blueberries; cell proliferation; flavonoids; PET; phenolic compounds; storage; TEAC.

INTRODUCTION

Epidemiological evidence suggests that diets rich in fruits and vegetables are associated with a reduced risk of cancer (1), coronary heart disease (2, 3), and stroke (4). Fruits and vegetables are excellent sources of phenolic compounds, including phenolic acids and flavonoids. Recently, much evidence has been accumulated to show that these compounds have inhibitory effects on mutagenesis and carcinogenesis (5). Plants produce phytochemicals for protection from herbivores, parasites and oxidative stress. More than 4000 of these compounds generally referred as flavonoids have been identified in both higher and lower plants. They can be subdivided into five classes: flavones, flavonones, isoflavones, flavonols (kaempferol, quercetin) and anthocyanins (6). Most of the phenolic compounds in plants occur as glycosides or as esters. The basic structure (7-8) for some of these flavonoids is given in **Figure 3.1**. Blueberry (*Vaccinium corymbosum*) of the family Ericuceae is reported to have high antioxidant activity compared to other fruits and vegetables (9-10). This is highly correlated with the anthocyanins and total polyphenolic content (11). Blueberries water extracts are similar to blueberry juice often converted into juice or concentrate for subsequent use in beverages, syrups and other food products.

Polyphenolic compounds including anthocyanins are not completely stable (12). After harvest these compounds undergo change on processing and storage (13-14), which may alter their biological activity. Anthocyanins and polyphenolics are readily oxidized because of their antioxidant properties, and are thus prone to degradation. The native

enzyme polyphenol oxidase (PPO), which is present in blueberry, is responsible for oxidation of polyphenolics to quinones, which produce brown pigments (15-17), and affect the color of the extract/juice/concentrate. Heating was shown to inhibit PPO activity (15). The significant deterioration of phenolic compounds in highbush blueberries when converted to juice has already been discussed in several reports (18-19). Oxygen, pH, and various storage conditions are shown to have marked effects on anthocyanin stability (20). Previous reports are available on pomegranate juice color and bioactive compounds during storage (21). However reports describing changes on antioxidant capacity or antiproliferation activity due to storage are rarely found.

The objectives of the present work were: (1) to study the effect of storage in glass bottles on phenolic compounds and their antioxidant capacity in blueberry extract and (2) to extract anthocyanin fractions from blueberry extract stored in glass bottles under different temperature conditions and evaluate their effect on cell proliferation activity using the HT-29 colon cancer cell line. Tifblue and Powderblue were the cultivars used. These are the most common berries used by food industries in prepration of different food products and Tifblue (Rabbiteye) is gaining attention due to its high anthocyanin content.

MATERIALS AND METHODS

Chemicals. Pure standards of gallic acid, *p*-hydroxybenzoic acid, (+)-catechin, caffeic acid, (−)-epicatechin, *p*-coumaric acid, ferulic acid, ellagic acid, quercetin, and kaempferol, were purchased from Sigma (St. Louis, MO). Anthocyanin standards were purchased from Polyphenols Laboratories (AS) (Sandnes, Norway). These standards

were: Dp-Glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-Gal (Cyanidin 3-*O*-β-galactopyranoside), Cy-Glc (Cyanidin 3-*O*-β-glucopyranoside), Pt-Glc (Petunidin 3-*O*-β-glucopyranoside), Pn-Gal (Peonidin 3-*O*-β-galactopyranoside), Pn-Glc (Peonidin 3-*O*-β-glucopyranoside), Mv-Glc (Malvidin 3-*O*-β-glucopyranoside), and Pn-Ara (Peonidin 3-*O*-α-arabinopyranoside). Folin-Ciocalteu reagent, dimethylsulfoxide (DMSO), and pectinase enzyme (Pectinex® ultra SP-L, ≥26,000 units/mL) were purchased from Sigma (St. Louis, MO). Acetone, acetonitrile, methanol, *O*-phosphoric acid (85% purity, HPLC grade), hydrochloric acid (analytical grade), sulfuric acid, formic acid, and water (HPLC grade) were purchased from Fisher Scientific (Norcross, GA). Glass bottles were purchased from Speciality Bottle Supply (Seattle, WA). MTT Cell Proliferation Assay kits were purchased from ATCC (Manassas, VA). The human hepatocellular carcinoma HT-29 cell line was purchased from ATCC (Manassas, VA).

Sample Collection. Mature blueberries were harvested from the farms in Tifton, Experiment Station, University of Georgia, GA in 2005. The Blueberry cultivars collected were Tifblue and Powderblue. Samples were frozen, and stored at -40°C until use.

Extract Preparation. Blueberries extract was prepared using using a modified method reported by earlier workers for juice preparation (14, 22). Frozen berries were thawed at 5 °C for 12 h. Berries were blanched using boiling water for 3 min. Blanched berries were milled in household blender in three lots of 650 g each. Pectinase enzyme (2.2 mL) was added to 2100g of crushed berries. The mix was stirred well manually and kept at room temperature for 1h. Crushed berries were centrifuged at 9740×g for 20 min

at 10 °C. The extract (supernatant) was collected and the residue was discarded. The extract (1.2 L) was gradually heated to 85 °C and was held at 85 °C for 2 min. Temperature was brought down to 75 °C using cold water (15 °C). Extract was filled in glass bottles, capped, and cooled to 30 °C using chilled water.

Storage Conditions. Extract was packed in glass bottles (30 mL) and kept at -20±1, 5±1, 23±1 and 35±1 °C and withdrawal time was set for 15, 30, 45 and 60 days. For cell proliferation assay, extract samples were withdrawn after 30, 60, and 90 days storage. All samples were analyzed in triplicate, and average results were reported.

Anthocyanin Extraction. The anthocyanin fraction from extract was obtained using a method reported by Yi et al. (23) after incubation, extract samples were applied to an activated Oasis HLB cartridge (Waters Corporation, Milford, MA). The 15% methanol fraction contained the phenolic acids, and the acidified methanol (5% formic acid in methanol) eluted the anthocyanins. The fraction containing the anthocyanins was freeze-dried using a UNITOP 600L freeze dryer (Virtis, Gardiner, NewYork). Extraction and hydrolysis for total polyphenols and total anthocyanins of blueberry fruit was done using the method reported by Sellappan et al. (24).

Total Soluble Solids (TSS) and pH. TSS and pH were measured using an Atago Abbey hand refractometer (0-32 ºBrix) and a pH meter-340, respectively.

Estimation of Total Anthocyanins. Total anthocyanin (TACY) content of the juice was estimated on a UV-visible spectrophotometer (Shimadzu UV-1601, Norcross, GA) by the pH-differential method using two buffer systems - potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). Samples were diluted in pH

1.0 and pH 4.5 buffers, and then measured at 520 and 700 nm. Absorbance was calculated as $A= (A_{510nm} - A_{700nm})_{pH1.0} - (A_{510nm} - A_{700nm})_{pH4.5}$

Monomeric anthocyanin pigment concentration in the extract was calculated as cyanidin-3-glucoside (25). Monomeric anthocyanin pigment (mg/L) =A ×MW × DF × 1000/ (€ ×1) where *A* = absorbance, MW= molecular weight (449.2); DF =dilution factor, € = molar absorptivity (26,900). The final concentration of anthocyanins (mg/100 g fruit) was calculated based on total volume of extract and weight of sample.

Estimation of Total Polyphenols. Total polyphenols (TPP) were estimated colorimetrically using the Folin-Ciocalteu method (26). Extract samples were filtered through a 0.2-μm nylon syringe filter. A sample aliquot of 20 μL was added to 800 μL of water, 1 mL of 0.2 N Folin-Ciocalteu reagent and 0.8 mL of saturated sodium carbonate solution (7.5%) and mixed well. The absorbance was measured at 765 nm with a Shimadzu UV-Visible spectrophotometer after incubation for 30 min at room temperature. Quantification was based on the standard curve generated with 100, 200, 300, and 400 mg/L of gallic acid.

Assay of Antioxidant Capacity. Antioxidant capacity was performed on the Shimadzu UV-Visible spectrophotometer in a kinetic mode based on the method of Re et al. (27). Briefly, $ABTS^{\cdot+}$ radical cation was produced by reacting 7 mM of 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2.45 mM potassium persulfate after incubation at room temperature in the dark for 16 h. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.70 ±0.1 at 734 nm. The filtered sample was diluted with ethanol so as to give 20-80% inhibition of the blank absorbance with 20 μL of sample. A 980 μL aliquot of $ABTS^{\cdot+}$ solution (absorbance of

0.70 ± 0.1) was read at 734 nm for a minute; after exactly 1 min, 20 μL of the sample was added and mixed thoroughly. Absorbance was continuously taken at every 6 s up to 7 min. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman- 2-carboxylic acid, a vitamin E analogue) standards of final concentration 0-15 μM in ethanol were prepared and assayed under the same condition. The Trolox-equivalent antioxidant capacity (TEAC) of the sample was calculated based on the inhibition exerted by standard Trolox solution at 6 min.

Hydrolysis. Hydrolysis was done as reported by Yi et al. (23). For the phenolic acid and flavonol analysis, fractions were dissolved in methanol containing 1.2 N HCl (40 mL methanol + 10 mL 6 N HCl). The samples were then placed in a water-bath at 80 ^{o}C with continuous shaking at 200 rpm for 2 h, to hydrolyze phenolic glycosides to aglycones. Anthocyanin fractions were dissolved in 50% methanol solution containing 2 N HCl (50 mL methanol + 33 mL water + 17 mL 37% HCl). Samples were placed in a water-bath at 80 ^{o}C with shaking at 200 rpm for 2 h, to hydrolyze anthocyanins to anthocyanidins.

HPLC Analysis. HPLC was performed with a Hewlett–Packard (Avondale, PA), model 1100 liquid chromatograph with quaternary pumps and a diode array UV–visible detector. For the analysis of phenolic acids and flavonols in blueberries juice, procedures previously reported by our laboratory were used (24, 28). A Beckman ultrasphere C18 ODS 4.6 x 250 mm column was used with column temperature at 40 ^{o}C. The mobile phases were, solvent A, methanol/acetic acid/water (10:2:88, v/v/v); solvent B, acetonitrile; and solvent C, water. The gradients were: at 0 min, 100% solvent A; at 5 min, 90% solvent A and 10% solvent B; at 25 min, 30% solvent A and 70% solvent B;

and at 30 min, 30% solvent A and 70% solvent B, with 5 min post-run with 100% solvent C. The flow rate was 1 mL/min. Phenolic compounds were detected at wavelengths of 260, 313, and 360 nm. For the anthocyanin and anthocyanidin analysis, the mobile phase was: Solvent A, O-phosphoric acid/methanol/ water (5:10:85, v/v/v); Solvent B, acetonitrile. The flow rate was 0.5 mL/min. The gradient for the separation was a linear gradient of 100–50% solvent A and 0–50% solvent B over 25 min, followed by 5 min post-run with HPLC-grade water. Anthocyanidins were detected at 520 nm.

Cell Cultures. The human hepatocellular carcinoma HT-29 cancer cells were cultured in ATCC McCoy's medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate (90%), and 10% fetal bovine serum. Cells were incubated under 37 ºC with 5% CO_2. Medium was changed 2–3 times per week.

Cell Proliferation Assay. After digestion with trypsin-EDTA, uniform amounts (~2 $\times 10^4$) of HT-29 cells in growth media were inoculated into each well of a 96-well flat-bottom plate. After 24 h of incubation at 37 ºC in 5% CO_2, the growth medium was replaced with 100 µL of medium containing anthocyanin extract from juice stored under different storage conditions. Based on the results of earlier reports from our laboratory (23), concentrations of each extract were kept at 100 µg/mL. Control cultures received everything but the anthocyanin fractions and blank wells contained 100 µL of growth medium and extract without cells. Anthocyanin fractions (water soluble) were directly dissolved in culture medium, and DMSO was added initially to the extracts/fractions to help dissolve the sample. The final DMSO content in the highest concentration of fraction treatment was 0.25%. Therefore, the control for these treatments also contained

the same amount of DMSO. After 48 h of incubation, cell population growth was determined using the ATCC MTT Cell proliferation assay at 570–655 nm with a Bio-Rad Model 680 Microplate Reader (Hercules, CA). Briefly, a mitochondrial enzyme in living cells, succinate dehydrogenase, reduced the yellow tetrazolium salt (MTT) to insoluble purple formazan crystals. Therefore, the amount of formazan produced was proportional to the number of viable cells (29). To better explain the inhibitory results, the inhibition of cell population growth was calculated based on the following formula:

Inhibition percentage

$$= \frac{(\text{Cell \# in control} - \text{cell \# in treatment})}{(\text{Cell \# in control} - \text{original cell \# before the extract was added})} \times 100$$

Statistical Analysis. Statistical analysis was done with the SAS software package (30). One-way analysis of variance (ANOVA) was performed to determine the difference among stored samples. When F values for the ANOVA were significant, differences in means were determined using Duncan's multiple range tests as a procedure of mean separation ($P < 0.05$).

RESULTS AND DISCUSSION

The contents of individual phenolic acids and flavonols of frozen blueberries are given in **Table 3.1** and individual anthocyanin is shown in **Table 3.2**. Catechin was the major flavonoid present in both cultivars: Tifblue (146.5 mg/100 g of berry) and Powderblue (75.5 mg/100 g of berry). Ferulic acid, caffeic acid, p-coumaric acid, and ellagic acid were the other predominant phenolic acids present in blueberry. These values are found higher than fresh berries reported by Sellappan et al. (24). The major

anthocyanidin found in Tifblue and Powderblue cultivers were malvidin followed by peonidin > cyanidin > delphinidin > petunidin **(Table 3.2).** Similar order was reported in highbush and lowbush varieties (31). The initial analysis of TPP, TACY, and TEAC of frozen blueberries, blueberry extract after pressing and heating is given in **Table 3.3**. Compared to the present study, our previous study showed 20% less TPP content and TEAC and 30% more TACY (11, 24). These variations may be due to difference in blueberry cultivars, storage conditions, stage of maturity, environmental factors such as light, temperature, agronomic practices, and various stresses. A single genotype of lowbush blueberries may differ in their anthocyanin content by 30% between two seasons (20). The Powderblue cultivar exhibited higher antioxidant capacity, TPP and TACY than Tifblue. The initial analysis of individual phenolic acids and flavonols and individual anthocyanidins of frozen blueberries, blueberry extract after pressing and heating of frozen blueberries, is given in **Table 3.4 and Table 3.5,** respectively. Recovery of gallic acid, catechin and quercetin was observed above 25 % in final extract after heating and removal of residue. Ferulic acid was not detected in the final extract in both the cultivars, Tifblue and Powderblue. Recovery of peonidin, malvidin and cyanidin was observed ~20 % in final extract in both the cultivars. These results suggest that most of the phenolic compounds are lost during removal of residue and during heating. Similar results were reported by earlier workers during juice preparations (14, 18-19).

Changes Observed in TPP, TACY and TEAC during Preparation of Extract. There was no significant change observed in TSS (11.8-12.5 ºBrix) and pH (3.2-3.4) in extract obtained after pressing and heating. Similar results were observed in both the cultivars (Tifblue and Powderblue). Recovery of TPP, TACY and TEAC in

blueberry extract after pressing and heating were ~25, ~29, and ~69%, respectively. The primary steps of preparation (thawing, crushing, depectinization and pressing) may have contributed to the considerable loss. Similar results were reported by other investigators (19, 32). This may be attributed to the oxidation of anthocyanins and polyphenols (19). Many researchers have suggested that native blueberry polyphenol oxidase (PPO) oxidizes polyphenolics to quinones, which produce brown pigments (15-17). However, after heating there was a slight increase in TPP in Powderblue. Slight increase in TACY was also observed in both the cultivars (Tifblue and Powderblue). This might be due to greater extraction due to fruit skin permeability/concentration during heating or complete inactivation of polyphenol oxidase (20).

Effect of Storage Conditions on TPP. TPP at different temperatures and time intervals are given in **Figure 3.2.** Cultivar type was not a significant predictor for the retention of TPP at all temperatures. However, interaction term, time×temperature was found to be a significant contributor to the degradation of TPP ($P < 0.05$). At -20±1 ºC, no statistically significant losses of TPP were observed until 30 days ($P < 0.05$). A slight reduction was observed by 30 days, but thereafter no significant degradation of TPP was observed. At 6±1 ºC there were significant losses observed from 15 to 30 days and then the retention became constant. Similar results were observed at 23±1 ºC and 35 ºC ($P < 0.05$). Earlier researchers reported no significant change in TPP for 12 months for frozen blueberry fruit (34), whereas high temperature and oxidative conditions were shown to significantly reduce TPP of lowbush blueberries (18, 20).

Effect of Storage Conditions on TACY. TACY at different temperatures and time intervals is given in **Figure 3.3.** Effect of storage on individual anthocyanins will be

discussed separately. All variables (time, temperature and interaction term, time×temperature) were significant contributors ($P < 0.05$) to the degradation of TACY. Statistically significant ($P < 0.05$) differences were observed for the two cultivars in the degradation of TACY. Duncan's multiple range tests showed that the highest value (34.7 mg/100 mL of extract) of TACY was observed at -20±1 ºC after 15 days storage. Lowest retention (17.0 mg/100 mL) was observed after 60 days at 35±1 ºC in extract obtained from the Tifblue cultivar. At all temperatures, significant differences in degradation were observed from 0 to 30 days ($P < 0.05$); thereafter it became constant. As discussed above, PPO is involved in the degradation of anthocyanins. There was no significant difference observed between 45 and 60 days. The chemical structure is a main factor affecting the stability of the anthocyanins. Stability and structure of individual anthocyanins are discussed later in this paper.

Effect of Storage Conditions on TEAC. TEAC at different temperatures and time intervals is given in **Figure 3.4**. Statistically significant ($P < 0.05$) differences were observed for the two cultivars with Tifblue having higher antioxidant activity than Powderblue. At -20±1 ºC, storage time did not affect the antioxidant activity. However, at 35±1 ºC there was significant loss of activity after 15 days which continued through 60 days. **Table 3.6** gives total retention of TPP, TACY, and TEAC after 60 days under all temperature conditions. Heat, storage time and oxidation contribute to the loss of antioxidant activity (18). Antioxidant activity of phenolic acids depends on the number and position of hydroxyl (-OH) groups and methoxy ($-OCH_3$) subsitituents in the molecules. Hydroxylation and glycosylation modulate the antioxidant properties of flavonoids (35). Prolonged storage at high temperature may affect the hydroxylation and

glycosylation of compounds and lead to gradual reduction in antioxidant activity (TEAC) as we observed. However, the rate of reduction was not similar to that of TPP and TACY, suggesting that there may be other compounds responsible, in part, to the antioxidant activity. A good example would be ascorbic acid which is present in blueberry and other fruits.

Effect of Storage Conditions on Phenolic Acids and Flavonols. Percent retention of phenolic acids and flavonols at different temperatures after 60 days of storage is given in **Table 3.7** (Tifblue) and **Table 3.8** (Powderblue). Temperature and time affected retention of phenolic acids and flavonols ($P < 0.05$). Phenolic acids in Tifblue and Powderblue were, gallic acid, caffeic acid, p-coumaric acid, and ellagic acid and flavonols were catechin, and quercetin. At -20±1 ºC retention of quercetin was 89.7% and 97.8% for Tifblue and Powderblue, respectively. However at 35±1 ºC these values reduced to 61.2% and 54.4%, respectively. There are reports available indicating no degradation of quercetin in whole fruit at 5 ºC for 9 months (36). Retention of ellagic acid varied from 87.5-48.2% in Tifblue and 46.3-60.0% in Powderblue at all temperature conditions. Rate of degradation of phenolic acid and flavonol at room temperature (23±1 ºC) is in the following order: quercetin > gallic acid > ellagic acid > catechin >caffeic acid > *p*-coumaric acid. Phenolic acids with higher hydroxyl group attachment may have contributed to the stability. Significant loss of caffeic may have occurred because caffeic acid is a good substrate for blueberry PPO (15).

Effect of Storage Conditions on Individual Anthocyanins. Percent retention of individual anthocyanidins at different temperatures after 60 days of storage is given in **Table 3.9** (Tifblue) and **Table 3.10** (Powderblue). Anthocyanins found in Tifblue and

Powderblue were: delphinidin, cyanidin, petunidin, peonididn, and malvidin. Temperature had a significant effect on retention of anthocyanidins. The proportion of Mv-Glc was highest in the extract obtained from both cultivars. The highest retention of Mv-Glc was observed when extract was stored at -20 ºC and 6 ºC for Tifblue and Powderblue, respectively. Delpinidin was not detected in the extract. Delphinidin is found most unstable. This was attributed to the methoxylation of the molecule, which increases the stability of anthocyanins (34). Stability of Mv-Glc and Pn-Glc increased due to the single hydroxyl group on the phenolic ring which makes them the least reactive anthocyanins and the least affected by PPO inactivation (14). There was a little change observed in peonidin-3-galactoside. A substantial decrease was observed in Cy-Glc. These results were in agreement with earlier studies (19). No anthocyanidins were detected at 35±1 ºC after 45 days. Increase in glycosidic substitution, acylation and methoxylation tend to improve the stability of anthocyanins. Methoxylation also increase the stability of anthocyanins (35).

Effect of Storage Conditions on Cell Proliferation. Inhibition of cell proliferation at different time intervals and temperatures is given in **Figure 3.5**. Highest inhibition (30.7%) was observed with anthocyanins extracted from the sample of Powderblue extract. Lowest inhibition (10.4%) was observed with anthocyanins extracted from Tifblue extract for 60 days at 6±1 °C. Interestingly, there was some cell growth (+7%) with anthocyanins from Tifblue extract stored for 90 days at 23±1 °C. Lower antiproliferation activity was associated with low or insignificant levels of delphinidin, petunidin, and cyanidin. Several researchers reported marked inhibitory effects of anthocyanins comprising cyanidin as main constituents on colorectal carcinogeneis (37)

and DNA damage (38), suggesting that the inhibition of anthocyanins on cell proliferation may be related to the number of hydroxyl groups on the B-ring. However, the molecular mechanisms are not clear. We observed initial inhibition percentage of 48.8 and 43.1% with anthocyanins from Tifblue and Powderblue, respectively. This decreased slightly in 30 days. However there were significant decreases after 30 to 90 days in both cultivars (Tifblue and Powderblue). We observed a slight decrease in inhibition percentage in cells treated with anthocyanin fraction from extract stored at different time intervals. This might be attributed to the high retention of malvidin in the initial 45 days which was followed by a significant reduction of this anthocyanin. As suggested, the presence of hindered phenol on the B ring via the presence of a methoxy group enhances the antioxidant activity or the H-donating activity. Malvidin has the phenolic group hindered by two methoxyl groups (39). Few studies have shown that the orthodihydroxyphenyl structure on the B-ring of anthcyanidins may be essential for the inhibitory action because pelargonidin, peonidin, and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show the inhibitory effects (40), which is contrary to our results. The effects of anthocyanidin on HepG2 cell viability have been reported. The estimated IC_{50} of cyanidin, delphinidin, and malvidin were 18.4, 10.8, and 50.4 μM (equivalent to 5, 3, and 17 μg/mL), respectively (41). This suggests that after heating, de-pectinization and storage there was a significant loss in antiproliferation activity of phenolic compounds.

Correlation between TPP, TACY and TEAC. The correlation between TEAC and total polyphenols or total anthocyanins contents of blueberry extract from different time intervals stored at 23±1 ºC is presented in **Figure 3.6**. The average values of TEAC

showed positive correlation with average values of total anthocyanins and total polyphenols. A linear relationship was observed between TEAC and TPP or TACY. In Tifblue the correlation coefficient, r^2 was 0.66 for TPP and and 0.56 for TACY and for Powderblue r^2 was 0.65 for TPP and and 0.52 for TACY. These values indicate that the antioxidant capacity is moderately related to TPP and TACY. Fresh blueberries had shown strong positive correlation between TEAC and TPP (24).

This study showed that prolonged storage of blueberry extract at room temperature significantly reduces the phenolic compounds and their biological activity. No difference in TPP, TACY and TEAC was observed between the two different cultivars. Retention of phenolic acids and anthocyanidins were least influenced by cold storage and frozen conditions.

ACKNOWLEDGMENT

Research was funded by the State of Georgia's Traditional Industries Program for Food Processing Research Grant. The authors would like to thank the Georgia Blueberry Growers Association for their support.

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Table 3.1 Individual Phenolic Acids and Flavonoids in Frozen Blueberries

	frozen blueberries							
	Phenolic acid (mg/100 g fruit weight)					Flavonoids (mg/100 g fruit weight)		
	gallic acid	caffeic acid	*p*-coumaric acid	ferulic acid	ellagic acid	catechin	quercetin	myricetin
Tifblue	5.5±3.5	32.5±4.9	29.5±4.9	69.0±11.3	11.0±5.7	146.5±10.6	8.5±0.7	3.5±2.1
Powderblue	31.0±1.4	56.5±0.7	65.5±6.4	11.5±0.7	36.0±1.4	75.5±6.4	6.5±3.5	4.0±1.4

Each value is expressed as mean±SD, n=3

Table-3.2. Individual Anthocyanidins in Frozen Blueberries

	frozen blueberries (mg/100 g of fruit weight)							
	Dp-Glc	Cy-Glc	Cy-Gal	Pn-Glc	Pn-gal	Pt-Glc	Pn-Ara	Mv-Glc
Tifblue	8.5±0.7	5.5±0.7	18.0±2.8	6.0±1.4	10.0±1.4	6.5±0.7	15.5±0.7	39.0±2.8
Powderblue	9.0±0.0	4.5±0.7	12.5±2.1	5.5±2.1	9.5±2.1	5.5±2.1	14.0±1.4	38.0±5.7

Abbreviations: Dp-Glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-Glc (Cyanidin 3-*O*-β-glucopyranoside), Cy-Gal (Cyanidin 3-*O*-β-galactopyranoside), Pn-Glc (Peonidin 3-*O*-β-glucopyranoside), Pn-Gal (Peonidin 3-*O*-β-galactopyranoside), Pt-Glc (Petunidin 3-*O*-β-glucopyranoside), Pn-Ara (Peonidin 3-*O*-α-arabinopyranoside), Mv-Glc (Malvidin 3-*O*-β-glucopyranoside).
Each value was expressed as mean±SD, n=3

Table 3.3 TPP, TACY, and TEAC Content of Frozen Blueberry, Blueberry Extract after Pressing and Heating

	Tifblue				Powder			
	Frozen berries	Extract after pressing	Extract after heating	Recovery (%)	Frozen berries	Extract after pressing	Extract after heating	Recovery (%)
TPP [1]	344.8±4.7	87.3±2.7	87.3±1.8	25.3	383.4±8.4	87.9±0.7	90.7±0.8	23.7
TACY [2]	114.5±3.2	29.9±0.3	33.6±0.5	29.3	120.9±3.7	33.4±1.8	36.1±0.3	29.9
TEAC [3]	26.1±1.1	17.0±0.9	17.2±0.1	65.9	27.3±1.2	17.5±0.6	17.4±0.3	63.7

Abbreviations: [1] TPP is total polyphenol (mg/100g of frozen blueberry or extract);
[2]TACY total anthocyanin mg/100g of frozen blueberry or extract); [3]TEAC trolox equivalent antioxidant capacity
Recovery percent was calculated based on frozen berries concentration as 100 %.
(μM/g whole frozen blueberry or extract). Each value was expressed as mean±SD, n=3.

Table 3.4 Phenolic Acids and Flavonols in Frozen Blueberry, Blueberry Extract after Pressing and Heating

	Tifblue				Powderblue			
Phenolic acids and flavonols	Frozen berries	Extract after pressing	Extract after heating	Recovery (%)	Frozen berries	Extract after pressing	Extract after heating	Recovery (%)
Gallic acid	5.5±3.5	1.8±0.4	2.0±0.2	35.5±3.9	31±1.4	27±5.7	12.7±1.1	41.0±3.7
Caffeic acid	32.5±4.9	21.5±1.8	4.4±0.6	13.4±2.0	56.5±0.7	19.1±1.2	5.4±2.2	9.5±3.9
p-Coumaric acid	29.5±4.9	6.0±0.4	4.7±0.5	15.8±1.7	65.5±6.4	8.5±0.92	5.5±0.9	8.3±1.4
Ferulic acid	69.0±11.3	7.6±0.4	nd	nd	11.5±0.7	4.9±0.3	nd	nd
Ellagic acid	11.0±5.7	5.9±1.0	2.8±0.1	24.4±1.2	36±1.4	17.2±1.0	12.1±1.3	33.5±3.7
Catechin	146.5±10.6	79.6±10.8	36.3±0.4	24.8±0.3	75.5±6.4	68.2±1.1	33.1±4.5	43.8±6.0
Myrecitin	3.5±2.1	0.6±0.4	nd	nd	4±1.3	0.5±0.1	nd	nd
Quercetin	8.5±0.7	2.2±0.4	2.9±0.4	33.5±4.2	6.5±3.5	2.7±0.6	2.3±0.5	34.6±7.6

Phenolic acids and flavonols are expressed in mg/100 g of frozen fruit and mg/100 mL of extract.
Recovery percent was calculated based on frozen berries concentration as 100 %.
Each value was expressed as mean±SD, n=3.

Table 3.5 Individual Anthocyanidins in Frozen Blueberry, Blueberry Extract after Pressing and Heating

	Tifblue				Powderblue			
Anthocynidins	Frozen berries	Extract after pressing	Extract after heating	Recovery (%)	Frozen berries	Extract after pressing	Extract after heating	Recovery (%)
Dp-glc	8.5±0.7	3.2±0.1	nd	nd	9.0±0.0	3.5±0.6	nd	nd
Cy-glc	5.5±0.7	6.8±0.7	1.1±0.2	19.9±3.2	4.5±0.7	8.3±0.2	1.1±0.2	25.1±5.3
Cy-gal	18.0±2.8	18.4±1.0	1.0±0.1	5.6±0.8	12.5±2.1	18.9±0.1	1.0±0.1	7.7±0.8
Pn-glc	6.0±1.4	3.6±1.0	3.2±0.1	52.7±0.9	5.5±2.3	2.8±0.1	3.4±0.1	62.1±2.2
Pn-gal	10.0±1.4	22.6±0.8	1.1±0.0	10.5±0.1	9.5±1.9	25.0±0.7	1.2±0.0	12.4±0.3
Pt-glc	6.5±0.7	20.9±3.1	nd	nd	5.5±2.0	24±1.3	nd	nd
Pn-ara	15.5±0.6	20.7±5.0	8.7±0.3	56.2±1.8	14±1.4	20.6±1.2	8.1±0.1	58.0±0.8
Mv-glc	39.0±2.8	38.8±14.5	7.7±0.0	19.7±0.0	38±5.7	33.3±7.9	8.6±0.2	22.7±0.5

Each anthocyanidins are expressed in mg/100 g of frozen fruit and mg/100 mL of extract. Recovery percent was calculated based on frozen berries concentration as 100 %. Dp-glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-glc (Cyanidin 3-*O*-β-glucopyranoside), Cy-gal (Cyanidin 3-*O*-β-galactopyranoside), Pn-glc (Peonidin 3-*O*-β-glucopyranoside), Pn-gal (Peonidin 3-*O*-β-galactopyranoside), Pt-glc (Petunidin 3-*O*-β-glucopyranoside), Pn-ara (Peonidin 3-*O*-α-arabinopyranoside), Mv-glc (Malvidin 3-*O*-β-glucopyranoside).
Recovery percent was calculated based on frozen berries concentration as 100 %.
Each value was expressed as mean±SD, n=3.

Table 3.6 Percent Retention of TPP, TACY and TEAC after 60 Days at Different Temperature Conditions

	Temperature (°C)							
	Tifblue				Powderblue			
	-20±1	**6±1**	**23±1**	**35±0.5**	**-20±1**	**6±1**	**23±1**	**35±0.5**
TPP [1]	78.3±3.5a	66.6±2.2b	49.4±2.5c	49.4±2.5c	75.9±0.1a	68.9±4.1b	35.6±1.1d	50.3±2.3c
TACY [2]	77.8±3.1a	67.5±1.1c	50.4±1.3e	50.7±2.3e	75.4±1.1b	68.5±0.4d	51.3±0.6e	31.5±0.5e
TEAC [3]	98±0.3a	95.7±0.5b	87.6±0.1e	76.6±0.4g	94.6±0.4c	91.2±0.4d	79.2±0.6c	79.2±0.6f

Abbreviations: [1] TPP is total polyphenol (mg/100mL of blueberry extract);
[2]TACY total anthocyanin mg/100mL of blueberry extract); [3]TEAC trolox equivalent antioxidant capacity (μM/ mL of blueberry extract).Retention percent was calculated after 60 days at a given temperature conditions.
Percent was calculated based on 0 day concentration as 100 %.
Each value was expressed as mean±SD, n=3.

Table 3.7 Retention of Blueberry Phenolic Acids and Flavonols in Blueberry Extract After 60 Days Under Different Temperature Conditions

	Tifblue								
	0 day	after 60 days							
		-20±1 ºC		**6±1 ºC**		**23±1 ºC**		**35±1 ºC**	
	[a]conc.	[a]conc	retn. %	[a]conc	retn. %	[a]conc	retn. %	[a]conc	retn. %
Gallic acid	2.0±0.2	0.5±0.0	23.5±1.4	1.2±0.1	61.3±2.5	1.1±0.1	53.3±2.5	1.2±0.0	59.8±1.7
Caffeic acid	4.4±0.6	1.7±0.1	39.2±2.4	0.6±0.1	13.6±3.2	0.7±0.1	14.8±1.6	0.9±0.1	19.3±1.6
p-Coumaric acid	4.7±0.5	1.3±0.1	27.6±1.7	0.3±0.1	6.4±2.1	0.6±0.1	12.8±3.0	0.4±0.1	8.5±3.0
Ferulic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ellagic acid	2.8±0.1	2.5±0.1	87.5±2.5	1.5±0.4	53.6±1.5	1.4±0.2	48.2±7.6	1.6±0.1	57.0±5.1
Catechin	36.3±0.4	5.6±0.4	15.4±1.2	5.9±0.6	16.1±1.8	3.7±0.3	10.2±0.9	5.4±0.3	14.9±0.8
Myrecitin	nd	nd	nd	nd	nd	nd	nd	nd	nd
Quercetin	2.9±0.4	2.6±0.3	89.7±9.8	2.1±0.1	70.1±2.4	2.2±0.2	74.1±7.3	1.8±0.3	61.2±11.0

Abbreviations: [a]conc. = Concentration of phenolic acids and flavonols expressed in mg/100 mL of extract; nd=not detected
Percent was calculated based on 0 day concentration as 100 %.
Each value was expressed as mean±SD, n=3.

Table 3.8 Retention of Blueberry Phenolic Acids and Flavonols in Blueberry Extract After 60 Days Under Different Temperature Conditions

	Powderblue								
	0 day	after 60 days							
		-20±1 ºC		**6±1 ºC**		**23±1 ºC**		**35±1 ºC**	
	[a]conc.	[a]conc	retn. %	[a]conc	retn. %	[a]conc	retn. %	[a]conc	retn. %
Gallic acid	12.7±1.13	5.0±1.6	18.9±1.1	7.3±0.3	57.7±2.3	8.0±1.3	63.0±1.0	7.0±0.1	54.7±0.6
Caffeic acid	5.34±2.18	1.8±0.4	34.0±8.0	0.8±0.1	14.2±1.3	0.8±0.1	15.1±2.7	0.7±0.1	12.3±1.3
p-Coumaric acid	5.45±0.92	1.3±0.1	23.4±0.9	0.3±0.0	6.3±0.3	0.4±0.0	8.0±0.8	0.7±0.1	12.0±1.3
Ferulic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ellagic acid	12.05±1.3	6.0±0.1	49.6±0.6	6.5±0.7	54.2±5.9	5.6±0.5	46.3±4.1	7.2±0.4	60.0±3.5
Catechin	33.1±4.53	6.3±0.4	17.4±1.0	3.7±0.3	11.8±0.0	7.8±0.2	23.4±0.6	4.0±0.2	11.9±0.6
Myrecitin	nd	nd	nd	nd	nd	nd	nd	nd	nd
Quercetin	2.25±0.49	2.3±0.4	97.8±1.5	1.1±0.3	47.8±1.2	1.6±0.4	67.4±1.5	1.3±0.5	54.4±2.1

Abbreviations: [a]conc. = Concentration of phenolic acids and flavonols expressed in mg/100 mL of extract; nd=not detected
Percent was calculated based on 0 day concentration as 100 %.
Each value was expressed as mean±SD, n=3.

Table 3.9 Retention of Individual Anthocyanidins in Blueberry Extract After 60 Days Under Different Temperature Conditions

	Tifblue								
	0 day	after 60 days							
		-20±1 ºC		**6±1 ºC**		**23±1 ºC**		**35±1 ºC**	
	[a]conc.	[a]conc	retn. %	[a]conc	retn. %	[a]conc	retn. %	[a]conc	retn. %
Dp-glc	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cy-glc	1.1±0.2	0.8±0.1	73.1±2.3	0.5±0.0	42.3±3.2	0.2±0.0	17.7±1.9	nd	nd
Cy-gal	1.0±0.1	0.5±0.0	44.5±3.5	0.4±0.0	37.2±0.1	0.1±0.0	15.0±5.8	nd	nd
Pn-glc	3.2±0.1	1.3±0.2	39.7±8.0	0.7±0.1	20.2±2.4	0.5±0.0	15.5±0.2	nd	nd
Pn-gal	1.1±0.0	0.9±0.0	78.6±1.9	0.7±0.1	64.6±3.9	0.5±0.1	41.4±5.6	nd	nd
Pt-glc	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pn-ara	8.7±0.3	4.1±0.1	46.6±1.0	2.1±0.0	24.3±0.2	1.5±0.1	17.1±1.5	0.1±0.0	17.8±3.1
Mv-glc	7.7±0.0	7.2±0.9	93.7±1.7	4.3±0.1	55.3±0.8	3.3±0.1	42.8±9.1	nd	nd

Abbreviations: [a]conc. = Concentration of phenolic acids and flavonols expressed in mg/100 mL of extract; nd=not detected
Each anthocyanidins are expressed in mg/100 g of frozen fruit and mg/100 mL of extract.
Dp-glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-glc (Cyanidin 3-*O*-β-glucopyranoside),
Cy-gal (Cyanidin 3-*O*-β-galactopyranoside), Pn-glc (Peonidin 3-*O*-β-glucopyranoside),
Pn-gal (Peonidin 3-*O*-β-galactopyranoside), Pt-glc (Petunidin 3-*O*-β-glucopyranoside),
Percent was calculated based on 0 day concentration as 100 %.
Each value was expressed as mean±SD, n=3.

Table 3.10 Retention of Individual Anthocyanidins in Blueberry Extract After 60 Days Under Different Temperature Conditions

	Powderblue								
	0 day	after 60 days							
		-20±1 ºC		**6±1 ºC**		**23±1 ºC**		**35±1 ºC**	
	[a]conc.	[a]conc	retn. %	[a]conc	retn. %	[a]conc	retn. %	[a]conc	retn. %
Dp-glc	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cy-glc	1.1±0.2	0.8±0.0	70.0±1.3	0.6±0.0	56.9±3.3	0.2±0.0	17.7±0.6	nd	nd
Cy-gal	1.0±0.1	0.6±0.0	74.5±4.9	0.8±0.0	59.5±2.1	0.3±0.0	27.5±0.7	nd	nd
Pn-glc	3.4±0.1	1.2±0.0	36.3±0.2	1.1±0.1	31.2±1.7	1.4±0.0	42.9±1.3	nd	nd
Pn-gal	1.2±0.0	0.8±0.0	69.4±4.4	0.6±0.0	47.1±1.8	0.2±0.0	16.7±0.0	nd	nd
Pt-glc	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pn-ara	8.1±0.1	2.7±0.2	33.7±2.6	2±0.0	24.6±0.2	1.6±0.1	19.9±0.5	1.6±0.3	19.1±3.3
Mv-glc	8.6±0.2	4.0±0.2	45.9±2.5	6.1±0.1	70.5±1.0	2.2±0.2	25.8±1.4	nd	nd

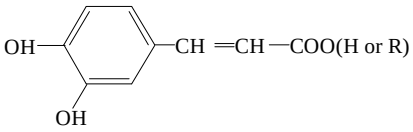
Abbreviations: [a]conc. = Concentration of phenolic acids and flavonols expressed in mg/100 mL of extract; nd=not detected
Each anthocyanidins are expressed in mg/100 g of frozen fruit and mg/100 mL of extract.
Dp-glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-glc (Cyanidin 3-*O*-β-glucopyranoside),
Cy-gal (Cyanidin 3-*O*-β-galactopyranoside), Pn-glc (Peonidin 3-*O*-β-glucopyranoside),
Pn-gal (Peonidin 3-*O*-β-galactopyranoside), Pt-glc (Petunidin 3-*O*-β-glucopyranoside),
Pn-ara (Peonidin 3-*O*-α-arabinopyranoside), Mv-glc (Malvidin 3-*O*-β-glucopyranoside).
Percent was calculated based on 0 day concentration as 100 %.
Each value was expressed as mean±SD, n=3.

Figure 3.1 Structure of phenolic compounds: (A) Phenolic acids: a) Caffeic acid (number of OH-2); b) *p*-Coumaric acid (number of OH-1); c) Gallic acid (number of OH=3); d) Ellagic acid (number of OH=4), (**B)** Flavonols a) (+)-Catechin (number of OH=5); b) Quercetin (number of OH=5), and **(C)** Anthocyanins; i) Pelargonidin (R^1= H, R^2= H); ii) Cyanidin (R^1= OH, R^2= H); iii) Peonidin (R^1= OMe, R^2= H); iv) Delphinidin (R^1= OH, R^2= OH); v) Petunidin (R^1= OMe, R^2= OH); vi) Malvidin (R^1= OMe, R^2= OMe).

Figure 3.1

(A)

a)



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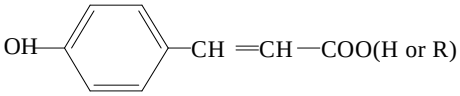

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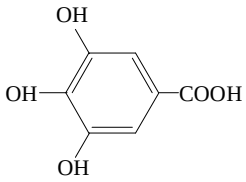

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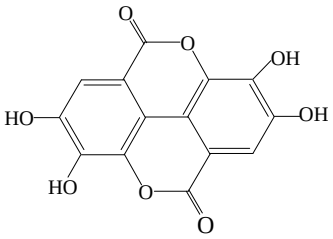
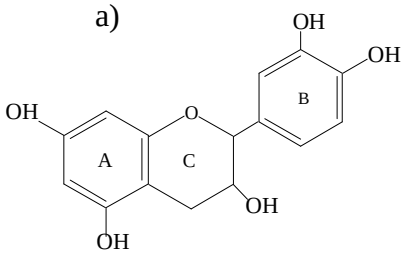


(B)

a)



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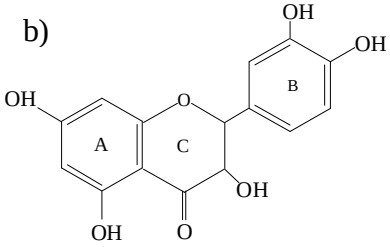


(C)

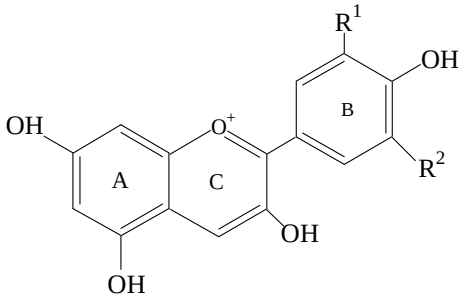
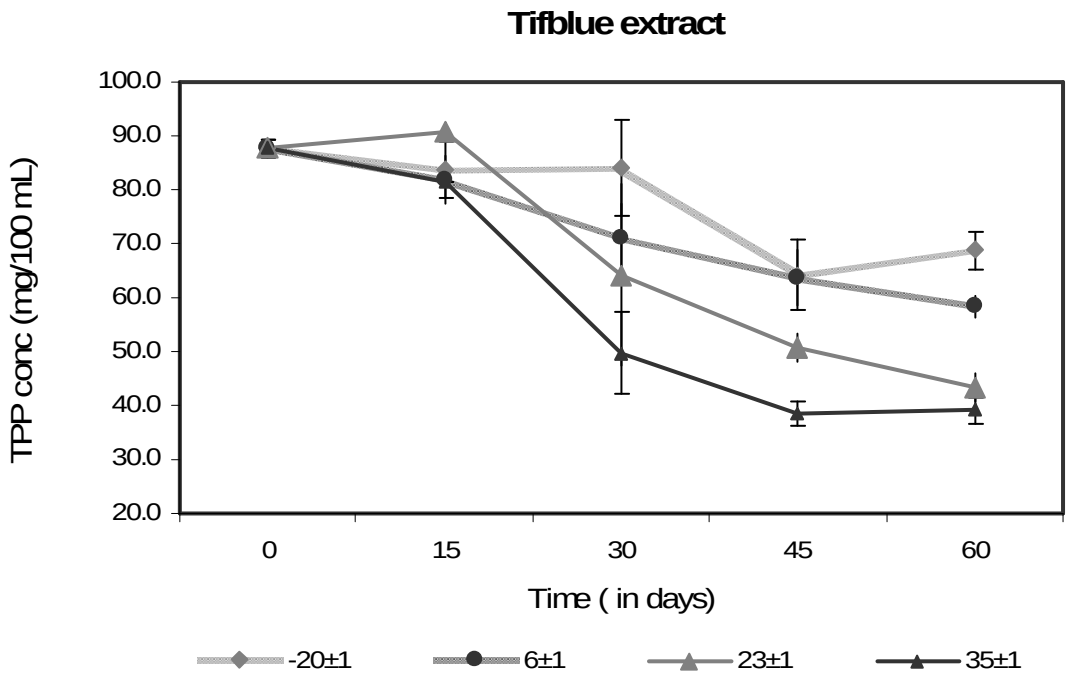


Figure 3.2 Changes in concentration of total polyphenol (TPP) in blueberry extract during storage at different temperatures. X axis represents time (in days). The Y-axis represents concentration of TPP (mg/100 mL of extract). Samples were analyzed in 15-day interval. Storage temperatures were at -20±1, 6±1 °C, 23±1 °C, and 35±1 °C. Cultivars were (A) Tifblue and (B) Powderblue.

Figure 3.2

(A)


Tifblue extract
TPP conc (mg/100 mL)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
0
15
30
45
60
Time (in days)
-20±1
6±1
23±1
35±1

(B)

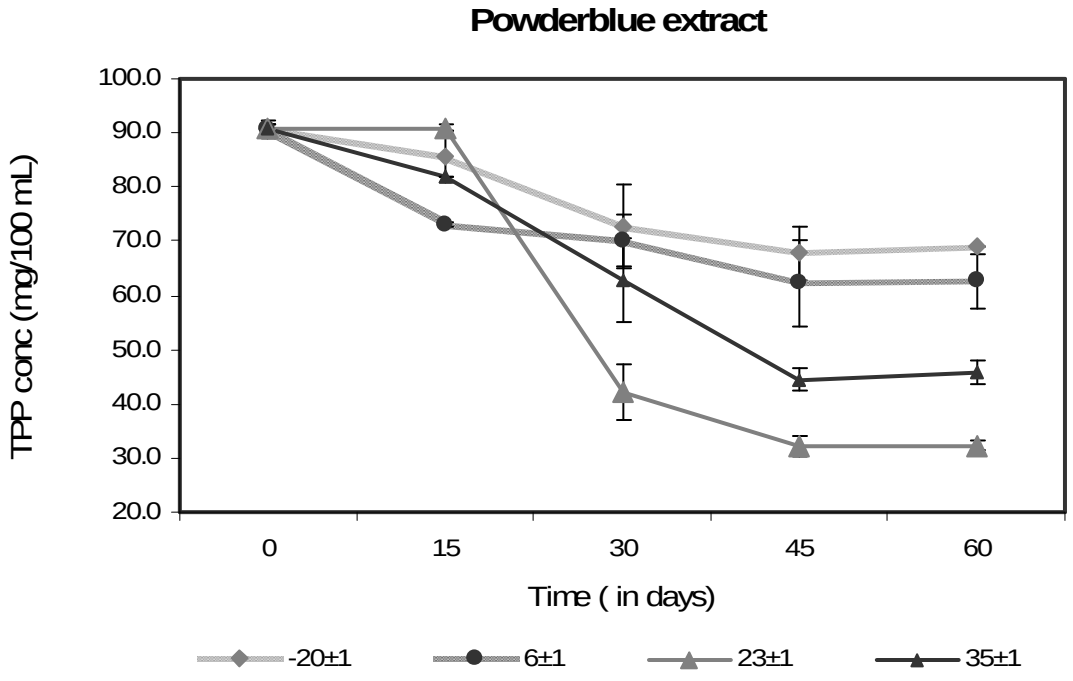
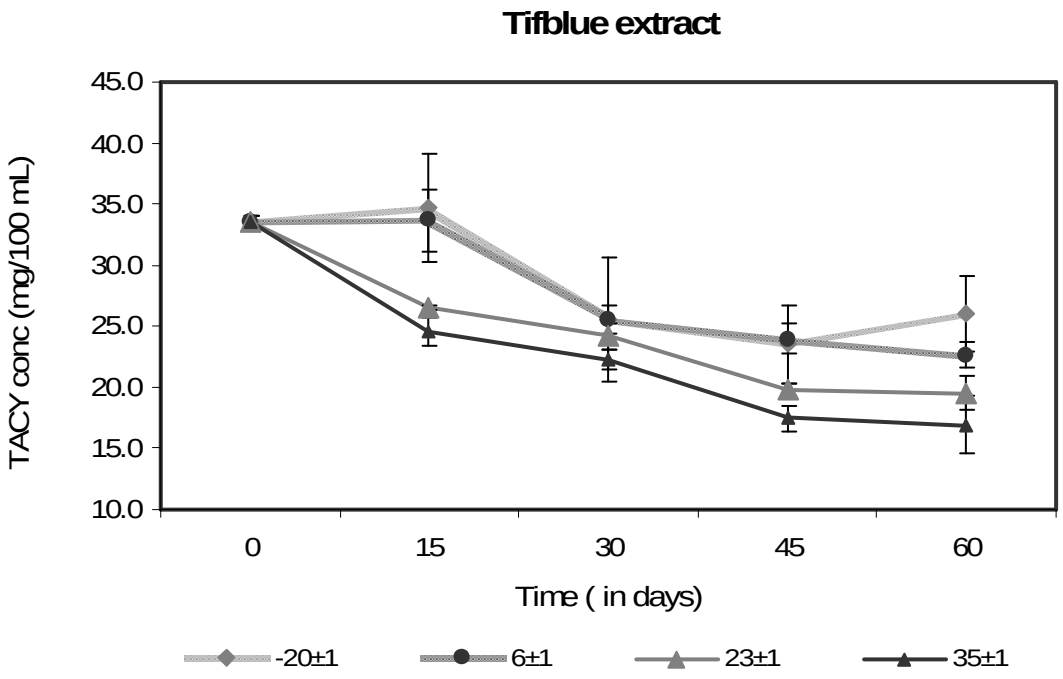

Powderblue extract
TPP conc (mg/100 mL)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
0
15
30
45
60
Time (in days)
-20±1
6±1
23±1
35±1

Figure 3.3 Changes in concentration of total anthocyanins (TACY) in blueberry extract during storage at different temperatures. X axis represents time (in days). The Y-axis represents concentration of TACY (mg/100 mL of extract). Samples were analyzed every 15 days. Samples were analyzed in 15-day interval. Storage temperatures were at -20±1, 6±1 ℃, 23±1 ℃, and 35±1 ℃. Cultivars were (A) Tifblue and (B) Powderblue.

Figure 3.3

(A)


Tifblue extract
TACY conc (mg/100 mL)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
0
15
30
45
60
Time (in days)
-20±1
6±1
23±1
35±1

(B)

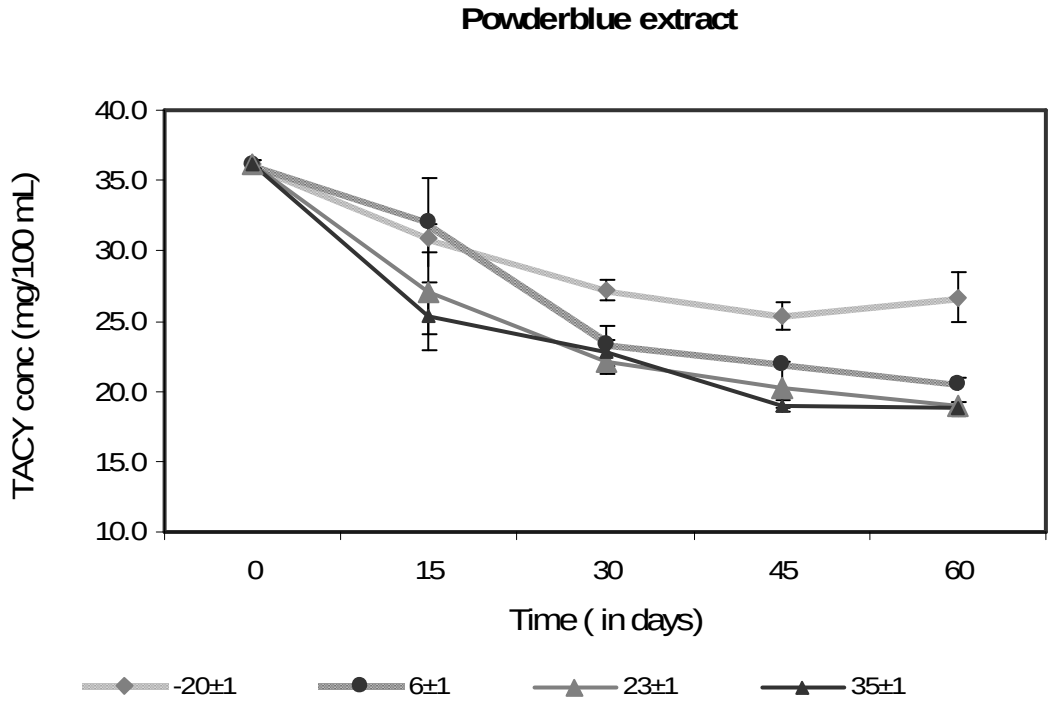
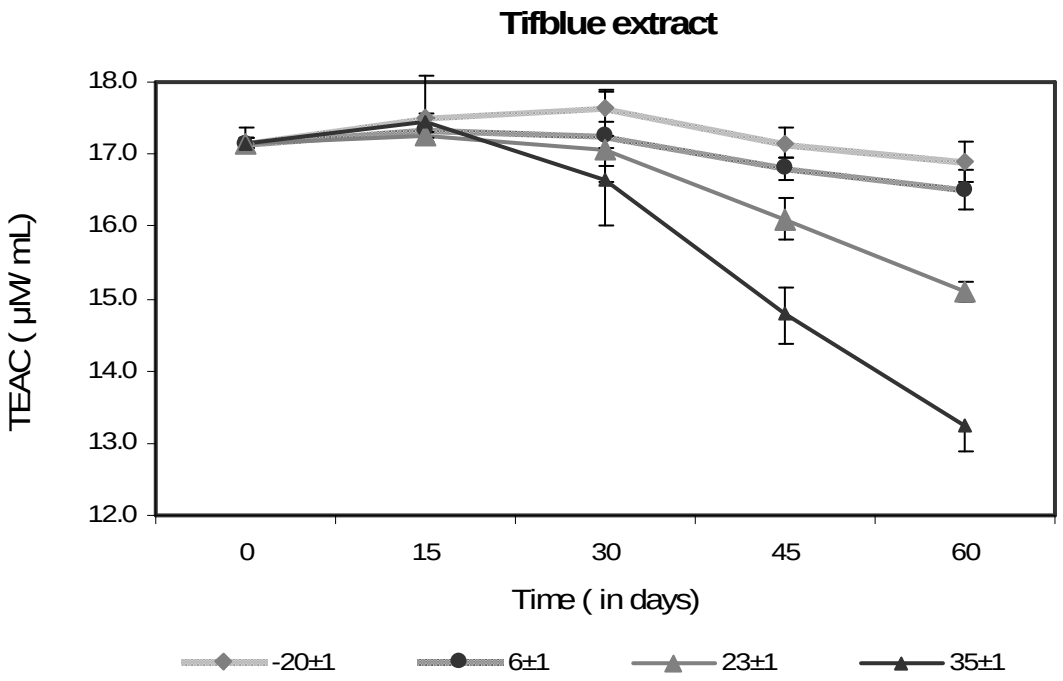

Powderblue extract
TACY conc (mg/100 mL)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
0
15
30
45
60
Time (in days)
-20±1
6±1
23±1
35±1

Figure 3.4 Changes in Trolox equivalent antioxidant capacity (TEAC) in blueberry extract during storage at different temperatures. X axis represents time (in days). The Y-axis represents TEAC in μM/mL of extract. Samples were analyzed every 15 days. Samples were analyzed in 15-day interval. Storage temperatures were at -20±1, 6±1 °C, 23±1 °C, and 35±1 °C. Cultivars were (A) Tifblue and (B) Powderblue.

Figure 3.4

(A)


Tifblue extract
TEAC (µM/ mL)
18.0
17.0
16.0
15.0
14.0
13.0
12.0
0
15
30
45
60
Time (in days)
-20±1
6±1
23±1
35±1

(B)

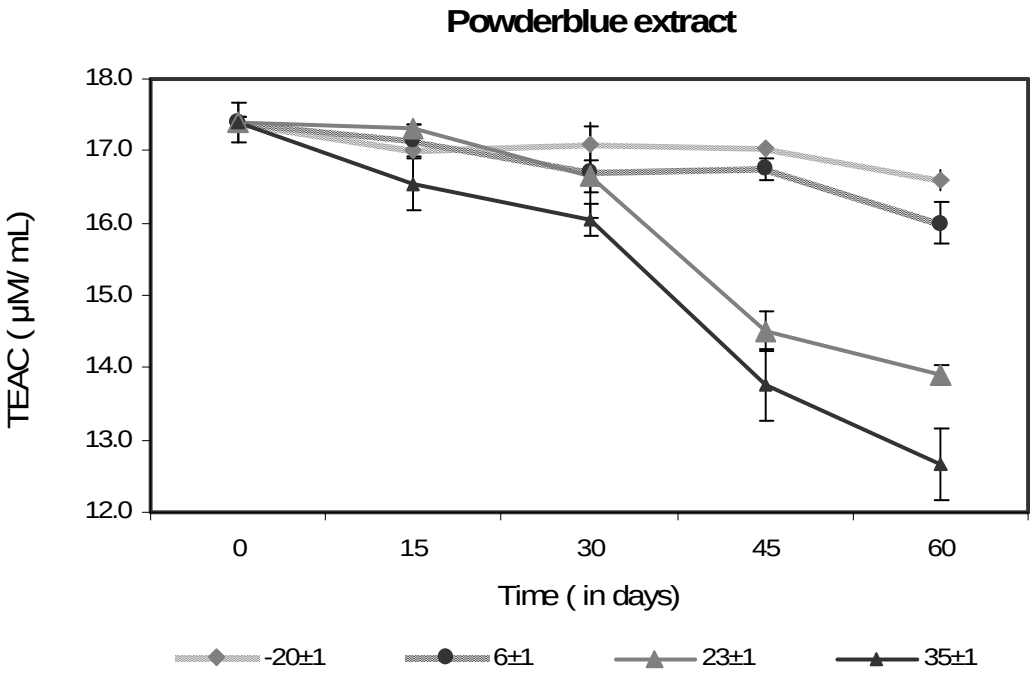

Powderblue extract
TEAC (µM/ mL)
18.0
17.0
16.0
15.0
14.0
13.0
12.0
0
15
30
45
60
Time (in days)
-20±1
6±1
23±1
35±1

Figure 3.5 Cell viability after treatment with anthocyanins fractions from extract stored under different temperature conditions. X axis represents time (in days). The Y-axis represents inhibition percentage of cells. Samples were analyzed every 30 days. Storage temperatures were at (A) 6±1 °C, and 23±1 °C. Packaging material were glass bottles. Cultivars were Tifblue and Powderblue. Abbreviations: Tif=Tifblue; Powder=Powderblue.

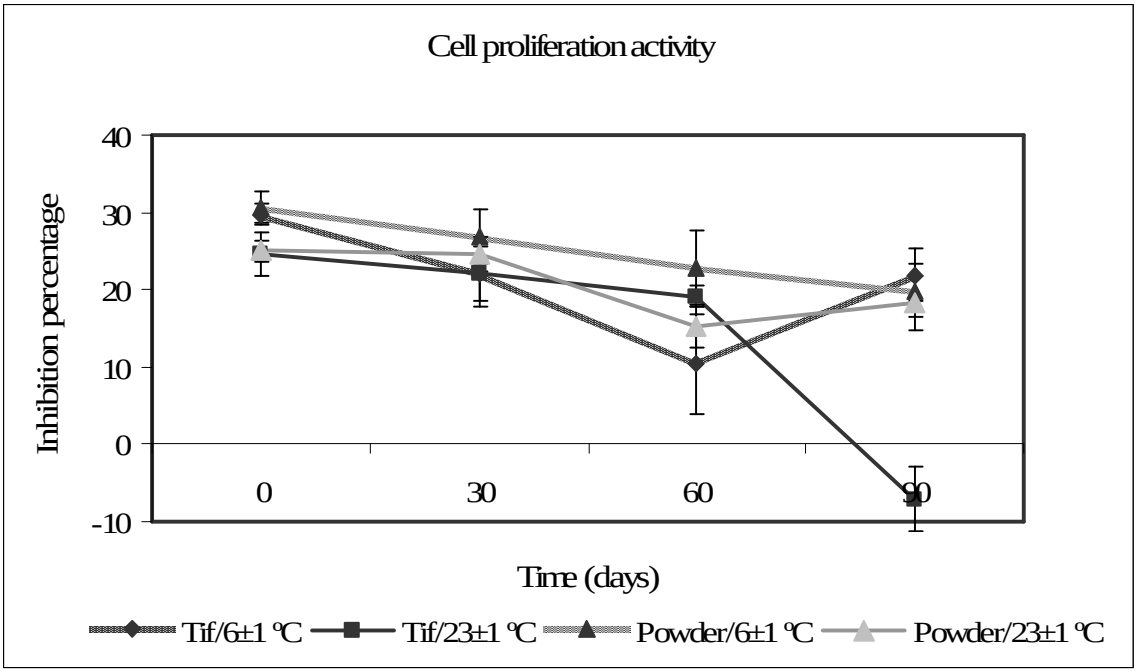
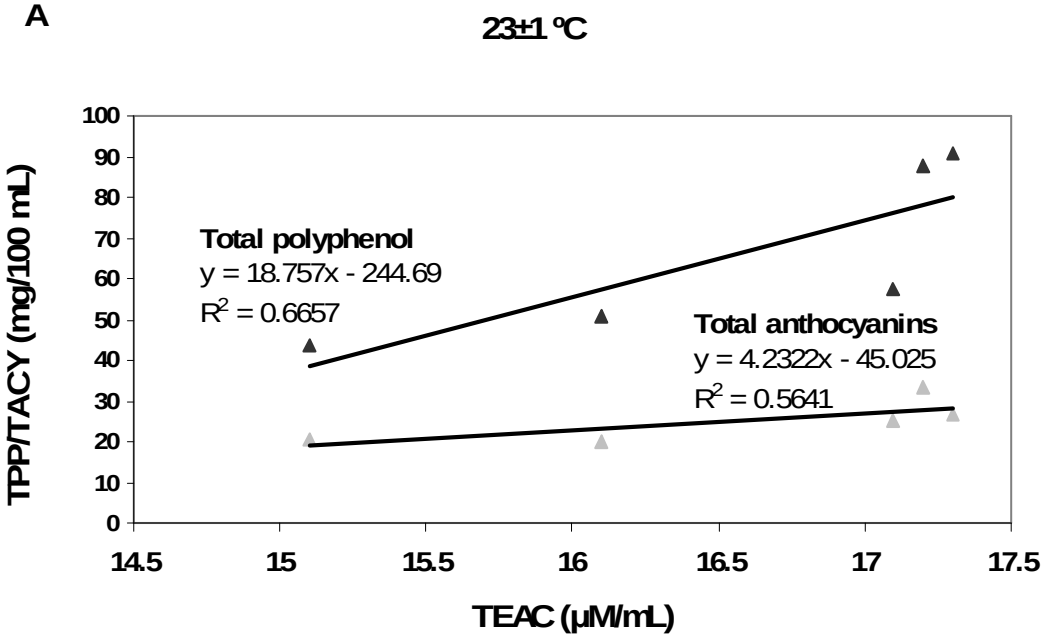
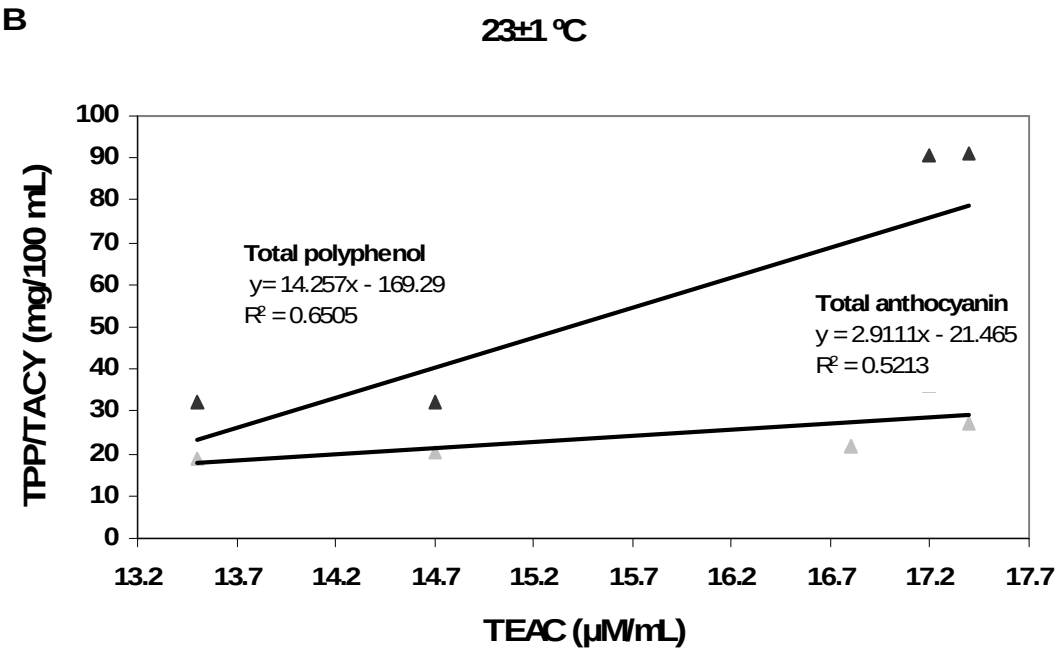

Cell proliferation activity
Inhibition percentage
40
30
20
10
0
-10
0
30
60
90
Time (days)
Tif/6±1 ℃
Tif/23±1 ℃
Powder/6±1 ℃
Powder/23±1 ℃

Figure 3.6 Correlation between total polyphenols, total anthocyanins, (*y*-axis), to TEAC value. The Y-axis represents total polyphenols and anthocyanins (mg/100 mL of extract). X-axis represents TEAC (μM/mL). Average values were used for plot at different time intervals. (A) Tifblue and (B) Powderblue at 23±1 °C**.**

A


23±1 °C
TPP/TACY (mg/100 mL)
TEAC (µM/mL)
Total polyphenol
y = 18.757x - 244.69
R2 = 0.6657
Total anthocyanins
y = 4.2322x - 45.025
R2 = 0.5641

B


23±1 °C
TPP/TACY (mg/100 mL)
TEAC (µM/mL)
Total polyphenol
y= 14.257x - 169.29
R² = 0.6505
Total anthocyanin
y = 2.9111x - 21.465
R² = 0.5213

CHAPTER 4

EFFECT OF ANTHOCYANIN FRACTIONS FROM SELECTED CULTIVARS OF GEORGIA-GROWN BLUEBERRIES ON APOPTOSIS AND PHASE-II ENZYMES: GLUTATHIONE-S-TRANSFERASE AND QUINONE REDUCTASE AS A MEASURE OF CHEMOPREVENTIVE PROPERTIES AND MECHANISMS

Anita Srivastava, Casimir C. Akoh, Joan Fischer, and Gerard Krewer
To be submitted to the Journal of Agricultural and Food Chemistry.

ABSTRACT

In recent years, considerable attention has been paid to anthocyanins due to their abilities both to inhibit oxidative stress and cell proliferation and to induce apoptosis in malignant cells. Regulation of phase-II enzymes a) glutathione-S-transferase (GST) and b) quinone reductase (QR) is another potential mechanism through which flavonoids prevent cancer. Our study confirmed apoptosis using two different methods: DNA fragmentation and caspase-3 activity. The effect of anthocyanins on the activity of detoxifying enzymes GST and QR was also determined. Major anthocyanidins identified were delphinidin, cyanidin, peonidin, petunidin, and malvidin. Malvidin was found to be the predominant anthocyanidin in all the cultivars, but Brightwell had peonidin as predominant anthocyanidin. The content of malvidin was 39.0, 38.0, 43.1, and 17.5 mg/100 g of frozen blueberry in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. Peonidin was the second largest anthocyanidin. The total content of peonidin was 31.5, 28.0, 35.0, and 50.5 mg/100 g of frozen blueberry in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. In anthocyanin fraction, malvidin was also found to be the predominant anthocyanidin in all the cultivars, but Brightwell had peonidin as predominant anthocyanidin. There was considerable loss in delphinidin content during extraction. In all cultivars DNA fragmentation increased with anthocyanin concentration from 50 to 150 μg/mL, but cells treated with anthocyanin fraction of Brighwell showed prominent ladder at 50 to 100 μg/mL when compared to cells treated with 150 μg/mL. There was a significant difference in the caspase-3 activity ($P < 0.05$)

between the control cells and the cells treated with anthocyanins from all the cultivars**.** Response correlated positively with dose. Highest activity (1.4 fold increase over control) was observed in cells treated with 150 μg/mL anthocyanin fraction from the Brightwell cultivar. Statistically no significant difference ($P < 0.05$) was observed in

caspase-3 activity between cells treated with Tifblue and Powderblue anthocyanin fractions at the same concentration (150 μg/mL)**.** QR activity was lower in all treated cells than in control cells (0.25 μM/mg protein); however, no statistically significant difference ($P < 0.05$) was observed in the QR activity of control cells and cells treated with the anthocyanin fraction from Brightblue (100 and 150 μg/mL) and Brightwell (50 μg/mL). Activity decreased gradually when treated with increased concentrations of anthocyanin fractions (50-150 μg/mL) in the Tifblue and Powderblue cultivars. A positive dose-response relationship was found in all the cultivars except Brightblue, where activity was the same for all three concentrations. GST activity was statistically higher ($P < 0.05$) in control cells than in cells treated with anthocyanin fractions from all the cultivars and at all levels of concentration. These results indicated that anthocyanins were not highly active in induction of detoxifying enzymes; however, apoptosis was confirmed in HT-29 cells when treated with anthocyanins consisting predominantly of malvidin.

KEYWORDS: Anthocyanins; blueberries; caspase-3; cultivars; detoxifying enzymes; DNA fragmentation; DNA ladder; glutathione-S-transferase; phase-II enzymes; quinone reductase

INTRODUCTION

Anthocyanins belong to a widespread class of phenolic compounds collectively named flavonoids. They are present in colored fruits and vegetables such as blueberries, red grapes, and red cabbages (1). Structure of individual anthocyanins differs in the number of hydroxyl groups, the nature and number of sugars, and the position of these attachments (2). In recent years, considerable attention has been paid to anthocyanins due to their ability both to inhibit oxidative stress, decrease cell proliferation, carcinogenesis, and to induce apoptosis in malignant cells (3-7). Apoptosis is a programmed cell death characterized by a series of distinct morphological and biochemical alterations (8-9). This process is essential for morphogenesis, tissue homeostasis, and host defense (9), and plays a significant role in the elimination of seriously damaged cells or tumor cells by chemopreventive agents (10-11). Accumulating evidence suggests that defects in apoptosis may lead to several pathologies, including some neurogenerative disorders, ischemic injury, and some forms of cancers (10).

Apoptosis is characterized by chromatin condensation, cytoplasmic blebbing, and DNA fragmentation (12-13). Nuclear alterations, the pre-eminent ultrastructural changes of apoptosis, are often associated with the internucleosomal cleavage of DNA (8), recognized as a ‘DNA ladder’ on conventional agarose gel electrophoresis and are considered to be a biochemical hallmark of apoptosis (14). Its measurement is simple and often used to determine whether a cell is apoptotic.

Caspase activity is responsible, either directly or indirectly, for the cleavage of

several intracellular proteins that are proteolyzed during apoptosis (14). The activation of caspases during this process results in the cleavage of critical cellular substrates, including poly (ADP-ribose) polymerase and lamins, thus precipitating the morphological changes in apoptosis (15).

Flavonoid intake, including anthocyanin, is reported to reduce the risk of colon cancer (16-17). Kang et al. (18) showed that tart cherry anthocyanin (cyanidin) reduced the growth of human colon cancer cell lines (HT-29). A well characterized defense mechanism may be contributing to this chemopreventive effect of flavonoids. This mechanism involves the induction of detoxification enzymes, including members of glutathione S-transferase (GST) family and NAD(P)H (quinone reductase) (QR).

The human body is constantly exposed to potential carcinogens in the environment. The body deals with these compounds through a system of xenobiotic-metabolizing enzymes, the phase-I and phase-II enzymes. Phase-I enzymes are members of the cytochrome P450 superfamily. They oxidize xenochemicals into electrophilic intermediates. These electrophilic intermediates are able to induce DNA damage and mutations. This accounts for the carcinogenic activity of many chemicals (19). Phase-II detoxification enzymes are responsible for metabolizing products of the phase-I metabolic reactions. They degrade reactive electrophilic intermediates through conjugation or reduction reactions, thereby protecting cells from oxidative DNA damage.

Glutathione-S-transferase (GST) detoxifies carcinogens and facilitates their excretion by promoting the conjugation of electrophilic compounds with glutathione and NAD(P)H (quinone reductase) (QR), another phase-II enzyme, works by catalyzing two-electron reductions on free radicals and toxic oxygen metabolites; this reduction

deactivates them and protects the surrounding tissues from mutagenesis and carcinogenesis.

More that 40 compounds from dietary sources that function as phase-II enzyme inducers have been identified (20-21). Many studies have shown that flavonoids such as anthocyanins can stimulate GST, a promising strategy for the prevention of colon cancer (22). Like GST, QR can be induced by dietary components, including the flavonoids (23).

For this study, we used moderately differentiated HT-29 colon cancer cells, which are widely used in research of colon cancer and to access the bioactivity of flavonoids (24). The objectives of the present study were the following: 1) to confirm the apoptosis caused by anthocyanin fractions extracted from four cultivars of blueberries (Tifblue, Powderblue, Brightblue, and Brightwell) using two methods: a) DNA fragmentation and b) caspase-3 activity; 2) to study the effect of anthocyanins on induction of phase-II enzymes GST and QR in cell culture as a measure of chemopreventive properties and mechanisms.

MATERIAL AND METHODS

Chemicals. Pure standards of anthocyanins were purchased from Polyphenol Laboratories (AS) (Sandnes, Norway). These standards were Dp-Glc (Delphinidin 3-O-β-glucopyranoside), Cy-Gal (Cyanidin 3-O- β -galactopyranoside, Pt-Glc (Petunidin 3-O-β -glucopyranoside), Pn-Gal (Peonidin 3-O- β -galactopyranoside), Mv-Glc (Malvidin 3-O-β-glucopyranoside). Acetone, acetonitrile, methanol, O-phosphoric acid (85% purity, HPLC grade), hydrochloric acid (analytical grade), sulfuric acid, formic acid, and water (HPLC grade) were purchased from Fisher Scientific (Norcross, GA). Caspase-3

colorimetric assay kits were purchased from Chemicon® International, Inc. (Temecula, CA). Apoptotic DNA ladder kits (Boehringer Mannheim, Roche) were purchased from Roche (Indianapolis, IN). Glutathione-S-transferase activity assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI). A BCA™ Protein assay kit was purchased from Pierce (Rockford, IL). Tween 20, FAD, NADPH, and dicoumarol, potassium chloride, Tris- HCl, monobasic sodium phosphate, dibasic sodium phosphate, and EDTA-disodium salt were purchased from Sigma (St. Louis, MO). The human hepatocellular carcinoma HT-29 cell line was purchased from ATCC (Manassas, VA).

Sample Collection. Mature blueberries were harvested from the Tifton field in 2005. The blueberry cultivars collected were Tifblue, Powderblue, Briteblue, and Britewell. The samples were frozen and stored at -40 °C until use.

Extraction and Fractionation. Anthocyanin fractions were obtained using a modified version of a procedure reported by Youdim et al. (25) and Oszmianski et al. (26). **Figure 4.1** shows a schematic diagram of the fractionation procedure. Crude extracts of blueberries were obtained through homogenization of whole blueberries in acetone: methanol: water: formic acid (40:40:20:0.1, v/v/v/v). Crude extracts were applied to an activated Oasis HLB cartridge (Waters Corporation, Milford, MA). They were washed with 15% methanol to remove the phenolic acids and then washed with acidified methanol (5% formic acid in methanol) which eluted the anthocyanins. The anthocyanin fraction was passed through a Sephadex LH20 column (Amersham Biosciences AB, Uppsala, Sweden). The column was then washed with 70% methanol acidified with 10% formic acid to elute the anthocyanins and flavonols. The LH20 column was then washed with 70% acetone to elute the tannins or procyanidins. The

anthocyanin and flavonol fraction were applied to the second Oasis HLB cartridge. The cartridge was washed with 5% formic acid, followed by ethyl acetate and then 10% formic acid in methanol. The ethyl acetate eluted the flavonols, and the acidified methanol eluted the anthocyanins. The anthocyanin fraction was collected and concentrated in rotatory evaloprator to remove the solvent at 48 ºC for 2 h and left for overnight at room temperature and freeze dried using a UNITOP 600L freeze dryer (Virtis, Gardiner, New York). Extraction and fractionation were repeated five times, and the fractions were pooled together to obtain a sufficient amount for the bioassay.

Hydrolysis. Anthocyanin fractions were hydrolyzed by dissolving in 50% methanol solution containing 2 N HCl (50 mL methanol + 33 mL water + 17 mL 37% HCl). The samples were placed in a water-bath at 80 ºC and shaken at 200 rpm for 1 h to allow for acid hydrolysis of anthocyanins to anthocyanidins.

HPLC Analysis. HPLC was performed with a Hewlett–Packard (Avondale, PA) model 1100 liquid chromatograph with quaternary pumps and a diode array UV–visible detector. The mobile phase was Solvent A, O-phosphoric acid/methanol/water (5:10:85, v/v/v) and Solvent B, acetonitrile. The flow rate was 0.5 mL/min. The gradient for the separation was a linear gradient of 100-50% for solvent A and 0-50% for solvent B over 25 min, followed by 5 min post-run with HPLC-grade water. Anthocyanin and anthocyanidin were detected at 520 nm.

Cell Culture. The human hepatocellular carcinoma HT-29 cancer cells were cultured in an ATCC McCoy's medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate (90%), and 10% fetal bovine serum. Cells were incubated at 37 ºC

with 5% CO_2 (Harris model # HWO 701T-ABA, Norwalk, CT). The medium was changed 2–3 times per week.

Induction of Apoptosis. Exponentially growing cells were harvested by centrifugation and resuspended in a fresh medium to achieve a culture density of 2×10^5 cells/mL. Apoptosis was induced with different concentrations of anthocyanins (50, 100, and 100 μg/mL) for 6 h. These cells were used for DNA fragmentation, a caspase-3 colorimetric assay, protein assay, GST and QR activity assays.

Electrophoretic Analysis of DNA Fragmentation. The untreated cells (control) and anthocyanin-treated cells (2×10^6) were harvested, washed in phosphate-buffered saline (PBS), and then lysed using lysis buffer. The samples were incubated at 15-25 °C for 10 min. The lysed sample was poured into a filter tube containing glass fiber fleece. Apoptotic DNA binds quickly to glass fiber fleece in the presence of a chaotropic salt, guanidine hydrochloride (guanidine HCl). After cellular impurities are washed off the fleece, the DNA is released from the fleece using a low salt buffer. The DNA quantification was done at 260 nm using UV-visible spectrophotometer (Shimadzu UV-1601, Norcross, GA). The DNA samples were electrophoresed at 100V for 1 h in 1.5% (w/v) agarose gels (Sigma) complemented with ethidium bromide (1 μg/mL, Sigma). Separated DNA fragments (DNA ladders) were visualized using UV transilluminator (254 nm, Ultra-Lum Electronic UV Transilluminator, Claremont, CA).

Caspase-3 Colorimetry Assay. The untreated cells (control) and anthocyanin-treated cells were harvested (2×10^6), washed in PBS, and centrifuged at 1500 rpm for 10 min. The pellet was resuspended in lysis buffer and incubated at room temperature for 10 min. After incubation, the samples were centrifuged for 5 min in a microcentrifuge

(10,000 × g). The cytosol collected was used for protein analysis and for caspase-3 activity. The assay mixture was prepared in a 96-well plate using cytosol from treated cells and untreated cells and a caspase-3 substrate. The plate was incubated at 37 ºC for 1 h, and the samples were read at 415 nm using Bio-Rad Model 680 Microplate Reader (Hercules, CA). Increases in caspase-3 activity were determined by comparing the OD reading from the induced apoptotic sample with the OD reading of the uninduced control.

Cell Preparation for Enzyme Activity Assays. Following trypsinization and harvesting, the cell suspension was centrifuged at 750 × g for 5 min (Beckman T-J6; Palo Alto, CA). The supernatant was discarded. The cells were resuspended in 2 ml PBS and centrifuged again at 750 × g for 5 min. The cells were then disrupted using a sonicator (Branson Sonifier 450, St. Louis, MO) for 30 sec at 20% power. The homogenate was combined with an equal amount of homogenizing buffer and centrifuged (Beckman J2HS, JS-7.5 swinging bucket rotor; Palo Alto, CA) at 10,000 × g for 20 min at 4 °C. The supernatant was transferred to a polycarbonyl centrifuge tube, and the weight-matched tubes were ultracentrifuged (Beckman Optima LE-80K Ultracentrifuge; Palo Alto, CA) at 100,000 × g for 1 h and 10 min at 4°C. The supernatant (cytosol) was divided into 3 tubes and frozen at –80°C until analysis.

Protein Assay. The amount of protein in the cells was measured using a BCA protein assay kit at 590 nm with a Bio-Rad Model 680 Microplate Reader (Hercules, CA). Briefly, this method combines the reduction of Cu^{++} to Cu^{+} using a protein in alkaline media with a highly sensitive and selective colorimetric detection of Cu^{+} using bicinchoninic acid. Enzyme activities for GST and QR were expressed per mg protein. All samples were run in duplicate.

Quinone Reductase Assay. Quinone reductase (QR) activity was also measured in the cytosol using methods described by Kore et al. (27) with 12 mmol/L 2,6-dichloroindophenol as the substrate (DPIP). QR activity was measured in triplicate with a spectophotometer (Beckman DU 650, Beckman Instruments Inc., Fullerton, CA). The total volume of the cuvette reached 1 mL. The contents included 0.06 to 0.28 mg cytosol protein incubated with 25 mmol/L Tris-HCl (pH 7.4), 0.7 mg bovine serum albumin, 1% Tween 20, 5 mol/L FAD, and 0.2 mmol/L NADPH and 0 or 10 mol/L dicoumarol at 25 °C. DPIP (40 mol/L) was added to initiate the reaction. The reduction of DPIP was measured at 600 nm every 15 sec for 3 min. The dicoumarol sensitive portion of the activity was taken as a measure of QR activity. The molar extinction coefficient for DPIP, at 600 nm, was 2.1 nmol/mL.

Glutathione-S-Transferase Assay. Glutathione-S-transferase (GST) activity was measured using 10 mM 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. An aliquot of cytosolic sample, potassium-phosphate buffer (0.1 mol/L), and the reduced form of glutathione (GSH; MW = 307.3) were added to each microplate well. To initiate the assay, 5.0 μL CDNB was added to each well. The rate of change in absorbance for each sample was read at 340 nm using a Bio-Rad Model 680 Microplate Reader (Hercules, CA). This assay indirectly measures enzyme activity by measuring the conjugation of CDNB with glutathione by GST. All samples were run in triplicate and average values reported.

Statistical Analysis. Statistical analysis was done with the SAS software package (28). One-way analysis of variance (ANOVA) was performed to determine differences in enzyme activity. When F values for the ANOVA were significant, differences in means

were determined using Duncan's multiple range tests as a procedure for mean separation ($P < 0.05$).

RESULTS AND DISCUSSION

Initial Analysis. The contents of individual anthocyanidins of frozen blueberries are given in **Table 4.1.** The major anthocyanidin found in all the cultivers (Tifblue, Powderblue, Brightblue, and Brightwell) was malvidin followed by peonidin > cyanidin > delphinidin > petunidin. Major anthocyanidins in four cultivars (Tifblue, Powderblue, Brightblue, and Brightwell) are shown in **Table 4.1**. The number of different anthocyanidins is expressed based on the specific weight of the anthocyanins, including delphinidin, cyanidin, petunidin, peonidin, and malvidin, because most of the anthocyanins in blueberry are monoglycosides (i.e., galactosides, glucosides or arabinosides) (29). HPLC chromatogram of the different cultivars is shown in **Figure 4.2 (A)**, **(B)**, **(C)**, and **(D)**. Malvidin was found to be the predominant anthocyanidin in all the cultivars, but Brightwell had peonidin as predominant anthocyanidin. The content of malvidin was 39.0, 38.0, 43.1, and 17.5 mg/100 g of frozen blueberry in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. Peonidin was the second largest anthocyanidin. The total content of peonidin (Pn-glc, Pn-gal, and Pn-ara) was 31.5, 28.0, 35.0, and 50.5 mg/100 g of frozen blueberry in Tifblue, Powderblue, Brightblue, and Brightwell respectively. There are not many reports available for anthocyanin content of the above cultivars; however malvidin was reported as predominant anthocyanidins in many blueberry varieties (30-31). Major anthocyanidins in the anthocyanin fractions of four cultivars (Tifblue, Powderblue, Brightblue, and Brightwell) are shown in **Table 4.2**. HPLC chromatogram of the different cultivars is shown in **Figure 4.3 (A)**, **(B)**, **(C)**, and

(D). Malvidin was found to be the predominant anthocyanidin in all the cultivars, but Brightwell had peonidin as predominant anthocyanidin. The content of malvidin was 40.5, 44.4, 44.4, and 20.0 mg/100 mg of anthocyanin fraction in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. Peonidin was the second largest anthocyanidin. The total content of peonidin (Pn-glc, Pn-gal, and Pn-ara) was 23.5, 25.2, 23.4, and 35.8 mg/100 mg of anthocyanin fraction in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. There was considerable loss in delphinidin content during extraction. There were certain unidentified peaks not considered for calculation. Delphinidin and cyanidin were lower in concentration than previously reported (32-33). These variations may be due to the fractionation procedure used or storage conditions or handling of raw material or due to environmental factors such as light, temperature, agronomic practices, and various stresses.

DNA Fragmentation. The induction of apoptosis in tumor cells has been shown to be the most common anti-cancer mechanism of many cancer therapies; therefore, finding potential therapeutic anti-tumor compounds with potent and selective apoptotic effects would be valuable (34). DNA fragmentation is a primary physiological characteristic of apoptosis and a relatively late event in apoptosis. Following agarose gel electrophoresis of HT-29 cells treated with anthocyanins from different cultivars, a typical ladder pattern of internucleosomal fragmentation was observed. **Figure 4.4** shows the DNA fragmentation in cells undergoing apoptosis. The characteristic cleavage of DNA into oligonucleosome fragments can be seen as DNA laddering. In all cultivars DNA fragmentation increased at anthocyanin concentrations of 50 to 150 μg/mL, but cells treated with anthocyanin fraction of Brighwell showed prominent ladder at 50 to

100 μg/mL when compared to cells treated with 150 μg/mL. Necrosis may be the predominant process at the highest anthocyanin concentrations, with fewer cells undergoing apoptosis. Peonidin was the predominant anthocyanidin in Brightwell; remaining cultivars had malvidin as predominant anthocyanidin. Some, but not all researchers have reported that malvidin stimulates apoptosis in HL-60 cells (35). In contrast to our results, a few researchers reported that malvidin is unable to induce apoptosis due to absence of dihydroxyphenyl structure on the B-ring of malvidin (36-37). The anthocyanin fraction in the current study contained malvidin as predominant anthocyanidin in anthocyanin fraction from all cultivars. Thus, it is possible that malvidin is an inducer for apoptosis. However, the molecular mechanism for this is not clear.

Caspase-3 Activity. Caspase-3 exists as an inactive pro-caspase-3 in the cytoplasm, is proteolytically activated by multiple cleavages of pro-caspase-3 to generate the cleave fragments in cells undergoing apoptosis. **Figure 4.4** shows the significant increase ($P < 0.05$) in caspase-3 activity in treated cells compared to control. There was a significant effect ($P < 0.05$) of anthocyanin on caspase-3 activity with a response that was dose dependent. The highest activity was observed in cells treated with the 150 μg/mL anthocyanin fraction from the Tifblue, Powderblue, and Brightblue cultivars. Cells treated with the Brightblue cultivar anthocyanin had less increase in caspase-3 activity at the 150 μg/mL concentration. The lowest response was observed with Brightblue anthocyanin. Similar results were reported in HT-29 cells when treated with natural and synthetic flavonoids (38).

Detoxifying Enzymes. Induction of the detoxification enzymes QR and GST is a well-characterized defense mechanism against carcinogens. In principle, the elevation of

these enzymes can reduce carcinogenesis due to an enhanced removal of reactive electrophiles.

Quinone Reductase. QR is one of several enzymes that inactivate electrophilic carcinogens, providing a mechanism for the inhibition of carcinogenesis. **Figure 4.6** shows QR activity in cells treated with anthocyanin fractions from different cultivars and control. QR was not increased by the addition of 50-150 μg/mL anthocyanin fractions from any cultivar. Further QR activity decreased when treated with 100-150 μg/mL anthocyanin fractions from Tifblue and Powderblue cultivars. The trend appeared to be a dose dependent relationship. Similar results have been reported by Ramanathan et al. (39). Bomser et al. (40) reported that crude extract of blueberries inhibit QR activity and ethylacetate extracts induce QR activity. Several researchers have suggested that enzyme activity is dependent on flavonoid structure (41-42). The presence of double bond in heterocyclic ring (C) linking position 2 and 3 **(Figure 4.5**) is required for QR induction capability (42), e.g., kaempferol and quercetin. The absence of double bond in C ring in anthocyanins may have contributed to low QR activity. However, the exact mechanism is still unclear.

Glutathione-S-transferase Activity. GST catalyzes reaction with the glutathione (GSH), thereby neutralizing electrophilic sites on carcinogens and rendering the products more water soluble. **Figure 4.7** shows GST activity in cells treated with anthocyanin fractions from different cultivars and control. There was statistically significant reduction in the activity of GST in treated cells when compared with control ($P < 0.05$). There was a dose-response relationship observed with all the cultivars. Our results suggest no induction in GST activity due to anthocyanins. Similar results were reported by earlier

researchers (43-44). The effect on enzyme activity is correlated with the structure of flavonoid and specific tissue (45). The GST activity was significantly induced in heart tisues but no activity was found in colon tissue cells (45). The attachment of the B-ring to C-2 position and a double bond between C-2 and C-3 may be contributing to the activity of flavonoids (47). The structure of anthocyanin may have contributed to low activity.

Our results show that anthocyanin compounds may not induce detoxification enzymes in colon carcinoma cells over the same concentration ranges that increase apoptosis. Anthocyanins from four cultivars Tifblue, Powderblue, Brightblue, and Brightwell were able to induce apoptosis in a dose-response manner. However, there was no increase observed in QR and GST activities. Instead, there was a decline in the activity of detoxifying enzymes when compared with control.

ACKNOWLEDGMENTS

This research study was funded by the State of Georgia's Traditional Industries Program for Food Processing Research Grant. The authors would like to thank the Georgia Blueberry Growers Association for their support.

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Table-4.1. Individual Anthocyanidins in Frozen Blueberries

	frozen blueberries (mg/100 g of fruit weight)							
	Dp-Glc	Cy-Glc	Cy-Gal	Pn-Glc	Pn-gal	Pt-Glc	Pn-Ara	Mv-Glc
Tifblue	8.5±0.7	5.5±0.7	18.0±2.8	6.0±1.4	10.0±1.4	6.5±0.7	15.5±0.7	39.0±2.8
Powderblue	9.0±0.0	4.5±0.7	12.5±2.1	5.5±2.1	9.5±2.1	5.5±2.1	14.0±1.4	38.0±5.7
Brightblue	8.5±0.6	7.5±0.7	10.5±2.1	7.5±2.1	13.3±2.1	3.8±2.1	14.0±1.4	43.1.0±5.7
Brightwell	15.5±2.1	4.5±3.5	16.1±2.6	12.8±2.1	8.0±0.0	5.0±1.4	30.0±2.8	17.5±0.7

Abbreviations: Dp-Glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-Glc (Cyanidin 3-*O*-β-glucopyranoside), Cy-Gal (Cyanidin 3-*O*-β-galactopyranoside), Pn-Glc (Peonidin 3-*O*-β-glucopyranoside), Pn-Gal (Peonidin 3-*O*-β-galactopyranoside), Pt-Glc (Petunidin 3-*O*-β-glucopyranoside), Pn-Ara (Peonidin 3-*O*-α-arabinopyranoside), Mv-Glc (Malvidin 3-*O*-β-glucopyranoside).
Each value was expressed as mean±SD, n=3

Table-4.2 Individual Anthocyanidins in Anthocyanin Fraction of Different Cultivars of Frozen Blueberries

	[a]Anthocyanin fraction								
	Dp-Glc	Cy-Glc	Cy-Gal	Pn-Glc	Pn-Gal	Pt-Glc	Pn-Ara	Mv-Glc	Total
Tifblue	1.8±0.3	2.1±2.1	13.5±2.1	3.5±0.7	6.0±1.3	6.1±0.1	14.0±1.5	40.5±1.3	87.4±0.1
Powderblue	2.6±0.8	2.0±0.1	7.7±1.2	3.7±0.6	8.2±0.3	4.7±0.6	13.3±0.9	44.4±1.9	86.0±0.1
Brightblue	4.6±0.4	3.7±0.1	6.7±1.7	4.3±0.6	8.5±0.8	2.0±0.1	10.6±1.0	44.4±0.9	87.7±0.4
Brightwell	8.0±0.1	2.2±1.3	9.4±0.9	6.9±2.6	6.3±1.1	4.7±0.4	21.8±1.9	20.0±1.0	79.2±0.4

Abbreviations: [a] each anthocyanidins are expressed in mg/100 mg of anthocyanin fraction.
Dp-Glc (Delphinidin 3-*O*-β-glucopyranoside), Cy-Glc (Cyanidin 3-*O*-β-glucopyranoside), Cy-Gal (Cyanidin 3-*O*-β-galactopyranoside), Pn-Glc (Peonidin 3-*O*-β-glucopyranoside), Pn-Gal (Peonidin 3-*O*-β-galactopyranoside), Pt-Glc (Petunidin 3-*O*-β-glucopyranoside), Pn-Ara (Peonidin 3-*O*-α-arabinopyranoside), Mv-Glc (Malvidin 3-*O*-β-glucopyranoside).
Each value was expressed as mean±SD, n=3.

Figure 4.1 Schematic diagram of separation of anthocyanin fraction from frozen blueberries. Abbreviations; Acet=acetone, MeOH=methanol, FA=formic acid (33)

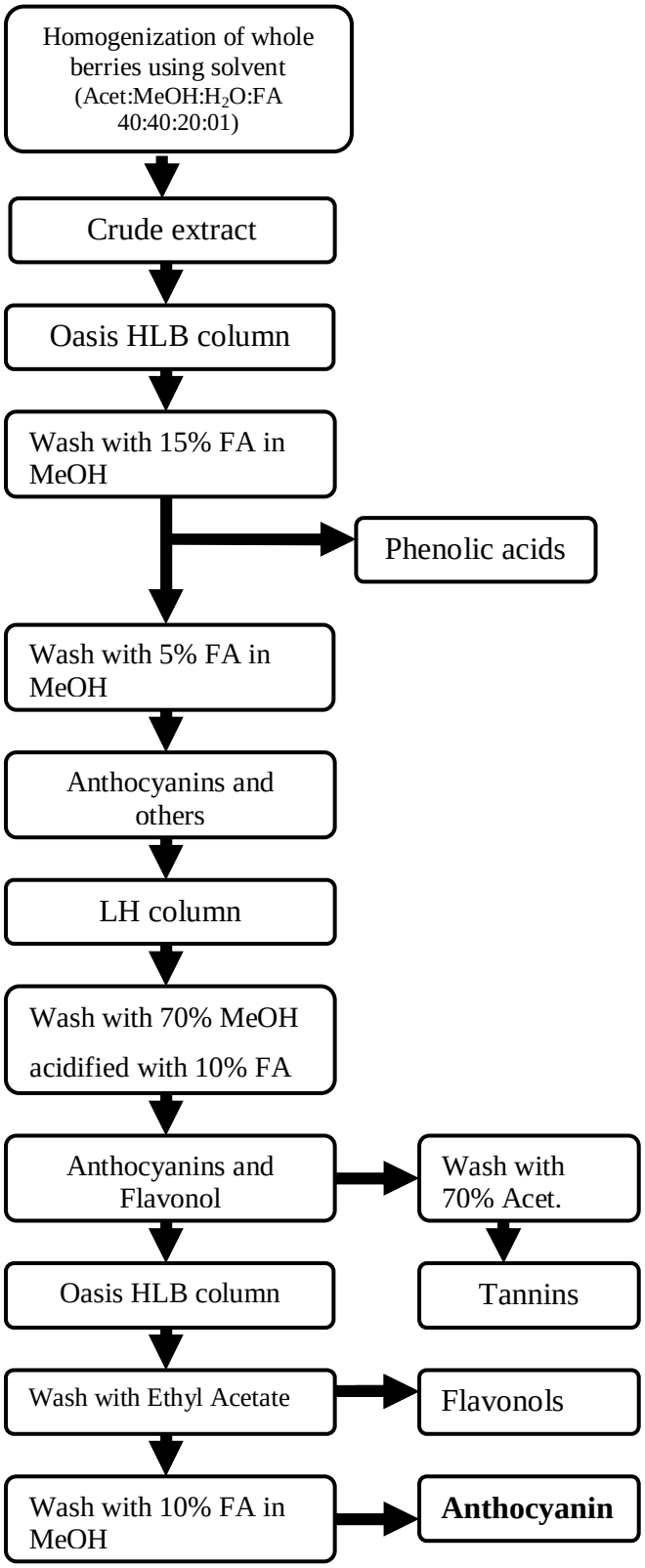

Homogenization of whole berries using solvent (Acet:MeOH:H_2O:FA 40:40:20:01)
Crude extract
Oasis HLB column
Wash with 15% FA in MeOH
Phenolic acids
Wash with 5% FA in MeOH
Anthocyanins and others
LH column
Wash with 70% MeOH acidified with 10% FA
Anthocyanins and Flavonol
Wash with 70% Acet.
Tannins
Oasis HLB column
Wash with Ethyl Acetate
Flavonols
Wash with 10% FA in MeOH
Anthocyanin

Figure 4.2 Analytical HPLC chromatogram of individual anthocyanidins in selected cultivars of frozen blueberries: (A) Tifblue, (B) Powderblue, (C) Brightblue, and D) Brightwell.

X-axis is time (min) and Y-axis is mAU.

Peaks: 1=Dp-Glc, 2=Cy-Glc, 3=Cy-Gal, 4= Pt-Glc, 5=Pn-Glc, 6=Pn-Gal, 7= Pn-Ara, and 8=Mv-Glc, a and b = unidentified peak

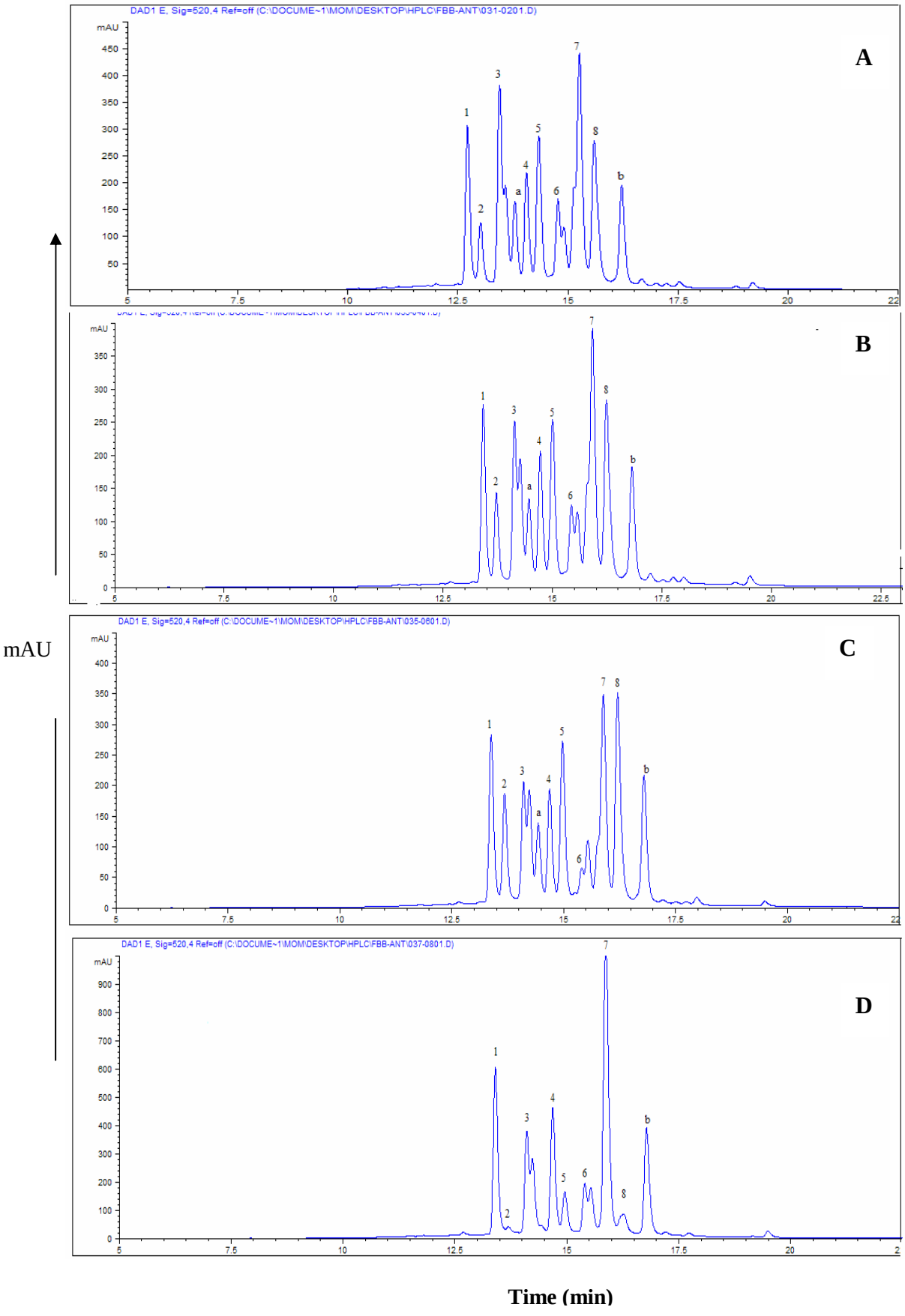

DAD1 E, Sig=520,4 Ref=off (C:\DOCUME~1\MOM\DESKTOP\HPLC\FBB-ANT\031-0201.D)
A
DAD1 E, Sig=520,4 Ref=off (C:\DOCUME~1\MOM\DESKTOP\HPLC\FBB-ANT\035-0601.D)
B
DAD1 E, Sig=520,4 Ref=off (C:\DOCUME~1\MOM\DESKTOP\HPLC\FBB-ANT\035-0801.D)
C
DAD1 E, Sig=520,4 Ref=off (C:\DOCUME~1\MOM\DESKTOP\HPLC\FBB-ANT\037-0801.D)
D
mAU
Time (min)

Figure 4.3 Analytical HPLC chromatogram of individual anthocyanidins in anthocyanin fraction of selected cultivars of frozen Blueberries (A) Tifblue fraction; (B) Powderblue fraction; (C) Brightblue fraction; (D) Brightwell fraction.

X-axis is time (min) and Y-axis is mAU.

Peaks: 1=Dp-Glc, 2=Cy-Glc, 3=Cy-Gal, 4= Pt-Glc, 5=Pn-Glc, 6=Pn-Gal, 7= Pn-Ara, and 8=Mv-Glc, a and b = unidentified peak

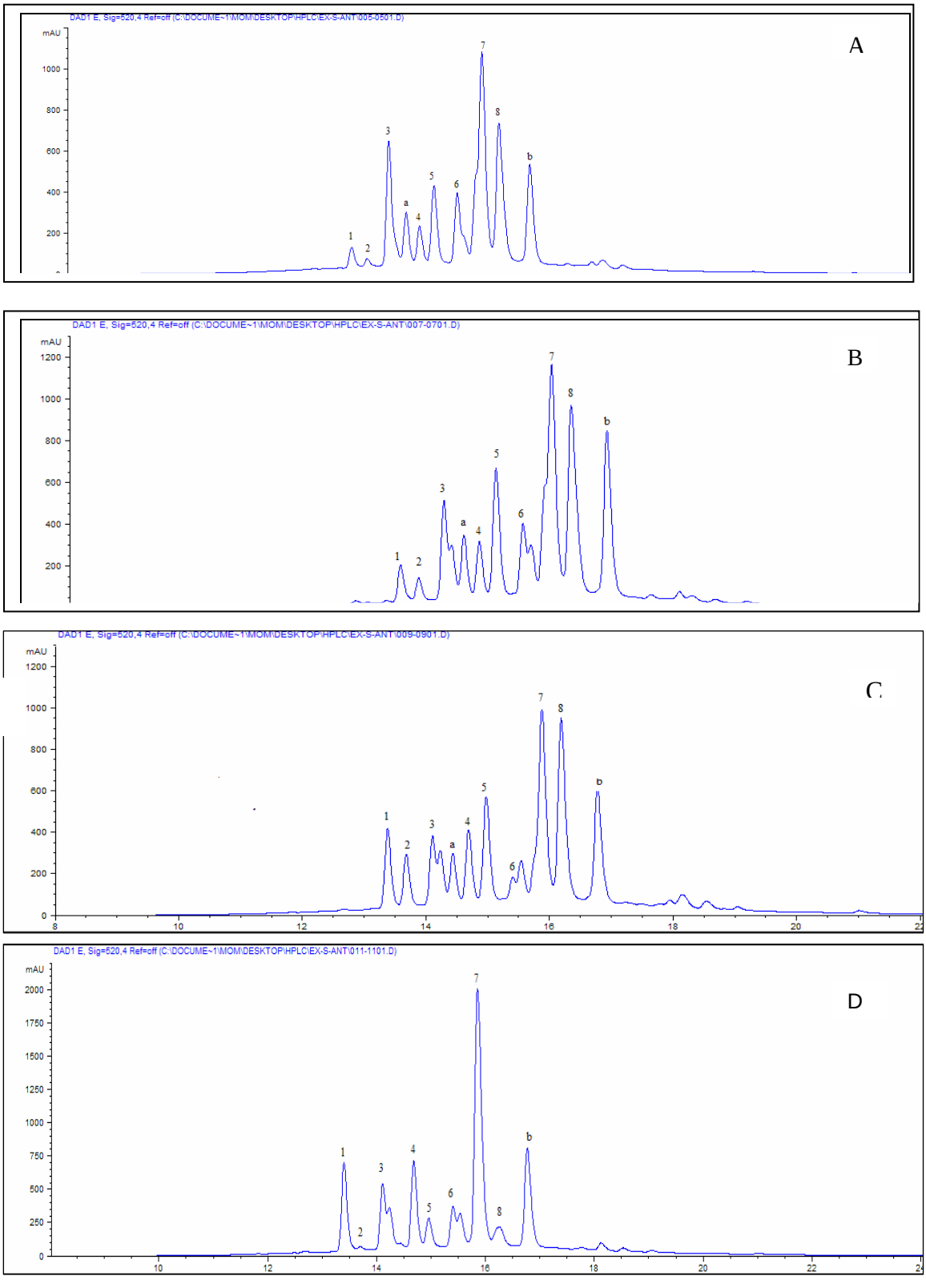
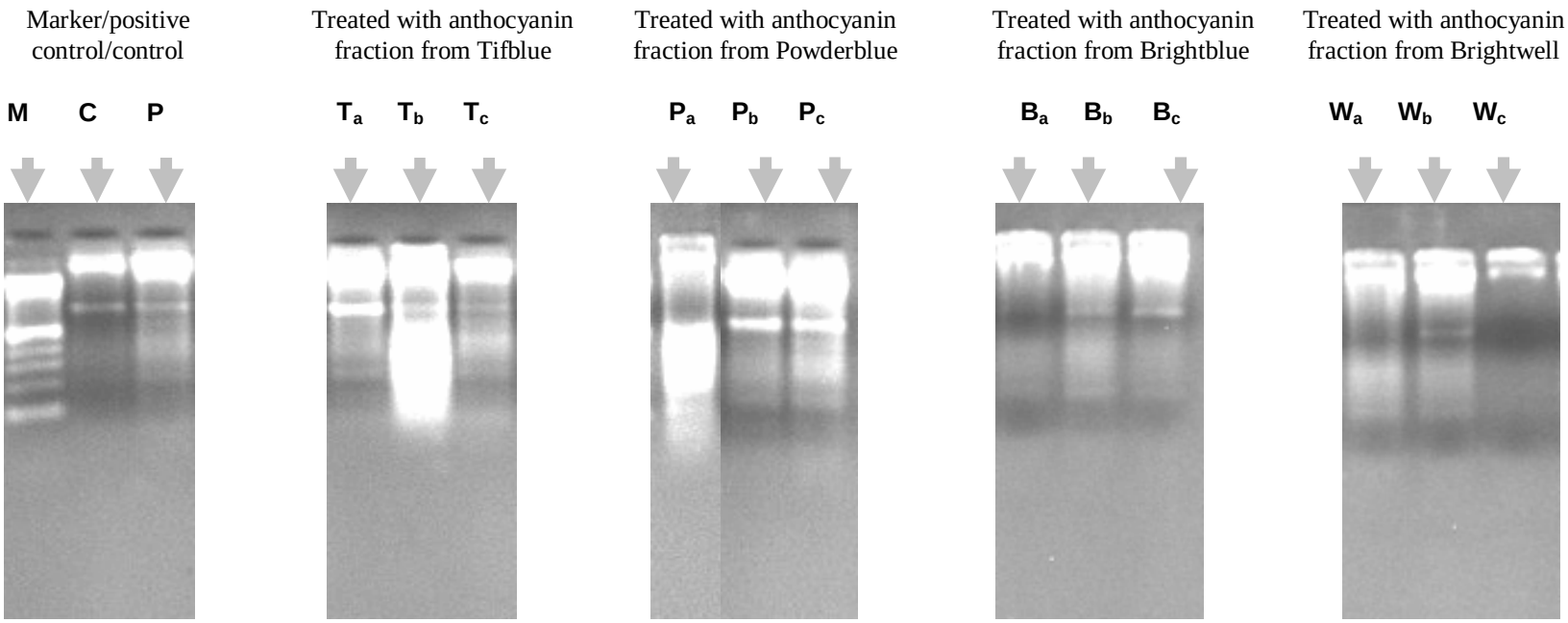

DAD1 E, Sig=520,4 Ref=off (C:\DOCUME~1\MOM\DESKTOP\HPLC\EX-S-ANT\005-0501.D)
A
DAD1 E, Sig=520,4 Ref=off (C:\DOCUME~1\MOM\DESKTOP\HPLC\EX-S-ANT\007-0701.D)
B
DAD1 E, Sig=520,4 Ref=off (C:\DOCUME~1\MOM\DESKTOP\HPLC\EX-S-ANT\009-0901.D)
C
DAD1 E, Sig=520,4 Ref=off (C:\DOCUME~1\MOM\DESKTOP\HPLC\EX-S-ANT\011-1101.D)
D
mAU
Time (min)

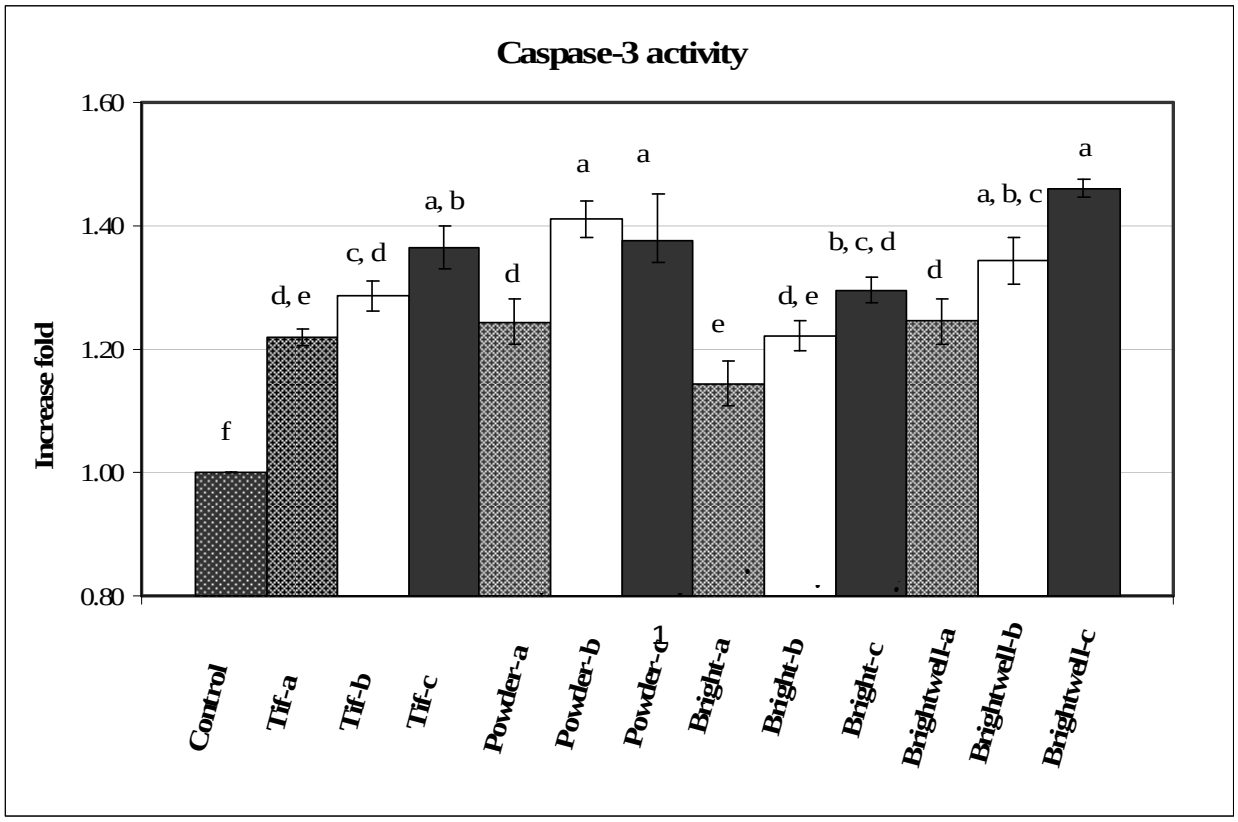
Figure 4.4 Apoptotic DNA fragmentation in HT-29 cells after 6 h treatment of anthocyanin fractions from four cultivars of blueberries: Tifblue, Powderblue, Brightblue, and Brightwell

.



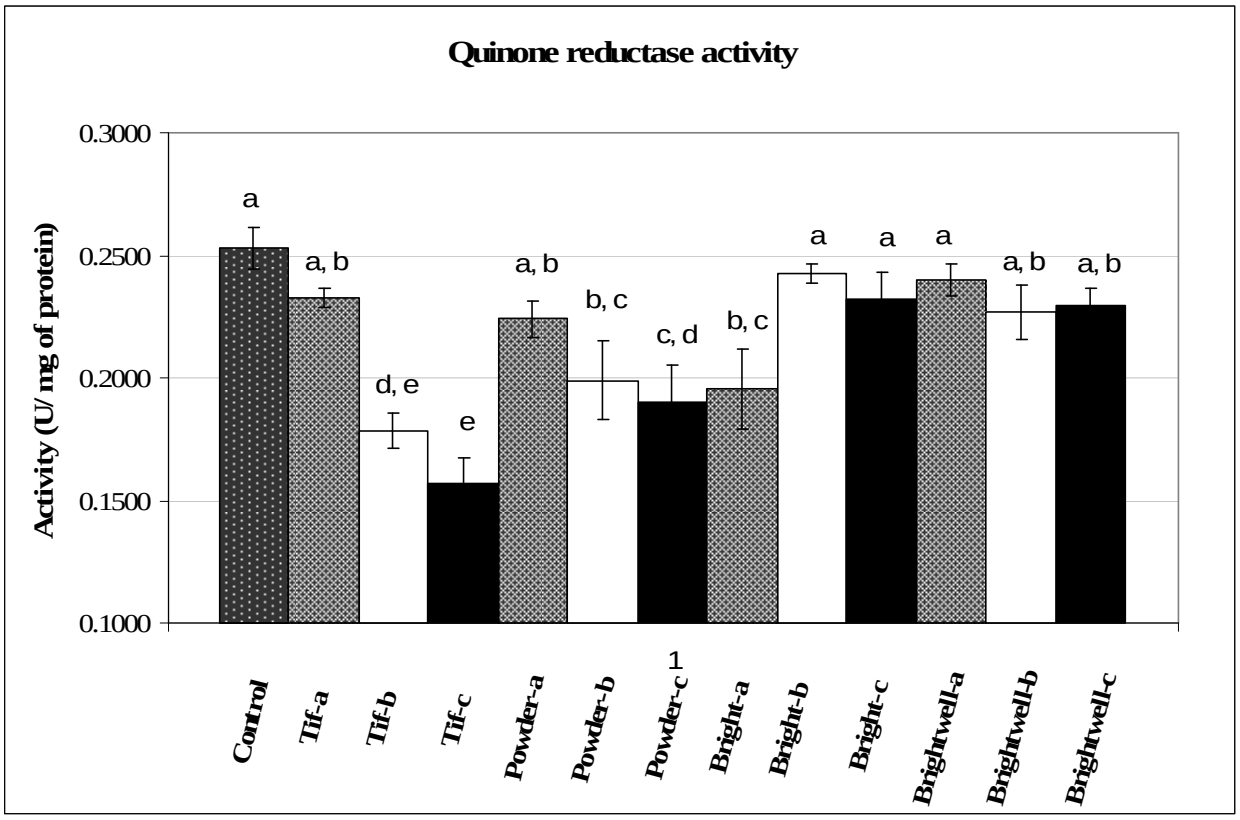
Concentrations of anthocyanins used were 50, 100, and 150 µg/mL of medium. Abbreviations: M=DNA marker, C=Control, P=Positive control, T_a, T_b, T_c=DNA from cells treated with Tifblue anthocyanin concentration of 50, 100, and 150 µg/mL of medium, respectively. P_a, P_b, P_c= DNA from cells treated with Powderblue anthocyanin concentration of 50, 100, and 150 µg/mL of medium, respectively, B_a, B_b, B_c= DNA from cells treated with Brightblue anthocyanin concentration of 50, 100, and 150 µg/mL of medium, respectively, and W_a, W_b, W_c= DNA from cells treated with Brightwell anthocyanin concentration of 50, 100, and 150 µg/mL of medium, respectively.

Figure 4.5. Caspase-3 activity in HT-29 cells after 6 h treatment of anthocyanin fractions from four cultivars of blueberries: Tifblue, Powderblue, Brightblue, and Brightwell.



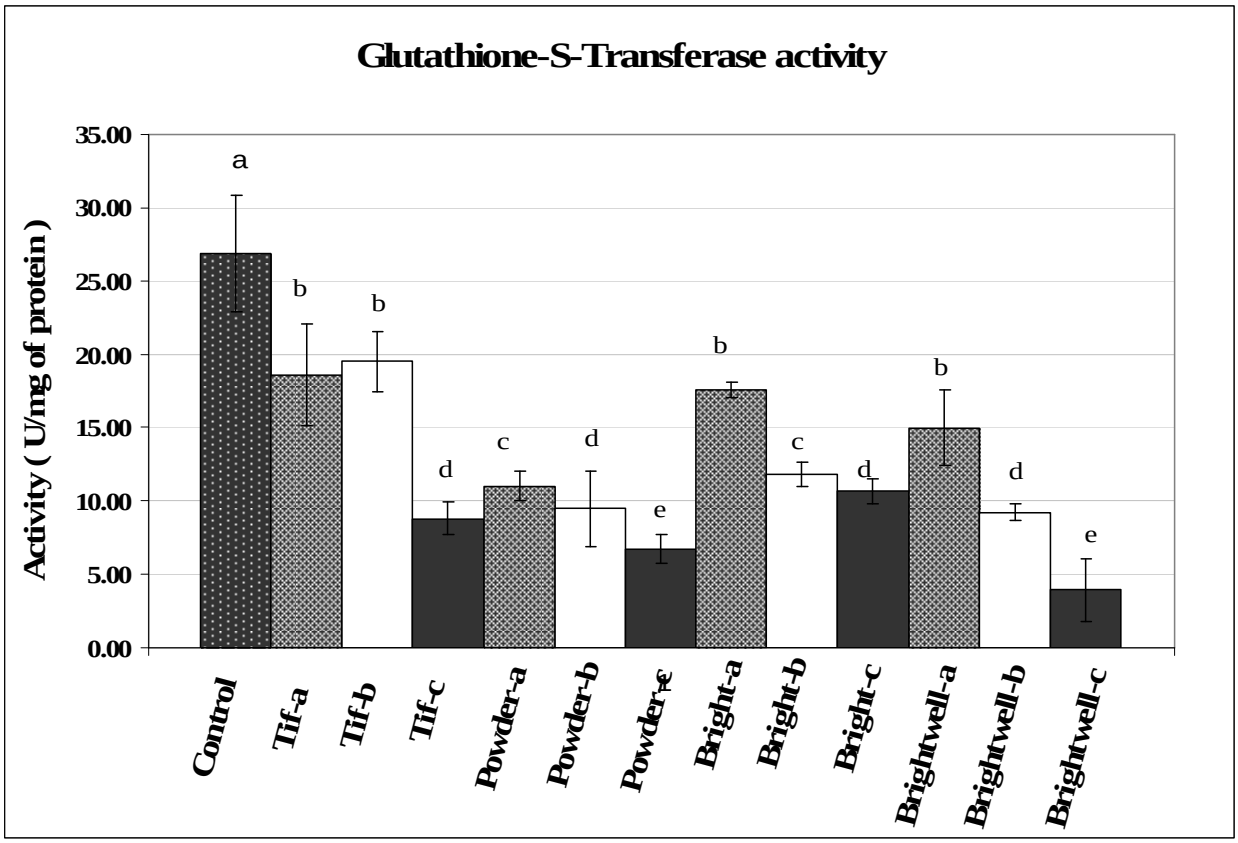
Concentrations of anthocyanins used were 50, 100, and 150 µg/mL of medium. The Y-axis represents the fold increase in caspase-3 activity compared to control. Abbreviations: Tif-a, b, and c= Cells treated with Tifblue anthocyanin concentration of 50, 100, and 150 µg/mL of medium, respectively. Powder-a, b, and c=Cells treated with Powderblue anthocyanin concentration of 50, 100, and 150 µg/mL of medium, respectively Bright- a, b, and c=Cells treated with Brightblue anthocyanin concentration of 50, 100, and 150 µg/mL of medium, respectively. Brightwell- a, b, and c=Cells treated with Brightwell anthocyanin concentration of 50, 100, and 150 µg/mL of medium, respectively.

Figure 4.6 Quinone reductase (QR) activity in HT-29 cells after 6 h treatment with anthocyanin fractions from four cultivars of blueberries: Tifblue, Powderblue, Brightblue, and Brightwell.



Concentrations of anthocyanins used were 50, 100, and 150 µg/mL of medium. The Y-axis represents QR activity (U/mg of protein). See figure 4.5 for abbreviations

Figure 4.7 Glutathione-S-transferase (GST) in HT-29 cells after 6 h treatment with anthocyanin fractions from four cultivars of blueberries: Tifblue, Powderblue, Brightblue and Brightwel



Concentrations of anthocyanins used were 50, 100, and 150 µg/mL of medium. The Y-axis represents GST activity (U/mg of protein). See figure 4.5 for abbreviations

CHAPTER 5

SUMMARY AND CONCLUSIONS

Blueberries are a good source of phenolic compounds, mainly flavonoids including anthocyanins, which are known to have potential health benefits. When blueberries are processed, phenolic compounds undergo various changes due to processing (thermal, mechanical and chemical). Stability of these compounds is also affected by various storage conditions such as light and temperature. We analyzed total polyphenols (TPP), total anthocyanin (TACY), Trolox-equivalent antioxidant capacity (TEAC), phenolic acids, flavonols, individual anthocyanins, and cell proliferation during storage, as a measure of antioxidant activity of blueberry extract.

After pressing of extract, recovery of TPP, TACY, and TEAC in blueberry extract, were ~25, ~29, and ~69%, respectively, for both cultivars. Recovery of gallic acid, catechin and quercetin was ~25 % in final extract. Ferulic acid was not detected in the final extract in both Tifblue and Powderblue cultivars. Recovery of peonidin, malvidin, and cyanidin was ~20% in final extract in both cultivars. Storage of extract for 60 days affected the phenolic compounds under all temperature conditions. Similar results were obtained for Tifblue and Powderblue cultivars. At -20±1 ºC, no statistically significant loss of TPP, TACY and TEAC was observed up to 30 days ($P < 0.05$). At 6±1 ºC storage, there was a significant loss of TPP, TACY and TEAC observed from 15 to 30 days. Similar results were obtained at 23±1 ºC and 35±1 ºC ($P < 0.05$). There was retention of more than 40% of ellagic and quercetin after 60 days at 35±1 ^{o}C.

Anthocyanins were not detected after 60 days of storage at 35±1 ^{o}C temperature conditions. Significant retention ($P < 0.05$) was observed for malvidin (42.8 and 25.8%) and peonidin (74.09 and 79.5%) after 60 days at 23±1 ^{o}C in glass bottles for Tifblue and Powderblue, respectively, when compared with other individual anthocyanins. A linear relationship was observed between TEAC values and total polyphenols and total anthocyanins.

Cell viability assay was performed using HT-29 cancer cell line and anthocyanins extracted from 30, 60, and 90 days stored extract at 6±1 and 23±1 ^{o}C. Significant cell proliferation inhibition percentage was observed in 30 days, although this was reduced significantly after 30-90 days. These results suggest that initial preparatory steps like washing, removal of residue mainly skin, heating and storage conditions were significantly affecting the phenolic compounds and their biological activity. Frozen and low temperature storage is suggested for blueberry extract in order to retain the bioactive components.

Human carcinoma cells, HT-29, were treated with anthocyanin extracts from different cultivars of Georgia-grown blueberries. Initial analysis of anthocyanins revealed that the major anthocyanidins identified were delphinidin, cyanidin, peonidin, petunidin, and malvidin. Malvidin was found to be the predominant anthocyanidin in all the cultivars, but Brightwell had peonidin as predominant anthocyanidin. The content of malvidin was 39.0, 38.0, 43.1, and 17.5 mg/100 g of frozen blueberry in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. Peonidin was the second largest anthocyanidin. The total content of peonidin was 31.5, 28.0, 35.0, and 50.5 mg/100 g of frozen blueberry in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. Major anthocyanidins in the anthocyanin fractions of four cultivars (Tifblue, Powderblue, Brightblue, and Brightwell) were delphinidin, cyanidin, peonidin, petunidin, and malvidin. Malvidin was found to be the predominant anthocyanidin in all the cultivars, but

Brightwell had peonidin as predominant anthocyanidin. The content of malvidin was 40.5, 44.4, 44.4, and 20.0 mg/100 mg of anthocyanin fraction in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. Peonidin was the second largest anthocyanidin. The total content of peonidin was 23.5, 25.2, 23.4, and 35.8 mg/100 mg of anthocyanin fraction in Tifblue, Powderblue, Brightblue, and Brightwell, respectively. There was considerable loss of delphinidin during extraction. DNA fragmentation and increase in caspase-3 activity in treated cells compared to the control confirmed the apoptosis. There was a significant difference in the caspase-3 activity ($P < 0.05$) between the control cells and the cells treated with anthocyanins from all the cultivars**.** Response correlated positively with dose. Highest activity (1.4 fold increase over control) was observed in cells treated with 150 μg/mL anthocyanin fraction from the brightwell cultivar.

There was no induction observed in quinone reductase and glutathione-S-transferase activity when cells were treated with anthocyanins. Contrary to this, activity decreased gradually when treated with increased concentrations of anthocyanin fractions (50-150 μg/mL) from the Tifblue and Powderblue cultivars. A positive dose-response relationship was found in all the cultivars except Brightblue, where activity was the same for all three concentrations. GST activity was statistically higher ($P < 0.05$) in control cells than in cells treated with anthocyanin fractions from all the cultivars and at all levels of concentration. These results indicated no induction of detoxifying enzymes; however, apoptosis was confirmed in HT-29 cells when treated with anthocyanins consisting predominantly of malvidin.

SUGGESTIONS FOR FUTURE WORK

Blueberry extract was affected by storage conditions, however there was drastic reduction in TPP, TACY and TEAC after initial steps of preparation of extract (washing, removal of

residue, and heating). These steps are also followed during juice preparation; therefore similar results are anticipated during preparation and storage of blueberry juice. However systematic studies are suggested in blueberry juice using various processing treatments and commonly used packaging materials. Anthocyanins are responsible for color of the blueberry extract. However due to poor stability, usage of anthocyanin is limited. It is suggested that a suitable method for extraction and encapsulation of anthocyanins with maximum purity be developed. There should be minimum loss of anthocyanidin, mainly delphinidin and cyanidin, in the fraction.

Total polyphenol and anthocyanins contents were reduced after processing but similar effect was not observed in TEAC. This suggests there are certain other antioxidants present in the blueberry, in addition to the phenolic acids and flavonoids standards used. Identification and characterization is required for these compounds by more sensitive and sophisticated instruments.

In vitro studies have confirmed apoptosis by anthocyanins, which is a single cell environment. Therefore *in vivo* studies are required to further confirm the process under complex biological environment.