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Examination of blueberry anthocyanins in prevention of age-related macular degeneration through retinal pigment epithelial cell culture study

Naomi Marie Sundalius
Louisiana State University and Agricultural and Mechanical College, nomie19251@hotmail.com

EXAMINATION OF BLUEBERRY ANTHOCYANINS IN PREVENTION OF AGE-RELATED MACULAR DEGENERATION THROUGH RETINAL PIGMENT EPITHELIAL CELL CULTURE STUDY

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DEDICATION

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ABSTRACT

This research investigated the ability of blueberry anthocyanins to inhibit the uptake of *N*-retinyl-*N*-retinylidene ethanolamine (A2E) by retinal pigment epithelial (RPE) cells as quantified by pigment epithelial derived factor (PEDF) levels in RPE cells. The A2E was synthesized and identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Freeze dried blueberry powder of 50/50 blend (Tifblue/Rubel) anthocyanins were extracted by acetone and concentrated through chromatography. A RPE cell culture study investigating four treatments using a combination of 100 µM A2E in DMSO and 100 µg of blueberry anthocyanins in DMSO was tested. The treatments included: (A) control RPE cells; (B) RPE cells incubated with A2E for 2 hours with blue light illumination for 20 minutes; (C) RPE cells incubated with blueberry anthocyanins for 24 hours and incubated with A2E for 2 hours with blue light illumination for 20 minutes; and (D) RPE cells incubated with blueberry anthocyanins for 24 hours, then incubated with A2E for 2 hours with blue light illumination for 20 minutes and then treated with blueberry anthocyanins again. All treatments were incubated for seven days at 37°C with 5% CO_2 and samples were collected on days 1, 3, 5 and 7. The supernatant of the RPE cells were collected and the protein concentration determined and normalized. The PEDF values were determined using a PEDF enzyme linked immunosorbant assay (ELISA). The average PEDF results indicated that blueberry anthocyanins promoted and maintained PEDF levels when compared to control RPE cells and RPE cells treated with A2E for 1, 3 and 5 collection days. Treatments C and D had significantly ($p<0.05$) higher PEDF levels of 10.17 and 10.14 ng PEDF per mg protein than treatment B of 5.750 ng PEDF per mg protein for day 1. Treatment D had a significantly ($p<0.05$) higher PEDF level of 13.76 ng PEDF per mg protein than the other treatments at day 3 and was significantly higher at 12.51 ng PEDF per mg

protein than treatment A at 8.947 ng PEDF per mg protein on collection day 5. There is evidence that blueberry anthocyanins protected RPE cells from oxidation as measured by PEDF values.

CHAPTER 1: INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world. This statement has opened so many papers about AMD that there is actually a review article entitled, "Age-related macular degeneration is the leading cause of blindness…" (Bressler, 2004). AMD is a painless degenerative disease that affects the macula and blurs the central vision needed for straight-ahead activities. The macula is located in the posterior portion of the retina and contains most of the eye's photoreceptors that are required for high-resolution visual acuity.

The retinal pigment epithelium (RPE) is responsible for the integrity of the photoreceptor cells (Lopez et al, 1990). Interference and disruption of the RPE cells occurs with the progressive accumulation of lipofuscin. Lipofuscin consists of a complex mix of compounds but the major component found within the RPE is pyridinium bisretenoid, *N*-retinyl-*N*-retinylidene ethanolamine, A2E (Sakai et al, 1996). A2E is a byproduct of the human visual cycle of vitamin A recycling. A2E cannot be enzymatically degraded and accumulates within the RPE with age and can have many adverse affects on the RPE. There are three different mechanisms in which A2E has been identified and outlined to be detrimental to RPE cells. (1) The RPE is responsible for the lysosomal degradation of the phagocytosed photoreceptor outer segments but A2E has been shown to inhibit this process (Bergmann et al, 2004). (2) A2E forms highly reactive and damaging A2E-epoxides upon blue light illumination and causes RPE apoptosis (Sparrow et al, 2003b). (3) In high concentrations, A2E behaves as a cationic detergent solubilizing the membrane (De and Sakmar, 2002).

Antioxidants are believed to help prevent some of the damage incurred by A2E since epidemiological studies have shown that elderly patients who regularly consume diets rich in

antioxidants are less likely to be afflicted by AMD (Shen et al, 2007). Anthocyanins are one of the major contributors of the antioxidant capacity of the fruits (Prior, 2003). Anthocyanins are the water soluble components of fruits and vegetables that impart the blue, purple and red pigments to the plants or plant products. According to Wu et al, (2006) the largest contributors of anthocyanins to the United States diet are the blueberry. Because of its high concentration of anthocyanins and high consumption, the blueberry is investigated in this study.

Angiogenesis is the multistep process that causes new vessels to develop from pre-existing vasculature. There are two major components to this process. The vascular endothelial growth factor (VEGF) is known to cause a wide-range of ocular diseases and pigment epithelial derived factor (PEDF) has been shown to play a key role in maintaining the avascularity of the ocular tissues. Blueberry anthocyanins could inhibit VEGF secretion and protect the stability of PEDF which may delay the progression of angiogenesis associated AMD. PEDF levels have been found to be sensitive to oxidative stress whereas VEGF levels remain the same (Ohno-Matsui, 2001). For this reason the PEDF levels were analyzed in this study to determine the effectiveness of anthocyanins in preventing oxidation within the RPE cells.

The main objective of this study was to determine whether anthocyanins from blueberries can prevent the accumulation of A2E in the RPE cells. Steps to test this objective were:

1. Synthesize lipofuscin A2E
2. Extract anthocyanins from blueberry powder concentrate
3. Conduct a RPE cell culture study to investigate the interactions between RPE, A2E and blueberry anthocyanin extract
4. Measure the anti-angiogenic PEDF by ELISA after various treatments in the cell culture study

CHAPTER 2: LITERATURE REVIEW

2.1 Age-Related Macular Degeneration

Age related macular degeneration (AMD) is a degenerative eye disease that is the leading cause of irreverable blindness in people over 50 years of age in developed countries (Bressler, 2004). According to a report written by the Eye Disease Prevalence Research Group in 2004 there are over 8 million Americans that are afflicted by AMD and this number is expected to double by 2020 (Eye Disease Prevalence Research Group, 2004). The etiology of AMD is complex but some well established risk factors include advanced age, white race, heredity and a history of smoking (Klein et al, 2004).

The degenerative eye sight can be caused by two different types of AMD; the dry form and the wet form. The most common type of AMD that affects 85-90% of the people who have AMD is the dry form. The dry or atrophic form of AMD is caused by geographic atrophy of the retinal cells and accumulation of lipofuscin (Bird et al, 1995). Lipofuscin is a complex aggregate of material that occurs in a variety of cells that primarily originates from the degradation of exhausted organelles with phagocytosis being the main cause of accumulation in the eye (Yin, 1996; Boulton et al, 2004; Sparrow and Boulton, 2006). The central visual loss caused by dry AMD is progressive and even after developing advanced dry AMD will continue over the course of months to years (Sunness et al, 1997). Wet AMD is an exudative or neovascular form of AMD. Wet AMD can have a serous or hemorrhagic detachement of the retinal pigment epithelium or sensory retina. There can also be the presence of subretinal, subpigment epithelium hemorrhage or fibrous scar (Jonasson et al, 2005). Patients with neovascular AMD can experience profound vision loss within days to weeks due to the hemorrhaging or fluid accumulation. Neovascular AMD only affects 10-15% of persons with AMD but is responsible

for 80% of cases of severe visual loss or legal blindness associated with AMD (Ferris et al, 1984). A patient can experience one form of AMD in one eye and the other form in the other eye and can also experience both forms in one eye (Sunness, 1999). According to an article published in 2001 by the Age-Related Eye Disease Study Research Group, there is a 43% chance that once advanced AMD develops in one eye there will be development in the other eye within 5 years.

Most people with advanced AMD do not become completely blind but their visual loss often markedly reduces the quality of life and is associated with disability. Clinical depression has been associated in up to a third of patients even if it only affects one eye (Jager et al, 2008). There is no generally accepted treatment for the dry form of AMD but there are a few treatment options for the wet form (Sunness, 1999). Treatments for the wet exudative form of AMD include anti-angiogenic intravitreal therapy, ocular photodynamic therapy, argon-laser photocoagulation therapy and vitreoretinal surgery (Jager et al, 2008). A localized injection of antiangiogenic agents into the vitreous is currently the primary therapy for wet AMD. There are rare but serious adverse reactions that can take place due to the intravitreous injections including endophthalmitis, retinal detachment, intraocular pressure, and even anaphylaxis (Jager et al, 2004). Photodynamic therapy is a treatment that causes localized choroidal neovascular thrombosis; it does not typically improve vision although it does limit visual loss due to wet AMD (TAP Study Group, 1999). The argon-laser photocoagulation therapy used to be the most common therapy for the wet form of AMD but since the treatment can create large retinal scars associated with permanent visual loss the treatment is reserved for neovascularization that extends by more than 200 μm from the center of the macula (Macular Photocoagulation Study Group, 1994). Vitreoretinal surgery involves the surgical extraction of choroidal

neovascularization but the efficacy is poor according to the Submacular Surgery Trials Research Group (2004). It is postulated by the Age-Related Eye Disease Study (report number 11) that if people that are at high risk for advanced AMD were to take supplements containing antioxidants plus zinc more than 300,000 persons could avoid development of advanced AMD with either forms of AMD (Bressler et al, 2003).

A person affected by AMD loses the ability to do many straight ahead activities due to the life time deposition of acellular, polymorphous debris in the macula that causes blurred and or blocked central vision (Jager et al, 2008). The macula is located in the posterior portion of the retina. The macula contains most of the eye's photoreceptors that are responsible for high-resolution visual acuity which allows a person to see fine detail necessary to read and recognize faces (Alfaro et al, 2006). The photoreceptors are light sensitive and the photoreceptor outer segments (POS) are shed and phagocytosed daily by the retinal pigment epithelial (RPE) cells (Young and Bok, 1969). These phagocytosed POS contain high concentrations of various proteins and polyunsaturated fatty acids (Brunk et al, 1995). The RPE cells degrade the phagocytosed POS through the action of lysosomal enzymes. The process is efficient but not complete and is therefore partially responsible for the slow accumulation of lipofuscin granuales in the RPE cell cytoplasm (Hayasaka, 1989). The retinal pigment epithelial cells are posterior to the photoreceptors and are part of the ocular-blood barrier and are responsible for nutrient transport and cytokine secretion as well as photoreceptor phagocytosis (Jager et al, 2008).

Analysis of the content of the lipofuscin contents revealed that the granules were residual lysosomal storage bodies since the constituents were that of the lysosomal enzymes (Berman, 1991). Further analysis of the lipofuscin showed that the granule also contained age pigments that were derivatives of vitamin A. The vitamin A derivative identified had two molecules of

retinaldehyde that were bonded with either one molecule of ethanolamine (A2E) or phosphatidylethalomine (A2-PE) (Eldred and Lasky, 1993). A2E has been shown to inhibit the lysosomal enzyme function of breaking down the phagocytosed POS and thus assist in the accumulation of lipofuscins (Eldred, 1995). When RPE cells contain A2E and are exposed to blue light, apoptosis of RPE cells has been shown to occur whereas cells devoid of A2E remain unaffected (Sparrow et al, 2003a).

2.2 Retinal Pigment Epithelium (RPE) and *N*-retinyl-*N*-retinylidene Ethanolamine (A2E)

The retinal pigment epithelium (RPE) is responsible for the integrity of the photoreceptor cells that are responsible for the high-resolution of straight ahead viewing. The demise of the RPE cells brings about the loss of the photoreceptors (Lopez et al, 1990). Interference and disruption of RPE cells occurs due to the progressive accumulation of lipofuscin (Sparrow et al, 1999). RPE lipofuscin consists of a complex mixture of compounds but the major hydrophobic component is the pyridinium bisretinoid, *N*-retinyl-*N*-retinylidene ethanolamine, A2E and thus named because there are two molecules of all-*trans* retinal with one molecule of ethanolamine (Sakai et al, 1996).

In December of 1998, Parish and his colleagues published a method outlining the isolation and preparation of A2E. The synthesized A2E was later shown to be able to be internalized by RPE cells in culture at levels comparable to levels in harvested human eyes (Sparrow et al, 1999).

The identification of A2E first took place because the lipofuscin in the RPE cells fluoresced an unusual golden yellow upon ultraviolet illumination (Feeney, 1978). Emissions of such long wavelengths are not typical for biochemical compounds of animal origin (Wagner et al, 1984). The unusual fluorescence of the lipofuscin elicited more interest in defining the source

as this could hold a potential key for the reason of formation and accumulation. Nutritional analysis suggested that dietary vitamin A was a possible contributor to the formation of the lipofuscin (Katz et al, 1987). The compound was proved to be a pyridinium bisretenoid of two molecules of retinaldehyde bonded with one molecule of ethanolamine (A2E) (Eldred, 1995).

The pyridinium bisretenoid A2E is a byproduct of the human visual cycle of vitamin A recycling (Sparrow et al, 2003). A2E is a product of the combination of all-*trans*-retinal and ethanolamine within the eye. The all-*trans*-retinal is released from the photoactivated rhodopsin which utilizes vitamin A to produce retinal. Ethanolamine is the head group of phosphatidylethanolamine which is abundant in the membrane phospholipid (Liu et al, 2000). Animals cannot synthesize vitamin A and rely on the photochemical regenerative pathways to recycle retinoid through a series of oxidation and reduction reactions called the visual cycle (Kuksa et al, 2003; Maeda et al, 2005). The biosynthesis of A2E within the eye is dependent upon the availability of the retinol released from the visual cycle (Radu et al, 2005). High intensity illumination can cause rapid generation of all-*trans*-retinal that would be available to interact with phosphatidyethanolamine to later form A2E after phosphate hydrolysis (Saari et al, 1998; Sparrow et al, 2003a).

A2E cannot be enzymatically degraded and accumulates in the RPE with age. The progressive accumulation of A2E in the RPE can have many adverse affects on the RPE cells. There are three different mechanisms in which A2E has been identified and outlined to be detrimental to RPE cells. (1) The RPE is responsible for the lysosomal degradation of the phagocytosed photoreceptor outer segments but A2E has been shown to inhibit this process (Bergmann et al, 2004). (2) A2E forms highly reactive and damaging A2E-epoxides upon blue

light illumination and causes RPE apoptosis (Sparrow et al, 2003b). (3) In high concentrations, A2E behaves as a cationic detergent solubilizing the membrane (De and Sakmar, 2002).

Light illumination of the eye can cause adverse reactions to occur in the retina. All-*trans*-retinal is first released from the visual cycle because of intense illumination and joined with ethanolamine to form A2E (Saari et al, 1998). The resultant compound, A2E, is a pyridinium bisretenoid consisting of a pyridinium polar head group and two hydrophobic retinoid tails (Sakai et al, 1996; Ren et al, 1997). A double bond isomer of A2E was identified when Parish et al determined the one-step preparation of A2E and named the isomer *iso*-A2E and found that they exist in photoequilibrium of 4:1 A2E:*iso*-A2E (1998). Both structures have been shown to accumulate intracellularly within RPE cells (Sparrow et al, 1999). The strong affinity of A2E to absorb the blue region of the spectrum of light was linked to RPE cells susceptibility to blue light damage when RPE cells containing A2E showed apoptosis and cells free of A2E did not (Sparrow et al, 2000). There is an electron excitation within the A2E molecule caused by the absorption of the photon from the blue light. The photoexcited A2E can transfer its energy to an adjacent oxygen molecule to form a singlet oxygen molecule and cause cell death (Sparrow et al, 2002). Blue light irradiation of A2E can cause photoxidation to occur to A2E and produce a series of epoxide rings along the retinoid side arms which are believed to be more damaging to cells because of their ability to diffuse further than singlet oxygen (only 10-20 nm) (Ben-Shabat et al, 2002; Sparrow et al, 2003b; Sparrow et al, 2003a). The initiating step in the formation of damaging species within the RPE starts with the initial blue light illumination and the formation of singlet oxygen. It has been shown that the singlet oxygen species can be quenched by antioxidants such as vitamins E and C before they attack the RPE or cause photoxidation within

A2E and therefore it is believed much of the damage caused by A2E can be restricted by antioxidants (Sparrow et al, 2003a).

2.3 Blueberry Anthocyanins as an Antioxidant

Epidemiological studies have shown that elderly patients who regularly consumed diets rich in antioxidants are less likely to be afflicted by age-related macular degeneration (AMD) (Shen et al, 2007). One cause of AMD is the accumulation of the lipofuscin A2E and the damage mediated by blue light illumination. A2E absorbs blue light and through the excitation forms singlet oxygen which can either cause damage to the retinal pigment epithelial (RPE) cells or the epoxidation of the A2E. If antioxidants are available to quench the singlet oxygen, it is believed that much of the oxidative damage that causes AMD could be restricted (Sparrow et al, 2003a). The Age-Related Eye Disease Study Research group found that early stage AMD patients who took supplements of high-dose antioxidants plus zinc and other vitamins had a reduced risk of progressing to advanced stages of either of the dry or wet forms of AMD (2001).

Evidence from several fields of science including epidemiology, human medicine and nutrition have outlined the important role that consumption of fruits and vegetables play in reducing the risk of degenerative diseases (Kalt et al, 1999; Keli et al, 1996; Negri et al, 1991; Steinmetz and Potter 1991). A diet rich in fruits and vegetables provide increased consumption of antioxidants including carotenoids, ascorbate, tocopherols, and phenolics (Ames et al, 1993). Antioxidant capacity has often been attributed to those first three compounds more commonly referred to as beta-carotene, vitamin C and vitamin E but it is the phenolics that are also believed to play an important antioxidant role (Prior 2003; Rice-Evans et al, 1996; Wang et al, 1996). One potent *in vitro* antioxidant fraction of phenolics is the flavonoids and that fraction is broken down further to include flavones, isoflavones, flavonones, catechins and anthocyanins (Moyer et

al, 2002). The antioxidant capacity in some fruits is attributed to the anthocyanin content (Prior 2003).

Anthocyanins are water soluble components of fruits and vegetables that impart the blue, purple and red pigments to the plants or plant products. Anthocyanins are glycosylated, polyhydroxy or polymethoxy derivatives of 2-phehylbenzopyrylium and contain two benzoyl rings separated by a heterocyclic ring (Mazza and Miniati, 1993). Over 600 naturally occurring anthocyanins have been reported with variations arising due to the number and position of hydroxyl and methoxyl groups on the anthocyanin base structure; the identity, number and positions at which the sugars attach; and the extent of and identity of the acylation to the sugar (Wu et al, 2006). Anthocyanins without the sugar molecule are referred to as an anthocyanidins. There are seventeen different anthocyanidins found in nature with only six found ubiquitously: cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin (Mazza and Miniati, 1993). Anthocyanins most frequently occur as 3-monosides, 3-biosides and 3,5-diglycosides and rarely 3,7-diglycosides and are associated with the sugars glucose, galactose, rhamnose, arabose and xylose (Mazza and Miniat, 1993).

Since anthocyanins are large, highly water soluble molecules, they were believed to have little absorption into cells or circulatory system (Hollman, 2004). Anthocyanins have since been shown to be absorbed from the gastrointestinal tract, transported through the circulatory system and excreted in urine (McGhie and Walton, 2007). Youdim and colleagues (2000) were the first to show that vascular endothelial cells can incorporate anthocyanins into the membrane and cytosol. It was also proved that the incorporation of anthocyanins into the endothelial cells significantly enhanced their resistance to the damaging effects of reactive oxygen species (Youdim at al, 2000). Both findings by Youdim et al are promising for cell culture studies since

anthocyanins have been found to incorporate into endothelial cells and provide antioxidant protection. Cell culture studies must be done at a neutral pH of 7 and anthocyanins may degrade at a neutral pH but may still provide some antioxidant activity (Prior, 2003). Since little is known about the mechanisms of absorption and transport of anthocyanins and the alterations that take place to incorporate anthocyanins within the endothelial cells, simple pH adjustment with sodium hydroxide is used to study anthocyanin efficacy (McGhie and Walton, 2007).

In the 1970s, anthocyanin consumption was estimated to be as much as 180-255 mg per day but a reexamination of the estimated consumption in the 2000s was found to be only 12.5 mg per day in the United States (Kuhnau, 1976; Wu et al, 2006). According to Wu et al, (2006) the largest contributors of anthocyanins from fruits, vegetables and beverages to the United States diet as determined by total anthocyanin in product and average daily intake are blueberries at an estimated consumption of 3.39 mg. Blueberries species in North America include the "wild," lowbush (*Vaccinium angustifolium* Aiton) and the "cultivated," highbush (*Vaccinium corymbosum* L. and *Vaccinium ashei*) (Prior et al, 1998). Both wild and cultivated blueberry species are grown throughout Eastern and Northeastern United States, Pacific Northwest, Wisconsin, Michigan, and much of Canada (Neto, 2007). The rabiteye cultivar of highbush blueberries are found in the Southern and Southeastern states of the United States (Trinka, 1997). Cultivated blueberries have had fifteen anthocyanins identified which include the 3-monoarabinosides, 3-monogalactosides, 3-monoglucosides of cyanidin, delphinidin, malvidin, peonidin and petunidin (Kader et al, 1996). Blueberries also have other phytochemicals that could possibly be of interest in future studies and are well described in the review article by Neto, 2007.

2.4 Angiogenesis and Anti-Angiogenic Pigment Epithelial Derived Factor (PEDF)

Angiogenesis is the multistep process that causes new vessels to develop from pre-existing vasculature. Angiogenesis is important for tissue homeostasis, embryonic development as well as other functions and supports wound and tissue repair (Folkman, 1972; Atherton 1977; Folkman and Haudenschild, 1980; Eichler et al, 2006). In the healthy adult eye, no new vessels are being formed (Dawson et al, 1999). There are several large components in the eye in which blood vessels are completely excluded and this exclusion is a continuous and active process (Bouck, 2002). Angiogenesis in the eye is believed to develop due to an imbalance between stimulators and inhibitors of angiogenesis (Folkman, 1995).

Angiogenesis is mitigated and controlled through stimulators and inhibitors that are commonly referred to as growth factors. The retinal pigment epithelium (RPE) is an important source of growth factors that are involved in tissue maintenance, homeostasis, inflammation and choroid neovascularization formation and is the central cell type regulating these processes (Schlingemann, 2004). RPE cells are crucial for the maintenance and survival of the neuroretina and choroid and destruction of RPE leads to atrophy of photoreceptors (Witmer et al, 2003).

Two growth factors that are found in the eye and exist in a dynamic relationship for the maintenance of normal retinal functions are vascular endothelial growth factor (VEGF) and pigment epithelial derived factor (PEDF) (Ohno-Matsui et al, 2001). Both VEGF and PEDF are expressed in the RPE cells (Matsuoka et al, 2004; Ohno-Matsui et al, 2001). Cultured RPE cells have been found to secrete PEDF to the media (Becerra et al, 1999). VEGF is the essential pro-angiogenic growth factor that has been found through laboratory and clinical studies to be the cause of a wide range of ocular diseases (Michels et al, 2006). Patients with age related macular degeneration (AMD) have been found to have high levels of VEGF in the RPE (Kvanta et al,

1996; Kliffen et al, 1997). PEDF is a 50 kDa glycoprotein that appears to play a key role in preventing angiogenesis in healthy ocular tissue (Jablonski et al, 2000; Dawson et al, 1999). PEDF has been shown to counteract the angiogenic potential of VEGF (Ohno-Matsui et al, 2001). PEDF has been shown to play a key role in maintaining the avascularity of the ocular tissues by preventing vessels from invading the cornea (Dawson et al, 1999; Bouck, 2002). A study by Tsao et al. (2006) revealed that PEDF concentration of 25 ng/ml or higher was cytoprotective and that the protection provided by the PEDF was time and dose dependent.

It has been found that increased oxidative stress leads to an overexpression of growth factors by the RPE (Beatty et al, 2000; Penfold et al, 2001). Ohno-Matsui et al (2001) were able to show that with oxidative stress there is a reduction in the expression of PEDF by RPE but that the expression of VEGF remains the same. With cellular senescence, there has been a decreased PEDF production observed in RPE cells as well as other cell types (Pignolo et al, 1993; Palmieri et al, 1999; Tresini et al, 1999). The combination of aging and oxidative stress contributes to the reduction of PEDF in the RPE cells. If caught and treated in the early stages, it is believed that antiangiogenic treatments can preserve or even regain vision (Michels et al, 2006).

CHAPTER 3: MATERIALS AND METHODS

3.1 Synthesis of A2E

3.1.1 Materials

All-*trans* retinal and ethanolamine were purchased from Sigma-Aldrich (St Louis, Missouri). Ethanol and acetic acid were purchased from Fisher (Hampton, New Hampshire).

3.1.2 Synthesis of A2E

The A2E was synthesized as outlined by Parish et al (1998) by mixing 100 mg (352 µM) all-*trans*-retinal and 9.5 mg (155 µM) ethanolamine in 3.0 ml ethanol in the presence of 9.3 µl (155 µM) acetic acid for two days (1998). Half of the ethanol was measured into a small amber bottle and the all-*trans* retinal was added to the ethanol. The ethanolamine was added to the ethanol mixture and then the remaining ethanol was added to the bottle. The acetic acid was added to the bottle. A magnetic stir bar was added to the bottle and the bottle was capped. The bottle was placed on a stir plate and covered with a box for darkness and allowed to stir on low for 48 hours. After 48 hours, the bottle was moved to a fume hood where nitrogen gas was used to flush off the ethanol.

3.1.3 Identification of A2E by MALDI

A small portion of the crude A2E was dissolved in methanol and was sent to the Department of Chemistry at Texas Agricultural and Mechanical University, College Station, Texas, for Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) identification. The A2E was dissolved in dimethyl sulfoixde (DMSO).

3.2 Extraction and Identification of Anthocyanins from Blueberry Concentrate

3.2.1 Materials

A freeze dried blueberry powder of a 50/50 blend of Tifblue/Rubel (*Vaccinium ashei/Vaccinium corymbosum*) concentrated from 7 to 1 fresh to freeze dried weight was

provided by the U.S. Highbush Blueberry Council (Folsom, California). Chemicals were purchased from Fisher (Hampton, New Hampshire). A XK 16/20 chromatography column was obtained from GE Healthcare with dimensions of 16 mm inner diameter and 20 cm in length (Piscataway, New York). The silica used in the column was purchased from Waters (Milford, Massachusetts) and was a Waters Preparative Chromatographic Bulk Packing Material WAT020594 with a specification of C18 125Å 55-105 µm.

3.2.2 Anthocyanin Extraction

One hundred grams of the blueberry powder was mixed with 500 ml of 70% (v/v) acetone in de-ionized water in a one liter seven speed stainless steel laboratory Waring blender (Torrington, Connecticut) on low for one minute. The speed of the blender was steadily increased to speed 3 and blended for 30 seconds. The blender was placed in a walk-in cooler overnight for further extraction from the blueberry concentrate.

The chilled blueberry and acetone mixture was filtered through five layers of cheesecloth. The filtrate was divided evenly by weight into two centrifuge bottles and stored at refrigeration conditions. The sediment trapped by the cheesecloth was blended with 500 ml of 70% (v/v) acetone in de-ionized water in the blender for a couple of minutes at speed 2. The blender was placed in the refrigerator overnight. The next day the chilled blueberry sediment in 70% (v/v) acetone in de-ionized water solution was filtered through five layers of cheesecloth. The filtrate was divided evenly by weight between two centrifuge bottles.

The four centrifuge bottles containing the filtrate were centrifuged at 5635 × g at 4°C for ten minutes. The supernatants from the four centrifuge bottles were poured into a Pyrex baking dish and placed under the fume hood to allow for the evaporation of the acetone. After acetone evaporation, 40 ml of de-ionized water was used to help remove the extract. All together 100 ml

of blueberry extract was collected. The pH of the blueberry extract was 3.02 and was adjusted to the physiological pH of 7.0 using 1.0 N sodium hydroxide (NaOH). The blueberry sample was lyophilized and kept at -20°C until use.

3.2.3 Anthocyanin Concentration through Chromatography

The chromatography column was rinsed with ethyl acetate and then filled a third of the way with ethyl acetate. Thirty grams of the reverse phase C18 packing silica was weighed out into a glass beaker and ethyl acetate was added until the silica was completely immersed. The mixture was poured into the column. The remaining silica in the beaker was washed with ethyl acetate and poured into the column. The column was set up under a fume hood capped while the silica settled out of solution. While the column's silica was allowed to settle, the freeze dried blueberry extract was reconstituted one to one by weight in de-ionized water.

The column was opened at the bottom and top to allow the excess ethyl acetate to drain out of the column until there was a thin layer of ethyl acetate above the silica to prevent the silica from drying out. The column was then equilibrated with 150 ml of methanol containing 0.01% hydrochloric acid and then with 60 ml of de-ionized water containing 0.01% hydrochloric acid. A layer of sand was added to the top of the silica in the column to prevent the silica from raising and a thin layer of the liquid was left above the top of the sand.

One and a half milliliters of the reconstituted blueberry extract was added to the top of the column. The sugars and acids were desorbed from the blueberry extract by running 150 ml of de-ionized water containing 0.01% hydrochloric acid through the column. The phenolics were desorbed from the blueberry extract by running 150 ml of ethyl acetate through the column. The anthocyanins were desorbed from the blueberry extract by running 150 ml of methanol containing 0.01% hydrochloric acid through the column and were collected. This process was

repeated a total of ten times with a cleaning process between each anthocyanin extraction as outlined in the next paragraph.

The column was cleaned between each anthocyanin extraction process by first running 250 ml of 70% (v/v) acetone in de-ionized water. The column was then equilibrated with 150 ml of methanol containing 0.01% hydrochloric acid and then with 60 ml of de-ionized water containing 0.01% hydrochloric acid.

The anthocyanin extraction collected from the column was left in the fume hood to allow the methanol to evaporate off. The pH of the anthocyanin extract was adjusted to 7.0 using 1.0 N NaOH. The sample was then transferred to a weigh boat and lyophilized. Two hundred and fifty milligrams of the lyophilized anthocyanin extract was combined with 5 ml of dimethyl sulfoxide (DMSO) and was stored with the remaining lyophilized sample in a -20°C freezer until use.

3.2.4 Identification of Monomeric and Polymeric Anthocyanin Concentration by pH Differential Method and HPLC

A sample of the lyophilized blueberry anthocyanin extract as well as a sample of the blueberry powder provided by the U.S. Highbush Blueberry Council was sent to the Department of Food Science and Technology at The Ohio State University, Columbus, Ohio for anthocyanin analysis. The work done by Pu Jing and M. Monica Giusti at The Ohio State as stated in their report as desrcibed below.

Approximately 0.010 grams of anthocyanin extract was mixed with 1 ml of methanol containing 0.1% hydrochloric acid and sonicated for 10 minutes. Then 1 ml of water containing 0.1% hydrochloric acid was added to the sample.

Approximately 0.010 grams of the blueberry powder was mixed with 25 ml of methanol containing 0.1% hydrochloric acid for 10 minutes. The sample was filtered through a Whatman No. 1 filter paper under vacuum condition using a Büchner funnel and the slurry was washed

with 10 ml of methanol containing 0.1% hydrochloric acid. The methanol was removed from the sample using a rotary evaporator at 40°C under vacuum. The extract was brought to a volume of 2 ml using a solvent of 50% water and 50% methanol containing 0.1% hydrochloric acid.

The total monomeric anthocyanin content was measured by the pH-differential method (Giusti and Wrolstad, 2000). A Shimadzu UV-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan) was used to measure absorbance at 420 nm, 510 nm, and 700 nm. Monomeric anthocyanins were calculated as cyanidin-3-glucoside equivalents, using the extinction coefficient of 26,900 L $cm^{-1}mg^{-1}$, and a molecular weight of 449.2 g/L. The polymeric color was calculated using absorption at 420 nm, 510 nm, and 700 nm before and after bisulfite treatment (Giusti and Wrolstad, 2000).

A reverse-phase high performance liquid chromatograph (HPLC) system (Shimadzu Corporation, Tokyo, Japan) consisted of LC-20AD prominence liquid chromatograph, a SPD-M20A prominence diode array detector, SIL-20AC prominence auto sampler at 4 ºC, and LCMS-2010EV liquid chromatograph mass spectrometer. LCMS solution Ver3.30 software was used to separate the anthocyanins present in the samples.

The reversed-phase 3.5 µm Symmetry C18 column (4.6×150mm, Waters Corporation, Massachusetts) fitted with a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corporation, Massachusetts) was used. Solvents and samples were filtered though 0.45 µm GE Magna* nylon membrane filters (Fisher Scientific, Fair Lawn, New Jersey). Separation was achieved by using a gradient mobile phase as following: 10% B, 0-3 min; 10% to 17% B, 3-13 min; 17-20% B, 13-13.01 min; 20% B, 13.01-20 min; 20%-10% B, 20-25. Solvent A was 5% (v/v) formic acid in water and B was 50% acetonitrile, 45% methanol and 5% formic acid. A 0.8 ml/min flow rate was used and injection volumes were 60 µl and 30 µl for the anthocyanin extract and blueberry

powder respectively. Spectral information over the wavelength range of 254-700 nm was collected

A 0.25 ml/min flow was diverted to a mass spectrometer. Mass spectrometry was conducted on a quadrupole ion-tunnel mass spectrometer equipped with an electrospray ionization (ESI) interface (Shimadzu Corporation, Tokyo, Japan). Mass spectrometric analyses were performed in a positive ion mode under the tuning auto mode and other specific conditions as following: nebulizing gas flow, 1.5 L/min; interface bias, +4.50 kV; Block temperature, 200 ºC; focus lens: -2.5 V; entrance Lens, -50.0 V; prerod bias, -3.6 v; main-rod bias, -3.5 V; detector voltage, 1.5 kV; scan speed, 2000 amu/sec. A full scan was performed with a mass range from 200-1500 *m/z*. An SIM scan was applied for anthocyanin aglycons: 271, 287, 301, 303, 317, and 331 *m/z*.

3.3 RPE Cell Culture Studies

3.3.1 Materials

Human retinal pigment epithelial (RPE) cells (ARPE-19 cell line) that are devoid of endogenous A2E were obtained from the American Type Culture Collection (Manassas, Virginia). The A2E used is outlined in section 3.1. The blueberry anthocyanin extract used is outlined in section 3.2.3.

3.3.2. Cell Culture Confluence

The RPE cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 supplemented with 10% fetal calf serum (FCS). The cells were grown at 37°C in a humidified 5% carbon dioxide condition and split twice a week when approximately 90% confluence was reached (Higgins et al., 2003). Cell cultures were grown to accumulate A2E in

the lysosomal compartment of the human RPE cell line to replicate as it does in the RPE of the eye.

3.3.3 Interaction Studies of RPE, A2E and Anthocyanin Extracts

Four different treatments to RPE cells with and without A2E and/or anthocyanins were investigated over seven days of incubation with collections on days one, three, five and seven. The four different interactions were as follows: (A) control RPE cells for the seven day study; (B) RPE cells incubated with 100 µM of A2E in DMSO for 2 hours and then exposed to blue light from a tungsten halogen source for 20 minutes and then the medium was changed for the seven day study; (C) RPE cells incubated with 100 µg of blueberry anthocyanins in DMSO for 24 hours and then incubated with 100 µM of A2E in DMSO for 2 hours and then exposed to blue light from a tungsten halogen source for 20 minutes and then the medium was changed for the seven day study; and (D) RPE cells incubated with 100 µg of blueberry anthocyanins for 24 hours and then incubated with 100 µM of A2E in DMSO for 2 hours and then exposed to blue light from a tungsten halogen source for 20 minutes and then the medium was changed and then 100 µg of blueberry anthocyanins in DMSO was applied to the cells and remained with the cells for the seven day study. Samples were collected from each treatment in triplicate on days one, three, five and seven. In the case of interactions C and D, where there was a 24 hour incubation period into the plan, the collection days were delayed by those 24 hours. On each collection day a digital photograph was taken of the cells using a Nikon camera at 40X magnification attached to a Leitz microscope.

The A2E level of 100 µM (50 µl) and blueberry anthocyanin extract of 100 µg anthocyanins (50 µl) was determined to be an effective level from previous work done. The A2E level was also determined by previous work done by others (Sparrow et al, 1999; Sparrow and

Cai, 2001; Jang et al, 2005). The cells treated with A2E were exposed to blue light illumination for 20 minutes from a tungsten halogen source for A2E photooxidation that causes apoptosis, cell damage and death within RPE cells (Sparrow and Cai, 2001).

3.3.4 Cell Culture Sample Collection

On the collection date of all the samples, the supernatant of the cells were collected into labeled eppendorf tubes. The tubes were placed in a -80°C freezer for later analysis. The flasks containing the cells were frozen as well for future use in other research projects.

3.4 PEDF by ELISA, Preparation and Quantification

3.4.1 Materials

The Bio-Rad *DC* Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, California). Pigment epithelium-derived factor (PEDF) enzyme linked immunosorbent assay (ELISA) was purchased from BioProducts MD (Middleton, Maryland).

3.4.2 Supernatant Protein Determination and Normalization

The protein content of all the collected supernatant was to be normalized. The *DC* Protein Assay by Bio-Rad (Hercules, California) was used to determine the protein content of all 64 samples. The test was done using the bovine serum albumin standard at 1.42, 0.71, 0.355, 0.1775, 0.08875 and 0.044375 mg/ml. Samples were diluted 1:10 in PBS. The test was run as outlined by the kit in triplicate for the standards and samples. The absorbance was read at 655 nm using Bio-Rad model 680 plate reader (Hercules, California).

The lowest average concentration was determined to be 3.11 mg/ml. All the samples' protein concentrations were normalized to a standard of 3.00 mg/ml in PBS using each of the samples' averages for the calculations.

3.4.3 PEDF Quantification by ELISA

The normalized supernatant of all samples were used for the Pigment Epithelium Derived Factor (PEDF) sandwich ELISA antigen detection kit from BioProducts MD. The samples were treated 1:1 with 8M urea for 30 minutes at 4°C with periodic mixing (every 5-10 minutes) to allow for lysing of the PEDF from the cellular proteins.

All standards and samples were tested in triplicate. Standards were prepared as suggested by the manufacturer. The kit recommends a dilution of at least 1:50 of the urea treated samples but a previous test showed the 1:50 dilution produced values outside of the standard curve. The 1:100 dilution of the urea treated samples fell within the standard curve. The samples were then diluted 1:100 in the diluents provided by the kit for a total dilution of 1:200.

Test procedure was followed as outlined by the kit with manual washing using a multichannel pipette. The plate was read at 450 nm with a reference of 655 nm using Bio-Rad model 680 plate reader (Hercules, California).

3.5 Data Analysis Method

3.5.1 Materials

Microsoft Office Excel 2007 by the Microsoft Corporation was used for data analysis. SigmaStat 3.5 by Systat Software, Inc was used for statistical analysis.

3.5.2 Determining Sample's Unknown Values from Standard Curve

For each plate used, a standard curve was drawn using Microsoft Excel from the average readings of the standards for both the protein determination and the ELISA for PEDF determination. All the R^2 values for the standard linear regressions were greater than 0.99. The unknown values for the samples were determined using the linear regression. For the protein

determination samples, the values were multiplied by 10 to negate the dilution. For the ELISA samples, the values were multiplied by 200 to negate the dilution.

3.5.3 Statistical Analysis of PEDF Results

A two way analysis of variance (ANOVA) was used to analyze the PEDF results. The interactions investigated were between the treatment (being A, B, C or D see section 3.3.3) and the day (collection days 1, 3, 5 or 7). SigmaStat uses the Kolmogorov-Smirnov test to test for normality at a $p < 0.05$. Multiple comparisons of the data were done using the Holm-Sidak test. The Holm-Sidak test is a more powerful test than the Tukey and Bonferroni tests and therefore can detect differences that these other tests cannot detect.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 A2E Analysis by MALDI

A2E was prepared as outlined by Parish et al (1998). As seen in Figure 4.1, the peak at 592.3208 m/z confirms that A2E was formed in the synthesis process outlined in section 3.1.2. The report by Parish et al (1998) indicates that the method produces A2E at a rate of 49% and the sample was used as is in the cell culture study.

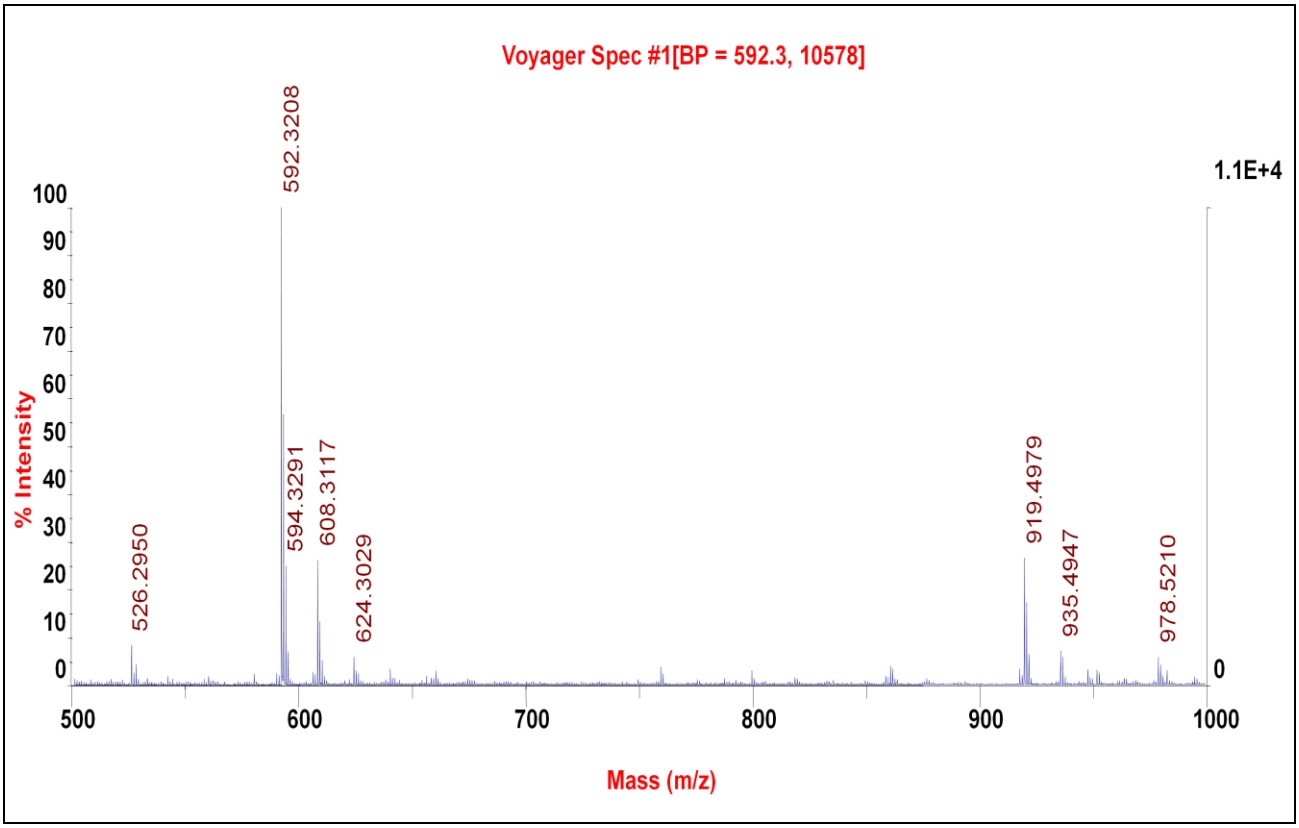


Figure 4.1: Mass Spectrometry Results of the A2E extraction

4.2 Anthocyanin Concentration Results

The blueberry powder provided by the U. S. Highbush Blueberry Council had, according to the specification sheet had 14.84 mg of anthocyanins per gram and was 58.90% sugar and 18.67% fiber (Table 4.2a and 4.2b).

Table 4.2a: Antioxidant profile of the freeze dried Tifblue/Rubel 50/50 blueberry blend powder as provided by the U.S. Highbush Blueberry Council

Composition		
ORAC*	356	µmole TE/g
Phenolics	32.48	mg/g
Anthocyanins	14.84	mg/g

*ORAC is oxygen radical absorbance capacity measured in TE trolox equivalents

Table 4.2b: Nutritional profile of the freeze dried Tifblue/Rubel 50/50 blueberry blend powder as provided by the U.S. Highbush Blueberry Council

Composition		
Calories	347.32	kcal/100 g
Carbohydrates	84.66	g/100 g
Total Sugars	58.90	g/100 g
Dietary Fiber	18.67	g/100 g
Vitamin C	172.21	mg/100 g
Calcium	30.39	mg/100 g
Iron	1.01	mg/100 g
Potassium	408.10	mg/100 g
Sodium	16.06	mg/100 g

The blueberry powder and the anthocyanin extract were sent to the Department of Food Science and Technology at The Ohio State University, Columbus, Ohio for anthocyanin analysis and quantification. The anthocyanin content, quality and profiles varied between the blueberry powder and the anthocyanin extract. The blueberry powder showed a high monomeric anthocyanin content with a low level of polymerization whereas the anthocyanin extract had a three times higher monomeric anthocyanin content but also a five times higher percentage of polymerization (Table 4.2c). Polymerization of anthocyanins is associated with degradation reactions during processing or storage of the samples. Anthocyanins are typically stable at a lower pH but at this lower pH the cells would be killed. In preparation of the anthocyanin extract, the pH was adjusted twice to the physiological pH of 7.0. In future studies, it is recommended that the pH only be adjusted to the physiological at the final step before treating cells with the anthocyanin extract. The threefold increase in monomeric anthocyanin content (Table 4.2c) does indicate that the extraction method used was effective in concentrating the

anthocyanin content but the fivefold increase in polymeric anthocyanins indicates the extraction method was a little detrimental since polymeric pigments are typically associated with degradation reactions during processing and storage of samples.

Table 4.2c: Monomeric anthocyanins and percentage of polymeric anthocyanins in blueberry powder and its extract

Samples	Monomeric anthocyanins (mg/g)	% Polymeric anthocyanins
Blueberry Powder	13.50 ± 0.37	11.21 ± 1.60
Anthocyanin Extract	39.78 ± 1.07	55.73 ± 1.11

Values are represented as mean ± standard error (n=2)

The high performance liquid chromatography (HPLC) procedure used by The Ohio State University, Columbus, Ohio was optimized to achieve separation of all the anthocyanins present in the two samples. A total of 13 anthocyanins were found in the two samples (Table 4.2d) in different proportions. The compounds were identified by their spectral characteristics as well as by their molecular ion mass and fragmentation patterns. The anthocyanin extract provided higher percentages of malvidin derivatives and lower percentages delphinidin derivatives when compared to the blueberry powder (Table 4.2d). Other minor differences were also observed (Table 4.2d). The chromatograph from the HPLC identification is shown in figure 4.2. The peaks in figure 4.2 correspond to the identification in table 4.2d.

The percentage of the anthocyanins in order of predominance in the anthocyanin extract was: malvidin-3-galactose (27.15%), malvidin-3-glucose (16.60%), malvidin-3-arabinose (12.16%), delphinidin-3-galactose (7.49%), petunidin-3-galactose (7.54%), cyanidin-3-galactose (5.31%), petunidin-3-glucose (4.63%), delphinidin-3-arabinose (4.42%), petunidin-3-arabinose (3.98%), delphinidin-3-glucose (3.08%), peonidin-3-galactose (2.77%), cyanidin-3-arabinose (2.57%), and cyanidin-3-glucose (2.31%) (Table 4.2d). Blueberries typically have fifteen anthocyanins but the report from The Ohio State only identified thirteen. The missing two

Table 4.2d: Anthocyanin percentage as determined by HPLC profiles of the blueberry powder and anthocyanin extract

Peak	Compound	λmax (nm)	M+, fractions *m/z*	Blueberry Powder	Anthocyanin Extract
1	Delphinidin 3-gal	524	465, 303	13.30	7.49
2	Delphinidin 3-glc	524	465, 303	5.24	3.08
3	Cyanidin 3-gal	516	449, 287	6.05	5.31
4	Delphinidin 3-ara	525	435, 303	7.64	4.42
5	Cyanidin 3-glc	517	449, 287	2.55	2.31
6	Petunidin 3-gal	528	479, 317	7.99	7.54
7	Cyanidin 3-ara	517	419, 287	2.80	2.57
8	Petunidin 3-glc	526	479, 317	4.94	4.63
9	Peonidin 3-gal	517	463, 301	2.69	2.77
10	Petunidin 3-ara	527	229, 317	4.17	3.98
11	Malvidin 3-gal	527	493, 331	21.12	27.15
12	Malvidin 3-glc	527	493, 331	12.15	16.60
13	Malvidin 3-ara	529	463, 331	9.34	12.16

Abbreviations: gal, galactose; glc, glucose; ara, arabinose

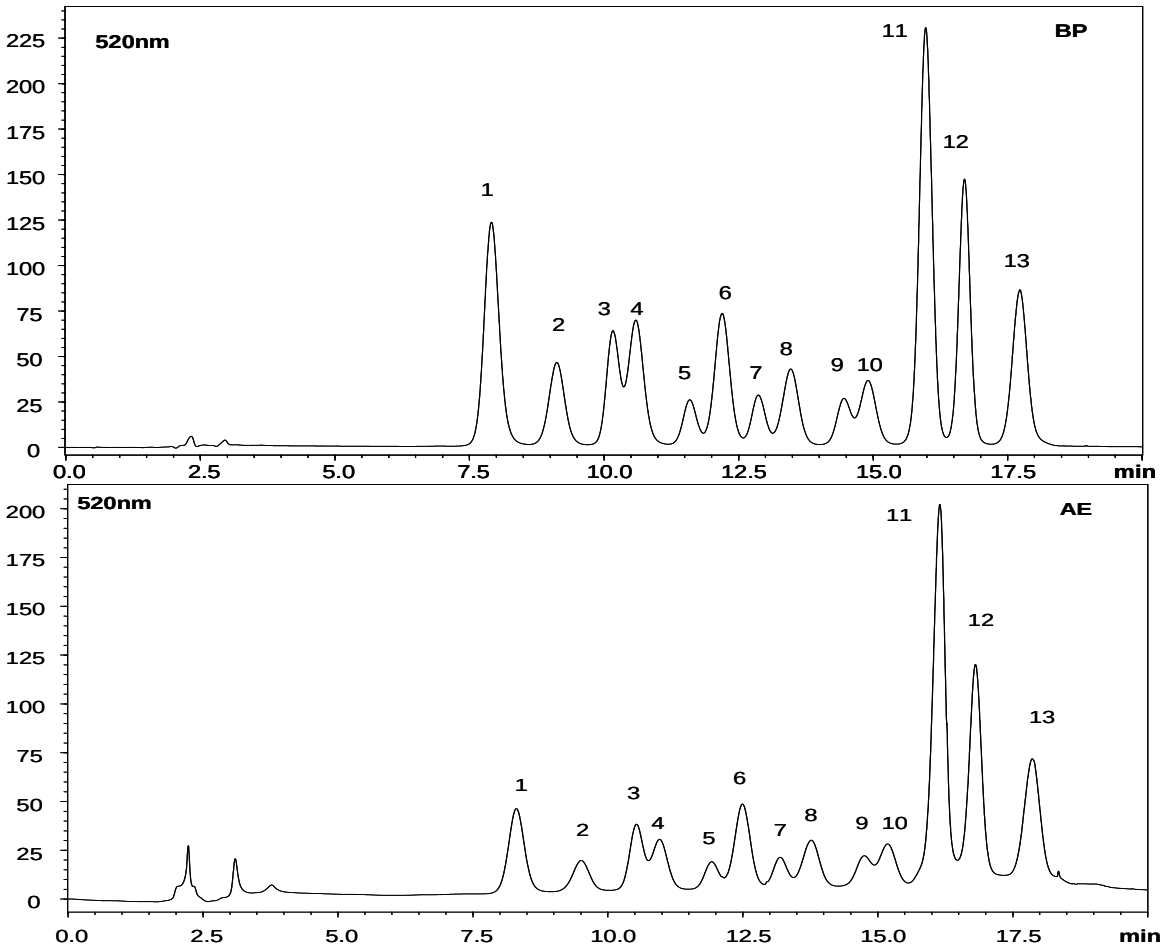


Figure 4.2: Anthocyanin profiles in blueberry powder (BP) top and anthocyanin extract (AE) bottom. Peak identities in Table 4.2d.

anthocyanins are peonidin-3-glucose and peonidin-3-arabinose. As reported by Kader et al using HPLC analysis for highbush blueberries, peonidin-3-glucose peaks between petunidin-3-arabinose and malvidin-3-galactose and peonidin-3-arabinose peaks between malvidin-3-galactose and malvidin-3-glucose which would fall between peak 10 and 11 and 12 and 13 respectively in Figure 4.2 (1996). This report also indicated the levels were low with total percentages at 1.1 and 0.1 respectively.

When compiling the anthocynidin derivative percentages of the anthocyanins it can be seen that the malvidin derivative concentration content increased in the anthocyanin extract (55.91%) from the blueberry powder (42.61%) due to a loss in concentration of delphinidin and cyanidin of 11.19% and 0.49% respectively (Table 4.2e). Jang et al reported findings looking at anthocyanins from bilberry (*Vaccinium myrtillus*) that showed that delphinidin, cyanidin, petunidin, and malvidin was able to protect RPE cells from A2E oxidation and that malvidin-3-glucose performed significantly (<0.001) better than the other anthocyanins (2005). The anthocyanidin malvidin is believed to be more hydrophobic than cyanidin and delphinidin since the base structure of malvidin has two methoxyl groups where cyanidin and delphinidin has two and three hydrophilic hydroxyl groups respectively (Chen et al, 1996). The side arms of the A2E molecule are hydrophobic and so it is speculated that the increased hydrophobicity of malvidin allows malvidin to neutralize the reactive oxygen species at the site of formation (Jang et al, 2005).

Table 4.2e: Percentage of anthocyanidin in total anthocyanin content of the blueberry powder and anthocyanin extract

Anthocyanidin	Blueberry Powder	Anthocyanin Extract
Malvidin	42.61	55.91
Petunidin	17.10	16.15
Delphinidin	26.18	14.99
Cyanidin	11.40	10.91
Peonidin	2.69	3.98

The percentage of 3-monosides of total anthocyanin content of the blueberry powder and anthocyanin extract remained about the same with galactose accounting for 51.15% and 50.26% respectively (Table 4.2f).

Table 4.2f: Percentage of 3-monosides in total anthocyanin content of blueberry powder and anthocyanin extract

3-monoside	Blueberry Powder	Anthocyanin Extract
Galactose	51.15	50.26
Glucose	24.88	26.62
Arabinose	23.95	23.13

4.3 Cell Culture Results

Four different treatments (A, B, C and D) were used to evaluate the interaction of A2E and blueberry anthocyanin extract in RPE cells over a seven day period. Treatment A was the control RPE cells. Treatment B was the control of RPE with A2E treatment and the degenerative affects of the A2E exposure. Treatment C was a simulation of prevention with anthocyanins in that the RPE cells were exposed to the anthocyanin extract for 24 hours before the cells were treated with A2E. Treatment D was a simulation of prevention and treatment in that the RPE cells were exposed to anthocyanin extract for 24 hours before the cells were treated with A2E and then anthocyanin extract was included in the growth medium of the cells.

The four different treatments were run in triplicate with collections on days 1, 3, 5 and 7. Pictures were taken under a microscope of the cells for each of the collection days. Representative images of the four different interactions are in Table 4.3a. Cells should look bright and free of granulation when healthy. As cells begin to die they become granulated and vacuolated until the point that they rupture and only debris is left. Treatments could have been analyzed using trypsin blue to determine the percentage of live versus dead.

Table 4.3a: Representative microscopy images of the RPE cells for each interaction on each collection day

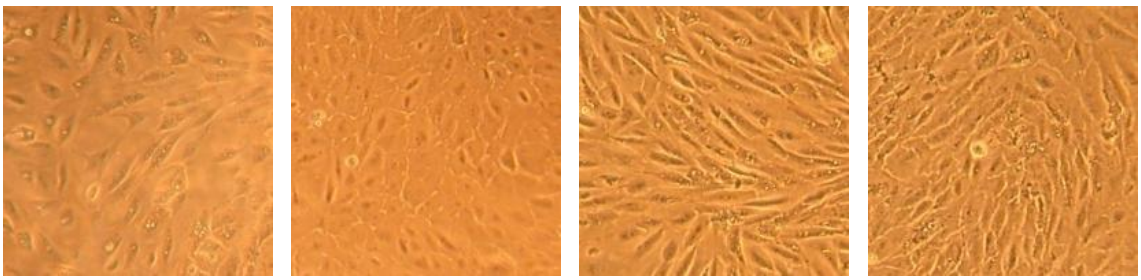
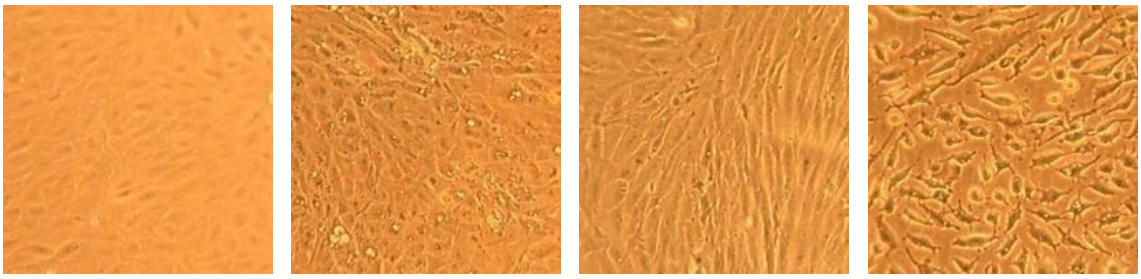
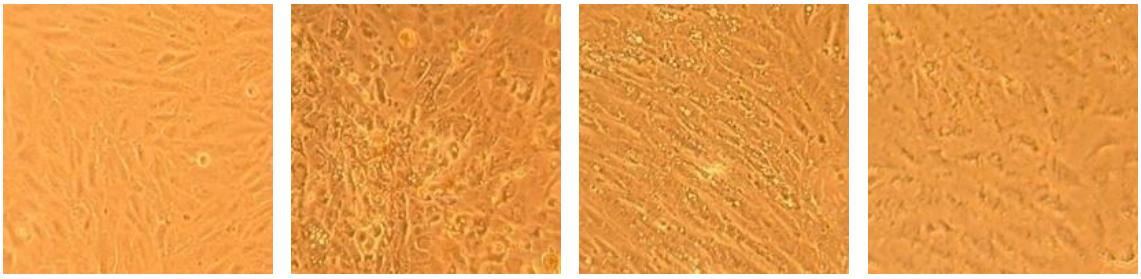
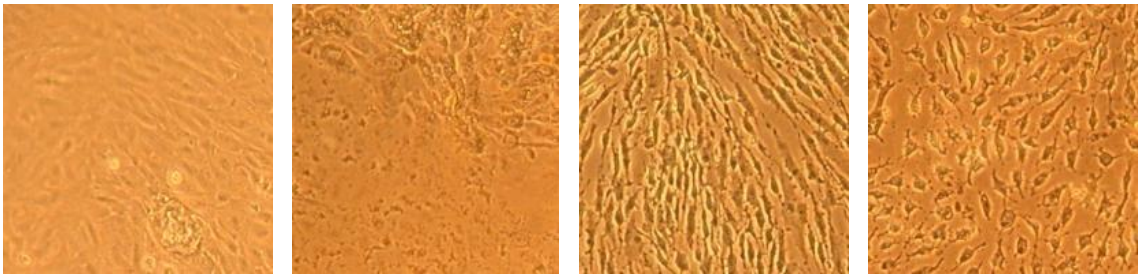
Days	Treatments (see Table 4.3b) A	B	C	D
1				
3				
5				
7				

Table 4.3b: Definition of cell culture Treatments

Treatment	Definition
A	RPE Control Cells
B	RPE with A2E for 2 hours with blue light (20 min)
C	RPE with Anthocyanin Extract for 24 hours, then with A2E for 2 hours with blue light (20 min)
D	RPE with Anthocyanin Extract for 24 hours, then with A2E for 2 hours with blue light (20 min), then Anthocyanin Extract

Treatment A is the control RPE cells over a 7 day incubation without any treatment and shows the confluent RPE cells. Treatment B shows the effects of the A2E on RPE cells over 7 day incubation. Treatments C and D both show RPE cells incubated with anthocyanin extract for 24 hours, treated with A2E for 2 hours, exposed to blue light from a tungsten halogen light for 20 minutes and then in treatment D the cells were treated with anthocyanin extract.

Treatment A shows RPE cells that remained confluent over the 7 day incubation. Treatment B shows cell death at day 3. Treatments C and D show a decline in cell life at day 7 but could have recovered if nutrients were available. Both treatments had 24 hour incubation so it had been eight days since the initial treatment and nutrients were applied to the cells.

4.4 PEDF Results

The protein concentration of the RPE cell supernatant was analyzed in triplicate using Bio-Rad *DC* Protein Assay kit. The average protein values ranged from 3.11 mg/ml to 8.99 mg/ml. All the supernatants were normalized to a concentration of 3.00 mg/ml in PBS.

The normalized supernatant of all samples were used for the Pigment Epithelial Derived Factor (PEDF) sandwich ELISA antigen detection kit. The unknown values for the samples were determined using the standard curve. The values were then multiplied by 200 to negate the dilution factor. Figures 4.4a, 4.4b, 4.4c and 4.4d show the average values of the triplicate testing of each of the replications of treatments A, B, C and D respectively with error bars of the standard deviation for the sample's readings. Figure 4.4e displays the average of all values for each treatment with the standard deviation for all samples shown as the error bars. Because of the large variation in averages for treatment D between sample a and samples b and c (Figure 4.4d), the a values were not used in determining the average and standard deviation for treatment D in Figure 4.4e. For treatments C and D, samples b and c represent replications that were done

separately from sample a. For treatments A and B, the triplicate testing was done at the same time and the identities of a, b and c were arbitrarily assigned for identification purposes.

For all treatments, the cell culture was run in triplicate and the PEDF results were run in triplicate for each triplicate. Treatment A had an overall average of 7.700 ± 1.282, 7.368 ± 1.896, 8.947 ± 1.710 and 9.318 ± 1.762 ng PEDF per mg protein for collection days 1, 3, 5 and 7 respectively. Treatment B had an overall average of 5.750 ± 2.688, 7.622 ± 1.886, 9.493 ± 0.7916, and 11.13 ± 1.481 ng PEDF per mg protein for collection days 1, 3, 5 and 7 respectively. Treatment C had an overall average of 10.17 ± 2.068, 9.633 ± 1.494, 11.26 ± 4.705 and 9.734 ± 1.577 ng PEDF per mg protein for collection days 1, 3, 5 and 7 respectively. Treatment D had an overall average of 7.458 ± 4.190, 10.71 ± 6.370, 9.114 ± 5.290 and 7.418 ± 4.377 ng PEDF per

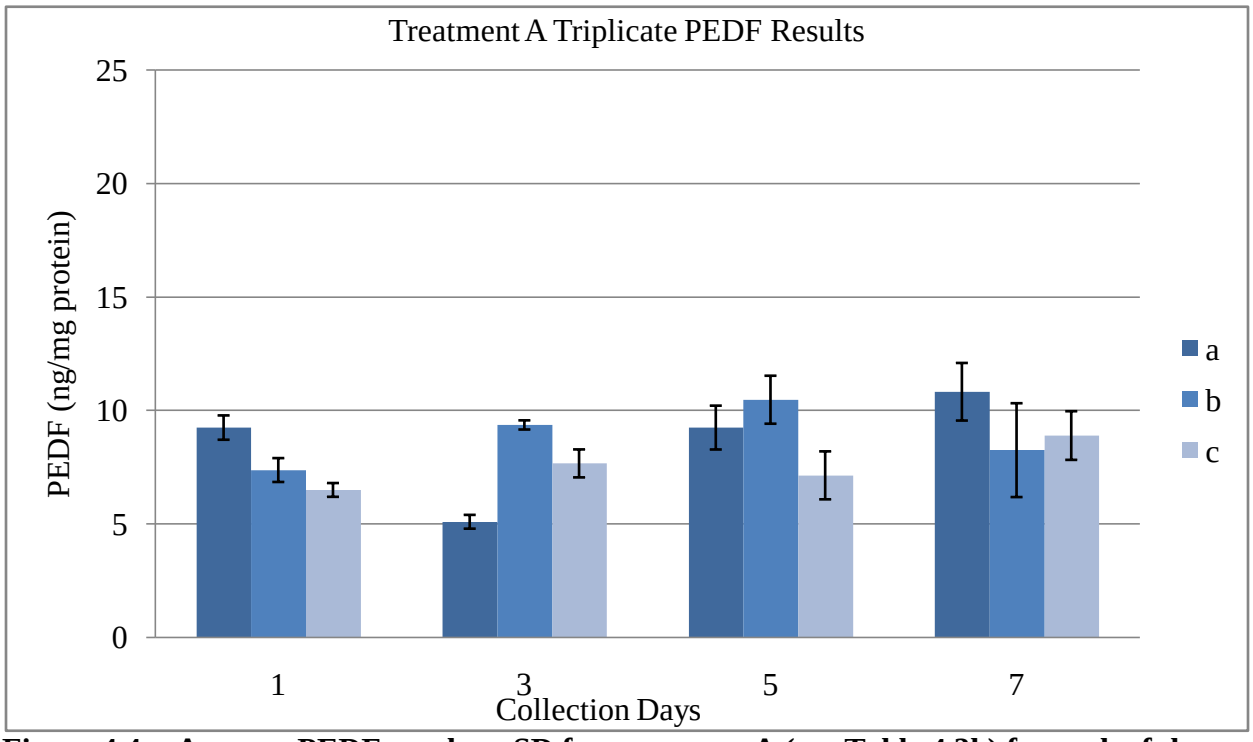


Figure 4.4a: Average PEDF results ± SD for treatment A (see Table 4.3b) for each of the triplicate treatments (a, b, and c) for each of the collection days as determined by ELISA.

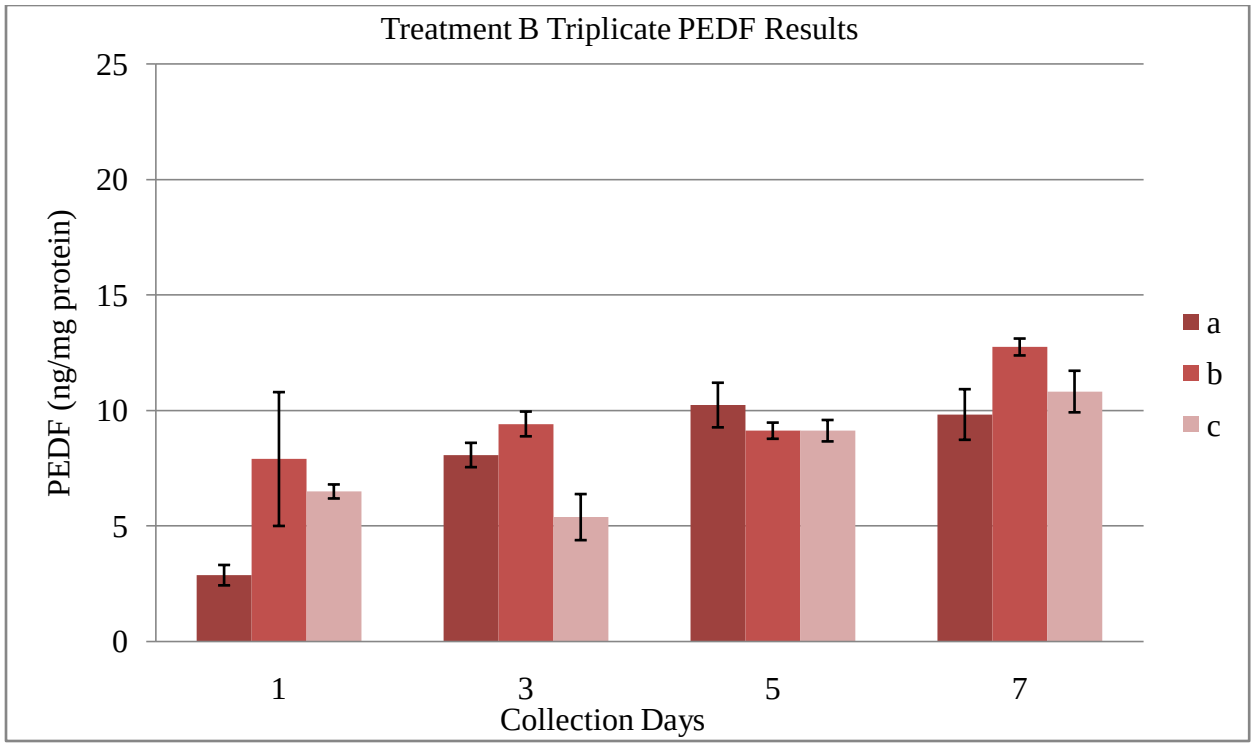


Figure 4.4b: Average PEDF results ± SD for treatment B (see Table 4.3b) for each of the triplicate treatments (a, b and c) for each of the collection days as determined by ELISA.

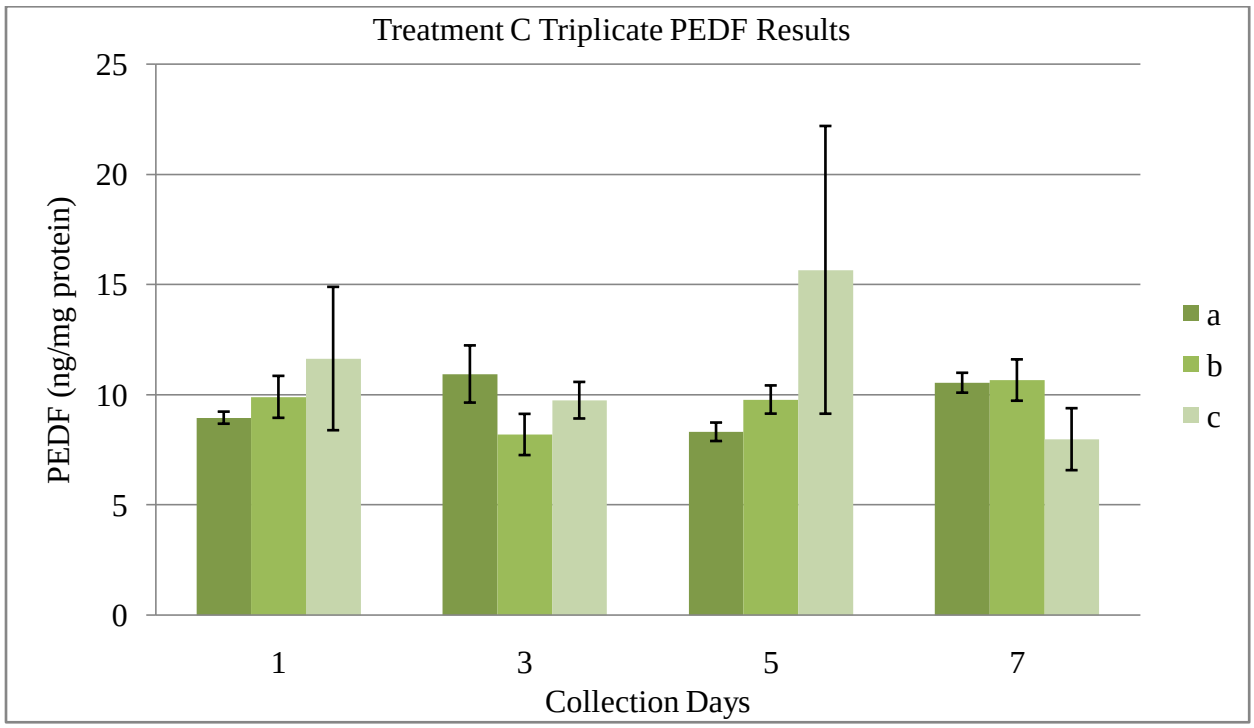


Figure 4.4c: Average PEDF results ± SD for treatment C (see Table 4.3b) for each of the triplicate treatments (a, b and c) for each of the collection days as determined by ELISA.

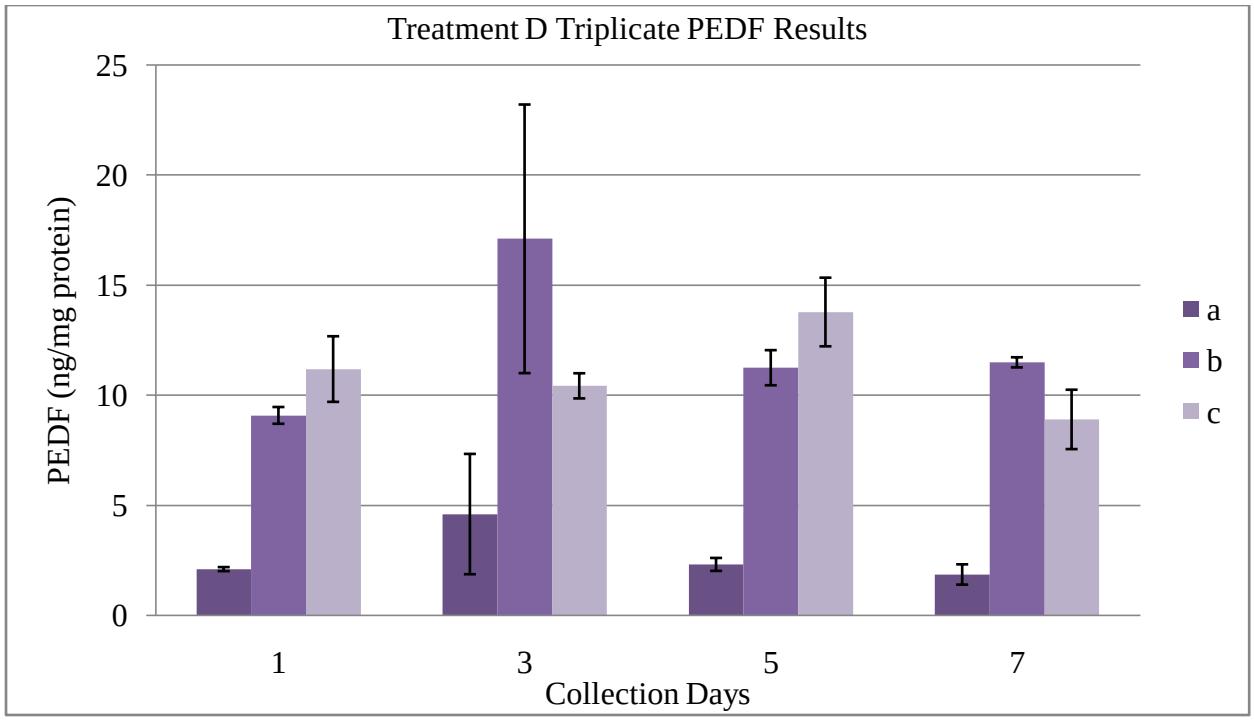


Figure 4.4d: Average PEDF results ± SD for treatment D (see Table 4.3b) for each of the triplicate treatments (a, b and c) for each of the collection days as determined by ELISA.

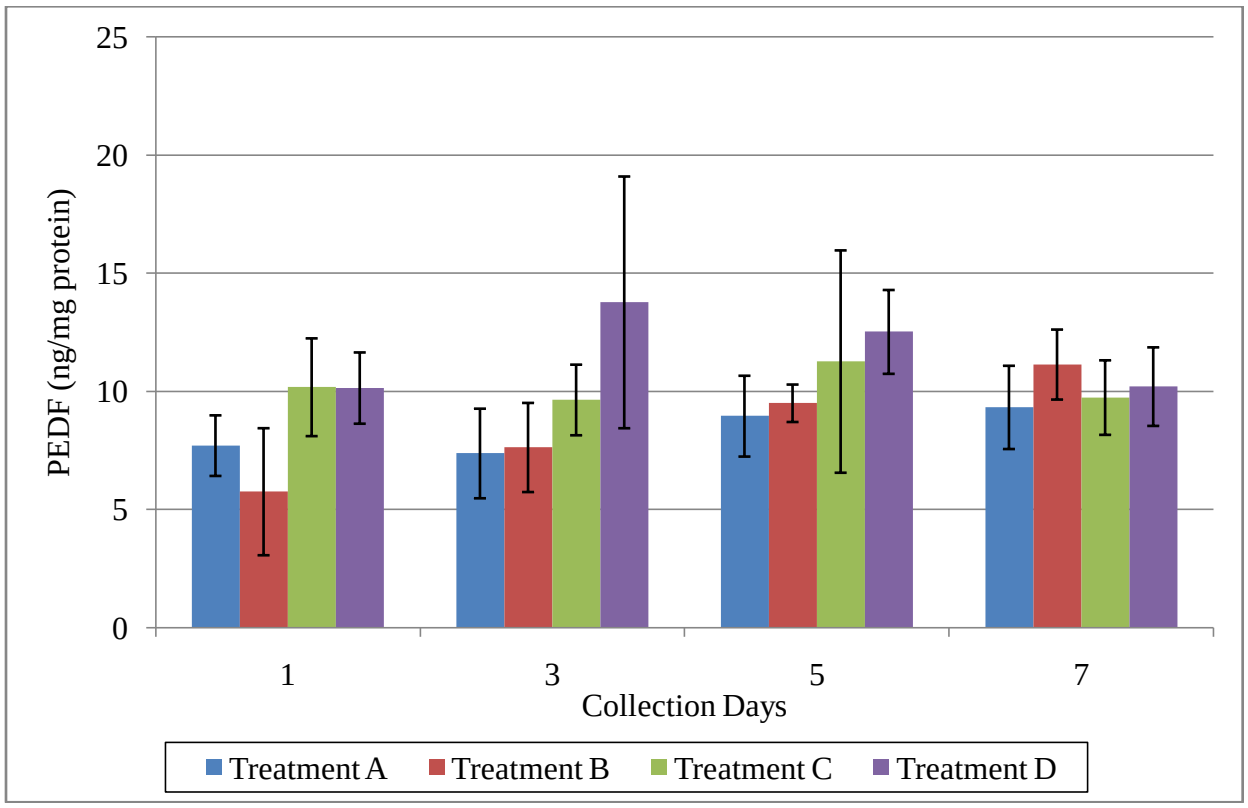


Figure 4.4e: Composite averages of Figures 4.4a, 4.4b, 4.4c and 4.4d of the PEDF ELISA results ± SD for treatments A, B, C and D (see Table 4.3b) for each collection day. Variable a's values were removed from treatment D.

mg protein for collection days 1, 3, 5 and 7. Since the replication of a for treatment D had PEDF values much lower than replications of b and c, the values were removed for statistical analysis and the overall average for days 1, 3, 5 and 7 were now 10.14 ± 1.508, 13.76 ± 5.327, 12.51 ± 1.774 and 10.20 ± 1.662 ng PEDF per mg protein respectively.

All the data points for treatments A, B, C and D for collection days 1, 3, 5 and 7 for the triplicates were analyzed by two way analysis of variance (ANOVA) by the program SigmaStat 3.5. When all data points were used in the analysis, the normality test as well as the equal variance test failed (both $P< 0.05$). The outlier factors of samples a for treatment D were removed from the data points and the data was reanalyzed. The data still failed the normality test but passed the equal variance test ($P=0.424$). Since the number of samples for each treatment and for each day is limited (n=9, n=6 for D), normality is harder to obtain. It is reasonable to assume that if there were more data that the data would have a normal distribution.

The expected results of this experiment were to have the PEDF values remain consistent over the seven day treatment for the control treatment A. The blueberry treated samples (treatments C and D) were expected to have higher PEDF values than the RPE cells treated with A2E (treatment B).

According to the two way ANOVA there was significant interactions ($p<0.05$) over the treatment and collection days. For treatment A there were no significant differences between the different collection days. The PEDF levels remained approximately the same over the collection days for the control RPE cells. For treatment B, there were significant differences between collection days. The values of 9.493 and 11.13 ng PEDF per mg protein for collection days 5 and 7 were significantly higher than the value of 5.750 ng PEDF per mg protein for collection day 1 and collection day 7 was significantly higher than the value of 7.622 ng PEDF per mg protein for

collection day 3. Over time the PEDF levels in the A2E treated RPE cells increased. Treatment C did not have significant differences in PEDF values for the collection days. The PEDF values in the RPE cells treated with blueberry anthocyanin for 24 hours and then plus A2E remained approximately the same over the collection days with no significant differences. For treatment D, the value of 13.76 ng PEDF per mg protein for collection day 3 was significantly higher than the values of 10.14 and 10.20 ng PEDF per mg protein for collection days 1 and 7. The RPE cells that were treated with blueberry anthocyanins for 24 hours and then plus A2E and then plus anthocyanin had significantly higher PEDF levels at collection day 3 as compared to days 1 and 7. The level of 12.51 ng PEDF per mg protein at day 5 did not differ significantly from day 1, 3 or 7.

Significant differences existed between the different treatments for the different collection days. Both treatments C and D had results of 10.17 and 10.14 ng PEDF per mg protein that were significantly higher than the 5.750 ng PEDF per mg protein for treatment B for collection day 1. Both the RPE cells that were treated with blueberry anthocyanin for 24 hours and then exposed to A2E and one of them treated with blueberry anthocyanin after had higher levels of PEDF than the RPE cells treated with A2E for day 1. Treatment D had results of 13.76 ng PEDF per mg protein that were significantly higher than the rest of the treatments for collection day 3. The RPE cells treated with blueberry anthocyanin for 24 hours and then plus A2E and then plus blueberry anthocyanin had significantly higher PEDF results at day 3 than the other three treatments. Treatment D at 12.51 ng PEDF per mg protein was significantly higher on collection day 5 than treatment A at 8.947 ng PEDF per mg protein. The PEDF results for the control RPE cells were significantly less than the RPE cells treated with blueberry anthocyanin for 24 hours and then plus A2E and then plus blueberry anthocyanin on collection day 5. On

collection day 7, there were no significant differences between all the treatments. The PEDF levels on collection day 7 were approximately the same for all the treatments with no significant differences existing. After seven days of incubation, all of the cells nutrients were used up and the treatments impacts could no longer be assessed.

The anthocyanins from blueberries proved effective in protecting the RPE cells from A2E cytotoxicity as apparent in Table 4.3a when comparing treatment B to treatments C and D. Treatment B was RPE cells that were exposed to A2E photooxidation and treatments C and D had blueberry anthocyanins to help prevent the uptake of A2E into the cells (Jang et al, 2005). The PEDF results of treatments C and D on collection day 1 was significantly higher than the treatment B. Treatment D, where the cells were incubated with anthocyanins for 24 hours, exposed to A2E photooxidation and then re-incubated with anthocyanins for seven days, had significantly higher PEDF results for collection day 3 over the A2E photooxidized cells (treatment B) without anthocyanins. This research was the first experiment that analyzed the oxidation protection provided by anthocyanins as a result of PEDF values in RPE cells and therefore cannot be compared to other literature at this time.

CHAPTER 5: CONCLUSION

The main objective of this study was to determine whether anthocyanins from blueberries can prevent the accumulation of the pyridinium bisretenoid, *N*-retinyl-*N*-retinylidene ethanolamine, A2E in retinal pigment epithelial (RPE) cells. To test this objective, A2E was synthesized, blueberry anothcyanins were extracted from blueberry powder, a RPE cell culture study was conducted to investigate four different interactions between RPE, A2E and blueberry anothcyanins and the anti-angiogenic pigment epithelial derived factor (PEDF) was measured by enzyme linked immunosorbant assay (ELISA) to quantify the protection allotted by the blueberry anthocyanins in two of the four different treatments in the cell culture study. The expected results of this study were that the blueberry anthocyanins would inhibit the A2E uptake by the RPE cells and reduce the cytotoxicity in RPE cells. The blueberry anthocyanins would also inhibit PEDF degradation in RPE cells.

The results for the control RPE cells (treatment A) remained consistent over the 7 day incubation with no significant differences. Treatment A had a consistent PEDF level which would be expected since there were no outside factors to cause a spike or decline. The results for RPE cells treated with A2E for two hours and with blue light illumination for 20 minutes (treatment B) produced PEDF values that increased significantly at days 5 and 7 over 1 and 3 respectively. These results could be explained by the RPE trying to recover from the insult caused by the A2E blue light mediated damage. In vivo, the accumulation of A2E is progressive whereas in this cell culture study the medium was changed after blue light illumination.

The two blueberry anthocyanin treatments (C and D) had significantly ($p<0.05$) higher PEDF levels of 10.71 and 10.14 ng PEDF per mg protein at day 1 than treatment B at 5.750 ng PEDF per mg protein. Both treatments C and D had RPE cells incubated with blueberry

anthocyanin for 24 hours then treated for 2 hours with A2E and exposed to blue light for 20 min and treatment D had blueberry anthocyanins added after the A2E treatment. Treatment D for collection day 3 had significantly ($p<0.05$) higher PEDF results at 13.76 ng PEDF per mg protein than the rest of the treatments. On day 5, treatment D had significantly ($p<0.05$) higher levels of PEDF at 12.51 ng PEDF per mg protein than treatment A at 8.947 ng PEDF per mg protein. The results show that the blueberry anthocyanins had a positive effect on the production and maintenance of PEDF within the RPE cells in the cell culture study for days 1, 3 and 5. The PEDF levels were statistically insignificant between the treatments at collection day 7.

The study does have room for improvement in future studies. It would be of interest to see the impact of anthocyanins in RPE cells without A2E treatment. The nutrients in the cells were not refreshed throughout the seven day incubation and should in future studies be refreshed every 2 to 3 days. An interesting assay to add to this study to have a better understanding of the values obtained for the PEDF results would have been to have values for vascular endothelial growth factor (VEGF) since they tend to be inversely related (Ohno-Matsui et al, 2003). VEGF is the essential pro-angiogenic growth factor found to cause a wide range of ocular diseases (Michels et al, 2006). Since VEGF is found at high levels in patients with age related macular degeneration, it would be of interest to determine if blueberry anothcyanins prevent the accumulation of VEGF.

The research did indicate that blueberry anothcyanins did help protect RPE cells from oxidation as measured by PEDF values.

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VITA

Naomi Marie Sundalius was born in September of 1983 in Chillicothe, Ohio. She grew up in Battle Creek, Michigan, and earned her Bachelor of Science in Food Science from Michigan State University, East Lansing, Michigan, in December of 2004. She joined the Department of Food Science at Louisiana State University, Baton Rouge, Louisiana, for her Master of Science in January 2006. In January of 2008 she moved to Minnesota, to work at Michael Foods, Incorporated in Gaylord, Minnesota, as a Food Technologist within Research and Development.