PHOSPHOLIPIDS AND TERPENES ENHANCE THE ABSORPTION OF POLYPHENOLICS IN A CACO-2 CELL MODEL

A Dissertation

by

JORGE ALFREDO CARDONA PONCE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Food Science and Technology

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ABSTRACT

Phospholipids and Terpenes Enhance the Absorption of

Polyphenolics in a Caco-2 Cell Model.

(December 2010)

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M.S., University of Florida

Chair of Advisory Committee: Dr. Stephen T. Talcott

Anthocyanins are the most important class of water-soluble pigments responsible for red to blue colors in various plants. Anthocyanins naturally occur in a broad range of plants and studies have shown associations between fruit consumption and reduction of certain diseases thought to be related to the presence of these and other polyphenolics. However, anthocyanin absorption is fairly poor which hinders their potential to be utilized in the human body.

Absorption of anthocyanins extracted from açaí puree and port wine was assessed. Various combinations of terpenes and phospholipids were added to anthocyanins to modulate and increase their transport within a model system. Açaí and port wine anthocyanins were poorly transported in the absence of phospholipids and terpenes. The

addition of terpenes and phospholipids significantly increased the transport of anthocyanins. Additionally, the presence of phospholipids and terpenes did not influence the way anthocyanins degraded over a 40 day period of time at three different temperatures. Transport of anthocyanins was not dependent on dosage since absorption results were similar at both concentrations of anthocyanins tested. Two methods to mix anthocyanins, phospholipids, and terpenes were also assessed (Sonication and French Press). Comparisons illustrated that both technologies created matrices that maintained the properties of phospholipids and terpenes as transport enhancers.

Finally, a study to determine the efficacy of phospholipids and terpenes on a different type of polyphenolic compound was assessed. Transport of gallic acid was enhanced by the use of these agents that cemented the idea that phospholipids and terpenes can enhance the transport of various types of polyphenolics.

The aiding effect of phospholipids and terpenes was well established and could play an important role in future investigation in this field. Further research needs to be conducted to reveal more information about the nature of these vesicles or associations that phospholipids and terpenes may have with anthocyanins. In vivo studies need to be considered to confirm these effects in rat models and, ideally, in humans. Nevertheless, these findings open a new line of investigation that could harvest promising results for the future of ingredient development for food products.

To my wife, family, and all the people that have believed in me

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I. INTRODUCTION

Polyphenlics are plant metabolites renowned for their antioxidant capacity and their contribution to flavor and color of several fruits and vegetables (Croft 1999). Recently, polyphenolic functionalities such as enzyme inhibition and radical scavenging have attracted consumer attention because of the potential association of these compounds activity with long-term human health (Parr and Bowell 2000). Anthocyanins represent a major group of polyphenolics responsible for the red to blue coloration in plants, flowers, roots, fruits, and vegetables (Bridle and Timberlake 1997). Several *in vitro* studies have shown anthocyanins antioxidant capacity to counter carcinogenic (Jing and others 2008; Kamei and others 1998), inflammatory (Lietti and others 1976; Šarić and others 2009), and atherosclerotic (Lapauld and others 1997; Miyazaki and others 2008) effects. In contrast, the bioavailability of polyphenolics is extremely low which hinders their potential to achieve potential health benefits as shown on *in vitro* models. Anthocyanins, specifically, are among the most common polyphenolics in nature but their absorption is very limited (Ichiyanagi and others 2008; Yi and others 2006).

This dissertation follows the style of Journal of Food Science.

The use of phospholipids and terpenes as transport/absorption enhancing agents could increase bioavailability of anthocyanins. Phospholipids are amphiphilic molecules commonly found in cell membranes. In addition, phospholipids can be found in foods containing membranes such as eggs, milk, and soybeans. Due to their structure, phospholipids act as surfactants by reducing surface tension and interacting with both lipophilic and hydrophilic regions of interfaces (McClements and Decker 2007). Studies have shown their potential to protect and absorb components both lipophilic and hydrophilic. Most of the research explaining absorption regulation using phospholipids has been conducted on lipophilic substances such as vitamin E (Koo and Noh 2000), carotenoids (Baskaran and others 2003; Sugawara and others 2001), and cholesterol (Homan and Hamelehle 1998; Rampone and Machida 1981) in nano-emulsions, but little is known about their potential to protect and transport anthocyanins through the gastrointestinal tract.

Terpenes might also be used as enhancers in the absorption of anthocyanins. Several investigations have demonstrated the effectiveness of terpenes in transdermal drug delivery (Cal 2005, Lim and others 2006; Lim and others 2008) that might be replicated at a transepithelial level. In addition, terpenes mixed with phospholipids could create a matrix where anthocyanins are not only protected against exogenous conditions, but could also enhance their absorption to deliver more of these components to the blood stream, increasing their potential to offset chronic diseases.

Increasing stability and bioavailability of phytochemicals would represent a breakthrough in the food industry since these compounds have gained so much attention

in recent years and any type of enhancement for these compounds would represent future steps in augmenting the quality of foods. In addition, the increase in absorption of phytochemicals would revolutionize the food industry creating opportunities to develop new products and shifting the trend towards optimization of phytochemicals absorption and stability. Most of the encapsulation research and use of phospholipids and terpenes has been done on a pharmaceuticals and this field is growing as technology becomes more available and cost effective, and as the demand for healthier foods increases. Encapsulation technology in the food industry has been related mainly to protection and delivery of enzymes and antimicrobials (Keller 2001; Mozafari and others 2008). Therefore, the objectives of this research were to investigate the potential impacts of phospholipids and terpenes on the stability and bioavailability of anthocyanins and; investigate the mechanisms by which terpenes and phospholipids may interact with anthocyanins.

This research study assessed the absorption/transport of anthocyanins with and without the presence of phospholipids and terpenes in the matrix. Furthermore, two techniques to homogenize samples and elaborate phospholipid-terpene-anthocyanin matrices were used, compared and assessed for bioavailability and physico-chemical stability.

II. LITERATURE REVIEW

2.1 Flavonoids

Flavonoids are phytochemicals recognized for their red, purple, and blue color and their association with health in diets rich in fruits and vegetables. Currently, more than 6,000 flavonoids have been identified from diverse plant species. Flavonoids comprise a wide group of compounds that share a similar diphenylpropane (C6-C3-C6) basic structure (Figure 2-1) and depending on the position of the association on the aromatic ring and the benzopyrano, flavonoids could be divided in three main groups: flavonoids, isoflavonoids, and neoflavonoids (Marais and others 2006; Winkel 2006).

Flavonoids are formed from the condensation of phenylpropane with coenzyme A to form chalcones that will then form other structures. Flavones, flavanones, flavonols, flavanonols, flavan-3-ols, and anthocyanidins are differentiated by the level of oxidation of the central pyran ring of the main diphenylpropane structure (Shahidi and Naczk 2003). Other differences within each flavonoid subgroup are dictated by the number and distribution of hydroxyl and carboxyl groups and the degree of alkylation or glycosylation (Le Marchand 2002).

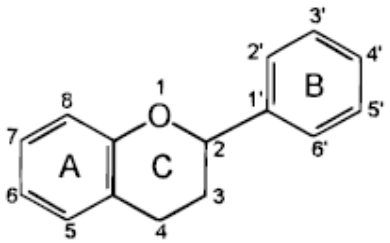


Figure 2-1. Basic flavonoid structure (Pietta 2000).

Flavones and flavonols comprise the majority of flavonoids in foods with approximately 100 flavones and 200 flavonols identified to date. The most common flavonols include myricetin, quercetin and kaempferol, found in many fruits and vegetables. Flavonols differ from flavones due to the presence of a hydroxyl group on the 3-position and are also known as 3-hydroxyflavones (Shahidi and Naczk 2003; Le Marchand 2002). Flavanones and flavanonols have a saturated C-ring. Flavanones are mainly found in citrus fruits and are frequently glycosylated in the 7-position with disaccharides (Tomás-Barberán and Clifford 2000). Catechins and anthocyanins are also known as flavans and are a significant group of flavonoids. Catechins are primarily found in tea and wine while anthocyanins are generally found in many berries, grapes, flowers, and other colored fruit and vegetable sources (Le Marchand 2002; Shahidi and Naczk 2003).

Due to their bright coloration, flavonoids can act as visual attractants for pollinating insects. Some flavonoids might also have protective mechanisms against predatory insects (Pietta 2000, Winkel 2006). In addition to their physiological functions in plants, flavonoids constitute a significant component of the human diet as they are present in most edible fruits and vegetables. Dietary intake of flavonoids varies significantly, ranging from 3 to 800 mg/day (Erlund 2004; Le Marchand 2002; Pietta 2000).

2.1.1 Anthocyanins

Anthocyanins are the most important class of water-soluble pigments responsible for the red, blue and violet colors in many fruits, vegetables, roots, tubers, bulbs,

legumes, cereals, leaves and flowers (Bridle and Timberlake 1997). Several fruits contain high concentrations of anthocyanins and studies have shown a relationship between fruit consumption and reduction of certain diseases attributable to the presence of antioxidant polyphenolics (Rommel and Wrosltad 1993; Parr and Bowell 2000; Aaby and others 2005). Anthocyanins are effective radical scavengers and can break free radical reactions through their electron donation, metal chelation, enzyme inhibition, and oxygen radical quenching capabilities (Kong and others 2003; Pastrana-Bonilla and others 2003; Pietta 2000).

2.1.1.1 Structure and occurrence

Anthocyanins are flavonoids formed by the cyclation of a chalcone molecule under acidic conditions (Shahidi and Naczk 2003). Variations on the hydroxylation (OH) and/or methoxylation (OCH_3) of their structure (B-ring) yield 17 naturally occurring anthocyanins. However, only six of them (Figure 2-2) are common in higher plants. From those six, Cyanidin (Cy), Delphinidin (Dp), and Pelargonodin (Pg) are the most widespread in nature (Kong and others 2003). Anthocyanins are exclusively found as glycosides in intact tissues of flowers and fruits. They can be bound to one or more molecules of sugar which yields more than 200 different anthocyanins that have been identified (Shahidi and Naczk 2003).

The flavylium cation arrangement (2-phenylbenzopyrilium) is the basic structure of the anthocyanins that has conjugated double bonds responsible for the color of these structures (Rein 2005). Depending on the presence of hydroxyl or carboxyl, the hue of the color will vary between blue and red, respectively (Shahidi and Naczk 2003).

Anthocyanins are usually glycosylated with glucose, galactose, arabinose, xylose, or rhamnose as 3-glycosides (monoglycosides), 3,3, 3,5 or 3,7-diglycosides (diglycosides) or triglycosides (Rein 2005). Anthocyanins can also be acylated with organic acids that are usually aromatic or aliphatic dicarboxyl acids bound to the anthocyanin through ester bonding. The most common acylating agents include hydroxycinnamic acids (*p*-coumaric, ferulic, caffeic and sinapic acids), hydroxybenzoic acids (gallic, *p*-hydroxy-benzoic, protocatechuic, and vanillic) and aliphatic acids including malonic, acetic, malic, succinic and oxalic acids (Francis 1989; Bruneton 1995; Cabrita and Andersen 1999).

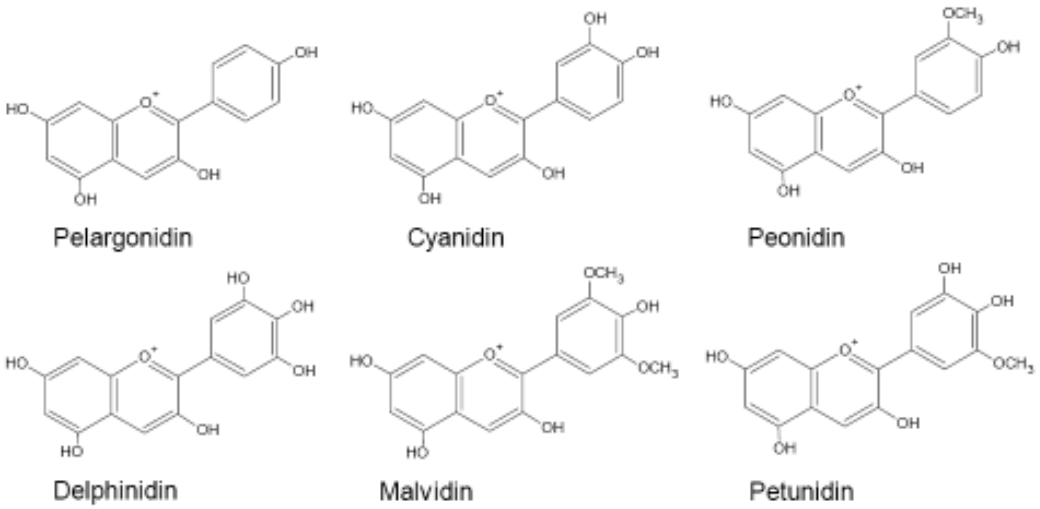


Figure 2-2. Chemical structures of anthocyanidins (Shahidi and Naczk 2003).

2.1.1.2 Color stability

The stability of anthocyanins is intimately related to self-association, concentration and structure, pH, organic chemicals, temperature, light, enzymes, oxygen, copigments, metallic ions, ascorbic acid, sugars, and processing (Shahidi and Naczk 2003; Stingzing

and others 2002). Glycosylation, acylation, methylation, and hydroxylation contribute to anthocyanin stability. It has been reported that acyl groups, such as aromatic and aliphatic acids, improve the juice color stability by interacting with anthocyanins. Increase in acylation of anthocyanin increases the stability of the molecule (Bassa and Francis 1987; Giusti and Wrosltad 2003; Rein and Heinonen 2004). In addition, hydroxylation in positions C-4 and C-5 prevents water addition that results in the formation of colorless species (Saito and others 1995; Rein 2005; Turker and others 2004; Shahidi and Naczk 2003). Copigmentation which is the association of enhances the perception of color by increasing the absorbance due to pigment concentration and association with other compounds by hydrophobic interaction between aromatic bases of the molecules involved (Shahidi and Naczk 2003). Anthocyanin copigmentation results in a stronger and more stable color than a singular anthocyanin molecule. Furthermore, overlapping association of copigmentation prevents the exposure of molecules to nucleophillic attack of water (Rein 2005). Copigments are colorless or slightly yellowish natural molecules in plant cells that exist along with anthocyanins. Copigments include flavonoids, organic acids, amino acids, and metal ions (Brouillard and others 1989).

Anthocyanins are extremely susceptible to pH shifts. Anthocyanins in solution exist in four different forms: blue neutral and ionized quinonoidial base, red flavylium cation or oxonium salt, colorless pseudobase, and colorless chalcone (Figure 2-3). Each of the four species has a variety of tautomeric forms and the chalcone could exist as *cis* or *trans* forms (Shahidi and Naczk 2003; Clifford 2000).

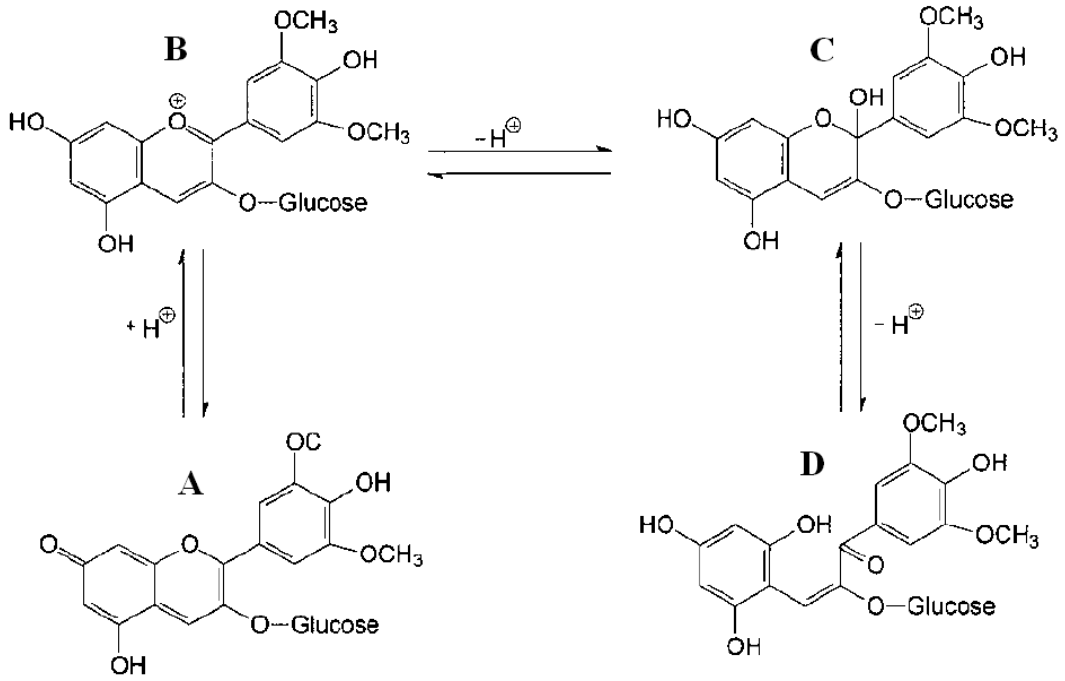


Figure 2-3. Anthocyanin equilibria: quinonoidal base (A), flavylium cation (B), carbinol base or pseudobase (C) and chalcone (D) (Clifford 2000; Shahidi and Naczk 2003).

Even though anthocyanins can exhibit a diversity of color tones in the pH range from 1-14, they are more stable at acidic media showing an intense red coloration in the pH range of 1 to 3 (Rein 2005; Shahidi and Naczk 2003). The increase of pH reduces the concentration of the flavylium cation, thus decreasing the intense red color to form the colorless carbinol base. As pH continues to increase (Figure 2-4), the colored quinonoidial form is produced by losing a hydrogen atom. If pH continues to rise, the cabinol base yields a colorless chalcone form (Rein 2005).

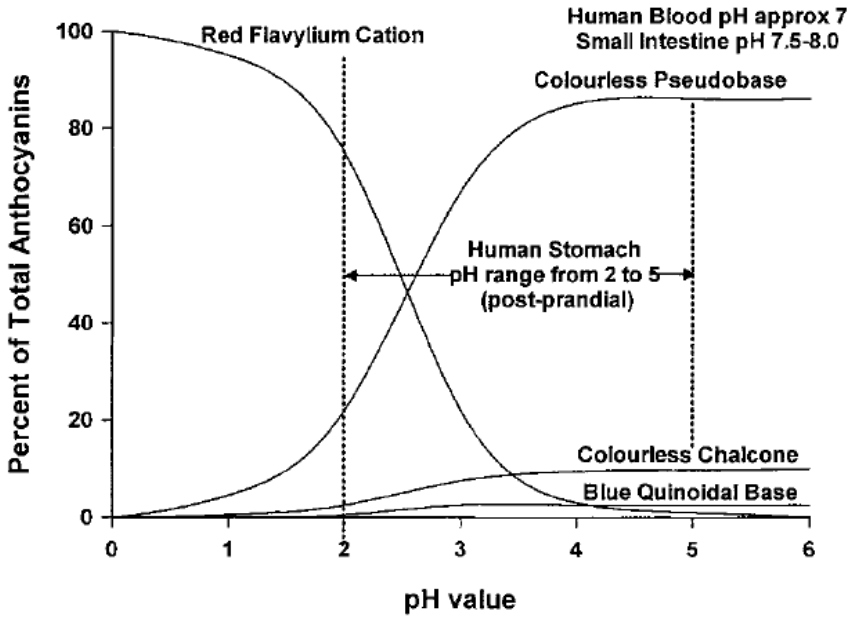


Figure 2-4. Effect of pH value on anthocyanin equilibria (Clifford 2000).

Anthocyanin stability is also affected by temperature. Thermal degradation follows first order kinetics (Kirca and others 2006). Elevated temperatures alter the anthocyanin equilibria or hydrolyze the glycosidic bonding to form unstable chalcones or aglycone forms, respectively. Ultimately, thermal degradation leads to the formation of brown pigments (Rein 2005; Clifford 2000). Conversely, extremely low temperatures also affect the quality of anthocyanins. Low temperatures favor quinonoidal base formation. Therefore, if a product was frozen red, it might appear blue after thawing due to the change of flavylium cation to quinonoidal form during that low temperature exposure (Bridle and Timberlake 1997).

Oxygen intensifies the degradation of anthocyanins. Even though the formation of unstable chalcones due to pH or thermal changes is reversible, the presence of oxygen

during these procedures impedes the normal reconversion of these compounds (Bridle and Timberlake 1997). The effect of oxygen on anthocyanins occurs as direct oxidative mechanisms or through indirect oxidation, yielding colorless or brown end products (Rein 2005). Visible and UV light are also harmful to anthocyanins (Laleh and others 2006). Although light is needed in the biosynthesis of anthocyanins, once formed light damages these compounds (Markakis 1982). In a photochemical study, Furtado and others (1993) found that aqueous solutions of anthocyanins submitted to irradiation help with the disappearance of the flavylium cation due to the formation of the chalcone form.

Enzymes can also contribute to the overall degradation of anthocyanins, thus inactivation of these compounds is important in the production of a variety of fruit and vegetable products (Fang and others 2006). The most common enzymes related to the degradation of anthocyanins are glycosidases. Glycosidases are not specific in the structural requirements of the aglycone portion of a molecule (Huang 1955); thus, they cleave the anthocyanins separating the sugar from the unstable aglycone form. Peroxidases (POD) and polyphenol oxidases (PPO) are enzymes naturally present in fruits that degrade phenolics compounds resulting in the formation of precursors of brown pigments (Kader and others 1997). PPOs degrade anthocyanins indirectly by the formation of quinones that subsequently will react with anthocyanins to form colorless products (Fang and others 2006; Kader and others 1998).

Sugars, ascorbic acid, and bisulfites also impact anthocyanin stability as they play a dual role in anthocyanin stability. Sugars and syrups could be used as cryoprotectants

by associating with plant water by osmosis. This process is known as osmotic dehydration (Wang 2006). In contrast, sugars can participate in the formation of browning products (furfural and Maillard products) which promote the degradation of anthocyanins (Tsai and others 2005). Ascorbic acid fortification has commonly been used in fruit juices as antioxidant protection and to increase the nutritional value. Ascorbic acid has proven to retard enzymatic browning by reducing *o*-quinones to *o*-diphenols that no longer produce brown pigments or degrade anthocyanins (Gregory 1996; Kader and others 1998). However, addition of ascorbic acid was shown to degrade anthocyanins in pomegranate (Marti and others 2001) and açaí juice (Pacheco-Palencia 2006). Bisulfite and other sulfurous compounds are also used to protect color and phenolic compounds. These compounds are usually used in wine production as antioxidants and bacteriostatic agents (Morata and others 2006). Bisulfite, like ascorbic acid, reacts with the *o*-quinone to eliminate the basic compound to form brown pigments (Lindsay 2007). Nevertheless, SO_2 prevents the formation of visitins. Visitins are compounds formed by condensation of anthocyanins and pyruvic acid or acetaldehyde released by certain strains of yeast. Visitins are more stable than anthocyanins and do not affect the desired color of these compounds (Morata and others 2006).

2.1.1.3 Anthocyanins as antioxidants

Anthocyanins are naturally occurring antioxidants that prevent oxidation of substrates. This prevention occurs not only in foods but also in humans, relating anthocyanins with the control of many degenerative diseases. Antioxidants protect oxidative substrates by reducing the concentration of oxygen, intercepting singlet

oxygen, or scavenging initial radicals to prevent the activity of reactive oxygen, nitrogen and chlorine species that are closely related to diseases such as arthritis, diabetes, and atherosclerosis (Shahidi and Naczk 2003; Le Marchand 2002). Even though the precise association of polyphenolics with some diseases is not fully understood, flavonoids have proven not only to inhibit enzymes directly related in the generation of reactive oxygen species, but also chelate metals that are important in the oxygen metabolism (Pietta 2000).

The most predominant method of antioxidant activity seems to be the hydrogen donation, also known as radical scavenging (Robbins 2003). Free radicals cause extensive damage to macromolecules in the body including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and cellular tissues (Khan and others 2008; Montoro and others 2005). Free radicals remove a proton from macromolecules, generating highly reactive radicals of high molecular weight creating a chain degradation reaction where radicals are trying to stabilize by removing a proton from a neighboring molecule. Anthocyanins may donate a hydrogen atom, breaking this degradation cycle. Furthermore, if anthocyanins can react with initial forms of free radicals, they could donate their proton, thus quenching the free radical and producing a less reactive radicals that will be subsequently stabilized by resonance delocalization (Parr and Bowell 2000; Shahidi and Naczk 2003). Many studies have suggested that the antioxidant properties of flavonoids are generally located in the B ring of the molecule, more specifically in the number of hydroxyl groups present in that ring (Reviewed by Pietta 2000).

2.1.1.4 Absorption of anthocyanins

Bioavailability of phytochemicals is an important issue when trying to correlate *in vitro* studies concerning chemopreventive and antioxidant properties of anthocyanins with *in vivo* results in disease prevention (Balimane and others 2000). Research has shown that anthocyanin absorption is extremely low since minute concentrations were detected in Caco-2 cell monolayer models, and plasma from rats and humans (Ichiyanagi and others 2008; Miyazawa and others 1999). Miyazawa and others (2009) also demonstrated the direct absorption of anthocyanins in rats and humans but due to their low stability at high pH, only trace amount could be found and quantified. To date investigations are still trying to understand the mechanisms by which anthocyanin are absorbed and only modest effort has been focused on improving this outcome.

2.2 Phospholipids

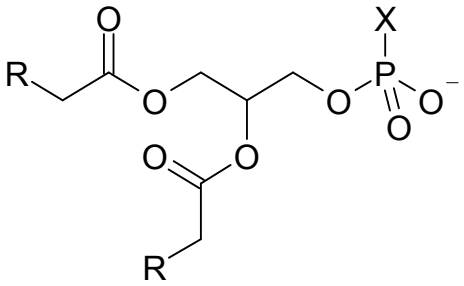
Phospholipids, also known as phosphoglycerides, are amphiphilic lipid molecules found as the major elements of biological membranes. Phospholipids act as surfactants (emulsifiers) by reducing the surface tension between hydrophilic and hydrophobic compounds. As with other surfactants, phospholipids are made of polar (hydrophilic) and non-polar (hydrophobic) structures. These amphiphilic compounds have been associated with disease prevention and human health due to their potential as agents to lower low-density lipoprotein cholesterol (LDL) in the blood stream (Koo and Noh 2000; Kirana and others 2005; Rampone and Machida 1981).

2.2.1 Structure and Occurrence

Phospholipids are commonly found organized in biological membranes. Phospholipids are triglycerides normally modified in position *sn*-3 where a phosphate group replaces a fatty acid chain (Silvius 1993). The presence of the polar phosphate groups make phospholipids surface active. This property allows them to form bilayers that are critical for biological properties in membranes (McClements and Decker 2007). Phospholipids are common in the human diet as a component of the food matrix and as an emulsifier or stabilizer (Yonekura and others 2006). Phosphatidylcholine (PC) is the most common phospholipid and is often called lecithin which can be commonly extracted from egg yolk or soybeans. Other major phospholipids include phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) depending on the organic molecule attached to the phosphate group. This class of compounds can also be found in organelles and membranes of mammals, fungi, bacteria, fish, and other living organisms (Neidleman 1993; Yorek 1993).

Phospholipids are synthesized from phosphatidic acid. Phosphatidic acid could result from three pathways: (1) stepwise acylation of *sn*-glycerol 3-phosphate; (2) acylation of dihydroxyacetonephosphate followed by reduction to 1-acyl-*sn*-glycerol-3-phosphate and a final acylation at position *sn*-2; and (3) by phosphorylation of a diacylglycerol (Longmuir 1993). Subsequently, organic bases (choline, ethanolamine, serine, inositol, or glycerol) attach to the phosphate group to yield a phospholipid. A single phospholipid is comprised of a diglyceride molecule, a phosphate group, and a simple organic molecule (Figure 2-5). Fatty acids of different chain length and in the

saturated and/or unsaturated forms are present at positions *sn*-1 and *sn*-2 (R) of the glycerol backbone. The phosphate group is commonly attached to position *sn*-3, attached to an organic molecule through ester bonding. Variations in the simple organic molecule, in addition to variations of fatty acids structure and positioning, yield a variety of phospholipids as previously mentioned (PC, PE, PI, PS). In addition, the removal of a fatty acid (normally from position *sn*-2) yields *lyso*-phospholipids. All these structural differences create a substantial amount of phospholipids found in nature that have specific characteristics and functions.



X = OH	Phosphatidic acid (PA)
X = O-$(CH_2)_2$-$N^+(CH_3)_3$	Phosphatidylcholine (PC)
X = O-$(CH_2)_2$-NH_2	Phosphatidylethanolamine (PE)
X = O-CH_2-$CH(NH_2)$-COOH	Phosphatidylserine (PS)
X = $C_6H_{12}O_6$	Phosphatidylinositol (PI)
R = Fatty acid chain	

Figure 2-5. Structure of phospholipids (McClements and others 2007).

2.2.2 Hydration of Phospholipids and Formation of Liposomes

Lipids can be conveniently classified in three groups depending on their behavior in water (McIntosh and Magid 1993). Insoluble, non swelling amphiphiles (cholesterol and waxes) which do not imbibe water (I), insoluble amphiphiles (II) which merely swell in water to form ordered "mesophases" (phospholipids with acyl chains longer than 8 carbons), and soluble amphiphiles (III) forming lyotropic liquid crystals at lower

water content (lysolecithins, short acyl chain phospholipids, and salts of long-chain fatty acids).

At concentrations above the critical micelle concentration (CMC) phospholipids form a variety of structures depending on the water content. Self-aggregation and polymerization of phospholipids is driven thermodynamically by the hydrophobic effect (Singh and Shnur 1993; Walstra and van Vliet 2007). In the presence of water, phospholipids organize into spherical, disc-like, or cylindrical micelles, or into bilayers and vesicles. Spheres or oblate spheroids are the simplest shapes that minimize surface area/volume ratio (McIntosh and Magid 1993; Singh and Schnur 1993). The polar head groups array on the surface and aggregate. Since one dimension of the head groups cannot embrace or exceed the fully extended chain length, phospholipids arrange in spheres. Phospholipids, with double acyl chains will tend to form double-layered vesicles rather than micelles because of the area required per lipid molecule (McIntosh and Magid 1993). Area per lipid molecule is lower for bilayers than any micellar form. In addition, bilayers can accommodate limitless number of chains without requiring a change in area per molecule (Barenholz and Crommelin 1994; McIntosh and Magid 1993). Once assembled, bilayers commonly form closed fluid-filled vesicles (Figure 2-6).

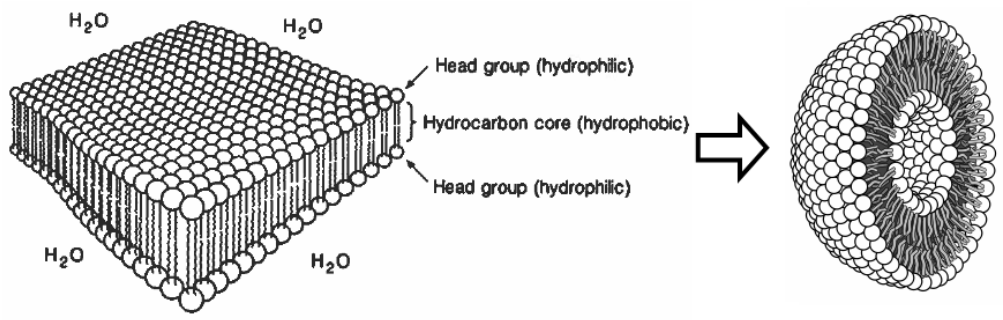


Figure 2-6. Structure of bilayers and formation of a liposome (Adapted from Reineccius 1995).

Phospholipids spontaneously hydrate in the presence of excess water forming closed membrane structures called liposomes (Figure 2-7) (Hauser 1993; Reineccius 1995). Liposomes of multiple layers are called multilamellar vesicles (MLVs) and are spherical particles consisting of concentrically arranged, equally spaced bilayers that are separated by water (Hauser 1993; McIntosh and Magid 1993). MLVs can range in diameter from 0.2 – 10 μm with a water holding capacity of about 4 μL per milligram of liposome (Yesair 1990). Multivesicular vesicles (MVVs) can also be formed and they consist of smaller vesicles formed inside larger vesicles (Barenholz and Crommelin 1994). Water is imbibed by the phospholipids up to a point and additional water forms an excess fluid phase. Lipid and water form separate layers. Water that is imbibed by phospholipids can be strongly associated with the head groups and also weakly associated within vesicle layers (Hauser 1993; McIntosh and Magid 1993).

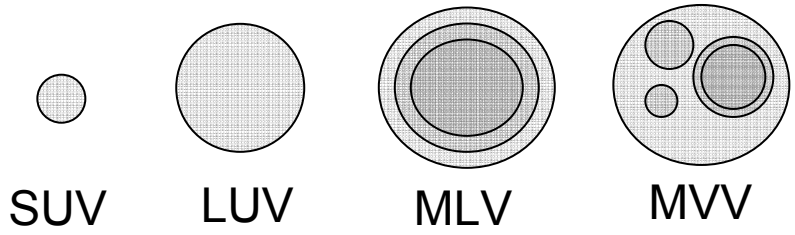


Figure 2-7. Various liposome structures (Adapted from Taylor and others 2005).

When energy (mechanical, electrical or chemical) is included in the system, MLVs and MVVs start breaking to form unilamellar vesicles of different sizes (SUVs and LUVs) (Taylor and others 2005). Vesicles undergo repeated disruption-resealing cycles that produce smaller, more homogeneous vesicles (Hauser 1993). Since energy is required to produce these vesicles, LUVs and SUVs are thermodynamically unstable mostly because of the vesicle's curvature that creates free energy (McIntosh and Magid 1993). Therefore, smaller vesicles will tend to coalesce and re-fabricate bigger multilamellar vesicles which are the most stable structures (Fan and others 2007).

2.2.3 Phospholipid and Liposome Stability

The chemical structure of phospholipids plays a significant role on their stability. Phospholipids can undergo hydrolytic splitting in strongly acidic and alkaline media. These compounds are only stable at neutral pH values. Splitting leads to the formation of glycerophosphates and free fatty acids. Further hydrolysis of glycerol phosphate will yield the separation of the organic base from the glycerophosphate molecule. Further degradation in alkaline media will yield the formation of 2- and 3-glycerolphosphates (Evstigneeva 1993).

Enzymes can also affect stability and structure of phospholipids. There are four types of phospholipases (A_1, A_2, C, and D) that react with phospholipids and are very region and stereo selective. Phospholipases A_1 and A_2 hydrolyze ester bonds at positions *sn*-1 and *sn*-2 of the glycerol backbone, respectively. Phospholipases C and D react in position *sn*-3 between the ester bond of the glycerol and phosphate and the phosphate and organic base, respectively. Pancreatic lipase can also affect phospholipids but with no specificity to the location of hydrolysis (Evstigneeva 1993). Similar to other lipids, phospholipids with unsaturated fatty acid chain in their structure undergo auto-oxidation and photooxygenation creating hydroperoxides. In addition, phospholipids are prone to decomposition during storage at high temperatures and with light and oxygen exposure (Evstigneeva 1993).

Instability of liposomes could happen by flocculation, coalescence, or Ostwald ripening (Walstra and van Vliet 2007). Flocculation consists of the aggregation of particles that remain intact. Particles tend to clump forming flocs or flakes which then tend to float over the liquid or precipitate depending on the nature of the system. Coalescence is produced by the rupture of thin films between close droplets. This rupture forces droplets to unite forming a larger particle. Ostwald ripening occurs more in water in oil emulsions and consists of the addition of smaller particles to larger particles. Small particles have high surface energy whereas large particles do not. This favors the inclusion of smaller particles into larger particles reducing the overall surface energy.

2.2.4 Liposome Encapsulation and Controlled Release of Active Ingredients

Liposome encapsulation has been a recognized technology in the pharmaceutical area for years but it is yet to be well established in the food industry (Gouin 2004; Reineccius 1995; Taylor and others 2005; Yesair 1990). Currently, efforts of liposome technology in the food industry are concentrated on encapsulation of enzymes to accelerate and optimize cheese processing (Kheadr and others 2006; Picon and others 1993), encapsulation of antimicrobials (Taylor and others 2008; Were and others 2003; Were and others 2004), and stabilization of vitamins (Kirby and others 1991). As liposome encapsulation is developing as a novel process, natural sources of phospholipids should be explored to reduce cost for food systems. The food industry differs from the pharmaceutical industry since it is very dependent on cost efficiency. For that reason, this technology's cost needs to be minimized to insure the widest use in large-scale food applications (Reineccius 1995). Other areas of improvement include the evaluation and optimization of flavor and aroma encapsulation and delivery (Taylor and others 2005). Finally, stability of liposomes to processing (temperature and pressure) should be improved. Some investigators (Filipovic-Grcic and others 2001; Guo and others 2003; Laye and others 2008) have been working on this area also known as release on demand. This area illustrates how liposomes could be enhanced to withstand processing and other hostile environments to deliver the desired payload to a specific site.

2.3 Terpenes

Terpenes also known as terpenoids and isoprenoids represent the largest class of metabolites with more than 40,000 structures. Many of these compounds possess essential physiologic, metabolic, and structural roles in numerous plants (Bohlman and Keeling 2008). In addition, terpenoids are responsible for aromas and flavors in fruits and herbs (Lindsay 2007). The chemical diversity of plant terpenoids may underlie their widespread biological activities. These activities drive interest in pharmaceutical, food, cosmetics, and pesticide applications (Sell 2003; Wang and others 2005).

2.3.1 Structure and Occurrence

Terpenes originate from the mevalonic acid (MEV) and the 2-C-methyl-D-erythritol-4-phosphate (MEP) metabolic pathways that yield two isomeric five-carbon structures, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) that serve as precursors for terpenoid synthesis (Cane 1999; Wang and others 2005). The smallest plant terpenoids are hemiterpenoids (C_5) which can be formed directly from DMAPP and IPP (Miller and others 2001; Sell 2003). Combination of one of more IPP and DMAPP molecules, catalyzed by enzymes (prenyltransferases), can synthesize linear prenyl diphosphates which serve as precursors of larger terpenoids (Miller and others 2001; Wang and others 2005). Thus, terpenoids can be classified in hemi (C_5), mono (C_{10}), sesqui (C_{15}), di (C_{20}), sester (C_{25}), tri (C_{30}), tetra (C_{40}), and polyterpenoids ($C_{n \times 5}$) based upon the chain length and the amount of isoprene units present in the molecule. After the formation of the backbone terpenoid structure, terpenoid modifying enzymes give raise to a variety of structures and classifications (Figure 2-8) with

distinctive characteristics and functions in plants (Bohlman and Keeling 2008; Wang and others 2005).

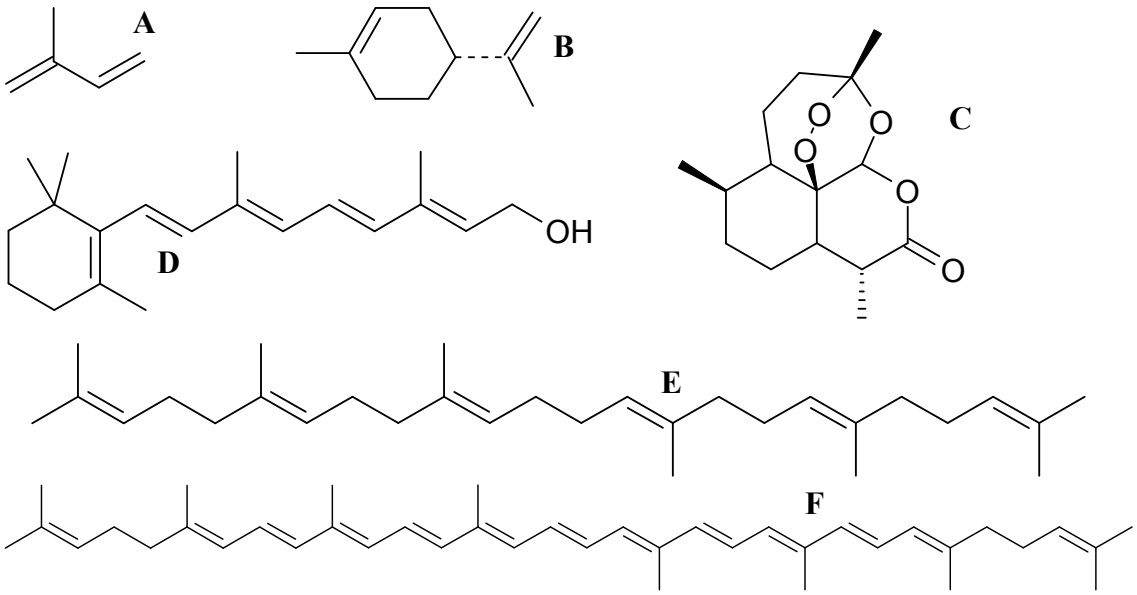


Figure 2-8. Structure of various terpenes: (A) isoprene (C_5), (B) limonene (C_{10}), (C) artemisinin (C_{15}), (D) retinol (C_{20}), (E) squalene (C_{30}), and (F) lycopene (C_{40}).

Terpenoids are widely distributed in almost 100 orders of plants. Due to the immense number of molecular forms that exist for these compounds, they are present in various sources such as oils, bitters, resins, green tissue, roots, fruits, and latex, among others (Banthorpe 1991). Monoterpenes are volatile compounds known as components of essential oils, floral scents, and resins of aromatic plants. Common examples of monoterpenes include limonene, menthol, pinene, carvone, and geraniol, among others (Charlwood and Charlwood 1991; Wang and others 2005). Sesquiterpenes comprise the largest class of terpenoids and occur in marine and terrestrial organisms. Artemisin, α-bisabolol, guaianolide, and eudomanolides are examples of this terpenoid group (Fischer 1991; Fraga 1991). Diterpenes are commonly found in plants, fungi, insects, and marine

organisms. Examples of diterpenes include phytol, taxol, and gibberelins (Beale and Willis 1991; Hanson 1991). Sesterterpenes are quite uncommon and are found primarily in marine organisms and fungi (Wang and others 2005). Triterpenes are a diverse terpenoid group derived from squalene. These compounds are very widespread making it difficult to generalize their distribution (Connoly and Hill 1991).

2.3.2 Terpene Stability

Stability of terpenes is closely related to the presence of oxygen and light in the environment (Sell 2003). Batterman and others (1998) assessed the stability of various terpenes exposed to humid air, humid nitrogen gas, and dry air. A strong effect was illustrated by dry air exposure on half-life of limonene, α-pinene, β-pinene, and 3-carene. Limonene, specifically, is very sensitive to oxidation and could convert to limonene oxide, limonene hydroperoxide, and carvone (Bertollini and others 2001; Clark and others 1981; Djordjevic and others 2008; Thomas and Bessière 1989). Terpenes are also susceptible to elevated temperature exposure. Yang and others (2007) demonstrated the effect of temperature treatment on the stability of certain terpenes. In addition, Cully and others (1991) developed a procedure to remove terpenes from fruit oils since they showed limited storage stability and thermolability. In contrast, terpenes in heated olive oil showed marked stability with a loss of only 23% in 72 hours at 180 ºC while more than 30% of total sterols were lost in the same time (Boskou 1978).

2.3.3 Terpenes as Absorption Enhancers

Research has demonstrated the effective mechanism of terpenes to interact with tissue and create openings for several drugs (Elgorashi and others 2008; Lim and others

2008) that could promote the access of anthocyanins into the blood stream. Transdermal efficiency of terpenes in drug delivery has been long established (Cal 2005. Lim and others 2006; Lim and others 2008) and this behavior could be replicated in the gastrointestinal tract if a product rich in terpenes is used. Another possibility is for terpenes to interact with anthocyanins and make them more lipophillic thus enhancing their absorptivity. There is a group of compounds known as prenylflavonoids that are combinations of isoprene units C-prenylated to ring A of flavonoid structures (Barron and others 1996). These compounds are commonly found in roots, barks, and seeds of *Leguminoseae*, *Moraceae*, and *Asterceae* plant families. Studies have been conducted to assess their impact on melanin biosynthesis by tyrosinase inhibition (Arung and others 2006; Son and others 2003) but no absorption/transport studies have been conducted with these compounds. Possibly due to their structure, they could illustrate better absorption than anthocyanins and flavonoids alone.

2.4 Cell Culture

2.4.1 Transepithelial Transport Assessed with Caco-2 Cells

Almost 40 years ago, a compilation of cell lines was obtained from gastrointestinal tumors, with the intention to analyze cancer mechanisms (Fogh and others 1977). Later, problems in the differentiation of intestinal cells for *in vitro* studies of intestinal transport of compounds and toxicity assessments lead to the use of tumor cells for this purpose. Among all tumor cells, Caco-2 cells showed spontaneous differentiation and expression of numerous morphological and biochemical characteristics observed in small intestine enterocytes making them a clear substitute in the development of *in vitro* modeling of

intestinal absorption and toxicity of compounds (Hidalgo and others 1989b; Pinto and others 1983).

2.4.1.1 Caco-2 cell morphology

Caco-2 cells (Fig 2-9) form a monolayer with a cylindrical polarized morphology, produce microvilli in the apical side, gain tight junctions between adjacent cells, and express small intestinal enzyme activity (Anderson and Van Itallie 1995; Chantret and others 1988; Matsumoto and others 1990). To further improve the steric conditions of Caco-2 monolayers (conditions that exist in the intestine), cells are seeded in permeable inserts which allowed nutrient access from both sides of monolayers. With all these charactiristics in mind, Caco-2 cells have been broadly employed for intestinal transport and toxicity analyses (Artursson 1990; Artursson and others 2001; Hidalgo and others 1989b).

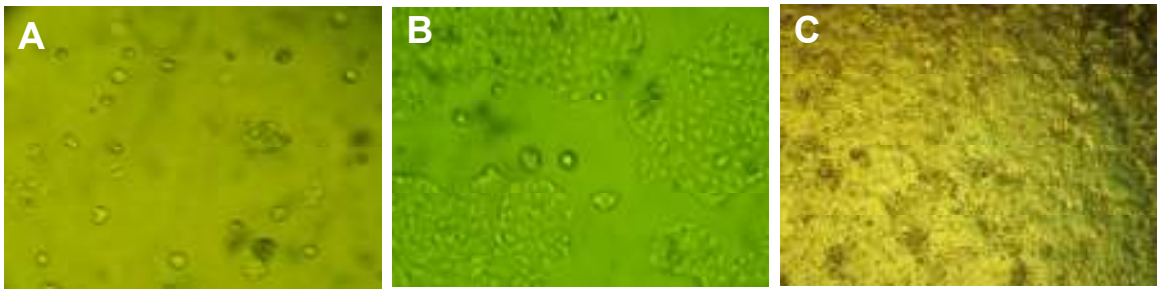


Figure 2-9. Caco-2 cells at different life cycle stages: (A) proliferation, (B) 75% confluency, (C) fully differentiated (21 days after confluency).

Caco-2 cells are morphologically similar to enterocytes once they have reached full differentiation evidenced by the presence of microvilli and the formation of tight

junctions that separate the apical from basolateral domains (Hidalgo and others 1989b). Monolayers created show formation of two distinct patterns. Some cells can form a thick layer of brush border with high density of microvilli while some can form clusters (Delie and Rubas 1997, Pinto and others 1983). Growth characteristic of Caco-2 cells are very similar to the regeneration of intestinal epithelium (Pinto and others 1983). The life cycle of Caco-2 cells is characterized by three steps: (1) proliferation, where cells are homogenously undifferentiated, (2) confluency, where cells have populated an area completely, and (3) differentiation, where cells are heterogeneously polarized and differentiated (day 0 to 20 after confluency) (Vachon and Beaulieu 1992). Cells differentiate and become polarized with specific expressions of lipids and proteins in the apical and basolateral membranes. Although differentiation is complete after 25-30 days, enzymatic activities are gradually increased, becoming maximal 15-21 days after confluency (Delie and Rubas 1997).

2.4.1.2 Differentiation of Caco-2 cells

Factors influencing differentiation include the support where cells are seeded (material and pore size) as well as growth conditions (medium used, cell line, passage number, presence of antibiotics, nature of support, temperature, and relative humidity) (Sambuy and others 2005). Enterocytes and other intestinal cells are equipped with a collection of proteins that assist uptake of compounds. Some of these transport systems include: peptide, amino acid, and nucleosides carriers as well as transporters for bile acids, sugars, and amino-acids. Acid-base transport systems, receptor and carrier mediated transport, and efflux pumps are also observed in intestinal cells (Delie and

Rubas 1997; Sambuy and others 2004). Coincidentally, these transporters are also identified in Caco-2 cells which allowed their use for drug carrier interactions which is not feasible *in vivo*. Caco-2 cells were shown to express glucose transporters (SGLT1, GLUT2, GLUT5), commonly observed in intestinal cells and have shown similar lipid metabolism with enterocytes (Blais and others 1987; Field and others 1987; Trotter and Storch 1991). Additionally, Caco-2 cells express insulin growth factors (IGF) and epidermal growth factors (EGF) which play an instrumental role in early development of Caco-2 cell monolayers (Hidalgo and others 1989a, McDonald and others 1993). Caco-2 cells have also been shown to express enzymes related with drug metabolism (Pinto and others 1983, Chantret and others 1994).

2.4.1.3 Assessment of polyphenolic absorption with Caco-2 cells

Due to the increased awareness about healthy diets and the perception that consumption of fruits and vegetables was related to prevention of chronic diseases, researchers started to use Caco-2 cell monolayer models to analyze phytochemical transport, absorption, and bioavailability. Investigations have been conducted in a series of polyphenolics including phenolic acids (Konishi and others 2003; Pacheco-Palencia and others 2008), anthocyanins (Yi and others 2006), kavalactones (Matthias and others 2007), catechins (Chan and others 2007; Vaidyanathan and Walle 2001), flavonols (Walgren and others 1998; Walgren and others 2000), and procyanindins (Déprez and others 2000; Déprez and others 2001) of various sources. Numerous investigations have illustrated that phenolic acids get transported by the monocarboxilic acid transporter (MCT) and via paracellular pathway (Konishi and others 2002; Konishi and Shimizu

2003; Konishi and others 2004; Konishi and Kobayashi 2004). In contrast, various mechanisms of transport/absorption of flavonoids have been elucidated and research has reported discrepancies on how they can be transported/absorbed in a Caco-2 cell monolayer model. Paracellular transport has been proposed as one of the mechanisms by which flavonols and procyanidins have been detected in the basolateral side of Caco-2 cell monolayer models (Walgren and others 1998; Déprez and others 2001). Additionally, bigger molecules could be broken down to phenolic acids that could then be absorbed (Déprez and others 2000). Milbury and others (2002) suggested that anthocyanin transport could also occur by glucose transport receptors which agreed with investigations that explained the involvement of SLGT1 transporter in the transport of quercetin glycosides and anthocyanins (Walgreen and other 2000; Gee and others 1998; Mulleder and others 2005). Additionally, Faria and others (2009) illustrated the enhancement of GLUT2, a glucose transporter, when malvidin glucoside was present in the apical side of the system. In contrast, transport of polyphenolics could be hindered by the action of efflux pumps in the basolateral side of the system (Chan and others 2007; Vaidyanathan and Walle 2001; Walgren and others 1998). Tea catechins were transported back from the basolateral to apical side by the action of multidrug resistance-associated protein-2 (MRP2) which is a multispecific transporter responsible for the efflux of exogenous materials from the basolateral side to the apical side, limiting their bioavailability (Chan and others 2007; Vaidyanathan and Walle 2001). In parallel, high efflux from basolateral to apical side of quercetin and its glucosides was also observed (Walgren and others 1998) illustrating the complexity of the Caco-2 monolayer to

control the transport/absorption of flavonoids. Onced flavonoids have been transported through the gastric epithelia, glucoronidation by UDP-glucoronosyl transferase (UGT) and *o*-methylation by catechol-*o*-methyl-transferase (COMT) have been observed as common metabolism pathways of flavanols and anthocyanins (Ichiyanagi and others 2008; Spencer 2003; Wu and others 2006).

In conclusion, Caco-2 cells shared outstanding resemblance with intestinal cells. They are characterized by the presence of tight junctions and the development of apical and basolateral domains with similar structures and characteristics as enterocytes. Regardless of their colonic origin, Caco-2 cells display many features that other colonic cell would not express making them an excellent candidate for intestinal absorption models for nutrients and other compounds. Nevertheless, research has shown cloning of Caco-2 cells in different laboratories depending on growing conditions (medium used, cell line, passage number, presence of antibiotics, nature of support, temperature, relative humidity) and from batch to batch which leads to believe that every laboratory works with different Caco-2 cell sub-clones (Walter and Kissel 1995; Herold and others 1994). This may explain the difficulty to compare results from different published reports using Caco-2 cells as a mean to assess intestinal absorption. Although research conducted in transport and/or absorption of polyphenolics has not yet fully explained specific routes for their uptake in the small intestine, Caco-2 cell model monolayer promises to be a useful technique to better understand the interaction of these compounds with the small intestine.

III. EFFECT OF PHOSPHOLIPIDS AND TERPENES ON THE THERMAL STABILITY AND ABSORPTION OF AÇAI ANTHOCYANINS

3.1 Introduction

There is extensive research that explains the benefits, both *in vivo* and *in vitro*, of anthocyanins on human health (Briviba and others 2002; Dillard and German 2000; Elattar and Viriji 1999; Khan and others 2008). However, anthocyanin absorption is fairly poor, and this hinders their potential to be utilized in the human body. Studies have shown the limited absorption (about 1%) of anthocyanins due to their highly polar properties such as glycosylation and multiple hydroxyl groups on their structure (Yi and others 2006; Ichiyanagi and others 2008). In addition, Yi and others (2006) suggested that anthocyanins might degrade under conditions in the gastric duodenum (pH 7 at 37ºC). Another investigation illustrated the direct absorption of anthocyanins in rats and humans but, due to their low stability at high pH, only trace amount could be found and quantified (Miyazawa and others 1999).

The absorption of anthocyanins could be enhanced by certain aiding agents that facilitate access or interact and carry compounds through the intestinal epithelia increasing the availability of target compounds for various applications in the body. Phospholipids could promote absorption of compounds to the blood stream making them more available (Williams and Barry 2004). Some mechanisms proposed for this application include the formation of micelles or liposomes (Taylor and others 2005). Liposomes are common structures built by phospholipids in excess water. Liposomes

are bilayered vesicles or vessels that entrap compounds in a solution similar to the surroundings. Large vesicles are spontaneously formed in water and energy is required to produce smaller more homogenous liposomes, thus requiring energy input to be formed (Barenholz and others 1977). Liposomes could entrap anthocyanins to carry them and possibly protect them from exogenous agents (Were and others 2003).

Improvement in the absorption of anthocyanins could also be accomplished by the use of terpenes. Drug delivery via transdermal transport aided by terpenes is well established (Cal 2005, Lim and others 2006; Lim and others 2008). This effect could be a mechanism by which terpenes aid in enhancing the bioavailability of anthocyanins. Transdermal transport mechanisms might be replicated in the gastrointestinal tract if a product rich in terpenes is used, thus, creating openings in the gastric epithelia by reacting with the tissue (Elgorashi and others 2008; Lim and others 2008). In addition, the mixture of terpenes and phospholipids may enhance anthocyanin absorption even further than they would do separately.

Therefore, the purposes of this study were to determine the efficacy of terpenes and/or phospholipids to aid in the absorption of anthocyanins through a Caco-2 cell monolayer model and to evaluate their effect on anthocyanin storage stability.

3.2 Materials and Methods

Clarified açaí concentrate from Brazil was obtained from Stiebs Pomegranate Products (Madera, CA). Anthocyanins from açaí were isolated by loading samples onto an activated 10g reversed phase Sep-Pak C18 20cc cartridge (Waters Corporation, Milford, MA) and allowed to adsorb by gravity feed. The cartridge was then washed

with water and ethyl acetate to remove organic acids, sugars, metals, phenolic acids, and flavonoids other than anthocyanins (Pacheco-Palencia and others 2007). Anthocyanin fractions were recovered with acidified methanol (0.01% HCl), concentrated following solvent evaporation under vacuum (40°C), and kept at -80°C until further analysis. Dubelcco's Phosphate Saline Buffer (PBS), Hank's Balanced salt solution (HBSS) and Dubelcco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen Inc. (Grand Island, NY).

Fractions were re-dissolved in a HBSS (pH 6.0), standardized to a final concentration of 500 mg cyanidin-3-glucoside equivalents/L (anthocyanin stock solution) which was determined spectrophotometrically by the pH differential method (Wrolstad 1976). Briefly, samples were appropriately diluted with buffer solutions at pH 1.0 and 4.5. Absorbance was read on a UV-Vis microplate reader (Molecular Devices Spectra Max 190, Sunnyvale, CA) at a fixed wavelength of 520 nm and total anthocyanin concentration calculated and reported in mg/L equivalents of cyanidin-3-glucoside with an extinction coefficient of 29,600 (Jurd and Asen 1966).

Subsequently, the anthocyanin stock solution was mixed with appropriate concentrations of phospholipids and/or terpenes based on concentrations found in previous investigations using these aiding agents. Samples were then sonicated for 10 minutes, vortexed for 1 minute, and sterile-filtered (220 nm) prior cell culture transport study. All handling and processing methods were compared to a control of the starting clarified extract for calculation of phytochemical recovery of changes due to process

techniques. Upon completion of each isolation or processing protocol, samples were held at -20^{o}C until analysis.

Antioxidant capacity was determined by the oxygen radical absorbance capacity (ORAC) method (Cao and others 1996), adapted to be performed with a 96-well Molecular Devices fmax® fluorescent microplate reader (485 nm excitation and 538 nm emission). The assay measures the ability of an antioxidant to inhibit the decay of fluorescein induced by the peroxyl radical generator 2,2-azobis (2-amidinopropane dihydrochloride) as compared to Trolox, a synthetic, water-soluble vitamin E analog. For analysis, samples were diluted in pH 7.0 phosphate buffer and 50μL of each sample was then transferred to a microplate along with a Trolox standard curve (0, 6.25, 12.5, 25, 50μM Trolox) and phosphate buffer blanks. 100μL of fluorescein and 50μL of peroxyl radical generator were added to all samples, standard curve, and blanks. Readings were taken every 2 min over a 70 min period at 37°C. Antioxidant capacity was quantified by linear regression based on the Trolox standard curve and results were expressed in μmol of Trolox equivalents per gram (μmol TE/g).

Transepithelial transport of anthocyanins was conducted using a Caco-2 colon carcinoma cells model. Caco-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained at 37ºC in an atmosphere of 5% CO2 and 95% relative humidity. Cells were cultured in DMEM, containing 4.5g/L of glucose, 20% fetal bovine serum, 1% nonessential amino acids, 100 units/ml of penicillin, 100μg/ml of streptomycin sulfate, and 1mM of sodium pyruvate (chemicals supplied by Sigma-Aldrich Co., St. Louis, MO). Cells grown in a 75 cm^2 T-flask were

passed after reaching 80-90% of confluence at a split ratio 1:7. The medium was changed every 2 days. Cells between passages 24-30 were seeded at a density of 100,000 cells/well onto a transparent 12 mm polycarbonated 0.4 μm pore diameter insert well plate (Transwell, Corning Costar Corp., Cambridge, MA). All volumes were kept constant at 0.5 ml at the apical side and 1.5 ml at the basolateral side and medium (DMEM) was changed every 2 days until enterocytic cell differentiation was achieved (18-21 days). Transepithelial electrical resistance (TEER) was monitored every seven days with an EndOhm Voltohmmeter equipped with a STX2 electrode (World Precision Instruments Inc., Sarasota, FL) to check cell confluence, integrity, and proper development of the monolayer. Only monolayers with TEER values above 350 Ω cm^2 were used for the experiment to assure monolayer integrity and strength. Transport of anthocyanins was conducted by modifying conditions explained elsewhere (Mertens-Talcott and others 2007). Monolayers were initially rinsed with PBS followed by the addition of HBSS previously adjusted to pH 7.4 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and to pH 6.0 with 2-(N-Morpholino)-ethanesulfonic acid solution (MES) for the basolateral side and apical side, respectively. After applying HBSS to both sides, cells were incubated (30 min) and treatments containing combinations of anthocyanins, phospholipids, and/or terpenes were then added to the apical side of the wells. Thereafter, sample aliquots (200 μL) were taken at time zero and every 30 min for 2 hours from the basolateral side, immediately acidified with a known volume of 4N HCl, kept refrigerated (5 ºC) and analyzed within hours after analysis. Basolateral volume was kept constant by adding fresh HBSS (200 μL)

after sampling. Resistance (TEER) was measured once again after assay was completed to insure monolayer integrity. Only monolayers with a final resistance above 350 Ω cm^2 were accepted for data analysis and samples collected from its basolateral side were considered for high performance liquid chromatography (HPLC) analysis.

Samples collected from the transport study were analyzed by reverse phase HPLC using modified chromatographic conditions (Talcott and Lee 2002) with a Waters 2690 Alliance HPLC system using a Water PDA detector. Separations were performed on a 250 x 4.6 mm Nova-Pak C_{18} column (Waters Corporation, Milford, MA) with a C_{18} guard column. Mobile phase A consisted of water acidified with *o*-phosphoric acid (pH 2.4) and Mobile phase B consisted of 60:40 methanol and water acidified with o-phosphoric acid (pH 2.4). The gradient solvent program run phase B from 0 to 30% in 1 min; 30 to 50% in 1 min, 50 to 70% in 2 min, 70 to 100% in 4 min and held at 100% for 8 min for a total run time of 16 minutes at a flow rate of 0.8 mL/min. Anthocyanins were identified by UV/VIS spectral interpretation, retention time and comparison to authentic standards (Sigma Chemical Co., St. Louis, MO). Data was reported as mg/L of each compound.

In parallel, a kinetic study was conducted for 40 days to assess the effect of phospholipids and terpenes on the degradation characteristics of anthocyanins at three different temperatures. Anthocyanin stock solution was appropriately mixed with known concentrations of phospholipids and/or terpenes based on results from the transepithelial transport study previously conducted. Samples were then sonicated for 10 minutes, vortexed for 1 minute, and sterile-filtered (220 μm) prior storage stability

assessment. Total anthocyanins were determined spectrophotometrically by the pH differential method (Wrolstad 1976) at day 0. Thereafter, samples were taken at days 10, 29 and 40, immediately acidified with a known volume of 4N HCl, kept refrigerated (5 ºC), and analyzed for total anthocyanins as explained earlier (spectrophotometrically) within hours after analysis.

Data from experiments were analyzed by one-way analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Data for each treatment represents the mean of three replicates. Mean separations were conducted using a Least Significant Differences (LSD) test ($P < 0.05$).

3.3 Results and Discussion

Anthocyanins from açaí puree were isolated, measured, characterized by HPLC analyses, and used for a unidirectional transport study assessed from the apical to the basolateral side of Caco-2 cells monolayers. Caco-2 cells have been widely characterized and used as an in-vitro model in the field of drug absorption and permeability (Hidalgo and others 1989) and were previously used to evaluate intestinal absorption and transport of various flavonoids (Chan and others 2006; Deprez and others 2001; Pacheco-Palencia and others 2008; Vaidyanathan and Walle 2001; Yi and others 2006).

3.3.1 Transepithelial Transport Study

Transport of açaí extracts was assessed from apical to basolateral side. Extracts were loaded into the apical side of the cells monolayer and presence of anthocyanin in the basolateral side was evaluated chromatographically and spectrophotometrically over

time for up to 2 hours. Extract transported in 2 hours and found in the basolateral side was compared to the initial concentration of anthocyanins loaded to the apical side (Fig. 3-1). According to Pacheco-Palencia and others (2007), Cyanidin-3-rutinoside (C3R) and Cyanidin-3-glucoside (C3G) are the two predominant anthocyanins in açaí. These anthocyanins were monitored through the transepithelial transport study and results are presented in Table 3-1. Açaí anthocyanins with no aiding agents were poorly transported as reported in previous studies illustrating polyphenolics transport through cell monolayers (Ichiyanagi and others 2008; Yi and others 2006; Pacheco-Palencia and others 2008). Transport of C3G (1.38%) was better than C3R (1.06%). Both terpenes and phospholipids significantly increased the transport of anthocyanins. Significant increases in transport ranged from 30 to 343% for C3G and 91 to 305% in the case of C3R. Best results were observed in mixtures of terpenes and phospholipids.

When soy lecithin (500 mg/L) was added to the matrix as a source of phospholipids, overall transport of C3G and C3R was increased by 29.6% and 30.6% respectively. Soy lecithin concentration was then increased (5 g/kg), and the transport of C3G and C3R was increased by 135% and 156% respectively. This increase might be attributed to the encapsulation of compounds by the formation of aggregates due to phospholipid interactions promoted by physical forces such as sonication, vortexing, and filtration (Lasch and others 2003; Walstra and van Vliet 2007). In addition increased presence of phospholipid molecules might have allowed formation of aggregates that potentially held, protected, and carried more anthocyanin molecules through the

monolayer. Besides, anthocyanins can also interact with outside of aggregates forming a thin layer of anthocyanins surrounding them which could also enhance transport.

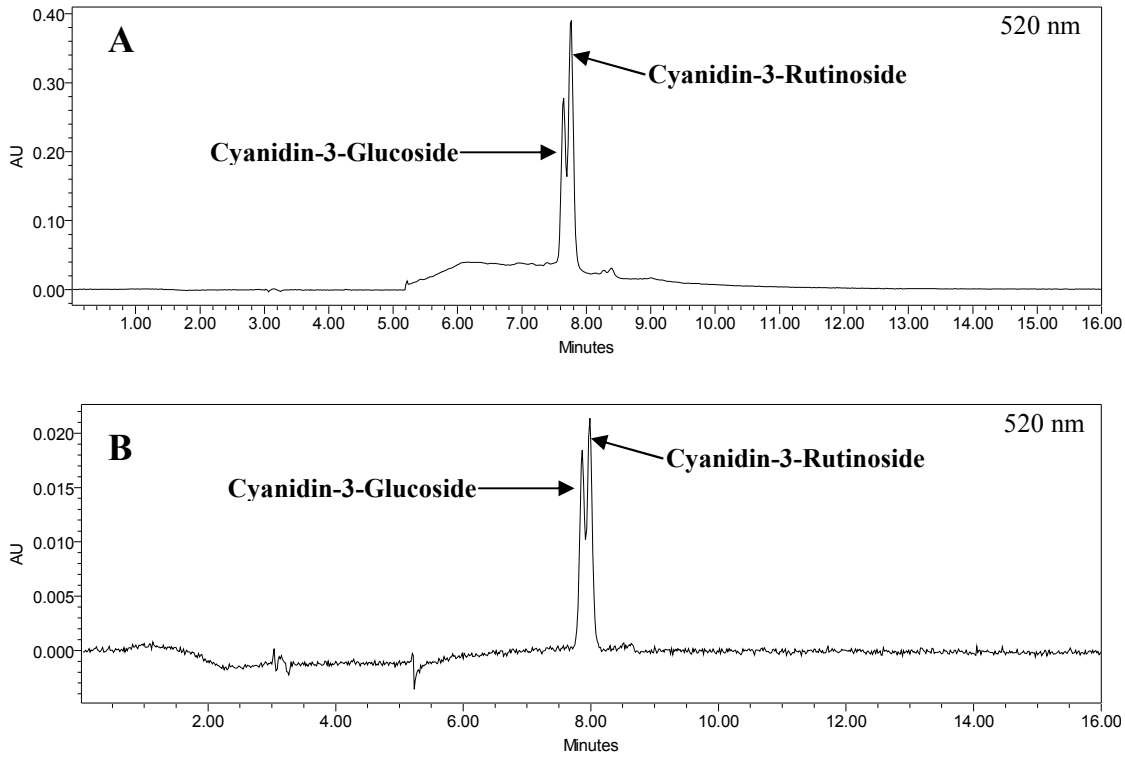


Figure 3-1. Chromatograms of anthocyanins from açaí extract in the apical side before analysis (A) and present in the basolateral side after an incubation period of 2 hours (B) I.

Filtration following sonication and vortexing was decisive at determining anthocyanin transport due to the potential formation of smaller and more homogenous liposomes. Although filtration reduced the anthocyanin concentration of samples before transport analysis, anthocyanin transport of filtered samples was significantly higher than non-filtered samples ($p<0.05$). Vortexing and sonication alone might have

produced a diverse group of vesicles of different sizes that demonstrated lower transport of compounds through the monolayer. When samples were not sterile filtered (0.22 μm), bigger aggregates might have been formed in the matrix and were not transported as efficiently as the filtered mixture of anthocyanins and PLs. Transport of the non-filtered sample was reduced by 10.2% for C3G and 10.8% in the case of C3R. Larger aggregates could not be transported and anthocyanins entrapped in these structures were not allowed to be transported through the monolayer. When samples were discarded after 2 hours, a red cloud was observed over the monolayer, suggesting that most of the compounds were still in the apical side.

Table 3-1. Percent transport of anthocyanins (500 mg/L) from apical to basolateral side of Caco-2 cell monolayers following incubation for 2 h.

Treatment	Concentration [mg/L]	% Anthocyanin Transport	
		Cyanidin-3-glucoside	Cyanidin-3-rutinoside
Control		$1.38 \pm 0.15^{gh\ 1}$	$1.06 \pm 0.12^{fg\ 1}$
Terpenes	50	4.08 ± 0.33^{b}	3.61 ± 0.39^{bc}
	500	2.41 ± 0.20^{f}	2.03 ± 0.12^{e}
	5000	2.60 ± 0.08^{def}	2.24 ± 0.05^{de}
Phospholipids	500	1.79 ± 0.15^{g}	1.38 ± 0.07^{f}
	5000	4.22 ± 0.35^{b}	3.55 ± 0.41^{bc}
NF[2] Terpenes	500	2.97 ± 0.36^{d}	2.22 ± 0.30^{de}
NF[2] Phospholipids	500	1.24 ± 0.05^{h}	0.95 ± 0.05^{g}
Terpenes / Phospholipids	50 / 50	2.88 ± 0.15^{de}	2.62 ± 0.23^{d}
	50 / 500	3.81 ± 0.33^{bc}	3.92 ± 0.35^{ab}
	50 / 5000	6.12 ± 0.17^{a}	4.29 ± 0.21^{a}
	500 / 50	3.52 ± 0.40^{c}	3.45 ± 0.43^{c}
	500 / 500	2.46 ± 0.28^{ef}	2.25 ± 0.17^{de}

[1]Values with different letters within the same column are significantly different (LSD test, $p<0.05$). [2]Non-filtere samples.

Valencia orange cold pressed oil was used as the source of terpenes. The major component of cold pressed oil from orange peels is d-limonene. Other components include α-pinene, sabinene, myrcene, linalool, and decanal (Temelli and others 1988). Terpenes also had a major impact on transport of açaí anthocyanins (Table 3-1). Even at very low concentrations (50 mg/L), anthocyanin transport was enhanced and resulted in higher values than samples containing more orange oil (0.5 and 5 g/kg). In the case of terpenes, micelles might have been formed due to the presence of inherent phospholipids and mono- and diglycerides present in the oil. However, terpenes might have interacted with the monolayer creating gaps for anthocyanins to passively be transported and this may be the principal method by which anthocyanin transport was enhanced. Terpenes are known to disrupt the stratum corneum to allow passage of compounds (Cal 2005; Mackay and others 2001; Anjos and others 2007). Although intestinal epithelia are structurally different from stratum corneum, the effect of terpenes could be similar due to the type of interaction they may exert on the intestinal surface. Filtration had no effects on the transport of samples containing terpenes, which increased the average values by 115% for C3G and 109% in the case of C3R. In addition, no negative effect from filtration may illustrate the possible lack of aggregate formation compared to treatments with soy lecithin, and substantiate the latter proposed method by which terpenes interact with the monolayer rather than with anthocyanins. Another possibility is that terpenes could interact with anthocyanins as it occurs in the formation of prenylflavonoids. Anthocyanins and terpenes could interact but not form aggregates of large molecular weight and filtration might not change this relationship.

The transport aiding effect of phospholipids and terpenes was enhanced when mixed together. Phospholipids at the highest concentration assessed (5000 mg/L) resulted in transport enhancements of 205% and 235% (C3G and C3G, respectively). When citrus oil (50 mg/L) was added to the matrix a 489% and 825% increase in transport compared to samples with only phospholipids were observed in C3G and C3R respectively (Table 3-1).

3.3.2 Anthocyanin Storage Stability and Antioxidant Capacity

Total anthocyanin concentration and antioxidant capacity were measured through a 40 day period at 5, 25, and 37ºC for samples that showed highest absorption/transport through the transport study. Samples with appropriate mixtures of anthocyanins, terpenes, and phospholipids were prepared and initial anthocyanin concentration was adjusted to ~500 mg/L. Filtration showed significant differences on anthocyanin concentration between treatments (Table 3-2). Anthocyanins mixed with terpenes (Ter) showed the highest concentration of anthocyanins after sterile filtration (477 mg/L) followed by the non-filtered sample (NF) containing 445 mg/L of anthocyanins. A significant loss was observed on the sample containing only anthocyanins (An) (394 mg/L). The highest losses were recorded in samples containing 5,000 mg/L of phospholipids (PL and PL+T). Such losses could be explained by the size of aggregates formed by phospholipids. Aggregates bigger than 0.22 μm were trapped in the filter and these aggregates trapped anthocyanins with them. Similar behavior was observed for antioxidant capacity with (Ter) showing the highest antioxidant value (27.7 μmol TE/ml)

and samples (PL) and (PL+T) containing phospholipids exhibited the highest losses after filtration (18.7 and 18.1 μmol TE/ml, respectively).

Table 3-2. Total anthocyanins and antioxidant capacity of filtered anthocyanin fractions from açaí juice mixed with terpenes and/or phospholipids.

Treatments[1]	Total Anthocyanins [mg/L]	Antioxidant Capacity [μmol/ml]
Control[2]	394 ± 12.1[c 3]	21.8 ± 0.89[c]
Phospholipids [5000mg/L]	332 ± 3.51[d]	18.7 ± 2.65[d]
Terpenes [50mg/L]	477 ± 13.8[a]	27.7 ± 0.64[a]
Phospholipids [500mg/L] NF[4]	445 ± 16.7[b]	25.2 ± 0.57[b]
Mix (Phospholipids + Terpenes)[5]	314 ± 8.41[e]	18.1 ± 0.26[d]

[1]All treatments were filtered unless mentioned in the table. [2]Control was adjusted to 500 mg/L of total anthocyanins before filtration and did not contain phospholipids or terpenes. [3]Values with different letters within the same column are significantly different (LSD test, $p<0.05$). [4]NF: Non-filtered sample. [5]Mix contained phospholipids (5000 mg/L) and terpenes (50 mg/L).

Color degradation followed a first-order kinetic model where degradation rate constants (β_1) and half-life ($t_{1/2}$) for anthocyanin loss were calculated (Cemeroğlu and others 1994; Kirca and Cemeroğlu 2003) as $\ln A_t/A_0 = -\beta_1 \times \text{time}$, and $t_{1/2} = \ln 0.5/\beta_1$, where A_0 is the initial color absorbance value at 520nm, and A_t is the absorbance value at a given time (Table 3-3). Anthocyanin degradation was similar within treatments at every temperature illustrating that the presence or absence of terpenes and/or phospholipids did not affect anthocyanins degradation kinetics. On the contrary, effects of storage temperature were evident. Higher temperature storage resulted in higher losses of anthocyanins and reduced shelf life of the samples. Refrigeration (5 ºC)

resulted in an average half-life of 97.7 days whereas higher temperatures (25 ºC and 37 ºC) resulted in a reduction of 43% and 59% in half-life respectively.

3.4 Conclusions

Addition of phospholipids and terpenes as absorption enhancers was effective. Transport values for anthocyanins with no enhancers on the matrix were 1.22% on average. When phospholipids were initially added to the matrix (500 mg/L) the increase in transport of anthocyanins was of about 30% while anthocyanins mixed with a higher concentration of phospholipids (5000 mg/L) increased absorption was almost three times more. In the case of terpenes, higher concentrations (500 and 5,000 mg/L) of this enhancer resulted in increased transport of anthocyanins (82% and 98% respectively). Best results were observed when the lowest concentration of terpenes was used (50 mg/L) which increased transport values 3.1 times from the control. Combinations of phospholipids and terpenes resulted in even greater transport of anthocyanins. The highest average transport values recorded (5.21%) occurred when phospholipids and terpenes were combined at 5,000 and 50 mg/L in the matrix, respectively. Anthocyanin degradation during storage followed first-order kinetics over a wide temperature range, and was unaffected by the presence of phospholipids or terpenes in solution. On the contrary, effects of storage temperature were evident. Anthocyanins under refrigeration (5 ºC) maintained a half life of almost 100 days while under temperatures above 25 ºC; anthocyanin half life resulted shorter than 50 days on average.

Table 3-3. Kinetic parameters of anthocyanin degradation during storage at 5, 25, and 37 ˚C in of filtered anthocyanin fractions from açaí juice mixed with terpenes and/or phospholipids.

Treatments[1]	5 ºC		25 ºC		37 ºC	
	β_1[2]	$t_{½}$[3]	B_1	$t_{½}$[4]	β_1	$t_{½}$[4]
An	6.96 ± 0.34[a 5]	99.7 ± 5.05[a]	12.1 ± 0.34[bc]	57.4 ± 1.65[ab] *	17.3 ± 0.06[a]	40.1 ± 0.13[a] *
PL	7.12 ± 0.89[a]	98.3 ± 12.7[a]	12.9 ± 0.32[a]	53.6 ± 1.33[c] *	17.2 ± 0.06[a]	40.2 ± 0.13[a] *
Ter	7.67 ± 1.27[a]	91.9 ± 14.1[a]	12.3 ± 0.19[b]	56.7 ± 0.85[b] *	17.4 ± 0.18[a]	39.8 ± 0.42[a] *
PL+T	6.87 ± 0.40[a]	101 ± 5.74[a]	13.2 ± 0.23[a]	52.5 ± 0.94[c] *	17.0 ± 0.90[a]	40.9 ± 2.24[a] *
NF	7.14 ± 0.64[a]	97.5 ± 8.32[a]	11.6 ± 0.30[c]	59.7 ± 1.51[a] *	17.4 ± 0.49[a]	39.8 ± 1.13[a] *

[1]Anthocyanin control (An), Anthocyanins mixed with 5000mg/L of soy lecithin (PL), Anthocyanins mixed with 50mg/L of cold pressed citrus oil (Ter), Anthocyanins mixed with 5000mg/L of soy lecithin and 50mg/L of cold pressed oil (PL+T), Unfiltered mixture of anthocyanins and 500mg/L of soy lecithin (NF). [2]Reaction rate constants ($\beta_1 \times 103$ days-1). [3]Half-life (days) of initial anthocyanin content. [4]Asterisk (*) for half-life indicates a significant effect (LSD test. $P<0.05$) due to storage temperature. [5]Values with similar letters within columns are not significantly different (LSD test. $P<0.05$).

These results demonstrated the potential of phospholipids and terpenes to increase absorption of anthocyanins. In addition, the stability and bioavailability of anthocyanins was illustrated and that phospholipids and terpenes did not promote the degradation of anthocyanins. The outcome of this investigation could represent a breakthrough for the food industry in future product development. Further analysis needs to be conducted to understand the concentration of enhancers used, find optimum concentrations, and attempt to explain how they are interacting with anthocyanin in the matrix.

IV. EFFECT OF PHOSPHOLIPIDS AND TERPENES ON THE ABSORPTION OF AÇAI ANTHOCYANINS AND ON THE PHYSICAL STABILITY OF THE MATRIX

4.1 Introduction

Anthocyanins are a major group of water-soluble pigments in plants and are the most consumed flavonoids in the US diet (Clifford 2000; Galvano and others 2004). The benefits of anthocyanins on human health have been elucidated in an extensive body of research (Briviba and others 2002; Galvano and others 2004; Khan and others 2008). However, studies have shown their limited absorption and susceptibility to gastric conditions (Miyazawa and others 1999; Ichiyanagi and others 2008; Yi and others 2006). As mentioned in Section 3, absorption of anthocyanins may be enhanced by some compounds (aiding agents) that facilitate access through the gastric epithelia increasing their bioavailability. Phospholipids and terpenes were selected as aiding agents due to the extensive literature explaining their use in absorption of compounds (Yonekura and others 2006; Keller 2001; Sugawara and others 2001; Cal 2005, Lim and others 2006; Lim and others 2008). However, the use of these compounds has not been previously assessed in potential absorption enhancement of anthocyanins. The use of phospholipids and terpenes was shown to increase absorption of anthocyanins in Caco-2 cells transepithelial model (Section 3). Nevertheless, improvement in the absorption of anthocyanins needs to be assessed further with additional combinations (terpenes-phospholipids) and anthocyanin dose-dependency for better knowledge of their distinctive absorption enhancement properties. In addition, particle size analysis might

be a useful tool to further understand the association of anthocyanins with terpenes and phospholipids in the matrix. Therefore, the purposes of this study were to further evaluate the efficacy of terpenes and/or phospholipids in the absorption aiding of anthocyanins through a Caco-2 cell model, assess dose response of anthocyanins, and evaluate the potential formation of vesicles in the matrix.

4.2 Materials and Methods

Clarified açaí concentrate from Brazil was obtained from Stiebs Pomegranate Products (Madera, CA). Anthocyanins from açaí were isolated by loading samples onto an activated 10g reversed phase Sep-Pak C18 20cc cartridge (Waters Corporation, Milford, MA) as explained in Section 3. Anthocyanin extract was then kept at -80°C until further analysis. Dubelcco's Phosphate Saline Buffer (PBS), Hank's Balanced Salt Solution (HBSS) and Dubelcco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen Inc. (Grand Island, NY).

Anthocyanins were re-dissolved in HBSS (pH 6.0) for analyses and standardized to a final concentration of 750 and 1,500 mg cyanidin-3-glucoside equivalents/L (anthocyanin stock solutions) which were determined spectrophotometrically by the pH differential method (Wrolstad 1976). Absorbance of samples diluted with pH 1 and 4.5 buffers were read on a UV-Vis microplate reader (Molecular Devices Spectra Max 190, Sunnyvale, CA) at a fixed wavelength of 520 nm and 700 nm, and expressed in mg/L equivalents of cyanidin-3-glucoside. Subsequently, the anthocyanin stock solutions were mixed with appropriate concentrations of phospholipids and/or terpenes. Samples

were then sonicated for 10 minutes, vortexed for 1 minute, and sterile-filtered (0.22 μm) prior cell culture transport study and particle size analysis.

Particle size analysis was conducted by dynamic light scattering (Walzem and others 1994). Diameter measurements were determined optically using a Microtrack® Series 9200 Ultrafine Particle Analyzer (Leeds and Northrup, Wales, PA). Samples were loaded in a well and system software and laser (3 mWatt, λ= 780nm) were activated. Light scattering was recorded for 30 seconds, adapted to an audio range, and deconvoluted. Polydispersity of particle populations was calculated as the width (nm) of the measured particle size distribution.

Transepithelial transport of anthocyanins was conducted using a Caco-2 colon carcinoma cells model as explained in Section 3. Samples collected from the transport study were analyzed by reverse phase HPLC using modified chromatographic conditions (Talcott and Lee 2002); detailed explanation of the chromatographic conditions can be found in section 3. Data was reported as ratios between compounds found in the basolateral compartments (mg/L) to compounds initially added to the apical compartments (mg/L).

Data from experiments were analyzed by one-way analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Data for each analysis represents the mean of three replicates. Mean separations were conducted using LSD test ($P < 0.05$).

4.3 Results and Discussion

Verification and further assessment of results from Section 3 was conducted on anthocyanins isolated from açaí puree. Characterized anthocyanins, Cyanidin-3-

Glucoside (C3R) and Cyanidin-3-Rutinoside (C3R), were used for a unidirectional transport study, assessed from the apical to the basolateral side of Caco-2 cells monolayers, and particle size analysis was conducted to possibly relate formation of liposomes to enhancement of anthocyanin absorption.

4.3.1 Transepithelial Transport Study

Transepithelial transport of açaí extract rich in anthocyanins was assessed through a Caco-2 cell model. Extracts properly mixed with various concentrations of terpenes and/or phospholipids were loaded into the apical side of the cells monolayer and presence of anthocyanins in the basolateral side was evaluated chromatographically and spectrophotometrically over time for up to 2 hours. Samples transported over 2 hours and found in the basolateral side were compared to the initial concentration of anthocyanins loaded to the apical side (Fig. 4-1). Samples were taken every 30 min, concentration of anthocyanins was calculated, and the total anthocyanin content found in the basolateral side was added and compared the concentration initially applied to the apical side (Appendix A). Cyanidin-3-glucoside (C3G) and Cyanidin-3-rutinoside (C3R) were detected in all samples as the main anthocyanins found in açaí which agreed with previous investigations conducted with this commodity (Pacheco-Palencia and others 2007; Pacheco-Palencia and others 2009). These anthocyanins were then monitored through the transepithelial transport study and results are presented in Tables 4-1 and 4-2 depending on the concentration of anthocyanins loaded initially (250 and 500 mg/L of anthocyanins, respectively).

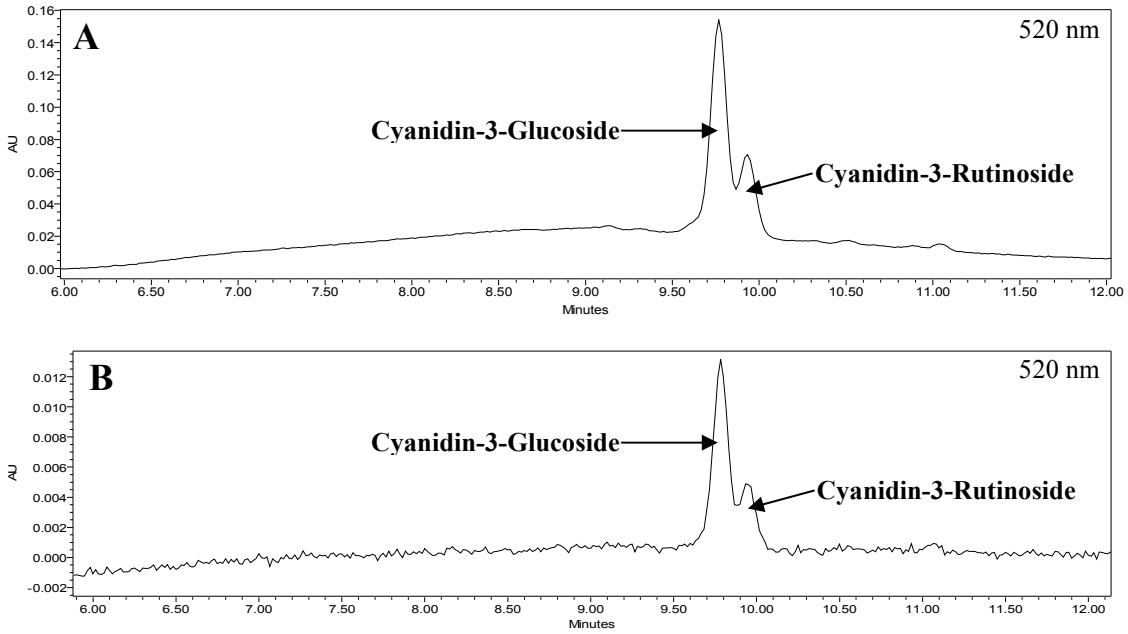


Figure 4-1. Chromatograms of anthocyanins from açaí extract in the apical side before analysis (A) and present in the basolateral side after an incubation period of 2 hours (B) II.

Açaí anthocyanins with no phospholipids or terpenes were transported inadequately at both concentrations (1.77%) agreeing with Section 3 and other investigations that demonstrated polyphenolic transport through cell monolayers (Pacheco-Palencia and others 2008; Walgren and others 1998). In general, addition of both terpenes and phospholipids increased the amount of anthocyanins found in the basolateral compartment. Increases in transport values ranged from 66 to 158% and 59 to 151% for C3G and C3R, respectively. In addition, relative transport of anthocyanins did not vary as a function of anthocyanin concentration since results were fairly similar and enhancement transport effect of phospholipids and terpenes did not change with change in anthocyanins content.

Table 4-1. Transport of anthocyanins (250 mg/L) from açaí juice extracts from the apical to the basolateral side of Caco-2 cell monolayers following incubation (120 min, 37ºC), as a function of phospholipid and terpene concentrations.

Terpene Concentration [mg/L]	% Transport of Anthocyanins from Açaí Juice Extracts					
	Cyanidin-3-Glucoside			Cyanidin-3-Rutinoside		
	Phospholipid Concentration [mg/L]			Phospholipid Concentration [mg/L]		
	0	5,000	10,000	0	5,000	10,000
0	$1.70 \pm 0.13f^{1}$	3.89 ± 0.35^{ab}	3.86 ± 0.16^{abc}	1.79 ± 0.04^{d}	4.01 ± 0.24^{a}	2.83 ± 0.30^{c}
25	4.00 ± 0.13^{a}	3.08 ± 0.25^{e}	3.56 ± 0.33^{bcd}	3.73 ± 0.37^{ab}	3.54 ± 0.34^{ab}	3.85 ± 0.37^{ab}
50	3.51 ± 0.14^{cd}	3.18 ± 0.25^{de}	3.64 ± 0.07^{abc}	3.50 ± 0.29^{b}	2.86 ± 0.12^{c}	3.59 ± 0.28^{ab}

[1]Values with different letters within anthocyanin transport data set (C3G and C3R) are significantly different (LSD test, $p<0.05$).

Table 4-2. Transport of anthocyanins (500 mg/L) from açaí juice extracts from the apical to the basolateral side of Caco-2 cell monolayers following incubation (120 min, 37ºC), as a function of phospholipid and terpene concentrations.

Terpene Concentration [mg/L]	% Transport of Anthocyanins from Açaí Juice Extracts					
	Cyanidin-3-Glucoside			Cyanidin-3-Rutinosidee		
	Phospholipid Concentration [mg/L]			Phospholipid Concentration [mg/L]		
	0	5,000	10,000	0	5,000	10,000
0	$1.80 \pm 0.15^{d\,1}$	4.58 ± 0.35^{a}	4.31 ± 0.24^{a}	1.80 ± 0.18^{e}	4.46 ± 0.45^{a}	4.26 ± 0.02^{ab}
25	3.59 ± 0.30^{b}	2.95 ± 0.30^{c}	3.57 ± 0.15^{b}	3.70 ± 0.38^{c}	3.10 ± 0.31^{d}	3.69 ± 0.18^{c}
50	3.47 ± 0.20^{b}	3.39 ± 0.16^{b}	3.74 ± 0.09^{b}	3.91 ± 0.05^{bc}	3.17 ± 0.05^{d}	3.88 ± 0.33^{bc}

[1]Values with different letters within anthocyanin transport data set (C3G and C3R) are significantly different (LSD test, $p<0.05$).

At the lowest concentration of anthocyanins (250 mg/L), differences between treatments were difficult to observe for either anthocyanin assessed. Highest transport values were observed in at least four different treatments. No trends were found that could guide selection of treatments that were better at enhancing transport of açaí anthocyanins. In contrast, differences were clear at higher concentration of anthocyanins (500 mg/L). Best results were observed when only phospholipids were present in the samples as aiding agents. Among all samples with phospholipids and terpenes present in the matrix, all combinations containing terpenes and 5,000 mg/L of phospholipids frequently resulted in the lowest transport of C3G and C3R despite anthocyanin concentration. These results illustrated a different position compared to previous studies which demonstrated that combinations of terpenes and phospholipids increased absorption of anthocyanins more than using these aiding agents separately. This could be attributed to the nature of the açaí extract used for theses studies which could vary slightly compared to the extract used in Section 3 which illustrates the complexity of the interactions between anthocyanins, phospholipids, and terpenes.

Transport rates are another significant assessment in a Caco-2 cell model. These values indicated the concentration of anthocyanins transported from the apical side to the basolateral side in a unit of time. Average transport rates (µg/mL·h) of açaí anthocyanins from the apical to the basolateral side were given in time depending on the concentration of anthocyanins (250 and 500 mg/L) initially mixed in the matrix (Tables 4-3 and 4-4, respectively). Individual anthocyanin transport rates (0.023 – 0.212 µg/mL·h) increased in a concentration-dependent matter (Fig. 4-2). When concentration

of anthocyanins was doubled, average transport rates of all samples were almost double for both C3G and C3R (1.96 and 1.95, respectively) which suggested that passive diffusion was occurring. In addition, average transport rates for C3G were significantly higher than for C3R (2.32 fold), which could be explained by the concentration difference of these two anthocyanins in the açaí extract. Concentration of C3G was almost 3.5 times higher than C3R in the extract utilized for these experiments. When evaluating C3G average transport rates, samples with no phospholipids had the highest recorded values regardless of terpene presence or concentration. An inverse relation was observed between phospholipid concentration and average absorption rates since transport rates decreased as phospholipid concentration increased. When C3G concentration was doubled, this effect was not as evident probably due to saturation of the system. A different scenario was observed for C3R. Samples with no phospholipids had the highest transport rates regardless of terpene concentration but rates of samples with no aiding agents were significantly lower. An inverse relation was also observed between phospholipid concentration and average transport rates but it was not as evident as for C3G.

Table 4-3. Average transport rates of anthocyanins (250 mg/L) from açaí juice extracts from the apical to the basolateral side of Caco-2 cell monolayers, as a function of phospholipid and terpene concentrations.

Terpene Concentration [mg/L]	Transport Rate (μg/mL·h) of Anthocyanins from Açaí Juice Extracts					
	Cyanidin-3-Glucoside			Cyanidin-3-Rutinoside		
	Phospholipid Concentration [mg/L]			Phospholipid Concentration [mg/L]		
	0	5,000	10,000	0	5,000	10,000
0	$0.111 \pm 0.008^{b\,1}$	0.091 ± 0.007^{c}	0.073 ± 0.002^{de}	0.034 ± 0.000^{d}	0.034 ± 0.001^{d}	0.023 ± 0.002^{e}
25	0.124 ± 0.009^{a}	0.075 ± 0.007^{de}	0.068 ± 0.006^{e}	0.047 ± 0.002^{ab}	0.038 ± 0.002^{cd}	0.037 ± 0.003^{cd}
50	0.112 ± 0.005^{b}	0.076 ± 0.002^{cd}	0.066 ± 0.000^{e}	0.052 ± 0.004^{a}	0.040 ± 0.002^{b}	0.041 ± 0.002^{bc}

[1]Values with different letters within anthocyanin transport data set (C3G and C3R) are significantly different (LSD test, $p<0.05$).

Table 4-4. Average transport rates of anthocyanins (500 mg/L) from açaí juice extracts from the apical to the basolateral side of Caco-2 cell monolayers, as a function of phospholipid and terpene concentrations.

Terpene Concentration [mg/L]	Transport Rate (μg/mL·h) of Anthocyanins from Açaí Juice Extracts					
	Cyanidin-3-Glucoside			Cyanidin-3-Rutinoside		
	Phospholipid Concentration [mg/L]			Phospholipid Concentration [mg/L]		
	0	5,000	10,000	0	5,000	10,000
0	$0.202 \pm 0.016^{a\,1}$	0.212 ± 0.014^{ab}	0.173 ± 0.007^{cd}	0.073 ± 0.009^{c}	0.077 ± 0.008^{c}	0.074 ± 0.002^{c}
25	0.202 ± 0.009^{ab}	0.141 ± 0.010^{de}	0.144 ± 0.008^{e}	0.110 ± 0.008^{a}	0.083 ± 0.008^{bc}	0.092 ± 0.005^{b}
50	0.182 ± 0.004^{bc}	0.156 ± 0.002^{cde}	0.152 ± 0.012^{de}	0.057 ± 0.005^{d}	0.049 ± 0.002^{d}	0.056 ± 0.011^{d}

[1]Values with different letters within anthocyanin transport data set (C3G and C3R) are significantly different (LSD test, $p<0.05$).

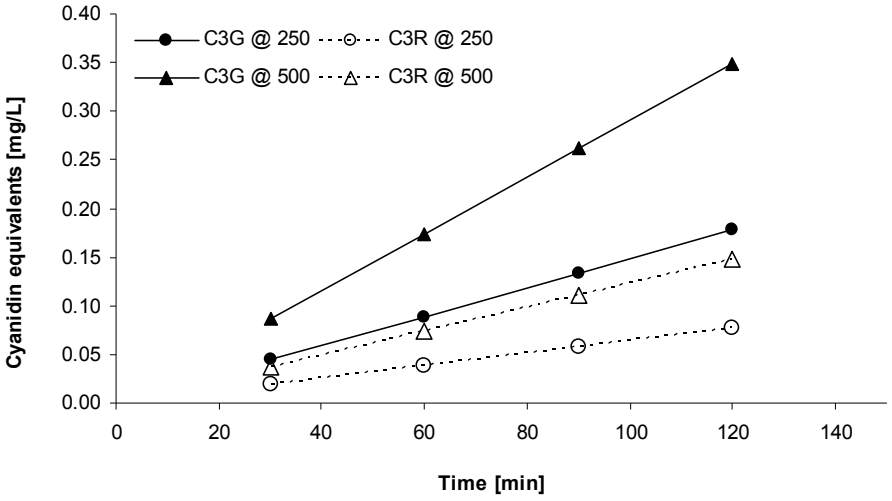


Figure 4-2. Average transport rates of açaí anthocyanins, cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R) at two different total anthocyanin concentrations (250 and 500 mg/L), from the apical to the basolateral side of Caco-2 cell monolayers over 120 min at 37ºC.

4.3.2 Particle Size Analysis

Particle size of açaí extract rich in anthocyanins was assessed by dynamic light scattering. Anthocyanin-rich extract properly mixed (as explained in the materials and methods section) with various concentrations of terpenes and/or phospholipids were subjected to two rounds of sonication and vortexing (~15 min) followed by sterile filtration (0.22 μm). Diameter measurements were determined. Light scattering was recorded for 30 seconds, adapted to an audio range, and deconvoluted by a software system. Sample particle populations were calculated as the width (nm) of the measured particle size distribution and are presented in Table 4-5.

Contrary to what was initially believed, there was no formation of vesicles except for samples containing no terpenes at the lowest concentration of anthocyanins assessed (250 mg/L). Perhaps vesicles created by the sonication/filtration method were too big to

be allowed through the filter (220nm). In contrast, transport of anthocyanins through a Caco-2 model was enhanced by terpenes and phospholipids regardless of vesicle formation, which suggested that the only presence of these absorption enhancers is necessary to see a significant increase in transport of polyphenolics rather than vesicle formation.

Table 4-5. Particle size of matrices as a function of anthocyanin, phospholipid, and terpene concentrations.

Anthocyanin Concentration [mg/L]	Terpene Concentration [mg/L]	Particle diameter [nm]		
		Phospholipid Concentration [mg/L]		
		0	5,000	10,000
250	0	0.92 ± 0.00[a]	185 ± 12.1[a]	416 ± 13.6[a]
	25	3.79 ± 0.57[a]	1.12 ± 0.13[b]	0.97 ± 0.02[b]
	50	0.94 ± 0.03[a]	0.92 ± 0.00[b]	0.92 ± 0.00[b]
500	0	1.00 ± 0.00[a]	0.92 ± 0.00[a]	0.92 ± 0.00[a]
	25	0.93 ± 0.02[a]	0.92 ± 0.00[a]	0.92 ± 0.00[a]
	50	0.92 ± 0.00[a]	0.92 ± 0.00[a]	0.94 ± 0.04[a]

[1]Values with different letters within the same column are significantly different (LSD test, $p<0.05$).

4.4 Conclusions

The ability of phospholipids and terpenes to serve as transport enhancers of anthocyanins was confirmed through these experiments. Transport values for anthocyanins with no enhancers on the matrix were 1.77% on average. Addition of phospholipids and terpenes resulted in average transport increase of 59 to 158% for both anthocyanins assessed (C3G and C3R). In addition, transport of anthocyanins was not dependent on dosage since absorption results were similar at both concentrations of anthocyanins tested. Contrary to results from Section 3, transport of anthocyanins was enhanced by the use of terpenes and phospholipids, but no significant differences were

observed between various concentrations of these compounds. Additionally, the additive effect of combining terpenes and phospholipids was not observed in these experiments. These results suggested that these compounds can achieve transport enhancement both individually and as a whole.

Transport rates were correlated with the concentration of anthocyanins initially applied to the Caco-2 cells monolayers. Additionally, transport rates for C3G were significantly higher than for C3R (2.32 fold), which also has to do with concentration difference of these two anthocyanins in the açaí extract. Different from overall absorption, which is a ratio of what is added to the apical side and what is found in the basolateral side, transport rates are only values found in the basolateral side which explains the differences related not only to treatment differences but also to concentration of analytes.

Particle size analysis revealed that vesicles over 50 nm in diameter were only found in samples with no terpenes in the matrix. Perhaps, terpenes together with phospholipids form a bigger liposome that is not allowed through the filter thus creating small vesicles and most of the anthocyanins are not encapsulated. Therefore, enhancement properties of terpenes and phospholipids could be attributed simply to the mere presence of these compounds.

Although increases in transport of anthocyanins were not as evident as in Section 3, the relevance of the increase still possesses tremendous implications for future research on this area. These results reinforced the idea of phospholipids and terpenes as transport enhancers for anthocyanin and other polyphenols. Further analysis needs to be

conducted to understand the mechanisms by which phospholipids and terpenes interact with anthocyanins and with the system to show enhanced transport of compounds through the epithelia.

V. EVALUATION OF TECHNOLOGIES TO MANUFACTURE MATRICES CONTAINING ANTHOCYANINS, TERPENES, AND PHOSPHOLIPIDS AND ITS EFFECTS ON ABSORPTION OF ANTHOCYANINS

5.1 Introduction

Interest in phytochemicals such as anthocyanins has increased in recent years due to their association with human health benefits (Talcott and Lee 2002; Wang 2006). The major mechanism by which these compounds enhance food quality and aid human health is radical scavenging which stops a degradation chain reaction caused by free radicals that are formed inside and outside the body (Robbins 2003). Around 100 radicals have been associated with degenerative diseases such as cancer, atherosclerosis, arthritis, and cataracts, and anthocyanins can donate a hydrogen atom, obstructing the development of such diseases (Shahidi and Naczk 2003; Parr and Bowell 2000). However, studies have illustrated the poor stability of anthocyanins exposed to the gastric environment and their limited absorption in the small intestine (Miyazawa and others 1999; Ichiyanagi and others 2008; Yi and others 2006). As mentioned in previous sections, transepithelial transport of anthocyanins could be enhanced by aiding agents that improve the absorption of compounds through the intestinal epithelia thus increasing their bioavailability. Research has illustrated the absorption/transport enhancement properties of phospholipids and terpenes in carotenoids, cholesterol, antimicrobials, and drug delivery (Yonekura and others 2006; Keller 2001; Sugawara and others 2001; Cal 2005, Lim and others 2006; Lim and others 2008). Yet, little work have been found in

the use of this technology to increase transport of polyphenolics. The use of phospholipids and terpenes was shown to increase transport of anthocyanins in Caco-2 cells transepithelial models (Section 3 and 4). Nevertheless, comparison of technologies to produce a matrix containing phospholipids, terpenes, and polyphenolics was not conducted in the past. Technologies commonly used to create vesicles from phospholipids include extrusion, sonication, French press, and ethanol injection. In addition, particle size analysis can be a useful tool to further understand the association of anthocyanins with terpenes and phospholipids in the matrix. Therefore, the purposes of this study were to further evaluate the efficacy of terpenes and/or phospholipids in the absorption of anthocyanins through a Caco-2 cell model, compare manufacturing technologies, and evaluate the potential formation of vesicles in the matrix.

5.2 Materials and Methods

Anthocyanins from açaí were isolated as explained in Section 3. Briefly, anthocyanins were allowed to adsorb onto an activated 10g reversed phase Sep-Pak C18 20cc cartridge (Waters Corporation, Milford, MA) cartridge by gravity feed followed by washes with water and ethyl acetate. Anthocyanin fractions were then recovered with acidified methanol (0.01% HCl), and kept at -80°C until further analysis. Anthocyanins were re-dissolved in Hank's Balanced Salt Solution (HBSS; pH 6.0) as explained is Section 3. Subsequently, the anthocyanin stock solutions were mixed with appropriate concentrations of phospholipids and/or terpenes. Samples were subjected to two different technologies (Sonication and French Press). One batch was bath sonicated (42 KHz) for 10 minutes, vortexed for 1 minute at 3000 RPM, and sterile-filtered

(Sonication) while two other batches were forced twice through a French needle-valve press (Fig. 5-1) at two different pressures (4,000 and 18,000 psi). More information on physical parameters and characteristics of this machine can be found in Appendix B. After processing, samples were assessed in a Caco-2 cell culture transport study and particle size analysis as explained in Sections 3 and 4, respectively.

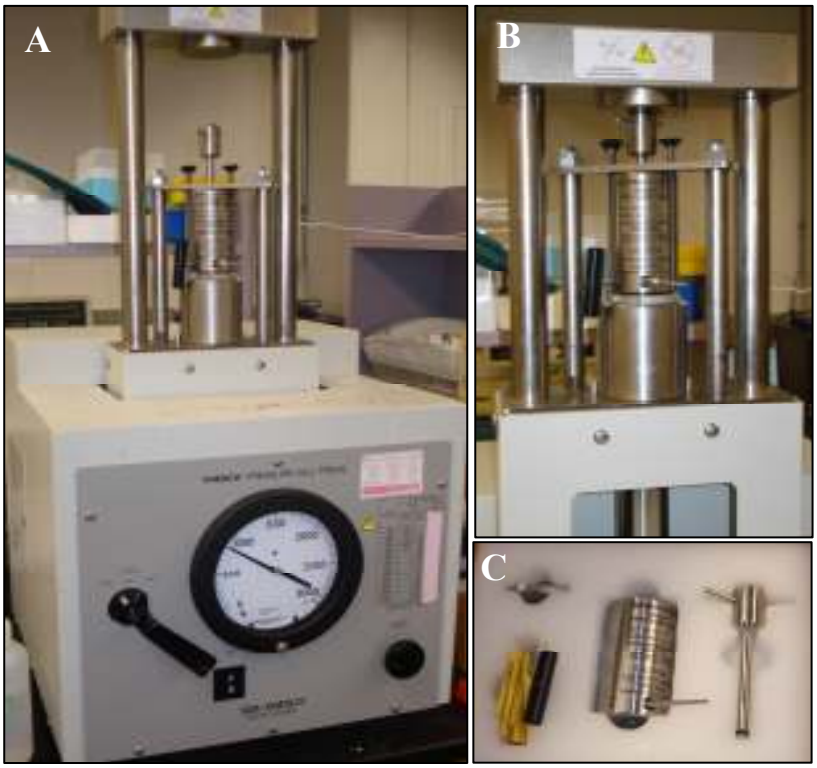


Figure 5-1. Photographs of French Press® and pressure cell used for experiments. (A) Position before sample collection. (B) Position for sample collection (C) Pressure Cell parts.

Data from experiments were analyzed by one-way analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Data for each analysis represents the mean of three replicates. Mean separations were conducted using LSD test ($P < 0.05$).

5.3 Results and Discussion

A comparison between technologies (French press and sonication-vortexing) to create a matrix comprising of anthocyanins, terpenes, and phospholipids was conducted. Selected combinations of these compounds were assessed based on their performance in anthocyanin transport previously assessed and to have representation of samples containing phospholipids, terpenes, and combinations of both. Samples were assessed at two different anthocyanin concentrations (250 and 500 mg/L). First sample set included 5,000 mg/L of phospholipids and no terpenes (T0 P5K); second set of samples contained 25 mg/L of terpenes and no phospholipids (T25 P0); third set of samples included 25 mg/L of terpenes and 10,000 mg/L of phospholipids (T25 P10K); and a final set combining 50 mg/L of terpenes and 10,000 mg/L of phospholipids (T50 P10K). Characterized anthocyanins, Cyanidin-3-Glucoside (C3R) and Cyanidin-3-Rutinoside (C3R), were used as analytes for a unidirectional transport study, assessed from the apical to the basolateral side of Caco-2 cells monolayers. Particle size analysis was also conducted to verify formation of vesicles (liposomes) that could be related to enhancement of anthocyanin transport.

5.3.1 Transepithelial Transport Study

Transepithelial transport of anthocyanins obtained from açaí was assessed through a Caco-2 cells model. Extracts properly mixed with various concentrations of terpenes and/or phospholipids were subjected to pressure (French press) or sonication before cell culture analysis. Subsequently, samples were loaded into the apical side of Caco-2 cells monolayers and presence of anthocyanins in the basolateral side was evaluated

chromatographically over time for up to 2 hours. Samples absorbed over 2 hours and found in the basolateral side were compared to the initial concentration of anthocyanins loaded to the apical side (Fig. 5-2). All samples contained C3G and C3R as the main anthocyanins found in açaí which agreed with results from previous sections. These anthocyanins were then monitored through the transepithelial transport study and results are presented in Tables 5-1 and 5-2 depending on the concentration of anthocyanins loaded initially (250 and 500 mg/L of anthocyanins, respectively).

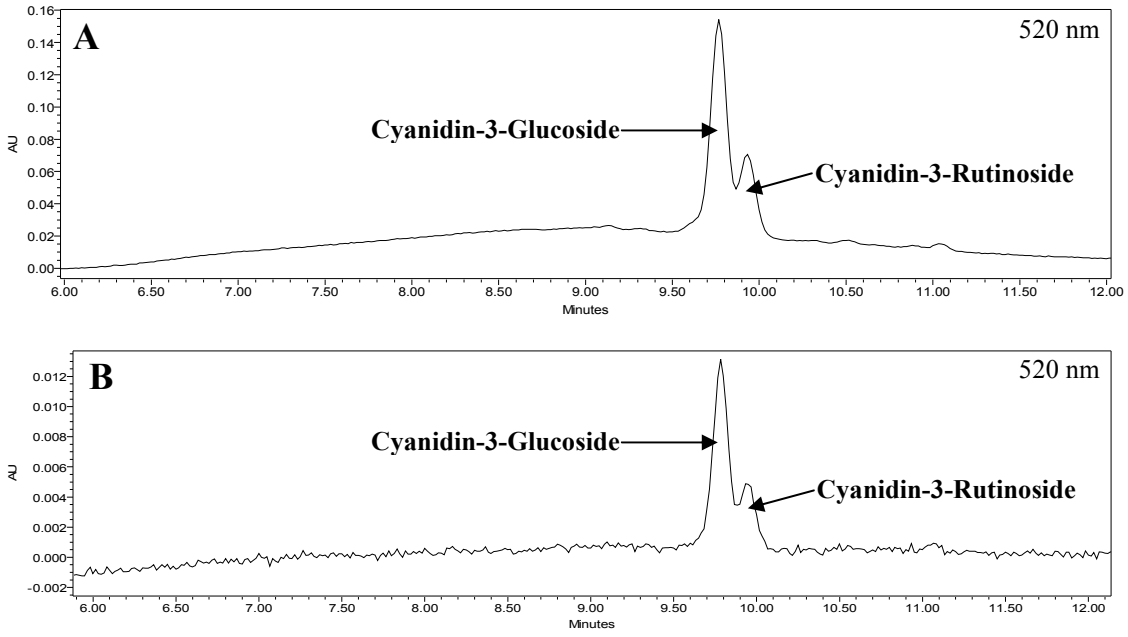


Figure 5-2. Chromatograms of anthocyanins from açaí extract in the apical side before analysis (A) and present in the basolateral side after an incubation period of 2 hours (B) III.

Table 5-1. Transport of anthocyanins (~250 mg/L) from açaí juice extracts from the apical to the basolateral side of Caco-2 cell monolayers following incubation (120 min, 37ºC), as a function of phospholipid and terpene concentrations and type of energy applied to the system.

		% Transport of Anthocyanins from Açaí Juice Extracts		
		Procedures		
	Treatments[1]	Pressure [psi]		Sonication/Vortexing
		4,000	18,000	/Filtration
Cyanidin -3- Glucoside	T0 P5K[2]	4.05 ± 0.16[a 3]	4.49 ± 0.40[a]	3.89 ± 0.35[a]
	T25 P0	3.70 ± 0.18[a]	3.89 ± 0.34[b]	4.00 ± 0.13[a]
	T25 P10K	3.89 ± 0.26[a]	4.41 ± 0.24[ab]	3.56 ± 0.33[a]
	T50 P10K	2.78 ± 0.22[b]	2.68 ± 0.17[c]	3.64 ± 0.07[a]
Cyanidin -3- Rutinoside	T0 P5K	4.02 ± 0.35[a]	4.08 ± 0.35[a]	4.01 ± 0.24[a]
	T25 P0	3.55 ± 0.32[ab]	3.73 ± 0.50[a]	3.73 ± 0.37[a]
	T25 P10K	4.09 ± 0.26[a]	4.20 ± 0.11[a]	3.85 ± 0.37[a]
	T50 P10K	3.08 ± 0.31[b]	2.78 ± 0.16[b]	3.59 ± 0.28[a]

[1]Combinations of terpenes (T) and phospholipids (P) subjected to three different procedures. [2]Treatments abbreviations: (T0 P5K) no terpenes and 5,000mg/L of phospholipids, (T25 P10K) 25 mg/L of terpenes and no phospholipids, (T25 P10K) 25 mg/L of terpenes and 10,000 mg/L of phospholipids, (T50 P10K) 50 mg/L of terpenes and 10,000 mg/L of phospholipids. [3]Values with different letters within the same column are significantly different (LSD test, $p<0.05$).

Table 5-2. Transport of anthocyanins (~500 mg/L) from açaí juice extracts from the apical to the basolateral side of Caco-2 cell monolayers following incubation (120 min, 37ºC), as a function of phospholipid and terpene concentrations, and type of energy applied to the system.

		% Transport of Anthocyanins from Açaí Juice Extracts		
		Procedures		
	Treatments[1]	French Press [psi]		Sonication/Vortexing
		4,000	18,000	/Filtration
Cyanidin -3- Glucoside	T0 P5K[2]	3.26 ± 0.10[b 3]	3.42 ± 0.18[b]	4.58 ± 0.35[a]
	T25 P0	3.49 ± 0.29[ab]	3.24 ± 0.16[b]	3.59 ± 0.30[b]
	T25 P10K	3.46 ± 0.03[ab]	3.79 ± 0.08[a]	3.57 ± 0.15[b]
	T50 P10K	3.63 ± 0.02[a]	2.92 ± 0.02[c]	3.74 ± 0.09[b]
Cyanidin -3- Rutinoside	T0 P5K	3.33 ± 0.15[ab]	3.65 ± 0.29[a]	4.46 ± 0.45[a]
	T25 P0	3.34 ± 0.12[ab]	3.19 ± 0.34[ab]	3.70 ± 0.38[b]
	T25 P10K	3.12 ± 0.14[b]	3.61 ± 0.16[a]	3.69 ± 0.18[b]
	T50 P10K	3.52 ± 0.06[a]	3.04 ± 0.23[b]	3.88 ± 0.33[ab]

[1]Combinations of terpenes (T) and phospholipids (P) subjected to three different procedures. [2]Treatments abbreviations: (T0 P5K) no terpenes and 5,000mg/L of phospholipids, (T25 P10K) 25 mg/L of terpenes and no phospholipids, (T25 P10K) 25 mg/L of terpenes and 10,000 mg/L of phospholipids, (T50 P10K) 50 mg/L of terpenes and 10,000 mg/L of phospholipids. [3]Values with different letters within the same column are significantly different (LSD test, $p<0.05$).

Increased transport of anthocyanins by the addition of terpenes and phospholipids was demonstrated once again. General increases in absorption ranged from 51 to 159% for C3G and 57 to 152% for C3R compared to transport of anthocyanins with no aiding agents. There were no differences in transport values for the lowest concentration of anthocyanins tested (250 mg/L) when the technology used to process the matrices was sonication and vortexing followed by filtration. In contrast, differences could be detected when anthocyanin concentration was doubled. Highest transport values were observed in T25 P10K samples for C3G (4.58%) and T0 P5K for C3R (4.46%).

Highest transport for C3G at the lowest concentration of anthocyanins analyzed (250 mg/L) were observed in T25 P10K and T0 P5K which occurred when French Press was the technology used at 18,000 psi of pressure (4.49 and 4.41%, respectively). This effect was observed in samples subjected to pressure (French Press) whereas no differences were observed in samples subjected to sonication/vortexing. For C3R, results illustrated that results from all technologies were fairly similar which ranged from 3.55% to 4.20%. Only T50 P10K samples subjected to French Press (at both pressures) were significantly lower. Although transport of anthocyanins was still enhanced compared to samples with no aiding agents, saturation of the system with a mixture of terpenes and phospholipids might have started antagonizing the enhancement of anthocyanin transport.

Results observed at the highest concentration of anthocyanins (500 mg/L) were different from the lowest concentration assessed (250 mg/L). Highest transport results for C3G and C3R were observed in samples subjected to sonication/vortexing (4.58%

and 4.46%, respectively). Transport of C3G in T25 P10K samples were among the highest when high pressure (18,000 psi) or sonication/vortexing was applied to the system. Lowest transport of anthocyanins (2.92%) was observed in T50 P10K only when high pressure was applied (18,000 psi). Conversely, transport of anthocyanins in T50 P10K was among the highest when less pressure (4,000 psi) was applied to the system. For analysis of C3R in samples subjected to pressure, results illustrated no important differences. When anthocyanin concentration was doubled, samples either maintained or decreased the level of anthocyanin transport. On the contrary, T50 P10K samples showed enhanced transport of anthocyanins when pressure was applied to the system.

5.3.2 Particle Size Analysis

Particle size of açaí extract rich in anthocyanins was assessed by dynamic light scattering (Walzem and others 1994). Anthocyanin-rich extract properly mixed with various concentrations of terpenes and/or phospholipids were subjected to two different encapsulation technologies. A set of samples was subjected to two rounds of sonication and vortexing (~15 min) followed by sterile filtration (0.22 μm) while two other sample sets were forced twice through a needle-valve press at two different pressure levels (4,000 and 18,000 psi). Diameter measurements were determined. Light diffusion was recorded for 30 seconds, modified to an audio range, and deconvoluted by a software system. Sample particle populations were calculated as the width (nm) of the measured particle size distribution and are presented in Table 5-3.

Table 5-3. Particle size of matrices as a function of anthocyanin, phospholipid, and terpene concentrations and manufacturing technologies.

Anthocyanin Concentration [mg/L]		Treatment[1]	Particle size [nm]		
			Procedures		
			French Press [psi]		Sonication / Vortexing / Filtration
			4,000	18,000	
250	MV[2]	T0 P5K[3]	1750 ± 178[b 4]	1760 ± 161[b]	185 ± 12.1[a]
		T25 P0	0.92 ± 0.00[d]	0.92 ± 0.00[d]	0.92 ± 0.00[b]
		T25 P10K	1054 ± 78.0[c]	2160 ± 7.77[a]	0.97 ± 0.02[b]
		T50 P10K	2190 ± 239[a]	1270 ± 118[c]	0.92 ± 0.00[b]
	MN	T0 P5K	386 ± 18.2[c]	937 ± 36.9[b]	179 ± 11.6[a]
		T25 P0	0.90 ± 0.00[d]	0.90 ± 0.00[d]	0.90 ± 0.00[b]
		T25 P10K	681 ± 84.7[a]	2130 ± 8.72[a]	0.94 ± 0.01[a]
		T50 P10K	501 ± 10.8[b]	514 ± 30.6[c]	0.90 ± 0.00[a]
	MA	T0 P5K	798 ± 73.3[b]	1170 ± 109[b]	183 ± 11.9[a]
		T25 P0	0.91 ± 0.00[c]	0.91 ± 0.00[d]	0.91 ± 0.00[b]
		T25 P10K	822 ± 90.1[b]	2150 ± 7.51[a]	0.95 ± 0.01[b]
		T50 P10K	1552 ± 115[a]	885 ± 95.5[c]	0.91 ± 0.00[b]
500	MV	T0 P5K	1730 ± 80.6[b]	2390 ± 62.5[a]	0.92 ± 0.00[a]
		T25 P0	1.63 ± 0.04[d]	1.33 ± 0.21[d]	0.93 ± 0.00[a]
		T25 P10K	2020 ± 183[a]	1760 ± 108[b]	0.92 ± 0.00[a]
		T50 P10K	794 ± 35.4[c]	1470 ± 140[c]	0.94 ± 0.04[a]
	MN	T0 P5K	500 ± 29.4[a]	502 ± 91.1[b]	0.90 ± 0.00[a]
		T25 P0	1.35 ± 0.13[d]	1.05 ± 0.04[d]	0.90 ± 0.00[a]
		T25 P10K	389 ± 29.5[b]	923 ± 34.5[a]	0.90 ± 0.00[a]
		T50 P10K	296 ± 10.3[c]	320 ± 11.8[c]	0.92 ± 0.03[a]
	MA	T0 P5K	1210 ± 61.5[b]	1270 ± 83.9[a]	0.91 ± 0.00[a]
		T25 P0	1.52 ± 0.07[d]	1.20 ± 0.13[c]	0.91 ± 0.00[a]
		T25 P10K	1490 ± 214[a]	1330 ± 135[a]	0.91 ± 0.00[a]
		T50 P10K	427 ± 14.4[c]	512 ± 39.6[b]	0.93 ± 0.03[a]

[1]Combinations of terpenes (T) and phospholipids (P) subjected to three different procedures. [2]Particle Size measurements: (MV) Volume distribution, (MN) Mean distribution, (MA) Area distribution. [3]Treatments abbreviations: (T0 P5K) no terpenes and 5,000mg/L of phospholipids, (T25 P10K) 25 mg/L of terpenes and no phospholipids, (T25 P10K) 25 mg/L of terpenes and 10,000 mg/L of phospholipids, (T50 P10K) 50 mg/L of terpenes and 10,000 mg/L of phospholipids. [4]Values with different letters within the same column are significantly different (LSD test, $p<0.05$).

Formation of vesicles over 220 nm confirmed the hypothesis proposed in the preceding Section. Vesicles created by any of the treatments (sonication/filtration or pressure) were too big to be allowed through the filter (220nm). Formation of vesicles was observed only in treatments subjected to pressure and T0 P5K in the case of sonicated samples. This is mostly due to the lack of a filtration process following French Press treatment. Little or no vesicle formation was observed in samples without phospholipids present on their matrix. Filtration created homogenous matrices since values for mean volume distribution (MV), mean area distribution (MA), and mean number distribution (MN) were similar within treatments.

In contrast, results from pressure treatments indicated that the population of vesicles created ranged broadly from 296 to 2,390 nm in diameter. Variations in particle size were more pronounced when anthocyanin concentrations were 500 mg/L. In addition, samples subjected to 18,000 psi of pressure created matrices with the least variability compared to a lower pressure (4,000 psi). Higher pressure applied to the system forces phospholipids to form a more homogenous population, which agreed with preliminary studies on understanding the effect of pressure on a system containing phospholipids. When no pressure is applied to the system, phospholipids naturally arrange but particle diameters are extremely variable. As pressure is applied, vesicles re-arrange and homogeneity is strongly related to force applied to the system. In addition, T25 P10K samples resulted in the least variability of all samples subjected to pressure. However, these differences did not affect the transport of anthocyanins as importantly as the presence of phospholipids and terpenes did in the matrix.

When particle size was related to transport of anthocyanins, weak correlations were observed in all samples subjected to sonication ($r < 0.42$). Correlations in samples subjected to pressure ranged from 0.25-0.64. Transport of anthocyanins was maintained or reduced when anthocyanin concentration was doubled except for T50 P10K samples, effect that was consistent among all samples. In contrast, changes in particle size were well correlated with changes in transport values due to modifications in anthocyanin concentration. The changes in transport of anthocyanins were partly related to particle size increment. This effect is most noticeable when the increase in transport of C3G and C3R in T50 P10K samples subjected to 4,000 psi of pressure is accompanied by a significant reduction in particle size (64%).

5.4 Conclusions

While previous Sections focused on illustrating the ability of phospholipids and terpenes as anthocyanin transport enhancers, the objective of this investigation was to assess two different technologies and their effect on construction of a matrix containing phospholipids, terpenes, and anthocyanins, and how this matrix could affect the transepithelial transport of anthocyanins. Comparisons illustrated that both technologies created matrices that maintained the properties of phospholipids and terpenes as transport enhancers. General increases in transport values ranged from 51 to 159% for C3G and 57 to 152% for C3R compared to transport of anthocyanins with no aiding agents. Best results in transport of anthocyanins at the lowest concentration tested (250 mg/L) were observed in T25 P10K and T0 P5K that occurred when French Press was the technology used at 18,000 psi of pressure. Additionally, T25 P10K and T0 P5K samples

presented the highest transport values in every technology. Lowest transport of anthocyanins was observed in T50 P10K. This effect was confirmed in all technologies with transports values of 2.68-3.56% for C3G and 2.78-3.59% for C3R. When anthocyanin concentration was doubled, best transports of anthocyanin were observed in T25 P10K for C3G and T0 P5K for C3R subjected to sonication and vortexing followed by filtration. Samples generally decrease in transport of anthocyanins as the concentrations of C3G and C3R were doubled except for T50 P10K samples which maintained or increased.

Particle size analysis illustrated the formation of vesicles that ranged broadly in diameter (296-2,390 nm). Variations in particle size were more pronounced when anthocyanin concentrations were 500 mg/L and when pressure applied to the system was low (4,000 psi). When water is initially added to a mixture, phospholipids spontaneously form vesicles of extremely variable diameters. As energy was applied to the system, vesicles were forced to re-arrange creating a more homogenous population.

Particle size was not well correlated with transport of anthocyanins except for C3G (500 mg/L) in samples subjected to pressure. Transport of anthocyanins was reduced when anthocyanin concentration was doubled except for T50 P10K samples and modifications in particle dimensions were well correlated with this effect in samples subjected to pressure (4,000 psi) or sonication/vortexing. The reduction in transport of C3G and C3R were partly related to particle size increment in samples subjected to low pressure (4,000 psi). This effect was noticeable since the increase in transport of C3G and C3R in sample T50 P10K was accompanied by a significant reduction in particle

size (64%). These aspects suggested that although the major contribution of these transport aiding agents is their presence in the matrix, the dimension and nature of the vesicles formed in it could impact the transport of compounds of interest. However, more research needs to be conducted to explore the effect of vesicle formation and anthocyanin encapsulation on the transport of these phytochemicals.

These results corroborate the idea of phospholipids and terpenes as transport enhancers for anthocyanin and other polyphenols. Further analysis needs to be conducted to understand the interaction of anthocyanins, phospholipids and terpenes in the matrix and confirm if encapsulation is a possible mechanism for enhanced transport of compounds through the epithelia.

VI. ADDITION OF PHOSPHOLIPIDS EXTRACTED FROM ACAI ENHANCES THE ABSORPTION OF ANTHOCYANINS EXTRACTED FROM ACAI AND WINE

6.1 Introduction

Interest in anthocyanins and other polyphenolics has increased in the past decade due to their potential health benefits related to their radical scavenging properties (Robbins 2003, Talcott and Lee 2002; Wang 2006). Anthocyanins are a major group of water-soluble pigments in plants responsible for the red, blue and violet colors in many fruits and vegetables (Bridle and Timberlake 1997). In addition, consumers in the US have an average daily intake of over 200 mg/L of anthocyanins (Galvano and others 2004). Although extensive research has suggested benefits of anthocyanin consumption on human health (Elattar and Viriji 1999; Khan and others 2008), absorption of these flavonoids is fairly poor. Their limited bioavailability may also limit their potential action against free radicals in the body. As established in previous Sections, transport of anthocyanins through a Caco-2 cells monolayer model was enhanced by the application of phospholipids and terpenes thus increasing their presence in the body. There is an extensive body of research illustrating the absorption/penetration enhancement properties of phospholipids and terpenes (Yonekura and others 2006; Keller 2001; Sugawara and others 2001; Cal 2005, Lim and others 2006; Lim and others 2008). Nevertheless, no investigations were conducted on the absorption enhancement properties of phospholipids extracted from açaí oil. Investigations have demonstrated the presence of phospholipids in crude oils from sunflower and soybean suggesting that

crude açaí oil may contain phospholipids (Carelli and others 1997; Mounts and Nash 1990). Therefore, the purpose of this study was to assess the efficacy of phospholipids extracted from crude açaí oil on the absorption/ transport of anthocyanins extracted from of açaí and grapes through a Caco-2 cell model.

6.2 Materials and Methods

Clarified açaí concentrate from Brazil was obtained from Stiebs Pomegranate Products (Madera, CA). Port wine was obtained from Messina Hof Winery (Bryan, TX). Anthocyanins from açaí and port wine were isolated by loading samples onto an activated 10g reversed phase Sep-Pak C18 20cc cartridge (Waters Corporation, Milford, MA) as explained in Section 3. Anthocyanin fractions were desorbed from the cartridge with acidified methanol (0.01% HCl), and kept at -80°C until further analysis.

In parallel, açaí oil was solvent extracted using a patent-pending process (Talcott 2007) from a water-insoluble processed juice by-product. Phospholipids were isolated from açai oil using a modified method from Carelli and others (1997) using a Hypersep Diol 5000 mg cartridge (Thermo-Fisher Scientific, Waltham, MA). Cartridge was previously washed with methanol, followed by a chloroform rinse. Subsequently, the cartridge was thoroughly cleaned with hexane before sample loading. Oil was dissolved in chloroform prior to loading onto the cartridge and allowed to adsorb to the cartridge. The cartridge was then rinsed with chloroform to assure all unbound compounds had been eluted. The fraction containing phospholipids was then desorbed with 100% methanol. Following evaporation to dryness, samples were stored at -40˚ C prior to analysis.

For analysis, methanol was completely removed from samples containing anthocyanins from açaí and wine. Anthocyanins were re-dissolved in Hank's Balanced Salt Solution (HBSS; pH 6.0) and standardized to a final concentration of 1,000 mg cyanidin-3-glucoside equivalents/L (anthocyanin stock solutions), which was determined spectrophotometrically by the pH differential method (Wrolstad 1976). In parallel, phospholipids were also re-dissolved in HBSS (pH 6.0) and standardized to a final concentration of 20,000 mg total phospholipids/L (phospholipid stock solution) determined spectrophotometrically according to a modified method by Totani and others (1982). The anthocyanin stock solutions from açaí and wine were mixed with appropriate concentrations of the phospholipid stock solution to generate various samples at different concentrations of both compounds. Samples were subjected to two rounds of bath sonication (5 min @ 42 MHz) and vortexing (30 seconds @ 3,000 RPM) prior to cell culture transport study.

Transepithelial transport of anthocyanins was conducted using a Caco-2 colon carcinoma cells model as illustrated in Section 3. Samples collected from the transport study were analyzed by reverse phase HPLC using modified chromatographic conditions (Talcott and Lee 2002) with a Waters 2690 Alliance HPLC system using a Water PDA detector as explained in Materials and Methods section of Section 3. Data was reported as ratios between compounds found in the basolateral compartments (mg/L) to compounds initially added to the apical compartments (mg/L).

Data from experiments were analyzed by one-way analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Data for each analysis represents the mean of three replicates. Mean separations were conducted using LSD test ($P < 0.05$).

6.3 Results and Discussion

Potential transport enhancement of anthocyanins from two different sources (açaí juice and port wine) by the use of phospholipids extracted form açaí oil was examined. Characterized anthocyanins from açaí, Cyanidin-3-Glucoside (C3R) and Cyanidin-3-Rutinoside (C3R), and from port wine, Anthocyanin 1 (An 1), Anthocyanin 2 (An 2), and Anthocyanin 3 (An 3), were used for a unidirectional transport study, assessed from the apical to the basolateral side of Caco-2 cells monolayers, and particle size analysis was conducted to possibly relate formation of liposomes to enhancement of anthocyanin absorption.

6.3.1 Transepithelial Transport Study

Transepithelial transport of açaí juice and port wine extracts rich in anthocyanins was assessed through a Caco-2 cells model. Extracts properly mixed with various concentrations of açaí oil phospholipids were loaded into the apical side of the cells monolayer and presence of anthocyanins in the basolateral side was evaluated chromatographically over time for up to 2 hours. Samples transported over 2 hours and found in the basolateral side were compared to the initial concentration of anthocyanins loaded to the apical side. C3G and C3R were detected in all samples as the main anthocyanins found in açaí, which agreed with results from previous sections (Fig. 6-1). Three predominant anthocyanins (An-1, An-2, An-3) were found and characterized in

Port wine (Fig. 6-2). These characterized anthocyanins from açaí juice and port wine were then monitored through the transepithelial transport study and results are presented in Tables 6-1 and 6-2, respectively.

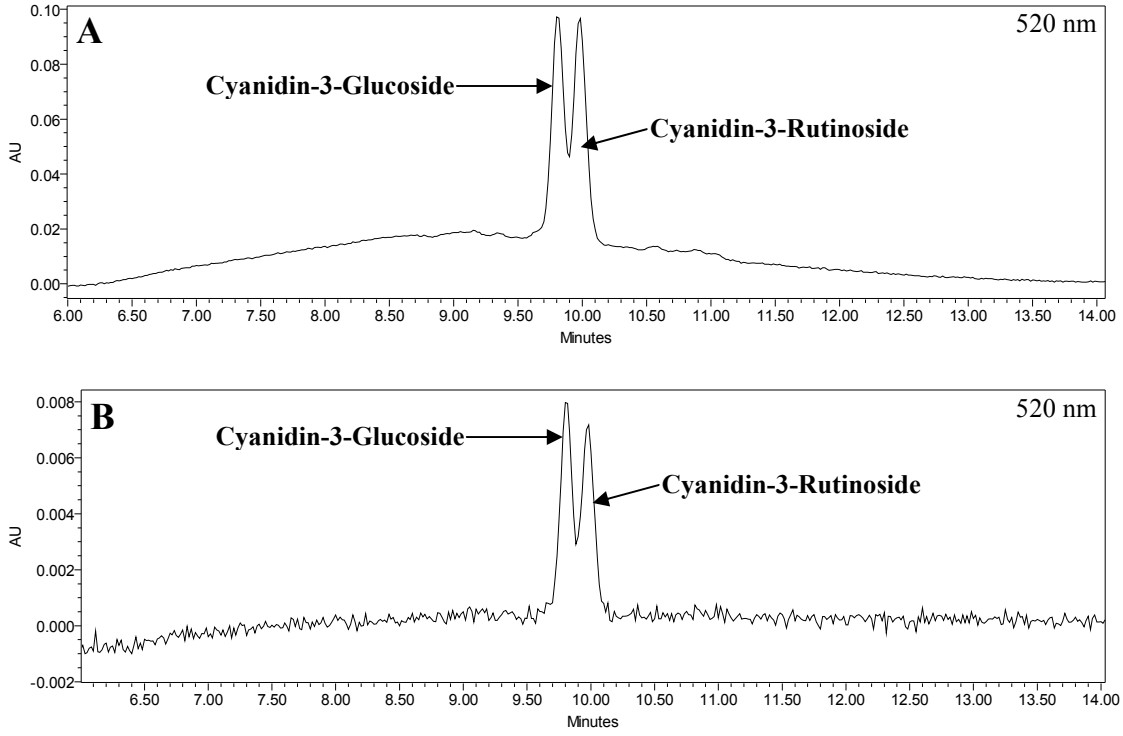


Figure 6-1. Chromatograms of anthocyanins from açaí extract in the apical side before analysis (A) and present in the basolateral side after an incubation period of 2 hours (B) IV.

Açaí anthocyanins with no phospholipids were transported inadequately at both concentration of anthocyanins (1.77%) agreeing with results from previous Sections and other investigations regarding polyphenolic transport through cell monolayers (Pacheco-Palencia and others 2008; Chan and others 2007). In general, addition of phospholipids

Table 6-1. Transport of anthocyanins from açaí juice extracts from the apical to the basolateral side of Caco-2 cell monolayers following incubation (120 min, 37ºC), as a function of anthocyanin and phospholipid concentrations.

Anthocyanin Concentration [mg/L]	Phospholipid Concentration [mg/L]	% Transport of Anthocyanins from Açaí Juice Extracts	
		Cyanidin-3-Glucoside	Cyanidin-3-Rutinoside
250	0	$1.70 \pm 0.13^{c\,1}$	1.79 ± 0.04^{b}
	5,000	4.12 ± 0.40^{a}	3.16 ± 0.22^{a}
	10,000	3.46 ± 0.17^{b}	3.02 ± 0.08^{a}
500	0	1.80 ± 0.15^{c}	1.80 ± 0.18^{b}
	5,000	$3.24 \pm 0.10^{b\,*\,2}$	3.11 ± 0.20^{a}
	10,000	3.29 ± 0.38^{b}	3.05 ± 0.18^{a}

[1]Values with different letters within the same column are significantly different (LSD test, $p<0.05$).
[2]Asterisk indicates significant difference in transport values of cyanidin-3-glucoside when concentration of anthocyanins fluctuated.

Table 6-2. Transport of anthocyanins from port wine extracts from the apical to the basolateral side of Caco-2 cell monolayers following incubation (120 min, 37ºC), as a function of phospholipids concentrations.

Anthocyanin Concentration [mg/L]	Phospholipid Concentration [mg/L]	% Transport of Anthocyanins from Açaí Juice Extracts		
		An-1	An-2	An-3
250	0	$ND^{d\,1}$	ND^{e}	ND^{e}
	5,000	$1.94 \pm 0.06^{a\,2}$	3.92 ± 0.10^{a}	1.94 ± 0.12^{a}
	10,000	1.68 ± 0.05^{b}	3.38 ± 0.23^{bc}	1.15 ± 0.11^{c}
500	0	$0.36 \pm 0.01^{c\,*\,3}$	$0.96 \pm 0.03^{d\,*}$	$0.32 \pm 0.00^{d\,*}$
	5,000	$2.07 \pm 0.22^{a\,*}$	3.53 ± 0.35^{b}	1.96 ± 0.19^{a}
	10,000	2.08 ± 0.03^{a}	3.20 ± 0.10^{c}	$2.03 \pm 0.04^{a\,*}$

[1]Compounds were not detected. [2]Values with different letters within the same column are significantly different (LSD test, $p<0.05$). [3]Asterisk indicates significant difference in anthocyanin transport values when concentration of anthocyanins fluctuated.

increased the anthocyanins found in the basolateral compartments. Increases in transport values ranged from 83 to 142% and 69 to 74% for C3G and C3R, respectively. Relative transport of anthocyanins did not vary as a function of anthocyanin concentration since results were fairly similar and enhancement transport effect of phospholipids did not change with change in anthocyanins content. The only variation detected due to

anthocyanin concentration change was observed in samples containing 5,000 mg/L of phospholipids were there was an inverse relation between concentration of C3G and transport of anthocyanins.

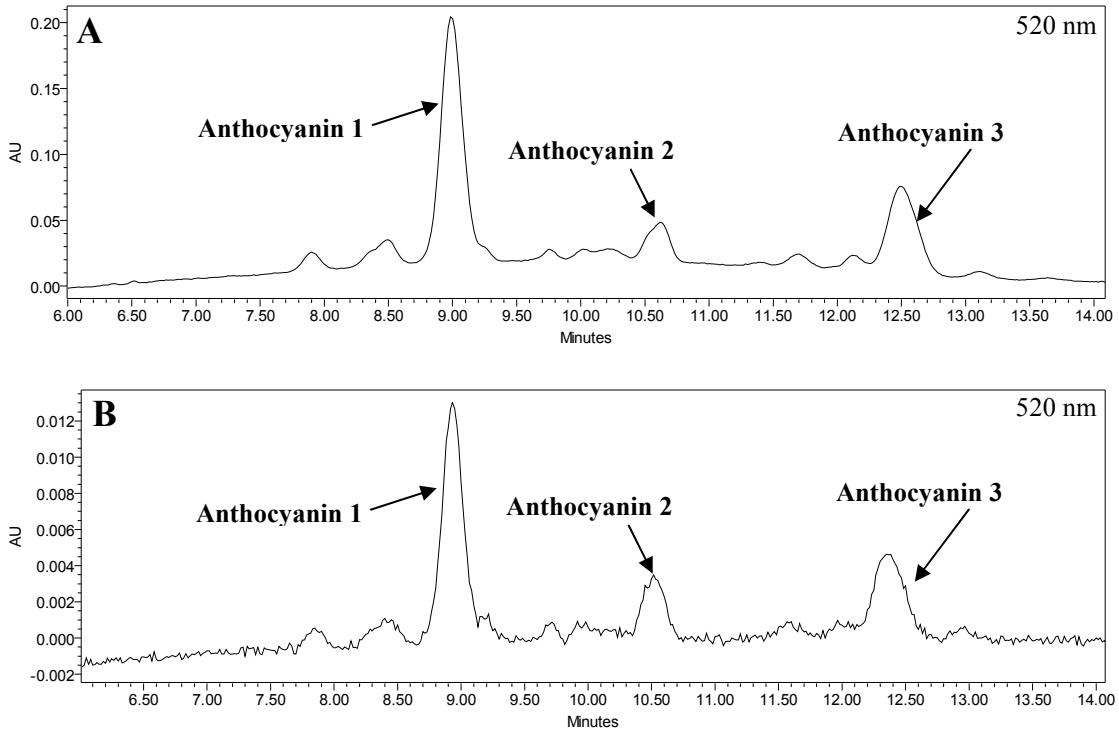


Figure 6-2. Chromatograms of anthocyanins from port wine extract in the apical side before analysis (A) and present in the basolateral side after an incubation period of 2 hours (B).

Differences in transport values were observed in C3G whereas no significant differences were observed for C3R when phospholipids where in the matrix. The highest anthocyanin transport values for C3G and C3R were observed in samples containing 5,000 mg/L (4.12% and 3.16%, respectively) elucidating the idea that 5,000

mg/L of phospholipids extracted from açaí are the best combination to promote absorption of açaí anthocyanins. Higher concentrations of phospholipids may hinder transport by interacting with anthocyanins and or monolayer too long hindering the passage of analytes through the Caco-2 monolayer thus making it unavailable for assessment after two hours.

In parallel, port wine anthocyanins with no phospholipids were also transported poorly which agreed with results from açaí samples in this and previous sections. No transport of anthocyanins was recorded after 2 hours of incubation at the lowest concentration of anthocyanins assessed (250 mg/L). Samples containing 500 mg/L of anthocyanins demonstrated the deprived transport of these compounds through a Caco-2 cell monolayer model. The transport values of the three main anthocyanins detected in port wine were lower than 1%.

Addition of phospholipids extracted from açaí oil significantly increased the anthocyanins found in the basolateral compartments. Increases in transport values ranged from 371 to 485%, 234% to 310%, and 264% to 543% for An-1, An-2, and An-3, respectively. Dissimilar to açaí anthocyanins, dependency on anthocyanin concentration was illustrated in relative transport of two of the three port wine anthocyanins assessed. An-2 did not show dependency on the concentration of anthocyanins tested. Changes were detected in all anthocyanins with no phospholipids in the matrix, and An-1 and An-3 in samples containing 10,000 mg/L of phospholipids where a positive relation between concentration and transport of anthocyanins was noticed.

Higher overall transport values were observed for An-2 compared to An-1 and An-3. Evident differences in transport values were observed in all three port wine anthocyanins. Highest anthocyanin transport values for An-1 and An-3 were observed in samples containing 10,000 mg/L at both concentrations of anthocyanins and when anthocyanins at the lowest concentration assessed was mixed with 5,000 mg/L of phospholipids. Additionally, best results in all anthocyanins were generally detected when 5,000 mg/L of phospholipids.

6.4 Conclusions

The capacity of phospholipids extracted from açaí oil as transport enhancers of anthocyanins was confirmed through these experiments. Transport values for açaí anthocyanins with no enhancers on the matrix were 1.77% on average. Addition of phospholipids resulted in average increase in anthocyanin transport from 83 to 142% and 69 to 74% for C3G and C3R, respectively. In addition, transport of anthocyanins was not dependent on dosage since absorption results were similar at both concentrations of anthocyanins tested except for samples containing 5,000 mg/L of phospholipids where an inverse relation between concentration and transport of anthocyanins was observed. Highest anthocyanin transport values for C3G and C3R were observed in samples containing 5,000 mg/L (4.12% and 3.16%, respectively) and C3G transport values were generally higher than those of C3R.

Similarly to açaí anthocyanins, port wine anthocyanins with no phospholipids were transported poorly. No transport of anthocyanins was detected after 2 hours of incubation when total anthocyanin concentration was 250 mg/L. When anthocyanin

concentration was doubled, transport values of the An-1, An-2, and An-3 were detected in samples assessed and the transport values were very low (0.36%, 0.96%, and 0.32%, respectively). Addition of phospholipids significantly increased the transport of anthocyanins demonstrating increases from 371 to 485%, 234% to 310%, and 264% to 543% for An-1, An-2, and An-3, respectively.

Açaí phospholipids illustrated their transport enhancement properties on two different matrices containing anthocyanins from açaí and port wine. These findings strengthen the idea of using phospholipids to improve bioavailability of different phytochemicals. This improvement possesses tremendous implications for future research on the area of modulation of phytochemical transport/absorption. Phospholipids were assessed on two anthocyanins sources (açaí and grape) and results illustrated anthocyanin transport enhancement on both. Additionally, anthocyanins from port wine were enhanced at a much higher proportion than açaí anthocyanins were which suggests that associations between phospholipids and polyphenolics are very sensitive not only to concentration of components and nature of the matrix (temperature, acidity, etc) but also to the type of components present in the matrix. These results confirmed that phospholipids could serve as transport enhancers for anthocyanin in various commodities. Further analysis needs to be conducted to demonstrate if phospholipids could enhance absorption of other polyphenolics in other commodities.

VII. EFFECT OF PHOSPHOLIPIDS AND TERPENES ON THE ABSORPTION OF GALLIC ACID AND THE SIZE OF PARTICLES FORMED

7.1 Introduction

Interest in polyphenolics has increased in recent years due to their potential health benefits (Robbins 2003, Talcott and Lee 2002). However, polyphenolic recognition is not only due to their bioactive properties but also for their contribution to flavor and color of several fruits and vegetables (Croft 1999). Gallic acid is a phenolic acid widely spread in nature and has been found in strawberries, lemons, grapes, and tea, among others (Chu and others 2002). Research has elucidated the antiproliferative, anti-tumorigenic, and pro-apoptotic properties of gallic acid (Kaur and others 2009; Loizzo and others 2009). In contrast, poor absorption of gallic acid has been illustrated and compared to other phenolic acids (Konishi and others 2004; Konishi and others 2006). Due to this limited bioavailability, gallic acid's potential action against free radicals is hindered.

As established in previous Sections, anthocyanin transport of various sources was enhanced by the application of phospholipids and terpenes. However, no investigations have elucidated the effect of phospholipids and terpenes on the transport/absorption of other polyphenolics. Gallic acid was selected as a marker compound to examine its transport through a Caco-2 cell monolayer model and how phospholipids and terpenes may modulate it. Gallic acid is a common phenolic acid found in nature that is structurally dissimilar to anthocyanins and other flavonoids. Additionally, polarity,

molecular weight, ionic strength, acidity, and other characteristics provide an opportunity to assess phospholipids and terpenes on transport of a different polyphenolic group and their contribution to the physical nature of the matrix. If enhancement properties of phospholipids and terpenes could be reproduced in gallic acid, it would cement the idea that phospholipids and terpenes can improve absorption of various polyphenolic groups. Therefore, the purposes of this study were to assess the efficacy of phospholipids and terpenes on the absorption/ transport of gallic acid through a Caco-2 cell model and the effect of these enhancers on the dimensions of particles created in the matrix.

7.2 Materials and Methods

Gallic acid was obtained from Fischer Scientific (Fair Lawn, NJ). Dubelcco's Phosphate Saline Buffer (PBS) and Hank's Balanced Salt Solution (HBSS) were purchased from Invitrogen Inc. (Grand Island, NY). Gallic acid was dissolved in HBSS (pH 6.0) and standardized to a final concentrations of 750 and 1,500 mg/L (gallic acid stock solutions) which were determined spectrophotometrically by the Folin-Ciocalteu assay (Singleton and Rossi 1965). Subsequently, the gallic acid stock solutions were mixed with appropriate concentrations of phospholipids and/or terpenes to generate various samples at different concentrations of all compounds. Samples were then subjected to two rounds of bath sonication for 5 minutes at 42 KHz (Branson Ultrasonic Corp. Danbury, CT) and vortexing (30 seconds at 3,000RPM) prior to cell culture transport study and particle size analysis.

Transepithelial transport of gallic acid was conducted using a Caco-2 colon carcinoma cells model as explained in Section 3. Briefly, monolayers were rinsed with PBS. Once PBS was removed from the plate, HBSS was added to the basolateral and apical side. After applying HBSS to both sides, cells were incubated (30 min) and treatments containing combination of gallic acid, phospholipids, and/or terpenes were added to the apical side of the wells. Thereafter, sample aliquots (200 μL) were taken at time zero and every 30 min for 2 hours from the basolateral side, immediately refrigerated (5 ºC) and analyzed within hours after analysis. Basolateral volume was kept constant by adding fresh HBSS (200 μL) after sampling. Resistance (TEER) was measured once again after assay was completed to insure monolayer integrity. Only compartments with TEER values over 350 Ω cm^2 were considered for data analysis.

Samples collected from the transport study were analyzed by reverse phase HPLC using modified chromatographic conditions (Talcott and Lee 2002) with a Waters 2690 Alliance HPLC system using a Waters PDA detector. Separations were performed on a 250 x 4.6 mm Nova-Pak C_{18} column (Waters Corporation, Milford, MA) with a C_{18} guard column. Mobile phase A consisted of water acidified with *o*-phosphoric acid (pH 2.4) and Mobile phase B consisted of 60:40 methanol and water acidified with *o*-phosphoric acid (pH 2.4). The gradient solvent program run phase B from 0 to 30% in 1 min; 30 to 50% in 1 min, 50 to 70% in 2 min, 70 to 96.2% in 3.5 min, 96.2 to 100% in 0.5 min and held at 100% for 1 min for a total run time of 9 minutes at a flow rate of 0.8 mL/min. Gallic acid was identified by UV/VIS spectral interpretation, retention time and comparison to an authentic standard (Fisher Scientific, Fair Lawn, NJ). Data was

analyzed as mg/L of gallic acid and reported as a ratio between gallic acid found in the apical and basolateral compartments.

Data from experiments were analyzed by one-way analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Data for each analysis represents the mean of three replicates. Mean separations were conducted using LSD test ($P < 0.05$).

7.3 Results and Discussion

7.3.1 Transepithelial Transport Study

Transport enhancement of gallic acid by the addition of phospholipids and terpenes was examined through a unidirectional transport study by assessing it from the apical to the basolateral side of Caco-2 cells monolayers. Gallic acid properly mixed with various concentrations of phospholipids and terpenes were loaded into the apical side of the cells monolayer and presence of gallic acid in the basolateral side was evaluated chromatographically over time for up to 2 hours (Fig. 7-1). Samples transported over 2 hrs and found in the basolateral side were compared to the initial concentration of gallic acid loaded to the apical side. Results are presented in Table 7-1.

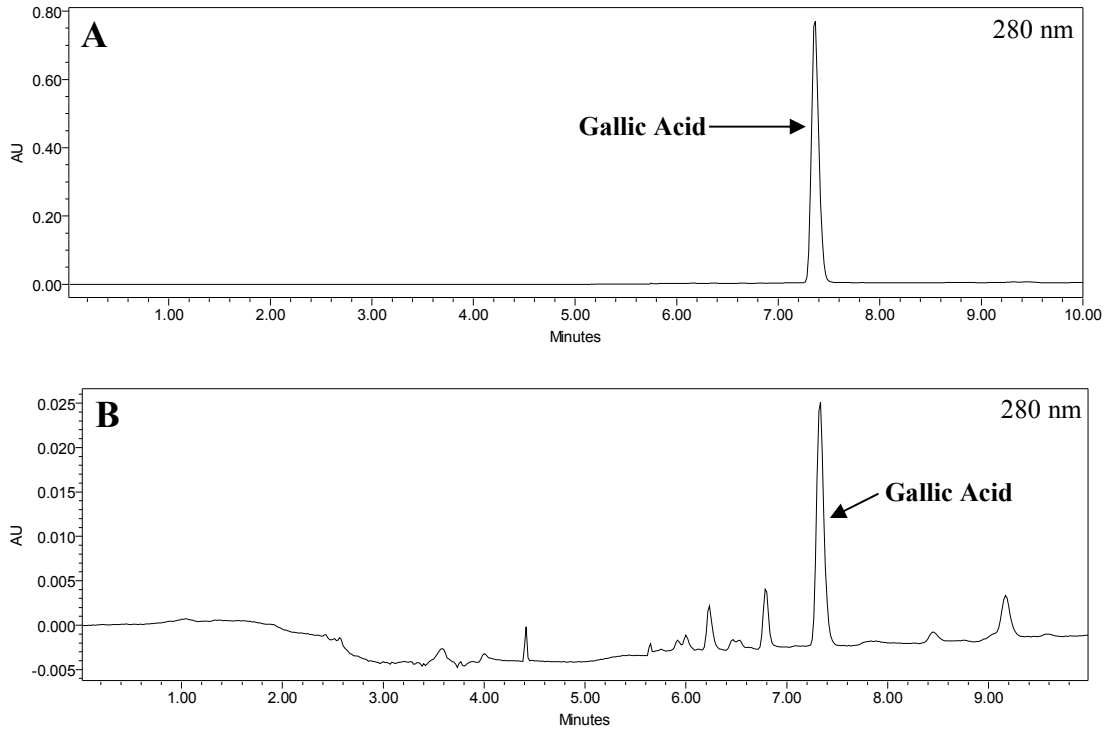


Figure 7-1. Chromatograms of gallic acid in the apical side before analysis (A) and present in the basolateral side after an incubation period of 2 hours (B).

Gallic acid with no aiding agents (phospholipids and terpenes) was poorly transported at both concentration assessed (0.13%) agreeing with other investigations regarding gallic acid absorption in Caco-2 monolayers and in rats after oral administration (Konishi and others 2003; Konishi and others 2004; Konishi and others 2006). In general, addition of phospholipids and terpenes increased the gallic acid found in the basolateral compartments which agreed with results from previous studies and confirmed the hypothesis that the use these aiding agents could improve absorption of polyphenolics other than anthocyanins through the intestinal epithelia. Increases in transport values ranged from 37 to 920%. Relative transport of gallic acid was dose

dependent when phospholipids and terpenes were present in the matrix. The increase in transport of gallic acid ranged from 1.4 to 9 fold when gallic acid increased from 125 to 250 mg/L.

Table 7-1. Transport of gallic acid from the apical to the basolateral side of Caco-2 cell monolayers following incubation (120 min, 37ºC), as a function of phospholipid and terpene concentrations.

Gallic Acid Concentration [mg/L]	Terpene Concentration [mg/L]	% Transport of Gallic Acid		
		Phospholipid Concentration [mg/L]		
		0	5,000	10,000
125	0	0.19 ± 0.01^{e} [1]	0.32 ± 0.03^{c}	0.22 ± 0.03^{e}
	25	-[2]	0.26 ± 0.01^{d}	0.98 ± 0.02^{a}
	50	-	0.43 ± 0.02^{b}	0.33 ± 0.03^{c}
250	0	0.18 ± 0.02^{e}	1.75 ± 0.29^{ab} * [3]	1.86 ± 0.24^{a} *
	25	-	1.73 ± 0.09^{ab} *	1.48 ± 0.15^{b} *
	50	-	0.81 ± 0.09^{d} *	1.12 ± 0.04^{c} *

[1]Values with different letters within the same column are significantly different (LSD test, $p<0.05$).
[2]Samples not assessed due to rupture of monolayer. [3]Asterisk indicates significant difference in transport values of gallic acid when concentration of anthocyanins fluctuated.

No trends were observed in samples containing 125 mg/L of gallic acid. The highest enhancement accomplished was 467% identified in samples containing 25 mg/L of terpenes and 10,000 mg/L of phospholipids. No transport enhancement was observed when only phospholipids (10,000 mg/L) were present in the matrix and only 35% increase in transport was observed in samples containing 25 mg/L of terpenes and 5,000 mg/L of phospholipids in the matrix. When gallic acid concentration was doubled, more pronounced increases in transport were observed. Highest gallic acid transport values were observed in samples containing no terpenes regardless of phospholipid concentration and samples containing 25 mg/L of terpenes and 5,000 mg/L of

phospholipids. Samples containing 50 mg/L of terpenes and 5,000 mg/L of phospholipids resulted in the lowest transport values of gallic acid. No results were recorded when terpenes were the only transport aides present in the matrix. Unfortunately, resistance of the monoloyers utilized for these treatments were lower than 120 Ω cm^2 hence data could not be used for analysis.

Transport rates are another important assessment in a Caco-2 cell model. These values indicated the concentration of gallic acid transported from the apical side to the basolateral side in a unit of time. Average transport rates (µg/mL·h) of gallic acid from the apical to the basolateral side were given in time depending on the gallic acid concentration initially mixed in the matrix and detected in the basolateral compartments assessed (Table 7-2). Individual gallic acid transport rates (0.026 – 0.305 µg/mL·h) increased in a concentration-dependent matter. When concentration of gallic acid was doubled, average transport rates of all samples were at least doubled and increases went up to almost 12 fold (11.5).

No trends were observed in samples regardless of gallic acid concentration. In samples containing 125 mg/L of gallic acid, the highest enhancement in transport rates accomplished (344%) was identified in samples containing 25 mg/L of terpenes and 10,000 mg/L of phospholipids. Improvement in gallic acid transport rates were also detected in samples containing 5,000 mg/L of phospholipids and no terpenes (64%). When gallic acid was doubled (250 mg/L), highest gallic acid transport rates were only observed in samples containing the highest concentration of terpenes and phospholipids assessed (50 and 10,000 mg/L, respectively).

Average transport rates were calculated and the increase was dependent on gallic acid concentration (Fig. 7-2). At the lowest gallic acid concentration analyzed (125 mg/L), average transport rate was 0.043 µg/mL·h. When gallic acid concentration was doubled, average transport rate was significantly increased (5.2 fold).

Table 7-2. Average transport rates of gallic acid from the apical to the basolateral side of Caco-2 cell monolayers, as a function of phospholipid and terpene concentrations.

Gallic Acid Concentration [mg/L]	Terpene Concentration [mg/L]	Transport Rate (µg/mL·h) of Gallic Acid Phospholipid Concentration [mg/L]		
		0	5,000	10,000
125	0	0.028 ± 0.002^{c} [1]	0.043 ± 0.003^{b}	0.026 ± 0.006^{c}
	25	-[2]	0.030 ± 0.002^{c}	0.116 ± 0.002^{a}
	50	-	0.030 ± 0.002^{c}	0.026 ± 0.004^{c}
250	0	0.055 ± 0.007^{c}	0.256 ± 0.052^{ab}	0.254 ± 0.053^{ab}
	25	-	0.230 ± 0.015^{b}	0.208 ± 0.035^{b}
	50	-	0.238 ± 0.033^{b}	0.305 ± 0.008^{a}

[1]Values with different letters within the same column are significantly different (LSD test, $p<0.05$).
[2]Samples not assessed due to rupture of monolayer.

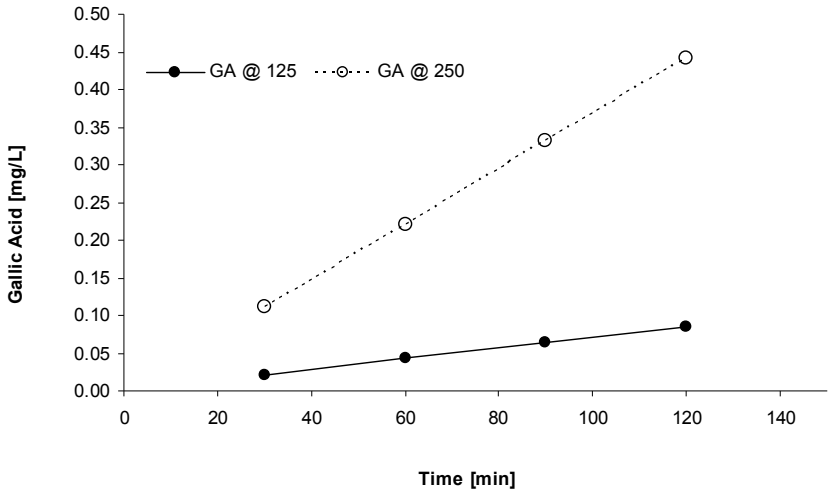


Figure 7-2. Average transport rates of gallic acid at two different gallic acid concentrations (125 and 250 mg/L), from the apical to the basolateral side of Caco-2 cell monolayers over 120 min at 37ºC.

7.3.2 Particle Size Analysis

Particle size of matrices containing gallic acid with terpenes and phospholipids was assessed as explained in previous sections. Gallic acid properly mixed with various concentrations of terpenes and/or phospholipids were subjected to two rounds of sonication and vortexing (~12 min). Diameter measurements were determined by light scattering that was recorded for 30 seconds, adapted to an audio range, and deconvoluted by a software system. Sample particle populations were calculated as the width (nm) of the measured particle size distribution and are presented in Table 7-3.

Vesicles formed ranged from 54.5 to 375 nm (MV) in diameter which confirmed the assumption that vesicles created with phospholipids are intimately related with the compounds found in the matrix. Vesicles formed in previous studies, with anthocyanins present in the matrix, were much bigger than the vesicles created in this investigation with gallic acid. Similarly to previous results, no vesicles were formed when phospholipids were not present in the matrix.

Particles formed, when concentration of gallic acid in the matrix was lower (125 mg/L), were generally bigger than the particles formed when gallic acid concentration was doubled. Correlations between particle size and transport of gallic acid were conducted. At the lowest concentration of gallic acid (125 mg/L), there was a high correlation ($r = 0.94$) between particle size and transport of gallic acid when phospholipid concentration fluctuated and terpene concentration was kept constant. In contrast, a very low correlation ($r = 0.18$) was observed when terpene concentration fluctuated and phospholipid concentration was kept constant.

Table 7-3. Particle size of matrices as a function of gallic acid, phospholipid, and terpene concentrations.

Gallic Acid Concentration [mg/L]	Terpene Concentration [mg/L]		Particle Diameter [nm]		
			Phospholipid Concentration [mg/L]		
			0	5,000	10,000
125	0	MV[2]	0.92 ± 0.00^{d} [1]	127 ± 22.3^{c}	375 ± 67.1^{a}
	25		0.92 ± 0.00^{d}	210 ± 14.0^{b}	329 ± 34.8^{a}
	50		0.96 ± 0.07^{d}	251 ± 9.71^{b}	100 ± 15.3^{c}
	0	MN	0.90 ± 0.00^{c}	63.0 ± 22.8^{ab}	62.2 ± 22.7^{ab}
	25		0.90 ± 0.00^{c}	71.8 ± 5.24^{a}	59.3 ± 16.7^{ab}
	50		0.92 ± 0.04^{c}	78.0 ± 22.1^{a}	35.8 ± 14.9^{b}
	0	MA	0.91 ± 0.00^{d}	92.4 ± 25.0^{bc}	143 ± 42.9^{a}
	25		0.91 ± 0.00^{d}	120 ± 5.90^{ab}	135 ± 27.9^{ab}
	50		0.94 ± 0.06^{d}	133 ± 21.4^{ab}	58.7 ± 21.5^{c}
250	0	MV	0.92 ± 0.00^{d}	100 ± 16.5^{a}	83.9 ± 5.66^{b}
	25		0.92 ± 0.00^{d}	70.1 ± 5.54^{b}	54.5 ± 8.55^{c}
	50		0.92 ± 0.00^{d}	83.7 ± 4.93^{b}	75.4 ± 9.32^{b}
	0	MN	0.90 ± 0.00^{d}	39.6 ± 12.4^{ab}	39.4 ± 5.05^{ab}
	25		0.90 ± 0.00^{d}	30.4 ± 5.02^{bc}	23.3 ± 3.40^{c}
	50		0.90 ± 0.00^{d}	33.7 ± 4.51^{abc}	43.5 ± 3.05^{a}
	0	MA	0.91 ± 0.00^{d}	62.7 ± 16.0^{a}	56.7 ± 5.86^{ab}
	25		0.91 ± 0.00^{d}	43.2 ± 4.68^{bc}	37.7 ± 8.53^{c}
	50		0.91 ± 0.00^{d}	53.7 ± 4.51^{ab}	55.9 ± 4.09^{ab}

[1]Values with different letters within the same columns and rows are significantly different (LSD test, $p<0.05$). [2]Particle Size measurements: (MV) Volume distribution, (MN) Mean distribution, (MA) Area distribution.

When concentration of gallic acid was doubled (250 mg/L), correlations were different. The highest correlation was reduced by 36.8% (r = 0.60) while the lowest correlation almost doubled (r = 0.36). Regardless of the changes in correlations due to the increase in gallic acid concentration, results illustrated that the relation between gallic acid transport and particle size was mostly dependent on occurrence of phospholipids rather than terpenes in the matrix. Additionally, high correlations between particle size and

transport rates as a function of phospholipid concentration illustrated the idea that vesicle formation played an important role in absorption of gallic acid.

7.4 Conclusions

Preceding Sections focused on illustrating the ability of phospholipids and terpenes as anthocyanin transport enhancers. In contrast, the objective of this investigation was to assess those capabilities of terpenes and phospholipids in the transport a different phenolic compound. Gallic acid transport was assessed through a Caco-2 monolayer model followed by a particle size analysis. Results illustrated the positive influence of terpenes and phospholipid had on the transport of gallic acid. Gallic acid alone was transported inadequately (0.19%). General increases in transport values ranged from 37 to 920% compared to transport of gallic acid alone. In addition, transport of gallic acid was dose-dependent with increases in transport of gallic acid ranging from 1.4 to 9 fold when gallic acid concentration was doubled. No results could be recorded for samples containing only gallic acid and terpenes. Final TEER of the monoloyers (after 2 hours of incubation) utilized for these treatments were lower than 120 Ω cm^2 and data could not be used for analysis. Gallic acid transport rates (0.026 – 0.305 $\mu g/mL \cdot h$) increased in a concentration-dependent matter. When concentration of gallic acid was doubled, average transport rates of all samples were significantly increased (2.0-11.5).

Vesicles formed ranged from 54.5 to 375 nm (MV) in diameter. These results cemented the idea that vesicles formation is closely related to the compounds found in the matrix. Gallic acid is a smaller molecule (m/z 170) than anthocyanins (m/z 449-

595), which leads to the formation of smaller vesicles. No vesicles were formed when phospholipids were not present in the matrix. Correlations between gallic acid transport and particle size illustrated a greater contribution from phospholipids presence in the matrix compared to terpenes which also suggested that encapsulation may play an important role in modulating absorption of gallic acid.

Results from this experiment confirmed the idea that phospholipids and terpenes can act as transport enhancers for various polyphenolics. Further analyses need to be conducted to understand the interaction of gallic acid, phospholipids, and terpenes in the matrix and confirm if encapsulation is a potential mechanism for enhanced transport of compounds through the epithelia. In addition, phospholipids and terpenes need to be assessed on some other phytochemicals to establish their positive effect on their bioavailability.

VIII. SUMMARY

Anthocyanins are the most important class of water-soluble pigments responsible for the red, blue and violet colors in many fruits and vegetables. Several fruits contain high concentrations of anthocyanins and studies have shown associations between fruit consumption and reduction of certain diseases attributable to the presence of antioxidant polyphenolics. Extensive research has elucidated the health benefits of anthocyanins. However, anthocyanin absorption is fairly poor which hinders their potential to be utilized in the human body.

The bioavailability of anthocyanins and other polyphenolics could be enhanced by compounds known to assist in the transport/absorption of phytochemicals through the gastric epithelia increasing the availability of these compounds for various applications in the body. Phospholipids could promote absorption of compounds to the blood stream making them more available. Improvement in the absorption of anthocyanins could also be accomplished by the use of terpenes. Transdermal transport could be replicated in the gastrointestinal tract if a product rich in terpenes is used, thus, creating temporary apertures in the gastric epithelia by reacting with the tissue.

Anthocyanins isolated from açaí puree were used for multiple unidirectional transport studies assessed from the apical to the basolateral side of Caco-2 cells monolayers. Cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R) were the two most predominant anthocyanins in açaí, thus, they were monitored through the transepithelial transport study. Açaí anthocyanins with no aiding agents were poorly

transported with transport values of 1.38% and 1.06% (C3G and C3R, respectively). Both terpenes and phospholipids significantly increased the transport of anthocyanins. Significant increases in transport ranged from 74 to 343% for C3G and 91 to 305% in the case of C3R. The transport aiding effect of phospholipids and terpenes was enhanced when mixed together. Phospholipids at the highest concentration assessed (5000 mg/L) resulted in transport value enhancement of 204% and 234% of C3G and C3R absorption, respectively. When terpenes (50 mg/L) were added to the matrix already containing phospholipids (5,000 mg/L), a 1.5 and 1.2 increase in transport values were observed for C3G and C3R, respectively.

Total anthocyanin concentration and antioxidant capacity were monitored through a 40 day period at various temperatures for samples that showed highest anthocyanin transport through the previous study. Color degradation followed first-order kinetics. There were no differences in anthocyanin degradation within treatments at every temperature assessed illustrating that the presence of terpenes and/or phospholipids did not affect anthocyanins degradation kinetics.

A confirmation transepithelial transport study was conducted to support the findings of the earlier investigations. A more complete dose-dependency study was carried out and particle size analysis was also assessed to understand the association of anthocyanins with terpenes and phospholipids in the matrix and possibly relate the formation of particles to enhanced transport of anthocyanins. Açaí anthocyanins with no phospholipids or terpenes were transported scantily at both concentrations of anthocyanins (1.77%) agreeing with results previously revealed. Addition of

phospholipids and terpenes resulted in average transport from 59 to 158% for both anthocyanins assessed (C3G and C3R). Furthermore, transport of anthocyanins was not dependent on dosage since absorption results were similar at both concentrations of anthocyanins tested. Contrary to previous results, no additive effect between combinations of terpenes and phospholipids could be detected in these experiments. This implied that the associations created in the confirmation studies were different from the previous investigation which could be attributed to an older sample of anthocyanins used which increase in the monomer-polymer ratio. Particle size analysis revealed that vesicles over 50 nm in diameter were only found in samples with no terpenes in the matrix. Perhaps, terpenes together with phospholipids formed a larger vesicle that was not allowed through the filter thus creating minute vesicles and most of the anthocyanins were not encapsulated. Therefore, enhancement properties of terpenes and phospholipids could be attributed simply to the mere presence of these compounds. Additionally, these results suggested that phospholipids and terpenes could achieve transport enhancement both individually and together as a whole.

A third study was conducted to compare three methods to produce matrices containing anthocyanins, terpenes and phospholipids. Furthermore, the effect of these technologies on matrix construction and transport of anthocyanins was assessed. Comparison between technologies (French press at two pressure levels and sonication-vortexing) was conducted. Selected combinations of phospholipid and terpenes at two different anthocyanin concentrations (250 and 500 mg/L) were assessed. Increased transport of anthocyanins by the addition of terpenes and phospholipids was, once again,

demonstrated. General increases in absorption ranged from 51 to 159% for C3G and 57 to 152% for C3R compared to transport of anthocyanins with no aiding agents. In addition, no dependency on anthocyanin concentration was observed. Comparisons illustrated that both technologies created matrices that maintained the properties of phospholipids and terpenes as transport enhancers. Particle size analysis demonstrated the formation of vesicles that ranged broadly in diameter (296-2,390 nm). Variations in particle size were more pronounced when anthocyanin concentrations were 500 mg/L and when pressure applied to the system was low (4,000 psi). When water is initially added to a mixture, phospholipids naturally arrange but particles arrangement and size are extremely variable. As energy is applied to the system, vesicles are forced to re-arrange creating a more homogenous population. Weak correlations were observed in all samples except those subjected to the highest pressure (18,000 psi) through the French Press when particle size was assessed against transport of anthocyanins. Only in certain samples, reduction in particle size was related to an increase in transport of anthocyanins suggesting that the main contribution of phospholipids and terpenes was their presence in the matrix rather than the dimension or nature of vesicles formed in it.

Since the absorption improving properties of phospholipids were well established in previous studies, phospholipids extracted from açaí oil were assessed in transport of anthocyanins from açaí and port wine to find similar effects as for phospholipid from soybeans. There is an extensive body of research illustrating the absorption/penetration enhancement properties of phospholipids from soybeans. Nevertheless, no investigations were conducted on the absorption enhancement properties of

phospholipids extracted from açaí oil. The ability of açaí phospholipids as transport enhancers of anthocyanins was confirmed through these experiments. Transport values for açaí anthocyanins with no enhancers on the matrix were lower than 3% on average. Addition of phospholipids resulted in average increase in anthocyanin transport from 83 to 142% and 69 to 74% for C3G and C3R, respectively. Moreover, transport of anthocyanins was not dependent on dosage except for samples containing 5,000 mg/L of phospholipids where an inverse relation between concentration and transport of anthocyanins was observed. In parallel, port wine anthocyanins with no phospholipids were also transported poorly. No transport of anthocyanins was detected after 2 hours of incubation when total anthocyanin concentration was 250 mg/L. When anthocyanin concentration was doubled, transport values were detected (0.32% to 0.96%) illustrating that poor absorbability of anthocyanins occurs in various commodities. Addition of phospholipids to the matrix increased the transport of anthocyanins demonstrating increases from 234% to 543%. These findings illustrated that phospholipids extracted from açaí also had transport enhancement properties on two different types of anthocyanins (açaí and port wine).

Finally, a study on the effects of phospholipids and terpenes on a non-anthocyanin polyphenolic was assessed. Gallic acid is a phenolic acid widely spread in nature and investigations have illustrated the health benefits of this compound. Unfortunately, poor absorption of gallic acid has also been demonstrated and compared to other phenolic acids that hinder gallic acid potential action against free radicals in the body. Consequently, gallic acid was selected for a transepithelial transport study where

phospholipids and terpenes acted as absorption enhancers. Additionally, a particle size analysis was conducted to understand the relation gallic acid might have with phospholipid and terpenes in the matrix. Gallic acid with no aiding agents was poorly transported at both concentrations assessed (0.13%). Addition of phospholipids usually increased the gallic acid found in the basolateral compartments suggesting that phospholipids and terpenes could promote absorption of various types of phytochemicals. Increases in transport values ranged from 37 to 920%. Relative transport of gallic acid was dose dependent when phospholipids and terpenes were present in the matrix. The increase in transport of gallic acid ranged from 1.4 to 9 fold when gallic acid increased from 125 to 250 mg/L. In contrast, no transport enhancement was observed when no terpenes and 10,000 mg/L of phospholipids were present in the matrix. Vesicles formed ranged from 54.5 to 375 nm (MV) in diameter which confirmed the idea that vesicle formation is closely related to the compounds found in the matrix. Gallic acid is a smaller molecule (m/z 170) than anthocyanins (m/z 449-595) which leads to the formation of smaller more homogenous vesicles. No vesicles were formed when phospholipids were not present in the matrix. Correlations between gallic acid transport and particle size illustrated that the relation between absorption and particle size was mostly dependent on phospholipids rather than terpenes presence in the matrix.

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APPENDIX A

CALCULATION OF COMPOUND TRANSPORT THROUGH THE CACO-2 CELL MONOLAYER IN 2 HOURS OF INCUBATION

Table A-1 explains the calculation process for transport values over a period of 120 min. These results could be areas under the curve or concentration of compounds. Since the calculation ends up in a ratio, the standard curve is used for both apical and basolateral values, thus, they cancel out in the calculation. The principle of these calculations relies on the idea of knowing the presence of compounds (area under the curve) at the different volumes used in the apical and basolateral side. It is only possible to compare presence of compounds in the apical and basolateral side when volume differences are taking into account.

To understand this example, the values observed in the table, will be treated as single molecules or units which could be then translated to weight (mg). In this specific example, it was assumed that 1 unit was absorbed per minute which yielded 30 units at every sampling time frame and 120 units overall. If 1,000 units were initially added to the apical side, the % absorption was 12%. These calculations will illustrate how to determine these results.

Table A-1. Calculation of the transepithelial transport of compounds from the Apical to the Basolateral Side of Caco-2 Cell Monolayers following Incubation (120 min, 37ºC).

		Apical Side	Basolateral Side				
	Time [min]	0	0	30	60	90	120
1	100 µL[a]	200	0	2	3.73	5.24	6.54
2	200 µL[b]	400	0	4	7.47	10.47	13.07
3	Total Volume[c]	1000	0	30	56.00	78.53	98.06
4	Area after sampling[d]		0	26	48.53	68.06	84.99
5	Area corrected[e]	1000	0	30	60.00	90.00	120.00
6	Area Transported in 30 min[f]	1000	0	30	30	30	30
7	%Trans per 30 min[g]	100.00%	0.00%	3.00%	3.00%	3.00%	3.00%
8	Total %Trans[h]	12.00%					

[a]Area under the curve (AUC) detected in a sample of 100 µL analyzed through HPLC.
[b]AUC which was removed from the basolateral side for HPLC analysis (200µL).
[c]Total volume found in the apical side (500µL) and in the basolateral side (1,500µL).
[d]AUC which was left after removing 200µL of sample.
[e]AUC that was found at a certain point in time taking into account losses due to sampling
[f]AUC which was transported in 30 min
[g]AUC compared to the initial AUC found in the apical side
[h]Sum of the %Abs at every time point

As shown in table A-1, 200 units were detected by HPLC analysis in 100µL of sample drawn by the autosampler. If 200 units are present in 100µL, 400 units would be present in 200µL. Similar thinking is used to calculate the next value. If 400 units were found in 200µL, 1,000 units will be found in the 500µL added to the apical side. Therefore, 1,000 units were initially added to the apical side and this was the value used to calculate the %Transport per 30 min and overall transport.

More calculations were used for data analysis from the basolateral side. The first three rows were calculated similarly, the only difference would be the volume from the basolateral side which was three times the volume found in the apical side (1,500µL). At time zero no units were found; thus the entire column is filled with zeros. At 30 min,

2 units were measured by the HPLC from a 100µL sample from the basolateral side. This meant that 4 units were removed from the basolateral compartment. If 4 units were sampled, it meant that at 30 min, 30 units were transported from the apical to the basolateral side. However, since 4 units were removed to assess transport by HPLC, only 26 units remain in the basolateral compartment. Since no units were detected in the previous time point (0 min) the units absorbed in 30 min are compared to 1,000 units initially added to the apical side, which resulted in a 3% transport in 30 minutes (30/1,000).

At the next time point (60 min), 3.73 units were detected through HPLC analysis in a 100µL sample. This means that 7.47 units were removed from the basolateral compartment (200µL). If 7.47 units were present in 200µL, 56 units would occur in 1,500µL. 26 units were transported in the first 30 min thus 30 units were transported in the second 30 min which is also 3.00% when compared to the 1,000 units initially added to the apical side. From those 56 units, 7.47 were subtracted for sampling so the remaining units after 60 min were 48.53.

In the next sampling at 90 min, 5.24 units were detected in the 100µL sample. This meant that 200µL sample extracted from the basolateral side had 10.47 units. Due to the sample, it was determined that 78.53 units were detected in 90 minutes. After subtraction of the units that were already in the basolateral since the last sampling (48.53), it was concluded that 30 units were absorbed in the last 30 min which was, once more, 3.00% of the total amount of units added to the apical side. From those 79.53

units, 10.47 were subtracted for sampling so the remaining units after 900 min were 68.06.

At the final sampling time, 6.54 units were identified in the HPLC sample, meaninig that 13.07 units were removed from the basolateral side. If 6.54 units were assessed, 98.06 units were found in the basolateral side after 120 min of analysis. After subtraction of the units that were already in the basolateral since the last sampling (68.06), it was concluded that 30 units were absorbed in the last 30 min which was also 3.00% of the total amount of units added to the apical side. If 3.00% was transported every 30 min, a total of 12.00% was transported throughout the Caco-2 cell monolayer model (2 hrs).

APPENDIX B

FRENCH PRESS DETAILS

The French Press is a hydraulic press which uses control valves and a motor-driven pump to vary hydraulic pressure generated by the press (Fig B-1). This method involves the extrusion of samples through a small orifice. Samples are subjected to enormous quantities of pressure that is quickly dropped when samples are released through the outlet tube. This pressure differential causes the phospholipids to rearrange forming smaller vesicles. It is important to maintain internal pressure, thus, sample should be drawn slowly (15 drops/min). This rate allows for a much slower decrease in internal pressure which will determine the homogeneity of the product.

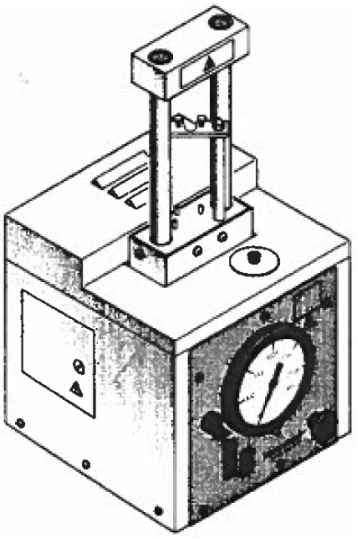
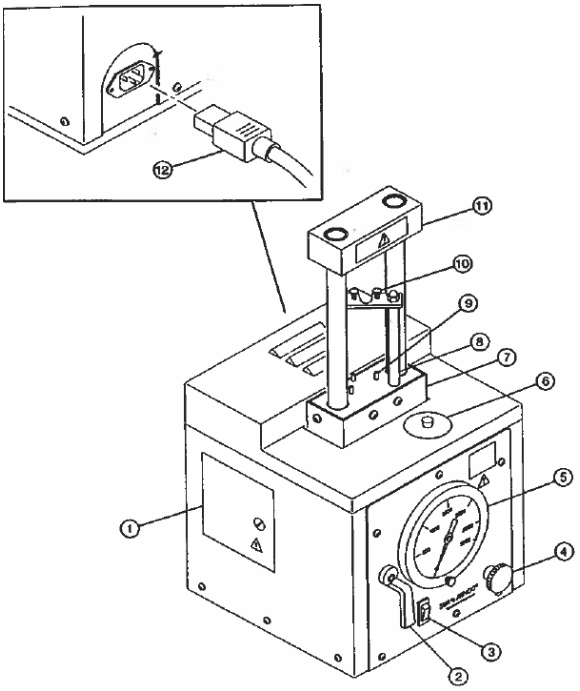


Figure B-1. French Press from Spectronic Instruments.

The French Press machinery is fairly simple (Fig. B-2) and equipment should be treatment with care. Dust and other exogenous materials should be cleaned before using the equipment since they could damage the pressure cell of any other parts subjected to high pressures.



1. Side panel access door
2. Ratio selector valve
3. Pump switch
4. Pressure increase control
5. Pressure gauge
6. Top panel access hole
7. Lower platen
8. Cell clamp
9. Aligning pins
10. Cell clump with thumb screws
11. Upper platen
12. Line cord

Figure B-2. Controls and Indicators of the French Pressure Cell Press.

The miniature pressure cell used for experiments had a cell piston diameter of 0.375 inches and a maximum sample capacity of 3.7 mL (Fig. B-3). The maximum pressure that could be applied was 20,000 psi. The first thing to before assembling the equipment is to decide on the pressure that will be applied to the samples by calculating the gauge pressure and relating that pressure to the internal pressure created by the cell.

This determination could be done by using Figure B-4 which is a chart relating internal pressure of the miniature pressure-cell and gauge pressure of the French Press.

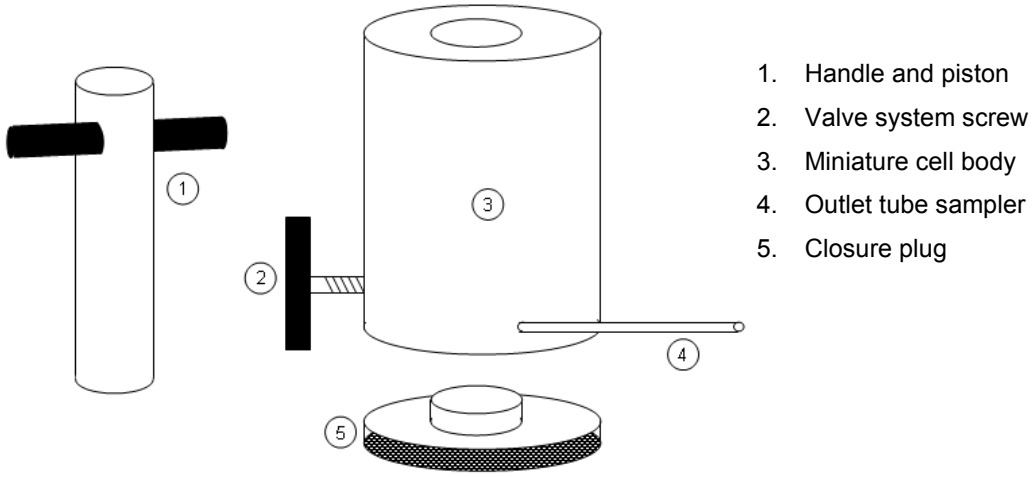


Figure B-3. Diagram of the Miniature Pressure Cell.

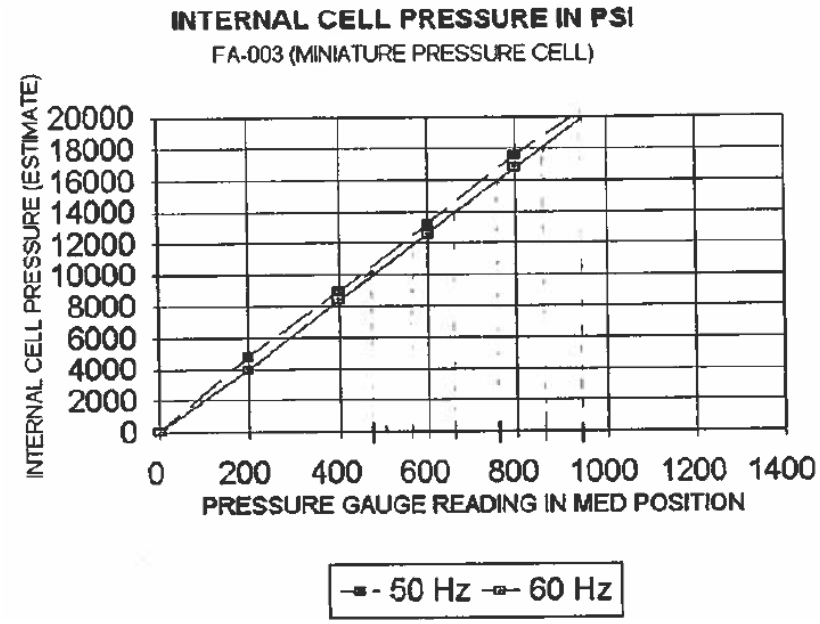


Figure B-4. Chart of the Internal Pressure in Miniature Pressure Cell Influenced by Gauge Pressure.

Once pressure is selected, press cell is assembled starting by putting the piston, outlet tube and handle on their respective places in the cell. Oil need to be check every time machine is being used to assure proper functioning of the pump and hydraulic system.

To add sample, pressure cell needs to be inverted, sample is introduced inside the cell and the closure plug is put to close the pressure cell. Place the pressure cell inside the French Press. Turn the ratio selector valve to start the hydraulic system. Once the pressure cell has been lifted towards the upper platen and desired pressure has been reached, open the flow valve slightly to allow slow release of sample.

VITA

Jorge Alfredo Cardona Ponce was born in Cochabamba, Bolivia. Before graduating from high school in 2000, he traveled to Devon, PA as an exchange student for 11 months with AFS exchange program. He went back to Bolivia to graduate from high school in 2001. After high school, he entered Zamorano University (Escuela Agrícola Panamericana) in Honduras, Central America to obtain his B.S. in Agroindustry in December 2005. He then pursued his graduate education at the Food Science and Human Nutrition Department at University of Florida and graduated with a M.S. in August 2007. Afterwards, he continued his graduate education by moving to Texas A&M University where he finally received a Ph.D. in Food Science and Technology in December 2010. Upon graduation, Jorge accepted an offer as an associate faculty member at Zamorano University where he can be reached by e-mail: jcardona83@gmail.com. He can also be reached at 1500 Research Pkwy Centeq A, 234 College Station TX, 77843. c/o Dr. Stephen Talcott.