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# In Vitro and In Vivo Antioxidant Capacity of Synthetic and Natural Polyphenolic Compounds Identified from Strawberry and Fruit Juices

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
with a concentration in Cell and Molecular Biology
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## DEDICATION

I dedicate this work to my family who made it possible for me to become who I am today. I am especially thankful for all the support you gave me throughout my journey here in the United States, for not giving up on me when times were difficult and for always being there for me when I needed you most. You always believed in me and let me shape my own future while still supporting me 100 percent of the time. Through the most difficult times, you stood by my side and did everything that was in your power to support me and help me continue to achieve my dream of earning an advanced degree in the United States. Even when you could not be with me I appreciate the countless hours of FaceTime to keep me mentally strong and help me through the most difficult times of my life. Without you I would not have been able to continue to pursue my education and I will forever be thankful for that. I am very proud to call you my family and hope that I can return the favor some day in the future to make you feel as appreciated as I did when I needed you most.

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## ABSTRACT

Strawberries can be considered a functional food because their consumption has been associated with several health benefits. They are important sources of bioactive compounds, such as vitamins and polyphenolic compounds, with recognized antioxidant capacity (AOC). However, strawberry overall quality and bioactive content are greatly affected by environmental conditions during pre- and post-harvest and, little is known about the stability of its bioactive compounds, specifically ascorbic acid (AA) and polyphenolics compounds. Furthermore, additional research that addresses the impact of polyphenolic compounds on *in vitro* and *in vivo* models is needed to understand the mechanisms behind their potential health benefits. Therefore, the objectives of the work presented in this thesis were to: 1) evaluate the impact of different disease control treatments on strawberry bioactive compounds and AOC; 2) understand the relationship between bioactive compounds and AOC in strawberries and fruit juices; 3) investigate the origin of AOC in strawberries by identifying their major polyphenolic compounds and, 4) explore the effects of polyphenol-rich fruits and fruit juices on the proliferation of cancer cells and lifespan of *Caenorhabditis elegans*.

Conscientious consumers are aware of the health benefits of substantial fruit and vegetable consumption but are also concerned about the amount of pesticide residues that can be found in conventionally grown produce, with pesticide-free produce (i.e., organic) becoming more popular. However, the market price for organic strawberries

can be more than twice that of conventionally grown fruit which discourages the average American from purchasing this fruit on a regular basis. Therefore, in the first study presented in this thesis, we hypothesized that reducing pesticide usage would provide the consumer with a "sustainable strawberry" that would have better or similar quality at a lower cost than organic fruit while it would also reduce environmental impact and risk to pesticide applicators. Results from this study showed that strawberries from a reduced fungicide treatment, had better or similar bioactive content and AOC than fruit from the conventional disease control treatment. After cold storage, strawberries from the reduced or conventional disease control treatments showed comparable amounts of bioactive compounds and AOC. These results indicate that growing strawberries with a reduced number of fungicide applications can be an alternative to the conventional disease control or organic practices as it may reduce residual fungicides in the fruit, decrease production costs while still retaining important bioactive compounds.

In order to understand the relationship between bioactive compounds and AOC in strawberries and fruit juices, 56 different types of commercial beverages were chosen for the second study presented in this thesis. Overall, results showed that the higher the total phenolic contents (TPC) in the beverage the higher their AOC. Amongst all beverages studied, aronia, blackcurrant, and pomegranate juices contained the highest amount of TPC and AOC. Furthermore, after opening the bottles, these juices were maintained for 14 days at 4 °C, to test the stability of their TPC which was in general relatively stable throughout storage.

Further investigation on individual polyphenolic compounds and their possible contribution to the overall AOC of fruits and fruit juices, led to a third study. Overall,

results showed that the AOC of major individual polyphenolic compounds found in strawberries (i.e., pelargonidin, cyanidin, ellagic acid, quercetin, kaempferol, catechin, epicatechin, caffeic acid, *p*-coumaric acid, ferulic acid) was significantly higher than that of mixtures of the same compounds. In addition, the AOC of strawberries correlated with its major bioactive compounds (i.e., polyphenolic compounds and ascorbic acid) in a form of a synthetic bioactive strawberry model ("Powerberry") composed of major strawberry polyphenolic compounds, vitamin C, fructose and glucose in the same ratios found in a real strawberry. These results suggest that even though strawberries contain many different polyphenolic compounds and vitamins, their AOC might only depend on few compounds that are found in significant quantities in the fruit.

Finally, using cell and worm models we were able to demonstrate that conventional and organic strawberry, raspberry and blueberry fruits, and aronia, blackcurrant and pomegranate juices successfully inhibited the proliferation of HeLa cervical cancer cell lines. In addition, when introduced in low doses (0.75 mg $ml^{-1}$ or lower) to the *C. elegans* diet, aronia, blackcurrant and pomegranate juices promoted longevity. Overall, results suggest that using whole fruit or fruit juices might constitute an alternative of treating cancer cells *in vivo* and that polyphenolic compounds contained in fruits and fruit juices displayed significant bioactivity in a worm model.

# CHAPTER ONE: INTRODUCTION

Strawberries can be considered a functional food because they contain biologically active compounds that provide clinically proven health benefits for the prevention, management or treatment of diseases. Strawberry bioactivity has been associated primarily with the high levels of vitamins (particularly vitamin C) and polyphenolic compounds such as flavonols, flavanols, anthocyanins and phenolic acids. These compounds, mainly polyphenolic compounds, are sought to contribute to the *in vitro* and *in vivo* antioxidant capacity (AOC) of strawberries (Azzini et al., 2010; Pineli et al., 2011; Singh et al., 2011). For example, recent studies demonstrated that polyphenolic compounds can decrease reactive oxygen species (Wang et al., 2011), reduce cancer by affecting the survival and metastasis of tumors (Chirumbolo, 2012), and inhibit the growth of various human cancer cells (Scalbert et al., 2005). The study of strawberry polyphenolic profiles has provided increased understanding about the AOC of each individual polyphenolic compound and their potential health benefits when included in a balanced diet. Although several studies suggested that AOC is significantly correlated with the total phenolic content (TPC) of various foods and beverages (Gardner et al., 2000; Long et al., 2001), there is a lack of information on how strawberry polyphenolic compounds are affected by pre- and post-harvest conditions because these compounds are not regulated or recognized as nutrients.

The polyphenolic profile of strawberries can vary tremendously with weather conditions, agricultural practices, and environmental conditions during supply chain

(Nunes et al., 2003; Nunes et al., 2006). Therefore, the composition of the fruit may significantly vary between harvests and it is also affected by the stage of ripeness at harvest. Pre-harvest conditions such as increased environmental stress, water availability and pesticide treatments used to reduce fruit rot may all negatively affect the amount of polyphenolic contents in strawberries (Laura et al., 2009; Pineli et al., 2011). In addition, poor post-harvest conditions (i.e., temperature and relative humidity) during storage have shown to decrease the overall quality and shelf life of strawberry fruit (Nunes and Emond, 1999; Nunes et al., 2005; Moraga et al., 2006). The pre- and post-harvest treatments may, therefore, have a major influence on the final “bioactive quality” of the fruit and its corresponding AOC.

The research work presented here is unique because it introduces a new insight about polyphenolic compounds and their AOC in strawberries as affected by pre- and post-harvest conditions. In addition, the identification of specific polyphenolic compounds in fruit juices will also bring a new understanding about the major polyphenol contributors to the AOC of processed fruit juices and the stability of such compounds during simulated consumer storage. Studies demonstrated that processing methods and/or enzymatic mechanisms can contribute to positive and negative physiochemical changes that will affect the overall polyphenolic content of juices (Dugo et al., 2005; Patras et al., 2010; Rodríguez-Roque et al., 2015). Overall, the identification and quantification of specific polyphenols will lead to a deeper understanding regarding the compounds responsible for the AOC in strawberries and in polyphenol-rich fruit beverages and will provide additional knowledge on their potential AOC once ingested. Furthermore, this work establishes a platform to identify and

evaluate the impact of other polyphenolic compounds both *in vitro* and *in vivo*. *In vivo* models already demonstrated that plant polyphenolic compounds are capable of inducing apoptosis-mediated cell death in cancer cells (Bulzomi et al., 2012; Kim et al., 2012). Finally, this research introduces a synthetic bioactive strawberry model composed of major strawberry polyphenolic compounds, vitamin C, fructose and glucose in the same ratios found in a real strawberry. This unique model can further be used to test other hypothesis related to, for example, the effect of abiotic stresses on the bioactive compounds of strawberry. Results from polyphenolic research are important to the field of food and beverages because they will help to better understand the function of these compounds and may also be used as a basis for future development of dietary supplementation that can possibly be used in the treatment of specific health problems.

The objectives of the work presented in this thesis were to: 1) evaluate the impact of different disease control treatments on strawberry quality, particularly on bioactive compounds; 2) determine the relationship between bioactive compounds and antioxidant capacity in strawberries and fruit juices; 3) investigate the origin of antioxidant capacity in strawberries and, 4) explore the effects of whole polyphenol-rich fruits and fruit juices on the proliferation of cancer cells and lifespan of *Caenorhabditis elegans*.

# CHAPTER TWO:

# REVIEW OF LITERATURE

## Polyphenolic Compounds

### Classification and Chemical Structure

Polyphenolic compounds are secondary plant metabolites that belong to the phenylpropanoid family. They are composed by a wide range of chemical structural classes and biological functions and are believed to accumulate in the vacuole of the plants, usually as glycosides or other conjugates (Strack and Sharma, 1985). The two major classes of polyphenolic compounds found in plant tissues are flavonoids and non-flavonoids. The flavonoid group includes the flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, anthocyanidins, isoflavones and proanthocyanidins. The non-flavonoids are the phenolic acids, phenols, benzoic acids, hydrolyzable tannins, acetophenones, phenylacetic acids, cinnamic acids, coumarins, benzophenones, xanthones, stilbenes, chalcones, lignans and secoiridoids (Laura et al., 2009). Structural diversity among these compounds is due to a variety of modifications including region specific hydroxylation, acylation, sulfation, glycosylation, prenylation and methylation (Dixon and Paiva, 1995).

The flavonoid compounds are composed of two aromatic rings (A and B) linked by an oxygenated heterocycle (C). The different subclasses of flavonoids depend on the degree of hydrogenation and substitution of the heterocycle (Laura et al., 2009).

Flavonoids, particularly anthocyanidins, are commonly found in nature conjugated to glucose and rhamnose but can also be linked to galactose, xylose, galactose, and arabinose or other sugars (Clifford 2000; Laura et al., 2009). The glycosylated anthocyanidins are then classified as anthocyanins. The only flavonoids found in nature in non-glycosylated (aglycone) monomer form, or proanthocyanidins, are catechin and epicatechin which are also known as flavan-3-ols. These flavonoids have a saturated three-carbon chain with a hydroxyl group in the C3 position (Laura et al., 2009). The action of the flavonoid 3'-hydroxylase determines the 3'-hydroxylation pattern of the B-ring of each flavonoid (Carbone et al., 2009).

Phenolic acids are classified into two different groups: hydroxybenzoic acids and hydroxycinnamic acids based on the C1-C3 and C3-C6 skeletons and their hydroxylation and methylation pattern of the aromatic cycle, respectively (Laura et al., 2009). These are further categorized according to the number of carbons in their chemical structure (Carbone et al., 2009; Laura et al., 2009). The structural base of these compounds include the trans-cinnamic acid, the p-coumaric acid and their derivatives, the phenylpropanoid lactones or coumarins, benzoic acid derivatives (monodyroxy, dihydroxy) and other complexes that form by additions of these basic carbon skeletons (Laura et al., 2009). Several simple phenylpropanoids with a basic C6-C3 carbon skeleton are produced through biosynthetic pathways from cinnamate by either hydroxylation, methylation or dehydration reactions. Those include p-coumaric, caffeic, ferulic and sinapic acids and simple coumarins (Dixon and Paiva, 1995).

## Biosynthetic Pathways in Plants

Even though there are hundreds of polyphenolic compounds found in nature, their main precursor is the amino acid L-phenylalanine (Heim et al., 2002). Depending on the environmental conditions during growth (i.e., light, water, nutrients, and presence of predators) the biosynthetic key enzyme, phenylalanine ammonia lyase (PAL), induces the production of different polyphenolic compounds at different time periods, in function of the demand for plant protection (Halbwirth, 2006).

The enzyme PAL catalyzes the first step in the biosynthetic pathway of phenylpropanoids, which with further synthesis results in a wide variety of compounds, including flavonoids, phenolic acids and hydrolysable tannins (Manach et al., 2004). The initial step of the phenylpropanoid pathway involves the conversion of phenylalanine or tyrosine to cinnamic acid involving an ammonia elimination reaction that is catalyzed by PAL (Jones, 1984; Laura et al., 2009). The biosynthetic pathway begins from phenylalanine to produce phenylpropanoids that are channeled into the flavonoid pathway by the enzyme chalcone synthase (Carbone et al., 2009). Further metabolism involves the enzymes chalcone isomerase, flavanone 3-beta-hydroxylase, dihydroflavonol 4-reductase and anthocyanidin synthase that lead to the synthesis of anthocyanidin pigments (Winkel-Shirley, 2001). Flavonol synthase produces flavonols while leucoanthocyanidin reductase and anthocyanidin reductase synthesize flavan-3-ols, the precursors of proanthocyanidin polymers (Aron and Kennedy, 2008). The concentration of PAL is usually low throughout most of the fruit developmental stages and starts to significantly increase at the beginning of anthesis or onset of blossom (Cheng and Breen, 1991). Halbwirth (2006) showed that the first PAL activity peak

corresponded to the formation of flavanols while the second peak was related to the accumulation of anthocyanin and flavonols. Accordingly, fruit has a developmental-dependent expression of PAL activity and accumulation of polyphenolic compounds derived from the phenylpropanoid pathway.

**Metabolism**

Plant stress has shown to be one of the major inducers of the phenylpropanoid pathway leading to specific biotransformation in plants. Biotic and abiotic stresses stimulate the phenylpropanoid pathway by translation and protein modification through increased transcription of PAL mRNA. Multiple genes encoding for PAL are only activated in specific tissues or under certain environmental signaling (Lincoln and Zeiger, 2006). Stress-induced phenylpropanoids are derived from the C15 flavonoid skeleton which is synthesized by chalcone synthase to yield a tetrahydroxychalcone. This compound can be further metabolized into other classes of flavonoids such as flavones, flavanones, flavanols and anthocyanins (Lincoln and Zeiger, 2006; Laura et al., 2009). In addition, these metabolites constitute the plant defense mechanisms and are usually synthesized in response to biotic or abiotic stresses such as pathogen invasion or adverse environmental conditions (Szajdek and Borowska, 2008). Coumarins and silbenes may also play a defense role in plants, exhibiting fungicidal properties and toxicity against insects and parasites (Lincoln and Zeiger, 2006). Dimerization of monolignols (i.e. 4-coumaric alcohol, coniferyl alcohol, and sinapyl alcohol) produces lignans which also have defense capabilities against bacteria and fungi (Laura et al., 2009). The polymerization of flavonoid molecules produces tannins

and phenolic acids which in high concentrations have an astringent taste (Quideau et al., 2011). Tannins are more resistant to water, microbes and heat because their structure includes collagen proteins bound together with phenolic groups (Laura et al., 2009).

Various abiotic and biotic stresses may also induce the synthesis of certain phenylpropanoids such as the phytoalexins (Laura et al., 2009). Other antimicrobial compounds, mostly synthesized in response to pathogen attack, include the pterocarpans, isoflavans, isoflavonoids, stilbenes, psoralens, coumarins, and flavonols, (Bailey and Mansfield, 1982; Dixon et al., 1995). These compounds are usually detected at elevated levels around infection sites to concentrations toxic to foreign pathogens (Laura et al., 2009). Anthocyanins and flavones have been detected in high levels in plants exposed to increased UV light. These compounds help reduce the amount of light that reaches the photosynthetic cells and also provide protection against damaging UV-B rays which cause DNA dimerization and breakage, leading to cell death (Beggs et al. 1987; Li et al., 1993). Cold stress and nutritional stresses also increase anthocyanin production (Christie et al., 1994).

**Importance in Human Health**

Recent studies have shown that the health benefits of polyphenolic compounds found in fruits, vegetables and beverages are not strictly related to their direct impact in the human metabolism but to their action as metabolites, formed in the small intestine and hepatic cells (Manach et al., 2004; Scalbert et al., 2005). For example, several studies have shown the ability of polyphenolic compounds to fight free radicals (Gong et

al., 2010), help against atherosclerosis by restoring endothelial function (Engler et al., 2003), display anticancer properties by inhibiting cancer growth and stimulating apoptosis of cancer cells (Seeram et al., 2006), and their capability of reducing inflammation by inhibiting inflammatory proteins and oxidative stress (La et al., 2009; Mukai and Sato, 2010). Others have also suggested that only approximately five percent of the polyphenols in the diet are absorbed into the bloodstream, acting as antioxidants, while the remaining seemed to reach the small and large intestine as digestible polyphenols or as indigestible condensed tannins and hydrolysable polyphenols. These compounds, which can benefit the health of bacterial microflora, only become bioavailable upon enzyme digestion and colonic fermentation (Clifford, 2004; Saura-Calixto, 2007).

Although the bioavailability of polyphenolic compounds in humans is poorly understood, it is now well established that they undergo extensive metabolism after being ingested (Manach et al., 2004; Donovan et al., 2006). Their metabolism seems to begin in the lumen of the small intestine where they are absorbed and modified to their metabolites in the liver and other organs (Manach et al., 2004; Mullen et al., 2006). In addition to some absorption in the small intestine, some flavonoids have shown to pass to the large intestine, where they are further metabolized and modified by colonic microflora. The degradation of these polyphenolic compounds by the colonic microflora to simple phenolic acids metabolites promotes their absorption into the blood stream (Parkar et al., 2008). Therefore, polyphenols and their catabolites may influence the microflora and impact the colonic health by increasing the total number of beneficial microorganism in the gut (Parkar et al., 2008; Tzounis et al., 2008).

Several studies have also shown that some plant polyphenolic compounds are capable of inducing apoptosis-mediated cell death in human cervical carcinoma (Scalbert et al., 2005; Chirumbolo, 2012; Kim, 2012). Results from these studies suggest that fruits and their corresponding juices may be an excellent source of bioactive compounds that, when ingested in generous amounts as part of a balanced diet, can help prevent various types of cancer (Scalbert et al., 2005). In addition, single synthetic polyphenol extracts could also constitute an emerging approach to reducing cancer growth. For example, studies showed that quercetin enhanced apoptosis and inhibited proliferation of various cancer cell lines (Mertens-Talcott et al., 2003; Bulzomi et al, 2012). In another study, the anticancer properties of polyphenolic extracts from several types of berries were displayed by inhibiting cancer growth and stimulating apoptosis of cancer cells (Seeram et al., 2006).

## Selected Food Models Particularly Rich in Polyphenolic Compounds

### Strawberry Fruit

**History and Origin**. Long before the production of the well-known garden strawberries, monks of Western Europe were incorporating strawberry fruits as seen in the wild into their religious paintings. Monks first began drawing strawberry plants because of their graceful form and pure colors making it a popular focal point of medieval art (Darrow, 1966). After this period, the French began transplanting wild strawberry plants from the forest to their gardens (Darrow, 1966) and, by the end of the 16th century three European strawberry species including *Fragaria vesca, Fragaria*

*moschata,* and *Fragaria viridis* were commonly grown for fruit production (Wilhelm and Saga, 1974).

The original garden strawberry (*Fragaria x ananassa*) was believed to be grown for the first time in France, towards the end of the 18th century, from a cross of *Fragaria virginiana* from eastern North America and *Fragaria chiloensis* from Chile (Darrow, 1996). The mild climate of Chile produced a larger fruit whereas the North American climate resulted in a smaller fruit that was better adapted to heat, drought and cold (Darrow, 1966). Throughout the years, the extensive crossing of these two plant varieties resulted in a strawberry fruit larger in size, with improved tasting and less sensitive to adverse growing conditions (Darrow, 1966).

When the *F. chiloensis* was first introduced to Europe, the plants grew vigorously but produced no fruit (Darrow, 1966). Later, it was discovered that the female plants could only be pollinated by plants that produced large fruit such as the *F. virginiana*. The Europeans then became aware that plants had the ability to produce male-only or female-only flowers. The modern strawberry plant is referred to as *Fragaria ananassa* because its fragrance and flavor resemble that of the pineapple fruit (Fletcher, 1917). In addition, the name *Fragaria* is derived from the Latin word *Frago*, which describes the delicate and sweet flavor of the fruit (Wilhelm and Saga, 1974).

Currently, the United States is the world's largest producer of strawberries followed by Turkey, Spain, Egypt and Mexico (FAO, 2013). California accounts for about 90% of the total annual production with Florida producing about 7% of the nation’s strawberries during the winter (Perez and Pollack, 2009). The mild, cool, coastal climate of California creates an ideal breeding ground for strawberries year-

round but the most productive months are from April to December. The main goal of the strawberry breeding programs in California was to produce a larger and firmer fruit which could be picked at three-quarter red, withstand shipping conditions and arrive full red to the east coast of the United States (Darrow, 1966). In 2015, California produced 2.55 billion pounds of strawberries (92% of the total U.S. production) and Florida produced around 243 million pounds of strawberries, representing 6% of the nation's production and virtually all the fruit grown during the winter (USDA-ERS, 2015). Thus, from late November to early April most of the nation's strawberries are grown in Florida, mainly in the Plant City area (Darrow, 1996).

In Florida, strawberries remain the most important small fruit crop which tend to be larger in size and superior in flavor compared to other berries (Peres et al., 2009). Today, there are many different strawberry cultivars grown worldwide, each having their own advantages and created for specific regions with particular soil and weather conditions. Some of the strawberry varieties commonly grown in Florida include 'Camarosa', 'Carmine', 'Camino Real', 'Gaviota', 'Strawberry Festival', 'Sweet Charlie', 'Treasure', 'Ventana', and 'Winter Dawn' (Peres et al., 2009). Currently, the major cultivars grown in Florida include 'Florida Radiance' with more than 75% of the total cultivated acreage, followed by 'Strawberry Festival' and the new cultivar Sweet Sensation® 'FL127'.

**Morphology and Physiology**. The strawberry fruit is not considered a true berry, but rather a fleshy receptacle bearing multiple fruits on its surface which are referred to as achenes (Szczesniak and Smith, 1969). Since strawberries belong to the

genus *Fragaria*, it makes them more closely related to the *Rosacea* family. The flowers appear hermaphroditic in structure but can also function as either male or female. *Fragaria* X *ananassa* plants have short, woody stems and a basal rosette of compound leaves (Darrow, 1996). The strawberry plants are characterized by stolons which are rooting runners that form new plantlets at their tip (Darrow, 1996).

The fleshy receptacle of the strawberry fruit accumulates sugars, vitamins, and polyphenolic compounds and ripens into a fruit that contains on average 92% water, 7% carbohydrates, 0.6% proteins and 2% fiber (Lundergan and Moore 1975; McCance and Widdowson 1978; USDA, 2010). The strawberry fruit is composed of five tissue zones: the epidermis, hypodermis, cortex, bundle zone and the pith (Szczesniak and Smith, 1969). The fruit's loosely bound structure and cells, that tend to be large with thin walls, make the tissue extremely fragile. Smaller cells are usually found near the periphery and larger cells toward the inside of the fruit (Avigdori-Avidov, 1986). The epidermis of the fruit consists of polygonal cells and stomata with thick-walled hairs while the hypodermis consists of meristematic cells with no intercellular spaces. The cortex of the fruit consists of rounded cells with intercellular spaces that are composed of pectin and cellulose (Harris, 2001). In addition, the fibrovascular bundles are comprised of cellulose that radiates out from the center of the fruit and connects the cortex to the achenes (Harris, 2001). The pith consists of thin-walled cells that usually separate during maturation, and may create smaller to large holes in the core of the fruit (Szczesniak and Smith, 1969). Nutrients and water are transported through long hollow strands of vessels that form spirals and nets referred to as xylem (Szczesniak and Smith, 1969). Vascular bundles are similar in function and may also transport water and

nutrients from the stem throughout the central cylinder to the flesh and the achenes (Suutarinen et al., 1998).

The achenes, produced by many species of flowering plants, are small one-seeded fruits with hard coverings that do not split open when ripe and, are considered the ovaries of the strawberry plant (Szczesniak and Smith, 1969). The achenes contain a single hard seed enclosed by an outer coat, located in the ripened receptacle of the strawberry flesh (Lyle, 2006). They are found on the outside of the receptacle of the strawberry and contribute to the overall fiber and polyphenolic content of the fruit (Avigdori-Avidov, 1986; Aaby et al., 2005). Lignin is the major fiber component of the achenes and vascular bundles (Suutarinen, 1998). It has also been shown that the achenes are critical to the normal fruit development, where their removal results in abnormal fruit coloration and shape (Avigdori-Avidov, 1986).

Variations in the morphology of different strawberry genotypes range from a very firm cortex and soft pith to a tender cortical layer and hard pith (Darrow, 1996). Therefore, genetic variations also significantly influence the overall quality of strawberries during ripening of the plant and after harvest. During development and ripening, strawberry fruit undergo a series of changes in their characteristic quality attributes, namely in color, texture, flavor, and chemical composition (Darrow, 1996).

**Quality Attributes.** The quality of strawberries is based primarily on the appearance, texture, flavor and chemical composition. The appearance of strawberries is primarily based on the color and overall freshness of the fruit which may directly affect the consumers' purchasing decisions (Brosnan and Sun, 2004). Additional factors such

as texture and flavor are equally important and play a major role in consumer acceptance of strawberries. These aspects are directly related to the composition of major chemical components such as water, sugar, and organic acids but also to minor bioactive components present in the fruit. Strawberries have been acclaimed as a rich source of bioactive compounds that include predominantly vitamins (i.e., vitamin C also known as ascorbic acid) and polyphenolic compounds (i.e., anthocyanins and phenolic acids). The quantity and quality of phenolic compounds have shown to be superior in fruit matrices such as strawberries when compared to vegetables (Vinson et al., 2001).

***Appearance.*** Strawberry appearance is a dominant aspect in the perception of the freshness of the fruit (Péneau et al., 2007). The external appearance of strawberries is also a good indicator of the internal quality of the fruit. Furthermore, the appearance of strawberry fruit plays a significant role in consumer acceptance because visual attributes give the first impression of quality experienced by the consumer (Hutchings et al., 2002). Therefore, fresh strawberries are highly appreciated for their attractive bright red color, which is associated with the preservation of their quality characteristics (Kovacevic et al., 2015).

The color of strawberries derives from major pigments namely, anthocyanins, carotenoids and chlorophyll (Woodward, 1972; Gross, 1982; Cheng and Breen, 1991). However, the pigments that have the biggest impact on strawberry color are the anthocyanins. Anthocyanins are the most abundant flavonoid compound in strawberries (Aaby et al., 2012), and are mainly located in the epidermal and hypodermic layers of the fruit, being also important indicators of fruit ripeness (Gross, 1987). The main anthocyanins of strawberry fruit are pelargonidin-3-glucoside and cyanidin-3-glucoside,

making up about 89-95 % and 3.9-10.6 % of the total anthocyanin content of the fruit, respectively (Lopes da Silva et al., 2002; da Silva Pinto et al., 2008). As the strawberry fruit ripens anthocyanin content increases and chlorophyll content decreases (Woodward, 1972; Nunes et al., 2006). An increase of about 31% in the total anthocyanin content of strawberry was observed as the strawberry fruit ripened (Nunes et al., 2006). The difference in shades of red between strawberry cultivars is due to the different concentrations of these two anthocyanin compounds in the fruit. Pelargonidin is responsible for a bright red color while cyanidin is responsible for the orange color shade of the fruit (Gössinger et al., 2009). As the strawberry matures and becomes overripe, the red color is replaced by a brownish or purplish coloration resulting from the loss of anthocyanins, caused by oxidation or by other natural chemical reactions (Hartmann et al., 2008; Gössinger et al., 2009; Holzwarth et al., 2012). Strawberry discoloration and thus anthocyanin degradation, is mainly attributed to temperature, light, oxygen, pH, irons, and to the action of certain enzymes (Bordignon-Luiz et al., 2007; Holzwarth et al., 2012) such as polyphenoloxidase and peroxidases (Grommeck and Markakis, 1963; Serradell et al., 2000). Since the color of anthocyanins is highly dependent on the pH, minor changes in pH values have a major impact on the quality of the pigments (Zhao et al., 2008). Variations in the color of different strawberry cultivars have been shown to occur due to the acidic (average pH values range from 3.70-4.15) environment within the vacuole of the cells (Markakis, 1982). Differences in the pH of the vacuole cause anthocyanins to undergo reversible structural transformation reactions that influence their color (Aaby et al., 2012). In addition, the color of strawberries can be influenced by condensation or co-pigmentation of anthocyanins

with other polyphenols leading to the development of compounds that have a higher stability than anthocyanin monomers (Markakis, 1982; Brouillard and Dangles, 1994).

***Texture and Flavor.*** The texture of strawberry fruits is mainly related to the structural integrity of the cell wall and middle lamella, as well as to turgor pressure (Jackman and Stanley, 1995). The firmness of strawberries is determined by the levels of cellulose, hemicellulose, and pectin which are three types of polysaccharides present in plant cell walls. Pectin is the most water-soluble polysaccharide in the cell wall, and thus the most susceptible to changes caused by enzymatic and non-enzymatic reactions during ripening and storage. These reactions can lead to transformations in the structure and composition of pectin resulting in fruit softening (Jackman and Stanley, 1995; Brett and Waldron, 1996).

Strawberries are one of the most popular fruits in the world mainly due to their unique taste and aroma (Chandler et al., 2012). Volatile compounds are responsible for the distinctive aroma of strawberries despite their minute quantities (Buttery, 1981). In addition to aroma volatiles, sugars and acids give sweetness and tartness to ripe strawberries, providing the characteristic strawberry flavor. The sugar/acid ratio is critical to the intensity of sweetness or acidity perception and, therefore, has a major effect on strawberry flavor (Zabetakis and Holden, 1997). Strawberry flavor volatiles comprise about 350 components, with 2,5-dimethyl-4-hydroxy-2H-furan-3-one being the most important strawberry flavor component (Zabetakis and Holden, 1997).

***Major Components: Water, Sugars and Acids.*** Strawberries are mostly composed of water (92%) and minor amounts of macro and micronutrients such as

sugars, acids, fiber, vitamins and minerals (Lundergan and Moore 1975; USDA, 2010), and also contain important polyphenolic compounds.

Sugars are the main soluble compounds in ripe strawberry fruit, with glucose, fructose, and sucrose accounting for almost 99% of total sugar content (Sturm et al., 2003). However, glucose and fructose which are normally present in a 1:1 ratio are predominant over sucrose. In fact, some studies have shown that the levels of sucrose in strawberry are normally very low, with some cultivars showing no presence at all (Sturm et al., 2003; Gündüz and Özdemir, 2014). In addition, daily temperatures during ripening have shown to directly affect the sugar content of strawberries, with cold nights and warm days promoting the increase in sugar content (Wang and Camp, 2000). Although during ripening of the plant, the levels of fructose, glucose and sucrose increase, they tend to decrease after harvest, particularly if the fruit is kept at temperatures higher than 0°C. For example, sugar content of 'Chandler' strawberries stored at 0, 5, and 10°C remained steady until five days of storage but decreased afterward especially in strawberries stored at 10°C (Ayala-Zavala et al., 2004). The decrease in sugars during storage at higher temperatures is due to higher respiration rates leading to depletion of sugars (Ayala-Zavala et al., 2004). Nunes et al. (2002) also reported a greater reduction in sugar content in strawberries stored at 10°C compared to fruit stored at 4°C.

Organic acids (i.e., malic, tartaric, citric acid and ascorbic acid) are minor components of strawberry fruit but they are important contributors to the flavor. In combination with sugars, organic acids have a significant impact on the overall sensory quality of strawberry fruit. Organic acids affect fruit flavor by regulating cellular pH and

by directly affecting the acidity of the fruit (Manning, 1993). In strawberries, citric acid is the major organic acid found in ripe-red fruit and is typically present at concentrations 10 times higher than ascorbic acid which is the second most abundant acid. Tartaric acid and malic acid are present at much lower concentrations (Koyuncu and Dilmaçünal, 2010). However, as the strawberry fruit matures a significant increase in ascorbic acid and a decrease in other organic acids is observed (Montero et al., 1996). For example, Cordenunsi et al. (2002) reported the decline in the acidity of strawberries during ripening but noted an increase in the levels of ascorbic acid.

***Bioactive Compounds: Vitamin C, Polyphenolic Compounds and Antioxidant Capacity.*** Strawberries are a good source of ascorbic acid (AA), a water-soluble vitamin (vitamin C), recognized for its plentiful health benefits (Loewus, 1987). Depending on the cultivar, maturity at harvest and pre-harvest conditions the levels of AA may range from 23 to 85 mg 100 $g^{-1}$ of fresh fruit (Szajdek and Borowska, 2008; Pineli et al., 2011; Crecente-Campo et al., 2012). In adults, the recommended dietary allowance (RDA) for AA is about 75 mg $day^{-1}$ which can easily be met with an average intake of about 100 g of strawberries a day (USDA, 2016). In fruits and vegetables, the total AA content is assumed to be the sum of L- ascorbic acid and dehydroascorbic acid (DHAA) (Combs, 1998). In general, the synthesis of AA increases with exposure of the fruit to sunlight and is better retained when the nights are cool and the metabolism of the fruit is lower (Hardenburg, 1986). The outer layer of the strawberry fruit was reported to contain more AA than the inner layers, and fruit ripened in the shade had less AA than those exposed to sunlight (Burkhart and Lineberry, 1942; Ezell, 1949). During development and ripening, AA content of strawberries increases. Therefore, the

levels of AA are normally higher in full red fruit compared to fruit at other color stages (Burkhart and Lineberry, 1942; Cordenunsi et al., 2002; Nunes et al., 2006).

Polyphenolic compounds in strawberries have also been extensively studied due to their acclaimed bioactive properties. The flavonoids are the major polyphenolic group in strawberries and are commonly found in the tissues conjugated to glucose or rhamnose (Clifford, 2000; Laura et al., 2009). For example, anthocyanidins are the non-glycosylated or aglycone form of the anthocyanins, with pelargonidin-3-glucoside and cyaniding-3-glucoside being the major anthocyanins found in strawberries (Lopes da Silva et al., 2002; da Silva Pinto et al., 2008). The only flavonoids found in strawberries in the non-glycosylated (aglycone) monomer form are catechin and epicatechin, also classified as flavan-3-ols. The flavonols quercetin and kaempferol, and their glycosylated counterparts have also been identified in different strawberry varieties (Vallejo et al., 2004; Lopes da Silva et al., 2007). Strawberries also contain flavanones and isoflavones and non-flavonoid compounds such as phenolic acids (Shier et. al, 2001; Laura et al., 2009). Phenolic acids present in significant amounts in strawberries include *p*-coumaric, ferulic and caffeic acids (Aaby et al., 2007). In addition, hydrolysable tannins which are mixtures of polygalloyl glucoses and/or poly-galloyl quinic acid derivatives containing gallic acid residues have also been isolated from strawberries (Ishikura et al., 1984). The most common hydrolyzable tannins occur in plant tissues as simple esters of glucose, tartaric acid and quinic acid (Rice-Evans et al., 1996; Laura et al., 2009). Ellagic acid has also been detected in strawberries in significant amounts (Häkkinen and Törrönen, 2000; Aaby et al., 2012). Plants may produce ellagic acid from hydrolysis of tannins such as ellagitannin and gallic acid

(Laura et al., 2009). Other non-flavonoid compounds found in minute quantities in strawberries include coumarins, benzophenones, xanthones, stilbenes, chalcones, lignans, secoridoids and acetophenones (Aaby et al., 2007).

Ascorbic acid and polyphenolic compounds are important components of strawberries because they not only contribute to the color and flavor of the fruit but are also highly correlated with their antioxidant capacity (Wang and Lin, 2000). Strawberries are considered photosynthetic plant systems, particularly vulnerable to reactive oxygen species (ROS). The fruit is often exposed not only to high levels of external oxygen but also to high levels of internal oxygen resulting from the production of ROS. In this case, in the chloroplasts, there is a transfer of high-energy electrons from the photosynthetic electron transport chain to the oxygen molecule instead of NADP (Dalton, 1995). Low-molecular-weight antioxidants such as AA, carotenoids, and polyphenolic compounds are able to interact directly with ROS on a non-enzymatic basis (Dalton, 1995; Foyer et al., 1997). Thus, in strawberries, AA may interact with damaging ROS at the enzymatic and non-enzymatic level. However, the biological importance of AA as an antioxidant is that unlike other low-molecular weight antioxidants (i.e., carotenoids and flavonoids), AA is able to reduce to non-radical products such as DHAA and diketogulonic acid (Dalton, 1995 and Seib and Tolbert, 1982). Further, since AA is only mildly electronegative, it can donate electrons to a wide range of substrates and, therefore, may play a more active role in antioxidant radical scavenging (Halliwell, 1996). Previous studies have shown that strawberries have high oxygen radical absorbance activity against peroxyl radicals, superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Wang and Jiao, 2000; Wang and Lin, 2000).

### Impact of Pre-Harvest Conditions on Strawberry Quality

Strawberries have exceptional health benefits due to their high levels of bioactive compounds, including AA, phenolic acids and flavonoids. However, the quality and quantity of these compounds can be greatly influenced by pre-harvest and post-harvest conditions. Strawberry growers in central Florida face a major challenge when it comes to keeping diseases and fruit rot as low as possible. The warm and humid climate of Florida and other southeastern regions of the U.S. appear to be highly conducive to fungicide resistance by plant pathogens, enabling fungi to thrive and multiply. Anthracnose fruit rot, caused by *Colletotrichum acutatum*, and Botrytis fruit rot, caused by *Botrytis cinerea*, are the most common diseases of strawberries in Central Florida and worldwide (Pavan et al., 2011). Even in well-managed fields, losses from fruit rots can exceed 50% when conditions favor disease development (Ellis and Grove, 1982). These diseases pose major challenges for strawberry growers because they may contribute to major crop losses. Therefore, in order to fight field fruit rots and avoid considerable crop losses, strawberry growers need to apply pesticides weekly throughout the season (Legard et al., 2001; Peres et al. 2010). However, well-informed consumers are gaining awareness of the health and environment risks of extensive pesticide use with alternative cultivation practices (i.e., organic), although more costly, gaining popularity. Organic agricultural practices constitute a good alternative to conventional production because they exclude the use of synthetic fertilizers and pesticides, but require soil building and biological pest control. In addition, labor requirements may be as much as twice those of a conventional system (Pritts and Handley, 1999).

In terms of fruit quality, controversial results have been published regarding the sensory and chemical characteristics of organic versus conventional strawberries. For example, some studies reported that organic strawberries have lower water content and acidity, and higher anthocyanins, phenolic and AA contents and also higher AOC compared to conventionally grown fruit (Olsson et al., 2004a; Abu-Zahara et al., 2007; Jin et al., 2011). Similarly, Reganold et al. (2010) reported that organic strawberries have higher dry matter, AA and phenolic contents, and longer shelf life, and some cultivars have also better taste and appearance than conventional strawberries. Nevertheless, other studies reported that the levels of polyphenolic compounds in organic strawberries were similar to that of conventionally grown fruit (Häkkinen and Törrönen, 2000) and, even though, organic strawberries had higher soluble solids content, no significant differences were found in the AA content between fruit from the two different agricultural practices (Kahu et al., 2010). Also, Hargreaves et al. (2008) found no significant differences in the sugar content and antioxidant capacity of organic compared to conventionally grown strawberries. Conversely, Cardoso et al. (2011) reported that AA content was significantly higher in conventionally grown strawberries than in organic fruit. Finally, Leskin et al. (2002) concluded that the quality of organic strawberries is similar to that of conventional grown strawberries.

An alternative approach to organic and conventional farming practices is the use of reduced pesticide applications based on disease forecast systems where, depending on the weather conditions (i.e., high field temperature and humidity), growers are advised to spray or not. Peres and Mackenzie (2009) showed that this system reduces the number of fungicide sprays by 50% without compromising disease control. By

reducing the amount of fungicides used to control diseases through accurately targeting the right application time, the overall postharvest quality of strawberry can be maintained, production costs reduced while providing an alternative to health-aware consumers. This type of approach has already been implemented by strawberry growers in Florida showing good results in terms of fruit yield and disease control.

### Impact of Post-Harvest Conditions on Strawberry Quality

Strawberries are very perishable fruits and their quality and shelf life greatly depends on the environmental conditions (i.e., temperature and relative humidity) to which the fruit is exposed from the field to the consumer. Poor storage and handling conditions may negatively affect the overall quality of the fruit, resulting in excessive loss of water and more specifically, reducing the amount of bioactive compounds in strawberry and potentially their antioxidant capacity.

***Temperature.*** Good temperature management is the most important and simplest way of delaying deterioration of strawberry fruit and extend shelf life (Kalt et al., 1993; Nunes et al., 2005; Nunes et al., 2006; Nunes, 2008). Optimum storage temperature, as close as possible to 0°C, has shown to delay senescence of strawberries due to ripening, softening, and textural and color changes, metabolic changes, moisture loss, and spoilage due to fungal invasion (Hardenburg et al., 1986; Nunes et al., 2003). Exposure of strawberry to temperatures higher than 0°C can drastically reduce shelf life and diminishes the quality of the fruit, with post-harvest life of strawberries being 7 to 8 days even at optimal conditions (Mitcham, 2004; Nunes, 2008). For example, when the storage temperature is raised from 0 to 10°C, the rate of

quality deterioration increases by two- to four-folds (Mitchell et al., 1996) due to an increase in the respiration rate and consequent depletion of sugars and acids (Moraga et al., 2006).

Temperature also has a negative impact on the bioactive quality of strawberry fruit, namely on ascorbic acid and polyphenolic compounds (Nunes et al., 2005; Shin et al., 2007; Laura et al., 2009). Shin et al. (2007) reported that total ascorbic acid concentrations in strawberry declined significantly when the fruit was stored at 0.5 and 20 °C, but remained unchanged at 10 °C. Ascorbic acid is readily oxidized, especially when exposed to elevated temperatures, increased storage time, cations (i.e. copper and iron), oxygen, alkaline pH, light, or degradative enzymes (Fennema, 1977; Gregory, 1996). The first oxidation product of AA is the radical monodehydroascorbate (MDHA), also known as semidehydroascorbate, or ascorbate free radical (Washko et al., 1992). Shin et al., (2007) also reported that AA has been shown to be readily oxidized when exposed to elevated temperatures and increased storage time. If further degradation occurs, two molecules of MDHA may also spontaneously disproportionate to AA and DHAA (Washko et al., 1992). The oxidation of AA to DHAA does not result in loss of biological activity since DHAA is readily reconverted into AA which makes the oxidation of AA to DHAA reversible (Deutsch, 1998). However, DHAA may also undergo irreversible hydrolysis to diketogulonic acid, which is not biologically active as AA (Padh, 1990; Russell, 2004).

In strawberries, AA losses during cold storage can be significant and normally increase as temperature increases. For example, AA content decreased by approximately 7% in ‘Oso Grande’ strawberries stored at 1°C for 8 days (Nunes et al.,

2006). When stored at 6°C for 6 days, ‘Oso Grande’ strawberries experienced a decrease of about 50% in their initial AA content (Cordenunsi et al., 2003). These results show that post-harvest storage temperature has a significant effect on the AA content of strawberries, even at a minimal storage temperature difference.

There is little information available about how temperature influences the bioactive content in strawberries. Shin et al. (2007) showed that the overall quality of ‘Jewel’ strawberry stored for 4 days at 0.5, 10 and 20 °C, declined more rapidly at 20 °C with total phenolic compounds showing higher stability at 20 °C than at other temperatures, and total AOC showing better stability at 10 °C than at 0.5 and 20 °C. In addition, Ayala et al., (2004) reported that strawberry fruit stored at 10°C or 5°C showed higher total phenolic and anthocyanin contents than those stored at 0°C. Finally, storage temperatures of 0°C were also associated with lower concentrations of anthocyanins and phenolic content and higher polyphenol oxidase (PPO) activity (Nunes et al., 2005). Jin et al. (2011) reported that although temperature can be manipulated for production of secondary metabolites, it may lead to a decrease in anthocyanins and polyphenolic compounds in general.

Since AOC can be used to estimate the potential health benefits of strawberries, it is of great interest to evaluate the impact of post-harvest environmental conditions on the antioxidant status of strawberries. Several studies have shown that the levels of bioactive compounds in strawberries and their AOC can vary tremendously depending on the cultivar, ripening stage and storage conditions (Olsson et al., 2004a; Shin et al., 2007). For example, Pineli et al. (2011) evaluated the antioxidant characteristics of strawberries at different ripening stages (green, pink, or ripe) and found that higher AOC

was observed at the pink stage which was also related to higher amounts of total phenolic and AA in 'Osogrande' and 'Camino Real' strawberry cultivars. Lower AOC during storage was associated with fruit harvested before the pink stage and with lower concentrations of total flavonoids and phenolic concentrations in strawberries stored at 10 °C compared to those stored at 3 °C (Shin et al., 2008). In general, strawberry fruit stored at 10°C or 5°C showed higher total phenolic and anthocyanin contents and higher AOC than those stored at 0°C (Ayala et al., 2004). Although previous studies showed a decrease in strawberry stored at lower compared to higher temperature, the authors did not express the data in terms of dry weight and therefore did not account for potential water loss during storage that may contribute to a concentration effect of polyphenolic compounds and AA rather than to an actual increase.

***Relative Humidity.*** In addition to optimum temperature conditions, relative humidity (RH) can also have a major effect on the quality and shelf life of fresh fruit and vegetables. However, when stored under the same temperature and RH conditions, not every fruit and vegetable lose water at the same rate. The rate of water loss differs mainly with the type of protective tissue (i.e., waxed versus non-waxed), skin thickness and surface area (Hardenburg, 1986). The amount of water lost by a fruit or vegetable is caused by the movement of water vapor which moves from higher to lower humidity concentrations until the equilibrium is attained. Since most fruits and vegetables have an internal RH higher than 90%, when the RH of the surroundings is lower, the fruit transpires and releases water into the atmosphere (Hardenburg, 1986). The rate of transpiration can be reduced by raising the RH, lowering the air temperature, reducing the movement of air around the product and by providing protective packaging.

Loss of weight after harvest is one of the major causes of quality deterioration in strawberries and other fresh fruits and vegetables. As weight loss increases, firmness decreases, and wilting, shriveling, or browning increase and render the fresh commodities unacceptable for sale (Nunes and Emond, 2007). It is well established that the maximum permissible water loss for strawberries before marketability is impaired is between 3 and 6% (Hardenburg, 1986; Nunes and Emond, 2007). Water loss higher than 5% may lead to excessive shriveling and a dull appearance of the epidermis, with negative impact on the appearance of the fruit (Hardenburg, 1986). Weight loss can also negatively affect the nutritional composition of strawberries. As the fruit loses water, water soluble vitamins and other bioactive compounds may also be easily lost (Lee and Labuza, 1975; Nunes et al., 1998; Ayala-Zavala et al., 2004; Shin et al., 2007). For example, Nunes et al. (2008) showed that 'Oso Grande' strawberries stored at 1°C for 8 days experienced a decrease of about 7% in total AA. At higher temperatures strawberries from the same cultivar experienced a decrease of about 50% of AA content when stored for 6 days at 6°C (Cordenunsi et al., 2003). These results suggest that post-harvest storage temperature has a significant effect on the AA content of strawberries, even at a minimal storage temperature difference.

Strawberry polyphenolic compounds are also significantly affected when the fruit water content decreases. Shin et al. (2007) reported little water loss but changes in total phenolic content and AOC of strawberry stored in 75, 85 or 95% RH. On the other hand, bioactive compounds such as polyphenolic compounds that are water soluble, have shown to significantly decrease when weight loss of strawberry increases above 5% or more (Nunes and Dea, 2015). In addition, Nunes et al. (2005) reported that during

storage of strawberries at 1°C, excessive water loss was associated with lower concentrations of anthocyanins and phenolic compounds and higher polyphenol oxidase (PPO) activity. PPO activity increased as a result of water loss and not only contributed to the development of surface browning but increased anthocyanin degradation and oxidation of soluble phenolic compounds.

In summary, in order to avoid loss of bioactive compounds during post-harvest storage, low temperatures and maintenance of high humidity are imperative (Nunes et al., 1998).

## Fruit Juices

### Composition and Processing of Commercial Beverages and Fruit Juices

Changes in lifestyles and increased consumer health awareness have been driving the food industry to develop new functional foods and beverages with added health benefits. Fruit juice beverages are extremely popular because they represent an easy and convenient way of consuming fruits, which are important sources of health-promoting compounds. In the United States, juice drinks are a $15.5 billion market with refrigerated juice and smoothie sales alone showing a 27% increase in 2012 (BNP Media, 2014). This rapid increase is most likely related to the addition of functional ingredients that are believed to have desired nutritional health benefits.

Commercial fruit juices are mainly composed of water (97%) and by a non-aqueous fraction that contains natural sugars such as glucose and fructose, and also starch, cellulose, vitamins, mineral and numerous polyphenolic compounds (Ashurst, 1995). Trace amounts of natural water-soluble organic acids such as acetic, ascorbic,

citric, malic, lactic and tartaric acids are responsible for the taste and character of fruit juices (Ashurst, 1995). In addition, fruit juices are high in polyphenolic compounds such anthocyanins, phenolic acids, and tannins, and also in carotenoids which provide color and astringency. In addition to polyphenolic compounds, juices can also be high in vitamins and minerals depending on the fruit used for juicing and are usually low in other nutrients such as lipids and proteins (Somogy et al., 1996).

The production of fruit juices involves several steps, from the raw material to the final juice product. The first step is to determine if the quality of the raw product is acceptable. Since the quality of the juice greatly depends on the quality of the raw product, fruit arriving at the processing plant should have an optimum overall quality. Prior to juicing, the fruit is washed, thoroughly inspected and sorted. Inspection and removal of unfit fruit are very important since bad units can cause contamination of an entire batch of juice (Wills et al., 1998). The goal of juice production is to remove as much as possible of the desirable components from the fruit without extracting any of the undesirable compounds such as seeds, skin, core, etc. In addition, to extend the shelf life of fruit juices, good sanitation practices, to reduce microbial load, and low temperature management throughout juice processing are key factors (Chen et al., 1993). In order to obtain a maximum storage life, the holding temperature of the fruit juice should be as close as possible to the freezing point (Chen et al., 1993; Wills et al., 1998). Good temperature management and the natural acidic pH (less than 4.5) of the juice are the most important factors in restricting microbial growth (Chen et al., 1993).

After sorting, the fruit is pressed to extract the juice. The pulpy fraction of the fruit either floats or sinks for easy separation which results in loss of all dietary fiber and

some phenolic compounds that are bound to the fibrous parts of the fruit (Brody et al., 2000). Juices, where turbidity is not acceptable, are further processed using centrifugation or filtration to yield a clear juice whereas juices with natural cloudiness generally do not require filtration (Somogy et al., 1996). There are many filtration systems used in the juice industry, ranging from plate and frame filters to plastic, ceramic, or metal membranes (Somogy et al., 1996). Extreme filtration such as sterile filtration filters juice through pores that are small enough to physically remove microorganisms from the juice (Chen et al., 1993; Somogy et al., 1996). Centrifugation may also be used to simplify subsequent filtration steps and thus it is an essential step used in many juice processing operations (Fellows and Hamptonnes, 1992). When processing techniques such as pressing, centrifugation and filtration are used, the fruit juice is subjected to considerable aeration. The inclusion of oxygen into the juice can promote enzymatic browning, destroy vitamins and phenolic compounds, modify flavor and damage the overall quality of the juice. To protect the fruit juice from exposure to too much oxygen careful handling and de-aeration (e.g., using a vacuum or an inert gas) of the juice may be necessary (Chen et al., 1993).

Until recently, to prevent microbial contamination and spoilage, fruit juices were preserved using exclusively a thermal treatment (TT) (Barbosa-Cánovas et al., 2005). Thermal treatment consists of heating the juice to about 70ºC to inactivate native enzymes and reduce the microbial load (Chen et al., 1993). The downside of this treatment is that delicate flavor compounds can be destroyed and unacceptable darkening due to enzymatic and non-enzymatic browning can occur. In addition, TT may also contribute to loss of many health promoting compounds. To overcome some

of these problems, rapid heating and cooling are usually necessary (Brody et al., 2000). In order to satisfy consumer demand for nutritious, healthy and safe products, non-thermal food preservation technologies, such as pulsed electric fields (PEF) and high-pressure processing (HPP), have been implemented as alternative methods to heat treatments (Barba et al., 2012).

The acidic nature of most juices allows the use of pasteurization which uses temperatures near 100ºC to reduce the amount of spoilage organisms (Brody et al., 2000). However, at a pH greater than 4.6, temperatures higher than 115ºC need to be used for extended time periods in order to reduce spoilage microorganisms (Brody et al., 2000). To reduce overall microbial load, the fruit also needs to be treated before processing through heat cleaning (1 minute at 80ºC). This method greatly reduces surface contamination without damaging the underlying flesh (Chen et al., 1993). Another process that is used in fruit juices is hot filling where the juice is heated to around 95ºC following aseptic packaging (Brody et al., 2000). Hot fill has the additional advantage of driving air from the juice and ensuring a partial vacuum in the sealed container. Processing technologies such as aseptic packaging may be preferable to maintain certain nutrients. In this case, the juice is pasteurized using high heat for a short time followed by rapid cooling before filling into sterile containers (Brody et al., 2000). This rapid heating and cooling of the juice guarantees microbial and enzyme destruction while preserving most of the health-promoting constituents (Brody et al., 2000). Other preservation methods include the use of preservatives such as sulphur dioxide which inhibits both microbial growth and enzymatic and non-enzymatic reactions. Other preservatives include benzoic acid, sorbic acid, sodium benzoate and

potassium sorbate that can be used individually or synergistically (Branen, et al., 1989). Benzoates and sorbates are often used in combination with low temperatures to extend the shelf life of minimally processed juice drinks (Somogyi, et al., 1996). Newer methods include high pressure processing (HPP) technologies that use high pressure at low temperatures or ionizing irradiation processing technologies (Thayer and Rajkowski, 1999).

Today, most juices that are available as ready-to-drink are composed by blends of juices. Blending different juices offers the opportunity to adjust sugar/acid ratios and compensate for other imbalances in the juice from single harvests or cultivars (Somogyi, et al., 1996). In addition to adjusting flavor, blending can also improve the content of important nutrients such as AA and polyphenolic compounds (Somogyi, et al., 1996). Finally, fruit juices are usually packaged in glass containers because of their impermeable nature and transparency which increases product appeal (Brody et al., 2000). However, the use of clear packaging may cause light-induced deterioration of polyphenolic compounds and AA (Pérez-Vicente et al., 2004).

### Fruit Juices Particularly Rich in Polyphenolic Compounds

The polyphenolic content of the juice is directly related to the composition of the raw fruit (Somogy et al., 1996). Amongst fruits, berries have shown to contain important amounts of polyphenolic compounds (Giusti and Jing, 2007). For example, pomegranate contains high levels of polyphenolic compounds, including anthocyanins, ellagic acid, punicalins, granatins, and different flavanols such as catechins and gallocatechins (González-Molina et al., 2009). These compounds are the main

contributors to the pomegranate juice sensory qualities (i.e., color, astringency, and bitterness). Several studies have reported a very high AOC in pomegranate juices (Gil et al., 2000; Noda et al., 2002) which significantly correlates with their polyphenolic composition, particularly with the levels of hydrolysable tannins and anthocyanins. Anthocyanins (i.e., delphinidin, cyanidin, and pelargonidin) are the main pigments responsible for the red color of pomegranates (Mousavinejad et al., 2009). However, the significant decrease in anthocyanin content of the juice observed after TT often results in undesirable changes in color and nutritional attributes of the juice. Nevertheless, TT of pomegranate juice have shown to decrease the percentage of polymeric anthocyanins and increase the levels of monomeric anthocyanins, resulting in increased AOC (Vegara et al., 2013). Furthermore, the intrinsic properties of pomegranate juice (i.e., pH, chemical structure, enzymes, co-pigments, metallic ions and sugars) may influence the degradation of polyphenolic compounds, depending on the magnitude and duration of heating, the storage time and temperature, and the presence of oxygen (Patras et al., 2010). Higher anthocyanin degradation was observed in pomegranate juices after storage at 25 °C compared to 5 °C (Vegara et al., 2013).

Blackcurrant juice also contains large amounts of polyphenolic compounds. The fruit and juice have a dark purple color because of the high levels of anthocyanins, the most prevalent of the flavonoids in these berries (Giusti and Jing, 2007). Blackcurrants contain various polyphenolic compounds, mainly anthocyanins, hydroxycinnamic acids, flavanols and flavonols, and AA. Anthocyanins are the major flavonoids, constituting approximately 90% of total polyphenols in blackcurrant juices. The major anthocyanins found in blackcurrant juice are conjugated forms of delphinidin and cyanidin (Schrage et

al., 2010). The high levels of polyphenolic compounds in blackcurrant juices seem to protect AA from oxidation (Miller and Rice Evans, 1997)

Aronia berry (also known as chokeberry) is one of the richest berries in polyphenolic compounds. However, the polyphenols that are present in the highest amounts in aronia juice (i.e., anthocyanins and proanthocyanidins) are easily lost during processing (Denev et al., 2012). Aronia berries are rich in chlorogenic acid, proanthocyanidins, containing also quercetin, flavonols, and anthocyanins such as cyanidin 3-galactoside, cyanidin 3-arabinoside, cyanidin 3-xyloside, and cyanidin 3-glucoside (Taheri et al., 2013; Wangensteen et al., 2014; Bolling et al., 2015). These polyphenols contribute to the high in vitro AOC of aronia extracts (Jakobek et al., 2011). However, anthocyanins and proanthocyanidins are known to be the least well absorbed polyphenolic compounds (Denev et al., 2012).

### Effect of Processing and Storage on Ascorbic Acid, Polyphenolic Compounds and Antioxidant Capacity

Fruit juices may contribute significant amounts of AA if consumed as part of a balanced diet. However, AA is extremely unstable and very susceptible to chemical and enzymatic oxidation during processing (Hotz and Gibson, 2007). Therefore, the quality of any fruit juice and its value as a source of AA depends on the type of processing, packaging material, and storage conditions. Storage duration and temperature are important variables that need to be controlled after the juice has been processed. To compensate for losses experienced throughout processing, fruit juices may be fortified with a synthetic form of AA. These may include esters of ascorbic acid, synthetic forms

such as 6-deoxy-l-ascorbic acid, isoascorbic acid (IAA) and oxidized compounds (Davey et al., 2000). The addition of IAA to beverages has shown to be only about 5% as active as the natural AA and may lead to an overestimation of the total AA content of the juice (Rodríguez-Roque, 2015).

Thermal treatment processing has shown to have the greatest impact on AA, and losses can be as high as 31%, compared to untreated beverages (Rodríguez-Roque, 2015). In addition, significant losses (11 to 16%) in AA bioavailability were observed in TT-treated orange, kiwi, pineapple and mango beverages (Rodríguez-Roque, 2015). On the other hand, TT has shown to promote nutrient release through cell rupture or cell separation which can, in turn, enhance the bioavailability of several other nutrients (i.e., phenolic compounds) (Wollstonecroft et al., 2008). However, the same TT may disrupt the beverage matrix and bring AA and oxidative enzymes (i.e., ascorbic acid oxidase and peroxidase) in contact accelerating degradation (Yeom et al., 2000). Non-thermal processing technologies such as PEF and HPP have shown to inactivate some of these oxidative enzymes and, therefore, prevent AA oxidation (Sánchez-Moreno et al., 2005). In fact, several studies showed that the use of PEF and HPP results in a higher retention of AA in juices (Yeom et al., 2000; Sánchez-Moreno et al., 2005; Torregrosa et al., 2006; Morales-de la Peña et al., 2010; Zulueta et al, 2013). For example, Torregrosa et al., (2006) showed that the remaining concentration of ascorbic acid after pasteurization of orange–carrot juice was 83%, whereas in the PEF-treated juice it was 90%.

Storage time and temperature after processing can also contribute to a decline in important components of the fruit juice. For example, storage of commercial fruit juices

(orange, peach, grapefruit, pineapple, apple, mango, kiwi, lemon and apricot) in closed containers for 4 months at room temperature resulted in AA losses ranging from 29 to 41% (Kabasakalis et al., 2000). When containers were opened for consumption and then stored in the refrigerator for 31 days, commercial orange juice lost up to 67% of its AA whereas, under the same conditions, AA losses in fresh orange juice were much lower (7–13%) (Kabasakalis et al., 2000). In the same study, when containers of commercial orange juice were kept for 10 days outside the refrigerator, AA losses were as high as 12.5%, decreasing to about 9% when the containers were refrigerated (Kabasakalis et al., 2000). In another study, non-pasteurized orange juice containing no preservatives lost 36% of its initial AA over a period of 14 days, while orange juice with added preservatives lost 21% of its AA content (Haddad, 1997).

Some types of beverages are considered functional foods because of the amount of polyphenolic compounds that they contain. The polyphenolic compounds in beverages are also believed to be more bioavailable compared to more complex food matrices (Clifford, 2004; Saura-Calixto, 2007). Furthermore, the effect of processing on polyphenolic compounds depends on the type of food, the nature and location of compounds in the food matrix, and the intensity and duration of treatment. Food processing may change some of the physicochemical properties of polyphenolic compounds and thus, increase or reduce their bioavailability. Several changes in the phenol structure or the formation of phenolic derivatives may occur during processing (Dugo et al., 2005). For example, handling and aeration may negatively affect the flavonoid content in fruit juices because these compounds are not bound to any fibrous tissue and thus are relatively unstable in pulp-free juices (Patras et al., 2010).

Oxygenation during processing may cause degradation of flavonoids and accelerate the degradation either through a direct oxidative mechanism and/or through the action of oxidizing enzymes such as polyphenol oxidase (Patras et al., 2010). In juices with a high concentration of anthocyanins, an unacceptable brownish coloration can develop during processing. This discoloration can, however, be prevented by the addition of chemical preservatives such as benzoic acid, sorbic acid, sodium benzoate or potassium sorbate (Maccarone et al., 1985; Marchese, 1995). In general, storage at high temperatures for long periods of time also has a negative effect on the anthocyanins and total polyphenolic contents of juices (Torres et al., 2011; Mazur et al., 2013). Therefore, current research suggests the use of a mild pasteurization, of less than 80 °C, to minimize the degradation of anthocyanins in fruit juices (Marchese, 1995). The stability of anthocyanins may also be influenced by their interaction with AA which may lead to mutual degradation in various fruit juices (Starr and Francis, 1968). Both the color and nutritional quality of juice products may decrease due to the interaction of AA with anthocyanin pigments. This may be caused by either excessive hydrogen peroxide formation through oxidation or condensation of AA with anthocyanin pigments (Markakis, 1982).

Phenolic acids seem to be more stable than anthocyanins to processing technologies. For example, TT resulted only in a slight change in phenolic acid concentrations as compared with untreated beverages (Rodríguez-Roque, 2015). Actually, the levels of phenolic acids significantly increased after PEF and HPP processing resulting most likely from the release of phenolic components from other food constituents or improving their extractability (Rodríguez-Roque, 2015). Wang et al.

(2014) reported that high pressure and high temperature increased the content of polyphenolic compounds due to the breakdown of the cell wall structure and hydrolysis of polysaccharides. Additionally, it is possible that these treatments inactivate enzymes that would otherwise contribute to the loss of phenolic substances (such as the polyphenol oxidase) or increase the activity of enzymes that participate in the biosynthesis of phenols (i.e., PAL), as shown in orange juice treated by PEF or HPP (Sánchez-Moreno e al., 2005; Morales-de la Peña et al., 2011). One problem that may arise from a too high phenolic content is the precipitation of ellagic acid in the juice. While ellagic acid is a desirable polyphenol in strawberries, in juice it forms an undesirable, powdery sediment. This precipitate which forms slowly in clear juices, even after microfiltration or sterile filtration, is accelerated by pasteurization (Musingo et al., 2001).

Beverages represent a good food model to measure the antioxidant capacity (AOC) of polyphenolic compounds because of less interference from dietary fibers, lipids and proteins that can be naturally found in other foods. Polyphenolic compounds in fruit juices exert antioxidant effects, and their level of activity is closely related to their synergistic interactions (Chandrasekara et al., 2012). Therefore, the decrease in the AOC of juices after processing could be explained by the oxidation of AA and polyphenolic compounds. Otherwise, it has also been suggested that processing could improve the AOC of fruit juices through the release of additional polyphenolic compounds from the food matrix or by inactivation of degradative enzymes (Morales-de la Peña et al., 2011; Rodríguez-Roque, 2015). Changes in AOC during storage of fruit juices usually parallels changes in the levels phenolic compounds, but not anthocyanin

or AA which suggests that the phenolic compounds are less modified during storage than anthocyanins and AA (Shin et al., 2007).

### Chemical and Biological Activity of Food Polyphenolic Compounds

Antioxidants are molecules that can scavenge unstable free radicals which are highly reactive compounds commonly produced by various cellular biochemical processes. Free radicals may behave as reactive oxygen species in mammal and in plant systems through cellular processes. These include superoxide, singlet oxygen, hydrogen peroxide, and the highly reactive hydroxyl radical (Halliwell, 1996). In these systems, the mitochondria produce ROS as byproducts of normal cellular metabolism. However, ROS production is enhanced by a variety of environmental stresses, including drought, starvation, wounding, excessive exposure to UV light and pollutants (Bowler et al., 1992; Dalton, 1995). In excessive stress situations, ROS may damage the polyunsaturated fatty acids in lipoproteins and in cell membranes, thereby disrupting the transportation of vital substances in and out of the cells. Therefore, ROS can cause damage by initiating a cascade of reactions that lead to the production of the hydroxyl radicals and other destructive species that can cause protein damage, lipid peroxidation, DNA damage and cell death (Dalton, 1995). Antioxidants can delay or inhibit this oxidation process by donating their own electrons or by hydrogen donation to become more stable free radicals themselves. Normally, organisms have the ability to cope with free radicals using both enzymatic and non-enzymatic mechanisms. The enzymes involved in this process include superoxide dismutases, ascorbate peroxidases and glutathione peroxidases (Bunkelmann et al., 1995). However, in some

cases, the organism may need dietary antioxidants such as vitamins and polyphenolic compounds to help reduce the levels of ROS. On the other hand, the production of ROS can also be beneficial because, when controlled, it can produce a cytotoxic effect to invading pathogens, and may also stimulate cell wall peroxidase activity to hinder pathogen penetration (Schopfer, 1996; Alvarez et al., 1998).

Polyphenolic compounds are defined as antioxidants when they have the ability to retard or prevent the autoxidation or free radical-mediated oxidation of certain compounds, and the ability to reduce a radical into a stable form through intermolecular hydrogen bonding that prevents further oxidation (Halliwell, 1990). The availability of the phenolic hydrogens as hydrogen-donating radical scavengers further predicts their AOC (Shahidi and Wanasundara, 1992). Overall, structurally important features that define the AOC of polyphenolic compounds include the hydroxylation patterns, in particular the 3', 4'-dihydroxyl cathecol group in the B-ring which gives the structure higher stability and participates in more electron delocalization; the planarity of the molecule or the presence of 2,3-unsaturation in conjugation with a 4-oxo-function in the C-ring which has shown to be responsible for electron delocalization from the B ring; and the 3- and 5-OH groups with 4-oxo function in the A and C rings (Halliwell, 1990; Shahidi and Wanasundara, 1992; Huang et al., 2005). For example, quercetin satisfies all of the properties mentioned above and thus has shown to be more effective at radical scavenging than the flavanols (i.e., catechin and epicatechin) which lack the additional 4-oxo function in the C ring (Zhou and Zheng, 1991). Furthermore, flavonoids are often found naturally glycosylated which makes them more water-soluble but less antioxidant effective. Common glycosylation positions include the 7-hydroxyl in flavones,

isoflavones and dihydroflavones, the 3- and 7- hydroxyl in flavonols and the 3- and 5-hydroxyl in anthocyanidins. Glucose, galactose, rhamnose, xylose, arabinose, as well as disaccharides, are usually constituents in the glycoside formation of anthocyanins. Glycosylation, however, tends to reduce their AOC when compared to their corresponding aglycones because the addition of sugar seems to block the 3-hydroxyl group in the C ring (Shahidi and Wanasundara, 1992). Conversely, AOC of phenolic acids and their esters depend on the number of hydroxyl groups in the molecule because the carboxylate group in the benzoic acid structure negatively influences hydrogen-donation (Rice-Evans et al., 1996). Regarding hydroxycinnamic acids, the addition of an ethylenic group between the phenyl ring carrying a *p*-hydroxyl group and a carboxylate group has a positive effect on AOC properties of the OH groups (Rice-Evans et al., 1996). Glycosylation of the carboxylate group in hydroxycinnamic acids such as in caffeic and *p*-coumaric acids does not result in a decrease in AOC as seen with anthocyanins (Grootveld and Halliwell, 1986). The AOC or hydroxybenzoic acids (i.e., gallic and salicylic acids) is strongly related to the relative positioning of the hydroxyl groups on the benzoic ring. Thus, dihydroxybenzoic acid derivatives showed higher AOC in either *ortho* and *meta* positions than monohydroxy benzoic acids in the same positions (Grootveld and Halliwell, 1986; Cuvelier et al., 1992).

Overall, it can be assumed that there is a positive correlation between AOC and the polyphenolic content of fruits and vegetables (Wang and Lin, 2000). However, there are no official recommendations for daily dietary antioxidant intake, data is limited, and developing a model for dietary intake is yet to be established (Prior, 2005). Nevertheless, several studies showed that the use of high doses of synthetic

antioxidants have an antagonistic effect on the AOC by decreasing the cell defense mechanisms and potentially inducing apoptosis of healthy cells (Hercberg et al., 1998). These findings suggest that antioxidants are only a minute component of the human diet and may have enhanced bioactive properties when ingested naturally from fruit and vegetables rather than from nutritional supplements.

### Chemical Assays: Antioxidant Capacity

Several analytical methods have been developed to measure the AOC of foods. These methods are typically classified according to the chemical reactions involved, which are basically hydrogen atom transfer (HAT) or electron transfer (ET) reactions (Huang et al., 2005). In HAT-based assays, the applied antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. These assays include the oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays (Huang et al., 2005). The ET-based assays measure the capacity of an antioxidant to reduce an oxidant, resulting in a change of color that can then be read with a spectrophotometer. ET-based assays include the total phenols assay by Folin-Ciocalteu reagent (FCR), Trolox equivalents antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), and 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH) (Huang et al., 2005). Depending on the food matrix, different methods, alone or in combination, are generally used to measure the AOC of a particular food product (Stratil et al., 2007). Specifically, these assays measure the same electron transfer reaction between the added oxidant and the antioxidants in a solution and give fast, reproducible

and comparable results (Huang et al., 2005). For example, because of the complex kinetics and potential interactions of multiple phenolic compounds in fruit samples, it is necessary to use more than one method to accurately measure AOC (Ozgen et al., 2006). The FRAP, DPPH and TEAC have been proven to accurately measure the AOC in fruits and vegetables as well as in individual phenolic compounds (Stratil et al., 2006; Thaipong et al., 2006).

### Cell Models: Cancer Cells

Numerous studies have shown that a high consumption of fruits and vegetables promotes health and is associated with a reduced risk of cancers and other degenerative diseases (Seeram et al., 2006; Parkar et al., 2008; Tzounis et al., 2008; La et al., 2009; Lewandowska et al., 2016). In the last decade, polyphenolic compounds found in fruits, vegetables and plants have received special interest because of their potential *in vitro* and *in vivo* AOC. These naturally occurring compounds can scavenge ROS, which might have the potential to damage cell components, such as DNA, proteins, and lipids (Lewandowska et al., 2016). Recent studies indicate that oxidative damage might be involved in initiating events in cancer, and free radicals may help to induce the initiation of apoptosis and help in the promotion and progression of carcinogenesis (Matés and Sánchez-Jiménez, 2000; Lewandowska et al., 2016). The increased cellular level of ROS is associated with key aspects of carcinogenesis, including induction of genetic alterations and cellular proliferation (Lewandowska et al., 2016).

Cancer cell proliferation is important in the progression of tumors. Unregulated cell proliferation together with suppressed apoptosis is common in cancer evolution and progression (Evan and Vousden, 2001). Individual polyphenolic compounds have shown to inhibit *in vitro* cancer cell proliferation of human colon cancer and breast cancer cells (Kuntz et al., 1999; Prakash et al., 2001). However, it is difficult to evaluate the relative importance of individual polyphenolic compounds as anticancer promoters. The protective effect might be due to additive or synergistic actions of several compounds, but most investigations on the inhibitory effects of polyphenolic compounds on the proliferation of cultured cancer cell focused on individual compounds (Lewandowska et al., 2016). The relative importance of the individual compounds in comparison with a complex mixture of polyphenolic compounds found naturally in fruits and vegetables is usually not determined. Seeram et al. (2006) published one of the few studies where fruit extracts (blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry) were used. The authors showed the ability of these complex polyphenolic fruit extracts in decreasing the proliferation of both colon cancer and breast cancer cells. In another study, Mertens-Talcott et al. (2003) reported enhanced apoptosis and inhibition of cell proliferation in human leukemia cell lines treated with quercetin plus ellagic acid and emphasized the importance of a synergistic rather than an additive effect.

**Worm Models: *Caenorhabditis elegans***

The nematode *Caenorhabditis elegans* is a multicellular organism, with the presence of tissue and organ systems which creates an ideal model for lifespan assays. Another advantage of using *C. elegans* as an *in vivo* model is the short lifespan of the worms, maturing into an adult in about 45 hours and their average lifespan of about 2-3 weeks (Félix and Braendle, 2010). At optimal conditions, the organism is also able to reproduce at a high rate and lay about 300-1000 eggs (Félix and Braendle, 2010). A significant percentage (60 to 80%) of human genes homologues have been identified in *C. elegans* (Kaletta and Hengartner, 2006). Thus, because of its morphological characteristics, its short life span and its well-studied cellular and genetic features, that show a strong correlation with mammals in cellular and molecular principles, the *C. elegans* has become an important *in vivo* model for the study of ageing, stress resistance, and degenerative diseases and an ideal model to test the biological efficacy and toxicity of certain metabolic compounds (Kaletta and Hengartner, 2006). In addition, *C. elegans* is easy to culture on solid and liquid media, is highly reproductive, and its small size allows easy storage and culturing on Petri dishes. *C.elegans* occupy various environments that contain different bacteria and nutrients. They feed on the bacteria such as *E.coli* but can survive on a diet of a variety of many kinds of bacteria and nutrients which makes them ideal *in vivo* models to test the effects of fruit extracts rich in polyphenolic compounds.

Various studies have shown that exposure of *C. elegans* to blueberry extracts rich in polyphenolic compounds and to individual polyphenols resulted in increased oxidative and thermal resistance and extended lifespan (Wilson et al., 2006; Kampkötter

et al., 2008). The authors attributed these responses to the AOC and to the ability of polyphenolic compounds to reduce the accumulation of intracellular ROS (Kampkötter et al., 2007). Xue et al. (2011) studied the impact of quercetin and rutin on *C. elegans* lifespan and showed that quercetin derivatives extended the worm's life by 12 to 20%. The mechanisms involved in the extension of lifespan by polyphenolic compounds are questionable. Some studies report that polyphenolic compounds are able to act as *in vivo* toxic pro-oxidants (Braeckman et al., 2002). Other studies associate the beneficial effect of polyphenolic compounds when used at lower dosages, to their ability to trigger light pro-oxidant mechanisms that in turn activate antioxidant defenses, leading to an overall cytoprotection (Halliwell, 2011).

# CHAPTER THREE:
# IMPACT OF DIFFERENT DISEASE CONTROL TREATMENTS ON ASCORBIC ACID, POLYPHENOLIC COMPOUNDS AND ANTIOXIDANT CAPACITY OF FLORIDA-GROWN STRAWBERRIES

## Introduction

Strawberries are an important crop to the Florida industry because of their high economic value and consumer demand. However, strawberry growers in central Florida face a major challenge when it comes to keeping fruit diseases and fruit rot as low as possible. This area of the country appears to be highly conducive for fungicide resistance development in plant pathogenic fungi because of the characteristic warm and humid climate enabling fungi to thrive and multiply. Therefore, in order to fight field fruit rots and avoid considerable crop losses, strawberry growers need to apply pesticides weekly throughout the season (Legard et al., 2001; Peres et al., 2010) which in turn may affect the overall quality of strawberries (Häkkinen and Törrönen, 2000; Magkos et al., 2003; Laura et al., 2009; Fernandes et al., 2012). Consequently, the U.S. Department of Agriculture has been directing agricultural research towards finding alternatives to conventional pesticide usage that are considered a potential environmental and health hazard (Kuchler et al., 1997). This is of special interest to the strawberry industry because growers apply ample amounts of pesticides weekly throughout the season to avoid fruit rot and crop losses which increase their production cost (Legard et al., 2001; Peres et al., 2010).

Poor post-harvest conditions (i.e., temperature and relative humidity) during storage have also shown to decrease the overall quality and shelf life of strawberry fruit (Nunes and Emond, 1999; Nunes et al., 2005; Moraga et al., 2006). Storage temperatures higher than 0°C during post-harvest storage greatly reduce strawberry quality, specifically the levels of fruit bioactive compounds. Even if kept at the optimum storage conditions, the post-harvest life of strawberries can be as short as seven to eight days (Mitcham, 2004; Nunes, 2008).

Pre- and post-harvest treatments, therefore, may have a major influence on the final “bioactive quality” of the fruit and its corresponding antioxidant capacity. However, it is still unclear if the sensory and compositional attributes are superior in organic strawberries compared to fruit grown under conventional or reduced pesticide disease control treatments. The controversial results are often attributed to the lack of direct comparative studies and to the great variability in the data available. Therefore, the objectives of this study were to: 1) determine the effect of repeated conventional, reduced or no fungicide applications (i.e., organic) on the overall quality of strawberry fruit, and specifically on the major bioactive compounds of strawberry namely, ascorbic acid, polyphenolic compounds and their AOC, and 2) the interaction between disease control treatments and cold storage on strawberry weight loss, and on the levels of ascorbic acid and polyphenolic compounds and their AOC.

## Materials and Methods

### Plant Material and Fungicide Treatments

‘Florida Radiance’ and ‘Strawberry Festival’ strawberry cultivars used in this study were grown under three different disease management conditions: conventional, reduced-fungicide using a disease forecasting system (Peres and MacKenzie, 2009) and organic. Strawberries grown conventionally or under a reduced-fungicide disease control treatment were harvested from commercial fields in Plant City and Floral City, respectively. ‘Strawberry Festival’ grown under organic conditions was obtained from a commercial field in Duette. ‘Florida Radiance’ strawberries grown under organic conditions were not available in Florida, therefore only organic ‘Strawberry Festival’ were evaluated against conventional and reduced-pesticide disease control treatments. The main commercial pesticides applied to the fruit were: Captan, Captec, QuiltXcel, Thiram, Switch, Elevate, Torino, and Fontelis. For conventionally grown strawberries, single or combinations of different pesticides were applied early in the season, during the bloom and late in the season, with up to 24 applications during the season. For reduced-pesticide fruit, fungicides were applied only when environmental conditions were favorable for disease. Organic strawberries were grown according to the USDA National Organic Program (NOP) guidelines.

### Postharvest Treatments

Strawberries from each cultivar and disease control treatment were harvested twice during the 2014 strawberry production season: ‘Florida Radiance’ from the two different disease control treatments (conventional and reduced-pesticide) were

harvested on January 21 (Harvest 1) and on February 18 (Harvest 2) and 'Strawberry Festival' from the three different disease control treatments (conventional, reduced-pesticide and organic) were harvested on February 7 (Harvest 1) and March 7 (Harvest 2). Fruit was brought to the Food Quality Laboratory at the University of South Florida in Tampa with minimal delay after harvest (30 min to 1 h, depending on the location of the field). Upon arrival to the laboratory, fruit were selected for uniformity of size, color and freedom of defects, carefully packed into 0.453 kg-clamshells (Wasserman Bag Co., Inc, New York, USA) and stored at 1.5°C and 85% RH inside temperature and RH-controlled chambers (Forma Environmental Chambers Model 3940 Series, Thermo Electron Corporation, OH, USA). These conditions simulated the lowest temperature and highest RH measured during strawberry handling (Nunes et al., 2009; Lai et al., 2011; Pelletier et al., 2011). Temperature was monitored throughout the study using Stow Away® XTI02 temperature loggers (-5 °C to +37 °C) (Onset Computer Corporation, Pocasset, Mass). RH was monitored with Stow Away® RH loggers (10 to 95% RH) (Onset Computer Corporation, Pocasset, Mass.).

**Visual Characteristics**

The fruit was evaluated at harvest and daily during a seven-day storage period. Visual subjective quality attributes were determined subjectively by internal and external appearance based on uniformity and intensity of red color. Pit size and appearance of the achenes were also evaluated subjectively.

### Weight Loss and Dry Weight

Weight loss of three replicated samples of 15 strawberries each was calculated from the initial weight of the fruit and every day during a seven-day storage period**.** Concentrations of chemical constituents were expressed in terms of dry weight in order to show the differences between cultivars and treatments that might be obscured by differences in water content. The following formula was used for water loss corrections: [chemical components (fresh weight) × 100 g / strawberry dry weight + weight loss during storage (g)]. Strawberry dry weight was determined by drying three weighed aliquots of homogenized strawberry tissue at 80 °C, and until weight stabilized.

### Total Ascorbic Acid Content

Total ascorbic acid content was quantified by mixing 2 g of homogenate with 20 mL metaphosphoric acid mixture (6% $HPO_3$ containing 2 N Acetic acid). Samples were then filtered (0.22 µm) prior to HPLC analysis. Ascorbic acid analysis was conducted using a Hitachi LaChromUltra UHPLC system with a diode array detector and a LaChromUltra C18 2µm column (2 × 50 mm) (Hitachi, Ltd., Tokyo, Japan). The analysis was performed under isocratic mode at a flow rate of 0.5 mL/min with a detection of 254 nm. Sample injection volume was 5 µL, each with duplicate HPLC injections. Mobile phase was buffered potassium phosphate monobasic ($KH_2PO_4$, 0.5%, w/v) at pH 2.5 with metaphosphoric acid ($HPO_3$, 0.1%, w/v). The retention time of the ascorbic acid peak was 2.5 min. After comparison of retention time with the ascorbic acid standard, the peak was identified. The amount of total ascorbic acid content in strawberry was quantified using calibration curves obtained from different concentrations (0.01 g $L^{-1}$,

0.02 g $L^{-1}$, 0.03 g $L^{-1}$, 0.05 g $L^{-1}$, 0.10 g $L^{-1}$, 0.15 g $L^{-1,}$ 0.20 g $L^{-1}$ and 0.30 g $L^{-1}$) of ascorbic acid standards. Total ascorbic acid content was expressed in terms of dry weight (g $kg^{-1}$) to compensate for water loss during storage.

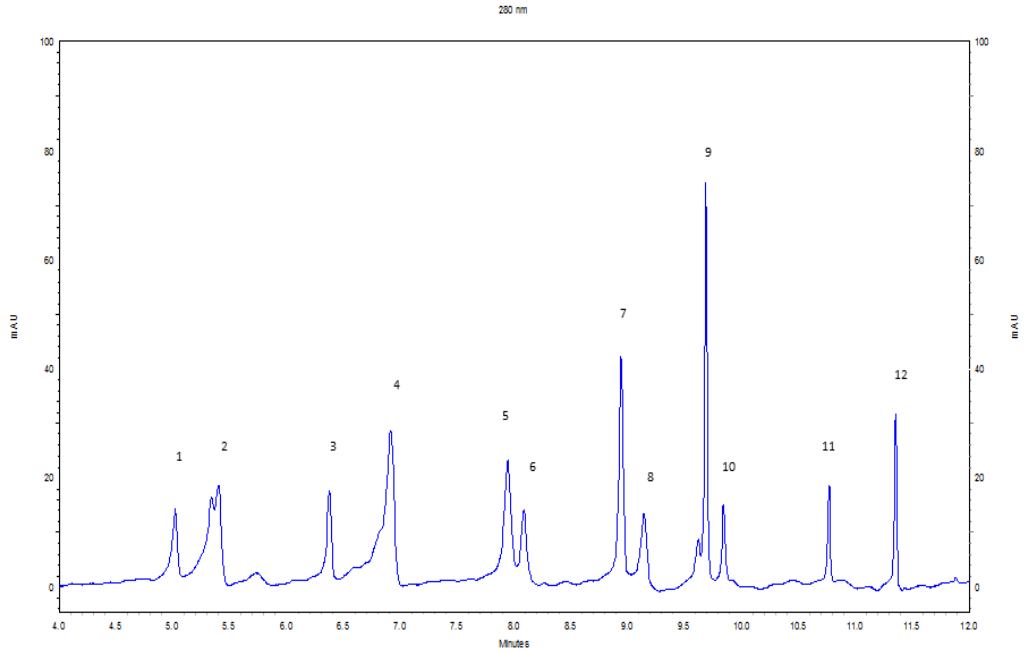


**Figure 1**. Identification of polyphenolic Standards at 280 nm. 1. Catechin 2.Chlorogenic acid 3. Caffeic Acid 4. Epicatechin 5. *p*-Coumaric Acid 6. Ferulic Acid 7. Ellagic Acid 8. Quercitin-3-glucoside 9. Kaempferol-3-glucoside 10. Myricetin 11. Quercitin 12. Kaempferol.

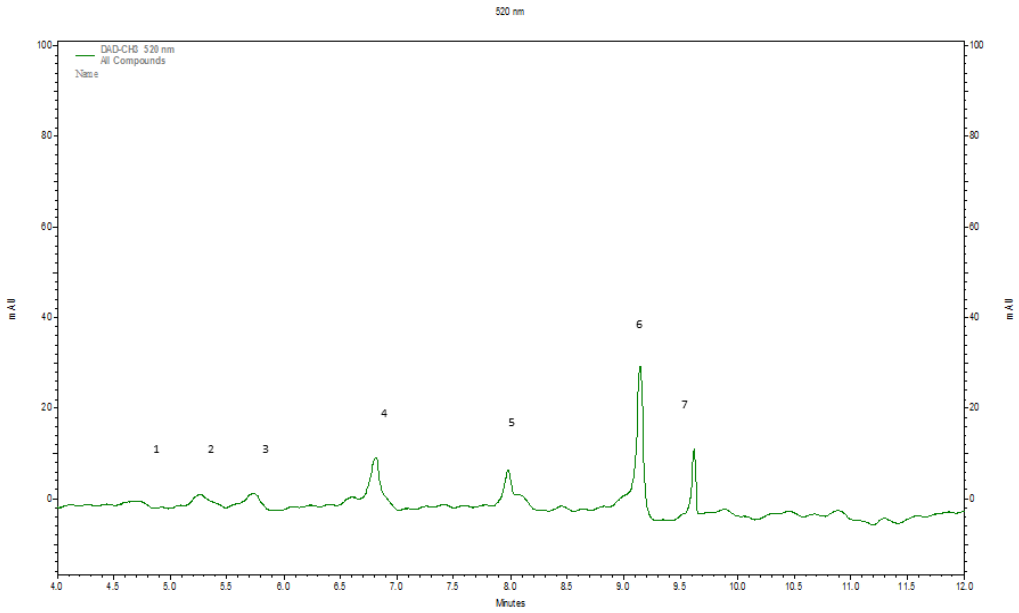


**Figure 2**. Identification of polyphenolic Standards at 520 nm. 1. Malvidin-3-glucoside 2.Cyanidin-3-glucoside 3. Delphinidin 4. Pelargonidin-3-glucoside 5. Cyanidin 6. Pelargonidin 7. Malvidin.

### Total Phenolic and Anthocyanin Contents

Total soluble phenolic compounds were measured using the Folin-Ciocalteau reagent as described by Nunes et al. (2005). Anthocyanins were extracted in 0.5% (v/v) HCl in methanol and measured using the procedure described by Nunes et al. (2005). The amount of total phenolic and anthocyanin were expressed in g $kg^{-1}$ on a dry weight basis.

### Polyphenolic Profiles

For polyphenol extraction, identification and quantification, frozen fruit puree (5 g) was blended with 15 mL of acetone using a Polytron, sonicated for 10 minutes and filtered through Whatman No.4 paper filter. The filtrate was concentrated to 5 mL in a rotary evaporator (Buchi Rotavapor R-114, Birkmann Instruments, Inc., USA) and

passed through a classic $C_{18}$ Sep-Pack cartridge (Waters Technologies Corp., USA) previously activated with methanol, followed by water and 3% formic acid. Acidified water (10 mL; 3% formic acid) was then passed through the cartridge to remove the sugars and the polyphenols were recovered by passing 1.8 mL of methanol containing 3% formic acid through the cartridge. The extract was finally filtered through a 0.20 µm syringe filter into auto-sampler vials and stored at -80°C until HPLC analysis.

Individual polyphenols were identified and quantified using the extracts prepared from fruit samples as described above. Polyphenol analysis was conducted using a Hitachi LaChroma Ultra system (Hitachi, Japan) with a diode array detector and a Hypersil Gold C18 1.9 µm, 100 × 2.1 mm column (Thermo Fisher Scientific Inc., USA). An isocratic solvent delivery of 0.5% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) was set at 0.6 µL/min with a detection of 280 nm (general polyphenols), and 520 nm (anthocyanins). Sample injection volume was 50 µL, each with duplicated HPLC injections. The retention time was set at 20 min. After comparison of retention times with those of polyphenolic standards, the peaks were identified at both 280nm (Fig. 1) and 520 nm (Fig. 2). Quantification of individual polyphenolic contents was based on surface area (%) of each individual peak.

**Antioxidant Capacity**

Antioxidant capacity was measured using the Trolox equivalents antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), and 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH) assays. The FRAP assay was conducted according to the method of Benzie and Strain (1996) with some

modifications. The reagent (160 µL) was mixed with 40 µL of sample in a 96 - well plate and then incubated at 37 °C for 30 min before measuring the absorbance at 593 nm. The DPPH assay was conducted according to the method of Brand-Williams et al. (1995) with some modifications. The reagent (950 µL) was mixed with 50 µL of sample and then incubated at room temperature for 1 hour in the dark. The absorbance of 200 µL of the mixture was read at 515nm. The TEAC assay was conducted according to the method of Arts et al. (2004) with some modifications. The diluted reagent (980 µL) was mixed with 20 µL of sample and the absorbance of 200 µL of the resulting mixture was read at 734 nm.

### Statistical Analysis

The Statistical Analysis System computer package (SAS Institute, Inc., 2004) was used for the analysis of the data from these experiments. The data was treated by two-way analysis of variance (ANOVA) with harvest, cultivar and disease control treatment as main effects. Significant differences between cultivars and disease control treatments were detected using the least significant difference (LSD) at the 5% level of significance.

## Results and Discussion

### Visual Characteristics

Significant differences were observed in the quality characteristics of strawberries from the different disease control treatments. The size of the fruit, external and internal flesh color, the pit size, and the appearance and density of the achenes in

conventional ‘Strawberry Festival’ were not significantly different from the reduced disease control treatment fruit (Fig 3). However, when compared to fruit from the conventional and reduced disease control treatments, organic ‘Strawberry Festival’ were smaller, had a darker internal flesh and a smaller pith size (Fig. 4). In addition, the achenes from organic ‘Strawberry Festival’ were denser (more achenes per surface area) and more protuberant towards the surface of the fruit.

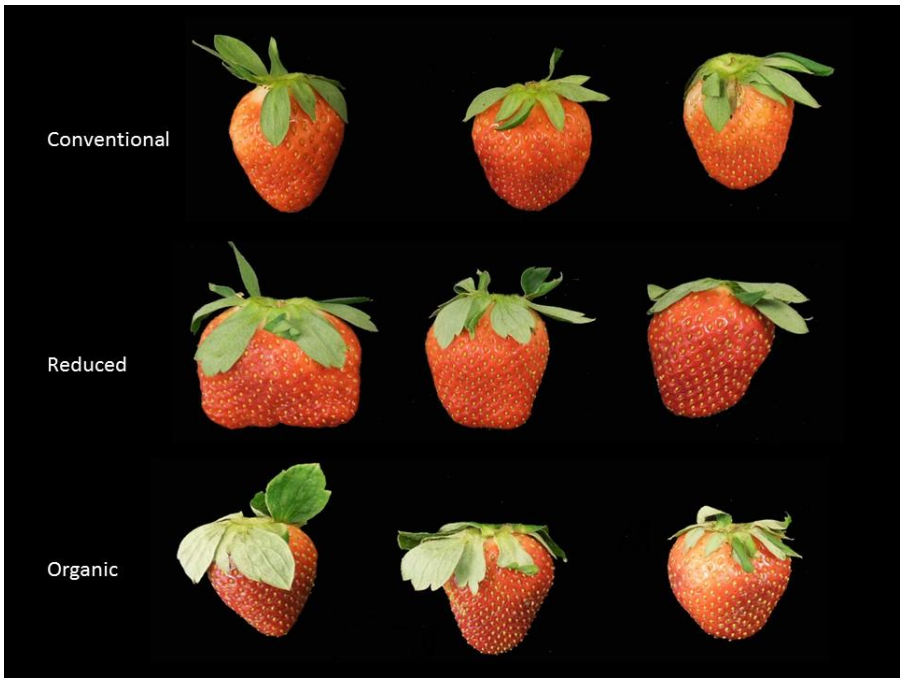


**Figure 3.** External appearance of ‘Strawberry Festival’ strawberries from different disease control treatments.

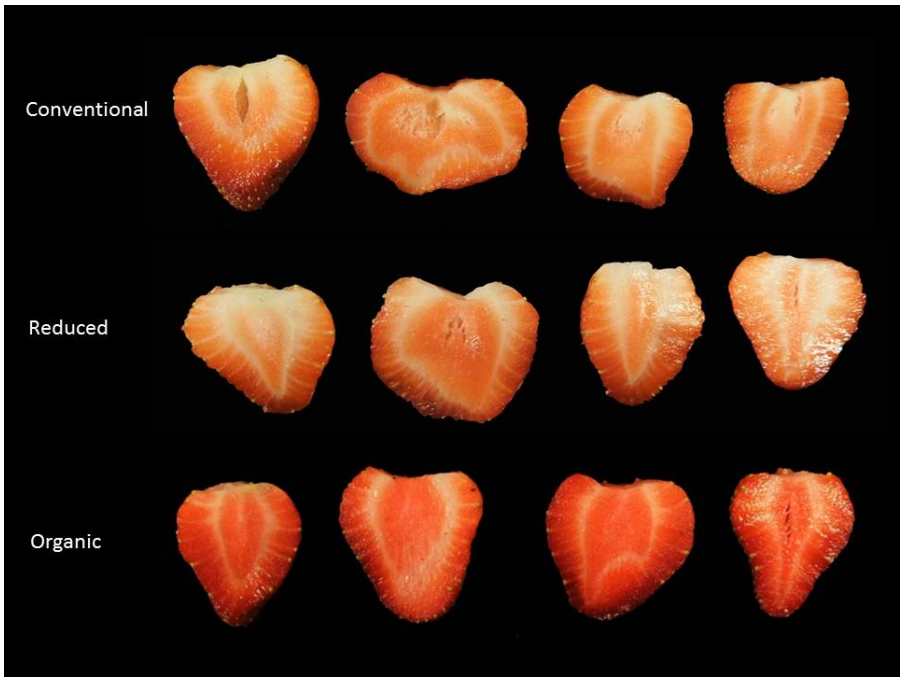


**Figure 4.** Internal appearance of ‘Strawberry Festival’ strawberries from different disease control treatments.

### Weight Loss

Weight loss is an important factor when evaluating postharvest strawberry quality because excessive loss of moisture results in accelerated quality deterioration and loss of economic value (Nunes and Emond, 2007). Most weight loss of stored fruit is caused by transpiration that results in strawberry fruit losing water and consequently their bioactive contents. Since bioactive compounds such as ascorbic acid and some polyphenolic compounds are water soluble, when weight loss of strawberry increases above a certain level (5% or more) the amounts of these compounds significantly decrease (Nunes and Dea, 2015).

In this study, there was a significant loss in weight of the fruit for both ‘Strawberry Festival’ and ‘Florida Radiance’ after seven days of storage, regardless of the treatment (Fig. 5). However, ‘Strawberry Festival’ showed on average a higher weight loss

compared to ‘Florida Radiance’ strawberries, regardless of the harvest or the disease control treatment used. In the first harvest, ‘Florida Radiance’ from the conventional and reduced disease control treatments showed on average 6% weight loss after seven days of storage, with no significant difference between treatments. On the other hand, after seven days of storage weight loss of ‘Strawberry Festival’ was significantly lower in fruit from the conventional treatment compared to that of fruit from the reduced treatment. Organic ‘Strawberry Festival’ showed significantly lower weight loss (7.9 %) compared to conventional ‘Strawberry Festival’ (9.4%) and reduced ‘Strawberry Festival’ (10.2%). In the second harvest, there was a less marked difference in the weight loss of fruit from the different treatments (Fig. 5). Nevertheless, weight loss of ‘Florida Radiance’ strawberry from the conventional treatment was significantly higher than that of fruit from reduced disease control treatment (9.1 and 7.7%, respectively). ‘Strawberry Festival’ from the reduced disease control treatment showed the lowest weight loss (7.5%) compared to the conventional and organic treatments (8.9 and 9.1%, respectively). On average, fruit from the reduced pesticide treatment tended to have similar or lower weight loss than that of strawberry from the conventional disease control treatment.

Differences in weight loss between cultivars and disease control treatments may be a result from variations in the morphological characteristics of the fruit. For example, the thickness of the skin may determine the amount of moisture lost during storage. That is, the thicker the skin of the fruit the lower the loss of moisture and thus the lower the weight loss (Nunes and Emond, 2007).

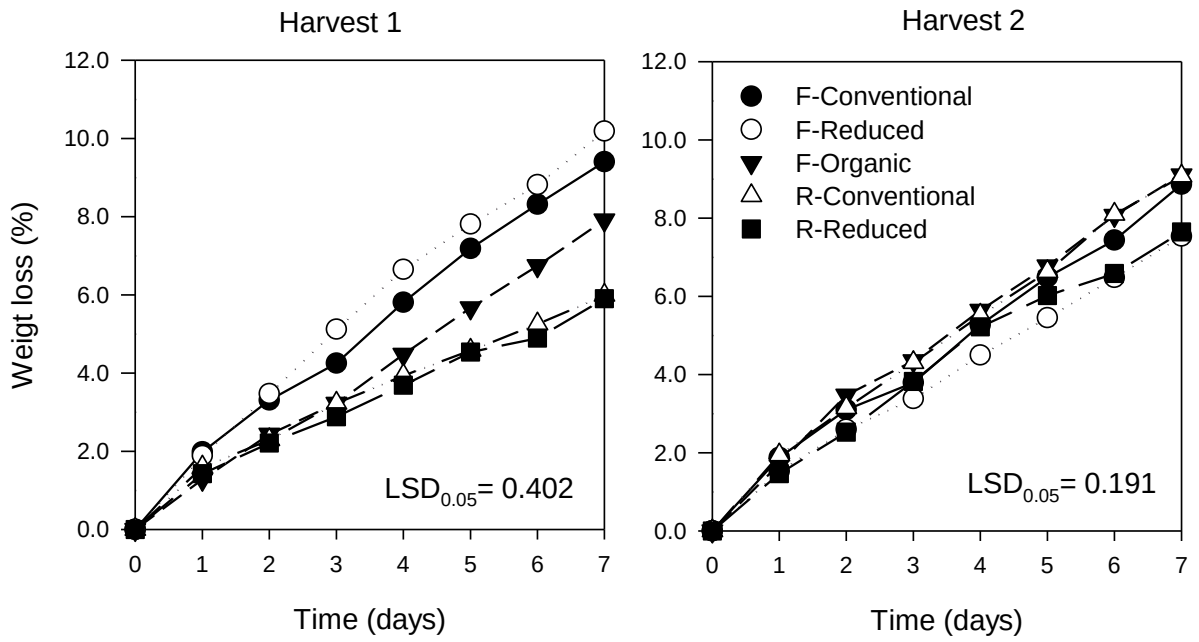


**Figure 5.** Changes in weight loss of 'Strawberry Festival' and 'Florida Radiance' strawberries from different disease control treatments during storage at 1.5°C and 85% RH. F = 'Strawberry Festival'. R = 'Florida Radiance'.

**Total Ascorbic Acid Content**

Unlike Pincemail et al. (2012) who reported that difference strawberry cultivars can differ significantly in their initial AA contents (51.0 to 184.7 mg/100g$^{-1}$ fruit fresh weight), AA content of strawberry cultivars from different disease control treatments used in this study was similar, ranging from 44.6 to 46.7 mg/100g$^{-1}$ fruit fresh weight. The data reported in the literature is somehow controversial in regards to the AA content of organic versus conventional fruit. For example, a significantly higher AA content has also been previously reported for organic (86.4 mg 100 g$^{-1}$ fruit fresh weight) versus conventional (71.2 mg 100 g$^{-1}$ fruit fresh weight) strawberry fruit (Abu-Zahra et al., 2007; Crecento-Campo et al., 2012). Cardoso et al. (2011) reported that AA content was significantly higher in conventionally grown strawberries than in organic

fruit whereas Häkkinen and Törrönen (2000) reported no difference in AA of organic versus conventional fruit.

In the present study, ‘Strawberry Festival’ from the second harvest and from the reduced pesticide treatment had higher AA content at harvest (557.6 mg $100g^{-1}$ fruit dry weight) compared to conventional fruit (Fig. 6). In addition, the levels of AA in ‘Strawberry Festival’ from the reduced disease control treatment were not significantly different compared to ‘Florida Radiance’ strawberry from the conventional disease control treatment; but were higher (516.1 mg $100g^{-1}$) than in ‘Florida Radiance’ strawberry from the reduced disease control treatment.

During storage, AA of strawberries significantly decreased, regardless of the cultivar, disease control treatment and date of harvest (Fig. 6). In the first harvest, there was on average a 59.6% decrease in the AA of ‘Florida Radiance’ strawberries from the reduced pesticide treatment, followed by a 57.8 and 52.1% decrease in ‘Florida Radiance’ and ‘Strawberry Festival’ from the conventional and organic treatments, respectively. Although at harvest organic ‘Strawberry Festival’ showed significantly higher AA content (632.7 mg $100g^{-1}$ fruit dry weight) when compared to the other treatments, after seven days of cold storage the AA levels were comparable to that of fruit from the other disease control treatments (Fig. 4). In fact, after storage, AA content of organic ‘Strawberry Festival’ was not significantly different from that of conventional or reduced ‘Florida Radiance’.

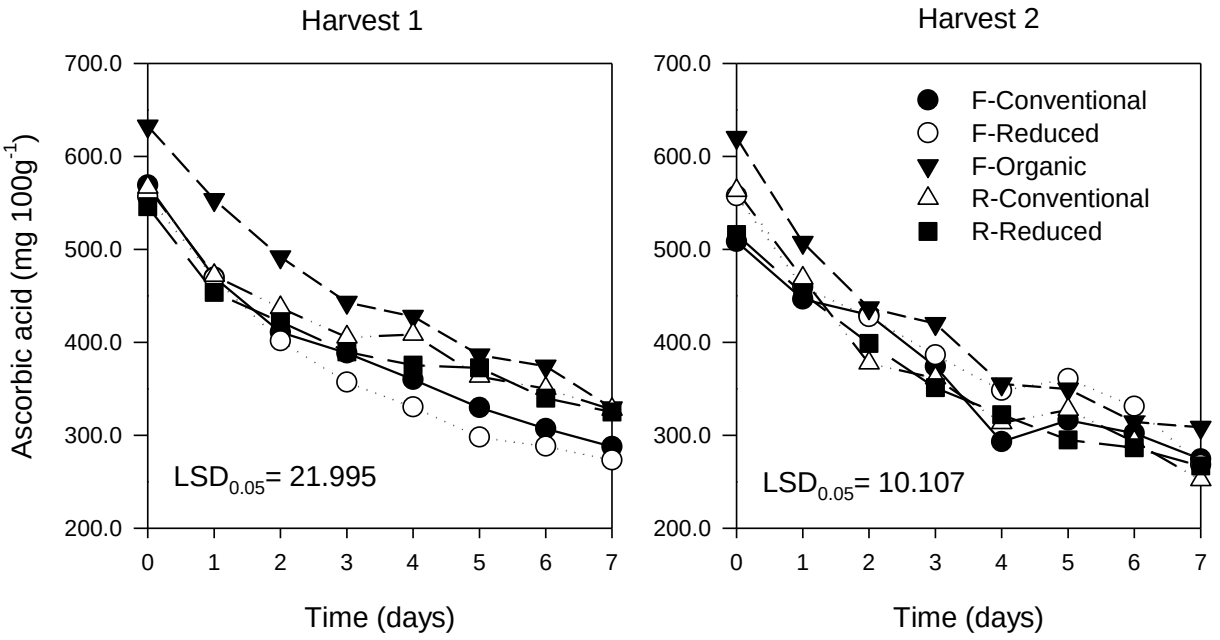


**Figure 6.** Changes in ascorbic acid content of 'Strawberry Festival' and 'Florida Radiance' strawberries from different disease control treatments during storage at 1.5°C and 85% RH. F = 'Strawberry Festival'. R = 'Florida Radiance'.

In the second harvest, there was on average a 53.9% decrease in the AA content of conventional Strawberry Festival', followed by a 51.8 and 49.8% decrease in 'Florida Radiance' and 'Strawberry Festival' fruit from the reduced and organic disease control treatments', respectively (Fig. 6). In addition, there was a similar trend with organic 'Strawberry Festival' showing significantly higher AA content (620.5 mg $100g^{-1}$ fruit dry weight) when compared to fruit from the conventional or reduced pesticide treatments, at harvest and after seven days of cold storage (308.7 mg $100g^{-1}$ fruit dry weight). Overall, after seven days of cold storage AA values were on average reduced by approximately 50%, with AA levels decreasing by similar amounts in fruit from the conventional and reduced disease control treatments. These results are in agreement with previously published data where AA in strawberries showed to be readily oxidized

when fruit was exposed to elevated temperatures and increased storage times (Torregrosa et al., 2006; Shin et al., 2007).

**Total Phenolic Content**

In the first harvest, organic 'Strawberry Festival' showed significantly higher total phenolic content (TPC) at harvest (2852.78 mg 100 $g^{-1}$ fruit dry weight) than fruit from any other treatment (Fig. 7). However, no significant difference in TPC was found between conventional or reduced 'Strawberry Radiance' and 'Strawberry Festival'. Results from the present study are in agreement with previously published data, where organic strawberries contained significantly higher TPC than conventional fruit (Asami et al., 2003; Crecente-Campo et al., 2012). The higher TPC in organic fruit could be related to an increased activity of the enzyme PAL in response to environmental stress (i.e., less protection from pesticide applications) which in turn results in the synthesis of secondary plant metabolites (Manach et al., 2004). These secondary plant metabolites may consist of a wide range of polyphenolic compounds including but not limited to hydroxycinnamic acids, phenolic acids, and flavonoid compounds. These polyphenolic compounds are synthesized in larger quantities in organic fruit since this type of growing method requires the synthesis of more compounds that are capable of acting as a defense mechanism for the plant since it does not have an extra layer of protection from pesticide applications.

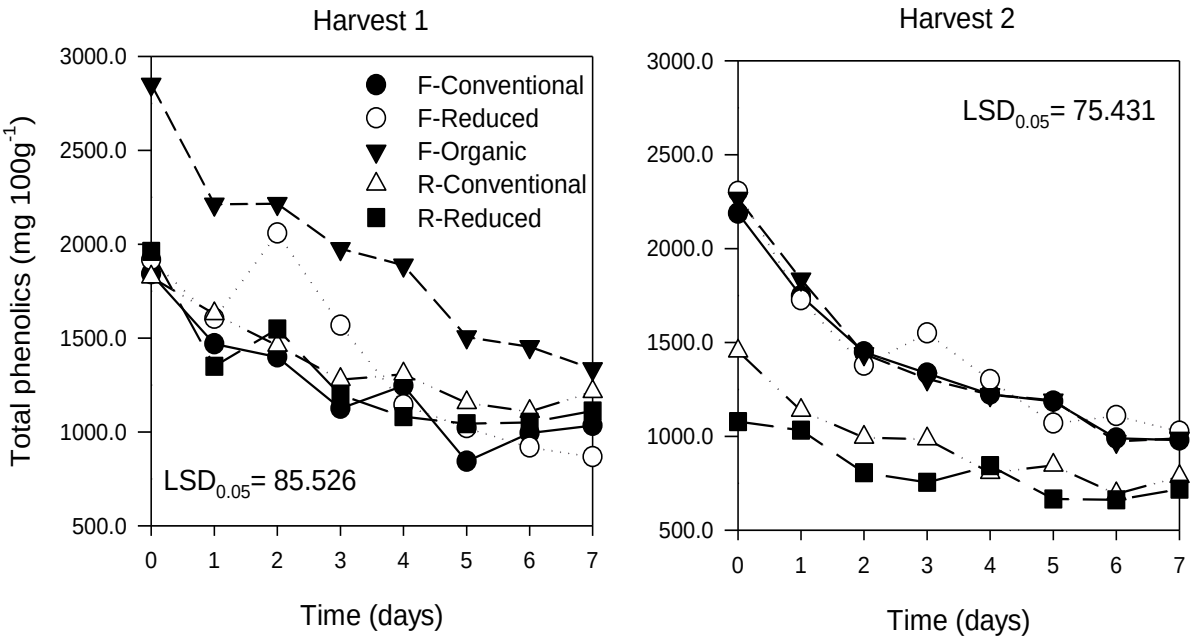


**Figure 7.** Changes in total phenolic contents of 'Strawberry Festival' and 'Florida Radiance' strawberries from different disease control treatments during storage at 1.5°C and 85% RH. F = 'Strawberry Festival'. R = 'Florida Radiance'.

In the second harvest, 'Strawberry Festival' strawberries from the conventional and reduced treatments showed at harvest the highest TPC (2303.9 and 2268.3 mg 100 $g^{-1}$ fruit dry weight, respectively). No significant difference in TPC between conventional and organic strawberries was found which is supported by previously published data (Häkkinen and Törrönen 2000). Although the fruit used in this study was harvested from the same area and sorted by color, the variations in TPC at harvest can be related to differences in strawberry cultivars, maturity at harvest as reported previously (Hakala et al., 2003). Conventional and reduced 'Strawberry Radiance' also showed a significantly lower TPC at harvest (1453.7 and 1079.2 mg 100 $g^{-1}$ fruit dry weight, respectively) when compared to 'Strawberry Festival' from the conventional and reduced disease control treatments (2188.6 and 2304.0 mg 100 $g^{-1}$ fruit dry weight, respectively).

After seven day storage, TPC decreased significantly for all cultivars, regardless of the disease control treatment used (Fig. 7). In the first harvest, there was on average a 56.1% decrease in TPC of conventional 'Strawberry Festival', followed by a 46.8 and 45.3% decrease in fruit from the organic and reduced treatments, respectively. This drastic change in TPC was most likely due to the significant loss of weight (i.e., water loss) during storage which may have contributed to an increased oxidation of polyphenolic compounds (Nunes et al., 2005). For organic strawberries, after seven days of storage, the TPC levels were within the range of the 'Florida Radiance' strawberries. All strawberry cultivars from the first harvest showed on average a 54.3% decrease in TPC after seven days of cold storage, regardless of the treatment. Although the TPC in 'Strawberry Festival' and 'Florida Radiance' strawberries from the conventional and reduced treatments were not significantly different at harvest, significant differences were observed between these two cultivars after seven days of cold storage.

For the second harvest, strawberries experienced on average a 50.7% decrease in TPC after seven days which agrees with results from previous studies (Nunes et al., 2005; Shin et al., 2007). After seven days of storage, conventional and reduced 'Strawberry Radiance' showed a significantly lower TPC (786.1 and 719.5 mg 100 $g^{-1}$ fruit dry weight, respectively) compared to 'Strawberry Festival'. There was no significant difference in TPC levels between 'Strawberry Festival' and 'Strawberry Radiance' from the conventional and reduced treatments. Organic 'Strawberry Festival' strawberries had similar TPC compared to fruit from conventional and reduced treatments after seven days of storage.

**Total Anthocyanin Content**

In the first harvest, strawberry cultivars had different total anthocyanin contents at harvest ranging from a minimum of 88.7 mg 100 $g^{-1}$ fruit fresh weight in conventional 'Strawberry Festival' to a maximum of 157.8 mg 100 $g^{-1}$ fruit fresh weight in conventional 'Florida Radiance' strawberry (Fig 8). Although Crecento-Campo et al. (2012) reported a higher anthocyanin content in organic strawberries than in conventional (193.0 versus 98.1 mg 100 $g^{-1}$ fruit fresh weight), in the present study organic strawberries from the first harvest had lower anthocyanin contents (132.4 mg 100 $g^{-1}$) than 'Florida Radiance' strawberries from the conventional treatment (157.8 mg 100 $g^{-1}$). Unlike in the first harvest, in the second harvest, total anthocyanin content at harvest, ranged from a minimum of 148.6 mg 100 $g^{-1}$ fruit fresh weight in 'Florida Radiance' strawberries from the reduced pesticide treatment to a maximum of 249.7 mg 100 $g^{-1}$ fruit fresh weight in organic 'Strawberry Festival' which agrees with results reported by Crecente-Campo et al. (2012). In addition, even though anthocyanin content has been shown to be higher in organic strawberries compared to conventional fruit (Reganold et.al., 2010), organic 'Strawberry Festival' from the first harvest had at harvest significantly lower anthocyanin contents than 'Florida Radiance' from the conventional disease control treatment (132.44 and 157.81 mg 100 $g^{-1}$ fruit fresh weight, respectively).

Overall, anthocyanin content decreased during cold storage regardless of the cultivar, disease control treatment or date of harvest (Fig. 8). In the first harvest, the total anthocyanin content fluctuated across cultivars and disease control treatments. A similar pattern was previously reported for strawberries and was suggested that the

production of secondary metabolites, that may lead to an increase or decrease of anthocyanins and/or phenolics (Jin et al., 2011; Ayala-Zavalaa et al., 2004) can be caused by specific antioxidant enzymes such as catalase and superoxide dismutase which activity was higher in strawberries stored at 0 or 5°C versus 10°C (Jin et al., 2011).

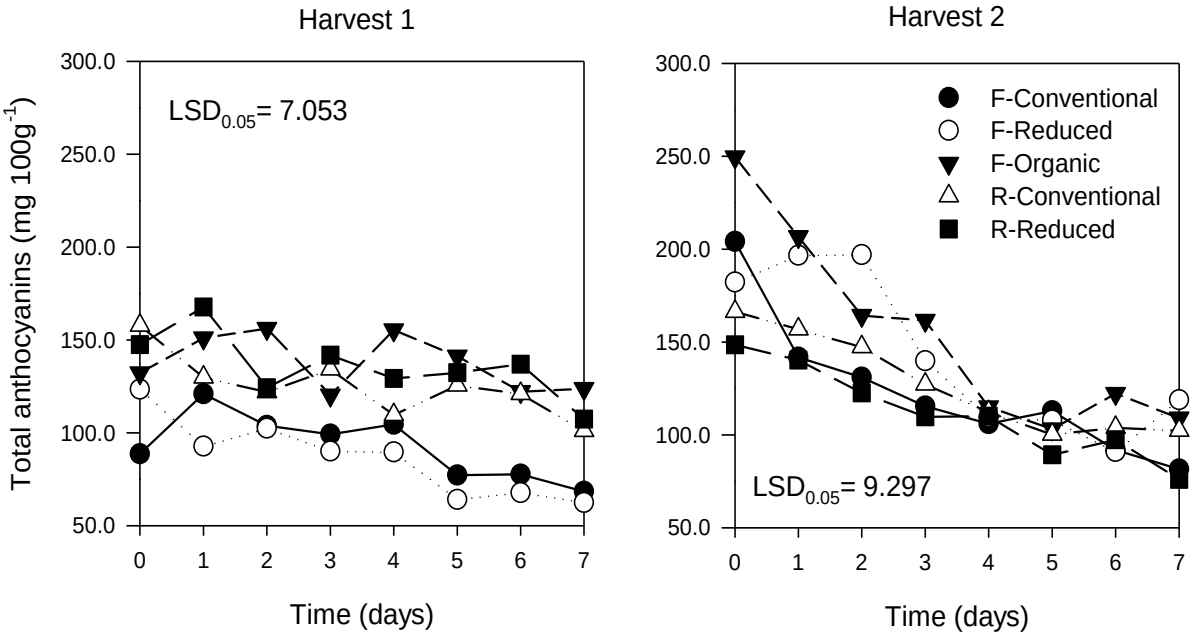


**Figure 8.** Changes in total anthocyanin contents of 'Strawberry Festival' and 'Florida Radiance' strawberries from different disease control treatments during storage at 1.5°C and 85% RH. F = 'Strawberry Festival'. R = 'Florida Radiance'.

In the present study, there was a significant decrease in anthocyanin content in fruit from both the conventional and reduced pesticide treatments for both cultivars stored for seven days at 1.5°C (Fig. 8). The anthocyanin content of organic 'Strawberry Festival' remained relatively stable throughout storage showing the smallest decrease (6.4%) after seven days, compared to the fruit from the conventional (22.9%) and reduced pesticide treatments (49.5%). For 'Florida Radiance', the decrease in the

anthocyanin content in fruit from the conventional treatment was higher (35.9%) compared to the decrease in fruit from the reduced pesticide treatment (27.2%). Although at harvest fruit from the reduced pesticide treatment had a lower anthocyanin content (147.7 mg 100 $g^{-1}$ fruit fresh weight) compared to fruit from the conventional treatment (157.8 mg 100 $g^{-1}$ fruit fresh weight), the decrease during cold storage was significantly higher in conventional fruit. In the second harvest, anthocyanin content also fluctuated throughout storage with a significant decrease after seven days of storage, regardless of the cultivar and disease control treatment (Fig. 8). Organic 'Strawberry Festival' experienced a significant decrease (56.3%) during storage when compared to fruit from the conventional (60.0%) and reduced (34.7%) treatments. ‘Florida Radiance’ from the conventional treatment showed a smaller decrease (38.4%) in anthocyanin content during storage compared to reduced ‘Florida Radiance' strawberry (48.8%). Fruit from the reduced pesticide treatment tended to have similar or significantly higher anthocyanin content compared to the conventional fruit whereas organic fruit had consistently higher anthocyanin contents compared to the other treatments after seven days at cold storage (Fig. 8). Overall, losses in anthocyanin content ranged from 6.4% for organic 'Strawberry Festival' from the first harvest to 60.0% for conventional 'Strawberry Festival' from the second harvest.

**Polyphenolic Profiles**

On the day of harvest, the major polyphenolic compounds identified from strawberries, regardless of the cultivar and disease control treatment, were pelargonidin-3-glucoside followed by quercetin and quercetin-3-glucoside (Fig. 7).

These results are in agreement with previously published studies where pelargonidin-3-glucoside was the major polyphenolic compound identified from strawberry tissue (Lopes da Silva et al., 2002). ‘Strawberry Festival’ fruit from the reduced pesticide treatment showed significantly higher pelargonidin-3-glucoside content (65.1%) than fruit from the conventional (60.0%) or organic (63.4%) diseases control treatments (Fig. 9). Significant differences in the polyphenolic profiles of strawberries from conventional and organic production was also reported by Jin et al. (2011) where pelargonidin-3-glucoside was found in significantly higher amounts in organic strawberries when compared to conventional fruit. The levels of quercetin-3-glucoside in ‘Strawberry Festival’ from the reduced disease control treatment (7.8%) was significantly higher than that of conventional and organic fruit (5.6% and 5.8%, respectively). The ferulic acid content showed the same pattern, where ‘Strawberry Festival’ from the reduced treatment contained significantly higher amount (6.5%) than conventional (5.0%) and organic strawberries (4.8%). On the other hand, organic ‘Strawberry Festival’ had significantly higher kaempferol-3-glucoside content (6.9%) than fruit from the conventional (4.1%) and reduced (4.2%) treatments. All other polyphenolic compounds identified were present in significantly lower amounts, regardless of the treatment. There was not a significant difference in the levels of catechin, cyanidin, epicatechin, kaempferol, caffeic acid, p-coumaric acid and ellagic acid, which were identified in minor amounts, between treatments.

Overall, the major polyphenolic compounds identified in highest concentrations from strawberries from different cultivars and disease control treatment belonged to the flavonoid family whereas phenolic acids and hydrolysable tannins were measured at

much lower concentrations. Results from this study are in agreement with previously published data (Lopes da Silva et al., 2002; Aaby et al., 2007; Aaby et al., 2012). In addition, the significant higher amounts of pelargonidin-3-glucoside, quercetin-3-glucoside and ferulic acid in 'Strawberry Festival' from the reduced treatment may possibly be related to an optimal level of pesticide applied that in turn protects the strawberry just enough from disease while enhancing the production and accumulation of polyphenolic compounds.

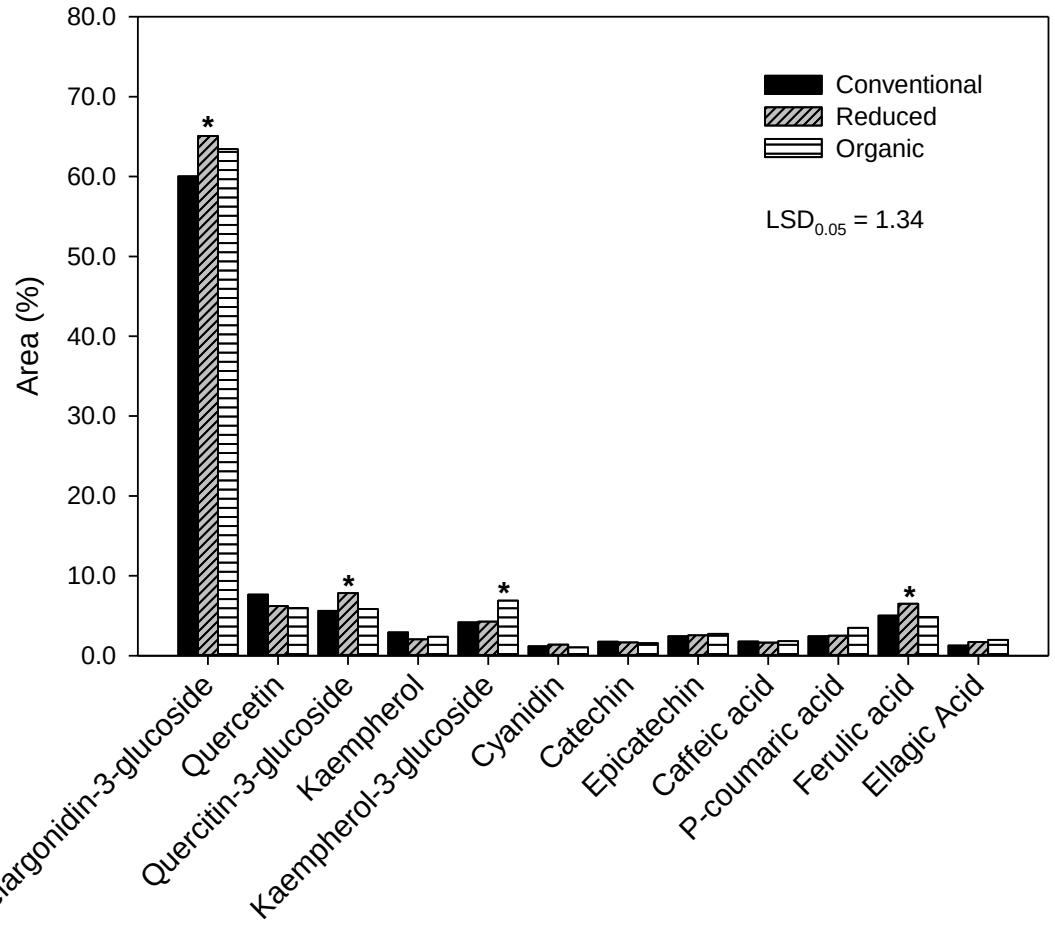


**Figure 9.** Polyphenolic profile for 'Strawberry Festival' from different diseases control treatments.

**Antioxidant Capacity**

In the first harvest, organic ‘Strawberry Festival’ showed consistently higher FRAP, DPPH and TEAC values when compared to the other treatments (Fig. 10). These results are in agreement with previously published studies, where TEAC was higher in organic strawberries when compared to conventional fruit (Reganold et. al., 2010). In addition, Jin et al. (2011) reported that organic strawberries have a significantly higher activity of antioxidant enzymes and AOC when compared to conventional fruit. ‘Strawberry Festival’ from the reduced pesticide treatment had, in general, similar or slightly lower FRAP, DPPH and TEAC values compared to conventional fruit which was also true for ‘Strawberry Radiance’. In the second harvest, there was a similar trend, with organic ‘Strawberry Festival’ showing consistently higher FRAP, DPPH and TEAC values compared to the conventional or reduced pesticide fruit (Fig. 10). In addition, ‘Strawberry Festival’ from the reduced pesticide treatment had, in general, similar or slightly lower FRAP, DPPH and TEAC values compared to conventional fruit which was also true for ‘Strawberry Radiance’.

The AOC of strawberries significantly decreased during storage, regardless of the cultivar, disease control treatment and date of harvest (Fig. 10). In addition, the different AOC assays used showed similar decreasing trends, regardless of the cultivar, disease control treatment and date of harvest. In the first harvest, there was on average a 57.9% decrease in the AOC in conventional ‘Strawberry Festival’, followed by a 56.3 and 52.1% decrease in fruit from the reduced and organic treatments, respectively. In general, there was no significant difference between the AOC of ‘Strawberry Festival’ from the conventional and reduced pesticide treatments. Similarly, conventional ‘Florida

Radiance' showed on average a higher decrease in AOC after cold storage (46.8%) when compared to fruit from the reduced pesticide treatment (40.7%). Overall, there was no significant difference in the AOC of 'Florida Radiance' strawberries from the conventional and reduced pesticide treatment. For the second harvest, 'Florida Radiance' strawberries from the conventional treatment showed on average a smaller decrease in AOC after seven days of cold storage compared to the reduced pesticide fruit (57.1 and 42.2%, respectively). However, there was no significant difference in the FRAP, DPPH and TEAC values between 'Florida Radiance' strawberries from the conventional or reduced pesticide treatments (Fig. 8). In addition, 'Strawberry Festival' from the organic treatment showed on average the least decrease in AOC throughout storage (52.9%) when compared to conventional (55.4%) and reduced pesticide fruit (56.1%).

Overall, after seven days of cold storage AOC values were on average reduced by approximately 50% (Fig. 10). Previous studies showed that strawberry anthocyanins and AOC vary significantly among growing environments (Wang and Lin, 2003; Wang and Zheng, 2001) but during cold storage both experience the same decrease. However, the decrease in AOC did not positively correlate with the total anthocyanin content that fluctuated from day to day but did not consistently decrease throughout cold storage. On the other hand, TPC was positively correlated with the decrease in AOC, both showing approximately 50% decrease in initial values after seven days of cold storage. These results further suggest that even though anthocyanins make up the majority of the polyphenols in strawberries, other compounds such as vitamin C and phenolic acids may also contribute to the overall AOC.

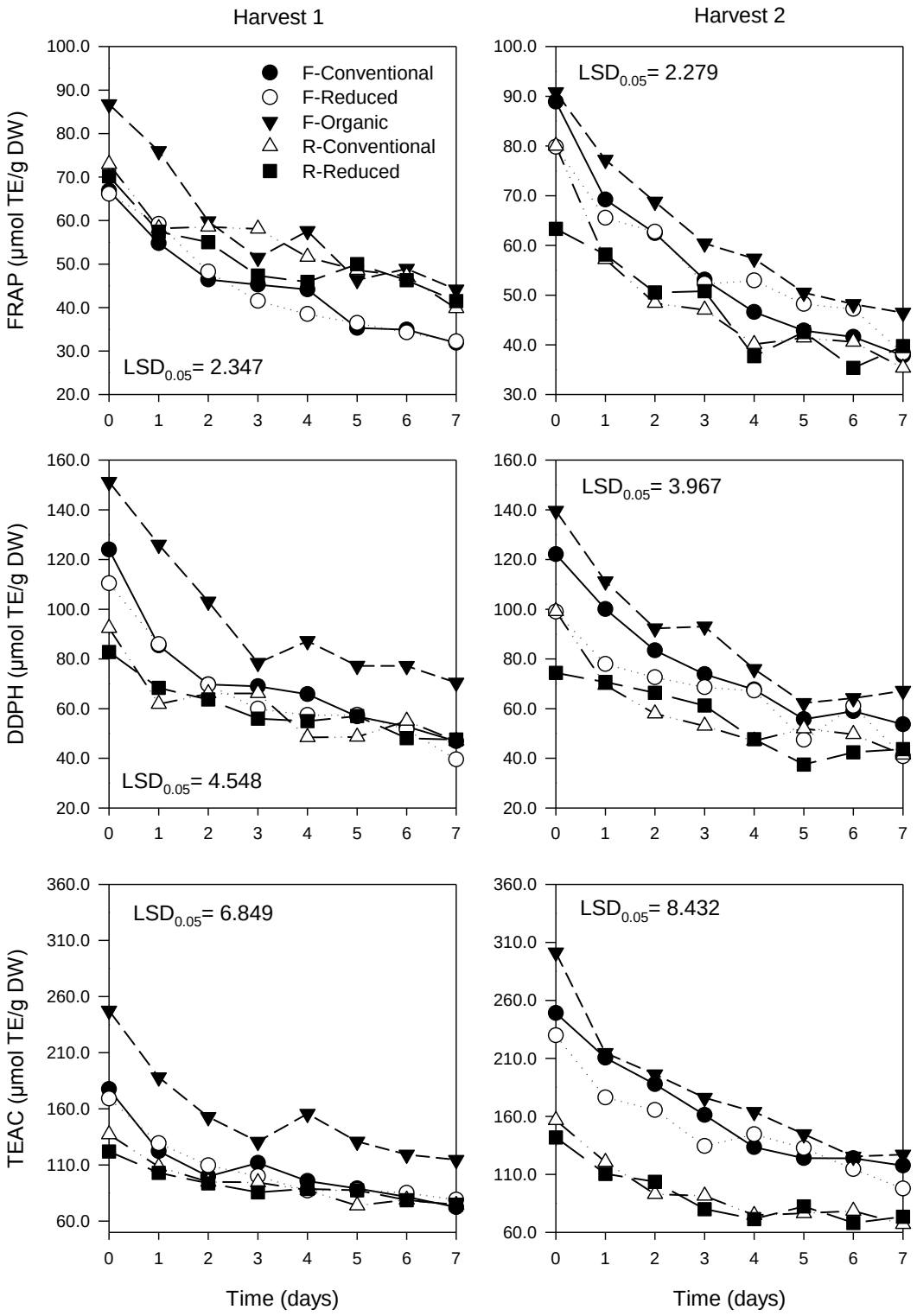


**Figure 10.** Changes in antioxidant capacity of 'Strawberry Festival' and 'Florida Radiance' strawberries from different disease control treatments during storage at 1.5°C and 85% RH. F = 'Strawberry Festival'. R = 'Florida Radiance'. TE = trolox equivalents.

## Conclusions

Results from this study showed that loss of weight during storage was cultivar dependent, as ‘Strawberry Festival’ tended to lose more weight during storage compared to ‘Florida Radiance’ strawberry. However, fruit from the reduced pesticide treatment tended to have on average similar or lower weight loss than strawberry from the conventional disease control treatment. Differences in weight loss between cultivars and disease control treatments may be a result from variations in the observed morphological characteristics of the fruit (i.e., fruit size, pith size or/and achene density). Results also showed that weight loss influenced significantly the levels of bioactive compounds in strawberries, regardless of the disease control treatments used. Although at harvest bioactive contents and AOC were significantly higher in organic fruit, after cold storage the differences between organic and reduced pesticide fruit were slight or non-significant. Furthermore, fruit from the reduced disease control treatment tended to have higher bioactive contents and higher AOC compared to conventional fruit. After storage, TPC decreased significantly for all cultivars with no differences between fruit from the conventional and the reduced treatments. As for anthocyanins, the conventional disease control treatment did not offer better protection from losses than the reduced treatment. The major polyphenol compounds found in strawberry fruit were flavonoids (i.e., pelargonidin-3-glucoside and quercetin-3-glucoside), regardless of the cultivar and disease control treatment used, and smaller amounts of non-flavonoid compounds were also detected. However, ‘Strawberry Festival’ from the reduced and organic disease control treatments had higher levels of pelargonidin-3-glucoside and quercetin-3-glucoside than fruit from the conventional disease control treatment.

Overall, strawberries from the reduced pesticide treatment, particularly ‘Florida Radiance’, showed a better or similar bioactive content and AOC than fruit from the conventional disease control treatment. After seven days of cold storage, both strawberry cultivars from the reduced or conventional disease control treatments showed comparable results for bioactive compounds and AOC. These results indicate that growing strawberries with reduced fungicide applications can be an alternative to conventional disease control or organic practices as it reduces fungicide residues and production costs while still retaining important bioactive compounds in the fruit.

# CHAPTER FOUR:
# ASCORBIC ACID AND MAJOR POLYPHENOLIC COMPOUNDS IN STRAWBERRIES AND FRUIT JUICES AND COMPOSITE ANTIOXIDANT CAPACITY

## Introduction

In the food industry, “antioxidant” foods and beverages have become increasingly popular because of their potential health benefits, attributed mostly to their bioactive compounds. Therefore, to attract consumers, companies design packages that include in their labels words such as "antioxidant", "polyphenol" and/or "superfruit". These words can be found on numerous beverages such as nutritional drinks, conventional juices, organic juices, smoothies and teas to make them stand out from competitors. However, the U.S. Food and Drug Administration (FDA) regulates the labeling of the word “antioxidant” and requirements include that the word can only be used for nutrients that have a Reference Daily Intake (RDI), recognized antioxidant activity and/or must be present as a Daily Reference Value (DRV) of 10% or higher (FDA, 2008). Therefore, since there is no established RDI, polyphenolic compounds found in foods and beverages cannot be used to claim antioxidant activity (FDA, 2008). Nevertheless, attributing the antioxidant activity (AOC) exclusively to vitamins (e.g., vitamin C) and, excluding the potential AOC of other compounds such as polyphenols, can mask the real antioxidant potential of a particular food or beverage.

There is current evidence that polyphenolic compounds not only are powerful radical scavengers but they are also involved in other biological mechanisms and are

thought to be more bioavailable when consumed in a beverage (Clifford, 2004; Saura-Calixto et al., 2007). Beverages, particularly fruit juices, also represent a good model to measure the AOC of polyphenols because of less interference from dietary fibers, lipids, and proteins that can be naturally found in other foods. Further, little data is available on the fate of polyphenolic compounds and AOC of fruit juices during traditional consumer storage. Finally, there is a need to determine the exact source of AOC in beverages and fruit juices, based on their individual polyphenolic profiles. Therefore, the objectives of this study were to: 1) determine the relationship between ascorbic acid, polyphenolic compounds and AOC in different types of beverages; 2) identify specific polyphenolic compounds in selected beverages, and 3) investigate the effect of consumer storage on total phenolic compounds and AOC of selected beverages.

## Materials and Methods

### Beverages

The beverages used in this study were purchased from three different major retail stores in Tampa, Florida, USA, during June and July 2014. A total of 56 beverages (3 samples each) including nine nutritional drink, 25 juices, seven organic juices, four smoothies and 11 teas were used in this study. Additionally, fresh juice from conventional and organic strawberries obtained from local farms in Florida was used as a control. The beverages were listed by category and the total volume of the sample used from each beverage is shown in parenthesis.

*Nutritional Drinks*: **N1** (591 mL), Vitamin Water XXX (The Coca Cola Company, Atlanta, GA); **N2** (591 mL), Vitamin Water XXX Zero (The Coca Cola Company, Atlanta,

GA); **N3** (591 mL), 365 Everyday Value Cranberry + Antioxidants (Whole Foods Market, Inc., Austin, TX); **N4** (591 mL), 365 Everyday Value Pomegranate + Polyphenols (Whole Foods Market, Inc., Austin, TX); **N5** (3 L), Propel Berry (PepsiCo Inc., Purchase, NY); **N6** (245 ml), Verve (Vemma Nutrition Company, Tempe, AZ); **N7** (245 ml), Verve Zero Sugar (Vemma Nutrition Company, Tempe, AZ) (245 ml); **N8** (960 mL), Vemma (Vemma Nutrition Company, Tempe, AZ); **N9** (245 ml), Vemma Renew Juice (Vemma Nutrition Company, Tempe, AZ).

*Fruit Juices*: **J1** (480 mL), POM Wonderful 100% Pomegranate (POM Wonderful LLC, Los Angeles, CA); **J2** (480 mL), POM Wonderful Blueberry (POM Wonderful LLC, Los Angeles, CA); **J3** (480 mL), Stanton Orchards Tart Cherry (Stanton Orchards, Leelanau, MI); **J4** (1.2 L), Welch's 100% Grape Juice (Welch Foods Inc., Concord, Massachusetts); **J5** (1.78 L), Ocean Spray Cranberry (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA); **J6** (1.78 L), Ocean Spray Diet Cranberry (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA); **J7** (976 mL), Sunsweet Prune Juice (Sunsweet Growers Inc., Yuba City, CA); **J8** (1.9 L), Northland Superfruits 100% Juice Blueberry Blackberry Acai Juice (Apple & Eve, Port Washington, NY) ; **J9** (500 mL), Fuze Slenderize Blueberry, Raspberry (The Coca Cola Company, Atlanta, GA); **J10** (960 mL), R.W. Knudson Cranberry (Knudsen *&* Sons*,* Inc., Chico, CA); **J11** (960 mL), R.W. Knudson Mega Antioxidant (Knudsen *&* Sons*,* Inc., Chico, CA); **J12** (960 mL), R.W. Knudson Just Aronia (Knudsen *&* Sons*,* Inc., Chico, CA); **J13** (355 mL), Tropicana Orange Juice (PepsiCo Inc., Purchase, NY); **J14** (1.75 L), Tropicana 50 Orange Juice (PepsiCo Inc., Purchase, NY); **J15** (480 mL), Trader Joe's Tart Cherry (Trader Joe's Company, Inc., Monrovia, CA); **J16** (960 mL), R.W. Knudson Cranberry Nectar

(Knudsen *&* Sons*,* Inc., Chico, CA); **J17** (960 mL), R.W. Knudson Just Blueberry (Knudsen *&* Sons*,* Inc., Chico, CA); **J18** (960 mL), R.W. Knudson Just Black Currant (Knudsen *&* Sons*,* Inc., Chico, CA); **J19** (960 mL), R.W. Knudson Mega C (Knudsen *&* Sons*,* Inc., Chico, CA; blend of apple, concorde grape and aronia juices); **J20** (960 mL), Lakewood Pure Pomegranate (Lakewood Juices Inc., Miami, FL); **J21** (1.75 L), Minute Maid Orange Juice (The Coca Cola Company, Atlanta, GA); **J22** (1.75 L), Minute Maid 50 Orange Juice (The Coca Cola Company, Atlanta, GA); **J23** (1.36 L), Tropicana Farmstand (PepsiCo Inc., Purchase, NY); **J24** (1.75 L), Tropicana 50 Pomegranate Blueberry (PepsiCo Inc., Purchase, NY); **J25** (1.75 L), Minute Maid Pomegranate Blueberry (The Coca Cola Company, Atlanta, GA).

*Organic Fruit Juices:* **O1** (960 mL), Sambazon Acai, Pomegranate, Blueberry (Sambazon Inc*.*, San Clemente, CA); **O2** (960 mL), Lakewood Pomegranate with Blueberry (Lakewood Juices Inc., Miami, FL); **O3** (960 mL), Lakewood Tart Cherry (Lakewood Juices Inc., Miami, FL); **O4** (960 mL), Lakewood Acai (Lakewood Juices Inc., Miami, FL); **O5** (960 mL), R.W. Knudson Organic Cranberry (Knudsen *&* Sons*,* Inc., Chico, CA); **O6** (960 mL), R.W. Knudson Acai Berry (Knudsen *&* Sons*,* Inc., Chico, CA); **O7** (1)(960 mL), Lakewood Cranberry (Lakewood Juices Inc., Miami, FL).

*Fruit Smoothies*: **S1** (480 mL), Naked Blue Machine (PepsiCo Inc., Purchase, NY); **S2** (480 mL), Bolthouse Farms (Campbell Soup Company Inc., Camden, NJ); **S3** (355 mL), Odwalla Blueberry B (The Coca Cola Company, Atlanta, GA); **S4** (960 mL), Trader Joe's Power Berry (Trader Joe's Company, Inc., Monrovia, CA).

*Teas*: **T1** (960 mL) Steaz Iced Green Tea Superfruit (The Healthy Beverage Company, Doylestown, PA); **T2** (960 mL), Steaz Iced Green Tea Blueberry

Pomegranate (The Healthy Beverage Company, Doylestown, PA); **T3** (960 mL), CelebriTea Pomegranate Blueberry (Celebrity Tea LLC, Tampa, FL) ; **T4** (960 mL), POM Wonderful Lychee Green Tea (POM Wonderful LLC, Los Angeles, CA); **T5** (960 mL), Bai 5 Pomegranate (Bai Brands LLC, Trenton, NJ); **T6** (960 mL), Bai 5 Blueberry (Bai Brands LLC, Trenton, NJ); **T7** (1 L), Yogi Super Antioxidant Green Tea (East West Tea Company LLC, Springfield, OR); **T8** (1 L), Trader Joe's Blueberry and Pomegranate Green Tea (Trader Joe's Company, Inc., Monrovia, CA); **T9** (1 L), Trader Joe's Green Tea (Trader Joe's Company, Inc., Monrovia, CA); **T10** (591 mL), Pure Leaf Sweet Tea (PepsiCo Inc., Purchase, NY); **T11** (591 mL), Pure Leaf Unsweet Tea (PepsiCo Inc., Purchase, NY).

*Fresh Strawberry Juice*: freshly harvested conventional (**J26**) and organic (**O8**) strawberries were obtained from commercial fields in Florida. Strawberries from each of the cultivation methods were harvested twice during the strawberry season in Florida (January through April 2014).

**Sample Preparation**

Samples for analysis were prepared by centrifuging triplicate samples of 20 mL of each beverage at 1600 $g_n$ for 20 min. The supernatant was then filtered through a two-layer Kimwipe and stored at -30 °C until use. Beverages that needed special preparation (i.e., dried teas or juice concentrates) were prepared following the instructions on the label. For dried teas, one tea bag was submerged into 240 mL of boiling water and soaked for three minutes. Juice concentrate (Stanton Orchards Tart Cherry) was prepared by mixing one part of juice concentrate with seven parts water.

Fresh strawberry juice was prepared using two replicated samples of 250 g of strawberries each. The calyces were removed, and the fruit was homogenized in a laboratory blender at high speed for 2 min and the resulting puree immediately frozen and kept at -30 °C until use. After thawing, the strawberry puree was again homogenized and centrifuged at 1600 $g_n$ for 20 min and then filtered through cotton cloth. The clear juice was used for analysis.

**Storage**

The impact of storage on the quality of the juices was determined only on fruit containing high levels of TPC. These juices were aronia, blackcurrant, and pomegranate. After purchase, bottles containing each of the three different juices were opened, samples were collected at day 0 and the remaining of the juice was kept at 4 °C for 14 days to simulate consumer storage at home. Samples were taken every two days and kept frozen at -30 °C until analysis.

**Total Ascorbic Acid, Total Phenolic Content, Polyphenolic Profiles and Antioxidant Activity**

Total ascorbic acid (AA), Total phenolic content (TPC), polyphenolic profiles and antioxidant activity (AOC) were determined using the methodology described previously (See Chapter Three: Material and Methods Section).

### Statistical Analysis

The Statistical Analysis System computer package (SAS Institute, Inc., 2004) was used for the analysis of the data. To determine the differences between beverages for each chemical attribute measured, the least significant difference (LSD) at the 5% significance level was used. LSD values were also used to compare the polyphenolic profiles of aronia, blackcurrant, and pomegranate juice, and for the comparison of changes in TPC and AOC after storage. For each beverage, the strength of the relationship between TPC or AA content and AOC was measured using the Pearson correlation coefficient ($r$) and the coefficient of determination ($r^2$) and, the significance of the relationship was expressed by probability levels ($p$ = 0.05).

## Results and Discussion

### Total Phenolic Compounds and Antioxidant Capacity

Ready-to-drink (RTD) beverages are complex mixtures containing water, sugar and various other ingredients that can contribute in different ways to the AOC of a beverage. For the beverages used in this study, total phenolic compounds (TPC) and total ascorbic acid (AA) were the main antioxidants measured. Nutritional drinks used in this study, in general, had low amounts of TPC with the exception of Vemma (N8) which had a significantly higher TPC compared to the other beverages in this category (Table 1). The TPC of fruit juices varied significantly, depending on the ingredients used to make the juice. For example, Minute Maid Orange Juice (J21) had the lowest TPC (77.2 mg 100 $mL^{-1}$) whereas R.W. Knudson Just Black Currant (J18) had the highest TPC (545. 3 mg 100 $mL^{-1}$). Other juices in this category with high TPC included R.W.

Knudson Just Aronia, followed by pure pomegranate (J20), POM Wonderful 100% Pomegranate, POM Wonderful Blueberry, Mega C (J19; contains a blend of apple, concorde grape, and aronia juices) and tart cherry (J15). Results from the present study are in agreement with those previously published by Seeram et al. (2008) where pomegranate juice also showed higher TPC than blueberry, cranberry, and orange juices.

Aronia (also known as chokeberry) and blackcurrant berries have been shown to contain major polyphenol compounds such as anthocyanins and proanthocyanidins (Denev et al., 2012; Wojdylo et al., 2013) thus these polyphenols most likely contributed to overall TPC of the beverages produced from these fruits. In addition, beverages containing pomegranate juice were also high in TPC probably due to the contribution of its major polyphenols, ellagitannins such as ellagic acid, gallic acid and punicalagins (Qu et al., 2012). Further, Lakewood Pure Pomegranate (J20) had a significantly higher TPC compared to POM Wonderful 100 % Pomegranate Juice (J1) due to the type of processing methods used in the production of POM Wonderful 100 % Pomegranate Juice. In fact, processing methods such as filtering or fining have been shown to lower TPC in pomegranate juices (Fischer et al., 2011). In addition, pomegranate juice showed significantly lower TPC than blackcurrant and aronia juice yet AOC values were not different when compared to the other two juices. These results suggest that polyphenolic compounds found in pomegranate juice may be more efficient at radical scavenging than polyphenols found in other beverages (Table 1). Besides, the TPC may also vary depending on which part of the fruit is used for juicing (Fischer et al., 2013). However, intrinsic properties of pomegranate juice (i.e., pH, chemical structure,

enzymes and co-pigments, metallic ions and sugars) in combination with the magnitude and duration of heating, storage temperature and time, and exposure to oxygen seem to have an influence on polyphenolic degradation (Patras et al., 2010). Even though Lakewood Pure Pomegranate (J20) showed higher TPC, higher anthocyanin degradation was observed in pomegranate juices after storage at 25 °C when compared to 5 °C (Vegara et al., 2013). Since all types of Lakewood and R.W. Knudson brand juices used in this study are usually commercialized at room temperature, one may expect that degradation in anthocyanins and other polyphenolic compounds are likely to occur from processing to consumption of the juice.

Among the organic juices evaluated, Lakewood Pomegranate with Blueberry (O2) had the highest TPC (184.9 mg 100 $mL^{-1}$) which was comparable to non-organic juices containing pomegranate and blueberry (J24 and J25). Lakewood Cranberry juice (O7) had the lowest TPC among the organic juices (75.8 mg 100 $mL^{-1}$) while non-organic juices containing cranberry showed similar (J5 and J16) or higher (J10) TPC. The differences between non-organic and organic juices containing similar ingredients may be due to several factors including formulation (i.e., water content) and processing techniques. Smoothies were, in general, low in TPC with the exception of Trader Joe's Power Berry (S4; 181.2 mg 100 $mL^{-1}$). Teas had variable TPC, with the highest value (99.0 mg 100 $mL^{-1}$) obtained for Trader Joe's Green Tea (T9). Green tea products contain mostly flavonoids, known as catechins, with (-) epigallocatechin-3-gallate representing about 50 to 80% of total catechin contents (Bansal et al., 2012).

Since strawberry was considered to be the most popular beverage flavor (Sloan, 2014) and because the fruit is considered an excellent source of vitamin C and

polyphenols, juice from fresh strawberries was used as a control. Strawberry juice from conventionally-grown fruit had similar TPC compared to Tropicana Orange Juice (J13) but significantly higher TPC than for example juices containing tart cherry (J3), cranberry (J5 and J16), superfruits (J8) and added antioxidants (J11). Strawberry juice from organic fruits had similar TPC compared to organic juice containing pomegranate and blueberry (O2) but significantly higher TPC when compared to strawberry from conventionally-grown fruit (J26) and to all other organic juices used in this study.

**Table 1**. Total phenolic content, total ascorbic acid content and antioxidant capacity of selected beverages.

| Beverage | TPC[a] (mg 100 $mL^{-1}$) | AA (mg 100 $mL^{-1}$) | FRAP (µmol TE[b] 100 $mL^{-1}$) | DPPH (µmol TE 100 $mL^{-1}$) | TEAC (µmol TE 100 $mL^{-1}$) |
|---|---|---|---|---|---|
| *Nutritional Drinks* | | | | | |
| N1 | ND[c] | 39.1 | 167.6 | 252.8 | 228.1 |
| N2 | ND | 41.2 | 193.1 | 368.3 | 258.6 |
| N3 | ND | 6.69 | 82.3 | 82.8 | 225.4 |
| N4 | ND | 5.61 | 75.9 | 62.5 | 219.8 |
| N5 | ND | 7.45 | 61.6 | 76.3 | 79.3 |
| N6 | 73.1 | 50.71 | 909.5 | 940.4 | 1097.7 |
| N7 | 72.9 | 120.9 | 961.8 | 1004.3 | 1049.1 |
| N8 | 229.3 | 129.6 | 1171.3 | 1082.3 | 1442.4 |
| N9 | 65.5 | 130.2 | 1001.9 | 1064.3 | 1188.8 |
| *$LSD_{0.05}$* | *3.7* | *3.3* | *89.4* | *90.7* | *225.1* |
| | | | | | |
| *Fruit Juices* | | | | | |
| J1 | 317.4 | ND | 1782.2 | 1719.6 | 2802.0 |
| J2 | 311.2 | ND | 1716.5 | 1441.4 | 2596.4 |
| J3 | 149.5 | ND | 437.1 | 527.2 | 460.5 |
| J4 | 218.0 | 34.9 | 859.1 | 778.4 | 1090.8 |
| J5 | 79.6 | 31.8 | 323.1 | 334.9 | 366.1 |
| J6 | ND | 32.1 | 240.5 | 253.9 | 269.8 |
| J7 | 226.1 | ND | 881.1 | 629.4 | 948.0 |
| J8 | 103.0 | 54.2 | 413.6 | 340.3 | 470.8 |
| J9 | ND | 60.7 | 267.3 | 325.6 | 325.8 |
| J10 | 176.2 | ND | 478.5 | 679.6 | 974.1 |
| J11 | 92.8 | 70.0 | 458.2 | 486.3 | 682.8 |
| J12 | 511.3 | ND | 3277.9 | 2983.3 | 4261.8 |
| J13 | 153.5 | 38.6 | 203.4 | 227.5 | 267.8 |
| J14 | 83.6 | 53.5 | 222.7 | 295.9 | 317.6 |
| J15 | 280.9 | ND | 906.3 | 942.5 | 1468.3 |
| J16 | 96.1 | ND | 261.8 | 342.0 | 306.5 |

Table 1 (continued)

| Beverage | TPC[a] (mg 100 mL$^{-1}$) | AA (mg 100 mL$^{-1}$) | FRAP (µmol TE[b] 100 mL$^{-1}$) | DPPH (µmol TE 100 mL$^{-1}$) | TEAC (µmol TE 100 mL$^{-1}$) |
|---|---|---|---|---|---|
| J17 | 220.0 | ND | 856.5 | 737.8 | 1180.0 |
| J18 | 545.3 | ND | 3140.4 | 2919.5 | 4139.6 |
| J19 | 294.5 | 460.6 | 2851.4 | 2558.0 | 3617.3 |
| J20 | 336.2 | ND | 3281.8 | 3138.3 | 4537.3 |
| J21 | 77.2 | 33.4 | 266.8 | 257.1 | 367.5 |
| J22 | 95.6 | 40.8 | 136.5 | 239.5 | 310.1 |
| J23 | 108.1 | 41.7 | 405.9 | 340.9 | 372.5 |
| J24 | 160.3 | 44.1 | 628.8 | 471.9 | 765.8 |
| J25 | 173.9 | 44.8 | 560.7 | 501.0 | 669.6 |
| J26 | 153.4 | 41.2 | 591.4 | 939.8 | 1618.2 |
| *$LSD_{0.05}$* | *5.2* | *7.4* | *68.9* | *145.4* | *359.4* |
| *Organic Fruit Juices* | | | | | |
| O1 | 153.7 | 76.01 | 667.8 | 906.6 | 824.0 |
| O2 | 184.9 | ND | 1260.2 | 1280.5 | 1352.9 |
| O3 | 169.3 | ND | 616.0 | 638.9 | 839.5 |
| O4 | 148.2 | ND | 563.4 | 438.2 | 591.3 |
| O5 | 146.0 | ND | 316.8 | 458.0 | 598.8 |
| O6 | 76.1 | ND | 189.5 | 198.2 | 242.7 |
| O7 | 75.8 | ND | 203.6 | 225.7 | 271.4 |
| O8 | 184.1 | 45.06 | 638.4 | 1045.6 | 1973.2 |
| *$LSD_{0.05}$* | *2.76* | *4.4* | *45.5* | *138.4* | *155.3* |
| *Fruit Smoothies* | | | | | |
| S1 | 70.1 | 26.2 | 323.4 | 294.7 | 370.3 |
| S2 | 71.1 | 27.8 | 333.7 | 259.4 | 339.7 |
| S3 | 75.8 | ND | 281.3 | 233.9 | 334.2 |
| S4 | 181.2 | ND | 749.8 | 711.8 | 972.0 |
| *$LSD_{0.05}$* | *3.3* | *1.2* | *37.8* | *90.2* | *149.1* |
| *Teas* | | | | | |
| T1 | ND | ND | 176.9 | 267.9 | 265.4 |
| T2 | ND | ND | 190.2 | 381.1 | 291.6 |
| T3 | 87.3 | ND | 426.1 | 521.4 | 700.3 |
| T4 | 93.3 | ND | 800.9 | 694.0 | 900.5 |
| T5 | ND | ND | 198.9 | 229.7 | 209.3 |
| T6 | ND | ND | 158.7 | 330.4 | 185.0 |
| T7 | 43.2 | ND | 280.9 | 331.1 | 363.3 |
| T8 | 88.6 | 7.1 | 457.2 | 541.1 | 801.6 |
| T9 | 99.0 | 4.3 | 496.8 | 594.2 | 896.6 |
| T10 | ND | ND | 198.0 | 322.8 | 269.4 |
| T11 | ND | ND | 189.0 | 302.8 | 286.2 |
| *$LSD_{0.05}$* | *8.5* | *0.6* | *14.4* | *121.9* | *108.2* |
| *$LSD_{0.05}$*[d] | *4.8* | *2.6* | *61.1* | *126.9* | *280.2* |

[a]TPC=Total Phenolic Content.
[b]TE = trolox equivalents.
[c]ND = Not detectable.
[d]LSD for all beverages, regardless of the category.

Chemical assays used to determine AOC of food and beverages give a good insight on how antioxidant vitamins and polyphenol compounds may act as in vivo radical scavengers (Lewandowska et al., 2016). Overall, the three different AOC assays used in this study gave similar results in terms of ranking the beverages according to their potential AOC (Table 1). AOC of nutritional drinks was considerably low except for all Vemma products (N6-N9). Vemma (N8) had the highest FRAP (1171.3 µmol TE 100 $mL^{-1}$) and TEAC (1442.4 µmol TE 100 $mL^{-1}$) values but the DPPH values obtained for Vemma (N8) were not significantly different from that of Verve Zero (N7) and Vemma Renew (N9). The fruit juice category contained the beverages with the highest AOC values overall, with pomegranate (J20) and aronia (J12) juices showing the highest values for both FRAP (3281.8 and 3277.9 µmol TE 100 $mL^{-1}$, respectively) and TEAC (4537.3 and 4261.8 µmol TE 100 $mL^{-1}$, respectively). However, DPPH values obtained for pomegranate juice (J20) were significantly higher (3138.3 µmol TE 100 $mL^{-1}$) than those obtained for all other juices. Results from the present study were similar to those previously reported by Seeram et al. (2008), where pomegranate juice also had higher FRAP, DPPH and TEAC values compared to grape, blueberry, cranberry and orange juices. The FRAP value of blackcurrant juice (J18; 3140.4 µmol TE 100 $mL^{-1}$) used in this study, was comparable to values previously reported for whole blackcurrant fruit (2993 ± 133 µmol TE 100 $mL^{-1}$) whereas other fruit juices such as pomegranate showed lower values than those previously published (Podsedek et al., 2014). The lowest FRAP values were obtained for orange juices (J22 and J13). DPPH and TEAC values were also low and not significantly different for beverages in the fruit juice category that contained either several different juice blends, orange juice or the least percentage of

juice reported in the label (Table 1). AOC of Pure Pomegranate (J20) was significantly higher than that of POM Wonderful (J1). These differences in AOC between juices containing 100% pomegranate juice were most likely due to the removal of ellagitannins during processing of POM Wonderful (J1). Because these polyphenolic compounds can contribute to an astringent taste, the juice is often processed to reduce the levels of ellagitannins (Quideau et al., 2011). However, gallic acid, ellagic acid and punicalagins which are also found in high quantities in POM Wonderful do not contribute to the astringent taste of the pomegranate beverage (Qu et al., 2012; Fischer et al., 2013). Furthermore, in the beverage POM Wonderful Blueberry (J2) the blend of 15 % blueberry juice with 85 % pomegranate juice did not contribute to differences in the FRAP or TEAC values. However, DPPH values were significantly higher in the 100% pomegranate juice (J1) compared to POM Wonderful Blueberry (Table 1).

AOC was also determined between products that were labeled with the same fruit name, for example, tart cherry products (J15, O3, J3), acai products (O4, O6), and cranberry products (J10, O5, O7). However, the AOC value could not be attributed to the fruit stated on the label since these beverages contained several other fruit concentrates that may have also influenced the total AOC of the juice blend (Somogyi, et al., 1996). The blending of juices during processing may cause an increase or decrease in desired nutrients (i.e., polyphenols and AA) and influence AOC accordingly.

Although juicing of strawberries has been shown to contribute to lower FRAP and TEAC values (Yvonne et al., 2005), FRAP and DPPH values of organic strawberry juice (O8) were similar to that of conventional strawberry juice (J26) but the TEAC values were higher in organic fruit juice. Conventional strawberry juice (J26) had similar FRAP

values compared to Tropicana 50 Pomegranate Blueberry (J25) but significantly higher AOC than products including 100 % orange juice (J13 and J21), tart cherry (J3), and cranberry (J5, J6, J10). In addition, conventional strawberry juice had similar DPPH and TEAC values to that of tart cherry (J15) but significantly higher AOC than grape juice (J3), prune juice (J7) and blueberry juice (J17). Organic strawberry juice (O8) had similar AOC when compared to beverages containing pomegranate and blueberry (O1, O2) and tart cherry (O3) but had significantly higher AOC values than products containing acai (O4, O6) and cranberry (O5, O7). When compared to conventional strawberry juice (J26), organic strawberry juice (O8) had similar FRAP and DPPH values but significantly higher TEAC values most likely due to their significantly higher TPC (Table 1).

Organic pomegranate with blueberry (O2) showed the highest values for FRAP (1260.2 µmol TE 100 $mL^{-1}$) and DPPH (1280.5 µmol TE 100 $mL^{-1}$) but organic strawberry juice (O8) had higher TEAC values (1973.2 µmol TE 100 $mL^{-1}$). Organic cranberry (O6) and organic acai berry (O7) juices had the lowest AOC amongst the organic juices. In the smoothie category, AOC of Power Berry (S4) was significantly higher than that of other smoothies. In the tea category, the POM Pomegranate Lychee Green Tea (T4) had significantly higher FRAP values (800.9 µmol TE 100 $mL^{-1}$) compared to other teas. However, DPPH or TEAC values were not significantly different than that of Trader Joe's Green Tea (T9) or Trader Joe's Blueberry and Pomegranate Green Tea (T8), probably due to their higher vitamin C content (7.1 and 4.3 mg 100 $mL^{-1}$).

Overall, regardless of the beverage category and the AOC assay used, the relationship between AOC and TPC was significantly high ($p$<0.0001). None of the beverages with high AOC contained significant amounts of AA but contained the highest amounts of TPC, except for beverage Fuze Slenderize Blueberry, Raspberry (J19) that did not contain polyphenolic compounds but significant amounts of AA (Table 1).

**Polyphenolic Profiles**

Out of the 56 beverages used in this study, the juices that contained the higher TPC and AOC (i.e., aronia, blackcurrant and pomegranate) were selected for further analysis. Aronia, blackcurrant and pomegranate fruits and their juices are of special interest considering the higher correlation between TPC and AOC with reported health benefits (Scalbert et al., 2005). Even though there are many studies that report the TPC and AOC of pomegranate juice, studies on aronia and blackcurrant are scarce.

In this study, several major polyphenol compounds were identified from blackcurrant, aronia and pomegranate juices (Fig. 11). The major polyphenolic compounds identified from aronia juice were myricetin (10 %), malvidin (6%), caffeic acid (4%), kaempferol-3-glucoside (4%), quercetin-3-glucoside (3%), chlorogenic acid (4%), pelargonidin-3-glucoside (2%) and delphinidin (1%). Taheri et al. (2013) also reported high amounts of anthocyanins and proanthocyanins in aronia juice. Other polyphenolic compounds were also identified but in quantities lower than 1% (i.e., catechin, epicatechin, *p*-coumaric acid, ferulic acid, kaempferol, cyanidin, malvidin-3-glucoside, cyanidin-3-glucoside, pelargonidin). In blackcurrant juice, the major polyphenolic compounds identified were myricetin (8%), pelargonidin (7%), kaempferol-

3-glucoside (6%), ferulic acid (5%), malvidin (5%), quercetin-3-glucoside (5%), pelargonidin-3-glucoside (4%) and delphinidin (2%) (Fig. 11). Other compounds were also identified but at levels below 2% (i.e. catechin, epicatechin, p-coumaric acid, caffeic acid, kaempferol, chlorogenic acid, cyanidin, malvidin-3-glucoside, cyanidin-3-glucoside). These results agree with previously published studies where high amounts of anthocyanins were also detected in blackcurrant juices (Parkar et al., 2014).

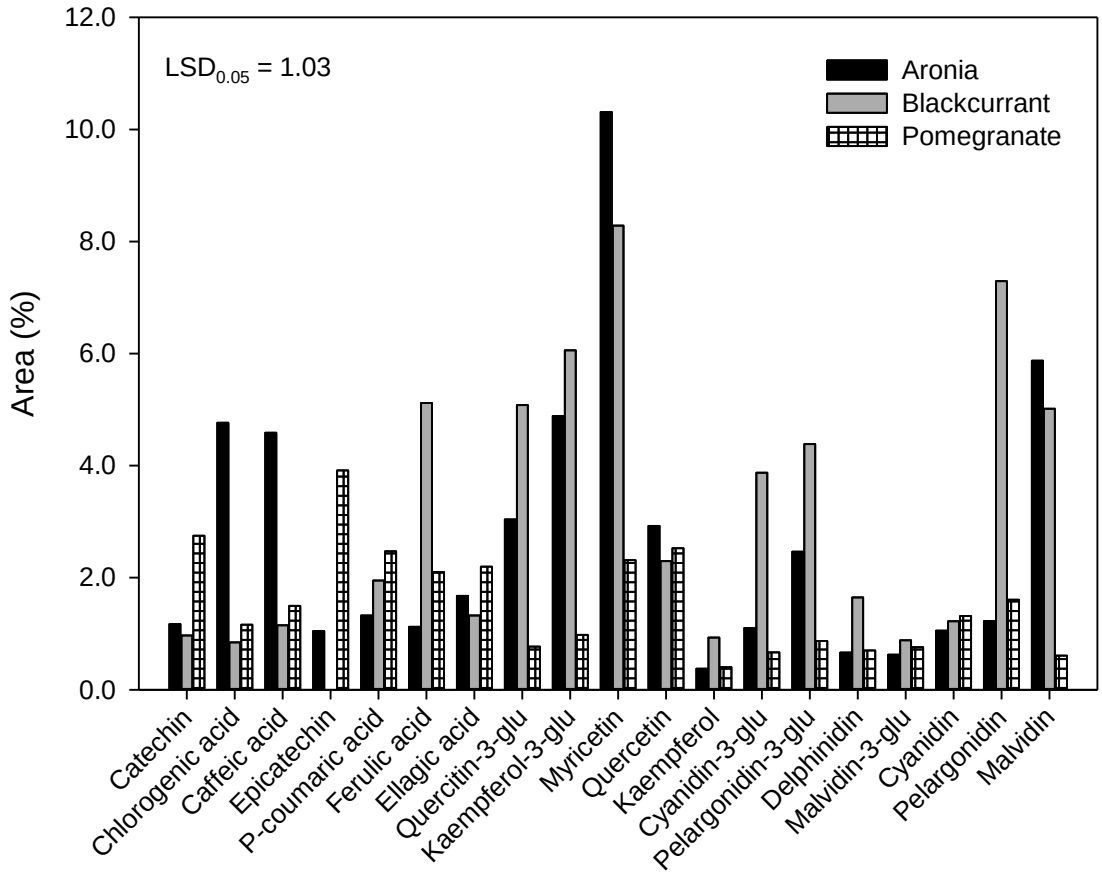


**Figure 11**. Polyphenolic profiles for aronia, blackcurrant, and pomegranate fruit juices.

In pomegranate juice, epicatechin (4%), catechin (3 %), ellagic acid (2%), ferulic acid (2%) myricetin (2 %), quercetin (2%), p-coumaric acid (2%), cyanidin (1%) and pelargonidin (2%) were the major polyphenolic compounds identified. Mousavinejad et al. (2009) also reported that the major compounds identified included from pomegranate juices were flavonols. Chlorogenic acid, kaempferol, delphinidin, malvidin-3-glucoside, cyanidin-3-glucoside, pelargonidin-3-glucoside, malvidin, kaempferol-3-glucoside quercetin-3-glucoside were identified at quantities below 1%.

Overall, aronia and blackcurrant juices contained significantly higher amounts of myricetin and malvidin compared to pomegranate juice (Fig. 11). Pomegranate juice contained significantly higher amounts of epicatechin and catechin than aronia or blackcurrant juices but lower amounts of other polyphenolic compounds. These results parallel those found for TPC, where pomegranate juice had significantly lower TPC than blackcurrant and aronia juice (Table 1). Thus, aronia and blackcurrant showed significantly higher TPC with a correspondingly higher amount of individual polyphenolic compounds (Fig. 11). Furthermore, these results suggested that AOC might not necessarily be related to overall TPC but rather to a synergistic effect between polyphenolic compounds at an ideal ratio that probably yields greater free radical scavenging potential. For example, pomegranate juice has significantly lower TPC than blackcurrant and aronia juices but did not show significantly different in AOC. The individual polyphenolic compounds found in higher quantities in pomegranate juice (catechin, epicatechin) may, therefore, be more efficient as radical scavengers by potentially creating a unique synergism with the other polyphenolic compounds identified.

### Total Ascorbic Acid and Antioxidant Capacity

Ascorbic acid (AA; vitamin C) has been shown to have powerful antioxidant properties (Loewus and Loewus, 1987). Normally found in whole fruits, adding vitamin C to beverages has become increasingly popular not only because of its known antioxidant activity but also to increase the nutrient content and/or to provide an extended shelf-life by delaying oxidation (Manso et al., 2001). In this study, AA was detected in the nutritional drink and juice categories, and also in beverages to which it was added during processing (Table 1). The addition of AA has been shown to positively affect AOC in apple and other fruit juices possibly due to its own AOC or through protecting juice's phenolic acids from oxidation (Miller and Rice-Evans, 1997; Kolniak-Ostek et al., 2013).

Results from the present study showed that AA content was not in agreement with the AA/vitamin C contents listed on the beverage nutrition label (data not shown). This disparity could have been caused by several factors including the complex matrices of the beverages, the form of vitamin C added to the beverage during production (i.e., L-ascorbic acid, sodium ascorbate, calcium ascorbate or ascorbyl palmitate), and/or to the method used for processing the juice. For example, pasteurization of juice has shown to significantly reduce vitamin C (Yvonne et al., 2005; Hiatt et al., 2011) whereas PEF or HPP treatments result in higher retention of AA and polyphenols in juice. Further, the addition of synthetic AA to beverages has shown to be only 5% active compared to the natural AA, and this may lead to a decreased AOC (Rodríguez-Roque et al., 2015). The low amounts of AA measured could also be related to the time-temperature history during storage which also negatively affects AOC

(Klimczak et al., 2007). In addition, since vitamin C is light sensitive, the type of packaging may also play an important role in its degradation as most of the beverages were filled into either clear glass or clear plastic bottles. However, the amounts of AA measured in the beverages used in this study seemed to only have had a minor contribution to the AOC (Table 1). This was obvious in the beverages with high TPC and AOC that contained insignificant amounts of naturally occurring vitamin C, and also in the beverages that had low AOC and contained an added form of vitamin C but had no detectable phenolic compounds. The beverage Vemma (N8) had significantly higher TPC and AOC values than its counterparts Verve Zero (N7) and Vemma Renew (N9) which contained similar AA but differentiated only in their TPC, suggesting that TPC was the main contributor for its AOC. Even beverages such as R.W. Knudson Mega C (J19; contains a blend of as apple, concorde grape, and aronia juices), that listed 600 mg of vitamin C per serving (240 mL), did not have considerably higher AOC than beverages that listed lower AA content.

In conventional strawberry juice (J26), vitamin C content was similar to that of orange juice (J13, J22) but significantly higher than grape (J4) and cranberry juice (J5, J6). Organic strawberry juice (O8) had significantly higher AA content than juice from conventional strawberries and was one of the only two beverages in the organic juice category (O1 and O8) with detectable AA. It is interesting to note that the juices with highest AOC did not contain any detectable AA but had the highest TPC. The contribution of AA to the total AOC of berries has shown to have only a minor effect when compared to polyphenolic compounds. For example, Aaby et al. (2007) reported that the contribution of AA to the AOC of strawberries was 24% whereas individual

polyphenols such as ellagitannins, anthocyanins and flavanols contributed 42%. In addition, polyphenolic content in juices from *Rubus* fruit, oranges, apples and grapes was positively correlated with the majority of their AOC whereas AA had only a minor contribution (Wang et al., 1996; Deighton et al., 2000).

### Effect of Storage on Total Phenolic and Antioxidant Capacity of Aronia, Blackcurrant and Pomegranate Juices

Commercial bottles of juices from brands such as Lakewood and R.W. Knudson normally contain about 960 mL of juice in a very concentrated form therefore, it is difficult for one consumer to use the contents of the entire bottle at once. Consequently, these juices are usually stored under refrigerated temperatures at home and consumed as pleased. However, the effect of refrigerated storage at home after opening the bottles as well as the contact with air may affect the TPC of the juices and their potential AOC. Furthermore, since polyphenolic compounds are not officially recognized as nutrients the expiration date on the bottle does not take these compounds into consideration.

In the present study, after opening the bottles, aronia, blackcurrant and pomegranate juices were stored under simulated consumer conditions (refrigerator at 4°C) for 14 days, to evaluate the effect of storage on TPC and AOC of the juices. Results showed significant losses in the TPC of aronia and blackcurrant juices during 14 days of storage (Fig. 12). Pomegranate juice, on the other hand, showed no significant losses after 14 days of storage. These results may be related to the lower concentration of anthocyanins in pomegranate juice compared to aronia and

blackcurrant juices. Overall, TPC in pomegranate juice was significantly lower than in aronia and blackcurrant juice which is in agreement with the results obtained for individual polyphenolic compounds (Fig. 11). In addition, results from this study showed that there is a significant variation in TPC between batches of juices, particularly for the aronia juice. Therefore, the amount of bioactive compounds that are available for consumption and their potential health benefits can vary tremendously between individual batches from each juice.

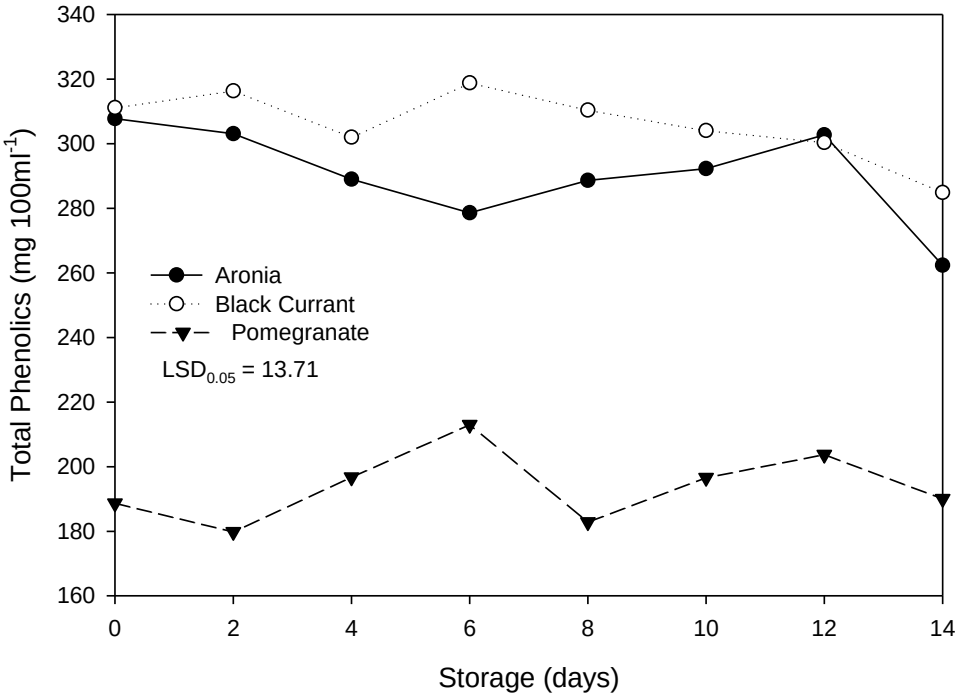


**Figure 12**. Total phenolic contents of aronia, blackcurrant, and pomegranate juices during 14 days of simulated consumer storage at 4°C.

AOC of the selected beverages showed a similar pattern to TPC (Fig. 13). For the aronia juice, the FRAP and DPPH assays showed significantly different results but the pattern was similar (Fig 13). That is, AOC values measured on day 0 and on day 14 were not significantly different in both assays. For the blackcurrant juice, the results

from the two AOC assays used were also significantly different but the pattern was similar in that there was little difference between day 0 and day 14 (Fig 13). For pomegranate juice, the results from the two AOC assays were not significantly different and the juice showed no significant reduction in AOC after 14 days of storage (Fig 13). The non-significant reduction of TPC in pomegranate juice was most likely related to the fact that fewer anthocyanins but more flavonols and phenolic acids are found in pomegranate juice when compared to blackcurrant and aronia juices (Giusti et al., 2007; González-Molina et al., 2009; Schrage et al., 2010; Denev et al., 2012).

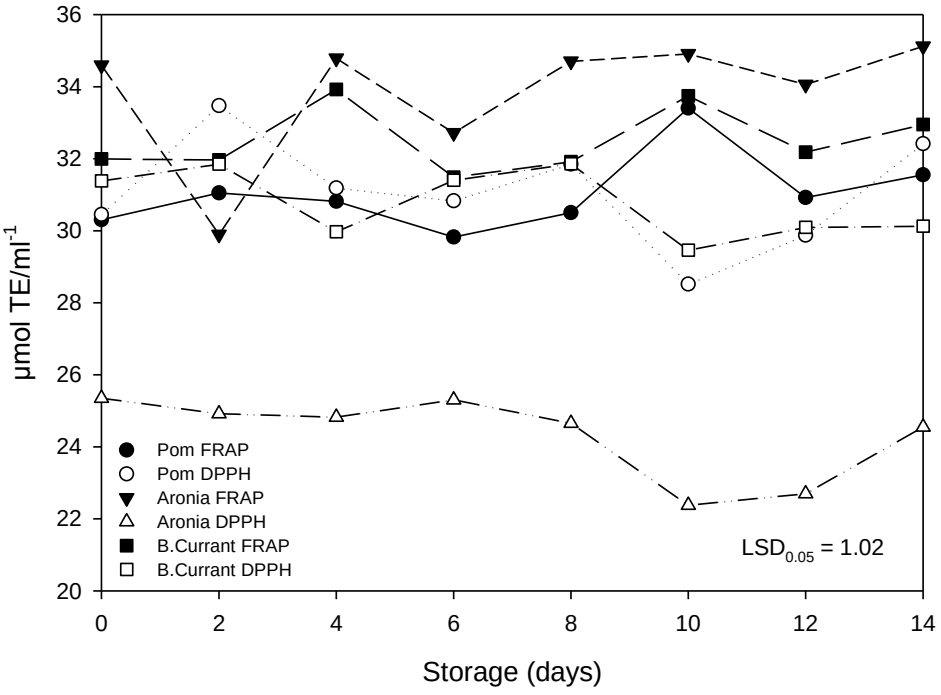


**Figure 13**. Antioxidant capacity of aronia, blackcurrant and pomegranate juices during 14 days of simulated consumer storage at 4°C.

There was no significant difference between batches of juices for FRAP whereas DPPH measurements showed a significant difference between batches of juices. FRAP and DPPH measurements were different for aronia and blackcurrant juices but not for

pomegranate juice. Therefore, these results emphasize the importance of using multiple assays to measure AOC in fruit juices and other foods since certain antioxidants have been shown to react differently with an introduced oxidant (Huang et al., 2005). Overall, the AOC assays used in this study showed a significant correlation with TPC ($p$<0.0001).

## Conclusions

Results from this study showed that beverages containing high amounts of TPC also have a higher AOC which can be related to a synergistic interaction between multiple polyphenolic compounds. Even though vitamin C has proven to be a powerful antioxidant, the addition of synthetic forms of the vitamin showed little effect on AOC but could potentially help stabilize the polyphenolic compounds and protect them from oxidation. The following fruit juices contained the highest amount of TPC and showed the highest AOC amongst all beverages evaluated: Blackcurrant (J18) > Aronia (J12) > Pure Pomegranate (J20) > POM 100% Pomegranate (J1) > POM Blueberry (J2) > Mega C (J19) > Tart Cherry (J15). Major polyphenolic compounds identified in aronia, blackcurrant, and pomegranate juice were anthocyanins, hydroxycinnamic acids, and flavonols. When held for 14 days at 4 °C, TPC of aronia, blackcurrant, and pomegranate juices showed to be relatively stable throughout storage. Overall, results from this study suggest that TPC and AOC in processed fruit juices are relatively stable during cold storage even when the bottles have been exposed to air. Finally, since numerous factors can influence the AOC of foods and beverages, further research needs to be done to establish a relationship between polyphenols and major food components such

as sugars, lipids, proteins and fiber that together make up the complex matrices of fruits and their beverages.

# CHAPTER FIVE:
# ANTIOXIDANT CAPACITY OF SYNTHETIC INDIVIDUAL POLYPHENOLIC COMPOUNDS AND THEIR MIXTURES COMPARED TO STRAWBERRY FRUIT

## Introduction

Dietary reference intake (DRI) is a system developed by the Institute of Medicine that provides the most current scientific knowledge of nutrient needs for healthy people. The recommended dietary allowance (RDA) guidelines list the average daily level of nutrients' intake sufficient to meet the requirements established by the DRI for healthy people (USDA, 2016). Strawberries provide several micronutrients including several vitamins and minerals in minute quantities. The most recognized nutrient in strawberries is ascorbic acid (vitamin C) which is well known for its health benefits (Loewus and Loewus, 1987). In healthy adults, the RDA for vitamin C is 60 mg $day^{-1}$ which can easily be met with an average intake of about 100 g of strawberries a day (USDA, 2016). Strawberries also contain polyphenolic compounds which in the last decade, have been acclaimed for their health promoting benefits. However, these compounds are not considered nutrients and thus so far there is no official recommendation for daily dietary intake. In addition, data is limited and so determining a model for polyphenolic dietary intake is difficult to establish (Prior et al., 2005). Studies that used high doses of synthetic polyphenolic compounds showed antagonistic, pro-oxidant rather than antioxidant effects resulting in a decrease in cell defense mechanisms and potentially induction of apoptosis of healthy cells (Hercberg et al., 1998; Metodiewa et al., 1999).

These results suggest that polyphenols are only a minute part of the diet and that their antioxidant properties in humans seem to be enhanced when these compounds are ingested in their natural form, from fruits and vegetables.

Several studies have shown the ability of polyphenolic compounds to fight free radicals (Gong et al., 2010), display anticancer properties by inhibiting cancer growth and stimulating apoptosis of cancer cells (Seeram et al., 2006), reduce inflammation by inhibiting inflammatory proteins and oxidative stress (La et al., 2009; Mukai and Sato, 2010) and even showed anti-microbial properties (Badjakov et al., 2008). Results from these studies suggest that polyphenolic compounds may have a potential to be used as emerging treatments of several health conditions. In addition, because of their acclaimed health benefits, there is a growing interest to increase the amount of polyphenolic compounds in the human diet either by using functional foods or dietary supplements. However, there is a lack of information available on the specific amounts necessary to promote antioxidant effects in humans or whether individual or multiple polyphenolic compounds should be used to positively impact human health. Reber et al. (2011) showed that there are unique interactions between polyphenolic compounds found in strawberries that may either lead to antioxidant or pro-oxidant effects. In addition, Hercberg et al. (1998) suggested that the optimal effect of antioxidant vitamins (e.g., beta-carotene, vitamins C and E) may be expected only when these are given in combination with other nutrients, at levels similar to those found in a healthy diet. This synergistic effect suggests that when included in functional foods or in dietary supplements, multiple polyphenolic compounds should be used since this is how they are found in nature. In fact, Grootveld and Halliwell (1986) suggested that some

polyphenolic compounds are unable to act as radical scavengers on their own and thus may have reduced AOC compared to when included in a mixture.

The chemical structure of polyphenolic compounds and whether these are present in glycoside or aglycone forms may also impact the AOC of foods (Fukumoto and Mazza, 2000). Structurally important features that define the AOC of polyphenolic compounds include the hydroxylation patterns, in particular the 3', 4'-dihydroxyl catechol group in the B-ring which gives the structure higher stability and participates in more electron delocalization; the planarity of the molecule or the presence of 2,3-unsaturation in conjugation with a 4-oxo-function in the C-ring which has shown to be responsible for electron delocalization from the B-ring; and the 3- and 5-OH groups with 4-oxo function in the A and C rings (Halliwell, 1990; Shahidi and Wanasundara, 1992; Huang et al., 2005). Glycosylation seems to reduce AOC by blocking the 3-hydroxyl group in the C-ring because of the addition of sugar to the polyphenol structure. On the other hand, aglycones have shown to have superior AOC since an additional sugar molecule is lacking in its structure (Shahidi, 1992).

Strawberries contain several polyphenolic compounds and also ascorbic acid which have been shown to possess antioxidant activity (Hannum, 2004). The major polyphenolic compounds in strawberries include pelargonidin, cyanidin, ellagic acid, quercetin, kaempferol, catechin, epicatechin, caffeic acid, *p*-coumaric acid and ferulic acid. Most of these compounds are naturally present in strawberry fruit in their glycosylated forms (Clifford, 2000; Laura et al., 2009). One study reported the AOC of some individual compounds found in strawberry fruit but the authors did not use the same concentrations naturally found in the fruit. Besides, this study did not consider

nutrients naturally occurring in the fruit (e.g., sugars, vitamins) and thus did not emulate the natural state and/or concentrations of polyphenolic compounds found in strawberries (Reber et al., 2011). Since anthocyanins, flavonols, and phenolic acids have shown to make up the majority of phytochemical compounds in strawberries, it is important to determine if they are the major contributors to the overall AOC of strawberry fruit. Therefore, the objectives of this study were to: 1) determine the relationship between chemical structure and AOC of major individual strawberry phytochemicals and mixtures of aglycones and glycosides, and 2) identify the specific source of AOC in strawberry fruit by creating a synthetic replica of a strawberry ("Powerberry") containing the same amounts of each major individual strawberry polyphenolic compounds as well as vitamin C, fructose and glucose and compare its AOC to that of different strawberry cultivars.

## Material and Methods

### Preparation of Fruit Extracts

Strawberry cultivars Sweet Sensation®, 'Strawberry Festival', 'Florida Radiance' and 'Winterstar' were harvested twice from commercial fields in Florida and sorted by color and freedom of defects upon arrival to the laboratory. Strawberry samples for analysis were prepared using two replicated samples of 250 g of strawberries each. The calyces were removed, and the fruit was homogenized in a laboratory blender at high speed for 2 min and the resulting puree immediately frozen and kept at -30 °C until use. After thawing, strawberry puree was again homogenized, centrifuged at 1600 $g_n$ for 20 min, filtered through cotton cloth and the clear juice used for analysis.

**Table 2**. Major polyphenolic compounds, vitamins, and sugars, and their amounts found in strawberries.

| Class | Group | Compound | Amount (mg100 $g^{-1}$) | References |
|---|---|---|---|---|
| Flavonoids | Anthocyanidins | Cyanidin | 0.3-18.5 | Määttä- Riihinen et al., 2004; Del Pozo-Insfran et al., 2006; Reber et al., 2011; Bhagwat et al., 2013; Phenol-Explorer, 2015 |
| | | Pelargonidin | 4.1-109.0 | Del Pozo-Insfran et al., 2006; Reber et al., 2011; Bhagwat et al., 2013; Phenol-Explorer, 2015 |
| | Anthocyanins | Cyanidin-3-glucoside | 0.1-6.7 | Määttä- Riihinen et al., 2004; Almeida et al., 2007; Lopes da Silva et al., 2007; Buendía et al., 2010; Kelebek and Selli, 2011; Aaby et al., 2012; Phenol-Explorer, 2015 |
| | | Pelargonidin-3-glucoside | 4.3-68.3 | Määttä- Riihinen et al., 2004; Almeida et al., 2007; Lopes da Silva et al., 2007; Buendía et al., 2010; Kelebek and Selli, 2011; Aaby et al., 2012; Phenol-Explorer, 2015 |
| | Flavonols | Quercetin | 0-4.4 | Häkkinen and Törrönen, 2000; Bhagwat et al., 2013 |

Table 2 (continued)

| Class | Group | Compound | Amount (mg100 $g^{-1}$) | References |
|---|---|---|---|---|
| | | Kaempferol | 0-2.3 | Häkkinen and Törrönen, 2000; Reber et al., 2011; Bhagwat et al., 2013 |
| | | Quercetin-3-glucoside | 0.3-5.2 | Almeida et al., 2007; Reber et al., 2011; Aaby et al., 2012 |
| | | Kaempferol-3-glucoside | 0.1-2.1 | Määttä- Riihinen et al., 2004; Almeida et al., 2007; Kelebek and Selli, 2011; Reber et al., 2011; Aaby et al., 2012; Phenol-Explorer, 2015 |
| | Flavanols | Catechin | 0-18.7 | Bhagwat et al., 2013; Phenol-Explorer, 2015 |
| | | Epicatechin | 0-2.2 | Mattila et al., 2006; Bhagwat et al., 2013; Phenol-Explorer, 2015 |
| Phenolic acids | Hydroxycinnamic acids | *p*-Coumaric acid | 0.9-4.9 | Häkkinen and Törrönen, 2000; Del Pozo-Insfran et al., 2006; Almeida et al., 2007; Reber et al., 2011; Phenol-Explorer, 2015 |

Table 2 (continued)

| Class | Group | Compound | Amount (mg100 $g^{-1}$) | References |
|---|---|---|---|---|
| | | Ferulic acid | 0.6-2.1 | Buendía et al., 2010; Kelebek and Selli, 2011 |
| | | Caffeic acid | 0.3-5.0 | Del Pozo-Insfran et al., 2006; Kelebek and Selli, 2011 |
| | Hydroxycinnamic quinic esters | Chlorogenic acid | 1.3-5.4 | Del Pozo-Insfran et al., 2006; Aaby et al., 2012 |
| Hydrolyzable tannins | Ellagitannins | Ellagic acid | 0- 52.2 | Häkkinen and Törrönen, 2000; Määttä- Riihinen et al., 2004; Del Pozo-Insfran et al., 2006; Almeida et al., 2007; Buendía,et al., 2010; Reber al., 2011; Aaby et al., 2012; Phenol-Explorer, 2015 |
| Vitamins | Water-soluble | Ascorbic acid | 23.8–84.7 | Szajdek and Borowska, 2008; Pineli et al., 2011; Crecente-Campo et al., 2012 |
| Carbohydrates | Sugars | Glucose | 1300-4260 | Kafkas et al., 2006; Giampieri et al., 2012 |
| | | Fructose | 1330-4200 | Kafkas et al., 2006; Giampieri et al., 2012 |

### Preparation of Synthetic Polyphenolic Compounds and Mixtures

Based on data from the literature, the major polyphenolic compounds, vitamins and sugars identified from strawberry fruit were selected for this study (Table 2). Pure polyphenolic compounds (cyanidin, pelargonidin, cyanidin-3-glucoside, pelargonidin-3-glucoside, quercetin, kaempferol, quercetin-3-glucoside, kaempferol-3-glucoside, catechin, epicatechin, *p*-coumaric acid, ferulic acid, caffeic acid, chlorogenic acid, ellagic acid, L-ascorbic acid, glucose, and fructose) used in this study were purchased from Fisher Scientific or Sigma-Aldrich. Individual compounds were prepared by mixing each individual compound with methanol to a concentration of 0.1 mg $ml^{-1}$. The mixtures of the different polyphenolic compounds are shown in Table 3. Ascorbic acid, fructose, and glucose were added to each mixture at same concentrations (0.1 mg $ml^{-1}$). In addition, based on the type and amounts of the major individual strawberry polyphenolic compounds, vitamin C, fructose and glucose reported in the literature, a synthetic replica of a strawberry ("Powerberry") was created based on the median amount from the lowest and highest values found in the literature (Table 4). After preparation, the solutions were stored at -30 °C until used.

### Antioxidant Capacity

Antioxidant capacity was determined using the assays previously described (See Chapter 3: Materials and Method Section). The three AOC assays used were repeated twice using three replicated samples for each compound/mixture analyzed. For ease of interpretation, results from the three assays used (FRAP, DPPH, and TEAC), and since

there was not a significant difference between the results from all assays, the AOC values were averaged.

**Table 3**. Composition of the mixtures containing flavonoids and flavonols in their aglycone or glycosylated forms plus flavanols, phenolic acids, ellagic acid, and ascorbic acid, fructose, and glucose.

| *Mixture A* | *Mixture B* | *Mixture C* | *Mixture D* |
|---|---|---|---|
| Cyanidin | Mixture A | Mixture A | Mixture A |
| Pelargonidin | Ascorbic acid | Fructose | Ascorbic acid |
| Quercetin | | Glucose | Fructose |
| Kaempferol | | | Glucose |
| Catechin | | | |
| Epicatechin | | | |
| *p*-Coumaric acid | | | |
| Ferulic acid | | | |
| Caffeic acid | | | |
| Chlorogenic acid | | | |
| Ellagic acid | | | |

| *Mixture E* | *Mixture F* | *Mixture G* | *Mixture H* |
|---|---|---|---|
| Cyanidin-3-glucoside | Mixture E | Mixture E | Mixture E |
| Pelargonidin-3-glucoside | Ascorbic acid | Fructose | Ascorbic acid |
| Quercetin-3-glucoside | | Glucose | Fructose |
| Kaempferol-3-glucoside | | | Glucose |
| Catechin | | | |
| Epicatechin | | | |
| *p*-Coumaric acid | | | |
| Ferulic acid | | | |
| Caffeic acid | | | |
| Chlorogenic acid | | | |
| Ellagic acid | | | |

| *Mixture I* | *Mixture J* | *Mixture K* | *Mixture L* |
|---|---|---|---|
| Mixture A | Mixture I | Mixture I | Mixture I |
| Mixture E | Ascorbic acid | Fructose | Ascorbic acid |
| | | Glucose | Fructose |
| | | | Glucose |

**Table 4.** Composition of the synthetic strawberry mixture "Powerberry".

| Compounds[a] | mg g100$^{-1}$[b] | mg mL$^{-1}$ |
|---|---|---|
| Cyanidin-3-glucoside | 3.4 | 0.03 |
| Pelargonidin-3-glucoside | 36.3 | 0.36 |
| Quercetin-3-glucoside | 2.6 | 0.03 |
| Kaempferol-3-glucoside | 1.1 | 0.01 |
| Catechin | 9.4 | 0.09 |
| Epicatechin | 1.1 | 0.01 |
| *p*-Coumaric acid | 2.6 | 0.03 |
| Ferulic acid | 1.6 | 0.02 |
| Caffeic acid | 2.3 | 0.02 |
| Chlorogenic acid | 3.0 | 0.03 |
| Ellagic acid | 26.1 | 0.26 |
| Ascorbic acid | 54.0 | 0.54 |
| Fructose | 2440.0 | 24.40 |
| Glucose | 2540.0 | 25.40 |

a = Bioactive compounds and sugars found in significant amounts in strawberry (see Table 2).
b = Median amounts found in strawberry (see Table 2).
c = Amounts used to prepare the "Powerberry" mixture; the compounds were diluted in methanol.

### Statistical Analysis

The Statistical Analysis System computer package (SAS Institute, Inc., 2004) was used for the analysis of the data. To determine the differences between the AOC of individual and mixtures of polyphenolic compounds and strawberries, the least significant difference (LSD) at the 5% significance level was used. The relationship between antioxidant assays and antioxidant capacity was measured using the Pearson correlation coefficient ($r$) and the coefficient of determination ($r^2$) and, the significance of the relationship was expressed by probability levels ($p$ = 0.0001).

## Results and Discussion

### Relationship between Chemical Structure and Antioxidant Capacity of major Individual Strawberry Polyphenolic Compounds

The most prevalent group of polyphenolic compounds in strawberries is the flavonoid group, which structures are composed of two aromatic rings (A and B) linked by an oxygenated heterocycle (C). The aromatic hydroxyl groups in flavonoids have been shown to be responsible for their AOC (Chen et al., 1996). The different subclasses of flavonoids are based on the degree of hydrogenation and substitution of the heterocycle. In this study, the AOC of specific anthocyanidins, anthocyanins, flavonols and flavanols found in strawberries was evaluated (Fig. 14). The AOC of anthocyanidins (cyanidin and pelargonidin) and anthocyanins (cyanidin-3-glucoside and pelargonidin-3-glucoside) were determined since the chemical structures of these compounds are similar. Cyanidin-3-glucoside contains a glycoside on 3' of the B-ring whereas cyanidin contains a hydroxyl group. Since the presence of hydroxyl groups at the 3' and 5' positions have been linked to a higher AOC, the lack of a hydroxyl group at that positions in the cyanidin-3-glucoside molecule could potentially be the reason for the significantly lower AOC when compared to cyanidin. The same trend was observed between pelargonidin-3-glucoside and pelargonidin (Fig. 14). Furthermore, the number of hydroxyl groups available for radical scavenging can possibly explain the higher AOC values obtained for cyanidin compared to cyanidin-3-glucoside (691.9 and 497.8 μmol TE $g^{-1}$, respectively). The same pattern was observed in pelargonidin and pelargonidin-3-glucoside (649.7 and 520.4 μmol TE $g^{-1}$, respectively). The lower AOC of the glycosides is most likely related to the addition of a sugar molecule at position 3' on the

C-ring. Similarly, using the DPPH assay, Fukumoto and Mazza (2000) showed that glycosylation of cyanidin, pelargonidin and quercetin resulted in lower AOC.

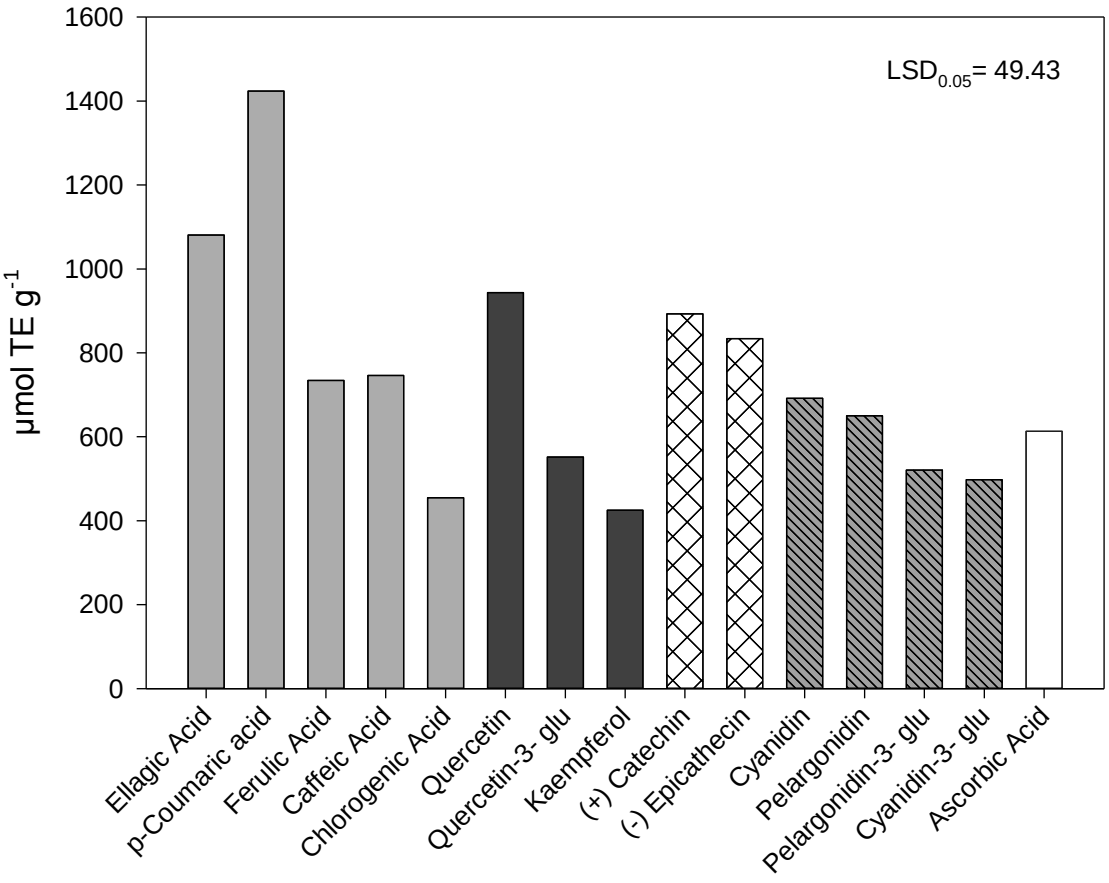


**Figure 14.** Antioxidant capacity of individual polyphenolic compounds at equal concentrations. TE = trolox equivalents.

From the flavonols found in strawberries, in addition, to cyanidin and pelargonidin which are the main pigments found in the fruit, quercetin and kaempferol can also contribute to the red color of the fruit (Mousavinejad et al., 2009). The chemical structure of quercetin and kaempferol differ only in the number of hydroxyl groups present in their molecules. Quercetin has a hydroxyl group on position 3' on the B-ring

which is essential for its electron donating properties (Moalin, 2011) whereas kaempferol contains only a hydrogen at position 3' on the B-ring which may further explain its significantly lower AOC when compared to quercetin (Fig. 14). The AOC of kaempferol-3-glucoside was not detected by any of the assays used in this study, possibly because of loss of one hydroxyl group due to glycosylation. This most likely resulted in the presence of only one hydroxyl group in the B-ring that alone, must have been too weak against the introduced oxidant. Further, these results can also be attributed to the fact that some polyphenolic compounds are inefficient radical scavengers without the synergistic action of other polyphenolic compounds (Lila and Raskin, 2005). The two flavanols found in significant amounts in strawberries were catechin and epicatechin. These two compounds are isomers and differ only in the arrangement of the hydroxyl group at position 3' of the C-ring. In epicatechin, this group lies below the plane and in catechin above the plane. This arrangement seemed to have had a significant impact on AOC because catechin showed significantly higher AOC compared to epicatechin (893.0 and 834.2 μmol TE $g^{-1}$, respectively).

Within the phenolic class, compounds are distinguished according to their underlying structure of either cinnamic or benzoic acids (Laura et al., 2009). The hydroxycinnamic acids present in significant quantities in strawberries include *p*-coumaric, ferulic and caffeic acids. Chlorogenic acid was the only hydroxycinnamic quinic ester found in significant quantities in strawberries (Fig. 9). Even though chlorogenic acid contains the most hydroxyl groups in its structure when compared to other cinnamic acids, the hydroxyl groups attached to the benzene ring showed to be more related to the AOC since caffeic acid had significantly higher AOC than

chlorogenic acid (746.4 and 454.7 µmol TE $g^{-1}$, respectively). Ellagic acid, a hydrolyzable tannin in the subclass of ellagitannins, had significantly higher AOC than cyanidin (1080.8 and 691.9 µmol TE $g^{-1}$, respectively) even though these two compounds both contain four hydroxyl groups. These results may be attributed to the structure of ellagic acid in which collagen proteins are often bounded together with phenolic groups making the structure more stable and more resistant to free radical stress (Laura et al., 2009). However, AA also with four hydroxyl groups in its structure, rated significantly lower in AOC (Wang and Lin, 2000).

**Mixtures of Polyphenolic Compounds and their Effect on Antioxidant Capacity**

In strawberries, glycosylated forms of polyphenolic compounds are naturally present in higher quantities than their aglycone counterparts (Mousavinejad et al., 2009). Unlike glycosides, aglycones are polyphenolic compounds that do not contain a sugar molecule linked to the carbon 3' on the C-ring. The major aglycones found in strawberries include cyanidin, pelargonidin, quercetin, kaempferol and, the major glycosides found in strawberries are cyanidin-3-glucoside, pelargonidin-3-glucoside, quercitin-3-glucoside and kaempferol-3-glucoside (Clifford 2000; Laura et al., 2009). In this study, aglycones showed on average a significantly higher AOC than glycosides (Fig. 14).

Since in nature, polyphenolic compounds are not typically found in their single form, a combination of multiple compounds was used to understand the effect of a mixture versus that of single compounds on the AOC. Overall, individual polyphenolic

compounds showed significantly higher AOC compared to their mixtures (Figs. 14 and 15). The aglycone mixture (mixture A: 708.9 µmol TE $g^{-1}$) showed significantly higher AOC than the glycoside mixture (Mixture E: 636.8 µmol TE $g^{-1}$) (Fig. 15). These results were somehow expected since, in previous studies, glycosides have also shown lower AOC compared to their aglycone forms (Fukumoto and Mazza, 2000). However, AOC of the aglycone-glycoside mixture (mixture I) was not significantly different from that of the aglycone mixture (mixture A) or to that of the aglycone-AA mixture (mixture B). Furthermore, the addition of AA to the aglycone or aglycone-glycoside mixtures did not have a major effect on AOC but it significantly lowered the AOC of the glycoside-AA mixture (Fig. 15). Even though AA is a well-established antioxidant, its addition to a mixture of polyphenolic compounds tended to diminish the overall AOC of the mixture.

In addition, to investigate the impact of sugars naturally found in strawberries in the overall AOC, mixtures containing glucose and fructose were also tested. Overall, the results showed significantly lower AOC for all mixtures containing sugars (Fig. 15). Therefore, the addition of fructose and glucose to a mixture of polyphenolic compounds seemed to lower the AOC possibly by interfering with the radical scavenging capacity of the hydroxyl groups (Shahidi, 1992). Since glucose and fructose have shown no AOC (data not shown), the significant lower AOC when glucose or fructose was present was most likely unrelated to the competition between sugar and polyphenols in scavenging free radicals but possibly due to a blockage of the hydroxyl groups. Further, when AA, glucose and fructose were added to the aglycone or glycoside mixtures, the AOC of the mixtures did not change significantly (Fig. 15).

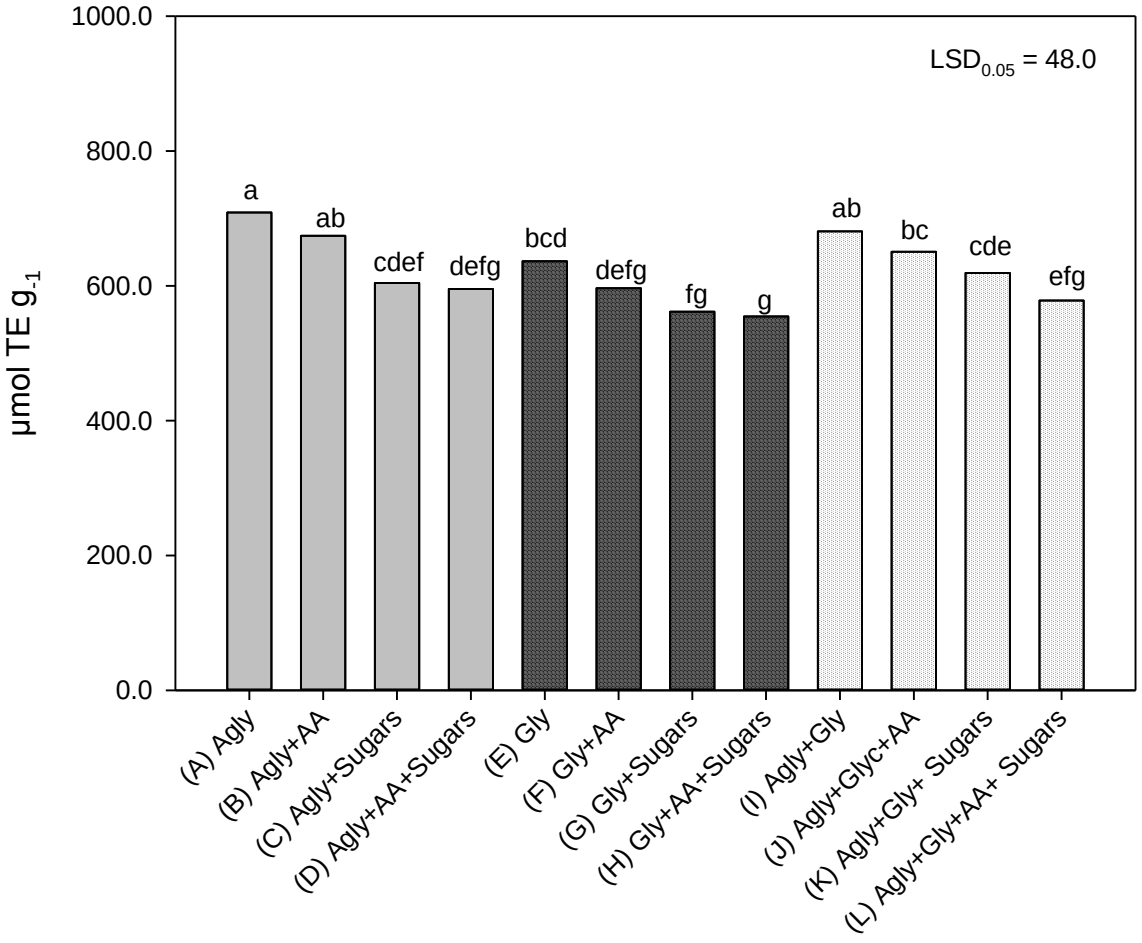


**Figure 15.** Antioxidant capacity of the mixtures containing flavonoids and flavonols in their aglycone or glycosylated forms plus flavanols, phenolic acids, ellagic acid, and ascorbic acid, fructose and glucose (see Table 3).TE = trolox equivalents.

### Antioxidant Capacity of Strawberry Compared to the "Powerberry" Mixture

The type and amount of each polyphenolic compound, vitamin C, glucose and fructose naturally found in strawberries was established from previously published data (Table 1). The polyphenolic compounds chosen were those measured in the highest amounts in strawberries (Fig. 9). Only these compounds were selected because strawberries are not considered a significant source of other nutrients such as fats,

proteins or other vitamins that could potentially affect the AOC (Giampieri et al., 2012). Vitamin C (AA) was chosen because strawberries are an excellent source of this vitamin with amounts ranging from about 23 to 85 mg 100 $g^{-1}$ in fresh fruit (Szajdek and Borowska, 2008; Pineli et al., 2011; Crecente-Campo et al., 2012). Glucose and fructose were chosen since these sugars make up almost 99% of total sugar content in strawberries (Sturm et al., 2003). Based on this information, a synthetic strawberry model ("Powerberry") was created and its AOC measured (Fig. 16). When compared to the AOC of strawberries from different cultivars, the average AOC of the "Powerberry" was not significantly different from that of 'Florida Radiance' and Sweet Sensation® (Fig. 16). Since the AOC values of the "Powerberry" were comparable to that of real strawberries, it is possible that these major polyphenolic compounds along with AA could be responsible for the overall AOC in strawberries, whereas glucose and fructose showed no AOC-increasing effect (Fig. 15).

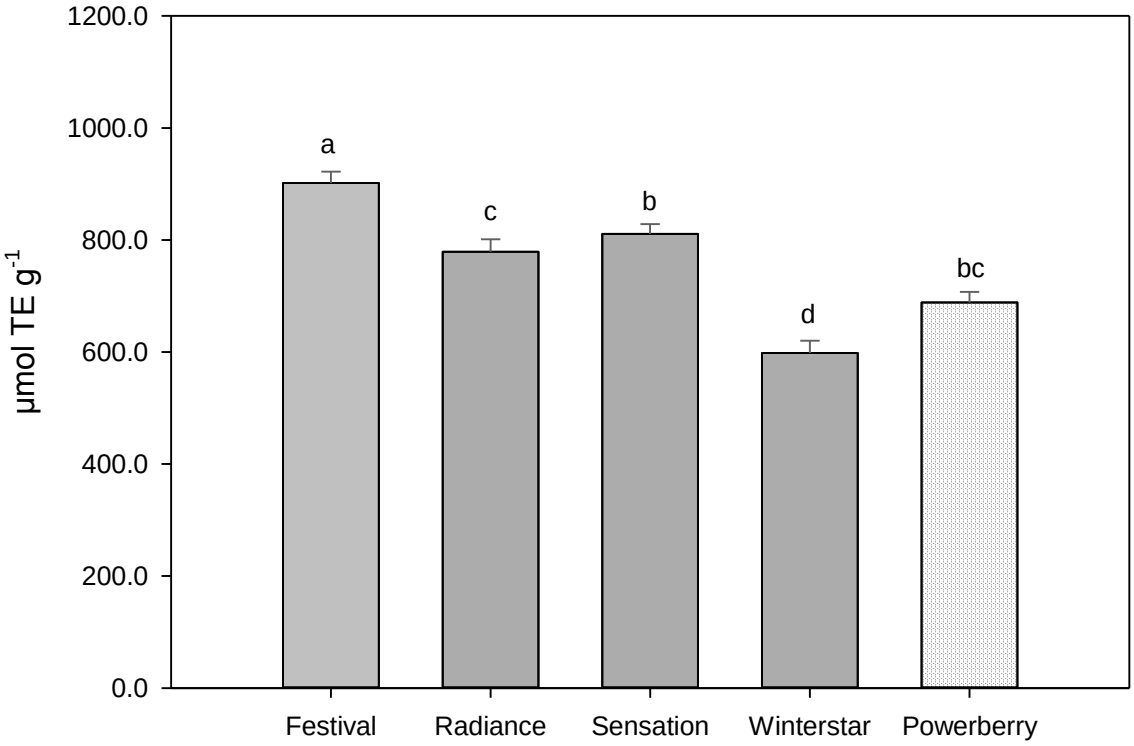


**Figure 16.** The antioxidant capacity of four different strawberry cultivars compared to that of the synthetic strawberry “Powerberry”. TE = trolox equivalents.

## Conclusions

Results from this study showed that the AOC of individual polyphenolic compounds (i.e., pelargonidin, cyanidin, ellagic acid, quercetin, kaempferol, catechin, epicatechin, caffeic acid, *p*-coumaric acid, ferulic acid) was significantly higher than that of mixtures of the same compounds (i.e., glycosides or aglycones) and, than that of the "Powerberry" mixture which also contained AA, fructose and glucose. The variation in the AOC obtained for individual compounds could be explained by the instability of polyphenolic compounds when in their isolated state which is not common in nature. For

the combination of compounds, results suggested that mixing individual polyphenolic compounds decreases their overall AOC. These results also suggest that when combined, polyphenols may compete for radical scavenging which in turn may reduce the overall AOC of the mixture. Further, the AOC of strawberries was correlated with its major bioactive compounds (i.e. polyphenolic compounds and ascorbic acid). Finally, although synthetic compounds may behave differently once ingested, in this study the AOC of these compounds as a mixture (“Powerberry”) was comparable to that of real fruit. These results suggest that even though strawberries contain many different polyphenolic compounds and vitamins, their AOC might only depend on few bioactive compounds that are found in significant quantities in strawberry fruit.

# CHAPTER SIX:
# THE EFFECTS OF POLYPHENOL-RICH FRUITS AND FRUIT JUICES ON THE PROLIFERATION OF CANCER CELLS AND LIFESPAN OF *CAENORHABDITIS ELEGANS*

## Introduction

The effect of dietary polyphenolic supplementation on human lifespan is difficult to assess since various factors such as lifestyle and genetic makeup of human subjects are highly variable, and the duration of the studies constitutes a major impediment. The aspects of aging are similar between nematodes and mammals where oxidative stress appears to be a major factor in limiting lifespan in *Caenorhabditis elegans* and humans (Finkel and Holbrook, 2000). As an alternative to human studies, *C. elegans* has been used as a model for lifespan studies because the worms mature into an adult in about 45 hours and have an average lifespan of about 2-3 weeks (Félix and Braendle, 2010). *C. elegans* occupy various environments that contain different bacteria and nutrients. They feed best on *Escherichia coli* but can survive on a diet of a variety of bacteria and nutrients which makes them ideal for testing the effect of polyphenolic compounds on their lifespan. For example, various studies showed that blueberry extracts rich in polyphenolic compounds as well as individual polyphenols extended *C. elegans* lifespan by increasing their oxidative and thermal resistance (Wilson et al., 2006; Kampkötter et al., 2008). Even when used individually, polyphenolic compounds such as quercetin and rutin were able to extend *C. elegans* lifespan by 12 to 20%, respectively (Xue et al.

2011). In addition, green tea extracts also extended the lifespan and enhanced stress resistance in *C. elegans* (Zhang et al., 2009). However, the exact mechanisms involved in the extension of *C. elegans* lifespan by polyphenolic compounds are still unknown. It is believed that at low dosage polyphenolic compounds have the ability to trigger light pro-oxidant mechanisms that in turn activate antioxidant defenses leading to an overall cytoprotection (Halliwell, 2011).

Several studies have shown that a high consumption of fruits and vegetables promotes health and reduces the risk of cancer and other degenerative diseases (Seeram et al., 2006; Tzounis et al., 2008; La et al., 2009; Mukai and Sato, 2011; Lewandowska et al., 2016). It has also been shown that some plant polyphenolics found in mangos are capable of inducing apoptosis-mediated death of HeLa human cervical cancer cells (Kim, 2012). These results suggest that fruits and their juices may be an excellent source of bioactive compounds and thus help to prevent various cancer types. Olsson et al. (2006) showed the ability of polyphenolic extracts from conventional and organic strawberry in decreasing the proliferation of both colon and breast cancer cells. Most of the current research focus on utilizing individual synthetic polyphenols to determine their potential impact on cell survival. Therefore, single synthetic polyphenolic compounds could also constitute an emerging approach in reducing cancer growth. For example, Mertens-Talcott et al. (2003) showed that quercetin and ellagic acid enhanced apoptosis and inhibited proliferation of human leukemia cell lines. Seeram et al. (2006) showed that polyphenolic extracts from several types of berries inhibited cancer growth and stimulated apoptosis of cancer cells (Seeram et al., 2006). In addition, polyphenolic

compounds isolated from apple peels showed a potent antioxidant and anti-proliferative activity against breast and liver cancer cells (He and Liu, 2007; He and Liu, 2008).

Although there are several published studies that explored the effects of plant polyphenolic extracts or single polyphenolic compounds on apoptosis of cancer cells, to our knowledge there are no published studies that have shown the potential of whole fruit or fruit juices in inhibiting cancer cell proliferation and extending *C. elegans* lifespan. Therefore, the objectives of this study were to: 1) determine the ability of selected polyphenolic-rich fruits and juices on inhibiting the proliferation of HeLa human cervical cancer cells and, 2) determine the impact of the same polyphenolic-rich juices on *C. elegans* lifespan.

## Material and Methods

### Fruits and Fruit Juices

Blueberry, raspberry, conventional and organic strawberries (3 replicated samples of 100g each) were dried in an oven at 80°C until weight stabilized. After drying, the samples were grinded using a pestle and mortar and the resulting powder was passed thru a sieve (U.S. Standard Test Sieve No. 45, Hogentogler & Co., Inc., Columbia, MD) to yield a finer powder. Aronia (R.W. Knudson Just Aronia), pomegranate (Lakewood Pure Pomegranate) and blackcurrant (R.W. Knudson Just Black Currant) juices (3 replicated samples of 250 mL each) were evaporated at 45°C to yield a concentrate of 50 mL. Before use, fruit powders and juice concentrates were dissolved in water at a concentration of 0.15g $mL^{-1}$ and then filter sterilized before a final concentration of 15ng $\mu L^{-1}$.

**Cell Models**

HeLa cells were maintained in DMEM medium (CellGro, cat#15-017-CV) supplemented with 10% fetal bovine serum (GIBCO, cat#10437-028) and 1% Pen-Strep-Glutamine (CellGro, cat#30-0090CI) at 37°C with 5% $CO_2$. For viability assays, HeLa cells were plated at a density of $1.5x10^5$ cells/well in a 96 well plate and allowed to adhere overnight. Dried blueberry, raspberry, conventional and organic strawberry, and concentrated aronia and blackcurrant juices were dissolved in water at a concentration of 0.15 g $mL^{-1}$ then filter and sterilized before addition to the cells. Puromycin was used at a dose of 5 µg $mL^{-1}$ as a positive control. After 24 hours, the viability reagent PrestoBlue® (ThermoFisher Scientific, cat#A-13261) was added at a 10% concentration to the cells and fluorescence was measured at an excitation of 525+/−20 nm and an emission of 590+/−35 nm.

**Worm Models**

*C. elegans* wild-type (N2) strain was maintained at 23°C on standard nematode growth medium plates seeded with *Escherichia coli* OP50. Synchronous worms were obtained by standard 20% hypochlorite treatment followed by a 24 hour rotation at 220 rpm in M9 buffer without food. For lifespan assays, synchronous wild-type worms were treated with no compound (control) or with various doses of concentrated aronia, blackcurrant, or pomegranate juice concentrates diluted in sterile water. Worms were picked to new plates daily to avoid progeny contamination during their reproductive period (~5 days). Standard NGM plates seeded with OP50 supplemented with sterile water were used as the control. Worms were scored for survival every other day starting

at day one of adulthood, and death was determined by lack of response to poking with a platinum wire pick. All lifespan assays were performed using at least 100 worms for each treatment condition in biological duplicates.

### Statistical Analysis

Statistical analysis for the HeLa cell viability assay was performed using GraphPad Prism (GraphPad Software, version 5.0, La Jolla, California, USA) using ANOVA followed by the Bonferroni post-test when an interaction term was significant. *C.elegans* survival was analyzed by the Log-rank Mantel-cox test using with GraphPad Prism (GraphPad Software, version 5.0, La Jolla, California, USA).

## Results and Discussion

### Ability of Whole Dried Fruits and Juice Concentrates in Inhibiting the Proliferation of HeLa Human Cervical Cancer Cells

Several studies have shown that plant polyphenolic compounds are capable of inducing apoptosis-mediated death in human cervical cancer cells (Kuntz et al., 1999; Prakash et al., 2001; Scalbert et al., 2005; Chirumbolo, 2012; Kim, 2012). Results from these studies suggest that fruits and their juices may help prevent various types of cancer when ingested in generous amounts as part of a balanced diet, mostly because of their bioactive compounds (Scalbert et al., 2005). In such studies, the decrease in cell proliferation rates was used as a marker for the effectiveness of polyphenolic compounds as putative cancer chemo-preventives (Joseph et al., 2005). In the present study, dried fruits and concentrated juices in a concentration of 0.15 g $mL^{-1}$ induced

cancer cell death (Fig. 17). Amongst the fruits, raspberry was the most effective in inhibiting cancer cell growth (34% cell survival) when compared to strawberry and blueberry. Organic strawberry showed a slightly higher effect in controlling cancer cell growth compared to conventional strawberry (73 and 78% cell growth survival, respectively). These results are in agreement with those previously published by Olsson et al. (2006) where polyphenolic extracts from conventional and organic strawberry decreased the proliferation of colon and breast cancer cells. This may due to the significantly higher levels of bioactive contents and AOC found in organic strawberries compared to conventional fruit (see Chapter 3).

Fruit juices were, in general, more effective than fruit in decreasing cancer cell proliferation, except for pomegranate juice that showed inferior results compared to raspberry fruit (Fig. 17). In addition, blackcurrant, and aronia juices showed more significant results (8 and 17% cell survival, respectively) compared to pomegranate juice (57% cell survival). Even though pomegranate juice had similar AOC when compared to blackcurrant and aronia juices (Table 1), its ability to inhibit cervical cancer cell growth was much lower than that of blackcurrant or aronia juices probably because of its significantly lower TPC. Since the AOC of pomegranate juice was comparable to that of aronia and blackcurrant juices the reduction of cancer cell survival was probably not related to an antioxidant effect but possibly to other mechanisms involving polyphenolic compounds and their secondary metabolites. It is also interesting that the blackcurrant and aronia juices had a higher impact on cancer cell survival compared to puromycin (30% cell survival) which has previously shown a high cytotoxicity in cancer cells (Young, 1966; Oguma et al., 2009).

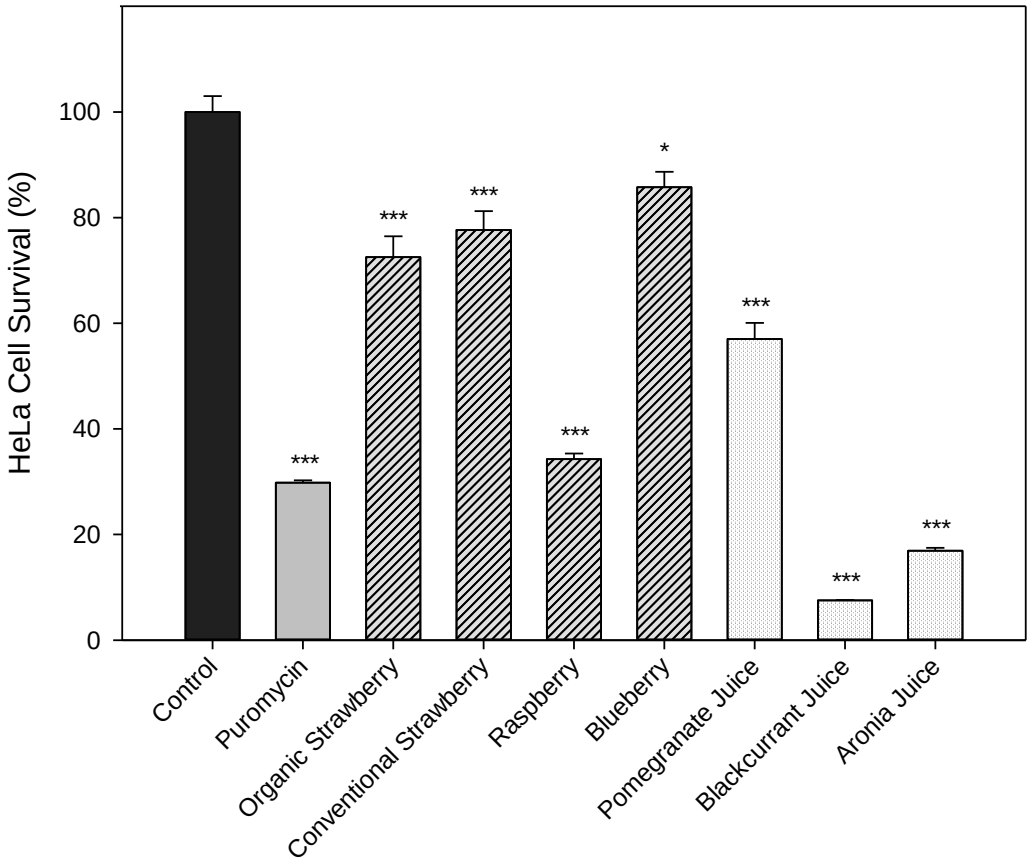


**Figure 17**. The effect of fruit (strawberry, raspberry and blueberry) and fruit juices (blackcurrant, aronia and pomegranate) on HeLa human cervical cancer cell survival compared to control (no treatment) or positive control (puromycin).

### Ability of Juice Concentrates in Extending the Lifespan of Wildtype *Caenorhabditis elegans*

Previous research suggested that the dietary consumption of polyphenolic compounds found in fruits and vegetables may be effective in reversing aging through anti-inflammatory and anti-oxidant effects (Joseph et al., 2005). In this study, pomegranate juice at doses higher than 0.75 mg $mL^{-1}$ (18-22 days) reduced *C. elegans* lifespan whereas doses lower than 0.75 mg $mL^{-1}$ increased the worm lifespan (26-30

days) when compared to the control (Fig. 18). Similarly, when added to the usual diet of the worm, aronia juice at doses of 0.75 mg $mL^{-1}$ or lower decreased lifespan of *C. elegans* (14-18 days) while concentrations of 0.375 mg $mL^{-1}$ or lower increased the worms' lifespan (26-30 days) when compared to the control (Fig. 18). The same pattern was observed for blackcurrant juice where a concentration of 0.75 mg $mL^{-1}$ or higher resulted in shorter lifespan (10-18 days) and a concentration of 0.375 mg $mL^{-1}$ or lower increased *C. elegans* lifespan (26-30 days) when compared to the control (Fig. 18).

Overall, all fruit juices at doses lower than 0.75 mg $mL^{-1}$ were efficient in extending the lifespan of *C. elegans* whereas higher doses were apparently cytotoxic as they decreased the lifespan of the worms. In fact, higher doses of synthetic polyphenolic compounds have shown to display antagonistic, pro-oxidant rather than antioxidant effects resulting in a decrease in cell defense mechanisms and potentially induction of apoptosis of healthy cells (Metodiewa et al., 1999) which could be the cause of the shorter lifespan in the *C. elegans*.

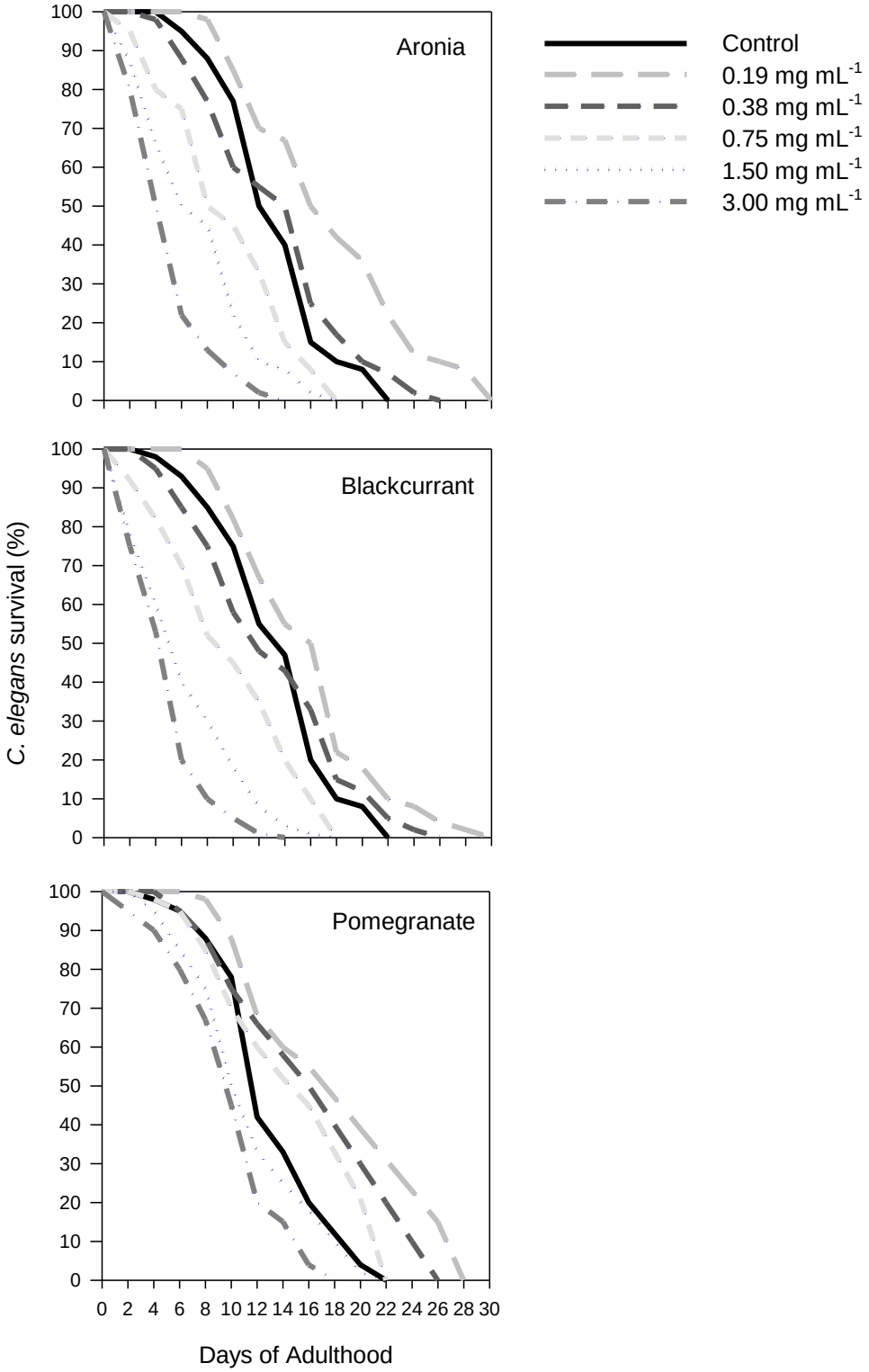


**Figure 18.** The effect of pomegranate, aronia and blackcurrant concentrated juices on the lifespan of *C. elegans*.

## Conclusions

Conventional and organic strawberry, raspberry and blueberry fruits, and aronia, pomegranate and blackcurrant juices were successful in inhibiting the proliferation of HeLa cervical cancer cells. Compared to fruits and pomegranate juice, aronia and blackcurrant juices displayed the most powerful effect towards inhibiting cancer cell proliferation most likely because of their significantly higher TPC and AOC. In addition, polyphenolic compounds in pomegranate, aronia and blackcurrant juices seem to have a significant bioactivity impact in the lifespan of the *C. elegans*. Thus, at doses lower than 0.75 mg $ml^{-1}$, all juices contributed to an increase of up to eight days in the lifespan of *C. elegans*. Higher doses were presumably toxic to the worms as their lifespan was reduced by four days, regardless of the juice used in their diet. Therefore, these results showed the importance of consuming an optimal amount of foods that are naturally high in polyphenolic compounds. Besides, these results raised a question about the safe dose of polyphenolic compounds when used as dietary supplements. In summary, careful doses should be considered when administering polyphenolic compounds in humans or other mammals because if given at too high concentrations may result in negative health effects.

# CHAPTER SEVEN: GENERAL CONCLUSIONS

Strawberries are greatly appreciated worldwide due to their pleasant flavor and nutritional qualities. However, pre- and post-harvest abiotic stresses may lead to deterioration of strawberry quality, reduce vitamin C and polyphenolic compounds and ultimately result in a decrease of its bioactivity. Overall, results from this work showed that strawberries treated with a reduced fungicide regime had similar quality to that of fruit treated with a conventional regime. In addition, specific polyphenolic compounds (i.e., pelargonidin-3-glucoside, quercetin-3-glucoside, and ferulic acid) were detected in significantly higher amounts in fruit from the reduced pesticide treatment, suggesting that this treatment may provide balanced stress conditions for the synthesis of bioactive compounds. These results suggest that reducing the use of fungicides in the field can be an alternative to conventional disease control treatments as it may help reduce residual fungicide levels while still retaining important bioactive compounds in the fruit.

The levels of polyphenolic compounds in foods seemed to be highly correlated with their potential antioxidant capacity (AOC) *in vitro* and *in vivo*. Thus, in order to show the relationship between AOC and total phenolic content (TPC), fruit juices as well as strawberries were used as food models. Results showed a positive significant correlation between TPC and AOC in beverages, specifically in fruit juices, whereas vitamin C showed little effect on AOC. Juices made from 100% blackcurrant, aronia, and pomegranate contained the highest amount of TPC and showed the highest AOC.

Major polyphenolic compounds identified from these juices included anthocyanins, hydroxycinnamic acids, and flavonols.

Numerous factors can influence the AOC of foods, namely vitamin C and sugar contents as well as the chemical structure of the individual polyphenolics compounds present in the food. Results showed that when compared at an individual level or when combined with other polyphenolic compounds, AOC was significantly higher in aglycone polyphenolic compounds than in their glycoside counterparts. In addition, combining individual polyphenolic compounds with glucose, fructose and/or vitamin C decreased their overall AOC, suggesting that polyphenols may compete for radical scavenging. Finally, a mixture containing polyphenolic compounds, vitamin C, glucose and fructose at levels found in real strawberries, showed similar AOC to that of real fruit. These results suggest that even though strawberries contain many different polyphenolic compounds and vitamins, their AOC might only depend on few bioactive compounds.

Many studies have shown that the potential health benefits of fruits and vegetables are highly correlated with their levels of TPC and consequently to their AOC. Results showed that fruits such as conventional and organic strawberry, raspberry and blueberry and fruit juices such as aronia, pomegranate, and blackcurrant inhibited the proliferation of HeLa cervical cancer cells. Compared to fruits and pomegranate juice, aronia and blackcurrant juices displayed the most powerful effect towards inhibiting cancer cell proliferation, most likely because of their significantly higher TPC and AOC. In addition, polyphenolic compounds in pomegranate, aronia and blackcurrant juices increased the lifespan of *C. elegans* worms at doses lower than 0.75 mg $ml^{-1}$ whereas higher doses reduced their lifespan. These results emphasize the importance of

consuming a well-balanced diet, rich in fruit and vegetables but providing just the right amount of polyphenolic compounds.

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