Quantifying Flesh Browning, Polyphenoloxidase, Total Phenolic Content and Vitamin C in Select Apple Varieties and Progeny

by Andrea Elaine Burke

QUANTIFYING FLESH BROWNING, POLYPHENOLOXIDASE, TOTAL PHENOLIC CONTENT AND VITAMIN C IN SELECT APPLE VARIETIES AND PROGENY

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Andrea Elaine Burke

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Andrea Elaine Burke, Ph.D.

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Apple (*Malus domestica* Borkh.) enzymatic browning is a significant problem in the fresh cut and processing industries. Quantifying flesh browning among a variety of cultivars can assist apple breeders in selecting low-browning parents to use in crosses. Polyphenoloxidase (PPO) is the catalyst of the browning reaction; phenolic compounds present in apples are the substrates. Rates of browning, PPO activity and total phenolic content were measured in 12 commercial cultivars and 5 advanced breeding selections. Vitamin C, an important antioxidant and metabolite in plants and for humans, was also quantified in these cultivars.

'Liberty', 'McIntosh', and selection #2 (a 'McIntosh' x 'Fuji' hybrid) were consistently the highest browning apples. 'AutumnCrisp' (previously 'NY674') and selection #5 ('AutumnCrisp' x 'Braeburn') were the lowest browning apples. For most cultivars, flesh browning decreased over time in cold storage and was higher in the core than near the peel. Selection #2, #3 ('Braeburn' x 'AutumnCrisp') and 'Braeburn' had the highest measured PPO activity; 'AutumnCrisp', 'Cortland', 'Zestar' and 'McIntosh' had the lowest. 'McIntosh', #4 ('AutumnCrisp' x 'Fuji'), #5 and #2 had the highest phenolic content; 'Braeburn' and #3 had the lowest. PPO activity and total phenolic content were correlated ($p<0.05$) to total color change only in some cultivars.

‘Braeburn’ had the highest mean vitamin C content, 21.7 mg/100g fresh weight. The five advanced breeding selections averaged higher than most of the commercial cultivars, with between 11.9 and 14.5 mg/100 g fresh weight. Harvest year and time in storage both affected vitamin C levels to varying degrees for different cultivars. There was a low but significant correlation between harvest date and vitamin C content.

BIOGRAPHICAL SKETCH

Andrea Burke grew up in her mother's garden in northern New Jersey, climbing her favorite cherry tree and counting the days until the first strawberries of spring. Her favorite part of gardening has always been harvest season, reaping the plentiful rewards of a season's full of work.

Andrea attended the University of North Carolina at Chapel Hill, where she majored in German and spent many hours exploring the school's on-campus arboretum. She was elected into Phi Beta Kappa her junior year and graduated with distinction. Following graduation, she worked as an investment banking analyst at Wachovia Securities. Andrea's favorite pastime during these years was building herself a backyard garden oasis on her quarter-acre lot in south Charlotte. Ultimately deciding to pursue her interest in things that grow, Andrea left Wachovia to attend North Carolina State University in Raleigh. She obtained her B.S. in Horticulture Summa Cum Laude. Andrea earned induction into the Pi Alpha Xi horticultural honor fraternity and was named 2006 ASHS Outstanding Horticulturalist for NC State University.

Inspired to continue further, Andrea joined Dr. Susan K. Brown's group at Cornell University's New York State Agricultural Experiment Station in Geneva, NY. She has been pursuing her Ph.D. in Horticulture with a minor concentration in Education. Her professional interests focus on apple flesh browning and nutrient content.

Andrea currently resides with her husband Patrick and two delightful sons, James (age 4) and Peter (age 2). She hopes in the future that she may inspire others to a love of Horticulture as she has been inspired.

For Jamie, Petey, and Pat, who give life meaning and make it all worthwhile

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LIST OF ABBREVIATIONS

1-MCP 1-methylcyclopropene

cDNA ..... complementary deoxyribonucleic acid

CO_2 ..... carbon dioxide

DHA ..... dehydroascorbate

GAE ..... gallic acid equivalents

GJIC ..... gap junction intercellular communication

H_2O_2 ..... hydrogen peroxide

L-AA ..... L-ascorbic acid

LG ..... linkage group

mRNA ..... messenger ribonucleic acid

MDHA ..... monodehydroascorbate

MRP ..... Maillard Reaction Products

PAL ..... phenylalanine ammonialyase

PCR ..... polymerase chain reaction

POD ..... peroxidase

PPO ..... polyphenoloxidase

QTL ..... quantitative trait loci

ROS ..... reactive oxygen species

TOSC ..... total oxyradical scavenging capacity

UV-C ..... ultraviolet-C

VCEAC ..... Vitamin C equivalent antioxidant capacity

CHAPTER 1

INTRODUCTION

An Overview of Enzymatic Browning

When an apple (*Malus domestica* Borkh.) is cut or injured, the flesh oxidizes and turns brown. This process is known as enzymatic browning, and is a reaction catalyzed by the enzyme polyphenoloxidase (PPO). In the reaction, phenolic compounds present in the apple flesh oxidize to form slightly colored *o*-quinones, which then polymerize to form pigments of varying hues and intensity (Le Bourvellec et al. 2004). The browning reaction is undesirable, leading to an unpleasant appearance, the possible development of off-flavors, and limiting the shelf life of fresh-cut or processed apples (Sapers 1993; Gámbaro et al. 2006). The rate and extent of apple flesh browning varies by cultivar, and may be affected by the activity level of the enzyme polyphenoloxidase as well as the total phenolic content of the fruit.

The basic enzymatic browning reaction involves the oxidation of colorless monophenols to diphenols, which are also colorless. These then further oxidize to form *o*-quinones, which are slightly colored. In the presence of amino acids and other proteins, these *o*-quinones polymerize into complex brown pigments (Grotheer et al. 2005). Additionally, quinones produced in the initial browning reaction may participate in coupled oxidation reactions, enabling them to oxidize other polyphenols that cannot be directly enzymatically oxidized (Cheynier et al. 1994). Figures 1.1 and 1.2 illustrate these reactions.

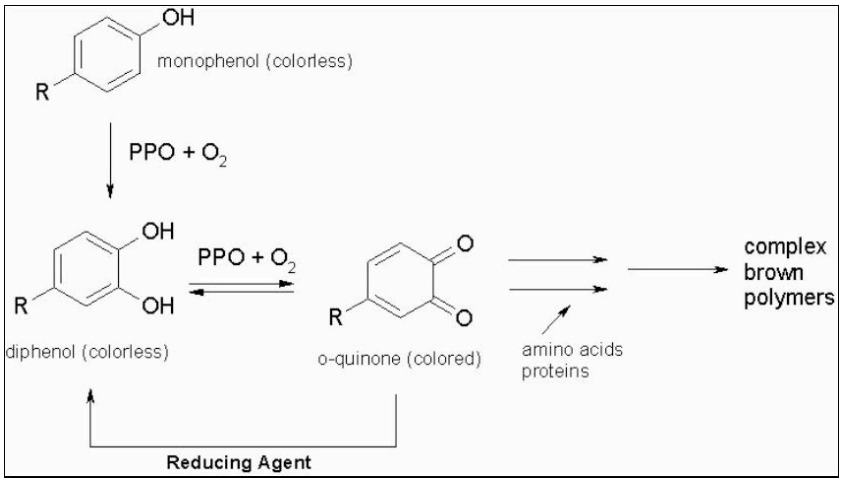

Figure 1.1. Simple diagram of the enzymatic browning reaction (Grotheer et al. 2005).

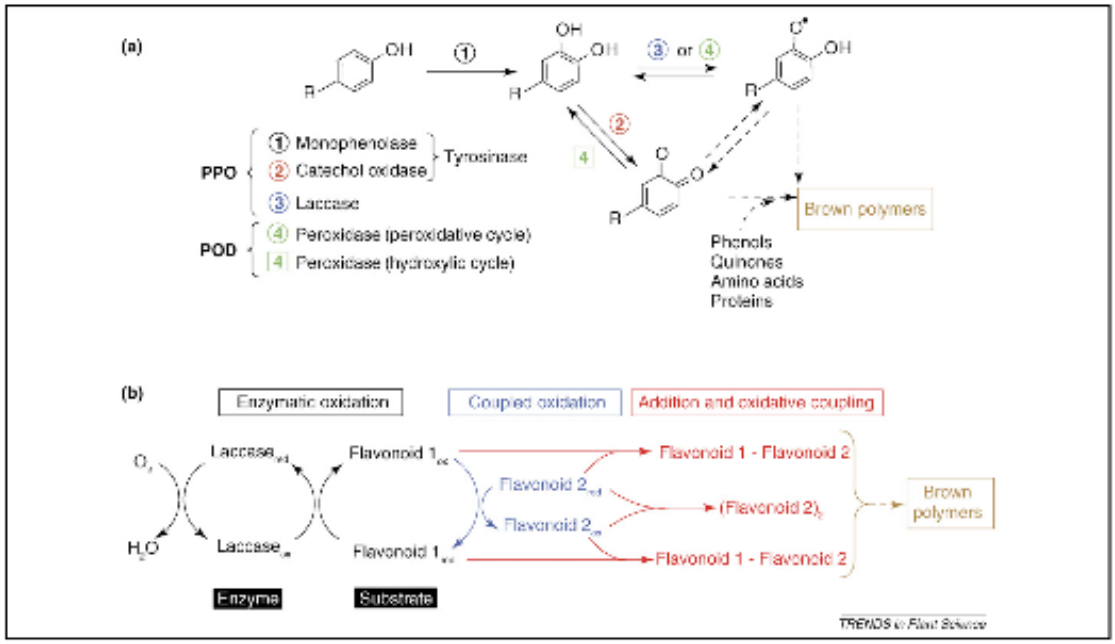

Figure 1.2. Comprehensive diagram of the enzymatic browning reaction, illustrating the initial conversion of monophenols to o-quinones, as well as the coupled oxidation reactions performed by o-quinones on other flavonoids (Pourcel et al. 2006).

Enzymatic browning is a significant problem in the juice, processing, dehydrated fruits and the fresh cut industries. Market reports estimate that increasing the availability of pre-cut apples could expand the apple market in the same way that baby carrots expanded the carrot market - doubling consumption, while at the same

time doubling its price (Brushett and Lacasse 2006; Jellie 2006). Approximately 65% of consumers said they would purchase fresh cut apple slices over whole apples; 55% would purchase both (Jellie 2006).

Currently, fresh cut apples are carefully processed to maintain fruit texture, flavor and appearance. The apples are washed and then sliced by a robotic slicing machine designed to minimize cell damage and therefore decrease browning and water loss. Slices are then treated with a calcium mineral solution such as NatureSeal^{TM} (calcium ascorbate) to preserve texture, crispness and color for up to 21 days. Finally, slices are packed in modified atmosphere packaging and shipped and handled at 34^{o}F to optimize storage life (Rupasinghe et al. 2005; FreshCut.com 2008; NatureSeal.com 2009). Modified atmosphere packaging consists of micro-perforated packaging films that allow for gas exchange with specified oxygen transmission rates (Bliss 2006). Gas exchange is critical, as vacuum packaging leads to a significant decrease in firmness over time and immediate browning upon opening (Lee and Smith 1995). Additionally, pre-slicing storage conditions have a significant effect on the quality of fresh-cut products. Apples stored under controlled atmosphere ("CA", low oxygen, controlled carbon dioxide storage) exhibited significantly lower browning than apples stored in refrigerated air (Chung and Moon 2009).

Several factors affect the rate and extent of apple enzymatic browning. Factors with a strong genetic component, such as specific enzyme activity and substrate availability, will vary at the cultivar level. Rocha & Morais found that the activity of polyphenoloxidase (PPO), the enzyme that catalyzes the reaction, was highly correlated to color changes on minimally processed 'Jonagored' apples over several days, with higher enzymatic activity leading to a greater overall color change on the fruit (Rocha and Morais 2002). Browning was directly correlated with PPO activity in the cultivars 'Classic Delicious', 'Rhode Island Greening', 'McIntosh', 'Cortland',

'Arengeh', and 'Granny Smith' (Coseteng and Lee 1987; Milani and Hamedi 2005). The activity of the enzyme peroxidase can also contribute to enzymatic browning (Nicolas et al. 1994; Pourcel et al. 2006). Additionally, the quantity and type of phenolic substrates available in the apple may affect the rate and extent of the browning reaction, with catechins, chlorogenic acids and caffeic acids most reactive (Amiot et al. 1992). A high positive correlation was found between the rate of browning and total phenolic concentration in the apple cultivars 'Empire', 'Rome', 'Golden Delicious', and 'Delicious' (Coseteng and Lee 1987; Milani and Hamedi 2005). In Japanese pear (*Pyrus pyrifolia*), immature fruits with high browning potential had high PPO activity and a high level of phenolics, while mature fruits with little browning had high PPO activity but a low level of phenolics (Nishimura et al. 2003). In peach (*Prunus persica*), the degree of browning was correlated with total phenolics ($r = 0.67$) (Lee et al. 1990). PPO activity and phenolic content tend to vary at the cultivar level because of the genetic makeup of the different cultivars as well as genotype by environment interactions.

Apple cultivars displaying relatively high levels of enzymatic browning include 'Red Delicious', 'Liberty', 'McIntosh', 'Macoun', 'Rome', 'Rhode Island Greening', 'Stayman', and 'Idared' (Coseteng and Lee 1987; Sapers and Douglas 1987; Kuczyński 1995; Lee and Smith 1995; Milani and Hamedi 2005). Cultivars with relatively lower rates of browning include 'Granny Smith', 'Jonagold', 'Empire', 'Cortland', 'Golden Delicious', 'AutumnCrisp' (NY674), and 'Gala' (Coseteng and Lee 1987; Sapers and Douglas 1987; Kuczyński 1995; Lee and Smith 1995; Milani and Hamedi 2005; DeEll et al. 2009). Cultivars such as 'Gala', 'Pink Lady', 'Granny Smith', and 'Empire' are some of the cultivars commonly used in the fresh-cut apple market due to their low browning rates and maintenance of firmness over time (PetersonFarms 2008). 'AutumnCrisp', a cultivar known for its low browning

potential, does not maintain firmness well in storage, so while not ideal for the fresh cut market, it is still being used (Lee and Smith 1995).

Environmental and cultural factors affecting the rate and extent of apple flesh browning include fruit ripening stage, time in storage and exposure to heat treatment. The earlier an apple is harvested, the higher the browning potential in that fruit (Lozano et al. 1994; Murata et al. 1995; Valentines et al. 2005). Unripe flesh or apple pulp browns much more rapidly than ripe flesh. Rojas-Grau et al. (2006) showed that storage time had a significant effect on the color change of fresh cut apples, with apples stored for two weeks exhibiting a greater overall color change than freshly picked apples. Other research showed that apples exposed to a heat treatment of 40^{o}C, 45^{o}C, or 50^{o}C typically developed flesh browning, perhaps due to accelerated respiration or altered apple metabolism from the adverse environment (Kim et al. 1993; Lee and Smith 1995). Although in-vitro inhibition of the enzyme PPO would not be expected between 40^{o}C and 50^{o}C, in-vivo conditions may vary because of differing micro-environments.

There are many topical treatments available for preventing browning of fresh-cut produce. Historically, sulfites were used, as they are highly effective anti-browning agents that inhibit PPO and react with intermediates to prevent pigment formation (Sapers 1993; Lee and Smith 1995). However, the FDA banned the use of sulfites on fresh fruits and vegetables and required that foods containing more than 10 ppm be labeled due to common allergies to sulfites (Lee and Smith 1995).

Ascorbic acid, or Vitamin C, is the most common sulfite alternative. It acts as a reducing agent, preventing quinone accumulation by reducing it back to the original phenolic form before they are able to polymerize and form pigments (Queiroz et al. 2008). Tested against cysteine, sodium chloride, calcium chloride, citric acid and sodium ascorbate, ascorbic acid showed the highest level of browning inhibition on

'Golden Delicious' apples (Tortoe et al. 2007). Ascorbic acid doesn't completely halt browning, but rather increases the lag period before browning begins from 0 seconds to 10.6 minutes, and decreases the amount of residual activity following the lag period (Wakayama 1994). Once ascorbic acid is fully oxidized, quinone buildup resumes, as does browning (Sapers 1993). Treatment with ascorbate may adversely affect texture and increase the incidence of certain diseases such as blue mold (*Penicillium expansum*), likely by reducing the accumulation of hydrogen peroxide, a compound used by plants in lignification and defense (Lee and Smith 1995; Lamikanra and Watson 2001; Valentines et al. 2005; Cocci et al. 2006). Total polyphenol content is higher in apples treated with ascorbate, likely due to its reducing action, which helps prevent phenolic degradation (Cocci et al. 2006). Ascorbic acid is often applied in the form of calcium ascorbate, an addition which helps prevent on a cellular level the loss of firmness that may result from the over-acidification of apple slices (Lee and Smith 1995).

Additional anti-browning alternatives have been studied. Many of these compounds also act as reducing agents. As with ascorbic acid, the effects of reducing agents are temporary, because they are irreversibly oxidized during the process (Martinez and Whitaker 1995). Competitive inhibitors of PPO that decrease rates of browning include cinnamic acid and 4-hexylresorcinol (Janovitz-Klapp et al. 1990; Son et al. 2001; Gacche et al. 2004; Rojas-Graü et al. 2006; Alvarez-Parrilla et al. 2007). When combined with ascorbic acid or maltosyl-β-cyclodextrin, 4-hexylresorcinol has a synergistic effect on decreasing PPO activity (Alvarez-Parrilla et al. 2007; Arias et al. 2007; López-Nicolás et al. 2007). Glutathione acts as a non-competitive PPO inhibitor and had a significant effect on maintaining color parameters of apple slices (Son et al. 2001; Gacche et al. 2004; Rojas-Graü et al. 2006). Treatment with green tea extract decreased the incidence of browning by

approximately 42% (Soysal 2009). Honey, while not as effective as ascorbic acid, provides some antioxidative benefit, both inhibiting enzymatic oxidation of polyphenols and converting a portion of the o-quinones formed back to their original polyphenols (Oszmiański and Lee 1990). Vacuum impregnation of honey is more effective in controlling browning than simple immersion (Jeon and Zhao 2005). Soluble Maillard Reaction products (MRPs), including those derived from cysteine/glucose or cysteine/ribose, inhibit enzymatic browning in apple pulp (Eissa et al. 2006). Oxalic acid derived from a 20% rhubarb solution (0.07% oxalic acid) effectively inhibited browning for over 2 hours, twelve times longer than a 1% ascorbic acid solution (Son et al. 2000). 'Idared' and 'Sampion' apple puree treated with rhubarb juice extract maintained 2.8 times higher phenolic content and significantly higher antioxidant activity (Oszmiański and Wojdyło 2008). Oxyresveratrol, a compound present in white mulberry (*Morus alba*), inhibited browning of cloudy apple juices at a concentration of 0.01%, but would need to be combined with ascorbic acid for use on fresh cut apples (Li et al. 2007). Sodium chlorite strongly inhibited apple PPO, most prominently at pH 4.5 (Lu et al. 2006).

Enzymatic browning is fairly widespread in plants, and although the enzymatic browning of apples is considered undesirable, browning serves important functions in other species (Pourcel et al. 2006). Enzymatic browning may have a protective function during plant growth and development. As a normal part of seed development, laccases present in *Arabidopsis* seed coats oxidize epicatechin, which might increase the ability of the seed to withstand physical and chemical stresses (Pourcel et al. 2006). Following the desiccation of pea (*Pisum elatius, P. fulvum, P. humile, P. sativum, and P. humile x P. sativum*), cotton (*Gossypium hirsutum* L.), and prickly sida (*Sida spinosa* L.) seeds, PPO-induced oxidation forms quinones and insoluble polymers on the seed coat, leading to browning and hardening of the seed

and impermeability to water (Werker et al. 1979; Halloin 1982; Egley et al. 1983). Browning of the seed coat may aid in coat-imposed dormancy, impeding the entrance of oxygen or water that might allow the plant to germinate, inhibiting leakage of substances such as abscisic acid or carbon dioxide, and scavenging oxygen and peroxides to protect the seed from deterioration during storage (Pourcel et al. 2006).

Enzymatic browning may be an important way for plants to react to environmental stresses. As an antioxidative mechanism, enzymatic browning enables the scavenging of reactive oxygen species, which has been shown to reduce UV radiation stress (Jansen et al. 2001). Some quinones produced by the browning reaction are antimicrobial (Walker and Ferrar 1998; Constabel et al. 2000). These polymers may form a protective barrier around a seed or bulb, which is toxic to some fungal or bacterial pathogens. Browning in onion (*Allium cepa* L.) scales, for example, produces an antifungal agent (3,4-dihydroxybenzoic acid) that protects the onion from fungal infection (Takahama and Hirota 2000). In ginseng (*Panax quinquefolius* L.), reddish brown areas on the root are a result of enzymatic browning and serve as a defense against fungal attack (Rahman and Punja 2005). These quinones may protect germinating embryos from pathogens (Pourcel et al. 2006). Enzymatic browning may serve important biological roles in a variety of plants.

Other Types of Browning

My research is primarily concerned with the enzymatic browning of apple, that is the conversion of phenolic compounds into o-quinones via the enzyme catalyst PPO. Apples are susceptible to several other types of browning as well, including non-enzymatic browning and some storage and physiological postharvest disorders.

The Maillard reaction is a type of non-enzymatic browning especially significant to the juice industry (Burdurlu and Karadeniz 2003). The Maillard reaction

is a non-oxidative reaction which occurs when a carbonyl group reacts with free amino groups, leading to the formation of melanoidin pigments (Bolin and Steele 1987; Sapers 1993). The process is desirable in some foods: the brown colors of cooked meat, coffee, and tea are all due to non-enzymatic browning (Sapers 1993). It can also be destructive, as the process can destroy essential amino acids and vitamins such as ascorbic acid (Vitamin C), which causes a loss in nutritional value (Burdurlu and Karadeniz 2003). Maillard reaction products (MRPs) may have antimicrobial, antioxidative, and mutagenic effects, and their use in the prevention of enzymatic browning in apple slices and pulp has been studied (Sapers 1993; Lee and Shibamoto 2002; Eissa et al. 2006).

Apple fruits are also susceptible to storage and physiological disorders that cause browning. Superficial scald is a physiological disorder which occurs after several months in cold storage and leaves the apple skin diffusely brown and occasionally roughened (Adams and Brown 2007). Scald susceptibility is greatest in less mature fruit and most extensive after several days at room temperature. Scald tends to develop primarily on green skinned apples and the non-blushed areas of red apples, likely because the concentration of simple phenols in the fruit peel at harvest affects the development of scald, and reddish anthocyanin pigments have a protective antioxidative role (Ju et al. 1996). Scald is caused when α-farnesene in the peel is oxidized to form trienols, which are toxic to the fruit. Ethylene is thought to have a major role in the development of superficial scald during cold storage, as treatment with 1-methylcyclopropene (1-MCP, a compound used to block ethylene reception in apple fruits) effectively reduces the incidence and severity of scald (Watkins 2008; Pesis et al. 2009). PPO and peroxidase also may be related to scald susceptibility (Piretti et al. 1996; Fernández-Trujillo et al. 2003). Postharvest drenching of apple

fruits with diphenylamine (DPA) is effective in preventing scald in some varieties (DeEll et al. 2005).

Carbon dioxide damage and internal browning are two apple storage disorders that result in browning. Carbon dioxide damage resembles scald, but is induced by high levels of CO_2 in controlled atmosphere storage (Adams and Brown 2007). Treatment with 1-MCP increases sensitivity to external CO_2 injury in CA storage at 2^oC (DeEll et al. 2005). Internal browning, or brown heart, is a disorder that develops in CA storage and is associated with high internal CO_2 levels in late-harvested, large and over-mature fruit (Adams and Brown 2007).

Finally, Braeburn browning disorder is a physiological disorder related to the harvest time of 'Braeburn' fruits and the concentration of superoxide dismutase. Late harvested 'Braeburn' fruit have a lower quantity of superoxide dismutase and a higher incidence of Braeburn browning disorder (Gong et al. 2001). The risk of the disorder is highest in growing seasons with lower temperatures and in fruit from cooler areas of the orchard, as both circumstances promote lower catalase activity and lower levels of lipid soluble antioxidants (Gong et al. 2001).

The Enzyme Polyphenoloxidase (PPO)

Polyphenoloxidase is the catalyst in the enzymatic browning reaction. As a class of enzymes, the polyphenoloxidases contain a dinuclear copper center bound to histidine residues and function to insert an oxygen in the *ortho*- position to an existing hydroxyl group of an aromatic ring (Mayer 2006; Ni Eidhin et al. 2006). Polyphenoloxidases are wide-spread in animals, plants, fungi and bacteria (Mayer 2006).

The two main classes of polyphenoloxidases are catechol oxidases and laccases (Pourcel et al. 2006). Peroxidase (POD) is a similar enzyme that belongs,

along with the polyphenoloxidases, to a larger grouping of enzymes known as the oxidoreductases (Pourcel et al. 2006). Catechol oxidases, also known as tyrosinase or monophenol oxygenase, are the class of PPO most common in apple fruit. They catalyze the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones, both of which consume molecular oxygen (Pourcel et al. 2006). Figure 1.3 illustrates the hydroxylation and oxidation catalyzed by PPO.

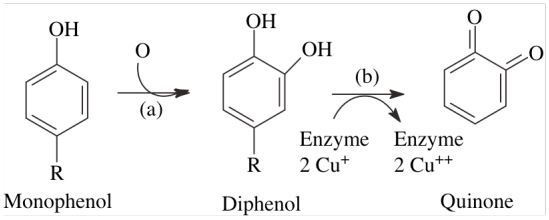

Figure 1.3. The (a) hydroxylation and (b) oxidation reactions catalyzed by PPO (Queiroz et al. 2008).

Laccases also catalyze the oxidation of diphenolic substrates in the presence of oxygen, but unlike catechol oxidases, which can only react with *o*-diphenols, laccases can oxidize both *o*- and *p*- diphenols (Pourcel et al. 2006). Laccases are mainly present in fungi and certain plants, such as peach (*Prunus persica*) and apricot (*Prunus armeniaca*), but absent in most other fruits and vegetables (Nicolas et al. 1994).

Peroxidases are not polyphenoloxidases, but both POD and PPO are oxidoreductases. Peroxidases are hemoproteins that catalyze the oxidation of phenolics through the reduction of hydrogen peroxide in the peroxidative cycle (Pourcel et al. 2006). Although hydrogen peroxide serves several important functions in the cell, including being involved in disease resistance, plant-pathogen interactions, and stress signaling responses, it can cause oxidative damage if left alone in the cell (Valentines et al. 2005). Peroxidases mitigate the potential harm from hydrogen peroxide by transferring its oxidative state to phenolic compounds, which take on the

role of antioxidants. Additionally, peroxidases are involved in lignification of apple fruit, a defense against pathogens (Valentines et al. 2005). Although peroxidases are not the primary enzyme involved in enzymatic browning, they are considered a contributing factor. They do not appear to be involved in enzymatic browning related to bruising or mechanical stress, but rather with slow processes such as internal browning (Nicolas et al. 1994).

PPO's primary function is to serve as a catalyst of the enzymatic browning reaction. In one study, transgenic apple calli with an antisense PPO gene had about half the amount and activity of PPO, and an estimated half of the browning potential of non-transgenic cells (Murata et al. 2001). Browning potential of transgenic 'Orin' and 'Fuji' apple shoots with antisense PPO, gauged by measuring PPO activity in the leaves, was much lower in transformed shoots than in non-transformed shoots (Murata et al. 2000).

Besides catalyzing the browning reaction, polyphenoloxidase serves several important functions in plants. Polyphenoloxidase can enhance the insect resistance of susceptible plants. In one study, the quinones generated by polyphenoloxidases limited available alkylatable dietary proteins to plant-feeding insects, causing their subsequent starvation (Felton et al. 1992). In free feeding experiments, leaves with the most PPO were generally avoided by caterpillars (Constabel et al. 2000). When some plants are wounded, they may shift their metabolism towards increased production of phenolics and enhanced PPO activity, providing more antioxidative and antimicrobial agents to protect the plant from potential harm. Constabel et al. showed that polyphenoloxidase was induced by plant herbivory in hybrid poplar (*Populus trichocarpa* X *Populus deltoides*), with PPO activity in the leaves detected eight hours after wounding (Constabel et al. 2000). Within 16 to 24 hours, the enzyme reached a peak of activity in both wounded and unwounded leaves, suggesting that it serves not

only to repair damage, but also to prevent further damage. In another study, transgenic tomato plants expressed 2 to 5.3 times higher PPO activity than antisense plants (Thipyapong et al. 2006). The increased PPO activity of these plants led to significantly higher mortality of common cutworm (*Spodoptera litura*) and cotton bollworm (*Heliothis armigera*) larvae. In another study of transgenic tomato plants, the growth rate of common cutworms was lower and larvae consumed less foliage on plants overexpressing PPO (Mahanil et al. 2008). PPO potentially made the foliage less nutritious to the leaf-eating insects.

PPO is also thought to be involved in increased disease resistance. The oxidation catalyzed by PPO appears to play an important role in restricting development of infection by the bacteria *Pseudomonas syringae* during early stages of infection in tomato plants (Li and Steffens 2002). Transgenic suppression of PPO resulted in enhanced susceptibility of tomato plants to infection with *P. syringae* (Thipyapong et al. 2004a). PPO-generated quinones and reactive oxygen species (ROS) were thought to play defensive roles against the bacteria. Additionally, direct microbial toxicity of hydrogen peroxide may restrict disease progression and alkylation of plant proteins may reduce the bioavailability of proteins to pathogens (Thipyapong et al. 2007).

Correlations between drought resistance and PPO expression were suggested when transgenic tomato plants with suppressed PPO expression exhibited more favorable water relations and decreased photoinhibition as compared to non-transformed controls (Thipyapong et al. 2004b). PPO may have role in the development of plant water stress and a plant's potential for photoinhibition (Thipyapong et al. 2004b).

Finally, PPO may be involved in decreasing the allergenicity of apples. Apple allergy affects approximately 2% of Northern and Central European populations

(García et al. 2007). IgE binding to Mal d 1, a major apple allergen, may be decreased by oxidative enzymes, especially PPO (García et al. 2007). This PPO-induced decrease in allergenicity is likely due to *o*-quinones, the products of the PPO-catalyzed reactions, cross linking with allergenic proteins (García et al. 2007). Mal d 2 is another apple allergen protein which reacts to almost 75% of apple allergic patient sera (Marzban et al. 2009). Serum IgEs showed a higher affinity to the reduced form of Mal d 2, indicating that the oxidation reaction mediated by PPO likely decreases the overall immuno-reactivity of this allergen as well (Marzban et al. 2009). PPO serves a variety of beneficial functions in plants and animals.

PPO is a significant enzyme with wide distribution among plants (Mayer 2006). Given such wide distribution, differences in the PPO present among various species might be expected. However, within the Rosaceae family PPO structure is highly conserved. Apple PPO has high homology with PPO from Japanese pear (*Pyrus pyrifolia*) (Nishimura et al. 2003). Antibodies derived from 'Fuji' apple PPO cross react with PPO from several other genera in the Rosaceae family, including pear (*Pyrus communis*), Japanese pear (*Pyrus pyrifolia*), peach (*Prunus persica*), Chinese quince (*Pseudocydonia sinensis*) and Japanese loquat (*Eriobotrya japonica*) (Haruta et al. 1999). Even in tomato, the function of PPO is conserved, and it has been linked to browning and degradation of the antioxidant lycopene (Spagna et al. 2005).

Polymerase chain reaction (PCR) cloned DNA fragments from 'Fuji' apple were discovered that encode for a 66-kDa precursor protein and a 56-kDa mature protein (Haruta et al. 1998). PPO from pear (*Pyrus pyrifolia*) was cloned, sequenced and found to have high homology with apple PPO (Nishimura et al. 2003). In potato (*Solanum spp.*), three quantitative trait loci (QTL) alleles for PPO were found, with a clear correlation between allele combinations and the degree of discoloration (Werij et al. 2007). Genotypes with two copies of the alleles had the highest level of gene

expression and the highest degree of discoloration (Werij et al. 2007). In wheat, the complete DNA sequences of two PPO genes were mapped on chromosomes 2A and 2D (He et al. 2007). In apple, a highly significant QTL for the rate of flesh browning, enough to explain 66% and 77% of population variance, was identified on linkage group (LG) 17 of 'Telamon' and 'Braeburn' in one year (Kenis et al. 2008). Unfortunately these data may be linked to environmental conditions during that year, as there were no QTL for browning detected the previous year (Kenis et al. 2008). This was surprising, as major QTL explaining more than 20% of variance tend to be year-stable (Kenis et al. 2008). Igarashi et al. developed markers to map PPO to the bottom of linkage group 10 of 'Red Delicious' (Igarashi et al. 2008).

As with all enzymes, PPO operates optimally under a specific range of conditions. PPO extracted from 'Golden Delicious' apples exhibited maximum activity at pH 5.5 using a 4-methylcatechol substrate (Soysal 2009). However, PPO from 'Monroe' apple peel had optimum activity at pH 4.6 using a 4-methylcatechol substrate, and at pH 5.0 with a catechol substrate (Zhou et al. 1993). PPO extracted from 'Bramley's Seedling' apples exhibited maximum activity at pH 6.5 in vitro (Ni Eidhin et al. 2006). Preferred substrates for apple PPO, in descending order, were: 4-methylcatechol, chlorogenic acid, catechol, pyrogallol and (-)-epicatechin (Zhou et al. 1993; Ni Eidhin et al. 2006). The enzyme follows a first order kinetic model, where PPO activity increases with increased temperature up to 30^{o}C, after which it declines (Sapers 1993; Zhou et al. 1993; Yemenicioğlu et al. 1997). The enzyme is heat stable up to 40^{o}C, but inactivates above 50^{o}C.

PPO tends to be distributed uniformly in immature apple fruits and mainly localized around the core in mature fruit (Murata et al. 1995). This concurs with research showing that PPO activity is highest in the core and lowest in the peel (Wakayama 1994). Apple PPO resides mostly in plastids within the cell (Murata et al.

1997). In the plastids, it is kept inactive and stabilized by chaperones; control of the activation process might be one way to up or down-regulate PPO activity (Marquès et al. 1995). In immature fruits, PPO is located in organelles other than the vacuole, most likely plastids, while in mature fruit PPO is located near the cell wall (Murata et al. 1997). This compartmentalization within plastids allows PPO to remain inactive and separate from the phenolic compounds stored in the vacuole, which it would otherwise oxidize (Queiroz et al. 2008). When tissues are damaged, the plastids rupture, causing the enzyme to come in contact with the phenolic compounds, which starts the browning reaction (Queiroz et al. 2008).

In addition to being isolated from its substrates in separate subcellular compartments, most plant PPOs are present in a latent form that requires activation (Pourcel et al. 2006). In vitro, latent catechol oxidase extracted from apple fruits and beets (*Beta vulgaris*) can be activated by the addition of sodium dodecyl sulfate (Marquès et al. 1994; Gandía-Herrero et al. 2005). In vivo, PPO is frequently latent, with both wounding and herbivore attack converting PPO into its active form by an unknown mechanism (Mayer 2006). PPO cDNA derived from apple peel was up-regulated in wounded tissues (Boss et al. 1995). PPO mRNA accumulated in wounded tissues and peel tissues showing signs of superficial scald, indicating that there is transcriptional control of PPO expression after wounding or physiological disorders (Boss et al. 1995; García et al. 2007).

PPO may be inactivated by a number of agents, such as acids, chelating agents, reducing agents and quinone couplers such as cysteine (Sapers 1993). Copper is a necessary cofactor for the enzyme's activity, so chelating any available copper inactivates PPO (Mayer 2006). Oxidized procyanidins, epicatechin and caffeoylquinic acid, which are the reaction products of enzymatic browning, also inhibit PPO activity (Le Bourvellec et al. 2004). Maillard reaction products, specifically those containing a

thiol group, effectively inhibit PPO as well (Billaud et al. 2005a; Billaud et al. 2005b; Eissa et al. 2006). However, the lack of stability of MRPs limits their application in food, and apple slices treated with MRPs have an unpleasant odor, which becomes more apparent after long term storage (Wu et al. 2008). PPO is also inactivated by ultra-violet-C (UV-C) light exposure in both model systems and food (Manzocco et al. 2009). UV-C light exposure caused a progressive decrease in PPO activity in apple juice, but the juice browned more extensively than under dark conditions, likely because of direct photo-oxidation of phenolic substrates (Manzocco et al. 2009). UV-C light exposure on fresh cut apple slices completely inactivated PPO, with no subsequent enzymatic browning of the slices nor degradation in quality, appearance or flavor (Manzocco et al. 2009). Pulsed electrical fields in combination with mild heat treatment have also been used to inactivate PPO and peroxidase in apple juice (Riener et al. 2008).

PPO activity is affected by apple maturity and time in storage. During maturation, the general trend is for PPO activity to decrease from the early stages of maturity through harvest time (Coseteng and Lee 1987). Murata et al. 1995 found that total PPO activity in 'Fuji' apples increased 2.5 to 4.5 months after flowering, but decreased from 4.5 to 6 months after flowering. During the same time, the absolute quantity of PPO per milligram of protein remained constant, implying that some of the PPO denatured over time. In another study, the PPO activity in 'Red Delicious' fruit declined steadily during the 4 weeks before and 4 weeks after commercial harvest (Janovitz-Klapp et al. 1989).

The effect of storage on PPO activity is subject to debate. PPO activity remained relatively constant in several cultivars ('Classic Delicious', 'Rhode Island Greening', 'McIntosh', 'Cortland', 'Empire', 'Rome', and 'Golden Delicious') during 3 months in cold storage (Coseteng and Lee 1987), while PPO activity in 'Jonagored'

apple slices increased over time in storage (Rocha and Morais 2002). Others observed a dramatic or gradual decrease in PPO activity over a period of time in storage (Barrett et al. 1991; Leja et al. 2003). Controlled atmosphere storage decreased the PPO activity of pre-sliced 'Golden Delicious' apples by 62% (Soliva-Fortuny et al. 2001). Chung & Moon observed that PPO in 'Tsugaru' apples was unaffected by varying storage conditions between regular cold storage and controlled atmosphere storage (Chung and Moon 2009).

Phenolic Compounds

Phenolic compounds are the substrates in the enzymatic browning reaction. In addition to serving this important role, they are also the primary contributors of antioxidative capability in apple fruits (Lee et al. 2003; Tsao et al. 2005). In a study comparing 34 different fresh fruits and vegetables most commonly consumed by Americans, apples contributed the second highest amount of phenolics and antioxidants to the American diet, second to oranges (Chun et al. 2005). Americans consume nearly 43.4 lbs of apple per person per year, more than any other fruit except for oranges, and although they are not the most antioxidant dense fruit (blueberries, plums, and strawberries are higher), they are among the most popular (Chun et al. 2005). Apples provide approximately 20 to 25% of the total antioxidants consumed by Americans (Vinson et al. 2001).

There are three main classes of phenolic compounds in apples: phenolic acids, dihydrochalcones and flavonoids (Nicolas et al. 1994; Podsędek et al. 2000). The phenolic acids, also known as the hydroxycinnamic acids, include primarily chlorogenic acid (3-*o*-caffeoyl-D-quinic) and its isomers, and comprise approximately 4 to 18% of polyphenols in apples (Vrhovsek et al. 2004; Cheynier 2005; Biedrzycka and Amarowicz 2008). Approximately 8% of chlorogenic acid is in the apple peel

(Łata et al. 2009). There is no significant correlation between chlorogenic acid and apple flesh browning (Song et al. 2007).

The dihydrochalcones include phloridizin and phloretin xyloglucoside, and comprise 2-6% of total apple phenolics (Vrhovsek et al. 2004). Phloridizin is a phenolic compound unique to apples (Cheynier 2005; Song et al. 2007). It represents 8% of the total phenolic concentration in apple peels; half of the phloridzin in apple is in the peel (Tsao et al. 2003; Łata et al. 2009).

Flavonoids are a class of compounds containing two phenolic rings and an oxygenated heterocycle (Cheynier 2005). They may be further subdivided into flavonols, flavan-3-ols and anthocyanins (Pourcel et al. 2006). The flavonols, which include quercetin-3-galactoside and its mono- and diglycerides, are colorless to pale yellow and contribute 1-11% of total apple phenolics and approximately 18% of phenolic concentration in apple peel (Tsao et al. 2003; Vrhovsek et al. 2004; Biedrzycka and Amarowicz 2008).

The flavonol quercetin is a particularly potent antioxidant, with much stronger antioxidative and anticarcinogenic activity than Vitamin C (Heo and Lee 2004). Quercetin is highly abundant in apple peel, and although quercetin glycosides are only slightly more abundant than Vitamin C, they contributed nearly three times the antioxidative power as Vitamin C (Lee et al. 2003). Quercetin structurally is highly advantageous as an antioxidant, because the o-dihydroxy group present on one of its rings adds stability to the resulting free radical form (Salah et al. 1995). Neuronal cells pre-incubated with quercetin and Vitamin C showed much greater resistance to peroxide induced toxicity in a dose-dependent manner (Heo and Lee 2004). Peroxidative damage may be particularly important in neurodegenerative diseases such as Alzheimer's and Parkinson's (Heo and Lee 2004). Quercetin decreased this oxidative stress more efficiently and at lower doses than did Vitamin C, indicating that

it might be useful in the fight against neurodegenerative diseases (Heo and Lee 2004). Quercetin glycosides provide protective effects against cancer, leukemia and heart disease, down-regulating the expression of mutant p53 in breast cancer cells and protecting Caco-2 colon cells from lipid oxidation, a carcinogenic phenomenon (Lamson and Brignall 2000; Peng and Kuo 2003).

The flavan-3-ols are the most common class of polyphenols in apples, and include (-)-epicatechin, (+)-catechin and procyanidins (Burda et al. 1990; Pourcel et al. 2006). The catechins differ from the procyanidins in that catechins are monomeric and procyanidins are polymers (Alonso-Salces et al. 2004). Flavan-3-ols are colorless and comprise approximately 65 to 90% of total apple polyphenol contents (Vrhovsek et al. 2004). Procyanidins dominate in both apple peel and flesh (Tsao et al. 2003). 24% of (+)-catechin and 32% of (-)-epicatechin are in the peel (Łata et al. 2009). Procyanidins are the only polyphenols that are true tannins: they form strong bonds with proteins, producing a bitter or astringent taste (Merwin et al. 2008).

Anthocyanins include cyanidin-3-galactoide and other cyanidin glycosides and comprise 1 to 3% of total apple phenolics (Vrhovsek et al. 2004). They are red to purple in color, occur only in red apple varieties, and accumulate in the skin towards the end of ripening (Cheynier 2005).

The relative concentration of different phenolic compounds, as well as the total concentration of phenolic compounds, varies by cultivar (Łata et al. 2005; Song et al. 2007; Łata et al. 2009). The polyphenols most correlated to browning are, in order: total polyphenols, proanthocyanidins, (+)-catechin, and phloridizin (Song et al. 2007). More than just the substrates of the browning reaction, phenolic compounds are important antioxidants in the cell. The activity of an antioxidant is determined by its reduction potential (reactivity in donating hydrogen or electrons), the stability of the radical resulting from the antioxidative reaction, its reactivity with other antioxidants,

and its transition metal-chelating potential (Rice-Evans et al. 1997). The redox potential of an apple corresponds to the total fruit phenolic content, and even low concentrations of phenolics may protect apples from oxidative damage (Kuczyński 1999; Podsędek et al. 2000). Phenolic compounds are powerful antioxidants that help protect against ultraviolet radiation (Jansen et al. 2001). Flavonoids in the epidermis absorb ultraviolet radiation, and act as scavengers of reactive oxygen species, preventing the peroxidation of liquids and protecting internal tissues from oxidative damage (Treutter 2006).

Phenolic compounds also exhibit defensive properties, protecting plants from certain pathogens and herbivores. Flavonoid synthesis is induced in plants in response to injury from pathogens and microbial invasion (Treutter 2006; Biedrzycka and Amarowicz 2008). Flavonoids are capable of targeting microbes at multiple levels by their ability to complex with extracellular and soluble proteins, bacterial cell walls and cell wall polypeptides, surface exposed adhesions and membrane-bound enzymes (Cowan 1999). High concentrations of flavonoids in fruits often correlate to a low incidence of pathogen infection, a phenomenon that explains why unripe fruits, typically higher in flavonoid content, are also more resistant to fungal decay than ripe fruits (Treutter 2006). The production of defensive plant flavonoids is metabolically expensive and occurs at the expense of plant growth, indicating that flavonoids likely serve valuable functions in plants (Treutter 2006).

Flavonoids may also restrict insect predation on fruits by acting as digestibility reducers, limiting the ability of herbivores to utilize dietary proteins and inhibiting the predators' digestive enzymes (Pourcel et al. 2006; Treutter 2006). They may also act as feeding deterrents and toxins to specifically targeted species (Treutter 2006). Some insects are capable of sequestering flavonoids in their cuticle as a defense against predators, or in their wings as a way to attract mates (Treutter 2006).

Flavonols may also account for defense against apple scab (*Venturia inaequalis*) (Treutter 2001). Scab resistant cultivars respond to scab infection by accumulating flavonols in surrounding cells during barrier formation (Treutter 2001). Inhibition of the enzyme phenylalanine ammonialyase (PAL), an enzyme necessary for the synthesis of flavanols in plants, leads to severe scab symptoms (Treutter 2001). Scab resistant apple cultivars have significantly higher content of certain phenolics such as chlorogenic acid, p-coumaric acid, catechin and epicatechin, when compared to scab susceptible cultivars (Petkovsek et al. 2007). Phloridzin is the phenolic compound most responsible for scab resistance (Leser and Treutter 2005). Environmental conditions conducive to excess vegetative growth, such as high nitrogen fertilization, also inhibit flavonoid biosynthesis and increase scab susceptibility in otherwise resistant cultivars (Leser and Treutter 2005).

Flavonoids also appear to play a role in environmental interactions and in signaling between plants and microbes (Winkel-Shirley 2001; Treutter 2006). The flavanone naringenin can stimulate colonization of wheat (*Triticum aestivum* L. cv. Canon) roots by the bacteria *Azorhizobium caulinodans*, leading to increased root growth (Webster et al. 1998). Nodulation of red alder (*Alnus rubra*) is thought to be influenced by flavonoids (Benoit and Berry 1997). There also appears to be some relationship between flavonoid activity and the symbiotic relationship of arbuscular mycorrhizal fungi with plants (Treutter 2006). Flavonoids may also be involved in providing protection against environmental stresses such as frost, drought and toxic metals in the soil (Treutter 2006). Some flavonoids may also provide reproductive benefits, attracting pollinators and seed dispersers, particularly the brightly colored anthocyanin pigments (Winkel-Shirley 2001).

Phenolic compounds serve several important functions in cider apples. The most predominant polyphenolic tannins in cider apples are epicatechin procyanidins,

chlorogenic acid, coumaroyl quinic phenolic acids and phloridzin (Merwin et al. 2008). In ciders, PPO reacts with phenols to create dark melanin pigments, a process which may be mitigated by eliminating oxygen or inactivating the PPO (Merwin et al. 2008). The additives ascorbic acid, sodium bisulfate and thiol compounds are all effective in inactivating PPO, as are heat and pasteurization treatments (Merwin et al. 2008).

Phenolic compounds also affect the balance of bitterness to astringency, or the "mouthfeel" of a cider (Merwin et al. 2008). Apples with greater total polyphenol content, especially flavan-3-ols and dihydrochalcones, are more bitter (Valois et al. 2006). The degree of polymerization of procyanidins affects how they taste, with polymer chains 2-5 units in length having a "bitter" or "hard tannin" taste, and polymer chains 6-10 units in length having an "astringent" or "soft tannin" taste (Alonso-Salces et al. 2004; Merwin et al. 2008). Bitterness may be defined as a sharp or stinging sensation on the sides or back of the tongue, while astringency is considered to be a drying or puckering of the tongue (Merwin et al. 2008). Phloridzin may also contribute to the overall bitterness of a cider apple (Merwin et al. 2008). Phenolics in ciders provide cider with aroma, colloidal stability, and help control fermentation and cider spoilage by inhibiting microbial growth (Merwin et al. 2008). They also may be involved as metabolites in alcoholic and malolactic fermentation, where sugar is fermented to form the cider alcohol (Valois et al. 2006). Polyphenolic contents tend to ensure balance, clarity, and astringency in ciders, all of which contribute to the complexity and fullness of flavor (Valois et al. 2006).

Phenolic compounds have significant benefits to human health. Phenolics may be involved in tumor suppression - nearly one third of cancer deaths might be avoided by positive changes in the diet (Liu et al. 2001). In an extensive review, 128 of 156 dietary studies showed that individuals consuming high amounts of phenolic-rich

fruits and vegetables have half the cancer risk as those who eat small quantities of them (Block et al. 1992). Apple polyphenols induce expression of at least 30 different genes, including those coding for specific enzymes related to tumor suppression, cell cycle arrest and regulation, apoptosis signaling, stress and signal transduction, and detoxification enzyme systems (Veeriah et al. 2008). These actions inhibit cell proliferation, induce cell differentiation and apoptosis and enhance anti-inflammatory responses in vitro (Veeriah et al. 2008). The anti-tumor activity of apples is likely due to this inhibition of inflammation as well as the modulation of gap-junction intercellular communication, or GJIC (Lee et al. 2005). GJIC is essential for maintaining the homeostatic balance of cells by modifying cell proliferation and differentiation in multicellular organisms, and inhibition of GJIC may be related to the carcinogenicity of hydrogen peroxide (Lee et al. 2005). The strong inhibition of tumor-cell proliferation in vitro could be due to the specific combination of phytochemicals found in apples (Eberhardt et al. 2000). The phenolic compounds gallic acid and quercetin inhibit the proliferation of Caco-2 cells (human colon cancer cells) in a dose-dependent manner (Lee et al. 2005). Other apple antioxidants do not directly inhibit tumor cell proliferation, but rather indirectly inhibit cell proliferation by generating H_2O_2 in cell culture media (Lapidot et al. 2002).

Phenolic and flavonoid concentration also correlated with overall antioxidative capacity. 'Gala' apples and plums exhibit a strong correlation between total phenolics and VCEAC (r^2=.938), as well as total flavonoids and VCEAC (r^2=.942) (Kim et al. 2003a; Kim et al. 2003b). Total oxyradical scavenging capacity, or TOSC, was also highly correlated to flavonoid content (r^2=.74) and total phenolics (r^2=.73) across several apple cultivars (Liu et al. 2001). Kim and Lee found a positive linear relationship between VCEAC and the number of free hydroxide groups around the flavonoid framework (Kim and Lee 2004). As antioxidants, phenolic compounds may

help prevent degenerative diseases associated with oxidative stress, such as cardiovascular disease (Wolfe and Liu 2003; Boyer and Liu 2004; Pourcel et al. 2006). Women consuming the highest amount of flavonoids in their diets showed a 35% reduction in cardiovascular disease (Sesso et al. 2003). Intake of catechin and epicatechin was inversely related to coronary heart disease death (Arts et al. 2001). Apple polyphenols help lower the ratio of non-HDL (high density lipoprotein) cholesterol to HDL cholesterol, which might lower an individual's risk of coronary heart disease (Lam et al. 2008). Apple pectin and a polyphenol-rich apple concentrate are more effective in tandem than separately in reducing cholesterol and triglycerides in plasma (Le Bourvellec et al. 2009).

Apple consumption is inversely linked with asthma and positively associated with good pulmonary health (Boyer and Liu 2004). Both apple consumption and selenium intake are associated with reduced incidence of asthma in the United Kingdom, with the healthiest subjects eating at least two apples a week (Shaheen et al. 2001). These benefits were linked to flavonoids, especially quercetin, hesperitin and naringenin. Consumption of apples has also been linked to a decreased risk of diabetes, with quercetin intake most significantly associated with lowering the incidence of type II diabetes (Knekt et al. 2002).

Although flavonoids provide significant health benefits, it is not possible to achieve these benefits simply by taking a supplement - the whole food must be consumed (Biedrzycka and Amarowicz 2008). There are additive and synergistic effects of the phytochemicals in fruits and vegetables that together provide these powerful antioxidative and anticancerous benefits (Biedrzycka and Amarowicz 2008). More *in vivo* studies are needed to determine the true bioavailability and physiological effects of flavonoids.

The total polyphenolic content varies in apples among different cultivars (Song et al. 2007). Cider apples, which traditionally have a high polyphenolic content and are generally considered unpalatable for fresh consumption, have phenolic content ranging from 2525 to 3512 mg/kg fresh weight (Song et al. 2007; Merwin et al. 2008). Bitter cider varieties have more phenols than sweet or acid apples, and these phenols tend to contribute complexity, body, and a well-rounded mouth-feel to a cider (Song et al. 2007; Merwin et al. 2008). Juice apples tend to have much lower polyphenolic content, ranging from 548 to 1085 mg/kg fresh weight (Song et al. 2007). Fresh and dessert apples tend to have high soluble solids content (sugars and sugar alcohols), low polyphenolic content and distinctive aromas or flavor (Valois et al. 2006).

Phenolic compounds vary by cultivar, both in quantity and type (Lee and Smith 2000; Liu et al. 2001). Cultivars with relatively higher phenolic content include 'Fuji', 'Red Delicious', 'Gala', 'Liberty', 'Braeburn', 'Idared' and 'Granny Smith' (Liu et al. 2001; Markowski and Płocharski 2006; Biedrzycka and Amarowicz 2008; Łata et al. 2009). Cultivars with relatively lower phenolic content include 'Jonagold', 'Empire', and 'AutumnCrisp' (NY674) (Liu et al. 2001). Total phenolics and total flavonoids show a high correlation with antioxidant activity (measured in Gallic Acid equivalents), with R^2 of 0.822 and 0.717, respectively (Yoo et al. 2008).

The apple peel has about two to nine times more phenolics than the pulp (Petkovsek et al. 2007). Phenolic compounds are highest in the peel, lowest in the outer part of the cortex, and increase gradually towards the core (Petkovsek et al. 2007). Total phenolics, expressed as mg gallic acid equivalent per 100 g fresh weight, are higher for nearly all apples with their skin than not, indicating that there is a higher concentration of phenolics in the peel than in the flesh (Liu et al. 2001). With the exception of chlorogenic acid, 50% or more of total phenolics were present in the peel of several apple cultivars (Łata et al. 2009). This is especially significant given that

the peel contributes only 10% of the total fruit weight, on average (Łata et al. 2009). This also varies by cultivar - 'Starking Delicious' had over 82% of its phenolic compound in the peel, while 'McIntosh and 'Prima' apples had just 26 to 29% of their total phenolics in the peel (Łata et al. 2009). Nearly all of the flavonol content (quercetin) is in the skin (Biedrzycka and Amarowicz 2008). Not surprisingly, the antioxidative and antiproliferative activity is significantly higher in apples with skin than in peeled apples (Tarozzi et al. 2004; Łata 2007).

Phenolic compounds are synthesized through a several step process from the precursor phenylalanine using the enzyme phenylalanine ammonia lyase (Treutter 2001). Over the course of development and maturation, the concentration of phenolics is high in young apple fruits, decreases during early development, and then remains constant during maturation and storage (Burda et al. 1990; Boyer and Liu 2004; Renard et al. 2007; Biedrzycka and Amarowicz 2008). The tendency for the fruit to brown likewise decreases throughout fruit development and maturation (Burda et al. 1990). Most polyphenols that will be synthesized are present 50 days after full bloom, which corresponds to the transition between cell proliferation and cell growth (Renard et al. 2007). At the intermediate (cell growth) phase, enzyme activity decreases, and synthesis persists but at low levels throughout fruit growth (Burda et al. 1990; Renard et al. 2007). When the fruits are nearly mature, most polyphenol synthesis enzymes are less active, but some increase, including those responsible for flavanol and anthocyanin production, which aid in color development (Burda et al. 1990; Treutter 2001).

Cultural and environmental conditions affect the overall level of phenolic content in an apple fruit. Fruit exposed to sunlight had significantly higher anthocyanins and quercetin glycosides than shaded fruits (Awad et al. 2000). Wounding and pathogen attack both increased the biosynthesis of phenolic

compounds, likely by activating the enzyme PAL, which increases production (Treutter 2001). Apples grown organically or using integrated methods of cultivation did not differ in phenolic content or antioxidative bioactivity (Tarozzi et al. 2004; Peck et al. 2009). Nutrient content and fertilization affect phenolic content. Increased nitrogen fertilization leads to an increase in overall shoot growth but a decrease in total phenolic production (Leser and Treutter 2005). Excess nitrogen fertilization is associated with a decrease in anthocyanins, catechins and total flavonoids, as well as a decreased percentage of fruit blush (Awad and De Jager 2002). Calcium fertilization of 'Elstar' is associated with an increase in anthocyanins and total flavonoids (Awad and De Jager 2002).

Treatment with 1-MCP increased the antioxidant potential of apple fruits, leading to lower levels of oxidative stresses and peroxidative markers, as well as higher peroxidase activity compared to non-treated fruits (Vilaplana et al. 2006). 1-MCP treated fruits stored in air had higher phenolic content in the peel, but slightly lower phenolic content in the flesh (Fawbush et al. 2009). Treated fruits stored in controlled atmosphere storage had much higher flavonoid concentration than untreated fruits (Fawbush et al. 2009). No correlation was found, however, between total phenolic content and antioxidant activity (Fawbush et al. 2009). Different flavonoids are affected differently by treatment with 1-MCP. While total flavonoid content was 5% greater in 1-MCP treated fruit, chlorogenic acid levels were 24% lower (MacLean et al. 2006). Untreated fruits had generally increasing levels of chlorogenic acid after storage. Late-harvested fruits were unaffected by 1-MCP treatment, suggesting that 1-MCP likely inhibits the activity of the flavonoid precursor enzyme PAL (MacLean et al. 2006). There was no net effect of 1-MCP on total flavonoids or total anthocyanin content of 'Starking' apples after 4 weeks in storage (Li et al. 2008a).

Postharvest storage and processing may affect the phenolic content of apple fruits. In some studies, storage decreased total phenolic content, while in others, total phenolic content increased. Total phenol concentration of 'McIntosh', 'Red Delicious', 'Jonagored', 'Empire', and 'Golden Delicious' remained relatively constant during three months in storage (Coseteng and Lee 1987; Rocha and Morais 2002; Napolitano et al. 2004). (Golding et al. 2001) found peel phenolic metabolism relatively stable over 9 months in regular cold storage, especially in apples treated with postharvest DPA. Flavonoids present in apple are stable after harvest, showing no losses during storage in regular and controlled atmosphere conditions (Awad and De Jager 2000). Quercetin glycosides, phloridzin, and anthocyanin content in several cultivars ('Jonagold', 'Golden Delicious', and 'Elstar') was not affected by 52 weeks in CA storage (Van der Sluis et al. 2001).

The phenolic compounds in fresh cut 'Tsugaru' apples tend to decrease during storage (Chung and Moon 2009). Total phenolics and total antioxidant activity decreased during three months in storage, but only in apples with skin, implying that cold storage rapidly deteriorates antioxidants in the skin but not the pulp (Tarozzi et al. 2004). The antioxidant bioactivity decreased significantly over six months in cold storage, so that one would need to consume two apples stored for six months to obtain the same benefit as one fresh apple (Tarozzi et al. 2004). There were no significant differences in total phenolic compounds, flavonoids, or chlorogenic acid between fruits stored in controlled atmosphere versus regular storage conditions (Awad and De Jager 2000; Chung and Moon 2009).

Processing may cause heavy losses of phenolic compounds. Only 19 to 40% of the original phenolics remain in clear apple juices, depending on the method of processing used (Markowski and Płocharski 2006). Freeze-dried and air dried apple

peels retain phenolic compounds better than oven-dried samples (Wolfe and Liu 2003).

Ascorbic Acid (Vitamin C)

Vitamin C, also known as L-ascorbate or L-ascorbic acid (L-AA), is an important antioxidant in apple fruits. As an antioxidant, it is capable of reacting with physiologically generated reactive oxygen species (ROS), such as photosynthetically derived hydrogen peroxide, singlet and triplet oxygen, superoxide, nitric oxide and hydroxyl radical, to inactivate free radicals (Davey et al. 2000; Grennan 2008). Continual damage by ROS contributes to the aging and senescence process in humans and plants (Grennan 2008). Quantitatively, Vitamin C and glutathione are the most common low-molecular-weight antioxidants in plants (Davey et al. 2007).

Vitamin C is structurally one of the simplest vitamins, related in form to the six-carbon sugars. A diagram of Vitamin C is given in Figure 1.4. L-ascorbic acid has two pK_as, at 4.13 and 11.6, and so it exists as the monovalent anion L-ascorbate at physiological pH.

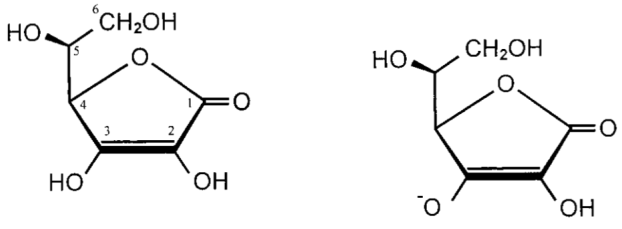

Figure 1.4. L-ascorbic acid and L-ascorbate (Davey et al. 2000).

L-ascorbate oxidizes readily, especially when there are trace amounts of copper, iron or alkali present (Davey et al. 2000). Ascorbic acid oxidizes via the

enzyme ascorbate peroxidase in a two step process, first converting into monodehydroascorbate (MDHA), and then into dehydroascorbate (DHA) (Li et al. 2010). L-AA can also be regenerated from these two products via reduction using electrons from NADPH and GSH (Li et al. 2010). Because of this, whether ascorbic acid is in the oxidized or reduced form can indicate the overall redox state of the cellular environment and, by extension, the extent of cell stress (Davey et al. 2000; Li et al. 2010).

Vitamin C has several additional roles in apple plants beyond being an antioxidant. The activation of L-AA and glutathione, along with other antioxidants, appears critical to a plant's ability to withstand stressful conditions (Davey et al. 2004; Li et al. 2010). Plants deficient in either of these antioxidants are highly sensitive to environmental stresses (Conklin et al. 1996; Cobbett et al. 1998). Moderate drought stress increased the ascorbate concentration in leaves of 'Jonagold' apples, making ascorbate concentration a reliable indicator of drought stress (Šircelj et al. 2007). Vitamin C also appears to contribute to postharvest disease resistance. A decrease in susceptibility to *Botrytis cinerea* infection is correlated with increasing Vitamin C levels in apple fruits (Davey et al. 2007). However, an excess of Vitamin C may increase disease incidence. Topical ascorbate on apple fruits was thought to inhibit oxidative processes that lead to an accumulation of hydrogen peroxide, a compound important in resistance to *Penicillium expansum* in apples (Valentines et al. 2005).

Vitamin C is involved metabolically in cell proliferation and elongation, as an enzymatic cofactor and as a donor/acceptor in electron transport. L-ascorbate modulates a number of enzymatic reactions for certain mono- or dioxygenase enzymes containing iron or copper at the active site. It functions as an enzymatic cofactor by maintaining the transition metal centers of these enzymes in a reduced form, thereby maximizing the enzyme's activity (Davey et al. 2000). Both ascorbate and the enzyme

ascorbate oxidase are involved in the modulation of cell growth and cell proliferation (Davey et al. 2000). MDHA and DHA, both products of ascorbate oxidation, directly increase cell expansion in onion root cells, likely from increased trans-membrane electron transport using MDHA as an electron acceptor (Smirnoff and Wheeler 2000).

Vitamin C also plays a critical role in human health as one of the most effective and least toxic antioxidants, providing protection against oxidative-stress related diseases such as cancer, cardiovascular disease, stroke, cataract formation, aging and neuro-degenerative diseases (Davey et al. 2000; Lee et al. 2002; Hancock and Viola 2005; Davey and Keulemans 2009). Vitamin C boosts immune system function by increasing the amount of available leucocytes, and promotes wound healing. It aids in the biosynthesis of some hormones, including folate, carnitine and adrenal hormones, and increases the bioavailability of adrenaline (epinephrine) (Kern and Bernards 1997; Davey et al. 2000). In meat-poor diets, Vitamin C increases the uptake of iron and zinc (Frossard et al.). It promotes collagen synthesis, including the formation and maintenance of cartilage, bones, gums, skin and teeth (Davey et al. 2000). Inadequate Vitamin C consumption leads to the disease scurvy (Davey et al. 2000). Since humans are unable to synthesize Vitamin C, they must rely on plants as a primary source of this important vitamin (Giovannoni 2007).

In some cultivars of apple, Vitamin C is one of the most abundant antioxidants (Lee et al. 2003). It is not, however, one of the most powerful ones. The relative effectiveness of an antioxidant can be measured using the Vitamin C equivalent antioxidant content scale (VCEAC), which compares radical scavenging activity of different compounds using Vitamin C as a standard. Because different antioxidants in an apple are found in differing concentrations, it is helpful to consider their antioxidative activity in terms of both scavenging activity as well as concentration (Kang et al. 2004). Some flavonoids have stronger antioxidant capacities against

peroxyradicals than Vitamin E, Vitamin C and glutathione (Chun et al. 2005). Polyphenolic antioxidants showed higher VCEAC compared to monophenolics (Kim and Lee 2004). In 'Empire' apples, the VCEAC values of several important antioxidants were, in order from greatest to least: quercetin (VCEAC of 3.91), epicatechin (2.44), procyanidin B2 (2.10), phloretin glycosides (1.64), chlorogenic acid (1.07), and finally, Vitamin C (1.00) (Kang et al. 2004). In 'Gala' apples, Vitamin C showed a greater VCEAC than chlorogenic acid, but less than quercetin, epicatechin, and catechin (Kim et al. 2002).

Vitamin C is not the greatest contributor to total antioxidant status in some apple cultivars due to its relatively low contribution to antioxidative capability. In 'Empire' apples, which contain 6.61 mg Vitamin C per 100 g of fruit, Vitamin C contributes only 11% of the total antioxidant capacity of the apple (Lee et al. 2003). Vitamin C accounts for only 0.4% of the antioxidant activity of 'Red Delicious', where it has a concentration of 5.7 mg/100 g (Liu et al. 2001). In cultivars containing higher concentrations of Vitamin C, some up to 25 mg/100g, Vitamin C would contribute a more significant portion of the overall antioxidative ability of the fruit.

Although some substances may have stronger antioxidant and antiproliferative effects, Vitamin C is still an important bioactive component. Vitamin C provides powerful preventative effects against peroxidative inhibition of gap-junction intercellular communication (GJIC), a phenomenon strongly related to carcinogenesis and tumor promotion (Lee et al. 2002). Inhibition of GJIC was prevented by pre-treatment with 100 umol/L of Vitamin C, while treatment with the known free radical scavengers propylgallate and trolox did not prevent inhibition of GJIC by H_2O_2. This indicates that Vitamin C may provide benefits to cells beyond simple free-radical scavenging (Lee et al. 2002).

Additionally, consumer acceptance of nutritional benefits can be an important factor in the marketability of fruits. Vitamin C is a familiar nutrient with well-advertised and well-known benefits among consumers. Functional ingredients in food that are familiar to consumers gain higher levels of acceptance than ingredients that have been in use for a short time only (Lampila et al. 2009). Novel food processing methods are often a concern to consumers, as are newer types of genetic improvement, such as genetic engineering. Consumers considered classical breeding to be the best way to enhance the nutritional value of fresh fruits and vegetables (Lampila et al. 2009).

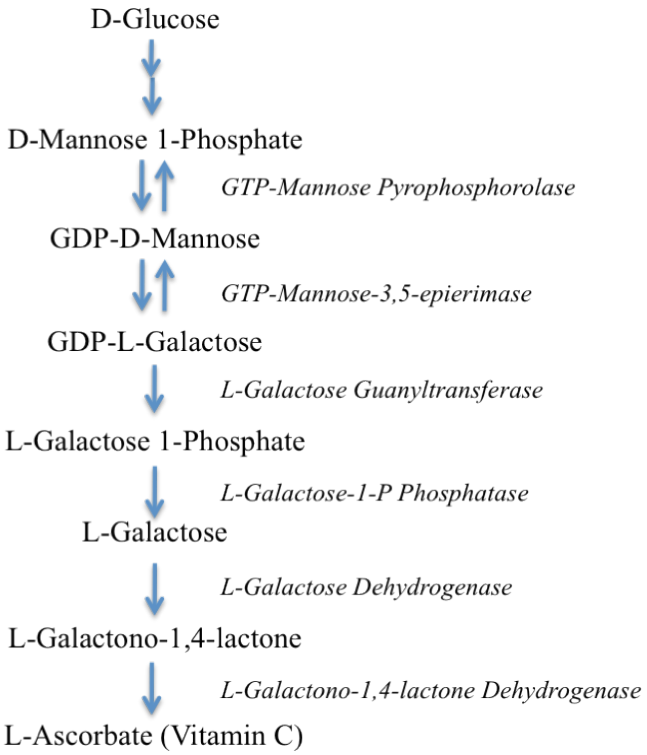

Figure 1.5. The L-galactose pathway of ascorbate synthesis in plants (Davey et al. 2000; Giovannoni 2007).

The current most widely supported theory of ascorbate biosynthesis is the L-galactose pathway, which involves a several step conversion of D-glucose to L-

ascorbate (Figure 1.5). The ability to significantly alter the rate of synthesis by increasing the quantity of L-galactose guanyltransferase (the enzyme responsible for converting GDP-L-galactose to L-galactose-1-phosphate) indicates that this may be the rate-limiting step of the reaction (Giovannoni 2007). Several of the genes for the enzymes involved in this process in *Arabidopsis* have been mapped (Giovannoni 2007). Older theories include the inversion pathway and the non-inversion pathway of synthesis (Isherwood et al. 1954; Loweus et al. 1990).

Apple seeds are capable of ascorbic acid synthesis via the L-galactose pathway, but apple fruit tissue appears to be incapable of *de novo* ascorbate biosynthesis (Davey et al. 2004; Li et al. 2008b). Foliar tissue on apple trees appears able to synthesize ascorbate using uronic acid substitutes (Davey et al. 2004). Ascorbate in apple flesh appears to be synthesized in the leaves and transported slowly to the flesh (Li et al. 2009). Some ascorbate is synthesized in the apple peel, and younger fruits accumulate ascorbate more quickly than more mature fruits (Li et al. 2009). Feeding seed, flesh, and peel cells with precursors leads to ascorbate synthesis, indicating that areas incapable of ascorbate synthesis likely lack the necessary metabolic precursors (Li et al. 2008b). Young, actively growing tissues have higher ascorbate levels than older tissue (Davey et al. 2000). During apple fruit development, there is a slight increase in total ascorbate content at physiological maturity, corresponding to an increase in internal ethylene concentration (Felicetti and Mattheis 2010).

Ascorbic acid is present in all metabolically active tissues of an apple and in all cellular compartments, including the apoplast (Davey and Keulemans 2009). Total ascorbate content is greatest in the leaves of an apple tree and lower in stems and roots (Zadeh et al. 2007; Li et al. 2009). The proportion of oxidized ascorbic acid, DHA, is

higher in the roots (60%) than in leaves and stems (2.5% and 34%, respectively) (Zadeh et al. 2007).

The concentration of ascorbic acid in apple fruits depends upon the location tested, and decreases exponentially from the exocarp to the core in 'Greenstar' and 'Golden Delicious' apples (Davey et al. 2004; Zadeh et al. 2007). The ascorbic acid content in the peel of 'Golden Delicious', 'Delicious' and 'Fuji' apples was greater than that in the cortex for all three cultivars at all sampling times from pre-harvest to six months in cold storage (Felicetti and Mattheis 2010). Vascular tissues of apple fruit have an abundance of ascorbic acid, suggesting that it is transported from source tissue to the fruit via the vascular network, or that the vascular tissues themselves are capable of synthesis (Li et al. 2008b). However, the ascorbate content in vascular tissues of apples may be cultivar specific, as it is present in 'Gala' but not in 'Fuji', 'Golden Delicious', or 'Red Delicious' (Li et al. 2008b).

Environmental, cultural, and genetic factors all affect the total concentration of Vitamin C in an apple. High light conditions increase ascorbate synthesis (Davey et al. 2000; Łata et al. 2005). Total ascorbate in 'Gala' apple peel was three times greater than the ascorbate in the flesh on the sun-exposed side of the fruit, but only two times greater on the shaded side of the fruit, indicating that ascorbate content in the peel is influenced by light intensity, but ascorbate content and recycling in the flesh is not (Li et al. 2008b; Li et al. 2009). Apple trees receiving 50% shading from the sun for 20 days had noticeably lower ascorbic acid levels in the leaves, peel, and flesh (Li et al. 2009). Shading only the apple fruit led to noticeable changes in the ascorbate levels in the peel, including an increased ratio of ascorbate to DHA, but no significant changes in the flesh ascorbate levels (Li et al. 2009). This is because the ascorbate in the apple flesh is at least partly dependent on translocation from source leaves, a slow process.

Some of the variation in ascorbate content of apples is cyclical. Seasonal variations in leaf ascorbate content correlate to ambient ozone conditions, oxidative damage, high light intensity, and magnesium deficiency (Davey et al. 2000). Different cultivars vary in their response to different growing years. An examination of ascorbate content of 56 different cultivars showed that the overall average ascorbate level in 2004 was considerably higher than in 2003, but that not all cultivars increased in that time (Łata et al. 2005). Environmental conditions conducive to increased ascorbate synthesis for one cultivar may not have the same effect on another cultivar.

Harvest date was significantly correlated with fruit ascorbate and total antioxidant activities, with later harvested cultivars such as 'Braeburn' and 'Ontario' having the highest ascorbate contents, and early harvested cultivars like 'Prima' and 'Retina' having the lowest (Davey and Keulemans 2004; Davey et al. 2007). Ascorbate was negatively correlated with mean pre-harvest daytime temperature, but it is not possible to separate the relative contributions of genetic and environmental factors (Davey et al. 2007). Varieties that ripen slowly or are picked early may be better able to maintain ascorbate biosynthetic capacity postharvest (Davey and Keulemans 2004).

As most apples are consumed after several months in cold storage, the postharvest metabolism of ascorbate is at least as important as its values at harvest. Ascorbic acid content gradually declined over six months in cold storage for 'Golden Delicious', 'Delicious', and 'Fuji' apples (Felicetti and Mattheis 2010). Three months of cold storage did not affect the Vitamin C levels of 'Golden Delicious' apples with or without skin (Tarozzi et al. 2004). Ascorbic acid decreased significantly in 'Golden Smoothee' apples during the first weeks of cold storage (Vilaplana et al. 2006). Both 1-MCP treated and non-treated fruit exhibited the same effects, indicating that the ascorbate pathway is not ethylene-dependent (Vilaplana et al. 2006). A broader study examining 31 different cultivars showed that the effects of 3 months of cold storage

(1°C) on Vitamin C concentration varied by cultivar (Davey and Keulemans 2004). Following storage, some cultivars showed no change in the total ascorbate content, while others showed a strong decrease or a moderate increase. The increase in the variability of these cultivars' ascorbate levels is likely due to the varying ability of each cultivar to preserve and regenerate their ascorbate pool. The cultivars 'Greenstar' and 'Braeburn' increased their ascorbate levels and retained their quality for up to six months at 1°C (Davey and Keulemans 2004). Storage at room temperature was far more damaging to antioxidant levels than cold storage (Davey and Keulemans 2004). Controlled atmosphere storage appears to have a detrimental effect on Vitamin C levels, which were lower for 'Tsugaru' apples stored under low oxygen conditions than those stored under air (Chung and Moon 2009).

There is broad variation in the quantity of Vitamin C in the peel and flesh of different apple cultivars. Davey and Keulemans (2004) found that the variation in mean ascorbate contents in apples at harvest in 2002 was approximately 3.6 fold across 31 different cultivars, from 401 to 1448 nmol/gram fresh weight (7.0 to 25.5 mg/100 g fresh weight). Different harvest years also had different levels of variation in vitamin C levels - the 2004 harvest had 4.8 fold variation across 12 cultivars, from 221 to 1061 nm/g fresh wt. (3.9 to 18.7 mg/100g fw) (Davey et al. 2007). Fruits with higher total ascorbate levels also had higher mean glutathione concentrations, but within different parts of the same fruit there tended to be an inverse relationship between ascorbate and glutathione levels (Davey and Keulemans 2004).

Davey et al. (2006) identified three highly significant QTLs regulating mean apple fruit ascorbate content in both 'Braeburn' and 'Telamon' on linkage groups (LG) 6, 10, and 11 of the *Malus* reference map. Together, these three QTLs account for 60% of the observed population variation, with a maximum individual contribution of 31% per QTL (Davey et al. 2006). A minor QTL for skin ascorbate content was found

on LG 9 (Davey et al. 2006). The following harvest year, QTL values were significantly lower, explaining a maximum of 47% of the observed population variance (Davey and Keulemans 2009). Major QTL clusters were located on LG 17, LG 11, and LG16, but clusters of co-segregating QTLs found on LG 6 were no longer present, and only one minor QTL was recovered from a cluster on LG 10 (Davey and Keulemans 2009). The cluster on LG 10 coincided with other physiological traits, including acidity, hardness, and degrees Brix, suggesting that this region may be involved in the regulation of plant growth and development (Davey and Keulemans 2009). Environmental conditions, including stress, along with the potential impact of tree maturity and juvenility may have played a role in the expression of these genes (Davey and Keulemans 2009). Further research is needed to determine the stability of these QTL across several years.

Significance

This research is unique in its comprehensive examination of 12 commercial cultivars and 5 advanced breeding selections both at harvest and after 4 months in cold storage. For studied cultivars, we measured rates and extent of browning over the period of an hour after slicing, total PPO activity and phenolic content, as well as Vitamin C content. Several parameters were measured over a period of two years to indicate some of the potential variation of these traits in different harvest years due to environmental conditions.

Decreasing the flesh browning and increasing the Vitamin C content of new apple varieties are among the goals of Cornell University's breeding program. There is a relative paucity of studies that have quantified the rate and extent of apple flesh browning across a large number of cultivars. Among these studies, the methods are not standardized, making apples-to-apples comparisons difficult. A goal of this

research was to quantify browning and vitamin C content in a broad range of cultivars, including several which have been used as parents in Cornell University's breeding program. Rates of browning were analyzed along with polyphenoloxidase activity and total phenolic content, investigating their roles as catalyst and substrate in the browning reaction. Vitamin C content was also measured in these same cultivars, for a separate analysis.

An awareness of the rates of browning and vitamin C content of new selections and existing cultivars is important, as it provides a basis for selecting parents for future crosses, as well as comparing new varieties to standard commercial cultivars. This is useful for marketing newer cultivars to growers and the public. Additionally, the methods developed and refined in this research may be used by Cornell University's apple breeding program to screen and compare the browning rates and vitamin C content of future new releases.

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CHAPTER 2

FLESH BROWNING, POLYPHENOLOXIDASE AND PHENOLIC CONTENT OF SELECT APPLE (*MALUS DOMESTICA* BORKH.) CULTIVARS AND ADVANCED BREEDING SELECTIONS

Abstract

Enzymatic browning of apples is a significant problem in the fresh cut and processing industries. Quantifying the rates and extent of flesh browning across a range of cultivars can assist breeders in selecting low-browning parents to use in crosses. The browning reaction uses phenolic compounds as the substrate and the enzyme polyphenoloxidase (PPO) as the catalyst. This study quantified flesh browning, PPO activity, and total phenolic content in twelve commercial cultivars and five advanced breeding selections. 'Liberty', 'McIntosh', and selection #2 (a 'McIntosh' x 'Fuji' hybrid) were consistently the highest browning apples. 'AutumnCrisp' (previously designated as NY674) and selection #5 ('AutumnCrisp' x 'Braeburn') were the lowest browning apples. Flesh browning tended to decrease over time in cold storage for most cultivars, and was lower near the peel than near the core. Cultivars 'AutumnCrisp', 'Cortland', 'Zestar', and 'McIntosh' had the lowest measured PPO activity. Selection #2, #3 ('Braeburn' x 'AutumnCrisp'), and 'Braeburn' had the highest PPO activity. 'McIntosh', #4 ('AutumnCrisp' x 'Fuji'), #5, and #2 had the highest phenolic content. Selection #3 and 'Braeburn' had the lowest phenolic content. Phenolic content and PPO were correlated to total color change only in some cultivars.

Introduction

When an apple (*Malus domestica* Borkh.) is cut or injured, the flesh turns brown in a process known as enzymatic browning. Enzymatic browning occurs when phenolic compounds oxidize in the presence of the enzyme polyphenoloxidase (PPO) to form slightly colored *o*-quinones, which then further polymerize to form pigments of varying hues and intensity (Le Bourvellec et al. 2004). The browning of apples is undesirable, leading to an unpleasant appearance, the possible development of off-flavors and affecting the shelf life of fresh-cut or processed apples (Sapers 1993; Gámbaro et al. 2006). The rate and extent of apple flesh browning varies by cultivar and may be affected by the activity level of the enzyme PPO as well as the total phenolic content of the fruit.

Enzymatic browning of apples is a significant problem in the juice processing, dehydrated fruit and fresh cut industries. Market reports estimated that increasing the availability of pre-sliced apples could expand the apple market in the same way that baby carrots expanded the carrot market: doubling consumption, while at the same time doubling price (Brushett and Lacasse 2006; Jellie 2006). Currently, fresh cut apples are processed carefully to maintain fruit texture, flavor and appearance. Slices are treated with products such as NatureSeal^{TM}, a calcium ascorbate solution that helps preserves color and texture of the sliced fruit (Rupasinghe et al. 2005). The cultivars 'Gala', 'Pink Lady', 'Granny Smith' and 'Empire' are used for fresh-cut apples because of their low rates of browning and ability to maintain firmness in storage (PetersonFarms 2008).

In ‘Granny Smith’, ‘Empire’ and ‘McIntosh’ apples, browning is most highly correlated with PPO activity, while in ‘Delicious’, ‘Golden Delicious’ and ‘Cortland’, the quantity and type of phenolic substrates has a more significant effect (Coseteng and Lee 1987; Milani and Hamedi 2005). This is typical of most enzymatic reactions,

which are limited by either the activity level of the catalyst or the quantity of substrate. Fruit ripening stage and time in storage can also alter a fruit's browning potential. Unripe apple flesh or pulp browns more rapidly than fully mature flesh, likely due to the higher proportion of PPO present in less mature fruit (Murata et al. 1995a; Valentines et al. 2005). Cold storage affects both PPO and phenolic content, although these effects are cultivar-dependent; some cultivars are able to maintain or increase their PPO activity during storage, while others have gradual or dramatic decreases (Coseteng and Lee 1987; Barrett et al. 1991; Rocha and Morais 2002; Leja et al. 2003). Likewise, cold storage caused no change in total phenolics in some cultivars, while in others storage resulted in a loss or gain in total phenolic content (Awad and De Jager 2000; Rocha and Morais 2002; Napolitano et al. 2004; Tarozzi et al. 2004; Chung and Moon 2009). Cultivars with the highest rates of browning include 'Liberty', 'McIntosh' and ‘Delicious', and those with relatively low rates of browning include 'Empire', 'Cortland', 'AutumnCrisp' (NY674) and 'Gala' (Coseteng and Lee 1987; Kuczyński 1995; Lee and Smith 1995; Milani and Hamedi 2005).

Genes for the browning enzyme polyphenoloxidase have been cloned in apple and transgenic antisense PPO apple shoots have been created (Haruta et al. 1998; Murata et al. 2001; Nishimura et al. 2003). Although decreasing the PPO activity of apple fruits may help reduce enzymatic browning, eliminating PPO activity entirely may also be detrimental. PPO-mediated oxidation is widespread in plants and serves several important functions in different species, including providing an antioxidative response to environmental stress as well as antimicrobial and anti-herbivory benefits (Pourcel et al. 2006; Thipyapong et al. 2006; Constabel and Barbehenn 2008). PPO is also known to decrease apple allergenicity by lowering the affinity of the apple allergens Mal d 1 and Mal d 2 to serum IgE (García et al. 2007; Marzban et al. 2009).

Allergy to apple is a problem affecting approximately 2% of Northern and Central European populations.

The phenolic substrates of the browning reaction also have significant and numerous benefits to plant and human health. Phenolic compounds are powerful antioxidants, and even low concentrations of phenolics may protect apples from oxidative damage (Kuczyński 1999; Podsędek et al. 2000; Jansen et al. 2001). Flavonoids protect plants from certain pathogens and microbes, including the fungal pathogen causing apple scab (*Venturia inaequalis*), and may restrict insect predation by acting as digestibility reducers (Pourcel et al. 2006; Treutter 2006; Biedrzycka and Amarowicz 2008). In humans, phenolic compounds provide antioxidative and tumor-suppressing benefits (Liu et al. 2001; Veeriah et al. 2008).

Quantitative trait loci (QTL) related to browning have been mapped in apple. A highly significant QTL for rate of flesh browning was identified on linkage group (LG) 17 of 'Telamon' and 'Braeburn' in one year, but environmental conditions also appear to have a role, as QTL for flesh browning were not detected the previous year (Kenis et al. 2008). PPO was mapped to the bottom of LG 10 in 'Delicious' apples (Igarashi et al. 2008). Identifying highly stable and significant QTL for browning may enable more directed breeding of this trait.

Cornell University's breeding program has as a goal decreasing the flesh browning of new apple varieties. Relatively few studies have quantified the rate and extent of apple flesh browning of a large number of cultivars, and among these studies methods are not standardized so side-by-side comparisons are difficult. The goal of this study was to comprehensively examine the rate and extent of apple flesh browning in a broad range of cultivars, which included twelve popular commercial cultivars and five advanced breeding selections used as parents in Cornell University's breeding program. Browning was measured both at the core and near the peel to determine how

those locations differed. In addition to the rate of browning, the two main factors thought to influence browning, polyphenoloxidase content and total phenolic content, were measured. When possible, we quantified values at harvest and after four months of cold storage to determine what effects cold storage had on these parameters. Advanced selections were compared with their parents.

Materials and Methods

Apple Cultivars. Twelve commercial apple cultivars and five advanced breeding selections were harvested: ‘Zestar’, ‘Gala’, 'AutumnCrisp' (NY674), ‘McIntosh’, ‘Honeycrisp’, ‘Cortland’, ‘Liberty’, ‘Golden Delicious’, ‘Braeburn’, ‘Fuji’, ‘Goldrush’, ‘Empire’ and selections #1 ('Honeycrisp' x NY selection), #2 ('McIntosh' hybrid x 'Fuji'), #3 ('Braeburn' x 'AutumnCrisp'), #4 ('AutumnCrisp' x 'Fuji'), and #5 ('AutumnCrisp' x 'Braeburn'). All cultivars were picked at commercial maturity during the 2008 and 2009 harvest seasons at the New York State Agricultural Experiment Station orchard in Geneva, NY. ‘Empire’ was only harvested and studied during the 2009 harvest season. Starch-iodine testing was used to assess maturity stage. Apples not analyzed immediately were kept in cold storage at 1^{o}C until use.

Chemicals. Polyvinylpolypyrrolidone (PVPP), sodium phosphate dibasic anhydrous, citric acid, catechol, Folin Ciocalteau Reagent, 2N, gallic acid, ethanol, and sodium carbonate, anhydrous were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All chemicals used were analytical grade.

Determination of Rate of Browning. A Konica-Minolta CR-400 colorimeter with Data Processor was used to measure and record the rate of browning for each cultivar (Konica Minolta Sensing Americas, Inc., Ramsey, NJ). Measurements were taken immediately after harvest and following approximately four months in cold storage. Fifteen apples were selected randomly from each cultivar. Apples were sliced in half

latitudinally with a stainless steel knife. The bottom half of each apple was placed cut side up, and a template indicating the placement of the colorimeter for measurement was placed on top of the cut apple. Each apple was measured at four locations – two directly inside the skin (the "outer" or "peel-proximate" locations), and two directly outside of the core (the "inner" or "core-proximate" locations). The approximate location of these measurements is illustrated in Figure 2.1. Colorimeter readings were taken immediately after slicing, every two minutes from 2 to 20 minutes, and every 10 minutes up to an hour after slicing. Data were recorded on the *L*, a*, b** color scale.

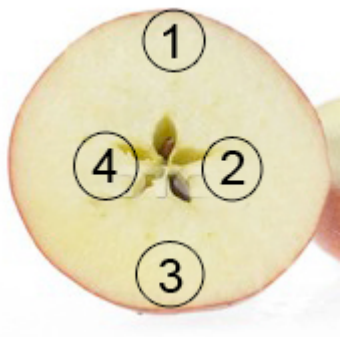

Figure 2.1. Approximate locations of the four browning measurements taken on each cut apple. Locations 1 and 3 are referred to as the "outer" or "peel-proximate" measurements. Locations 2 and 4 are referred to as the "inner" or "core-proximate" measurements.

Calculations and Statistical Analysis. ΔL^*, Δa^*, and Δb^* were calculated as the reading at time t minus the initial reading. Total color change ΔE^* was calculated using the formula $\sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$. Data were analyzed statistically using R (R Foundation, Vienna, Austria) and JMP version 8.0.1, Copyright © 2009 SAS Institute Inc. (Cary, NC).

Quantification of Polyphenoloxidase. PPO activity was determined using a Heλiosγ v.4.60 spectrophotometer with internal software (Thermo Fisher Scientific Inc., Waltham, MA). Measurements were taken after four months of storage for the 2008

harvested cultivars, and both at harvest and after storage for the 2009 harvested cultivars. Ten apples were randomly selected from each cultivar and divided into five groups of two apples each. The apples were sliced, and every third radial slice was selected and weighed. Slices were added to approximate 100 g. The weighed slices were placed in a chilled Waring blender jar with 1.5 mL of chilled Extraction Buffer per gram of fresh weight. The extraction buffer was comprised of 10.0 g PVPP in 1L of pH 5.0 Citric-Phosphate Buffer base. The citric-phosphate buffer, also known as "McIlvane's Buffer", contains 0.2M sodium phosphate and 0.1M citric acid in water. The mixture was blended at a rheostat setting of 100 for 2.0 minutes. The homogenized extraction was then filtered using Kendall milk filters for exactly 2.0 minutes. Samples were kept on ice during the filtration process. Immediately following this filtration, 0.2 mL of extract was added to 2.8 mL of 0.05M catechol solution (in Citric-Phosphate buffer base) in a 1-cm-pathlength cuvette. The cuvette was covered with Parafilm and inverted once, then immediately placed in the spectrophotometer for measurement. Spectrophotometer readings were taken at 420 nm at 1 second intervals for 120 seconds. The change in absorbance per minute was calculated as the slope of the line generated between 2 and 62 seconds. In some cases, when a change in absorbance was delayed for several seconds, the calculation was adjusted to determine the slope on the linear portion of the curve. One unit of enzyme activity was defined as a change in absorbance of 0.001 units per minute per gram fresh weight.

Quantification of Total Phenolics. Total phenolic content was measured in gallic acid equivalents using the Folin-Ciocalteau method. Twenty-four hours in advance, a sodium carbonate solution was prepared by dissolving 20g of anhydrous sodium carbonate in 80 mL of water and boiling. The solution was allowed to cool and a few additional crystals of sodium carbonate were added. After 24 hours, the solution was

filtered and water added to bring the volume to 100 mL. A calibration curve was prepared by dissolving 0.5 g of dry gallic acid in 10 mL of ethanol. This was diluted to 100 mL. Then 0, 1, 2, 3, 5 and 10 mL aliquots of this stock solution were added to different 100 mL flasks, which were diluted to volume with water. The resulting standard solutions had concentrations of 0, 50, 100, 150, 250, and 500 mL/L gallic acid. Ten apples were randomly selected from each cultivar and divided into groupings of three, three and four apples. Each group was juiced separately and three samples were selected from each juicing. Samples were diluted 1:1 in water so that they would fit within the calibration curve. 20 μL of sample was combined with 1.58 mL of water and 100 μL of Folin-Ciocalteau reagent. After 1 minute, 300 μL of sodium carbonate solution was added to this mixture. This solution was left undisturbed for 2 hours, after which the absorbance of each solution was measured at 765 nm. Resultant total phenolics were calculated using the calibration curve.

Results and Discussion

Rates and Extent of Browning. The most rapid initial browning of an apple occurs within the first 10 to 20 minutes after cell disruption (López-Nicolás et al. 2007). The rate of browning decreases exponentially, such that most of the browning potential of an apple is achieved within 60 minutes (Lozano et al. 1994; López-Nicolás et al. 2007). Measurements were taken every two minutes during the first twenty minutes after slicing the fruit to capture this initial rapid increase in total color change, then every ten minutes up to an hour to determine the overall total color change. Since most apples completed the majority of their browning within the first hour after slicing, this technique best captured the browning curve. Although apples for the fresh cut market may be stored for days or weeks before consumption, the initial time period between slicing and treatment with antibrowning agents is the most critical in

determining overall level of fruit browning. Apples not treated or specially packaged will brown most extensively in the first hour after slicing. Measuring the rate of browning in the first hour was therefore most significant in determining the overall browning potential, and allowed us to do so in a reasonable period of time across numerous cultivars with a large sample size.

The sample sizes were determined using a simple statistical calculation. Previous work by Brown, McLellan, and Barnard (unpublished) tested 30 apples and found a pooled estimate of variance (s^2) of 11.43 on 290 df. Originally, we specified a difference to be detected $\delta = 5$, power (the probability of picking up difference when the 'true' difference is ≥ 5) $P' = 90\%$, and significance level $\alpha = 5\%$. Using P' and α, Snedecor & Cochran's approximation (p.104), gives a multiplier m of 10.5 (Snedecor and Cochran 1989). The formula for sample size is: $m * 2s^2/\delta = 10.5*22.83/25 = 9.6$. Rounding up and adding 1, we get a recommended sample size of 11. This number was further increased to a sample size of 15 to maximize the validity of our results and minimize sampling error, and also because a wider range of cultivars was used on this study than in the original study done to determine variance. Measurements were taken at two opposite points of the apple for both the inner and outer measurements, to provide four measurements per apple and assess the known tendency for the core area to be more prone to browning. The actual sample variance of the ΔE^* values in this study measured at 60 minutes and separated by season and location in the fruit, averaged 4.43 across all seasons and all cultivars. Given that the actual sample variance was much lower than the assumed variance, we were well within the limits of an adequate sample size. Using the above formula and same assumed power and significance level, with a sample size of 15, we narrowed the difference detected to approximately 2.5 units, enough to separate the high, middle, and low browning fruits from one another.

The total extent of browning for a cultivar was calculated using a fitted regression curve of the total color change ΔE^*. Total color change was used because it incorporates the changes in both lightness and red-green chromicity, better reflecting the total color difference as it appears to the eye than either measurement alone. Davey et al. (2006) used only visual changes to rate the total level of browning in identifying quantitative trait loci (QTL) for this trait (Davey et al. 2006). A visual rating is not as precise as the use of a colorimeter, which quantifies the actual fruit color on a standardized, quantitative scale. QTLs based on visual rankings may therefore not be as accurate as QTLs determined from colorimeter measurements. Figure 2.2 depicts "before" and "after" photographs of the apples studied in 2009 after harvest, taken one hour apart. From these images, the difficulty in determining nuances of color change in different cultivars becomes apparent. Although it is clear from these images that some cultivars brown a lot and others do not brown very much, a more precise analysis than that is quite difficult. The use of a standardized instrument to measure color is important in a study seeking to elucidate all but the most obvious of color differences.

Figures 2.3, 2.4, 2.5, 2.6 and 2.7 depict the color change results by cultivar for 2008 and 2009 both at harvest and after four months in cold storage. Several trends are repeated consistently. For all four measurement periods, 'Liberty' had the highest initial rate and overall extent of browning. This is consistent with studies noting 'Liberty' as one of the highest browning apple cultivars (Kuczyński 1995; Lee and Smith 1995). 'McIntosh' and selection #2 ('McIntosh' hybrid x 'Fuji') were the next highest browners, typically experiencing a ΔE^* of 8 to 12 on the color scale. In 2008, 'Gala' browned only moderately, but in 2009, 'Gala' browned as extensively as #2 and 'McIntosh'.

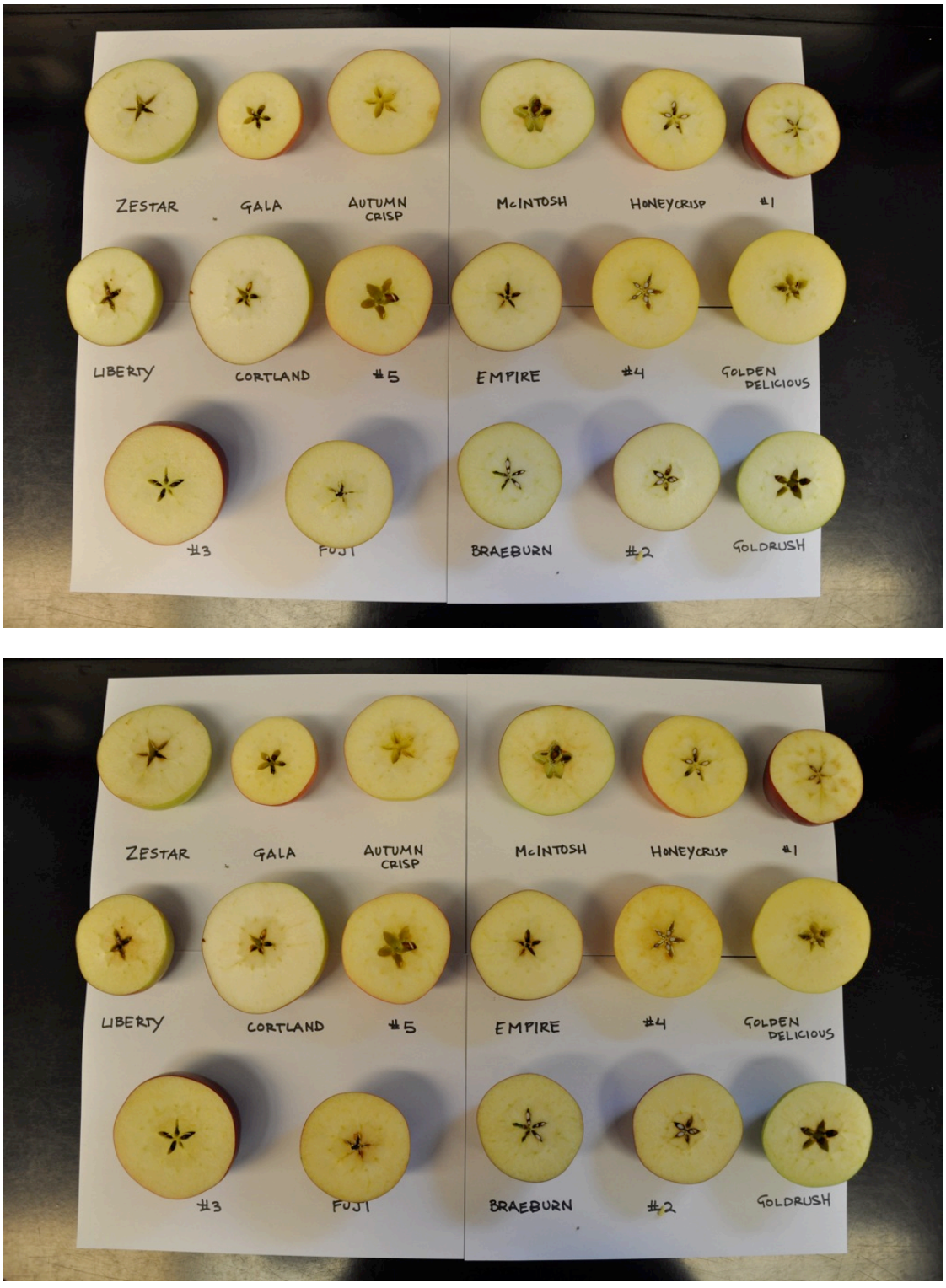

Figure 2.2. Photographs showing the apples studied in 2009, taken after four months in cold storage. The image at top shows the apples immediately after slicing, while the lower image shows the apples an hour after slicing.

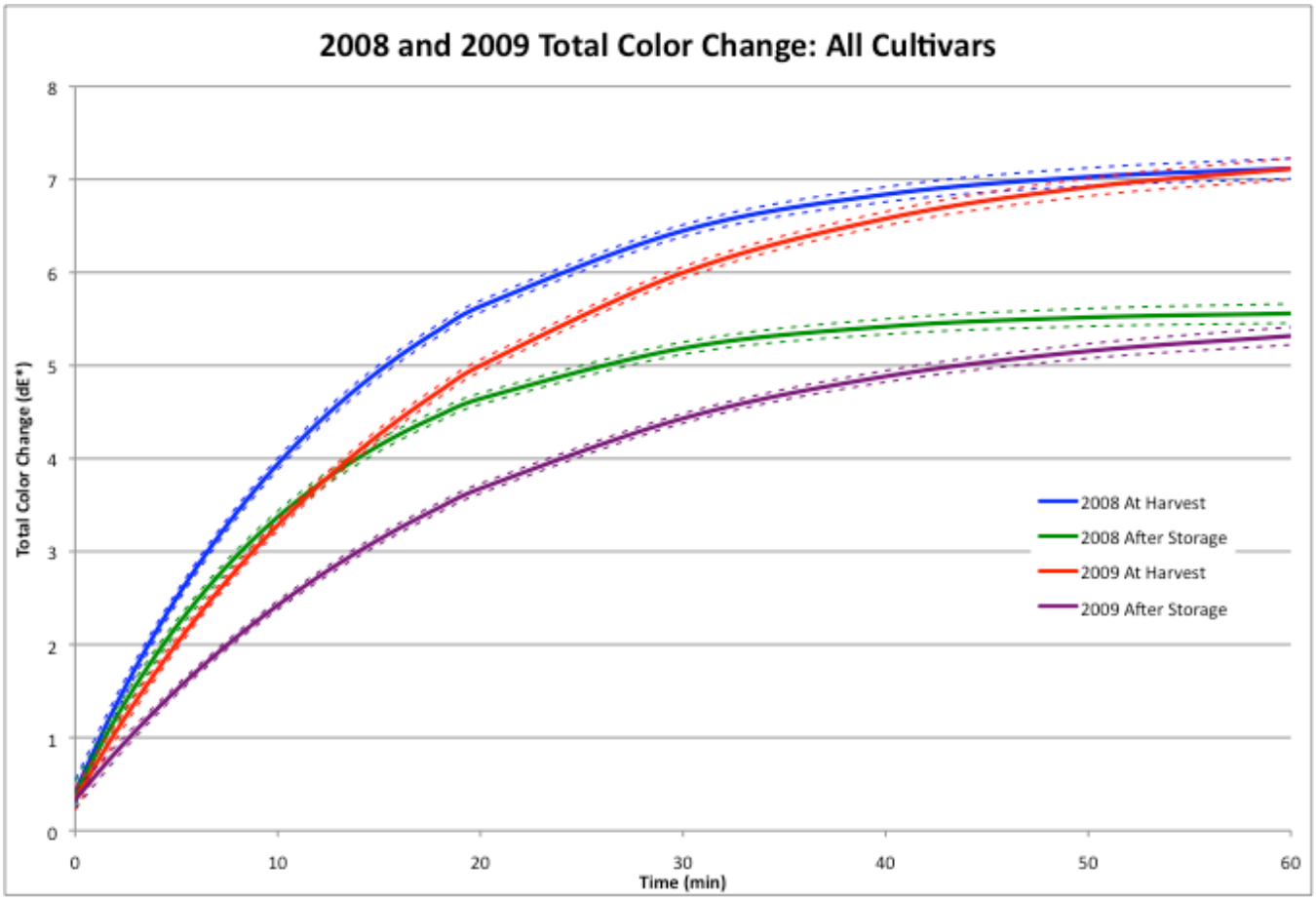

Figure 2.3. Overall color change (ΔE*) averaged across all cultivars for 2008 and 2009 at harvest and after four months in cold storage. 'Empire' is only included in 2009 data. Cultivars 'Zestar' and 'Gala' were not available for measurement in 2008 after storage. Dashed lines indicate standard error of the regression; overlapping dashed lines indicate lack of significant difference between two regression lines.

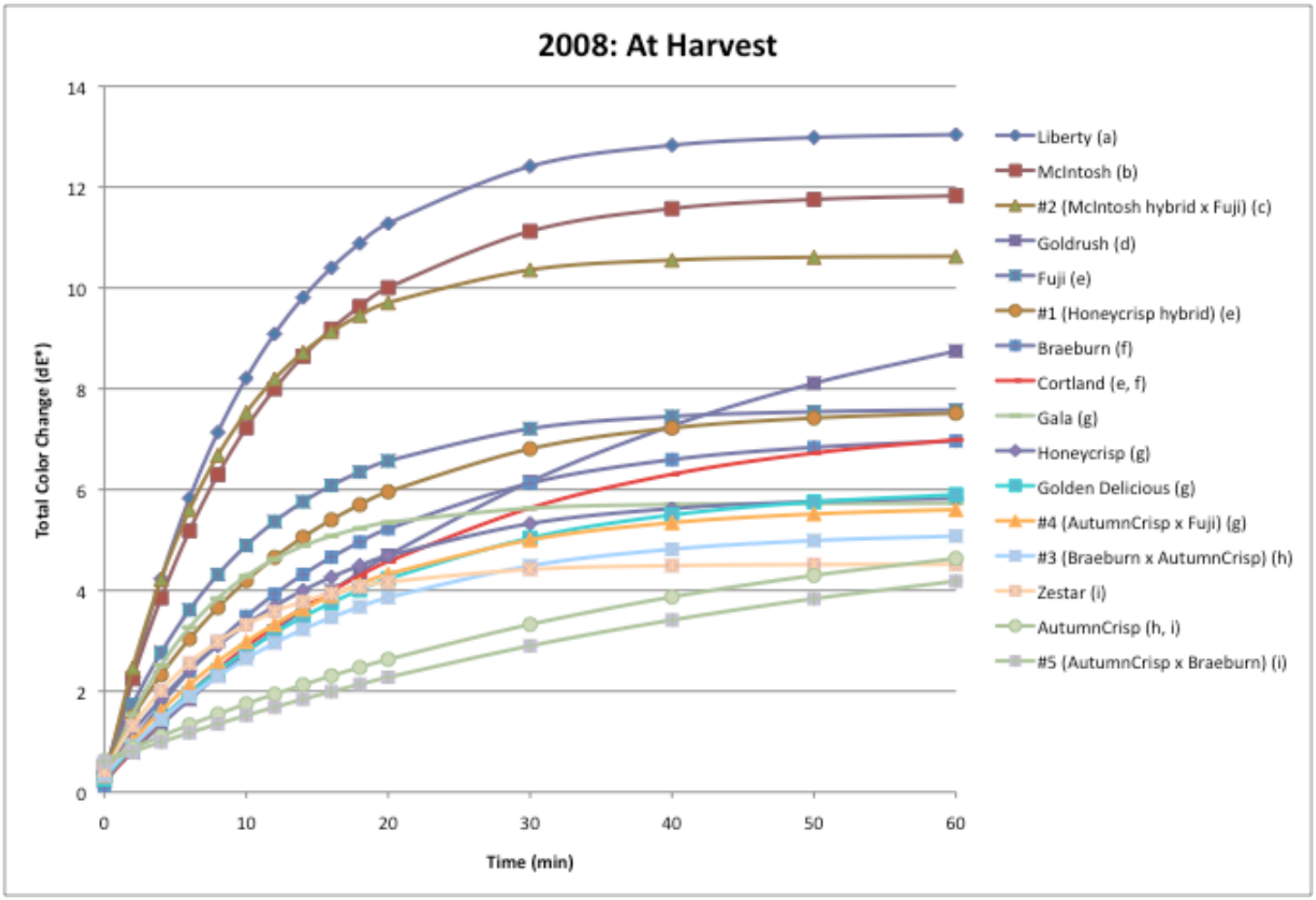

Figure 2.4. 2008 total color change (ΔE*) at harvest by cultivar. Lines shown are fitted regression lines. Letters in parentheses following cultivar name indicate which cultivars are not significantly different at 60 minutes with α=0.05.

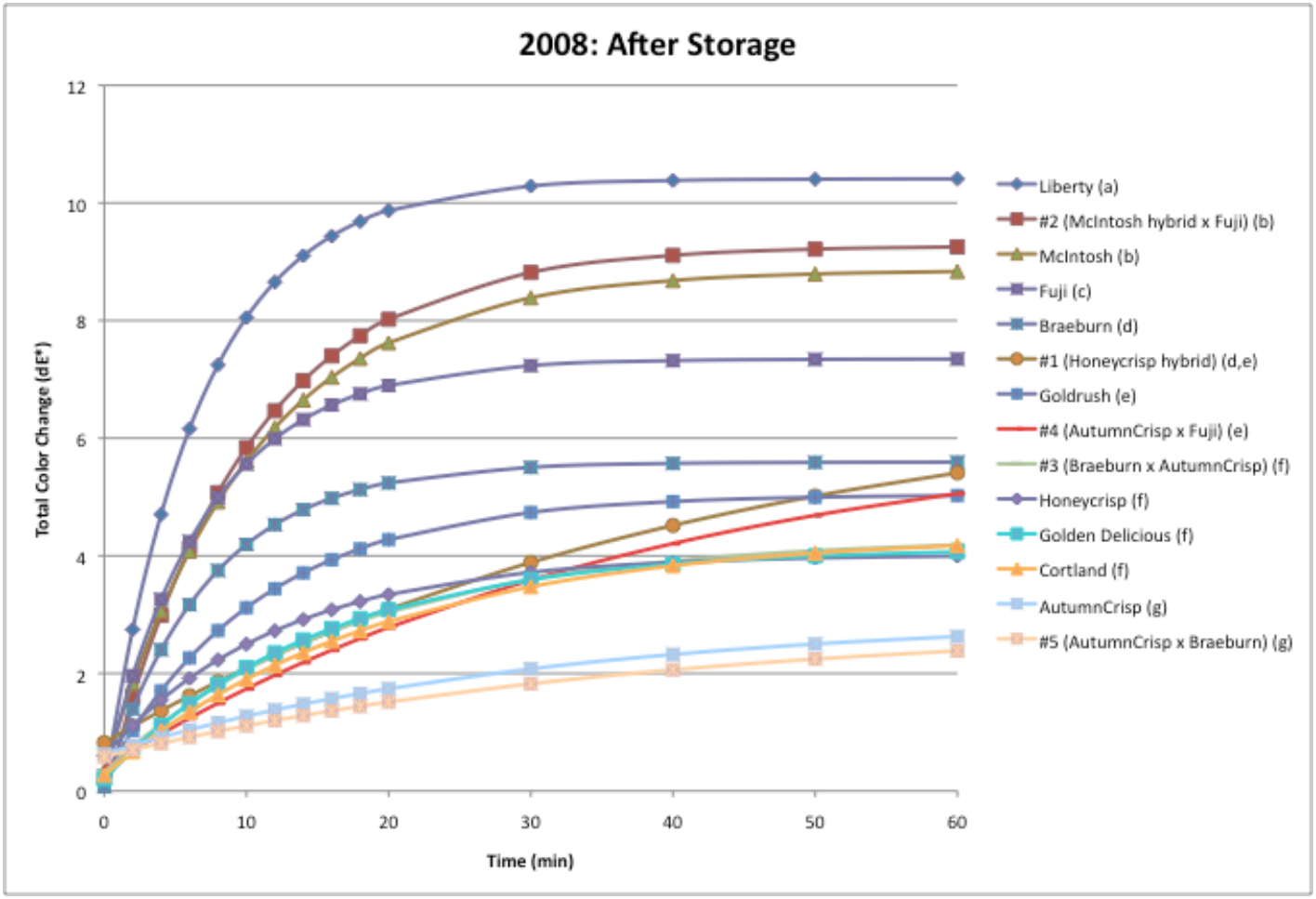

Figure 2.5. 2008 total color change (ΔE*) after four months in cold storage by cultivar. Lines shown are fitted regression lines. Letters in parentheses following cultivar name indicate which cultivars are not significantly different at 60 minutes with α=0.05.

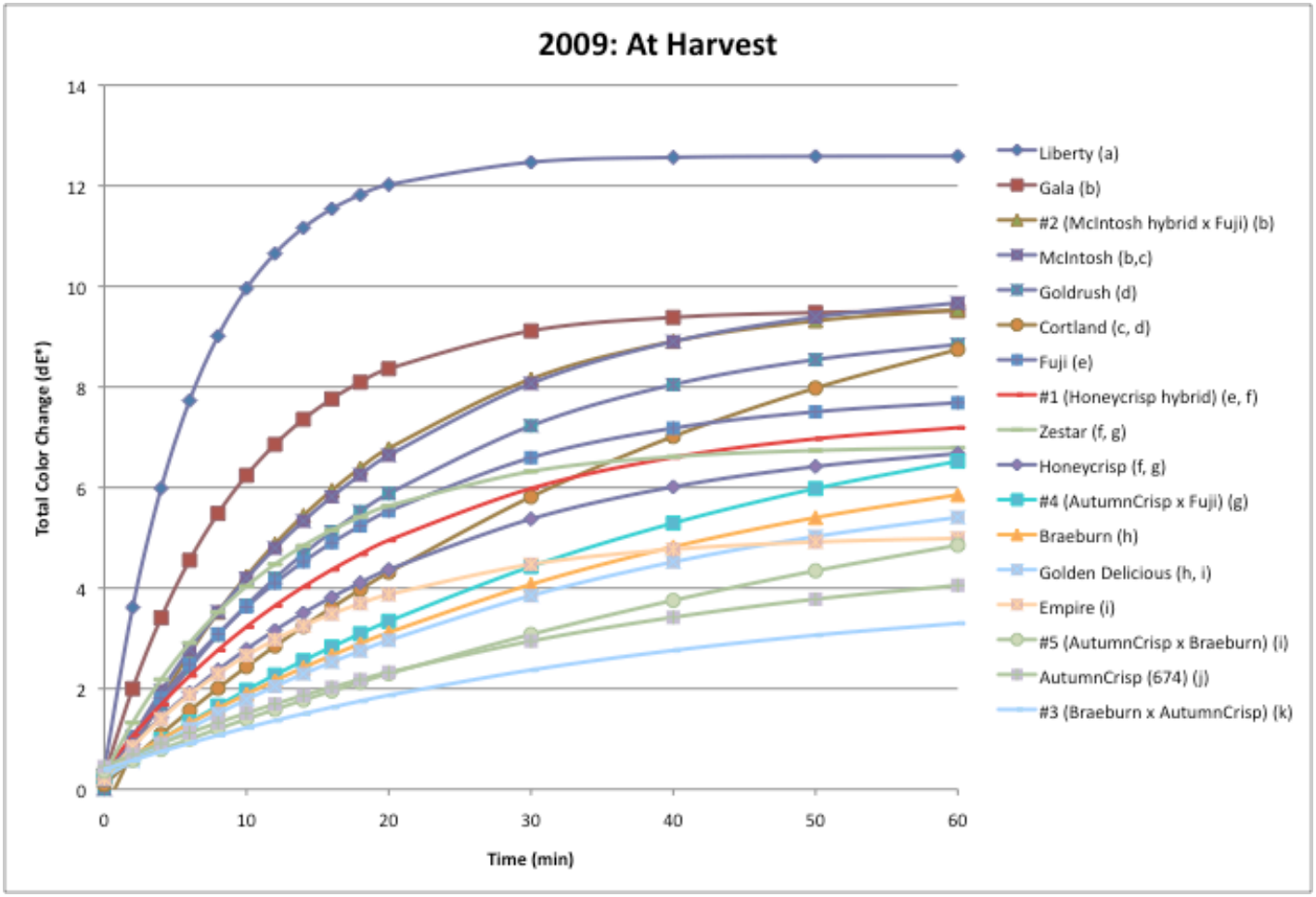

Figure 2.6. 2009 total color change (ΔE*) at harvest by cultivar. Lines shown are fitted regression lines. Letters in parentheses following cultivar name indicate which cultivars are not significantly different at 60 minutes with α=0.05.

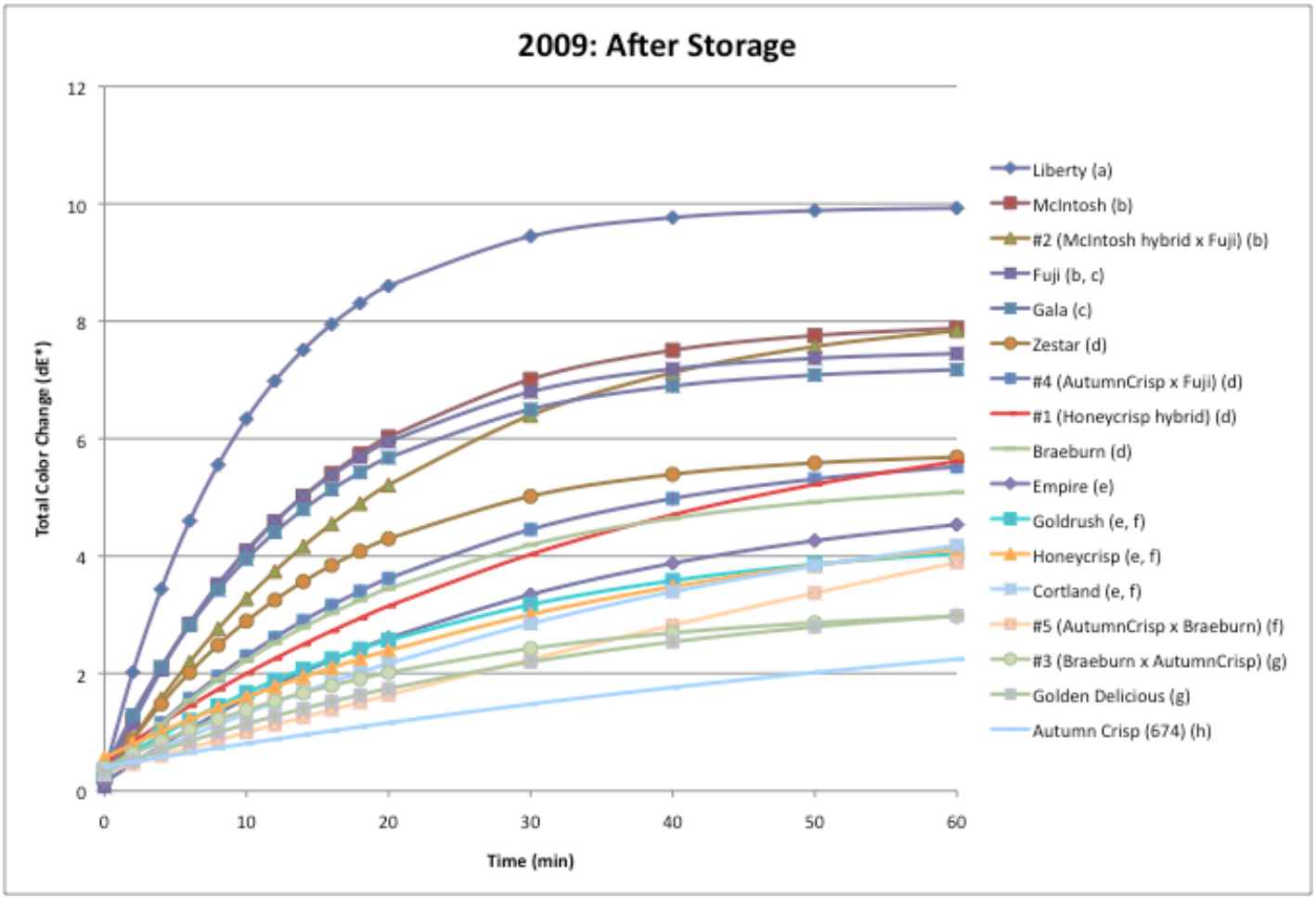

Figure 2.7. 2009 total color change (ΔE*) after four months in cold storage by cultivar. Lines shown are fitted regression lines. Letters in parentheses following cultivar name indicate which cultivars are not significantly different at 60 minutes with α=0.05.

Selection #4 ('AutumnCrisp' x 'Fuji') was among the moderate browning apples in both years, and tended to have a browning curve roughly intermediate to its two parents. Selection #1 ('Honeycrisp' x NY selection) tended to brown very similarly to its parent 'Honeycrisp', and also browned moderately.

The lowest browning cultivars were consistently low browning throughout the experiment. In 2008, AutumnCrisp and #5 ('AutumnCrisp' x 'Braeburn') were the lowest browning cultivars. They remained among the low browners in 2009 as well, and #5 browned similarly to its parent 'AutumnCrisp' during all four measurement periods. #3, another 'Braeburn' x 'AutumnCrisp' progeny, was among the lowest browners in 2008 at harvest, and in 2009 both at harvest and after storage. #3 behaved more like its parent 'AutumnCrisp' for these three time periods, but more like 'Braeburn', a moderately-high browner, in 2008 after storage.

Some varieties, such as ‘Gala’, ‘McIntosh’, and selections #2 (‘McIntosh’ hybrid x ‘Fuji’) and #3 (‘Braeburn’ x ‘AutumnCrisp’) have significantly different browning from one year to the next, while others, such as ‘AutumnCrisp’, ‘Cortland’, ‘Fuji’, ‘Liberty’, and selection #1 (‘Honeycrisp’ hybrid) browned similarly in 2008 and 2009. The tested fruits were harvested from the same set of trees from one year to the next. Therefore, cultivars that displayed wider differences in browning from one season to the next are likely influenced by weather conditions to a greater degree than those with consistent yearly values.

One particularly surprising result showed that ‘Cortland’, generally considered to be a low-browning standard in the industry, was among the moderately-high browners in at-harvest measurements. Yet after a period of time in cold storage, ‘Cortland’ was among the lower browning apples. Studies that did not measure apples both at harvest and after storage, or that used apples of unknown origin or growing conditions, such as those purchased at a grocery store, would not be able to account

for these factors, which appear significant in the relative rate of browning of this cultivar. If previous studies were performed on apples that had been stored for a period of time, ‘Cortland’ may have appeared among the lower browning fruits, accounting for the discrepancy between these results and previous research. All cultivars used for this study were grown in the same orchard under similar conditions and stored in the same cooler.

The initial rate of browning was determined as the slope of the fitted regression line from 0 to 4 minutes after slicing the apple. These results are depicted in Figure 2.8. Cultivars with the highest initial rates of browning include 'Liberty', #2 ('McIntosh' hybrid x 'Fuji'), 'McIntosh', 'Gala', and 'Fuji'. Many of these apples also exhibited the greatest extent of browning.

The cultivars with the lowest initial rate of browning include 'AutumnCrisp' and #5 ('AutumnCrisp' x 'Braeburn'). 'Cortland', 'Empire', #3 ('Braeburn' x 'AutumnCrisp') and #4 ('AutumnCrisp x 'Fuji') also showed consistently low initial rates of browning throughout the experiment.

A few cultivars had a low initial rate of browning but a high overall extent of browning, as occurred with 'Goldrush' in 2008 at harvest and 'Cortland' in 2009 at harvest. In general, though, the cultivars with the lowest initial rate of browning tended to have a lower overall extent of browning, and vice versa.

The trends in total color change in 2008 and 2009 at harvest and after storage are depicted in Figure 2.3 and Table 2.1. The initial rate of browning was 38% higher in 2008 than in 2009. Although the initial rate of browning was significantly higher in 2008, there were not significant differences between the two years 60 minutes after the fruit was cut. The average extent of browning for all cultivars was not significantly different from one year to the next. This varied by cultivar. Some cultivars, such as #1 (‘Honeycrisp’ hybrid), 'AutumnCrisp', 'Liberty' and 'Fuji', displayed virtually no

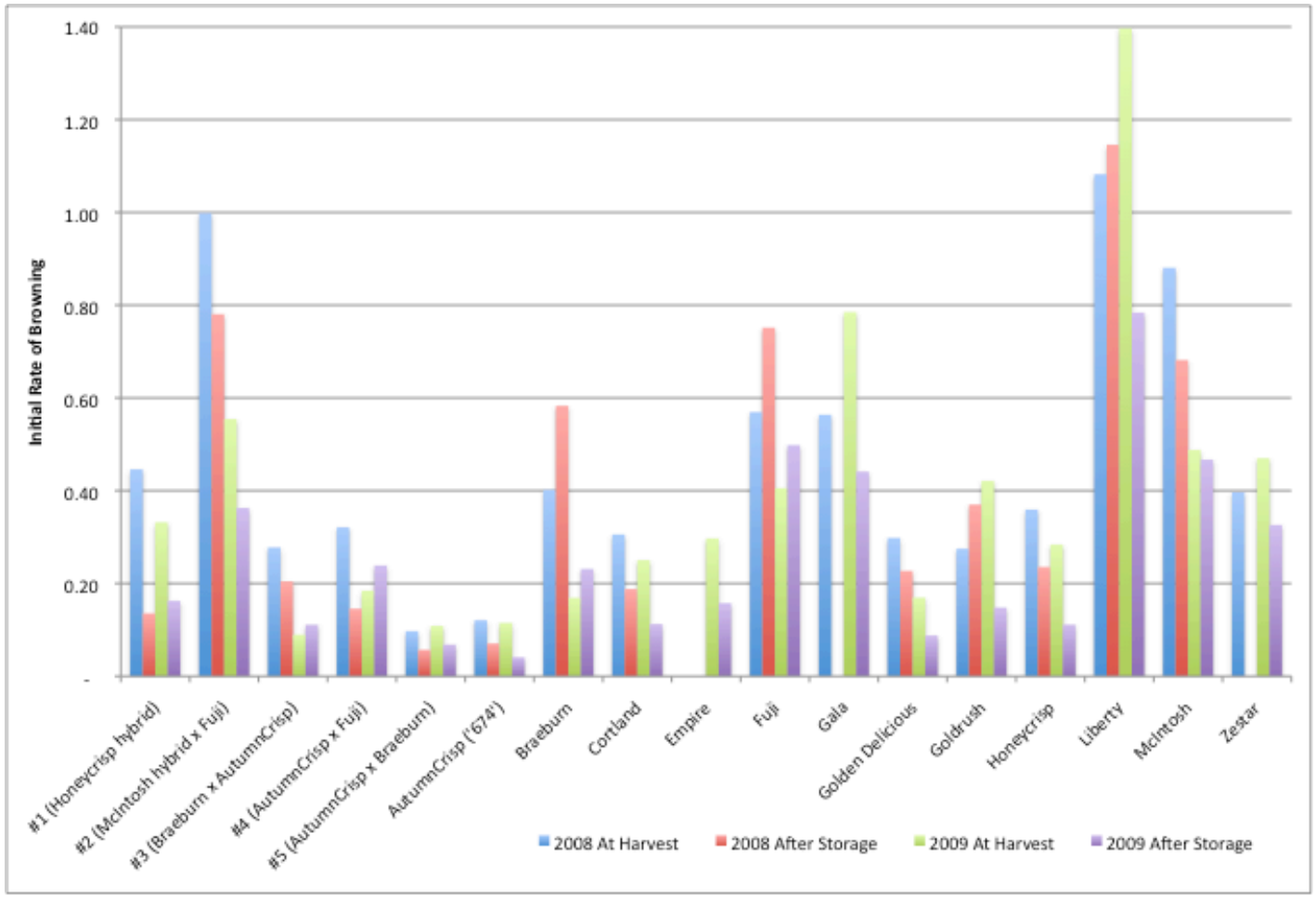

Figure 2.8. Initial rates of browning for all cultivars, 2008 and 2009 at harvest and after storage. Rates were calculated as the per minute total color change of the browning regression curves for the first four minutes after slicing the apple. 2008 data not available for 'Empire'. 2008 after storage data not available for 'Gala' and 'Zestar'.

significant difference in rates of browning from one year to the next, while for other cultivars, such as #2 (‘McIntosh’ hybrid x ‘Fuji’), #3 (‘Braeburn’ x ‘AutumnCrisp’) and 'McIntosh', the differences from one year to the next were far greater.

Table 2.1. Initial slopes of the ΔE* regression lines illustrated in Figures 3 and 9 from 0 to 4 minutes after slicing. Values shown are an average of all cultivars measured in that time period, and are divided by inner and outer locations in the apple fruit. Higher values indicate a more rapid initial rate of browning. In all cases, the inner location on the apple browned more rapidly than the outer location.

Season	Date	Inner	Outer	Overall
2008	At Harvest	0.51	0.37	0.43
2008	After Storage	0.44	0.32	0.37
2009	At Harvest	0.43	0.27	0.34
2009	After Storage	0.31	0.18	0.24

When comparing data between two harvest seasons, it is important to consider the differences in weather conditions during those two years, as they may be responsible for some of the differences in browning, PPO and phenolic content. 2009 in particular was considered a fairly atypical year in the Geneva, NY area. Data from NEWA shows that 2009 had 3.44” more rain in May and June than 2008, but was drier from July through the end of October, with 4.62” less rain in those months than in 2008 (NEWA 2010). 2009 also accumulated 146 fewer growing degree days (base 50^{o}F) than 2008 from May 1 through October 31, and was on average 0.71^{o}F cooler per day (NEWA 2010). These differences may have had a significant effect on all parameters studied in these years.

On average, both the initial rate and overall extent of browning decreased significantly in the four months between harvest and storage. The initial rate of browning decreased 13% in 2008 and 30% in 2009 after four months in cold storage. The overall extent of browning decreased 22% in 2008 and 25% in 2009 following storage. This again varied by cultivar. 'Fuji' and selection #3 (‘Braeburn’ x

‘AutumnCrisp’) were not significantly different in their at-harvest and after-storage rates of browning. Yet the majority of cultivars had a significant decrease in the extent of browning after four months in cold storage. This discrepancy led to 'Fuji' appearing to be moderately browning in the at-harvest cultivar comparison, to standing out as one of the highest browning cultivars after storage. The metabolic reasons for ‘Fuji’ maintaining its rate of browning after storage are not known, as it does not maintain its PPO or phenolic content better than other cultivars after storage. Since fruit storage duration before the browning measurement may be a significant factor in the rate and extent of browning, storage effects should be a part of future browning studies.

Cold storage may have a significant effect on factors affecting browning. Given how different cultivars respond differently to storage, studies examining only one or two cultivars should not be generalized to apply to the majority of apple cultivars. Broader studies examining a wider range of cultivars would be likely to pick up trends applicable to apples in general.

If a breeding program were to adapt this method for more rapid and efficient calculation of browning, the most important time measurements would be at 0 min, 5 min, 10 min, 20 min and 1 hour. By taking only these five measurements, the amount of labor involved would be significantly reduced while still covering the typical browning curve. Depending on the desired difference in total color change to be detected, sample size could also be reduced. At our current sample size of 15 and average variance of 4.43, a difference of 2.5 on the ΔE^* scale may be detected. Table 2.2 lists the sample size needed to detect various levels of difference in ΔE^*. If one were comfortable with a difference detected of 3 or 4, sample size could be reduced to 11 or 6 fruits per cultivar, respectively, while still obtaining statistically meaningful results. If the program were only concerned with flesh browning in the fruit as it is

consumed or used in fresh-cut, it would be possible to eliminate the measurements taken on the core. These steps would decrease the total number of measurements on a single cultivar from 900 at each season to 10x fruits per cultivar per season, where x is the sample size used for each apple, as determined from Table 2.2. Maintaining a difference detected of 2.5 in ΔE^*, but taking these other steps, would reduce the total number of measurements per cultivar by a factor of 6. Although it was important to capture these data for the sake of determining the outline of the browning curve and to be certain that it applied to a variety of cultivars, a breeding program could use these results to simplify their protocol, obtaining an overview of a single cultivar with less intense labor.

Table 2.2. Sample size required for different levels of detected difference in ΔE*. Assumed are an average intra-cultivar variance of 4.43, power of 90% and alpha significance level of 5%.

Difference Detected in dE*	Required Sample Size
1	94
2	24
2.5	15
3	11
4	6
5	4

Browning In the Core and Near the Peel. We measured the rates of browning in two locations on the cut apple fruit: directly outside the core ("inner") and directly inside the peel ("outer"). These locations were chosen because although PPO is uniformly distributed within the immature apple fruit, it tends to be localized near the core in mature apples (Murata et al. 1995b). This should cause higher rates of browning near the core than elsewhere in the apple. Some cultivars are also prone to post-harvest core browning disorders.

These results are illustrated in Figure 2.9 and Table 2.1. For both seasons, both at harvest and after storage, the core location tended to have a higher initial rate of browning than the outer location. The core-proximate browning rate was between 38% and 79% higher than the peel-proximate browning rate, at an average of 55% higher. This was expected, given the known higher concentration of PPO in the core of an apple versus the flesh.

Over the period of an hour, the overall extent of browning tended to converge. In 2008 both at harvest and after storage, the total extent of browning after 60 minutes as measured by the fitted ΔE* regression line was not significantly different for the inner and outer locations. In 2009, the at-harvest values converged, but remained significantly different for the two locations. The 2009 after storage values converged less and displayed more drastic differences between the two locations.

Since the quantity of polyphenoloxidase becomes less rate-limiting the longer the browning reaction progresses, differences in the extent of browning due to PPO concentration differences at the inner and outer locations should decrease over time. However, the concentration of phenolic substrates also affects the rate and extent of browning. Phenolic compounds are highest in the peel, lowest in the outer part of the cortex, and increase gradually towards the core (Petkovsek et al. 2007). A higher extent of browning near the core than in the outer portion of the cortex is suggested. Although there were no significant differences between the two locations in 2008, differences in 2009 were significant, and likely due to differences in PPO, phenolics or both.

Polyphenoloxidase Content. The PPO content by cultivar is presented in Figure 2.10. PPO is measured in enzyme activity units, where one AU is defined as a change

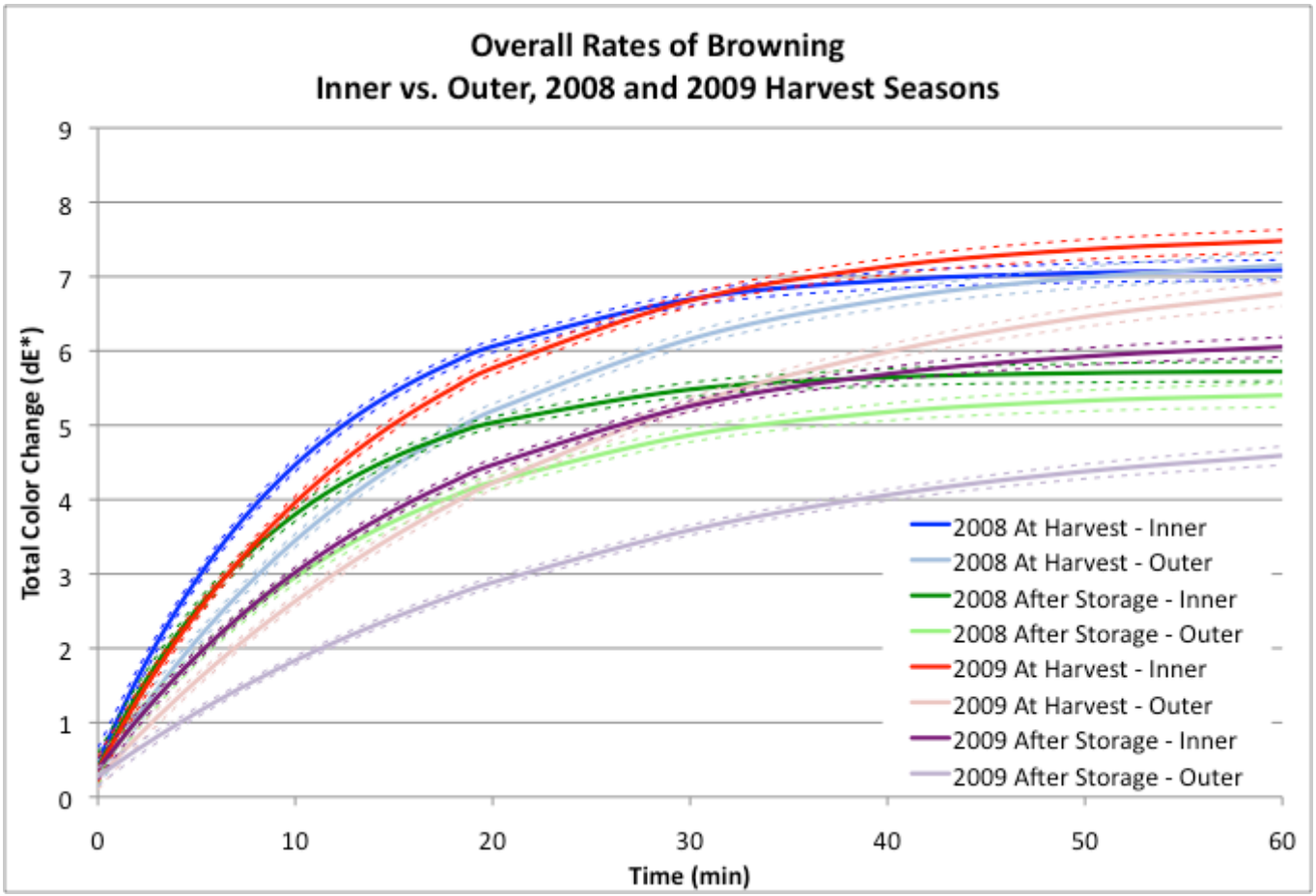

Figure 2.9. Fitted regression lines illustrating overall rates of browning (ΔE*) for the 2008 and 2009 harvest seasons both at harvest and after four months in cold storage. Core-proximate (inner) measurements are shown as darker lines, while peel-proximate (outer) measurements are shown lighter. Dashed lines indicate standard error of the regression; overlapping dashed lines indicate a lack of significant difference between two regression lines.

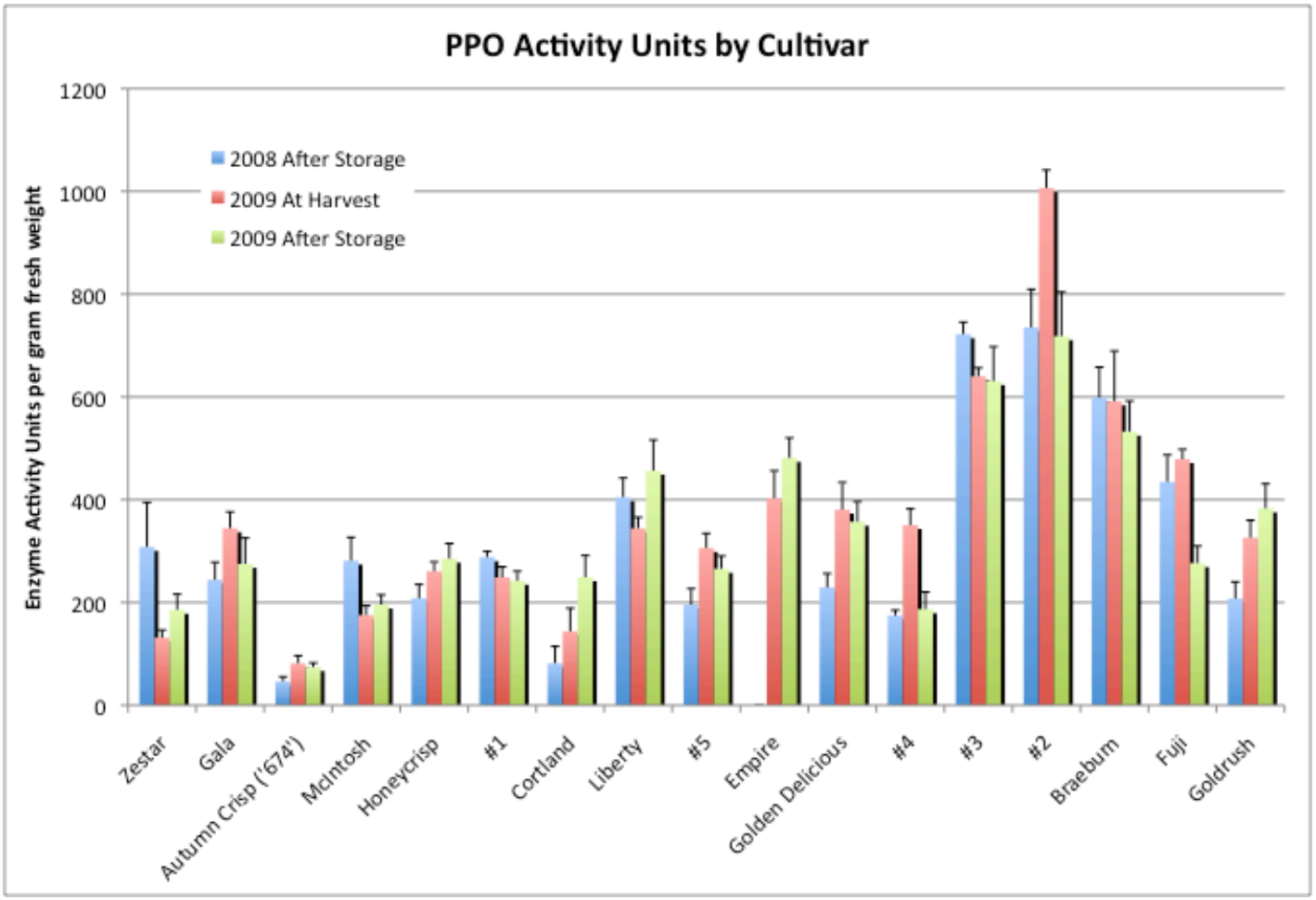

Figure 2.10. Total polyphenoloxidase (PPO) activity units by cultivar. One enzyme activity unit is defined as a change in absorbance of 0.001 units per minute per gram fresh weight.

in absorbance of the catechol standard of 0.001 units per minute per gram of fresh weight. PPO was not measured at harvest in 2008.

Cultivars with the lowest levels of PPO across the three measurement periods include 'AutumnCrisp', 'Cortland', 'Zestar', 'McIntosh', and selection #4 ('AutumnCrisp' x 'Fuji'). Although 'AutumnCrisp' is among the lowest browning cultivars, and 'Zestar' is a low to moderate browner, the others listed tended to be high browners despite their low PPO levels. Selection #4 and 'McIntosh' have very high phenolic content (Figure 2.11), which may explain their high level of browning despite fairly low PPO levels.

Cultivars showing the highest levels of PPO were selections #2 ('McIntosh' hybrid x 'Fuji'), #3 ('Braeburn' x 'AutumnCrisp'), 'Braeburn', 'Fuji', 'Empire', and 'Liberty'. Among these, selections #2, 'Fuji', and 'Liberty' were also among the highest browning fruits, likely due at least in part to their high PPO content. Selection #3, however, despite having relatively high levels of PPO, was among the lowest browning cultivars both years.

In vivo, PPO is compartmentalized in plastids within the cell, where it is kept inactive and stabilized by chaperones until tissue disruption enables it to contact phenolic compounds stored in the vacuoles (Marquès et al. 1995; Queiroz et al. 2008). Cultivars with high PPO may possess different or better mechanisms to stabilize and inactivate the enzyme such that they would behave as low-PPO cultivars.

Most of the advanced selections exhibited PPO activity either intermediate to their two parents or very similar to one of their parents. Selection #1, the 'Honeycrisp' hybrid, had PPO activity not statistically different than its parent 'Honeycrisp'. Selections #3 and #5, the 'Braeburn' x 'AutumnCrisp' hybrids, were intermediate to their parents, as was selection #4 ('AutumnCrisp' x 'Fuji'). Selection

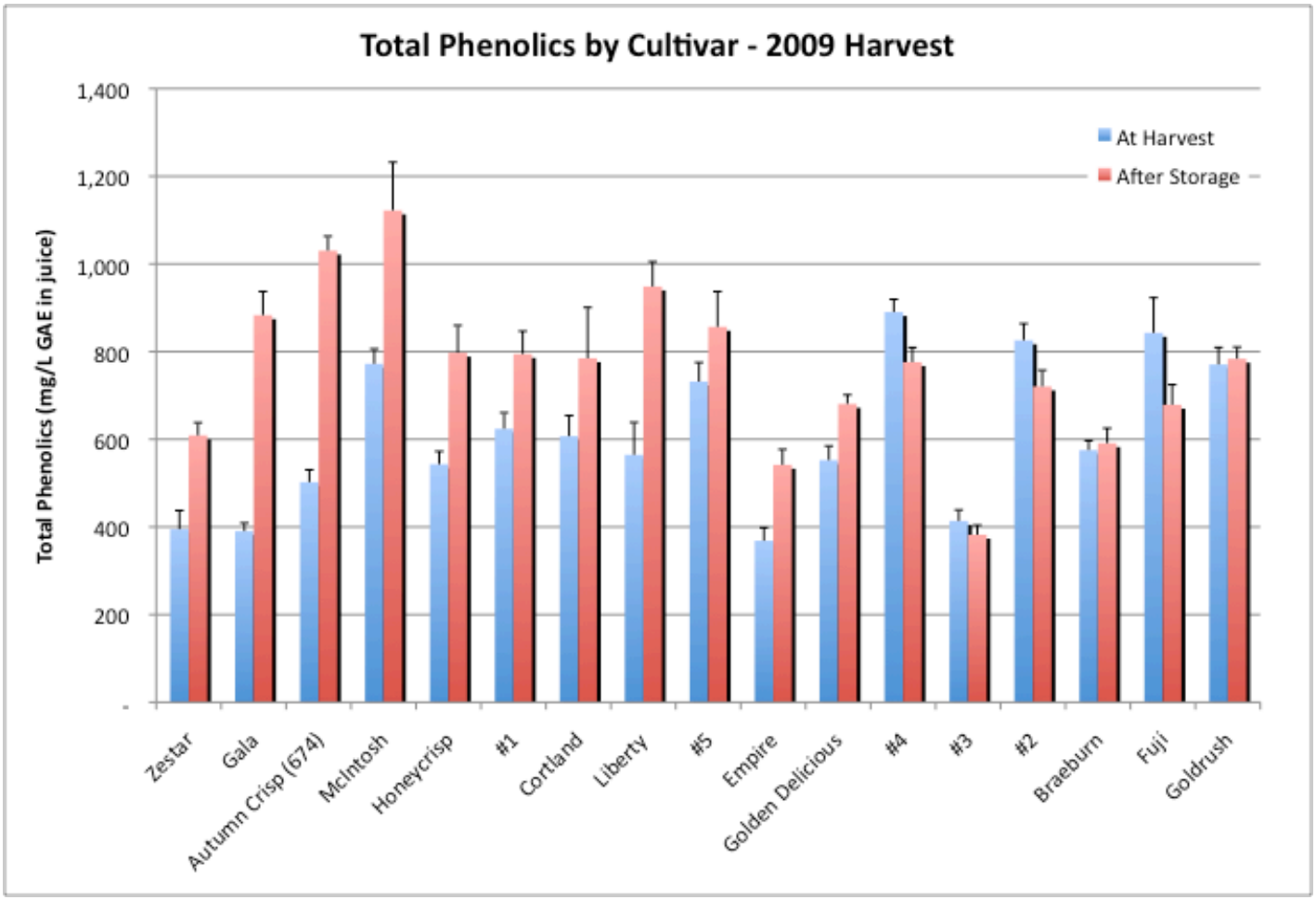

Figure 2.11. Total polyphenolic concentration by cultivar. Phenolic concentration is given in Gallic Acid Equivalents (GAE). Total phenolic content data unavailable for the 2008 harvest season.

#2 (‘McIntosh’ hybrid x ‘Fuji’), however, had significantly higher PPO activity than either ‘McIntosh’ or ‘Fuji’.

For the majority of cultivars, there was no difference between at-harvest and after-storage PPO in 2009. Only three cultivars, ‘Fuji’ and selections #2 (‘McIntosh’ hybrid x ‘Fuji’) and #4 (‘AutumnCrisp’ x ‘Fuji’), differed significantly in at-harvest and after-storage values. Ironically, ‘Fuji’ is the cultivar that differed least in its at-harvest and after-storage rates of browning, despite drastically reduced PPO values over this time period. There may be metabolic processes that caused the enzyme to degrade in these cultivars over time in storage.

Similarly, the majority of cultivars showed no significant differences between 2008 after-storage and 2009 after-storage measurements. Only ‘Cortland’ and ‘Goldrush’ were significantly different from one year to the next; both cultivars increased their PPO activity. Because we were unable to obtain 2008 at-harvest measurements for these cultivars, an at-harvest comparison between years was not possible. PPO after-storage was fairly stable from one year to the next in the majority of cultivars.

Total Phenolic Content. Total phenolic content (Figure 2.11) was measured against a gallic acid standard. Phenolic content was not measured for the 2008 harvest season. The average phenolic content for all cultivars was 610 mg/100 g fresh weight at harvest and 764 mg/100g fresh weight after storage. Phenolics ranged from 368 to 890 mg/100 g fresh weight at harvest and 382 to 1,122 mg/100 g fresh weight after storage.

As previously noted with rate of browning and PPO content, total phenolic contents of the advanced breeding selections were highly related to the phenolic content of one or both of their parents. Selection #1 (‘Honeycrisp’ hybrid) was nearly identical to ‘Honeycrisp’ both at harvest and after storage. Selections #2 (‘McIntosh’

hybrid x 'Fuji'), #4 ('AutumnCrisp' x 'Fuji') and #5 ('AutumnCrisp' x 'Braeburn') were intermediate to their respective parents. Selection #3 ('Braeburn' x 'AutumnCrisp') had slightly lower phenolic content than either of its two parents.

The phenolic content for many cultivars in this study increased after three months in cold storage, consistent with some previous research. Napolitano et al. (2004) reported that 'Annurca', 'Golden Delicious', and 'Empire' had an increase in total phenolics after 3 months in cold storage, possibly due to the activity of phenylalanine ammonia lyase, an enzyme that stimulates the biosynthesis of phenolic compounds. Other studies found that apple phenolic content was not greatly affected by cold storage (Coseteng and Lee 1987; Awad and De Jager 2002; Boyer and Liu 2004).

The cultivars with the highest average phenolic content included 'McIntosh', selections #4 ('AutumnCrisp' x 'Fuji'), #5 ('AutumnCrisp' x 'Braeburn'), and #2 ('McIntosh' hybrid x 'Fuji'), 'Goldrush', 'AutumnCrisp', 'Liberty', and 'Fuji'. Among these, 'Liberty', 'McIntosh, #2, and 'Fuji' were all consistently among the highest browners. Selection #4 tended to brown moderately. However, selection #5 and 'AutumnCrisp' were among the lowest browning cultivars. 'AutumnCrisp' has an extremely low level of PPO, which would help explain its low incidence of browning. Selection #5 also had a moderately low level of the enzyme polyphenoloxidase. Cultivars with the lowest total level of phenolics included selection #3 ('Braeburn' x 'AutumnCrisp') and 'Braeburn'. Although #3 tended to be fairly low browning, 'Braeburn' was among the moderate to high browning cultivars, indicating that total phenolic content is not controlling its rate of browning. These results highlight the complicated nature of the browning reaction, where several factors together determine the overall rate and extent of browning.

Correlations. To determine a baseline correlation, comparisons were run between the 2008 at-harvest and 2008 after-storage total color change values, as well as the 2009 at-harvest and after-storage values (Figure 2.12). The 2008 harvest to storage values were highly correlated, with an R^2 of 0.87 and p-value < 0.0001, while the 2009 harvest to storage values had an R^2 of 0.70 and p-value <0.0001.

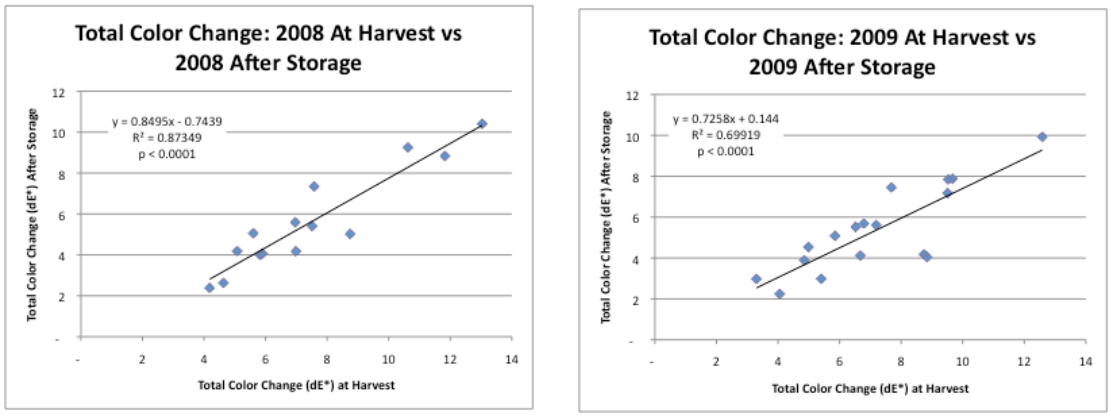

Figure 2.12. Total color change (ΔE*) after 60 minutes at harvest vs. after storage, 2008 and 2009.

PPO activity was compared to total color change across the three time periods in which PPO was measured (Figure 2.13). In 2008 after storage, the PPO values were correlated positively to total color change, with an R^2 of 0.24 and a p-value of 0.077. This is a very low correlation and is not significant at $\alpha = 0.05$. However, when three outliers were excluded ('Liberty', 'McIntosh', and selection #3), the correlation between PPO activity and browning after-storage is strongly positive, with an R^2 of 0.70, and significant ($p = 0.0013$). Although statistically there is not a significant reason for excluding these three outliers, there were many studies done in the past using far fewer than the 17 cultivars that we used in our analysis, that reported correlations between PPO activity and total extent of browning in apples. If our study

had by chance chosen to exclude these three cultivars, we would have had a fairly strong correlation in 2008 after-storage between PPO and total color change. Therefore, studies that use a smaller variety of cultivars may not necessarily be extrapolated to represent apples as a group. With some cultivars there is a correlation between PPO activity and color change in specific seasons, but this is not true for all cultivars and all years. PPO activity and total color change were not correlated in 2009 at harvest nor after storage (Figure 2.13).

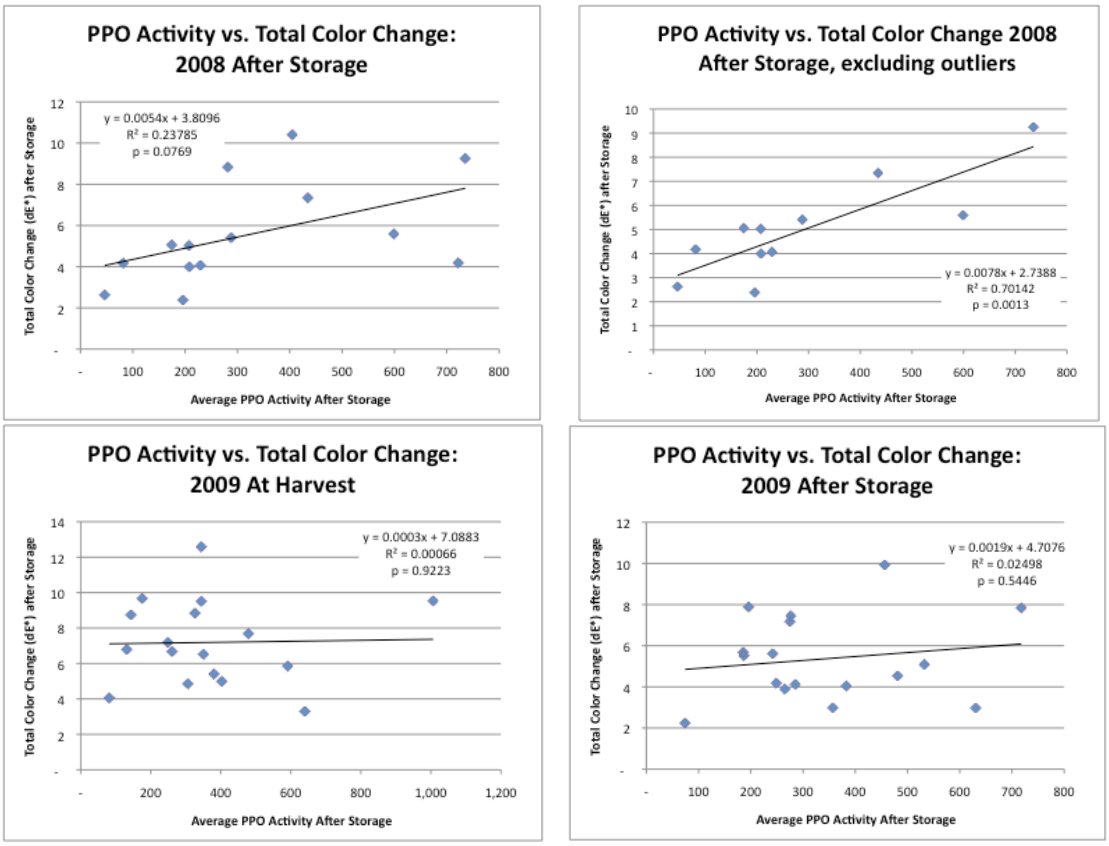

Figure 2.13. Correlation between PPO and total color change (ΔE*) after 60 minutes by season. Outliers excluded in 2008 were selection #3, 'Liberty', and 'McIntosh'.

Total phenolic content was analyzed for the 2009 harvest season (Figure 2.14). Both at harvest and after storage, there was a low correlation between total phenolic content and total color change (R^2 of 0.09 and 0.10 and p-values of 0.24 and 0.21, respectively).

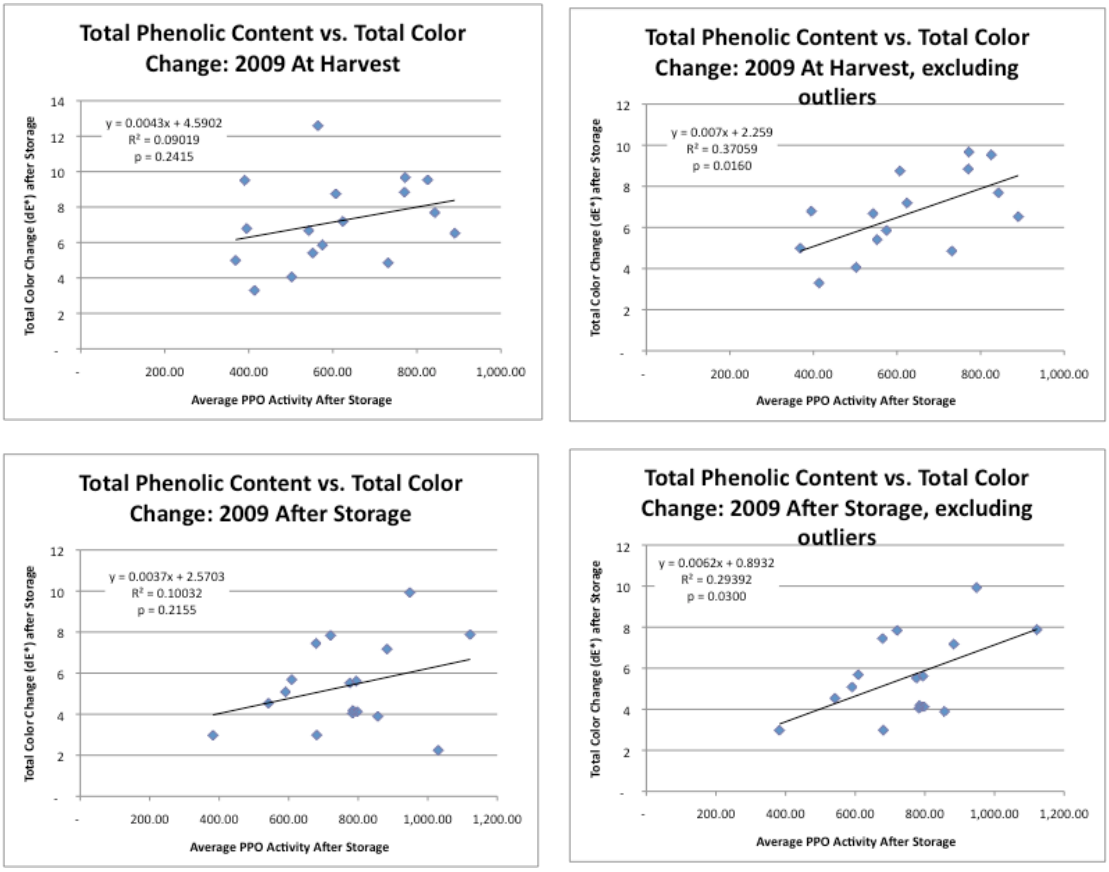

Figure 2.14. Correlation between total phenolic content and total color change (ΔE*) after 60 minutes by season. 'Gala' and 'Liberty' were the outliers excluded in 2009 at harvest. 'AutumnCrisp' was the outlier excluded in 2009 after storage.

Several multivariate models were run in an attempt to clarify relationships between polyphenoloxidase, total phenolics, rate and extent of browning and vitamin C content as reported in Burke et al. (2010). No model fit the data at a parameter significance level α=0.05.

Conclusion

This study provides a solid, quantitative baseline for the relative rates of browning, PPO activity, and phenolic content of twelve important commercial apple cultivars and five advanced breeding selections. Given that only some cultivars correlated their rates of browning to their PPO and/or phenolic content, future studies could expand on this work by exploring further the causative factors for differences in browning activity over different harvest years and after time in storage. The role that environmental conditions such as temperature, rainfall, sunlight, etc. might play in the development of browning potential could be explored. Given that there appears to be more to the browning equation than simply high PPO and high phenolic content yield high browning fruit, studies that are able to further elucidate the causative factors would be beneficial in ultimately developing lower browning apple cultivars.

The method used to quantify browning in this study can be broadly applied to different cultivars to measure the rate of browning on new varieties in a standardized way. Being able to thus quantify the rate of flesh browning is a highly useful tool for a breeding program seeking to manipulate this trait. When characterizing the rate of browning of a cultivar, it is important to note the different ways in which varieties react to environmental conditions, growing year, and time in storage, and account for these factors in any study. Since PPO activity and phenolic content are only correlated to browning in some cultivars, they should not be used in place of browning measurements to determine the browning potential of a fruit. Although the browning reaction involves more than simply the relative quantities of substrate and enzyme, it is useful to know the relative quantities of PPO and phenolics in apple fruit, especially given the other beneficial effects these compounds have on human and plant health.

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CHAPTER 3

VITAMIN C CONTENT OF SELECT APPLE (*MALUS DOMESTICA* BORKH.) CULTIVARS AND ADVANCED BREEDING SELECTIONS

Abstract

Vitamin C (L-ascorbic acid or L-AA) is an important antioxidant and metabolite in plants and for humans. Apples have the potential to be a significant source of vitamin C in the human diet. This study quantified the vitamin C levels of twelve commercial cultivars and five advanced breeding selections to assess differences and examine the potential to increase Vitamin C in new varieties. 'Braeburn' had the highest mean vitamin C content, 21.7 mg/100g fresh weight. The advanced breeding selections averaged between 11.9 and 14.5 mg/100 g fresh weight, significantly higher than most of the commercial cultivars measured. Harvest year and time in storage both had effects on vitamin C, with cultivars able to maintain their vitamin C levels during storage to varying degrees. A low but significant correlation was found between harvest date and vitamin C content.

Introduction

Apples (*Malus domestica* Borkh.) are the second most widely consumed fruit in America, with per capita consumption of 43.4 lbs per year (Chun et al. 2005). Vitamin C (L-ascorbic acid or L-AA) is an important antioxidant in apple fruits. Humans are unable to synthesize L-AA and must rely on plants as the primary source of this vitamin (Giovannoni 2007). Currently, the US Recommended Daily Allowance (RDA) of vitamin C ranges between 75 mg and 120 mg per day for adults (Medicine 2000). Although the ascorbate content of most commercial apple cultivars

is low, typically ranging between 2 and 10 mg/100 g fresh weight (FW), some cultivars reportedly have as much as 26 mg/100 g FW (Davey et al. 2000; Brown et al. 2004; Davey and Keulemans 2004; Planchon et al. 2004). In comparison, citrus fruits (grapefruit, orange, and lemon) range from 30 to 55 mg/100 g FW and strawberries contain 55 to 60 mg/100 g FW (Davey et al. 2000; Proteggente et al. 2002; Szeto et al. 2002). Although some other fruits contain higher concentrations of vitamin C, the high consumption of apples makes them a significant contributor to overall vitamin C intake in the American diet.

Vitamin C and other antioxidants protect cells from oxidative stress by inactivating reactive oxygen species (ROS), cell-damaging byproducts of metabolism. L-AA is one of the most common antioxidants in plants and is essential for all plant tissues (Davey et al. 2006; Davey et al. 2007). Dehydroascorbate (DHA) is the product of ascorbate oxidation, a reversible reaction. The amount of ascorbate present as DHA can indicate the overall oxidation-reduction state of the cellular environment, with higher proportions of DHA indicating higher levels of experienced oxidative stress (Li et al. 2010).

Vitamin C also has several functions in plants beyond antioxidant protection. Metabolically, vitamin C is involved in the modulation of plant cell growth and proliferation, as an enzymatic cofactor for some oxygenase enzymes and as a donor/acceptor in electron transport (Davey et al. 2000; Smirnoff and Wheeler 2000). Plants produce L-AA as a response to stress; those deficient in L-AA are highly sensitive to environmental stresses (Conklin et al. 1996; Cobbett et al. 1998; Šircelj et al. 2007). Increased vitamin C content may improve post-harvest pathogen resistance and overall storage properties of fruits (Franck et al. 2003; Hancock and Viola 2005; Davey et al. 2006; Davey et al. 2007).

In humans, vitamin C is one of the most effective and least toxic antioxidants, providing protection against oxidative stress related diseases such as cancer, cardiovascular disease and neurodegenerative diseases (Lee et al. 2002; Hancock and Viola 2005; Davey and Keulemans 2009). Vitamin C is multifunctional and valuable beyond its antioxidative ability. Vitamin C may have more powerful anti-cancer effects than other free radical scavengers, such as propylgallate and trolox, by acting through a different mechanism than these antioxidants (Lee et al. 2002). Vitamin C increases the uptake of iron and zinc in meat-poor diets by increasing absorption of nonheme iron, the form of iron present in plant-based foods (Frossard et al. 2000). It promotes collagen synthesis and the formation and maintenance of cartilage, bones, gums, skin, and teeth (Davey et al. 2000; Hancock and Viola 2005). Inadequate consumption of L-AA causes scurvy, a disease characterized by fatigue, widespread connective tissue damage and blood vessel fragility (Li and Schellhorn 2007).

The benefits of vitamin C are well known to consumers. Widespread marketing strategies emphasize the importance of adequate vitamin C intake as well as the vitamin C content of certain foods (Hancock and Viola 2005). Bioactive ingredients in food that are familiar to consumers are more likely to be understood and accepted than more novel ingredients (Lampila et al. 2009). In the fresh cut apple industry, calcium ascorbate is widely added to preserve texture, crispness and color in packaged apple slices (Rupasinghe et al. 2005). Processed foods can easily be fortified with synthetic vitamin C, but increased awareness and concern among consumers regarding additives in food, and the tightening of legislation on fortification make modification on the whole plant level more desirable (Hancock and Viola 2005). Several apple breeding programs have increasing the vitamin C content of new cultivars as a goal (Brown et al. 2004; Davey and Keulemans 2004).

L-AA content may vary by 3.6 to 4.8 fold across different cultivars grown in similar environments, indicating that it may be possible to breed for enhanced L-AA content (Davey and Keulemans 2004; Davey et al. 2007). L-AA is inherited quantitatively in apple (Davey and Keulemans 2009). Three highly significant, major quantitative trait loci (QTL) clusters were located on linkage groups (LG) 17, 11 and 16 in a 'Braeburn' x 'Telamon' mapping population (Davey et al. 2006; Davey and Keulemans 2009). Identifying highly significant and stable QTLs enables more directed breeding of a trait.

Understanding and characterizing environmental effects is a challenge in breeding for enhanced L-AA content. L-AA levels varied 25 to 30% from one year to the next and contained significantly different ratios of L-AA to DHA, indicating environmental conditions may mask underlying trait QTLs (Davey and Keulemans 2009). Light is one of the most significant environmental conditions affecting ascorbate content in apples. High light exposure may significantly increase the ascorbate content in the peel, but may have little effect on ascorbate content and recycling in the flesh (Łata et al. 2005; Li et al. 2008; Li et al. 2009). Harvest date may also factor into ascorbate content, as later harvested cultivars had higher levels of L-AA than early harvested cultivars (Davey and Keulemans 2004; Davey et al. 2007). Postharvest storage may also significantly affect vitamin C content of fruits, although the effect varies at the cultivar level (Davey and Keulemans 2004; Tarozzi et al. 2004; Vilaplana et al. 2006; Felicetti and Mattheis 2010). Since most apples are consumed after several months in cold storage, postharvest metabolism of ascorbate is as important as values at harvest.

An awareness of the vitamin C content of new selections is important, as it provides a basis for comparing new varieties with existing cultivars. This is useful both for marketing newer cultivars to the public and for selecting parents for future

breeding crosses. This study examined the vitamin C content of twelve commercial apple cultivars and five advanced breeding selections from Cornell University's apple breeding program that are being used as parents. We quantified vitamin C at harvest and after four months of storage to determine the effect of storage on levels of this nutrient. Several advanced selections will be compared to their parents. Our results will be compared with those of other researchers.

Materials and Methods

Cultivar Selection. Twelve commercial apple cultivars and five advanced breeding selections were harvested: 'Zestar', 'Gala', 'AutumnCrisp' (NY674), 'McIntosh', 'Honeycrisp', 'Cortland', 'Liberty', 'Golden Delicious', 'Braeburn', 'Fuji', 'Goldrush', 'Empire', and selections #1 ('Honeycrisp' x NY selection), #2 ('McIntosh' hybrid x 'Fuji'), #3 ('Braeburn' x 'AutumnCrisp'), #4 ('AutumnCrisp' x 'Fuji'), and #5 ('AutumnCrisp' x 'Braeburn'). All cultivars were picked at commercial maturity during the 2008 and 2009 harvest seasons at Cornell University's New York State Agricultural Experiment Station orchard in Geneva, New York. 'Empire' was only harvested and studied during the 2009 harvest season. Starch-iodine testing was used to assess maturity stage. Apples not analyzed immediately were kept in cold storage at 1^{o}C until use.

Chemicals and Solvents. Metaphosphoric acid (MPA), polyvinylpolypyrrolidone (PVPP), ethylenediaminetetraacetic acid, sodium salt, 0.1M in water (EDTA), o-phosphoric acid, methanol, L-ascorbic acid, dithiothreitol (DTT), and tris (2-carboxy-ethyl) phosphine hydrochloride base (Tris) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Acetonitrile was obtained from GFS Chemicals, Inc. (Powell, OH). All chemicals used were analytical or HPLC grade.

Quantification of Vitamin C. Ascorbic acid levels in the apple cultivars were determined using the "Rocket" HPLC method outlined by Davey et al. (Davey et al. 2003). This method allows for rapid and accurate quantification of Vitamin C across multiple samples. Measurements were taken after four months of storage for the 2008 harvested cultivars, and both at harvest and after four months of storage for the 2009 harvested cultivars. Ten apples were randomly selected from each cultivar, and subdivided into groups of three, three, and four apples. The apples were sliced with an apple corer-slicer and every third radial slice was selected. Slices were weighed and placed in a blender with 2 mL of extraction buffer (6% MPA, 2mM EDTA and 1% insoluble PVPP in water) per gram of fresh weight, and homogenized at high speed for 60 seconds. The resulting slurry was vacuum filtered using Whatman filter paper (Piscataway, NJ). Three aliquots of clarified extract were selected from the filtrate and further filtered using a Grace 0.45 micron, 13 mm diameter nylon HPLC syringe filter. Samples were refrigerated until analysis, which occurred within 12 hours of extraction.

A reduction procedure was performed to determine the total dehydroascorbic acid (DHA) concentration in the extract. Then 0.5 mL of extract was added to 0.25 mL of a stock solution comprised of 200 mM DTT in a 400 mM Tris base. The reduction was allowed to run for 15 minutes, then was halted by acidification with 0.25 mL of 8.5% o-phosphoric acid. Extracts were then analyzed by HPLC, with the resultant concentration doubled to account for the dilution of the original extraction. DHA was calculated as the difference between the HPLC readings of the non-reduced and reduced extractions.

HPLC analyses were performed on an Agilent Technologies 1200 Series HPLC system (Santa Clara, CA) with a variable length UV detector. The system was controlled and data were gathered and analyzed by Agilent ChemStation Version

A.10.02[1757] software on a PC. Samples were separated on a 53 x 7-mm "Rocket" HPLC column (Grace Davison Discovery Sciences, Deerfield, IL). The column was custom packed with a LiChrosorb RP C18 end-capped 3-µm spherical particle size stationary phase and fitted with a guard column packed with 5-µm sized particles of the same material. The column was eluted at 3.0 ml/min, with a 3-min linear gradient of 0-30% acetonitrile in a mobile phase comprised of 400 µl/L o-phosphoric acid, 0.1 mM EDTA, and 0.25% methanol (pH ~2.5). Following column regeneration, total analysis time was 6 minutes per sample. Ascorbic acid was quantified at its UV absorption maximum of 243 nm. Blanks were run to cancel out noise. The identity of the ascorbic acid peak was confirmed by elution of a standard. Stock solutions were prepared daily in 3% MPA/1 mM EDTA, stabilized with 2.5 mM DTT, and used to calibrate the machine.

Statistical Analysis. Statistical analysis was performed in JMP version 8.0.1, Copyright © 2009 SAS Institute Inc. (Cary, NC).

Results and Discussion

Total Vitamin C Content. Total vitamin C content for the 17 cultivars is presented in Figure 3.1, Table 3.1 and Table 3.2. In 2008, ascorbic acid was only measured after four months in storage. Values averaged 9.54 mg/100g fresh weight (FW) and ranged from 3.48 to 22.69 mg/100 g FW, a variation of 6.5 fold. Cultivars measured at harvest in 2009 averaged 10.25 mg/100 g FW and ranged from 3.60 to 24.50 mg/100 g FW, a 6.8 fold variation. The 2009-harvested apples measured after four months in cold storage averaged 10.43 mg/100 g FW and ranged from 2.46 to 24.42 mg/100 g FW, a variation of 9.9 fold. This is slightly higher variation than that found by Davey et al., who found a range of 3.6 to 4.8 fold variation (Davey et al. 2007). The HPLC method used to quantify L-AA in our study is nearly identical to that used by Davey et

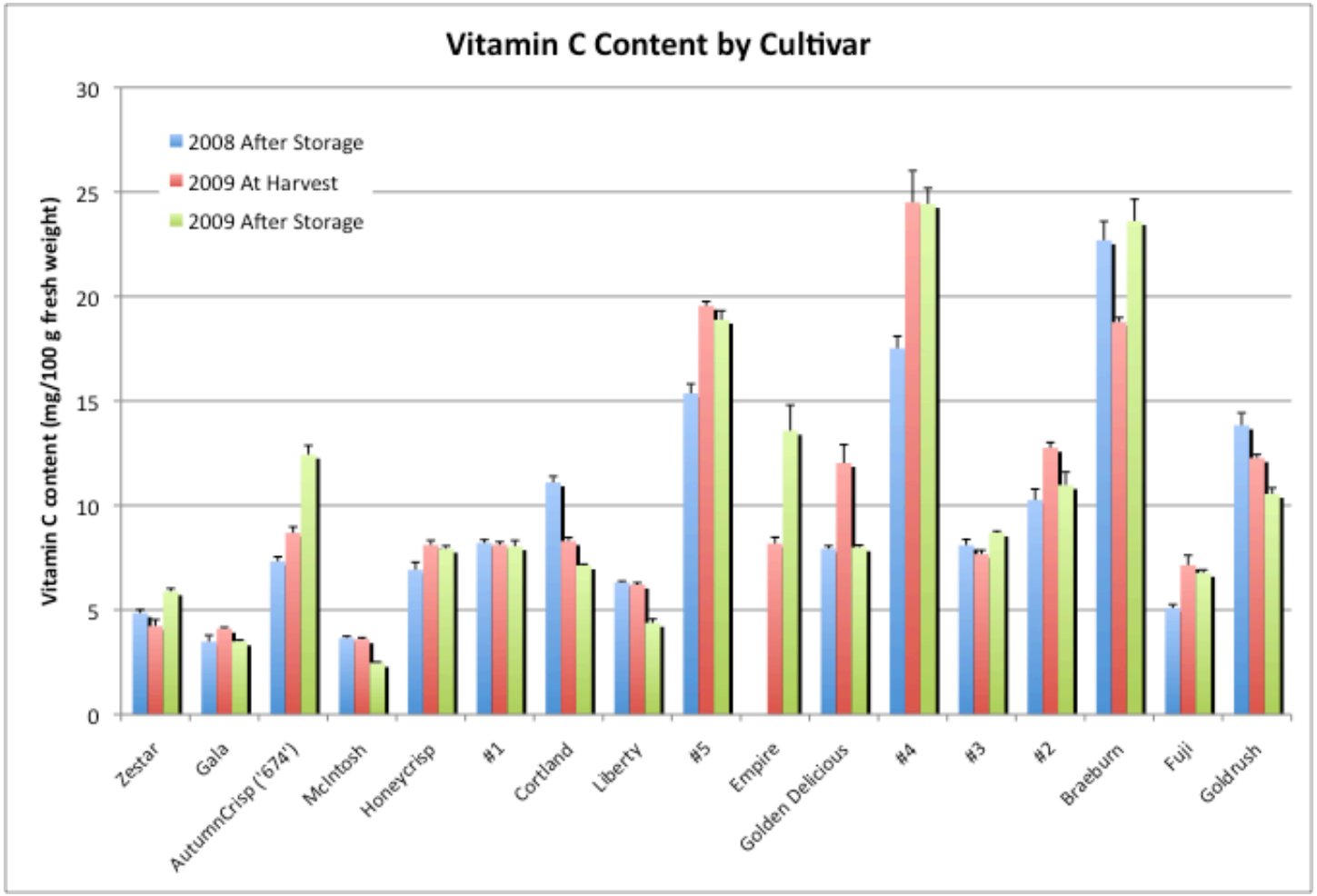


Figure 3.1. Vitamin C content for 17 apple cultivars for the 2008 and 2009 harvest seasons.

Table 3.1. Concentrations of Vitamin C and DHA for 17 apple cultivars. Measurements were taken in the 2008 harvest season after four months in cold storage and in the 2009 season both at harvest and after four months in cold storage. All values are given in mg/100g fresh weigh

	2008 - After Storage				2009 - At Harvest				2009 - After Storage			
Cultivar	Vitamin C	SD	DHA	SD	Vitamin C	SD	DHA	SD	Vitamin C	SD	DHA	SD
Zestar	4.85	± 0.48	0.98	± 0.44	4.22	± 0.94	0.00	± 1.03	5.89	± 0.41	0.23	± 1.03
Gala	3.48	± 0.92	0.82	± 1.37	4.10	± 0.21	0.25	± 0.30	3.49	± 0.19	0.18	± 0.30
AutumnCrisp ('674')	7.32	± 0.65	0.98	± 0.84	8.69	± 0.86	0.00	± 0.97	12.43	± 1.31	0.27	± 0.97
McIntosh	3.66	± 0.23	0.58	± 0.72	3.60	± 0.22	0.15	± 0.13	2.46	± 0.15	1.11	± 0.13
Honeycrisp	6.92	± 1.09	0.89	± 0.72	8.11	± 0.66	0.00	± 0.73	7.93	± 0.41	0.07	± 0.73
#1 (Honeycrisp x NY selection)	8.21	± 0.45	4.56	± 2.46	8.12	± 0.41	0.00	± 1.04	8.05	± 0.83	0.17	± 1.04
Cortland	11.10	± 0.88	0.31	± 1.35	8.29	± 0.50	0.00	± 0.51	7.13	± 0.17	0.00	± 0.51
Liberty	6.32	± 0.18	0.39	± 1.25	6.21	± 0.27	0.52	± 0.19	4.38	± 0.55	0.82	± 0.19
#5 (AutumnCrisp x Braeburn)	15.36	± 1.37	0.81	± 1.37	19.55	± 0.61	0.00	± 0.53	18.88	± 1.29	0.00	± 0.53
Empire					8.17	± 0.92	0.51	± 0.58	13.57	± 3.68	0.35	± 0.58
Golden Delicious	7.93	± 0.41	0.76	± 0.32	12.03	± 2.63	1.13	± 2.31	7.99	± 0.31	0.00	± 2.31
#4 (AutumnCrisp x Fuji)	17.51	± 1.75	0.85	± 1.71	24.50	± 4.56	0.00	± 3.59	24.42	± 2.28	0.00	± 3.59
#3 (Braeburn x AutumnCrisp)	8.10	± 0.82	1.55	± 0.79	7.67	± 0.57	0.68	± 0.61	8.70	± 0.21	0.00	± 0.61
#2 (McIntosh hybrid x Fuji)	10.26	± 1.58	1.01	± 1.80	12.75	± 0.78	0.94	± 0.71	10.97	± 1.87	0.00	± 0.71
Braeburn	22.69	± 2.73	0.15	± 2.76	18.77	± 0.67	0.10	± 1.07	23.61	± 3.12	0.00	± 1.07
Fuji	5.09	± 0.51	0.54	± 0.75	7.14	± 1.42	0.48	± 1.32	6.77	± 0.44	0.00	± 1.32
Goldrush	13.83	± 1.80	1.38	± 2.29	12.26	± 0.50	0.31	± 0.66	10.55	± 0.87	0.00	± 0.66

Table 3.2. Summary statistics: Vitamin C concentration in mg/100g fresh weight by time measured and cultivar type.

	2008 - After Storage		2009 - At Harvest		2009 - After Storage	
All Cultivars						
Mean	9.54	± 5.37	10.25	± 5.84	10.43	± 6.47
Range	3.48	- 22.69	3.60	- 24.50	2.46	- 24.42
Fold Variation	6.5	fold	6.8	fold	9.9	fold
Commercial Cultivars						
Mean	8.47	± 5.65	8.47	± 4.28	8.85	± 5.74
Range	3.48	- 22.69	3.60	- 18.77	2.46	- 23.61
Cornell's Advanced Selections						
Mean	11.89	± 4.31	14.52	± 7.35	14.21	± 7.16
Range	8.10	- 17.51	7.67	- 24.50	8.05	- 24.42

al. in previous studies. Differences in the range of variation can likely be accounted for by differences in cultivars selected for study and differing growing conditions. Values for specific cultivars were very similar to those obtained by Davey et al. (Table 3.3).

The twelve commercially available cultivars had an average vitamin C content ranging from 8.47 to 8.85 mg/100g FW across the three measurement periods, with all but 3 cultivars below 10 mg/100g FW. Among the commercial cultivars, 'Braeburn' had the highest vitamin C content, with a mean of 21.69 mg/100g FW. These values compare favorably with those from previous studies (Table 3.3). The L-AA content of 'Braeburn' apples in Belgium was 18.7 and 22.6 mg/100g for different harvest seasons using the same HPLC method as used in this study (Davey and Keulemans 2004; Davey et al. 2007). Partly due to its high vitamin C status, 'Braeburn' has been used as a parent in Cornell University's breeding program in a number of crosses, including advanced selections numbers 3 and 5. 'Goldrush', 'Empire' and 'AutumnCrisp' were the commercial cultivars with the next highest levels of vitamin C, containing 12.2, 10.9, and 9.5 mg/100g on average, respectively.

Table 3.3. Comparable vitamin C values from studied apple cultivars as reported in the literature. Included on the right are the values obtained in this study for comparison.

		L-AA in			L-AA Values Obtained in This Study		
Cultivar	L-AA as reported	mg/100g FW	Method used	Reference	08 After Storage	09 At Harvest	09 After Storage
Braeburn	1061 +/- 110 nmol/g FW	18.7	RP-HPLC	Davey et al. 2007	22.7	18.8	23.6
	1283 +/- 32 nmol/g FW	22.6	RP-HPLC	Davey et al. 2004			
Cortland	12.17 mg/100g	12.2	spectrophotometric	Lee et al. 2003	11.1	8.3	7.1
Empire	13.22 mg/100g	13.2	spectrophotometric	Lee et al. 2003	N/A	8.2	13.6
Fuji	471 +- 74 nmol/gFW	8.3	RP-HPLC	Davey & Keulemans 2004	5.1	7.1	6.8
Gala	415 +/- 138 nmol/gFW	7.3	RP-HPLC	Davey & Keulemans 2004	3.5	4.1	3.5
Golden Delicious	586 +/- 17 nmol/g FW	10.3	RP-HPLC	Davey et al. 2007	7.9	12.0	8.0
	937 +/- 246 nmol/g FW	16.5	RP-HPLC	Davey et al. 2004			
	16.6 mg/100 g	16.6	spectrophotometric	Lee et al. 2003			
Goldrush	782.6 +/- 69 nmol/g FW	13.8	RP-HPLC	Davey & Keulemans 2004	13.8	12.3	10.6
Liberty	677 +/- 138 nmol/g FW	11.9	RP-HPLC	Davey & Keulemans 2004	6.3	6.2	4.4
AutumnCrisp (NY674)	11.62 mg/100g	11.6	spectrophotometric	Lee et al. 2003	7.3	8.7	12.4

Cornell University's advanced breeding selections compared favorably to the twelve studied commercial cultivars, averaging between 140% to 171% higher vitamin C content overall. The mean vitamin C content ranged from 11.89 mg/100 g FW to 14.52 mg/100 g FW in these selections. Several of the advanced selections were very similar to one or both of their parents. Cultivar #1 had a nearly identical amount of vitamin C as its parent 'Honeycrisp'. Cultivar #5 was intermediate to its parents 'AutumnCrisp' and 'Braeburn'. Cultivars #2 ('McIntosh' hybrid x 'Fuji') and #4 ('AutumnCrisp' x 'Fuji'), however, contained significantly higher level of vitamin C than their respective parents. Cultivar #3 had significantly lower vitamin C than its parent 'Braeburn', but was similar to its parent 'AutumnCrisp'.

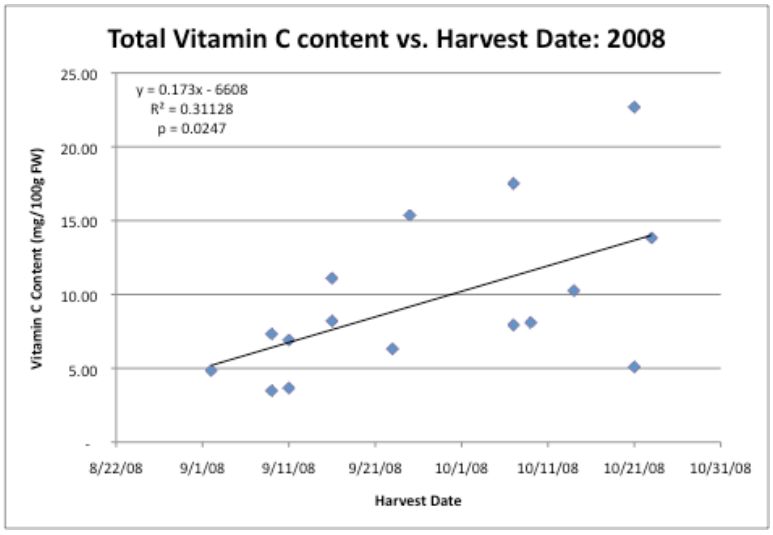
There does appear to be an effect of harvest year on Vitamin C levels. Since 2008 at harvest data was not available, comparisons were made between the after storage values of both years. The 2009 after-storage values averaged 7.2% higher than the 2008 after-storage values, excluding the cultivar ‘Empire’, for which data were not obtained in 2008. Many cultivars had nearly identical amounts of vitamin C after storage in both years: ‘Zestar’, ‘Gala’, ‘Honeycrisp’, #1 (a ‘Honeycrisp’ hybrid), ‘Golden Delicious’, #3 (‘Braeburn’ x ‘AutumnCrisp’), #2 (‘McIntosh’ hybrid x ‘Fuji’), and ‘Braeburn’. Other cultivars, however, differed quite significantly. ‘AutumnCrisp’, #5 (‘AutumnCrisp’ x ‘Braeburn’), #4 (‘AutumnCrisp’ x ‘Fuji’), and ‘Fuji’ all dramatically increased from 2008 after storage to 2009 after storage. ‘Goldrush’, ‘Cortland’, and ‘Liberty’ showed decreases over this time. These results illustrate how different cultivars may react differently to changing environmental conditions from one year to the next, with some cultivars being more heavily influenced by differences in the environment than others. Weather conditions in 2008 were dramatically different from those in 2009, which was considered a fairly atypical year for the Geneva, NY area. Overall, 2009 had 3.44” more rain in May and June

than 2008, but was drier from July through the end of October, with 4.62" less rain in those months in 2009 than in 2008 (NEWA 2010). 2009 accumulated 146 fewer growing degree days base 50^{o}F from May 1 through October 31, and was on average 0.71^{o}F cooler each day during that time period (NEWA 2010).

Davey et al. found a potential correlation between harvest date and vitamin C content of apple fruits, consistent across several harvest years (Davey et al. 2007). Later harvested fruit tended to have higher L-AA concentrations, and L-AA content was negatively correlated to mean daytime temperature. Since daytime temperature and harvest date are themselves often highly correlated, the researchers were unable to separate the contributions of temperature and genetic background, i.e. cultivar harvest date (Davey et al. 2007).

Our study found a significant, but not very high, correlation between fruit harvest date and vitamin C content for either 2008 or 2009 (Figure 3.2). In 2008, harvest date and L-AA content after three months in cold storage shared a correlation R^2 of 0.31 with a p-value of 0.025. In 2009, L-AA content at harvest shared an R^2 of correlation to harvest date of 0.25 with a p-value of 0.0397. It is interesting to note that with the 2009 data, if one excludes the two largest outliers - advanced selections #4 ('AutumnCrisp' x 'Fuji') and #5 ('AutumnCrisp' x 'Braeburn') - the strength of the correlation grows significantly, from 0.25 to 0.53, with a strengthening of the p-value from 0.0397 to 0.0020 (Figure 3.3). This highlights the impact that cultivar selection can have on determining relationships in apple fruits. If, by chance, our study had excluded these two selections, we would have reported this second, much higher, correlation between harvest date and Vitamin C content. It is likely that the L-AA levels of some cultivars are more highly influenced by factors related to harvest date, such as daytime temperature or even genetic differences, than others. Although our study did not find a high correlation between harvest date and L-AA content across

these 17 cultivars, that does not mean that such a correlation would not exist in a different grouping of cultivars.



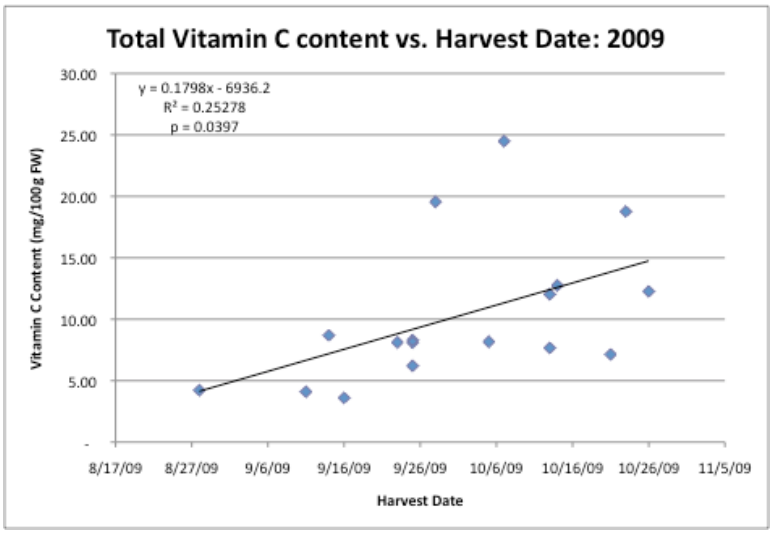


Figure 3.2. Correlation between harvest date and Vitamin C content for 2008 and 2009 harvest seasons. Vitamin C content was measured after 3 months in cold storage in 2008, and at harvest in 2009.

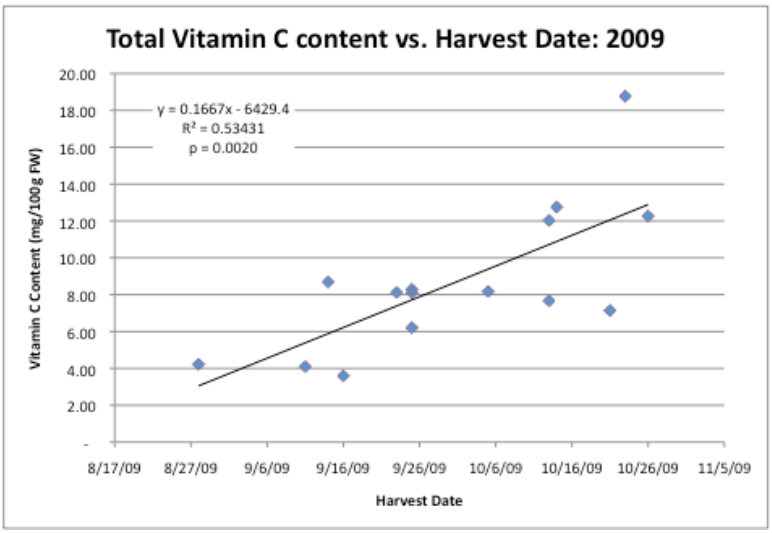


Figure 3.3. Correlation between harvest date and Vitamin C content for the 2009 harvest season, excluding two outliers. Vitamin C content was measured at harvest. Advanced selections #4 ('AutumnCrisp' x 'Fuji') and #5 ('AutumnCrisp' x 'Braeburn') were excluded from this analysis for illustrative purposes.

Total DHA Content. Total DHA content was measured in addition to ascorbate content and is presented in Table 3.1. Since DHA is both the end product of ascorbate oxidation and may be converted back to ascorbate, the quantity of DHA present in an apple fruit can indicate its overall oxidation state. As DHA is the oxidized form of L-AA, fruit with little to no DHA present have undergone less oxidative stress. A higher percentage of total ascorbate present as DHA indicates that the cellular environment has withstood more oxidative stress. A mean proportion of DHA below 25% of the total L-AA content indicates that significant oxidation did not occur during sample preparation (Davey and Keulemans 2004).

The percent of total ascorbate present in the fruit as DHA at harvest in 2009 ranged from 0 to 9%, and averaged 3%. After four months in cold storage, the percentage of L-AA present as DHA was in the range of 1 to 36% (average 11%) and 0 to 31% (average 4%) for the 2008 and 2009 harvest seasons, respectively. Overall,

fruit from the 2009 harvest had a lower quantity of ascorbate present as DHA after storage than fruit from the 2008 harvest. Fruit stored for four months had a higher proportion of its total ascorbate content present as DHA than fruit measured directly at harvest. Oxidative stress that cells experience in storage may cause more L-AA to oxidize to form DHA, causing this increase.

Davey and Keulemans found that the average DHA across 31 different cultivars was 1.7 mg/100g, with a range of 0 to 4.7 (Davey and Keulemans 2004). DHA levels in our cultivars were determined using a method modeled on that used by the Belgian team, and averaged 1.0, 0.3, and 0.19 across the three measurement periods (2008 after storage, 2009 at harvest and 2009 after storage). In some cases, the DHA present was very high. This is expected, given previous research in which only 3 of 31 cultivars studied had DHA above 4.5 mg/100g (Davey and Keulemans 2004). In other cases, DHA measured very low, at or near zero. Several cultivars in the Davey and Keulemans study also exhibited very low or no DHA.

Effect of Storage on Vitamin C Content. Data are available both at harvest and after storage for the 2009 harvest season. Because most apples are consumed after at least some time in cold storage, the ability of a fruit to maintain its vitamin C levels during storage greatly affects overall consumed levels of the vitamin. Davey et al. found that the differing ability of individual cultivars to maintain fruit ascorbate content post harvest led to an increase of cultivar differences from 4.7 to 18.7 fold (Davey et al. 2007). Our study revealed significant, but not as marked differences, with an increase from 6.8 fold to 9.9 fold. This could be due to the cultivars selected for study or differing growing or storage conditions.

The vitamin C levels of the commercial cultivars after storage was on average 102% of the fruit levels at harvest. The ability of different cultivars to maintain their vitamin C content varied widely, and 2009 post-storage levels ranged from 66% to

166% of 2009 at-harvest values. 'Golden Delicious', 'McIntosh' and 'Liberty' lost the most vitamin C during storage in 2009, losing 34%, 32%, and 29% of their at-harvest vitamin C, respectively. 'Empire', 'AutumnCrisp', 'Zestar' and 'Braeburn' had the most significant increases in their vitamin C levels at 166%, 143%, 139%, and 126%, respectively.

These results are similar to those of Davey and Keulemans, who found that certain early-harvested cultivars with poor storage characteristics lost up to 80% of their L-AA in storage, while other cultivars were able to maintain or increase L-AA levels in storage (Davey and Keulemans 2004). The ability of an apple to maintain its ascorbate levels in storage may be a marker for storage quality and the suitability for long-term storage. An increase in L-AA during cold storage is likely due to acclimation of the fruit to low temperature and other stresses. Davey and Keulemans (2004) suggested that since this response was observed in only a few middle- to late-ripening varieties, that it may be related to the fruit climacteric. They hypothesized that developing fruits are capable of L-AA biosynthesis, and that slower ripening varieties could conceivably maintain L-AA biosynthetic capacity postharvest (Davey and Keulemans 2004). We observed this response not only in several mid- and late-ripening varieties, but also in the early ripening varieties ‘Zestar’ and ‘AutumnCrisp’. Therefore, further research is needed to develop an understanding of the mechanism by which fruits may increase their vitamin C levels during storage.

Cornell University's advanced selections maintained between 86% and 113% of their 2009 at harvest vitamin C levels in storage. Selections #1 ('Honeycrisp' x NY selection), #4 ('AutumnCrisp' x 'Fuji'), and #5 ('AutumnCrisp' x 'Braeburn') had similar levels at harvest and after storage. Selection #3 ('Braeburn' x 'AutumnCrisp') gained 13% more vitamin C, while selection #2 ('McIntosh' hybrid x 'Fuji') lost 14%.

The majority of these advanced selections maintained or increased their vitamin C content in storage, a beneficial trait that makes them superior parents in crosses.

Conclusion

Vitamin C is an important antioxidant and metabolite in both plants and humans. The vitamin C content of apple fruits is a marker of fruit quality and a highly marketable trait. Quantifying the vitamin C levels of specific advanced breeding selections allows for more directed breeding of the trait. Breeding for increased vitamin C in apple fruits is feasible given the level of diversity among cultivars, but selection of parents should take into account vitamin levels at harvest and after storage as well as across different years. Further work is needed to determine why some cultivars gain vitamin C in storage and why others lose this nutrient.

Cornell University's advanced breeding selections average higher levels of vitamin C than many popular commercial cultivars, indicating that these selections may be nutritionally superior to apples currently on the market. There appears to be significant potential to improve the Vitamin C status of apple fruits, given the range of variation between different cultivars. The offspring of any given breeding cross may have similar, greater, or lesser Vitamin C than either of its parents, so it is an important trait for breeders to assay, especially over periods of time in storage and across several harvest years. A cross between two parents that are both high in ascorbic acid would likely produce the highest ascorbate offspring, but given the variability in inheritance, any breeding program seeking to enhance this trait would need to evaluate offspring to determine their status. Note that the advanced selections numbers 1 through 5 were chosen by the breeding program at Cornell based on their superior fruit quality, but they also exhibited high levels of Vitamin C. In the future, progeny of these selections could also be analyzed for their fruit quality.

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CHAPTER 4

CONCLUSION

This research provides a quantitative baseline for the rates and extent of browning, polyphenoloxidase activity, phenolic content and vitamin C for twelve commercial apple cultivars and five of Cornell University's advanced breeding selections. These values can be used as a basis of comparison for newly developed selections in the future. Likewise, the method used to quantify browning in this study can be broadly applied to new or different cultivars to measure their rates of browning. The ability to quickly characterize rates of browning in a standardized way is a valuable new development in efforts to breed for low browning cultivars.

Given that polyphenoloxidase activity and phenolic content are only correlated to browning in specific cultivars, these parameters should not be assumed to be indices of the browning potential of new or untested cultivars. Knowing the total phenolic content of a fruit is still valuable given the additional benefits that phenolic compounds provide.

It is apparent that environmental conditions present in the orchard from one season to the next have some effects on a cultivar's potential extent of browning. Future studies might explore in more depth how parameters such as temperature, rainfall and sunlight might more specifically affect the rates of browning. Additionally, given that cold storage appears to cause a significant decrease in the overall extent of browning, further research could expand on these findings, particularly exploring if the use of the storage compound 1-MCP effects this decrease of browning in any way.

The vitamin C content of apple fruits is another marker of fruit quality. This study used methods developed by other researchers to measure the vitamin C content of popular New York varieties, along with several advanced breeding selections. When breeding for the enhancement of vitamin C, researchers should measure levels both at harvest and after storage and across different years to determine variation in this trait due to storage and environmental conditions. More work is needed to determine the biochemical reasons why some cultivars gain and some cultivars lose this vitamin during storage.

Cornell's advanced breeding selections compared favorably to commercial cultivars that were measured in both rates of browning and vitamin C status. Lowering the rates of browning and increasing the vitamin C content of apple fruits both enhance the quality and marketability of new cultivars. This research helped to develop and refine methods that can be broadly applied in a breeding program to simplify and standardize the measurements of these important traits.