STARCH METABOLISM IN APPLE FRUIT AND ITS RELATIONSHIP WITH MATURATION AND RIPENING

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Franziska Clara Doerflinger

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STARCH METABOLISM IN APPLE FRUIT AND ITS RELATIONSHIP WITH MATURATION AND RIPENING

Franziska Clara Doerflinger, Ph. D.

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Harvest timing of apples, an important factor determining fruit quality after storage, is often based on maturity assessments that include the starch pattern iodine (SPI) test. The SPI test provides a visual indicator of starch degradation in the equatorial region of the fruit. SPI and starch concentrations in apple cultivars, and the effects of factors such as aminoethoxyvinylglycine (AVG) and 1-methylcyclopropene (1-MCP), have been investigated. SPI values increased as starch concentrations declined in 'Gala, 'Honeycrisp', 'McIntosh', and 'Empire' apples during maturation. The two factors have a curvilinear relationship for all cultivars. Declines in percentage of amylose were found to be linear and cultivar dependent. Computer-based image analysis of SPI-based staining revealed a wide range of values, and a linear correlation was found between SPI value and percentage stained area. Starch concentrations in stem-end tissues were lower than in equatorial and calyx-end tissues of 'Empire' and 'Gala' apples. AVG and 1-MCP applied preharvest to inhibit ethylene production and perception, respectively, had cultivar as well as application timing-dependent effects on maturation. Effects of these treatments on starch degradation were limited in both 'McIntosh' and 'Empire' fruit. Weak correlations were found for 'Empire' apples between harvest indices and differences in absorbance (I_{AD}) readings, which reflect chlorophyll concentrations in the skin. However, preharvest treatment of fruit with AVG and 1-MCP altered the

relationships between I_{AD} and other harvest indices, especially the internal ethylene concentration (IEC). 'Empire' apples are susceptible to firm flesh browning when stored at temperatures close to 0 °C, or after treatment with postharvest 1-MCP. This storage disorder causes major losses for the apple industry every year and no storage regime has been found so far to alleviate the problem. A full economic analysis indicated that there is an economic tradeoff between harvest date, occurrence of flesh browning, and likely net profits. Overall maturity assessment at harvest could be used as an indicator for storage disorders and/or storage length if factors such as differences in fruit maturation between cultivars and within the fruit are better understood.

BIOGRAPHICAL SKETCH

Franziska C. Doerflinger graduated from University of Applied Science Wiesbaden with a Bachelor of Science in 2009 and with a Masters in Science in 2010. During her Master's program she visited Lincoln University in New Zealand for course work. The Master thesis by Alan Lakso (Cornell University) and Peter Braun (University Geisenheim, Germany) was conducted at the NY State Agricultural Experiment Station, Cornell University, in Geneva New York. In January 2011 Franziska started a Ph.D. program at Cornell University with the thesis topic "Starch metabolism in apple fruit and its relationship to maturity and ripening" working with Chris Watkins and Lailiang Cheng (Department of Horticulture), and Bradley Rickard (Charles H. Dyson School of Applied Economics and Management). During her time at Cornell University Franziska enjoyed being in the Graduate and Professional Student Assembly (GPSA) Advocacy committee for 3 years, and chairing the committee for one year. Furthermore activities in the department such as SoHo and committees were an important part of a well-rounded Graduate School experience at Cornell.

I would like to dedicate this dissertation to my family for all their support, especially my husband Vinay Pagay, who has helped me through this time and supported me in every possible way.

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LIST OF ABBREVIATIONS

1-MCP	1-methylcyclopropene
3PGA (G3P)	3-phosphoglyceraldehyde or glyceraldehyde 3-phosphate
A	absorption coefficient
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
ADP	adenosine diphosphate
ADPG	ADP-glucose
ADPGPPase	ADP-glucose pyrophosphorylase
AGP	ADP-glucose phosphorylase
AIV	soluble acidic invertase
AM	amylose
AMY(3)	α-amylase
A/N-INV	alkaline/neutral invertase
ANOVA	analysis of variance
AP	amylopectin
ATP	adenosine triphosphate
AVG	aminoethoxyvinylglycine
Avg.	average
BAM	β-amylase
C	calyx or control (Chapter 3), cubic fit (Chapter 4), and Commercial (Chapter 6)

CA	controlled atmosphere
chl a	chlorophyll a
CO_2	carbon dioxide
CWINV	cell-wall invertase
d	day
DA	Delta Absorbance
DAH	days after harvest
DPE(1)	disproportionating enzyme
dwt	dry weight
E	equatorial
EC	enzyme commission numbers
EST	expressed sequence tag
F6P	fructose-6-phosphate
FBP	fructose-1,6-bisphosphate
FRK	fructokinase
ExFy	U.S. Extra Fancy
G1P	glucose-1-phosphate
G6P	glucose-6-phophate
GBSSI, II	granule-bound starch synthase I, II
GWD	glucan water dikinase
ha	hectare
Fy	U.S. Fancy
HPLC	high-performance liquid chromatography

HXK	hexokinase
I_2	iodine
I_{AD}	difference in absorbance (index of absorbance difference)
IC	ionic chromatography
IEC	internal ethylene concentration
ISA1, 2, 3	isoamylase 1, 2, 3
Kl	potassium iodine
kPa	kilopascal
L	linear fit
Log_{10}	logarithm base 10
MdACO1	1-amino-cycolopropane-carboxylase oxidase
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
$NADP^+$SDH	$NADP^+$-sorbitol dehydrogenase
ns	non-significant
O_2	oxygen
P	propylene; fraction of AM (within calculation of percentage amylose)
P	*P*-value, significance probability
PGI	phosphoglucose isomerase
PGM	phosphoglucomutase
PGR	plant growth regulator
PHS	glucan phosphorylase
PUL	pullulanase

PWD	phosphoglucan water dikinase
Q	quadratic fit
S	stem
S6P	sucrose-6-phosphate
S6Pase	sorbitol-6-phosphate phosphatase
S6PDH	sorbitol-6-phosphatedehydrogenase
SAM	S-adenosylmethionine
SBEI, IIa, IIb	starch branching enzymes I, IIa, IIb
SDH	NAD-dependent sorbitol dehydrogenase
SI	starch index
SOX	sorbitol oxidase
Sor-6P	sorbitol-6-phosphate
Sor-PP	sorbitol-6-phosphate phosphatase
SPI	starch pattern index
SPP	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
SUS	sucrose synthase
SS	starch synthase
SSI, II, III, IV	starch synthase I, II, III, IV
SSC	soluble solids concentration
TA	titratable acidity
TP	triose phosphate
R	ratio of absorbance

r	radius or Pearson correlation coefficient
UDP	uridine diphosphate
UDPG	UDP-glucose
UGP	UDP-glucose pyrophosphorylase
w	week
wt	weight

CHAPTER 1

INTRODUCTION

1.1 General introduction

Apples are an important part of the human diet in the United States and are the second most consumed fruit per capita after bananas (USDA, 2012). According to the U.S. Apple Association (2014), there are over 100 commercially-grown cultivars in the U.S., and the 15 most popular cultivars account for almost 90% of the total tonnage. At least 10,000 different apple cultivars have been developed over time, but due to practices such as seed propagation, many of these cultivars have been lost (Collett, 2011).

Apple fruit [*Malus sylvestris* (L.) Mill var. *domestica* Borkh.], a member of the family *Rosaceae,* subfamily of Pomoideae, and it is a temperate species with fleshy fruit. The fleshy fruit of apples, classified as pome, developed from an epigynous floral structure (Grierson, 2002; Masters, 1871). The cortical (hypanthial) tissue is derived from the floral tube (hypanthium portion fused to the gynoecium) (Pratt, 1988; Rudell et al., 2000) (Fig. 1.1). By definition fruit is the end product of the mature ovary (Grierson, 2002); the flesh of most fruit functions as a seed vessel (Thoreau, 1863), and in some cases protects the seeds (Janzen, 1977). Therefore, an important part of fruit development are the seeds, and fruit maturation is a major step between seed development and ripening, the coordination of these events being very important to guarantee seed dispersal (Janssen et al., 2008).

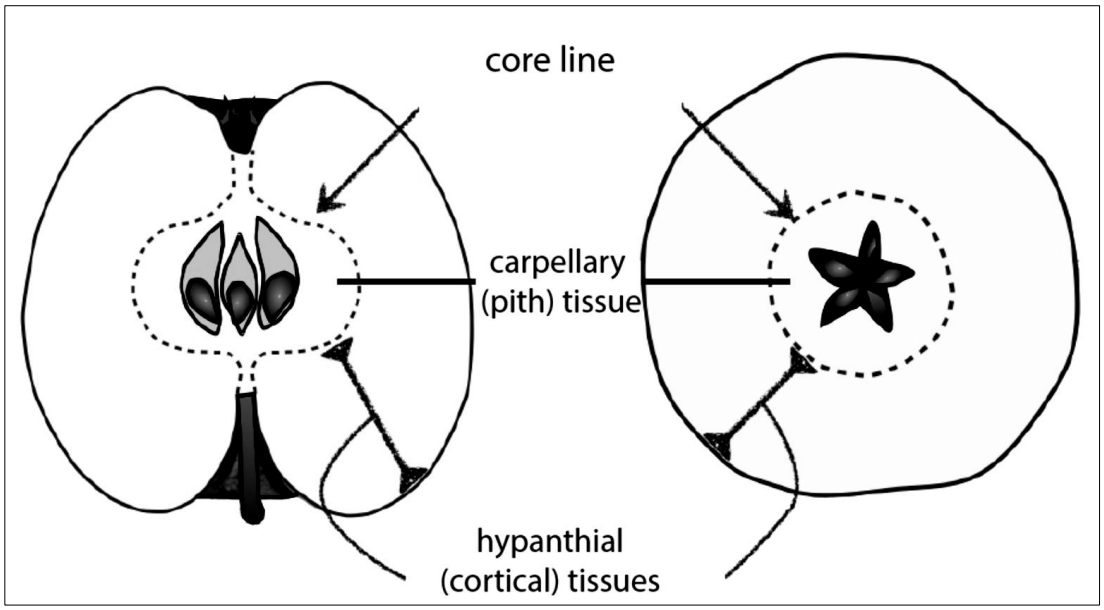

Fig. 1.1. Longitudinal and transverse sections of an apple fruit showing the skin, hypanthial (cortical) tissues, the core line, and the carpellary (pith) tissue (modified from Rudell et al., 2000)

For climacteric fruit, the physiological difference between maturation and ripening is not always clear; changes are ascribed to both events, e.g. ethylene production, the respiratory climacteric, change of background or ground color from green to yellow, loss of acidity, conversion of starch to sugar, formation of cuticular waxes and synthesis of aromatic compounds (Watkins, 2003). Fruit development can be divided into four phases: 1) ovary development in the flower following anthesis; 2) a period of rapid cell division; 3) growth through cell enlargement, a phase in which most fruit reach their final shape and size and food reserves are accumulated; and, 4) the fruit is mature and ripening can occur (Srivastava, 2002).

Strictly speaking, maturation refers to competency to ripen as an immature fruit cannot ripen. Fruit ripening is a highly coordinated, irreversible process which leads to soft edible fruit (Prasanna et al., 2007). Maturation can, therefore, be defined as the

period of growth and seed development, whereas ripening is a process that happens after seed maturation. However, in a commercial sense, maturation refers to the physiological stage at which the ripened fruit will have quality characteristics acceptable to the consumer (Bouzayen et al., 2010). Therefore, only at the point of seed maturation will the apple fruit ripen. At the onset of ripening, a climacteric rise occurs and other processes such as cell wall degradation, color change, and a change in carbohydrates occur (Srivastava, 2002), all of which contribute to typical ripe fruit characteristics (aroma, flavor and textural).

Apple fruit can be harvested mature from the pre-climacteric stage, i.e. before the respiratory climacteric and autocatalytic ethylene production occurs, to the post-climacteric stage. The window of optimum fruit harvest is a function of the target market as well as the storage period. Fruit destined for long-term storage in air or controlled atmosphere (CA) have to be harvested at a less mature state than fruit marketed soon after harvest. Different methods have been developed to aid harvest decisions and assess fruit maturity. A commonly used tool to evaluate fruit maturity is the starch pattern index (SPI), and many different charts for cultivars and growing regions are available (Blanpied and Silsby, 1992; Chu, 1988; Hanrahan, 2012; Reid et al., 1982; Smith et al., 1979; Travers et al., 2002) (see section 1.5). Another modern tool used to evaluate fruit maturity is the so-called Difference in Absorbance (DA) meter (Sinteleia, Bologna, Italy) which measures the difference in absorbance at 670 and 720 nm, calculating the I_{AD} (Costamagna et al., 2013). The I_{AD} is calculated as $I_{AD}=A_{670} - A_{720}$ where A_{670} and A_{720} are the A values measured at 670 and 720 nm, respectively (Bertone et al., 2012; Zanella et al., 2013; Ziosi et al., 2008). From the interaction (I)

spectra of the two wavelengths, fruit absorbance (A) is calculated using the Lambert's Beer law ($A=\log_{10} I^{-1}$ (Bertone, et al., 2012; Zanella, et al., 2013; Ziosi, et al., 2008).

Fruit maturity at harvest and therefore harvest date are the most important factors influencing fruit quality after storage and at the market place. Consumer willingness to pay and purchase fruit again is dependent on many factors. Disappointment in a product through blemish, off flavor or internal disorders can cause consumers to not purchase a product again for a long time. For apples, consumers find fruit firmness (or crunchiness) one of the most important quality factors (Rickard et al., 2013; Yue and Tong, 2011).

1.2 Carbohydrate transport

During development, fruit are mostly non-autotrophic and need to import most of their carbon from adjacent leaves (Blanke and Lenz, 1989). Partitioning of carbohydrates into source and sink tissue might be regulated through transcriptional regulation of starch in the tissues (Janssen et al., 2008).

In apple trees, as in most fruit trees within the *Rosaceae* family, the majority of the transported carbohydrates in the phloem are in form of the sugar alcohol sorbitol (Bieleski, 1969, 1977; Bieleski and Redgwell, 1985; Teo et al., 2006). Sorbitol is the 'masked' form of photoassimilates. Masking of transport carbohydrates is necessary to make the sugar less active and less susceptible for enzymatic breakdown or transformation (Büttner and Sauer, 2000). The number of carbon atoms in sorbitol per molecule is six, half the amount of sucrose, and storage ability of sorbitol might consequently be less than that of sucrose (Kanayama et al., 2008). However, the biosynthesis of sorbitol requires only two enzymes unlike sucrose, which requires four

(Kanayama et al., 2008). Sorbitol accounts for approximately 80% of the soluble carbohydrates in apple leaves, spurs, and pedicels, but in fruit, sorbitol accounts for less than 8% of soluble carbohydrates throughout the season (Cheng et al., 2005; Yamaki and Moriguchi, 1989). The sorbitol in the fruitlet is, therefore, not stored as such but converted into other sugars and starch (Yamaki and Moriguchi, 1989).

Sorbitol is synthesized in the leaves via reduction of glucose-6-phosphate (G6P) to sorbitol-6-phosphate (S6P) by sorbitol-6-phosphate dehydrogenase (S6PDH, EC 1.1.1.200) (Bieleski and Redgwell, 1985; Cheng et al., 2005) (Fig. 1.2), modeled in Shangguan et al. (2014) (Fig. 1.I. – supplementary material, in the end of Chapter 1). In the fruit sorbitol is converted into fructose by sorbitol dehydrogenase (SDH, EC 1.1.1.14) (Zhang et al., 2010), and sucrose is metabolized by invertase (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13) (Nguyen-Quoc and Foyer, 2001). The highest expression of SDH in apples is found about 2 to 3 weeks after bloom, a period when carbon starvation is especially critical (Teo et al., 2006). If fruit are deprived of carbon at this stage, growth slows, and the consequence of not reaching a minimum growth rate is fruit abscission (Bepete and Lakso, 1998; Dash et al., 2013; Grappadelli et al., 1994). Fruit size and quality (primarily color) are both economically important for growers since fruit are sorted based on these two parameters and marketed at prices according to fruit size (USDA, 2002). After sorbitol is translocated from the leaves into the fruit, carbon from sorbitol can be used to assimilate organic acids, lipids or oligosaccharides to provide an energy source for respiratory processes within the fruit (Blanke and Lenz, 1989) (Fig. 1.2). Part of the influx of sugars is also stored as starch, especially during maturation.

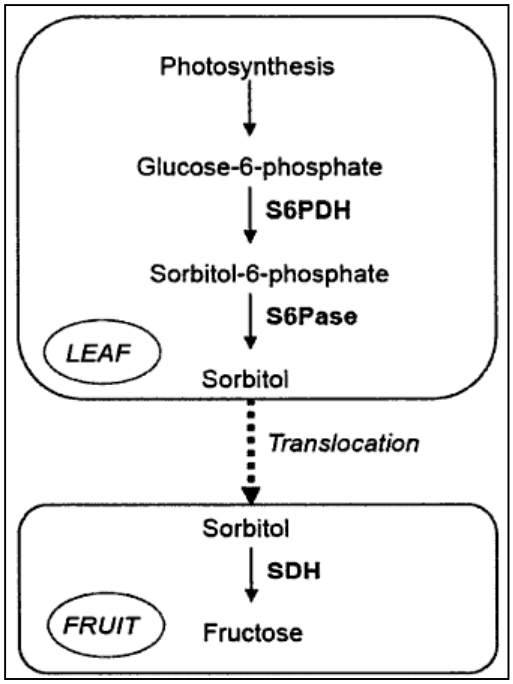

Fig. 1.2. Proposed sorbitol metabolic pathway in *Rosaceae* fruit trees. Sorbitol-6-phosphate dehydrogenase (EC 1.1.1.140) - S6PDH; sorbitol-6-phosphate phosphatase (EC 3.1.3.50) - S6Pase; NAD-dependent sorbitol dehydrogenase (EC 1.1.1.200) - SDH (Kanayama et al., 2008).

1.3 Starch synthesis

Sorbitol is converted to starch as soon as it reaches the developing fruit. Starch being the storage form of sugar in plant cells and can be found in all organs of most higher plants including pollen, leaves, stems, woody tissue, roots, tubers, bulbs, rhizomes, fruit, flowers, as well as in the pericarp, cotyledon, embryo and endosperm of seeds (Pérez and Bertoft, 2010; Shannon et al., 2009). Starch is very unreactive and not osmotically active (Kötting et al., 2010; Preiss, 2009), and therefore the optimal energy storage form for carbohydrates in developing fruits. Starch is made up of the glucose polymers amylose (AM) and amylopectin (AP) (Smith, 2001, 2007; Wang et al., 1998) (Fig. 1.3).

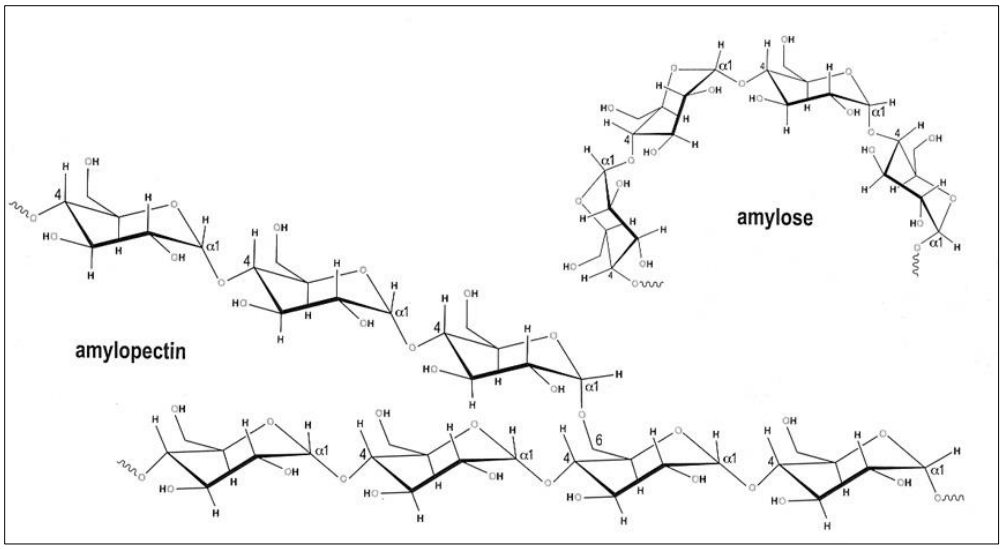

Fig. 1.3. Molecular structure of the starch molecules amylopectin (AP) and amylose (AM) (Rebel, 2014).

Starch is relatively stable in storage organs. Starch accumulation in storage organs is achieved through the crystalline packing of glucosyl units within the starch granule and the exclusion of water (Jane, 2009; Keeling and Myers, 2010; Pérez et al., 2009). The crystallinity of a starch granule varies between 15 to 45%, at a density of approximately 1.5 g crystalline cm^3 (Pérez and Bertoft, 2010). Starch granules in apple fruit are spherical with a smooth surface, and are sometimes cracked (Kovács and Eads, 1999; Ohmiya and Kakiuchi, 1990). The appearance of granules is similar in all parts of the apple, but differ in size in the different tissues of the fruit (Ohmiya and Kakiuchi, 1990). Starch is stored in all tissues of the fruit, but not at same rate or amount (Pérez et al., 2009; Smith, 2001). Size of the starch granules differ between inner flesh, mid-flesh and outer flesh, with the largest granules occuring in the outer regions (Kovács and Eads, 1999; Ohmiya and Kakiuchi, 1990).

Starch synthesis processes as well as hydrolysis are highly regulated through the activity of specific enzymes. Starch biosynthesis and metabolism catalyzing enzymes exist often in different isoforms; differences are either protein translated from different mRNAs, produced from different genes, or by differential splicing of mRNA from the same DNA (Beck and Ziegler, 1989). Multiple forms are mostly understood as derived from different posttranslational modifications (Beck and Ziegler, 1989). Understanding the orchestra of biosynthesis and degradation, and the inter linkage of enzymatic reaction will give clarification of how and when starch is produced, accumulated, and hydrolyzed. Gathering knowledge of partitioning of enzymes between the cytosol, amyloplast and starch granules can provide insight into the mechanisms of starch synthetic and degradative reactions in the amyloplast (Stensballe et al., 2008).

Starch synthesis involves several enzymes, including ADP-glucose pyrophos-phorylase (AGPase - EC 2.7.7.27), which catalyses the first committed step towards starch synthesis (Blennow et al., 2002; Zeeman et al., 2010; Zeeman et al., 2007) (Fig. 1.4) - G1P + ATP → ADPglucose + PP_i. AGPase is encoded by the *glgC* gene (Ball and Morell, 2003). Starch synthesis further includes chain elongation through transfer of glucosyl unit from ADP-glucose onto the growing chain and creating a new α-1,4-glucosidic bond through starch synthases (SS – EC 2.4.1.21) (Blennow et al., 2002; Santelia and Zeeman, 2011; Zeeman et al., 2010). Further involved in starch synthesis is chain transfer, which is catalyzed by the starch branching enzyme (SBE – EC 2.4.1.18) introducing α-1,6-linkage via a glucanotransferase reaction, through cutting of existing α-1,4-glucan chains and transferring chains of six or more glucose units to the C6 position of another glucan chain (Zeeman et al., 2010), and hydrolytic activities from

enzymes such as starch-debranching enzyme to facilitate formation of crystalline structure within the granule (Blennow et al., 2002; Preiss, 1982; Santelia and Zeeman, 2011; Smith, 2001). SS has five isoforms (Zeeman et al., 2007), granule-bound SS (GBS - EC 2.4.1.242) mediates amylose synthesis within the granule and is purely granule bound (Ball and Deschamps, 2009), and the other four are SSI to SSIV based on their different amino acid sequences (Zeeman et al., 2007). Each SS family elongates preferably a specific amylopectin chain length (Zeeman et al., 2007).

The major limiting factor for starch biosynthesis is the pyrophosphorylase reaction leading to ADP-glucose synthesis (Zeeman et al., 2010; Zeeman et al., 2007). In chloroplasts, this reaction is mainly controlled by the levels of 3-phosphoglyceric acid (3PGA) and inorganic phosphate (P_i) (Avigad and Dey, 1997). Since in photosynthetically-active cells, starch is stored in chloroplasts. Apple fruit do not have chloroplasts in the majority of the tissue, and therefore store starch in special plastids called amyloplasts. In amyloplasts, the ratio of 3GPA/P_i translocators is of less importance than in chloroplasts (Beck and Ziegler, 1989).

Storage organs and fruits are mostly supplied with sucrose (Avigad and Dey, 1997) or other transport forms of carbohydrate such as sorbitol. The transport sugar sucrose is converted to hexoses and hexose phosphates in the fruit by invertase, sucrose synthase, and hexokinase (Avigad and Dey, 1997). In apple, fruit sorbitol is converted into fructose by sorbitol SDH (Teo et al., 2006; Zhang et al., 2010). The metabolites glucose-6-phosphate (G6P) and to a lesser extent glucose-1-phosphaste (G1P) are transported into the amyloplast through 'modified' 3PGA/P_i translocators (Avigad and Dey, 1997). In the amyloplast, they are immediately used to synthesize ADP-glucose

and starch, bypassing gluconenogenesis, which is required when starting with triose phosphate (Avigad and Dey, 1997).

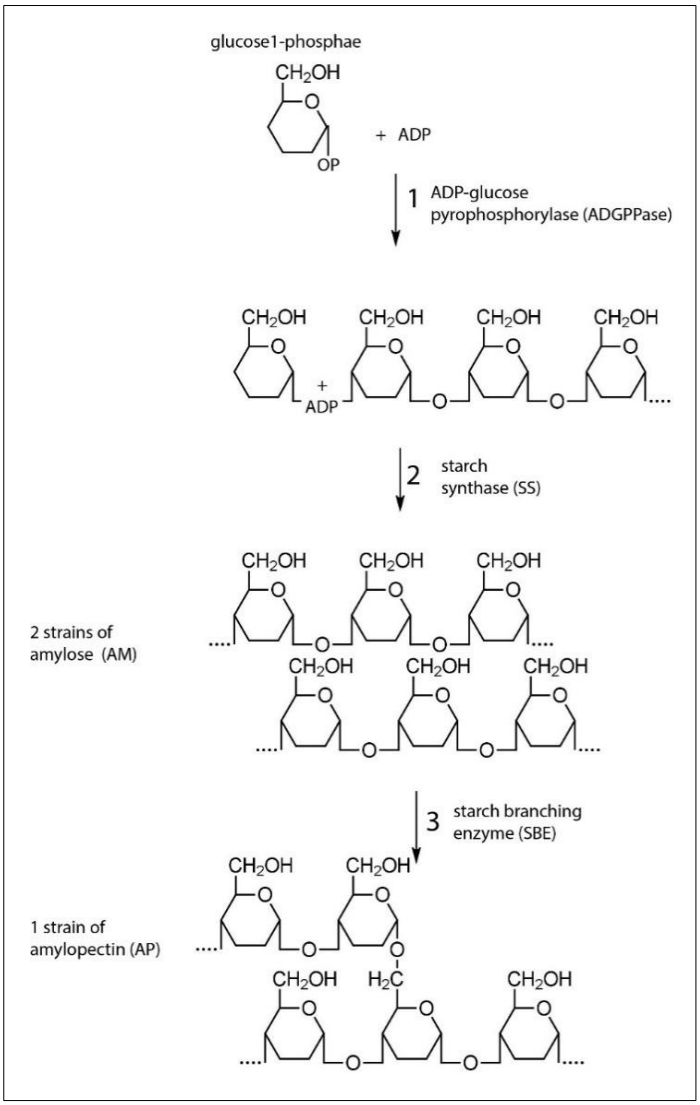

Fig. 1.4. Steps of starch biosynthesis. ADP-glucose pyrophosphorylase (ADPGPPase) catalyzes the formation of ADPglucose and inorganic pyrophosphate from glucose-I-phosphate and ATP (1). Starch synthases (SS) add glucose units from ADPglucose to the non-reducing end of a growing α(l-4)-linked glucan chain by an α(l-4) linkage and release ADP (2). Starch-branching enzymes (SBE) cut an α(l-4)-linked glucan chain and form an α(1-6) linkage between the reducing end of the cut chain and the C6 of another glucose residue in an α(l-4)-linked chain, thus creating a branch (3); modified from Martin and Smith (1995).

Much of the research concerning starch has been done on starch storage organs (Smith, 2001), such as potato (Blennow and Engelsen, 2010; Fernie et al., 2002; Geigenberger, 2003; Tauberger et al., 2000), grain, mostly wheat, barley (Beck and Ziegler, 1989; Bowsher et al., 2007; Kurma and Singh, 1983), and rice (Asatsuma et al., 2005), although transient starch in chloroplasts has been a major area of investigation (Caspar et al., 1985; Chen and Cheng, 2004; Niittylä et al., 2004; Santelia and Zeeman, 2011; Zeeman et al., 2007).

In fruit such as apple that synthesizes sorbitol in the leaves, the initial step might be slightly different and some enzyme reactions altered, but a general similarity between fruit starch accumulation and accumulation of starch in storage organs can be assumed. The enzymatic composition of apple fruit and leaves accounting for soluble sugar metabolism was found to be very different in the leaves than in the fruit (Yamaki and Ishikawa, 1986). But newer research has shown that starch synthesis and degradation in transitory systems such as leaves has close biological similarity to storage systems such as tubers and seed endosperm with similar enzymatic functions (Santelia and Zeeman, 2011).

Starch is generally stored in most plant organs in the form of granules, and the formation of such starch granules only happens in the presence of amylopectin (AP) (Pérez et al., 2009). The morphology of a granule is not influenced by the amount of AM, and is similar over a wide range of AM concentrations (Keeling and Myers, 2010). The formation of starch granules, including in grains and potato, is poorly understood (Zeeman et al., 2010).

In apple fruit, the highest concentration of starch granules is found in the cell layers directly underneath the skin (Brookfield et al., 1997; Ohmiya and Kakiuchi, 1990). Smaller granules have a more regular surface compared to larger granules (Kovács and Eads, 1999). Therefore, granules in the outer flesh of the fruit should have a more irregular surface compared to granules closer to the core. Starch granules have a semi-crystalline structure (Blennow and Engelsen, 2010). This semi-crystalline structure serves the purpose of being stable for storage and has the ability to be made available within the cell upon demand for soluble carbohydrates (Blennow and Engelsen, 2010). The structure of the granule is unique and might play an important role *in vivo* (Pérez et al., 2009). The hydrolysis of starch could be highly influenced by the unique patterns of the grains and the way starch is structured (Pérez et al., 2009). The differences in starch structure can have a big influence on the degradation and could possibly be a method to control the production of sugar during fruit ripening (Luengwilai et al., 2010). In apple fruit, it is known that during hydrolysis AM decreases faster within total starch and also faster than AP (Carter and Neubert, 1954; Fan et al., 1995; Potter et al., 1949; Stevenson et al., 2006). The amount of AM deposited within the starch granule could therefore influence the speed of starch degradation within the fruit. This degradation rate, in turn, could change the reading patterns of starch pattern iodine (SPI) test used for assessment of apple maturity.

1.4 Starch degradation

Detached apple fruit no longer have an influx of carbohydrate through the phloem and need to provide their energy needs from stored carbohydrates in the form of starch,

acids, or sugars. Also during fruit development on the tree, fruit start to degrade starch as they reach maturity and start to ripen. Apple fruit typically reach maturity and initiate ripening approximately 150 days after full bloom, the specific timing influenced by cultivar, the period being marked by starch degradation, and fruit sweetening (Brookfield et al., 1997). Some of the energetic balance during ripening is derived from malic acid utilization causing decreasing acidity of apples over time; malate is used as a substrate in gluconeogenesis and the tricarboxylic acid (TCA) cycle (Bai et al., 2012; Berüter, 2004). Soluble sugars as well as starch are used for respiration, as well as for fruit aroma (Dixon and Hewett, 2000).

Starch can be hydrolyzed and the stored sugars become available to provide needed energy. Starch is degraded either by phosphorolytic or hydrolytic cleavage reactions (Asatsuma et al., 2005) (Figs. 1.5 and 1.6). Phosphorolytic cleavage is done by starch phosphorylase (glucosidase) and hydrolytic cleavage is done by amylases (Asatsuma et al., 2005). During starch degradation the starch granule undergo erosion when degraded, the chief agents being amylases (α (AMY – EC 3.2.1.1) and β (BAM – EC 3.2.1.2)). The core of the grain is less-resistant to the amylases than their surface, consequently, α-amylases have to 'eat' their way into the starch grain (Ernst et al., 1999). β-amylase attacks the granule directly and releases dextrins, which are then hydrolyzed into smaller components (Gawęda and Ben, 2010). Starch granules, therefore, seem to be hydrolyzed from the inside out since the surface of the granule stays intact as starch degradation proceeds (Ohmiya and Kakiuchi, 1990).

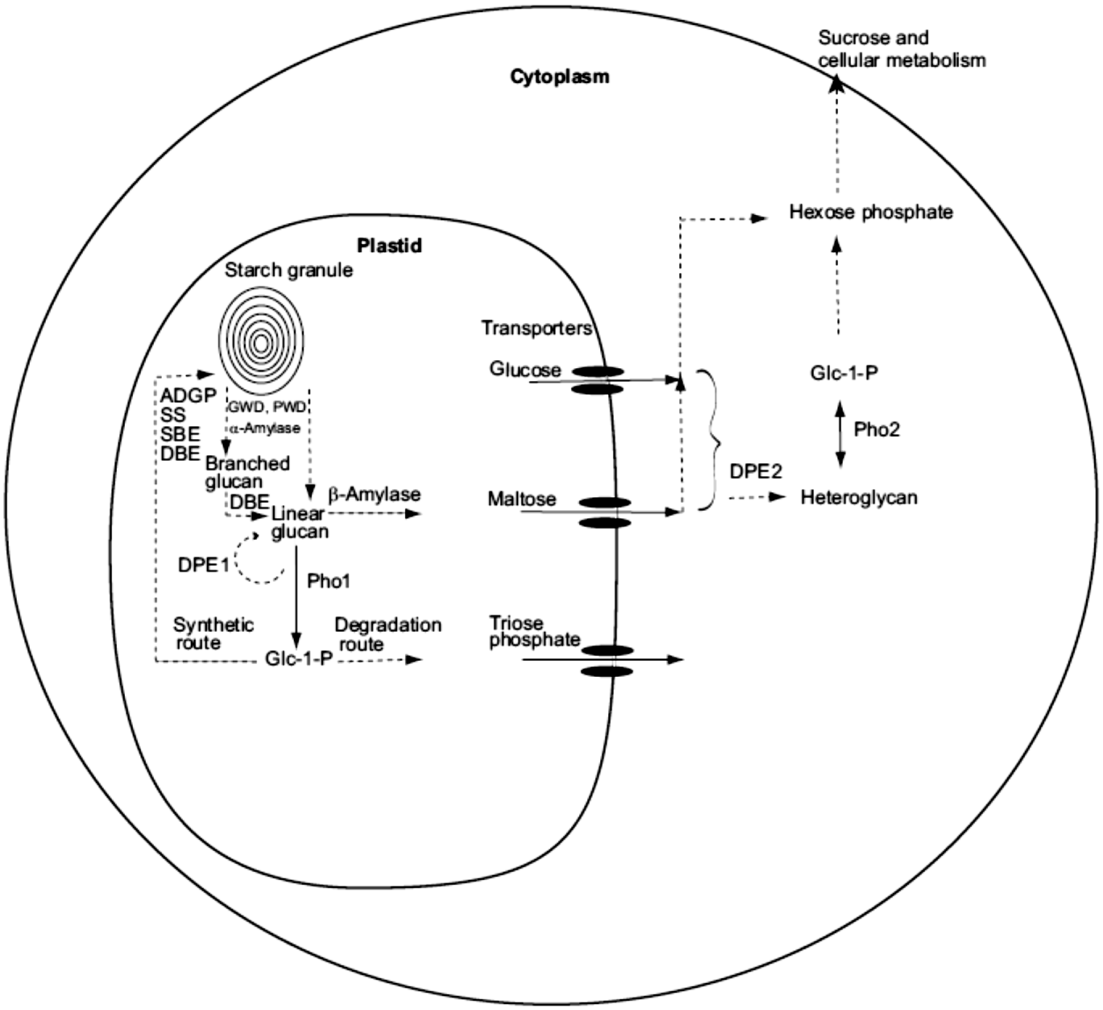

Fig. 1.5. A diagram illustrating the putative roles of plant phosphorylases in starch metabolism (storage organ starch). The dashed lines indicate that there may be intermediate steps in the pathways. Abbreviations: ADGP, ADP-glucose pyrophosphorylase; SS, starch synthases; SBE, starch branching enzymes; DBE, starch debranching enzymes, isoamylase, and limit-dextrinase; DPE1, disproportionating enzymes (Rathore et al., 2009). For apple fruit the export of maltose is not expected.

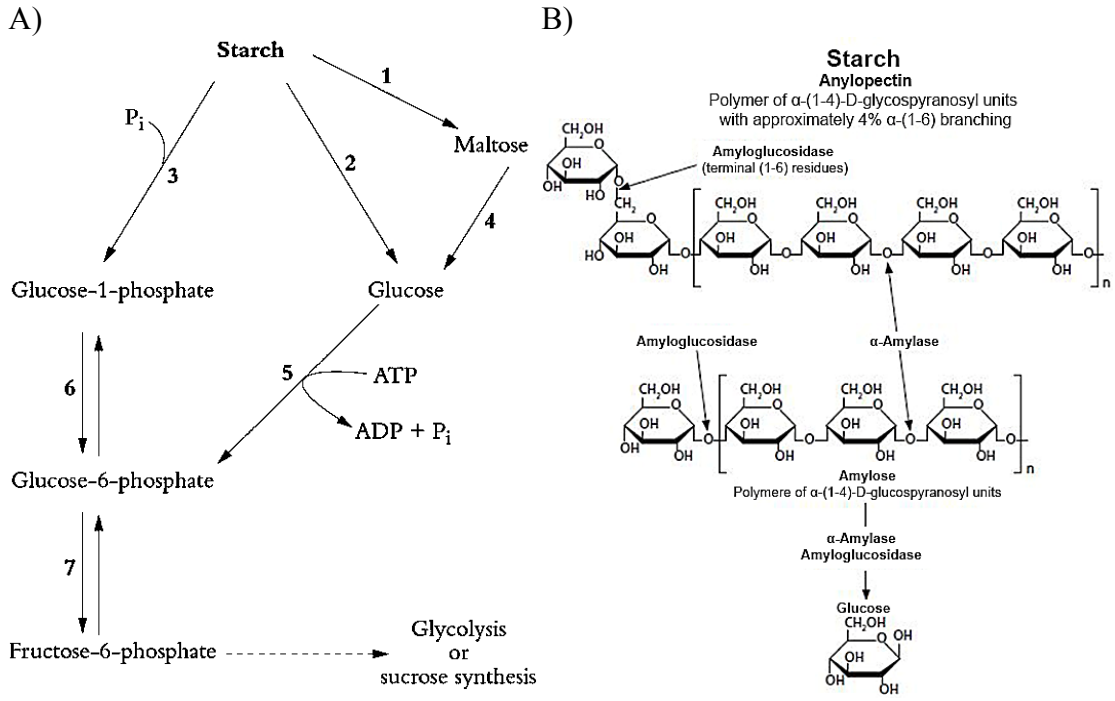

Fig. 1.6. A) Pathways of starch metabolism. Numbers refer to the following enzymes: 1, ß-amylase; 2, α-amylase; 3, starch phosphorylase; 4, glucosidase; 5, hexose kinase; 6, phosphoglucomutase; 7, glucose 6-phosphate isomerase. (Original drawing courtesy David Day) (Atwell et al., 1999). B) Enzymatic starch degradation by amyloclucosidase and α-amylase schema (Sigma-Aldrich, 2014).

Starch granule digestion in tomato fruit was modeled using four different but not mutually exclusive techniques with data taken from other publications (Luengwilai and Beckles, 2009). Digestion was proposed to happen by: A) "centrifugal digestion" breakdown of starch from the center of the granule; B) "centripetal digestion" suggests an attack of the granule from the surface; C) a centripetal digestion and granule aggregation is assumed; and the last model, D) is based on continued synthesis of starch during ripening with a combination of centrifugal digestion (Luengwilai and Beckles, 2009). Ohmiya and Kakiuchi (1990) found apple fruit starch grains to be digested from the inside out leading to a grain size unspecific reaction.

In apple fruit, the concentration of granules is initially highest in the outer cortex and lowest in the core (Brookfield et al., 1997; Ohmiya and Kakiuchi, 1990). Whether starch degradation happens differentially in different parts of the fruit is unclear. Some research suggests that degradation starts simultaneously but happens slower in the core than in the other tissue zones (Peirs et al., 2002). Others found that starch degradation occurred simultaneously in all tissues, but decreased fastest in the core and slower towards the outside of the fruit (Brookfield et al., 1997).

1.5 The starch pattern index (SPI)

For testing maturity of apple fruits, the SPI test is one the most widely-used in the industry and in research alike. Although the test was generally rejected as being variable in early studies (Haller and Smith, 1950; Tiller, 1934), the indexing of the patterns based on visual assessment of starch staining has gained wide acceptance. The SPI visualizes starch presence and progress of degradation, but does not measure the starch concentration in the fruit (Cho and Gil, 2004; Peirs et al., 2002; Travers et al., 2002).

For this test, the apple fruit is cut horizontally at the equatorial region and I_2-Kl solution is applied (Blanpied and Silsby, 1992). Starch, more precisely amylose, will form a polyiodine complex with I_2-Kl that is black-blue (Fan et al., 1995). The amylopectin-polyiodine complex produces a light purple-red pigment rather than the typical black-blue of the amylose-polyiodine stains (Cho and Gil, 2004). This test is inexpensive and quick, making it suitable for field use, but it only provides an indication of starch distribution but does not measure the starch content (Cho and Gil, 2004; Peirs et al., 2003; Peirs et al., 2002; Travers et al., 2002). It also does not allow for incomplete

accumulation of starch during fruit development (Watkins, 2003). Other disadvantages of the test include its subjective nature, and the wide array of charts and grading systems. Starch also changes in composition during development, and the amount of AM in total starch declines (Carter and Neubert, 1954; Fan et al., 1995; Potter et al., 1949), therefore the staining pattern changes and becomes less intense as starch hydrolysis progresses. AM percentage in apple starch is higher compared to other storage starches (Smith, 2001; Smith et al., 1995).

The SPI approach to measure starch content is also made more complicated since there are many different charts, which have different grading systems, and different techniques to estimate coloration, leading to different and inconsistent results. In Europe, the commonly-used generic scale for starch iodine staining is from 1 to 10 where 1 is all stained – no starch degradation therefore 100% stained area – and 10 no stains, hence fully degraded (Peirs et al., 2002). The Cornell chart is generic across all cultivars (Blanpied and Silsby, 1992) (Fig. 1.7). The optimum harvest window is cultivar-dependent. Based on this chart, recommendations for long-term storage made in 1992 were for air storage at 0 °C until January – approximately 3 to 4 months after harvest, depending on harvest date.

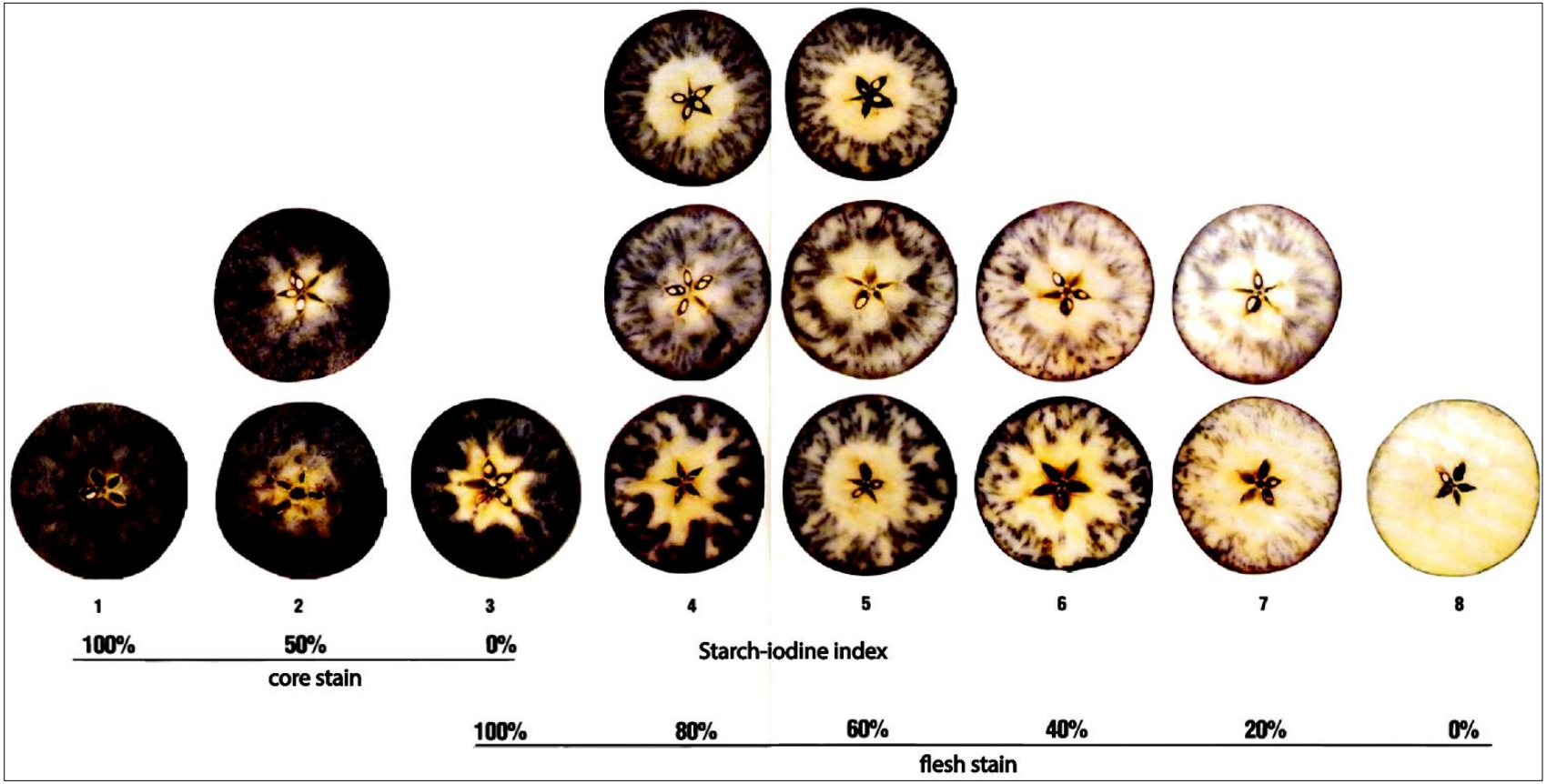

Fig. 1.7. Starch pattern index (SPI) Cornell generic chart (Blanpied and Silsby, 1992).

Other charts include one that was developed for 'Granny Smith' grown in New Zealand to optimize estimation of harvest dates (Reid et al., 1982), the optimum for air storage of fruit for export being staining pattern 3. SPIs also have been developed for the cultivars 'Northern Spy' and 'Delicious' (Smith et al., 1979). This chart suggests the best harvest window to store apples for "normal" commercial intervals in cold storage is 0 °C followed by an approximately one week shelf period. Travers et al. (2002) present a chart developed at the Centre Technique Interprofessionnel des Fruits et Lėgumes (CTIFL) for European cultivars; this chart has the classical 10-stage scale, and a curvilinear relationship between starch staining (darkened area) and SPI was found for the 10-point chart (Travers et al., 2002). The correlation between SPI and the percentage of iodine staining showed that in the beginning of starch degradation the overlap between what is scored 1 to 5 is greater than for the index numbers 6 to 10.

Another chart, with 10 levels, was created by Streif (1984). According to this chart, the values of best harvest for the cultivars 'Gala', 'Elstar', 'Jonagold', and 'Golden Delicious' are between steps 4 to 6, 2 to 3, 7 to 9, and 6 to 8, respectively (Neuwald et al., 2010). Priest and Lougheed (1981) developed a nine-point scale for 'McIntosh' and a separate nine-point scale for 'Red Delicious'. The best harvest according to the chart is at 4, 5, and 6 for long-term storage of 'McIntosh', and a staining index greater than 6 should be marketed without storage. 'Red Delicious' should be harvested at the stage of staining 2.5 to 3 (Priest and Lougheed, 1981). A chart for 'Honeycrisp' was developed recently by Hanrahan (2012).

Different cultivars have distinct patterns of starch hydrolysis as indicated by staining. In 'Golden Delicious', hydrolysis is initially indicated by lightening of the core region, but as starch degradation progresses, the pattern is more actiniform and the state of optimal starch degradation for harvest differs between the different cultivars (Streif, 1984). The difference in starch degradation progression is the reason why different charts were developed. Furthermore, each industry has their own standards and guidelines. For example, most fruit from New Zealand is destined for export and is, therefore, required to survive longer periods of shipping compared with fruit that travels locally. The importing country will also influence standards within an industry.

'Northern Spy', 'Delicious', and 'Empire' and late-ripening cultivars are harvested in late September and early October. 'McIntosh' is mostly harvested around early September and, therefore, relatively early in the season. 'Granny Smith' is a cultivar that needs an extended growing season, and, therefore, cannot be ripened easily in the cooler climate of northeastern US including NY State. Optimum harvest date recommendations made on the basis of SPIs seem to somewhat correlate to the amount of starch recommended.

All SPI charts are developed to estimate the harvest window and best quality fruit of apple cultivars. One of the issues with this approach is, that many of the suggested harvest maturities are not based on modern quality requirements and are often based on limited experimental results. For instance the recommendations in the Cornell chart are based on very minimal sensory evaluations after only three months in storage and are, therefore, not very valuable for long-term CA storage conditions, yet the harvest windows assessments are based on this very chart for air and CA storage alike.

1.6 Ethylene production, relation to ripening, and inhibition of ethylene

Ethylene (C_2H_4) is one of the simplest organic molecules having biological activity (Ecker, 1995; Theologis, 1992). It is a hormone, which as such is defined as a naturally produced substance and active in the organism elsewhere from where it was produced (Abeles, 1972). Plant hormones are active at very low concentrations and by strict definition do not have to be transported to a point of action (this definition is based on mammalian hormones) (Davies, 2010). Generally, hormones are effectors and are not metabolized or degraded during the course of the activation process, and degradation occurs as regulation and to prevent accumulation in the tissue (Abeles, 1972). Ethylene is a gaseous plant hormone (Klee and Clark, 2010), whose production is inhibited before climacteric by feedback inhibition during fruit maturation (system 1 – immature fruit) and autocatalytic production of ethylene after onset of climacteric during fruit ripening (system 2 – mature fruit) (Bouzayen et al., 2010; Klee and Clark, 2010). At the molecular level, the two systems differ by introduction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which only happens during system 2 activation (Klee and Clark, 2010). Ethylene is produced from methionine through Yang's cycle, which produces ACC from S-adenosylmethionine (SAM) through ACC-synthase (ACS – EC 4.4.1.14) (Pech et al., 2010). ACC is oxidized by ACC-oxidase (ACO – EC 1.14.17.4) and ethylene is produced (Pech et al., 2003; Pech et al., 2010) (Fig. 1.8).

In apple, ethylene is less vital for maturation (system 1) than for ripening (system 2). Ripening is accompanied by physiological changes due to the effects of ethylene. Ethylene production of apple fruit tissue has been found to be highest in the core area (Rudell et al., 2000) and, concentrations of the precursor of ethylene, ACC, were much

higher in core tissues, than the inner cortex and peduncular (shoulder) tissue, while calyx and outer cortex tissues were lowest (Mansour et al., 1986). Other differences between tissue zones are related to cell structure and tissue density (Bain and Robertson, 1951; James and Jobling, 2009; Leshem et al., 1984) and are unrelated to ethylene.

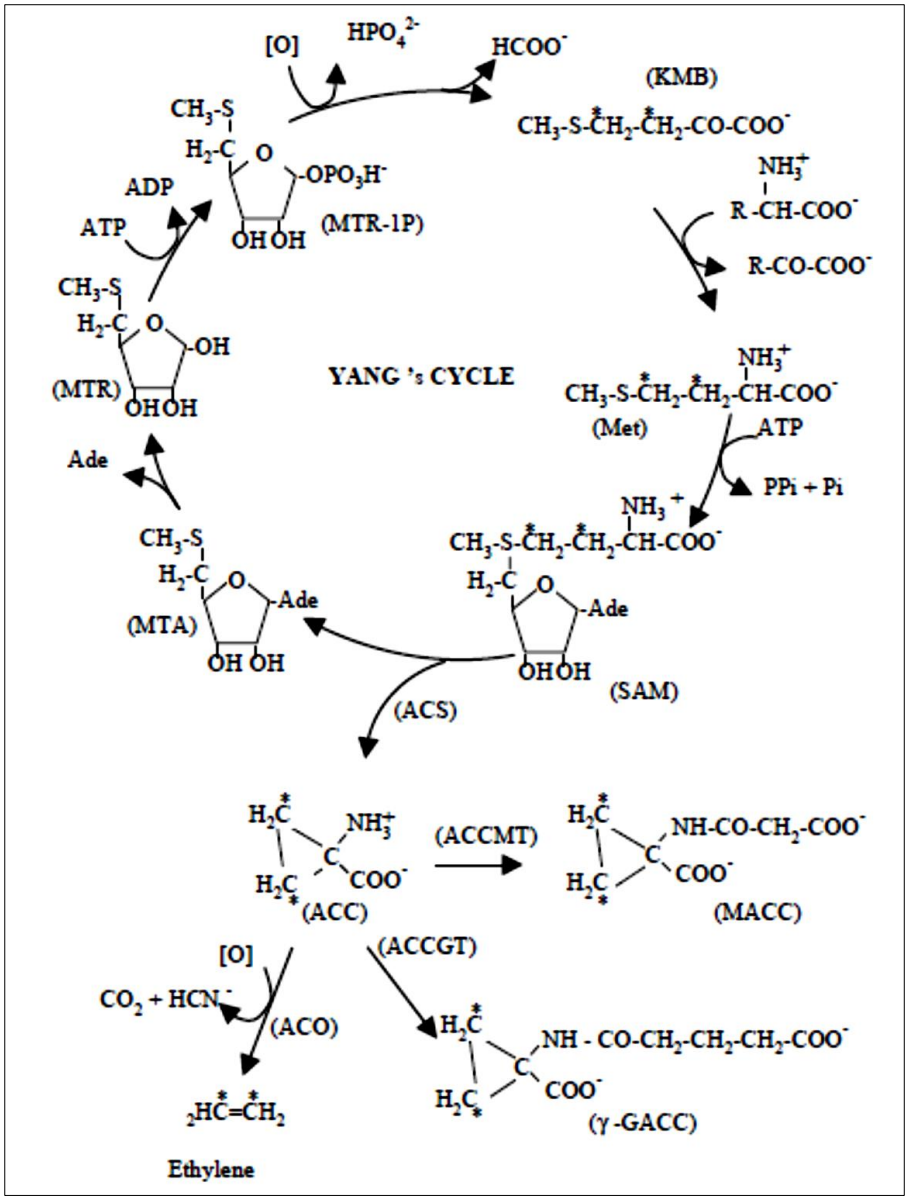

Fig. 1.8. The Yang's cycle and ethylene production in plant tissue (Pech et al., 2010).

For storage of apple fruit, especially for extended storage and storage in CAs, mature but preclimacteric fruit are needed. Ethylene is responsible for many aspects of fruit maturation such as change in background color (Dandekar et al., 2004; Johnston et al., 2009), fruit softening (Wilkinson et al., 2008), and aroma development (Dandekar et al., 2004; Johnston et al., 2009; Song and Bangerth, 1996). If fruit are too ripe at harvest, excessive flesh softening and other problems can occur during storage (Watkins et al., 2000). Starch degradation is one of the first indicators of fruit ripening, since it is very sensitive to ethylene (Johnston et al., 2009). The high sensitivity to ethylene makes the rate of starch degradation less dependent on ethylene, the onset as well as the progress of starch degradation might possibly be influenced mostly by other factors rather than just ethylene itself (Johnston et al., 2009).

To control ethylene-mediated ripening, fruit are treated with plant growth regulators (PGRs) in the field, or with ethylene perception inhibitor 1-methylcyclo-propene (1-MCP) after harvest. 1-MCP inhibits ethylene perception by binding to ethylene receptors (Sisler et al. 1996a,b). The effectiveness of inhibiting some of the ripening-related physiological changes in the fruit with postharvest 1-MCP has been documented previously (Blankenship and Dole, 2003; Watkins, 2006; Watkins, 2008). Inhibiting ethylene response in the field is carried out with applications of PGRs, either with preharvest 1-MCP or with aminoethoxyvinylglycine (AVG). AVG inhibits ethylene production by inhibiting ACS activity (Adams and Yang, 1979; Boller et al., 1979). Preharvest ethylene suppressors are applied to manage fruit harvest and fruit size, prevent premature and excessive fruit drop, as well as enhance fruit response to postharvest 1-MCP treatment (Byers et al., 2005; Elfving et al., 2007; Watkins et al.,

2010; Yuan and Carbaugh, 2007).

The effect of ethylene on starch degradation was investigated by Thammawong and Arakawa (2007), who found no significant decrease in starch breakdown in immature apple fruit after 1-MCP treatment for the cultivars 'Tsugaru' and 'Gala'. In riper fruit, however, 1-MCP affected starch contents of 'Tsugaru' but not in 'Gala' (Thammawong and Arakawa, 2010). The starch in fruit of both cultivars disappeared in 'Tsugaru' after 8 days and after 12 days, respectively, in the stored 'Gala' fruit (Thammawong and Arakawa, 2010). There seemed to be no ability of the immature fruit to respond to internal or external ethylene vis-à-vis starch degradation. However, in ethylene treated fruit, the increase in sugar content did not correlate with an increase of respiration, and ethylene production induced starch hydrolysis, sugar accumulation was not induced through ethylene treatment and after 10 days reached the initial level measured in the control fruit (Thammawong and Arakawa, 2010).

1.7 Objectives

This dissertation focuses on changes in starch concentration during the late stages of fruit maturation and ripening. The overall goal is to understand cultivar-specific differences in ripening and starch degradation, the effects of 1-MCP both pre- and post-harvest on starch degradation, and the effects of AVG on starch degradation.

Cultivar-related differences in starch degradation are described in Chapter 2. In addition, changes in amylose concentration in total starch and related changes in SPI are also given. Included is a computer-based quantitative assessment of staining patterns and fruit image analysis. Objectives of this chapter are: i) Assess changes in SPIs and

starch concentrations of ‘Gala’, ‘Honeycrisp’, ‘McIntosh’, and ‘Empire’ over their maturation periods. (ii) Measure in % AM of total starch to investigate its effect on accurate assessment of SPIs for these cultivars. (iii) Use computer based image analysis using MATLAB® to assess relationships between percentage stained areas and assigned SPI values.

Differences in fruit ripening through differences in starch concentration in different zones of apple fruit tissue are described in Chapter 3 in order to verify the popular belief that an apple ripens from the stem down. This chapter begins by describing patterns of ethylene accumulation within the fruit. Other aspects of ripening might therefore also be different between zones of the same tissue within a fruit. A ripening gradient along the latitudinal axis is known in tomato (Nguyen et al., 2014); likewise visible maturation differences can be observed from green at the stem end to red towards the calyx of the fruit. In banana, ripening occurs from the distal end. Differential patterns of ripening within the apple fruit can therefore be assumed but has hitherto not been proven. Furthermore, the effect of 1-MCP and propylene, which can influence internal ethylene concentrations (IECs) of fruit, on postharvest starch degradation at 20 °C was investigated.

Ethylene is the ripening hormone in apple fruit, and pre-harvest alterations of IEC might change ripening patterns both on and off the tree. Effects of pre-harvest PGRs on starch hydrolysis during tree maturation of ‘Empire’ and ‘McIntosh’ fruit are investigated in Chapter 4, including the postharvest effects of AVG and preharvest application of 1-MCP on ‘Empire’ storage quality after air storage. The objective was to assess the relationship between IEC, SPI, and starch concentration of preharvest AVG

and 1-MCP treated fruit in contrast to untreated control fruit. Also assessed were the effects of application timing of both PGRs on maturity and starch loss at harvest and after storage.

Effects of AVG and 1-MCP applied in the field on changes in chlorophyll a concentration in the skin (differences in absorbance; I_{AD}) were investigated for 'Empire' fruit in Chapter 5. The objective of this study was to establish correlations between I_{AD} and other maturity indices, and how the correlations change through the use AVG and 1-MCP.

Effects of fruit maturity on storability and the related economic implications of 'Empire' fruit was investigated in Chapter 6. This experiment focused on harvest date effects on firm flesh browning during CA storage at 0.5 °C and 2 °C, as well as the influence of postharvest applied 1-MCP. The economic analysis was done to determine the effect of harvest timing on potential revenue for fruit marketed at harvest and after long term storage.

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Supplementary material

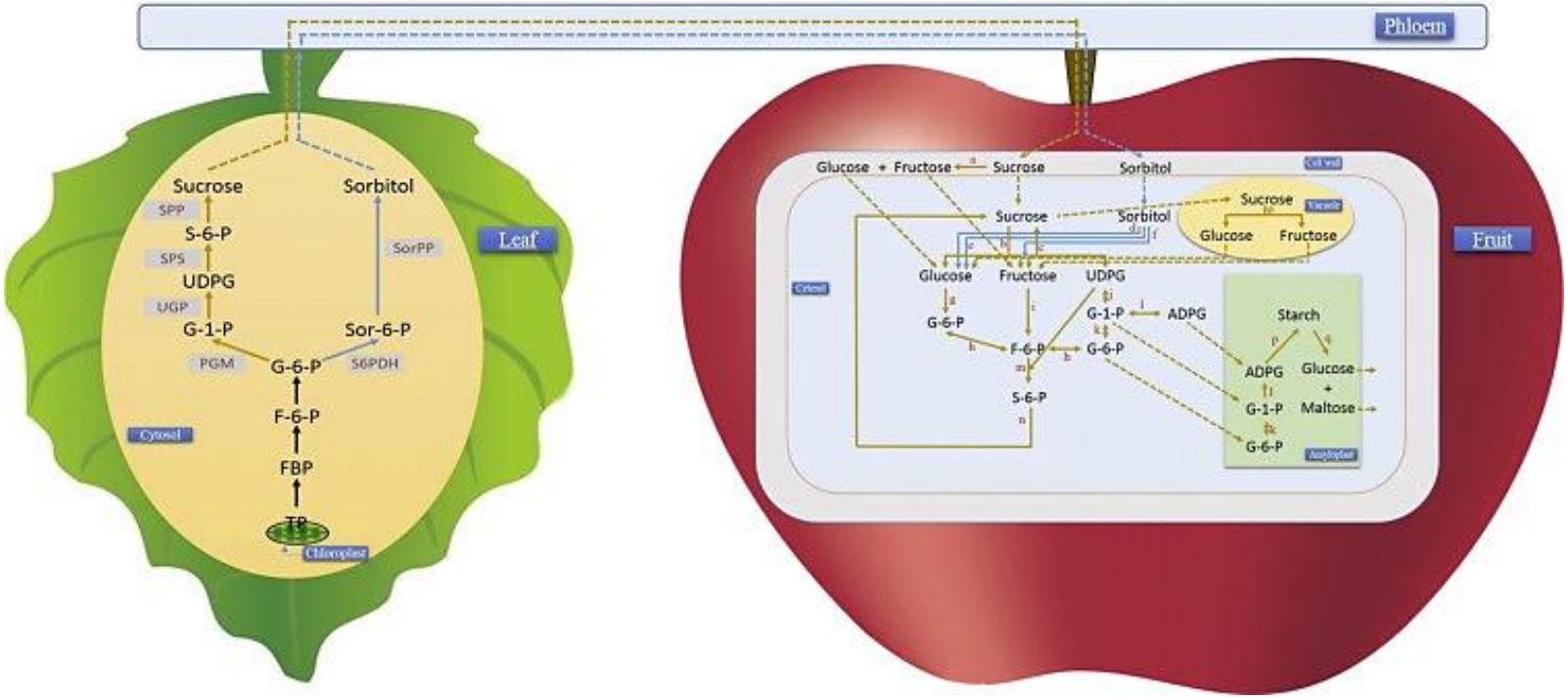

Fig. 1.I. A comprehensive and simplified model of the sugar biosynthesis pathway in a plant. The sugar biosynthesis steps are marked by solid lines, and the trans-membrane transport is marked by dashed lines. The sorbitol and sucrose biosynthesis pathways are represented in blue lines and yellow lines, respectively. The tissues and organelles are highlighted in blue boxes, and the enzymes are highlighted in gray boxes (in leaf) or lowercase letters (in fruit). The legend is as follow: ADPG, ADP-glucose; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; S6P, sucrose-6-phosphate; Sor-6P, sorbitol-6-phosphate; TP, triose phosphate; PGM, phosphoglucomutase; S6PDH, sorbitol-6-phosphatedehydrogenase; Sor-PP, sorbitol-6-phosphate phosphatase; SPP, sucrose phosphate phosphatase; SPS, sucrose phosphate synthase; UDPG, UDP-glucose; UGP, UDP-glucose pyrophosphorylase; a, cell-wall invertase (CWINV); b, alkaline/neutral invertase (A/N-INV); c, sucrose synthase (SUS); d, $NADP^+$-sorbitol dehydrogenase ($NADP^+$SDH); e, sorbitol oxidase (SOX); f, NAD^+-sorbitol dehydrogenase (NAD^+SDH); g, hexokinase (HXK); h, phosphoglucose isomerase (PGI); i, fructokinase (FRK); j, UDP-glucose pyrophosphorylase (UGP); k, phosphoglucomutase (PGM); l, ADP-glucose pyrophosphorylase (AGP); m, sucrose phosphate synthase (SPS); n, sucrose phosphate phosphatase (SPP); o, soluble acidic invertase (AIV); p, granule-bound starch synthase I, II (GBSSI, II), isoamylase 1, 2 (ISA1, 2), starch branching enzymes I, IIa, IIb (SBEI, IIa, IIb), starch synthase I, II, III, IV (SSI, II, III, IV); q, α-amylase (AMY3), β-amylase (BAM), glucan water dikinase (GWD), phosphoglucan water dikinase (PWD), isoamylase 3 (ISA3), pullulanase (PUL), disproportionating enzyme (DPE), and glucan phosphorylase (PHS) (Shangguan et al., 2014).

Table 1.I. Enzymes involved in starch metabolism with enzyme commission numbers (EC #) *Arabidopsis thaliana* gene names and comparable apple genes (representative EST on the array is shown for the best apple gene match to the *Arabidopsis* gene) expanded from Janssen et al. (2008)

Enzyme	EC #	*A. thaliana* gene	Genbank acc.
Sucrose synthase (SUS)	2.4.1.13	At3g43190 At4g02280 At5g20830 At5g37180 At5g49190	EB144194 CN8977963
UDP-glucose pyrophosphorylase (UGP)	2.7.7.9	At5g17310	EG631379
Starch synthase (SS)	2.4.1.21	At1g32900 At3g01180	EE663720 EB121923
ADP-glucose phosphorylase (AGP)	2.7.7.27	At1g27680 At2g21590 At4g39210 At5g48300 At1g05610	CN884033
Starch phosphorylase (glucosidase)	2.4.1.1	At3g29320 At3g46970	EE663644 EB108842
Sucrose-phosphate synthase (SPP)	2.4.1.14	At5g20280 At1g04920 At5g11110 At4g10120	EB112628 EB123469
β-amylase (BAM)	3.2.1.2	At4g15210 At4g17090	EB114557 EG631202
α-glucosidase	3.2.1.20	At3g45940 At5g11720 At5g63840	EE663791 EE663790
Sucrose phosphate phosphatase (SPP)	3.1.3.24	At2g35840	EB156512
α-amylase (AMY3)	3.2.1.1	At1g69830	XP_008376500.1
Glucan, water dikinase (GWD)	2.7.9.4	AtGWD3 (Baunsgaard et al., 2005)	
Phosphoglucan, water dikinase (PWD)	2.7.5.9	STARCH-EXSESS SEX4; At3g52180 (Kötting et al., 2009) SEX1; At1g10760 (Kötting et al., 2005)	
Isoamylase 3 (ISA3)	2.3.1.68	At2g39930 (AtISA1) At1g03310 (AtISA2) At4g09020 (AtISA3) At5g04360 (AtPU1) (Wattebled et al., 2005)	
α-glucan-branching (starch branching enzyme) (SBE)	2.4.1.18		

CHAPTER 2

RELATIONSHIPS BETWEEN STARCH PATTERN INDICES AND STARCH CONCENTRATIONS IN FOUR APPLE CULTIVARS

Abstract

One of the most widely used measurements to assess apple [*Malus sylvestris* (L.) Mill var. *domestica* (Borkh.) Mansf.] maturity is the starch pattern index (SPI), but relatively little is known about changes in its relationship with starch concentration in the fruit during maturation and ripening. Relationships between SPI and starch concentrations in 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire' fruit harvested over several weeks have been investigated. While SPI values increased and starch concentration decreased over the sampling period, the relationship between the two factors was curvilinear for all cultivars. The binding capacity of iodine to starch is determined by the starch composition and consequently by the amylose (AM) concentration. Therefore, changes in AM concentration in total starch were assessed to evaluate whether changes in AM account for discrepancies between SPI values and changes in starch concentration. Low levels of AM at later stages of fruit development account in part for the discrepancy between SPI and starch concentration, due to the low staining potential of the remaining starch (amylopectin (AP)). Evaluation of SPI values was also carried out by calculation of percentage stained area by means of image analysis; changes in SPI and % staining were linear for all cultivars but to different degrees depending on cultivar. Computational analysis of SPI images have the potential to be more consistent, but interpretation of the index number assigned still relies heavily

on a general understanding of fruit maturation. Understanding cultivar specific differences in the starch iodine staining patterns and the influential factors on starch during ripening can lead to an overall better understanding of SPI as a harvest index.

2.1. Introduction

Harvest management to ensure that apples are picked at the optimum time to meet consumer expectations for quality is straightforward if fruit are consumed immediately, but is more difficult if fruit are to be stored. Fruit harvested at full maturity will not store for extended periods whereas successful long term storage requires harvest of less mature fruit. Therefore, development of maturity indices to aid harvest decisions has been a major research emphasis for many years, and many different tests have been proposed, e.g. days from full bloom, ethylene production, the respiratory climacteric, change of background or ground color from green to yellow, loss of acidity and the progress of conversion of starch to sugar measured by starch pattern index (SPI) and soluble solids concentration (SSC) (Watkins, 2003). More recently, there has been interest in the development of non-destructive maturity tests such as the Delta Absorbance meter that can be used to assess chlorophyll a changes of the fruit (Nyasordzi et al., 2013; Toivonen and Hampson, 2014). However, the primary tests used by apple industries at present are the SPI, flesh firmness and SSC, and where equipment is available, ethylene production, usually assessed as the internal ethylene concentration (IEC). The term harvest indices, rather than maturity indices, is preferred because firmness and SSC are more measures of quality than maturity per se.

The SPI test is one of the simplest and easiest harvest indices to perform; apple fruit are cut horizontally at the equatorial region and I_2-Kl solution is applied to stain the starch and thereby indicate the degree of starch hydrolysis. Although the test was generally rejected as being too variable in early studies (Haller and Smith, 1950; Tiller, 1934), the indexing of the patterns based on visual assessment of starch staining has gained wide acceptance. The SPI test can be based on cultivar-specific charts such as for 'Delicious' and 'Northern Spy' (Smith et al., 1979), 'Granny Smith' (Reid et al., 1982), 'Empire', 'Idared' and 'Spartan' (Chu, 1988), 'Honeycrisp' (Hanrahan, 2012), or generic charts such as the Cornell 8-point chart (Blanpied and Silsby, 1992) and the European 10-point chart (Travers et al., 2002).

The reliability of the SPI as a harvest index can be affected by preharvest factors. A light cropping tree will have fruit that stain darker due to higher starch concentrations in the fruit but does not indicate a delay of maturation (Blanpied and Silsby, 1992). It is likely that within a cultivar, climate and position in the tree can affect maximum starch concentrations in fruit (Brookfield et al., 1997; Poapst et al., 1959; Smith et al., 1979). Also, the SPI only provides an indication of starch distribution and does not measure the starch content as precisely as other analytical methods (Cho and Gil, 2004; Peirs et al., 2003; Peirs et al., 2002; Travers et al., 2002).

Starch, more precisely amylose (AM), forms a polyiodine complex with I_2-Kl that is black-blue (Fan et al., 1995). The amylopectin (AP)-polyiodine complex produces a light purple-red pigment rather than the typical black-blue of the AM-polyiodine stains (Cho and Gil, 2004) and contributes less to staining. The ratio of AM/AP within the starch granule therefore influences the staining due to the unequal binding of AM and

AP with iodine. Some parts of the granule are crystalline and others amorphous which creates a physically heterogeneous grain (Donald et al., 2001; Gallant et al., 1997). Starch grains also contain small amounts of non-carbohydrate compounds, such as lipids, proteins, and ash (Ernst et al., 1999; Potter et al., 1949).

Surprisingly there has been relatively little research on the starch metabolism of apple fruit in relation to the SPI. Travers et al. (2002) found a linear relationship between staining patterns and starch concentration of two cider cultivars. Seasonal variation in correlation between starch concentrations and SPI were found in 'Fuji' and the AM to AP ratio of the starch may influence the ability to assess starch with iodine (Fan et al., 1995). The amount of AM in apple fruit starch has been studied (Carter and Neubert, 1954; Potter et al., 1949; Stevenson et al., 2006). The capacity of binding with iodine was determined to be about 20 to 21% (w/w) for AM compared to 0.3 to 2.6% for AP (Fan et al., 1995; Magein and Leurquin, 2000). Overall, AM concentration in total starch has been measured in many ways and for different purposes (Smith et al., 1995).

Assigning SPI values to staining patterns of each fruit is a subjective assessment, and errors for different inspectors were as high as 60% (Peirs et al., 2002). That same study also demonstrated that differences in subjective interpretation were related to the number of indices in the chart. Development of objective measurements of SPIs has been attempted. Travers et al. (2002) obtained a strong regression with staining patterns between 0 and 100% for 'Bisquet' and 'Binet Rouge'. Cho and Gil (2004) found a good linear correlation between starch concentration in the dry matter and staining pattern in 'Fuji'.

The objectives of our study were to:

(i) Assess changes in SPIs and starch concentrations of 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire' over their maturation periods.

(ii) Measure in % AM of total starch to investigate its effect on accurate assessment of SPIs for these cultivars.

(iii) Use computer based image analysis using MATLAB® to assess relationships between percentage stained areas and assigned SPI values.

2.2. Materials and methods

2.2.1 Plant material

'Gala', 'Honeycrisp', 'McIntosh', and 'Empire' apples [*Malus sylvestris* (L.)Mill var. *domestica* (Borkh.) Mansf.] were harvested in 2011 from trees grown on M.9 rootstock at the Cornell Orchards, Ithaca, New York. Six fruit of each cultivar were harvested randomly from ten marked trees at one week intervals from August 16 to September 14. Thereafter, bi-weekly harvests were carried out until most of the sampled fruit of each cultivar had reached a SPI of 7 or higher on the Cornell generic chart (Blanpied and Silsby, 1992).

2.2.2 Fruit and tissue sampling

Each fruit was treated as an individual sample throughout the experiment. The IEC of all fruit was measured at all harvests except August 16 by injecting 1 mL of gas sample taken from the core cavity using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) as described by Watkins et al. (2000). The GC was equipped with a flame ionization detector and fitted with a stainless

steel column packed with 60/80 mesh Alumina F-1 (2 m x 2 mm i.d.).

Wedges of flesh, approximately 1/8th of the fruit, were cut longitudinally from the blushed (exposed) and unblushed (shaded) side of each apple, the skin removed quickly, and the combined flesh samples flash frozen in liquid N_2. The tissue samples were kept frozen at -20 °C until they were lyophilized. The freeze-dried samples were ground with a Willey mill (mesh size 40) to fine powder for carbohydrate and starch extraction (Miller and Langhans, 1989; Ranwala and Miller, 2008). Each apple was then cut equatorially, dipped into iodine solution as described by Blanpied and Silsby (1992), and rated on a scale of 1 to 8, where 1 = 100% starch-iodine-complex stain coverage and 8 = 0% starch stain coverage. All SPI readings were carried out by the same person.

2.2.3 Starch analysis

Freeze dried samples (50 mg) were extracted with 80% ethanol three times with 30 min incubation in a 70 °C water bath. After removal of the liquid phase, the samples were dried overnight at 50-60 °C to evaporate the remaining alcohol. The starch was digested for 48 h at 55 °C with amyloglucosidase (AGSA; Sigma, A7255) (50 units enzyme per mL) in 100 mM sodium acetate buffer (pH 4.5) (Arĕas and Lajolo, 1981; Miller and Langhans, 1989; Ranwala and Miller, 2008). To solubilize the starch before enzymatic digestion, the starch was first boiled for 30 min with sodium acetate buffer and cooled to room temperature before addition of the enzyme solution.

The amount of glucose released from starch was determined colorimetrically using an enzymatic indicator solution of glucose oxidase (5 units/mL), peroxidase (1 unit/mL) and o-dianisidine (0.04 mg/mL) in 100 mM sodium phosphate buffer (pH 7). The

reaction was stopped after 30 min at 30 °C by addition of 1.0 mL 1 N HCl, and the absorption read at 450 nm in a spectophotometer (Spectronic 20, Genesys, Rochester, USA).

The amount of starch represented by the measured glucose was calculated as follows:

$$\textbf{Starch}\ {}^{\textbf{mg}}/_{\textbf{g DW}} = \Big(\big(\mathrm{S} * \mathrm{Abs}(450\ \mathrm{nm})\big) + \mathrm{I}\Big) * 0.18 * 0.9 * \left(\frac{1000}{\text{sample weight (mg)}}\right) * \left(\frac{5000}{\text{sample volume}(\mu\mathrm{L})}\right)$$

The S (slope) and I (intercept) of the standard curve were determined for each batch of extractions using the same enzyme solution as for the samples. The standard curve was developed with 10 glucose dilution samples starting at 0 µmol glucose in 100 µL H_2O to 0.8 µmol glucose in 100 µL H_2O.

2.2.4 Percent amylose of total starch

The percentage amylose (% AM) in total starch was determined using a semi-quantitative method as described by Magel (1991) and Fan et al. (1995). This method is an improved approach of the Hovenkamp-Hermelink et al. (1988) method. Freeze dried tissue (60 mg) was weighed into 15 mL Falcon tubes. To solubilize the starch 1 mL 18% HCl was added and the sample vortexed and incubated for 30 min at 20 °C. Then 9 mL distilled water was added and mixed to dilute the sample to 1.8% HCl. Samples were centrifuged in an Eppendorf Model 5810 (Eppendorf North America,

Inc., Westbury, NY) for 10 min at 4000 rpm, and 500 µL of the supernatant was then pipetted into a 13x100 glass culture tube, and 5 mL of 1.8% HCl added. Addition of 200 µL I_2-Kl solution (2 mg I_2 and 20 mg KI per mL H_2O) leads to a color change from blue to red, depending on the AM concentration. The absorption at 530 and 606 nm was read after 10 min. A standard curve with different percentages of potato amylose (Sigma) to amylopectin (Maize; Sigma) showed that absorption for the standard curve was linear from 0 µg to 100 µg total solids. The standard curves were carried out with 10% steps from 100% AM to 0% AM to AP. The % AM in the sample was based on the calculation from the 100% and 0% AM standard curve absorption coefficient (A) in OD units x ml x μg^{-1} x cm^{-1} of $A_{(AM\ 530\ nm)} = 9.43$, $A_{(AM\ 606\ nm)} = 14.47$, $A_{(AP\ 530\ nm)} = 6.57$, $A_{(AP\ 606\ nm)} = 4.31$. The calculation was based on the ratio of absorbance (R) as described by Hovenkamp-Hermelink et al. (1988) and Magel (1991) as:

$$R = \frac{P * C * A(AM_{605\ nm}) + (1 - P) * C * A(AP_{605\ nm})}{P * C * A(AM_{530\ nm}) + (1 - P) * C * A(AP_{530\ nm})}$$

where C = starch concentration (µg mL^{-1}) and P = fraction of AM. Since R is known and P is the unknown factor for the calculation, the following was used:

$$P = \frac{(6.7R - 4.31)}{(10.16 - 2.18R)} * 100$$

2.2.5 Iodine stained area computational analysis

Photographs in the Cornell generic staining chart (Blanpied and Silsby, 1992) as well as photographs of each fruit after staining with iodine were taken and analyzed for percentage stained area. Photographs were taken at high resolution (Canon, Powershot SX170 IS) without direct light to minimize reflection for computer based analysis in

MATLAB® (R209A, The Math Works Inc.) and analyzed with a mathematic code.

Image analysis first required the color image to be transformed to grey scale based on the blue color of the image. A square was drawn around the fruit with the edges as close to the fruit outline as possible. The number of pixel rows and columns in the square were used to define a center. The distance from the center to the square to the edge was defined as the radius (r) of a circle within the square. After the square was cropped out the image was binarized. For binarization three different threshold levels were used. The threshold levels set the cut off between whether a pixel is marked white (0) or black (1). The program then counted the total number of pixels within the square and the number of black pixels. To account for round fruit, a circle around the center was calculated with the radius r. To account for the missing wedges, which had been taken previously from the apple for chemical analysis, the missing parts were cropped out. The program then calculated the area of pixels within the cropped area and subtracted those from the total and black pixel count. The ratio of black to white pixels for the circle without the two wedges was calculated.

2.2.6 Statistics

All statistical analyses were carried out using JMP® Pro 11 (SAS Institute Inc.). Statistical models applied to data were least square fit, Tukey (HSD) and Pearson correlations. Comparison of slope of regressions was used to compare daily rate of starch hydrolysis between cultivars. IEC data were transformed with logarithms for analysis, but back-transformed means are presented within the text and graphs. Data was also subjected to regression analysis to define linear (L) and quadratic (Q) terms.

2.3. Results

2.3.1 Internal ethylene concentration (IEC)

The IEC in fruit of each cultivar was relatively low at the time of first sampling, and the patterns of change over time were affected by cultivar (Fig. 2.1). 'Gala' apples averaged 1.1 μL L^{-1} on August 26, but the IEC increased only gradually over time, with higher concentrations found in only a few fruit. IECs in 'Honeycrisp' were initially low, but varied greatly by and within harvest date. 'McIntosh' IECs also varied greatly by harvest date but concentrations were much higher than in other cultivars with a maximum single fruit value of 302 μL L^{-1} on September 27. On September 27, the average IEC for 'McIntosh' was 72.5 μL L^{-1}. 'Empire', the latest maturing cultivar in this study, had a long lag phase until the IEC increased above the climacteric point of ~1.0 μL L^{-1}, and higher concentrations were found only after October 14. The R^2 values of individual fruit for linear regressions ranged from 0.07 for 'Empire' to 0.21 for 'Gala'. The association between log IEC and harvest date improved the R^2 values slightly with 0.31, 0.39, 0.2, and 0.23 for 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire', respectively.

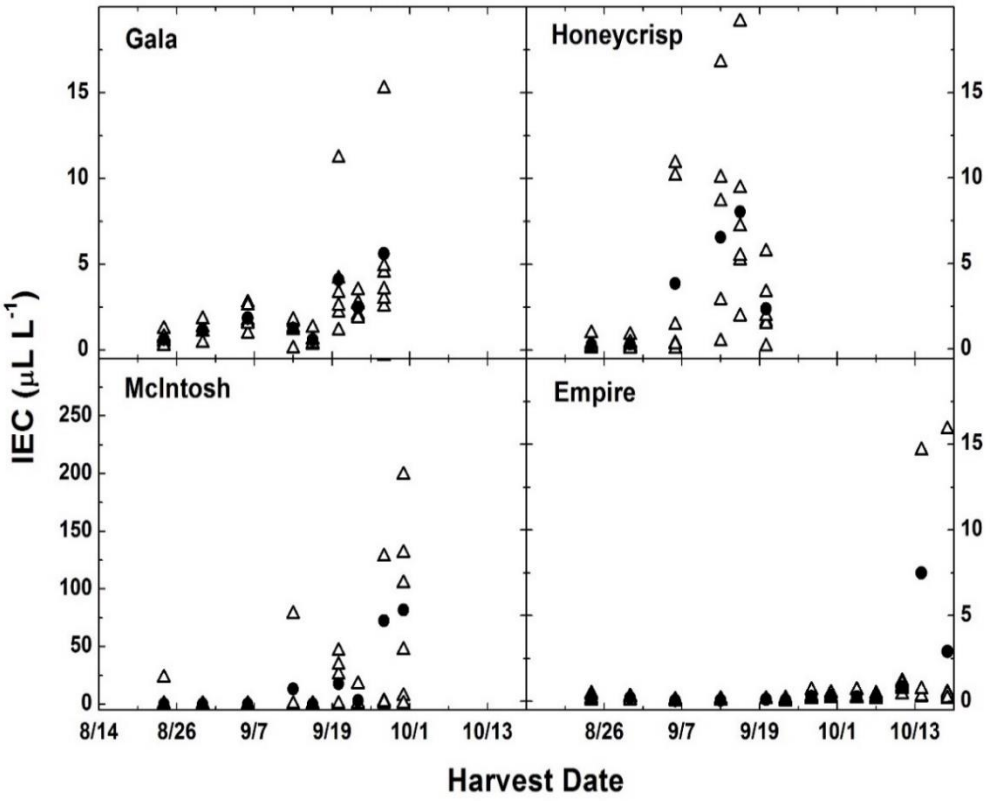

Fig. 2.1. Internal ethylene concentration (IEC) of individual fruit of 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire', apples. Note the change in y axis for 'McIntosh' (range to 300 µL L^{-1}) compared with 20 µl L^{-1} for the other cultivars; fruit with similar IEC value or within a small range of values may appear as one symbol. The filled circle symbols represent the average (Avg.) for each harvest date.

2.3.2 Starch pattern index (SPI)

SPI values increased for all cultivars over the sampling period (Fig. 2.2). The weakest association was found for 'Gala', reflecting a wide variation of SPI values especially among fruit within each harvest date. However, using averages for each date increased the R^2 for all cultivars. Linear relationships were found for 'Gala' and 'McIntosh' (Table 2.1) and quadratic relationships, indicating a lag phase, for 'Honeycrisp' and 'Empire' fruit. With the removal of the first early harvest date the relationship is linear for 'Gala', 'Honeycrisp' and 'McIntosh' and only the late ripening

cultivar ‘Empire’ still had a quadratic increase.

The most rapid increase for SPI was 0.19 units day^{-1} was seen with ‘Honeycrisp’ and the slowest increase for ‘Empire’, 0.08 units day^{-1}. ‘Gala’ and ‘McIntosh’ increased by 0.14 and 0.10 units day^{-1}, respectively.

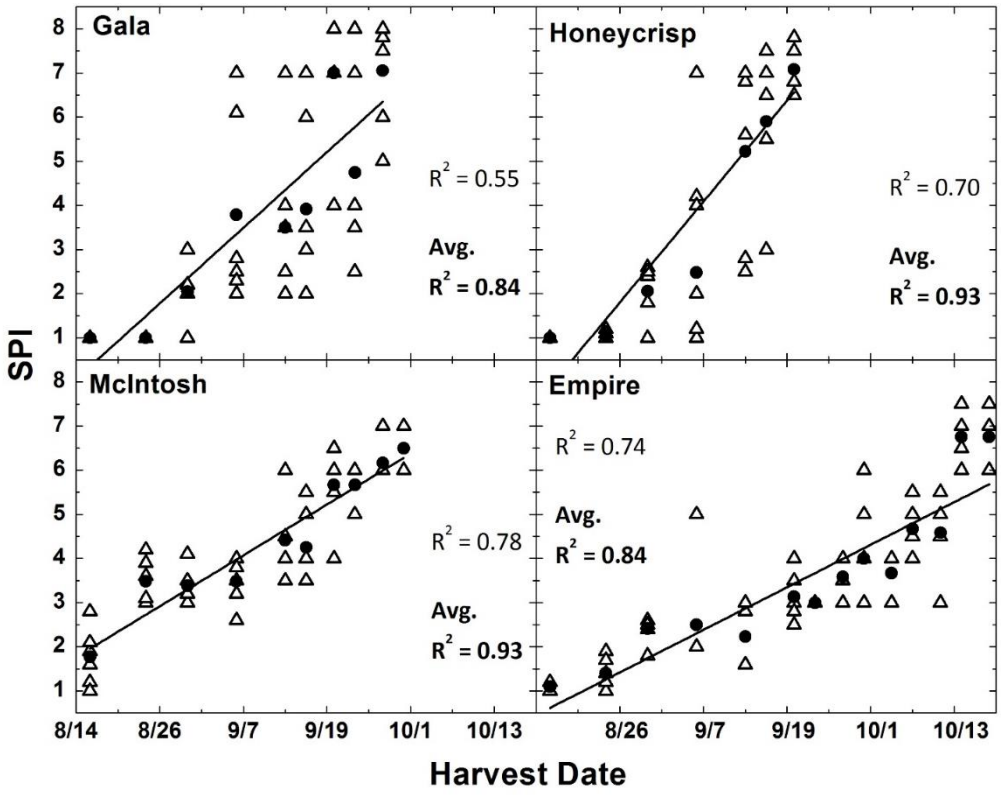

Fig. 2.2. Starch pattern index (SPI) of individual fruit of ‘Gala’, ’Honeycrisp’, ‘McIntosh’, and ‘Empire’. R^2 represents the regression for individual fruit of each cultivar, while the filled circle symbols represent the average (Avg.) reading for each harvest date. The Avg. R^2 in bold font represents the regression for combined data for each harvest date.

Table 2.1. Regression analysis for best fit linear (L) or quadratic (Q), R^2 and *P*-values for the effects of harvest date on starch concentration and starch pattern index (SPI), and of SPI on starch concentration, of individual fruit and harvest date averages, for 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire' with and without the first harvest date (Sept 16).

Variable	Effect	'Gala'	without Aug 16	'Honeycrisp'	without Aug 16	'McIntosh'	without Aug 16	'Empire'	without Aug 16
SPI:	harvest date	**L***** R^2 = 0.55 *P* <0.0001	**L***** R^2 = 0.50 *P* <0.0001	**Q*** R^2 = 0.70 *P* = 0.0106	**L***** R^2 = 0.71 *P* <0.0001	**L***** R^2 = 0.78 *P* <0.0001	**L***** R^2 = 0.47 *P* <0.0001	**Q***** R^2 = 0.74 *P* <0.0001	**Q***** R^2 = 0.78 *P* <0.0001
SPI: (Averages)	harvest date	**L**** R^2 = 0.84 *P* = 0.0013	**L**** R^2 = 0.82 *P* = 0.0068	**Q**** R^2 = 0.93 *P* = 0.0029	**L***** R^2 = 0.99 *P* <0.0001	**L***** R^2 = 0.93 *P* <0.0001	**Q*** R^2 = 0.96 *P* = 0.0293	**Q*** $R^{2=}$ 0.84 *P* = 0.0112	**Q**** R^2 = 0.91 *P* = 0.0087
Starch concentration:	harvest date	**L***** R^2 = 0.87 *P* <0.0001	**L***** R^2 = 0.80 *P* <0.0001	**L***** R^2 = 0.81 *P* <0.0001	**L***** R^2 = 0.77 *P* <0.0001	**L***** R^2 = 0.68 *P* <0.0001	**L***** R^2 = 0.56 *P* <0.0001	**L***** R^2 = 0.79 *P* <0.0001	**L***** R^2 = 0.73 *P* <0.0001
	SPI	**Q***** R^2 = 0.75 *P* <0.0001	**Q***** R^2 = 0.65 *P* <0.0001	**Q*** R^2 = 0.61 *P* = 0.0289	**L***** R^2 = 0.72 *P* <0.0001	**Q***** R^2 = 0.77 *P* <0.0001	**L***** R^2 = 0.58 *P* <0.0001	**Q**** R^2 = 0.72 *P* = 0.0055	**L***** R^2 = 0.58 *P* <0.0001
Starch concentration: (Averages)	harvest date	**Q*** R^2 = 0.92 *P* = 0.0167	**L***** R^2 = 0.90 *P* <0.0001	**Q*** R^2 = 0.92 *P* = 0.0169	**L**** R^2 = 0.92 *P* = 0.0067	**L**** R^2 = 0.78 *P* = 0.0018	**L***** R^2 = 0.87 *P* = 0.0003	**L***** R^2 = 0.90 *P* <0.0001	**L***** R^2 = 0.87 *P* <0.0001
	SPI	**L**** R^2 = 0.74 *P* = 0.0010	**L**** R^2 = 0.79 *P* = 0.0074	**Q*** R^2 = 0.67 *P* = 0.0243	**Q*** R^2 = 0.98 *P* = 0.0361	**Q**** R^2 = 0.74 *P* = 0.0081	**L**** R^2 = 0.72 *P* = 0.0048	**Q*** R^2 = 0.80 *P* = 0.0361	**L***** R^2 = 0.80 *P* <0.0001

2.3.3 Starch concentrations

Starch concentrations were higher in 'Gala', 'Honeycrisp', and 'Empire' than in 'McIntosh' at the first harvest date (Fig. 2.3; $P = 0.002$). A marked decline in starch concentrations occurred at second harvest date, but overall cultivar differences in concentration were maintained. Starch concentrations in all cultivars decreased linearly over time (Fig. 2.3; Table 2.1), but faster in 'Gala' and 'Honeycrisp' at 4.1 and 4.5 mg starch g^{-1} day^{-1}, respectively, than in 'Empire' and 'McIntosh', both at 2.1 mg g^{-1} day^{-1}.

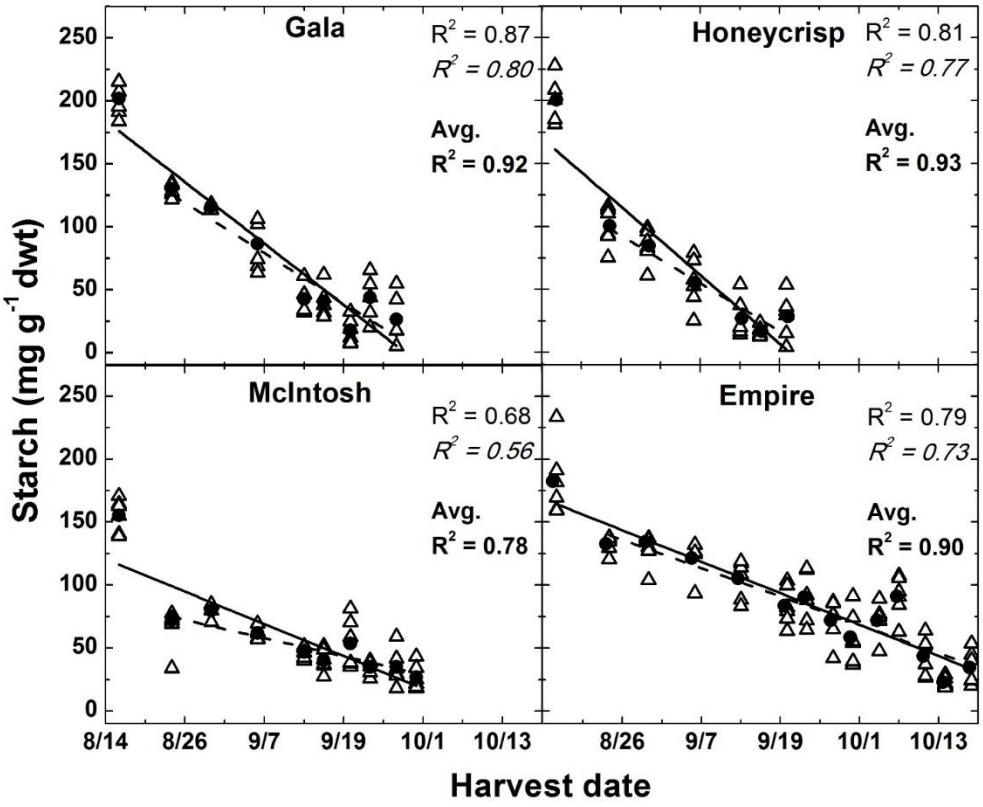

Fig. 2.3. Starch concentration (mg g^{-1} dry wt) of individual fruit of 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire', straight line and R^2. Dashed lines and cursive R^2 are best fits when harvest date 1 (August 16) is excluded. The filled circle symbols represent the average (Avg.) reading for each harvest date. The Avg. R^2 in bold font represents the regression for combined data for each harvest date.

Because starch concentrations in the fruit at the first harvest date were generally markedly higher than at the second harvest date, regression analysis were also performed with starch concentrations from the first harvest date removed (Fig. 2.3; Table 2.1). The overall relationship between cultivars remained constant and the starch decline remained linear with and without August 16 (8/16). The rates of starch loss were still greater in 'Gala' and 'Honeycrisp' than in 'Empire' and 'McIntosh', but they were lower overall, being 3.3, 3.2, 1.9 and 1.2 mg g^{-1} day^{-1}, respectively.

2.3.4 SPI and starch concentration

The relationship of SPI and starch concentration was curvilinear in fruit of all cultivars (Fig. 2.4, Table 2.1) when harvest date one (8/16) was included into the regressions, and the starch concentration at a single index varied a great deal. However, if harvest one was removed all cultivars but 'Gala' present a linear relationship between starch concentration and SPI values (Table 2.1). Including the higher starch concentrations on the first sampling date altered the overall progression of starch concentration compared to SPI values.

'Honeycrisp' represented an extreme case of the wide spread of starch values on at a given SPI with starch concentrations ranging from 61 to 228 mg g^{-1} at a starch index reading of 1. Also, changes in starch concentrations over a wide range of SPI units were often relatively small. In 'Gala' and 'Honeycrisp', for example, there was little change in starch concentrations from SPI of 3 to 8. At the last sampling date, which was based on an average SPI of 7, starch concentrations were similar across the cultivars; 26.2, 28.6, 27.0 and 34.6 mg g^{-1} for 'Gala', 'Honeycrisp', 'McIntosh' and, 'Empire' respectively.

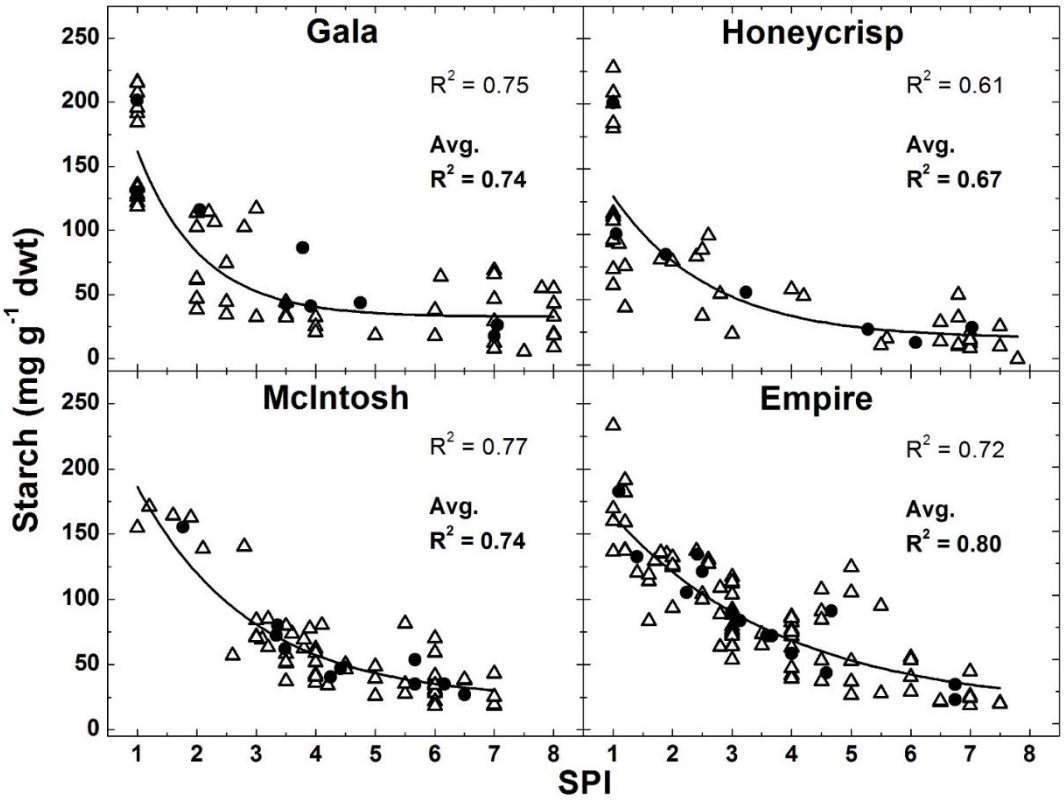

Fig. 2.4. Starch concentrations (mg g^{-1} dry wt) in fruit at each starch pattern index (SPI) value for individual fruit of 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire'. R^2 represents the regression for individual fruit of each cultivar, while the filled circle symbols represent the average (Avg.) reading for each harvest date. The Avg. R^2 in bold font represents the regression for combined data for each harvest date.

2.3.5 Percentage of amylose in total starch (% AM)

The % AM to total starch varied greatly among cultivars (Fig. 2.5). 'Gala' had the greatest decrease in % AM over the sampling period with a decline from maximum of 62.4% to as low as 0.01%. The overall decline of the regression was largest for 'Honeycrisp' which 1.05 % day^{-1}. Overall the rate of decline was slower for 'Empire' than 'McIntosh', 'Gala' and 'Honeycrisp' ($P < 0.001$).

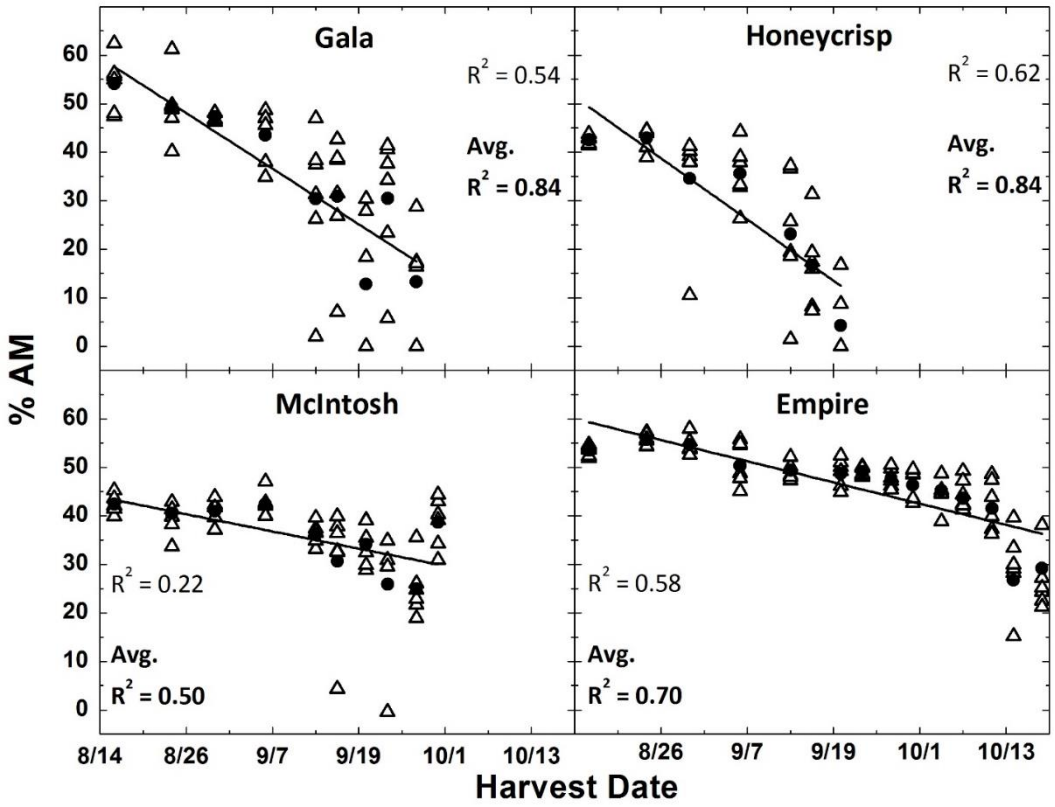

Fig. 2.5. Percentage amylose (AM) of total starch of individual fruit of 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire' over time (Harvest Date). R^2 represents the regression for individual fruit of each cultivar, while the filled circle symbols represent the average (Avg.) reading for each harvest date. The Avg. R^2 in bold font represents the regression for combined data for each harvest date.

2.3.6 Relationship of starch index (SPI) and starch concentration to computed starch-iodine stained area (% staining)

Alteration of images to binarized forms is illustrated in Fig. 2.6 using the Cornell generic chart. Computational calculation of iodine stained area of the fruit is shown in Fig. 2.7. The computed stained areas from binarized images of Cornell generic chart are between 85% at SPI 1 and 1.9% at SPI 8, indicating that the stain area of binarized images are slighter lower than those presented at Cornell Chart. Additionally the chart is referring to core stained area and flesh stained area separately, whereas the image

analysis always refers to the entire apple surface. Therefore, while a direct comparison of staining percentage is not possible, it has to be assessed with the difference in reference in mind. Still a staining of 85% total area computed is lower than the indication of the whole core and flesh stained.

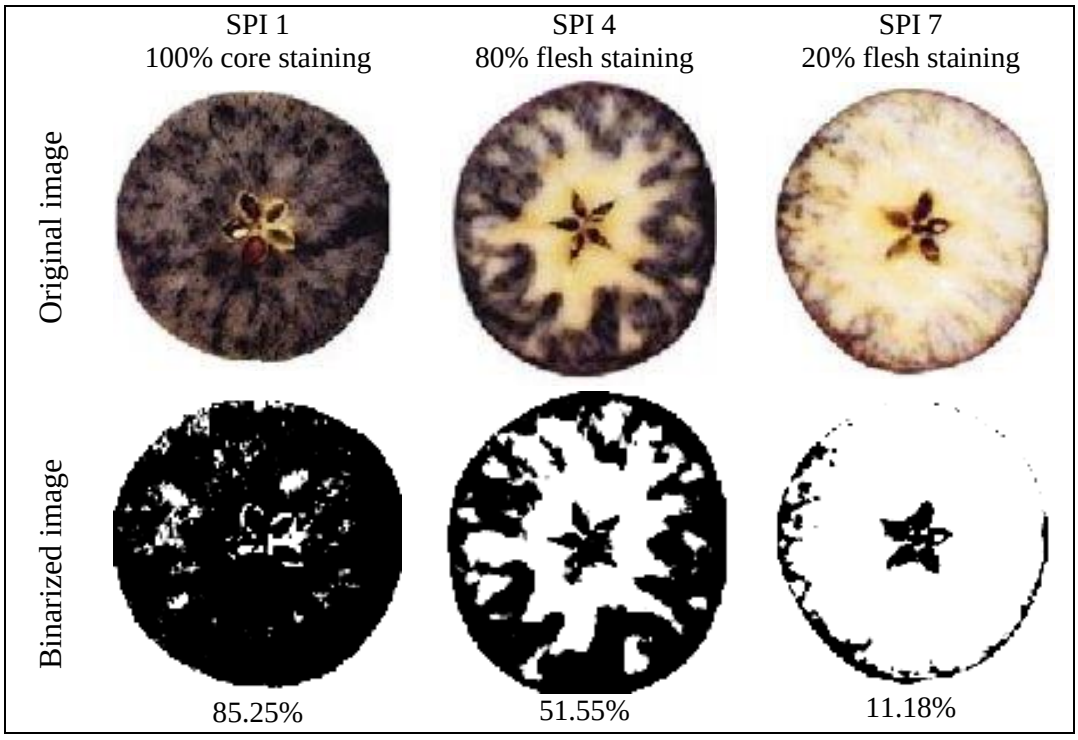

Fig. 2.6. Original images from the Cornell SPI chart by Blanpied and Silsby (1992) for starch indices (SPIs) 1, 4, and 7 and their respective descriptions in the chart, as well as for comparison with the MATLAB® binarized images used for the calculation of stained area; the percentage below indicates the calculated amount of staining for that apple.

However, while all cultivars showed the expected decrease in stained area with increasing SPI, the relationships were poor for 'Gala', 'Honeycrisp', and 'McIntosh'. The best fit was found for 'Empire'. The % staining values for 'Empire' are generally slightly higher than those of the comparison chart, mostly due to the fact that the Cornell

chart distinguishes between core and flesh staining but the MATLAB® code calculates staining for the entire area. The computed images give lower stain area than Cornell chart for lower SPI but not for higher SPI, being 85.3% and 70% at SPI 1 and 2 respectively, compared to that of 'Empire' with averages of 91.6% and 76.8% at SPI 1 and 2 respectively. At SPIs of 6 and 7, 'Empire' has average values of 23.1% and 16.6%, respectively, compared with the Cornell which has 16.2% and 7.4% respectively. The staining area of 'Empire' at SPI 7 is therefore very similar to the SPI 6 average on the Cornell chart, but overall relatively similar compared with the other three cultivars.

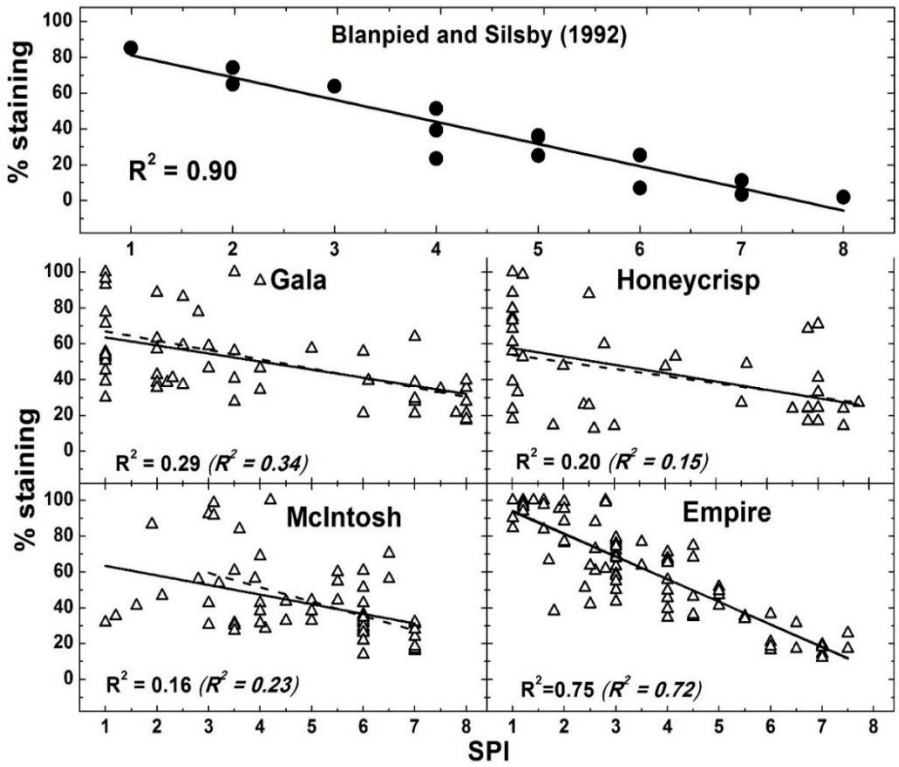

Fig. 2.7. Calculated % iodine staining using MATLAB® compared with assigned SPI values for the images of the Cornell SPI chart of Blanpied and Silsby (1992), and four cultivars. The straight line linear fit and bold R^2 for all harvest dates, dashed line and italics R^2 indicate the fit without the first harvest date (Aug 16).

2.3.7 Pearson correlations

The Pearson correlation coefficient r measures the strength of linear association between two variables. A good linear relationship for all four cultivars between Log_{10}IEC and SPI as well as for IEC and SPI was detected (Table 2.2), and was strongest for 'Honeycrisp'. 'Empire', with the very slow increase of IEC over time and the early start of sampling, compared with the other cultivars, has the weakest linear correlation for Log_{10}IEC and IEC with SPI. 'McIntosh' also has a relatively robust linear correlation between IEC and SPI. The relationship is not as strong for 'Gala' and 'Empire'.

Table 2.2. Pearson correlation coefficient r for comparison of relationships of internal ethylene concentration (IEC), or Log_{10}IEC, to starch pattern index (SPI), starch concentration, percent amylose (% AM), and percent computed starch-iodine stained area (% staining) as well as all variables to each other.

Comparison	**'Gala'**	**'Honeycrisp'**	**'McIntosh'**	**'Empire'**
IEC and SPI	0.53***	0.66***	0.56***	0.45**
IEC and starch concentration	- 0.29*	- 0.65***	- 0.37*	- 0.39**
IEC and % AM	- 0.53***	- 0.51**	0.52***	- 0.47***
Log_{10}IEC and SPI	0.67***	0.78***	0.77***	0.62***
Log_{10}IEC and starch concentration	- 0.45**	- 0.80***	- 0.59***	- 0.58***
Log_{10}IEC and % AM	- 0.54***	- 0.60***	- 0.47**	- 0.59***
Starch concentration and SPI	- 0.71***	- 0.75***	- 0.80***	- 0.81***
Starch concentration and % AM	0.73***	0.67***	0.43***	0.75***
Starch concentration and % staining	0.22^{ns}	0.44**	0.27^{ns}	0.74***
SPI and % AM	- 0.90***	- 0.88***	- 0.53***	- 0.88***
SPI and % staining	- 0.49***	- 0.47**	- 0.42**	- 0.87***
% staining and % AM	0.38**	0.53**	0.27*	0.73***

[*P*-values: ≤0.001 (***), ≤0.01 (**), ≤0.05 (*), non-significant (^{ns})]

Starch concentration and SPI show good curvilinear regressions (Fig. 2.4), and also high linear correlations (Tables 2.1 and 2.2). SPI and % AM, and starch concentration and % AM are well correlated for all cultivars. Linear correlations between starch concentration, SPI and % AM with percent computed starch-iodine stained area (% staining) are low for 'Honeycrisp', and 'McIntosh'. 'Empire' had a stronger relationship for % staining and other factors than the other cultivars.

2.4. Discussion

The apple cultivars used in this study varied greatly in the timing of the increase and amount of ethylene production as measured by IEC (Fig. 2.1), but the SPIs in all cultivars followed either a linear or quadratic increase over the time, albeit with varying degrees of fit (Fig. 2.2; Table 2.1). The lowest R^2 values for SPIs were found for 'Gala', using either all data points or means, reflecting the greatest variation of SPIs on any single harvest date, a wide variation of 1 to 3 units around the mean reading not being unusual. At the same time the decrease in % AM in the starch is much greater in 'Gala' and 'Honeycrisp' fruit compared with the other two cultivars, possibly indicating a greater chance of misreading the SPI staining through weaker binding of the iodine to the remaining starch early on in the development. 'Gala' and 'Honeycrisp' may be picked multiple times depending on the grower operation, using criteria of red color intensity and coverage on fruit to meet required commercial standards. The relationship between the SPI and red color of the fruit was not measured in this experiment; however, no consistent relationship between these factors has been detected in our other studies, and SPIs in 'Gala' appear to be consistently more variable than in other cultivars

(unpublished data).

In commercial practice a minimum of 10 fruit is usually used to assess any orchard block and the mean values are used for decision making (Blanpied and Silsby, 1992; Lau, 1985). In this study, only six fruit were used as the focus was on relationships between the SPI and starch concentration within single fruit. R^2 values were higher with harvest date averages than for individual fruit (Fig. 2.2; Table 2.1). Although a linear change of SPI over time is a common feature of apple fruit during maturation (Blanpied and Silsby, 1992; Reid et al., 1982; Watkins et al., 1982), effects of cultivar and starch reference chart, perhaps interrelated, have been found. For example, patterns of change in 'Fuji' appear to have at least two phases (Brookfield et al., 1997; Fan et al., 1995), while those for 'Gala' are linear (Brookfield et al., 1997). Non-linear changes may reflect unsuitability of a given starch reference chart for a cultivar. The quadratic fit of 'Empire' fruit over time for individual data as well as harvest date averages (Table 2.1) may be a result of the extended sampling period compared with the other cultivars. With the same number of harvest dates (nine in total) as for the other cultivars, the regression becomes linear for average harvest date data (*P*-value 0.0132) but remains quadratic for individual fruit data (*P*-value 0.0004), therefore still indicating a lag phase before the rapid decline in starch.

Starch concentrations declined linearly over time for all cultivars (Fig. 2.3; Table 2.1), concentrations being similar to those reported by Fan et al. (1995) and Travers et al. (2002). However, we observed a much greater difference between starch readings on the first harvest date of August 16 and the second harvest date of August 24 than subsequently observed (Fig. 2.3). There may have been unknown environmental

reasons responsible for this marked change, but it is interesting that 'McIntosh', in which the core does not necessarily fully fill with starch visible with the SPI test (personal observation), still changed greatly over that time period. The initial amount of starch early in the season possibly varies with growing season and region. Starch in the chloroplast is synthesized and hydrolyzed on a diurnal basis to supply carbohydrates to the plant at night (Chen and Cheng, 2004). Starch in storage organs is more permanent, and hydrolysis of potato or kernel starch is only needed after a period of vernalization to initiate growth in the spring (Smith et al., 1995). Starch in apple fruit is possibly less like starch in these storage organs as it is more transitory, reaching a peak of accumulation prior to maturation.

The relationship between SPI and starch concentration is curvilinear (Fig. 2.4), but becomes linear for three of the cultivars when the first harvest date is eliminated from the regression calculation (Table 2.1), indicating a greater reduction in starch concentration in the early compared with the late ripening stages of the fruit. At a SPI of 1, the variance of starch concentrations is especially high for 'Gala' and 'Honeycrisp' reflecting the differences in concentrations that are related to harvest date even at the minimum SPI. During the early phase of starch degradation the decline in % AM does not influence the staining pattern since the binding capacity with iodine remains high. The decline in stained area therefore in the early stages represent decline of starch, perhaps predominantly AM, in the core area of the fruit, but generally the % AM within the fruit remains sufficiently high to not reduce staining. In contrast, Travers et al. (2002) found a linear relationship between SPI and starch concentration. Differences in cultivars as well as differences in SPI charts are to be considered when interpreting

starch pattern data in comparison with starch concentration.

The 10-point chart as used by Planton (1994) has a non-linear scale (Piers et al., 2002). Piers et al. (2002), and Travers et al. (2002) proposed a simplified scale of 5 points to improve accuracy of SPI readings to avoid the subjectivity of higher point scales. Charts with greater number of indices, such as the 10-point chart used in Europe (Streif, 1984; Peirs et al., 2002; Travers et al., 2002) or the 9-point chart used by Priest and Lougheed (1981), possibly lead to a change in relationship compared with a lower scoring chart, with tighter intervals leading to a linear rather than a curvilinear relationship between starch concentration and SPI. Having too many intervals, however, requires charts to be more cultivar specific in order to provide clear guidance, and a greater number of indices are subject to greater error in interpretation (Peirs et al., 2002). Lower numbered indices such as used by Reid et al. (1982) have greater differences between the index steps and are hence easier to compare.

The binding capacity of iodine to starch molecules differs depending on the starch composition. Lower binding capacity of AP and much higher binding capacity of AM leads to changes in staining intensity depending on the composition of starch. Early in the season, steady constant minimum value (SPI 1) from one week to another does not mean starch is not being hydrolyzed. On the contrary, later in the season, when % AM is low, a small reduction of AM concentration has great effect on reduction of stained area (increase of SPI), despite the considerable amount of starch (AP) remaining within the fruit. The % AM of all cultivars declined over the harvest period, although at differing rates. Overall, the average values of % AM of 33, 27, 35, and 46% for 'Gala', 'Honeycrisp', 'McIntosh' and 'Empire', respectively, were all slightly higher than for

other cultivars. These cultivars include 'Newtown Pippin' 25% AM (Potter et al., 1949), 'Delicious' and 'Golden Delicious' 26% and 'Jonathan' and 'Winesap' 25% (Carter and Neubert, 1954), six cultivars with % AM ranging between 40-48% with 'Gala' at 40% (Stevenson et al., 2006), and 'Fuji' fruit with 25% AM, declining later in the season (Fan et al., 1995). The low % AM in starch later in the season might explain why at a SPI of 8 with "no starch remaining", still starch concentrations between 69 and 5.4 mg g^{-1} DW were measurable (Fig. 2.4). The differences between the cultivars, especially 'Empire' might be explained more by the extended sampling time of this cultivar compared with the others. Overall the change in the later ripening cultivars 'McIntosh' and 'Empire' is less with only a decrease of the % AM average from 43 to 25% and 56 to 27%, respectively. The changes in 'Gala' and 'Honeycrisp' were from 54 to 13% and 42 to 4%, respectively. Changes in % AM therefore are greater in earlier ripening cultivars compared with slower maturing cultivars.

The patterns of IEC change vary significantly among cultivars. While correlations between SPIs and either IEC or $\log_{10}$IEC were detected (Table 2.2), these correlations may be an artifact in that they result from changes in factors over time that may not necessarily be physiologically linked. Over the sampling period, SPI values increased (Fig. 2.2) reflecting starch hydrolysis (Fig. 2.3) in the absence of 'significant' ethylene or changes thereof. However, starch hydrolysis can be slowed by treatment of fruit on the tree with aminoethoxyvinylglycine (ReTain, Valent Biosciences) and 1-methylcyclopropene (Harvista, AgroFresh, Inc.), inhibitors of ethylene production and perception, respectively (Elfving et al., 2007; Watkins et al., 2010; Yuan and Carbaugh, 2007). Johnston et al. (2009) found that some maturity parameters such as starch are

very ethylene sensitive, and therefore, even very low amounts of IEC may cause the onset of starch hydrolysis.

We also investigated computer assessment of the SPI. In contrast to the findings of Travers et al. (2002), the % staining area of the four cultivars declines linearly over SPI values (Fig. 2.7). The decline within the Cornell chart was also linear. Perhaps the difference of an 8 point scale at Cornell verses the 10 point scale in Europe results in the linear rather than curvilinear decline. For the four cultivars the linear fit is not great as presented in the low R^2 values for 'Gala', 'Honeycrisp', and 'McIntosh', but fits acceptably for 'Empire'. The major flaw of SPI is that it is evaluated subjectively, and not as consistent in determining the exact value as a chemical analysis or any other objective evaluation. Therefore, the comparison of the stained area and the assigned value cannot always line up. Knowing that starch should be declining over the period of ripening could influence the evaluation even subconsciously. Another problem of the poor fit between the assigned SPI values and the calculated stained area could be caused by the analysis itself. The calculation done in MATLAB® is based on a perfect circle within the square placed around the fruit, which does not always fit the apple perfectly due to natural variation in shape. Since the assumption was made for all fruit however, the error should even out. In the description for the Cornell chart distinctions between core staining and staining after the core has cleared are made. Therefore, since this distinction between which area was stained was not made for the calculations, a one to one comparison between the percentage named within the chart and the results from MATLAB® was not possible.

In summary, the experiment investigated changes in starch concentration and SPI over time for four cultivars. The observed changes progressed in the same fashion for 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire' but at different rates unique to the ripening pattern of each cultivar. Changes in the AM concentration in the analyzed starch also changed in a manner unique for each cultivar but were linear over time. Assessment of staining patterns with MATLAB® showed that the calculated stained area and the assigned SPI value do not always match up well. Therefore, the image analysis not only underlines the subjectivity of SPI which can always be biased, but also shows that analyzing a complex image such as the apple, which was neither perfectly round nor had well defined differences between stained and unstained areas, can bear its own set of problems. Overall the SPI is still a widely utilized test which can have its bias but as long as it is performed by the same person generally gives a good indication of progressing maturation of apple fruit. SPI does not always represent the full image of how much starch is in the fruit, but for its purpose the exact determination of starch concentration is not needed. Since changes in % AM concentration can change starch visibility in the SPI test, how those changes are influenced by ethylene and general seasonal influences cannot be assessed with this data set. Therefore, other factors such as the use of preharvest ethylene regulators and the general season should be kept in mind when decisions for harvest based on the SPI are made.

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CHAPTER 3

STARCH CONCENTRATIONS IN DIFFERENT TISSUE ZONES OF APPLES DURING MATURATION AND IN RESPONSE TO PROPYLENE AND 1-METHYLCYCLOPROPENE (1-MCP) AFTER HARVEST

Abstract

Patterns of starch hydrolysis in stem, equatorial and calyx zones of 'Honeycrisp' and 'Empire' during maturation and ripening, and in 'Gala' in response to propylene or 1-methylcyclopropene (1-MCP) treatments after harvest, were studied. Differences in zonal starch concentrations were found for 'Empire' and 'Gala' fruit, with highest concentrations in the calyx end, but not for 'Honeycrisp'. During maturation and ripening of 'Empire', on the tree, the concentration of starch was highest in the calyx and lowest in the stem region. No differences in rates of starch hydrolysis among zones were detected. Postharvest treatment with propylene treatment did not affect the internal ethylene concentration of the fruit but 1-MCP markedly inhibited it. Starch concentrations were highest in the calyx end but gradients of starch among zones were not changed by postharvest treatment of the fruit. Soluble carbohydrate concentration variation between zones was inconsistent between on and off tree ripening. 'Honeycrisp' and 'Empire' had higher sorbitol concentrations in the calyx region, whereas postharvest 'Gala' fruit had lower concentrations in the calyx end. The distribution differences of glucose, fructose and sucrose were similar in all three cultivars, and showed higher concentrations in the stem region for fructose and glucose, and higher sucrose concentrations in the calyx end of the fruit. Postharvest treatments

influenced sorbitol, glucose and fructose concentrations, but an interaction between zone and treatment could not be detected. The differences in starch concentration between the zones did not confirm differences in ripening but rather is along the lines of a previously proposed assumption that starch is not evenly distributed throughout the fruit during development. Therefore measured differences in zonal starch are most likely related to starch accumulation during fruit development, rather than differences in fruit ripening during starch degradation.

3.1. Introduction

It is generally assumed that ripening is initiated in different parts of the fruit depending on species. Fruit such as tomato undergo uneven ripening across the latitudinal gradient (Nguyen et al., 2014), but based on color changes it is assumed that ripening starts in different zones e.g. tomatoes from the calyx and bananas from distal end. In the case of apples, popular belief is that apples ripen first at the stem end, although such differences among tissues have not been validated. In some respects it depends on the definition of ripening, the process by which a product becomes ready to eat or able to disperse seeds, and therefore the initiating zone may be not be critical per se in terms of consumer preferences. However, it is important in terms of understanding how ripening is controlled.

Fruit developing on the tree utilize photoassimilates to accumulate carbohydrates in the form of starch, and once detached from the tree the starch is used to provide soluble sugars for metabolic processes of ripening and senescence. Also on the tree, when a certain stage of maturity is reached, starch is hydrolyzed into soluble carbohydrates.

The first steps of starch hydrolysis occur in the plastids, and conversion to sucrose and other sugars occurs in the cytosol (Smith, 2007). Depending on the type (α or β) of amylase activity, maltose, maltotrioses or other carbohydrates, containing three or more glucose units, are released first (Garcia and Lajolo, 1988) and are further converted into glucose by α-glucosidase (Beck and Ziegler, 1989). α-Amylase hydrolyses α-1,4 linkage and releases both linear and branched oligosaccharides from the starch granule (Beck and Ziegler, 1989; Smith, 2007). β-amylase attacks the granule directly and releases dextrins, which are then hydrolyzed into smaller components (Gawęda and Ben, 2010). Branched chains are cleaved by debranching enzyme, to release linear chains, which in turn are attacked by amylase to release maltose (Smith, 2007). Sugars released from starch are ultimately glucose, fructose and sucrose, which are used for respiration as well as to enhance sweetness of the ripening fruit.

The apple fruit is comprised of hypanthial (cortical) and carpillary (core) tissues surrounded by a waxy cuticle covering the epidermal and hypodermal layers (Pratt, 1988). Rudell et al. (2000) found that ethylene production was generally highest in the core tissues. Also, concentrations of the precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), were much higher in core tissues, than the inner cortex and peduncular (shoulder) tissue, while calyx and outer cortex tissues were lowest (Mansour et al., 1986). These studies support a view that the ripening signal might be initiated in the core tissues, either as a seed-controlled mechanism and/or as a "tree factor".

Brookfield et al. (1997) found that starch concentrations were highest in outer cortical tissues than in the core before the onset of hydrolysis. While the rate of starch hydrolysis was slower in the core than in the cortex, its onset was simultaneous in all

tissues, and dissimilarities in starch concentration between the ends of the fruit were found in 'Fuji', which had higher starch concentrations in the calyx region, but not in 'Royal Gala' (Brookfield et al., 1997). The onset of starch hydrolysis started before increases in internal ethylene concentration (IEC). Differences in structure and size of cells could lead to developmental differences within the fruit, although such differences vary by cultivar (Bain and Robertson, 1951; Leshem et al., 1984). Starch granules were more abundant in the cytoplasm of the cells in the outer flesh of 'Jonagold', and granule size differences between the mid and outer flesh compared with the inner flesh, might lead to starch composition differences between the tissue zones (Ohmiya and Kakiuchi, 1990). Distribution of starch within kiwifruit (*Actinidia deliciosa* (A. Chev.) C. F. Liang et A. R. Ferguson cv. Hayward) (MacRae et al., 1989) is similar to that found in apple. Kiwifruit, similar to apple, accumulate starch during development and only release soluble sugars after the fruit has reached maturity (Nardozza et al., 2013).

Progress of starch degradation in apple fruit is commonly assessed by the starch pattern index (SPI) and used as an indicator of when to harvest fruit for short or long term storage (Blanpied and Silsby, 1992; Hanrahan, 2012; Reid et al., 1982; Smith et al., 1979; Travers et al., 2002). While the SPI is used as a means of assessing starch hydrolysis in fruit, few studies are available on starch concentration changes. Neuwald et al. (2010) compared on- and off-tree changes in the SPI in different cultivars and showed that starch loss was typically slower at 1 °C and faster at 20 °C than on the tree. Treatment of fruit with 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, resulted in little effect on SPIs at 20 °C, but marked inhibition of fruit softening. 1-MCP treatment was found to reduce starch loss based on SPIs (Fan et al.,

1999; Pre-Aymard et al., 2003). However, Thammawong and Arakawa (2007) found little influence of 1-MCP, and a cultivar dependent influence of exogenous ethylene on starch degradation. Starch concentrations declined faster in 'Tsugaru' fruit treatment with ethylene than in untreated fruit of the same cultivar (Thammawong and Arakawa, 2007; Thammawong and Arakawa, 2010). In contrast, rates of starch decline in 'Fuji' fruit, which generally produce low amounts of ethylene, were not affected by 1-MCP or ethylene treatments (Thammawong and Arakawa, 2007). 'Tsugaru' apples also showed few differences among treatments in total sugar concentrations among zones within the cortex tissue (Thammawong and Arakawa, 2010).

Little research exists about differences in metabolism in stem and calyx end tissues of apples. My interest is in changes of starch concentrations in these tissues during ripening as part of an overall study on starch in apple fruit (Chapter 2). In addition to presumed metabolic differences, physiological differences include higher tissue density in calyx-end than stem-end tissues (James and Jobling, 2009), and collectively may have relevance to development of flesh browning in 'Empire' and 'Gala' apples, which is first visible in the stem end part of the fruit (Lee et al., 2012a,b; Lee et al., 2013).

The objective of this study was to investigate changes in starch concentrations in stem-end, equatorial and calyx end of apple fruit. Two approaches have been taken. In the first, concentrations in different tissue zones of 'Honeycrisp' and 'Empire' fruit during maturation and ripening on the tree were measured. In the second, postharvest changes were measured in 'Gala' fruit kept at 20 °C after treatment with propylene or 1-MCP.

3.2. Material and Methods

3.2.1 Maturation and ripening on the tree

Fruit were obtained from 'Honeycrisp' and 'Empire' trees grown on the Cornell University Orchard at Ithaca, NY. Five fruit of each cultivar were harvested weekly between August 16 and September 13 2011, and thereafter twice a week until September 23 and October 18 respectively. The IEC of each fruit was measured on each sampling date except the first (August 16), by injecting 1 mL of gas sample taken from the core cavity using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) (Watkins et al., 2000).

Tissue samples were then taken for analysis of sugars and starch. Each fruit was cut latitudinally parallel to the core to leave an approximately 1 cm wide section around the core, stem and calyx. Tissue samples were taken from the shoulder tissue (stem), from tissue in the equatorial cortex tissue as centered between core and skin, as well as on the calyx end of the fruit, using a core borer (diameter 1.9 cm). Tissues from both sides of the fruit were combined to provide three samples per fruit: stem, equatorial or calyx. The samples were frozen in liquid nitrogen and stored at -20 °C until they were lyophilized. Dried samples were ground in a Wiley mill through a 20-mesh screen (Miller and Langhans, 1989).

3.2.2 Postharvest manipulation of ripening

'Gala' fruit were harvested from potted trees at the Cornell University Orchard in Ithaca, NY, on August 29, 2012. In the lab they were split equally into three lots of ~60 fruit, and either untreated of treated with 1-MCP or propylene on the day of harvest.

One set of fruit were put into a 4,000 L plastic tent and treated with 1 μL L^{-1} 1-MCP (SmartFresh, AgroFresh Inc., Springhouse, PA) for 24 h using a release and fan system supplied by the manufacturer. A second set, also directly after harvest, was treated for 24 h with 200 μL L^{-1} propylene in a 280 L gas tight plexiglass chamber. Both treatments and the untreated control fruit were kept at 20 °C. After removal from the chambers the fruit were allowed to vent before sampling the first time on August 30 (1 day after harvest (DAH)) and then at 2, 3, 5, 7, 9, and 13 DAH. Five randomly selected fruit were used at each sampling date. The IEC was measured as described above. I_{AD} was measured using a handheld non-destructive instrument (DA meter, Sinteleia, Bologna, Italy) (Costamagna et al., 2013). Readings were taken on opposite sides of the fruit on the green and blushed side and the average noted (Nyasordzi et al., 2013). Flesh samples were taken by cutting two wedges of the fruit, approximately $1/8^{th}$ each. The wedge was pealed and placed on the edge of the cutting board where a 1 cm wide area was marked. In the area between the marks the core was placed to ensure even cutting of the sections. The sections were frozen in liquid nitrogen and stored at -20 °C until they were lyophilized and ground as described above.

3.2.3 Carbohydrate and starch determination

For determination of sugars and starch, 50 mg of ground samples were washed with 80% ethanol and incubated for 30 min at 70 °C three times (Miller and Langhans, 1989; Ranwala and Miller, 2008). The aqueous phase containing all the soluble carbohydrates was run through columns of 1 mL Dowex 50-W, 100-200 mesh layered on 1 mL Amberlite IRA-45, 16-50 mesh (Miller and Langhans, 1989; Ranwala and Miller,

2008). For the maturation experiment (2.1), the aqueous phases were vacuum evaporated (Rapidvap Vacuum Evaporation System, Labconco, Kansas City, MO) (Hou et al., 2011). The dry sugar samples were kept at -20 °C until preparation for ionic chromatography (IC). Samples were diluted in 10 mL with high-performance liquid chromatography (HPLC)-grade water. In the postharvest experiment, aqueous phases were evaporated using an Evapo-Mix (Buchler Instruments, Fort Lee, NJ). The dried samples were stored in the freezer, and diluted with 20 mL HPLC-grade water for analysis.

In both experiments, the samples were further diluted by adding 100 µL sample with 400 µL HPLC-grade water in the sample vials. Samples were analyzed on Dionex (Sunnyvale, CA) high-performance anion exchange chromatography with pulsed amperometric detection (ED50; Dionex) equipped with a Carbopac PA-1 column (CarboPac PA1 Analytical, 4 × 250 mm; Dionex). Carbohydrates were eluted with 100 mM NaOH at a flow rate of 1.0 mL min^{-1} for 20 min (Hou et al., 2011). Sorbitol, glucose, fructose and sucrose were quantified by comparison with known standards.

The non-soluble carbohydrates obtained after extraction with ethanol were dried overnight. Enzymatic starch determination was performed as described previously in Chapter 2.

3.2.4 Statistics

All statistical calculations were done in JMP® Pro 11.0.0 (SAS Institute) using simple least square models with inclusion of interactions. For regressions and ANOVA individual samples were used as replicates. ANOVA was performed on log transformed IEC data.

3.3. Results

3.3.1 Maturation and ripening on the tree

Generally low levels of IECs were measured in 'Honeycrisp', except for a spike in ethylene on September 6 (Fig. 3.1). 'Empire' IECs remained low throughout the experiment, and never exceeded 2.8 μL L^{-1} with the exception of one fruit on September 27 (120 μL L^{-1}). This fruit was probably a 'push off' and was not included in the analysis.

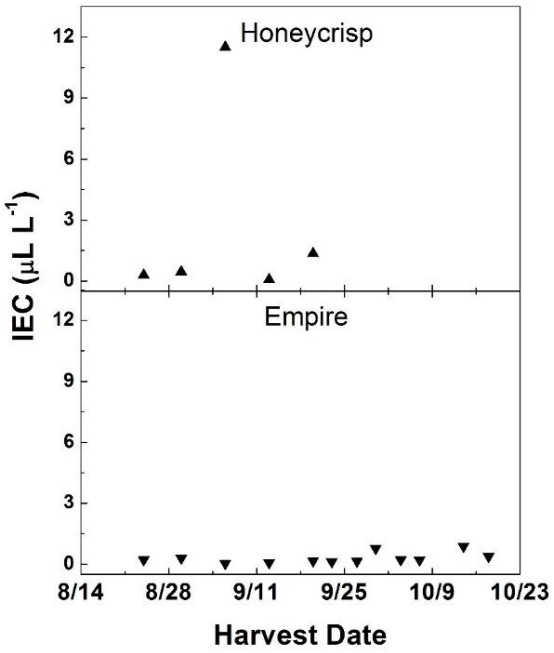

Fig. 3.1. Internal ethylene concentration (IEC) in fruit of 'Honeycrisp' and 'Empire' during harvest. Effect of harvest date for each cultivar P <0.0001.

Starch concentrations in stem end, equatorial, and calyx end zones of 'Honeycrisp' and 'Empire' decreased linearly during maturation and ripening (Fig. 3.2). Differences in starch concentrations between zones were detected only for 'Empire' fruit. In this cultivar, concentrations were higher overall in the calyx end (180 mg g^{-1} dry wt) than in the equatorial and stem end zones (171 and 141 mg g^{-1} dry wt, respectively). The rate

of decrease of starch was similar in all zones for each cultivar, ranging from -6.33 to -6.96 mg g^{-1} day^{-1} for 'Honeycrisp' and -4.24 to-4.29 mg g^{-1} day^{-1} for 'Empire' (Fig. 3.2).

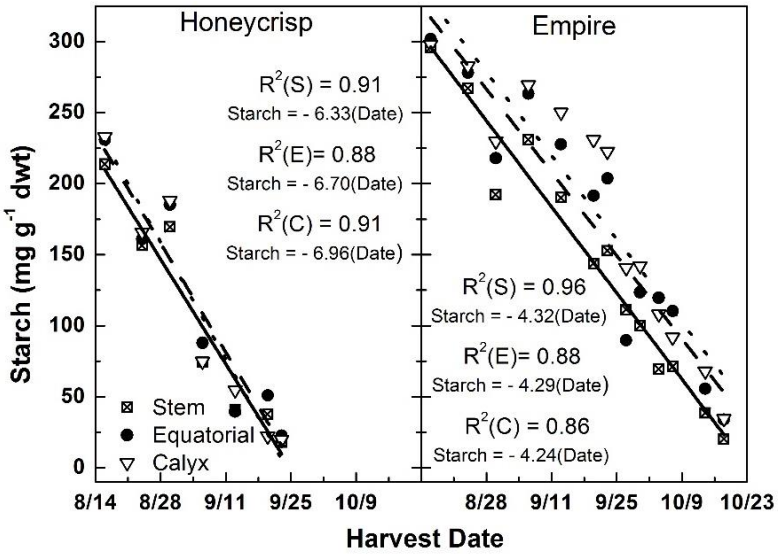

Fig. 3.2. Starch concentration (mg g^{-1} dry wt) of 'Honeycrisp' and 'Empire' of stem (S; solid line), equatorial (E; dashed line), and calyx (C; dotted line) at each harvest date. The R^2 represents the linear fit of the data over harvest date; 'Honeycrisp' effects of harvest date $P<0.0001$, no interaction detected; 'Empire' effect of harvest date $P<0.0001$, and zone $P<0.0001$, no interaction detected.

Although there was variability among harvest dates, overall the concentrations of sorbitol, glucose and sucrose increased in 'Honeycrisp' tissue zones over the harvest period, while those of fructose decreased (Table 3.1). Sorbitol and sucrose concentrations were highest in the calyx tissues whereas glucose and fructose were highest in stem tissues (Table 3.1). Similarly, the concentrations of sorbitol, glucose and sucrose increased in 'Empire' tissue zones over the harvest period (Table 3.2). However, fructose concentrations changed over time with a quadratic relationship being detected. Sorbitol and sucrose concentrations were highest in the calyx tissues whereas glucose was highest in stem tissues, and fructose was not affected by tissue zone (Table 3.2).

Table 3.1. Sorbitol, glucose, fructose and sucrose in stem (S), equatorial (E), and calyx (C) tissues of 'Honeycrisp' at each harvest date.

Harvest date (2011)	Sorbitol			Glucose			Fructose			Sucrose		
	(mg g^{-1} dry wt)											
	S	E	C	S	E	C	S	E	C	S	E	C
8/16	8.2	10.4	10.3	45.3	29.9	17.3	303.7	293.1	281.9	202.7	196.8	228.3
8/24	8.9	10.1	13.7	57.8	51.6	30.7	274.7	286.0	280.0	215.1	225.8	252.2
8/30	11.9	14.7	15.2	52.0	40.8	34.4	282.1	270.5	272.9	241.9	259.3	272.6
9/06	12.7	15.2	18.7	60.4	42.4	30.5	318.7	285.5	274.1	288.9	292.3	291.0
9/13	11.7	15.0	18.7	61.8	39.9	31.3	282.5	273.1	288.2	279.0	319.6	357.7
9/20	14.6	21.0	22.6	48.4	40.6	33.5	266.9	247.4	253.9	324.4	333.2	350.5
9/23	10.2	12.0	13.4	62.6	51.0	43.3	249.2	236.6	239.2	379.8	397.1	407.3
Overall means	11.2	14.1	16.4	55.0	40.0	31.7	284.6	271.8	271.8	269.7	284.2	305.9
Regression	L*	L*	L*	ns	L*	L**	L*	L**	L*	L***	L***	L***
P-values:												
Date	0.0009			0.0120			<0.0001			<0.0001		
Zone	0.0004			<0.0001			0.0198			<0.0001		
date × zone	ns			ns			ns			ns		

Table 3.2. Sorbitol, glucose, fructose and sucrose in stem (S), equatorial (E), and calyx (C) tissues of 'Empire' at each harvest date.

Harvest date (2011)	Sorbitol			Glucose			Fructose			Sucrose		
	(mg g^{-1} dry wt)											
	S	E	C	S	E	C	S	E	C	S	E	C
8/16	10.5	12.3	14.4	44.2	23.2	22.3	266.1	277.7	263.1	137.1	154.5	164.0
8/24	10.6	14.6	17.7	43.6	32.5	31.4	301.0	299.7	286.8	149.2	165.0	169.0
8/30	12.7	14.8	17.7	61.0	43.0	40.4	279.0	283.8	278.1	165.1	170.1	176.9
9/06	9.5	12.6	13.9	59.8	39.2	35.8	280.7	281.6	272.3	164.9	192.3	200.8
9/13	15.5	16.7	17.7	53.5	38.6	37.4	253.4	287.1	278.9	154.6	190.6	196.9
9/20	18.6	24.6	25.6	75.6	56.7	46.6	256.1	282.4	260.2	209.5	236.7	240.3
9/23	11.2	16.4	16.1	54.9	35.5	30.5	275.6	281.7	274.3	220.6	239.4	251.6
9/27	13.6	17.4	20.7	63.1	46.1	43.1	279.2	267.9	279.6	232.2	236.3	261.3
9/30	12.3	14.7	15.0	55.0	42.3	36.9	277.9	256.0	255.3	237.0	254.6	258.1
10/04	13.6	19.4	20.2	78.6	55.7	48.6	322.6	320.3	310.4	216.8	255.8	278.8
10/07	16.5	25.4	24.6	69.6	57.2	49.8	309.5	302.0	285.2	254.4	287.0	288.8
10/14	15.5	24.9	23.6	64.8	51.1	46.6	257.0	274.6	251.4	252.8	293.2	289.8
10/18	17.1	18.2	18.2	56.4	52.7	46.1	248.7	245.0	229.7	287.0	284.0	282.8
Overall means	13.7	17.8	18.8	60.2	43.9	39.5	279.5	281.5	271.4	208.3	227.6	236.2
regression	L***	L***	L**	L**	L***	L***	Q*	Q*	Q*	L***	L***	L***
P-values:												
Date	<0.0001			<0.0001			0.0390			<0.0001		
Zone	<0.0001			<0.0001			ns			<0.0001		
date × zone	ns			ns			ns			ns		

3.3.2 Postharvest manipulation of ripening

Propylene and 1-MCP were applied to 'Gala' apples with the objective of increasing and decreasing the rate of ripening, respectively. The IECs of fruit from all treatments remained low for the first 5 DAH (Fig. 3.3). The IECs of untreated and propylene-treated fruit then increased, but no differences between them were detected. In contrast, the IECs of 1-MCP treated fruit remained low throughout the experiment.

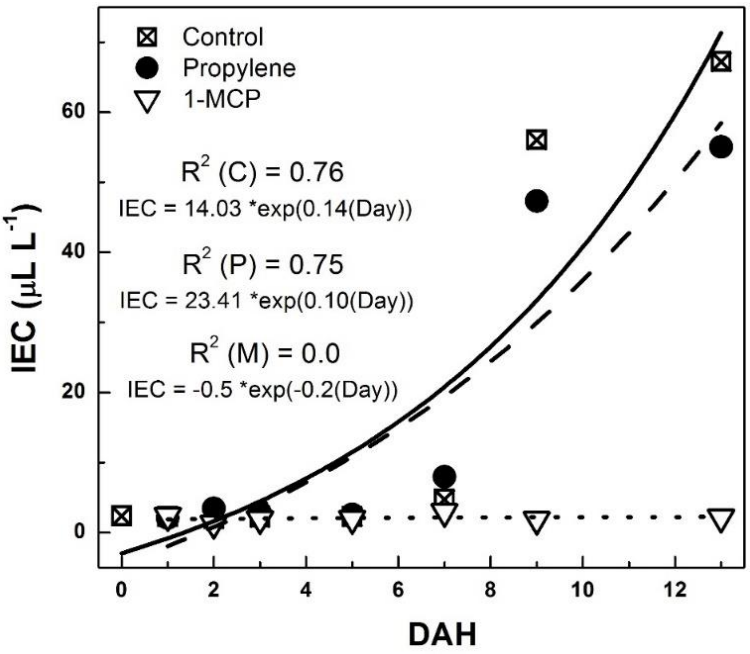

Fig. 3.3. IEC (µL L^{-1}) of 'Gala' in untreated (Control (C) straight line), propylene treated (P; dashed line) or 1-MCP treated (M; dotted line) fruit from at harvest (0) to 13 days after harvest (DAH). The R^2 describes the exponential fit; *P*-values: treatment = 0.0001, DAH = 0.0001, and treatment × DAH = 0.0001.

The decline in I_{AD} values was slower in 1-MCP treated fruit, indicating slower loss of chlorophyll levels, compared with untreated fruit and those treated with propylene (Fig. 3.4).

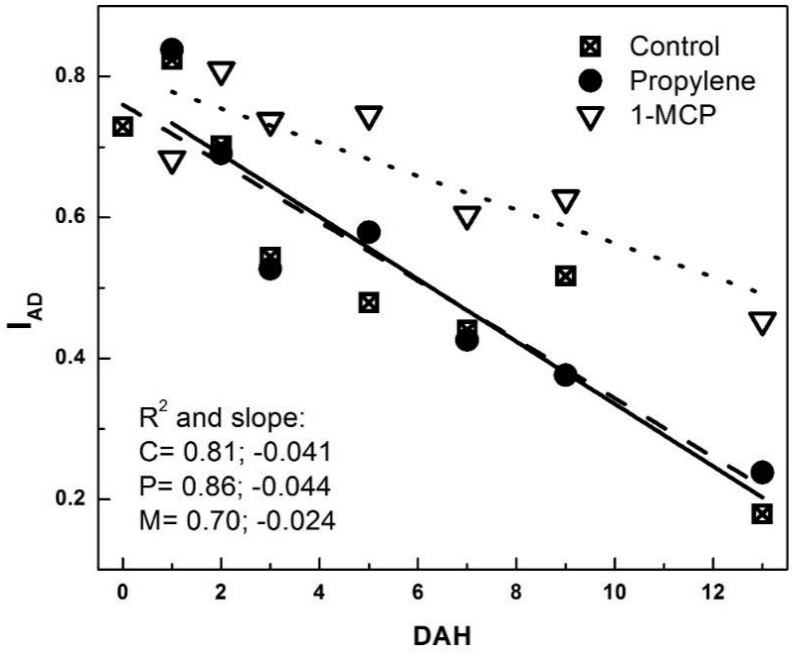

Fig. 3.4. I_{AD} of 'Gala' in untreated (Control (C) straight line), propylene treated (P; dashed line) or 1-MCP treated (M; dotted line) fruit from at harvest (0) to 13 days after harvest (DAH). The R^2 describes the exponential fit; *P*-values: treatment = 0.0097, DAH <0.0001, no interaction detected.

Regardless of treatment, starch concentrations were lowest in the stem-end tissues and highest in the calyx-end tissues, and these decreased linearly over time (Fig. 3.5). 1-MCP treated fruit generally had the highest concentration in each zone, while there was little difference between control and propylene treated fruit. Treatment with propylene lead to lower starch concentrations combined for all zones and sampling dates with 53 mg g^{-1} dry wt compared with 57 and 69 mg g^{-1} dry wt for control and 1-MCP treated fruit, respectively. But a statistical difference between the propylene treated fruit and the untreated could not be detected. Starch concentrations in the calyx end of the fruit over all treatments were higher by 34% compared with the equatorial zone, and 74% compared with the stem end tissues.

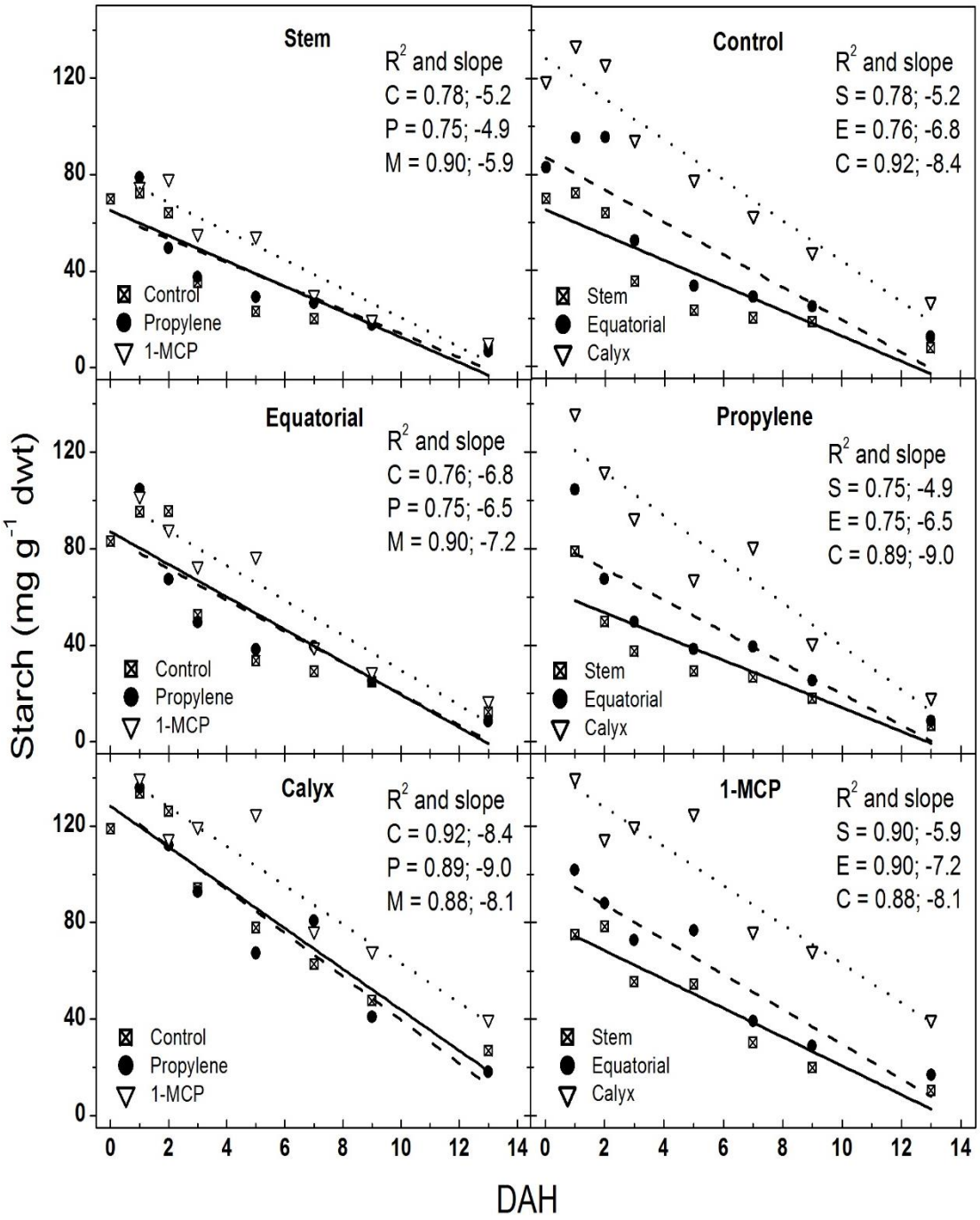

Fig. 3.5. Starch concentration (mg g^{-1} dry wt) in stem, equatorial and calyx tissues of 'Gala' in untreated (Control (C) straight line), propylene treated (P; dashed line) or 1-MCP treated (M; dotted line) fruit from at harvest (0) to 13 days after harvest (DAH) (left side) and (ride side) starch concentration in fruit zones of either untreated (Control), propylene or 1-MPC treated fruit; stem-end (S), equatorial (E) and calyx-end (C). The R^2 describes the linear fit of the data; *P*-values: treatment <0.0001, zone <0.0001, DAH <0.0001, zone × DAH = 0.0003, no other interactions detected.

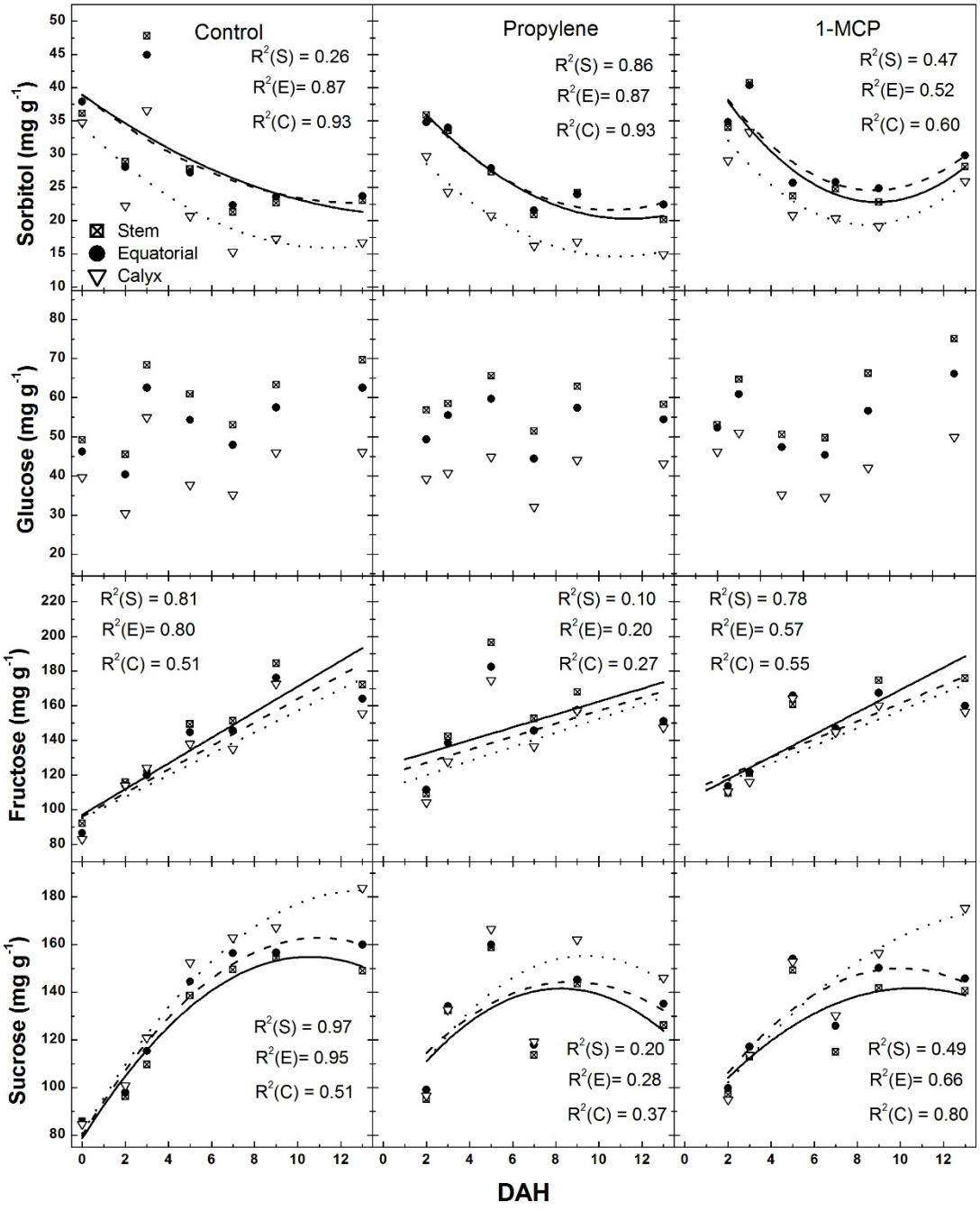

Fig. 3.6. Sorbitol, glucose, fructose and sucrose in stem (S; straight line), equatorial (E; dashed line) and calyx-end (C; dotted line) tissues of 'Gala' in untreated (Control), propylene or 1-MCP treated fruit from at harvest (0) to 13 days after harvest (DAH). The R^2 describes the exponential or linear fit of the data.

Table 3.3. ANOVA for sorbitol, glucose, fructose and sucrose tissue zones (stem, equatorial and calyx) of 'Gala' with treatment (untreated (control), propylene or 1-MCP), zone, and days after harvest (DAH).

	Sorbitol	Glucose	Fructose	Sucrose
Treatment	0.0005	0.0310	0.0491	ns
Zone	<0.0001	<0.0001	0.0107	0.0018
DAH	<0.0001	<0.0001	<0.0001	<0.0001
Treatment × zone	ns	ns	ns	ns
Treatment × DAH	<0.0001	ns	<0.0001	<0.0001
Zone × DAH	ns	ns	ns	0.0009
Treatment × zone × DAH	ns	ns	ns	ns

Concentrations of sorbitol generally decreased, while those of sucrose generally increased over time, although the patterns were curvilinear (Fig. 3.6; Table 3.3). Sorbitol in untreated fruit decreased between 1 DAH to 13 DAH in the stem end region by 36%, equatorial and calyx zone decreased by 67 and 50% respectively. Propylene treated fruit had a decrease in sorbitol by 43, 37, and 50% for stem, equatorial, and calyx end zone. Sorbitol in 1-MCP treated fruit decreased until 9 DAH but increased on the last sampling point (13 DAH). In contrast to the general trend of a continuous concentration gradient, the equatorial zone sorbitol was slightly higher than stem end concentrations on most sampling days for all treatments. Glucose increased in untreated control and 1-MCP treated fruit, but remained relatively level in propylene treated fruit. The glucose concentrations of control and 1-MCP treated fruit on day 13 were higher (average of zones being 60 and 64 mg g^{-1} dry wt, respectively) compared with that in propylene treated fruit (52 mg g^{-1} dry wt). Fructose concentrations increased linearly over time. Zonal effects on fructose concentrations were most pronounced in 1-MCP treated fruit at day 13 with equatorial and calyx at 160 and 157 mg g^{-1} dry wt compared with the stem end with 176 mg g^{-1} dry wt. Control and propylene treated fruit had little

differences in fructose concentration by day 13 but overall had higher concentrations in the stem end zone compared to equatorial and lowest levels in calyx, averaging 135, 135 and 130 mg g^{-1} dry wt in stem, equatorial and calyx zone of control respectively, and 146, 141 and 135 mg g^{-1} dry wt in the zones of propylene treated fruit. Sucrose concentrations varied between zones with the highest concentration in the calyx. Sucrose concentrations reached a maximum curve at different times depending on treatment. Zones of propylene treated fruit all reached the maximum 9 DAH. Equatorial and calyx zone of control and 1-MCP treated fruit also reached highest sucrose concentrations on 9 DAH but the calyx end concentration increased until 13 DAH. The effect of tissue zone was significant for all sugars and an interaction between tissue zone and DAH was detected for sorbitol, fructose and sucrose (Table 3.3).

3.4. Discussion

Starch concentrations in all tissue zones declined in a linear fashion over time, regardless of cultivar, and whether fruit were on or off the tree. These patterns of change are therefore consistent with those found for starch concentrations in whole fruit (Chapter 2). Rates of decrease are similar between the same zones in the different treatments, but are different with in fruit of the same treatment, never the less this might not support differences in rates of ripening per se, but rather may reflect differences in starch accumulation in different tissues zones during fruit development. Although starch is known to accumulate during fruit development to a maximum before net starch loss occurs (Berüter and Studer Feusi, 1997; Brookfield et al., 1997), little is known about rates of deposition in the different tissues. Starch concentration in the calyx end tissue

in all treatments of ‘Gala’ was higher at 1 DAH compared with those in the equatorial and stem zones. ‘Empire’ fruit also had a higher starch concentration in the calyx than in stem end region. Higher amounts of starch in the outer cortex compared to the core and the vascular bundle region were previously found by Brookfield et al. (1997).

A faster decline in starch with higher levels of ethylene has been shown previously (Johnston et al., 2009). Thammawong and Arakawa (2007) found that ethylene levels influenced starch hydrolysis only in ‘Tsugaru’ not in ‘Fuji’ fruit, relative independence of SPI changes from ethylene was also observation by Blankenship and Unrath (1988) for ‘Golden Delicious’. Starch concentration in ‘Gala’ treated with propylene were slightly lower in all three tissue zones compared to the control, but an effect of propylene treatment compared with untreated fruit on starch concentration could not be detected (Table 3.3). Propylene treatment did not elevate IEC, and a higher dose of, or prolonged exposure to, propylene might have been needed to increase IEC sufficiently and affect starch hydrolysis. Little effect of 1-MCP on starch degradation was detected in ‘Jonagold’ and ‘Golden Delicious’ apples suggesting that starch hydrolysis is relatively ethylene independent (Neuwald et al., 2010). Initiation of starch hydrolysis is very ethylene sensitive however, and therefore even a very small increase in IECs can trigger the onset of starch hydrolysis (Johnston et al., 2009). In ‘Gala’, low IECs in the 1-MCP treated fruit appear sufficient to trigger progression of starch degradation and hydrolysis may have been already initiated on the tree. The effects of 1-MCP on decreasing starch hydrolysis in ‘Gala’ were stronger than those observed by the German group in ‘Jonagold’ and ‘Golden Delicious’ (Neuwald et al., 2010), but those observations were based on SPI readings and not chemical analysis of starch.

Starch hydrolysis has been shown to initiate and proceed simultaneous throughout the fruit (Brookfield et al., 1997) and higher initial values of starch in the calyx end of the fruit (Fig. 3.2 and 3.5) supports the assumption that starch is not evenly distributed within the fruit during development (Ohmiya and Kakiuchi, 1990). Such distributional differences within the fruit earlier in development with changes in granule size or granule structure have been documented (Pérez et al., 2009; Smith, 2001). Granules are degraded from the inside out (Ohmiya and Kakiuchi, 1990) leading to a size unspecific rate of degradation. The actual structure and composition of starch was not analyzed in my study, but previously changes in starch composition of all three cultivars was performed for the whole fruit (Chapter 2). The percentage amylose (linear starch) in total starch decreases as the fruit mature. This also supports the assumption that differences within the starch granule cause differences in starch degradation (Ohmiya and Kakiuchi, 1990). Therefore, the differences in granule size and/or number of starch granule in different fruit tissue zones, could lead to differences of starch distribution within the fruit, even if starch degradation is initiated simultaneously in the entire fruit. Hence, the measured differences could be artifacts from earlier events during fruit development rather than differentiated starch degradation.

Low glucose and sorbitol, and increasingly higher concentrations, of sucrose and fructose during fruit ripening and storage have been described (Ackermann et al., 1992). Changes in sugar concentrations in the whole fruit (on a fresh weight basis) of ‘Honeycrisp’ during development (Zhang et al., 2010). Similar trends of sugar accumulation were measured in the current study; all three cultivars had low concentrations of sorbitol and glucose, and much higher concentrations of fructose and

sucrose, which increased as the fruit ripened. Sorbitol is the preferred transport sugar in *Rosaceae* fruits trees (Bieleski and Redgwell, 1985), but generally represents a relatively small proportion of the total carbohydrates in the fruit (Thammawong and Arakawa, 2007; Yamaki and Ishikawa, 1986). Sorbitol is not stored as such but is converted into other sugars, mostly fructose, by the enzyme NAD-dependent sorbitol dehydrogenase (Cheng et al., 2005; Kanayama et al., 2008; Teo et al., 2006). Therefore, the already low levels of sorbitol decrease after harvest, since the influx of sorbitol no longer exists. Fluctuations in glucose levels could be explained, since glucose is used for respiration as well as its transformation into fructose and consequently sucrose (sucrose-6-phosphate-syhthase). This could explain the small increase in glucose compared with the hydrolysis of starch and the much greater increase in fructose and sucrose. Glucose is not as sweet in taste as fructose and sucrose (Moskowitz, 1970) and therefore, does not contribute to flavor characteristics of the fruit. The sucrose concentration gradient between tissue zones were similar to starch concentration gradient with higher concentrations in the calyx region. The other sugars show an opposite trend in 'Gala' with higher levels in the stem region. Differences in sugar concentration between parts of the apple fruit have been documented previously (Harding, 1936; Smock, 1950), with higher total sugar concentrations in the stem end vs. the calyx end of the fruit (Archbold and Barter, 1934), similar to those sugars concentrations found in 'Honeycrisp', 'Empire' and 'Gala'. But it has also been pointed out that variations between fruit are much greater than variance of total sugars within the fruit (Harding, 1936; Smock, 1950).

'Gala' fruit treated with 1-MCP retained higher values of I_{AD} which indicates retardation of chlorophyll degradation by the treatment. Hue angle of skin has been found to have low sensitivity, and therefore stronger dependence on ethylene (Johnston et al., 2009). Propylene treated fruit decreased in I_{AD} at a similar rate as untreated fruit, but the values of IEC were also very similar. Delay of chlorophyll degradation by 1-MCP during storage have been found previously (Toivonen and Hampson, 2014; Zanella, 2003).

3.5. Conclusion

A zonal gradient of starch and sugar concentration between the stem and calyx end of the fruit was shown in 'Empire' and 'Gala' fruit. The results for 'Honeycrisp' were not as conclusive. Whether these differences are purely due to a distributional difference in starch accumulation during development and therefore an artifact of starch distribution during fruit growth or whether this proves a differentiation in maturation cannot be answered based on these data alone. More research needs to be done in order to understand the developmental differences between the stem and calyx region of the fruit. Changes in starch and soluble solutes sugars cannot verify true maturity differences but could as well be artifacts from earlier development of larger starch granule in the lower part of the fruit. Also IECs of 'Gala' fruit treated with propylene or 1-MCP alone could not explain the differences in starch decline between the zones and/or treatments, there still could be a linkage between the two factors. As shown previously very small amounts of ethylene trigger the onset of starch hydrolysis (Johnston et al., 2009), and increasing the amount of ethylene even hastens the

progression. Such effects of ethylene could not be detected in this study but the propylene treatment, without effecting overall IECs, did lead to a lower starch concentration after 13 days at 20 °C. The differences between zones might be due to accumulation differences earlier in development rather than differences in hydrolysis of starch, and the differences between treatments propylene and untreated fruit on the one hand and 1-MCP treatment on the other, could be due to miniscule changes in ethylene concentrations within the fruit.

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CHAPTER 4

PREHARVEST AMINOETHOXYVINYLGLYCINE (AVG) AND 1-METHYLCYCLOPROPENE (1-MCP) EFFECTS ON ETHYLENE AND STARCH CONCENTRATION OF 'EMPIRE' AND 'MCINTOSH' FRUIT

Abstract

Aminoethoxyvinylglycine (AVG; ReTain®) and 1-methylcyclopropene (1-MCP; Harvista™) are used to delay fruit maturation and ripening, and thereby reduce fruit drop and manage harvest. In this study, 'McIntosh' and 'Empire' fruit were treated with AVG four or one week, or 1-MCP one week, prior to the anticipated first harvest date, to assess effects of these chemicals on maturation and ripening in relation to starch degradation. In a second season, 'Empire' fruit were treated with either AVG or 1-MCP four and one week prior to anticipated first harvest. Fruit from this trial were also harvested to investigate the effects of treatment on ripening in air storage. Cultivar and timing of application influenced the efficacy of both AVG and 1-MCP in delaying the increase of internal ethylene concentration (IEC) and the starch pattern index (SPI), and decrease of starch concentrations in the fruit. Little effect of treatment was found for the high ethylene producing 'McIntosh', only the SPI being affected on the date of first harvest. 'Empire' fruit from trees treated with 1-MCP or AVG had lower IEC and were greener (higher I_{AD} values), and had lower SPIs and higher starch concentrations, but the effects were inconsistent and limited to only some harvest dates. In storage, only 1-MCP applied 10 d before harvest markedly slowed the increase in IEC and the rate of softening. AVG treatment effects on IEC were intermediate between the one week 1-

MCP treatment and the untreated controls and 4 week 1-MCP treatment, but did not affect softening. No treatments affected the rate of starch concentration loss during storage.

4.1. Introduction

Aminoethoxyvinylglycine (AVG) and 1-methylcyclopropene (1-MCP) inhibit ethylene production by inhibiting 1-aminocyclopropane-1-carboxylase (ACC) synthase activity (Adams and Yang, 1979; Boller et al., 1979) and ethylene perception by binding to ethylene receptors (Sisler et al. 1996a,b), respectively. These products are used extensively as plant growth regulators (PGRs) in North America and elsewhere under the commercial names of ReTain® and Harvista™, respectively, to prevent premature fruit drop, delay harvest to manage harvest and increase fruit size, and to improve responses of fruit to postharvest 1-MCP (Elfving et al., 2007; Watkins et al., 2010; Yuan and Carbaugh, 2007). In northeast U.S. it is now common to find blocks within orchards untreated or sprayed with either PGR.

Time of harvest is the most important factor determining fruit quality after storage. Prediction of the harvest window is very important, especially for long term storage, and the starch pattern index (SPI) is commonly used for this purpose (Blanpied and Silsby, 1992). Therefore, if the SPI is used to assess the effects of PGRs on fruit maturity and ripening, it is important to understand how changes in the production and perception of the ripening hormone ethylene influences starch hydrolysis. Whether PGR applications delay maturation overall or affects only some physiological changes is not fully understood. Differences in sensitivity and dependency of such factors to

ethylene have been shown in fruit with antisense suppression of 1-amino-cycolopropane-carboxylase oxidase (MdACO1) (Johnston et al., 2009). Sensitivity to ethylene is negatively correlated to dependence on ethylene, therefore, the less sensitive a maturity aspect, the more dependent it is on ethylene (Johnston et al., 2009), e.g. low levels of ethylene (0.01 μL L^{-1}) initiated the degradation of starch and increasing ethylene to 0.1 μL L^{-1} hastened starch degradation, but other factors, such as skin background color, were not affected by low levels of ethylene and only increased at climacteric ethylene levels (1 μL L^{-1}).

Neuwald et al. (2010) found little effect of preharvest 1-MCP on postharvest SPI changes in a comparison of on-tree maturation and postharvest ripening. A delay of starch degradation by preharvest1-MCP was found in 'Golden Delicious' (McArtney et al., 2008) and 'Bisbee Delicious' (Yuan and Li, 2008) but to varying degrees. The effectiveness of slowing starch degradation was dependent on concentration of preharvest 1-MCP in 'Golden Delicious', although no effect was detected for 'Law Rome' (McArtney et al., 2008). In a follow up trial, preharvest 1-MCP did not affect SPI or IEC over all of 'Golden Delicious' or 'Law Rome', but an effect of 1-MCP on SPI was found at the earlier application (September 26) three days after application and 'Delicious' fruit firmness was improved through 1-MCP three and six days after application with the later application (September 9) (McArtney et al., 2009). Watkins et al. (2010) found a reduction of IEC by preharvest 1-MCP in both 'McIntosh' and 'Delicious', but effects on SPI in treated fruit of both cultivars were only found at the second delayed harvest (14 d after the anticipated first harvest date). Preharvest 1-MCP effects on IEC after storage were concentration as well as application timing dependent

(Elfving et al., 2007; Watkins et al., 2010). Effects of preharvest 1-MCP on delta absorbance (I_{AD}), a measure of chlorophyll a concentration (Ziosi et al., 2008), have not yet been reported, but postharvest 1-MCP delayed chlorophyll loss in 'Arora Golden Gala' and 'Fuji' fruit during storage (Toivonen and Hampson, 2014).

AVG delayed some aspects of maturation in 'Gala' and 'Fuji' fruit (do Amarante et al., 2002). Slower increases of the SPI occurred in 'Gala', with a calculated delay of 34 days, than in 'Fuji', which was delayed by 10 days (do Amarante et al., 2002). A delay in starch degradation with AVG application was also shown in 'McIntosh' (Schupp and Greene, 2004). Stover et al. (2003) found the effectiveness of AVG on internal quality parameters of 'McIntosh' to be dependent on region; effects on starch hydrolysis were found for fruit grown in Champlain, but not for Mid-Hudson growing regions of NY. AVG delayed the onset of ethylene climacteric (Schupp and Greene, 2004; Yuan and Carbaugh, 2007; Yuan and Li, 2008). Effectiveness of AVG on IEC was linearly correlated to the applied concentration (Schupp and Greene, 2004). Carry over effects of AVG in reducing IECs after storage was dependent on cultivar (Elfving et al., 2007).

Timing and concentration of both AVG and 1-MCP did not affect efficacy in reducing ethylene production in 'Bisbee Delicious', but were important for the persistence of the repression (Yuan and Li, 2008). Effects of both PGRs inconsistently reduced ethylene production and/or starch degradation.

The relationships between IECs, SPIs and starch concentrations are still unclear. The objective of this study therefore was to investigate the effects of preharvest AVG and 1-MCP sprays, including application time, on maturity and starch concentration losses in fruit before harvest and during air storage.

4.2. Material and methods

4.2.1 Preharvest plant growth regulator applications

In 2012 trees of 'McIntosh' and 'Empire' cultivars in the Lansing Orchard in NY were sprayed with AVG (Valent BioSciences Corporation, Libertyville, IL) or 1-MCP (AfxRD038, AgroFresh Inc., Springhouse, PA). For 'McIntosh', AVG was applied on August 9th (4w AVG) and 23rd (2w AVG), 1-MCP on August 30th (1-MCP). For 'Empire' AVG was applied on August 21st (4w AVG) and September 4th (2w AVG), and 1-MCP on September 11th (1-MCP). AVG was applied at 823 g ha^{-1} and 1-MCP at a concentration of 125 µL L^{-1}. Applications were made using a CO2 pressure backpack sprayer (Bellspray, Opelousas, LA) calibrated to deliver the spray at 276 kPa and fitted with a TeeJet 8004VS flat fan nozzle (Spraying Systems, Wheaton, IL). 1-MCP was mixed as described by McArtney et al. (2008), but without the addition of oil. Three replicate sets of 12 trees each arranged in a random block design were assigned for each treatment with buffer trees. Four fruit from each replicate were harvested weekly starting for untreated control on the day of the first spray application – 'McIntosh' 8/9 and 'Empire' 8/21. The experiment ended one week (9/13) and two weeks (10/12) after anticipated commercial harvest for 'McIntosh' and 'Empire', respectively.

In 2013, 'Empire' trees in the Cornell Orchard in Ithaca NY were sprayed with AVG or 1-MCP on August 26th and September 16th, 31 and 9 days before harvest, for consistence the treatments remain the names 4w and 1w before harvest, with three single tree replicates randomly assigned to a treatment with buffer trees on either side. The trees were sampled starting 8/26, day of spray application, for untreated control trees only, following weekly sampling until October 14th. Four fruit were harvested from each tree.

The same replicate trees were used to obtain fruit for storage experiments.

4.2.2 At harvest maturity assessments

The IEC, I_{AD}, SPI and total starch concentration was measured on each fruit. IEC was measured using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) by injecting 1 mL of gas sample taken from the core cavity as described by Watkins et al. (2000). The I_{AD} was measured using a handheld Delta Absorbance (DA) meter (Sinteleia, Bologna, Italy) (Costamagna et al., 2013). The SPI was determined using the Cornell generic chart where 1 = 100% staining and 8 = 0% starch (Blanpied and Silsby, 1992). The flesh was sampled by taking two wedges from each fruit of approximately 1/8th each, the skin removed, and frozen in liquid nitrogen. The frozen samples were kept at -20 °C until freeze dried. The lyophilized samples were ground with a Willey mill mesh size 40 to fine powder for carbohydrate and starch extraction (Miller and Langhans, 1989; Ranwala and Miller, 2008).

4.2.3 At harvest and air storage

Fruit for air storage was harvested from untreated trees and trees treated with AVG or 1-MCP on August 26th and September 16th, on September 25th, nine days after the last application date. Approximately 50 fruit were harvested from each replicate tree. Fruit were stored at 0.5 °C in air storage for 56, 112, and 168 days. On the day of harvest, and for each removal, ten fruit were taken from each replicate and assessed after one day at 20 °C. Flesh firmness, IEC, and SPI (at harvest only) were assessed at harvest and after storage. Firmness was measured with an 11.1 mm diameter probe (Guss Manufacturing (Pty) Ltd., Strand, South Africa) on opposite peeled sides of the

fruit, on the blushed and un-blushed side. Flesh samples were taken as 1/8th of each fruit and combining the ten fruit samples of each replicate. Each sample was peeled and frozen in liquid N_2, stored at -20 °C, freeze dried and ground as described above.

4.2.4 Starch determination

For starch concentration analysis 50 mg of dried powder for each sample was washed three times with 80% ethanol to extract soluble sugars. The aqueous phase with soluble carbohydrates was discarded. Starch was determined enzymatically after drying the non-soluble pellet overnight in the drying oven (60 °C) as described previously (Chapter 2).

4.2.5 Statistics

All statistical analyses were done with JMP® Pro 11. (SAS Institute Inc., Cary, NC, USA) simple least square model ANOVA, Pearson product-moment correlation, and regressions. Single fruit were treated as replicates in all harvest maturity analyses. For the storage experiment, data were based on three replicates of bulked 10 fruit samples. Analyses of IEC data were performed on log-transformed data. Regressions for graphs were calculated in Origin 8 SRO (v8.0724 (B724), OriginLab Co., Northampton, MA, USA). For all statistical analysis in JMP harvest date was used as month/day/year (continuous variable) or days after harvest (DAH) for storage.

4.3. Results

4.3.1 Effects of AVG and 1-MCP at harvest

'McIntosh'

The anticipated first harvest date for 'McIntosh' fruit in this block was September 6, 2012. IECs were consistently preclimacteric (<1 μL L^{-1}) on August 23rd and then increased in fruit of all treatments (Table 4.1). Variability of IEC in fruit in each treatment, e.g. a range from 1 μL L^{-1} to over 880 μL L^{-1} in control fruit on August 30th, and fruit with high IECs were found in all treatments; 19, 17, 14, and 14% of fruit were over 1 μL L^{-1} in control, 4 week AVG, 2 week AVG and 1 week 1-MCP, respectively. Consequently, no differences among treatments were detected. Similarly, no differences among treatments were detected for loss of chlorophyll (I_{AD}). Effects of treatment were detected on for fruit harvested on September 6, with highest SPIs and lowest starch concentrations tending to be in the untreated fruit compared with the AVG treatment.

The correlation between SPI and starch concentrations was -0.75, similar in magnitude for that for SPI and starch over harvest date (Table 4.2).

Values of I_{AD} decreased linearly for the untreated control, and fruit treated with AVG at four and two weeks before harvest. Differences between decreases in chlorophyll a among the treatments could not be detected.

Table 4.1. Internal ethylene concentration (IEC), difference in absorbance (I_{AD}), starch pattern index (SPI) and starch concentration of 'McIntosh' either untreated (Control), or treated with AVG on August 9 (4w AVG) or August 23 (2w AVG) or 1-MCP on August 30 2012 (1 w 1-MCP), n = 12. Effects of harvest date, P <0.0001 for Log_{10}IEC, I_{AD}, SPI and 0.0081 for starch concentration. Differences within a harvest date for any factor ($P \leq 0.05$) are indicated by different letters. IECs are shown as back-transformed means.

			IEC (µL L^{-1})				
Treatment	8/9	8/16	8/23	8/30	9/6	9/13	Regression
Control	0.06	0.12	0.08	73.8	25.8	16.0	L***
4w AVG	-	0.09	0.3	7.1	9.3	60.4	L***
2w AVG	-	-	-	10.7	45.2	17.4	L*
1w 1-MCP	-	-	-	-	11.5	13.9	-
			I_{AD}				
Treatment	8/9	8/16	8/23	8/30	9/6	9/13	Regression
Control	1.82	1.87	1.74	1.55	1.49	1.52	L***
4w AVG	-	1.89	1.74	1.65	1.56	1.40	L***
2w AVG	-	-	-	1.66	1.62	1.49	L***
1w 1-MCP	-	-	-	-	1.43	1.56	-
			SPI (1-8)				
Treatment	8/9	8/16	8/23	8/30	9/6	9/13	Regression
Control	3.2	2.9	3.9	4.3	5.8 a	6.0	L***
4w AVG	-	3.2	3.8	4.4	4.6 b	6.3	L***
2w AVG	-	-	-	4.6	4.5 b	6.1	L**
1w 1-MCP		-	-	-	4.9 ab	5.9	-
			Starch concentration (mg g^{-1} dry wt)				
treatment	8/9	8/16	8/23	8/30	9/6	9/13	Regression
Control	98.1	96.1	75.8	60.1	38.9 b	34.0	L***
4w AVG	-	91.5	84.2	65.2	45.0 ab	37.0	L***
2w AVG	-	-	-	62.6	48.8 ab	38.2	L***
1w 1-MCP	-	-	-	-	51.9 a	42.8	-

Table 4.2. Pearson product-moment correlation coefficients (r) for factors of 'McIntosh' and 'Empire' 2012 and 2013 sprayed with AVG or 1-MCP. Significance probability (P) for all correlations <0.0001.

Pearson (r)	**'McIntosh'**	**'Empire' 2012**	**'Empire' 2013**
Date vs. SPI	0.69	0.67	0.86
Date vs. Log_{10}IEC	0.46	0.59	0.74
Date vs. starch	-0.79	-0.77	-0.86
Date vs. I_{AD}	-0.52	-0.62	-0.82
SPI vs. Log_{10}IEC	0.64	0.67	0.68
SPI vs. starch	-0.75	-0.84	-0.86
SPI vs. I_{AD}	-0.41	-0.50	-0.67
Starch vs. Log_{10}IEC	-0.60	-0.67	-0.68
Starch vs. DA	0.42	0.47	0.67
I_{AD} vs. Log_{10}IEC	-0.31	-0.54	-0.53

'Empire'

The anticipated first harvest date for 'Empire' fruit in this block was September 18, 2012. The IECs of 'Empire' remained below 1 μL L^{-1} until September 25th when concentrations increased in untreated fruit (Table 4.3). IECs of AVG treated fruit did not increase above 1 μL L^{-1} until the last sampling date of October 2nd. I_{AD} values were similar between treatments at each harvest date except September 25th when they were higher (more chlorophyll a) in the AVG treatments than in the control fruit, while that in the 1-MCP treatment was intermediate between the two (Table 4.3).

SPIs increased, and starch concentrations decreased over time in either a linear and quadratic fashion (Table 4.3). Quadratic fits typically indicated a delayed change before a rapid change in response to a PGR, as opposed to linear change over time alone. However, SPIs were affected by treatment only on September 25th, when all PGRs delayed the increase in indices. Significant effects of treatment on starch concentrations were detected on three harvest dates (Table 4.3). The effects on September 11th, probably reflect the earlier (2 weeks) application of AVG, while on September 25th and October 2nd, the starch concentrations were generally higher than that of the controls. SPI and starch concentrations were highly correlated at -0.85 (Table 4.2).

Table 4.3. Internal ethylene concentration (IEC), difference in absorbance (I_{AD}), starch pattern index (SPI) and starch concentration of 'Empire' either untreated (Control), or treated with AVG on August 20 (4w AVG) or September 4 (2w AVG) or 1-MCP on September 11 2012 (1w 1-MCP), n = 12. Effects of harvest date, P <0.0001 for Log_{10}IEC, I_{AD}, SPI and starch concentration. For Log_{10}IEC effect of treatment P =0.0097 and treatment × harvest date 0.0041. Effect of treatment for SPI P <0.0001 and for starch concentration 0.0006. Differences within a harvest date for any factor (P ≤0.05) are indicated by letters. IECs are shown as back-transformed means.

IEC (µL L^{-1})								
Treatment	8/21	8/28	9/4	9/11	9/18	9/25	10/2	Regression
Control	-	0.04	0.65	0.12	1.11	2.70	7.51 a	L***
4w AVG	-	0.04	0.08	0.12	0.10	0.31	1.48 b	L***
2w AVG	-	-	-	0.10	1.25	0.15	1.59 b	ns
1w 1-MCP	-	-	-	-	0.13	0.10	0.50 b	L***
I_{AD}								
Treatment	8/21	8/28	9/4	9/11	9/18	9/25	10/2	Regression
Control	1.68	1.56	1.46	1.37	1.23	1.15 b	1.09	L***
4w AVG	-	1.50	1.41	1.38	1.29	1.35 a	1.14	L***
2w AVG	-	-	-	1.34	1.20	1.35 a	1.02	L**, Q**
1w 1-MCP	-	-	-	-	1.26	1.23 ab	1.10	L***
SPI (1-8)								
Treatment	8/21	8/28	9/4	9/11	9/18	9/25	10/2	Regression
Control	2.0	2.3	3.0	3.2	4.1	5.3 a	5.3	L***
4w AVG	-	2.0	2.4	2.8	3.0	3.6 b	4.5	L**, Q*
2w AVG	-	-	-	2.8	4.2	3.8 b	4.8	L***
1w 1-MCP	-	-	-	-	3.0	3.0 b	4.5	L**
Starch concentration (mg g^{-1} dry wt)								
Treatment	8/21	8/28	9/4	9/11	9/18	9/25	10/2	Regression
Control	131.5	132.5	121.1	118.3 ab	100.2	60.5 b	45.1 b	L***, Q***
4w AVG	-	124.9	122.0	129.3 a	117.1	78.0 ab	64.7 a	L***, Q***
2w AVG	-	-	-	112.6 b	92.4	83.1 a	71.7 a	L***
1w 1-MCP	-	-	-	-	96.3	92.1 a	71.0 a	L**

Table 4.4. Internal ethylene concentration (IEC), difference in absorbance (I_{AD}), starch pattern index (SPI) and starch concentration of 'Empire' either untreated (Control), or treated with AVG on August 26 (4w AVG) or September 16 (1w AVG) or treated with 1-MCP on August 22 (4w 1-MCP) or September 16 (1w 1-MCP), n = 12. Effects of harvest date, P <0.001 for Log_{10}IEC, I_{AD}, SPI and starch concentration. Effects of treatment, P <0.001 for I_{AD}, SPI, starch concentration and 0.0079 for Log_{10}IEC. Effects of the interaction (harvest date × treatment) for I_{AD} 0.0152, SPI 0.0072, and for starch concentration 0.0006. Differences within a harvest date for any factor ($P \leq 0.05$) are indicated by letters. IECs are shown as back-transformed means.

				IEC (μL L^{-1})					
Treatment	8/26	9/2	9/9	9/16	9/23	9/30	10/7	10/14	Regression
Control	0.05	0.03	0.11 a	0.13 a	0.10	0.18	3.76	3.98 a	L***, Q*
4w AVG	-	0.06	0.06 b	0.07 b	0.30	0.17	0.28	0.64 ab	L***
4w 1-MCP	-	0.07	0.06 b	0.06 b	0.20	0.15	0.26	0.58 ab	L***, Q***
1w AVG	-	-	-	-	0.13	0.22	0.26	0.37 b	L***
1w 1-MCP	-	-	-	-	0.15	0.20	0.30	0.49 ab	L***
				I_{AD}					
Treatment	8/26	9/2	9/9	9/16	9/23	9/30	10/7	10/14	Regression
Control	1.72	1.66	1.64	1.53 ab	1.57	1.37 ab	1.12 b	1.09 b	L***, Q***
4w AVG	-	1.70	1.63	1.61 a	1.57	1.49 a	1.30 a	1.29 a	L***, Q**
4w 1-MCP	-	1.69	1.67	1.46 b	1.50	1.36 b	1.23 ab	1.24 ab	L***
1w AVG	-	-	-	-	1.55	1.42 ab	1.26 ab	1.21 ab	L***
1w 1-MCP	-	-	-	-	1.52	1.39 ab	1.24 ab	1.27 a	L***, Q*
				SPI (1-8)					
Treatment	8/26	9/2	9/9	9/16	9/23	9/30	10/7	10/14	Regression
Control	1.9	2.0	2.5	2.3	3.9 ab	5.1 ab	5.3 ab	5.6 ab	L***, Q*, C***
4w AVG	-	1.8	2.5	2.6	4.2 a	5.3 a	5.4 a	6.3 a	L***
4w 1-MCP	-	1.8	2.3	2.0	3.5 ab	4.5 ab	4.7 bc	5.6 ab	L***, Q*, C*
1w AVG	-	-	-	-	3.1 b	4.3 b	4.6 c	5.7 ab	L***
1w 1-MCP	-	-	-	-	3.1 b	4.3 b	4.6 c	5.0 b	L***, Q*
				Starch concentration (mg g^{-1} dry wt)					
Treatment	8/26	9/2	9/9	9/16	9/23	9/30	10/7	10/14	Regression
Control	124.6	133.9 ab	119.5	78.4 b	90.9 ab	66.5 a	58.7	36.0	L***
4w AVG	-	118.6 a	105.7	93.8 b	75.1 ab	49.0 b	42.5	27.2	L***
4w 1-MCP	-	140.6 b	114.4	112.8 a	84.9 b	57.3 ab	49.6	29.0	L***
1w AVG	-	-	-	-	89.0 ab	62.3 ab	54.2	41.1	L***
1w 1-MCP	-	-	-	-	94.4 a	63.8 ab	54.5	38.8	L***, Q*

The anticipated first harvest date for 'Empire' fruit in the block used in year 2 was September 23rd, 2013. The IECs were very low in fruit at the first two harvests (4 and 3 weeks before September 23rd); thereafter increasing significantly in untreated fruit compared with 4 week AVG and 1-MCP treatments on September 9th and 16th, though still preclimacteric (Table 4.4), IECs in the untreated fruit then increased. Those of treated fruit remained low, and a significant difference was only detected between control fruit and those treated with AVG one week before harvest. Inability to detect differences between the remaining treatments was probably due to low number of fruit and close similarity in fruit IECs amongst treatments. I_{AD} values were variable among treatments but tended to be lowest in the untreated fruit compared with those treated with AVG or 1-MCP (Table 4.4).

SPI values increased in linear, quadratic, and cubic fashion. Control and 4w 1-MCP treatment both had a good fit of linear but also quadratic and cubic relationships were detected, indicating a lag phase before the increase and a leveling out of the values at the later part of the season. Control and 4w 1-MCP fruit SPIs remained at levels around 2 and 2.5, respectively, until September 16 after which SPIs increased more rapidly, and then slowed down again after the initial increase to values around five and high fours for control and 4w 1-MCP, respectively. The quadratic increase of 1w 1-MCP fruit also shows a rapid change in SPI in the earlier sampling dates and a slower increase between the later harvests.

Starch concentrations decreased linearly in all treatments over the period of the experiment (Table 4.4). Fruit treated at one week before harvest with 1-MCP also showed a tendency toward a quadratic decline with a faster decline between September

23 and October 7 and a slowing of decrease thereafter. A strong negative linear correlation (r = -0.84) between starch concentration and SPI was also found (Table 4.2). For individual treatments starch concentration and SPI as well as starch concentration and sampling date both between correlated with -0.78 and -0.95

4.3.2 Storage effects of PGRs

For the storage experiment, fruit were harvested 3 days after the anticipated optimum harvest date 31 and 10 d after application of AVG and 1-MCP; the same nomenclature of 1 week and 4 weeks is used for clarity, but in actual days after treatment were slightly longer.

The IECs of untreated fruit and those treated with 1-MCP four weeks before harvest increased progressively during storage (Fig. 4.1A). In contrast, IEC of fruit treated with 1-MCP 10 days before harvest remained low throughout storage with a slight increase after day 112. The IECs of fruit treated with AVG, either 10 or 31 d before harvest, were intermediate between the one week 1-MCP treatment and the others. Fruit softened over the storage period with no treatment differences, except for those treated with 1-MCP one week before harvest (Fig. 4.1B). The softening of the 1 week 1-MCP treated fruit was slow until day 112, thereafter softening more rapidly. Starch declined rapidly from concentrations of 95 to 57 mg g^{-1} dry wt at harvest to less than 9 mg g^{-1} dry wt at day 56 (Fig. 4.1C). Starch concentrations decreased exponentially during storage without effect of treatment (Fig. 4.1C). At 56 DAH, starch concentrations were lowest in the 4 week AVG treatment and highest in the untreated and 1 week 1-MCP treatment. Little differences between treatments were found at subsequent removals from storage.

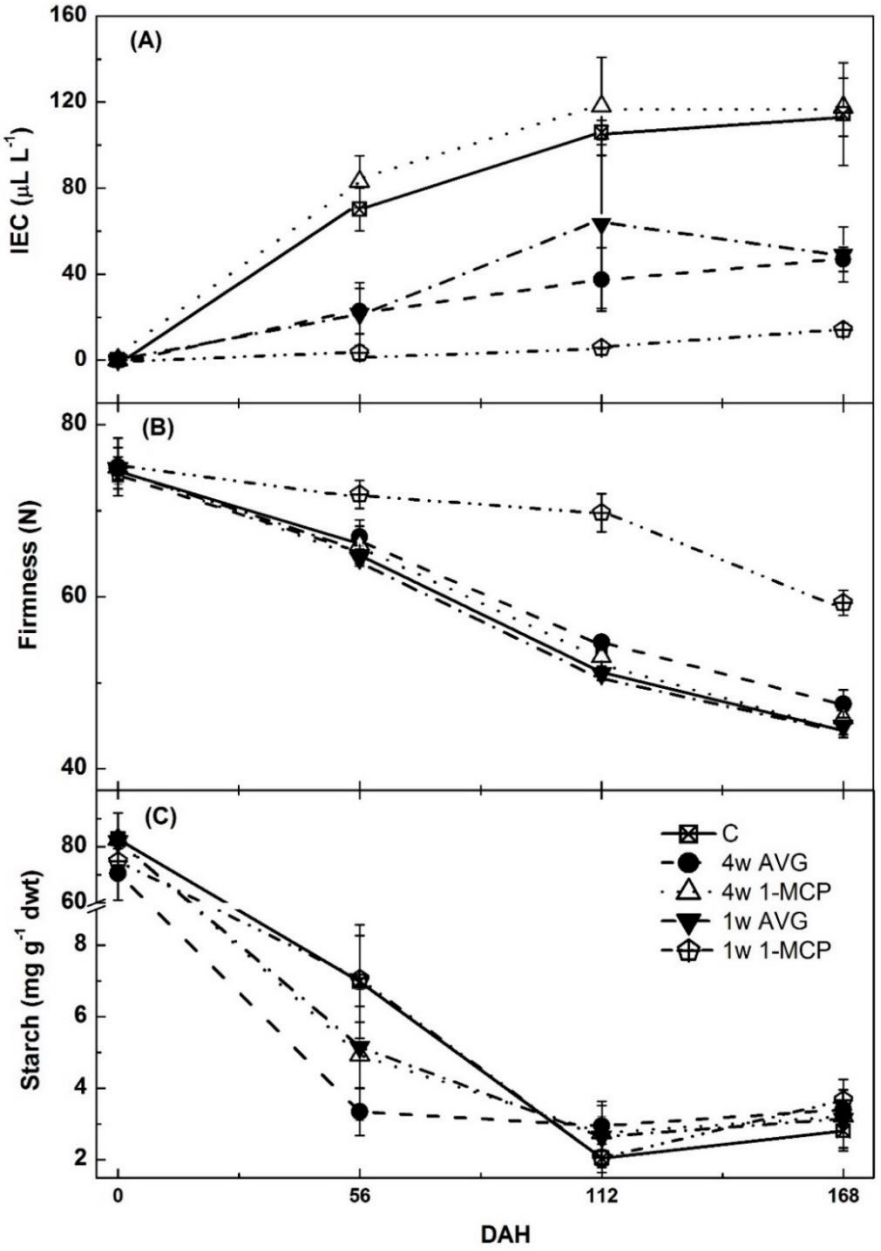

Fig. 4.1. Internal ethylene concentration (IEC [µL L^{-1}]) (A), fruit firmness (N) (B), and starch concentration (mg g-1 dry wt) (C) of 'Empire' either untreated (Control), or treated with AVG on August 22 (4w AVG) or September 16 (1w AVG), or treated with 1-MCP on August 22 (4w 1-MCP) or September 16 (1w 1-MCP), for an assumed harvest date of September 23 (Actual harvest date was September 25). Fruit were assessed at 56, 112, and 168 days after harvest (DAH) at 0.5 °C plus 1 day at 20 °C; Vertical bars represent standard error (n = 3). Effects of DAH, P <0.0001 for Log_{10}IEC, starch concentration and fruit firmness. Effect of treatment Log_{10}IEC 0.0037 and firmness <0.0001. Interaction (treatment × DAH) for firmness <0.0001. Regressions: Log_{10}IEC and Firmness L***, and starch concentration Q***, C***. IECs are shown as back-transformed means.

4.4. Discussion

Manipulation of ethylene production does not affect all aspects of maturation evenly. Some aspects are more affected by, or sensitive to, higher endogenous and exogenous ethylene concentrations than others (Johnston et al., 2009). The onset of the climacteric rise is typically delayed if fruit are treated with AVG and 1-MCP prior to harvest (do Amarante et al., 2002; McArtney et al., 2009; Schupp and Greene, 2004; Watkins et al., 2010; Yuan and Carbaugh, 2007). IEC of 'McIntosh' was lower in fruit treated four weeks before harvest at the first harvest date (September 9th) compared with the control and the other treatments, but the effectiveness was not detectable one week later. For 'McIntosh' fruit, therefore, precise timing of application of AVG, and application before climacteric rise, is important to achieve all the beneficial effects of the treatments.

Background color changes of apple fruit is due to the degradation of chlorophyll during ripening (Knee, 1972). I_{AD} values correlate with chlorophyll a concentration in the skin (Toivonen and Hampson, 2014). Postharvest 1-MCP has been shown to delay chlorophyll degradation during storage (Toivonen and Hampson, 2014; Zanella, 2003), but effects of AVG or preharvest 1-MCP on I_{AD} values have not been documented. In my study AVG and 1-MCP treatment of 'McIntosh' fruit delayed the decline of I_{AD} values until harvest on September 6th but not if harvest was delayed. 1-MCP applied one week before harvest delayed chlorophyll degradation for the delayed harvest but not on the estimated first harvest date. 'Empire' fruit I_{AD} values were higher in fruit treated with AVG four weeks before harvest in both years compared with the untreated control or 1-MCP treated fruit.

Starch concentrations in treated 'McIntosh' and 'Empire' in the first year remained higher compared with the controls, even though starch hydrolysis has been shown to be sensitive to ethylene (Johnston et al., 2009). But 'Empire' fruit in the second year of the study did not show the same trend in starch concentrations with lower concentration in fruit treated with AVG four weeks before harvest compared with untreated fruit. A delay in starch degradation of 'McIntosh' fruit by AVG was found to only last for only about three weeks and fruit maturation to be as fast as untreated control thereafter (Greene and Schupp, 2004). Application of AVG was shown to slow down starch decline in 'McIntosh' fruit, but with effectiveness depended on growing region (Stover et al., 2003). Silverman et al. (2004) showed AVG to be effective in reducing starch loss in 'Delicious' fruit, and demonstrated higher levels of amylose and reduced activity of amylase in treated fruit. Amylose, as the linear starch molecules, is generally digested faster, compared with amylopectin (branched starch) (Fan et al., 1995). If the delay of starch degradation is mainly dependent on amylase activity as suggested by Silverman et al. (2004) the effectiveness of AVG at slowing down starch degradation will be affected by many other factors which in turn influence starch composition in the fruit. Starch is made up of two glucose polymers amylose and amylopectin, and most storage starch contains about 70-80% amylopectin (Smith, 2007). But during starch degradation the percentage changes within the fruit (Chapter 2) and amylose is digested first, which means the percentage of amylose decreases within total starch to about 20 to 40%. Factors effecting starch composition and may influence the AVG effectiveness delaying starch degradation are: developmental differences between cultivars or single fruit within a cultivar; starch composition changes during maturation; as well as other factors, which

influence the amount of storage carbohydrates in the fruit, such as growing region and climate. Changes in amylose concentration are important, since amylose binds stronger to iodine and therefore changes in percentage of amylose influence SPI reading values. The capacity of binding with iodine was determined to be about 20 to 21 g per 100 g for AM compared with 0.3 to 2.6 g for AP (Fan et al., 1995; Magein and Leurquin, 2000).

An effect of PGRs on the SPI was not detected for 'McIntosh', but they affected SPI in 'Empire' in both years. Differences in effectiveness of AVG on delaying visible starch degradation was found for 'Gala' and 'Fuji', with greater effects on the earlier ripening 'Gala' (do Amarante et al., 2002). 'Fuji' as a generally lower ethylene producing fruit had less effects on ripening through manipulation of IEC (Elfving et al., 2007). Therefore, it is surprising that no effect on SPI was found in the higher IEC producing cultivar 'McIntosh', but an effect was found for the later ripening and lower ethylene producing apple 'Empire'. Whether preharvest PGRs affect SPI through manipulation of ethylene production or perception in the fruit, therefore, still remains unanswered. Strong correlations between starch concentration and SPI were found for individual treatments in both 'McIntosh' and 'Empire' fruit. Linear regression was detected ($P \leq 0.005$) for both cultivars for starch concentration over SPI. Whereas for the four cultivars 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire' in Chapter 2, the correlation of starch concentration and SPI were found to be curvilinear ($P \leq 0.005$). Therefore, treatment with 1-MCP or AVG may have caused a change in relationship of SPI and starch concentration. Untreated control fruit, however, also showed a changed relationship compared with relationships found in other cultivars (Chapter 2), which leads to the possibility that the changes are more due to season and growing condition

rather than treatment with PGRs alone.

During storage, 1-MCP applied four week before harvest did not inhibit IEC as much as the later application. 1-MCP has been found very effective if applied postharvest on preclimacteric fruit and vegetables (Blankenship and Dole, 2003; Watkins, 2006; Watkins, 2008). The effectiveness and longevity of applications of preharvest 1-MCP have been found to be affected by time of application, with greater effects if applied closer to harvest (Elfving et al., 2007) and a decline in efficacy during storage of the preharvest 1-MCP treatments has been found for prolonged periods between application and harvest (McArtney et al., 2009). Decreasing efficacy of 1-MCP treatment was found for pre- and postharvest application in some cultivars and has been thought to be caused by either insufficient binding of 1-MCP to the ethylene receptors or by the formation of new ethylene receptors in the fruit tissue (Sisler et al., 1996b). The decrease in effectiveness of earlier applied 1-MCP (4w) during cold storage (Fig. 4.1) indicates that a decrease of effectiveness could have been observed at harvest, if the sampling period would have been extended. The effect of AVG application timing on IEC for stored fruit was low, resulting in similar IECs in fruit treated ten days and four weeks before harvest. AVG, similar to preharvest 1-MCP (Fig. 4.1), has been shown to wear off in efficacy during storage (Schupp and Greene, 2004). Starch decline during storage was rapid regardless of preharvest treatment (Fig. 4.1) and the rate of starch loss more dependent on initial starch concentration and time in storage than preharvest treatment.

Fruit firmness after storage was found to be highest in fruit treated one week before harvest with 1-MCP. Treatment with AVG had no effect on fruit firmness. Effects of

AVG on fruit firmness have been observed in different cultivars, and were often found to be either timing or concentration dependent (Johnson and Colgan, 2003; Watkins et al., 2010). Timing of application especially for 1-MCP was important with little effect of the treatment applied four weeks before harvest on any of the measured fruit quality parameter. Beneficial effects on fruit quality of lowering IECs were shown to only carry over into the postharvest period if levels were kept sufficiently low (Williams, 1980), and fruit treated with 1-MCP four weeks before harvest had IEC as high or higher as the untreated control (Fig. 4.1).

Whether the use of AVG and 1-MCP prior to harvest affects the reliability of the SPI as a harvest index cannot satisfactory be answered. Many studies have shown that the effects on starch hydrolysis of both PGRs are dependent on factors such as cultivar, season, application timing and concentration. Therefore, studies specifically addressing regional and cultivar specific changes in starch hydrolysis due to preharvest PGR applications which alter ethylene concentrations in the fruit might be needed to clearly assess the changes in SPI as well as starch concentration in the fruit.

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CHAPTER 5

NON-DESTRUCTIVE MATURITY ASSESSMENT OF 'EMPIRE' APPLES TREATED WITH PREHARVEST INHIBITORS OF ETHYLENE PRODUCTION WITH A DELTA ABSORBANCE (DA) METER

Abstract

Harvest indices for apple fruit are usually based on destructive measurements such as starch pattern staining, firmness and internal ethylene concentration (IEC). One recent tool to estimate harvest windows is a DA meter, which provides a reading of difference in absorbance (I_{AD}). The I_{AD} estimates chlorophyll a (chl a) content based on absorbance differences between 670 and 720 nm. In 2013 a trial was carried out on a commercial orchard in which 'Empire' trees were either untreated or sprayed with Harvista (preharvest 1-methylcyclopropene; 1-MCP), or ReTain (aminoethoxy-vinylglycine; AVG). Fruit were harvested at three one week intervals, and I_{AD}, IEC, firmness and starch pattern indices (SPIs) measured. Correlations between individual fruit and harvest indices were weak, but when data were grouped and ranked by I_{AD} readings the relationships were stronger. However, preharvest treatment with chemicals that modulate ripening of fruit altered the relationships between I_{AD} and other harvest indices, especially IEC.

5.1. Introduction

Measurement of maturity is a critical part of harvest management that enables fruit growers and storage facility managers to harvest and store fruit optimized for their storage performance. Harvest indices used commonly for apple fruit include internal ethylene concentration (IEC), starch pattern index (SPI), firmness, soluble solids concentration and background (ground) color (Watkins, 2003). Although changes of pigment contents, such as loss of chlorophyll (chl) (Knee, 1972; Zude-Sasse et al., 2002), can be used as a harvest index, traditional methods such as use of color cards can be difficult in many red cultivars and high coloring strains.

A small hand-held delta absorbance (DA) meter (Sintéleia, Bologna, Italy) has been commercialized. The DA meter measures the absorption differences (I_{AD}) of wave-lengths at 670 and 720 nm and functions essentially as electronic color chart. From the interactance (I) spectra, fruit absorbance (A) is calculated using the Lambert's Beer law ($A=\log_{10} I^{-1}$). The I_{AD} is calculated as $I_{AD}=A_{670} - A_{720}$ where A_{670} and A_{720} are the A values measured at 670 and 720 nm, respectively (Bertone et al., 2012; Zanella et al., 2013; Ziosi et al., 2008). Recent research has detected good correlations between I_{AD} values and other harvest indices in various apple cultivars such as 'Starking', 'Granny Smith' and 'Pink Lady' (Nyasordzi et al., 2013), 'Gala' (Costamagna et al., 2013), 'Braeburn' and 'Cripps Pink' (Zanella et al., 2013) and 'Honeycrisp' (DeLong et al., 2014). However, Toivonen and Hampson (2014) found that while good relationships between I_{AD} values and other harvest indices existed for 'Aurora Golden Gala^{TM}', 'Fuji' and 'Royal Gala', these became weaker for any indices other than chl a content if 1-methylcyclopropene (1-MCP) or controlled atmosphere (CA) storage was applied after harvest.

Preharvest applications of the plant growth regulators (PGRs) ReTain (aminoethoxyvinylglycine; AVG) and Harvista (1-MCP), which inhibit ethylene production and perception, respectively, are used extensively in North America and other countries to stop fruit from dropping, to delay harvest to increase fruit size, and to improve responses of fruit to SmartFresh (postharvest 1-MCP) (Elfving et al., 2007; Yuan and Carbaugh, 2007; Watkins et al., 2010). It is now common to find orchard blocks within a single growing region which are untreated, or treated with either ReTain or Harvista. Therefore it is important to understand the effects of PGRs on the relationships between I_{AD} values and other harvest indices.

In this study, the effects of ReTain and Harvista on harvest indices of 'Empire' apples have been investigated. Fruit were harvested at three intervals over the harvest period, and were analyzed for correlations between individual fruit and harvest indices, and with grouping of data within I_{AD} categories.

5.2. Material and methods

'Empire' fruit from a 2007 high density orchard on M9 rootstock in Western New York (Wolcott, NY) were harvested September 26, October 3 and October 10 in 2013. The trees were untreated or treated with ReTain (Valent BioSciences Corporation, Libertyville, IL) or Harvista (AgroFresh Inc., Springhouse, PA). The treatments were carried out according to standard industry rates of 823 g ha^{-1} and 12.6 kg ha^{-1} for ReTain and Harvista (AF-2005), respectively. ReTain and Harvista were applied four weeks and one week, respectively, prior to first harvest on August 27 2013. Both PGRs were applied commercially on approximately 0.4 hectares of orchard.

At harvest 40 fruit from each treatment were taken into the lab for assessment of IEC, flesh firmness, I_{AD} readings, and SPI ratings. Each apple was assessed individually to allow correlation of I_{AD} to the other harvest indices. All assessments were done within 24 hours of harvest.

IEC was measured by injecting 1 mL of gas sample taken from the core cavity using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) as described by Watkins et al. (2000). Flesh firmness was measured on opposed sides of the fruit on the blushed and un-blushed side with an 11.1 mm diameter probe (Guss Manufacturing (Pty) Ltd., Strand, South Africa). The SI was determined according to Blanpied and Silsby (1992), where 1 = 100% staining and 8 = 0% starch.

All statistical calculations were done in JMP® Pro 10.0.0 (SAS Institute) using simple least square models with inclusion of interactions.

5.3 Results

Over the three harvest dates IECs and SPIs increased, while I_{AD} values and firmness decreased, but harvest indices were not consistently affected by treatment across harvest dates (Table 5.1). At the first harvest (September 26), only the I_{AD} values were affected by treatment, with the Harvista values higher (greener fruit) than ReTain values. There were no significant differences between treatments on October 3 but on October 10, ReTain had lower I_{AD} values than Control or Harvista treated fruit. Ethylene production, as indicated by IECs, increased in Control fruit by the time of the second harvest and was significantly lower in the Harvista treatment than the Control. The IECs

of Control and ReTain treated fruit were statistically higher than the Harvista treated fruit on October 10. Overall, fruit treated with Harvista remained firm throughout, whereas ReTain treated fruit dropped to the level of Control fruit by October 10. By the third harvest, the lowest IEC was found in Harvista treated fruit, while the lowest I_{AD} values were found in the ReTain treatment. The SPI were similar for all three treatments at the first and second harvests. At the third harvest, Harvista was lower than ReTain.

Table 5.1. Harvest indices of 'Empire' apples that were untreated or treated with Harvista or ReTain before harvest. Means are average values of 40 apples, and different letters indicate differences at $P \leq 0.05$.

Harvest Date (2013)	Treatment	IEC ($\mu L\ L^{-1}$)	I_{AD}	SPI (1-8)	Firmness (N)
September 26	Control	0.20 c	1.42 ab	4.1 e	76.7 a
	Harvista	0.16 c	1.51 bcd	3.9 e	75.4 a
	ReTain	0.84 c	1.37 a	4.0 e	75.9 a
October 3	Control	13.37 ab	1.36 bcd	5.5 cd	71.1 bc
	Harvista	0.27 c	1.39 bc	5.2 cd	75.4 a
	ReTain	4.35 bc	1.30 cd	5.2 cd	73.1 ab
October 10	Control	19.36 a	1.29 cd	6.1 ab	65.1 de
	Harvista	5.52 bc	1.28 d	5.8 bc	68.9 cd
	ReTain	16.02 a	1.18 e	6.4 a	64.6 e

The data were first analyzed by plotting IEC, SPI and firmness against I_{AD} values. Only results for IEC are shown here (Fig. 5.1) as they are representative of the relationships with other harvest indices. Relationships between factors were poor. When the data were further analyzed by grouping harvest indices data by categorizing I_{AD} values by 0.1 units from 1.0 to 1.7 within each treatment, stronger relationships were generally observed (Figs. 5.2-5.4). However, the PGR affected the correlations individually.

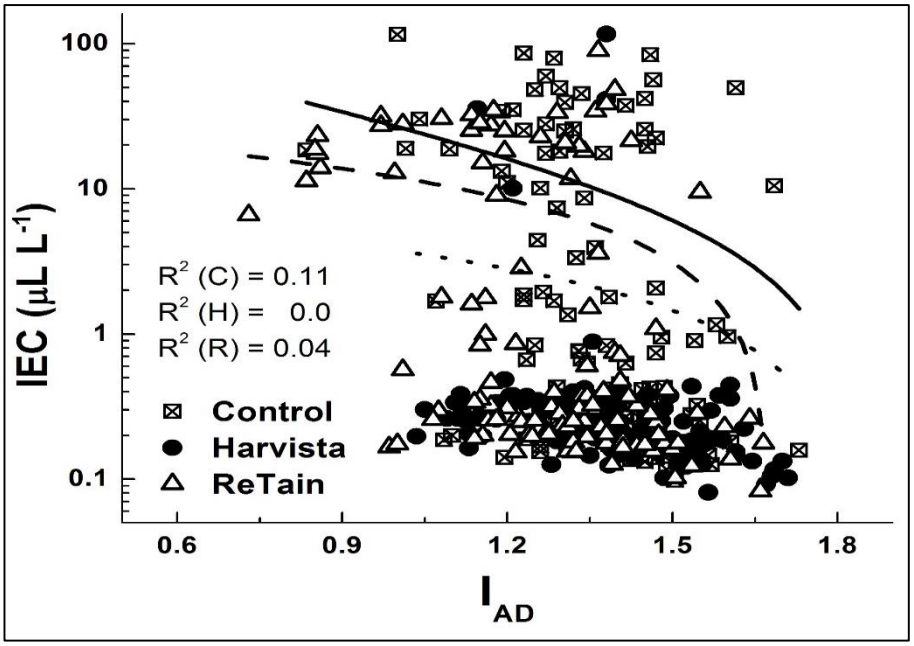

Fig. 5.1. Individual fruit internal ethylene concentration (IEC [µL L^{-1}]) plotted against I_{AD} values – letters after the R^2 values indicate control = C, Harvista = H, and ReTain = R.

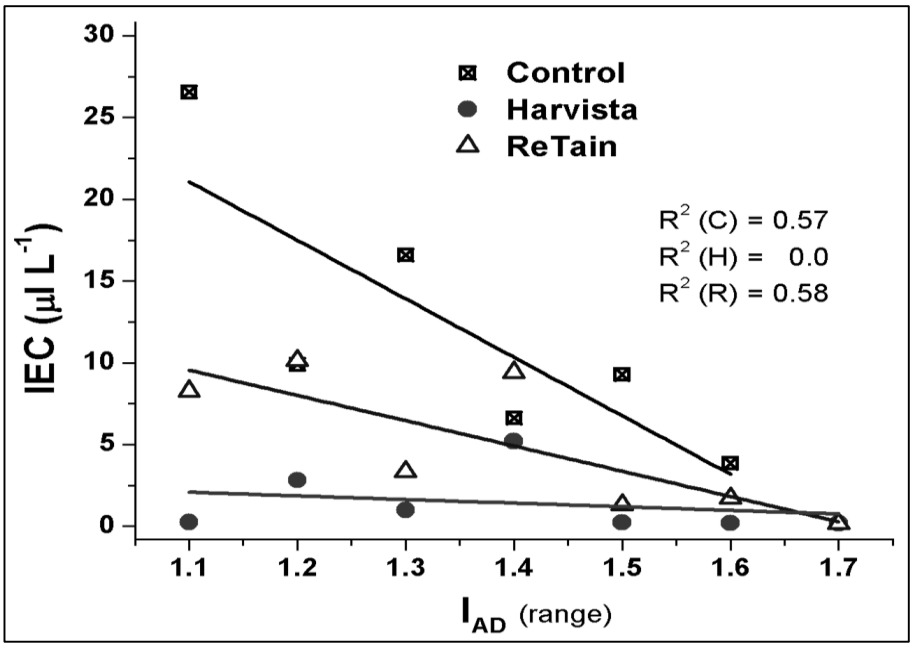

Fig. 5.2. Internal ethylene concentration (IEC) plotted against I_{AD} categories; Data within each I_{AD} range are a combination of all three harvest dates.

The R^2 values for the relationship between IEC and I_{AD} were similar for Control and ReTain treatments, but very low for Harvista because of the limited changes in IEC over time (Fig. 5.2). The highest average IEC in Control and ReTain were 26.6 (range 1 to 1.1) and 10.2 µL L^{-1} (range 1.1 to 1.2), but only 5.2 µL L^{-1} (range 1.2 to 1.3) in Harvista treated fruit.

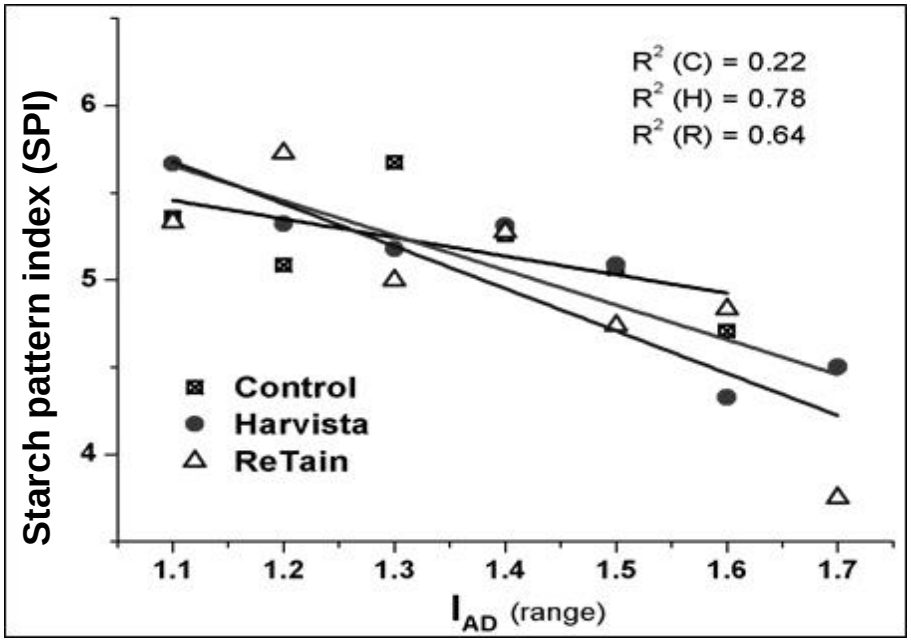

Fig. 5.3. Starch pattern index (SPI) values plotted against I_{AD} categories; Data within each I_{AD} range are a combination of all three harvest dates.

Fitted lines of SI (Fig. 5.3) and firmness (Fig. 5.4) against I_{AD} were not affected as much by PGRs. However, the R^2 values for these harvest indices were markedly lower in the Control treatment than either Harvista or ReTain treatments, and generally lower for firmness than for SI. These weaker relationships for Control may be a result of a smaller range of values. Control had no high I_{AD} value (1.6-1.7). SI values only ranged

from 4.7 to 5.7 for Control compared to 3.8 to 5.7 and 4.3 to 5.7 for ReTain and Harvista treated fruit respectively. Firmness ranged between 66.3 and 75 N for Harvista and 69.3 and 76.7 N for ReTain treated fruit, while Control fruit ranged from 68.4 to 71.7 N.

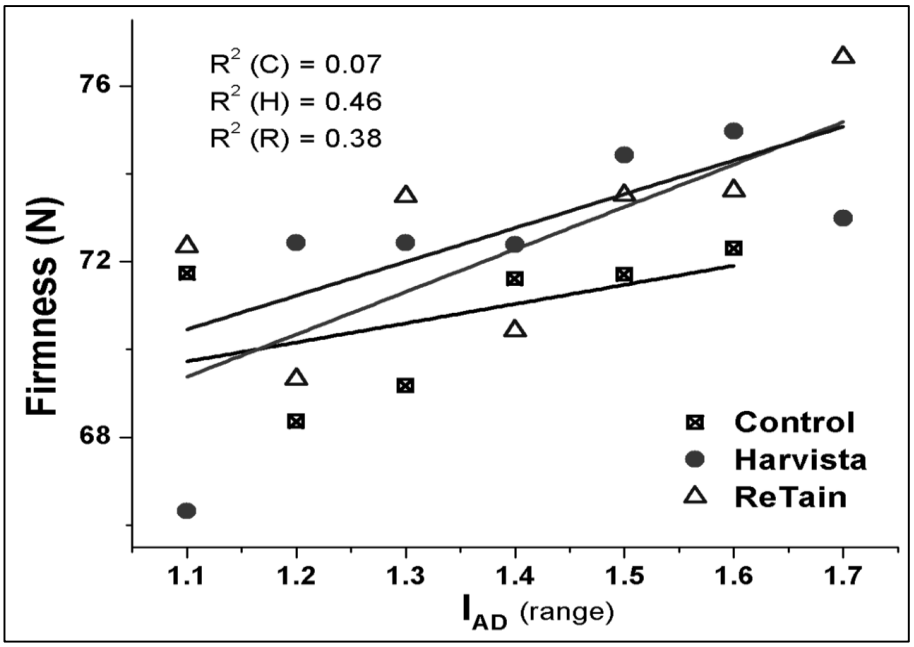

Fig. 5.4. Firmness plotted against I_{AD} categories; Data within each I_{AD} range are a combination of all three harvest dates.

Table 5.2. Internal ethylene concentration (IEC) of 'Empire' apples untreated or treated with Harvista or ReTain on September 19, and September 5, 2013, respectively, and harvested on October 3, 2013. Fruit were assessed by I_{AD} reading before measurement of IEC.

I_{AD} value	Control	Harvista	ReTain
	IEC (µL L^{-1})		
1.1-1.2	16.0	0.3	2.3
1.2-1.3	9.4	0.3	0.8
1.3-1.4	6.1	0.2	7.0
1.4-1.5	16.7	0.3	3.1
1.5-1.6	12.3	0.2	0.2
1.1-1.2	16.0	0.3	2.3

The relationships between harvest indices and I_{AD} values were analyzed separately (Table 5.2). Within each category from 1.1–1.2 to 1.5–1.6, a clear dissociation between IECs and I_{AD} values becomes visible as a result of PGRs is shown (P=0.05). However, effects on relationships between SI and firmness with I_{AD} values were small (data not shown).

5.4 Discussion

The DA meter is a new technology that is receiving considerable interest because of its possible utility as a non-destructive estimator of apple quality at harvest (Nyasordzi et al., 2013; Costamagna et al., 2013; Zanella et al., 2013; DeLong et al., 2014). In our study, I_{AD} values decreased over time, while the standard harvest indices of IEC and SPI values (higher values = less starch) increased and firmness decreased (Table 5.1). However, the relationship between IEC and I_{AD} values was poor (Fig. 5.1) as was also found for other indices (data not shown). This is in contrast to another study with 'Starking' in which individual fruit were analyzed (Nyasordzi et al., 2013). The difference may in part be to the limited range of I_{AD} values in 'Empire' apples over a 3 week harvest period (1.1 to 1.6 units) compared with approximately 0.4 to 2.0 units in 'Starking' apples harvested over a 40 day period, where the wider range of values might have contributed to better relationships.

When fruit were analyzed by categorizing by I_{AD} value, relationships were stronger for IEC (Fig. 5.2), though not for SPI or firmness (Figs. 5.3 and 5.4). Nyasordzi et al. (2013) found for 'Starking' individual fruit a strong correlation for SPI and firmness but over a much longer harvest period. A positive and strong correlation between firmness and I_{AD} was found by Zanella et al. (2013) for 'Braeburn' fruit, but in the same study results

for ‘Cripps Pink’ were not as conclusive, with the correlation being positive or negative, depending on harvest date and shelf life. But for comparison the studies named here, Zanella et al. (2013) and Nyasordzi et al. (2013), present data for individual fruit rather than grouped by range.

A focus of this study was the effect of PGRs on relationships between I_{AD} values and other harvest indices. Two modulators of harvest maturity that are used extensively in New York are ReTain and Harvista, and each operates by affecting ethylene production. For a non-destructive method of apple quality to work in the field, it is necessary for I_{AD} values to relate reliably with the physiological indicator of ripening, ethylene, and other factors such as starch hydrolysis, as well as quality indicators such firmness. Therefore, any given I_{AD} value should relate to a similar range of IEC, SI, firmness or any other harvest index if it is going to be a reliable harvest index across an industry with mixed management practices. Our data (Tables 5.1 and 5.2; Figs. 5.1-5.4) show clearly that the relationships are dissociated by both ReTain and Harvista. Interestingly, Toivonen and Hampson (2014) have identified a similar dissociation between I_{AD} values and other quality factors when fruit are treated after harvest with 1-MCP and/or stored in CA storage.

In conclusion, the utility of I_{AD} values as a harvest index for ‘Empire’ apples is not supported by our study. Other researchers have found varying degrees of relationship between I_{AD} values and other harvest indices and relationships with specific indices are affected by cultivar (Costamagna et al., 2013; DeLong et al., 2014; Nyasordzi et al., 2013; Toivonen and Hampson, 2014; Zanella et al., 2013). Most importantly, these relationships are further affected by PGRs such as ReTain and Harvista which influence fruit maturity

independently from I_{AD}. Further investigations of relationships of harvest indices to each other and their relationships to PGRs and potentially other management practices are needed.

Overall, however, the data indicate that the relationship between I_{AD} and other harvest indices can be greatly influenced by PGR applications. If, for example, data for these indices at each harvest date are tabulated against three I_{AD} groupings, it is possible to see what relationships are lost as shown in table 5.2. Especially the relationship between IEC and I_{AD} is greatly altered for Harvista treated fruit. Data for harvest one and three are not shown but are overall similar.

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CHAPTER 6

AN ECONOMIC ANALYSIS OF HARVEST TIMING TO MANAGE THE PHYSIOLOGICAL STORAGE DISORDER FIRM FLESH BROWNING IN 'EMPIRE' APPLES

Abstract

Firm flesh browning of 'Empire' apple [*Malus sylvestris* (L.) Mill var. *domestica* Borkh.] fruit is a major cause of revenue loss for growers and storage operators. In this study, the economic impact of harvest date on revenue based on percentage pack-out (fruit size/weight and red coloration) and disorder incidence has been investigated using a budgeting model. Fruit were harvested from a commercial orchard weekly for three weeks; H1 (one week before normal harvest), H2 (normal harvest), and H3 (one week after normal harvest). Fruit samples were either untreated or treated with 1-methylcyclopropene (1-MCP) and stored under controlled atmosphere (CA) conditions (2 kPa O2; 2 kPa CO2) at 0.5 °C and 2 °C. Field bins of fruit were used to assess pack-out percentages. Flesh browning was low at H1 and unaffected by 1-MCP treatment, whereas incidence was highest in fruit from H3, and especially in 1-MCP treated fruit stored at 2 °C. The enterprise budget model shows that net profits for a one hectare high density planting vary slightly between fresh-marketed fruit in the fall, and for fruit stored after nine months in CA storage, both with and without 1-MCP treatment. Net profits from one hectare are higher for H3 when fruit are marketed in the fall, in comparison to the normal harvest (H2), but at harvest, pack-out was substantially lower for fruit from H1. Fruit quality and pack-out percentages after storage depended not only on the harvest date but also on storage temperature and 1-MCP treatment. Therefore, there is an

economic tradeoff between harvest date, occurrence of flesh browning, and likely net profits to producers of 'Empire' apples.

6.1 Introduction

In the United States, apples are the second most consumed fruit per capita after bananas (USDA, 2012). However, there is much product differentiation within the apple category providing consumers with many choices. Producers need to be vigilant, therefore, to produce high quality fruit to remain profitable in increasingly competitive markets. Profitability depends on maintaining low costs, selling fruit for the best possible price point (aiming for higher prices linked to higher grades and color requirements), and having fruit to sell all year round.

The 'Empire' apple cultivar, a cross between 'McIntosh' and 'Delicious', was released in 1966 (Derkacz et al., 1993). It is a major cultivar in the Northeastern U.S. particularly in New York State as well as in Canada. 'Empire', at almost 1,860 hectares, was the second most planted cultivar after 'McIntosh' in the northeast in 2006 (NASS, 2012), and is the fifth most important cultivar in the U.S. with a total production of 170,000 tons in 2011 (Lehnert, 2012). The popularity of the cultivar is due to its fresh eating qualities with an excellent sugar/acid balance and good texture. 'Empire' is also an ideal cultivar for the fresh cut slice industry (Kim et al., 1993) and increasing production has been diverted to meet this market segment.

Four general quality grades are used in the U.S.: "U.S. Extra Fancy" (ExFy), "U.S. Fancy" (Fy), "U.S. no. 1" (#1), and "Commercial" (C) by §51.300 and §51.301 of the U.S. standards for grading of apples (USDA, 2002). ExFy is the highest grade and,

therefore, brings the highest price. Standards for fruit size and red coverage for this grade are high, and often require later harvest to reach the desired size and color, which is especially difficult for a bicolored cultivar. Whole fruit prices are determined by grade as well as size, which ranges between 72 and 163 fruit per box. This refers to an average fruit weight of approximately 265 to 116 g. Fresh cut requires from 100 to 115 count boxes (18.1 kg), with individual fruit weight between 190 to 170 g. Modern high density plantings have the advantage of allowing earlier color development. The fruiting wall in a high density system allows most fruit to be well exposed and color is developed more evenly and uniformly (Dorigoni et al., 2011). Diameter increases around harvest time are relatively rapid (Tukey and Young, 1942), and therefore later harvests result in larger fruit that generate higher prices.

'Empire' apples are air stored to meet market demands until about December with fruit for marketing beyond this time usually being controlled atmosphere (CA) stored. Both air-stored and CA-stored fruit are often treated with the inhibitor of ethylene perception, 1-methylcycopropene (1-MCP) (Watkins, 2008). A storage period of at least 10 months is desired by the whole fruit and fresh cut industries, but the cultivar is susceptible to several physiological disorders that limits its storage potential (Watkins et al., 1997; Watkins and Liu, 2010).

Incidence of fruit to external carbon dioxide injury can be increased by 1-MCP (Fawbush et al., 2008), but the risk of injury can be controlled with diphenylamine (DeEll et al., 2007; DeEll et al., 2005). Another serious disorder is firm flesh browning (Watkins and Liu, 2010). Symptoms typically become visible in May or June in the northern hemisphere, but can occur earlier in some years. Flesh browning is not externally visible

and mostly starts at the stem end of the fruit in the shoulder region (Lee et al., 2012). The disorder is thought to be a chilling injury (Jung and Watkins, 2011; Lee et al., 2012; Snowden, 1990) and a recommended storage temperature of 1 to 2 °C reflects a compromise between chilling injury at lower temperatures and faster softening at warmer temperatures. However, browning can be enhanced after postharvest treatment with 1-MCP at temperatures from 3 °C to 4 °C (Fawbush et al., 2008; Jung and Watkins, 2011; Watkins, 2008). The presence of flesh browning has been especially problematic for the fresh cut industry as only apples with no internal browning – even slight browning in the stem end region (shoulder) – are acceptable. Minor flesh browning in whole fruit may not be detected by the consumer, but modern sorting lines with internal quality assessment will detect internal defects regardless of fruit use.

No postharvest treatments or storage regimes have successfully controlled flesh browning (DeEll and Ehsani-Moghaddam, 2012; Fawbush et al., 2009). However, preliminary trials have indicated that browning occurs more frequently in later- than earlier harvested 'Empire' apples (unpubl. data). The objective of this research is to investigate the effects of harvesting fruit a week earlier than 'normal' to control development of the disorder in comparison with two subsequent harvests. A 'normal' harvest date was defined by the participating grower as optimal on past orchard block experience. Fruit were stored at two storage temperatures with or without treatment with 1-MCP. A full economic analysis was carried out to compare the effects of early harvest having lower quality, as judged by pack-outs based on fruit size and color, with later harvests with higher pack-outs but potentially greater loss of fruit due to disorder development.

6.2 Material and methods

6.2.1 Plant material and harvest

'Empire' trees grown in a commercial orchard in Wolcott, New York, were used for this experiment. 'Empire' on M9 rootstocks were planted in 2007 in a 0.6 m by 3 m spacing high density planting (5,313 trees per hectare). The 'Empire' trees were planted in sets of four rows with 12 rows of other cultivars separating the next block of 'Empire' apples. Within each row, poles that held the wire were used as markers throughout the block. At each harvest only one-third of each row was harvested by strip-picking all fruit; at harvest 1, sections 1, 4, 7 etc. and at harvest 2, sections 2, 5, 6 etc. and so on. Fruit were picked by a commercial harvest crew. The total area harvested was 0.4 hectare per harvest.

Fruit were harvested on September 21th (H1), 28th (H2), and October 5th (H3) in 2012. At each harvest, approximately 11 metric tons (25 harvest bins) were harvested. The harvested bins were labeled and stored in commercial cold storage at 2 ºC in Wolcott, NY, until they were sorted on a commercial sorting line in November. During the commercial harvest, 16 plastic containers of approximately 40 fruit each plus four replicates of 10 fruit for maturity analysis, were taken randomly throughout each set of trees and transported to the postharvest laboratory at Ithaca for analysis and storage.

6.2.2 Harvest assessments

Internal ethylene concentration (IEC), firmness, titratable acidity (TA), soluble solids content (SSC), delta absorbance (I_{AD}), and starch pattern index (SPI) measurements were carried out on each replicate of ten fruit. IEC was measured by injecting 1 ml of gas

sample taken from the core cavity using a Hewlett-Packard 5890 series II gas chromatograph (Wilmington, DE) equipped with a flame ionization detector and fitted with a stainless steel column packed with 60/80 mesh Alumina F-1 (2m x 2mm i.d.) (Alwan and Watkins, 1999). I_{AD}, a measure of chlorophyll a concentration (Ziosi et al., 2008), was measured on opposite sides of the fruit, on the blushed and unblushed side, unsing using a handheld Delta Absorbance (DA) meter (Sinteleia, Bologna, Italy) (Costamagna et al., 2013). Flesh firmness was measured on opposite peeled sides of each fruit using a fruit texture analyzer (Guss Manufacturing (Pty) Ltd., Strand, South Africa) fitted with an 11.1 mm probe. TA was measured using a bulked sample of all 10 fruit per replica with $1/8^{th}$ of each fruit juiced together and analyzed using an autotitrator (Mettler DL12, Hightstown, NJ). Aliquots of 10 mL were titrated to pH 8.1 with 0.1 N NaOH and the results expressed as % malic acid. The juice was also used to measure SSC with a pocket refractometer (Pal-1, Atago U.S.A Inc., Bellevue, WA). SPI was measured by cutting the fruit in half along the equatorial and dipping on half into iodine (I_2-Kl) solution and evaluating the pattern by comparison to the Cornell generic SPI chart (Blanpied and Silsby, 1992).

6.2.3 Postharvest and Storage treatments

Fruit were divided into treatment replicates consisting of four containers for each ±1-MCP treatment and storage temperature. Fruit were then cooled overnight at either 0.5 or 2 °C. Half the fruit at each temperature were treated with 1 µL L^{-1} 1-MCP for 24 h in a gas tight 4,000-L plastic tent using a release and fan system supplied by the manufacturer for 1-MCP treatment (SmartFresh, AgroFresh, Springhouse, PA). After another 24 h, the containers of fruit were placed into experimental CA chambers (volume

of 0.9 m^3) fitted with a circulating fan system (Storage Control Systems, Sparta, MI) and stored at 2 kPa CO_2 and 2 kPa O_2. Partial pressures were established within 48 h, checked hourly and maintained within 0.2 kPa of target values with an ICA 61/CGS 610 CA Control System (International Controlled Atmosphere Ltd., Kent, U.K.) modified with flow controllers for the experimental chambers (Storage Control Systems, Sparta, MI). Fruit in the field bins (25 per harvest date) were stored in commercial air storage at 2 ºC on side in Wolcott, NY, until they were sorted in November.

6.2.4 Quality evaluation after storage

Fruit firmness, SSC, and TA were measured as before on four replicate sets of 10-fruit at 1 and 7 days after storage at 20 ºC. IEC was measured after 7 days at 20 ºC only. On day 7, all remaining fruit were assessed for external and internal disorders. For this purpose each fruit was assessed externally and sliced at least five times from the shoulder to the calyx end of each fruit to reveal the presence or absence of internal disorders. The percentage of fruit with any visible injury was calculated.

6.2.5 Fruit grading

The fruit from the commercial cold storage were graded on a sorting line GREEFA Geosort (Geldermalsen, Netherlands) was equipped with iQS III for external defects and iFA for internal defects. Exact numbers of fruit in each grade was recorded separately for each of the three harvest dates (Table 6.5) to evaluate changes in potential income due to changes in fruit size and color over the time of the experiment.

6.2.6 The economic model

We developed an enterprise budgeting model that considers the revenue flows and costs of producing, storing, and marketing apples. The model employed a spreadsheet framework to quantify the revenue and cost implications for fruit harvested in three different time periods. An enterprise budgeting model allows for a comparison between production or management alternatives (Bradford and Debertin, 1985; Dillon, 1993). Such a budget model is ideal for the present study as it documents all possible sources of revenue as well as all associated variables and fixed costs (Peabody, 2007); it also facilitates the use of a sensitivity analysis to understand how small changes in model parameters impact the financial results. Our model was designed following, and borrowed some parameters from, similar frameworks used by agricultural economists to study orchard profitability (DeMarree et al. 2010; Gallardo and Galinato 2012).

The model takes into account production costs such as pest and weed management, costs for hand and machine labor; and several more aspects of production per hectare (Table 6.4). Approximate costs were calculated based on costs per hectare taken from a workbook developed by DeMarree et al. (2010) for a high density 'Gala' planting in New York State and adjusted for 'Empire' production with 5,313 trees per hectare. For simplification purposes, costs of establishment were not considered as they are assumed to be constant across the three production systems (that differ only by harvest date). Therefore, the costs reported here represent average maintenance costs for a fully-established high-density 'Empire' planting. Pack-out data from 25 field bins per harvest date was therefore obtained in November, while actual losses due to internal and external

disorders were estimated based on sample fruit stored for nine month in CA storage at two storage temperatures.

6.2.7 Statistics

Data was subjected to ANOVA to analyze effects storage temperature and 1-MCP treatment. IEC and percentage disorder data were transformed with logarithms and arcsine, respectively, for analysis, but back-transformed means are presented within the tables. Data for each replica of ten fruit, as well as extra fruit within the replica assessed on day 7 after removal for disorders, was assessed as a replicate for statistical analysis. All statistical analysis were performed using with JMP® Pro 11. (SAS Institute Inc., Cary, NC, USA).

6.3 Results and discussion

6.3.1 Harvest and storage quality

The IECs in fruit indicated that they were preclimacteric at the first harvest, but then increased at each subsequent week (Table 6.1). Over this time period, fruit firmness and acidity decreased, and starch hydrolysis continued as indicated by higher SPI values. I_{AD} values did not changed between H1 and H2 and only decreased slightly between H2 and H3, indicating a loss of chlorophyll a over the harvest period. Earlier harvested fruit were greener compared to fruit harvested at the latest harvest date. SSC was not affected by harvest date.

Table 6.1. Internal ethylene concentration (IEC), flesh firmness, soluble solids concentration (SSC), titratable acidity (TA), starch pattern index (SPI) and delta absorbance (I_{AD}) of 'Empire' apples at three harvests.

Harvest date (2012)	**IEC** (µL L^{-1})	**Firmness** (N)	**SSC** (%)	**TA** (%)	**SPI** (1-8)	**I_{AD}**
Sept 21 (H1)	0.329	77.3	13.6	0.50	3.4	1.22
Sept 28 (H2)	1.28	74.6	14.2	0.42	3.7	1.22
Oct 5 (H3)	18.83	72.0	14.2	0.43	4.7	0.95
P-values	0.0103	0.0032	ns	0.0033	0.0006	ns

After nine months in CA storage plus 1 d shelf life (Table 6.2), firmness was higher in H1 and H2 harvested fruit compared with H3. Marketing to a fresh cut provider would not be possible for the non 1-MCP treated fruit from H3 since the fresh cut industry requires a minimum firmness of 62.3 N to guarantee good quality cut produce (Toivonen and Hampson, 2009). Even 1-MCP treated fruit of H3 only barely reached the minimum required firmness after storage. Other guidelines such as for certain export markets also imply minimum standards for fruit firmness, for example the Canadian guidelines (CFIA, 2011), which would most likely not be reached by fruit harvested at H3. Storage regime and temperature has a strong influence on SSC with a decline towards H3. TA decreased towards the later harvest.

After a 7 d shelf life, the IEC was lower in fruit that were harvested early, treated with 1-MCP, and stored at the lower storage temperature (Table 6.2). Quality factors such as flesh firmness were influenced by time of harvest, length of storage, storage temperature, and whether or not 1-MCP was applied prior to storage. Lower TAs were found in untreated than 1-MCP treated fruit.

Table 6.2. Internal ethylene concentration (IEC) measured only after 7 days at 20 ºC, flesh firmness, soluble solids concentration (SSC) and titratable acidity (TA) of 'Empire' apples with and without 1-MCP treatment after 9 months in CA storage plus 1 d and 7 d at 20 °C.

Harvest date (2012)	Treatment	IEC (µL L-1)	Firmness (N)	SSC (%)	TA (%)
			1 d		
Sept 21 (H1)	2 °C	-	73.2	14.5	0.305
	2 °C + 1-MCP	-	73.7	14.1	0.294
	0.5 °C	-	75.9	14.5	0.300
	0.5 °C + 1-MCP	-	75.4	14.5	0.309
Sept 28 (H2)	2 °C	-	68.5	14.6	0.268
	2 °C + 1-MCP	-	69.3	14.3	0.264
	0.5 °C	-	71.5	14.2	0.318
	0.5 °C + 1-MCP	-	72.2	14.6	0.287
Oct 5 (H3)	2 °C	-	50.7	13.9	0.251
	2 °C + 1-MCP	-	63.6	13.9	0.252
	0.5 °C	-	50.7	14.3	0.253
	0.5 °C + 1-MCP	-	63.6	14.2	0.289
Harvest date		-	<0.0001	0.009	0.028
Storage temperature		-	0.0378	0.009	ns
1-MCP treatment		-	ns	0.034	ns
Harvest date × temperature		-	ns	<0.0001	ns
Harvest date × 1-MCP		-	<0.0001	0.042	ns
Temperature × 1-MCP		-	ns	0.007	ns
Harvest date × temperature × 1-MCP		-	ns	0.007	ns
			7 d		
Sept 21 (H1)	2 °C	26.6	70.0	14.5	0.299
	2 °C + 1-MCP	0.44	71.1	14.6	0.283
	0.5 °C	11.2	74.9	14.4	0.292
	0.5 °C + 1-MCP	0.36	74.6	14.3	0.293
Sept 28 (H2)	2 °C	54.6	70.8	14.4	0.247
	2 °C + 1-MCP	2.13	74.3	14.3	0.271
	0.5 °C	105	68.3	14.1	0.279
	0.5 °C + 1-MCP	0.44	74.3	14.4	0.296
Oct 5 (H3)	2 °C	92.2	43.5	14.3	0.224
	2 °C + 1-MCP	5.28	62.0	14.1	0.239
	0.5 °C	86.2	51.5	14.4	0.232
	0.5 °C + 1-MCP	0.85	65.3	14.4	0.279
Harvest date		<0.0001	<0.0001	ns	ns
Storage temperature		<0.0001	0.0008	ns	ns
1-MCP treatment		<0.0001	ns	ns	0.044
Harvest date × temperature		<0.0001	0.0008	ns	ns
Harvest date × 1-MCP		<0.0001	<0.0001	ns	ns
Temperature × 1-MCP		<0.0001	ns	ns	ns
Harvest date × temperature × 1-MCP		<0.0001	ns	ns	ns

Table 6.3. Percentage external CO_2 injury (Ext CO_2), decay, core browning (CB), firm flesh browning (FFB), other losses (Others) and total fruit loss (total loss) of 'Empire' apples with and without 1-MCP treatment after 9 months in CA storage plus 7 d at 20 °C.

Harvest (2012)	Treatment	Ext. CO_2 (%)	Decay (%)	CB (%)	FFB (%)	Others (%)	Total loss (%)
Sept 21	2 °C	1.3	0	0.4	5.1	2.2	9.0
(H1)	2 °C + 1-MCP	2.4	1.9	0	9.5	1.4	15.2
	0.5 °C	0	0.5	0	1.4	0	1.9
	0.5 °C + 1-MCP	0	2.8	0.9	2.8	1.4	7.9
Sept 28	2 °C	0	1.0	0	9.5	2.1	12.6
(H2)	2 °C + 1-MCP	1.6	0	0	19.5	0	21.1
	0.5 °C	0	0	2.0	9.2	0	11.2
	0.5 °C + 1-MCP	0	1.8	0.7	12.4	2.0	16.9
Oct 5	2 °C	0	9.9	0.8	16.5	5.3	32.5
(H3)	2 °C + 1-MCP	0	4.0	3.6	51.6	3.0	62.2
	0.5 °C	0	10.7	2.1	20.9	0.9	34.6
	0.5 °C + 1-MCP	0	5.3	5.7	24.6	2.1	37.7
Harvest date		0.0415	<0.0001	0.0002	<0.0001		<0.0001
Storage temperature		0.0037	ns	ns	0.0067		0.0030
1-MCP treatment		ns	ns	ns	0.0254		0.0033
Harvest date × temperature		ns	ns	ns	ns		ns
Harvest date × 1-MCP		0.0402	ns	ns	ns		ns
Temperature × 1-MCP		ns	ns	ns	ns		ns
Harvest date × temperature × 1-MCP		ns	ns	ns	ns		ns

Losses due to flesh browning increased with later harvest and with the application of 1-MCP (Table 6.3). Increased losses due to treatment with 1-MCP has been found previously (DeEll et al., 2007; Jung et al., 2010; Jung and Watkins, 2011). Jung et al. (2010) found more browning in 'Empires' stored at 0.5 °C than at 2 °C, but there was less browning at 0.5 °C compared to the warmer storage temperature when treated with 1-MCP. However, storage at cooler temperatures does not consistently affect flesh browning as shown in this study (Table 6.3). Additional fruit losses were caused by low incidence of external carbon dioxide injury, while decay and core browning incidences tended to increase as harvest dates progressed (Table 6.3). Overall fruit losses also account for minor losses due to core rot, water core breakdown and senescence

breakdown (data not individually shown). Increased levels of flesh browning in 1-MCP treated fruit may be associated with inhibited ethylene production by the fruit (Jung and Watkins, 2011). Total percentage fruit loss was higher at H3 and losses were exacerbated with 1-MCP treatment at both 0.5 °C and 2 °C.

6.3.2 Cost model and fruit pricing

The information shown in Table 6.4 provides much of the detail used in our spreadsheet model to calculate the profits associated with each of the three harvest dates. The production costs are based on those used in the analysis by DeMarree et al. (2010) for high density 'Gala' production; the costs for marketing and storage follow those used by the Ag Profit budget template 1022 (Oregon State University) and Gallardo and Galinato (2012). In our model the costs per hectare are specific to the number of trees per hectare and for the 'Empire' cultivar. However, the spreadsheet model can be generalized and extended to consider the costs for alternative orchard systems and for different varieties.

Table 6.4. Estimated costs for management, production, harvest, storage, and marketing of 28 tons production for a one-hectare orchard block with approximately 5313 trees (costs for 1-MCP treatment are not considered in base costs).

Name	Costs per unit ($)	Units per hectare	Costs per hectare ($)
Labor (ton)	100	28	2,254
Seasonal quality control (h)	11.47	12	137.64
FT* truck/tractor driver (h)	17.54	12	210.48
FT tractor driver (h)	14.37	12	172.44
PT** truck/tractor Driver (h)	12.71	12	152.52
Interest on operating capital (ha)	1,790	-	1,790
Disease control (Fungi, Insect/mite, Herbicides) (ha)	1,329	-	1,329
Chemical thinners (ha)	279	-	279
Fruit thinning/return bloom (ha)	1,483	-	1,483
Fertilizer (ha)	543	-	543
Total overhead expense (ha)	919	-	919
Average equipment investment replacement-1 year-1 (ha)	692	-	692
Operators' management only (ha)	704	-	704
Total annual equipment expense (ha)	514	-	514
Marketing, & packaging (ton)	24.76	28	693.75
Sorting & Storing Bins (Ap) (ton)	13.18	28	369.42
1-MCP (SmartFresh) (1.4 m3)	9.50	62	589
Costs per ha for fresh marketed fruit:			$11,874
Costs per ha for fruit marketed after 9 months CA-storage:			$12,243
With 1-MCP treatment:			$12,832

[*Full time (FT); **Part time (PT)]

To calculate revenue, we calculated average prices for each grade and size using 10 recent years of 'Empire' market prices. Because of the low yields in 2012, we assumed that fruit prices were 150% of the average 10-year values that we calculated (Table 6.5). By comparing the 10 year average price data to the available 2012 'Empire' market price data, we see the 2012 prices were between 135 and 175% higher. Data suggest that this is a conservative assumption for the 2012 prices, but was performed to prevent

overestimation of revenue in 2012; furthermore, this assumption was made in the analysis for all three harvest dates and adjustments in this assumption do not change the general pattern of our results. The costs per hectare and per ton are shown in Table 6.4. Costs for sorting, storing, marketing and packaging the fruit were calculated per ton; when needed we adjusted cost information that is commonly recorded per box to the per ton equivalent. The calculations are carried out based on 28 tons per hectare to account for the 50% lower yield in 2012. A 5,313 trees per hectare planting of 'Empire' averages approximately 52 tons in "normal" years.

The costs for one hectare are $11,875 without application of storage or 1-MCP application. Including costs for storage materials increases the total costs to $12,832 and $12,243 with and without 1-MCP, respectively (Table 6.4). The costs shown for spray amendments for pests, diseases and weeds are average values but are held constant across the costs for the three harvest dates. These costs might not reflect the exact costs for all growers in 2012, but provide a general idea of the application costs per hectare in a typical year in New York State. The costs for thinning and return bloom sprays are very high, but represent a reasonable investment in a high-density orchard given that correcting the crop load in a given year will ensure a good crop load in the following year (Denne, 1960) even though 2012 was a year in which return bloom spray applications might not have been needed.

Table 6.5. Unit prices per box (18.1 kg) for an average year and 2012, a low cropping year, and pack-out for fruit at harvest.

# fruit box^{-1} (unit)	**Average fruit weight (g)**	**\$ Unit^{-1} (10 year average)**	**\$ Unit^{-1} (2012)**	**Pack-out Sept 21 (H1)** (# of fruit)	**Pack-out Sept 28 (H2)** (# of fruit)	**Pack-out Oct 5 (H3)** (# of fruit)
Extra Fancy (ExFy)						
163	116	13.02	19.39	5,529	5,569	4,244
150	128	14.13	21.05	7,186	7,664	6,704
138	136	13.37	19.93	7,676	8,837	8,650
125	153	13.64	20.32	7,697	9,378	10,551
113	167	18.00	26.82	6,080	8,031	9,688
100	190	17.38	25.90	3,301	4,628	5,939
88	215	18.08	26.94	1,103	1,651	2,255
80	238	18.68	27.84	304	473	773
72	264	18.87	28.12	84	107	182
64	298	20.86	31.08	8	18	22
1.1 kg bags	126	14.12	21.05	2,823	2,525	1,437
1.25 kg bags	114	12.48	18.59	3,091	2,821	1,935
% total fruit				62.6	73.1	79.8
Fancy (Fy)						
138	136	8.55	12.74	2,966	1,884	1,118
125	153	8.30	12.37	3,113	1,923	1,288
113	167	6.43	9.58	2,102	1,379	1,071
100	190	7.96	11.86	984	749	575
88	215	6.57	9.78	361	225	167
80	238	7.54	11.23	35	52	47
1.1 kg bags	126	10.60	15.79	4,997	3,008	1,388
% total fruit				20.3	13	8.6
Commercial/Juice (C)						
1000		4.00	5.96	12,280	9,815	7,574
% total fruit				17.1	13.9	11.5
Total number of fruit:				71,720	70,737	65,608

6.3.3 Changes in apple size and grade distribution

Fruit grade prices per box were based on the average price between 2002 and 2011, and the average price was then used to estimate the market prices in 2012 (based on a 50% premium on the 10-year average price). The increase of 50% is based on the available pricing for 2012, which in average was close to 55% higher compared with the average 10 year price. To guarantee no over estimation of the income a slightly lower increase was assumed. Using a 10 year average price is a reasonable approach here given the small range of fluctuation observed across apple grades and sizes over this time period, and reflects a general approach employed by other researchers studying the effects of production and management systems in orchards (Baritelle and Price, 1974). Variation in fruit prices depends largely on seasonal changes in supply and demand; years with low supplies lead to increases in fruit prices and 2012 is a good example of such a case given the amount of frost damaged fruit and yield losses due to frost. In our analysis, we assume two sets of prices for fruit that comes out of storage. In the first case, we assume that prices are constant throughout the year using the prices shown in Table 6.5 (that is, prices are the same for fruit sold in the fall or for fruit that had been stored and sold in spring); in the second case, we consider the effects when the prices for fruit that come out of storage are 10% higher than fruit sold in the fall (see Table 6.6).

Fruit size and color changes over the time of the experiment are shown by the distribution of ExFy and Fy fruit (Table 6.5). At the third harvest (H3) almost 80% of the fruit from the air stored field bins were in ExFy, compared with 63% and 73% for H1 and H2 respectively. This is mainly due to the fact that apple fruit are still increasing in

diameter late in the season (Bain and Robertson, 1951; Denne, 1960; Schechter et al., 1993). Fruit color is influenced by many factors such as cultivar strain (genetics), as well as light conditions and day/night temperatures (Lancaster and Dougall, 1992; Lancaster et al., 1994). Therefore, the time a fruit grows on the tree will influence fruit size and coloration of the fruit and, therefore, influence the pack-out for a given crop. Based on pack-out data at harvest, or shortly thereafter, the increase in size and color, increased amount of fruit within the category of ExFy, is desirable. However, there is a potential economic tradeoff from delaying harvest. Delaying harvest to obtain larger fruit may increase the likelihood that the stored fruit will experience postharvest physiological disorders, and this has the capacity to decrease the overall value of the crop.

The increase of fruit in the ExFy category, with a bigger diameter and lower fruit count per box, is greatest in the last harvest date (Table 6.5). Smaller fruits as well as fruit of good size, with slight defect/imperfections of shape or coloration, typically are packed in pre-packed bags of 1.1 kg; the amount of fruit sorted into this category was much higher in H1 and decreased over the next two harvests. The outlet of fruit through pre-packed bags is used for slightly lower quality fruit with less coloring. The fruit still may qualify as ExFy but market demand for less colored fruit (that only marginally reach a minimum color standard) is lower and often leads to this fruit being marketed in the pre-packed bags.

6.3.4 Economic effects of harvest dates and storage methods

The net economic effects of delayed harvest for one hectare high density 'Empire' orchard block are shown in Table 6.6. If fruit are marketed directly in the fall or after a relatively short period of time in storage (air or CA), the results show that there are net benefits associated with later harvest dates. More specifically, our results show an increase of more than $3,000 for fruit from H3 compared with that from H1, due to the higher amount of larger fruit in the ExFy. This assumption is based on calculations assuming every fruit could be sold in its category for the assumed price as stated in Table 6.5, for example: an individual fruit sorted into ExFy 100 count box would theoretically be sold for $0.259 per fruit, due to the assumption of box price = $25.90 and 100 fruit per box = $0.259 per fruit. The results also show that there would be a net loss from marketing late harvested fruit after long term storage (Table 6.6). Even when we assume higher prices for fruit stored long term, we still see a significant net loss in H3. The long-term stored fruit from H3 were very soft, and this is an important determinant of prices for stored fruit as firmness (or crunchiness) is one of the factors that are most important to consumers (Rickard et al., 2013; Yue and Tong, 2011). Fruit quality as such was not incorporated into the model, since specific parameters such as fruit minimum firmness can change depending on the market. Never the less marketing poor quality fruit with suboptimal flavor can lead to consumer disappointment that can last for a long time. The income increase from H1 to H2 is relatively small after storage. But flesh browning incidences are much lower for fruit harvested early and stored at the lower temperature.

Table 6.6. Revenue due to harvest date and marketing strategy; marketing strategies at harvest or after nine months in CA storage based on percentage loss due to flesh browning for fruit storage at 0.5 or 2 °C with and without 1-MCP treatment; for losses due to storage disorders it was assumed that all grades and counts would be affected evenly. A 10% price premium is assumed for fruit marketed in June

	Revenue per hectare*** ($)		
Fruit marketed	**Sept 21 (H1)**	**Sept 28 (H2)**	**Oct 5 (H3)**
At harvest	10,558	12,988	13,815
After 9 month in storage (June)			
2 °C	8,223	9,558	5,260
2 °C + 1-MCP	6,273	6,898	-2,818
0.5 °C	9,775	9,898	4,730
0.5 °C + 1-MCP	7,870	7,918	3,355
Assuming 10% price increase in June			
2 °C	10,268	11,735	7,000
2 °C + 1-MCP	8,178	8,863	-1,843
0.5 °C	11,980	12,110	6,415
0.5 °C + 1-MCP	9,940	9,990	4,960

*** An area of 0.4 hectare were harvested at each harvest and the numbers extrapolated

Decreased revenue for 1-MCP treated fruit compared with untreated fruit is the result of two factors. First, there is a slightly higher percentage of flesh browning and other disorders in the 1-MCP treated fruit, and second, there are slightly higher costs (an increase of approximately $693 per hectare) associated with 1-MCP treatment. However, the treated fruit that were not affected by the disorders exhibited more marketable levels of acidity and firmness, and therefore were of higher quality and may have received higher prices in the market. Although we did not model the effects of this higher quality fruit with higher prices, we expect that this consideration may generate additional revenue that may equal or exceed the increased cost ($693 per hectare) of treating the fruit. Additional research that provides specific measures of fruit quality is needed to better

understand the relationship between 1-MCP treatments and grower profitability for selected harvest dates across different varieties.

In summary, the results from this research show that there is an economic tradeoff between harvest date, the occurrence of flesh browning, and profitability for 'Empire' apples. There are net benefits associated with later harvest dates for fruit that is marketed earlier in the season. However, these benefits disappear for fruit that is stored long-term because of greater risk of flesh browning development; the revenue earned for more saleable fruit with less size and less color outweighs the benefits of greater size and more color. Overall, harvesting the fruit as early as one week prior to the conventionally accepted harvest date sacrifices skin color and fruit size, but can yield higher profits for industry stakeholders if fruit are stored for extended periods (nine month) in CA storage.

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CHAPTER 7

SUMMARY AND FUTURE WORK

Apple fruit maturity is one of the most important factors that influence post-storage quality of fruit. Some storage disorders such as browning of 'Gala' and 'Empire' or senescent breakdown are enhanced by greater maturity, while others such as external CO_2 injury are less severe. Nevertheless ultimately consumer preferences for aroma, color and fruit firmness, determine the maturity at harvest of any cultivar required to meet market demands for quality.

Each cultivar has specific maturation and ripening patterns, and these have not been fully characterized for most cultivars. A key change during this development stage is starch hydrolysis, often assessed using the SPI after the flesh has been stained with iodine. In the research described in this thesis, the relationships between the SPI and starch concentration in fruit has been investigated during maturation and ripening. In Chapter 1, starch degradation during maturation and ripening of four cultivars, 'Gala', 'Honeycrisp', 'McIntosh', and 'Empire', was studied. The rate of starch degradation alone does not explain the differences among cultivars during ripening but the onset of degradation happened at similar time points while progressing slower in the later ripening cultivars. Starch concentrations declined linearly in all cultivars and the decline between the first harvest date (August 16) and the second harvest date (August 24) was large for all four cultivars. Even the later maturing cultivar 'Empire' had a greater decline between those two dates than during any other week throughout the remainder of the season. The subsequent changes in starch concentration over time were generally much smaller.

Changes in starch concentrations are accompanied by changes in starch composition, as shown previously in 'Fuji' (Fan et al., 1995). This could be due to differences in digestibility of starch within the starch granule, or to levels of enzyme activity within the cells. Therefore, future research could focus on changes in gene expression and activity of enzymes associated with starch accumulation and degradation during development to help understand developmental differences between cultivars. Such knowledge might provide a better understanding of the events leading to, and controlling, starch degradation.

MATLAB® based image analysis showed a linear relationship between calculated stained areas and SPI values of the four cultivars. Future work could focus on developing a version of this calculation to assess SPI patterns with a phone or on the computer, and therefore, eliminate some of the subjectivity associated with visual evaluation of SPI. Furthermore, improved consistency of SPI readings could lead to better prediction models for harvest timing and susceptibility of fruit to storage disorders.

Maturation and ripening differences between the stem- and calyx-end of the fruit were investigated in Chapter 3. 'Empire' fruit show differences in starch concentration between the stem and the calyx of the fruit, while differences were not detected for 'Honeycrisp'. These limited differences could have been due to a comparatively later start during maturation of 'Honeycrisp' fruit. Postharvest treatments of 'Gala' fruit with propylene or 1-methylcyclopropene (1-MCP) did not change the overall difference in starch concentration between the ends of the fruit, but 1-MCP slowed down starch degradation in all three zones compared with the untreated control and propylene treated fruit. The rate of starch degradation was not significantly different between fruit treated

with propylene and the untreated control. In future research, the analysis of developmental differences throughout fruit development, from a stage earlier such as 60 days after full bloom (DAFB), might provide insight into developmental changes within the fruit. Previous studies have shown that starch deposition during development starts near the skin – the outer part of the cortex (Kovács and Eads, 1999; Ohmiya and Kakiuchi, 1990) and that degradation was either simultaneous throughout the fruit or slower or faster in the core compared with the cortex. Understanding of processes associated with starch deposition and degradation between the zones of the fruit therefore is not entirely clear. A better understanding of timing of events during development could be achieved by following starch synthesis and degradation throughout the growing season of fruit in three or four cultivars with different ripening patterns; for example an early ripening cultivar such as 'Gala' or 'Minnewashta' (Zestar!®), a mid-season ripening one such as 'McIntosh' or 'CrimsonCrisp®' (Co-op 39), and a relatively late cultivar such as 'Empire' or 'Red Delicious'. Sampling should be started simultaneously early in the season, after about 60 DAFB and changes in gene expression, enzyme activity, and starch concentration investigated, The stem and calyx-ends of each fruit, as well as core and flesh tissues should be sampled to discern differences between zones of a fruit..

Treatment of fruit with AVG (ReTain®) is common to manage harvest timing, and 1-MCP (Harvista™) is being used increasingly. The effect of these treatments on the SPIs and starch concentrations within 'McIntosh' and 'Empire' fruit was investigated in Chapter 4. Effects on starch degradation as well as SPI were minimal in both cultivars and effectiveness of the treatments in prolonging the onset of climacteric rise cultivar and application timing dependent. Effects of PGRs on starch degradation could also be

included in the above proposed experiment and therefore include more insights into the effects of ethylene on starch degradation. As shown by Johnston et al. (2009), starch degradation is very ethylene sensitive, and a better understanding of the whole system will lead to clarification about inhibition of starch metabolism through ethylene manipulation and its effects on maturation.

Non-destructive methods are being developed to measure maturation. Such methods can be used on many more fruit and even on every fruit at harvest so that storage potential could be identified. Measurements of difference in absorbance (I_{AD}) with the DA meter provide an index number which correlates well with chlorophyll a (chl a) concentration in the skin. Yet what the number exactly represents in means of harvest maturity needs to be assessed for each cultivar, and possibly for each growing region. In Chapter 5, correlations between I_{AD} values and other maturity indices such as IEC, fruit firmness, and SPI were evaluated for 'Empire' fruit treated with preharvest AVG and 1-MCP. I_{AD} values of individual fruit had poor correlations with the other assessments, but fruit grouped by I_{AD} range prove to have better correlations with other indices. Most critically, PGRs altered the relationships between I_{AD} values and other factors, most importantly ethylene. Therefore, variable use of PGRs by farmers could have a major effect on interpretation of data. A more extensive study on changes of I_{AD} values over time, how they relate to other indices such as IEC and firmness, as well as SPI needs to be obtained in order to fully understand the I_{AD} values. Fruit from different regions, orchards, and growing systems need to be used to understand the changes in chl a concentration in the peel and I_{AD} measurements. Incorporation of chemical changes, such as chl a and anthocyanin concentration in the peel, would give a full picture of ripening differences

and how it can be measured through changes within the skin.

'Empire', an important cultivar in New York, often develops firm flesh browning during long-term storage in CA of about seven to nine months. No storage regime that controls the disorder has been identified. Earlier studies suggested that warmer storage temperatures such as 3 °C might cause less browning, but faster softening of the fruit leads to unmarketable quality. However, 1-MCP applied postharvest can enhance browning at these higher temperatures. Earlier harvest of less mature fruit seems promising in controlling the issue to a certain extent. However, fruit are sorted and marketed according to the US grading system, and more colored, larger fruit bring higher revenue to the producer. Therefore, in Chapter 6, a full economic analysis of the effects of harvest one week prior and one week post optimum commercial harvest for fruit in this block was performed. Fruit were stored with and without 1-MCP treatment at 0.5 °C and 2 °C. The results show that fruit harvested later than estimated optimal commercial harvest date for CA storage are larger, and have a greater amount of red color. Therefore, if fruit are marketed soon after harvest the income can be higher, for fruit harvested at enhanced maturity. If fruit are stored for up to nine or ten months in CA storage fruit with lower maturity develop less browning and other disorders. Revenues for the first (early) and "normal" harvest had enhanced quality after storage compared with the later harvested fruit and differences in revenue were mostly compensated through greater amounts of marketable fruit. A follow-up project could develop a spreadsheet that allows growers and storage operators to calculate the potential change in revenue through earlier harvest. The calculations would have to be based on estimates for the changes in fruit color and size from proposed earlier to the regular harvest. In addition, collecting storage

browning data to create a model which is based on seasonal data such as growing degree days (GDDs), amount and/or distribution of precipitation, could help clarify for the grower and storage operator whether to expect a severe or less severe browning year. The spreadsheet combined with the model could function as decision-making tools of when to harvest and for how long to store the fruit.

Three seasons of research has yielded much new information and knowledge on starch concentration changes within apple fruit and its relation to fruit maturation. Effects of preharvest and postharvest applied ethylene suppressors have proven to be application timing and cultivar dependent especially for changes related to the SPI. With analyzing effects of harvest time not only on storage disorder development but also on the potential revenue changes for the grower, the study in chapter 6 took on a whole different aspect of fruit storage research. In summary, using starch as a relatively easy measure of indicator of fruit maturity and therefore as harvest index certainly has proven to be a useful tool.

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