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# INTERACTIONS OF AVG, MCP AND HEAT TREATMENT ON APPLE FRUIT RIPENING AND QUALITY AFTER HARVEST AND COLD STORAGE

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# ABSTRACT OF DISSERTATION

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The Graduate School

University of Kentucky

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By
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Lexington, Kentucky

Director: Dr. Douglas D. Archbold, Professor of Horticulture

Lexington, Kentucky
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# ABSTRACT OF DISSERTATION

## INTERACTIONS OF AVG, MCP AND HEAT TREATMENT ON APPLE FRUIT RIPENING AND QUALITY AFTER HARVEST AND COLD STORAGE

The effects of AVG, an inhibitor of ethylene synthesis, in combination with MCP or heat treatment (HT) on quality traits of several apple cultivars after harvest (AH) or cold storage (ACS), and the involvement of ethylene in the regulation of SDH activity during the last weeks of fruit development were studied. AVG was applied to 'Royal Gala', 'Lodi', 'Senshu', 'Redchief Delicious' and 'Red Fuji' trees 4 weeks before normal harvest (H1). Control and AVG-treated (AVG) fruit were harvested at H1 and treated with MCP or air-heated. Fruit were ripened at room temperature (RT) AH or ACS. Some AVG fruit were harvested at H1 and 1 to 2 weeks after H1 (H2), or at H2 only. Ethylene production (EP), respiration rate (RR), firmness, starch index (SI), titratable acidity (TA), volatile production (VP) and AAT activity, among others, were measured AH and ACS. Peel and cortex of 'Gala' were alcohol-fed and ester production quantified. EP and SDH presence and activity were measured at various harvest dates on control and AVG 'Lodi', 'Red Delicious' and 'Fuji' apples. AVG plus MCP was more effective in reducing HEP, RR, firmness and TA loss than either treatment alone; it did not provide further control on SI and did not repress 'Gala' red skin color development more than AVG alone, though it consistently repressed VP. AVG plus HT was generally more effective than single treatments in reducing HEP, RR and firmness loss during storage. It was not different than the single treatments on TA, SI, and VP. The effect of AVG plus HT on fruit quality ACS was cultivar-dependent. AVG plus HT was not enough to maintain the quality of the early-harvest cultivars, and it did not improve fruit quality of late-harvest cultivars. The effects of AVG plus MCP but not of AVG plus HT were evident at H2. Precursor availability was the major factor limiting VP, suggested by the low VP when RR was low, the increase in ester production in alcohol-fed samples, and the lack of correlation between AAT and ester production. SDH activity or expression was not affected by a reduction in ethylene production.

KEYWORDS: *Malus domestica* Borkh, alcohol-acyl tranferase, sorbitol dehydogenase, aminoethoxyvinylglycine, 1-methylcyclopropene

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# DISSERTATION

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# DEDICATION

This is dedicated to my grandmother Maria Julia Vitale de Escalada, who will always live in my heart, and to my friend Mariel Betina Orlando who was a constant support from Argentina during all these years.

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# CHAPTER 1

# LITERATURE REVIEW

## Apple production and utilization in the United States

Apple *(Malus sylvestris var. domestica)* is the most economically important fruit crop in the United States. According to the U.S. Apple Association (2005), the record apple crop in the U.S. occurred in 1998 and reached 277.3 million bushels. The apple crop in 2004 was 248.1 million bushels of which almost 52% was produced in Washington State. Fresh market apple varieties represented 75.4% of the total crop harvested in 2004, while production of dual-purpose varieties accounted for only 14.9%. 'Red Delicious' is the most widely grown cultivar in the U.S., representing 24.9% of the total production in 2004, followed by 'Golden Delicious' at 11.4%, 'Gala' at 9.2% and 'Fuji', 'Granny Smith', and 'McIntosh', all at 4.4%. The production of 'Red Delicious' and 'Golden Delicious' is expected to decrease, while that of 'Gala', 'Granny Smith' and 'Fuji' is expected to increase. Nationally, 'Gala' production was forecast to increase up to 4% in 2005. The 2005 U.S. apple utilization per capita was 49.5 pounds per person, 6.4% higher than in 2003. Of the 2004 apple crop, 62.8% was sold in the fresh market, 36% was processed into apple juice, canned, dried and frozen apples, and apple slices, and only 0.9% was not marketed.

## Harvest and postharvest handling systems of apples

### *Generalities and harvest maturity index*

Both quantitative and qualitative losses occur in horticultural crops between harvest and consumption, and in the United States postharvest losses of fresh fruit and vegetables can range between 2% and 23%, depending on the commodity (Kader, 2005). In 1995, fresh fruit and vegetable losses accounted for almost 20% of consumer and foodservice losses, due mainly to product deterioration, excess perishable products that were discarded, and food not consumed by the purchaser, the latter often linked to consumer dissatisfaction with product quality, especially flavor (Kantor et al., 1997). For example, Washington State 'Delicious' apple sales and market shares have experienced significant losses in recent years, and this has been partially attributed to lack of post-storage quality and its influence on consumer acceptability (Fellman et al., 2003). Biological causes of deterioration include high respiration rate, excessive ethylene production

and action, high rates of compositional changes (associated with color, texture, flavor and nutritive value), mechanical injury, water stress, physiological disorder and pathological breakdown. The rate of biological deterioration depends on environmental factors, including temperature, relative humidity, air velocity, atmospheric composition and sanitation procedures (Kader, 2005).

The development of many biological disorders depends on the stage of fruit maturity at harvest. Suggested maturity indices include skin color, seed color, cortex firmness, soluble solids content, starch content, titratable acidity content, respiratory rate and ethylene production, as well as days after full bloom, accumulated heat units as degree days above 7$^{o}$C, and various combinations of these. All possible maturity indices have limitations. For example, measurable ethylene production may occur too late or is too variable to be a useful tool for timing of harvest. The most commercially used maturity indexes include days from full bloom as a rough guide and firmness and starch index as more time-refining tools. Maturity standards used for harvest depend on future handling of the fruit, like immediate marketing, short-term air storage or long-term controlled atmosphere storage (Mitcham and Mitchell, 2002). Early harvest, together with cold storage, can slow down ripening but with the cost of smaller, poorly-colored and poorly-flavored fruit which are more susceptible to bitter pit (Peirs et al., 2002). The causal relationship between bitter pit, a physiological disorder, and early harvest has not been established. Late harvested fruit are softer and mealy, with a higher risk of internal breakdown (Peirs et al., 2002) and controlled atmosphere-related disorders (Mitcham and Mitchell, 2002). Starch index is currently used as the harvest guide of 'Granny Smith' apples in California, Washington and New Zealand, but unfortunately the scales used in different places are not uniform (Mitcham and Mitchell, 2002). The few obstacles previously mentioned provide insight into the problems associated with developing a reliable, practical standard maturity index for each apple variety and region. General guidelines for harvest maturity of some apple cultivars are listed in Table 1.1.

***Regular vs. controlled atmosphere storage***

A fraction of the annual apple production is immediately marketed, while the rest is stored for later utilization. The U.S. Apple Association (2005) anticipated that by December, 2005, 59% of the predicted 2005 apple crop, representing 139 million bushels, would have been held in cold storage, and about 79% of these holdings would have been in controlled atmosphere

(CA) storage. Regular air storage of apples consists of holding the fruit at 0°C to 4.5°C depending on the cultivar, though most cultivars are safely stored at 0°C. Controlled atmosphere consists of lowering oxygen partial pressure and/or increasing $CO_2$ partial pressure in the cooled chambers. Adequate concentrations of $O_2$ and $CO_2$ are cultivar-dependent and may vary from 1% to 2.5% $O_2$ and 1% to 4% $CO_2$. Maturity at harvest is an important characteristic to consider in CA storage. Fruit that are too ripe are more susceptible to developing CA-related disorders (Mitcham and Mitchell, 2002). Controlled atmospheres are often used when apples are stored for over three months, but benefits might be seen even in shorter storage times, depending on the cultivar.

**Table 1.1:** General guidelines for harvest maturity of some apple cultivars. Adapted from Mitcham (1998).

| | Firmness (Newtons) | Starch Index (0-6, high to none starch) | Ground color |
|---|---|---|---|
| Red Delicious | | | |
| Storage | 76 to 80 | 2 to 3 | Light green or white |
| Immediate market | 71 | 4 | White |
| Golden Delicious | | | |
| Storage | 71 to 76 | 2 to 3 | Greenish – white |
| Immediate market | 67 | 4 | Yellow-green |
| Fuji | | | |
| Storage | 71 to 76 | 4 to 5 | Light green |
| Immediate market | 67 | 5 to 6 | Light green to white |
| Gala | | | |
| Storage | 76 to 80 | 1 to 2 | Light green |
| Immediate market | 67 | 3 to 4 | Light yellow to white |
| Granny Smith | | | |
| Storage | 71 to 76 | 2 to 3 | Not applicable |
| Immediate market | 67 to 71 | 4 to 5 | Not applicable |

**Acceptability and edibility of apple fruit**

Given the increasing demand for high quality produce, maintaining the quality of apples during short and long term postharvest storage would result in great benefits for both growers and consumers. There are several quality factors influencing the acceptability and edibility of apple fruit, such as appearance, texture and flavor. First-time purchases are often based on

appearance and firmness, but repeat buys are determined by internal quality traits such as mouth-feel and flavor (Baldwin, 2002). Maturity at harvest will influence the later quality of the fruit and consumer acceptability.

Color is a major characteristic of appearance, and its development is cultivar and environment-dependent (Layne et al., 2002). Non-destructive measurements of color are based on determination of the characteristics of light that are transmitted or reflected by the fruit. Objective measurements can be made with chromameters which measure color parameters in different color space-coordinates, such as x y z, L* a* b*, L a b and L C h. Of these scales, L* a* b* is one of the most popular, where L* indicates lightness and a* and b* are the chromaticity coordinates (red-green and yellow-blue, respectively). Subjective visual color scales can also be used to assess color. For example, Layne et al. (2002) classified apple fruit on a 1-4 scale representing different percentages of red color covering on the surface, and Wang and Dilley (2001) established an index for development of ground color, where 1 and 5 corresponded to green and yellow, respectively.

Firmness is an important component of texture (Kader, 2002), and is also closely related to internal textural qualities like crispness and mealiness (Saftner et al., 2002). Texture attributes that define the feel of the fruit in the mouth are experienced during mastication, which causes a breakdown of the tissue composed mainly by parenchyma cells. The presence and structural integrity of the cell wall plays a major role in the perception of texture (Redegwell and Fischer, 2002). The strength of primary cell walls and wall-to-wall adhesion between cells are the main factors affecting the integrity of the fruit cell, and the latter is considered to be the most critical one influencing the perception of fruit texture (Diehl and Hamann, 1980; Pitt and Chen, 1983).

Flavor is a complex trait composed of sweetness, sourness, bitterness, saltiness and aroma (Baldwin, 2002). Of these traits, sweetness can be evaluated through total soluble solids content (SSC), sourness (acidity) through total titratable acidity, and aroma through the quantification and identification of volatile compounds (Kader, 2002). The sourness-acidity was related to apple-fruity flavor, acceptability of flavor, and overall acceptability by a test panel (Saftner et al., 2002). According to Hulme and Rhodes (1971), taste in pome fruits is principally based on acid-sugar balance, evaluated through sugar:acid ratio. However, phenolics compounds may also contribute to flavor (Paillard, 1990). There exists an increasing concern of consumers about fruit sensory quality deficiencies. When comparing harvest times, panelists assigned high

ratings for sourness and firmness to early harvested ‘Gala’ and ‘Fuji’ apples, but these were always associated with a low rating for overall desirability, sweetness and flavor, implying that high firmness and sourness do not necessarily correspond to high consumer acceptability (Plotto et al., 1997). Table 1.2 lists the suggested Washington Grade Standards for marketing different apple cultivars, based on consumer response to apple quality.

**Table 1.2:** Washington grade standards for apples, adapted from Kupferman and Harker (2001). Minimum standards were set with 10% tolerance based on consumer acceptability.

| Cultivar | Firmness (N) | Soluble Solids (%) | Starch Index (0-6) |
|---|---|---|---|
| Red Delicious | 58 | 12.5 | 4.0 |
| Golden Delicious | 49 | 12.5 | 4.0 |
| Fuji | 56 | 13.0 | 4.0 |
| Gala | 49 | 12.5 | 4.0 |

**Apple ripening**

The ripening of apples is a period of physiological and structural changes characterized by softening of the cortex, starch hydrolysis, increases in soluble sugars, changes in acid composition, chlorophyll degradation, enhanced color development, membrane changes, specific protein synthesis, and increased ethylene ($C_2H_4$) production, respiration rate, and aroma volatile synthesis (Brown et al., 1966; Brady, 1987; Lurie, 1998a, b).

Cortex softening during ripening occurs in almost all fruits and involves cell wall changes not seen in leaf senescence. It is commercially very important because increasing softness limits the postharvest life of the fruit by enhancing physical deterioration during handling and increasing the fruit susceptibility to diseases (Brady, 1987). Fruit softening could be due to loss of turgor, degradation of starch or breakdown of fruit cell walls. Loss of turgor is associated with the postharvest dehydration of the fruit and starch degradation is important in fruit like banana, where starch accounts for a large percentage of the fresh weight. In general, however, fruit softening is the result of partial cell wall dissassembly (Tucker, 1993).

Apple cell walls consist mainly of cellulose and pectin, with some hemicellulose and a very small amount of extensin (Knee and Bartley, 1981). During ripening, compositional changes in cell walls are restricted to the pectic polymers, with no changes documented in cellulose or hemicellulose (Bartley, 1976). Changes mainly involve the dissolution of the pectin-

rich middle lamella (Tucker, 1993). During ripening neutral sugars are lost, mostly galactose and some arabinose, which are the main components of the cell wall's neutral pectin. Additionally, changes can be observed in the acidic portion of cell wall pectin, rhamnogalacturonan, which can be progressively depolymerized (Tucker and Grierson, 1987). Of the cell wall hydrolases, pectinesterase, exopolygalacturonase, β-galactosidase and β-1,4 glucanase or cellulase are the only ones that have been convincingly proven to be active in apple fruit, whereas no endo-polygalacturonase has yet been found, in agreement with the high molecular weight soluble pectin found in ripening apples (Knee, 1993).

In fruit, sugars and organic acids contribute to the fruit taste and are used as respiratory substrates. The most common sugars are glucose, fructose and sucrose, accounting for 2, 6 and 4 % of the fresh weight, respectively (Tucker, 1993). Carbohydrates in developing apple fruits are imported as sorbitol and converted mainly to sucrose and starch, though some sorbitol might persist in mature fruit (Berüter, 1985). Starch is hydrolyzed into soluble sugars during the last stages of fruit growth increasing the concentration of free sugars (Berüter, 1985; Knee et al., 1989), and later more glucose and fructose are formed from sucrose (Whiting, 1970). A number of enzymes could be involved in the conversion of starch to sugars towards the end of the pome fruit growing phase, like β- and α-amylase (Knee, 1993), though it is possible that starch phosphorylase, detected in apples, may be more important than the amylases (Preiss, 1982). Sugar content, expressed as SSC, is an important factor in apple quality. Sorbitol dehydrogenase (SDH) is a key enzyme that converts sorbitol (the main imported sugar in the fruit) into fructose, and its activity in 'Fuji' apple has been found to be low in young fruit and increase close to harvest, remaining high from 160 to 207 days after bloom (Yamaguchi et al., 1996; Yamada et al., 1999). The rise in activity might depend upon the expression of the *SDH* gene (Yamada et al., 1999), but it is not known whether ethylene is involved in the regulation of this process.

Organic acids could accumulate through metabolism of imported carbohydrates and amino acids, as well as by fixation of $CO_2$ through the action of phosphoenol pyruvate carboxylase. Organic acids can also be metabolized (Knee, 1993), and in general decline during ripening due to their utilization as respiratory substrates (Ulrich, 1970). The major organic acid in apples is malate, which can be found at concentrations of 3-19 μeq 100 $g^{-1}$ (Tucker, 1993), and serves as an important substrate for respiration, falling by 50% during the life of a fruit (Knee, 1993). Some apples can also contain appreciable amounts of citric acid (Ulrich, 1970).

In general, color change is associated with ripening, although many varieties of apple remain green. Anthocyanins, chlorophylls and carotenoids are the major pigments of pome fruit. Anthocyanins are a diverse range of pigments localized within the vacuoles of the cells and give rise to colors from blue to red. Red skin coloration of apples is directly related to the proportion of red-pigmented cells in the epidermis and the size of vacuoles containing the anthocyanin pigments (Lancaster et al., 1994), of which cyanidin-3-galactoside is the most abundant (Sun and Francis, 1967). These pigments are synthesized from the aromatic amino acid phenylalanine. Two key enzymes in the synthesis of anthocyanins are phenylalanine ammonia lyase (PAL) and flavanone synthase (Tucker, 1993). Anthocyanin synthesis in apple fruit skin is light- (Dong et al., 1995) and temperature-dependent (Saure, 1990). Doud and Ferree (1980) observed that, depending on the location, the average irradiance during the growing season may vary and have a significant impact on skin color.

Chlorophyll, the major pigment associated with photosynthesis, is embedded in the thylakoid membranes within the chloroplasts. The precise mechanism of chlorophyll degradation is unclear, but may involve both enzymatic and chemical reactions. Carotenoids are not as diverse as anthocyanins and are localized within the chloroplast, often named chromoplast (Tucker, 1993). They are terpenoid compounds that are derived from acetyl-CoA via the mevalonic acid pathway. The main anthocyanin in apple is idaein (cyanidin-3-galactoside, Knee, 1993), whereas the typical carotenoids are β-carotene, lutein, violaxanthin, neoxanthin and cryptoxanthin. Carotenoids are normally synthesized in green tissue. Carotene declines during ripening, but lutein and violaxanthin increase substantially. Anthocyanin synthesis occurs as fruits grow, but color changes during ripening are revealed by the simultaneous disappearance of chlorophylls *a* and *b* (Knee, 1972, 1980), which unmask previously present or newly synthesized pigments (Tucker, 1993).

Membrane changes take place during apple ripening. Galactolipids with their associated linolenyl moieties, typical components of chloroplast membranes, are lost (Gaillard, 1968), though phospholipids and fatty acyl groups typical of other cell membranes remain constant or even increase (Knee, 1993). Turnover of lipids may greatly increase in ripening fruit, based on studies made by Bartley (1985) where [$^{14}$C]acetate incorporation by apple cortex into various phospholipids increased ten-fold.

**Synthesis of ethylene in ripening apples**

Apple is a climacteric fruit presenting a typical peak in the respiration rate that precedes or is parallel to an autocatalytic rise in ethylene ($C_2H_4$) production. The ripening of climacteric fruit is regulated by the hormone ethylene (Lurie, 1998a, b), and the ripening process is irreversible once autocatalytic ethylene production increases to a certain level (McGlasson, 1985). Adams and Yang (1979) elucidated the $C_2H_4$ biosynthetic pathway. $C_2H_4$ is synthesized from S-adenosyl-L-methionine (SAM) which is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS). The oxidation of ACC by ACC oxidase (ACO) is the last step in the production of this hormone (Yang and Hoffman, 1984). ACS requires pyridoxal phosphate for maximal activity (Yu et al., 1979). During the climacteric ethylene production of apples and tomatoes there is a simultaneous increase in the activity of ACS and ACO, as well as in the abundance of their transcripts (Ross et al., 1992; Nakatsuka et al., 1997; Dong et al., 1991). ACS activity exhibits a major positive feedback regulatory step in ethylene biosynthesis (Yang, 1980; Atta-Aly et al., 2000), and can be inhibited by inhibitors of pyridoxal phosphate-linked enzymes, like aminooxiacetic acid (AOA) and aminoethoxy-vinylglycine hydrochloride (AVG) (Adams and Yang, 1979; Boller et al., 1979; Yu et al., 1979).

It has been documented in apple that an increase in lipoxygenase (LOX) activity precedes the evolution of ethylene until it reaches detectable levels, and 24 hours later the respiratory climacteric occurs, preceding the ethylene peak (Meigh et al., 1967). C18 free fatty acids rapidly accumulate in the peel during the climacteric, and after reaching a pronounced peak the level declines at a high rate (Meigh and Hubble, 1965). How autocatalytic ethylene production is triggered remains unknown, but Lara and Vendrell (2000) proposed the involvement of ABA. They noticed that in very immature preclimacteric apples, the application of exogenous ABA and not of $C_2H_4$ would induce the transcription of ACO, leading to a final $C_2H_4$ production similar to that of fruit harvested at commercial harvest. However, the authors could not discriminate among ACO isoforms and consequently could not verify an increase in the synthesis of the ACO isoform linked to fruit ripening. The change in tissue sensitivity to endogenous ethylene is also believed to play an important role in the onset of ripening. This sensitivity increases with advancing age of the fruit and is affected by several factors, including the balance of endogenous plant growth regulators (Reid, 1985).

**Ethylene signaling pathway**

As with other hormones, ethylene binds to a receptor and forms an activated complex that triggers a chain of reactions that includes modification of gene expression and cause an array of physiological responses. Depending on plant species, type of tissue and/or developmental timing, $C_2H_4$ regulates several processes, including fruit ripening, cell elongation, flower senescence, leaf abscission, sex determination (Abeles et al., 1992), and wound response (O'Donnell et al., 1996). The current knowledge of $C_2H_4$ perception and signal transduction has arisen from molecular genetic approaches using *Arabidopsis thaliana* mutants. The most characterized $C_2H_4$ receptor is ETHYLENE RECEPTOR1 (Etr1), which gene was isolated from $C_2H_4$-insensitive mutants (Schaller and Bleecker, 1995; Schaller et al., 1995). ETR1 is a protein homolog to the bacterial histidine kinase two-component system of signal transduction (Chang, et al., 1993). The two-component systems often involve a first component, a sensor, comprising a signal-input domain and a catalytic (histidine kinase) transmitter domain, and a second component, a response regulator, that includes a receiver domain. After ligand binding, there is autophosphorylation of a conserved histidine in the transmitter domain, followed by phosphate transfer to a conserved aspartate residue in the receiver domain of the second component. The basic two-component architecture is conserved in the ETR1 protein. The *Arabidopsis ETR1* gene represents a small family currently composed by five members: *ETR1*, *ETHYLENE RESPONSE SENSOR1* (*ERS1)*, *ETHYLENE RECEPTOR2 (ETR2)*, *ETHYLENE INSENSITIVE4* (*EIN4)* and *ETHYLENE RESPONSE SENSOR2* (*ERS2*), which present structural differences and therefore are grouped in subfamilies (Bleecker, 1999). An ETR1 homolog has been found in tomato, called NEVER RIPE (NR) (Wilkinson et al., 1995). Because ethylene is a simple molecule and is effective in the nM range, a transition-metal cofactor would be required to provide the necessary chemistry for high affinity between receptor and ligand (Burg and Burg, 1967). A copper ion is required for the binding of $C_2H_4$ to the receptor (Rodriguez et al., 1999), thus copper may play the role of the transition-metal cofactor (Bleecker, 1999).

Several components acting downstream of ETR1 have been identified, like COSNTITUTIVE RESPONSE1 (CTR1), ETHYLENE-INSENSITIVE2 *(*EIN2) and ETHYLENE-INSENSITIVE3 (EIN3). CTR1 acts between ETR1 and EIN2, and is a negative regulator of the $C_2H_4$ response since a loss of function in its gene results in a constitutive $C_2H_4$-response phenotype (Kieber et al., 1993). *CTR1* gene codes for a putative serine/threonine kinase

related to the MAPKK kinase family (Kieber et al., 1993), often involved in regulating transcription factors (Treisman, 1996). CTR1 negatively regulates EIN2, which is required for $C_2H_4$ signaling and acts between CTR1 and the EIN3 family of transcriptional regulators. EIN3 and several related proteins like ETHYLENE-INSENSITIVE3-LIKE1 (EIL1), ETHYLENE-INSENSITIVE3-LIKE2 (EIL2) and ETHYLENE-INSENSITIVE3-LIKE3 (EIL3) are positive regulators in the $C_2H_4$ signaling pathway in Arabidopsis and are localized to the nucleus (Chao et al., 1997). Loss-of-function *ein3* mutants cause insensitivity to $C_2H_4$ in seedlings and adult plant tissues, suggesting a central role for EIN3 in this signaling pathway (Roman et al., 1995).

**Role of ethylene in fruit ripening**

The large increase in endogenous $C_2H_4$ biosynthesis regulates genes with products linked to fruit ripening. The availability of transgenic plants and of naturally-occurring mutants like tomato *Nr* has facilitated the discrimination between ethylene-dependent and -independent pathways. Fruit softening is known to be one of the ripening processes that are most sensitive to ethylene. Endo-polygalacturonase (PG), an important cell wall degrading enzyme, requires ethylene for its translation (Theologis et al., 1993), and an ethylene antagonist has prevented the expression of two endo-β-1,4-glucanase genes (Cel1 and Cel2, Lashbrook et al., 1994). Color changes can be ethylene-dependent or -independent, according to the types of pigments involved and fruit species (Lelièvre et al., 1997). For example, lycopene synthesis was impaired in transgenic (Oeller et al., 1991; Klee, 1993) and *Nr* tomato mutants (Tigchelaar et al., 1978), but carotenoid accumulation was not affected, and chlorophyll degradation was prevented in transgenic melons (Ayub et al., 1996). Volatile ester production can be negatively affected by ethylene, as will be discussed later in this chapter.

Four methods can be used to manipulate $C_2H_4$ responses (Yang, 1985): (a) changing the level of $C_2H_4$ in the tissue by addition/removal of $C_2H_4$, (b) changing the level of $C_2H_4$ in the tissue by stimulating or inhibiting its biosynthesis, (c) manipulating the $C_2H_4$ response by modifying the $C_2H_4$-receptor interaction or by modifying the amount of the receptor, and (d) acting downstream of the signaling pathway by manipulating $C_2H_4$-dependent gene expression.

**Respiratory activity in ripening apples**

Respiration supplies energy and carbon skeletons for the *de novo* synthesis of mRNAs, proteins, pigments and flavor compounds that takes place during ripening. As mentioned before, apples display a characteristic respiratory peak during ripening and are therefore classified as

climacteric fruit. The magnitude and timing of the respiratory peak varies among species. In apples, the respiration rate generally varies between 5 and 40 mL $CO_2$ kg $FW^{-1}h^{-1}$, depending on cultivar and environmental conditions. Sugars and organic acids, the main respiratory substrates, are sequestered in the vacuole and presumably released in a controlled way into the cytosol to become available for respiration. The respiratory pathways for the oxidation of sugars in the fruit, glycolysis, the oxidative pentose phosphate pathway (OPP) and the tricarboxylic acid (TCA) pathway, are common to all plant tissues. The increased respiration of sugars in climacteric fruit seems to be mediated by an increased flux through glycolysis, though it is not known how glycolysis is regulated during ripening. A role for ethylene in the increase of respiration rate in climacteric fruit has been proposed since even non-climacteric fruit, which normally produce low amounts of ethylene, will respond to exogenous ethylene with increased respiration (Tucker, 1993). Interestingly, the calculated energy demand for metabolic activity in most fruit during ripening is much less than that produced during the climacteric (Solomos, 1983), and non-climacteric fruit ripen without any increase in respiration. Even though the metabolic importance of the respiratory rise is uncertain, keeping respiration rate low and sugar utilization at minimum levels is desirable for maintaining fruit quality (Watkins, 2002).

**Volatile production in apple fruit**

Most volatile compounds produced by apple fruit are esters, alcohols, aldehydes, acids, ketones and terpenes (Lurie et al., 2002; Fan and Mattheis, 1999). The majority of aroma-related volatiles are esters (78-82%) and alcohols (6-16%), and the most abundant are even-numbered 2-6 carbon chains (Paillard, 1990). At the onset of ripening there is an accumulation of alcohols and aldehydes or green notes (Mattheis et al., 1991). The largest change in volatile compound production during ripening is an increase in ester production (Brown et al., 1966), which partly accounts for the development of the characteristic flavor and aroma of apples (Mattheis et al., 1991). This is regulated by ethylene action (Fan and Mattheis, 1999). A continuous ethylene presence is required to stimulate the synthesis of some esters (Fan et al., 1998). Three esters, butyl acetate, 2-methylbutyl acetate and hexyl acetate, are thought to be the main contributors to apple-like aroma (Lurie et al., 2002; Dimik and Hoskin, 1983). Volatile compounds are produced in greater quantity in peel tissue than apple cortex or intact fruit, apparently because of an abundance of fatty acid substrates (Guadagni et al., 1971). Additionally, Rudell et al. (2000) found that the respiration rate in the skin of 'Fuji' apples was approximately 100 % to 200 %

higher than in the hypanthial tissue and 24 % to 100 % higher than in the carpellary tissue. They proposed that volatile synthesis, while dependent on ethylene presence, also occurs to a greater extent in cells with a high rate of respiration.

Fatty acids are the major precursors for the synthesis of aliphatic volatiles (Fellman et al., 2000; Harb et al, 2000) and are metabolized through β-oxidation and LOX action (Bartley and Hindley, 1980; Brackmann et al., 1993; Yahia, 1994; Sanz et al., 1997; Rowan et al., 1999). β-oxidation is believed to be the major contributor to alcohols and acyl-CoAs (Sanz et al., 1997; Paillard, 1979), but as apples ripen, lipid synthesis, membrane fluidity and chloroplast breakdown increase and release linoleic (18:2) and linolenic (18:3) acids, substrates for LOX action (Sanz et al., 1997). LOX activity increased in ripening 'Fuji' apples, and this could be an alternative pathway to β-oxidation (Echeverria et al., 2004b). Free fatty acids could also be synthesized *de novo* during ripening, when ethylene induces an increase in respiration rate and greater ammounts of ATP are available for the synthesis of linoleic and linolenic acids, precursors for aroma volatiles (Song and Bangerth, 2003). A strong support for this theory is the similar patterns of change of linoleic and linolenic acids, respiration and volatile production (Song and Bangerth, 1996), and concentration of ATP (Tan and Bangerth, 2001) in pre-climacteric and climacteric apple fruit.

Amino acids, leucine, isoleucine, and valine, among others, also contribute to the aroma of fruits and vegetables as they are transformed to branched-chain alcohols and ethanol, substrates for ester synthesis, through a pathway that involves the activity of alcohol dehydrogenase (ADH) (Sanz et al., 1997; Tressl and Drawet, 1973; Rowan et al., 1996). Acyl-CoAs are reduced to aldehydes by acyl-CoA reductase, and a further reduction by ADH transforms these aldehydes into alcohols (Sanz et al., 1997). Echeverria et al. (2004a) found that ADH activity declined while ethanol concentration increased in peel and pulp as 'Fuji' apples ripened, suggesting that low ADH activity might be sufficient for ethanol production. The last step in the synthesis of esters is the transfer of acyl moieties to alcohols from acyl –CoAs. This step, catalyzed by the enzyme alcohol-acyl transferase (AAT), is probably regulated by ethylene through an induction in the transcription of *AAT* (Fan and Mattheis, 1999; Defilippi et al., 2005a). Substrate specificity of AAT differs among fruit (Olias et al., 1995) and apple varieties and tissues (Holland et al., 2005). The esterification of aliphatic alcohols is preferred over that of

branched-chained alcohols (Olias et al., 1995), probably determining the differential volatile profile of each fruit (Dixon and Hewett, 2000).

Volatile production can be limited by precursor availability like $C_2$-$C_8$ acids and alcohols (De Pooter et al., 1981; Knee and Hatfield, 1981; Mattheis et al., 1991), though this may not be the only limiting factor (Fan and Mattheis, 1999) and there could be a tissue-specific response. Defilippi et al. (2005b) proposed that in cortex AAT is a more important step for ester formation than in peel, where the supply and metabolism of amino acids and fatty acids seems to be more critical. Echeverria et al. (2004a) found that, overall, precursor availability is a more significant factor than enzyme activity for the development of aroma during on-tree maturation of 'Fuji' apples. They also found a steady increase in 2-methylbutyl acetate and hexyl acetate after storage due mainly to the availability of alcohol precursors, given AAT showed no major changes in activity (cortex) or the activity even decreased (peel). The relative amounts of acids and alcohols present also influence the final composition of the volatile esters (Paillard, 1979; De Pooter et al., 1981; Souleyre et al., 2005), and the contribution of each compound to the aroma depends on its particular sensory threshold and presence of other compounds (Buttery, 1993). Apple aroma after storage depends on maturity stage at harvest (Dirinck and Schamp, 1989), $O_2$ and $CO_2$ concentrations in the storage atmosphere, storage period and seasonal variation (Echeverria et al., 2004b).

**Postharvest manipulation of apple ripening**

***Generalities and CA storage***

Reducing metabolic rate of any fruit from the moment of harvest is the main practice that will maintain quality during storage and post-storage shelf life. Techniques that slow ripening of climacteric fruit such as application of inhibitors of ethylene synthesis, like aminoethoxyvinylglycine (AVG), and of ethylene action, like 1-methylcyclopropene (MCP), heat treatment after harvest and controlled atmospheres (CA), are valuable tools that may maintain fruit quality in cold storage. Abeles et al. (1992) listed techniques that minimize ethylene action in storage, such as low $O_2$, high $CO_2$ and low temperature. Apple fruit can be stored from a few weeks to at least 11 months, depending on variety, type of storage (regular cold storage or CA), storage temperature, and speed of cooling (Kader, 2002). Fruits in cold storage tend to soften, lose water, and lower their rate of volatile synthesis, affecting the final quality of the product. 'Gala' apple, a variety that quickly loses firmness in cold storage, retained

firmness when stored in CA thus extending the marketing period (Boylston et al., 1994; Cliff et al., 1998). CA can also reduce volatile ammounts, respiration, and ethylene production rates compared with regular cold storage (Saftner et al., 2002), but this technology is often used when apples are stored for longer then 3 months (Kader, 2002). CA-stored apples have a highly reduced aroma-related volatile production in post storage (Bangerth and Streif, 1987). This could be due to low rates of alcohol and fatty acid synthesis and/or degradation caused by minimal $O_2$ availability during storage (Argenta et al., 2004). Bangerth et al. (1998) proposed that under low $O_2$ and/or high $CO_2$ storage conditions, the resulting low respiration leads to the depletion of metabolites (ATP, NADPH) required for the synthesis and desaturation of fatty acids, and that effect could not be reversed at ambient temperature if the fruit had been stored for more than 5 months.

***Thermal treatments***

Interest in thermal treatments of fruit began with the increasing demand to find alternatives to the use of postharvest chemicals for control of pathogens and insects. Later on, heat treatments were studied as possible tools to maintain fruit quality in storage. Salveit (1991), working with tomato disks, showed that a heat stress could reduce tissue sensitivity to chilling injury when it was applied before cold storage. Lurie et al. (1995) found that the plasma membrane of apples heated at 38$^{o}$C for 4 days and later stored at 4$^{o}$C for 4 months had more unsaturated fatty acids than control fruit, resulting in a more fluid membrane that is known to reduce the risk of chilling injury (Lyons, 1973). Whitaker et al. (1997) found greater fatty acid unsaturation in heated apples, which corresponded with lower indiscriminate membrane leakage. Even though apples are normally thought to be insensitive to low temperatures, they can develop superficial scald in cold storage, which is a form of chilling injury (Bramlage and Meir, 1990). Superficial scald derives from the oxidation of α-farnesene, a component of the apple wax that causes peel browning (Huelin and Coggiola, 1970). The biosynthesis of α-farnesene in apples is mediated by ethylene-regulated gene expression during fruit ripening (Ju and Curry, 2000). Heat-treated apples later stored at 0$^{o}$C for one month had both lower contents of α-farnesene and reduced superficial scald incidence (Lurie et al., 1990).

Of the different thermal treatments, hot air (placing fruit in a heated chamber) heats more slowly than hot vapor (heat transfer by condensation of water vapor on the cooler surface of the fruit) but avoids the potential deterioration resulting from the excessive humidity of the latter

(Lurie, 1998a, b). In climacteric fruit, heat might inhibit ripening through its effect on the enzymes involved in the synthesis of ethylene. ACO activity is the first to be inhibited, followed by ACS activity (Yu et al., 1980; Klein, 1989; Atta Aly, 1992). A major problem can be that after the fruit is removed from the treatment and held at room temperature, ethylene production recovers to equal or higher levels than those of control fruit (Klein and Lurie, 1990; Lurie and Klein, 1992a). Transcripts of *ACO* and ACO protein accumulated during the recovery from a hot air treatment at 38$^{o}$C (Lurie et al., 1996a). Paull and Chen (2000) found that protein denaturation by heat treatment can be reversed at certain temperatures but can be permanent at excessively high ones, causing heat injury. They listed many factors that could influence the response of fruit to heat in terms of ripening, like field-induced thermotolerance, cultivar, fruit size and morphological characteristics, ripeness level (physiological state), heat transfer rate and energy balance, final temperature, and duration of exposure at different temperatures.

Heat treatments have yielded differing results. For example, it decreased firmness loss after 6 months in regular cold storage at 0$^{o}$C according to Porrit and Lidster (1978), but Saftner et al. (2002) found that quality and sensory characteristics of heated and non-heated fruit did not differ significantly after the same period in cold storage. 'Anna' and 'Granny Smith' apples that were heated at 46$^{o}$C for 12 hours or at 42$^{o}$C for 24 hours before storage were firmer at the end of storage, had a higher SSC:TA and a lower incidence of superficial scald than unheated fruit, results similar to heating apples at 38$^{o}$C for 4 days (Klein and Lurie, 1992). According to Tu and De Baerdemaeker (1997), heated 'Golden Delicious' and 'Jonagold' apples maintained firmness better than unheated ones, though the effects of heat treatments on apple quality were cultivar-dependent. However, internal browning after 4 months of cold storage was more obvious for heat-treated apples. Heated apples (38$^{o}$C for 4 days) were found crispier and sweeter by a taste panel than unheated ones (Lurie and Nussinovitch, 1996). The authors attributed that effect to a possible decrease in the activity or synthesis of cell wall degrading enzymes based on previous studies by Klein et al. (1990) and Ben Shalom et al. (1993) that showed lower soluble pectin and higher insoluble pectin contents in heated fruit compared to control fruit. Additionally, more calcium is bound to cell walls and less to water-soluble pectin of heated fruit (Lurie and Klein, 1992b). Calcium ions are chelated by de-esterified regions of pectic polymers, forming 'egg-boxes' that in sufficient number would be expected to hold adjacent polymers firmly together (Tucker, 1993). Fallik et al. (1997) found that volatile production was first inhibited, but then

recovered to even greater activity than non-heated fruit after 6 weeks of cold storage. Hot air chambers for thermal treatments are not commercially used, but have been utilized to study physiological changes in fruits and vegetables in response to heat (Lurie, 1998a, b).

***Chemical treatments***

Chemical methods are less expensive and require less sophisticated equipment than CA facilities (Bangerth, 1978). Some chemicals inhibit ethylene synthesis, like aminoethoxyvinylglycine (AVG), while others interfere with ethylene action, like silver ions, diazocyclopentadiene (DACP), and 1-methylcyclopropene (MCP).

AVG is a plant growth regulator commercially registered as ReTain® Plant Growth Regulator by Valent Biosciences Corporation to control preharvest drop in apples and pears. The recommended application rate for apple is 124 g a.i. $ha^{-1}$. It competitively inhibits the synthesis of ACC and, therefore, of ethylene (Green, 2003). AVG inhibits pyridoxal phosphate-linked enzymes such as ACS (Boller et al., 1979), and was found to delay apple fruit ripening and decrease preharvest drop (Bangerth, 1978; Stover et al., 2003), inhibit ethylene and volatile production when applied to pre-climacteric fruit (Fan et al., 1998; Harder-Doll and Bangerth, 1987), retard production of ethylene and ripening-related volatiles during storage (Bangerth and Streif, 1987), and reduce volatile production, including acetate esters, of fruit harvested before the climacteric peak (Fan et al., 1998; Mir et al., 1999). Greene (2002) found that 124 g a.i. $ha^{-1}$ was effective for delaying fruit maturity and preharvest drop of 'McIntosh'. Greene and Schupp (2004) established that one application of AVG 4 weeks before harvest was more effective in preventing fruit drop, and economically more efficient, than 2 half dose applications 4 and 2 weeks before harvest. Trees treated with AVG have increased vegetative growth the following year (Williams, 1980). Beneficial effects of AVG could also be seen after CA storage. Fruit that had been treated with AVG had lower internal ethylene concentration (IEC) and higher retention of cortex firmness and shelf-life than non-treated fruit after 6 months in CA storage and 7 days at room temperature (Wang and Dilley, 2001). Autio and Bramlage (1982) found that early-season cultivars were less affected than late-season ones, conflicting with results by Byers (1997) showing that there may be not be such a tendency. There is some evidence that AVG does not affect the SSC of apple fruit at harvest (Autio and Bramlage, 1982; Byers, 1997; Fan et al., 1999; Knee, 1976), but because AVG is applied one month prior to harvest and prevents ethylene

production in apple, it is not known whether SDH activity and expression and resulting sorbitol metabolism would be negatively affected by AVG.

Silver is a transition metal that mimicks the effect of copper in allowing the binding of $C_2H_4$ to the receptor (Rodriguez et al., 1999), with an inhibitory effect on $C_2H_4$ responses *in vivo* (Abeles et al., 1992), suggesting that it can replace copper and interact with $C_2H_4$ but it does not transmit the signal to downstream effectors (Bleecker, 1999). Another antagonist of $C_2H_4$ action, DACP, occupies the $C_2H_4$ binding site binding tightly to the receptor, and requires light for permanent attachment (Sisler and Blankenship, 1993a). DACP can inhibit ripening in tomatoes (Sisler and Blankenship, 1993b) and reduce $C_2H_4$ production and firmness loss in apples (Blankenship and Sisler, 1993).

MCP, a synthetic cyclic olefin that is a vapor under physiological conditions, has been registered by AgroFresh Inc. for application on edible products and labeled in the U.S. for use in apples (DeLong et al., 2004). MCP is an inhibitor of ethylene action that irreversibly forms a complex with a metal in ethylene receptors and blocks ethylene binding (Sisler et al., 1996; Sisler and Serek, 1997). MCP effectively slowed ripening of several apple cultivars ranging from summer apples (i.e., 'Ginger Gold') to long-term storage types (i.e., 'Fuji') by reducing ethylene production, respiration rate, and firmness and titratable acidity loss (Fan et al., 1999). It considerably reduced the rate of softening and delayed superficial scald development but repressed total volatile production of 'Red Delicious' and 'McIntosh' apples after cold storage (Rupasinghe et al., 2000). 'Anna' apples treated with MCP retained more alcohols and aldehydes and had lower ethylene, ester volatiles and total volatile production than untreated fruit. Untreated fruit, in turn, developed more fruity, ripe, and overall aromas, and were less accepted than treated apples in a sensory evaluation (Lurie et al., 2002). SSC was not clearly affected by MCP (Rupasinghe et al., 2000; Fan et al., 1999), suggesting that ethylene might not influence final SSC. Watkins et al. (2000) listed several factors governing the efficacy of MCP, such as concentration and duration of treatment, type and cultivar of fruit and stage of ripening at the moment of the treatment, storage conditions, and rate of *de novo* synthesis of receptors. They suggested that high ethylene-producing cultivars might be less responsive to MCP especially at their climacteric phase due to a higher concentration of ethylene receptors.

**Objectives of the work**

AVG is commercially used as a preharvest treatment to control fruit drop in apple and, due to its fungal or natural origin, a re-formulation of product might be recognized as an organic treatment. Heat treatment could be used as an organic postharvest treatment to maintain shelf life. MCP is commercially available as a postharvest treatment. There is information about the effects of each of these treatments (AVG, heat, MCP) on ripening traits of apple, but not much is documented about their combined effects. The combination of preharvest AVG with postharvest application of MCP or heat treatment may have a strong negative effect on post-storage ripening and fruit volatile production, though the different responses may be genotype-specific.

The overall objectives of this work were:

1) To study quality traits, especially volatile production and its biochemical basis, of apple fruit following AVG and/or MCP treatments.
2) To assess the effect of AVG and heat treatment, alone or combined
   a) on ripening traits, postharvest storage quality of apples, and possible genotypic variation in response to the treatment.
   b) on volatile production after harvest and cold storage.
3) To assess the effect of AVG on development of SDH activity and its relation to sugar accumulation during ripening.

# Chapter 2

## EFFECT OF AVG AND 1-MCP ON FRUIT QUALITY AND AROMA PRODUCTION OF 'ROYAL GALA' APPLES HELD IN REGULAR ATMOSPHERE COLD STORAGE

## INTRODUCTION

'Gala' (*Malus domestica* Borkh) is an apple cultivar of increasing popularity in the U.S. It has become the fourth most cultivated apple cultivar in the country, representing 9.2 % of the total apple production in 2004 with an expected increase of up to 4 % in 2005 (U.S. Apple Association, 2005). The 'Royal Gala' apple is a natural sport of 'Gala' with a strong aroma when ripe, but consumer feedback has indicated that there is a loss of flavor after storage. There exists an increasing concern by consumers about deficiencies in sensory quality of fruit, and given the increasing demand for high quality produce, maintaining the quality of these apples during short and long term postharvest storage has become a priority for the fresh apple industry.

Apple is a climacteric fruit, and during ripening it undergoes softening of the cortex, increased sugar:acid ratio, enhanced color development, and presents a typical peak in the respiration rate that precedes or is parallel with an autocatalytic rise in ethylene production. Ethylene ($C_2H_4$), the hormone that regulates ripening in apple and all climacteric fruit, is synthesized from S-adenosyl-L-methionine (SAM) which is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS) which is then oxidized by ACC oxidase (ACO) to produce ethylene (Yang and Hoffman, 1984). ACS is a major positive feedback regulatory step in ethylene biosynthesis (Yang and Dong, 1993). Some but not all the processes during ripening are regulated by ethylene action.

There are several quality factors influencing the acceptability and edibility of apple fruit, such as appearance, texture and flavor. First-time purchases are often based on appearance and firmness, but repeat buys are determined by internal quality traits such as mouth-feel and flavor (Baldwin, 2002). Color is a major characteristic of appearance, and its development is cultivar- and environment-dependent (Layne et al., 2002). Firmness is an important component of texture

(Kader, 2002), and is also closely related to textural qualities like crispness and mealiness (Saftner et al., 2002). Flavor is a complex trait composed of sweetness, sourness, bitterness, saltiness and aroma; the mix of sugars, acids and volatile compounds play a primary role in quality composition (Baldwin, 2002). Fruit taste is usually associated with sugar and organic acid content, measured through soluble solids content (SSC) and titratable acidity (TA), respectively (Ferguson and Boyd, 2002), and sugar:acid ratio is related to overall acceptability by consumers (Saftner et al., 2002).

Volatiles are key components affecting fruit flavor. Most volatile compounds produced by apple fruit are esters, alcohols, aldehydes, acids, ketones and terpenes (Lurie et al., 2002; Fan and Mattheis, 1999). The majority of aroma-related volatiles are esters (78-82%) and alcohols (6-16%) and the most abundant are even-numbered 2-6 carbon chains (Paillard, 1990). The largest change in volatile compound production during ripening is from an increase in ester production (Brown et al., 1966), which partly accounts for the development of the characteristic flavor and aroma of apples (Mattheis et al., 1991). This is regulated by ethylene action (Fan and Mattheis, 1999). The most important volatiles for aroma of 'Gala' apple fruit are hexyl acetate, butyl acetate, 2-methylbutyl acetate and β-damascerone, and after cold storage the most abundant volatiles are hexyl acetate, butyl acetate, butyl 2-methylbutyrate and hexyl 2-methylbutyrate (Plotto et al., 2000).

Fatty acids are the major precursors for the synthesis of aliphatic volatiles (Fellman et al., 2000; Harb et al., 2000) and are metabolized through β-oxidation and lipoxygenase (LOX) action (Brackmann et al., 1993; Yahia, 1994; Sanz et al., 1997; Rowan et al., 1999). β-Oxidation is believed to be the major contributor to alcohols and acyl-CoAs (Sanz et al., 1997; Paillard, 1979). Amino acids, leucine, isoleucine, and valine, among others, also contribute to the aroma of fruits and vegetables as they are transformed to branched-chain alcohols and ethanol, substrates for ester synthesis (Sanz et al., 1997; Tressl and Drawet, 1973; Rowan et al., 1996). Alcohol dehydrogenase (ADH) catalyzes the reduction of straight and branched-chain aldehydes to alcohols. LOX and ADH activities do not seem to be regulated by ethylene, and they are not considered a limiting step for the synthesis of aroma volatiles (Defilippi et al., 2005a, 2005b; Echeverria et al., 2004a) The last step in the synthesis of esters is the transfer of acyl moieties to alcohols from acyl –CoAs. This step, catalyzed by the enzyme alcohol-acyl transferase (AAT), is probably regulated by ethylene through an induction in the transcription of *AAT* (Fan and

Mattheis, 1999; Defilippi et al., 2005a). Echeverria et al. (2004a) found that precursor availability is a more significant factor overall than enzyme activity for the development of aroma during on-tree maturation of 'Fuji' apples.

Techniques that slow down ripening of climacteric fruit such as application of inhibitors of ethylene synthesis, like aminoethoxyvinylglycine (AVG), and action, like 1-methylcyclopropene (MCP), and controlled atmospheres (CA) are valuable tools that may maintain fruit quality in cold storage. Fruit in cold storage tend to soften, lose water, and lower their rate of volatile synthesis affecting the final quality of the product. 'Gala' is a variety that quickly loses firmness in cold storage, but it retained firmness when stored in CA, extending the marketing period (Boylston et al., 1994; Cliff et al., 1998). CA can also reduce respiration and ethylene production rates, with the adverse effect of repressing volatile production compared with regular cold storage (Mattheis et al., 1998; Saftner et al., 2002).

Chemical methods are less expensive and require less sophisticated equipment than CA facilities (Bangerth, 1978). MCP, a synthetic cyclic olefin that is a vapor under physiological conditions, has been registered by AgroFresh Inc. for application on edible products and is labeled in the U.S. for use on apples (DeLong et al., 2004). It is an inhibitor of ethylene action that irreversibly forms a complex with a metal, possibly copper, in ethylene receptors and blocks ethylene binding (Sisler et al., 1996; Sisler and Serek, 1997). It considerably reduced the rate of softening and total volatile production of 'Red Delicious' and 'McIntosh' apples in cold storage, delayed superficial scald development (Rupasinghe et al., 2000), reduced the rate of ethylene production and respiration, and maintained high fruit acidity. MCP effectively slowed ripening of several apple cultivars ranging from summer apples (i.e., 'Ginger Gold') to long-term storage types (i.e., 'Fuji') by reducing ethylene production, respiration rate, and firmness and titratable acidity loss (Fan et al., 1999). 'Anna' apples treated with MCP retained more alcohols and aldehydes, and had lower ethylene, ester volatiles and total volatile production than untreated fruit. Untreated fruit, in turn, developed a fruitier, ripe aroma, and were less accepted than treated apples in a sensory evaluation because they were perceived as too ripe (Lurie et al., 2002). Watkins et al. (2000) listed several factors governing the efficacy of MCP, such as concentration and duration of treatment, type and cultivar of fruit, and stage of ripening at the moment of the treatment, storage conditions, and rate of *de novo* synthesis of receptor.

AVG is an inhibitor of ethylene biosynthesis that is commercially used to stop fruit drop as a preharvest application one month before harvest. It inhibits pyridoxal phosphate-linked enzymes such as ACS (Boller et al., 1979), and was found to delay apple fruit ripening and decrease preharvest drop (Bangerth, 1978; Stover et al., 2003), inhibit ethylene and volatile production when applied to pre-climacteric fruit (Fan et al., 1998; Halder-Doll and Bangerth, 1987), retard production of ethylene and ripening-related volatiles during storage (Bangerth and Streif, 1987), and reduce volatile production, including acetate esters, of fruit harvested before the climacteric peak (Fan et al., 1998; Mir et al., 1999). Greene (2002) found that 124 g a.i. $ha^{-1}$ was effective for delaying fruit maturity and preharvest drop of 'McIntosh', and Greene and Schupp (2004) recommended one application 4 weeks before harvest compared to 2 half dose applications 4 and 2 weeks before harvest. Trees treated with AVG have increased vegetative growth the following year (Williams, 1980). Beneficial effects of AVG could also be seen after CA storage. Fruit that had been treated with AVG, and presented a delay in fruit maturation and ripening at harvest, had lower internal ethylene concentration (IEC) and higher retention of cortex firmness and shelf-life than non-treated fruit after 6 months in CA storage (Wang and Dilley, 2001).

AVG and MCP are commercially used as preharvest and postharvest treatments, respectively. There is information about the effects of the individual chemicals on ripening traits of apple, but there is only limited data about their combined effects. As they may both be used understanding their potential interaction is essential to their effective use and to avoiding potential adverse interactions. Based on their separate effects, the combination of AVG and MCP may have a strong negative effect on fruit volatile production, but a positive effect on firmness retention after cold storage. In this study the effect of AVG and MCP, alone or combined, on major quality traits of 'Royal Gala' apples at harvest and after short-term regular atmosphere storage, with emphasis on aroma volatile production was assessed.

## MATERIALS AND METHODS

### Treatments and harvest

At the University of Kentucky Horticultural Research South Farm in Lexington, Kentucky, four whole trees of 'Royal Gala/M7a', planted in 1993, were treated with an aqueous

solution of AVG (ReTain, Valent Biosciences, Libertyville, IL) containing 500 ppm Silwet L-77 (Helena Chemical Co., Collierville, TN) as surfactant 4 weeks before the expected normal harvest at the commercial rate of 124 g a.i. $ha^{-1}$ (Commercial Tree Fruit Spray Guide, 2003) in 2004 and 2005. The solution of AVG was applied to leaves and fruit with a hand pump sprayer to the point of runoff. Non-treated fruit (controls) were harvested at the beginning of ripening (H1) based on starch index (SI) and ethylene production. Fruit from H1 were harvested 119 days after full bloom (DAFB) in 2004 and 123 DAFB in 2005. AVG-treated apples were harvested with the untreated fruit (H1) and also two weeks later in 2004, and one week later in 2005 (H2). Fruit from H2 were harvested 133 DAFB in 2004 and 130 DAFB in 2005. After harvest fruit were allowed to equilibrate at room laboratory temperature (21 $\pm$ 0.5 $^{o}$C) (RT) for three to five hours. Half of each lot was then placed in 26 L plastic containers for 20 h with MCP (EthylBloc powder, Biotechnologies for Horticulture, Burr Ridge, IL) in a solution at pH 8.2 at an estimated final headspace concentration of 1µL/L. There were four treatments at H1 (control, AVG, MCP, and AVG plus MCP) and two treatments at H2 (AVG and AVG plus MCP). Fruit were ripened at RT for 7 days after postharvest treatment or were stored in regular atmosphere (RA) cold storage for 6 or 12 weeks at 4$^{o}$C (6WCS, 12WCS) and later ripened at RT for 7 days. An ethylene treatment was applied to half of the AVG plus MCP-treated fruit stored for 12 weeks at 4$^{o}$C in 2005. Fruit were equilibrated at RT for three hours and then dipped for thirty seconds in an aqueous solution containing 300 ppm a.i. ethephon (2-chloroethyl)phosphonic acid (Florel®, Southern Agricultural Insecticides, Inc., Hendersonville, NC) and 500 ppm Silwet L-77 as surfactant.

**Internal ethylene concentration**

Internal ethylene concentration (IEC) was measured on different lots of ten fruit per treatment on days 1 and 7 AH, 6WCS, and 12WCS. A gas sample was taken from the seed cavity by inserting a needle attached to a 10 mL syringe through the calyx end, and a 0.2 mL sub-sample was analyzed with a gas chromatograph (HP 5890, Agilent Technology, Wilmington, DE) equipped with a flame ionization detector (FID) and an alumina capillary column (AT-Alumina Plot GC Column, 30 m, 0.53 cm i.d.) containing activated alumina and $N_2$ as the carrier gas. Temperatures were 35$^{o}$C, 175$^{o}$C and 125$^{o}$C for oven, injector and FID, respectively. An external standard (100 ppm Ethylene/Helium, Alltech Associates Inc., Deerfield, IL) was diluted and used to quantify the amounts of detected ethylene.

**Respiration and headspace ethylene production**

Ethylene concentration in the headspace of the RA refrigerator was measured weekly to ensure that the level was negligible. Respiration rate (RR) and headspace ethylene production (HEP), nondestructive measurements, were assessed on days 1, 3, 5 and 7 after harvest, 6WCS or 12WCS on five replicate samples of two fruit each. Respiration rate was quantified by placing the two fruit in sealed 2 L glass jars, taking direct 10 mL samples from the headspace through a rubber septum in the lid after 4 h, and measuring each sample with an $O_2/CO_2$ analyzer (Model ZR 892 HS, Illinois Instruments Inc., McHenry, IL). For headspace ethylene production, 0.2 mL headspace samples were analyzed as above.

**Starch index, cortex firmness, color and soluble solids content**

Starch index (SI), cortex firmness and soluble solids content (SSC) were assessed on the same 10 fruit per lot used for IEC measurements at days 1 and 7 after harvest, 6WCS, and 12WCS. Starch index (SI), a quantification of cortex starch degradation, was assessed by cutting fruit in half perpendicular to the stem-blossom axis, and the halves were soaked in iodine solution (0.1% iodine, 1% potassium iodide in water). The degree of staining was rated on a visual scale of 1 to 9, where 1= staining the entire cut surface (high starch content) and 9= no staining (no starch; Cowgill et al., 2005). Cortex firmness was measured using a penetrometer (Model DF M10, John Chatillon & Sons, Inc. Greensboro, NC) equipped with an 8 mm diameter probe after a disk of skin was removed from opposite sites on the equatorial plane of the stem halves. To convert firmness values from 8 mm to that more commonly reported using an 11 mm diameter probe, firmness was measured with both probes on opposite sites of 30 apples of different cultivars and varying firmness. A regression was derived between the average firmness of each fruit measured with both probes. Firmness (N) with 11 mm probe = 8.5202 + 1.5703 x firmness (N) with 8 mm probe ($r^2 = 0.77$). Color was measured on whole fruit before the other traits were assessed. A visual scale from 1 to 5 was used to measure percentage of red coloration covering the fruit surface, where 1= 0-20 %, 2= 20-40%, 3= 40-60 %, 4= 60-80 % and 5= 80-100 % of the surface as red. Soluble solids content (SSC) was determined on a fresh juice sample from each fruit using a temperature compensated hand refractometer (Model 10430, Reichert Scientific Instruments, Buffalo, NY).

**Titratable acidity and sugar:acid ratio**

Titratable acidity (TA) was assayed on three composite samples per treatment. Each sample was composed of 5 g of cortex from three different fruit that had been kept at room temperature for 1 or 7 days after harvest, 6WCS and 12WCS, and collected and frozen at -20 $^{o}$C. Frozen samples were thawed on the day of the analysis, macerated with a mincer/chopper (Handy Chopper Plus, Applica Consumer Products Inc., Miami Lakes, FL) and filtered trough two layers of cheesecloth separated by a layer of Miracloth (Calbiochem, EMD Biosciences Inc., La Jolla, CA). One mL of juice from each sample was mixed with 14 mL of deionized water and titrated to pH 7.0 with 0.1 N NaOH. Results were expressed as mg malic acid 100 mL$^{-1}$ juice. Using SSC and TA from the same fruit, sugar:acid ratio was calculated as SSC/ TA. A composite average value of SSC was derived for the three fruit used for TA measures. Each averaged value of SSC corresponded with the TA value from the same three fruit. TA was re-calculated as g malic acid 100 mL$^{-1}$ juice for the ratios.

**Volatile production**

Volatile production was measured on three peel and three cortex composite samples per treatment of three apples each. Approximately 9 g-cortex samples were frozen at -20 $^{o}$C 7 days after harvest, 6WCS and 12WCS and were then measured according to Hamilton-Kemp et al. (2003). Briefly, samples were thawed in 30 mL glass jars sealed with Teflon-lined plastic screw caps containing a 3-layer septum. Samples were equilibrated in a water bath to 26 $^{o}$C for 3 h and then placed at ambient temperature. The headspace in the bottle was sampled for 15 min using solid phase microextraction (SPME) employing a 100 μm poly(dimethylsiloxane) (PDMS) fiber. The SPME fiber was removed and inserted into a GC (Model Hewlett Packard 5890 Series II, Agilent Technology, Wilmington, DE) equipped with a DB-5 column (60 m x 0.32 mm i.d., 1 μm film thickness) and a flame ionization detection (FID) detector. Volatiles were desorbed in the GC injection port for 5 min. Conditions for analysis were as follows: injection port temperature, 220 $^{o}$C; FID detector, 240 $^{o}$C; initial oven temperature, 35 $^{o}$C held for 5 min and then increased to 184$^{o}$C at 2$^{o}$C min$^{-1}$; injector splitless for 5 min. A modified splitless injection port was used so that both the septum and inlet purges were interrupted during SPME injections. Volatiles were identified from retention times matching those of authentic standards.

**AAT activity**

Alcohol acyl-CoA transferase (AAT) activity was separately assayed on peel and cortex tissue of three composite samples per treatment from three apples each. Samples were frozen at -80 $^{o}$C 7 days after harvest, 6WCS and 12WCS, and were later analyzed using a methodology adapted from Echeverria et al. (2004a). Basically, 3 g of frozen cortex tissue were pulverized and then homogenized in 6 mL of extraction solution (0.1 M potassium phosphate, 1 mM ethylene-diaminetetraacetic acid (EDTA), 0.1% (w/v) Triton X-100 and 1% (w/v) polyvinylpolypyrrolidone (PVPP), pH 8.0). For peel, 2 g of frozen tissue were pulverized and extracted using the same solution as with cortex, but with 10% PVPP to prevent oxidative reactions. The homogenate was centrifuged at 25,000 x *g* for 20 min at 4 $^{o}$C. The supernatant was recovered and placed on ice as crude enzyme extract. AAT was assayed by mixing 1000 µL enzyme extract with 10 µL of 1 M $MgCl_2$, 50 µL of butanol solution (0.2 M butanol in 0.1 M potassium phosphate buffer, pH 8.0) and 300 µL of acetyl-CoA solution (2.5 mM acetyl-CoA in 0.1 M potassium phosphate buffer, pH 8.0) and incubating the solution at 35 $^{o}$C for 10 min. Then, 100 µL of 5.5'-dithiobis(nitrobenzoic acid) (DTNB) was added to the mixture and immediately analyzed spectrophotometrically (Model Cary 50 Bio, Varian Analytical Instruments, Walnut Creek, CA) to measure the production of the yellow thiophenol product, from DTNB reacting with free CoA, through an increase in absorbance at 412 nm over time. AAT activity was expressed as mU x mg protein$^{-1}$ where U, activity unit, is the increase in one unit of absorbance per minute. Total protein content of the enzyme extract was determined spectrophotometrically at 595 nm using the Coomassie Plus$^{TM}$ Protein Assay Kit (Pierce, Rockford, IL) following the manufacturer's instructions and using bovine serum albumin (Fisher Scientific, Fair Lawn, NJ) as a standard.

**Substrate feeding experiment**

Alcohol substrates were separately fed to apple peel and cortex tissue to assess capacity for volatile synthesis. The feeding experiment was conducted in 2005 using control and AVG plus MCP-treated fruit stored for 12 weeks at 4$^{o}$C. Fruit were retrieved from storage and equilibrated at laboratory RT for three hours. Peel strips 10-12 mm-wide and cortex disks 5 mm diameter and 50-80 mm length were sampled from each fruit and combined into nine 3 g three-fruit composite samples per treatment and tissue type. All samples were placed on three layers of water-saturated filter paper in 15 mL glass jars sealed with Teflon-lined plastic screw caps.

Three samples per treatment and tissue type were fed with 5 µl of 1-butanol or 1-hexanol (Fisher Scientific, Fair Lawn, NJ), and three samples not fed with any alcohol were used as controls. Alcohol substrates were placed in small open glass containers inside the glass jars and allowed to evaporate to be available for tissue uptake. The sealed containers were placed in an incubator at 22 $^{o}$C for 24 h. Samples were then frozen at -20 $^{o}$C. For volatile analysis, samples were thawed in 15 mL glass jars sealed with Teflon-lined plastic screw caps containing a 3-layer septum. Samples were equilibrated in a water bath to 26 $^{o}$C for 2 h and then placed at laboratory RT. The headspace in the bottle was sampled for 15 min using a 100 µm PDMS SPME fiber, and volatiles were analyzed as previously.

**Experimental design and statistical analysis**

Each experiment was conducted using a completely random design. All data were subjected to analysis of variance. Means were compared by Fisher's protected least significance difference (LSD, $p$=0.05) using S.A.S. version 9.1 software (S.A.S. Institute Inc., Cary, N.C.).

## RESULTS

### GENERAL RIPENING TRAITS

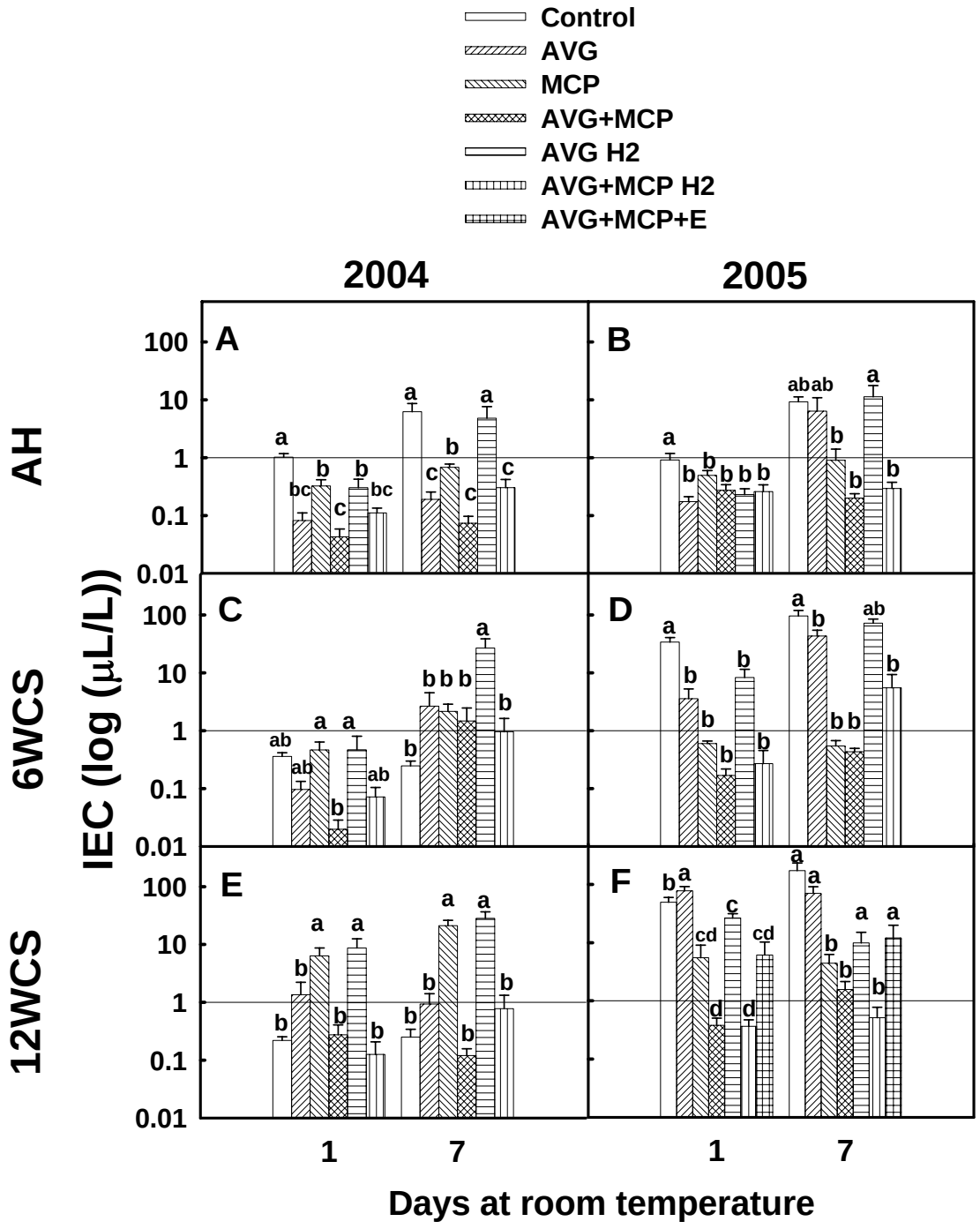
**Internal ethylene concentration**

**Harvest:** One day after harvest control fruit had an IEC close to 1 µL/L (or ppm) in both 2004 (Figure 2.1.A) and 2005 (Figure 2.1.B), whereas all treated fruit had a lower IEC. After 7 days at RT, only control fruit and AVG-treated fruit from H2 had IEC values

higher than 1 µL/L in 2004, and control and AVG-treated apples from H1 and AVG from H2 had IEC values over 1 µL/L in 2005. IEC of fruit treated with MCP or AVG plus MCP were low through 7 days at RT both years. AVG plus MCP-treated fruit from H1 and H2 had IEC below 0.1 ppm and close to 0.3 ppm, respectively, in both years.

**6WCS:** In 2004, all fruit retrieved from a 6-week cold storage had IEC values lower than 1 ppm after 1 day at room temperature (Figure 2.1.C). Fruit from H1 treated with AVG plus MCP had the lowest IEC levels (0.02 $\pm$ 0.01 ppm). After 7 days at RT, AVG-treated fruit from H2 had the highest IEC (27 $\pm$ 11 ppm), and AVG plus MCP had IEC values close to 1 ppm from both harvests. In 2005, control fruit had a significantly higher IEC than the treatments 1 day after retrieval from a 6-week cold storage (Figure 2.1.D). After fruit had been at RT for 7 days,

**Figure 2.1:** Effect of AVG and/or MCP on internal ethylene concentration (IEC) of 'Royal Gala' apple in 2004 (A, C, E) and 2005 (B, D, F). Analyses were performed on different lots of apples ripened at room temperature at 1 and 7 days after harvest (AH, A, B) and after 6 (6WCS, C, D) and 12 weeks (12WCS, E, F) in cold storage at 4°C. H2: fruit harvested 1 or 2 weeks after normal harvest; E: ethephon treatment. Different letters indicate significant differences separated by LSD at *p=0.05* within date.



control and AVG-treated fruit from H2 had a high IEC (96 ± 24 and 72 ± 14 ppm, respectively), while values for other treatments were lower than controls, though statistically similar to those of AVG. AVG plus MCP-treated fruit had IEC of 0.04 ± 0.06 for H1 and 5.5 ± 3.9 for H2.

***12WCS:*** After 12 weeks in cold storage in 2004 and 1 or 7 days at RT, MCP-treated fruit and AVG-treated fruit from H2 had the highest IEC, and all other treatments had lower IEC (Figure 2.1.E). In 2005, control and AVG-treated fruit had a high IEC after 1 and 7 days at RT (26 to 173 ppm, Figure 2.1.F). MCP and AVG plus MCP-treated fruit from both harvests had lower IEC (0.5 to 5.5 ppm). Immediately after treatment, ethephon did not have any effect on IEC of AVG plus MCP-treated fruit, though by day 7 IEC increased almost 8 fold. Overall, AVG plus MCP consistently showed a trend for having the lowest IEC, independent of harvest dates.

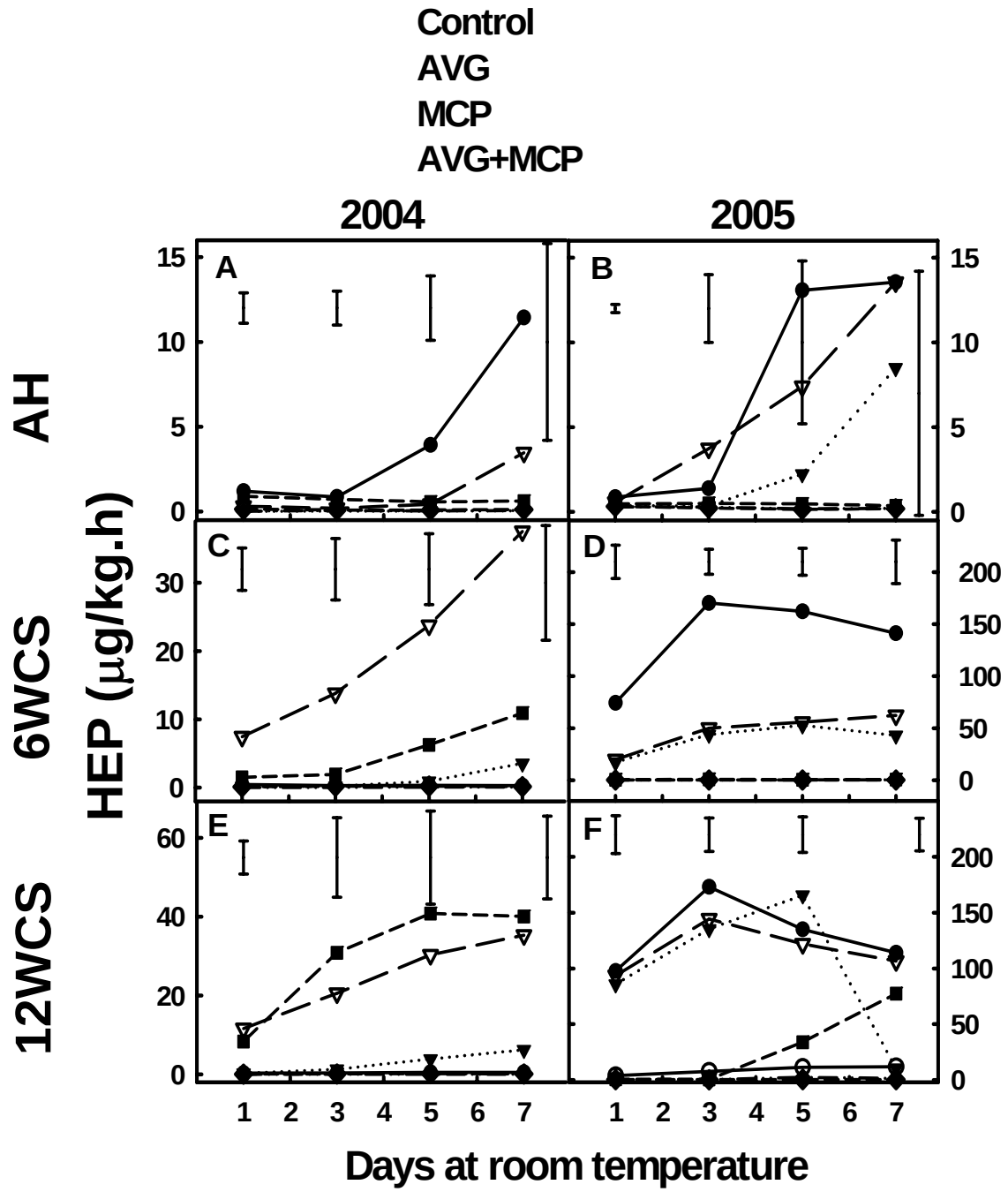
**Headspace ethylene production**

***Harvest:*** Immediately after harvest control fruit had the highest HEP, which increased over time when fruit were kept at RT in both 2004 and 2005 (Figure 2.2.A, 2.2.B). AVG plus MCP-treated fruit from H1 and H2 had very low HEP with no increase over time either season. HEP by MCP-treated fruit decreased slightly over time in 2004 and remained nearly constant over 7 days in 2005. AVG greatly repressed HEP in fruit from H1 in 2004, but showed an increase by 5 days in 2005. AVG-treated fruit from H2 had low HEP in 2004 but showed an increasing HEP in 2005 with levels similar to those of control fruit.

***6WCS:*** After 6 weeks in cold storage, control fruit had lower HEP than at harvest and even lower than AVG-treated fruit from H2, which had the highest HEP in 2004 (Figure 2.2.C). AVG plus MCP-treated fruit from both harvests generally had the lowest HEP, though the levels of HEP were statistically different from those of AVG-treated fruit from H2 only. In 2005, control apples had the highest HEP, approximately 10 times higher than at harvest (Figure 2.2.D). AVG-treated fruit from both harvest dates had HEP values lower than control apples but still much higher than apples treated with MCP or AVG plus MCP.

***12WCS:*** After 12 weeks in cold storage in 2004 fruit behaved similarly as at 6 weeks of storage (Figure 2.2.E), with the only difference that MCP-treated fruit had a HEP as high as that of AVG-treated fruit from H2. In 2005, control and AVG-treated fruit from H1 and H2 had the highest HEP, which was high from the first day after removal from storage (Figure 2.2.F). MCP-treated fruit had an initially low HEP, but it increased over time and by 7 days and reached values comparable to those of control and AVG-treated fruit. AVG plus MCP-treated fruit

**Figure 2.2:** Effect of AVG and/or MCP on headspace ethylene production (HEP) of 'Royal Gala' apple in 2004 (A, C, E) and 2005 (B, D, F). Fruit were ripened at room temperature for 7 days immediately after harvest (AH, A, B) and after 6 (6WCS, C, D) and 12 weeks (12WCS, E, F) in cold storage at 4°C. Closed symbols correspond to H1 and open symbols to H2. Open circles in Figure F represent AVG plus MCP fruit from H1 treated with ethephon. Least significant differences (LSD) at *p=0.05* within date are shown as vertical bars. Note that left and right Y axes show different scales, for storage, time and year.



always had low HEP. Treating AVG plus MCP fruit from H1 with ethephon did not affect HEP for the following 7 days. HEP of control and AVG-treated fruit was much higher in 2005 than in 2004 after cold storage.

**Respiration rate**

**Harvest:** Control fruit presented a respiratory climacteric 5 days after harvest in 2004 (Figure 2.3.A), and RR was still increasing by day 7 at RT in 2005 (Figure 2.3.B). In general control fruit had the highest RR, and AVG plus MCP reduced it the most. Fruit treated with AVG and harvested at H1 had a respiratory peak by day 5 in 2004 and in 2005 the peak occurred by day 3 with values similar to controls. RR of AVG-treated fruit from H2 increased over time in both seasons. RR of H1 and H2 fruit treated with AVG plus MCP had similar RR in 2004, but H1 fruit had initially higher RR in 2005. MCP also had a negative effect on RR that was more evident in 2005, and there was no aparent respiratory climacteric.

***6WCS:*** After 6 weeks in cold storage in 2004, RR of controls was very low and similar to to that of AVG plus MCP fruit from H1 (Figure 2.3.C). In 2005, RR of control fruit increased up to 5 days at RT and later decreased (Figure 2.3.D). AVG plus MCP consistently reduced RR of H1 fruit in 2004 and 2005, and RR of AVG-treated fruit from H2 was one of the highest both years.

**12WCS:** After 12 weeks in cold storage, control and AVG plus MCP-treated fruit had low RR during 7 days at RT (Figure 2.3.F), while MCP-treated and AVG-treated fruit had higher RR by day 5. In 2005, control and AVG-treated fruit from H1 and H2 had similar and higher RR through 5 days, though RR of AVG-treated fruit from H2 later decreased (Figure 2.3.F). In general, all AVG plus MCP-treated fruit had the lowest RR regardless of harvest date, and treating this fruit with ethephon induced an increase in RR at day 7 only.

**Starch index**

***Harvest:*** Fruit from H1 treated with AVG or AVG plus MCP showed the least starch degradation immediately after harvest both seasons, $3.1 \pm 0.5$ being the highest values, less than 50 % of control fruit (Table 2.1), and the difference persisted for 7 days. Fruit from H2 treated with AVG or AVG plus MCP had lower starch degradation than control or MCP-treated fruit 1 day after harvest in 2004 but not in 2005, and the effect was partially lost by 7 days.

***6WCS:*** One day after removal from a 6-week RA storage AVG-treated fruit from H1 had the lowest starch degradation both years ($5.6 - 7.4 \pm 0.5$). AVG plus MCP-treated fruit had starch

**Figure 2.3:** Effect of AVG and/or MCP on respiration rate of 'Royal Gala' apple in 2004 (A, C, E) and 2005 (B, D, F). Fruit were ripened at room temperature for 7 days immediately after harvest (AH) (A, B) and after 6 (C, D) and 12 weeks (E, F) in cold storage at 4°C (6WCS and 12WCS, respectively). Closed symbols correspond to H1 and open symbols to H2. Open circles in Figure F represent AVG plus MCP fruit from H1 treated with ethephon. Least significant differences (LSD) at *p=0.05* within date are shown as vertical bars.

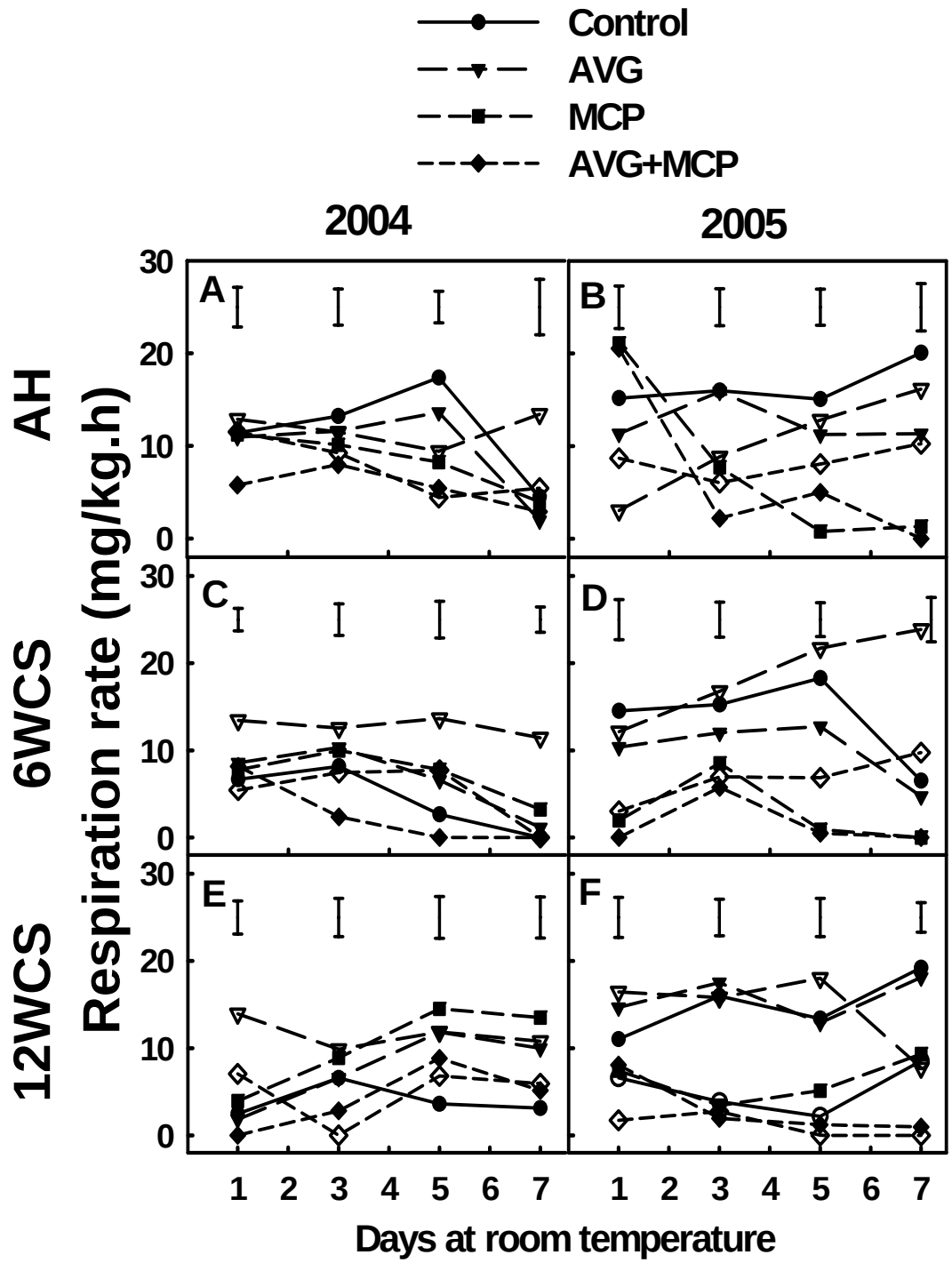


**Table 2.1:** Effect of preharvest AVG and/or postharvest MCP on starch index (SI), firmness and color (1 = green; 5 = red) of 'Royal Gala' apples ripened at room temperature for 1 and 7 days after harvest (AH), and 6 (6WCS) and 12 (12WCS) weeks in cold storage in 2004 and 2005. Different letters indicate significant differences within storage temperature and year separated by LSD at *p=0.05*; *ns:* no significant differences among means. H2 indicates fruit harvested 1 or 2 weeks after the normal harvest date. E= Poststorage ethephon treatment at 12 weeks only.

| | *DAY 1* | | | | | | *DAY 7* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **SI (1-9)** | | **Firmness (N)** | | **Color (1-5)** | | **SI (1-9)** | | **Firmness (N)** | | **Color (1-5)** | |
| ***AH*** | *2004* | *2005* | *2004* | *2005* | *2004* | *2005* | *2004* | *2005* | *2004* | *2005* | *2004* | *2005* |
| Control | 7.3 a | 5.7 a | 76.3 *ns* | 71.1 c | 4.8 a | 2.6 *ns* | 8.8 ab | 7.8 a | 59.3 c | 62.3 c | 4.4 a | 2.1 *ns* |
| AVG | 3.1 de | 2.0 b | 75.6 | 92.7 a | 2.6 cd | 1.9 | 3.5 c | 5.8 b | 80.7 a | 80.7 ab | 3.6 ab | 2.5 |
| MCP | 6.9 ab | 4.9 a | 71.3 | 79.6 bc | 4.4 ab | 1.9 | 9.0 a | 7.4 ab | 69.1 b | 73.9 abc | 4.3 a | 2.3 |
| AVG+MCP | 2.3 e | 2.6 b | 76.7 | 85.7 ab | 2.3 d | 1.8 | 4.3 c | 5.6 b | 80.2 a | 85.7 a | 2.9 b | 1.9 |
| AVG *H2* | 5.5 bc | 4.8 a | 73.5 | 80.9 bc | 3.3 cd | 2.0 | 7.7 b | 6.8 ab | 68.4 b | 72.4 bc | 3.6 ab | 2.4 |
| AVG+MCP *H2* | 4.6 cd | 3.7 ab | 71.0 | 88.5 ab | 3.6 bc | 1.9 | 7.7 b | 5.5 b | 75.9 a | 83.3 ab | 3.1 b | 2.2 |
| *LSD* | *1.8* | *2.0* | *6.8* | *10.5* | *1.2* | *1.1* | *1.1* | *2.0* | *5.7* | *12.7* | *1.0* | *1.2* |
| ***6WCS*** | | | | | | | | | | | | |
| Control | 9.0 a | 8.6 a | 60.1 d | 60.8 b | 4.5 a | 2.9 ab | 9.0 *ns* | 9.0 *ns* | 68.2 bc | 52.3 c | 4.9 a | 2.7 *ns* |
| AVG | 7.4 b | 5.6 c | 77.8 ab | 89.2 a | 3.8 ab | 3.4 a | 9.0 | 9.0 | 75.2 a | 68.4 ab | 3.5 b | 2.9 |
| MCP | 8.5 a | 8.7 a | 69.7 c | 61.0 b | 4.4 a | 2.1 b | 9.0 | 9.0 | 64.5 c | 62.7 bc | 4.8 a | 2.7 |
| AVG+MCP | 7.3 b | 7.5 ab | 78.7 a | 82.6 a | 4.1 ab | 2.4 ab | 9.0 | 8.9 | 76.9 a | 72.8 ab | 3.5 b | 2.6 |
| AVG *H2* | 7.9 ab | 7.6 ab | 70.8 bc | 67.6 b | 3.3 b | 3.1 ab | 9.0 | 9.0 | 64.1 c | 65.2 b | 4.1 ab | 3.3 |
| AVG+MCP *H2* | 8.2 ab | 6.9 b | 71.7 bc | 79.1 a | 4.0 ab | 3.2 ab | 9.0 | 9.0 | 71.9 ab | 78.3 a | 3.7 b | 2.8 |
| *LSD* | *1.5* | *1.0* | *7.1* | *10.5* | *1.1* | *1.2* | *NA* | *NA* | *6.8* | *10.8* | *0.9* | *0.8* |
| ***12WCS*** | | | | | | | | | | | | |
| Control | 9.0 *ns* | 9.0 *ns* | 56.6 b | 55.7 c | 4.5 ab | 3.0 *ns* | 9.0 *ns* | 9.0 *ns* | 51.0 b | 40.2 d | 4.8 a | 2.2 *ns* |
| AVG | 9.0 | 9.0 | 75.4 a | 66.4 bc | 2.7 d | 2.4 | 9.0 | 9.0 | 74.8 a | 58.2 c | 3.1 c | 1.9 |
| MCP | 9.0 | 9.0 | 60.6 b | 65.8 bc | 4.6 a | 2.3 | 9.0 | 9.0 | 54.7 b | 66.9 b | 4.4 ab | 2.4 |
| AVG+MCP | 9.0 | 9.0 | 79.4 a | 80.2 a | 3.7 bc | 2.4 | 9.0 | 9.0 | 79.8 a | 71.0 ab | 3.0 c | 2.2 |
| AVG+MCP+E | | 9.0 | | 79.8 a | | 2.3 | | 9.0 | | 79.1 a | | 2.6 |
| AVG *H2* | 9.0 | 9.0 | 64.5 b | 58.4 c | 3.3 cd | 3.0 | 9.0 | 9.0 | 55.5 b | 49.7 c | 3.7 bc | 2.3 |
| AVG+MCP *H2* | 9.0 | 9.0 | 73.0 ab | 72.4 ab | 4.0 abc | 2.0 | 9.0 | 9.0 | 74.8 a | 77.0 a | 4.1 ab | 2.4 |
| *LSD* | *NA* | *NA* | *10.8* | *11.0* | *0.9* | *1.2* | *NA* | *NA* | *8.4* | *9.4* | *0.7* | *0.9* |

degradation similar to AVG fruit in 2004 and slightly higher in 2004. After 7 days at room temperature all fruit lost all the starch regardless of the treatment.

**12*WCS:*** After a 12-week RA storage, all fruit had lost all the starch.

**Firmness**

***Harvest:*** In 2004, after 7 days at RT, all treated fruit were firmer than controls (Table 2.1), especially fruit from H1 treated with AVG (21 N firmer) and fruit from H1 and H2 treated with AVG plus MCP (21 and 17 N firmer, respectively), even though treatments did not differ at day 1. The same treatments that reduced firmness loss the most in 2004 were still effective in 2005, and the firmest fruit were the ones treated with AVG plus MCP (approximately 11 N firmer than control fruit).

***6WCS:*** After 6 weeks in cold storage and 1 day at RT in 2004 all treated fruit were firmer than controls. After 7 days at RT, only fruit from H1 treated with AVG or AVG plus MCP had greater firmness, with values 11 to 13 N above the least firm treatment,followed by AVG plus MCP fruit from H2. In 2005, the same treatments were the most effective in reducing firmness loss 1 and 7 days after the fruit were retrieved from cold storage.

***12WCS:*** After 12 weeks in cold storage both years, AVG plus MCP-treated fruit from both harvests were the firmest, even after 7 days at RT. AVG-treated fruit from H1 was as firm as AVG plus MCP fruit in 2004. In 2005, the application of ethephon to AVG plus MCP-treated fruit had no effect on firmness after 1 or 7 days at RT.

**Color**

Fruit color was very different between the 2 years (Table 2.1). Though color was good on controls in 2004, in 2005 the red coloration of all fruit was extremely poor, and differences among treatments were not evident after harvest or after 7 days at RT following 6 or 12 weeks of cold storage. Ethephon did not induce a significant increase in red coloration of AVG plus MCP-treated apples. Thus, we will only describe color in 2004.

***Harvest:*** Control fruit with an average color score of 4.8, 80-100 % of red coloration, had markedly more red-covered skin than AVG-treated fruit, especially of H1 fruit at less than 40-60 % average red coloration. After 7 days at RT only fruit treated with AVG plus MCP did not reach a red color similar to that of control fruit. By 7 days at RT there were no differences between harvest dates of fruit that received the same treatment.

***6WCS:*** After 6 weeks in storage and 7 days at RT AVG-treated fruit from H1 and all AVG plus MCP-treated fruit had approximately 20 % less red color than the rest. Control, MCP-treated, and AVG-treated fruit from H2 had average scores from 4.1 to 4.9, representing 80-100 % red color.

***12WCS:*** After 12 weeks in storage and 7 days at RT, only AVG fruit from both harvests and AVG plus MCP-treated apples from H1 had less red color than the other treatments.

**Soluble solids content**

SSC values did not change much within year during the study, though SSC was higher in 2005 than in 2004. In general, the variation in SSC within date did not exceed 13% of the lowest value (Table 2.2).

**Harvest:** SSC of AVG fruit from H1 and AVG plus MCP-treated apples from H1 and H2 were low 1 day after harvest in 2004 (9.8 $\pm$ 0.2 to 10.1 $\pm$ 0.3 %), but there was no treatment effect in 2005. After 7 days at RT there were no differences among treatments in 2004, whereas in 2005 AVG-treated fruit from H2 was up to 7.5% greater than the rest of treatments.

***6WCS:*** In 2004 there were no differences among treatments one day after removal from storage, and after 7 days at RT control fruit had higher SSC, while AVG-treated fruit from H2 had the lowest SSC. In 2005 control and MCP-treated fruit had approximately 5 % lower SSC than the rest of the treatments.

***12WCS:*** After 12 weeks in cold storage and 7 days at RT in 2004 treatments did not differ, though there were small differences at 1 day. In 2005 fruit that had been harvested later (H2) or treated with ethephon had values of SSC 8-13 % relatively higher than the other treatments.

**Titratable acidity**

***Harvest:*** One day after harvest in 2004 fruit from H2 had lower TA than treatments from H1, except MCP-treated samples (Table 2.2). TA of control fruit was 233 $\pm$ 5 mg malic acid/100 mL, approximately 32 % higher than those for H2 fruit. In 2005 there were no differences among treatments on day 1. Fruit from H2 had low levels of TA 7 days after harvest in 2004, with values 200 mg malic acid/100 mL, though TA of control fruit was also low at 179 $\pm$ 20 mg malic acid/100 mL. The combined treatment had high acidity for fruit from H1 in both years (around 267 mg malic acid/100 mL) after fruit were kept at RT for 7 days, and in 2005 that was also true for fruit from H2.

**Table 2.2:** Effect of preharvest AVG and/or postharvest MCP treatment on soluble solids content (SSC), titratable acidity (TA), and sugar:acid ratio (S:A) of 'Royal Gala' apples ripened at room temperature for 1 and 7 days after harvest (AH), and 6 (6WCS) and 12 (12WCS) weeks in cold storage in 2004 and 2005. Different letters indicate significant differences within date separated by LSD at *p=0.05*; *ns:* no significant differences among means. H2 indicates fruit harvested 1 or 2 weeks after the normal harvest date. E= Poststorage ethephon treatment at 12 weeks only.

| | *DAY 1* | | | | | | *DAY 7* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **SSC** *%* | | **TA** ***mg malic acid/100 mL*** | | **S:A** | | **SSC** *%* | | **TA** ***mg malic acid/100 mL*** | | **S:A** | |
| ***AH*** | *2004* | *2005* | *2004* | *2005* | *2004* | *2005* | *2004* | *2005* | *2004* | *2005* | *2004* | *2005* |
| Control | 11.1 a | 12.6 *ns* | 233 a | 246 *ns* | 47.9 ab | 51.8 *ns* | 11.8 *ns* | 13.3 b | 179 d | 210 d | 68.7 a | 63.9 a |
| AVG | 9.8 b | 12.9 | 224 ab | 268 | 43.9 b | 48.2 | 11.5 | 13.6 b | 252 ab | 250 bc | 46.3 cd | 54.6 bc |
| MCP | 11.0 a | 12.6 | 211 bc | 244 | 51.9 a | 51.8 | 11.6 | 13.4 b | 226 bc | 247 bc | 51.3bcd | 54.3 bc |
| AVG+MCP | 9.9 b | 12.5 | 236 a | 267 | 42.4 b | 47.3 | 11.1 | 13.6 b | 268 a | 267 ab | 41.9 d | 50.8 c |
| AVG *H2* | 10.4 ab | 12.5 | 199 c | 239 | 53.0 a | 52.5 | 11.5 | 14.5 a | 189 d | 242 c | 53.0 ab | 60.0 ab |
| AVG+MCP *H2* | 10.1 b | 12.8 | 200 c | 260 | 51.0 a | 49.7 | 11.5 | 14.1 ab | 207 cd | 283 a | 51.0 bc | 50.2 c |
| *LSD* | *0.9* | *0.9* | *21* | *30* | *6.5* | *7.8* | *0.9* | *0.8* | *34* | *23* | *13.8* | *6.8* |
| ***6WCS*** | | | | | | | | | | | | |
| Control | 12.2 *ns* | 13.3 b | 202 *ns* | 215 c | 60.8 *ns* | 62.3 a | 12.2 a | 13.5 c | 215 ab | 213 b | 56.9 abc | 64.4abc |
| AVG | 11.6 | 13.7 a | 226 | 260 ab | 51.6 | 52.6 b | 11.5 bc | 14.2 abc | 213 ab | 215 b | 54.0 bc | 67.2 ab |
| MCP | 11.6 | 13.3 b | 213 | 246 bc | 55.3 | 52.6 b | 11.9 ab | 13.4 c | 186 ab | 233 b | 64.6 ab | 58 bcd |
| AVG+MCP | 11.5 | 13.9 a | 229 | 257 ab | 50.6 | 53.4 b | 11.3 bc | 13.8 bc | 226 a | 278 a | 50.3 c | 49.5 d |
| AVG *H2* | 11.5 | 14.4 a | 216 | 262 ab | 53.4 | 55.5 ab | 11.1 c | 14.6 ab | 169 c | 218 b | 66.1 a | 67.8 a |
| AVG+MCP *H2* | 11.6 | 14.2 a | 208 | 292 a | 57.1 | 49.0 b | 11.7abc | 14.9 a | 182 bc | 273 a | 64.4 ab | 55.0 cd |
| *LSD* | *0.8* | *0.9* | *42* | *38* | *11.7* | *7.3* | *0.6* | *0.9* | *37* | *37* | *11.5* | *9.6* |
| ***12WCS*** | | | | | | | | | | | | |
| Control | 11.8 a | 13.1 d | 224 a | 205 e | 54.1 b | 64.6 a | 12.5 *ns* | 13.7 b | 181 bc | 187 b | 70.2 a | 75.0 ab |
| AVG | 11.5 ab | 13.5 cd | 226 a | 210 de | 50.7 b | 64.9 a | 11.9 | 14.0 b | 177 bc | 187 b | 67.2 ab | 75.8 a |
| MCP | 11.7 ab | 13.0 d | 177 b | 254 bc | 67.4 a | 51.5 bc | 12.2 | 13.6 b | 179 bc | 267 a | 67.4 ab | 51.1 c |
| AVG+MCP | 11.8 a | 14.3 abc | 221 a | 262 bc | 53.8 b | 54.5 bc | 12.0 | 14.5 ab | 202 ab | 254 a | 59.4 bc | 58.3 bc |
| AVG+MCP+E | | 14.0bcd | | 236 cd | | 59.5 ab | | 15.3 a | | 268 a | | 56.7 bc |
| AVG *H2* | 10.9 b | 14.7 ab | 189 ab | 278 b | 59.0 ab | 53.8 bc | 11.9 | 15.1 a | 165 c | 202 b | 73.3 a | 76.2 a |
| AVG+MCP *H2* | 11.8 ab | 15.1 a | 218 ab | 330 a | 54.2 b | 45.5 c | 11.9 | 15.4 a | 223 a | 247 a | 54.3 c | 63.4abc |
| *LSD* | *0.9* | *1.1* | *43* | *27* | *12.8* | *9.2* | *0.7* | *1.2* | *26* | *43* | *10.4* | *16.9* |

***6WCS:*** After 6 weeks at 4 $^{o}$C and 7 days at RT, fruit from H2 had lower acidity regardless of treatment in 2004 though they did not differ at H1, while fruit from H1 and H2 treated with AVG plus MCP had the highest acidity in 2005, reaching values of 278 $\pm$ 3.2 mg malic acid/100 mL.

***12WCS:*** After 12 weeks in cold storage in 2004, MCP-treated fruit had the lowest acidity on day 1 at RT, whereas the combined treatment had TA similar to control and AVG-treated fruit. After 7 days at RT AVG-treated apples from H2 tended to have the lowest TA and AVG plus MCP-treated fruit tended to have the highest. In 2005, control fruit had the lowest TA 1 day after removal from storage, and control and AVG-treated fruit from H1 and H2 had the lowest TA after 7 days at RT, with values as low as 187 $\pm$ 17 mg malic acid/100 mL. AVG plus MCP had high TA values, up to 35 % higher than control fruit, regardless of harvest time. Ethephon did not have an effect on TA of AVG plus MCP-treated fruit.

**Sugar:acid ratio**

***Harvest:*** By **7** days at RT after harvest control fruit had the highest S:A ratio in both 2004 and 2005 (Table 2.2) with values of 68.7 $\pm$ 9.3 and 63.9 $\pm$ 1.5, respectively, while AVG plus MCP-treated fruit from H1 the lowest ratio, ranging from 41.9 $\pm$ 1.9 to 50.8 $\pm$ 1.5. In 2005, AVG plus MCP-treated fruit from H2 also had low S:A.

***6WCS:*** After 6 weeks in cold storage and 7 days at RT, AVG plus MCP-treated fruit from H1 tended to have the lowest S:A values in both years, and the same treatment had similar values in 2005 when fruit was harvested later. AVG-treated fruit from H2 differed from that of H1 only in 2004, when S:A ratio was higher at H2 (66.1 $\pm$ 3.7 vs. 54 $\pm$ 1.6, respectively).

***12WCS:*** In 2004, after 7 days at RT, control fruit and AVG-treated fruit from H2 had the highest, and all AVG plus MCP-treated fruit had the lowest, S:A ratio (73.3 $\pm$ 6.6 and 54.3 $\pm$ 1.4 to 59.4 $\pm$ 1.8, respectively). In 2005, control and all AVG-treated fruit had the highest S:A ratio at 7 days, while MCP-treated fruit had the lowest. Ethephon did not change the S:A ratio of AVG plus MCP-treated fruit. Control and all AVG-treated fruit consistently had the highest S:A ratio by the end of the study in both years, reaching values up to 75 $\pm$ 6.8).

**VOLATILE PRODUCTION**

**Total volatile production**

Fifteen straight and branched-chain volatiles were identified in peel and thirteen were detected in cortex of 'Royal Gala' apples, comprised of 3 alcohols and 12 (peel) or 10 (cortex) esters. Total volatile production (TVP), the sum of all of the identified volatile compounds, by

untreated fruit was higher by peel than by cortex both seasons, though in 2004 that difference was less evident in fruit stored for 6 or 12 weeks at 4°C. In 2004 TVP of control peel and cortex tissue decreased over time in cold storage (TVP = 2301.4 – 385.7 x + 21 $x^2$ with $R^2$ = 0.94 for peel, and TVP = 1024.2 – 177.7 x + 9.5 $x^2$ with $R^2$ = 0.89 for cortex, where x is time in months; n=9 in both equations). In 2005 TVP increased after 6 and 12 weeks in RA storage for both peel and cortex (TVP = 2874.5 - 196.6 x + 37.5 $x^2$ with $R^2$ = 0.83 for peel, and TVP = 1565.9 – 32.2 x + 15.9 $x^2$ with $R^2$ = 0.95 for cortex, where x is time in months). All the quadratic regression curves were significant at $P < 0.01$ and accurately represent trends in the data for the studied range only.

***Peel tissue***

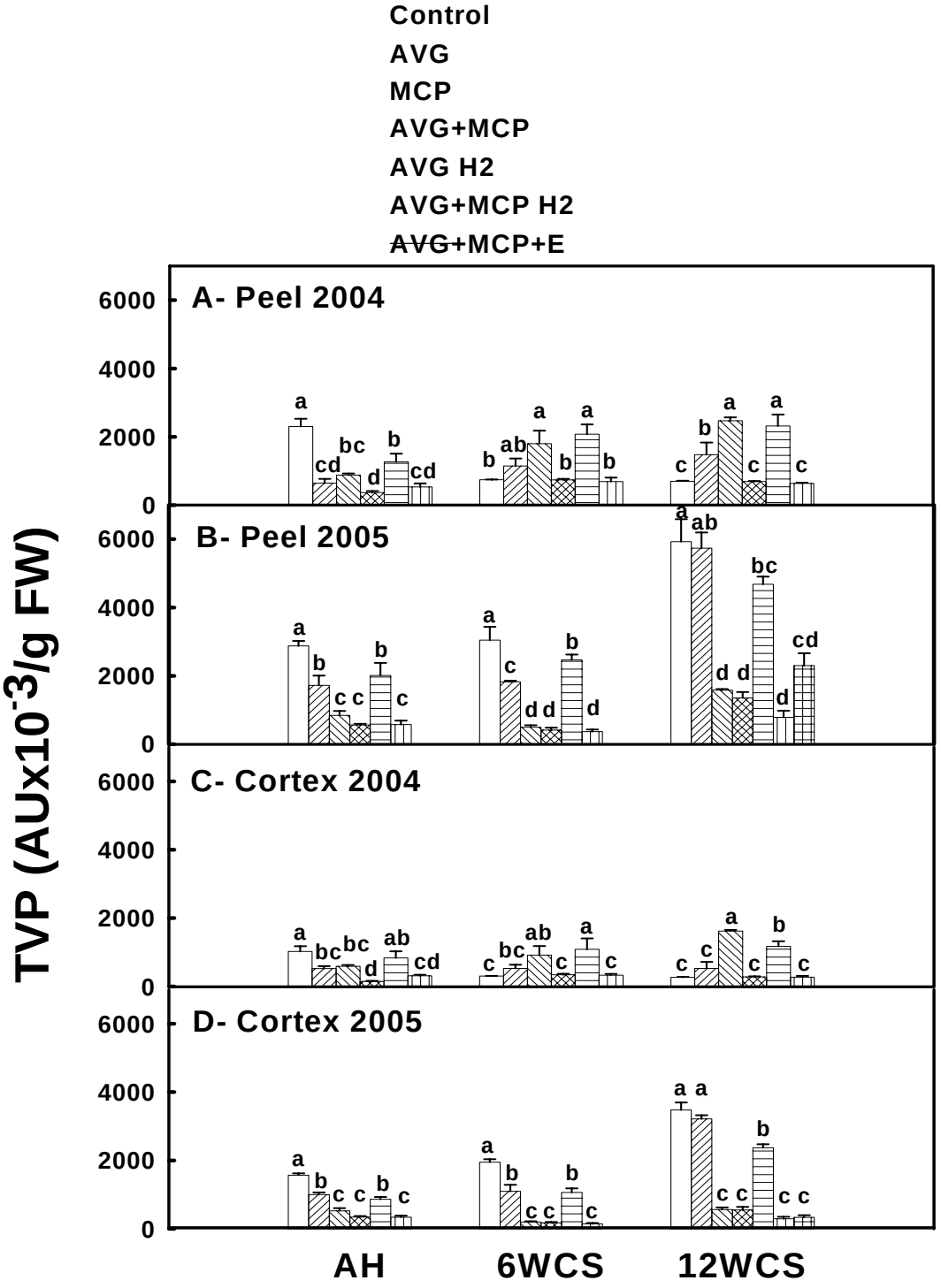
***Harvest:*** Peel of control fruit had the highest TVP at harvest both in 2004 (Figure 2.4.A) and 2005 (Figure 2.4.B). However, after fruit were kept in cold storage for up to 12 weeks, TVP decreased 75 % in 2004 but approximately doubled in 2005. All treatments

reduced TVP immediately after harvest by 45-84 % in 2004 and by 30-80 % in 2005. AVG plus MCP was the only treatment that consistently and greatly reduced TVP across years. In 2004 TVP by AVG-treated peel from H2 was higher than from H1, reaching 55 % of TVP of controls, though in 2005 TVP was similar from both harvests, and higher than the rest of the treatments (60-70 % of control TVP). The later harvest did not affect TVP of AVG plus MCP-treated peel in any year.

***6WCS:*** In 2004, peel of MCP-treated fruit kept in 6-week cold storage showed greater volatile production than control fruit, as did levels by peel of AVG-treated fruit from H2. In 2005, AVG-treated samples from H1 and H2 had TVP slightly lower than control fruit and MCP alone greatly repressed TVP, similar to AVG plus MCP. AVG plus MCP repressed TVP the most both years and for both harvests.

**12WCS:** The same treatment effects seen after 6WCS were also noted after 12WCS with a couple of exceptions. AVG plus MCP was still the treatment that consistently and greatly repressed TVP across years and harvests. However, TVP in AVG-treated peel from H1 reached values higher than (2004) or equal to (2005) controls. Ethephon did not affect TVP in AVG plus MCP-treated fruit.

**Figure 2.4:** Effect of AVG and/or MCP on total volatile production (TVP) by peel (A, B) and cortex (C, D) of 'Royal Gala'. TVP was measured in 2004 (A, C) and 2005 (B, D) on tissue samples of fruit ripened at room temperature for 7 days immediately after harvest (AH) and after 6 and 12 weeks in cold storage at 4°C (6WCS, 12WCS) that had been frozen and then thawed. Different letters indicate significant differences within date separated by the least significant difference (*LSD*) at *p=0.05*.



***Cortex tissue***

Total volatile production by cortex of control fruit behaved similarly to that of peel both in 2004 (Figure 2.4.C) and in 2005 (Figure 2.4.D). Cortex tissue was affected by the treatments in the same way as peel tissue. Overall, TVP by cortex was 31 to 57 % that by peel tissue on a per g FW analysis.

**Grouped volatile production**

Volatile compounds were grouped by primary functional groups, alcohols and esters. Total esters comprised 95 to 100 % of TVP in peel and cortex both years and therefore they are not discussed further in this section. Ester grouping was based on their major contributors; thus they were grouped by acid or alcohol moiety.

***Peel tissue***

Volatile alcohol levels were less than 2.3 % of TVP. As with TVP, volatile alcohol levels in peel of control fruit were highest at harvest in 2004 (Table 2.3) and 2005 (Table 2.4), decreasing after storage in 2004, and increasing in 2005. AVG plus MCP consistently repressed volatile alcohol levels the most at all sampling times regardless of harvest dates, to values 80% to 99% lower than controls. Volatile alcohol levels were higher in both years when AVG-treated fruit was harvested later (H2). Ethephon did not affect AVP of AVG plus MCP-treated fruit.

Acetate esters were the most abundant after harvest, comprising 42.1 % and 35.3 % of the total volatiles in 2004 (Table 2.3) and 2005 (Table 2.4), respectively, followed by methylbutanoate esters. Butanoate and hexanoate esters were the least abundant. In 2004, the relative production of acetate esters decreased in cold storage, though there was a retative increase in butanoate and especially in methylbutanoate ester production. Hexanoate ester production was always the lowest. In 2005, acetate esters were the most abundant after cold storage at levels greater than at harvest followed by methylbutanoate esters. Butanoate and hexanoate esters were the least abundant after harvest, and the production of hexanoate esters only increased after cold storage. In general, ester groups responded to the treatments in the same way as TVP. Only butanoates were more abundant in ethephon-treated AVG plus MCP fruit compared to their untreated counterparts.

Ethyl esters were the most abundant in control fruit immediately after harvest in 2004 and 2005 (Table 2.3, 2.4) as 34 to 38 % of TVP, respectively, and were followed by hexyl esters. Methylbutyl esters were the least abundant. In 2004, relative ethyl ester production almost

**Table 2.3:** Grouped volatile production by peel and cortex tissue from 'Royal Gala' fruit harvested in 2004. Volatiles were grouped as alcohol, acid moiety of esters and alcohol moiety of esters. Fruit were treated with AVG and/or MCP and ripened for 7 days at room temperature after harvest or cold stored for 6 or 12 weeks and then ripened. Some AVG-treated fruit were harvested with controls, and some were harvested 2 weeks later (H2). Different letters indicate significant differences within date separated by the least significant difference (*LSD*) at *p=0.05*; *ns:* no significant differences among means.

| | | AU x10-3/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| | Treatment | Peel | Cortex | Peel | Cortex | Peel | Cortex |
| Alcohol volatiles | Control | 51 a | 46 a | 19 b | 16 bc | 18 bc | 19 bc |
| | AVG | 2 c | 2 c | 15 b | 13c | 31 b | 24 b |
| | MCP | 16 b | 19 b | 36 ab | 40 ab | 62 a | 63 a |
| | AVG+MCP | 1 c | 0 c | 4 b | 3 c | 3 c | 2 c |
| | AVG H2 | 23 b | 28 b | 64 a | 46 a | 82 a | 71 a |
| | AVG+MCP H2 | 2 c | 3 c | 23 b | 10 c | 3 c | 3 c |
| | LSD | 10 | 11 | 33 | 26 | 21 | 19 |
| Acid moiety | | | | | | | |
| Acetate esters | Control | 969 a | 636 a | 156 bc | 109 bc | 141 bc | 98 c |
| | AVG | 25 d | 23 d | 186 bc | 160 bc | 350 b | 263 c |
| | MCP | 315 bc | 271 bc | 648 ab | 539 ab | 1004 a | 1166 a |
| | AVG+MCP | 16 d | 12 d | 33 c | 29 c | 24 c | 23 c |
| | AVG H2 | 419 b | 384 b | 765 a | 670 a | 891 a | 766 b |
| | AVG+MCP H2 | 99 cd | 69 cd | 90 c | 66 c | 26 c | 24 c |
| | LSD | 220 | 241 | 498 | 466 | 322 | 261 |
| Methylbutanoate Esters | Control | 621 a | 185 bc | 308 b | 89 c | 233 d | 77 c |
| | AVG | 618 a | 498 a | 794 a | 352 a | 793 b | 235 b |
| | MCP | 469 a | 257 b | 845 a | 330 a | 992 a | 378 a |
| | AVG+MCP | 175 b | 62 c | 466 b | 174 b | 431 c | 139 c |
| | AVG H2 | 619 a | 412 a | 888 a | 363 a | 821 b | 326 a |
| | AVG+MCP H2 | 256 b | 131 bc | 339 b | 125 bc | 347 cd | 120 c |
| | LSD | 198 | 133 | 204 | 80 | 118 | 68 |
| Butanoate esters | Control | 326 ab | 152 c | 219 b | 87 d | 234 b | 69 c |
| | AVG | 324 ab | 274 a | 513 a | 191 a | 461 a | 119 b |
| | MCP | 307 b | 167 bc | 481 a | 180 ab | 485 a | 190 a |
| | AVG+MCP | 170 c | 66 d | 231 b | 133 bcd | 229 b | 113 c |
| | AVG H2 | 400 a | 224 ab | 426 a | 153 abc | 446 a | 134 bc |
| | AVG+MCP H2 | 155 c | 106 cd | 225 b | 127 cd | 254 b | 121 b |
| | LSD | 91 | 66 | 109 | 52 | 66 | 48 |
| Hexanoate Esters | Control | 291 a | 6 a | 39 bc | 0.9 c | 69 c | 1.7 b |
| | AVG | 4 c | 0 c | 127 bc | 1.5 bc | 242 b | 1.5 b |
| | MCP | 58 c | 2 bc | 211 ab | 2.8 ab | 313 ac | 4.1 a |
| | AVG+MCP | 1 c | 3 bc | 6 c | 0.4 c | 7 c | 0 c |
| | AVG H2 | 160 b | 4 ab | 474 a | 3.1 a | 382 a | 4.1 a |
| | AVG+MCP H2 | 17 c | 0 c | 15 bc | 0 c | 6 c | 0.4 bc |
| | LSD | 96 | 3 | 203 | 1.6 | 135 | 1.4 |
| Alcohol moiety | | | | | | | |
| Ethyl esters | Control | 788 a | 321 bc | 514 c | 175 b | 453 d | 146 d |
| | AVG | 618 ab | 498 a | 772 a | 351 a | 731 b | 233 c |
| | MCP | 456 bcd | 291 bc | 817 a | 325 a | 936 a | 371 a |
| | AVG+MCP | 345 d | 128 d | 696 abc | 306 a | 660 bc | 252 bc |
| | AVG H2 | 591 abc | 407 ab | 797 ab | 358 a | 739 b | 319 ab |
| | AVG+MCP H2 | 402 cd | 237 cd | 560 bc | 252 ab | 599 c | 241 bc |
| | LSD | 205 | 117 | 194 | 108 | 90 | 79 |

| 2.3 (continued) | | AU x$10^{-3}$/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| | Treatment | *Peel* | *Cortex* | *Peel* | *Cortex* | *Peel* | *Cortex* |
| *Hexyl esters* | Control | 616 a | 298 a | 75 b | 38 bc | 89 c | 40 b |
| | AVG | 24 c | 20 c | 171 b | 79 bc | 341 b | 136 b |
| | MCP | 119 c | 71 bc | 370 ab | 238 ab | 565 a | 485 a |
| | AVG+MCP | 13 c | 12 c | 26 b | 20 c | 23 c | 18 b |
| | AVG H2 | 319 b | 185 ab | 801 a | 314 a | 623 a | 360 a |
| | AVG+MCP H2 | 45 c | 21 c | 66 b | 28 c | 26 c | 17 b |
| | *LSD* | *162* | *127* | *348* | *208* | *204* | *131* |
| *Butyl esters* | Control | 480 a | 153 a | 66 bc | 27 c | 97 bc | 36 c |
| | AVG | 3c | 2 c | 115 bc | 44 bc | 239 b | 80 c |
| | MCP | 85 bc | 54 bc | 375 ab | 211 ab | 603 a | 510 a |
| | AVG+MCP | 1 c | 1 c | 5 c | 2 c | 4 c | 1c |
| | AVG H2 | 190 b | 115 ab | 665 a | 239 a | 592 a | 304 b |
| | AVG+MCP H2 | 23 c | 7 c | 32 c | 19 c | 2 c | 2 c |
| | *LSD* | *131* | *88* | *311* | *179* | *225* | *102* |

**Table 2.4:** Grouped volatile production by peel and cortex tissue from 'Royal Gala' fruit harvested in 2005. Volatiles were grouped as alcohol, acid moiety of esters and alcohol moiety of esters. Fruit were treated with AVG and/or MCP and ripened for 7 days at room temperature after harvest or cold stored for 6 or 12 weeks and then ripened. Some AVG-treated fruit were harvested with controls, and some were harvested 1 week later (H2). E= Ethephon. Different letters indicate significant differences within date separated by the least significant difference (*LSD*) at *p=0.05*; *ns:* no significant differences among means.

| | | AU $x10^{-3}$/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| | Treatment | Peel | Cortex | Peel | Cortex | Peel | Cortex |
| Alcohol volatiles | Control | 21 ab | 22 a | 53 a | 31 a | 200 a | 896 a |
| | AVG | 12 bc | 9 b | 33 b | 23 b | 189 a | 85 a |
| | MCP | 2 c | 2 bc | 6 c | 6 c | 35 b | 26 c |
| | AVG+MCP | 0 c | 1 c | 4 c | 5 c | 30 b | 22 c |
| | AVG+MCP+E | | | | | 41 b | 0 d |
| | AVG H2 | 32 a | 24 a | 61 a | 34 a | 174 a | 52 b |
| | AVG+MCP H2 | 2 c | 2 bc | 1 c | 5 c | 21 b | 18 c |
| | LSD | 17 | 7 | 14 | 7 | 54 | 17 |
| Acid moiety Acetate esters | Control | 1013 a | 734 a | 1367 a | 1554 a | 2988 a | 2962 a |
| | AVG | 463 bc | 292 bc | 690 c | 804 b | 2369 b | 2740 a |
| | MCP | 197 c | 141cd | 127 d | 83 c | 474 cd | 316 c |
| | AVG+MCP | 74 c | 58 d | 95 d | 68 c | 387 cd | 272 c |
| | AVG+MCP+E | | | | | 672 c | 216 c |
| | AVG H2 | 755 ab | 501 b | 1057 b | 823 b | 2012 b | 2080 b |
| | AVG+MCP H2 | 134 c | 86 cd | 84 d | 423 c | 222 d | 132 c |
| | LSD | 400 | 222 | 242 | 302 | 414 | 294 |
| Methylbutanoate Esters | Control | 756 a | 446 a | 818 a | 198 a | 1185 a | 252 a |
| | AVG | 698 a | 406 a | 532 b | 151 b | 1537 a | 233.a |
| | MCP | 398 c | 238 b | 180 c | 54 d | 347 b | 123.bc |
| | AVG+MCP | 309 c | 175 b | 180 c | 57 d | 394 b | 1567b |
| | AVG+MCP+E | | | | | 652 b | 73 c |
| | AVG H2 | 548 b | 196 b | 571 b | 108 c | 1150 a | 157 b |
| | AVG+MCP H2 | 281 c | 156 b | 161 c | 49 d | 282 b | 88 c |
| | LSD | 123 | 92 | 237 | 389 | 450 | 62 |
| Butanoate esters | Control | 527 a | 358 a | 261 a | 156 a | 364 a | 162 a |
| | AVG | 332 b | 292 a | 200 b | 109 b | 379 a | 143 a |
| | MCP | 214 cd | 141 b | 110 c | 50 c | 245 bc | 94 b |
| | AVG+MCP | 172 cd | 110 b | 88 c | 41 c | 189 cd | 93 b |
| | AVG+MCP+E | | | | | 274 b | 42 d |
| | AVG H2 | 248 bc | 132 b | 194 b | 93 b | 324 ab | 69 c |
| | AVG+MCP H2 | 138 d | 91 b | 89 c | 40 c | 126 d | 52 cd |
| | LSD | 85 | 68 | 36 | 16 | 79 | 23 |
| Hexanoate Esters | Control | 517 a | 6 ab | 465 a | 7.3 a | 1032 a | 10 ab |
| | AVG | 198 b | 2 b | 329 b | 4.3ab | 1091 a | 12 a |
| | MCP | 27 bc | 1 b | 73 c | 1.8bc | 457 cd | 2 c |
| | AVG+MCP | 10 c | 1 b | 44 c | 0 c | 336 cd | 4 bc |
| | AVG+MCP+E | | | | | 617 bc | 0 c |
| | AVG H2 | 389 a | 10 a | 535 a | 3.1bc | 864 ab | 14 a |
| | AVG+MCP H2 | 16 c | 2 b | 27 c | 1.5bc | 126 d | 1 c |
| | LSD | 134 | 7 | 96 | 3.5 | 340 | 7 |

| 2.4 (continued) | | AU x$10^{-3}$/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| Alcohol moiety | Treatment | Peel | Cortex | Peel | Cortex | Peel | Cortex |
| Ethyl esters | Control | 1111 a | 789a | 465 a | 317 a | 684 ab | 379 a |
| | AVG | 920 b | 694 a | 423 ab | 242 b | 743 a | 326 a |
| | MCP | 588 c | 378 b | 272 c | 104 d | 524 c | 210 b |
| | AVG+MCP | 469 cd | 284 b | 248 c | 99 d | 529 c | 245 b |
| | AVG+MCP+E | | | | | 653abc | 108 d |
| | AVG H2 | 588 c | 316 b | 365 b | 187 c | 560 bc | 179 bc |
| | AVG+MCP H2 | 400 d | 246 b | 238 c | 88 d | 374 d | 136 cd |
| | LSD | 174 | 152 | 75 | 35 | 137 | 68 |
| Hexyl esters | Control | 728 a | 288 a | 1329 a | 751 a | 2854 a | 1381 a |
| | AVG | 429 a | 135 b | 697 b | 409 b | 2541 ab | 1363 a |
| | MCP | 78 b | 38 bc | 116 c | 39 c | 416 c | 142 b |
| | AVG+MCP | 31 b | 22 c | 78 c | 29 c | 371 c | 141 b |
| | AVG+MCP+E | | | | | 708 c | 85 b |
| | AVG H2 | 720 a | 254 a | 1207 a | 463 b | 2061 b | 1210 a |
| | AVG+MCP H2 | 51 b | 21 c | 60 c | 23 c | 210 c | 71 b |
| | LSD | 327 | 111 | 296 | 169 | 691 | 238 |
| Butyl esters | Control | 629 a | 203 a | 823 a | 531 a | 1766 a | 1247 a |
| | AVG | 228 bc | 77 bc | 468 c | 256 b | 1599 ab | 916 b |
| | MCP | 43 c | 19 c | 59 d | 20 c | 419 cd | 71 d |
| | AVG+MCP | 18 c | 7 c | 46 d | 18 c | 288 cd | 60 d |
| | AVG+MCP+E | | | | | 561 c | 54 d |
| | AVG H2 | 409 ab | 125 b | 642 b | 264 b | 1375 b | 562 c |
| | AVG+MCP H2 | 34 c | 12 c | 28 d | 8 c | 106 d | 25 d |
| | LSD | 260 | 76 | 160 | 124 | 325 | 142 |

doubled that of harvest after 6 and 12 weeks in cold storage, while the rest of the esters decreased. In 2005, hexyl esters were the most abundant after storage followed by butyl esters, while the relative production of ethyl esters decreased. There was a similar response to the treatments as with TVP, though hexyl and butyl esters were greatly affected, more than ethyl esters. In 2005, AVG-treated peel from H2 had lower ethyl ester production than from H1, both after harvest and after 12WCS, which wasopposite to the general trend for the later harvest. Ethephon did not have an effect on any group.

***Cortex tissue***

Volatile alcohol levels were similar in cortex and peel of control fruit in absolute values, though they were twice as high in cortex than in peel when they were expressed as percentage of TVP both in 2004 (Table 2.3) and 2005 (Table 2.4), reaching values close to 5 %. Volatile alcohol levels in cortex were similarly affected by the treatments as in peel, though we could not detect volatile alcohols in AVG plus MCP-treated fruit that were further treated with ethephon.

Of the volatiles grouped by acid moiety, acetate esters were the most abundant in both years immediately after harvest, comprising 62.1% and 46.9 % of the total volatiles in 2004 and 2005, respectively. Acetate esters were also the most abundant after storage, though their relative production decreased in 2004 but increased in 2005, reaching 36.1 % and 85.2 % of TVP after 12 weeks at 4 $^{o}$C, respectively. Hexanoate esters were the least abundant, and their production was very limited at all times. Butanoate and methylbutanoate esters had similar production levels, and greatly decreased in 2005 with time in storage. There were no major differences between cortex and peel in how they responded to the treatments, though methylbutanoate esters increased in AVG-treated cortex and butanoate esters increased in AVG plus MCP-treated cortex in 2004 when fruit were harvested later, whereas peel tissue did not differ between harvests. Ethephon did not induce an increase in the production of any group of volatiles.

Of the esters grouped by alcohol moiety, ethyl esters were the most abundant in cortex of control fruit immediately after harvest in 2004 (Table 2.3) and 2005 (Table 2.4),
comprising 31.3 % and 50.4 % of TVP, respectively. The relative production of butyl and hexyl esters after 6 and 12 weeks in storage decreased in 2004 and increased in 2005, parallel to a decrease in ethyl esters. 2-Methylbutyl acetate was present in lower amounts at all times. The same effects of treatments observed in peel were evident in cortex of ‘Royal Gala’ apples and again, ethephon did not affect the production of any group of volatiles.

**Individual volatile production**

**Peel tissue**

**Harvest:** Seven days after harvest esters were the most abundant volatile compounds, and the most abundant ester produced by peel tissue of control fruit was ethyl 2-methylbutanoate (E2MB) both in 2004 (Table 2.5) and 2005 (Table 2.6) with 21.3 % and 34.8 % of TVP, respectively. In 2004, the next most abundant volatiles were hexyl acetate, 2-methylbutyl acetate, ethyl butanoate and butyl hexanoate comprising 16.9 %,
16.5 %, 13.0 %, and 9.3 % of TVP, respectively. In 2005, ethyl butanoate, hexyl acetate, 2-methylbutyl acetate, and butyl hexanoate followed E2MB, representing 17.3 %, 15.0 %, 13.7 % and 12.5 % of TVP. Butyl acetate was relatively less abundant, being 6 to 8 %
of TVP. Hexanol, butanol and 2-methyl-1-butanol were 1 %, 0.9 % and 0.3 % of TVP in 2004 and 0.3 %, 0.3 % and 0.1 % of TVP in 2005, respectively.

**6WCS:** After 6 weeks in cold storage, the most abundant ester in 2004 was E2MB at close to 40.6% of TVP, followed by ethyl butanoate, 2-methylbutyl acetate, and hexyl acetate with production levels 28.6 %, 9.9 % and 7 % of TVP, respectively. In 2005, the most abundant volatile was hexyl acetate with 21.6 % of TVP, followed by hexyl-2- methylbutanoate, 2-methylbutyl acetate, butyl hexanoate, butyl acetate, E2MB, and ethyl butanoate, comprising 14.2 %, 13.5 %, 11.4 %, 9.7 %, 7.8 % and 7.4 % of TVP, respectively. Alcohol volatile levels were similar to those at harvest in 2004, though there was a small increase in 1-butanol to 1.2 % of TVP. In 2005 butanol and hexanol levels increased to 0.5 % ad 1.2 % of TVP, respectively, and 2-methyl-1-butanol remained at the same relative concentration as at harvest.

**12WCS:** After 12 weeks in cold storage in 2004, the most abundant volatiles were ethyl butanoate and E2MB as 32.7 % and 32.3 % of TVP, followed by hexyl acetate, butyl hexanoate and 2-methylbutyl acetate representing 8.6 %, 7.5 % and 6.6 % of TVP, respectively. In 2005, hexyl acetate was the most abundant volatile (26.8 % of TVP), followed by butyl acetate, butyl hexanoate, hexyl 2-methylbutanoate and 2-methylbutyl acetate (14.2 %, 11.8 %, 10.8 % and 9.4 % of TVP). Individual alcohol concentrations remained the same as after 12 weeks in cold storage in 2004, and doubled in 2005.

***Treatment effects:*** In 2004 AVG plus MCP repressed volatile production the most at harvest and after 6 and 12 weeks in cold storage, while AVG and MCP alone had variable effects. At harvest,

**Table 2.5:** Individual volatile production by peel and cortex tissue from 'Royal Gala' fruit harvested in 2004. Fruit were treated with AVG and/or MCP and ripened for 7 days at room temperature after harvest or cold stored for 6 or 12 weeks and then ripened. Some AVG-treated fruit were harvested with controls, and some were harvested 2 weeks later (H2). Different letters indicate significant differences within date separated by the least significant difference (LSD) at p=0.05; ns: no significant differences among means.

| | | AU x$10^{-3}$/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| Compound | Treatment | *Peel* | *Cortex* | *Peel* | *Cortex* | *Peel* | *Cortex* |
| **Alcohols** | | | | | | | |
| *Hexanol* | Control | 23.9 a | 18.8 a | 7.2 ab | 4.8 bc | 6.8 cd | 5.6 bc |
| | AVG | 2.1c | 1.5 cd | 7.4 ab | 6.0 bc | 14.3 bc | 9.8 b |
| | MCP | 4.2 c | 5.3 c | 13.7 ab | 13.6 ab | 20.1 b | 19.5 a |
| | AVG+MCP | 1.0 c | 0.4 d | 2.6 b | 1.2 c | 2.4 d | 1.7 c |
| | AVG H2 | 11.8 b | 13.1 b | 28.6 a | 16.5 a | 36.4 a | 24.3 a |
| | AVG+MCP H2 | 1.6 c | 2.0 cd | 20.2 ab | 4.4 c | 3.1 b | 2.3 c |
| | *LSD* | *4.5* | *4.5* | *22.5* | *9.0* | *8.2* | *6.2* |
| *1-Butanol* | Control | 20.4 a | 21.3 a | 9.1 bc | 8.9 bc | 9.0 bc | 11.1 bc |
| | AVG | 0 c | 0 c | 6.1 c | 6.4 c | 13.8 b | 12.2 b |
| | MCP | 6.6 b | 8.0 b | 19.0 ab | 23.4 ab | 36.4 a | 38.6 a |
| | AVG+MCP | 0 c | 0 c | 1.0 c | 2.0 c | 0.5 c | 0 c |
| | AVG H2 | 9.5 b | 12.1 b | 25.6 a | 24.8 a | 39.6 a | 41.7 a |
| | AVG+MCP H2 | 0 c | 0 c | 2.8 c | 3.37 c | 0 c | 0.8 bc |
| | *LSD* | *4.8* | *6.4* | *5.1* | *5.0* | *12.5* | *12.1* |
| *2-Methyl-1-butanol* | Control | 6.7 a | 6.1 a | 2.5 b | 2.6 ab | 1.8 bc | 2.0 b |
| | AVG | 0 c | 0 c | 1.7 b | 1.0 b | 2.8 b | 2.2 b |
| | MCP | 4.8 a | 5.2 a | 3.3 b | 3.0 ab | 5.4 a | 4.9 a |
| | AVG+MCP | 0 c | 0 c | 0 c | 0 b | 0 c | 0 c |
| | AVG H2 | 2.4 b | 2.6 b | 9.5 a | 4.4 a | 6.1 a | 5.2 a |
| | AVG+MCP H2 | 0 c | 1.3 bc | 0.4 b | 1.98 ab | 0 c | 0 c |
| | *LSD* | *2.3* | *2.3* | *3.6* | *3.2* | *2.1* | *1.3* |
| **Ethyl esters** | | | | | | | |
| *Ethyl-2-methylbutanoate* | Control | 490 a | 169 bc | 302 b | 88 c | 225 c | 77 c |
| | AVG | 324 b | 274 a | 492 a | 189 a | 413 a | 117 bc |
| | MCP | 287 bc | 165 bc | 428 a | 175 a | 399 a | 182 a |
| | AVG+MCP | 175 c | 62 d | 465 a | 174 a | 431 a | 139 b |
| | AVG H2 | 367 ab | 218 ab | 339 b | 147 ab | 337 b | 129 b |
| | AVG+MCP H2 | 252 bc | 131 cd | 337 b | 125 bc | 345 b | 120 b |
| | *LSD* | *124* | *69* | *89* | *46* | *48* | *42* |
| *Ethyl butanoate* | Control | 299 a | 152 bc | 212 c | 87 c | 228.0 d | 69 c |
| | AVG | 293 a | 225 a | 280 abc | 162 ab | 318.0 bc | 117 bc |
| | MCP | 169 b | 126 c | 389 a | 151 abc | 536.5a | 189 a |
| | AVG+MCP | 170 b | 66 d | 231 bc | 133bc | 228.9 d | 113 bc |
| | AVG H2 | 225 ab | 189 ab | 375 ab | 211 a | 402.4 b | 190 a |
| | AVG+MCP H2 | 150 b | 106 cd | 223 c | 127 bc | 254.0 cd | 121 b |
| | *LSD* | *109* | *59* | *145* | *68* | *85.3* | *51* |
| **Hexyl esters** | | | | | | | |
| *Hexyl acetate* | Control | 389 a | 268 a | 52 abc | 33 bc | 60 bc | 34 c |
| | AVG | 20 c | 19 c | 89 bc | 72 bc | 166 b | 125 c |
| | MCP | 81 c | 64 bc | 275 ab | 219 ab | 381 a | 458 a |
| | AVG+MCP | 11 c | 9 c | 21 c | 19 c | 16 c | 17 c |
| | AVG H2 | 222 b | 165 a | 357 a | 292 a | 392 a | 329 b |
| | AVG+MCP H2 | 33 c | 19 c | 39 bc | 23 c | 16 c | 15 c |
| | *LSD* | *126* | *119* | *242* | *195* | *134* | *123* |

| 2.5 (continued) | | AU x$10^{-3}$/g FW Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
|---|---|---|---|---|---|---|---|
| Compound | Treatment | *Peel* | *Cortex* | *Peel* | *Cortex* | *Peel* | *Cortex* |
| *Hexyl 2-methylbutanoate* | Control | 82 a | 11 a | 3.5 b | 1.4 *ns* | 4 b | 0.4 b |
| | AVG | 0 c | 0 c | 22.4 ab | 1.0 | 62 a | 1.5 b |
| | MCP | 13 bc | 1 bc | 27.7 ab | 4.8 | 56 a | 7.1 a |
| | AVG+MCP | 1 c | 0 c | 0 b | | 0 b | 0 b |
| | AVG H2 | 27 b | 4 b | 64.3 a | 5.0 | 82 a | 6.8 a |
| | AVG+MCP H2 | 4 c | 0 c | 1.5 b | | 1 b | 0 b |
| | *LSD* | *23* | *5* | *46.5* | *5.1* | *40* | *3.3* |
| *Hexyl hexanoate* | Control | 77 a | 0.9 b | 119 b | 0 *ns* | 16 b | 0 *ns* |
| | AVG | 2 c | 0.4 b | 47 ab | 0 | 82 a | 0 |
| | MCP | 17 c | 0.5 b | 43 ab | 0 | 86 a | 0 |
| | AVG+MCP | ND c | 3.0 a | 3 b | 0 | 5 b | 0 |
| | AVG 2H | 45 b | 2.1 a | 92 a | 0 | 80 a | 0.4 |
| | AVG+MCP 2H | 5 c | 0.4 b | 5 b | 0 | 6 b | 0 |
| | *LSD* | *26* | *1.1* | *55* | | *45* | *0.5* |
| *Hexyl propionate* | Control | 43 a | 0 *ns* | 2 a | 0 *ns* | 2 c | 0 *ns* |
| | AVG | 0 b | 0 | 5 b | 0 | 16 b | 0 |
| | MCP | 4 b | 0 | 11 b | 0 | 22 ab | 0 |
| | AVG+MCP | 0 b | 0 | 0 b | 0 | 0 c | 0 |
| | AVG H2 | 13 b | 0 | 47 a | 0 | 33 a | 0 |
| | AVG+MCP H2 | 0 b | 0 | 0 b | 0 | 0 c | 0 |
| | *LSD* | *15* | | *12* | | *12* | |
| **Butyl esters** | | | | | | | |
| *Butyl hexanoate* | Control | 214 a | 5.2 a | 28 bc | 0 c | 52 cd | 1.7 b |
| | AVG | 2 c | 0 b | 80 bc | 1.5 bc | 160 bc | 1.5 bc |
| | MCP | 41 bc | 1.4 b | 169 ab | 2.8 ab | 227 ab | 4.1 a |
| | AVG+MCP | 1 c | 0 b | 3 c | 0.4 c | 3 d | 0 d |
| | AVG H2 | 115 b | 1.8 a | 258 a | 3.1 a | 302 a | 3.7 a |
| | AVG+MCP H2 | 11 c | 0 b | 10 c | 0 c | 0 d | 0.4 cd |
| | *LSD* | *74* | *1.9* | *152* | *1.6* | *114* | *1.1* |
| *Butyl acetate* | Control | 190 a | 143 a | 29 b | 26 c | 35 b | 34 c |
| | AVG | 2 c | 2 c | 35 b | 43 bc | 79 b | 78 c |
| | MCP | 44 bc | 52 bc | 206 a | 208 ab | 376 a | 506 a |
| | AVG+MCP | ND c | 1 c | 2 b | 2 c | 1 b | 1 c |
| | AVG H2 | 75 b | 110 ab | 219 a | 236 a | 290 a | 300 b |
| | AVG+MCP H2 | 7 c | 7 c | 19 b | 19. c | 2 b | 2 c |
| | *LSD* | *53* | *86* | *162* | *177* | *121* | *102* |
| *Butyl-2-methylbutanoate* | Control | 49 a | 5 a | 3 b | 0 b | 4 cd | 0 b |
| | AVG | ND c | 0 c | 12 b | 1 ab | 36 bc | 1.5 b |
| | MCP | 9 bc | 1 bc | 28 ab | 2 ab | 57 ab | 3.8 a |
| | AVG+MCP | ND c | 0 c | 0 b | 0 b | 0 d | 0 b |
| | AVG H2 | 16 b | 2 b | 48 a | 3 a | 77 a | 4.2 a |
| | AVG+MCP H2 | 0 c | 0 c | 1 b | 0 b | 0 d | 0 b |
| | *LSD* | *13* | *2* | *35* | *3* | *34* | *2.0* |
| *Butyl butanoate* | Control | 28 a | 0 *ns* | 7 bc | 0 *ns* | 6 bc | 0 *ns* |
| | AVG | 0 d | 0 | 7 bc | 0 | 12 b | 0 |
| | MCP | 11 b | 0 | 20 ab | 0 | 24 a | 0 |
| | AVG+MCP | 0 d | 0 | 1 c | 0 | 0 c | 0 |
| | AVG H2 | 17 bc | 0 | 26 a | 0 | 33 a | 0 |
| | AVG+MCP H2 | 5 cd | 0 | 2 c | 0 | 0 c | 0 |
| | *LSD* | *9* | | *15* | | *10* | |

| 2.5 (continued) | | AU x$10^{-3}$/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| Compound | Treatment | *Peel* | *Cortex* | *Peel* | *Cortex* | *Peel* | *Cortex* |
| **Methylbutyl esters** | | | | | | | |
| *2-Methylbutyl* | Control | 380 a | 216 a | 74 abc | 49 abc | 46 bc | 30 cd |
| *Acetate* | AVG | 3 d | 3 c | 61 bc | 43 bc | 105 b | 59 c |
| | MCP | 191 b | 155 b | 162 ab | 108 ab | 243 a | 198 a |
| | AVG+MCP | 5 d | 3 c | 10 b | 8 c | 7 c | 6 d |
| | AVG H2 | 122 bc | 103 b | 188 a | 139 a | 208 a | 135 b |
| | AVG+MCP H2 | 59 cd | 43 c | 31 c | 24 bc | 9 c | 7 cd |
| | *LSD* | *102* | *59* | *125* | *95* | *86* | *51* |
| **Others** | | | | | | | |
| *t-2-Hexenyl* | Control | 10 a | 9 a | 2.1 b | 0.9 bc | 1.5 b | 0 b |
| *acetate* | AVG | 0 b | 0 b | 1.5 bc | 1.9 abc | 0 c | 0.7 b |
| | MCP | 0 b | 0 b | 5.2 a | 3.5 a | 3.6 a | 4.3 a |
| | AVG+MCP | 0 b | 0 b | 0.4 c | 0 c | 0 c | 0 b |
| | AVG H2 | 0 b | 5 ab | 0.8 bc | 2.5 ab | 0 c | 1.4 b |
| | AVG+MCP H2 | 0 b | 0 b | 0.4c | 0 c | 0 c | 0 b |
| | *LSD* | *1* | *6* | *1.6* | *2.2* | *0.2* | *1.9* |

**Table 2.6:** Individual volatile production (AU x$10^{-3}$/g FW) by peel and cortex tissue from 'Roya Gala' fruit harvested in 2005. Fruit were treated with AVG and/or MCP and ripened for 7 days at room tempearture or stored and then ripened as in 2004. Some AVG-treated fruit were harvested with controls, and some were harvested 1 week later (H2). E= Ethephon. Different letters indicate significant differences within date separated by the least significant difference (*LSD*) at *p=0.05*; *ns:* no significant differences among means.

| | | AU x$10^{-3}$/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| Compound | Treatment | *Peel* | *Cortex* | *Peel* | *Cortex* | *Peel* | *Cortex* |
| **Alcohols** | Control | 8 ab | 8 b | 35 a | 12 b | 140 a | 27 ab |
| *Hexanol* | AVG | 6 b | 4 c | 23 b | 12 b | 123 a | 28 a |
| | MCP | 0 b | 1 cd | 5 c | 5 c | 21 b | 11d |
| | AVG+MCP | 0 b | 1 cd | 3 c | 3 c | 19 b | 12 cd |
| | AVG+MCP+E | | | | | 28 b | 0 e |
| | AVG H2 | 18 a | 12 a | 44 a | 17 a | 123 a | 19 bc |
| | AVG+MCP H2 | 0 b | 0 d | 0 c | 4 c | 14 b | 10 d |
| | *LSD* | *10* | *4* | *11* | *4* | *43* | *7* |
| *1-Butanol* | Control | 11 a | 10 a | 14 a | 16 a | 52 a | 55 a |
| | AVG | 4 ab | 4 b | 8 b | 10 b | 53 a | 48 a |
| | MCP | 1 b | 0 c | 1 c | 2 c | 10 b | 10 c |
| | AVG+MCP | 0 b | 0 c | 1 c | 2 c | 8 b | 8 cd |
| | AVG+MCP+E | | 3 b | | | 8 b | 0 d |
| | AVG H2 | 10 a | 9 a | 15 a | 15 a | 42 a | 27 b |
| | AVG+MCP H2 | 1 b | 1 bc | 1 c | 1 c | 5 b | 6 cd |
| | *LSD* | *6* | *3* | *6* | *4* | *12* | *9* |
| *2-Methyl-1-Butanol* | Control | 3.4 a | 3.3 a | 3.3 a | 3.1 a | 8 b | 6.5 b |
| | AVG | 1.5 abc | 0.6 b | 2.4 a | 1.6 b | 14 a | 9.1 a |
| | MCP | 1.3 bc | 1.4 c | 0 b | 0 c | 4 c | 4.0 cd |
| | AVG+MCP | 0 c | 0 c | 0 b | 0 c | 3 c | 2.7 de |
| | AVG+MCP+E | | | | | 5 c | 0.0 f |
| | AVG H2 | 3.2 ab | 3.1 a | 2.1 a | 2.1 ab | 9 b | 5.5 bc |
| | AVG+MCP H2 | 0.8 c | 1.0 bc | 0 b | 0 c | 2 c | 2.0 ef |
| | *LSD* | *2.0* | *3.0* | *1.4* | *1.14* | *3* | *1.7* |
| **Ethyl esters** | | | | | | | |
| *Ethyl-2-Methylbutanoate* | Control | 614 a | 431 a | 2383 a | 161 a | 371 abc | 217 a |
| | AVG | 600 a | 403a | 241 a | 133 b | 427 a | 183 ab |
| | MCP | 381 b | 237 b | 164 b | 54 d | 296 cd | 117 cd |
| | AVG+MCP | 304 b | 175 b | 165 b | 57 d | 352 abcd | 153 bc |
| | AVG+MCP+E | | | | | 405 ab | 66 d |
| | AVG H2 | 362 b | 185 b | 198 ab | 93 c | 304 bcd | 110 cd |
| | AVG+MCP H2 | 270 b | 155 b | 150 b | 48 d | 253 d | 85 d |
| | *LSD* | *146* | *97* | *55* | *25* | *103* | *55* |
| *Ethyl butanoate* | Control | 498 a | 358 a | 227 a | 156 a | 313 ab | 162 a |
| | AVG | 320 b | 292 a | 182 b | 109 b | 316 a | 143 a |
| | MCP | 208 cd | 141 b | 108 c | 50 c | 228 cd | 93 b |
| | AVG+MCP | 165 cd | 110 b | 84 c | 41 c | 176 de | 93 b |
| | AVG+MCP+E | | | | | 247 bc | 42 d |
| | AVG H2 | 226 c | 132 b | 168 b | 93 b | 256 abc | 69 c |
| | AVG+MCP H2 | 130 d | 91 b | 89 c | 40 c | 121 e | 52 cd |
| | *LSD* | *81* | *68* | *37* | *16* | *68* | *23* |

| 2.6 (continued) | | AU x$10^{-3}$/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| Compound | Treatment | *Peel* | *Cortex* | *Peel* | *Cortex* | *Peel* | *Cortex* |
| **Hexyl esters** | | | | | | | |
| *Hexyl acetate* | Control | 431 a | 270 a | 659 a | 720 a | 1586 a | 1335 a |
| | AVG | 247 a | 128 bc | 309 b | 388 b | 1059 b | 1308 a |
| | MCP | 49 b | 36 cd | 54 c | 32 c | 197 c | 126 b |
| | AVG+MCP | 24 b | 20 d | 38 c | 25 c | 176 c | 124 b |
| | AVG+MCP+E | | | | | 235 c | 80 b |
| | AVG H2 | 361 a | 229 ab | 578 a | 436 b | 960 b | 1164 a |
| | AVG+MCP H2 | 36 b | 18 d | 34 c | 17 c | 105 d | 59 b |
| | *LSD* | *191* | *102* | *139* | *161* | *224* | *2300* |
| *Hexyl hexanoate* | Control | 156 a | 1.1 *ns* | 118 b | 1.9 *ns* | 335 a | 2.0 *ns* |
| | AVG | 76 ab | 0.5 | 105 b | 0.7 | 323 a | 2.4 |
| | MCP | 10 b | 0.7 | 40 c | 1.8 | 142 bc | 0 |
| | AVG+MCP | 3 b | 1.4 | 22 c | 0 | 125 bc | 1.8 |
| | AVG+MCP+E | | | | | 214 ab | 0 |
| | AVG H2 | 157 a | 4.8 | 229 a | 0 | 215 ab | 2.9 |
| | AVG+MCP H2 | 5 b | 1.9 | 14 c | 1.1 | 56 c | 0 |
| | *LSD* | *88* | *4.6* | *45* | *2.0* | *144* | *3.1* |
| *Hexyl 2-methylbutanoate* | Control | 93 a | 9 a | 433 a | 18 a | 637 a | 17 a |
| | AVG | 78 ab | 2 b | 218 b | 10 b | 862 a | 24 a |
| | MCP | 15 bc | 1 b | 16 c | 0 c | 35 b | 5 b |
| | AVG+MCP | 5 c | 0 b | 13 c | 0 c | 33 b | 3 b |
| | AVG+MCP+E | | | | | 186 b | 5 b |
| | AVG H2 | 144 a | 8 a | 303 ab | 10 b | 609 a | 23 a |
| | AVG+MCP H2 | 9 bc | 1 b | 11 c | 1 c | 26 b | 2 b |
| | *LSD* | *70* | *5* | *158* | *6* | *344* | *7* |
| *Hexyl propionate* | Control | 41 a | 0 | 83.8 a | 0 | 1563 a | 0 |
| | AVG | 22 b | 0 | 41.7 b | 0 | 174 a | 0 |
| | MCP | 5 bc | 0 | 1.2 c | 0 | 21 b | 0 |
| | AVG+MCP | 0 c | 0 | 3.4 c | 0 | 19 b | 0 |
| | AVG+MCP+E | | | | | 45 b | 0 |
| | AVG H2 | 40 a | 0 | 52.1 b | 0 | 154 a | 0 |
| | AVG+MCP H2 | 3 c | 0 | 2.2 c | 0 | 8 b | 0 |
| | *LSD* | *17* | | *23.5* | | *46* | |
| **Butyl esters** | | | | | | | |
| *Butyl hexanoate* | Control | 360 a | 5.3 a | 348 a | 5.4 a | 698 a | 8 a |
| | AVG | 122 bc | 1.4 b | 224 b | 3.6 ab | 768 a | 10 a |
| | MCP | 18 c | 0.4 b | 32 c | 0 c | 315 b | 2 b |
| | AVG+MCP | 7 c | 0 b | 22 c | 0 c | 210 bc | 2 b |
| | AVG+MCP+E | | | | | 403 b | 0 b |
| | AVG H2 | 232 ab | 5.2 a | 306 a | 3.1 b | 650 a | 11 a |
| | AVG+MCP H2 | 12 c | 0 b | 13 c | 0.4 c | 71 c | 1 b |
| | *LSD* | *133* | *3.0* | *65* | *1.9* | *216* | *4* |
| *Butyl acetate* | Control | 190 a | 191.9 a | 295 a | 505.7 a | 840 a | 1221 a |
| | AVG | 75 bc | 74.7 bc | 153 b | 244.8 b | 521 b | 881 b |
| | MCP | 17bc | 18.1 c | 25 c | 20.1 c | 72 c | 68 d |
| | AVG+MCP | 4 c | 6.9 c | 18 c | 17.5 c | 57 c | 57 d |
| | AVG+MCP+E | | | | | 72 c | 53 d |
| | AVG H2 | 114 ab | 116.4 b | 240 a | 255.6 b | 422 b | 528 c |
| | AVG+MCP H2 | 13 c | 12.3 c | 14 c | 7.6 c | 27 c | 243 d |
| | *LSD* | *99* | *73.3* | *70* | *124.0* | *109* | *144* |

| 2.6 (continued) | | AU x$10^{-3}$/g FW | | | | | |
|---|---|---|---|---|---|---|---|
| | | Harvest | | 6 weeks in cold storage | | 12 weeks in cold storage | |
| Compound | Treatment | *Peel* | *Cortex* | *Peel* | *Cortex* | *Peel* | *Cortex* |
| *Butyl-2-methylbutanoate* | Control | 50 a | 5.3 a | 143 a | 19.5 a | 176 a | 19 a |
| | AVG | 19 ab | 1.2 c | 73 b | 7.6 b | 248 a | 26 a |
| | MCP | 2 b | 0 c | 1 c | 0 b | 16 b | 2 b |
| | AVG+MCP | 0 b | 0 c | 2 c | 0 b | 9 b | 1 b |
| | AVG+MCP+E | | | | | 60 b | 2 b |
| | AVG H2 | 42 a | 3.1 b | 70 b | 5.1 b | 236 a | 23 a |
| | AVG+MCP H2 | 2 b | 0 c | 1 c | 0 b | 3 b | 1 b |
| | *LSD* | *31* | *1.4* | *43* | *8.4* | *97* | *9* |
| *Butyl butanoate* | Control | 29 a | 0 | 34 a | 0 | 52 a | 0 |
| | AVG | 12 b | 0 | 18 c | 0 | 64 a | 0 |
| | MCP | 6 b | 0 | 2 d | 0 | 18 bc | 0 |
| | AVG+MCP | 7 b | 0 | 4 d | 0 | 12 bc | 0 |
| | AVG+MCP+E | | | | | 26 b | 0 |
| | AVG H2 | 22 a | 0 | 26 b | 0 | 68 a | 0 |
| | AVG+MCP H2 | 7 b | 0 | 1 d | 0 | 5 c | 0 |
| | *LSD* | *8* | | *8* | | *20* | |
| **Methylbutyl esters** | | | | | | | |
| *2-Methylbutyl acetate* | Control | 393 a | 272.4 a | 413 a | 328.0 a | 554 bc | 395 b |
| | AVG | 141 bc | 88.5 bc | 227 b | 171.2 b | 784 a | 543 a |
| | MCP | 131 bc | 86.5 bc | 47 c | 31.2 d | 203 de | 121 c |
| | AVG+MCP | 46 c | 31.0 c | 39 c | 24.9 d | 153 de | 90 c |
| | AVG+MCP+E | | | | | 363 cd | 83 c |
| | AVG H2 | 281 ab | 155.3 b | 238 b | 131.6 c | 625 ab | 382 c |
| | AVG+MCP H2 | 85 c | 55.6 c | 37 c | 18.2 d | 88 e | 48 c |
| | *LSD* | *153* | *93.7* | *69* | *36.1* | *216* | *95* |
| **Others** | | | | | | | |
| *t-2-Hexenyl acetate* | Control | 0 | 0 | 0 | 0 | 7.4 a | 10.4 a |
| | AVG | 0 | 0 | 0 | 0 | 4.4 b | 7.8 b |
| | MCP | 0 | 0 | 0 | 0 | 1.1 cd | 1.4 d |
| | AVG+MCP | 0 | 0 | 0 | 0 | 0 d | 0.9 de |
| | AVG+MCP+E | | | | | 1.8 c | 0 e |
| | AVG H2 | 0 | 0 | 0 | 0 | 4.5 b | 5.7 c |
| | AVG+MCP H2 | 0 | 0 | 0 | 0 | ND d | ND e |
| | *LSD* | | | | | ***1.1*** | ***1.3*** |

E2MB production by fruit from H1 was similarly reduced by AVG and MCP. AVG repressed the production of butyl acetate, 2-methylbutyl acetate and butyl butanoate more than MCP, data consistent with the slightly lower TVP by AVG-treated fruit compared to MCP-treated apples. 1-Butanol and 2-methyl-1-butanol production was repressed more by AVG than MCP, though hexanol was equally affected. In general, AVG-treated fruit from H2 was the least affected immediately after harvest. After 6 and 12 weeks in cold storage, AVG plus MCP-treated fruit from both H1 and H2 had the lowest levels of production of all volatiles, levels compared to those of control fruit, and E2MB was more affected by the combined treatment in H2 than in H1 after 6WCS. In general, AVG-treated fruit from H1 had lower production of alcohol and ester volatiles than treated fruit from H2, though E2MB production was lower in AVG-treated fruit from H2 after 6 and 12 weeks in cold storage. Hexanol was more abundant in AVG-treated fruit from H2 than in any other treatment.

In 2005, MCP and AVG plus MCP repressed the production of major individual volatile esters and alcohols the most after harvest and cold storage. AVG showed less reduction of individual volatile production than MCP, except E2MB and ethyl butanoate in H2 fruit. Volatile alcohols seemed to be less affected by AVG than ester volatiles. Only ethyl butanoate was significantly and negatively affected by ethephon in AVG plus MCP-treated fruit stored for 12 weeks at $4^{o}$C.

***Cortex tissue***

***Harvest:*** Seven days after harvest, the most abundant ester produced by cortex tissue of control fruit was hexyl acetate in 2004 (Table 2.5) and E2MB on 2005 (Table 2.6) with 26.1 % and 27.5 % of TVP, respectively. In 2004, the next most abundant volatiles were 2-methylbutyl acetate, E2MB, ethyl butanoate and butyl acetate comprising 21.1 %, 16.5 %, 14.8 % and 14.0 % of TVP, respectively. In 2005, ethyl butanoate, 2-methylbutyl acetate, hexyl acetate and butyl acetate followed E2MB, representing 22.9 %, 17.4 %, 17.2 % and 12.3% of TVP, respectively. Butanol, hexyl alcohol and 2-methyl-1-butanol were 2.1 %, 1.8 % and 0.6 % of TVP in 2004 and 0.7 %, 0.5 % and 0.2 % of TVP in 2005.

***6WCS:*** After 6 weeks in cold storage in 2004 the most abundant ester was E2MB at 29.2 % of TVP, followed by ethyl butanoate, 2-methylbutyl acetate, hexyl acetate and butyl acetate with production levels 28.8 %, 16.3 %, 10.8 % and 8.7 % of TVP, respectively. In 2005, the most abundant volatile was hexyl acetate with 37.0 % of TVP, followed by butyl acetate, 2-

methylbutyl acetate, E2MB and ethyl butanoate, comprising 26.0 %, 16.8 %, 8.3 % and 8.0 % of TVP, respectively. Butanol and 2-methyl butanol levels were slightly higher than at harvest in 2004 and similar in 2005, and hexanol levels were similar both years.

***12WCS:*** After 12 weeks in cold storage, the most abundant volatile esters were the same as after 6 weeks in storage in both 2004 and 2005, with small changes in their relative concentrations. Some esters with high molecular weight were detectable in peel but not in cortex of control apples, like butyl butanoate and hexyl propionate. Butanol and hexanol concentrations increased in both years, while 2-methyl-1-butanol remained the same. In general, individual volatiles were similarly affected by the treatments both in peel and cortex tissue. Alcohols, E2MB and ethyl butanoate were less abundant in ethephon-treated cortex, and no changes were detected in other esters.

**AAT activity**

AAT activity per mg protein in peel and cortex tissue of 'Royal Gala' apples was similar and ranged approximately from 100 to 200 mU/mg protein in 2004 (Figure 2.5.A, 2.5.C) and 2005 (Figure 2.5.B, 2.5.D).

***Peel tissue***

***Harvest:*** Immediately after harvest, AAT activity was equally reduced by AVG, MCP and their combination for all harvests in 2004, but there were no differences among treatments in 2005.

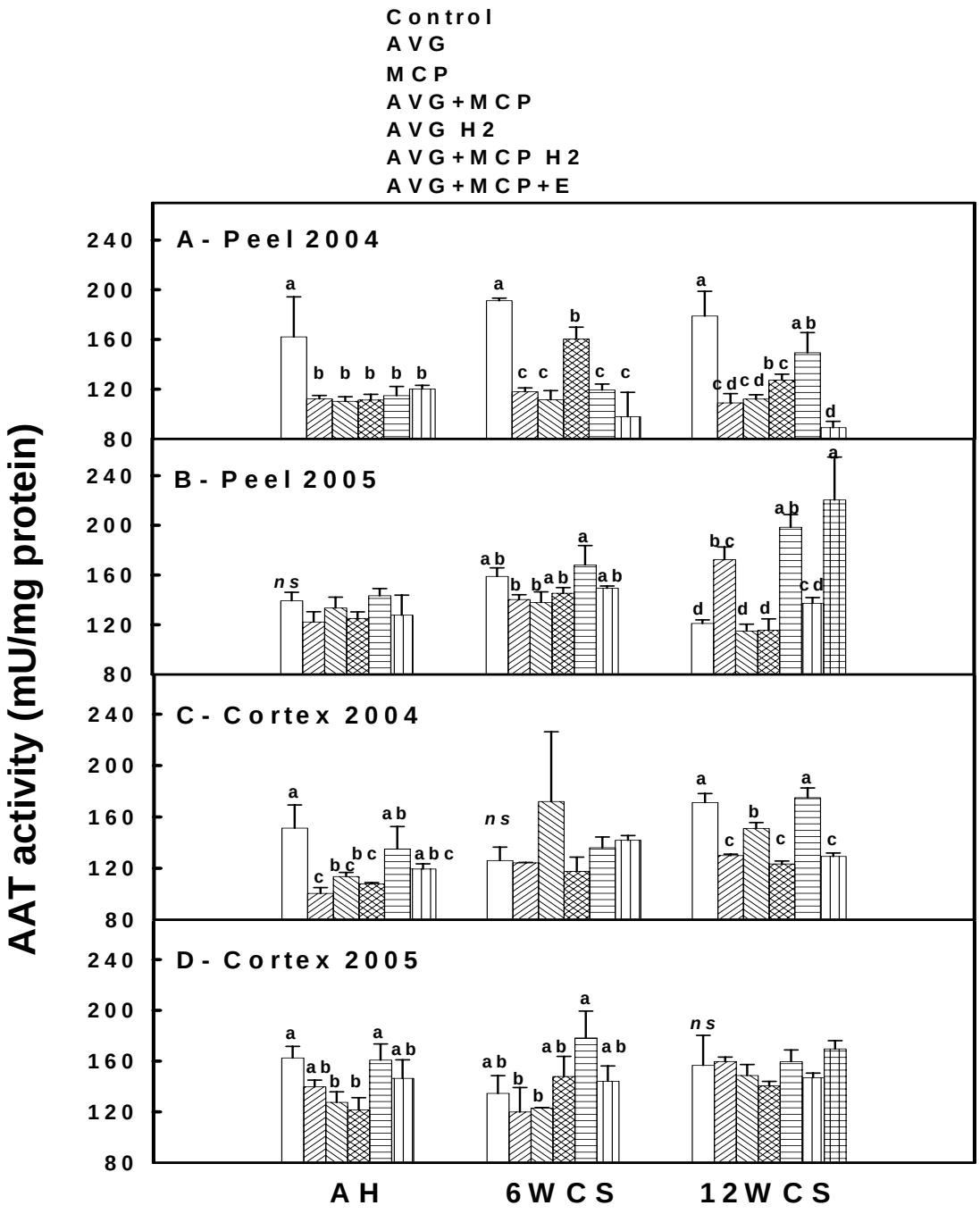
***6WCS:*** In 2004 all treatments reduced peel AAT activity, but AVG plus MCP had a less negative effect on fruit harvested on H1. In 2005, peel AAT activity in AVG-treated fruit from H2 was higher than that of AVG- and MCP-treated fruit from H1, but all other treatments were comparable.

***12WCS:*** In 2004, all treatments reduced peel AAT activity except AVG in fruit from H2. In 2005, peel tissue of control, MCP and AVG plus MCP-treated fruit from both harvest dates had the lowest AAT activity, whereas AVG plus MCP-treated fruit dipped in an ethephon solution tended to have the highest AAT activity of all, followed by all AVG-treated fruit.

***Cortex tissue***

AAT activity in cortex did not respond to the treatments in the same way as peel. **Harvest:** Immediately after harvest, cortex from control apples had high AAT activity in 2004 (Figure 2.5.C) and in 2005 (Figure 2.5.D). Cortex from all treated H1 fruit had the lower AAT activity values in both years except AVG-treated fruit in 2005.

**Figure 2.5:** Effect of AVG and/or MCP on the activity of alcohol acyl-CoA transferase (AAT) per unit protein in peel (A, B) and cortex (C, D) of 'Royal Gala'. AAT activity was measured in 2004 (A, C) and 2005 (B, D) from tissue samples of fruit ripened at room temperature for 7 days immediately after harvest (AH) and after 6 and 12 weeks in cold storage at 4°C (6WCS and 12WCS, respectively) that had been frozen and then thawed. AAT activity was expressed as mU x mg protein$^{-1}$ where U, activity unit, is the increase in one unit of absorbance per minute due to the production of a yellow thiophenol product with increasing free CoA. Different letters indicate significant differences separated by LSD at *p=0.05* within date; *ns*: no significant differences.



In general, the effect of AVG and AVG plus MCP was lost when fruit were harvested later at H2.

***6WCS:*** After 6 weeks in cold storage there were no differences among treatments in 2004. In 2005, AVG-treated fruit from H2 had the highest AAT activity, and as in peel, it was significantly higher than that of AVG or MCP-treated fruit from H1. There were no differences between harvests for AVG plus MCP-treated fruit.

***12WCS:*** After 12 weeks in cold storage, cortex from control and AVG-treated H2 fruit had the highest AAT activity in 2004, and AVG plus MCP-treated fruit had low AAT activity, regardless of harvest dates. There were no significant differences among treatments in 2005. Ethephon did not affect AAT activity in 2005 in contrast to the response in peel.

**Volatile production when fed alcohols**

***Total ester production when fed alcohols***

Peel and cortex tissue of control and AVG plus MCP-treated fruit stored at 4 $^{o}$C for 12 weeks were fed butanol or hexanol, and their ester production compared. Overall, total ester production (TEP) was higher from peel than cortex tissue. TEP by non-fed peel tissue from control fruit was almost 4 times higher than from AVG plus MCP-treated fruit (Figure 2.6.A), while there were no statistical differences between non-fed control and AVG plus MCP-treated cortex tissue (Figure 2.6.B). TEP by peel of control and AVG plus MCP-treated samples were similar when they were fed with either butanol or hexanol. Only hexanol-fed control peel had higher TEP than non-fed tissue (5 times more), whereas peel of AVG plus MCP-treated fruit fed with both butanol and hexanol had higher TEP than non-fed samples (5.5 and 25 times, respectively). When cortex tissue was fed butanol, control samples had higher TEP than AVG plus MCP-treated samples, but when samples were fed hexanol TEP by cortex of control and treated fruit was similar. TEP by both peel and cortex was higher when hexanol was fed to the samples compared to butanol. The ratio of TEP of hexanol-fed: butanol-fed samples was 4 and 4.5 by controls and AVG + MCP peel, respectively, and 7.7 and 23 by controls and AVG + MCP cortex, respectively.

***Grouped volatile production when fed alcohols***

Grouped volatile production by peel and cortex of control and AVG plus MCP treated apples is shown in Table 2.7. Across tissue types, alcohol volatiles increased 11-147 times when butanol was fed to the samples, and 82-728 times when hexanol was fed. When volatile esters

**Figure 2.6:** Total ester production (TEP) by peel (A) and cortex (B) of 'Royal Gala' apples fed with alcohol substrates. In 2005, peel and cortex tissue of fruit that had been stored for 12 weeks at 4°C were incubated with no alcohol (control), 1-butanol or 1-hexanol for 24 h, and volatile ester profiles were subsequently measured. Different lower case letters indicate significant differences between control and AVG plus MCP peel or cortex tissue within alcohol substrate, separated by ANOVA at *p=0.05*; different upper case letters indicate significant differences among alcohol substrates categories within tissue type and treatment (control or AVG plus MCP), separated by LSD at *p=0.05* within date. *ns*: no significant difference.

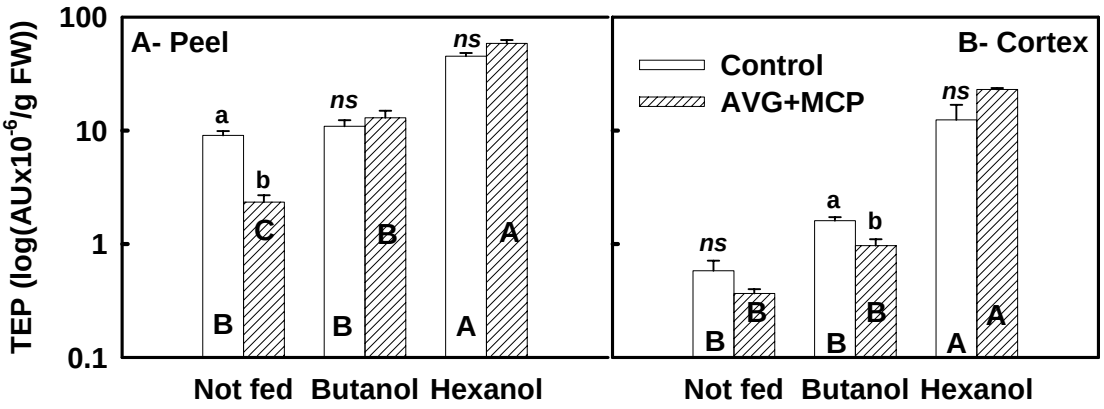


**Table 2.7:** Grouped volatile production (AU x$10^{-3}$/g FW) by peel and cortex tissue of control and AVG plus MCP-treated apples fed with alcohol substrates. Apples had been stored for 12 weeks in cold storage and equilibrated at laboratory room temperature for 3 h before headspace sampling. Samples were incubated for 24 h with 1-butanol, 1-hexanol or no alcohol. An asterisk (*****) indicates a significant difference between control and AVG plus MCP within tissue type and alcohol substrate separated by ANOVA at *p=0.05*.

| | | AU x$10^{-3}$/g FW | | | |
|---|---|---|---|---|---|
| | Alcohol | Peel | | Cortex | |
| | substrate | Control | AVG+MCP | Control | AVG+MCP |
| *Alcohol volatiles* | None | 771 | 167 * | 211 | 62 * |
| | 1-Butanol | 8399 | 17006 | 13879 | 9136 |
| | 1-Hexanol | 63154 | 43450 | 131937 | 45269 |
| *Acetate esters* | None | 4469 | 926 * | 455 | 150 * |
| | 1-Butanol | 1275 | 1192 | 667 | 523 |
| | 1-Hexanol | 28390 | 46289 * | 11197 | 20182 |
| *Hexanoate esters* | None | 1564 | 637 * | 9 | 3 |
| | 1-Butanol | 2558 | 1786 | 149 | 43 |
| | 1-Hexanol | 10396 | 8110 | 132 | 1574 * |
| *Methylbutanoate esters* | None | 1811 | 453 * | 44 | 123 * |
| | 1-Butanol | 1415 | 869 | 93 | 86 |
| | 1-Hexanol | 2746 | 1005 * | 165 | 294 |
| *Butanoate esters* | None | 645 | 269 * | 70 | 89 |
| | 1-Butanol | 5585 | 9041 | 693 | 314 * |
| | 1-Hexanol | 1960 | 2797 | 892 | 849 |
| *Hexyl esters* | None | 5480 | 1031 * | 248 | 88 |
| | 1-Butanol | 1145 | 683 | 22 | 19 |
| | 1-Hexanol | 39914 | 51216 | 11002 | 21543 |
| *Butyl esters* | None | 1892 | 487 * | 177 | 21 * |
| | 1-Butanol | 8681 | 11239 | 1095 | 700 * |
| | 1-Hexanol | 3397 | 4208 | 920 | 392 |
| *Ethyl esters* | None | 983 | 575 * | 114 | 211 |
| | 1-Butanol | 1036 | 953 | 478 | 240 |
| | 1-Hexanol | 1806 | 3192 | 429 | 968 |

were grouped by acid moiety, acetate esters were the most abundant in all non-fed samples, with relative amounts from 37 % to 58 % of TEP. When butanol was fed to the samples, butanoate esters increased the most (4-34 times of non-fed samples), followed by hexanoates (2-16 times). Acetate esters remained the same, except for peel of control apples that showed a reduction of 70 %. When hexanol was fed to the samples, acetates showed the largest increase, up to 135 times the production of non-fed samples, followed by hexanoate esters that in cortex tissue of AVG plus MCP samples showed an increment of 477 times, compared to non fed samples. Butanoate and methylbutanoate esters increased 3-13 and 2-4 times, respectively.

When grouped by alcohol moiety, hexyl esters were the most abundant in peel and cortex of control apples, with relative amounts from 43 % to 65 %, and also in peel of AVG plus MCP-treated fruit (44 % of TEP), while ethyl esters were the most abundant in cortex (58% of TEP). When samples were fed with butanol, hexyl ester production decreased, butyl esters increased up to 33 times and ethyl esters increased up to 4 times. Hexyl esters were up to 244 times higher in hexanol-fed than non-fed samples, butyl esters up to 19 times higher and ethyl esters up to 5 times higher. The relative increments were steeper in AVG plus MCP than control samples. Ethyl esters showed the smaller increments, and the greater increments occurred in hexyl esters when hexanol was fed.

***Individual volatile production when fed alcohols***

Fifteen straight and branched-chain volatiles, twelve esters and three alcohols, were detected in peel and cortex tissue of 'Royal Gala' apples (Table 2.8). Of the alcohol volatiles, hexanol was the most abundant in control and AVG plus MCP peel samples, followed by butanol. Similar amounts of hexanol and butanol were detected in cortex tissue. 2-Methylbutyl acetate was always the least abundant volatile alcohol. When butanol was fed to the samples, butanol increased 58 and 506 times in peel and cortex of control tissue, respectively, and increased 123 and 330 times in peel and cortex of AVG plus MCP-treated tissue. When hexanol was fed to the samples, hexanol increased 103 and 345 times in peel and cortex of control tissue, respectively, and 1403 and 1463 times in peel and cortex of AVG plus MCP-treated tissue. However, when samples within tissue type and treatment were compared in terms of hexanol detected when hexanol was fed and of butanol detected when butanol was fed, the ratio hexanol:butanol was 8-10 in peel and 3-5 in cortex, and this would be an indication of how much alcohol was absorbed by the tissue.

**Table 2.8:** Individual volatile production (AU x$10^{-3}$/g FW) by peel and cortex tissue of control and AVG plus MCP-treated apples fed with alcohol substrates. Apples had been stored for 12 weeks in cold storage and equilibrated at laboratory room temperature for 3 h before headspace sampling. Samples were incubated for 24 h with 1-butanol, 1-hexanol or no alcohol. An asterisk (*****) indicates a significant difference between control and AVG plus MCP within tissue type and alcohol substate separated by ANOVA at *p=0.05*.

| | | AU x$10^{-3}$/g FW | | | |
|---|---|---|---|---|---|
| | Alcohol substrate | Peel | | Cortex | |
| | | Control | AVG+MCP | Control | AVG+MCP |
| **Alcohols** | | | | | |
| *Hexanol* | None | 609 | 125 * | 93 | 31 * |
| | 1-Butanol | 456 | 211 | 213 | 59 * |
| | 1-Hexanol | 62952 | 43120 | 131426 | 45069 |
| *1-Butanol* | None | 137 | 33 * | 110 | 28 * |
| | 1-Butanol | 7914 | 16776 | 13649 | 9069 |
| | 1-Hexanol | 161 | 316 | 155 | 193 |
| *2-Methyl-1-butanol* | None | 24 | 9 * | 7 | 4 |
| | 1-Butanol | 28 | 18 | 18 | 8 |
| | 1-Hexanol | 40 | 14 | 356 | 6 * |
| **Hexyl esters** | | | | | |
| *Hexyl acetate* | None | 3156 | 551 * | 244 | 85 * |
| | 1-Butanol | 195 | 68 | 23 | 11 |
| | 1-Hexanol | 28247 | 45920 * | 10898 | 20134 |
| *Hexyl 2-methylbutanoate* | None | 1112 | 99 * | 0 | 0 |
| | 1-Butanol | 610 | 110 | 0 | 0 |
| | 1-Hexanol | 2301 | 510 * | 92 | 59 |
| *Hexyl hexanoate* | None | 663 | 327 * | 3 | 3 |
| | 1-Butanol | 267 | 489 | 0 | 8 * |
| | 1-Hexanol | 7678 | 4317 * | 0 | 1332 * |
| *Hexyl propionate* | None | 547 | 53 * | 1 | 0 |
| | 1-Butanol | 73 | 16 * | 0 | 0 |
| | 1-Hexanol | 1687 | 468 * | 12 | 18 |
| **Butyl esters** | | | | | |
| *Butyl hexanoate* | None | 900 | 309 * | 6 | 0 * |
| | 1-Butanol | 2291 | 1297 | 150 | 35 * |
| | 1-Hexanol | 2717 | 3793 | 132 | 241 |
| *Butyl acetate* | None | 631 | 131 * | 171 | 21 * |
| | 1-Butanol | 1037 | 1095 | 637 | 506 |
| | 1-Hexanol | 79 | 316 | 252 | 36 * |
| *Butyl-2-methylbutanoate* | None | 284 | 26 * | 0 | 0 |
| | 1-Butanol | 508 | 296 | 9 | 7 |
| | 1-Hexanol | 72 | 11 * | 0 | 0 |
| *Butyl butanoate* | None | 75 | 22 * | 0 | 0 |
| | 1-Butanol | 4843 | 8551 | 300 | 153 |
| | 1-Hexanol | 527 | 88 | 536 | 116 |

| | | AU x$10^{-3}$/g FW | | | |
|---|---|---|---|---|---|
| | Alcohol substrate | Peel | | Cortex | |
| | | Control | AVG+MCP | Control | AVG+MCP |
| **Alcohols** | | | | | |
| *Hexanol* | None | 609 | 125 * | 93 | 31 * |
| | 1-Butanol | 456 | 211 | 213 | 59 * |
| | 1-Hexanol | 62952 | 43120 | 131426 | 45069 |
| *1-Butanol* | None | 137 | 33 * | 110 | 28 * |
| | 1-Butanol | 7914 | 16776 | 13649 | 9069 |
| | 1-Hexanol | 161 | 316 | 155 | 193 |
| *2-Methyl-1-butanol* | None | 24 | 9 * | 7 | 4 |
| | 1-Butanol | 28 | 18 | 18 | 8 |
| | 1-Hexanol | 40 | 14 | 356 | 6 * |
| **Hexyl esters** | | | | | |
| *Hexyl acetate* | None | 3156 | 551 * | 244 | 85 * |
| | 1-Butanol | 195 | 68 | 23 | 11 |
| | 1-Hexanol | 28247 | 45920 * | 10898 | 20134 |
| *Hexyl 2-methylbutanoate* | None | 1112 | 99 * | 0 | 0 |
| | 1-Butanol | 610 | 110 | 0 | 0 |
| | 1-Hexanol | 2301 | 510 * | 92 | 59 |
| *Hexyl hexanoate* | None | 663 | 327 * | 3 | 3 |
| | 1-Butanol | 267 | 489 | 0 | 8 * |
| | 1-Hexanol | 7678 | 4317 * | 0 | 1332 * |
| *Hexyl propionate* | None | 547 | 53 * | 1 | 0 |
| | 1-Butanol | 73 | 16 * | 0 | 0 |
| | 1-Hexanol | 1687 | 468 * | 12 | 18 |
| **Butyl esters** | | | | | |
| *Butyl hexanoate* | None | 900 | 309 * | 6 | 0 * |
| | 1-Butanol | 2291 | 1297 | 150 | 35 * |
| | 1-Hexanol | 2717 | 3793 | 132 | 241 |
| *Butyl acetate* | None | 631 | 131 * | 171 | 21 * |
| | 1-Butanol | 1037 | 1095 | 637 | 506 |
| | 1-Hexanol | 79 | 316 | 252 | 36 * |
| *Butyl-2-methylbutanoate* | None | 284 | 26 * | 0 | 0 |
| | 1-Butanol | 508 | 296 | 9 | 7 |
| | 1-Hexanol | 72 | 11 * | 0 | 0 |
| *Butyl butanoate* | None | 75 | 22 * | 0 | 0 |
| | 1-Butanol | 4843 | 8551 | 300 | 153 |
| | 1-Hexanol | 527 | 88 | 536 | 116 |

Of the ester volatiles, hexyl acetate was the most abundant in cortex and peel samples of control fruit as 30.8 % and 32.2 % of TVP, respectively, and in peel tissue of AVG plus MCP-treated fruit as 22.9 % of TVP. E2MB was the most abundant ester in cortex of AVG plus MCP-treated fruit comprising 28.8 % of the TVP. Butyl acetate, ethyl butanoate and 2-methyl butyl acetate were the next most abundant esters in cortex of control and AVG plus MCP-treated apples, present as 5 % to 20 % of TVP, each. The same esters were also present in peel tissue of control and AVG plus MCP-treated fruit, and two other volatiles that were barely present in cortex tissue were detected: hexyl propionate and butyl hexanoate with relative abundances of 2.1 and 12.3 % of TVP, respectively. The production levels of all volatiles in non-fed peel of AVG plus MCP-treated fruit was lower than that of control fruit, but this was evident for only about half of the compounds from cortex tissue.

When tissue samples were fed butanol, butyl butanoate production greatly increased in all samples, and was present at levels 16 and 56 times more in peel than in cortex tissue of control and AVG plus MCP-treated apples, respectively. The production of volatile esters with a butyl alcohol moiety, a butanoic acid moiety or both increased in peel fed with butanol, reaching similar levels for control and AVG plus MCP-treated apples. Also, the production of esters without either of these moieties decreased when peel was fed with butanol, exept for hexyl hexanoate in AVG plus MCP samples. The production of hexyl acetate was greatly reduced, and also that of E2MB, 2-methylbutyl acetate, hexyl propionate and hexyl-2-methylbutanoate. The effect of feeding butanol to cortex tissue was not very marked, though esters with a butyl and/or butanoic moiety increased compared to non-fed cortex, and the production of hexyl acetate was greatly reduced. Of all volatile compounds, only butyl hexanoate and hexanol were present in significantly larger amounts in butanol-fed cortex of control than AVG plus MCP-treated fruit.

Hexyl acetate was the most abundant ester volatile in peel and cortex of control and AVG plus MCP-treated fruit fed with hexanol, with production levels as high as 237 times those of non-fed samples. There were no significant differences in the amounts of hexyl acetate present in cortex of control and treated fruit, though peel of AVG plus MCP-treated fruit produced much more hexyl acetate than that of control fruit. In peel of control fruit fed with hexanol there was a significant increase in the amounts of esters with hexyl or hexanoic acid moieties. 2-Methyl-1-butanol and butyl butanoate also increased, though to a lesser extent. Conversely, the amounts of butyl acetate, 2-methylbutyl acetate and butyl-2-methylbutanoate were lower in control peel

tissue fed with hexanol than non-fed tissue. A similar response to hexanol-feeding was detected in peel of AVG plus MCP-treated apples, though the production of various ester volatiles was higher in control than treated peel. Except for hexyl acetate, there were no major changes in ester production by cortex of control or AVG plus MCP-treated apples. Interestingly, 2-methyl-1-butanol was significantly higher in hexanol-fed cortex of control than AVG plus MCP-treated apples, while the opposite trend was more evident for hexyl hexanoate. Methylbutyl esters always decreased when butanol or hexanol were fed to the samples (Table 2.8).

# DISCUSSION

### GENERAL RIPENING TRAITS

#### Internal ethylene concentration

The IEC threshold that triggers ripening in apples is 1 μL/L (or ppm) (Canada Ministry of Agriculture, Food and Rural Affairs, 2003). In this study, control fruit were harvested with IEC levels close to 1 μL/L in both years, and so were at the beginning of ripening (Figure 2.1.A, 2.1.B). IEC increased approximately ten-fold in control fruit after 7 days at RT both years. After 6 and 12 weeks in cold storage, fruit had lower than 1 μL/L IEC in 2004 (Figure 2.1.C, 2.1.E), whereas in 2005 IEC was much higher than immediately after harvest (Figure 2.1.D, 2.1.F). Though fruit was harvested at similar calendar dates in both years, control fruit could have been more ripe at harvest in 2004 than in 2005, as suggested by the high starch index at harvest (Table 2.1), and the very low respiration rate (Figure 2.3.C, 2.3.E) and volatile production (Figure 2.4.A, 2.4.C) at 6WCS and 12WCS. In support of this hypothesis, the average temperature from April 20 through August 15, the growing season for 'Royal Gala' in Kentucky, was 10 $^{o}$F higher in 2004 than in 2005 (University of Kentucky Agricultural Weather Center, 2006). Results by Singh et al. (2004) showed that 'Royal Gala' apples stored in air or CA at 2.5 $^{o}$C had higher ethylene production 1 to 14 days after retrieval from a 120 to 240 day-storage than their counterparts stored at 0 $^{o}$C. Therefore, the increase in IEC after 6WCS and 12WCS in 2005 could be due to an ongoing ripening process at the storage temperature of 4 $^{o}$C set in the present work.

AVG inhibits $C_2H_4$ synthesis in apples and can keep IEC at low levels (Autio and Bramlage, 1982; Johnson and Colgan, 2003; Schupp and Greene, 2004; Silverman et al., 2004),

and though several studies have shown an important repression of the autocatalytic apple $C_2H_4$ production by MCP (DeLong, et al., 2004, Fan et al., 1999, Rupasinghe et al., 2000; Mattheis et al., 2005), there have also been variable results (positive effect or no effect; Watkins et al., 2000). CA storage may prolong the effect of MCP (Mattheis et al., 2005; DeLong et al, 2004) and enhance the effect of AVG (Mir et al., 1999) in apples, but little information is available about the effects of combining both chemicals. Drake et al. (2006) measured similar levels of internal $C_2H_4$ concentration in MCP-treated fruit irrespective of AVG treatment, and these levels were lower than those of AVG only-treated fruit. In the present study, AVG plus MCP had a greater negative impact on $C_2H_4$ production than either alone. The combined treatment consistently and greatly reduced IEC of apples across harvest dates, and storage lengths in both years. Immediately after harvest, the combined treatment had an effect similar to or greater than the best single treatment with H1 fruit, and an effect greater than AVG alone in H2 fruit. AVG plus MCP was the only treatment to consistently keep IEC at low levels even after fruit had been stored for 12 weeks. These results suggest that there may be an additive effect between AVG and MCP, possibly due to their different modes of action.

While AVG may decrease or delay climacteric $C_2H_4$ production, MCP would interfere with the action of the residual $C_2H_4$ present in the fruit by binding to the $C_2H_4$ receptors. Much of the work on fruit $C_2H_4$ receptors has been done with tomato. *NEVER RIPE* (*NR)* is a tomato homolog of the *Arabidopsis thaliana* ethylene receptor *ETHYLENE RECEPTOR1* (*ETR1)* and can be induced by exogenous ethylene in wild-type fruit that have reached the mature-green stage of development (Wilkinson et al., 1995). Also, *NR* transcripts greatly accumulate from the mature green wild type tomato fruit on, reaching maximal levels at the breaker stage of ripening, parallel with increasing ethylene production (Yen et al., 1995). Based on these findings, Lelièvre et al. (1997) suggested that the number of ethylene-binding sites may increase during fruit ripening, and that *NR* transcript accumulation appears to be both developmentally-regulated and ethylene-dependent. Fruit treated with AVG was at a less ripe stage than control fruit at H1, had lower initial IEC, and may have had a lower concentration of $C_2H_4$ receptors. The exposure of these fruit to MCP could have bound most if not all the receptors present at that time, further repressing ethylene action. It is possible that AVG-treated fruit had more ethylene receptors at H2 than at H1, but fewer than controls harvested at H1, and therefore MCP was able to bind to most if not all the receptors even if fruit was harvested later. Exposure to a source of ethylene

greatly stimulated $C_2H_4$ production in AVG plus MCP-treated fruit that had been stored for 12 weeks. The effect of AVG had probably disappeared, as $C_2H_4$ production by AVG-treated fruit was equal to or greater than control fruit. Given that MCP irreversibly binds to the $C_2H_4$ receptors (Sisler et al., 1996; Sisler and Serek, 1997), new receptors could have been present and active by the time ethephon was applied, overcoming the remaining effect of MCP.

The effect of AVG on $C_2H_4$ production can be reduced with delayed harvests (Greene and Schupp, 2004). The effect of AVG on fruit from the second harvest was partially or completely lost when fruit were held for 7 days at RT AH, 6WCS or 12WCS in 2004 (Figure 2.1.A, 2.1.C, 2.1.E), though in 2005 fruit from both harvests had similar and high IEC (2.1.B, 2.1.D, 2.1.F). One possible explanation is that in 2005 AVG was applied to trees approximately 5 weeks before H1 because the expected harvest date was one week earlier. The effectiveness of AVG depends, among other things, on timing of application (Schupp and Greene, 2004). Thus, it is posible that the effect of AVG in 2005 had been partially lost by the fruit by the time of the first harvest. Additionally, AVG may be more effective in cooler climates where the evolution of $C_2H_4$ production would be slower during fruit maturation, resulting in a more uniform and prolonged AVG suppression of $C_2H_4$ production (Stover et al., 2003). The average temperature during the last weeks of 'Gala' maturation was 8 $^{o}$F higher in 2005 compared to 2004 (University of Kentucky Agricultural Weather Center), possibly reducing the efficacy of AVG on suppression of $C_2H_4$ production. As mentioned above, AVG plus MCP was effective after harvest and up to 12 weeks in cold storage, even if fruit were harvested later. This is another indicator that AVG and MCP complement each other in repressing $C_2H_4$ production in apple fruit.

**Headspace ethylene production**

IEC is considered a more accurate measurement than HEP of how much ethylene is present and acting in the fruit. However, measurement of IEC deteriorations the fruit and this was undesirable for repeated measurements. Thus, HEP was measured repeatedly instead on many samples. HEP did not greatly differ from IEC in fruit response to the treatments, though the occurrence of a peak in $C_2H_4$ production was not evident (Figure 2.2). Only control and AVG-treated fruit from the second harvest consistently showed increasing HEP immediately after harvest, suggesting that these fruit were more advanced in the ripening process. Changes in HEP were relatively smooth rather than abrupt and paralleled the changes in IEC, suggesting that

HEP was a good indicator of IEC trends. One possible drawback for measuring HEP instead of IEC is the higher variability observed in the current study by 7 days after harvest both in 2004 and 2005. This variability did not result in statistical discrimination among treatments with large differences among means (Figure 2.2.A, 2.2.B).

**Respiration rate**

A lower $C_2H_4$ production, induced by the application of inhibitors of $C_2H_4$ synthesis like AVG (Bangerth, 1978), or action like MCP (Mattheis et al., 2005), could result in lower RR (Bangerth et al., 1998; Song and Bangerth, 1996). A role for ethylene in the increase in respiration rate in climacteric fruit has been proposed, because even non-climacteric fruit which normally produce low amounts of ethylene, will respond to exogenous ethylene with increased respiration (Tucker, 1993). In the present studies, the RR increase followed that for $C_2H_4$ production in most cases (Figure 2.3), suggesting that any treatment that reduces ethylene action or production would negatively affect RR. Thus, it is not surprising that fruit treated with AVG plus MCP had consistently low RR in both years, and that control fruit had both low RR and $C_2H_4$ production after 12WCS in 2004 but they had high RR and $C_2H_4$ production in 2005 (Figure 2.3.E, F).

When fruit were treated with ethephon, the increase in RR was not parallel to, but rather followed that of, $C_2H_4$ production by 7 days. These results differ from those immediately after harvest, when the increase in RR was parallel to that in $C_2H_4$ production. It is possible that, in apples stored for 12 weeks, metabolic activity was reduced by both AVG plus MCP and the cold storage temperature, and therefore the response of RR to exogenous ethylene could have been delayed.

**Starch index**

Apple fruit ripening involves starch degradation, and SI is a common parameter used to assess maturity at harvest. A SI of 4-6 at harvest is considered appropriate for early CA storage (not long term CA storage), and a SI of 6 or above is suitable for RA storage or immediate marketing (Cowgill et al., 2005). SI of control fruit at harvest was higher in 2004 than in 2005 (Table 2.1), indicating a higher level of starch degradation and a more advanced stage of ripening in 2004. Accordingly, control fruit from 2004 had very low RR and IEC after 12WCS, consistent with a possible ongoing post-ripening senescing process. A summary with the effects of all the treatments on apple quality traits is shown in Table A1 in the Applendix. Control fruit

harvested in 2005 had a slightly higher SI than that appropriate for long-term storage. Both MCP and AVG slow down ripening of apples (Bangerth, 1978; Byers, 1997; Johnson and Colgan, 2003; Fan et al., 1999), but several factors can influence their effectiveness (Watkins et al., 2000; Autio and Bramlage, 1982; Greene and Schupp, 2004). AVG delayed starch degradation immediately after harvest both years, while MCP did not. AVG was applied to immature fruit, but MCP was applied to harvested fruit with more advanced starch degradation, and this could have reduced its efficacy in preventing a further increase in SI. The combined treatment was, at most, as effective as AVG alone in preventing starch degradation at harvest, and that effect was lost in later harvests and with storage. Accordingly, inhibitors of ethylene synthesis or action were found to delay starch degradation immediately after harvest (Layne et al., 2002; Johnson et al., 2003; Silverman et al, 2004; Drake et al., 2006) but not after cold storage (Fan et al., 1999). Thus, it is likely that ethylene regulates the beginning of starch degradation, but it has little to no effect after fruit are detached from the trees.

**Firmness**

Firmness is a major quality trait of apples, and changes in this parameter after harvest depend on the postharvest handling of the fruit and on firmness at the moment of harvest. Firmness of control fruit at harvest was slightly higher in 2004 than in 2005 (Table 2.1). However, all treated fruit were more firm in 2005 than in 2004. Control fruit were harvested within the appropriate harvest window for short-term storage for this particular cultivar and growing area. Accordingly, fruit firmness was acceptable for short-term but not for long-term storage in both years (firmness of control fruit was 76.3 N and 71.1 N in 2004 and 2005, respectively), considering that 'Royal Gala' harvest standards are 76 to 80 N for storage and as low as 67 N for immediate marketing. Firmness in control fruit decreased 8 to 19 N when they were ripened for 7 days at RT immediately after harvest or 6WCS, but these fruit would still be marketable. Plotto et al. (1997) reported that taste panelists gave high ratings for tartness and firmness of early harvested 'Gala' apples but these values were always associated with low ratings for overall acceptability, sweetness and flavor. They concluded that firmness did not necessarily imply high quality and acceptability by consumers if fruit had not developed full flavor. Kupferman and Harker (2001) established a minimum standard of 49 N for marketing Washington 'Gala' apples based on consumer acceptability. Control apples ripened at RT after

12WCS were at or below the minimum firmness threshold presenting a drawback for a possible extended marketability.

Firmness is one of the ripening processes most sensitive to ethylene (Lelièvre et al., 1997). Previous studies show a reduction in apple cortex firmness loss by AVG (Layne, 2002; Wang and Dilley, 2001; Williams, 1980) and MCP (DeEll, 2002; Mir et al., 2001; Rupasinghe et al., 2000), though the effect of AVG declined with later harvests (Greene and Schupp, 2004; Johnson and Colgan, 2003) and that of MCP with increasing storage temperature (Mir et al., 2001). AVG alone seemed to be more effective than MCP alone in reducing firmness loss, though this effect was lost when fruit was harvested later. The combination of AVG plus MCP resulted in greater firmness retention across years and storage duration, even for later harvests.

Crouch et al. (2005) studied the effect of commercially-applied MCP on fruit quality of various apple cultivars and suggested that the most important factors affecting the efficacy of this product were starch levels followed by cortex firmness at the moment of harvest. Specifically, for 'Royal Gala' apples, they found that the optimum maturity range for treatment was 20 – 60 % starch breakdown, and that fruit with over 65 % starch breakdown did not maintain firmness in response to the treatment. In the current study, 'Gala' fruit had starch breakdown higher than 65 % in 2004 and lower than, though close to, 65 % in 2005 (Table 2.1), and this could be an explanation for the low efficacy of MCP alone in maintaining cortex firmness. AVG-treated fruit had much lower starch breakdown at the moment of the MCP treatment (Table 2.1) making possible the effective action of MCP. This could be part of the prolonged reduction of the ripening process also reflected in $C_2H_4$ production and RR. Exposure of AVG plus MCP-treated fruit to ethephon did not induce further firmness loss, probably due to lower metabolic activity suggested by the delayed increase in RR.

In general, low IEC corresponded with higher firmness retention, though firmness loss (Table 2.1) was greater in MCP-treated fruit with low IEC after 6WCS both years (Figure 2.1.C, 2.1.D) than in AVG-treated fruit from H1 that had equal or lower IEC than MCP-treated fruit. Fruit softening can be divided into two major stages (Bennet, 2002). The first stage occurs in early fruit ripening and is associated with the coordinated disassembly of the cell wall hemicellulose. The second stage occurs in the more ripe stage and is associated with disassembly of the pectin network. Expansins seem to play a critical role during the first stage of ripening. They are proteins that cooperate with cell wall hydrolases by disrupting hydrogen bonds at the

cellulose/hemicellulose interface to allow 'cell wall creep' in expanding cells (Cosgrove, 1997), though a new subclass of expansins has been found to be specifically expressed in fully expanded and ripening fruit (Rose et al., 1997), and expansin mRNA transcripts are suppressed in transgenic lines with low ethylene production (Rose et al., 1997). Pectinesterase, exo-polygalacturonase (Bartley, 1978) and β-galactosidase (Bartley, 1974) could account for losses of pectin during the second stage of apple ripening. A possible explanation for the results of the present work is that AVG-treated fruit was harvested at the beginning of the first ripening stage, whereas control fruit were harvested during that stage or at the beginning of the second stage, so MCP was applied to fruit at different stages of cell wall disassembly. Low $C_2H_4$ production in AVG-treated fruit from H1 and in AVG plus MCP fruit from any harvest would be affecting the first stage, slowing down softening, while low $C_2H_4$ production in MCP-treated fruit might have affected the second stage of fruit softening only, and was thus less effective in maintaining firmness even at low IEC.

**Color**

Environmental and genetic factors interact to determine red color in fruit (Layne et al., 2002). Apple skin color is caused by the pigments chlorophyll and carotenoids in plastids and by anthocyanins, phenolic pigments, in vacuoles. Red skin coloration in apples is proportional to anthocyanin content in epidermal cells (Lancaster et al., 1994). Anthocyanin synthesis is light- (Dong et al., 1995) and temperature-dependent (Saure, 1990). 'Gala' may not develop good red color in a hot, summer climate such as Kentucky (Layne et al., 2002), and the application of AVG can delay the development of red color even further (Byers, 1997; Layne et al., 2002; Drake et al. 2006) by delaying the loss of chlorophyll (Bangerth, 1978; Wang and Dilley, 1998). Results of this study agree with these observations (Table 2.1). Control 'Royal Gala' fruit developed good red skin color in 2004, when temperatures were mild. In 2005, the temperature was low for several weeks followed by an abrupt increase during the last weeks before harvest. The average temperature during the last 20 days before H1 was 71 $\pm$ 4 $^{o}$F in 2004 and 80 $\pm$ 3 $^{o}$F in 2005 (University of Kentucky Agricultural Weather Center). The high temperatures close to harvest in 2005 could have accelerated the ripening process, leaving limited time for anthocyanin accumulation, resulting in poor red color of the fruit.

AVG negatively affected red skin color of the fruit in 2004 (Table 2.1). Red color developed later in AVG-treated fruit during ripening at RT, and during and after cold storage,

though treated fruit from H1 did not reach the same final color as controls. Applying MCP to AVG-treated fruit did not further repress the development of red color 7 days after harvest or after 6WCS or 12WCS. The lower color rate in some AVG plus MCP- treated fruit on day 7 after harvest or cold storage compared to day 1 may be due to sampling variation, which may have been avoided by working with larger samples. MCP alone did not affect color development because this treatment was applied to control fruit with visible red skin color. The delayed development of red color in AVG and AVG plus MCP-treated fruit was probably due to the slower degradation of chlorophyll (Knee, 1972, 1980), which would unmask the already present and newly-synthesized anthocyanin pigments. Fruit from H2 reached nearly the same final color as control fruit, probably due to greater chlorophyll loss and/or higher accumulation of anthocyanins and more chlorophyll degradation during the longer on-tree maturation.

In 2005, poor red color of controls was not affected by AVG or any other treatment, and color did not even develop after 12 weeks in cold storage in any treatment group (Table 2.1). This suggests that anthocyanins did not accumulate in the fruit prior to harvest, and that even though chlorophyll was degraded during storage, there were no anthocyanin pigments present to give the fruit the characteristic red coloration. Thus, it is not surprising that ethephon did not induce any change in the color of AVG plus MCP-treated fruit.

**Soluble solids content**

SSC is a measure of sugar content in fruit. Previous studies on the effect AVG or MCP on quality traits of apples have reported no changes in SSC (Autio and Bramlage, 1982; DeEll et al., 2002; Rupasinghe et al, 2000), variable results (Johnson and Colgan, 2003; Schupp and Greene, 2004; Fan et al., 1999), and higher (Greene and Schupp, 2004; DeLong et al., 2004) or lower SSC (Bangerth, 1978; Layne et al., 2002) in treated fruit. No treatment in this study consistently changed SSC of the fruit (Table 2.2). This would imply that the evolution of total soluble sugars in apple fruit is independent of ethylene action, as it has been previously suggested (Fan et al., 1999; Knee, 1976).

**Titratable acidity**

Sugars and organic acids contribute to fruit taste and are used as respiratory substrates. TA mainly reflects the abundance of malic acid, the most conspicuous organic acid in apples (Tucker, 1993). Malic acid is a major respiratory substrate and can fall by 50% during the ripening of a fruit (Knee, 1993). A major reduction in TA was not noted (Table 2.2), but TA

generally declined more in fruit with higher RR and was less affected in fruit with lower RR. Autio and Bramlage (1982) did not detect any change in TA when fruit were treated with AVG, in contrast to findings by Bangerth (1978). Silverman et al. (2004) did not detect a significant change in the amounts of malate, citrate or ascorbate, the principal organic acids in apple juice, when fruit were treated with AVG. DeLong et al. (2004) and Fan et al. (1999) measured higher acidity in apples of different cultivars treated with MCP and later stored in low temperature air or CA, and in those cases $C_2H_4$ production was greatly suppressed. In the current study no consistent response to any single treatment was found, but there was less TA loss in fruit exhibiting large reductions in $C_2H_4$ production, most likely through the subsequent decrease in RR. AVG plus MCP-treated fruit had generally low RR and high TA, even after 12 weeks in cold storage. Ethephon did not stimulate acidity loss of fruit treated with AVG plus MCP, again possibly due to generally low metabolic activity. RR in fruit treated with ethephon increased only by 7 days, so it is possible that these fruit could have responded to the treatment if TA had been measured several days later.

### Sugar:acid ratio

Immediately after harvest control fruit had the highest SSC, the lowest TA and the highest S:A ratio (Table 2.2). The increase in S:A ratio in any treatment group after cold storage was parallel to a decrease in TA rather than due to a change in SSC. Fruit treated with MCP plus AVG generally had a lower S:A ratio due to less reduction in acidity, and this did not change with the post-storage application of ethephon. A low S:A ratio might lead to a greater overall acceptability by consumers, given that fruit acidity can influence flavor perception (Malundo et al., 2001; Stampaoni, 1993) by increasing the sensitivity to flavor-related compounds (Saftner et al., 2002).

## VOLATILE PRODUCTION

### Total volatile production

Overall, peel and cortex tissue showed similar responses to the treatments, and therefore volatile production will be discussed in general terms, commenting on differences between tissues when necessary. The production of volatile esters in apple is regulated by ethylene (Mattheis et al., 1991), so treatments that reduce or eliminate ethylene action may have a negative impact on volatile production (Halder-Doll and Bangerth, 1987; Streif and Bangerth, 1988). Inhibitors of $C_2H_4$ production or action could delay chloroplast degradation, reducing the

amounts of free fatty acids available for volatile synthesis (Sanz et al., 1997; Paillard, 1979). However, Song and Bangerth (2003) suggested that, in climacteric fruit, a reduction in ethylene action would result in a lower RR that could limit the amount of ATP available for *de novo* synthesis and reduction of free fatty acids and thus affect volatile production. Diazocyclopentadiene (DACP), a compound that irreversibly inhibits ethylene action in the light (Sisler and Blankenship, 1993a) as well as production (Blankenship and Sisler, 1993), was found to inhibit ester production, probably due to a low RR as a result of inhibition of $C_2H_4$ action (Fan et al., 1998).

Overall, TVP by peel and cortex of 'Gala' apples showed trends similar to ethylene production and respiration rate (Figure 2.4). Control fruit had high volatile production when $C_2H_4$ production and RR were high, and lower volatile production when $C_2H_4$ production and RR were low, and the same was generally true for any treated fruit. AVG plus MCP reduced RR and ester production the most at each measurement time and in both years. In support of the hypothesis by Song and Bangerth (2003), there seemed to be chloroplast degradation in AVG plus MCP-treated apples stored at 4 $^{o}$C in 2004 since they developed red skin color, and if the major source of fatty acid substrates was membrane degradation, an increase in volatile ester synthesis may be expected. However, TVP in these apples was very low, as were RR and IEC. The high and consistent reduction of volatile production in AVG plus MCP-treated fruit could be due to a reduction in ATP availability for *de novo* synthesis of fatty acids.

TVP on a per g fresh weight basis was consistently higher from peel than from cortex of untreated 'Gala' apples. This is in agreement with findings by Guadagni et al. (1971) and Defilippi et al. (2005b) who attributed this effect to a higher abundance of amino acid and fatty acid precursors in the peel. Ester synthesis can be limited by the concentration of alcohol precursors (Berger and Drawert, 1984). Volatile alcohols were detected, and these may be considered a proportional representation of the alcohols present in the tissue. However, alcohol volatiles were present in similar amounts in peel and cortex, except for stored samples in 2005 that showed higher hexanol content in peel. The higher volatile production by peel in spite of similar alcohol levels in cortex suggests that there is another limiting factor. Another explanation could be that the methodology used in the current study did not yield a fair representation of the alcohols available as substrates for the enzymes involved in ester production. The air-to-water partition of alcohols is very low, and therefore alcohols would be not proportionally represented

in the headspace. Additionally, the SPME fiber has a higher affinity for non-polar compounds, and alcohols are more polar than esters, which in turn were more abundant. Thus, it is possible that alcohols were trapped in relatively lesser amounts than esters.

Defilippi et al. (2005b) proposed that in the cortex AAT is more important for ester formation than in peel, where the availability of amino acids and fatty acids seems to be more critical. No major differences were detected between peel and cortex AAT activity per mg protein (Figure 2.5). Low RR may have affected ester synthesis in cortex versus peel. Rudell et al. (2000) found that the respiration rate in the skin of 'Fuji' apples was approximately 100 to 200 % higher than in the hypanthial tissue and 24 to 100 % higher than in the carpellary tissue. A lower RR in cortex tissue could have a negative impact on ATP availability, and therefore on the availability of fatty acid-derived substrates for volatile synthesis.

In a report from Plotto et al. (1999), a decrease in sensory scores for fruitiness of controlled atmosphere apples was correlated with a decrease in volatile levels. AVG plus MCP greatly repressed volatile production (TVP after 6WCS and 12WCS was 7-15 % and 12-23 % of controls in cortex and peel, respectively; Figure 2.4), and that could be a factor negatively affecting consumer acceptability. Treating these fruit with ethylene could help overcome their impaired volatile production. Exposure of cold-stored AVG plus MCP-treated fruit to ethylene had a different impact on cortex than on peel of 'Gala' apples. Both $C_2H_4$ production (Figure 2.1) and RR (Figure 2.2) increased in whole apples by the 7$^{th}$ day after treatment, possibly inducing an increase in substrate availability. However, volatile production showed a tendency to increase only in peel tissue (Figure 2.4). Accordingly, alcohols were not detected in cortex of ethephon-treated apples but they increased in peel, implying that substrate availability is a limiting factor for the synthesis of esters in both peel and cortex tissue. Defilippi et al. (2005b) found that exposing transgenic 'Greensleeves' apples with repressed $C_2H_4$ production to exogenous ethylene induced an increase in the availability of amino acid and fatty acid substrates in peel but not in cortex tissue, and continuous $C_2H_4$ action was required to stimulate volatile ester synthesis. Continuous $C_2H_4$ action and production was also necessary for the synthesis of some esters in 'Super Red Delicious' apples (Fan et al., 1998). In the current study $C_2H_4$ was applied as a one-time treatment, and it may not have been enough to induce a significant increase in ester production by 7 days after treatment. The non-responsiveness of cortex tissue found in the current and in previous studies (Defilippi et al., 2005b), together with the lower RR found in

cortex compared to peel (Rudell et al., 2000), suggests that metabolic activity is much lower in cortex than in peel, and that might negatively affect its ability to respond to ethylene action.

**Grouped volatile production**

It is not surprising that acetate esters were the most abundant volatiles after harvest in both peel and cortex (Table 2.3, 2.5), given that the major volatiles reported to be quantitatively related to the aroma of 'Royal Gala' apples are acetate esters (Young et al., 1996; Mattheis et al., 1998; Plotto et al., 2000). 2-Methylbutanoate esters were abundant in peel but not in cortex tissue, with varying relative amounts over time in storage. Hexanoate esters were the least abundant in both tissues and at all times, and butanoate esters were quantitatively similar to hexanoate esters in peel and to 2-methylbutanoate esters in cortex. The production of all esters is reduced when inhibitors of $C_2H_4$ production or action are applied to pre-climacteric fruit, but synthesis of some esters is not inhibited when the same treatments are applied to post-climacteric fruit, implying that the initiation of ester production associated with fruit ripening requires $C_2H_4$ action, but only some esters need continuous $C_2H_4$ action for their synthesis after ripening has started (Fan et al., 1998). The combination of AVG plus MCP repressed the production of all groups of esters, while the individual treatments showed less reduction, and sometimes there was no effect on the production of esters grouped by acid moiety. This suggests that the application of a repressor of $C_2H_4$ synthesis followed by treatment with a repressor of $C_2H_4$ action might have kept the fruit in a pre-climacteric state in terms of aroma-related volatile production. The low levels of IEC in AVG plus MCP-treated fruit (Figure 2.1) may not have been enough to trigger the normal production of volatiles during ripening.

Ethyl esters were the most abundant of the volatiles grouped by alcohol moiety. Ethanol was not detected with the GC conditions used, but it is likely that the availability of this alcohol was high. Acetate esters were abundant throughout the study, and acids can be reduced to their corresponding alcohols in apple tissue (De Pooter et al., 1983; Echeverria et al., 2004a), so acetic acid could be one of the sources of ethanol. 2-Methyl-1-butanol was less abundant than hexanol in both tissues and at all times, and hexanol was relatively abundant in peel tissue in 2005, as were their corresponding methylbutyl and hexyl esters in peel tissue. This is evidence of the importance of substrate availability for the synthesis of esters. Defillipi et al. (2005b) found that the presence of the immediate substrates, butanol, hexanol and 2-methylbutanol, increased parallel to ester production. Accordingly, in the present work there was a positive correlation

between the levels of volatile alcohols and the respective esters that were detected, though the increment in ester levels with increasing alcohol levels was tissue type-, alcohol-, and year-dependent (Figure A.2, A.5 in Appendix). AVG plus MCP had the same effect on the production of esters grouped by alcohol moiety as on esters grouped by acid moiety. Ethephon seemed to stimulate the production of all but methylbutyl esters in peel of AVG plus MCP-treated apples, and 2-methyl-1-butanol was not detected, explaining the lack of methylbutyl esters.

**Individual volatile production**

The aroma volatile profile of apples is cultivar-dependent (Dixon and Hewett, 2000), and the relative amounts of acids and alcohols present influence the final composition of the volatile esters (Paillard, 1979; De Pooter et al., 1981; Souleyre et al., 2005). Substrate specificity of AAT differs among species (Olias et al., 1995) and among apple varieties and tissues (Holland et al., 2005). The esterification of aliphatic alcohols is preferred over that of branched-chained alcohols (Olias et al., 1995), probably determining the differential volatile profile of each fruit (Dixon and Hewett, 2000), and perhaps of each tissue. In the present study, ethyl 2-methylbutanoate (E2MB) and hexyl acetate were the most conspicuous ester volatiles in peel and cortex of untreated 'Royal Gala' apples, followed by 2-methylbutyl acetate, ethyl butanoate and butyl acetate, while alcohols were detected in low amounts. Young et al. (1996) determined that the major aroma volatile components in 'Royal Gala' were 2-methylbutyl acetate, butyl acetate, hexyl acetate, butanol, 2-methylbutanol and hexanol. 2-Methylbutyl acetate was the volatile with the greatest impact on the characteristic apple aroma and flavor for this cultivar, while butyl acetate was the least important. Interestingly, Young et al. (1996) did not mention E2MB or ethyl butanoate, both conspicuously found in this study, though it can contribute to the aroma of 'Gala' fruit after a 20-week RA storage (Plotto et al, 2000) and is responsible for the underlying sweet and fruity aroma of 'Gala' apple (Plotto et al., 1999). Different sampling methodologies might be part of the cause for the differences in volatile profile among these studies. The amounts of alcohols that were detected were very low in this work compared to the study by Young et al. (1996), though they used vacuum vapor distillation as the extraction method which they stated would detect higher amounts of alcohols relative to the headspace sampling. As mentioned before, the SPME extraction method used in this study might favor esters versus alcohols compared to other methods, amplifying even more the differences between methods. Berger (1991) suggested that studies may differ greatly in the sensory importance they attach to single compounds, due to

biological fluctuations and different methods of quantitative analysis and sensory evaluation. It is also possible that the profile of aroma volatiles produced differed among studies due to different geographical locations of fruit production.

The contribution of each compound to the aroma depends on its particular sensory threshold and presence of other compounds (Buttery, 1993). According to Flath et al. (1967), alcohols have the highest odor thresholds while aldehydes and esters have the lowest. E2MB has the lowest odor threshold of all apple aroma volatile compounds, and has a typical apple-like aroma. Of the other esters, butyl acetate has the highest odor threshold, while hexyl acetate and ethyl butanoate have the lowest. Based on relative compound abundance and odor thresholds, the major esters detected in this study would contribute significantly to the aroma and flavor of 'Royal Gala' apples. In addition, treatments that repressed volatile production like AVG plus MCP would significantly affect the acceptability of the treated fruit to consumers, given that all volatiles were affected in the same way as TVP.

Two esters were quantitatively important in peel but not in cortex, butyl hexanoate and hexyl 2-methylbutanoate, and two esters were detected only in peel, butyl butanoate and hexyl propionate. It appeared that, in cortex of 'Royal Gala' apples, the synthesis of esters of higher molecular weight was restricted compared to peel, even though the corresponding alcohol substrates were present in both tissues. There are at least 12 acyl transferases in apple, and Souleyre et al. (2005) suggest that some of these might have differing substrate preferences, regulation, and kinetic characteristics with different substrate concentrations. Thus, it is possible that different AAT isomers are active in cortex and peel tissue, resulting in differing ester profiles.

**AAT activity**

AAT has been shown to respond to inhibitors of ethylene action (Defilippi et al., 2005a, 2005b), and no other enzymes upstream in the volatile biosynthetic pathways seem to be limiting for volatile production during on-tree ripening (Echeverria et al, 2004a) or when ethylene production or action is impaired (Defilippi et al., 2005b). In the present study, the activity of AAT per mg protein study was similar in peel and cortex tissue (Figure 2.5). Alcohol substrate availability was generally similar in both tissues, and yet volatile production was higher in peel than in cortex tissue. It is likely that AAT activity was not a limiting factor for the synthesis of aroma volatiles which depended more on substrate availability (Echeverria et al., 2004a).

Therefore, one of two different causes for the lower ester production in cortex compared to peel, or their combination, are possible: a) AAT activity per g FW might have been higher in peel, as suggested by the higher protein per g FW for peel tissue (data not shown), or b) substrate availability was higher in peel compared to cortex tissue, related to its higher RR, lipid and protein content, and metabolic activity in general.

The treatments did not affect peel and cortex AAT activity in the same way, but no effects were consistent with the variations in TVP. Ethephon induced a significant increase in peel AAT activity, suggesting the involvement of ethylene in the regulation of AAT. AAT activity did not follow TVP, in agreement with results by Defilippi et al. (2005b) and Echeverria et al. (2004a), which would indicate that aroma precursors are the main limiting factor for volatile synthesis.

**Volatile production when fed alcohols**

***Total ester production when fed alcohols***

The feeding experiment provided evidence that substrate availability may be limiting in peel and cortex of AVG plus MCP-treated fruit with impaired ethylene production and respiration rate. A significant increase in total ester production of butanol- and hexanol-fed peel and cortex of treated and control fruit was observed (Figure 2.6). In general, treated and control samples had similar ester production when they were fed alcohols, implying that substrate availability is a major limiting step for the synthesis of aroma compounds when $C_2H_4$ action and production are impaired. The consistently higher ester production by hexanol-fed samples compared to those fed with butanol could be due to a higher rate of esterification of hexanol (Knee and Hatfield., 1981), or to lower uptake of butanol by the samples, evident through the lower levels of butanol in butanol-fed samples compared to hexanol in hexanol-fed samples (Table 2.8). The ratio hexanol:butanol in hexanol- versus butanol-fed samples was 8-9.6 for control tissue and 3-5 for AVG plus MCP tissue, and the ratio in TEP was 4-8 and 4.5-23, respectively, implying that substrate availability might have been lower in butanol-fed samples, contributing to their lower TEP. Alcohols fed to apple tissue may be oxidized into their respective acids incrementing the availability of acyl-CoAs (Knee and Hatfield, 1981). Thus, the possible higher tissue uptake of hexanol compared to butanol may have led to a higher oxidation rate of hexanol to aldehyde and then to acid incrementing the availability of acyl groups in hexanol-fed more than in butanol-fed samples.

Peel tissue seemed to have a higher rate of ester formation compared to cortex tissue, in agreement with previous findings (Knee and Hatfield, 1981; Rudel et al., 2002). Peel tissue might have a 'more active esterifing system than cortex', though the system itself is qualitatively similar in both tissue types (Knee and Hatfield, 1981). Rudell et al. (2002) provided more evidence for that hypothesis and suggested that skin tissue might have increased amounts of fatty acids, a greater capacity to oxidize alcohol substrates, and more capacity to synthesize esters. As mentioned before, there might be less AAT enzyme in cortex compared to peel that might result in lower AAT activity per unit FW in this tissue. The possibly lower AAT activity per unit FW together with a possibly lower availability of acyl-CoAs could limit volatile synthesis in cortex tissue. There seems to be a complex regulation system in cortex, given AVG plus MCP-treated fruit did not reach the production levels of control cortex when butanol was fed, though ester production was similar in control and treated cortex when hexanol was fed. Additionally, TEP was 23 times higher in cortex of AVG plus MCP samples fed with hexanol versus butanol but only 8 times higher in cortex of control samples, with no difference in TEP ratio between control and AVG plus MCP-treated peel samples. Thus, lower levels of $C_2H_4$ in AVG plus MCP-treated fruit could have negatively affected a cortex AAT isoform that has more affinity for butanol.

***Individual and grouped ester production when fed alcohols***

The acetate esters of butanol and hexanol increased the most when the corresponding alcohol was fed to the samples (Table 2.7), results similar to those of Bartley et al. (1985). The particular increase in acetate esters could be due to a higher relative presence of acetyl-CoA (Paillard, 1979; de Pooter et al., 1981; Mattheis et al., 1991) and/or to a higher affinity of AAT for the acetate substrate. Hexyl acetate was the ester that showed the most significant increase in all cortex and peel tissues fed hexanol, though this was not true for butyl acetate in butanol-fed samples, where butyl butanoate was the most abundant ester. A higher production of the acetate ester by hexanol-fed compared to butanol-fed samples has been observed before (Knee and Hatfield, 1981), and may be partially due to the high AAT *Vmax/Km* for hexanol, three times higher than for butanol (Souleyre et al., 2005), and to lower alcohol availability in butanol-fed samples, as previously discussed. Holland et al. (2005) measured different apple AAT substrate specificities *in vitro* and also suggested that there might be more than one AAT involved in the formation of volatile esters in this fruit. Cortex tissue fed with butanol exhibited a smaller increase in ester production compared to butanol-fed peel tissue and all hexanol-fed tissue. This

could indicate the presence of different AAT isoforms in peel and cortex of apple fruit and a lower AAT affinity for butanol in cortex, assuming both alcohols were abundantly plentiful within the tissues.

The production of esters with the alcohol or acid moiety corresponding to the fed alcohol increased at the expense of other esters, and that could be due to a higher binding rate of the enzyme to relatively more abundant substrates, and to changes in preference for substrates at different concentrations (Souleyre et al., 2005). Butyl butanoate increased 64- to 388-fold in butanol-fed versus control peel tissue while other butyl esters increased 2- to 11-fold only (Table 2.8). This effect was not as evident in cortex tissue, which had a lower ester production. Hexyl hexanoate increased more than any other hexyl ester but hexyl acetate when hexanol was fed to peel tissue (Table 2.8). It was interesting that butanoate esters increased when hexanol was fed. This suggests that alcohols can not only be oxidized to aldehydes and then to acids, but that these acids can also undergo β-oxidation to yield shorter chain Acyl-CoAs. Tissues fed with aldehydes have converted them into acids in previous studies (De Pooter el al., 1983), and a further oxidation might take place.

## CONCLUSIONS

During recent years the importance of the effect of several treatments and storage regimes on fruit aroma has increased in response to consumer concerns of flavor loss of stored fruit. 'Royal Gala' is gaining increasing popularity in the U.S., but it stores poorly and easily loses aroma compared to other cultivars. The impact of either AVG or MCP alone on the quality of various apple cultivars has been thoroughly studied, but little has been published about their combined effect. Neither AVG nor MCP alone was consistently effective in reducing firmness or acidity loss of 'Royal Gala' apples after short-term cold storage, while soluble sugar content was shown to be independent of ethylene control.

AVG has been used to provide a wider harvest window, and it appears it can also extend the window for an effective postharvest application of MCP from the present work. The combination of AVG and MCP was more effective than either alone in reducing $C_2H_4$ production, RR, firmness loss and acidity loss; it did not reduce starch degradation nor repress red skin color development more than AVG alone, though volatile production was consistently

repressed by the combined treatment. Sugar-to-acid ratio was lower in AVG plus MCP-treated fruit due to higher acidity retention as soluble sugar content did not change. Overall, ripening was slowed in fruit treated with AVG plus MCP after harvest and cold storage, but at the expense of a greatly reduced aroma-related volatile production. The major ester volatiles that contribute to the typical aroma and flavor of 'Royal Gala' were greatly reduced by AVG plus MCP. Precursor availability seemed to be the major factor limiting ester production in peel and cortex of 'Royal Gala' apples, though other factors might interact to drive volatile production in cortex.

An external source of $C_2H_4$, like ethephon, failed to stimulate the reduced volatile production in AVG plus MCP-treated fruit, though it did not negatively affect other quality traits such as firmness and acidity. Continuous $C_2H_4$ action might be needed to stimulate the synthesis of some volatiles, and therefore the treatment should be applied for a longer time to assess this possibility. Different thresholds of $C_2H_4$ concentration that trigger responses of several fruit quality traits should be taken into consideration when $C_2H_4$ availability and/or action are manipulated, either repressed or increased. Consumer acceptability was not studied in our work, and it would be important to determine whether the loss of volatile production by AVG plus MCP treatment and sugar-to-acid ratio, and the increase in volatile production in peel but not in cortex of this fruit, in response to post-storage ethylene treatment, would be detected by consumers.

# CHAPTER 3

# EFFECT OF AVG AND HEAT TREATMENT ON FRUIT QUALITY DURING POSTHARVEST STORAGE OF DIFFERENT APPLE VARIETIES

## INTRODUCTON

Apple *(Malus sylvestris var. domestica)* is the most economically important tree fruit crop in U.S. Given the increasing demand for high quality fruit throughout the year, maintaining quality during short and long term postharvest storage is essential to both growers and consumers. There are several quality factors influencing the acceptability and edibility of apple fruit, such as appearance, texture and flavor. First-time purchases are often based on appearance and firmness, but repeat buys are determined by internal quality traits such as mouth-feel and flavor (Baldwin, 2002). Color is a major characteristic of appearance, and its development is cultivar and environment-dependent (Layne et al., 2002). Firmness is an important component of texture (Kader, 2002) that is also closely related to textural qualities like crispness and mealiness (Saftner et al., 2002). Flavor is a complex trait composed of sweetness, sourness, bitterness, saltiness and aroma (Baldwin, 2002). Of these traits, sweetness can be evaluated by total soluble solids content (SSC), sourness by total titratable acidity, and aroma by the quantification and identification of volatile compounds (Kader, 2002). The combination sourness-acidity was related to apple-fruity flavor, acceptability of flavor, and overall acceptability by a test panel (Saftner et al., 2002). There exists an increasing concern by consumers about deficiencies in sensory quality of fruit. Maturity at harvest can affect fruit flavor (Fellman et al., 1993). Early harvest is a tool that slows down ripening during cold storage, at the cost of getting smaller, poorly colored fruit with less flavor that is more susceptible to bitter pit, while later harvested fruit may be softer and mealier, with a higher risk of internal breakdown during storage (Peirs et al., 2002). When comparing harvest dates, panelists assigned high ratings for tartness and firmness to early harvested ‘Gala’ and ‘Fuji’ apples, but these were always associated with low

ratings for overall desirability, sweetness and flavor, implying that high firmness and tartness do not necessarily correspond with high consumer acceptability (Plotto et al., 1997).

Of the volatiles produced by apples, esters and alcohols are the major compounds responsible for the characteristic aroma (Lurie et al., 2002; Fan and Mattheis, 1999). Three esters: butyl acetate, 2-methylbutyl acetate and hexyl acetate are thought to be the main contributors to apple-like aroma (Lurie et al., 2002; Dimik and Hoskin, 1983). Alcohol-acyl transferase (AAT) catalyzes the transfer of acyl moieties to alcohols from acyl–CoAs, the last step in the synthesis of esters, and appears to be regulated by ethylene (Fan and Mattheis, 1999; Defilippi et al., 2005a).

Ethylene ($C_2H_4$) regulates several processes in climacteric fruit such as apple, including softening of the cortex, acidity loss, color development and respiration rate (Lurie, 1998a, b). Techniques that slow down ripening of climacteric fruit such as application of inhibitors of ethylene synthesis like aminoethoxyvinylglycine (AVG), ethylene perception like 1-methylcyclopropene (1-MCP), heat treatment after harvest, and controlled atmospheres (CA) are valuable tools that may maintain fruit quality in cold storage. Apples can be stored from a few weeks to 11 months, depending on variety, type of storage (regular cold storage or CA), storage temperature, and speed of cooling (Kader, 2002). Fruit in cold storage tend to soften, lose water, and lower their post-storage rate of volatile synthesis, affecting the final quality of the product. CA can reduce respiration and ethylene production rates compared with regular cold storage at the cost of reducing aroma volatile levels (Saftner et al., 2002). This technology is often used when apples are stored for longer then 3 months (Kader, 2002).

AVG is an inhibitor of ethylene biosynthesis that is commercially used to stop fruit drop as a preharvest application. It inhibits pyridoxal phosphate-linked enzymes such as ACC synthase (Boller et al., 1979), a key enzyme in the ethylene biosynthetic pathway (Yang and Hoffman, 1984), and was found to delay apple fruit ripening and decrease preharvest drop (Bangerth, 1978; Stover et al., 2003), inhibit ethylene and volatile production when applied to pre-climacteric fruit (Fan et al., 1998; Halder-Doll and Bangerth, 1987), retard production of ethylene and ripening-related volatiles during storage (Bangerth and Streif, 1987), and reduce volatile production, including acetate esters, of fruit harvested before the climacteric peak (Fan et al., 1998; Mir et al., 1999). Greene (2002) found that 124 g a.i. $ha^{-1}$ of AVG was effective for delaying fruit maturity and preharvest drop of ‘McIntosh’. Greene and Schupp (2004)

established that one application of AVG 4 weeks before harvest was more effective in preventing fruit drop, and economically more efficient, than 2 half dose applications 4 and 2 weeks before harvest. Trees treated with AVG have increased vegetative growth the following year (Williams, 1980). Beneficial effects of AVG have been seen after CA storage. Fruit that had been treated with AVG, and presented a delay in fruit maturation and ripening at harvest, had lower internal ethylene concentration (IEC) and higher retention of cortex firmness and shelf-life than non-treated fruit after 6 months in CA storage and 7 days at room temperature (Wang and Dilley, 2001). Autio and Bramlage (1982) found that early-season cultivars were less affected by AVG than late-season ones, conflicting with results by Byers (1997) who showed that there may not be such a tendency.

Interest in thermal treatments of fruit began with the increasing demand to find alternatives to the use of postharvest chemicals against pathogens and insects. Later, heat treatments were studied as a possible tool to maintain fruit quality in storage. Salveit (1991), working with tomato disks, showed that a heat stress could reduce tissue sensitivity to chilling injury when it was applied before cold storage. Lurie et al. (1995) found that the plasma membrane of apples preheated at 38$^{o}$C for 4 days and later stored at 4$^{o}$C for 4 months had more unsaturated fatty acids than control fruit, resulting in more fluid membranes that reduces the risk of chilling injury (Lyons, 1973). Of the different thermal treatments, hot air (placing fruit in a heated chamber) heats more slowly than hot vapor (heat transfers by condensation of water vapor on the cooler surface of the fruit) but avoids the potential deterioration resulting from the excessive humidity of the latter (Lurie, 1998a, b). In climacteric fruit, heat might inhibit ripening through its effect on enzymes involved in the synthesis of ethylene. ACC oxidase (ACO) converts ACC to ethylene (Yang and Hofman, 1984). ACO activity is the first to be inhibited, followed by ACS activity (Yu et al., 1980, Klein, 1989 and Atta-Aly, 1992). Although heat treatment itself inhibits ripening, after the fruit is removed from the treatment ethylene production recovers to equal or higher levels than those of control fruit (Klein and Lurie, 1990; Lurie and Klein, 1992a). ACO protein and transcripts accumulated during the recovery from a hot air treatment at 38$^{o}$C (Lurie et al., 1996a). Paull and Chen (2000) found that protein denaturation by heat treatment can be reversed at certain temperatures but can be permanent at excessively high ones, causing heat injury. They listed many factors that could influence the ripening response to heat, like field-induced thermotolerance, cultivar, fruit size and

morphological characteristics, ripeness level (physiological state), heat transfer rate and energy balance, final temperature, and duration of exposure at different temperatures.

Heat treatments have yielded different results. To name a few, it decreased firmness loss after 6 months in regular cold storage at 0 $^{o}$C according to Porrit and Lidster (1978), but Saftner et al. (2002) found that quality and sensory characteristics of heated and non-heated fruit did not differ significantly after the same period in cold storage. 'Anna' and 'Granny Smith' apples that were heated at 46 $^{o}$C for 12 hours or at 42 $^{o}$C for 24 hours before storage were firmer at the end of storage, had a higher sugar:acid ratio and a lower incidence of superficial scald than unheated fruit, results similar to heating apples at 38 $^{o}$C for 4 days (Klein and Lurie, 1992). Superficial scald is a chilling injury caused by the oxidation of α-farnesene, a component of the apple wax, and heat-treated apples stored at 0 $^{o}$C for one month had both lower α-farnesene content and reduced superficial scald incidence (Lurie et al., 1990). The biosynthesis of α-farnesene in apples is mediated by ethylene-regulated gene expression during fruit ripening (Ju and Curry, 2000). According to Tu and De Baerdemaeker (1997), 'Golden Delicious' and 'Jonagold' heated apples maintained firmness better than unheated ones, though the effects of heat treatments on apple quality were cultivar dependent. However, internal browning after 4 months of cold storage was more obvious for heat-treated apples. Heated apples (38 $^{o}$C for 4 days) were found crispier and sweeter by a taste panel than unheated ones (Lurie and Nussinovitch, 1996). The authors attributed that effect to a possible decrease in the activity or synthesis of cell wall degrading enzymes based on previous studies by Klein et al. (1990) and Ben Shalom et al. (1993) that showed lower soluble pectin and higher insoluble pectin content in heated fruit compared to control fruit. Additionally, more calcium is bound to cell walls and less to water-soluble pectin of heated fruit (Lurie and Klein, 1992b). Calcium ions are chelated by de-esterified regions of pectic polymers, forming 'egg-boxes' that in sufficient number would be expected to hold adjacent polymers firmly together (Tucker, 1993). Fallik et al. (1997) found that volatile production was first inhibited, but then recovered to even higher levels than non-heated fruit after 6 weeks of cold storage.

AVG is used commercially as a preharvest treatment, though postharvest effects are now known. Heat could be used as an additional postharvest treatment for maintaining fruit quality in storage. There is information about the effects of the individual treatments on ripening traits of apple, but nothing has been reported about their combined effect. Based on apple response to

each treatment alone, the combination of AVG and heat may have a strong negative effect on fruit volatile production but a positive effect on firmness retention during cold storage, though this response may be genotype-specific. In this study the response of different varieties of apple to AVG and heat treatment, alone or combined, was assessed in terms of ripening and postharvest storage quality.

## MATERIALS AND METHODS

### FRUIT QUALITY AND CULTIVAR RESPONSES

#### Treatments and harvest

At the University of Kentucky Horticultural Research South Farm in Lexington, Kentucky, whole trees of 'Lodi/M7' and 'Senshu/M26' –early harvested apples- and 'Redchief Red Delicious/M7' and 'Red Fuji/M7a' –later harvested apples-, all planted in 1993, were treated in 2003 with an aqueous solution of AVG (ReTain, Valent Biosciences, Libertyville, IL) containing 500 ppm Silwet L-77 (Helena Chemical Co., Collierville, TN) as surfactant, at the commercial rate of 124 $g.ha^{-1}$ a.i. (Commercial Tree Fruit Spray Guide, 2003). AVG was applied 32, 28, 31 and 35 days before harvest of 'Lodi', 'Senshu', 'Red Delicious' and 'Fuji', respectively. In 2004 the same treatment was applied but only to 'Senshu' and 'Lodi' trees 28 days before harvest. The solution of AVG was applied to leaves and fruit with a hand pump sprayer to the point of runoff. In order to determine the best harvest time for AVG-treated fruit, and due to low yield and high drop rate of some varieties, even from trees treated with AVG, different cultivars were harvested at different times after treatment with AVG. In 2003 and 2004, control and AVG-treated 'Lodi' and 'Senshu' apples were harvested at the beginning of contol fruit ripening ripening based on starch index and headspace ethylene production. In 2003, AVG-treated 'Redchief Red Delicious', 'Red Fuji' and a second group of 'Senshu' apples were harvested 10, 14 and 14 days after control apples, respectively. Because 'Senshu' had a high crop load and was a summer variety which has not stored well, it was chosen to test early versus late harvest dates. Fruit were equilibrated at laboratory room temperature ($21 \pm 0.5\ ^{o}C$) (RT) for three hours immediately after harvest. Half of each lot was then heat-treated in trays placed in an incubator at $38^{o}C$ for 4 days inside plastic bags to reduce weight loss. A source of water was also placed in the incubator to maintain a high relative humidity. Fruit from the four treatments -

control, AVG, heat and AVG plus heat- were ripened at room temperature for five days after harvest or after completion of heat treatment, or placed in regular atmosphere cold storage at 4$^{o}$C for one month, and then ripened at RT for five days. In 2003, 'Lodi' yield was low so fruit were not ripened after harvest but were only cold-stored.

**Respiration and headspace ethylene production**

Ethylene ($C_2H_4$) concentration in the cold storage chamber was measured weekly to ensure that the level was negligible. Individual respiration rate (RR) and headspace ethylene production, both non-destructive measurements, of five to eight fruit were assessed on days 1, 3 and 5 after harvest (AH) or heat treatment or after removal from 4-week cold storage (4WCS). RR was quantified by placing individual fruit in sealed 2 L glass jars, taking direct samples from the headspace through a rubber septum in the lid after 4 h, and measuring each sample with an $O_2/CO_2$ analyzer (Model ZR 892 HS, Illinois Instruments Inc., McHenry, IL). For headspace ethylene production, 0.2 mL of headspace samples after 4 h were analyzed with an HP 5890 gas chromatograph (HP 5890, Agilent Technology, Wilmington, DE) equipped with a flame ionization detector (FID) and an alumina capillary column (AT-Alumina Plot GC Column, 30 m, 0.53 cm i.d.). Temperatures were 35 $^{o}$C, 175 $^{o}$C and 125 $^{o}$C for oven, injector and FID detector, respectively. An external standard (100 ppm Ethylene/Helium, Alltech Associates Inc., Deerfield, IL) was used to quantify the amounts of detected ethylene.

**Starch index, cortex firmness and soluble solids content**

Starch index (SI), cortex firmness, titratable acidity and soluble solids content, all destructive measurements, were taken on four to eight fruit at 1 and 5 days AH or heat treatment and after 4WCS. Measurements were taken on the same fruit used for $C_2H_4$ and $CO_2$ production. To assess SI, a quantification of starch degradation, fruit were cut in half perpendicular to the stem-blossom axis and the halves were soaked in iodine solution (0.1% iodine, 1% potassium iodide in water). The degree of staining was rated on a visual scale of 1 to 9, where 1= staining the entire cut surface (high starch content) and 9= no staining (no starch, Cowgill et al., 2005). For 'Fuji' a 1-6 scale, with 1=all starch and 6= no starch, was used (Cowgill et al., 2005). Cortex firmness was measured using a penetrometer (Model DF M10, John Chatillon & Sons, Inc. Greensboro, NC) equipped with a 8 mm diameter probe after a disk of skin was removed from opposite sites on the equatorial plane of the stem halves. To convert firmness values from 8 mm to that more commonly reported using an 11 mm diameter probe, firmness was measured with

both probes on opposite sites of 30 apples of different cultivars and varying firmness. A regression was derived between the average firmness of each fruit measured with both probes. Firmness with 11 mm probe (N) = 8.5202 + 1.5703 x firmness with 8 mm probe (N), $R^2 = 0.77$. Soluble solids content (SSC) was determined on a fresh-squeezed juice sample from each fruit using an automatically temperature-compensated hand refractometer (Model 10430, Reichert Scientific Instruments. Buffalo, NY).

**Titratable acidity and sugar:acid ratio**

For titratable acidity (TA), approximately 15 g of cortex cut from the apples used for previous analyses were frozen at -20 $^{o}$C. Samples were later thawed, macerated with a mincer/chopper and filtered through two layers of cheesecloth separated by a layer of Miracloth (Calbiochem, EMD Biosciences Inc., La Jolla, CA). One mL of each sample was mixed with 14 mL of deionized water and titrated to pH 7.0 with 0.1 N NaOH. Results were expressed as mg malic acid 100 $mL^{-1}$. Using SSC and TA from the same fruit, sugar:acid ratio (S:A) was calculated as SSC/ TA, where TA was re-calculated as g malic acid/100mL.

**Physical deterioration and weight loss**

Physical deterioration from bruising and cracking was assessed after 4WCS on 'Lodi' fruit in both years and quantified as the percentage of fruit bruised or cracked after retrieval from cold storage. Weight loss (WL) during cold storage was measured in 2004 on both 'Lodi' and 'Senshu' and assessed by measuring the weight of individual apples before heat treatment or storage and immediately after cold storage, and differences expressed as percentage of the initial weight.

**VOLATILE PRODUCTION**

A separate group of control, AVG, heat-treated and AVG plus heat-treated 'Redchief Red Delicious' apples from those used for the other monitored quality measurements was used in this experiment in 2003.

**Internal ethylene concentration**

Internal ethylene concentration (IEC) was measured on different lots of ten fruit each on days 1, 3 and 5 AH or heat treatment and after 4WCS. A gas sample was taken from the seed cavity by inserting a needle attached to a 10 mL syringe through the calyx end, and a 0.2 mL sub-sample was injected into the GC with the same column and settings used to quantify headspace ethylene production, as above.

**Volatile production**

Volatile production (VP) was measured on three individual apples per treatment, taken from the lots used for IEC assessment. Approximately 9 g peeled cortex samples were frozen at -20 $^{o}$C 5 days AH or heat treatment and after 4WCS and then measured according to Hamilton-Kemp et al. (2003). Briefly, samples were thawed in 30 mL glass jars sealed with Teflon-lined plastic screw caps containing a 3-layer septum. Samples were equilibrated in a water bath to 26 $^{o}$C for 3 h and then placed at ambient laboratory temperature. The headspace in the bottle was sampled for 15 min using solid phase microextraction (SPME) employing a 100 μm poly(dimethylsiloxane) (PDMS) fiber. The SPME fiber was removed and injected into a GC (Model Hewlett Packard 5890 Series II, Agilent Technology, Wilmington, DE) equipped with a DB-5 column (60 m x 0.32 mm i.d., 1 μm film thickness) and a flame ionization detection (FID) detector. Volatiles were desorbed in the GC injection port for 5 min. Conditions for chromatography were as follows: injection port temperature, 220 $^{o}$C; FID detector, 240 $^{o}$C; initial oven temperature, 35 $^{o}$C held for 5 min and then increased to 184 $^{o}$C at 2 $^{o}$C min$^{-1}$; injector splitless for 5 min. A modified splitless injection port was used so that both the septum and inlet purges were interrupted during SPME injections.

**AAT activity**

Alcohol acyl-CoA transferase (AAT) activity was assayed on cortex tissue of three individual fruit per treatment frozen at -80 $^{o}$C 5 days AH or heat treatment and after 4WCS, using a methodology adapted from Echeverria et al. (2004b). Three g of frozen tissue were pulverized and then homogenized in 6 mL of extraction solution (0.1 M potassium phosphate, 1 mM ethylene-diaminetetraacetic acid (EDTA), 0.1% (w/v) Triton X-100 and 1% (w/v) polyvinylpolypyrrolidone (PVPP), pH 8.0). The homogenate was centrifuged at 25,000 x *g* for 20 min at 4$^{o}$C. The supernatant was recovered and placed on ice as crude enzyme extract. AAT was assayed by mixing 1000 μL enzyme extract with 10 μL of 1M $MgCl_2$, 50 μL of butanol solution (0.2 M butanol in 0.1 M potassium phosphate buffer, pH 8.0) and 300 μL of acetyl-CoA solution (2.5 mM acetyl-CoA in 0.1 M potassium phosphate buffer, pH 8.0) and incubating the solution at 35$^{o}$C for 10 min. Then, 100 μL of 5.5'-dithiobis(nitrobenzoic acid) (DTNB) were added to the mixture and immediately placed in a spectrophotometer (Model Cary 50 Bio, Varian Analytical Instruments, Walnut Creek, CA) to measure the production of the yellow thiophenol product from DTNB reacting with free CoA through an increase in absorbance at 412

nm over time. AAT activity was expressed as mU x mg protein$^{-1}$ where U, activity unit, is the increase in one unit of absorbance per minute. Total protein content of the enzyme extract was determined spectrophotometrically at 595 nm using the Coomassie Plus$^{TM}$ Protein Assay Kit (Pierce, Rockford, IL) following the manufacturer's instructions and using bovine serum albumin (Fisher Scientific, Fair Lawn, NJ) as a standard.

To accurately determine tissue protein content, an exhaustive protein extraction was made on 3 control and 3 heated samples from 5 days AH that had been used for assaying AAT activity. Three grams of frozen tissue were pulverized and then homogenized in 10 mL of extraction buffer (0.6 M Tris pH 7.0, 0.2 % ascorbic acid, 0.1% (w/v) Triton X-100 and 1% (w/v) (PVPP). The homogenate was centrifuged at 25,000 x *g* for 20 min at 4$^{o}$C. The supernatant was recovered and placed on ice as crude protein extract. The pellet was re-suspended in 10 mL of extraction buffer and centrifuged at 25,000 x *g* for 10 min at 4$^{o}$C, the supernatant was recovered and mixed with the previously recovered crude protein, and this procedure was repeated one more time. Total protein content of the crude extract was determined spectrophotometrically at 595 nm, as described above.

**Experiment design and statistical data analysis**

Each experiment was conducted using a completely random design. All data were subject to analysis of variance. Means were compared with Fisher's protected least significance difference (LSD, $p$=0.05) using SAS version 9.1 software (SAS Institute Inc., Cary, N.C.).

# RESULTS

### FRUIT QUALITY AND CULTIVAR RESPONSES

**Headspace ethylene production**

After harvest, AVG significantly inhibited HEP of 'Lodi' apples in 2003 (Figure 3.1.A), and AVG plus heat treatment reduced it the most in 2004 (Figure 3.1.B). After 30 days in cold storage ethylene production of AVG-treated 'Lodi' fruit was statistically similar to that of control fruit in both seasons (Figure 3.1.C, D). After harvest, HEP of AVG-treated 'Senshu' fruit was highly repressed in 2003 when fruit were harvested at the same time as control fruit (H1) and that effect was partially lost by H2 (Figure 3.2.A), but there was no effect of AVG up to 5 days after harvest in 2004 (Figure 3.2.B). After 4WCS, ethylene production of 'Senshu' fruit was

**Figure 3.1:** Effect of AVG and/or heat treatment on headspace ethylene production (HEP) of 'Lodi' ripened at room temperature for 5 days immediately after harvest or heat treatment (A, B) and after 4 weeks of cold storage at 4°C (C, D) in 2003 and 2004. Least significant differences (LSD) at *p=0.05* within date are shown as vertical bars. Note that Y axes are shown with different scales.

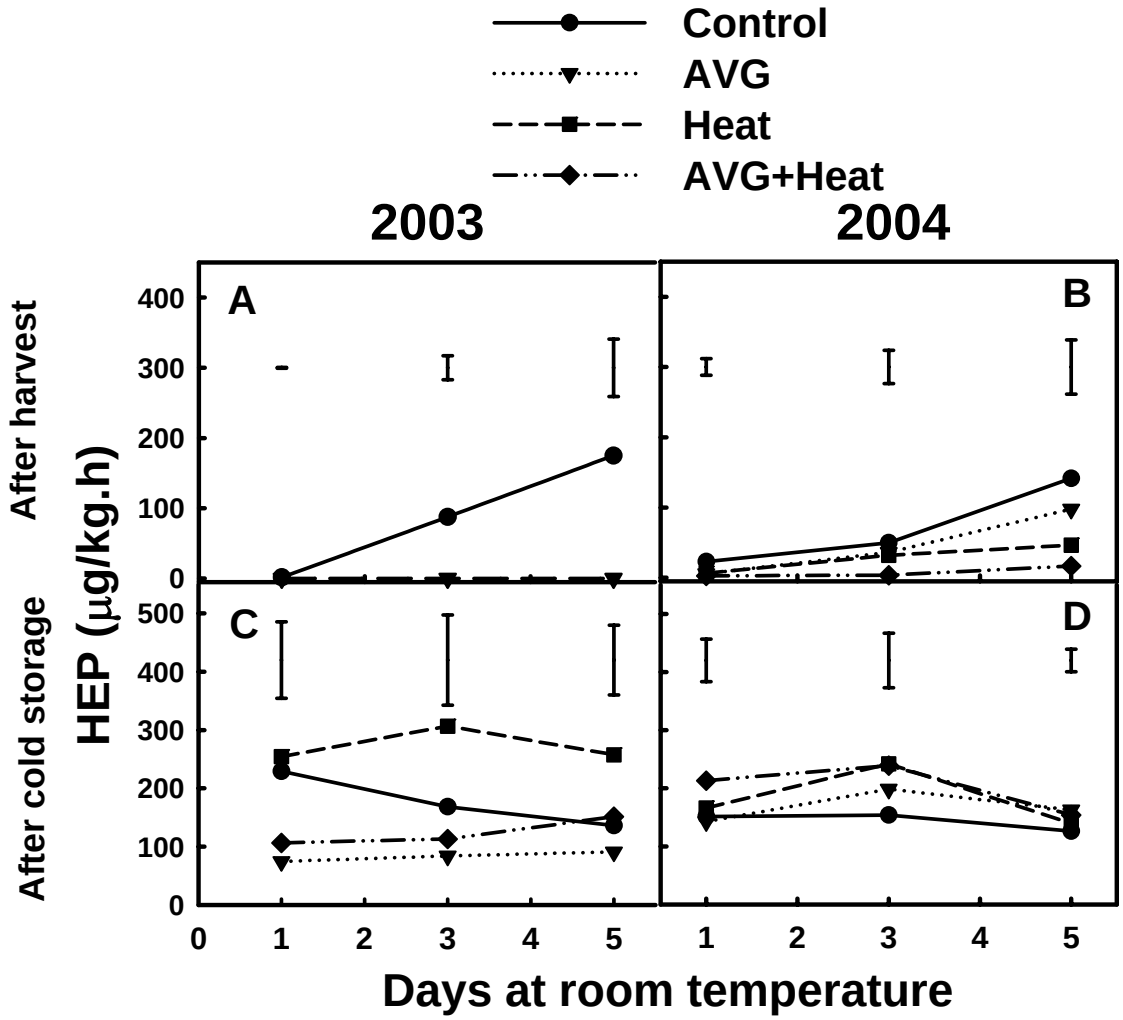
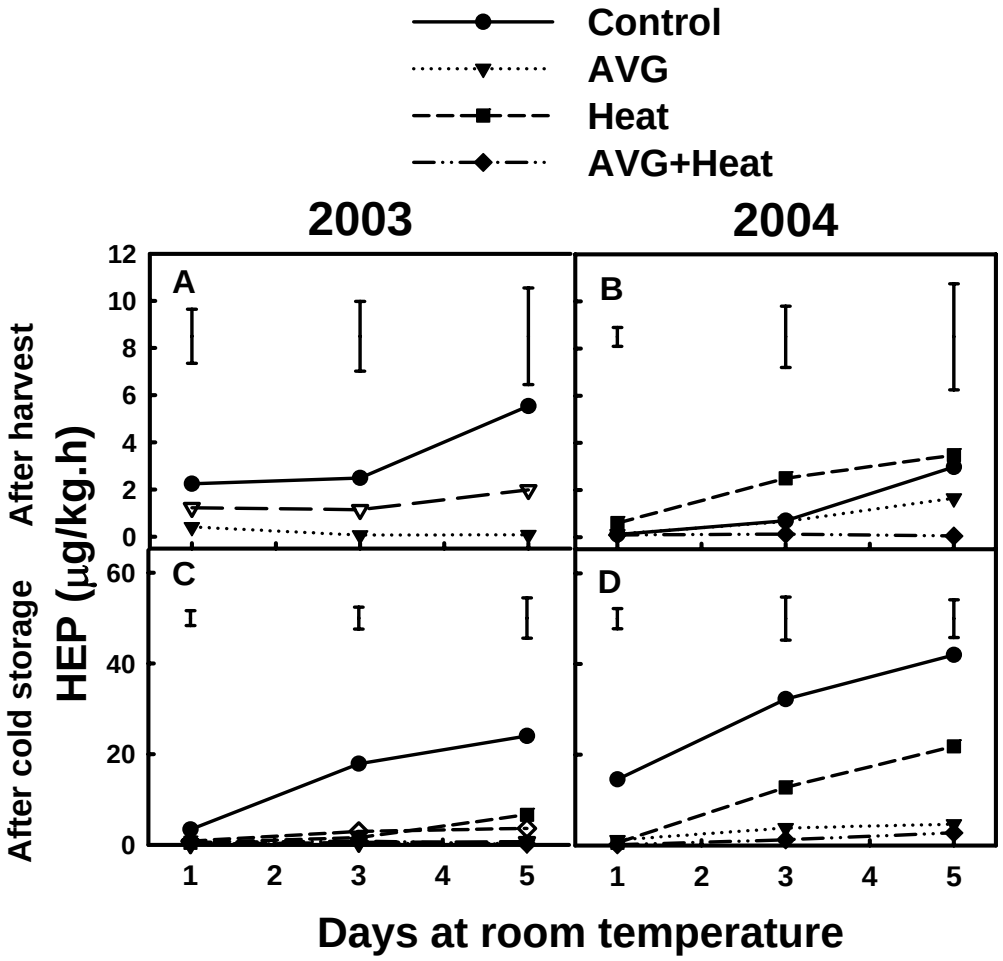


**Figure 3.2:** Effect of AVG and/or heat treatment on headspace ethylene production (HEP) of 'Senshu' ripened at room temperature for 5 days immediately after harvest or heat treatment (A, B) and after 4 weeks of cold storage at 4°C (C, D) in 2003 and 2004. Closed symbols represent data from the normal harvest (H1), and open symbols represent data from the late harvest (H2) in 2003 only. Least significant differences (LSD) at *p=0.05* within date are shown as vertical bars. Note that Y axes are shown with different scales.



consistently reduced by all the treatments in both seasons (Figure 3.2.C, D), though heat treatment alone had a lesser effect in 2004. After harvest, ethylene production by 'Red Delicious' was reduced by AVG and AVG plus heat, and increased by heat treatment alone (Figure 3.3.A). After cold storage ethylene production of 'Red Delicious' fruit was reduced by all the treatments, with AVG plus heat repressing it the most (Figure 3.3.B). After harvest, 'Fuji' had the lowest HEP of all cultivars, and all the treatments repressed it to even lower levels (Figure 3.3.C) and afer 4WCS, the combined treatment repressed HEP of 'Fuji' apples the most (Figure 3.1.D). 'Lodi' had the highest ethylene production of all cultivars in 2003 and 2004 (Figure 3.1), showing HEP 7- to almost 400- fold higher than the cultivar with the next highest ('Red Delicious'; Figure 3.3) and with the lowest ('Fuji'; Figure 3.3) HEP 5 days AH, respectively.

**Respiration rate**

Respiration rate did not present a clear pattern and seemed to be less affected by the treatments than ethylene production (Figure 3.4, 3.5, 3.6). Immediately after harvest, AVG did not affect RR of 'Lodi' fruit in any season (Figure 3.4.A, B). In 2004 heat treatment repressed RR the most, and AVG plus heat treatment had a similar effect only 5 days after harvest. The respiration rate of 'Senshu' apples was reduced by AVG in 2003 even in fruit from H2 (Figure 3.5.A), and by the combined treatment in 2004 (Figure 3.5.B). After 30 days in cold storage, the combined treatment repressed RR of 'Lodi' and 'Senshu' fruit in both seasons (Figure 3.4.C, D, 3.5.C, D).

AVG alone or combined with heat treatment greatly reduced RR of 'Redchief Red Delicious' fruit (Figure 3.6.A). All treatments reduced RR of 'Fuji' apples (Figure 3.6.C). After 4WCS, heat treatment slightly reduced RR in 'Red Delicious' (Figure 3.6.B) and the combined treatment reduced RR in 'Fuji' (Figure 3.6.D).

**Firmness**

At harvest, AVG did not significantly affect firmness loss of 'Lodi' fruit in 2003, but AVG plus heat reduced loss in 2004 when fruit was ripened at room temperature for 5 days (Table 3.1). After cold storage, fruit firmness was reduced by 50% or more compared to harvest. In 2004 firmness was greatly reduced during cold storage. However, after 4WCS and 5 days at RT, AVG alone or combined with heat reduced firmness loss to some extent while heat treatment alone increased it compared to controls. After harvest, AVG reduced firmness loss of H1 'Senshu' apples held for 5 days at room temperature in 2003, though that effect was lost in fruit

**Figure 3.3:** Effect of AVG and/or heat treatment on headspace ethylene production (HEP) of 'Redchief Delicious' (A, B) and 'Red Fuji' (C, D) ripened at room temperature for 5 days immediately after harvest or heat treatment (A, C) and after 4 weeks of cold storage at 4°C ( B, D) in 2003. AVG-treated fruit were harvested 1 or 2 weeks after the controls. Least significant differences (LSD) at *p=0.05* within date are shown as vertical bars. Note that Y axes are shown with different scales. Inset in Figure C shows means and LSDs at a scale 0 to 0.6 µg/kg.h.

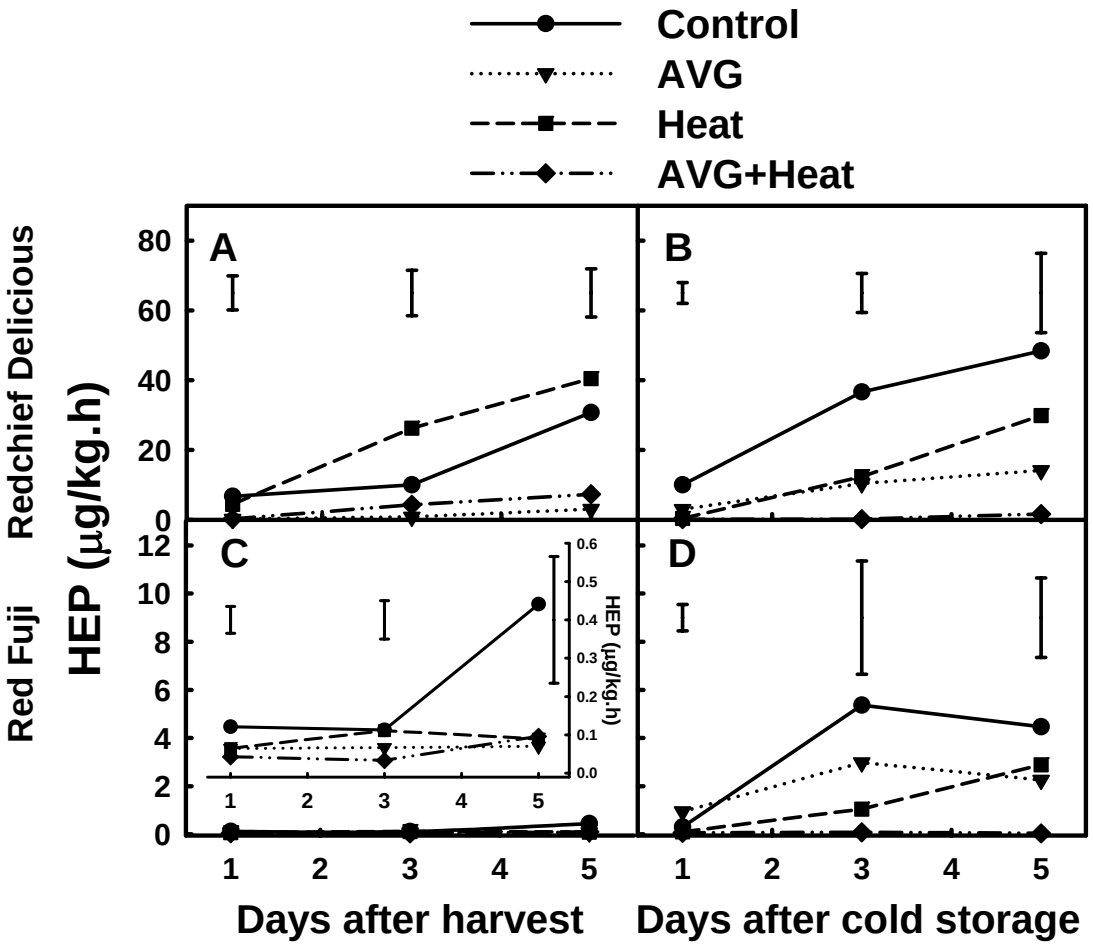


**Figure 3.4:** Effect of AVG and/or heat treatment on respiration rate (RR) of 'Lodi' ripened at room temperature for 5 days immediately after harvest or heat treatment (A, B) and after 4 weeks of cold storage at 4°C (C, D) in 2003 and 2004. Least significant difference (LSD) at *p=0.05* within date are shown as vertical bars.

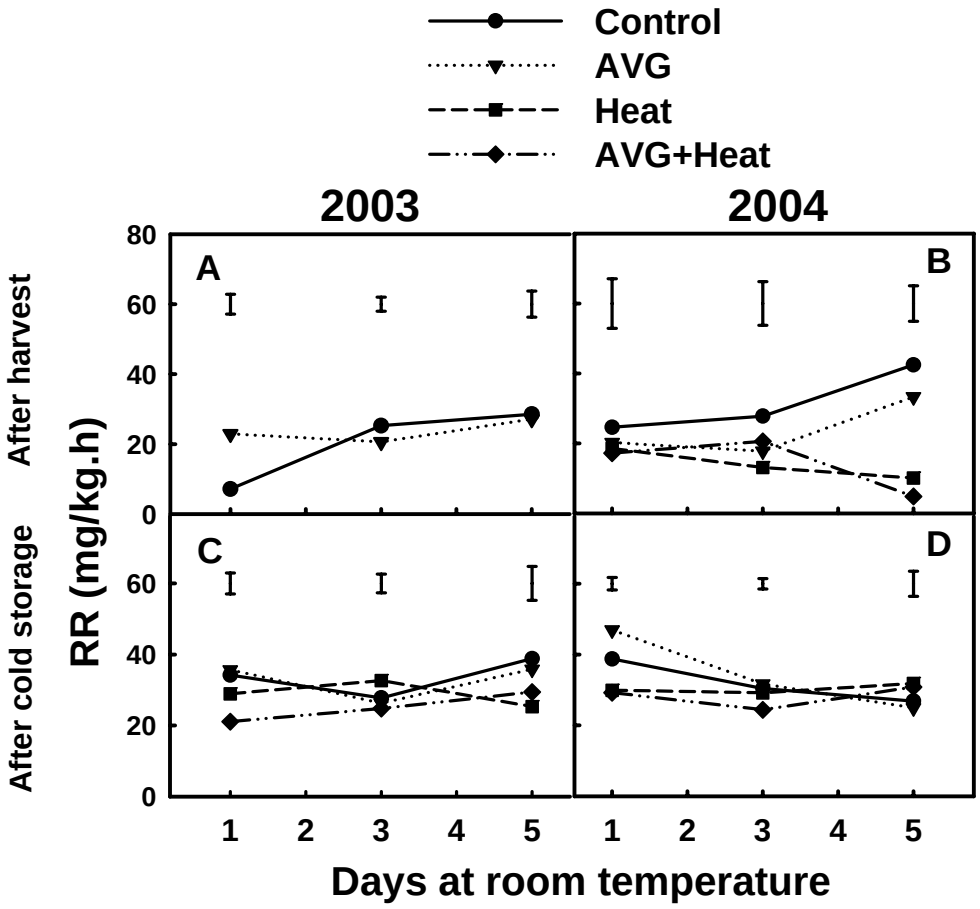


**Figure 3.5:** Effect of AVG and/or heat treatment on respiration rate (RR) of 'Senshu' ripened at room temperature for 5 days immediately after harvest or heat treatment (A, B) and after 4 weeks of cold storage at 4°C (C, D) in 2003 and 2004. Closed symbols represent data from the normal harvest (H1), and open symbols represent data from the late harvest (H2) in 2003 only. Least significant differences (LSD) at *p=0.05* within date are shown as vertical bars. Note that Y axes are shown with different scales.

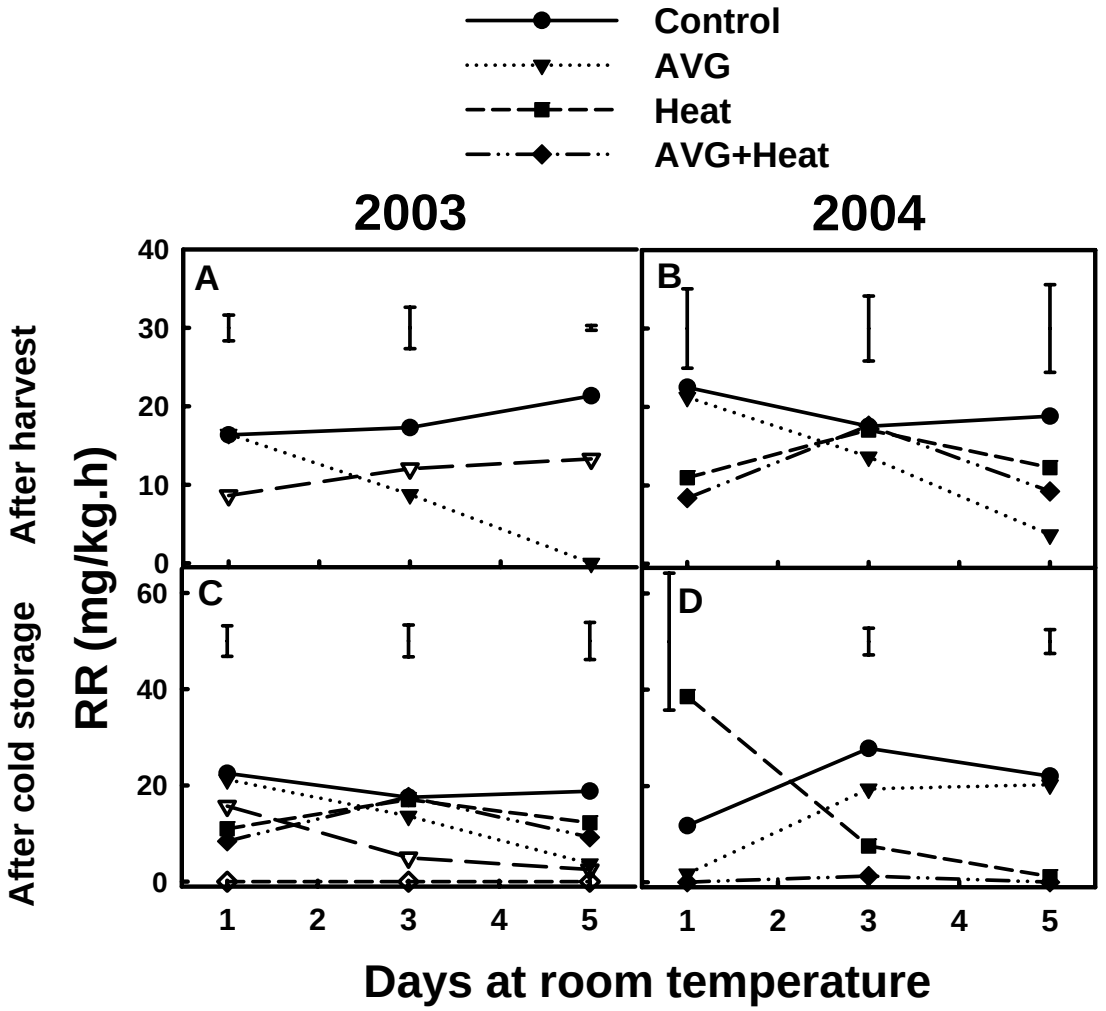


**Figure 3.6:** Effect of AVG and/or heat treatment on respiration rate (RR) of 'Redchief Delicious' (A, B) and 'Red Fuji' (C, D) ripened at room temperature for 5 days immediately after harvest or heat treatment (A, C) and after 4 weeks of cold storage at 4°C ( B, D) in 2003. AVG-treated fruit were harvested 1 or 2 weeks after the controls. Least significant differences (LSD) at *p=0.05* within date are shown as vertical bars. Note that Y axes are shown with different scales.

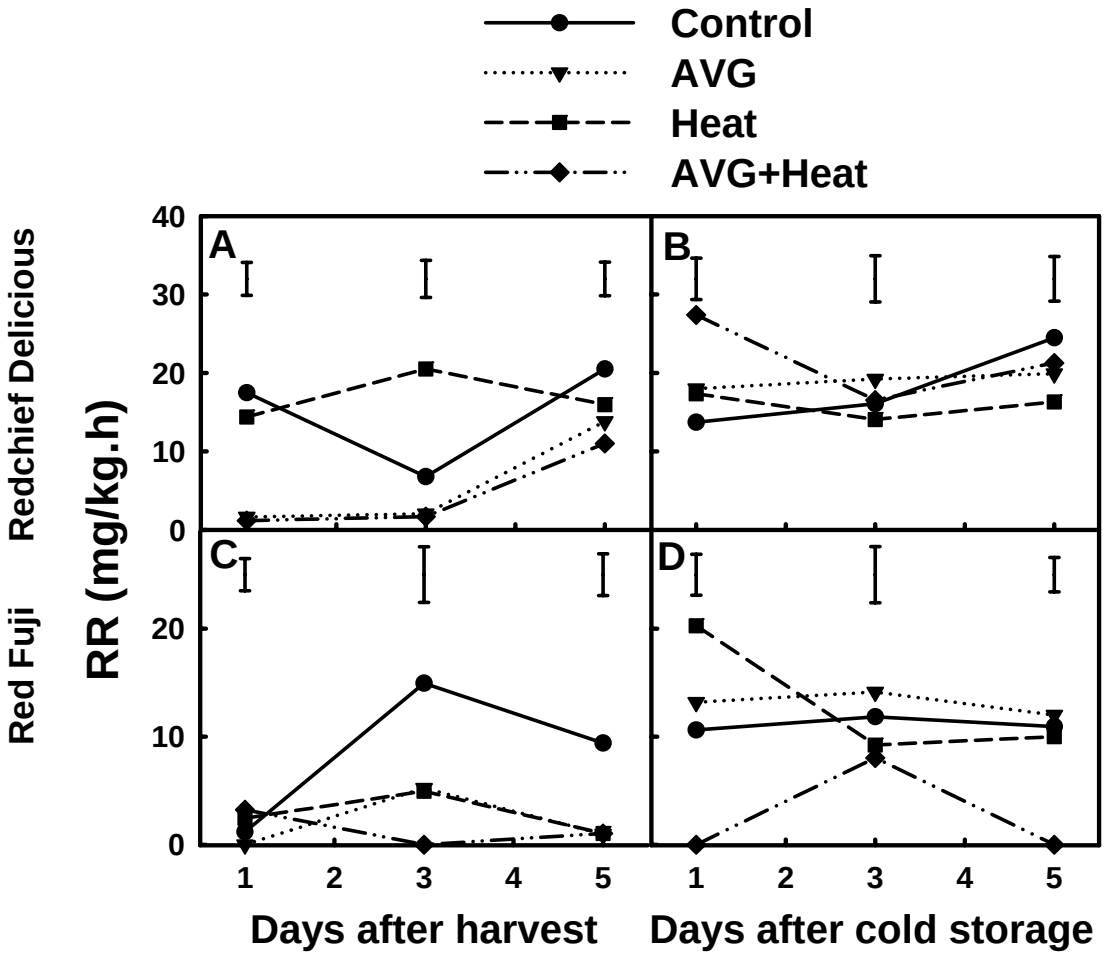


**Table 3.1:** Effect of AVG and/or heat treatment on quality traits of 'Lodi' apples. Fruit were ripened at room temperature for 1 and 5 days immediately after harvest or heat treatment and after 4 weeks in cold storage at 4°C in 2003 and 2004. SI: starch index; TA: titratable acidity; SSC: soluble solids content; F: cortex firmness, S:A: sugar to acid ratio. Different letters indicate significant differences within date separated by the least significant difference (*LSD*) at *p=0.05*; *ns:* no significant differences among means. NC: data not collected.

| After Harvest | | | | | After Cold Storage | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 Day | | | | | 1 Day | | | | |
| F *N* | SI *(%)* | TA *mg malic acid/100mL* | SSC % | S:A | F *N* | SI *(%)* | TA *mg malic acid/100mL* | SSC % | S:A |
| 50.7 *ns* | 7.4 *ns* | 974 *ns* | 10.2 *ns* | 10.8 *ns* | 23.6 *ns* | NC | 1080 a | 9.4 *ns* | 8.7 b |
| 72.3 | 6.2 | 1016 | 10.9 | 10.9 | 22.3 | | 1143 a | 9.9 | 8.7 b |
| | | | | | 21.2 | | 791 b | 10.1 | 13.0 a |
| | | | | | 28.4 | | 832 b | 9.9 | 11.9 a |
| *33.3* | *0.9* | *241* | *2.17* | *3.8* | *19.9* | | *170* | *0.8* | *2.7* |
| 1Day | | | | | 1 Day | | | | |
| 61.4 bc | 2.4 b | 797 a | 9.9 a | 13.2 b | 26.9 c | 9.0 *ns* | 770 a | 9.0 *ns* | 19.2 a |
| 75.2 a | 1.8 b | 665 ab | 9.1 b | 14.0 b | 36.1 ab | 9.0 | 587 b | 9.1 | 13.2 b |
| 52.3 c | 3.8 a | 547 b | 9.5 ab | 17.1 a | 31.3 bc | 9.0 | 558 b | 9.2 | 18.9 a |
| 72.8 ab | 3.9 a | 493 b | 9.7 ab | 21.6 a | 40.0 a | 9.0 | 516 b | 9.4 | 23.5 a |
| *12.5* | *0.8* | *197* | *0.7* | *6.9* | *6.2* | *NA* | *105* | *1.1* | *5.5* |
| 5 Days | | | | | 5 Days | | | | |
| 48.8 b | 4.2 b | 548 ab | 10.7 a | 14.4 b | 26.1 b | 9.0 *ns* | 568 a | 10.5 *ns* | 17.5 b |
| 59.5 ab | 5.7 ab | 694 a | 9.7 b | 16.7 ab | 31.3 a | 9.0 | 573 a | 9.8 | 17.4 b |
| 56.0 ab | 6.2 a | 524 bc | 10.6 a | 19.0 a | 21.5 c | 9.0 | 454 b | 9.7 | 22.0 b |
| 68.7 a | 5.9 ab | 422 c | 10.1 ab | 20.3 a | 31.9 a | 9.0 | 336 c | 10.1 | 29.1 a |
| *16.7* | *1.7* | *112* | *0.7* | *4.4* | *2.7* | *NA* | *78* | *0.9* | *7.0* |

harvested later (Table 3.2). In 2004, AVG and AVG plus heat-treated fruit were firmer than the rest 1 day after treatment, though no differences among treatments were found 4 days later. AVG plus heat reduced firmness loss of 'Senshu' after 4WCS in both seasons and heat alone reduced it in 2003. AVG, alone or combined with heat treatment, reduced firmness loss of 'Red Delicious' (Table 3.3) and heat treatment reduced it in 'Fuji' (Table 3.4). We found no differences among treatments in 'Red Delicious' and 'Fuji' apples after cold storage.

**Physical deterioration**

'Lodi' fruit exhibited bruising and cracking after cold storage (Figure 3.7). Heat treatment dramatically improved 'Lodi' apple storability by decreasing the number of cracked and bruised fruit from 72.7% to 20 % in 2003 (data not shown). In 2004, deterioration was reduced from 13.6% to 7.1% by heat, though cracking still occurred in some apples. AVG decreased deterioration to 38% in 2003, and in 2004 some AVG-treated fruit showed bruises (9.1%) but not cracking. The combination of heat and AVG showed the best results in 2003, with only 15.4% of the fruit affected, but in 2004 it was similar to heat treatment alone.

**Weight loss**

Wight loss of heated 'Lodi' during storage was close to 3 % of the initial weight, and significantly higher than that of AVG-treated fruit with a WL of 1 % (Figure 3.8). For 'Senshu' fruit, heat treatment, alone or combined with AVG, increased fresh weight loss compared to the other treatments, with values close to 3 % (heated fruit) and 1.5 % (non-heated fruit; Figure 3.8).

**Starch index**

SI of 'Lodi' fruit in 2004 was increased (i.e., starch degradation was more) by heat, alone or combined with AVG, immediately after the treatment, but 5 days later that was only true for heat treatment alone (Table 3.1). AVG reduced SI of 'Senshu' apples immediately after harvest, but there were no differences among treatments 5 days later (Table 3.2). 'Red Delicious' fruit that had been treated with AVG had a lower SI even after 5 days at room temperature (Table 3.3). All treated 'Fuji' fruit had a higher SI immediately after harvest, but 5 days later there no differences among treatments (Table 3.4). After 30 days in cold storage and 5 days at room temperature starch degradation was almost complete and none of the varieties showed any difference among treatments except for a lower SI of AVG-treated 'Senshu' fruit from H1 in 2003, heated or not.

**Table 3.2:** Effect of AVG and/or heat treatment on quality traits of 'Senshu' apples ripened at room temperature for 1 and 5 days after harvest or heat treatment and after 4 weeks of cold storage in 2003 and 2004. SI: starch index; TA: titratable acidity; SSC: soluble solids content; F: cortex firmness; S:A: sugar:acid ratio. H2: fruit harvested 2 weeks later than normal harvest. NC: data not collected. Different letters indicate significant differences within date separated by *LSD* at *p=0.05*; *ns:* no significant differences among means.

| After Harvest | | | | | After Cold Storage | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 Day | | | | | 1 Day | | | | |
| F *N* | SI *(%)* | TA *mg malic acid/100mL* | SSC % | S:A | F *N* | SI *(%)* | TA *mg malic acid/100mL* | SSC % | S:A |
| 67.8 a | 4.3 b | 560 a | 12.3 a | 22.9 bc | 60.8 c | NC | 505 a | 11.9 ns | 24.8 d |
| 64.9 ab | 3.1 b | 576 a | 10.6 c | 18.4 c | 65.2 c | | 429 a | 10.9 | 26.4 cd |
| 71.9 a | | 485a | 12.6 ab | 25.5 bc | 67.8 a | | 324 b | 12.1 | 38.2 ab |
| 72.6 a | | 495 a | 10.8 c | 22.0 c | 70.2 ab | | 333 ab | 12.0 | 37.1abc |
| 56.0 b | 7.3 a | 347 b | 10.8 c | 31.5 b | 62.8 bc | | 370 ab | 11.0 | 30.1bcd |
| 57.1 b | | 218 c | 11.3 bc | 53.3 a | 65.2 c | | 287 b | 11.9 | 41.9 a |
| *9.5* | *2.1* | *105* | *1.2* | *9.0* | *5.3* | | *123* | *1.4* | *10.9* |
| 5 Days | | | | | 5 Days | | | | |
| 60.4 b | NC | 437 ab | 12.3 a | 29.0 b | 60.8 c | 8.8 a | 485 ab | 12.1 bc | 26.6 ab |
| 71.9 a | | 445 a | 11.4 b | 25.2 b | 65.2 abc | 8.6 ab | 500 a | 11.6 c | 24.0 b |
| | | | | | 67.8 ab | 7.9 bc | 471 ab | | |
| | | | | | 70.2 a | 7.3 c | 409 ab | | |
| 61.1 b | | 338 b | 12.1 ab | 37.1 a | 62.8 c | 8.7 ab | 331 b | 12.4 ab | 38.9 a |
| 61.0 b | | | | | 65.2 abc | 8.7 ab | 344 ab | 13.0 a | 39.0 a |
| 5.3 | | *109* | *0.7* | *8.0* | *5.3* | *0.8* | *161* | *0.7* | *13.5* |
| 1 Day | | | | | 1 Day | | | | |
| 61.4 c | 6.9 a | 331 a | 10.0 *ns* | 29.6 b | *59.5 ns* | 8.3*ns* | 284 a | 10.3 *ns* | 38.9 ns |
| 68.6 ab | 4.3 b | 330 a | 9.8 | 31.0 b | 66.9 | 8.0 | 268 ab | 10.6 | 39.7 |
| 63.2 bc | 7.7 a | 265 ab | 11.0 | 41.3 ab | 61.4 | 8.7 | 208 b | 10.2 | 47.3 |
| 70.4 a | 6.7 a | 243 b | 11.1 | 48.2 a | 63.2 | 7.8 | 231 ab | 10.5 | 47.7 |
| *7.1* | *1.8* | *80* | *1.4* | *14.7* | *7.1* | *0.9* | *69* | *1.4* | *14.3* |
| 5 Days | | | | | 5 Days | | | | |
| 65.1 *ns* | 8.4 *ns* | 350 a | 11.0 a | 31.4 b | 57.9 b | 8.8 *ns* | 298 a | 10.5 *ns* | 35.1 b |
| 63.1 | 8.6 | 271 b | 9.6 b | 34.8 b | 63.1 ab | 8.8 | 251 ab | 9.9 | 41.6 ab |
| 63.3 | 8.3 | 235 b | 10.8 a | 48.3 a | 63.2 ab | 8.9 | 242 b | 10.8 | 44.6 ab |
| 63.2 | 8.2 | 228 b | 10.5 ab | 47.4 a | 65.1 a | 8.8 | 202 b | 10.1 | 52.6 a |
| *5.3* | *0.6* | *48* | *0.9* | *8.8* | *5.3* | *0.3* | *53* | *1.0* | *13.1* |

**Table 3.3:** Effect of AVG and/or heat treatment on quality traits of 'Redchief Red Delicious' apples. Fruit were ripened at room temperature for 1 and 5 days immediately after harvest or heat treatment and after 4 weeks in cold storage at 4°C in 2003. SI: starch index; TA: titratable acidity; SSC: soluble solids content; F: cortex firmness, S:A: sugar to acid ratio. Different letters indicate significant differences within date separated by the least significant difference (*LSD*) at *p=0.05*; *ns:* no significant differences among means.

| After Harvest | | | | | After Cold Storage | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 Day | | | | | 1 Day | | | | |
| F *N* | SI *(%)* | TA *mg malic acid/100mL* | SSC % | S:A | F *N* | SI *(%)* | TA *mg malic acid/100mL* | SSC % | S:A |
| 70.4*ns* | 4.8 ab | 211 *ns* | 11.6 a | 57.7ab | 68.7 *ns* | 5.2 *ns* | 234 a | 11.0 *ns* | 47.3 c |
| 75.9 | 3.5 b | 212 | 10.2 b | 49.0 b | 70.4 | 5.7 | 182 bc | 11.4 | 63.6 b |
| 74.1 | 5.2 a | 175 | 11.5 a | 66.8 a | 70.4 | 5.6 | 199 ab | 10.9 | 57.4 bc |
| 74.1 | 4.4 ab | 183 | 10.8 ab | 60.1ab | 66.9 | 5.7 | 144 c | 11.1 | 79.6 a |
| *5.7* | *1.4* | *42* | *1.0* | *14.3* | *7.1* | *0.6* | *43* | *0.7* | *12.5* |
| 5 Days | | | | | 5 Days | | | | |
| 68.6 b | 6.5 a | 171 *ns* | 11.5 ab | 69.1 ab | 68.6 *ns* | 7.6 *ns* | 240 a | 11.1*ns* | 46.6 c |
| 75.9 a | 4.3 b | 203 | 10.8 b | 54.1 b | 70.4 | 7.1 | 168 b | 11.9 | 71.5 b |
| 65.1 b | 6.0 ab | 176 | 11.8 a | 70.6 a | 70.4 | 7.1 | 134 c | 11.4 | 86.5 a |
| 74.1 a | 5.0 ab | 215 | 11.1 ab | 55.0 ab | 66.9 | 6.8 | 150 c | 10.6 | 72.2 ab |
| *5.3* | *2.0* | *54* | *0.8* | *16.4* | *5.3* | *0.9* | *41* | *1.4* | *14.7* |

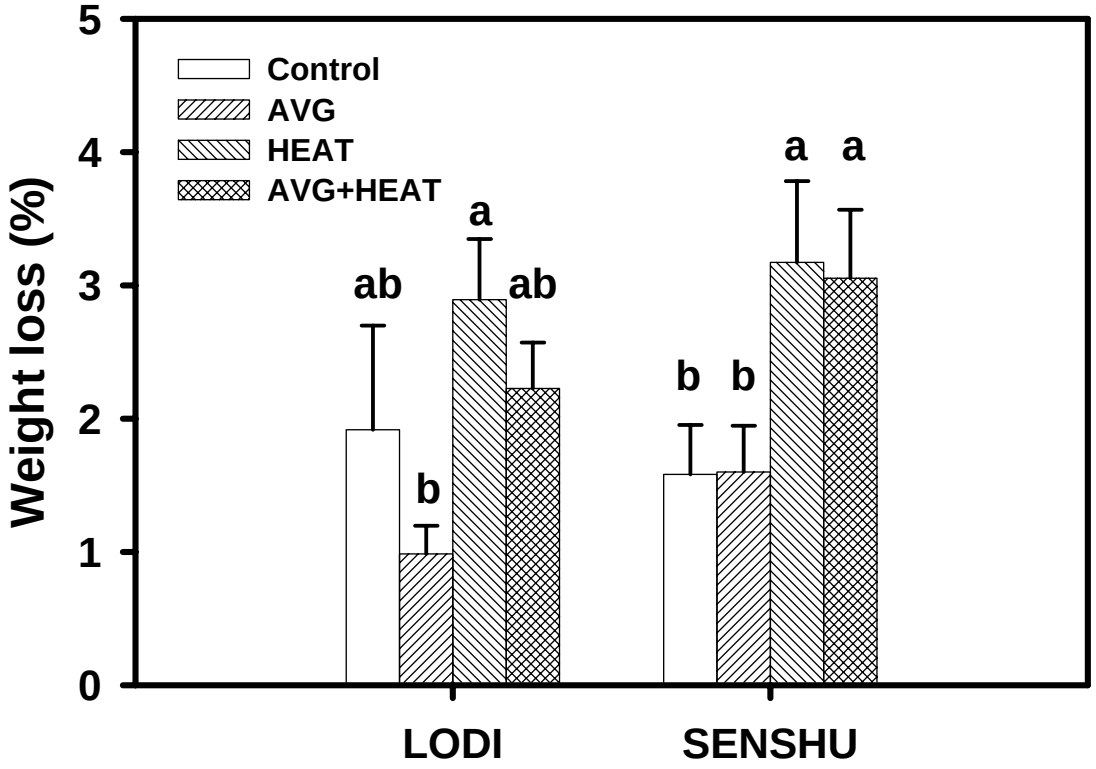
**Table 3.4:** Effect of AVG and/or heat treatment on quality traits of 'Red Fuji' apples. Fruit were ripened at room temperature for 1 and 5 days immediately after harvest or heat treatment and after 4 weeks in cold storage at 4°C in 2003. SI: starch index; TA: titratable acidity; SSC: soluble solids content; F: cortex firmness, S:A: sugar to acid ratio. Different letters indicate significant differences within date separated by the least significant difference (*LSD*) at *p=0.05*; *ns:* no significant differences among means.

| After Harvest | | | | | After Cold Storage | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 Day | | | | | 1 Day | | | | |
| F *N* | SI *(%)* | TA *mg malic acid/100mL* | SSC % | S:A | F *N* | SI *(%)* | TA *mg malic acid/100mL* | SSC % | S:A |
| 56.9 *ns* | 2.4 b | 385 ab | 12.3*ns* | 32.5 b | 70.4 a | 3.9 c | 370 ab | 13.4 ab | 37.4 b |
| 66.9 | 3.4 a | 399 a | 13.1 | 33.7 b | 68.6 a | 4.8 a | 443 a | 12.9 b | 30.8 b |
| 70.4 | 4.1 a | 332 ab | 13.1 | 39.4 ab | 63.2 b | 4.0 bc | 246 b | 13.9 ab | 39.1 b |
| 68.6 | 4.0 a | 331 b | 13.1 | 40.9 a | 65.1 b | 4.6 ab | 270 ab | 14.2 a | 54.0 a |
| *7.1* | *0.9* | *67* | *1.0* | *7.0* | *3.6* | *0.6* | *96* | *1.1* | *11.4* |
| 5 Days | | | | | 5 Days | | | | |
| 72.3 ab | 3.5 b | 466 a | 13.6 *ns* | 29.7 c | 68.6 *ns* | 5.4 *ns* | 373 a | 13.8*ns* | 37.7 b |
| 74.1 a | 3.9 ab | 470 a | 13.6 | 29.4 c | 67.1 | 5.6 | 316 a | 14.6 | 46.4 a |
| 74.0 a | 3.9 ab | 343 b | 14.0 | 43.2 b | 66.5 | 5.1 | 192 b | 12.6 | 43.9ab |
| 68.6 b | 4.8 a | 252 c | 13.3 | 53.1 a | 66.9 | 5.1 | 280 ab | 13.9 | 50.4 a |
| *5.3* | *1.0* | *76* | *1.3* | *9.3* | *3.6* | *0.6* | *61* | *2.2* | *7.8* |

**Figure 3.7:** Physical deterioration of 'Lodi' apples after retrieval from 4-week cold storage at 4°C in 2003**.** A: Control apple showing cracks and bruises. B: Detail of control apple showing cracks. C: AVG-treated apple showing bruises

**A** **B** **C**

**Figure 3.8:** Effect of AVG and/or heat treatment on weight loss of 'Lodi' and 'Senshu'. In 2004 fruit were weighed immediately before and after 4 weeks of cold storage at 4°C. Means and SE are shown. Different letters indicate significant differences separated by LSD at *p=0.05* within cultivar.



**Titratable acidity**

'Lodi' had the highest TA of all cultivars (Table 3.1), followed by 'Senshu' (Table 3.2), and 'Red Delicious' had the lowest TA (Table 3.3). After harvest, all 'Lodi' fruit that had been heated had lower TA than control or AVG treated fruit in both seasons, exept for 1 day after harvest in 2004 (Table 3.1). In 2003, TA decreased in 'Senshu' apples harvested in H2 (Table 3.2). In 2004, all treated fruit had lower acidity than control fruit 5 days after harvest. After retrieval from cold storage, heated 'Lodi' fruit, with or without AVG, had lower acidity in both seasons. TA of heated 'Senshu' fruit was lower compared to the rest of the treatments 1 and 5 days after 4WCS in both seasons.

There were no differences in TA of 'Red Delicious' fruit (Table 3.3) immediately after harvest, whereas heated 'Fuji' fruit had generaly lower TA than the non-heated ones (Table 3.4). All treatments reduced TA of 'Red Delicious' fruit 5 days after removal from storage, and the lowest acidity corresponded to heated fruit, pre-treated with AVG or not. Heat treatment alone significantly reduced the acidity of 'Fuji' fruit after cold storage, and AVG showed a tendency to lessen this effect.

**Soluble solids content**

There were few consistent effects of any treatment on fruit SSC. After harvest, AVG reduced SSC of 'Lodi' in 2004 (Table 3.1), and of 'Senshu' both years (Table 3.2). SSC of 'Red Delicious' was also reduced by AVG in 2003 (Table 3.3), but it did not affect the SSC of 'Fuji' apples (Table 3.4). After 30 days in cold storage and 5 days at room temperature there were no differences among treatments for any of the varieties studied.

**Sugar: acid ratio**

'Lodi' S:A was 3- to 5- fold lower than in the rest of the cultivars (Table 3.1), and that of 'Senshu' was also low (Table 3.2), while 'Red Delicious had the highest S:A of all (Table 3.3). One day after completion of heat treatment, heat-treated and AVG plus heat-treated 'Lodi' fruit had the highest S:A compared to the other treatments in 2004, and that difference was still evident for the combined treatment after 5 days at RT (Table 3.1). The combined treatment increased S:A of 'Senshu' apples in 2004 but not 2003. Fruit from H2 treated with both AVG and heat had the highest S:A of all, and S:A was higher in fruit treated with AVG and harvested at H2 compared to fruit from H1 (Table 3.2). After cold storage, the combined treatment

increased S:A of 'Lodi' the most. S:A of 'Senshu' was higher in AVG plus heat-treated fruit, though only in 2004 and after fruit were held for 5 days at RT.

S:A of 'Red Delicious' was reduced by AVG compared to heated apples (Table 3.3). The combined treatment increased S:A of 'Fuji' at days 1 and 5, followed by heat treatment alone at day 5 (Table 3.4). After cold storage and 5 days at RT, 'Red Delicious' fruit that had only been heated had the highest S:A, followed by AVG-treated fruit, heated or not, while control fruit had the lowest S:A. One day after 'Fuji' apples were removed from cold storage, AVG plus heat was the only treatment to increase S:A (Table 3.4), while after 5 days at RT AVG, alone or combined with heat, had higher S:A than control fruit.

## VOLATILE PRODUCTION

### Internal ethylene concentration

AVG alone was the most effective treatment in reducing IEC of 'Red Delicious' apples immediately after harvest, and heat treatment alone increased it compared to AVG-treated fruit only (Figure 3.9). Control fruit produced more ethylene than AVG-treated or heated fruit up to 3 days after retrieval from cold storage, but by the 5$^{th}$ day at room temperature there were no differences among treatments.

### Volatile production

Twelve major volatiles were detected, 11 straight and branched-chain esters and 1 alcohol. TVP of control fruit increased over time both after harvest and after retrieval from cold storage (Figure 3.10). There was no difference among treatments in TVP 1 day after harvest, while TVP of AVG and AVG plus heat treated fruit was significantly reduced after 5 days at room temperature. One day after retrieval from cold storage all treated fruit were producing less volatiles than control fruit, and by the 5$^{th}$ day at room temperature AVG-treated fruit were the only ones to recover their volatile production to levels close to, though lower than, those of control fruit. Heated fruit showed the lowest TVP.

Of the esters grouped by acid moiety (Table 3.5), 2-methylbutanoates were the most abundant, comprising 30% to 50% of the total volatiles, and had the same response to the treatments as TVP. The production of acetate esters by control and heat-treated fruit greatly increased over time after harvest. In constrast, AVG, alone or combined with heat treatment, repressed the production of acetate esters even after 5 days at room temperature. The production of acetate esters after 30 days in cold storage followed a similar pattern to TVP. Butanoate ester

**Figure 3.9:** Effect of AVG and/or heat treatment on internal ethylene concentration (IEC) of 'Redchief Delicious' apples in 2003. Fruit were ripened at room temperature for 1, 3 and 5 days immediately after harvest or heat treatment (A) and after 4 weeks of cold storage at 4°C (B). AVG-treated fruit was harvested 1 week after controls. Least significant differences (*LSD*) at *p=0.05* within date are shown as vertical bars.

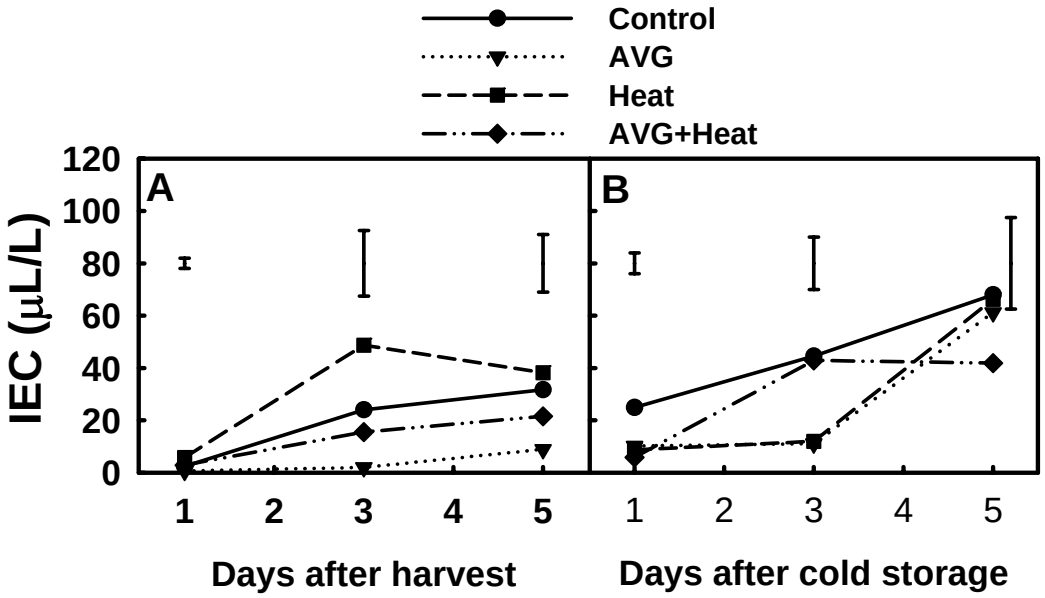


**Figure 3.10:** Effect of AVG and/or heat treatment on total volatile production (TVP) and on the activity of alcohol acyl-CoA transferase (AAT) of 'Redchief Delicious' apples in 2003. TVP was measured on samples of fruit ripened at room temperature for 1 and 5 days immediately after harvest or heat treatment (A) and after 4 weeks of cold storage at 4°C (B) that had been frozen and then thawed. More than 99 % of TVP were volatile esters. AAT activity was measured on tissue from the same samples after 5 days at room temperature only and is expressed as mU x mg protein$^{-1}$ where U, activity unit, is the increase in one unit of absorbance per minute due to the production of a yellow thiophenol product with increasing free CoA. For TVP, different letters indicate significant differences within date separated by the least significant difference (LSD) at *p=0.05*; *ns:* no significant differences among means. Means and standard errors of AAT activity are shown; there were no significant differences among treatments at harvest or after cold storage.

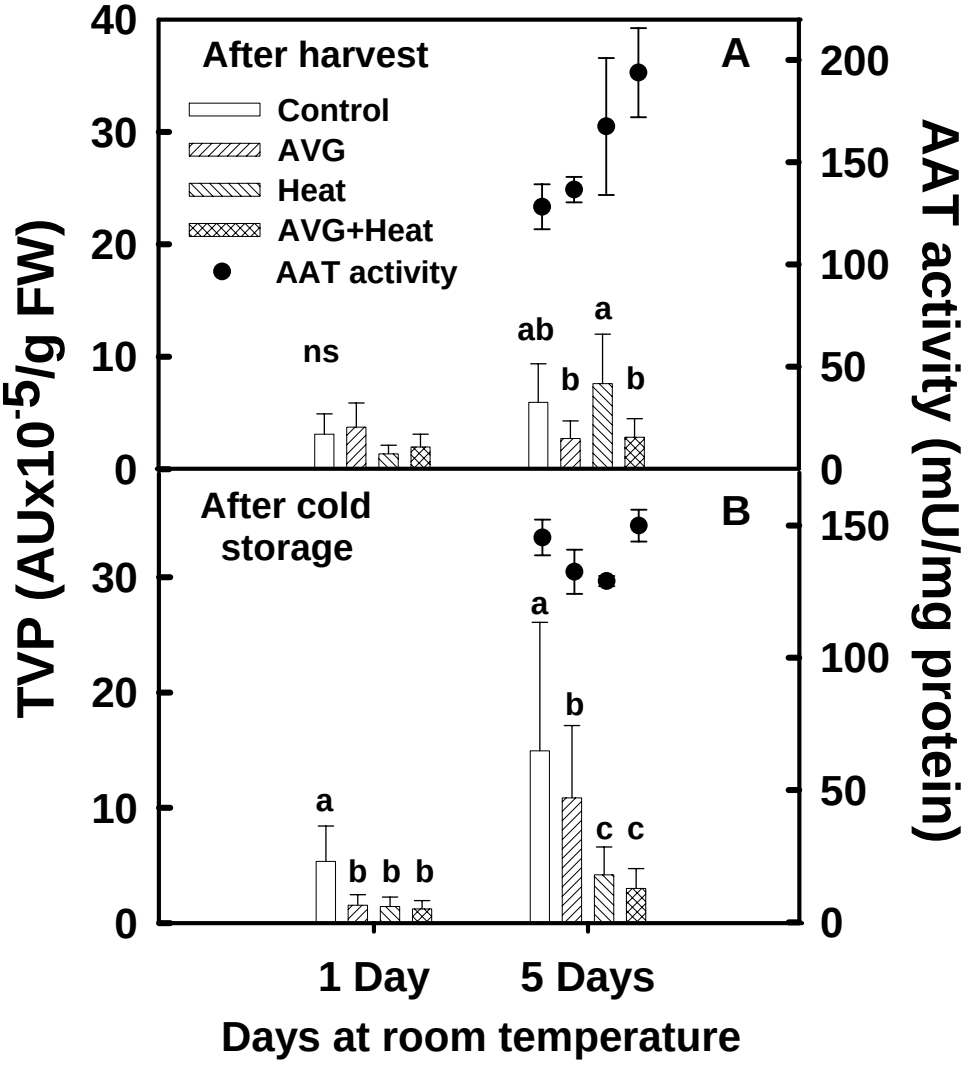


**Table 3.5:** Effect of AVG and/or heat treatment on volatile production of 'Redchief Red Delicious' apples. Fruit were ripened at room temperature for 1 and 5 days immediately after harvest or heat treatment and for 1, 3 and 5 days after 4 weeks in cold storage at 4°C in 2003. Different letters indicate significant differences within date separated by the least significant difference (*LSD*) at *p=0.05*; *ns:* no significant differences among means.

| | | AU x $10^{-3}$ g FW | | | |
|---|---|---|---|---|---|
| | | Harvest | | Cold Storage | |
| Compound | Treatment | D1 | D5 | D1 | D5 |
| **Total volatile production** | Control | 311 *ns* | 595 ab | 534 a | 1931 a |
| | AVG | 374 | 272 b | 155 b | 1 085 b |
| | Heat | 135 | 761 a | 143 b | 417 c |
| | AVG+Heat | 197 | 284 b | 123 b | 298 c |
| | *LSD* | *261* | *420* | *270* | *525* |
| **Esters grouped by acid moiety** | | | | | |
| *Acetate esters* | Control | 80 *ns* | 174 a | 102 a | 370 a |
| | AVG | 93 | 73 ab | 23 b | 173 b |
| | Heat | 13 | 148 ab | 11 b | 33 b |
| | AVG+Heat | 10 | 40 b | 6 b | 51 b |
| | *LSD* | *92* | *119* | *34* | *154* |
| *Butanoate esters* | Control | 75 *ns* | 125 ab | 77 *ns* | 157 a |
| | AVG | 62 | 47 c | 23 | 65 b |
| | Heat | 59 | 190 a | 45 | 63 b |
| | AVG+Heat | 85 | 79 bc | 32 | 66 b |
| | *LSD* | *42* | *69* | *54* | *70* |
| *2-Methylbutanoate esters* | Control | 139 ab | 203 ab | 168 a | 937 a |
| | AVG | 178 a | 109 b | 66 b | 479 b |
| | Heat | 60 b | 255 a | 61 b | 172 c |
| | AVG+Heat | 99 ab | 85 b | 56 b | 88 c |
| | *LSD* | *93* | *137* | *77* | *307* |
| *Hexanoate esters* | Control | 10 *ns* | 78 ab | 182 a | 429 a |
| | AVG | 35 | 36 b | 40 b | 339 b |
| | Heat | 2 | 152 a | 23 b | 123 c |
| | AVG+Heat | 1 | 75 ab | 27 b | 85 c |
| | *LSD* | *45* | *114* | *136* | *82* |
| **Individual volatiles** | | | | | |
| *Ethyl butanoate* | Control | 74 *ns* | 122 ab | 68 *ns* | 144 a |
| | AVG | 61 | 46 c | 21 | 59 b |
| | Heat | 59 | 187 a | 44 | 61 b |
| | AVG+Heat | 85 | 78 ab | 32 | 64 b |
| | *LSD* | *41* | *67* | *52* | *68* |
| *Butyl acetate* | Control | 2 *ns* | 6 a | 11 a | 30 a |
| | AVG | 3 | 4 ab | 4 b | 7 b |
| | Heat | 0 | 7 a | 2 b | 1 b |
| | AVG+Heat | 0 | 2 b | 1 b | 2 b |
| | *LSD* | *3* | *4* | *5* | *13* |
| *Ethyl-2-methylbutanoate* | Control | 103 a | 136 a | 105 a | 101 a |
| | AVG | 131 a | 69 bc | 52 b | 71 ab |
| | Heat | 59 b | 122 ab | 42 b | 36 b |
| | AVG+Heat | 99 ab | 57 c | 52 b | 43 b |
| | *LSD* | *40* | *56* | *48* | *58* |

Table 3.5 (continued)

| Compound | Treatment | AU x $10^{-3}$ g FW | | | |
|---|---|---|---|---|---|
| | | Harvest | | Cold Storage | |
| | | D1 | D5 | D1 | D5 |
| *Hexanol* | Control | 1.0 *ns* | 2.2 ab | 3.4 a | 7.1 a |
| | AVG | 1.2 | 1.5 b | 1.4 b | 4.1 b |
| | Heat | 0.4 | 3.6 a | 0.8 b | 2.7 bc |
| | AVG+Heat | 1.3 | 1.5 b | 0.7 b | 2.1 c |
| | *LSD* | *1.0* | *1.4* | *1.2* | *1.7* |
| *2-Methylbutyl acetate* | Control | 71 *ns* | 143 a | 54 a | 232 a |
| | AVG | 78 | 53 ab | 7 b | 130 b |
| | Heat | 11 | 103 ab | 7 b | 27 c |
| | AVG+Heat | 8 | 23 b | 1 b | 37 bc |
| | *LSD* | *75* | *100* | *21* | *97* |
| *Hexyl acetate* | Control | 7 ab | 25 ab | 37 a | 108 a |
| | AVG | 12 a | 16 b | 12 b | 35 b |
| | Heat | 1 b | 38 a | 4 b | 6 b |
| | AVG+Heat | 3 b | 15 b | 6 b | 12 b |
| | *LSD* | *7* | *19* | *14* | *54* |
| *Hexyl propionate* | Control | 6.1 *ns* | 12 *ns* | 2 *ns* | 32 a |
| | AVG | 4.8 | 6 | 3 | 26 a |
| | Heat | 1.7 | 12 | 3 | 24 ab |
| | AVG+Heat | 1.0 | 3 | 2 | 8 b |
| | *LSD* | *5.1* | *14* | *2* | *17* |
| *Butyl hexanoate* | Control | 6 *ns* | 38 *ns* | 81 a | 230 a |
| | AVG | 14 | 20 | 19 b | 156 b |
| | Heat | 2 | 65 | 8 b | 55 c |
| | AVG+Heat | 1 | 34 | 8 b | 40 c |
| | *LSD* | *18* | *50* | *58* | *65* |
| *Hexyl-2-methylbutanoate* | Control | 34 *ns* | 61 *ns* | 56 a | 77 a |
| | AVG | 44 | 37 | 12 b | 373 b |
| | Heat | 1 | 121 | 18 b | 125 bc |
| | AVG+Heat | 1 | 26 | 4 b | 40 c |
| | *LSD* | *68* | *107* | *37* | *65* |
| *Hexyl hexanoate* | Control | 5 *ns* | 40 ab | 101 a | 199 a |
| | AVG | 21 | 17 b | 21 b | 183 a |
| | Heat | 1 | 87 a | 15 b | 68 b |
| | AVG+Heat | 1 | 41 ab | 19 b | 45 b |
| | *LSD* | *25* | *65* | *79* | *42* |
| *Butyl butanoate* | Control | 0.5 *ns* | 2.3 *ns* | 9 a | 12 a |
| | AVG | 1.1 | 1.2 | 3 b | 7 b |
| | Heat | 0.1 | 2.7 | 1 b | 8 c |
| | AVG+Heat | ND | 1.3 | 1 b | 1 c |
| | *LSD* | *1.4* | *2.3* | *6* | *3* |
| *Butyl-2-methylbutanoate* | Control | 2 ns | 7 ab | 7 a | 64 a |
| | AVG | 3 | 4 b | 2 b | 35 b |
| | Heat | ND | 12 a | 1 b | 11 c |
| | AVG+Heat | ND | 3 b | 1 b | 5 c |
| | *LSD* | *4* | *8* | *4* | *21* |

production had the same trends as TVP both after harvest and retrieval from cold storage, with heated fruit producing high amounts 5 days after the treatment. The production of hexanoate esters showed a similar pattern to that of TVP, though it was not as affected by the combined treatment any time after harvest. The repression in acetate ester production by the treatments was more evident after 4WCS than afer harvest, especially for heat and AVG plus heat.

Immediately after harvest, the most abundant ester produced by control fruit was ethyl-2-methylbutanoate at 33.1% of TVP. Ethyl butanoate and 2-methylbutyl acetate followed, comprising 23.9% and 22.8% of TVP, respectively. Butyl acetate and hexyl acetate were only 0.6% and 2.3% of TVP. By the 5$^{th}$ day at room temperature, the most abundant ester was 2-methylbutyl acetate, 24% of TVP. Immediately after cold storage, the most abundant ester was ethyl-2-methylbutanoate at close to 19.6% of TVP, followed by hexyl hexanoate and butyl hexanoate with production levels 18.8% and 15.1% of TVP. Four days later, the most conspicuous volatile was hexyl-2-methylbutanoate with a production of 40% of the total volatiles. 2-Methylbutyl acetate and hexyl-2-methylbutanoate were still present in high concentrations, 12.0% and 11.9% of TVP, respectively. Volatile alcohol production was insignificant compared to that of esters (less than 1% of TVP).

**AAT activity**

There were no significant differences among treatments in AAT activity (Figure 3.10). Heated fruit had lower protein content than control fruit 5 days after harvest with values of 315 $\pm$ 12.1 versus 362 $\pm$ 3.2 mg protein/ g FW, respectively, different at $p = 0.10$.

# DISCUSSION

### FRUIT QUALITY AND CULTIVAR RESPONSES

**Headspace ethylene production**

Ripening in apples occurs when the sensitivity of the fruit to $C_2H_4$ reaches a threshold at which autocatalytic $C_2H_4$ production is initiated (Harkett et al., 1971). Some AVG-treated fruit were harvested with the controls when these were at the beginning of or during the climacteric ethylene rise, and some were harvested 1 to 2 weeks later. HEP of control fruit increased over time in all varieties, both at harvest and after 4 weeks in cold storage (Figure 3.1 - 3.3). AVG has

a strong affinity toward ACS (Capitani et al., 2002); it irreversibly binds to this enzyme inhibiting its activity, delaying ripening (Boller et al., 1979). AVG repressed HEP in all cultivars immediately after harvest and also after cold storage, with the exception of 'Lodi'. 'Lodi' was the cultivar with the highest HEP and had a variable response to AVG. Autio and Bramlage (1982) worked with several cultivars and found that 'McIntosh', the earliest cultivar with highest intrinsic ethylene production, responded less to the application of AVG than the others. These authors suggested that early cultivars may have greater ACS activity and therefore might need greater concentrations of AVG to delay ripening. However, another early cultivar, 'Senshu', in this study exhibited reduced HEP both after harvest and after cold storage in response to AVG. 'Senshu' fruit produced considerably less HEP than 'Lodi' (Figure 3.2). Thus, it seems more likely that the effect of AVG on $C_2H_4$ production of different cultivars may be linked to the levels of $C_2H_4$ production, but this may not necessarily correlate with harvest date. In agreement with this, Byers (1997), after working with cultivars of different harvest dates, suggested that there might not be early vs. late cultivar responses.

When two harvest dates for the same variety, 'Senshu', were compared, AVG was more effective in reducing HEP for the earlier harvest (Figure 3.2), results similar to those of Greene and Schupp (2004) and to our results with 'Royal Gala' (Chapter 2). Additionally, fruit from 'Red Delicious' and 'Fuji' that had been treated with AVG but were harvested later than their controls showed reduced $C_2H_4$ production immediately after harvest, but this effect was less important after 4 weeks in cold storage (Figure 3.3). Johnson and Colgan (2003) reported that AVG applied to 'Cox's Orange Pippin' apples caused a delay in ripening close to 13 days. Thus, it is possible that in the later-harvested AVG-treated fruit the ongoing preclimacteric ripening process would have partially overcome the effect of this compound.

Heat can repress ethylene production during the treatment, but production levels may recover if fruit are later kept at room temperature (Klein and Lurie, 1990; Lurie and Klein, 1992a). ACO is more sensitive to heat than ACS (Klein, 1989; Atta Aly, 1992), and increasing levels of ACO mRNA and protein have been seen during recovery from heat treatment (Lurie et al., 1996a). In our study, heat treatment alone was less effective than AVG alone in maintaining a low ethylene production both after harvest and after retrieval from cold storage. Heat even stimulated HEP of 'Lodi' (Figure 3.1). These results might be due to the reactivation of the synthesis of ACO to levels close to or even higher than before the treatment. The synthesis of

ACO may have been slowed down at the lower temperatures of cold storage, but to avoid membrane deterioration fruit sat at room temperature for 3 to 4 hrs after heat treatment before placing them in cold storage. ACO synthesis may have been reactivated, thus stimulating ethylene synthesis and resulting in the high levels of ethylene production seen after the fruit was retrieved from cold storage.

The combination of AVG with heat treatment reduced ethylene production the most, and was the most consistent treatment across varieties and sampling times, even when AVG-treated fruit was harvested later than their controls (Figure 3.1 - 3.3). There might be an additive effect between both treatments. ACS is less sensitive to heat than ACO, but protein levels can still decrease after a heat treatment (Klein, 1989; Atta-Aly, 1992). Maybe the internal concentration of AVG was high enough to bind to all the ACS that was left and/or that was synthesized *'de novo'* after the heat treatment, preventing or delaying the rise in ethylene production.

**Respiration rate**

RR should decrease with lower ethylene production (Bangerth et al., 1998; Song and Bangerth, 1996), induced, for example, by a preharvest application of AVG (Bangerth, 1978). Heat can also repress respiration during the treatment (Porrit and Lidster, 1978), though Saftner et al. (2002) measured RR levels of previously-heated fruit similar to or higher than those of control apples. There was no clear pattern of RR in response to the treatments in the present study, though the combined treatment reduced RR of 'Senshu', 'Red Delicious' and 'Fuji' the most, at least immediately after harvest (Figure 3.5, 3.6). The combined treatment was also the only one to reduce RR of 'Lodi' after the fruit had been at 4 $^{o}$C for 4 weeks (Figure 3.4). Several factors can influence RR; for example, low internal ethylene concentrations would fail to stimulate it (Bangerth, 1978). Even though fruit treated with AVG plus heat had, in general, the lowest ethylene production, this effect was not as evident with RR. An increase in metabolic activity linked to a reactivation in the synthesis of proteins, lipids and other components of cell membranes after a heat treatment might increase RR, attenuating the effects of low ethylene levels.

**Firmness**

AVG-treated fruit are generally more firm than non-treated ones immediately after harvest and after cold storage (Bangerth, 1978; Layne et al., 2002; Wang and Dilley, 2001), though there have also been variable effects of AVG depending on cultivar and type of storage

(Autio and Bramlage, 1982; Bramlage et al., 1980). In this study, firmness of AVG-treated fruit was equal to or greater than that of control fruit. 'Senshu' fruit harvested on H2 showed no AVG effect (Table 3.2), results similar to those of Greene (2004). AVG-treated fruit from the later harvest had lower initial firmness than that of control fruit harvested 2 weeks earlier. However, firmness loss during storage was more obvious for control than AVG-treated fruit. These results are similar to those of Johnson and Colgan (2003) who concluded that the firmness benefits associated with AVG were not entirely due to their less mature and firmer status at the time of harvest. Autio and Bramlage (1982) found that the effectiveness of AVG in reducing firmness loss during storage of 'McIntosh' and 'Red Delicious' was reduced when storage temperature was higher (3.3$^{o}$C vs. 0$^{o}$C). The lack of response of 'Red Delicious' (Table 3.3) and of 'Fuji' (Table 3.4) to AVG during storage could be due the storage temperature used here (4$^{o}$C), and/or to the delayed harvest of the treated fruit or both. Based on consumer acceptability, Kupferman and Harker (2001) established a minimum firmness of 49 N for marketing 'Gala' apples grown in Washington State. Even though AVG-treated 'Lodi' fruit was firmer than non-treated fruit, at least in 2004, this effect may not be enough to maintain firmness at marketable levels after storage (Table 3.1). In 2004, AVG-treated 'Lodi' apples had higher HEP than in 2003, with levels similar to control fruit but lower firmness loss even during storage. These results are opposite to what was expected, and are probably related to the less mature stage of the fruit in 2004, given that in that year starch degradation at harvest was much lower (Table 3.1). A summary of treatment effects on several quality traits of the studied cultivars is shown in Table A2 in the Appendix.

Heat treatment has been found to decrease firmness loss in the past. This could be due to a decrease in either synthesis or activity of cell wall degrading enzymes (Klein and Lurie, 1992; Lurie and Nussinovitch, 1996), as heated apples may retain more insoluble pectin during ripening (Ben Shalom et al., 1993; Porrit and Lidster, 1978). However, heat has also shown no effect on this trait (Saftner et al., 2002). Tu and De Baerdemaeker (1997) found that heat treatments had differing effects on firmness among different apple varieties. They also considered, based on nondestructive firmness measurements, that heating apples at 38$^{o}$C for 4 days was a good time-temperature combination treatment for maintaining apple firmness. 'Anna' is an apple that produces high amounts of ethylene, stores very poorly and rapidly loses firmness both at room temperature and in cold storage; 'Anna' apples that received a pre-storage heat

treatment were firmer than controls after 6 weeks in cold storage (Klein and Lurie, 1992). 'Lodi' and 'Anna' share similar characteristics, but in the present experiment heat alone did not greatly reduce firmness loss in 'Lodi', though heated 'Senshu' apples tended to be firmer than controls after cold storage (Table 3.2). For 'Lodi' and 'Senshu' the combined treatment was more effective than heat treatment alone in reducing firmness loss, with results similar to or better than AVG alone. These results suggest that there could be a mild additive effect of both treatments. Heat can not only attenuate ethylene synthesis and therefore have similar effects to AVG, but it can affect protein synthesis and catabolic enzyme activity independently of $C_2H_4$ (Klein, 1989; Lurie and Klein, 1990). One possible mode of action of heat to maintain firmness would be through the activation of pectin methylesterase, which could then demethylate pectic substrates that would allow cross-linking of the freed carboxylic groups by internal calcium ions (Kim et al., 1994), though Lurie (1998a) suggested that loss of neutral sugar side chains during the heat treatment (Ben Shalom et al., 1993) may lead to closer packing of the pectin strands and obstruct enzymic cleavage during and after storage.

**Physical deterioration**

Early season apple cultivars tend to ripen very rapidly, have a short postharvest life (Autio and Bramlage, 1982), and maintain quality poorly in cold storage compared to later season cultivars. 'Anna', an early summer cultivar like 'Lodi', is more sensitive to chilling injury in cold storage than other cultivars (Klein and Lurie, 1992). Summer apples have fewer and larger cells compared to late season cultivars (Fallik, personal communication); they also have more intercellular space which would make them more vulnerable to bruises caused by the normal handling of the fruit. Also, given the not-so-cohesive nature of the peel, this tissue would be more prone to break during storage with changes in temperature and relative humidity that might not affect other later season cultivars. The higher percentage of deteriorated fruit in 2003 (Figure 3.7) could be due to the lower firmness and a more mature stage at harvest, which would make the fruit even more sensitive to bruising during harvest and handling. The large reduction in physical deterioration by heat treatment (Figure 3.5) is in agreement with experiments by Lurie et al. (1996b), who noted a reduction in the number of cracks of apple peel related to changes in cuticle structure. AVG-treated fruit could have been less ripe than control fruit and, therefore, less susceptible to handling deterioration. For any treatment, there were more fruit with both cracks and bruises than with any individual deterioration. This might indicate that fruit

that had been bruised during handling could have deteriorationd tissue at the beginning of cold storage, making it more susceptible to cracking.

**Weight loss**

Weight loss was increased the most by heat treatment alone in 'Lodi' and 'Senshu' (Figure 3.8), in general agreement with results published by Klein and Lurie (1990). The greatest firmness loss in heated apples might have occurred during the heating process (Lurie et al., 1996b). Although this seems to be a negative side-effect of heat treatment, a taste panel gave heated fruit higher sensory evaluation scores than control fruit (Lurie and Nussinovitch, 1996). Hatfield and Knee (1988) conducted a sensory evaluation of fruit under controlled weight loss, which consisted of a 5 % weight loss during 20-30 days in CA storage and in the presence of a desiccant (anhydrous calcium chloride), followed by CA storage with no desiccant for up to 6 months. The fruit with higher weight loss were rated as firmer, tougher and less mealy than control apples. However, Tu and De Baerdemaeker (1997) suggested that an increase in weight loss would lead to a decrease in expressible juice content. In the current study heated fruit seemed to be mealier than control and AVG-treated fruit, and produced less juice when squeezed for SSC measurements. Conversely, fruit treated with AVG alone were juicer than any other fruit after 4 weeks in cold storage, which together with their higher firmness may have a positive impact on consumer acceptability.

Heated 'Lodi' fruit had the highest weight loss and a low percentage of cracks, in agreement with previous studies using 'Golden Delicious' apples (Roy et al., 1994; Lurie et al., 1996b). Lurie et al. (1996b) found lower wax content, higher calcium content and a re-distribution of the remaining wax that filled cracks, evident at low magnification microscopy, in peel of heated apples. They suggested that water loss increased water movement towards the fruit surface and mobilized ions to the skin, including calcium which prevented the development of physiological disorders during storage. The lower number of cracks in heated fruit in the present study may be related to higher calcium content in the skin wax, and to the filling of the cracks with wax during heat treatment, which would repress the further and deeper development of cracks.

**Starch index**

Starch index is used to assess the maturity of the fruit, especially at harvest. The levels of maturity at harvest varied among cultivars and years (Table 3.1-3.4), but all harvested fruit were suitable for short-term regular atmosphere storage. The difference in the rate of starch degradation as a response to the treatments varied among cultivars and was evident only immediately after harvest or heat treatment. In general, AVG-treated fruit had lower starch degradation at the moment of harvest even at delayed harvests, as would be expected since AVG slows down ripening (Bangerth, 1978; Byers, 1997; Johnson and Colgan, 2003), though this response was cultivar-dependent. Silverman et al. (2004) observed lower starch degradation immediately after harvest in 'Red Delicious' apples treated with AVG compared to controls, though they did not find differences in the activity of starch phosphorylase, the only starch degrading enzyme that increased during the last weeks of apple ripening. How ethylene regulates starch degradation remains to be unravelled. Heat treatment stimulated starch degradation in all cultivars immediately after the treatment. The higher starch degradation might be related to an induced increment in metabolic activity that would also include the conversion of starch into soluble sugars. All fruit ripened at room temperature for several days or placed in cold storage lost most of, if not all, their starch. It was unexpected that in 2003 AVG-treated 'Lodi' apples had almost no ethylene production after harvest, a general indicator of less ripe fruit, but yet starch degradation was not different from control fruit.

**Titratable acidity**

The acidity of apples depends on the concentration of organic acids. Malic acid, the most conspicuous acid in apple fruit, is an important substrate for respiration and may fall by 50% during the life of a fruit (Knee, 1993). AVG did not have an important effect on TA, in agreement with findings by Autio and Bramlage (1982) and Silveran et al. (2004). Heat treatment greatly reduced the acid content of 'Lodi', 'Senshu' and 'Fuji' apples with or without AVG when fruit were ripened at room temperature for 5 days immediately after the treatment (Table 3.1, 3.2, 3.4). After 4 weeks in cold storage the effect of heat treatment on TA was also evident in 'Red Delicious' (Table 3.3). These results are comparable to those of Klein and Lurie (1990). The combined treatment had effects similar to those of heat alone, though sometimes AVG seemed to accentuate the reduction in TA. The mild negative effect of AVG on TA when combined with heat treatment is difficult to explain, given that a reduction in TA loss by AVG

would be a more expected outcome (Bangerth, 1978). Acid content of 'Senshu' apples harvested on H2 was lower compared to H1 (Table 2.2). It could be that more organic acids were utilized during the longer on-tree maturation of fruit harvested later. Even with some acidity loss during storage, this effect was independent of the treatments, results similar to those of Porrit and Lidster (1978). Fruit stored at 4$^{o}$C continued to ripen and probably had an active metabolism that may have caused a partial depletion of organic acids. After cold storage fruit had lower TA and similar or higher RR than immediately after harvest. This would suggest that the concentration of organic acids available for respiration, even if reduced during cold storage, was not a limiting factor for the respiratory activity of the fruit.

**Soluble solids content**

There have been variable effects of AVG on SSC of apples, including no response (Autio and Bramlage, 1982), a decrease (Bangerth, 1978; Layne et al., 2002), an increase (Greene, 2004; Wang and Dilley, 2001) and inconsistent results (Johnson and Colgan, 2003; Schupp and Greene, 2004), suggesting that this is not an ethylene-dependent trait. Accordingly, in this study, SSC was not affected by most treatments (Table 3.1-3.4). Only at harvest did AVG show a negative effect on this variable in all cultivars when treated fruit were harvested with controls and in all cultivars but 'Fuji' when treated fruit were harvested later. Heat treatment has also produced inconsistent results in other studies (Klein and Lurie, 1992; Porrit and Lidster, 1978; Tu and De Baerdemaeker, 1997). Therefore, it is not surprising that we could not detect a clear change in SSC in fruit treated with heat or AVG plus heat.

**Sugar: acid ratio**

Apples with higher sugar:acid ratio were described as sweeter and less tart by taste panelists (Lurie and Nussinovitch, 1996). The sugar:acid ratio increased with heat due to the decrease in TA and little change in SSC, and this could be translated to greater acceptability by consumers.

Differences in TA among cultivars led to major differences in S:A (Table 3.1-3.4). For example, 'Lodi' had the highest TA and the lowest S:A of all cultivars, while differences in SSC could not explain the variability in S:A. The combined treatment increased S:A in all cultivars, especially after cold storage, though the reasons seemed to differ among cultivars. S:A of 'Lodi' increased due to a decrease in TA when fruit were heated, an effect that was not lessened by the application of AVG (Table 3.1). S:A of 'Senshu' was more affected by a delayed harvest than by

any treatment, which caused an increase in SSC and a decrease in TA (Table 3.2). Changes in S:A of 'Red Delicious' and 'Fuji' may be the result of both treatments and of a delayed harvest, rather than of the AVG treatment alone, based on the comparison between H1 and H2 AVG-treated 'Senshu' fruit. For 'Red Delicious', treatments may have been a major factor influencing S:A (Table 3.3), where AVG reduced SSC and heat treatment increased it while reducing TA. After cold storage both the delayed harvest of AVG-treated fruit and heat treatment negatively affected TA resulting in higher S:A ratio. Heat treatment and probably the delayed harvest of AVG-treated apples additively affected TA in 'Fuji', so S:A of AVG plus heat-treated apples was the highest.

### VOLATILE PRODUCTION

#### Internal ethylene concentration

The lower IEC of AVG-treated 'Red Delicious' apples immediately after harvest (Figure 3.9) was expected based on previous work (Bramlage et al., 1980; Fan et al., 1998; Greene and Schupp, 2004; Silverman et al., 2004). The data also support the idea that the effectiveness of AVG can be reduced by low temperature storage (Bramlage et al., 1980) or that the effect of AVG is lost over time, given that the IEC of AVG-treated and non-treated fruit were very similar 5 days after retrieval from cold storage. This increase in IEC by AVG-treated fruit suggests an ongoing ripening process during cold storage. There was no effect of heat or heat plus AVG on IEC.

#### Volatile production

As expected from previous studies (Defilippi et al., 2005b; Dixon and Hewett, 2000; Echeverria et al. 2004a), most of the volatiles detected were esters (Table 3.5). Of the three esters that typically contribute to apple-like aroma, only 2-methylbutyl acetate was present in large amounts. However, ethyl-2-methylbutanoate (E2MB), one of the most abundant esters after harvest and storage, is also considered a main player in the aroma of apples (Flath et al., 1967; Paillard, 1990). Factors that affect the contribution of individual volatiles to aroma are compound concentration, presence of other compounds and odor threshold (Flath et al., 1967; Buttery, 1993). In studies conducted by Flath et al. (1967), E2MB had the lowest odor threshold, but butyl acetate, 2-methylbutyl acetate and hexyl acetate had very low odor thresholds as well. Therefore, it is possible that these compounds could have contributed to the overall aroma of the fruit, even though some of them were present in lower amounts.

Reductions in volatile production due to AVG or heat treatment immediately after harvest or after short-term cold storage have been reported (Fallik et al., 1997; Fan et al., 1998; Mir et al., 1999; Saftner et al., 2002). In the present study, TVP was reduced by AVG and AVG plus heat 5 days after harvest (Figure 3.10). TVP of AVG-treated fruit recovered over time after fruit had been taken out of cold storage and ripened at ambient temperature. By the 5$^{th}$ day, TVP was 75% of control fruit levels. Continuous ethylene production is required for a high rate of ester synthesis (Fan et al., 1998; Fan and Mattheis, 1999; Defilippi et al., 2005b). Thus, it is possible that the increase in IEC of AVG-treated stored fruit triggered this recovery in ester production. Heat treatment did not affect fruit TVP immediately after harvest, though it reduced it considerably after storage. These results are different from those of Fallik et al. (1997), where TVP was inhibited by heat treatment immediately after but recovered with time in cold storage. However, differences may have been the result of cultivar variation since they worked with 'Golden Delicious' apples, or of fruit maturity stage at the time heat treatment was applied, since they stored the fruit for 5 to 6 weeks at 1 $^{o}$C before heat treatment. Maturity indexes such as SI and firmness were not recorded by Fallik et al., thus it is not possible to thoroughly compare both studies.

Given that heated fruit had IEC values similar to those of AVG-treated apples, the inhibition of volatile production by heat seems to be an ethylene-independent process. Substrate availability seems to be the main limiting factor for ester synthesis (Argenta et al., 2004; Berger and Drawert, 1984; De Pooter et al., 1981, 1983; Echeverria et al, 2004a). Heated fruit had lower amounts of hexanol, a substrate for the synthesis of some straight-chain esters, compared to control and AVG-treated fruit. Heat treatment may have affected metabolic activity in a way that would reduce the concentration of alcohols and other substrates for ester synthesis. The synthesis of aliphatic and branched-chain esters was also repressed, indicating that several pathways may have been affected including those related to amino acids and fatty acids (Sanz et al., 1997). Low levels of ATP and NADPH resulting from low respiration rates could be a limiting factor for substrate availability and, therefore, for volatile synthesis (Bangerth et al, 1998). In our study, RR after cold storage was not affected by heat treatment (data not shown), so this depletion of metabolites does not seem to be the factor limiting ester synthesis.

Contrary to expectations, AVG and heat treatment did not have an additive or synergistic negative effect on volatile production. After harvest, fruit that received the combined treatment

behaved similarly to those receiving AVG alone, whereas after cold storage the combined treatment had an effect similar to that of heat alone. AVG had the most negative impact on volatile production after harvest, while heat showed this same effect after storage. Thus, it seems that fruit that received both treatments responded in the same way as fruit with the single treatment that caused the most negative effect, while neither treatment was clearly additive or synergistic with the other.

**AAT activity**

AAT activity can be reduced when ethylene production is impaired (Defilippi et al., 2005a, 2005b). There is no reported information about the effect of heat treatment on the activity of AAT. The activity of AAT was not significantly affected by any treatment (Figure 3.10), and therefore no correlation between AAT activity and ester production was found, in agreement with findings by Defilippi et al. (2005b). Lower protein content was detected in heated versus control fruit kept at RT for 5 days after harvest, though the difference was significant at $p=0.10$, not at $p=0.05$. Heat treatment denatures proteins, and it is possible that protein synthesis could not completely re-establish the levels of protein that were present before the heat treatment. After cold storage there were no differences in AAT activity, and these results further support the idea that volatile production is not limited by enzyme activity but by substrate availability.

## CONCLUSIONS

The effect of AVG plus heat treatment on the storability of different apple cultivars was studied. The combined treatment was generally more effective than single treatments in reducing ethylene production, respiration rate, firmness loss and physical deterioration during storage. However, the combined treatment had the same effect on other traits as the single treatments, like titratable acidity, volatile production, starch degradation, and weight loss in storage. The effect of AVG plus heat on fruit quality after harvest and short-term RA storage was cultivar-dependent and was reduced as fruit was harvested at a more ripe stage. AVG plus heat treatment had a positive effect on early-harvested cultivars with low storability, though it was not enough to maintain marketable fruit quality of 'Lodi', the cultivar with the poorest storability. Later harvested cultivars did not show enhanced storability with heat treatment when AVG-treated fruit was harvested two weeks later than their controls, though it increased sugar:acid ratio in an

additive way with heat treatment. However, assessments need to be made with fruit harvested earlier.

AVG plus heat did not reduce volatile production of 'Red Delicious' apples below that by a single treatment, though it equaled the most negative effects after harvest and after cold storage. Production of all volatile compounds was repressed by the treatments, including the major flavor-related esters, and volatile production recovered in AVG-treated fruit after RA storage only when they were not heated. The results of this study support the idea that substrate availability is a more limiting factor than AAT activity for the production of esters in apples treated with AVG and heat.

Based on the present results, AVG plus heat treatment did not prove to be a commercially desirable treatment to maintain apple fruit quality during short-term cold storage of early- or late-harvest cultivars. AVG plus heat slowed firmness loss and reduced physical deterioration of early-harvest cultivars, the ones that would benefit most from this treatment, during cold storage. However, the effects were not significant enough to maintain fruit quality above marketable standards and showed the additional negative impact of heat treatment on volatile production.

# CHAPTER 4

# STUDY OF ETHYLENE ACTION ON THE REGULATION OF NAD-DEPENDENT SORBITOL DEHYDROGENASE ACITIVITY DURING LATE FRUIT DEVELOPMENT OF VARIOUS APPLE CULTIVARS

## INTRODUCTION

Soluble sugars determine the sweetness of apples and other fruit. Sweetness is an important flavor component that influences the acceptability of fruit by consumers. Therefore understanding carbohydrate metabolism in apples is important for maintaining or improving the quality of this fruit before and after harvest. Sorbitol is the primary photosynthetic product and the major translocated sugar in many species of the family Rosaceae, including apples (Loescher, 1987). Sorbitol accounts for approximately 80 % of the total soluble carbohydrate in apple leaves, spurs and peduncles but only 3 to 8 % in the fruit, where the most common sugars are fructose, glucose and sucrose, which comprise 45-60 %, 20 and 10 % of the total soluble sugars, respectively (Berüter, 1983). The main enzymes involved in the metabolism of imported sugars in apple are NAD-dependent SORBITOL DEHYDROGENASE (SDH, Ec.1.1.1.14) and sorbitol oxidase for sorbitol, and acid invertase for sucrose (Berüter, 1985; Yamaki and Ishikawa, 1986; Yamaki, 1995). The difference between sorbitol content in the fruit and the high content in most of the rest of the tree has been attributed to the high activity of SDH (Yamaki and Ishikawa, 1986), the enzyme that catalyzes the oxidation of sorbitol and reduction of fructose (Negm and Loescher, 1979). SDH favors sorbitol conversion to fructose in apple fruit (Yamaguchi et al., 1994).

Apple is a climacteric fruit and its ripening is triggered by an autocatalytic increase in the production of the hormone ethylene (Lurie, 1998 a, b). Ethylene is involved in the regulation of several ripening processes, like softening of the cortex, color development (Abeles et al., 1992) and volatile production (Fan and Mattheis, 1999). Total soluble sugar content in the fruit is measured as soluble solids content (SSC), and this trait is independent of ethylene action when it is measured on harvested fruit (Autio and Bramlage, 1982; Byers, 1997; Fan et al., 1999; Knee,

1976). Sucrose and fructose levels may be affected by ethylene possibly through a reduction of starch hydrolysis (Defilippi et al., 2004), though Silverman et al. (2004) found that sucrose and sorbitol but not sucrose levels were lower in apples treated with an inhibitor of ethylene synthesis. Little is known about the possible involvement of ethylene in the regulation of sugar metabolism of apples during the last stages of development on the tree. SDH activity in 'Fuji' apple was reported to be low in young fruit and increase close to harvest, remaining high from 160 to 207 days after bloom (Yamaguchi et al., 1996; Yamada et al., 1999). The rise in activity appears to depend upon the expression of the SDH gene in 'Fuji' (Yamada et al., 1999), but not in 'Orin' apples where SDH protein accumulated before the rise in SDH activity (Yamaguchi et al., 1996), suggesting that posttranslational modifications of the enzyme may also play a role in the regulation of its activity, and this could be cultivar-dependent. Yamada et al. (1999) observed that *SDH* transcription in 'Fuji' apples occurred before the climacteric rise indicating that ethylene may not be involved in the regulation of SDH expression, but no information is available about the role of ethylene on the regulation of SDH activity. Aminoethoxyvinylglycine (AVG) is a plant growth regulator that inhibits the synthesis of ethylene (Greene, 2003). AVG was found to delay apple fruit ripening and decrease preharvest drop (Bangerth, 1978), and to inhibit ethylene and volatile production when applied to pre-climacteric fruit (Fan et al., 1998; Harder-Doll and Bangerth, 1987) one month prior to harvest. Thus, it could be a useful tool to study the possible involvement of ethylene in the regulation of SDH expression and activity. The objective of the present work was to determine if ethylene influences SDH expression and/or activity of various apple cultivars during the last weeks of on-tree fruit development.

## MATERIALS AND METHODS

### Treatments and harvest

At the University of Kentucky Horticultural Research South Farm in Lexington, Kentucky, whole trees of 'Lodi/M7', 'Redchief Red Delicious/M7' and 'Red Fuji/M7a', planted in 1993, were treated in 2003 and 2005 with an aqueous solution of AVG (ReTain, Valent Biosciences, Libertyville, IL) containing 500 ppm Silwet L-77 (Helena Chemical Co., Collierville, TN), as surfactant, 4 weeks before the expected normal harvest at the commercial rate of 124 $g.ha^{-1}$ a.i. The AVG solution was applied to leaves and fruit with a hand pump

sprayer to the point of runoff. In 2003, control and treated apples were harvested one week before the expected harvest (H-1), at normal harvest (H), and one week after H (H+1). In 2005 control and treated fruit were harvested 3, 2, and 1 week before the expected normal harvest (H-3, H-2, H-1, respectively), at normal harvest and 1 week after normal harvest (H and H+1, respectively).

**Quality traits at harvest**

To evaluate the effect of AVG on ethylene production, headspace ethylene production (HEP) was measured on 4-6 individual fruit per cultivar and treatment in 2003, and on six composite samples of two fruit each in 2005. HEP was assessed on days 1, 3 and 5 after H, and 1 day after H-1 and H+1. Fruit held at room temperature (21 $\pm$ 0.5 $^{o}$C) were placed in sealed 2 L glass jars, and 0.2 mL samples were taken from the headspace through a rubber septum in the lid after 4 h. Samples were analyzed with a gas chromatograph (HP 5890, Agilent Technology, Wilmington, DE) equipped with a flame ionization detector (FID) and an alumina capillary column (AT-Alumina Plot GC Column, 30 m, 0.53 cm i.d.) containing activated alumina and $N_2$ as the carrier gas. Temperatures were 35$^{o}$C, 175$^{o}$C and 125$^{o}$C for oven, injector and FID, respectively. An external standard (100 ppm Ethylene/Helium, Alltech Associates Inc., Deerfield, IL) was diluted and used to quantify the amounts of detected ethylene.

Starch index (SI) and firmness were used to quantify apple fruit maturity on the harvest day. To assess SI, fruit were cut in half perpendicular to the stem-blossom axis, and the halves were soaked in iodine solution (0.1% iodine, 1% potassium iodide in water). The degree of staining was rated on a visual scale of 1 to 9, where 1= staining the entire cut surface (high starch) and 9= no staining (no starch) (Cowgill et al., 2005). Cortex firmness was measured using a penetrometer (Model DF M10, John Chatillon & Sons, Inc. Greensboro, NC) equipped with an 11 mm diameter probe after a disk of skin was removed from opposite sites on the equatorial plane of the stem halves.

**Sorbitol dehydrogenase extraction and activity assay**

Fruit sampled for SDH activity were peeled, cut and frozen in liquid nitrogen in the orchard immediately after detaching them from the trees, and samples were held at -80 $^{o}$C until the activity assays were performed. Enzyme was extracted from cortex of apple fruit and assayed as in Archbold (1999), with the exception that 2.5 g instead of 5 g of cortex tissue were used in each assay, 0.6 M Tris buffer (pH 7) was used to extract the tissues in place of K-phosphate

buffer, and samples were not filtered before centrifugation. The protein content of the Sephadex-purified extracts was determined spectrophotometrically (Model Cary 50 Bio, Varian Analytical Instruments, Walnut Creek, CA) at 595 nm using the Coomassie Plus$^{TM}$ Protein Assay Kit (Pierce, Rockford, IL) following the manufacturer's instructions and using bovine serum albumin (Fisher Scientific, Fair Lawn, NJ) as a standard. There were 3 replicate extractions of single fruit samples per weekly sampling date, cultivar and treatment in 2003 and of 2-fruit composite samples in 2005. Enzyme is reported as nmol $NAD^+$ reduced • $min^{-1}$ • mg $protein^{-1}$.

**Western analyses**

Western blots were performed with protein from one sample per cultivar, treatment and harvest date in 2003. In 2005, two samples per treatment of 'Lodi' and 'Red Delicious' fruit harvested at H in 2005 were used, each corresponding to the lowest and the highest SDH activity found within treatment and cultivar. Western blots were performed using the ImmunoPure ABC Phosphatase Staining Kit (Pierce, Rockford, IL) at room temperature. Western blots were obtained after transferring protein from 12.5% SDS-PAGE gels, run with 50 μg of apple protein per lane, to nitrocellulose membranes which were incubated first with 15 mL blocking buffer (Pierce, Rockford, IL) for 1 h and later with primary antibody (immunopurified SDH antibody as in Nosarzewski et al., 2004) for 30 min. After washing, blots were exposed to 10 mL of secondary antibody (biotinylated affinity purified goat anti-rabbit IgG 1.5 mg/mL, Pierce, Rockford, IL) for 30 min. After washing, wells were incubated with 10 mL ABC solution (Avidin, biotinylated alkaline phosphatase, Pierce, Rockford, IL) for 30 minutes. The signal was developed using a NBT/BCIP solution (Pierce, Rockford, IL). Purified antibody interacted with the recombinant SDH recovered from *E. coli* and sheep liver SDH equally well (Nosarzewski et al., 2004), so the sheep liver SDH was used as a visual standard as its molecular mass was close to apple SDH.

**Experimental design and statistical analysis**

Each experiment was conducted using a completely random design. HEP, SI and firmness data were subjected to analysis of variance. Means of different treatments within cultivar were compared with ANOVA ($p=0.05$) using SAS version 9.1 software (SAS Institute Inc., Cary, N.C.).

# RESULTS

## Quality traits at harvest

'Lodi' had the highest and 'Red Fuji' the lowest HEP, respectively. HEP over time after harvest H increased in control samples and remained low in AVG-treated apples of all cultivars in 2003 (Figure 4.1.A), though AVG significantly repressed HEP of 'Lodi' only. In 2005, 'Lodi' and 'Red Delicious' apples showed an increase in HEP over time after harvest with control 'Lodi' fruit having the highest HEP of all, while HEP of all 'Fuji' samples remained low (Figure 4.1.B). HEP of AVG-treated 'Lodi' fruit was consistently repressed in both seasons, while that of 'Red Delicious' was significantly repressed 5 days after harvest in 2003 only. 'Fuji' HEP was the least affected by AVG. HEP measurements one day after H-1 and H+1 harvests showed the same effects, with 'Lodi' the most significantly affected by AVG, followed by 'Red Delicious', and with 'Fuji' presenting the lowest HEP of all cultivars in control and AVG-treated fruit (data not shown). AVG reduced SI at harvest of 'Fuji' in 2003 and of 'Red Delicious' in 2005 (Table 4.1), whereas firmness at harvest was not affected by AVG in any cultivar or season (Table 4.1).

## SDH activity

SDH in cortex of control versus AVG-treated fruit was similar across varieties, harvest dates and years (Figure 4.2). 'Red Delicious' had the highest SDH activity in 2003 and 2005 (Figure 4.2.C, 4.2.D), though it was considerably lower in 2005 than 2003. 'Lodi' and 'Fuji' had similar levels of SDH activity in 2003 (Figure 4.2.A, 4.2.E), but in 2005 'Lodi' SDH activity was lower than in 2003 (Figure 4.2.B), while 'Fuji' remained at similar or even greater levels (Figure 4.2.F). SDH activity did not show a significant trend when plotted against HEP (Figure 4.3). For example, SDH activity in 'Red Delicious' apples was lower than 10 nmol NADH $min^{-1}$ mg $protein^{-1}$ at HEP below 1 μg $kg^{-1}$ $h^{-1}$, though this was not true for all samples, and 'Lodi' SDH had similar activity independent of HEP. 'Fuji' SDH activity was similar for all plotted samples, as was HEP. The regression coefficients within each variety reflected this pattern (Figure A.2 in Appendix). When SDH activity of all control and AVG-treated samples from H-1,

H and H+1 was compared blocking by year, cultivar and treatment, no difference between treatments was found at any harvest date, and only 'variety', 'year' and their interaction were significantly different across harvest dates (data not shown).

**Figure 4.1:** Effect of AVG on headspace ethylene production (HEP) of 'Lodi', 'Redchief Red Delicious' (RD) and 'Red Fuji' apples in 2003 (A) and 2005 (B). Fruit were ripened at room temperature for 5 days immediately after harvest H. Significant differences (*) between treatments and within cultivar and date separated by ANOVA at *p=0.05*.

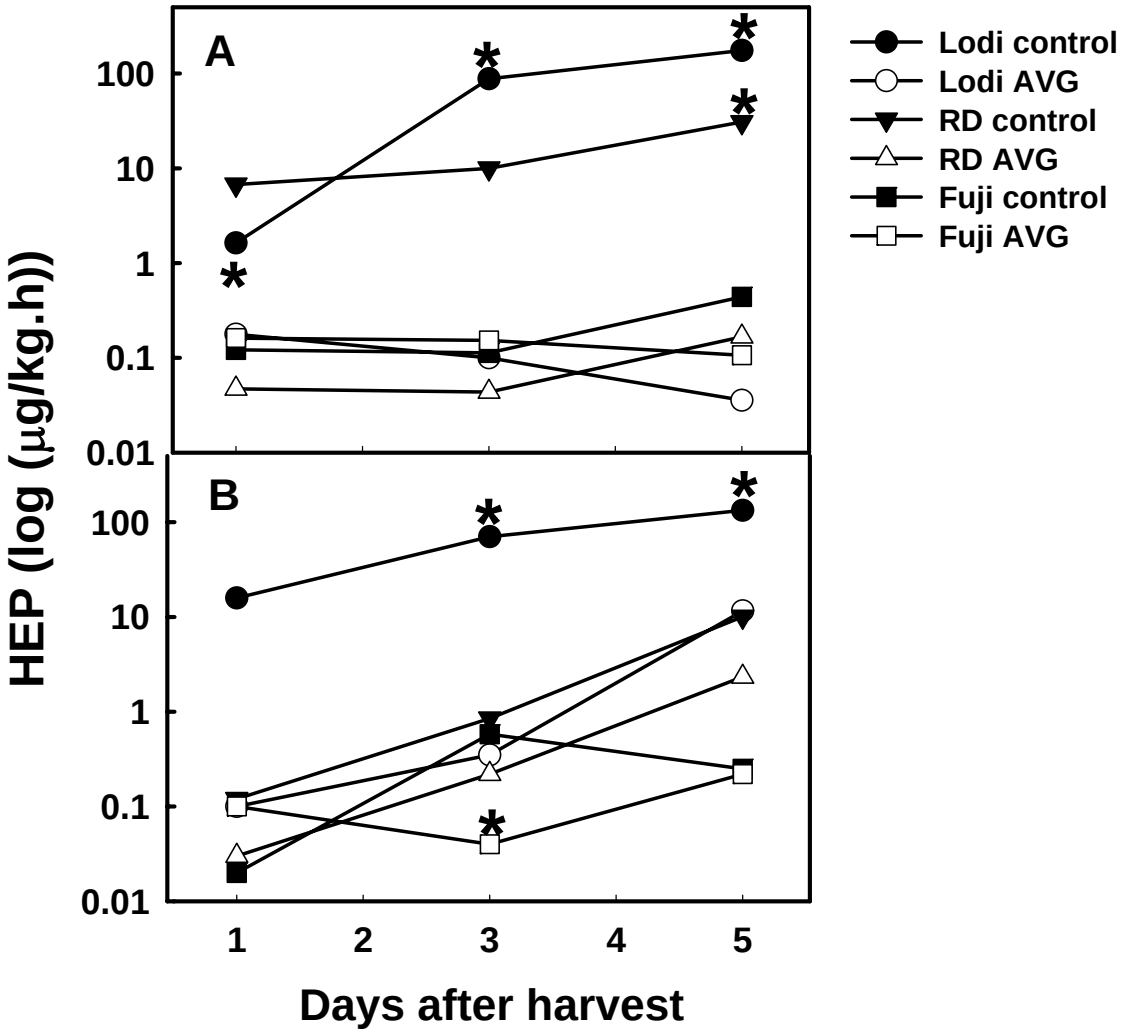


**Table 4.1:** Starch index (SI) and firmness of 'Lodi', 'Redchief Red Delicious' and 'Red Fuji' apples at the expected normal harvest time, not treated (Control) and treated with AVG 4 weeks before harvest (AVG) in 2003 and 2005. Significant differences (*) between treatments by ANOVA at *p=0.05*.

| Cultivar | Treatment | ***2003*** | | ***2005*** | |
|---|---|---|---|---|---|
| | | SI (%) | Firmness (N) | SI (%) | Firmness (N) |
| Lodi | | | | | |
| | Control | 7.4 | 50.7 | 4.8 | 67.6 |
| | AVG | 6.2 | 72.3 | 5.0 | 69.9 |
| Redchief Red Delicious | | | | | |
| | Control | 4.8 | 70.6 | 4.2 * | 71.7 |
| | AVG | 3.6 | 70.6 | 1.8 | 72.4 |
| Red Fuji | | | | | |
| | Control | 5.1* | 66.9 | 4.3 | 80.0 |
| | AVG | 3.6 | 68.4 | 6.0 | 77.8 |

**Figure 4.2:** SDH activity in cortex of AVG-treated (AVG) and control 'Lodi' (A, B), 'Redchief Red Delicious' (C, D) and 'Red Fuji' (E, F) fruit in 2003 (A, C, E) and 2005 (B, D, F). Fruit were harvested 1 week before (-1H) the normal harvest (H), at H, and 1 week after H (H+1) in 2003, and 3, 2, 1 weeks before H (H-3, H-2, H-1), at H, and one week after H (H+1) in 2005. Samples were not available to assay SDH activity of 'Lodi' at H+1 in 2005 or of control 'Redchief Red Delicious' at H+1 in 2003. Means and SE are shown. There were no significant differences between treatments at any harvest date and in any year. Means were separated by ANOVA at *p=0.05*.

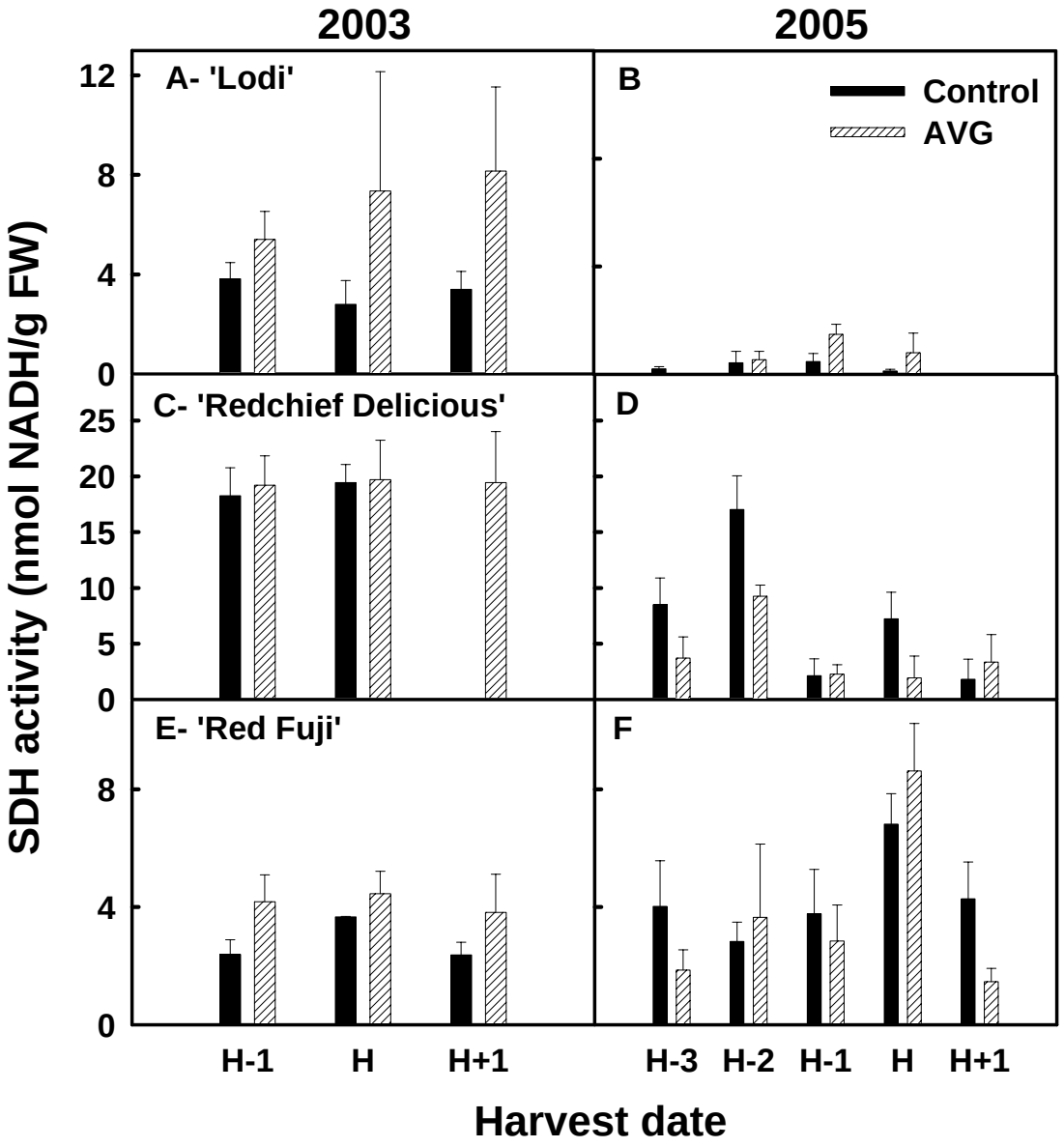
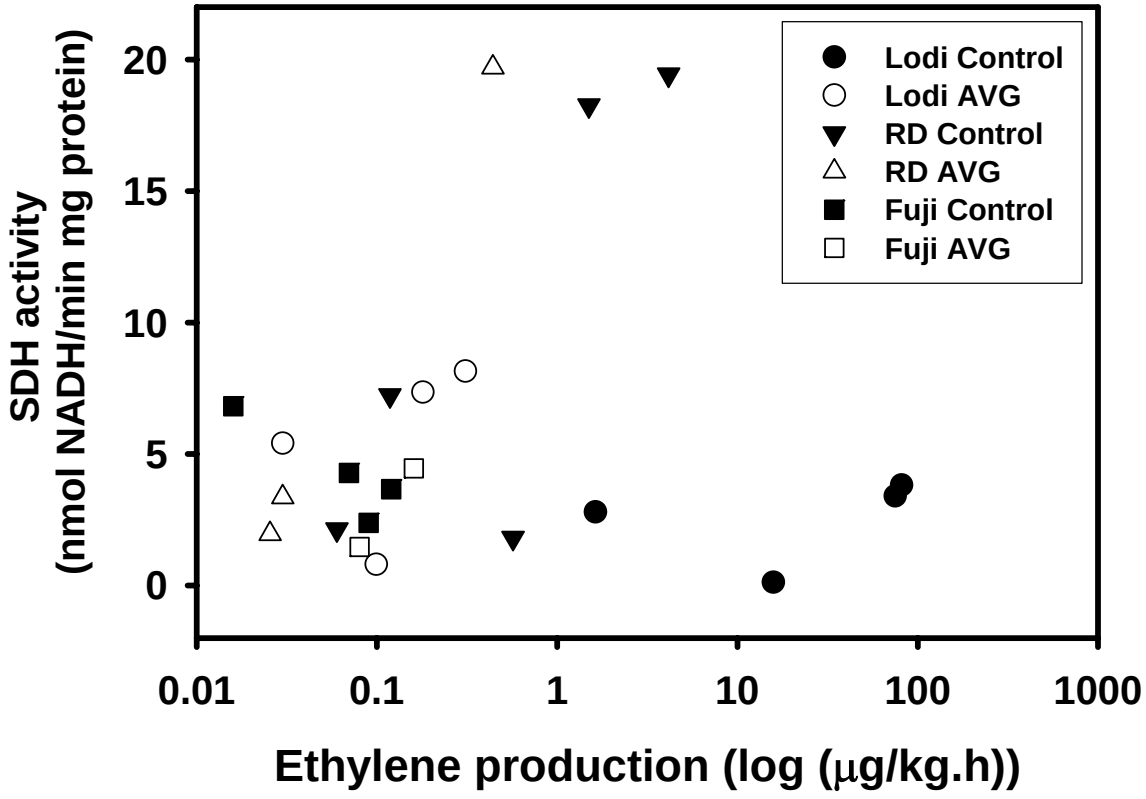


**Figure 4.3:** Mean SDH activity in cortex of control (closed symbols) and AVG-treated (open symbols) 'Lodi', 'Redchief Red Delicious' and 'Red Fuji' fruit vs. mean headspace ethylene production within cultivar, harvest date and year. Data from 1 week before, at, and 1 week after normal harvest in 2003 and 2005.



**Western analyses**

Figure 4.4.A shows roughly comparable SDH presence in cortex of control and AVG-treated 'Fuji' apples from H-1, H and H+1 in 2003. SDH presence in cortex of 'Lodi' and 'Red Delicious' was also confirmed by Western blots in 2003, but these results are not shown as they were similar to those for 'Fuji'. Figure 4.4.B shows SDH presence in cortex of control and AVG-treated 'Lodi' and 'Red Delicious' fruit from H in 2005. Samples that presented the highest and the lowest SDH activity within cultivar and treatment were used, and SDH was present in all samples, regardless of treatment, cultivar and level of SDH activity. The double line corresponding to SDH observed in Figure 4.3.B may be due to protein degradation in the samples kept for several months at -80 $^{o}$C.

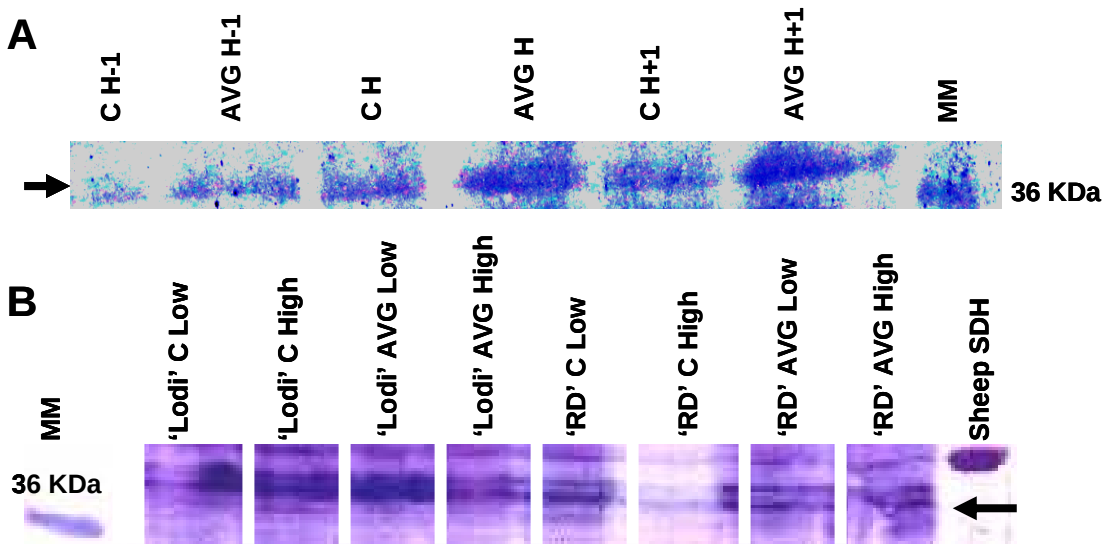
## DISCUSSION

AVG affected HEP of 'Lodi' apples the most (Figure 4.1), data contradicting findings by Autio and Bramlage (1982) who suggested that cultivars with high ethylene production are less likely to be affected by AVG. The effect of AVG on HEP was not reflected in starch degradation or firmness (Table 4.1), but other studies (Chapter 3) show that the repression of firmness loss by AVG was more evident in this cultivar when fruit was held in cold storage for 4 weeks. 'Red Delicious' exhibited an intermediate response to AVG in terms of ethylene production, and AVG did not affect HEP of 'Fuji' apples, though this cultivar exhibited a low HEP even in untreated fruit. That 'Red Delicious' and 'Fuji' did not show a significant response to

AVG in HEP, firmness or SI values suggests that AVG affected 'Lodi' more than the other cultivars.

Because significant differences in SDH activity were not observed between control and AVG-treated fruit in 2003 (Figure 4.2), we considered the possibility that samples from single fruit were perhaps too variable, and therefore composite samples were used in 2005. However, no differences were detected that year either, even with samples of different cultivars and from both years combined for the statistical analysis to increase the number of replications (data not shown). Additionally, there was no consistent trend between the effect of AVG on HEP and on SDH activity (Figure 4.3). One could argue that AVG failed to reduce ethylene production in 'Red Delicious' and 'Fuji' sufficiently, and that was the reason for the lack of response in SDH

**Figure 4.4:** Western blots of SDH in cortex of different cultivars. Each lane was loaded with 50 µg protein. MM is the molecular marker. Sheep liver SDH was used as visual standard. A: 'Red Fuji' apples treated with AVG versus controls (C) and harvested at H-1, H and H+1 in 2003, determined by Western blot. B: 'Lodi' and 'Redchief Red Delicious' (RD) apples treated with AVG versus controls (C) and harvested at H in 2005. Two samples, each representing the lowest or highest SDH activity (Low, High) within cultivar and treatment were chosen.



activity. However, AVG greatly reduced HEP in 'Lodi' fruit, and yet SDH was not affected in 'Lodi'. Thus, it is likely that ethylene is not involved in the regulation of SDH activity, or that the threshold for ethylene action is very low.

SDH protein was present in cortex of control and AVG-treated fruit across years, harvest dates and cultivars, and regardless of the levels of SDH activity measured (Figure 4.4). The double line in Figure 4.4.B may be due to protein degradation during the long sample storage at -80$^{o}$C. The data from this study indicates that ethylene is not involved in the regulation of SDH expression or activity. Yamaguchi et al. (1996) suggested that SDH activity may be regulated through post-translational modifications. A role for ethylene in this type of regulation is not likely though, because there was no clear response of SDH activity to different intrinsic levels of ethylene production, as shown in Figure 4.3. Interestingly, total soluble sugar content in apples is believed to be an ethylene-independent trait, though levels of individual sugars decrease in transgenic fruit with impaired ethylene production (Defilippi et al., 2004). During apple ripening a major source for soluble sugars is starch degradation. Thus, it is possible that ethylene may be affecting other enzymes involved in carbohydrate metabolism.

Several factors might influence SDH activity, like source/sink relationships and sugar availability. The availability of sorbitol and glucose, though not of fructose, induced SDH activity in apple cortex (Archbold, 1999). Thus, reduced amounts of sorbitol transported to a fruit due to low photosynthetic rates and/or competition among sinks might have a great impact on SDH activity (Archbold, 1999). Geiger et al. (1996) suggested that sink strength and activity would be regulated to maintain a balance between carbohydrate supply and utilization. SDH activity of all cultivars, especially 'Lodi' and 'Fuji', varied between years supporting the idea that environmental conditions may affect SDH activity through carbohydrate availability.

## CONCLUSIONS

In order to understand carbohydrate metabolism of apple fruit, the activity of SDH, the main enzyme responsible for the conversion of sorbitol into fructose in the fruit, was measured during the last weeks of on-tree fruit ripening. The objective was to determine if ethylene was involved in the regulation of SDH activity by using an inhibitor of the synthesis of ethylene. The

inhibitor AVG did not significantly reduce ethylene production of all cultivars, though all AVG-treated fruit had ethylene production levels below 1 μg $kg^{-1}$ $h^{-1}$ both in 2003 and 2005. Control and AVG-treated fruit were at similar ripening stages at harvest. Overall, reducing ethylene production did not seem to affect SDH activity or presence. This study provides evidence that inhibitors of ethylene synthesis or action should not inhibit sugar accumulation in the fruit.

# APPENDIX

**Abreviations**

4WCS – 4 weeks in cold storage
6WCS – 6 weeks in cold storage
12WCS – 12 weeks in cold storage
AAT – ALCOHOL-ACYL TRANSFERASE
ACC – 1-Aminocyclopropane-1-carboxylic acid
ACO – ACC OXIDASE
ADH – ALCOHOL DEHYDROGENASE, involved in the reduction of aldehydes to alcohols
AH – After harvest or treatment
AVG – Aminoethoxyvinylglice, inhibitor of ACC SYNTHASE involved in ethylene synthesis. Retain®.
DACP – Diazocyclopentadiene
HEP – Headspace ethylene production
HT – Heat treatment
H1 – Normal harvest
H2 – 1 or 2 weeks after normal harvest
IEP – Internal ethylene production
LOX - LIPOXIGENASE
MCP – 1-Methylcyclopropene
OPP – Oxidative pentose phosphate pathway
PAL – PHENYLALANINE AMONIA LYASE
PG – ENDOPOLIGALACTURONASE
RA – Regular atmosphere storage
RR – Respiration rate
RT – Room temperature ($21 \pm 0.5\ ^{o}C$)
SDH – SORBITOL DEHYDORGENASE
S:A – Sugar to acid ratio
SAM – S-adenosyl-L-methionine
SI – Starch index
SSC – Soluble solids content
TVP – Total volatile production
TA – Titratable acidity
TCA – Tricarboxylic acid pathway
TEP – Total ester production
WL – Weight loss

**Table A.1:** Effect of AVG and/or MCP on several quality traits of 'Royal Gala' apple in 2004 and 2005. Effects shown are relative to controls. A+M = AVG plus MCP. Double arrows indicate greater effect.

| Quality trait | AVG | MCP | A+M |
|---|---|---|---|
| Ethylene production | ↓ | ↓ | ⇊ |
| Respiration rate | ↓ | ↓ | ⇊ |
| Firmness | ↑ | = | ⇈ |
| Starch index | ↓ | = | ↓ |
| Color | ↓ | = | ↓ |
| Soluble solids content | V | V | V |
| Titratable acidity | = | = | ↑ |
| Sugar:acid ratio | = | = | ↓ |
| Volatile production | ↓= | ↓ | ⇊ |
| AAT activity | V | ↓= | ↓= |

V - Variable results: decrease, increase or similar

↓ - Decrease

↑ - Increase

= - Similar

**Table A.2:** Effect of AVG and/or heat treatment on several quality traits (QT) of different apple cultivars in 2003 and 2004. Effects shown are relative to controls. L = 'Lodi'; S = 'Senshu'; RD = 'Redchief Red Delicious'; F = 'Fuji'; HEP = headspace ethylene production; RR = respiration rate; Firm = firmness; SI = starch index; SSC = soluble solids content, TA = titratable acidity; S:A = sugar to acid ratio; **H1** = normal harvest; H2 = fruit harvested 1 or 2 weeks after normal harvest. Larger arrows indicate greater effect. 'Senshu' AVG-treated fruit were harvested at **H1** and H2.

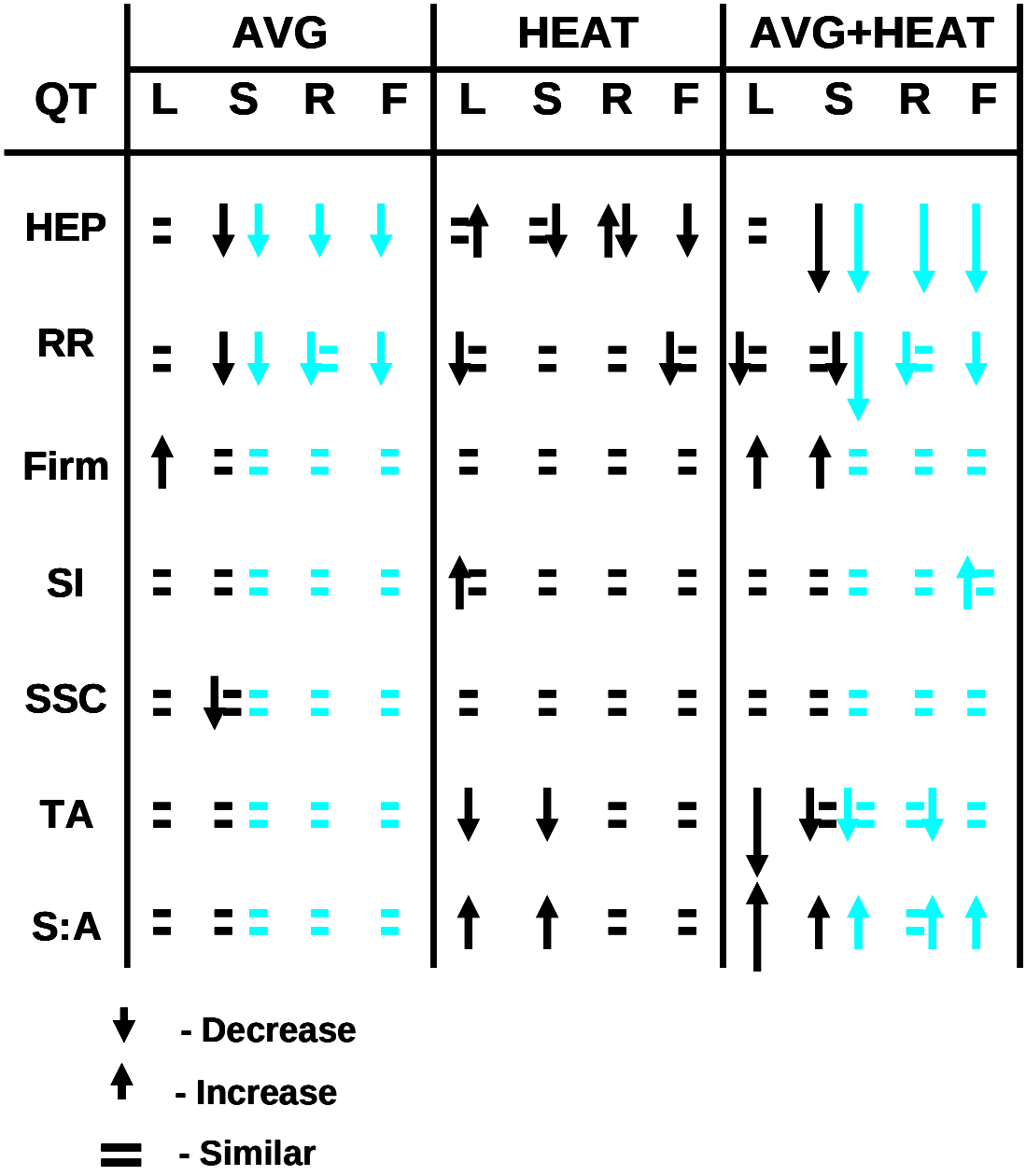


**Figure A.1:** Effect of AVG and/or MCP on headspace ethylene production (HEP) of 'Royal Gala' apple in 2004 (A, C, E) and 2005 (B, D, F). Log scale. Fruit were ripened at room temperature for 7 days immediately after harvest (A, B) and after 6 (C, D) and 12 weeks (E, F) in cold storage at 4°C. Closed symbols correspond to H1 and open symbols to H2. Open circles in Figure F represent AVG plus MCP fruit from H1 treated with ethephon. Least significant differences (LSD) at p=0.05 within date are shown as vertical bars. Note that Y axes show different scales.

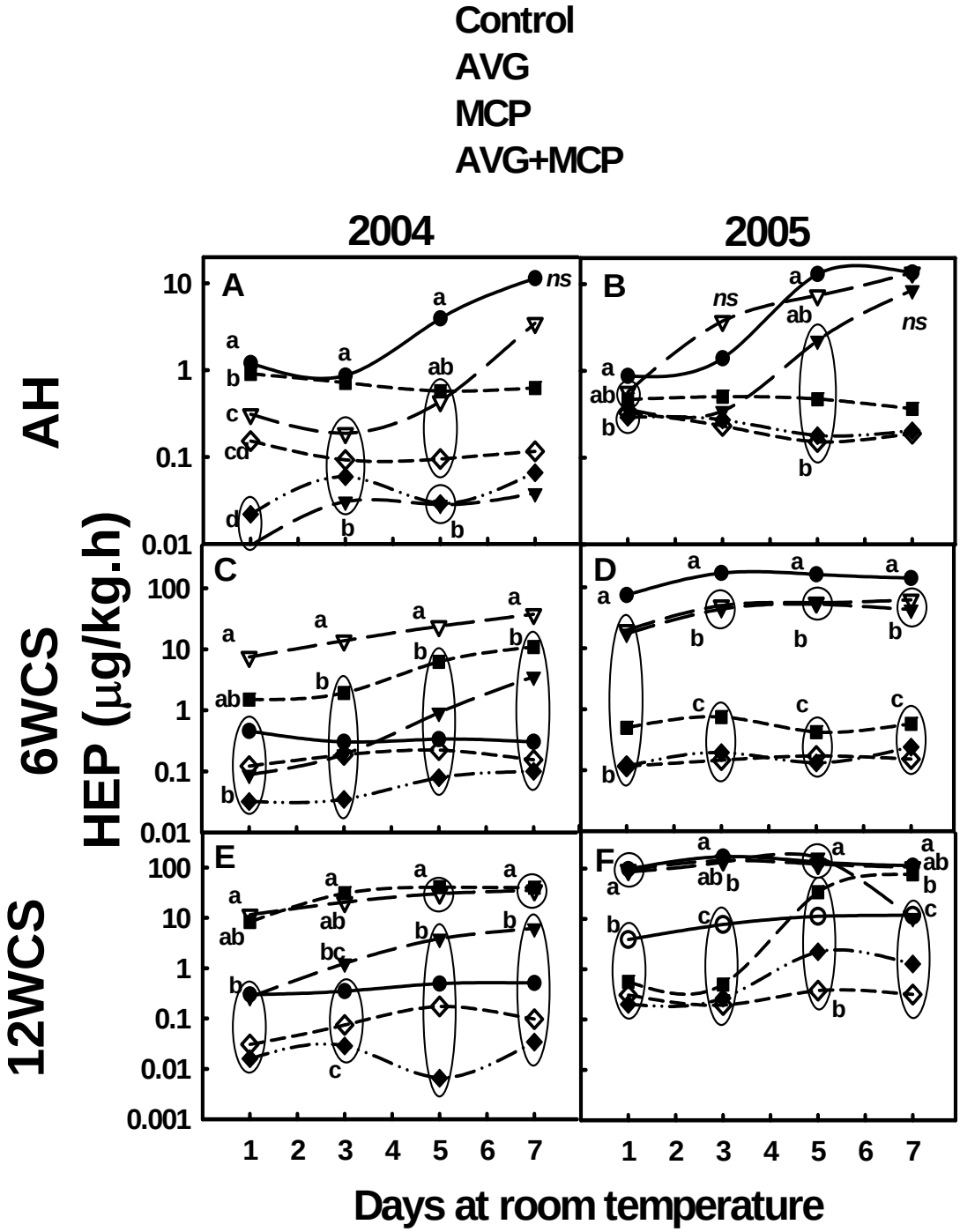


**Figure A.2:** Mean volatile ester production in cortex of control 'Royal Gala' apples vs. mean total volatile alcohol production in 2004 (closed symbols) and 2005 (open symbols). Coefficients for each year are shown, where ester production ($AUx10^{-3}$ g $FW^{-1}$) = b [0] + b [1] x alcohol production ($AUx10^{-3}$ g $FW^{-1}$); n = 9.

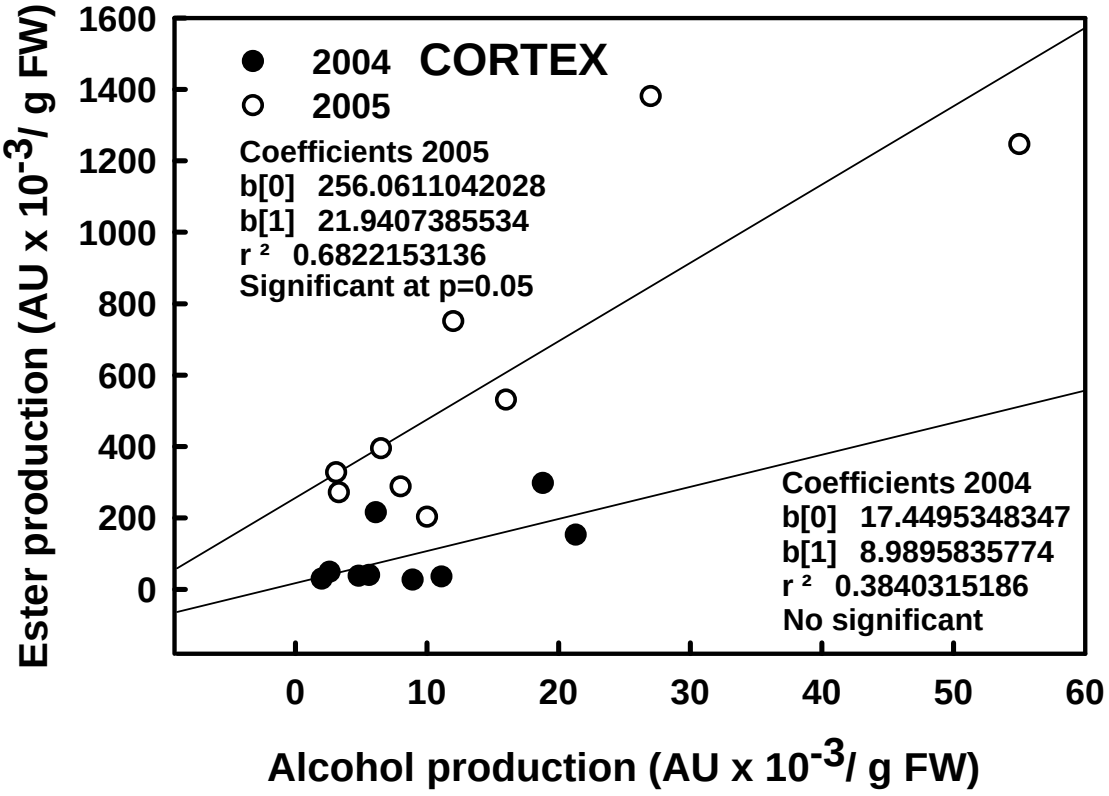
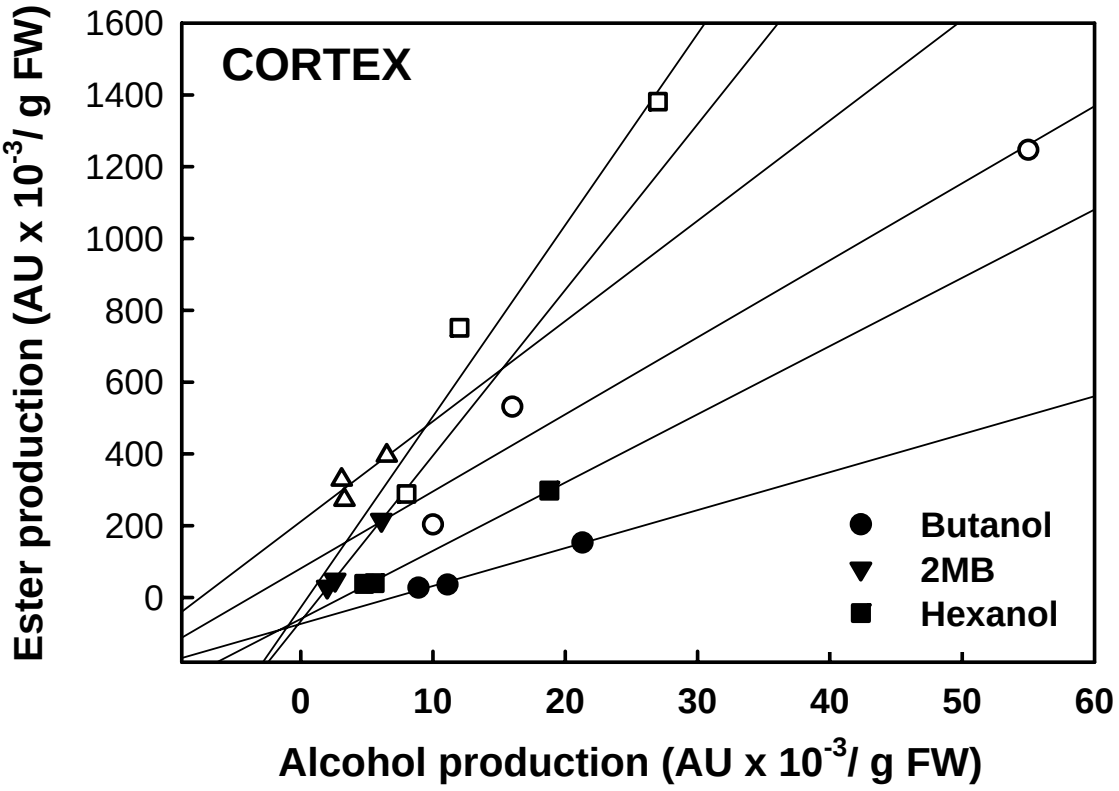


**Figure A.3:** Mean volatile ester production grouped by alcohol moiety in cortex of control 'Royal Gala' apples vs. corresponding mean individual volatile alcohol production in 2004 (closed symbols) and 2005 (open symbols). Hexyl esters where plotted vs. hexanol, butyl esters vs. butanol, and 2-methylbutyl esters vs. 2-methylbutanol (2MB). Coefficients for each year are shown in the table below, where individual ester production ($AUx10^{-3}$ g $FW^{-1}$) = b [0] + b [1] x individual alcohol production ($AUx10^{-3}$ g $FW^{-1}$). With n=3, $r^2$ 0.95 or higher represent significant differences at p=0.05.




| | Fed Alcohol | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Butanol | | | 2-Methylbutanol | | | Hexanol | | |
| Year | b [0] | b [1] | $R^2$ | b [0] | b [1] | $R^2$ | b [0] | b [1] | $R^2$ |
| 2004 | -73.5 | 10.5 | 0.989 | -66.4 | 46.2 | 0.998 | -59.6 | 19.0 | 0.998 |
| 2005 | 80.9 | 21.4 | 0.964 | 211 | 27.9 | 0.749 | -27.9 | 53.3 | 0.946 |

**Figure A.4:** Mean volatile ester production in peel of control 'Royal Gala' apples vs. mean total volatile alcohol production in 2004 (closed symbols) and 2005 (open symbols). Coefficients for each year are shown, where ester production ($AUx10^{-3}$ g $FW^{-1}$) = b [0] + b [1] x alcohol production ($AUx10^{-3}$ g $FW^{-1}$); n = 9.

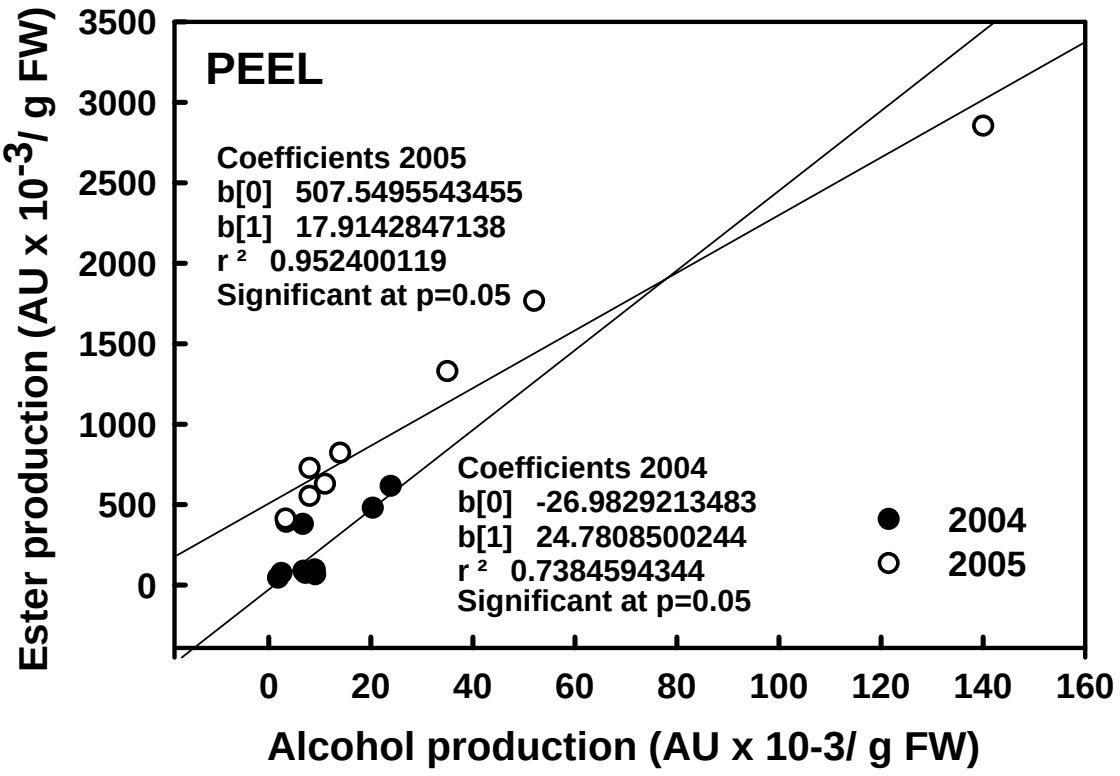
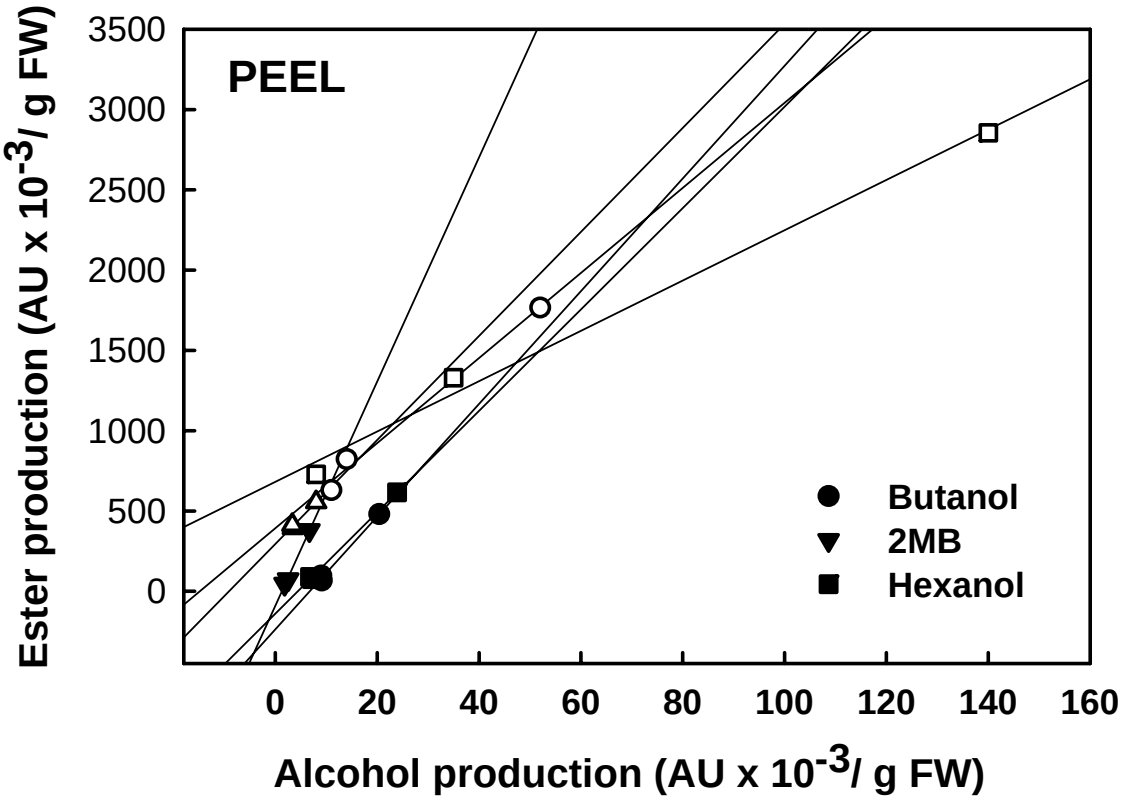
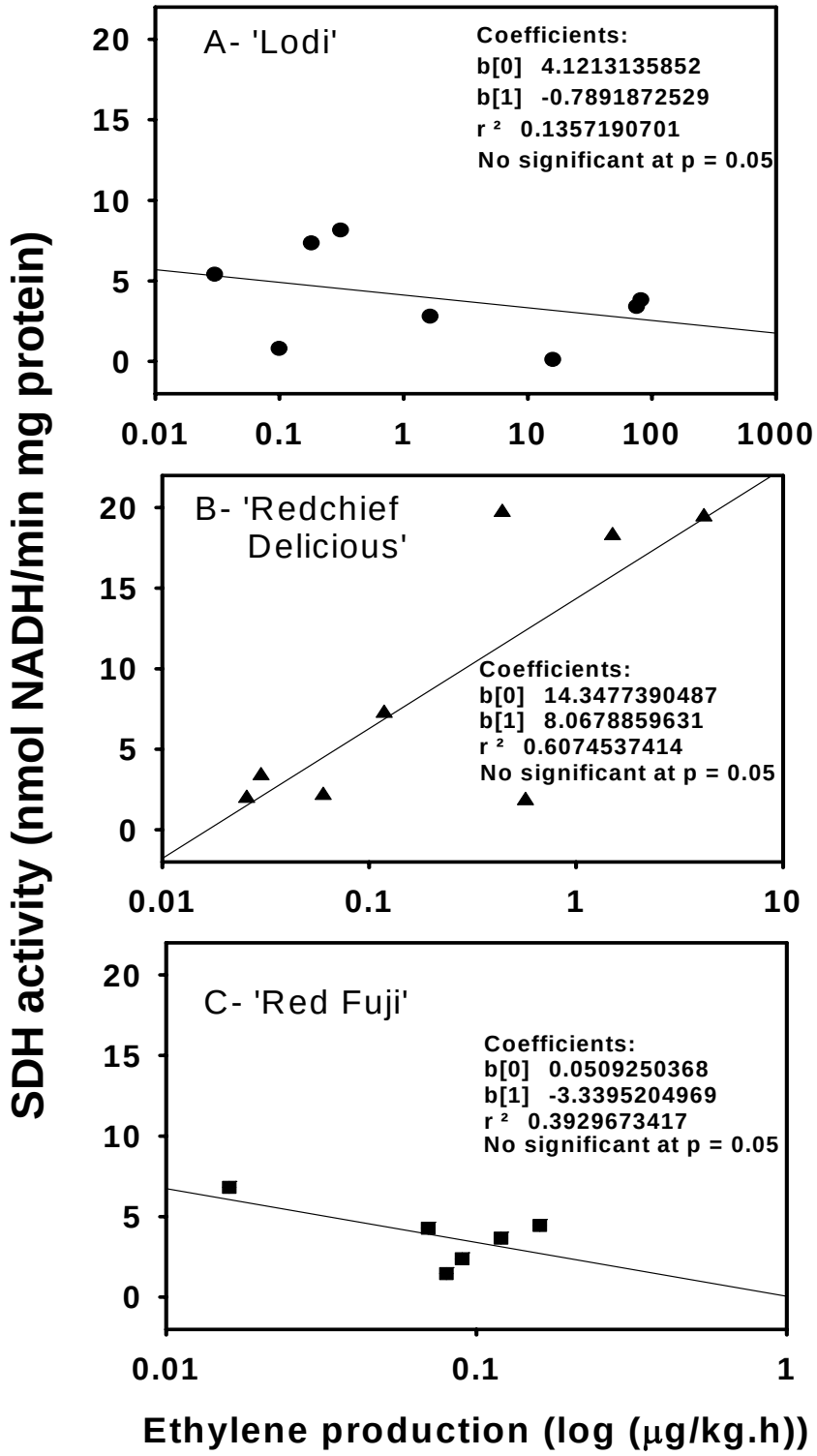


**Figure A.5:** Mean volatile ester production grouped by alcohol moiety in peel of control 'Royal Gala' apples vs. corresponding mean individual volatile alcohol production in 2004 (closed symbols) and 2005 (open symbols). Hexyl esters where plotted vs. hexanol, butyl esters vs. butanol, and 2-methylbutyl esters vs. 2-methylbutanol (2MB). Coefficients for each year are shown in the table below, where grouped ester production ($Aux10^{-3}$ g $FW^{-1}$) = b [0] + b [1] x individual alcohol production ($AUx10^{-3}$ g $FW^{-1}$). With n=3, $r^2$ 0.95 or higher represent significant differences at p=0.05.




| | Fed Alcohol | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Butanol | | | 2-Methylbutanol | | | Hexanol | | |
| Year | b [0] | b [1] | $R^2$ | b [0] | b [1] | $R^2$ | b [0] | b [1] | $R^2$ |
| 2004 | -236 | 35.1 | 0.994 | -89.3 | 69.8 | 0.997 | -138 | 31.6 | 0.998 |
| 2005 | 393 | 26.5 | 0.991 | 295 | 32.4 | 0.982 | 682 | 15.7 | 0.993 |

**Figure A.6:** SDH activity versus headspace ethylene production (HEP) of 'Lodi' (A), 'Redchief Red Delicious' (B) and 'Red Fuji' (C). Mean SDH activity within harvest date and year are plotted against their corresponding mean HEP. Note that X axes are shown with different scales.



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