EFFECT OF 1-METHYLCYCLOPROPENE (1-MCP) ON THE FLAVOUR METABOLITES OF APPLE JUICE

by

Bizuayehu Mengstie Muche

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Dedication

To my mother, Alemitu Tafere (Emye). Emye, you paid incredible sacrifice to send me to school and this is the result of your strength, loving care and perseverance.

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Abstract

This study determined the effect of 1-methylcyclopropene (1-MCP), storage atmosphere storage time and harvest maturity on the content of sugars, titratable acidity (TA), malic acid, volatile compounds, total phenolic content (TPC), total antioxidant activity (TAA), and physiochemical juice quality parameters of cloudy, clear and fresh juices prepared from 'McIntosh' and 'Honeycrisp' apples. The effectiveness of 1-MCP treatment was confirmed by the suppressed ethylene production of intact fruits. 1-MCP treatment had a significant ($p<0.05$) effect on TA, malic acid content, volatile aroma concentration, TPC and TAA of juice samples from both apple cultivars. Generally, juice samples from 1-MCP treated apples had significantly ($p<0.05$) higher TA, malic acid content, TPC and TAA, but with reduced content of esters, aldehydes and total volatile aroma compounds. Improved acidity retention in 1-MCP treated 'McIntosh' was attributed to the downregulated MdcyME and upregulated MdcyPEPC genes, which regulate malic acid degradation and biosynthesis, respectively. Similarly, acidity retention in 'Honeycrisp' was associated with the upregulated V-ATPase and MdcyMDH genes that regulate the vacuolar transport and cytosolic malic acid biosynthesis, respectively. Fructose, glucose and sucrose were the major sugars in all juice samples tested. In contrast to acidity, the content and composition of these sugars were not influenced by 1-MCP treatment; instead, it was substantially influenced by storage time. During long-term fruit storage, the content of sucrose decreased with a corresponding accumulation of glucose, fructose and total sugar. Juice processing techniques had a significant ($p<0.05$) effect on the concentration of volatile aroma, TPC, TAA and juice colour. As compared to clear juices, cloudy juice samples had significantly ($p<0.05$) higher content of most volatiles, TPC and TAA as compared to clear juice counterpart. The cloudy juice prepared from both cultivars could able to meet the expected yellowish colour. Turbidity and cloud stability values obtained from late harvested 'McIntosh' fruit fulfilled the requirement of stable cloudy juice. However, 'Honeycrisp' apples were not suitable for the production of stable cloudy juice as the turbidity (<250 NTU) and cloud stability (<50%) values were much lower than the minimum requirement.

List of abbreviations and symbols used

$+a/-a$	Redness/greenness
$+b/-b$	Yellowness/blueness
α	Alpha
β	Beta
1-MCP	1-Methylcyclopropene
AAFC	Agriculture and Agri-Food Canada
AAT	Alcohol acyltransferase
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
ADH	Alcohol dehydrogenase
ATP	Adenosine triphosphate
BI	Browning index
CA	Controlled atmosphere
CAT	Catalase
CD	Cyclodextrin
cDNA	Complementary DNA
CT	Cycle threshold
DPPH	1-1-diphenyl-2-picrylhydrazyl
DACP	Diazocyclopentadiene
Eq.	Equation

F1,6BP	Fructose 1,6-biphosphate
F6P	Fructose-6-phosphate
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
Fru	Fructose
G6P	Glucose-6-phosphate
GAE	Gallic acid equivalent
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry system
Glu	Glucose
HCN	Hydrogen cyanide
HPLC	High performance liquid chromatography
HS-SPME	Headspace solid phase microextraction
IEC	internal ethylene concentration
L	Lightness or darkness
LC_{50}	Lethal concentration (50%)
LOX	Lipoxygenase
MDH	Malate dehydrogenase
MdcyMDH	Gene encoding NAD-MDH
MdcyME,	Gene encoding NADP-ME
MdcyPEPC	Gene encoding PEPC
MdVHA-A	Gene encoding V-ATPase-A

MTA	Methylthioribose
NAD	Nicotinamide adenine dinucleotide (oxidized)
NAD-MDH	NAD-dependent cytosolic malate dehydrogenase
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate
NADP-ME	NADP dependent cytosolic malic enzyme
NTU	Nephelometric turbidity units
OAA	Oxaloacetic acid
PE	Pectin esterase
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PG	Polygalacturonase
PL	Pectinlyase
POX	Peroxidase
PPi	Inorganic pyrophosphate
PPO	polyphenol oxidase
RA	Regular air
RH	Relative humidity
RI	Retention index
RT-qPCR	Real-time quantitative PCR

SAM	S-adenosyl methionine
SDH	Sorbitol dehydrogenase
SS	Sucrose synthase
STS	Silver thiosulfate
Suc	Sucrose
TA	Titratable acidity
TAA	Total antioxidant activity
TCA	Tricarboxylic acid
TE	Trolox equivalent
TP	Triphosphate
TPC	Total phenolic content
TSS	Total soluble solids
UDP	Uridine diphosphate
V-ATPase-A	Vacuolar pyrophosphatase subunit A

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Chapter 1: Introduction

According to FAO, about 80 million tonnes of apple fruit was produced in 2013, accounting about 13% of the total world fresh fruit production (FAO, 2015). In Canada, apples are the number one fruit in terms of both production volume and value, representing 25% of the total fruit farm cash receipts (Agriculture and Agri-Food Canada, 2010). Although the varietal production in Canada is dominated by 'McIntosh', which accounts for about 30% of the total Canadian apple production, Canadian consumers and growers are moving to new apple cultivars such as 'Honeycrisp' and 'Ambrosia' which command a premium price compared to the traditional cultivars (Agriculture and Agri-Food Canada, 2010). 'Honeycrisp', developed in Minnesota and released in 1991 (DeLong et al., 2004) has a crispy and juicy texture that has made it very popular among consumers and growers (Zhang et al., 2010). While older apple cultivars such as 'Red Delicious' are losing market share, 'McIntosh' is still popular particularly for juice production (Agriculture and Agri-Food Canada, 2010).

Juice is the primary processed product from apple; worldwide apple juice is the second most widely consumed juice following orange (Statistics Canada, 2010). Although apple juice is still widely consumed in Canada, its per capita consumption (7.23 L) is less than orange juice (10.16 L) (Statistics Canada, 2010). In a marketing analysis study conducted in Nova Scotia, apple juice had only 25% share of all juices consumed in 2005 (MacIntosh et al., 2006). According to this survey, the major reasons that influence apple juice purchase include taste (acidity, sweetness), health benefits, and price (MacIntosh et

al., 2006). Thus, studying the factors that affect the taste and nutritional quality of apple juice could improve juice quality and its market share.

Apple juice is processed and sold in many forms, including sweet cider (American apple cider), European apple cider (apple cider), and shelf stable apple juice. Shelf stable juice types include clear juice, cloudy juice, and juice from concentrate (Oke and Paliyath, 2006). Commercially the most popular apple juice in the North American market is a clarified apple juice, which has a typical amber-like hue colour (Oke and Paliyath, 2006). Cloudy apple juice, also called opalescent or natural juice, is unclarified apple juice with yellowish or greenish colour containing a remarkably higher portion of pulp in suspension, which results in greater turbidity and higher nutritional quality than clear juice (Nagel, 1992; Markowski et al., 2009). Cloudy apple juice can be considered a 'minimally processed' product since the process does not involve the rigorous enzymatic clarification or membrane filtration, which are common processing procedures in clear apple juice production (Oszmianski et al., 2007). Several studies have demonstrated that the consumption of either whole apples or cloudy apple juices may be more beneficial to human health than the consumption of clear apple juices (Barth et al., 2005; Barth et al., 2007; Oszmianski et al., 2007; Matthes and Schmitz-Eiberger, 2008). Thus, cloudy apple juice is becoming a fast growing sector, especially in European countries such as Germany where cloudy apple juice accounts for about 30% of their apple juice consumption (Will et al., 2008). To the authors knowledge there are no published reports regarding cloudy juice processing, production and consumption in Canada, which indicates the need of further study.

Currently, the apple industry in the North America and other parts of the world has adopted the extensive use of a potent ethylene action inhibitor, named 1-methylcyclopropene (1-MCP), as a means to extend the storage and shelf life of apples, thus allowing extended supply of seasonal fruits to a global market (Watkins, 2006b). This is achieved by the ability of 1-MCP to block the action of ethylene (Serek et al., 1994) thereby improving flesh firmness retention and preventing superficial scald of apples during, or after, storage (Watkins, 2008; Watkins, 2006a; Blankenship and Dole, 2003). Previous studies on 1-MCP treatment have focused mostly on the relation between 1-MCP and external fruit quality attributes including shelf life, flesh firmness, colour, appearance, postharvest diseases and physiological disorders to support marketing and improve postharvest handling (Watkins, 2007; Seok-Kyu Jung and Watkins, 2009; Lu et al., 2013; Sabban-Amin et al., 2011; Moggia et al., 2010; DeEll and Ehsani-Moghaddam, 2010; Lippert, 2006; DeEll et al., 2002; Rupasinghe et al., 2000). Nevertheless, little information is available regarding the effect of 1-MCP on juice quality parameters such as individual sugars, malic acid, volatile aroma, phenolics and other juice physiochemical properties, which could affect the flavour perception of cloudy and clear juices.

Flavour perception is a combination of taste, aroma, and mouth feel (Baldwin, 2002). The four main juice quality components contributing to the flavour of apple juice include sugars organic acids, phenolics, and volatile aroma compounds (Baldwin, 2002). The major sugars in ripe apples include fructose, sucrose, and glucose with minor amount of the sugar alcohol, sorbitol. Of these, fructose is the most predominant sugar (Hecke et al., 2006; Dever et al., 2006; Eisele and Drake, 2005; Lee and Wrolstad, 1988; Karadeniz and Ekşi, 2002; Schols et al., 1991). As the sweetness level of individual sugars is different,

the change in relative composition as well as total amount of these sugars could play a key role in determining the sweetness sensation of apple juice (Itai and Tanahashi, 2008). In addition, the balance between sugars and organic acids is crucial for the sweet and sour tastes of apple juice (Hecke et al., 2006). Moreover, knowledge of the exact qualitative and quantitative distribution of the characteristic sugars and organic acids is important to detect adulteration in juices, to control changes during processing and storage and to evaluate the impact on the health of consumers (Hecke et al., 2006). Volatile aroma is one of the pivotal organoleptic quality parameters of apple juice used by consumers to evaluate product acceptability and preference (Nikfardjam and Maier, 2011). The content and composition of volatile aroma components of food and beverages is one of the most important quality attributes evaluated when a new product is developed (Roberto et al., 2005).

The consumption of apple and apple products has been linked to a reduced risk of several chronic illnesses including cardiovascular disease, neurodegenerative disease, diabetes, obesity and cancer (Boyer and Liu, 2004; Gerhauser, 2008; Barth et al., 2007; Barth et al., 2005; Will et al., 2008; Kujawska et al., 2011). These health benefits are associated with the major phenolic compounds identified in apple fruits or their products (Wojdyło et al., 2008; Vrhovsek et al., 2004). Antioxidant capacity, which is the ability to inhibit oxidative degradation (Roginsky and Lissi, 2005) is a common characteristic of phenolic compounds resulting from their ability to scavenge and reduce the production of reactive oxygen species (ROS), suppress enzymes involved in oxidative stress, and chelate transition metal ions that promote oxidation (Dangles, 2012). Moreover, phenolic compounds are responsible for the mouthfeel, bitterness, astringency and colour of fresh and processed apple products (Lea, 1992). Thus studying the change of these flavour

metabolites associated with postharvest storage treatments and juice processing techniques could help to improve the organoleptic quality and the health benefit of the final product.

The final concentration of malic acid in ripe fruits is determined by the balance of malate biosynthesis, degradation, and vacuolar storage (Yao et al., 2009; Chen et al., 2009; Khan et al., 2013). A number of studies have shown that 1-MCP could significantly improve acidity retention in ripening apples (Watkins, 2008; Watkins, 2006a; Blankenship and Dole, 2003). However, studies are yet to unequivocally determine the molecular mechanisms by which malate degradation is reduced in response to 1-MCP treatment.

Based on the literature presented it is evident that the potential impacts of 1-MCP treatment on the flavour, nutritional and other quality parameters of apple juice have not been thoroughly investigated. As will be shown in Chapter 2, it has been well established that 1-MCP treatment could suppress the autocatalytic ethylene production and respiration rate essentially maintaining the fruit in a perpetual pre-climacteric stage thereby delaying ripening, senescence and the associated biochemical and physiological quality changes. Therefore, it was hypothesized that inhibition of ethylene action by 1-MCP treatment could improve the juice quality by delaying fruit senescence, maintaining fruit quality, and consequently preventing the development of undesirable flavour and nutritional quality changes.

Hence, in this study we hypothesized that 1-MCP treatment of apples could inhibit ethylene production, delay fruit ripening and senescence and consequently maintain the flavour, nutritional and other quality attributes of cloudy or clear juices prepared from ‘Honeycrisp’ and ‘McIntosh’ apples stored up to 7 months. Recognizing the dynamic biochemical and physiological changes involved in the postharvest storage of the fruit, as

well as the numerous organoleptic considerations in the finished juice, the following research objectives were established to further elucidate the mechanisms of action of 1-MCP and optimum conditions for apple juice production.

1.1 Objectives

1. To evaluate the effect of 1-MCP, storage atmosphere [controlled atmosphere (CA) and regular air (RA)], storage time (4 and 7 months), and harvest maturity (commercial and late) on the changes in ethylene production, flesh firmness, and volatile aroma production of intact 'McIntosh' and 'Honeycrisp' apples.
2. To determine the effect of 1-MCP, storage atmosphere, storage time, and harvest maturity on the acidity and sugar content (fructose, sucrose and glucose) of cloudy and fresh apple juices prepared from 'McIntosh' and 'Honeycrisp' apples.
3. To further investigate the effect of 1-MCP and storage atmosphere on the expression of the genes encoding the key enzymes involved in malic acid metabolism.
4. To determine the effect of 1-MCP treatment, storage atmosphere and harvest maturity on the content and composition of major volatile aroma compounds of cloudy and clear juice samples extracted from 'McIntosh' and 'Honeycrisp' apples.

5. To determine the effect of 1-MCP treatment, storage atmosphere and harvest maturity on the total phenolic, total antioxidant content and physiochemical properties of cloudy and clear juice prepared from ‘McIntosh’ and ‘Honeycrisp’ apples.

Chapter 2: Literature review

Abstract

1-MCP (1-methylcyclopropene) is a strong ethylene action inhibitor, which is thought to act by binding to ethylene receptors. The apple industry in North America has implemented the extensive use of 1-MCP as a means to extend the storage and shelf life of apples. In addition to its physiological effect, 1-MCP is reported to affect the flavour components of apples. The overall flavour of apple fruit and its products is influenced by the composition of sugars, organic acids, phenolics, and volatile aroma compounds as well as by the sugar-acid ratio. The sugar-acid balance is a crucial factor for the sweet and sour taste of apple and is important driver of consumer preference. Several studies confirmed the remarkable inhibition of volatile aroma production and loss of titratable acidity (TA) in 1-MCP treated apples during and after long-term short-term storage. In order to assess the change in acidity and sweetness of apples, measurements of TSS (^{o}Brix) and TA are often included in most studies. However, only few studies have been conducted to uncover the impact of 1-MCP on the content of individual sugars (fructose, glucose, and sucrose) and organic acids and how 1-MCP regulates the changes in sugars or malic acid at the molecular level during long-term storage. This review highlights the effect of 1-MCP treatment on the content and metabolism of the major flavour components of apple fruit or juice including sugars, organic acids, phenolics, and volatile aroma with special focus on fructose, sucrose, glucose and malic acid.

Keywords: 1-MCP, flavour, malic acid, fructose, glucose, sucrose.

2.1 Ethylene and fruit ripening

Ethylene (C_2H_4), the simplest unsaturated olefin, with one double bond and a molecular weight of 28.05, is a gaseous plant hormone that regulates many aspects of metabolic and developmental processes in plants including fruit ripening, senescence of plant organs, root initiation, floral development, seed germination, and response to various abiotic and biotic stresses (Abeles et al., 1992). Because of its crucial commercial and agronomical importance, ethylene's role as regulator of fruit ripening has been studied most intensively.

Ethylene is biologically active in trace amounts (as little as 0.01 μL/L air) (Pech et al., 2010) and plays a double-edged sword role in the ripening process of most climacteric fruits. Besides stimulating ripening, which is important to develop a desirable organoleptic property of most fruits, ethylene also induces senescence, which results in reduced shelf life and substantial postharvest loss of fruits. Hence, minimizing or avoiding the effect of ethylene during storage, transport, and handling of fruits is becoming a common commercial practice (Bai et al., 2005; Watkins et al., 2000). In most apple cultivars, concentrations of ethylene between 0.01 to 10 μL/L are physiologically active to stimulate ripening (Johnston et al., 2009). To slow ripening, storage recommendations are to maintain ethylene concentrations <0.1 μL/L (Stow et al., 2000).

Ethylene is biosynthesized by a pathway that uses the amino acid methionine as a precursor (Wang et al., 2002; Pech et al., 2010; Zarembinski and Theologis, 1994; Kende, 1993; Yang and Hoffman, 1984) (Figure 2.8-1). The first reaction of the pathway involves the transformation of methionine to s-adenosyl methionine (SAM) mediated by the enzyme SAM synthatase at the expense of one ATP (adenosine triphosphate) molecule. In the second step, SAM is further transformed to 1-aminocyclopropane-1-carboxylic acid (ACC)

by the enzyme ACC synthase (ACS). The second reaction also produces methylthioribose (MTA). The MTA generated during this reaction is recycled back to form methionine, which serves to preserve methionine for another round of ethylene production (Kende, 1993). Finally, ACC, which is the immediate precursor of ethylene, is oxidized by ACC oxidase (ACO) to generate ethylene, CO_2, and hydrogen cyanide (HCN). The conversion of ACC to ethylene requires oxygen; hence, in the absence of oxygen ethylene biosynthesis is suppressed. This is why CA storage with very low oxygen concentrations (1-3%) can reduce the production of ethylene. The rate limiting steps in ethylene biosynthesis involves ACC and ACO (Wang et al., 2002). Hence, inhibiting the biosynthesis of ACS/ACO could reduce ethylene production and ethylene-induced ripening events. After its synthesis, ethylene binds to a family of membrane-localized receptors. In apple, three ethylene receptors have been identified, these are ETR1, ERS1 and ERS2 (Wiersma et al., 2007; Tatsuki and Endo, 2006).

Based on the level of ethylene production during ripening and responsiveness to exogenous ethylene, fruits are generally categorized into two ripening groups: climacteric (apple, pear, banana, tomato, avocado) and non-climacteric fruits (orange, lemon, pineapple, etc.) (Paliyath and Murr, 2006). Climacteric fruits are characterized by a pronounced burst in ethylene production and respiration rate during ripening (Klee and Clark, 2010), as well as the enhancement of fruit ripening after exogenous ethylene treatment (Klee and Clark, 2010). The concurrent rise in respiration rate with ethylene production is referred to as the respiratory climacteric (Biale et al., 1954).

During ripening, climacteric fruit ethylene production reaches levels of 30-500 $\mu Lkg^{-1}h^{-1}$ (Paliyath and Murr, 2006). However, in non-climacteric fruit, ethylene

production is only in the range of 0.1- 0.5 $\mu L kg^{-1} h^{-1}$. The ethylene production pattern can further be categorized into system I and system II. The production of low level of ethylene by immature climacteric fruits (pre-climacteric stage) or ripening non-climacteric fruits is termed system I ethylene production (Barry et al., 2000). The massive production of ethylene occurring only in ripening climacteric fruits is termed system II (autocatalytic) ethylene production. In system II, once ethylene is produced in trace amounts, ethylene promotes its own biosynthesis and the internal ethylene production rapidly increases and triggers the onset of ripening (Paliyath and Murr, 2006). In system I ethylene production scheme, ethylene supresses its own biosynthesis. At the molecular level, the two systems are further distinguished by the requirement of ACS and ACO induction in system II but not in system I (Lin et al., 2009; O'Malley et al., 2005; Johnston et al., 2009).

2.1.1 Ethylene-dependent and independent biochemical changes

The role of ethylene in climacteric fruit ripening has been reviewed extensively by several authors and is therefore covered only briefly here. As with other climacteric fruit, ethylene is central in apple fruit ripening by inducing a climacteric burst of respiration accompanied by increased ethylene production and other important changes including higher content of aroma volatile and reduced flesh firmness (Schaffer et al., 2007). Suppressing ethylene biosynthesis through the down regulation of genes responsible for ethylene biosynthesis has led to a better understanding of the role of ethylene in ripening related changes.

Most of the research in this regard has been directed towards the genetic manipulation of the two key genes that control the expression of ACS and ACO (Pech et al., 2010). A study on ‘Greensleeves’ apples that are silenced for either ACS or ACO genes

and stored for 12 days at 20 ºC and 95% RH found no significant difference in total sugar (measured as total soluble solids, TSS) and acid accumulation (measured as titratable acidity, TA) between transgenic apples, which produced 0.004-0.008 $\mu Lkg^{-1}h^{-1}$ of ethylene, and non-transgenic apples that produced 0.04-0.1 $\mu Lkg^{-1}h^{-1}$ of ethylene (Dandekar et al., 2004). Hence, it was concluded that total sugar and acid accumulation are ethylene-independent events (Dandekar et al., 2004). On the other hand, the volatile aroma production and firmness retention were found to be ethylene-dependent ripening events, where higher ethylene production rates in non-transgenic fruit, (0.04-0.1 $\mu Lkg^{-1}h^{-1}$) lead to a significant increase in volatile production and reduction of firmness (Dandekar et al., 2004).

A similar study in determining ethylene-dependant and -independent changes in transgenic 'Greensleeves' apples and 1-MCP treated non-transgenic 'Greensleeves' apples, stored for 14 days at 20 ºC and 90-95% RH, indicated inhibited ethylene production in both transgenic (<5 $\mu Lkg^{-1}h^{-1}$) and 1-MCP treated (5 $\mu Lkg^{-1}h^{-1}$) (Defilippi et al., 2004) fruits as compared to the non-transgenic 'Greensleeves' (50-60 $\mu Lkg^{-1}h^{-1}$) . This is contrary to Dandekar et al. (2004) and Defilippi et al. (2004), who found a significant retention of TA when ethylene production or action was suppressed and suggested that change of TA during ripening is an ethylene-dependant event. As well, Defilippi et al., (2004) reported a low ethylene dependency of TSS accumulation and starch loss.

Defilippi et al. (2004) also studied the ethylene dependency of individual sugars (sucrose, fructose, glucose, and sorbitol) and individual organic acids (malic and citric) and found increased accumulation of sucrose and fructose with little change in glucose when apples were ripened with ethylene. However, after 1-MCP treatment, reduced sucrose

accumulation was observed. Hence, the authors suggested that while the changes in sucrose level were ethylene-dependent, the level of glucose and fructose were not.

A study on transgenic 'Royal Gala' apples containing *ACO1* antisense genes that only produce <0.00013 $\mu L g^{-1} h^{-1}$ (Schaffer et al., 2007) levels of ethylene at full climacteric stage and stored for 14 days at 20 or 4 ºC revealed that ethylene mediated ripening is regulated through ethylene dependency and sensitivity (Johnston et al., 2009). According to Johnston et al. (2009), ethylene dependency refers to the ratio of the maximum response with the presence of ethylene relative to the response in the absence of ethylene as is calculated as given in Eq. 2.1 to give percentage ethylene dependency values from 0 to 100% (0 = independent, 100 = dependent).

$$\text{Ethylene dependency } (\%) = \left[\frac{(\text{Ethylene response at 1000 } \mu L/L - \text{Ethylene response at 0 } \mu L/L)}{\text{Ethylene response at 1000 } \mu L/L}\right]100 \qquad \text{Eq. 2.1}$$

Ethylene sensitivity denotes the minimum concentration of ethylene needed to attain a significant response and is calculated as the ethylene concentration required for a 50% response (Johnston et al., 2009). According to this study (Johnston et al., 2009), early ripening events such as the conversation of starch to sugar, TSS accumulation and loss of TA had a weak ethylene dependency but are highly sensitive to low concentration of ethylene (≤ 0.01 µL/L). On the other hand, later ripening events such as firmness loss and volatile production were found to be strongly ethylene dependent but less sensitive to low concentration (≥ 0.1 µL/L) for a response. The authors found irrespective of the difference in internal ethylene concentration (< 0.1 µL/L in transgenic and 100 µL/L in control), both the transgenic and control 'Royal Gala' apples had a large degree of starch degradation, increase in TSS and a loss of TA during the 14-day ripening period (Johnston et al., 2009).

This supports the weak ethylene dependency of these ripening parameters. However, significant loss of flesh firmness, increase in total volatiles and loss of background colour occurred only in control fruit where internal ethylene concentration (IEC) was close to 100 µL/L, which leads to the conclusion that these traits are ethylene dependent. The authors investigated ethylene sensitivity of different ripening events of apple by exposing transgenic 'Royal Gala' to ethylene concentrations from 0.01-1000 µl/L for 14 days at 20 ^{o}C. They found that the majority of the response that occurred between 0.1 and 10 µL/L for loss of firmness and background colour and increase in total volatiles, while in contrast, a lower range of 0.01 to 1.0 µL/L was required for starch loss (Johnston et al., 2009). In other words, starch degradation started at 0.01 µL/L and reached a maximum at 1.0 µl/L with little or no change thereafter at which point the process was saturated (Johnston et al., 2009). In summary, fruit ripening is a complex developmental process where ethylene is acting as a modulator of ripening rather than a ripening trigger (Johnston et al., 2009).

2.2 Sugar and organic acid metabolism after harvest

2.2.1 Importance of sugar and acids

The overall flavour of apple fruit and its products is influenced by the composition of sugars, organic acids, phenolics, and volatile aroma compounds, and the sugar-acid balance (Baldwin, 2002). Of these, the sugar-acid balance is a key component for the sweet and sour taste of the fruit and therefore is of major significance for consumers (Hecke et al., 2006).

The knowledge of the exact qualitative and quantitative distribution of the characteristic sugars and organic acids is important to evaluate quality, detect adulteration

in juices or control changes that may occur during processing and storage. Understanding the sugar and acid content of fruits is also crucial since it contributes for the health of consumers (Hecke et al., 2006). A low level of sucrose in apple is an advantage for diabetic patients since it helps to keep the blood sugar level constant (Hecke et al., 2006). The regular consumption of fruit acids is helpful in preventing illness by lowering the postprandial blood glucose and insulin responses (Hecke et al., 2006). Moreover, it is known that malic acid dissolves uric acid and is therefore an important source of relief when someone suffers from rheumatism (Hecke et al., 2006). However, people who react to fruit acids and breastfeeding mothers are recommended to eat apple cultivars with low acid concentrations (Hecke et al., 2006). This might be related to the prevention of dental erosion due to the acid in the apple juice. As the critical pH at which enamel erosion occurs is 5.5, acidic products like apple juice with a pH below 4.0 can result significant enamel erosion (Mita et al., 2013).

2.2.2 Sugar metabolism

Apples are harvested commercially at the pre-climacteric stage and are commonly cold stored to delay ripening and maintain availability during off seasons (Knee, 1993). Since harvested apples are living biological entities, metabolic processes are maintained by respiration, which mainly consists of three interconnected pathways, glycolysis, tricarboxylic acid (TCA) cycle, and the electron transport chain (Abeles et al., 1992). Respiration in higher plants is the oxidative breakdown of complex substrates, mainly sugars and organic acids, to produce energy and intermediate compounds that are required to sustain the different metabolic reactions essential for the maintenance of cellular

organization and membrane integrity in the living cells (Bapat et al., 2010). Since sugars and malic acid are the main substrates involved in aerobic respiration of harvested apples, their catabolism will continue without a supply from photosynthesis, which results in the depletion or change in the composition of sugars and acids in the fruit (Ackermann et al., 1992). This could lead to noticeable alterations in fruit flavour, which is an important, non-visual quality attribute of consumers. Hence understanding sugar and acid metabolism in harvested apple is valuable for maintaining fruit and juice quality in the postharvest period.

Unlike some fruits where carbon is stored in the form of starch, in apples, a mixture of starch and soluble sugars (fructose, sucrose, glucose) is accumulated during early fruit development (Duque et al, 1999). As the development stage advances, there is steady accumulation of fructose, sucrose, and glucose with dramatic degradation of starch (Berüter, 1985; Zhang et al., 2010). Starch disappears just before harvest (Berüter, 1985; Zhang et al., 2010) and at harvest. The major sugars in ripe apples include fructose, sucrose, and glucose, with minor amount of sorbitol. Of these, fructose is the most dominant sugar (Hecke et al., 2006; Dever et al., 2006; Eisele and Drake, 2005; Lee and Wrolstad, 1988; Karadeniz and Ekşi, 2002; Schols et al., 1991). In many fruits, sucrose is a well-known translocatable carbohydrate from source (leaves) to sink (fruit) tissues. However, apples and other fruits in the Rosaceae family synthesize sorbitol, in addition to sucrose, in source leaves and both are translocated to the fruit (Webb and Burley, 1962; Loescher et al., 1982; Kanayama et al., 2008; Teo et al., 2006). The translocated carbohydrates in the sink tissue are comprised of about 70% sorbitol and 30% sucrose (Berüter, 2004; Klages et al., 2001). In the sink tissue, almost all of the sorbitol and half of the sucrose molecules are converted to fructose (Li et al., 2012b) consequently fructose is the major hexose sugar accumulated

in apple fruit. After sorbitol is translocated into the cell wall, space in apple fruit (Zhang et al., 2004) it will be taken up into the cytosol of parenchyma cells and then converted to fructose by sorbitol dehydrogenase (SDH, EC 1.1.1.14). After translocation, sucrose is either directly taken up into parenchyma cells and then broken to fructose and glucose by cytoplasmic neutral invertase (Li et al., 2012) or first broken into glucose and fructose by cell wall invertase and then transported into the parenchyma cells (Zhang et al., 2004) (Figure 2.8-2).

A fruit development study in 'Honeycrisp' and 'Greensleeves' apples indicated that while glucose remains unchanged there was a considerable accumulation of fructose and sucrose during fruit maturity (Li et al., 2012; Zhang et al., 2010). The accumulation of fructose during fruit development is mainly attributed to the conversion of sorbitol to fructose via SDH (Zhang et al., 2010; Li et al., 2012), which is associated with the exponential reduction of sorbitol in 'Honeycrisp' apples (Zhang et al., 2010). The decreased expression and activity of fructokinase (FK EC 2714), which leads to less fructose catabolism, was also observed in 'Greensleeves' apples (Li et al., 2012). Moreover, up-regulation of carrier proteins is responsible for the active transport of fructose from the cytosol contributed for the accumulation of fructose during fruit development (Li et al., 2012). The sustained accumulation of sucrose towards fruit maturity was ascribed to the sucrose transported from the leaves, newly re-synthesised sucrose via sucrose phosphate synthase (SPS, EC 2.4.1.14) or sucrose synthase (SS, EC. 2.4.1.13), as well as starch break down (Li et al., 2012; Zhang et al., 2010). Li et al (2012) observed higher activity and gene expression level of sugar metabolizing enzymes including SDH, invertase, SUS, and FK during the early stage of fruit development (Li et al., 2012), to

facilitate rapid metabolism of imported sugars to satisfy the high demand for energy and other intermediates at this stage (Li et al., 2012). However, as development stage advances the requirement of energy decreases which results decreased expression levels and activities of these enzymes that in turn leads to reduced metabolism of imported sugars (Li et al., 2012). This suggests that sugar metabolism before storage of apple fruit is highly regulated by developmental processes.

The metabolism of sugars in harvested apple is different in such a way that, catabolism of sucrose via invertase or SS becomes more active (Mao et al., 2007; Zhu et al., 2013). Fructose and glucose are catabolized through the two respiratory pathways; glycolysis and the TCA cycle (Figure 2.8-3 and Figure 2.8-4). Before heading to glycolysis and TCA cycles glucose and fructose are phosphorylated to glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) by hexokinase (HK, EC 2.7.1.1) and FK and enter to glycolysis and TCA cycle to generate energy and intermediates for other processes (Zhu et al., 2013). Glycolysis is the first stage of respiratory metabolism by which glucose undergoes partial oxidation to produce pyruvate, ATP and NADH (Nicotinamide adenine dinucleotide, reduced). Under anaerobic condition, pyruvate changes to ethanol, while under aerobic condition it enters to the TCA cycle for complete oxidation and production of energy and CO_2 (Paliyath and Murr, 2006). Sugars that have not metabolized will be stored in the vacuole. Once sucrose is stored in the vacuole, it can be hydrolysed to fructose and glucose by vacuolar invertase or SS (Li et al., 2012). The re-synthesis of sucrose after cleaved by SS or invertase is still debatable (Li et al., 2012).

In ripening mangos the activity of SPS increased about 10-fold during the phase of rapid sucrose accumulation (Castrillo et al., 1992). Similarly, in apple that activity of SPS

increased by 2-fold during the gradual accumulation of sucrose in 60 days of storage at 4 ^{o}C (Duque and Arrabaça, 1999). During the same period, hexose sugars (glucose-1-phosphate, G6P, fructose-1,6-bisphosphate and fructose-1-phosphate) declined considerably, which suggested the production of sucrose from these hexoses through SPS activity (Duque and Arrabaça, 1999). On the other hand, Drake and Eisele, (1999) reported reduced level of sucrose in 'Gala' apples after storage in RA conditions for 45-90 days. This might be due to hydrolysis of sucrose to glucose and fructose during RA storage (Eisele and Drake, 2005). As indicated in Figure 2.8-2, during storage, sucrose is irreversibly cleaved to fructose and glucose by invertases (EC 3.2.1.26) or reversibly converted to fructose and uridine diphosphate glucose (UDP-glucose) by SS (EC 2.4.1.13) (Itai and Tanahashi, 2008).

Recently Zhu et al. (2013) investigated changes in sugar content in 'Fuji' apples that were stored in RA or CA condition up to 8 months. This study also determined the biochemical and molecular basis for changes in sugar content during storage (Zhu et al., 2013). The authors observed a significant degradation of sucrose during storage either under CA or RA conditions (Zhu et al., 2013). Nevertheless, the loss of sucrose was significantly lower in CA than RA conditions (Zhu et al., 2013). Even though the authors did not observe a significant change in fructose and glucose during storage, higher glucose and sucrose content were found in CA than RA stored fruits (Zhu et al., 2013). The higher sucrose content in CA stored fruit is attributed to greater SS activity as well as its promoted gene expression level in CA than RA conditions (Zhu et al., 2013). Similar trends were reported in peaches (Lara et al., 2011) and potato tubers sored in a reduced oxygen environment (Bologa et al., 2003). Interestingly, the activity and gene expression level of

invertase were lower under CA. This suggests the degradation pathway of sucrose may vary depending on the storage atmosphere. As discussed earlier, there are two alternative biochemical pathways for sucrose degradation that involves invertase or SS. These pathways have different energy requirements (Lara et al., 2011; Stitt, 1998). While the hydrolysis of sucrose by invertase requires two molecules of ATP, its catabolism by SS requires only one molecule of inorganic pyrophosphate (PPi) (Lara et al., 2011; Stitt, 1998). Consequently, the hydrolysis of sucrose through the energetically less costly route provided by SS pathway might be a favorable pathway in CA stored apples. This will save more energy or decrease oxygen consumption in CA storage conditions (Bologa et al., 2003; Lara et al., 2011). Moreover, the activity and expression level of SPS, FK and HK were promoted in apples stored in CA, suggesting that CA storage could delay the hydrolysis of sucrose (Zhu et al., 2013). Hence, the authors concluded that CA storage could enhance sucrose synthesis and delay hydrolysis of sucrose.

2.2.3 Malic acid metabolism

Malic acid, the major organic acid in apples, is responsible for the sourness and acidity of apples (Dandekar et al., 2004). It has been demonstrated that in the high acid mutant of 'Usterapfel' apples the concentration of malic acid increased by 10 fold up to 800 µmol/g (dry weight) as compared to a sweet-tasting (low-acid) form of 'Usterapfel' apples (Berüter, 1998). Malic acid accounts for about 90% of the acid in apples, while citric, succinic and traces of several other acids make up the rest (Ackermann et al., 1992). Malic acid accumulates up to 100 µmol/g (fresh weight) in young fruits and gradually decreases

during ripening (Yao et al., 2009); this reduction is due to malic acid being the major substrate of respiration metabolism (Berüter, 2004).

Malic acid is synthesized mainly during fruit maturation and development, most likely from carbohydrate precursors such as glucose, fructose, and sucrose, which are stored in the fruit. The two key enzymes that regulate malic acid accumulation include cytosolic phosphoenolpyruvate carboxylase (PEPC) and NAD dependant cytosolic malate dehydrogenase (NAD-MDH), which catalyse the conversion of phosphoenolpyruvate (PEP) to oxaloacetic acid (OAA) and then to malate (Berüter, 2004). NAD-MDH catalyses the reversible conversion of malic acid to OAA, the most likely direction being the synthesis of malic acid (Yao et al., 2011; Sweetman et al., 2009). As depicted in Figure 2.8-2, these reactions take place in the cytosol (Sweetman et al., 2009). Yao et al. (2011) demonstrated the contribution of NAD-MDH for the increased content of malic acid in acidic apples. As compared to the mitochondrial form, cytosolic NAD-MDH represents 70-80% of the total NAD-MDH and therefore given greater consideration than the mitochondrial form (Abou-Zamzam and Wallace, 1970).

During the ripening process, malic acid could be degraded through one of three pathways (Figure 2.8-2). The first option is the conversion of malic acid into pyruvate by the activity of NADP (Nicotinamide adenine dinucleotide phosphate) dependent cytosolic malic enzyme (NADP-ME). NADP-ME catalyses the reversible conversion between pyruvate and malic acid, and is potentially involved in both malate synthesis and degradation (Zheng et al., 2013). Pyruvate could then be converted either into PEP or directly headed to TCA cycle in the mitochondrion or to a partial oxidation pathway through anaerobic respiration route.

The second catabolism pathway could be conversion of malic acid to PEP via OAA by the action of PEP carboxykinase (PEPCK), which leads to the gluconeogenesis pathway to produce glucose and then fructose and glucose (Figure 2.8-5). The involvement of NADP-ME to reduce malic acid in the ripe flesh of low acid apple cultivars has been demonstrated (Yao et al., 2009). The third option could be the direct involvement of the TCA cycle. Finally, extra malic acid may be accumulated in the vacuole of pulp cells that contain 85-90% of the total malic acid (Yao et al., 2009). Some studies demonstrated that the change in malic acid concentration during fruit development could mainly be controlled at the level of vacuolar storage (Etienne et al., 2013; Khan et al., 2013). However, the regulation of vacuolar malate storage during postharvest storage of apples remain to be clarified

2.3 1-Methylcyclopropene (1-MCP)

2.3.1 Discovery and commercialization of 1-MCP

As mentioned earlier, ripening of climacteric fruits is ethylene regulated. Thus in order to circumvent the harmful effects of ethylene, inhibiting its biosynthesis and/or removing it from the atmosphere surrounding perishable products should be achieved. Different technologies have been attempted to achieve this objective (Martinez-Romero et al., 2007). More recently, the apple industry in the North America, including Canada, has adopted the extensive use of 1-MCP as a means to extend the storage life of apples (Watkins, 2006). This is due to the effectiveness of 1-MCP in blocking the action of ethylene, thereby improving the retention of firmness and reducing superficial scald of stored apples (Watkins, 2008; Tsantili et al., 2007; DeEll et al., 2002). A non-volatile 1-MCP named

N,N-dipropyl (1-cyclopropenyl-methyl) amine (DPCA) has also been recently produced and used to protect several ornamental plants against ethylene action (Seglie et al., 2010).

1-MCP is a potent inhibitor of ethylene action in plant tissues and is deemed to act by binding to ethylene receptors (Sisler et al., 2006; Sisler and Serek, 1999). The breakthrough that led to the development of 1-MCP was the result of an effort to identify the ethylene binding site protein (Reid and Staby, 2008). The use of 1-MCP to inhibit ethylene action was discovered and patented by Sisler and Blankenship in 1996 at North Carolina State University. The commercialization and application of 1-MCP was first started in 1999 for ornamental plants using the product called EthylBlock™ that is formulated by Floralife Inc. (Watkins and Nock, 2005; Blankenship and Dole, 2003; Watkins, 2006). Later, in 2002 AgroFresh Inc. (a wholly owned subsidiary of the Dow Chemical Company) assumed the commercial postharvest application of 1-MCP to fresh fruits and vegetables under the trade name of SmartFresh™. Currently 1-MCP has been accepted and registered by the US Environmental Protection Agency (EPA) and European Union (EU) as reduced risk product for use on several fruits and vegetables 37 countries, including Canada (Watkins, 2006; Watkins, 2009). The registered fruits and vegetables include apple, banana, broccoli, date, cucumber, apricot, avocado, kiwifruit, mango, melon, nectarine, papaya, peach, pear, pepper, persimmon, pineapple, plantain, plum, squash, and tomatoes (Watkins, 2009). In the retail market, 1-MCP products for postharvest fruit and vegetable treatment are available in different trade names with various level of the active ingredient 1-MCP (Table 2.6-1). Recently new formulations of 1-MCP have been developed for application as a sprayable preharvest treatment (Table 2.6-1). The

sprayable 1-MCP is registered in the US, Argentina and Chile for commercial use as Harvista™ for the tree fruit products and Invinsa™ for field crops but it is still under research (Nock et al., 2009). Some studies have demonstrated the effectiveness of sprayable 1-MCP in reducing preharvest fruit drop, soft scald development, IEC and starch hydrolysis (DeEll and Ehsani-Moghaddam, 2010; Elfving et al., 2007).

2.3.2 What is 1-MCP?

Structurally, 1-MCP is a four carbon cyclic olefin with a three-membered ring to which a methyl group is attached at the C-1 position. At standard temperature and pressure, 1-MCP is a gaseous cyclopropene with molecular weight of 54 and a molecular formula of C_4H_6 (Neoh et al., 2007). In the gaseous state, it is chemically unstable and begins to self-react immediately. Furthermore, it is also presents an explosive hazard when compressed (Neoh et al., 2007).

Due to the previously mentioned difficulties, 1-MCP is formulated as a powder or tablet by encapsulating with cyclodextrin (CD) compounds with alpha-cyclodextrin being the most preferred (Daly and Kourelis, 2001). The encapsulation improves the stability of the product during transportation and storage. CDs are water soluble cyclic oligosaccharides made up of 6, 7, 8 D-glucopyranose units linked through α-1,4 glycosidic bonds and are produced from natural starch by the enzymatic degradation of cyclodextrin glycosyltransferase (Neoh et al., 2008). CDs have a truncated molecular structure, which is hydrophilic (polar) on the exterior and relatively hydrophobic (less polar) in the interior (Neoh et al., 2007). This unique molecular structure imparts an ability to encapsulate a less polar 1-MCP molecule within their hydrophobic interior part (Neoh et al., 2007). The

release of 1-MCP gas from the 1-MCP/CD complex is accomplished by simply adding water, which can dissolve the hydrophilic part of the CD complex (Kostansek, 2002). This reaction is faster with heating or changing the pH of the water (Daly and Kourelis, 2001). Release from the CD is typically complete within an hour or two and venting is required after 24 h exposure (Daly and Kourelis, 2001).

As compared to other ethylene response inhibitors such as silver thiosulfate (STS), silver nitrate, diazocyclopentadiene (DACP), and other cyclopropenes such as cyclopropene, 3-methylcyclopropene (3-MCP), 3,3-Dimethylcyclopropene (3,3-DMCP), 1-MCP has several interesting merits. 1-MCP is environmentally friendly and a safe product due to its low animal toxicity (acute inhalation toxicity in rat; LC_{50} >160 mg/L, category IV toxicity) with negligible residue (0.002-0.4 µg/L) (EPA, 2002). The distribution of 1-MCP in plant tissues is fast and no noticeable levels are found in the core of apples treated with 1-MCP after 8 h of treatment (Blankenship and Dole, 2003).

Silver nitrate, STS, and DACP have been shown to be inhibitors of ethylene action. However, neither of these compounds can be used in foods since silver is a heavy metal and a potential pollutant and DACP is explosive at high concentrations (Sisler and Serek, 1997). 1-MCP has higher efficacy at lower concentration (1.9 nL/L) and short exposure time compared to other cyclopropenes (Blankenship and Dole, 2003). Moreover, 1-MCP is known to be an effective ethylene antagonist that induces long lasting insensitivity to ethylene upon a single exposure, while other synthetic ethylene antagonists require continuous exposure (Grichko, 2006). Finally, 1-MCP is available in stable and easily applied formulations including powder and tablets (Bai et al., 2005; Watkins and Miller, 2002).

2.3.3 Mode of action of 1-MCP

It has been suggested that 1-MCP blocks the action of ethylene in two ways, by binding with ethylene receptors and suppressing ethylene biosynthesis. The structural similarity of 1-MCP with ethylene allows its interaction with ethylene receptors. As compared to ethylene, 1-MCP has much greater (10 times) affinity to ethylene receptors and hence it can displace ethylene from its receptor sites irreversibly and plants will not respond to ethylene until new ethylene receptors are regenerated (Watkins and Nock, 2005; Blankenship and Dole, 2003; Watkins, 2006). The greater affinity of 1-MCP to the ethylene receptors is ascribed to the strained cyclic alkenes, which form very stable adduct with the Cu (I) ion found on ethylene receptors (Grichko, 2006).

It has also been found that 1-MCP treatment could suppress the expression of genes regulating ethylene receptors (ETR1, ETR2, ETR5, ERS1, and ERS2) in 'Orin', 'Fuji' (Tatsuki and Endo, 2006) and 'Golden Delicious' apples (Yang et al., 2013). In addition to blocking ethylene receptors, the ability of 1-MCP to strongly suppress the expression of genes controlling ethylene biosynthesis has been demonstrated (Li and Yuan, 2008; Tatsuki et al., 2007).

2.3.4 Factors affecting effectiveness of 1-MCP

Several interconnected factors such as cultivar, harvest maturity, temperature, treatment delay, IEC at harvest, storage conditions, method of application, and concentration can affect the efficacy of 1-MCP.

Significant cultivar effects were reported in different studies. Recently, Lu et al. (2013a) reported that the effectiveness of 1-MCP on inhibiting softening, loss of TA and

superficial scald development was much greater for ‘Delicious’ than for ‘Cortland’ apples. Similarly, previous studies demonstrated the enhanced success of 1-MCP in cultivars such as ‘Delicious’, ‘Granny Smith’, and ‘Law Rome’ (Fan et al., 1999; Rupasinghe et al., 2000; Watkins et al., 2000; Magazin et al., 2010; Jung and Watkins, 2014) as compared to ‘McIntosh’ and ‘Cortland’ apples. In all the above studies, the effectiveness of 1-MCP treatment appears to be related to whether or not inhibition of ethylene production is maintained during long-term storage.

For most fruits, 1-MCP is effective when it is applied at temperatures ranging from 20-25 ^{o}C (Mir et al., 2001). Several studies reported that at a given concentration of 1-MCP, lower temperature treatments yielded less effectiveness (Mir et al., 2001). The reason for the reduced effectiveness of 1-MCP at lower temperature is attributed to the possible lower affinity of the binding sites to 1-MCP. DeEll and others studied the effect of various temperatures (3, 13, 23 ^{o}C) and treatment durations (0 to 48 h) in ‘Cortland’ and ‘Empire’ apples and indicated that the lower the temperature the longer the treatment duration was required (DeEll et al., 2002). For instance, in ‘Cortland’ improved retention of firmness was observed in 6 h at 13-24 ^{o}C as compared to 9 h at 3 ^{o}C. Similarly, Mir et al. (Mir et al., 2001) studied the effect of different treatment temperatures (0, 5, 10, 15, 20 ^{o}C) on 1-MCP treated ‘Redchief Delicious’ apples and found that as temperature decreased below 15 ^{o}C the benefits of 1-MCP became less pronounced. Likewise, Toivonen and Lu (2005) reported a reduced effect of 1-MCP in early ripening summer apples as treatment temperature goes below 15 ^{o}C.

Several studies demonstrated that the level of IEC in fruit at the time of 1-MCP treatment are a crucial factor for the effectiveness of 1-MCP in apples (Watkins and Nock,

2005; Mir et al., 2001; Nock and Watkins, 2013; Jung and Watkins, 2014). The higher IEC, which is commonly observed in late harvested apples (Mir et al., 2001) could compete for the binding sites on ethylene receptors that make the fruits less responsive to 1-MCP application. Cooling the fruit before 1-MCP application has a beneficial effect by reducing the IEC at harvest (Watkins and Nock, 2012).

DeEll and others evaluated the effects of 1-MCP concentration by comparing 0.625 µL/L vs. 1 µL/L combined with treatment time (3, 7, 10 days after harvest) on 'McIntosh' apples that were then stored under RA (0-1 ºC for 3-6 months) or in CA (3 ºC for 6-9 months) conditions (DeEll et al., 2008). The study observed better firmness retention in apples treated with 1 µL/L 1-MCP, especially for the earliest harvest (first harvest). The same study (DeEll et al., 2008) indicated the benefit of minimizing treatment delays following harvest. Their result supports the SmartFresh™ label in Canada, which states the application must be within 3 days of harvest. 1-MCP concentration less than 1 µL/L can reduce the beneficial effect in 'McIntosh' apples, especially with treatment delays and long storage periods (DeEll et al., 2008). Longer delay periods before 1-MCP treatment increased IEC and consequently reduced effectiveness of the treatment (Watkins and Nock, 2005).

Storage condition is another crucial factor for the effectiveness of 1-MCP. Several studies indicated the synergistic effect of CA and 1-MCP in reducing ethylene concentration, loss of firmness, and incidence of superficial scald (Rupasinghe et al., 2000; Johnson, 2002; Watkins et al., 2000; Zanella, 2003). However, Akbudak et al. (2009) indicated that 1-MCP could maintain the quality of apples in both CA (1% CO_2 + 1% O_2) and RA (0.03% CO_2 + 21% O_2) storage condition to a higher degree than untreated apples

over 4 months of storage. This suggests that applying 1-MCP without CA storage might be a workable alternative to use the 1-MCP technology in areas where there is no CA facility. However, irrespective of 1-MCP treatment, loss of TA increased after 6 months of storage in both CA and RA being greatest in RA storage. Even if 1-MCP binds permanently to receptors present at the time of treatment, the possibility of regenerating new receptors has been suggested (Akbudak et al., 2009). This could explain why 1-MCP treated fruits become sensitive to ethylene after long-term storage (Blankenship and Dole, 2003a; Sisler and Serek, 1997). Hence, repetition of 1-MCP treatments in long-term storage is recommended for the continuity of 1-MCP activity (Akbudak et al., 2009). A study which compared the efficacy of 1-MCP applied in water (dissolved) and air (gaseous) in 'Golden Delicious' apples found that both application methods could prevent the deleterious effect of ethylene, nevertheless treatments in water requires a 700-fold higher amount of 1-MCP compared to treatments in air (Argenta et al., 2007).

2.4 Effects of 1-MCP on apple fruit and juice quality

2.4.1 Physiological effect

As discussed earlier, 1-MCP could block ethylene binding sites (Sisler et al., 2006) and thereby inhibiting and delaying ethylene dependent responses including respiration, ripening rate, IEC, loss of firmness and the incidence of some physiological disorders. Of these, the most important effects of 1-MCP have been its ability to maintain flesh firmness and reduce superficial scald of apples during and after storage (Watkins, 2008; DeEll et al., 2002; Watkins, 2007; Tsantili et al., 2007; Rupasinghe et al., 2000).

2.4.2 Effect of 1-MCP on sugar and malic acid metabolism

It has been reported that 1-MCP increased, decreased, or had no effect on the content of total soluble sugar (TSS) in different apple cultivars (Table 2.6-2). Several studies found that 1-MCP has no or little effect on TSS content of 'Granny Smith' (Akbudak et al., 2009), 'McIntosh', and 'Delicious (Rupasinghe et al., 2000), 'Cortland' and 'Empire' (DeEll et al., 2002), and 'Golden Smoothee' (Larrigaudiere et al., 2008) apples. On the other hand, higher TSS was observed in 1-MCP treated 'Honeycrisp' apples stored under CA for 6-months (DeEll, 2005), while Watkins et al.(2006) reported lower TSS level in 1-MCP treated 'McIntosh' and 'Law Rome' apples. The above findings indicated the inconsistent effect of 1-MCP on the TSS content of apple and the reason is still uncertain. Fan et al (1999) suggested that the accumulation of soluble solids in apple might not depend on ethylene perception or action.

As indicated in Table 2.6-2, a number of studies demonstrated the effectiveness of 1-MCP to suppress or delay the loss of total acidity (measured as TA) during storage of apples. However, the extent of TA loss suppression depends on time, storage condition and other related postharvest conditions (Jemrić et al., 2013; Rupasinghe et al., 2000; Larrigaudiere et al., 2008; DeEll, 2005; Watkins et al., 2000; Mir et al., 2001; DeLong et al., 2004; Lu et al., 2012; DeEll and Ehsani-Moghaddam, 2013; DeEll and Ehsani-Moghaddam, 2010; Çelikel et al., 2009; Fan et al., 1999).

As mentioned earlier, several studies evaluated the change of sugars and acidity in terms of TSS and TA; however, there is very little information on the effects of 1-MCP on the major specific sugars (fructose, glucose and sucrose) and organic acids (malic acid). Sucrose, with a sweetness value of 100, is considered as the standard in comparing the

sweetness level of different sugars (Charalambous, 1982). Accordingly, the respective relative sweetness value of fructose, glucose and sorbitol is 150-170, 70-80, and 55-70 (Pancoast and Junk, 1980). As the sweetness level of individual sugars is different, the composition and total amount of these sugars play a key role in determining the sweetness sensation of apple juice (Itai and Tanahashi, 2008). The sweetness index of apple juice, an estimate of total sweetness perception can be calculated based on the amount and sweetness levels of individual sugars in the juice (Sánchez et al., 2014). For instance, a juice with 48% sucrose, 48% fructose and 4% glucose will have a relative sweetness index of

Sweetness index = [(48 x 100) + (48 x 150) + (4 x 70)]/100 = 114.4.

On the other hand, if sucrose is reduced to 5%, fructose, and glucose elevated to 75 and 25%, respectively, the sweetness index will be 150. Meaning that the juice samples with sweetness index of 114.4 and 150 will be 1.14 and 1.5 times sweeter than sucrose

Defilippi et al. (2004) studied sugar and malic acid changes in transgenic 'Greensleeves' that were silenced for either ACS or ACO as well as 1-MCP-treated, non-transgenic 'Greensleeves' apples. After storing apples for 14 days at 20 ^{o}C and 90-95% RH, the authors reported that ethylene production was inhibited in both transgenic (95%) and 1-MCP-treated, non-transgenic (70%) apples. The study found a significant reduction of malic acid degradation when ethylene production or action was inhibited by 1-MCP, suggesting that malic acid accumulation is an ethylene-dependant event. Regarding sugars, the same study reported increased accumulation of sucrose and fructose with little change in glucose when apples were ripened with ethylene. However, after 1-MCP treatment, reduced sucrose and total sugar accumulation was observed.

A recent study investigated the impact of 1-MCP application on the content of individual sugars, organic acids, phenolics and ascorbic acid in 'Idared' apples that were stored in ultra-low oxygen for 6-months and then exposed to room temperature for 16-days (Bizjak et al., 2012). Unlike Defilippi et al. (2004), this study reported that 1-MCP treatment resulted in higher amount of total sugars, fructose, glucose, and malic acid during storage than untreated fruit. Their result also indicated that the 1-MCP treatment had no effect on the significant loss of sucrose. The reduced loss of malic acid, fructose and glucose in 1-MCP treated apples is attributed to suppressed respiration, which is a consequence of 1-MCP treatment.

Limitations of the above studies (Bizjak et al., 2012; Defilippi et al., 2004) include, the effect of 1-MCP on the composition of individual sugars and acids in apples only being measured at room temperature for a short-term storage time as well as no evidence at the molecular level was presented. A study conducted on Japanese pear found a contrasting result that 1-MCP treated pears had more sucrose and less fructose and glucose (Itai and Tanahashi, 2008) and 1-MCP was found to regulate the genes controlling sucrose metabolizing enzymes including SS, acid invertase and SPS. They found that 1-MCP prevented the loss of sucrose by reducing the activity of invertase or by increasing the activity of SPS. Other studies have also indicated the key role of sucrose-metabolizing enzymes in regulating the sugar content of ripening apples (Duque and Arrabaça, 1999), mango (Castrillo et al., 1992), kiwi (MacRae et al., 1992) and melon (Hubbard et al., 1989). Deng et al. (Deng et al., 2013) also reported the key role of sucrose in the carbohydrate economy of apple. On the other hand, 1-MCP was reported to cause negligible changes in the activity of enzymes catalysing the key steps of the glycolysis pathway including

phosphoenolpyruvate kinase, phosphoenolpyruvate, and pyruvate kinase in ripening peach (Borsani et al., 2009), and stored banana (BALL and Tom, 1988). Hence, it was postulated that enzymes involved in glycolysis and the TCA cycle might not affect sugar accumulation during storage.

2.4.3 Effects on phenolics, ascorbic acid, and volatile aroma

A number of studies indicate that the content of most phenolic compounds were not changed significantly in 1-MCP treated apples (DeEll et al., 2005a; DeEll et al., 2005b; Fawbush et al., 2009; Qiu et al., 2009; Vilaplana et al., 2006; MacLean et al., 2006; Hoang et al., 2011), except for chlorogenic acid (MacLean et al., 2006; Hoang et al., 2011), which showed significant reduction.

Treatment with 1-MCP did not significantly affect ascorbic acid concentration in 'Empire' (Fawbush et al., 2009) or 'Jonagold' (Kevers et al., 2011) apples stored under RA or CA. On the other hand, in 'Golden Smoothee' apples, 1-MCP treatment resulted in reduced ascorbic acid content (Vilaplana et al., 2007; Vilaplana et al., 2006). The reason for this reduction of ascorbic acid in 1-MCP treated fruits is still uncertain. Contrary to the above findings, Lu et al. (2013) found higher total ascorbic acid content in 'Fuji' apples treated with 1-MCP as compared to the non-treated ones.

Numerous studies have confirmed a significant reduction of total aroma volatiles in 1-MCP treated apples (DeEll et al., 2005a; Defilippi et al., 2005; Kondo et al., 2005; Lurie et al., 2002; Fan and Mattheis, 1999; Fan et al., 1998). The loss of volatiles was also greater under CA than RA condition (Akbudak et al., 2009). Hence, to maintain the natural aroma of apples, 1-MCP under RA was recommended as a better alternative to CA storage.

2.5 Conclusion

This article highlighted the effect of 1-MCP treatment on the flavour component of apple including sugars, organic acids, phenolics, and volatile aroma with special focus on sugars and malic acid. Since sugars and malic acid are the main substrates involved in aerobic respiration of harvested apples, their catabolism will continue during long-term storage, which could easily lead to noticeable alterations in fruit flavour. A number of studies confirmed that postharvest 1-MCP treatment of apples significantly suppressed the loss of acidity, reduced production of volatile aroma, and had no effect on the content of phenolic compounds after short or long-term storage. However, only few studies have been done to uncover effect of 1-MCP on the change of individual sugars (fructose, glucose, and sucrose) during long-term storage of apples. The studies reviewed had inconsistent results and most importantly, they did not give adequate evidence to ascertain how 1-MCP regulates the changes in malic acid at the molecular level. Hence, further study is warranted in this area.

2.6 Tables

Table 2.6-1. Different commercial formulations of 1-MCP.

Product Name	**Percentage of active ingredient (1-MCP)**	**Commercial use**
EthylBlock® Powder	0.14	Floral and nursery crops
EthylBlock™ Sachet	0.014	Fresh-cut flowers and potted flowering and foliage plants
SmartFresh™ powder	3.30	Fruits (postharvest): apples, melons, tomatoes, pears, avocadoes, mangoes, papayas, kiwifruit, plums, apricot and persimmons
SmartFresh™ SmartTabs	0.63	Food commodities derived from apples, melons, pears, avocados, mango, papaya, kiwifruit, plum, apricots and persimmons
Harvista™	3.80	Tree fruits (preharvest spray)
Invinsa™	3.80	Field crops (foliar spray)

Adapted from: AgroFresh (2014).

Table 2.6-2. Effects of 1-MCP treatment on the content of sugars, malic acid, ascorbic acid, and phenolics in stored apples.

Parameters	Effect	Apple cultivars	References
Total soluble solids (TSS) accumulation	No or little effect	'Granny Smith'	Akbudak et al. (2009)
		'McIntosh' and 'Delicious'	Rupasinghe et al. (2000)
		'Empire' and 'Golden Smoothee'	Larrigaudiere et al. (2008)
	Increased	'Honeycrisp'	DeEll, (2007)
	Decreased	'McIntosh' and 'Law Rome'	Watkins et al. (2000)
Titratable acidity (TA) retention	No or little change	'Granny Smith'	Jemrić et al. (2013)
	Significantly improved	Gala', `Delicious', `Granny Smith' and `Fuji'	Mir et al. (2001)
		'Redchief Delicious'	DeLong et al. (2004)
		'Golden Delicious'	Lu et al. (2012)
		'Honeycrisp'	DeEll and Ehsani-Moghaddam (2010)
		'Fuji', 'Gala', 'Ginger Gold', 'Jonagold', and 'Delicious'	Fan et al. (1999)
		'McIntosh' and 'Delicious'	Rupasinghe et al. (2000)
		'McIntosh' and 'Spartan'	DeEll and Ehsani-Moghaddam (2013)
		'Granny smith'	Çelikel et al. (2009)
		'Anna'	Pre-Aymard et al. (2003)
Malic acid retention	Significantly improved	Transgenic	Defilippi et al. (2004)
		'Greensleeves'	Bizjak et al. (2012)
		'Idared'	

Continued from Table 2.6-2

Parameters	Effect	Apple cultivars	References
Fructose	No/little change	‘Empire’ apples	Lee et al. (2012a)
	Increased	‘Idared’	Bizjak et al. (2012)
	No or little effect	‘Greensleeves’	Defilippi et al. (2004)
		‘Empire’	Lee et al. (2012a)
Sucrose	No or little effect	‘Idared’	Bizjak et al. (2012)
		‘Empire’	Lee et al. (2012a)
	Decreased	‘Greensleeves’	Defilippi et al. (2004)
Glucose	Increased	‘Idared’	Bizjak et al. (2012)
	No or little effect	‘Greensleeves’	Defilippi et al. (2004)
		‘Empire’	Lee et al. (2012a)
Total phenolics	No or little effect	Several apple cultivars	(Fawbush et al., 2009, Qiu et al., 2009, Vilaplana et al., 2006, MacLeanet al., 2006, Hoang et al., 2011, Tardelli et al., 2013)
Ascorbic acid	No or little effect	‘Jona Gold’	Fawbush et al. (2009)
		‘Empire’	Kevers et al. (2011)
		‘Ambrosia’	Bizjak et al. 2012)
	Reduced	‘Golden Smoothee’	(Vilaplana et al., 2007, Vilaplana et al., 2006)
		‘Empire’	Lee et al. (2012b)
	Increased	‘Fuji’	Lu et al. (2013b)
		‘Krameri, Tuviõun’ and ‘Talvenauding’	Moor et al. (2007)
		‘Elstar’ and ‘Jonagold’	Neuwald and Streif, (2009)
Volatile aroma	Reduced	Several apple cultivars	(Defilippi et al., 2005, Kondo et al., 2005; Lurie et al., 2002, Fan and Mattheis, 1999, Fan et al., 1998, Neuwald, Streif, 2009)

2.7 Figure captions

Figure 2.8-1. Ethylene biosynthesis pathway in higher plants. Adapted from (Hopkins and Huner, 2004). ATP, adenosine triphosphate; SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; HCN, hydrogen cyanide; PPi, inorganic pyrophosphate

Figure 2.8-2. Simplified presentation of sugar and organic acid metabolism in ripening apple. Adapted from (Hopkins and Huner, 2004) and (Itai and Tanahashi, 2008). The enzymes, which catalyse the numbered steps are: (1) sorbitol dehydrogenase;(2) acid invertase and neutral invertases; (3) sucrose synthase; (4) fructokinase; (5) hexokinase; (6) uridine diphosphate glucose pyrophosphorylase (UDPGlcPPase); (7) phosphoglucose isomerase; (8) phosphoglucomutase; (9) sucrose phosphate synthase; (10) phosphoenolpyruvate; (11) phosphoenolpyruvate kinase; (12) phosphoenolpyruvate carboxylase; (13) phosphoenolpyruvate carboxykinase; (14) malate dehydrogenase; (15) dependent malic enzyme; (16) pyruvate kinase; (17) pyruvate decarboxylase; (18) lactate dehydrogenase; and (19) alcohol dehydrogenase. The key enzymes are circled with black. Glu, glucose; Fru, fructose; Suc, sucrose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F-1,6BP, fructose-1,6-bisphosphate; TP, triphosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetic acid; UDP-Glu, uridine diphosphate glucose

Figure 2.8-3. Glycolysis pathway in plants. Adapted from (Hopkins and Huner, 2004). The enzymes in the glycolysis pathway includes: (A) hexokinase, (B) phosphofructokinase (1) trios phosphate isomerase, (2) glyceraldehyde phosphate dehydrogenase, (3) phosphoglycerate kinase, (4) phosphoglycerate mutase, (5) enolase, and (6) pyruvate

kinase. The hexose-phosphate pool includes glucose-1-phosphate, glucose-6-phosphate, fructose1,6-biphosphate, and fructose-1-phosphate.

Figure 2.8-4. The citric acid cycle in plants. Adapted from (Hopkins and Huner, 2004). The enzymes in the numbered reactions include: (1) pyruvate dehydrogenase, (2) citrate synthase, (3) aconitase, (4) isocitrate dehydrogenase, (5) α-ketoglutarate dehydrogenase, (6) succinyl-CoA synthetase, (7) succinate dehydrogenase, (8) fumarase, and (9) malate dehydrogenase.

Figure 2.8-5. Simplified presentation of malic acid conversion to simple sugars in ripening apple. The enzymes that catalyze the numbered steps are: (1) malate dehydrogenase; (2) phosphoenolpyruvate; (3) phosphoenolpyruvate kinase; (4) sucrose phosphate synthase; (5) hexokinase; (6) phosphoglucomutase; (7) uridine diphosphate glucose pyrophosphorylase (UDPGlcPPase); (8) sucrose synthase, (9) invertase, (10) fructokinase; and (11) phosphoglucose isomerase. The shaded area indicates the pathway of malate catabolism to sucrose, glucose, and fructose. Sorbito, sorbitol; Glu, glucose; fru, fructose; suc, sucrose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose 1,6-bisphosphate; UDP-Glu, uridine diphosphate glucose

2.8 Figures

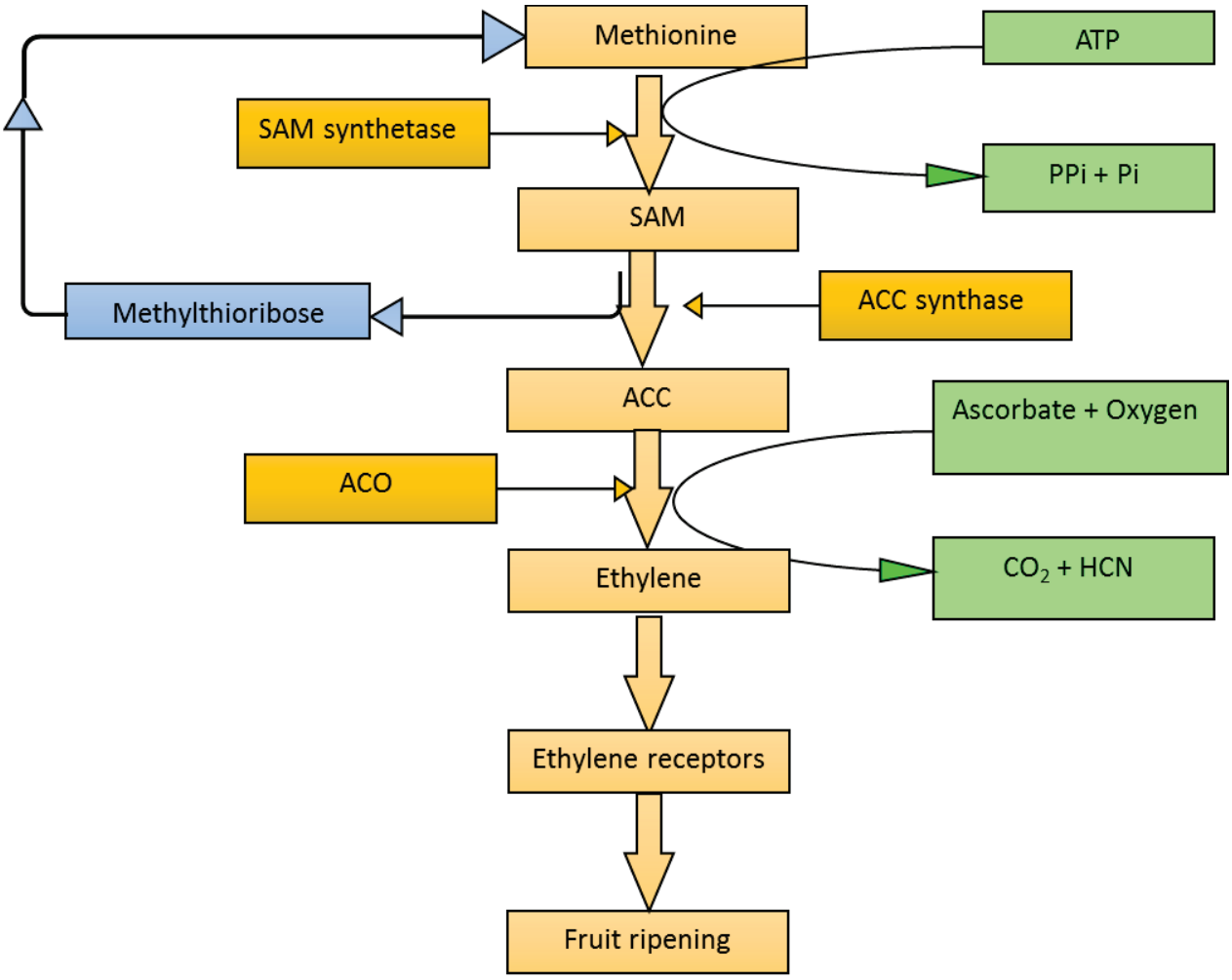
Methionine
ATP
SAM synthetase
PPi + Pi
SAM
Methylthioribose
ACC synthase
ACC
Ascorbate + Oxygen
ACO
Ethylene
CO_2 + HCN
Ethylene receptors
Fruit ripening

Figure 2.8-1

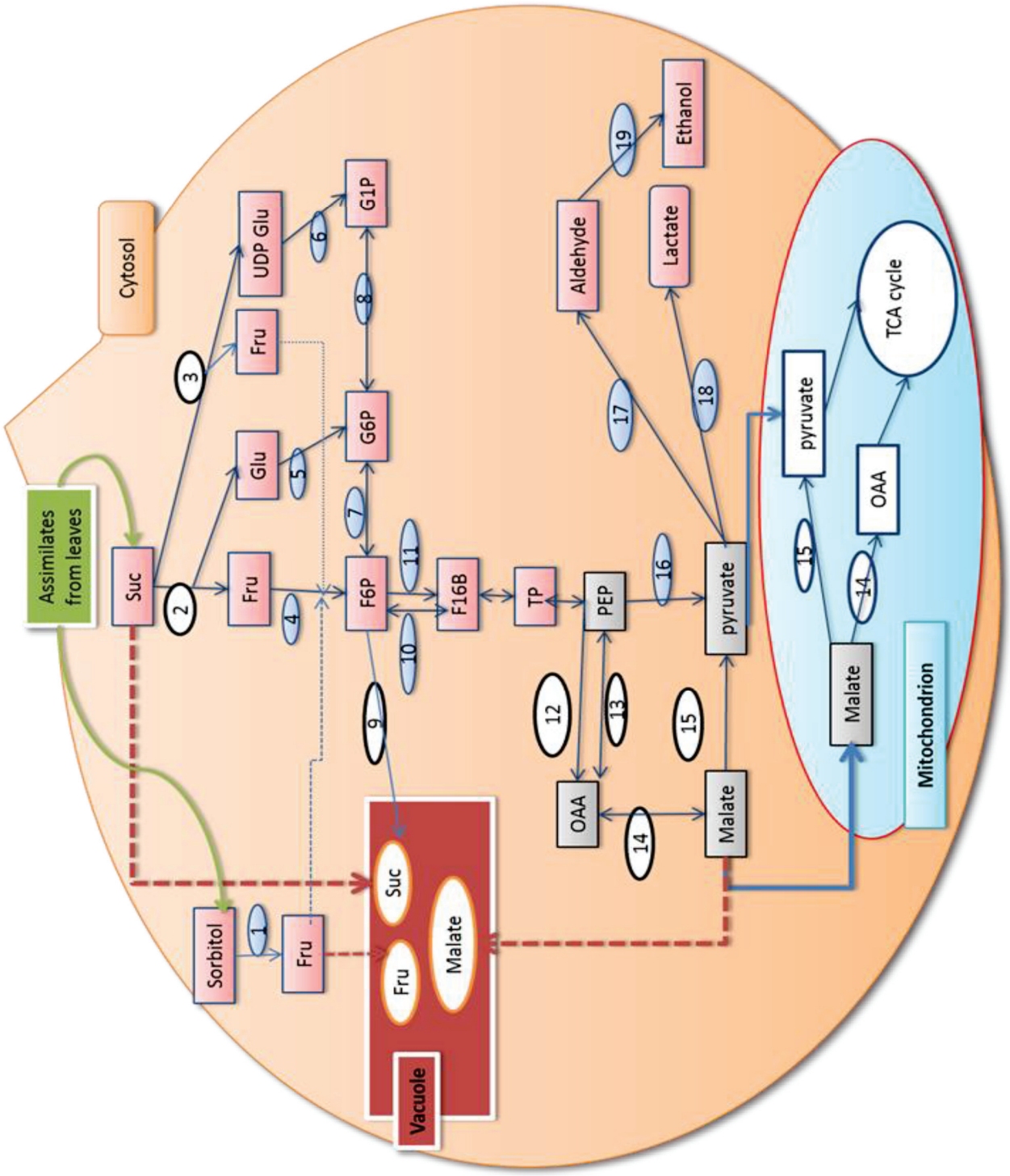
Cytosol
Assimilates from leaves
Suc
Fru
Glu
UDP Glu
G1P
G6P
F6P
F16B
TP
PEP
pyruvate
Aldehyde
Ethanol
Lactate
OAA
Malate
Sorbitol
Vacuole
Suc
Fru
Malate
Mitochondrion
TCA cycle
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Figure 2.8-2

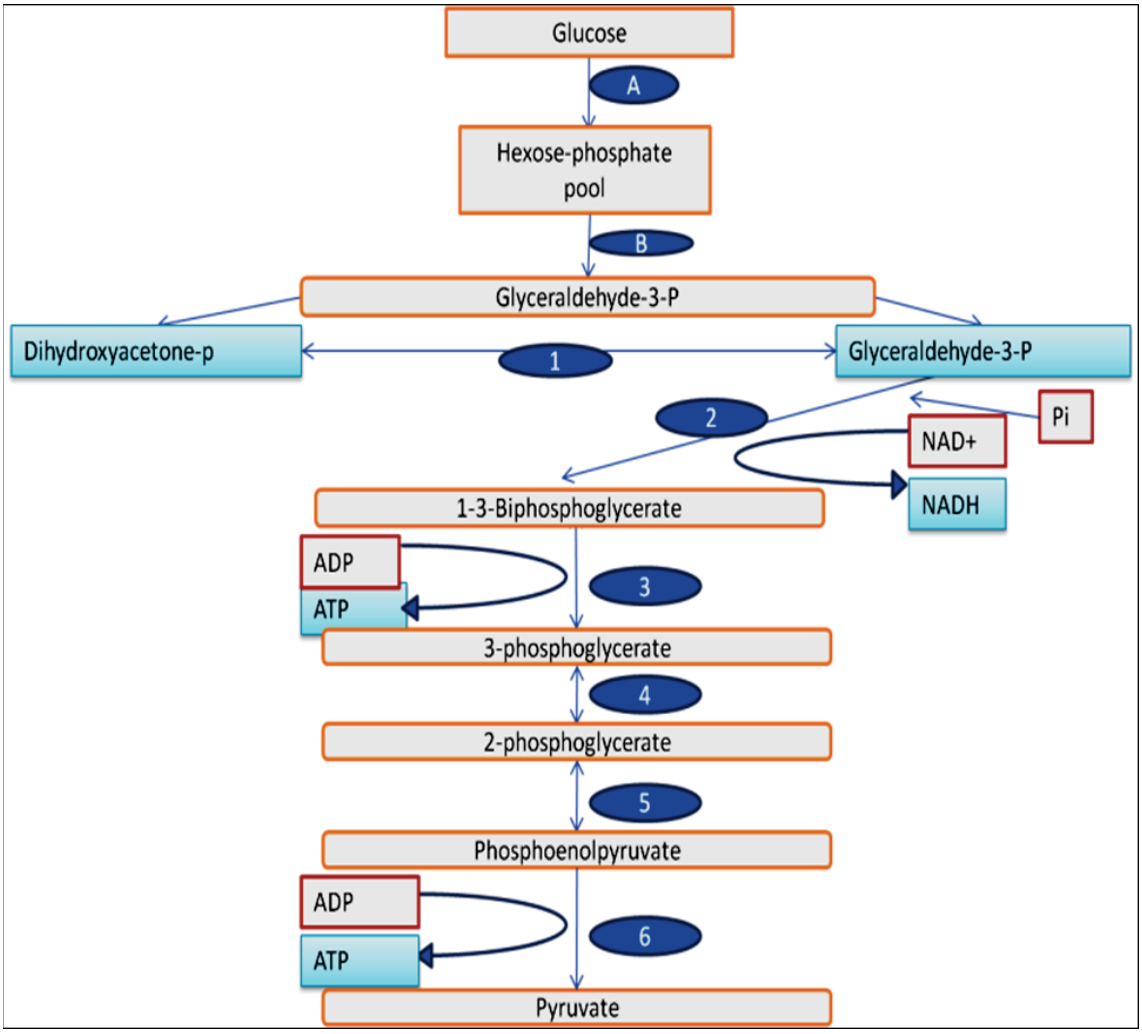
Glucose
A
Hexose-phosphate pool
B
Glyceraldehyde-3-P
Dihydroxyacetone-p
1
Glyceraldehyde-3-P
2
Pi
NAD+
NADH
1-3-Biphosphoglycerate
ADP
ATP
3
3-phosphoglycerate
4
2-phosphoglycerate
5
Phosphoenolpyruvate
ADP
ATP
6
Pyruvate

Figure 2.8-3

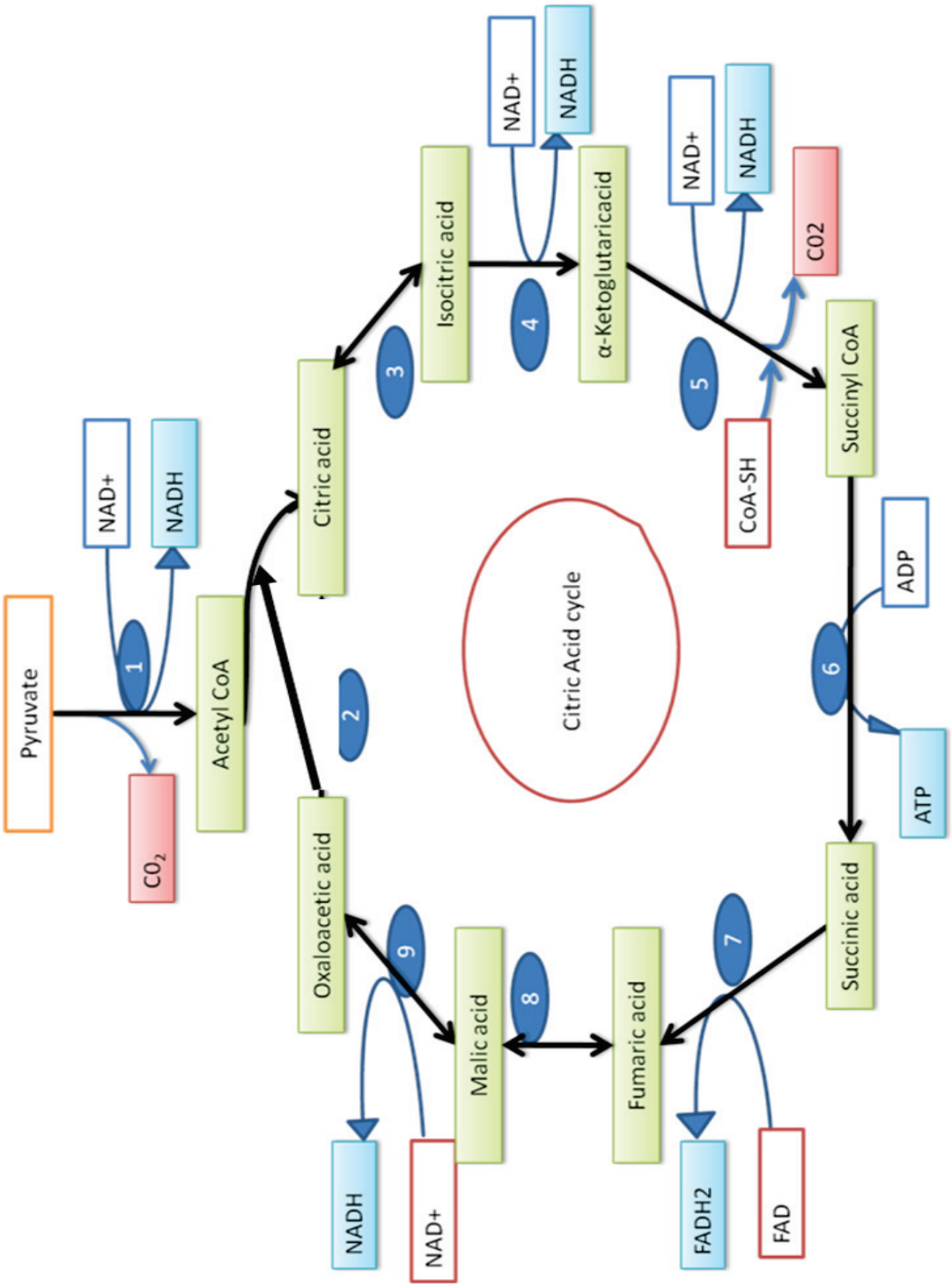
Pyruvate
NAD+
NADH
CO_2
Acetyl CoA
Citric acid
Isocitric acid
NAD+
NADH
α-Ketoglutaricacid
NAD+
NADH
C02
CoA-SH
Succinyl CoA
ADP
ATP
Succinic acid
FADH2
FAD
Fumaric acid
Malic acid
NADH
NAD+
Oxaloacetic acid
Citric Acid cycle
1
2
3
4
5
6
7
8
9

Figure 2.8-4

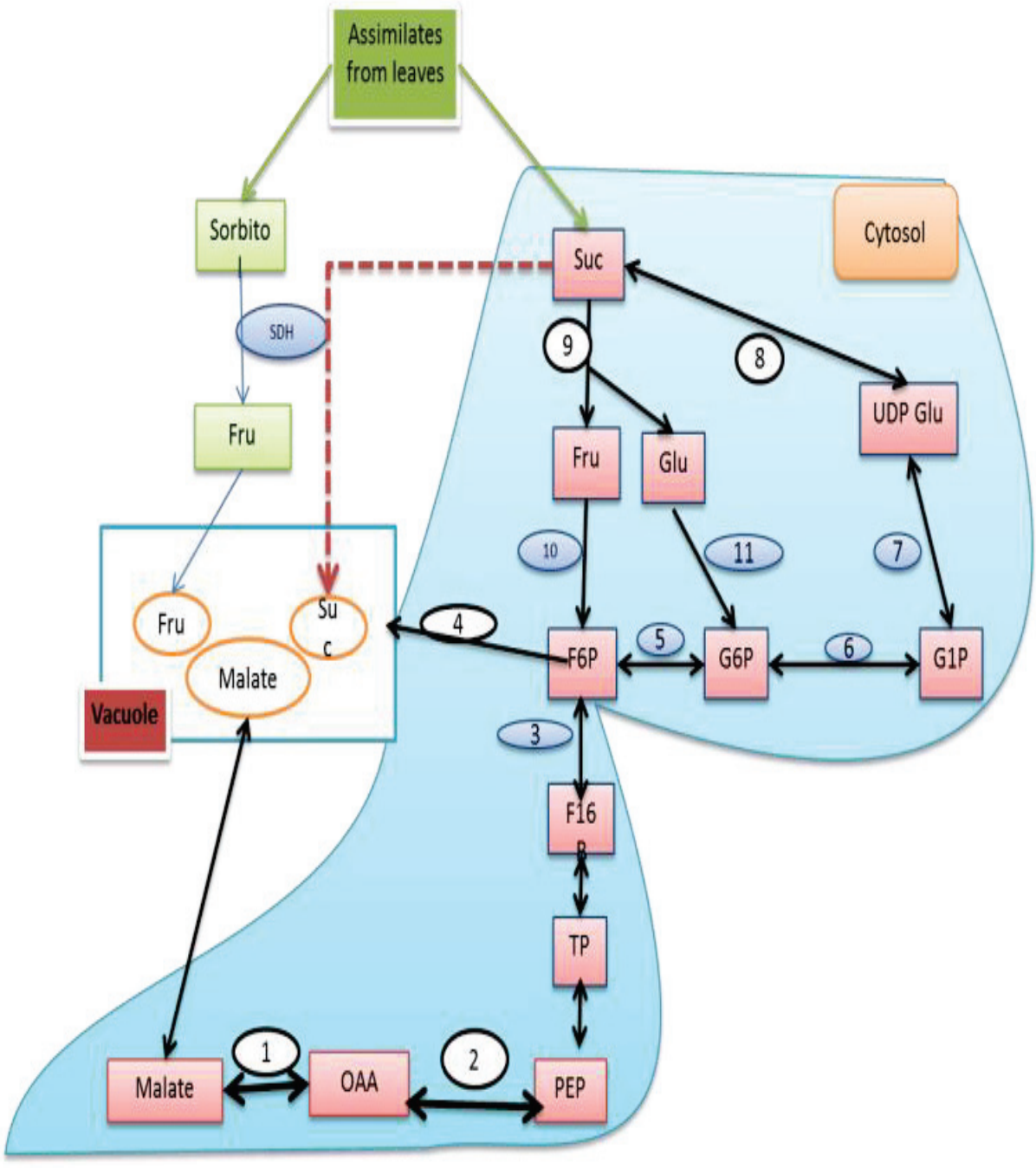
Assimilates from leaves
Sorbito
SDH
Fru
Cytosol
Suc
9
8
UDP Glu
Fru
Glu
10
11
7
Fru
Su c
4
F6P
5
G6P
6
G1P
Malate
Vacuole
3
F16 B
TP
1
2
Malate
OAA
PEP

Figure 2.8-5

Chapter 3: Effect of 1-methylcyclopropene (1-MCP), storage atmosphere, storage time and harvest maturity on acidity and sugar content of cloudy and fresh juice of 'McIntosh' and 'Honeycrisp' apples

Abstract

This study investigated the effect of 1-methylcyclopropene (1-MCP) treatment, storage atmosphere [controlled atmosphere (CA) and regular air (RA)], storage time (4 and 7 months), and harvest maturity (commercial and late) on the acidity and sugar content of cloudy and fresh apple juices prepared from 'McIntosh' and 'Honeycrisp' . Acidity and sugar content were assessed through the analyses of titratable acidity (TA), pH, total soluble solids (TSS), TSS/TA ratio, malic acid, and individual sugars (fructose, glucose and sucrose). 1-MCP treatment combined with CA storage suppressed ethylene production in both 'McIntosh' (0.6-5.5 $\mu Lkg^{-1}h^{-1}$) and 'Honeycrisp' (1.1-2.1 $\mu Lkg^{-1}h^{-1}$) apples harvested at commercial and late maturity stage and stored for 4 months, respectively. Juice samples from 1-MCP treated 'McIntosh' apples had 6.9-30.2% higher TA, 25-30% higher malic acid, and 0.06-0.07 units lower pH as compared to those from untreated fruit kept under RA and CA condition, respectively. Similarly, juices from 1-MCP treated 'Honeycrisp' fruit had 5.9-7.9% higher TA, 37.9-61.7% higher malic acid, and 1.6-1.8%

lower pH as compared to those from untreated fruit kept under RA and CA, respectively. The higher acidity retention was attributed to the reduced expression of MdcyME in 'McIntosh' or the increased expression of V-ATPase in 'Honeycrisp', which regulate the degradation and vacuolar transport malic acid, respectively. Fructose, glucose and sucrose were identified as the major monosaccharide and disaccharide sugars in all juice samples tested. Regardless of 1-MCP treatment and storage conditions, juices from stored apples had substantially lower content of sucrose with increased level of fructose and glucose. Neither CA storage nor 1-MCP treatment prevented the loss of sucrose during long-term storage. Juice processing technique had a significant effect on the sugar content where cloudy juice samples yielded higher content of all individual sugars.

Key words: 1-MCP, storage atmosphere, cloudy juice, sugars, malic acid, gene expression, 'McIntosh', 'Honeycrisp'.

3.1 Introduction

1-Methylcyclopropene (1-MCP) is a strong ethylene action inhibitor, which is thought to act by binding to ethylene receptors (Sisler et al., 2006; Sisler and Serek, 1999). The apple industry in North America has adopted the extensive use of 1-methylcyclopropene (1-MCP) as a means to extend the storage life of apples (Watkins, 2006). In addition to its physiological effect, 1-MCP is reported to affect the flavour components of apples (Bizjak et al., 2012; Defilippi et al., 2004). The overall flavour of apple fruit and its products is influenced by the composition of sugars, organic acids, phenolics, and volatile aroma compounds as well as by the sugar acid ratio (Baldwin, 2002). The sugar-acid balance is a crucial factor for the sweet and sour taste of apple and is an important driver of consumer preference. Previous studies confirmed the remarkable inhibition of volatile aroma production and loss of titratable acidity (TA) in 1-MCP treated apples after long-term or short-term storage (Rupasinghe et al., 2000; DeEll and Ehsani-Moghaddam, 2013). In order to assess the change in acidity and sweetness of apples, measurements of total soluble solids (TSS) and TA are often included in most studies. However, only few studies have been conducted to uncover the impact of 1-MCP on the content of individual sugars (fructose, glucose, and sucrose) and organic acids. Moreover, molecular level evidence is lacking to elucidate how 1-MCP regulates the changes in sugars or malic acid during long-term storage.

Flavour perception is a combination of taste, aroma, and mouth feel (Baldwin, 2002). The major components contributing to the flavour of apple juice include sugars, organic acids, phenolics, and volatile aroma compounds (Baldwin, 2002). The balance

between sugars and organic acids is responsible for the sweet and sourness of apple (Hecke et al., 2006). Reduced level of aroma volatiles after 1-MCP treatment, have been reported in many apple cultivars (Defilippi et al., 2005). Improved retention of total acidity in 1-MCP treated and cold stored 'Honeycrisp' (DeEll and Ehsani-Moghaddam, 2010), 'McIntosh' (Rupasinghe et al., 2000) as well as in many other apple cultivars (Lu et al., 2013) has been demonstrated. However, there is still little published information about the effect of 1-MCP on the gene expression of the key enzymes involved in the malic acid metabolism after long-term storage under RA or CA conditions.

Recent studies on stored 'Empire' (Lee et al., 2012) and 'Idared' apples (Bizjak et al., 2012) have indicated the potential effect of 1-MCP on the content and composition of simple sugars. However, published information is still scarce about the change of major individual sugars (fructose, sucrose and glucose) in 'Honeycrisp' and 'McIntosh' apples after long-term storage. Moreover, all the aforementioned researchers have only focused on the relationship between 1-MCP and fruit quality parameters of apples. Nevertheless, to the authors' knowledge a study that integrates 1-MCP treatment and apple juice quality has not been reported yet.

Commercially, the most popular apple juice in the North American market is a clarified apple juice, which has a typical amber-like hue colour (Oke and Paliyath, 2006). However, cloudy apple juice is becoming a fast growing sector, especially in European countries such as Germany where cloudy apple juice accounts for about 30% of their apple juice consumption (Will et al., 2008). Cloudy apple juice, also called opalescent or natural juice, is unclarified apple juice with yellowish or greenish colour containing remarkably

higher portion of pulp in suspension, which results in greater turbidity and better nutritional quality than in clear juice (Markowski et al., 2009). It has been demonstrated that the health benefit associated with apple juice is much higher for cloudy apple juice than the clear juice (Oszmianski et al., 2007). This is attributed to the low content of phenolic compounds in clear apple juice due to the rigorous clarification process, which reduces the content of proanthocyanidins by about 10 to 30% (Hubert et al., 2007). Cloudy apple juice can be considered as a minimally processed product since the process does not involve the enzymatic treatments, rigorous membrane filtration and clarification, which are common procedures in clear apple juice production (Oszmianski et al., 2007). With the increasing consumer preference to minimally processed products, it seems that there is increasing demand for quality over quantity (Jaros et al., 2009).

The purpose of this study was to investigate the effect of 1-MCP, storage atmosphere (CA and RA), storage time (4 and 7 months), and harvest maturity (commercial and late) on the acidity (malic acid) and sugar content (fructose, sucrose and glucose) of cloudy and fresh apple juice prepared from 'McIntosh' and 'Honeycrisp' apples. The effect of 1-MCP and storage atmosphere on the gene expression of the key enzymes involved in malic acid metabolism was also examined. Moreover the change in ethylene production, flesh firmness, and volatile aroma production were assessed from whole fruit as an index for 1-MCP effectiveness.

3.2 Materials and Methods

3.2.1 Fruit harvesting, 1-MCP treatment and storage

'Redmax McIntosh' (hereafter referred to as 'McIntosh') and 'Honeycrisp' apples were harvested from the commercial farm J. W. Mason and Sons Ltd. located at Windsor, Nova Scotia. Harvesting was performed from Sep. 14 to Oct. 09, 2010 at ether optimum (commercial) or late maturity stages. Good quality (disease and damage free) apples were selected, packed in carton boxes, wrapped with plastic, and transported to the Agriculture and Agri-Food Canada (AAFC) Research Centre in Kentville, Nova Scotia. Within 6-8 h of harvest, 'McIntosh' fruit were stored at 10 ^{o}C overnight, and 'Honeycrisp' fruits were held at room temperature (20 ± 1 °C). Fruit were divided randomly into control and 1-MCP treatment groups. Fruit were treated with 1-MCP (1 µL/L) within 24 h of harvest in a 2 m^3 gas tight chamber at room temperature (20 ± 1 °C) for 24 h. 1-MCP was generated by dissolving SmartFresh™ Research Tablets (four purple and two yellow) with a Blue Activator Tablet (AgroFresh, Inc. Philadelphia, PA, USA) in a plastic release vial containing 18 mL activator solution according to the manufacturer's instructions. The release vial was placed adjacent to a battery operated fan (2000001021, Coleman, Wichita, KS) to facilitate circulation of 1-MCP throughout the chamber. After 1-MCP treatment (24 h at room temperature) and venting, fruit, which were in plastic crates (24" x 16" footprint, AF2416-10 NPL 655, ORBIS Corporation) were loosely covered with a single layer of polyethylene film to reduce water loss and placed in CA or RA storage. 'Honeycrisp' apples were held at 20 ± 1 ^{o}C for one week prior to being placed in RA or CA storage.

Keeping 'Honeycrisp' apples in at room temperature prior to cold storage is referred to as delayed cooling and is mainly used to inhibit soft scald development and internal breakdown during storage (Nichols et al., 2008). Control and 1-MCP treated fruits were stored at 3 ± 1 ^{o}C and 95% RH in air (RA) or CA, which was comprised of 2.5% CO_2, and 2.5% or 2% O_2 for 'McIntosh' and 'Honeycrisp' apples, respectively. The gas concentration for CA storage were determined based on the recommendations from AAFC (DeEll and Prange, 1994; Nichols et al., 2008). The higher oxygen concentration for 'McIntosh' apples was due to its sensitivity to lower oxygen levels (<1.5%) (DeEll and Prange, 1998). The O_2 and CO_2 concentrations within the CA chambers were established within 3 to 4 h of sealing, after flushing with N_2 and CO_2 (99.99%) gases (Praxair, Dartmouth, NS, Canada). Gas levels and storage temperatures were monitored daily using Fruit Store Analyzer fitted with Controlled Atmosphere System (David Bishop Instrument, Type 770, Sussex, UK).

Samples were removed from CA or RA storage after 4 and 7 months. The two apple cultivars were stored in two different rooms. A total of 16 bushels or 1600 (one bushel = ca 100 apples) apples were used for this experiment. At each removal, 100 fruit per experimental unit were taken. Of these, 50 fruit were frozen and the rest processed into juice.

3.2.2 Juice preparation

Cloudy apple juice was prepared by washing six medium sized apples with tap water, cutting each into 12 pieces and pressing them using a laboratory scale juice extractor

(Supreme Juicerator, USA). Juice samples were collected into 250 mL beakers containing 0.5 g/L ascorbic acid (Oszmianski et al., 2009). The juice was filtered with four layer cheesecloth and heated up to 80 ^{o}C using a water bath, held for 5 min at that temperature (Benitez et al., 2007; Genovese and Lozano, 2006), collected in sterile 50 mL centrifuge tubes (0553849 Fisher Scientific, Ottawa, ON, Canada), cooled in an ice bath (Oszmianski et al., 2009), and stored at -20 ^{o}C until analysis. The main purpose of juice heating was to inhibit further enzymatic (PPO) activity and browning (Benitez et al., 2007; Genovese and Lozano, 2006). The holding time (5 min) was recorded after the juice reached the desired temperature (80 ^{o}C). The time required to reach the set temperature was about 5 min. Juice preparation was done separately for the three biological replications. Fresh apple juice was prepared in the same way as above, but simply pressed and filtered with four layer cheesecloth without ascorbic acid addition and pasteurization.

3.2.3 Experimental design and statistical analysis

The experimental design was a split plot factorial with four factors for the fruit quality study and five factors for the juice quality study with three biological replications. The independent variables include, harvest date (commercial and late harvest), 1-MCP treatments (control and treated), storage atmosphere (CA and RA), storage time (4 and 7 months) and juice type (fresh and cloudy). Since storage time, 1-MCP treatment and storage conditions can be randomized with less restriction; they were assigned at the sub plot level. However, harvest date was a hard to change factor and hence it was assigned as a whole plot treatment. Statistical analyses were accomplished using SAS 9.3 (SAS 9.3,

Cary, NC, USA) and Minitab (Release 17, Minitab Inc. State College, PA, USA) software Analysis of variance (ANOVA) was performed using PROC GLIMMIX procedure. PROC GLIMMIX performs estimation and statistical inference for generalized linear mixed models (GLMMs), which consider both fixed and random, factors (Schabenberger, 2005). As our experimental design involves both fixed and random factors this model was employed for the proper analysis of the data. Whenever there were significant interactions among factors, multiple mean comparisons were employed using adjusted Tukey's method at $\alpha = 0.05$. If the adjusted *p*-value for a comparison was less than or equal to 0.05, the difference between the means was considered significant. For each response, the validity of model assumptions, namely normal distribution and constant variance of the error terms, were verified by examining residual plots as indicated in the Appendix A, Figure 1 to Figure 9. In some data sets power transformations had to be used to achieve normality (Montgomery, 2008).

3.2.4 Reagents and standards

Iodine and ethylene standards were purchased from Sigma Aldrich (Oakville, ON, Canada). Buffer solutions (pH 7.0 and pH 4.0) were purchased from Fisher Scientific, (Ottawa, ON, Canada). Sodium hydroxide solution, L-ascorbic acid 99%, HPLC grade sulfuric acid, and HPLC grade standards (glucose, fructose, sucrose, and L-malic acid) were purchased from Sigma Aldrich (Oakville, ON, Canada).

3.2.5 Ethylene production analysis

Apple samples were warmed at room temperature for 24 h, placed in 4 L glass jars, labeled, weighed, and sealed. One hour later 1 mL sample of headspace was taken and analyzed for ethylene. Ethylene was analyzed using a Carle gas chromatograph (GC) (Carle Instruments Inc., Anaheim, California) equipped with a flame ionization detector and activated alumina column (1 .9 m long × 3.2 mm outer diameter). Hydrogen gas was used as a carrier with a flow rate of 50 mL/min. Quantifications were completed by comparison of GC response of the sample to that of certified ethylene standards of 4.3 mg/L and 53 mg/L, with two injections per level. The analyses were done on three replicates each with 1.0 ± 0.1 kg apples.

3.2.6 Volatile analysis from whole apples

Apples were held in a 4 L, sealed glass jar with a Teflon lid for 1 h at 20 ^{o}C. A 100 mL sample of head space then was captured on an adsorption tube (89 mm long x 6.4 mm outer diameter) containing Carbopack B (155 mg) and Carboxen 1000 (70 mg), both from Supelco Inc. (Oakville, ON, Canada). Adsorption tubes were held at -86 ^{o}C until time of analysis.

Volatiles were removed from the adsorption tube using a Turbo Matriz 650 ATD thermal desorber (PerkinElmer Like and Analytical Sciences, CT, USA). Tubes were heated to 250 ^{o}C using an outlet split of 1:2 for sample introduction into a Varian 4000 gas chromatograph mass spectrometry system (GC-MS) (Varian Inc., Walnut Creek, Calif., USA). Volatile analysis was conducted using a VF-WAXms column (0.32 mm, internal

diameter x 30 m, length x 1.00 µm, film thickness, Varian Inc., Lake Forest, Calif., USA). The column was held at 35 ºC for 5 min, increased 10 ºC per min to 240 ºC, and held for 4.5 min. Column flow rate was 2.5 mL/min of helium while temperatures of the transfer line from the GC to the MS and the MS were 180 and 220 ºC respectively. Detection by MS was carried out in electron ionization (EI) mode with a mass range of 35-400 amu, emission current of 25 µAmps, and a scan rate of 0.60 s (4 µscans). Temperatures of the transfer line, trap, manifold and ion source were 170, 100, 50, and 180 ºC, respectively.The analyses were done on three replicates each with 1.0 ± 0.1 kg.

3.2.7 Starch index measurement

Fruit starch index was determined after slicing the apples in half, pouring iodine solution (2.5 g/L iodine and 10 g/L potassium iodide in distilled water) on the cut surface until it is covered fully and observing colour change after 60 s. The starch index was then determined by rating the staining pattern according to Cornell University starch-iodine index chart (Blanpied and Silsby, 1992) using a 1-8 scale (1 = 100% starch and 8 = 0% starch). The measurements were done on three replicates each with five apples.

3.2.8 Flesh firmness measurement

Fruit firmness was measured using a fruit texture analyser (GS 15, GUSS, Cape Town, South Africa) by removing the skin at the red side of the fruit and penetrating the flesh with

an 11 mm diameter blunt probe at a speed of 10 mm/sec to a penetration depth of 8.5 mm. The measurements were done on three replicates each with 10 apples.

3.2.9 Titratable acidity (TA), pH, and totals soluble solid (TSS) measurement

A semi-automated titrator (785 Metrohm, Herisau, Switzerland) was used to measure TA (expressed as mg equivalent of malic acid per 100 mL juice) of 2 mL of juice mixed with 28 mL water using 0.1 N NaOH as titrant to an end point pH of 8.2. The content of TA was expressed in terms of malic acid equivalents, which is the most prominent organic acid in apples. Juice pH of 15 mL juice sample was measured using a digital pH meter (Accumet 10, Denver Instruments, NY, USA) that had been calibrated using pH-7 and pH-4 standard buffer solutions. TSS was measured with a digital refractometer (300016, Sper Scientific, Scottsdale, AZ, USA) on 200 μL of juice at room temperature. The measurements were done on three replicates.

3.2.10 HPLC analysis of fructose, sucrose, and glucose

The major sugars (fructose, sucrose and glucose) were analysed using a Waters Alliance 2695 HPLC system (Waters, Milford, malic acid, USA) equipped with an auto sampler, temperature control for the column, a degasser system, a refractive index detector and QuanLynx software (Micromass, Cary, NC) for system control and data acquisitions. A Rezex ROA organic acid analytical column (300 × 7.8 mm; Phenomenex, Torrance, USA) and a Carbo-H4 x 3.00 mm, 8/Pk guard column (Phenomenex, Torrance, CA, USA) were used for sugar analysis. Juice samples were diluted (100 fold), centrifuged (8500 rpm 10

min), and filtered using 0.45 micron filters (Chromaspec, Chicago, IL, USA). HPLC analysis was employed in the following set up: injection volume 20 µL, detector temperature 30 ºC, column temperature 30 ºC, run time 30 min, isocratic mode, eluting order of glucose (12.8 min), fructose (13.9 min), and sucrose (11.4 min). The mobile phase used for this analysis was 0.005 N H_2SO_4 with a flow rate of 0.6 mL/min. Calibration curves were obtained using standard solutions of glucose, fructose and sucrose (50-500 mg/L).

3.2.11 HPLC analysis of organic acids

An HPLC system similar to that described for sugar analyses was used for the analysis of malic acid. Juice samples were diluted 5-10 fold, centrifuged at 8500 rpm for 10 min, and filtered using 0.45-micron filters (Chromaspec, Chicago, IL, USA). The analytical column used was a Synergy Polar RP (4 µm, 150 mm x 4.6 mm) (Phenomenex, Torrance, CA, USA). HPLC analysis was conducted at 60 ºC using 0.1 N H_2SO_4 as the mobile phase at a flow rate of 0.5 mL/min. Spectral measurements were made over the range of 200-600 nm with 210 nm as the detection wave length. Calibration curves were obtained using standard solutions of L-malic acid (10-250 mg/L).

3.2.12 Total RNA extraction and cDNA synthesis

Frozen apple tissue samples were prepared after selecting six blemish and disease free apples from each experimental unit, which were then washed with tap water, cut into eight pieces, frozen in liquid nitrogen (-196 ºC) and finally packed in sterile zipper lock plastic bags and stored at -80 ºC until analysis. Total RNA was isolated using RNAqueous-4PCR

kit (Ambion, AM1914 Burlington ON, Canada) following the manufactures instructions. The quality, quantity, and integrity of RNA were monitored using NanoDrop 2000 spectrophotometer (Thermo Scientific, Ontario, Canada) and gel electrophoresis using denaturing agarose (1.5%) that contains 6.5 µL formaldehyde, 11 µL formamide and 32 µL 10X MOPS buffer. In order to remove any genomic DNA, the total RNA was treated with DNase I (RNase free), which came with the RNAqueous-4PCR kit, following the manufacturer's instructions.

A single strand complementary DNA (cDNA) was synthesized using iScript^{TM} cDNA synthesis kit (Bio-Rad 1708890) using 2 µg RNA in 40 µL reaction mix. The reaction mix contained 8 µL 5x iScript reaction mix (Oligo dT and random hexamer), iScript reverse transcriptase) and nuclease free water in final volume of 40 µL. Reverse transcription was performed for a total of 45 min (5 min at 25 ^{o}C, 30 min at 42 ^{o}C, 5 min at 85 ^{o}C and 5 min at 4 ^{o}C) using Bio-Rad DNA Engine® Peltier PCR Thermal cycler (PTC-200 DNA Engine Cycler, Canada). The synthesized cDNA was kept at -20 ^{o}C until use.

3.2.13 Optimization of Real-time PCR analysis

The oligonucleotide primers used for real-time quantitative PCR (RT-qPCR) analysis were adopted from the literature (Table 3.6-9). The concentration of c-DNA was determined by testing various c-DNA dilutions (1/2, 1/4 1/10, 1/100) using a pooled c-DNA reference sample collected from all samples. The optimum c-DNA dilution (1/4) was chosen with a between 22-25 cycle threshold (CT) values. Primer efficiencies were calculated from the standard curve using the equation $E = 10^{-1/slope}$ (Table 3.6-10). The standard curve was

generated using a two-fold dilution series of five data points (1/2, 1/4, 1/8, 1/6, 1/32). The stock solution had 10 ng/µL RNA concentration based on the NanoDrop spectrophotometric result after DNase cleanup.

3.2.14 Real-time q-PCR analysis

The gene expression analysis was performed in triplicate using LightCycler® 480 System. Samples were loaded inside an automated liquid handling system using epMotion 5075 LH apparatus (Eppendorf, Hamburg, Germany). Reactions were performed in three technical replicates (for each of the three biological replicate) and contained 5 µL iQ™SYBR® Green Supermix, 1.25 µL diluted cDNA, 0.6 µL of each forward and reverse primers and 2.55 µL PCR-grade water in a final volume of 10 µL reaction mix. In addition, each plate contained no-template controls to control reagent contamination. PCR cycling reactions comprised one denaturation cycle at 95 ºC for 3 min, 40 amplification cycles at 95 ºC for 10 sec and one annealing cycle at 60 ºC for 30 sec. The specificity of the PCR reaction was determined with a heat dissociation protocol from 65-95 ºC following the final PCR cycle. Melting curves were analysed to check the specificity of PCR reactions (Appendix A Figure 10). The transcript abundance level (normalized relative gene expression) for each target gene was quantified efficiency corrected **ΔΔ**CT method. The housekeeping gene, 18S rRNA was used as a reference gene to normalize the CT values of the target genes. Pooled cDNA was used as a calibrator normalized relative gene expression (NRGE) was calculated as:

$$NRGE = Et^{[CT\,(target)\,calibrator - CT\,(target)\,sample]} / Er^{[CT\,(reference)\,calibrator - CT\,(reference)\,sample]} \quad \text{Eq. 3.1}$$

Where, RGE, normalized relative gene expression; Et and Er are efficiencies for the target and the reference gene respectively; CT, cycle threshold value

3.3 Results

3.3.1 Maturity at harvest

'McIntosh' apples from commercial and late harvests were at optimum or acceptable maturity stage for long-term storage. As shown in (Table 3.6-1), the starch index of 'McIntosh' fruit was between 2.7 (commercial) and 5.9 (late) which is the recommended range for CA storage of 'McIntosh' fruit (Brandied and Silsby, 1992). Late harvested 'McIntosh' fruit had significantly lower ($p<0.05$) firmness and higher TSS, levels as compared to those harvested at commercial stage (Table 3.6-1). The optimum harvest window for long-term storage of 'Honeycrisp' apples in Atlantic Canada generally occurs between September 25 and October 10, which coincided with timing of the harvest dates in this experiment (Nichols et al., 2008). The starch index of 'Honeycrisp' fruit harvested at commercial and late harvest dates was 7.3 and 8.0, respectively (Table 3.6-1). The respective average TSS and flesh firmness of 'Honeycrisp' fruit at harvest were 11.9 ^{o}Brix and 61.5 N and no significant ($p>0.05$) differences in starch index, TSS or firmness were detected in 'Honeycrisp' between the two harvest dates (Table 3.6-1). The later harvested 'McIntosh' fruit had significantly ($p<0.001$) higher starch index, TSS with lower firmness than those harvested at commercial harvest. The effects of this maturity difference on fruit and juice quality will be discussed in the later sections.

3.3.2 Ethylene production, fruit firmness and volatile aroma

3.3.2.1 Ethylene production

Ethylene production of 'McIntosh' and 'Honeycrisp' apples (Table 3.6-2) after long-term storage was significantly ($p<0.05$) affected by a four-way interaction of harvest maturity, storage atmosphere, 1-MCP treatment and storage duration. Irrespective of storage atmosphere and harvest maturity, 1-MCP treated 'McIntosh' apples had considerably lower ethylene production (11.3-20.2 $\mu Lkg^{-1}h^{-1}$) in comparison to untreated fruit (31.9-26.9 $\mu Lkg^{-1}h^{-1}$) following 4 and 7 months of storage, respectively (Table 3.6-2). In 'McIntosh' apples, the minimum ethylene production was observed in 1-MCP treated fruit kept under CA, harvested at commercial (0.63 $\mu Lkg^{-1}h^{-1}$) or late stage (5.5 $\mu Lkg^{-1}h^{-1}$) and stored for 4 months (Table 3.6-2). In 'McIntosh' apples, maximum ethylene production was detected in untreated fruit harvested at commercial stage and stored under CA (46.09 $\mu Lkg^{-1}h^{-1}$) for 7 months or RA (40.97 $\mu Lkg^{-1}h^{-1}$) for 4 months (Table 3.6-2).

Considerably lower ethylene production in 'Honeycrisp' apples was detected under treatment conditions including: commercial +1-MCP + CA (1.1-2.9 $\mu Lkg^{-1}h^{-1}$); late + 1-MCP + CA (2.1-1.9 $\mu Lkg^{-1}h^{-1}$) and commercial + CA (5.1-5.3 $\mu Lkg^{-1}h^{-1}$) stored for 4 and 7 months (Table 3.6-2). Unexpectedly, 1-MCP treated 'Honeycrisp' fruit held under RA had significantly higher (17.9 - 49.0 $\mu Lkg^{-1}h^{-1}$) ethylene production as compared to untreated (13.8- 27.8 $\mu Lkg^{-1}h^{-1}$) fruit harvested at commercial and late stages, respectively following 4 months of storage (Table 3.6-2).

While ‘McIntosh’ apples had extremely elevated ethylene production as storage time advanced to 7 months, even with 1-MCP treatment, such extreme elevations were not observed in ‘Honeycrisp’ fruit. For instance, ‘McIntosh’ fruit harvested at commercial maturity, treated with 1-MCP and stored in CA, ethylene production increased by about 26-fold after 7 months of storage (Table 3.6-2). However, the same treatment in ‘Honeycrisp’ fruit yielded only a 2.7-fold increase (Table 3.6-2). ‘Honeycrisp’ and ‘McIntosh’ apples not treated with 1-MCP had less ethylene production after 7 months than 4 months of RA storage, suggesting that these fruit were post-climacteric.

A synergistic effect of 1-MCP + CA combination in reducing ethylene production was detected in both ‘McIntosh’ and ‘Honeycrisp’ apples. In ‘McIntosh’ apples, harvested at commercial stage and stored 4 months, 1-MCP + CA treatment had 45.2 and 25.2 times lower ethylene production compared to CA or 1-MCP + RA, respectively (Table 3.6-2). Similarly, in ‘Honeycrisp’ apples harvested at commercial stage, 1-MCP + CA treatment had 4.7 and 16.57 times lower ethylene production compared to CA or 1-MCP + RA treatments, respectively (Table 3.6-2). Unlike ‘McIntosh’ apples, late harvested ‘Honeycrisp’ apples also benefited from the 1-MCP + CA combination, where this treatment lowered ethylene production by 5.52 and 23.6-fold in comparison to CA or 1-MCP + RA treatments, respectively (Table 3.6-2).

3.3.2.2 Flesh firmness

Flesh firmness of ‘McIntosh’ fruit was significantly ($p<0.05$) affected by a two-way interaction of 1-MCP with storage time and 1-MCP with harvest maturity. In addition,

storage atmosphere had a highly significant effect ($p<0.001$) on firmness. Untreated 'McIntosh' fruit kept under RA softened and lost about 47 to 54% of their initial (66.7 N) postharvest firmness after 4 and 7 months of storage, respectively (Table 3.6-3). However, 1-MCP treatment (7.5-15.8%) and/or CA (7.6-13.15%) storage significantly improved flesh firmness retention of 'McIntosh' fruit during storage (Table 3.6-3). The 1-MCP + CA combination resulted in higher (10-20%) firmness retention as compared to either 1-MCP or CA alone (Table 3.6-3). Moreover, the presented results indicated that 1-MCP treatment in 'McIntosh' fruit was more effective with commercial than late harvested fruit. Irrespective of 1-MCP treatment or storage conditions, 'Honeycrisp' apples maintained their flesh firmness up to 7 months of storage. Main effects of 1-MCP treatment or CA storage on firmness retention was not significant. However, the significant combined effect of 1-MCP with storage atmosphere and harvest maturity suggest that 1-MCP was more effective in commercial than late harvest fruit and in CA than RA stored fruit (Table 3.6-3).

3.3.2.3 Volatile aroma

A total of 16 volatile compounds were detected in 'Honeycrisp' and 'McIntosh' apples, including esters (butanoates and acetates), ethanol, alkenes (alkene I, alkene II, and 3-heptene), and isoprenoid (alpha-farnesene) (Table 3.6-4). Of these, esters (79-80%) followed by ethanol (6-8%) were the dominant volatile compounds in both cultivars. Even though, there is a diverse range of compounds in the volatile profile of apples, the majority are esters (78-92%) and alcohols (6-16%), (Paillard, 1990), which is consistent with our

study. As presented in Figure 3.8-1, the major aroma volatiles from intact 'McIntosh' fruit included ethyl-2-methylbutanoate (46.9%), 3-methyl-1-butyl acetate (10.6%), alpha-farnesene (9.7%), 1-methylethyl acetate (8.9%), ethanol (7.6%), and butyl acetate (4.9%). Similarly, Figure 3.8-2 depicts the main aroma volatiles identified in intact 'Honeycrisp' apples; these include ethyl acetate (35.8%), butyl acetate (32.7%), 3-methyl-1-butyl acetate (10.6%), and ethanol (6.1%).

The composition and abundance of volatile compounds varies according to cultivar, 1-MCP treatment, storage atmosphere and storage time. Irrespective of storage period and other treatments, 'Honeycrisp' apples had 2.5 times higher total volatile content than 'McIntosh' fruit. In both cultivars irrespective of harvest maturity, the content of major and total volatile compounds was tremendously supressed by 1-MCP + CA and CA alone as compared to control fruit held in RA (Figure 3.8-1 and Figure 3.8-2). However, the suppression of volatile compounds in 'McIntosh' fruit kept under 1-MCP + CA or control + CA condition did not last up to 7 months of storage, which is indicated by the accumulation of volatile aroma production after extended storage (Figure 3.8-1). On the other hand, the production of volatile compounds in 'Honeycrisp' fruit kept under the same treatment condition was suppressed up to 7 months of storage (Figure 3.8-2)

In both cultivars, the 1-MCP + CA combination had the most substantial suppression effect compared to either treatment alone. The extent of volatile aroma suppression by 1-MCP alone (1-MCP + RA) was generally lower than that of CA. In addition, not all volatile aroma compounds were suppressed by the 1-MCP + RA combination in 'Honeycrisp' fruit. For instance, a higher concentration of butyl acetate and

hexyl acetate were observed under the 1-MCP + RA treatment than in the control fruit (Figure 3.8-2). Exceptionally, 3-methyl-1-butyl acetate in 'Honeycrisp' apples was not supressed by either 1-MCP + CA or CA storage alone, rather its production was supressed under RA storage and increased under CA condition (Figure 3.8-2)

3.3.3 Juice acidity and sugar content

The combined effect of 1-MCP, storage atmosphere, storage time, harvest maturity and juice type on the acidity and sugar content of apple juice prepared from 'McIntosh' and 'Honeycrisp' apples was assessed through the analyses of TA, pH, TSS/TA ratio, malic acid, TSS, and individual sugars (fructose, glucose and sucrose) (Table 3.6-5 to Table 3.6-8).

3.3.3.1 TA, pH, TSS, and TSS/TA ratio

The effect of 1-MCP and other postharvest conditions on juice TA, pH, and TSS/TA ratio from 'McIntosh' and 'Honeycrisp' apples is summarized in Table 3.6-5 and Table 3.6-6, respectively. The results indicated that these quality parameters were not affected by juice type, but rather they were significantly affected by 1-MCP treatment, storage conditions, storage time as well as harvest maturity. Generally, the presented results showed that as storage time advanced, major changes occurred that included: reduced TA and malic acid, increased pH and TSS/TA with a subtle variation of juice TSS.

Juice TA from 'McIntosh' fruit was significantly ($p<0.05$) affected by the two-way interaction of 1-MCP with storage atmosphere as well as harvest maturity with storage time. Irrespective of juice type, a substantial loss of TA (42.5%, 54.8%) was observed after

4 and 7 months of storage, respectively (Table 3.6-5). This loss was significantly higher ($p<0.05$) in commercial (50%) than late (5.4%) harvested 'McIntosh' fruit (Table 3.6-5). As indicated in Table 3.6-5, significantly ($p<0.05$) higher juice TA was observed in 1-MCP treated 'McIntosh' as compared to control fruit held either in CA or RA condition. This suggests that the loss of acidity was substantially reduced by 1-MCP treatment, which was more intensive in RA (21.1%) than in CA (6.9%) storage fruit. The change in pH and TSS/TA ratio reflected that of TA, thus significantly lower ($p<0.05$) juice pH and TSS/TA levels were found in 1-MCP + CA (3.54, 26.3) and 1-MCP + RA (3.7, 31.8) as compared to control + CA (3.6, 28.9) or control + RA (3.9, 37.9) (Table 3.6-5).

As indicated in Table 3.6-6, TA, pH, and TSS/TA ratio in 'Honeycrisp' juice samples were significantly ($p<0.05$) affected by 1-MCP treatment, harvest maturity and the two-way interaction effect of storage time and storage atmosphere. TSS/TA ratio was calculated as TSS in ^{o}Brix divided by TA in g/100 mL. In 'Honeycrisp', regardless of harvest maturity, 1-MCP treatment, storage atmosphere and juice type a substantial loss of TA (38-45%) was observed after 4 and 7 months of storage respectively when compared to the initial content at harvest (Table 3.6-6). However, the loss was reduced by the 1-MCP treatment and/or CA storage. Accordingly, significantly higher ($p<0.05$) juice TA (0.37 g/100 mL) was found from 1-MCP treated 'Honeycrisp' as compared to the control (0.33 g/100 mL). The change in pH (3.7, 3.7) and TSS/TA ratio (29-31) in 1-MCP and control treatments followed similar trend (Table 3.6-6). As that of 'McIntosh', in 'Honeycrisp' juices, significantly higher ($p<0.05$) TA retention was observed under 1-MCP + CA (0.38 g/100 mL) and 1-MCP + RA (0.34 g/100 mL) as compared to control + CA (0.35 g/100

mL), and control + RA (0.32 g/100 mL) treatments (Table 3.6-6). This suggests that 1-MCP treatment in 'Honeycrisp' fruit improved juice acidity retention under both CA (5.9%) and RA (7.8%). Correspondingly, lower juice pH and TSS/TA levels were found in 1-MCP + CA (3.6, 27.1) and control + CA (3.7, 29.5) as compared to 1-MCP + RA (3.7, 31.1) and control + RA (3.8, 32.9) treatments (Table 3.6-6). 'Honeycrisp' fruit from commercial harvest had higher (18.9%) juice TA as compared to those from the late harvest. Our results also indicated the synergistic effect of 1-MCP + CA to improve the retention of acidy in 'Honeycrisp' and 'McIntosh' juices.

Irrespective of all other factors, juice from 1-MCP treated 'McIntosh' fruit resulted in higher TSS levels both in RA (11.7 ^{o}Brix) or CA (11 ^{o}Brix) as compared to control + CA/RA (10.5-10.6 ^{o}Brix) treatments (Table 3.6-5). Nonetheless, in 'Honeycrisp' juices, there was very little change in TSS associated with storage time, harvest stage, 1-MCP treatment and storage atmosphere (Table 3.6-6).

3.3.3.2 Malic acid

In both 'McIntosh' (Table 3.6-5) and 'Honeycrisp' (Table 3.6-6) juices, the content of malic acid was significantly ($p<0.05$) affected by the 1-MCP treatment, storage time, storage conditions and harvest maturity of the apples, but not by the juice type. Regardless of juice type, harvest maturity and storage atmosphere, a considerable degradation of malic acid was detected in juices prepared from stored 'McIntosh' (36.9-79.3%) and 'Honeycrisp' (50.6-72.4%) fruit after 4 and 7 months of storage, respectively when compared to the initial value at harvest (Table 3.6-5 and Table 3.6-6). In both cultivars the

content of malic acid was significantly ($p<0.05$) affected by the two-way interaction of 1-MCP and storage atmosphere. For 'McIntosh', significantly higher malic acid was observed in juices produced from 1-MCP treated apples (0.24 g/100 mL) as compared to juices from control fruit (0.22 g/100 mL) kept under either CA or RA (Table 3.6-5). Similarly, in 'Honeycrisp', significantly higher ($p<0.05$) malic acid was observed in juices produced from 1-MCP treated apples held in CA (0.38 g/100 mL) or RA (0.31 g/100 mL) as compared to juices from untreated fruit held under CA (0.34 g/ 100 mL) or RA (0.33 g/100 mL) conditions (Table 3.6-6). This indicates the benefit of 1-MCP treatment in delaying loss of malic acid, which is more pronounced in CA than RA storage. In 'Honeycrisp', juice produced from fruit subjected to 1-MCP + CA treatments had 21% more malic acid content than from fruit subjected to 1-MCP + RA treatments (Table 3.6-6). Similar to the TA pattern, the malic acid content of 'Honeycrisp' juices was influenced by fruit harvest maturity, where fruit harvested at commercial maturity had 26.7 to 27.6% higher malic acid level following 4 and 7 months of storage respectively than late harvest (Table 3.6-6).

3.3.3.3 Fructose, glucose and sucrose

The content of fructose, glucose, sucrose and total sugar from cloudy and fresh apple juices prepared from 'McIntosh' (Table 3.6-7) and 'Honeycrisp' (Table 3.6-8) apples stored under CA/RA conditions up to 7 months were assessed. Total sugar refers to the sum of the three individual sugars (fructose, glucose, and sucrose).

In ‘McIntosh’ apples, the change in sugar content during storage involves the loss of sucrose (72-80%) and the accumulation of fructose (50-65%), and glucose (83-86%) after 4 and 7 months of storage, respectively when compared with the initial content at harvest. When the change of individual sugars after storage are compared, juice from ‘McIntosh’ apple stored for 7 months had higher fructose (31%), glucose (22%), and reduced sucrose content (28%) (Table 3.6-7).

The content of fructose and glucose from stored ‘McIntosh’ fruit was significantly affected by the interaction of harvest and storage atmosphere. For fruit from the commercial harvest, there was greater accumulation of fructose and glucose in CA (39.9% fructose, 39.8% glucose), than RA storage (20.9% fructose, and 5% glucose). On the other hand, in late harvested ‘McIntosh’ apples, juice from RA stored fruit had higher content of fructose and glucose (36.8% fructose, 29.7% glucose) than from CA stored fruit (19.5% fructose, 16.7% glucose). In juices from ‘Honeycrisp’ fruit, CA storage, regardless of harvest maturity, caused lower content of fructose and glucose, especially when it was not combined with the 1-MCP treatment. The content of total sugar in both ‘Honeycrisp’ and ‘McIntosh’ juices followed the same trend as that of fructose.

The content of sucrose in juice made from stored ‘McIntosh’ apples was significantly ($p<0.05$) affected by the three way interaction of 1-MCP, storage atmosphere and storage time, as well as by harvest maturity, storage atmosphere and storage time (Table 3.6-7). In both CA and RA storage, the content of sucrose was higher in juice samples from late than commercially harvested ‘McIntosh’ fruit (Table 3.6-7). The results also indicated that juice samples from RA stored ‘McIntosh’ with or without 1-MCP

treatment, yielded higher sucrose content as compared to juice from CA stored fruit, which is more prominent after 7-month storage (Table 3.6-7).

When compared with the initial content, the change of sugar content in juices from stored 'Honeycrisp' fruit includes considerable degradation of sucrose (54-72%) with substantial accumulation of glucose (82-83%) and fructose (29-34%) following 4 and 7 months of storage respectively (Table 3.6-8). If we consider the change of sugars between 4 and 7 months, there was a significant loss of sucrose (38.3%) with a small accumulation of fructose (7%) and glucose (7.5%).

Sucrose content from 'Honeycrisp' juice samples was significantly ($p<0.05$) affected by the two-way interaction of harvest maturity and storage as well as 1-MCP and storage atmosphere. After 4 months of storage, juices from late harvest had higher sucrose content but the opposite was true after 7 months of storage (Table 3.6-8). As with 'McIntosh' fruit, juice samples from RA stored 'Honeycrisp' fruit with or without 1-MCP treatment yielded higher sucrose, content as compared to juice from CA stored fruit. In this regard, the highest (2.07 g/100 mL) and the lowest (1.61 g/100 mL) sucrose was found in control + RA and control + CA treatments, respectively (Table 3.6-8). Thus, the results suggest that in both cultivars neither 1-MCP treatment nor CA storage prevented the loss of sucrose over long-term storage time.

Juice type had a significant effect on the sugar content of 'McIntosh' and 'Honeycrisp' juices where cloudy juice samples yielded higher content of all individual sugars.

3.3.4 Expression of genes regulating malic acid metabolism

The effect of storage time, 1-MCP treatment and storage atmosphere on the expression of the genes which encode the activity of NADP-ME (MdcyME), NAD-MDH (MdcyMDH), PEPC (MdcyPEPC) and subunit A of V-ATPase (MdVHA-A) is shown in Figure 3.8-3 ('McIntosh') and Figure 3.8-4 ('Honeycrisp'). In this experiment '0 month control' or at harvest treatments refers to samples that were neither treated with 1-MCP nor stored under CA or RA condition. Note that, as 'McIntosh' apples stored under RA condition without 1-MCP treatment were over ripe and in a very advanced ripening stage (even started to shrivel and wilt) the RNA extraction process was not successful and hence it was not possible to consider the expression of the target genes in control + RA treated 'McIntosh' fruit samples.

3.3.4.1 MdcyME

In 'McIntosh' fruit, the expression of MdcyME increased as the ripening stage gets to a more advanced stage and hence its expression level was considerably higher (4-fold) in stored than at harvest fruit samples. The results also indicated significant differences among postharvest treatments where higher MdcyME expression was observed in 1-MCP + RA treatments as compared to 1-MCP/control + CA storage conditions (Figure 3.8-3).

A different trend of MdcyME expression was found in 'Honeycrisp' fruit (Figure 3.8-4). Generally, the expression level of MdcyME in 'Honeycrisp' fruit was considerably lower (0.08-0.52) than 'McIntosh' (5.6-8.8). Moreover, in contrast to 'McIntosh' the expression level of MdcyME in 'Honeycrisp' fruit decreased with advanced

fruit ripening stage (Figure 3.8-4). Consequently, the lowest MdcyME expression was found in the control + RA treatment which had 10.6, 6.5, 5.6 and 5.1 times lower expression level as compared to samples at harvest, 1-MCP + CA, 1-MCP + RA and control + CA treatments, respectively. For RA stored 'Honeycrisp' fruit, 1-MCP treatment yielded higher (5.6-fold) MdcyME expression as compared with control samples; however, 1-MCP treatment in CA storage condition did not affect the expression level of this gene.

3.3.4.2 MdcyMDH and MdcyPEPC

The expression of MdcyMDH in 'Honeycrisp' decreased as the fruit gets to a more advanced ripening stage and the difference was most prominent between samples at harvest and those kept under control + RA treatment. Among stored fruit, the level of MdcyMDH expression differed depending on 1-MCP treatment and storage condition. Thus, 1-MCP + CA, 1-MCP + RA, and control + CA treatments had 5.4, 3.4, and 3.0 times higher MdcyMDH expression than control fruits kept under control + RA condition, respectively (Figure 3.8-4).

The expression of MdcyPEPC in 'McIntosh' fruit showed a similar trend as that of MdcyMDH in 'Honeycrisp'. Thus, the expression of MdcyPEPC decreased considerably as 'McIntosh' fruit gets to a more advanced ripening stage during the 4-month storage period (Figure 3.8-3). This difference is clearly observed between samples at harvest and samples stored under RA conditions. Accordingly, 'McIntosh' fruit at harvest had 5-fold higher MdcyPEPC expression as compared to those stored under RA storage condition. In stored 'McIntosh' fruit, 1-MCP + CA and control + CA treatments had 1.5-

2.5 times, higher MdcyPEPC expression as compared to those kept under 1-MCP + RA treatment (Figure 3.8-3). Note that the transcript levels of MdcyMDH in 'McIntosh' and MdcyPEPC in 'Honeycrisp' were very low (CT values >35) and consequently their expression level were similar in all samples irrespective of the treatments applied (data not shown).

3.3.4.3 MdVHA-A

MdVHA-A was expressed in both 'McIntosh' and 'Honeycrisp' apples but with different trend. In 'McIntosh' apples, little or no significant differences were found among treatments (Figure 3.8-3). However, relatively higher MdVHA-A expression was observed in samples at harvest as compared to stored samples. In 'Honeycrisp', considerably higher MdVHA-A expression (2.46-2.83) was observed in samples at harvest as well as in stored fruits under 1-MCP + CA condition as compared to other treatments (Figure 3.8-4). In this cultivar, 1-MCP treatment in both CA (3.4-fold) and RA (1.8-fold) storage regimes resulted in higher level of MdVHA-A expression as compared to control treatments.

3.4 Discussion

3.4.1 Change in ethylene production, volatile aroma and firmness

3.4.1.1 Ethylene production

As expected a remarkable inhibition of ethylene production was observed in 'McIntosh' fruit treated with 1-MCP especially when fruit were harvested at commercial stage and stored under CA condition. Under CA storage, the inhibition of ethylene production due to 1-MCP treatment ranges from 81% (late harvest) to 98% (commercial harvest) at the end

of 4 months storage period. Even if it was not as remarkable as CA, 1-MCP treatment under RA condition could also inhibit ethylene production by about 60% when 'McIntosh' fruit were harvested at commercial maturity stage and stored for 4 months. It must be highlighted that the 1-MCP treatment was more effective when 'McIntosh' fruit were harvested at commercial maturity. This might be ascribed to the higher IEC of late harvested apples at the time of treatment (Jung and Watkins, 2014).

The results also suggests the synergistic positive effect of 1-MCP and CA combination to reduce ethylene production and maintained at its pre-climacteric level (0.6 and 5.0 $\mu L kg^{-1}h^{-1}$) in 'McIntosh' fruit stored for 4 months and harvested at commercial and late harvest stage, respectively. This is in agreement with a previous study (Defilippi et al., 2004), where 1-MCP treatment of 'Greensleeves' apple resulted in reduction of ethylene production below 10 $\mu L kg^{-1}h^{-1}$ (about 70% inhibition), which caused in a remarkable reduction of respiration rate and loss of TA during the 14 day storage at room temperature. A similar rate of ethylene suppression (>90%) has been reported in transgenic 'Greensleeves' apples suppressed for ACC synthase (ACS) or ACC oxidase (ACO) (Dandekar et al., 2004). This supports that 1-MCP treatment in our study was effective in supressing autocatalytic ethylene production during long-term storage of apples.

In agreement with previous studies, which concluded that ethylene production in 'Honeycrisp' apples is different from many other apple cultivars (Wargo and Watkins, 2004; Watkins et al., 2004; DeEll and Ehsani-Moghaddam, 2010; Harb et al., 2012) our results also showed different trends of ethylene production in 'Honeycrisp' and 'McIntosh' fruits. While, suppressed ethylene production (5-11 $\mu L kg^{-1}h^{-1}$) was found in untreated and

CA stored ‘Honeycrisp’ fruit harvested at commercial stage and stored up to 7 months, the CA storage alone in ‘McIntosh’ fruit caused considerably higher ethylene production (28.8-46 $\mu Lkg^{-1}h^{-1}$). Unlike ‘McIntosh’, where 1-MCP + CA combination had a much greater ethylene suppression effect (0.6-20 $\mu Lkg^{-1}\ h^{-1}$) as compared to control + CA treatment (28-46 $\mu Lkg^{-1}\ h^{-1}$) such huge difference was not observed in ‘Honeycrisp’ apples. In ‘Honeycrisp’ apples, CA storage alone had similar effect in lowering ethylene production (5.09 and 5.27 $\mu Lkg^{-1}\ h^{-1}$) as compared to 1-MCP + CA combination (1.08 and 2.97 $\mu Lkg^{-1}\ h^{-1}$) over 7 and 4 months of storage, respectively. Another striking difference was observed in regard to the effect of 1-MCP to inhibit ethylene production under RA storage condition. While 1-MCP treatment alone (1-MCP + RA) in ‘McIntosh’ apples inhibited ethylene production better than the control + CA/RA treatments, the same treatment in ‘Honeycrisp’ produced the highest ethylene (49.03 $\mu Lkg^{-1}\ h^{-1}$) level, which was even more than in control fruit (17 $\mu Lkg^{-1}\ h^{-1}$). This suggests that incorporating 1-MCP treatment might not be a cost effective option for the long-term storage of ‘Honeycrisp’ apples.

Moreover, it has to be highlighted that while inhibition of ethylene production in 1-MCP treated ‘Honeycrisp’ fruit stored under CA condition lasted up to 7 months of storage, elevated ethylene biosynthesis was restored in ‘McIntosh’ fruit after 7 months storage under CA or RA condition, irrespective of 1-MCP treatment. Thus, the effects of 1-MCP was shorter lived in ‘McIntosh’ than ‘Honeycrisp’. Thus, a second 1-MCP application after 4 months of storage might be needed as recommended by DeEll et al., (2016). It has been demonstrated that cultivars differed in their response to 1-MCP

treatment, effects being short-lived in some cultivars, such as 'McIntosh' and 'Cortland' than in others e.g. 'Delicious', 'Empire' and 'Honeycrisp' (Jung and Watkins, 2014; Watkins et al., 2000; Watkins 2008). Jung and Watkins, 2014 compared the effect of 1-MCP in 'McIntosh', 'Cortland', and 'Empire', apples, with highest, medium and lower IEC at harvest, respectively. Their results confirmed that the degree of the inhibition of ethylene production during long-term storage of these apples is a function of IEC of fruit at the time of treatment. Moreover, the reasons for differences in cultivar responses in terms of ethylene receptor stability and expression was investigated by Tatsuki et al. (2007) and Tatsuki et al. (2009). These authors compared 'Orin' and 'Fugi', fast, slow ripening cultivars, respectively, and suggested that cultivar variations in response to 1-MCP were associated with a combination of ethylene production, expression of ethylene receptor genes as well as protein stability of these receptors.

3.4.1.2 Flesh firmness

In agreement with previous studies (Watkins, 2008; DeEll et al., 2002; Watkins, 2007; Rupasinghe et al., 2000) our results indicated that in 'McIntosh' fruit inhibited ethylene production by 1-MCP and or CA storage was accompanied by better flesh firmness retention, which is most pronounced in fruits harvested at commercial maturity and stored for 4 months. In both harvest dates, 1-MCP + CA combination could maintain the flesh firmness of 'McIntosh' apples within acceptable firmness level (> 44.5 N) for Canadian consumers who find apples with a firmness less than 44.5 N too soft (DeEll et al., 2001). The underlying biochemical mechanism for the effect of 1-MCP in delaying flesh softening

of ‘McIntosh’ fruit is attributed to the change in the enzymatic cell wall disassembly. 1-MCP reduces or delays the expression and accumulation of cell wall modifying enzymes including polygalacturonases, pectinmethylesterase, beta-galactosidase (β-Gal) (Zheng et al., 2013) and the down regulation of their gene expression level (Johnston et al., 2002), which is directly linked to the softening of many apple cultivars.

Unlike ‘McIntosh’, which softens dramatically after prolonged storage, ‘Honeycrisp’ apple maintained its flesh firmness close to at harvest levels over 7 months of storage regardless of elevated ethylene production. This is in agreement with previous studies that confirmed the ability of ‘Honeycrisp’ to maintain their flesh firmness during short or long-term storage (Wargo and Watkins, 2004; Watkins et al., 2004; DeEll and Ehsani-Moghaddam, 2010; Harb et al., 2012). The maintenance of flesh firmness in ‘Honeycrisp’ during long-term RA or CA storage has been attributed to the maintenance of high turgor potential and cell wall integrity (Tong et al., 1999). Harb et al. (2012) studied the molecular mechanism responsible for the slow softening of ‘Honeycrisp’ fruit as compared with a rapidly softening cultivar, ‘McIntosh’. Their finding demonstrated very low expression of genes regulating the two cell wall catalysing enzymes including polygalacturonases and pectate lyase in ‘Honeycrisp’ than ‘McIntosh’ and hence it was suggested that restricted activity of these cell wall metabolizing enzymes as a possible reason for the slow softening of ‘Honeycrisp’ apples.

3.4.1.3 Volatile aroma production

In both cultivars, inhibited ethylene production was followed by a substantial reduction of volatile aroma production including esters, ethanol and total volatile aroma compounds. A similar pattern of ester, ethanol and total volatile aroma inhibition has been reported in other apple cultivars where ethylene production was supressed by 1-MCP treatment and or CA storage (Lurie et al., 2002; Defilippi et al., 2004). Thus, the results of the present study confirm that ester production is under ethylene regulation. The expression of alcohol acyl-CoA transferase (AAT), the rate-limiting enzyme in ester biosynthesis, is regulated by ethylene in apple (Defilippi et al., 2004) which could explain the inhibition of esters and total volatile aroma production in 'McIntosh' and 'Honeycrisp' apples where ethylene production was suppressed.

Interestingly, higher ethylene production was not always followed by elevated volatile aroma production. For instance, our results indicated reduced level of total volatile aroma production in untreated 'McIntosh' fruit stored under CA, where ethylene production was higher. Similar patterns were found in 'Honeycrisp' fruit kept under 1-MCP + RA, where higher ethylene production was accompanied by decreased production of ethyl acetate and ethanol. This may be attributed to the feedback inhibition response due to the excessive amount of ethylene (Balbontín et al., 2007). It might also suggest that the production of some volatile compounds might not be regulated by ethylene.

3.4.2 Change of juice acidity and sugar content

Most research to date regarding apple and 1-MCP has focused mainly on its effect on fruit quality parameters such as appearance, flesh firmness, physiological disorders. However, the effect of 1-MCP on the internal fruit quality attributes after juice processing is still poorly characterized. To our knowledge, this is the first study to report the effect of 1-MCP on the acidity and sugar content of cloudy apple juice prepared from 'Honeycrisp' and 'McIntosh' apples after long-term storage under CA or RA conditions. In the present study, acidity and sugar content of apple juice was determined by measuring pH, TA, malic acid content, TSS, TSS/TA ratio and HPLC quantification of major simple sugars (fructose, glucose, and sucrose).

3.4.2.1 TA and pH

Juice TA measures the concentration of both bound and free hydrogen ions in a solution and is a better predictor of acid's impact on the flavour of a food system than pH that only measures the content of free hydrogen ions (-log $[H^+]$) (Da Conceicao Neta et al., 2007). Yet, juice pH is an important juice quality as it affects the growth and development of microorganisms that in turn affects the safety and quality of the product (Da Conceicao Neta et al., 2007).

The level of TA from freshly harvested and stored apples in the present work is in agreement with previously reported ranges for apple juices extracted from fruit harvested at commercial maturity (Wu et al., 2007; Elkins et al., 1996). At harvest, juice samples from 'McIntosh' were more acidic (TA, 0.73 g/100 mL, pH, 2.92) than 'Honeycrisp' (TA,

0.60 g/100 mL, pH, 3.18). However, after extended fruit storage the cultivar difference was negligible and juice acidity was substantially reduced, which was indicated by reduced TA (0.33 g/100 mL) and increased pH (3.8).

In spite of the significant loss of acidity during storage, 1-MCP treatment of fruit resulted in higher TA and lower pH in both cloudy and fresh juice samples, but its effectiveness varied between cultivars, harvest maturity, and storage atmospheric conditions. Juice samples from 1-MCP treated 'McIntosh' apples had 6.9% to 21% higher TA and 0.05-0.25 lower pH units as compared to those from untreated fruit kept under RA and CA, respectively. Similarly, juice from 1-MCP treated 'Honeycrisp' fruit had higher TA [5.9% (RA), 7.9% (CA)] and lower pH units [0.06, (CA), 0.07 (RA)] as compared to untreated fruit. As pH = -log[H+], a change of pH by 1.0 unit is equivalent to a 10-fold change in hydrogen ion concentration and hence, an apparently smaller changes in pH such as 0.05 units would result 0.89-fold difference in terms of hydrogen ion concentration. Our results from both cultivars suggest that the combination of 1-MCP + CA yielded better acidity retention as compared to either treatment alone. Moreover, the presented results clearly showed that under RA storage condition, 1-MCP is more effective in 'McIntosh' than 'Honeycrisp' apples. This cultivar difference was also noticed in other studies (Watkins et al., 2000; Magazin et al., 2010; Fan et al., 1999; Jung and Watkins, 2014). The reduced effectiveness of 1-MCP in RA stored 'Honeycrisp' could be attributed to the higher ethylene production found in this treatment. In both cultivars, higher acidity retention was associated with the suppressed ethylene production especially under 1-MCP + CA condition. This confirms the ethylene dependency of TA as suggested by previous

studies in other apple cultivars (Watkins et al., 2000; Magazin et al., 2010; Fan et al., 1999; Jung and Watkins, 2014).

The strong positive correlation between TA and tart, sour or acid taste sensations of apples has been demonstrated in a number of studies as reviewed by Da Conceicao Neta et al. (2007). According to Harker et al. (2002), TA differences as low as 0.08% (0.08 g/100 mL) evoked a response in perceived acid taste. As presented in our results, the TA difference between 1-MCP treated and control apples ranged from 7-21%, this implies that juice samples from 1-MCP treated apples could evoke more acidic or sour taste perception than those from untreated fruit. From the technological point of view, a juice with higher acidity or lower pH is an advantage for juice processors. As lower pH and high TA enhances microbial inactivation, products with lower pH (<4.6) will receive less heating process, which in turn, prevents the loss of heat sensitive nutrients and flavour compounds (Beales, 2004). The immense loss of malic acid in juices from control apples stored under RA would be a disadvantage as it resulted in loss of flavour and limited shelf life. Thus, juices from 1-MCP treated apples could have better overall quality.

From the dental health point of view, however, frequent and excessive consumption of acidic beverages such as apple juice is suggested to pose dental erosion, which is caused by acid dissolution of dental hard tissues without the involvement of bacteria (Stefański and Postek-Stefańska, 2014). In the present study 1-MCP + CA/RA or control + CA treatments in ‘Honeycrisp’ or ‘McIntosh’ fruit following 4 months of storage yielded juice pH <3.5. Untreated fruit kept under RA for 4 months yielded a juice pH

between 3.5 and 3.7. However, after 7 months of storage the juice pH increased above 3.7 to a maximum value of 4.2 irrespective of any treatment. This indicates that the acid profile of stored apples depends on both storage time and postharvest treatments. Hence, by considering these differences, juice processors can have a wider range of products with different pH ranges for different market segments or purposes. For instance, those fruit with a pH of 4.0 would be suitable to those consumers who are concerned about dental erosion. Similarly, those fruit, which have a pH, range of <3.5 could be appropriate for long-term storage, long distance transport or for blending with low acid beverages.

3.4.2.2 TSS

TSS also called Brix value is a good approximation for total sugar content measurement of fruit juices and it is one of the basic tests carried out by fruit juice producers to assess the soluble solids content of fruit juice samples. Even though our results found little or no change of TSS during storage, juices from 1-MCP treated 'McIntosh' fruit had 0.56-1.04 ^{o}Brix higher TSS level in comparison to those from untreated fruit held in CA and RA storage, respectively. Harker et al. (2002) suggested that TSS is the best objective predictor of sweetness, which can predict a difference in sweetness taste when apple TSS value differed by more than 1% or 1 ^{o}Brix. Accordingly, juices from 1-MCP treated 'McIntosh' apples could possibly have sweeter taste perception; however, this needs to be confirmed by a sensory taste experiment. Previous studies on the effect of 1-MCP on quality traits of whole apples have reported no or little change (Akbudak et al., 2009), higher (DeEll, 2007), or lower TSS (Watkins et al., 2000) values in 1-MCP treated apples. The inconsistent effect

of 1-MCP on the TSS content of different cultivars might indicate that the change of TSS in apple fruit is independent of ethylene action.

3.4.2.3 TSS/TA ratio

TSS/TA ratio has been found to be an important parameter and is frequently used to establish standard sensory or taste qualities for predicting consumer acceptance of apple juice (Jaros et al., 2009). The higher the TSS value in relation of the acid content of the juice, the higher the TSS/TA ratio and 'sweeter' the taste. In our study, the change in TSS/TA ratio in 1-MCP treated and control fruit followed the same trend as that of TA but in reverse order. The lower TSS/TA ratio in any treatment group after 1-MCP treatment and or CA storage was related with the higher retention of TA rather than due to a change in TSS. Our TSS/TA ratios (16-34 for 'McIntosh, 20-32 for 'Honeycrisp') are comparable to literature values given for single variety apple juices (Jaros et al., 2009; Rødbotten et al., 2009).

Sensory analysis conducted in Germany using single-cultivar cloudy apple juice from 'Golden Delicious', 'Elstar', 'Idared' and 'Jonagold' apples found that less sweet and the more acidic products with sugar acid ratio of 15 to 21 were significantly preferred; however, there was a sub-fraction of approximately one-third of the panel who preferred the samples with a higher sugar acid ratio (21-28) (Jaros et al., 2009). In another study conducted in Norway and Spain, consumers preferred the sweeter apple juice samples with a sugar acid ratio ranging from 21 to 25 as compared to other samples with lower sugar acid ratios (10-21) (Rødbotten et al., 2009). In the present study, juices made from apples from 1-MCP + CA or control + CA treatments yielded a TSS/TA ratio ranging from 27.1 to 29.5, which is in a favourable consumer preference range as compared to juice samples from control fruit kept under RA with higher TSS/TA ratio (31 to 37). As the higher TSS/TA ratio might render an excessively sweet sensory sensation, 1-MCP treatment might yield a juice that is not excessively sweet or sour.

3.4.2.4 Malic acid

Malic acid ($C_4H_6O_5$), the major organic acid in apples that accounts for about 90% of total acids, is responsible for the sourness and acidity of apples (Ackermann et al., 1992). It has been demonstrated that in acid testing, high acid mutant 'Usterapfel' apples the concentration of malic acid 10-fold greater compared to a sweet tasting or low-acid form of 'Usterapfel' apples (Berüter, 1998).

Even though there was a substantial degradation of malic acid during fruit storage, regardless of juice type, storage time and other postharvest treatments, juices from

1-MCP treated ‘McIntosh’ and ‘Honeycrisp’ apples had considerably higher malic acid content as compared to those from untreated fruit. Juices from 1-MCP treated ‘McIntosh’ apples had 25% to 30% higher malic acid content as compared to untreated fruit kept under either RA or CA conditions, respectively.

Similarly, juice samples from 1-MCP treated ‘Honeycrisp’ apples retained noticeably higher malic acid content as compared to those from untreated fruit particularly in CA versus RA storage. In contrast to ‘McIntosh’, where CA storage alone did not prevent the loss of malic acid, in ‘Honeycrisp’ fruit CA storage was as effective as 1-MCP treatment in preventing the loss of malic acid during storage. This discrepancy is attributed to the different ethylene production trend of the two cultivars as previously discussed in section 3.4.1. The lower malic acid concentration from the 1-MCP + RA treatment versus the 1-MCP + CA or control + CA treatments in ‘Honeycrisp’ apples was expected since the fruit under this treatment had the highest ethylene production rate especially in the late harvest maturity. In ‘Honeycrisp’, 1-MCP + RA treatment also yielded lower TA, higher pH and higher TSS/TA ratio as compared to 1-MCP/control + CA treatments. The presence of organic acids in many beverages plays a key role for their refreshing taste and storage stability. Thus improved retention of malic acid in juices prepared from 1-MCP treated and or CA stored apples would be an advantage for juice processors and consumer satisfaction.

Malic acid is the major substrate involved in aerobic respiration of harvested apples (Yao et al., 2009). Thus, the reduced level of acidity in juices extracted from control apples could be attributed to the degradation of malic acid due to the increased ethylene production rate, which in turn leads to higher respiration rate. During apple ripening, malic

acid is degraded and decarboxylated into either pyruvate or PEP by the action of cytosolic NADP-ME and PEPCK, respectively (Etienne et al., 2013). The decarboxylation of malic acid into pyruvate or PEP leads to its consumption in the TCA cycle and the production of glucose (gluconeogenesis) (Etienne et al., 2013). Gluconeogenesis is the metabolic pathway that generates glucose from PEP, which occurs mostly during fruit ripening when sugars accumulate rapidly (Sweetman et al., 2009). However, in stored apples, rapid accumulation of sugars is not an apparent phenomenon, thus the most probable pathway of malic acid degradation is possibly through its decarboxylation into pyruvate through the action of NADP-ME. A genetic and enzymatic study indicated higher levels of NADP-ME activity in low acid apples than in high acid ones suggests the possible role of NADP-ME in malic acid degradation of ripening fruit (Yao et al., 2009). However, since ethylene production was neither controlled nor measured, this study could not elucidate how ethylene regulates the activity or gene expression of NADP-ME in ripening apple fruit. A recent proteomic study on 'Golden Delicious' apples stored for 20 days at room temperature, reported a considerable reduction of TA corresponding to increased NADP-ME activity and elevated ethylene production rate of ethylene-treated fruit, which proposes the regulation of NADP-ME by ethylene production (Zheng et al., 2013).

The improved malic acid retention in 1-MCP treated or CA stored 'McIntosh' and 'Honeycrisp' fruit, where ethylene production is suppressed, suggests the ethylene dependency of malic acid metabolism during long-term storage of apples. These results are in agreement with previous studies that found improved retention of malic acid in 1-MCP treated apples stored for short or long-term storage periods. Defilippi et al. (2004) studied

the change of malic acid in transgenic 'Greensleeves' (silenced for either ACS or ACO) and non-transgenic 'Greensleeves' 1-MCP treated apples stored for 14 days at 20 ^{o}C and 90-95% RH, and found a significant reduction of malic acid degradation when ethylene production was inhibited below 10 $\mu Lkg^{-1} h^{-1}$. A recent study (Bizjak et al., 2012) on 1-MCP treated 'Idared' apples stored for 6 months under ultra-low oxygen condition found similar results as that of Defilippi et al. (2004). However, none of the above studies conducted genetic or enzymatic studies to explain the mechanism by which 1-MCP regulates malic acid metabolism in apple fruit. In our study, efforts were made to address this issue as discussed in section 3.4.1

3.4.2.5 Sugars

In the present study, fructose, glucose and sucrose were identified as the major monosaccharide and disaccharide sugars in all juice samples tested. The change of these major sugars during long-term storage of 'Honeycrisp' or 'McIntosh' apples mainly involves reduction of sucrose, with a corresponding accumulation of glucose and fructose. Both in stored and freshly harvested fruit, fructose level was always higher than glucose and sucrose. The dominance of fructose was also observed in many other apple cultivars (Fuleki et al., 1994; Hecke et al., 2006; Lee and Wrolstad, 1988). At harvest, the major sugars in juices from 'McIntosh' and 'Honeycrisp' fruit were fructose (47.6-48.1%) and sucrose (44.8-48.3%) with a minor proportion of glucose (3.2-3.6%). However, after storage, the main sugars in 'McIntosh' and 'Honeycrisp' juices were fructose (69.9-79%) and glucose (15.5-17.8%) with smaller fraction of sucrose (5.5-12.2%). This indicates that

the accumulation of fructose and glucose is at the expense of sucrose degradation, which is most pronounced in 'McIntosh' juices. The significant reduction of sucrose with a concomitant accumulation of monosaccharide sugars during storage of apples has been demonstrated in several other cultivars as well (Ackermann et al., 1992; Fuleki et al., 1994; Suni et al., 2000).

Our results suggest that neither CA storage nor 1-MCP treatment prevented the loss of sucrose during long-term storage of both apple cultivars. In agreement with our findings, other studies also demonstrated that 1-MCP treatment did not prevent the significant loss of sucrose in 'Idared' (Bizjak et al., 2012) and 'Empire' apples (Lee et al., 2012) stored for 6 to 10 months under CA conditions. Hence, it can be suggested that sucrose metabolism might not be regulated by ethylene production. The increased fructose, glucose and total sugar regardless of 1-MCP treatment or CA storage was consistent with a study in 'Empire' apples stored under CA for 10 months (Lee et al., 2012). 1-MCP was reported to cause negligible changes in the activity of enzymes catalysing the key steps of the glycolysis pathway in ripening peach (Borsani et al., 2009) and in stored banana (Ball and Tom, 1988), which supports the conclusion that sugar metabolism is not ethylene dependent.

Recently, Zhu et al. (2013) investigated changes in sugar content in 'Fuji' apples that were stored in RA or CA for up to 8 months. This study also determined the biochemical and molecular basis for changes in sugar content during storage. In agreement with our finding, the authors observed a significant degradation of sucrose during storage in both CA and RA (Zhu et al., 2013). In their study, the degradation of sucrose followed

two different pathways depending on the storage atmosphere. The two alternative biochemical pathways for sucrose degradation involve invertase or SS. These pathways have different energy requirements (Lara et al., 2011; Stitt, 1998). The study found, lower activity and gene expression level of invertase in CA than in RA stored fruit. While the hydrolysis of sucrose by invertase requires two molecules of ATP, its catabolism by SS requires only one molecule of PPi (Lara et al., 2011; Stitt, 1998). Consequently, the hydrolysis of sucrose through the energetically less costly route provided by the SS pathway might be a favorable pathway in CA stored apples. This would save energy and decrease oxygen consumption under CA storage conditions (Lara et al., 2011).

The variation in the content and proportion of major sugars may have implications on the functional, sensory and nutritional perspectives of the product in our case apple juice. Due to growing health concerns and nutritionists' recommendation to decrease sucrose intake, many food companies are interested in reducing the sucrose content of fruit juices and nectars (Rødbotten et al., 2009). Since regular fruit juices contain a significant quantity of sugars, most beverage industries are concerned in reducing the sugar content of their products. Recently, there was an initiative by the European Collective Research Project (Contract 030379) to find techniques to produce a novel apple juice with up to a 50% reduction of sugars (measured by calories) without affecting consumers' preference for the juice (Rødbotten et al., 2009). Cultivar selection (Eisele and Drake, 2005), membrane filtration (Fukumoto et al., 1998) and use of artificial sweeteners (Al-Dabbas and Al-Qudsi, 2012; Pimentel et al., 2014) were some of the proposed ways to achieve this goal. Our results suggest that using stored fruit (4 to 7 months) could also be

an alternative to reduce the content of sucrose by about 50-80%. However, since sucrose has been part of the human diet for centuries and the sweet taste that it provides to food products is naturally preferred by consumers (Al-Dabbas and Al-Qudsi, 2012). Thus, juices that are dominated by fructose and glucose might have unfavourable acceptability. Compared to sucrose solution (10% w/v) with a given (standard) sweetness value of 100, the relative sweetness values of fructose and glucose are: 150-170, 70-80, respectively (Pancoast and Junk, 1980). Consequently, the high fructose content in juices from stored apples may increase the sweetness sensation in samples of similar TSS/TA ratio due to the high sweetness index of fructose. The difference in sugar content between fresh and cloudy juice might be related to the heat treatment delivered to cloudy juice, which results in hydrolysis of sucrose and starch, and the accumulation of more hexose sugars such as glucose and fructose.

3.4.3 Gene expression of key enzymes regulating malic acid metabolism

As previously discussed in section 3.4.2, 1-MCP treated 'McIntosh' and 'Honeycrisp' fruits yielded considerably higher juice acidity, as evidenced from higher TA, malic acid content and lower pH, as compared with untreated fruit samples. The effectiveness of 1-MCP treatment in preventing the loss of acidity was most pronounced in fruits harvested at commercial maturity and stored for 4 months. Hence, this work investigated the expression of four genes including MdcyMDH, MdcyME, MdcyPEPC, MdVHA-A that encode the activities of cytosolic NAD-MDH, NADP-ME, PEPC and subunit A of V-ATPase, respectively. The aim of this section of our study was therefore to examine the

effects of 1-MCP treatment on the expression of the genes encoding malic acid biosynthesis (MdcyMDH and MdcyPEPC), degradation (MdcyME) and vacuolar transport (MdVHA-A) in 'McIntosh' and 'Honeycrisp' apples stored for 4 months under CA or RA condition.

The final concentration of malic acid in ripe fruits is determined by the balance of malate biosynthesis, degradation and vacuolar storage (Yao et al., 2009; Chen et al., 2009; Khan et al., 2013). However, studies are yet to unequivocally determine the biochemical and molecular mechanisms by which reduced malate degradation occurs in response to 1-MCP treatment and or CA storage condition. Hence, this work investigated the expression of four genes including MdcyMDH, MdcyME, MdcyPEPC, MdVHA-A that encode the activities of cytosolic NAD-MDH, NADP-ME, PEPC and subunit A of V-ATPase, respectively. Malate biosynthesis mainly occurs in the cytosol and catalysed by PEPC and NAD-MDH (Yao et al., 2011; Yao et al., 2009). The loss of malate during fruit ripening and storage has usually been attributed to its degradation by the action of cytosolic NADP-ME (Yao et al., 2009). Previous investigations on apples and other fruits demonstrated the possible role of these enzymes in regulating the biosynthesis and degradation of malic acid in fruit cells, thus acidity of fruits (Yao et al., 2009; Yao et al., 2011; Chen et al., 2009). It has also been suggested that the vacuole transporters such as V-ATPase could play a key role in determining the fruit acidity (Etienne et al., 2013; Etienne et al., 2002; Schumacher and Krebs, 2010). However, these studies focused at early developmental stages of apples and little published information is available to explain the relation between the changes of malic acid content and the gene expression level of the

enzymes regulating malate metabolism and transport during the postharvest storage life of apples.

3.4.3.1 Change of MdcyME

The concentration of malic acid in harvested fruits is mainly affected by the rate of its degradation associated with the ripening process during storage (Berüter, 2004). Malic acid degradation is the result of its decarboxylation into pyruvic acid and CO_2 via the action of cytosolic NADP-ME, which allows malate to be used as respiratory substrate (Sweetman et al., 2009).

The higher gene expression of MdcyME observed in stored 'McIntosh' fruit as compared to samples at harvest, suggests the possible role of cytosolic NADP-ME for the degradation of malic acid during storage. In stored 'McIntosh' apples, the 1-MCP + CA treatment had substantially reduced MdcyME (about 2-fold) expression as compared to control + CA and 1-MCP + RA treatments. As discussed earlier (section 3.4.2) 'McIntosh' fruit kept under 1-MCP + CA treatment had, higher malic acid content as compared to those stored under 1-MCP + RA treatments. Thus, the reduced expression of MdcyME in 1-MCP + CA treatment might explain the mechanism by which 1-MCP treatment prevented the loss of malic acid during storage. The role of cytosolic NADP-ME to the lack of malate has also been demonstrated in low acid apple (Yao et al., 2009) and loquat genotypes (Chen et al., 2009). As 1-MCP + CA treatment had the lowest ethylene production (section 3.4.1), the lower expression level of MdcyME in this treatment suggests the possible role of ethylene to regulate the expression of MdcyME and

consequently the level of malic acid during ripening. Similarly, Shi et al. (2014) reported a positive association between NADP-ME and ethylene production. The study observed increased activity of cytosolic NADP-ME during ripening, which in turn coincided with the climactic rise of ethylene production and respiration (Shi et al., 2014).

In contrast to 'McIntosh', the expression level of MdcyME in 'Honeycrisp' fruit decreased with advanced fruit ripening stage. Similar trend of MdcyME expression was reported for 'Fugi' and 'Jonatan' apples where the expression of MdcyME reduced as the ripening stage advances from 30 to 175 days after bloom, regardless of the rapid decline of malic acid during the same time (Sun et al., 2015). Yao et al. (2009) studied the expression of the genes controlling malic acid degradation (MdcyME), biosynthesis (MdcyPEPC) and transport (MdVHA-A) in low and high acid apple genotypes and found that while the expression levels of MdcyPEPC and MdVHA-A matched well with the respective enzyme activities at most stages of fruit development, the expression of MdcyME fluctuated with a quite different way with cytosolic NADP-ME activity. Hence, the results for our study as well as other related studies suggest that the activity of cytosolic NADP-ME might be regulated by other genes (Yao et al., 2009).

3.4.3.2 Change of MdcyMDH and MdcyPEPC

Malate biosynthesis mainly occurs in the cytosol and catalysed by cytosolic PEPC and NAD-MDH (Yao et al., 2009; Yao et al., 2011). Our results show that the transcript level of MdcyMDH (in 'Honeycrisp') and MdcyPEPC (in 'McIntosh') were considerably higher in fruit samples before storage and decreased as the fruit gets to a more advanced ripening

stage. This trend is parallel with the content of malic acid that decreased during storage. Hence, the lower transcript level of MdcyMDH or MdcyPEPC and therefore reduced malic acid biosynthesis in stored fruits as compared with those at harvest might be one cause as to why the content of malic acid in 'Honeycrisp' or 'McIntosh' apples was reduced during storage.

In addition, our results indicated that the 1-MCP + CA treatment in 'Honeycrisp' fruit had the highest MdcyMDH expression as compared to other treatments. Similarly, 1-MCP/control + CA treatment in 'McIntosh' yielded considerably higher MdcyPEPC expression as compared to 1-MCP + RA treatments. As discussed earlier (section 3.4.2) apple samples kept under 1-MCP + CA treatment condition had, higher malic acid content as compared to those stored under 1-MCP/control + RA treatments. Thus, the upregulation of the genes encoding malic acid biosynthesis (MdcyMDH in 'Honeycrisp' and MdcyPEPC in 'McIntosh') for 1-MCP + CA treated samples might indicate the possibility of malic acid biosynthesis in stored apples. Moreover, as mentioned in section 3.4.1, fruit samples kept under 1-MCP + CA treatment had suppressed ethylene production rate as compared with other treatments. Thus, the upregulation of MdcyMDH in the 1-MCP + CA treatment condition might suggest the possible role of ethylene in malate metabolism.

Similarly, in apple (Yao et al., 2009) and loquat (Chen et al., 2009; Yang et al., 2013) fruits the changes in the expression level of MdcyPEPC correlated well with the concentration of malate. A positive role of MdcyMDH towards the biosynthesis of malic acid has been reported in transgenic apple callus and tomato fruit, where MdcyMDH

overexpression resulted in higher MDH activity and malate content (Yao et al., 2011). In contrast, a different trend of MdcyMDH expression patterns were reported in low-acid and high-acid apple genotypes, where MdcyMDH transcript level and MDH activity increased with increasing fruit developmental stage while the malic acid content declined during the same time (Yao et al., 2011); suggesting the need of further study to uncover the roles of other gens/enzymes in the change of malate during fruit development.

3.4.3.3 Change of MdVHA-A

V-ATPase, a vacuolar pyrophosphatase, which transfer protons from cytosol to vacuole and generate an electrical potential gradient across the tonoplast, is believed to play an important role in in the accumulation of malic acid in the vacuole by providing driving force during their transport (Sweetman et al., 2009). The proton pumping system helps to maintain acidic pH in the vacuole and nearly natural pH (ca = 7) in the cytosol (Etienne et al., 2013). Some studies demonstrated that the change in malic acid concentration during fruit development could mainly be controlled at the level of vacuolar storage (Yao et al., 2009; Etienne et al., 2002; Berüter, 2004; Khan et al., 2013). However, the regulation of vacuolar malate storage during postharvest storage of apples remain to be clarified.

In our experiment, substantially higher MdVHA-A expression was found in 1-MCP treated ‘Honeycrisp’ apples as compared to untreated samples and the effect was more pronounced in CA than RA storage condition. Consequently the higher malic acid content in 1-MCP treated ‘Honeycrisp’ fruit especially those under CA storage condition might be attributed the higher expression level of MdVHA-A. As discussed in section 3.4.1

‘Honeycrisp’ fruit kept under 1-MCP + CA treatment had reduced loss of malic acid with a suppressed ethylene production rate as compared to those stored under 1-MCP/control + RA treatments. Thus, the results of the present study suggests the possible positive role of malic acid transporter gene in maintaining higher malic acid content during long-term storage of apples. The suppressed ethylene production and the upregulated MdVHA-A in 1-MCP + CA treated apples suggests the possible association between ethylene production and malic acid accumulation during storage.

In agreement with our study, Yao et al. (2009) found a positive association between increased MdVHA-A expression and higher malate concentration in high acid apple genotypes. More recently, a study on hybrid apples (‘Prima’ × ‘Fiesta’ cross) demonstrated the direct association between the expression of malic acid transporter gene (Ma1) and fruit acidity (Khan et al., 2013). In their study, apples with higher expression of Ma1 gene had 3-fold higher malic acid as compared to those having lower Ma1 gene expression (Khan et al., 2013). Similarly, in peach fruit the expression of different genes encoding the vacuolar proton pumps was more consistent with the patterns of organic acid accumulation (Etienne et al., 2002).

Generally, our study provides new insights on the potential role of the genes regulating malic acid degradation, biosynthesis and vacuolar storage to determine malic acid concentration not only during fruit development but also during the postharvest storage life of apples.

3.5 Conclusion

In conclusion, this is the first study to report the effect of 1-MCP on the acidity and sugar content of cloudy and fresh juices prepared from 'Honeycrisp' and 'McIntosh' apples after long-term storage under CA or RA conditions. The study also provided a new insight on how 1-MCP treatment and storage conditions regulate the genes encoding malic acid accumulation and degradation during storage.

The effectiveness of 1-MCP treatment was confirmed by the suppressed ethylene production, inhibited volatile aroma development, and improved flesh firmness retention of intact fruits. In both cultivars, the combination of 1-MCP treatment and CA storage was more effective in supressing ethylene production and delaying the loss of flesh firmness as compared to either 1-MCP treatment or CA storage alone. Ethylene production in 'McIntosh' fruit was considerably suppressed by 1-MCP + CA and 1-MCP + RA treatments, but not by CA storage alone. On the other hand, suppressed ethylene production in 'Honeycrisp' fruit was achieved by 1-MCP + CA combination or CA storage alone but not in 1-MCP + RA conditions. Moreover, the effects of 1-MCP treatment was short lived in 'McIntosh' than 'Honeycrisp'. Thus, the present study confirms the distinctive ethylene production trend of 'Honeycrisp' apples as suggested by previous studies (Wargo and Watkins, 2004; Watkins et al., 2004; DeEll and Ehsani-Moghaddam, 2010; Harb et al., 2012).

1-MCP treated 'McIntosh' or 'Honeycrisp' fruits yielded considerably higher juice acidity, as evidenced from higher TA, malic acid content and lower pH, as compared with untreated fruit samples. In both cultivars, higher acidity retention was associated with the

suppressed ethylene production, which are most pronounced in fruits harvested at commercial maturity, kept under 1-MCP + CA treatment and stored for 4 months. The positive association between suppressed ethylene production and higher malic acid retention might confirm the ethylene dependency of malic acid metabolism as proposed by previous studies in other apple cultivars (Watkins et al., 2000; Magazin et al., 2010; Fan et al., 1999; Jung and Watkins, 2014).

The higher acidity retention in 1-MCP + CA treated 'McIntosh' apples was attributed to the downregulated MdcyME and upregulated MdcyPEPC genes, which regulate malic acid degradation and biosynthesis, respectively. Similarly, higher acidity retention in 'Honeycrisp' was associated with the upregulated V-ATPase and MdcyMDH genes, which regulate the vacuolar transport and cytosolic malic acid biosynthesis, respectively. Thus, our study provides new insights about the role of the genes regulating malate biosynthesis, degradation or vacuolar transport in determining the content of malic acid during the long-term storage of apples.

Fructose, glucose and sucrose were the major monosaccharide and disaccharide sugars in all juice samples tested. In contrast to acidity, the changes of these sugars were not influenced by 1-MCP treatment; instead, it was considerably influenced by the extended storage time. The change of these sugars during the long-term storage of 'Honeycrisp' or 'McIntosh' apples mainly involves reduction of sucrose with a corresponding accumulation of glucose and fructose. Hence, in agreement with previous studies in other apple cultivars (Bizjak et al., 2012, Lee et al., 2012) our data suggests that the changes of individual sugars during ripening and long-term storage of apples might not

be regulated by ethylene production. The changes of sugars may have implications on the functional, sensory and nutritional perspectives of the final product in our case apple juice. Due to growing health concerns and nutritionists' recommendation to decrease sucrose intake, many food companies are interested in reducing the sucrose content of juice products. Accordingly, the lower sucrose content of juices from stored apples could be advantageous in this respect. Our results suggest that using stored apples (4 to 7 months) could be an alternative to reduce the content of sucrose by about 50-80%. Thus, the juice processor might consider using stored apples as an alternative to produce a product line with lower sucrose content. On the other hand, the higher fructose content in juices from stored apples may increase the sweetness sensation due to the higher sweetness index of fructose, which warrants further sensory analysis for the consumer acceptability of the product.

3.6 Tables

Table 3.6-1. Starch index, TSS and flesh firmness of 'McIntosh' and 'Honeycrisp' apples at harvest.

Cultivar	Harvest date	Firmness (N)	TSS (°Brix)	Starch Index (1-8 scale)
'McIntosh'	Comm[A] (Sep. 14)	70.66 ± 1.06[a]	10.03 ± 1.14[c]	2.72 ± 0.12[c]
'McIntosh'	Late (Oct. 06)	62.72 ± 1.06[b]	11.05 ± 1.14[b]	5.97 ± 0.35[b]
'Honeycrisp'	Comm (Sep. 28)	62.72 ± 1.06[b]	11.75 ± 1.14[a]	7.3 ± 0.21[a]
'Honeycrisp'	Late (Oct. 09)	61.37 ± 1.06[b]	12.04 ± 1.14[a]	8.0 ± 0.00[a]
Statistical[B]	Cv[C]	**	***	***
significance	H[D]	**	***	***
	Cv x H	*	**	***

All the values represent mean ± standard error (n = 3) three biological replicates of five (starch index) or ten (firmness and TSS) fruits each. Means followed by the same letter within a column are not significantly different.

[A] Comm = commercial.

[B] Statistical significance = *, **, *** = significant at *p<0.05, p<0.01 or p<0.001,* respectively.

[C] Cv = cultivar.

[D] H = harvest date.

Table 3.6-2. Ethylene production (µL kg^{-1} h^{-1}) in 'McIntosh' and 'Honeycrisp' fruit treated with 1 µL/L of 1-MCP and stored under CA or RA conditions for 4 and 7 months.

Treatment combinations				**Ethylene production ($\mu L kg^{-1} h^{-1}$)**	
				McIntosh	**Honeycrisp**
Harvest (H)	**1-MCP (M)**	**Atmosphere (A)**	**Storage months (S)**		
Comm[A]	-	-	0[B]	NA[C]	NA
Late	-	-	0	NA	NA
Comm	Control	CA[D]	4	28.58 ± 0.04^{edc}	5.09 ± 0.001^{gf}
Comm	Control	CA	7	46.09 ± 0.04^{a}	5.27 ± 0.001^{gf}
Comm	Control	RA[E]	4	40.97 ± 0.04^{b}	13.77 ± 0.001^{de}
Comm	Control	RA	7	13.77 ± 0.04^{hgf}	6.63 ± 0.001^{f}
Comm	1-MCP	CA	4	0.63 ± 0.04^{i}	1.08 ± 0.001^{h}
Comm	1-MCP	CA	7	15.94 ± 0.04^{gf}	2.97 ± 0.001^{gfh}
Comm	1-MCP	RA	4	15.90 ± 0.04^{gf}	17.90 ± 0.001^{c}
Comm	1-MCP	RA	7	22.34 ± 0.04^{edf}	16.07 ± 0.001^{dc}
Late	Control	CA	4	31.57 ± 0.04^{bdc}	11.49 ± 0.001^{e}
Late	Control	CA	7	36.57 ± 0.04^{bac}	4.05 ± 0.001^{gfh}
Late	Control	RA	4	26.60 ± 0.04^{ed}	27.84 ± 0.001^{b}
Late	Control	RA	7	11.29 ± 0.04^{hg}	6.48 ± 0.001^{f}
Late	1-MCP	CA	4	5.52 ± 0.04^{hi}	2.09 ± 0.001^{gh}
Late	1-MCP	CA	7	20.82 ± 0.04^{egf}	1.88 ± 0.001^{gh}
Late	1-MCP	RA	4	23.20 ± 0.04^{edf}	49.03 ± 0.001^{a}
Late	1-MCP	RA	7	21.69 ± 0.04^{edf}	12.65 ± 0.001^{de}
Statistical significance[F]				H×M×A×S**	H×M×A×S***

All the values represent mean ± standard error (n = 3) of three biological replicates. Means followed by the same letter within a column are not significantly different.

[A] Comm = commercial.

[B] Apples at harvest, were neither treated with 1-MCP nor stored under CA/RA.

[C] Ethylene production at harvest was not measured.

[D] CA = controlled atmosphere.

[E] RA = regular atmosphere.

[F] Only significant main and interaction effects are reported for each response. *, **, *** significant at $p<0.05$, $p<0.01$ or $p<0.001$ respectively. M, 1-MCP; H, harvest date; A, atmosphere; S, storage time in months

Table 3.6-3 Flesh firmness (N) of 'McIntosh' and 'Honeycrisp' fruit treated with 1 µL/L of 1-MCP and stored under CA or RA conditions for 4 and 7 months.

Treatment combinations		**Flesh Firmness (N)**	
		McIntosh	**Honeycrisp**
Storage months (S)			
0		66.69 ± 0.75[a]	62.04 ± 0.75[a]
4		43.08 ± 0.68[b]	62.98 ± 0.53[a]
7		35.95 ± 0.68[c]	62.73 ± 0.53[a]
Harvest (H)			
Comm[A]		40.24 ± 0.68[a]	65.0 ± 0.75[a]
Late		38.79 ± 0.68[a]	60.7 ± 0.75[b]
1-MCP (M)			
1-MCP		42.06 ± 0.68[a]	63.27 ± 0.75[a]
Control		36.97 ± 0.68[b]	62.44 ± 0.75[a]
Atmosphere (A)			
CA[B]		43.01± 0.68[a]	63.68 ± 0.75[a]
RA[C]		36.02 ± 0.68[b]	62.03 ± 0.75[b]
H × M			
Comm	1-MCP	43.83 ± 0.95[a]	65.23 ± 1.07[a]
Comm	Control	36.64 ± 0.95[c]	64.76 ± 1.07[a]
Late	1-MCP	40.28 ± 0.95[b]	61.31 ± 1.07[b]
Late	Control	37.29 ± 0.95[c]	60.31 ± 1.07[b]
M × S			
1-MCP	4	46.78 ± 0.95[a]	64.16 ± 1.07[a]
Control	4	39.38 ± 0.95[a]	61.81 ± 1.07[b]
1-MCP	7	37.34 ± 0.95[b]	62.39 ± 1.07[ab]
Control	7	34.56 ± 0.95[c]	63.07± 1.07[ab]
H × M × A			
Comm	1-MCP	47.29 ± 1.35[a]	66.86 ± 1.52[a]
Comm	1-MCP	40.39 ± 1.35[b]	63.62 ± 1.52[bdc]
Comm	Control	40.20 ± 1.35[b]	64.00 ± 1.52[bac]
Comm	Control	33.10 ± 1.35[d]	65.52 ± 1.52[ba]
Late	1-MCP	43.93 ± 1.35[b]	62.01± 1.52[dc]
Late	Control	40.63 ± 1.35[b]	61.87± 1.52[dc]
Late	1-MCP	36.65 ± 1.35[d]	60.6 ± 1.52[ed]
Late	Control	33.96 ± 1.35[d]	58.37 ± 1.52[e]

Continued from Table 3.6-3

	Flesh Firmness (N)	
	McIntosh	**Honeycrisp**
Statistical significance[D]	S*** H*** M*** A*** H×M* M×S*	H* A* H×M×A*

All the values represent mean ± standard error (n = 3) of three biological replicates. Means followed by the same letter within a column are not significantly different.
[A] Comm = commercial.
[B] CA = controlled atmosphere.
[C] RA = regular atmosphere.
[D] Only significant main and interaction effects are reported for each response. *, **, *** significant at $p<0.05$, $p<0.01$ or $p<0.001$ respectively. M, 1-MCP; H, harvest date; A, atmosphere; S, storage time in months.

Table 3.6-4. Volatile compounds detected from intact 'Honeycrisp' and 'McIntosh' apples treated with 1 µL/L of 1-MCP and stored under CA or RA conditions for 4 and 7 months.

Compound[A]	**Retention time (min)**	**Experimental Retention index (RI)**[B]
Ethyl acetate	6.62	902
Ethanol	7.78	950
1-Methylethyl acetate	8.74	991
Methyl butanoate	9.00	1002
Ethyl butanoate	10.07	1055
Ethyl 2-methylbutanoate	10.38	1070
Butyl acetate	10.80	1091
3-methyl-1-butyl acetate	11.72	1140
Alkene I	12.02	1156
Alkene II	13.09	1216
Butyl butanoate	13.40	1235
Butyl 2-methylbutanoate	13.62	1249
Hexyl acetate	14.27	1290
3-heptene, (e)-	15.36	1363
Hexyl butanoate	16.32	1430
Alpha-farnesene	20.57	1759

[A] Apples were removed from storage and volatile samples were collected from intact fruit after 24 h at 20 ^{o}C.
[B] RI was calculated based on the retention times of a series of alkane standards.

Table 3.6-5 Acidity, total soluble solids and their ratio of apple juice from 'McIntosh' fruit treated with 1-MCP (1 µL/L) stored under CA or RA conditions for 4 and 7 months prior to processing into fresh or cloudy juices.

Treatment combinations	TA (g/100 mL)	pH	Malic Acid (g/100 mL)	TSS (°Brix)	TSS/TA[A]
Storage months (S)					
0	0.73 ± 0.02[a]	2.92 ± 0.02[c]	0.58 ± 0.01[a]	10.54 ± 0.08[a]	16.17 ± 0.81[c]
4	0.42 ± 0.01[a]	3.50 ± 0.02[b]	0.35 ± 0.01[a]	11.23 ± 0.08[a]	27.39 ± 0.59[b]
7	0.33 ± 0.01[b]	3.83 ± 0.02[a]	0.12 ± 0.01[b]	10.90 ± 0.08[b]	34.73 ± 0.59[a]
Harvest (H)					
Comm[B]	0.37 ± 0.01[a]	3.62 ± 0.02[b]	0.24 ± 0.01[a]	10.90 ± 0.08[a]	31.58 ± 0.59[a]
Late	0.38 ± 0.01[a]	3.71 ± 0.02[a]	0.24 ± 0.01[a]	11.23 ± 0.08[a]	30.55 ± 0.59[a]
1-MCP (M)					
1-MCP	0.40 ± 0.01[a]	3.59 ± 0.02[b]	0.26 ± 0.01[a]	11.35 ± 0.08[a]	29.05 ± 0.59[b]
Control	0.35 ± 0.01[b]	3.75± 0.02[a]	0.22 ± 0.01[b]	10.79 ± 0.08[b]	33.07 ± 0.59[a]
Atmosphere (A)					
CA[C]	0.41 ± 0.01[a]	3.56 ± 0.02[b]	0.25 ± 0.01[a]	10.98 ± 0.08[b]	27.25 ± 0.59[b]
RA[D]	0.34 ± 0.01[b]	3.78 ± 0.02[a]	0.23 ± 0.01[b]	11.15 ± 0.08[a]	34.87 ± 0.59[a]
Juice (J)					
Cloudy	0.38 ± 0.01[a]	3.67 ± 0.02[a]	0.24 ± 0.01[a]	11.02 ± 0.08[a]	31.00 ± 0.59[a]
Fresh	0.37 ± 0.01[a]	3.67 ± 0.02[a]	0.23 ± 0.01[a]	1.12 ± 0.08[a]	31.13 ± 0.59[a]

Continued from Table 3.6-5

Treatment combinations		TA (g/100 mL)	pH	Malic Acid (g/100 mL)	TSS (°Brix)	TSS/TA
M × A						
1-MCP	CA	0.43 ± 0.01[a]	3.54 ± 0.02[d]	0.24 ± 0.01[a]	11.02 ± 0.09[b]	26.31 ± 0.83[c]
1-MCP	RA	0.38 ± 0.01[b]	3.65 ± 0.02[b]	0.27 ± 0.01[a]	11.67 ± 0.09[a]	31.79 ± 0.83[b]
Control	CA	0.40 ± 0.01[b]	3.59 ± 0.02[c]	0.22 ± 0.01[b]	10.94 ± 0.09[b]	28.19 ± 0.83[c]
Control	RA	0.30 ± 0.01[c]	3.90 ± 0.02[a]	0.22 ± 0.01[b]	10.63 ± 0.09[c]	37.95 ± 0.83
H × S						
Comm	4	0.45 ± 0.01[a]	3.45 ± 0.02[a]	0.36 ± 0.01[a]	11.25 ± 0.09[a]	25.64 ± 0.83[d]
Comm	7	0.30 ± 0.01[d]	3.80 ± 0.02[a]	0.12 ± 0.01[a]	10.56 ± 0.09[a]	37.54 ± 0.83[a]
Late	4	0.39 ± 0.01[b]	3.56 ± 0.02[a]	0.34 ± 0.01[a]	11.21 ± 0.09[a]	29.03 ± 0.83[c]
Late	7	0.37 ± 0.01[c]	3.87 ± 0.02[a]	0.13 ± 0.01[a]	11.24 ± 0.09[a]	32.16 ± 0.83[b]
A × S						
CA	4	0.45 ± 0.01[a]	3.44 ± 0.02[d]	0.33 ± 0.01a	11.17 ± 0.09a	25.36 ± 0.83[c]
CA	7	0.38 ± 0.01[a]	3.68 ± 0.02[b]	0.12 ± 0.01a	10.79 ± 0.09a	29.14 ± 0.83[b]
RA	4	0.39 ± 0.01[a]	3.56 ± 0.02[c]	0.37 ± 0.01a	11.29 ± 0.09a	29.43 ± 0.83[b]
RA	7	0.28 ± 0.01[a]	3.99 ± 0.02[a]	0.13 ± 0.01a	11.02 ± 0.09a	40.32 ± 0.83[a]
Statistical Significance[E]		S***	S***	S***	S**	S***
			H*		H*	M***
		M***	M***	M***	M***	A***
		A***	A***	A***	A**	
		M × A*	M × A**		M × A***	M × A**
		H × S***				A × S***
						H × S***
Lambda [F]				0.01		

All the values represent mean ± standard error (n = 3) three biological replicates. Means followed by the same letter within a column are not significantly different.

[A] TSS/TATA ratio was calculated as TSS in °Brix divided by TA in g/100 mL.

[B] Comm = commercial.

[C] CA = controlled atmosphere.

[D] RA = regular atmosphere.

[E] Only significant main and interaction effects are reported for each response. *, **, *** significant at *p<0.05, p<0.01* or *p<0.001* respectively. M, 1-MCP; H, harvest date; A, atmosphere; S, storage time in months.

[F] Malic acid data was transformed by with lambda value of 0.01 and back transformed by expiating to 100. The lambda values indicates the power to which the data should be raised.

Table 3.6-6. Acidity, total soluble solids and their ratio of apple juice from 'Honeycrisp' fruit treated with 1-MCP (1 µL/L) stored under CA or RA conditions for 4 and 7 months prior to processing into fresh or cloudy juices.

Treatment Combinations	TA (g/100 mL)	pH	Malic acid (g/100 mL)	TSS (°Brix)	TSS/TA[A]
Storage months (S)					
0	0.60 ± 0.02[a]	3.18 ± 0.02[c]	0.87 ± 0.02[a]	11.90 ± 0.08[a]	20.21 ± 0.81[c]
4	0.37 ± 0.01[b]	3.55 ± 0.01[b]	0.43 ± 0.01[a]	10.32 ± 0.12[b]	27.85 ± 0.50[b]
7	0.33 ± 0.01[c]	3.86 ± 0.01[a]	0.24 ± 0.01[b]	10.31 ± 0.12[b]	32.39 ± 0.50[a]
Harvest (H)					
Comm[B]	0.37 ± 0.01[a]	3.67 ± 0.01[b]	0.39 ± 0.01[a]	10.45 ± 0.12[a]	28.75 ± 0.50[a]
Late	0.33 ± 0.01[b]	3.74 ± 0.01[a]	0.29 ± 0.01[b]	10.19 ± 0.12[a]	31.49 ± 0.50[a]
1-MCP (M)					
1-MCP	0.37 ± 0.01[a]	3.67 ± 0.01[b]	0.35 ± 0.01[a]	10.42 ± 0.12[a]	29.04 ± 0.50[b]
Control	0.33 ± 0.01[b]	3.74 ± 0.01[a]	0.33 ± 0.01[a]	10.21 ± 0.12[a]	31.20 ± 0.50[a]
Atmosphere (A)					
CA[C]	0.37 ± 0.01[a]	3.65 ± 0.01[b]	0.36 ± 0.01[a]	10.31 ± 0.12[a]	28.23 ± 0.50[b]
RA[D]	0.33 ± 0.01[b]	3.77 ± 0.01[a]	0.32 ± 0.01[b]	10.33 ± 0.12[a]	32.01 ± 0.50[a]
Juice (J)					
Cloudy	0.35 ± 0.01[a]	3.67 ± 0.01[b]	0.35 ± 0.01[a]	10.36 ± 0.12[a]	30.26 ± 0.50[a]
Fresh	0.35 ± 0.01[a]	3.74 ± 0.01[a]	0.34 ± 0.01[a]	10.28 ± 0.12[a]	29.98 ± 0.50[a]
M × A					
1-MCP CA	0.38 ± 0.01[a]	3.62 ± 0.01[a]	0.38 ± 0.02[a]	10.44 ± 0.16[a]	27.08 ± 0.50[a]
1-MCP RA	0.34 ± 0.01[a]	3.73 ± 0.01[a]	0.31 ± 0.02[b]	10.45 ± 0.16[a]	31.13 ± 0.50[a]
Control CA	0.35 ± 0.01[a]	3.68 ± 0.01[a]	0.34 ± 0.02[b]	10.15 ± 0.16[a]	29.54 ± 0.50[a]
Control RA	0.32 ± 0.01[a]	3.80 ± 0.01[a]	0.33 ± 0.02[b]	10.27 ± 0.16[a]	32.86 ± 0.50[a]

Continued from Table 3.6-6

Treatment Combinations		TA (g/100 mL)	pH	Malic acid (g/100 mL)	TSS (°Brix)	TSS/TA
H × A						
Comm	CA	0.39 ± 0.01[a]	3.61 ± 0.01[a]	0.41 ± 0.02[a]	10.60 ± 0.16[a]	27.41 ± 0.50[a]
Comm	RA	0.35 ± 0.01[a]	3.74 ± 0.01[a]	0.36 ± 0.02[a]	10.26 ± 0.16[ab]	30.09 ± 0.50[a]
Late	CA	0.35 ± 0.01[a]	3.69 ± 0.01[a]	0.30 ± 0.02[a]	9.98 ± 0.16[b]	29.05 ± 0.50[a]
Late	RA	0.32 ± 0.01[a]	3.80 ± 0.01[a]	0.28 ± 0.02[a]	10.40 ± 0.16[a]	33.93 ± 0.50[a]
A × S						
CA	4	0.38 ± 0.01[a]	3.53 ± 0.01[d]	0.45 ± 0.02[a]	10.26 ± 0.16[a]	27.17 ± 0.50[c]
CA	7	0.35 ± 0.01[b]	3.77 ± 0.01[b]	0.26 ± 0.02[a]	10.35 ± 0.16[a]	29.44 ± 0.50[b]
RA	4	0.37 ± 0.01[ab]	3.57 ± 0.01[c]	0.42 ± 0.02[a]	10.39 ± 0.16[a]	28.50 ± 0.50[bc]
RA	7	0.30 ± 0.01[c]	3.96 ± 0.01[a]	0.23 ± 0.02[a]	10.27 ± 0.16[a]	35.48 ± 0.50[a]
Statistical Significance[E]		S***	S***	S***		S***
		H*	H*	H*	H × A**	
		M***	M***			M***
		A***	A***	A*		A***
		A× S**	A × S***	M × A*		A×S**

All the values represent mean ± standard error (n = 3) three biological replicates. Means followed by the same letter within a column are not significantly different.
[A] to [E] = refer to Table 3.6-5.

Table 3.6-7 Fructose, glucose and sucrose content of apple juice from 'McIntosh' fruit treated with 1 µL/L 1-MCP and stored under CA or RA conditions for 4 and 7 months prior to processing into fresh or cloudy juices.

Treatment combinations			Fructose (g/100 mL)	Glucose (g/100 mL)	Sucrose (g/100 mL)	Total (g/100 mL)
Storage months (S)						
0			3.92 ± 0.26^{c}	0.23 ± 0.08^{c}	3.97 ± 0.23^{a}	8.12 ± 0.26^{c}
4			7.94 ± 0.24^{b}	1.74 ± 0.07^{a}	1.10 ± 0.03^{b}	10.77 ± 0.3^{b}
7			11.43 ± 0.24^{a}	2.24 ± 0.07^{a}	0.79 ± 0.03^{c}	14.46 ± 0.3^{a}
Harvest (H)						
Comm^{A}			9.45 ± 0.24^{a}	1.97 ± 0.07^{a}	0.79 ± 0.03^{a}	12.20 ± 0.3^{a}
Late			9.92 ± 0.24^{a}	2.01 ± 0.07^{a}	1.10 ± 0.03^{a}	13.03± 0.3^{a}
1-MCP (M)						
1-MCP			9.86 ± 0.24^{a}	2.01 ± 0.07^{a}	0.95 ± 0.03^{a}	12.82± 0.3^{a}
Control			9.50 ± 0.24^{a}	1.97 ± 0.07^{a}	0.94 ± 0.03^{a}	12.41± 0.3^{a}
Atmosphere (A)						
CA^{B}			9.49 ± 0.24^{a}	2.05 ± 0.07^{a}	0.88 ± 0.03^{a}	12.42 ± 0.3^{a}
RA^{C}			9.87 ± 0.24^{a}	1.93 ± 0.07^{b}	1.01 ± 0.03^{a}	12.82 ± 0.3^{a}
Juice (J)						
Cloudy			11.31 ± 0.24^{a}	2.35 ± 0.07^{a}	1.01 ± 0.03^{a}	14.67 ± 0.3^{a}
Fresh			8.06 ± 0.24^{b}	1.63 ± 0.07^{b}	0.88 ± 0.03^{b}	10.57 ± 0.3^{b}
H x A x S						
Comm	CA	4	6.97 ± 0.48^{d}	1.51 ± 0.12^{e}	0.86 ± 0.07^{cd}	9.34 ± 0.61^{d}
Comm	CA	7	11.60 ± 0.48^{ab}	2.45 ± 0.12^{a}	0.64 ± 0.07^{e}	14.72±0.61 ^{ab}
Comm	RA	4	8.28 ± 0.48^{dc}	1.90 ± 0.12^{cd}	0.97 ± 0.07^{bc}	11.16 ± 0.61^{c}
Comm	RA	7	10.93 ± 0.48^{b}	1.99 ± 0.12^{bc}	0.68 ± 0.07^{e}	13.61 ± 0.61^{b}
Late	CA	4	8.65 ± 0.48^{c}	1.94 ± 0.12^{c}	1.28 ± 0.07^{a}	11.87 ± 0.61^{c}
Late	CA	7	11.45 ± 0.48^{ab}	2.52 ± 0.12^{a}	0.73 ± 0.07^{ed}	14.70 ± 0.61^{ab}
Late	RA	4	7.85 ± 0.48^{cd}	1.59 ± 0.12^{de}	1.28 ± 0.07^{a}	10.73 ± 0.61^{cd}
Late	RA	7	12.79 ± 0.48^{a}	2.27 ± 0.12^{ab}	1.08 ± 0.07^{b}	16.12 ± 0.61^{a}

Continued from Table 3.6-7

Treatment combinations			Fructose (g/100 mL)	Glucose (g/100 mL)	Sucrose (g/100 mL)	Total sugar (g/100 mL)
M × A × S						
1-MCP	CA	4	7.78 ± 0.48[a]	1.73 ± 0.12[a]	1.03 ± 0.07[ab]	10.53 ± 0.61[b]
1-MCP	CA	7	11.61± 0.48[a]	2.45 ± 0.12[a]	0.72 ± 0.07[d]	15.15 ± 0.61[a]
1-MCP	RA	4	8.30 ± 0.48[a]	1.68 ± 0.12[a]	1.20 ± 0.07[a]	11.18 ± 0.61[b]
1-MCP	RA	7	11.77 ± 0.48[a]	2.19 ± 0.12[a]	0.83 ± 0.07[cd]	15.54 ± 0.61[a]
Control	CA	4	7.84 ± 0.48[a]	1.72 ± 0.12[a]	1.11 ± 0.07[ab]	10.68 ± 0.61[b]
Control	CA	7	10.74 ± 0.48[a]	2.29 ± 0.12[a]	0.64 ± 0.07[d]	13.67 ± 0.61[a]
Control	RA	4	7.84 ± 0.48[a]	1.83 ± 0.12[a]	1.04 ± 0.07[ab]	10.70 ± 0.61[b]
Control	RA	7	11.58 ± 0.48[a]	2.04 ± 0.12[a]	0.97 ± 0.07[bc]	14.58 ± 0.61[a]
Statistical Significance[D]			S*** J*** H × A × S**	S*** J*** A* H × A × S**	S*** J*** A* H × A × S** M × A × S*	S*** J*** H × A × S**

All the values represent mean ± standard error (n = 3) three biological replicates. Means followed by the same letter within a column are not significantly different.

[A] Comm = commercial.

[B] CA = controlled atmosphere.

[C] RA = regular atmosphere.

[D] Only significant main and interaction effects are reported for each response. *, **, *** significant at $p<0.05$, $p<0.01$ or $p<0.001$ respectively. M, 1-MCP; H, harvest date; A, atmosphere; S, storage time in months

Table 3.6-8 Fructose, glucose and sucrose content of apple juice from 'Honeycrisp' fruit treated with 1 µL/L 1-MCP and stored under CA or RA conditions for 4 and 7 months prior to processing into fresh or cloudy juices.

Treatment combinations		Fructose (g/100 mL)	Glucose (g/100 mL)	Sucrose (g/100 mL)	Total sugar (g/100 mL)
Storage months (S)					
0		5.12 ± 0.26^{c}	0.34 ± 0.08^{c}	4.81 ± 0.23^{a}	10.74 ± 0.26^{b}
4		7.30 ± 0.21^{b}	1.85 ± 0.06^{a}	2.22 ± 0.08^{b}	11.37± 0.31^{a}
7		7.85 ± 0.21^{a}	2.00 ± 0.06^{a}	1.37 ± 0.08^{c}	11.22± 0.31^{a}
Harvest (H)					
Comma^{A}		7.76 ± 0.21^{a}	1.96 ± 0.06^{a}	1.75 ± 0.08^{a}	11.48 ± 0.31^{a}
Late		7.39 ± 0.21^{a}	1.89 ± 0.06^{a}	1.83 ± 0.08^{a}	11.11 ± 0.31^{a}
1-MCP (M)					
1-MCP		7.80 ± 0.21^{a}	1.96 ± 0.06^{a}	1.74 ± 0.08^{a}	11.51 ± 0.31^{a}
Control		7.35 ± 0.21^{a}	1.89 ± 0.06^{a}	1.84 ± 0.08^{a}	11.09 ± 0.31^{a}
Atmosphere (A)					
CA^{B}		7.66 ± 0.21^{a}	2.05 ± 0.06^{a}	1.63 ± 0.08^{b}	11.36 ± 0.31^{a}
RA^{C}		7.49 ± 0.21^{a}	1.80 ± 0.06^{a}	1.94 ± 0.08^{a}	11.23 ± 0.31^{a}
Juice (J)					
Cloudy		7.86 ± 0.21^{a}	2.03 ± 0.06^{a}	1.85 ± 0.08^{a}	11.76 ± 0.31^{a}
Fresh		7.28 ± 0.21^{b}	1.82 ± 0.06^{b}	1.73 ± 0.08^{b}	10.83 ± 0.31^{b}
H × S					
Comm	4	7.21 ± 0.30^{b}	1.84 ± 0.08^{b}	2.12 ± 0.10^{b}	11.18 ± 0.44^{b}
Comm	7	8.56 ± 0.30^{a}	2.15 ± 0.08^{a}	1.39 ± 0.10^{c}	12.11 ± 0.44^{a}
Late	4	7.72 ± 0.30^{ab}	1.96 ± 0.08^{ab}	2.37 ± 0.10^{a}	12.04 ± 0.44^{a}
Late	7	7.02 ± 0.30^{b}	1.84 ± 0.08^{b}	1.26 ± 0.10^{c}	10.13 ± 0.44^{b}
M × S					
1-MCP	4	7.25 ± 0.30^{b}	1.85 ± 0.08^{a}	2.12 ± 0.10^{a}	11.23 ± 0.44^{ab}
1-MCP	7	8.22 ± 0.30^{a}	2.06 ± 0.08^{a}	1.36 ± 0.10^{b}	11.99 ± 0.44^{a}
Control	4	7.68 ± 0.30^{ab}	1.95 ± 0.08^{a}	2.36 ± 0.10^{a}	11.57 ± 0.44^{ab}
Control	7	7.36 ± 0.30^{b}	1.93 ± 0.08^{a}	1.28 ± 0.10^{b}	10.66 ± 0.44^{b}
M × A					
1-MCP	CA	8.24 ± 0.30^{a}	2.24 ± 0.08^{a}	1.65 ± 0.10^{b}	12.14 ± 0.44^{a}
1-MCP	RA	7.23 ± 0.30^{ab}	1.67 ± 0.08^{c}	1.76 ± 0.10^{b}	10.66 ± 0.44^{b}
Control	CA	7.28 ± 0.30^{b}	1.94 ± 0.08^{b}	1.61 ± 0.10^{b}	10.84 ± 0.44^{b}
Control	RA	7.77 ± 0.30^{ab}	1.95 ± 0.08^{b}	2.12 ± 0.10^{a}	11.83 ± 0.44^{ab}

Continued from Table 3.6-8

Treatment combinations	Fructose (g/100 mL)	Glucose (g/100 mL)	Sucrose (g/100 mL)	Total sugar (g/100 mL)
Statistical Significance[D]		A***	A***	
	J**	J**	J*	J**
	H x S***	M x A**	M x A**	H x S***
	M x S**	H x S***	H x S*	M x S**
	M x A**			M x A**

All the values represent mean ± standard error (n = 3) three biological replicates. Means followed by the same letter within a column are not significantly different.
[A] to[D] = refer to Table 3.6-7

Table 3.6-9. Primers used for RT-qPCR experiment.

Target genes[A]	**Forward primer (5′–3′)**	**Reverse primer (5′–3′)**	**References**
MdcyME	GTACAGCCCTTCCTA TCGAAGTTT	TCTTGAACCCGGTACAA CCAA	Yao et al. (2009)
MdcyMDH	GTTCGYGTYCTYGTY ACYGG	CTNACYGNCCRTNAYGA NTG	Yao et al. (2011)
MdcyPEPC	CGAGAGACGGGTTG TGTCC	TGCACAGCAATCTGATC ACAG	Yao et al. (2009)
MdVHA-A	AAACCCGGTGATTGA ACATCT	CCCTAACACTGTCCTTC TTCGT	Yao et al. (2009)
18S rRNA	AAACGGCTACCACAT CCA	CACCAGACTTGCCCTCC A	Yao et al. (2009)

[A] MdcyMDH, MdcyME, MdcyPEPC, MdVHA-A are the genes that encode the activities of cytosolic NAD-MDH, NADP-ME, PEPC and subunit A of V-ATPase, respectively. A housekeeping gene, 18S rRNA was used to normalize the CT values of the target genes.

Table 3.6-10. RT-qPCR efficiency (%), correlation coefficient (R^2) and related values of target and reference genes

Genes[A]	**Efficiency**[B]	**Slope**	**R^2**	**Y-intercept**	**E (Mean square error)**[C]
18S rRNA	1.941	-3.47	0.99	6.55	0.012
Beta Actin[D]	1.945	-3.46	0.99	25.92	0.019
MdcyME	1.928	-3.51	0.99	27.14	0.015
MdcyMDH	1.919	-3.53	0.99	27.22	0.019
MdcyPEPC	1.976	-3.38	0.99	27.14	0.018
MdVHA-A	1.889	-3.62	0.99	27.51	0.009

[A] MdcyMDH, MdcyME, MdcyPEPC, MdVHA-A are the genes that encode the activities of cytosolic NAD-MDH, NADP-ME, PEPC and subunit A of V-ATPase, respectively. A housekeeping gene, 18S rRNA was used to normalize the CT values of the target genes.
[B] A two-fold dilution series was built to estimate the amplification efficiency of each primer pair, using a pooled cDNA from fruit samples. For each dilution, RT-qPCR was run in triplicate.
[C] E is a measure of the accuracy of the quantification result based on the standard curve. An acceptable value is <0.2.
[D] This reference gene was not used as its expression varied among samples.

3.7 Figure captions

Figure 3.8-1. Volatile abundance (area counts) of 'McIntosh' apples treated with 1-MCP (1 μL/L) or not (control) and stored under CA or RA conditions at 3 ^{o}C for up to 7 months. 4 and 7 = Storage time in months. Atmos., storage atmosphere; Comm, commercial harvest date. Error bars represent mean ± standard error (n = 3, where 'n' represents the number of biological replicates).

Figure 3.8-2. Volatile abundance (area counts) of 'Honeycrisp' apples treated with 1-MCP (1 μL/L) or not (control) and stored under CA or RA at 3 ^{o}C for up to 7 months. 4 and 7 = Storage time in months. Atmos., storage atmosphere; Comm, commercial harvest date. Error bars represent mean ± standard error (n = 3, where 'n' represents the number of biological replicates).

Figure 3.8-3. Gene expression in 'McIntosh' apples stored for 4 months under CA or RA storage condition after 1-MCP treatment. The label '0' stands for the control sample (untreated sample at harvest) used as a calibrator. Error bars represent mean ± standard error (n = 3, where 'n' represents the number of biological replicates). The transcript abundance level (normalized relative gene expression) for each target gene was quantified efficiency corrected **ΔΔ**CT method. The housekeeping gene, 18S rRNA was used as a reference gene to normalize the CT values of the target genes. Pooled cDNA was used as a calibrator.

Figure 3.8-4. Gene expression in ‘Honeycrisp’ apples stored for 4 months under CA or RA storage condition after 1-MCP treatment. The label ‘0’ stands for the control sample (untreated sample at harvest) used as a calibrator. Error bars represent mean ± standard error (n = 3, where ‘n’ represents the number of biological replicates). The transcript abundance level (normalized relative gene expression) for each target gene was quantified efficiency corrected **ΔΔ**CT method. The housekeeping gene, 18S rRNA was used as a reference gene to normalize the CT values of the target genes. Pooled cDNA was used as a calibrator.

3.8 Figures

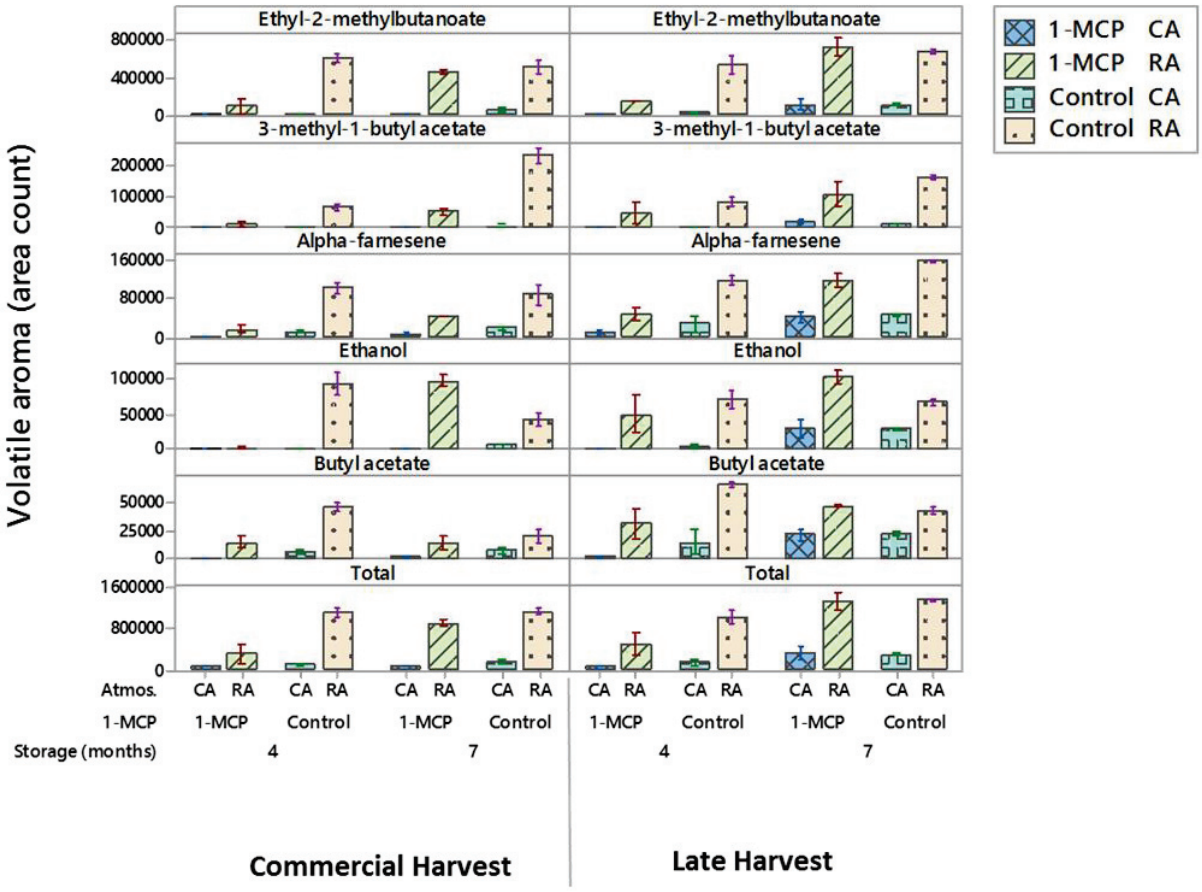
Ethyl-2-methylbutanoate
3-methyl-1-butyl acetate
Alpha-farnesene
Ethanol
Butyl acetate
Total
Volatile aroma (area count)
1-MCP CA
1-MCP RA
Control CA
Control RA
Atmos.
1-MCP
Storage (months)
Commercial Harvest
Late Harvest

Figure 3.8-1

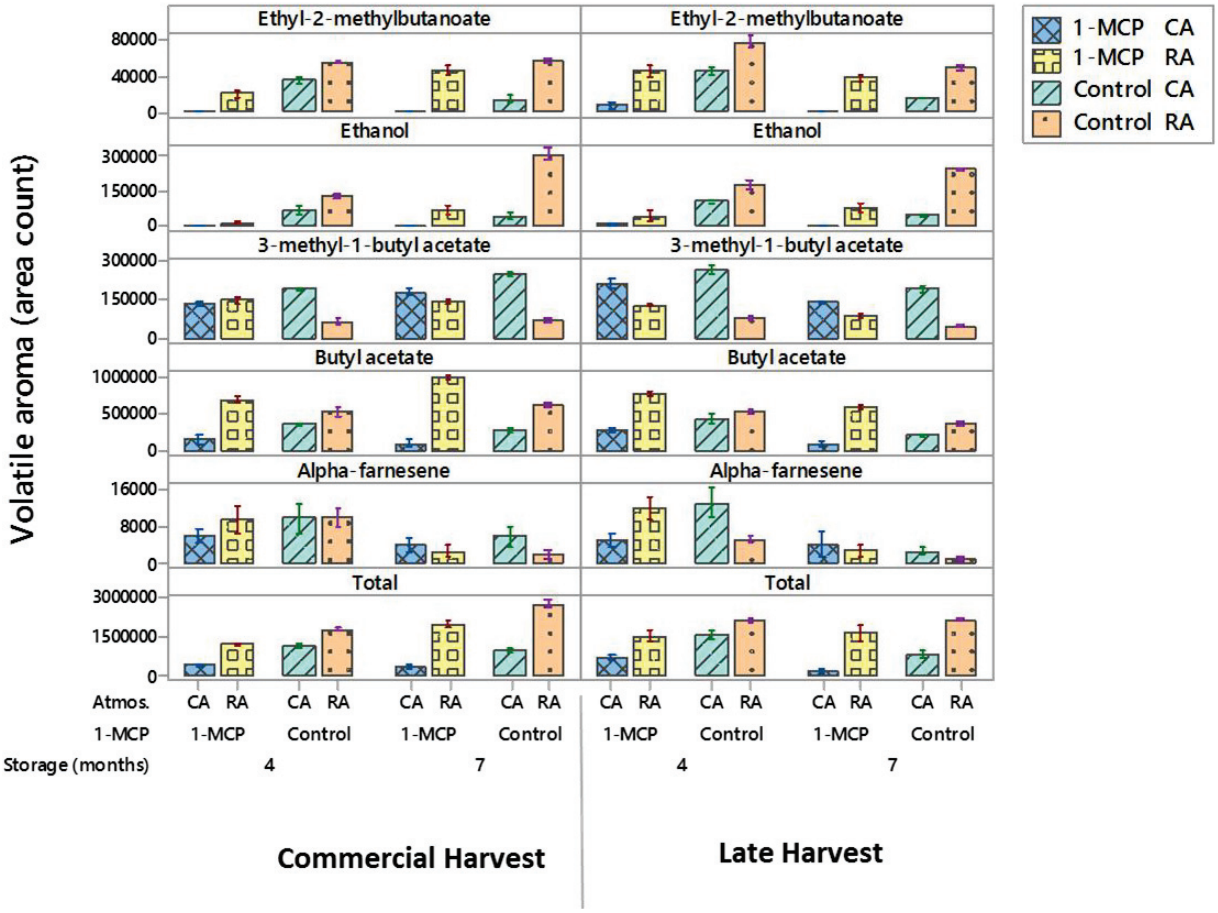
Ethyl-2-methylbutanoate
Ethanol
3-methyl-1-butyl acetate
Butyl acetate
Alpha-farnesene
Total
Volatile aroma (area count)
1-MCP CA
1-MCP RA
Control CA
Control RA
Atmos.
1-MCP
Storage (months)
Commercial Harvest
Late Harvest

Figure 3.8-2

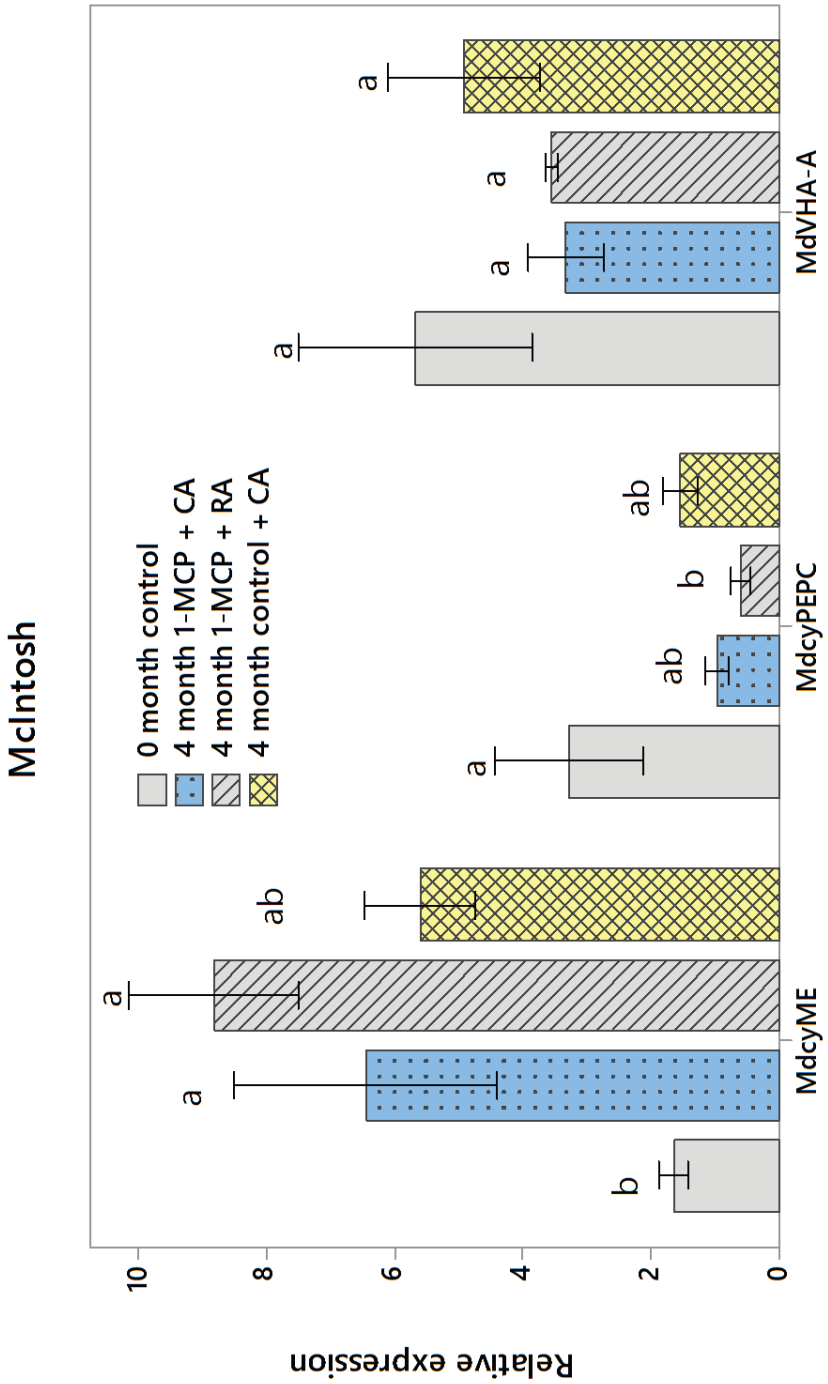
McIntosh
0 month control
4 month 1-MCP + CA
4 month 1-MCP + RA
4 month control + CA
Relative expression
0
2
4
6
8
10
MdcyME
MdcyPEPC
MdVHA-A
a
a
ab
a
b
ab
b
ab
a
a
a

Figure 3.8-3

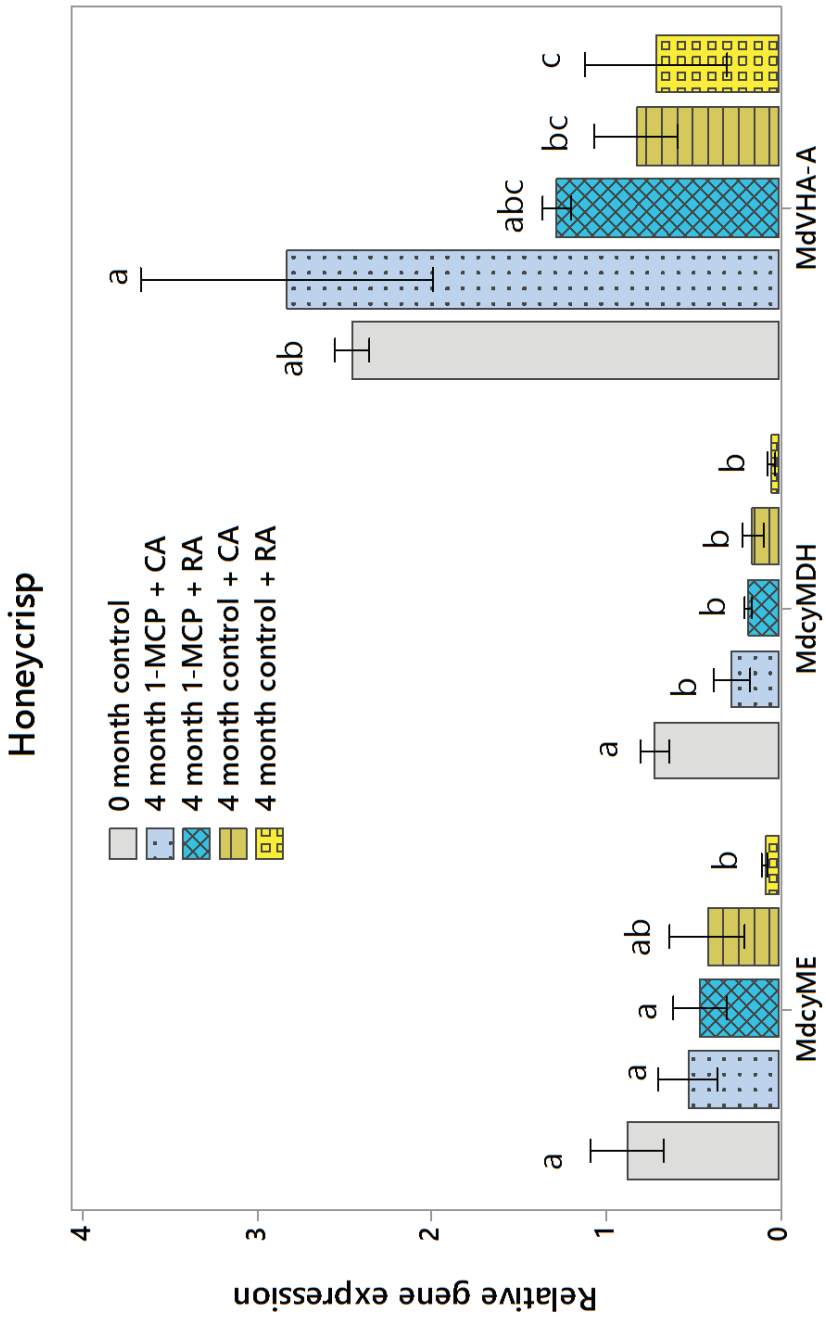
Honeycrisp
0 month control
4 month 1-MCP + CA
4 month 1-MCP + RA
4 month control + CA
4 month control + RA
Relative gene expression
0
1
2
3
4
a
a
a
ab
b
a
b
b
b
b
ab
a
abc
bc
c
MdcyME
MdcyMDH
MdVHA-A

Figure 3.8-4

Chapter 4: Effect of 1-methylcyclopropene (1-MCP) and storage atmosphere on volatile aroma of clear and cloudy juice from 'McIntosh' and 'Honeycrisp' apples

Abstract

The aim of this study was to investigate the effect of 1-methylcyclopropene (1-MCP) treatment, storage atmosphere, harvest maturity, and juice processing on the composition and content of volatile aroma compounds from 'McIntosh' and 'Honeycrisp' juices using gas chromatography-mass spectrometry (GC-MS). Fourteen volatile aroma compounds were identified from clear and cloudy juice samples in each cultivar. Esters, aldehydes and alcohols were the major groups of volatile compounds identified in the juice samples of both cultivars. Compared to 'McIntosh', 'Honeycrisp' juices had considerably higher content of total esters (2-fold), and total volatiles compounds (6-fold). While acetate esters (butyl acetate, 2-methyl butyl acetate, ethyl acetate and hexyl acetate) were the predominant ester form in 'Honeycrisp' juices, 'McIntosh' juices were dominated by butanoate esters (ethyl, butyl, and hexyl butanoate). In 'McIntosh' juices, 1-MCP treatment resulted in a remarkable reduction of all types of esters, aldehydes and total volatile compounds regardless of juice type and storage atmosphere. Unlike 'McIntosh', 1-MCP treatment in 'Honeycrisp' apples did not suppress the content of volatile compounds except

ethyl acetate and ethanol. Considerable reduction of major esters and total volatile compounds was also observed in juices from controlled atmosphere (CA) stored ‘McIntosh’ as well as ‘Honeycrisp’ apples as compared to those in regular atmosphere (RA) storage condition, regardless of harvest maturity, 1-MCP treatment and juice type. The content of volatile aroma compounds was also strongly affected by juice processing techniques. As compared to clear juices, cloudy juice samples from both cultivars had considerably higher content of total volatiles, most notably esters and aldehydes.

Keywords: 1-methylcyclopropene, storage atmosphere, GC-MS, volatile, cloudy, clear, ‘McIntosh’, ‘Honeycrisp’.

4.1 Introduction

Apple juice with its per capita consumption of 7.23 L per year is the second most widely consumed juice in Canada (Statistics Canada, 2010). Aroma is one of the pivotal organoleptic quality parameters of apple juice used by consumers to evaluate its acceptance and preference (Nikfardjam and Maier, 2011). Volatile aroma components of food and beverages is one of the main quality attributes evaluated when a new product is developed (Roberto et al., 2005).

The content and composition of volatile compounds responsible for the aroma of apple juice can be influenced by many factors including variety, harvest maturity, postharvest and storage treatments, and juice processing techniques (Rupasinghe et al., 2000; Dixon and Hewett, 2000; Echeverrıa et al., 2004; Eisele and Drake, 2005). Currently, the apple industry in North America has adopted the extensive use of a potent ethylene action inhibitor, 1-methylcyclopropene (1-MCP), as a means to extend the storage and shelf life of apples, thus allowing extended supply of seasonal fruits to a global market (Watkins, 2006). However, the delay or inhibition of fruit ripening associated with 1-MCP treatment with, or without, CA storage could have a deleterious effect on volatile aroma production of climacteric fruits including apples (Bangerth et al., 2012; Sigal Escalada and Archbold, 2009; Graell et al., 2008; López et al., 2007; Kondo et al., 2005; Dandekar et al., 2004; Raffo et al., 2009).

There is a considerable amount of literature that has examined the effect of different postharvest treatments and storage conditions on the volatile composition of fresh apple

fruit. Nevertheless, to the author's knowledge a study that integrates 1-MCP treatment and apple juice volatile aroma composition has not yet been reported. Moreover, information about the volatile composition of apple juice from the relatively newer apple cultivars such as 'Honeycrisp' is still scarce. The aim of this study was to determine the effect of 1-MCP treatment, in combination with harvest maturity, storage atmosphere and juice processing techniques, had on the content and composition of volatile aroma compounds of cloudy and clear juice samples extracted from 'McIntosh' and 'Honeycrisp' apples.

4.2 Materials and Methods

4.2.1 Apple harvesting, 1-MCP treatment and storage

Refer to chapter 3. For this experiment only 4-month old samples were used.

4.2.2 Juice preparation

Cloudy apple juice was prepared by washing six medium sized apples with tap water, cutting each into 12 pieces and pressing them using a laboratory scale juice extractor (Supreme Juicerator, USA). Juice samples were collected into 250 mL beakers containing 0.5 g/L ascorbic acid (Oszmianski et al., 2009). The juice was filtered with four layer cheesecloth and heated up to 80 ^{o}C using a water bath, held for 5 min at that temperature (Benitez et al., 2007; Genovese and Lozano, 2006), collected in sterile 50 mL centrifuge tubes (0553849 Fisher Scientific, Ottawa, ON, Canada), cooled in an ice bath (Oszmianski et al., 2009), and stored at -20 ^{o}C until analysis. The main purpose of juice heating was to inhibit further enzymatic (PPO) activity and prevent enzymatic browning (Benitez et al.,

2007; Genovese and Lozano, 2006). The holding time (5 min) was recorded after the juice reached the desired temperature (80 ^{o}C). The time required to reach the set temperature was about 5 min. Juice preparation was done separately for the three biological replications. Fresh apple juice was prepared in the same way as above, but simply pressed and filtered with four layer cheesecloth without ascorbic acid addition and pasteurization.

Clear apple juice was prepared as above without ascorbic acid addition. Filtered juice samples were treated with Ultrazym 100 G (Novozymes Ultrazym 100 G, 0.04 g/L) for 3 h at 25 ^{o}C. Ultrazym, a clarification aid enzyme, acts only on soluble pectin (Scaman et al., 2004) and is reported to contain activities of pectinesterases, polygalacturonases, and pectinlyases. At the end of the enzyme treatment, the enzyme in the sample was inactivated (to prevent further enzyme activity which can reduce juice clarity) by heating at 90 ^{o}C for 5 min (Rai et al., 2004). The juice was allowed to settle overnight at 4 ^{o}C, centrifuged at 8500 rpm for 15 min and stored at -20 ^{o}C until analysis. The same procedure was repeated separately for each of the three replicates of the different apple cultivars.

At the end of the enzyme treatment, the enzyme in the sample was inactivated by heating the suspension at 90 °C for 5 min in a water bath.

4.2.3 Experimental design and statistical analysis

The experimental design was a four-factor factorial with three replications. The independent variables included (i) 1-MCP treatments (control and treated), (ii) storage atmosphere (CA and RA), (iii) harvest maturity and (iv) juice type (clear and cloudy). Statistical analyses were performed using Minitab software (Release 17, Minitab Inc. State

College, PA, USA). Analysis of variance (ANOVA) was done using the general linear model (GLM) procedure. GLM is an ANOVA procedure in which the statistical difference between one or more factors are performed using a least square (minimizing sum of squares or residuals) linear regression approach. Whenever there are significant main or interaction effects, multiple mean comparisons were employed using Tukey's method at significance level, $\alpha = 0.05$. For each response, the validity of model assumptions, namely normal distribution and constant variance of the error terms, were verified by examining residual plots as indicated in the Appendix B-Figure 1 to Appendix B-Figure Figure 4. In some data sets transformations had to be used to achieve normality (Montgomery, 2008).

4.2.4 Reagents and standards

Ultrazym 100 G was purchased from Novozymes (Franklinton, NC, USA). Buffer solutions (pH 7.0 and pH 4.0), 2-octanone standard and NaCl were from Fisher Scientific (Ottawa, ON, Canada). Sodium hydroxide solution, L-ascorbic acid (99%), and GC grade standards (for individual volatile analysis) were purchased from Sigma Aldrich (Oakville, ON, Canada).

4.2.5 Volatile sampling from juice

Frozen juice samples were defrosted overnight in a cold room (4 ^{o}C) before the volatile analysis. Volatile compound analysis of the juices used headspace solid phase microextraction (HS-SPME) followed by GC-MS. Juice samples (10 mL) were pipetted into a new 20 mL vial followed by the addition of 2 g of NaCl and 100 µL of internal

standard [2-octanone (0.524 µg/mL 100 methanol). Blanks were prepared using 10 mL water with 2 g NaCl in a 20 mL vial. During SPME extraction, the juice sample was stirred at 250 rpm. A 20 mm DVB/CAR/PDMS HS-SPME fibre (Supelco Analytical, Bellefone, PA) was exposed to the headspace of the juice sample for 30 min at 30 ^{o}C. Volatiles sampled by the fibre were desorbed and analysed by immediate injection into the GC-MS using a splitting ratio of 1:3 and an injector temperature of 250 ^{o}C. The fibre was held in the injector for 5 min.

4.2.6 Volatile analysis

Volatile analysis was employed using a 4000 Varian GC-MS system (Varian Chromatography Walnut Creek, Calif., USA), equipped with a Varian 1177 injector (Varian Chromatography Systems, Walnut Creek, CA) and a CombiPAL auto sampler (CTC Analytics AG, Zwingen, Switzerland). Volatile compounds were separated on a VF-WAXms capillary column of 30 m length and 0.32 mm internal diameter with 1.0 µm film thickness (Agilent Technologies, Netherlands). The temperature program was 35 °C for 5.0 min, from 35 °C to 240 °C at the rate of 10 °C/min and 240 °C for 4.5 min. The injector was maintained at 250 ^{o}C and the carrier gas was helium with the flow rate of 2.5 mL/min. Detection by MS was carried out in electron ionization (EI) mode with a mass range of 35-400 amu, emission current of 25 µAmps, a scan rate of 0.60 s (4 µscans) and total run time of 30 min. Temperatures of the transfer line, trap, manifold and ion source were 170, 100, 50, and 180 ^{o}C, respectively. All the peak areas were normalized using the peak are of the internal standard that was run on the day of the analysis (i.e. sample peak area count divided

by the peak area count of the internal standard). The volatile compounds were identified based on the retention index (RI) and by comparing their mass spectra with spectral data from the National Institute of Standards and Technology library matched and confirmed where possible with standards. The RI values were calculated based on the retention times of a series of alkane standards.

4.3 Results

4.3.1 Volatile aroma composition

GC-MS analysis of the volatile fraction in fresh, clear and cloudy juice samples from 'McIntosh' and 'Honeycrisp' apples allowed the identification of the 14 most abundant volatile aroma compounds (Table 4.6-1, Figure 4.8-1 and Figure 4.8-2). The volatile compounds from 'McIntosh' juice were composed of about 42% aldehydes, 37% esters, and 19% alcohols (Figure 4.8-3, A). The major aldehyde detected in 'McIntosh' juice was 2-methyl-4-pentenal (38.4%) with a minor content of (E)-2-hexenal (3.4%) (Figure 4.8-3, A). The major esters detected in 'McIntosh' juice included ethyl butanoate, butyl butanoate, hexyl butanoate and 2-methyl butyl acetate, which contribute 11.8%, 8.1%, 7.8% and 2.8% of the total volatile compounds, respectively (Figure 4.8-3, A). An unidentified branched chain alcohol and 2-hexen-1-ol, which comprised 12.6% and 6.0% of the total volatiles, were the major alcohols detected in the 'McIntosh' juice (Figure 4.8-3, A).

The aroma volatiles in 'Honeycrisp' juice were dominated by esters, which comprised 78.9% of the total volatiles followed by aldehydes (11.8%) and alcohols (8.7%)

(Figure 4.8, B). The major esters found in 'Honeycrisp' juice were butyl acetate (31.3%), 2-methyl butyl acetate (22.9%), ethyl acetate (10.3%) and hexyl acetate (8.0%) (Figure 4.8-3, B). As in 'McIntosh', the major aldehyde in 'Honeycrisp' juice was 2-methyl-4-pentenal (10.4%) with minor amounts of (E)-2-hexenal (1.4%) (Figure 4.8-3, B). The unidentified branched chain alcohol (5.7%) and 2-hexen-1-ol (2.1%) were the major alcohols detected in 'Honeycrisp' juice. In both cultivars, ethanol was a minor constituent accounting for < 1% of the total volatile compounds (Figure 4.8-3, B).

4.3.2 Effect of 1-MCP, storage conditions and harvest maturity on volatile composition

4.3.2.1 'McIntosh' juices

All the major esters and total volatile content from 'McIntosh' juice were significantly affected by harvest maturity ($p<0.001$), the two-way interaction of storage atmosphere and harvest maturity ($p<0.001$) and harvest maturity and 1-MCP treatment ($p<0.01$) (Table 4.6-2). A considerable difference among volatile compounds was also caused by juice processing technique especially for hexyl butanoate ($p<0.001$).

Juice produced from late harvested fruit had higher concentrations of all the major esters than the commercial maturity harvested fruit averaging 2.7- to 6.5-fold greater. There was a significant interaction between 1-MCP and harvest maturity on their effects on the content of total volatiles and all major esters (Table 4.6-2). The 1-MCP treatment increased the total volatile content of juice produced from commercial harvest fruit by 69% but reduced total volatiles in juice from late harvested fruit by 32% (Table 4.6-2). All major

esters demonstrated this same effect with 1-MCP treated fruit being 2.3- to 4.9-fold greater in commercial harvest fruit but 1.4- to 2.7-fold less in late harvest fruit (Table 4.6-2). There was also a significant interaction between harvest maturity and storage atmosphere on their effects on total and major ester composition of 'McIntosh' juice (Table 4.6-2). Juice made from commercial maturity harvested fruit tended to have higher concentrations of total volatile and major ester from CA stored than RA stored fruit, but with late harvested fruit, volatiles were greatest in juice made from RA stored fruit. Regarding the effect of juice type, cloudy juice tended to have higher concentrations of total volatiles and major ester than clear juice, with hexyl butanoate averaging 6.9-fold greater (Table 4.6-2).

The major aldehydes and alcohols identified in 'McIntosh' juices were 2- to 3-fold more abundant in juice produced from late harvested fruit than from commercial maturity fruit with the exception of (E)-2-hexenal that had a 2.3-fold greater concentration in juice from the commercial harvest maturity (Table 4.6-3). The 1-MCP treatment had a significant interaction with harvest maturity that affected the aldehyde and alcohol content of 'McIntosh' juice. In juices made from commercial maturity fruit, concentrations of 2-methyl-4-pentenal, the unknown branched chain alcohol and (Z)-2-hexen-1-ol were 2.3-, 3.9- and 1.2-fold greater in juice made from 1-MCP treated fruit than untreated fruit (Table 4.6-3). However, in late harvested fruit concentrations were lower in juice from 1-MCP treated fruit except for (E)-2-hexenal, which was 1.7-fold higher. There was also a significant interaction between harvest maturity and storage atmosphere. In juice made from commercial maturity fruit, concentrations of 2-methyl-4-pentenal and the unknown

branched chain alcohol were 2.5- and 6.3-fold greater in juice made from CA than RA stored fruit (Table 4.6-3). However, in late harvested fruit, these concentrations were lower in juice from CA stored fruit except for (E)-2-hexenal, which was 1.6-fold higher (Table 4.6-3). The concentration of (E)-2-hexenal was1.6 to 1.9-fold higher in clear juice than in cloudy juice produced from both commercial maturity and late harvested fruit, respectively. The concentration of (Z)-2-hexen-1-ol was also about 1.5-fold higher in clear juice than cloudy juice from commercial maturity fruit, but in juice from late harvested fruit, concentration in cloudy juice was 1.5-fold higher than in clear juice (Table 4.6-3).

4.3.2.2 'Honeycrisp' juice

Regardless of the postharvest treatment and storage conditions, 'Honeycrisp' juice had about 4-fold higher ester and total aroma volatile content than 'McIntosh' juice. In 'Honeycrisp', significant differences in the level of esters and total aroma volatiles were mainly caused by storage atmosphere and juice processing techniques ($p<0.001$) but not by 1-MCP treatment (Table 4.6-4). Overall, cloudy juice produced from 'Honeycrisp' fruit had a 3- to 4-fold higher content of all the major esters and total volatile compounds than in clear juice. Similar to 'McIntosh', CA storage of 'Honeycrisp' fruit resulted in juices that exhibited a 27 to 51% reduction of most straight chain esters as compared to RA storage (Table 4.6-4). However, concentration of the branched chain ester 2-methyl butyl acetate was 2.6-fold higher in juice from CA stored fruit than RA stored fruit (Table 4.6-4). This was similar to juice made from commercial maturity 'McIntosh' fruit, but not late

harvested fruit, which had higher 2-methyl butyl acetate concentration in juice from RA stored fruit than CA stored fruit. Ethyl acetate was the only volatile compound that was significantly reduced in juice made from 1-MCP treated 'Honeycrisp' apples, being only 8 to 21% of that from untreated fruit (Table 4.6-4).

The major aldehydes and alcohols in 'Honeycrisp' juice were significantly affected by storage atmosphere ($p<0.001$). The concentration of 2-methyl-4-pentenal and the unknown branched chain alcohol in juice made from CA stored fruit were 29% and 74%, respectively, less than in juice from RA stored fruit, while (E)-2-hexenal and (Z)-2-hexen-1-ol were 40% and 51% greater (Table 4.6-5). The 1-MCP treatment reduced the concentration of the unknown branched chain alcohol and ethanol by 91% and 84%, respectively, in 'Honeycrisp' juice. Aldehyde content was 31% and 50% less for 2-methyl-4-pentenal and (E)-2-hexenal, respectively, in juice produced from late harvested fruit compared to commercial harvest fruit (Table 4.6-5). On average, cloudy juice from 'Honeycrisp' fruit had 33.5 to 66.9% higher content of all the major aldehydes and alcohols as compared to clear juice with the difference being most prominent in late harvested fruit samples (Table 4.6-5).

4.4 Discussion

4.4.1 Change of volatile aroma between cultivars

As to the authors knowledge this is the first study to report the volatile profile from 'Honeycrisp' juices. Substantiating our results, esters, aldehydes and alcohols have been

reported as major groups of volatile compounds in juices from different apple cultivars including 'McIntosh' (Sapers et al., 1977), 'Jonagold' (Komthong et al., 2007), 'Fuji' and 'Royal Gala' (Dixon and Hewett, 2001), 'Golden Delicious' and 'Red Delicious'(Schmutzer et al., 2014) 'Holsteiner Cox', 'Ingrid Marie', and 'Rajka'(Martínez Vega et al., 2014) which concur with the present results. As indicated in Table 4.6-1 each group of volatile compounds contribute a typical odour characteristic to the apple juice. While esters are responsible for the sweet and fruity odour of apple juice, C-6 aldehydes have been described as the main contributor to the green-grassy (associated with odour of freshly cut green grass or hexanol) odour of apple fruit and apple juice (Sapers et al., 1977; Dixon and Hewett, 2000; Komthong et al., 2007).

In line with previous studies (Fellman et al., 2000; Dixon and Hewett, 2000; Zhu et al., 2008) our results indicated quantitative and qualitative differences in the level of volatile compounds between the two cultivars. Compared to 'McIntosh', 'Honeycrisp' juices had considerably higher content of total esters (2-fold), and total volatile compounds (6-fold). While 'McIntosh' juices contained esters and aldehydes (2-methyl-4-pentenal) in almost equal ratio, 'Honeycrisp' juices were dominated by volatile esters. The higher content of 2-methyl-4-pentenal in 'McIntosh' juice samples could offer a desirable green grass and fruit aroma note (Sampaio et al., 2011). The dominance of esters might be ascribed to the high expression of alcohol acyltransferase (AAT) genes, which regulate the last step of volatile ester biosynthesis (Zhu et al., 2008). Zhu and others (2008), found greater expression of AAT genes in the higher ester producing cultivar 'Golden Delicious'

compared to the low ester producing cultivar ‘Granny Smith’. Esters are known for their desirable aromatic notes in apple beverages, which are often described as ‘fresh apple’, ‘fruity’ and ‘sweet’, it is reasonable to assume that the higher amount of total esters in ‘Honeycrisp’ juices would increase its overall aroma and flavour (Poll, 1983). Moreover, while acetate esters (butyl acetate, 2-methyl butyl acetate, ethyl acetate and hexyl acetate), comprised the largest proportion of esters in ‘Honeycrisp’ juices, ‘McIntosh’ juices were dominated by butanoate esters (ethyl, butyl, and hexyl butanoate). Despite the greater content of total esters in ‘Honeycrisp’ juices, the higher level of ethyl acetate in ‘Honeycrisp’ juices may lead to the development of undesirable flavour as excessive amounts of fermentation volatiles including ethyl acetate can generate off-flavours and aroma (Dixon and Hewett, 2000). A study conducted in Germany (Nikfardjam and Maier, 2011) compared the volatile composition of apple juice samples prepared ‘from concentrate’ and ‘not from concentrate’ demonstrated the association between higher concentrations of ethyl butanoate and ethyl-2-methyl butanoate with a flavour impression of ‘fruity, ripe sweet’ that stands for the typical aroma of ripe apples. The same study also indicated that juices ‘not from concentrate’ had fruity, ripe and sweet aroma impression that was preferred by sensory panelists. Hence, the higher concentration of butanoate esters in ‘McIntosh’ juices could provide juice with a favourable fruity, ripe and sweet aroma. Due to the low odour threshold values (Table 4.6-2) of acetate and butanoate esters, these groups of esters could generate a significant contribution to the final aroma of the juice samples. Accordingly, it has been demonstrated that acetate esters, including butyl acetate,

2-methyl butyl acetate, hexyl acetate and ethyl butanoate, are major contributors to the typical apple-like aroma and flavour in many apple cultivars (Echeverrıa et al., 2004; Aaby et al., 2002; Song and Bangerth, 1996; Dimick et al., 1983). Similarly, hexyl acetate, butyl acetate and ethyl butanoate were reported as the most important volatiles in 'Jonagold' juice (Komthong et al., 2006; Kato et al., 2003).

In addition to straight chain esters, a branched chain ester, 2-methyl butyl acetate, was one of the most abundant volatile compound detected in 'Honeycrisp' and 'McIntosh' juices. Owing to its lower threshold value, 2-methyl butyl acetate has been described as one of the most significant odour active volatile compounds in other apple cultivars including 'Golden Delicious' (Plotto et al., 2000; Song and Bangerth, 1996) and 'Gala' (Young et al., 1996; Plotto et al., 2000).

It has been indicated that C-6 aldehydes, particularly (E)-2-hexenal and hexenal are responsible for the green fresh aroma in apple juice (Dimick et al., 1983). Excessive concentration of C-6 aldehydes (>2430 µg/L) in apple juice from concentrate has been associated with negative odour impressions and thus led to lower sensory scores and is often denoted by sensory descriptors such as 'artificial flavour, too green, and shampoo-like'(Nikfardjam and Maier, 2011). Thus, it is reasonable to assume the relatively high content of these compounds in 'McIntosh' juices might lead to the development of negative organoleptic properties.

4.4.2 Change of volatile aroma whole fruit vs. juice

The volatile compounds identified from 'McIntosh' and 'Honeycrisp' juices are a combination of primary (synthesized by the intact fruit) and secondary (synthesized in response to cellular disruption during juice processing) volatile compounds (Dimick et al., 1983). As mentioned in Chapter 3, the volatile compounds detected from intact fruit are mainly composed of esters and ethanol (Figure 4.8-3, C and D). These primary volatiles are synthesized by controlled enzymatic reactions mainly from fatty acid metabolism (Schreier et al., 1978). It is well known that fatty acids are major precursors of aroma volatiles in several fruits including apple, and the biosynthetic pathway includes beta-oxidation (primary volatiles) and lipoxygenase (LOX) action (secondary compounds) (Schreier et al., 1978). The beta-oxidation pathway provides alcohols and acyl co-enzyme, acyl (CoA,), which are the main precursors for volatile ester production. Acyl CoAs are reduced by acyl CoA reductase to produce aldehydes, which in turn are reduced by alcohol dehydrogenase (ADH) to form alcohols that are converted to esters via the action of AAT (Song and Bangerth, 2003; Dimick et al., 1983).

Secondary volatiles, which are mainly C-6 aldehydes and the associated alcohols, are formed by the LOX pathway from unsaturated fatty acids (linoleic and linolenic acids) when fruit are crushed and exposed to oxygen (Schreier et al., 1978). In our experiment, C-6 and C-5 aldehydes such as (E)-2-hexenal and 2-methyl-4-pentenal were detected only in juice samples but not in whole apple samples (refer to chapter 3, Figure 3.8-1 and Figure 3.8-2). This is in agreement with other studies, which reported the higher content of C-6

aldehydes in apple juice compared to intact fruit (Paillard and Rouri, 1984; Su and Wiley, 1998). As discussed earlier, the presence of aldehydes in juice samples but not in intact 'Honeycrisp' and 'McIntosh' apples is attributed to the oxidation of unsaturated fatty acids (linoleic and linolenic) during juice processing.

4.4.3 Change of volatile aroma by 1-MCP treatment and storage atmosphere

According to the results presented, the content and composition of volatile compounds from clear and cloudy juice were strongly influenced by the different combination of 1-MCP treatment, storage atmosphere, harvest maturity, and juice type.

Since each group of volatile compound has a typical odour characteristic (Table 4.6-2), the difference in their abundance associated with postharvest treatments and juice processing steps could affect the subsequent organoleptic quality of the juice. The resultant impact on the levels of esters and aldehydes on the juice odour depends on its odor threshold value (i.e. the detection or recognition values, above which the compound can be detected by smell) and concentration (Echeverrıa et al., 2004).

Based on the threshold values of volatile compounds summarized from the literature (Table 4.6-2), esters and aldehydes have considerably lower threshold values as compared with alcohols. This means esters and aldehydes may have a key role to influence the odour of the juice even at low concentrations. On the other hand, volatile compounds with higher threshold values (notably ethanol, Table 4.6-2), might not have a large impact on the odour of apple juice. Hence, our discussion will focus on aldehydes and esters.

4.4.3.1 Effect of 1-MCP

In 'McIntosh' juices, whether it is clear or cloudy, our results indicated a remarkable reduction of all types of esters, aldehydes, most alcohols and total volatile compounds when juices are extracted from 1-MCP treated fruit stored in CA or RA. This is consistent with previous studies that found a substantial suppression of volatile aroma compounds in several apple cultivars that had been treated with 1-MCP prior to storage in RA or CA (Lurie et al., 2002; Kondo et al., 2005; Bai et al., 2005; DeEll et al., 2005; Rupasinghe et al., 2000). The reduction of volatile compounds following 1-MCP treatment could be associated with the suppressed ethylene production as observed in 1-MCP treated 'McIntosh' fruit kept under RA or CA condition (see chapter 4). Hence, our study confirms the regulatory role of ethylene for the biosynthesis of volatile compounds in apples as suggested by previous studies (Schaffer et al., 2007; Fan et al., 1998; Defilippi et al., 2005).

Unlike 'McIntosh', 1-MCP treatment alone (1-MCP + RA) in 'Honeycrisp' apples did not alter the content of most volatile compounds except ethyl acetate and ethanol, which were substantially suppressed by the treatment. This effect might be attributed to the unusual response of this cultivar to 1-MCP treatment. As mentioned in chapter 4, while 1-MCP + RA treatment in 'McIntosh' apples inhibited ethylene production better than the control + CA/RA treatments, the same treatment in 'Honeycrisp' produced the highest ethylene (49.03 $\mu L\ kg^{-1}\ h^{-1}$) level, which was present at higher levels than in control fruit (17 $\mu L\ kg^{-1}\ h^{-1}$). As observed in our study and reported in other cultivars (Lurie et al., 2002; Kondo et al., 2005; Bai et al., 2005; DeEll et al., 2005; Rupasinghe et al., 2000) elevated

ethylene production is usually accompanied by increased level of volatile compounds and vice versa. Nevertheless, as observed in our results, this trend did not occur in 'Honeycrisp' apples. As there is no published information regarding the volatile profile of 'Honeycrisp' fruit or juice especially none focusing on 1-MCP treatment, it is difficult to account for the unusual response of this cultivar to 1-MCP treatment. However, based on our observation it is reasonable to speculate that the volatile production in 'Honeycrisp' apples might not be fully regulated by ethylene production. To explain the actual mechanism of this unexpected phenomenon further research in this area is warranted.

4.4.3.2 Effect of storage atmosphere

Our results indicated a considerable reduction of major esters and total volatile compounds in juices from both CA stored 'McIntosh' and 'Honeycrisp' fruit as compared to those kept in RA storage, regardless of harvest maturity, 1-MCP treatment and juice type. The observed inhibitory effect of CA storage on the content of volatile compounds is consistent with previous studies in different apple cultivars (Plotto et al., 2000; Both et al., 2014; Raffo et al., 2009; Lara et al., 2007; Mattheis et al., 2005). Reduced sensitivity to ethylene (Kader, 1988) or suppressed ethylene production of CA stored fruit (Yang and Hoffman, 1984; Kader, 1988) has been suggested as a mechanism by which volatile production could be inhibited in CA stored apples. The inhibitory effect of the lower oxygen concentration on the biosynthesis pathway of volatile compounds has also been suggested (Dimick et al., 1983). The biosynthesis of volatile compounds via beta-oxidation or LOX pathway needs

oxygen and therefore their production could be slowed down by CA condition where the oxygen level is much lower than the RA atmosphere (López et al., 2007).

Even though CA storage suppressed the content of most volatile compounds, it also enhanced some branched chain esters detected from intact 'Honeycrisp' apples (3-methyl-1-butyl acetate, see chapter 4) as well as from 'Honeycrisp' juices (2-methyl butyl acetate). In agreement with our observation, other studies also reported the increased level of branched chain acetate esters in 'Delicious' (Fellman et al., 2003), 'Gala' (Plotto et al., 2000; Mattheis et al., 2005) and 'Fuji' (Echeverrıa et al., 2004) apples that have been kept under low oxygen storage conditions. None of the above studies explained the reason behind this unique enhancement of branched chain esters. A recent study in pear fruit found increased level of branched chain esters associated with the higher level of amino acids, which are the main precursors of branched chain esters (Zhang et al., 2013). In addition to branched chain esters, (E)-2-hexenal was enhanced by CA storage and/or 1-MCP treatment. The higher concentration of (E)-2-hexenal in juices from 1-MCP and/or CA treated 'Honeycrisp' apples might be attributed to the suppressed ripening of the apples associated with lower ethylene production (Mattheis et al., 2005)

Contrary to the results observed in late harvested 'McIntosh' juices, the suppressive effect of 1-MCP and/or CA storage was not clearly observed in juices extracted from fruit harvested at commercial maturity. These results are unexpected and no explanation or corresponding results were found in the literature.

4.4.3.3 Effect of juice processing

In addition to harvest maturity, 1-MCP treatment and storage condition, a significant effect of juice processing (cloudy versus clear) on the content of volatile compounds, particularly esters and aldehydes were observed. As compared to clear juices, cloudy juice samples from both 'McIntosh' and 'Honeycrisp' apples had considerably higher levels of all the major esters, aldehydes and total volatiles. Even though there is lack of literature pertaining to the volatile composition of cloudy apple juices, one recent study reported higher levels of total esters in apple juice with pulp as compared to juice from concentrate (Schmutzer et al., 2014). The reduction of esters in clear juice samples can be explained by the hydrolysis of esters by the action of esterase that is present in the commercial enzyme preparation (Schreier et al., 1978). In addition to esters, a higher content of aldehydes was observed in cloudy apple juice samples as compared to the clear counterpart. As mentioned in the methodology part, one of the major differences between the two juices is the absence (clear) or presence (cloudy) of ascorbic acid. The higher content of aldehydes in cloudy juices, which is processed with ascorbic acid addition, is consistent with a previous study (Komthong et al., 2007) that investigated the changes in the aroma value (the ratio of volatile concentration to odour threshold) of volatile compounds due to the addition of ascorbic acid (0.2% w/v) to the apple juice. Komthong et al. (2007) found considerably higher (4 to 5-fold) aroma value of (E)-2-hexenal and hexanal in juices treated with ascorbic acid than the control. Even though there is limited information about the exact mechanism of ascorbic acid reaction with volatile compounds, the reduced concentration

of aldehydes in clear juice samples has been associated with the action of ADH during the clarification process. Lower content of aldehydes in clear juice samples was ascribed to the conversion of aldehydes to alcohols by the action of ADH during the enzymatic incubation (Poll, 1988; Schreier et al., 1978). The longer incubation period (about 3 h at 25 ^{o}C, in our case) would give additional time for different enzymatic reactions activated via endogenous enzymes including ADH. In our experiment, the long incubation period was not part of cloudy apple juice preparation; instead, the juice was immediately cooled and then pasteurized. This immediate cooling, which is followed by pasteurization, could slow down and inactivate the action of the indigenous enzymes such as ADH. Hence, the preservation of aldehydes in cloudy apple juice could be attributed to the inhibition of ADH activity during processing (Schreier et al., 1978).

The higher concentration of aldehydes has been found to correspond with increased green/grassy odour of apple juice (Komthong et al., 2007). Similarly, in another study the decrease in aldehyde and the increase in esters have been corresponded well with the low intensity of green odour and the high intensity of sweet odour, respectively (Komthong et al., 2006). Komthong et al. (2007) who studied the odour of 'Jonagold' apple juice in relation to ascorbic acid (0-0.2% w/v) addition using GC and sensory analysis reported the undesirableness of green odour in the apple juice samples associated with higher concentration of ascorbic acid which resulted increased level of t-(E)-2-hexenal. A similar study by Komthong et al. (2006) studied the changes in the odours of apple juice during enzymatic browning. The authors indicated the improvement of odour in the first 2

h of browning and the deterioration thereafter. The improved juice odour was attributed to the decrease in the volatile release of (E)-2-hexenal and the increased content of acetate esters which were well corresponded with the green and sweet aroma intensity, respectively (Komthong et al., 2006). On the other hand, the deterioration of juice odour after 2 h of browning could not be associated with the content of esters (unchanged) or aldehydes (continued to decline) rather the authors suggested the production of some other compounds as the probable cause of this undesirable odour (Komthong et al., 2006). In agreement with the above studies, Kato et al. (2003) reported that apple juice with higher levels of fresh, sweet, and fruity aroma and low levels of green odours is preferable by consumers.

4.5 Conclusion

The results of this study demonstrated that the content and composition of volatile aroma compounds in juices from 'McIntosh' and 'Honeycrisp' apples varied depending on several interrelated factors such as cultivar, harvest maturity, storage atmosphere, postharvest 1-MCP treatment and juice processing technique. As 'Honeycrisp' juices had considerably higher content of esters (2-fold), and total volatiles compounds (6-fold), these cultivars could be the best candidate to produce apple juice with intense fruity and sweet aroma. Our study also found a substantial and differential effect of 1-MCP treatment and storage atmosphere on the individual and total volatile content. While most volatile compounds were suppressed in juices from 1-MCP treated 'McIntosh' apples, 1-MCP treatment in 'Honeycrisp' apples had a little or no effect on the content of most volatile compounds

except ethyl acetate and ethanol, which were suppressed by 1-MCP treatment. Regarding the effect of storage atmosphere, considerable reduction of major esters and total volatile compounds was observed in juices from CA stored 'McIntosh' as well as 'Honeycrisp' fruit as compared to those in RA storage, regardless of harvest maturity, 1-MCP treatment and juice type. In addition to storage atmosphere and 1-MCP treatment, the content of volatile compounds was strongly influenced by juice type. Regardless of all other factors considered, cloudy juice samples had substantially higher amount of most esters, aldehydes and total volatile compounds as compared to the clear counterpart. Hence, our study suggests that the content and composition of volatile aroma compounds in apple juice could be strongly influenced by the fruit quality and juice processing techniques. However, sensory evaluation is warranted to assess the consumers' perception associated with the change of volatile aroma compounds observed in this study.

4.6 Tables

Table 4.6-1. Volatile compounds isolated from clear and cloudy apple juice of 'McIntosh' and 'Honeycrisp' apples.

Volatile Compounds	Retention time (min)	Experimental retention index (RI)[A]	Odour Propety[B]	Odour Threshold (mg/L)[C]
Esters				
Ethyl acetate	6.83	898	Ether like (0)	7.50 [0]
Ethyl butanoate	10.15	1049	Sweet fruity (1)	0.001 [1]
Butyl acetate	10.91	1087	Sweet fruity (1)	0.066 [0, 1]
2-Methyl butyl acetate	11.83	1137	Fresh (1)	0.011 [1]
Butyl butanoate	13.46	1233	Fresh (1)	0.10 [1]
Hexyl acetate	14.32	1287	Sweet fruity (1)	0.002 [1]
Hexyl butanoate	16.41	1433		
Aldehydes				
2-(E)-hexenal	13.64	1244	Green apple like (0)	0.011 [0]
2-Methyl-4-pentenal	15.49	1367	Desirable, green grass, fruity (2)	

Continued from Table 4.6-1

Volatile Compounds	Retention time (min)	Experimental retention index (RI)[a]	Odour propety[b]	Odour Threshold (mg/L)[c]
Alcohols				
Ethanol	7.88	944	Sweet (0)	716 [0]
(Z)-2-hexen-1-ol	11.09	1096	Fresh leaf green (1)	0.07 [2]
Unidentified branched chain alcohol	16.56	1444		
Acids				
Ethyl 2-methylbutanoic acid	10.43	1063		
Hydrocarbon				
Alkene I	13.16	1214		

[A] RI was calculated based on the retention times of a series of alkane standards.
[B] Odour properties were cited from (0) (Nikfardjam and Maier, 2011); (1) (Komthong et al., 2007); (2) (Sampaio et al., 2011).
[C] Odour thresholds were cited from [0] (Flath et al., 1967), [1] (Jennings and Tang, 1967), [2] (Young and Suffet, 1999; Azhu Valappil et al., 2009).

Table 4.6-2. Relative amounts of major ester volatile compounds identified in clear and cloudy juice samples from 'McIntosh' apples harvested at commercial or late harvest maturity, treated or untreated with 1-MCP and stored under CA or RA conditions for 4 months.

Treatment combinations		Normalized peak area counts[A]				
		Ethyl butanoate	Butyl butanoate	Hexyl butan oate	2-Methyl butyl acetate	Total Volatile
Harvest (H)						
Comm[B]		0.59 ± 0.06[b]	0.31 ± 0.05[b]	0.20 ± 0.29[b]	0.43 ± 0.13[b]	20.31 ± 3.28[b]
Late		2.47 ± 0.06[a]	2.01 ± 0.05[a]	0.89 ± 0.29[a]	1.16 ± 0.13[a]	37.14 ± 3.28[a]
1-MCP (M)						
1-MCP		1.48 ± 0.06[a]	0.94 ± 0.05[a]	0.41 ± 0.29[a]	0.79 ± 0.13[a]	27.87 ± 3.28[a]
Control		1.09 ± 0.06[a]	0.79 ± 0.05[a]	0.43 ± 0.29[a]	0.80 ± 0.13[a]	29.58 ± 3.28[a]
Atmosphere (A)						
CA[C]		0.99 ± 0.06[a]	0.64 ± 0.05[a]	0.31 ± 0.29[a]	0.42 ± 0.13[b]	21.07 ± 3.28[a]
RA[D]		1.62 ± 0.06[a]	1.14 ± 0.05[a]	0.57 ± 0.29[a]	0.16 ± 0.13[a]	36.38 ± 3.28[a]
Juice (J)						
Clear		0.94 ± 0.06[a]	0.61 ± 0.05[a]	0.16 ± 0.29[b]	0.72 ± 0.13[a]	24.37 ± 3.28[a]
Cloudy		1.69 ± 0.06[a]	1.18 ± 0.05[a]	1.10 ± 0.29[a]	0.87 ± 0.13[a]	33.08 ± 3.28[a]
H x M						
Comm	1-MCP	1.22 ± 0.08[ab]	0.62 ± 0.07[bc]	0.31± 0.42[ab]	0.61 ± 0.18[bc]	25.80 ± 4.64[b]
Comm	Control	0.25 ± 0.08[b]	0.14 ± 0.07[c]	0.13 ± 0.42[b]	0.26 ± 0.18[c]	14.82 ± 4.64[b]
Late	1-MCP	1.77 ± 0.08[a]	1.37 ± 0.07[ab]	0.55 ± 0.42[ab]	0.97 ± 0.18[ab]	29.95 ± 4.64[ab]
Late	Control	3.40 ± 0.08[a]	2.87 ± 0.07[a]	1.46 ± 0.42[a]	1.34 ± 0.18[a]	44.33 ± 4.64[a]
H x A						
Comm	CA	1.64 ± 0.08[b]	0.72 ± 0.07[b]	0.41 ± 0.42[b]	0.58 ± 0.18[b]	25.54 ± 4.64[b]
Comm	RA	0.16 ± 0.08[c]	0.12 ± 0.07[c]	0.09 ± 0.42[b]	0.28 ± 0.18[b]	15.08 ± 4.64[b]
Late	CA	0.56 ± 0.08[bc]	0.57 ± 0.07[b]	0.24 ± 0.42[b]	0.26 ± 0.18[b]	16.60 ± 4.64[b]
Late	RA	7.86 ± 0.08[a]	5.50 ± 0.07a	3.40 ± 0.42[a]	2.05 ± 0.18[a]	57.86 ± 4.64[a]

Continued from Table 4.6-2.

	Ethyl butanoate	Butyl butanoate	Hexyl butanoate	2-Methyl butyl acetate	Total Volatile
Statistical Significance[E]	H ***	H ***	H ***	H ***	H ***
			J ***	A **	
	H x M *	H x M *	H x M *	H x M *	H x M **
	H x A ***	H x A ***	H x A***	H x A ***	H x A **
Lambda[F]	0.2	0.2	0	1	1

All the values represent mean ± standard error (n = 3) three biological replicates. Means followed by the same letter within a column are not significantly different.

[A] Ratio of sample peak area counts to the peak area count of the internal standard, 2-octanone.

[B] Comm = commercial maturity for long-term storage.

[C] CA = controlled atmosphere.

[D] RA = regular atmosphere.

[E] Statistical significance = only significant main or interaction effects are reported for each response. *, **, *** = significant at $p<0.05$, $p<0.01$ or $p<0.001$, respectively. A, atmosphere; H, harvest; M, 1-MCP; J, juice

[F] The lambda values other than zero indicates the power to which all the data should be raised to the lambda value (e.g. Lambda = 0.2, means Y^0.2). Zero lambda values indicate the natural log transformation (Log Y, where Y is the response value). All mean values were back transformed to their original values.

Table 4.6-3. Relative amounts of major aldehydes and alcohols identified in clear and cloudy juice samples from 'McIntosh' apples harvested at commercial or late harvest maturity, treated or untreated with 1-MCP and stored under CA or RA conditions for 4 months.

Treatment combinations		Normalized peak area counts[A]			
		2-Methyl 4-pentenal	**(E)-2-hexenal**	**Branched chain alcohol**	**(Z)-2-hexen-1-ol**
Harvest (HA)					
Comm[B]		6.37 ± 0.19[b]	1.15 ± 0.05[a]	0.32 ± 0.37[b]	1.06 ± 0.07[b]
Late		12.69 ± 0.19[a]	0.49 ± 0.05[b]	0.98 ± 0.37[a]	2.07 ± 0.07[a]
1-MCP (MA)					
1-MCP		9.73 ± 0.19[a]	0.84 ± 0.05[a]	0.55 ± 0.37[a]	1.40 ± 0.07[a]
Control		8.81 ± 0.19[a]	0.62 ± 0.05[b]	0.58 ± 0.37[a]	1.57 ± 0.07[a]
Atmosphere (A)					
CA[C]		8.26 ± 0.19[a]	0.78 ± 0.05[a]	0.37 ± 0.37[a]	1.65 ± 0.07[a]
RA[D]		10.33 ± 0.19[a]	0.66 ± 0.05[a]	0.85 ± 0.37[a]	1.33 ± 0.07[b]
Juice (J)					
Clear		7.67 ± 0.19[a]	0.97 ± 0.05[a]	0.51 ± 0.37[a]	1.50 ± 0.07[a]
Cloudy		11.01 ± 0.19[a]	0.55± 0.05[b]	0.66 ± 0.37[a]	1.46 ± 0.07[a]
H x M					
Comm	1-MCP	9.30 ± 0.27[a]	1.12 ± 0.07[a]	0.63 ± 0.53[ab]	1.20 ± 0.09[bc]
Comm	Control	4.00 ± 0.27[b]	1.19 ± 0.07[a]	0.16 ± 0.53[b]	0.94 ± 0.09[c]
Late	1-MCP	10.17 ± 0.27[a]	0.65 ± 0.07[b]	0.48 ± 0.53[ab]	1.63 ± 0.09[b]
Late	Control	15.50 ± 0.27[a]	0.38 ± 0.07[c]	2.01 ± 0.53[a]	2.63 ± 0.09[a]
H x A					
Comm	CA	9.54 ± 0.27[b]	1.01 ± 0.07[a]	0.82 ± 0.53[b]	1.16 ± 0.09[b]
Comm	RA	3.84 ± 0.27[c]	1.33 ± 0.07[a]	0.13 ± 0.53[c]	0.97 ± 0.09[b]
Late	CA	7.07 ± 0.27[bc]	0.63 ± 0.07[b]	0.17 ± 0.53[c]	2.35 ± 0.09[a]
Late	RA	19.96 ± 0.27[a]	0.39 ± 0.07[c]	5.76 ± 0.53[a]	1.83 ± 0.09[a]
H x J					
Comm	Clear	6.13 ± 0.27[b]	1.41 ± 0.07[a]	0.31 ± 0.53[b]	1.34 ± 0.09[b]
Comm	Cloudy	6.62 ± 0.27[b]	0.96 ± 0.07[ab]	0.33 ± 0.53[b]	0.85 ± 0.09[c]
Late	Clear	9.38 ± 0.27[a]	0.70 ± 0.07[b]	0.85 ± 0.53[a]	1.69 ± 0.09[b]
Late	Cloudy	16.51 ± 0.27[a]	0.36 ± 0.07[c]	1.12 ± 0.53[a]	2.53 ± 0.09[a]

Continued from Table 4.6-3

	2-Methyl 4-pentenal	(E)-2-hexenal	Branched chain alcohol	(Z)-2-hexen-1-ol
Statistical Significance[E]	H ***	H *** M ** J ***	H *	H ***
	H x M *** H x A ***	H x M ** H x A *** H x J *	H x M *** H x A ***	H x M *** H x A *** H x J ***
Lambda[F]	0.5	0.5	0	0

All the values represent mean ± standard error (n = 3) three biological replicates. Means followed by the same letter within a column are not significantly different.
[A] to [F] refer to Table 4.6-2.

Table 4.6-4. Relative amounts of major ester volatile compounds identified in clear and cloudy juice samples from 'Honeycrisp' apples harvested at commercial or late harvest maturity, treated or untreated with 1-MCP and stored under CA or RA conditions for 4 months.

Treatment combinations	Normalized peak area counts[A]				
	Butyl acetate	2-methyl butyl acetate	Ethyl acetate	Hexyl acetate	Total Volatile
Harvest (H)					
Comm[B]	16.17 ± 0.09[a]	14.26 ± 0.20[a]	3.57 ± 0.03[a]	4.77 ± 0.06[a]	58.44 ± 8.17[a]
Late	16.46 ± 0.10[a]	12.44 ± 0.21[a]	4.24 ± 0.03[a]	3.36 ± 0.06[a]	48.31 ± 8.43[a]
1-MCP (M)					
1-MCP	15.64 ± 0.10[a]	15.22 ± 0.20[a]	1.41 ± 0.03[b]	4.08 ± 0.06[a]	50.09 ± 8.17[a]
Control	17.02 ± 0.10[a]	11.58 ± 0.20[a]	9.33 ± 0.03[a]	3.96 ± 0.06[a]	56.37± 8.43[a]
Atmosphere (A)					
CA[C]	13.95 ± 0.10[b]	19.78 ± 0.20[a]	2.69 ± 0.03[b]	2.82 ± 0.06[b]	57.91± 8.17[a]
RA[D]	19.09 ± 0.10[a]	8.15 ± 0.20[b]	5.51 ± 0.03[a]	5.54 ± 0.06[a]	48.75± 8.43[a]
Juice (J)					
Clear	9.08 ± 0.10[b]	6.48 ± 0.20[b]	2.53 ± 0.03[b]	1.79 ± 0.06[b]	30.90 ± 8.17[b]
Cloudy	29.33 ± 0.10[a]	22.63 ± 0.20[a]	5.81 ± 0.03[a]	7.72 ± 0.06[a]	91.36 ± 8.43[a]

Continued from Table 4.6-4

Treatment combinations		Butyl acetate	2-methyl butyl acetate	Ethyl acetate	Hexyl acetate	Total Volatile
H x M						
Comm	1-MCP	14.70 ± 0.14[a]	16.70 ± 0.28[a]	0.90 ± 0.04[b]	4.23 ± 0.08[a]	53.62 ± 11.6[a]
Comm	Control	17.79 ± 0.14[a]	12.02 ± 0.28[a]	11.01± 0.04[a]	5.36 ± 0.08[a]	63.70 ± 11.6[a]
Late	1-MCP	16.64 ± 0.15[a]	13.81± 0.30[a]	2.13 ± 0.04[b]	3.95 ± 0.09[a]	46.79 ± 11.6[a]
Late	Control	16.29 ± 0.15[a]	11.14± 0.30[a]	7.86 ± 0.04[a]	2.84 ± 0.09[a]	49.88 ± 12.3[a]
H x J						
Comm	Clear	11.95 ± 0.14[c]	9.61 ± 0.28[b]	3.19 ± 0.04[b]	2.56 ± 0.08[b]	45.07 ± 11.6[b]
Comm	Cloudy	21.88 ± 0.14[b]	19.84 ± 0.28[a]	4.00 ± 0.04[a]	8.06 ± 0.08[a]	75.77 ± 11.6[a]
Late	Clear	6.89 ± 0.15[d]	3.97 ± 0.30[b]	2.00 ± 0.04[b]	1.19 ± 0.09[b]	21.19 ± 12.3[c]
Late	Cloudy	39.30 ± 0.15[a]	25.60 ± 0.30[a]	8.28 ± 0.04[a]	7.39 ± 0.09[a]	110.17± 1.6[a]
Statistical Significance[E]		A * J *** H x J ***	A *** J *** H x J **	M *** A *** J *** H x M ** H x J **	A *** J ***	M* J *** H x J ***
Lambda[F]		0	0.5	0.2	0.3	0

All the values represent mean ± standard error (n = 3) three biological replicates. Means followed by the same letter within a column are not significantly different.
[A] to [F] refer to Table 4.6-2.

Table 4.6-5. Relative amounts of major aldehydes and alcohols identified in clear and cloudy juice samples from 'Honeycrisp' apples harvested at commercial or late harvest maturity, treated or untreated with 1-MCP and stored under CA or RA conditions for 4 months.

Treatment combinations	Normalized peak area counts[A]				
	2-methyl-4-pentenal	(E)-2-hexenal	Branched chain alcohol	(Z)-2-hexen-1-ol	Ethanol
Harvest (H)					
Comm[B]	6.62 ± 0.10[a]	1.02 ± 0.13[a]	1.07 ± 0.02[a]	1.33 ± 0.18[a]	0.56 ± 0.11[a]
Late	4.57 ± 0.10[b]	0.51 ± 0.14[b]	1.11 ± 0.02[a]	1.61 ± 0.19[a]	0.71 ± 0.12[a]
1-MCP (M)					
1-MCP	5.22 ± 0.11[a]	0.70 ± 0.14[a]	0.39 ± 0.02[b]	1.52 ± 0.19[a]	0.17 ± 0.11[b]
Control	5.81 ± 0.11[a]	0.74 ± 0.14[a]	3.69 ± 0.02[a]	1.41 ± 0.19[a]	1.09 ± 0.11[a]
Atmosphere (A)					
CA[C]	4.64 ± 0.11[b]	0.93 ± 0.14[a]	0.58 ± 0.02[b]	1.97 ± 0.19[a]	0.50 ± 0.11[a]
RA[D]	6.52 ± 0.11[a]	0.56 ± 0.14[b]	2.23 ± 0.02[a]	0.97 ± 0.19[b]	0.77 ± 0.11[a]
Juice (J)					
Clear	3.37 ± 0.11[b]	0.54 ± 0.14[b]	0.66 ± 0.02[b]	0.92 ± 0.19[b]	0.41 ± 0.11[b]
Cloudy	8.98 ± 0.11[a]	0.96 ± 0.14[a]	1.88 ± 0.02[a]	2.02 ± 0.19[a]	0.86 ± 0.11[a]
H x M					
Comm 1-MCP	5.59 ± 0.15[a]	0.85 ± 0.19[a]	0.31 ± 0.02[b]	1.38 ± 0.26[a]	0.10 ± 0.16[a]
Comm Control	7.85 ± 0.15[a]	1.22 ± 0.19[a]	5.10 ± 0.02[a]	1.29 ± 0.26[a]	1.02 ± 0.16[a]
Late 1-MCP	4.87 ± 0.15[a]	0.58 ± 0.19[b]	0.51 ± 0.02[b]	1.67 ± 0.27[a]	0.24 ± 0.17[a]
Late Control	4.29 ± 0.15[a]	0.45 ± 0.19[b]	2.72 ± 0.02[a]	1.54 ± 0.27[a]	1.18 ± 0.17[a]
H x A					
Comm CA	5.74 ± 0.15[a]	1.48 ± 0.19[a]	0.62 ± 0.02[a]	1.82 ± 0.26[a]	0.45 ± 0.16[a]
Comm RA	7.64 ± 0.15[a]	0.47 ± 0.19[b]	1.97 ± 0.02[a]	0.84 ± 0.26[a]	0.67 ± 0.16[a]
Late CA	3.75 ± 0.15[a]	0.58 ± 0.19[b]	0.54 ± 0.02[a]	2.11 ± 0.28[a]	0.55 ± 0.17[a]
Late RA	5.57 ± 0.15[a]	0.45 ± 0.19[b]	2.53± 0.02 [a]	1.11 ± 0.28[a]	0.87 ± 0.17[a]

Continued from Table 4.6-5

Treatment ombinations		2-methyl - 4-pentenal	(E)-2-hexenal	Branched chain alcohol	(Z)-2-hexen-1-ol	Ethanol
H x J						
Comm	Clear	5.68 ± 0.15^{b}	0.47 ± 0.18^{b}	0.87± 0.02^{bc}	1.07 ± 0.26^{b}	0.55 ± 0.16^{ab}
Comm	Cloudy	7.73 ± 0.15^{ab}	1.48 ± 0.18^{a}	1.34 ± 0.02^{b}	1.60 ± 0.26^{ab}	0.57± 0.16^{ab}
Late	Clear	2.00 ± 0.15^{c}	0.41 ± 0.19^{b}	0.51 ± 0.02^{c}	0.76 ± 0.28^{b}	0.26 ± 0.17^{b}
Late	Cloudy	10.44 ± 0.15^{a}	0.62 ± 0.19^{b}	2.69 ± 0.02^{a}	2.45 ± 0.28^{a}	1.16 ± 0.17^{a}
M x A						
1-MCP	CA	4.03 ± 0.15^{a}	1.04 ± 0.18^{a}	0.20 ± 0.02^{d}	2.18 ± 0.26^{a}	0.08 ± 0.16^{b}
1-MCP	RA	6.75 ± 0.16^{a}	0.37± 0.18^{c}	0.91 ± 0.02^{c}	0.87 ± 0.26^{b}	0.26 ± 0.16^{b}
Control	CA	5.34 ± 0.16^{a}	0.83 ± 0.19^{ab}	2.25 ± 0.02^{b}	1.75 ± 0.28^{a}	0.92 ± 0.17^{a}
Control	RA	6.31 ± 0.15^{a}	0.57 ± 0.19^{bc}	6.35 ± 0.02^{a}	1.08 ± 0.28^{b}	1.27 ± 0.17^{a}
Statistical		H*	H***	M***		M***
Significance[E]		A*	A***	A***	A***	
		J***	J***	J***	J**	J***
			H x M ***	H x M ***		
			H x A ***			
		H x J *	H x J **	H x J **	H x J **	H x J *
			M x A**			
Lambda[F]		0	0	0	1	1

All the values represent mean ± standard error (n = 3) three biological replicates. Means followed by the same letter within a column are not significantly different.
[A] to [F] refer to Table 4.6-2.

4.7 Figure captions

Figure 4.8-1 A typical chromatogram for cloudy apple juice from ‘Honeycrisp’ apples.

Figure 4.8-2. A typical chromatogram for cloudy apple juice from ‘McIntosh’ apples.

Figure 4.8-3. Relative abundance of volatile compounds identified in fresh, clear and cloudy apple juices from ‘McIntosh’ (A) and ‘Honeycrisp’ (B) apples as well as from whole ‘McIntosh’ (C) and ‘Honeycrisp’ (D) apples stored for 4-months under CA or RA condition and harvested at commercial or late maturity stage. The value for each bar represents the mean of 48 samples each with three biological replicates.

4.8 Figures

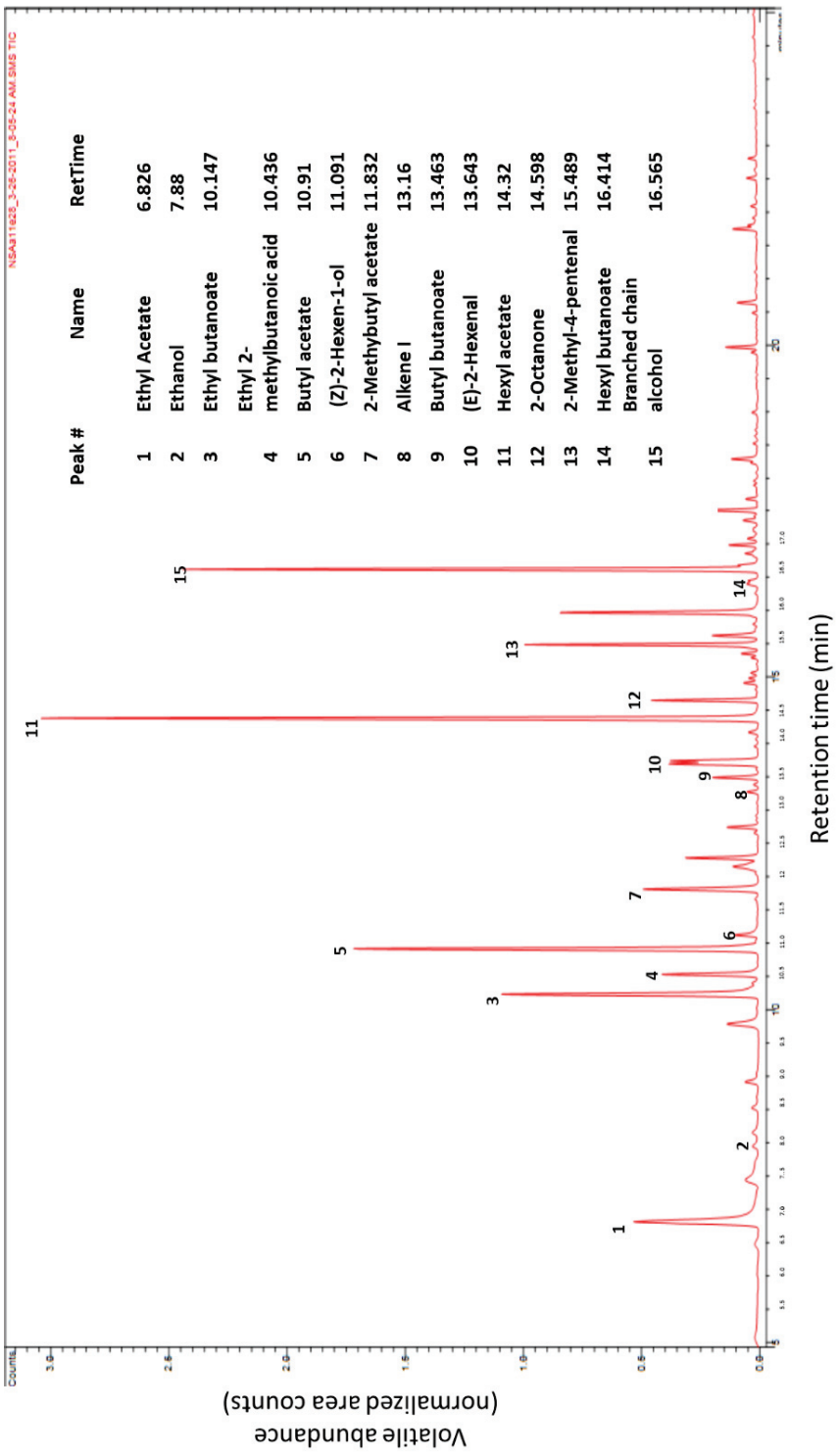
Peak # Name RetTime
1 Ethyl Acetate 6.826
2 Ethanol 7.88
3 Ethyl butanoate 10.147
4 Ethyl 2-methylbutanoic acid 10.436
5 Butyl acetate 10.91
6 (Z)-2-Hexen-1-ol 11.091
7 2-Methybutyl acetate 11.832
8 Alkene I 13.16
9 Butyl butanoate 13.463
10 (E)-2-Hexenal 13.643
11 Hexyl acetate 14.32
12 2-Octanone 14.598
13 2-Methyl-4-pentenal 15.489
14 Hexyl butanoate 16.414
15 Branched chain alcohol 16.565
Volatile abundance (normalized area counts)
Retention time (min)

Figure 4.8-1

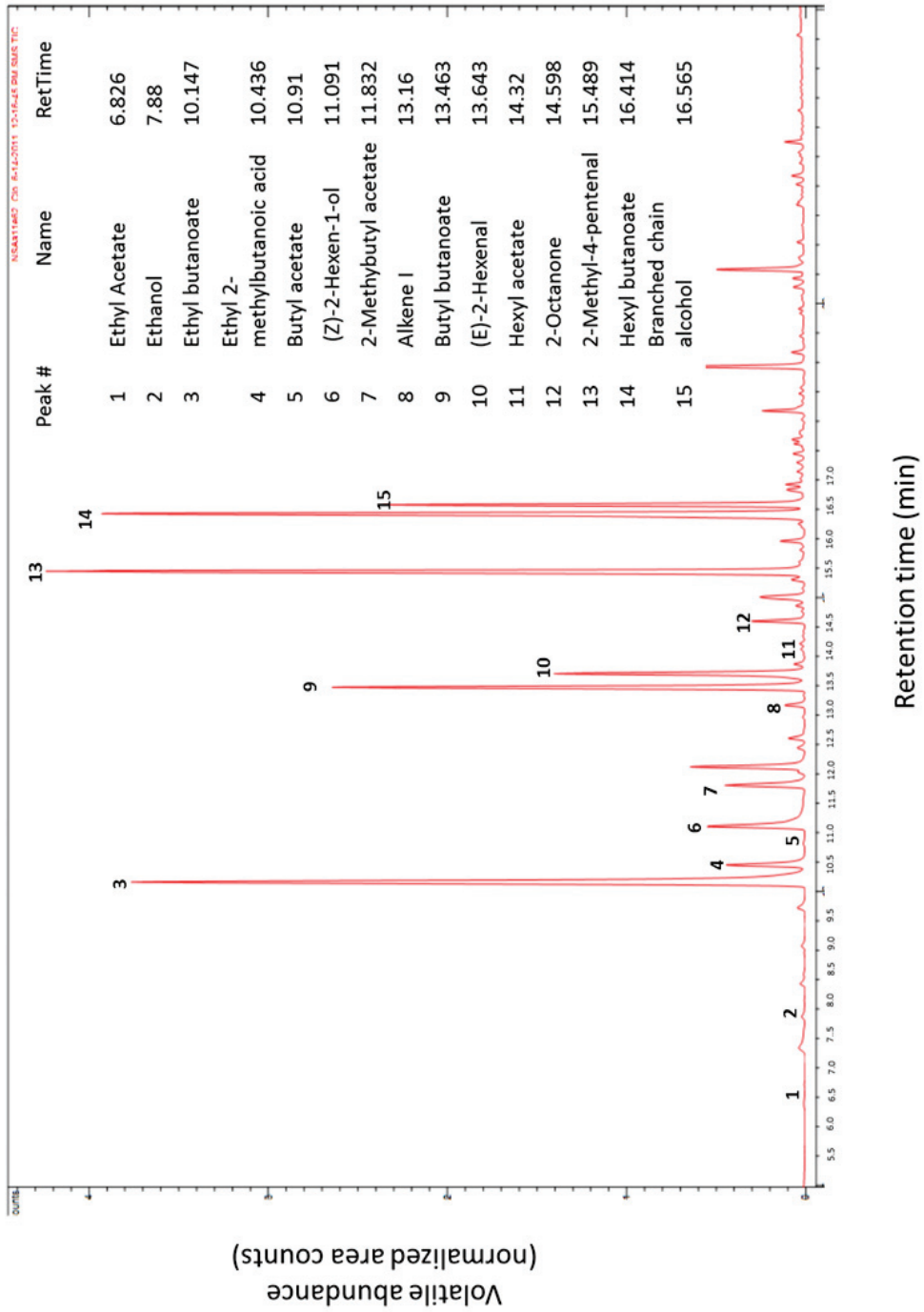
Peak # Name RetTime
1 Ethyl Acetate 6.826
2 Ethanol 7.88
3 Ethyl butanoate 10.147
4 Ethyl 2-methylbutanoic acid 10.436
5 Butyl acetate 10.91
6 (Z)-2-Hexen-1-ol 11.091
7 2-Methybutyl acetate 11.832
8 Alkene I 13.16
9 Butyl butanoate 13.463
10 (E)-2-Hexenal 13.643
11 Hexyl acetate 14.32
12 2-Octanone 14.598
13 2-Methyl-4-pentenal 15.489
14 Hexyl butanoate 16.414
15 Branched chain alcohol 16.565
Volatile abundance
(normalized area counts)
Retention time (min)

Figure 4.8-2

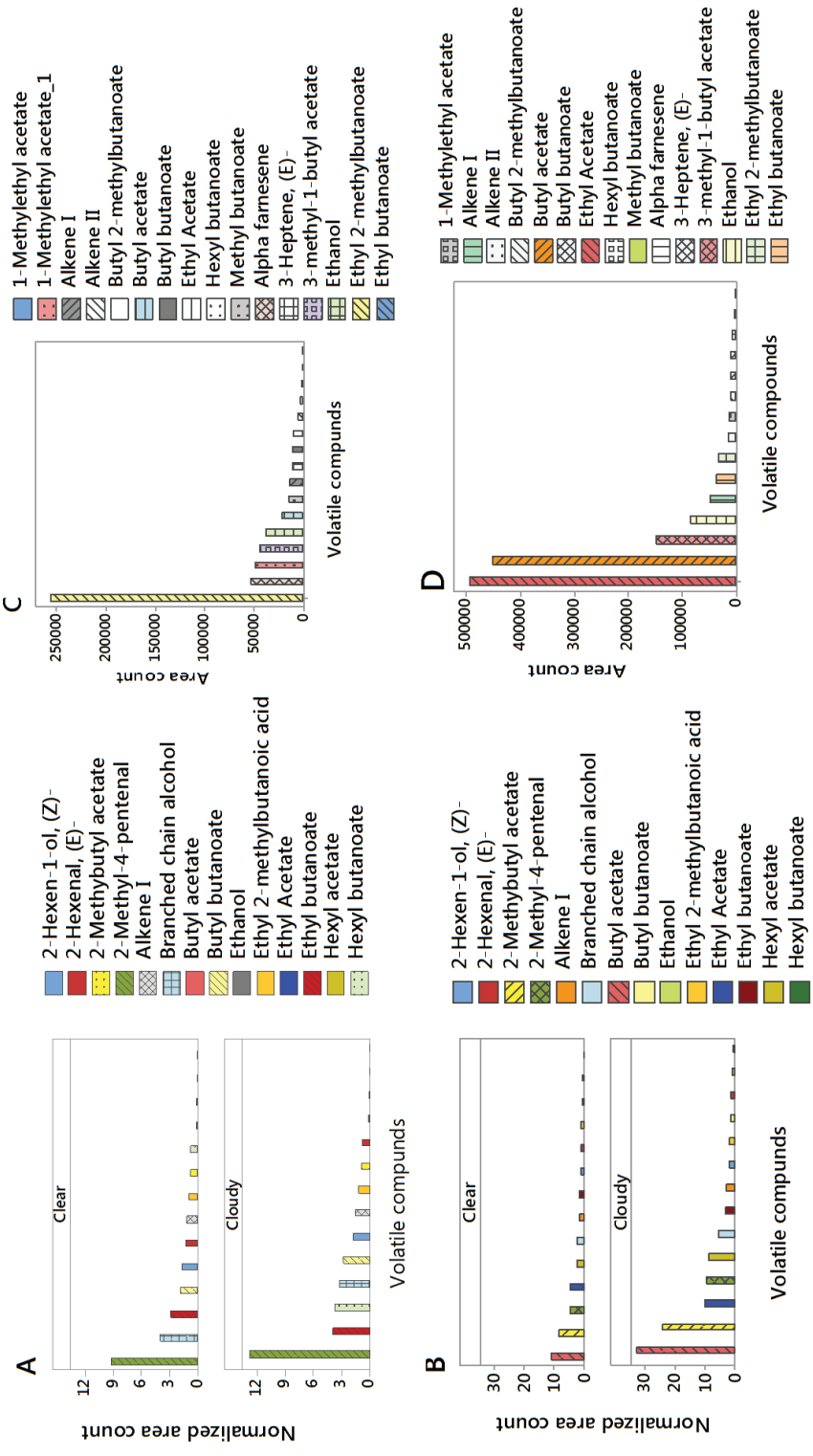
A
Clear
Cloudy
Normalized area count
Volatile compunds
2-Hexen-1-ol, (Z)-
2-Hexenal, (E)-
2-Methybutyl acetate
2-Methyl-4-pentenal
Alkene I
Branched chain alcohol
Butyl acetate
Butyl butanoate
Ethanol
Ethyl 2-methylbutanoic acid
Ethyl Acetate
Ethyl butanoate
Hexyl acetate
Hexyl butanoate
B
Clear
Cloudy
Normalized area count
Volatile compunds
2-Hexen-1-ol, (Z)-
2-Hexenal, (E)-
2-Methybutyl acetate
2-Methyl-4-pentenal
Alkene I
Branched chain alcohol
Butyl acetate
Butyl butanoate
Ethanol
Ethyl 2-methylbutanoic acid
Ethyl Acetate
Ethyl butanoate
Hexyl acetate
Hexyl butanoate
C
Area count
Volatile compunds
1-Methylethyl acetate
1-Methylethyl acetate_1
Alkene I
Alkene II
Butyl 2-methylbutanoate
Butyl acetate
Butyl butanoate
Ethyl Acetate
Hexyl butanoate
Methyl butanoate
Alpha farnesene
3-Heptene, (E)-
3-methyl-1-butyl acetate
Ethanol
Ethyl 2-methylbutanoate
Ethyl butanoate
D
Area count
Volatile compounds
1-Methylethyl acetate
Alkene I
Alkene II
Butyl 2-methylbutanoate
Butyl acetate
Butyl butanoate
Ethyl Acetate
Hexyl butanoate
Methyl butanoate
Alpha farnesene
3-Heptene, (E)-
3-methyl-1-butyl acetate
Ethanol
Ethyl 2-methylbutanoate
Ethyl butanoate

Figure 4.8-3

Chapter 5: Effect of 1-methylcyclopropene (1-MCP) and storage atmosphere on polyphenol content, antioxidant capacity and visual quality attributes of cloudy apple juice from 'McIntosh' and 'Honeycrisp' apples

Abstract

The effects of 1-methylcyclopropene (1-MCP) treatment, storage atmosphere and juice processing on total phenolic content (TPC) and total antioxidant activity (TAA) of cloudy and clear juices prepared from 'McIntosh' and 'Honeycrisp' apples harvested at optimal maturity and stored for 4-months were studied. The influence of 1-MCP treatment, storage atmosphere and harvest maturity on juice yield, turbidity, cloud stability and colour of cloudy juice samples was also investigated. TPC and TAA measured as FC (Folin-Ciocalteu) and FRAP (ferric reducing antioxidant power) were significantly ($p<0.001$) affected by juice type and 1-MCP treatment. Irrespective of 1-MCP treatment and storage atmosphere, considerably higher FRAP (3- to 5-fold) and FC (2- to 4-fold) values were present in cloudy as opposed to clear juice samples from 'McIntosh' and 'Honeycrisp' apples, respectively. In addition, higher FRAP values were observed in juices from 1-MCP treated McIntosh' (30-50%) and 'Honeycrisp' (13%) apples as compared to untreated fruits. Juices from 1-MCP treated 'McIntosh' fruit stored under controlled (CA) or regular (RA) atmosphere storage condition had considerably higher (40%) FC values than those

juices from untreated fruit. 1-MCP treatment in 'Honeycrisp' fruit yielded higher FC when combined with CA (47%) than RA (14%), which suggests a synergistic increase of FC in the presence of the 1-MCP + CA combination. The juice yield from 'McIntosh' fruit ranged from 33% to 81% depending on harvest maturity, storage atmosphere and 1-MCP treatment. Harvesting 'McIntosh' apples at optimal maturity stage or storing them under CA condition improved the juice yield by about 11-21%, relative to those harvested late and stored under RA condition. Moreover, 1-MCP treatment enhanced the juice yield by about 4-8% in CA and RA stored 'McIntosh' apples, respectively. On the other hand, the juice yield from 'Honeycrisp' fruit (60-70%) was not considerably influenced by harvest maturity, 1-MCP treatment and storage conditions. Juice turbidity and cloud stability values from 'Honeycrisp' juices were substantially less than those in the 'McIntosh' juices studied and were not changed with harvest maturity, storage atmosphere and 1-MCP treatment. Regarding colour, all cloudy juice samples prepared from both cultivars could meet the expected yellowish colour and the colour variation among cloudy juice samples was not affected by harvest maturity, 1-MCP treatment or storage atmosphere.

Keywords: 1-MCP, atmosphere, polyphenol, antioxidant, cloudy, clear, juice, 'McIntosh', 'Honeycrisp'.

5.1 Introduction

Compared to many other commonly consumed fruits in the North America, apples have the second highest level of total phenolic content and antioxidant capacity after cranberry (Sun et al., 2002). The consumption of apple and apple products has been linked to a reduced risk of several chronic illnesses including cardiovascular disease, neurodegenerative disease, diabetes, obesity and cancer (Boyer and Liu, 2004; Gerhauser, 2008; Barth et al., 2007; Barth et al., 2005; Will et al., 2008; Kujawska et al., 2011). These health benefits are associated with the major phenolic compounds identified in apple fruits or their products including flavan-3-ols (catechins and proanthocyanidins, 71-90%), hydroxycinnamic acids (4-31%), flavonols (2-11%), dihydrochalcones (2-6%), and anthocyanins (1-3%) (Wojdyło et al., 2008; Vrhovsek et al., 2004).

Antioxidant capacity, which is the ability or capability to inhibit oxidative degradation (Roginsky and Lissi, 2005), is a common characteristic of these phenolic compounds because of their ability to scavenge and reduce the production of reactive oxygen species (ROS), suppress enzymes involved in oxidative stress, and chelate transition metal ions that promote oxidation (Dangles, 2012). Moreover, phenolic compounds are responsible for the mouthfeel, bitterness, astringency and colour of fresh and processed apple products (Lea, 1992). Hence, the change in phenolic compounds associated with postharvest storage treatments and juice processing techniques could affect the nutritional attributes as well as organoleptic quality of the final product.

Worldwide, apple juice is the second most widely consumed juice following orange (Statistics Canada, 2010). Apple juice is processed and sold in many forms, including sweet

cider (American apple cider), European apple cider (apple cider), and shelf stable apple juice. In many countries, apple cider refers to naturally fermented apple juice but in the USA, apple cider refers to fresh apple juice that is not fermented and or pasteurized (Root, 1996). Sweet apple cider refers to a fresh apple juice, which has been pressed, bottled and refrigerated without preservatives or pasteurization (Root, 1996). This type of juice is turbid, brown in colour and tends to sediment during storage (Lea, 1999). Shelf stable juice types include clear juice, cloudy juice, and 'juice from concentrate'. Commercially the most popular shelf stable apple juices include clear and concentrated apple juice, which have a typical amber like hue and clearer appearance (Root, 1996).

However, owing to its sensory and nutritional components, cloudy apple juice has become a fast growing sector especially in European countries such as Germany where cloudy apple juice now accounts for about 30% of their juice consumption (Will et al., 2008). Cloudy apple juice, also called opalescent or natural juice, is unclarified apple juice, which has a yellowish or greenish colour with a hazy/cloudy appearance (Nagel, 1992). As compared to clear juice, cloudy juices contain a remarkably higher portion of pulp, which is associated with the higher turbidity (Oszmianski et al., 2007; Oszmiański et al., 2009). Some studies have demonstrated that the health benefits associated with apple juice are more substantial for cloudy apple juice than the clear counterpart (Barth et al., 2005; Barth et al., 2007; Oszmianski et al., 2007; Matthes and Schmitz-Eiberger, 2008). Relative to clear juice, the greater health benefit of cloudy apple juice is attributed to the higher content of polyphenols, especially procyanindins (Huemmer et al., 2008; Oszmianski et al., 2007; Candrawinata et al., 2012). The lower content of phenolic compounds in clear apple juice

mainly result from the extensive clarification and filtration processes, which use enzymatic treatments to remove pectin and fibrous substances as well as phenolic compounds (Oszmianski et al., 2007; Olk et al., 2010). The loss of phenolic compounds during clear juice processing is also attributed to the enzymatic oxidation of phenolic compounds catalysed by polyphenol oxidase (PPO) (Oszmianski and Lee, 1990). As cloudy apple juice is not clarified and is processed under non-oxidative conditions including the use of ascorbic acid (to reduce enzymatic browning), the loss or alternation of polyphenol compounds and pectin substances is substantially minimized (Oszmianski et al., 2007). Consequently, cloudy apple juice could be considered as a minimally processed product since the process does not involve either enzymatic treatment or the rigorous filtration or clarification procedures, which are commonly used in clear apple juice production (Oszmianski et al., 2007). With the increasing consumer interest in minimally processed products, cloudy juice could become more popular (Jaros et al., 2009).

In addition to the juice processing method, the concentration of phenolic compounds depends on cultivar, storage conditions and postharvest treatments (Will et al., 2008; van der Sluis et al., 2002; van der Sluis et al., 2004). The apple industry in North America has adopted the extensive use of 1-methylcyclopropene (1-MCP) as a means to extend the storage life of apples (Watkins, 2006). In addition to its physiological effect, recent studies have focused on the influence of 1-MCP on the nutritional quality retention of apples, especially the antioxidant phenolic compounds (Awad and de Jager, 2000; Fawbush et al., 2009; Kolniak-Ostek et al., 2014; MacLean et al., 2006). Regarding the effect of 1-MCP, inconsistent results have been reported in the literature and the responses

were cultivar dependent. According to Watkins (2008), the existing information about the commercial trials of 1-MCP is still inadequate; in order to understand the full potential of 1-MCP treatment in different apple cultivars further studies are warranted.

The aim of the present study was to determine the effect of 1-MCP treatment, storage atmosphere, and juice type on the total phenolic and antioxidant content, of cloudy and clear juice prepared from 'McIntosh' and 'Honeycrisp' apples. The effects of 1-MCP, storage atmosphere on the yield, turbidity, cloud stability and colour of cloudy juice were also studied to evaluate the potential application of stored 'McIntosh' and 'Honeycrisp' apples in cloudy juice preparation.

5.2 Materials and Methods

5.2.1 Apple harvesting, 1-MCP treatment and storage

Refer to chapter 3, section 3.21. For this experiment only 4-month old samples were used.

5.2.2 Juice preparation

For cloudy and clear juice preparation, Refer to chapter 4, section 4.2.2. Fresh apple juice was prepared in the same way as cloudy juice, but simply pressed and filtered with four layer cheesecloth without ascorbic acid addition and pasteurization. The reason for the fresh juice preparation was to evaluate the effectiveness of ascorbic acid treatment in preventing enzymatic browning.

5.2.3 Experimental design and statistical analysis

The experimental design was a three-factor factorial with three replications. The independent variables included 1-MCP treatments (control and treated), storage atmosphere (CA and RA), juice type (clear and cloudy, used only for TPC and TAA) and

harvest maturity (used only for visual quality response parameters. Statistical analyses were accomplished using Minitab software (Release 17, Minitab Inc. State College, PA, USA). Analysis of variance (ANOVA) was performed using general linear model (GLM) procedure. GLM is an ANOVA procedure in which the statistical difference between one or more factors are performed using a least square (minimizing sum of squares or residuals) linear regression approach. Whenever there are significant main or interaction effects, multiple mean comparisons were employed using Tukey's method at significance level, $\alpha = 0.05$. For each response, the validity of model assumptions, namely normal distribution and constant variance of the error terms were verified by examining residual plots as indicated in the Appendix C-Figure 2 to 4 (Montgomery, 2008).

5.2.4 Reagents and standards

Ultrazym 100 G was purchased from Novozymes (Franklinton, NC, USA). Buffer solutions (pH 7.0 and pH 4.0) were obtained from Fisher Scientific (Ottawa, ON, Canada). Gallic acid, 2, 4, 6-tris (2-pyridyl)-S-triazine (TPIZ), Trolox, fluorescein, Folin-Ciocalteu reagent, quercetin, 1-1-diphenyl-2-picrylhydrazyl (DPPH), sodium carbonate, acetate buffer, ferric chloride and phosphate buffer were obtained from Sigma Aldrich (Oakville, ON, Canada). Hydrochloric acid and 96-well microplates were purchased from Fisher Scientific (Ottawa, ON, Canada).

5.2.5 Juice yield measurement

Juice yield was recorded for cloudy juice only and the measurement was done in triplicates. Juice yield in percentage was calculated as:

$$\text{Juice yield } (\%, \text{volume/weight}) = \left(\frac{\text{Juice volume}}{\text{Fruit weigh}}\right) \times 100 \quad \text{Eq. 5.1}$$

5.2.6 Turbidity measurement

Turbidity measurements were carried out using a digital turbidimeter (AQ4500, Thermo Scientific Orion, and USA). An aliquot of 5 mL juice was poured into the sample holder. Turbidity measurements were taken, after gently mixing the sample to resuspend settled solid particles. Results were expressed in Nephelometric Turbidity Units (NTU) (Tajchakavit et al., 2001).The measurements were done on three biological replicates.

5.2.7 Cloud stability

Percentage of cloud stability for cloudy juice was calculated as:

$$\text{Cloud stability (\%)} = \frac{T_c}{T_o} \text{x } 100 \quad \text{Eq. 5.2}$$

where T_o and T_c are turbidity measurement before and after centrifugation (4200 x g for 15 min), respectively (Oszmianski et al., 2009; Stahle-Hamatschek, 1989). The measurement was performed on three biological replicates.

5.2.8 Colour measurement

The colour of juice samples was determined using a reflectance colourimeter (CR300, Minolta Camera Co. Ltd, Japan) based on Hunter Lab, colour space system. The *L, a, b* values represent lightness or darkness (*L*), redness or greenness (+*a*/-*a*), yellowness or blueness (+*b*/-*b*). The colourimeter was calibrated using a white plate (CR-A43, Minolta Camera Co. Ltd, Japan) with the reference values of X = 92.30, Y = 0.3137 and Z = 0.3195. Juice samples (30 mL) were poured into 100 mL capacity white weighing dishes (juice depth of about 1 cm) and placed on a white background, the measuring head was partially

immersed in the juice sample and the *L, a, b* values were recorded. Triplicate colour readings were taken for each of the three biological replicates. While measuring, the colourimeter was held firm and perpendicular to the juice surface.

The total colour difference (ΔE) and browning index (*BI*) were calculated from *L, a, b* values as indicated in Eq. 5.3 and Eq. 5.4 (Maskan, 200; Bozkurt and Bayram, 2006).

$$\Delta E = \sqrt{(L_o\text{-}L)^2 + (a_o\text{-}a)^2 + (b_o\text{-}b)^2} \quad \text{Eq. 5. 3}$$

where *L, a, b* values represent lightness or darkness (*L*), redness or greenness (+*a*/-*a*), yellowness or blueness (+*b*/-*b*), the subscript 'o' refers to the Hunter Lab values of fresh juice samples as these juices were used as the reference.

$$BI = 100 \text{ x } \left(\frac{X-0.31}{0.17}\right) \quad \text{Eq.5.4}$$

where, $X = \left(\frac{a+1.75L}{5.645L + a - 3.012b}\right)$

5.2.9 Total phenolic content (TPC) and total antioxidant activity (TAA) assays

Both TPC and TAA in apple juice extracts were analysed using BMG FLUOstar OPTIMA micro-plate reader with an injection port system (BMG LABTECH, Offenburg, Germany). TPC was quantified using the Folin-Ciocalteu (FC) method (Singleton et al., 1999). Total antioxidant activity (TAA) was measured using two different chemical approaches: DPPH (1, 1-diphenyl-2-picrylhydrazyl) was used to determine the free radical scavenging activity (Yen and Chen, 1995) and FRAP which was used to measure the ferric reducing capability of the samples (Benzie and Strain, 1996). While the results of DPPH and FRAP were expressed in μmol of Trolox equivalent (TE) per liter of juice, the results of FC was

expressed as μmol of gallic acid equivalent (GAE) per liter. The detail of each assay is explained below.

5.2.9.1 FC assay for TPC

The FC assay was performed to estimate the total phenols present in the juice sample. The assay was performed as described by Singleton et al. (1999) and modified by Rupasinghe et al. (2010). Briefly, 20 μL of diluted juice (1.5-fold for 'Honeycrisp' juices and 5-fold for 'McIntosh' juices) sample was mixed with 100 μL of 0.2 N FC reagent in the wells of a clear 96-well microplate (COSTAR 9017, Fisher Scientific, Ottawa, ON, Canada) and left to stand for 5 min. After 5 min, 80 μL of a 7.5% sodium carbonate solution was added and the microplate was covered with aluminum foil (to prevent light exposure) for 2 h at ambient temperature before reading the absorbance values at 760 nm. To protect the integrity of the samples, the reagent solutions were made fresh and the analysis was carried out under dark condition. The total phenolic content was calculated using a series of gallic acid standards. The calibration graph (Appendix C-Figure 1) was linear in the range 59 to 1470 μmol of gallic acid with the correlation coefficient of 0.9996.

5.2.9.2 FRAP assay for TAA

The FRAP assay, which is based on the principle of the reduction of the ferric (Fe^{3+}) ion to ferrous (Fe^{2+}) ion under low pH conditions, was used to determine the electron donating potential of antioxidant compounds in the juice samples. Under low pH conditions, the non-coloured ferric-tripyridyltriazine complex changed to a bright blue colour ferrous tripyridyltriazine complex. The FRAP procedure used was that described by Benzie and Strain (1996) and subsequently modified by Rupasinghe et al. (2010). The working reagent

consisting of 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) solution , and 20 mM ferric chloride solution, was prepared in the ratio of 10:1:1 and preheated to 37 ^{o}C. These solution were prepared fresh daily. An aliquot of 20 µL of diluted juice sample (5-fold for 'Honeycrisp' juices and 8-fold dilution for 'McIntosh' juices) and 180 µL of the working solution were placed into the wells of the 96-well clear micro-plate (Fisher Scientific, Ottawa, ON, Canada). The absorbance was read at 593 nm after 6 min reaction time and the antioxidant capacity was calculated based on Trolox standards. The calibration graph (Appendix C-Figure 1) is linear in the range 20 to 900 µmol of Trolox with the correlation coefficient of 0.9968.

5.2.9.3 DPPH assay for TAA

Antioxidant activity in terms of free radical scavenging capability of the samples was measured using the stable, free radical scavenger DPPH (1, 1-diphenyl-2-picrylhydrazyl) as described by Yen and Chen (1995). The DPPH method is a well-established method used to determine TAA and is based on the capability of a stable free radical (2, 2-diphenyl-1-picrylhydrazyl) to react with H-donor phenolic compounds (Roginsky and Lissi, 2005). A stock solution of 0.2 mM (in methanol) DPPH solution was prepared fresh daily. The DPPH solution (1 mL) was added to 1 mL of 8-fold diluted juice (in methanol) and 3 mL of absolute methanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 10 min. An aliquot of 200 µL of the mixture was placed into the wells of the 96-well clear micro-plate (Fisher Scientific, Ottawa, ON, Canada) and the absorbance readings were recorded at 517 nm. Trolox standard solutions were used to generate the standard curve. The standard curve (Appendix C-Figure 1) was linear in the

range from 5 to 80 μmol of Trolox concentration with the correlation coefficient of 0.9913. The percentage of inhibition of the DPPH was calculated and plotted as a function of the concentration of Trolox and results were expressed as TE antioxidant capacity in μmol/L (Oszmianski et al., 2007).

5.3 Results

5.3.1 Change of TPC and TAA

The effect of 1-MCP treatment, storage atmosphere and juice type on TPC (measured as FC) and TAA (measured as FRAP and DPPH) of cloudy and clear juice prepared from 'McIntosh' and 'Honeycrisp' apples which had been harvested at optimal maturity and stored for 4-months is presented in Figure 5.8-1, A to F.

5.3.1.1 TPC

Juice FC values from both 'McIntosh' and 'Honeycrisp' fruit were significantly ($p<0.001$) affected by juice type. Regardless of 1-MCP treatment and storage atmosphere, the results indicated markedly higher FC values in cloudy than clear juice samples from both 'McIntosh' (2-fold) and 'Honeycrisp' (4-fold) apples (Figure 5.8-1, A and D). In addition to juice processing, 1-MCP and/ or storage atmosphere had a significant effect on the FC values from both cultivars. Juices from 1-MCP treated 'McIntosh' fruit stored under CA or RA had 40% higher FC values than those from untreated fruit (Figure 5.8-1 , A). Unlike that of 'McIntosh' where 1-MCP treatment was similarly effective under both RA and CA conditions, 1-MCP treatment in 'Honeycrisp' fruit yielded higher FC when combined with CA (47.3%) than RA (13.6%), which suggests a synergistic effect of the 1-MCP + CA combination to improve the content of TPC in 'Honeycrisp' juices (Figure 5.8-1, D).

5.3.1.2 TAA-FRAP

In 'McIntosh' juice samples FRAP values were remarkably affected by juice type and 1-MCP ($p<0.001$) and to a smaller extent by the two-way interaction of 1-MCP treatment and juice type ($p=0.041$) (Figure 5.8-1, B). Similarly, the FRAP value in 'Honeycrisp' juice samples were significantly affected by juice type ($p<0.001$) and marginally influenced by 1-MCP treatment ($p=0.041$) (Figure 5.8-1, E). Irrespective of 1-MCP treatment and storage atmosphere, the results indicated higher FRAP values in cloudy than clear juice samples from both 'McIntosh' (3-fold higher) and 'Honeycrisp' (5-fold higher) apples (Figure 5.8-1, B and E). In addition to juice processing, the results also demonstrated, higher values of FRAP in juices from 1-MCP treated apples as compared to untreated fruits. This difference is more pronounced in 'McIntosh' (30-50%) than 'Honeycrisp' fruit (12.7%) (Figure 5.8-1, B and E).

5.3.1.3 TAA-DPPH

Unlike FRAP, juice DPPH values from both cultivars were not significantly influenced by 1-MCP treatment. Rather juice processing was the major factor that noticeably affected the DPPH value of the juice samples from both cultivars (Figure 5.8-1, C and F). In 'McIntosh' juices a considerably higher (8.5%) DPPH values were observed in cloudy juices as compared to clear juice samples especially when juices were extracted from CA stored fruit. Such pronounced differences associated with juice processing were not observed in RA stored 'McIntosh' apples (Figure 5.8-1, C). Similarly, cloudy juices from 'Honeycrisp' apples contained 10.3% higher DPPH response as compared to clear juices, regardless of 1-MCP treatment and storage atmosphere (Figure 5.8-1, F).

5.3.1.4 Correlation among FC, FRAP and DPPH

Our results exhibited significant ($p<0.01$) and positive correlations between FC and FRAP ($r^2 = 0.97, 0.90$), FC and DPPH ($r^2 = 0.50, 0.67$) as well as between FRAP and DPPH ($r^2 = 0.53, 0.76$) in 'McIntosh' and 'Honeycrisp' juice samples, respectively (Table 5.6-1).

5.3.2 Change of physical quality parameters of cloudy apple juice

The influence of 1-MCP treatment, storage atmosphere and harvest maturity on physical quality parameters including juice yield, turbidity, cloud stability and colour of cloudy juice samples prepared from 4-month old 'McIntosh' and 'Honeycrisp' fruit is presented in Figure 5.8-2, A-F.

5.3.2.1 Juice yield

The juice yield from 'McIntosh' fruit ranged from 30% to 80% (Figure 5.8-2). The highest juice yield was obtained from 1-MCP treated and CA stored fruit harvested at optimal maturity stage. In contrast, the lowest juice yield was from untreated and RA stored 'McIntosh' fruit, which had been harvested at late maturity stage. In 'McIntosh' juices, significantly ($p<0.001$) different juice yield was observed due to harvest maturity and storage atmosphere conditions. Substantially higher juice yield was observed from fruit harvested at optimal harvest maturity (65.3%) as compared to those from late harvest date (54.4%). Regarding the effect of storage atmosphere, considerably higher juice yielded was observed from CA (70.5%) than RA (49.2%) stored 'McIntosh' fruit. Even though, 1-MCP treatment alone did not have a significant effect on the juice yield of 'McIntosh' fruit, the combination of 1-MCP with CA resulted higher juice yield (10%) than CA alone especially in late harvested fruits.

The juice yield from 'Honeycrisp' fruit ranged from 60-70%. Unlike 'McIntosh', juice yield from 'Honeycrisp' fruit was not significantly influenced by harvest maturity, 1-MCP treatment and storage conditions (Figure 5.8-2, D).

5.3.2.2 Juice turbidity and cloud stability

Juice turbidity and cloud stability of the cloudy juice from 'McIntosh' fruit was significantly affected by harvest maturity ($p<0.001$). As indicated in Figure 5.8-2, B and C, late harvested 'McIntosh' fruit yielded considerably higher juice turbidity and cloud stability (1369-3021 NTU, 21.8-57.3%) as compared to those harvested at commercial maturity (1019-1100 NTU, 4.9-5.7%) stored under CA and RA condition, respectively. In addition to harvest maturity, juice turbidity and cloud stability were significantly different due to 1-MCP treatment ($p<0.05$) and storage atmosphere ($p<0.001$). The higher juice turbidity and cloud stability values were observed in juices prepared from control fruits kept under RA as compared to 1-MCP treated and CA stored fruits and these differences were prominent in late harvested 'McIntosh' apples (Figure 5.8-2, B and C).

Juice turbidity (358-546 NTU) and cloud stability (5.9-12.2%) values of 'Honeycrisp' juices were substantially lower than 'McIntosh' juices (Figure 5.8-2, E-F). Unlike 'McIntosh' juices, harvest maturity and postharvest storage or 1-MCP treatment conditions had little effect on juice turbidity and cloud stability of 'Honeycrisp' apples.

5.3.2.3 Juice colour

The significant ($p<0.05$) effect of juice processing and harvest maturity on the Hunter Lab colour parameters (L, a and b) of juice samples from 'McIntosh' and 'Honeycrisp' apples is shown in Figure 5.8-3 and Figure 5.8-4. The visual colour difference between cloudy

(whitish yellow) and fresh/raw (dark brown) apple juice was also shown (Figure 5.8-5). In either cultivar, postharvest 1-MCP treatment or storage atmosphere had little or no effect on the colour properties measured. Even though, the interaction effect of juice processing and harvest maturity was statistically significant ($p<0.05$) the observed colour differences were mainly due to juice type ($p<0.001$).

Our results clearly indicated that cloudy juice samples had considerably higher *L* (43.9, 47.9) and *b* (16.9, 27.9) values as compared to fresh juice samples [*L* (29.8, 34.2), *b* (10.8, 16.4)] in 'McIntosh' and 'Honeycrisp' juices, respectively. As expected, cloudy juice samples had dramatically lower *a* values (0.92, 1.74) as compared to fresh juice samples (9.25, 10.32) extracted from 'McIntosh' and Honeycrisp' fruit, respectively. In relation to lower '*a'* values, cloudy juice samples had considerably lower *BI* as compared to fresh juices. The visual colour difference between cloudy and fresh juice sample reflected the higher *BI* and *a* values of fresh juice samples (Figure 5.8-5). The total colour change (ΔE) between cloudy and fresh juice samples was also calculated and the values ranged from 9.5-25.8 units.

5.4 Discussion

5.4.1 Changes of total phenolic content (TPC)

The present results indicate a substantial influence of juice processing techniques on TPC, measured as FC. Substantially higher (2 to 4- fold) TPC was found in cloudy juice rather than clear juice samples irrespective of 1-MCP treatment, storage atmosphere and harvest maturity and cultivars used. These results are in a good agreement with previous studies which found substantially higher TPC in cloudy juices as compared to the clear counterpart prepared either in a commercial (Candrawinata et al., 2012; Kahle et al., 2005) or laboratory level (Markowski et al., 2005; Oszmianski et al., 2007). Even though individual phenolic compounds were not determined in this study, other studies have established a significant positive association between TPC reduction and the decreased content of major individual phenolic compounds including proanthocyanidins, chlorogenic acid, dihydrochalcones, coumaric acid, quercetin glycosides and anthocyanins (Kahle et al., 2005; Oszmianski et al., 2007; Candrawinata et al., 2012).

The clarification and filtration process used to remove most of the pulp and suspended particles has been suggested as the main reason for the loss of phenolic compounds during clear juice processing (van der Sluis et al., 2002). As most phenolic compounds are localized in the apple pulp (Karaman et al., 2013; Wolfe et al., 2003; van der Sluis et al., 2002; van der Sluis et al., 2004) and since the clarification, process removes most of pulp suspended in the juice; the process could drastically reduce the TPC of the clarified juice. Moreover, phenolic compounds such as polymerized proanthocyanidins that have greater affinity for cell wall polysaccharide compounds and are retained in the

pulp, could also be discarded in the waste stream during clear juice processing (Guyot et al., 2003)

Another potential reason for the loss of phenolic compounds during clear juice processing is the enzymatic oxidation of phenolic compounds (enzymatic browning) via the action of PPO in the presence of oxygen (Oszmianski and Lee, 1990; Rupasinghe, 2008). PPO catalyses the oxidation of phenolic compounds into a very reactive compounds called o-quinones, which in turn react with phenolic compounds (e.g. catechins, proanthocyanidins, and dihydrochalcones) resulting in polymerization of phenolic compounds and the formation of brown pigments (Oszmianski and Lee, 1990). Polymerized phenols would increase particle density and particle size by both binding to particles and by providing cross linkages between particles, which in turn would hasten the sedimentation of the particles (Beveridge, 2002). Beveridge et al. (1998) demonstrated the increase in proanthocyanidins particles during storage of oxidized juices. However, proanthocyanidins from un-oxidized juices showed no corresponding particle development.

In our experiment, the oxidation of phenolic compounds in cloudy juice samples was prevented by the addition of ascorbic acid (0.5 g/L). Ascorbic acid inhibits the enzymatic browning reaction by reducing o-quinones back to the original phenolic compounds, thereby inhibiting the polymerization of phenolic compounds and their subsequent sedimentation (Komthong et al., 2007; Kolniak-Ostek et al., 2013). The striking increment (2 to 4-fold) of TPC in cloudy juices observed in our study demonstrated the effective inhibition of enzymatic browning reaction during cloudy juice processing.

Similarly, a recent study by Kolniak-Ostek et al. (2013) demonstrated the effectiveness of ascorbic acid addition for the preservation of phenolic compounds in cloudy juice samples prepared from several apple cultivars. These authors reported that cloudy apple juices pressed with 0.5 g/kg of ascorbic acid contained considerably higher amount of phenolic compounds (65%) with increased antioxidant capacity (300-433%) (Kolniak-Ostek et al., 2013) compared to those without supplemental ascorbic acid.

The higher TPC observed in juices from 1-MCP treated 'McIntosh' or 'Honeycrisp' apples is in agreement with previous studies. These found higher amounts of individual or total phenolic compounds in the peel or flesh tissue of fresh apples that have been treated with 1-MCP (Fawbush et al., 2009; MacLean et al., 2006). Fawbush et al. (2009) found higher TPC in the peel tissue of 'Empire' apples stored for 7-months. In line with this, a study on 'Delicious' apples demonstrated a greater quantity of anthocyanin and flavonoid compounds in 1-MCP treated apples as compared to those without 1-MCP treatment (MacLean et al., 2006). According to MacLean et al. (2006), the higher content of phenolic compounds in 1-MCP treated apples is attributed to the preservation of phenolic compounds, since the researches did not observe any *de novo* synthesis of these phenolic compounds throughout the storage and shelf life period. The improved retention of phenolic compounds after 1-MCP treatment is associated with a concurrent increase in the activity of antioxidant enzymes including peroxidase (POX) and catalase (CAT) (Cao et al., 2011; Eberhardt et al., 2000). A study on 'Golden Smoothee' apples demonstrated the ability of 1-MCP treatment to reduce the generation of ROS by enhancing the activity level POX and CAT, which catalyse the elimination of ROS including hydrogen peroxide and

superoxide (Vilaplana et al., 2006). Similarly, increased levels of enzymatic antioxidant activity have been reported in 1-MCP treated loquat fruit (Cao et al., 2009) and 'Conference' pears (Chiriboga et al., 2012). It has also been suggested that reduced electrolyte leakage in 1-MCP treated fruits could explain the higher content of TPC in 1-MCP treated apples (Eberhardt et al., 2000). In many fruits, loss of membrane integrity results the de-compartmentalization of oxidative enzymes and substrates (phenolic compounds) that initiates enzymatic browning which in turn leads to the oxidative degradation of phenolic compounds (Jiang et al., 2001). A reduced electrolyte leakage is generally associated with better cell membrane integrity and improved flesh firmness (Larrigaudiere et al., 2004).

5.4.2 Change of total antioxidant activity (TAA)

As the antioxidant activity of apples is the consequence of synergistic and additive affects phenolic compounds (Liu, 2003) the determination of TAA could provide a better estimate of the overall contribution of antioxidant compounds in achieving health benefits (Eberhardt et al., 2000; Liu, 2003).

The present results showed the remarkable impact of juice processing on TAA measured as FRAP. In both cultivars, cloudy juices had considerably higher (3- to 5-fold) FRAP values when compared to clear juices. DPPH values for clear and cloudy juice differed significantly but only by about 11%. This minor difference between DPPH values is consistent with a study in 'Champion' and 'Idared' apples where the DPPH values of cloudy and clear juice samples differed only by 10-25% while the difference in the content of proanthocyanidins was about 3- to 5-fold (Oszmianski et al., 2007). As discussed earlier,

the higher TAA in cloudy juices should be attributed to the preservation of phenolic compounds, which in turn is associated with the prevention of enzymatic browning by the addition of ascorbic acid. The potent antioxidant activity of the added ascorbic acid itself could also have some contribution (Davey et al., 2000).

In addition to juice processing, TAA was also strongly influenced by 1-MCP treatment. The obtained results indicated substantially higher content of FRAP values for juices from 1-MCP treated fruits especially from 'McIntosh' apples. This is in agreement with a pervious study that found significantly higher FRAP values (50%) in cloudy juices from 1-MCP treated 'Idared' apples (Kolniak-Ostek et al., 2014). In addition, higher TAA (measured as total oxyradical scavenging capacity) was reported in the peel tissue of 'Empire' and 'Delicious' apples, which have been treated with 1-MCP (MacLean et al., 2003). In line with MacLean et al. (2003), 1-MCP treatment yielded higher TAA (DPPH) in the peel and flesh tissue of 'Fuji' and 'Empire' (Fawbush et al., 2009) apples.

In agreement with previous studies on different apple cultivars (MacLean et al., 2003; Fawbush et al., 2009; Kondo et al., 2005; Hoang et al., 2011), our results indicated the insignificant influence of 1-MCP treatment on DPPH values of both 'McIntosh' and 'Honeycrisp' juices. In contrast, reduced DPPH values were reported in the peel tissue of 1-MCP treated 'Cripps Pink' apples (Hoang et al., 2011). The inconsistent effect of 1-MCP on TAA measured as DPPH could also be attributed to different apple cultivars, storage conditions, and analytical methods used. The DPPH assay, according to the present results, seems to be less useful to indicate the difference in the antioxidant power due to 1-MCP treatment or juice type. This is probably due to the selectivity of the assay, as it does not

react with flavonoids that contain OH-groups in the B-ring nor to aromatic acids containing only one OH-group (Roginsky and Lissi, 2005).

5.4.3 Correlation between total phenolic and antioxidant activity

The present results indicated significant positive correlations between TPC and TAA measured as FC, DPPH and FRAP. In agreement with our results, positive relations between TPC and TAA were found in 'Cripps Pink' apples (Hoang et al., 2011), as well as in other fruits including cranberry, red grape, strawberry, pineapple, banana, peach, lemon, orange, pear and grape fruit (Sun et al., 2002). The strong positive correlation between TPC and TAA confirms the synergistic and additive effects phenolic compounds for the antioxidative properties of apples and its products (Liu, 2003).

5.4.4 Change of physical juice quality parameters

5.4.4.1 Juice yield

Juice yield is an important parameter in the juice processing industry as it affects the profitability of the juice production. The differences in juice yield from 'McIntosh' fruit was mainly attributed to harvest maturity and storage condition. The results suggest that by harvesting fruits at optimal maturity stage or by storing them under CA condition, it is possible to improve the juice yield by about 11-21%, respectively. Moreover, the presented results indicated the combined benefit of CA storage with 1-MCP treatment in improving the juice yield especially in 'McIntosh' apples. Accordingly, 1-MCP treatment increased the juice yield by about 4-8% in CA and RA stored 'McIntosh' apples, respectively. The improved juice yield observed in 1-MCP treated 'McIntosh' apples is consistent with Kolniak-Ostek et al. (2014) who found improved juice yield (about 10%) in 1-MCP treated

'Idared' and 'Topaz' apples stored under RA condition (2 ºC) up to 6 months. The observed difference in juice yield from 'McIntosh' juices is probably the result of differences in flesh firmness associated with harvest maturity, 1-MCP treatment and storage atmosphere. As mentioned in chapter 4, 1-MCP treatment (7.5-15.8%), CA storage (7.6-13.15%) or 1-MCP + CA combination (10-20%) significantly improved flesh firmness retention of 'McIntosh' fruit during storage. On the other hand, 'Honeycrisp' apples maintained their flesh firmness even up to 7 months of storage, irrespective of 1-MCP treatment or storage conditions. This might explain the similar juice yield values observed from this cultivar. Reduced juice yield associated with long-term storage and decreased flesh firmness retention was also reported in other apple cultivars stored for a longer period of time (Beveridge and Rao, 1997; Gerard and Roberts, 2004; Kolniak-Ostek et al., 2014). The loss of juice yield in stored apples is associated with the change in pectin structure. As ripening advances during long-term storage, various pectolytic enzymes (PG, PE, PL) cause intact pectin polymers bound to cellulose microfibrils to depolymerize, de-esterify and as a result increase pectin solubility (Kashyap et al., 2001). The presence of disintegrated pectin could lead to tissue softening, gel formation, and increased viscosity, which makes the pulp slippery and difficult to press, in turn resulting in lower juice yield (Kader and Barrett, 1996; Jong et al., 1999; Huber et al., 2001).

Commercially, enzymatic mash treatment is a common practice to improve the juice yield from stored apples through the hydrolysis of the pectin gel by pectinases (Hohn, 1996) thus leading to lower viscosities, rapid flow of juice (Mehrlander et al., 2002) and improved juice yield (Mantovani et al., 2005). Since, enzymatic pre-treatment and

clarification steps are omitted, in the production of cloudy apple juices (Mrakowski et al., 2012); juice yield improvement could be achieved by keeping apples firm and fresh until processing time. Thus, 1-MCP treatment and or CA storage would be good alternative to get cloudy juice with higher juice yield without using enzymatic treatment. By doing so, we could also minimize the detrimental aspects associated with enzymatic treatment including high cost as compared to the additional juice yield; storage haze development, in case of partial pectin breakdown; high methanol content due to demethylation (ca. 400 mg/L, normal level is ca. 50 mg/L); increased acidity due to solubilized polygalacturonic acid (Schols et al., 1991); and reduced polyphenol content (Mehrlander et al., 2002). Hence, minimizing or elimination of the enzyme usage could be useful in terms of quality improvement as well as reducing cost.

5.4.4.2 Turbidity

The most recognisable parameter that differentiates cloudy apple juice from clear juice is its turbidity. Cloudy apple juice is a colloidal dispersion where the continuous phase (juice serum) is composed of water soluble polysaccharides mainly pectin, sugars and malic acid and the dispersed phase (pulp) is compose of cellular tissue which are commuted during processing (Benitez et al., 2007; Genovese and Lozano, 2006). The light scattering of the dispersed particles in suspension is referred as juice turbidity (Mollov et al., 2006).

Cloudy apple juice is characterized by having an average turbidity of ≥ 250 NTU and cloud stability of greater or equal to 50% (Dietrich et al., 1996). As indicated in our results, the turbidity and cloud stability values from 'McIntosh' juice samples matched these reference values. In our study, the turbidity of cloudy apple juice from 'McIntosh'

apples was in the range of 859 and 3436 NTU. In agreement with our findings, a recent study on cloudy apple juice from 'Shampion', 'Idared' and 'Topaz' apples found turbidity values ranged from 540-2860 NTU. Irrespective of harvest maturity, the turbidity values of cloudy juices from 'Honeycrisp' were considerably lower (274-637 NTU) than 'McIntosh' juices and hence this cultivar may not be as suitable for stable cloudy juice production. This varietal difference was also reported in other apple cultivars where the turbidity of cloudy juice from 'Idared' was substantially lower than 'Topaz' and 'Shampion' juices (Kolniak-Ostek et al., 2014).

In addition to cultivar type, harvest maturity had a crucial influence on turbidity of cloudy juices prepared from 'McIntosh' apples. Juices from late harvested and RA stored 'McIntosh' apples had up to 3-times higher turbidity as compared with those from optimal harvest maturity. Other studies also found higher turbidity values in cloudy juices extracted from stored than freshly harvested apples (Markowski, 1998; Kolniak-Ostek et al., 2014). The higher juice turbidity from late harvested and RA stored 'McIntosh' apples is attributed to the increased level of soluble pectin substances that are less bounded to cellulose microfibrils and lead to softening of the fruit tissue in more ripe fruits (Huber et al., 2001; Kashyap et al., 2001). An increased proportion of soluble pectin leads to higher juice viscosity, which in turn leads to better stability of suspended particles and increased turbidity (Beveridge, 2002). The smaller particle size of suspended particles from more mature apples was also related with more turbid cloudy juices (Dietrich et al., 1996). Smaller particles scatter light more strongly at wide angles than larger particles that in turn leads to higher nephelometer readings (Lozano, 2006) and hence juice samples from stored

apples might contain more small sized particles as compared to those from less ripe apples. Moreover, higher starch content from less ripe fruits could reduce turbidity and cloud stability via the gelatinization and retrogradiative flocculation of native starch molecules during the heating and cooling process of juice preparation (Dietrich et al., 1996). Hence, in order to produce a stable cloudy juice from 'McIntosh' apples, fully ripe apples with lower starch content should be selected.

5.4.4.3 Cloud stability

Cloud stability is one of the visual quality attributes that is crucial for consumer acceptance and shelf life of cloudy juice (Beveridge, 2002). As cloudy apple juice is expected to be free of settled sediments, a phase separated juice or evidence of container ringing are considered serious quality defects (Beveridge, 2002). In this study, cloud stability was predicted using the centrifugation method that separated the juice cloud into course (sediment) and fine (suspended) cloud (Stahle-Hamatschek, 1989). The cloud stability measured by this method has been reported to be well correlated with the visual cloud loss recorded one year after the date of manufacture and hence it provides a good predictor of cloud stability for one-year shelf life at 20 ^{o}C (Stahle-Hamatschek, 1989).

Similar to juice turbidity, cloud stability was also influenced by cultivar, harvest maturity and postharvest storage conditions. Generally, the presented results indicated that the factors that make the fruit riper, such as late harvest dates, RA storage and lack of 1-MCP treatment yielded considerably higher cloud stability, which is more prominent in 'McIntosh' juices. As mentioned earlier, cloud stability before and after centrifugation at 4200 g, 15 min, should be equal or greater than 50%. This criterion was met only for

'McIntosh' juices, which suggests the unsuitability of this 'Honeycrisp' for the production of stable cloudy juice. Moreover, the cloud stability from 'Honeycrisp' apples did not differ by ripening stage and this should be ascribed to the lower turbidity values observed in 'Honeycrisp' juices.

Factors that affect the particle size, structure, particle density and the serum viscosity, could affect the cloud stability of apple juice (Beveridge, 2002; Markowski, 1998). Loss of cloud stability could be caused by adherence of cloud particles to form floc, or the change of particles to a much denser aggregate (coagulation) that results in settling of the dispersed particle and the development of sediment that is considered undesirable visual quality parameter for cloudy juice products (Genovese and Lozano, 2000). Particle size greater than 0.5-0.65 μm is unstable and settles out (Beveridge, 2002). It has been found that cloud particles from stored fruit are much more open and less compact or less structured, suggesting the disintegration of the cell wall structure during maturation (Markowski, 1998). This structural change could reduce the particle density and increase particle hydration, which results better particle stability (Markowski, 1998).

5.4.4.4 Juice colour

In addition to cloud stability, colour is another important quality attribute for the visual quality of cloudy apple juice (Gerard and Roberts, 2004). Cloudy apple juice is expected to have whitish yellow colour, which represents the natural colour of the fresh product (Nagel, 1992; Niu et al., 2010; Mihalev et al., 2004). The mean *L* values (43.8-47.9) of cloudy juice samples obtained in our study are in good agreement with other studies on cloudy apple juice from 'Golden Delicious' (41.1), (Özoğlu and Bayındırlı, 2002),

'Shampion' (50.03) (Kolniak-Ostek et al., 2013) and 'Fugi' (55.03) (Gui et al., 2006). Our results demonstrated the substantial inhibition of enzymatic browning in cloudy juices as compared to the fresh counter parts. This was confirmed by the considerably higher *L (lightness),* lower *a (redness)* values as well as the reduced *BI* in cloudy than fresh juice samples. The cloudy juice samples from both cultivars met the expected yellowish colour, which was confirmed by the higher *b (yellowness)* and *L* values as compared to the fresh juice samples. The visual observation confirmed this as well. The observed yellowish colour was similar to the findings described previously by Nagel (1992) and Niu et al. (2010). In our study, the inhibited enzymatic browning in cloudy juice samples was achieved by the addition of ascorbic acid during processing. Moreover, cloudy juice samples were pasteurized at 80 oC for 5 min there by PPO activity could be inhibited. Ascorbic acid inhibits enzymatic browning reaction by reducing o-quinones back to the original phenolic compounds thereby prevents the formation of brown pigments (Özoğlu and Bayındırlı, 2002).

Total colour change is very important for the juice industry as it expresses the human eye's ability to discriminate the colour difference between two samples, in our case cloudy and fresh juice samples. It is generally accepted that human eye could only differentiate the colours of two juices through glass containers if delta $\Delta E \geq 5$ (Pérez-Magariño and González-Sanjosé, 2003). In this study, ΔE values for all juice samples were higher than five that confirms the visible colour difference between cloudy and fresh juice samples.

Even though previous studies on cut fruits reported that application of 1-MCP inhibited enzymatic browning in 'Empire' (Rupasinghe et al., 2005), 'Braeburn', and 'Pacific Rose' apples (Vilas-Boas and Kader, 2007) our results indicated no effect of 1-MCP on the measured colour properties of fresh or cloudy apple juice. The observed effect of 1-MCP treatment and storage atmosphere demonstrates that the colour change of cloudy apple juice was mainly affected by juice processing but not by fruit maturity and storage treatment. This is consistent with previous studies, which demonstrated the major role of processing for the colour change of cloudy apple juice products (Oszmiański et al., 2011; Mihalev et al., 2004).

5.5 Conclusion

The results of the present study demonstrated that juice processing technology and postharvest 1-MCP treatment could have a major impact on the total phenolic and antioxidant capacity of juice samples prepared from both 'McIntosh' and 'Honeycrisp' apples. In both cultivars, cloudy juice had extremely higher TPC (2 to 4-fold) and TAA (3 to 5-fold) as compared with clear juice samples, irrespective of 1-MCP treatment, and storage atmosphere. Our results also revealed the favourable effect of 1-MCP treatment in improving the TPC and TAA of cloudy or clear juice samples prepared from 'McIntosh' and 'Honeycrisp' apples. Regardless of juice type and storage atmosphere, juices from 1-MCP treated 'McIntosh' fruit had considerably higher TPC and TAA as compared to those from control fruit. Similarly, 1-MCP treatment in 'Honeycrisp' apples yielded higher TPC and TAA especially when combined with CA storage. Thus, our results suggests that to produce apple juice with improved content of health protecting compounds one needs to give due attention to the juice processing steps as well as to the source of the raw material. Our results confirm the conclusions of Oszmianski et al. (2007) and Markowski et al. (2015) who found considerably lower phenolic content and as a result, reduced potential health value in clear juice compared with cloudy juice.

In addition to the nutritional quality, turbidity, cloud stability and juice colour of cloudy apple juice were considered and found to be influenced by cultivar and fruit ripening. The turbidity (859-3436 NTU) and cloud stability (42-69%) values obtained from late harvested 'McIntosh' juices fulfilled the requirement for stable cloudy apple juice. Generally, factors such as late harvest dates, RA storage and lack of 1-MCP treatment that

lead to advanced fruit ripening yielded noticeably higher juice turbidity and cloud stability, which is more pronounced in ‘McIntosh’ juices. On the other hand ‘Honeycrisp’ apples were not found appropriate for the production of stable cloudy juice as the turbidity (<250 NTU) and cloud stability (<50%) values were much lower than the minimum requirement for stable cloudy juice production. Regarding colour, the cloudy juice prepared from both cultivars could be able to meet the expected yellowish colour. The colour difference among the juice samples was mainly affected by juice processing but not by 1-MCP treatment or storage atmosphere. In summary, our results suggests that by incorporating ascorbic acid (0.5 g/L), and selecting well ripe fruits as well as soft apple cultivars like ‘McIntosh’ one could produce cloudy juice with acceptable colour, turbidity and cloud stability.

5.6 Tables

Table 5.6-1. Correlations among FRAP, FC and DPPH in juices from 'McIntosh' and 'Honeycrisp' apples.

Cultivars	**Correlation values**		
		FRAP	FC
'McIntosh'	FC	0.968***	
	DPPH	0.531**	0.497**
'Honeycrisp'	FC	0.904***	
	DPPH	0.736***	0.674***

** Significant at $p<0.01$.
*** Significant at $p<0.001$

5.7 Figure captions

Figure 5.8-1. FRAP, FC and DPPH values of cloudy and clear juice prepared from 'McIntosh' and 'Honeycrisp' apples stored for 4-months under CA or RA condition after treated or untreated with 1-MCP (1 µL/L). Capital and small letters for FC, under 'McIntosh' indicate the main effect of 1-MCP and juice type, respectively. Capital and small letters for FC under 'Honeycrisp' indicate the main effect of juice type and interaction effect of 1-MCP and storage atmosphere (Atmos.), respectively. Capital and small letters for FRAP, under 'Honeycrisp' indicate the main effect of juice type and 1-MCP treatment, respectively. Means followed by the same letter are not significantly different at $p<0.05$. Multiple mean separation was assessed using Tukey's multiple range test ($\alpha = 0.05$). The error bars designate mean ± Standard deviation (n = 3).

Figure 5.8-2. Juice yield, turbidity and cloud stability of cloudy apple juice from 'McIntosh' and 'Honeycrisp' apples harvested at commercial (comm.) and late harvests stages and stored for 4- months under CA or RA condition after treated or untreated with 1-MCP (1 µL/L). Capital and small letters for juice yield of 'McIntosh' indicate the main effect of harvest maturity and storage atmosphere (atmos.) on juice yield, respectively. Capital and small letters for juice turbidity of 'McIntosh' indicate the interaction effect of harvest with atmos. and harvest with 1-MCP, respectively. Means followed by the same letter are not significantly different at $p<0.05$. Multiple mean separation was assessed using Tukey's multiple range test ($\alpha = 0.05$). The error bars designate mean ± Standard deviation (n = 3).

Figure 5.8-3. Hunter Lab colour properties of cloudy and fresh apple juices extracted from 'McIntosh' apples harvested at commercial (comm.) and late harvests stages, stored for 4 months under CA or RA condition after treated or untreated with 1-MCP (1 µL/L). *L, a, b,* and *BI* values represent lightness or darkness (*L*), redness or greenness (+*a*/-*a*), yellowness or blueness (+*b*/-*b*) and browning index. Means followed by the same letter are not significantly different at $p<0.05$. Multiple mean separation was assessed using Tukey's multiple range test ($\alpha = 0.05$). The error bars designate mean ± Standard deviation (n = 3).

Figure 5.8-4. Hunter Lab colour properties of cloudy and fresh apple juices extracted from 'Honeycrisp' apples harvested at commercial (comm.) and late harvests stages and stored for 4 months under CA or RA condition after treated or untreated with 1-MCP (1 µL/L). *L, a, b,* and *BI* values represent lightness or darkness (L), redness or greenness (+a/-a), yellowness or blueness (+b/-b) and browning index. Capital and small letters for '*a'* colour values indicate the main effect of juice type and harvest maturity, respectively. Means followed by the same letter are not significantly different at $p<0.05$. Multiple mean separation was assessed using Tukey's multiple range test ($\alpha = 0.05$). The error bars designate mean ± Standard deviation (n = 3).

Figure 5.8-5. Fresh (A) and cloudy (B) juices prepared from 'McIntosh' apples. Cloudy juice was prepared with ascorbic acid (0.5 g/L) and pasteurized at 80 oC for 5 min. Fresh or raw juice was prepared by simply pressing and filtering with four-layer cheesecloth without ascorbic acid addition and pasteurization.

5.8 Figures

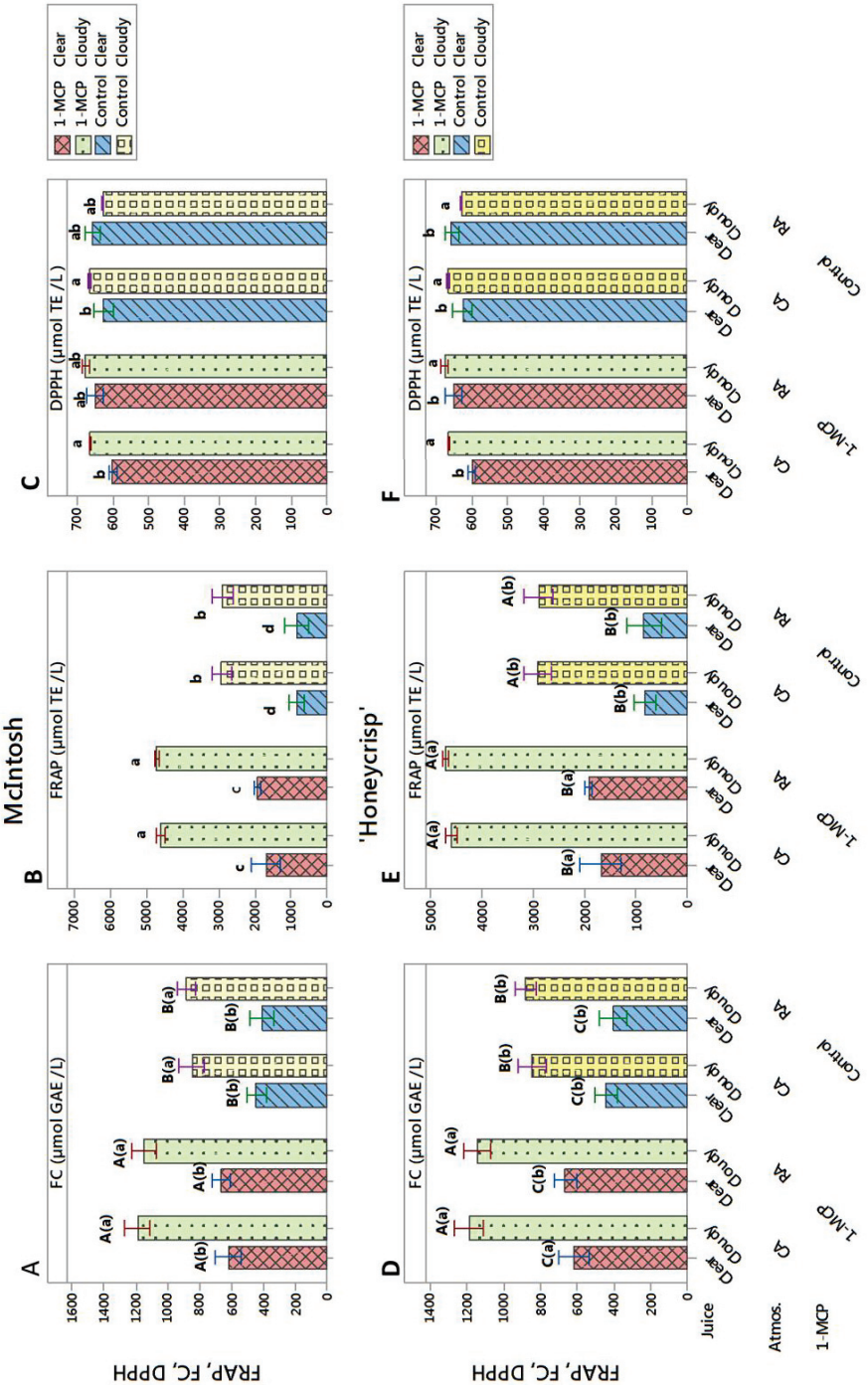
McIntosh
'Honeycrisp'
A
B
C
D
E
F
FC (µmol GAE /L)
FRAP (µmol TE /L)
DPPH (µmol TE /L)
FRAP, FC, DPPH
1-MCP Clear
1-MCP Cloudy
Control Clear
Control Cloudy
Juice
Atmos.
1-MCP
CA
RA
Control
Clear
Cloudy

Figure 5.8-1

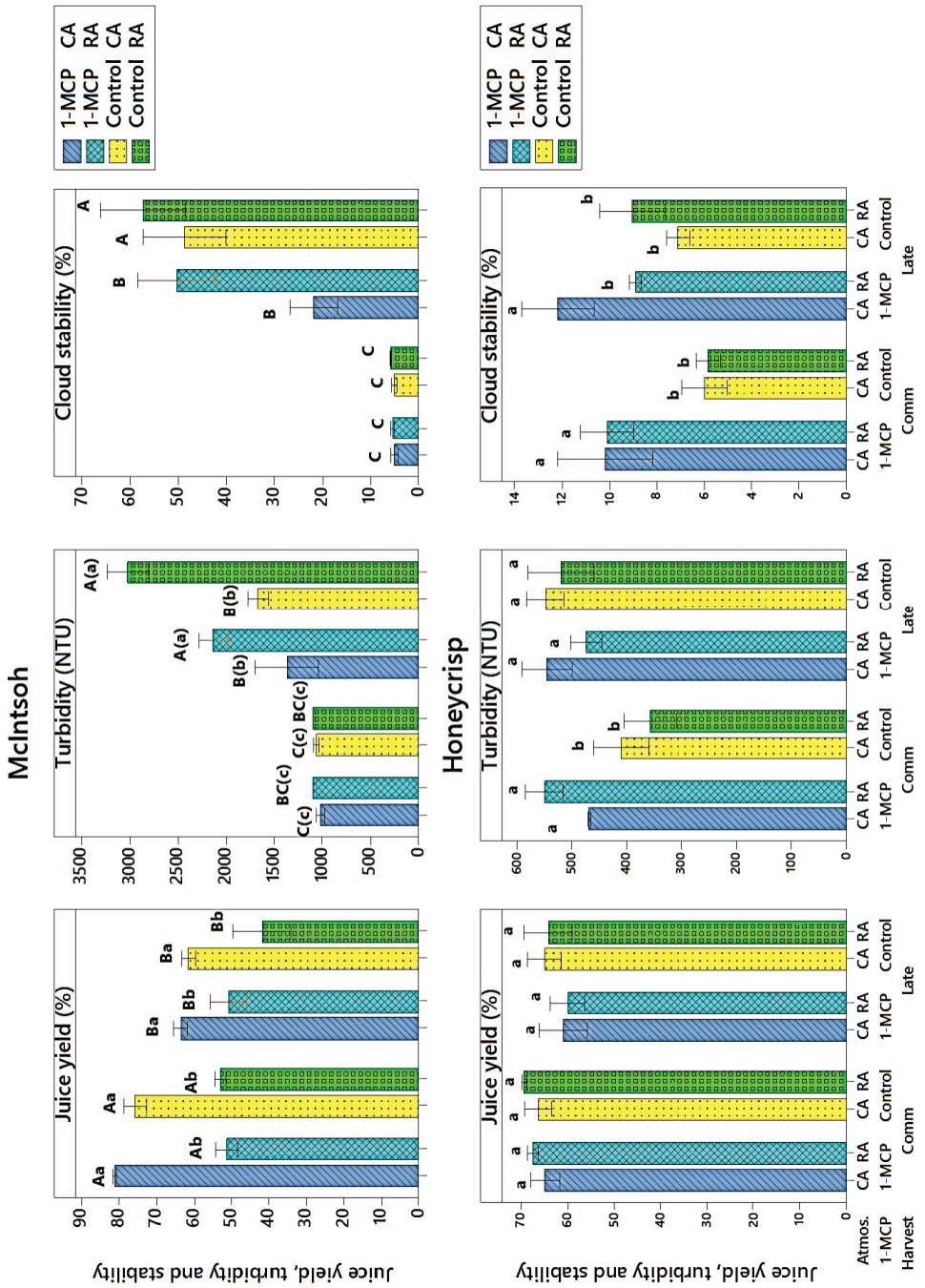
McIntsoh
Honeycrisp
Juice yield (%)
Turbidity (NTU)
Cloud stability (%)
Juice yield, turbidity and stability
1-MCP CA
1-MCP RA
Control CA
Control RA
Atmos.
1-MCP
Harvest
CA RA
1-MCP
Control
Comm
Late

Figure 5.8-2

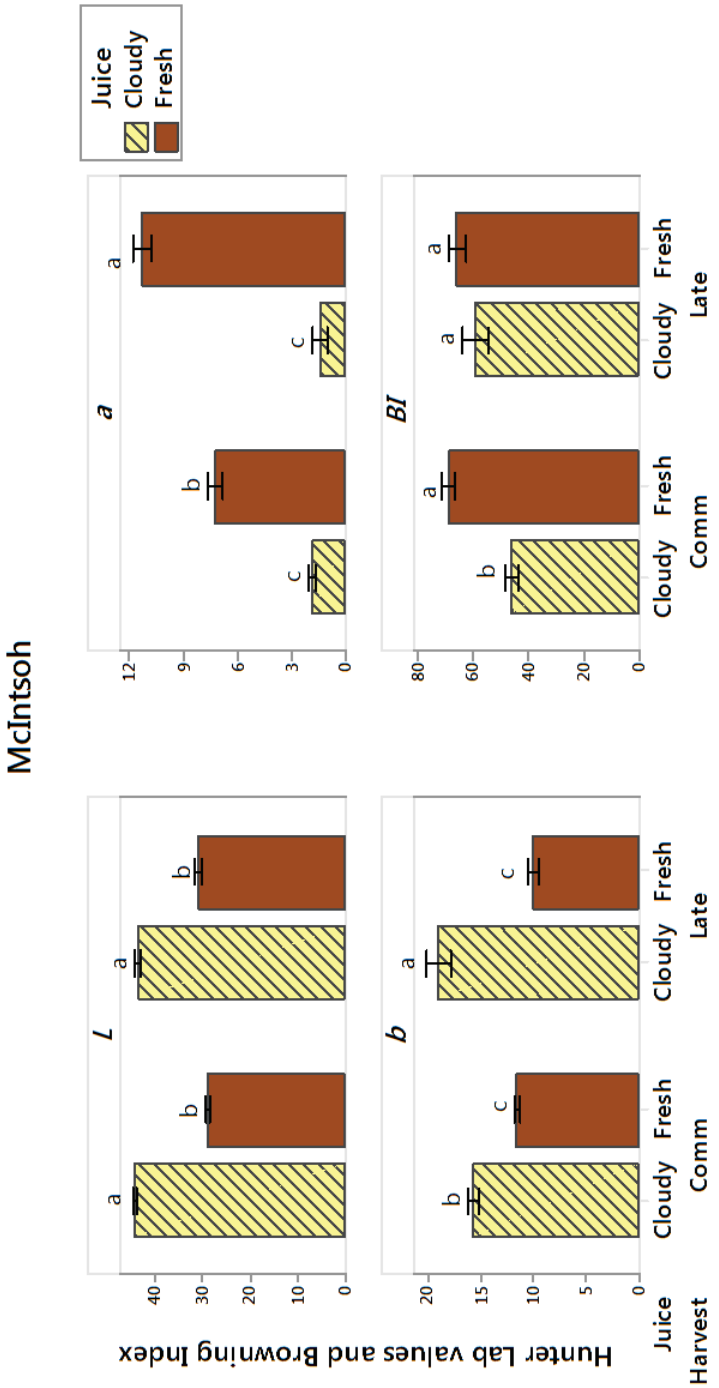
McIntsoh
Juice
Cloudy
Fresh
L
a
b
BI
Hunter Lab values and Browning Index
Juice
Harvest
Cloudy
Fresh
Comm
Late

Figure 5.8-3

Honeycrisp
Hunter Lab values and Browning Index
Juice
Cloudy
Fresh
L
a
b
BI
Juice
Harvest
Cloudy
Fresh
Comm
Late

Figure 5.8-3

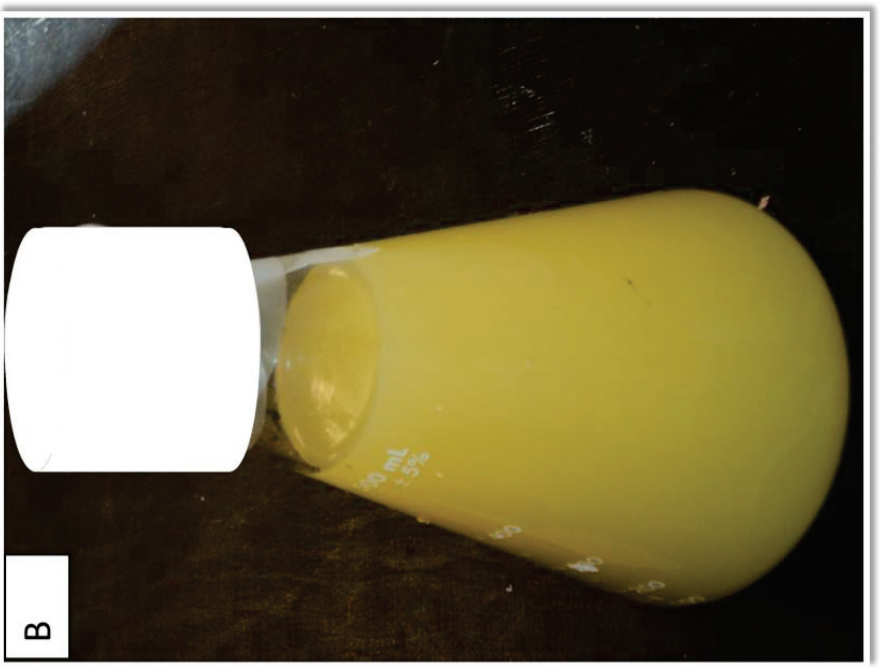

Figure 5.8-5

Chapter 6: General conclusion and recommendation

This study evaluated the combined effect of postharvest 1-MCP treatment, storage atmosphere, storage duration and harvest maturity on the flavour metabolites of cloudy and clear juices prepared from 'McIntosh' and 'Honeycrisp' apples. As sugars, organic acids, phenolics, and volatile aroma compounds are the major quality components influencing the flavour (taste, aroma and mouth feel) of apple juice (Baldwin, 2002) our study investigated the change of these flavour metabolites associated with harvest maturity and different postharvest treatments. The potential health benefits associated with the consumption of either whole apples or apple juices has been linked to the antioxidant phenolic compounds (Barth et al., 2005; Barth et al., 2007; Oszmianski et al., 2007; Matthes and Schmitz-Eiberger, 2008). Hence, in addition to the organoleptic quality parameters, our study evaluated the influence of 1-MCP and storage atmosphere on total phenolic content and total antioxidant capacity of cloudy and clear juices. The study also provided a new insight on how 1-MCP treatment and storage conditions regulate the genes encoding malic acid accumulation and degradation during storage.

In our study, the effectiveness of 1-MCP treatment was confirmed by suppressed ethylene production, which in turn was accompanied by inhibited volatile aroma development and improved flesh firmness retention of intact fruits. 1-MCP treatment in 'McIntosh' fruit effectively suppressed ethylene production, which is most pronounced in CA (80-98%) than RA (60%) storage conditions. However, this suppression effect did not last up to 7 months of storage. Thus, our results indicated the short-lived effect of 1-MCP

treatment in ‘McIntosh’ apples. Hence, a second 1-MCP application after 4 months of storage is recommended as suggested by other recent studies (DeEll et al., 2016).

In ‘Honeycrisp’ apples, suppressed ethylene production was achieved in CA stored samples whether treated (1.08 to 2.97 $\mu Lkg^{-1} h^{-1}$) or untreated (5 to 11 $\mu Lkg^{-1}h^{-1}$) with 1-MCP and the inhibition lasts up to 7 months of storage. However, under RA storage, both 1-MCP treated and untreated ‘Honeycrisp’ fruit had elevated ethylene production level, even higher than untreated samples. Thus, our study confirms the unique ethylene production trend of ‘Honeycrisp’ apples as suggested by previous studies (Wargo and Watkins, 2004; Watkins et al., 2004; DeEll and Ehsani-Moghaddam, 2010). As 1-MCP is thought to work by irreversibly binding to the ethylene receptors, further research is recommended on the regulation of ethylene perception related genes or enzymes during postharvest ripening of these cultivars.

In both cultivars, suppressed ethylene production was accompanied by higher juice acidity as evidenced from higher TA, malic acid content and lower pH value. The positive association between suppressed ethylene production and higher acidity retention suggests the ethylene dependency of malic acid metabolism. To explain this at molecular level, the expression of the genes regulating the metabolism of malic acid were investigated. According to our results, the higher acidity retention in ‘McIntosh’ apples was attributed to the downregulation of the genes regulating malic acid degradation (MdcyME). Likewise, higher acidity retention in ‘Honeycrisp’ was associated with the upregulated V-ATPase genes, which regulate the vacuolar transport and accumulation of malic acid. Thus, our study offers a new insight into the important role of the genes regulating malate degradation

and vacuolar transport in determining the content of malic acid during the long-term storage of apples.

Significant differences in the level of juice acidity was mainly caused by storage duration, postharvest 1-MCP treatment and storage atmosphere. Our results indicated that, in order to prepare apple juice with higher acidity (TA 0.6-0.7 mg/100 mL, pH 2.9-3.2, malic acid 0.58-0.87 g/100 mL) one has to use freshly harvested apples instead of stored fruits. The results also indicated that in seasons when freshly harvested apples are not available, it is still possible to produce apple juice with substantially higher acidity (6-21% TA, 25-30% malic acid) by using 1-MCP treated apples as compared with untreated fruits. As tartness and sourness sensations of apples have been associated with, higher malic acid and TA values (Da Conceicao Neta, Edith Ramos et al., 2007) juice from freshly harvested apples, or those from 1-MCP treated apples, will have more acidic or sour taste perception than those from untreated fruit. As lower pH and higher TA enhances microbial inactivation, juices with higher acidity values will need less heating process, which results reduced loss of heat sensitive nutrients and flavouring compounds (Beales, 2004). Moreover, juices with lower pH are appropriate for long-term storage or for blending with low acid beverages. Nonetheless, as frequent and excessive consumption of acidic beverages could pose dental erosion (Stefański and Postek-Stefańska, 2014) higher juice acidity (pH< 3.5) would not be a desirable quality parameter for those consumers who are concerned about dental erosion. Our results indicates that, it is possible to produce low-acid juices (pH, 3.7-4.2) by using stored apples (7-month old), especially those kept under RA storage condition without 1-MCP treatment. Thus, our study suggests that by

segregating apple lots based on the storage conditions and 1-MCP treatment, juice processors can produce wider range of products with diverse TA and pH ranges for different market segments or purposes.

Fructose, glucose and sucrose were the major monosaccharide and disaccharide sugars identified in all juice samples tested. In contrast to acidity, the changes of these sugars were not influenced by 1-MCP treatment, storage condition and the associated change in ethylene production. Instead, it was considerably influenced by storage time. During the long-term storage of 'Honeycrisp' or 'McIntosh' apples, there was an immense degradation of sucrose with a corresponding accumulation of glucose and fructose. Hence, in agreement with a previous study in 'Idared' apples (Bizjak et al., 2012) our data confirms the weak ethylene dependency of this these quality traits during long-term storage of apples. Due to growing health concerns and nutritionists' recommendation to decrease sucrose intake, many food companies are interested in reducing the sucrose content of juice products. Based on our results using stored apples (4-7 months old) it is possible to produce apple juice that has markedly lower (50-80%) level of sucrose. Hence, juice processing industries would use stored apples as an alternative to produce a product line with lower sucrose content. As stored apples had lower TA and malic acid content, in order to produce low-acid and low-sugar apple juice, it is suggested to use stored than freshly harvested apples. On the other hand, the increased fructose level in juices from stored apples will increase the sweetness perception of the product and thus its consumer acceptability should be evaluated.

As volatile aroma compounds play a crucial role in determining the flavour of apple juice, the change of these compounds related to 1-MCP treatment, storage atmosphere, harvest maturity and juice processing was evaluated. The results of this study demonstrated that the content and composition of volatile aroma compounds in juices from 'McIntosh' and 'Honeycrisp' apples was influenced not only by 1-MCP treatment but also by several interrelated factors including harvest maturity, storage atmosphere and juice processing techniques. In general, as compared to 'McIntosh, 'Honeycrisp' juices had considerably higher content of total esters (2-fold) and total volatile aroma compounds (6-fold). This suggests that 'Honeycrisp' apples are preferable to produce apple juice with intense fruity and sweet aroma. In 'McIntosh' juices, whether it is clear or cloudy, 1-MCP treatment alone, 1-MCP with CA or CA storage alone resulted in a remarkable reduction of esters, aldehydes, most alcohols and total volatile compounds. As with 'McIntosh' CA storage alone or with 1-MCP treatment caused a remarkable inhibition of most volatile compounds in 'Honeycrisp' juices. Thus to produce apple juice with an intense aroma profile it is generally recommended to use untreated apples stored under RA conditions.

The results found in 'McIntosh' are consistent with previous studies that found a substantial suppression of volatile aroma compounds in several apple cultivars that had been treated with 1-MCP and stored under CA or RA condition (Lurie et al., 2002; Kondo et al., 2005; Bai et al., 2005; DeEll et al., 2005; Rupasinghe et al., 2000). As 1-MCP treatment in 'McIntosh' lead to suppressed ethylene production, our study confirms the regulatory role of ethylene for the biosynthesis of volatile compounds as suggested by previous studies (Schaffer et al., 2007; Fan et al., 1998; Defilippi et al., 2005). As there is

no published information regarding the volatile profile of 'Honeycrisp' fruit or juice especially none focusing on 1-MCP treatment, it is challenging to account for the unusual response of this cultivar to 1-MCP treatment. To explain the actual mechanism of this unexpected phenomenon further research is warranted.

In addition to storage atmosphere and 1-MCP treatment, the content of volatile compounds was strongly influenced by juice processing techniques. Regardless of all other factors considered, cloudy juice samples had substantially higher amount of most esters, aldehydes and total volatile compounds as compared to the clear juice samples. The hydrolysis of esters via the action of esterase, which is present in most commercial clarifying enzymes (Schreier et al., 1978) could explain the reduction of esters in clear juice samples. The lower content of aldehydes in clear juices could be due to the conversion of aldehydes to alcohols by the action of ADH during the long incubation period needed for clear juice processing (Poll, 1988; Schreier et al., 1978). Hence, our study suggests that by using cloudy juice processing technique it is possible to prevent the loss and undesirable change aroma volatiles during juice processing.

The effects of 1-MCP, storage atmosphere and juice processing methods on the phytochemical and physiochemical juice quality parameters was also accessed. The results demonstrated the substantial impact of juice processing technology on the total phenolic content and consequently antioxidant capacity of juice samples prepared from both 'McIntosh' and 'Honeycrisp' apples. In both cultivars, cloudy juice processing yielded extremely higher phenolic content (2 to 4-fold) and antioxidant capacity (3 to 5-fold) as compared with clear juice samples, regardless of 1-MCP treatment and storage atmosphere.

These results confirm the conclusions of previous studies in other apple cultivars, which found considerably higher phenolic content and as a result, increased potential health value in cloudy juices compared with clear juice samples (Oszmianski et al., 2007; Markowski et al., 2015). The clarification and filtration steps in clear juice processing which removes most of the pulp and suspended particles could be the main reasons for the reduced level of phytochemical compounds in clear juice samples (van der Sluis et al., 2002). Another potential reason for the loss of phenolic compounds during clear juice processing is the enzymatic oxidation of phenolic compounds via the action of PPO in the presence of oxygen (Oszmianski and Lee, 1990). Thus, based on our results cloudy juice processing technique is recommended to prevent the loss of phenolic compounds and for the production of apple juice rich in phenolic content and better potential health value than the traditional clear juice processing technology. Our results also revealed the favourable effect of 1-MCP treatment in preserving the phenolic and antioxidant content of both 'McIntosh' and 'Honeycrisp' juices. Higher flesh firmness in 1-MCP treated fruits could result improved membrane integrity and de-compartmentalization of oxidative enzymes and substrates (phenolic compounds) thereby reducing the initiation of enzymatic browning reaction and the oxidative degradation of phenolic compounds (Jiang et al., 2001). Thus, our results suggests that to produce apple juice with improved content of health protecting compounds one needs to give due attention to the juice processing technology as well as to the source of the raw material.

Cloud stability, turbidity and colour are crucial for consumer acceptance and shelf life of cloudy juice (Beveridge, 2002). The turbidity (859-3436 NTU) and cloud stability

(42-69%) values obtained from ‘McIntosh’ juices fulfilled the requirement for stable cloudy apple juice. On the other hand ‘Honeycrisp’ apples were not found appropriate for the production of stable cloudy juice as the turbidity (<250 NTU) and cloud stability (<50%) values were much lower than the minim requirement for stable cloudy juice production. In ‘McIntosh’, higher juice turbidity and improved cloud stability could be achieved by using late harvested and RA stored fruits without 1-MCP treatment. Regarding colour, cloudy juices prepared with the addition of ascorbic acid from both cultivars could meet the expected yellowish colour. The colour difference among the juice samples was mainly affected by juice processing. Hence, the present study suggests that to produce cloudy juice with acceptable colour, turbidity and cloud stability it is necessary to incorporate ascorbic acid, to select well ripe fruits or to use soft apple cultivars such as ‘McIntosh’.

Based on the results of the current study further research is recommended on the following areas. Conducting sensory evaluation to determine the preferences and overall acceptability of cloudy juices for Canadian consumers; evaluating the influence of non-thermal processing technologies on the shelf life and other quality changes of cloudy apple juice; developing a pilot scale cloudy juice processing and introducing the product to the Canadian apple juice producers; investigating the health benefits of cloudy apple juice using cell culture, animal studies and clinical trials. Further study is also suggested to examine the potential health benefit and consumer acceptance of juices obtained from stored apples as these juice samples have higher fructose and very low sucrose content.

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Appendix captions

Appendix A-Figure 1. Normal probability and residual plots used to validate normality and constant variance of data for flesh firmness and ethylene production from (A) 'McIntosh' and (B) 'Honeycrisp' apples.

Appendix A-Figure 2 and 3. Normal probability and residual plots used to validate normality and constant variance of data for major volatile aroma compounds identified from whole fruit samples of 'McIntosh' apples

Appendix A-Figure 4 and Figure 5. Normal probability and residual plots used to validate normality and constant variance of data for major volatile aroma compounds identified from whole fruit samples of 'Honeycrisp' apples.

Appendix A- Figure 6. Normal probability and residual plots used to validate normality and constant variance of data for TSS, TA, pH and TSS: TA ratio of 'McIntosh' juices.

Appendix - Figure 7. Normal probability and residual plots used to validate normality and constant variance of data for fructose, glucose, sucrose and malic acid content of 'McIntosh' juices.

Appendix A- Figure 8. Normal probability and residual plots used to validate normality and constant variance of data for TSS, TA, pH and TSS: TA ratio of 'Honeycrisp' juices.

Appendix A-Figure 9. Normal probability and residual plots used to validate normality and constant variance of data for fructose, glucose, sucrose and malic acid content of 'Honeycrisp' juices.

Appendix A-Figure 10. Melting cures for target and referee genes.

Appendix B-Figure 1 and 2. Normal probability and residual plots used to validate normality and constant variance of data for major volatile aroma compounds identified from juice samples of 'McIntosh' apples

Appendix B-Figure 3 and 4. Normal probability and residual plots used to validate normality and constant variance of data for major volatile aroma compounds identified from juice samples of 'Honeycrisp' apples.

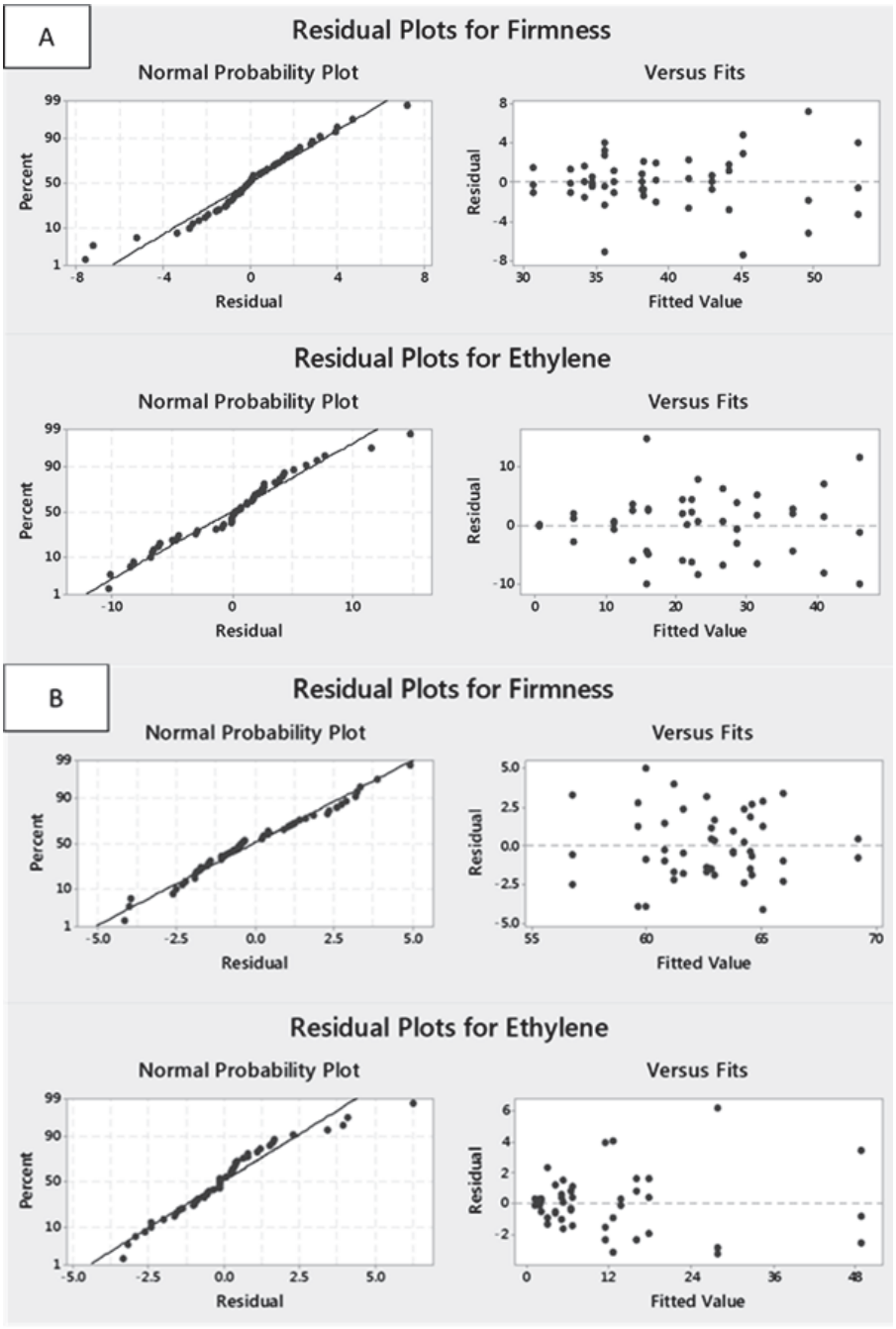
Appendix C-Figure 1. Standard curves for FRAP, FC and DPPH used for cloudy and clear juice total antioxidant and total phenolic assay.

Appendix C- Figure 2. Normal probability and residual plots used to validate normality and constant variance of data for FRAP, FC and DPPH from 'McIntosh' (A) and 'Honeycrisp' (B) juices.

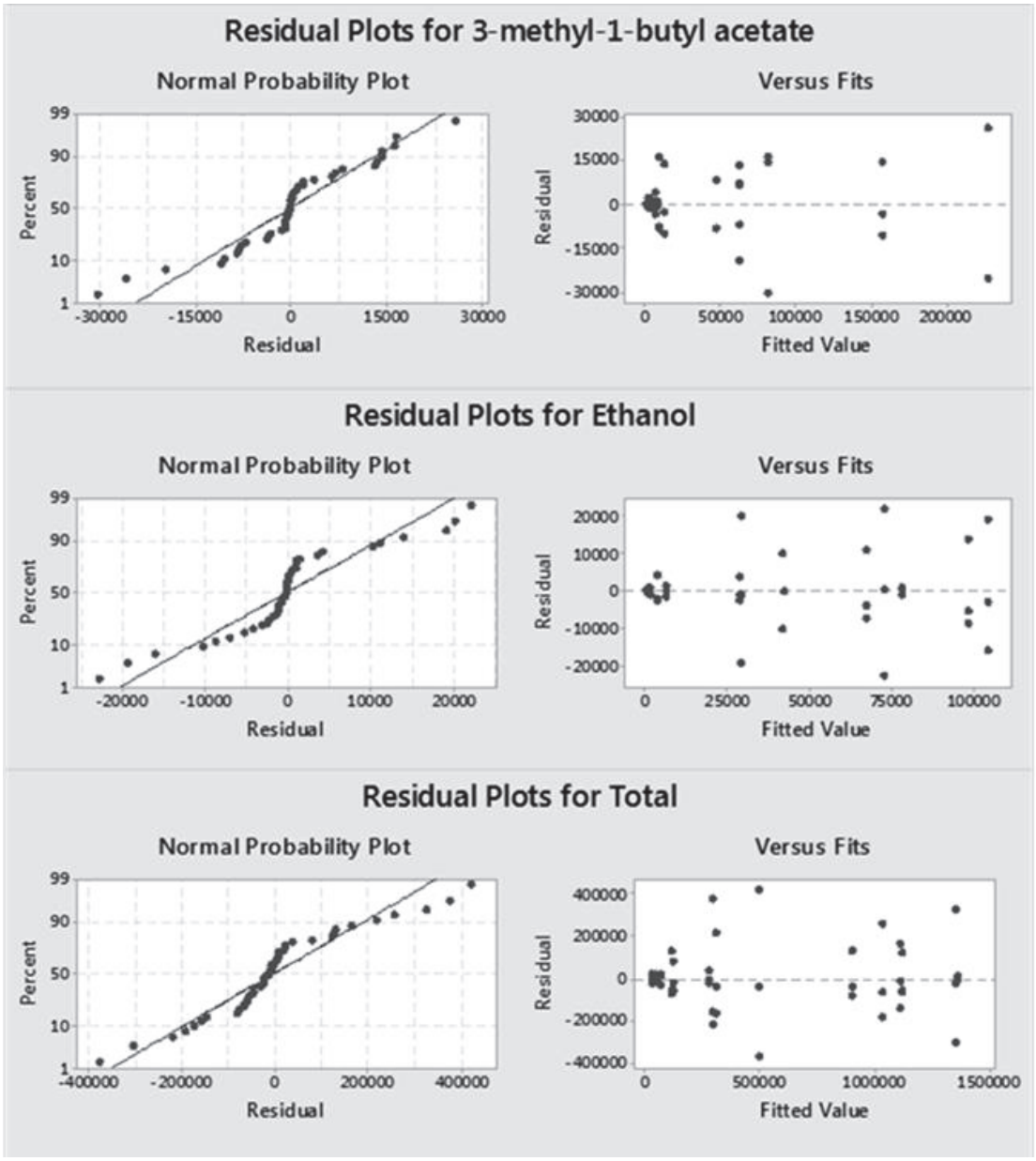
Appendix C-Figure 3. Normal probability and residual plots used to validate normality and constant variance of data for turbidity, juice yield and cloud stability of 'McIntosh' (A) and 'Honeycrisp' (B) juices.

Appendix C-Figure 4. Probability and residual plots used to validate normality and constant variance of data for Hunter Lab values and browning index of 'McIntosh' (A) and 'Honeycrisp' (B) juices.

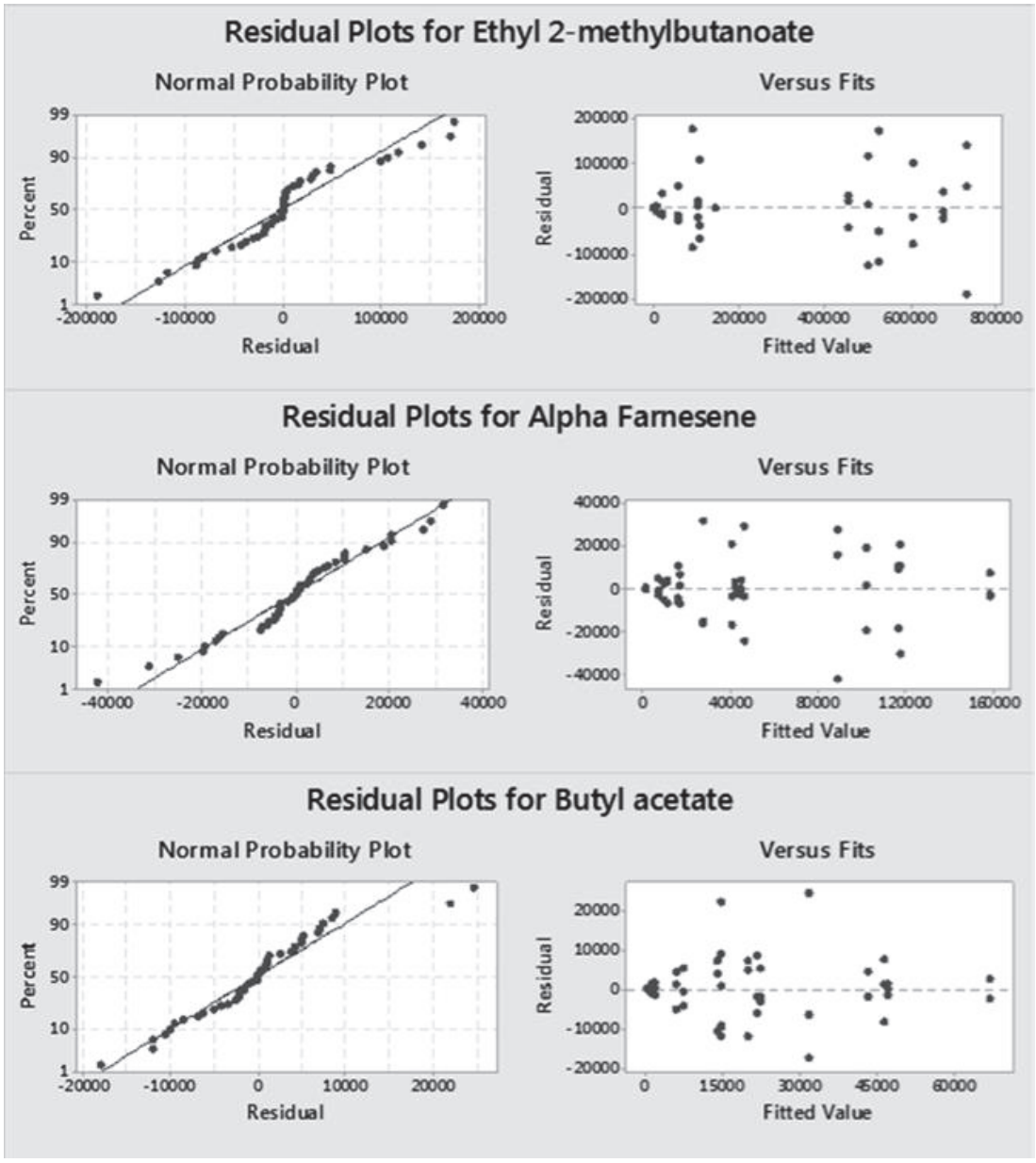
Appendix A



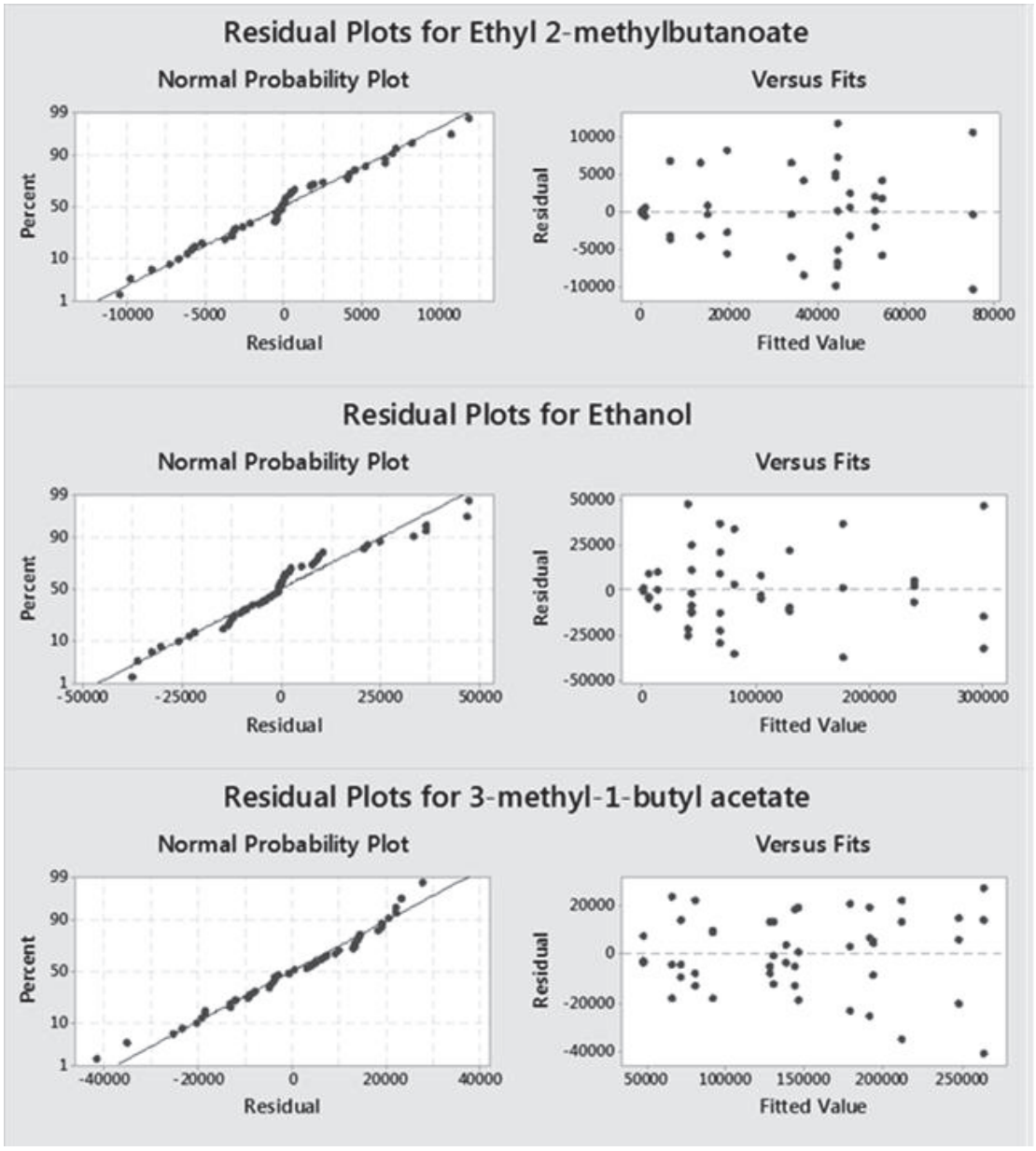
Appendix A Figure 1



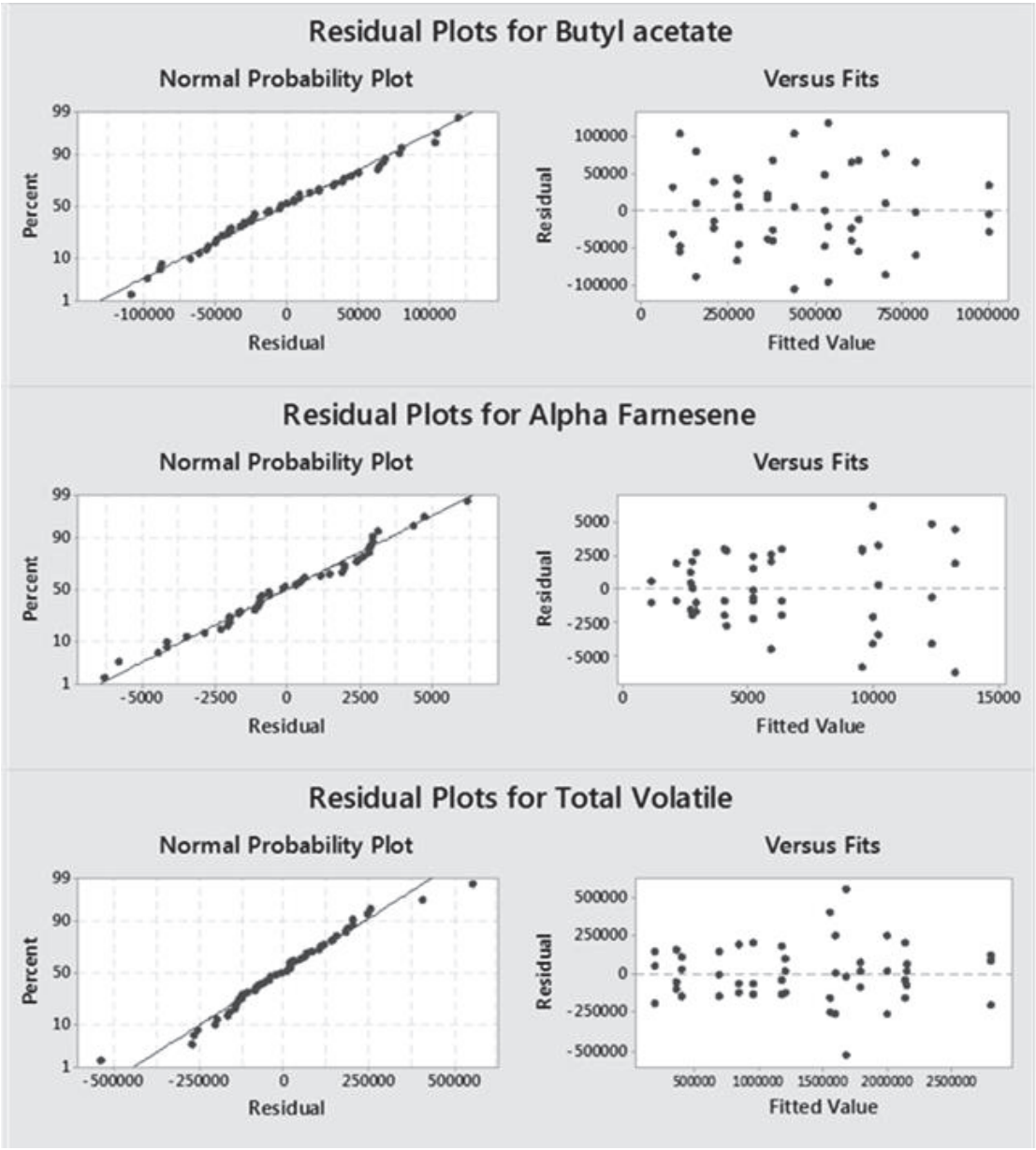
Appendix A Figure 2



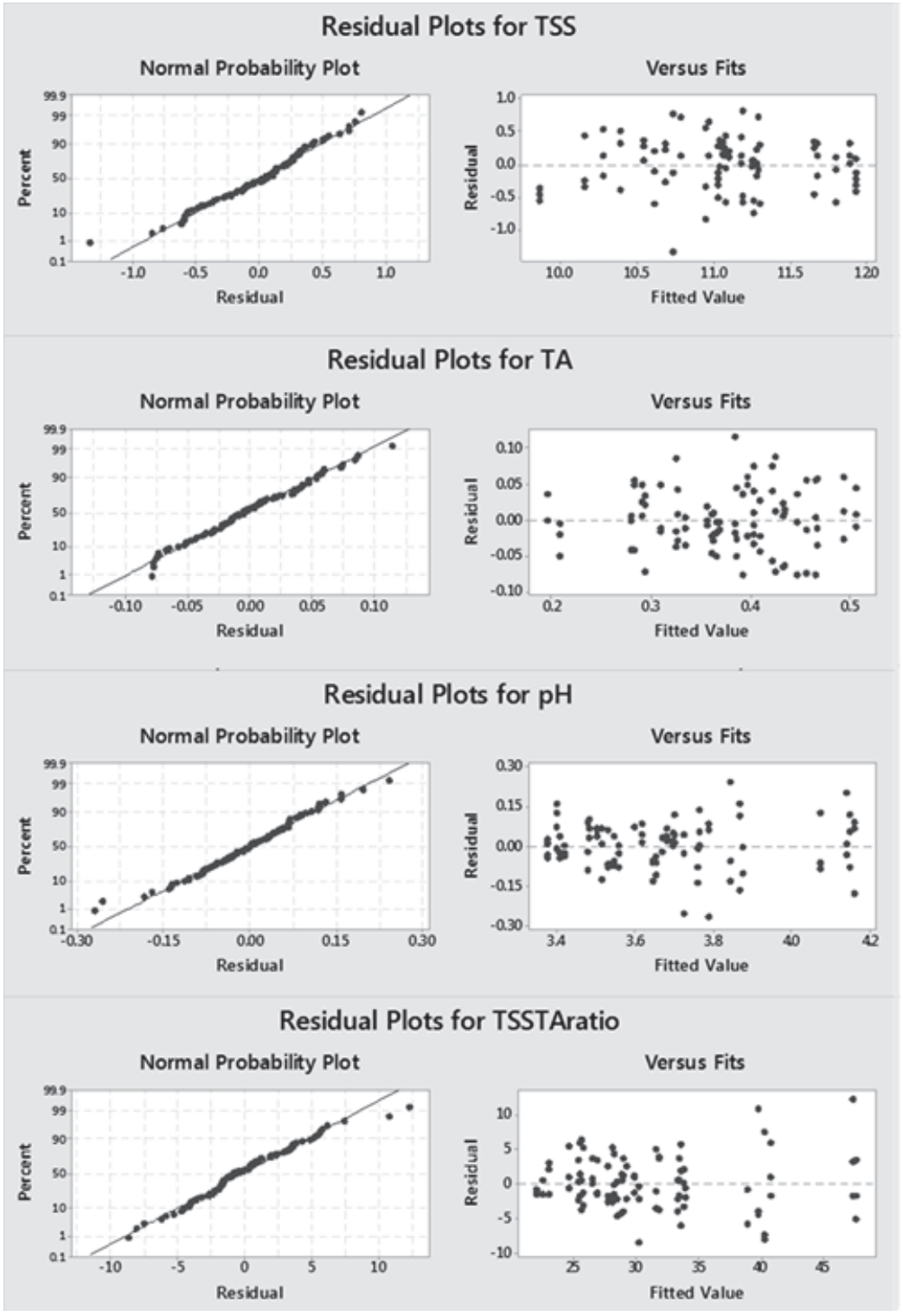
Appendix A Figure 3



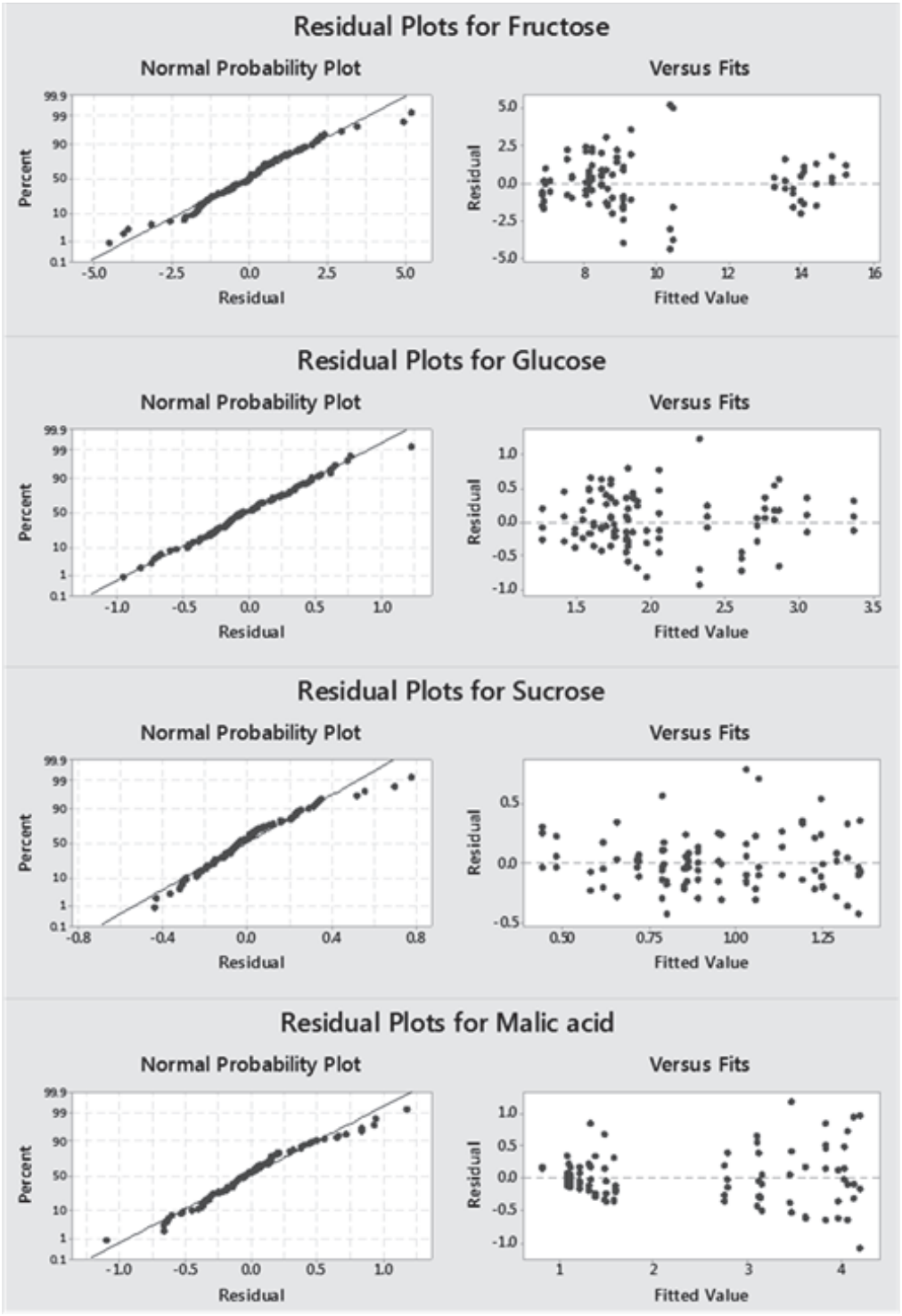
Appendix A Figure 4



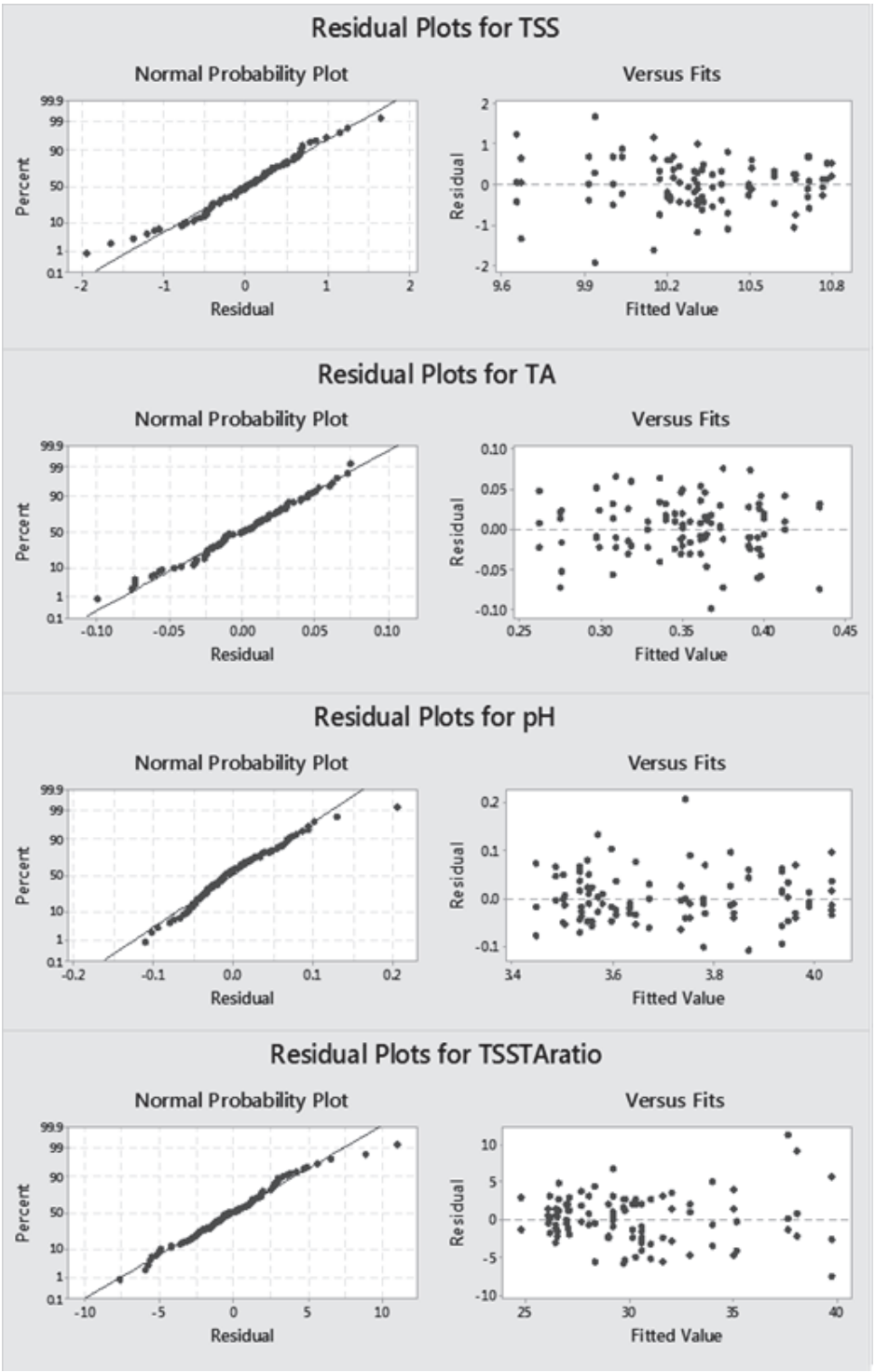
Appendix A Figure 5



Appedix I Figure 6



Appendix A Figure 7



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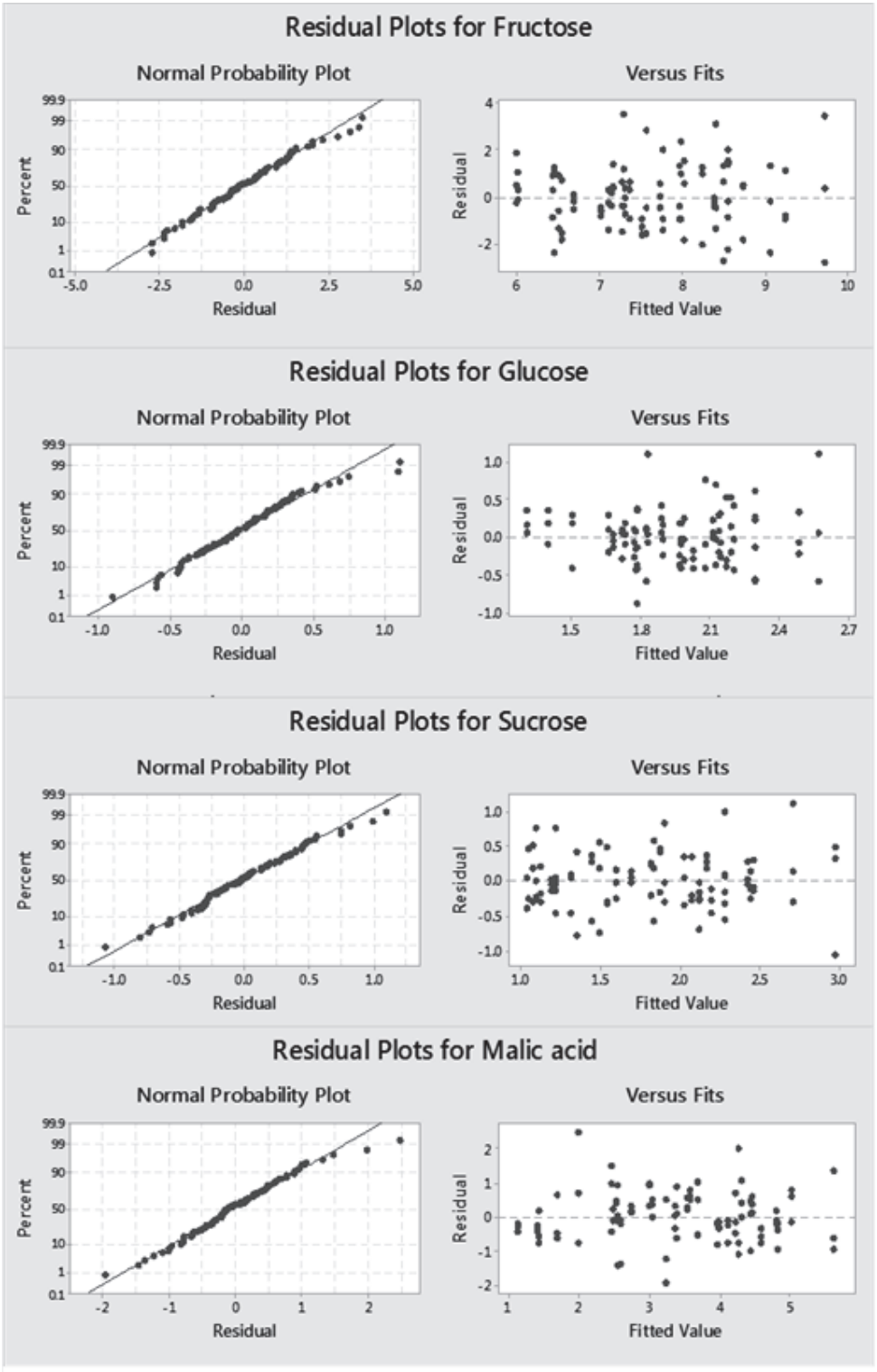

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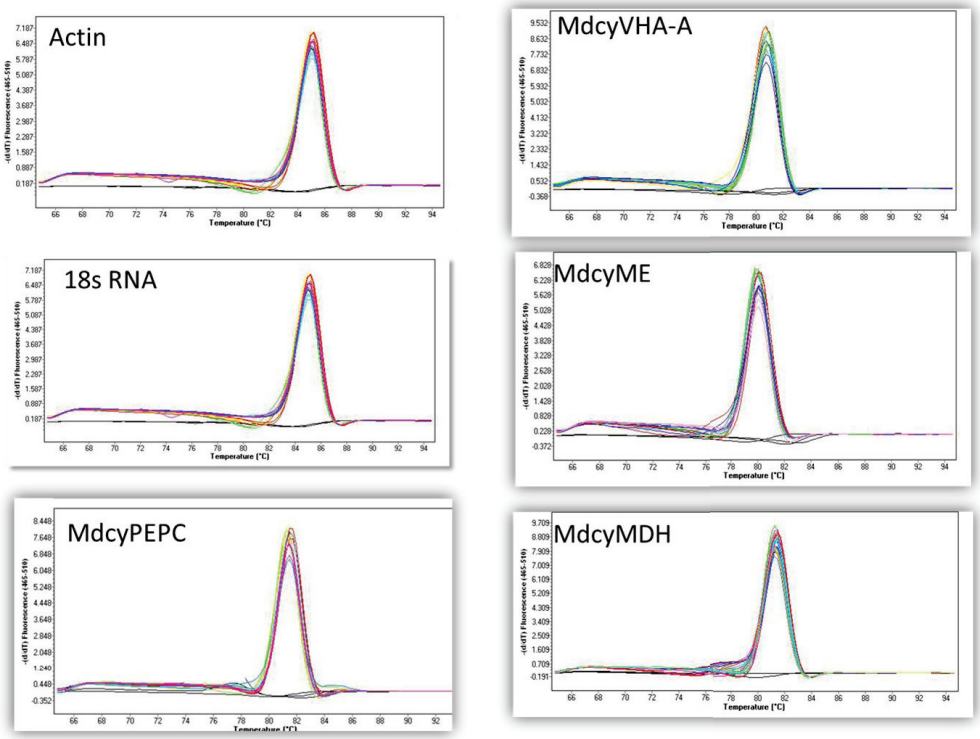
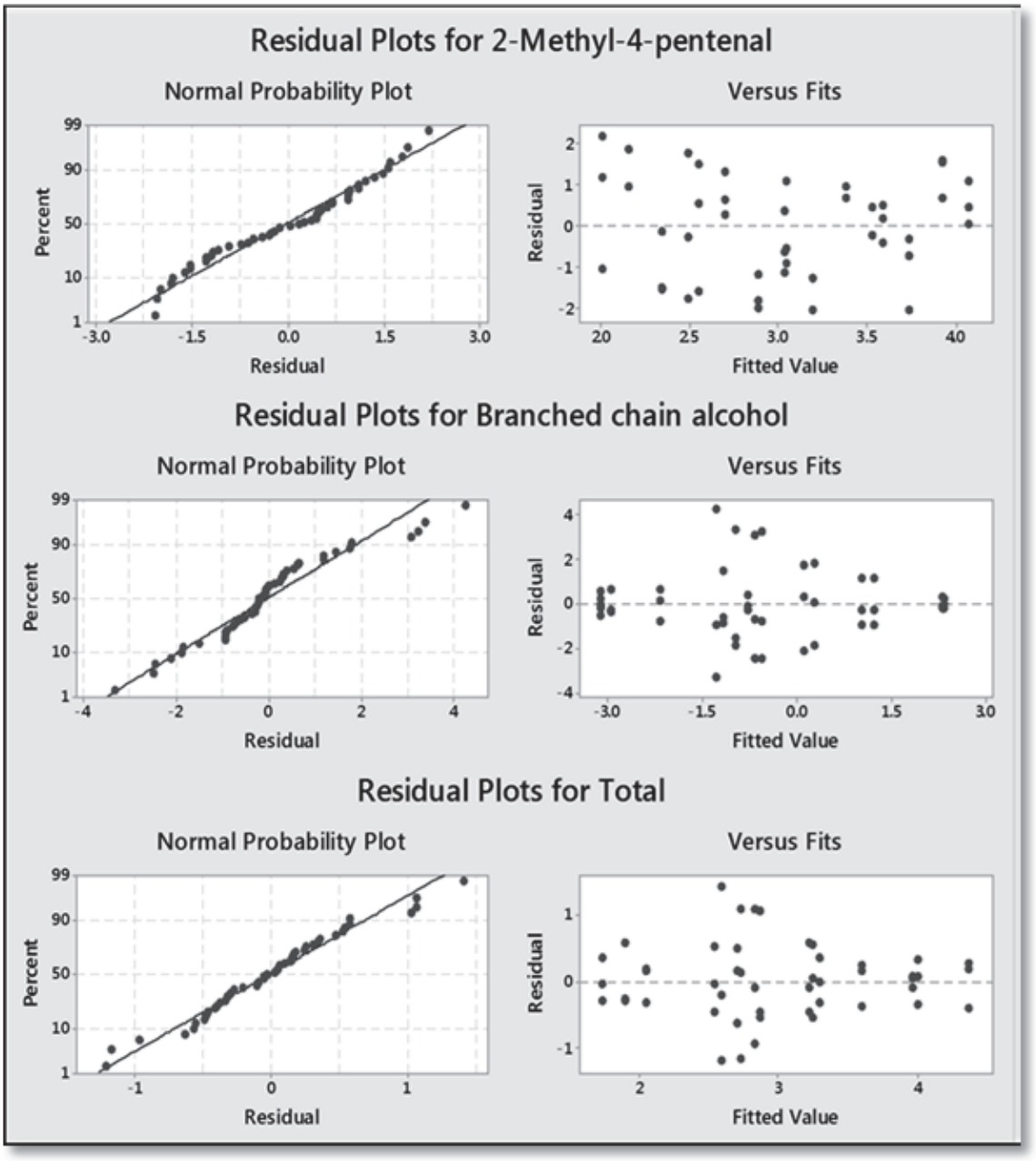
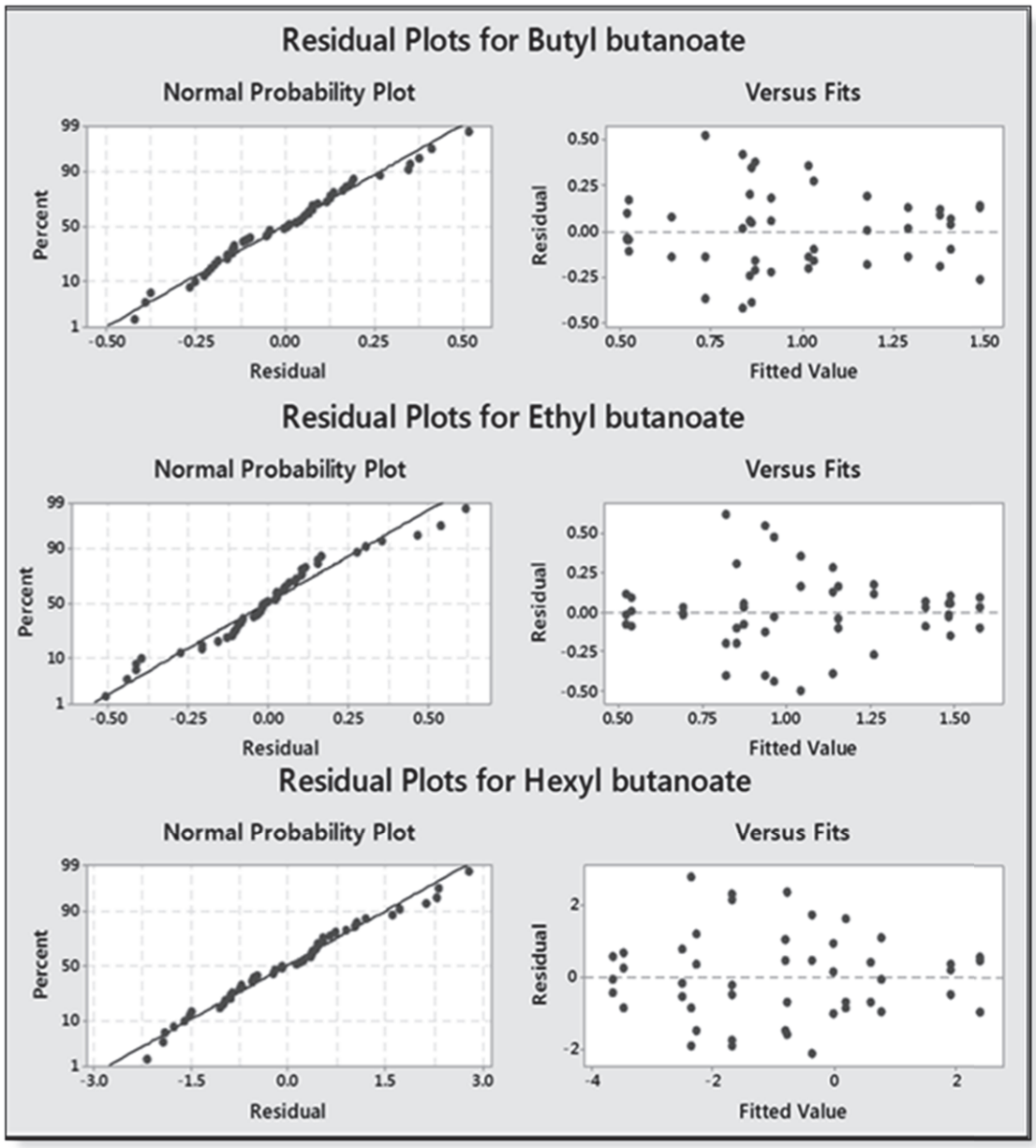


Appendix A-Figure 10

Appendix B



Appendix B Figure 1



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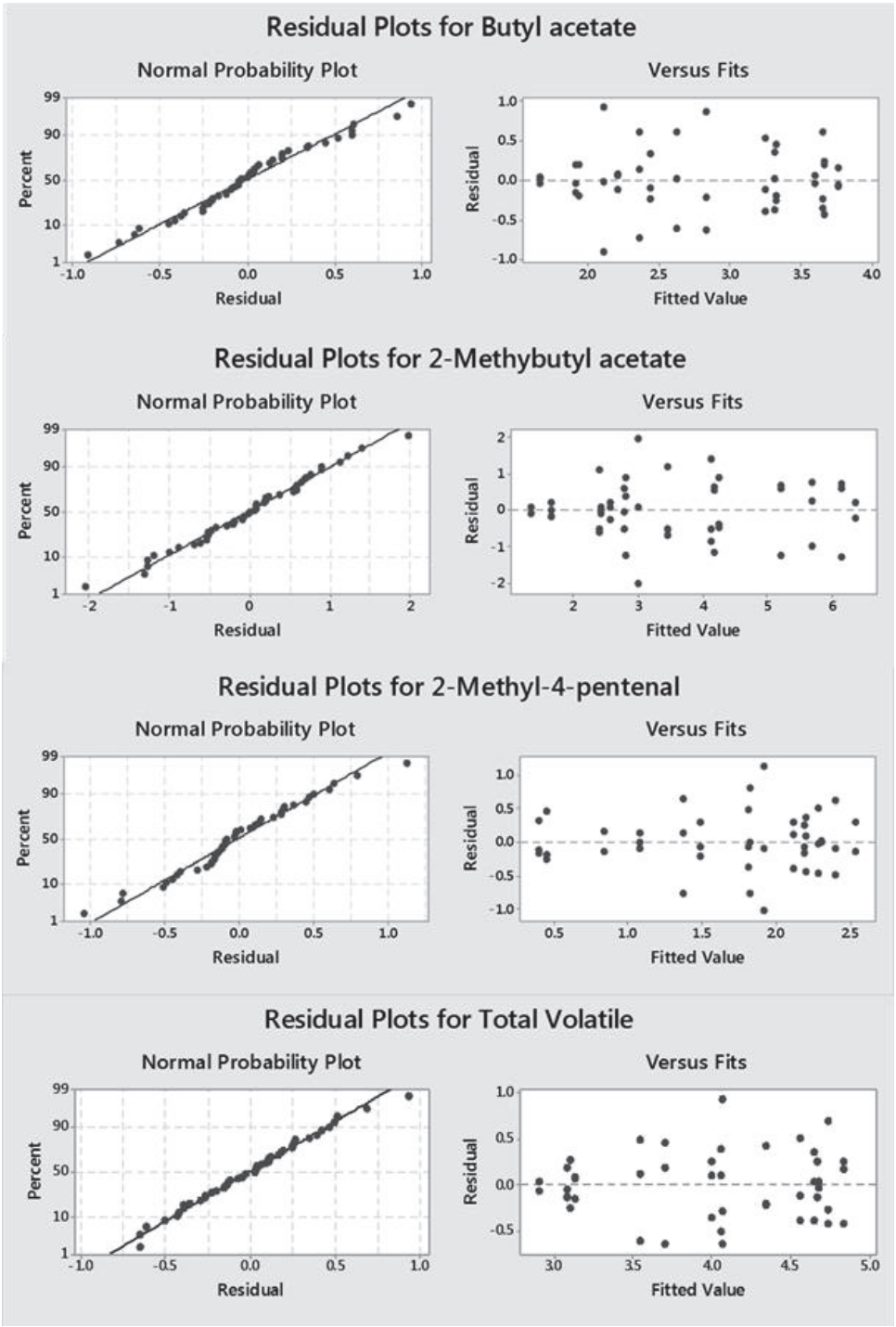

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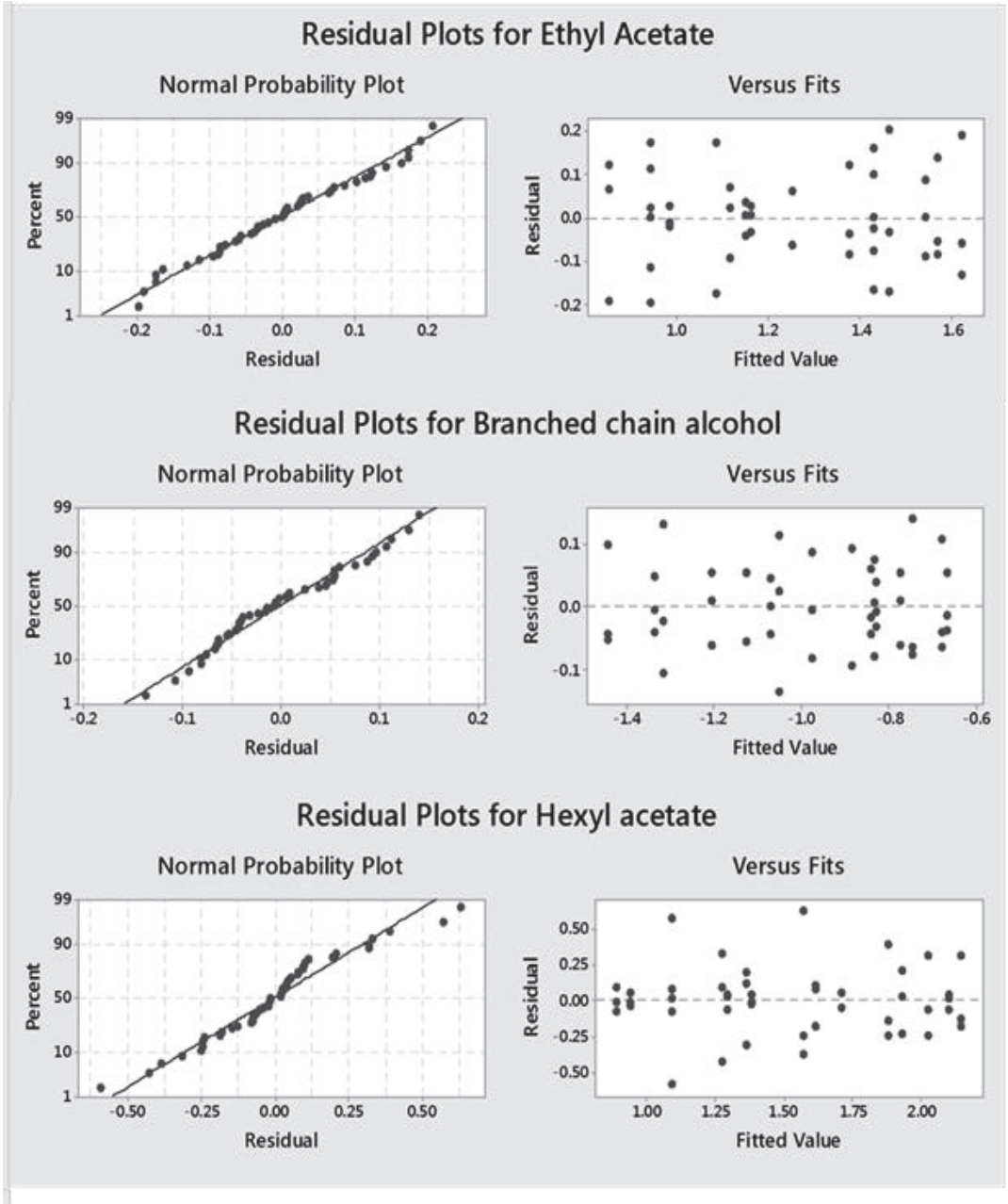
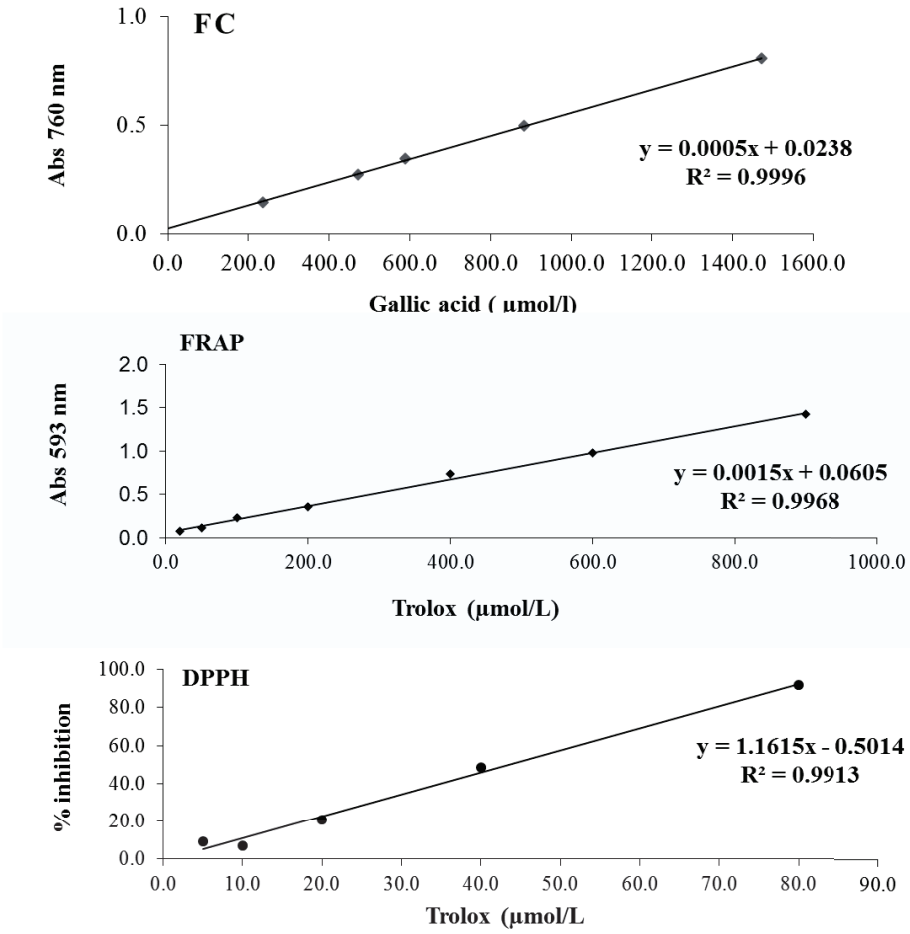
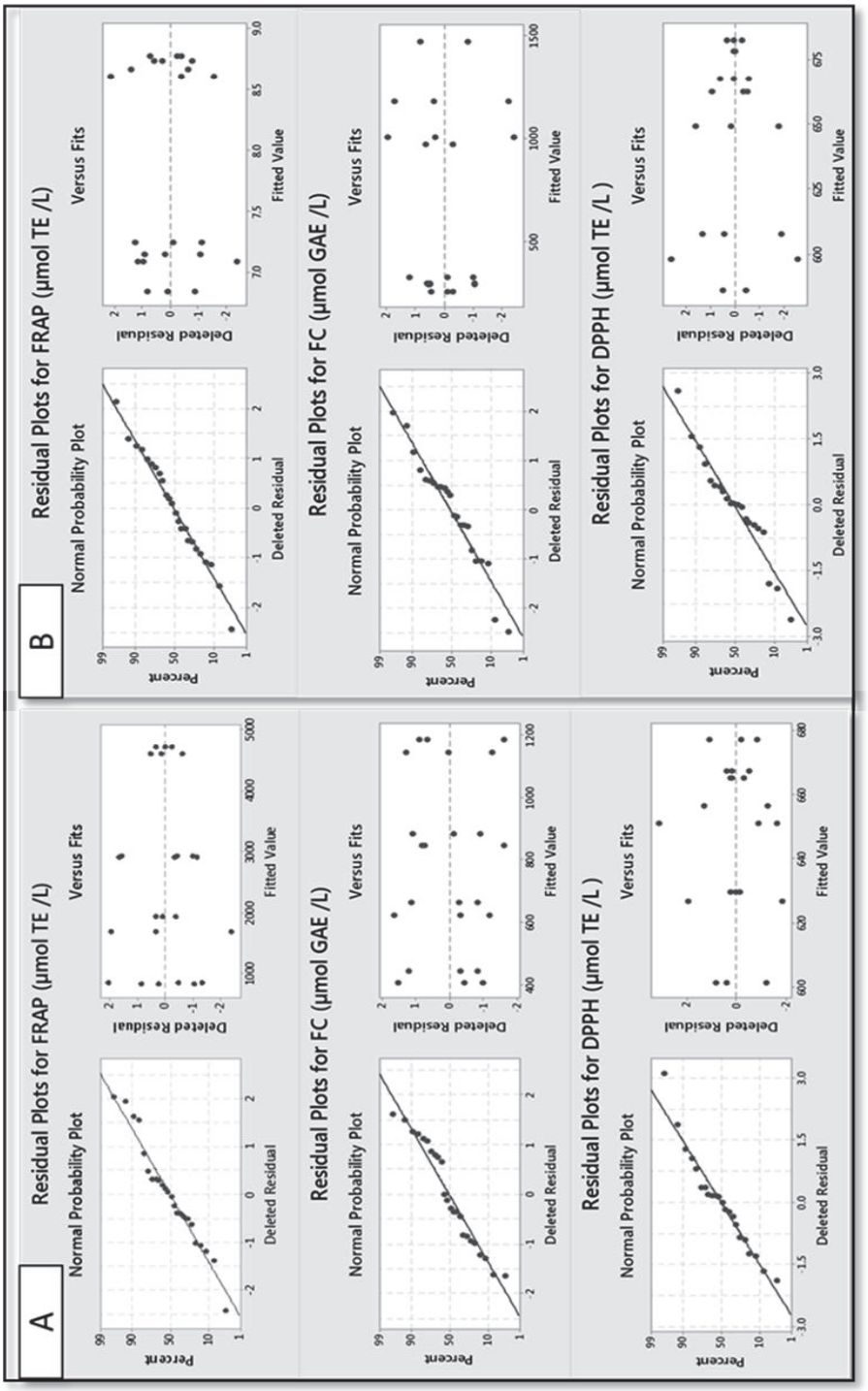


Appendix B Figure 4

Appendix C



Appendix C Figure 1



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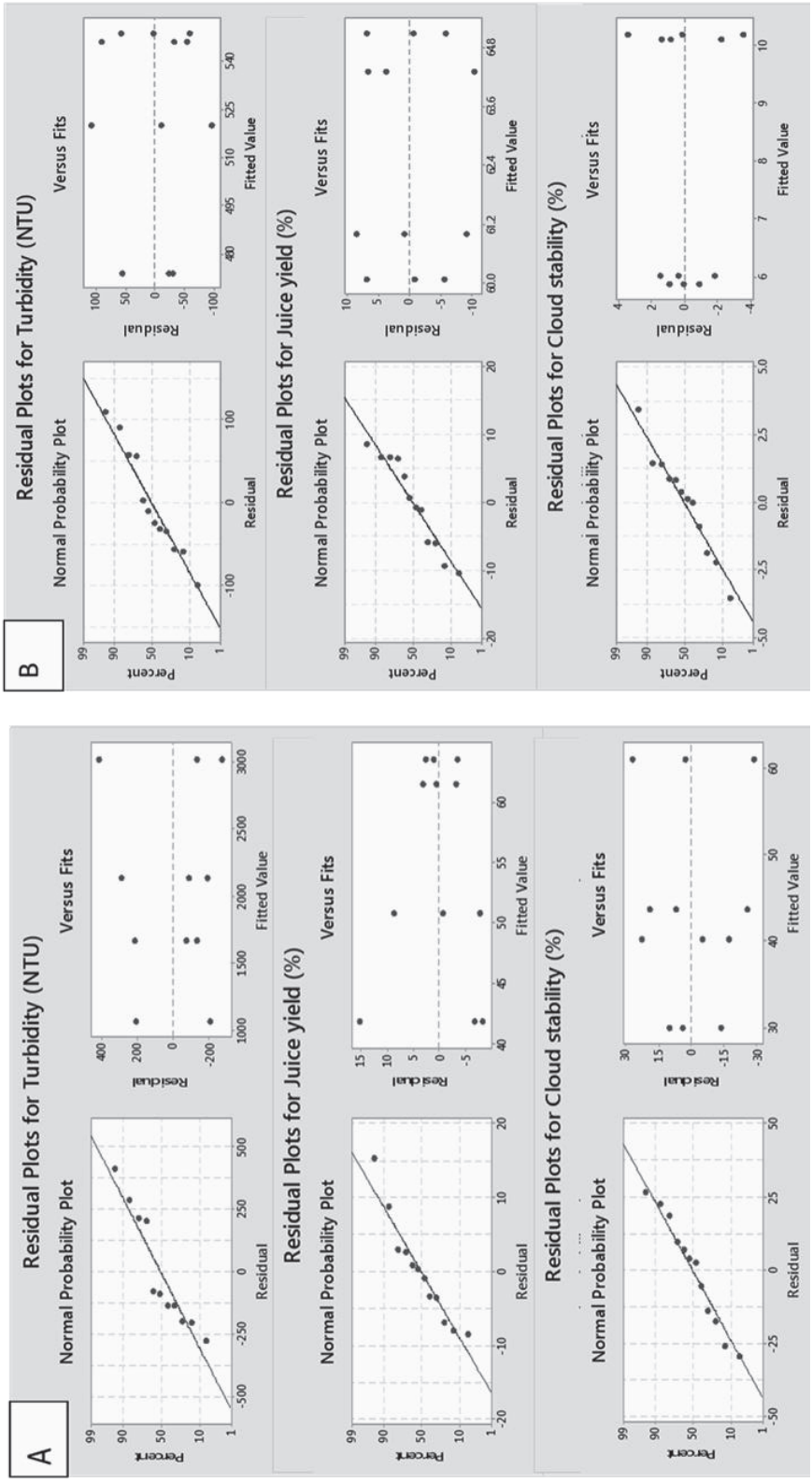

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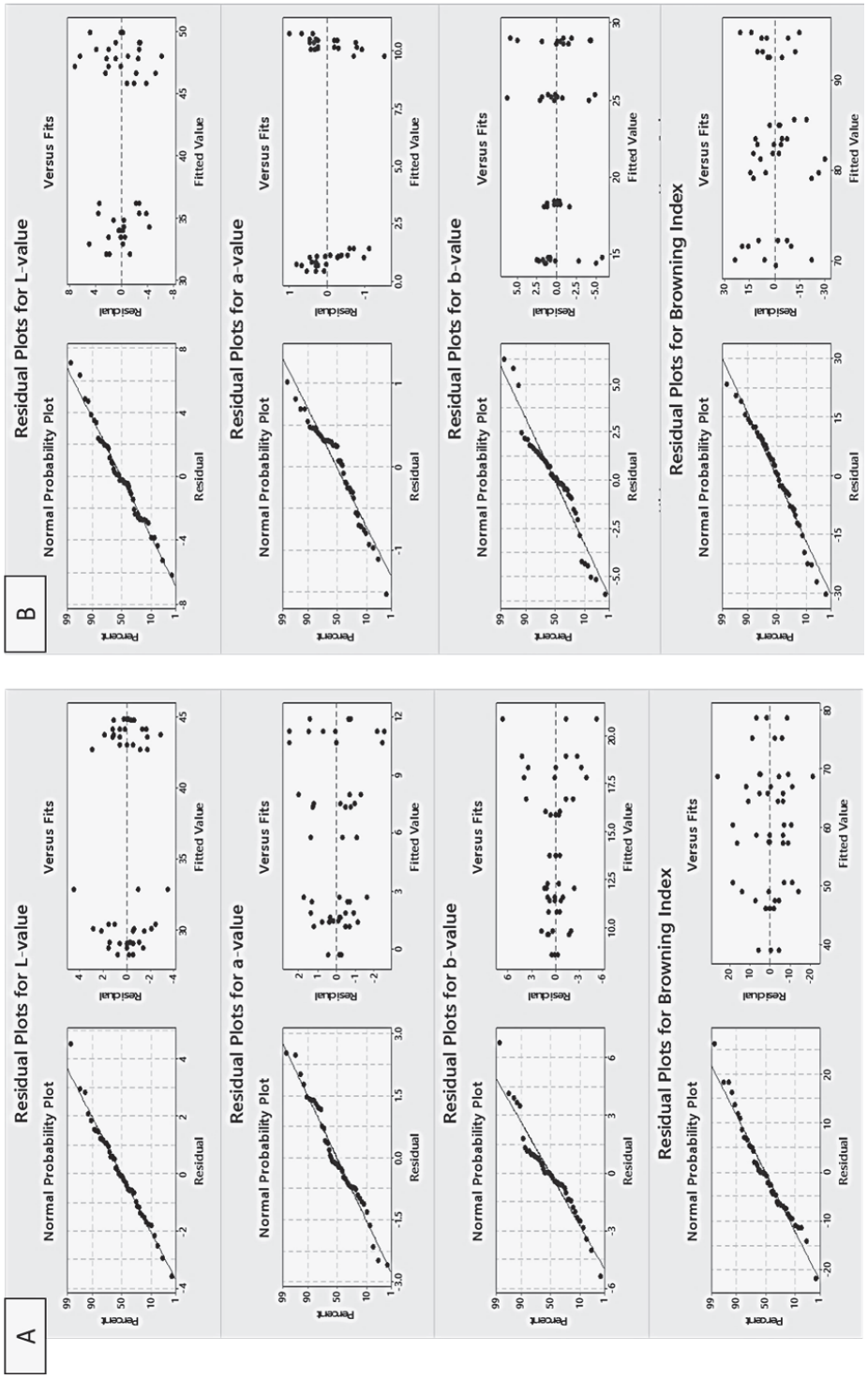

Appendix C Figure 4.