Enhancement of Post-Harvest Shelf Life of Nectarines (*Prunus persica (L.)* Batsch var. *Nectarina*) using Hexanal

by

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Plant Agriculture

Guelph, Ontario, Canada

Abstract

ENHANCEMENT OF POST-HARVEST SHELF LIFE OF NECTARINES (*PRUNUS PERSICA (L.)* BATSCH VAR. *NECTARINA*) USING HEXANAL

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University of Guelph, 2018

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This research investigated the effects of an 'Enhanced Freshness Formulation' (EFF) with hexanal as the key ingredient to improve the shelf life of nectarines. Pre-harvest sprays of EFF on 'Fantasia' nectarines, conducted at two commercial orchards in the Niagara region, ON, showed a general improvement in shelf life. Application of EFF delayed the incidence of chilling injury symptoms (internal browning and mealiness/woolliness) by one week. Treated fruit maintained significantly higher firmness until 38 d post-harvest. qRT-PCR was conducted to evaluate the expression levels of 22 genes potentially involved in ripening, to understand the regulatory effects of the hexanal formulation. EFF application induced a highly significant reduction in transcript levels of three Phospholipase D genes, five N-glycoprotein group genes, and other genes involved in ripening and softening processes. These findings indicate that a delay in the ripening process caused by EFF may be associated with the modulation of the expression of key ripening-related genes, enhancing shelf life and quality of nectarines.

Acknowledgements

This project was made possible by the generous support from Global Affairs Canada through their Canadian International Food Security Fund (CIFSRF), the Ontario Ministry of Agriculture, Food and Rural Affairs' Highly Qualified Personnel Scholarship (OMAFRA HQP). I would also like to acknowledge the department and kind donors for the following scholarships: Walter and Laura Scott Horticultural Scholarship, Mrs. Fred Ball Graduate Scholarship, Arthur Richmond Memorial Scholarship, OAC '60 Leadership Development Scholarship, Vineland Centennial Horticultural Scholarship, Patricia Harney Graduate Scholarship and the OAC Richards Trust Professional Development Grant.

First and foremost, I would like to thank my advisor Dr. Jay Subramanian for his impeccable supervision. His constant support, encouragement and direction helped me go a long way in achieving my goals for this project. His advice and sense of humor were always a source of inspiration and enjoyment, even during rigorous times in the project. His questions were always thought provoking and he encouraged me to be involved in activities across campus. I would also like to thank my co-advisor Dr. Alan Sullivan for his continuous support and willingness to help. He went above and beyond what was necessary to ensure that my career was progressing in the right path. Thanks is also due to my committee member Dr. Gopi Paliyath, whose vast knowledge was always a treasure trove for enthusiasts like me. His patience and kindness always led me in the right direction from the beginning.

I would like to thank Glen Alm for teaching me to prepare for any and every eventuality when conducting field and lab experiments. His expertise in field work came to the fore when we were doing pre-harvest sprays for our project. I could not have conducted many of the experiments if not for the help from our Research Associate Dr. Walid El-Kayal, who taught me everything about molecular

biology and especially took the time to explain the theory behind why we do what we do. Thanks is also due to Dr. Dave Liscombe, Thomas Hern, and Rosalie Zielinski who helped with the volatile collection, analysis and interpretation. I would also like to thank the summer students Varsha Jayasankar, Olivia Colling, Shannon Colling and Haley Shelton for their timely help with field work. Dr. Stephen Bowley and Dr. Michelle Edwards, our statisticians were also of critical help in regard to my thesis. Many thanks to Bob Troupe and Jamie Warner, who provided plots for my field experiment, and helped me with their knowledge that came with many years of experience as a grower.

My lab members and colleagues Robert Brandt, Syndhiya Ranjan, Jack Mackenzie, Renu Chandrasekaran, Ranjeet Shinde, Karthika Sriskandarajah, Erika DeBrouwer, Dr. Amritpal Singh, Gurleen Sidhu, Derek Plotkowski, Edward Flaherty, and Travis Cranmer were instrumental in helping me whenever I needed a hand, and also for their instruction and advice. Thanks is due to Mrs. Sivagamasundhari Sikamani, whose delicious meals filled with love, care and advice lit up many a day during my master's program. I would like to thank my housemate and dear friend Akansha Saxena for always keeping my spirits high, motivating me to get working and also enjoy life at the same time. I will be ever grateful to my extended Guelph and Vineland families for enriching my stay in Ontario.

Last but not the least, I would like to extend gratitude to my family for their continual support. My father Krishnakumar who always encouraged me to reach greater heights, provided me with a lot of opportunities, advice and support whenever necessary while always looking out for me. My mother Vijayalakshmi, for always encouraging me, especially when obstacles arose. Her love and support were invaluable and crucial to finish my masters program. Thanks to my brother Dr. Siddharth for his constant support, encouragement and care. Thanks is also due to my grandparents, uncle, aunt and cousins for their support.

I dedicate this Thesis to my parents, who have steadfastly believed in my ability and constantly encourage me to reach for the stars.

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List of Abbreviations

AO-Ascorbate Oxidase

AFS - Arabinofuranosidase

DAB – Days After Bloom

EFF-Enhanced Freshness Formulation

EXP -Expansin

FSC- Food Supply Chain

GC-Gas Chromatography

HEX-Hexosaminidase

HPLC-High Performance Liquid Chromatography

MAN - Mannosidase

MS-Mass Spectrometry

Mg - Milligram

EI-Electron Ionization

PC-Principal Component

PE – Pectin esterase

PLD – Phospholipase D

PG - Polygalacturonase

TA-Titratable Acidity

TSS-Total Soluble Solids

XTH – Xyloglucan endotrnasglycosylase

Chapter 1

Introduction

1.1 General Introduction

Food wastage is an important global concern, especially since the world population is predicted to reach 9 billion by 2050. An average of one third of all the edible food produced for human consumption is wasted, which amounts to 1.3 billion tons (Gustavvson et al., 2011). The food is wasted in different stages of the food supply chain (FSC) right from agricultural production to individual household wastage. In developed countries, most of the loss and waste occurs in the latter stages of the FSC. However, the food losses in developing countries occur mainly in the early and mid-stages of the FSC (Fig. 1.1). The world average food loss per capita ranges from 280-300 kg in Europe and North America to 50-100 kg per capita in certain regions of Asia and Africa (Gustavvson et al., 2011)

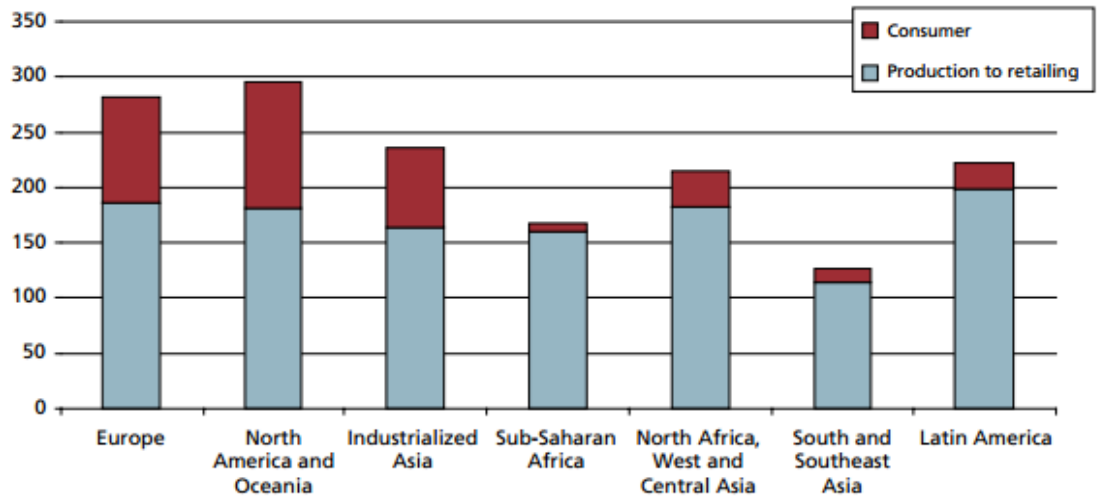


Fig 1.1 Per capita food losses and waste, from consumption and pre-consumption stages, in different regions of the world (Gustavvson et al., 2011).

Among the different food categories, fruits and vegetables form a very important part of the human diet as they provide the primary source of nutrients, fiber and antioxidants which are essential for maintaining healthy body functions or homeostasis. However, fruits are highly perishable commodities. Globally, post-harvest losses in fruits and vegetables vary from 35% to 60% of production (Gustavvson et al., 2011). This represents one of the most chronic wastage scenarios among all the agricultural product categories. Most of the losses in developing countries occur during the post-harvest and distribution phases due to deterioration in quality of the produce and also due to seasonality resulting in unsaleable gluts (Fig. 1.2). In addition to the above-mentioned losses, grading and sorting of produce and wastage by the consumer, especially in developing countries, contributes to the overall loss (Gustavvson et al., 2011).

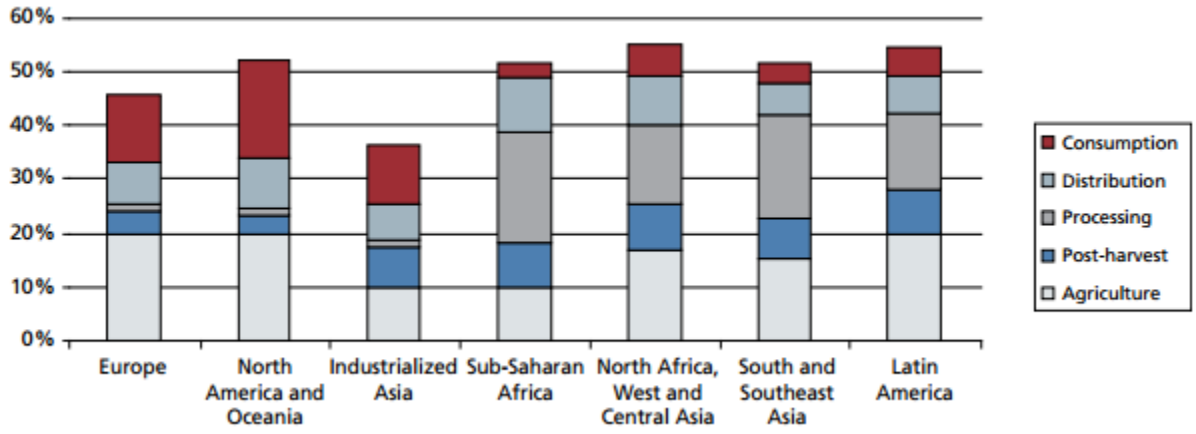


Fig 1.2 Production losses and wastage at different stages of the food supply chain of fruits and vegetables in different regions of the world. (Gustavvson et al., 2011)

Enhancement of the post-harvest shelf life is essential to ensure nutritional security of rural communities all over the world. The storage of fruits poses a challenge and is a drain on resources,

especially in developing countries (Subramanian et al., 2014). Existing storage solutions are geographic region and crop specific, and are often not adaptable or affordable to small-scale growers. In order to provide price and scale-suitable solutions, researchers are working towards increasing the post-harvest storage life of many fruits to increase their marketability and reduce loss/wastage.

1.2 Research Problem

Peach and nectarine have a very short shelf life of 2-3 weeks, even under ideal storage conditions. Sustainable and cost-effective technologies to increase the harvest window and shelf life of peach and nectarine is an important consideration in the production of these crops. These fruit typically start losing their market and nutraceutical appeal as a result of membrane breakdown leading to loss of phytochemical content and increased post-harvest diseases. Though peach and nectarine can be stored in cold storage, they are prone to cold injury and lose their palatability. Other storage options such as controlled, modified atmosphere storage and 1-MCP treatments have their respective shortfalls and have shown variable results. Hence, there is a need to develop new methods to extend post-harvest storage and nutrition content of the fruits

Recently advances in post-harvest storage technology have been made with the advent of Phospholipase D (PLD) inhibiting technology, the key enzyme responsible for lipid breakdown in the cell membrane during ripening (Paliyath and Droillard, 1992). A C6 aldehyde, hexanal is a GRAS (generally regarded as safe) compound (Waddell et al., 2010) that is responsible for downregulating the expression of the PLD enzyme which initiates a series of catabolic reactions resulting in the degradation of the phospholipid bilayer (Paliyath and Murr, 2003). These treatments are hypothesized to delay ripening in peach and nectarine. This technology enables the treated fruit to retain the market quality for significantly longer periods due to the retention of

membrane integrity- a signature effect of hexanal treatment. Further, it is hypothesized that when membrane integrity is retained, it will favorably impact the phytochemical content.

1.3 Hypothesis

Pre-harvest applications of 'Enhanced Freshness Formulation', containing hexanal, will enhance shelf life and improve/maintain different quality attributes (physiological and biochemical properties) of the nectarine fruit by downregulating the expression of PLD and other ripening related genes.

1.4 Research Objectives

1. To determine the effect of EFF on fruit retention on nectarine trees.
2. To delay and reduce the incidence of physiological disorders such as mealiness and internal browning, thus increasing shelf life.
3. To improve and/or maintain the physiological properties (marketable and visual characteristics) of the harvested fruit such as firmness, colour, total soluble solid content, and titratable acidity.
4. To determine the effect of EFF on the volatile profiles of the fruit
5. To analyze the effect of EFF on expression of genes involved in the ripening process of nectarines.

Chapter 2

Literature Review

2.1 Peach and Nectarine-Origin and Production in Canada

Peach (*Prunus persica var. persica (L.*) Batsch) and nectarine (*Prunus persica (L.)* Batsch var. *nectarina*) are tender fruit native to China. It was introduced to North America by European travelers in the 16th century. Nectarine is very similar to peach. They only differ subtly, by their taste and aroma, and the absence of pubescence. Peach and nectarine are economically important crops with an expanding world production estimated at 21.6 million tons in 2013 (FAO, 2013). In Canada, peach and nectarine are grown in the Niagara region and Southwestern Ontario, the Okanagan Valley of British Columbia, along with small acreage in Nova Scotia. In Canada, peach and nectarine are grown in an area of 2874 ha with an annual production of 25.6 thousand metric tons and a farm gate value of approximately 40 million CAD during 2013 (AAFC, 2015). Ontario occupies the major share of the production of peach (82%) and nectarine (77%) (AAFC, 2015). In Ontario alone, the farm gate value of peach and nectarine increased to approximately 35 million CAD in 2016 (OMAFRA, 2017).

2.2 Peach and Nectarine Fruit Constituents

Peach and nectarine are characteristically soft-fleshed and have a very short life span post-harvest. The fruit has a water content of approximately 87% and it consists of carbohydrates, organic acids, pigments, vitamins, volatiles, phenolic and antioxidants. The main soluble sugars that constitute 75% of the total are sucrose, glucose and fructose. Soluble sugars contribute about 7-18% of total weight and fibre provides about 0.3% of the fresh weight (FW) of the fruit. Organic acids contribute to about 0.4-1.2% FW and the ratio of soluble solids to titratable acidity determines the flavor and consumer perception of the fruit (Crisosto and Valero, 2008). Malic acid

and citric acid constitute the main organic acids. Acidity usually decreases about 30% during ripening. Proteins (0.4-0.8 FW) and lipids (0.1-0.2 FW) are two other components with major functions. Proteins catalyze the chemical reactions responsible for compositional changes and lipids make the surface wax that improves the cosmetic appearance of the peel. The cuticle which is mainly made up of lipids also protects against water loss and pathogens. Lipids also influence the physiological activities of fruit, as constituents of the cell membrane. The minerals present in the fruit include Ca, Mg, K, Na, P, Cl and S. Ca is a very important mineral which is responsible for activating many catabolic processes such as lipid breakdown post-harvest changes in mineral content are very negligible. Esters, alcohols, aldehydes, ketones and acids, all contribute to the aroma of the fruit (Crisosto and Valero, 2008).

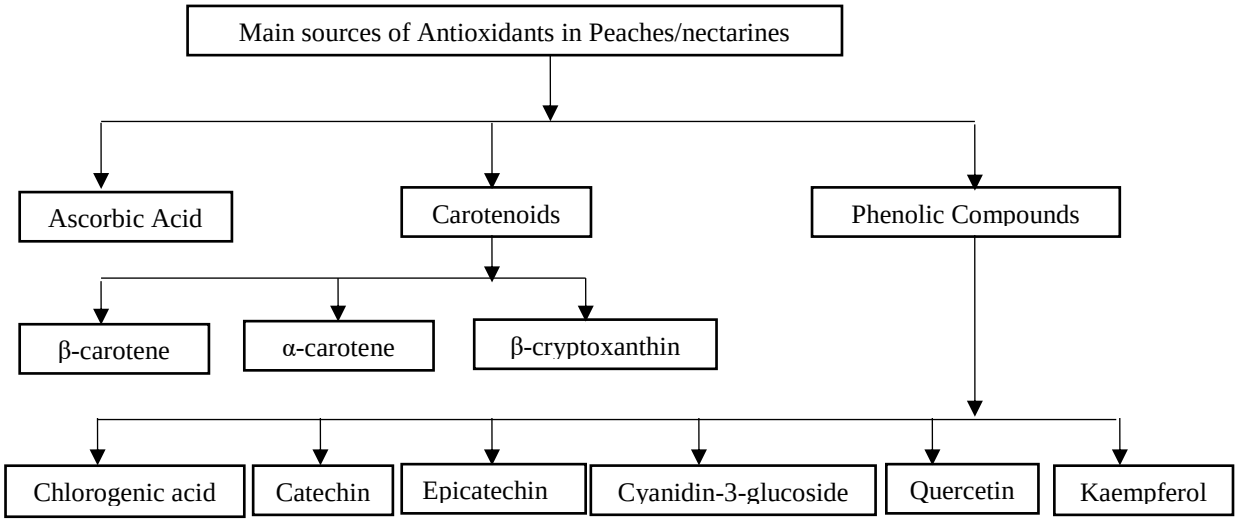


Fig 2.1 Schematic diagram representing the major potential sources of antioxidants in peach and nectarine (Byrne 2002; Gil et al., 2002; Crisosto and Valero, 2002).

The antioxidant capacity of the fruit varies according to cultivar and the important antioxidants are presented in Fig 2.1. However, Crisosto and Valero (2002) have observed that white-fleshed had slightly higher antioxidant capacities than yellow-fleshed peach. Most of the antioxidant compounds are highly abundant and restricted to the fruit peel. Gil et al. (2002) observed that ascorbic acid content ranged from 4-13mg/100g in different white and yellow-fleshed varieties grown in North America. The average carotenoid concentration for white-fleshed and yellow-fleshed peach cultivars was in the range of 71-210 µg/100 g FW and 7-20 µg/100 g FW respectively (Gil et al., 2002). The phenolic content varied from 28-111 and 21-61mg/100g FW for white fleshed and yellow fleshed cultivars respectively. The fruit phenolics also contributes to the colour and astringency, apart from the beneficial antioxidant properties (Tómas-Barberán et al., 2001). The higher content of antioxidants present in the white-fleshed varieties in-turn enabled a markedly higher antioxidant capacity. The total antioxidant content ranged from 13-107.3 mg of ascorbic acid equivalents when evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical method and from 19-119.6 mg of ascorbic acid equivalents when evaluated by FRAP (Ferric reducing ability plasma) method (Tómas-Barberán et al., 2001). It is comparable to the antioxidant capacities for pear and apple, and are significantly lower than those observed in strawberry, raspberry and plums (Proteggente et al., 2002).

2.3 Post-harvest life and deterioration of nectarine

Once harvested, nectarine has a very short lifespan which can be influenced by a variety of factors such as environmental conditions, maturity at harvest and other post-harvest handling procedures. Nectarines are usually harvested based on maturity indices, including background colour of the fruit peel (Crisosto, 1994), fruit firmness or maximum maturity index (Crisosto et al., 2001). Maximum maturity index is defined as the lowest firmness at which the fruit are

harvested to avoid bruising during the post-harvest chain and storage (Crisosto and Valero, 2008). Shortage of skilled labour during harvest is a serious issue faced by many growers in Ontario. The shortage of labour results in a more dispersed harvesting season which may result in harvesting of under-ripe or over-ripe fruit. Harvesting under-ripe or overripe fruit results in loss of flavor/marketability (Subramanian et al., 2014). Overripe fruits also soften quickly reducing the post-harvest life and rendering the fruit susceptible to other injuries and diseases. Decay, internal breakdown (IB) or chilling injury (CI) are the main caused for post-harvest losses in peach and nectarine (Ceponis et al., 1987; Mitchell and Kader, 1989). Storage of these fruit at a temperature of 2.2°-7.6°C seemed to further aggravate the internal browning disorder characterized by a dry, mealy texture of the fruit, flesh browning and loss of flavor (Harding and Haller, 1934; Smith, 1934; Mitchell and Kader 1989). Fungal pathogens also prove to be a major cause of post-harvest loss. *Botrytis cinerea* (Pers.) and *Monilinia fructicola* (G.Winter) Honey which cause botrytis rot and brown rot respectively, are the most devastating pathogens causing huge losses of fruit, with severe economic implications (Crisosto and Valero, 2002).

2.4 Post-Harvest Physiology of Peach and Nectarine

Ripening is a complex process which involves many biochemical changes in the fruit. It is usually characterized by reduction of organic acid content, increase in sugar content, breakdown of pectin and starch leading to softening of the fruit, changes in colour and production of a characteristic aroma. As is the case with many stone fruit, fruit development and ripening in peach is seen as a double sigmoid curve with 4 distinct stages as described by Tonutti et al. (1991) for the peach cv. Red Haven. Stage 1 ranges approximately from 23-37 days after bloom (DAB), characterized by fast fruit growth. The fruit increase their size three-fold within these 15 days. The Stage 2 is a lag phase where growth slows down and lignification of the endocarp occurs (38-68

DAB). This is followed by another log phase from 67-94 DAB where fruit size increases three to four-fold within 30 days. Stage 4 represents the ripening stage at 94 DAB, where the fruit has already reached its full size and is undergoing the process of ripening followed by senescence. The structural breakdown of different cellular components and other changes that occur during ripening of peach and nectarine is mentioned below.

2.5 Structural breakdown of different cellular components

2.5.1 Cell Wall Carbohydrate Metabolism

Cell wall degradation is responsible for softening of most fruits (Paliyath et al., 2008). Many laboratories and review articles have studied softening of fruits especially due to their economic implications (Brownleader et al., 1999; Brummel and Harpster, 2001). Tender fruit are more susceptible to softening injury during transportation and storage which may lead to secondary issues such as pathogen attacks. It can be assumed that softening of peach is one of the most important deciding factors in the shelf life of the produce. During softening, cell separation occurs due to a loss of link between adjacent cells caused due to weakened cell wall structure (Roberts et al., 2002). This process involves the expression of different enzymes that initiate a cascade of reactions which irreversibly degrades the mechanical properties of the cell wall. Cellulase (EC 3.2.1.4) degrades cellulose and pectin methyl-esterase, polygalacturonase and β-galactosidase are involved in pectin degradation (Paliyath and Murr, 2008). These enzymes are described in more detail below.

Cellulose has been found at increased levels during ripening in peach (Hinton and Pressey, 1974). The fruit softened markedly on further ripening, after the maximum expression of cellulose. Further research by Bonghi et al. (1998) indicated that endo-β-1,4-glucanase (EC 3.2.1.4; EGase) has two isoforms with isoelectric points (pI) of 6.5 and 9.5. The enzyme with the pI of 9.5 was

expressed at its maximum level during ripening (climacteric and early softening stages) whereas the enzyme with pI of 6.4 is expressed during early fruit growth (preclimacteric stages). The authors suggested that different EGase genes operate during the late and early developmental stages of peach fruit ripening and senescence. The peach EGase expressed during ripening (pI-9.5) has a molecular mass of 54 kDa which is similar to the EGase of tomato (51-53 kDa, Lashbrook et al., 1994). Studies conducted by Trainotti et al., (1997) isolated and characterized the gene (ppEG1) of which the 9.5 pI Egase was a fragment. However, Trainotti et al. (1997) concluded that the 9.5 pI EGase could be regulated by a different gene throughout the fruit development as the EGase.

There are two forms of polygalacturonase (PG) that have been identified from peach fruit extracts (Exo and Endo-polygalacturonase) (Pressey and Avants, 1973). The endo PG's (EC 3.2.1.15) are predominant in the freestone type of peach and exo-PG's (EC 3.2.1.67) are present in the mesocarp of both the freestone and clingstone varieties of peach (Paliyath et al., 2008). Exo-PG's remove the galacturonic acid moieties from the terminal reducing end of the chain, whereas the endo-PG's cleaves the pectin chain at random. PG activity is absent in unripe peach, with peak activity just after the advent of the softening of the fruit. The functioning of this enzyme parallels the formation of water soluble pectin, indicating that PG had a major role to play in ripening and degradation of the pectin present in cell wall and middle lamella. The endo-PG acts on de-esterified pectate and catalyzes the solubilization of protopectin from cell walls (Pressey and Avants, 1973). Along with exo-PG, they provide a system for complete hydrolysis of pectin. The detection of free galacturonic acid in ripe peach indicated the extensive and complete degradation of pectin by PGs. In peach, the high gene expression of endo-PG after the climacteric rise of ethylene is regarded as the main cause for the softening of peach (Lester et al., 1994). However, it seems that the loss of

firmness and the textural changes are different interconnected cellular mechanisms. (Ghiani et al., 2011). They reported that endo-PG is required for the textural changes that are imperative for the development and maintenance of the structure of the melting fruit texture, one of the most important factors determining the consumer's perception of fruit quality (Bruhn, 1995). However, they also proved that endo-PG's role in contributing to loss of fruit firmness may not be essential as loss of firmness occurs in the non-melting peach varieties as well, with minimal presence of endo-PG's.

Expansins are proteins that have been shown to contribute to fruit ripening and softening in many fruits, most notably, in tomato and peach (Hayama et al., 2003). Several expansins show a general increase during ripening, however, it has been documented that there are some expansin genes that are more closely related to fruit softening than others. There are 3 peach expansin genes that have been isolated thus far. Among them the *PpEXP3* gene (AB047519) seemed to be the gene most closely related to fruit softening. Pectin methyl esterases (EC 3.1.1.11) has been shown to have an indirect influence on ripening and softening of the peach fruit. It is believed to de-esterify the pectin predisposing it to further degradation by the polygalacturonases (Brummell et al., 2004).

2.5.2 Regulation of ripening by N-glycoprotein group

The peach fruit softening occurs in tandem with degradation of cell wall polysaccharides by cell wall degrading enzymes. However, inhibition of the activities of these enzymes does not result in successful regulation of ripening (Brummel and Harpster, 2001). Recently, Ghiani et al. (2011) demonstrated that endo-PG, the key enzyme responsible for pectin polysaccharide breakdown does not influence peach softening. Hence, there is a need to look further to examine the key genes responsible for fruit softening in fruit. N-Glycoproteins play an important role in

various physiological processes of ripening. During ripening of tomatoes, N-glycan accumulation in the pericarp occurred either as precursors of N-glycosylation or due to degradation of N-glycoproteins (Priem et al., 1993). These free glycans can also stimulate fruit ripening by activating the ethylene synthesis pathway (Priem and Gross, 1992). Among the N-glycoprotein enzymes, mannosidase, β-hexosaminidase and xyloglucan endo-transglycosylase/hydrolase play prominent roles in the N-glycosylation pathway. Inhibition of these enzymes in tomato fruit delays softening of fruit (Meli et al., 2010). β-hexosaminidase (β-hex; EC 3.2.1.52) is involved in the cleavage of the terminal N-acetyl-D-hexosamine (GlcNAc) residues from N-glycoproteins at the later stages of the N-glycosylation pathway (Cao et al., 2014). Cao et al. (2014) cloned two β-hexosaminidases gene homologs, *Pphex1* (JQ979080) and *Pphex2* (JX069961), both of which showed different expression patterns but with peaks in expression during storage of fruit. The peach fruit treated with 2-acetaindo-1, 2-dideoxynojirimycin inhibited the activity of both these hexosaminidases. This translated into increased fruit firmness and inhibition of enzymes involved in cell wall degradation and ethylene synthesis pathway. This research indicated that β-hexosaminidases could be involved in the regulation of fruit softening in peach. Xyloglucan endotransglycosylase/endohydrolase (EC 2.4.1.207) is a predominant N-glycan enzyme which can result in rapid cell wall loosening in a two-step process. Xyloglucan endotransglycosylase/endohydrolase enzymes have been described in closely related species such as apple (Atkinson et al., 2009; Miedes et al., 2004), pear (Fonseka et al., 2005) and strawberry (Opazo et al., 2010). Miedes et al. (2010) states that the role of Xyloglucan endotransglycosylase/endohydrolase could be related to maintenance of structural integrity of the cell wall and its decrease in activity during ripening contributes to fruit softening. Hence, there is potential for examination of peach xyloglucan endotransglycosylase/endohydrolase genes to

observe any changes during ripening. α-mannosidase genes (α-Man; EC 3.2.1.24) are regulated by the RIN (ripening inhibitor)-mediated direct transcriptional regulation (MADS box family transcription factor). Numerous studies have indicated the increase in fruit firmness by inhibition of this enzyme (Ghosh, et al., 2011; Meli, et al., 2010) indicating its potential role in the softening process.

2.5.3 Starch Metabolism

The conversion of starch to fructose and glucose is an integral part of fruit ripening. The enzymes amylase degrade starch into sucrose. Starch phosphorylase is also involved in generating glucose-1-Phosphate, a metabolite which is involved in further downstream reactions. The resulting products such as maltose, dextrin are converted to glucose and fructose through the action of invertase (Paliyath et al., 2008). The sugars generated during starch degradation are metabolized through glycolysis and the citric acid cycle. Sugar phosphates can also be channeled through the pentose phosphate pathway which provides reducing power (NADPH) for biosynthesis reactions and also the framework for synthesis of secondary plant metabolites. Gluconeogenesis utilizes the organic acids stored in the vacuole through a reversal of the respiratory pathway (Paliyath et al., 2008). Recent ripening studies on peach indicated that the cell wall breakdown and other catalytic metabolisms occur alongside starch synthesis which helps to maintain the structure of cell wall and other membranes (Trainotti et al., 2003). Thus, ripening occurs as a balance between catabolism and starch synthesis (sucrose cycle).

2.5.4 Lipid Metabolism

Nectarines have a low lipid content. However, lipids play an important role during the ripening process. As in many other fruit, the content of phospholipase decrease during senescence. This decline is accompanied by increases in the content of neutral lipids, primary diacyl glycerols, free

fatty acids, fatty aldehydes and sterols. The change in the ratio of sterols: phospholipids results in the formation of micelles (gel phase or non-lipid bilayer) and eventually results in leaky membranes. Loss of compartmentalization and complete senescence follows (Paliyath and Droillard, 1992).

Lipid degradation progresses in sequential steps. Phospholipase D (PLD) (EC 3.1.4.4) is the first and key enzyme in the pathway which degrades the phospholipase into phosphatidic acid. This product is converted to diacyl glycerol and then to free and peroxidized fatty acids. These fatty acid products from the lipid degradation pathway are important precursors for the production of aroma volatiles and flavor compounds (Paliyath and Murr, 1998). The very low specificity of enzymes downstream of PLD such as the activity of phosphatidate phosphatase, lipolytic acyl hydrolase, and lipoxygenase is characteristic and is a regulatory mechanism of this pathway (Paliyath and Murr, 2008). Therefore, it can be hypothesized that the measure of membrane lipid catabolism is determined by the extent to which PLD is activated.

During the natural ripening process, lipid degradation leads to destabilization of the membrane. This, in turn leads to leakage of Ca^{2+} and H^{+} ions from the cell wall space with simultaneous inhibition of Ca^{2+} and H^{+} ATPases which regulate the physiological Ca^{2+} and H^{+} levels in the cytoplasm. This leads to increased Ca^{2+} concentration in the cytoplasm (greater than 10µL), which when coupled with low pH, activates PLD and the cascade of lipid catabolic reactions which follow, leading to substantial degradation of the lipid membranes (Paliyath and Murr, 2008). There are several isoforms of PLD such as PLD-α, PLD-β, PLD-γ (Wang, 2001). PLD-α is by far the most abundant enzyme and hence, considered as the housekeeping enzyme. PLD is a soluble enzyme under normal circumstances. Part of PLD enzymes named as the C2 domain which contains a segment of 130 amino acids at the N-terminal end, is very similar to many enzymes and

proteins that take part in the signal transduction pathway. The increase in cytosolic calcium concentration due to environmental and hormonal signaling results in the Ca^{2+} being bound to the C2 domain, which then transports the PLD from the cytoplasm to its site of action (Paliyath and Droillard, 1992). Research on the relationship between ethylene receptors and PLD activity is not fully understood. Antisense inhibition of PLD-α in tomatoes resulted in good control of membrane degradation and hence, increased the shelf life (Oke et al., 2003; Pinhero et al., 2003). Hexanal application on tomatoes heavily downregulated the expression of PLD-α with substantial increase in the shelf life of tomatoes (Tiwari and Paliyath, 2011a).

In peach and nectarine, there are 12 potential genes which code for Phospholipase D (International Peach Genome Initiative, 2013). Among all PLD's, it has been shown that PLDα plays a major role in fruit ripening (Tiwari and Paliyath, 2011b; Paliyath et al., 2008). One PLDα gene from peach *α-PLD1* (EU925810) has been cloned and isolated in peach in 2008 (Wan et al., unpublished data). Extensive cloning of tomato PLD genes (Paliyath et al., 2008) and strawberry PLD genes (El Kayal et al., 2017) provide good references and information regarding mechanism of action of PLD and potential pathways for PLD inhibition mechanism.

2.5.5 Protein Metabolism

Chloroplasts form the major proteinaceous compartments in the fruit. During senescence, the protein content gradually degrades by disorganization of the grana lamellar stacks. The degradation of chlorophyll unmasks the other colour pigments. The enzyme chlorophyllase acts on the pigment and degrades it into chlorophyllide and phytol chains (Kräutler and Hörtensteiner, 2013). The phytol chains contain the building blocks for isoprenoid biosynthesis and their products accumulate in the plastoglobuli. The phytol chains also degrade to produce volatile compounds

responsible for the flavor and aroma of the fruit. During fruit ripening, the proteins degrade and enrich the soluble fractions with amino acids (Kräutler and Hörtensteiner, 2013)

2.6 Peach Volatilomics

During ripening, the lipid degradation pathway results in the production of many volatile and aroma compounds, which contribute to flavor. The protein degradation pathway, secondary metabolism pathways (isoprenoid and polyphenolic pathways) also contribute to the production of volatile organic compounds (VOC's). Aroma volatiles are one of the most important factors contributing to peach quality and acceptability by consumers (Bruhn, 1995). Approximately 100 volatiles have been found in peach and nectarine, including alcohols, esters, terpenoids, ketones and lactones (Wang et al., 2009). Lactones are the major contributors to the peach-like aroma (Eduardo et al., 2010; Horvat et al., 1990). Among them, γ-decalactone is the major contributor. Horvath et al. (1990) described terpenoids and C6 aldehydes as minor contributors to the peach aroma. Esters ((Z)-3-hexenyl acetate, (E)-2-hexen-1-ol acetate) and ethyl acetate contribute to the 'fruity' aroma in peach while the 'floral' aroma is provided by terpenoid compounds such as linalool and β-ionone (Sanchez et al., 2014; Eduardo et al., 2010). 2-hexanal and 3-hexanal provide the aroma that is characteristic of unripe fruit (Horvath et al., 1990). Recent research has focused on identifying the genes and loci that control aroma production and increasing the content of volatile components which improve peach aroma and flavor, an important characteristic that consumers perceive to be important for a good quality peach fruit. Microarray based RNA profiling and QTL studies have been done to understand the mechanism of control in the volatile production systems (Sánchez et al., 2013; Pirona et al., 2012; Eduardo et al., 2010). Based on the observations of these experiments, it is now a real possibility to develop peach varieties with an increase in favorable volatiles through marker assisted breeding techniques.

2.7 Methods to reduce post-harvest loss and enhance post-harvest life

Many ethylene inhibition chemicals such as 1-MCP (methylcyclopropene) treatment are successful in the delay of ripening and senescence in climacteric fruits such as apples, pear, banana and plums (Blankenship and Dole, 2013). Controlled atmosphere storage and modified atmosphere storage have been shown to have minimal and variable enhancement of the quality of peach/nectarine. Some of the ethylene inhibition techniques have been mentioned below.

2.7.1 1-MCP treatment for peach and nectarine

1-MCP has been shown to be effective in fruits which maintain their firmness after ripening such as in apples. The use of 1-MCP has not been very effective in fruit which soften during ripening (Watkins et al., 2006). Softening is accompanied by various changes in total soluble solids, acidity and aroma. A combination of concentration and exposure time of 1-MCP affects the ripening response in fruit. Peach and nectarine fall under the latter category and there have been varied reports on the effectiveness of 1-MCP in delaying post-harvest ripening and senescence. The effective concentration of 1-MCP for peach/nectarine ranges from 0.4 to 5 $\mu L.L^{-1}$ as described in previous experiments (Liquori et al., 2004; Liu et al., 2005). However, only ephemeral changes were observed in the delay of softening in many studies conducted on many peach and nectarine varieties all over the world (Bregoli et al., 2005; Dal Cin et al., 2006; Dong et al., 2001; Fan et al., 2002; Liu et al., 2005). The maintenance of firmness and ground colour is short-lived and requires multiple applications to provide a prolonged effect (Liu et al., 2005). However, this method was found to be uneconomical in a cost benefit analysis (Hayama et al., 2005; Liu et al., 2005). Inability of the fruit to ripen following 1-MCP treatment and off-taste are other problems associated with the use of this chemical (Watkins et al., 2006). Moreover 1-MCP has been found to increase the effect of chilling injury during low temperature storage leading to increased occurrence of

mealiness, internal bleeding and other physiological disorders (Dong et al., 2001, Fan et al., 2002). Hence, limitations exist with the use of this chemical in low temperature below 4°C (Bregoli et al., 2005). It also emphasizes the need for ethylene-mediated ripening processes to maintain the quality of the fruit during cold storage. Deterioration of quality has also been observed in peach. The total soluble solids (TSS) of peach/nectarine either declines (Fan et al., 2002; Bregoli et al., 2005) or there is a delay in the development of sugars during ripening (Liu et al., 2005) following 1-MCP application. The titrable acidity (TA) also declines in high acid cultivars (Bregoli et al., 2005; Liu et al., 2005, Fan et al., 2002). Though 1-MCP can increase the shelf life of peach and nectarine at room temperature (Hayama et al., 2005), it is ineffective in low temperatures which limits its use in extending the post-harvest shelf life of peach and nectarine.

2.7.2 Phospholipase D Inhibition Technology

Most of the technologies used to enhance the shelf life of fruit have taken advantage of the ethylene biosynthesis pathway to delay the ripening process. However, both cell wall and lipid metabolism are important pathways to exploit in order to enhance the shelf life. The membrane degradation process also behaves in an autocatalytic manner due to the development of suitable conditions for the process to advance. As discussed earlier, PLD is the key enzyme in that pathway.

Many compounds developed to inhibit PLD activity have been shown to enhance the shelf life of fruit, vegetables and flowers. PLD inhibition by lysophosphatidylethanolamine was developed by Palta and Farag (1992) and it was effective for fruits such as apple, cranberry and tomato. Lysophosphatidylethanolamine is a non-competitive inhibitor of PLD. High concentrations of N-acylethanolamine have inhibitory action against PLD (Chapman and Shea, 2007).

The active site of PLD consists of 2 HKD motifs which hydrolyze phospholipids in a 2-step process generating a PLD-phosphatidate intermediate (Koonin, 1996). The intermediate complex then reacts with water or primary alcohol to produce phosphatidic acid or phosphatidyl alcohol respectively. Many aliphatic primary alcohols and aldehydes inhibited PLD activity (Paliyath et al., 2003). These compounds inhibited soluble and membrane forms of PLD (Paliyath et al., 1999).

2.8 PLD inhibition by Hexanal and EFF (Enhanced Freshness Formulation) Application

Recent advancements in increasing the shelf life of fruit, vegetables and flowers is associated with the use of hexanal formulations which inhibits the PLD induced membrane catabolism. Hexanal downregulates the expression of the PLD enzyme and other catabolic ripening reactions resulting in enhanced shelf life and quality parameters of the fruit such as firmness, colour and TSS (Paliyath and Subramanian, 2008). It is also approved by the United States Food and Drug Administration as a GRAS compound (Waddell et al., 2010). Hexanal is naturally produced in the lipid peroxidation pathway mediated by lipoxygenase and hydroperoxide lyases. Hexanal is also naturally produced as a defense response to different biotic stresses. It has an odour which resembles freshly cut grass or cut cucumber and beans (Paliyath et al., 2008).

Hexanal is more advantageous than other PLD inhibiting compounds because of its volatility. It is in many ways similar to the application of 1-MCP. Hexanal has been combined with other ingredients to form an 'Enhanced Freshness Formulation' (EFF) consisting of 1% (v/v) geraniol, 1% (w/v) α tocopherol, 1% (w/v) ascorbic acid, 0.1% (w/v) cinnamic acid, 0.1% (w/v) Tween 80 dissolved in ethanol (10% v/v). This formulation along with the PLD inhibiting hexanal technology have been patented (US Patent # 6,514,914; 7,198,811). Hexanal treatment has shown promising results in fruits such as apple, banana, cherry, peach, and strawberry. (Paliyath and

Subramanian, 2008). Recent studies have also shown considerable improvement in quality and shelf life of cherries, guavas, and greenhouse tomatoes (Gill et al., 2015; Cheema et al., 2014; Sharma et al., 2010). Hexanal treatment offers several advantages over 1-MCP treatment as it does not impair colour and flavor development during ripening of the fruit (Cliff et al. 2009; Kondo et al., 2005). Extensive gene expression studies have been undertaken to understand the mode of action of hexanal. Oligoarray studies on tomatoes sprayed with hexanal have shown interesting results (Tiwari and Paliyath, 2011a). Hexanal application, like 1-MCP, cause a moderate downregulation of key ripening related genes. Hexanal treatment does not inhibit any of the secondary metabolite synthesis pathways (eg. isoprenoid, carotenoid and aromatic acid synthesis pathways) in comparison with 1-MCP which downregulates key enzymes in those pathways. Hexanal delays but does not inhibit the full expression of colour pigments in the fruit. Hexanal treatment also upregulates the expression of genes involved in certain flavors compounds such as terpenes and alcohol (Tiwari and Paliyath, 2011a). Apart from enhancing the shelf life of fruit, hexanal treatments on peach, nectarine, mango and other tree fruits increases the retention period of fruit on the tree (Anusuya et al., 2016). This characteristic is advantageous especially with regards to labour shortage. With the increased retention time, growers can allocate workers more efficiently and the fruit could be picked at proper maturity to ensure consistent quality.

This technology shows great promise in enhancing the shelf life and quality attributes of fruits and vegetables. The mode of action of hexanal is not fully understood and is currently being investigated in more detail. Further research to optimize hexanal applications and enhancement of quality attributes have to be studied individually for nectarine/peach.

This research aims to understand the improvement in shelf life, quality and sensory attributes of ‘Fantasia’ nectarine in response to hexanal applications. It also investigates the expression of ripening related genes to further understand the mode of action of hexanal.

Chapter 3

Quality and Gene Expression Studies

This chapter is a more detailed and reformatted version of the following publication.

Kumar, S. K., El Kayal, W., Sullivan, J. A., Paliyath, G. and Jayasankar, S. 2018. Pre-harvest application of hexanal formulation enhances shelf life and quality of 'Fantasia' nectarine by regulating membrane and cell wall catabolism-associated genes. *Scientia Horticulturae*, *229*, pp.117-124. https://doi.org/10.1016/j.scienta.2017.10.031

Contributions

Kumar, S. K., Jayasankar, S., Paliyath, G., and Sullivan, J. A., conceived and contributed to the design of the experiment. El-Kayal, W., provided impetus with the methods and protocols for qRT-PCR experiments. Kumar, S. K. conducted, analyzed and wrote the manuscript. All authors provided insights and edits to the final manuscript.

3.1 Introduction

Nectarine is a characteristically soft-fleshed tender fruit with a short post-harvest shelf life. In ambient temperatures, they quickly deteriorate in quality, hence, post-harvest technologies are essential to prolong their shelf life and maintain quality attributes of the fruit. Cold storage is the most commercially adopted technique, and a cold chain has been integrated into the post-harvest management systems (Lurie and Crisosto, 2005). However, chilling injury symptoms such as internal browning and mealiness/woolliness have been reported especially when the fruit were stored in the chilling temperature range of 2.2°C to 7°C range (Crisosto et al., 1999). Further, these symptoms develop during advanced stages of ripening after cold storage, when the fruit reach the consumer (Bruhn et al., 1991; Crisosto et al., 1995).

Extensive research has been conducted to combat the incidence of these chilling injury disorders. Various techniques such as fertilizer practices, irrigation regimes, crop load adjustments, fruit size, canopy position, application of plant growth regulators, controlled atmosphere storage, use of ethylene inhibitors, intermittent warming (IW), and controlled delayed cooling have been used with varying levels of success (Lurie and Crisosto, 2005). Among them, 1-MCP has been successfully used to enhance shelf life of many fruits such as apples and pear to maintain firmness, however, it is ineffective at low temperatures which limits its use in extending the post-harvest shelf life of peach and nectarine (Watkins, 2006). Hence, there is a need to develop more efficient strategies to combat the chilling injury disorders.

Nectarine fruit ripening is a complex process and is detailed in Section 2.4. Cell wall and membrane degradation are two highly complex interrelated processes responsible for softening of most fruit (Paliyath and Murr, 2008). Much attention has been focused on softening of fruit especially due to their economic implications (Crisosto et al., 1995; Brummel and Harpster, 2001). Tender fruit, when softening, are more susceptible to injury during transportation and storage, which may lead to secondary issues such as pathogen attack (Crisosto and Valero, 2008). During softening, the cell wall structure weakens causing a loss of linkage between adjacent cells and cell separation. This process involves the expression of different enzymes that initiate a cascade of reactions which irreversibly degrades the mechanical properties of the cell wall. Several enzymes have been implicated in this process. β-1,4-glucanase degrades cellulose; Pectin methyl-esterase de-esterifies pectin predisposing it to further degradation by polygalacturonase and β-galactosidase (Brummell et al., 2004). Expansins are proteins which facilitate loosening of the cell wall (Rose et al. 1997; Brummell and Harpster 2001) and a general increase in their expression during ripening of peach has been recorded (Hayama et al., 2003).

Membrane degradation during ripening has been studied in great detail (Paliyath and Droillard, 1992). During the natural ripening process, lipid degradation leads to destabilization of the membrane. This can result in progressively increasing Ca^{2+} concentration in the cytoplasm due to leakage and inactivation of calcium pumps, which activates PLD, the key enzyme involved in membrane lipid catabolism. This in turn has been proposed to drive the autocatalytic cascade of lipid catabolic reactions leading to progressive degradation of the lipid membranes (Paliyath and Droillard, 1992). Subsequent studies on PLD (Wang, 2001) have revealed several isoforms of PLD, such as PLD-α, PLD-β and PLD-γ, which are involved in several interrelated processes and are activated by different levels of calcium (Paliyath and Murr, 2008).

N-Glycoproteins also play an important role in various physiological processes of ripening. (See Section 2.5.2 for more details regarding the role played by N-glycoproteins). Most of the technologies used to enhance the shelf life of fruit have focused on ethylene biosynthesis and cell wall degradation pathways to delay the ripening process. However, inhibition of lipid metabolic pathways has also been used to inhibit PLD in order to enhance the shelf life of fruits, vegetables and flowers (Paliyath et al., 2003). (See Section 2.8 for more details on hexanal and PLD). At present, several studies are being conducted to study the ability of hexanal based technologies in enhancing post-harvest characteristics of fruits and vegetables.

The main objective of this study was to determine the effect of a pre-harvest spray of Enhanced Freshness Formulation, a hexanal based formulation, in enhancing post-harvest shelf life of Fantasia nectarine. Further, we studied the expression pattern of 22 genes, encoding proteins involved in lipid and cell wall metabolism, to better understand the mechanism of regulation of ripening by the hexanal formulation on nectarine fruit.

3.2 Materials and Methods

3.2.1 Trial Locations

Experiments were conducted at two commercial nectarine orchards in the Niagara region of Ontario, to determine the effect of the EFF as a pre-harvest spray in enhancing post-harvest shelf life of 'Fantasia' nectarine. The nectarine orchard sites were located at Beamsville, ON (Site A) (43°11'32"N, 79°29'3"W) and Jordan, ON (Site B) (43°10'14"N, 79°90'54"W). The trees at Site A were ~15 of age and the trees at Site B were 5-6 years of age.

3.2.2 Plant Material and Pre-harvest Treatments

The 'Fantasia' nectarine trees grafted on Bailey rootstocks received two pre-harvest sprays of EFF approximately 15 and 10 days prior to harvest. The experiment was arranged as a randomized complete block design with four blocks (replicates) and two treatments consisting of a water control and EFF (0.02% v/v hexanal), as described previously (El Kayal et al., 2017; Anusuya et al., 2016; Misran et al., 2015). Each experimental unit consisted of 5 trees. Hence, the experiment was conducted on 40 trees in each farm (2 treatments*4 replicates*5 trees per experimental unit). The constituents of the Enhanced Freshness Formulation 2 are mentioned in section 2.8. The stock solution was mixed with water to provide 2% (1litre stock in 50 litres of water) and sprayed at the rate of approximately 5l per tree from both sides of the tree using a mechanical pickup truck mounted sprayer, custom built by Rittenhouse Sprayers, St. Catharines, ON. The sprayer consisted of a 5.5 hp Honda motor (Model DBO) with an adjustable pattern hand-held nozzle (green garde-JD9-C) and was calibrated with a standard pressure of 1378.95 KPa to deliver a uniform spray volume of 15 L per minute. Care was taken to ensure that all foliage and fruit received a good coating of the spray. Spray contamination was avoided by using alternate rows of trees for the experiment and a 2 tree gap between treatments in the same row of trees. The

trees at Site B were sprayed on 12th and 19th August and the trees at the Site A were sprayed on 15th and 22nd August 2016.

3.2.3 Fruit retention Studies

Two branches from each tree were tagged and the number of fruit on the branch were counted at regular intervals from the date of harvest. Care was taken to ensure uniform distribution of branches in the tree. Diseased and decaying branches were not used for this experiment. The retention time was measured as the number of days after commercial maturity that the trees are able to hold on to the fruit before they detach from the tree. Commercial maturity is defined as the maturity of the fruit at which it is harvested by the growers, such that the fruit are fresh and not over-ripe by the time they reach the market. Usually, commercial maturity occurs prior to horticultural maturity and is region specific. The commercial maturity date for 'Fantasia' nectarine was set at Sept 1st in 2016. Retention was calculated using the following formula at the different measurement dates and it was expressed in percentage.

$$Retention\ \% = 100 - [((initial\ fc - final\ fc)\ /\text{initial fc}) \text{ x } 100] \quad \text{fc = fruit count}$$

3.2.4 Harvest and Storage of Fruit

Fruit of similar size and developmental stage were harvested at commercial maturity. They were immediately transported to the post-harvest storage facility and stored at 2 ± 1°C. The fruit to be used in the study were sorted for uniform size, maturity and to remove damaged fruit. Three fruit from each experimental unit were randomly picked to collect data for each individual experiment and measurements were taken approximately every 5 days to measure chilling injury disorders and firmness. Samples were taken every 15 days for measurements of TSS and TA. Samples were taken every 30 days for color measurements. Samples for gene expression studies were taken at -20, -10, 0, 10, 20 and 30 days post-harvest.

3.2.5 Standard Quality Assessment

3.2.5.1 Colour

The peel colour of the fruit was measured at 0, 20, 40 and 60 d after harvest using a Minolta Chroma meter (Konica Minolta Sensing Americas Inc., NJ, USA) and was expressed according to the *CIE Lab* System (*L*-rightness, *a*-red/green and *b*-yellow/blue). Anthocyanin (bright red colour) and background colour (yellow colour) of 3 individual fruit per replicate were measured to provide a comprehensive picture of the changes in fruit colour of nectarine. The L, a and b values were then converted to *L, C, H°* [(lightless indicative of brightness), Chroma, a measure of colour clarity $(a2 + b2)1/2$, and hue angle $(tan - 1(b/a))$] using a software available at http://www.easyrgb.com.

3.2.5.2 Firmness

A penetrometer (Effegi pressure tester, Facchini 48011, Alfonsine, Italy) with a 10mm diameter tip was used to measure firmness. The tip was pushed into the fruit after removal of a small patch of skin, and a standard pressure was used to puncture the fruit to obtain uniform application of force and firmness. Firmness measurements were taken on both the cheeks of every fruit. The firmness reading was measured in N force.

3.2.5.3 Total Soluble solids

A prism refractometer (Fischer Scientific, Mississauga, Canada) was used for measurement of TSS. Frozen nectarine tissue (-20°C) was allowed to thaw and reach room temperature. Nectarine juice was then extracted from fruit using a lemon press with a cheesecloth filter and used for the soluble solids measurement and expressed as % soluble solids.

3.2.5.4 Titratable Acidity

The protocol established by Garner et al. from UC Davis, California, USA (http://fruitandnuteducation.ucdavis.edu/files/162035.pdf) was used with modifications to estimate the titratable acidity in the sample. Frozen nectarine tissue (-20°C) was allowed to thaw and reach room temperature (25°C). Nectarine juice was then extracted from fruit using a lemon press fitted with a cheesecloth filter. Two grams of the juice was added to 48 mL of water in a 100 mL beaker. The initial pH of the sample was recorded using a pH meter. The sample was titrated against a 0.1 N solution of NaOH until the pH of the sample reached 8.1. The amount of NaOH used in the reaction was also recorded. The titratable acidity was calculated using the following formula and expressed as g/L of malic acid.

$$\%\, TA = [(mL\ of\ NaOH\ used) \text{ x } (0.1\ N\ NaOH) \text{ x } (milliequivalent\ factor) \times (100)]/(grams\ of\ sample)$$

3.2.6 Chilling Injury Assessment

Fruit stored at 2°C were visually assessed for incidence of physiological disorders such as mealiness and internal browning at regular intervals of 5-6 days, immediately after removal from cold storage. The mealiness symptoms were observed through presence of thread formation in the fruit flesh and lack of juice when squeezed. In order to fully visualize the internal browning symptoms, fruit peel were removed in order to observe its onset, as they were not evident when the fruit was cut in half. If 25-50% of the fruit was damaged, half the fruit was considered to be affected by the chilling injury symptom. If more than 50% of the fruit was damaged, the whole fruit was considered to be affected by the physiological disorder. Based on this premise, % mealiness and % internal browning were calculated to express the onset of incidence of physiological disorders.

3.2.7 RNA extraction and gene expression assays

Nectarine fruit samples for gene expression studies were collected at 20 and 10 days pre-harvest and 0, 10, 20 and 30d post-harvest, frozen in liquid N_2 and stored at -80°C for further analyses. Samples were ground using an arbor press and 2 g of RNA was used for extraction. Total RNA extraction and cDNA synthesis were performed as described by El-Sharkawy et al. (2014). A total of 23 genes which represent a combination of biological processes occurring during fruit development, including ripening and senescence, were used for quantitative reverse transcriptase PCR (qRT-PCR). Seven of these genes represented PLD, a major class of genes and the key enzymes which regulate the cascade of reactions that result in membrane degradation and softening of the fruit (International Peach Genome Initiative, 2013). The other four groups were N-glycoproteins (α-mannosidase, β-hexosaminidase and xyloglucan endotransglycosylase-7 genes), expansins (3 genes), pectin esterases (2 genes) and polygalacturonase (1 gene). In order to develop a comprehensive picture of the transcriptional response occurring during ripening, a representative from the following groups were also added to the gene expression mix: ascorbate oxidase and α-L-arabinofuranosidase. Gene-specific primers were designed using Primer Expression (v3.0, Applied Biosystems, Carlsbad, CA, USA) (Table 3.1). Primer pairs were validated using the serial dilution technique and only primers which had more than 90% efficiency were used for the experiment. qRT-PCR was conducted in a 10 μL volume, consisting of 5 μL of SYBR Green master mix [0.2 mM dNTPs, 0.3U Platinum Taq Polymerase (Invitrogen, Burlington, ON, Canada), 0.25 x SYBR Green, and 0.1 x ROX], 50 ng of cDNA and 400 nM of each primer. Three biological replicates for each sample were analyzed in triplicate on a CFX96 Real-Time PCR Detection System (Biorad, Mississauga, ON, Canada) with the protocol described by El Kayal et al. (2017). A geometric mean of two genes, glyceraldehyde 3-phosphate (GAPDH-

XM_007222412.1) and polyubiquitin (UBQ-XM_007211547.1:77-1450) were selected as endogenous controls. They were selected based on experiments conducted by Tong et al. (2009) and were validated for reliability, since their expression did not show significant changes between treatments. The sample's gene expression was normalized to the endogenous controls, and to the calibrator sample to obtain relative changes in gene expression using the $2^{-\Delta\Delta Ct}$ procedure (Livak and Schmittgen, 2001).

Table 3.1 Genes and gene specific primers used for qRT-PCR in this study.

Primer name	Sequence	Accession number	Gene
αPLD1-Fw	TATGCAACCGTTGATCTGGA	NC_034011.1	α Phospholipase D
αPLD1-Rv	CCAAAATCTCAGCCCACCTA		
αPLD4-Fw	ATGCTTTGGGATGACGAAAC	NC_034011.1	
αPLD4-Rv	GGTTTTCTGATGGTGGGAGA		
βPLD-Fw	GGGAGCAGCATTTTGATGTT	NC_034013.1	β Phospholipase D
βPLD-Rv	GCTGGCATTAAGGATTGGAA		
γPLD-Fw	TTCCCATAGTGGTCCTCTGC	NC_034010.1	γ Phospholipase D
γPLD-Rv	GAGTGAGGAGGCACGAGTTC		
δPLD-Fw	GGCCGGAATATAACGGAGTT	NC_034009.1	δ Phospholipase D
δPLD-Rv	TTCCCAGCACTTTCTTTGCT		
ζPLD1-Fw	TAAAATCGGGCACACAACAA	NC_034009.1	ζ Phospholipase D
ζPLD1-Rv	TCGCTCTCATTGAACACAGG		
ζPLD2-Fw	CACGGTTGAACACAAAGTGG	NC_034014.1	
ζPLD2-Rv	AAGGGCACATTGGACATCAT		
XTH1-Fw	TCTTCGTGGACGACATACCA	NC_034009.1	Xyloglucan endotransglucosylase/hydrolase
XTH1-Rv	TTGGACCAATCGGTCTTTTC		
XTH2-Fw	TCAGCGCATCATATTCTTGG	NC_034011.1	
XTH2-Rv	TCTTCACAAGGCCACCTCTT		
αMAN1-Fw	CCATAGAAGTTGGGCAAGGA	NC_034011.1	α mannosidase
αMAN1-Rv	AGGCCTGAGGATCTCTGTCA		
αMAN2-Fw	CTCCAAGTGTGACCCCAAGT	NC_034013.1	
αMAN2-Rv	TTCCTGGCCCAACTTCTATG		
αMAN3-Fw	ATATTATTTGGCGGCACGAG	NC_034015.1	
αMAN3-Rv	AGGCGCTTTGCATAGTCATT		
αMAN4-Fw	GACTCTTGGGCATCGGTTTA	NC_034011.1	
αMAN4-Rv	TTTTGCGTTTATCCCTGTCC		
PG4-Fw	CAAAATGCTCTCCCAGTCGT	NC_034013.1	Polygalacturonase
PG4-Rv	GGCGATCTTGATCCTGTCAT		

Primer Name	Sequence	Accession Number	Gene
EXP-A1-Fw	GTGGGTCTTCTTGCAATGGT	NC_034009.1	Expansin
EXP-A1-Rv	TATTTGTCCCGTACCCCTGA		
EXP-A8-Fw	TCACTGCCACAAACTTCTGC	NC_034010.1	
EXP-A8-Rv	CCCTCCCTTTTTCACACAAG		
EXP-A4-Fw	GCATGTGGGTATGGGAACTT	NC_034014.1	
EXP-A4-Rv	AATGATGCTTCCAGGACGAC		
PE2-Fw	AACGCCAATTCTACCGTGAG	NC_034009.1	Pectin esterase
PE2-Rv	CACGCGAGCTATGGATAACA		
PE3-Fw	GGTGCAGCTGTGCTACAAAA	NC_034014.1	
PE3-Rv	TATGTTGGCTGAGTGGCAAG		
AO1-Fw	AAGCTGGCGGACTACTTCAA	NC_034009.1	Ascorbate oxidase
AO1-Rv	TGCCAGCTCTGAATGATGTC		
AFS1-Fw	ACGTGCGTATCCAGACATCA	NC_034015.1	α-L-Arabino-furanosidase
AFS1-Rv	CAAAAGCCTTGGGACCACTA		
βHAM2-Fw	GTGGCCAGATGGAGTTGATT	NC_034010.1	β hexosaminidase
βHAM2-Rv	CTCCAGCGTGGTAAAATGGT		

3.2.8 Statistical Analysis

Data were collected for analysis of quality parameters of the fruit and was analyzed using repeated measures ANOVA in SAS (SAS Version 9.4, SAS Institute, Raleigh, NC) using the PROC GLIMMIX (Generalized Linear Mixed Model) procedure. An F test was used to test the equality of the variance of the fixed effects and a likelihood ratio test to validate the significance of the random effects. The variation was partitioned into fixed effects of treatment, day and their combination, and the random effects of block, site and block*site*treatment. 'Day' was considered as the repeated measure sequence in the analysis. Based on the data obtained, a suitable distribution function was fit for the analysis. The distribution function was also selected based on fit statistic values (lower AICC value). The spatial power (sp (pow)) covariance type was used for most of the analyses such as firmness, TSS, TA and physiological disorders assessment data, as was able to account for the unequal spacing of time measures, homogenous error variances and an exponential decline in correlation over time. The colour measurements and gene expression studies were equally spaced, hence, the compound symmetric (cs) distribution function which accounts for homogenous variance and constant correlations was used. Error assumptions of the variance analysis (random, homogenous, normal distribution of error) were tested using the studentized residual plots and the Shapiro-Wilk normality test. In order to fit the assumptions of ANOVA, some of the measurements were log transformed before analysis. In those cases, back-transformed values have been provided in parenthesis or mentioned in the legend. Means for the analyses was determined using the LSMEANS statement and means separation conducted using Tukey-Kramer adjusted multiple means comparison test. Outliers were identified using the studentized residuals to check for deviation by more than ± 3.4 from the expected values. The type 1 error rate was set at 0.05 for all statistical comparisons.

3.3 Results

3.3.1 Fruit Retention Studies

The retention time was measured as the number of days after commercial maturity that the trees are able to hold on to the fruit before they detach from the tree. The difference in retention times was measured and expressed as a percentage. Figs 3.1 and 3.2 illustrate the fruit drop in the different treatments during the retention experiments.

Fig 3.1 Effect of sequential pre-harvest applications of 2% Enhanced Freshness Formulation (EFF) on retention of fruit on the trees in 'Fantasia' nectarine cultivar (*Prunus persica* [L.] Batsch var. *nectarina*), 23 days after harvest maturity, conducted at Site A. L-Control, R-EFF.

Fig 3.2 Effect of sequential pre-harvest applications of 2% Enhanced Freshness Formulation (EFF) on retention of fruit on the trees in 'Fantasia' nectarine cultivar (*Prunus persica* [L.] Batsch var. *nectarina*), 28 after harvest maturity, conducted at Site B. L- Control, R- EFF

Table 3.2 Effect of sequential pre-harvest applications of 2% Enhanced Freshness Formulation (EFF) on retention of fruit on the trees in 'Fantasia' nectarine cultivar (*Prunus persica* [L.] Batsch var. *nectarina*) conducted at two orchards in the Niagara region, ON.

Days after Harvest	Retention of fruit on the tree (%)-Site A	
	Control	EFF
10	78 ± 6.4[a]A[b]a[c]	93 ± 6.4Aa
15	65 ± 7.1Aa	82 ± 6.4Aa
	Retention of fruit on the tree (%)-Site B	
15	93 ± 2.2[a]A[b]a[c]	91 ± 2.2Aa
20	82 ± 2.2Ba	78 ± 2.2Ba

[a]Values represent the mean ± standard error of retention percentage of five trees.
[b]Means within columns followed by the same upper-case letter(s) for each experimental run are not significantly different based on Tukey's means comparison at α= 0.05.
[c]Means within rows followed by the same lower-case letter(s) are not significantly different based on Tukey's means comparison at α= 0.05.

For unknown reasons, the trends in retention of fruit were quite different in both the sites, hence they were analyzed separately due to the significant effect of site (P = 0.0001). At both Site A and Site B, there was no difference observed between the treatments (Table 3.2).

3.3.2 Firmness

The results showed a significant effect of the repeated measure 'days post-harvest' (P < 0.0001) and treatment (P = 0.0009). The day*treatment effect was not significant indicating the consistency of the trends seen in the firmness results. This also shows that there was no interaction between the treatments and storage period. Fruit firmness decreased over time in both treatments, but EFF treated fruit always maintained a significantly higher firmness level at most time points of at least 5 N/cm from 8d post-harvest (Table 3.3). The benefit of the EFF treatment was maintained, until about d 38 post-harvest. Firmness levels were not altered thereafter (Table 3.3).

Table 3.3 Effect of sequential pre-harvest applications of 2% Enhanced Freshness Formulation (EFF) on firmness of 'Fantasia' nectarine fruit tested at two commercial orchards in the Niagara region and stored at 2°C.

Days post-harvest	Firmness (N)	
	Control	EFF
0	60.0 ± 1.77[a]a[b]	65.3 ± 1.77a
3	58.7 ± 1.77a	63.6 ± 1.77a
8	60.4 ± 1.77b	67.6 ± 1.77a
14	57.8 ± 1.77b	64.9 ±1.77a
20	55.6 ± 1.77a	60.4 ± 1.77a
26	55.1 ± 1.91b	63.1 ± 1.91a
32	53.3 ± 1.91b	59.6 ± 1.77a
38	52.4 ± 1.91b	59.6 ± 1.77a
45	43.7 ± 1.77a	49.3 ± 1.91a

[a]Values expressed as mean ± standard error and represent means of four replicates.
[b]Means within rows followed by the same lower case letter(s) are not significantly different based on Tukey's means comparison at $\alpha = 0.05$.

3.3.3 Assessment of internal browning

There was a highly significant effect of the repeated measure 'days post-harvest' ($P < 0.0001$) and treatment ($P < 0.0001$) on the internal browning parameter, compared to the control. EFF treatments delayed the onset of internal browning symptoms by 7- and 13d at Sites A and B respectively, as compared to the control. The magnitude and onset of internal browning in EFF treated fruit was also significantly lower than their control counterparts (Fig 3.3, Table 3.4). Approximately 90% of control fruit were affected by internal browning at d 45 of storage in comparison to only 40% in the EFF treated fruit. Peripheral browning was more prevalent than inner tissue browning and the spread of browning in affected fruit was also uneven (Fig 3.3).

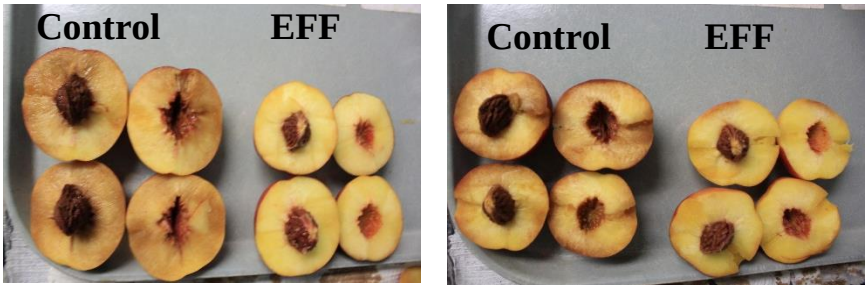


Fig 3.3 Incidence of internal browning symptoms in nectarine fruit harvested from Site A (L) and Site B (R) and stored at 2°C for 58 and 54 days respectively.

Table 3.4 Effect of sequential pre-harvest applications of 2% Enhanced Freshness Formulation (EFF) on incidence of internal browning and mealiness in 'Fantasia' nectarine fruit (*Prunus persica* [L.] Batsch var. *nectarina*) tested at two commercial orchards in the Niagara region and stored at 2°C.

Days post-harvest[a]	Control	EFF
	% fruits exhibiting internal browning (Sample size: 12 fruit)	
32	25.0 ± 14.00[b]a[c]	-
38	41.6 ± 10.67a	8.3 ± 10.67b
45	10.0 ± 10.67a	29.1 ± 10.67b
53	10.5 ± 10.67a	41.6 ± 10.67b
	% of fruits exhibiting mealiness (Sample size: 12 fruit)	
45	62.5 ± 6.34a	-
53	62.5 ± 6.34a	8.3 ± 6.34b
58	95.8 ± 6.34a	29.1 ± 6.34b

[a]The incidence of internal browning and mealiness occurred at different time points and hence, the variation in the days at which the measurements are represented. The experiment was concluded when 90% or more of the fruit were affected by the disorder.
[b]Values expressed as mean ± 1 standard error and represent a mean of 4 replicates. Values obtained are rounded to the nearest half or whole number.
[c]Means within rows followed by the same lower case letter(s) are not significantly different based on Tukey's means comparison at $\alpha = 0.05$.

3.3.4 Assessment of Mealiness

Visual symptoms of mealiness were first observed at 45 d after harvest following the incidence of visual internal browning symptoms. Mealiness could be observed through the formation of thread like leathery surfaces and lack of juice (Fig 3.4). EFF treatments significantly delayed the onset of mealiness symptoms by 8 d at both orchard locations, as compared to the control ($P < 0.0001$). The magnitude and onset of mealiness in EFF treated fruit was also significantly lower than their control counterparts (Fig 3.4, Table 3.4). At d 53 of storage, EFF showed a percentage decrease in incidence of mealiness symptoms by 86%, as compared to the control. At 58d post-harvest, greater than 95% of the control fruit had high levels of mealiness while only 30% of EFF treated fruit showed symptoms of mealiness.

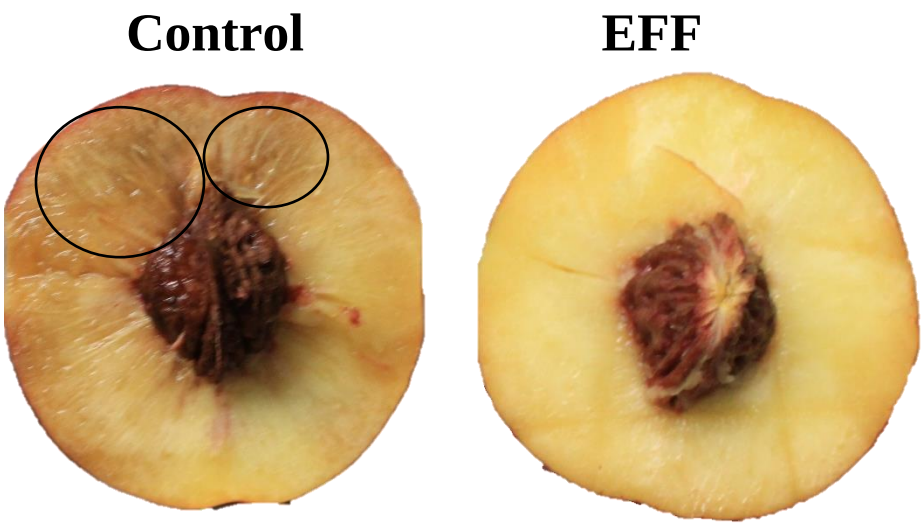


Fig 3.4 Incidence of mealiness symptoms in nectarine fruit harvested from Site A and stored at 2°C for 58 days. Note the formation of thread like leathery structures in the control fruit (black circles).

3.3.5 Quality parameters-TA, TSS and Colour

TA did not vary between treatments. However, there was a gradual decline in acidity of the fruit in all treatments (Table 3.5). The TSS did not change over both the post-harvest measurement period and between treatments for the most part (Table 3.5). Similarly, background colour measurement of lightness, hue and chroma also did not change over time and between treatments (data not shown). However, the lightness and hue angle of peel colour of the fruit gradually declined over the measurement period, even though there were minimal differences between the treatments. The chromaticity parameter of the anthocyanin colour remained constant for 30 d post-harvest and slightly decreased following that time point. Overall, there were no differences between the treatment and control in the TSS, TA and colour measurements.

Table 3.5 Effect of sequential pre-harvest applications of 2% Enhanced Freshness Formulation (EFF) on titratable acidity, total soluble solids and anthocyanin colour in 'Fantasia' nectarine fruit (*Prunus persica* [L.] Batsch var. *nectarina*) tested at two commercial orchards in the Niagara region and stored at 2°C.

Days post-harvest	Control	EFF
	Titratable acidity (g/100mL malic acid)	
0	1.9 ± 0.13Aa	1.8 ± 0.13Aa
15	1.7 ± 0.13Aa	1.4 ± 0.13Bb
30	1.2 ± 0.13Ba	1.1 ± 0.13Ca
45	0.8 ± 0.13Ca	0.9 ± 0.13Ca
	TSS (°Brix)	
0	10.8 ± 0.16Aa	11.0 ± 0.16Aa
15	10.7 ± 0.16Aa	10.7 ± 0.16Aa
30	10.7 ± 0.16Aa	10.7 ± 0.16Aa
45	10.8 ± 0.19Aa	10.5 ± 0.16Ba
	Anthocyanin colour-Lightness (L°)	
0	3.35 ± 0.025Aa (28.6)[d]	3.30 ± 0.025Aa (27.1)
30	3.27 ± 0.025Ba (26.4)	3.22 ± 0.025Ba (25.0)
60	3.20 ± 0.025Ca (24.7)	3.20 ± 0.025Ba (24.7)
	Anthocyanin colour-Chroma (C)	
0	3.50 ± 0.034Aa (33.2)	3.42 ± 0.034Aa (30.7)
30	3.50 ± 0.034Aa (33.3)	3.43 ± 0.034Aa (30.9)
60	3.34 ± 0.034Ba (28.2)	3.32 ± 0.034Ba (27.7)
Days post-harvest	Anthocyanin colour-Hue Angle (H°)	
0	2.79 ± 0.036Aa (16.4)	2.77 ± 0.036Aa (16.0)
30	2.73 ± 0.036Ba (15.3)	2.67 ± 0.036Ba (14.4)
60	2.62 ± 0.036Ca (13.7)	2.62 ± 0.034Ca (13.7)

[a]Values expressed as mean ± 1 standard error and represent means of four replicates.
[b]Means within columns followed by the same upper-case letter(s) for each experimental run are not significantly different based on Tukey's means comparison at $\alpha = 0.05$.
[c]Means within rows followed by the same lower-case letter(s) are not significantly different based on Tukey's means comparison at $\alpha = 0.05$.
[d]Values were log transformed to fit the assumption of ANOVA. Back transformed values are presented in parenthesis.

3.3.6 Gene expression during pre- and post-harvest stages of ripening

Gene expression pattern was monitored for a set of 24 genes which encode for cell wall and membrane modifying enzymes (Table 3.1) using qRT-PCR. The gene expression studies were conducted on both pre-harvest (-20 and -10d) and post-harvest (0, 10, 20 and 30d) time points. Both the control and EFF treatments were used for the experiment.

Based on the expression pattern during pre- and post-harvest stages of nectarine fruit ripening, 11 genes were selected and separated into four categories. Transcript levels at all storage time points were expressed relative to their level at the d after harvest. The first category consisted of *αPLD4* and *ζPLD2*, both of which exhibited a transcript abundance at 20 d post-harvest (between 25-50-fold change relative to control) (Fig 3.5A). The second category consisting of γPLD and ζPLD1 and PE2 genes, where a 5-8-fold change in transcripts was apparent by d 20 of storage in EFF- relative to control fruit sampled at harvest (Fig 3.5B). The third category consisted of two genes that had a distinct expression pattern. Xyloglucan endotransglucosylase (*XTH1*) transcript levels in fruit sampled 10 d prior to harvest were 30-35-fold higher than those at harvest, and declined to negligible levels thereafter (Fig 3.5C). α-L-Arabinofuranosidase (*AFS1*) expression was at near zero levels until 0d post-harvest and increased consistently during the following time period of storage, and peaked at 30d post-harvest (fold change between 45-50), (Fig 3.5C). The fourth category consisted of genes which did not show any marked change in their expression until they reached very low levels (near zero) at 30d post-harvest (fold change less than 0.01) (Fig 3.5D). The rest of the genes whose transcript levels did not show any significant difference are presented in Fig 3.6.

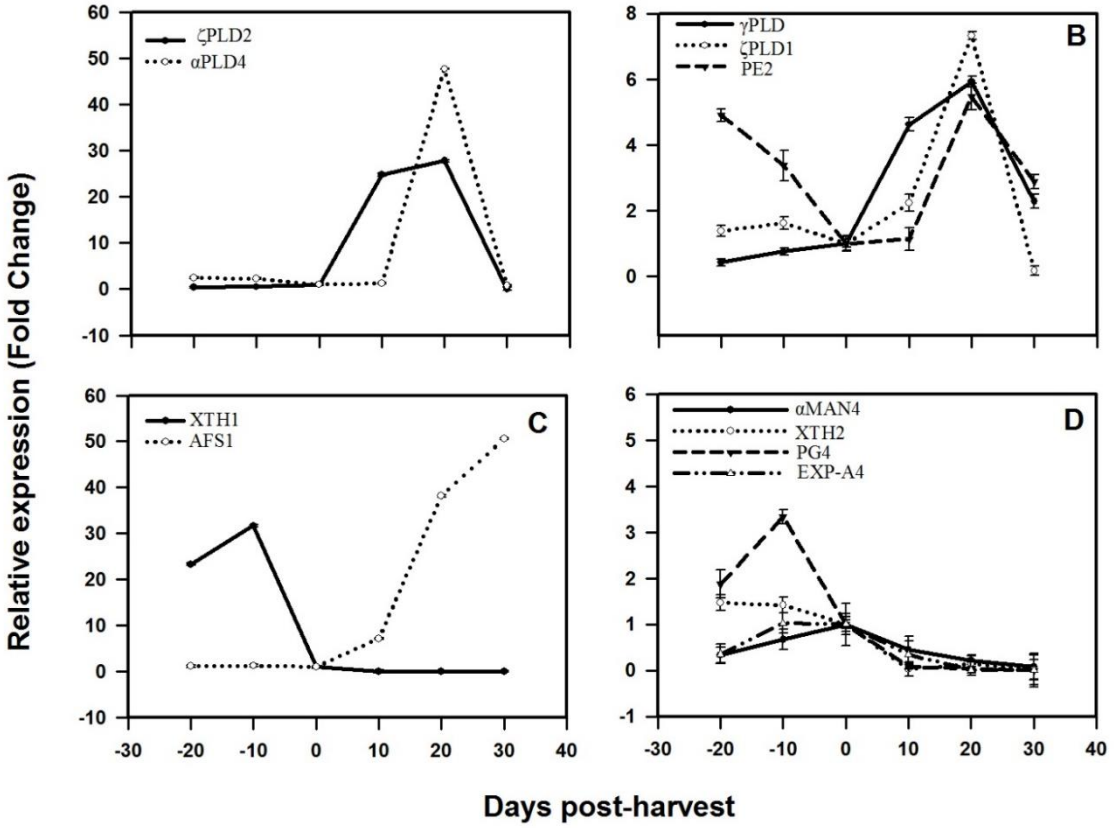


Fig 3.5 Relative expression of selected genes (relative to their 0-day expression), that showed significant changes during developmental and ripening stages of nectarine fruit sprayed with EFF formulation. All times points are significantly different from their respective 0 day expression except for the following *αPLD4* (-10d), *γPLD* (-20, -10, 30d), *ζPLD1* (-20), *PE2* (-10, 10, 30d), *AFS1* (-20,-10d), *XTH2* (-20,-10d), *PG4* (-20d), *EXP-A4* (-10d). In Fig. D, the 30d post-harvest time period witnessed significant decrease in expression of all the genes presented (<0.01).

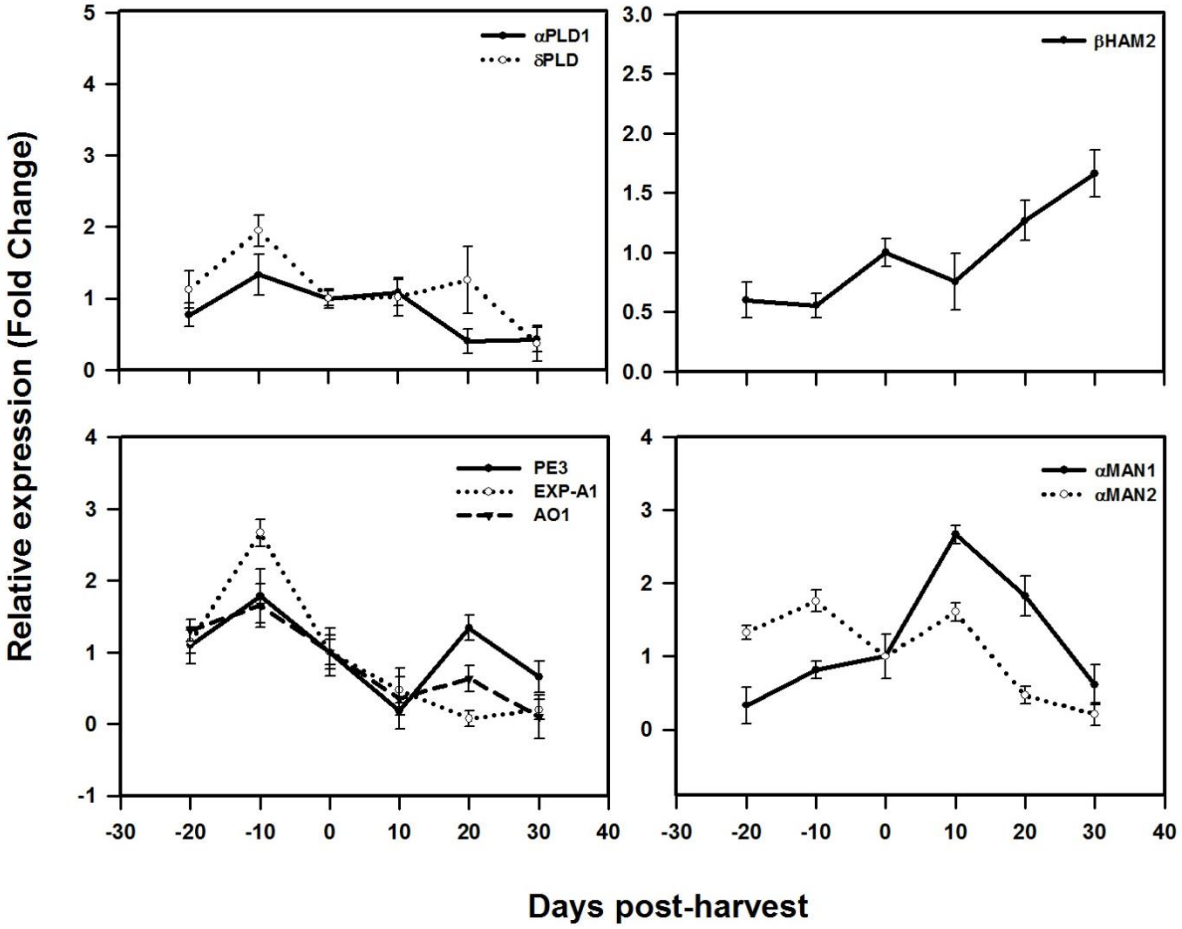


Fig 3.6 Relative expression of selected genes (relative to their 0-day expression), that showed insignificant changes during developmental and ripening stages (fold change less than 3) of nectarine fruit sprayed with EFF formulation

3.3.7 Regulation of ripening by the hexanal formulation

In order to understand the effect of the hexanal formulation on the expression pattern of ripening related genes, the fold change of control and treated fruit at every time point relative to their -20d base expression, were plotted next to each other (Fig 3.7, 3.8). Twelve genes were selected based on significant differences between the treatments (Fig 3.8). The other genes which did not respond to the hexanal treatment are shown in the Fig 3.7. Most of the genes except *XTH1* and *XTH2* were downregulated during the post-harvest stage.

Three PLD genes were significantly downregulated by hexanal formulation application at different post-harvest stages. *αPLD4* and *γPLD* were significantly downregulated at 20d post-harvest ($P < 0.05$ and $P < 0.01$ respectively) while *ζPLD2* was downregulated at the 10d post-harvest stage ($P < 0.05$) (Fig 3.8).

α-mannosidase genes (*αMAN1 and αMAN4*) were downregulated at all time points of measurement between -10d pre-harvest until 30d post-harvest. The genes β-hexosaminidase (*βHAM2*) and α-L-arabinofuranosidase (*AFS1*), both exhibited significant downregulation at the 20d and 30d post-harvest stages ($P < 0.01$ and $P < 0.05$ respectively). The transcript abundance of xyloglucan endotransglucosylases (*XTH1 and XTH2*) showed a significant reduction at the -10d and 0d post-harvest stages respectively ($P < 0.05$) (Fig 3.8), while expansins (*EXP-A1* and *EXP-A8*) were significantly downregulated at the 0d and 10d post-harvest stages respectively ($P < 0.01$ and $P < 0.05$ respectively) (Fig 3.8).

Interestingly, there was a highly significant upregulation of *γPLD* and *XTH1* genes at 30d post-harvest ($P < 0.01$ and $P < 0.0001$ respectively). However, the global pattern of regulation of gene expression was downregulation at varying time points from 0d until 30d post-harvest, indicating a potential delay or inhibition of certain ripening related genes, thus resulting in enhanced shelf life of the nectarine fruit.

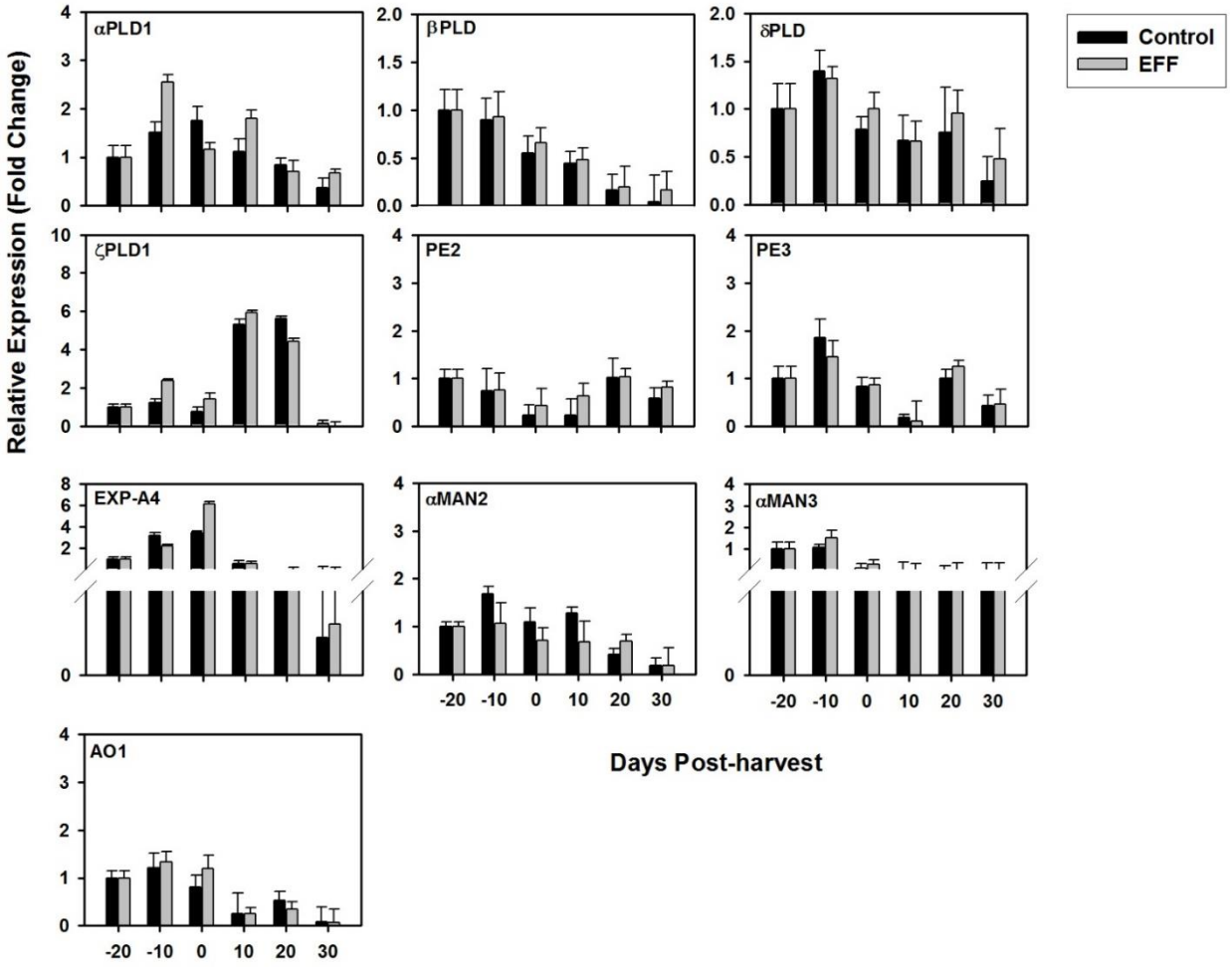


Fig 3.7 Relative expression of PLD genes and other selected genes that showed insignificant changes during pre- and post-harvest stages of development. The expression of genes is normalized to their -20 day expression. Bars represent the means ± standard error of three biological and three technical replicates each.

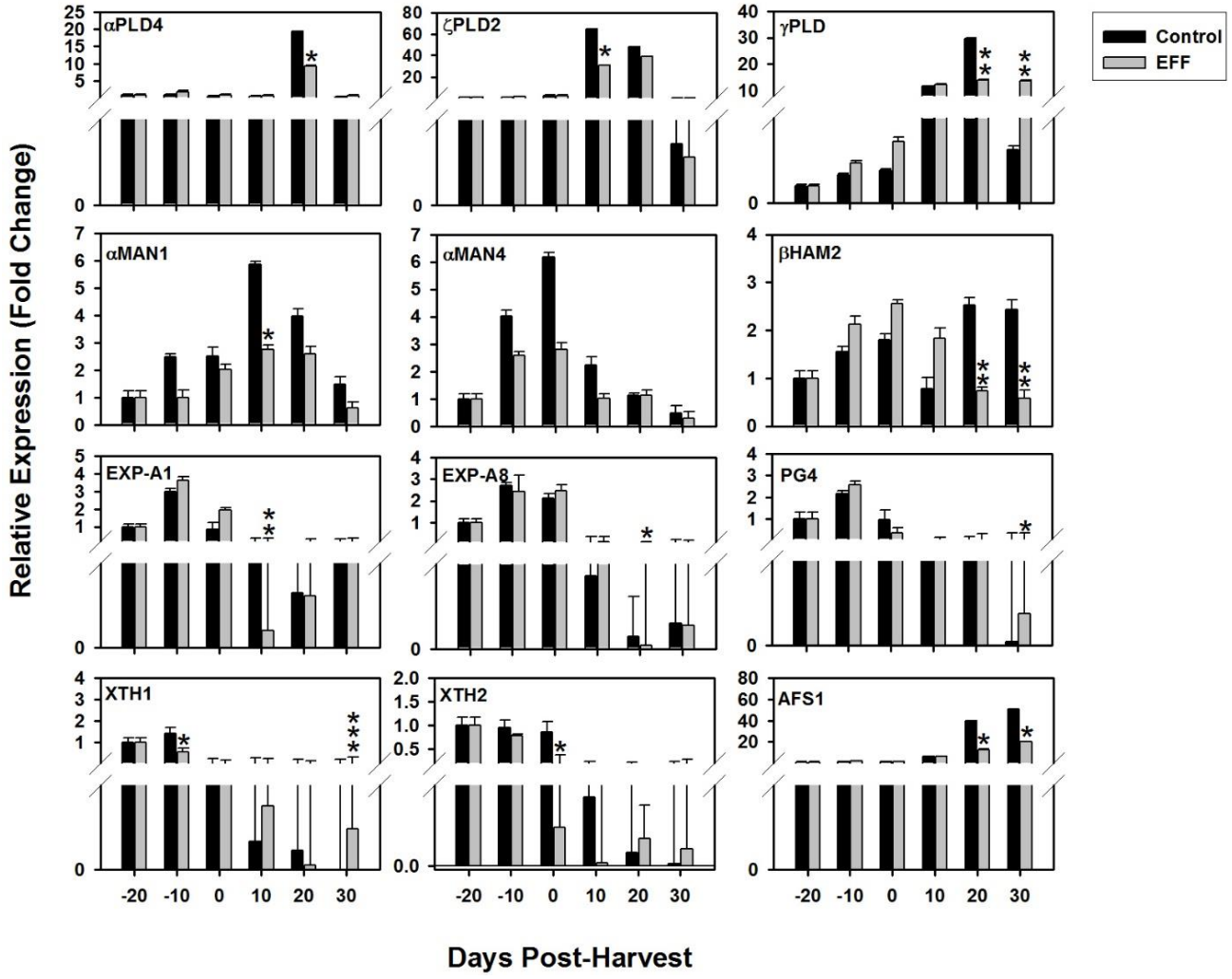


Fig 3.8 Relative expression of PLD genes and other selected genes that showed significant changes during pre- and post-harvest stages of development. The expression of genes is normalized to their -20 day expression. Bars represent the means ± standard error of three biological and three technical replicates each.

3.4 Discussion

Peach and nectarine are highly perishable soft fleshed fruit with a short market life. Cold storage at near 0°C has been integrated into the post-harvest storage chain to enhance shelf life and ensure the freshness of fruit until they reach the market (Crisosto and Valero, 2008). However, the fruits are susceptible to the development of chilling injury symptoms such as internal browning and mealiness, thus reducing the taste, flavor and consumer acceptance of these fruit (Lurie and Crisosto, 2005). In this study, EFF treatment drastically reduced the magnitude and onset of these physiological disorders. This is consistent with hexanal enhancing shelf life and reducing post-harvest decay and disorders in strawberries, mango, guava, tomato and sweet cherries (El Kayal et al., 2017; Anusuya et al., 2016; Gill et al., 2015; Cheema et al., 2014; Sharma et al., 2010).

Progress in ripening and senescence leading to tissue deterioration and changes in membrane permeability results in leakage of compartmentalized phenolic components, leading to a reaction between the phenols and polyphenol oxidase, generally present in different parts of the cell (Lurie and Crisosto, 2005). The reaction between these compounds catalyzed by PPO results in the development of internal browning. Hexanal, the key ingredient of EFF delays the degradation of the lipid membranes by inhibiting PLD and related genes, thus retaining membrane integrity (Paliyath and Murr, 2003). In this study, EFF delayed the onset of browning, and reduced its incidence and severity thereafter. Further research into the relation between hexanal and the browning related pathways is necessary to understand the mechanism of action of hexanal in reducing the browning symptoms exhibited by the fruit.

EFF-treated fruit consistently maintained higher levels of firmness beginning from d 8 until 38 d of storage. This could be due to the effect of hexanal, which inhibited the expression of PLD and other ripening related genes, thus ensuring membrane integrity and enhancing shelf life. It is

interesting to note that a narrowing of the firmness range between the treatments was observed at 45d post-harvest or longer. This could be attributed to the development of mealiness in the control fruit which resulted in the formation of a leathery surface devoid of juice, and altering the readings of the penetrometer. Although, the EFF treated fruit maintained higher firmness and did not soften, they were affected by chilling injury symptoms rendering them unmarketable. This trend was also observed by Ben Arie and Sonego (1980), wherein the fruit did not soften to edible firmness during the entire storage period. Thus, the firmness data is reliable only until the onset of physiological disorders in fruit that are subjected to internal browning.

Unlike the severity of internal browning, the occurrence of mealiness symptoms was delayed and much less severe in EFF treated fruit. Crisosto and Labavitch (2002) determined that mealiness symptoms tend to precede, or occurred together with internal browning symptoms. This was in contrast with the current results obtained. This difference could be attributed to the fact that mealiness develops before the visual symptoms can be observed, thus potentially developing before internal browning starts. Externally, the fruit remains firm even after the onset of mealiness and internal browning, and is a property known as 'leatheriness' (Luza et al., 1992). The results from this study closely follow the pattern of occurrence of chilling injury symptoms described by Brummell et al. (2004) who observed that leatheriness is associated with a lack of free juice, in addition to other mealiness symptoms and increased browning.

The fruit retention studies exhibited different trends in both the orchards. Site B did not exhibit any difference in retention between treatments whereas Site A exhibited a statistically insignificant numerical increase in retention of fruit on trees sprayed with EFF formulation. Thus, EFF had no impact on fruit retention on trees sprayed in the orchard. These results are in contrast with superior retention of fruit on treated trees observed in mango (Anusuya et al., 2016). It was

expected that hexanal would have delayed abscission by dilution and slowing of peroxidases, RNA and protein synthesis in the abscission zone, as was observed by Goren (1993) for a similar shelf life enhancing organic compound. Further research into the molecular mechanism of abscission of nectarine fruit is necessary to understand the exact role of hexanal, if any, to delay abscission.

There were no differences in TSS levels between the treatments and these were comparable to previous studies on the same cultivar (DeEll et al., 2008). There are contrary views to development of sugars during maturation and ripening. Some studies have reported an increase in TSS over the storage period (Crisosto, 1994), whereas others indicate that TSS of peach and nectarine did not change after harvest (Powell et al., 1999). The present study followed the latter pattern as TSS values did not considerably increase during the storage period. There was a steady decrease in TA throughout storage, although there were no differences observed between the treatments. There was a considerable decrease in TA between 30 and 45d post-harvest, indicating the progression of ripening after an initial delay due to the effect of cold storage.

The background colour of the nectarine fruit did not change with storage or treatment, as was evident in the assessment of hue, chroma or lightness values. These results confirm observations made by Crisosto (1994), who concluded that background colour could be used as an effective maturity index for harvesting peach and nectarine, as they do not change during the storage period. The anthocyanin colour exhibited a different trend of slowly declining hue, chroma and lightness values over time. The decreasing hue angle indicated an increasing red intensity which could result from the development of anthocyanins in the skin of the fruit during ripening. This also resulted in reduced chroma and lightness values during the storage period. These results are in contrast with EFF spray studies conducted on other fruit such as tomatoes and sweet cherries, where the colour development was delayed in EFF treated fruit, indicative of freshness and delayed

ripening (Cheema et al., 2014, Sharma et al., 2010). However, the colour development in nectarine happened prior to and during harvest. (Crisosto, 1994). Only incremental changes could be seen during post-harvest storage. Hence, colour is not as important a parameter when it comes to indications regarding ripening and freshness of nectarine fruit.

Gene expression studies were conducted to better understand the molecular mechanisms behind the ripening process in nectarine fruit and regulation of these processes by the hexanal formulation. Paliyath and Subramanian (2008) described the maintenance of membrane integrity in several fruit due to hexanal application. In the present study, 22 genes encoding various classes of cell wall and membrane metabolism proteins, were analyzed including PLD. PLD is the key enzyme in the membrane degradation pathway (Paliyath and Droillard, 1992). PLD action generates phosphatidic acid which undergoes a set of tandem reactions catalyzed by enzymes such as phosphatidic acid phosphatase, lipolytic acyl hydrolase and lipoxygenase, finally resulting in membrane degradation and softening. However, none of the downstream enzymes can act on membrane phospholipid directly. Hence, inhibition of PLD could potentially result in a delay in the progression of the membrane lipid catabolic pathway (Paliyath and Murr, 2008). In the present study we explored the expression levels of several PLD genes including PLD α, β, γ, δ and ζ isoforms. The changes in expression levels of these genes provide potential insight into the role of PLDs in the initiation and propagation of senescence. The expression levels of *αPLD4* and *γPLD* showed progressive increase starting from ripening initiation to post harvest storage, peaked 20 days postharvest, and declined during further storage. Both *αPLD4* and *γPLD* are localized on plasma membrane and endomembranes, even though *αPLD* possesses only 40% sequence similarity when compared to that of *γPLD* (Paliyath et al., 2008). But, both possess a C2 domain that drive their membrane binding. By contrast, *ζPLD* possesses a Phox-Pleckstrin domain at their

N-terminal end. While *γPLD* is involved in catabolism of phospholipids, *ζPLD* is probably involved in metabolic processes. In contrast to these two isoforms, αPLD1 expression level was relatively low before harvest and continued to remain low during postharvest storage. In a similar way, the *δPLD* isoform, which is believed to be involved in hormone signaling and stress responses (Paliyath et al., 2008), also showed a lower expression, at all stages of development and storage. EFF treatments did not influence the expression levels of *αPLD1* and *δPLD* delta, which coupled with the downregulation of *αPLD4* and *γPLD*, could have translated into increased membrane preservation and enhanced shelf life. More studies are needed in terms of protein level and activity to delineate the function of the PLD isoforms.

The transcript level of the studied *βHAM2* gene exhibited upregulation during post-harvest ripening, whereas pre-harvest application of the hexanal formulation significantly downregulated their expression at 20d and 30d of storage. These results are consistent with studies conducted by Meli et al. (2010) on tomato and Cao et al. (2014) on peach, where *βHAM2* inhibition using either RNAi interference or a ripening inhibitor, resulted in delayed ripening and softening of the fruit. Similar to *βHAM2*, α-mannosidase genes have also been found to be upregulated during ripening, and their inhibition by hexanal results in delayed ripening as observed in strawberry (El Kayal et al., 2017). Similarly, xyloglucan endotransglucosylase was also downregulated by hexanal in fruit sampled 10 d prior to harvest and at harvest indicating a potential delay in their function, resulting in delayed ripening and softening.

Transcript levels of α-L-arabinofuranosidase dramatically increased during post-harvest storage, consistent with previous studies conducted by Di Santo et al. (2009). Hexanal increased *AFS1* transcript levels at 20 and 30 d of storage. Expansin genes which are involved in cell wall loosening (Rose et al., 1997; Brummel and Harpster, 2001) were found to be downregulated at 10

and 20 d post-harvest. The general trend for expansin gene expression included a slow decline to near zero transcript levels starting on d 10 of storage, consistent with previous findings that expansin levels decline during ripening prior to the visual observations of chilling injury symptoms such as mealiness (Obenland et al., 2002). Interestingly, hexanal's inhibition of expansin gene expression was limited to fruit stored for 10 d or longer. After d 20 of storage, the transcript level of treated fruit reached near control levels or higher. This trend was similar to the transcript levels of genes such as *γPLD, PG4* and *XTH*, were upregulated in hexanal treated fruit by d 30 of storage. It seems that hexanal's temporary downregulation during ripening and subsequent upregulation of transcript levels to near control levels or more helped to delay ripening, but at the same time, delay the development of chilling injury symptoms such as mealiness and internal browning. This is consistent with previous research, demonstrating that hexanal treated tomato fruit eventually soften, unlike fruit treated with 1-MCP which did not soften or develop all of the favorable characteristics of a ripe tomato fruit (Tiwari and Paliyath, 2011). Further research on this topic is necessary to understand the mechanisms underlying the delay of chilling injury symptoms induced by pre-harvest application of the hexanal formulation.

In conclusion, EFF treated fruit maintained a significantly higher firmness over the storage period of 45 d at 2°C. The EFF treated fruit also delayed the onset of mealiness and internal browning by ~ 8 d and reduced their incidence by almost 50% even at 45 days after harvest. There were no adverse effects in other fruit quality parameters such as TSS, TA and fruit colour. Gene expression studies also support the physico-chemical analyses, where three PLD genes, five N-Glycoprotein group genes and expansin genes among others, were downregulated indicating their potential regulation by hexanal. These results indicate the formulation's potential in enhancing shelf life of nectarine and reducing the incidence of chilling injury symptoms, to benefit both

growers and consumers, although further research regarding the molecular basis of its mode of action needs to be investigated.

Chapter 4

Role of pre-harvest hexanal based EFF in modulating the volatile profiles of nectarine fruit

4.1 Introduction

Nectarines are highly aromatic, soft-fleshed fruit belonging to Rosaceae. They are widely appreciated for their bright colour, smooth skin, unique taste, aroma and sweetness. Aromatic volatiles along with colour and firmness are the most important factors contributing to nectarine and peach quality according to consumers (Bruhn, 1995). Previous studies on nectarine fruit have revealed over 100 volatiles, including various alcohols, esters, terpenoids, ketones and lactones (Sevenants and Jennings, 1966; Do et al., 1969; Spencer et al., 1978; Engel et al., 1988; Takeoka et al., 1988; Horvat and Chapman, 1990; Horvat et al., 1990; Narain et al., 1990; Chapman et al., 1991), with each contributing to the overall flavor of the fruit.

During ripening, the lipid degradation pathway results in the production of many volatile and aromatic compounds, which contribute to flavor. The protein degradation pathway and secondary metabolism pathways (i.e. isoprenoid and polyphenolic pathways) also contribute to the production of volatile organic compounds. Each volatile compound is characterized by an odor threshold from a few parts per billion to several parts per million. Once the threshold has been surpassed, the compound contributes to the aroma. Regardless of whether the qualitative compositions of the fruit appear similar, their aroma properties might differ. Nectarine aroma is not attributable to one compound, but is considered to result from an integrated response of the olfactory organ to a series of flavor contributing compounds (Sevenants and Jennings, 1966). Please refer to section 2.7 for detailed information regarding contribution of major peach/nectarine volatiles to flavor and aroma.

Differences in volatile constituents are also found among cultivars. The concentrations of hexanal, E-2-hexenal, linalool, phellandrene, and 6-decalactone are significantly higher for white-fleshed cultivars than yellow-fleshed ones. In addition, nectarines produce more y-hexalactone, y-octalactone and 6-decalactone than peach (Engel et al., 1988; Horvat et al., 1990; Robertson et al., 1990a). Hence, there is a lot of variability among the concentrations of volatiles in nectarines.

From a post-harvest perspective, nectarines have a very limited shelf life. In ambient temperatures, they quickly deteriorate in quality and hence, cold storage has been used to extend the shelf life of these fruit (Lurie and Crisosto, 2005). However, this could result in chilling injury symptoms such as mealiness and internal browning wherein the flesh becomes leathery and flavorless, and the juice becomes bound, especially when fruit are stored in the temperature range of 2 °C to 8 °C (Lurie and Crisosto, 2005). In order to avoid these chilling-related disorders, many post-harvest technologies have been developed to enhance shelf life. For example, 1-MCP has been shown to increase the shelf life of peach and nectarine at ambient temperature (Hayama et al., 2005), however, it is ineffective at low temperatures and its use is limited in extending their post-harvest shelf life. Techniques such as controlled and modified atmosphere packaging have also been used with moderate success to enhance the shelf life of nectarine (Lurie, 1993).

Recent advances in enhancing the shelf life of fruit have resulted from the use of hexanal. Hexanal inhibits the enzyme PLD that is involved in lipid degradation and this results in the delay of cell membrane breakdown during ripening, thus resulting in delayed softening and improved shelf life (Paliyath et al., 2003). Hexanal has been used extensively to enhance shelf life of fruit and reduce post-harvest decay and disorders in strawberries, mango, guava, tomato and sweet cherries (El Kayal et al., 2017; Anusuya et al., 2016; Gill et al., 2015, Cheema et al., 2014 and Sharma et al., 2010). Application of a 2% (v/v) EFF results in a delayed in incidence of internal

browning and mealiness disorders in peach and nectarine (See section 3.3.3 and 3.3.4). Furthermore, EFF application reduced transcript levels of three PLD genes, five N-glycoprotein group genes, and other genes involved in ripening and softening processes (See section 3.3.7). In earlier studies, hexanal has been shown to decrease the abundance of several volatiles including esters, ketones and lactones in strawberries (Misran et. al., 2014).

Given the importance of nectarine fruit volatiles, this study was performed to understand the regulatory effects of 2% EFF, containing hexanal as the main ingredient. It is hypothesized that external application of a 2% EFF will increase and retain the internal hexanal concentration for a longer period of time, thus delaying the ripening process and enhancing the shelf life of nectarine. In the present study, we investigated the changes in volatile profiles due to the application of 2% EFF containing hexanal to enhance the post-harvest shelf life of 'Fantasia' nectarine. This study will help us to understand the changes in the production of volatile compounds, one of the most noticeable changes during nectarine ripening and an important quality factor influencing consumer acceptability.

4.2 Materials and Methods

4.2.1 Trial Location

Please refer to thesis section 3.2.1 for a description of trial location.

4.2.2 Plant Material and Pre-harvest Treatments

Please refer to thesis section 3.2.2 for a description of the plant material and pre-harvest treatments used. The only difference in the treatments was the use of a carrier treatment (EFF without hexanal) instead of the water control in order to separate the effect of hexanal from the EFF formulation.

4.2.3 Harvest and Storage of Fruit

Nectarine fruit of similar size and developmental stage were harvested at commercial maturity. They were immediately transported to the lab and stored at ambient temperature (25 °C). The fruit were sorted for uniform size and maturity, and to remove damaged fruit. Samples for volatile studies were obtained at 0, 3, 5, 7 and 11 d post-harvest.

4.2.4 Standard Quality Assessment

Colour, firmness, total soluble solids (TSS) and titratable acidity (TA) were measured according to procedures listed in thesis sections 3.2.5.2, 3.2.5.3, 3.2.5.4 and 3.2.5.5, respectively.

4.2.5 Volatile Concentration Estimation

4.2.5.1 Sample preparation

Nectarine fruit volatiles were collected and concentrated from chopped fruit similar to the method described by Tieman et al. (2006) using nonyl acetate as an internal standard for Gas Chromatography – Electron Ionization Mass Spectrometry (GC-EIMS) analysis, respectively. Chopped fruit (100 g) was enclosed in glass tubes, and filtered air (BHT-4 hydrocarbon trap; Agilent, Palo Alto, CA) flowed through the tubes over the samples for 1 h with collection of the volatile compounds on HayeSep Q resin (Sigma-Aldrich, St Louis, MO, USA). Volatiles were eluted from the HayeSep resin into a GC vial with dichloromethane after the addition of internal standards and subsequently separated and analyzed by GC-EIMS.

4.2.2 Targeted volatile profiling by GC-EIMS

Analysis of aroma volatile composition of nectarine was performed with a Bruker 436 gas chromatograph coupled to a Scion SQ mass detector fitted with an EI source (70 eV). An aliquot (1 μL) of the liquid sample was injected into the GC using an 8400 autosampler (Bruker, Milton,

ON, Canada). The injector was operated in splitless mode at 280°C. Chromatographic separations were achieved using a Bruker BR-5ms column (60 m length, 0.25 mm i.d., 0.25 µm film thickness). Initial oven temperature was 40°C, followed by a ramp to 45°C at 2°C min^{-1}, then increasing at 5°C min^{-1} to 180°C, ending with a final ramp at 30°C min^{-1} to 275°C with a 2-min hold. Data acquisition in centroid format was initiated after a 4-min solvent delay. The source and transfer line were operated at 300°C and 290°C, respectively. Volatile peaks were putatively identified by comparison of experimental spectra to reference spectra in the NIST database (version 11), and unambiguous identification was achieved by comparison to authentic standards (Sigma Aldrich, St. Louis, MO, USA) (Appendix Table 1, Fig 1A-L). Linalool was a gift from Dr. Tariq Akhtar (U. of Guelph, Dept. of Molecular and Cellular Biology), which was obtained from Sigma Aldrich, St. Louis, MO, USA.

4.2.6 Statistical Analysis

Please refer to section 3.2.8 for statistical analysis conducted in this experiment. Apart from previously conducted analyses, a principal component analysis (PCA) was conducted in R (v. 3.4.3, Wirtschaftsuniversität Wien, Welthandelsplatz 1, 1020 Vienna, Austria) using all the quality analyses and targeted volatile data to understand the complex correlations and trends between the different variables. The number of principal components (PC) to be used for analysis and interpretation was restricted based on the Kaiser criterion (i.e., the eigenvalues of the correlation matrix greater than 1) as described by Valle et al. (1999).

4.3 Results

4.3.1 Quality Parameters: Firmness, TSS, TA and Colour

The firmness results show a significant effect in the interaction between the repeated measure ‘Days post-harvest’ and ‘Treatment’ (P = .0328), indicative of a complex relationship

between variables as seen in Fig. 4.1. The EFF-treated fruit had higher firmness levels at 0, 7 and 11 d post-harvest (Fig 4.1). At 3 and 5 d post-harvest, there was no difference between the treatments (Fig 4.1). The TSS results indicate a significant effect of the 'Days post-harvest' parameter ($P = 0.0082$). Generally, there was an increase in TSS values as the fruit ripened from harvest until d 11 post-harvest (Fig 4.1). The TA results indicate a significant effect in the interaction between the 'Days post-harvest' and 'Treatment' measures ($P = 0.0098$). There was a general decrease in TA values from 0 to 11 days post-harvest (Fig 4.1). At 11 d post-harvest, fruit from the carrier treatment, had higher TA levels as compared to the EFF treatment (Fig 4.1). The peel and ground colour parameters of lightness, hue and chroma did not exhibit any difference between treatments (Fig 4.1).

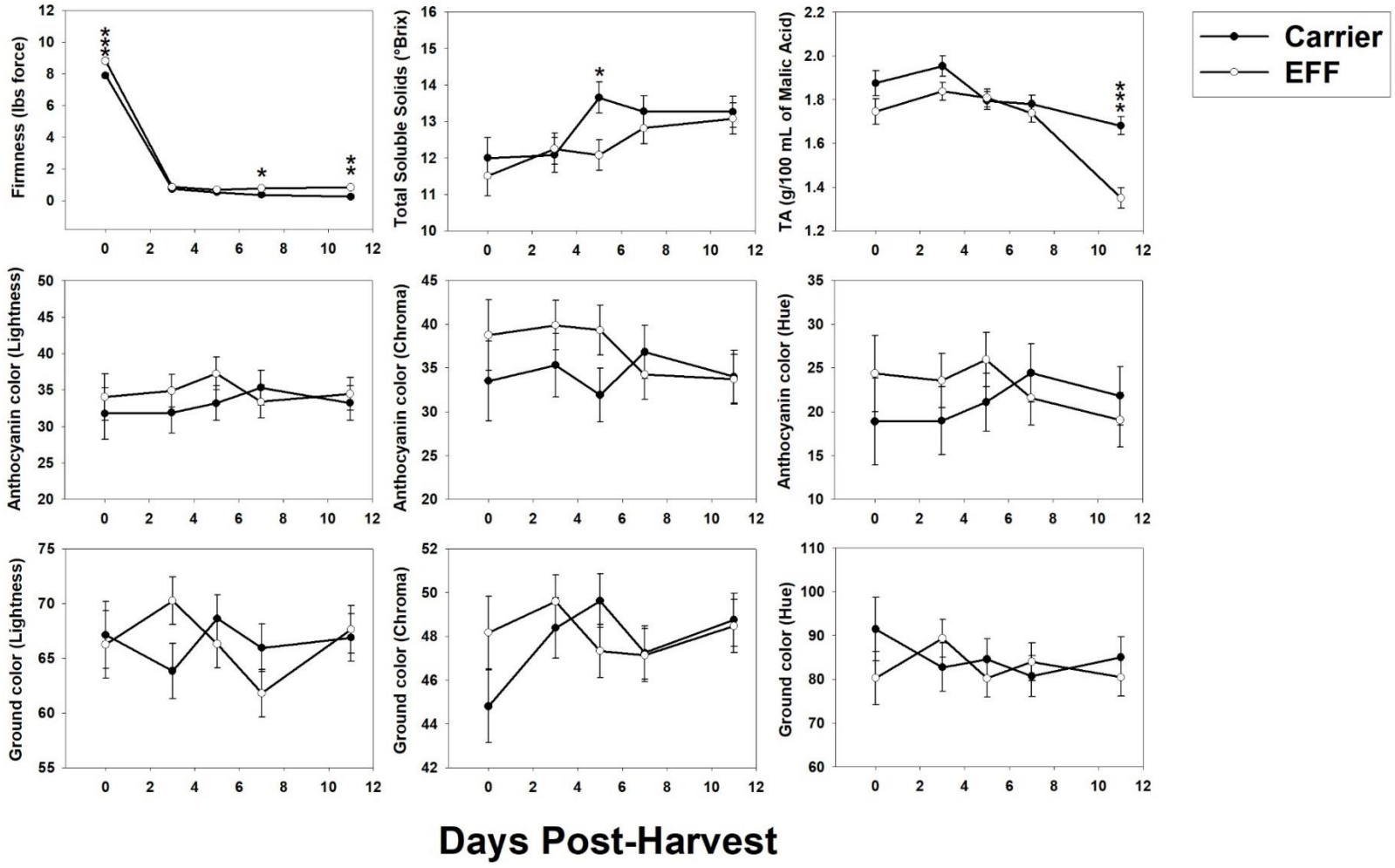


Fig. 4.1 Firmness, TSS, TA and colour measurements of carrier and EFF nectarines treated with 2% EFF sprays (15 and 7 days prior to harvest) at 0, 3, 5 and 11 days post-harvest. Nectarine trees were located at two commercial orchards in the Niagara Region, ON. Values represent means ± standard error of 4 biological replicates

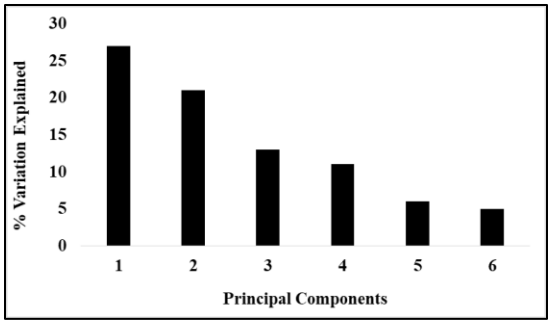
4.3.2 Targeted Analysis: A Principal Component Analysis (PCA)

A PCA was conducted to understand the relationship between the targeted volatiles compounds and quality parameters that were measured. The variation explained by the PCs is detailed in Fig. 4.2. PC 1 explained about 27% of the variation observed, whereas PC6 explained about 4% of the variation observed. The combined variation explained by all six of the PCs was about 81%.

With respect to the PCs, the volatile compounds segregated according to the class of compounds (i.e., lactones, C6 alcohols and C6 acetates) as seen in Table 4.1. The first PC is strongly correlated with eight of the variables in the data studied. These variables could be split into two groups: C6 alcohols and C6 acetates, both of which tend to change in concentrations together and fall under the green leaf volatiles group. Also, it is important to note that decreasing values of the C6 alcohols and esters is associated with increasing values of lactones as indicated by the lacotone's positive values for PC1 (Table 4.1). Thus, increasing PC1 values are associated with decreasing concentrations of C6 aldehydes and alcohols. The second PC is an exclusive measure of the lactone group; hence, it could be interpreted a measure of characteristic peach/nectarine aroma. The third and fifth PCs are measures of anthocyanin and ground colour components, respectively. Ground colour is commonly used as a measure of ripening in nectarine. The fourth PC is a measure of acetates and aldehydes. The sixth PC is a measure of firmness and titratable acidity and they have an inverse relationship. For the 6 PCs, only one score and loading plot comparing PC 1 and PC 2 is presented (Fig. 4.3). Together, PC1 and PC2 account for 47% of the variation in this analysis and they also incorporate all of the major volatile compounds quantified in the targeted volatile analysis. In Fig. 4.3, the longer arrows represent a greater influence of that particular variable on the data set. The closeness of arrows indicates greater

association or correlation between the variables. The distance of an orthogonal projection from the variables to any particular score indicates the closeness to the score of the variable. Using these three tenets of the PCA, the results from the scores and loading plot comparing PC1 and PC2 are presented in the following text.

The three lactones measured (γ octalactone, γ hexalactone and γ decalactone) had the greatest influence on this model and were very closely associated with each other in a directly proportional manner (Fig. 4.3). The TSS variable was very closely associated with lactone concentration. The TSS variable was negatively associated with firmness and TA, both of which were closely associated with each other, although, having a slightly lower influence on the model. Firmness was also closely related to ground colour measurements of lightness, chroma and hue. The anthocyanin and ground colour components segregated separately and are moderately associated with firmness and TA. Hexanal is negatively associated with the lactone and TSS variables and is closely associated with the other C6 alcohols, aldehydes and acetates. N-propyl acetate is neither closely associated with the other acetates nor is it closely associated with the lactones. However, it is negatively associated with TSS, TA and ground colour parameters. There is a clear progression of scores that are initially associated with C6 alcohols, aldehydes and acetates during the 1-5 day post-harvest period to scores being more closely associated with lactones, propyl acetate and TSS variables during the 7-11 day post-harvest period.



Legend	
PC's	Significant Loadings
1	C6 alcohols, aldehydes and esters
2	Lactones
3	Anthocyanin color components
4	Acetates and Aldehydes
5	Ground color components
6	Firmness and Titratable Acidity

Fig. 4.2 Percent variation explained by the first 6 PC's in the PCA analysis of volatile compounds of 'Fantasia' nectarine fruit sprayed with 2% EFF at two commercial orchards in the Niagara region in 2016 and after storage at 25 °C for 11 d post-harvest. The X-axis represents PC's 1 through 6 and the Y-axis represents the variance explained by each of the PC's.

Table 4.1 Six principal components of volatiles and other quality parameters described by the loadings of the parameters for each individual component for 'Fantasia' nectarine sprayed with 2% EFF at two commercial orchards in the Niagara region in 2016 and after storage at 25 °C for 11 d.

Variables	Principal Components					
	1	**2**	**3**	**4**	**5**	**6**
Eigenvalues	*6.12*	*4.74*	*2.88*	*2.50*	*1.38*	*1.10*
(E)-2-hexen-1-ol	**-0.32**[a]	0.15	-0.12	0.17	-0.14	0.05
(Z)-3-hexen-1-ol	**-0.36**	0.04	-0.14	0.18	-0.02	-0.08
1-Hexanol	**-0.33**	0.19	-0.09	0.13	-0.09	-0.12
(Z)-2-hexen-1-ol-acetate	**-0.27**	0.14	0.04	**-0.33**	-0.18	0.04
(Z)-3-hexen-1-ol-acetate	**-0.32**	0.04	-0.09	**-0.30**	-0.12	0.02
hexyl.acetate	**-0.25**	0.25	0.07	-0.21	-0.17	-0.18
n-propyl.acetate	-0.11	0.28	0.06	**-0.25**	-0.06	0.25
3-Hexanal	**-0.24**	-0.10	-0.11	**0.36**	0.18	0.16
E-2-Hexenal	**-0.28**	-0.05	0.05	0.20	0.20	0.21
Hexanal	**-0.32**	0.03	-0.15	**0.29**	0.10	0.05
γ decalactone	0.10	**0.41**	0.04	0.03	0.09	0.04
γ hexalactone	0.02	**0.42**	-0.02	-0.06	0.04	-0.07
γ octalactone	0.11	**0.40**	0.08	0.06	0.08	0.04
Linalool	-0.16	-0.22	-0.15	**-0.34**	0.02	-0.05
Firmness	-0.09	-0.22	-0.14	**-0.32**	0.05	**0.47**
Total soluble solids	0.14	0.29	0.11	0.00	-0.08	0.01
Titratable acidity	-0.08	-0.16	-0.17	-0.16	-0.05	**-0.65**
Anthocyanin.Colour Lightness	-0.15	-0.03	**0.49**	0.00	0.21	-0.10
Anthocyanin.Colour Chroma	-0.18	-0.11	**0.41**	-0.08	0.29	-0.10
Anthocyanin.Colour Hue	-0.18	-0.06	**0.46**	-0.06	0.22	-0.12
Ground.Colour Lightness	-0.06	-0.09	0.29	0.07	**-0.52**	0.19
Ground.colour Chroma	0.02	-0.10	0.14	0.30	**-0.47**	-0.21
Ground.colour Hue	-0.02	-0.15	0.28	0.03	**-0.35**	0.21

[a]A values in bold letters indicate important loadings of that variable on that principal component (i.e., within one column).

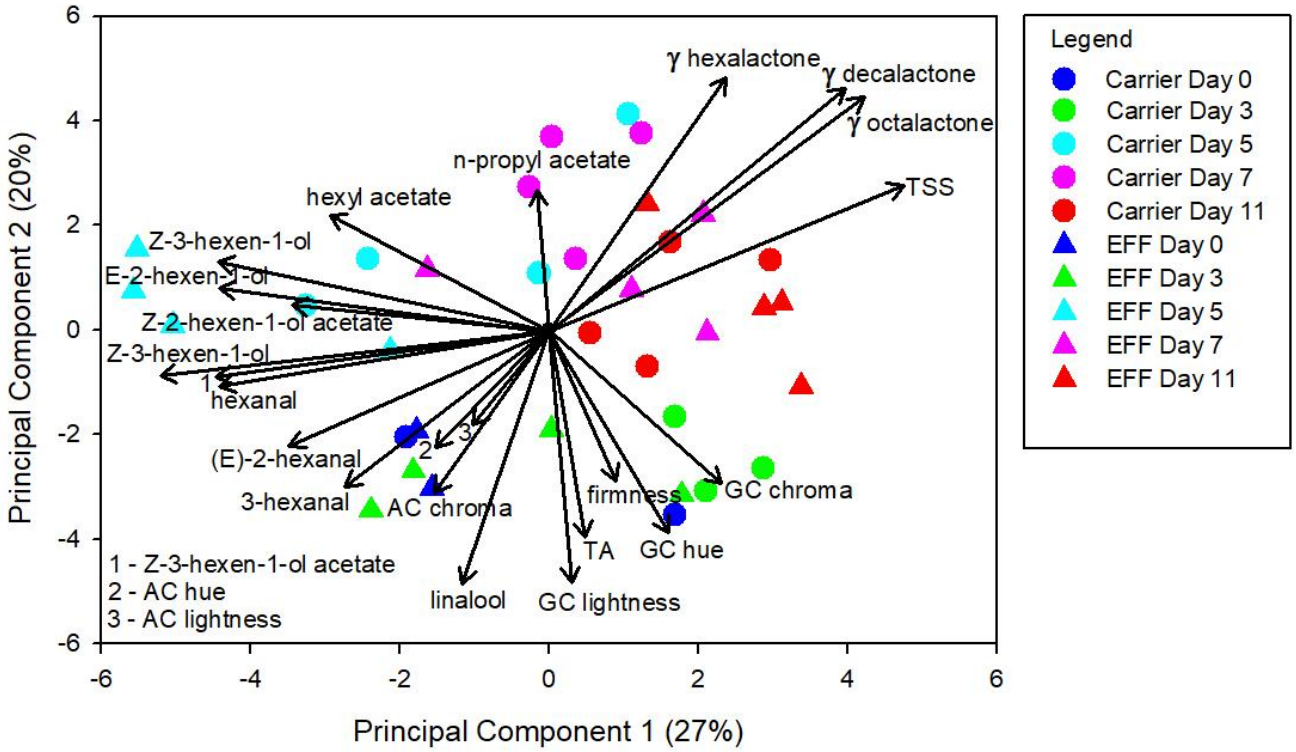


Fig. 4.3 Principal component analysis of the targeted nectarine volatiles in relation to quality parameters for 'Fantasia' nectarine sprayed with 2% EFF at two commercial orchards in the Niagara region in 2016 and after storage at 25 °C for 0, 3, 5, 7 and 11 days. AC: anthocyanin colour, GC: ground colour, TSS: total soluble solids and TA: titratable acidity.

4.3.3 Correlation Between Hexanal and Other Volatile Compounds

Pearson's correlation coefficients were generated to understand the correlation between hexanal and other volatile compounds (Fig 4.4). Increased hexanal concentrations were very highly and positively correlated ($p < .001$) with corresponding increases in the concentrations of all the C6 alcohols, aldehydes and esters, which include Z-3-hexen-1ol, 1-hexanol, E-2-hexen-1-ol, E-2-hexanal, 3-hexanal, Z-3-hexen-1-ol acetate and Z-2-hexen-1-ol acetate. Hexanal was also highly and positively correlated ($p < .01$) with hexyl acetate. Increased hexanal concentrations were also associated with corresponding increases in linalool, n-propyl acetate and inversely correlated with all the three lactones measured (ie. γ hexalactone, γ octalactone and γ decalactone).

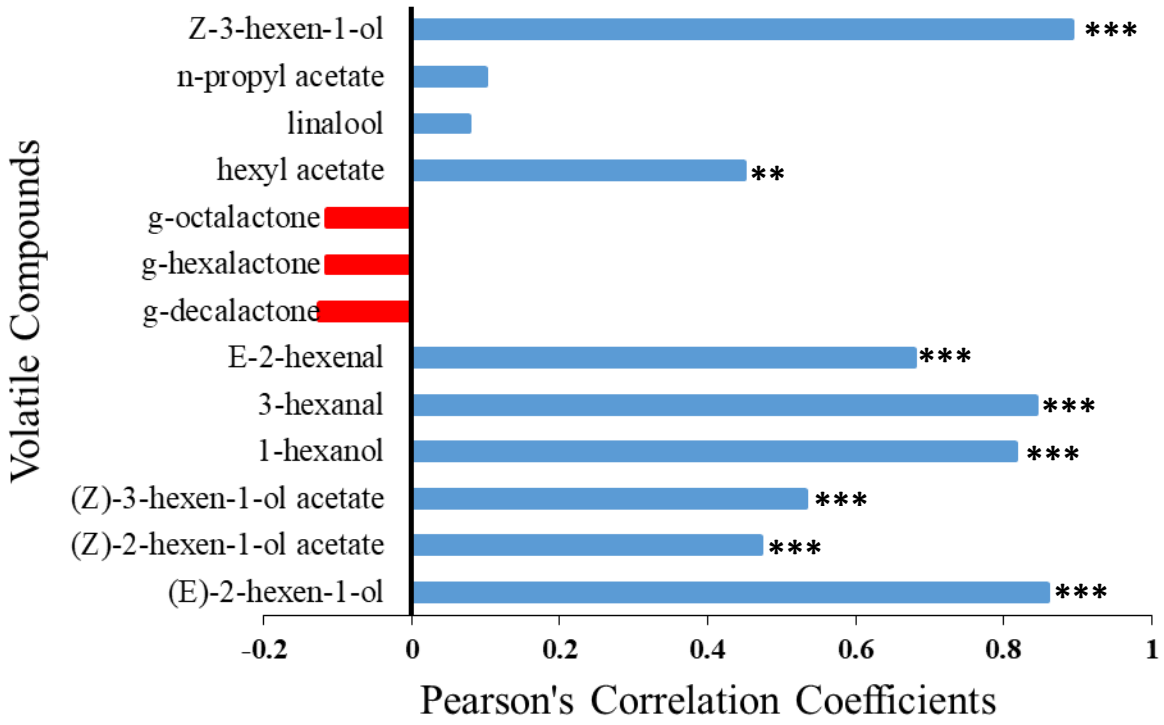


Fig 4.4 Targeted volatile compounds correlated with hexanal abundance in 'Fantasia' nectarine fruit sprayed with 2% EFF at two commercial orchards in the Niagara region in 2016 and after storage at 25 °C for 11 days. * indicates significance of $p < .05$, ** indicates a significance of $p < .01$ and *** indicates a significance of $p < .001$.

4.3.4 Targeted Volatiles: ANOVA

An ANOVA conducted on the targeted volatile compounds revealed changes in EFF-treated fruit in comparison to the carrier or control fruit (Table 4.2). At 0 days post-harvest, the concentration of (Z)-2-hexen-1-ol-acetate, (Z)-3-hexen-1-ol-acetate and linalool were significantly higher in EFF treated fruit in comparison to carrier fruit ($P < .05$). The concentrations of other C6 alcohols, aldehydes and esters were also generally higher in EFF treated fruit, albeit not significant (all $P \geq .05$). Also, the concentration of lactones in EFF treated fruit were slightly lower in EFF fruit than in carrier fruit. At 3 days post-harvest, there was a significant increase in the concentrations of (Z)-3-hexen-1-ol, 3-hexanal and E-2-hexanal in EFF treated fruit in comparison to carrier-only fruit. Similar to 0-day post-harvest measurements, EFF-treated fruit exhibited higher concentrations of many acetates and C6 alcohols than their carrier counterparts. Day 5 post-harvest witnessed highly significant increases in concentrations of all of the C6 alcohols and most of the C6 aldehydes measured. There was little to no difference in the concentration of acetates between the treatment and carrier fruit at day 5 post-harvest. Up until day 5 post-harvest, EFF-treated fruit had lower concentrations of lactones compared to the carrier fruit. This difference in lactones further increased at 7 days post-harvest, but at day 11 post-harvest, there were no differences between the treated fruit and controls with regards to the concentration of the measured volatiles.

Table 4.2 An ANOVA of relative expression for volatile aroma compounds extracted post-harvest using GC-EIMS methods from 'Fantasia' nectarine (*Prunus persica* [L.] Batsch var. *nectarina*) sprayed with a carrier (the control) or 2% EFF at two commercial orchards in the Niagara region in 2016 and after storage at 25 °C for 0, 3, 5, 7 and 11 days. Values are represented as means ± standard error of 4 biological replicates.

Volatile Compounds	**Days post-harvest**									
	0		**3**		**5**		**7**		**11**	
	Carrier	**EFF**	**Carrier**	**EFF**	**Carrier**	**EFF**	**Carrier**	**EFF**	**Carrier**	**EFF**
Lactones										
δ decalactone	0.005 ± 0.0084	0.002 ± 0.0084	0.007 ± 0.0071	0.002 ± 0.0063	0.031 ± 0.0063	0.015 ± 0.0063	0.063 ± 0.0063	0.041 ± 0.0063*****	0.030 ± 0.0063	0.033 ± 0.0063
δ hexalactone	0.005 ± 0.0011	0.006 ± 0.0011	0.005 ± 0.0009	0.004 ± 0.0008	0.010 ± 0.0008	0.008 ± 0.0008	0.013 ± 0.0008	0.009 ± 0.0008******	0.008 ± 0.0008	0.007 ± 0.0008
δ octalactone	.0004 ± 0.0003	.0001 ± 0.0003	.0003 ± 0.0003	.0001 ± 0.0002	0.001 ± 0.0002	.0008 ± 0.0002	0.002 ± 0.0002	0.001 ± 0.0002*****	0.001 ± 0.0002	0.001 ± 0.0003
Acetates										
(Z)-2-hexen-1-ol-acetate	0.096 ± 0.0279	0.182 ± 0.0243*****	0.051 ± 0.0219	0.077 ± 0.1721	0.160 ± 0.0180	0.165 ± 0.0172	0.139 ± 0.0179	0.106 ± 0.0172	0.089 ± 0.0179	0.069 ± 0.0172
(Z)-3-hexen-1-ol-acetate	0.210 ± 0.0242	0.285 ± 0.0230*****	0.119 ± 0.0194	0.163 ± 0.0162	0.254 ± 0.0167	0.251 ± 0.0162	0.194 ± 0.0167	0.144 ± 0.0162	0.111 ± 0.0167	0.089 ± 0.0162
hexyl acetate	0.143 ± 0.0697	0.241 ± 0.0661	0.081 ± 0.0563	0.121 ± 0.0470	0.469 ± 0.0485	0.495 ± 0.0470	0.387 ± 0.0485	0.301 ± 0.0470	0.216 ± 0.0485	0.176 ± 0.0470
n-propyl acetate	0.008 ± 0.0028	0.011 ± 0.0028	0.004 ± 0.0023	0.003 ± 0.0019	0.013 ± 0.0019	0.008 ± 0.0019	0.011 ± 0.0019	0.008 ± 0.0019	0.006 ± 0.0019	0.008 ± 0.0019
Terpenoids										
Linalool	0.092 ± 0.0077	0.112 ± 0.0057*****	0.055 ± 0.0053	0.046 ± 0.0040	0.042 ± 0.0045	0.057 ± 0.0040*****	0.032 ± 0.0046	0.032 ± 0.0040	0.009 ± 0.0046	0.008 ± 0.0040
C6 alcohols										
(E)-2-hexen-1-ol	0.004 ± 0.0020	0.005 ± 0.0020	0.001 ± 0.0017	0.005 ± 0.0014	0.008 ± 0.0014	0.013 ± 0.0014*****	0.005 ± 0.0014	0.005 ± 0.0014	0.005 ± 0.0014	0.003 ± 0.0014
1-hexanol	0.015 ± 0.0119	0.022 ± 0.0119	0.008 ± 0.0098	0.023 ± 0.0085	0.056 ± 0.0085	0.098 ± 0.0085******	0.038 ± 0.0085	0.039 ± 0.0085	0.024 ± 0.0085	0.017 ± 0.0085
(Z)-3-hexen-1-ol	0.003 ± 0.0011	0.004 ± 0.0011	0.002 ± 0.0009	0.005 ± 0.0008*****	0.006 ± 0.0008	0.010 ± 0.0008******	0.003 ± 0.0008	0.002 ± 0.0008	0.002 ± 0.0008	0.001 ± 0.0008
C6 aldehydes										
3-hexanal	0.001 ± 0.0017	0.004 ± 0.0017	0.001 ± 0.0015	0.005 ± 0.0013*****	0.001 ± 0.0013	0.006 ± 0.0013*****	0.001 ± 0.0013	0.001 ± 0.0013	0.001 ± 0.0013	0.001 ± 0.0013
E-2-hexanal	0.006 ± 0.0030	0.011 ± 0.0030	0.002 ± 0.0025	0.016 ± 0.0021**	0.007 ± 0.0021	0.012 ± 0.0021	0.006 ± 0.0021	0.007 ± 0.0021	0.006 ± 0.0021	0.004 ± 0.0021
Hexanal	0.007 ± 0.0056	0.018 ± 0.0056	0.003 ± 0.0049	0.016 ± 0.0044	0.009 ± 0.0044	0.041 ± 0.0044*******	0.010 ± 0.0044	0.013 ± 0.0044	0.010 ± 0.0044	0.006 ± 0.0044

4.4 Discussion

The unique flavor of nectarine fruit is determined by a complex blend of esters, acids, alcohols, aldehydes, lactones and terpenoids (Sevenants and Jennings, 1966; Do et al., 1969; Bayonove, 1974; Spencer et al., 1978; Engel et al., 1988a, Engel et al., 1988b; Takeoka et al., 1988; Horvat and Chapman, 1990; Horvat et al., 1990; Narain et al., 1990; Chapman et al., 1991; Sánchez et al., 2011). Bruhn (1995) has shown with survey data that about 70% of consumers considered that the aroma of nectarine was "very important to extremely important" at the time of purchase. Hence, it is important to quantify the effects of hexanal on the volatile network of nectarine. There were 14 compounds identified through the targeted GC-EIMS analysis, all of which were validated with standards. Hexanal was found to be naturally occurring within the fruit. This agrees with previous studies conducted by Chapman et al. (1991) who identified the presence of high levels of hexanal in unripe peach. High levels of hexanal, (E)-2-hexenal, and (E)-2-hexenol from volatile fractions of nectarine have been associated with lipoxygenase and alcohol dehydrogenase activities (Tressl et al., 1980; Eriksson, 1975). Linoleic acid is thought to be a precursor for these compounds (Tressl et al., 1981; Engel et al., 1988) and it has been found to be one of the major fatty acids in peach seed and endocarp (Takenaga et al., 1984). Chapman et al. (1991) identified that the levels of hexanal and (E)-2-hexanal C6 were highest between 90 and 106 d after flowering and that this may indicate the time during maturation when lipoxygenases were the most active, following which the levels of these compounds dropped to a minimum. By contrast, our studies indicate a delay in this ripening process as there was a significant increase in the concentration of these C6 aldehydes and alcohols at 5 d post-harvest, rather than at the maturity phase. This can be explained due to hexanal's effect on inhibiting PLD and delaying the ripening process (Paliyath and Subramanian, 2008). PLD is involved in lipid breakdown and disintegration

during ripening, and hexanal has been shown to be an effective inhibitor of PLD action, thus delaying the ripening and senescence process in multiple fruits and vegetables (Paliyath et al., 2003). PLD inhibition results in the delay of downstream processes such as the breakdown of phospholipids, the conversion of phosphatidic acid to diacylglycerols, the formation of free and peroxidized fatty acids, and finally, the production of volatile C6 aldehydes, alcohols, and esters which contribute flavor to the fruit (Paliyath and Murr, 2008). The delay in ripening in EFF treated nectarines is in agreement with previous studies that analyzed hexanal's effect on volatiles in strawberry (Misran et al., 2014) where elevated levels of several C6 esters occur in control fruit and increased concentration of C6 alcohols and aldehydes are evident in EFF-treated fruit. Further, hexanal has been discovered to increase in concentration during plant injury and act as a chemical signal in terms of plant distress (Yan and Wang, 2006). Increased concentrations and longer durations of presence of hexanal and related compounds could cause enhanced fruit protection, although phytopathological studies have to be conducted to understand the effects of enhanced presence of hexanal in fruit on disease and pest pressures.

The EFF-treated fruit exhibited lower concentrations of lactone fractions troughout the storage period. Lactones are involved in ripening (Sánchez et al., 2011; Visai and Vanoli, 1997) and provide the characteristic peach/nectarine flavor indicative of ripened fruit (Horvat et al., 1990; Derail et al., 1999; Eduardo et al., 2010). Lactones have also been positively correlated with ground colour and negatively correlated with flesh firmness in a study involving non-targeted analysis of peach and nectarine volatile fractions (Sánchez et al., 2011). These studies along with our observed data indicate a delay in ripening caused by EFF application. The EFF treated fruit followed the trends observed by Horvat et al., 1990; Chapman et al., 1991 with lower overall lactone concentration.

The PCA clearly displayed a segregation of volatile fractions according to their class such as lactones, acetates, C6 alcohols and C6 aldehydes. This is in accordance with previous studies conducted on volatile profiles of peach and nectarine (Sánchez et al., 2011; Wang et al., 2009; Eduardo et al., 2010). There was a negative correlation between the C6 aldehydes and alcohols in PC1 with lactones in PC2. This is consistent with a decrease in "green volatiles" (i.e., C6 aldehydes and alcohols) and an increase in lactones associated with the characteristic peach/nectarine flavor (Chapman et al., 1991; Horvat et al., 1990). Further, Pearson's correlations helped to provide insight into the association between hexanal and other volatiles. As expected, hexanal was strongly correlated with other C6 aldehydes, alcohols and esters and negatively correlated with lactone fractions. These results also point to a delay in ripening caused by hexanal in the EFF formulation.

This research study was conducted at room temperature to test the effects of hexanal on volatile production in nectarines. Due to the room temperature storage, the firmness of the fruit dropped considerably after 3 days of storage, from 9 lb to 2.5 lb, hence the effect of hexanal to retaining firmness was masked or reduced. It is expected that the delay in volatile compound production and increased firmness in treated fruit would be much more pronounced if these studies were conducted in cold storage, as observed in Chapter 3. There was a predictable increase in TSS values as the study progressed from 0 to 11 days post-harvest, and it was accompanied by a decrease in TA. There were no differences in colour between the treatments. This is consistent with previous studies analyzing hexanal's effect on fruit such as nectarine, raspberry, mango and guava (Kumar et al., 2018; El Kayal et al., 2017; Anusuya et al., 2016; Gill et al., 2015). Unlike treatments such as 1-MCP which results in an irreversible change by blocking certain ethylene receptors (Watkins et al., 2006), the hexanal treatment has been shown to result in a reversible

delay in ripening and senescence without affecting other quality characteristics such as colour, TSS and TA (Paliyath and Subramanian, 2008).

Overall, this study indicated that hexanal's effect on shelf life of fruit did not have detrimental effects on the volatile production of the fruit. There was a clear downregulation of compounds associated with ripening fruit such as lactones and an upregulation of unripe fruit volatiles such as C6 alcohols, aldehydes and esters, indicating hexanal's effect in delaying volatile synthesis and the ripening process. More research has to be conducted with nectarine fruit stored at 0-1 °C to more closely replicate commercial storage conditions and to understand the comprehensive effect of hexanal's action in enhancing the shelf life of nectarines. This study validates the promising effect of hexanal as a shelf life-enhancing compound for nectarines.

Chapter 5

Summary and Future Directions

Tender fruit such as nectarines have a very limited shelf life of two to three weeks, after which they are too soft to be commercially acceptable (Crisosto and Valero, 2008). Hence, cold storage has been incorporated into the post-harvest storage system in order to enhance the shelf life of these fruit. However, cold storage leads to many disorders collectively called chilling injury. Among them, mealiness is a major factor that decreases the quality of the fruit and causes consumers to stop purchasing the fruit (Bruhn, 1995). Mealiness is characterized by a leathery texture with no juice. Apart from that, internal browning is also a consistent and common disorder in nectarines (Lurie and Crisosto, 2005). These disorders are prominent at the temperature range of 2-8°C. Nectarine growers in Ontario following best practices, transfer harvested fruit to 0-1°C cold storage, immediately after sorting/grading (Ontario Tender Fruit Marketing Board, personal communication). However, existing cold storage infrastructure does not have the luxury of accurate temperature manipulation and maintenance and costs for maintaining the temperature at 0-1°C is prohibitory. Hence, there is rampant presence of mealiness/internal browning in nectarines due to inappropriate storage temperatures.

Extensive research has been done to understand the process of ripening and senescence in order to find ways to enhance shelf life. Several metabolic pathways, especially the ethylene pathway, have been elucidated in considerable detail to understand the molecular mechanisms underlying ripening (Watkins et al., 2006). It is desirable to maintain elevated levels of health-related compounds after harvest and during storage of these fruit. Fruit undergo several changes after harvest, which in most cases results in a decrease in nutritional compounds (Macheix et al.,

1990). The majority of research into enhancing shelf life has focused on the ethylene pathway and chemicals which block ethylene, such as 1-MCP.

However, the current research explored other avenues such as membrane and lipid degradation that occurs during ripening. PLD is the key enzyme which enhances the lipid degradation in fruit resulting in senescence (Paliyath and Droillard, 1992). This lipid degradation leads to softening of the fruit, which can provide easy access for secondary pathogens. Many C6 alcohols and aldehydes have been shown to inhibit PLD in its actions, thus delaying the disintegration of lipid membranes leading to production of fatty acids and volatiles. However, hexanal is the most effective in delaying the membrane degradation process (Paliyath and Subramanian, 2008). Further EFF with hexanal as the key ingredient (Paliyath et al., 2003), is effective for enhancing shelf life in a variety of fruit, vegetables and flowers (Paliyath and Subramanian, 2008).

In experiments conducted at commercial nectarine orchards in the Niagara region, EFF treated fruit maintained a significantly higher firmness over the storage period of 45 d at 2°C. The EFF treated fruit also delayed the onset of mealiness and internal browning by ~8 days and reduced their incidence by almost 50% even at 45 days after harvest. There were no adverse effects on other fruit quality parameters such as TSS, TA and fruit colour. Gene expression studies also support the physico-chemical analyses, where three PLD genes, five N-Glycoprotein group genes and expansin genes among others, were downregulated indicating their potential regulation by hexanal. These results indicate the formulation's potential in enhancing shelf life of nectarines and reducing the incidence of chilling injury symptoms, to benefit both growers and consumers, although further research regarding the molecular basis of its mode of action needs to be investigated.

The fruit retention study on nectarines did not provide significant results. This is in contrast with hexanal's effect on enhancing retention in other fruit such as mango and raspberry (El-Kayal et al., 2017; Anusuya et al., 2016). Future steps include a detailed molecular analysis in the fruit stalks to understand how the abscission mechanisms works in nectarines, including analysis of genes involved with ABA and ethylene pathways.

The volatile studies in Chapter 4 indicated that hexanal is present within the fruit. Further, it became clear that pre-harvest hexanal application delayed ripening-related aroma formation, as was indicated by a decrease in lactone concentration followed by a corresponding increase in C6 alcohol and aldehyde concentration. Generally, lactone concentration is associated with ripening fruit, where they provide a very characteristic peach flavor and C6 alcohols and aldehydes provide the aroma of unripe fruit, as they are yet to be converted to esters and other volatile compounds to enhance the floral and fruity aroma. Future studies should include volatile analysis of nectarines stored at 0-1°C to replicate the conditions that these fruit go through before reaching the market. This study was conducted primarily to benefit nectarine growers and for increasing consumer experience when eating the fruit. Enhanced shelf life of fruit provides the grower an economic edge during marketing of their fruit to grocery chains and individual stores. The better quality of fruit helps to increase the overall quality and acceptability of nectarines in the market, hopefully leading to an increase in market share of this fruit.

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Appendix

Table 1. Targeted analysis of nectarine aroma volatiles by GC-MS.

Compound	Retention time (min)
n-propyl acetate	5.1
3-hexenal	7.0
Hexanal	7.1
(Z)-3-hexen-1-ol	8.6
(E)-2-hexenal	8.6
(E)-2-hexen-1-ol	8.9
1-hexanol	9.0
(Z)-3-hexen-1-ol-acetate	13.4
hexyl acetate	13.6
(Z)-2-hexen-1-ol-acetate	13.7
gamma-hexalactone	14.9
Linalool	16.5
gamma-octalactone	21.1
gamma-decalactone	26.8

Fig 1A. N-propyl acetate: confirmed by comparison to authentic standard.

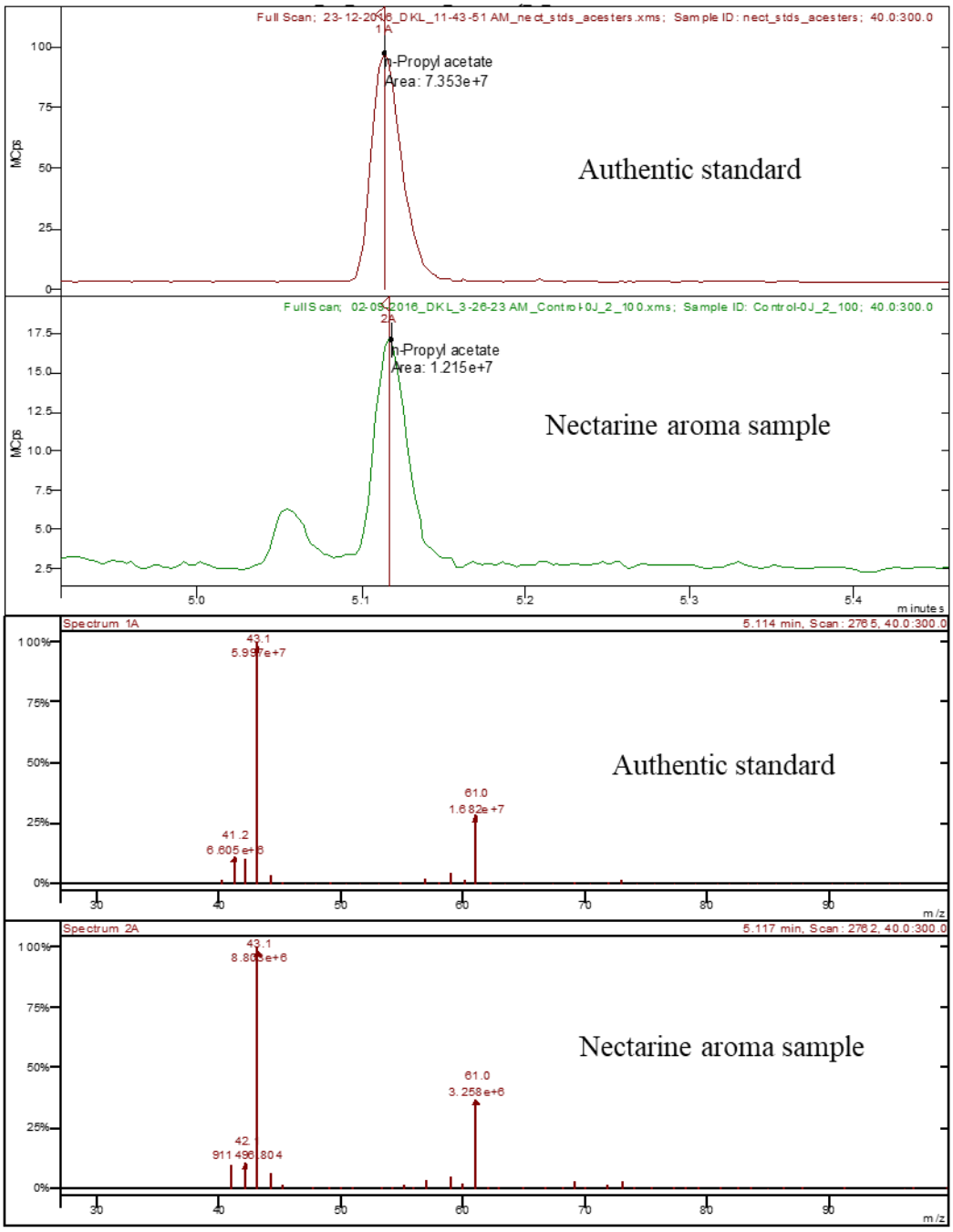

Fig 1B. Hexanal: confirmed by comparison to authentic standard.

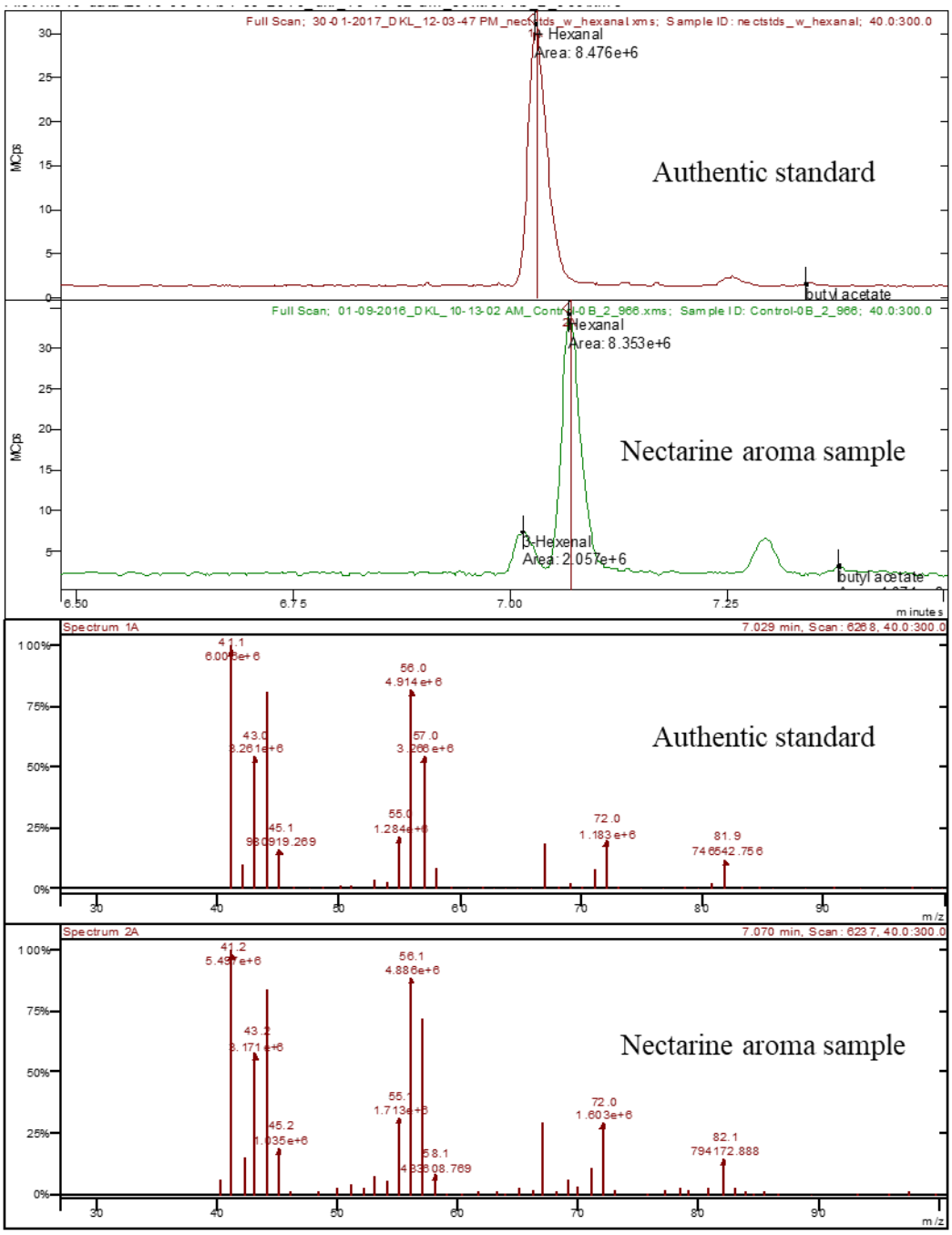

Fig 1C. (Z)-3-hexen-1-ol: confirmed by comparison to authentic standard.

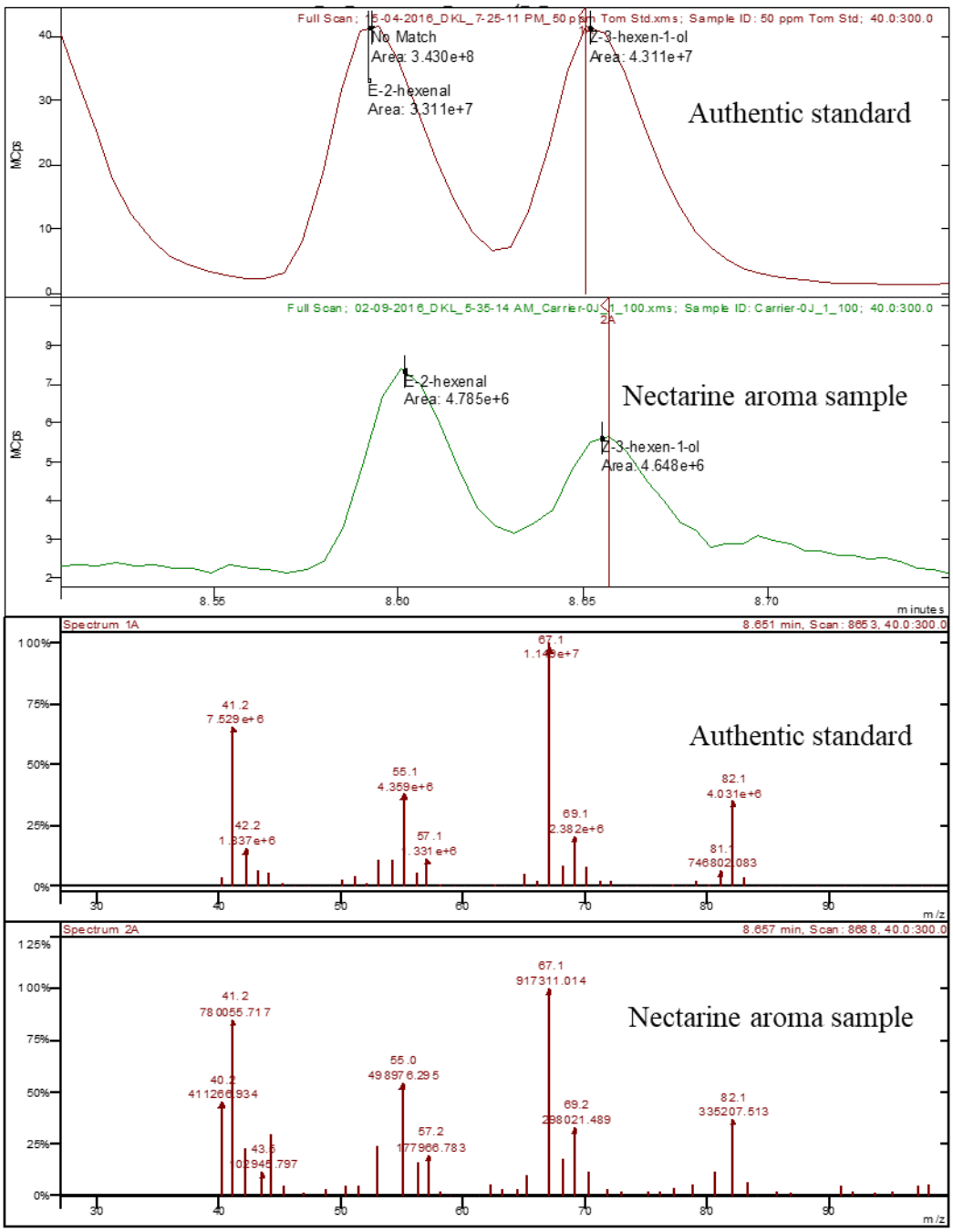

Fig 1D. (E)-2-hexenal: confirmed by comparison to authentic standard.

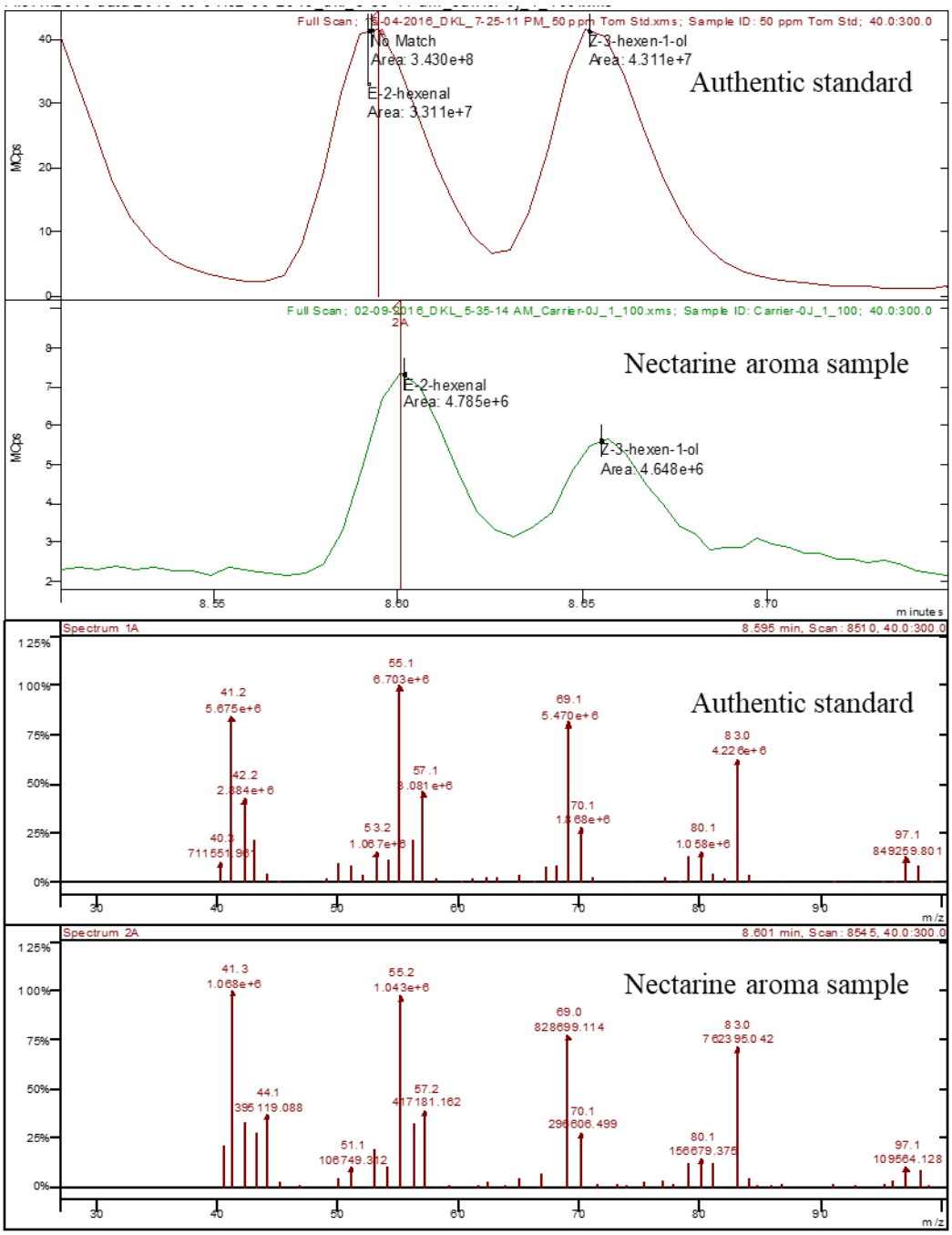

Fig 1E. 1-hexanol: confirmed by comparison to authentic standard.

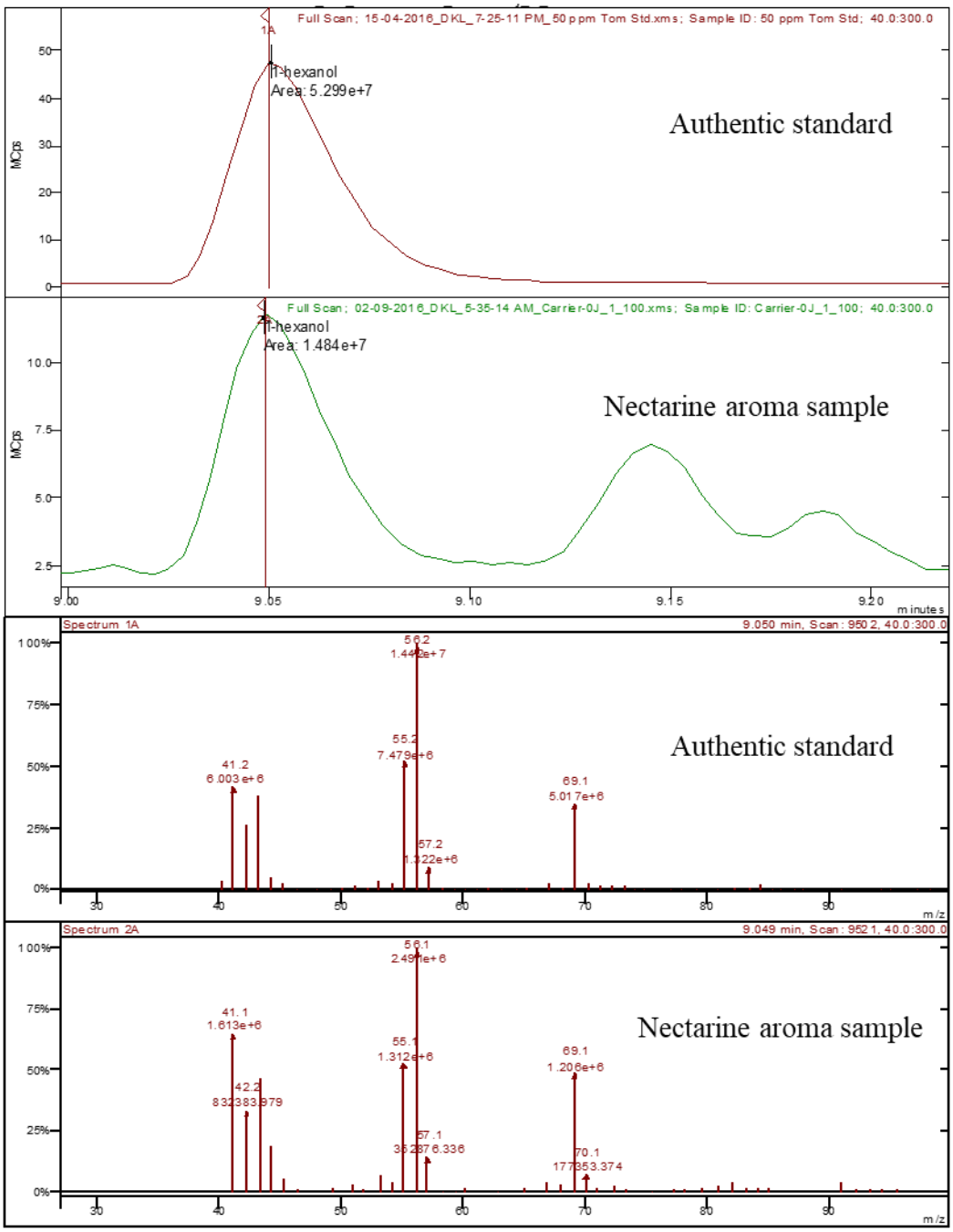

Fig 1F. (Z)-3-hexen-1-ol-acetate: confirmed by comparison to authentic standard.

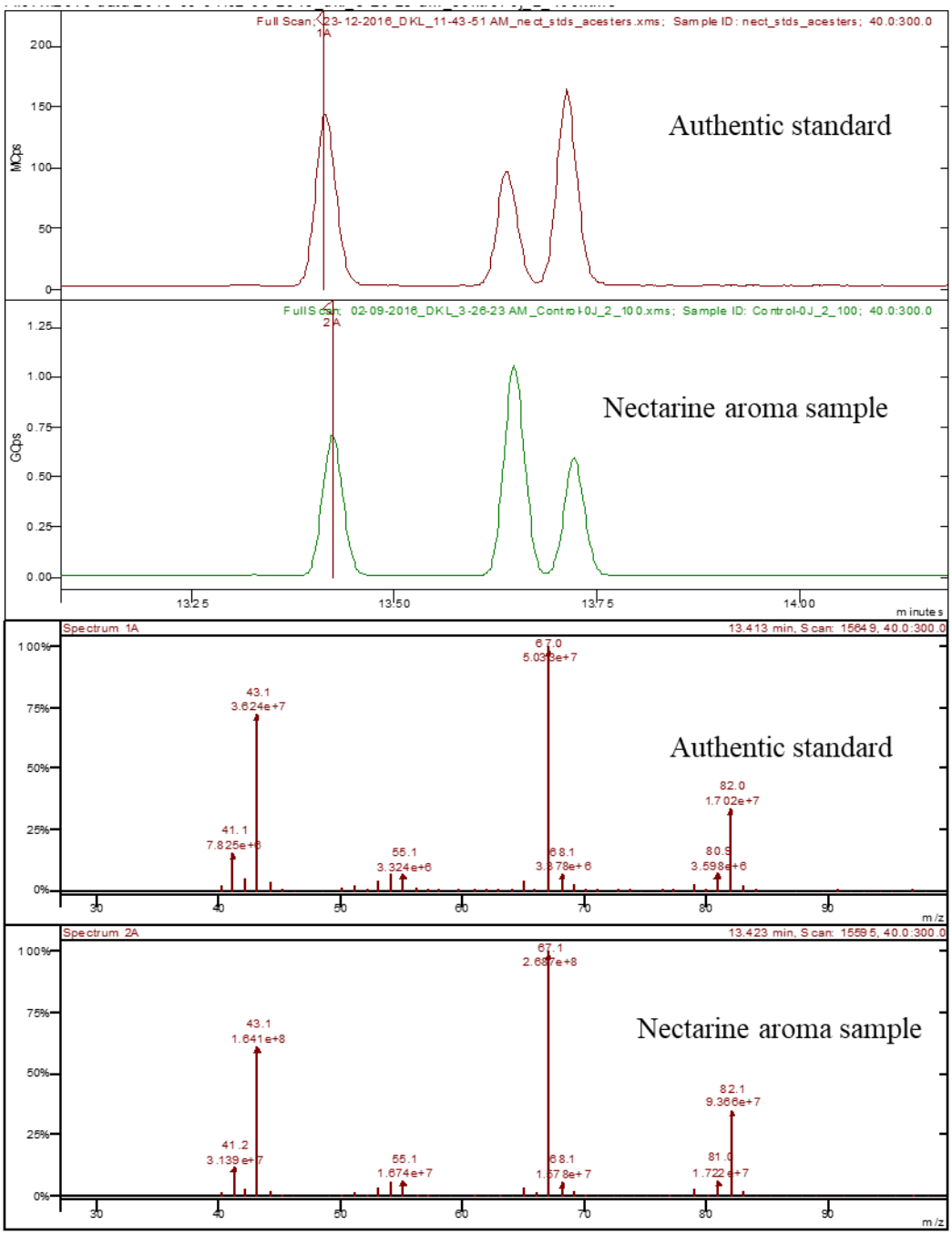

Fig IG. Hexyl acetate: confirmed by comparison to authentic standard

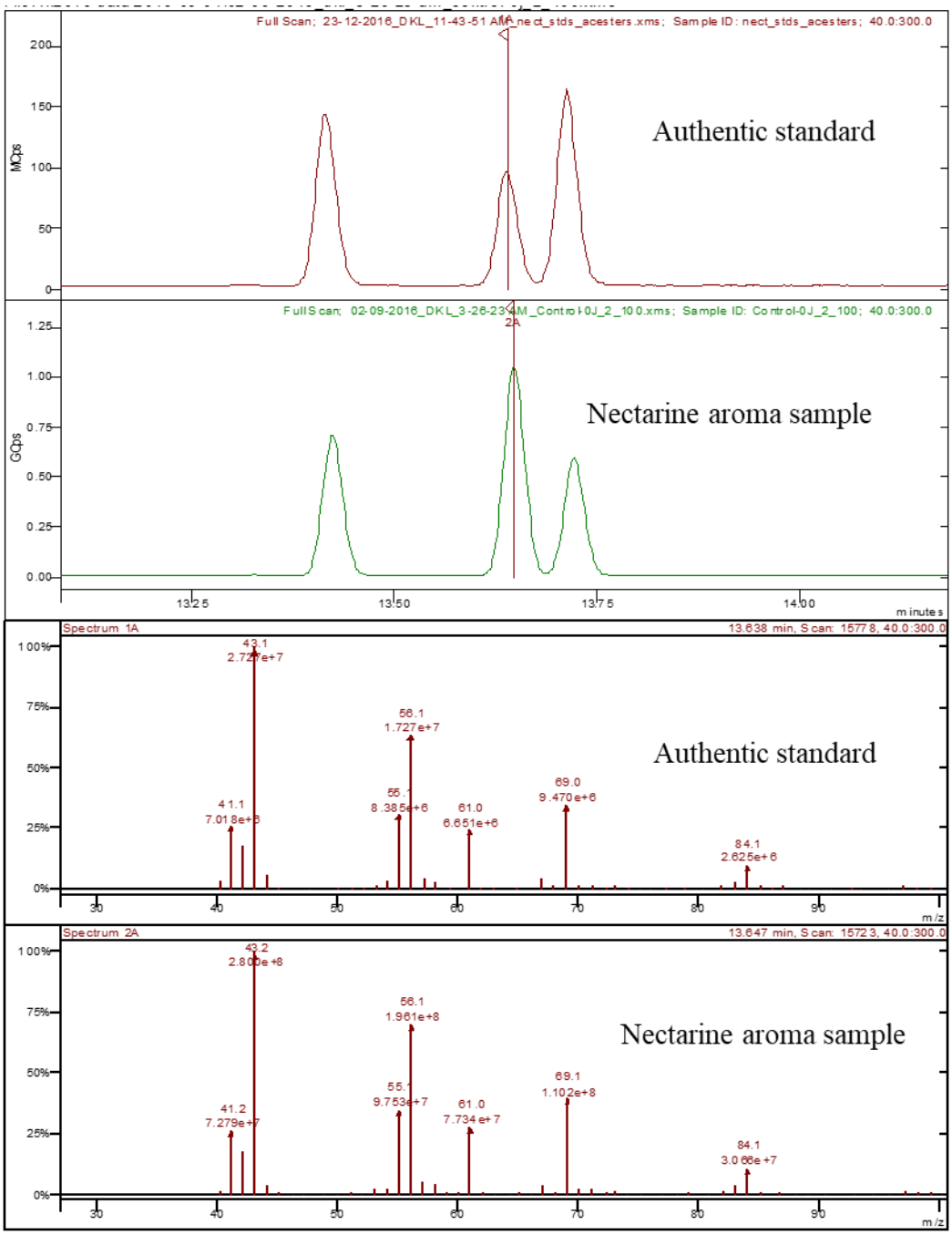

Fig 1H. (Z)-2-hexen-1-ol-acetate: confirmed by comparison to authentic standard.

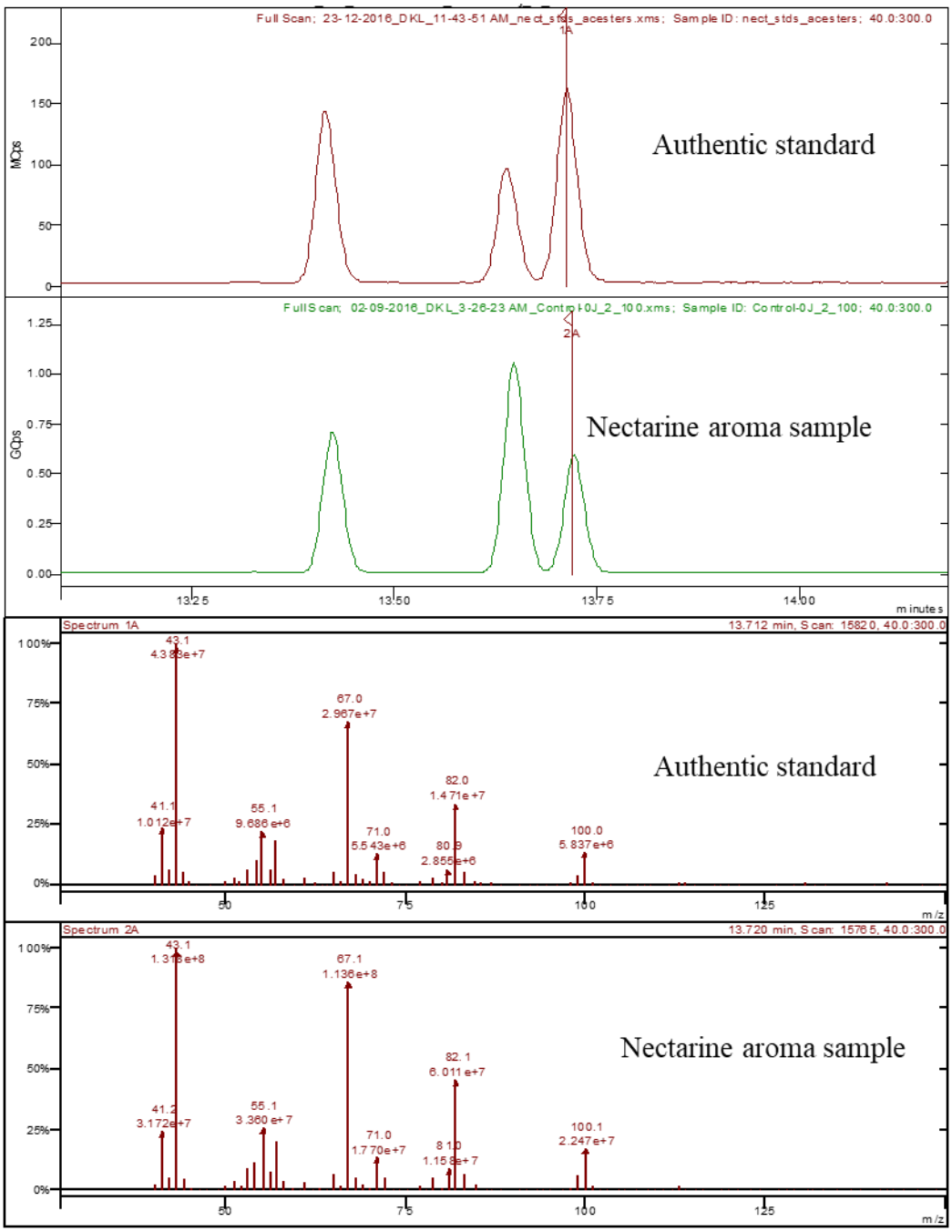

Fig 1I. gamma-hexalactone: confirmed by comparison to authentic standard.

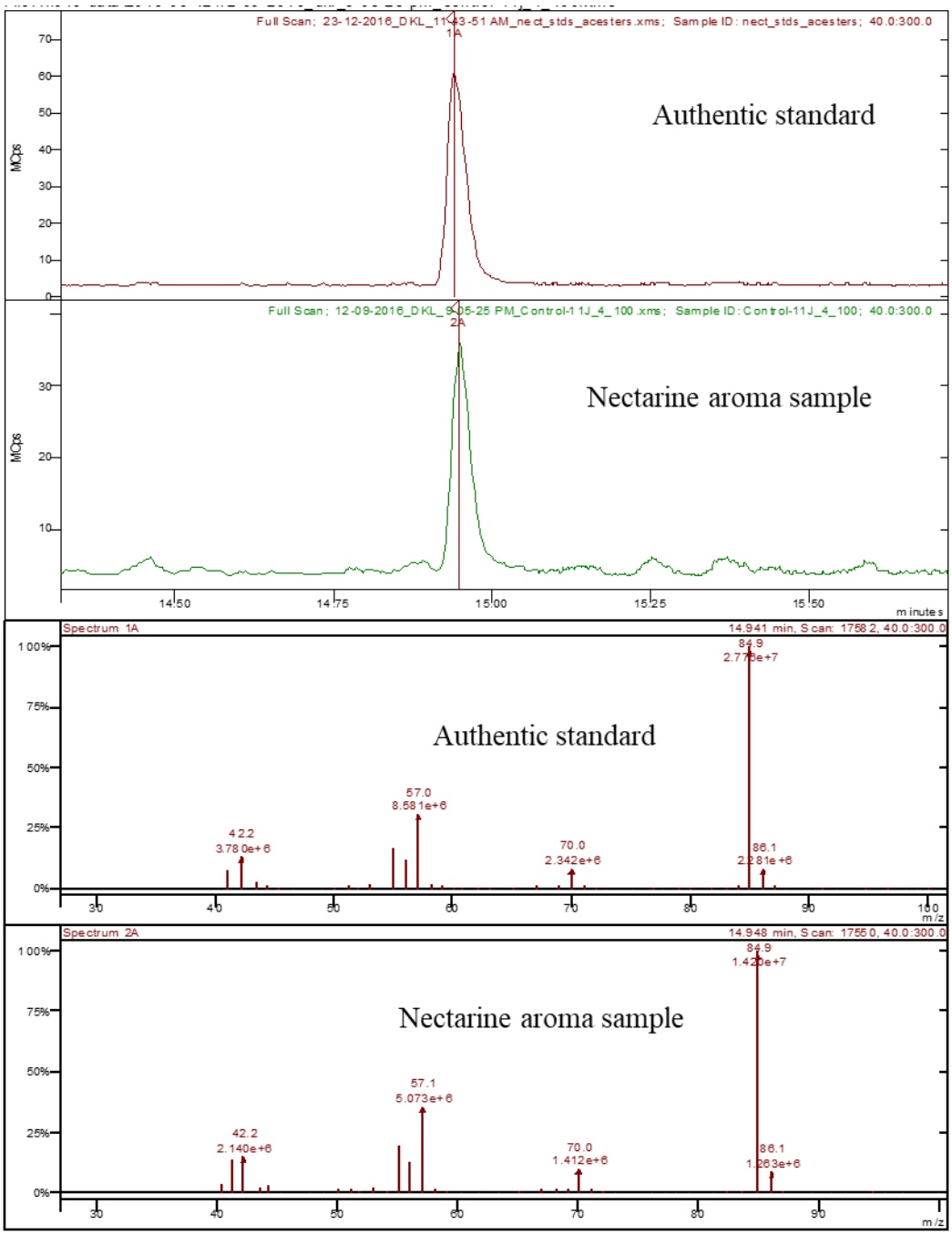

Fig 1J. linalool: confirmed by comparison to authentic standard.

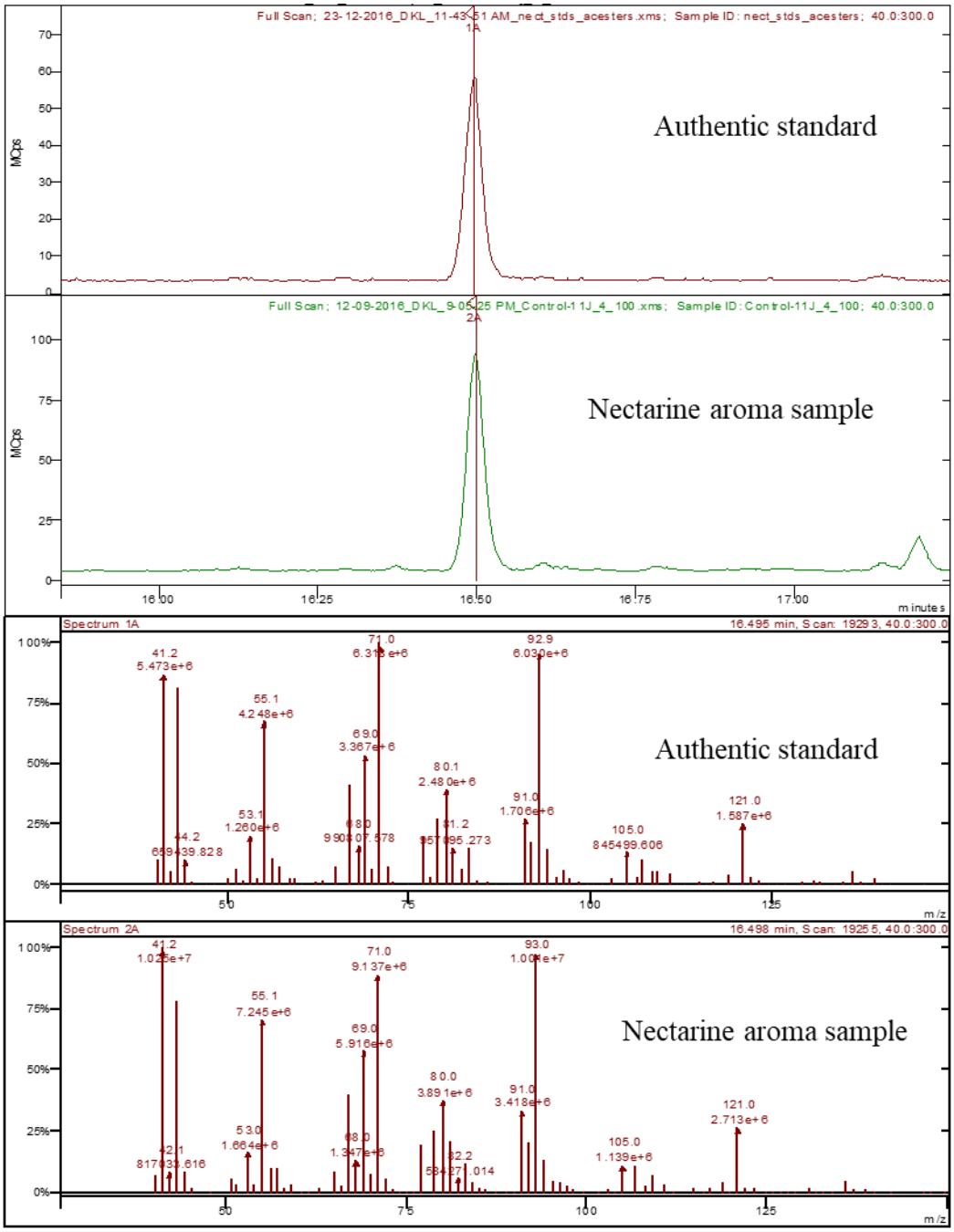

Fig 1K. gamma-octalactone: confirmed by comparison to authentic standard.

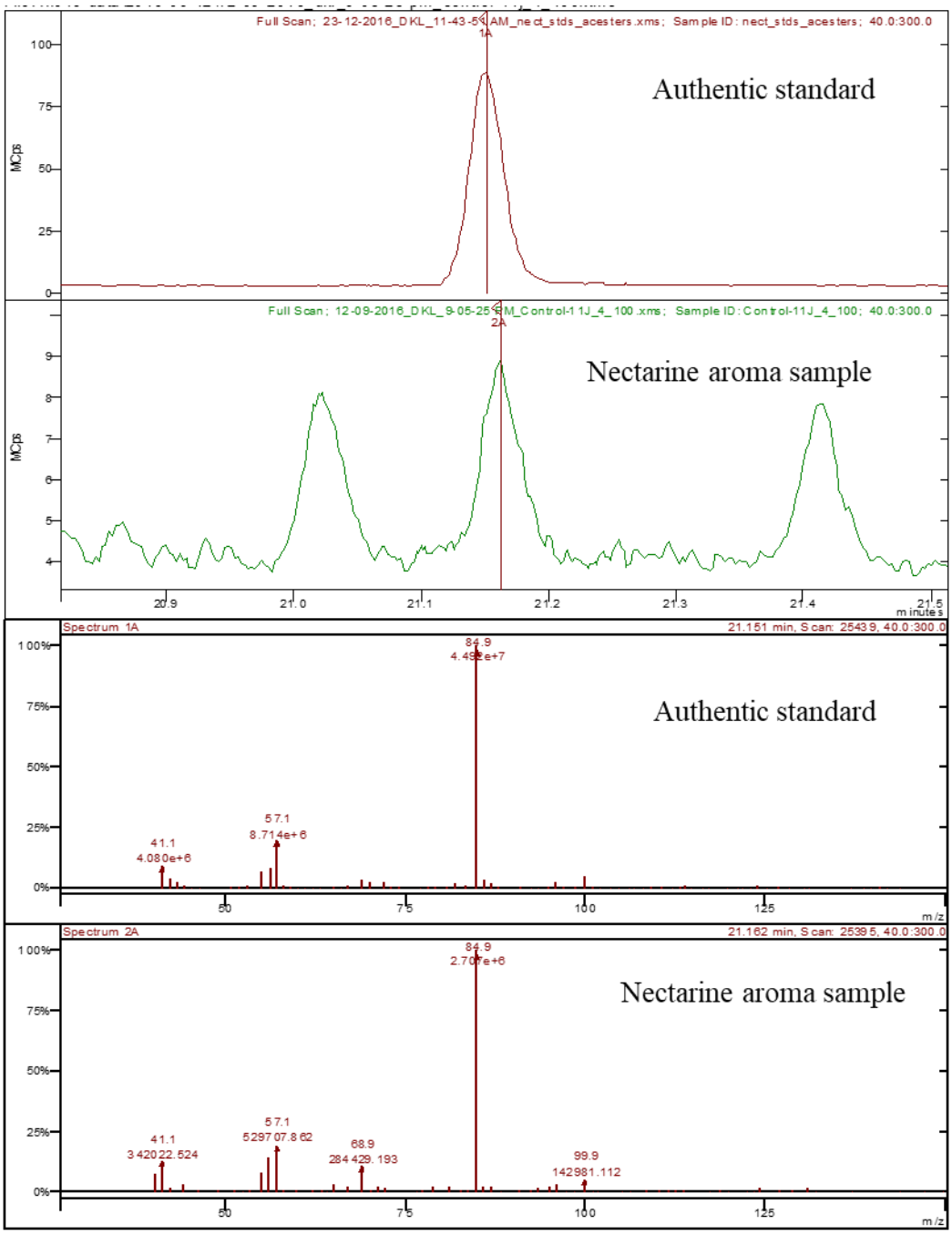

Fig 1L. gamma-decalactone: confirmed by comparison to authentic standard.