Development of Predictive tests for Determination of Shelf Life for Citrus Fruits

Thesis submitted for the fulfilment of the requirements for the degree of Doctor of hilosophy

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Doctor of Philosophy Declaration

I, Murad Yusuf, declare that the PhD thesis entitled “Development of Predictive tests for Determination of Shelf Life for Citrus Fruits”, is no more than 80,000 words in length, including quotes and exclusive of tables, figures, appendices, bibliography references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work. I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University’s Higher Degree by Research Policy and Procedures.

01-03-2022

Signature Date

Abstract

The main focus of this project has been on the development of better understanding of the potentially devastating damage caused by cold winter freeze to growing citrus fruits. Fruit such as oranges are extremely important to the Australian fruit industry, and the establishment of such an investigation to address the current losses in this area is overdue. Any significant advancement towards better understanding and improvement in this problem area will be welcomed, and this work, using methods based in scientific analysis and visual investigation, has been mounted to contribute to this need.

The cultivars investigated during the period 2007-13 were Navel and Valencia sweet oranges, two of the most important species in the citrus fruit industry. Every experimental method used in this investigation consists of two parallel investigations. The first study involves the development of simulated treatment in the laboratory, through techniques based on controlled freeze treatment with various sub-zero temperatures.

In summary, the study found that emission of ethanol and other compounds could be observed following the freeze treatment of orange fruit, and these emissions were demonstrated to be predictive of damage to the fruit that will be present following a three-week period of storage. This finding strongly suggests that one or more of these volatiles could be used as analytical indicators to evaluate the degree of damage that has been caused during the frost damage that has occurred in a damaging freeze. The detection of ethanol emanations and the observations of internal and external changes that have resulted because of frost-damage conditions will also help to formulate quality control process needed for confident retailing of these fruits. It is recommended that further research needs to more closely ascertain whether these volatiles can be useful in the evaluation of damage to naturally frozen oranges.

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I am immensely grateful to the Colour Vision Australia for providing me an Australia Postgraduate Scholarship for this research programme and I also appreciate the extensive contribution offered by Colour Vision Systems as industry partner of the above scholarship and industrial partner. I am eternally grateful to Allah (God) for his enabling grace and for fixing in my life such a momentous destiny including a PhD programme at Victoria University, Australia. I am unaware of what tomorrow might bring, but I rest my case and my life in his enabling, safe and secure hands.

Dedication

This thesis, being an initial meaningful contribution to the body of scientific knowledge, is dedicated to my beloved late grandmother Mrs Amatula Adus and to Mrs Sara Ahmed, for their immense sacrifices and prayers for the many years since I was a child. Their courage and inspiration was unshakeable at the time I was in need of someone to foster me as a mother. My grandmother indeed was my only life-line who raised me, taught me to be disciplined, educated me and paved the way for who I am today. Without her I would be lost. During my studies for the last few years, my family has made an enormous sacrifice; this act needs to be respected, and has made this endeavour possible and worthwhile.

List of Publications, Conferences Presentations and Awards

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M. Yusaf, A. McGill and M. Millikan, Spectrophotometric Analysis of Selected Wheat Cultivars from Phytic Acid Content, proceedings of 54th Australian Cereal Chemistry Conference and 11th Wheat Breeders Assembly. Ed(s). C.K. Black, J.F. Panozzo and G. J. Rebetzke, RACI, North Melbourne, Victoria, 429 – 431, 2004.

M. Yusuf and M. Millikan, Analysis of Oranges Stored at Range of Low Temperatures by Near Infrared Spectroscopy. Abstracts: 13th Australian Near Infrared Spectroscopy Conference: no. 25, 2008.

M. Yusuf and M.B. Millikan, Near Infrared Analysis of Volatile Oils from Citrus Fruit Stored at Low Temperatures. Abstracts 14th Australian Near Infrared Spectroscopy Conference: Abstracts, 2010, p30

M. Yusuf, G. Thorpe, M.B. Millikan Determination of the rate of decay of several pesticides on fresh fruit. Abstracts: RACI, 13th National Convention in conjunction with the 12th IUPAC International Congress of Pesticide Chemistry, 2010 p253

Yusuf`1 M, and Millikan M, Shelf life-studies of oranges stored at a series of low temperatures. Abstracts: AIFST, 43rd Annual Convention, 2010, p53

M. Yusuf and M. Millikan "Analysis of navel oranges stored at a range of low temperatures" Abstracts: 16th International Conference on Near Infrared Spectroscopy, 2013, p234

Student Awards

ANISG Student Conference Award 2008 13th Australian Near Infrared Spectroscopy Conference, Hamilton, Victoria, 6 – 10 April, 2008

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Conferences and Presentations

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M. Yusuf, M. Millikan, Effect of cold damage on orange fruits texture and firmness, Analytica Chimica Acta, 2013.

M. Yusuf, M. Millikan, Identification of volatile compounds from cold damaged orange fruits by Gas Chromatography, Analytica Chimica Acta, 2013.

Justification of this Study

The Australian citrus fruit industry is one of the important sectors of local and international agricultural market, and, on average, Australia produces 0.5% of the world's citrus fruit per annum. Indeed, orange production, at 80 % of the total, is by far the highest compared to the rest of the citrus fruits, which is the reason for its pre-eminence in the industry. Of relevance, is that approximately 310.2 billion kg oranges are produced commercially in 114 countries worldwide (Australian Bureau of Statistics, 2008), and it is thus vitally important that Australia improve it is quality of production and upgrade it is technology if it is to continue to compete in this global market. In this respect, it is vital that we give support to farming communities by researching and solving some of the difficulties and problems that the industry continually faces.

However still there are some global issues that required attention when it comes to reduction of orange fruit production due to issues such as bad weather, post-harvest, storage and microbial problems. According to a report by United States Department of Agriculture and Foreign Agricultural Service 2015, Brazil indicated a reduction of 16.3 million tons and when fresh consumption is expected to remain nearly unchanged whilst fruit for processing is expected to drop 5% to 10.8 million tons. EU's production is forecasted to drop 500,000 tons to 6.2 million, South Africa's production shows a 1.6-million-ton loss and Morocco's production is forecast to fall 25% to 750,000 tons (United States Department of Agriculture and Foreign Agricultural Service 2015).

Furthermore, when it comes to internal injury and internal defect they are difficult for the farmers to identify and isolate sound oranges from freeze-damaged fruit unless there are visual differences, and there will be a problem once they are stored together after harvested, contamination can quickly spread to the sound fruit. In addition, Microbiological contamination, caused by deterioration of the fruit due to handling, frost damage and storage issues, is one of the greatest threats to the fruit industry in general (Tournas and Katsoudas 2005). Moreover, it is possible for the fruit to be attacked by bacteria, yeast and mould which are the main cause of spoilage during both the immediate post-harvest stage and long-term storage. In such cases harvested fruit contains living tissues which evidence continuing metabolic changes, and are thus subject to respiration and water loss. Because of this exchange with the environment, they can easily be contaminated by microorganisms (Kamal Rai *et al*. 2014).

In this research, frost damage orange fruits were investigated in order to find a solution to the problem that many farmers meet during winter. Every year millions of dollars' worth of citrus fruits is destroyed by chilling injury, due to cold winters. Especially the Navel variety is more likely to be hit with adverse cold weather conditions than Valencia verity. Indeed, Obenland (2008) has

confirmed that Navel oranges have been found to be the most sensitive to cold temperature and are therefore more vulnerable to frost damage than the Valencia cultivar.

Therefore, if damaged fruit can be identified early, they can be sent to juicing, which can offset some of the potential economic losses. But generally, Values of fresh and juicing orange varieties are varied due to very seasonal in nature, and the quality specifications based on production level, the suppliers and market demand (Department of Environment and Primary Industries 2010). The cost of orange fruits as well as orange juice kg/L in Australia averagely shows as follows, Price of Valencia orange coasts $2.50 per kg and Navel orange coasts $3.90 per kg compare to juicing price of much lower than normally expected and it is $1.00 per L. This is due to high challenges in fruit marketing especially for the fruit does not "meet retail specifications" has become more apparent to be processed and sold as juice (Australian Computation and Consumer Commission 2016).

As a consequence, to reduce the uncertainty which currently exists in the orange fruit industry regarding freeze damage, we believe it is necessary to conduct some scientific investigation into the issue, and help to resolve this problem. Victoria University has conducted this current study to identify and isolate sound fruits from the freeze damaged fruits by analysing both the internal damage and chemical change that takes place during the storage period after the orange fruit has been freeze damaged.

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List of Abbreviations

DCM Dryness cut methods
CHI Chilling injury
VI Visual inspection
UCCERT University of California Cooperative Extension research team
OAV Odour activity value
NFPP National Foods' processing plant
USAPMR US Agricultural product and marketing report
UCANRP University of California Agriculture and Natural Resource publication EPR
Ethylene Production Rates CHE
Chilling Exposure CHI
Chilling Injury VPBA
Various Physiological and Biochemical Alterations FAO
Foreign Agricultural
Organizations
FAS Foreign Agricultural Service
WCFP World Citrus Fruit production
USDFAS United States Department of Foreign Agricultural Service
HCP Horticulture /Citrus Publications
AAB Australian Agri-Business Report
FD Freeze Damage
RCA Responses to Controlled Atmospheres
ABP Anti-Bacterial property
CCC Chemical Composition of Citrus Fruit
VE Volatile Extracts
FC Flavour Compounds
EO Essential oil
GAT Green Alternative Technique
GC Gas Chromatography
GC-MS Gas Chromatography mass spectrometry
NIRS Near-Infrared Spectroscopy
LED Light-emitting diodes
CCD Charge Coupled Device (silicon Based)
UV Ultraviolet
VFC Volatile Flavouring Constituents
DCT Development of Column technology

VE Volatiles Emanated

WCAC Waxes Coating Appealing Characters

FPCF Fermentation Process of Citrus Fruits

QQV Qualitative and Quantitative Variation

OA Odormetrology Analysis

SPME Solid Phase Micro-extraction

SC Sensory Characteristics

IP Insecticidal Potency

ABS Australian Bureau of Statistics

IQF Individually Quick Freezing

Chapter One

General Introduction

Oranges are one of the most important fruits in our diet. When consumed, they can contribute to our nutritional needs and are a major source of Vitamin C (National Academy Press 2000). However, as a perishable commodity, they need proper care to preserve their quality and visual appeal before reaching consumers. In order to meet the required food standards, there are guidelines and specifications that producers need to follow to make sure consumers are receiving good quality fruit. As a consequence, there are food-handling procedures, represented by a number of important parameters, which represent food quality that should be instituted in order to provide high-quality oranges to commercial markets (Watada *et al.,* 1984).

1.1 The Complexity of Global Citrus Fruit production

Before describing the current project in detail, an understanding of the importance of the orange market in the global food chain through an appreciation of the forecast global production of orange fruits, and the level of complexity of the market structure, is absolutely crucial. In the year 2011, orange fruit production rose to a record level of 51.3 million metric tons. However, according to a report from the United States Department of Foreign Agricultural Service (2013), global orange production for 2012/13 was reported to have dropped sharply due to a decline in US production by 4% reflecting the unavailability of oranges from Florida.

Furthermore, International orange production forecasts for 2014/15 shows more drops for countries such as Brazil, China, and the European Union (EU) compared to a 4% decline from the year 2012/13 to 48.8 million metric tons (United States Department of Agriculture and Foreign Agricultural Service, 2015). As a result, fruit processing will also be expected to drop over 7% with exports dropping by 3% as a result of US orange production still showing a reduction of 6.1 million metric tons which is the same as the previous year (United States Department of Agriculture and Foreign Agricultural Service, 2015). Overall production was reduced by 16.3 million tonnes for Brazil and fresh consumption is expected to remain nearly unchanged whilst fruit for processing is expected to drop 5% to 10.8 million tonnes. EU's production is forecast to drop 500,000 tonnes to 6.2 million, South Africa's production shows a 1.6 million tonne loss and Morocco's production is forecast to fall 25% to 750,000 tonnes due to issues such as bad weather, post-harvest, storage and microbial problems (United States Department of Agriculture and Foreign Agricultural Service, 2015). In addition, the South African government has put strict control measures on orange exports in order to avoid

quality problems. Russia's ban on certain agricultural imports was another factor that was mentioned in the report contributing to why exports declined from United States, EU, Canada, Australia and Norway, especially in 2014 (United States Department of Agriculture and Foreign Agricultural Service, 2015). In this somewhat complicated scenario, there is a mix of economic, political and natural drivers which are affecting production and distribution of oranges. This thesis will focus upon natural issues, particularly those involved with the identification of damaged fruit after cold weather events.

1.2 Australian Horticulture

Within the Australian horticultural industry, citrus fruit represents an important product, and is the largest fresh fruit export in Australia, which is worth in excess of $200 million annually. According to the Australian Bureau of Statistics' (ABS) report of 2014, Australia citrus fruit production was estimated at 617,000 tonnes, with 75% of this total being oranges (Navel, 238,500 tonnes and Valencia, 194,300 tonnes), and mandarins being 18%. The report also indicates that of the oranges produced in 2012/13, New South Wales produced 52%, South Australia 33%, Victoria 12%, Western Australia 2% and Queensland 1%. NSW produces around 250,000 tons of citrus fruit annually representing 40% of Australian production and 36% of citrus fruit exports. Citrus fruit are sold either fresh or as processed citrus fruit products such as juice. Generally, most citrus fruit production is accounted for by oranges; however, grapefruits, mandarins, pomelos, lemons and limes are also produced at the more local level (Department of Environment and Primary Industries, 2010).

According to the report from the Australian Horticulture Factsheet (2013-14), Australia's horticulture industry, which produces a significant amount of orange fruit, has long enjoyed a domestic and international reputation for quality. This reputation is due to the high standards which have been maintained across all stages of the supply chain, from farm to consumer. In 2009-10, and again in 2011-12, Australia's horticultural industry was the nation's third largest agricultural industry based on gross value of production (Horticulture Factsheet, 2012). The major horticulture growing areas in Australia include the Goulburn Valley of Victoria, the Murrumbidgee Irrigation area of New South Wales, the Sunraysia district of Victoria/NSW, the Riverland region of South Australia, northern Tasmania, southwest Western Australia and the coastal strip of both northern New South Wales and Queensland. In Victoria, with which this investigation is primarily concerned, a report from Department of Environment and Primary Industries (2010) shows that fruit production is widely geographically spread across the State. Key production areas are in the Mallee, the Goulburn Valley, Port Phillip, Westernport and the North Central regions, and these fruit industries make a significant contribution to their local economies. Of particular interest here is that in 2009-10, the Mallee region of Victoria

accounted for 93% of the value of Victorian orange production during that period (Department of Environment and Primary Industries, 2010). The economic importance of the orange industry is reflected in the following figures; Australian orange export in the year 2013 was forecast to be approximately 77% (133,409 tonnes) of total citrus export volume. These exports were: 26% to Japan, 24% to Hong Kong, 9% to United States, 9% to Malaysia and 6% to Singapore. China was an exceptional figure in this period with exports to China being increased by 288% (2,371 tonne to 9,195 tonnes (Australian Bureau of Statistics, 2014).

1.3 Comments on the Contemporary Orange Industry

In Australia, oranges are produced in a number of different varieties such as early, middle and late ripening varieties. These varieties commonly share a typical fruit colour, with a distinction being drawn between blond, blood and late oranges (Department of Environment and Primary Industries, 2010). Valencia and Navel were the most common varieties, with Valencia becoming available from November to February and Navels available during the winter from June to October. Furthermore, the Navel variety is more likely to be damaged by freezing temperatures than Valencia cultivars (Obenland, 2008).

1.3.1 Cost of orange fruit and juiced products

Generally, the value of fresh and juicing orange varieties varies due to them being seasonal in nature, and due to quality specifications based on production level, the suppliers and market demand (Department of Environment and Primary Industries, 2010). The average cost of orange fruits as well as orange juice in Australia are as follows; Valencia orange costs $2.50 per kg and Navel orange costs $3.00 per kg while the juicing price which is much lower and is $1.90 – 2.50 per L. This is due to challenges in fruit marketing especially for fruit that does not "meet retail specifications" which can only be processed and sold as juice (Australian Computation and Consumer Commission, 2016).

1.3.2 Common problems in the citrus fruit industry

In order for Australia to preserve and expand its traditional markets, strict measures must be taken to improve and maintain the quality of the fruit (Australian Trade Commission, 2013). The importance of this issue is reflected in the possible saving of millions of dollars that are currently lost due to quality defects and from natural disasters during pre- and post-harvest conditions (Judith, 1998), together with the concomitant protection of the national food chain. To contribute to this important issue, clearly an understanding of the steps needed to protect fruit attributes, represented by standard quality parameters of citrus fruit, is urgently needed.

Citrus fruits are very prone to losing flavour quality during storage and, as a result, often have an unacceptably short shelf life. To better understand the basis of this flavour loss, Obenland *et al*. (2008) have reported that temperature and time have great influence on the sensory quality of the fruit during storage and therefore the storage conditions must be carefully controlled for the fruit to be acceptable to consumers. It has been suggested that a change in the flavour of citrus fruit is due to changes in a range of volatile components and other key attributes. In this respect, the volatile components of citrus fruits were investigated by De-Sousa *et al*. (2004) who reported that the main components in the citrus peel essential oils are limonene, which accounts for 72.5-76.4%, followed by β-pinene 11.6-18.7%, monoterpene hydrocarbons, namely terpinene 2.88-8.26%, α-pinene 1.4-1.5% and myrcene 0.95-1.12%. Identification of a wide array of novel bioactive compounds such as flavonoids, phenolics and limonoids from citrus fruits, was also reported by Divaio *et al*. (2010). Similar work has been reported during the storage of mandarins by Tietel *et al*. (2010), and according to Obenland (2003) post-harvest damaged mandarins often develop off-flavours due to loss of volatile compounds and microbial attacks during storage that impact consumer acceptance. In addition, ethanol has long been identified as being a compound identified with flavour loss in stored citrus fruit.

1.4 Microbiological Issues

Microbial issues are one of the biggest concerns to the fresh orange fruit industry. Beuchat, (1996) and Beuchat and Ryu (1997) stated that the fruits normally carry no pathogens unless the fruit is damaged, injured or has some internal/external changes due to some environmental issues. As well, it is possible for fruit to be contaminated on the farm or during stages of product handling to the point of sale (Ryu, 1997).

In addition to the above issues, microbial contamination of bacteria, yeasts and moulds are the main cause of spoilage of orange fruit. In most cases old practices such as the use of raw manure and contaminated soil amendments, dirty irrigation water, wild animals and birds, and dirty farming equipment as well as working in unhygienic environment and storage environment can also contribute to deterioration to the orange fruit (Beuchat 1996).

In addition, penetration of the skin, which can be caused by external damage such as bruising, cracks, and punctures, creates sites for establishment and outgrowth of the spoilage microbes. This spoilage can be relatively rapid, occurring within days of the trigger damage (Kader, 1997). As a consequence of the harvesting and storage process, fruits and vegetables constitute nearly ideal conditions for the survival and growth of many types of microorganisms. However, whilst the internal tissues of fruit and vegetables are nutrient rich (Mandrell *et al*.,

2006), fruits and vegetables possess an outer protective epidermis, typically covered by a natural waxy cuticle layer containing the polymer cutin (Lequeu *et al.,* 2003). Microbial communities must cause deterioration to the fruit in order to exploit the nutrient (Miedes and Lorences, 2004), and consequently, spoilage microorganisms help to exploit the host by using extracellular lytic enzymes to degrade the fruit and vegetable's structural polymers, which in turn releases water and the fruit's other intracellular constituents for use as nutrients for their growth (Bartz, 2006).

In this Project, however, to provide appropriate focus and depth to the work, only the effect of frost damage on orange fruits and methods to predict damage in advance of the emergence of physical and chemical symptoms was investigated. Current losses for this problem alone often mount to millions of dollars. In one season in 1988, in California alone the freeze damage to citrus fruit amounted to US $700.4 million (California Department of Food and Agriculture, 1999). In a separate report on the above incident, Guillaume *et al.* (2013) concluded that the severity of this damage was such that it warranted detailed future research, since with the potential onset of extreme weather conditions due to global climate change, it is anticipated that these losses will increase significantly because of the effect of cold weather on citrus fruit.

During cold winters, particularly when the temperature falls below average, frosts and freezes can occur and present a significant concern to citrus fruit growers (Arce *et al*., 2007). Such cold snaps occur from time to time throughout the world; therefore, this study has global, as well as local, significance. Indeed, according to a California Food and Agriculture Department report, freezing has been a major issue in the US. As mentioned above, in 1998 alone, the California citrus industry suffered a US $700 million loss after a three-day freeze in which 85% of the crop was lost. Rodrigo (2000) made an investigation into how subsequent frost damage is caused at the cellular level as well as its anatomical and morphological consequences in fruits. He concluded that freeze injury is one of the main limiting factors to crop production and distribution of horticultural crops, and it still accounts for greater losses of fruits and vegetables.

In the citrus industry, whilst frosts can damage current crops, they can also cause long term harm or death of orange trees. As a further example of the severity of this phenomenon, the last major freeze which occurred in Florida in December 1989 which followed four other significant freezes throughout the 1980s, has consequently caused the Florida orange growing industry to physically migrate further south, through the plantation of replacement groves where the risks posed by freezes and frosts are somewhat reduced (Bancroft, 1994). Although this aspect will not play a key part of this study, as mentioned earlier, the importance of climate

change on citrus production is going to be significant factor in the near future regarding the siting, development and preservation of the industry.

As it is common to see unpredictable freeze injuries to orange fruit being caused by prolonged low temperatures in the field, this is an ongoing challenge in the export citrus industry around the world (Kader and Arpaia, 1992). Early symptoms of freeze injury include a 'water-soaked' appearance in regions of the interior of fruit segments and the presence of hesperidin (Hesperetin 7-rutinoside) crystals in the membranous areas between the segments of the orange fruit (Kader and Arpaia, 1992). Drying of the internal flesh and the development of open spaces between the segments occurs between several days to weeks after the freeze event, depending upon the severity and duration of the freeze (David, 2007). It is also possible to find peel damage in the form of brown staining and pitting in the case of more frost damage. It makes the evaluation process difficult as the initial freeze symptoms are in the interior of the fruit rather than being visible from outside. The samples need to be cut for examination and evaluation, which is a labour-intensive and unreliable method. It is imperative, therefore, for the protection and development of the industry, to develop a fast, effective and reliable method of detection of frost damage (Kader and Arpaia, 1992).

After experiencing extremely low temperatures, orange fruit survival may depend on their stage of growth and development. Whereas low temperatures inevitably cause some damage to the fruits, it appears that the degree of chilling and frost injuries depends on the duration of the cold temperatures and how quickly the temperature dropped during the onset of the cold spell (Bancroft, 1994).

1.5 Previous Investigations on the Impact of Cold Weather on Citrus Industry

Investigations have been previously conducted on freeze damage to fruit in relation to reducing chilling injury and decay (Arce *et al.,* 2007). Once the fruit is injured by frost damage, the fruit loses its volatiles during the storage periods which in turn cause quality deterioration in the fruit. According to Askar *et al*. (1973), it is these volatile components which are important factors within the plant which gives the fruit its aroma. A similar study was also carried out by Aroujalian and Raisi (2007) in relation to chilling injury and fruit quality. During the investigation of the quality of orange juice taken from freeze-impacted fruit, this group found that unacceptable changes in the aroma of the fruits occurred. Experimental analyses showed that samples lost most of their volatiles, including linalool and limonene, during storage time (Aroujalian and Raisi, 2007). One further study, carried out by Zhang *et al*. (2011), involved an investigation of fruit stored at different low temperatures for 7, 14 and 21 days and after a subsequent shelf-life of three days. At each of those storage times it was reported that

reduction of volatiles was markedly influenced by storage temperature and time. In general, 90% of fruits were sensitive to chilling injury (CI) and had the lowest levels of volatile compounds, especially in respect to esters and lactones.

Physical freeze damage on citrus fruit occurs when water inside the fruit and leaves from a tree freeze, causing rupturing of cell membranes during the expansion of the water on solidification (Aroujalian and Raisi, 2007). In addition, frost has long been believed to impair the storage quality of apparently undamaged citrus fruits unless they are harvested shortly after the foliage is killed. Several hypotheses, but few data, have been submitted to explain this impairment or even to substantiate the injury. Nevertheless, chilling injury symptoms such as pitting, necrosis and staining may be seen as soon as the fruits are brought to room temperature (Aroujalian and Raisi, 2007). Furthermore, these authors note that chilling damage can also be identified, in citrus fruits in particular, by brown spots on the peel, and fruit usually has a bitter taste and unpleasant odour, with evidence of rot and cell wall collapse. Other indications are that the glossiness of the peel will be lost, the white albedo layer of the orange turns to a dark colour, and when inspected the fruit evidences loss of juice content compared to the sound fruit after storage time (Aroujalian and Raisi, 2007). These physical symptoms clearly present a problem for the economic value of affected fruit.

1.6 Current Industry Practices

Fresh fruit suppliers and packers are the main channel of fruit export and domestic consumption sources. Large fresh fruit packers may contract with growers in several different production regions to ensure that fresh fruits are available throughout the year according to the season of fruit. These packers generally contract only in regions with a large number of growers to make sure they fulfil market demand. Price and quality are very important in fruit production. Buyers and consumers always expect the highest quality grade of fruit, which is categorized by its flavour, ripeness, odour, cleanliness, and the absence of insects and foreign material. To ensure that these conditions are met, special attention must be given to adequate post-harvest handling, storage and distribution. This includes detection of fruits containing non-authorized pesticides, other pesticide residues exceeding permissible limits, products having inadequate labelling and packaging requirements, and with contaminants exceeding regulatory levels, without the required nutritional information and/or with inadequate general quality in order to complete the demand of industry practices (Luz Berania, 2004).

At the present time, oranges are sorted by density but this might only occur after a period of up to six weeks after a frost event. Sound oranges are sent to fresh fruit markets both locally and overseas, while the orange fruits that were damaged, and cannot be utilised by fresh fruit

markets, are processed to be juiced and sold to consumers in this form to save farmers from further losses (Aroujalian and Raisi, 2007).

1.6.1 Fruit processing

The industry makes possible for the creation of an enormous diversity of fruit based or fruit-flavoured food and beverages. It also can be used to convert the perishable fresh fruit into more stable processed products with longer storage lives and individually quick freezing (IQF) of fruit pieces, aseptic pulps and juices. Clearly, the processes must assure food safety, compliance with regulations, with nutritional quality and product quality to meet consumer expectations. There are implications here again for frost damaged fruits, which must be carefully monitored for nutritional damage before processing and removal of physical marks (Aroujalian and Raisi, 2007).

1.7 The Need for Further Research

In conclusion, it is important to emphasize the importance of the citrus/orange market to the Australian (and global) food economy, and therefore the essential nature of investigations into the problems that face citrus/orange industry due to during, pre and post-harvest, storage, frost and freeze damage. In this particular study, particular focus has been placed on the critical problem of low temperate damage during a cold winter, and what needs to be done to maximize the opportunities to identify the damaged fruit and sound fruits immediately after damage. As noted earlier, the Australian citrus industry is the largest fresh fruit exporter in the Australian horticultural industry, and this suggests that research into frost damage can play an important role in relation to production practices, resulting in increasing yields and quality (James, 2010).

1.8 Aims and Objectives of the Project

The overall aim was to develop a chemical method that would replace the current physical based (density) methodology to improve quality control (accuracy and efficiency) for identification of different degree of damage in orange fruit.The focus is to realise a practical method for testing freeze damaged oranges more accurately and which can be achieved in a short time frame. Thus, a number of analytical methods will be considered, all of which can be completed in 2 – 24 hours, as follows. Once samples are prepared for a test using gas chromatography (GC) or gas chromatography mass spectrometry (GC-MS) methods take typically around 1-2 hours, a hand-held ethanol test takes 24 hours, an Instron firmness test can be done within 10-30 minutes, and a microbiology test takes 1-2 days. In the case of GC and GC-MS multiple samples can be tested, since such systems typically employ an auto-sampler system.

These involve the following aims:

To simulate frost damage by subjecting oranges to different low temperatures and thawing times using a laboratory freezer.

To investigate the change in pH and TSS (Total Soluble Solid) in damaged oranges after freezing.

To study volatile components such as terpenes, in particular limonene, and ethanol by GC (Gas Chromatography) and GC/MS (Gas Chromatography-Mass Spectroscopy) in freeze damaged oranges.

To evaluate the effect of freeze damage on the fruit and investigate changes that take place during three weeks storage periods.

To study the types of bacteria, yeast and mould that grow in freeze damaged oranges.

To investigate the degree of external damage caused to oranges after freeze damage using visual assessment and general quality investigations such as a firmness test (using Instron equipment).

To present the findings to the Citrus Industry in a seminar and in journal articles to the scientific community.

Figure 1.8 presents a flow chart of the three phases and five steps that enable the various aspects of this research Project to be appreciated. Pre-experimental phase (Phase 1) for the development of the material, a pilot study and to test the suitability of the material and procedures on the freeze treated Valencia and Navel orange fruit as well as to organizing the materials and methods in (Phase 2), and an experimental phase of (Phase 3), which follows five experimental steps and finally the presentation and interpretation of data as Figures and Tables, as well as report writing.

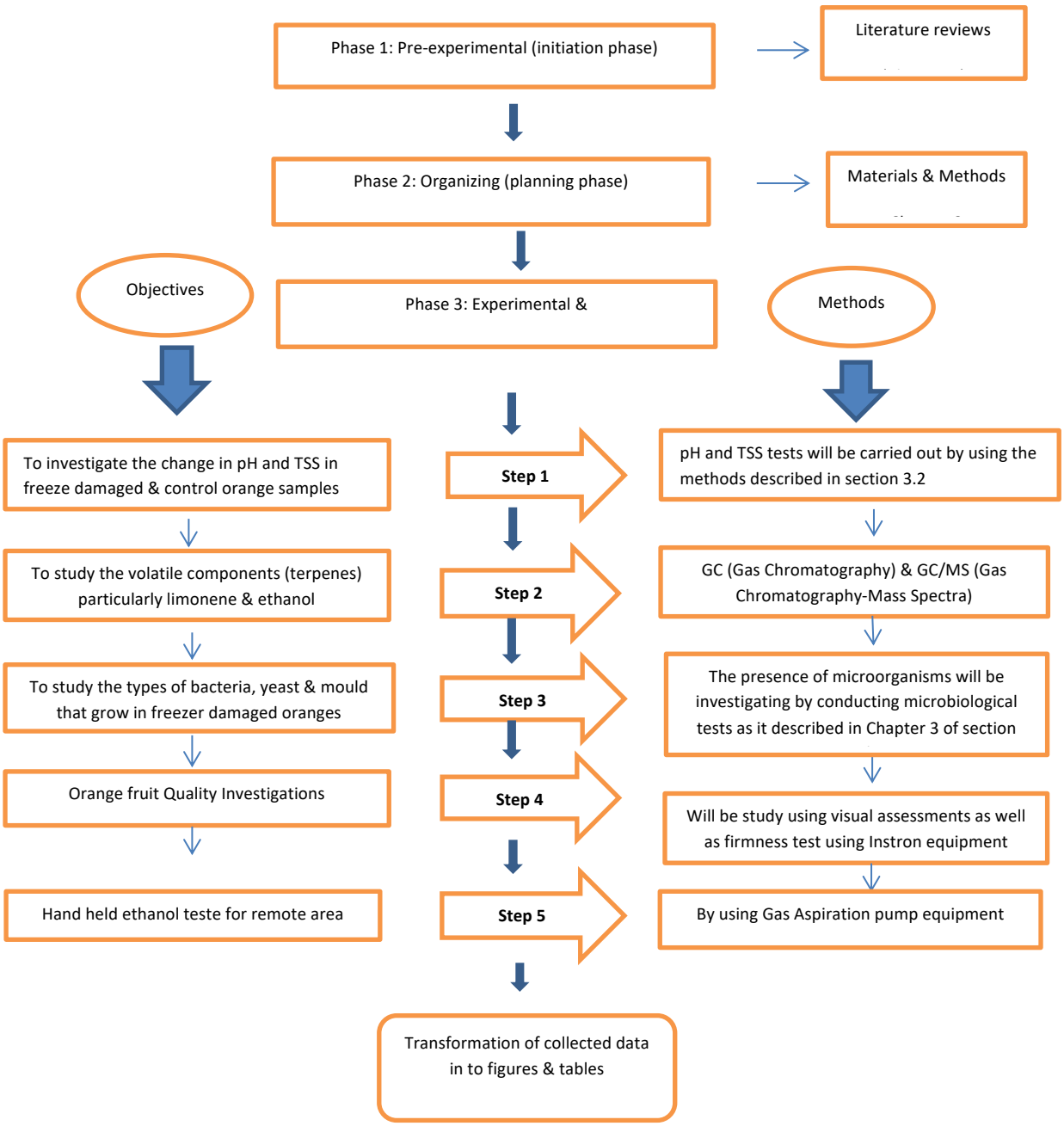

Figure 1.8 presents a flow chart the phases and steps of the research Project

1.8.1 Research Design

Quantitative research study (Cone & Foster, 1993).

The Research design presented in the study indicates the structure and procedure that follow to answer the research questions listed below based on the premise. The purpose of this study is described in abstract and Justification section of page (iv and ix), the thesis outline is also presented in section 1.9, and figure 1.8 on page 10 shows a flowchart that explains the stages and steps of the project.

As it was stated by Cone & Foster, (1993), the above project phases were designed as a collection of scientifically related project activities that concludes when one or all objectives of the project are achieved. The project was also divided into a number of phases and steps that are required for the specific work to be performed uniquely to a piece of the project and are typically linked to the development of particular major issues that needs to be resolved.

This research study was conducted in a sequence process of three phases and five steps as described in Figure 1.8 of (flow chart) above. According to the flow chart, sequential processes of the three phases are described as follows: phase 1 represents project requirements or Initiation, whereas phase 2 is project planning and phase 3 is constructing the project or executing. Furthermore, the project focuses in three major areas of investigations as listed below.

1. General fruit quality, pH and TSS tests to both freeze damaged and control orange fruit and juice samples.

2. To study volatile compounds of orange fruit, particularly terpenes (limonene and ethanol) using oil collected from orange skin.

3. To study types of bacteria, yeast and mould that grow in freeze damaged oranges and the level of damage that takes place during three weeks of the storage periods.

Moreover, quantitative research methodology focused on fruit quality testing and volatility analysis using experimental methods that described in Chapter three of section 3.1 Maturity levels, project factors and number of repeats, section 3.1.5, pH and TSS analysis, section 3.2, volatility test, in section 3.3, microbiological test, section 3.5, fruit firmness in section 3.8 and fruit quality test in section 3.8.1 and 3.1.6, fruit firmness test in section 3.5 was described.

1.8.2 Research Questions

The following sub-questions were investigated in this study subsequently:

Are there any changes to pH and TSS levels to frost damage fruits during storage time? If there are changes what are the effect on the quality of orange fruit and can the changes be used as indictor for a purpose of identifications of fruit deteriorations? How do we perform fruit quality tests when it comes to volatility study and which volatile compounds can be used to investigate the level of damage to the fruit?, in order to answer the above questions the decision made was influenced by investigators such as (Creswell, 2003; Creswell et al., 2003; Onwuegbuzie & Teddlie, 2003; Cone & Foster, 1993; Teddlie & Tashakkori, 2003; Johnson & Onwuegbuzie, 2004, October; Phillips, 2004) who have more knowledge in project design methods, These investigators assert that researchers who prefer to use any of the design should do it with respect to their underlying research questions and type of the project study they are dealing with.

1.9 Outline of the Thesis

Chapter One has introduced the quality parameters of oranges and gives the reader a basic understanding of the project as well as outlining the approaches that will be taken to predict frost damage and details of the investigation to be undertaken. In **Chapter Two**, a review of the literature is given regarding the effect of frost damage in orange fruit. It also examines the laboratory techniques used by researchers in relation to a broad range of topics such as: quality parameters of frost damage fruit, textural quality, physical property, antimicrobial activity, volatility, chemical property, marketing and instrumental analysis of citrus. The material and methods that will be used throughout the experiments will be covered in **Chapter Three**, which also gives detailed information on experimental analysis of pH and TSS, Limonene and ethanol tests. The chapter also discusses practical work that was done on peel oil (collected by steam distillation) and analysed using GC/GC-MS and hand-held ethanol testing equipment, some microbiology tests.

Chapter Four is the results and discussion, including figures and the tables, relating to pH and TSS that were conducted in this investigation. **Chapter Five** provides a summary of the GC and GC-MS Chromatographic experiments that yielded information about the presence of volatile compounds including limonene and ethanol. Details in **Chapter Six** are concerned with an ethanol test that was conducted using hand-held equipment; in this experiment, ethanol was tested on both freeze damage orange fruits and control samples, and the results and relevant discussion are also provided in this chapter. In the following chapter, **Chapter Seven**, microbiological analysis of freeze damaged orange fruits are presented. This chapter

explains in detail the experimental investigation into yeast, mould and bacterial presence in freeze damaged orange fruit samples. Results and discussions of the practical findings are also presented throughout this chapter. The work in **Chapter Eight** deals with a demonstration of a textural test of orange fruit firmness using Instron equipment, and presents an experimental analysis of freeze damaged and sound orange fruits. **Chapter Nine** concludes the study by presenting an overview of the research findings and suggesting some ideas on possible future research to further explore aspects of frost damage in orange fruits while **Chapter Ten** is the reference list of the thesis.

Chapter Two

Literature Review

2.0 Orange Fruit

When it comes to the importance of evaluating orange fruit quality, considerable time and investment is required to investigate and improve the level of quality specifications (Kader and Arpaia, 1992). Despite the economic and nutritional importance of oranges, very little research has been done in terms of developing easy, accurate and simple test methods that enable farmers to assess the extent of damage that has been caused during a cold winter to the orange fruits (Kader and Arpaia, 1992).

As described in the previous Chapter, this investigation will focus on the determination of the quality of the fruit in regards to the effect of low temperature damage and storage time. It has become obvious that the influences of environmental differences for the duration of the fruit-crop years, combined with episodes of severe climatic conditions, can produce major quality differences in the fruit-crop year depending on the different level of fruit maturity (Scora *et al.,* 1967). Quantitative and qualitative changes to the composition of the essential rind oils as well as low temperature damage that is associated with mature fruits and handling should be quality control issues that require routine monitoring (Scora *et al.,* 1967).

In this research, it has been intended to provide efficient and simple detection methods to detect freeze damaged orange fruits which are based on chemical and physical changes. It is important to develop such techniques in the hope that it will help the industry to maintain the quality of the marketed fruit. As explained in Chapter One, frost damage has been recognised as a serious problem that causes much loss to the citrus fruit industry; consequently, research needs to be conducted in many areas of this industry to ensure that acceptable quality fruit, up to world standard, can be confidently selected from frost damaged crops. Citrus fruits are an important part of our daily life since they provide important nutritional needs. Components also include essential oil compounds which can be used for medicinal purposes and which can be extracted using a variety of methods. According to Selli and Kelebek (2011), aromatic extracts were obtained by using liquid-liquid extraction to achieve compounds representative of blood orange juice odour. These aromatic compounds included alcohols, esters, terpenes, aldehydes, acids, ketones, volatile phenols and lactones, which were all identified in the Moro and Sanguinello variety juices. From these identified compounds, 15 volatile components presented odour activity values (OAVs) greater than 1, with d-limonene, nootkatone ($C_{15}H_{22}O$) and linalool being those with

Many research projects have been carried out studying the volatility of components in citrus fruit and research groups such as those of Montero *et al*. (2011) pointed out that these volatile components of the fruits can be used as an indicator of the control quality parameters. In this respect, volatile elements of citrus fruit in correlation to post-harvest handling issues were studied by Ramesh Yadav *et al.* (2004), who mentioned that the quality of the fruit depends on post-harvesting treatment and flavour levels which are also subject to environmental factors. As mentioned earlier, low temperature damage to the fruit is a very important factor that needs to be understood, which implies knowledge of the mechanisms which lead to the destruction of parts of the fruits. When the external temperature drops to freezing level or lower, particularly 2 ^{o}C or less, it appears that there are large amounts of damage that can be caused. The degree of damage can vary between cells or tissues of various fruits, and it can differentially damage fruit between groves or within individual groves (Ting and Blair, 1965).

In most cases for harvested crops, freeze damaged fruit can be inadvertently mixed with sound materials as the level of damage varies with the level of maturity of the individual pieces. Sorting the fruits after weather damage prior to storage is a challenging issue for industry (Bellon *et al.,* 1999). For many years, the citrus industry has been implementing use of specific gravity separation methods, a technique that has been acceptable to industry practices in many countries (Sala, 1998). However, this practice has become out of date, and there is need of a more accurate predictive mechanism to facilitate a system that could use automated sorting technology (Bellon *et al.,* 1999). A previous attempt to bring better quality fruit products into the market using automatic sorting methods was investigated by Steve and Frigola (2007), who reported that quality is also dependent on the way fruit is produced, processed, handled, sorted and stored.

The implementation of accurate and efficient isolation methods is desperately needed to help to distinguish freeze-damaged fruit from sound fruit, since this stops damaged fruit from reaching the fresh fruit market as well as international markets (Sinclair, 1994). It will also allow the grower and packing section to make decisions on whether to pack the fruit for the fresh market or to divert them for alternative use, such as processing into juice (Wardowski *et al.,* 1999), which has clear economic implications.

According to Artes and Eschiche (1994), investigation into frost damaged plants allows an explanation of what happens to plants when they go through a frost attack. These authors noted that the survival of the plant itself was a critical issue. It may survive depending on the amount of damage caused by the frost or an environmental change, but this may lead to an internal change in the fruit. In addition, Roger (2003) has mentioned some important proactive steps that can be taken in order to prevent damage. During the frost season, when

temperatures fall below -4 ^{o}C, preventative measures include insulating the tree trunks and building soil banks to help reduce the rate at which the trees can lose heat in sub-zero conditions. Insulating an orange sapling with a fibreglass insulation wrap-around for the sapling-rod system was also considered to be effective (Roger, 2003), while ignoring these methods may cause (irreversible) damage to orange fruits (Roger, 2003).

Chilling injury has a significant effect on citrus yield, growth, fruit quality and economic returns. A study of citrus fruits shows a significant variation in volatiles due to differences in environment and region (Bampidis and Robinson, 2006). An earlier study by Bazemore *et al.* (1999) noted that while citrus fruits contain volatile compounds that can be used for a variety of purposes, proper care needs to be taken during fruit maturation in cool climates since there is a required quality before the pieces can be marketed. In addition, chilling injury was studied by Wang (1993) who noted that this issue is one of the major problems when it comes to loss in the citrus industry. According to Wardowski and Harland (1999), fruit that had been through frost damage showed interior suffering with extreme damage even though the peel appears normal. However, this may not happen in all cases or in situations where the fruit suffers moderate damage. In any case, the frozen interior of the fruit will dry out, and the fruit will become hollow over time (Wardowski and Harland, 1999).

Furthermore, many fruits and vegetables are sensitive to damage when exposed to extreme temperatures (David *et al.,* 2013). In particular, fruits can be injured after a period of exposure to freezing conditions, and as a result they can go through internal changes which become evident in a short time after they are removed to warmer temperatures. Fruit and vegetables that have been chilled may go off easily due to physiological and biochemical changes (Oberoi *et al.,* 2011; David, 2004), and the tissues weaken because they are unable to carry on normal metabolic processes. Various physiological and biochemical alterations occur in the sensitive species in response to low-temperature exposure, this includes internal cellular enlargement (Chien, 2009). The development of chilling injury symptoms to the fruit tissues are among the common signs of freeze damage to many fruits (David *et al.,* 2003). In general, as a result of injury from freezing temperatures, it is possible that the fruit loses important volatile compounds and can produce ethanol and other compounds as by-products (Nursten, 1970).

2.1 Citrus Fruit Industry

According to the Foreign Agricultural Service (2014), individual countries' global production of citrus fruit is increasing and the top 11 producers, as listed in the Report, are presented in Table 2.1.1. Current annual worldwide citrus production has been increased in Brazil by 29% followed by USA with 18%, and more than half of this total is oranges. The rise in citrus production been noticeable in recent years, and is thought to be mainly due to the increase in cultivation areas, improvements in transportation and packaging and consumer preference for healthy foods.

Table 2.1.1 Orange Producing Countries by Foreign Agricultural Service (FAS)

Top 11 Countries (% of world citrus fruit production)	
1. Brazil (29%)	7. Italy (3%)
2. USA (18%)	8. Iran (3%)
3. Mexico (6%)	9. Egypt (3%)
4. China (6%)	10. Pakistan (2%)
5. India (5%)	11. Australia (0.5%)
6. Spain (4%)	

2.1.1 Orange Fruit Consumption

The Foreign Agricultural Service (FAS) reported that world consumption of oranges grew at a compound rate of 3.5% over the period 1987 - 2000. Consumption of fresh oranges grew at an annual rate of 2.8%, and at the same time processed orange consumption grew at the rate of 4.4% every year (FAS, 2000). It is reported that orange product consumption has increased due to the expansion in the bottling industry. According to the report by Horticultural and Tropical Product Division of the United States Department of Foreign Agricultural Service (USFAS, 2004), the consumption of fresh oranges in Europe declined from 12.6 to 9.5 kg per capita, at the same time processed orange consumption increased to 28 kg (fresh fruit equivalent) in the United States and in Canada. Fresh orange consumption has also decreased in both countries. The report additionally indicated that orange juice consumption increased in developed countries such as North America and Europe. Markets for processed orange products also appear to be developing in Latin American countries such as Brazil and Mexico, while some third world countries are showing great decline in orange juice consumption due to the problem in availability of advance refrigeration, transportation and storage faculties (FAO and FAS Horticulture, 2004). Furthermore, according to the USFAO

(2004) report, citrus farmers are facing a decline in fruit prices due to great competition from international producers and reduction in production because of frost, drought, pests and diseases. These have tended to disrupt production by causing a severe shortage in supply that encourages buyers to look for foreign products with a more economical price (USFAO, 2004).

2.1.2 The Australian Agribusiness

According to a report from Australian Agribusiness (AAB), there are indications of how Australia's citrus industry is important in the world market. Its production of oranges, mandarins, lemons, limes and grapefruit was estimated to be 0.5% of the world's citrus production in 2007. There was an increase over the years 2005 – 2006 because the total Australian harvest increased by 16% (AAB, 2007). Given that citrus farming was only introduced into Australia after 1788, it is remarkable that it has become Australian's second largest horticultural industry (AAB, 2007). It is suggested that Australia's diverse climate helps the farming community in producing fresh fruits for local markets as well as exporting to international markets (AAB, 2007). The production of oranges was 80% of the total citrus fruit crop produced in 2004 and 2006 (AAB, 2007). Within this harvest, Navel orange fruits are grown for local and export market, with 80% of the fruit consumed as fresh fruit and 20% of low quality as fruit juice (AAB, 2007). In addition, it has been found that Valencia oranges are more suitable for juicing purposes. As a result, 55% of harvested fruit is processed as juice whereas 45% of the fruit is consumed fresh (AAB, 2007). Within the Agribusiness, there is a long process before the fruits arrive to consumers since it involves a relatively long supply chain. It begins with growers, after that to packing houses, wholesalers, agents, brokers, transport agents, processors, import/export agents, retailers, before it reaches the consumer (AAB, 2007). It is during this extended supply chain period that frost damage becomes evident, and damaged fruit must be removed from the rest of the crop to prevent further spoilage (AAB, 2007).

2.1.3 The Economic Effects of Low Temperature and Losses

According to a US Agricultural Product Marketing Report (USAPMR, 1990/91), differences in total citrus fruit production between seasons is not remarkable, but can have large economic implications for the industry. Conditions in the major orange producing states contrast sharply, and examples quoted include Florida's orange crop in 1989/90 which was up to 3.72 million kg less due to freeze-damage (US Agricultural Report, 1990), while Californian production fell 3.33 million kg because of similar freeze-damage in December 1990. The freeze of 1989 clearly impacted orange production in Florida's processing market, while the fresh market also felt the impact of that 1990 California freeze. The freeze-damaged 1989/90 Florida crop

resulted in an increase of imported frozen concentrate orange juice (US Agriculture Report, 1990). Much less juice was imported during the 1990/91 season, as processed utilization of Florida's oranges was up to 32 % from the 1989/90 season (US Agriculture Report, 1990). In the 5 years prior to the Report, 65 % of US orange production was processed into juices (chilled, canned, or frozen concentrate) and 35 % was utilized fresh. Nearly 90 % of US oranges used for processing have been from Florida, and almost 80 % of fresh oranges have been supplied by California.

According to Terzahewing and Stacy's report from The Wall Street Journal (29 December 1998), the freezing weather that devastated orange and other citrus crops in California could have a two-sided impact on one of the nation's most popular fruits; the first impact would be an increase the price of orange fruit. That is because California growers, who normally sell most of their orange crops as fresh fruit were possibly sending the damaged fruits for juicing, adding to an already ample supply worldwide. Also, juice prices rose sharply earlier the same year in response to bad weather and wildfires in Florida. On the other hand, the shortage of storage quality of orange fresh fruit, could lead to higher produce prices (Wall Street Journal, 29 December 1998).

The California Farm Bureau Federation in Sacramento estimated that losses to the state's citrus crops would be approximately US $591 million in 1998. The actual loss for that particular year was 50% to 75% of their Navel-orange crops, and these were mainly due to freeze damage according to a report by the growers (Wall Street Journal, 29 December 1998). More than 80% of the state's orange crops are produced in the freeze-hit counties of Tulare, Fresno and Kern; about two-thirds of those are Navel oranges, the crop most affected by the cold snap. There also is some concern about California's other orange variety, Valencia oranges, which are harvested in the spring and summer and are just starting to grow in the cold weather (Wall Street Journal, 29 December 1998).

2.2 Changes to orange fruits from freezing events

According to Millikan *et al*. (1991), freeze-damaged oranges display changes that make them unsuitable for consumption. Immediate visible changes include the appearance of spots over the surface of the fruit and the formation of white ice crystals in the interior of the fruit (Millikan *et al.,* 1991). Ice crystal formation disrupts the pulp cells in frozen fruit, and this creates pathways for the fluid to escape from the fruit; consequently, dehydration is the ultimate negative result of freeze-damaged oranges (Sylvertsen, 1982).

An adverse chemical change in frost damaged orange fruits includes a change in volatile composition, together with the formation of new volatile compounds and a reduction of some

important terpenes from the fruit (Njoroge, 2005). Changes in volatile compositions could be the main reason for the fruit to develop an unpleasant and unusual bitter taste which will be unacceptable to consumers (Pallottino, 2011), but this could also be due to simultaneous reduction in soluble solids and total sugar content of the fruit (Sinclair, 1984). Freeze damaged orange fruit symptoms include pitting, brown staining, discoloration and increased susceptibility to decay. Internal symptoms include brown discoloration of the white membranes separating segments of the fruit. The concept of a minimum safe temperature has been suggested, but this often depends on the particular cultivar; for most fruits this is 4-5 °C (Elyatem *et al.,* 1984). Other factors such as production area and maturity-ripeness stage at harvest are all factors which will determine the degree of damage to the fruit (Khairi, 2001). It was also reported that respiration and ethylene production rates (EPR) increase, and when other chilling injury symptoms appear, their severity increases with lower temperatures and longer durations of chilling exposure (CHE). Therefore, an important procedure is harvesting the fruit as early as possible after freeze damage event to prevent further damage. Moreover, external and internal browning is related to oxidation of phenolic compounds (Kader *et al.,* 1984).

However, it has been suggested that there are ways to slow down the chilling damage process. According to Khairi, (2001), the severity of symptoms can be reduced if water loss is minimized by waxing or film wrapping, and decay caused by fungi can be controlled by use of fungicides and/or biological antagonists. Most fruits show sensitivity to low temperatures (Kader *et al.,* 1984). The fruits get injured after a period of exposure to chilling temperatures, -2 °C and below, which is below their freezing points (Kader *et al.,* 1984). Certain horticultural products are also susceptible to chilling injury at lower temperatures (Elyatem *et al.,* 1984). As the fruit is faced with chilling temperatures, the tissues inside the fruit get weaker as a result, and they are unable to carry on normal metabolic processes (Wang, 1994).

According to Wang (1990) various physiological and biochemical alterations (VPBA) and cellular dysfunctions occur in chilling-sensitive species in response to chilling stress. When chilling stress is prolonged, these alterations and dysfunctions will lead to the development of a variety of chilling injury symptoms, which includes surface lesions, internal abnormalities, water-soaking of the tissue, and failure to ripen normally. The longer the fruit has remained unharvested, the greater will be the extent of damage (Saltveit and Morris, 1990). One particular problem is that, often, products that are chilled will still look sound when remaining at low temperatures, but symptoms of the chilling injury become evident in a short time after they are removed to warmer temperatures (McColloch, 1962).

Fruits and vegetables that have been chilled may be particularly susceptible to biological decay. Weak pathogens such as the *Alternaria* species do not grow readily on healthy tissues, but can attack tissues which have been weakened by low temperature exposure (McColloch *et al.,* 1962). Both temperature and duration of exposure are involved in determining the extent of chilling injury. Damage may occur in a short time if temperatures are considerably below a fruit's threshold level (McColloch *et al.,* 1962), but in some cases, a product may be able to withstand temperatures a few degrees in the critical zone for a longer time before an injury become irreversible (McColloch *et al.,* 1962). In this case, the maturity at harvest and the degree of ripeness are important factors in determining chilling sensitivity, particularly in some fruits like avocados (McColloch *et al.,* 1962).

2.2.1 Freezing injury

This occurs when ice crystals form within the tissues, with the type of cultivars, the locations, and the growing conditions all affecting the freezing point (Whiteman, 1957). The most common symptom of freezing injury is a water-soaked appearance, and the tissues injured by freezing generally lose rigidity and become soft when the fruit is thawed (McColloch, 1953). The susceptibility of different fresh fruits and vegetables to freezing injury varies widely, with some commodities able to be frozen and thawed a number of times with little or no injury, whereas others are permanently injured by even slight freezing (McColloch, 1953). All fruits and vegetables can be categorized into three groups based on their sensitivity to freezing. The first group are those likely to be injured by one light freezing; the second group is moderately susceptible, and which can recover from one or two light freezing events, and the third group is the least susceptible as they can be lightly frozen several times without serious damage (Bramlage and Meir, 1990).

2.2.2 Albedo breakdown

Albedo, or mesocarp, is the white spongy material located between the fruit segments and the outer leathery peel called the flavedo. Albedo breakdown is the loss of cohesion in the cells; if this white layer under the skin has any stress imposed on it and, as a result of the expansion of the pulp, this layer may rot (Fernando and Jacqueline, 2003). According to Treeby (2002), albedo breakdown is a major rind disorder of orange fruit that results in significant economic cost to the Australian citrus industry. The industry has had a history of using calcium sprays as a control measure for albedo breakdown. However, a report from US Department of Agriculture and Food (2014), citing research by Treeby (2002), indicated that severe cases of albedo or pith layer beneath the skin could be much better controlled using gibberellic-acid sprays compared with calcium sprays.

2.3 Warming Frozen Products

Plant tissues are very sensitive to bruising while frozen, and this sensitivity is an important reason for leaving commodities undisturbed until they have warmed. Selecting a suitable thawing temperature involves a number of compromises (Bramlage and Meir, 1990). Fast thawing damages tissues, but very slow thawing (between 0 to 1 °C) allows ice to remain within the tissues too long and causes injury. Research on the rate of thawing has suggested that thawing at 4 °C for 4 hours causes the least damage for most frozen commodities (Lutz, 1936).

2.4 Evaluating Frost Damaged Oranges

Figure 2.4.1A and B shows the dryness cut method (DCM) in citrus fruit which is typically used to estimate the volume percentage of freeze damage in a fruit. In this method, one or more slices is removed from the stem end of the fruit to expose the flesh. The level of damage is given a letter (A-D) that is based on the first slice for which freeze damage is visible. The dryness cut method relies on visual inspection and it is subjective, inaccurate and destructive (Wardowski *et al.,* 1999). The instructions given for the use of this test may be expressed as follows:

First cut a thin slice off the stem end to expose the flesh;

(1) Then remove a 0.64 cm slice and examine the orange. If internal damage does not extend below this slice the fruit is graded “A”;

(2) If no damage is noted below this slice, it is graded as “B”. Otherwise, another 0.64 cm cut is made;

(3) An additional cut may be made as needed to determine the full extent of damage down to the middle of the orange (resulting in the grade “C”);

(4) In some cases, even lower inspections must consider the extent of damage within a slice (grade “D”). A 10% tolerance is allowed on fruit graded for the fresh market. Oranges for concentrate must be ‘wholesome”, (advice as cited by Wardowaski *et al*. (1999), from Annon 1983).

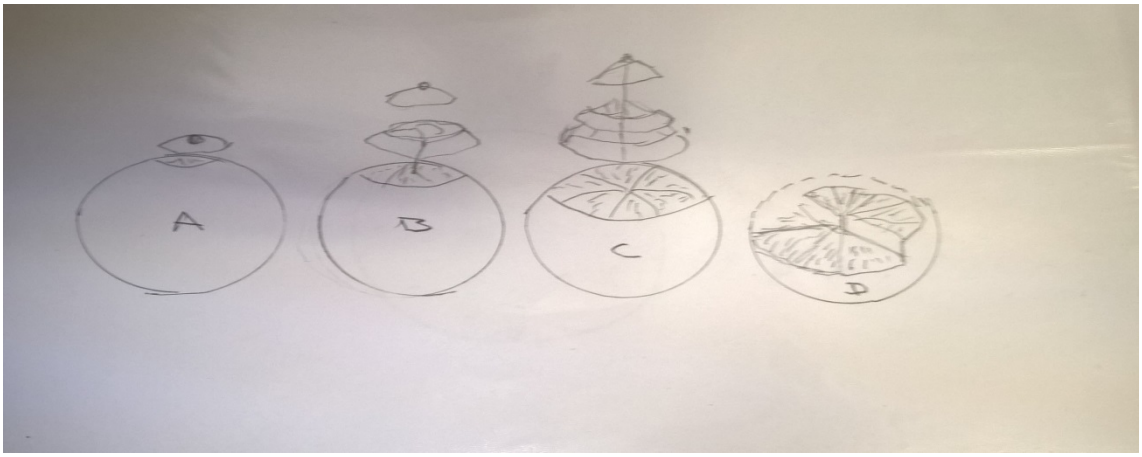

Figure 2.4.1A Details of the dryness cut method (DCM); hand drawing (from our work)

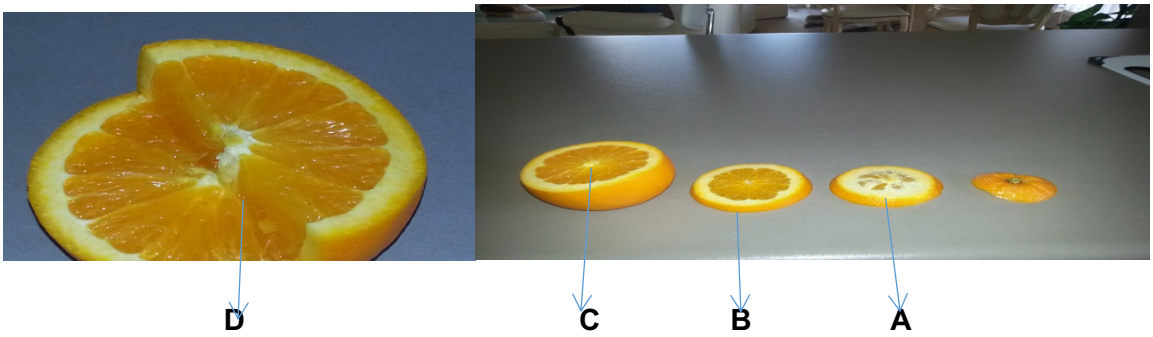

Figure 2.4.1B, Evaluating, extent of freeze damage using dryness cut method (DCM) in citrus fruit (from our work)

An alternative method was investigated by Davis (1973). In this work, Davis conducted a density separation of freeze-damaged fruit. Since the specific gravity of the damaged fruit decreases as a result of dehydration, the difference in density can be used for determination of frost damage. The report cautioned that this method is only effective between a few weeks to two months after a freeze event, during which time when the difference in specific gravity between damaged and undamaged fruit becomes apparent. Several other techniques have also been used to study the quality of oranges. Recently, Natale *et al*. (2007) used an electronic 'nose' to evaluate the quality of oranges. This device is designed to be sensitive to a range of volatiles and aromatic compounds such as alcohols and aldehydes, which helps in detecting damage, and it is hoped that this method will improve to provide an efficient and convenient method of volatile detection. Bellon *et al*. (1992) showed how sensors could be used to enable the use of automatic fruit sorting methods to reduce the complication of manual sorting methods.

According to a report of David *et al*. (2009), US Citrus Research Board established a task force seeking new and improved methods of detecting freeze-damaged fruit following the 1998 freeze when some poor-quality fruit reached the fruit market. The aim became to provide a non-destructive, objective, rapid, inexpensive, and usable test regime which can be used immediately after a freeze. UC and USDA researchers found that freeze-damaged fruit tends to produce high levels of ethanol, and the peel exterior often has bright yellow dots when exposed to black light. This report added that nuclear magnetic resonance techniques can also be used to sense freeze damage.

2.5 Controlling Orchard Temperatures

When winter brings cold temperatures that can damage fruit or foliage and thereby pose an economic threat to citrus production, the facilitation of warmer temperatures in orchards can decrease the threat of frost damage. For example, wind machines can be used to protect commercial acreage from frost by mixing warmer air with colder air near the surface of the orchard (Snyder and O'Connell, 1999). By using temperature forecast models that adjust the floor cover, citrus fruits growers can use these wind machines more efficiently (Natale *et al*., 2007).

In locations where wind machines are not cost effective, management of the orchard floor is even more important. According to Arnal *et al.* (2005), orchard floor management practices can impact positively on frost problems encountered in the orchard. The ideal orchard floor, from a frost protection standpoint, is firm, bare, moist soil, without any vegetation. This type of orchard floor absorbs more heat during the day than other floor management plans. In this respect, Snyder and O'Connell (1999), who were part of the University of California Cooperative Extension research team (UCCERT), ran an orchard floor management trial established in a commercial Valencia orange orchard in northern Kern County on Feb. 3, 1995 and results were found to be 'satisfactory'.

Heaters may also be used to warm the air under the inversion layer. They should be capable of raising the temperature above freezing point; however, the success of this practice depends on the height and strength of the inversion and on the presence or not of wind. The objective of heating is to distribute heat across the orchard to keep all areas above the critical lower (freezing) temperature (Arnal *et al.,* 2005). In most cases, when fruit buds progress from a fully dormant condition to bloom, they lose their ability to tolerate cold temperatures without being injured or killed. In this context, the critical temperature is defined as the temperature that buds, flowers or fruits will tolerate for 30 minutes or less (Arnal *et al.,* 2005).

2.6 Botanical Description and Beneficial Nature of Citrus Fruits

The fruit of Citrus *sinuses* is called sweet orange and Citrus *aurantium* is the bitter orange, and generally oranges are referred to with these Latin names (Mitiku 2000; Sawamura *et al.,* 2004). Fruits of all members of the genus *Citrus* are considered as berries because they have many seeds, they are fleshy, soft and they are derived from a single ovary. An orange seed is called a pip and the white thread-like material attached to the inside of the peel is called pith November to February (Millikan, 1991). Dietary carotenoid antioxidants from fruits and vegetables have long been known to play an essential role in human health (Agocs *et al.,* 2007). These positive influences on human health have significantly increased the citrus consumption in the last few years, and it is continuously increasing with an estimated world production of citrus fruits up to 72 million tonnes in 2007 – 2008 (Khan *et al.,* 2010). Fruits belonging to the citrus group are described as “hesperidium." Hesperidium is a scientific term to describe the fruit structure characteristic of the citrus group even though, as noted above, citrus fruit is a modified berry with tough, leathery rind. The interior flesh of citrus fruit is composed of segments, called carpels, made up of numerous juice-filled vesicles that are actually specialized hair cells (Baldwin 1993).

In seeded citrus cultivars, fruit development is linked to the presence of seeds and, therefore, it depends upon pollination and fertilization. Fruit development can be divided into a series of stages (Peter, 2012). Early in development, fruit are enlarging rapidly and are small, hard, green and accumulating organic acids. The seeds become mature prior to ripening. During ripening, fruit become soft textured, and accumulate soluble sugars, pigments and aroma volatiles. Eventually fruit will become over-ripe, cell structures will deteriorate and the fruit will become susceptible to pathogens (Peter, 2012). A number of carotenoids are found in citrus fruit, together with secondary compounds with pivotal nutritional properties such as vitamin E, pro-vitamin A, flavonoids, limonoids, polysaccharides, lignin, fibre, phenolic compounds and essential oils (Davies and Albrigo, 1994). Despite previous work on the nature of citrus fruit by various groups (Wardowaski *et al.,* 1999; David *et al.,* 2009; Bellon *et al.,* 1992), Giovannoni (2004) stated that there is still a major need to improve fruit quality to meet current consumers’ demands.

Chemical and physical properties of these fruits are somewhat dependent on cultivation of the fruit and more detailed knowledge about the variability of the compositions of different parts of fruit will be beneficial in the future selection of orange materials with improved and suitable processing characteristics for subsequent manufacture (Anna *et al.,* 2002). For example, terpenes and terpenoids are present in the flavedo sacks, and therefore peel oil is a volatile part of the fruit which can be collected by extraction. Water-soluble components are located

in the vesicles of the endocarp and pulps are also part of the fruit which are rich in soluble sugars (Ceccarelli, 2004).

2.7 Oranges in General

Oranges constitute the bulk of citrus fruit production accounting for more than half of global citrus production in 2004, and thus the quantities of generated by-products are also very important (Rezzoug and Louka, 2009). According to Sawamura *et al.* (2011), we are exposed to a great number of scented products, not only in food stuffs but in many aspects of our daily life, for example, in medicines, cosmetics and household products. As a result, it has become increasingly important to study the functions and reactions of fragrances and flavouring in order to ensure that safe eating conditions are maintained. Oranges generally grow in warm climates, and the ambient temperature for optimum development should be kept between 15.5 – 29 °C. Flavours of oranges vary from sweet to sour, and this property depends upon the types of the fruit, a number of which are given here:

2.7.1 Sweet orange (common orange)

Common sweet oranges are the Valencia, Hart's Tardiff Valencia, and the Hamlin. The Valencia orange is one of the sweet oranges used for juice extraction. It is a late-season fruit, thinner skin, less seeds and popular (Selli and Kelebek, 2011).

2.7.2 Blood or pigmented orange

The two types blood orange are the light blood orange and the deep blood orange (Selli and Kelebek, 2011). Blood oranges are a natural mutation of *C. sinensis,* where high amounts of anthocyanin give the entire fruit its deep red colouration. In the blood orange category, varieties of orange fruit include Maltese, Moro, Sanguinelli, Scarlet Navel and Tarocco (Selli and Kelebek, 2011).

2.7.3 Navel orange

The most common types of Navel orange are the Cara Cara, Bahia, Dream Navel, Late Navel and Washington or Californian Navel. Navel oranges are the most common eating variety of oranges. They are sweet, seedless, and classic orange-sized (Selli and Kelebek, 2011).

2.7.4 Acid-less orange

Acid-less oranges have very little acid, hence they exhibit little flavour. Acid-less oranges are early season fruit and are also called "sweet" oranges. Also included amongst the sweet common orange varieties is an original citrus species, the mandarin. Amongst its many cultivars are Satsuma, Tangerine and Clementine (Selli and Kelebek, 2011).

2.7.5 Bitter orange varieties

These are the Seville orange (*C. Aurantium)*, Bergamot orange (*C. Bergamia Risso* and C. *Poncirus Trifoliata*) and Trifoliate orange (Selli and Kelebek, 2011)

2.7.6 Cross section of orange fruit

The cross sections of orange fruit in Figure 2.7.1 and Figure 2.7.2 are presented to illustrate anatomical descriptions of damaged and undamaged orange fruit, respectively.

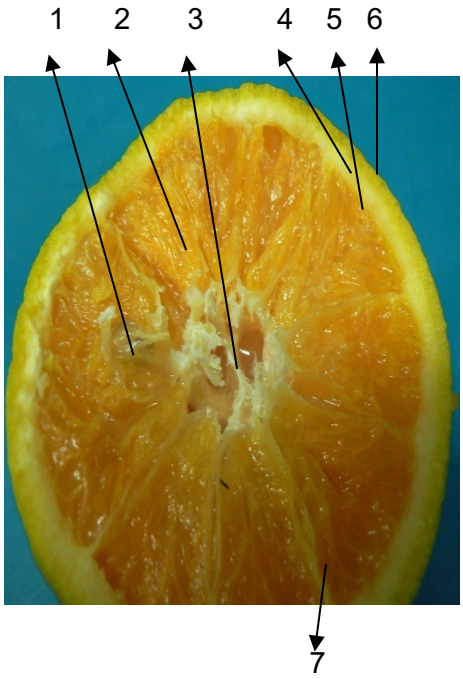

Figure 2.7.6A Cross-section of frost damaged orange fruit, showing more loss of juices and water (from current work). (Key: 1. seed; 2. juice sacs; 3. central core; 4. albedo; 5. endoderm; 6. oil sacs in flavedo; 7. segment membrane)

Figure 2.7.6B Cross-section of control orange fruit shows more intact, firm and structured (from current work)

2.8 Growing Conditions

Citrus fruit prefers subtropical or Mediterranean climates and slightly acidic soil. Citrus farming in Australia uses extensive irrigation systems, and a water intake of 900 to 1,200 mm/hectare per annum is required for a mature plant (AAB, 2007). The production of citrus fruits varies from state to state. NSW is the highest in production compared with other states. NSW produced 33% of Australia's 2005/06 citrus production; followed by South Australia, which provided 27%, Victoria 24% and Queensland had 14% of national production (AAB, 2007). The growing seasons of the orange fruits in Australia are dependent on the type and variety of the fruits. Navel oranges can be grown from January to October, and Valencia can be grown between September and January (AAB, 2007).

2.9 Mobile Ethanol Tester Equipment

Ethanol testing equipment can be handy for remote orange fruit testing. The equipment is easily available and can be managed by farmers. As volatile compound like ethanol can be detected in damaged oranges the test is more helpful in detecting defects. Furthermore, the findings of Obenland *et al.* (2008), shows that volatiles were trapped within the fruit for up to 7 days following cold exposure at -7 °C. They claimed that volatile chemicals and compounds were present in the trapped head space, among those being ethanol, ethyl butanoate, methyl hexanoate, and ethyl octanoate, and these were only released after day 7 of a storage period. The group also clearly noted that little or none of the above compounds were present in oranges that had not been frozen (Obenland *et al.,* 2008). These authors also stated that volatiles were emitted in substantial amounts following all of the storage durations, indicating that these volatiles may remain as viable markers of freeze injury for a considerable time after the freeze event (Obenland *et al.,* 2008).

Comparisons with our current work have been made by some of the investigations that were conducted with fruit samples in relation to the detection of ethanol from damaged or deteriorated fruits. In an early work (Kimball, 1991), the emanation of volatile compounds from plant tissues have been influenced by exposure to extreme freezing temperatures which had a pronounced effect on the structure of the fruit. Other researchers such as Ruiz-Colorado *et al*. (2010, have studied the ethanol production from banana fruit as a by-product of fermentation. In a different development, Kinab *et al*. (2014) studied the possibility of finding alcohol from fruits that were internally decayed and achieved similar results. Paul *et al*. (1990) found the production of ethanol from freeze-damaged papaya, and it was commented that the finding of ethanol and other compounds emitted from damaged fruits is a common scenario once yeast has established itself in the interior of the fruits (Lombard *et al.,* 2013). Lim *et al.*

(2013) also investigated guava fruit, and noted that bacterial production might be due to damage to the internal membrane of the fruits.

According to James et al. (2014), it is suggested that freezing is not suitable for all foods, since freezing does cause physical and chemical changes in many foods. This is perceived as reducing the quality of the food once it is thawed. Sumonsiri *et al*. (2013) investigated strawberries and other similar fruits, and suggested that there is a higher rate of enzyme activity and increased volatility as a result of frozen storage. In summary, when fruits are freeze damaged and deteriorated such that it opens way for internal chemical changes of the fruits, this can facilitate fermentation.

Several investigations have been reported in relation to the fruits that have been damaged due to a cold winter, where the temperature has fallen below 0 ^{o}C resulting in frosts and freezes. These instances are a major concern for citrus fruit growers, resulting in damage to fruit from production of unwanted by-products and cellular damage (Bakkali *et al.,* 2008). Investigations have shown that not only can the fruits be damaged, but long-term harm to the orange trees can occur in an extremely cold temperature (Eriksson and Nummi, 1982). As a consequence, in the face of climate change, the orange growing industry may be compelled to move, establishing plantations of replacement groves in different area where the risks posed by freezes and frosts are somewhat reduced (Bancroft, 1994). However, in the short term, interim measures will have to be taken to protect fruit from damage, and evaluation procedures will need to be developed to assess the quality of harvested fruit.

According to Burt (2004), these volatile components are important factors within the plant which gives the fruit its attractive qualities. Similar studies were also carried out by David (2003) and Aroujalian (2007) in relation to chilling injury and degradation of fruit quality and these included changes in the aroma of the fruits. Bramlage (1990); Meir (1995); Syvertsen, (1982) and Slaughter *et al.* (2008), reported that oranges subjected to freezing conditions are frequently unsuitable for consumption because they develop ‘off’ flavours or have dehydrated flesh. It appears that intracellular ice formation permanently damages the fruit’s cells thereby creating quality issues. Moreover, this damage leads to the production of unwanted compounds such as ethanol, which can be found during fermentation of sugars in the fruit ($C_6H_{12}O_6$ $\rightarrow$*fermentation*$\rightarrow$ 2 C_2H_5OH + 2 CO_2). The important point here is that ethanol production has been found to be self-limiting, and occurs only in anaerobic environments (Jordano, 2000; McCarty *et al.,* 2002). Functionally, this suggests that fruits will harbour internal colonies of ethanol producing yeasts, and in the right conditions (for example after frost damage) ethanol will be an observed emission from fruit because sugars, common in fruit, are readily fermented (Ingram and Dombek, 1987). To date there have not been any

reports of testing frost damaged oranges with a hand-held ethanol detector that can give an indication of damage after 24 hours of freezing events, harvest and thawing. Development of such an instrument would be a considerable advantage to the industry since six weeks is the usual time for sorting oranges by density at present.

2.10 Quality of the Fruits

Fruit quality plays an important role in relation to consumer choice. The word "quality" refers to the attribute property or basic nature of an object. However, nowadays it can also be defined as the "degree of superiority" (Kader *et al.,* 1985). Quality refers to characteristics and demands that are common worldwide, and is important to understand the requirements of the average global consumer (Kader *et al.,* 1986).

There is a world tendency towards a greater consumption of fruits due to a growing concern for a more balanced diet, with a lower proportion of carbohydrates, fats and oils and with a higher proportion of dietary fibre, vitamins, and minerals (McCarthy, 2000). This trend is influencing consumption patterns and is increasing market segmentation through the expansion in shapes, colours, flavours, ways of preparation, and/or packaging in which a product is presented. Amongst other issues, there is a growing demand for higher quality, referring to external as well as internal quality. External aspects (presentation, appearance, uniformity, ripeness, and freshness) are the main components in the decision to purchase, which is usually taken when the consumer sees the product exhibited at the sales point (McCarthy, 2000). Internal quality (flavour, aroma, texture, nutritional value, and absence of biotic and non-biotic contaminants) is linked to aspects not generally perceived externally, but are equally important to many consumers (McCarthy, 2000). Some of the important quality indices are presented in the next sub section.

2.10.1 Quality indices

According to Wang (1993), there are three main indices of concern. These are:

1. Colour intensity of the fruit, uniformity, firmness, size, shape, smoothness, freedom from decay.
2. Absence of defects including physical damage such as bruising, skin blemishes and discoloration, freezing damage and insect damage.
3. Maintenance of flavour quality, which is related to soluble solids/acid ratio and absence of off-flavour-causing compounds including fermentative metabolites.

With respect to quality indices, there are some lessons which have been learnt from previous work. For example, the optimum temperature of storage has been found to be 3 - 8 °C for up to three months, depending on the cultivar, maturity-ripeness stage at harvest and production area. Also, there is optimum relative humidity for storage of orange fruit which is 90-95% (Cohen and Cohen, 1999). In addition, an atmosphere combination of 5-10% O_2 and 0-5% CO_2 can be useful for delaying senescence and for firmness retention (Cohen and Cohen, 1999). It has been found that the rate of respiration of stored orange fruit is temperature dependant, as indicated in Table 2.13.1, and it is thought that his may have implications for this investigation.

Table 2.13.1 Rate of respiration in orange fruits (Cohen and Cohen, 1999).

Temperature (°C)	5	10	15	20
mL CO_2 (kg/hr)	2-4	3-5	6-12	11-17

2.11 Chemical constituents in oranges

There are many chemical constituents of citrus fruits such as essential oils, in relation to its volatility and flavouring that effect their storage and quality attributes (Agnes 2012). Like all organic compounds, essential oils are made up of hydrocarbon molecules which can further be classified in this context as terpenes, alcohols, esters, aldehydes, ketones and phenols (Agnes, 2012). Those constituents are characteristic of citrus species within different parts of the fruit and they are useful for determining the authenticity of the product; in other words they help to detect the quality of citrus products and, in addition, most of these chemical constituents are useful as by-products of the peel extract or pulp (Agnes, 2012).

2.11.1 Essential Oils

Citrus fruits such as oranges, mandarins, limes, lemons and grapefruits contain many essential oils. These are widely used as food additives since they are generally recognized to be safe, and many foods tolerate their presence. In fact, among the great variety of essential oils, citrus fruit essential oils and their major components have gained wide acceptance in the food industry (Tonder *et al.,* 1998). Orange peel is used for the extraction of its volatile oils, and it plays an important economic role in the food and other industries. Literature reviews from other researchers are presented below to highlight the importance of citrus essential oil in general (Tonder *et al.,* 1998).

Orange oil is an essential oil derived from the glands of the orange peel (Tonder *et al.,* 1998). It has a strong, fragrant aroma that is uplifting to the senses and it can be extracted using

variety of methods including cold pressing, which refers to extraction by various mechanical devices. This process ruptures the oil sacks in the flavedo, and expresses the oil as an aqueous emulsion from which it is separated by centrifuging (Verzera *et al.,* 2004). Volatile oils can be collected by steam distillation of citrus fruits followed by decantation or centrifuging the condensate (Muccilli *et al.,* 2009). Orange peel is high in monoterpenes and has d-limonene as its major component, which makes up about 90% of the oil. The components of the terpene fractions do not differ greatly among the different kinds of citrus fruit, but orange oils are distinguished by the presence of valencene as the principal sesquiterpene, a compound which is probably derived from the cuticular wax (Attaway *et al.,* 1968).

It is likely, however, that another α-unsaturated aldehyde, α-sinensal, contributes significantly to orange aroma since it has a sweet pungent penetrating aroma and a very low odour threshold. It has found to be present in cold-pressed orange oil at a concentration of about 0.1% (Stanley, 1962). While citral is also found to be a minor component of orange oils, limonene presents in greater amounts in both navel orange oil and in Valencia orange oil (Hunter and Brogden, 1965). Citrus fruits possess unique aromas rarely found in other fruit species. Fruit flavour is composed of complex combinations of soluble and volatile compounds, and orange, lemon and mandarin peels contain an aroma which distinguishes them from other citrus fruits (Macleod, 1988). According to Macleod, sixteen volatile compounds that have not previously been reported as orange volatiles are present. These include traces of sabinol, 4-methylacetophenone, hexyl hexanonate, gamma-selinene and bisabolene, with limonene (52%), linalool (15.8%), geranial (3.5%), [beta]-copaene (4.5%) and decanal (2.2%) as the major components.

The essential oils of six citrus fruit samples were also investigated by Lan-Phi *et al.* (2010). In their findings, the researchers clearly indicated that limonene, α-pinene, β-fernesene and linalool were the main volatile compounds. The peels of citrus fruit such as mandarins were also investigated by Lota (2001) using gas chromatography (GC) and combined gas chromatography and mass spectrometry (GC-MS). In their findings, limonene and terpinene were found in citrus peel oil samples, together with sabinene, linalool, γ-terpinene, linalool and methyl N-methylanthranilate, which were observed in leaf oils (Lota 2001). The peel essential oils from four selected Tunisian citrus fruits of sweet orange and sour orange fruits were also studied by Hosni *et al.* (2010), using GC-MS. The essential oils' content ranged from 1.06% to 4.62% (w/w) in mandarin, and qualitative and quantitative analysis led to the identification of 70 components in all oil samples. The analysed oils consist mainly of monoterpene hydrocarbons (97.59 - 99.3%), with limonene (92.52 - 97.3%) and β-pinene (1.37 - 1.82%) being the major constituents (Hosni *et al.,* 2001).

Mitiku *et al.* (2000) reported 20 constituents from a study carried out from cold-pressed orange peel oil for its volatile components, whereas Moshonas (1994) made quantitative determination of 46 volatile constituents in orange peel. In similar studies, three major chemotypes, limonene, limonene/γ-terpinene and linalyl acetate/limonene, were also distinguished from peel oil of a citrus fruit (Lota *et al.,* 2001). In addition, similar results were obtained by Flamini (2003; 2007; 2010). Essential oils from orange, lemon and mandarin fruit peel were also investigated for volatile compounds by Blanco *et al.* (1995) by using Gas Chromatography.

2.12 Citrus Leaf

Aroma compounds are important for citrus fruit, not only as a critical attribute of fruit quality, but also as valuable commercial products which are used extensively in some related industries such as the food and cosmetic areas. Citrus leaves have also been studied for their volatility, because of their richness in volatile compounds, rapid growth and large biomass, and because they are available throughout the year (Lota, 2002). Leaf oil composition is more diverse than in fruit, and is not over dominated by limonene or γ-terpinene, which commonly constitute over 70% of total volatiles in fruit peel (Hosn, 2010). In general, however, the number of citrus leaf volatile studies is limited; in particular, there is a lack of information for the comparative study of young and mature leaf volatiles from different citrus cultivars (Lota *et al.,* 2001). Although volatile changes during the opening of leaf buds and development from young to mature leaves have been reported previously, the study was limited to grapefruit and lemon only (Flamini, 2007).

Historically, leaf volatiles were analyzed by hydrodistillation (Hosni, 2010) and solvent extraction (Gancel, 2004) which takes a long time for analysis. Recently, solid phase microextraction (SPME) integrated with GC-MS has been shown to be much more sensitive, reproducible and efficient for metabolomics studies of volatiles, and has been widely used in plant research (Verdonk, 2003). Azam (2013) reported on a citrus leaf investigation where the study concentrated on major volatiles from young and mature leaves of different citrus types, and analyzed by headspace-solid phase microextraction (HS-SPME)-GC-MS. Results show a total of 123 components were identified from nine citrus cultivars, including nine aldehydes, 19 monoterpene hydrocarbons, 27 oxygenated monoterpenes, 43 sesquiterpene hydrocarbons, eight oxygenated sesquiterpenes, two ketones, six esters and nine miscellaneous. Young leaves produced higher amounts of volatiles than mature leaves in most cultivars. The percentage of aldehyde and monoterpene hydrocarbons increased, whilst oxygenated monoterpenes and sesquiterpenes compounds decreased during leaf

development. Linalool was the most abundant compound in young leaves, whereas limonene was the chief component in mature ones.

Citrus leaf contains reasonable amount of volatile compounds, depending on the considered citrus species, and the leaf volatile compounds show different relative distributions in hydrocarbons and oxygenated compounds (Gancel, 2003). In the research study conducted by Blanco *et al.* (1995), comparison was made between citrus leaves and citrus peel in relation to its volatility content. The concentration of volatile secondary metabolites showed a maximum value when the citrus fruits were at an intermediate maturation stage characterized by a greenish-yellow coloration (with 45 - 75% green). Citrus peel oils contained from 94.01 - 98.66% of monoterpenes ($C_{10}H_{16}$) with limonene as a major component and from 0.82 - 5.84% of oxygenated compounds, whilst the extracts from citrus leaves contained only 65.26, 31.23 and 79.43% of monoterpenes ($C_{10}H_{16}$) in lemon, mandarin and orange, respectively (Blanco *et al.,* 1995) with oxygenated compounds in these oils being 33.08, 68.47 and 16.38% (Blanco *et al.,* 1995), respectively.

2.13 Separation of Volatile Constituents

The essential oils from citrus fruit contains volatile organic compounds (components of pleasant sensory characteristics), and in order to collect those compounds there are a variety of methods which have been developed. In relation to identifying the most important constituents, scientists were able to achieve more results by using separation methods such as spinning band, molecular distillation, column and thin-layer chromatography (Keefford and Chandler, 1970). In gas chromatography, the development of column technology (DCT), stationary phases, detectors, carrier gas pressure and the programming of column temperature perfected the techniques (Keefford and Chandler, 1970).

Kawaii (1999) applied extraction techniques to investigate Valencia oranges from California. In the separated oil, he found more than 50 components including seven terpene fractions. A further 36 were oxygenated compounds and there were an additional 14 more compounds identified that had not been previously reported (Bernhard, 1961). According to an investigation by De Pasquale (2006) involving comparative tests on lemons, it has been found that the peel oil extracted contained 18.9% oxygenated compounds present when extracted using petroleum, while the oil extracted with processes using water contained only 12.5% (De Pasquale, 2006).

Volatile compounds presented in the citrus fruits can be separated as juice oils by distillation and direct centrifuging methods. These oils are derived mainly from oil glands in the peel that are broken during extraction of the juice (Huet, 1969). Scott *et al.* (1985) reported some

important observations regarding the state of the essential oil in orange juice. When the juice is centrifuged immediately after extraction, the juice oil is recovered in the lightest phase of the effluents. However, if the juice is held for a few hours and then centrifuged, the oil is found in the main sediment. Scott *et al.* (1985) regards this occurrence as evidence that the essential oil enters the lipid fraction in the cloud components of the juice, and conjectured that the subsequent decrease in volatility might explain the loss of fresh aroma from orange juice, which takes place soon after extraction. Work reported by Huet (1999) stated that most of the essential oil in citrus juices is attached to the solid particles in the suspension. When citrus juices are evaporated, the volatile flavouring compounds are removed to differing extents. They may be recovered from the evaporate or condensate, either as an oil phase or as an aqueous phase, commonly called “essence” (Coleman *et al.,* 1999).

2.14 Volatile Compounds

Citrus fruits are a potential source of valuable oil with important volatile components which might be utilized for edible and other industrial applications (Anwar *et al.,* 2008). These are complex mixtures whose composition may include volatile terpenic compounds, which have the formula $(C_5H_8)_n$, where the compounds are monoterpenes if n = 2, sesquiterpenes when n = 3, and diterpenes when n = 4. The terpenoids are oxygenated derivatives of terpenes, which may contain hydroxyl or carbonyl groups (Smith *et al*., 2001). Each citrus fruit has particular components present in minor quantities, and these components differ between fruits and can be used in identifying the various oils and controlling their quality (Mondello *et al.,* 2003).

Some of the constituents are hydrocarbons, alcohols, aldehydes, esters, and ketones (Nisperos, 1990). Among these compounds, terpenes are the most important constituents that are found in citrus fruit (Smadja, 2005). d-limonene is the most abundant compound in citrus fruit (Cozzolino *et al.,* 2000), which also contain a variety of volatile molecules such as terpenoids, phenol-derived aromatic components and aliphatic components (Bakkali *et al.,* 2008) with traces of oxygenated components (Espina *et al.,* 2011). Terpenes were predominantly present in the essential oil and accounted for 61.3 – 76.0% of the essential oil with an average result of 69.1%. The most abundant eight compounds were p-cymene > β-pinene > β-phellandrene > limonene > cryptone > α-pinene > 4-terpineol + γ-muurolene (Jianbo *et al.,* 2010).

The volatile compounds in orange juice are generally similar to those in orange oil (Razzaghi, 2009), with the exception that there is greater representation of saturated and unsaturated alcohols, aldehydes and esters with up to six carbon atoms (Wolford *et al.,* 1963). Ethyl

butyrate concentration has been shown to increase with advancing maturity of the fruit (Attaway *et al.,* 1994b). Ohta (1992) extracted samples from two different commercial orange essences, by continuous liquid-liquid extraction, with analyses by glass capillary gas chromatography that enabled the identification and evaluation of volatile components.

Citrus fruit in general contain some similar compounds in variable quantities, and in most cases, investigations can be carried to study which fruit contains which compounds and the level of concentration. Perez-Jabalpurwala *et al*. (2009) studied volatile compounds in sweet orange, sour orange, mandarin and lemon using GC-MS. In their findings, the major volatiles consisted of linalool β-myrcene, α-myrcene, limonene, E-ocimene, methyl anthranilate and indole. A similar study was also conducted by Hognadottir (2003), and orange essential oil was investigated for its volatile compounds using GC-MS and GC. In their results, they found that 95 volatile components were detected, and among this limonene was 94.5%, myrcene 1%, valencene 0.8%, linalool 0.7%, and octanal, decanal and ethyl butyrate were 0.3%. The most intense aromas were produced by octanal, wine lactone, linalool, decanal, β-ionone, citronellal, and β-sinensal (Hognadottir, 2003).

Regnier *et al.* (2010) studied the odour thresholds of the volatile compounds in which their sensory characterization was determined by dilution analysis. In the findings, benzaldehyde, 2-heptanone, hexanal, hexanol, limonene, 3-methylbutanal, 3-methylbutanol, 2-nonanone, octano and pentanol were identified. Volatile components extracted from two different commercial orange essences by continuous liquid-liquid extraction were analysed by glass capillary gas chromatography (Ohta *et al.,* 1992). Fifty-three volatile constituents of Micro-citrus inodora were identified or tentatively identified in the juice by GC and GC–MS analyses (Shaw *et al.,* 2000; 2001). Yu *et al.* (2010) studied Jinchen, a native sweet cultivar of Citrus *sinensis*, which is one of the most important varieties used in orange juice processing in China.

Citrus fruits send a special aroma to the atmosphere which originates from volatile compounds of the fruit itself. According to a study of oranges by Norman *et al*. (1998), the number of volatiles emanated (VE) increased at lower temperatures, and increased greatly also when the peel was injured in such a way as to rupture oil sacks; the components identified were d-limonene, β-myrcene, α-pinene, and acetaldehyde. From the work by Terada *et al*. (2009), 72 volatile flavouring and aroma components were identified from Florida's orange fruit. According to Kugler and Kovats (1983) investigation of lime and mandarin fruit shows more than 100 constituents of volatile compounds that gives citrus fruit its aroma. Furthermore Goretti *et al*. (1987) studied and separated around 180 compounds from lemon and 170 from oranges.

The volatile compounds of citrus juices have been studied by Barboni *et al.* (2009) using GC and GC-MS and according to their findings 90.2 - 99.8% of the volatile compounds were identified from 44 samples in the group tested. Among the findings, limonene was (56.8 - 93.3%) and γ-terpinene (0.1 - 36.4%) and were the major components in all samples. Clementine juice was characterised by the pre-eminence of limonene (90.0%) and a minor amount of Y-terpinene (1.2%) while mandarin juice exhibited high amount of limonene (66.3%) and Y-terpinene (21.1%) (Barboni *et al.,* 2009). A GC analysis of linalool and α-terpineol in orange peel juice was conducted to detect unpleasant flavours in the juice. The concentration of linalool at 23 ppm and α-terpineol at 8.5 ppm in juice was high enough to contribute to unpleasant flavour in a taste test (Gomez, 2004). Another constituent of citrus juice volatiles associated with microbial deterioration is diacetyl, which imparts a buttermilk off-flavour (Hill *et al*., 1990). According to Gomez (2004), investigation of several volatile compounds such as ethyl butanoate, limonene, linalool, and α-pinene, geranial, neral and α-terpineol) has been conducted and reported that they play central role in determining orange juice flavour. Furthermore, Limonene is one of the most dominant terpenes in citrus fruit, and gives citrus fruit their familiar aroma. In some cases, this aroma could be less than expected due to the composition varying from region to region, and also because of seasonal changes (Attaway *et al.,* 1968).

In another study, Ukeda *et al.* (2002) investigated the volatile components of *Citrus sphaerocarpa tanaka* (Kabosu) cold-pressed peel oil were investigated by chemical and sensory analyses. Monoterpene hydrocarbons (more than 94.6%) were predominant in Kabosu peel oil, with limonene and myrcene accounting for the major proportions (70.5% and 20.2%, respectively). The Kabosu oxygenated fraction was characterized by quantitative abundance in aldehydes and a relatively wide variety of alcohols. In a study conducted by Angerosa (2004), it was stated that both citrus fruits and olive oil contain volatile compounds that give the fruit greater aroma. In the report, the volatile compounds, the biogenesis of sensory characteristics (SC) delicate and fragrant aroma composition were briefly studied and illustrated.

According to the study by Arce (2007), citrus essential oil was simulated as the binary mixture formed from limonene and linalool, and equilibrium data for the more complex limonene + linalool + 1-ethyl-3-methylimidazolium ethylsulfate mixture have also been experimentally measured. Linalool distribution ratios and selectivity have been calculated from experimental data and had slightly larger values than those parameters found at the lowest temperature. According to Yu *et al*. (2010) Jinchen is a native sweet cultivar of Citrus *sinensis*, and one of the most important varieties used in orange juice processing in China.

The study of flavour components of Jinchen orange juice were carried out in relation to the colour characteristics, pH value, total soluble solids, total acids, as well as organic acids and sugars. In addition, flavours from different parts of the fruit such as peeled juice, pulp juice and whole fruit juice were also determined. It was reported that the colour characteristics were significantly different among three types of Jinchen orange juice, and the level of vitamin C and total soluble solids/total acids ratio (TSS/TA) is higher in whole fruit juice. In addition, pulp juice was rich in organic acids and sugars, showing the highest amounts. Volatiles from three juices were also studied using the solid phase micro extraction (SPME) combined with gas chromatography/mass spectrometry. The whole fruit juice has the highest number of volatile compounds (53.07 mg/L) followed by peeled fruit juice (51.04 mg/L) and pulp juice (27.10 mg/L).

Natural orange essence obtained during the concentration of orange juice is also used as an important flavour addition to some citrus products and other food and beverage products. According to Shaw *et al*. (2000), 53 volatile constituents from the juice and 20 from the peel have been identified by gas chromatographic and mass spectral analysis. All except seven had been reported earlier as citrus constituents.

2.15 Volatile Emissions

Even though orange oil was mostly used in the manufacture of perfumes, cosmetics, soap, and flavourings for food and drink (Bauer, 2001).For many years, commercial standards and specifications for citrus fruit's essential oils have included a number of physical and chemical parameters (Wang et al., 2008), and researchers have been working hard to investigate the properties of essential oil (Temime *et al.,* 2006). According to Terada *et al.* (2009), citrus fruits contain an essential oil that can be used for variety of purposes, but in some cases it has been illustrated that there are aroma and volatility differences which emerge due to damage (Tu *et al.,* 2002; 2003), and the nature of volatile emissions can also depend on processing methods of the orange fruits (Verzera *et al.,* 2004).

Fruit and vegetable breeders select market produce on the basis of colour, size, disease resistance, yield and other easily quantified horticultural traits (Bellon *et al., 2006*). Clearly, improvements in these quality attributes should be developed to meet industry standards, but in some cases, there are difficulties during the time of processing of the product. In the study conducted by Federica *et al.,* (2011), a sample of lemon oil was taken directly from an oil sac by means of a glass capillary to compare with oil extracted commercially. It was shown that in freshly extracted commercial oils there was some loss of aldehydes and esters and some

oxidation and isomerization of terpenes when compared directly with that obtained from an oil sac (Federica *et al.,* 2011).

In the study of Obenland (2003) volatile emissions from navel oranges (*Citrus sinensis L. Osbeck* cv. Washington) were evaluated as a means for predicting and gauging freeze damage. The fruits were subjected to -5 or -7 °C treatments in a laboratory freezer for various time periods. It was found that, corresponding to the loss in fruit quality, there were large increases in the emissions of ethanol, ethyl butanoate, methyl hexanoate, and ethyl octanoate. As a consequence, the measurement of volatile emissions appears to be a useful approach to identify freeze damage.

Volatile emission of citrus fruit was also investigated by Flamini (2003; 2007), and Hwan *et al.* (2003) used a GC injector solid phase micro extraction to investigate the volatile components that were responsible for the aroma of the fruit. The orange fruit pulps are rich in soluble sugars (Pourmortazavi *et al.,* 2007, and they also contain essential oils that have antioxidant properties (Angerosa *et al.,* 2004). Research on the aroma compounds in orange fruit has been carried out over many years, and different types of volatile compounds of fruit have been analysed using different analytical methods (Sawamura, 2005). In some cases, high or low temperature treatments can affect the presence of volatile constituents, in case of freezing or heating some of the terpenes can be easily lost (Nguyen *et al.,* 2009).

2.16 Volatility and Quality of the Fruit

A study of quality parameters such as permeability, roughness of the fruit and wax coating appealing characters (WCAC) were investigated by Chen (2001). He stated that any changes that we make to the fruit or changes that take place during processing the fruit have a direct effect on the volatile constituents (Chen 2001). In a similar study, essential oil showed significant changes in volatile components which mostly depend on the types the fruit, processing methods, and quality of the fruit (Pourmortazavi *et al.,* 2007).

As qualitative and quantitave analysis of volatile oil is very important, these characteristics were studied by Jahouach *et al.* (2008), Jerkovic (1989), Schieberle (1988), Sciarrone (2010), Selli (2004), Shaw *et al.* (1992), Jerkovic (2003) and Smadja (2005). Steffen *et al* (1996) reported that citrus fruit contain a strong aroma that was produced by limonene, octanal, linalool, decanal, β-ionone, citronellal, and β-sinensal. These are flavour compounds that have been seen as one of the important appealing character of the fruit (Nisperos *et al.,* 1990). However, yields of aroma fractions are different from region to region and cultivar (Angerosa *et al.,* 2004; Raeissi, 2002; 2004).

The aromatic quality of the fruit is primarily dependent on the aroma active compounds and they are easily detectable using modern equipment. For example, gas chromatography olfactometer (GC-OA) analysis is a valuable method for the detection of aroma active compounds (Lin *et al.,* 2002). Using this method, Jianbo *et al.* (2010) carried out identification and quantification of the flavonoid, carboxylic amino acid and sugar constituents of citrus fruit juices and identified a total of 33 compounds.

The most important determinant of fruit quality is the internal chemistry and external appearance of the fruit (Christensen *et al.,* 2000). It is well documented that linalool and limonene are significant components (67.8%) and the main constituents of the terpenes group. In addition, several compounds such asgeranial (3.5%), β-copaene (4.5%) and decanal (2.2%) as well as sabinol, 4-methylacetophenone, hexyl hexanonate, γ-selinene and bisabolene have been reported (Sakurada *et al.,* 2011). According to the study by Hosni *et al*. (2010) mono-terpenes, limonene and β-pinene were identified. Limonene, however, was described as a major constituent of several citrus oils and is shown by the following results: total monoterpene hydrocarbon (97.59-99.3%), which consists of limonene (92.52-97.3%) and β-pinene (1.37-1.82).

Often volatilities present in the fruit are different in quantities and qualities, which are largely dependent on the types and cultivates. Eric *et al.* (1998) compared citrus fruits such as lemons in terms of production quantities within the same fruit. According to this group's investigation, production of volatile oil from the whole yellow lemon was greater than the green lemon (Norman *et al.,* 1998). Lemon oil is different from orange oil and the rest of citrus group in the composition of the oxygenated fractions (Ukeda *et al.,* 2002).

In order to assist with this investigation regarding freeze damage, it is clear that the volatile compounds responsible for the aroma and flavour of fruits which present require further studies. In this respect, emission of volatiles from citrus fruits was also investigated by Flamini (2003; 2007), Ikeda *et al.* (1990; 2000) and Ahmed (1978), and it was noticed that fewer volatile compounds were recorded after long storage of the fruit. Hwan *et al*. (2003) used a GC injector solid phase microextraction to investigate the volatile components that were responsible for the aroma of the fruit. Qualitative and quantitative analysis of volatile oil from citrus fruit was also investigated by Jahouach (2008), as well as Jerkovic (2001) who both found that volatile components require favourable conditions at pre and post-harvest periods to survive in the fruits.

As mentioned in detail earlier, citrus fruit production and quality are influenced by many factors, including weather, climatic conditions and production practices. Postharvest quality of

a fruit was investigated by Li *et al. (*1997), who stated that maintaining fruit quality requires good systems and communication throughout the supply chain, as each step is influenced by its previous history since it is a chain of interdependent activities. In this respect, manual sorting was the main sorting method for quality of fresh produce, and has traditionally always been based on external characteristics of size, colour, and absence of surface defects.

Aroma differences, processing methods and quality parameters were investigated by Buettner and Schieberle (2001). In the study, Valencia and Navel orange juice that was extracted by hand squeezing was evaluated for its aroma difference from other juicing methods, and the finding noted great differences in the percentage of volatile components present in relation to processing methods.

2.17 Volatilities and Storage

Different types of fruits have different abilities to survive and maintain their quality. Some types have very short storage lives because they have high metabolic rates and high rates of water loss, and therefore must be kept under reasonably cool conditions (Peter, 2012). Fruit continue to develop (ripen) when detached from the plant. Their ripening program may not be completed if the handling and storage conditions disrupt the program. Excess heat or cold may inactivate essential enzymes required for ripening to progress or cause temperature injury, resulting in permanent loss of eating quality (Peter, 2012).

In order to obtain a clearer understanding of the relationship between storage and volatile compounds, experiments were conducted by Raymal *et al.* (1998). Volatile flavour of orange juices stored for 27 months at 4 and 27 °C were compared during this study, and the findings showed that the principal changes occurring at a higher temperature was the conversion of much of the d-limonene to α-terpineol by acid-catalyzed hydration and disappearance of most of the linalool and accumulation of furfural, probably from non-volatile precursors such as ascorbic acid (Raymal *et al.,* 1998).

There are possible variations in volatile compounds due to the conditions of preservation and storage conditions of citrus fruit (Biolatto *et al.,* 2005). This group also performed studies on the effect of relative humidity, storage temperatures and storage period including evaluation of chemical composition in the fruit by measuring the level of acetaldehyde, ethanol and d-limonene contents, in addition to the sensory characteristics, such as sweet, acid and bitter taste, the typical flavour intensity that was also investigated. The finding shows acetaldehyde, ethanol and d-limonene contents were not affected in fruit stored under treatments that included cold quarantine. In some cases, treatments that included temperature conditioning

significantly increased acetaldehyde and ethanol levels; however, the amounts detected were comparable with fresh citrus fruit juice (Biolatto *et al.,* 2005).

2.18 Volatilities and Ethanol

As ethanol is a by-product of the fermentation process of citrus fruits, it was investigated by (Pourbafrani *et al.* (2010) along with the production of bio-fuels, limonene and pectin from citrus waste. Ethanol serves as an indicator and a sign to off-flavour citrus fruit caused by micro-organisms. Qualitative and quantitative variation (QQV) of volatile aroma compounds in citrus fruit was studied by Razzaghi (2009). Flavour compounds of citrus fruits have been seen as one of the important compounds that gave citrus fruit its natural aroma (Razzaghi *et al.,* 1990). The investigation of Alan *et al.* (2001) of the number of volatile compounds found in Valencia orange found the following mean average values: limonene (0.25 µL/kg), pinene (1.90 µL/kg), linalool (10.81 µL/kg), acetaldehyde (1.60 µL/kg), ethylene (1.92 µL/kg), octanol (10.7 µL/kg), hexanol (2.9 µL/kg), terpinene (5.9 µL/kg) and ethyl butyrate (0.93 µL/kg).

Mitiku *et al.* (2000) investigated cold-pressed orange peel oil for its volatile components. General information on the importance of terpenes in citrus fruit was described by Moshonas (1994), who made a quantitative determination of 46 volatile constituents in orange fruits and the investigation of flavour and chemical comparison. These constituents can simply be destroyed by unfavorable temperatures of heating or chilling. According to Pourbafrani *et al.* (2010), during the above unfavourable conditions, the fruit undergoes a chemical change that accelerates the destruction of volatile compounds and ethanol as a by-product of fermentation. Of key relevance to the current project are the findings of Cronje (2011), Schirra (1993) and Forney and Jordan (1996). They found that besides effects of anaerobiosis and other factors, the longer the period of low-temperature freeze treatment and the longer the storage time, the greater becomes the stress and production of ethanol and losses of volatile compounds. Results in this work to freeze damaged oranges also agree with those of Corrales and Tlapa (1999), who found an increase in the production of ethanol and other metabolites in their study on avocado fruits is a manifestation of chilling injury.

2.19 Microbial spoilage in citrus fruits

According to Kader (2002, cited by Abdurrab *et al.,* 2012), it is estimated that post-harvest diseases destroy 10-30% of the total yield of crops. However, in perishable crops, especially in developing countries, diseases ruin more than 30% of the crop yield (Kader, 2002). Of particular interest to this investigation is that it has also been reported that disease incidence in the storage of sweet orange fruit increases with increasing storage duration (Dhallewin and

Schirra, 2000), and it has been suggested by Snowdon (1990) that, in some instances, losses to microbial spoilage could be as high as 50%.

According Arpaia and Kadar (2009), pathogens are most concerning quality issues among the farming community, especially where there are no proper storage facilities. Penicillium rot, penicillium digitatum (green mould) and penicillium italicum (blue mould) are the most common diseases due to the predominant pathogens of citrus fruits (Snowdon, 1990). It has been noted that once oranges are subjected to chilling for a prolonged duration, this may actually damage the tissue to the level where it may become highly sensitive to infections (Ritenour *et al.,* 2004). In most situations, the damage can be assessed during the storage period of the fruit, as in most cases it becomes visible, since the most common post-harvest disease of oranges is by *Penicillium digitatum,* a bright green mould which occurs on the surface of the citrus fruit (Brown and Eckert, 1988).

According to a Centre for Food Safety and Applied Nutrition report by Margaret *et al*. (2009), intensive tests were made to investigate the presence of yeast and mould in recently harvested fruit. In these experiments, 251 fresh fruit samples such as grapes, strawberries, blueberries, raspberries, blackberries, and various citrus fruits were surface-disinfected and incubated at room temperature for up to 14 days without supplemental media then subsequently examined for mould and yeast growth. The levels of contamination reported were as follows; 33% - 100% contaminations were recorded for raspberry and blackberry samples, and 95% contamination for blueberry samples was found. Mould growth at levels between 10% and 100% was found for all of the tested berries (Tournas and Katsoudas, 2005). The most common moulds isolated from fruits were Botrytis Cinerea, Rhizopus (in strawberries), Alternaria, Penicillium, Cladosporium and Fusarium followed by yeasts, Trichoderma and Aureobasidium. 35% of the grape samples tested were contaminated and supported growth of microorganisms, with the level of contamination ranging from 9% to 80% (Tournas and Katsoudas, 2005).

Citrus juices are acidic beverages with a pH of 3 to 4.2, and they are high in sugar content (15° TSS). Under these conditions, acidolactic bacteria, molds, and yeasts comprise the typical microbiota present in citrus juices. It has been found that lactic acid bacteria are the primary spoilage bacteria in fruit beverages (Hocking and Faedo, 1992). Moulds and yeasts tolerate high-osmotic and are capable of growing at refrigeration temperatures; consequently, they can therefore cause spoilage in the processed product (Dudley, 2002).

Typical yeast species found in citrus juices are *Candida parapsilosis*, *Candida Zygosaccharomyces, Saccharomyces, Torulaspora delbrueckii*, *rouxii*, *stellata*, *cerevisiae*,

and although species from the genus *Rhodotorula*, *Pichia*, *Hanseniaspora*, and *Metschnikowia* are also common (Hocking and Faedo, 1992). Despite the economic importance of citrus fruits and the fruit juices, there are few reports investigating the yeast species associated with them (Thyagaraja and Hosono, 1994). It is also clear that a detailed study of citrus juice microbiota is needed so that factors involved in spoilage can be assessed and methods can be developed to aid in rapid identification of spoilage microorganisms (Spencer *et al.,* 2002).

According to Lanciotti *et al.* (1999), 22% of the fruit juice samples tested showed fungal contamination. Yeasts were the predominant contaminants ranging from < 1.0 to 6.83 $\log_{10}$ cfu/mL. Yeasts commonly found in fruit juices were *C. lambica*, *C. sake*, and *Rhodotorula rubra. Geotrichum* spp. Thyagaraja and Hosono (1994), examined microbiological properties of fruit juices in which the number of microorganisms using the total count method was studied. In this work, they also investigated related yeasts using potato dextrose agar (Thyagaraja and Hosono, 1994). In a second study, Rajendran and Ohta (1998) determined the number of microorganisms in fruit juices also using the total count method. Their results showed that orange juice was likely to contain more microorganisms than other fruit juices.

2.20 Fruit Firmness

Firmness is one of the most important textural properties of the fruit, and it is an important sensory attribute of the orange, which generally determines the fruit's acceptance to discerning consumers. Over the years, many instruments have been used to evaluate these particular properties, since firmness of fruit is also used as an indication of ripeness and maturity as well as a means of determining quality (David, 2007). The effect of various harvesting, handling, storage, and processing techniques all play their part in assuring that the fruit has the desired level of firmness. This parameter can be measured using a compression/extension instrument. In the past, firmness of fruits has been studied and links to quality have been demonstrated by White *et al.* (1984), Lu (2004), Harker *et al.* (1971), Feng *et al*. (2011) and Maria *et al*. (2010).

Fruit that does not pass firmness checks will not be sent to fruit markets due to strict quality procedures in most countries around the world (White *et al.,* 1984). Often, less firm fruits show soft, mealy flesh and have inferior flavour quality; they are also susceptible to bacterial attack (Subedi and Walsh, 2009). The effect of fruit quality during post-harvest was studied by Abbot (1998), and in this study fruit firmness and related textural properties were measured. Abbot commented that these structural properties are most important as they provide psychological

appeal and give tactile satisfaction to the consumers. The term ‘quality’ implies the degree of excellence of a product and the appropriate weight must be given to its contributing attributes (Cavaco *et al.,* 2009). Consequently, firmness is an essential test that should be assessed mechanically (objectively) rather than manually (subjectively) (Paolo *et al.,* 2008).

The susceptibility of fruit to mechanical damage and infection by microorganisms is indicated by firmness assessment of fruit quality, and this typically occurs prior to shipping and then upon arrival at market, in an effort to ensure that acceptable fruit quality standards are met (Meir *et al.,* 1996). Current industry practices for assessing fruit firmness have been largely subjective, consisting of compression of the fruit between the fingers. Strong demand exists within the fruit industry for an alternative, more objective method of determining firmness (Larsen *et al.,* 1995).

In the research studies of Peng *et al*. (2006; 2008), fruits are described as notoriously variable in terms of the quality of individual fruits, particularly where these differences in firmness were due to handling the fruits in different ways. Data obtained by these authors showed significant differences in average results when compared to individual results (Peng, 2008). However, in some cases, firmness results may show similarities if the fruits have been treated in a similar environment are similar in weight and are the same cultivar (Qing and Zude, 2007). Firmness of the fruit is largely determined by the ripeness or softness of the fruits due to changes that take place during or before storage (Peng *et al.,* 2006). In determining quality, special attention should be given to the aspects of size, ripening stage, growing conditions, sensory attributes, nutritive value, chemical constituents, mechanical properties, functional properties and defects (Abbot, 2004).

Data analysis is important when comparing devices that measure fruit firmness (Doving *et al.,* 2005), and comparisons between devices are at the within-fruit level of variability (Gomez *et al.,* 2006). More precise equipment, therefore, should be used rather than inaccurate devices or placing reliance upon subjective operators when testing fruits (Qing *et al.,* 2007). A report from Chen and Opara (2013) also discussed how the mechanical properties of employed devices may affect results, and Doving *et al. (*2005) considered implications of well-known equipment for its practical use in the experiments that they conducted. According to White *et al.* (1984), firmness of Kiwifruit was investigated in relation to the genotype of three varieties of the fruit. The results showed that the softening of tissue zones including the outer pericarp, inner pericarp and core, generally followed the same pattern as that found during whole-fruit firmness measurements.

The most reliable method of assessing when the fruit is ripe to eat is by firmness (White *et al.,* 1984). The firmness at which a fruit is consumed or analysed for its quality is very important in relation to its internal quality during the latter stages of fruit ripening (Zude *et al.,* 2006). In this respect, research groups have used a range of different methods to assess firmness of fruits. A firmometer was used by Harker *et al.* (1996); whole fruit compression was used by Swarts (1981); and a mechanical test was used by Davis *et al.* (2008) and Peng and Lu (2008) to study the firmness of fruit. According to their results, all confirm that fruit firmness shows great appealing character that can be affected due to post-harvest handling, temperature, and storage of the fruits and a ripening period of 10 days. The results in general showed, as the ripening progressed, the textural property of peel, pulp and fruit decreased.

Huanggua melons were measured for firmness by Nourain *et al*. (2005), and they reported that the vibrational characteristics of fruits and vegetables are governed by their elastic modulus (firmness), mass and geometry. Therefore, they suggested that it is possible to evaluate firmness of fruits and vegetables based on their vibrational characteristics. Avocado ripeness was evaluated by Landahl and Terry (2012) using destructive firmness assessment, but they found quality is notorious for being heterogeneous within a consignment. This problem, which is especially true for imported avocado fruit, lends itself to searching for non-destructive methods for firmness evaluation.

One of the most important quality indicators for fruit still is firmness, which is highly correlated with maturity and storage time (Kim *et al.,* 2006). The group conducted an investigation in order to evaluate the potential use of ultrasonic parameters for the determination of apple firmness. Parameters such as ultrasonic velocity and attenuation were analysed based on the storage time of the fruit. Correlation analyses among ultrasonic parameters and fruit firmness were performed and a multiple linear regression model describing the relationship between firmness and ultrasonic parameters was proposed. Consequently, calibration equations for measurement of apple firmness were developed and validated.

Firmness tests were carried out using a penetrometer by Abbot, (2004). However, the wide use and appeal of firmness measurements has led to the periodic development of new devices for measuring firmness and evaluation of their performance (Fan *et al.,* 2009). Two different cultivars of apple fruit were also tested for firmness by Lu (2004), who suggested that firmness is one quality aspect. Fruit that were harvested at pre-climacteric stage and left untreated as a control, or treated 24 h after harvest, were also tested by Harker *et al*. (1997), and the results showed different firmness values to treated samples. Deihl *et al*. (1979) and Zude *et al. (*2006) also investigated initial fruit firmness and at ripe maturity stages. In another development

Subedi and Walsh (2009) conducted experiments with three major commercial apple fruits to investigate firmness.

Furthermore, fruit quality has always been a key issue for growers (Sun, 1991), and retailers throughout the history of the industry have noticed that the quality demand for fruits has been growing rapidly over the last decade (Meyer, 2009; Shyam *et al.,* 2011; Ding *et al.,* 2007). Whilst good quality standards are used throughout the supply and distribution chain, they mainly rely on subjective measurement techniques rather than using available (objective) technologies (Taniwaki *et al.,* 2010). Firmness is regarded as one of the very important quality aspects relating to fruit consumption, and is always analysed for its internal quality during the latter stages of fruit ripening (Hopkirk *et al.,* 1994). Therefore, a firmness test is also needed.

2.21 Conclusion

This literature review has covered the most important aspects of orange fruits in general, including the quality of orange fruits, the effect of freezing temperature on orange fruits, the harvesting and growing conditions including orchard temperatures, essential oils as well as chemical constituents and volatile compounds in orange. Furthermore, the marketing and challenges of orange fruit industry have also been discussed. This review included comments on freeze damage, relevant volatiles and peel oil from oranges and other citrus fruit, marketing considerations, chilling injury, storage, and managing the orchard floor. Therefore, whilst there have been various research attempts that sought to understand the problem of frost damage and its impact on citrus fruit, there is a considerable need for systematic research on the subject in which many aspects of the effect of freezing conditions on fruit properties are investigated simultaneously.

Chapter Three

3.0 Material and Methods

In the previous section, a review of the literature was presented which dealt with a range of points in regard to the importance of evaluating orange fruit quality and research works. In this Chapter, the equipment, material and methods used in this thesis to explore some of the issues involved in determining the extent of freeze damage will be presented. As discussed in the literature review there is a lack of systematic research on the problem of frost damage. Therefore, the objective of the experimental analysis of this was the study of a number of physical and chemical changes that are due to frost damage. The method used was similar to that of David *et al.* (2003).

3.1 The Fruits and their Maturity

Mature fruit is easily damaged during a freeze incident, and so this Project was focused on mature fruits to investigate the types of changes that such an incident produces. Previously, El Otmani *et al.* (2000) noted that during maturation, when the rind of oranges changes from green to orange, they begin to soften and they continue to soften as the fruit matures. 'Legal maturity' actually refers to the state that occurs after rind-softening begins, but rind continues to soften slowly for some time after this (Agusti *et al.,* 2002). A wider investigative study that addresses the occurrence of frost damage on different maturity levels of orange fruits, leaves and tree structure, is also of interest, and could be the subject of future research studies.

To address the broad aims of this Project many mature oranges were required, especially as there was likelihood that oranges might become mouldy prior to experimentation. Thus, a total of 159 boxes (60 pieces in each) of ripened/matured Navel orange and the same number of Valencia orange fruit was used; i.e. a total for both cultivars of 318 boxes. These had been harvested in August 2009, 2010, 2011, 2012 and 2015 from Mildura (Victoria) and in November 2009, 2010, 2011, 2012 and 2015 from Leeton (NSW), and then they were packed in a box, un-waxed and untreated. In each of the stated years, these boxes were received at Victoria University, Werribee campus.

3.1.1 Weighing and measuring

The samples were weighed individually using a top loading balance (Selby Anax model B3100P-Sartorius), and their axial (as opposed to their equatorial) circumference was measured using a tailor's tape measure.

3.1.2 Freeze treatment and storage

These functions were performed according to the methods of David *et al*. (2003). The fruits were freeze-treated with -2, -4, -6, -8 and -10 °C treatments using a Fiocchetti Scientific Refrigerator VITH with variable temperature (±2 °C) for periods of 2, 4, 6, 8, 10 or 24 hours, and with storage times of 1, 2, 4, 8, 14 or 21 day periods at 4 ˚C until the batch trials were finished. Thus, in total there were 180 conditioning combinations. The fruit was then allowed to warm normally to room temperature. During this storage treatment, the levels of volatile compounds were investigated. It should be noted that some oranges were untreated so that they could be used as controls.

3.1.3 Pictures of freeze treated and untreated Navel oranges

Samples are displayed in Figure 3.1.3, showing control Navel samples and freeze damaged orange fruit samples. Figure 3.1.3A shows control Navel samples that were not freeze treated whereas samples in Figure 3.1.3B shows Navel samples that were freeze treated at (-4 to -6 °C) for 10 hours for comparison with Figure 3.1.3C showing Navel samples that were freeze treated at (-8 to -10 °C) for 10 hours. Figure 3.1.3D shows the freeze treated Navel samples that were thawed for 48 hours, and it can be seen that the fruit was badly damaged with the colour having been changed to dark yellow compared to the (control) bright yellow in Figure 3.1.3A. Some black spots are also visible on the skin of freeze damaged Navel samples that had been freeze treated. According to Figure 3.1.3E, the half sliced Navel oranges fruits that were frost damaged show loose segments and become very juicy within 48 hours of thawing time after freeze treatment at -10 °C for 10 hours.

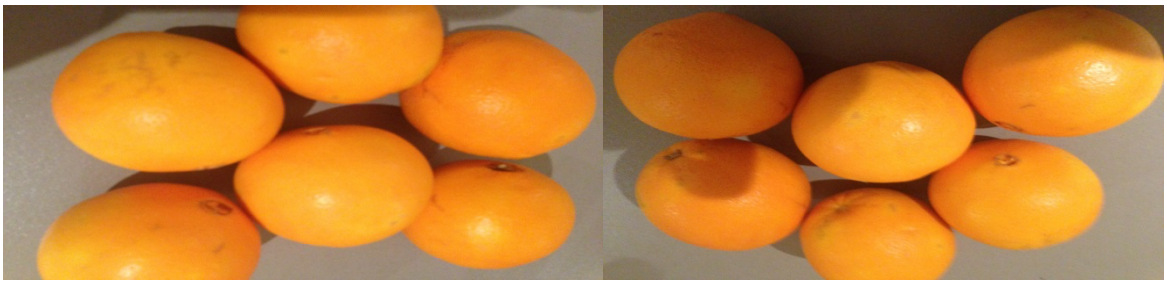

Figure 3.1.3A Control orange sample (Navel Orange from our work)

Figure 3.1.3B freeze treated Navel samples (-4 °C to -6 °C)

Figure 3.1.3C Freeze treated Navel samples (-8 to -10 °C)

Figure 3.1.3D Orange fruit after frost damage and thawed for 48 hours

Figure 3.1.3E internal part of frost damage Navel orange fruit after 48 hours

3.1.4 Processing and labelling

After freezing, the fruit was scanned for the internal quality defect check as described in (3.1.4.1), stored (at 4 ˚C fridge as described below) and the fruit was thawed (for 48 hours' time as described in section 3.1.3), the fruit was then cut in half along the equatorial axis allowing for juicing using an orange squeezer with minimal twisting to reduce the amount of pulp in the juice. Normally around 160 - 180 mL of juice was obtained from one orange fruit and the juice was transferred to a container and labelled according to the orange fruit type and sample number, the date of trial and the analyses to be undertaken. 20 mL of juice was used for measuring the pH and TSS. The remaining orange juice was kept in its tube, labelled and stored at 4 ˚C for further tests.

3.1.4.1 Scanning process (internal fruit quality check)

Following freeze treatment (-2, - 4, - 6, - 8 and – 10 ˚C), the fruits were scanned for internal quality defection analysis using a Colour Vision Systems NIRS DIODE ARRAY Spectrometer model. This NIRS instrument was a prototype constructed by Colour Vision for their work and was kindly loaned by Colour Vision for this research. The particulars of the instrument (wavelength and software) were proprietary information and were not disclosed to the researchers. The instrument was calibrated against untreated oranges, both Navel and Valencia, as it was the difference between treated and untreated samples that was critical not the absolute values. The fruit were scanned twice, and the orange fruit was positioned directly opposite each other at a position perpendicular to the stem axis of the fruit, on the equator of the fruit, each measurement was recorded by the software with the file number given and saved, moreover the fruit was identified as severely damaged, lightly damaged and no damage was detected.

3.1.4.2 Project factors and guide

This project investigated three major factors (freeze temperatures, duration of freeze treatment times and storage periods) relating to two cultivars, mainly Valencia and Navel orange fruits, the treatment details are provided in Section 3.1.2. All of the experiments were conducted using multiple oranges that had been subject to the same treatment conditions and typically each experimental test was repeated 30 times so that suitable data's can be obtained for further statistical analysis of (ANOVA) experiments. Damaged and control samples of whole oranges were used for fruit firmness testing and for ethanol testing using handheld equipment. The skins were used for limonene and ethanol testing using GC and GC-MS analysis and the juice extracted from an orange fruit was used for pH, TSS and microbiology tests. The project chart showing all the steps of this investigation is also presented in section 1.9 of page 10.

3.2 Measurement of pH and TSS

Previously, Harrill (1998) discussed how pH and TSS are useful indicators of fruit quality, and the measurement of these quantities is discussed here.

3.2.1 pH measurement

pH was measured using a pH meter (Type: Benchtop pH Meter – Brand: Hanna, Model: HI-2211) and calibration was performed according to the manufacturer's instructions. The electrode was placed in a buffer solution of the required pH, the display was allowed to stabilize, and then the display was set to the buffer pH by adjusting the calibration. The electrode was then removed from the buffer; it was rinsed with deionized water and blotted dry using soft paper tissue (designed for lens). The electrode was then placed in orange juice that was in a 50 mL beaker and the pH recorded.

3.2.2 TSS measurement

The TSS was measured using a hand-held electronic refractometer (Type: AS200 MISCO Products Division, Cleveland, Ohio, USA).

The standard way to determine TSS was by measurement of the refractive index of the sample, since this depends upon the sugar concentration. (Total soluable solid (TSS) Total soluble solids content of a solution is determined by the index of refraction and is also referred to as the degrees Brix. -Brix is the term used when a refractometer equipped with a scale, based on the relationship between refractive indices at 20°C and the percentage by mass of total soluble solids of a pure aqueous sucrose solution (Pesquisa B. 2006).

The test was undertaken using an electronic refractometer (Type: AS200 MISCO Products Division, Cleveland, Ohio, USA). Small drops of orange juice sample were placed on the lens

of the refractometer using small plastic pipettes and the TSS values were recorded (from refractometer reading). After each test the lens was cleaned using water and dried with lens tissue. Each sample was analysed 30 times and the average recorded.

3.2.2.3 Number of samples for pH and TSS tests

The experiments for TSS and pH were repeated 30 times, with the total of 5400 x 2 =10800 orange juice samples, plus an additional 360 control orange juice samples were tested for both (Valencia and Navel orange).

3.3 Extraction of oils

Volatile compounds were extracted using steam distillation from orange peel, and the oil obtained was mostly composed of terpenes (Bauer, 2001).

Even though we are generally looking at a common type of compounds in our investigation, from the oil that was collected using steam distillation, the focus was clearly concentrated on volatile compounds such as limonene and ethanol.

3.3.1 Steam distillation of orange peel and extraction of oil

Standard steam distillation and distillation apparatus were set up as described by Vogel (1996). Orange peel (100 - 150 g) was placed in a 500 mL round-bottomed flask with 250 mL of distilled which was replenished during the distillation. Adding too much water was avoided to prevent the orange peel and water rising up in the neck of the steam distillation apparatus while boiling. A few boiling chips were added to the mixture to prevent bumping.

The separating funnel was filled with water and the still head was wrapped with aluminium foil to assist the steam mixture to travel as a vapour to the condenser. The hot plate was turned on and left for 15-20 minutes to bring the orange peel mixture to boil. As the mixture was continuously boiling, the water level was closely monitored and prevented from dropping further by continually adding small volumes of water via a separating funnel. If the water level is allowed to fall too low, due to the high sugar concentration of orange, the sugar will caramelize and burn.

About 20 - 40 mL of distilled samples were collected and placed in a beaker. NaCl (0.5 g) was added to the distillate to raise the ionic strength of the water, thus increasing the separation of the two layers. Droplets or a film of the terpenes were observed on the surface of the distillate. Separation of the essential oil from the oil-water mixture was carried out using a (500 mL) separatory funnel as shown in Figure 3.3.1. Subsequently, by draining one of the solvent layers away, the oil samples were collected in beaker and stored in a fridge for further analysis

Figure 3.3.1 Collection of essential oil (from our work)

3.3.2 SPE preparation and loading samples

Following collection of orange peel oil from the extraction steam distillation, the samples were processed using a Manifold, which is shown in Figure 3.3.2, for injection into the GC-MS or GC and the detailed proceders are described below.

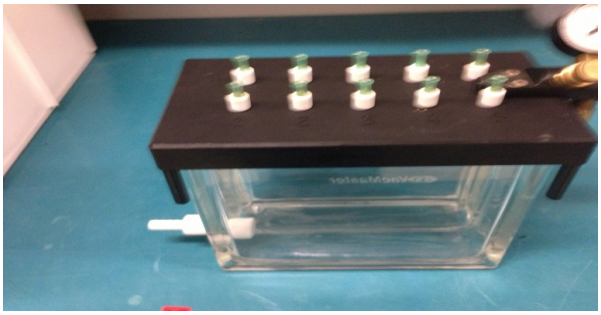

Figure 3.3.2 Manifold for solid-phase extraction (from our work)

The 10 port, Hyper Sep Glass Block Vacuum Manifold was purchased from Thermo Chemicals for solid-phase extraction (SPE). SPE extraction was carried out on the orange peel oil samples that were collected from the steam distillation process using a 10-port manifold (Waters HLB 3CC). The cartridge was prepared with 2 mL of 50/50 methanol/water and 25 mL of orange oil extract. A 15 drop/min rate was loaded to the cartridge, and the sample

washed with 5% methanol in water. Air was allowed to pass through the cartridge for 30 mins for drying. In the elution process, 1 mL of acetone followed by 3 mL of dichloromethane was washed through the cartridge and the eluant transferred to a 10 mL glass vial. The samples were evaporated gently with nitrogen and dissolved in 1 mL methanol and transferred to a 1 mL glass vial for further GC and GC-MS analysis (Hosni *et al.,* 2010).

3.3.3 Standard sample and the Calibration Curve (case no 64-17-5 10% v/v Stock solution from Merk)

A limonene standard was prepared with a concentration of 1000 µL/L was diluted to 100 µL/L by a 10:1 dilution with methanol and at 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 µL/L. The linear regression analysis of the calibration curve in Figure 5.7.1 for limonene and 5.8.1 for ethanol was used to calculate the concentration of limonene and ethanol accordingly in the sample. These were then used to calculate the concentrations of the orange oil samples that were freeze treated at different temperature, duration and storage times (Michiko *et al.* 1995).

3.3.4 Gas Chromatography Mass Spectrometry (GC-MS)

For this work, a GC-MS QP 2010 system, composed of a Shimadzu Gas Chromatography-Mass Spectrometer with Shimadzu OC-20i Auto sampler, was used. Operation conditions are described in section 3.3.5. GC-MS data were recorded in combination with a GC-MS Time Analyser using GC-MS software. GCMS data and identification of volatile compounds were performed using this GC-MS apparatus that is displayed in Figure 3.3.4.

Figure 3.3.4 Shimadzu GC-MS equipment (from our work)

3.3.5 Gas Chromatography Flame Ionization Detector (GC/FID)

A quantitative analysis of the volatiles from the orange peel samples was conducted by gas chromatography with a Varian Star GC Chromatograph 3400 CX series, which is depicted in Fig 3.3.5. The auto sampler of Varian model 8200 auto sampler was also used to assist in processing samples in this test. The chromatography apparatus was equipped with a flame

ionization detector (FID), and a 30 m, DB 5 x 0.22 µm x 0.25 mm ID fused silica capillary column coated with a stationary polar liquid (CP-Wax 52 CB). The operating conditions were as follows: injector and detector temperature, 200 ˚C; He carrier gas flow rate, 1.0 mL/min; oven temperature program, 45 ˚C for 1 minute, rise at 3 ˚C/minute to 230 ˚C and hold for 25 minutes. The results were analysed using Chromatography work station software.

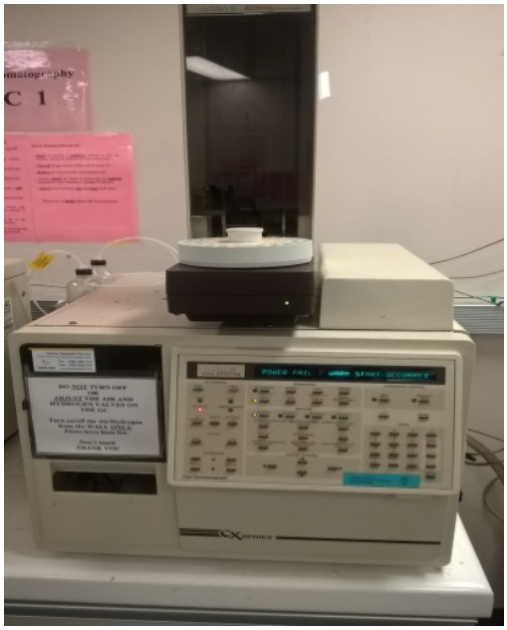

Figure 3.3.5 GC equipment used in the study of freeze treated orange skin oil and control sample (from our work).

3.3.6 GC conditioning

The chromatography apparatus was used with an injection volume of 1 µmL and a split injection mode. An oven temperature program of 45 °C for 1 minute, then a temperature rise of 3 °C/minute was used. The carrier gas at was set at 10 psi, the injector temperature at 230 °C, and the electron ionization detector was set at 70 electron-volts (Hosni *et al.,* 2010).

3.3.7 Sample injection

An auto sampler was used to inject a 1 µmL liquid sample through a rubber septum into a high temperature detector port at the head of the column.

3.3.8 Analysis of Volatile Compounds

The previous Section provided details of the materials and methods for measuring the TSS and pH of juice from orange that had been frozen to determine the extent of changes that occurred during the periods of storage. In this Section the material and methods relating to the use of Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography (GC)

for studying volatile elements of orange peel oil extracted by steam distillation will be described. The GC-MS and GC equipment used are described in Sections 3.3.4 and 3.3.5, respectively. Furthermore for GC-MS analysis of orange oil samples were tested in order to investigate the volatile compounds of limonene and ethanol from Valencia and Navel orange fruits. using (-2 and -10 °C for 24 hours freeze treated oranges) as well as control samples . However the number of samples used for GC tests for limonene and ethanol are similar to that for pH and TSS tests, (5400 x 2 = 10800 freeze treated orange samples and 360 control samples) . Also more information was given on the volume used in section 3.2.1 and 3.2.2 for pH and TSS respectively.

The experiment followed the work of David *et al.* (2003), in relation to volatility, physical and chemical changes that took place during freeze damage to the fruit, the emission of volatile compounds such as ethanol, ethyl butanoate, methyl hexanoate and ethyl octanoate and terpenes. This is to find out, if these emissions were found to be strongly enhanced by freezing and are thus thought to correspond to subsequent damage to the fruit.

3.4 Ethanol and limonene tests from (orange skin oil)

This Study examined the level of volatile compounds using the oil from the orange fruit skin that had been freeze treated and stored for up to 21 days in order to investigate the level of ethanol and limonene production arising from freeze damaged oranges. In this study, fresh, mature and ripe Valencia and Navel variety orange fruits, sourced as described in Section 3 was used, the fruits for analysis were selected at random; the weights of orange fruits were determined by using an electronic balance (Selby Anax model B3100P-Sartorius) which had an accuracy of 0.01 g prior to the treatment. Fresh orange fruits were put in a laboratory freezer and treated at the specified temperatures, as described in Section 3.1.2, respectively, and the procedures are followed as described in section 3.3.1 to 3.3.8 and the concentrations of the samples were calculated using the standards as described in section 3.3.3. The experiments were repeated 30 times and total of 3600 tests were conducted for both limonene and ethanol tests.

Furthermore, the samples were monitored on a daily basis during the storage time of 3 weeks and the mean average value was recorded. In addition to the treated sample the control samples were also prepared (total of 180) for each cultivar and 360 in total.

3.5 Ethanol Analysis of whole orange (hand held)

In this section ethanol test was conducted using the whole orange fruit that was placed in the plastic bag with ethanol tester, using the pump as described in section 3.5.2 and the ethanol readings were recorded, after 24 hours of storage time. Experiments were perfomed 30 times

and average was taken. In this study chemical and physical changes of orange fruit that takes place after the fruit was freeze treated and stored for up to 21 days.

In this study, fresh, mature and ripe Valencia and Navel variety orange fruits, sourced as described in Section 3.1, were used. The fruits for analysis were selected at random; the weights of orange fruits were determined (as described in section 3.1.2) and fresh orange fruits were put in a laboratory freezer and treated at the specified temperatures, as described in Section 3.1.2, respectively, and the test was repeated 30 times and the total number of tests conducted was 360 control orange samples and 2160 freeze treated orange samples in total for both Valencia and Navel. And after the treatment the samples were stored and monitored on a daily bases and results were recorded.

3.5.1 Gas aspiration equipment (hand held)

The gas aspiration pump (AP-20 Gas Aspiration Pump) used was purchased from Geneq Inc. Scientific Instruments (Canada) and delivered to Victoria University Werribee campus with suitable ethanol test tubes, and is shown in Figure 3.5.1. The pump was fitted with a gas detector tube and was placed together with freeze treated orange fruits packed in a polyethylene bag (25 × 50 cm) and tightly closed using a plastic strip. The fruit was stored for a variable number of days (1, 3, 7, 14 and 21) all at 4 °C, before the readings were taken and recorded.

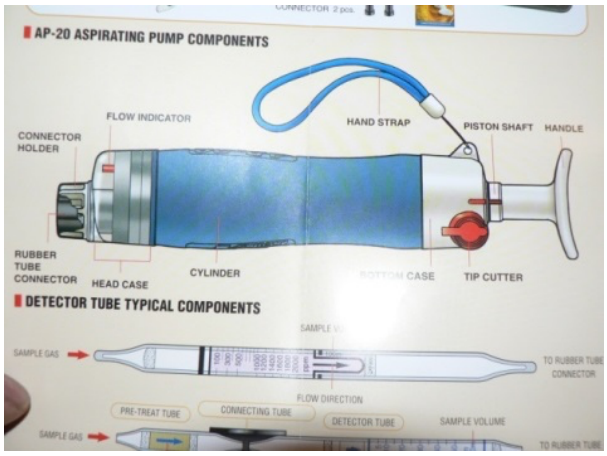

Figure 3.5.1 Ethanol tester equipment used in this experiment (Kitagawa AP-20, Gas Aspiration pump) from our work.

3.5.2 Preparation of the testing equipment

The pump was checked for leaks prior to assembly. To assist the experiment, the gas detector tube was cut by putting it into the tip cutter and scratching the tip of the tube by rotating it for one revolution. The tube was then connected to the aspirating pump in order to draw ethanol sample gas through the gas detector tube in the correct direction from orange fruit. This was

ensured by inserting the gas detector into the rubber tube connector with the tube's directional arrow pointing towards the pump. The handle was pulled by aligning the red line on the bottom case and that on the shaft, then the pump was pulled with the pump handle until it was in the fully 100% counter-clockwise lock position.

After the sample had been stored for the required time, the pump was removed from the bag, and the handle was turned to 90° position and the concentration indicated on the gas detector tube was recorded. Figure 3.5.1 is a diagram of the compartments of the AP-20 Gas Aspiration Pump, together with tubes that were fitted to analyse the ethanol content of the orange samples.

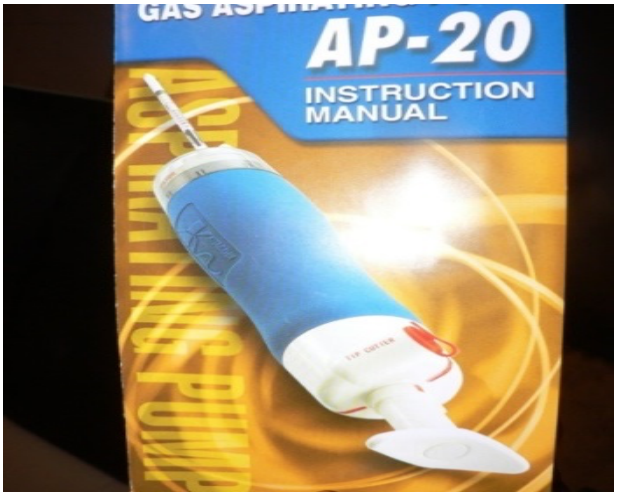

Figure 3.5.2 Gas aspiration pump with the testing tube fitted (from our work)

3.5.3 Freezing regimes, number of samples and storage

In this test 3 variable temperature regimes (-2, -6 and -10 °C), 3 different freeze treatment times (2, 8 and 24 hours) and 6 storage times were conducted. Moreover the number of samples prepared were slightly different than previous tested as it was requires of placing the whole fruit in a plastic bag and monitor them accordingly. For this practical work the total of 3240 freeze treated and 360 control orange fruit was used

3.6 Microbiology Test

A total plate count (TPC) of yeast and mould counts was carried out for microbiological analysis of the freeze-treated and untreated samples during the 21 days of storage. TPC was performed using the pour plate method. Two types of agars were prepared to further investigate bacteria, yeast and mould growth on the orange samples that were freeze treated

and tested for microbial growth. These experiments followed the approach of Lee *et al.* (2003), and this is similar to the methods of Talibi *et al*. (2014) and Sharpe and Smith (1966).

3.6.1 Chemicals

All chemicals and agars used in this experiment were supplied by Oxoid microbiology product suppliers, Melbourne, Vic, Australia.

3.6.2 Bacterial test

The procedure of Sharpe and Smith, 1966, was used, as follows: 52 g of MRS broth was added to 1 litre of distilled water at approximately 60 °C and mixed until completely dissolved and dispensed into final containers and sterilise by autoclaving at 121 °C for 15 minutes.

3.6.2.1 Description of MRS (bacterial test)

According to the label from the product, MRS Broth used for tests was in the identification of lactobacilli, such as temperature dependence, growth in 4% NaCl and growth in 0.4% Teepol.

3.6.2.2 Description of the medium

According to the manufacturer, the MRS formulation used for this experiment was developed to support good growth of lactobacilli bacteria in general, MRS formulation provided and used were (Rogosa and Sharpe 1 type) with pH being 6.2 ± 0.2 at 25 °C.

3.6.2.3 General technique

The media, apparatus and glassware were sterilised by heating in an autoclave at 121 °C for 20 minutes before use. A fume hood with UV and aseptic techniques were used to reduce the likelihood of bacterial contamination. Disinfection of working areas to minimise possible access by bacteria from the air to exposed media was carried out, and a flame was used to kill bacteria which might enter vessels as they are opened.

3.6.3 Yeast and Mould Test

3.6.3.1 Material and methods (microbiology)

A total plate count (TPC) of yeast and mould counts was carried out for microbiological analysis of the freeze-treated and untreated samples during the 21 days of storage. TPC was performed using the pour plate method. Section 3.4.3 provided the full details of all samples used in these experiments. In this experiment 720 control samples and 6480 freeze treated orange juice samples were used for bacteria, yeast and mould test to both cultivars.

3.6.3.2 Preparation of Agar for yeasts and moulds (potato dextrose agar)

Potato dextrose Agar (CM0139), a recognised suitable medium for the isolation and count of yeasts and moulds, was used. It was purchased from Sigma-Aldrich. 39 g of potato Dextrose Agar was added to 1 litre of purified water, which was brought to the boil to dissolve the agar completely. The resultant solution was mixed well before pouring onto plates which were sterilised by autoclaving at 121 °C for 15 minutes. In order to suppress bacterial growth, the medium was acidified to pH 3.5. This was done by adding 1 mL of lactic acid 10% (SR0021) to each 100 mL of sterilised medium at 50 °C. The medium was not heated after the addition of the acid to prevent hydrolysis of the agar and consequently destroying its gelling properties. After cooling, the agar was poured into Petri dishes. Yeast and moulds were determined using spread plate methods. Furthermore, all microbiological analysis was carried out and the results were expressed as $\log_{10}$ (colony forming units per gram ($\log_{10}$CFU/mL). General technique relating to the media, apparatus and glassware sterilisations methods, sampling methods and plating, and serial dilution was performed as described in Section 3.10.5.

3.6.4 Serial dilutions

The objective of the serial dilution method was to estimate the concentration (number of organisms, bacteria or colonies) of an orange juice that was extracted from freeze treated orange fruit.

It was done by enumeration of the number of colonies cultured from serial dilutions of the sample. By diluting a sample in a controlled way, it is possible to obtain incubated culture plates with an easily countable number of colonies (around 30–100) and calculate the number of microbes present in the sample.

During the process, 1 ml of properly shaken orange juice sample (freeze treated or untreated orange juice) drawn into the pipette and then added to the first tube that contain 9 ml of peptone to make the total volume of 10 ml. This provides an initial dilution of 10^{-1}.

The same process is then repeated for the remaining tube, taking 1 ml from the previous tube and adding it to the next 9 ml diluents. This resulted in serial dilutions of 10^{-1} to 10^{-8}.

Sterilized molten agar (45 °C) was poured into dishes and left to set and 1 mL of serially diluted orange samples were added to the dishes accordingly. and then approximately 15 mL of medium, cooled to 50 °C, was added to each dish. The sample was mixed gently, turning the plates three times clockwise and three times counter clockwise. The medium was allowed to gel, and then the Petri dishes were turned upside down and stacked conveniently to incubate.

3.6.5 Incubation method

Using CM0359, the mixture was stirred gently then autoclaved at 121 °C for 15 minutes. After cooling to 50 °C, it was poured into the Petri dishes. For this work a 25 °C psychotropic incubation was carried out for three days at 25 ºC, under anaerobic (microaerophilic conditions) to identify the presumptive Lactobacillus colonies.

3.6.6 Statistical Analysis

One-way analysis of variance (ANOVA) was performed to compare mean values of freeze treated samples and storage periods between the orange cultivars (Valencia and Navel). Differences were considered to be significant when p values were < 0.05.

3.7 Fruit Firmness Test

Orange samples were obtained as described in Section 3.1. In this trial the total number of tests, types of treatment and In this test 4 variable temperature regimes (-2, -4, -6 and -10 °C), with 24 hours freeze treatment times and 6 storage times (1, 3, 5, 7, 14, and 21 days), were conducted. The experiments were repeated 30 times for each type of test in total 3240 treated orange fruit samples and 1080 control orange samples were tested. Moreover, the weight ranges of orange used were between 300 - 350 g. The measurements for firmness tests were carried out, after the freeze treatment, and kept at 4°C for maximum of 21 days.

3.7.1 Weight loss and fruit quality test

As noted in Section 3.1.1 all fruits were weighed prior to investigations. Fruits were re-weighed after the storage period of 21 days to determine the effect of freeze treatment**.** Weight loss due to freeze treatment was recorded, and the percentage weight loss was calculated from the difference between the initial and final weights divided by the initial weight multiplied by 100 (Dhar *et al.,* 2008). In this practical work 120 control samples and 720 freeze treated oranges were used to both cultivar. Similarly, circumference tests were conducted using the same fruit that was used that was used for weight loss assessment.

3.7.2 Testing equipment

Figure 3.7.2 Instron Universal Testing Equipment used

The Universal Testing Equipment used was an Instron unit (Model 4465, capacity 5 x N, wt. 130 kg; Blue Hill 2 version 2.5 Software was used to analyse the results of the Instron measurements. The procedure was adapted from the method of Doving *et al*. (2002). Samples were compressed at a speed of 8 – 9 mm/sec with a full-scale load. The Instron was cycled to give two successive measurements for each sample at intervals of 30 seconds. The diameter of the plunger and sample cup were 11.5 cm, and the diameter of the oranges ranged from 7.5 to 9 cm. Orange fruit diameter was also measured in both treated and untreated samples using a tape measure (cm). in this experiment the total of 720 orange fruit and 1440 (-2, -4, -6 and -10 ºC freeze treated orange fruits were used.

3.8 Conclusion

The various instrumentation and techniques presented here enable a comprehensive study and the effect of freezing of oranges, with the results are also presented in the following chapters.

Chapter Four

Experiments, Results and Discussions

4.0 Orange quality, Total Soluble Solid (TSS) and pH Tests on Orange Juice

The previous Chapter discussed the materials, equipment and methods used in this investigation. This Chapter starts with simple quality assessments and further provides the detailed results and comments which relate to the investigations into TSS and pH analyses which are relevant to the study of the effects of frost damage to orange fruits during a cold winter. As it was indicated in Section 3.1, the orange samples were delivered to Victoria University undipped and un-waxed in order to ensure that no external chemical influence would be introduced to affect the results.

The samples were freeze-treated in a laboratory freezer (at temperatures from -2 to -10 °C), thawed on a lab bench and stored at 4 °C (for up to 21 days), as outlined in Section 3.1.2. Subsequently, TSS and pH tests were conducted on samples of juice that had been extracted from the fruit, as described in Section 3.1.4. The extents of damage inside the fruit, via TSS and pH analyses of orange juice, are presented below as graphs of either TSS or pH plotted as a function of storage time. A separate graph is provided for each freeze temperature (and cultivar), with graphs having a set of straight lines drawn between data points for the same freeze time to guide the eye in ascertaining trends. Summary discussions are provided about these investigations.

4.1 Fruit Quality Test

Quality of the fruit is an important factor that the buyer, wholesalers, retailers and consumers are looking for. However, the appealing character comes from the appearance of the fruit in the initial purchase and subsequent purchases may be more related to texture and flavour (Chen, P. et al., 1991); (Harker, F., 1997) therefore it becomes important for the fruit to be tested for the presence of defects.

Furthermore, consumers are demanding fresh fruits not only in terms of their appeal, such as bright colour and perfect shape, but also tasty fruits with significant nutritional values. The quality of fresh oranges depends upon the supply chain, i.e. growers, producers, and transportations. Therefore, quality controllers and researchers, who work on different aspects of the industry, need to do further investigations to improve nutritional content, external and

internal quality based on °Brix or TSS, colour, shape and other important requirements (Heinz,1983; Gnanasekharan, 1993).

This Section reports fruit quality assessments that were conducted before and after freeze treatment on orange fruits of Valencia and Navel cultivars that were assessed for weight loss, appearance, skin colour and damage.

In this experiment an investigation of visual Assessment to orange fruit samples were carried out prior to experimental work started as illustrated on Table 8.9.1 below.

Table 4.1 lists the orange fruit quality checks to samples that were freeze treated and stored for 3 weeks periods and shows that defects such as Colour change (from normal yellow to dark yellow), Softness (less firm), Skin blemishes, Discoloration, freezing damage and Internal dryness (loss of fluid) were observed whereas Insect damage, Bruising, Uniformity and Smoothness were not found in any freeze-treated samples. But no defects were observed before the samples under went through freeze treatment.

Table 4.1 Orange fruit quality checks of samples that were freeze treated and stored for 3 weeks periods. (Short hand) V = Valencia, N = Navel and hr = hours

Temp.	-2 ºC/24 hr		-4 ºC/ 24 hr		-6 ºC/ 24 hr		-8 ºC/ 24 hr		-10 ºC/ 24 hr	
Cultivar	V	N	V	N	V	N	V	N	V	N
Color change	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Uniformity	x	X	x	x	x	x	x	x	x	x
Softness	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Smoothness	x	X	x	x	x	x	x	x	x	x
Bruising	x	X	x	x	x	x	x	x	x	x
Skin blemishes	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Internal dryness (loss of fluid)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Discoloration	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Freezing damage	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Insect damage	x	X	x	x	x	x	x	x	x	x

The context of this work is that citrus fruit can be damaged due to exposure to a cold winter, particularly if the temperature falls too far. It is more likely that the fruit could be exposed to repeated frosts, where damage can be caused but the effects may not be immediately obvious externally for some weeks after the cold snap. Ideally, the internal as well as external quality of fruit needs to be evaluated to identify the extent of the frost or cold damage before the fruit can be transferred to the market (Oberoi *et al.,* 2011). It is also common practice that harvested fruit is not usually sorted until a few weeks after the frost, so it is important that sound and damaged fruit be identified and separated to prevent damaged fruit causing secondary damage to sound fruit (Oberoi *et al.,* 2011).

It is clear that growers and retail inspection services would benefit from this rapid assessment technique to determine suspect fruit before external damage becomes visibly obvious. Because of the quantity of fruit involved in harvesting and the generally low skill levels of harvesting staff, the assessment device should be easy to operate and used for random and quick assessment of fruit samples at the orchard.

4.1.1 Weight loss assessment (Dhar et al., 2008)

Freeze treated and control orange samples were used for this experiment. The initial weight of each fruit was recorded using the electronic balance as described in Section 3.1.1 the percentage weight loss was calculated at the end of 21 days of storage as:

$$\text{Weight loss (\%)} = \frac{\text{Initial weight - final weight fruit}}{\text{Initial weight}} \times 100$$

Control Sample

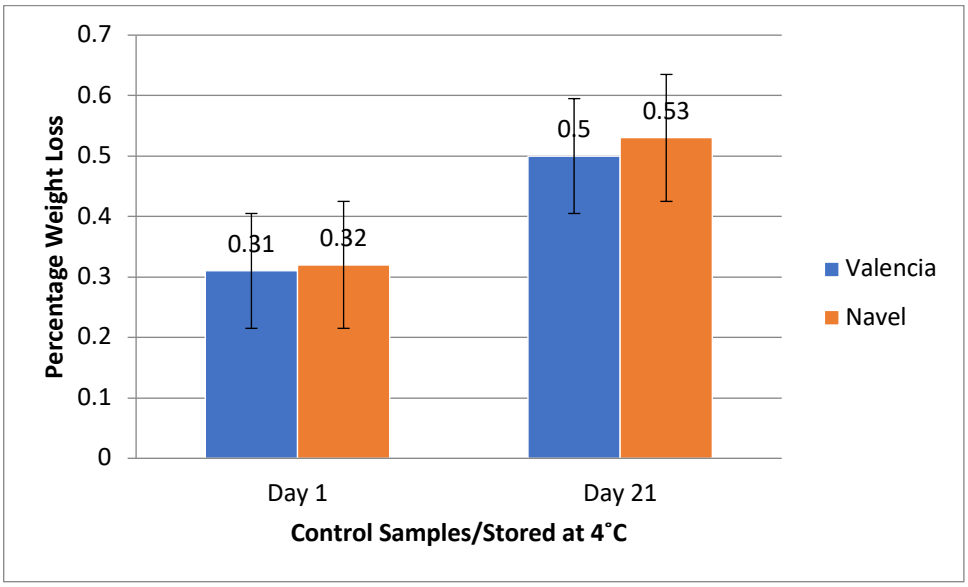

Figure 4.1.1 Weight loss of Valencia and Navel control orange fruit samples for storage time of day 1 and day 21. Error bars represent the standard error of two different types of treatment with respect to storage times expressed in days.

Figure 4.1.1 weight loss for control samples shows that not much change during the storage time of 21 days. For Valencia orange samples it shows a decrease of 0.31% for day 1 and 0.5% for day 21 whereas for Navel orange samples the result shows 0.32% for day 1 and 0.53% for day 21. According to the above result Navel samples were damaged more than the Valencia samples however the difference was 0.01 % in day 1 and 0.03 % in day 21.

The weights of Valencia and Navel samples were recorded before and after freeze treatment at -2 °C for 2/24 hours and at -10 °C for 2/24 hours followed by storage for 3 weeks. Similarly, control samples of both varieties were recorded after 3 weeks storage. Figures 4.1.1 to 4.1.4 show the weight loss and circumference decrease of freeze treated and control Valencia and Navel orange fruit samples, respectively, with the error bar representing the standard error.

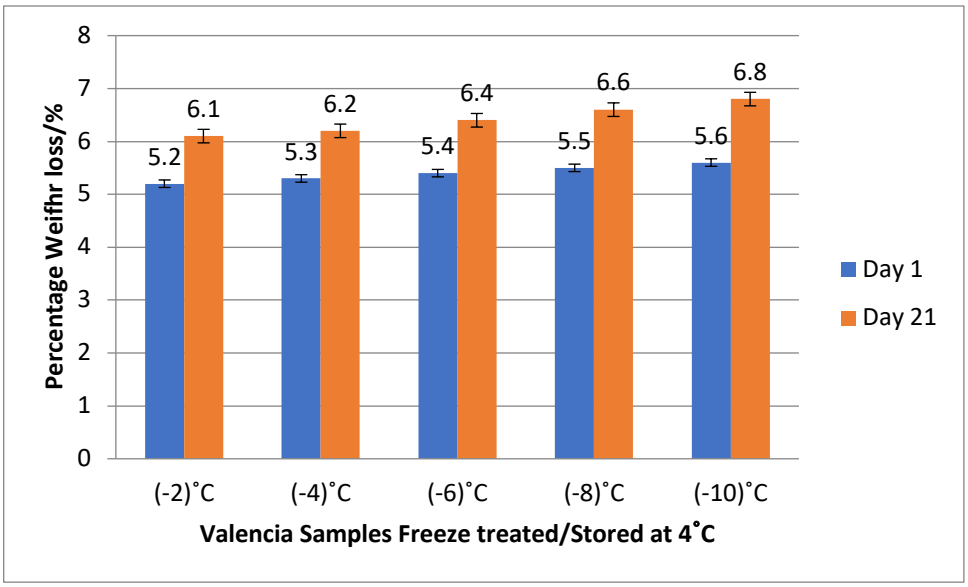

Figure 4.1.1.2 shows weight loss of Valencia orange fruit samples after freeze treatment at -2 ºC, -4 ºC, -6 ºC, -8 ºC and -10 ºC for 24 hours and stored from 1 to 21 days. Error bars represent the standard error of five different types of treatment with respect to storage times expressed in days.

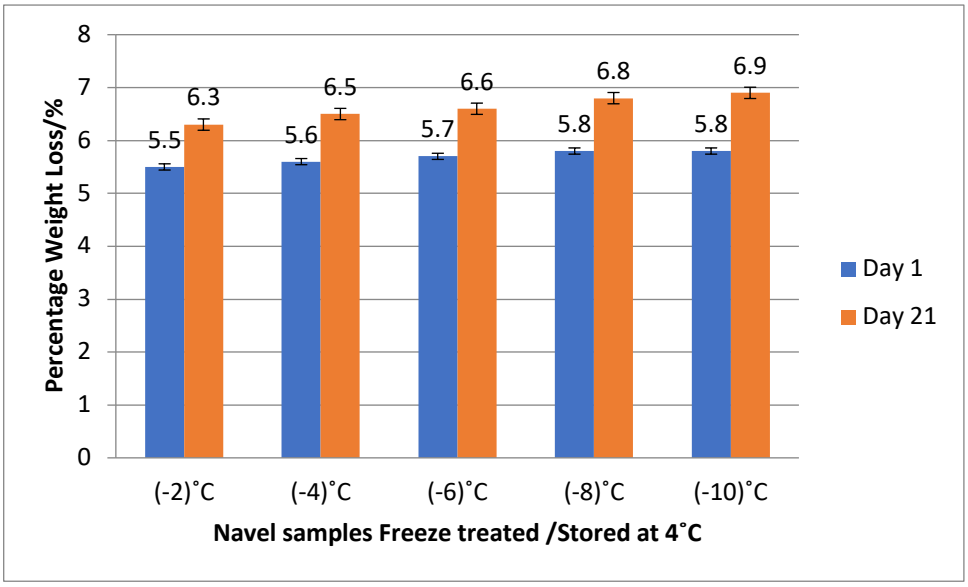

Figure 4.1.1.3 shows Weight loss of Navel orange fruit samples after freeze treatment at -2 ºC, -4 ºC, -6 ºC, -8 ºC and -10 ºC for 24 hours and stored from 1 to 21 days. Error bars represent the standard error of five different types of treatment with respect to storage times expressed in days.

According to the results in Figure 4.1.2 in day 1 weight loss of Valencia orange fruit samples that was freeze treated for 24 hours at -2 ºC shows (5.2%), -4 ºC (5.3%), -6 ºC (5.4%), -8 ºC (5.5%) and at -10 ºC (5.6%) for 24 hours

Similarly in Figure 4.1.3 Navel orange samples that was freeze treated for 24 hours at -2 ºC shows (5.5%), -4 ºC (5.6 (%), -6 ºC (5.7%), -8 (5.8%) ºC and at -10 ºC (5.9%) for 24 hours in day 1. However in Figure 4.1.2 after 3 weeks of storage of Valencia orange fruit samples that were freeze trated for 24 hours at -2 ºC shows (6.1%), -4 ºC (6.2%), -6 ºC (6.4%), -8 ºC (6.6%) and -10 ºC (6.8%) for 24 hours.

In Figure 4.1.3 for Navel samples that were freeze treated at -2 ºC shows (6.3%), -4 ºC (6.5%), -6 ºC (6.6%), -8 ºC (6.8%) and at -10 ºC (7.06%) for 24 hours.

4.1.2 Circumference measurement test

In section 4.1 weight loss assessment tests were carried out a in relation to freeze treatment and storage effect and the results (average values in %) are displayed above. Similarly in this study an investigation was carried out in terms of fruit shrinkage or reduction in size due to similar freeze treatment. The circumference of Valencia and Navel orange fruits were measured and the change in circumference (average values in cm) are displayed below. This value for circumferences differences were calculated using mean value of **X1 (before treatment and storage/original value/cm) – X2 (after the treatment and storage/final value/cm) = differences/cm.**

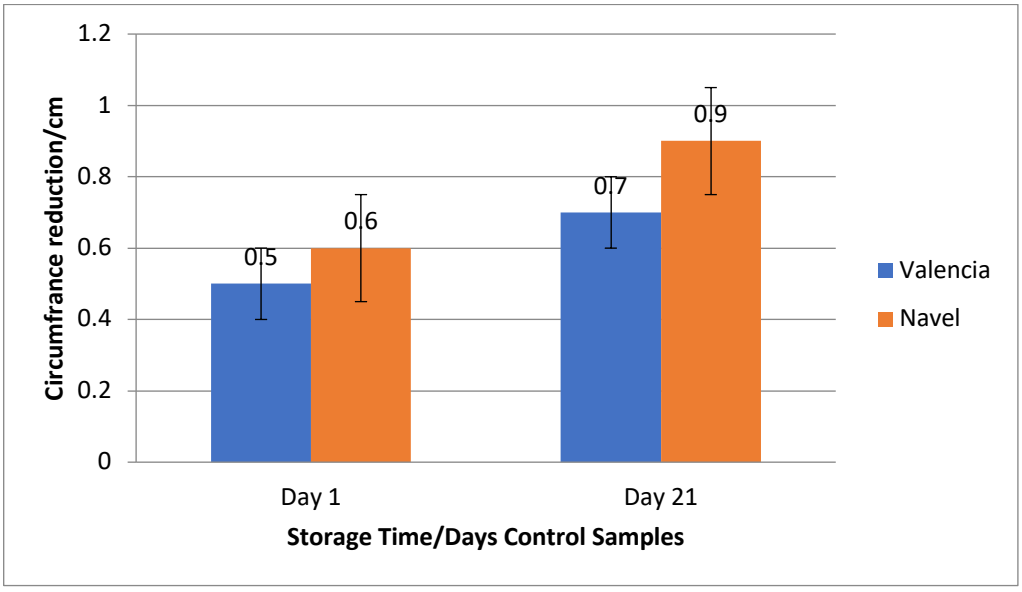

Figure 4.1.2.1 shows change in circumfrence of Valencia and Navel control orange fruit samples for storage time of day 1 and day 21. Error bars represent the standard error of two different types of treatment with respect to storage times expressed in days.

In Figure 4.1.2.1 the circumference measurement for control samples indicated 0.5 cm on day 1 and 0.7 cm on day 21 for Valencia samples however for Navel samples the results show 0.6 cm on day 1 and 09 cm on day 21 samples

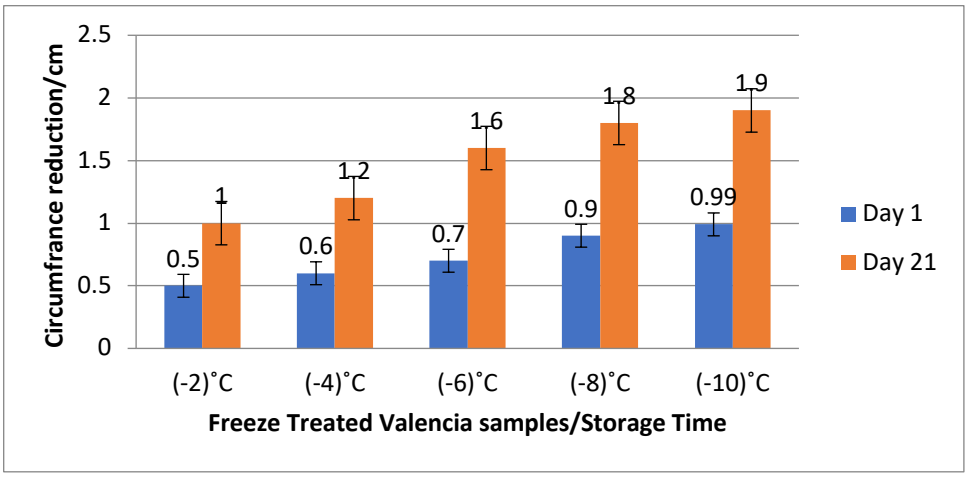

Figure 4.1.2.2 shows change in circumference of Valencia orange fruit samples after freeze treatment at -2 ºC, -4 ºC, -6 ºC, -8 ºC and -10 ºC for 24 hours and stored from 1 to 21 days. Error bars represent the standard error of five different types of treatment with respect to storage times expressed in days.

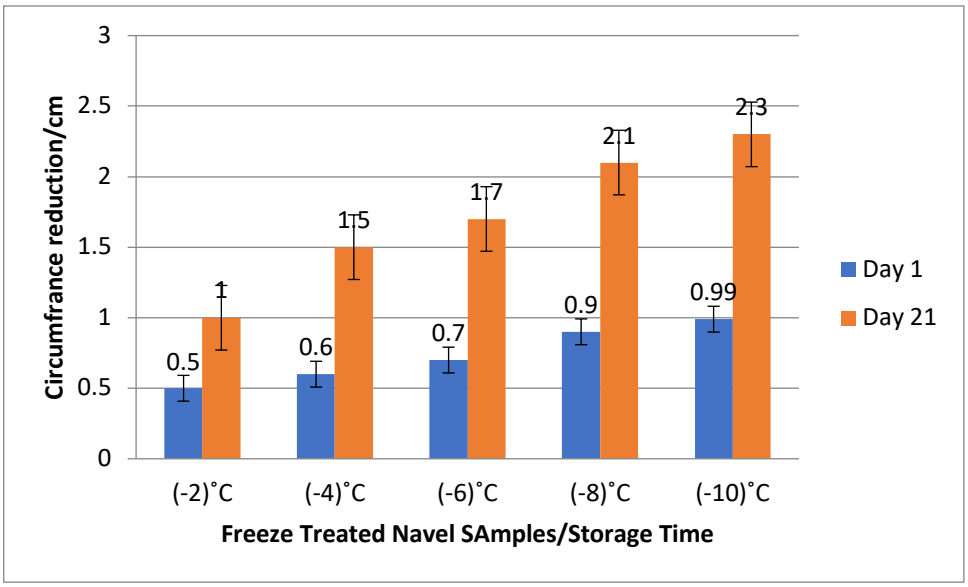

Figure 4.1.2.3 Mean circumference in cm value of Navel orange fruit samples after freeze treatment at -2 ºC, -4 ºC, -6 ºC, -8 ºC and -10 ºC for 24 hours and stored from 1 to 21 days. Error bars represent the standard error of five different types of treatment with respect to storage times expressed in days.

Figure 4.1.2.2 shows the circumference measurements in cm for Valencia freeze treated orange samples at -2 ºC shows (0.5 cm), -4 ºC (0.6 cm), -6 ºC (0.7 cm), -8 ºC (0.9 cm) and -10 ºC (0.99 cm) for 24 hours duration on day 1 storage. Similarly in Figure 4.1.2.3 for Navel orange samples freeze treated at -2 ºC shows (0.5 cm), -4 ºC (0.6 cm), -6 ºC (0.7 cm), -8 ºC (0.9 cm) and -10 ºC (0.99 cm) for 24 hours They show 0.5 – 0.99 cm shirink in circumferencein day 1. Figure 4.1.2.2 shows the circumference/cm for Valencia orange samples due to the effect of freeze treatment at -2 ºC shows (1 cm), -4 ºC (1.2 cm), -6 ºC (1.6 cm), -8 ºC (1.8 cm) and -10 ºC (1.9 cm) for 24 hours duration in day 21 storage. Similarly in Figure 4.1.2.3 for Navel orange samples freeze treated at -2 ºC shows (1 cm), -4 ºC (1.5 cm), -6 ºC (1.7 cm), -8 ºC (2.1 cm) and -10 ºC (2.3 cm) for 24 hours They show 1.0 – 2.3 cm shirink in circumferencein day 21.

4.2 Further discussion of weight loss analysis

The above weight loss investigations were carried out by measuring all orange samples using weight scale and recorded the values before and after the freeze treatments and storage time. The weight loss of Valencia and Navel orange fruits were investigated by freeze treating orange samples at -2 ºC, -4 ºC, -6 ºC, -8 ºC and -10 ºC in order to understand the nutritional content of orange fruit. The dryness of the fruit and sogginess with less watery content was observed compare to control samples that were not received freeze treatment. As Figure 4.1.1 shows not much change was observed during day 1 storage (3%) despite small fraction, which was (4%) change during 21 days of storage. However, changes were observed to both

Valencia and Navel orange samples that were freeze treated for 24 hours and stored up to 3 weeks. According to Figure 4.1.2 of Valencia samples shows 6.1 – 6.8% weight loss for day1 to day 21 stored samples.

Similar action was performed for Navel Orange samples and Figure 4.1.3 show 6.3 – 7.06% increase in % weight loss from day 1 to day 21 storage periods for 24 hours freeze treated samples. Furthermore, Navel orange samples in Figure 4.1.2.1 show 6.3% – 7.06% more damage than Valencia orange samples this was due to the structure and thin layer of Valencia orange (Eskin, 1991). Further investigations were carried out by measuring the circumference of both Valencia and Navel orange samples using type measure in (cm) before and after freeze treatments and this was done prior to any experiments were conducted to this practical study concerning orange fruit storage quality issues that was described above.

Moreover, it was observed during investigation that once the fruit showed weight loss and less circumference measurements than original measurements as well as lost its internal watery/fluid contents (which can be observed by pressing or pushing the fruit in), and the loss of weight from its our original weight recorded before the treatment/storage. Furthermore, the fruit become softer and soggy as well as it showed body shrinkage compare to control samples (not freeze treated). Fruit becoming soggy due to loss of volatility as well as damages was also mentioned by (Eskin, 1991). He also added that harvested horticultural products are living tissues with continuing metabolism after harvest. They are subject to respiration, water loss and cell softening throughout the postharvest system. The storage life of a product varies with species, variety and pre-harvest conditions particularly with quality and maturity.

According to our results in Figure 4.1.2.2 and 4.1.2.3, it shows reductions in circumference measurements for the both Valencia and Navel orange samples after freeze treatment and storage of 3 weeks. In Figure 4.1.2.2 Valencia orange samples showed circumference reductions of 1 cm – 1.9 cm and in Figure 4.1.2.3 Navel orange samples show values of 1 – 2.3 cm reductions.

4.3 TSS and pH Tests on Orange Juice

The previous Chapter discussed the materials, equipment and methods used in this investigation. This Section provides the detailed results and comments which relate to the investigations into TSS and pH analyses which are relevant to the study of the effects of frost damage to orange fruits during a cold winter.

As was indicated in Section 3.1, the orange samples for investigation were delivered to Victoria University undipped and un-waxed in order to ensure that no external chemical influence

would be introduced to affect the results. The samples were freeze-treated in a laboratory freezer (at temperatures from -2 to -10 °C), thawed on a lab bench and stored at 4 °C (for up to 21 days), as outlined in Section 3.1.2. Subsequently, TSS and pH tests were conducted on samples of juice that had been extracted from the fruit, as described in Section 3.1.4.

The extents of damage inside the fruit, via TSS and pH analyses of orange juice, are presented below as graphs of either TSS or pH plotted as a function of storage time. A separate graph is provided for each freeze temperature (and cultivar), with graphs having a set of straight lines drawn between data points for the same freeze time to guide the eye in ascertaining trends. Summary discussions are provided about these investigations.

4.3.1 Total Soluble Solid (TSS) Test for Valencia and Navel Orange Fruits

The following Figures (4.3.1A & B – 4.3.5A & B) show the average (mean) TSS reading of freeze-exposed orange juice samples for the 5 different freeze temperatures chosen for these investigations, for the different freeze times and storage times. Similar measurements were conducted on control samples for the different storage times and the results a given in Table 4.3.1. All results show decreasing TSS values in the damaged fruit with increased storage time, which is in line with the findings of David *et al.* (2003).

All results show a decrease in TSS values in the damaged fruit with increased storage time compared with the control samples, which is in line with the findings of David *et al.* (2003). Those authors reported significant differences in TSS and internal chemical compositions between healthy fruit and orange fruit that was freeze treated.

The changes in TSS value for Valencia species are displayed in Figure 4.3.1A, and those for Navel species are in Figure 4.3.1B. According to these figures, it appears that minor changes in TSS value were observed in Valencia samples on day 1. The results further show values drop from an initial 8.3 to 7.8 on day 21 for 2 hours treatment, and from 8.2 on day 1 to 7.40 on day 21 for the 24 hours treatment. Similarly for the Navel variety, it was 12.6 on day 1 and 12.5 on day 21 for the 2 hours treatment, and 12.5 on day 1 and 11.0 on day 21 for the 24-hour treatment. The reduction in TSS in both Valencia and Navel oranges are similar for the -2 °C for 2 hours treatment. The change in TSS is minimal for both orange species at this temperature as expected, with the sweetness being very slightly reduced in both samples. In Figures 4.3.1 A, Valencia, and 4.3.1 B, Navel, it can be seen that there is a small but significant difference between the -2 ^{o}C treatment temperature and storage time of 21 days. This is again to be expected as the temperature is only just below 0 ^{o}C.

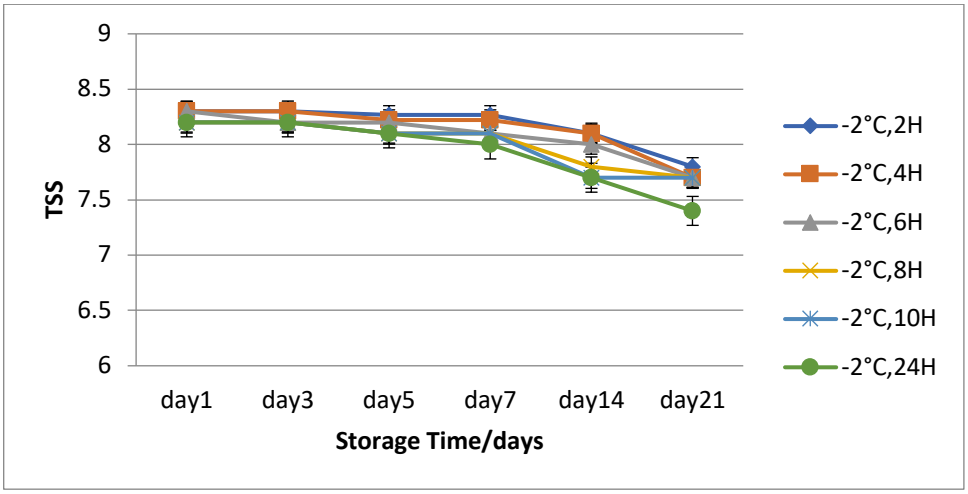

Figure 4.3.1A Variation in TSS of pre-freezed Valencia orange fruit at -2 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard deviation of six different types of treatment with respect to storage times expressed in days and hours (H).

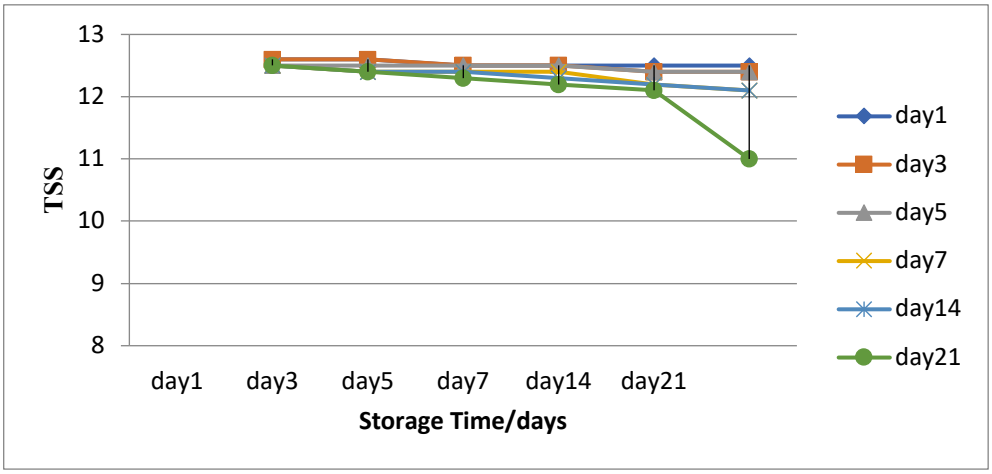

Figure 4.3.1B Variation in TSS of pre-freezed Navel orange fruit at -2 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times expressed in days and hours (H).

Figures 4.3.2A (Valencia) and Figure 4.3.2B (Navel) show the effect of pre-freeze treatment on TSS values at -4 °C with treatment times from 2 - 24 hours followed by storage time of 1 to 21 days. Results for Valencia samples show a minor decrease in TSS value from 8.3 on day 1 for 2 hours treatment compared to 7.7 on day 21 for 24 hours treatment. Similarly, the result for Navel samples showed TSS value changed from 12.6 at day 1 for 2 hours treatment to 10.0 on day 21 for 24 hour treatment. Again, the sweetness for Navel oranges has been reduced slightly more than for Valencia fruit from day 1 to day 21.

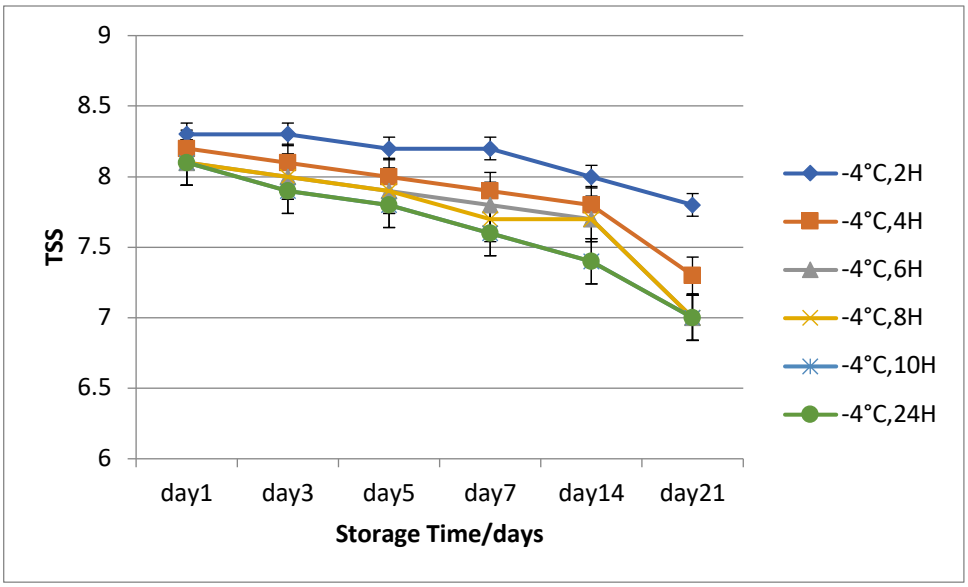

Figure 4.3.2A Variation in TSS for pre-freezed Valencia orange fruit at -4 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times expressed in days and hours (H).

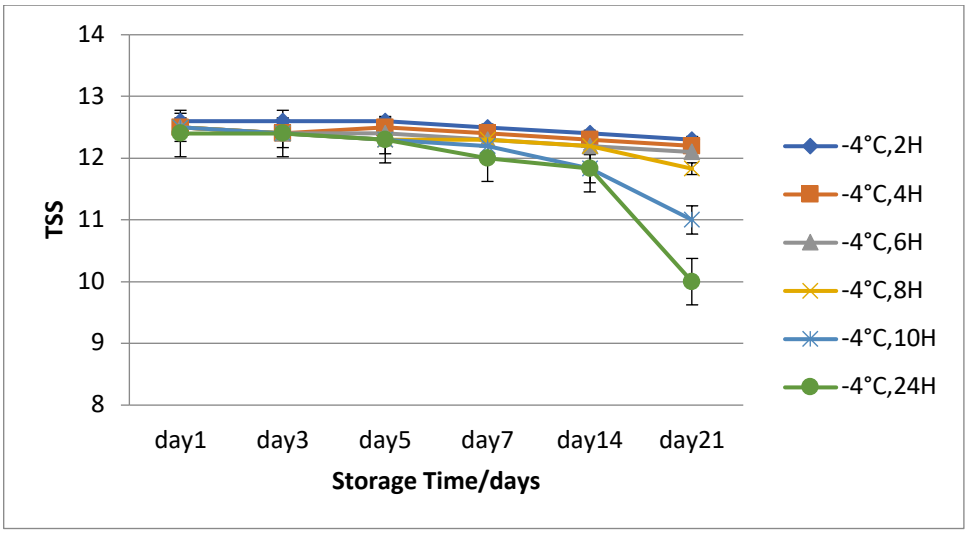

Figure 4.3.2B Variation in TSS of pre-freezed Navel orange fruit at -4 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times expressed in days and hours (hr).

In Figure 4.3.3A (Valencia) and Figure 4.1.3B (Navel), the change in freeze treatment with respect to storage time for samples treated at -6 °C can be seen. The data in Figure 4.3.3A for Valencia samples gives a TSS value of 8.3 on day 1 for 2 hour treatment compared to 6.8 on day 21 for 24 hours treatment. In contrast, for Navel samples, the TSS value changes from 12.6 on day 1 to 9.0 on day 21 for the 24 hour treatment. These values show the change in Navel samples is greater than for Valencia samples; this follows the previous trends, but the

increase is at a greater rate at the lower temperature, and this implies greater damage has ensued.

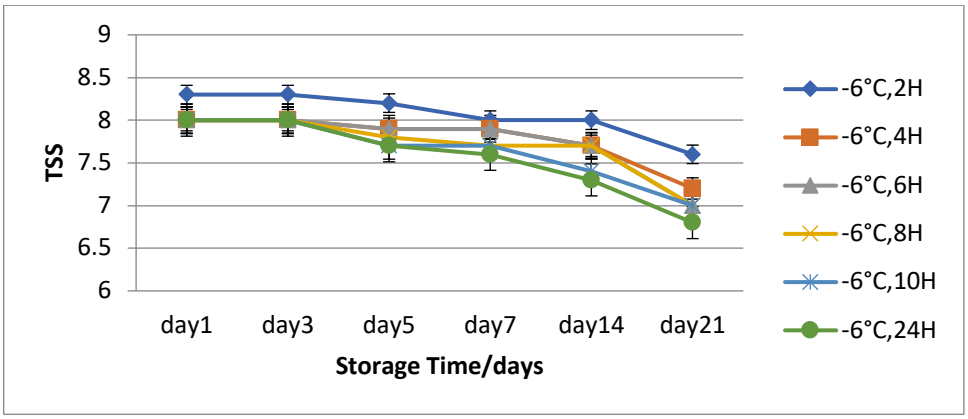

Figure 4.3.3A Variation in TSS of pre-freezed Valencia orange fruit at -6 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times that expressed in days and hours (H).

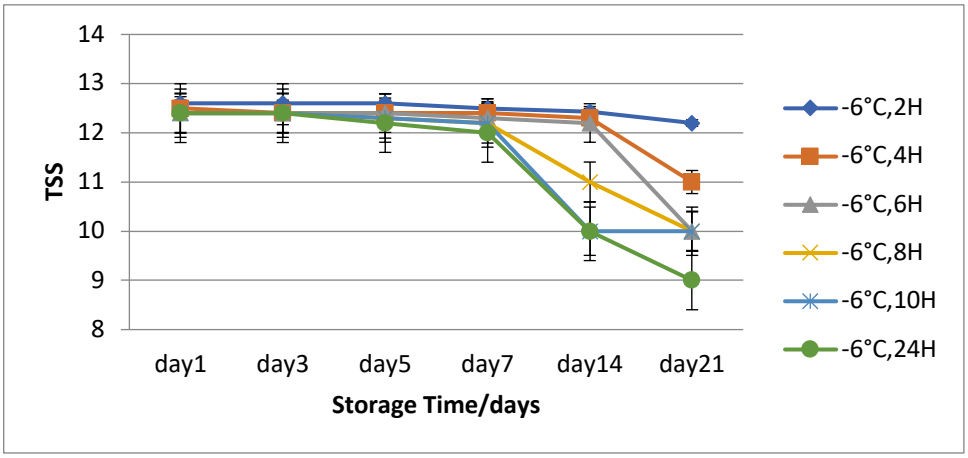

Figure 4.3.3B Variation in TSS of pre-freezed Navel orange fruit at -6 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times expressed in days and hours (H).

Figure 4.3.4A and Figure 4.3.4B show the samples of Valencia and Navel fruit that have been treated in a laboratory freezer at -8 °C for 2 hours to 24 hours and stored from day 1 up to day 21 to observe the changes in sugar content. According to the findings, the results show a greater decrease in TSS, compared with the previous results (at higher freeze temperatures). The change in Figure 4.3.4A, Valencia fruit, is seen to be from 8.0 at day 1 with 2 hours treatment compared to 6.8 on day 21 with 24 hours treatment, whereas the Navel samples in

Figure 4.3.4B shows a change from 12.5 on day 1 with 2 hours treatment to 9 on day 21 with 24 hours treatment. The change in TSS for Navel samples is greater than for Valencia oranges, which indicates greater damage for Navel samples, this being 3.5 compared to 1.2 on day 21 for 24 hours treatment for Valencia samples, and meaning that Navel oranges are more affected by cold damage.

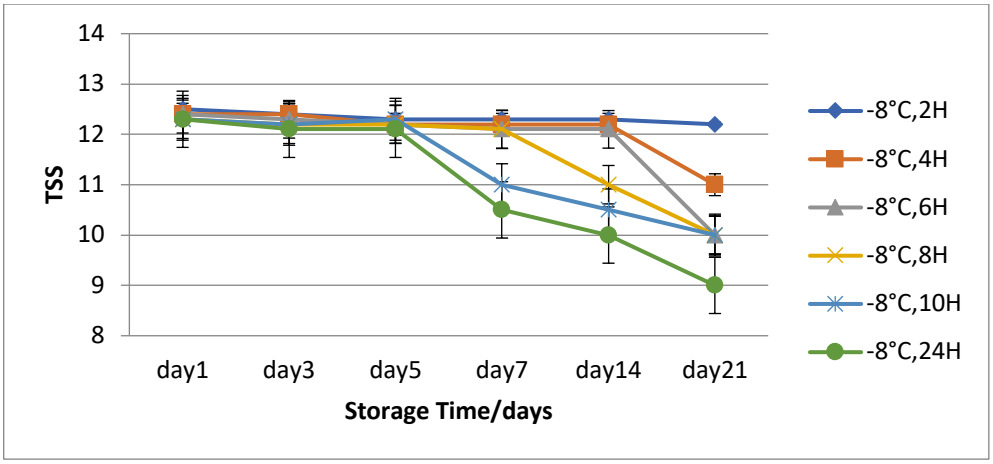

Figure 4.3.4A Variation in TSS of pre-freezed Valencia orange fruit at -8 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times expressed in days and hours (H).

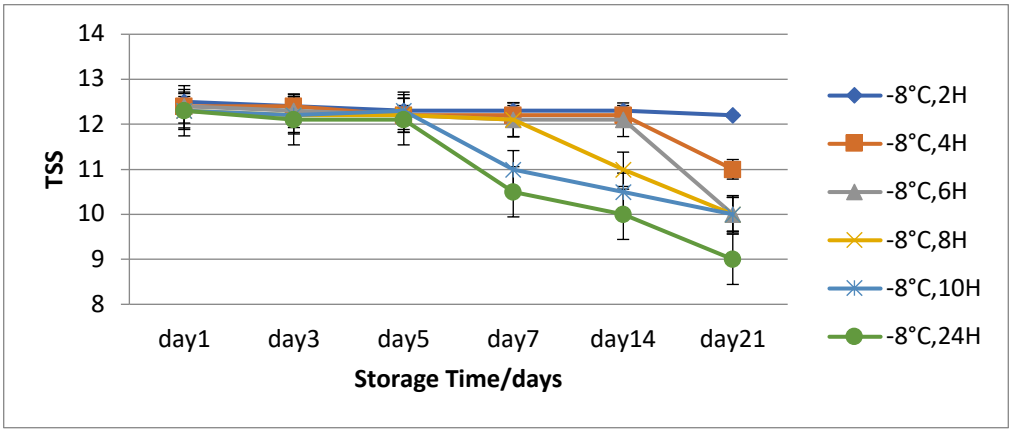

Figure 4.3.4B Variation in TSS of pre-freezed Navel orange fruit at -8 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times expressed in days and hours (H).

The results in freeze treated samples for -10 °C and storage time up to three weeks was assessed for sweetness, TSS, and the results are given in Figures 4.3.5A and 4.3.5B, for Valencia and Navel varieties, respectively. The values found for Valencia oranges changed from 8.0 on day 1 with 2 hours treatment to 5.5 on day 21 for 24 hours treatment, while the Navel samples decreased from 12.4 on day 1 with 2 hours treatment to 8.0 on day 21 for 24 hours treatment. The change in TSS is 2.5 for Valencia and 4.4 for Navel samples, which

represents a 0.16% to 0.54% change for freeze-damage treatment. These results clearly show that Valencia fruit are more resistant to cold damage than Navel oranges, which could particularly occur in a transport storage container. In addition, Valencia oranges grow in the warmer months whereas Navel oranges are grown in the winter, and therefore are often subject to mild to severe frosts.

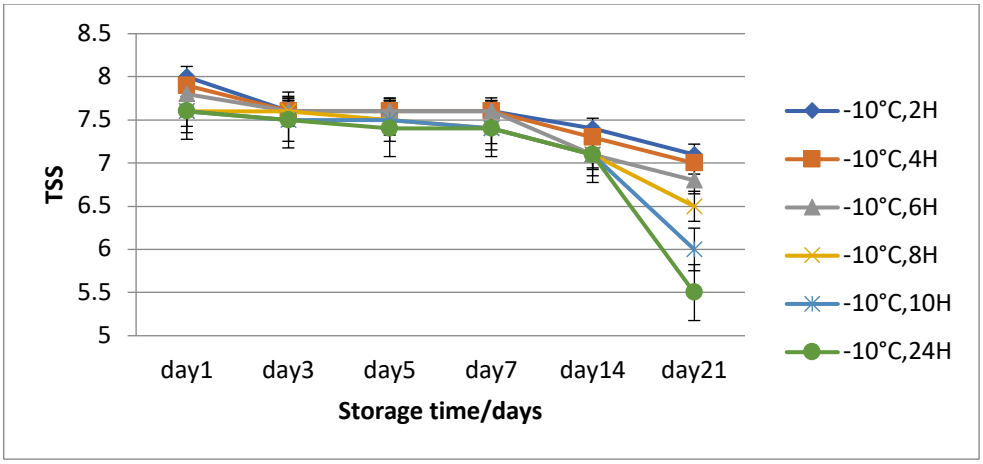

Figure 4.3.5A Variation in TSS of pre-freezed Valencia orange fruit at -10 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times expressed in days and hours (H).

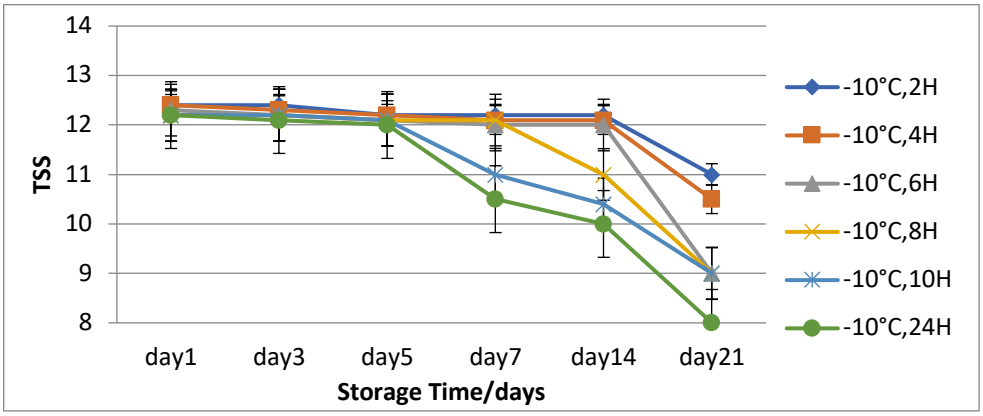

Figure 4.3.5B Variation in TSS of pre-freezed Navel orange fruit at -10 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard error of six different types of treatment with respect to storage times expressed in (days) and hours (H).

The TSS readings for the Valencia and Navel control orange samples that were not freeze-treated are given in Table 4.3.1, and it can be seen that TSS remained virtually unchanged.

Table 4.3.1 TSS readings in Valencia and Navel control orange samples with up to 21 days of storage at 4 °C.

TSS Test of control sample	Valencia	Navel
Storage time/days	Mean±SD	Mean±SD
Day 1	9.0^{a}±0.00	12.9^{a}±0.00
Day 3	9.0^{a}±0.00	12.9^{a}±0.00
Day 5	9.0^{a}±0.00	12.9^{a}±0.00
Day 7	8.9^{a}±0.00	12.9^{a}±0.00
Day 14	8.9^{a}±0.00	12.5^{a}±0.00
Day 21	8.9^{a}±0.00	12.5^{a}±0.00

Results expressed as means ± standard deviation (n=6). Statistical analysis by means of one way ANOVA

^{abcd} Means in the same row with different lower case are significantly different ($p<0.05$)

^{ABCD} Means in the same column with different upper case are significantly different ($p<0.05$)

Statistically analysis by means of one-way ANOVA

4.4 pH Test of Valencia and Navel Orange Fruits

The pH level of a food helps to understand whether bacteria can grow or not. Acidity is measured using what is known as the pH scale which goes from 0 to14, where pH 7.0 is neutral, a pH less than 7 is acidic and a pH greater than 7.0 is basic or alkaline. Foods with a pH close to 7.0 are ideal for most bacterial growth. Every microorganism has a minimum, optimum and maximum pH for growth. The optimum growth range is about 6.0 to 7.5, but growth can occur slowly at lower or higher pH levels. A pH of 4.6 and below or a pH above 11 will prevent pathogen growth. Change in pH levels plays a primary role in the preservation of fruits and foods in general (International Commission on Microbiological Specification for Foods. 1996). Considering the above information our investigations in the project were focused on pH changes to study the vulnerability and deterioration stage of Valencia and Navel orange fruit which were damaged and stored for specific time frame compared with control samples. The results in pH measurements including changes in pH are presented in the next section.

Results of pH measurements for freeze-treated Valencia and Navel samples which were stored for up to 3 weeks at 4 °C are shown in Figures 4.4.1 to 4.4.5, for the 5 chosen freeze temperatures and the various treatment times. Table 4.4.1 provides the pH readings for the control orange samples. Figure 4.4.1 A and Figure 4.4.1 B shows the pH recordings for

treatments for variable periods at -2 °C followed by the storage of the sample at 4 °C for a maximum of 21 days. In Figure 4.4.1 A, a pH of 4.17 was recorded on day 1 with 24 hours treatment whilst the pH was 4.21 on day 21 for Valencia oranges. Results for Navel species are given in Figure 4.4.1B, and these showed correlation with the Valencia results; pH was recorded at 4.19 on day 1 and 4.21 on day 21 for 24 hours treatment. Correlation was noticed between the two cultivars, with a very slight increase in absolute value.

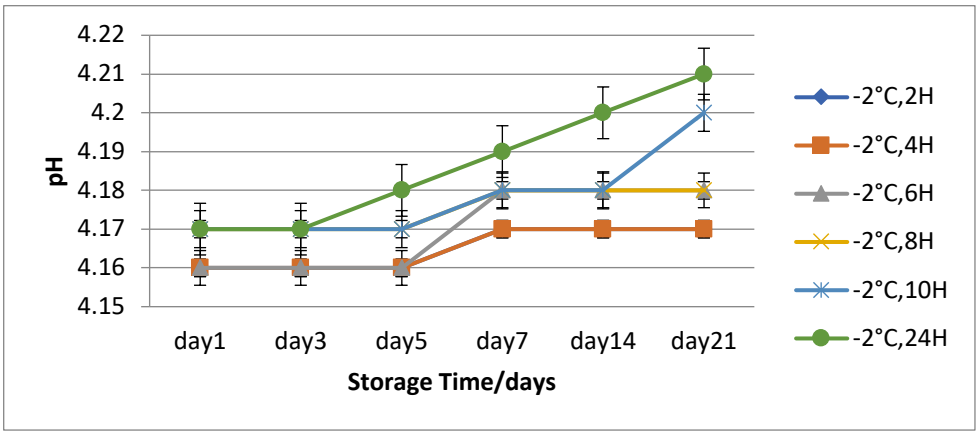

Figure 4.4.1A Variation in pH of pre-freezed Valencia orange fruit at -2 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

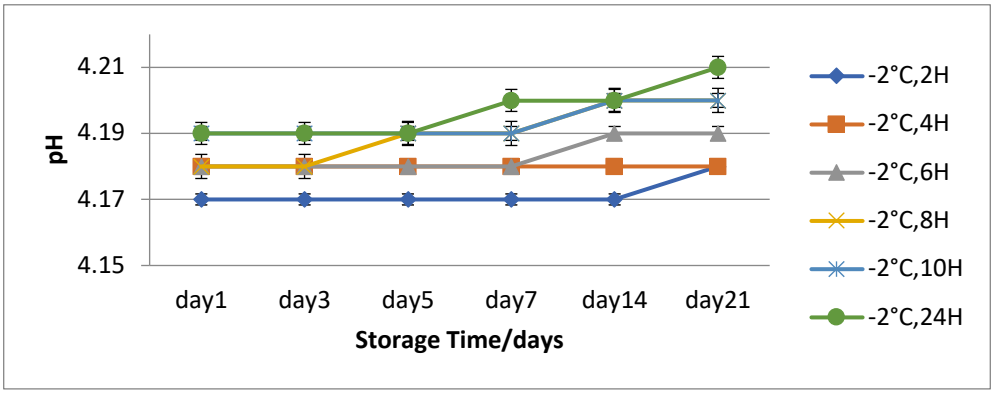

Figure 4.4.1B Variation in pH of pre-freezed Navel orange fruit at -2 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

Results for -4 °C treatment are given in Figure 4.4.2 A and B. Figure 4.4.2A shows that the pH of Valencia samples on day 1 was 4.17 for the 2-10 hours treatment time, compared with a pH of 4.19 for 24-hour treatment. All treatments showed an increase in the pH after day 21 with the 24 treatment showing the greatest increase.

Considering Figure 4.2.2 B (Navel samples) on day 1 at treatment times of 2 hours the pH is 4.19 compared with the 24 hours treatment the pH value of 4.20. Again all treatments indicated an increase in pH after 21 days storage.

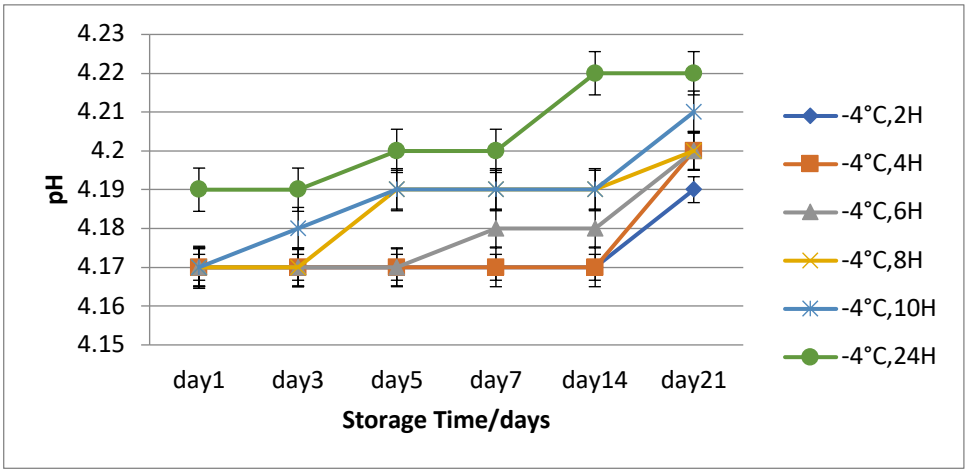

Figure 4.4.2A Variation in pH of pre-freezed Valencia orange fruit at -4 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

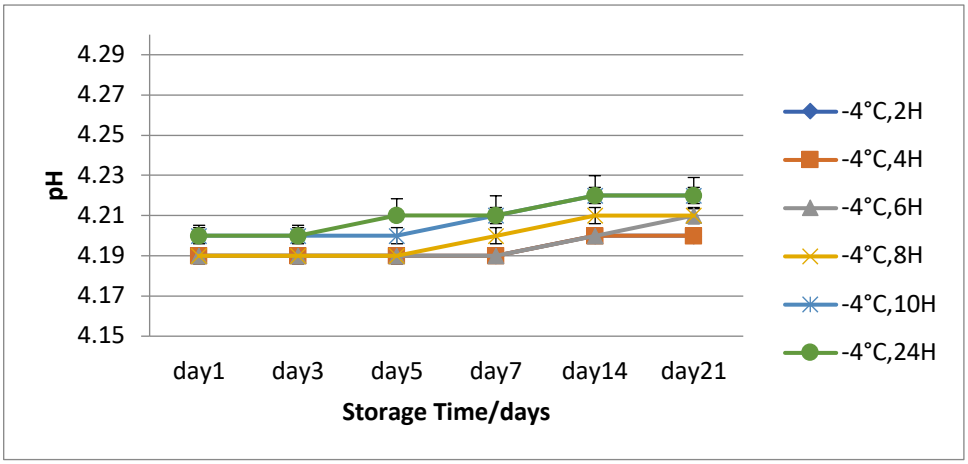

Figure 4.4.2B Variation in pH of pre-freezed Navel orange fruit at -4 °C for 2-24 hours and stored at 4 °C for up to 3 weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

Figures 4.4.3A and 4.4.3B are results for both samples that were freeze treated at -6 °C. Figure 4.4.3A (Valencia) shows that for day 1 and treatment time of 2, 4 and 6 hours, the pH was the same at 4.19. The pH increased steadily for treatment times of 8, 10 and 24 hours from day 1 to day 21. Similarly, results for Navel oranges in Figure 4.4.3B show a pH of 4.19

on day 1 and pH 4.21 for day 21 of 2 hours treatment, whereas for 24 hours treatment the value was 4.21 on day 1 and 4.24 on day 21.

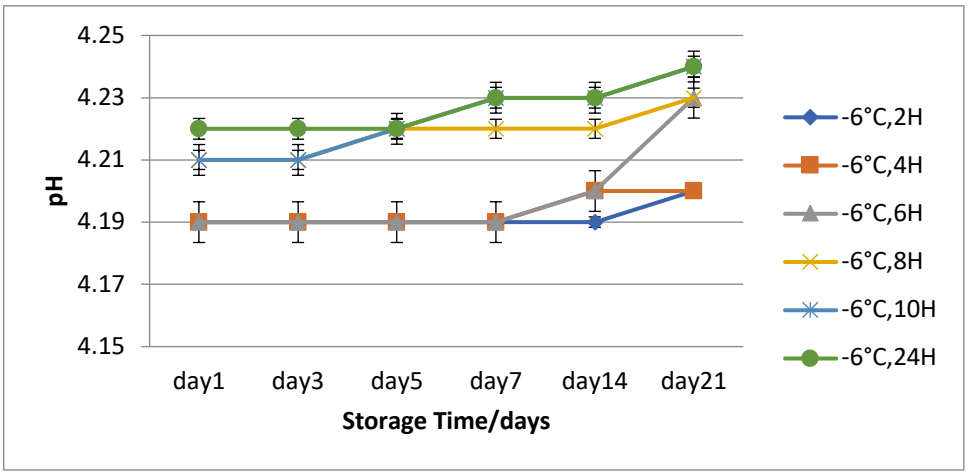

Figure 4.4.3A Variation in pH of pre-freezed Valencia orange fruit at -6 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

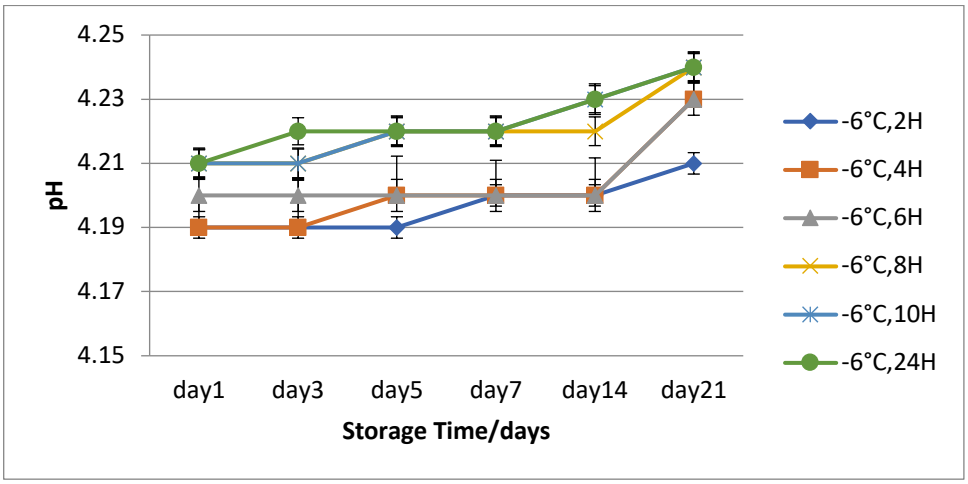

Figure 4.4.3B Variation in pH of pre-freezed Navel orange fruit at -6 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

Figure 4.4.4A and Figure 4.4.4B illustrate the change in pH of Valencia and Navel orange fruits in response to freeze treatment at -8 °C for variable hours and storage of 4 °C for up to 21 days. Both varieties showed a slight increase in pH on day 1 with increased treatment times from 4.19 with 2 hours freeze treatment and 4.22 - 4.23 with 24 hours freeze treatment. All treatment times showed an increase in pH after 21 days storage.

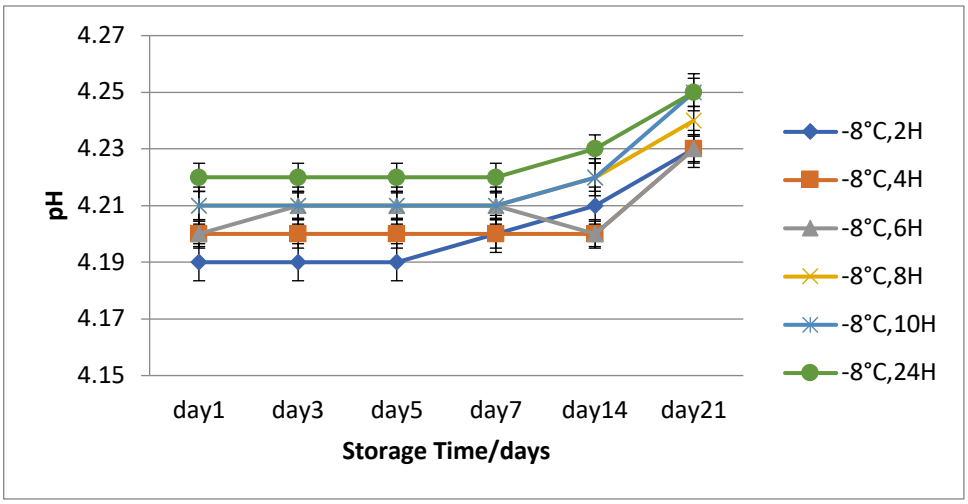

Figure 4.4.4A Variation in pH of pre-freezed Valencia orange fruit at -8 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

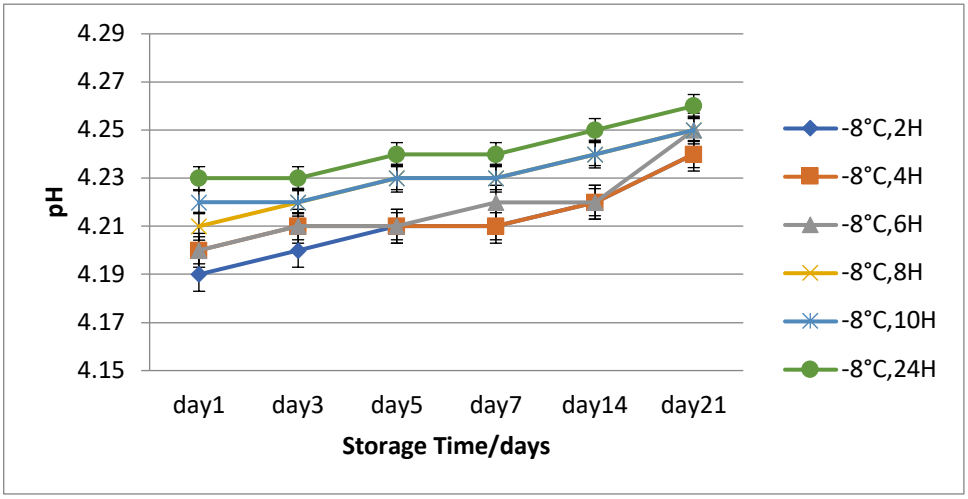

Figure 4.4.4B Variation in pH of pre-freezed Navel orange fruit at -8 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

Shown in Figure 4.4.5A Valencia and 4.4.5B Navel are the results for both varieties for freeze treatment at -10 °C and various storage times. The results also show a gradual increase in pH during three weeks of storage for both cultivars at all treatment times. Valencia samples ranged from 4.20 on day 1 for 2 hours freeze treatment to 4.26 on day 21 for the samples freeze treated for 24 hours. For Navel samples the range was 4.21 on day 1 for the samples freeze treated for 2 hours and 4.27 on day 21 for the samples that were treated for 24 hours.

The measurements of pH have been found to yield useful information to inspect the quality of the fruit and the data were analyzed to determine the degree of relatedness between TSS and pH. Correlation between these two factors can also be anticipated in terms of their relationship.

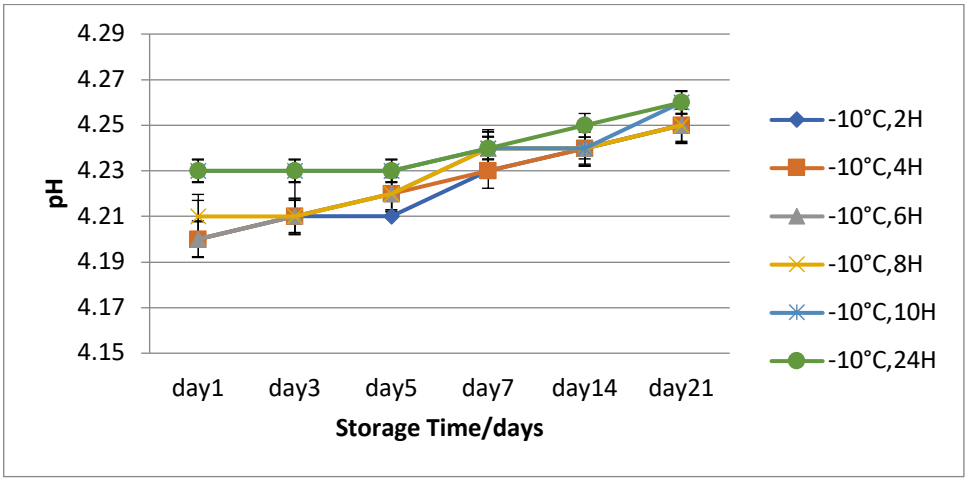

Figure 4.4.5A Variation in pH of pre-freezed Valencia orange fruit at -10 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

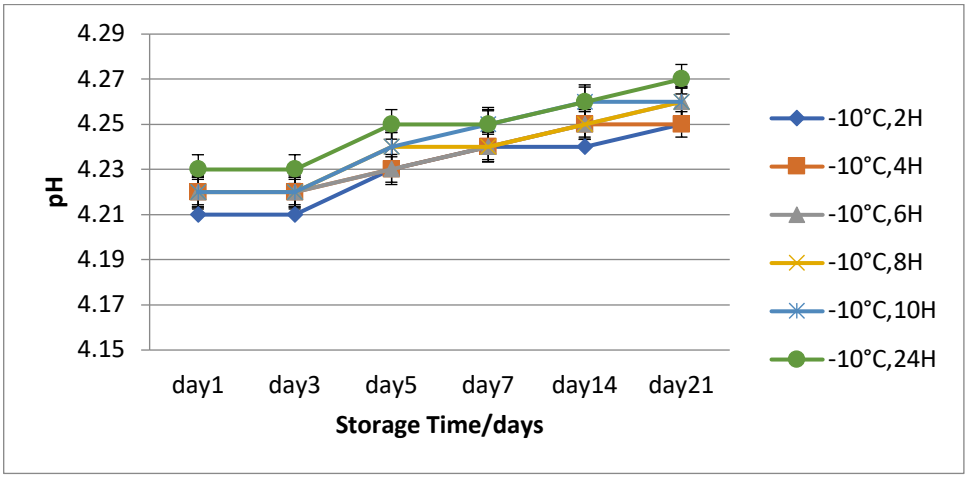

Figure 4.4.5B Variation in pH of pre-freezed Navel orange fruit at -10 °C for 2-24 hours and stored at 4 °C for up to three weeks. Error bars represent the standard errors of six different types of treatment with respect to storage times expressed in days and hours (H).

Table 4.4.1 pH values of Valencia and Navel control orange samples that stored for up to 21 days at 4 °C.

pH Test of Control sample	Valencia	Navel
Storage time/days	Mean±SD	Mean±SD
Day 1	3.9^{a}±0.00	4.1^{a}±0.00
Day 3	4.1±0.00	4.1±0.00
Day 5	4.0^{a}±0.00	4.1^{a}±0.00
Day 7	4.0±0.00	4.0±0.00
Day 14	3.9^{a}±0.00	4.0^{a}±0.00
Day 21	4.4^{a}±0.00	4.2^{a}±0.00

Results expressed as means ± standard deviation (n=6). Statistical analysis by means of one way ANOVA
^{abcd} Means in the same row with different lower case are significantly different ($p<0.05$)
^{ABCD} Means in the same column with different upper case are significantly different ($p<0.05$)
Statistically analysis by means of one-way ANOVA

4.4.1 Discussion of pH and TSS results

Freeze damage is clearly a major problem of fruits, bringing severe damage to the fruits which create a financial loss to the industry. It limits the ability of producers to generate the required volume and creates quality issue to high-volume buyers of fresh produce (Cutting *et al.,* 1992). Therefore, understanding the mechanisms of how to keep the quality parameters of orange fruit is essential. The focus here has been on the effects of frost damage, and an understanding of the nature of such damage, when it happens, how it damages plants and how it can be detected earlier so the fruit can be saved from deterioration. Tests such as TSS and pH, with developed limonene and ethanol levels, are anticipated to lead us to clearer insights on these issues.

From these results it can be seen that the results show that there are variations between the two cultivars; the Valencia samples showed less TSS reading than the corresponding Navel samples at the end of day 21. This difference may be due to growing conditions of the fruit pre-harvest, but it is clear that some fruits lose their volatilities more readily than others. Pre-harvest frost damage and post-harvest chilling injury in most fruits are associated with increased water loss, and it appears that some cultivars are more sensitive to this water loss (Cutting *et al.,* 1992). Water loss in storage has also been associated with reduced incidence

of physiological disorders (Bower *et al.,* 1990). This latter scenario can be seen particularly when fruits have gone through rapid freezing treatment; deteriorative processes can take place at a very low rate during storage periods (Thane and Reddy, 1997).

Furthermore, glucose and fructose levels are normally high at harvest time when the fruit has reached full ripeness (Nora *et al.,* 2005). However, in relation to TSS values after harvest, an investigation was carried out and results were provided. For control samples of (Valencia and Navel) with average (mean) and standard deviation results in Table 4.3.1 shows a TSS value of 9.0 to 8.9 with standard deviation of 0.00 for Valencia and 12.9 – 12.5 with standard deviation of 0.00 for day 1 to day 21 for Navel. However in Figure 4.4.1, of pH the reading for Valencia control orange samples with the storage time of day 1 to 21 shows a mean value of 4.4 – 3.9 with the standard deviation of 0.00. At the same time Navel control samples show pH readings of 4.1 – 4.2 with a standard deviation of 0.0.

By comparison with the results of TSS and pH values for -2 °C for 24 hours freeze treated Valencia oranges samples that stored for 21 days shows a reduction of in TSS reading from 8.20 to 7.40 and increase in pH value from 4.17 to 4.21, whereas the Navel samples show a reduction from 12.50 to 11.00 in TSS and an increase of pH from 4.19 to 4.21. Values for control samples were almost the same (Table 4.3.1). Considering the above results for -2 °C 24 hours, generally TSS levels were 9.8% lower for Valencia and 12% lower for Navel after three weeks of storage periods than samples stored and tested on day 1. In our investigation control samples did not show an obvious change in pH level in any of the cultivars (Table 4.4.1), whereas the pre-freezed treated samples showed an increase in pH. Moreover, the work by Harrill (1998) indicated obvious changes in acidity level compared to the control samples during investigation. The above statement clearly showed that Harrill (1998) used investigations based on acidity level whereas our study focused in pH changes.

When control results were compared to pre-freeze treated Navel samples, results for the Navel cultivar declined slightly with the TSS dropping from 12.6 - 10.0 which is 20.6 % drop after freeze treatment at -4 °C from day 1 - 21 with 2 - 24 hours treatment. Similarly, Valencia samples show a TSS decline of 8.3 to 7 from day 1 - 21 with 2 - 24 hours treatment pH values in Fig 4.4.3A of Valencia orange samples that were freeze-treated at -6 °C was observed and showed an increase from 4.19 to 4.23 with standard deviation of 0.001 whereas Fig 4.4.3B Navel samples increased from 4.19 to 4.24 between day 1 of 2 hours and day 21 of 24 hours with standard deviation of 0.002.

Furthermore, an increase in pH was also noticed at -10 °C freeze-treatment, which was the lowest temperature we have applied to the experiment. For Valencia, there was an increase

from 4.20 to 4.26, compared to a pH increase of 4.21 to 4.27 for Navel samples (Figures 4.4.5A and 4.4.5B). Whilst it is suspected that the reason for the above observations is that these relatively low freezing temperatures have been shown to increase the chilling-induced degradation of the membrane (Wang *et al.,* 1992). In a related experiment conducted by Jianbo *et al.* (2010), it was noted that the acidity level of tomato fruits subjected to chilling have shown changes after fruits were exposed to ambient conditions for three days (Jianbo *et al.,* 2010).

Figures 4.4.2A and B show a pH increase for Valencia and Navel (-10 °C/24 hours), freeze-treated samples over 21 days of storage time. The result was similar to those that have been reported by Nkansah *et al.* (2003). Others have also reported increases in pH and a decrease in TSS, and suggested that these were due to a loss of nutrition and the occurrence of chemical changes that takes place in the fruit during storage time for those fruit that were damaged (Simmonds *et al.,*1969; Kumah *et al.,* 2011).

Selected soluble sugars and organic acids were analysed in strawberry, sweet cherry, and mulberry fruits at different ripening stages by HPLC in their studies. The strawberry, sweet cherry and mulberry mainly contained tartaric, citric and ascorbic acids fructose and glucose were established to be the major sugars in all the tested fruit (Simmonds 1969). While citric and ascorbic acid were the predominant organic acids in strawberry and mulberry tartaric acid was mainly present in sweet cherry. The tested fruits mostly showed an increase in the concentration of sugars and organic acids with ripening (Simmonds *et al.,* 1969).

Furthermore, the amount of organic acids usually decreases during maturity, because they are a substrate of respiration. pH rises to a maximum at or soon after the climacteric and then usually shows a slight fall as ripening progresses (Simmonds 1969). It also was observed that, in these experiments, the pH of three selected cultivars were 4.20 before the experiment and after exposure to low temperature, the pH was 4.21, and increased gradually to 4.27 for Valencia at -10 °C. As stated by Simmonds *et al.,* (1969) It was also noted that environmental conditions and soil type can affect the pH of a product. Moreover, the effects are mostly depending on the genotype, the soil type for cultivation and the level of fertilizer application.

Albertini *et al.,* (2006) indicated how acids and sugars in lemon, lime, and orange from the fruit that changes during development. Their results provided the first complete report on sugar and organic acid accumulation during the early stages of fruit development in several citrus varieties. In addition, Tzur et al. (1992) reported that the sugars of citrus fruit pulp are fructose, glucose, and sucrose and fructose were detected.

In a further investigation by Salunkha *et al*. (1974), it was noticed that the fruits lost their quality attributes, with less firmness, and showed visible discoloration of skin with black spot. These changes are due to enzymatic activities that were not totally stopped and were somewhat reduced so that starch was still being converted to sugar.

4.5 Statistical Analysis of TSS and pH Results

A brief discussion of the statistical analyses which were carried out on these experimental results will be presented here.

One-way Analysis of Variance (ANOVA):

One-way ANOVA is used to compare the means of populations that are classified in two different cultivar samples and the mean responses in an experiment were used. We also fitted one-way ANOVA models using p values for comparing the significance of the outcomes of these experiments.

The study was conducted to analyse the effect of frost damage to the orange fruit samples. The samples were treated according to material and methods provided in Chapter 3 and the results of lower and higher temperature to TSS and pH of each cultivar are displayed in Figures 4.3.1 A and B to Figure 4.3.5A and B (TSS) and Figures 4.4.1 A and B to Figure 4.4.5A and B (pH). The results of this investigation are aimed at finding the answers to the following questions: Does freeze damage to the orange fruits have an effect on the level of pH and TSS? The statistical analysis was used to compare the results obtained from orange fruit samples that were freeze treated at variable temperature and stored for several days. Significant differences to the extent of damage among the fruits within each treatment type are indicated and discussed in regards to freeze treatment compared with sound orange fruits, and storage periods with respect to temperature. The overall results shows decreased in TSS values while the pH values were increasing.

4.5.1 TSS statistical report

Based on the statistical analysis of TSS from Figure 4.3.1A (Valencia) treated at -2 °C from 2 hours to 24 hours shows no significant differences to the storage periods such as (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 3-day 5), (day 3-day 7), (day 3-day 14) and (day 5-day 7) (day 5-day 14), (day 7-day 14) with $p > 0.05$.

Valencia samples that was treated at -2 °C from 2 hours to 24 hours which showed significant differences are the those on (day 1-day 14), (day 1-day 21), (day 3-day 21), (day 5-day 21), (day 7-day 21) and (day 14-day 21). These results show a significant statistical difference with $p < 0.05$. Similarly Figure 4.3.1 B (TSS reading of Navel) treated at -2 °C from 2 hours to

24 hours shows no significant differences to some of the storage periods such as (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 3-day 5), (day 3-day 7), (day 3-day 14), (day 5-day 7) and (day 7-day 14) with $p > 0.05$.

In contrast, there was significant difference been found to the Navel samples at the same freezing regimes on (day 1-day 14), (day 1-day 21), (day 5-day 14), (day 5-day 21), (day 7-day 21) and (day 14-day 21) with $p < 0.05$.

Figure 4.3.5 A TSS tests of (Valencia) samples treated at -10 °C from 2 hours to 24 hours. That was performed statistical comparison to the effect of storage periods after freeze treatment and shows no significant differences to some of the samples on (day 1-day3), (day 1-day 5), (day 1-day 7), (day 1-day 14), (day 3-day 5), (day 3-day 7), (day 5-day 7), (day 3-day 14), (day 5-day 14) (day 7-day 14) with $p > 0.05$. But significant difference were found at the same freezing regimes on (day 1-day 21), (day 3-day 21), (day 5-day 21), (day 7-day 21) and (day 14-day 21) with $p < 0.05$.

Similarly, according to the ANOVA one way analysis in Figure 4.3.5 B (Navel), when treated at -10 °C from 2 hours to 24 hours, the TSS test shows no significant differences for some of the storage periods such as (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 1-day 14), (day 3-day 5), (day 3-day 7), (day 3-day 14), (day 5-day 7) and (day 5-day 14) with $p > 0.05$.But a significant difference was found at the same freezing regimes on (day 3-day 21), (day 5-day 21), (day 7-day 21) and (day 14-day 21) with $p < 0.05$

4.5.2 pH Statistical report

Based on that statistical analysis of pH from Figure 4.4.1 A (Valencia) treated at -2 °C from 2 hours to 24 hours shows no significant differences to the storage periods of (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 1-day 14), (day 1-day 21), (day 3-day 5), (day 3-day 7), (day 3-day 14), (day 3-day 21), (day 5-day 7), (day 5-day 14) (day 5-day 21), (day 7-day 14), (day 7-day 21) and (day 14-day 21) with $p > 0.05$.

Similarly statistical analysis of pH from Figure 4.4.1 B (Navel) treated at -2 °C from 2 hours to 24 hours shows no significant differences to all storage periods of (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 1-day 14), (day 1-day 21) (day 3-day 5), (day 3-day 7) and (day 3-day 14), (day 3-day 21), (day 5-day 7), (day 5-day 14), (day 5-day 21), (day 7-day 14), (day 7-day 21) and (day 14-day 21) with $p > 0.05$. Figure 4.4.5A pH tests of (Valencia) samples treated at -10 °C from 2 hours to 24 hours. The statistical comparison between the effect of storage periods shows no significant differences to some of the samples on (day 1-day 3), (day 1-day 5), (day 5-day 14), (day 1-day 7), (day 3-day 5), (day 3-day 14), (day 5-day 7) and (day 7-day 14) with $p > 0.05$.

However, there are significant difference to the following samples treated same freezing regimes as above on, (day 1-day 14), (day 1-day 21), (day 3-day 21), (day 5-day 21), (day 7-day 21) and (day 14-day 21) and that show a significant statistical difference with $p > 0.05$. Similarly Figure 4.4.5B pH tests of (Navel) samples treated at -10 °C from 2 hours to 24 hours. That was performed ANOVA statistical comparison between the samples shows no significant differences to some of the storage periods such as (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 1-day 14), (day 3-day 5), (day 3-day 7), (day 3-day 14), (day 5-day 7) and (day 7-day 14) with $p > 0.05$.

Moreover, there was some significant difference found to the same freezing regimes and cultivar on (day 1-day 21), (day 3-day 21), (day 5-day 14), (day 5-day 21), (day 7-day 21) and (day 14-day 21) with $p < 0.05$

4.6 Conclusion

Tests for pH and TSS have been described, and the ANOVA statistical application was used in relation to the results for the effect of freeze treatment temperatures treatment time and storage periods of orange fruit samples of the two cultivars, Valencia and Navel fruit. The TSS readings decreased when the storage time was longer and also for lower freeze-treatment temperature. On the other hand, pH values increased when the storage time was longer and also for lower freeze-treatment temperature.

Chapter Five

5.0 GC-MS and GC Tests of Orange Peel Oil

In the previous chapter results and discussion were presented in relation to pH and TSS, including tables and figures. Similarly, in this chapter GC-MS and GC Chromatographic spectra will be presented and discussed. These will enable the concentrations of volatile compounds, namely limonene and ethanol, which are of central importance to the analysis and determination of the extent of freeze damage, to be determined.

As explained previously much of this work was mainly focused on two particular compounds, namely limonene and ethanol. As a result, multiple number of orange skin oil samples were tested using GC-MS and GC, as discussed in Sections 5.1 and 5.3, respectively. The results for the most important terpenes and related compounds are provided in Section 5.2 for the GC-MS and 5.4 for the GC tests.

5.1 GC-MS test results

The type and conditions of Gas Chromatography-Mass Spectroscopy (GC-MS) used, as described in Section 3.3.2, is an analytical technique that imply two techniques that are combined to form a single method of analysing mixtures of chemicals (Wang *et al.,* 2008). While Gas chromatography separates the components of a mixture, mass spectroscopy characterizes each of the components individually. The combinations of two techniques make qualitative and quantitative analysis possible. The mass spectra also show a specific pattern corresponding to compounds which helps to identify unknown compounds from the peak. The traditional way of comparing a measured spectrum and a database is an effective identification method (Wang *et al.,* 2008) and was used in this experimental analysis.

The following GC-MS figures represent the Chromatogram of samples that were injected. Each of the peaks in the chromatogram represents the signal created when a compound elutes from the GC column into the detector. The x-axis displays retention time (RT), and the y-axis shows the intensity (abundance) of the signal. In these figures, there are several peaks labelled with their names and numbers. Each peak represents an individual compound that was separated from an orange skin oil sample that was collected from distillations of orange skin.

5.1.1 Gas Chromatography-Mass Spectrometry (GC-MS)

The following section will detail some explanation on analytical instruments (GC and GC-MS) used in the preceding analyses.

Gas chromatography-mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different compounds and matches them to a standard software library. Applications of GC-MS include food analysis, drug detection, fire investigation, environmental analysis and identification of unknown samples. GC was used to investigate the level of volatile compounds in orange citrus fruits, taking advantage of the benefit of using the equipment as discussed by Pavia 2006; Mas (2011); Hajji *et al*. (2008) and Higson (2004).

When a sample is injected onto the GC, it vaporizes and passes through the column using helium as the carrier gas. The compound comprising the mixture of interest are separated by the column stationary phase and carrier gas and then enters the ion source where compounds eluting from the column are converted to ions and separated on the basis of their mass to charge ratio (Higson, 2004).

5.2 Results and Discussion (GC-MS)

Figures 5.2.1 - 5.2.6 are results for various compounds that were identified from orange oil samples that were extracted from the orange skin. Include test sample was run of standards and identified by retention time This was done in order to understand the compound present or lost from control samples with respect to treatment temperature, time or duration of treatment and storage time, as noted. The vertical (Y) axis represents area counts and horizontal (X) axis represents retention time in minutes.

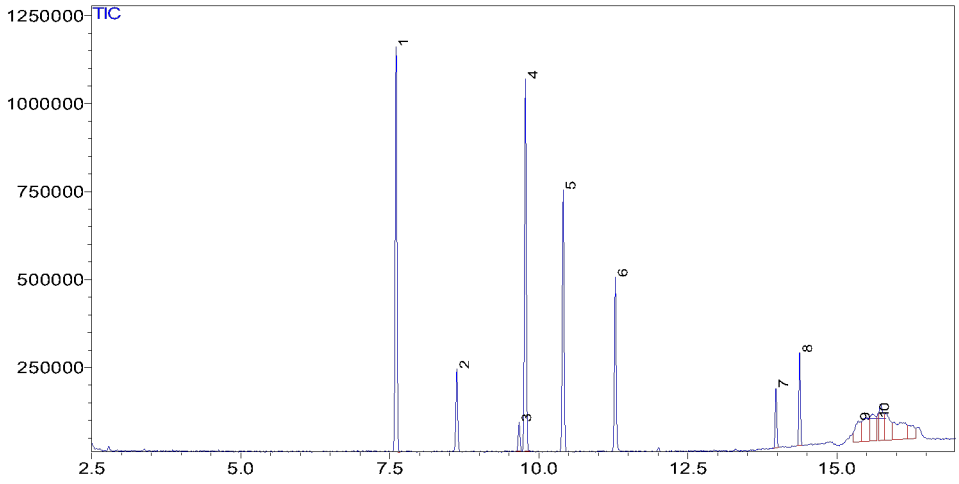

Figure 5.2.1 (GC-MS) chromatograph of the control Valencia sample

A number of volatile compounds were detected in the control Valencia sample (Figure 5.2.1) including, (β-Pinene) on No. 1, at 7.7 minutes, (α -Pinene) on No. 2 at 8.74 minutes and (α-Terpenes) on No. 3. at 9.53 minutes, (d-Limonene) on No. 4. at 9.80 minutes, (Terpenes) on

No. 5, at 10.40 minutes, (Linalool) on No. 6, at 11.35 minutes, (β-Citral) on No. 7, at 14.00 minutes and (α-Citral) on No. 8, at 14.50 minutes.

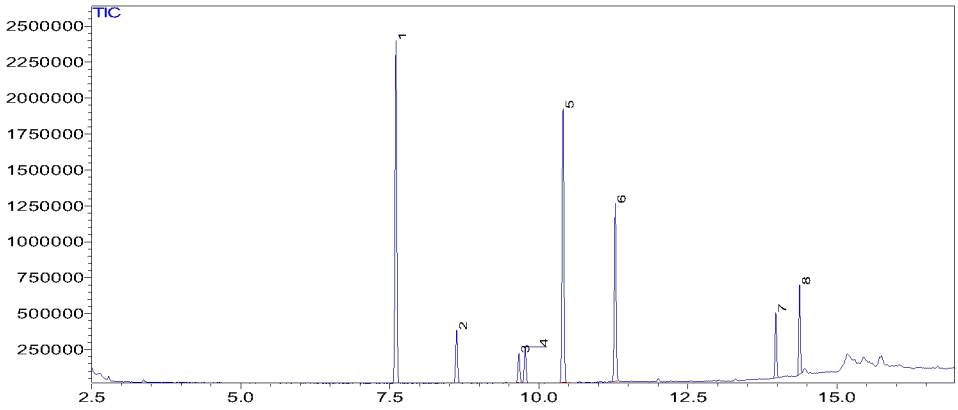

Figure 5.2.2 GC-MS chromatograph of the control Navel sample

A number of volatile compounds were detected in the control Navel sample (Figure 5.2.2) including, (α-Pinene) on No. 1, at 7.62 minutes, (β-Pinene) on No. 2 at 8.74 minutes and minutes, (d-Limonene) on No. 4 at 9.80 minutes, (Terpene) on No. 5, at 10.40 minutes, (Linalool) on No. 6, at 11.35 (β-Citral) on No. 7, at 14.00 minutes and (α-Citral) on No. 8, at 14.50 minutes.

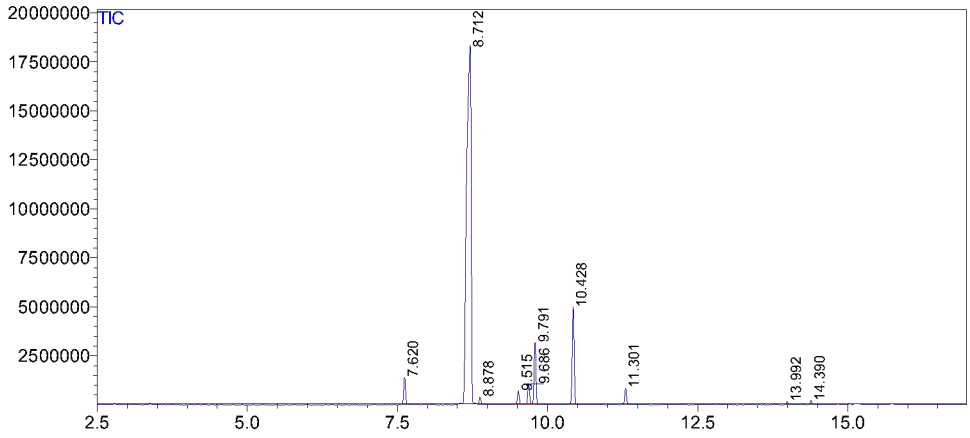

Figure 5.2.3 GC-MS chromatograph of the Valencia orange sample treated -2 °C

A number of volatile compounds were detected in the Valencia sample treated at 2°C (Figure 5.2.3) including, (α-Pinene) at 7.62 minutes, (Pinene) at 8.712 minutes and (α-Terpene) at 7.91 minutes, (Terpinene) at 10.428 minutes, (Linalool) at 11.375 minutes, (myrcene) at 13.992, (Citral) at 14.59.

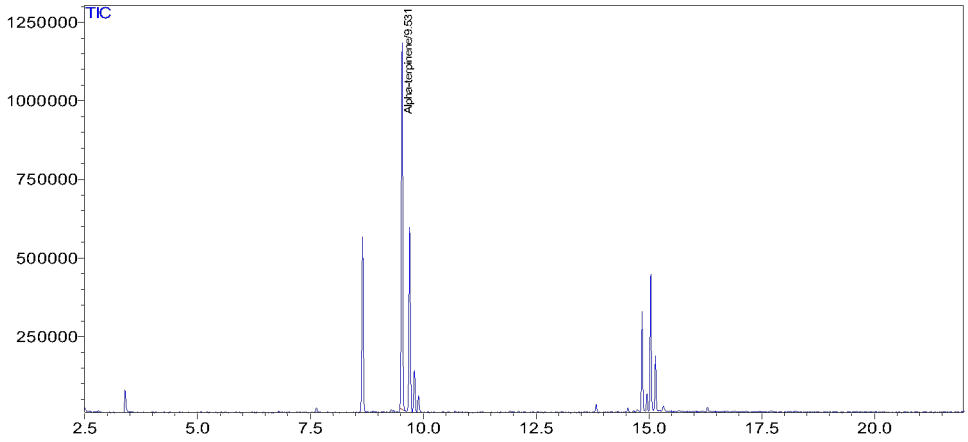

Figure 5.2.4 GC-MS chromatograph of the Navel orange sample freeze treated at -2 °C

A number of volatile compounds were detected in the Navel sample treated at 2°C (Figure 5.2.4) including, (α-Pinene) at 7.62 minutes and (α-Terpene) at 9.53 minutes, (d-Limonene) at 9.80 minutes, (Linalool) at 14.90 minutes, (Citic acid) at 15.00 minutes and (Citral) at 15.10 minutes.

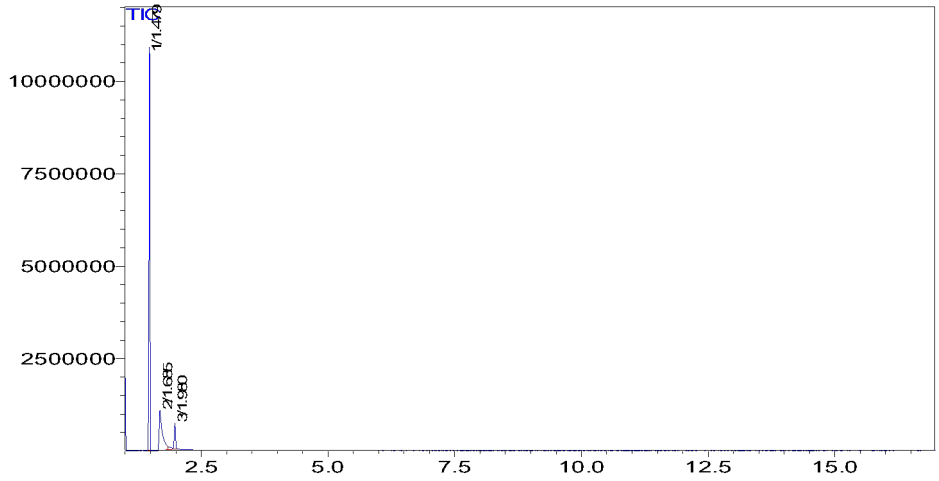

Figure 5.2.5 GC-MS chromatograph of Navel a sample that was freeze treated at -10°C for 2 hours and stored for three weeks

A number of volatile compounds were detected in the Navel sample treated at -10°C (Figure 5.2.5) including, acetaldehyde at 1.47 minutes, Ethanol at 1.685 minutes and ethyl acetate at 1.980 minutes. Most of the volatile compounds observed in the earlier samples have disappeared.

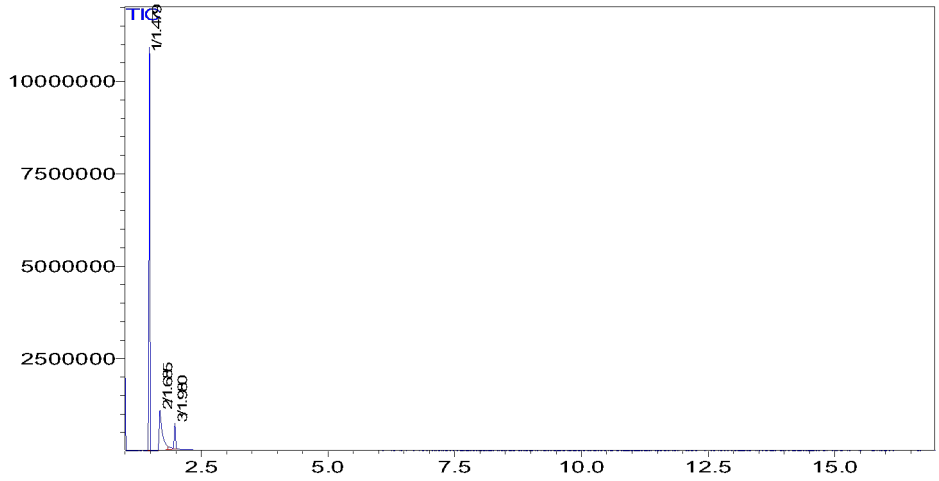

Figure 5.2.6 GC-MS) chromatograph of Valencia samples that was freeze treated at -10 °C for 24 hours and stored for three weeks

A number of volatile compounds were detected in the Valencia sample treated at -10°C (Figure 5.2.6) including, acetaldehyde at 1.47 minutes, Ethanol at 1.685 minutes and ethyl acetate at 1.980 minutes and most of the volatile compounds observed earlier have disappeared.

5.2.1 Discussion of GC-MS test

According to the GC-MS result in Figure 5.2.1 control Valencia sample was injected to see the difference and it shows the peak of (β-Pinene) on No. 1, at 8.74 minutes, α -Pinene on No. 2 at 8.74 minutes and α-Terpenes on No. 3 at 9.53 minutes, d-Limonene on No. 4 at 9.80 minutes, Terpenese on No. 5, at 10.40 minutes, Linalool on No. 6, at 11.35 β-Citral on No. 7, at 14.00 minutes and α-Citral on No. 8, at 14.50.

Compare to Figure 5.2.2 similarly Control Navel sample shows the peak of (α-Pinene) on No. 1, at 7.62 minutes, β-Pinene on No. 2 at 8.74 minutes and D-Limonene on No. 3 at 9.80 minutes, (d-Limonene) on No. 4 at 9.80 minutes, Terpine on No. 5, at 10.40 minutes, (Linalool) on No. 6, at 11.35, β-Citral on No. 7, at 14.00 minutes and α-Citral on No. 8, at 14.50 minutes.

Figure 5.2.3 (Valencia -2°C freeze treated for 24 hours samples) clearly shows the presence of some of important terpenes such as α-Pinene at 7.62 minutes, Pinene at 8.71 minutes, α-terpene at 7.91 terpene at 10.42 minutes, Linalool at 11.37 minutes (myrcene) at 13.99 minutes and Citral at 14.59 minutes. Even though most of the volatile compounds are still

present, it is clear that limonene was missing from the peak as the concentration was too low to see it with the rest of the terpenes.

Results in Figure 5.2.4 (Navel orange sample freeze treated at -2°C for 24 hours) shows the peak for α-Pinene at 3.50 minutes, Pinene at 8.80 minutes and α-Terpene at 9.53 minutes, d-Limonene at 9.80 minutes, Linalool at 14.90 minutes, Citic acid at 15.00 and Citral at 15.10 minutes.

Figure 5.2.5 Valencia samples and in Figure 5.2.6 for Navel samples that were freeze treated at -10 °C for 24 hours and stored for three weeks results shows uniquely different features from all the above figures. In Figure 5.2.5 the result indicated that the peak of acetaldehyde at 1.47 minutes on No. 1, Ethanol at 1.685 minutes and ethyl acetate at 1.980 minutes and most of the volatile compounds are disappeared. Similarly Figure 5.2.6 Navel samples shows similar to Valencia orange Figure 5.2.5.

In general, the comparison samples with different freeze treatment show different results for Fig 5.2.1 – 5.2.6 as described above.

In addition, the above experimental results of Figure 5.2.1 to 5.5.6 show different freeze treatment as well as control samples. Figure 5.2.1 and Figure 5.2.2 control sample of Valencia and Navel orange samples shows more than 7 peaks were displayed that includes Pinene and Limonene. However, ethanol wasn't detected with the samples that were not freeze treated.

Furthermore, freeze treated samples lost much of their volatiles according the temperature they are treated. In Figure 5.2.5 the result indicated that the peak of acetaldehyde at 1.47 minutes on No. 1, Ethanol at 1.685 minutes and ethyl acetate at 1.980 minutes shows the severity of damage to the fruit and the production of unwanted compound such as ethanol.

Moreover, the general understanding of the practical work from GC and GC-MS tests are using those tests as an indicator to identify freeze damage sound orange fruit by analysing the degree of volatilities as shown in Figure 5.2.1 to 5.5.6.

5.3 Gas Chromatography Test Results

The Varian Chromatographic system, as described in Section 3.3.3, was used to separate and visual extract the components of Citrus essential oils collected using stem distillation, as described in Section 3.3.4. Extracts were examined using Hexane as a mobile phase and separated using column with conditions that were described in Section 3.3.3.1.

5.3.1 Quantification methods

The internal standard method of quantification was employed for both Limonene and Ethanol respectively. The GC peak area ratios of both Limonene and Ethanol were obtained by plotting the peak area ratio for both at different concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 µg/mL. A line of best fit was drawn then the total ethanol and limonene content was determined from the slope of the graph, by calculating the ratio of total peak area in the sample, against the standard peak area in the sample. Formulas are provided for both limonene and Ethanol as (Y = 18650X - 15674 with R^2 value of 0.9884 and Y = 14007X with R^2 value of 0.9682 respectively.

The results of this study show the importance of balance in flavour composition and how freeze damage and storage can affect the quality of orange fruits. Producers can take steps to assess the specific fresh aroma active compounds lost during storage, while designing the detection methods to minimize storage off-flavours and limiting off-flavour compounds through fortification. Finally, orange fruit that was noticed to determine possible off-flavour have been observed with the aroma compounds such as ethanol, acetaldehyde and ethyl acetate were detected in the studies. Furthermore, all the compounds appeared with different retention times according to their atomic weight.

5.4 GC Spectral Test Result

Figures 5.4.1 - 5.4.6 are GC Chromatographic peak of Valencia and Navel orange samples. The vertical (Y) axis represents area counts and horizontal (X) axis represents retention time.

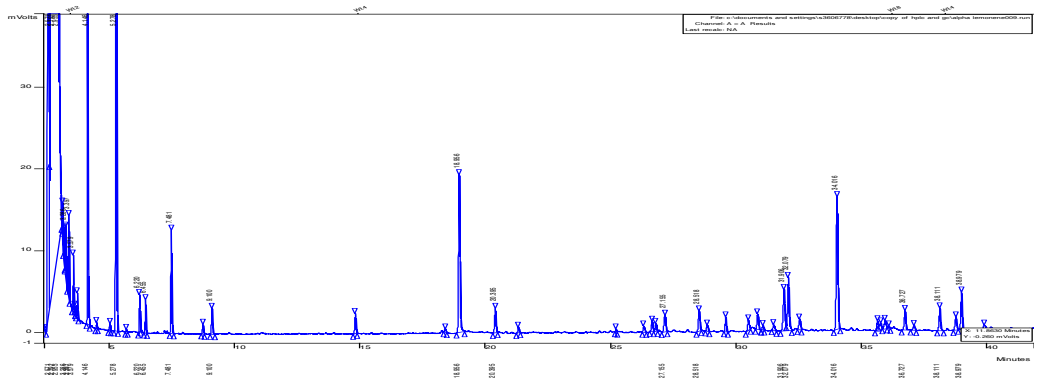

Figure 5.4.1 Gas Chromatography (GC) of control Valencia sample

A number of volatile compounds were detected in the Valencia control sample (Figure 5.4.1) including, (*Dichloromethane*) at 3.45 minutes, (Hexanol) at 2.79 minutes, (β-Pinene) at 4.15 minutes, (α-Pinene) at 5.28 minutes, (Trpinene) at 6.22 minutes, (d-Limonene) at 7.48 minutes, (Terpenese) at 9.10 minutes, (β-Myrcene) at 18.96 minutes, (β-Citral) at 20.40 minutes, (α-Citral) at 27.16 minutes, (Linalool) at 28.52 minutes, (Nonanal) at 31.91 minutes,

(Geranial) at 32.08 minutes, (4-terpineol) at 34.02 minutes, (Santalol) at 36.73 minutes, (β-Santalol) at 38.11 minutes, and (α-Santalol) at 38.98 minutes.

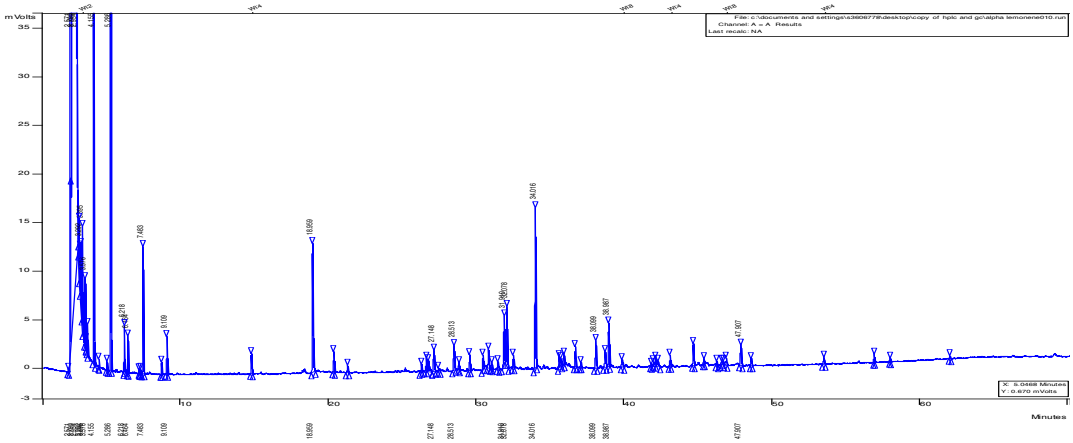

Figure 5.4.2 Gas Chromatography (GC) of control Navel sample

A number of volatile compounds were detected in the Navel control sample (Figure 5.4.2) including, (methanol) at 2.57 minutes, (*Dichloromethane*) at 2.79 minutes, (β-Pinene) at 4.15 minutes, (α-pinene) at 5.28 minutes, (Trpinene) at 6.22 minutes, (D-Limonene) at 7.48 minutes, (Terpenese) at 9.11 minutes, (β-Myrcene) at 18.96 minutes, (β-Citral) at 27.15 minutes, (Linalool) at 28.52 minutes, (Nonanal) at 31.91 minutes, (Geranial) at 32.08 minutes, (4-terpineol) at 34.02 minutes, (Santalol) at 38.10 minutes, (Santalol) at 38.99 minutes, (Santalol) at 47.91 minutes

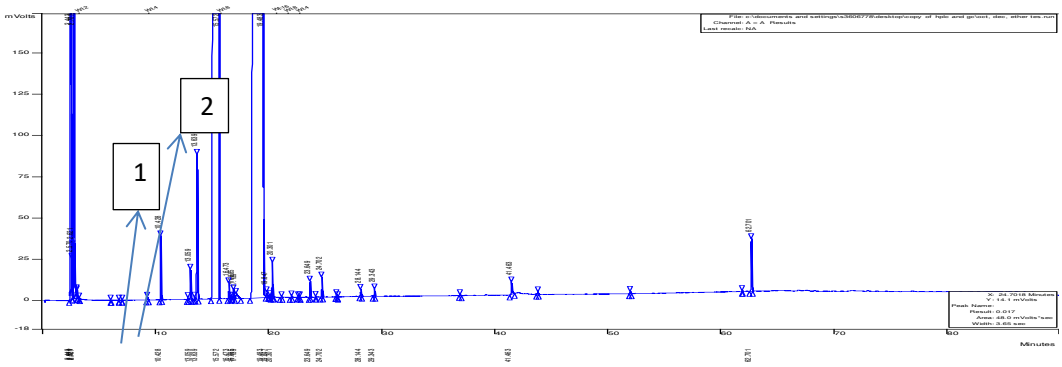

Figure 5.4.3 Gas Chromatography (GC) of Valencia sample that was -2 °C for 2 hours freeze treated and stored for 2 weeks

A number of volatile compounds were detected in the Valencia sample that was freeze treated at -2°C for two hours (Figure 5.4.3) including, (*Acetaldehyde)* at 2.45 minutes, (Ethanol) at 2.71 minutes, (Dichloromethane) at 3.45 minutes, (Ethyl acetate) on No 1 at 7.40 (Limonene) on No 2 at 7.48 minutes, minutes, and (β-Bisabolol) at 29.34 minutes.

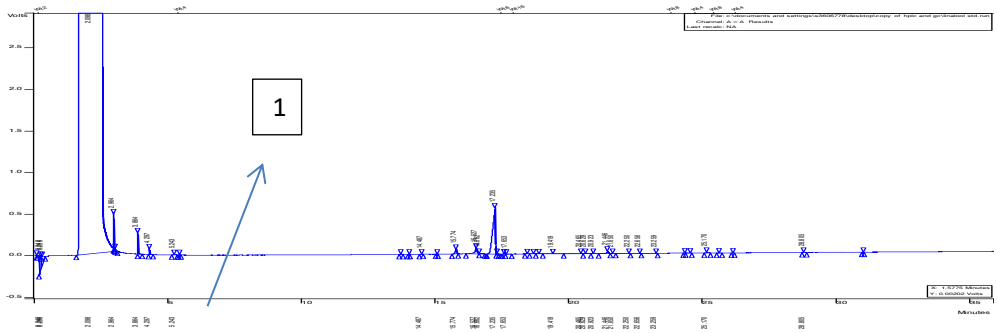

Figure 5.4.4 Gas Chromatography (GC) of Navel sample that was -2 °C for 2 hours freeze treated and stored for 2 weeks

A number of volatile compounds were detected in the Navel sample that was freeze treated at -2°C for two hours (Figure 5.4.4) including, (*Acetaldehyde)* at 2.45 minutes, (Ethanol) at 2.71 minutes, (*Dichloromethane*) at 3.45 minutes, (Ethyl acetate) at 7.40 minutes and (Limonene) at No 1 at 7.48 minutes.

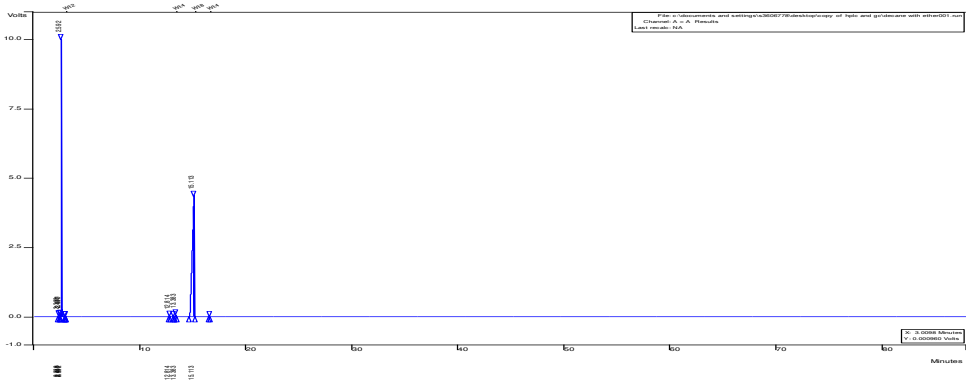

Figure 5.4.5 Gas Chromatography (GC) of Valencia sample that was -10 °C for 24 hours freeze treated and stored for 3 weeks

A number of volatile compounds were detected in the Valencia sample that was freeze treated at -10°C for 24 hours (Figure 5.4.5) including, (*Acetaldehyde)* at 2.31 minutes, (Ethanol) at 2.71 minutes, (*Dichloromethane*) at 3.45 minutes, and most of terpenes are disappeared and at the same time other impurity have been noticed at 12.81 minutes, 13.38 minutes and 15.11 minutes.

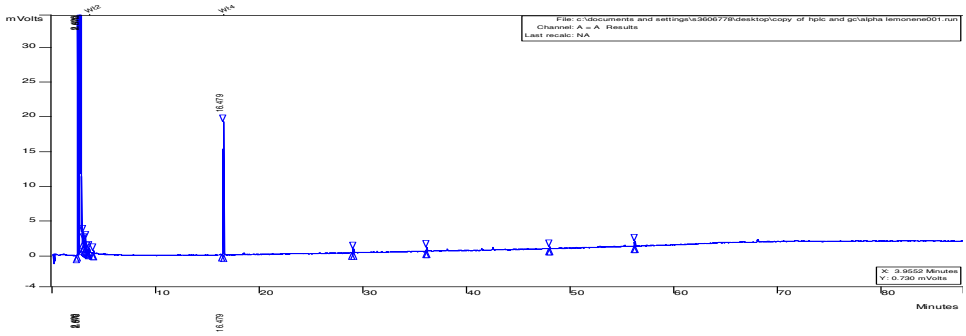

Figure 5.4.6 Gas Chromatography (GC) of Navel sample that was -10 °C for 24 hours freeze treated and stored for 3 weeks

A number of volatile compounds were detected in the Navel sample that was freeze treated at -10°C for 24 hours (Figure 5.4.6 including, (*Acetaldehyde)* at 2.31 minutes, (Ethanol) at 2.71 minutes, (*Dichloromethane*) at 3.45 minutes, and most of terpenes are disappeared and at the same time other impurity have been noticed at 12.81 minutes, 13.38 minutes and 15.11 minutes.

5.5 Discussion of GC Spectral Tests

In the present study the GC method has been applied to the analysis of citrus essential oils and the results are presented in Figure 5.4.1 - 5.4.6 with analyses that were focused on main compounds of limonene and ethanol as well as the presence and disappearance of volatile compounds including some flavour compounds. Further figures of limonene and ethanol test results and discussions are presented in section 5.7 and 5.8.

The results in Figure 5.4.1 clearly explains that the control Valencia sample contain most of the volatile the gives citrus fruit its important flavour that is needed, Figure 5.4.1 shows the results from Gas Chromatography (GC), Chromatographic peak of (*Dichloromethane*) at 3.45 minutes, whereas (β-Pinene) at 4.15 minutes, (α-pinene) at 5.28 minutes, (Trpinene) at 6.22 minutes and (D-Limonene) at 7.48 minutes, followed by (Terpenese) at 9.10 minutes, (β-Myrcene) at 18.96 minutes, (β-Citral) at 20.40 minutes, (α-Citral) at 27.16 minutes, also important terpenes such as (Linalool) at 28.52 minutes, additionally others compounds including (Nonanal) at 31.91 minutes, (Geranial) at 32.08 minutes, (4-terpineol) at 34.02 minutes, (Santalol) at 36.73 minutes, (β-Santalol) at 38.11 minutes, and (α-Santalol) at 38.98 minutes have been detected.

Similarly Figure 5.4.2 represents the control Navel sample that shows results from Gas Chromatography (GC), Chromatographic peak of (methanol) at 2.571 minutes, *(Dichloromethane)* at 2.79 minutes. Furthermore, in this result (β-Pinene) at 4.14 minutes, (α-pinene) at 5.27 minutes, (Trpinene) at 6.21 minutes, (D-Limonene) at 7.48 minutes, were detected. Also (Terpenese) at 9.10 minutes, (β-Myrcene) at 18.95 minutes, additionally (β-

Citral) at 27.148 minutes and (Linalool) at 28.513 minutes, followed by (Nonanal) at 31.91 minutes, (Geranial) at 32.07 minutes, (4-terpineol) at 34.01 minutes, (Santalol) at 38.099 minutes, (Santalol) at 38.987 minutes and (Santalol) at 47.90 minutes.

Furthermore, the above results were compared with samples in Figure 5.4.5 Valencia sample that was -2 °C for 2 hours freeze treated and stored for 2 weeks shows results from Gas Chromatography (GC), Chromatographic peak of (*Acetaldehyde)* at 2.45 minutes, (Ethanol) at 2.71 minutes, (*Dichloromethane*) on No 1 at 3.45 minutes, (Limonene) on No 2 at 7.40 minutes, (Ethyl acetate) on No 2 at 7.40 minutes, and (*β-Bisabolol)* at 29.34 minutes.

Moreover, it can be seen that the missing of some terpenes was common among the Valencia sample in Figure 5.4.5 and Figure 5.4.4 Navel sample that was at -2 °C for 2 hours freeze treated and stored for 2 weeks shows the peak of (*Acetaldehyde)* at 2.52 minutes, (Ethanol) at 2.90 minutes, (*Dichloromethane*) at 3.45 minutes, (Limonene) on No 1 at 7.40 minutes, (Ethyl acetate) at 7.40 minutes.

More harshly treated samples such as -10 °C was also injected and the Figure 5.4.5 Valencia sample that was -10 °C for 24 hours freeze treated and stored for Three weeks shows results from Gas Chromatography (GC), Chromatographic peak of (*Acetaldehyde)* at 2.31 minutes, including (Ethanol) at 2.58 minutes was detected in this test and (*Dichloromethane*) at 3.45 minutes have been seen however at the same time most of the terpenes disappeared and other impurities have been noticed during investigations.

Similarly the sample in Figure 5.4.5 has lost most of its volatilities during storage after freeze treatment and the result shows Figure 5.4.6 Navel sample that was -10 °C for 24 hours freeze treated and stored for three weeks shows results from Gas Chromatography (GC), Chromatographic peak of (*Acetaldehyde)* at 2.20 minutes and in indication to damage to the sample again (Ethanol) was detected at 2.67 minutes, (*Dichloromethane*) at 3.45 minutes, and most of terpenes have disappeared and at the same time other impurity have been also noticed similar to Valencia samples at 12.81 minutes, 13.38 minutes and 15.11 minutes.

It was understandable to know when different chromatographic profiles were observed for the compounds that were extracted to the samples that were prepared with different freeze treatment and storage time shave shown extensively differences in relation to the concentration levels, the presence and the missing of volatile compounds from the sample. For this reason, the investigation illustrates the importance of those volatile compounds to identify the wellbeing of the orange fruit or the extent of damage already caused from frost damage. This points the usefulness of quality assessment by relying on chemical methods rather than visual or density inspection.

5.5.1 Conclusion

Tests for GC and GC-MS have been described. The effect of freeze treatment, temperatures treatment time and storage periods of orange fruit samples of the two cultivars mainly Valencia and Navel fruits were tested and the spectra are presented above. More picks were discovered for the samples that were treated with higher and control temperatures and lower storage time however, when the storage time was longer and also for lower freeze-treatment temperature the results were totally opposite.

5.6 Analysis of Limonene and Ethanol Test (Orange Fruit Skin Oil)

The following two tests (Sections 5.7 and 5.8) were concerned with the volatile compounds, limonene and ethanol, which are of central importance to the analysis and determination of the extent of freeze damage. These tests relate to the orange oil samples that were collected from samples of orange skin that were subsequently analyzed using GC and GC-MS equipment. In this empirical work, simulated laboratory freezing equipment was used to subject the fruits to various temperature regimes as described in Section 3.1.3. The purpose of this analysis was to gauge the loss or build-up of indicator compounds, which is done in order to help to identify and sort damaged fruits from sound fruits. This approach, using chemical identification tests rather than using simple physical and weight/density assessments, has been described in detail in Chapter 1.

The recorded values noted the concentration levels of the chemical constituents, including the gradual loss of limonene (a flavour constituent) and the parallel buildup of ethanol (a defect indicator). The cause of the above concentration changes indicates severe effects on the freeze damaged fruit arise because of internal damage caused by both freezing and the length of the storage period. From the results, it was clearly obvious that the longer the freeze affected samples were stored, the more was the damage observed. In terms of this analysis, it is thought that even though the GC and GC-MS chromatography results noted in the next section interpret some of the important chromatogram peaks as commonly occurring terpenes, it is important to provide additional discussions to clarify this complex area.

5.7 Limonene Test

The chemicals present in the oil peel samples were explored, and as a result changes were observed in some of the terpene levels which showed reductions during this investigation of the effect of freeze treatment. In particular, limonene was given high attention due to its general availability and dominance in citrus fruits. In addition, its initially high percentage meant that it could be easily detected in the citrus fruit samples, and was therefore a useful indicator of changes due to external damage. In order to further demonstrate the above claims,

the oil samples collected control samples GC results of Valencia and Navel are presented in section 5.7.1. In addition, results for samples which were freeze treated and examined over various lengths of time are presented in Figures 5.7.2A and B to 5.7.6A and B (for Valencia and Navel cultivars) which were treated at -2 °C for 2 and 24 hours, to -10 °C for 2 hours and 24 hours.

5.7.1 Limonene test results

Table 5.7.1 Limonene value of Valencia and Navel control sample influenced by up to 21 days of storage at 4 °C.

Limonene Test of Control sample	Valencia/ μL/L	Navel/ μL/L
Storage time/days	Mean±SD	Mean±SD
Day 1	0.250^{aA}±0.00	0.262^{aA}±0.00
Day 3	0.250^{aA}±0.00	0.262^{aA}±0.00
Day 5	0.248^{aA}±0.00	0.261^{aA}±0.00
Day 7	0.248^{aA}±0.00	0.261^{aA}±0.00
Day 14	0.248^{aA}±0.00	0.260^{aA}±0.00
Day 21	0.248^{aA}±0.00	0.260^{aA}±0.00

Results expressed as means ± standard deviation (n=6). Statistical analysis by means of one way ANOVA

^{abcd} Means in the same row with different lower case are significantly different ($p<0.05$)

^{ABCD} Means in the same column with different upper case are significantly different ($p<0.05$)

Statistically analysis by means of one-way ANOVA

Limonene standards were prepared using a limonene stock solution made from a 1:100 dilution with hexane. The standard solutions were prepared by serial dilutions resulting in final limonene concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 µL/L , and the corresponding peak areas measured by GC.

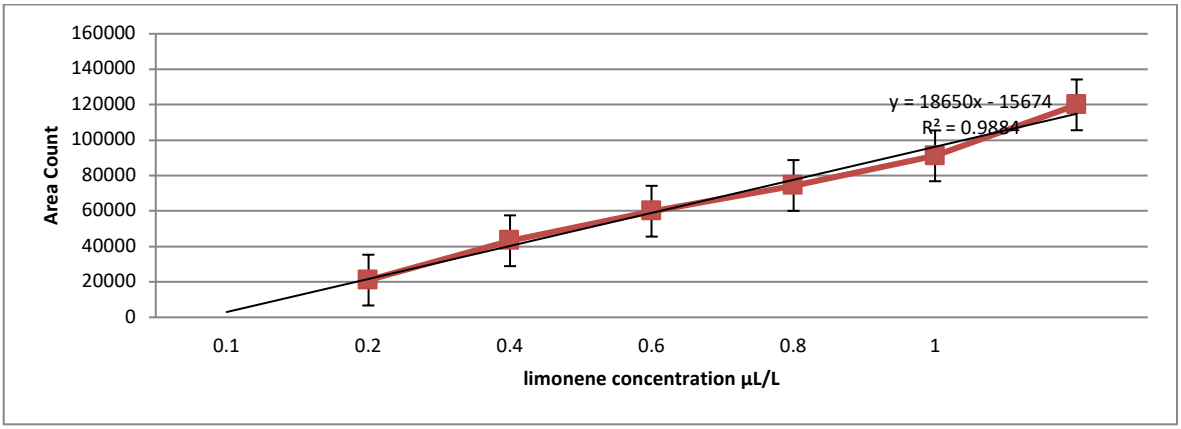

Figure 5.7.1 GC results for limonene standards at concentrations of 0.1, 0.2, 0.4, 0.8 and 1.0 µL/mL the above plot and the equation of the fitted curve have been used to find the readings of unknown limonene concentrations in this work and the error bars is represents standard error.

In Figure 5.7.2A the concentration of limonene for Valencia oranges at -2 ^{o}C on day 1 for treatment times of 2 and 4 hours is 0.151 µL/L. However, for day 1 and treatment times of 6, 8, 10 and 24 hours, the limonene value was slightly less but remained constant for these times at 0.141 µL/L. At day 21 the concentration of limonene steadily decreased from 0.10 to 0.085 for 6 and 8 hours freeze treatment times. Then a further decrease to 0.080 to 0.075 µL/L occurred for treatment times of 10 and 24 hours. In Figure 5.7.2B the concentration of limonene in Navel samples for day 1 and treatment time of 2 to 24 hours remained steady between from 0.162 to 0.16 µL/L. For day 21, there was a gradual decrease in limonene concentration from 0.12 to 0.07 µL/L for the treatment times of 2 to 24 hours.

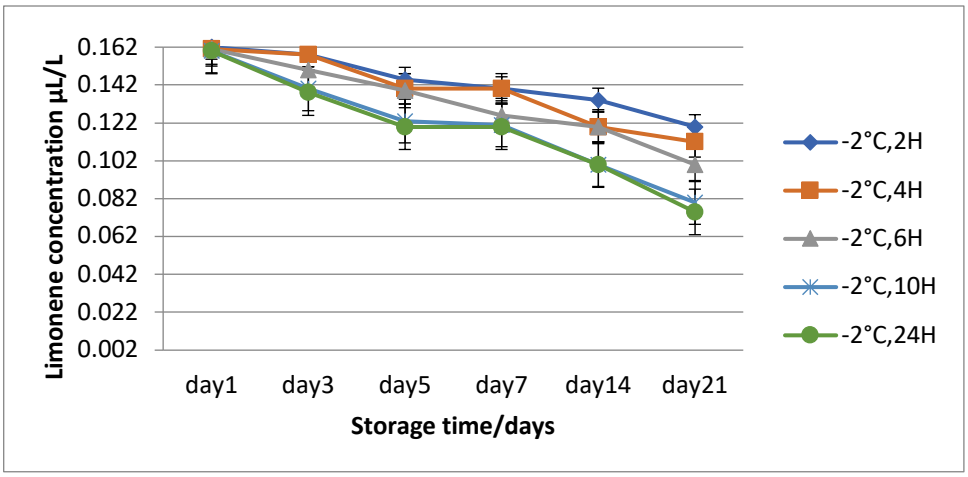

Figure 5.7.2A variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -2 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

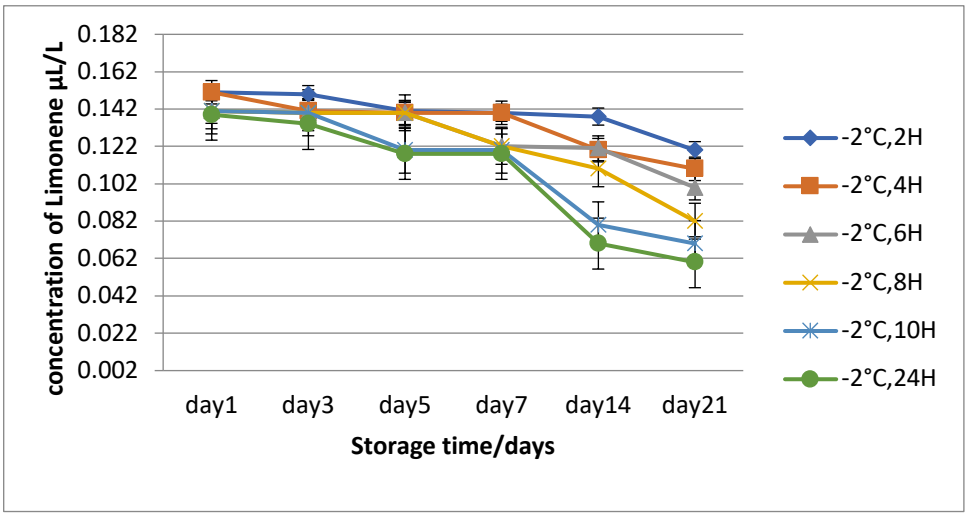

Figure 5.7.2B Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -2 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

In Figure 5.7.3A, the limonene values changed very slightly from 0.132 to 0.118 µL/L on day 1 for treatment time 2 to 24 hours in Valencia oranges. On day 21 the concentration of limonene decreased from 0.10 to 0.05 µL/L for 2 hours and 4 hours at 6 hours treatment time on day 21 then dropped from 0.04 and 0.028 µL/L for 10 hours and 24 hours. Meanwhile in Figure 5.7.3B similar results were observed in Navel oranges with the value of 0.160 µL/L on

day 1 for 2 hours treatment time and day 21 for 2 hours treatment time shows 0.111 µL/L whereas for 2 hours treatment on day 21 shows a sharp drop of from 0.1 µL/L to 0.02 µL/L to 24 hours treatment.

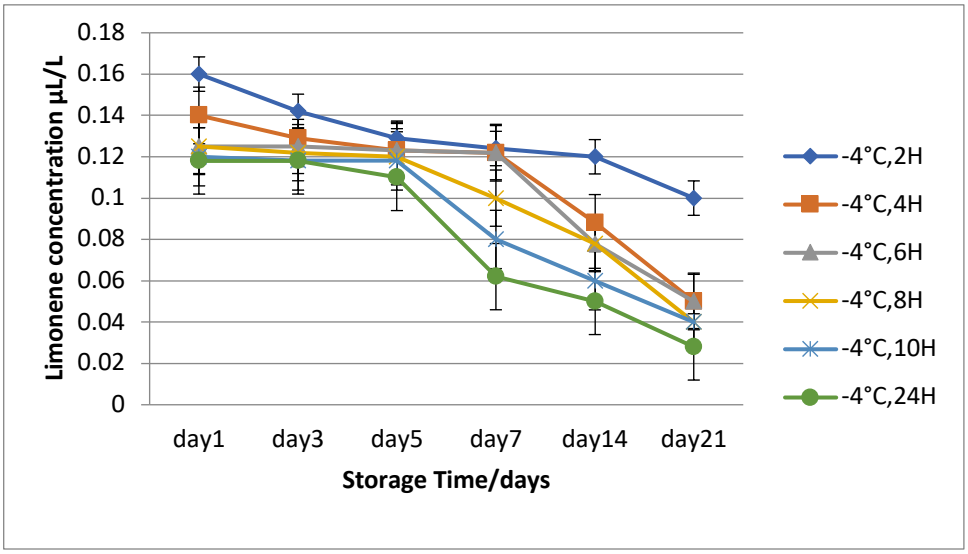

Figure 5.7.3A Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -4 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

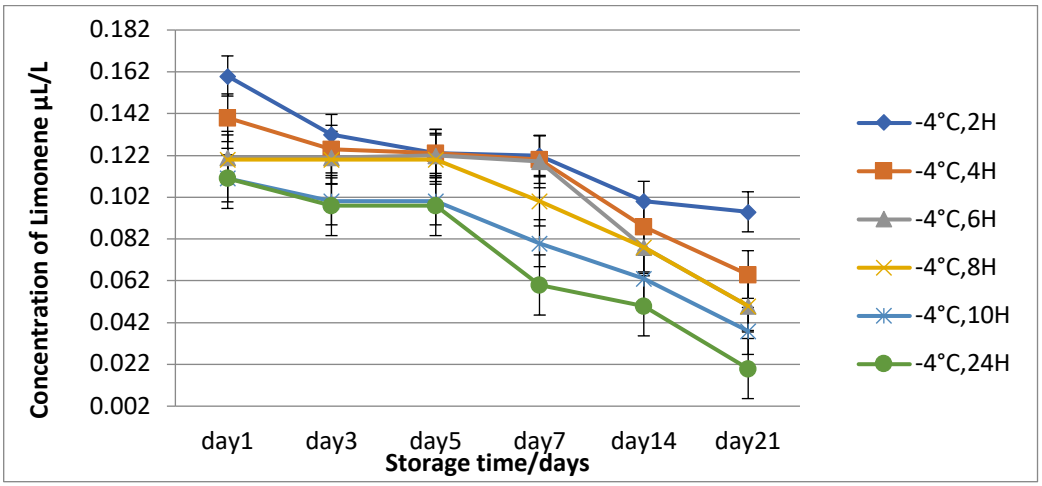

Figure 5.7.3B Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -4 °C for 2-24 hours and stored at 4 °C for up to three

weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

In Figure 5.7.4A it can be seen that for a storage time of -6 °C, the decrease in limonene concentration for Valencia samples is becoming more pronounced. For day 1, the decrease in limonene concentration is from 0.128 to 0.095 µL/L. This is a sharper decrease than was observed for -4 °C storage time. The decrease in limonene value is much greater for day 21 storage times with the value of 0.075 µL/L for 2 hours and 0.030 µL/L for 24 hours freeze treatment. In Figure 5.7.4B the Navel concentration changed from 0.12 for day 1 and treatment time of 2 hours 0.055 µL/L for 24 hours freeze treatment. Considering day 21, the change in limonene concentration decreased on day 21 with the reading dropping from 0.055 to 0.02 µL/L for 2 hours to 24 hours treatment.

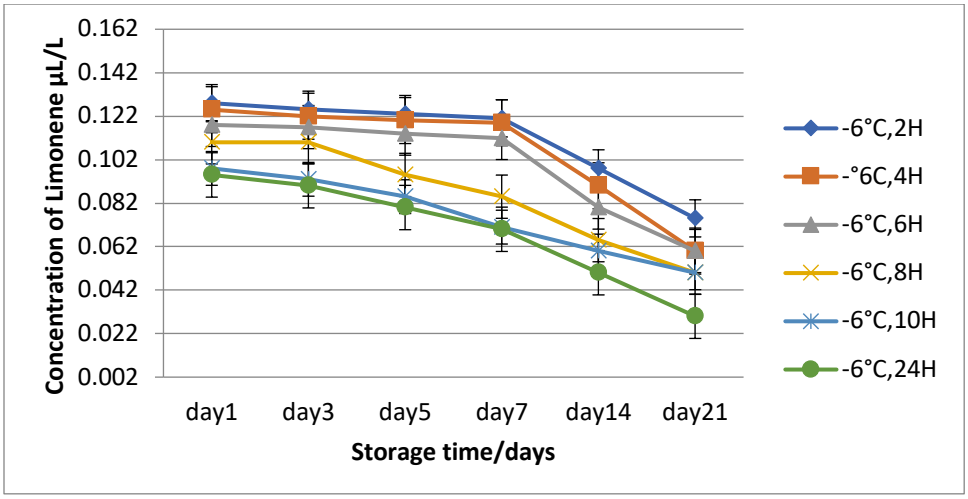

Figure 5.7.4A Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -6 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

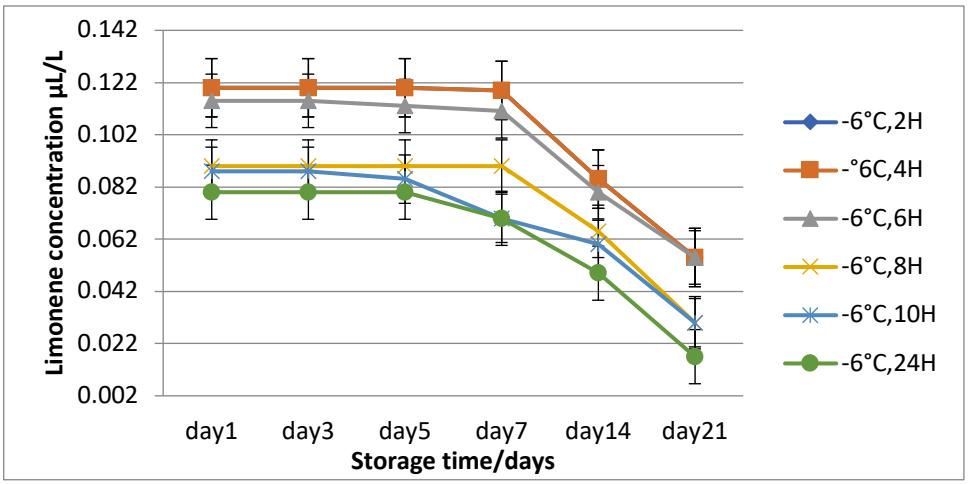

Figure 5.7.4B Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -6 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

Figure 5.7.5A illustrates results for a treatment temperature of -8 °C, where the decrease in limonene concentration for Valencia samples is becoming obvious. For day 1, the decrease in limonene concentration for 2 hours and 24 hours were from 0.128 to 0.095 µL/L. This shows more decrease than was observed for -6 °C storage time. The decrease in limonene value is more for day 21 at all storage times when compared to 2 hours treatment times. The concentration was down from 0.075 to 0.03 µL/L for 24 hours freeze treatment.

In Figure 5.7.5B the limonene concentration for Navel oranges changed from 0.120 for day 1 and treatment time of 2 hours and 0.08 µL/L for 24 hours freeze treatment. Considering day 21, the change in limonene concentration for 2 hours treatment time was 0.055 which decreased to 0.02 µL/L for 24 hours freeze treatment.

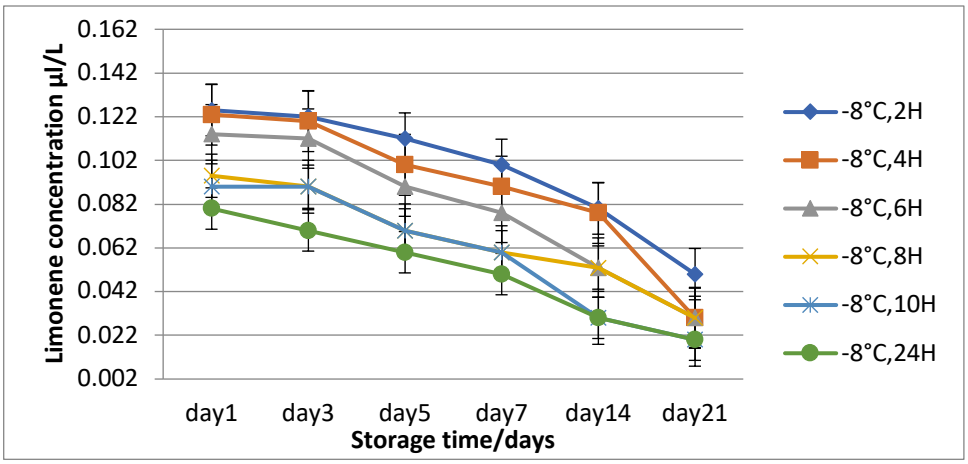

Figure 5.7.5A Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -8 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

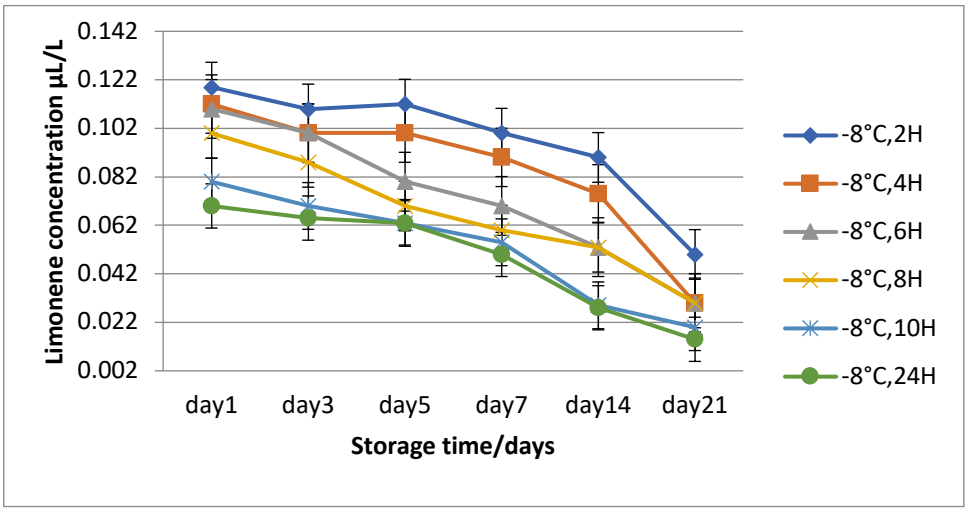

Figure5.7.5B Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -8 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

In Figure 5.7.6A, the decrease in limonene concentration is much greater over the treatment times 2 to 24 hours. For day 1 the change in concentration is from 0.125 to 0.08 µL/L. This is not as marked as the changes for day 21. It can be seen that on day 21 for 2 hours treatment time the concentration is 0.05 µL/L and this changes to a very small value of 0.002 µL/L for

24 hours that is only just detectable. In Figure 4.5.6B, again the trend is similar for day 1 change in the concentration was 0.119 to 0.07 µL/L for 2 hours treatment times. This change is gradual but for day 21 the value for 2 hours is much less than for day 1 being 0.05 µL/L. The concentration decreased for 24 hours to an extremely small value of 0.002 µL/L and it was similar to Valencia sample in Figure 5.7.6A.

This large decrease in limonene value for the more severe temperature treatment at -10 °C and treatment time of 24 hours shows that these damaged oranges have very little or no limonene.

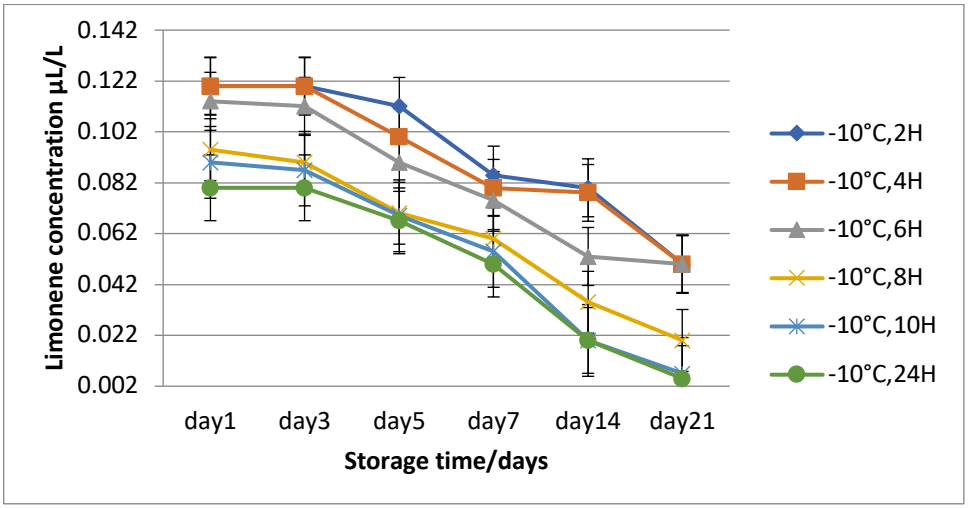

Figure 5.7.6A Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -10 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

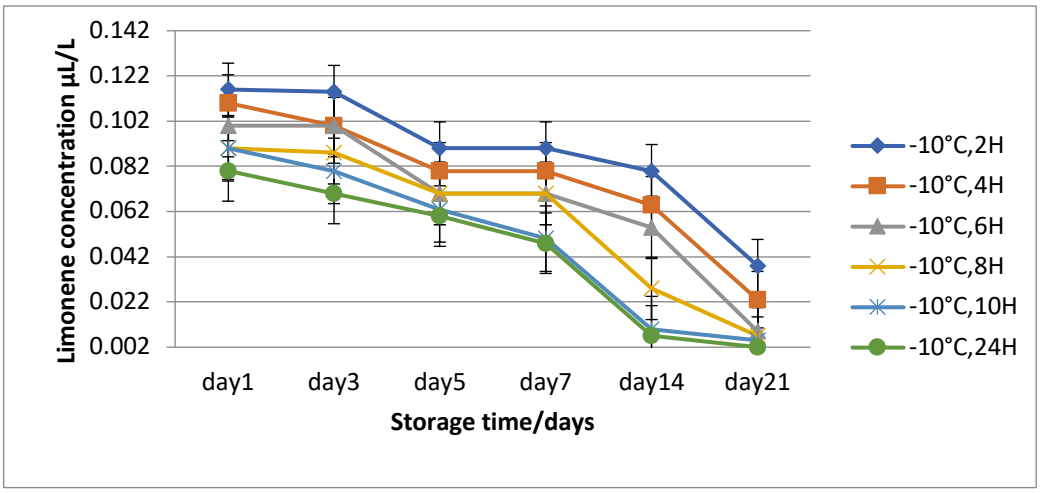

Figure 5.7.6B Variation in limonene concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -10 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

5.7.2 Discussions of limonene test

Oranges are considered to be in danger of freezing when the temperature fall below 0 °C during cold winters. It was necessary, in this study to simulate these natural freezing conditions by exposing oranges to freezing temperatures in the laboratory. Temperatures were used from −2 to −10 °C, as described in Chapter 3. Exposure of oranges to these temperatures caused visible damage to the peel surface, whilst drying of the internal fruit vesicles and peel injury was also discovered for most cases. Freeze damage caused intracellular ice formation and internal drying of the orange juice vesicles. According to Kader *et al.* (1984), these symptoms are most common for freeze damage fruits, and have been noticed during visual inspection of the fruits that were tested in this investigation.

Limonene investigations were carried out using oil collected from the distillation process of orange skins, as described in Section 3.3.5. The samples were freeze treated as described in section 3.1.3, and analysed using GC and GC-MS. During the investigation, it was found that there was a continual emission of limonene during the freeze injury and storage periods, and these results are in line with the experiments conducted by David *et al.* (2003), who also noted loss of volatile compounds in damaged fruit, which was largely due to increased vapour emissions.

Figure 5.7.2A shows the difference in concentration for limonene values in Valencia samples of 0.162 – 0.120 µL/L for samples held at -2 °C for 2 hours and 0.160 – 0.075 µL/L for samples held at -2 °C for 24 hours treatment for the limonene percentages that were lost, Figure 5.7.2A indicates that 25.9% for 2 hours freeze treatments at -2 °C and freeze treatment at -10 °C show 52.8% lost. While Figure 5.7.2B Navel samples pre-freeze treated at -2 °C indicated decreases from 0.151 – 0.120 µL/L for 2 hours treatment and 0.139 – 0.060 µL/L for at for 24 hours treatment and in percentage loss, they shows a 26.1% decrease for -2°C and 67% decrease at -10 °C for 2 hours freeze treatments.

5.7.3 Statistical analysis/discussion of limonene

To test for any significant difference in the limonene concentration between Valencia and Navel orange fruit, ANOVA statistical tests were used. A t-test was also performed to check for a significant difference between different freeze treatments and 1-Three weeks storage assessments, as well the level of limonene lost during the above conditions of treatment. No significant difference was found to the fruits that were treated at higher temperature, less storage time and control samples during the test of this experiment. More than 80% of limonene lost was detected to most of -10 °C and longer storage periods more than 2 weeks. Significance as defined by a p value of less than 5% ($p < 0.05$).

Limonene concentrations were recorded during three weeks of storage after simulated freeze damage was conducted, and subsequent statistical analysis of the results from Figure 5.7.2A (Valencia) which were treated at -2 °C from 2 hours to 24 hours, shows no significant differences to some of the storage periods that are listed as follows. These periods included: (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 3-day 5), (day 3-day 7), (day 5-day 7), (day 5-day 14) and (day 7-day 14) with $p > 0.05$.

However, there was slight difference noticed at the same freezing regimes on (day 1-day 14), (day 1-day 21), (day 3-day 14), (day 3-day 21), (day 3-day 21), (day 7-day 21) and (day 14-day 21) with mean difference of 0.030 µL/L. These results show a significant statistical difference with $p < 0.05$. Similarly statistical analysis of limonene from Figure 5.7.2B Navel) treated at -2 °C from 2 hours to 24 hours shows no significant differences to bellow storage periods such as (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 3-day 5), (day 3-day 7) and (day 5-day 7), (day 5-day 14), (day 7-day 14) with $p > 0.05$.

However, there was slight significant difference noticed at the same freezing regimes on (day 1-day 14), (day 1-day 21), (day 3-day 14), (day 3-day 21), (day 5-day 21), (day 7-day 21), (day 14-day 21). The above results show a significant statistical difference with $p < 0.05$

Figure 5.7.6A shows limonene tests of (Valencia) samples treated at -10 °C from 2 hours to 24 hours. That was performed ANOVA statistical comparison between the effect of storage periods and at much lower temperature of -10 °C shows no significant differences to some of the storage periods such as (day 1-day 3), (day 1-day 5), (day 1-day 14), (day 3-day 5) and (day 5-day 7) with $p > 0.05$.

But there are some significant difference to the same freezing regimes as above on (day 1-day 21), (day 3-day 7) (day 3-day 14), (day 3-day 21), (day 5-day 21), (day 5-day 14) (day 7-day 14), (day 5-day 21), (day 7-day 21) and (day 14-day 21), and that show a significant statistical difference with $p < 0.05$ with $p < 0.05$

Similarly Figure 5.7.6B limonene tests of (Navel) samples treated at -10 °C from 2 hours to 24 hours. The ANOVA statistical comparison between the effect of storage periods and at much lower temperature of -10 °C shows no significant differences to some of the storage periods such as (day 1-day 3), (day 1-day 5), (day 1-day 7), (day 3-day 5), (day 3-day 7), (day 3-day 5), (day 5-day 7) and (day 5-day 14) with $p > 0.05$.

However, there was some significant difference found to the same freezing regimes on (day 1-day 14), (day 1-day 21), (day 3-day 14), (day 3-day 21), (day 5-day 21), (day 1-day 21), (day 7-day 14), (day 7-day 21) and (day 14-day 21), (day 3-day 21), (day 5-day 21), (day 7-day 21) and (day 14-day 21) and that show a significant statistical difference with $p < 0.05$.**5.8**

5.8 Ethanol Test of Valencia and Navel extracted from (Orange Fruit Skin Oil)

Ethanol standards were prepared using ethanol stock solution with a 1:100 dilution with H_2O. The remaining solutions were prepared by serial dilutions resulting in final ethanol concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 µl/L.

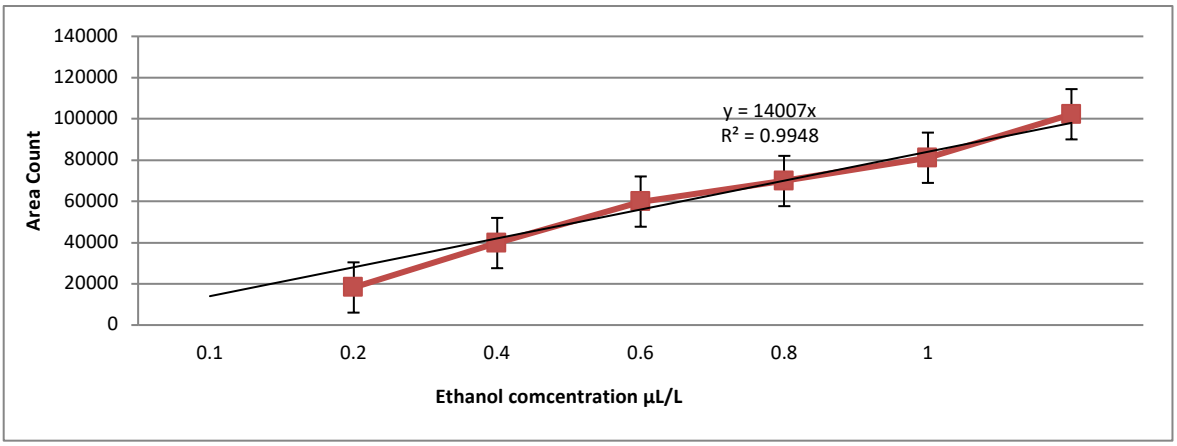

Figure 5.8.1 Ethanol standards at the concentrations level of 0.1, 0.2, 0.4, 0.8 and 1.0 µL/L. The above plot and the equation of that curve have been used to find the readings of unknown ethanol concentrations in this work and the error bar represents standard errors.

The results in Figure 5.8.2A & B to 5.8.6A & 5.8.2B are changes in ethanol concentration oil samples are collected by distillation and tested using GC analysis.

The ethanol tests were conducted from the oil that was collected from steam distillation of orange skin. The samples were pre-freeze treated and stored up to 21 days to investigate the level of ethanol detected due to the above conditions.

The emanation of volatile compounds from plant tissues has been shown to be influenced by extreme temperatures such as freezing. It has been found to have a particularly pronounced effect on the emanation of acetaldehyde and ethanol (Forney *et al.,* 2000). The effect of freezing of two apple cultivars were tested by Forney *et al.* (2000), and this group have detected increased emanations of ethanol and ethyl acetate from the fruit during storage, an observation which is in line with our investigative results discussed below.

In addition to making these observations, Forney's group have gone further to suggest that these two volatile compounds could be usefully employed as a qualitative sign of fruit damage, since emanations seem to arise from severe internal damage due to frost exposure (Forney *et al.,* 2000). This suggestion thus supports our attempts to quantify the relation between severity of freeze treatment and the extent of damage caused to the samples of Valencia and Navel oranges that we have tested.

Furthermore, no ethanol emission was detected from undamaged orange fruits (the skin oil) that were collected using steam distillation of orange skin. As previously noted, we have conjectured that volatile compound emissions can be used as an indicator of frost damage, and this notion was also held by Espina (1998), who suggested that hydrogen sulphide can

be used as the indicator of frost damage fruits, but their studies did not perform measurements for volatiles other than hydrogen sulphide.

Freeze damaged oranges have been analysed for ethanol production after the orange fruits were stored from 1 day to 21 days. Results show positive indications of ethanol production except during very early stages of storage periods for damaged fruits. This observation increases our confidence that ethanol can be used as an indicator for the differentiation of frost damaged oranges from sound orange fruits. GC and GC-MS tests were conducted on control samples and freeze-damaged items, using analysis methods as described in Chapter 3 (Material and Methods). Moreover, we were aware of the investigation of James (2004), who stated that "freeze damage can be detected in batches of oranges based on ethanol production of the thawed fruit", and he clearly noted that "Freshly harvested unfrozen oranges never produce headspace ethanol levels above 0.01 mL/L" (James, 2004).

The following ethanol tests were conducted from freeze treated Valencia and Navel orange skin oil in order to investigate the level of ethanol concentration in comparison to the control samples tested. Results are presented from Table 5.8.1A & B and Figure 5.8.2A & B to Figure 5.8.6A & B. Furthermore, the project is also trying to answer questions like "does frost damage initiate the production of ethanol and does it impact on the limonene level? Is there an interaction between frost damage and deterioration of the fruit?"

5.8.1 Ethanol test results

No ethanol was detected in either the Valencia or Navel oranges that were stored at 4°C for up to 21 days.

Figure 5.8.2A is results for Valencia samples that were freeze treated at -2 °C for 2 to 24 hours. On day 1, day 3 and day 5 for 2 and 24 hours treatment time, there was not any ethanol detected. However, ethanol was detected in very small concentrations on day 5 increasing to day 21. The readings were 0.1 µL/L for 2 hours and 0.2 µL/L for 24 hours on day 5. Up to day 21, there was a steady increase in ethanol production of 0.28 µL/L for 2 hours and 2.7 µL/L for 24 hours freeze treatment.

For Figure 5.8.2B, similarly for Navel oranges there was not any ethanol detected for day 1 and day 3 samples. However, on day 5 at 2 hours treatment 0.2 µL/L and 0.4 µL/L were observed. This value increased steadily for treatment times up to 24 hours; on day 21 the reading was increased to 0.33 µL/L for 2 hours treatment and 3.5 µL/L for 24 hours freeze treatment.

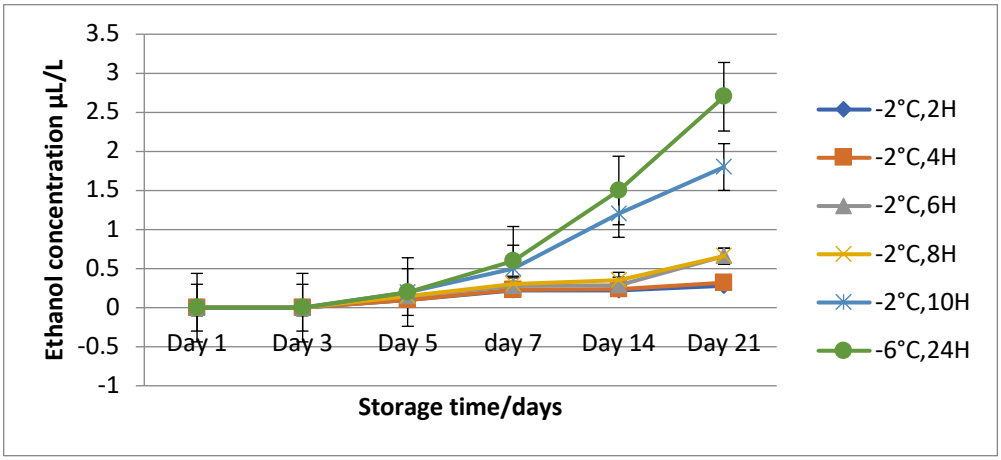

Figure 5.8.2A Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -2 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

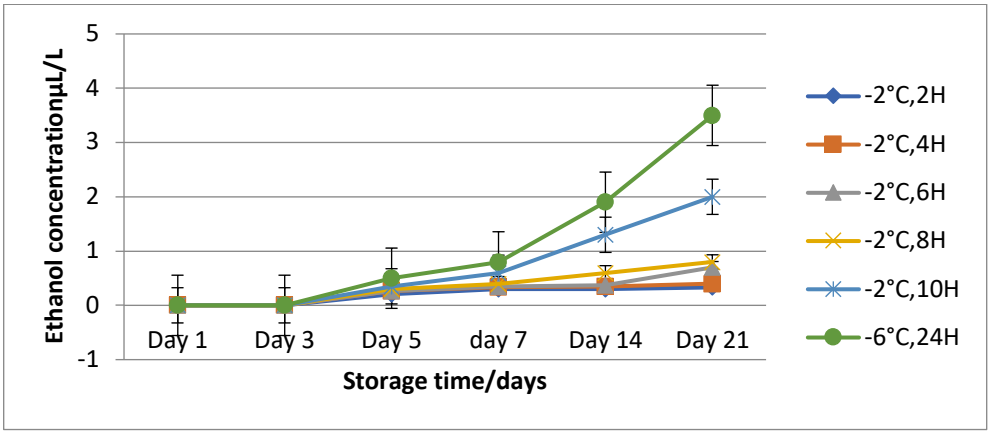

Figure 5.8.2B Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -2 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

Figure 5.8.3A, for Valencia samples treated at -4 °C show that no ethanol was detected from day 1 to day 3. On day 5, the value of ethanol concentration was increased to 0.1 µL/L for 2 hours freeze treated samples and further 0.21µL/L for 24 hours freeze treat samples. An

increase in ethanol level was observed on day 21, showing values of 0.35 µL/L for 2 hours and 0.3.5 µL/L for 24 hours.

Navel samples in Figure 5.8.3B show no ethanol was detected from day 1 to day 5 which is similar to the situation for Valencia samples. There was a small increase for 2 hours treatment on day 5 from 0.25 to 0.72 µL/L, which increased from 0.4.4 to 4.2 µL/L on day 21 for 24 hours freeze treatment.

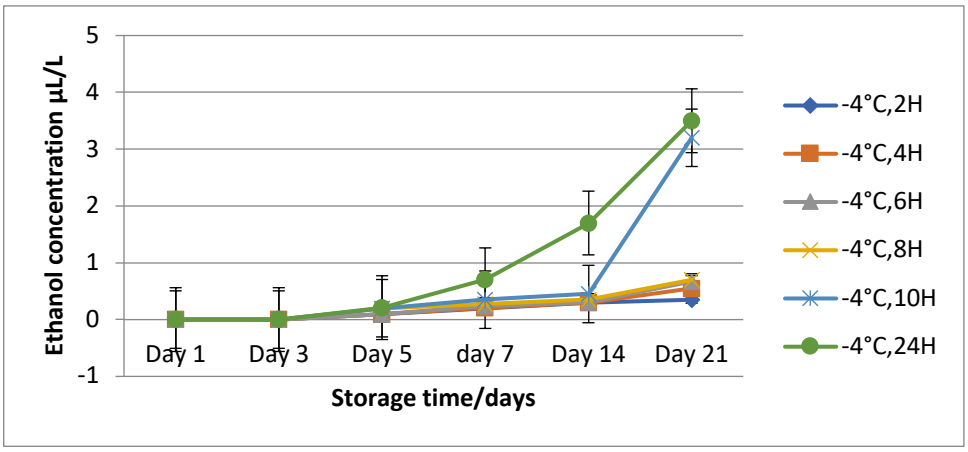

Figure 5.8.3A Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -4 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

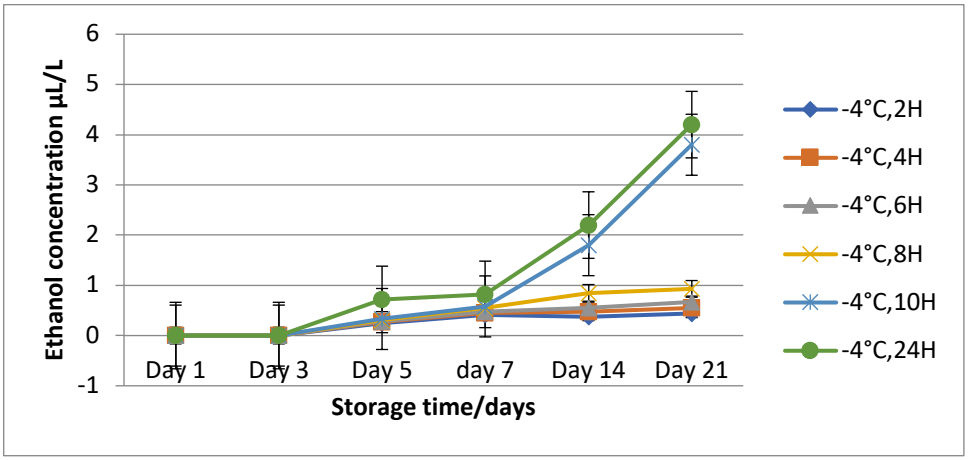

Figure 5.8.3B Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -4 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

Figure 5.8.4A which shows Valencia sample treated at -6 °C, shows that there was no ethanol present on day 1 and day 3, however there was ethanol present on day 5 for 2 hours treatment with the value of 0.1 µL/L increasing to 0.3 µL/L and it show 0.5 µL/L to 4 µL/L on day 21 for the 2 - 24 hour freeze treatment, on day 7 for 2 - 24 hours freeze treatment there was 0.2 – 0.8 µL.

For Figure 5.8.4B the variation of Navel samples sample treated at -6 °C shows no ethanol detected to day 1 and day 3 but a small increase in ethanol was detected on day 5 for all treatments. On day 5 there was 0.29 µL/L for 2 hours treated samples compared with 0.7 µL/L for the 24 hours freeze treated samples. By day 21, there was 0.5 µL/L for the 2 hours treatment time and an increase of 0.4.3 µL/L for the 24 hours treatment.

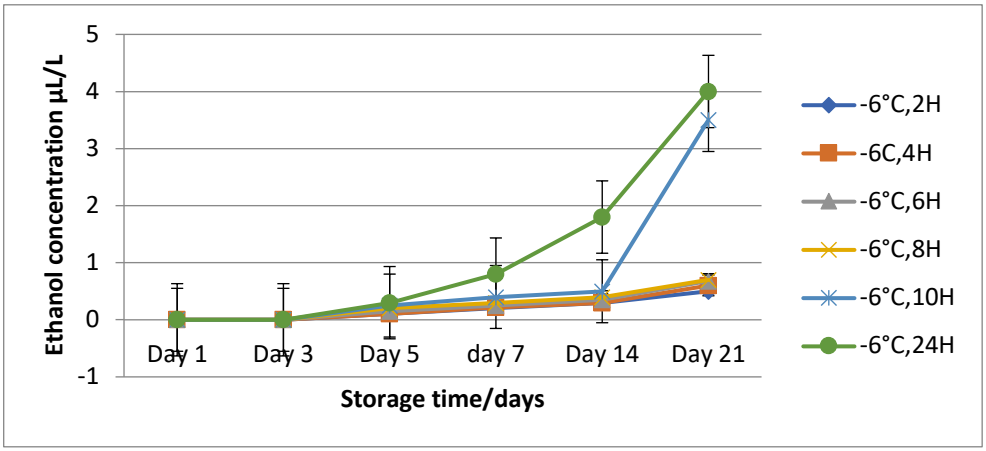

Figure 5.8.4A Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -6 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

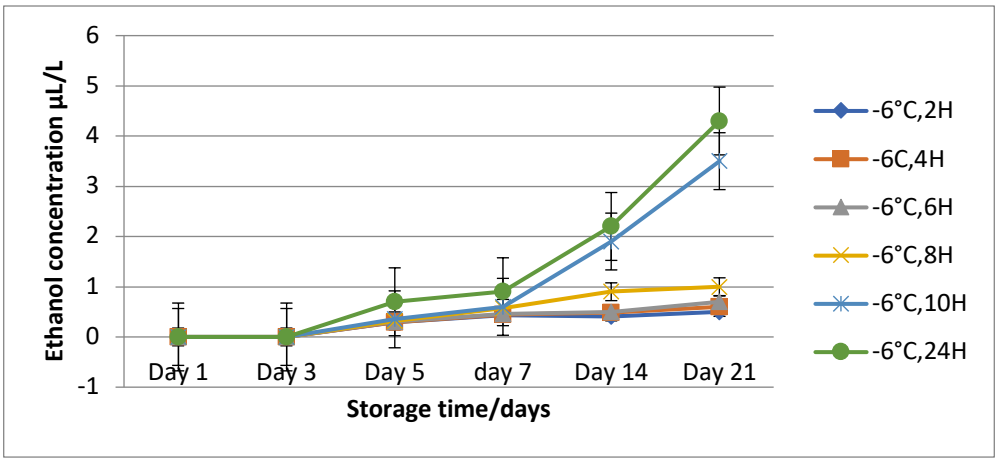

Figure 5.8.4B Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -6 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

In Figure 5.8.5A, similarly no ethanol was observed for Valencia samples treated at -8 °C from day 1 to day 3 and it can be seen that on day 5 the concentration of ethanol shows 0.2 µL/L for 2 hours treatment and 0.38 µL/L for 24 hours. This increased on day 21 for 2 hours with the value of 0.55 µL/L compare to 4.2 µL/L for day 21 storage periods and 24 hours freeze treated samples.

Figure 5.8.5B also shows for Navel samples that there was no ethanol found from day 1 to day 3. However, on day 5 the value for Navels showed 0.4 µL/L to 0.9 µL/L for 2 hour treatment times and 0.8 µL/L to 4.4 µL/L for 24 hours treatment.

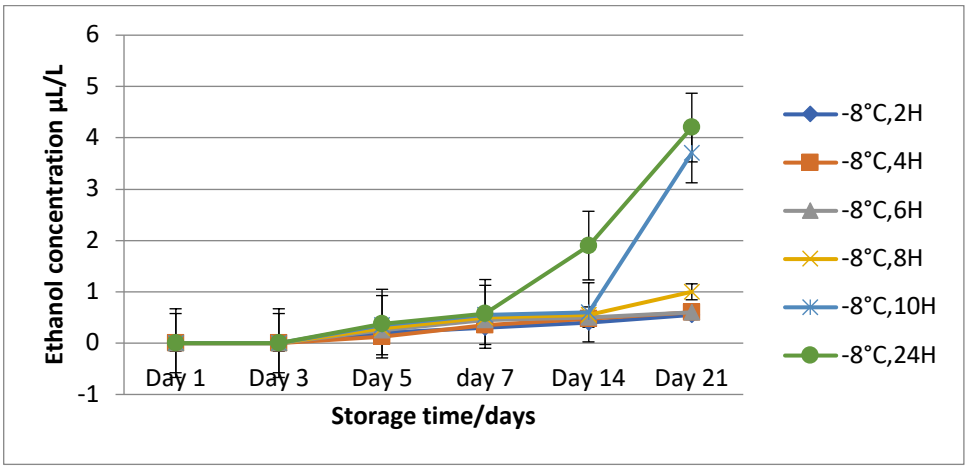

Figure 5.8.5A Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Valencia orange fruit at -8 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the

variability of data and used on the above figure to indicate the standard error in a reported measurement.

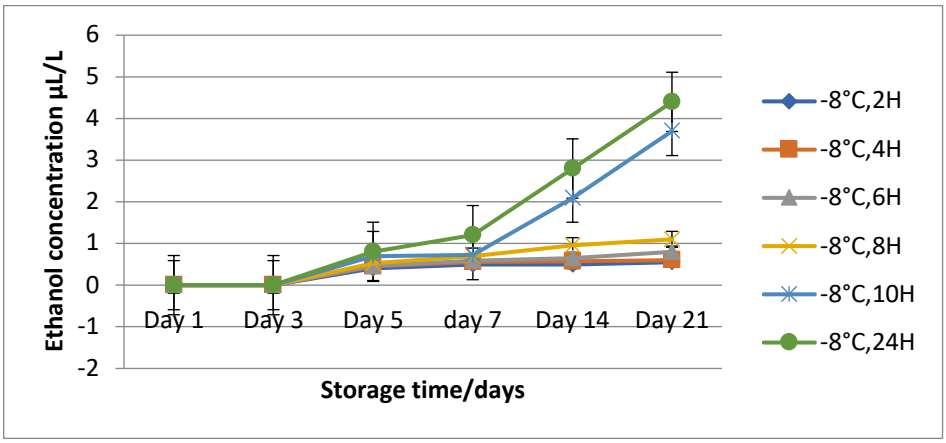

Figure 5.8.5B Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -8 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

From Figure 5.8.6A, for the Valencia sample that was treated at -10 °C, similar to the lower freeze regimes, no ethanol emanation was detected from day 1 to day 3. Again, on day 5 ethanol appeared, showing for 2 hours freeze treatment time a concentration of ethanol at -10 °C for 24 hours 0.2 µL/L and 0.4 µL/L. This had risen by 21 days to 0.67 µL/L for 2 hours treatment and 4.3 µL/L for 24 hours at -10 °C treatment. In Figure 5.8.6B, it can be seen that no ethanol was detected from day 1 to day 3 for Navel samples which was the same trend as that for Valencia. Ethanol was discovered on day 5 onward.

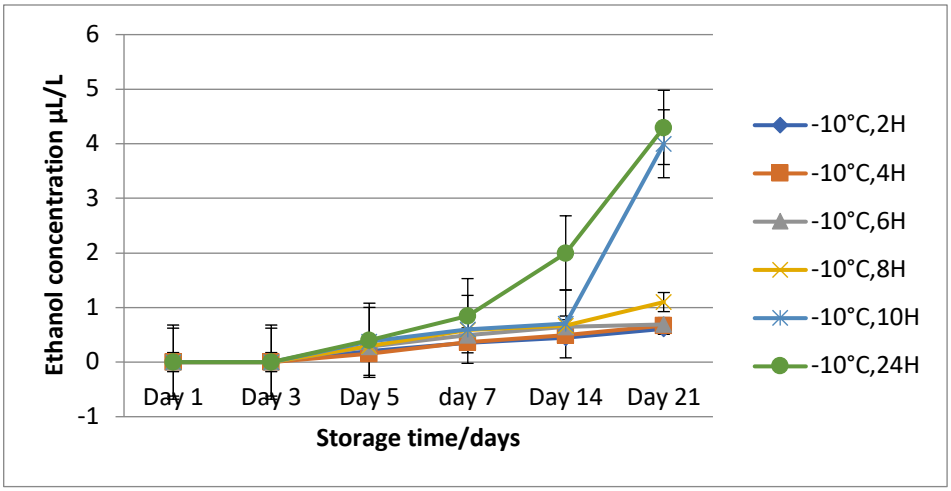

Figure 5.8.6A Variation in ethanol concentration of the oil ollected from distillation of orange skin of pre-freezed Valencia orange fruit at -10 °C for 2-24 hours and stored at 4 °C for up to

three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

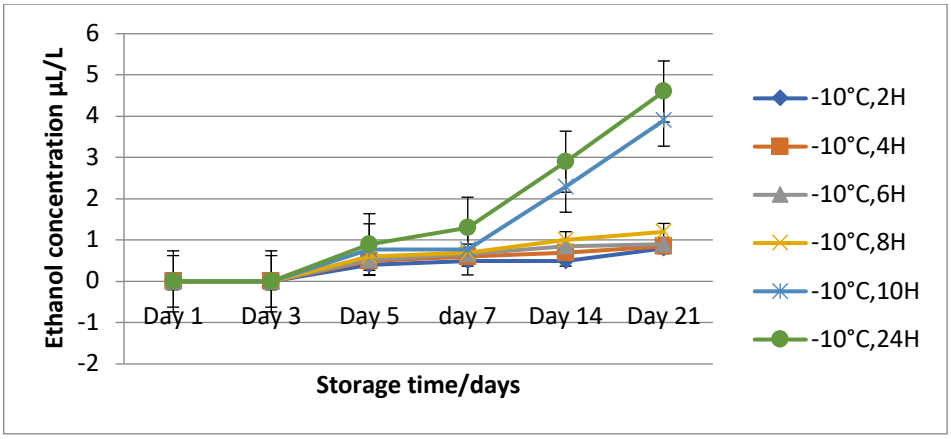

Figure 5.8.6B Variation in ethanol concentration of the oil collected from distillation of orange skin of pre-freezed Navel orange fruit at -10 °C for 2-24 hours and stored at 4 °C for up to three weeks, obtained via GC analysis. Error bars are graphical representations of the variability of data and used on the above figure to indicate the standard error in a reported measurement.

5.8.2 One way ANOVA statistical test (ethanol)

ANOVA statistical tests were used to examine if there was any significant difference in the ethanol concentration that was detected between Valencia and Navel orange fruit which had been subjected to the same external conditions. This analysis is based on the search for a significant difference in emanation of ethanol between cultivars for each freeze treatment and for 21 days storage assessments. In addition, and of central importance to this thesis, an analysis of ethanol emanations for each cultivar at different freezing regimes was carried out. This was aimed at detecting what were the critical conditions leading to significant damage of the orange fruit.

To begin this analysis, it was observed that there was no significant difference to be found between control sample cultivars for any of the storage times, since no ethanol emanation was detected. This gives confidence to the following analyses which look at differences between cultivars for various treatment regimes. In general, it is clear that the moderate temperature decreases and the smaller storage times are less likely to cause measurable damage, and thus are least likely to show an appreciable difference between cultivars. Interest will focus therefore on the more severe conditions and longer storage times. In the following discussions significance of difference is defined by a p value of less than 5% ($p < 0.05$), which means that

the difference in observed ethanol emanations are due to a real effect rather than by chance variation.

Our results show that there was a significant difference found in some of the conditions which have caused levels of freeze damage to Valencia and Navel orange fruits. This is an important finding because it will alert growers and fruit retailers to be aware of varietal difference for storage conditions. The discussion will begin with an analysis of the conditions which lead to significant freeze damage.

5.8.3 Discussion of Ethanol statistical test

As described in Chapter 3, ethanol concentrations were recorded during three weeks of storage after freeze damage was conducted. Subsequently the statistical analysis recorded in Figure 4.6.2A for Valencia treated at -2 °C from 2 hours to 24 hours shows no significant differences to the samples after storage periods of (day 1 – day 3), (day 1 – day 5), (day 3 - day 5) and (day 7 - day 14), (day 14 - day 21) with $p > 0.05$. But there was slight difference that noticed at the same freezing regimes on (day 1 - day 7), (day 1 - day 14), (day 1 - day 21), (day 3 - day 7), (day 3 - day 14), (day 3 - day 21), (day 5 - day 7), (day 5 - day 14) and (day 5 - day 21), (day 7 - day 21), and these show a significant statistical difference with $p < 0.05$.

Similarly Figure 4.6.2B (Navel) treated at -2 °C from 2 hours to 24 hours shows no significant differences to some of the storage periods such as (day 1 – day 3), (day 1 - day 5), (day 3 - day 5) and (day 7 – day 14) with $p > 0.05$. In contrast there was significant difference for some of the samples on (day 1 - day 7), (day 1 - day 14), (day 1 - day 21), (day 3 - day 7), (day 3 - day 14) and (day 3 - day 21), (day 5 - day 7), (day 5 - day 14) and (day 5 - day 21), with $p < 0.05$.

Figure 5.8.6A (Valencia) samples treated at -10 °C from 2 hours to 24 hours report ANOVA statistical comparison between the effect of storage periods and at much lower temperature of -10 °C. These show no significant differences to some of the storage periods such as (day 1 – day 3), (day 7 - day 14), with $p > 0.05$. But significant differences were found on (day 1 - day 5), (day 1 - day 7), (day 1 - day 14), (day 1 - day 21), (day 3 - day 5), (day 3 - day 7), (day 3 - day 14), (day 3 - day 21), (day 5 - day 7), (day 5 - day 14), (day 5 - day 21), (day 7 - day 21) and (day 14 - day 21) with $p < 0.05$

Similarly, according ANOVA one way analysis in Figure 5.8.6B (Navel) treated at -10 °C from 2 hours to 24 hours, the ethanol test shows no significant differences to some of the storage periods on (day 1 - day3), (day 7 - day 14) with $p > 0.05$. But significant difference was found for some of the samples on (day 1-day 5, (day 1 – day 7), (day 1 – day 14), (day 1 – day 21),

(day 3 – day 5), (day 3 – day 7), (day 3 – day 14), (day 3 – day 21), (day 5 – day 7), (day 5 – day 14) and (day 5 – day 21) (day 7 – day 21) and (day 14 – day 21), with $p < 0.05$.

Food storage is one of important methods of food preservation however many problems can be arisen due to storage conations and storage time which affects the shelf life of the fruit. Consequently, loss of quality characteristics seen during storage can markedly affect the shelf life of the fruits (Gomez and Garcia 2004). In this practical study the effect of storage of those freezes treated orange samples have been carefully evaluated and the above results indicated that the length of storage time shows more effect on the frost damage fruits. More significant differences were noticed in the fruits that were treated at much lower temperature and stored longer. Similarly, loss of volatilities and unacceptable quality characteristics were obvious in affected fruits.

The experiment that was evaluated for prediction and assessment to freeze damage by David *et al.* (2003), used fruit that was subjected to -5 or -7 °C treatments in a laboratory freezer for various periods of between 2 to 9.5 hours, then the group stored the fruits at 23 °C for 1 day, 2 days and 7 days. The emission of volatiles from the fruit was then measured. The fruits were subsequently stored at 5 °C for an additional 2-3 weeks and then evaluated for fruit quality characteristics. According to the group assessment, peel injury, drying of the juice vesicles, a decline in acidity, and a loss of flavour were observed in freeze damage oranges. Losses in fruit quality were also largely increased due to emissions of ethanol, ethyl butanoate, methyl hexanoate, and ethyl octanoate (David *et al.,* 2003).

Our findings were in line with David *et al.* (2003) in relation to physical and chemical changes that took place during freeze damage to the fruit, the emission of compounds such as ethanol, ethyl butanoate, methyl hexanoate and ethyl octanoate and that was found to be strongly enhanced by freezing and to correspond to subsequent damage to the fruit (David *et al.,* 2003).

5.9 Conclusion

The tests presented in this Chapter were for limonene and ethanol, and complement those presented in Chapter 4 for pH and TSS. Again the ANOVA statistical application was used in relation to the effect of freeze treatment temperatures (-2 °C to -10 ^{0}C), treatment time and storage periods of orange fruit samples of the two cultivars, Valencia and Navel fruit. Limonene concentrations decreased when the storage time was longer and also for lower freeze-treatment temperature. On the other hand, ethanol concentrations increased when the storage time was longer and also for lower freeze-treatment temperature.

Chapter Six

6.0 Detection of Ethanol in Oranges using Portable Equipment

6.1 Introduction

The following experimental work focused on mobile equipment that is used for the assessment of ethanol level in fruit. This is in order to understand the changes that takes place during storage period and this can be perform remotely and easily when citrus fruits are damaged due to exposure to a cold winter, particularly when the temperature falls too far, it is more likely, however, that fruit could be damaged, contaminated or be of low quality standard. As described in Section 3.5.2, a gas aspiration pump (Kitagawa AP-20) was purchased. The unit is designed to detect the ethanol that is produced and escapes from the fruit at the storage, and is mobile and robust, and can be operated by non-technical personnel, allowing a convenient method of generating a rapid estimate of cold damage.

The study was conducted in order to evaluate the use of such a small hand-held cost-effective ethanol testing device that will allow the practical detection of freeze or cold damaged oranges. This work presents an experimental investigation of levels of ethanol released from freeze-damaged orange fruits of Valencia and Navel cultivars.

Further advantage of this instrument is that other volatile compounds can also be tested by inserting a unique test tube that is designed for that particular compound. Ethanol, however, has been shown to be the most appropriate chemical to monitor the changes in oranges. This claim is based on another study, not yet in print, as well as other indirect work reported in the literature. It is noted here that similar tests for volatiles were conducted by James and David (2007), but they used a significantly different approach, and it is believed that this current work represents a novel investigation of ethanol released from freeze-damaged orange fruits (Valencia and Navel).

6.2 Methodology

This study involved the method explained in Section 3.4.1, and number of samples, treatment times and how to place the samples in the bag were also reported in Section 3.4.2. For these investigations fewer samples were tested compared with the worth presented in previous chapters; freeze temperature of -2, -6, -10°C and freeze times of 2, 8, and 24 hours were chosen for both cultivars. The procedures used for the preparation of the testing equipment were presented in section 3.4.3.

6.3 Results

The figures shown below are present ethanol levels in a test tube marked in blue for each sample that was tested with different combinations as well as tested under different conditions.

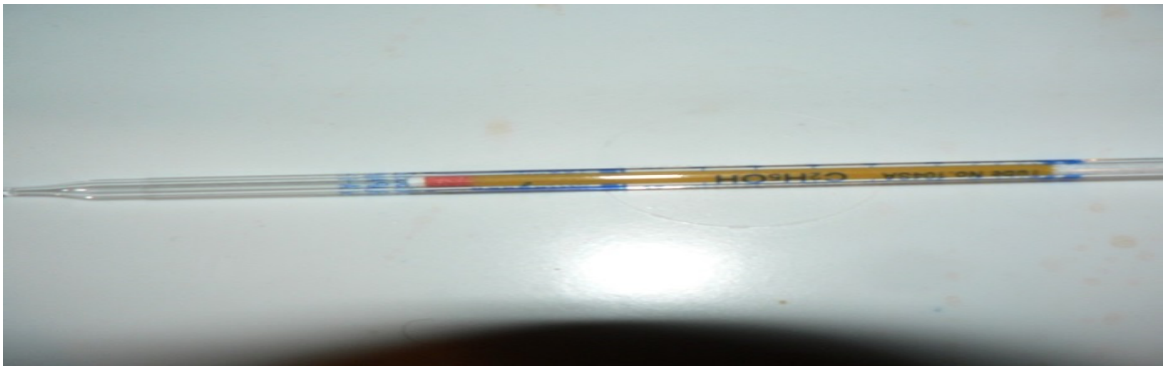

Figure 6.3.1 Ethanol testing tube stored with control Valencia sample; no ethanol was detected as shown by the clear yellow colour.

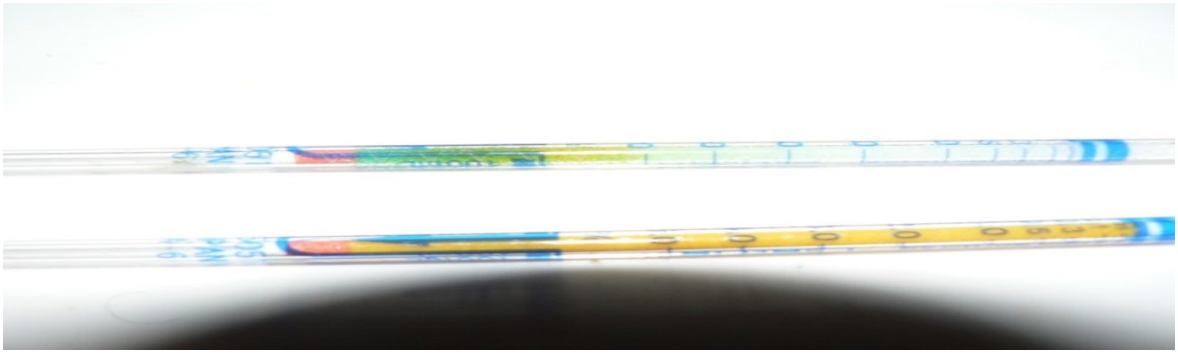

Figure 6.3.2 Comparison a control Valencia sample (test tube on the bottom, yellow only) and a freeze- treated Valencia sample at -2 ºC with ethanol recorded (test tube on the top, green colouration shows 3.5 % ethanol concentration for a single test).

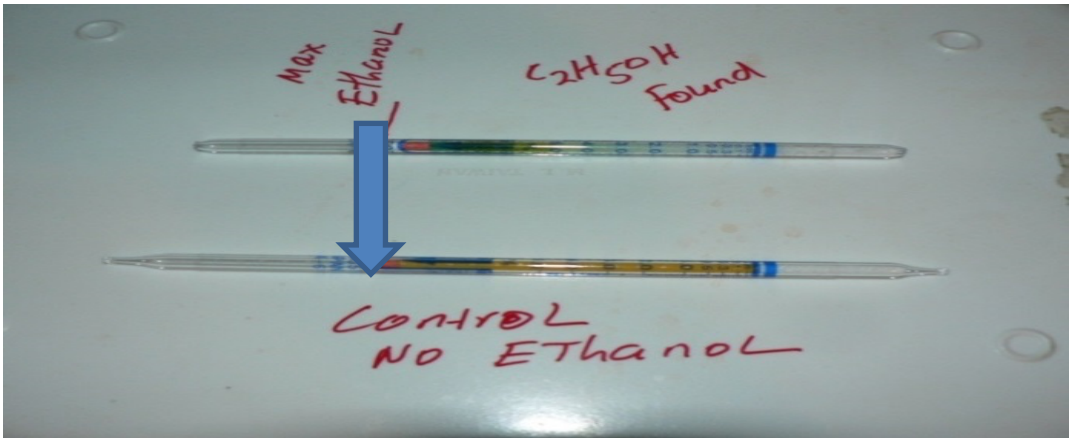

Figure 6.3.3 ethanol test for Navel orange sample treated at -6 ºC for 24 hours and stored for 3 weeks, showing 4.8 % ethanol concentration found from (test tube on the top) and control Navel samples with no ethanol found on (test tube on the bottom).

In this comparative test, the top test tube shows a bluish dark colour after the red line indicating the presence of ethanol; the tube on the bottom shows yellow colour indicating no ethanol.

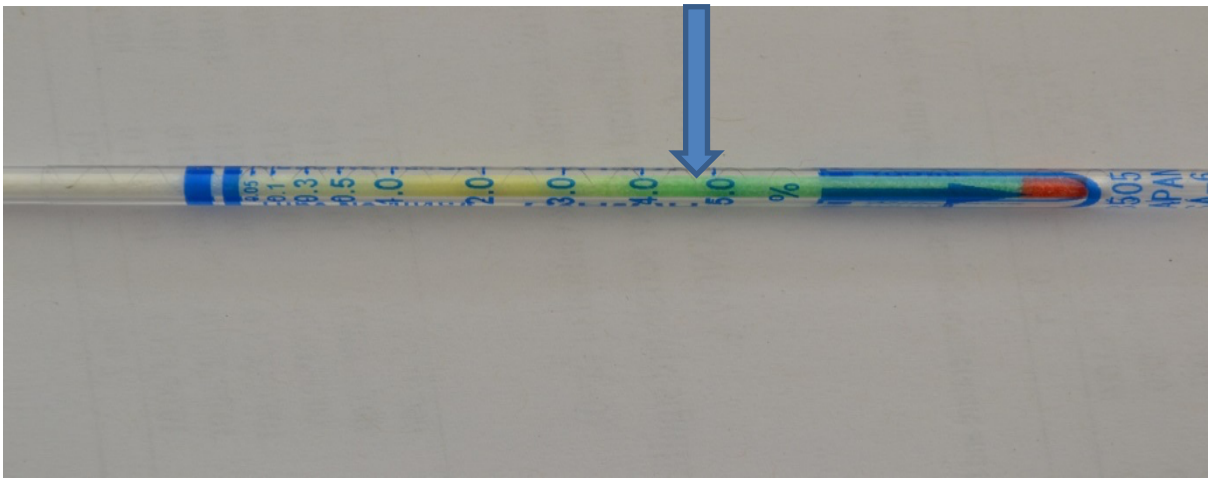

Figure 6.3.4 ethanol test for Navel orange fruit sample treated at -10 °C/24 hours and stored for two weeks, which indicates the presence of max of 5% ethanol as given by the bluish colour in the above test tube for a single test.

The results in Table 6.3.1 show hand held equipment used for ethanol tests of control orange samples and Table 6.3.1 - Table 6.3.4 are showing -2 °C, -6 °C and -10 °C freeze treated for 24 hours and tested using the above equipment of ethanol tester for both Valencia and Navel orange samples.

Results expressed as means ± standard deviation (n=6).

[abcd] Means in the same row with different lower case are significantly different ($p<0.05$)

[ABCD] Means in the same column with different upper case are significantly different ($p<0.05$)

Statistically analysis by means of one-way ANOVA

No ethanol was detected in either the Valencia or Navel oranges that were stored at 4°C for up to 21 days.

Table 6.3.2 Ethanol concentrations of Valencia orange fruit samples freeze-treated at (-2°C for 2, 8 and 24 hours) with three weeks storage time.

Mean value of Ethanol concentration expressed in µL/L			
Treatment	-2 °C/2 hour	-2 °C/8 hour	-2 °C/24 hour
Storage	Mean ±SD	Mean ±SD	Mean ±SD
Day 1	0	0	0
Day 3	0	0	0.1^{aA}±0.00
Day 5	0.1^{aA}±0.00	0.2^{aA}±0.00	0.3^{aA}±0.00
Day 7	0.2^{aA}±0.00	0.3^{aA}±0.02	0.7^{bB}±0.00
Day 14	0.2^{aA}±0.00	0.4^{bA}±0.03	1.7^{bcBC}±0.10
Day 21	0.3^{aA}±0.02	0.7^{bB}±0.06	3.0^{bcBCD}±0.20

Results in Table 6.3.2 (Valencia) orange samples that were freeze treated at -2 °C for 2 hr shows 0 – 0.3 µL/L shows ethanol concentration from day 1 to day 21, -2 °C/8 hr shows ethanol concentration of 0 – 0.7 µL/L from day 1 to day 21 and -2 °C/24 hr shows ethanol concentration of 0 – 3.0 µL/L from day 1 to day 21.

Table 6.3.3 Ethanol concentrations of Valencia orange fruit samples freeze-treated at (-6 °C for 2, 8 and 24 hours) with three weeks storage time.

Mean value of Ethanol concentration expressed in µL/L			
Treatment	-6 °C/2 hour	-6 °C/8 hour	-6 °C/24 hour
Storage	Mean ±SD	Mean ±SD	Mean ±SD
Day 1	0	0	0
Day 3	0	0	0.2^{aA}±0.00
Day 5	0.1^{aA}±0.00	0.2^{aA}±0.00	0.3^{aA}±0.00
Day 7	0.2^{aA}±0.00	0.3^{aA}±0.00	0.8^{bB}±0.00

Day 14	0.3^{aA}±0.00	0.4^{aA}±0.00	1.9^{bBC}±0.00
Day 21	0.5^{aB}±0.00	0.7^{bB}±0.00	4.0^{bcBCD}±0.01

Results on Table 6.3.3 (Valencia) orange samples that were freeze treated -6 °C/2 hr shows 0 – 0.5 µL/L shows ethanol concentration from day 1 to day 21, -2 °C/8 hr shows ethanol concentration of 0 – 0.7 µL/L from day 1 to day 21 and -6 °C/24 hr shows ethanol concentration of 0 – 4.0 µL/L from day 1 to day 21.

Table 6.3.4 Ethanol concentrations of Valencia orange fruit samples freeze-treated at (-10 °C for 2, 8 and 24 hours) with three weeks storage time.

Mean value of Ethanol concentration expressed in µL/L			
Treatment	-10 °C/2 hour	-10 °C/8 hour	-10 °C/24 hour
Storage	Mean ±SD	Mean ±SD	Mean ±SD
Day 1	0	0	0
Day 3	0.1^{aA}±0.00	0.2^{aA}±0.00	0.3^{aA}±0.00
Day 5	0.2^{aA}±0.00	0.3^{aA}±0.00	0.4^{bA}±0.02
Day 7	0.4^{aB}±0.03	0.5^{aB}±0.07	0.9^{bB}±0.04
Day 14	0.5^{aB}±0.04	0.7^{bBC}±0.05	2.0^{bcBC}±0.16
Day 21	0.7^{aBC}±0.06	1.2^{bBCD}±0.14	4.5^{bcBCD}±0.25

Results on Table 6.3.4 (Valencia) orange samples that were freeze treated -2 °C/2 hr shows 0 – 0.7 µL/L shows ethanol concentration from day 1 to day 21, -2 °C/8 hr shows ethanol concentration of 0 – 1.2 µL/L from day 1 to day 21 and -2 °C/24 hr shows ethanol concentration of 0 – 4.5 µL/L from day 1 to day 21.

Table 6.3.5 Ethanol concentration of Navel orange fruit samples freeze-treated at (-2 °C for 2, 8 and 24 hours) with three weeks storage time.

Mean value of Ethanol concentrations expressed in µL/L			
Treatment	-2 °C/2 hour	-2 °C/8 hour	-2 °C/24 hour
Storage	Mean ±SD	Mean ±SD	Mean ±SD
Day1	0	0	0
Day3	0.1^{aA}±0.00	0.1^{aA}±0.00	0.2^{aA}±0.00
Day5	0.2^{aA}±0.00	0.3^{aA}±0.00	0.5^{bB}±0.03

Day7	0.3^{aA}±0.00	0.4^{aA}±0.03	0.8^{bBC}±0.06
Day14	0.3^{aA}±0.00	0.6^{bB}±0.05	1.9^{bcBCD}±0.20
Day21	0.5^{aB}±0.04	0.8^{bB}±0.06	3.5^{bcBCDE}±0.31

Results on Table 6.3.5 (Navel) orange samples that were freeze treated -2 °C/2 hr shows 0 – 0.5 % shows ethanol concentration from day 1 to day 21, -2 °C/8 hr shows ethanol concentration of 0 – 0.8 µL/L from day 1 to day 21 and -2 °C/24 hr shows ethanol concentration of 0 – 3.5 µL/L from day 1 to day 21.

Table 6.3.6 Ethanol concentrations of Navel orange fruit samples freeze-treated at (-6 °C for 2, 8 and 24 hours) with three weeks storage time.

Mean value of Ethanol concentration expressed in µL/L			
Treatment	-6 °C/2 hour	-6 °C/8 hour	-6 °C/24 hour
Storage	Mean ±SD	Mean ±SD	Mean ±SD
Day1	0	0	0
Day3	0.1^{aA}±0.00	0.1^{aA}±0.00	0.2^{aA}±0.00
Day5	0.3^{aA}±0.00	0.3^{aA}±0.00	0.7^{bB}±0.00
Day7	0.4^{aA}±0.00	0.5^{aB}±0.03	0.9^{bB}±0.05
Day14	0.4^{aA}±0.00	0.8^{bBC}±0.06	2.1^{bcBC}±0.15
Day21	0.6^{aB}±0.04	0.9^{bBC}±0.06	4.2^{bcBCD}±0.14

Results on Table 6.3.6 (Navel) orange samples that were freeze treated -6 °C/2 hr shows 0 – 0.6 µL/L shows ethanol concentration from day 1 to day 21, -6 °C/8 hr shows ethanol concentration of 0 – 0.9 µL/L from day 1 to day 21 and -2 °C/24 hr shows ethanol concentration of 0 – 4.2 µL/L from day 1 to day 21.

Table 6.3.7 Ethanol concentrations of Navel orange fruit samples freeze-treated at (-10°C for 2, 8 and 24 hours) with three weeks storage time.

Mean value of Ethanol concentration expressed in µL/L			
Treatment	-10 °C/2 hour	-10 °C/8 hour	-10 °C/24 hour
Storage	Mean ±SD	Mean ±SD	Mean ±SD
Day1	0	0	0
Day3	0.2^{aA}±0.00	0.2^{aA}±0.00	0.3^{aA}±0.00
Day5	0.4^{aA}±0.00	0.4^{aA}±0.00	0.8^{bB}±0.04
Day7	0.5^{aA}±0.04	0.7^{bB}±0.05	1.2^{bcBC}±0.12
Day14	0.5^{aA}±0.04	0.9^{bB}±0.06	2.8^{bcBCD}±0.25
Day21	0.8^{aB}±0.05	1.2^{bBC}±0.14	4.6^{bcBCDE}±0.36

Results on Table 6.3.7 (Navel) orange samples that were freeze treated -10 °C/2 hr shows 0 – 0.8 µL/L shows ethanol concentration from day 1 to day 21, -10 °C/8 hr shows ethanol concentration of 0 – 1.2 µL/L from day 1 to day 21 and -10 °C/24 hr shows ethanol concentration of 0 – 4.6 µL/L from day 1 to day 21.

6.4 Discussions

The ethanol detection tube results were displayed in Figures 6.3.1- 6.3.4. In Figure 6.5.1, which is a test sample, ethanol is visible as a bluish colour compared to the yellowish colour of (no ethanol) control samples. This test is easy and simple to perform and, ideally, brings an option of mobile testing methods that can be applied to the industry. In return, the consumer can be confident of receiving an end product of fresh orange fruits that meet required specifications embodied in government regulations.

In the experimental investigation, samples were kept at a number of temperatures below zero for 24 hours to replicate different freeze-damage conditions. For our experiments, we selected -2 ºC to be the highest temperature, -6 ºC of intermediate temperature and -10 ºC as the lowest temperature for 2, 8 and 24 hours freeze treatment. In all cases, the treated fruit was then stored for 1, 3, 5, 7, 14 and 21 days to enable progressive deterioration to be detected. Furthermore, control samples were also tested for comparison reason and tests for levels of emitted ethanol were conducted using mobile handheld ethanol testing equipment. In Table 6.3.2 the results of freeze treatment at -2 ºC for 24 hours, Valencia samples showed a lower emitted ethanol level of between 0 to 3 µL/L than Table 6.3.5 for the Navel samples which

showed a value of between 0 to 3.5 µL/L. Table 6.3.3 also freeze treated at -6 °C for 24 hours were also show ethanol level of (0 to 4.0 µL/L) for Valencia and Table 6.3.6 treated at -6 °C for 24 hours show (0 to 4.2 µL/L) for Navel. Similarly, in Table 6.3.4 to the samples that were treated with the lower temperature (-10 °C/24 hours), Valencia cultivars samples in Table 6.3.4 show emitted ethanol losses of between 0 to 4.5 µL/L, and in Table 6.3.7 Navel treated at -10 °C for 24 hours show 0 to 4.6 µL/L however, samples that were treated at (-10 °C/8 hours) show 0 -1.2 µL/L.

In this investigation, the test tubes presented in Figures 6.3.2, 6.3.3 and 6.3.4, describes the samples that were treated at -2 °C, -6 °C and -10 °C, and tested for possible ethanol present. The results show the differences of (5%) in Figure 6.5.4 (Valencia orange fruit) compared to control orange samples in (Figure 6.5.1) with a control sample of (0%), Similarly -2 °C treated samples in Figure 6.3.2 show 3.5 µL/L and in Figure 6.3.3 show 4.5 µL/L for -6 °C compare to control sample in Figure 6.5.1 with 0% and this show a significant difference between the samples. More multiple tests were conducted and mean average value have been calculated for all samples that were freeze treated at -2, -6 and -10 °C and results are displayed in Table 6.3.2 to 6.3.7 accordingly.

These results show that the samples that were treated at -2 °C 24 hours for Valencia in Table 6.5.3 ethanol levels of 3 µL/L on day 21 compare to 1.7 µL/L on day 14 and 0.7 on day 7. For the Navel samples, (Figure 6.3.6, treated at the same temperature of (-2 °C 24 hours) show ethanol levels of 3.5 µL/L for 21 days, 1.9 µL/L for day 14 and 0.8 µL/L for day 7 samples. No ethanol was recorded on day 1 for both cultivars at -2 °C/2 to 24 hours, or for any control samples.

Similarly, at -6 °C 24 hours for Valencia in Table 6.5.4 return ethanol levels of 4 µL/L on day 21, 1.9 µL/L on day 14 and 0.8 µL/L on day 7. For the Navel samples, Figure 6.3.7, treated at the same temperature of (-6 °C 24 hours) show ethanol levels of 4.2 µL/L for 21 days, 2.1 µL/L for day 14 and 0.9 µL/L for day 7 samples. No ethanol was recorded on day 1 for both cultivars, or for any control samples in Table 6.3.1 and Table 6.3.2.

By comparison, for the samples that was treated with the much lower temperature of -10 ºC/24 hours, Valencia cultivars in Figure 6.3.5 show 4.5 µL/L to day 21 samples, 2 µL/L for day 14 and 0.9 µL/L on day 7. And in Figure 6.3.8 for Navel cultivars treated at the same conditions show 4.7 µL/L for day 21 samples, 2.8 µL/L for day 14 samples and 1.2 µL/L for day 7 orange samples, which represents a marked increase over Valencia samples.

The results on both figures show an increase in ethanol level after freeze damage and consequent storage of 7 days and over. Prior to the three days storage periods no ethanol was detected from both cultivars at -2, -4 and -10 °C.

6.5 Further Discussions

6.5.1 Comparing GC and mobile hand-held ethanol tester

Comparisons were made to the result achieved in Gas Chromatography (GC) and hand-held mobile testing methods, the goal was to identify ethanol compound that was detected in damaged oranges and compare their concentrations across and within the samples.

To achieve this goal, mobile ethanol tester must fit with correct tube designed for ethanol compound as the mobile ethanol tester have different functions and can be used to test different compounds. Similarly, Gas Chromatography (GC) data processing must fulfil three criteria: (1) using correct processing methodology (2) it must correctly determine the mass spectrum of the individual compounds for identification and; (3) it must accurately calculate the abundance of chromatographic peaks corresponding to those compounds in each sample. These three tasks are often challenging and time consuming mainly due to the co-elution of chromatographic peaks within a single chromatogram, as well as retention time (RT) of different compounds.

Comparing the results, the majority of graphs displayed in section 5.8 and 6.3 of page 124 - 126 on mobile hand-held equipment test and page 113-118 on GC tests are related. As many as 85% of graphs and table presented for ethanol test using mobile hand held equipment show similarities in terms of releasing ethanol compound due to the defect on the freeze damaged orange tested whereas the control samples of both tests did not release ethanol.

According to Fig 5.8.2 A of GC ethanol test on page 113 at -2˚C/2hr treatment and 21 days storage time show the value of 0.1 µL/L at -2˚C/2hr and 0.2 µL/L at -2˚C/24hr on day 5 and the value of 0.28 µL/L at -2˚C/2hr and 2.7 µL/L at -2˚C/24hr on day 21 for Valencia samples. Similarly in Fig 5.8.2 B of GC ethanol test on page 113 show 0.2 µL/L at -2˚C/2hr and 0.5 µL/L at -2˚C/24hr freeze treatment on day 5 and 21 days samples show the value of 0.33 µL/L at -2˚C/2hr and 3.5 µL/L at -2˚C/24hr on day 21for Navel samples.

Moreover in Fig 6.3.2 of mobile hand held equipment test on page 124 show the value of 0.1 µL/L at -2˚C/2hr and 0.3 at -2˚C/24hr treatment show 0.3 µL/L at -2˚C/2hr and 3.0 µL/L at -2˚C/24hr treatment on 21 days storage for Valencia. Also, in a mobile hand-held equipment test of Fig 6.3.4 for Navel samples on page 126 show the value of 0.2 µL/L at -2˚C/2hr and

0.5 at -2˚C/24hr treatment show 0.5 µL/L at -2˚C/2hr and 3.5 µL/L at -2˚C/24hr treatment on 21 days storage

Furthermore, in Fig 5.8.2 A of further low temperature treatments at -10˚C/2hr of Fig 5.8.6 A of GC test on 118 show the value of 0.2 µL/L at -10˚C/2hr and 0.4 µL/L at -10˚C/24hr on day 5 and the value of 0.61 µL/L at -10˚C/2hr and 4.3 µL/L at -10˚C/24hr on day 21 for Valencia samples. Similarly in Fig 5.8.2 B of GC ethanol test on page 113 show 0.4 µL/L at -10˚C/2hr and 0.9 µL/L at -10˚C/24hr freeze treatment on day 5 and 21 days samples show the value of 0.8 µL/L at -10˚C/2hr and 4.6 µL/L at -10˚C/24hr on day 21for Navel samples.

Moreover, in Fig 6.3.4 mobile hand-held equipment on page 126 with lower temperature show the value of 0.2 µL/L at -10˚C/2hr on day 5 and 0.4 at -10˚C/24hr treatment show 0.7 µL/L at -10˚C/2hr and 4.5 µL/L at -2˚C/24hr treatment on 21 days storage for Valencia. As well as in Fig 6.3.7 mobile hand-held equipment of Navel samples test on page 126 show the value of 0.4 µL/L at -10˚C/2hr and 0.8 at -10˚C/24hr treatment on day 5 similarly it show 0.8 µL/L at -10˚C/2hr and 4.6 µL/L at -2˚C/24hr treatment on 21 days storage.

Even though many other kinds of testing equipment are exist, using and knowing how to fully operate a hand held mobile equipment can be used and helpful to the person in laboratory filed as well as to the vast majority of individuals with limited scientific knowledge and who is working in the warehouse or packing shed environment or any fruit quality testing area.

Furthermore, the Table on page 124 and 126 show both the short and long term treatments within the data during the storage time. On the Figure 6.3.2 and 6.3.4 of Valencia and Figure 6.3.5 and 6.3.7 of Navel samples, it is easy to see that the concentration of ethanol steadily raised over time, from a high temperature of -2˚C to lower temperature of -10˚C.

In most cases it would be difficult for individuals to imagine what is going on inside the fruit that was already damage unless tests are conducted in terms of quality of the fruit with in that specific storage periods even a trained individuals had to use some of scientific equipment or need to perform some sort of visual and quality inspections to sort things out. However with modern technology using computer program it is possible for defected fruits and can be picked from conveyer belt during packing by applying special ray system but by then the fruit would be too late to save once it arriving to the packaging stage. Those are sensor based sorting machine that work based on colour, size and shape or other parameters that designs in relations to fruit quality parameters. Grading sorting peeling however they are expensive equipment and need someone who can operate them properly and maintenances would be costly and therefore mainly used by big companies.

The graphs on page 113-118 of section 5.8 contain representations of scientific information and a measure of error bars based on calculated standard errors. Moreover Figure 5.8.2 A & B – 5.8.6 A & B presents mean measurements of ethanol emissions from an orange fruit at various times over the course of up to 21 day and the error bars on each vertical bar provide the standard errors of measurements.

Chapter Seven

7.0 Microbiology: The Effects of Bacteria, Yeast and Moulds on Oranges

7.1 Introduction

In this Chapter the issue of Microorganisms mainly Yeast, Moulds **bacteria** will be investigated in freeze treated and control orange samples. Microorganisms play an important role in the post-harvest physiological changes occurring in fruits and vegetables, and considerable research has been conducted over the years in order to limit and prevent post-harvest spoilage of fruits by microorganisms (Garcia *et al.,* 1989). Orange is a high value fruit due to its taste and its contribution to traditional commodities throughout the world (Abdurrab *et al.,* 2012) where, generally, fruits are preferred and consumed for their highly recognised nutritional needs (Dougo *et al.,* 2003) and possibly for their therapeutic properties (Sajia, 1994). Generally, most fruits, including citrus species, are susceptible to both pre and post-harvest pathogens (Pienaar, 1969), and as a result citrus fruits lose their qualities during post-harvest storage. This has been one of the common problems with fruit as a nutritional source, and, most importantly, biological issues such as mould, yeast and bacteria are becoming increasing concerns to important quality parameters of the fruit industry (Kinay *et al.,* 2001).

7.2 Aim

The aim of this study was to investigate the susceptibility to microbial growth of frost damaged orange fruits, mainly Valencia and Navel cultivars, together with measurement of the reduction of TSS, change in pH, and the rate of ethanol production, in regard to the effects of microbial growth such as bacteria, yeasts and moulds.

7.3 Material and methods

The total plate count method used in this investigation, together with the source of chemicals used in the practical experiments, were described in Section 3.5. In particular, for the bacterial tests, the methods were described and the directions were given in Section 3.5.2, details of the MRS are in Section 3.5.2.1, the medium used is noted in section 3.5.3; the incubation methods using CM0359 were detailed in Section 3.5.4. General techniques used on bacterial testing described in section 3.5.5, serial dilutions are also explained in Section 3.5.5.1 and freeze treatment and storage periods are similar to section 3.4.3.

In addition, a general overview of mould and yeast experiments was explained in Section 3.6 and equipment and methods used were illustrated in Section 3.6.1 and instructions related to the directions for preparation of agar (CM0139) are given in Section 3.6.2.

7.4 Results

Figures 7.4.1 and 7.4.2 show moulds arising in this work, specifically of samples that were freeze treated and stored for more than three weeks. Blue Mould pathogen, *Penicillium italicum* (Figure 7.4.1), is the most common defect, and is usually the most destructive type of mould found on fruits. Factors that influence the development and spread of blue mould rot may be classed as follows: (1) the extent of the spore load on the fruits, (2) the condition of the fruits, and (3) environmental conditions (Washington State University, 2005). Green mould, Penicillium italicum (Figure 7.4.2), that was found on treated samples, also causes a destructive fruit rot on citrus fruit. Early symptoms include a soft water-soaked area on the peel, followed by development of a circular colony of mould, where the fruit rapidly spoils and collapses (Brown and Eckert, 1988). In Figure 7.4.3, an orange fruit, which was detected with Blue Mould pathogen (*Penicillium italicum),* is shown to be completely destroyed by the mould after a few weeks' storage post-freeze damage.

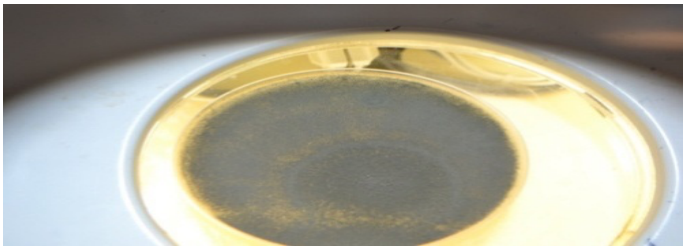

Figure 7.4.1 Blue mould pathogen *Penicillium italicum* (From this work)

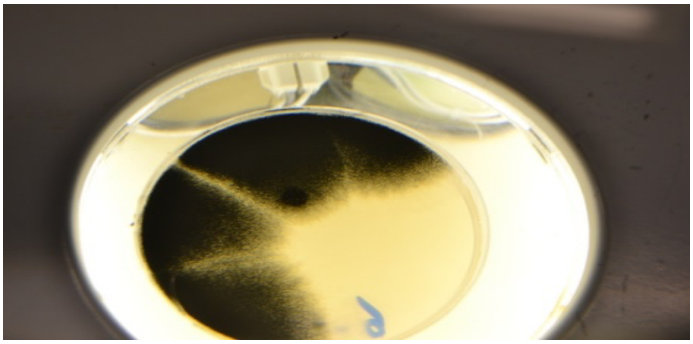

Figure 7.4.2 Green mould Penicillium italicum (From this work)

Figure 7.4.3 Orange fruit destroyed by Blue mould pathogen (From this work)

7.4.1 Total soluble solid (TSS) results

Total soluble solid value for Valencia and Navel orange fruit presented in Figures 4.1.1 (A and B) to 4.1.5 (A and B) show a steady decrease in TSS value over time, for freeze treated oranges of both cultivars, is shown reduction in TSS over the time during storage periods due to chemical changes in the fruit initiated by microbial issues (Zubbair, 2009).

7.4.2 Microbiology tests results

Tables 7.4.2 to 7.4.7 are the results of yeast and mould tests that were conducted on orange juice extracted samples and show significant increase in yeast and mould count on freeze damaged sample as the storage time was increased up to three weeks compared to the control samples. Valencia and Navel orange that shows no yeast or mould were detected in either the Valencia or Navel oranges that were stored at 4°C for up to 21 days. However, results in Tables 7.4.2 – 7.4.7 shows yeast and mould growths. Results in the tables are expressed as mean ± standard deviation with (n=6).

^{abcd} Means in the same row with different lower case are significantly different ($p<0.05$)
^{ABCD} Means in the same column with different upper case are significantly different ($p<0.05$)

Statistically analysis by means of one-way ANOVA

Table 7.4.2 Microbiology tests results (yeast and mould count) of pre-freezed Valencia orange fruit at -2 °C for 2, 8 and 24 hours and stored at 4 °C for up to three weeks

	-2 °C/2 hr		-2 °C/8 hr		-2 °C/24 hr	
Storage	Mould counts (log CFU/mL)	Yeast counts (log CFU/mL)	Mould counts (log CFU/mL)	Yeast counts (log CFU/mL)	Mould counts (log CFU/mL)	Yeast counts (log CFU/mL)

	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Day1	0	0	0	0	0	0
Day3	1.00^{aA}±0.00	1.11^{aA}±0.00	1.30^{bA}±0.33	1.40^{bA} ±0.81	1.80^{bcA}±0.00	2.00^{bcA}±0.11
Day5	1.01^{aA}±0.00	1.22^{bA}±0.15	1.95^{bB}±0.00	2.35^{bB}±0.12	2.74^{bcB}±0.00	3.00^{bcB}±0.25
Day7	1.50^{aB}±0.20	1.72^{bB}±0.21	2.00^{bB}±0.00	2.40^{bB}±0.00	3.65^{bcBC}±0.12	4.00^{bcBC}±0.00
Day14	2.62^{aBC}±0.25	3.01^{bBC}±0.20	4.00^{bBC}±0.00	4.22^{bBC}±0.23	4.77^{bcBCD}±0.16	5.00^{bcBCD}±0.30
Day21	3.00^{aBBCD}±0.10	3.51^{bBCD}±0.21	4.78^{bBCD}±0.15	5.00^{bBCD}±0.00	5.18^{bcBCDE}±0.00	5.55^{bcBCDE}±0.00

Table 7.4.2 show the mean values of different types of treatment with respect to storage times. Results increased from 1.00 ± 0.00 CFU/mL on day 3 of 2 hours treatment to 5.18 ± 0.00 log CFU/mL for day 21 of 24 hours treatment for mould and the yeast experiments show a result of 1.11 ± 0.00 CFU/mL on day 3 of 2 hours treatment and 5.55 ± 0.00 log CFU/mL on day 21 of 24 hours treatment.

Table 7.4.3 Microbiology tests results (yeast and mould count) of pre-freezed Valencia orange fruit at -6 °C for 2, 8 and 24 hours and stored at 4 °C for up to three weeks.

	-6 °C/2 hr		-6 °C/8 hr		-6 °C/24 hr	
Storage	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD
Day 1	0	0	0	0	0	0
Day 3	1.04^{aA}±0.00	1.20^{aA}±0.22	1.40^{bA}±0.00	1.70^{bcA}±0.21	2.00^{bcdA}±021	2.20^{bcdeA}±0.00
Day 5	1.11^{aA}±0.00	1.41^{bA}±0.00	2.30^{bB}±0.00	2.70^{bcB}±0.25	3.02^{bcdB}±0.21	3.12^{bcdeB}±0.00
Day 7	2.00^{aB}±0.12	2.10^{B}±0.00	2.50^{bB}±0.24	2.88^{bcBC}±0.04	4.14^{bcdBC}±0.16	4.23^{bcdeBC}±0.00
Day 14	3.72^{aBC}±0.23	4.00^{bBC}±0.00	4.20^{bcBC}±0.27	4.98^{bcdBCD}±0.00	$5.88^{bcdeBCD}$±0.00	$6.70^{bcdeBCD}$±0.12
Day 21	4.12^{aBCD}±0.00	4.72^{bBCD}±0.17	5.01^{bcBCD}±0.25	$6.00^{bcdBCDE}$±0.12	$6.68^{bcdeBCDE}$±0.00	$7.82^{bcdeBCDE}$±0.10

Table 7.4.3 show the mean values of three different types of treatment with respect to storage times. Results increased significantly from 1.04 ± 0.00 CFU/mL on day 3 of 2 hours treatment to 6.68 ± 0.00 log CFU/mL for day 21 of 24 hours treatment for mould similarly yeast experiments show a result of 1.20 ± 0.22 CFU/mL on day 3 of 2 hours treatment and 7.82 ± 0.10 log CFU/mL on day 21 of 24 hours treatment

Table 7.4.4 Microbiology tests results (yeast and mould count) of pre-freezed Valencia orange fruit at -10 °C for 2, 8 and 24 hours and stored at 4 °C for up to three weeks.

	-10 °C/2 hr		-10 °C/8 hr		-10 °C/24 hr	
Storage	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD
Day 1	0	0	0	0	0	0
Day 3	1.20^{aA}±0.00	1.30^{bA}±0.00	1.60^{bcA}±0.00	1.80^{bcd}±0.21	2.13^{bcde}±0.16	2.20^{bcde}±0.00
Day 5	1.20^{aA}±0.00	1.50^{bB}±0.00	2.80^{bcB}±0.00	3.10^{bcd}±0.20	4.11^{bcde}±0.33	4.10^{bcde}±0.00
Day 7	2.14^{aB}±0.02	2.54^{bBC}±0.02	3.14^{bcBC}±0.02	3.14^{bcd} ±0.02	5.14^{bcde}±0.02	5.39^{bcdef}±0.34
Day 14	3.98^{aBC}±0.31	3.98^{bBCD}±0.20	4.98^{bcBCD}±0.00	5.98^{bcd}±0.00	6.98^{bcde} ±0.31	7.24^{bcdef}±0.25
Day 21	4.42^{aBCD}±0.38	4.42^{bBCDE}±0.12	5.42^{bcBCDE}±0.00	6.42^{bcd}±0.00	8.42^{bcde} ±0.38	8.96^{bcdef}±0.09

Table 7.4.4 shows the mean value of three different types of treatment with respect to storage times. Results for mould experiment increased significantly from 1.20 ± 0.00 log CFU/mL on day 3 of 2 hours treatment to 8.42 ± 0.38 log CFU/mL for day 21 of 24 hours treatment and yeast experiments show a result of 1.30 ± 0.00 CFU/mL on day 3 of 2 hours treatment and 8.96 ± 0.09 log CFU/mL on day 21 of 24 hours treatment.

Table 7.4.5 Microbiology tests results (yeast and mould count) of pre-freezed Navel orange fruit at -2 °C for 2, 8 and 24 hours and stored at 4 °C for up to three weeks

	-2 °C/2 hr		-2 °C/8 hr		-2 °C/24 hr	
Storage	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD
Day 1	0	0	0	0	0	0
Day 3	1.12^{aA}±0.00	1.22^{aA}±0.31	1.52^{bcA}±0.33	1.71^{bcdA}±0.31	1.89^{bcdeA}±0.00	2.40^{bcdefA}±0.00
Day 5	1.22^{aA}±0.00	1.55^{bB}±0.00	1.99^{bcB}±0.00	2.46^{bcdB}±0.00	2.87^{bcdeB}±0.00	3.10^{bcdefB}±0.02
Day 7	1.62^{aB}±0.22	1.99^{bBC}±0.00	2.44^{bcBC}±0.00	2.55^{bcdB}±0.00	3.99^{bcdeBC}±0.02	$4.20^{bcdefBC}$±0.14
Day 14	2.81^{aBC}±0.16	3.22^{bBCD}±0.01	4.53^{bcBCD}±0.00	4.77^{bcdBC}±0.22	$4.96^{bcdeBCD}$±0.00	$5.00^{bcdefBCD}$±0.30
Day 21	3.12^{aBCD}±0.22	3.81^{bBCDE}±0.12	4.92^{bcBCDE}±0.01	$5.66^{bcdBCDE}$±0.13	$5.69^{bcdeBCDE}$±0.22	$5.55^{bcdefBCDE}$±0.22

Table 7.4.5 shows the mean value of three different types of treatment with respect to storage times. Mould experiment results show an increase from 1.12 ± 0.00 log CFU/mL on day 3 of 2 hours treatment to 5.69 ± 0.22 log CFU/mL for day 21 of 24 hours treatment and yeast experiments show a result of 1.22 ± 0.31 CFU/mL on day 3 of 2 hours treatment and 5.55 ± 0.22 log CFU/mL on day 21 of 24 hours treatment.

Table 7.4.6 Microbiology tests results (yeast and mould count) of pre-freezed Navel orange fruit at -6 °C for 2, 8 and 24 hours and stored at 4 °C for up to three weeks

	-6 °C/2 hr		-6 °C/8 hr		-6 °C/24 hr	
Storage	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD
Day 1	0	0	0	0	0	0
Day 3	1.53^{aA}±0.00	1.22^{bA}±0.00	1.73^{bcA}±0.33	1.91^{bcdA}±0.00	2.23^{bcdeA}±0.13	2.62^{bcdefA}±0.00
Day 5	1.61^{aA}±0.00	1.65^{bB}±0.00	2.01^{bcB}±0.22	2.62^{bcdB}±0.00	2.92^{bcdeB}±0.11	3.22^{bcdefB}±0.00
Day 7	1.77^{aB}±0.00	1.99^{bBC}±0.00	2.62^{bcBCD}±0.00	2.69^{bcdB}±0.11	4.01^{bcdeBC}±0.00	$4.55^{bcdefBC}$±0.12
Day 14	2.92^{aBC}±0.00	3.22^{bBCD}±0.00	4.71^{bcBCDE}±0.00	4.92^{bcdBC}±0.00	$4.99^{bcdeBCD}$±0.00	$5.22^{bcdefBCD}$±0.31
Day 21	3.73^{aBCD}±0.23	3.81^{bBCDE}±0.31	$4.99^{bcBCDEF}$±0.00	5.88^{bcdBCD}±0.00	$5.88^{bcdeBCDE}$±0.00	$5.93^{bcdefBCDE}$±0.35

Table 7.4.6 shows the mean value of three different types of treatment with respect to storage times. Results increased significantly from 1.53 ± 0.00 log CFU/mL on day 3 of 2 hours treatment to 5.88 ± 0.00 log CFU/mL for day 21 of 24 hours treatment for mould trail and yeast experiments show a result of 1.22 ± 0.00 CFU/mL on day 3 of 2 hours treatment and 5.33 ± 0.35 log CFU/mL on day 21 of 24 hours treatment.

Table 7.4.7 Microbiology tests results (yeast and mould count) of pre-freezed Navel orange fruit at -10 °C for 2, 8 and 24 hours and stored at 4 °C for up to three weeks.

	-10 °C/2 hr		-10 °C/8 hr		-10 °C/24 hr	
Storage	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD	Mould counts (log CFU/mL) Mean ±SD	Yeast counts (log CFU/mL) Mean ±SD
Day 1	0	0	0	0	0	0
Day 3	1.59^{aA}±0.00	1.72^{aA}±0.00	1.81^{aA}±0.00	2.01^{bA}±0.00	2.61^{bcA}±0.00	2.88^{bcdA}±0.00
Day 5	1.87^{aB}±0.00	1.89^{bB}±0.00	2.92^{bcA}±0.00	3.51^{bcdB}±0.00	4.77^{bcdeB}±0.00	$4.45^{bcdefBC}$±0.00
Day 7	2.39^{aBC}±0.02	2.45^{bBC}±0.22	3.34^{bcB}±0.00	3.55^{bcdB}±0.00	5.82^{bcdeBC}±0.00	$5.61^{bcdefBCD}$±0.00
Day 14	3.99^{aBCD}±0.31	4.02^{bBCD}±0.42	6.01^{bcBC}±0.35	6.07^{bcdBC}±0.12	$7.02^{bcdeBCD}$±0.32	$7.74^{bcdefBCDE}$±0.15
Day 21	4.49^{aBCDE}±0.38	4.55^{bBCDE}±0.45	6.67^{bcBCD}±0.41	6.81^{bcdBCD}±0.24	$8.88^{bcdeBCDE}$±0.41	$8.99^{bcdefBCDEF}$±0.42

Table 7.4.7 shows the mean value of three different types of treatment with respect to storage times. Results increased significantly from 1.59 ± 0.00 log CFU/mL on day 3 of 2 hours treatment to 8.88 ± 0.41 log CFU/mL for day 21 of 24 hours treatment for mould similarly yeast experiments show a result of 1.72 ± 0.00 CFU/mL on day 3 of 2 hours treatment and 8.99 ± 0.642 log CFU/mL on day 21 of 24 hours treatment

7.5 Discussion

The effects of microbial contamination from freeze-treated orange samples investigated in this Chapter have a relation to fermentation process and the production of ethanol that were studied using a portable ethanol tester equipment on Chapter six, Moreover, they show indirect dependence to TSS level and they are in line with the work to (Dudley 2000). Microbiology work is one of important task for fruit testing, this is as frost-damaged fruits are more susceptible to bacteria, mould and yeast attack than are sound fruits. It has also been postulated that the observed drop in TSS value is due to utilization of nutrients by microorganisms during their growth through these storage periods (Nielsen 2005). In most cases, the presence of yeast, mould and cold-loving bacteria are of the most common concern to refrigerated fruits. Wang *et al.* (1981) stated that psychotropic microorganisms are able to grow at low temperatures (3 to 7 °C) by hydrolysing and using large molecules of proteins and lipids for growth. In addition, according to Cousin (1982), pH is one of the major factors associated with microbial growth (Cousin, 1982), and it has been observed that yeast and mould have an ability to grow at lower pH levels (Kamal *et al.,* 2014).

In this practical work, fruits were freeze-damaged using simulated freezing in the laboratory at various temperatures, then thawed and stored at 4 °C before tests were conducted for possible microorganism detection after the storage of up to three weeks. The results showed that the fruits that were treated with much lower temperatures of -10 °C for 24 hours) and stored over for periods of (3 weeks) developed mould attack (see Figures 7.5.1, 7.5.2 and 7.5.3 for mould). Psychotropic microorganisms represent a substantial percentage of bacteria in frozen-food products, with pseudomonads and related aerobic, Gram-negative, rod-shaped bacteria being the predominant groups (Garcia *et al.,* 1989). Microorganisms such as aerobic psychotropic Gram-negative bacteria, yeasts and moulds have been found to be the main growths on orange fruits that had frost damage and stored at 4 °C more than three-week periods. This caused wide variety of metabolic by-products, including off-odours and flavours, in addition to that of visible changes in colour or textural change (Nielsen, 2005).

The reduction in pH creates favourable conditions for the growth to microbial communities (Wang and Frank, 1981). According to Wang and Frank (1981), yeasts can grow well at low pH, and can produce off-flavours. It has been observed that yeasts are a major cause of spoilage to food, and low pH provides a selective environment for their growth (Fleet, 1990). Signs of gas appearance are often detected when yeasts grow to 105–106 CFU, and it was also suggested that positive strains of yeasts such as *Saccharomyces cerevisiae* is the most common species (Giudici *et al.,* 1996). In another relevant development, Hocking and Fadeo (1992) stated that moulds grow well on the surfaces of fruits when oxygen is present, with low

pH being a suitable environment; in these conditions, it has been found that *Cladosporium spp* is the most common type of mould that can be found.

Mould and yeast experiments were carried out to further investigate their effects on frost damaged fruits. Microbial defects were discovered during the storage of the fruits samples from day 1 to day 21. It was suspected that the observed decrease in TSS value is due to the process of fermentation, which produces ethanol and other compounds within the damaged fruits where yeast has permeated the protective skin. Fermentation is the conversion of a carbohydrate such as sugar into an acid or alcohol; in yeast, fermentation changes sugars such as glucose into alcohol (Dudley 2000).

Fermentation process (Dudley 2000)

According to Rajendran and Ohta (1998), microbes, like any living organisms, require food and water to carry on their life processes. Nutrients must be in solution before they can be transported into the cells. Most food preservation techniques can be affected by bacterial growth if the conditions are favorable; however, growth can also be controlled by limiting the availability of nutrients, lowering the temperature, restricting water, adjusting the pH of the medium, using chemical inhibitors, and providing an inert atmosphere (Rajendran and Ohta, 1998). It has been found that an essential factor that affects the growth of bacteria is temperature, and microbial growth can occur over a wide range of temperatures. Indeed, organisms can be divided into three groups based on their temperature growth range; Pschrophiles (cold loving microbes), Mesophiles (moderate temprature loving microbes) and Thermophiles heat loving microbes) (Eriksson *et al.,* 2000; Jacobe *et al.,* 1973).

We have indicated previously that the presence of yeast facilitates the production of ethanol (Haskard *et al.,* 2000). As ethanol is a naturally occurring substance resulting from the fermentation by yeast of fruit sugars, ethanol plumes can potentially be used to locate ripe and over-ripe fruit. The widespread occurrence of fermentative yeasts in ripening and ripe fruits indicates potential co-option of benefits associated with ethanol production (Dudley, 2000). It has been suggested that ethanol expression by fermentative yeasts appears to have specifically evolved to inhibit the activity of bacterial competitors within the ripe fruit. In anaerobic situations, when oxygen is not available, sugars break down to lactic acid, but with the presence of yeasts, sugars break down to carbon dioxide and ethanol, which is called

alcoholic fermentation (Ingham and Buttke, 1995). Damage to fruits from the breaking of the protective skin cover, acts to accelerate these natural phenomena, and freeze damage to fruit, followed by interjection of yeast to the sugary interior, thus acts in this way. Table 7.4.7 records values for samples that were deteriorated due to freeze damage followed by long term storage. TSS values showed a decrease in values in Section 4.2 of -10 °C, furthermore our results show similarities with the comments made by Ingham and Buttke (1995), suggesting that sugar was consumed by a microbial community and used up as energy during fermentation, and alcohol was then produced (Ingham and Buttke, 1995; Hossain 1985). The above scenarios were observed for freeze-treated and damaged orange fruit samples which were treated at lower temperature (-10, -8 and -6 °C) but not to the control samples in this experiment. The pH indicates how acidic the fruit interior is, and most bacteria do not grow very well in an acidic environment (Coallier *et al.,* 1994).

Foods with a pH of 4.6 and below are considered acid foods, and this includes most fruit juices, whilst foods with a pH above 4.6 are said to be low acid. It is found that bacteria (gram posative) thrive at pH 4.0 to 8.5, bacteria (gram negative) at pH 4.5 to 9.0, moulds at pH 1.5 to 9.0 and Yeast at a pH of 2.0 to 4. 5. Clearly, microorganisms can only grow at certain pH levels, but mould and yeast can grow over a broad range of pH. Bacteria, being micro-organisms, are more restricted, and the difference between gram-positive bacteria and gram-negative bacteria shown above indicates that pH can be used to control the growth of bacteria (Coallier *et al.,* 1994). According to our results, pH was changing with the extent of damage, and showed a small decrease of pH reading from 4.17 (undamaged) to 4.27 (damaged) orange fruits. This was due to chemical changes in the fruit, and this fits with the observed growth of bacteria, yeast and mould in damaged fruit.

Further investigations, including microbiological tests, have been conducted in order to understand the production of yeast and mould results found during the storage of freeze-treated samples are displayed in table format and some of moulds are presented in Figures 7.4.1, 7.4.2 and 7.4.3. The results from this work are in line with the finding of Dudley (2000; 2002) and a study reported by Ingham and Buttke (1995).

Further results are presented in Table 7.4.1 to 7.4.7 for yeast and mould counts of freeze treated Valencia and Navel orange samples (from control samples to the lowest of (-10 °C/24 hours) held for a storage period of up to 3 weeks. These are recorded as mean ±SD of log CFU/mL and the results show significant ($p < 0.05$) differences of the plate count of yeast and mould that were found between freeze-treated fruit samples held for different storage times. There was no significant ($p > 0.05$) differences in results between cultivars.

Similarly, the yeast count did not show any considerable differences between changes to Valencia or Navel samples. For both, however, longer storage time after freeze damage led to significantly ($p < 0.05$) higher levels of deterioration.

7.6 Statistical Analysis

To reinforce the above findings, results were expressed as mean ± SD (standard deviation) of plate count. All tests were done using UV and aseptic techniques. Plate count of mould and yeast colonies are carried and statistical significance was determined by Repeated Measures One-way Analysis of Variance (ANOVA). This test was performed to compare mean values of freeze-treated samples and control samples. As normal, differences were considered to be significant when $p < 0.05$.

There was no significant difference in pH between the samples over time – indeed there was only small decrease of pH from (4.07 to 3.90) for -2 °C day 1 to day 21 storage and day 2 to 24 hours treatment of Valencia orange and from (4.06 to 3.70) for Navel orange in (Figure 4.2.1A and B), for the samples that were treated at -8 °C shows the pH reading from (3.90 to 3.46) for Valencia and (3.65 to 3.43) for Navel in (Figure 4.2.4A and B) however -10 °C treatment in (Figure 4.2.5A and B) shows (3.47 to 3.30) for Valencia and (3.37 to 3.30) for Navel during the 3 week trial. Figure 1.1.1A and B show sharp comparison, with a significant reduction in TSS of the freeze-treated samples over time in Section 4.1 Table 7.4.1 records the obtained values of 0 for up to day 21 storage trial respectively for control Valencia and Navel. This means that there is no significant difference between the cultivars. However when the results were compared with freeze treated samples, it was observed that untreated sample did not evidence any TSS reduction over the testing period.

Similar results were also found by Last and Price (1969), Cipollini and Stiles (1997) and Spencer and Spencer (2000). According to the investigation by Last and Price (1969), among the microbial community they investigated, yeast and moulds were commonly observed. Mycotoxinogenic moulds such as Aspergillus, Fusarium and Penicillium play an undeniable role in the deterioration of the marketable quality and hygiene of foodstuffs by synthesizing highly toxic metabolites known as mycotoxins. Several of these toxins have been identified, but many more could be responsible for significant problems in foodstuffs (Tournas and Katsoudas, 2005).

In acid foods and foods of low water activity, bacteria often grow causing spoilage losses, especially if the products (e.g., fresh fruit and vegetables, frozen or dried foods) are improperly stored. Additionally, there is also the potential hazard from production of mycotoxins by moulds (Tournas and Katsoudas, 2005). This investigation was mounted to study the spoilage of

freeze-treated orange samples by yeast and mould moreover, blue mould, green mould and orange fruits fully destroyed blue mould were presented in Figures 7.4.1, 7.4.2 and 7.4.3 respectively.

7.7 Conclusion

In conclusion, mould and yeasts were discovered on freeze-treated samples, and more deterioration was observed at the surface of orange fruits than for samples that were treated at less extreme cold temperature and the control samples. As agents of both microbial decay and fermentative activity, yeasts were widespread both on outside and inside the fruits which were stored for more than 3 weeks. Overall, moulds were present in counts greater than 1 log CFU/mL in all the freeze-treated samples that were tested.

There was a significant increase ($p < 0.05$) shown in Table 7.4.2 for the mould count from Valencia orange samples, -2 ^{0}C for 8 hours shows the results of 1.30 ± 0.33 to 4.78 ± 0.15 log CFU/mL for mould and 1.40 ± 0.0.81 to 5.00 ± 0.00 log CFU/mL for yeast. Corresponding results in Table 7.5.6 for Navel orange samples were 1.52 ± 0.33 to 4.92 ± 0.01 log CFU/mL and 1.71 ± 0.31 to 5.66 ± 0.13 log CFU/mL.

Furthermore, results at intermediate temperature of -6 ^{0}C were also observed closely and discussions are provided below. Table 7.4.3 for the mould count from Valencia orange samples, -6 ^{0}C for 8 hours shows the results of 1.40 ± 0.00 to 5.01 ± 0.25 log CFU/mL for mould and 1.70 ± 0.0.21 to 6.00 ± 0.12 log CFU/mL for yeast. Corresponding results in Table 7.5.7 for Navel orange samples were 1.73 ± 0.33 to 4.99 ± 0.00 log CFU/mL and 1.91 ± 0.00 to 5.88 ± 0.00log CFU/mL of significant increases for both mould and yeast experiments.

Moreover, significant increases were observed for the much lower temperature of -10 ^{0}C for 8 hours for both tests. In Table 7.4.4 for mould count of Valencia orange samples, shows the results of 1.60 ± 0.00 to 5.42 ± 0.00 log CFU/mL for mould and 1.80 ± 0.0.21 to 6.42 ± 0.00 log CFU/mL for yeast. Corresponding results in Table 7.5.8 for Navel orange samples were 1.81 ± 0.00 to 5.67 ± 0.41 log CFU/mL and 2.01 ± 0.00 to 6.81 ± 0.24 log CFU/mL.

Chapter Eight

8.0 Physical Attributes and Firmness Tests of Fruit

The assessment of the quality of fruit is an essential part of maintaining the traditional standards of the fruit industry. As a result of fruit defects which can be caused by a range of different reasons including frost damage, several tests of orange fruit quality were conducted. Such tests can help in the detection of internal defects, and this will assist to improve the fruit industry's competiveness, profitability and assure consumer satisfaction.

Firmness is an important characteristic of fruit, and it is one of the most reliable and quickest methods of determining fruit quality. In many instances, during harvest or post-harvest, after a few days of storage damaged fruits show less firmness when tested, e.g. using firmness testing equipment (Harker *et al*., 1996). Firmness tests, which were conducted on a range of samples that had been freeze treated to see whether results were influenced by sample location, prior compression and the size of the fruit, are presented in this Chapter. This Chapter also reports fruit quality assessments, which were conducted before and after freeze treatment on orange colour and damage.

These investigations of the effect of cold damage on the quality of Valencia and Navel orange fruit also used orange samples obtained from Leeton (NSW) and Mildura (Victoria), as described in Chapter Three, with freeze treatments conducted as outlined in Section 3.1.3. This was done in order to compare freeze-treated fruit with untreated control orange fruit samples.

8.1 Aim

The objective of the current study was to investigate the firmness of Valencia oranges before and after freeze treatment in relation to storage time. This was to simulate the possible occurrence of storage temperatures falling too low in transportation to markets, and in the investigation, we used an intermediate temperature of -6 °C.

8.2 Material and Methods

In this study, a universal testing instrument was used to evaluate orange fruit firmness, and evidence was sought that would show if results were influenced by sample location, prior compression and the size of the fruit. Methodological information on these experiments has previously been provided in Section (3.8).

8.3 Firmness Test Results

The objective was to investigate the firmness of oranges before and after freeze treatment in relation to storage time as well as its quality. A universal testing instrument was used to evaluate orange fruit firmness, and information about these experiments, which were carried out using Instron equipment, was provided in Section 3.8. The use of an Instron instrument for firmness test provides a measure of the maximum stress that a material can sustain under crush loading. Whilst the compressive strength of a material that fails by shattering fracture can be defined within fairly narrow limits as an independent property, the compressive strength of materials that do not shatter in compression must be defined as the amount of stress required to distort the material by an (agreed) arbitrary amount.

The compressive load strength was calculated to determine the required capacity of the load cell. This can be done for most materials by multiplying the ultimate strength by the cross-sectional area of the sample (the load cell should have a maximum load capacity greater than the maximum strength of the sample). The compressive load is expressed as the calculated amount of load in newton applied during the compression test. The compressive extension is the distance in cm that the sample moves downward, and the compressive energy expresses how much energy in joule was applied during compression to the sample.

Instron tests for Valencia and Navel cultivars were carried out on 6 occasions during 21 days of storage for control samples, and for samples that had been freeze treated at 4 different temperatures, namely -2, -4, -6 and -10 ºC, for 24 hours. All results are shown in tables 8.3.1 – 8.3.8, as mean ± standard deviation, with Statistical analysis by means of one-way ANOVA. ^{ABCD} Means in the same column with different upper case are significantly different ($p<0.05$)

Results for the Valencia and Navel control samples, in relation to compressive extension, compressive load and compressive energy, are in Tables 8.3.1 and 8.3.2, respectively.

Table 8.3.1 Firmness test of control Valencia orange fruit samples

Storage time	Compressive extension (mm/sec)	Compressive load (newton)	Compressive energy (joule)
	Mean ±SD	Mean ±SD	Mean ±SD
Day 1	2.22^{A}±0.00	76.20^{A}±0.00	0.01^{A}±0.00
Day 3	2.22^{A}±0.00	75.20^{A}±0.00	0.01^{A}±0.00
Day 5	2.23^{A}±0.00	73.10^{A} ±0.00	0.01^{A}±0.14
Day 7	2.23^{A}±0.03	71.10^{A}±0.15	0.01^{A}±0.00
Day 14	2.23^{A}±0.00	71.10^{A}±0.22	0.02^{A}±0.13
Day 21	2.24^{A}±0.05	71.10^{A}±0.21	0.02^{A}±0.12

Table 8.3.2 Firmness test of control Navel orange fruit samples

Storage time	Compressive extension mm/sec	Compressive load/newton	Compressive energy/ joule
	Mean ±SD	Mean ±SD	Mean ±SD
Day 1	2.31^{A}±0.00	76.20^{A}±0.00	0.01^{A}±0.00
Day 3	2.42^{A}±0.00	75.20^{A}±0.00	0.02^{A}±0.00
Day 5	2.40^{A}±0.00	73.12^{A} ±0.00	0.02^{A}±0.00
Day 7	2.43^{A}±0.00	70.17^{A} ±0.00	0.02^{A}±0.00
Day 14	2.43^{A}±0.03	70.19^{A} ±0.10	0.03^{A}±0.09
Day 21	2.45^{A}±0.03	69.19^{A} ±0.14	0.03^{A}±0.13

As seen in Table 8.3.1 and 8.3.2, the firmness test for Valencia and Navel control orange fruit samples didn't show any changes compare to freeze treated samples in terms of Compressive extension, load, and energy during the investigations as it displayed above compare to treated samples presented below in Table format.

The results for Valencia fruit types that were investigated for the effect of pre-freeze treatment at -2, -4, -6 and -10 °C for 24 hours are in Tables 8.3.3 - 8.3.5 for compressive extension, compressive load and compressive energy, respectively.

Table 8.3.3 Compressive extension test result for Valencia orange fruit samples freeze treated at -2, -4, -6 and -10 °C for 24 hours.

Compressive extension in mm/sec (mean value)				
Storage time	-2°C/24 hr	-4°C/24 hr	-6°C/24 hr	-10°C/24 hr
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Day1	2.23^{aA}±0.05	4.02^{bA}±0.00	7.12^{bcA}±0.02	9.13^{bcdA}±0.09
Day3	2.62^{aB}±0.00	4.50^{bB}±0.00	7.35^{bcB}±0.00	9.15^{bcdA}±0.01
Day5	2.81^{aB}±0.00	4.50^{bB}±0.00	7.39^{bcB}±0.00	9.35^{bcdB}±0.01
Day7	2.89^{aB}±0.00	4.56^{bB}±0.03	7.53^{bcB}±0.00	9.66^{bcdBC}±0.01
Day14	2.90^{aB}±0.03	4.61^{bB}±0.02	8.59^{bcBC}±0.03	10.76^{bcdBCD}±0.00
Day21	3.98^{aBC}±0.01	5.70^{bBC}±0.03	8.72^{bcBC}±0.02	10.93^{bcdBCD}±0.00

As seen in Table 8.3.3 the compressive extension shows a gradual increase in all samples as follows: at -2 °C from 2.23 ± 0.05 to 3.98 ± 0.01 mm/sec, at -4 °C from 4.02 ± 0.00 to 5.70 ± 0.03 mm/sec, at -6 °C from 7.12 ± 0.02 to 8.72 ± 0.02 mm/sec, at -10 °C 9.13 ± 0.09 to 10.93 ± 0.00 mm/sec of day 1 to 21 storage periods.

Table 8.3.4 Compressive load test results of Valencia orange fruit samples freeze

treated at -2, -4, -6 and -10 °C for 24 hours.

Compressive load in newton				
Storage time	-2 °C/24 hr	-4 °C/24 hr	-6 °C/24 hr	-10 °C/24 hr
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Day1	76.2^{aA}±0.12	48.81^{bA}±0.00	41.61^{bcA}±0.00	34.10^{bcdA}±0.00
Day3	65.2^{aA}±0.00	47.81^{bA}±0.00	40.60^{bcA}±0.00	34.02^{bcdA}±0.00
Day5	63.1^{aA}±0.00	45.72^{bA}±0.00	38.52^{bcA}±0.11	33.80^{bcdA}±0.00
Day7	53.0^{aA}±0.00	41.71^{bA}±0.00	36.42^{bcA}±0.35	33.01^{bcdA}±0.00
Day14	51.0^{aA}±0.00	40.62^{bA}±0.24	34.55^{bcA}±0.22	32.62^{bcdA}±0.31
Day21	48.0^{aB}±0.00	39.58^{bB}±0.20	30.21^{bcB}±0.30	29.60^{bcdB}±0.29

The results for compressive load in Table 8.3.4 shows a gradual decrease for all samples, i.e. at -2 °C from 76.2 ± 0.12 to 48.0 ± 0.00 N, at -4 °C from 48.81 ± 0.00 to 39.58 ± 0.20 N, at -6 °C from 41.61 ± 0.00 to 30.21 ± 0.30 N, at -10 °C 34.10 ± 0.00 to 27.60 ± 0.29 N over the 21 day storage period.

Table 8.3.5 Compressive energy test results of Valencia orange fruit samples freeze treated at -2, -4, -6 and -10 °C for 24 hours

Compressive energy in joule				
Storage time	-2 °C/24 hr	-4 °C/24 hr	-6 °C/24 hr	-10 °C/24 hr
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Day1	0.33^{aA}±0.21	0.21^{bA}±0.00	0.20^{bA}±0.19	0.11^{bcA}±0.00
Day3	0.33^{aA}±0.20	0.20^{bA}±0.21	0.20^{bA}±0.21	0.10^{bcA}±0.00
Day5	0.33^{aA}±0.20	0.19^{bA}±0.00	0.19^{bA}±0.00	0.10^{bcA}±0.18
Day7	0.31^{aA}±0.18	0.18^{bA}±0.17	0.19^{bA}±0.00	0.10^{bcA}±0.00
Day14	0.29^{aA}±0.00	0.17^{bA}±0.16	0.18^{bA}±0.00	0.09^{bcA}±0.16
Day21	0.21^{aB}±0.00	0.16^{bB}±0.00	0.16^{bB}±0.19	0.09^{bcB}±0.20

As shown in Table 8.3.5, the compressive energy showed a gradual decrease at -2 °C from 0.33 ± 0.21 to 0.21 ± 0.00 J, at -4 °C from 0.21 ± 0.00 to 0.16 ± 0.00 J, at -6 °C from 0.20 ± 0.19 to 0.16 ± 0.19 J, at -10 °C 0.11 ± 0.00 to 0.09 ± 0.20 J for the 21 day storage period.

The results for Navel fruit types that were investigated for the effect of pre-freeze treatment at -2, -4, -6 and -10 °C for 24 hours are in Tables 8.3.6 - 8.3.8 for compressive extension, compressive load and compressive energy, respectively.

Table 8.3.6 Compressive extension test result for Navel orange fruit samples freeze treated at -2, -4, -6 and -10 °C for 24 hours

Compressive extension in mm/sec				
Storage time	-2 °C/24 hr	-4 °C/24 hr	-6 °C/24 hr	-10 °C/24 hr
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Day1	2.25^{aA}±0.05	4.22^{bA}±0.06	7.68^{bcA}±0.0	9.22^{bcdA}±0.09
Day3	2.81^{aB}±0.00	4.59^{bB}±0.03	7.89^{bcB}±0.00	9.46^{bcdB}±0.01
Day5	3.01^{aB}±0.00	4.59^{bB}±0.00	7.91^{bcB}±0.00	9.55^{bcdB}±0.00
Day7	3.20^{aBC}±0.00	4.80^{bBC}±0.00	7.93^{bcB}±0.04	9.69^{bcdB}±0.00
Day14	3.22^{aBC}±0.03	4.87^{bBC}±0.00	8.67^{bcBC}±0.03	10.80^{bcdBC}±0.00
Day21	4.01^{aBCD}±0.01	5.60^{bBCD}±0.00	8.92^{bcBC}±0.02	11.00^{bcdBC}±0.04

The results in Table 8.3.6 shows that the compressive extension gradually increased at -2 °C from 2.25 ± 0.05 to 4.01 ± 0.01 mm/sec, at -4 °C from 4.22 ± 0.06 to 5.60 ± 0.00 mm/sec, at -6 °C from 7.68 ± 0.0 to 8.92 ± 0.02 mm/sec, and at -10 °C from 9.22 ± 0.09 to 11.00 ± 0.04 mm/sec over the 21 day storage period.

Table 8.3.7 Compressive load test results of Navel orange fruit samples freeze treated at -2, -4, -6 and -10 °C for 24 hours.

Compressive load in newton				
Storage time	-2 °C/24 hr	-4 °C/24 hr	-6 °C/24 hr	-10 °C/24 hr
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Day1	75.31^{aA}±0.00	47.61^{bA}±0.00	40.61^{bcA}±0.00	38.22^{bcdA}±0.00
Day3	63.22^{aB}±0.00	45.51^{bB}±0.00	38.44^{bcB}±0.00	33.12^{bcdB}±0.00
Day5	61.21^{aB}±0.00	43.42^{bB}±0.00	36.59^{bcB}±0.00	32.72^{bcdB}±0.61
Day7	51.11^{aB}±0.20	41.73^{bB}±0.00	34.46^{bcB}±0.00	31.31^{bcdB}±0.53
Day14	50.09^{aB}±0.14	41.65^{bB}±0.22	33.39^{bcB}±0.61	26.12^{bcdB}±0.61
Day21	46.07^{aB}±0.16	41.59^{bB}±0.23	33.29^{bcB}±0.43	24.10^{bcdB}±0.55

As shown in Table 8.3.7, the compressive load showed a gradual decrease at -2 °C from 75.31 ± 0.00 to 46.07 ± 0.16 N, at -4 °C from 47.61 ± 0.00 to 41.59 ± 0.23 N, at -6 °C from 40.61 ± 0.00 to 33.29 ± 0.43 N, at -10 °C 33.22 ± 0.00 to 31.10 ± 0.55 N over the 21 day storage period.

Table 8.3.8 Compressive energy test results of Navel orange fruit samples freeze treated at -2,-4 , -6 and -10 °C for 24 hours.

Compressive energy in joule				
Storage time	-2 °C/24 hr	-4 °C/24 hr	-6 °C/24 hr	-10 °C/24 hr
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Day1	0.31^{aA}±0.15	0.20^{bA}±0.14	0.19^{bA}±0.00	0.10^{bcA}±0.13
Day3	0.31^{aA}±0.17	0.19^{bA}±0.14	0.19^{bA}±0.00	0.10^{bcA}±0.13
Day5	0.30^{aA}±0.00	0.19^{bA}±0.18	0.18^{bA}±0.00	0.10^{bcA}±0.15
Day7	0.30^{aA}±0.19	0.17^{bA}±0.00	0.18^{bA}±0.19	0.10^{bcA}±0.00
Day14	0.27^{aA}±0.19	0.16^{bA}±0.00	0.18^{bA}±0.19	0.08^{bcB}±0.00
Day21	0.20^{aB}±0.19	0.15^{bB}±0.19	0.14^{bB}±0.19	0.08^{bcB}±0.00

The results in Table 8.3.8 show that the compressive energy has a gradual decrease at -2 °C from 0.31 ± 0.15 to 0.20 ± 0.19 J, at -4 °C from 0.20 ± 0.14 to 0.15 ± 0.19 J, at -6 °C from 0.19 ± 0.00 to 0.14 ± 0.19 J, at -10 °C 0.10 ± 0.13 to 0.08 ± 0.00 J during the 21 day storage period.

8.4 Discussion

In this experimental work, firmness analysis of orange fruit texture was performed under various conditions using a compression/extension instrument. The samples were compared on the basis of the severity of their damage to freeze treatment with control samples and storage time of one to 21 days. In this investigation it was found that the orange fruit firmness was influenced by freeze injury of the fruit, mainly shown by depth of compression and compressive load of the sample. The control orange samples had a higher mean value of firmness measurement for both Valencia and Navel with 76.20 ± 0.00 N, and 76.20 ± 0.20 respectively, compared to the (-10 °C) cold damaged Navel samples which shows the of mean value of 34.10 ± 0.00 N, regardless of location of measurement. Compressive extensions of Navel control samples show speed of 2.31 ± 0.00 to 2.45 ± 0.03 mm/sec compare to (-10°C) freeze treated Navel samples and shows values of 9.22 ± 0.09 mm/sec on day 1 to 11.00 ± 0.04 mm/sec in day 21 with decreased compressive energy from 0.10 ± 0.13 J on day 1 to 0.08 ± 0.00 J on day 21.

Orange fruit samples were freeze-treated to investigate the effect of chilling injury to the firmness of the fruits. In order to understand the difference between freeze treated and control fruit, Instron measurement and firmness analysis of orange fruit texture was performed under various conditions. The samples were compared on the bases of severity of their damage to freeze treated samples and storage time of 1-21 days. It was found that orange fruit firmness was influenced by water loss of the fruit, depth of compression and by the circumference of the sample. Tables 8.1.1 – 8.1.2 for the control samples shows a higher mean value in firmness measurement (76.20 N on day 1, and 71.10 N) on day 21 for Valencia and 76.20 N on day 1, and 69.19 N) on day 21 for Navel accordingly, when the sample was compressed at speed of 8 - 9 mm/sec and compared with frost damaged samples (Tables 8.3.4 and 8.3.7) shows mean value of 48.00 N to 29.6 N and 46.10 N to 27.60 N of -2 °C to 10 °C for Valencia and Navel respectively regardless of location of measurement.

Table 8.3.1 (control sample) shows the statistical analyses of the data with the experimental limits of uncertainly. To investigate the fruit firmness, the fruit was compressed between 8 mm and 9 mm at the top centre and the bottom of the centre. In contrast, however, for the compressive load there is a small difference between day 1 and day 21, where the values change from 76.20 ± 0.72 N to 62.10 ± 0.60 N for Valencia and 76.20 ± 0.72 N to 60.19 ± 0.50 for Navel, ($p < 0.05$) in Table 6.1.1 and 6.1.2 respectively. However, in Table 8.1.4 and 8.1.7 shows value of 34.10 ± 0.51 N to 27.60 ± 0.29 N for Valencia and 33.22 ± 0.55 to 31.10 ± 0.55 N for Navel (respectively) Therefore, the test confirms the impact of freeze damage in combination with the storage, indicating that there is a considerable decrease of quality to freeze treated fruit as the storage time is increased. At this stage, the orange fruit has almost lost most of it is volatile compounds and water level.

It can be seen for the compression extension in Table 8.3.1 and 8.3.2, that the value on day 1 is 2.23 ± 0.04 cm and this increases very slightly to a value of 2.22 ± 0.05 cm on day 21 which is not significant ($p > 0.05$) for Valencia and 2.31 ± 0.05 on day 1 and 2.45 ± 0.03 ($p < 0.05$) in day 21 for Navel samples. This indicated that the fruit was stronger and undamaged fruit, that was not freeze treated, was firm and filled with juices so more energy was required during the compressive energy test however freeze treated fruits indicated light and soft with less energy was recorded during compression test.

In summary, the depth of compression and compressive load is indicative of the cold damage. Compression gives a lesser firmness value and the compressive load is reduced, indicating that there is a significant effect of damage, and the fruit is softer than sound fruit. Orange fruit firmness in these tests was measured by the size of the fruit, the depth of compression and the circumference of the sample. The control samples had a higher measurement when

compressed at 8 mm/sec compared with damaged fruits regardless to location of measurement. The higher values indicate the firmness of the fruit, the control samples showed greater resistance to the compression, and no difference was noted in values obtained when measurements were taken at a different location in all samples.

Comparison was also made with the study conducted by Yankun and Renfu (2006). In their experiment, 100 Apple samples with two different types of apples were investigated, and the statistical result shows the mean firmness of 58.12 N for RD (Red Delicious apples) and 50.79 N for GD (Golden Delicious apples). The standard deviation (SD) = 14.56 N for RD and 13.44 N for GD; minimum firmness = 21.17 N for RD and 25.06 N for GD; and maximum firmness = 99.24 N for RD and 81.51 N for GD. Red Delicious apples showed less firmness than Golden Delicious apples due to the inherent conditions and type of cultivars.

Similarly, in our results, the orange fruit samples that were freeze-treated show less firmness while the untreated (control samples) which were stored at 4 ºC (normal storage temperature) showed consistent of 76.74 N. These figures were less than the maximum of 99.24 N for the apple samples that were tested by Yankun (2007), which simply indicates the difference in rigidity of the fruit type. In another development, Renfu (2004) has also conducted an experiment with apples that was stored in controlled environment of 2% of O_2 and 3% of CO_2 at 0 ºC for about five months, and suggested that better firmness results of correlation coefficient (r) of 0.896 standard error of 6.50 N for firmness were found when compared to untreated samples.

Firmness was also studied by various investigators such as (Nourain et al., 2005; Kim *et al.,* 2005; Døving *et al.,* 2005; Sun 1991). There were great similarities with the work we have conducted in terms of testing for the firmness character of the fruit, and they have reported that firmness can be affected by various types of factors such as ripeness, sogginess, loss of its water and nutrients, and post-harvest handling.

Other similarities to the reports by Nourain *et al.* (2005), Kim *et al.* (2005), Døving *et al.* (2005) and Sun (1991) were found. We noticed, as did these authors, that the effect of frost damage on orange fruit firmness was obvious. In order to understand the effect orange fruit was freeze damaged and stored up to three weeks to investigate the firmness of the fruit during these periods.

With regard to the importance of firmness to the acceptability of apples, Bernd and Jean (2006) investigated apple fruits on trees which were specifically built for predicting the fruit flesh firmness, and the result show coefficients of determination (R2) and standard errors of cross-validation (SECV) of R^2 = 0.93/0.81 and SECV = 7.73/10.50 [N/cm^2]. SSC prediction of freshly

harvested apples using NIR spectrometry was obtained with R^2 = 0.20/0.41 and SECV = 1.29/0.94. This group suggested that these instruments seem certain to remain as key determinants in the determination of apple quality. In our study into the effect of freeze injury, we used Instron tester equipment rather than the Bernd and Jean choice of NIR, therefore there some differences in the results obtained.

Constantino and Carlos (2007) also investigated the correlations between destructive and non-destructive firmness measurements of fruit firmness, and achieved a significant difference (p = 0.0001), even though the values were too low for commercial applications. The group suggested that the values varied from R^2 = 0.60–0.71, according to fruit type. It seems that firmness values are more sensitive in detecting treatment differences between types of fruits. In the study that was carried by Crisosto et al. (2004), plums were segregated into two classes ("ready to eat" and "ready to buy") by using a UCF firmness threshold of 13 N. This threshold was chosen based on sensory work that demonstrated that plum consumers' acceptance increased for fruit with a UCF firmness < 13 N (Crisosto et al., 2004). While this is a good firmness value for plums, results cannot be directly compared with orange due to the fruit size difference. It is understandable that we should expect higher values in the orange samples.

According to Subedi and Walsh (2009), most cultivars of almost 100 fruit were investigated and the result from statistical analyses for 'Ivory Princess' peaches shows that 41 samples gave results lower than desired. Destructive firmness ranged from 13.3 N to 124.5 N for fresh peaches, 35.6 – 133.3 N for nectarines and 4.4 -115.6 N for plums. The non-destructive firmness values ranged from 0 to 18 SFI for peaches, 1 – 16 SFI for nectarines and 0 – 13 SFI for plums. These firmness ranges covered low maturity to ripe fruit, including the standard commercial firmness range.

In a different investigation, Subedi and Walsh (2008) also studied the assessment of fruit firmness in banana, mango and peach fruits using a penetrometer (Fpen). Results were linearly correlated (R^2 > 0.8) with sound velocity in mango, but not in peaches or bananas. Spectra were related to the penetrometer and sound velocity readings, using a partial least squares regression. A cross-validation result of R^2 = 0.92, 0.86 and 0.79 for the penetrometer reading and R^2 = 0.88, 0.77 and 0.58 for the sound velocity reading was achieved for banana, mango and peach fruit, respectively. These results are in disagreement with our results since the group used penetrometer while our investigation is based on an Instron tester.

Ali Batu (1998) also investigated the firmness of tomatoes using a Universal Instron, and noted that "during destructive measurement of firmness, fruits should have firmness values above 1.45 N/mm but the Instron values of the tomato in the consuming stage at home, should be

higher than 1.28 N/mm. The firmness of tomatoes is closely associated with acceptability levels of the fruits. Subjective evaluation scores based on finger-feel were highly and positively correlated (0.96 and 0.98) (Ali Batu, 1998). Even though we have used orange fruits, our results are well above 1.28 N as (Ali Batu, 1998) was suggesting. According to experiments that were conducted by Marcio *et al.* (2013), three major commercial avocado cultivars grown in Florida were tested for fruit firmness based on whole fruit texture, respiration and ethylene evolution. The result show that firmness was reduced, with the recorded measurement of 118 N for the samples that were treated with ethylene due to ripening and (softness) compare to control samples which showed more firmness with a value of 268 N. As Marcio *et al*. (2013) indicated, our practical results also experienced similar scenarios with freeze-treated samples showing a drop in firmness value of 65.53 N in day 1 to 48.81 N in day 21.

In a similar study, a laser Doppler vibrometer (LDV) was used for non-contact recording. Firmness decreased exponentially as expected (304.1 N to 2.1 N) over six days. The LDV results showed significant differences between days, treatments and laser-location. The resonant frequency of the fruit decreased linearly until day 4 and then decreased more slowly from 1671 to 476 Hz (Rosalia 2013). White *et al.* (2005) also studied firmness of kiwifruit, and suggested that the results were dependent on the equipment used, treatment type, the type and size of the fruit.

Comparisons were made with our results which agree with a statistical result of the mean firmness value of compressive load 72.23 N. for day 1 samples. day 3 samples showed less firmness, with the value of compressive load of 65.53 N in day 3, and a result of 65.29 N was obtained in day 5, Less firmness was found with the compressive load of 58.64 N on day 7 samples, 51.46 N on day 14 and 48.81 N for day 21 samples. Our results are different from Subedi and Walsh (2009) due to types of fruits and treatment type; however, our day 21 values (48.81 N), shows much closer relation to peaches with 35.6 - 133.3 N.

Chapter Nine

9.1 Summary of Findings and Future Recommendations

It is well known that fruits are universally recommended as being an important part of a healthy diet (Lamikanra *et al.,* 2005). However, maintaining the quality of the harvested product and instituting proper post-harvest handling in order to preserve the appealing characters of the fruit, requires significant depth of knowledge of post-harvest product physiology. Quality is of underlying importance since (i) a poor-quality product will not be acceptable for consumption, and (ii) some of the healthy attributes of the fruit may be degraded by poor handing practices (Bartz and Brecht, 2003).

However, even if one is equipped with a complete knowledge of proper fruit handing, it is inevitably difficult to avoid environmental challenges because harvested fruits are 'living' entities. They, therefore, continue to perform metabolic functions even though they are in the post-harvest state. Quality deterioration is common in harvested fruits as a result of a combination of physiological, mechanical, microbiological and environmental factors and storage conditions (Sinha, 2011). It is believed that intracellular ice formation due to freeze damage causes huge damage to the fruit harvest and accounts for millions of dollar losses within the post-production chain (Sinha, 2011). Improvement of post-harvest handling practices to minimize these losses will not only contribute to increasing the income of farmers, but would also ensure the availability of superior quality produce to the consumer at a reasonable price.

In this Study, the effects of low temperature were investigated on Valencia and Navel orange fruit cultivars. During this study, the quality of the fruit treated with different conditions was closely monitored and the experimental results and consequent discussions were presented in the Thesis. The investigations were focused on internal and external damage and quality changes that are influenced by the simulated treatment of the different freezing regimes of -2 to -10 °C for 2 - 24 hours, and up to three weeks storage. The parameters examined were pH, TSS, volatile compounds including limonene, and production of ethanol; these were observed for freeze damaged as well as control orange samples. When freeze injury was incurred at low temperature to most of the orange samples due to ice formation, the quality of the fruits was totally affected both internally and externally during the storage periods.

The investigation was particularly focused on four of the above parameters in identifying the sound fruits from the damage ones using chemically identification methods rather than traditional methods. The older approach of density separation or the later density sorting technology to separate frost-damaged produce using accurate diameter and weight readings

for individual samples of produce, takes at least six weeks before it can be used to pick up damage. However, in our practical work, it has been shown that the damaged fruits can be sorted and identified during a storage period of just over a week after harvest by using chemical identification methods.

Change in pH, reduction in TSS values, internal chemical changes that trigger the loss of important volatile compound such as limonene from the orange fruit, as well as the production of ethanol, have been seen as one the important and key identification methods of sorting frost damage fruit before it reaches the packaging process. This uses GC and GC-MS analysis. The weight loss, visual inspection and diameter of the fruits were also measured and investigated to categorise the fruits' internal and external fruit quality.

Sorting for fruit quality is a usual manual operation in the packing of agricultural produce. The process has considerable economic implications for the industry and significantly influences the quality of the marketed commodity. Therefore, it would be of great benefit to the industry if the sorting process could be revised by the introduction of innovative methods that are rapid, simple and affordable. Furthermore, the industry can be assisted by the modification of some of the scientific study results similar to this investigation that can reduce the amount of labour input that the farmers put in the sorting of citrus fruits. This sorting is dependent on, and is expressed in terms of, certain quality parameters of the produce. On the basis of the series experiments that were performed in this investigation, it is possible that a model can be developed to express the interdependence of the quality parameters and intensive nature of work input.

In addition to the above reports, a number of experiments such as microbiological tests and fruit firmness tests were also conducted in order to evaluate important aspects of quality parameters. On the bases of a fruit-damage investigation, these practical works were able to develop easy and simple methods of using hand-held ethanol test equipment for determination and sorting the damaged orange fruits from sound orange fruits which can be used in remote areas as well as in storages. Practical procedures were provided in Chapter Six.

This practical work was solely focused on mature oranges of both Valencia and Navel cultivars, as maturity at harvest is the most important determinant of storage-life and final fruit quality. Immature fruit are highly susceptible to shrivelling and are of inferior flavour quality when ripe. Overripe fruit are likely to become soft and mealy with insipid flavour soon after harvest and become highly susceptible to damage during subsequent post-harvest handling operations. Harvesting of tree fruits at the correct stage of maturity is important in preserving quality and for minimizing post-harvest losses.

9.2 Future Research Directions

The nature of the freeze-damage of Valencia and Navel orange fruits used in this study, in terms of verifying the extent of damage using mature fruits, are based on the concept that mature fruit are more vulnerable to freeze damage than immature fruits. This is due to the level of rigidity and softness as well stronger and softer internal structures. A further project that might be mounted is a further investigation of fruits that are in different maturity levels to determine how much damage can arise at different levels. In order to do this, it would be necessary to follow up the effect of maturation levels in the field as well as the variations caused by freezing events and the freezing temperatures.

As our practical work focused on freeze treated orange samples, and this analysis enabled us to investigate the variable temperature ranges that can give a clearer understanding of the freeze-damage effects, and this project could be modified and repeated in the field, where frost damage mostly occurs. Industry is keen that machine-sorting technology, that would be placed next to a conveyer belt, to be developed in conjunction with software that allows the system to intuitively recognize produce types and aspects of damage, and to adjust grading accordingly using mathematical algorithms. This can improve mechanical packaging systems in a reduced time frame to allow detection of damaged orange fruits within a few seconds. Indeed, Colour Vision Pty Ltd is hoping to achieve an infra-red orange sorting machine for detecting damaged fruits and has a prototype infra-red detection device. It would be useful to compare results with their equipment and the findings from this thesis to verify the accuracy of both approaches. Our methods can be further improved by the introduction of simple GC equipment with a small gas cylinder that can be easily transported and moved around. This can facilitate testing involving affordable prices rather than relying on the currently used large, heavy and expensive GC equipment.

Chapter Ten

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