THE NATURE OF CHILLING INJURY IN AVOCADO FRUIT

by
PHATLANE WILLIAM MOKWALA

Submitted in partial fulfilment of the requirement for the degree

MAGISTER SCIENTIAE

in BOTANY

in the Faculty of

MATHEMATICS AND NATURAL SCIENCES

at the

UNIVERSITY OF THE NORTH

SUPERVISOR: PROF. D.C.J. WESSELS
CO-SUPERVISOR: PROF. J.J.M. MEYER

1999

I, the undersigned declare that the thesis for the Degree of Masters in Botany at the University of the North hereby submitted, has not been previously submitted by me for a degree at this or any other University, that it is my own work in design and that all material contained herein has been duly acknowledged.

When you are making a success of something, it's not work. It's a way of life. You enjoy yourself because you are making a contribution to the world.

- Andy Granatelli

CONTENTS

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 4

RESULTS

CHAPTER 5

DISCUSSION AND CONCLUSION

CHAPTER 6

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 The avocado fruit

The avocado, *Persea americana* (Mill) (Family Lauraceae) is a subtropical fruit which originated in central America, and is cultivated in most tropical and subtropical areas of the world. Avocado fruit varies in shape from spherical to oval to pearshaped. There are three ecological races of the avocado namely; Mexican, Guatemalan and West Indian. The Mexican race can be regarded as subtropical, the Guatemalan as semitropical and the West Indian as tropical. Fruits of the Mexican race are smaller with a smooth skin and are adapted to the cooler parts of the tropics and subtropics. They are thus cold tolerant and mature early. Fruits of the West Indian race are medium to large, with thick, soft and smooth skin, and are adapted to tropical conditions of high humidity accompanied by high temperatures. Fruits of the Guatemalan race are of intermediate characteristics (De Arriola *et al.* 1979; Salunkhe & Desai 1984).

The avocado is a climacteric fruit with the respiration rate declining after harvest, before the climacteric rise (Bennett *et al.* 1987). According to Koster (1986), the different parts of the fruit (stem end, middle and blossom end) have different rates of respiration with the slowest rate of respiration at the stem end and the highest rate at the blossom end. Koster (1986) further states that after storage at 5.5°C, the respiration rate of the fruit deviates from the normal pattern, being high after removal from cold storage and decreases steadily with ripening. The avocado fruit does not

ripen on the tree and according to Salunkhe and Desai (1984), Hass and Fuerte fruit can remain on the tree after reaching maturity for six months or longer. In general, fruit of summer cultivars remain attached to the tree for shorter periods compared to winter cultivars. Cell division proceeds in the fruit even after harvest.

1.2 Uses of the avocado fruit

The avocado fruit is of high nutritional value. It is rich in vitamin B6; has a high oil content ranging from 3 to 30%; its total protein concentration ranges between 1.21 and 2.26% and the total carbohydrate concentration ranges from 1.82 to 7.80% (Salunkhe & Desai 1984). Because of their low sugar content, avocados are often recommended as a high energy food for diabetics. The avocado fruit is normally consumed fresh as thermal processing gives it a bitter after-taste (De Arriola *et al.* 1979). Avocado fruits are also used in salads, soup, spreads, purees and guacamole. Its oil is used in the cosmetic industry, and the non-saponifiable fraction of the oil has a dermatological use (De Arriola *et al.* 1979).

1.3 World cultivation of the avocado

Major avocado producing areas are the North American continent and the Caribbean Islands, which jointly account for 86% of world production. Four countries, namely; Mexico, U.S.A., Dominican Republic and Brazil dominate world avocado production with more than 100 000 tons per annum each in 1983 (Van Zyl & Groenewald 1986). In 1994 Mexico alone produced 718 000 metric tons equalling the production of the next seven largest producers combined (Anon 1995). South Africa produces less than 2% of world production. Major producers are not necessarily major exporters. The export-import dynamics change with season, improvement in production and increase in consumption. The major exporters are Israel, South Africa and the United States of America in that order. Israel and South Africa account for about 90% of the world's avocado export (Van Zyl & Groenewald 1986; Anon 1995). In 1994 the U.S.A. imported 23 932 metric tons avocados while at the same time exporting 9 669 metric tons

avocados (Anon 1995). Europe constitutes about 80% of the world's importers, while the United Kingdom and France make up about 85% of the European market.

The Israeli harvest period starts in September and ends in May, while the United States of America (USA) harvests throughout the year. South Africa has its peak harvest period from June to July. At this time it competes with the USA and to a lesser extent the Ivory Coast and Kenya for the European market (Van Zyl & Groenewald 1986). During this period competition from other avocado producing countries on the European markets is less intense. However, it is still a difficult time for the South African avocado exporters as the avocado has to compete with asparagus and a multitude of different fruits and vegetables on the European market (LeClercq 1989).

Varieties or cultivars cultivated in South Africa include: Fuerte, Hass, Ettinger, Pinkerton, Ryan, Duke 7, Bacon, Rinton and G6. In South Africa, as in the rest of the world, Fuerte was initially the most widely cultivated cultivar (Köhne 1991; Kremer-Köhne *et al.* 1991). However, this situation has changed where in 1994 the most widely cultivated variety was Hass (Anon 1995). Fuerte is a hybrid between Mexican and Guatemalan races, while Hass belongs to the Guatemalan race (De Arriola *et al.* 1979).

South Africa exports mainly Fuerte and Hass fruits; Fuerte accounting for about 56% of the South African export (Kremer-Köhne *et al.* 1991). Hass fruits are, however, more popular in France. According to Kremer-Köhne and co-workers (1991), Fuerte fruits are more prone to chilling injury than other cultivars. If the shelf life and resulting quality of this cultivar can be improved, exporters will receive better returns on their produce, as poor quality of one cultivar influences the sale of others from the same country. Buyers in the overseas market are conscious of the country of origin of the fruit, and they consequently switch from one country to another in the event of poor quality fruit (LeClercq 1989).

1.4 Cold storage of fruit during transportation

South African avocados take about twenty eight days to reach the European market by sea. The challenge facing South African exporters is to place fruit of good quality on the overseas market. The ideal is to export firm fruit with no external cold damage and no physiological disorders. Low temperature is the major commercial method used to prolong the shelf life of South African export avocados (Vorster *et al.* 1990; Vorster *et al.* 1991). Temperature management integrates various principles including a specific temperature regime dependent on the maturity and chilling sensitivity of the fruit at a specific stage of maturity.

Storage temperatures that are too high result in soft fruit on arrival overseas, while temperatures that are too low result in fruit with external cold damage. Accepted standards of avocado fruit quality are fruit firmness lower than 35 units (as measured with a firmometer) and cold damage of less than 1% (Truter *et al.* 1991). South African avocados are stored at a temperature of 5.5°C during shipment to the European market. Avocado fruit, however, develop physiological disorders when stored at this low temperature. These physiological disorders include: softness, skin discolouration, grey pulp, chilling injury, pulp spot and vascular browning (LeClercq 1989; Eksteen & Henning 1992; Bezuidenhout 1992).

Controlling the storage atmosphere (5.5°C; 10% CO_2; 2.0% O_2 and 88% N_2) can also prolong the shelf life of avocados (Ginsburg 1985). Such fruit must be cooled rapidly, held at optimum temperature and shipped under these controlled atmospheric conditions to arrive at the market in a sound and attractive condition. However, a lack of controlled atmosphere container availability makes this service impossible (Bower *et al.* 1989).

Carbon dioxide treatment can also be used to prolong the storage life of fruit. This treatment involves exposure of fruit to 25% CO_2 in a normal oxygen atmosphere for three days. This treatment is said to lower polyphenol activity and total phenol

content (Bower *et al.* 1989). According to Truter *et al.* (1991), CO_2 treatment can extend the storage life and maintain the quality of avocado fruit. This treatment decreases the potential of post harvest disorder development and is relatively easy to apply.

In Hawaii where cold treatment of avocado fruit at 2.2°C is carried out as a disinfection treatment against Mediterranean fruit fly, Nishijima *et al.* (1995) found that a heat pretreatment at 38°C for ten hours induces chilling tolerance in Sharwil avocado fruit. Pesis *et al.* (1994) found that prestorage treatment of Fuerte avocado fruit with a low oxygen atmosphere (3% O_2 and 97% N_2) for twenty four hours significantly reduces chilling injury symptoms after storage at 2°C for three weeks. This treatment was found to lower respiration and ethylene production rates during cold storage and after cold storage when fruits are maintained at 17°C.

CHAPTER 2

LITERATURE SURVEY

2.1 Plant responses to chilling temperatures

Plants grow under various environmental conditions. Different types of plants have different minimum and maximum ranges of environmental conditions under which they grow. Each type flourishes under a set of optimal conditions; outside this range a stressful situation arises. Stress can be defined as any environmental factor potentially unfavourable to living organisms. It can result in an aberrant change in physiological processes and has the potential to produce injury. Factors that can bring about stress are water, temperature, nutrients, salt and radiation (Levitt 1980; Hale & Orcutt 1987).

Organisms that can survive a stressful situation are said to be resistant to that type of stress. Stress resistance refers to the ability of a plant to survive an unfavourable factor and even grow in its presence. This ability involves various combinations of tolerance and avoidance strategies. Stress avoidance occurs when a plant does not come to equilibrium with the stress factors or can exclude the stress by means of a physical or metabolic barrier. Stress tolerance on the other hand occurs when a plant comes to equilibrium with the stress factors without injury (Levitt 1980).

A low temperature stress usually results in chilling injury. Chilling injury occurs when the temperature drops to some point above freezing, but low enough to cause

damage to cells, tissues, or organs. Various physiological and biochemical alterations occur in response to chilling stress. The extent of the alterations and ability of the plant to withstand them determines the sensitivity of the plant to the chilling stress.

The primary response of plants to chilling stress occurs in membrane alterations. Cellular membranes undergo a physical phase transition from a flexible liquid-crystalline to a solid-gel structure (Wang 1982). Physical phase changes in membranes may lead to secondary and/or irreversible changes depending on the temperature, length of exposure and susceptibility of the plant to chilling at that temperature. Secondary responses include loss of membrane integrity, leakage of solutes, loss of compartmentation, decrease in the rate of mitochondrial oxidative activity, increase in activation energy of membrane-associated enzymes, cessation of protoplasmic streaming, reduction in energy supply and utilisation, decrease in photosynthetic rate, metabolic imbalance and accumulation of toxic products (Graham & Patterson 1982; Wang 1982).

2.2 Chilling injury

Chilling injury is a physiological disorder that develops in tropical and subtropical plants grown at low but non-freezing temperatures (1°C to 10°C) that would normally not harm plants from temperate zones (Somerville & Browse 1991). Tropical plants prone to chilling injury include; citrus, cotton, maize, avocados, rice, sorghum, soybean, sugarcane and sweet potatoes. Symptoms of chilling injury are: reduction in rates of growth and development, altered chloroplast structure, development of lesions, browning of the flesh in fruits, skin darkening in fruits, cessation of protoplasmic streaming and stimulation of ethylene production (Roughan 1985; Zauberman *et al.* 1985; Wise & Naylor 1987a and 1987b; Eaks 1990; Luza *et al.* 1992).

2.3 Causes of chilling injury

Chilling injury is believed to be a result of membrane disorganisation in cells. This results in loss of cell compartmentation, increased membrane permeability and leakage

of ions and small electrolytes (Murata & Yamaya 1984; Wang *et al.* 1992). Membrane disorganisation may result from (1) phase transition in the membrane and from (2) peroxidation of lipids in the membrane (Platt-Aloia & Thomson 1987; Wise & Naylor 1987a and 1987b).

2.3.1 Chilling injury as a result of phase transition in membranes

As temperature is lowered, lipids in some cellular membranes solidify, thereby changing from a liquid-crystalline to a solid-gel state. This is due to re-ordering of some membrane phospholipids (Raison & Orr 1986a; Platt-Aloia & Thomson 1987; Wang *et al.* 1992). This then results in the chilling injury symptoms discussed above. The temperature at which phase separation occurs is determined by the ratio of saturated to unsaturated fatty acids in the lipids. A relatively high saturated fatty acid content induces chilling sensitivity to the membranes. On the other hand, a high amount of unsaturated fatty acids induces chilling resistance to the membranes.

By differential scanning calorimetry Raison and Orr (1986a) found that thermotropic phase transition of thylakoid lipids occurs at temperatures similar to those at which chilling injury occurs. The calorimetric exotherms occurred at 7°C, 10°C and 12°C for oleander, mung bean and tomato respectively. They concluded that thermotropic phase transition takes place at lower temperature limits, below which chilling injury develops in plants, and that transition in the physical and functional properties of cell membranes occurs at the same temperature. They found that in mitochondrial membranes thermotropic phase transition takes place at a few degrees above the temperature below which chilling injury often occurs. According to these authors phase transitions in mitochondrial membrane lipids occur at: 15°C, 16°C, 15°C, 14°C and 15°C for soybean hypocotyl, tomato fruit, cucumber fruit, mung bean hypocotyl and sweet potato respectively. Phase transition could not be observed in lipids of mitochondria from chilling-resistant plants at temperatures above 0°C.

Murata and Yamaya (1984) employed fluorescence polarisation to show that phosphatidylglycerols from leaves of chilling-sensitive plants went into a state of phase

separation at a temperature of about 30°C. Phosphatidylglycerols from chilling-resistant plants went into a phase separation state at much lower temperatures. The other classes of lipids (phosphatidylycholine, phosphatidylethanolamine, phosphatidylinositol) remained in a liquid crystalline state at temperatures between 5°C and 40°C. These findings suggest that phosphatidylglycerol from chilling-sensitive plants undergoes phase transition at room temperature and above. This leads to ion leakage and diminishing ion gradients across membranes that are essential for the maintenance of the physiological activities of the cell. They concluded that only phosphatidylglycerol can induce phase transition.

2.3.1.1 Phosphatidylglycerol and phase transition in membranes

Chilling-resistant plants have phosphatidylglycerols with a lower proportion of disaturated molecular species (phosphatidylglycerol having two saturated fatty acids), while in chilling-sensitive plants disaturated molecular species account for 50 to 60% of the total phosphatidylglycerol (Roughan 1985; Raison & Wright 1983).

A phospholipid with two molecules of saturated fatty acids has a phase transition temperature higher than room temperature (Murata *et al.* 1982). A 18:0/18:0 phosphatidylcholine has a phase transition at 58°C; 16:0/16:0 phosphatidylglycerol has a phase transition at 42°C; 18:0/18:1 at 3°C; and 16:0/18:1 phosphatidylcholine has a phase transition at -5°C. A saturation bond decreases the temperature at which phase transition takes place; a *trans* unsaturation bond is less effective than a *cis* unsaturation bond, and an unsaturation bond near the end of a hydrocarbon is much less effective than one at the centre (Murata *et al.* 1982). This information indicates that a molecular species having at least one *cis* unsaturation bond will undergo the gel to liquid crystalline phase transition below room temperature, and therefore cannot form a gel in the cellular membranes of higher plants (Murata *et al.* 1982).

2.3.1.2 Acclimation in chilling injury caused by phase transition

Exposure of chilling-sensitive plants to a low nonchilling temperature (15°C) before exposure to chilling temperatures (5°C) induces the plant to become resistant to chilling injury. This treatment is referred to as preconditioning, cold hardening or acclimation (Gilmour *et al.* 1988; Wang *et al.* 1992).

Acclimation induces some adaptive response to chilling stress. Some physiological responses to acclimation are: increase in sugar and starch content, changes in mRNA and a decrease in protein content and lipid soluble phosphate. Acclimation prevents the loss of phospholipids that usually occurs during storage at chilling temperatures in nonacclimated plants. The degree of unsaturation of phospholipids also increases in acclimated plants (Gilmour *et al.* 1988; Wang *et al.* 1992). Generally, acclimation before exposure to chilling temperatures contributes to the maintenance of high membrane fluidity, resulting in normal membrane functions during the chilling exposure (Wang *et al.* 1992).

2.3.2 Chilling injury as a result of lipid peroxidation

Chilling temperatures, together with high light intensities, cause photooxidative lipid peroxidation in chilling-sensitive plants. Wise and Naylor (1987a and 1987b) found that in cucumber, maximum lipid peroxidation occurs at 5°C and a light intensity of 1 000 microeinstein m^{-2} s^{-1}. Symptoms of chilling injury in light are; rapid appearance of photosynthetic dysfunction, altered chloroplast ultrastructure and cellular lipid degradation (Wise & Naylor 1987a and 1987b; Parkin & Kuo 1989; Prasad *et al.* 1994a and 1994b).

Wise and Naylor (1987b) suggest that light reactions of photosynthesis leaks some energy to molecular oxygen, forming toxic oxygen species. These toxic oxygen species are the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$). The toxic oxygen species cause peroxidation of fatty acids esterified to phospholipids as well as those that are not esterified to lipids. This produces free

hydrocarbon radicals. The free hydrocarbon radicals cause further peroxidation of fatty acids resulting in extensive degradation of membrane lipids, proteins and DNA. Polyunsaturated fatty acids like linolenic acid are more susceptible to peroxidation (Vick & Zimmerman 1987).

Lipid peroxidation once initiated can continue on its own, a process called auto-oxidation. The process progresses through three phases namely the initiation phase, propagation phase and termination phase (Larson 1988). In the initiation phase a hydrogen atom is abstracted from an organic compound to form an organic free radical ($R^{\cdot}$).

During the propagation phase the organic free radical reacts with oxygen to form a peroxy radical ($ROO^{\cdot}$). Free radicals of fatty acids are highly reactive with amino acids and proteins and can cause damage to membrane structure and organisation. The peroxy radicals can abstract hydrogen from other organic molecules, forming more organic free radicals (Halliwell 1984).

In the termination phase, usually in the absence of oxygen, the free radicals recombine with each other to form inactive, nonradical products.

$ROO^{\cdot} + R \rightarrow ROOR$

$2ROO^{\cdot} \rightarrow ROOR + O_2$

Acclimation or preconditioning induces the production of enzymes and antioxidants which scavenge toxic oxygen species and free hydrocarbon radicals. Glutathione increases in acclimated tomato (Walker & McKersie 1993) and catalase increases in acclimated maize seedlings (Prasad *et al.* 1994b). Croft *et al.* (1993) believe that induction of catalase by acclimation may be the first line of defence against oxidative damage throughout the cell. The induction of peroxidases in cell walls leading to elevated lignin content in acclimated maize seedlings suggests that lignification may be a component of acclimation-induced tolerance to chilling.

2.3.3 Oxygen toxicity and lipid peroxidation

Oxygen is an essential gas for living organisms but can unfortunately also be toxic. Oxygen toxicity is mainly due to the production of reactive oxygen species under normal and stressful situations. Atmospheric oxygen is regarded as the principal agent for the deterioration of organic material exposed to air. The toxic oxygen species are: the singlet oxygen ($^1O_2$), the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\cdot$). Toxic oxygen species are by themselves less reactive (except for the hydroxyl radical) with organic molecules but can form damaging species (Daub & Hangarter 1983; Halliwell 1984; Doke & Ohashi 1988). Cellular components susceptible to toxic oxygen species are lipids (peroxidation of unsaturated fatty acids in membranes) and nucleic acids (mutation).

Molecular oxygen can also be toxic. Polyunsaturated fatty acid side chains are prone to undergo lipid peroxidation when oxygen reacts with conjugated dienes to produce lipid peroxides (Halliwell 1984). Lipid peroxides decompose to give aldehydes and volatile hydrocarbons. Peroxides and their degradation products cause damage to enzymes and membranes, causing a decrease in electrical resistance and membrane fluidity. Loss of compartmentation may lead to the release of hydrolytic enzymes and organic acids which then cause cell damage.

The rate of lipid peroxidation is increased by the presence of transition metal ions like iron and copper (Fe^{2+} and Cu^{2+}). Lipid peroxidation is autocatalytic and the peroxide radicals produced will cause further lipid peroxidation and production of more peroxide radicals. Scavengers like α-tocopherol, and ß-carotene trap peroxide radicals, thereby breaking the chain of reactions (Dumelin & Tappel 1977; Hudson & Mahgoub 1980; Halliwell 1984).

2.3.3.1 The singlet oxygen

Chlorophyll can transfer excitation energy to molecular oxygen changing it to a reactive singlet oxygen ($^1O_2$). The singlet oxygen can survive longer in hydrophobic

environ-ments where it causes lipid peroxidation. It attacks several biological molecules including amino acids and fatty acids. Its reaction rate with polyunsaturated fatty acids increases with an increase in the degree of unsaturation (Larson 1988).

The singlet oxygen can also be formed when cercosporin, a fungal toxin, is activated by light. Cercosporin kills plant cells in the presence of light (Daub & Hangarter 1983) and does not damage the host plant by itself. In the presence of light and the excited triplet form of oxygen, cercosporin reacts directly with molecular oxygen to produce singlet oxygen and /or a superoxide radical (Scandalios 1993), which then causes damage.

2.3.3.2 The superoxide anion

A superoxide anion is formed when electrons are misdirected and donated to oxygen. Some cellular oxidation reactions can transfer an electron to molecular oxygen to produce the superoxide anion (O_2^-). Such reactions are catalysed by enzymes like galactose oxidase, xanthine oxidase, and nitropropane oxidase (Halliwell,1984). Auto-oxidation of some compounds can also transfer electrons to oxygen, producing a superoxide anion.

Superoxide is not reactive with most organic compounds but may give rise to more reactive species which are potentially toxic. It can for instance be converted into hydrogen peroxide, a reaction catalysed by the enzyme superoxide dismutase (SOD) (Halliwell 1984).

The superoxide anion can be formed in normal unstressed illuminated chloroplasts as well as under conditions of oxidative stress. When cercosporin is illuminated, it produces both the singlet oxygen and the superoxide anion. In tobacco leaves infected with the tobacco mosaic virus (TMV), superoxide generation is activated at the TMV multiplication site. Necrotic lesions are formed when NADPH-dependant superoxide generating reactions are activated following infection by the virus (Doke & Ohashi 1988).

Hypersensitive reactions of cells to bacterial infection induces superoxide initiated lipid peroxidation (Keppler & Baker 1989). Normal plant cell membrane structure and function are altered during the hypersensitive reaction to the pathogen.

2.3.3.3 Hydrogen peroxide

Some oxidase enzymes transfer two electrons to oxygen to produce hydrogen peroxide. Examples of such enzymes are: glycolate oxidase; urate oxidase; and amino acid oxidase (Halliwell 1984). Oxidative stress causes the production of hydrogen peroxide and other reactive oxygen species. The superoxide anion can be converted into hydrogen peroxide, a reaction catalysed by superoxide dismutase.

2.3.3.4 The hydroxyl radical

The hydroxyl radical is the most reactive of the active oxygen species. It reacts indiscriminately with organic compounds. The hydroxyl radical can abstract hydrogen from fatty acids to produce hydrocarbon radicals, thereby causing lipid peroxidation. It can be derived from molecular oxygen by the sequential univalent reduction of oxygen to water, a pathway that involves the formation of both the superoxide anion and hydrogen peroxide.

$$O_2 + e^- \rightarrow O_2^- \quad (1)$$

$$O_2^- + e^- + 2H^+ \rightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + e^- + H^+ \rightarrow OH^\cdot + H_2O \quad (3)$$

$$OH^\cdot + e^- + H^+ \rightarrow H_2O \quad (4)$$

Although the hydroxyl radical can be converted into water, it is very reactive and can cause damage to cells. An effective way of preventing the damage it causes is by removing the intermediates superoxide and hydrogen peroxide. Some enzymes, superoxide dismutase and catalase, as well as some organic antioxidants can effectively do this (Foyer & Halliwell 1976; Fridovich 1978; Halliwell 1984).

2.3.4 Lipid peroxidation caused by toxic oxygen species

The singlet oxygen can react directly with free unsaturated fatty acids and fatty acid side chains of lipids to produce hydroperoxides.

$$RH + {}^1O_2 \rightarrow ROOH \qquad (5)$$

R = the alkyl chain of the fatty acid or lipid.

ROOH = a hydroperoxide

In this way the singlet oxygen causes lipid peroxidation without forming free hydrocarbon radicals.

The hydroxyl radical abstracts a hydrogen from free fatty acids or fatty acid side chains to produce a conjugated diene hydrocarbon radical and water.

$$RH + OH^{\cdot} \rightarrow R^{\cdot} + H_2O \qquad (6)$$

The hydrocarbon radical then reacts with molecular oxygen to produce a peroxide radical.

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \qquad (7)$$

The peroxide radical abstracts a hydrogen atom from another free fatty acid or fatty acid side chain of a lipid to produce a hydroperoxide and a hydrocarbon radical.

$$ROO^{\cdot} + R^1H \rightarrow ROOH + R^{1\cdot} \qquad (8)$$

The hydrocarbon radical can react with oxygen to produce a peroxide radical which can abstract a hydrogen from fatty acids or fatty acid side chains. This indicates that once initiated, peroxidation can carry on as long as enough oxygen is available. The hydroperoxide produced can fragment to produce aldehydes like malondialdehyde and volatile gases like ethane and pentane (Dumelin & Tappel 1977; Halliwell 1984; Vick & Zimmerman 1987).

2.3.5 Lipoxygenase activity and lipid peroxidation

The enzyme lipoxygenase, linoleate: O_2 oxidoreductase (LOX) catalyses the incorporation of molecular oxygen into polyunsaturated fatty acids. It has a molecular mass of about 1000 dalton, and contains one iron or copper atom. The most preferred substrates are those that contain *cis-cis* 1,4-pentadiene structures like linoleic and

linolenic acids (Vick & Zimmerman 1987). Oxygen is incorporated into number 6 and number 10 positions to produce conjugated hydroperoxydienes.

2.3.5.1 Distribution of lipoxygenase in plants

Lipoxygenase is widely distributed in plants, especially in dicotyledons (apple, cotton, gooseberry, grapes, pear, strawberry, sunflower and tea leaves) (Vick & Zimmerman 1987). It also occurs in some fungi (e.g. bakers' yeast). It is found in storage parenchyma cells in epidermal and hypodermal cells as well as cells surrounding vascular bundles. Inside the cell it occurs in the cytoplasm, vacuoles and chloroplasts. There are two types of lipoxygenase, namely, Type 1 and Type 2. Type 1 has an optimum activity at pH 9.0 and Type 2 has an optimum activity between pH 6.5 and 7.0 (Vick & Zimmerman 1987).

2.3.5.2 Activity of lipoxygenase

The native form of the enzyme (the one in which the iron atom is divalent) is inactive. This native form is activated by complexing with a fatty acid hydroperoxide in a reaction that does not require oxygen (anaerobic). Products of the reaction are an active lipoxygenase (E-Fe(III)), a hydroxyl ion and an alkoxy radical.

$$\text{E-Fe(II)}_{\text{native}} + \text{LOOH} \rightarrow \text{E-Fe(II)-LOOH}$$

$$\text{E-Fe(II)-LOOH} \rightarrow \text{E-Fe(III)}_{\text{active}} + \text{LO}^{\cdot} + \text{OH}^{-} \qquad (9)$$

LOOH = fatty acid hydroperoxide

The alkoxy radicals recombine to produce dimers (LOOL/ROOR), pentane, ethane, and oxodienoic acids. The active enzyme then reacts with a fatty acid (LH), releasing a hydrogen ion and forming an enzyme-fatty acid complex.

$$\text{E-Fe(III)}_{\text{active}} + \text{LH} \rightarrow \text{E-Fe(II)-L}^{\cdot} + \text{H}^{+} \qquad (10)$$

In the presence of oxygen, the enzyme-fatty acid complex forms an enzyme-hydroperoxide radical complex.

$$\text{E-Fe(II)-L}^{\cdot} + \text{O}_2 \rightarrow \text{E-Fe(III)-LOO}^{\cdot}$$

$$\text{E-Fe(III)-LOO}^{\cdot} \rightarrow \text{E-Fe(III)-LOO}^{-} \qquad (11)$$

The complex then releases a hydroperoxide ion and a free active enzyme.

$$E\text{-}Fe(III)\text{-}LOO^{-} \rightarrow E\text{-}Fe(III)_{active} + LOO^{-} \quad (12)$$

The hydroperoxide ion can take up a hydrogen ion to produce a hydroperoxide. This hydroperoxide is capable of activating more lipoxygenase enzymes. In this way lipid peroxidation initiated by the activity of lipoxygenase is autocatalytic.

In the absence of oxygen, the enzyme-fatty acid complex releases the fatty acid radical and an inactive native enzyme.

$$E\text{-}Fe(II)\text{-}L^{\cdot} \rightarrow E\text{-}Fe(II)_{native} + L^{\cdot} \quad (13)$$

The fatty acid radicals released can recombine to form inactive nonradical dimers (Bidlack & Tappel 1973; Dillard & Tappel 1973; Halliwell 1984; Vick & Zimmerman 1987).

$$L^{\cdot} + L^{\cdot} \rightarrow L\text{-}L \quad (14)$$

2.3.5.3 Lipoxygenase in avocado fruit

Marcus *et al.* (1988) extracted lipoxygenase from avocado fruit peel of the cultivar Fuerte. The enzyme has a molecular mass of 47 000 dalton. Its optimum activity was found to be at pH 7.1 and it is therefore a Type 2 enzyme. The enzyme is competitively inhibited by epicatechin. According to Karni *et al.* (1989), lipoxygenase activity is not correlated with enzyme concentration, but with the concentration of its inhibitor, epicatechin. A decrease in epicatechin concentration leads to increased lipoxygenase activity. The activity of lipoxygenase in the peel of ripening avocado fruit is regulated by endogenous epicatechin.

An avocado lipoxygenase catalyses the breakdown of the antifungal compound *cis, cis* 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (Prusky & Kobiler 1985). It catalyses the breakdown of the compound making the fruit vulnerable to attack by the fungus, *Colletotrichum gloeiosporioides*. Epicatechin decreases to noninhibitory levels at a faster rate in cultivars that are susceptible to this fungus as compared to resistant cultivars.

2.3.6 Membrane structure and lipid peroxidation

Any membrane with phospholipids rich in polyunsaturated fatty acids are susceptible to lipid peroxidation. Lipoxygenase is not known to attack esterified fatty acids (Halliwell 1984; Vick & Zimmerman 1987). In the plasmamembrane, therefore, damage can be caused by hydroperoxides that are formed from lipid peroxidation that has already occurred elsewhere.

Chloroplast membranes are composed almost entirely of glycerolipids and sulpho-quinovosyl diglycerides. The lipids are highly unsaturated and composed of about 70% trienoic fatty acids (Somerville & Browse 1991). According to Halliwell (1984), chloroplast lipids form about 35% of chloroplast dry weight. The chloroplast envelope differs in composition from that of thylakoids. Monogalactosyl diglycerides make up the largest proportion in thylakoids while digalactosyl diglycerides are the most abundant in the chloroplast envelope. Phosphatidylglycerol is only found in small quantities in both the envelope and thylakoids. α-Linolenic acid is a major component of chloroplast lipids. It comprises about 90% of all esterified fatty acids (Halliwell 1984) and is a 18-carbon, trienoic, all *cis* unsaturated fatty acid.

In the chloroplast, chlorophyll can transfer some excitation energy to oxygen, forming the reactive singlet oxygen. The electron transport system of chloroplasts is also a rich source of the superoxide anions. Singlet oxygen and hydroxyl radicals formed from superoxide anions can react with esterified linolenic acid to form hydroperoxides. Hydroperoxides can cause damage to the membranes in which they occur. Chloroplast membranes are therefore prone to lipid peroxidation. This is due to the formation of both singlet oxygen and the superoxide anion in the chloroplast and the presence of a high proportion of linolenic acid esterified to the thylakoid membranes (Raison & Orr 1986a).

Phosphatidylcholine and phosphatidylethanolamine are the major lipids in mitochondrial membranes, comprising about 80% of the phospholipids (Daum 1985). Galactolipids are absent in mitochondria. The major fatty acids of mitochondrial

membranes are linoleic acid and arachidonic acid. Linoleic acid is a 18-carbon dienoic polyunsaturated fatty acid, while arachidonic acid is a 20-carbon tetraenoic fatty acid.

The mitochondrial electron transport system produces large amounts of the superoxide anion. The singlet oxygen, together with the polyunsaturated fatty acids present in the mitochondrial membranes, make the mitochondrial membranes prone to damage through lipid peroxidation Raison & Orr (1986b).

2.3.7 Protection against the toxic oxygen species

Organisms have evolved some mechanisms to protect themselves from damage caused by toxic oxygen species. Protection occurs by means of two mechanisms, one is enzymatic and the other is through production of organic antioxidants.

2.3.7.1 Enzymatic protection

Two enzymes present in plant cells play a major role in the removal of toxic oxygen species from cells. These enzymes are superoxide dismutase (SOD) and catalase.

Superoxide dismutase is ubiquitous and widely distributed among oxygen-consuming organisms, aerotolerant anaerobes and some obligate aerobes (Scandalios 1993). It is a multimeric metalloprotein that is efficient in scavenging superoxide radical. Different forms of superoxide dismutase contain copper, zinc, manganese, or iron. It catalyses the conversion of the superoxide anion to hydrogen peroxide.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The enzyme occurs in chloroplasts where some of it is bound to thylakoids. In this way it effectively removes the superoxide formed during electron transport.

Catalase is mainly found in peroxysomes and glyoxysomes. It catalyses the reduction of hydrogen peroxide to water and oxygen. In the peroxisomes, it helps remove hydrogen peroxide formed during photorespiration.

$$H_2O_2 \rightarrow H_2O + O_2$$

Hydrogen peroxide readily diffuses through membranes. Therefore, it can be effectively maintained below toxic levels by diffusing from chloroplasts where it is formed from superoxides and by conversion into water (Halliwell 1984; Scandalios 1993).

2.3.7.2 Organic antioxidants

Organic antioxidants can be classified into two groups; the lipid-soluble and the water-soluble. Lipid-soluble antioxidants contain long hydrocarbon tails. They occur embedded in membranes. Water-soluble antioxidants contain many oxygen or nitrogen atoms in their structures. They occur in the cytosol, inside organelles and in the vacuole.

2.3.7.2.1 Lipid-soluble antioxidants

2.3.7.2.1.1 α-Tocopherol

α-Tocopherol is the most active of the vitamin E-activity group of compounds. It occurs in large quantities in thylakoid membranes where it blocks chain propagating reactions of lipid peroxidation. It quenches singlet oxygen and peroxy radicals (Bowler *et al.* 1992; Walker & McKersie 1993). According to Larson (1988), α-tocopherol inactivates two equivalents of the chain-carrying peroxy radicals, thereby terminating two potential radical chain reactions per mole of inhibitor. Its long phytyl tail enables it to penetrate into lipophilic membranes of cells and organelles where it exerts its antioxidant activity, thereby preventing oxidative damage to membranes. At low free radical concentrations, α-tocopherol can be regenerated from vitamin C. It is said to be more effective than commercial butyryl hydroxytoluene (BHT) (Larson 1988).

2.3.7.2.1.2 ß-Carotene

Carotenes occur together with chlorophylls and xanthophylls in thylakoid membranes in chloroplasts. ß-Carotene is the most effective antioxidant of the carotenoids. It quenches singlet oxygen produced in the chloroplast and excess chlorophyll excitation

energy that is not readily passed to the photosystems for photosynthesis (Halliwell 1984).

2.3.7.2.2 Water-soluble antioxidants

2.3.7.2.2.1 Glutathione

Glutathione is a tripeptide which contains a thiol group and can be synthesised in the cytosol and chloroplasts. Glutathione acts in concert with ascorbic acid to scavenge free hydrocarbon radicals and hydrogen peroxide (Alscher 1989). It also prevents the oxidation of thiol groups in certain enzymes. The oxidised form of glutathione (GSSG), can be converted to the reduced form (GSH) by the enzyme glutathione reductase (Foyer & Halliwell 1976).

2.3.7.2.2.2 Ascorbic acid

Ascorbic acid can react with the superoxide anion to produce hydrogen peroxide and in turn react with the hydrogen peroxide to form water (Foyer & Halliwell 1976). It occurs in chloroplasts where it helps protect the chloroplast membranes against toxic oxygen species. Ascorbic acid is regenerated from dehydroascorbic acid through the Halliwell-Asada pathway (Foyer & Halliwell 1976) which involves glutathione.

2.3.7.2.2.3 Flavonoids

Flavonoids are widely distributed in plants, most commonly in leaves, flowering tissues and pollen. Flavonoids with either 3', 4' B-ring substitution or flavonols with the 3-hydroxy C-ring substitution are active lipid peroxidation inhibitors (Larson 1988). Examples of flavonoid peroxidation inhibitors are: myricetin, ribonetin, quercetin, dihydroquercetin and fisetin (Hudson & Mahgoub 1980).

The antioxidant activity of flavonoids is attributed to their ability to donate a hydrogen atom to peroxy radicals. They inhibit O_2^- promoted redox reactions within

chloroplasts. Some flavonoids are inhibitors of lipoxygenase, the enzyme that catalyses the peroxidation of free polyunsaturated fatty acids. Flavonoids that absorb in the 300 - 400 nm region act as internal light filters and protect chloroplasts and other organelles from ultraviolet light damage (Hudson & Mahgoub 1980).

Phenolic acids such as caffeic acid, chlorogenic acid and ferulic acid as well as their lipid-soluble esters are also active antioxidants. Most organic antioxidants occur in the chloroplast (Halliwell 1984), and a chloroplast is therefore better protected than a mitochondrion.

2.3.8 Low temperature and lipid peroxidation

Low temperature, even in the absence of light, induces an oxidative stress to chilling-sensitive plants by producing toxic oxygen species (Parkin & Kuo 1989; Prasad *et al.* 1994a). Chilling-resistant plants have evolved some mechanism to remove toxic oxygen species and free hydrocarbon radicals. Some enzymes and organic antioxidants scavenge toxic anions and free radicals. Superoxide dismutase (SOD) converts the superoxide into molecular oxygen and hydrogen peroxide. Catalase converts hydrogen peroxide to molecular oxygen and water. The antioxidants (ascorbic acid, glutathione, α-tocopherol and carotenoids) scavenge both toxic oxygen species and free hydrocarbon radicals (Foyer & Halliwell 1976; Hudson & Mahgoub 1980; Larson 1988).

2.3.9 Chilling injury in avocado fruit

Different avocado cultivars exhibit different sensitivities to chilling. Fruit of chilling-tolerant species store better at 4.4°C while those of chilling-sensitive species store better at 7.2°C (Vakis 1982). Symptoms of chilling injury in avocado fruit are brown patches on the fruit, mesocarp discolouration, abnormal ripening, and vascular browning. According to Kahn (1975), browning is caused mainly by the oxidation of polyphenols and subsequent polymerisation to quinones by both enzymatic and nonenzymatic reactions.

Phase separations in membranes were also observed in avocado fruit (Platt-Aloia & Thomson 1987). These phase separations were found to be reversible but reversibility was lost after chilling injury had occurred. This indicates that chilling injury is due to some secondary effects, following the presence of gel phase lipids in the membrane. Gel phase lipids are thought to aggregate into microdomains within the plane of the fluid bilayer, leading to the loss of selective permeability and change in activation energy of membrane-bound enzymes.

Mesocarp discolouration and vascular browning in avocados also occurs without chilling (Golan *et al.* 1977; Kahn 1977; Sharon-Raber & Kahn 1983). Browning potential was found to be positively correlated with total phenols, polyphenol oxidase and peroxidase activity (Golan *et al.* 1977; Kahn 1977; Van Lelyveld *et al.* 1984).

However, Zauberman *et al. (1985)* found that peroxidase activity does not increase in the mesocarp of Fuerte avocado fruit which showed chilling injury symptoms. They concluded that peroxidase activity in avocado mesocarp plays no role in the development of chilling injury disorder, which manifests itself as dark patches on the skin of the fruit. Sharon-Raber and Kahn (1983) found that there was no relationship between catalase, peroxidase and polyphenol activity and browning potential. Furthermore there was no inverse relationship between carotenoid content and browning potential. They believe that chilling injury is associated with a low carotenoid content.

Reactions that lead to tissue browning can either be enzymatic or nonenzymatic (Sapers 1993). Enzymatic browning discolouration results when monophenolic compounds are hydroxylated to *o*-diphenols by polyphenol oxidase. The diphenols in turn are oxidised to *o*-quinones. Polyphenol oxidase (PPO) is also known as tyrosidase *o*-diphenol oxidase and also as catechol oxidase (Sapers 1993). The quinones condense and react nonenzymatically with other phenolic compounds and amino acids to produce pigments of indeterminate structure. Important substrates of PPO are catechins, cinnamic acid esters, 3,4-dihydroyphenylalanine, and tyrosine. The optimum pH for PPO is between pH5.0 and pH7.0. It is relatively heat labile; can be inhibited

by acids, halides, phenolic acids, sulfites, chelating agents, reducing agents (ascorbic acid), quinone couplers (cysteine) and various substrate-binding compounds. Enzymatic browning is not always a defect; it contributes to the desirable colour and flavour in such products as raisins, prunes, coffee, tea, and cocoa. In food processing enzymatic browning can be controlled by blanching, exclusion of oxygen and by the application of browning inhibitors (Sapers 1993).

In nonenzymatic browning, discolourations result from the reaction of carbonyl compounds and free amino groups that lead to the formation of brown maladion pigments (brown nitrogenous polymers and copolymers). Nonenzymatic browning may also result from sugar degradation or from oxidative degradation of ascorbic acid and further reaction of the carbonyl compounds via aldol concentration with amino groups to form brown pigments. Phenolic compounds can also undergo nonenzymatic oxidation to yield brown pigments. In addition to discolouration, nonenzymatic browning reactions can also result in destruction of nutrients such as essential amino acids and ascorbic acid, reduced protein digestibility, inhibition of digestive enzymes and interference with mineral metabolism. Nonenzymatic browning is a desirable attribute in some products like bakery products, snack foods, nuts and roasted meats (Sapers 1993).

Sulfites act as browning inhibitors by inhibiting polyphenol oxidase and by reacting with intermediates of the browning reactions to prevent pigment formation. The sulfites also inhibit nonenzymatic browning by reacting with carbonyl intermediates. Ascorbic acid is a highly effective inhibitor of enzymatic browning primarily because of its ability to reduce quinones back to phenolic compounds. Other inhibitors of polyphenol oxidase are: cinnamic acid, benzoic acid and copper-chelating compounds (Sapers 1993).

2.3.9.1 Fatty acids and chilling injury in avocado fruit

In an ontogenetic study of the lipid composition of Fuerte and Hass avocados, Eaks (1990) found that oleic, linolenic and linoleic fatty acids decreased during development

of the fruit. Palmitic and palmitoleic acids remained relatively constant as percentage of total fatty acids. Oleic acid was found to be a major fatty acid in both cultivars. Fatty acid composition did not change significantly during two, four, and six weeks of storage at 0°,5° and 10°C or even after ripening at 20°C. In mature fruit, palmitoleic and linoleic (both unsaturated fatty acids) accounted for 80% of the total fatty acids. Palmitic acid accounts for less than 20% of the total fatty acids. Eaks (1990) concluded that the degree of unsaturation may not be related to chilling sensitivity in avocado fruits.

The literature does not point to a single cause of chilling injury. It gives conflicting results concerning the involvement of fatty acids. Membrane phase transition indicates that saturated fatty acids confer chilling sensitivity to plants while lipid peroxidation implicates unsaturated fatty acids. Wu and Browse (1995) found that phosphatidylglycerols rich in saturated fatty acids do not induce chilling sensitivity to a mutant of *Arabidopsis* which contains lipids with a high melting point.

With respect to the avocado fruit, there are conflicting results about the cause of chilling injury and the role of polyphenol oxidases and peroxidases in chilling injury. The polyphenol oxidases and peroxidases are believed to cause oxidation of phenolics to quinones, which then react with other compounds to form dark-coloured complexes. Fruit mesocarp browning potential was found to be positively correlated with the total phenolic content and polyphenol activity (Kahn 1975; Kahn 1977; Golan *et al.* 1977). However, Sharon-Raber and Kahn (1983), did not find a similar correlation.

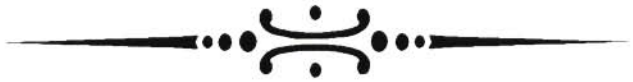
2.4 Aims of this study

In the light of contradicting findings in the literature about the role of phenolics in chilling injury, this study investigates:

- The level of phenolics in the exocarp of avocado fruits stored at a low temperature.
- Whether there is a correlation between fruit phenolic level and sensitivity to chilling injury.
- Whether there could be a change in phenolic concentration as chilling injury

advances.

- The possible presence of cellular damage in the exocarp of chilling injured fruits.
- Whether differential cooling between the exocarp and mesocarp of fruits does not lead to chilling injury.



CHAPTER 3

MATERIALS AND METHODS

3.1 Material

Mature fruit, picked for packing, were collected at the Westfalia Estate, Tzaneen, Northern Province. Fruit of two avocado cultivars were collected namely, chilling-susceptible Fuerte and the more resistant Hass cultivar. The first batch of fruit was collected on the 1st of June 1994 (early batch) and the second batch on the 13th of July 1994 (late batch).

3.2 Methods

3.2.1 Ripening

On arrival at the laboratory, forty fruit of both cultivars, except early Hass, were left at room temperature (20°C) to ripen. Avocado fruit become soft on ripening without any colour change. Fruit are fully ripe if they form a dent when pressed lightly with a finger. Three fruit were sliced into thin sections (5 mm) every three days (0, 3, 6, 9, 12 days) until the fruit were fully ripe on the twelfth day. The exocarp was removed from the sections, freeze-dried, and kept at a temperature of -20°C until used in phenolic analyses.

3.2.2 Cold storage

Sixty fruit of each cultivar were stored at a temperature of 4.5°C in a Conviron incubator, model E 15. The fruit were examined weekly for chilling injury symptoms

and sampled once a week for a period of four weeks. The sampled fruit were also sliced into thin sections (5 mm). The exocarp was removed from the sections, freeze-dried, and stored at a temperature of -20°C until used in phenolic analyses.

3.2.3 Rate of cooling

To determine the rate and evenness of cooling of avocado fruit, three thermocouples were inserted in randomly selected chilling-sensitive Fuerte fruit in a pre-chilled Conviron. A Kane May hand held temperature measuring instrument (KM10000) connected to K-type thermocouples of 0.5 mm diameter was used to measure fruit temperatures. One thermocouple was closely attached to the surface of the fruit with "Prestik" manufacred by Genkem. A second was inserted just below the exocarp and a third into the mesocarp. Temperature in the Conviron was set at 4.5°C. The thermocouples were lead through an instrument port in the Conviron and temperatures recorded every two hours until all the temperatures stabilised at 4.5°C.

3.2.4 Light microscopy

Thin exocarp sections (20 µm) were cut from chilling-injured and nonchilling-injured cold-stored fruit. These were sectioned by means of a Reichert sledge microtome (Nr. 307 198) connected to a cryostat (Leitz KRYOMAT 1700). The sections were stained with ruthenium red (Johansen 1940). Mounted slides were observed under a light microscope (Reichert Univar).

3.2.5 Scanning electron microscopy

Thin exocarp sections (5 x 20 mm and 3 mm thick) from chilling-injured and nonchilling-injured cold-stored fruit were quenched in liquid nitrogen and fractured using a chilled scalpel (Robards 1978). The sections were fractured transversely or paradermally (across the surface). These sections were then dehydrated in a series of ethanol solutions, coated with gold and examined under a scanning electron microscope (JEOL

JSM 6100).

3.2.6 Histochemical localisation of phenolics

Small blocks measuring 5 x 5 x 15 mm were cut from the fruit. Each block included exocarp and mesocarp. These were fixed in formalin-acetic acid-alcohol (5:5:90) containing ferrous sulphate (Johansen 1940). Thin sections (20 µm) were cut from frozen blocks by means of a sledge microtome (Reichert Nr. 307 198) connected to a cryostat. Prepared slides were examined under a light microscope (Reichert Univar).

3.2.7 Total phenolics

Freeze-dried material was covered with liquid nitrogen and ground to a powder with pestle and mortar. Duplicate 100 mg samples were weighed into 150 ml Erlenmeyer flasks to which 15 ml of methanol was added. The flasks were stoppered and shaken for two hours at room temperature on a Griffin flask shaker. These samples were then filtered into 50 ml volumetric flasks through Whatman No.1 filter paper. The residue was washed three times with 10 ml volumes of methanol and made up to volume with methanol.

Triplicate 500 µl aliquots were used for the determination of total phenolics according to the method of Torres *et al.* (1987) as follows:
A 500 µl aliquot of extract was pipetted into a test tube and 5 ml distilled water was then added. To this mixture 0.5 ml of Folin-Ciocalteau reagent was added, thoroughly mixed and allowed to stand for five minutes at room temperature, whereafter 1.5 ml of 20% sodium carbonate was added. The mixture was made up to a final volume of 10 ml with distilled water, thoroughly mixed and incubated for two hours at 50°C. After this treatment the mixture was again mixed thoroughly on a vortex (FISONS whirlmixer). Absorbance was then read at 765 nm with a spectrophotometer (Hitachi 100-60) against a blank which contained all the reagents but no plant extract.

A standard curve (Figure 6) was prepared using gallic acid as a standard. From this curve the concentration of phenolics in the samples were determined as gallic acid equivalents. Gallic acid solutions for the standard curve were prepared as follows: An amount of 0.200 g gallic acid was dissolved in methanol and made up to a final volume of 100 ml in a volumetric flask. This served as a stock solution with a gallic acid concentration of 2 000 mg l^{-1}. A dilution series was then prepared from the stock solution, by adding various amounts of methanol to aliquots of the gallic acid stock solution to make a final volume of 10 ml. The dilution series is given in Table 2.

3.2.8 Thin layer chromatography of phenolics

The remainders of the duplicate 50 ml extracts from the procedure discussed under 3.2.7 were combined and evaporated to dryness under reduced pressure at a temperature of 30°C in a rotary evaporator (BUCHI Rotavapor- Re). The residue was dissolved in 2 ml methanol to which 0.2 ml distilled water was added to complete dissolution (Mabry *et al. 1970).* Extracts (10 µl) were spotted on 10 x 10 cm HPLC thinlayer silica gel 60 F_{254} plates and developed one-dimensionally in two solvent systems. The first solvent system was acetic acid: chloroform (1:9) and the other was: benzene: methanol: ethyl acetate (45:4:1) (Harborne 1984; Torres *et al.* 1987). With the second solvent system, 5 µl of extracts were applied on similar TLC plates (60 F_{254}) with a concentration zone. The plates were allowed to air dry and viewed under ultraviolet light at a wavelength of 366 nm. Fluorescent compounds were first marked and thereafter the plates were sprayed with Folin-Ciocalteau reagent for phenolics (Harborne 1984).

3.2.9 Purification of the fluorescent compound

A fluorescent compound which appeared to increase with storage at low temperature was consistently observed in the chilling-susceptible cultivar, Fuerte. This compound did not react with the Folin-Ciocalteau reagent which is specific for phenolics and, therefore, necessitated further analysis to identify the compound.

Duplicate 20 g samples of exocarp of the chilling-susceptible cultivar Fuerte, showing chilling injury symptoms, were extracted in 100 ml volumes of a methanol-chloroform (2:1) mixture. The extracts were evaporated to dryness under reduced pressure at 30°C. The residues were dissolved in 10 ml volumes of the methanol-chloroform mixture. The extracts were applied to preparatory TLC silica gel 60 F_{254} plates and developed in the acetic acid-chloroform solvent system. The developed plates were viewed under UV light at 366nm and the fluorescent compound encircled. The circled area was then carefully scraped off and dissolved in the methanol-chloroform mixture. This extract was then filtered through Whatman No.1 filter paper, evaporated to dryness under reduced pressure at 30°C and dissolved in 5 ml of the methanol-chloroform mixture. The extract was then applied to 10 x 10 cm HPLC thin layer silica gel 60 F_{254} plates, developed in the ethyl acetate-benzene mixture, viewed under UV-light and the fluorescent compound encircled and scraped off once again, redissolved in the methanol-chloroform mixture, evaporated to dryness and finally dissolved in 5 ml methanol.

3.2.10 Paper chromatography of the fluorescent compound

The fluorescent compound dissolved in methanol (3.2.8 above) was developed two-dimensionally by paper chromatography in tertiary butanol: acetic acid: water (3:1:1) (TBA) in the first dimension and in 15% acetic acid in water (15%HOAc) in the second dimension (Mabry *et al.* 1970). R_f values were determined for both dimensions.

3.2.11 UV-VIS absorption spectrum of the fluorescent compound

The ultraviolet-visible light absorption of the fluorescent compound, dissolved in methanol (method described under 3.2.9 above), was determined using a Beckman DU-65 spectrophotometer at a wavelength range from 200 to 700 nm .

3.2.12 Absorption spectra of the compound with shift reagents

Shift reagents were used according to the method of Markham (1982), to determine whether the fluorescent compound was a flavonoid and if it was, which type of flavonoid it was. The following procedures were followed:

- Sodium methoxide (supplied by the company Fluka) 30% in methanol was added to 2 ml of the fluorescent compound in methanol in a 10 mm cuvette. After thorough mixing the UV-VIS absorption spectrum was determined in the wavelength range of 200 to 700 nm. The absorption spectrum was again determined after five minutes.
- Sodium acetate (powdered anhydrous, analar, supplied by Hopkins and Williams) was added to 2 ml of the fluorescent compound solution in a cuvette until a 2 mm layer settled at the bottom. The UV-VIS absorption spectrum was determined as described above.
- Aluminium chloride (5 g powdered anhydrous supplied by Fluka) was dissolved in 100 ml Univar methanol supplied by Saarchem. Six drops of this solution were added to 2 ml of the fluorescent compound solution in a cuvette. The UV-VIS absorption spectrum was determined. Three drops of 10% HCl were added, mixed and the absorption spectrum determined.

3.2.13 Purification of the fluorescent compound by high pressure liquid chromatography

The fluorescent compound was further purified by High Pressure Liquid Chromatography (HPLC) at the University of Pretoria. The eluent was water-methanol-acetic acid (67:12:1) and a Spherisorb C18 250 x 4 mm column was used. A UV detector with a filter at 276 nm was used. A LKB 2150 HPLC pump supplying a pressure of 120 bars, at a rate of 1.5 ml min^{-1} was used. The column temperature was kept constant at 30°C.

About 500 mg of the dry fluorescent compound prepared as described under 3.2.9 was dissolved in a minimum amount of chloroform. Aliquots of 100 µl of this solution were injected into the HPLC and eluent was collected in fractions. Combined fractions were evaporated under reduced pressure at a temperature of 30°C, the residue dissolved in a minimum amount of chloroform and again evaporated to dryness under reduced pressure. The compound was finally dissolved in chloroform for infra red spectroscopy and in deuterated chloroform for nuclear magnetic resonance spectroscopy.

Nuclear magnetic resonance spectroscopy (NMR) is an important tool in the interpretation of structures of chemical compounds. The basis of NMR is to subject molecules to radiation which will result in transition of a nucleus from one spin at a lower energy state to another at a higher energy state (Pasto & Johnson 1969; Furnis *et al.* 1989). The difference in energy levels between the two spin orientations is dependent on the particular location of the atom in the molecule. Processional frequency of the nucleus is equal to the frequency of the electromagnetic radiation required to induce transition from one spin to another e.g. from +1/2 to -1/2. Two forms of NMR that are commonly used to determine structures of organic compounds are proton nuclear magnetic resonance (^{1}H *NMR)* and carbon-13 nuclear magnetic resonance (^{13}C *NMR).*

Infrared spectroscopy is also a valuable tool for the determination of structural information concerning organic molecules. It uses the radiant energy absorption property of molecules in the wave number range 700 to 4000 cm^{-1}. Absorption of radiation in this region results in excitation of bond deformation - either stretching or bending deformation. The amount of energy required depends on the atomic masses of the bonded atoms, the bond order (single, double or triple bond) and on the environment in which the bonds and the molecule exists. This energy is indicated by the position of the absorption band on the infrared spectrum (Nakanishi 1962; Cowley 1966; Pasto & Johnson 1969). The absorption spectrum of a given substance is generally specific for that substance (Cowley 1966).

3.2.14 Exposure of fruit to the isolated fluorescent compound

Ten randomly selected fruit of each of the cultivars Fuerte and Hass from the late batch, were vacuum-infiltrated with 20 mg l^{-1} of the isolated fluorescent compound in 10% methanol at a pressure of -0.14 MPa for one minute in a large desiccator at room temperature. Ten fruit of each cultivar were treated in the same way with 10% methanol to act as a control. After treatment the fruit were stored at 4.5°C for four weeks and examined for chilling injury.

CHAPTER 4

RESULTS

4. 1 Chilling injury in cold-stored fruit

Chilling injury was assessed on the basis of brown patches on the surface of the fruit only (Figure 1). Injury was rated on a scale of 0 to 5, where 0= no injury; 1= slight injury; 3= moderate injury; and 5= severe injury according to Vakis (1982).

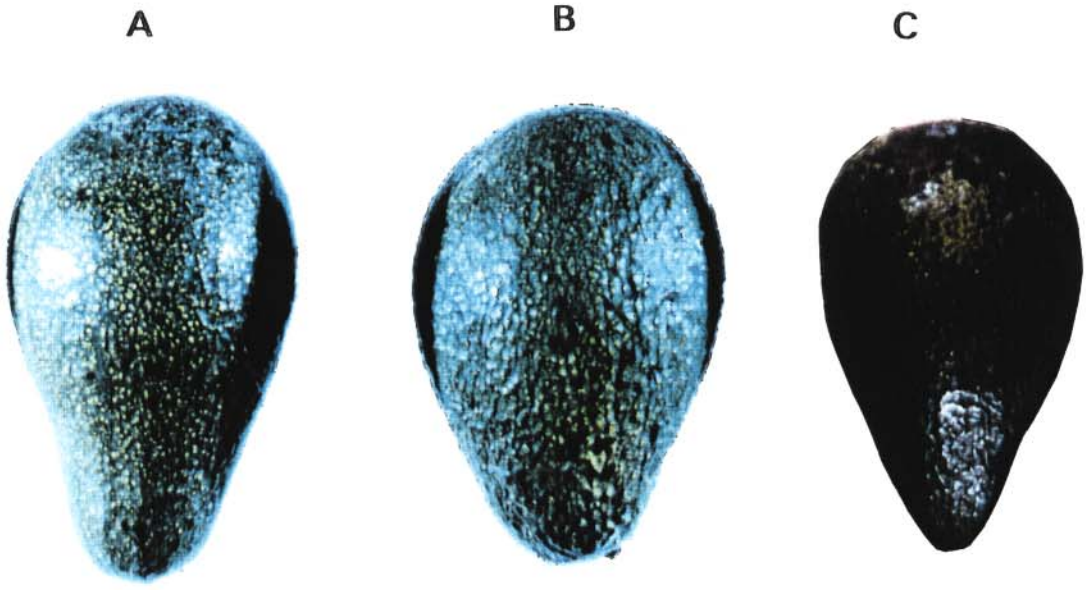


Figure 1 Morphology of avocado fruit, A= normal Fuerte; B= normal Hass; C= Fuerte showing chilling injury symptoms.

No fruit showed any sign of chilling injury after one week of cold storage. After two weeks of storage only early Fuerte fruit showed slight injury. Chilling injury was moderate in these fruit at the end of the four weeks of storage. The late Fuerte fruit showed slight chilling injury only after four weeks of storage (Table 1).

Table 1 Chilling injury in Fuerte and Hass avocado fruit stored at 4.5°C for four weeks (0= no injury; 1= slight injury; 3= moderate injury; and 5= severe injury).

Fruit Sample	Week 1	Week 2	Week 3	Week 4
Early Fuerte	0	1	3	3
Late Fuerte	0	0	0	1
Early Hass	0	0	0	0
Late Hass	0	0	0	0

4.2 Cooling rate of Fuerte fruit stored at 4.5°C

The rationale of this study was to determine:

- the rate of cooling of avocado fruit after introduction into a cooling chamber.
- whether differential fruit-cooling might not be the cause of chilling injury.

The three parts of the fruit (at which temperature was measured) took about twelve hours to reach the ambient storage temperature of 4.5°C. Temperatures in all three parts of the fruit fell less rapidly during the last six hours than during the first six hours of cold storage (Figure 2). During this period the exocarp surface temperature declined more rapidly than that below the exocarp and that of the mesocarp. The rate of temperature decline on the surface slowed down after eight hours to fall at the same rate as that below the exocarp and in the mesocarp. The highest average decline was between the second and fourth hours after placement in cold storage, at 4.1°C per hour. During this period temperatures on the exocarp surface and below the exocarp declined rapidly; the decline in mesocarp temperature slightly trailing that below the exocarp. During the first two hours the average decline was 2.3°C per hour and the slowest decline occurred between the tenth and the twelfth hours (0.25°C per hour).

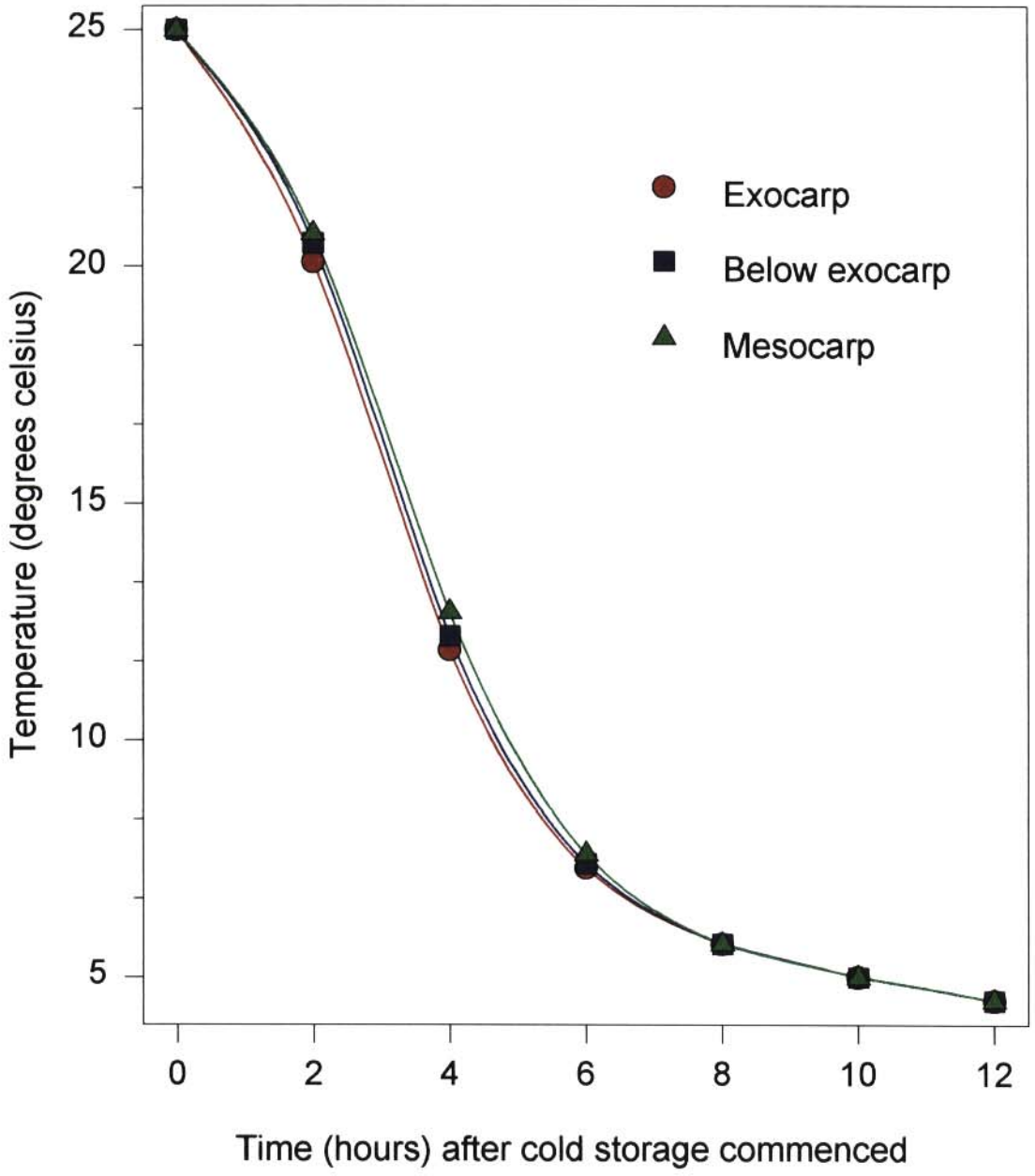


Figure 2 The rate of cooling of Fuerte avocado fruit stored at 4.5^{o}C.

4.3 Light microscopy

As shown in Figure 3, exocarp cells in fruit showing chilling injury symptoms have collapsed, leaving only cell wall remnants. The remains of the cell walls are thick and look gelatinous as if the cellulose in the cell walls has been digested. However, epidermal cells above these damaged exocarp cells are still intact (Figure 3B). The epidermal layer sags in when the exodermal tissue below it collapses. The mesocarp cells below the exocarp do not collapse, indicating that chilling damage is localised in the exocarp.

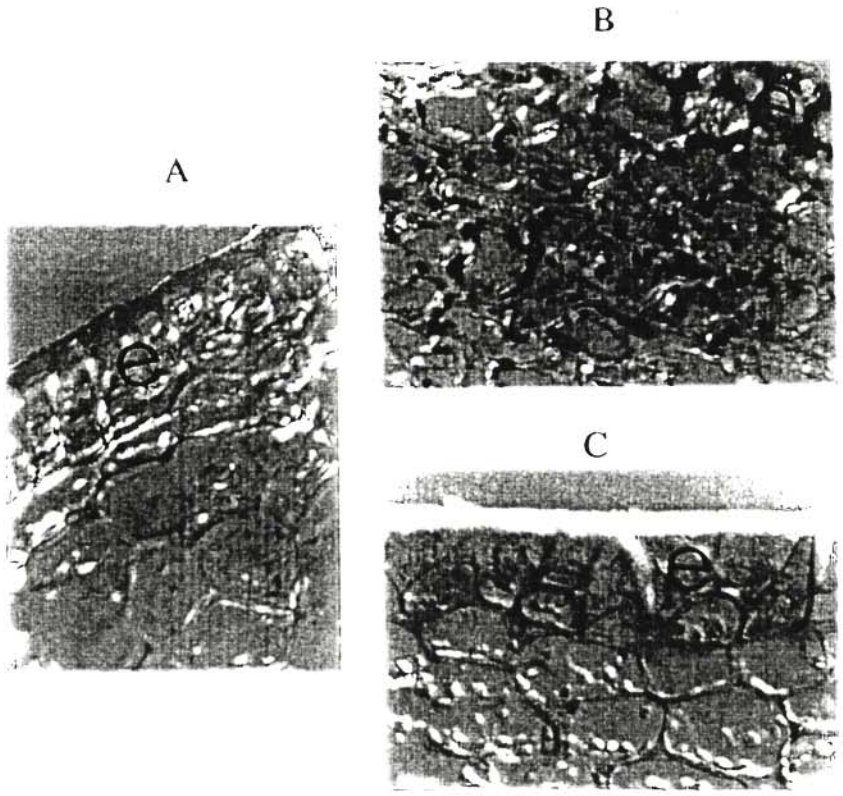


Figure 3 Anatomy of avocado fruit exocarp. e= epidermis; A= normal Fuerte fruit exocarp; B= chilling-injured Fuerte fruit exocarp; and C= normal Hass fruit exocarp. Note that in A and C the exocarp cells below the epidermis are expanded while in B, where chilling injury has set in, the exocarp cells have collapsed.

4.4 Scanning electron microscopy

Scanning electron micrographs of the exocarp of cold-stored fruit (Figure 4) show that the exocarp of the fruit of Fuerte (Figure 4 D) have large extensive lenticels or intercellular spaces as compared to those of Hass (Figure 4 C). The exocarp cells of both cultivars contain granules which resemble starch granules (Figures 4 A and 4B). These seem to be invaginations of the plasmalemma, as well as coagulation of small vacuoles or membrane bound vesicles (Figure 4 E). The plasmalemma and vesicles shrink and disintegrate as chilling injury advances (Figure 4 F). When the cells are fully chilling-injured, in Fuerte, their walls collapse (Figure 4 G and H). Most of these collapsed cells are in the vicinity of lenticels.

4.5 Localisation of phenolics in the exocarp of avocado fruit.

Ferrous sulphate stain forms dark complexes with all classes of phenolic compounds (Johansen 1940). Figure 5 shows that in both Fuerte and Hass, the phenolics are mainly concentrated in the exocarp. In the epidermal cells they occur as large globular structures. Lower down in the exocarp, they occur as smaller droplets (possibly membrane-bound) aligned along the plasmalemma. In chilling-injured cells the small phenolic droplets combine into larger globules that fill up the whole cell. Where the cell has completely disintegrated the remains of the cell stain black for phenolics. The phenolics in the epidermal cells do not seem to change during injury. Most of the chilling-injured cells were observed in the vicinity of lenticels.

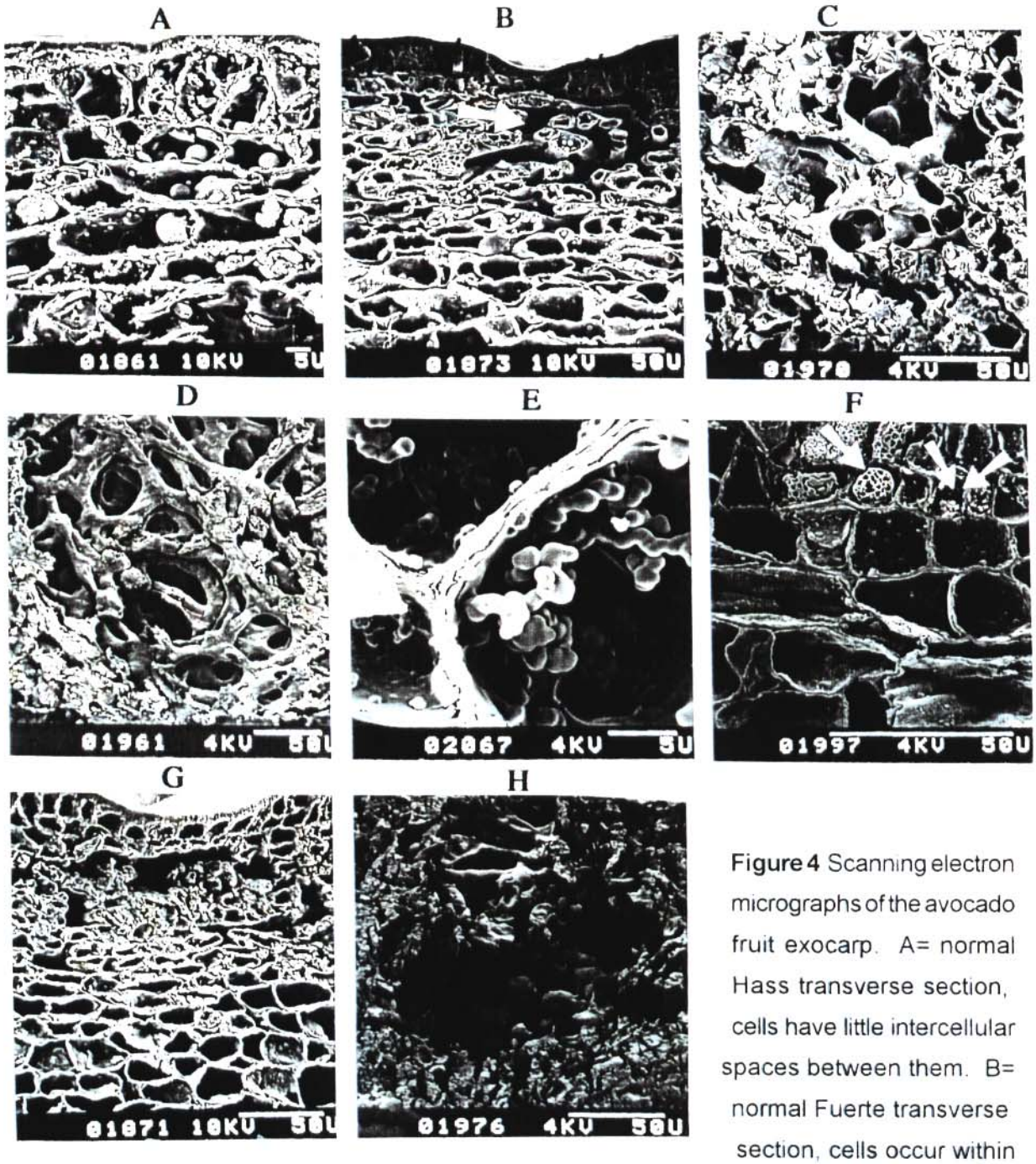


Figure 4 Scanning electron micrographs of the avocado fruit exocarp. A= normal Hass transverse section, cells have little intercellular spaces between them. B= normal Fuerte transverse section, cells occur within large intercellular spaces (arrow) that can be regarded as lenticels. C= normal Hass tangential section, intercellular spaces are visible among cells. D= normal Fuerte tangential section, the large intercellular spaces or lenticels viewed from above. E= plasmalemma invaginations and vesicle coagulation that form in Fuerte during the early stages of chilling injury. F= shrinking (arrow) and disintegration (double arrow) of plasmalemma during chilling injury in Fuerte fruits. G= chilling-injured Fuerte fruit transverse section, cells within lenticels are damaged and have collapsed. H= chilling-injured Fuerte tangential section, the damaged cells and lenticels viewed from the top.

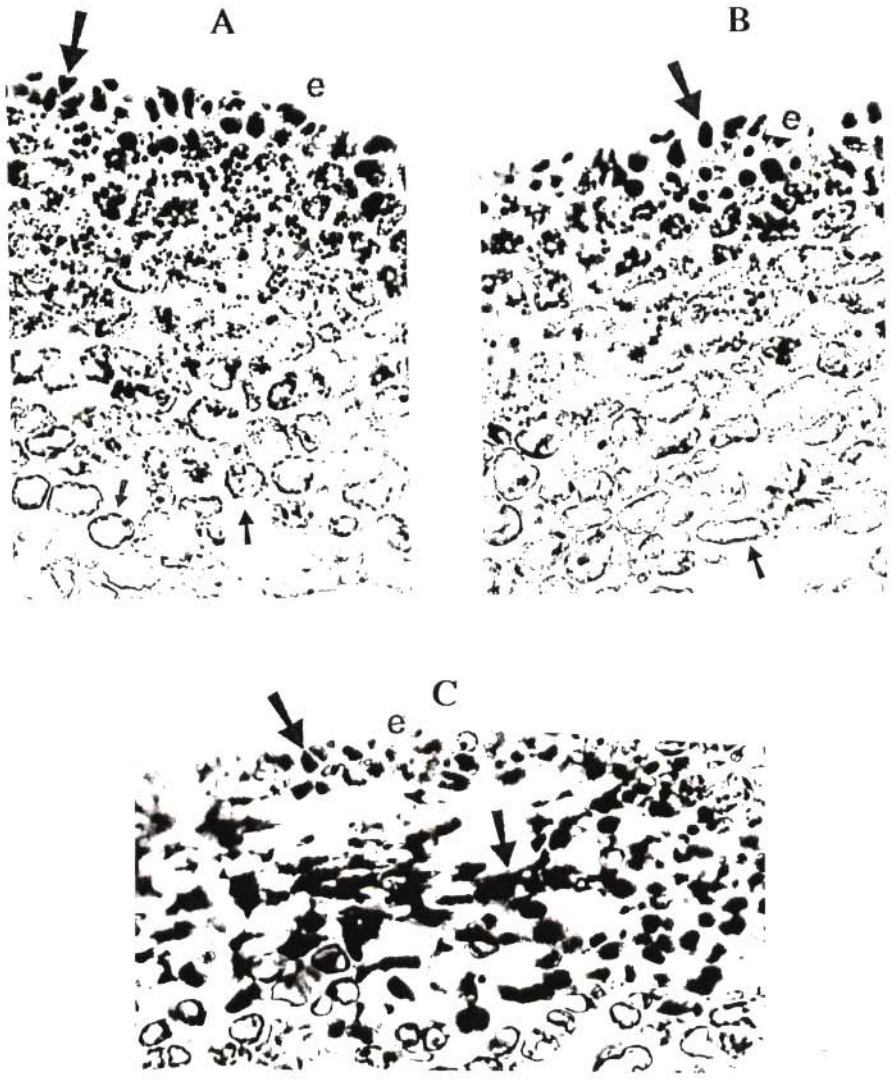


Figure 5 Histochemical localisation of phenolics in the exocarp of avocado fruit. A= Fuerte fruit without chilling injury symptoms. B= Hass fruit without chilling injury symptoms. C= Fuerte fruit with chilling injury symptoms. The black spots indicate phenolics (arrows). In the epidermis, (e), the black spots are large and almost fill the entire cell, while in the exocarp cells they occur as small droplets at the periphery of the cells except in C where chilling injury has already set in and the phenolics fill the cells.

4.6 Phenolic concentrations in the exocarp of avocado fruit

A series of gallic acid solutions were prepared and their absorbance determined at 765 nm after reaction with Folin-Ciocalteau reagent (Table 2). A graph of gallic acid concentration versus absorbance was plotted (Figure 6). The graph was used to determine the concentration of phenolics in the samples as gallic acid equivalents. Duplicate samples were extracted and the analyses were done in triplicate (described under 3.2.7). From each sample six values were obtained from which the mean standard deviation and standard error were calculated.

4.6.1 Phenolic concentration in the exocarp of ripening avocado fruit

For this investigation fresh fruit were left at room temperature to ripen (described under 3.2.1). It was observed that Fuerte fruit ripened earlier than Hass fruit. By the tenth day the Fuerte fruit were all ripe while those of Hass were ripe only by the twelfth day. The phenolic content of early harvested fruit was relatively lower than that of late harvested fruit (fruit that was collected early in June are designated as early fruit while those that were collected in mid July are designated as late fruit as described in 3.1 (Table 3). The phenolic concentrations of Fuerte fruit were 49.82 and 106.79 mg g^{-1} dry matter (as gallic acid equivalents) for early and late fresh fruit respectively. The phenolic concentration of freshly harvested fruit increased more than two fold from early to late fruit. For Hass, the concentrations were 56.80 and 60.77 mg g^{-1} dry matter (as gallic acid equivalents) for early and late fresh fruit respectively. Compared to fresh Fuerte fruit, the increase in phenolic concentration in fresh Hass fruit was very low.

The results show that the phenolic concentration in avocado fruit changed during ripening. The change in phenolic content fluctuated, but in general increased with fruit ripening (Figure 7). In early Fuerte fruit, the phenolic concentration after three days of ripening remained similar to that of fresh fruit. Thereafter, phenolic concentration increased to 78.22 mg g^{-1} after six days and then dropped to 48.39 mg g^{-1} after nine days of ripening.

Table 2 Absorbance of standard gallic acid solutions at 765 nm.

Sample	Gallic acid Aliquot (ml)	Gallic Acid Conc.mg l^{-1}	Absorbance at 765 nm	Average Absorbance
Blank	0.00	0.00	0.00	0.00
1	0.01	2.00	0.15	
1	0.01	2.00	0.21	0.18
2	0.02	4.00	0.42	
2	0.02	4.00	0.47	0.45
3	0.03	6.00	0.63	
3	0.03	6.00	0.65	0.64
4	0.04	8.00	0.88	
4	0.04	8.00	0.86	0.87
5	0.05	10.00	1.05	
5	0.05	10.00	1.05	1.05
6	0.06	12.00	1.34	
6	0.06	12.00	1.26	1.30
7	0.07	14.00	1.50	
7	0.07	14.00	1.44	1.47
8	0.08	16.00	1.72	
8	0.08	16.00	1.66	1.69
9	0.09	18.00	1.83	
9	0.09	18.00	1.90	1.87

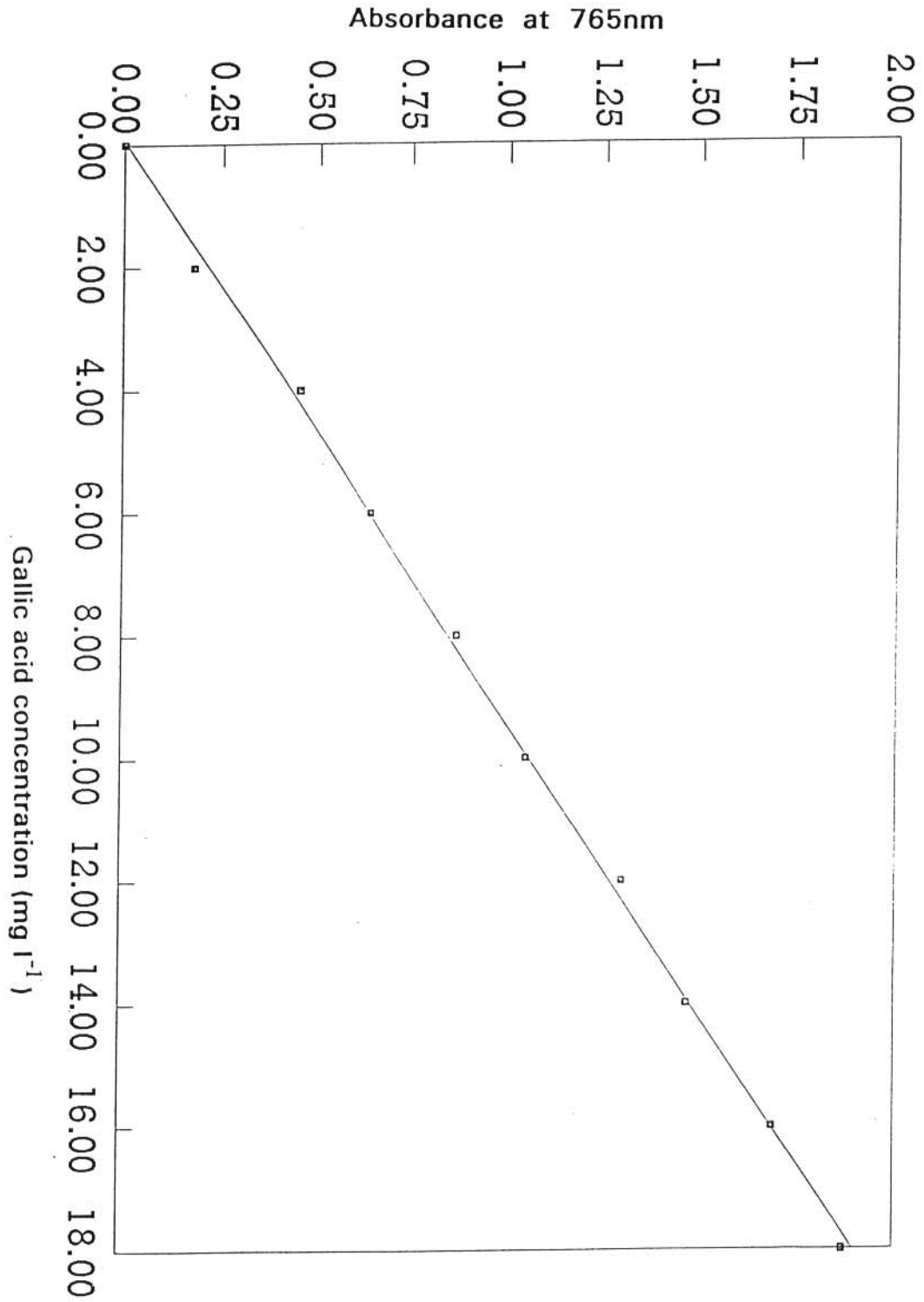


Figure 6 Standard curve for the estimation of phenolic concentration as gallic acid equivalents.

The phenolic concentration then increased again to a high of 89.17 mg g^{-1} after twelve days when the fruit were fully ripe (Table 3 and Figure 7). In late Fuerte fruit, changes in phenolic concentration increased from 106.79 to 116.63 mg g^{-1} during the first three days of ripening, then dropped to 113.46 mg g^{-1} after six days, increased to 155.51 mg g^{-1} after nine days and dropped again to 116.95 mg g^{-1} when the fruit were fully ripe (Table 3 and Figure 7).

The change in phenolic concentration in ripening late Hass fruit showed a trend similar to that described for late Fuerte fruit. The phenolic concentration increased from 60.77 mg g^{-1} to 77.51 mg g^{-1} during the first three days of ripening, it thereafter decreased to 71.54 mg g^{-1} by day six. Another increase on the ninth day of ripening (88.19 mg g^{-1}) was followed by a decrease up to day twelve when the fruit were fully ripe (Table 3 and Figure 7).

Table 3 Phenolic concentration in mg g^{-1} dry mass (as gallic acid equivalents) in the exocarp of ripening Fuerte and Hass fruit given as mean with standard deviation and standard error. E= early and L= late.

Sample	Statistic	Fresh	3 Days	6 Days	9 Days	12 Days
E. Fuerte	Mean	49.82	49.82	78.22	48.39	89.17
	Std.dev.	2.08	8.49	9.86	7.21	9.53
	Std. err.	±0.85	±3.47	±4.02	±2.94	±3.89
L. Fuerte	Mean	106.79	116.63	113.46	155.51	116.95
	Std.dev.	2.89	10.92	10.85	3.5	1.92
	Std. err.	±1.18	±4.45	±4,42	±1.43	±0.78
L. Hass	Mean	60.77	77.57	71.54	88.19	84.84
	Std.dev.	4.00	10.62	6.52	8.68	9.35
	Std. err.	±1.63	±4.34	±2.66	±3.54	±3.93

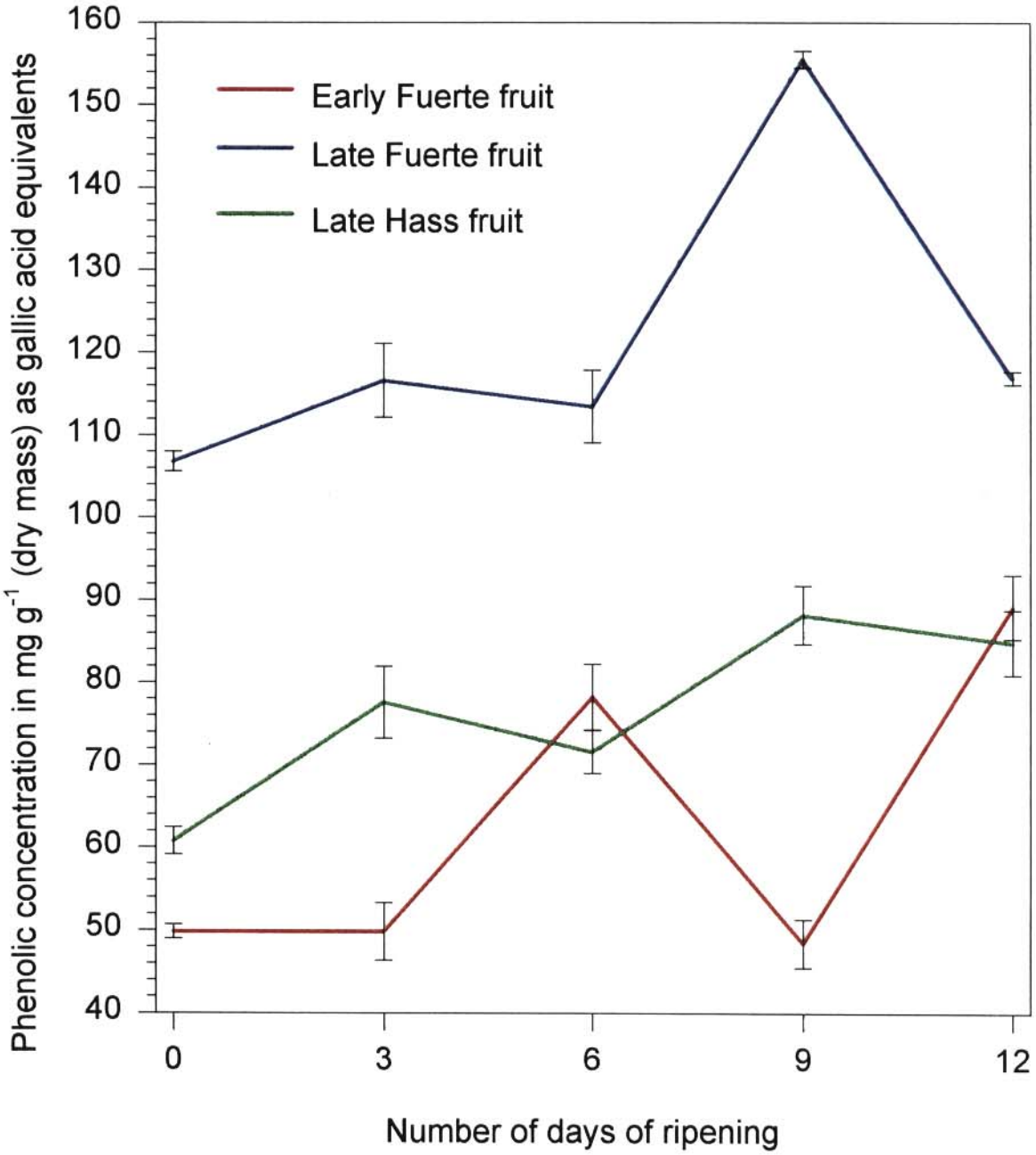


Figure 7 Comparison of the change in phenolic concentrations (average values) in the exocarp of Fuerte and Hass avocado fruit during ripening at room temperature.

4.6.2 Phenolic concentration in the exocarp of cold-stored avocado fruit

In both early and late Fuerte fruit, phenolic concentrations showed a general increase during cold storage, though the concentration fluctuated. The same was observed with fruit kept at room temperature (Table 4 and Figure 8). In the case of early Fuerte fruit, the highest phenolic concentration (97.27 mg g^{-1}), was reached during the third week of storage. In late Fuerte fruit the highest phenolic concentration(155.56 mg g^{-1}), was reached during the second week of storage. The change in phenolic concentration during cold storage of early and late Fuerte fruit followed dissimilar patterns during the second and subsequent weeks of storage (Figure 8). In late Fuerte fruit, the phenolic concentration increased from 124.56 mg g^{-1} to 155.56 mg g^{-1} after the second week of storage. After the same period of storage, the phenolic concentration in early Fuerte fruit had decreased from 71.56 mg g^{-1} to 55.20 mg g^{-1}. The phenolic concentration in late Fuerte fruit then decreased to 120.12 mg g^{-1} by the third week of storage and then increased to 129.80 mg g^{-1} by week four. During the same period of storage the phenolic concentrations in early Fuerte fruit increased to 97.27 mg g^{-1} then decreased to 85.36 mg g^{-1}. Results for cold storage (discussed under 4.1 and shown in Table 1), show that fruit of early Fuerte developed moderate chilling injury after the third and fourth weeks of storage. The phenolic concentration of early Fuerte fruit during the same period (third week) increased to twice the concentration in fresh fruit. Fruit of late Fuerte which did not develop chilling injury symptoms, after three weeks of storage (Table 1), showed a decrease in phenolic concentration during the same period (third week) (Figure 8).

In Hass fruit the change in phenolic concentrations show a similar overall trend in both early and late harvested fruit (Figure 8). In early Hass fruit the highest phenolic concentration of 74.74 mg g^{-1} was reached during the third week of storage, after which the phenolic concentration declined to 66.32 mg g^{-1} by the fourth week. The phenolic concentration in late Hass fruit also reached the highest concentration of 78.22 mg g^{-1} during the third week of cold storage after which the phenolic concentration decreased to 76.96 mg g^{-1} by week four.

In general the phenolic content of the exocarp of avocado fruit of both Fuerte and Hass cultivars increase during ripening at room temperature and cold storage. Where these characteristics decrease, they never drop to levels below those present in fresh fruit (Figures 7 and 8).

Table 4 Total phenolic concentration in mg g^{-1} dry mass (as gallic acid equivalents) in the exocarp of cold-stored Fuerte and Hass fruit, given as mean with standard deviation and standard error. E= early and L= late.

Sample	Statistic	Fresh	1 Week	2 Weeks	3 Weeks	4 Weeks
E.Fuerte	Mean	49.82	71.56	55.20	97.27	85.36
	Std.dev.	2.06	6.06	4.12	7.21	7.79
	Std. err.	±0.85	±2.47	±1.68	±2.94	±3.18
L.Fuerte	Mean	106.79	124.56	155.56	120.12	129.80
	Std.dev.	2.89	12.12	6.59	4.54	9.48
	Std.err	±1.18	±4.95	±2.69	±1.85	±3.87
E.Hass	Mean	56.80	68.23	69.02	74.24	66.23
	Std.dev.	3.00	1.92	1.40	2.17	2.14
	Std.err.	±1.23	±0.78	±0.57	±0.88	±0.87
L.Hass	Mean	60.77	71.40	76.96	78.22	76.96
	Std.dev.	4.00	5.47	7.78	6.00	6.89
	Std.err.	±1.63	±2.23	±3.18	±2.45	±2.81

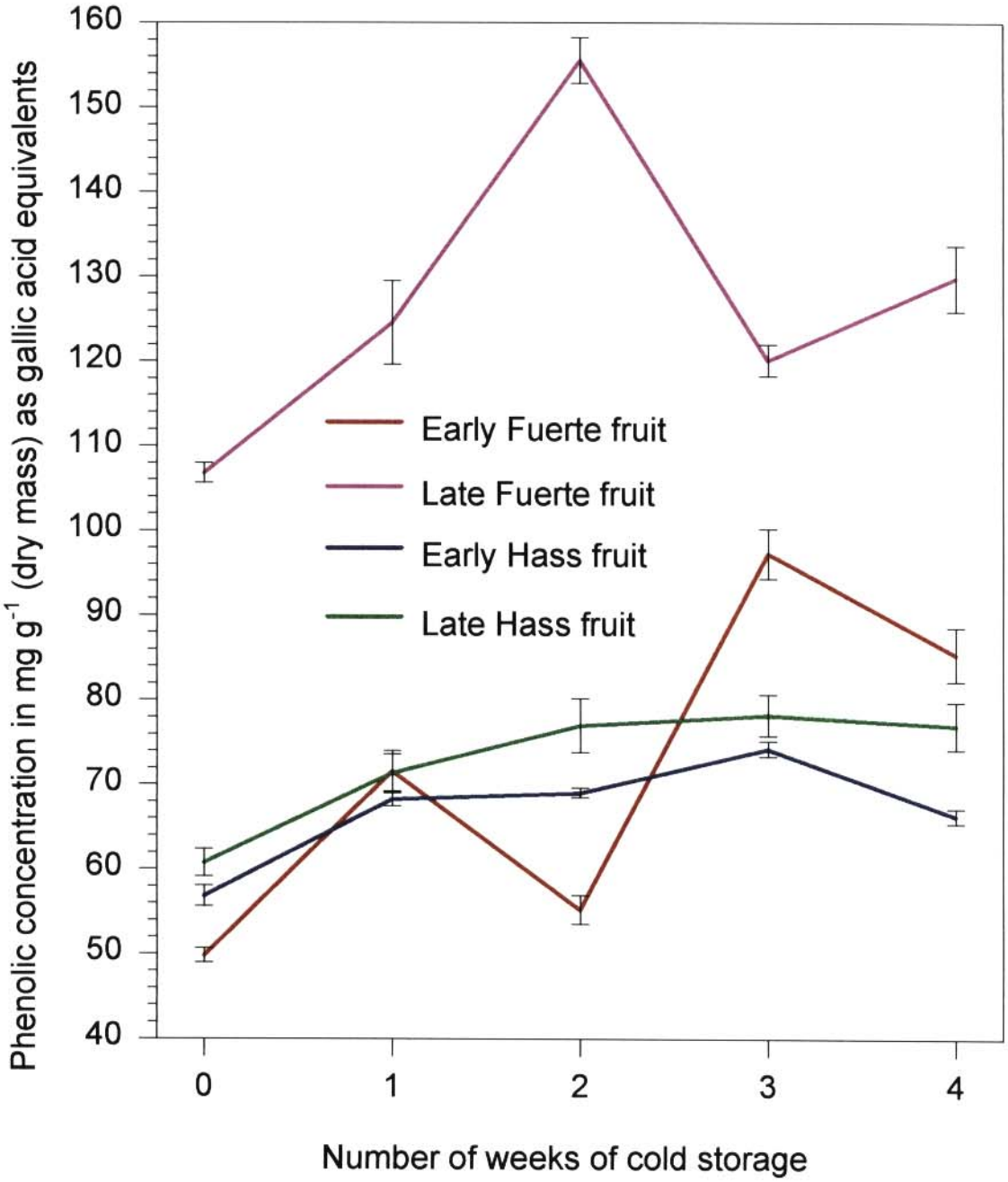


Figure 8 Comparison of the changes in phenolic concentrations (average values) in the exocarp of Fuerte and Hass avocado fruit during storage at 4.5°C.

4.7 Thin layer chromatography of phenolics

Thin-layer chromatographic patterns of the phenolics extracted from the exocarp of Fuerte and Hass fruit, developed in ethyl acetate-benzene (9:11), appear similar, except for the presence of a fluorescent compound that does not react with the phenolic reagent - Folin-Ciocalteau (encircled compound 5 in Figure 9). This implies that it is not a phenolic compound. If it is a phenolic compound, it should be completely substituted, that is, it is lacking a free hydroxyl group to which the phenol reagent can complex. The compound appears in extracts of the exocarp of chilling-sensitive Fuerte fruit, where it increases with storage at low temperature as shown by an increase in spot size (Figures 9 and 10). Traces of this compound appear in chromatograms of extracts of ripening early harvested fruit as well as in cold stored late harvested fruit.

Most of the phenolic spots change in intensity or size with increased cold storage and duration of ripening. Compound 8 decreases with storage in Fuerte fruit (Figure 9). In late harvested Hass fruit this compound (compound 8) decreases during both cold storage and ripening. Compounds 4 and 6 decrease during storage in both Fuerte and Hass fruit (Figure 9). Some compounds seem to increase in concentration while others decrease between early and late harvested fruit. It is shown in Figure 9 that in early harvested Fuerte fruit the spot of compound 7 is intense and the spot size of compound 9 is small. In late harvested Fuerte fruit spot of compound 7 is faint while spot size of compound 9 is larger than in early harvested fruit. In Hass fruit spot of compound 7 also becomes faint in late harvested fruit as compared to early harvested fruit, and spot of compound 9 increases in size in late harvested fruit. Spot size of compound 4 decreases between early and late harvested fruit in both cultivars. The presence of compound 9 in chilling-resistant early and late Hass, as well as late Fuerte fruit that became chilling-resistant, might be associated with resistance to chilling injury.

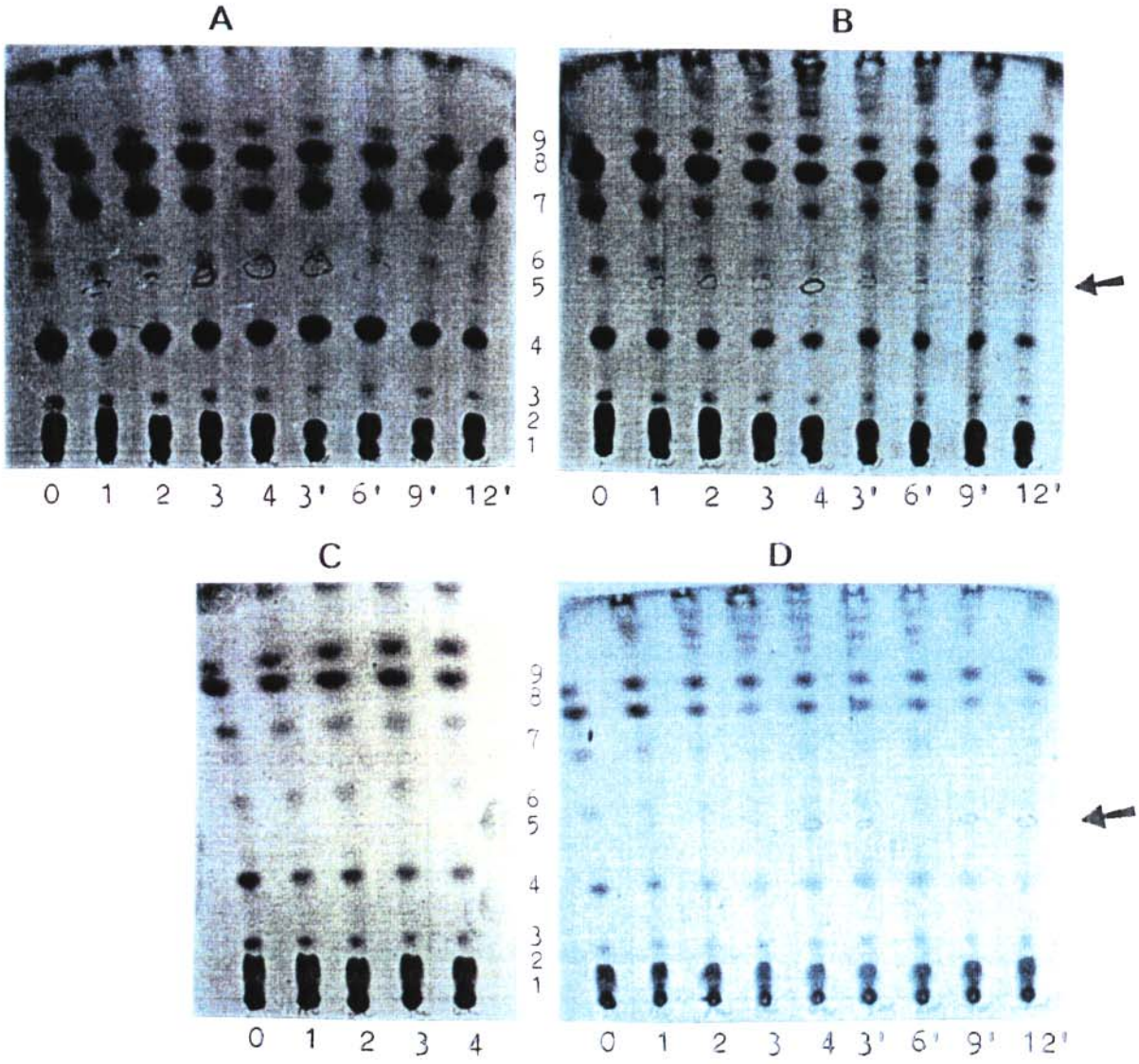


Figure 9 Thin layer chromatograms of phenolics in early and late harvested fruit of both Fuerte and Hass developed in ethyl acetate-benzene (9:11). The numbers 0 to 4 = number of weeks in cold storage and the numbers 3' to 12' are the number of days during ripening at room temperature. The circles represent the compound that did not react with the phenol reagent (arrow). The size of this spot increases with cold storage in A. A= Early Fuerte cold storage and ripening fruits. B= Late Fuerte cold storage and ripening fruits. C= Late Hass cold storage fruits. D= Late Hass cold storage and ripening fruits.

Development of chromatograms in benzene-methanol-ethyl acetate (45:4:1) show a similar pattern (Figure 10). Spot size of compound 14 is relatively small in late harvested when compared to early harvested fruit in both cultivars. Spot size of compound 12, though small, seems to increase between early and late harvested fruit in both cultivars.

A high concentration of phenolic compounds represented by spot sizes of compounds 4 and 7 in Figure 9 could be associated with susceptibility to chilling injury. The reason being that the spots representing these phenolic compounds are large in fruit that were injured by chilling and small in those that were not injured by chilling. A possible physiological effect of the two trends could be that of conferring chilling resistance to the fruit. The two trends are: a decrease in concentration in phenolic compounds represented by spots 4, 5, and 7 in Figure 9 and spot 14 in Figure 10 as well as the increase in compounds represented by spot 9 in Figure 9 and spot 12 in Figure 10. These have respectively decreased and increased in fruit that are or have become resistant to chilling injury.

4.8 Determination of the structure of the fluorescent compound

4.8.1 Paper chromatography

The compound that was observed on thin layer chromatography which did not react with the Folin-Ciocalteau reagent, fluoresces in ultraviolet light (366 nm), which is a property of most flavonoids. Paper chromatography was carried out to verify whether the compound is a flavonoid or not. The compound has R_f values of 0.74 and 0.58 in tertiary butanol-acetic acid-water (3:1:1) (TBA) and 15% acetic acid (HOAc) respectively (Figure 11). The R_f values are similar to those of isoflavone aglycones, flavone aglycones or dihydroflavone aglycones (Markham 1982). On exposure to ammonia fumes under UV light (366 nm), the fluorescence changes to blue-green. This reduces the selection of possible compounds to isoflavones that lack a free 5-OH group (Mabry *et al.* 1970; Markham 1982), suggesting that it could be fully substituted.

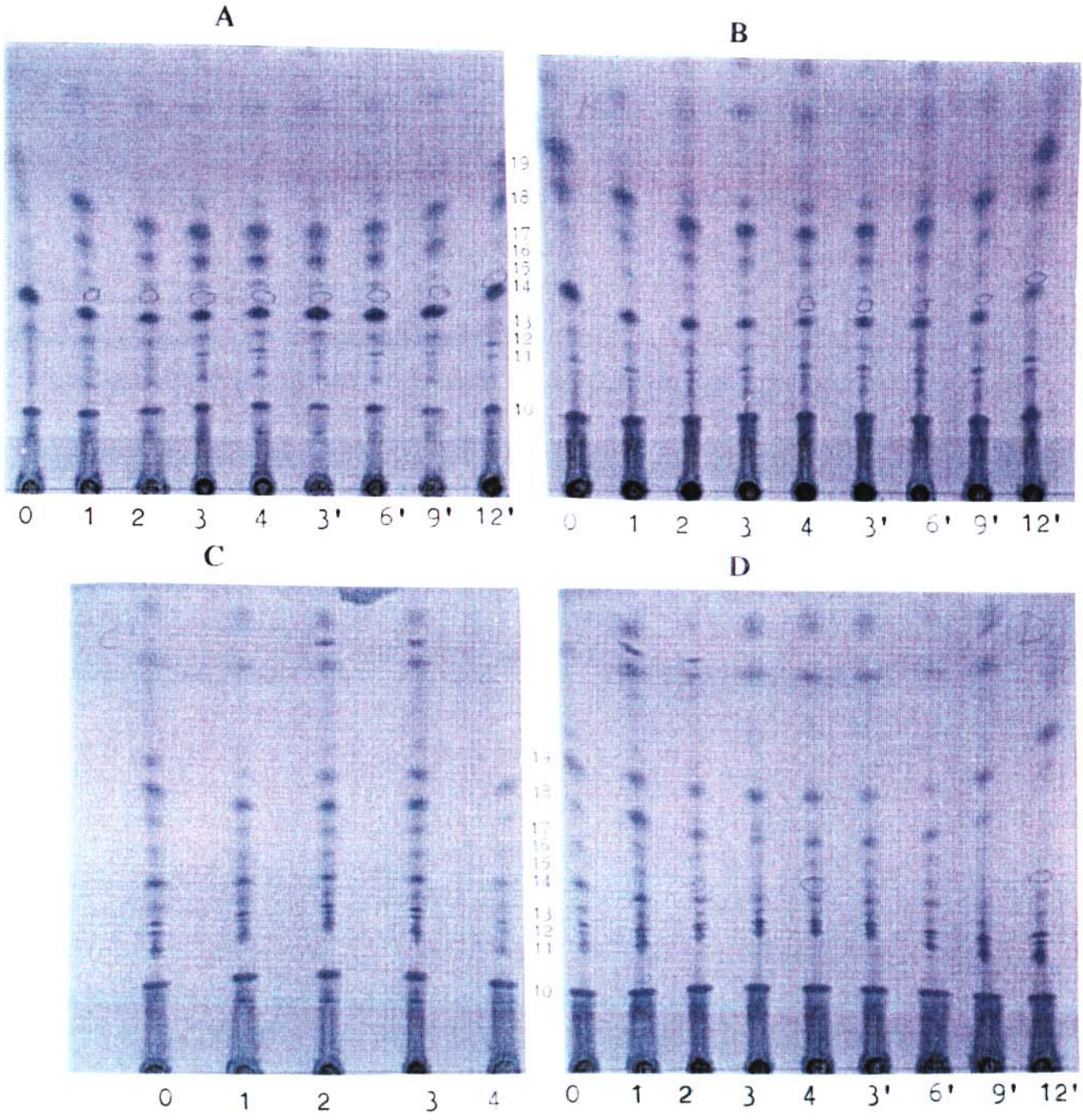


Figure 10 Thin layer chromatograms of early and late harvested Fuerte and Hass fruits developed in benzene-methanol ethyl acetate (45 4 1). A= Early Fuerte cold storage and ripening fruits. B= Late Fuerte cold storage and ripening fruits. C= Early cold storage Hass fruits. D= Late Hass cold storage and ripening fruits.

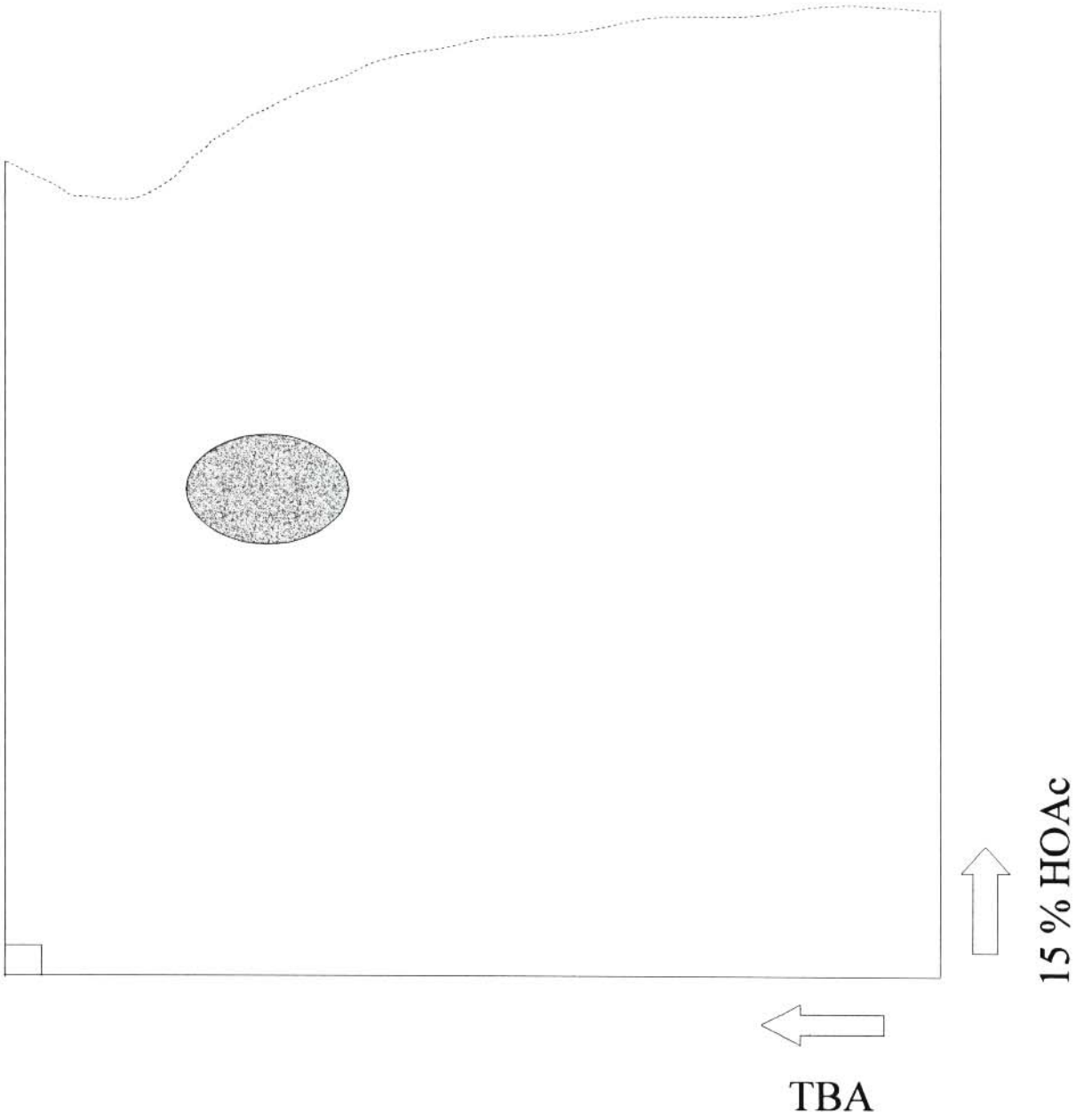


Figure 11 Results of two-dimensional paper chromatography of the fluoresecnt compound developed in tertiary butanol-acetic acid-water (3:1:1) in the first dimension and 15% acetic acid in the second dimension.

A lack of a free hydroxyl group could explain why the compound does not react with the phenolic reagent. Another possibility is that the compound could be an aglycone, thus suggesting that the substitution is methylation and not glycosylation.

4.8.2 Ultraviolet light absorption of the compound

The fluorescent compound has a single absorption peak at 239 nm (Figure 12), the region in which isoflavones absorb ultra violet light (Markham 1982). Absorption of light in this region suggests that the compound is not a long polyunsaturated hydrocarbon as most of these compounds absorb light in the blue light region (Silverstein & Bassler 1967; Pasto & Johnson 1969). However, if it is a polyunsaturated hydrocarbon, it could have some auxochromes that produce some hypsochromic shifts, that is shifting absorption towards the lower wavelengths.

4.8.2.1 Ultraviolet light absorption with shift reagents

Shift reagents are usually used in the identification of flavonoid structure through their shifting of ultraviolet light absorption peaks of the flavonoids (Mabry *et al.* 1970; Markham 1982). Flavonoids have two major absorption peaks in the ultraviolet range, namely, band I in the range 300 to 380 nm and band II in the range 240 to 280 nm (Mabry *et al.* 1970). Addition of shift reagents to flavonoid solution in methanol shifts flavonoid absorption bands either to the shorter wavelengths (hypsochromic), to the longer wavelengths (bathochromic) or may not cause a shift. The presence and direction of a shift depends on the presence and position of the hydroxyl groups in the rings or their substitutions. Shift reagents may also increase or decrease the intensity of the absorption bands (Mabry *et al.* 1970; Markham 1982).

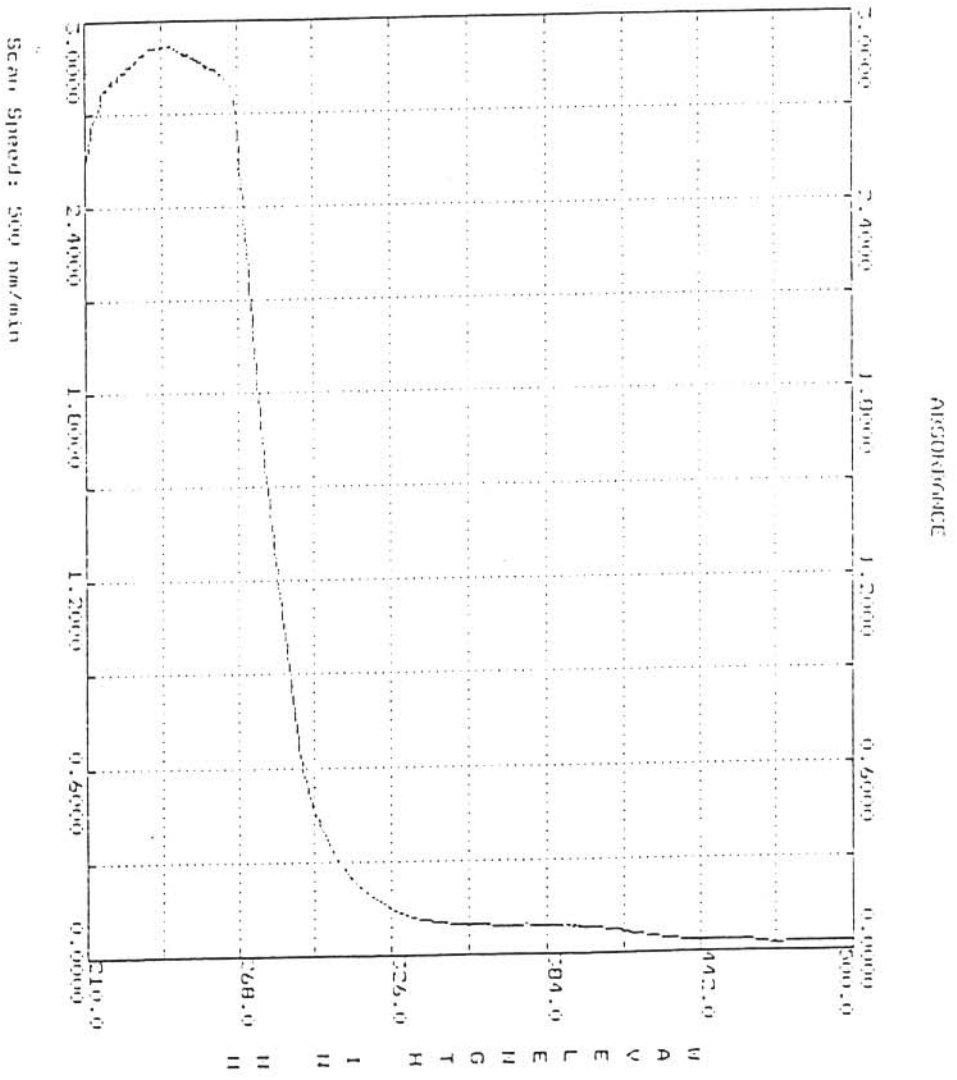


Figure 12 UV absorption spectrum of the fluorescent compound dissolved in methanol.

Isoflavones have spectra which exhibit intense band II absorption with only a shoulder of low intensity peak representing band I. Band II occurs in the region 245 to 270 nm and is relatively unaffected by increased hydroxylation of the B-ring. The basic structures of flavonoids and isoflavones are shown in Figure 13. According to Mabry *et al.* (1970) oxidation and substitution patterns in isoflavones have the following effects on their spectra:

- A-ring trioxygenated isoflavones have their band II absorption in the 265 to 270 nm range.
- 6,7 dioxygenated isoflavones have intense band I absorption.
- Methylation, glycosylation or absence of 5-hydroxyl groups causes a hypsochromic shift of band II.

According to Mabry *et al.* (1970) and Markham (1982) shift reagents have the following effects on flavonoid absorption spectra:

- Sodium methoxide causes bathochromic shifts of both band I and band II in isoflavones containing A-ring hydroxyl groups. Sodium methoxide is a strong base and it ionises the hydroxyl groups on the flavonoid nucleus. Degradation of the spectrum with time indicates the presence of alkali hydrolisable hydroxyl groups (3- or 4'-OH groups)
- Sodium acetate specifically ionises the 7-hydroxyl group in isoflavones and causes a bathochromic shift in band II.
- Aluminium chloride/Hydrochloric acid causes bathochromic shifts in 6,7- and or 7,8- orthodihydroxy isoflavones.

The shift reagents did not produce any hypso- or bathochromic shifts in the position of the absorption band of the compound and there was also no decrease in intensity (Figures 14A to Figure 14 C). A small shoulder (arrow in Figure 14 A) was produced after reaction with the $AlCl_3$ reagent. Results with shift reagents show the following:

- A lack of a shift with sodium methoxide indicates the absence of a free -OH group in the A-ring.

- A lack of a shift with sodium acetate indicates absence of a 7 - OH group.
- The absence of decrease in intensity over time with sodium methoxide indicates the absence of 3' and 4' -OH groups.

However, the production of a shoulder could mean that the compound is methylated. This conclusion was arrived at by comparing the absorption spectrum of the compound with those given in Mabry *et al.* (1970).

From the basic structure of isoflavones (Figure 13), the above results would imply that the fluorescent compound, if its an isoflavone, does not have free hydroxyl groups and if the hydroxyl groups are substituted, they are not substituted with sugar molecules.

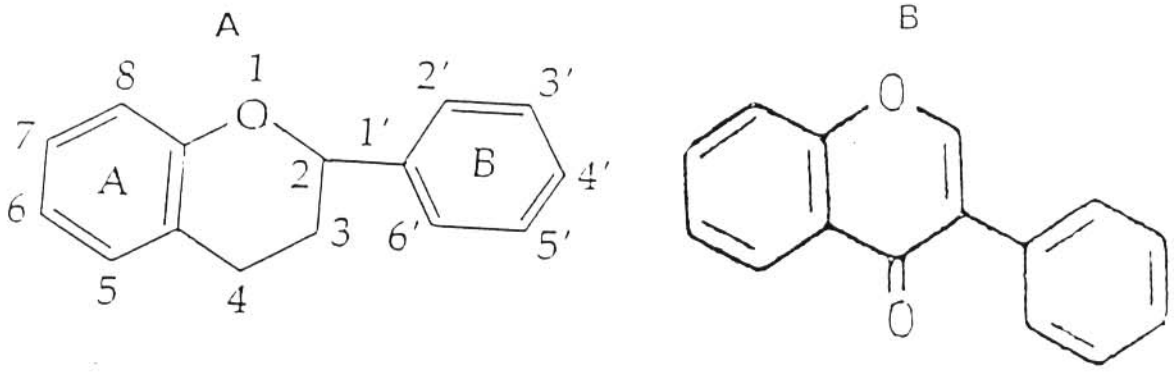


Figure 13 Structures of the flavonoid nucleus (A) and the basic isoflavonoid (B).

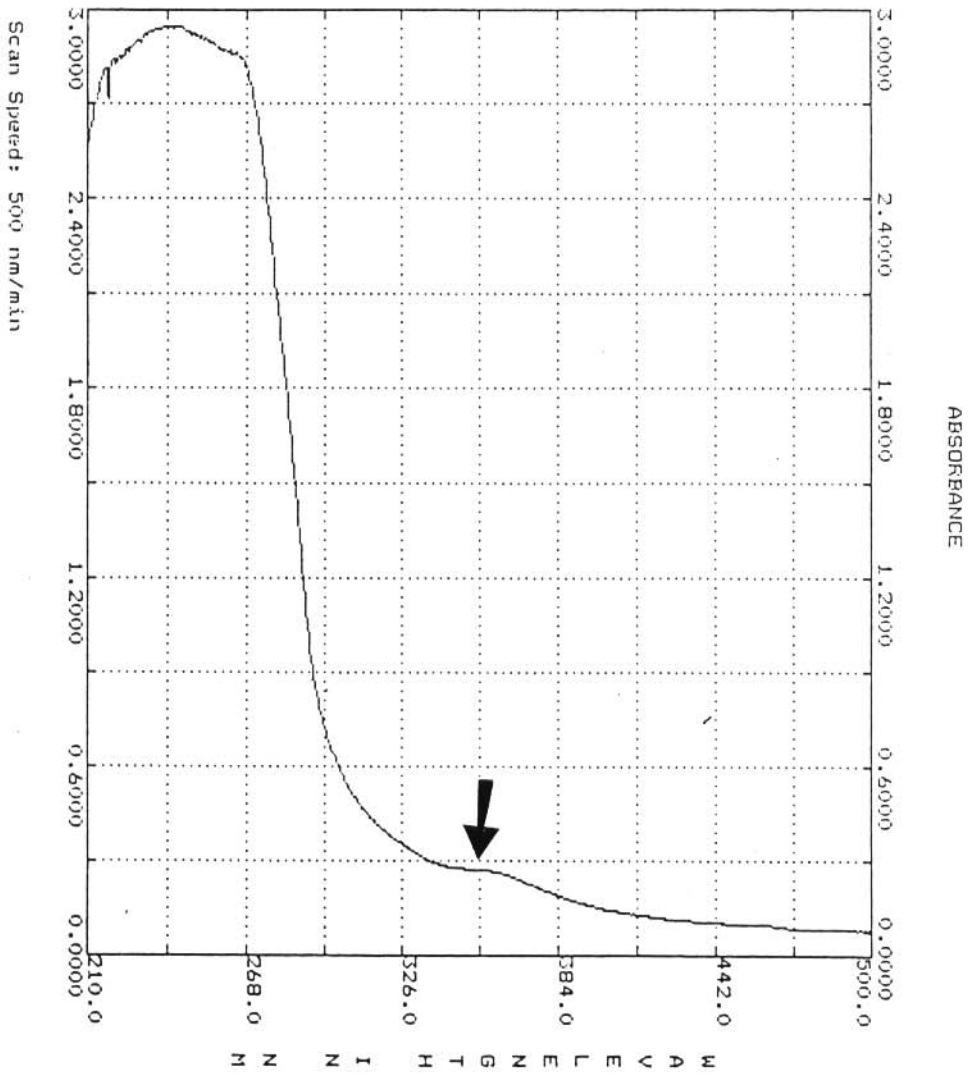


Figure 14 A Ultraviolet light absorption of the fluorescent compound after reaction with aluminium chloride. The slight shoulder produced (arrow) suggests methylation of the compound.

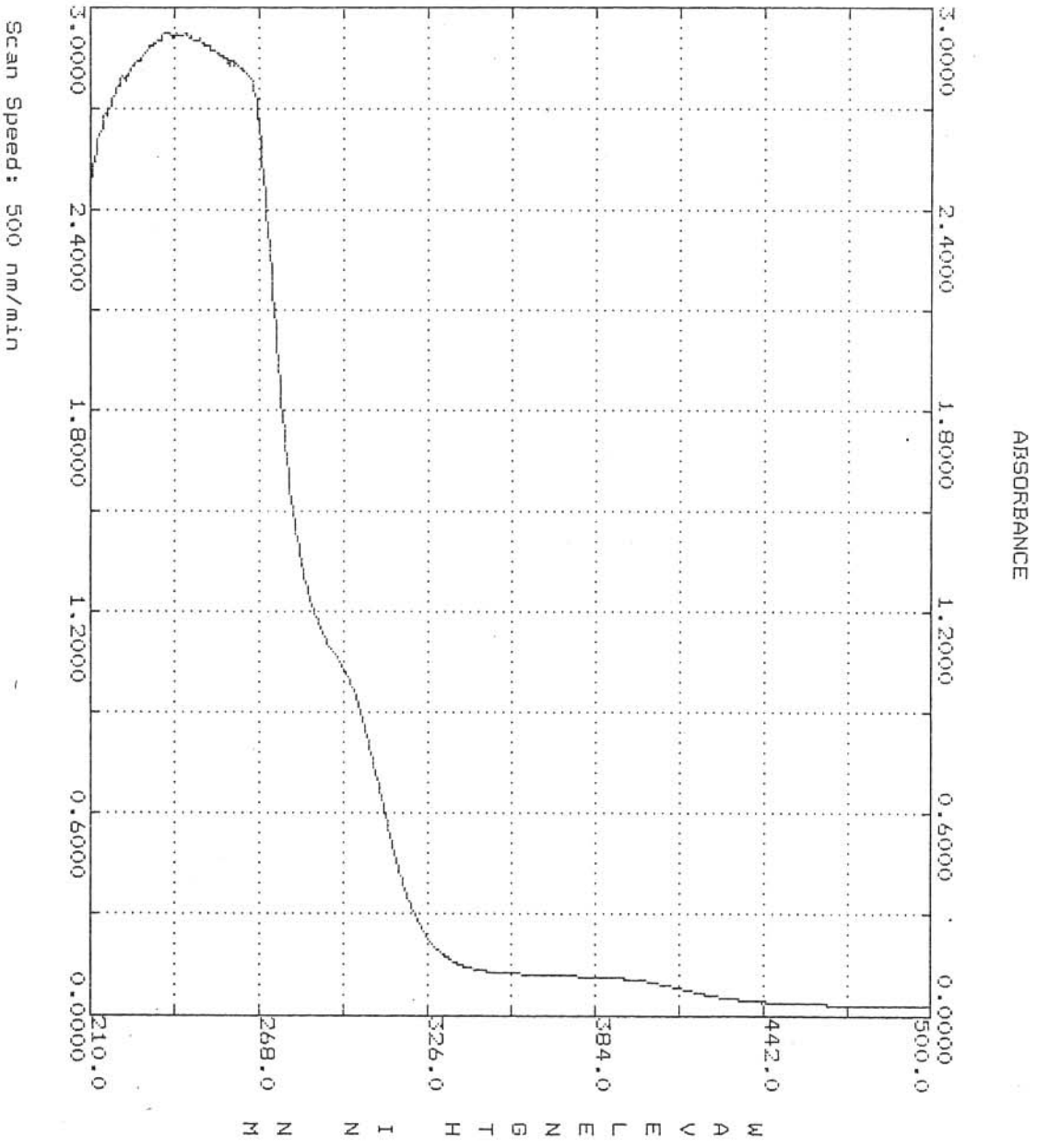


Figure 14 B Ultraviolet absorption spectrum of the fluorescent compound after addition of sodium methoxide.

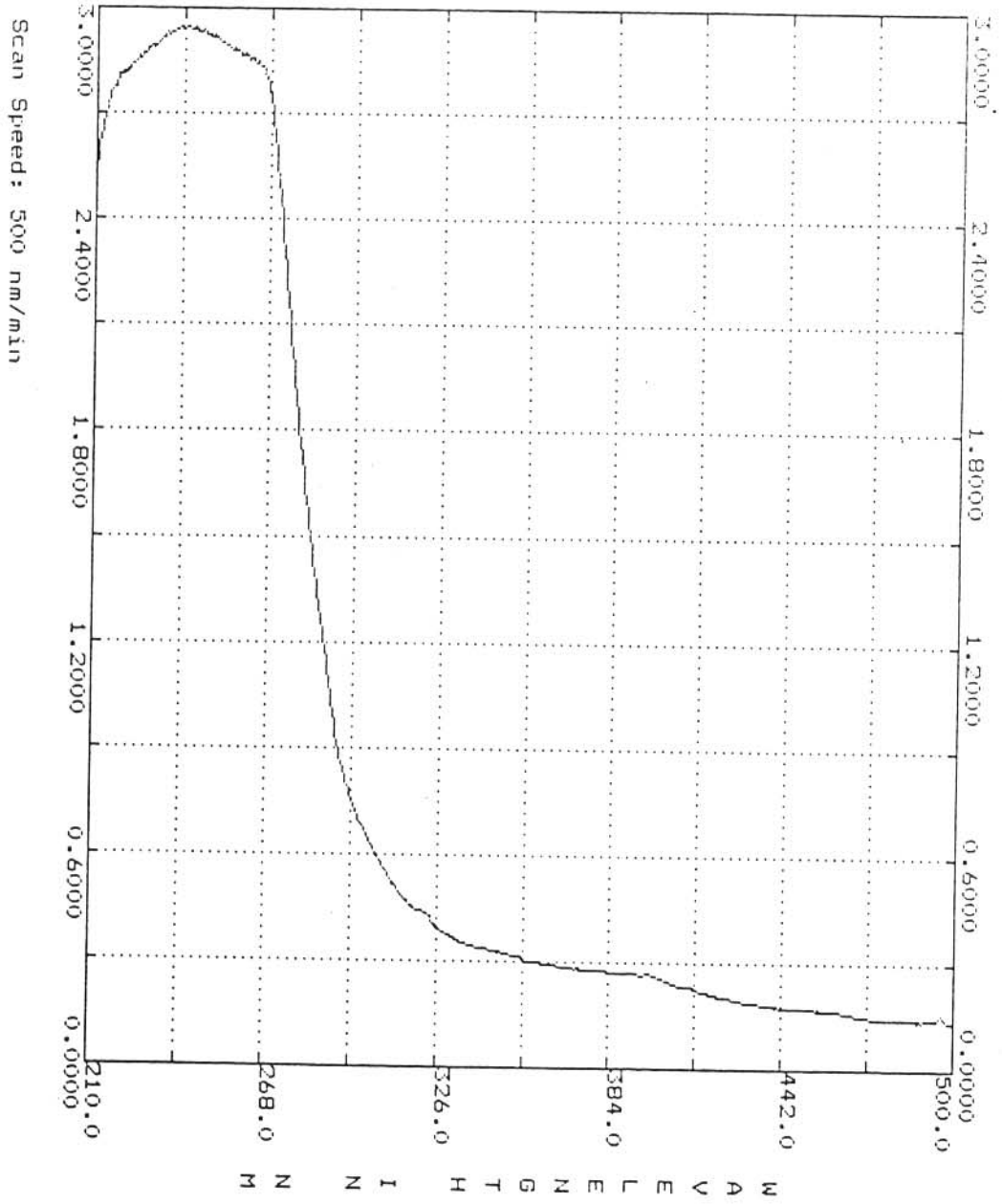


Figure 14 C Absorption spectrum of the fluorescent compound after reaction with sodium acetate.

4.8.3 High pressure liquid chromatography of the fluorescent compound

About 500 mg of the dried fluorescent compound prepared as described under 3.2.9, was dissolved in a minimum amount of chloroform. Aliquots of 100 µl of this solution were injected into the HPLC. The compound produced a peak after 9.05 minutes (Figure 15). The eluent was collected at this point. Combined eluents were evaporated to dryness under reduced pressure at a temperature of 30°C, the residue dissolved in a minimum amount of chloroform and again evaporated to dryness under reduced pressure. The compound was finally dissolved in chloroform for infra red spectroscopy and in deuterated chloroform for nuclear magnetic resonance spectroscopy.

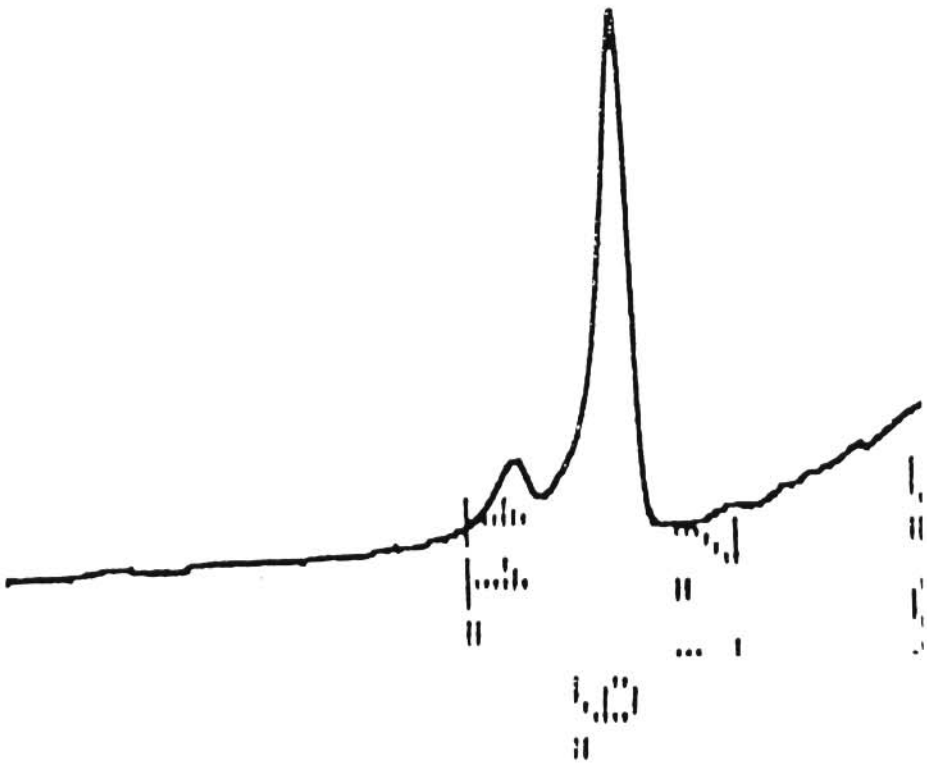


Figure 15 HPLC chromatograph of the fluorescent compound.

4.8.4 Nuclear magnetic resonance spectroscopy of the fluorescent compound

4.8.4.1 ^{1}H NMR spectrum of the fluorescent compound

Figure 16 shows the *^{1}H NMR* spectrum of the compound with signals at δ 0.86, 1.23, 1.58, 2.07, 3.93, 4.92, 5.76 and 7.23. The spectrum does not resemble that of a flavonoid as the flavonoid hydrogen atoms produce signals in the region δ 6.0 - 8.0 (Markham 1982).

The signal at δ 0.86 indicates that the compound contains a methyl group (-CH_3) (Pasto & Johnson 1969; Furnis *et al.* 1989; Gunstone 1992). As this value is below δ 0.9, it indicates that the methyl group is not influenced by functional groups like carbon-carbon double bonds or oxygen atoms (Pasto & Johnson 1969; Gunstone 1992). Therefore, the methyl group belongs to a long hydrocarbon chain. The signal at δ 1.23 is due to methylene hydrogen atoms. It also seems not to be influenced by oxygen atoms or carbon-carbon double bonds. The one at δ 1.58 could be due to methylene hydrogen atoms close to a carbonyl carbon atom (-CH_2CO-). The signal at δ 2.07 could indicate the presence of allylic hydrogen atoms (-C*H_2*CH=CHC*H_2*-). Finally the signal at δ 4.92 could be due to the presence of olefinic hydrogen atoms (-CH=CH) in the molecule.

This information from the ^{1}H NMR spectroscopy suggests that the compound could be a long hydrocarbon chain with a carbon-carbon double bond or bonds, a methyl group and a ketone group.

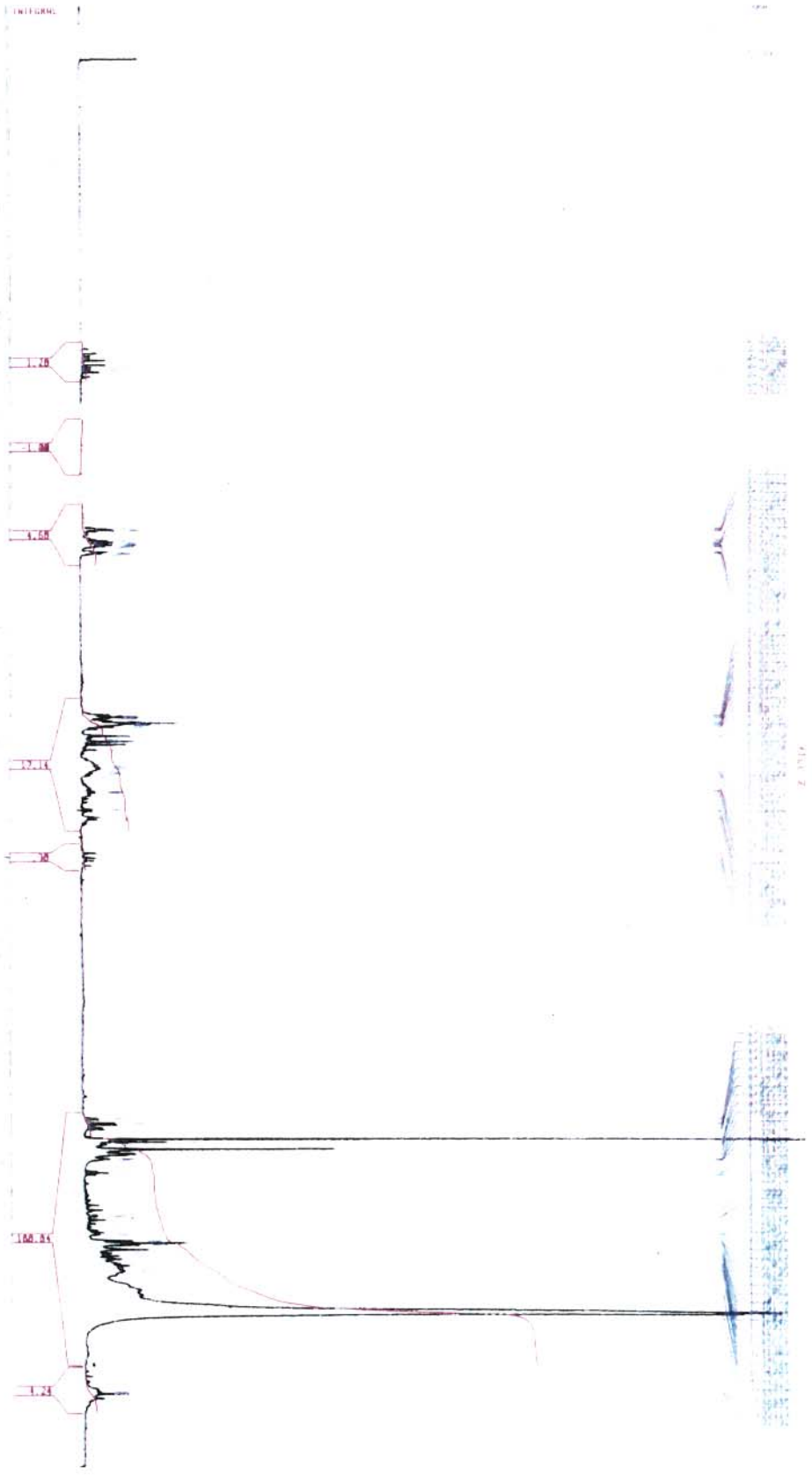


Figure 16 ^{1}H NMR spectrum of the fluorescent compound

4.8.4.2 ^{13}C NMR spectroscopy of the fluorescent compound

The ^{13}C NMR spectrum of the compound shows many signals (Figure 17). The signal at 14.0 ppm indicates the presence of a methyl group ($-CH_3$) (Gunstone 1990; Gunstone 1992). The group of signals from 18.04 - 22.7 ppm possibly indicate a methylene group bonded to a methyl group ($-CH_2-CH_3$). The bands from 25.2 to 25.3 ppm indicate a methylene group bonded to an olefinic group $-CH_2-CH=CH-$). However, the absence of a signal at 129 ppm indicates the absence of a carbon-carbon double bond. The band at 171 ppm indicates the presence of a carbonyl carbon. This information suggests that the compound does not contain carbon-carbon double bonds.

4.8.5 Infrared spectroscopy

The IR spectrum of the compound shows several absorption bands (Figure 18). The compound contains a methyl group ($-CH_3$), as indicated by the absorption bands at 2960, 2829.35, and 1360 cm^{-1}. Absorption peaks at 2928.35, 2855.07 and 1440 cm^{-1}, indicate the presence of methylene groups(-CH-) (Nakanishi 1962; Cowley 1966; Pasto & Johnson 1969). The intensity of the peaks, especially at 2928.35, shows that the compound could be a long hydrocarbon chain.

The absorption band at 1731.14 cm^{-1}, indicates the presence of a ketone group (-C=O) or an aldehyde group (-CHO). The absence of an absorption band in the region 2820 to 2720 cm^{-1}, implies that the group is not an aldehyde (Cowley 1966; Pasto & Johnson 1969). The shift of the ketone band, down from 1715 cm^{-1} to 1731.14 cm^{-1} shows that the ketone group is in an ester bond. The presence of an ester bond is also indicated by absorption bands at 1206.74, 1221.12, and 1248.45 cm^{-1}.

The diffuse broad band around 3440 cm^{-1} could be attributed to absorption by an alcoholic -OH group. This is supported by the band at 1065.95 cm^{-1}. Absorption in this region is characteristic of carbon-oxygen bond in a C-O-H group (Cowley 1966; Pasto & Johnson 1969). Bands at 3040 and 1632 cm^{-1} could be due to the absorption

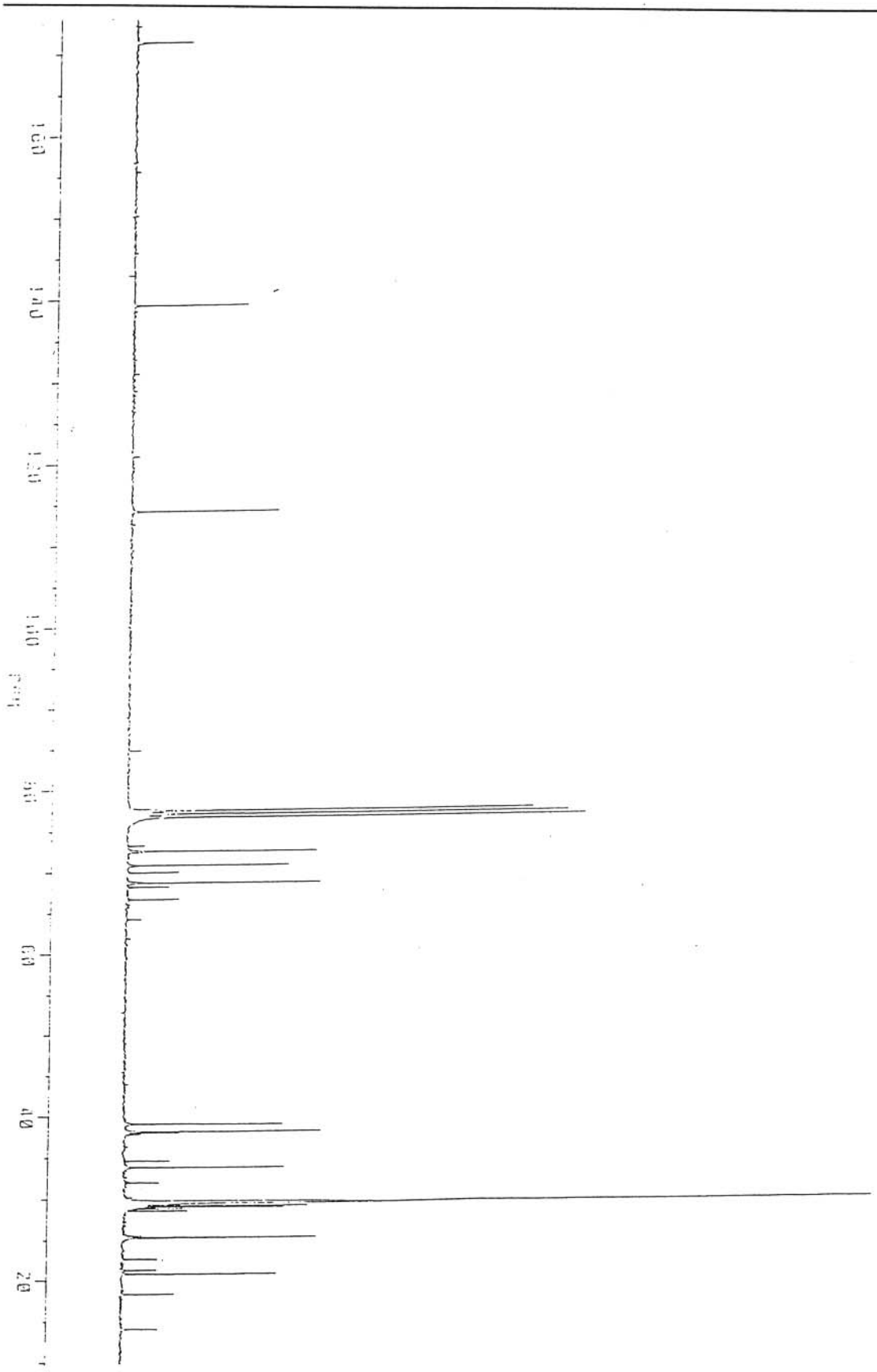


Figure 17 ^{13}C NMR spectrum of the fluorescent compound.

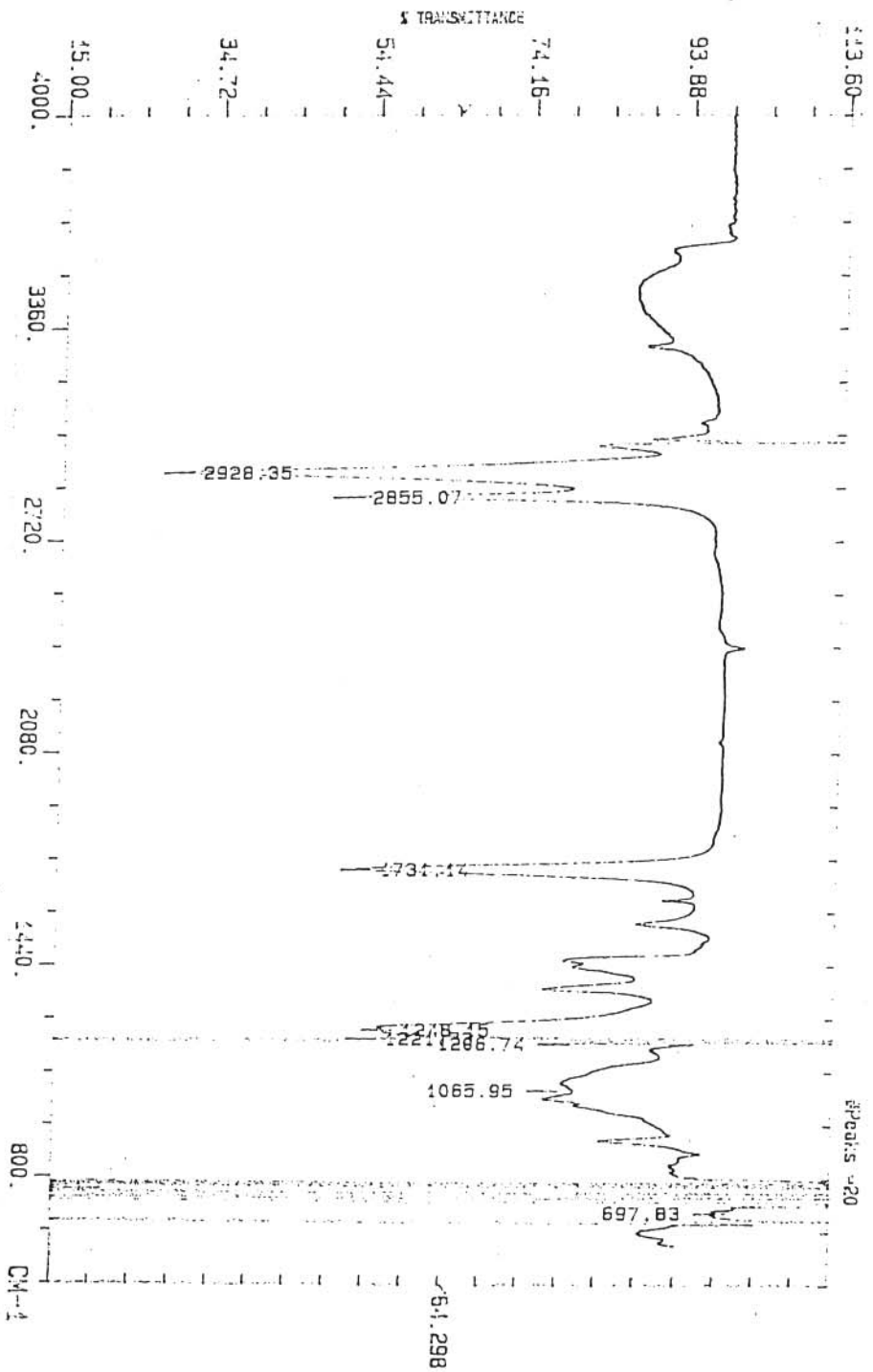


Figure 18 Infrared spectrum of the compound.

of the carbon-hydrogen bond in an olefinic group (=CH-). The band at 880 cm^{-1} could indicate the presence of a terminal methylene group (=CH-). IR spectroscopy indicates the compound to be a long hydrocarbon chain with a ketone, an alcoholic and an olefinic group.

The spectra discussed above indicate that the compound is a long hydrocarbon chain containing a methyl, a long methylene chain, a keto group (possibly in an ester bond) and an alcoholic or hydroxyl group. However, the spectra disagree on the presence of olefinic groups (carbon-carbon double bonds). According to the ^{13}C NMR spectrum, there are no olefinic groups in the molecule. Olefinic groups absorb in the 127 to 132 ppm range (Gunstone 1990 and 1992) of which there is no absorption peak in Figure 17. On this basis it can be concluded that the fluorescent compound does not contain an olefinic group.

The band at δ 0.86 in Figure 16 represents a methyl group. If the integral given in the Figure, namely 4.24 represents the three hydrogen atoms in a methyl group and also taking into consideration the other integrals, the fluorescent compound could have one hundred and thirty three (133) hydrogen atoms.

4.9 Chilling injury in fruit treated with the fluorescent compound

The fruit used in this investigation were of both Fuerte and Hass from the late batch which were found to have an increased tolerance to chilling during cold storage as discussed under 4.1. Brown patches appearing on the surface of the fruit were used as a criterion for the assessment of chilling injury at the end of four weeks of storage at 4.5°C. A scale of 0 to 5 was used to categorise the degree of chilling injury; 0 = no injury, 1 = slight injury, 3 = moderate injury, and 5 = severe injury. Fuerte fruit treated with the fluorescent compound showed slightly more chilling injury symptoms than untreated Fuerte fruit. Treated Hass fruit also showed chilling injury symptoms while untreated Hass fruit did not show any injury symptoms (Table 5).

Table 5 Chilling injury in vacuum-infiltrated fruit. Samples consisted of ten fruit. 0= no injury, 1= slight injury, 3= moderate injury, and 5= severe injury.

Chilling injury rating	No. of Fuerte fruit		No. of Hass fruit	
	Treated	Control	Treated	Control
0	4	5	7	10
1	5	4	3	0
3	1	1	0	0
5	0	0	0	0

CHAPTER 5

DISCUSSION AND CONCLUSIONS

South Africa is one of the major exporters of avocado fruit. Together with Israel, they constitute 90% of the avocado fruit export to the European market (Van Zyl & Groenewald 1986: Anon 1995). South African avocado fruit take about a month by sea to reach the overseas market. The fruit are stored in transit at a temperature of 5.5°C. However fruit of some cultivars develop chilling injury symptoms when kept at such a low temperature, which results in financial losses to the fruit farmers (LeClercq 1989; Eksteen & Henning 1992; Bezuidenhout 1992). The understanding of the nature and cause of chilling injury will help the fruit farmers to reduce their losses.

Some of the causes of chilling injury are believed to be phase transition in membranes, peroxidation of membrane lipids and oxidation of phenolics (Golan *et al.* 1977; Kahn 1977;Raison & Orr 1986a; Platt-Aloia & Thompson 1987; Gilmour *et al.* 1988; Wang *et al.* 1992). In this study the following were investigated on the avocado fruit: sensitivity of fruit to chilling, differential cooling between the surface and the internal of fruit, the anatomical aspect of chilling injury and the involvement of phenolics in chilling injury.

5.1 Sensitivity of avocado fruit to chilling

Experimental fruit of two avocado cultivars, Fuerte and Hass, were kept at a temperature of 4.5°C to induce chilling injury. The results show that fruit of Fuerte are more susceptible to chilling injury than those of Hass, with 5% of the fruit showing severe chilling injury symptoms at the end of the storage period (Table 1). Fruit of

Fuerte that were picked later in the harvest season (13th July) became more resistant to chilling than the early-picked (1st June) fruit, showing very little severe chilling injury symptoms. It has been established that exposure of chilling-sensitive plants to low nonchilling temperatures before exposure to chilling temperatures induces the plants to be resistant to chilling, a process called acclimation (Gilmour *et al.* 1988; Wang *et al.* 1992). It could be that the chilling resistance in the late-picked fruit is due to acclimation by the low winter temperature. The same could apply to the fruit of Hass which mature and are picked later than those of Fuerte.

The following conclusions can be drawn from this investigation:

- Avocado fruit of the cultivar Fuerte are more sensitive to chilling than those of Hass.
- Late harvested fruit of Fuerte are more resistant to chilling than early harvested fruit.

5.2 Differential cooling of the avocado fruit surface and fruit flesh

This was done to find out whether the fruit flesh does not stay warm while the surface has already cooled to storage temperature because of the size of the fruit and its high oil content. The cooling rates of the skin of the fruit (exocarp) and the flesh (mesocarp) differed only during the first eight hours of cold storage. Thereafter, the cooling rate became uniform until the cold storage temperature of 4.5°C was maintained (Figure 2).

The following conclusion can be drawn from this investigation:

- A differential cooling rate is, therefore, ruled out as a cause of chilling injury in avocado fruit.

5.3 The anatomical aspect of chilling injury in avocado fruit

An anatomical investigation was carried out on the experimental fruit to determine the kind of damage that occurs in the fruit. The results show that chilling injury causes damage to exocarp cells (Figures 3 & 4). The mesocarp cells were not damaged indicating that damage caused by chilling is localised to the exocarp. In fruit that show chilling injury symptoms, the epidermal cells remain intact while the exocarp cells beneath them collapse. The remains of the exocarp cells become gelatinous, suggesting that the cell walls have been digested. The collapse of the exocarp cells below the intact epidermis explains why surface browning is followed by pitting. Scanning electron microscopy show that the plasmalemma firstly forms invaginations followed by coagulation of small vacuoles (Figure 4), these then shrink and disintegrate. Collapse of the cell walls follow disintegration of membranes. It could be that membrane disintegration releases enzymes that digest cell walls. The sequence of events that lead to chilling injury is in agreement with the findings of Ishikawa (1996). Using mung been cells cultured at 0°C, Ishikawa (1996) found that chilling injury occurs over three stages. According to his findings, the early stage of chilling injury is marked by swelling of cells accompanied by appearance of rough vacuolar membranes, development of vacuoles inside plastids, swollen mitochondria and enlargement of Golgi bodies. According to him the second stage involves shrinkage of the plasma-membrane while the third stage involves the destruction of the cytoskeleton which is a very slow process.

The anatomical investigations further show that fruit of Fuerte have extensive lenticels as compared to those of Hass (figure 4). In chilling injured fruit the most damaged cells occur in the vicinity of lenticels. Pesis *et al.* (1994) found that treatment of Fuerte avocado fruit with a low oxygen atmosphere, for twenty four hours, before cold storage reduced chilling injury symptoms. This could imply that molecular oxygen aggravates chilling injury. Molecular oxygen is known to produce reactive oxygen species (singlet oxygen, superoxide anion, hydrogen peroxide and the hydroxyl radical)

under normal and stressful conditions (Daub & Hangarter 1983; Halliwell 1984; Doke & Ohashi 1988). Aeration of the exocarp cells, due to the presence of lenticels could aggravate chilling injury if cold stress involves formation of toxic oxygen species. This might contribute to susceptibility of the Fuerte fruit to chilling injury.

Pesis *et al.* (1994) found that a low oxygen atmosphere treatment lowers the respiration rate and ethylene production during cold storage (2°C) and subsequently at 17°C. Ethylene activates cellulase which degrades cell walls. In turn, oxygen is required for the synthesis of ethylene (Salisbury & Ross 1992). This could mean that in addition to producing toxic oxygen species, molecular oxygen may cause increased levels and activity of cellulase which may then cause cell wall digestion. This implicates that, more ventilated cells, due to the presence of lenticels, produce more ethylene which in turn activates cellulase which may thus lead to cell wall digestion.

The following conclusions can be drawn from the anatomical investigation:

- Chilling injury in avocado fruit is confined to the exocarp.
- The epidermal cells in the exocarp remain intact while the other exocarp cells collapse.
- The mesocarp is not damaged during chilling injury.
- Membranes in damaged cells coagulate and then disintegrate.
- Cell walls of damaged cells become gelatinous.
- Most damaged cells occur in the vicinity of lenticels.
- The lenticels are larger in the fruit of Fuerte compared to those of Hass.
- Chilling injury might be aggravated by aeration due to the presence of lenticels.
- Sensitivity of fruit of the cultivar Fuerte might be linked to the presence of large lenticels.

5.4 The involvement of phenolics in chilling injury of avocado fruit

Phenolics are a diverse group of compounds which are normally produced by plants under conditions of stress (Goodwin & Mercer 1983; Dey *et al.* 1997). They have in common an aromatic ring or rings with hydroxyl or substituted hydroxyl groups. They are synthesised through the shikimic acid pathway which leads to the formation of other compounds. According to Goodwin and Mercer (1983) accumulation of phenolic compounds can be due to activation/inactivation of some enzymes in the shikimic acid pathway. Phenylalanine ammonia-lyase (PAL), is a key enzyme in phenolic metabolism that leads to the synthesis of lignin, phenolics and alkaloids. However, this enzyme is not a rate-limiting step in phenolic metabolism due to its high concentrations in plants.

Histochemical localisation of phenolic compounds in the experimental avocado fruit show that more phenolics occur in the exocarp than in the mesocarp (Figure 5). In noninjured fruit the phenolics occur as small droplets arranged along the periphery of the cell. In damaged cells they occur as large droplets that fill the entire cell and in completely damaged cells they are embedded in the remainder of the cell wall material. This suggests that phenolics occur in small bodies that are bound by membranes. Damage to the membrane leads to leakage of the phenolic compounds, indicating that chilling injury involves damage to membranes. The leaked phenolics could possibly be acted on by polyphenol oxidases or complex directly with other compounds to produce the symptomatic brown colouration.

Quantitative investigation of phenolics in avocado fruit, show that fruit of the chilling sensitive Fuerte, have a higher phenolic content than those of Hass (Tables 3 & 4 and Figures 7 & 8). The late harvested fruit of Fuerte have a higher phenolic content than the early harvested fruit, containing twice as much phenolics as the latter (Figure 7). A high phenolic content cannot be associated with resistance to chilling injury as the late harvested fruit of Fuerte is more resistant to chilling and have a higher

phenolic content while the chilling-resistant fruit of Hass have a lower phenolic content than both early and late fruit of Fuerte. The phenolic concentration of early Fuerte fruit increased two-fold after three weeks of cold storage corresponding with the appearance of chilling injury symptoms in the fruit. The phenolic conentration in late Fuerte, both early and late Hass increased only slightly during this period. It could be that the amount of increase, or increase in a particular type of phenolic compound, and not the phenolic content as such leads to chilling injury.

The type of phenolics present in fruit may play a role in determining sensitivity of the fruit to chilling. Thin layer chromatography shows that the amount of some phenolics (estimated by spot size) increased in late harvested fruit that became resistant to chilling (compound 9 in Figure 9 and compound 12 in Figure 10). Corresponding compounds are present in Hass, which is resistant to chilling, and these do not change between early and late harvested fruit. The presence of these compounds can therefore be associated with resistance to chilling injury. Other phenolics decreased in late harvested Fuerte fruit that have become resistant to chilling, when compared to those in early harvested fruit (compound 7 in Figure 9 and compound 14 in Figure 10). The combined effect of the increase and decrease in the amount of the different phenolics may play a role in conferring sensitivity to chilling injury. One of the explanations could be that the decreasing phenolics inhibit and the increasing ones activate some of the processes involved in inducing resistance to chilling injury.

The results do not support the idea of a general oxidation of phenolics catalysed by polyphenol oxidases and peroxidases and phenolic oxidation does not seem to be associated with chilling injury in avocado fruit. If it was, the total phenolic content should have decreased as chilling injury became evident. The fact that the phenolic content increases and decreases during cold storage (Figures 7 and 8), indicates that low temperature does not completely stop the metabolism of phenolic compounds.

In general, the total phenolic concentration in the two cultivars increased both

during ripening and cold storage at 4.5°C (Figures 7 & 8). This differs from the findings of Van Rensburg (1984), who found that the total phenolic and flavonoid concentrations in general decreased during cold storage at 7°C. However, his investigations were done on the mesocarp and not on the exocarp.

The following conclusions can be drawn from the above investigation:

- Fruit of the avocado cultivar Fuerte have a higher phenolic content than those of Hass.
- There is no relationship between phenolic content and sensitivity of avocado fruit to chilling.
- A general oxidation of phenolics is not a cause of chilling injury in avocado fruit.
- In general the phenolic content increases during cold storage of avocado fruit.
- In chilling injured fruit the increase in phenolics during storage corresponds with the appearance of chilling injury symptoms.
- The exocarp of avocado fruit contain a higher concentration of phenolics than the mesocarp.
- Phenolics in avocado fruit occur in membrane-bound bodies and then disperse following chilling injury.
- The dispersal of phenolics suggests that chilling injury involves damage to membranes.

5.5 The fluorescent compound

On the thin layer chromatograms a certain compound that fluoresces when exposed to ultraviolet light was found to increase with increase in chilling injury in Fuerte fruit (Figure 9). This compound does not react with the phenol reagent Folin-Ciocalteau, and is therefore not a phenolic. Nuclear magnetic resonance and infrared spectroscopy analyses of this compound(Figures 16, 17 & 18) show that it is a large organic compound with a methyl group, a long methylene chain, a ketone group and

an alcoholic or hydroxyl group. Lipid peroxidaton usually produces some fluorescent compounds (Halliwell 1984; Vick & Zimmerman 1987; Larson 1988). Lipid peroxidation products are short volatile or gaseous hydrocarbons and carbonyl compounds. Such products include ethane, pentane, hexanal and malondialdehyde. They are often used as indicators in lipid peroxidation studies (Bidlack & Tappel 1973; Dillard & Tappel 1973; Dillard and Tappel 1979; Dumelin & Tappel 1977). The compound is therefore also not a lipid peroxidation product.

Carbonyl compounds produced during lipid peroxidation are highly reactive and combine with amino acids, amino groups of proteins, phospholipids and nucleic acids to produce conjugated chromophoric Schiff base systems (Dey *et al.* 1997). These are yellowish in colour and have excitation bands around 360 nm (Dillard & Tappel 1973; Fletcher *et al.* 1973; Meir *et al.* 1992). The fluorescent compound identified in this study is a long hydrocarbon and lacks the conjugated system. However, this statement does not rule out the production of such conjugated systems in chilling-injured avocado fruit; they were not investigated in this study. According to Faubion and Kader (1995), lipid peroxidation products increased during cold storage of Hass fruit at 5°C in air and under a controlled atmosphere. They also found that glycolipid and phospholipid fatty acid unsaturation decreased with development of chilling injury. Meir *et al.* (1991) found that fluorescent lipid peroxidation products in the peel of Fuerte avocado fruit increase with ripening. These products were also found to increase with storage at 5°C for one to two weeks. The sources cited above (Faubion & Kader 1995; Meir *et al.* 1991) indicate that lipid peroxidation takes place during cold storage of avocado fruit. However, their findings do not indicate whether lipid peroxidation is a cause or a result of chilling injury.

The fluorescent compound identified could be a product resulting from the recombination of two hydroperoxy radicals or of a hydroperoxy radical and a fatty acid radical. In either case the components should not contain conjugated unsaturated systems, because such unsaturated systems could not be identified in the compound.

When fruit are treated with this compound and stored at a low temperature, they become more susceptible to chilling injury. Treatment of the fruit of Fuerte and Hass cultivars followed by storage of the fruit at 4.5°C increased chilling injury in both cultivars (Table 5). However, only slightly so in chilling-sensitive Fuerte.

The other possibility is that the compound could be a long hydrocarbon chain that is a monomer of cutin. According to Koster (1986), the cell walls of the hypodermal cells of avocado fruit become cutinised just before the fruit reach maturity. This might explain why the compound does not increase in late-picked fruit; suggesting that by then the fruit have reached maturity. Furthermore, cell walls in avocado fruit thicken during ripening and storage at 5.5°C (Koster 1986). According to Salunkhe and Desai (1984), the cuticular wax of the avocado fruit contains C_{20} to C_{27} long-chain fatty acids. This does not necessarily mean that the avocado increases cutin production in order to protect itself from damage during storage at low temperatures, but that cutin production is a normal maturation process. The reason for the high phenolic level in late Fuerte fruit could be that cell walls are by then lignified and phenolics are not used for lignin production at this stage of development. However, Anderson *et al.* (1995) found that acclimation increases lignin content in the mesocotyl of maize seedlings. They suggested that lignification may be a component of acclimation-induced chilling tolerance which would increase cell wall rigidity, allowing cells to remain intact while nonacclimated cells become desiccated and mechanically weak in response to chilling.

Lipid peroxidation normally results from the activity of the reactive oxygen species and the enzyme lipoxygenase on polyunsaturated fatty acids. Both produce hydroxyl and hydroperoxy radicals. These further cause peroxidation of other lipids. Avocado oil is rich in the polyunsaturated fatty acids, oleic acid, linoleic acid and linolenic acid (Eaks 1990; Meir *et al.* 1991; Hiero *et al.* 1992), although it might not be the case with the membrane lipids. Major free fatty acids of the avocado are: oleic, palmitic and linoleic acid. The exocarp cells in Fuerte are well aerated due to the presence of relatively large lenticels. This provides a chance for the formation of toxic

oxygen species at low temperatures, which might cause lipid peroxidation. The involvement of singlet oxygen can be ruled out since it is formed during illumination and the avocado fruit used in the study were stored in the dark.

Chromatographic and spectroscopic analyses of the fluorescent compound show that:

- It is not a phenolic.
- It is not a lipid peroxidation product.
- It has a long mehtylene chain with a methyl group, a ketone group and a hydroxyl group.
- Treatment of fruit with the compound slightly increases chilling injury.

5.6 Possible involvement of lipoxygenase

The activity of lipoxygenase was not investigated in this study. However, it is not known to catalyse the peroxidation of nonesterified fatty acids (Halliwell 1984). Some of the activators of lipoxygenase are free peroxy radicals which are themselves products of lipid peroxidation. This means that lipid peroxidation should somehow be initiated before lipoxygenase can be actively involved in causing damage to membranes. Therefore, in order to cause lipid peroxidation, lipoxygenase can act on free unsaturated fatty acids to produce hydrocarbon radicals. The other possibility is that the low temperature oxidative stress should cause the sequential univalent reduction of oxygen which ultimately produces the free hydroxyl and hydroperoxy radicals. The free radicals will then activate lipoxygenase into causing lipid peroxidation.

5.7 General conclusions

Chilling injury in avocado fruit involves disintegration of membranes followed by digestion of cell walls. The study could not show a relationship between phenolic content and sensitivity of avocado fruit to chilling injury. Chilling injury occurs in

exocarp cells that are highly aerated, that is, those cells that occur in the vicinity of lenticels. There are larger lenticels in fruit of the cultivar Fuerte than in those of Hass. This suggests that chilling injury in avocado fruit could involve lipid peroxidation, because both lipoxygenase activity and formation of toxic oxygen species require molecular oxygen. Lipid peroxidation would then lead to membrane disintegration. Membrane-bound enzymes, including cell wall hydrolysing ones, would then be released leading to cell wall digestion. Cellulase, a cell wall hydrolysing enzyme, is indirectly activated by molecular oxygen through ethylene synthesis. In this way, lenticels would further contribute to chilling injury.

The fluorescent compound that increases with chilling is a large organic compound with a methyl group, a long methylene chain, a ketone group and an alcoholic or hydroxyl group. It could result from the recombination of two hydroperoxy radicals or of a hydroperoxy radical and a fatty acid radical following lipid peroxidation. The other possibility is that it could be a long hydrocarbon monomer used for the synthesis of cutin.

The following aspects can be further investigated to resolve chilling injury in avocado fruit:

- The final identification of the fluorescent compound and its influence on fruit be determined in greater detail.
- An analysis of lipid peroxidation products in early and late harvested fruit of Fuerte and an investigation as to whether these are a cause or a result of chilling injury.
- Investigation of oxidative stress in cold stored fruit, that is whether cold storage leads to the production of the super oxide anion or hydrogen peroxide.
- Localisation of hydrogen peroxide production in the avocado fruit exocarp cells.
- An investigation of the biochemical changes that occur in the vascular areas of the mesocarp in both early and late harvested Fuerte fruit.
- Analysis of antioxidants present in avocado fruit exocarp.
- Both qualitative and quantitative analyses of the phenolics is necessary to

determine their possible effect.

CHAPTER 6

SUMMARY

The aim of this study was to investigate:

- The level of phenolics in the exocarp of avocado fruit stored at a low temperature.
- Whether there is a correlation between fruit phenolic level and sensitivity to chilling injury.
- Whether there could be a change in phenolic concentration as chilling injury advances.
- The possible presence of cellular damage in the exocarp of chilling injured fruit
- Whether differential cooling between the exocarp and mesocarp of fruit does not lead to chilling injury.

The following anatomical changes were observed:

- During chilling injury the plasmamembrane forms some invaginations and vesicles and small vacuoles within the cell coagulate.
- Disintegration of these membranes is followed by digestion of the cell wall.

Phenolic content of fruit of two avocado cultivars were compared; namely chilling-sensitive Fuerte and chilling-tolerant Hass. The following results were obtained:

- Fruit of Fuerte have a higher phenolic content than those of Hass.
- Late harvested fruit of Fuerte are more chilling resistant than early harvested fruit.
- In both cultivars late harvested fruit have a higher phenolic content than early

harvested fruit.

- In general the phenolic content of fruit increases with fruit ripening and during cold storage at 4.5°C.
- Two trends were observed, namely, a decease in concentration in phenolic compounds represented by spots 4, 5, and 7 in Figure 9 and spot 14 in Figure 10 as well as an increase in compounds represented by spot 9 in Figure 9 and spot 12 in Figure 10.

This study showed no direct correlation between phenolic content of fruit and fruit sensitivity to chilling.

A fluorescent compound which increases in amount with the development of chilling injury was identified in fruit of the cultivar Fuerte. The compound does not react with the phenol reagent, indicating that the it is not phenolic. Analyses of the purified compound by infra red and nuclear magnetic resonance spectroscopy indicate that it is a long hydrocarbon chain with a methyl group, a ketone and a hydroxyl group. The study could not conclusively determine whether the compound is the cause or the result of chilling injury in avocado fruit.

REFERENCES

Alscher, R.G. 1989. Biosynthesis and antioxidant function of glutathione in plants. *Physiol. Plant.* 77: 457 - 464.

Anon, 1995. World market for avocado. *RAP Market Information Bulletin.* No. 10: 1 - 7. www.agroindia.org/world/rap/avocado.htm

Anderson, M.D., Prasad, T.K. and Tewart, C.R. 1995. Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant Physiol.* 109: 1247 - 1257.

Bennett, B.A., Smith, G.M. and Nichols, B.G. 1987. Regulation of climacteric respiration in ripening avocado fruit. *Plant Physiol.* 83: 973 - 976.

Bezuidenhout, J.J. 1992. Analysis of transit temperature and fruit condition of South African export avocados. *S. A. Avocado Growers' Assoc. Yrbk.* 15: 39 - 40.

Bidlack, W.R. and Tappel, A.L. 1973. Fluorescent products of phospholipids during lipid peroxidation. *Lipids* 8: 203 - 207.

Bower, J.P., Cutting, J.G.M. and Truter, A.B. 1989. Modified atmosphere storage and transport of avocados - what it means. *S. A. Avocado Growers' Assoc. Yrbk.* 8: 17 - 20.

Bowler, C., Van Montagu, M. and Inze, D. 1992. Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43: 83 - 116.

Cowley, R.T. 1966. Infrared Spectroscopy. Allyn and Bacon Inc., Boston.

Croft, K.P.C, Jutner, F. and Slurasenko, A. 1993. Volatile products of lipoxygenase pathway evolved from *Phaseolus vulgaris (L.)* inoculated with *Pseudomonas syringae* pv *phasiolicola. Plant Physiol.* 101: 13 - 24.

Daub, M.E. and Hangarter, R.P. 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. *Plant Physiol.* 73: 855 - 857.

Daum, G. 1985. Lipids of mitochondria. *Biochim. Biophys. Acta* 822: 1 - 42.

De Arriola, M.C., Menchu, J.F. and Rolz, C. 1979. The Avocado. In: Tropical Foods: Chemistry and Nutrition, Vol. 2, eds. G.E. Inglett & G. Charalombous. Academic Press, New York.

Dey, P.M.; Brownleader, M.D. and Harborne, J.B. 1997. The Plant, the Cell and its Molecular, Components. In: Plant Biochemistry, eds, P.M. Dey and J.B. Harborne. Academic Press, San Diego.

Dillard, C.J. and Tappel, A.L. 1973. Fluorescent products from reaction of peroxidising polyunsaturated fatty acids with phosphatidylethanolamine and phenylalanine. *Lipids* 8: 183 - 189.

Dillard, C.J. and Tappel, A.L. 1979. Volatile hydrocarbon and carbonyl products of lipid peroxidation: A comparison of pentane, ethane hexanal and acetone. *Lipids* 4: 989 - 994.

Doke, N. and Ohashi, Y. 1988. Involvement of an O_2^- generating system in the induction of necrotic lesions on tobacco leaves infected with tobacco mosaic virus. *Physiol. Mol. Plant Pathol.* 32: 163 - 175.

Dumelin, E.E. and Tappel, A.L. 1977. Hydrocarbon gases produced during *in vitro* peroxidation of polyunsaturated fatty acids and decomposition of preformed hydroperoxides. *Lipids* 12: 894 - 900.

Eaks, I.L. 1990. Change in fatty acid composition of avocado fruit during ontogeny, cold storage and ripening. *Acta Hortic.* 269: 141 - 152.

Eksteen, G.J. and Henning, B. 1992. 1991 Temperature survey of export avocados. *S. A. Avocado Growers' Assoc. Yrbk.* 15: 41 - 44.

Faubion, D.F. and Kader, A. 1995. Biochemical bases for controlled-atmosphere effects on reducing chilling injury of "Hass" avocado fruit. *HortScience* 30: 809.

Fletcher, B.L., Dillard, C.J. and Tappel, A.L. 1973. Measurement of fluorescent lipid peroxidation products in biological systems and tissues. *Anal. Biochem.* 52: 1 - 9.

Foyer, C.H. and Halliwell, B. 1976. The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. *Planta* 133: 21 - 25.

Fridovich, I. 1978. The biology of oxygen radicals. *Science* 201: 875 - 880.

Furnis, B.S., Hammaford, A.J., Smith, P.W. and Tatchell, A.R. 1989. Vogel's Textbook of Practical Organic Chemistry. Longman, New York.

Gilmour, S.H., Hajela, R.K. and Thomashow, M.F. 1988. Cold acclimation in *Arabidopsis thaliana*. *Plant Physiol.* 87: 745 - 750.

Ginsburg, L. 1985. Post harvest physiological problems of avocados. *S. A. Avocado Growers' Assoc. Yrbk.* 8: 8 - 11.

Golan, A., Kahn, V. and Sadovski, A.Y. 1977. Relationship between polyphenols and browning in avocado mesocarp. Comparison between the Fuerte and Lerman cultivars. *J. Agric. Fd. Chem.* 25: 1253 - 1261.

Goodwin, T.W. and Mercer, E.I. 1983. Introduction to Plant Biochemistry, 2nd edn. Pergamon Press, Oxford.

Graham, D. and Patterson, B.D. 1982. Responses of plants to low, nonfreezing temperatures: Proteins, metabolism and acclimation. *Ann. Rev. Plant Physiol.* 33: 347 - 372.

Gunstone, F.D. 1990. Fatty Acids - Structural Identification. In: Methods in Plant Biochemistry, Vol. 4, eds. J.L. Harwood & J.R. Bowyer. Academic Press, London.

Gunstone, F. D. 1992. High-resolution ^{1}H and ^{13}C NMR. In: Lipid Analysis, eds. R.J. Hamilton & S. Hamilton. IRL Press, Oxford.

Hale, M.G. and Orcutt, D.M. 1987. Physiology of Plants Under Stress. John Wiley and Sons, New York.

Halliwell, B. 1984. Chloroplast Metabolism. Clarendon Press, Oxford.

Harborne, J.B. 1984. Phytochemical Methods. 2nd edn. Chapman and Hall, London.

Hiero, M.T.G., Tomas, M.C., Fernandez-Martin, E. and Santa-Maria, G. 1992. Determination of the triglyceride composition of avocado oil by high-performance liquid chromatography using a light-scattering detector. *J.*

chromatogr. 607: 329 - 338.

Hudson, B.J.F. and Mahgoub, S.E.O. 1980. Naturally-occurring antioxidants in leaf lipids. *J. Sci. Fd. Agric.* 31: 646 - 650.

Ishikawa, H.A. 1996. Ultrastructural features of chilling injury: Injured cells and early events during chilling of suspension-cultured mung bean cells. *Amer. J. Bot.* 83: 825 - 835.

Johansen, D.A. 1940. Plant Microtechnique. McGraw-Hill, New York.

Kahn, V. 1975. Polyphenol oxidase activity and browning of three avocado varieties. *J. Sci. Fd. Agric.* 26: 1319 - 1324.

Kahn, V. 1977. Latency properties of polyphenol oxidase in two avocado cultivars differing in their rate of browning. *J. Sci. Fd. Agric.* 28: 233 - 239.

Karni, L., Prusky, D., Kobiler, I., Bar-Shira, E. and Kobiler, D. 1989. Involvement of epicatechin in the regulation of lipoxygenase activity during activation of quiescent *Colletotrichum gloeosporeoides* of ripening avocado fruits. *Physiol. Mol. Plant Pathol.* 35: 367 - 374.

Keppler, L.D. and Baker, C.J. 1989. O_2^- -Initiated lipid peroxidation in a bacterium-induced hypersensitive reaction in tobacco cell suspensions. *Phytopathol.* 79: 555 - 562.

Köhne, J.S. 1991. Performance of Hass on three clonal rootstocks. *S.A. Avocado Growers' Assoc. Yrbk. 14: 39.*

Koster, S.A. 1986. Aspekte van sommige ultrastrukturele en fisiologiese veranderinge van avocadovrugte (*Persea americana* Mill kultivar Fuerte) gedurende koel-

opberging en rypwording. Masters thesis. University of Pretoria, Pretoria.

Kremer-Köhne, S., Köhne, J.S. and Kirkman, B. 1991. Yield and fruit quality of avocado cv Fuerte as influenced by paclobutrazol foliar applications. *S.A. Avocado Growers' Assoc. Yrbk.* 14: 22 - 23.

Larson, R.A. 1988. The antioxidants of higher plants. *Phytochemistry 27: 969 - 978.*

LeClercq, H.Y.H. 1989. Observations of avocados on the overseas market. *S. A. Avocado Growers' Assoc. Yrbk.* 12: 14 - 16.

Levitt, J. 1980. Responses of Plants to Environmental Stress. 2nd edn. Academic Press, Orlando.

Luza, J.G., Van Gorsel, R., Polito, V.S. and Kader, A.A. 1992. Chilling injury in peaches: A cytochemical and ultrastructural cell wall study. *J. Amer. Soc. Hort. Sci.* 117: 114 - 118.

Mabry, T.J., Markham, K.R. and Thomas, M.B. 1970. The systematic identification of Flavonoids. Springer-Verlag, Berlin.

Marcus, L., Prusky, D. and Jacoby, B. 1988. Purification and characterisation of avocado lipoxygenase. *Phytochemistry* 27: 323 - 327.

Markham, K.R. 1982. Techniques of flavonoid identification. Academic Press, London.

Meir, S., Philosoph-Hadas, S., Zauberman, G., Fuchs, Y., Akerman, M. and Aharoni, N. 1991. Increased formation of fluorescent lipid-peroxidation products in avocado peels precedes other signs of ripening. *J. Amer. Soc. Hort. Sci.* 116: 823 - 826.

Meir, S., Philosoph-Hadas, S. and Aharoni, N. 1992. Ethylene-increased accumulation of fluorescent lipid-peroxidation products detected during senescence of parsley by a newly developed method. *J. Amer. Soc. Hort. Sci. 117: 128 - 132.*

Murata, N., Sato, N., Takahashi, N. and Hamazaki, Y. 1982. Compositions and positional distributions of fatty acids in phospholipids from leaves of chilling-sensitive and chilling-resistant plants. *Plant and Cell Physiol.* 23: 1071 - 1079.

Murata, N. and Yamaya, J. 1984. Temperature-dependent phase behaviour of phosphatidylglycerols from chilling-sensitive and chilling-resistant plants. *Plant Physiol.* 74: 1016 - 1024.

Nakanishi, K. 1962. Infrared Absorption Spectroscopy. Holden-Day Inc., San Fransisco.

Nishijima, K.A., Chan, H.T., Sanxter, S.S. and Linse, E.S. 1995. Reduced heat shock period of 'Sharwil' avocado for tolerance in quarantine cold treatment. *Hortscience* 30: 1052 - 1053.

Parkin, K.L. and Kuo, S.J. 1989. Chilling-induced lipid degradation in cucumber (*Cucumis sativus L. cv. Hybrid C)* fruit. *Plant Physiol.* 90: 1049 - 1056.

Pasto, D.J. and Johnson, C.R. 1969. Organic Structure Determination. Prentice Hall, Englewood Cliffs.

Pesis, E., Marinansky, R., Zauberman, G. and Fuchs, Y. 1994. Prestorage low-oxygen atmosphere treatment reduces chilling injury symptoms in 'Fuerte' avocado fruit. *HortScience* 29: 1042 - 1046.

Platt-Aloia, K.A. and Thomson, W.W. 1987. Freeze fracture evidence for lateral phase separations in the plasmalemma of chilling-injured avocado fruit. *Protoplasma* 136: 71 - 80.

Prasad, T.K., Anderson, M.D., Martin, B.A. and Stewart, C.R. 1994a. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *The Plant Cell* 6: 65 - 74.

Prasad, T.K., Anderson, M.D. and Stewart, C.R. 1994b. Acclimation, hydrogen peroxide and abscisic acid protect mitochondria against irreversible chilling injury in maize seedlings. *Plant Physiol.* 105: 619 - 627.

Prusky, D. and Kobiler, I. 1985. Inhibitors of avocado lipoxygenase: their possible relationship with the latency of *Colletotrichum gloeosporioides*. *Physiol. Plant Pathol.* 27: 269 - 279.

Raison, J.K. and Orr, G.R. 1986a. Phase transitions in thylakoid polar lipids of chilling-sensitive plants. *Plant Physiol.* 80: 638 - 645.

Raison, J.K. and Orr, G.R. 1986b. Phase transitions in liposomes formed from the polar lipids of mitochondria from chilling-sensitive plants. *Plant Physiol.* 81: 807 - 811.

Raison, J.K. and Wright, L.C. 1983. Thermal phase transitions in the polar lipids of plants: Their induction by disaturated phospholipids and their possible relation to chilling injury. *Biochim. Biophys. Acta* 731: 68 - 78.

Robards, A.W. 1978. Scanning Electron Microscopy. In: Electron Microscopy and Cytochemistry of Plant Cells. Ed. J.C. Hall. Elsevier Biomedical Press, Amsterdam.

Roughan, P.G. 1985. Phosphatidylglycerol and chilling sensitivity in plants. *Plant Physiol.* 77: 740 - 746.

Salisbury, F.B. and Ross, C.W. 1992. Plant physiology. 4th edn. Wadsworth, Belmont.

Salunkhe, D.K. and Desai, B.B. 1984. Postharvest biotechnology of fruits. Vol.2. CRC Press, Boca Raton.

Sapers, G.M. 1993. Browning of foods: control by sulfites, antioxidants and other means. *Food Technol.* 47: 75 - 84.

Scandalios, J.G. 1993. Oxygen stress and superoxide dismutase. *Plant Physiol.* 101: 7 - 12.

Sharon-Raber, O. and Kahn, V. 1983. Avocado mesocarp; browning potential, carotenoid content, polyphenol oxidase, catalase and peroxidase activities: Comparison between six avocado cultivars. *J. Fd. Sci.* 48: 1874 - 1875.

Silverstein, R.M. and Bassler, G.C. 1967. Spectrometric identification of organic Compounds. 2nd edn. John Wiley and Sons Inc., New York.

Somerville, C. and Browse, J. 1991. Plant lipids: Metabolism, mutants and membranes. *Science 252: 80 - 87.*

Torres, A.M., Mau-Lastovicka, T. and Rezaaiyan, R. 1987. Total phenolics and high-performance liquid chromatography of phenolics of avocado. *J. Agric. Food Chem.* 35: 921 - 925.

Truter, A.B., Cutting, J.G.M., Bower, J.P. and Van Eeden, S.J. 1991. Effect of modified atmospheres on internal physiological browning of Fuerte avocados.

S. A. Avocado Growers' Assoc. Yrbk. 14: 50 - 52.

Vakis, N.J. 1982. Storage behaviour of Ettinger, Fuerte and Hass avocados grown on Mexican rootstocks in Cyprus. *J. Hort. Sci.* 57: 221 - 226.

Van Lelyveld, L.J., Gerrish, C. and Dixon, R. 1984. Enzyme activities and polyphenols related to mesocarp discolouration of avocado fruit. *Phytochemistry* 23: 1531 - 1534.

Van Rensburg, E. and Engelbrecht, A.H.P. 1986. Effects of calcium salts on susceptibility to browning of avocado fruit. *J. Fd. Sci.* 51: 1067 - 1068.

Van Zyl, J. and Groenewald, J.A. 1986. The South African export market for avocado and exchange rate: effects on profitability. *S. A. Avocado Growers' Assoc. Yrbk. 9: 67 - 71.*

Vick, B. and Zimmerman, D.C. 1987. Oxidative systems for modification of fatty acids: The lipoxygenase pathway. In: The Biochemistry of Plants, Vol. 9 eds. P.K. Stumpf, and E.E. Conn. Academic Press Inc., Orlando.

Vorster, L.L., Toerien, J.C. and Bezuidenhout, J.J. 1990. Temperature management of avocados - an integrated approach. *S. A. Avocado Growers' Assoc. Yrbk.* 13: 43 - 46.

Vorster, L.L., Bezuidenhout, J.J. and Toerien, J.C. 1991. The principles of temperature management - commercial results. *S. A. Avocado Growers' Assoc. Yrbk.* 14: 44 - 46.

Walker, M.A. and McKersie, B.D. 1993. Role of the ascorbate-glutathione antioxidant system in chilling resistance of tomato. *J. Plant Physiol.* 141: 234 - 239.

Wang, C.Y. 1982. Physiological and biochemical responses of plants to chilling stress. *HortScience* 17: 173 - 186.

Wang, C.Y., Kramer, G.F., Whitaker, B.D. and Lusby, W.R. 1992. Temperature preconditioning increase tolerance to chilling injury and alters lipid composition in zucchini squash. *J. Plant Physiol.* 140: 229 - 235.

Wise, R.R. and Naylor, A.W. 1987a. Chilling-enhanced photooxidation. *Plant Physiol.* 83: 272 - 277.

Wise, R.R. and Naylor, A.W. 1987b. Chilling-enhanced photooxidation. *Plant Physiol.* 83: 278 - 282.

Wu, J. and Browse, J. 1995. Elevated levels of high-melting-point phosphatidyl-glycerols do not induce chilling sensitivity in an *Arabidopsis* mutant. *The Plant Cell* 7: 17 - 27.

Zauberman, G., Fuchs, Y. and Akerman, M. 1985. Peroxidase activity in avocado fruit stored at chilling temperatures. *Sci. Hortic.* 26: 261 - 265.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to the following:

1. Prof. D.C.J. Wessels, my supervisor, for his inspiration, advice and constructive comments.
2. My co-supervisor, Prof. J.J.M. Meyer for his guidance and constructive comments.
3. Staff members of Westfalia Estates for freely supplying experimental material.
4. The Departments of Botany and Chemistry, University of Pretoria for their assistance and use of their instruments in analysing the fluorescent compound.
5. The Department of Biochemistry, University of the North for the use of their instruments.
6. The Electron Microscope Unit at the University of the North for the use of the scanning electron microscope.
7. Mr. R. Sandrock of the Instructional Support Services at the University of the North for taking photographs of study material.
8. Mr. M.J. Potgieter, Department of Botany, University of the North, for his assistance.
9. The staff at the library of the University of the North for interlibrary loans, without their assistance this study would not have been a success.