Superficial Scald in Apples

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A thesis presented in fulfilment of the requirement for the degree of Doctor of Philosophy

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July 1999
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
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Comparative physiological and biochemical studies were conducted with apple varieties which were both susceptible to superficial scald, ‘Granny Smith’ and ‘Lady Williams’, and resistant to scald, ‘Crofton’, to understand the basic mechanisms of scald aetiology. This provided a physiological basis for the development of alternatives to diphenylamine (DPA) to control the disorder in cool stored apples. The research included a re-examination of the pioneering work on scald histology by Bain (1956) and Bain and Mercer (1963), using newer methods of tissue preparation and instrumental techniques. The results confirmed their work, which showed that the typical scald symptoms were the result of the deposition of brown pigments on the tonoplast in hypodermal and epidermal cells. It has been generally accepted that these pigments were formed by the action of polyphenol oxidase (PPO) following its release from chloroplasts, as a result of membrane damage caused by the oxidation products of α-farnesene. However, intact and functional chloroplasts were observed in severely scalded peel tissue. It was proposed that peroxidase (POD) might play a more important role in scald development.

The concept that the concentration of α-farnesene or its volatile breakdown products was related to the severity of scald was examined in two ways. In the first set of experiments, α-farnesene was applied in vapour form in the headspace, topically or to the underside of peel discs of ‘Granny Smith’ apple stored at 0°C. In the second series of experiments at 0°C, ‘Granny Smith’ apples (with and without a pre-storage DPA application) and ‘Crofton’ apples were ventilated with air streams that had passed over a large mass of ‘Granny Smith’ or ‘Crofton’ apples. None of these treatments increased the incidence of scald in ‘Granny Smith’ and no scald was observed in ‘Crofton’ or DPA treated ‘Granny Smith’ apples. Research on the role of endogenous α-farnesene has been hindered by the lack of a rapid specific method for its quantitative analysis. A reliable direct gas chromatography method was developed for the measurement of α-farnesene in the peel and waxy cuticle. Application of this method showed that there was a positive linear relationship between internal ethylene and peel α-farnesene concentrations in both ‘Granny Smith’ and ‘Crofton’ apples. However ‘Granny Smith’ produced significantly more α-farnesene than ‘Crofton’. Treatment of ‘Granny Smith’ fruit with the ethylene analogue, propylene, confirmed that the increase in α-farnesene production in harvested apples was dependent on endogenous ethylene production. The concentration of 6-methyl hepten-2-one, a major oxidation product of α-farnesene, was high in scalded peel tissue but essentially absent in DPA treated and scald resistant peel. It was also observed that delayed application of DPA had no significant
effect on the accumulation of $\alpha$-farnesene, and that the occurrence of scald during storage in late harvested ‘Lady Williams’ fruit occurred without the accumulation of large concentrations of $\alpha$-farnesene or its oxidation products. These results support the proposal that differences in scald susceptibility were related to the ability of apple tissue to cope with oxidative stress at low storage temperatures rather than the concentration of $\alpha$-farnesene per se. The oxidation of $\alpha$-farnesene is a possible consequence of oxidative stress that may occur in its absence. If $\alpha$-farnesene has a scald promoting role, it could be as a participant in a free radical chain reaction rather than an initiator.

Detailed analytical studies were conducted on the phenolic compounds present in apple peel because of their dual roles as both substrates for browning reactions and as scavengers of active oxygen species and free radicals. The electron-dense pigments deposited on the tonoplast in scalded tissue result from the polymerisation of phenolics. The polymerisation reactions involved in the production of the brown pigments remain unknown, but are likely to be complex, involving numerous phenolic coupling oxidations. However, in scalded tissue there was a substantial change in phenolic metabolism that resulted in the production of benzoyl-$\beta$-$D$-glucose. This compound has not previously been reported in apples and appears to be an end product, as it was absent in scald resistant ‘Crofton’ and DPA-treated ‘Granny Smith’. HPLC studies of the peel phenolics showed that there were large differences in the relative concentrations of phenolics among varieties and it was found that many of these compounds are substrates for POD and not PPO. Due to its high concentrations in scald resistant and non-scalded peel tissue, chlorogenic acid was identified as a potentially important compound. This leads to the proposal that phenolics, such as chlorogenic acid, might confer scald resistance by conserving other antioxidants (eg $\alpha$-tocopherol) and preventing peroxidation of membrane lipids. Based on these results, an holistic model of scald development was proposed. According to this model, scald results from a chilling stress since scald only occurs at temperatures $<$4°C and only in apples in which the capacity to protect membranes from oxidative stress is low. The production of high concentrations of $\alpha$-farnesene and its autoxidation products in scald susceptible varieties may overwhelm the natural defence mechanisms resulting in lipid peroxidation and irreversible membrane damage. This damage is manifest in the extensive oxidation and polymerisation of vacuolar phenolics on the tonoplast resulting in the characteristic scald symptoms, and the production of benzoyl-$\beta$-$D$-glucose. The model proposes that POD may have a more important role than PPO in scald development than previously thought. This was in part, due to the lack of preferred substrates for PPO and the presence of suitable substrates for POD in apple peel.
Declarations and Publications

The research reported in this thesis has not been submitted for a higher degree at any other university or institution.

J.B. Golding

Parts of this thesis have been published, presented, or are being prepared for publication.


The role of phenolics in superficial scald in apples- Preliminary findings - Australian Society of Plant Physiologists, 35th Annual meeting, Sydney New South Wales 1995.

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**Abbreviations**

A  absorption  
CA  controlled atmosphere  
CO₂  carbon dioxide  
CT  conjugated triene  
Da  dalton  
DPA  diphenylamine  
EM  electron microscopy  
ER  endoplasmic reticulum  
ESEM  environmental scanning electron microscopy  
FAB-MS  fast-atom bombardment mass spectrometry  
FDA  fluorescein diacetate  
FID  flame ionisation detector  
\( F_v / F_m \)  chlorophyll fluorescence yield  
GC  gas chromatograph  
GT  glucosyl transferase  
HCl  hydrochloric acid  
HP  Hewlett Packard  
HPLC  high performance liquid chromatography  
HRP  horse radish peroxidase  
\( H_2O_2 \)  hydrogen peroxide  
HS-GC  headspace gas chromatography  
IEC  induced ethylene climacteric  
IPP  isopentenyl diphosphate  
LOX  lipoxygenase  
MeOH  methanol  
MHE  multiple headspace extraction  
MHS  multiple headspace  
MRI  magnetic resonance imaging  
mRNA  messenger RNA  
MS  mass spectrometry  
MVA  mevalonate
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<td>N</td>
<td>Newton</td>
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<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>nitrogen</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NSW</td>
<td>New South Wales</td>
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<td>O&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<td>OsO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>osmium tetroxide</td>
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<tr>
<td>PAL</td>
<td>phenylalanine ammonia lyase</td>
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<td>POD</td>
<td>peroxidase</td>
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<tr>
<td>PPO</td>
<td>polyphenol oxidase</td>
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<td>photosystem II</td>
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<td>uridine 5’phosphate</td>
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<tr>
<td>UFGT</td>
<td>UDP glucose:flavonoid 3-O-glycosyltransferase</td>
</tr>
<tr>
<td>ULO</td>
<td>ultra low oxygen</td>
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<tr>
<td>UV</td>
<td>ultra-violet</td>
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<td>ε</td>
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Chapter 1  Introduction

Superficial scald (scald) is a physiological storage disorder that affects some apple and pear varieties. Scald is characterised by a superficial browning of the peel, which only affects the epidermal and hypodermal layers in the peel (Figure 1.1). The unsightly browning of the peel significantly reduces the appearance and hence utility and value of scald affected fruit. The cause(s) of this disorder have not been established despite more than 80 years of research.

Both apples (*Malus domestica* Borkh.) and pears (*Pyrus communis* L.) are members of the Rosaceae family and are thought to have evolved in a cool climate (Hulme and Rhodes, 1971) where the fruit are expected to store best at about 0°C. Indeed, maximum storage life of most varieties is achieved by storage at -1°C to 0°C, however it is well known that some apple varieties suffer chilling injury in the form of flesh browning when stored at < 4°C (Bramlage and Meir, 1990). Chilling injury is defined as a physiological disorder that develops in susceptible plants in the temperature range 0°C to about 10°C. There is no single critical chilling temperature for all susceptible species below which injury occurs. Chilling injury usually occurs in plants of subtropical and tropical origin when stored at low temperatures. However scald is also reported to be a form of chilling injury because it only develops in susceptible varieties during long term cool storage (Watkins *et al.*, 1995).

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*Figure 1.1  Superficial scald symptoms in ‘Granny Smith’ apples*

The peel has been removed to show that the scald symptoms are localised in the peel.
Commercially important varieties such as ‘Granny Smith’, ‘Delicious’, ‘Lady Williams’ and ‘Gala’ are susceptible to scald and require postharvest treatment to prevent scald symptoms. Scald is currently controlled by postharvest application of diphenylamine (DPA), registered as ‘Shield-Brite’ and ‘No ScalD DPA’. DPA can be applied as postharvest dips or drenches and have become the major tools in reducing scald. Thermal fogging or thermo-nebulisation of apples with DPA has also successfully been investigated (Sive and Resnizky, 1989; Bertolini et al., 1995). Because international health authorities have some concerns about the safety of DPA, it is necessary to find alternative methods for controlling scald. Although DPA (N-phenylphenate, N-phenyl aniline) is a secondary aromatic amine and has long been thought of as an antioxidant (Ingle and D’Souza, 1989), its mode of action in controlling scald is unknown. The action of DPA is controversial, with DPA seemingly having numerous and often contradictory antioxidant functions (Lurie et al., 1989a; Sugihara et al., 1993). They suggest that DPA have simultaneous antioxidant and pro-oxidant effects. Studies of DPA analogues have shown that the antioxidant effects of DPA are associated with its secondary amine function and its reactivity with peroxyl radicals (Sugihara et al., 1993). However, the intermediate product of free radical quenching by DPA results in the oxidation of a lipid peroxyl radical, hence DPA actually promotes the generation of lipid hydroperoxides (LOOH). These suggestions are rationalised by the fact that DPA appears to react well with peroxyl radicals and undergoes redox cycling in the process. Presumably this results in intermediate formation of a nitrogen-based radical so that redox cycling of this aromatic amine stimulates further peroxidation (Sugihara et al., 1993). This contradicts the traditional view that the nitrogen centred radical formed by aromatic amine antioxidants is not sufficiently reactive to promote further peroxidation because of delocalisation of the unpaired electron into the aromatic ring structure (Kaur and Perkins, 1991). This is also contrary to Bors and Saran (1981) and Van der Zee et al. (1989) who showed DPA strongly inhibited lipid peroxidation, and was a strong inhibitor of alkoxyl radicals (RO•) but not peroxyl radicals (RCOO•). As alkoxyl and peroxyl radicals may have different reactivities (Van der Zee et al., 1989), elucidation of the various mechanisms is crucial for the better understanding of the role of hydroperoxides under physiological conditions.

Investigation of the mechanism of scald development and the action of DPA should assist the development of safe alternative control measures. If an alternative to DPA is not found and it is banned, the expected that monetary losses to the apple industry could exceed 50%. The history of scald in apples parallels the introduction of long term cool storage more than 80 years ago. Brooks et al. (1919) and Kidd and West (1935) described the biology of scald and the factors
predisposing apples to this disorder. The work of Brooks et al. (1919) led to the wrapping of apples in paper impregnated with various oils. Oil wraps reduced the severity of scald but effective control of scald in bulk handled fruit was not achieved until the advent of DPA (Smock, 1955).

As a physiological disorder, the susceptibility of the fruit to scald is significantly affected by factors influencing the physiology of fruit growth and maturation, which in turn is strongly affected by genetic, environmental and management factors. Therefore numerous pre- and postharvest factors affect the development of scald. Preharvest orchard factors include variety, fruit temperature during growth and maturation and maturity at harvest (Smock, 1961; Emonger et al., 1994). Susceptibility to scald varies widely among varieties and is the over-riding factor in the development of scald (Meigh, 1970). Apple varieties such as ‘Granny Smith’ are highly susceptible, whilst others such as ‘Crofton’ are resistant. Immature fruit are more susceptible to scald (Meigh, 1970; Wilkinson and Fidler, 1973; Anet, 1974). Other preharvest factors that have been reported to influence scald development include fruit size, nutrition, rootstocks and climatic factors (Smock, 1961; Wilkinson and Fidler, 1973; Emonger et al., 1994). In general, apples that mature in warm dry environments are more prone to scald (Wilkinson and Fidler, 1973). In some production regions an inverse relationship was found between the number of preharvest hours below 10°C and the development of scald (Merritt et al., 1961; Bramlage and Watkins, 1994).

Postharvest factors that affect the development of scald during storage include the rapidity of cooling, ventilation of coolrooms (Smock, 1961; Emonger et al., 1994), duration and storage temperature (Meigh, 1970; Watkins et al., 1995) and the composition of the storage atmosphere (Chen et al., 1985; Little et al., 1985; Lau et al., 1998). These factors can be manipulated to reduce the incidence of scald in storage; for example, ultra low oxygen storage (ULO, ~1% O₂) has been successfully used to control scald (Little et al., 1982; Little, 1985). One further factor that has emerged from earlier work is the importance of applying treatments to reduce scald as soon as possible after harvest. Alternative measures for controlling scald include ethanol vapours (Scott et al., 1995a), prestorage hot water treatments (Lurie et al., 1990), natural oils (Scott et al., 1995b) and a range of antioxidants other than DPA (Wills and Scott, 1974; Wills et al., 1977; Wills and Scott, 1990; Bauchot et al., 1995).
According to the current hypothesis, scald is caused by the products of autoxidation of a naturally occurring sesquiterpene, α-farnesene (Huelin and Coggiola, 1968, 1970a). These oxidation products are principally conjugated trienes (CT), which have since been characterised (Brimble et al., 1994a; Rowan et al., 1995). More recent research suggests there are additional unknown factors involved in the development of scald symptoms (Du and Bramlage, 1993, 1994a).

This thesis provides an account of the outcomes of a re-examination of the histology of scald, comparative physiological and biochemical studies of susceptible and resistant varieties and offers a model to guide the systematic development of alternative control measures.
Chapter 2  Aims

The overall aim of this thesis was to develop a greater understanding of the aetiology of scald in apples. Experiments were conducted on two susceptible varieties, ‘Granny Smith’ and ‘Lady Williams’ and the resistant variety ‘Crofton’. ‘Granny Smith’ is a green skinned variety, which was a chance seedling found near Sydney, Australia. It was thought to have been derived from French crab apples grown in Tasmania (Dr. Jill Campbell, pers. comm.). ‘Lady Williams’ is a red striped variety that originated as a chance seedling in Western Australia. The parents are thought to be ‘Granny Smith’ and ‘Rokewood’ (Cripps, 1991). ‘Crofton’ is a striped variety that was bred by Joseph Cato at Mt. Stewart, Hobart, Tasmania over 100 years ago. The parents of ‘Crofton’ are ‘Pomme d’Neige’ (= ‘Fameuse’) and ‘Scarlet Pearmain’ (= ‘Long Red Pearmain’) (Dr. Jill Campbell, pers. comm.; Beach, 1905).

Specific aims of this study were to:

- Re-examine the histology of scald in apple peel using imaging techniques, such as environmental scanning electron microscopy (ESEM), magnetic resonance imaging (MRI) and conventional transmission electron microscopy (TEM).

- Develop a reliable and reproducible method of inducing scald. This would provide a valuable tool for the study of the development of scald in peel tissue.

- Examine the volatiles hypothesis of scald development by comparing the physiological and biochemical responses in the peel of ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apples.

- Investigate the role of phenolics, as both substrates and antioxidants in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during 9 months storage at 0°C.

- Compare the possible role of polyphenol oxidase (PPO) and peroxidase (POD) in scald development.

- Develop a holistic model of scald development that might lead to new ways of scald control.
Chapter 3    Histology of Superficial Scald

3.1    Literature Review and Introduction

Superficial scald is a physiological disorder of apples that is restricted to the peel of susceptible apple varieties during long term storage. Therefore, it is important to understand the anatomy and morphology of the apple fruit in relation to the development of scald during storage. Roth (1977) and Westwood (1978) described fruit morphology and patterns of fruit development, while MacDaniels (1940) provided a comprehensive survey of the histology of the apple fruit. This brief review outlines the histology of scald development in apple fruit. Reviews of the specific techniques used to examine scald are given at the beginning of each section. It is important to note that scald normally occurs during long term storage in fruit of susceptible varieties that have been harvested at a preclimacteric stage when starch reserves are at a maximum and cell division and growth are essentially complete (Bain and Robertson, 1951).

3.1.2    Apple Fruit Anatomy

The apple is classified as a pome fruit and is a member of the Rosaceae family (Westwood, 1978). The apple flower is inferior and the subtending fruit comprises the ovary and accessory tissue that can be divided into several regions or zones. Figure 3.1 outlines the basic anatomy of an apple fruit and the peel.

**Core**   The core and pericarp of the apple are derived from the ovary wall and comprise two kinds of tissue: parenchyma tissue in the median and lateral carpellary bundles, and cartilaginous tissue made of sclereids lining the locules (Esau, 1977). Both of these tissues are considered to be pericarp, with the endocarp restricted to cartilaginous tissue. The elongated sclereids have very thick walls, which almost completely obliterate the cell lumen. The cartilaginous endocarp and the fleshy mesocarp extend toward the core-line. There is no clear definition of what the coreline represents or how it originates, but it is suggested that it is the demarcation between the floral cup and the ovary (MacDaniels, 1940). It includes the median bundles of the carpels but excludes the vascular bundles assigned to the floral tube and is anatomically distinguished by smaller tangentially elongated cells with a greater amount of intercellular space.
Figure 3.1  Transverse section through mature ‘Granny Smith’ apple fruit
The insert is an ESEM apple peel section showing cuticle, epidermis, hypodermis
and fleshy parenchyma

**Flesh**  There has been some confusion regarding the origin of the flesh (MacDaniels, 1940). One interpretation of the development of the apple fruit is that the part of the apple flower that makes up the fruit (except the ovary) is axial or receptacular in origin and therefore, part of the stem or axis (Kraus, 1913, 1916). The accessory tissue is regarded as part of the receptacle and thus designates the outer fleshy part of the fruit, is referred to as the cortex.

An alternative interpretation of the origin of the apple fruit is that the outer fruit tissue is derived from fused floral parts and is appendicular in origin (MacDaniels, 1940). This is where a mature, ripened inferior ovary in which the pericarp of the gynaeicum plus the receptacle tissue becomes fleshy (Roth, 1977). Five ovaries of the apple flower are fused at the base, which along with the receptacle becomes the fruit (Esau, 1977). This appendicular interpretation of the origin of the apple fruit is now widely held (Esau, 1977).

Cell division in the developing apple fruit is continuous until about 28-35 days after anthesis, and subsequent growth is by cell expansion (Bain and Robertson, 1951; Westwood, 1978). As the cells expand and are pushed apart, the volume of the air spaces increases. The parenchyma cells have very thin walls with large intercellular spaces. These large intercellular spaces vary greatly
in size and are up to 2,000 μm in length and 100-200 μm in diameter (Reeve, 1953). The mean volume of intercellular spaces is between 22-40 % and does not appear to be related to cell size (Reeve, 1953). Bain and Robertson (1951) estimated the intercellular spaces in 'Granny Smith' apples to be about 27%. Within the parenchyma there are ten primary vascular bundles which originate in the vascular cylinder of the pedicle near the flower base, five belonging to the sepals and five to the petals. These vascular bundles are branched and penetrate into the parenchyma where they form an anatomising system. A detailed explanation of the vascular system in apples is given by MacDaniels (1940).

Skin

The skin of the apple fruit is composed of the epidermis and the hypodermis (Bell, 1937). These outer cell layers can be distinguished from the fleshy parenchyma by having heavy wall thickening and tangential cell elongation (Roth, 1977). The skin is usually 4 - 7 cells deep and contains chloroplasts and anthocyanins. The epidermis consists of a single layer of elongated thick walled cells which overlays the hypodermis that consists of 3 - 5 layers of cells. The hypodermal layer, as the outer most portion of the fleshy hypanthium, is characterised by closely packed collenchyma with small intercellular spaces. The collenchyma cells have thick cell walls with thickened corners (Bell, 1937).

Lenticels

Stomata and trichomes are present on young immature fruit, but are replaced by lenticels in mature fruit (Roth, 1977). Roth (1977) reports that in the very young fruit there are about 2-10 stomates.mm⁻². The walls of the guard cells of the old stomates become thickened and the sub-stomatal cavities are blocked by suberisation of the cells. Roth (1977) discusses the morphology and biogenesis of the three types of lenticels distinguishable in the apple skin. One is developed by epidermal breaks, a second arises below the stomata through cork formation from sub-stomatal cells and the final type of lenticel is formed below the trichome bases. In mature fruit, lenticels are functionally closed by the formation of a cuticle or suberisation of the sub-epidermal cells (Roth, 1977).

Cuticle

The cuticle is an external, non-cellular membrane and is thought to consist of various layers. These layers are not discrete as their boundaries merge. The outermost layer (cuticle proper) consists entirely of cutin and wax, whilst the inner layer (cuticular layer) consists of cutin, wax and cellulose. These two layers are referred to as the cuticular membrane. A pectic layer lies immediately below the cuticular membrane and is immediately above the epidermis (Esau, 1977). Cuticular development correlates well with the general growth of the apple fruit, ie
the outside gradually increases in thickness and complexity concomitant with the gradual increase in fruit volume (Miller, 1982). It is presumed that the precursors of both the cutin and wax components are synthesised in the epidermis (and possibly in the adjacent parenchyma cells) and that they move to the outer surface of the cell through the cell wall (Miller, 1982). However, the precise pathways through the cell wall and cuticular layer are unknown.

Gough and Shutak (1972) suggested that cuticle structure rather than cuticle thickness was related to scald. Using scanning electron microscopy (SEM), they showed that in three apple varieties there was a correlation between the wax structure, the occurrence and nature of surface breaks in the cuticular wax complex and the susceptibility to scald. The cuticle in the low scald susceptibility variety, ‘Golden Delicious’, was heavily encrusted in wax and had large ultramicroscopic breaks, which appeared to facilitate gas exchange. In contrast in ‘Cortland’, a highly scald susceptible variety, the surface wax was granular and had no surface breaks. This structure in ‘Cortland’ did not appear to aid gas exchange. The cuticle of an intermediate scald susceptible variety, ‘McIntosh’, was found to be composed of wax platelets and would likely to be intermediate in gas exchange capacity. However, a major deficiency of this work was that no gas exchange measurements were made.

Miller (1982) surveyed a range of apple varieties for the existence of discrete pores and canals in the fruit cuticle. Miller (1982) found in dewaxed isolated cuticles the ubiquitous presence of pores and canals. However, it seems that neither cuticle thickness, pore diameter nor pore numbers were correlated with scald susceptibility. There were few cuticular differences between the relatively scald resistant variety, ‘Golden Delicious’, and the scald susceptible varieties, ‘Granny Smith’ and ‘McIntosh’. Miller (1982) also showed that there were different topographical aspects of cuticles when viewed using light microscopy or by SEM. Using normal optical microscopy the outer cuticle surface appeared smooth, whilst using SEM the microtopography of the cuticular surface becomes apparent, revealing pits, pores and granulations. However, Miller (1982) found few differences with SEM in cuticular surfaces among varieties but did not discuss these in any detail. The differences in observations by Gough and Shutak (1972) and Miller (1982) should be examined in closer detail in conjunction with measurements of gas permeability of the cuticle. The development of scald in relation to cell types will be discussed in more detail in the following sections.
3.1.3 Ultrastructural Changes During Scald Development
Differentiating the ultrastructural changes associated with scald development from those associated with the progression of normal senescence is essential to understand the development of scald.

3.1.3.1 Senescence and ethylene
Senescence is a controlled degradative process leading to the loss of normal cellular function and ultimately cell death (Nooden, 1988). Membranes play an essential role in the regulation of cell biochemistry and physiology, however with the onset of senescence, membranes become less fluid. This disruption to membranes alters their normal regulatory function that ultimately results in a total loss of membrane integrity and compartmentation (Thompson et al., 1982). Butler and Simon (1971) described the normal ultrastructural changes that occur in senescing plant cells. The first detectable changes are usually a decrease in ribosomes and the start of chloroplast breakdown. The endoplasmic reticulum swells, vesiculates and disappears as do the golgi dictyosomes. As senescence is a controlled process, the essential cellular functions are retained until the very last stages of senescence (Thompson and Platt-Aloia, 1987). Therefore, the relatively late ultrastructural changes in senescence occur in the mitochondrial, nuclear and vacuolar membranes (Woolhouse, 1984). The tonoplast breaks down long before the organelles have completely degraded. Finally when all the internal membranes are broken, the plasmalemma loses its integrity (Harris and Arnott, 1973).

Ethylene does not appear to have a direct effect on plant membranes but it does influence senescence (Abeles et al., 1992). Ethylene, at least in part, is responsible for an increase in membrane permeability in senescing plant tissue that leads to cell death (Suttle and Kende 1980; Faragher et al., 1987). Increasing membrane permeability is a direct result of phospholipid degradation, presumably caused by an increase in activity of pre-existing phospholipases. However, the effect of ethylene is thought to be a secondary effect which is mediated by other cellular processes requiring both RNA and protein synthesis (Suttle and Kende, 1980).

Legge et al. (1986) showed that the plastid membrane bilayers of tomato fruit remain unchanged with fruit ripening. This is in contrast to the ultrastructural changes associated with ripening and exposure to ethylene, where the most notable changes occur in chloroplasts viz, gradual loss of granal thylakoid membranes and extensive vesiculation along the inner envelope membrane (Spurr and Harris, 1968; Toyama, 1980). Ethylene is widely and effectively used to promote chlorophyll degradation in early season citrus. Purvis and Barmore (1981) showed that ethylene
was necessary for chlorophyll degradation, but its primary role is not solely the induction of chlorophyllase. They suggest the breakdown of the thylakoid membrane is enzyme mediated and induced by ethylene. Although ultrastructural studies provide a valuable insight into cell senescence, it is important to be aware of artefacts induced in the preparation of tissue sections.

3.1.3.2 Chilling injury

Chilling injury confounds the progression of senescence in stored apples (Bramlage and Meir, 1990). Chilling injury is a physiological condition that develops at a non-freezing temperatures (Wang, 1982). Scald is considered to be a low temperature storage disorder (Meigh, 1970; Watkins et al., 1995) and chilling injury has been implicated its development (Bramlage and Meir, 1990). There are several theories on the cause and nature of chilling injury, however, there has been little agreement (Wang, 1982; Parkin et al., 1989). Symptoms of chilling injury appear similar to normal senescence. The swelling of organelles, due to the destruction of osmotic regulation, the disorganisation of the membranes and the appearance of lipid bodies throughout the cell are common symptoms of chilling injury and can be explained as a consequence of the degradation of membranes (Wang, 1982).

Chilling injury is thought to be due to a phase change in the fluidity of the membranes (Wang, 1982), and can result in a series of indirect injuries and dysfunctions. Rather than a uniform change across membranes, fluidity changes probably occur in micro-domains within the membrane. These changes subsequently affect the regulation of ion transport and the catalytic properties of membrane bound enzymes (Platt-Aloia and Thompson, 1987). This can lead to further secondary responses such as a loss of membrane integrity, loss of compartmentment and ultimately cell death. Other theories postulate that chilling injury results from a direct effect of reduced temperature on enzymes or an indirect effect of membrane perturbations (Graham and Patterson, 1982). Minorsky (1985) suggested that the cellular redistribution of calcium is the primary transducer of chilling injury. However, the physical phase change of the membranes may or may not lead to secondary or irreversible changes, depending on the chilling temperature and length of exposure (Wang, 1982).

The primary site of chilling injury damage in healthy tissue is thought to be the chloroplasts (Marangoni et al., 1989). However, Niki et al. (1978; 1979) suggested that disruption of the tonoplast might be responsible for irreversible chilling damage in callus tissue of Cornus stolonifera. The irreversible loss of integrity of the tonoplast would release toxic substances and
enzymes that could degrade cellular components. Wang and Baker (1979) showed that the application of free radical scavengers (eg ethoxyquin) maintained relatively high concentrations of unsaturated fatty acids in polar lipids and reduced the severity of chilling injury in cucumbers and sweet peppers. Ethoxyquin is an antioxidant and inhibitor of scald (Meigh, 1970). Wang (1982) suggested a relationship between scald and chilling injury and speculated that the use of free radical scavengers might reduce chilling injury by retarding the degradation of unsaturated fatty acids in the membrane polar lipids. However, there is no direct proof for a mechanism involving these free radical scavengers and Wang and Baker (1979) suggested that the tolerance to chilling is more complicated than a simple inhibition of fatty acid peroxidation.

3.1.3.3 Scald development

While the morphology of the apple is well known, there have been few histological studies of scald. Bain (1956) and Bain and Mercer (1963) conducted the most comprehensive investigations of scald in ‘Granny Smith’ apples. These studies showed that scald was restricted to the skin of scald susceptible apple varieties with the hypodermis being the primary site of symptoms (Bain, 1956). Using light microscopy, Bain (1956) found that the development of scald in ‘Granny Smith’ apples was associated with a progressive browning of the contents of the hypodermal cells. In severely scalded fruit, the affected cells collapse in a radial direction so that the brown area becomes sunken. Using transmission electron microscopy, Bain and Mercer (1963) showed that the first ultrastructural symptom of scald was the formation of an electron dense material in close association with a normal constituent of the vacuoles of hypodermal cells. As the disorder became more severe, additional material accumulated on the tonoplast and the protoplast became disorganised. They also showed that scald was distinct from normal aging, in that the scald symptoms were superimposed on the disorganisation associated with senescence. They observed vesicle formation in the chloroplasts and throughout the cytoplasm in all senescing tissue but superimposed on this were the ultrastructural changes associated with scald. It was suggested that the breakdown in cell structure and the homogeneous nature and density of the protoplasts in the scald affected areas was due to an uncontrolled polyphenol oxidase (PPO) system. They postulated that cell breakdown was caused by an increase in permeability of membranes, resulting in the loss of cell turgor. This would result in release of the vacuolar contents, containing the polyphenols, into the cytoplasm. The resultant oxidation of the polyphenols and tannins would result in the common macroscopic browning symptoms of scald.
The aim of this chapter was to re-examine and expand knowledge of histological and ultrastructural changes associated with the development of scald in apples. The following sections will describe the results of the examination of scalded and non-scalded peel:

- optical microscopy, including ultra violet fluorescence and cell viability tests
- transmission electron microscopy, with different fixation techniques
- environmental scanning electron microscopy
- magnetic resonance imaging
3.2 Optical Microscopy

The classical work of Bain (1956) remains one of the few studies on the histology of scald. Newer techniques are now available that permit a closer examination of changes in scalding cells. These include UV fluorescence techniques for examining cell viability.

3.2.1 Cell Viability

The integrity of membranes ensures selective permeability and is essential to the maintenance of living processes in cells. An important question in relation to scald development is whether symptoms develop before or after the death of hypodermal and epidermal cells. Thompson (1988) discussed some of the techniques that have been used to examine membrane integrity. These include measurements of membrane leakiness, loss of specialised membrane function, electron spin resonance, changes in lipid composition and phase properties. In addition, numerous dyes and probes have been used to determine plant cell viability (Gahan, 1989).

The integrity of the plasmalemma can be tested using dyes that are either excluded or accumulated by the cells. In the dye exclusion techniques, dye molecules will not enter intact living cells and only dead or damaged cells stain. Examples of this type of stain include Evans Blue and ethidium homodimer (Gahan, 1989).

Other dyes are accumulated only by living cells. Fluorescein diacetate (FDA) is a common vital stain of this type (Heslop-Harrison and Heslop-Harrison, 1970), and relies upon an active esterase activity in living cells. FDA is non-polar, non-fluorescing and when it permeates the intact plasmalemma, esterases cleave the acetate from FDA. The resultant product, fluorescein, is fluorescent and polar, and does not move freely out of living cells. Fluorescein accumulates only in viable cells. An analogue of FDA is 6-carboxy-fluorescein diacetate (CFDA). CFDA also freely enters the cells where it is retained following enzyme conversion to a hydrophilic fluorophore, 6- carboxyfluorescein. CFDA provides better results than other fluorescein esters which hydrolyse to a much less hydrophilic fluorophore that may leak from the cells (Goodall and Johnson, 1982).

A similar test that utilises the intracellular esterase activity and gives high levels of dye within living cells employs calcein-AM. The substrate is virtually non-fluorescent and permeates living cell membranes, whilst the enzymatic hydrolysis product, calcein is fluorescent (intense uniform green) and is retained by the cell. Calcein-AM is utilised in a two colour fluorescence cell viability assay called ‘Live/Dead CytologyTM’ (Molecular Probes Inc., Eugene, OR, USA). Ethidium homodimer can be used to detect dead cells. Ethidium homodimer is a nucleic acid
stain that is excluded from living cells. The nuclei of cells with damaged membranes stain rapidly and fluoresce red.

The specific aims of this section were:

- re-examine the histology of scald with optical microscopy
- examine chloroplast fluorescence in scalding peel tissue, and
- explore the use of cell viability tests (FDA, calcein-AM / ethidium homodimer) on scalding and aging peel tissue to determine the membrane changes associated with the development of scald

3.2.2 Materials and Methods

3.2.2.1 Optical microscopy
Immature, preclimacteric ‘Granny Smith’ apples were harvested in April 1992 from a commercial orchard in Bilpin, NSW. They were immediately stored in air at 0°C. Scald is a disorder where the symptoms may not be obvious upon removal from storage at 0°C, but the characteristic browning symptoms develop at 20°C (Meigh, 1970). The development of scald and aging of the fruit was followed by transferring apples at successive intervals to 20°C. Scald was allowed to develop for seven days. To distinguish between normal aging and scalding cells, non-scaled tissue from scalded apples were used as controls. Transverse hand cut sections of peel tissue were examined under light and ultra-violet (UV excitation range 460 - 490 nm), using an Olympus BH light microscope.

3.2.2.2 Chlorophyll fluorescence
‘Granny Smith’ apples were removed from cold storage 10, 7 and 3 days prior to measurement of chlorophyll fluorescence and stored at 20°C. Fluorescence was measured on areas of skin with scald (light bronze, medium scald development or severe sunken dark brown scald symptoms) and compared to skin from non-scaled regions on the same apple (Figure 3.2). Chlorophyll fluorescence was determined using a method modified from Conroy et al. (1988). The apples were dark adapted for at least 10 min and a tungsten light source filtered through a Corning 4-96 filter to give a photosynthetic photon density flux of 20 μm⁻²s⁻¹. This was directed onto the surface of the peel and the resulting chlorophyll fluorescence was directed via a
Figure 3.2  Example of the scalded and non-scaled peel regions in ‘Granny Smith’ apples measured for whole apple chlorophyll fluorescence measurements
second arm onto a photomultiplier tube protected by a Bausch and Lomb 693nm interface filter and a Corning 3-64 red cut-off filter. The output was interfaced to a HP 8926 computer. The fluorescence yield ($F_v / F_m$) was used to evaluate Photosystem II (PSII) electron transport activity and was determined from measurements of $F_o$ (constant yield chlorophyll fluorescence) and $F_m$ (maximum chlorophyll fluorescence = constant plus variable fluorescence) (Conroy et al., 1988).

3.2.2.3 Determination of cell viability

**Fluorescein Diacetate** Transverse hand cut sections of apple tissue were bathed in a 0.1% FDA or CFDA in water solution (by dilution from a stock solution of 5 mg.mL$^{-1}$ of acetone) for 10 minutes at room temperature in darkness. The sections were thoroughly rinsed and examined for fluorescence with UV / blue light (excitation range 460 - 490 nm) using an Olympus BH light microscope.

**Live / Dead Cytology$^{TM}$** Transverse hand cut sections of apple peel tissue were incubated in various concentrations of calcine-AM (0.2 - 50µM) and ethidium homodimer (0.4 - 50µM) for varying lengths of time (0.25 - 12 hours) in darkness at room temperature. The sections were thoroughly rinsed and examined under UV / blue light (excitation range 460 - 490 nm) using an Olympus BH light microscope. Incubating sections of the peel for 6 hours at concentrations of 4 µM calcine-AM and 8 µM ethidium homodimer, respectively, achieved best results.

3.2.3 Results and Discussion

No visible browning was observed in non-scalded peel tissue when viewed with light or UV microscopy, although normal senescence and disorganisation of the cell contents were observed over the storage period (Figure 3.3a). The FDA viability test of the non-scalded peel tissue showed that the plasmalemma maintained its integrity, with all intact cells in the tissue sections fluorescing under blue excitation (Figure 3.3b). Also, chloroplasts fluoresced under blue excitation. This indicates the presence of chlorophyll, which is associated with PS II activity and viable intact chloroplasts.

Light microscopy of scalded tissue confirmed many of the observations of Bain (1956), Bain and Mercer (1963) and Kang and Lee (1987). Successive observations of scalded peel tissue
Figure 3.3  Transverse section of non-scalded 'Granny Smith' peel tissue.

Figure 3.3a Brightfield. Figure 3.3b Blue excitation (λ 450-490nm) showing fluorescent chloroplasts. Bar = 100 μm
revealed that the scald symptoms consisted of general browning of the affected cells, which are chiefly confined to the hypodermis.

The first symptoms of scald appeared as a slight browning of the outer hypodermal cells. As scald became more severe, browning of the hypodermal cells became more intense and developed throughout the hypodermis (Figure 3.4 a and b). Eventually, all the cells of the hypodermis and epidermis were scalded. In the later stages of scald, the epidermal cells collapsed to give the peel its characteristic sunken appearance. In very severe cases, scald symptoms appeared in a few cells of the outer cortex underlying the hypodermis.

An interesting observation was the presence of fluorescing chloroplasts in scalding hypodermal cells (Figure 3.4 c and d). This indicates the presence of chlorophyll and suggests that the chloroplast membranes were still intact in damaged brown cells. This was further examined by using chlorophyll fluorescence techniques on intact whole apple fruit. ‘Granny Smith’ apples were removed from storage at different times, assessed for scald development and the levels of chlorophyll fluorescence measured. Chlorophyll fluorescence ($F_v / F_m$) is a measure of Photosystem II (PS II) activity. PS II is an essential component of photosynthesis involving chlorophyll $a$ and is dependent on functioning thylakoids. Wilson and Greaves (1990) showed that chlorophyll fluorescence analysis was a useful tool to detect chilling injury or damage to thylakoid membranes in a range of plants. De Ell et al. (1996) further suggested that chlorophyll fluorescence in ‘Delicious’ apples at harvest may be a predictor of scald development. However in this case, chlorophyll fluorescence measurements after storage showed that chlorophyll fluorescence ($F_v / F_m$) in apples declined gradually after removal from cold storage ($0^\circ$C in air) indicating a decline in PS II activity (Table 3.1). More importantly, Table 3.2 shows that while non-scaled peel tissue and lightly scalded tissue had higher levels of chlorophyll fluorescence than more heavily scalded peel areas, there was significant chlorophyll fluorescence in even severely scalded tissue, indicating that there is at least some PS II activity in scalded tissue. This suggests that the chloroplasts in scalded tissue are, to some extent still functional. This has some significant implications for the role of PPO in scald development that will be discussed in Section 3.5.
Figure 3.4  Transverse section of scalded ‘Granny Smith’ peel tissue.

Figure 3.4a Brightfield, Bar = 100μm. Figure 3.4b Brightfield, Bar = 20μm.

Figure 3.4c Blue excitation (λ 450-490nm) showing fluorescent chloroplasts, Bar = 100 μm. Figure 3.4d Fluorescent chloroplasts in severely scalded hypodermal tissue, Bar = 10 μm
Table 3.1  Influence of time at 20°C on the changes in chlorophyll fluorescence ($F_r / F_m$) in the peel of ‘Granny Smith’ apples stored for 6 months at 0°C. Apples were removed from storage 10, 7 and 3 days before measurement of chlorophyll fluorescence at 20°C. Different letters signify statistical differences (P<0.05)

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<tr>
<td>10</td>
<td>0.62$^a$</td>
</tr>
<tr>
<td>7</td>
<td>0.62$^a$</td>
</tr>
<tr>
<td>3</td>
<td>0.70$^b$</td>
</tr>
</tbody>
</table>

Table 3.2  Interaction of scald intensity with the chlorophyll fluorescence ($F_r / F_m$) in the peel of ‘Granny Smith’ apples stored for 6 months at 0°C and 1 week at 20°C. Different letters signify statistical differences (P<0.05)

<table>
<thead>
<tr>
<th>Scald Severity</th>
<th>$F_r / F_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>severe scald</td>
<td>0.56$^a$</td>
</tr>
<tr>
<td>medium scald</td>
<td>0.65$^b$</td>
</tr>
<tr>
<td>green healthy tissue</td>
<td>0.65$^b$</td>
</tr>
<tr>
<td>light scald</td>
<td>0.70$^b$</td>
</tr>
</tbody>
</table>

The viability tests on the scalding cells showed that it took some time for the plasmalemma membranes to lose integrity. Eventually, the plasmalemma became non-functional and the scalded hypodermal cells did not fluoresce (Figure 3.5 a and b). An interesting observation was that some epidermal cells remained viable while the underlying hypodermal tissue was functionally dead (Figure 3.5c). This was not an artefact of cutting as the epidermal and hypodermal cells had approximately the same dimensions (thickness) and cut sections comprised a similar number of cells of both regions. This raises some interesting questions regarding cellular physiology and biochemistry of apple peel, where a functionally dead hypodermal layer is sandwiched between living epidermal and fleshy parenchyma cells.
Figure 3.5  Transverse section of 'Granny Smith' peel tissue illustrating FDA viability
test viewed with blue excitation(λ. 450-490nm). Figure 3.5a Non-scalded peel tissue.
Figure 3.5b Scalded peel tissue; h, hypodermal cells. Figure 3.5c Scalded peel tissue
showing some 'viable' epidermal cells; h, hypodermal cells. Bar = 100 μm
3.3 Electron Microscopy

3.3.1 Introduction

3.3.1.1 Chemical fixation

Bain and Mercer (1963) conducted the first electron microscopy study of scald in apples. This classic EM work was conducted using osmium tetroxide as the fixative and embedding in 'Araldite'. However, since the early 1960s there have been considerable advances in EM technology, which throw new light on the interpretation of classical chemical fixative EM work.

Chemical fixation is the routine method for preserving biological specimens for electron microscopy (EM). However, the fixation and preservation of living cells produce artefacts. For example, cellular components may be moved from their in vivo position, completely extracted, their chemical nature may be changed, or rapid events may not be stopped quickly enough to be studied (Robards, 1991). The study of plant cells containing large amounts of phenolic compounds (such as apple cells) is further complicated because after fixation in glutaraldehyde and osmium tetroxide, these cells have a dense osmophillic appearance in which some of the cytoplasmic details are lost. This effect is most marked in aging cells, where the vacuole occupies a large portion of the cell volume. In such cells, the tonoplast is apparently susceptible to damage during the fixation process (Mueller and Greenwood, 1978). During fixation, the phenolics from the vacuoles can leach into the cytoplasm where they combine with osmium. This situation would be obvious in aging apple peel tissue, where the cells are becoming disorganised. However, this problem can be partly overcome with the application of caffeine before fixation with osmium (Mueller and Greenwood, 1978). Caffeine has long been used in light microscopy to precipitate tannins in vivo while not causing injury to the cell (Flasch, 1955). The caffeine reacts with the phenolics in the vacuoles and prevents leaching and subsequent darkening of the cytoplasm after post fixation with osmium.

3.3.1.2 Cryofixation

Cryofixation, or rapid freezing of the biological sample can physically stabilise cellular components within a few milli-seconds, preserving the cell structures in their natural state. The fixation time is estimated to be at least 10,000 times faster than chemical fixation by immersion or perfusion (Sitte et al., 1987). However, cryofixation produces its own range of difficulties and
artefacts (Sitte et al., 1987; Robards, 1991). Ice crystal formation in the specimen is an obvious and major problem with cryofixation especially in plant preparations (Robards, 1991). The rate of crystal growth and ultimate ice crystal size is strongly correlated with the rate of heat exchange from the specimen. Therefore, the freezing procedure should remove the heat from the specimen as quickly as possible in order to minimise crystal size, thereby obtaining optimal fixation quality.

Cryofixation at normal pressures is not widely used due to the difficulty in routinely achieving rapid freezing of plant tissue beyond a depth of 10-20 μm (Ding et al., 1991). Beyond this depth of fixation the mixed plastid phases segregate. However, cryofixation is a valuable technique to complement chemical fixation. There are various methods for cryofixing biological specimens. In this study, methods included plunging into a liquid cryogen and slamming the specimen against a cold metal surface. Plunge freezing is probably the simplest method of freezing biological specimens. It involves plunging the specimen into a liquid cryogen. The cryogen is usually sub-cooled liquid nitrogen at about -210°C, or liquid propane at -185°C. Besides selection of the cryogen, the specimen holder and the entry velocity of the specimen into the cryogen are critical in determining the quality of cryofixation (Sitte et al., 1987). Slam freezing involves slamming the specimen against a cold metal block. Excellent fixation can be achieved using this method if bouncing of the specimen from the cold metal plate can be avoided. A major benefit of slam freezing is that heat transfer across a solid metal interface is more rapid than from a specimen in a liquid cryogen (Sitte et al., 1987). Freeze substitution and low temperature embedding are routinely used on cryofixed samples. The frozen samples are first dehydrated by dissolving the ice in organic liquids and then the samples are infiltrated with liquid resins that are subsequently polymerised. With careful selection of solvents, resins and conditions, it is possible to preserve the ultrastructure of biological specimens without serious artefacts.

3.3.1.3 Environmental scanning electron microscopy (ESEM)

Various microscopy and imaging techniques have been used to study the structure of plant tissue. These studies are vitally important in providing structural and histological information of the biological processes occurring in fruit; however a disadvantage of most EM imaging systems used is the production of artefacts. To maintain the electron beam, conventional EM requires that the specimen be in a high vacuum (10⁻⁷ Torr). However, biological samples contain high percentages of water and to prevent the collapse of the sample, it is necessary to pre-treat or fix
the sample before observation. As discussed previously, these preparation procedures can cause morphological changes in cellular structures and delicate samples can be further distorted in a high vacuum (Feder and O'Brien, 1968).

Environmental SEM (ESEM) is a relatively new SEM technique that overcomes the limitations of high vacuum SEM imaging by allowing the whole sample to be held at a high vacuum (up to 20 Torr, cf. $1 \times 10^{-6}$ Torr). However at the same time, a high vacuum is maintained in the gun and the electron optical areas (Peters, 1994). ESEM maintains a relatively low pressure environment around the specimen to prevent gasses in the chamber scattering and dissipating the electron beam and reducing quality of the images. This allows the imaging of fresh, fully hydrated samples in their natural state. Specimen temperature, moisture content and the gas environment can be manipulated whilst continuously observing the sample, thus allowing direct imaging and video recording of dynamic processes. The sample does not require fixation or conductive coating therefore there are no fixation artefacts. However, volatile components especially water, may be lost by evaporation, which can lead to dimensional changes in the specimen and ultimate structural collapse. In addition, the water vapour can lead to poor resolution, charging and other interference with the image forming processes. Although this progressive dehydration of the specimen is impossible to stop, it can be minimised by maintaining the sample in a fully hydrated state by careful manipulation of temperature and pressure. This minimises vapour pressure deficits and allows greater imaging times.

To further examine the development of scald in ‘Granny Smith’ apples at the sub-cellular level, a range of fixation techniques were prepared for TEM. A comparison of fixation techniques is essential to assist recognition of artefacts and to understand changes that occur in scalding apple cells. These fixation techniques included:
- chemical fixation (with and without caffeine)
- cryofixation (plunge and slam freezing)

In addition, low vacuum ESEM was conducted on 'Crofton' peel and in a range of scalded and non-scalded regions of ‘Granny Smith’, and ‘Lady Williams’ apples to examine the morphological changes in the scalding apples.
3.3.2 Materials and Methods

3.3.2.1 TEM
Representative peel sections from scalded and non-scalded tissue from ‘Granny Smith’ apples stored at 0°C for 6 months were fixed using two chemical and two cryofixation techniques. At least 6 sections from either scalded or nonscalded peel tissue were used for each fixation technique.

Chemical Fixation
1. Overnight fixation using 3% glutaraldehyde in 0.066M cacodylate buffer (pH 6.8)
2. Overnight fixation with 1% caffeine (Mueller and Greenwood, 1978) and 3% glutaraldehyde in 0.066M cacodylate buffer pH (6.8)

The tissues fixed by both methods were washed in 0.066M cacodylate buffer and postfixed in 1% OsO₄ in the same buffer for 6 hours. Dehydration was conducted in a graded series of methanol, and were embedded in hard grade Spurr resin.

Cryofixation
Cryofixation was conducted using two different cryopreparation techniques:
1. Cutting peel tissue sectors (< 0.5mm²) and slam freezing them onto a liquid N₂ cooled polished sapphire mirror using a LifeCell CF100 cryofixation unit.
2. Plunging peel sections (< 0.5mm²) into liquid propane cooled to -196°C with liquid N₂ using a Reichert Jung KF80 cryofixation apparatus.

After cryopreservation the specimens were stored in liquid N₂ until cryosubstitution, when the frozen specimens were treated using a custom built unit. The tissue was exposed to 2% OsO₄ in acetone (molecular sieve included) at -90°C for 48 hours. The temperature was gradually raised to -30°C over 16 hours. The specimens were held at 4°C for 2 hours and then washed with acetone, 4 times each for 20 minutes. These sections embedded in Spurr resin.

Ultrathin sections were cut using a Reichert Ultracut E ultramicrotome and stained with ethanoic uranyl acetate and Reynolds lead citrate. The sections were observed in Hitachi H800 and Phillips 400 electron microscopes.

3.3.2.2 ESEM
The apple material used was the same as that in Section 3.3.2.1. Apple sections (5mm³) were hand cut and directly mounted onto a low temperature (4°C) stage for observation with the ElectroScan environmental scanning electron microscope. The samples were imaged in the ESEM operating at 15kV using the environmental secondary electron detector. The samples were kept fully hydrated during imaging by maintaining a temperature of 4 - 10°C and a chamber pressure of 6 - 9.2 Torr.
3.3.3 Results

3.3.3.1 Optical Microscopy of TEM Preparations
The TEM preparations revealed there was a significant difference between the scalded and non-scalded peel sections. The hypodermal layer in the scaled sections appeared collapsed (Figure 3.6 b and d) in comparison to the control non-scalded tissue (Figure 3.6 a and c). In addition, the scalded hypodermal cells appeared to contain more dense dark deposits (Figure 3.6 b and d).

3.3.3.2 TEM
The plunge freeze fixation method revealed more detail of the internal cell structures (Figure 3.7a). Conventional chemical fixation also gave satisfactory results, however, subcellular artefacts did not aid interpretation (Figure 3.7b). Slam freezing presented special problems for fixing apple tissue, as the peel did not fix well and was difficult to cut (Figure 3.7c).

Non-scalded hypodermal cells showed aging (Figure 3.8 b and d). The cellular structure was disintegrating, the cytoplasm contained pieces of plastids and osmophillic bodies and the vacuoles contained aggregates of a dense material that were finely dispersed throughout the vacuole. In the scalded hypodermal cells, aging symptoms were also present but superimposed was a significant deposit of an electron dense material on the tonoplast (Figure 3.8 a and c). In addition to the finely dispersed material in the vacuole, there were larger and darker aggregates of the dense material present. This material looked physically similar to the material deposited on the tonoplast.

The addition of caffeine to the chemical fixative bound the phenolics and tannins in the vacuoles into black osmophillic droplets (Figure 3.9 a, b and c). This tended to clear the vacuole contents, however traces of finely dispersed material were still present. The electron dense deposit on the tonoplast was present in the scalded hypodermal cells (Figure 3.9b). The epidermal cells in the scalded peel tissue did develop scald symptoms (Figure 3.10a), whilst the underlying fleshy parenchyma cells did not show any scald like symptoms (Figure 3.10b).
Figure 3.6  Transverse sections of TEM preparations viewed with brightfield optical microscopy of non-scalded (Figure 3.6 a and c) and scalded (Figure 3.6 b and d) ‘Granny Smith’ peel tissue, showing epidermis and hypodermal cells
Figure 3.7  TEM sections of non-scalded 'Granny Smith' hypodermal cells using different fixation methods. Figure 3.7a Plunge frozen.
Figure 3.7b Chemical fixation. Figure 3.7c Slam frozen. Bar = 5 μm
Figure 3.8  TEM sections of scalded (Figure 3.8a and c) and non-scalded (Figure 3.8b and d) ‘Granny Smith’ hypodermal cells. The arrow indicates the electron dense deposit on the tonoplast in scalded cells. Hypodermal cells in Figure 3.8a and b were plunge frozen, while those in Figure 3.8c and d, were chemically fixed without caffeine pre-treatment. Bar = 5 μm
Figure 3.9  TEM sections of non-scalded (Figure 3.9a) and scalded (Figure 3.9b) 'Granny Smith' hypodermal cells chemically fixed with a 1% caffeine pre-treatment. Bars = 5 μm. Figure 3.9c Optical view of caffeine pre-treated TEM scalded section; e epidermis, h hypodermis, p parenchyma.

Bar = 25 μm
Figure 3.10  TEM sections of epidermal and hypodermal cells in scalded 'Granny Smith' apples; e epidermis, h hypodermis (Figure 3.10a). Figure 3.10b shows a fleshy parenchyma cell in scalded tissue; p parenchyma cell. Both sections were plunge frozen. Bars = 5 μm
An interesting feature was the structural integrity of the chloroplasts in the hypodermal layer (Figure 3.11). In both scalded and non-scalded senescing hypodermal cells, the chloroplasts appeared fragile but still intact. The chloroplast illustrated in Figure 3.11, was in a severely scalded hypodermal cell that lacked cellular structure. Although there were osmiophillic bodies in this chloroplast, it clearly shows an intact chloroplast structure.

3.3.3.3 ESEM

Figure 3.12 shows that the anatomical differences between scalded (Figure 3.12a) and non-scalded (Figure 3.12b) peel appear to be minor. All cells (epidermal, hypodermal and fleshy parenchyma) seemed remarkably similar considering the actual physical severity of scald. Only minor collapsing of the hypodermal cells in scalded tissue differentiates the two peel samples. Figure 3.13 shows a transverse section of non-scalded ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apples of approximately the same age. Note the anatomical similarity between the varieties. However, in ‘Crofton’ the hypodermal layer and the fleshy parenchyma tended to be more distinct. A new observation was the presence of protuberances on the surface of the walls lining the large fleshy parenchyma cells (Figure 3.14). These protuberances were approximately 5 μm in diameter.

Figure 3.15 is a cross section of a lenticel in a ‘Crofton’ apple. It shows that the lenticels were functionally closed, which would restrict gas exchange, to diffusion through the waxy cuticle. This has significant implications for the movement of volatiles from the apple. This will be discussed further in Chapter 5. An interesting aside was the presence of rounded starch grains (Figure 3.16) in the fleshy parenchyma cells of a new season ‘Granny Smith’ apple. This dramatically illustrates the presence of starch in new season fruit.

3.3.4 Discussion

3.3.4.1 ESEM

The anatomical differences between scalded and non-scalded ‘Granny Smith’ peel when viewed with ESEM were minor. The severely scalded peel showed only minor collapse of the hypodermis compared to the non-scalded control. This contrasts with conventional TEM preparations, where there was a clear difference between scalded and non-scalded tissue (Figure 3.12). This difference between the ESEM and TEM preparations was probably an artefact of the
Figure 3.11  TEM of a severely scalded hypodermal cell in ‘Granny Smith’ peel tissue showing an intact chloroplast. Bar = 1 μm
Figure 3.12  Transverse sections of ‘Granny Smith’ peel tissue viewed with ESEM.

(A) Severe scald symptoms, (B) No visual scald symptoms, healthy tissue.
c, cuticle; e, epidermis; h, hypodermis; p, parenchyma.

Scale bar in both plates represents 50µm
Figure 3.13  Transverse ESEM non-scalded sections (A) 'Granny Smith',
(B) 'Lady Williams' and (C) 'Crofton' peel. The white scale bar units are \( \mu \text{m} \).
c, cuticle; e, epidermis; h, hypodermis; p, parenchyma
Figure 3.14  Fleshy protuberances on the surface of the walls lining the large parenchyma cells in 'Granny Smith' apples. The white scale bar represents $10\mu m$
Figure 3.15  Transverse section of a wax encrusted lenticel in the skin of ‘Crofton’.

The white scale bar represents 50μm

Figure 3.16  Rounded starch grains in fleshy parenchyma cells of new season ‘Granny Smith’ apples. The white scale bar represents 20μm
TEM preparation, as ESEM does not require any pre-treatment of the tissue. Feder and O'Brien (1968) found that dehydrating biological specimens, commonly causes irreversible damage to cell structures. They found that the least distortion occurred when there was a gradual change of polarity of the medium, from water to the final medium. In this case, the TEM dehydration / fixation treatment probably exaggerated the collapse of hypodermal cells in scalded tissue. This illustrates the major advantage of ESEM because there are no fixation or dehydration artefacts that are normally associated with conventional EM.

ESEM also showed that in ‘Crofton’ apples, there was a clear distinction between the hypodermis and the fleshy parenchyma, ie no intermediate parenchyma (Figure 3.13). This observation is probably not linked to the scald resistance of ‘Crofton’, as the hypodermis of ‘Crofton’ appears to be otherwise similar to scald susceptible varieties. The hypodermal cells in ‘Crofton’ apples contain plastids (chloroplasts and chromoplasts) as do the susceptible varieties.

The presence of intercellular protuberances has not been reported previously in apple fruits (Figure 3.14). Carr and Carr (1975) first described the protuberances on the walls lining intercellular spaces of the Marattiaceae and other ferns, and they have only rarely been reported in fruits (Reeve, 1959). Potgieter and van Wyk (1992) recently reviewed the development, distribution and function of intercellular pectic protuberances in over 200 plant species. They showed that intercellular protuberances have a widespread distribution and are morphologically diverse. These protuberances maybe derived from remnants of pectic strands between cells. Carr and Carr (1975) described rows of pectic strands connecting palisade mesophyll cells in regular configurations in some species of Eucalyptus. They suggested that the strands are formed by rearrangement of materials in the middle lamella during cell separation, however, their biological function (if any) is unknown. Carr and Carr (1980) also discussed the role of calcium in the formation of these protuberances. Potgieter and van Wyk (1992) speculated that pectic protuberances may be involved in apoplastic transport, cell wall hydration, storage (eg carbohydrates, potassium), cell adhesion and defence against pathogens. The intercellular protuberances observed in the ‘Granny Smith’ parenchyma were pronounced and prolific, and it is remarkable that they have not been described previously in apples since they were first described in plant cells more than 100 years ago (Potgieter and van Wyk, 1992).
Fixation of plant cells is notoriously difficult because of their large vacuoles (Feder and O'Brien, 1968). These problems are further compounded by the senescent nature of the stored peel tissue. Indeed, Bain and Mercer (1963) could only use a very small proportion of their sections for TEM (Joan Bain, pers. comm.). However, the fixation and preparation techniques used in this study were superior to those available to Bain and Mercer (1963). Although it is presumed that the better fixation techniques should preserve greater structural detail (Feder and O'Brien, 1968), this may not be the case, as other artefacts in fixation may make interpretation meaningless (Robards, 1991).

The plunge freezing method revealed greater structural detail (Figure 3.8). Obvious fixation artefacts, such as ice crystal formation, only seemed to affect the vacuole in those cells deep in the peel section, but did not significantly obstruct structural details in the sections examined. However, conventional chemical fixation showed typical chemical fixation artefacts (Figure 3.8). For example, dense material aggregated throughout the vacuole. During the fixation process, the phenolics can leach from the vacuoles into the cytoplasm where they combine with osmium. This results in dense osmiophillic staining in which some of the cytoplasmic detail is lost. This effect is most marked in aging cells, where the vacuole occupies a large portion of the cell volume. In such cells, the tonoplast is apparently very susceptible to injury during the fixation process (Mueller and Greenwood, 1978). This situation probably applied to the work of Bain and Mercer (1963). However, interference from phenolics can be partly overcome by application of caffeine before fixation with osmium (Mueller and Greenwood, 1978). Caffeine has long been used in light microscopy to precipitate tannins in vivo while not causing injury to the cell (Flasch, 1955). The addition of caffeine precipitated the phenolics and tannins in the vacuole into large dense osmiophillic aggregates (Figure 3.9), thus preventing a general darkening of the cytoplasm after post-fixation with osmium (Mueller and Greenwood, 1978). This tended to clear the vacuole contents, however traces of finely dispersed material were still present. However, an electron dense deposit on the tonoplast was still apparent in scalded hypodermal cells (Figure 3.9b). In slam frozen specimens, the peel tissue did not fix well and was difficult to cut. This resulted in significant structural damage to the cells. In addition there were numerous artefacts in the slammed tissue, which included ‘chattering’ in the cytoplasm.

All fixation methods confirmed the TEM observations of Bain and Mercer (1963), in that the brown symptoms of scald were associated with an electron dense deposit on the tonoplast. The
epidermal cells in the scalded peel tissue did develop scald symptoms (Figure 10a), whilst the underlying fleshy parenchyma cells did not show any scald-like symptoms (Figure 10b).

Non-scaled ‘Granny Smith’ hypodermal cells showed aging symptoms that were similar to those reported by Bain and Mercer (1963) and de Barsy et al. (1989) (Figure 3.8b and d). There was evidence of a disintegrating cellular structure, and the cytoplasm contained pieces of plastids and osmiophillic bodies. The vacuoles contained aggregates of a dense material which were finely dispersed throughout the vacuole. In the scalded hypodermal cells, aging symptoms were also present but superimposed on them was a significant deposit of an electron dense material on the tonoplast (Figure 3.8 a and c). In addition to the finely dispersed material in the vacuole, there were larger and darker aggregates of the dense material present. This material looked physically similar to the material deposited on the tonoplast and was responsible for the typical brown scald symptoms.

It is a common ultrastructural observation that the chloroplast is the organelle most susceptible to the onset of senescence or chilling injury. This was shown by Butler and Simon (1971) who described the ultrastructural changes in tobacco leaves during senescence and by Marganoni et al. (1989) who examined chilling sensitive mature green tomato fruit cells. Indeed, Bain and Mercer (1963) reported that even in lightly scalded apple hypodermal tissue, the chloroplast membranes became disorganised, dispersing fragments of lamellae, vesicles, starch grains and osmiophillic bodies into the cytoplasm. However, in non-scaled peel tissue of the same age, Bain and Mercer (1963) showed that the chloroplasts were slightly swollen but were clearly visible and appeared quite normal. Other ultrastructural changes observed in non-scaled cells due to senescence were in agreement with de Barsy et al. (1989).

Contrary to observations by Bain and Mercer (1963), the chloroplasts in both scalded and non-scaled senescing hypodermal cells, appeared fragile but still relatively intact. The chloroplast shown in Figure 3.11 was in a severely scalded hypodermal cell which lacked a well defined cellular structure. Although there were osmiophillic bodies in the chloroplast, its structure appeared normal with well defined grana (Figure 3.11). The presence of chlorophyll auto fluorescence in the chloroplasts in scalded tissue under blue excitation (Figure 3.4 c and d) was a further indication that at least the chlorophyll was still intact. Some of the earliest changes related to senescence occur in the chloroplast and that these ultrastructural changes are centred in the thylakoids (Butler and Simon, 1971). The early changes in senescing apple peel include swelling and gradual disintegration of the stroma thylakoids and appearance of lipids droplets
and plastoglobuli (Bain and Mercer, 1963; de Barsy et al., 1987). Subsequent to the loss of integrity of the stroma thylakoids, the grana thylakoids swell and undergo gradual disintegration (Huber and Newman, 1976). The nature and significance of the plastoglobuli in senescing tissue is unclear. During the early stages of senescence in leaf chloroplasts, the plastoglobuli are rich in tricylglycerols, hydroquinones and α-tocopherol, but as the leaf senesces, the tricylglycerols decline and esterified carotenoids dominate the composition of the plastoglobuli (Tevini and Steinmuller, 1985).

Since photosystem II (PS II) is mainly located in the grana and PS I is mainly distributed in both the grana and the stroma lamellae, the early loss of stromal lamellae in senescing chloroplasts explains why PS I declines faster than PS II (Bricker and Newman, 1982). The double membrane envelope that encloses the chloroplast, retains its integrity until very late in the senescence process when all the internal membranes are broken (Harris and Arnott, 1973). The chloroplast envelope is not only a structural barrier between the stroma and the cytosol, but it also plays a pivotal role in the regulation and function of the chloroplast (Hoober, 1987). The presence of intact chloroplasts in scalded cells of ‘Granny Smith’ apples raises questions about the rate of senescence and injury of the scalding hypodermal cell and its contents. Perhaps the presence of intact chloroplasts in scalded hypodermal cells reflects a physiological response to the storage history of the fruit. The apples used in this study were stored in air for six months at 0°C, and under these storage conditions, respiration and the sequence of programmed senescence are dramatically slowed. Perhaps the chloroplasts may be chilling resistant or may senesce at a slower rate than other cellular structures. However, this is unlikely because it is well established that the thylakoid membrane is often the first subcellular membrane to lose its integrity during senescence (Butler and Simon, 1971). In addition, the chloroplast is also thought to be the primary site of chilling injury (Marangoni et al., 1989; Abe, 1990).

The presence of intact and viable chloroplasts in scalded tissue has important implications in the interpretation of the cause of the browning reactions involved in scald development. Bain and Mercer (1963) suggested that the formation of the electron dense materials in scalded ‘Granny Smith’ hypodermal cells was a result of uncontrolled PPO activity following the breakdown of the tonoplast. This allowed mixing of the vacuolar contents containing the phenolic substrates with the PPO resulting in a dense deposit on the tonoplast. This process is not simply normal senescence as not all the epidermal and hypodermal cells in scald susceptible varieties undergo browning. Indeed, the incidence and severity of scald is variable and subject to seasonal and
orchard conditions (Emongor et al., 1994). The apparent random nature of scald indicates that perhaps some membranes (chloroplast and / or vacuolar) remain intact, whilst others (often neighbouring cells) become disorganised, exposing the phenolic substrates to PPO. Possible reasons for the variable distribution of scald symptoms may include antioxidants protecting the membranes from oxidation or differential expression or activity of PPO.

Phenolics are the primary substrates for PPO activity and are localised in the vacuole (Mayer and Harel, 1979). The tonoplast in scalding apple cells appears relatively intact although there seems to be some occasional breaks in the membrane (Figure 3.8 a and c). Indeed, the tonoplast in senescing cells usually remains intact until late into senescence (Wittenbach et al., 1982; Woolhouse, 1984).

PPO is found extensively throughout the plant kingdom and there is now little doubt that PPO is a plastid enzyme localised in a range of plastid types (Mayer and Harel, 1979). However in most tissues, PPO is localised at the thylakoid membrane within the chloroplast in association with the PSII complex (Lax and Vaughn, 1991). The chloroplast localisation of PPO ensures that the enzyme is physically separate from its phenolic substrates located in the vacuole. Under normal senescence, Butler and Simon (1971) reported that the thylakoid membrane is the first subcellular membrane to lose its integrity. Although the outer chloroplast membrane envelope of the chloroplasts in the scalded hypodermal cells appeared fragile, the chloroplasts in the scalded cells seemed to have a regular internal structure, eg intact grana and thylakoid membrane (Figure 3.11). However, even if the outer chloroplast envelope is intact there still maybe some passive movement of PPO from the chloroplast into the cytosol resulting in ‘free’ PPO in the cytoplasm. Vaughn and Duke (1984) reported that PPO can apparently exist free in the cytoplasm of degraded or senescing tissue, such as ripening fruit, but this is thought to be due to artefacts in the membranes in senescing cells which are exceptionally fragile and are difficult to prepare during fixation of tissue for EM (Thompson and Platt-Aloia, 1987). Nevertheless, the presence of free PPO in the cytoplasm of senescing cells cannot be totally dismissed.

A non-plastid source of PPO would also result in an uncontrolled mixing of the phenolic substrates and PPO. However, if there is no movement of PPO from the chloroplast, and there is no other cellular source of free and active PPO, then the electron dense deposits in scalded tissue must be generated by another mechanism. Perhaps peroxidases, which are found in either a soluble or bound form in the cytoplasm, endoplasmic reticulum, chloroplast, plasmalemma and
cell wall are partly responsible for the browning symptoms (Barz and Koster, 1981). Peroxidases can catalyse the oxidation of the major polyphenols in apples e.g. catechins, hydroxycinnamic acid derivatives and flavonols (Richard and Nicolas, 1989). The primary oxidised products of peroxidases are quinones, which can similarly polymerise (Barz and Koster, 1981) and could also form dense complexes on the tonoplast.

Although peroxidases are widely distributed in the peel of apples (Richard and Nicolas, 1989) they are often not considered to be involved in browning because the internal level of H$_2$O$_2$ is limited (Richard and Nicolas, 1989). However, their involvement in slow physiological processes, such as the development of scald is possible. This may also be possible as the membrane breakdown associated with the breakdown of the chloroplast and vacuolar membrane is thought to be free radical initiated (Anet, 1969). Although gross peroxidase activity does not seem to be directly involved in the development of scald (Du and Bramlage, 1995), there still remains the possibility that specific peroxidases may be related to specific and crucial events in the development of scald. This is discussed in more detail in Chapter 6.

More work is required on enzyme (PPO and POD) localisation and kinetics in hypodermal cells. This should include comprehensive immunocytochemistry and labelling work particularly over the ripening and storage life of scalding apples. In addition, further characterisation and quantification of the phenolic substrates and oxidised phenolic products will help in the understanding of the nature of the dense deposits on the tonoplast, typical of the scald symptoms.
3.4 Magnetic Resonance Imaging

3.4.1 Introduction

Magnetic resonance imaging (MRI) is a relatively new and powerful technique for examining the physical and chemical composition of scalp development in situ. MRI is a non-invasive and non-destructive method for visualising the internal structure of complex objects. It allows flexibility through direct examination of a range of both physical and chemical parameters and by eliminating uncertainties due to biological variation. In addition to obtaining data with micrometer resolution, MRI can also enable observations in two or three dimensions. Callaghan (1991) gives a thorough discussion of the physics of MRI, while the biological and food applications of MRI are discussed by Schrader et al. (1992). The current MRI technique is an extension of NMR spectroscopy by incorporating linear magnetic fields to give data on spatial position. It relies on the interaction between low power radio frequency radiation and the magnetic moments of (H) hydrogen nuclei to produce images related to the proton distribution in the sample. Hydrogen is one of the most suitable elements for MRI because of its abundance and favourable magnetic moment. When a sample containing water is placed in a magnetic field the hydrogen nuclei in the sample becomes polarised. A small net magnetisation is established which precesses around the axis of the magnetic field at a frequency given by the Larmor relationship:

\[ \omega_0 = \gamma B_0 \]

where \( \omega_0 \) is the angular frequency of the precession; \( B_0 \) is the magnetic field strength and \( \gamma \) is a constant for the particular nuclei (Callaghan, 1991).

A brief pulse of radio frequency radiation applied at the precession frequency momentarily disturbs the equilibrium for a given length of time. The relaxation processes and the time constants associated with the return of the nuclei to their equilibrium, are exploited in MRI. These relaxation processes are governed by a number of processes. The re-establishment of the magnetisation along the magnetic field is referred to as \( T_1 \) or spin-lattice relaxation. This occurs as a result of the dissipation of energy from the excited nuclear spins to the surrounding material. The second type of relaxation is referred to as \( T_2 \) or spin-spin relaxation and leads to dephasing of the nuclear spins. Instead of the energy of the nuclei being transferred to the lattice, the nuclei exchange energy with each other. These two types of relaxation processes can be exploited to highlight differences in chemical and physical composition as they are a function of the chemical
environment, mobility, temperature, diffusion, species, concentration and structure. Callaghan
(1991) gives a discussion and interpretation of these relaxation parameters.

MRI has been used to examine the internal changes occurring during the development of
watercore in apple (Wang et al., 1988) and core breakdown in pears (Wang and Wang, 1989).
The greatest advantage of MRI is its non-destructive imaging and hence it can be used to follow
the in vivo changes in the same fruit. This is important for storage disorders (such as scald)
which are random in nature, as the development of the disorder can be followed non-
destructively and permits a reduction in the level of replication, as it can image non-destructively
repeatedly on the same fruit. However in this study due to time restrictions, MRI was confined
to imaging at one time interval.

The objective of this section was to investigate the in vivo physical and chemical changes that
occur in scalding ‘Granny Smith’ apples by MRI. Two imaging experiments were conducted
using :

- whole apple MRI, and
- three dimensional imaging experiments on apple pieces

In addition, the relative water content of scald affected peel was determined.
3.4.2 Materials and Methods

'Granny Smith' apples were stored in air at 0°C for 9 months. Upon removal and simulated shelf life (seven days at 20°C), apples were separated into scalded and non-scalded fruit.

3.4.2.1 MRI

Imaging was conducted at the Centre for Magnetic Resonance at the University of Queensland, St. Lucia, Queensland.

3.4.2.2 Whole apple imaging experiment

Proton microscopy was performed using a modified Bruker MSL200 console interfaced to a 4.7 Tesla 40 cm bore magnet system. The radiofrequency probe was a custom built device and was conducted using a 20 cm birdcage RF resonator. Typically 4-8 averages were used with the total sequence time taking about 40 minutes. The in-plane resolution was 90 μm and the slice thickness was 500 μm. The in-plane resolution was 200 μm and the slice thickness was 1.5 mm. The imaging sequence was a multi-echo spin-echo experiment with the echo time being multiples of 18 ms.

3.4.2.3 Three dimensional imaging experiment

Proton microscopy was performed using a Bruker AMX300 spectrometer interfaced to a 7 Tesla 89 mm bore vertical magnet system. A resonator permitting an access diameter of 22 mm, and the second a solenoidal coil with 3 mm access were used. Shielded gradients capable of producing 80 G/cm were used for the imaging experiments. These gradients, when used in conjunction with mild $Z_0$ compensation produced no temporal or spatial distortion of the required field 0.4 ms after a gradient pulse. A three-dimensional Fourier imaging sequence was used where TE = 12 ms (Figure 3.17) (Callaghan, 1991). In these solenoidal experiments using a vertical bore magnet, the in-plane resolution was 35 μm and the slice thickness was 200 μm.

3.4.2.4 Water content of scalded peel tissue

Ten peel sections were sliced from both non-scalded and scalded areas from the same scalded 'Granny Smith' apples. The sections were sliced by hand and were approximately 0.5 cm$^2$ x 2 mm deep. The fresh weights were immediately determined before freeze drying. Dry weights were determined and relative water contents calculated.
3.4.3 Results

3.4.3.1 Whole apple imaging

Figure 3.18 illustrates whole apple images of $T_1$ weighted, multi slice-spin echo images, with a slice thickness of 3mm. Figure 3.18a is a $T_1$ weighted image of a non-scalded, control 'Granny Smith' apple, whilst Figure 3.18b had slight scald symptoms. Proton density images of the same apples are shown in Figure 3.18 c and d. The diffuse margin indicated in Figure 3.18 b and d was a scalded region. The bright regions represent regions of the fruit with a different water state. A bright region is considered to have higher water content.

3.4.3.2 Three dimensional imaging experiment

Scalded apples were clearly different to non-scalded control apples. Differences were detectable by NMR imaging approximately 200 - 600 μm below the skin surface. The 'Granny Smith' apple section chosen to illustrate this imaging work was on the edge of a severely scalded region. Approximately 2/3 of the apple piece was scalded. The four images shown in Figure 3.19, are four slices of the same piece of 'Granny Smith' apple peel, which shows four slices of a 32 slice three-dimensional image. The brightness near the peel was the scald affected region and
Figure 3.18  Whole apple imaging of ‘Granny Smith’ apples showing the development of scald. (A) $T_1$-weighted image of a non-scalded control; (B) $T_2$ weighted image of an apple with slight scald symptoms; (C) Proton density image of a non-scalded control; (D) Proton density image of an apple with slight scald symptoms
Figure 3.19  Four slices of a partly scalded ‘Granny Smith’ peel tissue.

NS indicates non-scalded peel tissue; S indicates scalded peel tissue
indicates a change in water state. This effect was noticed on 10 scalded ‘Granny Smith’ apple peel samples compared with 8 non-scaled control sections from the same apples. It is important to note that these apples exhibited a short (<50 ms) $T_2$ values at 7 Tesla. Therefore, in imaging sequences that are $T_2$ weighted, the signal-to-noise ratio is low and therefore have low spatial resolution.

3.4.3.3 Water content of scalded peel tissue
Scalld peel tissue had a significantly lower relative water content than non-scaled tissue (Table 3.3).

Table 3.3 Relative water content (fresh weight / dry weight x 100) of scalded and non-scaled peel tissue of ‘Granny Smith’ apples determined by freeze drying

<table>
<thead>
<tr>
<th>Peel</th>
<th>Relative water content</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (non-scaled)</td>
<td>80.0 *</td>
</tr>
<tr>
<td>severely scalded</td>
<td>75.6 *</td>
</tr>
</tbody>
</table>

$P \leq 0.05$

3.4.4 Discussion
The results showed that scald in ‘Granny Smith’ apples is detectable by NMR micro-imaging. Whole apple imaging showed that scald appeared as an irregular outline of the fruit surface with little visible damage interior to the fruit surface. The changes in proton density suggest a change in water content. This was further investigated by the three-dimensional imaging. The results showed that the NMR effect was visible in the scalded region of the peel approximately 200 - 600 $\mu$m below the skin surface. This correlated well with the dimensions of the hypodermal cells where scald is known to occur.

To determine whether the change in water state observed by MRI was due to actual loss of water from the affected areas, the relative water content of scalded regions was compared to non-scaled regions of ‘Granny Smith’ peel. The results showed that scalded peel tissue had significantly reduced relative water content. The reduced relative water content of the scalded tissue does not contradict the current scald hypothesis proposed by Huelin and Coggiola (1968), and Du and Bramlage (1993). However scald is sometimes considered to be a chilling disorder
(Bramlage and Meir, 1990; Watkins et al., 1995). A common symptom of chilling injury is the loss of membrane integrity resulting in cellular breakdown and an increase in the relative water content (Salveit and Morris, 1990; Walker et al., 1991). This can eventually lead to a water-soaked lesion typical of chilling injury (Salveit and Morris, 1990). However, in severely scalded peel, the hypodermal cell contents become disorganised and the cells eventually collapse, but the surface of the scalded peel remains dry and firm. As there is a decrease in the relative water content of the scald affected peel, the water from the collapsing hypodermal cells is either adsorbed into the fleshy underlying parenchyma, or is lost by evaporation to the storage atmosphere.

In conclusion, this work has shown that MRI can detect scald and that the change in water state was due to water loss from scald affected peel tissue. Further MRI investigations into the relaxation and diffusive behaviour in apples developing scald may provide greater insights into the molecular mechanisms inherent in non-scalded and scalded apple tissue.
3.5 Discussion

Although scald only occurs in physiologically immature scald susceptible varieties stored for long periods at low temperatures (0°C), these apples have completed cell division and cell growth (Bain and Robertson, 1951). Consequently incomplete cell growth and expansion should not be a factor in scald development.

The first symptoms of scald appear as a slight browning of the outer hypodermal cells. As scald becomes more severe, the browning of the hypodermal cells becomes more intense and develops throughout the hypodermis. Eventually, all the hypodermis and epidermal cells become scalded. In the later stages of scald, the epidermal cells collapse to give the peel its characteristic sunken appearance. In very severe cases, scald symptoms appear in a few cells of the outer cortex underlying the hypodermis. MRI results showed that scald could be detected by NMR micro-imaging, and showed differences 200 - 600 μm below the skin, dimensions that correlate to the size of the hypodermal layer. These MRI differences were found to be due the lower water content of scalded tissue. Presumably the water in the collapsing hypodermal cells in scalded tissue is absorbed into the fleshy underlying parenchyma, or is lost by evaporation to the storage environment. However the anatomical differences between scalded and non-scalded 'Granny Smith' peel when viewed with ESEM were minor. The severely scalded peel showed only minor collapse of the hypodermis compared to the non-scalded control. This illustrates the major advantage of ESEM for avoiding fixation or dehydration artefacts, which are commonly associated with conventional EM.

Using a range of both chemical and cryofixation methods, ultrastructural examination of scalded 'Granny Smith' peel tissue showed that the brown symptoms were associated with an electron dense deposit on the tonoplast. This confirmed the observations of Bain and Mercer (1963) who used 'Araldite' and osmium tetroxide when preparing cells for EM. In this current study, the plunge freezing technique was the 'best' fixation method as it showed more detailed internal cell structure. The addition of caffeine before chemical fixation bound the phenolics and tannins in the vacuoles into black osmiophilic droplets. This tended to clear the vacuole contents and clearly showed the electron dense deposit on the tonoplast in scalded hypodermal cells.
These results support Bain and Mercer’s hypothesis of scald development in which the formation of the electron dense materials in scalded hypodermal cells is considered to be a result of uncontrolled PPO activity following the breakdown of the tonoplast. Subsequent mixing of the vacuolar contents containing the phenolic substrates with the PPO in the cytoplasm results in a dense deposit on the tonoplast. However there were several interesting features of scalded hypodermal cells. For example the relatively long period in which the scalding epidermal cells remained viable whilst the tissue developed scald symptoms. This lead to the situation where the epidermal cells remained viable while the underlying hypodermal cells were functionally dead (ie plasmalemma is unable to contain fluorescein). This raises further questions about the functionality and sequence of scald development. For example, only hypodermal cells are affected by scald, but the overlying epidermis is initially unaffected.

Another interesting feature was the presence of relatively intact chloroplasts within hypodermal cells that had severe scald symptoms. These chloroplasts had well defined grana and structure, appeared normal, and auto-fluoresced under blue excitation. In addition, significant chlorophyll fluorescence ($F_v / F_m$) was detected in severely scalded tissue. This suggests that the chloroplasts in scalded tissue are, to some extent, functional. This is surprising considering the chloroplast membranes are usually the first to lose their integrity during senescence (Butler and Simon, 1971) and are the first to be affected by chilling injury (Marangoni et al., 1989).

The current scald hypothesis suggests that plastid bound PPO is released from the chloroplast and oxidises phenolics released from the tonoplast, resulting in the characteristic brown deposits on the tonoplast (Bain and Mercer, 1963). However, if the chloroplasts are relatively intact and PPO is not released into the cytoplasm, and there is no other source of PPO, then the oxidation of phenolics on the tonoplast must be via another mechanism. A possible candidate is peroxidase (POD). Although POD is widely distributed in the peel of apples (Richard and Nicolas, 1989), it is not usually considered to be involved in browning because it is thought that the internal level of $\text{H}_2\text{O}_2$ is limited. However, their involvement in slow physiological processes, such as the development of scald maybe possible, particularly when the membrane breakdown associated with the breakdown of the chloroplast and vacuolar membrane is thought to be initiated by free radicals (Anet, 1969). Although total peroxidase activity does not seem to be directly involved
in the development of scald (Du and Bramlage, 1995), there still remains the possibility that specific peroxidases may be related to specific and crucial events in the development of scald.

In conclusion, these results confirm the classic histological work of Bain (1956) and Bain and Mercer (1963). However some new observations, for example the presence of 'intact' chloroplasts, and viable epidermal cells in scalding tissue raise further questions about the development of scald. These questions include a possible role for specific peroxidases in the browning reactions. This possibility led to a re-assessment of the role of α-farnesene in scald (Chapter 5) and to a detailed examination of the phenolic profiles in scald susceptible and resistant apple varieties (Chapter 6).
Chapter 4  Induction of Superficial Scald

4.1 Introduction

Superficial scald is an unpredictable physiological disorder. The storage disorder is affected by numerous pre- and postharvest conditions (Emongor et al., 1994; Ingle and D’Souza, 1989). The current hypothesis on the cause of scald implicates the sesquiterpene, α-farnesene, and its oxidation products (conjugated trienes) (Huelin and Coggiola, 1970 a,c; Anet, 1972a). If this hypothesis is correct, increasing the concentration of α-farnesene or its oxidation products in apple skin should increase the levels of scald. Development of a model system for inducing scald on demand should greatly facilitate understanding of the physiology and biochemistry of the disorder.

Dilley et al. (1963) induced scald-like symptoms in ‘McIntosh’ and ‘Red Rome’ apples by anaerobic treatment at 20°C. Although the symptoms following the anaerobic treatment were reported to be similar to those naturally occurring scald, it is difficult to imagine a similar mechanism of action, since the condition was induced at 20°C. Scald only occurs during long term storage at low temperatures (0 - 4°C), and is thought to be induced by chilling (Watkins et al., 1995). Although Dilley et al. (1963) showed that a pre-treatment application of diphenylamine (DPA) inhibited the development of scald in anaerobic treatments, DPA has numerous other physiological actions in apples (Lurie et al., 1989a) and the function of DPA is unclear.

Huelin and Coggiola (1970c) attempted to induce scald in apples by applying a range of chemicals, including α-farnesene, in ethanol solutions onto the peel of apples. Their results were inconsistent and inconclusive, however the conjugated trienes (α-farnesene oxidative products) gave a relatively higher correlation with scald. In this study, some different ways of applying α-farnesene or its oxidation products to apple peel were investigated to develop a reliable model system for the induction of scald in ‘Granny Smith’ apples. Varied concentrations of α-farnesene were applied to ‘Granny Smith’ peel tissue either topically to the patches on the fruit, to the underside of peel discs or via a vapour to excised peel discs.
4.2 Materials and Methods

Preclimacteric ‘Granny Smith’ apples were harvested from a commercial orchard at Bilpin, NSW. Fruit were placed in air storage (0°C) on the day of harvest. When apples were required for experiments, the apples were removed from storage and allowed to warm to room temperature (20°C). Several techniques were used to apply α-farnesene to the peel of apples. These included:

- topical application of a range of concentrations of α-farnesene and oxidised α-farnesene on patches to the peel of the apples
- application of a range of concentrations of α-farnesene in patches to the underside of aseptically prepared apples peel discs
- application of α-farnesene in vapour phase to aseptically prepared apple peel discs

4.2.1 Topical application

Alpha-farnesene is extremely labile when exposed to oxygen (Anet, 1969), so a suitable solvent (light paraffin oil, Sigma Chemicals) was necessary to retain α-farnesene on the peel surface. A range of concentrations of α-farnesene (0, 50, 100, 250, 500 and 1000 μg.cm⁻² apple peel) was applied in patches to ‘Granny Smith’ apples (Figure 4.1). Oxidised α-farnesene was prepared by bubbling oxygen through a α-farnesene solution for 10 min (Anet, 1969). The solutions of α-farnesene and its oxidised form in paraffin were applied to glass microfibre filter paper patches (Whatman GF/F) on the peel of ‘Granny Smith’ apples. Control patches were treated with paraffin only. To help retain the α-farnesene over the peel, a polyethylene (60 μm) cover patch was placed between the α-farnesene patch and the sticky plaster (BDF Leukopor tape). Four patches were attached to the apple around the equator with sticky plaster. There were 5 apple replicates per treatment. The apples were then stored at either 0°C or 20°C.

Samples were removed from storage at 3-weekly intervals for 3 months and assessed for scald and changes in colour (L, a, b) were measured using a colorimeter (Minolta CR-2000, Japan) after 5 days at 20°C. To examine any histological changes that had taken place, tissue transverse sections of peel were cut by hand from a representative sample of treatments and examined by optical microscopy (Section 3.2.1).
Figure 4.1  Application of $\alpha$-farnesene by patches attached to the skin of
'Granny Smith' apples

Figure 4.2  Filter paper patches impregnated with $\alpha$-farnesene
were placed on the underside of excised 'Granny Smith'
apple peel discs that were stored at 0°C
4.2.2 Application to the underside of peel discs

Disc preparation and subsequent manipulations were carried out aseptically in a laminar flow cabinet using methods adapted from Campbell et al. (1990). 'Granny Smith' apples were surface sterilised in 1% sodium hypochlorite solution and rinsed thoroughly with sterile water. Peel discs (2cm²) were prepared using a sharp cork borer and excess parenchyma tissue was removed with a scalpel to leave uniform peel discs, about 2 - 4 mm thickness. The discs were briefly rinsed with sterile distilled water, drained and blotted, with sterile tissue paper to remove excess water.

Glass filter paper discs were impregnated with various concentrations of α-farnesene (Section 4.2.1), however the 100 µg.cm⁻² α-farnesene treatment was omitted from this experiment. Two solvents (hexane and paraffin) were used to transfer the α-farnesene to the glass filter paper discs. The excised aseptic apple peel discs (epidermis side-up) were then placed on the glass filter paper discs. The apple peel discs plus the glass filter discs were placed on raised platforms and stored in sterile glass petri dishes (Figure 4.2). There were 6 apple peel discs / farnesene disc combinations per treatment. Sterile distilled water was placed in the bottom of the petri dishes to maintain high humidity. The petri dishes were stored at 0°C and the peel discs were assessed for scald development at 3 weekly intervals for 3 months and colour changes (L, a, b) were measured using a colorimeter (Minolta CR-2000, Japan). Transverse sections of the peel discs were examined under a compound microscope for any ultrastructural changes.

4.2.3 Vapour phase treatment

Apples were surface sterilised and peel discs were excised aseptically (Section 4.2.2). The peel discs were placed epidermis side-up in multiwell tissue culture plates ('Falcon' Multiwell 3047) and sealed with 'Dow Corning' 3140 RTV Sealant (USA). [A previous experiment used a commercial domestic sealant ('Selleys' bathroom and kitchen sealant), but caused rapid and excessive tissue damage]. Each plate contained 16 peel discs. Water was added between the wells containing the discs to maintain a saturated atmosphere. Four plates of apple discs were connected in series with teflon tubing to make a bank of apple peel discs over which α-farnesene in a humidified air stream was passed at about 1 mL.min⁻¹ (Figure 4.3). A source of α-farnesene was enclosed in a flask upstream from the apple discs. A control bank of apple discs were similarly prepared, and ventilated with humidified air stream. The discs were stored at 0°C and after 1 month were assessed for scald symptoms and changes in colour (L, a, b) were measured using a colorimeter (Minolta CR-2000, Japan). Transverse sections of representative peel discs were examined under a compound microscope for any ultrastructural changes.
Figure 4.3 System of introducing α-farnesene in vapour form to 'Granny Smith' apple peel discs (A). A humidified air stream transfers α-farnesene from an external source, over a series of four sets of peel discs (B). Control discs were ventilated with humidified air only. Figure C shows 'poisoning' of the discs from the solvent in initial studies with a commercial sealant ('Selleys' bathroom and kitchen sealant) used to seal the lids onto the wells.
4.3 Results

All fruit held at 20°C developed fungal rots before any scald symptoms could develop. The severity of scald symptoms under the α-farnesene or oxidised α-farnesene patches was similar to the surrounding untreated peel tissue irrespective of the severity of the scald symptoms on untreated skin (Figure 4.4 A - D). It was noted that the peel under the patches lost chlorophyll at a slower rate than the surrounding exposed peel, but this was not related to α-farnesene concentration. This is probably due to exposure to light in the coolrooms and in the laboratory, where the chlorophyll in the surrounding exposed peel tissue was degraded at a greater rate than under the light-proof patches.

The effects of α-farnesene patches applied to the underside of excised peel discs are shown in Figure 4.5. No scald symptoms were induced, even with the highest concentration of α-farnesene application. The application of α-farnesene in hexane solvent was extremely deleterious to excised peel discs, resulting in whole tissue browning and death. Induction of scald by application of α-farnesene in the vapour phase was unsuccessful. In all cases, no apple discs developed scald symptoms (Figure 4.6).

4.4 Discussion

The availability of a model system for reliably inducing scald should greatly assist studies on this disorder. All of these initial attempts to induce scald on ‘Granny Smith’ apples were unsuccessful. These negative results could be dismissed on the grounds that the methodology was unsuitable. Alternatively, the results raise questions about the validity of the prevailing hypothesis that α-farnesene or its oxidation products are the primary cause. Questions that remain unanswered include; the time course of α-farnesene production, presence of natural anti-scald agents and precisely when the disorder is induced during storage. These aspects are investigated in later chapters.
Figure 4.4  Scald symptoms after 3 months storage at 0°C and one week at 20°C.

(A) Shows the level of scald under a α-farnesene impregnated patch which is not different to the surrounding tissue. The other figures show the level of scald incidence and severity not related to the α-farnesene patch on lightly (B), moderately (C) and severely (D) scalded peel tissue.
Figure 4.5  Comparison of excised ‘Granny Smith’ peel discs after 3 weeks (A,C,E,G,I) and 12 weeks (B,D,F,H,J) storage at 0°C. Each peel was placed on a source of α-farnesene at 0 (A,B), 50 (C,D), 250 (E,F), 500 (G,H) and 1000 (I,J) µg α-farnesene.cm² peel. No scald symptoms were observed on any peel disc.
Figure 4.6  Figure A shows the green healthy peel tissue at the beginning of the volatile scald induction experiment. Figure B is an example of the symptoms of senescent apple peel discs after 4 weeks storage at 0°C. The tissue was senescent and the peel did not develop any scald symptoms.
It is important to note that most of these experiments were conducted with stored apples, except the vapour phase experiment. Perhaps these results were not surprising since significant amounts of α-farnesene were found in stored ‘Granny Smith’ apples (Section 5.2), and suggests that other factors, other than α-farnesene alone, are responsible for the development of scald. For example the role of α-farnesene in the development of scald may be time dependent, i.e. α-farnesene may only be effective in inducing scald within the first few months of storage. It is known that the scald inhibitor DPA is most effective when it is applied within 24 hours after harvest (Meigh, 1970). DPA is a well known but unusual antioxidant (Sugihara et al., 1993) that prevents the oxidation of α-farnesene (Huelin and Coggiola, 1970a; Anet and Coggiola, 1974a). However, DPA also affects respiration, ethylene production and the activities of PPO, POD and LOX (Lurie et al., 1989a).

The lack of quantitative measurements of α-farnesene or its oxidation products in the apple peel tissue or in vapour phase over the apple discs was a deficiency of these experiments. This would have confirmed that α-farnesene was present at the site of scald development. In addition, measurements would have shown whether there was an increase in the level of α-farnesene in the headspace from the first to the last multiwell and this should have been reflected in differences in the incidence and severity of scald development among the discs in series. However, there were no differences in the levels of scald in any peel tissue among the wells.
Chapter 5  Role of Volatiles in the Development of Superficial Scald

5.1 Introduction

Brooks et al. (1919) observed that loosely packed or well ventilated apples developed less scald than tightly packed or unventilated apples. This led to the use of oil impregnated wraps as the first practical control measure but also to the theory that scald was induced by volatile or gaseous compounds (other than CO₂) produced by the apple. The search for the volatile compound(s) responsible for scald was interrupted in the mid-1950's when Smock (1955, 1957) showed that pre-storage applications of diphenylamine (DPA), and ethoxyquin were effective in controlling scald. The nature of the substance responsible for scald was subject to conjecture for 45 years until Murray et al. (1964) and Huelin and Murray (1966), identified α-farnesene in the coating of 'Granny Smith' apples.

Following this discovery, there was renewed interest in the causes of scald, with particular attention focussed on α-farnesene and its oxidation products (conjugated trienes) (Huelin and Coggiola, 1970a,c; Meir and Bramlage, 1988; Du and Bramlage, 1993). However, after over 30 years of research, our current knowledge of the role of α-farnesene in scald development remains incomplete. The proposed role of α-farnesene or its oxidation products in scald development has not been challenged despite evidence of poor correlations between scald development and α-farnesene concentration (Huelin and Coggiola, 1970c; Chen, et al., 1990b; Gallerani and Pratella, 1992; Du and Bramlage, 1994a).

The objective of this work was to re-examine the role of apple peel volatiles, in particular, α-farnesene in the development of scald.
The specific aims of this section were:

- reliably separate, identify and quantify apple peel volatiles using more specific methods than the current spectrometric methods,

- measure changes in α-farnesene and other apple volatiles in scalding (‘Granny Smith’ and ‘Lady Williams’) and non-scalding (DPA pre-treatment and ‘Crofton’) apple peel during storage,

- quantify differences in volatiles from scalded and non-scalded peel tissue,

- compare changes in volatiles during storage between fruit harvested immature and at optimum maturity,

- re-examine the role of apple volatiles in the development of scald, by examining the volatile profile during storage in both DPA treated and non treated ‘Granny Smith’ and ‘Crofton’ apples subjected to a constant challenge of volatiles from both ‘Granny Smith’ and ‘Crofton’ apples, and

- examine the relationship between ethylene, α-farnesene and DPA applications in ‘Granny Smith’ and ‘Crofton’ apples stored at both 0°C and 10°C.
5.2 Materials and Methods

5.2.1 Apple Fruit Maturity Indices and Assessment

5.2.1.1 Introduction

The majority of scald and apple work, has been conducted with little regard to the an accurate measure of the physiological age of the fruit. This section describes the methods used for the determination of fruit maturity and scald assessment.

5.2.1.2 Internal ethylene concentration

Internal concentrations of ethylene were measured on a gas sample withdrawn via a syringe and hypodermic needle inserted in the calyx end of the fruit (Jobling, 1993). Ethylene was determined with a GowMac Model 580 GC fitted with an activated alumina column (2m x 2mm ID, stainless steel) and FID detector with nitrogen carrier gas at 28 mL.min⁻¹, hydrogen 20 mL.min⁻¹, air 300 mL.min⁻¹. One mL gas samples were used and the lower detection limit was 0.01 μL.L⁻¹.

5.2.1.3 Induced ethylene climacteric

The induced ethylene climacteric (IEC) is a procedure for estimating when apples on the tree will enter their climacteric and begin to ripen (Dilley and Dilley, 1985). Five 6L polyethylene buckets were filled with blemish-free fruit (about 10) and the buckets were sealed with lids fitted with serum caps for gas sampling. A paper bag containing about 100g hydrated lime (Ca(OH)₂) was enclosed in each bucket to absorb carbon dioxide. The ethylene concentration from a headspace sample (1mL) from each bucket was analysed by GC as above (Section 5.2.1.2). The buckets were sampled daily until 3 of the 5 buckets had reached an ethylene concentration of 0.5 μL.L⁻¹. The estimated harvest date for long term CA storage was determined by multiplying the number of hours taken to accumulate 0.5 μL.L⁻¹ ethylene in the sealed container by 0.125 days.hour⁻¹. The product of this multiplication is the number of days from the time the fruit were sealed in the containers to the predicted harvest date (Dilley and Dilley, 1985). The buckets were regularly opened to prevent significant decreases in oxygen levels in the buckets. Aerating the buckets does not disrupt the time taken to accumulate the critical level of 0.5 μL.L⁻¹ of ethylene in the container (Jobling, 1993).
5.2.1.4  **Fruit firmness**
Flesh firmness was measured with an Effegi penetrometer (11mm tip) mounted on a drill press (Blanpied and Blak, 1977). Two readings were taken on peeled areas on opposite sides of the fruit and the units used were Newtons (Kgf x 9.807 = Newton (N)) (Kader, 1982).

5.2.1.5  **Soluble solids content**
Soluble solids content (SSC) were determined from a sample of juice and measured at 20°C using a hand held refractometer (Atago, Japan).

5.2.1.6  **Starch index**
Starch content was estimated by staining with potassium iodine. Fruit were cut transversely in the equatorial region and dipped in a solution of potassium iodine (2.54 g) and iodine (1.27 g) in water (1L) for 30sec. The reagent stains starch blue. The area of the stained flesh tissue was assessed according to the ‘Granny Smith’ starch chart (0 = all cortex blue and three quarters of the core blue = full starch, to 6 = all surface white = no starch) (Beattie and Wild, 1973).

5.2.1.7  **Scald assessment**
Upon removal of fruit from cold storage, the apples were transferred to air at 20°C for 1 week, when they were assessed for the occurrence of scald. Scald was subjectively assessed using the scale of; 0 = no scald, 1 = trace 1% to 5%, 2 = 6% - 26%, 3 = 26% to 50%, 4 = 51% - 75%, 5 = 76% to 100% of the surface area affected (Figure 5.1).

A mean score was calculated for each experimental unit of 25 fruit, and treatment differences were compared by analysis of variance as reported by Hall *et al.* (1961a). Experiments with many extreme values resulted in non-homogeneous variances. Homoscedasticity is one of the underlying assumption of analysis of variance and was tested with Bartlett’s test of homogeneity of variances, and if appropriate transformations were not successful, treatments were compared using the Kruskall Wallis ranking system.
Figure 5.1  Subjective scald assessment scoring system

0 = no scald  →  5 = severe scald
5.2.2 \(\alpha\)-Farnesene and Other Apple Volatiles: Extraction and Quantification

5.2.2.1 Introduction

The current spectroscopic method of estimating the concentration of \(\alpha\)-farnesene (Figure 5.2) in the peel of apples relies on measuring a specific UV absorption (\(\lambda = 232\) nm). Apart from being time consuming, this spectroscopic method has some inherent problems, which include possible interference from UV absorbing contaminants, and the difficulties associated with handling large volumes of solvent used to extract the apple peel.

In addition to \(\alpha\)-farnesene, it is important to identify and quantify other apple volatiles. There are numerous techniques for determining the composition and concentration of volatiles (Kolb, 1985). However, there are many problems associated with identifying and quantifying the volatiles in a complex biological system. These include the inherent complexity of apple volatiles and the high water and wax content of apple peel that may significantly affect analytical procedures.

![Structure of \(\alpha\)-farnesene]

Figure 5.2 Structure of \(\alpha\)-farnesene

\([3E,6E]-3,7,11\text{-trimethyl-}1,3,6,10\text{-dodecatetraene} \text{ (C}_{15}\text{H}_{24})\]

The ability to reliably and accurately monitor the changes in \(\alpha\)-farnesene and other fruit volatiles during storage is essential for the study of their role in scald development. The following procedures were compared:

- the existing spectroscopic method for estimating \(\alpha\)-farnesene with a direct measure of \(\alpha\)-farnesene in a hexane extract of ground peel tissue using a flame ionisation detector GC (FID-GC), and
- volatile collection and analytical techniques (static headspace, including multiple headspace extraction, dynamic headspace and solid phase microextraction) to quantify apple volatiles.
5.2.2.2 Direct GC method for \( \alpha \)-farnesene quantification

Ground apple peel (1g) was extracted in hexane (5mL, HPLC grade) for 10 min in a sonicating bath (Soniclean 120T) at room temperature. Dodecane (20 \( \mu \)g C12.mL\(^{-1}\) hexane) was added as an internal standard. The extract was injected directly into a Hewlett Packard (HP) 5890 GC with an injector temperature of 250°C. The samples were separated on a fused silica SE-30 capillary column and detected with a flame ionisation detector held at 280°C. The column was maintained at 60°C for 1 min then programmed at 10°C.min\(^{-1}\) to 200°C and held for 5 min. Data was acquired with HP 3365 series II ChemStation software. Quantification was obtained using an external standard of pure \( \alpha \)-farnesene which was kindly provided by Dr. Daryl Rowan, The Horticulture and Food Research Institute of New Zealand at Palmerston North, New Zealand.

5.2.2.3 Spectroscopic method for \( \alpha \)-farnesene quantification

Individual apples were dipped in HPLC grade hexane (100 - 300 mL) for 3 min with occasional agitation of the fruit. The UV absorption of aliquots of this extract were measured with a Cary Spectrometer. Samples were diluted to allow reliable UV measurements. \( \alpha \)-Farnesene absorbs at 232 nm \((e_{232} = 27,700)\) and conjugated trienes were measured as \( \Delta OD_{281-290} \) \((e_{281-290} = 25,000)\) (Anet, 1972a; Meir and Bramlage, 1988). These values were related to the surface area of the fruit, which was calculated by peeling the fruit and measuring the surface area with an imaging system (Delta-T; Cambridge, UK).

5.2.2.4 Comparison of \( \alpha \)-farnesene extraction and quantification methods

Ten ‘Granny Smith’ apples were removed from cold storage after 5 months storage at 0°C and after 1 week at 20°C, the fruit were used to compare \( \alpha \)-farnesene extraction and quantification procedures. The regular spectrometric method for the estimation of \( \alpha \)-farnesene was used on one half of the apple (Section 5.2.2.3), whilst the other half was used for direct \( \alpha \)-farnesene extraction and quantification after grinding the peel tissue (Section 5.2.2.2). In addition, the levels of conjugated trienes (CT) were determined (Section 5.2.2.3) and the volatiles in the headspace of the non-hexane washed apples were measured (Section 5.2.2.5). Two previously hexane washed apples were peeled and re-analysed for \( \alpha \)-farnesene. A further two previously hexane washed apples were re-washed with hexane and this wash and peel were re-analysed for \( \alpha \)-farnesene.
5.2.2.5 Static headspace

The headspace - gas chromatograph (HS-GC) system consisted of a HP 5890 GC equipped with a 19395A headspace sampler. Ground apple peel (1g) was incubated in a 10mL sealed vial at 100°C for 35 min. After equilibration, the sealed vial was pressurised for 10 sec, then allowed to vent for 30 sec during which the 3mL headspace sample loop was filled. The sample was then injected for 1 min at an injector temperature of 200°C. The samples were separated on a J&W DB1 capillary column (30m x 0.32 x 0.32μm; J&W Scientific, Rancho Cordovan, CA, USA) and detected with a flame ionisation detector set at 220°C. The column was maintained at 40°C for 5 min, then programmed to rise at 10°C.min⁻¹ to 200°C. Data were acquired with HP 3365 Series II ChemStation software. The components were identified by their mass spectra and comparison of their retention times with known standards. After determining the concentration of α-farnesene in the peel samples (Section 5.2.2.2), the other volatiles in the peel headspace were quantified using α-farnesene as the internal standard (assuming a response factor of 1).

5.2.2.6 Multiple headspace

The volatile preparation and analysis for multiple headspace extraction was conducted as per Section 5.2.2.5. However, successive injections (at least 3) were taken from the same sample vial and individually analysed.

5.2.2.7 Dynamic headspace

Tenax traps (300 mg of 60/80 mesh Tenax-TA® (Alltech Associates, Deerfield, IL, USA) were packed into 5 mm i.d. glass tube between plugs of silanised glass wool. Before use, Tenax traps were cleaned overnight by flushing with about 10 mL.min⁻¹ of nitrogen at 220°C. Volatiles were collected from air exiting from drums containing apples at 0°C. Restriction capillaries were used to regulate the flows to about 10 mL.min⁻¹. Tenax traps were attached for a measured time and a known volume of headspace was collected for analysis. Three mL of diethyl ether was washed through the Tenax trap and collected in a glass vial. The eluate was concentrated to about 400 μL by evaporation at room temperature in a gentle stream of purified nitrogen. The concentrated ether extracts were injected into a HP 5890 Series II gas chromatograph interfaced with a HP 5971A mass selective detector. The compounds were separated on a DB-1 column (J&W Scientific, Rancho Cordovan, CA, USA; 30m, 0.25mm). Operating conditions of the GC were: oven temperature 50°C for 2 min then increased to 200°C at 5°C.min⁻¹ and held for 5 min. The other instrumental parameters were the same as those in Section 5.2.2.9.
5.2.2.8 Solid phase microextraction

‘Granny Smith’ apples were individually sealed in 1L containers and ventilated with air (1L.hr$^{-1}$). Volatiles were collected on a SPME fibre (Supelco Co., USA) equipped with a poly(dimethylsiloxane) (PDMS) fibre (100 μm). The SPME fibre was introduced into the exiting air streams from the apples for up to 1 hour. Volatiles were thermally desorbed in a GC injector port at 250°C. The GC instrumentation and other parameters are the same as in Section 5.2.2.5.

5.2.2.9 Apple volatile determination - Cryo-focus GC/MS

The volatile samples (5mL) were collected from a sealed vial containing apple peel that had equilibrated for 35 min at 100°C using a gas tight glass syringe. Mass Spectra were obtained on a HP 5971A mass selective detector interfaced with a HP 5890 Series II gas chromatograph. The compounds were separated on a DB-1 column (J&W Scientific, Rancho Cordovan, CA, USA; 30m, 0.25mm). The volatiles were introduced into the injector set at 250°C. The column was initially maintained at -5°C with liquid CO$_2$ for 2 min, then programmed at 20°C.min$^{-1}$ to 40°C for 5 min. A second temperature ramp at 5°C.min$^{-1}$ to 200°C for 1 min completed the analysis. The inlet pressure was 10 psi using the split injection mode (ratio 1:30). Inlet helium pressure was 17 psi with a splitless injection mode. The detector was held at 280°C and the ion source was at 70 eV scanning the mass range m/z 40-650 every 0.7 sec. All data was collected using Mustang software (Hewlett Packard). Sample mass spectra were compared to known standards and the MS-Wiley library (John Wiley and Sons, New York).
5.2.3 Results and Discussion

5.2.3.1 Comparison of α-farnesene extraction and quantification methods

As α-farnesene is the only major hydrocarbon in apple peel, its identification and quantification using a direct GC measurement is relatively straightforward. Knee and Hatfield (1981a) used a FID-GC to quantify α-farnesene from surface ether washes, however this method of α-farnesene quantification has not been adopted by other workers. The GC method overcomes many of the problems encountered using current spectrometric methods for estimating the concentration of α-farnesene (Murray, 1969; Anet, 1972a; Meir and Bramlage, 1988). Apart from the tedious nature of the current α-farnesene isolation procedure there are inherent problems associated with the method. For example, this method relies on the UV absorption due to conjugation within α-farnesene. However, there may be other compounds in the hexane wash which absorb at 232nm that could produce significant interference. This would lead to a lack of accuracy and reproducibility. In addition, there is the perennial safety problem in handling large volumes of hexane. Whitaker et al. (1997b) has since developed a HPLC method for the quantification of α-farnesene and its major oxidation products in peel hexane washed using UV detection at 232 and 269nm.

Table 5.1 summarises of the results of a comparison of the current procedures for estimating α-farnesene in apple peel.

Table 5.1 Comparison of the extraction (hexane wash and hexane extraction of ground peel) and quantification methods (direct GC measurement and spectrometric methods) of measuring α-farnesene and its conjugated trienes (CT) in ‘Granny Smith’ apple peel. SEM = standard error of the means (n = 10)

<table>
<thead>
<tr>
<th>Direct GC Measurement</th>
<th>Spectroscopic Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-farnesene</td>
</tr>
<tr>
<td></td>
<td>(nmol.cm⁻²)</td>
</tr>
<tr>
<td>α-farnesene mean SEM</td>
<td>15,586 1,207</td>
</tr>
<tr>
<td>hexane wash</td>
<td></td>
</tr>
<tr>
<td>ground peel</td>
<td>53,872 4,359</td>
</tr>
</tbody>
</table>
This comparison of the extraction and quantification methods relies on the assumption that both halves of the apples had similar levels of \( \alpha \)-farnesene. Although Barden and Bramlage (1994a) showed that low light had no effect on the levels of \( \alpha \)-farnesene or CT in fruit peel, there have been reports that there are a considerable differences in physiology between the shady side and the exposed side of the fruit (Johnson and Andrews, 1997). However, the allocation of the halves of each fruit in this experiment was randomised.

The results showed that the direct measurement of \( \alpha \)-farnesene by GC (irrespective of the extraction method) gave significantly greater concentrations of \( \alpha \)-farnesene than those obtained by spectrometric methods. The direct GC method uses a direct measure of \( \alpha \)-farnesene in the hexane extract and an internal standard (20 \( \mu \)g dodecane.mL\(^{-1}\) hexane), where the relative standard deviation of the internal standard was 0.62%. Whereas the spectrometric methods relies on the UV absorption at 232 nm and Beer’s Law to estimate the levels of \( \alpha \)-farnesene.

Comparison of the extraction methods showed that extracts of the ground peel yielded significantly higher concentrations of \( \alpha \)-farnesene than the regular hexane wash of the apple surface. These results showed that significant concentrations of \( \alpha \)-farnesene remain in the peel, after a single hexane wash. This was further examined when hexane washed fruit were subject to a second hexane extraction. Recoveries of \( \alpha \)-farnesene from previously extracted apples are shown in Table 5.2.

A possible difficulty in the use of GC determination of \( \alpha \)-farnesene is that some \( \alpha \)-farnesene oxidation products may have the same retention time as \( \alpha \)-farnesene. However, in the development of this GC method, numerous non-polar columns and oven temperature gradients were tested and no obvious impurities were observed.
Table 5.2  Comparison of the extraction (hexane wash and hexane extraction of ground peel) and quantification methods (direct GC measurement and spectrometric methods) for measuring α-farnesene and its conjugated trienes (CT) in ‘Granny Smith’ apple peel after an initial hexane wash (3 min).

A shows the levels of α-farnesene and its CT in ground peel of fruit which had been washed once in hexane for 3 min.

B shows the levels of α-farnesene and its CT extracted from apples with a second hexane wash.

C shows the levels of α-farnesene and its CT extracted from ground peel recovered from apples in treatment B.

SEM = standard error of the means (n = 2)

<table>
<thead>
<tr>
<th></th>
<th>Direct GC Measurement</th>
<th>Spectroscopic Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-farnesene</td>
<td>α-farnesene</td>
</tr>
<tr>
<td></td>
<td>(nmol.cm⁻²)</td>
<td>(nmol.cm⁻²)</td>
</tr>
<tr>
<td></td>
<td>mean SEM</td>
<td>mean SEM</td>
</tr>
<tr>
<td>A</td>
<td>2,222 134</td>
<td>1,484 115</td>
</tr>
<tr>
<td>B</td>
<td>348 0.2</td>
<td>254 19.6</td>
</tr>
<tr>
<td>C</td>
<td>564 21.6</td>
<td>599 137</td>
</tr>
</tbody>
</table>

Although the data in Table 5.2 were derived from only two apple replicates, they clearly show that there were significant amounts of α-farnesene remaining in the peel after a 3 min hexane wash (Table 5.2A). A second hexane extraction removed significant levels of α-farnesene from the peel (Table 5.2B), but high levels still remained in the peel after a second hexane wash (Table 5.2C).
Table 5.3 shows that no further \( \alpha \)-farnesene was recovered from the ground peel after one initial hexane extraction. This extraction procedure therefore gives more reliable and consistent results than the whole apple hexane wash procedure.

<table>
<thead>
<tr>
<th>Hexane Extraction</th>
<th>( \alpha )-farnesene Concentration (( \mu )g.g(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>158</td>
</tr>
<tr>
<td>2</td>
<td>trace</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

The ground peel extraction procedure and the direct GC technique for extracting and analysing \( \alpha \)-farnesene overcomes many problems inherent in other methods and it is efficient and reliable, and can be easily automated. In addition, the current method for estimating \( \alpha \)-farnesene ignores the fact that scald develops within the hypodermis below the epidermis, while the hexane wash only removes \( \alpha \)-farnesene present in the waxy cuticle. Huelin and Coggiola (1970c) showed that up to 20\% of the total \( \alpha \)-farnesene was located within the peel tissue. Thus the GC technique described here gives an accurate measure of \( \alpha \)-farnesene in both the cuticle and peel. Although this method does extract the wax and peel tissue, it does not directly measure the non-volatile oxidation products of \( \alpha \)-farnesene, which are normally estimated by UV spectrometry (Huelin and Coggiola, 1968; Whitaker et al., 1997b). The volatiles in the apple peel, including the volatile oxidation products of \( \alpha \)-farnesene are examined in the following section.
5.2.3.2 Identification and quantification of apple volatiles

Volatile analysis using headspace - gas chromatography (HS-GC) of apple peel was examined. A static headspace system relies on analyte equilibrium being established in a sealed container. In this case, equilibration was hastened by heating. The headspace method was optimised with regard to several factors that contribute to the concentration of volatiles in the headspace. Although the apple peel was heated to 100°C for 35 min before each sampling, the volatile profiles from a range of equilibration temperatures (80 - 100°C) showed little variation in volatile composition. The higher the equilibration temperature, the greater the concentration of volatiles at equilibrium. However there were some change in the volatiles profile > 120°C.

Equilibration times up to several hours were examined, but volatile degradation occurred after long periods at high incubation temperatures. In addition, a range of sample sizes (0.1 - 5 g peel samples) and sample types (ground peel tissue of different size, whole intact peel tissue, with and without water solvent) were examined. Although larger samples gave higher concentrations of volatiles needed for GC detection, they took longer to equilibrate. A one g ground sample of peel tissue in a sealed 10 mL vial was found to be optimal. In addition, several instrumental headspace conditions were examined (eg pressurisation of the vial and vent time) to optimise volatiles transfer to the GC. A typical chromatogram of a static headspace analysis of apple peel volatiles is shown in Figure 5.3, and Table 5.4 reports the major volatiles identified and quantified from apple peel.

![Gas Chromatogram](image)

**Figure 5.3** Typical GC chromatogram of apple peel volatiles collected by static headspace, as outlined in Section 5.2.2.5
### Table 5.4  Apple peel volatiles identified and quantified using static headspace GC

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Compound name</th>
<th>RT (min)</th>
<th>std RT</th>
<th>lit ref</th>
<th>Mass Spec</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ethyl acetate</td>
<td>2.1</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>2</td>
<td>ethyl propanoate</td>
<td>2.2</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>3</td>
<td>2-methyl butanol</td>
<td>2.2</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>4</td>
<td>methyl 2-methyl butanoate</td>
<td>2.3</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>5</td>
<td>hexanal</td>
<td>2.3</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>6</td>
<td>ethyl butanoate</td>
<td>2.6</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>7</td>
<td>ethyl 2-methyl butanoate</td>
<td>2.9</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>8</td>
<td>hexenal</td>
<td>3.2</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>9</td>
<td>hexanol</td>
<td>3.4</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>10</td>
<td>6-methyl hepten-2-one</td>
<td>5.3</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>11</td>
<td>hexyl butanoate</td>
<td>11.3</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>12</td>
<td>hexyl 2-methyl butanoate</td>
<td>12.7</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>13</td>
<td>hexyl hexanoate</td>
<td>16.6</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>14</td>
<td>α-farnesene</td>
<td>18.3</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
</tbody>
</table>

**RT (min)**: Retention time (minutes) on J&W DB1 capillary column (30m x 0.32 x 0.32μm) (as per method 5.2.2.5)

**std RT**: Comparison of retention time with known standard

**lit ref**: Literature reference, i.e isolated from apple peel (Paillard, 1990)

**Mass Spec**: Mass spectrometry (Section 5.2.2.9)

This headspace method was rapid and the samples could be prepared and frozen with few artefacts, except for introduction of high levels of lipid oxidation products, particularly hexanal. Hexenal, hexanal and hexanol are found in considerably lower concentrations in whole intact fruit and are thought to be the result of lipid oxidation, via lipoxygenase (Sanz et al., 1997). Figure 5.4 shows the effect of freezing peel tissue on the volatiles profile. Volatiles were identified by comparison with standards and the use of cryo-focus GC-MS. The use of a range of compounds as internal standards for quantification was examined (e.g. octan-2-ol, and 6-methyl hepten-2-one) under a range of experimental conditions (e.g. different concentrations, solvents, emulsifiers, equilibrium temperature and time). In the static headspace system used, none of the compounds or conditions gave reproducible results. The most suitable internal standard was the already quantified α-farnesene (Section 5.2.2.2).
Figure 5.4  Comparison of the GC/MS chromatograms of volatiles from 'Lady Williams' tissue following freezing. Figure 5.4A Fresh non-frozen tissue. Figure 5.4B Frozen then thawed peel tissue showing the high concentration hexanal.
Another potentially elegant method for quantifying headspace is the use of multiple headspace extraction (MHE) GC. MHE involves dynamic gas extraction, carried out step-wise with the establishment of equilibrium conditions at each step. The concentrations of the volatiles in the headspace decrease exponentially during the series of extraction steps. The original concentration of volatiles can be accurately extrapolated using the following equation:

$$\Sigma A_n = \frac{A_1}{1 - e^{-k}}$$

where $\Sigma A_n$ is the total peak area of the original concentration, $A_1$ is the experimental value of the peak area of the first injection, and $k$ is a constant depending on both the instrumental and chemical parameters (Kolb and Ettre, 1991). MHE has been found to be very useful in determining volatile concentrations in complex matrices that may have a high water content (Maggio et al., 1991).

MHE promised to avoid hexane extraction of the peel for measuring α-farnesene, and was examined under a range of experimental conditions. Figure 5.5 shows the general principles of MHE determination of volatiles. Figure 5.5A shows the results of a series of injections from the same sealed vial containing a vapour standard sample of 6-methyl hepten-2-one. The graph shows that successive injections from the same sample resulted in a linear (log integrated peak area) decrease in concentration. The original concentration of the volatile can then be determined by extrapolation (Kolb and Ettre, 1991). However, in the case of apple peel, the decrease in α-farnesene with successive extractions was not linear (Figure 5.5B), ie the equilibrium conditions required between injections could not be satisfied. Several experimental conditions, eg incubation temperature, equilibrium time, sample size and composition, were examined in an attempt to overcome this problem, however no procedure was satisfactory. The major problem was the complex nature of the apple peel matrix, consisting of cells, sugars, cuticle, waxes and large amounts of water. Although MHE is supposed to be independent of the sample matrix (Kolb and Ettre, 1991), the large proportion of waxes in the peel may have interfered with equilibration and was probably the major reason why differences were observed from model extractions (Figure 5.5A).

A widely used dynamic headspace technique involves collecting volatiles from an airstream by the use of absorbent traps, such as Tenax. Tenax is a porous polymer based on 2,6-diphenyl-p-phenylene oxide and is an excellent trapping agent for organic compounds. Volatiles can be desorbed from the Tenax traps with solvents, such as diethyl ether (Section 5.2.2.7) or thermally
Figure 5.5  Determination of apple headspace volatile concentrations with multiple headspace extraction (MHE). Figure 5.5A 6-Methyl hepten-2-one standard in vapour phase. Figure 5.5B α-Farnesene in ‘Granny Smith’ apple peel.
(Golding *et al.*, 1999), before analysis by GC-FID or MS. Analysis of volatiles with Tenax trapping and solvent desorption was successful in determining the composition of volatiles from apples, however quantification was difficult. Quantification was attempted by the addition of an external standard, however the required removal of the ethyl ether solvent after desorption was too variable to allow accurate quantification, but was useful for qualitative comparisons. It is claimed by the manufacturers that the Tenax (Tenax-TA 60/80) used in this analysis has a low affinity for water (Alltech Associates, Deerfield IL USA), and the water vapour did not seem to interfere with Tenax trapping and desorption. Break-through of volatiles from the Tenax traps was not detected and was regularly tested by attaching Tenax traps in series.

Solid phase microextraction (SPME) is a system in which volatiles are collected from the headspace onto a polymer coated fibre. The volatiles are thermally desorbed directly into the GC injector port. SPME is a rapid qualitative procedure for the determination of volatiles and has been used for analysis of a variety of fruit volatiles (Song *et al.*, 1997b; Matich *et al.*, 1996). It has the potential to reduce the time required for sampling and works well in combination with rapid separation and detection systems. In addition, it provides complete extraction and transfer of volatiles in a complex mixture within 5 min, compared to up to 60 min for the standard purge and trap analysis (Song *et al.*, 1997b).

SPME was successful in isolating and identifying apple fruit volatiles including α-farnesene. However, there were some irregularities in equilibration and quantification, and this procedure was not further utilised in this work. The use of SPME has since been examined in the quantification of volatile production by ‘Granny Smith’ apples during cold storage by Matich *et al.* (1996). They showed that while lower molecular weight compounds equilibrate rapidly between the fruit and the fibre, quantification of higher molecular weight volatiles such as α-farnesene by SPME was hindered by the slow transport into the gaseous phase, which results in long equilibrium times and headspace depletion during sampling (Matich *et al.*, 1996). They concluded that SPME is ideal for rapid, qualitative determination of apple headspace volatiles but the slower equilibration of higher molecular weight volatiles limits its use for quantification.
5.2.4 Conclusions

α-Farnesene can be readily isolated and quantified using a hexane extraction of ground apple peel and direct quantification by GC. The measure of α-farnesene from the ground apple peel tissue includes both the waxy cuticle and the hypodermal and epidermal layers where scald occurs. This method is superior to the current spectroscopic method for quantifying α-farnesene from hexane washes of the apple surface. Apple peel volatiles were most readily quantified by analysing volatiles released from ground apple peel into a static headspace and quantified using the known concentrations of α-farnesene in the peel.
5.3 Evolution of Apple Peel Volatiles During Storage

5.3.1.1 Introduction

The participation of fruit volatiles in the development of scald was first proposed by Brooks et al. (1919), and since the isolation of farnesene in ‘Granny Smith’ peel tissue (Murray et al., 1964), the role of α-farnesene in scald development has remained unchallenged. However, there is no information on the evolution of α-farnesene and other apple volatiles during cold storage of the scald sensitive ‘Lady Williams’, and the scald resistant ‘Crofton’ that would enable comparative studies with the highly susceptible, ‘Granny Smith’.

5.3.1.2 Aims of experiment

Three varieties of apples were chosen for this study: the standard green coloured peel and scald susceptible ‘Granny Smith’, a red coloured skin and susceptible variety, ‘Lady Williams’ and ‘Crofton, a variety which is a resistant to scald development and has striped peel. These varieties provided a range of scald susceptibilities for the examination of the role of volatiles in the development of scald. Several treatments were included to both enhance and inhibit scald development. A low oxygen stress treatment (< 0.5% O₂) was included to promote scald development. Dilley et al. (1963) showed that the severity of scald symptoms was proportional to the duration of anaerobiosis (N₂ storage). Treatments to inhibit scald included application of DPA and pre-storage heating. Application of DPA immediately after harvest to scald susceptible varieties is well known to inhibit scald development (Smock, 1961). Klein and Lurie (1990) and Lurie et al. (1990) showed that a pre-storage heat treatment of 38°C for four days inhibited scald development in ‘Granny Smith’ apples.

The objective of this set of experiments was to re-examine the role of volatiles in the development of scald. Specific aims were:

- examine the changes in volatiles composition in the peel of scald susceptible (‘Granny Smith’, and ‘Lady Williams’) and scald resistant (‘Crofton’) apples during air storage at 0°C,
- examine the role of pre-storage treatments comprising application of DPA, a low oxygen storage atmosphere, and heat treatment on the changes in peel volatiles during storage and during the development of scald, and
- examine the production of volatiles by scalded and non-scalded peel tissue from the same fruit.
5.3.2 Materials and Methods

5.3.2.1 Evolution of apple volatiles during storage

‘Granny Smith’ and ‘Crofton’ apples were harvested from a commercial orchard at Orange, NSW. ‘Lady Williams’ apples were harvested in both May and June at Harcourt Vic, and transported overnight to UWSH (1,000 km).

Upon arrival at UWSH, ‘Granny Smith’ and ‘Lady Williams’ were dipped in DPA at 3,000 mg.L\(^{-1}\) (‘Shield-Brite®’, Washington USA). Another pre-storage treatment included applying an ultra low oxygen (ULO) atmospheric stress, where ‘Granny Smith’ and ‘Lady Williams’ apples were subject to a <0.5% O\(_2\) atmosphere for 2 days at 0°C. The ULO treatment comprised of sealed 50L steel drums which were ventilated continuously with <0.5% O\(_2\) at a rate of 1 L.min\(^{-1}\). The ULO was created by mixing metered flows of air and nitrogen. Nitrogen was generated with a Permea Air Separator (Model CPA-2, Monsanto, USA). Other treatments included a pre-storage heat treatment (4 days at 38°C) to both ‘Granny Smith’ and ‘Lady Williams’ apples. Apples were packed in boxes and treated in 38°C incubator for 4 days. Control fruit were not given any pre-storage treatments.

After the application of each treatment, four replicates of each variety and treatment were stored in plastic lined boxes in air at 0°C for up to nine months. Each replicate consisted of 25 fruit. At monthly intervals ten apples from each replicate were removed from storage, allowed to warm to room temperature (20°C). A representative sample of the peel (50g) was carefully removed with an apple peeler and excess parenchyma tissue was scrapped from the peel tissue before the peel was frozen and ground in liquid nitrogen then stored at -75°C for later analysis. \(\alpha\)-Farnesene and other apple peel volatiles from the frozen tissue were extracted and quantified using the methods outlined in Sections 5.2.2.2 and 5.2.2.5. Each replicate of 25 apples were removed from each treatment at 3, 6 and 9 months and assessed for scald development after 7 days at 20°C (Section 5.2.1.7).

The absorbance of 6-methyl hepten-2-one of known concentrations in hexane was measured using a Carey Spectrometer. The extinction coefficient (\(\varepsilon\)) was calculated with Beers Law (Bladon, 1964)

5.3.2.2 Volatiles profile in scalded peel tissue

The volatiles in scalded and non-scalded peel tissue from the same ‘Granny Smith’ and ‘Lady Williams’ apples were compared to non-scalded ‘Crofton’ peel tissue. All apples were stored in
air at 0°C for six months with 1 week at 20°C and a representative sample (>4 apples) were prepared for volatile analysis. Volatiles were measured by headspace analysis (Section 5.2.2.5) and determined with cryo-focus GC-MS (Section 5.2.2.9).

5.3.3 Results

5.3.3.1 Maturity indices at harvest

The results of the maturity tests at harvest of the three varieties of apples used in the experiment are shown in Table 5.5. The results indicate that all apples were suitable for long term storage. Although the ‘Granny Smith’ apples were still considered preclimacteric (<0.1 mL.L⁻¹) (Dilley et al., 1989), the conversion of starch to sugar was advanced (Stage 4, half of cortex is sugar) indicating the ‘Granny Smith’ apples were physiologically more advanced.

Table 5.5 Maturity indices of ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apples. Standard error of the means are shown in brackets (n = 15)

<table>
<thead>
<tr>
<th></th>
<th>‘Granny Smith’</th>
<th>‘Lady Williams’</th>
<th>‘Crofton’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>May harvest</td>
<td>June harvest</td>
<td></td>
</tr>
<tr>
<td>internal ethylene (µL.L⁻¹)</td>
<td>0.08 (0.03)</td>
<td>0.03 (0.006)</td>
<td>0.65 (0.03)</td>
</tr>
<tr>
<td>flesh firmness (N)</td>
<td>73.4 (1.34)</td>
<td>108.3 (1.8)</td>
<td>108.7 (1.7)</td>
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<tr>
<td>SSC (%)</td>
<td>11.4 (0.16)</td>
<td>12.9 (0.15)</td>
<td>14.4 (0.08)</td>
</tr>
<tr>
<td>starch index*</td>
<td>4.1 (0.3)</td>
<td>0 (0)</td>
<td>1.5 (0.4)</td>
</tr>
</tbody>
</table>

* starch index 0 = full starch; 8 = no starch

5.3.3.2 Scald development

Table 5.6 summarises the development of scald in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apples during air storage at 0°C, and shows that scald did not develop in fruit treated with DPA or in ‘Crofton’ fruit. Scald developed on control (non-treated fruit) of ‘Granny Smith’ and ‘Lady Williams’ apples after six months air storage at 0°C (Figure 5.6). Pre-storage heat treatment and ULO stress did not significantly affect the development of scald. The pre-storage heat treatment (38°C for four days) was unsatisfactory, as the fruit became shrivelled from the heat treatment. The apples were treated in boxes in an incubator at 38°C, where the RH was very low, resulting in excessive water loss from the fruit. The levels of scald in both the heat treatment and ULO are not presented or referred to in any other section as these treatments were not successful and further measurements were not significantly different from the control.
Figure 5.6  Incidence of scald on ‘Granny Smith’ (A and B), ‘Lady Williams’ harvested early (May) (C and D), and late (June) (E and F) and ‘Crofton’ apples after six months storage at 0°C and one week at 20°C. Apples were either not treated (A, C, E and G), or treated (B, D and F) with DPA before storage.
Table 5.6  Development of scald in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apples during the storage period in fruit treated with and without a pre-storage DPA dip. ‘Lady Williams’ apples were harvested early and late. Apples were subjectively assessed for scald at 3, 6 and 9 months after the fruit had been held at 20°C for seven days after removal from storage

<table>
<thead>
<tr>
<th>Time (months)</th>
<th>Granny Smith</th>
<th>Lady Williams</th>
<th>Crofton</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>early harvest control</td>
<td>late harvest control</td>
</tr>
<tr>
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<td>DPA</td>
<td>DPA</td>
<td>DPA</td>
</tr>
<tr>
<td>3</td>
<td>0.1a</td>
<td>0.3a</td>
<td>0a</td>
</tr>
<tr>
<td>6</td>
<td>0.9b</td>
<td>3.0cd</td>
<td>0.6b</td>
</tr>
<tr>
<td>9</td>
<td>2.7c</td>
<td>3.3d</td>
<td>1.0c</td>
</tr>
</tbody>
</table>

A scald score of 0 = no scald; 5 = very severe scald
Different letters indicate statistically significant differences (P<0.05)

### 5.3.3.3 α-Farnesene production

The concentrations of α-farnesene in ‘Granny Smith’ (Figure 5.7) and early harvest ‘Lady Williams’ (Figure 5.9) peel tissue were generally greater than in the later harvest ‘Lady Williams’ (Figure 5.10) and ‘Crofton’ (Figure 5.8). There were high levels of α-farnesene (91 μg.g⁻¹) in the later harvested ‘Lady Williams’ apples and the concentrations of α-farnesene increased in all varieties and treatments during the first few months of storage in all apple varieties. The concentration of α-farnesene in ‘Lady Williams’ peel peaked during the first two months of storage at 0°C in air, whilst in ‘Granny Smith’ and ‘Crofton’ peel tissue, α-farnesene levels peaked later, at 3 - 5 months after harvest.

A pre-storage application of DPA to ‘Granny Smith’ apples significantly lowered the levels of α-farnesene during the early stages of storage. However in ‘Lady Williams’, this difference was less obvious, and in late harvested ‘Lady Williams’ the levels of α-farnesene in DPA treated fruit were higher than in control fruit.
Figure 5.7  α-Farnesene concentrations in the peel of ‘Granny Smith’ apples stored in air at 0°C. Bars show the standard errors of the means (SEM, n = 4; when absent SEM bars fall within the dimensions of the symbol)

Figure 5.8  α-Farnesene concentrations in the peel of ‘Crofton’ apples stored in air at 0°C. Bars show the standard errors of the means (SEM, n = 4; when absent SEM bars fall within the dimensions of the symbol)
Figure 5.9  $\alpha$-Farnesene concentrations in the peel of ‘Lady Williams’ apples harvested in May and stored in air at 0°C. Bars show the standard errors of the means (SEM, $n = 4$; when absent SEM bars fall within the dimensions of the symbol)

Figure 5.10  $\alpha$-Farnesene concentrations in the peel of ‘Lady Williams’ apples harvested in June and stored in air at 0°C. Bars show the standard errors of the means (SEM, $n = 4$; when absent SEM bars fall within the dimensions of the symbol)
Figures 5.11 - 5.23 show the evolution of representative apple volatiles during 9 months storage of ‘Lady Williams’, ‘Granny Smith’ and ‘Crofton’ apples in air at 0°C. The concentrations of volatiles were often low and variable.

The results show that there were differences in volatile concentrations among the varieties during storage. The scald tolerant ‘Crofton’ had lower concentrations of most volatiles but this difference was greatest in the concentrations of hexenal, 6-methyl hepten-2-one and hexyl hexanoate (Figures 5.18, 5.20 and 5.23). The concentration of volatiles in ‘Lady Williams’ peel during storage were generally greater than in the other apple cultivars, although the concentrations of hexenal in ‘Granny Smith’ were high (Figure 5.18). Ethyl acetate was an exception to these generalisations, where high concentrations were detected in ‘Crofton’ and late harvested ‘Lady Williams’ apples (Figure 5.11).

In most cases the pre-storage application of DPA resulted in lower concentrations of volatiles. The concentrations of ethyl acetate, ethyl propanoate, hexanal, hexenal, hexanol, 6-methyl hepten-2-one, hexyl butanoate, hexyl 3-methyl butanoate and hexyl 2-methyl butanoate (Figures 5.11-12, 5.15, 5.18-22) were generally suppressed by the pre-storage application of DPA.

There were high concentrations of ethyl acetate in the peel of both ‘Crofton’ and late harvested ‘Lady Williams’ (Figure 5.11). Ethyl acetate remained relatively constant in ‘Crofton’, whilst in late harvested ‘Lady Williams’ the concentration of ethyl acetate increased from harvest until very late into the storage period. Control fruit generally had higher concentrations of ethyl acetate than DPA treated fruit. The levels of ethyl acetate in both ‘Granny Smith’ and early harvested ‘Lady Williams’ were relatively low and variable compared to ‘Crofton’ and late harvested ‘Lady Williams’.

The concentration of ethyl propanoate in the peel was relatively low in all apple varieties, however, ‘Lady Williams’ generally had greater levels than either ‘Granny Smith’ or ‘Crofton’ (Figure 5.12). The levels of ethyl propanoate were generally greater in control fruit than in DPA treated fruit.

The concentrations of 2-methyl butanol and methyl 2-methyl butanoate in the all apple varieties were very low and inconsistent (Figure 5.13 and 5.14), however their concentrations tended to
Figure 5.11  Concentration of ethyl acetate (μg.g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 μg.g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.12  Concentration of ethyl propanoate (µg.g fresh weight⁻¹) in the peel of
‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early
(May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000
µg.g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady
Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.13  Concentration of 2-methyl butanol (µg g⁻¹ fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 µg g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.14  Concentration of methyl 2-methyl butanoate (μg.g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 μg.g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
increase after about two months storage in ‘Granny Smith’ and early harvested ‘Lady Williams’ apples. The levels of methyl 2-methyl butanoate in late harvested ‘Lady Williams’ gave variable results, with higher levels of methyl 2-methyl butanoate at harvest than during storage (Figure 5.14).

The concentrations of hexanal in ‘Lady Williams’ peel tissue increased throughout the storage period, with late harvested ‘Lady Williams’ producing more hexanal than earlier harvested fruit (Figure 5.15C and 5.15D). The level of hexanal in ‘Crofton’ was low, but peaked at 3 months storage, whilst apart from a few outliers, the levels of hexanal in ‘Granny Smith’ was very low.

The concentrations of ethyl butanoate were relatively low, but generally increased during the storage period (Figure 5.16). The differences in concentration of ethyl butanoate between DPA treated and control fruit were not large. The concentrations of ethyl 2-methyl butanoate in the peel of ‘Lady Williams’ and ‘Granny Smith’ were higher than in ‘Crofton’ peel (Figure 5.17). The levels of ethyl 2-methyl butanoate generally increased from harvest, and the differences between control and DPA treated fruit were not great. Large concentrations of ethyl 2-methyl butanoate were found in some samples of control ‘Granny Smith’ peel tissue, but these measurements had very large standard errors.

The concentrations of hexenal in the peel of ‘Granny Smith’ and early harvested ‘Lady Williams’ peel were greater than in later harvested ‘Lady Williams’ and ‘Crofton’ apples (Figure 5.18). There were fewer differences in the concentration of hexenal between DPA and control fruit, but DPA generally suppressed the concentration of hexenal. The concentrations of hexenal tended to decline late in storage, except in late harvested ‘Lady Williams, where they remained relatively constant during storage.

The concentration of hexanol in the peel of ‘Lady Williams’ apples tended to increase during storage, with higher levels of hexanol detected late in storage (Figure 5.19). There were few differences between DPA and control fruit, however when there were differences in concentrations of hexanol, control fruit tended to have higher levels of hexanol than DPA treated fruit. The concentrations of hexanol in ‘Crofton’ and ‘Granny Smith’ were low and showed no trends in production.

High concentrations of 6-methyl hepten-2-one were found in control ‘Granny Smith’ and ‘Lady Williams’ but no 6-methyl hepten-2-one was detected in DPA treated ‘Granny Smith’ fruit and
Figure 5.15  Concentration of hexanal (μg.g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 μg.g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.16  Concentration of ethyl butanoate (µg.g fresh weight⁻¹) in the peel of 'Granny Smith' (A), 'Crofton' (B) and 'Lady Williams' apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 µg.g⁻¹) was applied as a pre-storage treatment in 'Granny Smith' and 'Lady Williams' apples. The bars indicate standard errors of the means (n=4)
Figure 5.17  Concentration of ethyl 2-methyl butanoate (µg g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 µg g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.18  Concentration of hexenal (μg.g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 μg.g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.19  Concentration of hexanol (μg.g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 μg.g⁻¹) was applied at a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.20  Concentration of 6-methyl hepten-2-one (µg·g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 µg·g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
only low concentrations were measured in DPA treated ‘Lady Williams’ fruit (Figure 5.20). Only low levels (<1 μg.g⁻¹) of 6-methyl hepten-2-one were detected in ‘Crofton’ fruit. 6-Methyl hepten-2-one did not begin to accumulate in ‘Lady Williams’ and ‘Granny Smith’ apples until after about 2 months in storage, although some 6-methyl hepten-2-one was detected at harvest in the late harvested ‘Lady Williams’ apples.

The patterns of accumulation of hexyl butanoate in ‘Lady Williams’ not treated with DPA from both harvests were similar to those observed for 6-methyl hepten-2-one, however a pre-storage DPA treatment had less effects (Figure 5.21). Hexyl butanoate was not measurable in ‘Granny Smith’ fruit until after 7 months storage and only trace amounts were detected in ‘Crofton’.

Like hexyl butanoate, appreciable concentrations of hexyl 2-methyl butanoate were found in ‘Lady Williams’ apples (Figure 5.22). Meanwhile concentrations of hexyl 2-methyl butanoate were only found late in ‘Granny Smith’ storage and only traces were detected in ‘Crofton’. When differences between DPA and control fruit were present, DPA treated fruit had lower concentrations of hexyl butanoate than control fruit. There were no differences in hexyl butanoate between early harvest or later harvested ‘Lady Williams’ apples. As with other hexanoate derivatives, concentrations of hexyl hexanoate in the peel of ‘Lady Williams’ were much higher than in ‘Granny Smith’ or ‘Crofton’ apples (Figures 5.23). Hexyl hexanoate began to accumulate in the early stages of storage and DPA treatment had no significant effect on the concentrations of hexyl hexanoate, or the pattern of accumulation. Appreciable concentrations of hexyl hexanoate began to accumulate in ‘Granny Smith’ apples after 7 months storage.

5.3.3.5 Volatiles in scalded peel tissue
A comparison of the volatiles evolved by DPA treated, non-scalded and scalded ‘Granny Smith’ peel tissue is shown in Figure 5.24. The relative amounts of volatiles from a range of apple peel sources is summarised in Table 5.7. The major volatiles in the aroma profile of the apples examined were ethyl acetate, hexanal, 6-methyl hepten-2-one and α-farnesene. Initial comparisons between scalded and non-scalded peel tissue from the same ‘Granny Smith’ apple showed that there were significant differences in the volatile profiles. The most significant volatile in scalded peel tissue is 6-methyl hepten-2-one (Figure 5.25C). This supports the data from Figure 5.20, where scalded control ‘Granny Smith’ apples had significantly more 6-methyl hepten-2-one than DPA treated or ‘Crofton’ fruit.
Figure 5.21  Concentration of hexyl butanoate (μg.g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 μg.g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.22  Concentration of hexyl 2-methyl butanoate (μg g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 μg·g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.23  Concentration of hexyl hexanoate (µg·g fresh weight⁻¹) in the peel of ‘Granny Smith’ (A), ‘Crofton’ (B) and ‘Lady Williams’ apples harvested early (May) (C) and late (June) (D) during nine months air storage at 0°C. DPA (3,000 µg·g⁻¹) was applied as a pre-storage treatment in ‘Granny Smith’ and ‘Lady Williams’ apples. The bars indicate standard errors of the means (n=4)
Figure 5.24  Comparison of the GC/MS chromatograms of volatiles from ‘Granny Smith’ peel tissue after 6 months storage.

Figure 5.24A DPA treated apple. Figure 5.24B Non-scallded peel.

Figure 5.25C Scalded peel showing the high concentration of 6 methyl hepten-2-one
Table 5.7  Relative amounts of volatiles in the peel of ‘Granny Smith’,
‘Lady Williams’ and ‘Crofton’ apples after 6 months air storage at 0°C

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<th>peak</th>
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<th>scald</th>
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- no peak detected
+ + trace, not integrated
++ integrated peak
+++ large peak
1 = largest peak
2 = second largest peak
3 = third largest peak
Figure 5.25  Comparison of the GC/MS chromatograms of volatiles from
‘Lady Williams’ peel tissue after 6 months storage.
Figure 5.24A Non-scalded peel. Figure 5.25B Scalded peel showing
the high concentration of 6 methyl hepten-2-one
A comparison of ‘Lady Williams’ scalded and non-scalded tissue is shown in Figure 5.25, while the levels of α-farnesene and 6-methyl hepten-2-one are shown in Table 5.8. The results show that there was significantly more α-farnesene than 6-methyl hepten-2-one in non-scalded peel tissue, whilst in scalded peel tissue there is significantly more 6-methyl hepten-2-one than α-farnesene.

Table 5.8 Comparison of α-farnesene and 6-methyl hepten-2-one levels in scalded and non-scalded ‘Lady Williams’ peel tissue from the same apple

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α-Farnesene was detected in other mature apple tissues such as the fleshy parenchyma and core, but was not detected in either apple flowers, leaves, nor in the peel, flesh or core of immature fruitlets harvested 4 months before physiological maturity.

5.3.4 Discussion

Besides ethylene, α-farnesene is the major volatile of stored apples with levels up to 500 μg.g⁻¹ peel fresh weight. Since it was first isolated from ‘Granny Smith’ and ‘Crofton’ apples in 1966, there have been numerous studies describing the relationship between α-farnesene and scald but these have been limited to only a few apple varieties, such as the scald susceptible, ‘Cortland’ and ‘Granny Smith’ (Huelin and Murray, 1966; Huelin and Coggiola, 1968; Anet and Coggiola, 1974a; Meir and Bramlage, 1988; Chen et al., 1990b; Gallerani and Pratella, 1992; Du and Bramlage, 1993). Only a few of these studies examined the concentration of α-farnesene during long-term storage (9 months) in scald susceptible varieties. The data reported in this thesis show that the scald susceptible ‘Granny Smith’ had much more α-farnesene than the scald resistant, ‘Crofton’ apple during 9 months storage at 0°C in air and a pre-storage application of DPA lowered the concentration of α-farnesene in the peel of ‘Granny Smith’ apples. In the other scald susceptible variety, ‘Lady Williams’, the earlier harvested fruit, which developed more scald had higher concentrations of α-farnesene than later harvested fruit. These observations indicate a direct relationship between scald and α-farnesene. However, the later harvested ‘Lady Williams’ fruit (ie more mature), had higher levels of α-farnesene at harvest (91 μg.g⁻¹ peel fresh weight), but did not develop severe scald symptoms. The picture is further confounded by the
observation that the later harvested 'Lady Williams' fruit treated with DPA had higher levels of α-farnesene than non-treated fruit and still did not develop scald. Thus, there appears not to be a direct relationship between α-farnesene and the occurrence of scald (Anet, 1972a; Chen et al., 1990b; Gallerani and Pratella, 1992; Du and Bramlage, 1993), rather it is believed that the oxidation products α-farnesene are more closely related to scald (Huelin and Coggiola, 1968; Anet, 1972a; Gallerani and Pratella, 1992; Du and Bramlage, 1993).

The structure of α-farnesene renders it very susceptible to free radical attack (Kaur and Perkins, 1991), and the rapid auto-oxidation of α-farnesene has been extensively studied (Anet, 1969; Coggiola, 1969; Filmer and Meigh, 1971; Anet, 1972a; Stanley et al., 1986; Spicer et al., 1993; Rowan et al., 1995). α-Farnesene can be attacked by free radicals in two ways; by abstraction or removal of a hydrogen atom or by addition of the radical to one of the unsaturated bonds (Anet, 1969). The central CH₂ in this diene unit undergoes hydrogen abstraction and the resulting free radical reacts with molecular oxygen to form the peroxy radical (ROO•). The peroxy radical can then rapidly undergo numerous reactions (Kaur and Perkins, 1991). Figure 5.26 shows the major oxidation pathways of α-farnesene. The initial monomer products are conjugated trienes (CT), a conjugated trienol (Figure 5.26, Compound II) and a conjugated triene endoperoxide (Figure 5.26, Compound III), which were isolated after reduction of the intermediate hydroperoxides (Anet, 1969) (Figure 5.26). The characteristic UV absorption spectra of these CT (λ max 259, 269 and 281 nm) are used to estimate CT concentrations in hexane washes from the skin of stored apples (Anet, 1972a).

It is not known why α-farnesene is so stable in vivo, since it is very labile to autooxidation in vitro (Anet, 1969), but endogenous antioxidants may slow α-farnesene oxidation (Anet, 1974a). Correlations between scald development and CT abound in the scald literature (Huelin and Coggiola, 1968, 1970a, 1970b, Anet and Coggiola, 1974a; Meir and Bramlage, 1988; Chen et al., 1990b; Gallerani and Pratella, 1992; Du and Bramlage, 1993). However, inconsistencies are commonly observed (Meir and Bramlage, 1988; Du and Bramlage, 1993). Du and Bramlage (1993, 1994) suggest that if CTs are associated with scald development, the metabolites / catabolites of CT281 are involved more directly in cellular disruption than CT281 itself, whilst CT258 seems to have a protective effect. They suggest several alternative sequences of α-farnesene oxidation via CT and unknown compounds, factors or conditions that lead to scald symptoms. All of these studies have used UV absorbance measurements from hexane washes of the fruit surface and are subject to inherent difficulties (Section 5.2.3.1) and therefore, unlikely to shed any further knowledge on the causes on scald.
Figure 5.26  Auto-oxidation of α-farnesene (I), showing some of the major oxidation products:

II  conjugated trienol [2,6,10-trimethyl dodeca-2,7(E),9(E),11-tetraen-6-ol],

III conjugated endoperoxide [α,α,6-trimethyl-6-(4-methylhexa-1,3,5-trienyl)-1,2-dioxan-3-methanol],

IV 6-methyl hept-5-en-2-one

(Adapted from Anet, 1969)
Rowan et al. (1995) recently showed that one of the CT oxidation products of $\alpha$-farnesene, 2,6,10- trimethyl dodeca- 2,7(E),9(E),11- tetraen-6-ol, was the major CT (88 - 95% of total) present in the apple skin by HPLC, but accounted for only 12 - 35% of the CT concentration of the apple skin washes as measured by UV spectroscopy. This major over-estimate of CT can have implications in interpretation of CT data and the measurement of trienes with HPLC should provide a more accurate measure of CT concentrations in the peel, particularly at the lower CT concentrations found early in scald development (Rowan et al., 1995). It is important to realise that a significant proportion of total $\alpha$-farnesene is located in the waxy cuticle and not the epidermal and hypodermal layers where scald occurs (Table 5.1). The role of this reservoir of $\alpha$-farnesene has yet to be elucidated. $\alpha$-Farnesene has been synthesised with radiolabels on the C1 and C4 carbons (Fielder et al., 1993), which should assist in elucidating $\alpha$-farnesene metabolism. Indeed Spicer et al. (1993) and Brimble et al. (1994a, b) have since characterised and synthesised a range of $\alpha$-farnesene oxidation products (allylic alcohols, diols, epoxides and bisepoxides) which are being studied in relation to scald development (Daryl Rowan, pers. comm.).

Filmer and Meigh (1971) and Anet (1972a) confirmed that 6-methyl hepten-2-one was the major product of $\alpha$-farnesene auto-oxidation (Figure 5.26, Compound IV). In addition, other simple and higher molecular weight carbonyl volatile compounds, such as acetone and 3 buten-2-one are also formed from $\alpha$-farnesene auto-oxidation (Table 5.9). Table 5.7 shows that when these compounds are detected, and when there are differences between scalded and non-scalded peel tissue, the volatile oxidation products of $\alpha$-farnesene were in higher concentrations in scalded tissue than in non-scalded or DPA treated fruit.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Identification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetone</td>
<td>GC/MS</td>
<td>Filmer and Meigh (1971)</td>
</tr>
<tr>
<td>2-methyl propenal</td>
<td>MS</td>
<td>Stanley et al. (1986)</td>
</tr>
<tr>
<td>2,3-epoxy-2-methyl pentane</td>
<td>MS</td>
<td>Stanley et al. (1986)</td>
</tr>
<tr>
<td>3-buten-2-one</td>
<td>MS</td>
<td>Stanley et al. (1986)</td>
</tr>
<tr>
<td>2-methyl but-3-en-2-ol</td>
<td>GC/MS</td>
<td>Filmer and Meigh (1971)</td>
</tr>
<tr>
<td>1,3,3-trimethyl-2,7-dioxabicyclo(2,2,1) heptane</td>
<td>GC/MS</td>
<td>Stanley et al. (1986)</td>
</tr>
<tr>
<td>2-methyl but-2-enal</td>
<td>MS</td>
<td>Stanley et al. (1986)</td>
</tr>
<tr>
<td>6-methyl hept-5-en-2-one</td>
<td>GC/MS</td>
<td>Anet (1972a)</td>
</tr>
</tbody>
</table>
The appearance of 6-methyl hepten-2-one in scalded tissue and non-DPA treated ‘Lady Williams’ and ‘Granny Smith’ apples (Table 5.7 and Figure 5.20), implies a high level of α-farnesene oxidation in scalding peel tissue. Conversely, the absence or low concentrations of 6-methyl hepten-2-one in the scald resistant ‘Crofton’ and DPA treated fruit, even though there are relatively high concentrations of α-farnesene (up to 350 µg g⁻¹ peel fresh weight), suggests an inhibition of α-farnesene oxidation and that this is related to scald development. The possible nature of the inhibitors of α-farnesene oxidation is discussed in Chapter 6. However, the relationship between the oxidation of α-farnesene, or CT and scald may not be as direct as initially indicated (Huelin and Coggiola, 1970), but may be an indicator of general cell oxidative stress that results in scald symptoms.

The presence of the volatile, 6-methyl hepten-2-one as a significant oxidation product of α-farnesene has often been missed in the literature due to the almost universal use of UV absorbance measurement of α-farnesene and its oxidation products (Du and Bramlage, 1993; Whitaker et al., 1997b). However, even where volatile analysis has been conducted on scalded and non-scalded fruit, 6-methyl hepten-2-one was not reported (Paliyath et al., 1997). The absence of 6-methyl hepten-2-one in these experiments may have been due to the method of volatile analysis used. The volatile collection technique used by Paliyath et al. (1997) discriminates against large molecular weight volatiles (such as α-farnesene) and favours the low molecular weight volatiles (Matich et al., 1996). 6-Methyl hepten-2-one is slightly lipophilic and would normally be differentially partitioned into the waxy cuticle. In the headspace methods used in these experiments the heating of the peel sample would encourage 6-methyl hepten-2-one into the volatile phase.

A significant shoulder in the UV spectrum of 6-methyl hepten-2-one occurs at 281 nm (Figure 5.27), and this may interfere with the determination of CT281 concentration. The extinction coefficient (ε) of CT281 (A₂₈₁ - A₂₉₀) = 25,000 (Anet, 1972a), whilst the ε for isolated carbonyl groups is generally around 10 - 50 (Bladon, 1964) and the ε₂₈₁ of 6-methyl hepten-2-one was measured to be just 28. This shows that the intensity of absorption of 6-methyl hepten-2-one is very low, in comparison to that reported for CT281 (Anet, 1972a), therefore the presence of 6-methyl hepten-2-one in a complex mixture of compounds in the apple hexane wash would not significantly contribute to the UV absorption at 281 nm.
Figure 5.27 UV spectra of 6-methyl hepten-2-one in hexane solvent showing a significant shoulder at $\lambda = 280$nm

Apart from $\alpha$-farnesene, the major components of apple aroma comprise esters. These are synthesised by the esterification of the respective acid and alcohol moieties which are themselves derived from amino acids and fatty acids (Sanz et al., 1997), whereas $\alpha$-farnesene is derived via glycolysis and isopentenyl diphosphate and its biosynthesis is discussed in some detail in Section 5.5. However, it is important to recognise that the biosynthetic pathways are significantly different and do not have a common derivation, except at the primary metabolic level. Therefore, it is not surprising that $\alpha$-farnesene and the other volatiles behave differently.

The branched chain acid moieties are thought to be derived from amino acids, whilst the straight chain moieties which predominate in the apple volatile profile are derived from the straight chain fatty acids which are catabolised through two main oxidative pathways, $\beta$-oxidation and lipoxygenase mediated degradation (Sanz et al., 1997). $\beta$-Oxidation involves the catabolism of fatty acids (acyl-CoA derivatives) to shorter chain acyl-CoAs by losing two carbons at every round of the oxidation cycle. The resulting acyl-CoAs are esterified by alcohol acyltransferase to produce esters. Bartley et al. (1985) showed there was an active $\beta$-oxidation pathway in stored ‘Cox’s Orange Pippin’ apples which significantly contributed to ester synthesis. Paillard (1979)
further demonstrated that varietal differences in the aroma composition (acetate and butanoate esters) depended on the last stage of the β-oxidation of fatty acids, ie the transformation of the butanoate into acetate, particularly in ‘Golden Delicious’ apples.

The lipoxygenase (LOX) pathway is activated by the disruption of the cell integrity and produces a wide variety of C₄ and C₃ volatile compounds (Schreier, 1984). LOX (EC 1.13.11.12) catalyses the production of hydroperoxides from polyunsaturated fatty acids and are highly substrate specific for the Z, Z-1,4-pentadiene structure (Siedow, 1991). Hexanal and trans-hex-2-enal are the principal products of LOX from polyunsaturated fatty acids in apples, with lesser amounts of hexanol, trans- hex-2-en-1-ol, cis- hex-3-enal and cis- hex-3-en-1-ol (Flath et al., 1967; Schreier, 1984). LOX is located in the cytoplasm, mitochondria and chloroplasts (O’Connor and O’Brien, 1991), whilst the polyunsaturated acids in apples are mainly present in the lamellar lipids (Galliard, 1968). Meigh and Hulme (1965) showed LOX activity increases dramatically during ripening and membranes become more permeable to different substrates allowing LOX to initiate volatiles production (Sanz et al., 1997). Brackmann et al. (1993) suggested that LOX is a more likely pathway for fatty acid metabolism in stored ‘Golden Delicious’ apples than β-oxidation. As scald is thought to be caused by membrane disruption (Bain and Mercer, 1963), it would be expected that the fatty acid metabolism (β-oxidation and LOX) would increase in scalded tissue. To some extent this is shown in Figures 5.13, 5.18 and 5.19 and Table 5.7, but the results shown in Table 5.7 are not strictly quantitative.

A pre-storage application of DPA has been shown to have numerous other postharvest effects (Lurie et al., 1989a), which may significantly alter normal volatile production. For example, DPA has been shown to lower LOX activity by 50% in treated ‘Granny Smith’ peel tissue after 6 months in air at 0°C (Lurie et al., 1989a). As LOX oxidation is in-part free radical mediated (Whitaker, 1991), the addition of the DPA, a free radical scavenger and antioxidant (Kaur and Perkins, 1991), may interfere with this pathway. The significantly lower levels of LOX activity in DPA treated fruit would be expected to affect volatiles production, particularly to lower levels of LOX related volatiles, eg C₄ based volatiles. Indeed, when there were differences in the levels of hexanal, hexenal and hexanol, between control and DPA treated fruit, the DPA treated fruit generally had lower levels of volatiles (Figures 5.15, 5.18, 5.19). The origins of the other C₄ esters eg hexyl -butanoate, -2-methyl butanoate, -hexanoate, (Figures 5.21, 5.22, 5.23) show similar trends, but the possibility of the β-oxidation of fatty acids providing alternative pathway for the production C₄ skeletons, means the results are not clear cut.
If hexanal, hexenal and hexanol are a measure of LOX activity, the generally lower levels of these volatiles in ‘Crofton’ apples, suggests that lower LOX activity (whether by suppression by DPA or varietal) may be related to resistance to scald. Feys et al. (1980) showed that LOX activity was higher in the peel than the cortex of ‘Schone van Boskoop’ apples and hypothesised that LOX oxidation of polyunsaturated fatty acids is involved in scald induction. Although no other evidence was presented, a role for LOX may be possible. Marcelle (1989) showed that LOX activity significantly increased after transfer from cool storage to 20°C, perhaps reflecting the significant changes that occur during the expression of scald. Interestingly LOX catalyses the first step of jasmonic acid biosynthesis (Mueller, 1997). Jasmonic acid is receiving more attention as a possible phytohormone that is involved in numerous fruit responses, including apple fruit development, ripening and senescence (Olias et al., 1992, Fan et al., 1997a and b). Alternatively, DPA could directly inhibit β-oxidation of fatty acids, either by direct antioxidant action on the polyunsaturated fatty acids, or via the inhibition of the enzymes involved in β-oxidation, such as acyl-CoA dehydrogenase or acetyl-CoA acetyltransferase.

The observation that the pre-storage application of DPA generally suppresses volatiles production can be rationalised on the basis of evidence from Song and Bangerth (1996) who suggested that a general non-specific increase in metabolic activity is a prerequisite for the stimulation of aroma production in ‘Golden Delicious’ apples. In addition, Song et al. (1997d) postulated that precursor supply was a factor that limits ester production in apples. It is well known that the pre-storage application of DPA significantly reduces respiration, as measured by CO₂ production and delays senescence in stored apples (Lurie et al., 1989a), therefore the reduced respiration in DPA treated fruit may result in lower volatiles production as observed in the experiments reported here.

As expected there were varietal differences in the volatiles analysed. ‘Crofton’ had fewer volatiles, whilst ‘Lady Williams’ generally had higher levels of volatiles. Paliyath et al. (1997) also compared the volatile ester profile of CA stored scald resistant ‘Empire’ and ‘Gala’ and scald susceptible ‘Red Delicious’ and ‘McIntosh’ varieties using SPME. Although only qualitative data was reported, they suggested there were varietal differences in both α-farnesene and ester production after 3 months storage. The scald resistant cultivars produced more esters and less α-farnesene than the scald susceptible varieties. In addition, they showed that the differences in the volatiles profiles between scalded and non-scalded ‘Red Delicious’ apples were minor. Similar results for these compounds are shown here. However, 6-methyl heptan-2-one was a very significant component of the volatiles from scalded peel tissue.
5.4 Role of Apple Volatiles in Scald Development

5.4.1 Introduction

The incidence of scald is known to be lower in fruit stored in well ventilated cold stores (Smock and Southwick, 1945; Hall et al., 1961b). The effectiveness of oil wraps in reducing scald was thought to be due to the absorption of naturally occurring fruit volatiles (Smock, 1961). In addition, Kidd and West (1938) were the first to show that mixed storage of apple varieties can increase scald in a susceptible variety. Smock and Southwick (1945) and Smock (1961) have also shown that when stored together, the volatiles from ‘McIntosh’ apples slightly increased scald on ‘Rhode Island Greening’ apples. This observation has been confirmed in recent studies at Michigan State University that showed susceptible apples ventilated with an atmosphere from other stored apple fruits had increased incidence and more severe scald (David Dilley, pers. comm.). These reports imply that naturally occurring fruit volatiles are involved in the development of scald. To further investigate this relationship it is necessary to identify fruit volatiles and any related biochemical changes in the peel that may be related to scald development.

The aim of this section was:

- examine the changes in scald development and volatile composition during storage in air at 0°C in the peel of scald susceptible (‘Granny Smith’) with and without a pre-storage application of DPA, and in the peel of scald resistant (‘Crofton’) apples exposed continuously to a stream of volatiles evolved by either ‘Granny Smith’ or ‘Crofton’.

5.4.2 Materials and Methods

Immature ‘Granny Smith’ and ‘Crofton’ apples were harvested on April 5 1993, from a commercial orchard at Orange, NSW, transported to UWSH (about 3h drive) and subjected to maturity tests (Section 5.2.1). Two 200L drums (containing either ‘Granny Smith’ or ‘Crofton’ apples) were used as a source of volatiles. These drums were ventilated (40 L.h⁻¹) with air at 0°C and the exiting streams then passed over fruit in two 50L drums in series. Fruit in the 50L drums included either ‘Granny Smith’ apples without a pre-storage application of DPA (control), ‘Granny Smith’ apples treated with DPA (3,000 mg.L⁻¹, ‘Shield-Brite’®, Washington USA)
before storage, or ‘Crofton’ apples (Figure 5.28). The 200L drums contained approximately 400 apples, whilst the 50L drums contained approximately 100 apples per drum.

Volatile s were collected at monthly intervals from the effluent at the end of each treatment series (Figures 5.28 and 5.29). Volatile s were analysed and identified by solvent desorption of the Tenax and semi-quantified by GC/MS, with the use of an internal standard, as described in Sections 5.2.2.7 and 5.2.2.9. Samples of apples were removed from the 50L drums at 3 monthly intervals and assessed for scald development after a further seven days at 20°C, as described in Section 5.2.1.7. Volatiles from the peel samples were measured by static headspace analysis (Section 5.2.2.5).

Figure 5.28  Diagrammatic representation of the flow system and the locations of the Tenax traps used for volatile collection
Figure 5.29  An illustration of the experimental ventilation system in a cool room at 0°C. Capillary manometers (rear shelf) were used to maintain a ventilation rate of about 40 L.h⁻¹ through the 200 L source drums. Air exiting from the sealed source drums was divided into three approximately equal streams of air, which were admitted to pairs of 50 L sealed drums arranged in series. Silicone rubber tubing was used for all connections.
5.4.3 Results

5.4.3.1 Maturity indices at harvest

The results of the maturity tests at harvest of ‘Granny Smith’ and ‘Crofton’ apples used in the experiment are shown in Table 5.10. The results show that both apple varieties were preclimacteric and suitable for long term storage.

Table 5.10 Maturity indices of ‘Granny Smith’ and ‘Crofton’ apples. Standard error of the means are shown in brackets ($n = 15$)

<table>
<thead>
<tr>
<th></th>
<th>Granny Smith</th>
<th>Crofton</th>
</tr>
</thead>
<tbody>
<tr>
<td>internal ethylene</td>
<td>0.04 (0.005)</td>
<td>0.08 (0.007)</td>
</tr>
<tr>
<td>flesh firmness (N)</td>
<td>85.9 (2.6)</td>
<td>98.6 (2.8)</td>
</tr>
<tr>
<td>SSC (%)</td>
<td>10.9 (0.06)</td>
<td>10.4 (0.12)</td>
</tr>
<tr>
<td>starch index</td>
<td>2.25 (0.13)</td>
<td>0.0 (0)</td>
</tr>
</tbody>
</table>

5.4.3.2 Scald development

The scald data were highly variable and did not conform to the assumptions of analysis of variance (normality and homoscedasticity). A Kruskall Wallis ranking system, a non-parametric comparison of treatment means, was employed to compare the development of scald between removal times (Table 5.11), source of volatiles (Table 5.12) and position of the treatment drum (Table 5.13).

No scald symptoms developed on either DPA treated ‘Granny Smith’ or ‘Crofton’ apples throughout the experiment. The occurrence of scald at 3, 6, and 9 months in ‘Granny Smith’ apples not treated with DPA at harvest is summarised in Table 5.11. Scald symptoms developed in fruit stored for 3 months and reached maximum severity after 6 months storage. Volatiles from either ‘Crofton’ or Granny Smith’ apples in the large 200L source drum had no significant effects on scald development in ‘Granny Smith’ apples not treated with DPA at harvest (Table 5.12).

There were no differences in severity of scald between the first and second treatment drums, except at 3 months in fruit connected to the ‘Granny Smith’ source drum (Table 5.13), where the apples in the second treatment drum had significantly more scald that those in the first drum. The severity of scald in air stored control apples, which were held separately in boxes were similar to those in the ventilation experiment (Table 5.13). However, the scalded areas in apples
from the ventilation system were more intense (darker) than in those apples stored separately in boxes. This was noted from the first removal and observed throughout the experiment. Although the scalded areas in air stored ‘Granny Smith’ apples had lighter brown symptoms than in the ventilation system, the background (green) colour of the peel in apples from both storage systems were similar.

Table 5.11 Development of scald in ‘Granny Smith’ apples not treated with DPA before storage at 0°C. Apples were assessed for scald after seven days at 20°C following removal from storage at 3, 6 and 9 months
The data from all treatments were combined into removal times

<table>
<thead>
<tr>
<th>Removal</th>
<th>Scald Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 month</td>
<td>2.34 a</td>
</tr>
<tr>
<td>6 month</td>
<td>3.87 b</td>
</tr>
<tr>
<td>9 month</td>
<td>3.99 b</td>
</tr>
</tbody>
</table>

A scald score of 0 = no scald; 5 = very severe scald.
Different letters indicate statistically significant differences (P<0.05)

Table 5.12 Development of scald in ‘Granny Smith’ apples not treated with DPA before storage and ventilated with air streams emulating from source drums containing either ‘Granny Smith’ or ‘Crofton’. Apples were subjectively assessed for scald following 3, 6 and 9 months storage at 0°C and seven days at 20°C after the fruit were transferred to air
The data obtained from the two treatment drums were combined

<table>
<thead>
<tr>
<th>Removal</th>
<th>Source Drum</th>
<th>Scald Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 month</td>
<td>Granny Smith</td>
<td>2.24 a</td>
</tr>
<tr>
<td></td>
<td>Crofton</td>
<td>2.44 a</td>
</tr>
<tr>
<td>6 month</td>
<td>Granny Smith</td>
<td>3.80 a</td>
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<tr>
<td></td>
<td>Crofton</td>
<td>3.94 a</td>
</tr>
<tr>
<td>9 month</td>
<td>Granny Smith</td>
<td>3.86 a</td>
</tr>
<tr>
<td></td>
<td>Crofton</td>
<td>4.12 a</td>
</tr>
</tbody>
</table>

A scald score of 0 = no scald; 5 = very severe scald.
Different letters within a removal indicate statistically significant differences (P<0.05)
Table 5.13  Development of scald in ‘Granny Smith’ (with and without pre-storage DPA treatment) and ‘Crofton’ apples during the storage. Apples were subjectively assessed for scald following 3, 6 and 9 months storage at 0°C and seven days at 20°C

<table>
<thead>
<tr>
<th>3 Months Storage</th>
<th>drum #1</th>
<th>drum #2</th>
<th>Air stored</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Granny Smith</td>
<td>Granny Smith</td>
<td>Granny Smith</td>
</tr>
<tr>
<td>source drum</td>
<td>GS Control 1.76 b</td>
<td>GS Control 2.72 a</td>
<td>1.6 b</td>
</tr>
<tr>
<td></td>
<td>GS DPA 0</td>
<td>GS DPA 0</td>
<td>Granny Smith</td>
</tr>
<tr>
<td></td>
<td>Crofton 0</td>
<td>Crofton 0</td>
<td>DPA 0</td>
</tr>
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<td></td>
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<td>Granny Smith</td>
<td>Crofton 0</td>
</tr>
<tr>
<td></td>
<td>GS Control 2.32 a</td>
<td>GS Control 2.56 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GS DPA 0</td>
<td>GS DPA 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crofton 0</td>
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<table>
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<td>Granny Smith</td>
</tr>
<tr>
<td>source drum</td>
<td>GS Control 3.72 a</td>
<td>GS Control 3.88 a</td>
<td>3.24 a</td>
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<tr>
<td></td>
<td>GS DPA 0</td>
<td>GS DPA 0</td>
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<tr>
<td></td>
<td>GS Control 3.92 b</td>
<td>GS Control 3.96 b</td>
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</tr>
<tr>
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<td>GS DPA 0</td>
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<tr>
<td></td>
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<table>
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<tr>
<td>source drum</td>
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<td>GS DPA 0</td>
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</tr>
<tr>
<td></td>
<td>Crofton 0</td>
<td>Crofton 0</td>
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</tr>
</tbody>
</table>

A scald score of 0 = no scald; 5 = very severe scald

Different letters within a row indicate statistically significant differences (P<0.05)
5.4.3.3 Apple volatiles

Quantification of apple volatiles using ethyl ether solvent desorption from Tenax is unreliable due to the inability to accurately apply an internal standard and quantitatively handle small volumes of ethyl ether. Therefore, data from this experiment are semi-quantitative and the concentrations shown are only a guide to the absolute levels of volatiles in the aroma profile.

Since there were no increases in scald severity after 6 months, only volatiles data from the 6 month measurement are shown as a guide to other monthly measurements (Table 5.17). A list of 71 volatiles collected from the treatment drums after six months storage is shown in Table 5.14. From this list, 23 of the most common volatiles were selected for more detailed comparison (Tables 5.15, 5.16 and 5.17). The results show that α-farnesene is a key component of the aroma profile, irrespective of the source drum or treatment. The levels of 6-methyl hepten-2-one were higher from control ‘Granny Smith’ apples than from DPA treated ‘Granny Smith’ fruit and ‘Crofton’ apples. However production of volatiles by ‘Crofton’ was low, compared to ‘Granny Smith’.

5.4.3.4 In situ concentration of peel α-farnesene and 6-methyl hepten-2-one

Figures 5.30, 5.31 and 5.32 show the concentrations of α-farnesene and 6-methyl hepten-2-one in the peel of ‘Granny Smith’ and ‘Crofton’ apples after 3, 6 and 9 months storage, respectively. The concentrations of α-farnesene in both ‘Granny Smith’ treatments were significantly greater than in ‘Crofton’ peel at 3 months storage (Figure 5.30), while the concentrations of 6-methyl hepten-2-one were significantly greater in ‘Granny Smith’ control fruit than either DPA treated ‘Granny Smith’ or ‘Crofton’ peel. Similar trends were observed at both 6 and 9 months, however the concentrations of α-farnesene in control ‘Granny Smith’ peel declined, while in DPA treated fruit, the concentrations of α-farnesene in the peel remained at high levels throughout storage (Figure 5.31). The concentrations of α-farnesene and 6-methyl hepten-2-one in the peel of ‘Crofton’ apples at all removal times were lower than in ‘Granny Smith’ peel. There were no major differences in the levels of α-farnesene and 6-methyl hepten-2-one in the peel of ‘Granny Smith’ and ‘Crofton’ apples between the two treatment drums in series. In addition, the source of ventilating air had no effect on the levels of α-farnesene and 6-methyl hepten-2-one in the peel of ‘Granny Smith’ or ‘Crofton’ apples. It is most noteworthy that the concentrations of these peel volatiles was not effected by the high concentrations emanating from the ‘Granny Smith’ source drum.
Table 5.14  Concentrations (µg.1000 L⁻¹ headspace) of 71 volatiles in the aroma profile of ‘Granny Smith’ and ‘Crofton’ apples after six months storage at 0°C. ‘Granny Smith’ and ‘Crofton’ apples were used as a source of volatiles which flowed over ‘Granny Smith’ apples with and without a pre-storage DPA treatment, and ‘Crofton’ apples

<table>
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<th>Crofton /</th>
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Total Volatiles

70 59 56 121 105 30

* Tentative GC/MS identification. Identification from MS Wiley Library
Table 5.15  Concentration (µg.1000 L⁻¹ headspace) of selected volatiles in the aroma profile of ‘Granny Smith’ and ‘Crofton’ apples after two months storage at 0°C. ‘Granny Smith’ and ‘Crofton’ apples were used a source of volatiles which flowed over ‘Granny Smith’ apples with and without a pre-storage DPA treatment, and ‘Crofton’ apples

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<th>Crofton /</th>
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<th></th>
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<td>Control GS</td>
<td>Crofton</td>
<td>DPA GS</td>
<td>Control GS</td>
<td>Crofton</td>
<td></td>
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Table 5.16  Concentration (μg·1000 L⁻¹ headspace) of selected volatiles in the aroma profile of ‘Granny Smith’ and ‘Crofton’ apples after three months storage at 0°C. ‘Granny Smith’ and ‘Crofton’ apples were used a source of volatiles which flowed over ‘Granny Smith’ apples with and without a pre-storage DPA treatment, and ‘Crofton’ apples

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<th>Crofton / DPA</th>
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<td>1.60</td>
<td>-</td>
<td>1.80</td>
<td>1.52</td>
<td>1.97</td>
</tr>
<tr>
<td>11 ethyl hexanoate</td>
<td>0.80</td>
<td>-</td>
<td>0.74</td>
<td>0.62</td>
<td>0.89</td>
</tr>
<tr>
<td>12 hexyl acetate</td>
<td>5.98</td>
<td>-</td>
<td>5.29</td>
<td>3.72</td>
<td>4.27</td>
</tr>
<tr>
<td>13 decane</td>
<td>0.50</td>
<td>-</td>
<td>0.38</td>
<td>0.65</td>
<td>0.75</td>
</tr>
<tr>
<td>14 hexyl 2-methyl butanoate</td>
<td>0.54</td>
<td>-</td>
<td>0.00</td>
<td>1.08</td>
<td>1.17</td>
</tr>
<tr>
<td>15 pentyl butanoate</td>
<td>0.40</td>
<td>-</td>
<td>0.00</td>
<td>0.00</td>
<td>0.37</td>
</tr>
<tr>
<td>16 hexyl propanoate</td>
<td>0.54</td>
<td>-</td>
<td>0.68</td>
<td>0.60</td>
<td>0.65</td>
</tr>
<tr>
<td>17 undecane</td>
<td>1.25</td>
<td>-</td>
<td>0.76</td>
<td>1.05</td>
<td>1.24</td>
</tr>
<tr>
<td>18 hexyl butanoate</td>
<td>11.59</td>
<td>-</td>
<td>18.84</td>
<td>12.02</td>
<td>13.64</td>
</tr>
<tr>
<td>19 hexyl 2-methyl butanoate</td>
<td>1.11</td>
<td>-</td>
<td>0.54</td>
<td>9.52</td>
<td>7.37</td>
</tr>
<tr>
<td>20 hexyl hexanoate</td>
<td>6.35</td>
<td>-</td>
<td>4.97</td>
<td>1.25</td>
<td>0.41</td>
</tr>
<tr>
<td>21 unknown</td>
<td>0.57</td>
<td>-</td>
<td>0.00</td>
<td>0.47</td>
<td>0.51</td>
</tr>
<tr>
<td>22 α-farnesene</td>
<td>208.11</td>
<td>-</td>
<td>24.92</td>
<td>120.35</td>
<td>92.31</td>
</tr>
<tr>
<td>23 6-methyl-2-bicyclo[3.1.1]heptane</td>
<td>0.00</td>
<td>-</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

- no data available
Table 5.17  Concentration (μg.1000 L⁻¹ headspace) of selected volatiles in the aroma profile of ‘Granny Smith’ and ‘Crofton’ apples after six months storage at 0°C. ‘Granny Smith’ and ‘Crofton’ apples were used a source of volatiles which flowed over ‘Granny Smith’ apples with and without a pre-storage DPA treatment, and ‘Crofton’ apples

<table>
<thead>
<tr>
<th>Volatile</th>
<th>Granny Smith /</th>
<th>Crofton /</th>
</tr>
</thead>
<tbody>
<tr>
<td>#</td>
<td>DPA GS</td>
<td>Control GS</td>
</tr>
<tr>
<td>1 ethyl butanoate</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>2 1-hexanol</td>
<td>0.29</td>
<td>0.24</td>
</tr>
<tr>
<td>3 1-butanol 3methyl acetate</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td>4 propyl butanoate</td>
<td>0.64</td>
<td>0.27</td>
</tr>
<tr>
<td>5 methyl hexanoate</td>
<td>0.08</td>
<td>1.23</td>
</tr>
<tr>
<td>6 unknown</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>7 2-methyl propyl butanoate</td>
<td>0.57</td>
<td>0.55</td>
</tr>
<tr>
<td>8 6-methyl 5-hepten-2-one</td>
<td>0.34</td>
<td>0.84</td>
</tr>
<tr>
<td>9 6-methyl-5-hepten-2-ol</td>
<td>0.00</td>
<td>0.31</td>
</tr>
<tr>
<td>10 butyl butanoate</td>
<td>0.97</td>
<td>0.30</td>
</tr>
<tr>
<td>11 ethyl hexanoate</td>
<td>0.15</td>
<td>0.21</td>
</tr>
<tr>
<td>12 hexyl acetate</td>
<td>1.21</td>
<td>0.95</td>
</tr>
<tr>
<td>13 decane</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>14 butyl 2-methyl butanoate</td>
<td>0.21</td>
<td>0.34</td>
</tr>
<tr>
<td>15 pentyl butanoate</td>
<td>0.12</td>
<td>0.11</td>
</tr>
<tr>
<td>16 hexyl propanoate</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>17 undecane</td>
<td>0.24</td>
<td>0.17</td>
</tr>
<tr>
<td>18 hexyl butanoate</td>
<td>4.02</td>
<td>2.11</td>
</tr>
<tr>
<td>19 hexyl 2-methyl butyrate</td>
<td>1.85</td>
<td>0.75</td>
</tr>
<tr>
<td>20 hexyl hexanoate</td>
<td>0.91</td>
<td>0.86</td>
</tr>
<tr>
<td>21 unknown</td>
<td>0.16</td>
<td>0.10</td>
</tr>
<tr>
<td>22 α-farnesene</td>
<td>52.71</td>
<td>44.23</td>
</tr>
<tr>
<td>23 6-methyl-2-bicyclo[3.1.1]heptane</td>
<td>0.17</td>
<td>0.27</td>
</tr>
<tr>
<td>Total Volatiles</td>
<td>65</td>
<td>53</td>
</tr>
</tbody>
</table>
Figure 5.30 Concentrations of α-farnesene and 6-methyl hepten-2-one in the peel of control ‘Granny Smith’ (A, B), DPA treated ‘Granny Smith’ (C, D) and ‘Croton’ (E,F) apples after 3 months storage. The fruit were ventilated with air streams from source drums containing either ‘Granny Smith’ (A, C and E) or ‘Croton’ apples (B, D and F)
Figure 5.31 Concentrations of α-farnesene and 6-methyl hepten-2-one in the peel of control ‘Granny Smith’ (A, B), DPA treated ‘Granny Smith’ (C, D) and ‘Crofton’ (E, F) apples after 6 months storage. The fruit were ventilated with air streams from source drums containing either ‘Granny Smith’ (A, C and E) or ‘Crofton’ apples (B, D and F)
Figure 5.32 Concentrations of α-farnesene and 6-methyl hepten-2-one in the peel of control ‘Granny Smith’ (A, B), DPA treated ‘Granny Smith’ (C, D) and ‘Crofton’ (E, F) apples after 9 months storage. The fruit were ventilated with air streams from source drums containing either ‘Granny Smith’ (A, C and E) or ‘Crofton’ apples (B, D and F)
Figure 5.33  Concentration of α-farnesene and 6-methyl hepten-2-one in the peel of control ‘Granny Smith’, DPA treated ‘Granny Smith’ and ‘Croton’ apples after 3 (A), 6 (B) and 9 (C) months stored separately in boxes in air at 0°C.
The concentrations of α-farnesene and 6-methyl hepten-2-one in the peel of the control ‘Granny Smith’ and ‘Crofton’ apples that had been stored in boxes in air at 0°C (Figure 5.33), were similar to those from the ventilation system (Figures 5.30, 5.31 and 5.32). The concentrations of α-farnesene in the peel of control ‘Granny Smith’ apples declined over the 9 month storage at 0°C, whereas the concentrations of α-farnesene remained relatively constant in DPA treated ‘Granny Smith’ and ‘Crofton’ apples. No 6-methyl hepten-2-one was found in the peel of Granny Smith’ apples treated with DPA, whilst only low concentrations of 6-methyl hepten-2-one were detected in ‘Crofton’ peel. The concentration of 6-methyl hepten-2-one in the peel of control ‘Granny Smith’ apples at 3 months was higher than in ‘Granny Smith’ treatments, but declined during storage and was undetectable by 9 months.

5.4.4 Discussion

The volatiles theory of scald development proposed by Brooks et al. (1919) suggests that volatiles from one variety can increase scald on another. The results in this experiment showed that scald did not develop in DPA treated ‘Granny Smith’ or ‘Crofton’ apples that were continuously challenged with the volatiles from either ‘Granny Smith’ or ‘Crofton’, indicating that volatiles from these apples did not overcome the natural resistance to scald in ‘Crofton’ or chemical inhibition of scald (in DPA treated ‘Granny Smith’). If the volatiles from one variety can affect the scald in another, the levels of scald should be greater in the second treatment drum in the ventilation series. However, the effect of apple volatiles on the development of scald was not significant, except after 3 months storage at which time the second drum of untreated control ‘Granny Smith’ apples had significantly more scald than those apples in the first drum. The lack of effects in volatile challenged apples was confirmed by the comparison with the apples that were loose stored in boxes in the same coolroom. Apples from the same harvest developed similar levels of scald to those in the volatiles challenged fruit, except in untreated ‘Granny Smith’ apples at 3 months where the second treatment drum had significantly more scald than ‘Granny Smith’ stored separately in boxes.

Similar studies by Kidd and West (1938), Smock and Southwick, 1945 and Smock (1961) gave either unconvincing or inconclusive results as some experiments were not strictly controlled, or the differences were not significantly different. However, David Dilley (per. comm.) recently found that the incidence and severity of scald increased in scald susceptible ‘Cortland’ and ‘Red
Delicious’ by exposing them to the volatiles produced by ‘McIntosh’ apples. He also reported that the severity of scald increased as a function of exposure to volatiles produced from ‘McIntosh’ apples. However Dilley’s experiments were conducted in controlled atmospheres (3% CO₂, 1.5 or 3% O₂) and hence cannot be compared directly to the results obtained in this experiment.

Although the volatiles trapping and analysis methods used in this experiment only yielded semi-quantitative data, some useful comparisons were made. The levels of volatiles exiting the treatment drums showed some differences among treatments with high levels of α-farnesene and hexyl derivatives collected from ‘Granny Smith’ apples. This generally resulted in higher levels of total volatiles collected from ‘Granny Smith’ apples than ‘Crofton’ apples. These results were similar to those reported in Section 5.4, and they complement the more detailed volatile biosynthesis studies of Harb et al. (1994) and Song and Bangerth (1994), who showed the production of similar volatile compounds by ‘Golden Delicious’ apples during storage.

The concentrations of α-farnesene and 6-methyl hepten-2-one in the peel of stored apples were not affected by the source of volatiles flowing over them, suggesting that at least α-farnesene and 6-methyl hepten-2-one are not differentially absorbed from other apples during storage. This is surprising considering the large quantities of volatiles (particularly α-farnesene) that emanated from the source drums that contained about 400 ripening fruit. It would be expected that the waxy apple cuticle should absorb the hydrophilic α-farnesene. This was true to some extent, as shown by the lower levels of α-farnesene in apples stored loosely in boxes compared to fruit stored in drums. However, the levels of scald in loose fruit were similar to those in the drums, suggesting that scald is not directly related to α-farnesene concentration per se.

The concentration of α-farnesene in control ‘Granny Smith’ peel tissue in ventilated drums and loose in boxes declined during storage, whilst the concentrations of α-farnesene in DPA treated fruit remained relatively high in both ventilated (> 400 μg·g⁻¹) and loose (200 μg·g⁻¹) storage throughout the storage. In ‘Crofton’ peel, the levels of α-farnesene remained low but constant during storage, except in the ventilated system where the concentration of α-farnesene declined by 9 months storage.

The concentrations of 6-methyl hepten-2-one in the peel were unaffected by the source of volatiles flowing over the apples. Furthermore the concentrations of 6-methyl hepten-2-one were
low in the peel of DPA treated ‘Granny Smith’ and ‘Crofton’ peel at all removal times. This
suggests that the pre-storage application of DPA and the natural resistance in ‘Crofton’ apples
inhibits α-farnesene autoxidation (i.e., 6-methyl hepten-2-one production).

The concentration of 6-methyl hepten-2-one in the peel of control ‘Granny Smith’ apples varied
between removals and source of volatiles. If α-farnesene and its oxidation products (represented
by 6-methyl hepten-2-one) had a role in scald development, the levels of 6-methyl hepten-2-one
would be expected to be higher in more scalded tissue and this was generally the case. However,
it surprising that after three months storage, the concentration of 6-methyl hepten-2-one in
‘Granny Smith’ peel tissue was lower in the first treatment drum compared to the second (Figure
5.31A), as the first treatment drum was the only treatment and removal that had significantly
more scald than in those apples in the second treatment drum.
5.5 Relation Between Evolution of Ethylene and $\alpha$-Farnesene, and the Timing of DPA Treatment

5.5.1 General Introduction

The role of ethylene in the development of scald has been widely examined, however the results have been variable. Little et al. (1985) and Lau (1990) showed that removal of ethylene did not control scald in ‘Granny Smith’ and ‘Delicious’ fruit stored in ultra low oxygen CA (1% O$_2$), while Knee and Hatfield (1981a) showed that removal of ethylene from the CA storage environment (5% CO$_2$ and 3% O$_2$) with permanganate, resulted in a slower rate of $\alpha$-farnesene accumulation and decreased the levels of scald. However, permanganate is a non-specific oxidising agent, oxidising many organic volatiles, including $\alpha$-farnesene (Saltveit, 1980). Knee and Hatfield (1981a) also showed that the addition of ethylene (1,100 $\mu$L.L$^{-1}$) to the storage environment caused an accumulation of $\alpha$-farnesene, and an earlier onset of scald.

Ethephon (2-chloroethyl phosphonic acid) is well known to accelerate ripening (Unrath, 1972) and has been applied before harvest to counteract the effects of early harvesting, thereby reducing scald development in ‘Delicious’, ‘Granny Smith’ and ‘Fuji’ apples during storage (Brohier and Faragher, 1984; Lurie et al., 1989b; Curry, 1994). The lower levels of scald development in ethephon treated fruit have been associated with higher levels of $\alpha$-farnesene after storage (Lurie et al., 1989b; Curry, 1994). However, other workers have reported increases in scald with ethephon application in ‘McIntosh’ and ‘Cortland’ (Greene et al., 1974; Windus and Shutak, 1977). Therefore the effect of ethephon application may vary according to variety (Curry, 1994), orchard factors (Lurie et al., 1989b), and timing (Lurie et al., 1989b) and the concentration (Brohier and Faragher, 1984) of ethephon applied. Accordingly a more defined procedure to synchronise and hasten ethylene production is required to re-examine the role of ethylene in scald development. Propylene is an active analogue of ethylene, which is not produced by the fruit and provides a convenient means of advancing the autocatalytic production of ethylene, whilst also allowing the measurement of endogenous ethylene production (McMurchie et al., 1972).

There is considerable evidence that DPA must be applied as soon as possible after harvest to successfully suppress scald development (Little, 1985). However, little is known about changes in peel physiology that occur during this critical time. It has been shown that $\alpha$-farnesene
increases with the ethylene climacteric (Du and Bramlage, 1994a) and that DPA reduces the oxidation of α-farnesene (Huelin and Coggiola, 1968). It can be postulated that the production and oxidation of α-farnesene is suppressed by the application of DPA, and this can only occur before the ethylene climacteric.

The aims of the experiments in this section were:

- examine the relationship between ethylene and α-farnesene peel concentrations after DPA application during the first few months of storage of ‘Granny Smith’ apples at 10°C,
- compare the responses in ethylene and α-farnesene production in ‘Granny Smith’ and ‘Crofton’ apples, to delayed DPA application at a scald inducing storage temperature of 0°C, and a non-scald inducing temperature of 10°C.

5.5.2 Relation Between Ethylene and α-Farnesene Production and the Timing of DPA Treatment in ‘Granny Smith’ Apples Treated with Propylene at 10°C

To test the hypothesis that α-farnesene is suppressed by the application of DPA only before the onset of the climacteric, DPA was applied at regular intervals before and during the climacteric and the concentrations of ethylene and α-farnesene were followed during storage. To accelerate changes in the fruit, this experiment was conducted at 10°C. At the commercial storage temperature (0°C), the respiratory climacteric and other associated changes are prolonged, and may take several months before significant changes are observed. Although scald does not develop at 10°C (Watkins et al., 1995), it was reasoned that it would be easier to compare ethylene and α-farnesene production at 10°C because the rates should be approximately twice those at 0°C.

A major deficiency in the majority of scald (and apple) experimental studies has been the lack of a consistent and reliable physiological marker of fruit age. Calendar date, starch content and firmness are general guides for commercial maturity but not of physiological age. The onset of the ethylene climacteric is a fundamental change that should be independent of climatic effects (Dilley and Dilley, 1985). Therefore propylene application was used to hasten and synchronise the climacteric and thereby reduce variability within the treated fruit population.
5.5.3 Materials and Methods

'Granny Smith' apples were harvested on March 23 1994, from a local commercial orchard at Bilpin, NSW, and transported to UWSH (about 30 min drive). Fruit were assessed for maturity (Section 5.2.1) and stored in air at 10°C. Eight fruit replicates per removal time were treated as outlined in Table 5.18.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Time of DPA application (days)</th>
<th>Propylene (100 μL.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>at harvest</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>5</td>
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</tr>
<tr>
<td>7</td>
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<tr>
<td>10</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>no DPA</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>no DPA</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

The fruit were dipped in DPA (3,000 mL.L⁻¹ ‘Shield-Brite’, Washington USA) and propylene was applied at 100 μL.L⁻¹ propylene (equivalent to about 1 μL.L⁻¹ ethylene) (Sfakiotakis and Dilley, 1973). The times when DPA was applied refer to the days after the onset of the climacteric. The first treatment of fruit were dipped in DPA before the onset of the ethylene climacteric. Eight single apple replicates per treatment were used for the measurement of internal ethylene concentration (Section 5.2.1.3). Four of these fruit were peeled for α-farnesene analysis (Section 5.2.2.2).

Apples from the same harvest were stored at 0°C for 6 months to assess scald potential of the fruit used in this experiment (Section 5.2.1.7).
5.5.4 Results

5.5.4.1 Maturity indices at harvest

The maturity data in Table 5.19 show that the fruit used in this experiment were preclimacteric when harvested.

Table 5.19 Maturity indices of ‘Granny Smith’ apples harvested from Bilpin, NSW on 23 March. Standard error of the means are shown in brackets (n = 15)

<table>
<thead>
<tr>
<th>Internal ethylene</th>
<th>not detectable (limit of detection = 0.01 µL.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flesh firmness (N)</td>
<td>74.7 (1.9)</td>
</tr>
<tr>
<td>SSC (%)</td>
<td>10.1 (0.13)</td>
</tr>
<tr>
<td>Starch index</td>
<td>1.0 (0.39)</td>
</tr>
<tr>
<td>IEC</td>
<td>51 days after harvest (= 13 May)</td>
</tr>
</tbody>
</table>

5.5.4.2 Concentrations of internal ethylene and peel α-farnesene

When DPA was applied immediately after harvest and before the apples were placed in the propylene ripening atmosphere (Figure 5.34), the patterns of internal ethylene and peel α-farnesene concentrations were similar. However the absolute concentrations of α-farnesene in the peel of fruit treated at harvest were lower, than in apples not dipped in DPA (Figure 5.35A) or those dipped during the climacteric (Figure 5.36).

In all cases, propylene treatment advanced the onset of both α-farnesene and internal ethylene production. Indeed α-farnesene concentrations began to increase before any significant internal ethylene was detected. However in fruit ripened in air, the increase in α-farnesene coincided with the increase in internal ethylene production (Figure 5.35B). This implies that endogenous ethylene production has a role in α-farnesene synthesis but that their biosynthetic pathways are independent. A water dip was used as a control and showed that the process of dipping did not affect peel physiology (data not shown).

The effects of delaying DPA application on the internal ethylene and peel α-farnesene concentrations in ‘Granny Smith’ apples at 10°C at intervals during the ethylene climacteric in a
propylene ripening environment are shown in Figure 5.36. These data also shows that α-farnesene concentrations in the peel began to increase rapidly before there was a significant increase in internal ethylene concentrations. α-Farnesene concentrations reached a maximum 35 - 45 days after harvest in most treatments, whereas ethylene continued to rise until at least until the experiment was terminated (day 50). Delaying the application of DPA after harvest did not affect the production of α-farnesene. Its effects on ethylene production were less clear. However, a slight delay in ethylene onset was observed in the initial stages of ethylene production and was more evident with the 10 and 14 day delay in DPA application (Figure 5.36 G and H).

A subsample of fruit from the same harvest were stored at scald inducing temperatures (0°C) to ensure they developed scald. After six months at 0°C and 1 week at 20°C, the average scald score was 3.6, indicating moderate to severe scald symptoms, whilst DPA treated fruit did not develop any scald symptoms.

![Graph showing ethylene and α-farnesene concentrations over time](image)

Figure 5.34  Internal ethylene and peel α-farnesene concentrations in ‘Granny Smith’ apples stored at 10°C. Apples were dipped in DPA at harvest, and ventilated continuously with propylene. Bars show standard error of the means (SEM, n(ethylene) = 8, n(α-farnesene) = 4, when absent the SEM bars fall within the dimensions of the symbol)
Figure 5.35  Internal ethylene and peel α-farnesene concentrations in the peel of untreated 'Granny Smith' apples stored at 10°C. Apples in Figure 5.35A were ventilated continuously with propylene, whilst apples in Figure 5.55B were ventilated with air. Bars show standard errors of the means (SEM, \( n(\text{ethylene}) = 8, n(\text{α-farnesene}) = 4 \), when absent the SEM bars fall within the dimensions of the symbol)
Figure 5.36  Internal ethylene and peel \( \alpha \)-farnesene concentrations in 'Granny Smith'
apples stored at 10°C. Apples were treated with DPA (3,000 \( \mu \)g.L\(^{-1} \)) at the
beginning of the ethylene climacteric (A = Time Zero), 1 day (B), 2 days (C),
3 days (D), 5 days (E), 7 days (F), 10 days (G) and 14 days (H) after the initiation
of the ethylene climacteric with 100 \( \mu \)L.L\(^{-1} \) propylene. Bars show the standard
error of the means (SEM; \( n \) (ethylene) = 8; \( n \) (\( \alpha \)-farnesene) = 4);
when absent the SEM bars fall within the dimensions of the symbol
Figure 5.36  Legend previous page
5.5.5 Relationship Between Ethylene and α-Farnesene Production and the Timing of DPA Treatment in ‘Granny Smith’ and ‘Crofton’ Apples at 0°C and 10°C

The results from the previous section showed that there was a relationship between endogenous ethylene production and peel α-farnesene and that propylene stimulated α-farnesene production before internal ethylene concentration. Only the DPA application at harvest lowered peel α-farnesene concentrations during storage. The aim of this experiment was to further examine the relationship between ethylene production and the timing of DPA application at both a scald inducing temperature (0°C) and a non-scald inducing temperature (10°C) on a scald resistant variety, ‘Crofton’ and a scald susceptible variety, ‘Granny Smith’.

5.5.6 Materials and Methods

‘Granny Smith’ and ‘Crofton’ apples were harvested from a commercial orchard at Orange, NSW on 25 April 1994, and transported to UWSH (about 3h drive). Fruit were assessed for maturity (Section 5.2.1). Eight fruit replicates were stored at either 0°C or 10°C in boxes lined with polyethylene film. DPA (3,000 mL L⁻¹ ‘No Scald’) was applied at harvest (time zero), 4 days, or 7 days after harvest. A control treatment (no DPA) was included at both storage temperatures for both varieties. Fruit were removed from cold storage at 2-weekly intervals from each treatment. Eight single apple replicates per treatment were used for the measurement of internal ethylene (Section 5.2.1.3). Four of these fruit were peeled for α-farnesene analysis (Section 5.2.2.2). Apples from the same harvest were stored at 0°C for 6 months to assess scald potential of the fruit used in this experiment (Section 5.2.1.7).

5.5.7 Results

5.5.7.1 Maturity indices

The fruit used in this experiment were preclimacteric at harvest (Table 5.20), although starch conversion was more advanced than in the previous experiment (Section 5.5.4.1).

Table 5.20 Maturity indices of ‘Granny Smith’ and ‘Crofton’ apples harvested from Orange, NSW on 15 April.

<table>
<thead>
<tr>
<th></th>
<th>Granny Smith</th>
<th>Crofton</th>
</tr>
</thead>
<tbody>
<tr>
<td>internal ethylene</td>
<td>0.09 (0.04)</td>
<td>0.19 (0.03)</td>
</tr>
<tr>
<td>flesh firmness (N)</td>
<td>84.3 (1.6)</td>
<td>94.1 (1.9)</td>
</tr>
<tr>
<td>SSC (%)</td>
<td>12.3 (0.3)</td>
<td>13.0 (0.2)</td>
</tr>
<tr>
<td>starch index</td>
<td>4.5 (0.17)</td>
<td>3.0 (0.26)</td>
</tr>
</tbody>
</table>
5.5.7.2 Concentrations of internal ethylene and peel α-farnesene

The effects of the timing of DPA application after harvest on internal ethylene concentration and the concentrations of α-farnesene in the peel are shown in Figures 5.37 - 5.40. The data show that variety and storage temperature significantly affected internal ethylene and peel α-farnesene concentrations. Figure 5.37 shows that the concentrations of internal ethylene and peel α-farnesene in ‘Granny Smith’ apples at 0°C increased at similar rates, but with relatively low rates of ethylene production and high concentrations of peel α-farnesene. DPA application had no significant affect on either internal ethylene or peel α-farnesene production. The concentrations of internal ethylene and peel α-farnesene followed similar trends in ‘Crofton’ apples stored at 0°C (Figure 5.39), except that DPA application at harvest suppressed α-farnesene production late in storage. The levels of ethylene in ‘Crofton’ apples were relatively high, whilst the levels of α-farnesene were low compared to ‘Granny Smith’ at 0°C. The concentrations of internal ethylene and peel α-farnesene in ‘Granny Smith’ and ‘Crofton’ apples stored at 10°C are shown in Figures 5.38 and 5.40. The data show that increasing α-farnesene concentrations in the peel were associated with increasing ethylene levels. DPA application generally suppressed α-farnesene production during storage but did not affect ethylene production and may have slightly increased ethylene production in ‘Crofton’ fruit at 0°C.

Figure 5.41 show the relationships between internal ethylene and peel α-farnesene concentrations when the data from all removal times were combined. At 0°C a positive linear relationship \( y = a + bx \) best describes the relationship between the concentration of peel α-farnesene and the internal ethylene concentrations (Figure 5.41 A and B), whilst an asymptotic relationship \( y = a + b/(hx) \) best describes the relationship at 10°C (Figure 5.41 C and D). However, there were significant differences between the two varieties. For example, ‘Granny Smith’ generally had lower internal ethylene concentrations and higher peel α-farnesene concentrations, whilst ‘Crofton’ had higher internal ethylene concentrations and significantly lower concentrations of peel α-farnesene. The higher storage temperature stimulated higher internal ethylene concentrations in both ‘Granny Smith’ and ‘Crofton’ apples, which resulted in high peel α-farnesene concentrations (up to 500 µg.g\(^{-1}\)) in ‘Granny Smith’ apples (Figure 5.41C), but significantly lower concentrations (< 70 µg.g\(^{-1}\)) in ‘Crofton’ peel (Figure 5.41D). Indeed the maximum concentration of α-farnesene in ‘Crofton’ apples was 77 µg.g\(^{-1}\), irrespective of storage temperature and internal ethylene concentration. A sample of fruit were stored at 0°C to ensure they developed scald. After six months at 0°C plus one week at 20°C, the average scald score of non-DPA treated ‘Granny Smith’ apples was 2.6, indicating moderate scald symptoms.
Figure 5.37  Concentrations of internal ethylene and α-farnesene in the peel of ‘Granny Smith’ apples stored in air at 0°C for 12 weeks. Apples were treated with DPA at harvest (B), four days (C) and seven days (D) after harvest. Control apples (A) were not treated with DPA. Bars show the standard errors of the means (SEM, n (ethylene) = 8, n (α-farnesene) = 4; when absent the SEM bars fall within the dimensions of the symbol)
Figure 5.38  Concentrations of internal ethylene and α-farnesene in the peel of ‘Granny Smith’ apples stored in air at 10°C for 12 weeks. Apples were treated with DPA at harvest (B), four days (C) and seven days (D) after harvest. Control apples (A) were not treated with DPA. Bars show the standard errors of the means (SEM, $n$ (ethylene) = 8, $n$ (α-farnesene) = 4; when absent the SEM bars fall within the dimensions of the symbol)
Figure 5.39 Concentrations of internal ethylene and α-farnesene in the peel of ‘Crofton’ apples stored in air at 0°C for 12 weeks. Apples were treated with DPA at harvest (B), four days (C) and seven days (D) after harvest. Control apples (A) were not treated with DPA. Bars show the standard errors of the means (SEM, \( n (ethylene) = 8, n (\alpha-farnesene) = 4 \); when absent the SEM bars fall within the dimensions of the symbol).
Figure 5.40  Concentrations of internal ethylene and α-farnesene in the peel of ‘Crofton’ apples stored in air at 10°C for 12 weeks. Apples were treated with DPA at harvest (B), four days (C) and seven days (D) after harvest. Control apples (A) were not treated with DPA. Bars show the standard errors of the means (SEM, n (ethylene) = 8, n (α-farnesene) = 4; when absent the SEM bars fall within the dimensions of the symbol).
Figure 5.41 Relationship between concentrations of internal ethylene and \(\alpha\)-farnesene in the peel of ‘Granny Smith’ (A and C) and ‘Crofton’ apples (B and D) stored in air at 0°C (A and B) and 10°C (C and D) for 12 weeks. The time of DPA application at each variety and storage temperature were combined and the regression of best fit (ranked \(r^2\)) was determined with Table Curve 2-D (Version 4, Jandel Scientific, San Rafael CA USA).

The regression of best fit for those apples stored at 0°C, irrespective of variety was \(y = a + bx\), whilst at 10°C the best fit regression was \(y = a + b (\ln x)^2\).
5.5.7.3 Diphenylamine residues

DPA residues in hexane extracts of peel were detected and confirmed with GC/MS (Figure 5.42). There were detectable levels of DPA found late in storage in the hexane peel extracts of both varieties not treated with DPA (Figure 5.43). Data for ‘Crofton’ apples showed similar trends (data not shown). The fruit were stored in separate boxes but all fruit were in the same small coolrooms. The time taken to detect DPA residues in peel extracts was dependent on the temperature at which the fruit were stored. Residues of DPA in fruit stored at 10°C were detected within 2 weeks of storage (Figure 5.43B), whilst at 0°C, DPA was detected after 6 weeks storage in ‘Granny Smith’ (Figure 5.43A) and 12 weeks in ‘Crofton’ apples (data not shown). However these low DPA residues did not prevent the development of scald symptoms in ‘Granny Smith’ apples not treated with DPA, because the average scald score in non-treated ‘Granny Smith’ apples stored for six months at 0°C plus one week at 20°C, was 2.3 indicating moderate scald symptoms. DPA residues in fruit treated at harvest declined during storage (Figure 5.43C and 5.43D), and prevented the development of any scald symptoms.

5.5.8 Discussion

Considering the universal importance of ethylene in ripening climacteric fruit and the apparent role of α-farnesene in scald development, it is surprising how little scald work has explored the direct relationship between ethylene and α-farnesene. As expected, the data reported in this section showed that, the application of the ethylene analogue, propylene to ‘Granny Smith’ apples at 10°C, significantly hastened ripening as indicated by internal ethylene production, and the onset of α-farnesene production. Significantly the rise in α-farnesene preceded ethylene production in propylene treated fruit but not in fruit stored in the absence of propylene.

Watkins et al. (1993) and Du and Bramlage (1994a) similarly showed that the preclimacteric application of ethephon (up to 500 µg.L−1) to ‘Cortland’ apples accelerated the rates of production of ethylene, α-farnesene and its oxidation products. However, the levels of scald in ethephon treated fruit and controls were similar. Watkins et al. (1993) reported that although ethephon treated apples developed slightly less scald than the controls, at least 90% of the fruit in all treatments developed scald. However, in the present experiments when the same fruit were ripened in air (ie in the absence of propylene), the onset of α-farnesene synthesis coincided with
Figure 5.42 Identification of DPA residues in hexane extracts of apple peel tissue.

Figure 5.42A GC chromatogram of a hexane extract of peel tissue showing the internal standard (C12), α-farnesene and DPA. Figure 5.42B MS of DPA peak in hexane extract. Figure 5.42C Comparison with known MS of DPA standard from Wiley MS library and structure.
Figure 5.43  The relative levels of DPA residues in hexane peel extracts of ‘Granny Smith’ apples stored at 0°C (A and C) or 10°C (B and D). Apples were either not treated with DPA (A and B), or dipped in DPA (3,000 mg/L⁻¹) (C and D) before storage in plastic lined boxes. Bars show the standard errors of the means (SEM, n = 4)
ethylene production suggesting that α-farnesene production is dependent on endogenous ethylene production. Meigh and Filmer (1969) also showed that a rapid rise in α-farnesene content coincided with the respiration and ethylene climacterics at 12°C, and the differences between the scald susceptible variety ('Edward VII') and the scald resistant apple ('Cox’s Orange Pippin') were small. The slightly earlier production of α-farnesene in the presence of propylene shows that the precursors of α-farnesene are available and its biosynthetic enzymes are triggered by autocatalytic ethylene. This shows that ethylene has a fundamental role in α-farnesene synthesis, although their biosynthetic pathways are independent. The biosynthetic pathways and enzymes for α-farnesene synthesis have been reported in immature apple fruit by Boeve et al. (1996). They showed that infestation of immature apples with European sawfly (Hopocampa testudinea) stimulated α-farnesene production, but that this was not simply a result of injury to the fruit.

Ethylene biosynthesis is thought to be located in the cytoplasm and proceeds from methionine, through S-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid to ethylene (Taiz and Zeiger, 1991). However, less is known about α-farnesene biosynthesis and histochemistry, but the sub-cellular location of α-farnesene biosynthesis and transport is crucial to understanding the possible role of α-farnesene in scald development. Several strategies for the in vitro synthesis of α-farnesene have been reported (Anet, 1970; Fielder et al., 1993; Rupasinghe et al., 1998). Until recently, the biosynthesis of α-farnesene, which proceeds via isopentenyl diphosphate (IPP), was thought to be derived exclusively from the well established acetate / mevalonate (MVA) pathway (Figure 5.44, Pathway B). However, a mevalonate independent pathway in chloroplasts has been elucidated (Eisenreich et al., 1997; Lichtenhaler et al., 1997). This pathway produces IPP via an alternative glyceraldehyde - pyruvate pathway (Figure 5.44, Pathway A). IPP is also the precursor of many plant growth regulators, such as abscisic acid, gibberellins and cytokinins, as well many other plant products, such as carotenoids and chlorophylls.
Figure 5.44  Biosynthesis of α-farnesene via glycolysis and isopentenyl diphosphate (IPP) via two different pathways.

A = glyceraldehyde - pyruvate pathway; B = mevalonic acid (MVA) pathway
PP = diphosphate residue

(adapted from Lichtenthaler et al., 1997)
The current hypothesis is that isoprenoids derived from mevalonic acid, such as the sterols, are located in the cytoplasm while the chloroplast bound isoprenoids, such as β-carotene and lutein, are derived from the glyceraldehyde - pyruvate pathway located in the plastids (Lichtenthaler et al., 1997). There has been little work published on the localisation of sesquiterpene biosynthesis since the discovery of the mevalonate independent pathway, although it is generally accepted that the initial step of all isoprenoid precursor production, glycolysis is carried out by a group of enzymes located in the cytosol (Taiz and Zeiger, 1991). Recently, Adam and Zapp (1998) showed that the sesquiterpene biosynthesis in Matricaria recutita was predominantly from the glyceraldehyde - pyruvate pathway but with significant levels of mevalonic acid derived isoprene units. These sesquiterpenes of mixed biosynthetic origin raise further interesting questions about the compartmentation of sesquiterpene biosynthesis that remain to be answered, and further work is required to determine the contribution, transport and compartmentation of the IPP biosynthesis pathways in the formation of sesquiterpenes, such as α-farnesene in fruit. However, any study of α-farnesene biosynthesis must consider the histological localisation and compartmentation of the different pathways.

As α-farnesene is ultimately derived from glucose it would be assumed that any factor that effects glycolysis and the supply of acetyl CoA or pyruvate, for example changes in ethylene production or respiration rates caused by DPA application (Lurie et al., 1989a) would ultimately affect α-farnesene biosynthesis. The regulation of α-farnesene biosynthesis by ethylene is unknown, but the experiments with propylene ripened fruit suggest there is an association between α-farnesene production and ethylene production, although their biosynthetic pathways are clearly independent. It is likely that the general increase in metabolism initiated by the onset of the endogenous ethylene climacteric (or application of propylene) may stimulate glycolysis and the supply of substrates and activate some of the enzymes in the α-farnesene biosynthesis pathway (Figure 5.44). Rupasinghe et al. (1998) showed that α-farnesene is derived directly from farnesyl pyrophosphate in ‘Delicious’ apples and is localised predominantly in the epidermal and hypodermal layers. They also suggested that α-farnesene is derived from the isoprenoid (MVA) pathway, however their radiolabelled precursor data do not support this, and should be re-examined in the light of reports of a mevalonate independent pathway (Lichtenthaler et al., 1997). Rupasinghe et al. (1998) further showed that the incorporation of radiolabelled farnesyl pyrophosphate into α-farnesene was nearly 3-fold lower in scalding skin tissue than in scald-free tissue. They suggested that at least, the final enzymatic step of α-farnesene biosynthesis is affected by scald development. This suggestion is not surprising
considering the obvious cellular damage to scalded peel tissue. If α-farnesene is synthesised via the glyceraldehyde - pyruvate pathway, which is located in the chloroplast (Lichtenthaler et al., 1997), then any dysfunction of the chloroplast during scald development, would result in lower levels of α-farnesene in scalding peel tissue. This has been consistently observed in ‘Granny Smith’ and ‘Lady Williams’ apples (Table 5.7 and 5.8) and also shown in ‘Delicious’ apples by Paliyath et al. (1997) and Rupasinghe et al. (1998). Well designed and carefully conducted experiments are required to understand the mechanism and role of α-farnesene biosynthesis and metabolism in apples.

DPA is universally used commercially to control scald. However little is known about its effects on ethylene and α-farnesene production. The application of DPA can either lower, have no effect or increase α-farnesene production (Huelin and Coggiola, 1968; Meigh and Filmer, 1969; Lurie et al., 1989a) and these responses seem dependent on variety, maturity and time in storage (Huelin and Coggiola, 1968). In the present experiments, the timing of the postharvest application of DPA had some differential effects on ethylene and α-farnesene production. For example the application of DPA at harvest when the apples were preclimacteric, reduced α-farnesene production during storage. Du and Bramlage (1994a) also showed that the application of DPA at harvest to ‘Cortland’ apples suppressed the rates of ethylene production, α-farnesene and its oxidation products, and that the maximum concentrations of these components were less than in the untreated controls. However, the effects of a delay in the timing of the DPA application on α-farnesene and ethylene production depended on the storage temperature, variety and ripening environment. Delayed DPA application to ‘Granny Smith’ and ‘Crofton’ apples stored at 10°C in air, suppressed α-farnesene production, whilst a delay in application to ‘Granny Smith’ apples at 10°C in a propylene atmosphere had no effect on α-farnesene production. This difference was probably due to the earlier initiation of α-farnesene biosynthesis by propylene so that application of DPA was too late to significantly affect α-farnesene production. At 0°C, DPA only slightly suppressed peel α-farnesene concentrations in ‘Crofton’ apples treated at harvest, and any delay in DPA application had no affect in α-farnesene production. This suggests the varietal and temperature response may be due to a change in α-farnesene biosynthesis and accumulation at 0°C and related to apple respiration and physiology. This is not consistent with the work of Watkins et al. (1993) and Du and Bramlage (1994a) who showed that even when ‘Cortland’ apples were well into their ethylene climacteric (10 and 20 weeks after harvest), the application of DPA reduced both ethylene and α-farnesene production. However, it should be remembered that DPA suppressed α-farnesene production by only 40% in fruit treated at harvest,
but no apple developed any scald symptom. This suggests that suppression of \( \alpha \)-farnesene production by DPA contributes little to its role in scald inhibition, particularly when the scald resistant, ‘Crofton’ still produces \( \alpha \)-farnesene, albeit in lower concentrations.

In general, DPA application had no significant effect on ethylene production, although there are some indicators of a delay in, or suppression, of ethylene production following DPA treatment. This was most clearly observed when DPA application was delayed until 10 and 14 days after the application of propylene, where the rise in ethylene was delayed approximately another 10 days (Figure 5.36). This delay was not observed with the DPA treatment at harvest as the apples were already preclimacteric and perhaps the ‘tree factor’ suppression of the ethylene response to the applied propylene was still very strong. However this delay in ethylene production was not observed in those fruit ripened in air (Section 5.6.5) and DPA may have even enhanced ethylene production (Figure 5.39). Du and Bramlage (1994a) reported that ethylene production and \( \alpha \)-farnesene accumulation increased when ‘Cortland’ apples were treated with DPA and ethephon at harvest. They suggested that ethephon accelerated rates of ethylene and \( \alpha \)-farnesene production and essentially counteracted the suppression of ethylene and \( \alpha \)-farnesene accumulation caused by DPA. However, the results of ethephon application to apples reported by Watkins et al. (1993) and Du and Bramlage (1994a) were from a single variety (‘Cortland’), and they reported that ethephon application to ‘Granny Smith’ had only slight effects. This suggests that the differences may result from much greater ethylene production in ‘Cortland’ than ‘Granny Smith’ (Watkins et al., 1993). This may also account for some of the varietal differences observed in these experiments, where ‘Granny Smith’ apples produced significantly less ethylene than ‘Crofton’ apples.

The large varietal differences in \( \alpha \)-farnesene and ethylene levels between ‘Granny Smith’ and ‘Crofton’ may be related to scald development. ‘Granny Smith’ always produced significantly higher concentrations of \( \alpha \)-farnesene than ‘Crofton’ apples, while ‘Crofton’ apples always produced higher rates of ethylene production than ‘Granny Smith’. As expected storage temperature had a significant effect on ethylene production. Interestingly the relationship between internal ethylene concentration and peel \( \alpha \)-farnesene concentration in ‘Granny Smith’ and ‘Crofton’ was similar at each storage temperature, although the absolute concentrations were significantly different. At a scald inducing temperature (0\(^\circ\)C) there was a positive linear relationship between internal ethylene concentration and peel \( \alpha \)-farnesene concentration. As there is a close association between internal ethylene concentration and peel \( \alpha \)-farnesene
concentration (Figure 5.34 and 5.35), the relatively lower ethylene response of ‘Crofton’ results in lower peel α-farnesene production, and supports the possibility that any factor that lowers ethylene production also lowers peel α-farnesene concentrations. At higher storage temperatures (10°C) a different relationship between internal ethylene concentration and peel α-farnesene concentration exists, suggesting a significant change in metabolism and biosynthesis. This notion is supported by observations with ‘Granny Smith’ apples, in which chilling is known to induce ethylene production (Jobling et al., 1991).

In this experiment, DPA residues were detected by GC-MS in hexane extracts of the peel of ‘Granny Smith’ and ‘Crofton’ that had not been treated with DPA. Presumably DPA from treated apples volatilised and was absorbed by non-treated fruit. The plastic lining of boxes used in this experiment was apparently insufficient to prevent cross-contamination of DPA. However the levels and timing of DPA residues produced in this way were insufficient to prevent the development of scald symptoms in non-treated but contaminated fruit. Huelin (1968) treated ‘Granny Smith’ apples with 0.01M DPA before storage at 1°C, and showed that more than 50% of the DPA residues were lost in 10 weeks, and after 30 weeks only about 5% remained. In this experiment, the rate of residue loss was dependent on storage temperature. As expected, the higher the storage temperature, the more rapid the loss. Conversely, the higher the storage temperature, the more rapidly DPA residues were detected in the peel in non-treated fruit. This suggests that DPA is volatile and capable of being absorbed by other apples in storage. Huelin (1968) found that the DPA residues were restricted to the peel (60% in the cuticle, 20% in the epidermis and 20% in the hypodermis). Presumably the waxy cuticle rapidly absorbs the volatile non-polar DPA.

The permitted maximum residue limit of DPA in apples in Australia is 5 mg.kg⁻¹ (DPIE, 1999) but some countries do allow any DPA. Therefore, residue levels may become a contentious issue in the marketing of apples, particularly when every hexane extraction of an apple fruit made by Bramlage et al. (1996) produced a positive DPA signal. They also detected DPA residues in the peel of range of apple varieties that had not been treated with DPA and confirmed that the apple storage rooms contained significant concentrations of DPA. In addition they were able to detect DPA or DPA like substances at much lower concentrations on the surfaces of freshly harvested fruit that were unlikely to have been subjected to contamination in the orchard. However there still may be a generalised source of contamination that was not identified. Although Bramlage et al. (1996) results were unclear, they raise the possibility of endogenous DPA in apples.
The high concentrations of \(\alpha\)-farnesene produced during storage (up to 500 \(\mu\)g.g\(^{-1}\) peel fresh weight) represents a substantial diversion of carbon from primary metabolism to the production of a secondary volatile metabolite. The rate of \(\alpha\)-farnesene production is surprising considering that \(\alpha\)-farnesene is not only the sole sesquiterpene in apples, but it is the only terpene and major volatile hydrocarbon. Furthermore the concentration of \(\alpha\)-farnesene far exceeds other apple volatiles. So what is the role of \(\alpha\)-farnesene in apples and why should \(\alpha\)-farnesene be involved in scalar development? The production of \(\alpha\)-farnesene may be a dysfunction of the IPP / MVA pathway, where the carbon skeletons destined for other IPP / MVA derivatives, such as carotenoids and chlorophyll are diverted to \(\alpha\)-farnesene production (Figure 5.44). This is an archaic view of secondary plant metabolites, but it still may be valid.

The evolutionary role of \(\alpha\)-farnesene in the apple volatile profile is unknown. However, it is important to note that large quantities of \(\alpha\)-farnesene are produced during and after ripening when apples attract herbivores to aid seed dispersal. In addition, a number of insect hormones are derived from sesquiterpenes, including the insect juvenile hormone and the male sex attractant. Since insects appear unable to manufacture sesquiterpenes, they presumably derive the carbon skeletons for these compounds from their plant food. Cavill et al. (1967) showed that \(\alpha\)-farnesene accounted for 4% of the live weight in the myrmicine ant, *Aphaenogaster longiceps*. Sutherland et al. (1974) showed that \(\alpha\)-farnesene plays an important part in the successful entry of young codling moth (*Lasseyresia pomonella*) into the apple fruit. \(\alpha\)-Farnesene has also been shown to be an attractant for newly hatched larvae of apple leaf roller (*Epiphyas postvittana*) and codling moth and as an oviposition stimulant for codling moths (Sutherland et al., 1974).

Since \(\alpha\)-farnesene has been implicated in numerous evolutionary functions in insects, it is possible its association with the physiological disorder during storage is ‘accidental’. The continuous long term cold storage, especially CA storage of apples is an ‘unnatural’ process that has been imposed in the last 100 years with the development of cool storage systems. Therefore the accumulation and metabolism of \(\alpha\)-farnesene under these conditions has not been subject to evolutionary pressures. Thus, it should not surprising that occasional physiological dysfunctions, such as scald occur during cold storage.
5.6 Discussion

Reliable and accurate methods for measuring apple volatiles are essential to study the role of apple volatiles in scald development. The current and almost universal method for α-farnesene quantification relies on UV absorption at 232nm of crude hexane washes from the surface of the apples. This method is inherently unreliable. Most importantly, α-farnesene is located in both the waxy cuticle and in the peel where scald develops. Although there may be roles for α-farnesene in the waxy cuticle, such as a reservoir of α-farnesene, a more accurate estimate of α-farnesene in the peel must include extraction of both the cuticle and the peel, not just surface washes. Interference from other UV absorbing compounds can also pose problems. Meaningful and reproducible methods for extracting, isolating and quantifying apple volatiles were therefore developed. α-Farnesene concentrations in hexane extractions of ground peel were determined by FID-GC. Other apple volatiles were measured using a calibrated static headspace GC system. These new methods were the basis for the re-examination of the volatiles theory in scald development.

The volatile theory of scald development was first proposed by Brooks et al. (1919) who suggested that a volatile compound(s) produced by apples is responsible for the development of scald. This hypothesis is now generally accepted (Ingle and D'Souza, 1989). Despite considerable work to investigate this theory, the published results are often contradictory and difficult to interpret (Smock, 1961; Huelin and Coggiola, 1968; Wilkinson and Fidler, 1973; Du and Bramlage, 1993). Numerous workers have reported that one sample of apples can influence the development of scald in another variety (Southwick and Smock, 1945; Smock, 1961). If the scald inducing factor was a volatile produced by apples, then the incidence of scald should be related to the concentrations of the volatiles in the atmosphere. It may then be assumed that more scald would develop on apples subject to volatiles from other apples, and perhaps scald may develop in a variety that does not normally develop scald symptoms when the volatiles of a scald susceptible cultivar were passed over them. The present results showed that scald did not develop on a scald tolerant variety ('Crofton') or DPA treated ‘Granny Smith’ fruit even though they were continuously ventilated with high concentrations of apple volatiles. Indeed, the level of scald in 'Granny Smith' apples did not change when apple were continuously challenged by volatiles from a scald tolerant or a scald susceptible variety. However other factors such as aeration, ethylene concentrations, relative humidity, fruit maturity may influence these observations.
Since its discovery in 1966, α-farnesene has been central to the examination of the causes of scald (Huelin and Murray, 1966; Anet, 1972a; Ingle and D'Souza, 1989). The present experiments have shown that it is important not to rely on generalisations on a single variety. For example, most research has focussed on one scald susceptible variety, such as ‘Cortland’ or ‘Granny Smith’, and important varietal differences that may affect scald development have been neglected. This study showed that although scald susceptible ‘Granny Smith’ produced more α-farnesene than the scald tolerant ‘Crofton’, another scald susceptible variety, ‘Lady Williams’ had intermediate concentrations of α-farnesene and was clearly affected by harvest maturity. Although late harvested ‘Lady Williams’ produced α-farnesene at harvest (91 μg.g⁻¹), the levels of scald were significantly lower than in early harvest fruit.

Inconsistencies between scald development and α-farnesene concentration are common, and are often better described by the oxidation products of α-farnesene, measured of UV absorbance (Huelin and Coggiola, 1968; Anet and Coggiola, 1974a; Chen et al., 1990, Gallerani and Pratella, 1992). However, even these correlations often do not hold (Meir and Bramlage, 1988; Du and Bramlage, 1993). Although these methods in examining scald have been useful, new approaches and techniques are necessary to re-examine the biochemical and physiological causes of scald. Recent research on the chemistry of α-farnesene (Spicer et al., 1993; Brimble et al., 1994a; Rowan et al., 1995) has begun to explore the role of non-volatile α-farnesene oxidation products in scald. The present experiments showed that the major volatile oxidation product of α-farnesene, 6-methyl hepten-2-one, is an excellent indicator of scald development. Indeed the differences in volatiles between scalded and non-scalded peel tissue are due mainly to the large concentrations of 6-methyl hepten-2-one and lower concentrations of α-farnesene. In contrast, Paliyath et al. (1997) showed that although non-scalded ‘Red Delicious’ apples evolved higher amounts of volatiles such as hexyl acetate, butyl hexanoate, propyl octanoate, hexyl hexanoate and α-farnesene, there were no major qualitative changes in volatiles with the development of scald. Perhaps the static headspace method used in the present experiments promoted the vapour equilibration of the lipophilic α-farnesene and 6-methyl hepten-2-one, which was not observed by Paliyath et al. (1997).

The oxidation of α-farnesene to 6-methyl hepten-2-one is free radical mediated and presumably involves both the peroxyl radical (ROO•) and the alkoxyl radical (RO•). The observation that 6-methyl hepten-2-one was either not detected or present in very low concentrations in peel tissue which did not show scald symptoms, ie DPA treated or ‘Crofton’ apples, suggests that DPA or
endogenous antioxidants prevent the in vivo oxidation of α-farnesene. This supports an association of α-farnesene oxidation with scald development, where the oxidation proceeds via free radical intermediate which can be deleterious to cell integrity. In tissue that does not develop scald, the oxidation of α-farnesene is presumably either quenched by the free radical scavenging DPA or by endogenous antioxidants.

Considering the apparent importance of α-farnesene in scald development, and ethylene in apple physiology, it is surprising how few comprehensive studies have explored this relationship. The results presented here have shown that α-farnesene production was stimulated by the application of propylene, an active analogue of ethylene, whilst in those fruit ripened in air, α-farnesene production coincided with ethylene. This suggests that α-farnesene production is dependent on ethylene, although their biosynthetic pathways are independent. If α-farnesene is synthesised in the chloroplast, it is likely that its oxidation products would eventually disrupt the chloroplast membrane leading to a breakdown of in chloroplast integrity and function. This would not only significantly affect α-farnesene production, but would support the current scald hypothesis where the oxidation of α-farnesene disrupts the chloroplast membranes allowing PPO to be released from the chloroplast. However our understanding of α-farnesene biosynthesis and metabolism is not complete, particularly the histology of its production and catabolism.

DPA is a secondary amine antioxidant with numerous postharvest effects (Lurie et al., 1989a). DPA applied at harvest significantly lowered α-farnesene production in ‘Granny Smith’ apples during storage, whilst in ‘Lady Williams’ apples the effects of DPA were less obvious and the concentration of α-farnesene in late harvested ‘Lady Williams’ were higher than in the controls. However, the timing of the DPA application has significant effects on α-farnesene production. DPA application at harvest suppressed α-farnesene production, but delaying DPA application into the climacteric phase affected both ethylene and α-farnesene production. However, these responses were dependent on the ripening environment, variety, storage temperature and time. Delaying DPA application in ‘Granny Smith’ apples at 0°C did not affect α-farnesene production, but at 10°C, a delay in DPA application to ‘Crofton’ and ‘Granny Smith’ apples suppressed α-farnesene production. These results show that DPA application does not significantly alter the internal concentrations of ethylene in ‘Granny Smith’ or ‘Crofton’ apples during storage.

Viable differences in α-farnesene and ethylene production were significant and maybe related to scald development. Whereas ‘Granny Smith’ apples produced very high concentrations of α-
farnesene and lower levels of ethylene, ‘Crofton’ produced lower levels of α-farnesene (maximum concentration of 77 μg α-farnesene per g fresh weight), and higher levels of ethylene. The production of α-farnesene in ‘Crofton’ apples during early storage, when scald is thought to be initiated in susceptible varieties, was significantly lower than in ‘Granny Smith’. This supports the current theory about the role of α-farnesene in scald development. However, the occurrence of scald in late picked fruit ‘Lady Williams’ without the accumulation of large concentrations of α-farnesene or its oxidation products contradicts this. Possibly the oxidation of α-farnesene is a consequence of oxidative stress and the initiation of free radical mediated oxidations that can occur in its absence. If α-farnesene has a scald promoting role, it could be as a participant in a chain reaction rather than an initiator.
Chapter 6  Phenolics and the Development of Superficial Scald

6.1 Introduction

Phenolics are important secondary plant metabolites that have a range of functions (Macheix et al., 1990). Phenolics are not only substrates for oxidative enzymes, such as polyphenol oxidase (PPO) and peroxidase (POD), but can also act as antioxidants, protecting the cell from deleterious oxidative damage. Phenolics have been implicated in the development of scald (Bain and Mercer, 1963). The current hypothesis on the cause of scald involves the disruption of intercellular membranes which allows the mixing of PPO and the phenolic substrates. This uncontrolled PPO system is thought to be responsible for the electron dense deposits that are a characteristic symptom of scald in the peel of scald affected tissue (Bain and Mercer, 1963). Given the general acceptance of this theory, it is surprising that the role of phenolics in the development of scald in apples is unknown.

As early as 1924, it was suggested that phenolics were related to the development of scald in ‘McIntosh’ apples (Sando, 1924). However, there have only been a few studies of the role of phenolics in the development of scald. Much of the early work on apple phenolics was unreliable due to uncertainties in their separation, identification and quantification. The early methods of separation and identification were generally based on crude extracts and gross spectroscopic assays. New techniques such as diode array high performance liquid chromatography (HPLC), $^1$H and $^{13}$C nuclear magnetic resonance (NMR) and mass spectrometry (MS) have enabled the accurate determination and quantification of apple phenolics. Recently Piretti et al. (1994, 1996) attempted to link the oxidation of $\alpha$-farnesene with the reduction of phenolics, which subsequently polymerise and produce the scald symptoms, while Abdallah et al. (1997) suggested some phenolic components were antioxidants preventing scald development. There is an obvious need for more detailed exploration of the role of phenolics in the development of scald, not only as substrates in browning reactions, but also their potential as antioxidants that protect the cell from oxidation.

The objective of this section of work was to examine the role of apple peel phenolics in the development of scald.
The specific aims of this work were:

- review published literature on the biochemistry of phenolics in apples,
- reliably separate, identify and quantify apple peel phenolics,
- measure changes in the concentration of phenolics in 'Granny Smith', 'Lady Williams' and 'Crofton' apple peel during long term storage in air at 0°C,
- quantify the differences in concentration of phenolics in scalded and non-scalded peel tissue,
- compare changes in peel phenolics during storage between fruit harvested immature and at optimum storage maturity,
- re-examine the activities of polyphenol oxidase and peroxidase in the peel of 'Granny Smith', 'Lady Williams' and 'Crofton' apples during storage, and
- examine a possible role of peroxidase in the metabolism of phenolics.
6.2 The Biochemistry and Physiology of Apple Phenolics

6.2.1 Introduction

Phenolics include a wide range of compounds that possess an aromatic ring with at least one hydroxyl group. The major classes of phenolics found in mature apple fruit are phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavonols, flavans, anthocyanins) and tannins. Appendix II contains a summary of the literature on phenolics identified in both the peel and flesh of mature apple fruit.

6.2.1.1 Phenolic acids

Phenolic acids include the hydroxybenzoic acids and the hydroxycinnamic acids and their derivatives.

*Hydroxybenzoic acids* Hydroxybenzoic acid derivatives arise directly from benzoic acid and have the general structure of C₆-C₁ (Figure 6.1A). The variations in structure result from hydroxylation and methoxylation of the aromatic ring. Hydroxybenzoic acids are not widely distributed in apples and are usually found in either a bound form or as free acids (Bilyk *et al.*, 1988; Delage *et al.*, 1991).

![Benzoic acid derivative](image)

**Figure 6.1A** Benzoic acid derivative

*p*-hydroxybenzoic acid

*Hydroxycinnamic acids* Hydroxycinnamic acid derivatives are derived from cinnamic acid and have the C₆-C₃ skeleton. There are four basic structures; *p*-coumaric, caffeic, ferulic and sinapic acids. Hydroxycinnamic acids are very rarely found as free acids and are usually found in association with other compounds (Möller and Herrmann, 1983). The coumaroyl quinic and caffeoyl quinic acid ester derivatives (such as chlorogenic acid) are the most common esters in both the flesh and the peel of apples (Figure 6.1B) (Möller and Herrmann, 1983; Risch and Herrmann, 1988). Ferulic acid is less common and is usually associated with the core and flesh (Mosel and Herrmann, 1974b; Möller and Herrmann, 1983) whilst sinapic acid is rarely found in apples (Möller and Herrmann, 1983; Bilyk *et al.*, 1988). Coumarins are also classified as
hydroxycinnamic derivatives, and more than 1,000 coumarins occur in nature (Macheix et al., 1990), however no coumarins have been isolated from apples.

![Figure 6.1B](image)

**Figure 6.1B** Hydroxycinnamic acid derivative

5'-caffeoylquinic acid = chlorogenic acid

6.2.1.2 Flavonoids

All flavonoids contain the diphenylpropane skeleton (C₆-C₃-C₆) in which the three carbon bridge between the phenyl groups is cyclised with oxygen (Figure 6.1C). The different classes of flavonoids differ in the oxidation level of the central pyran ring (except in chalcones where it is open). The three most common classes of flavonoids found in apples include anthocyanins, flavonols and flavan-3-ols, the latter being present as monomers and in condensed forms (procyanidins).

![Figure 6.1C](image)

**Figure 6.1C** Generalised flavonoid structure

6.2.1.3 Flavans

Flavans are flavonoids incorporating a saturated 3-C chain. There are two types of flavans; flavan-3,4-diols (leucoanthocyanidins) and flavan-3-ols. Apparently the monomeric flavan-3,4-diols are unstable and are rarely found in fruit (Lea and Timberlake, 1974). The flavan-3-ols particularly [+]catechin (Figure 6.1D) and [-]-epicatechin are widespread in fruits and are generally found in the free form as monomers, rather than glycosylated or esterified.

![Figure 6.1D](image)

**Figure 6.1D** Monomeric flavan-3-ol

(+)-catechin
6.2.1.4 Tannins

There are two types of tannins; hydrolysable and non-hydrolysable or condensed tannins. The hydrolysable tannins may be degraded under hydrolytic conditions into simpler fragments, mainly sugars and some phenolic acids. Hydrolysable tannins are complex polyphenols that are polymers based on gallic acid and/or hexahydrodiphenic acid and their derivatives. Mature ‘Granny Smith’ apple fruit has a complete absence of hydrolysable tannins, whereas the condensed tannins are plentiful (Foo and Porter, 1981).

Proanthocyanidins are the only major oligomeric group of flavonoids. Procyanidins (condensed or non-hydrolysable tannins) possess a dihydroxy B ring and are found in mature apple fruit (Foo and Porter, 1981; Guyot et al., 1997). They are oligomeric structures based on flavan-3-ols in which the flavan bonds are most commonly C-4 to C-8 interflavan linkages (Figure 6.1E). Oligomeric forms of procyanidins are built up by the addition of additional flavan-3-ol units. Guyot et al. (1996a) showed that the procyanidins in the peel of the cider apple, ‘Kermerrrien’, comprise of mainly (-)-epicatechin units with up to 17 monomeric units. The study of procyanidins is extremely complex due to the ambiguous nature of the mass spectra and NMR data (Spanos et al., 1992). Foo and Porter (1980), Haslam (1989) and Porter (1993) discuss the biochemistry of the procyanidins. The three major dimeric procyanidins, B1, B2 (Figure 6.1E) and B5 are widespread in fruit and are commonly found in apple fruit (Lea and Timberlake, 1974; Mayr et al., 1995).

![Figure 6.1E Procyanidin](attachment:image.png)

**Figure 6.1E Procyanidin**

(-)-epicatechin-4-β6-epicatechin

procyanidin B2
6.2.1.5 Flavonols

Flavonols are flavonoids characterised by an unsaturated 3-C chain with a double bond between C-2 and C-3 and by the presence of the hydroxyl group in the 3-position. The flavonols in apples exist only as glycosides of quercetin (3',4' diOH) (Dick et al., 1987; Oleszek et al., 1988). There have been some reports of kaempferol (4'OH) glycosides (Herrmann, 1976) but the supporting data are very limited. Glycosylation occurs enzymatically and allows the flavonoids to be more soluble, permitting storage in the vacuole (Macheix et al., 1990). The monoglycosylation preferentially occurs on the hydroxyl group in the 3' position, where glucose is the most common glycone. Other sugars such as galactose, rhamnose, xylose and arabinose are glycosylated as mono-saccharides, or linked as di- and tri-sacccharides. The most widespread flavonol diglycosides are the 3-rutinosides (rutin) in which a rhamnose and a glucose molecule are joined by a 1-6 bond (Figure 6.1F). Triglycoside flavonols have been found in fruits however they have not been identified in apples (Macheix et al., 1990).

![Flavonol](image)

**Figure 6.1F** Flavonol

**quercetin rhamnoglucoside = rutin**

6.2.1.6 Chalcones

Chalcones are characterised by a C_{15} skeleton with an open 3-C chain and are rarely found in fruit. Dihydrochalcones such as phloretin derivatives (Figure 6.1G) are virtually unique to the genus *Malus* in the plant kingdom. Although the majority of dichalcones are located in the leaves, bark and seeds (Hunter and Hull, 1993), phloretin glycosides have been identified in the fruit of numerous apple varieties (McRae et al., 1990; Oleszek et al., 1988; Coseteng and Lee, 1987). Lu and Foo (1997) recently confirmed the presence of 3 different phloretin glycosides in apples.

![Dihydrochalcone](image)

**Figure 6.1G** Dihydrochalcone

**phloretin 2'-glucose = phloridizin**
6.2.1.7 Anthocyanins

The pigments responsible for the red colouration of apple peel are predominantly anthocyanins, although chlorophyll and carotenoids dominate the background colour. Non-red apples such as ‘Granny Smith’ and ‘Golden Delicious’ accumulate similar levels of flavonoids (Burda et al., 1990), but seldom synthesise anthocyanidins. Anthocyanins are glycosides that release aglycone forms (anthocyanidins) by hydrolysis. Anthocyanidins are themselves hydroxylated and methoxylated derivatives of phenyl-2-benzopyrrole (flavylium salt structure). Cyanidin is the most ubiquitous anthocyanidin in the Rosaceae and is the only phenyl-2-benzopyrrole found in apple fruit (Figure 6.1H). Although acylated anthocyanins have been isolated from the red peel of some apple varieties (Timberlake and Bridle, 1971), the acylated forms have not been further identified in other studies.

![Figure 6.1H Anthocyanidin cyanidin 3-galactoside](image)

6.2.2 Phenolic Biosynthesis and Metabolism

6.2.2.1 Biosynthesis

The biosynthesis of phenolics in fruit is generally well known and was reviewed by Macheix et al. (1990) and Mol et al. (1988), and discussed by Stafford (1990) and Heller and Forkman (1993). Phenylalanine is an aromatic amino acid that is produced by the shikimate pathway and is the precursor for most of the phenolic compounds in fruit synthesised via the phenylpropanoid pathways. The hydroxycinnamic acids and their coenzyme A esters, are common structural elements to the benzoic acids, flavonols, flavan-3-ols, dihydrochalcones and procyanidins. These relationships are represented in Figure 6.2. The basic skeleton of the flavonoids is derived from three molecules of malonate, in the form of malonyl-CoA which combine with cinnamic acid (cinnamyl-CoA) to form a chalcone, after which ring closure gives the base structure for the flavonoids.
Although the vacuoles are probably the site of anthocyanin and flavonoid accumulation they are not the sites of biosynthesis. 
Hrazdina and Wagner (1985) suggest that the biosynthesis of phenylpropanoids and flavonoids occurs on a complex consisting of membrane associated, consecutively assembled enzymes in the endoplasmic reticulum (ER). In their model, Hrazdina and Wagner (1985) suggest that the products are then sequestered in specific regions of the ER that is destined for vesiculation to form transport vesicles. The glycosylated flavonoids are then transported and stored in the vacuole.

![Phenolic biosynthesis and metabolism diagram]

**Figure 6.2** Phenolic biosynthesis and metabolism
6.2.2.2 Glycosylation

An important part of phenolic metabolism is the glycosylation of phenolics (Hosel, 1981; Heller and Forkmann, 1993). Phenolics are glycosylated to increase their water solubility and are then sequestered in the vacuole as glycosidic esters. Phenolic glycosylation is discussed in more detail in Section 6.8.1.2. UDP glucose:flavonoid 3-O-glycosyltransferase (UFGT) is an enzyme that is associated with the cytoplasm and adds a glucose molecule to the C3 position of the C ring (Hosel, 1981). The flavonoid glycosyltransferases have been reported to play a regulatory role in phenolic metabolism (Heller and Forkman, 1993; Lancaster, 1992), and may serve as an important recognition signal for trans-membrane transport (Heller and Forkmann, 1993. However Ju et al. (1995) showed that UFGT may only play a minor role in apple peel phenolic metabolism and is probably dependent on the stage of fruit development.

6.2.2.3 Glycosidases

Glycohydrolases (glucosidases and galactosidases) enzymatically remove secondary heterosides from aglycones and are necessary in the metabolism of phenolics and sugars. Hosel (1981) reviews the extent of specificity. Since nearly all naturally occurring plant glycosides are β-linked, and glycosidases show an absolute specificity with regard to the configuration of the linkage hydrolysed, only β-glycohydrolases are discussed (Hosel, 1981). Hosel (1981) suggests that some β-glycosidases are substrate specific, but many have broad substrate specificity even between phenolic classes. Hosel (1981) and Marty et al. (1991) suggest the β-glycosidases are generally associated in the vacuoles or tonoplast.

6.2.2.4 Metabolism

Several factors play an important part in phenolic metabolism and biosynthesis. These factors include; light (Dong et al., 1995), temperature (Faragher, 1983), ethylene (Faragher and Brohier, 1984; Tomas-Barberan et al., 1997), wounding (Ke and Saltveit, 1988; Tomas-Barberan et al., 1997), growth regulators (Macheix et al., 1990; Gomez-Cordoves et al., 1996), cellular sugars (Gianfagna and Berkowitz, 1986), mineral and water status (Lea and Beech 1978), and the postharvest storage environment (Blankenship and Richardson, 1985; Burda et al., 1990).

With respect to the co-ordination of biosynthesis, Lister et al. (1994) suggested that there was a coordination of enzymes in the flavonoid biosynthetic pathway during fruit development. Mayr et al. (1995) showed that the pool of hydroxycinnamic acids and the monomeric flavanols decreased in favour of the flavonols and procyanidins. Mayr et al. (1995) suggested that the shift in flavanol pools from monomeric to oligomeric structures during fruit growth indicated the
biosynthetic tendency towards the formation of procyanidins at the end of the growing period. This was confirmed by Lister et al. (1994) who showed that cyanidin glycoside synthesis in 'Splendour' apples is accompanied by a corresponding increase in quercetin glycoside and procyanidin synthesis. Ju et al. (1995) further showed that some regulatory enzymes (eg UFGT) of quercetin glycosides and anthocyanin synthesis were closely related, and that the formation of the final storage phenolics is due to the presence of their precursors rather than final enzymatic activity. Mayr et al. (1995) concluded that the diversity of phenolics and the developmental variability indicates that the interrelation between phenolics is more complex than simply a linear relationship. This may have some implications for the development of scald, as it is well known that earlier harvested fruit tend to have higher levels of scald than fruit harvested later.

6.2.3 Previous Work on Apple Phenolics

Much of the early work regarding the identification, separation and quantification of apple fruit phenolics has been inconsistent and unreliable due to uncertainties in identification and quantification. For example, total phenolics have conventionally been measured using a colorimetric assay with Folin-Ciocalteu's reagent (Slinkard and Singleton, 1977; Cilliers et al., 1990). This assay is very sensitive but not specific as compounds such as enediols and reductones interfere with the quantification (Spanos and Wrolstad, 1992).

There are numerous methods for estimating the concentration of procyanidins (Matthews et al., 1997). Most of these methods rely on colorimetric assays based on reaction with an aromatic aldehyde (vanillin and dimethyl amino-cinnamaldehyde) (Lees et al., 1995) or on their oxidative depolymerisation into anthocyanidins. Acid catalysed degradation of procyanidins is the basis of the butanol / HCl assay in which the chain extender flavan-3-ol units are oxidised to give anthocyanidins absorbing in the visible region. These reaction mechanisms are complex and must be considered with caution because the colour yield depends on the type of phenolic material and its polymerisation of the procyanidin and the selected phenolic standard (Lees et al., 1995). For example, the colorimetric assay (Vanillin - HCl) used by Lees et al. (1995b) for the quantification of procyanidins (polymeric flavan-3-ols) also detects monomeric flavan-3-ols (catechin). The flavan-3-ols are a significant component of the phenolic profile of apples, particularly during the initial stages of storage following harvest (Burda et al., 1990). Therefore this common colorimetric method does not give a reliable quantitative measure of procyanidins. Mathews et al. (1997) has since developed a quantitative method that depolymerises proanthocyanidins, which is more specific and sensitive than the colorimetric methods.
Bilyk et al. (1988) concluded that the use of single wavelength detection in HPLC could lead to faulty assumptions about the peak identification and incorrect quantification. They also suggested that instead of relying solely on the retention time for identification, the comparison of absorbance ratios helped eliminate inaccurate identifications and faulty assumptions about the homogeneity of peaks. However, even with this procedure it is difficult to assign meaningful absorption ratios. The development and availability of new techniques such as diode array HPLC, $^1$H-nuclear magnetic resonance (NMR), $^{13}$C-NMR and fast-atom bombardment mass spectroscopy (FAB-MS) has enabled the accurate identification of apple phenolics (Dick et al., 1987; Oleszek et al., 1988). Section 6.3 describes the extraction, identification and quantification of phenolics in apple peel.

6.2.4 Role of Phenolics in Apple Storage

Phenolic compounds are important constituents of apples, providing a range of important biological functions, and have been implicated with numerous metabolic processes (usually inhibitors) (Macheix et al., 1990; Larson, 1988; Shahidi and Wanasundara, 1992). However, most of these studies have been conducted in vitro with little regard to the compartmentation of the phenolics and enzymes at the cellular and tissue level. Therefore, the precise role(s) of phenolics in apples is unknown. It is possible that they can act as antioxidants, pro-oxidants and substrates for browning reactions. In addition phenolics have also been implicated in fruit quality and physiological disorders.

6.2.4.1 Antioxidants

Phenolics are well known to have antioxidant properties and are discussed in detail by Hudson (1990), Shahidi and Wanasundara (1992) and Rice-Evans et al. (1996). Phenolics can interfere with the oxidation process by reacting with peroxy radicals (Torel et al., 1986), chelating catalytic metals (Pratt and Hudson, 1990) and by acting as $\text{O}_2^-$ (superoxide ion) scavengers (Takahama, 1984; Jovanovic et al., 1994) and $^1\text{O}_2$ quenchers (Takahama, 1983). There have been numerous studies into the relationship between phenolic structure and antioxidant activity (Dziedzic and Hudson, 1983; Rice-Evans et al., 1996). The antioxidant efficacy of the phenolic acids and their esters often depend on the number of hydroxyl groups in the molecule (Dziedzic and Hudson, 1983).
The major value of the flavonoids and hydroxycinnamic acid derivatives as antioxidants, is their primary antioxidant activity (i.e., free radical acceptors and as chain breakers) and they may have a protective role, scavenging and quenching free radicals in the chloroplast and other organelles generated either thermally or by light (Caldwell et al., 1983). Indeed, catechins and quercetin are more effective antioxidants than α-tocopherol and serve as powerful antioxidants against lipid peroxidation when phospholipid bilayers are exposed to aqueous oxygen radicals (Terao et al., 1994).

There have been numerous studies examining the role of antioxidants in the development of scald in apples (Anet, 1974b; Meir and Bramlage, 1988; Watkins et al., 1988; Gallerani et al., 1990; Barden and Bramlage, 1994b, c). Sal’kova and Zvyagintseva (1981) isolated natural antioxidant compounds from the wax coating of ‘Antonovka’ apples by thin layer chromatography (TLC) that were capable of inhibiting and suppressing the oxidation of α-farnesene. These compounds were of terpenoid and polyphenolic nature, and included α-tocopherol. Anet (1974b) also isolated eleven lipid soluble antioxidants from the cuticle of 16 varieties of apple by TLC but only identified α, δ, and γ-tocopherol. He suggested that scald did not occur during storage if the antioxidant content remained adequate to prevent or limit the extent of α-farnesene autoxidation. However, these antioxidants and the autoxidation were only studied in the cuticle, whereas the vital changes concerned with scald are probably occurring in the hypodermal cells where scald develops (Bain and Mercer, 1963). Barden and Bramlage (1994b, c) determined both lipid soluble and water soluble reducing compounds in apple peel and showed that water soluble antioxidants and anthocyanidins declined during storage and lipid soluble antioxidants generally increased. They showed that in general there was an inverse relationship between antioxidant concentrations and scald development, however no single antioxidant was consistently associated with scald development. Unfortunately, gross spectroscopic assays were used for total flavonol and anthocyanin concentrations (Lees and Francis, 1972), therefore making interpretation of their results more difficult. Gallerani et al. (1990) showed that the levels of antioxidant were significantly reduced in scald affected tissue, compared to healthy green tissue of the same age.

*Pro-oxidants* There are numerous sources of free radicals in the cell which are capable of oxidative damage in the cell, including phenolic oxidation, which may cause irreversible membrane damage. The autoxidation of quinones occurs via a semi-quinone and hydroquinone (Smith et al., 1985). During this autoxidation process, dioxygen is often the oxidising agent and
is itself reduced to the superoxide anion radical ($O_2^-$), a potentially toxic species. Most semi-quinone radicals react rapidly with dioxygen to form superoxide. Thus the one electron reduction of quinones to semi-quinones and the subsequent autoxidation of the semi-quinone to the quinone can yield high quantities of $O_2^-$ (Smith et al., 1985). The enzymatic or spontaneous dismutation of $O_2^-$ yields $H_2O_2$ and $O_2$. The $O_2^-$ and $H_2O_2$ can react together in a process catalysed by certain metal ions, to form even more deleterious oxygen species such as hydroxyl radical (HO*) and singlet oxygen (Smith et al., 1985). These active oxygen species are capable of inflicting damage to membranes by processes such as lipid peroxidation.

6.2.4.2 Fruit quality

Phenolics have been suggested to play a role in the growth and development of apple fruit and may be an indication of ripening (Macheix et al., 1990). In addition, phenolics play an important role in fruit quality. Anthocyanins produce the colour in the peel that is an important factor in the appearance of apples. Procyanidins and dihydrochalcones can contribute to the astringency and ‘mouth feel’ in apples by binding to the glycoproteins of the salivary and mouth mucosa (Lea and Timberlake, 1974). In addition, phenolics can also play a role in the aroma of some ripe fruits (Knudsen et al.), but have a limited role in the impact of the apple aroma profile.

6.2.4.3 Physiological disorders

Phenolics have been correlated with the other physiological disorders in pome fruit. Wang and Mellenthin (1973a) and Wang and Mellenthin (1973b) correlated cork spot a handling problem in ‘d’Anjou’ pears, with frictional discolouration and phenolics. In addition, phenolics have also been suggested to be involved in resistance to many mechanical and biological stresses (Macheix et al., 1990).

Phenolics are known to possess anti-microbial properties, and their content in fruit after infection can increase. They may act as phytoalexins being biosynthesised de novo after infection (Macheix et al., 1990). The high levels of hydroxycinnamic acid derivatives ($p$-coumaryl - quinic esters and chlorogenic acid) in ‘Red Delicious’ fruit, particularly young immature fruit, is correlated ($r^2 = 0.78$) with the inhibition of germination of Botrytis cinerea and mycelial growth of B. cinerea, Penicillium expansum and Alterneria sp. (Nbudizu, 1976). Nbudizu (1976) suggested that the resistance to fungal infection is due to a number of factors including the high level of endogenous phenolic inhibitors. Procyanidins have also been implicated in plant defence mechanisms (Stafford, 1988).
Appel (1993) argues that although phenolics have several modes of action, the oxidised phenolics are most common. She suggests it is phenolic oxidation, more than the phenolics themselves, which has a significant effect on physiological and ecological responses and this suggestion can be seen to have some merit from re-examination of the phenolic literature. Examples include PPO browning (Mayer and Harel, 1979) and POD oxidation of esterified phenolic acids on cell walls ( Cvikrova et al., 1993), procyanidin polymerisation and haze formation (Lea, 1982).

6.2.4.4 Browning

The formation of brown pigments in bruised fruit, apple juice and the formation of hazes and sediments in juices are all associated with phenolics (Amiot et al., 1992; Spanos et al., 1990), and are thought to be associated with scald. These browning reactions are a result of the oxidation of phenolics often via PPO or POD (Vamos-Vigyazo, 1981; Mayer and Harel, 1991). These browning enzymes are discussed in more detail in sections relating to PPO and POD oxidation (Section 6.7).

6.2.5 Browning Reactions

The oxidation and polymerisation of phenolics is thought to be a key factor in the development of the brown pigmentation in scald development. Polyphenol oxidation and polymerisation have been implicated in numerous other situations. For example, the formation of haze in beer (Beart et al., 1985a), postharvest browning in persimmon fruit (Oshida et al., 1996), wine aging (Saucuer et al., 1997) and cloud formation in apple juice (Lea, 1982; Beveridge, 1997). These oxidation and polymerisation reactions may have similar modes of actions to those occurring in scalding apple peel, and have yet to be fully examined. For example the browning associated with the premature leaf blackening in Protea has been linked with PPO and POD, and non-enzymatic phenolic oxidation (McConchie et al., 1994), and similar to scald, this blackening of the leaves can be inhibited with low oxygen, controlled atmosphere storage (1% O₂, 5% CO₂) (Jones and Clayton-Green, 1992) and with antioxidant dips such as diphenylamine (1.5 mg.L⁻¹) (Jones and Clayton-Green, 1992). The following is a brief review of the phenolic polymerisation reactions that may be occurring in scalding peel tissue.
6.2.5.1 Oxidative polymerisation

*Ortho*-quinones are very reactive compounds that are the primary products of phenolic oxidation, particularly by PPO (Mayer and Harel, 1979). They can be involved in a range of different reaction pathways and have been widely reviewed (Vamos-Vigyazo, 1981; Rouet-Mayer et al., 1993). They have been studied in relation to apple browning and polymer formation by Richard-Forget et al. (1992a) and Rouet-Mayer et al. (1993).

Hathaway and Seakins (1957) showed that prolonged autooxidation of catechin leads to the formation of polymers via a head to tail polymerisation (repeated condensation reactions between the A ring of one unit and the B ring of another) through quinones. The reactivities of the o-quinones differ widely from one phenolic to another (Richard-Forget et al., 1992a), and can react with both phenolic and non-phenolic compounds, both enzymatically and non-enzymatically, to form a variety of products and co-polymers, including pigments (Rouet-Mayer et al., 1993). All of these secondary o-quinone reactions are very rapid and the stability of the o-quinones is variable, depending on the oxidised parent phenolic and the oxidation conditions (Rouet-Mayer et al., 1990). For example, the pH of the solution (Cillers and Singleton, 1989; Fulcrand et al., 1994), other phenolics (Goupy et al., 1995) and the concentration of the reactants (Richard-Forget et al., 1992a; Rouet-Mayer et al., 1993) all have significant effects on phenolic oxidation. The polymeric pigments produced differ widely in hue and intensity depending on the reactivity of the o-quinone and the environment of the oxidation reaction (Fulcrand et al., 1994; Richard-Forget et al., 1995). In addition, as the o-quinones are powerful electrophiles, they can suffer nucleophilic attack from water (Rouet-Mayer et al., 1990; Richard-Forget et al., 1992a).

6.2.5.2 Coupled oxidation

Owing to their oxidative properties, the o-quinones are able to oxidise molecules presenting lower redox potentials, such as other phenolics (Cheynier et al., 1995). The o-quinones react with other phenolics, leading to a co-polymer or re-generating the original phenolic and giving a different o-quinone by coupled oxidation (Cheynier et al., 1994). The o-quinones can react with phenolics to produce dimers, which can be subject to re-oxidation either enzymatically or by another o-quinone, resulting in the formation of larger oligomers with different colour intensities (Rouet-Mayer et al., 1993). Catechin and procyanidin polymers have been suggested to be the precursors of phenolics haze formation (Johnson et al., 1968). Lea (1982) suggested the oxidation of these precursors results in high molecular weight polymeric forms and virtually complete loss of monomeric forms of procyanidins.
6.2.5.3 Reverse disproportionation

Ortho-quinones can also lead to the formation of semi-quinone radical anions through a mechanism of reverse disproportionation (Nilges et al., 1984). These radical species are widely involved in coupling reactions of phenolics (Cilliers and Singleton, 1991; Young et al., 1987). For example, in the absence of other substrates, o-quinones may condense with the corresponding hydroquinone through a mechanism involving semi-quinone radical intermediates and polymerise (Figure 6.3). Cilliers and Singleton (1991) and Fulcrand et al. (1992) suggested that the presence of unoxidised substrate in quinone solution, might favour the reaction leading to the formation of semi-quinones. These semi-quinone radicals may combine and form dimers arising from carbon-carbon or carbon-oxygen coupling. There may be sufficient conjugation present to extend into the visible region and produce brown products (Cilliers and Singleton, 1989). In addition, these dimers may further polymerise to produce large oligomers.

![Figure 6.3](image)

**Figure 6.3 Reverse disproportionation reaction** (Cilliers and Singleton, 1991)

6.2.5.4 Other polyphenol interactions

Phenolics such as procyanidins can react with proteins and carbohydrates to form insoluble deposits (Beart et al., 1985a; Refsgaard et al., 1996). In addition, o-quinones can react with non-phenolic compounds, such as ascorbic acid, sulfites, amino acids, small peptides and large proteins, leading to Michael-type addition products (Pierpoint, 1966; Rouet-Mayer et al., 1993).

6.2.6 Phenolics and Superficial Scald

The current hypothesis on the cause of scald is that a disruption of membranes allows the mixing of PPO and the phenolic substrates. This uncontrolled PPO system is thought to be responsible for the electron dense deposits that are a characteristic symptom of scald in the peel of scald affected tissue (Bain and Mercer, 1963). Phenolics as the substrates to browning and the antioxidant properties of apple peel phenolics have not fully been studied. There have been a few studies into the role of phenolics in the development of scald. Duvenage and De Sward
(1973) examined the changes in the concentration of the total phenols, flavonols and leucoanthocyanins in the peel of a scald resistant variety, ‘White Winter Pearmain’, and the susceptible variety, ‘Granny Smith’. Using relatively crude identification and quantification procedures they showed that the concentration of lower molecular weight (absolute methanol extractable) flavonols and leucoanthocyanins (flavan-3,4-diols) increased initially, and then declined during cool storage. They concluded that DPA inhibited both the synthesis and oxidation of flavonols during storage. However their evidence for this conclusion is limited. Duvenage and De Sward (1973) also found an initial decrease in the higher molecular weight (50% aqueous methanol extractable) flavonols and leucoanthocyanins during storage. They suggested that this was a function of the polymerisation of oxidised flavonols and leucoanthocyanins. After transferring the fruit from cool storage to room temperature, Duvenage and De Sward (1973) concluded that there was a close relationship between the leucoanthocyanin content of the apple peel and the degree of browning or susceptibility to scald. However the evidence they presented does not appear to fully support their generalised conclusions.

A major problem with their results was the extraction, identification and quantification procedures used. For example leucoanthocyanins are rare in fruit and have not been described in apple fruit (Macheix et al., 1990). However, the quercetin glycosides are a prominent phenolic in the peel of stored apples and may have been in the leucoanthocyanin fractions examined by Duvenage and De Sward (1973). This still does not explain the polymerisation and production of the complex tannins thought to be responsible for the brown pigments associated with scald, as there is no evidence to support the direct coupling of flavonol glycosides to polymerisation reactions.

In other apple peel phenolic studies, Lee (1992) reported that the scald resistant ‘Empire’ apple had levels of total phenolics in the skin similar to those in the scald susceptible variety, ‘Cortland’. However, there tended to be greater levels of flavan-3-ols in ‘Cortland’ and greater levels of flavonols in ‘Empire’ peel. Burda et al. (1990) showed the levels of total phenolics in the peel of ‘Empire’ were similar to ‘Golden Delicious’, but significantly lower than those in ‘Rhode Island Greening’, and this was reflected in the trends in epicatechin and quercetin glycoside concentrations.

Piretti et al. (1994) showed in the peel of stored ‘Granny Smith’ apples there was a general decline in the concentration of the major polyphenols (epicatechin, quercetin glycosides,
procyanidin B2, and other unidentified polyphenols) with time. They also showed that there were no significant differences among the phenolic profiles of ‘Granny Smith’ apples subject to a postharvest DPA treatment, temporary storage at 20°C for 10 days until yellowing (over-ripening) prior to cold storage, low oxygen storage (1% O₂ and 2% CO₂) and untreated control apples during and after storage at 0°C and 95% RH for 6 months. They concluded from storage experiments with ‘Granny Smith’ apples, that the oxidative coupling of the o-diphenols in scald affected peel is the most likely explanation of the browning associated with scald. Piretti et al. (1994) found no evidence to involve flavonols, condensation between flavonoid glycosides and gallic acid or polymerisation of flavan-3,4-diols with scald development.
6.3 Identification and Quantification of Apple Peel Phenolics

6.3.1 Introduction

Much of the early apple literature regarding the identification, separation and quantification of apple fruit phenolics is inconsistent and unreliable due to the uncertainties in identification and quantification. However, the development and availability of new techniques such as diode array HPLC, $^1$H-nuclear magnetic resonance (NMR), $^{13}$C-NMR and fast-atom bombardment mass spectroscopy (FAB-MS) has enabled the accurate identification of apple phenolics (Dick et al., 1987; Oleszek et al., 1988). This section describes the extraction, identification and quantification of phenolics in apple peel.

6.3.2 Materials and Methods

6.3.2.1 Semi-preparative HPLC

Ground apple peel (10g) was extracted in 80% methanol (50 mL). The peel was sonicated (Soniclean 120T) for 20 min at room temperature, then centrifuged at 4,000 g for 10 min in a bench top centrifuge with swing out heads. The extract was passed through a C-18 Sep-Pak column (Waters, Millipore) to remove waxes and chlorophyll, then concentrated on a rotary evaporator.

A Dynamax semi-prep C-18 column and Varian 9012 HPLC was used to separate the phenolics. A 540 μL sampling loop and Rheodyne injector were used to introduce the sample into the system. A solvent program which used (A) 0.1% trifluoroacetic acid (TFA) in water and (B) 0.1% TFA in acetonitrile as the mobile phase, with a flow rate of 4 mL.min$^{-1}$ was employed. After equilibration and injection, the column was eluted for 15 min at 10% acetonitrile, and then the concentration was increased to 15% after 25 min. The level of acetonitrile remained at 15% until 40 min, when it was increased to 50% over 10 min. The compounds were monitored at 280 nm, and the fractions of interest were collected. Five to eight semi-preparative runs were conducted per apple extract to collect sufficient sample for further analysis. To check the retention time and purity of the collected fractions, each fraction was re-injected into the analytical HPLC (Section 6.3.2.7). The solvent was removed by freeze drying.
6.3.2.2 Semi-preparative HPLC of flavonols

As the flavonols (RT > 40 min) were not well separated on the semi-preparative HPLC column, the flavonol fractions were collected and re-run with a second semi-preparative method. Acetonitrile was initially 15% for 40 min, when it was increased to 20% for the next 10 min. Acetonitrile was then increased to 50% over 5 min and finally was reduced to 15% over the last 5 min of the separation. To check the retention time and purity of the collected fractions, each fraction was re-injected into the analytical HPLC (Section 6.3.2.7).

6.3.2.3 Determination of flavonol aglycone

To confirm that the flavonols in the phenolic profile were aglycones of quercetin, a combined apple extract was acid hydrolysed. Excess HCl (2M) was added to a combined ‘Granny Smith’ peel methanol extract and boiled for 45 minutes (Mabry et al., 1970). The resultant product was taken up in ethyl acetate and analysed by the analytical HPLC procedure above (Section 6.3.2.2).

6.3.2.4 Nuclear magnetic resonance

$^1$H-NMR and $^{13}$C-NMR was conducted at the University of Western Sydney, Nepean with a Varian Unity Plus 300, at 300 MHz. The software used was Varian VNMR 5.3b.

6.3.2.5 Mass spectrometer

Electrospray mass spectrometry was conducted at the University of Wollongong using a VG Quattro™ mass spectrometer equipped with an electrospray ionisation source (VG Biotech (Micromass), Altrincham, UK). This instrument has a quadrupole /hexapole /quadrupole mass analyser configuration and a mass range of 4000 Da for singly-charged species. Solvent delivery for the spray was achieved through fused silica capillary tubing by means of an ISCO (Lincoln, NE, USA) SFC-500 microflow syringe pump. A flow rate of between 5-15 μL.min$^{-1}$ was employed using a solution of 50% aqueous acetonitrile containing 1% formic acid. Samples were de-salted with an on-line small Michrom C-18 cartridge placed in the sample introduction loop of a 7125 Rheodyne injector. Samples (10μL) were loaded with the injector in the load position and samples were then delivered directly onto the C-18 cartridge. The cartridge was then flushed with several mL of MilliQ™ water to remove excess salt from the samples. The injector would then be turned to the inject position allowing the electrospray solvent to pass through the C-18 column and thereby elute the samples directly into the electrospray source.
The formation of a stable spray was assisted by a flow of nitrogen nebulising gas concurrent to the stainless steel capillary in the probe at a flow rate of \(\sim 10 \text{ L.hr}^{-1}\). In order to enhance droplet evaporation, a flow of dry, warm, nitrogen bath gas was applied counter-current to the solvent flow at a rate of \(\sim 350 \text{ L.hr}^{-1}\). The potential on the electrospray probe tip was typically -3.5 kV with -0.5 kV on the chicane counter electrode (for negative ions) and +3.5 kV and +0.5 kV (for positive ions). The photomultiplier was set to 650 V.

All MS spectra were acquired in the negative ion mode (ES\(^-\)) or positive by multi-channel analysis (MCA) at a scan rate of 1 second per 100 \(m/z\) units and between 10-30 scans were summed to obtain a representative spectrum.

### 6.3.2.6 Quantitative extraction

Apple peel (2g) samples were extracted with methanol (80%, 10mL) containing naringenin (25 \(\mu\text{g.mL}^{-1}\)) as an internal standard. The peel was sonicated for 20 min, then centrifuged at 4,000 g for 10 min. The extract was passed through a C-18 Sep-Pak column (Waters, Millipore) to remove unwanted waxes and chlorophyll.

### 6.3.2.7 Analytical HPLC

A Hewlett Packard 1090 HPLC system with diode array detection was used to separate and quantify the apple peel phenolics. A C-18 column (ODS-2, 250mm, Spherisorb) was maintained at 40°C during analysis. A solvent program which used (A) 0.1% trifluoroactic acid (TFA) in water and (B) 0.1% TFA in acetonitrile as the mobile phase, with a flow rate of 1 mL.min\(^{-1}\). The solvents were regularly sparged with helium. The column was equilibrated with 10% acetonitrile and held for 5 min following injection of 20 \(\mu\text{L}\) sample. The acetonitrile was then increased from 10% to 20% over 35 min. A second gradient from 20% to 50% acetonitrile over 5 min completed the run. The phenolics were detected using a diode array detector (from 200 to 400 nm) but were monitored at 254 nm and 280 nm.
6.3.2.8  Quantification of phenolics in apple peel

The phenolics were classified into their appropriate phenolic classes (e.g. hydroxycinnamic acid derivatives, flavonols etc). To quantify the phenolics using the internal standard, naringenin, the relative response factor for each phenolic class was calculated, using a representative phenolic from each class:

<table>
<thead>
<tr>
<th>Phenolic Class</th>
<th>Standard phenolic used for quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>dihydrochalcone</td>
<td>phloridizin</td>
</tr>
<tr>
<td>flavan-3-ol</td>
<td>epicatechin</td>
</tr>
<tr>
<td>flavonol</td>
<td>quercetin rhamnoglucoside</td>
</tr>
<tr>
<td>hydroxycinnamic acid</td>
<td>chlorogenic acid</td>
</tr>
</tbody>
</table>

The individual procyanidins (procyanidin B2, B5 and trimer) were quantified from individual procyanidin standards kindly provided by Dr. Jane Lancaster, New Zealand Institute for Crop and Food Research Limited, Christchurch, New Zealand. The unknown benzoic acid derivatives were quantified from the hydroxycinnamic acid derivatives.

The linearity of the relative response of each of the standard phenolics was examined over the range of concentrations observed in apple peel. 20 μL samples of 6.25 - 100 μg phenolic standard.mL⁻¹ in 80% methanol were injected into the analytical C-18 column using the standard analytical HPLC method (Section 6.3.2.7) and relative responses for each phenolic standard determined.

6.3.3  Results

6.3.3.1  Quantitative analytical HPLC

A typical analytical HPLC chromatogram is shown in Figure 6.4. The chromatogram was monitored at 254 nm and 280 nm. The internal standard, naringenin (25 μg.mL⁻¹) elutes at 46.4 minutes. Acid hydrolysis confirmed that the only flavonol aglycone present in apple peel is quercetin (Appendix III). Quercetin has only been found glycosylated in apple peel (Oleszek et al., 1988; Lister et al., 1994). Table 6.1 is a summary of the phenolics identified and quantified in apple peel.

The linearity of the relative response of each of the standard phenolics was confirmed over the range of concentrations observed in apple peel, i.e. 6.25 - 100 μg phenolic standard.mL⁻¹ in 80% methanol (Appendix III).
Figure 6.4  Analytical HPLC chromatogram of a ‘Granny Smith’ peel extract monitored at $\lambda = 254\text{nm}$ and $\lambda = 280\text{nm}$, as per Section 6.3.2.7.

Naringenin ($25 \mu\text{g.mL}^{-1}$) is the internal standard (RT 46.4 min)
Table 6.1  A summary of the phenolics in apple peel separated by HPLC and identified from NMR, MS and UV spectra and comparison with known standards

<table>
<thead>
<tr>
<th>#</th>
<th>phenolic class</th>
<th>phenolic name</th>
<th>RT (min)</th>
<th>lit ref</th>
<th>std RT</th>
<th>UV</th>
<th>NMR</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>benzoic acid derivative</td>
<td>unknown benzoic 1</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>benzoic acid derivative</td>
<td>unknown benzoic 2</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>benzoic acid derivative</td>
<td>unknown benzoic 3</td>
<td>6.3</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>flavan-3-ol derivative</td>
<td>unknown flavan-3-ol derivative 1</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>flavan-3-ol</td>
<td>catechin</td>
<td>8.0</td>
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<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>flavan-3-ol derivative</td>
<td>unknown flavan-3-ol derivative 2</td>
<td>8.8</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>cinnamic acid derivative</td>
<td>chlorogenic acid</td>
<td>9.7</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>benzoic acid derivative</td>
<td>1-O-benzoyl-β-D-glucose</td>
<td>9.7</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>9</td>
<td>procyanidin</td>
<td>procyanidin B2</td>
<td>10.5</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>cinnamic acid derivative</td>
<td>caffeic acid</td>
<td>11.1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>flavan-3-ol</td>
<td>unknown flavan-3-ol derivative 3</td>
<td>12.0</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>flavan-3-ol</td>
<td>epicatechin</td>
<td>12.8</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>flavan-3-ol</td>
<td>unknown flavan-3-ol derivative 4</td>
<td>13.4</td>
<td>-</td>
<td>-</td>
<td>✓</td>
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</tr>
<tr>
<td>14</td>
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</tr>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>unknown phenolic 2</td>
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<td>-</td>
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<td>ferulic acid</td>
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<td>✓</td>
<td>-</td>
<td>-</td>
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<td>unknown</td>
<td>unknown phenolic 3</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>unknown</td>
<td>unknown phenolic 4</td>
<td>25.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>procyanidin</td>
<td>procyanidin B5</td>
<td>28.3</td>
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<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>flavonol</td>
<td>quercetin galactoside</td>
<td>29.2</td>
<td>✓</td>
<td>-</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>23</td>
<td>flavonol</td>
<td>quercetin rhamnoglucoside (rutin)</td>
<td>30.0</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>24</td>
<td>flavonol</td>
<td>quercetin glucoside</td>
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<td>✓</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
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<td>unknown flavonol 1</td>
<td>32.2</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>flavonol</td>
<td>quercetin xyloside</td>
<td>33.7</td>
<td>✓</td>
<td>-</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>flavonol</td>
<td>quercetin arabinopyranoside</td>
<td>35.1</td>
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<td>-</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td>flavonol</td>
<td>quercetin arabinofuranoside</td>
<td>35.9</td>
<td>✓</td>
<td>-</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>chalcone</td>
<td>phloridizine</td>
<td>36.5</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>flavonol</td>
<td>unknown flavonol 2</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
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<td>unknown flavonol 3</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>flavonone</td>
<td>naringenin (internal standard)</td>
<td>46.4</td>
<td>-</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**RT(min)**: retention time on analytical C18 column (minutes) (Section 6.3.2.7)

**lit ref**: literature reference, ie isolated in apple peel (see Appendix II)

**std RT**: retention time of pure standard (minutes)

**UV**: ultra-violet spectra (λ, 200-400, nm)

**NMR**: nuclear magnetic resonance (¹H and ¹³C, 300 MHz) (Section 6.3.2.4)

**MS**: electrospray mass spectroscopy (Section 6.3.2.5)

Flavonol, cinnamic acid derivates and flavan-3-ol standards were obtained from Sigma Chemical Company (St.Louis, USA). The procyanidin standards were kindly provided by Dr. Jane Lancaster, New Zealand Institute for Crop and Food Research Limited, Christchurch, New Zealand.
6.3.3.2 Semi-preparative HPLC

As some of the compounds in the apple extracts were unknown, semi-preparative HPLC was conducted to isolate purified compounds of sufficient concentration for further identification. The resultant concentrates of the 'Granny Smith' control and DPA peel extracts after extraction and concentration were amber coloured oils. However, the scalded 'Granny Smith' peel concentrate was yellow in colour, and foamed very readily and required several methanol washes to remove water from the extract. Appendix III shows typical semi-preparative HPLC chromatograms of non-scalded, scalded and DPA treated 'Granny Smith' peel and 'Crofton' peel and the fractions collected for further analysis.

An unknown phenolic compound (RT 9.7 min) was consistently identified in scalded 'Granny Smith' peel tissue. This contributed over 25% of the total phenolics in scalded 'Granny Smith' peel tissue after 6 months storage. The retention time of the unknown phenolic was similar to chlorogenic acid but had a significantly different UV spectra (Figure 6.5 and 6.6). The UV spectra of the unknown peak was relatively pure (99.6%) (Appendix III).
Figure 6.5  A typical analytical HPLC chromatogram of non-scaled ‘Granny Smith’ peel tissue showing chlorogenic acid (RT 9.7 min). Figure 6.5B Comparison of the UV spectra of the chlorogenic acid peak (RT 9.7) and a pure chlorogenic acid standard which confirms the identity of chlorogenic acid in the apple peel extract.
Figure 6.6  A typical analytical HPLC chromatogram of scalded ‘Granny Smith’ peel tissue showing RT of unknown peak at 9.7 min. Figure 6.6B UV spectra of the unknown peak with characteristic UV$_{\text{max}}$ at $\lambda = 234$ nm. Figure 6.6C Comparison of UV spectra of chlorogenic acid standard and the unknown peak.
6.3.3.3 Nuclear magnetic resonance

After freeze drying, the purified unknown scald fraction (RT 9.7 min in Figure 6.6) consisted of hydrophobic white crystals which readily absorbed water and did not dissolve in chloroform, suggesting that the unknown compound was polar, possibly glycosylated. \(^1\text{H}\)- and \(^{13}\text{C}\)-NMR spectroscopy were conducted on the unknown compound using methanol (CH\(_3\)OD), DMSO-d\(_6\), water (D\(_2\)O) and pyridine-d\(_4\). The unknown compound was soluble in all these solvents.

The NMR data for the unknown peak are shown in Figure 6.7. The \(^1\text{H}\) NMR data for the scald peak run in D\(_2\)O (Figure 6.7A) and DQ COSY NMR run in DMSO-d\(_6\) (Figure 6.7B). The \(^{13}\text{C}\) NMR spectrum run in DMSO is shown in Figure 6.7C. Additional confirmational NMR data of other compounds / fractions are in Appendix III.

6.3.3.4 Mass spectra

The MS data are shown in Figure 6.8. There was consistent contamination of the MS samples (Fragment 302, 586 in the +\(^{1}\text{ve}\) mode and 329 in the -\(^{1}\text{ve}\) mode), however a consistent and strong parent ion (284) and corresponding spectra were observed in the scald fraction. Additional confirmational mass spectra of other collected fractions are presented in Appendix III.

6.3.3.5 Identification of 1-\(O\)-benzoyl-\(\beta\)-D-glucose

From the UV, NMR and MS data the unknown peak was identified as 1-\(O\)-benzoyl-\(\beta\)-D-glucose (Figure 6.9). Benzoyl-\(\beta\)-D-glucose has been identified in the fruit of some Vaccinium species including cranberries and red whortleberries (Heimhuber et al., 1990) and the UV, NMR and MS data correspond to those presented here.

![Diagram of 1-\(O\)-Benzoyl-\(\beta\)-D-glucose](image_url)

Figure 6.9 1-\(O\)-Benzoyl-\(\beta\)-D-glucose
Figure 6.7  NMR spectra of unknown scald peak (RT 9.7min)

Figure 6.7A $^1$H NMR in D$_2$O solvent. Figure 6.7B DQCOSY NMR in DMSO-d$_6$.
Figure 6.7C $^{13}$C NMR in DMSO. 300MHz NMR run as per Section 6.3.2.4.
Figure 6.8  Electrospray Mass Spectra of unknown scald peak (RT 9.7 min) in the +ve and -ve mode, as per Section 6.3.2.5. The parent ion is 284.
6.3.4 Discussion

The use of diode array HPLC to successfully isolate, identify and quantitate apple peel phenolics is crucial for accurate description of the changes in phenolics in apple peel. Previous methods such as the colorimetric Vanillin - HCl assay (Lees et al., 1995a), Folin - Ciocalteu’s reagent (Cilliers et al., 1990) and general spectroscopic assays (Ju et al., 1996) are inherently flawed due to their non-specific nature and the chemical similarities of the phenolics found in apples. Therefore, any phenolic data from non-specific assays must be treated with caution. Indeed, only regular and careful inspection of the UV spectra of all peaks was able to confirm that the compound responsible for the peak at RT 9.7 min in the ‘Granny Smith’ scalded peel extract was not chlorogenic acid, even though the RTs were the same, and chlorogenic acid is found widely in apples. The unknown compound was identified as 1-O-benzoyl-β-D-glucose.

The presence of benzoyl-β-D-glucose in scalded peel tissue is intriguing as this compound has not previously been reported in apples. Heimbuber et al. (1990) identified and quantified significant quantities of benzoyl-β-D-glucose in the fruit of some Vaccinium species including cranberries and red whortleberries while Horsley and Meinwald (1981) found benzoyl-β-D-glucose in the leaves of black cherry (Prunus serotina). The proposed pathways of benzoyl-β-D-glucose synthesis are discussed in Section 6.8.

The biological role or function of benzoyl-β-D-glucose is unknown. Horsley and Meinwald (1981) suggested that the benzoyl-β-D-glucose in black cherry leaves is a potential source of benzoic acid that has allelopathic effects by inhibiting the growth of red maple seedlings. However benzoic acid derivatives in apples, eg benzoic acid, 4 hydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid, have also been associated with pathological stresses (Brown and Swinburne, 1971; Fawcett and Spencer, 1967). Benzoates are also well known preservatives (Swanson, 1989), having a broad spectrum of anti-microbial activity (Wedzicha, 1993). Therefore Macheix et al. (1990) believes benzoic acid to be the main phytoalexin in apples.

Benzoic acid has been found to be associated with resistance of immature ‘Bramley’s Seedling’ apples to Nectria galligena (Brown and Swinburne, 1971). They showed that immature apple fruit accumulate benzoic acid to concentrations that inhibit the growth of N. galligena. Swinburne and Brown (1975) later showed that PAL activity was induced by fungal infection and that maximum benzoic acid concentration was attained approximately 3 days after maximum
PAL concentration had been reached. This suggests that the synthesis of benzoic acid is an anabolic phytoalexin response, and not a catabolic mechanism. Indeed, the biosynthetic pathways of phenolic phytoalexins are identical to naturally accumulated phenolic compounds (Stoessel, 1982).

Janovitz-Klapp et al. (1990b) showed that benzoic acid was a competitive inhibitor of apple PPO and a more effective inhibitor than sorbic acid. Pifferi et al. (1974) further showed that the esterification of the carboxyl group greatly decreased the inhibition of Prunus avium PPO, but did but suppress its affinity for the enzyme. Although benzyol-β-D-glucose may confer phytochemical resistance to PPO, it would be ironic if a competitive inhibitor of PPO is only identified in scalding tissue which is thought to be associated with the uncontrolled action of PPO (Bain and Mercer, 1963). The production of benzoyl-β-D-glucose after the appearance of the browning symptoms indicates that it is a product of scald affected tissue.

Brown and Swinburne (1971) used an ethyl acetate extraction of necrotic tissue to purify benzoic acid from the apple tissue. After acid hydrolysis (HCl, pH 2.5) and ether extraction, they found benzoic acid to be associated with a brown high molecular weight polyphenolic compound. Presumably the benzoic acid was present in an esterified form before acid hydrolysis. This parallels the situation where benzoyl-β-D-glucose was associated with browning in scalded tissue. Brown and Swinburne (1971) found the mean concentration of benzoic acid to be 220 µg.g⁻¹ fresh weight of necrotic tissue of N. galligena infected ‘Bramley’s Seedling’ apples. This is similar to the levels of benzoyl-β-D-glucose found in the peel of scalding apples, 250 -310 µg.g⁻¹ fresh weight (Figure 6.38). It is possible that the production of benzoic derivatives in apple fruit is a general response to stress, where there is a change or diversion in phenolic metabolism to the synthesis of benzoic acid derivatives. The preferential formation of glucose esters is well known (Hanson, 1966), where glucose esters are thought to be the primary detoxification products of aromatic acids (Schlepphorst and Barz, 1979).

Ndubizu (1976) also showed that phenolic compounds in apple fruit possess anti-microbial properties and their content increases after infection. He showed that extracts from maturing ‘Red Delicious’ apple fruit containing chlorogenic and p-coumaryl-quinic acids were inhibitory to germination of Botrytis cinerea spores and mycelia growth of B. cinerea, Penicillium expansum and Alternaria sp. In addition, the location of the phenolics is an important factor in phytochemical resistance. For example the high concentrations of phenolics in the peel of apples
in the epidermal layers may play a role as a barrier to natural infection (Ndubizu, 1976). This also has a parallel in apple fruit where benzoyl-\(\beta\)-\(D\)-glucose was isolated from the apple peel. Although the relationship between phenolic content and microbial resistance is sometimes poor this maybe a general phytochemical role of phenolics in fruits (Macheix et al., 1990). The development of scald and the production of benzoyl-\(\beta\)-\(D\)-glucose maybe a variation of this phytochemical response.

The de novo synthesis of phytoalexins has some parallels to the conventional scald hypothesis. Macheix et al. (1990) described the biosynthesis of phytoalexins, where healthy untreated cells contain material capable of initiating synthesis of the appropriate enzymes, but they remain inactive. When the cells are injured, these materials become active and thus enzyme synthesis and subsequently phytoalexin synthesis occurs in the injured cells and in adjacent cells which remain healthy. It has been suggested that the action of these materials is facilitated by their release as a direct consequence of membrane dysfunction (Macheix et al., 1990). Perhaps membrane dysfunction associated with the development of scald (Bain and Mercer, 1963) is responsible for an up-regulation of these phytochemical enzymes. Further work is required on the role of benzoyl-\(\beta\)-\(D\)-glucose in scalding apple peel. Preliminary work conducted in Section 6.8 examines the role of peroxidative enzymes in the production of benzoyl-\(\beta\)-\(D\)-glucose in apple peel extracts.
6.4 Changes in Peel Phenolics During Cool Storage

6.4.1 Introduction

To understand the role of phenolics in scald, it is important to fully understand and accurately describe the phenolic profile of scalding and non-scalding apple peel during cold storage. Three varieties of apples were chosen for this study. The standard green coloured peel and scald susceptible ‘Granny Smith’, a red coloured skin and susceptible variety, ‘Lady Williams’ and ‘Crofton, which is a resistant to scald development and has striped peel. These varieties provided a range of scald susceptibilities and skin colour for comparison of peel phenolic profiles in relation to scald development. In addition, the application of DPA immediately after harvest to the scald susceptible variety inhibited scald development and allowed the examination of peel phenolic profiles during storage in peel which did not develop scald.

The aims of this section were:

- examine the changes in phenolic composition during air storage at 0°C in the peel of scald susceptible (‘Granny Smith’, and ‘Lady Williams’) and scald resistant (‘Crofton’) apples
- examine the role of a pre-storage application of DPA to ‘Granny Smith’ and ‘Lady Williams’ apples on the changes of peel phenolics during storage

6.4.2 Materials and Methods

‘Granny Smith’ and ‘Crofton’ apples were harvested from a commercial orchard at Orange, NSW. ‘Lady Williams’ apples were harvested at Harcourt Vic. and transported overnight to UWS (1,000 km). The fruit of ‘Granny Smith’ and ‘Lady Williams’ were dipped in diphenylamine at 3,000 mg.L\(^{-1}\) (‘Shield-Brite\(^{®}\), Washington USA), on receipt of the fruit at UWSH. Four replicates of 25 fruit of each variety and treatment were stored in plastic lined boxes at 0°C for up to nine months.

At monthly intervals 10 apples from each replicate were removed from storage and a representative sample of the peel (50g) was carefully removed with an apple peeler, excess parenchyma tissue was scrapped from the peel tissue before the peel was frozen in liquid nitrogen, ground and stored (-75°C) for future analysis. Twenty apples were removed from each treatment at 3, 6 and 9 months and assessed for scald development after 7 days at 20°C (Section
5.2.1.7). Apple peel phenolics from the frozen tissue were extracted and quantified using the methods outlined in Section 6.3.2.7.

6.4.3 Results

6.4.3.1 Scald development

Table 5.6 summarises the incidence and intensity of scald in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apples during air storage at 0°C and shows that scald did not develop in any DPA treated fruit or ‘Crofton’. Scald developed on control (non-DPA treated) fruit of ‘Granny Smith’ and ‘Lady Williams’.

6.4.3.2 Total phenolics

Over the 9 month storage period in air at 0°C, the concentration of total phenolics in the peel of ‘Lady Williams’ was always greater than in ‘Crofton’ (except at 6 and 9 months where the differences were not significant) (Figure 6.10). The concentration of total phenolics in the peel of ‘Granny Smith’ was always lower than either ‘Crofton’ or ‘Lady Williams’. Over the storage period and across all treatments the average concentration of total phenolics in the peel of ‘Lady Williams’ (4,036 μg.g⁻¹) was higher than ‘Crofton’ (2,668 μg.g⁻¹) and in turn was higher than in ‘Granny Smith’ (1,275 μg.g⁻¹) apples. In both ‘Lady Williams’ and ‘Granny Smith’ apples, the peel of the fruit treated with DPA at harvest had more total phenolics than control fruit, but these differences were not often significant. In general, total phenolics increased during the first few months of storage and plateaued or declined during the remainder of the storage period at 0°C.

6.4.3.3 Benzoic acid derivatives

The concentrations of total benzoic acid derivatives in the peel were variable and relatively low, contributing less than 5% of the total phenolics. The concentrations of benzoic acid derivatives in the peel of ‘Lady Williams’ were generally higher than in ‘Granny Smith’ and ‘Crofton’ apples during air storage over 9 months of storage (Figure 6.11). Total benzoic acid derivatives of all apple varieties generally peaked at 2 months and slowly declined with time, except for ‘Lady Williams’ DPA peel, which showed an increase in total benzoic acid derivatives at six months, then subsequently, declined. By 9 months all apples and treatments were similar, except ‘Lady Williams’ DPA (Figure 6.11A). There were no differences in the concentrations of total benzoic acid derivatives among the DPA treatment and control fruit for both ‘Granny Smith’ and ‘Lady Williams’ over the storage period, except for ‘Lady Williams’ DPA treatment from 5 months onward, which were greater than ‘Lady Williams’ control fruit.
Figure 6.10  Concentrations of total phenolics (µg·g⁻¹ fresh weight) in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during storage in air at 0°C. DPA was applied to ‘Granny Smith’ and ‘Lady Williams’ apples before storage. Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.11 Concentrations of benzoic acid derivatives (µg·g⁻¹ fresh weight) in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during storage in air at 0°C. DPA was applied to ‘Granny Smith’ and ‘Lady Williams’ apples before storage. (A) Total benzoic acid derivatives; (B) Unknown benzoic acid derivative 1; (C) Unknown benzoic acid derivative 2; (D) Unknown benzoic acid derivative 3. Concentrations accompanied by different letters differ significantly at P<0.05.
The concentrations of the individual unknown benzoic acid derivatives were variable. There were generally few differences in the concentrations of Unknown Benzoic 1 among the apple varieties, treatments and during storage (Figure 6.11B). The concentrations of Unknown Benzoic 2 were greater in ‘Lady Williams’ than in ‘Crofton’ or ‘Granny Smith’ peel (Figure 6.11C). The significant increase in Unknown Benzoic 2 at 6 months in the ‘Lady Williams’ DPA peel was responsible for the increase in the total benzoic acid derivatives at 6 months. There was little or no Unknown Benzoic 2 in ‘Granny Smith’ (Figure 6.11C). Similarly, Unknown Benzoic 3 was only detected in ‘Lady Williams’ (Figure 6.11D). There were no differences in the levels of Unknown Benzoic 3 between the DPA treated and control peel until late in storage, where DPA treated fruit had more Unknown Benzoic 3 than control ‘Lady Williams’. Unknown Benzoic 3 was not detected in ‘Granny Smith’ and ‘Crofton’ apples (Figure 6.11D).

6.4.3.4 Cinnamic acid derivatives

All major cinnamic acid derivatives were identified in the apple peel, except sinapic acid. No coumarins were identified. The concentrations of total cinnamic acid derivatives were always higher in the peel of ‘Crofton’ throughout the storage period (Figure 6.12). The total concentration of cinnamic acid derivatives in ‘Crofton’ peaked at 2 months storage, where the maximum concentrations of cinnamic acid derivatives were more than 4 and 12 times greater than at the same storage time in ‘Lady Williams’ and ‘Granny Smith’, respectively. After 2 months storage, the levels of total cinnamic acid derivatives declined in ‘Crofton’. However, even after 9 months storage, ‘Crofton’ still had about 3 and 12 times more total cinnamic acids in the peel than in ‘Lady Williams’ and ‘Granny Smith’ respectively. The concentrations of total cinnamic acid derivatives in the peel of ‘Lady Williams’ were higher than ‘Granny Smith’ peel throughout the storage period. There were generally no differences between DPA treatment and controls for either apple variety. However, when significant differences did occur, the DPA treated apples had more total cinnamic acid derivatives than the control fruit (Figure 6.12). The only exception was with ‘Lady Williams’, where after 9 months storage the concentrations of total cinnamic acid derivatives in the control ‘Lady Williams’ peel were significantly greater than in DPA treated fruit. Total cinnamic acids remained relatively constant over the storage period in the peel of ‘Granny Smith’, but in ‘Lady Williams’ the total concentrations of total cinnamic acids increased until 3 months.
Figure 6.12  Concentrations of total cinnamic acid derivatives (µg.g⁻¹ fresh weight) in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during storage in air at 0°C. DPA was applied to ‘Granny Smith’ and ‘Lady Williams’ apples before storage. Concentrations accompanied by different letters differ significantly at P<0.05
The high concentrations of total cinnamic acid derivatives in the peel of ‘Crofton’ were mainly due to the high concentrations of chlorogenic acid (up to 412 µg.g⁻¹ fresh weight) (Figure 6.13A). The concentrations of chlorogenic acid in ‘Lady Williams’ and ‘Granny Smith’ peel were relatively low, with a maximum of 80 µg.g⁻¹ fresh weight in the peel of ‘Lady Williams’ DPA after 6 months storage. However, ‘Lady Williams’ peel always possessed significantly more chlorogenic acid than ‘Granny Smith’. In addition, DPA treated fruit had higher concentrations of chlorogenic acid than the control fruit in both varieties. The only exception was at 9 months storage where the concentration of chlorogenic acid in the DPA ‘Lady Williams’ peel was significantly lower than in the control fruit. The general trends in the concentrations of chlorogenic acid in the peel during storage were similar to those of the total cinnamic acid derivatives. In ‘Crofton’ peel, the concentration of chlorogenic acid peaked after 2 months storage and slowly declined over the storage period. However in ‘Lady Williams’ peel, the concentrations of chlorogenic acid gradually rose then remained constant for the entire storage period, whilst in ‘Granny Smith’ the concentrations of chlorogenic acid remained relatively constant (Figure 6.13A).

Changes in the concentration of caffeic acid and ferulic acid during storage were similar to the trends of chlorogenic acid, although the absolute concentrations were lower, particularly in the peel of ‘Crofton’ (Figure 6.13B, 6.13D). No caffeic or ferulic acid was detected in ‘Granny Smith’ peel over the entire storage period. Similarly, the concentrations of caffeic and ferulic acid in DPA treated ‘Lady Williams’ were generally higher than in control ‘Lady Williams’, except for the final few months of storage. By 9 months storage, the concentration of caffeic acid was higher in the control ‘Lady Williams’ peel than in the DPA treated fruit. There were no significant differences in the concentrations of ferulic acid between DPA and control ‘Lady Williams’, however by 7 months storage the concentrations of ferulic acid in the peel of control fruit were higher than in the DPA treated fruit.

The concentrations of coumaric acid were always higher in the peel of ‘Lady Williams’ than in ‘Granny Smith’ throughout the storage period. No coumaric acid was detected in ‘Crofton’ throughout the storage period (Figure 6.13C). The levels of coumaric acid remained relatively constant throughout the storage period in ‘Lady Williams’, whilst in ‘Granny Smith’ the concentration of coumaric acid declined from harvest. There were few differences among DPA and control fruit, however when significant differences in the concentration of coumaric acid occurred, DPA treated fruit had higher concentrations in the peel than control fruit.
Figure 6.13  Concentrations of cinnamic acid derivatives (μg·g⁻¹ fresh weight) in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during storage in air at 0°C. DPA was applied to ‘Granny Smith’ and ‘Lady Williams’ apples before storage. (A) Chlorogenic acid; (B) Caffeic acid; (C) Coumaric acid; (D) Ferulic acid. Concentrations accompanied by different letters differ significantly at P<0.05.
6.4.3.5 Flavan-3-ols

The three varieties contained significantly different concentrations of flavan-3-ols across the entire storage period (Figure 6.14). The highest concentrations of total flavan-3-ols in the peel were in ‘Lady Williams’, followed by ‘Crofton’ then ‘Granny Smith’ (Figure 6.14A). For most of the storage period, there were few differences between the DPA and control fruit within a variety. However, when significant differences between treatments did occur, the DPA treated ‘Lady Williams’ had higher concentrations of total flavan-3-ols than the control fruit. In the ‘Granny Smith’ peel, when there was a significant difference between treatments, the control apple peel had higher concentrations of total flavan-3-ols than ‘Granny Smith’ DPA. The total flavan-3-ols for all varieties remained relatively constant, or gradually declined over the storage time.

The concentration of catechin in ‘Lady Williams’ peel was about 3 times higher than in ‘Crofton’ and ‘Granny Smith’ (Figure 6.14B). ‘Crofton’ had significantly more catechin than ‘Granny Smith’ during the first four months of storage, but this difference became less clear over time. There were no differences in catechin concentrations among the DPA and control fruit, for either ‘Lady Williams’ or ‘Granny Smith’. Although variable, catechin concentrations in ‘Lady Williams’ generally peaked at 3 - 4 months then declined, whilst the concentration of catechin in ‘Crofton’ and ‘Granny Smith’ remained relatively constant after an initial rise for the first few months.

The concentrations of epicatechin in the peel were approximately double those of catechin, and followed similar trends to catechin (Figure 6.14C). The concentrations of epicatechin in ‘Lady Williams’ were always greater than in ‘Granny Smith’ and ‘Crofton’. When there were significant treatment differences in epicatechin concentration, they were higher in DPA ‘Lady Williams’ than on control fruit. However, DPA had no effect in ‘Granny Smith’ apples. The concentrations of epicatechin in ‘Granny Smith’ were higher than those in ‘Crofton’ peel during the early stages of storage, but these differences disappeared during the later stages of storage. In general, there was a gradual decline in the levels of epicatechin for all varieties and treatments during cold storage.

The concentrations of Unknown Flavan-3-ol Derivative 1 were statistically similar for all varieties and treatments during the first months of storage (Figure 6.15A). However, the
Figure 6.14  Concentrations of flavan-3-ol derivatives (µg g⁻¹ fresh weight) in 'Granny Smith', 'Lady Williams' and 'Crofton' apple peel during storage in air at 0°C. DPA was applied to 'Granny Smith' and 'Lady Williams' apples before storage. (A) Total flavan-3-ol derivatives; (B) Catechin; (C) Epicatechin. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.15  Concentrations of unknown flavan-3-ol derivatives (µg.g⁻¹ fresh weight) in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during storage in air at 0°C. DPA was applied to ‘Granny Smith’ and ‘Lady Williams’ apples before storage. (A) Unknown flavan-3-ol derivative 1; (B) Unknown flavan-3-ol derivative 2; (C) Unknown flavan-3-ol derivative 3; (D) Unknown flavan-3-ol derivative 4. Concentrations accompanied by different letters differ significantly at P<0.05.
concentration of Unknown Flavan-3-ol Derivative 1 in ‘Crofton’ peel gradually increased during the storage period, whereby after 9 months storage, the concentration of Unknown Flavan-3-ol Derivative 1 was more than 7 times the concentrations in ‘Lady Williams’ and ‘Granny Smith’ peel. During this time the levels of Unknown Flavan-3-ol Derivative 1 in ‘Lady Williams’ and ‘Granny Smith’ remained low and there were no differences among the 2 susceptible varieties, or DPA treatment.

There were no differences in the concentrations of Unknown Flavan-3-ol Derivative 2 or Unknown Flavan-3-ol Derivative 3 as they were low and very variable over the storage time (Figure 6.15B and 6.15C). No Unknown Flavan-3-ol Derivative 2 or Unknown Flavan-3-ol Derivative 3 were detected in ‘Granny Smith’ over the storage period. Unknown Flavan-3-ol Derivative 4 was only detected in ‘Crofton’ apples, in which its concentration remained relatively high (around 80 μg.g⁻¹ fresh weight) and constant over the entire storage period (Figure 6.15D). Unknown Flavan-3-ol Derivative 4 was generally not detected in either ‘Granny Smith’ or ‘Lady Williams’.

6.4.3.6 Flavonols

The concentrations of total flavonols in the peel of all apples were affected by time and treatment (Figure 6.16). The concentrations of total flavonols were greatest in ‘Lady Williams’ peel, reaching a maximum of up to 3,000 μg.g⁻¹ fresh weight and lowest in ‘Granny Smith’, with ‘Crofton’ having intermediate levels (Figure 6.16). Although DPA treated fruit generally had higher concentrations of total flavonols, these differences were not significant. The trends in total flavonol concentration over the storage time were similar for all apple varieties. The concentrations of total flavonols increased during the first 2 - 4 months of storage, and then either plateaued or slowly declined. This is reflected in the trends in the individual flavonols.

Quercetin rhamnoglucoside contributed around 30% of the total flavonols. ‘Lady Williams’ had significantly higher concentrations of quercetin rhamnoglucoside than ‘Granny Smith’ (Figure 6.17A). The concentrations of quercetin rhamnoglucoside in ‘Crofton’ were higher than in ‘Granny Smith’, and this difference only became statistically significant after 3 months of storage and remained significantly higher for the entire storage period. In most cases, DPA treated ‘Lady Williams’ and ‘Granny Smith’ apples had higher concentrations of quercetin rhamnoglucoside in the peel than control fruit. However the data were quite variable and there
Figure 6.16  Concentrations of total flavonols (μg.g⁻¹ fresh weight) in 'Granny Smith', 'Lady Williams' and 'Crofton' apple peel during storage in air at 0°C. DPA was applied to 'Granny Smith' and 'Lady Williams' apples before storage. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.17  Concentrations of flavonol derivatives (µg·g⁻¹ fresh weight) in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during storage in air at 0°C. DPA was applied to ‘Granny Smith’ and ‘Lady Williams’ apples before storage. (A) Quercetin rhamnogluicoside; (B) Quercetin glucoside; (C) Quercetin xyloside; (D) Quercetin arabinofuranoside

Concentrations accompanied by different letters differ significantly at P<0.05
were no significant differences in the concentrations of quercetin rhamnoglucoside among DPA treated and control fruit.

The concentrations of quercetin xyloside, quercetin arabinofuranoside and Unknown Flavonol 3 followed similar trends to quercetin rhamnoglucoside and were significantly higher in ‘Lady Williams’ peel throughout the storage period (Figure 6.17C, 6.17D and 6.18D). DPA treatment had no affect on these flavonol concentrations. The concentrations of quercetin xyloside, quercetin arabinofuranoside and Unknown Flavonol 3 increased from harvest, then plateued or declined during the storage period for all apple varieties and treatments.

The concentrations of quercetin glucoside were generally lower in the peel of ‘Granny Smith’ and greater in the peel of ‘Lady Williams’ and ‘Crofton’ (Figure 6.17B). In general, there was no difference between DPA treated and control fruit in either ‘Lady Williams’ and ‘Granny Smith’ apples. Across all treatments there was an increase in the concentration of quercetin glucoside during the first few months of storage. At most sampling times, the concentrations of Unknown Flavonol 1 were lower in ‘Granny Smith’ apples than either ‘Lady Williams’ or ‘Crofton’ (Figure 6.18C). There were no differences in the concentration of Unknown Flavonol 1 between ‘Lady Williams’ and ‘Crofton’ over the storage period. In addition, there were no differences between DPA treated or control apples for either apple variety. Quercetin arabinopyranoside and Unknown Flavonol 2 were found only in relatively low concentrations in the peel of ‘Lady Williams’. Quercetin arabinopyranoside and Unknown Flavonol 2 were undetectable or low concentrations in ‘Granny Smith’ or ‘Crofton’ throughout storage (Figure 6.18A, 6.18D). The levels of quercetin arabinopyranoside and Unknown Flavonol 2 increased during the first few months of storage and there were few differences between DPA and control ‘Lady Williams’ during most of the sampling period. However, the untreated peel had higher concentrations of quercetin arabinopyranoside and Unknown Flavonol 2, than DPA treated fruit in the latter part of the storage period, and these differences became significant towards the end of the storage period.

6.4.3.7 Procyanidins

The concentrations of total procyanidins were highest in ‘Lady Williams’ apple peel, and lowest in ‘Crofton’ (Figure 6.19A). The concentrations of total procyanidins in 'Granny Smith' peel was about 50% lower than in ‘Lady Williams’, but still significantly greater than ‘Crofton’, except after 6 months storage when the concentrations of total procyanidins in ‘Crofton’ and ‘Granny Smith’ were similar. In general, the concentrations of total procyanidins were higher in
Figure 6.18  Concentrations of flavonol derivatives (µg.g⁻¹ fresh weight) in 'Granny Smith', 'Lady Williams' and 'Crofton' apple peel during storage in air at 0°C. DPA was applied to 'Granny Smith' and 'Lady Williams' apples before storage. (A) Unknown flavonol 2; (B) Unknown flavonol 3; (C) Unknown flavonol 1; (D) Quercetin arabinopyranoside
Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.19 Concentrations of procyanidins (μg g⁻¹ fresh weight) in 'Granny Smith', 'Lady Williams' and 'Crofton' apple peel during storage in air at 0°C. DPA was applied to 'Granny Smith' and 'Lady Williams' apples before storage. (A) Total procyanidins; (B) Procyanidin B2; (C) Procyanidin trimer; (D) Procyanidin B5. Concentrations accompanied by different letters differ significantly at P<0.05
the peel of DPA treated fruit than control fruit, however these differences were not always significant. The concentrations of total procyanidins in ‘Granny Smith’ peel generally declined during the storage period, however in ‘Lady Williams’ peel, after an initial increase in the levels of procyanidins, the concentrations of total procyanidins remained relatively constant during storage. The concentration of total procyanidins remained relatively constant in ‘Crofton’ peel until 6 months when the levels of total procyanidins increased and remained relatively high for the rest of the storage period (Figure 6.19A).

Procyanidin B2 and procyanidin trimer made up the majority of the total procyanidins (Figure 6.19B, 6.19C). Consequently, the trends in variety and treatment over time are very similar to the total procyanidins. ‘Lady Williams’ had higher concentrations of procyanidin B2 and procyanidin trimer in the peel than either ‘Granny Smith’ or ‘Crofton’. ‘Granny Smith’ had very low concentrations of procyanidin B2 and procyanidin trimer for the first 6 months of storage, but after this time, these differences were generally not significant. The concentrations of procyanidin B2 and procyanidin trimer remained relatively constant or declined during storage. Procyanidin B5 was only detected in ‘Lady Williams’ (Figure 6.19D), but its concentration was significantly lower than for the other procyanidins. In general, there was no difference between the control and DPA treated ‘Lady Williams’.

6.4.3.8 Chalcones
Phloridzin is the only chalcone in the peel of apples and was present in relatively low concentrations in the peel during storage (Figure 6.20). ‘Lady Williams’ peel always contained more phloridzin than ‘Granny Smith’, whilst the concentrations of phloridzin in ‘Crofton’ peel were intermediate. DPA treatment had no effect on phloridzin concentrations. The concentrations of phloridzin remained relatively constant in ‘Granny Smith’ over the 9 month storage period, whilst in ‘Crofton’ and ‘Lady Williams’ peel the concentration of phloridzin increased after harvest and generally peaked at 6 months before declining.

6.4.3.9 Unknown phenolics
The total concentrations of unknown phenolic compounds were relatively low, except in ‘Crofton’ peel where the concentrations were much higher than in the peel of ‘Granny Smith’ and ‘Lady Williams’ (Figure 6.21A). The concentrations of total unknown phenolics in ‘Lady Williams’ were generally higher than in ‘Granny Smith’, but these differences were often not
Figure 6.20 Concentrations of phloridizin (μg g⁻¹ fresh weight) in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during storage in air at 0°C. DPA was applied to ‘Granny Smith’ and ‘Lady Williams’ apples before storage. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.21  Concentrations of unknown phenolics (µg g⁻¹ fresh weight) in ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apple peel during storage in air at 0°C. DPA was applied to ‘Granny Smith’ and ‘Lady Williams’ apples before storage. (A) Total unknown phenolics; (B) Unknown phenolic 1; (C) Unknown phenolic 2; (D) Unknown phenolic 3; (E) Unknown phenolic 4. Concentrations accompanied by different letters differ significantly at P<0.05.
significant. Indeed, there were few unknown phenolics in ‘Granny Smith’ peel. DPA treatment had no effects on the concentrations of unknown phenolics.

The concentration of Unknown Phenolic 3 in ‘Crofton’ peel was higher than in either ‘Lady Williams’ or ‘Granny Smith’ apples and remained relatively constant during the storage period (Figure 6.21D). The concentrations of Unknown Phenolic 3 in the peel of ‘Lady Williams’ and ‘Granny Smith’ were low and generally not significant. DPA had little or no effect on the concentration of Unknown Phenolic 3 in ‘Lady Williams’. No Unknown Phenolic 4 was detected in ‘Granny Smith’ or ‘Lady Williams’ (Figure 6.21E). The concentration of Unknown Phenolic 4 in ‘Crofton’ increased after harvest, reaching a maximum at 4 months before declining in storage. This increase in the concentrations of Unknown Phenolic 4 was mainly responsible for the increase in total unknown phenolics at this time. The concentrations of the unknown phenolics, Unknown Phenolic 1 and Unknown Phenolic 2, were low and variable. These unknowns generally did not reveal any variety or treatment effects (Figure 6.21B, 6.21C).

6.4.4 Discussion

Phenolic metabolism is a complex process. Phenolics undergo constant turnover and degradation. The recycling of flavonoids was thoroughly discussed by Stafford (1990) and Barz and Köster (1981). There have been relatively few studies that examined the fate of apple fruit phenolics during storage and even fewer that examined peel phenolics. In general, the concentrations of total extractable phenolics in the peel increased during the first few months of storage before remaining constant or slowly declining. Some phenolics declined or remained constant during storage. For example, the concentrations of cinnamic acid derivatives and epicatechin in the peel of scald susceptible varieties and total procyanidins in ‘Granny Smith’ peel remained relatively constant or declined during storage. Burda et al. (1990) showed that the major phenolics (epicatechin, procyanidin B2 and the phloretin glycosides) in the peel of ‘Rhode Island Greening’, ‘Empire’ and ‘Golden Delicious’ apples remained relatively constant over a normal air storage period of six months. Mosel and Herrmann (1974a) showed that the concentrations of all cinnamic acid derivatives and flavan-3-ols consistently decreased in ‘Schöner von Boskoop’ apples during the 5 month air storage period at 4°C. Coseteng and Lee (1987) also showed that the changes over the storage period (4 months regular air store at 0°C, 90% RH) were small in the 9 apple varieties examined. Barrett et al. (1991) observed that CA storage (0°C, 2%O₂, 3% CO₂) of ‘Delicious’ apples and high CO₂ storage conditions had little
effect on the peel concentration of total phenolics. Burda et al. (1990) concluded that there are large varietal differences in the peel phenolics but the trends in the individual phenolics during storage remain relatively constant.

In the present experiments, the concentrations of most of the peel phenolics generally increased during the initial stages of storage before slowly declining. For example, benzoic acid derivatives increased for the first few months of storage then gradually declined, however these changes during storage were minor. Similarly the concentration of total procyanidins in ‘Lady Williams’ apple peel increased then remained relatively constant during storage. In addition, the concentrations of catechin in all varieties increased during the first few months of storage and then remained constant or declined during storage. Hulme and Rhodes (1971) also observed a small rise in the total phenolics during the ripening of ‘Cox’s Orange Pippin’ apples. Burda et al. (1990) found that there was an initial rise in the levels of epicatechin in the peel of ‘Rhode Island Greening’ apples during the first two months of storage that then decreased during storage. These results show that the phenolic profile of apple peel is dynamic, and phenolic turnover, degradation and biosynthesis must be considered when examining the role of phenolics in scald development.

A significant example of these general trends was the production of cinnamic acid derivatives, particularly chlorogenic acid in the peel of ‘Crofton’ apples that significantly increased for the first few months of storage and then declined. Also, during the initial stages of storage, the concentrations of total flavonols increased. Dick (1986) suggested that the 2 - 3 fold increase in the levels of quercetin glucosides observed during cold storage in ‘McIntosh’ apples was coincident with the respiratory climacteric, however he did not report any physiological measurements. Blankenship and Richardson (1985) suggested that there may be a relationship between ethylene production and the quantity as well as the composition of phenolics present during the storage of mature ‘Beurre d’Anjou’ and ‘Beurre Bosc’ pear fruit. This has also been proposed in the ripening of cherry tomato fruit (Fleuriet and Macheix, 1981). However, the role of ethylene and ripening on phenolic biosynthesis has yet to be fully explored in apple peel.

The first step of phenolic biosynthesis involves the production of trans-cinnamate from phenylalanine with phenylalanine ammonia lyase (PAL) (Figure 6.2). PAL activity has been shown to have a direct influence on total flavonoids in apples (Lister et al., 1996b), although it does not necessarily control anthocyanin production (Ju et al., 1995b; Lister et al., 1996b).
Increased PAL activity and ethylene production have been shown to coincide with ripening in 'Red Delicious' and 'Golden Delicious' apples (Blankenship and Unrath, 1988). This is not surprising since ethylene exposure increased the levels of PAL mRNA and the RNA of other enzymes involved in metabolism of phenolic compounds in carrots (Ecker and Davis, 1987). Phenylpropanoid metabolism has also been shown to be induced by ethylene (Ke and Saltviet, 1989; Tomas-Barberan et al., 1997). Tomas-Barberan et al. (1997) found that there were significant changes in individual phenolic compounds in response to ethylene exposure in iceberg lettuce. They showed that chlorogenic acid and isochlorogenic acid increased 5 and 10 fold respectively, while the caffeoyltartaric derivatives were not significantly affected by ethylene treatment. These observations may be relevant to phenolic turnover in climacteric fruit such as apple, and may help explain differential phenolic metabolism in apples during cold storage. More work is required to explore these relationships.

The concentrations of total extractable phenolics in the peel did not appear to be related to scald susceptibility, as the scald susceptible variety, 'Lady Williams' contained over three times the levels of total phenolics in the peel than in the other scald susceptible variety, 'Granny Smith', whilst the scald resistant variety, 'Crofton' had intermediate levels. In other apple peel phenolic studies, the concentrations of total phenolics varied with scald susceptibility. For example Lee (1992) reported that the scald resistant 'Empire' apple had concentrations of total phenolics in the skin similar to those in the scald susceptible variety, 'Cortland'. Burda et al. (1990) showed that the levels of total phenolics in the peel of the scald resistant 'Empire' were similar to the moderately scald resistant 'Golden Delicious' but significantly lower than those in the scald susceptible 'Rhode Island Greening'. Kang and Lee (1987) further showed that the total phenolic content in the peel of both the scald resistant, 'Fuji', and the scald susceptible 'Mutsu' variety, were similar and remained relatively constant during 8 months of storage. However, Kang and Lee (1987) used the unreliable Folin-Ciocalteu method to estimate total phenolics (Macheix et al., 1990). In addition, whilst the 'Fuji' apples were stored continuously at 0°C, the 'Mutsu' apples were stored separately in a cellar where the temperature varied from 0 - 10°C. These storage conditions can have significant effects on the storage behaviour of apples including phenolic metabolism. However, the 'Mutsu' apples developed scald symptoms within 4 months suggesting that the storage temperatures were sufficiently low to induce scald (Huelin and Coggiola, 1970c; Watkins et al., 1995). These large discrepancies between total phenolic concentration and scald susceptibility clearly show that generalisations about total phenolics cannot be made.
Within the various classes of phenolics there were large differences in the quantities of peel phenolics. The concentrations of total benzoic acid derivatives were low. ‘Lady Williams’ peel contained significantly more total benzoic acid derivatives than the other varieties, and this was mainly due to high levels of Unknown Benzoic 2 and Unknown Benzoic 3 in the peel of ‘Lady Williams’ (Figure 6.11). Benzoyl-glucose was not identified in this time course study probably because the retention time of benzoyl glucose is the same as chlorogenic acid (Section 6.3). The concentrations of benzoyl glucose would be expected to be relatively low, in comparison to chlorogenic acid, as the levels of benzoyl-glucose would be diluted in a non-discriminative peel sample (ie no separation of scald and non-scalded tissue) compared to a pure scald peel sample (Section 6.5). Therefore the UV spectra of chlorogenic acid dominated the spectra of the peak, and the compound was identified and quantified as chlorogenic acid. However the concentrations of chlorogenic acid in ‘Granny Smith’ and ‘Lady Williams’ apple peel were very low, in comparison to ‘Crofton’ peel. Indeed, ‘Crofton’ always had more total cinnamic acids (including caffeic and ferulic acid) than the scald susceptible varieties, ‘Granny Smith’ and ‘Lady Williams’. Conversely, Burda et al. (1990) showed that the concentrations of chlorogenic acid in the peel of another scald resistant variety, ‘Empire’ were similar to those in the peel of ‘Golden Delicious’ and ‘Rhode Island Greening’ apples (Burda et al., 1990).

Total concentrations of cinnamic acid derivatives in ‘Crofton’ were always greater than in ‘Lady Williams’ and ‘Granny Smith’ apple peel. ‘Crofton’ contained at least 3 and 12 times more total cinnamic acids in the peel than in ‘Lady Williams’ and ‘Granny Smith’ respectively. These large concentrations of cinnamic acids (especially chlorogenic acid) in ‘Crofton’ peel may have a role in protecting ‘Crofton’ from scald development. Possibly chlorogenic acid acts as an antioxidant to protect the cell from scald damage. This will be discussed in more detail in Section 6.7 and 6.9.

There were no significant trends in the concentrations of flavan-3-ols between scald resistant and scald susceptible varieties. The concentrations of flavan-3-ols in the peel of ‘Lady Williams’ were greater than in ‘Crofton’ that in turn had higher concentrations than ‘Granny Smith’. The trends in the concentrations of epicatechin and catechin were similar and dominated the flavan-3-ol profile. ‘Lady Williams’ had significantly more epicatechin and catechin than either ‘Crofton’ or ‘Granny Smith’. Similarly Burda et al. (1990) showed that the concentration of epicatechin in the peel of the scald susceptible ‘Rhode Island Greening’, were greater than in the scald resistant
‘Empire’. However, there may be phenolics such as Unknown Flavan-3-ol 1 and 4, and Unknown Phenolic 3 and 4, that were unidentified in the peel of ‘Crofton’ and may impart scald tolerance. For example, the high concentrations of Unknown Phenolic 3 (150 μg.g⁻¹) and Unknown Phenolic 4 (50 μg.g⁻¹) in the peel of ‘Crofton’ apples may act as antioxidants protecting the cell against oxidative stress. However more work is required to identify and determine their roles.

Flavonols are an important component of the phenolic profile and comprise about 80% of the total extractable phenolics during storage. The concentrations of the flavonols in the peel of ‘Lady Williams’ were higher than in ‘Granny Smith’ with the concentration reaching a maximum of about 3,000 μg.g⁻¹ in the peel of ‘Lady Williams’ whilst the maximum in ‘Granny Smith’ peel was only 991 μg.g⁻¹ at 2 months. The concentrations of flavonols in the peel of ‘Crofton’ were intermediate. Quercetin glycosides were the only flavonols identified in the peel, with quercetin rhamnoglucoside making up the majority of the flavonols in the peel, around 30% of the total phenolics. Other flavonols, such as quercetin glucoside, quercetin xyloside and quercetin arabinofuranoside are present in lower concentrations but behaved similarly to quercetin rhamnoglucoside. Quercetin arabinopyranoside and Unknown Flavonol 2 exhibited similar behaviour in that both flavonols were only detected in the peel of ‘Lady Williams’. The concentration of Unknown Flavonol 1 was variable across varieties, but were significantly lower in ‘Granny Smith’.

In other peel studies, the overall concentrations of total flavonols in ‘Empire’ apple peel were lower than in ‘Golden Delicious’ and ‘Rhode Island Greening’ peel, but these differences were not significant (Burda et al., 1990). In contrast, Lee (1992) reported that ‘Empire’ apple peel had greater concentrations of flavonols than the scald susceptible ‘Cortland’. These observations show that the concentrations of phenolics vary widely among varieties that have different scald susceptibilities, but the qualitative differences among varieties are small. However, as outlined in Section 6.2.3, the methods for the extracting, identification and quantification of apple phenolics can often be misleading.

The concentrations of total procyanidins in the peel of the scald susceptible varieties were generally higher than in the scald resistant, ‘Crofton’. Procyanidin B2 and procyanidin trimer comprise a significant proportion of the total procyanidins, and their relative concentrations and trends over time were very similar to those of total procyanidins. This was also shown by Burda
et al. (1990) where the levels of procyanidin B2 in ‘Empire’ peel were significantly lower than in ‘Rhode Island Greening’ peel. In this study procyanidin B5 was only detected in the peel of ‘Lady Williams’.

In general, the concentrations of all phenolics (i.e. benzoic acid derivatives, cinnamic acid derivatives, flavan-3-ols, flavonols and procyanidins) and consequently total phenolics in peel of stored fruit treated with DPA at harvest were higher than in the control (non-treated) peel, however these differences were not often statistically significant (P<0.05). Thus peel from scald susceptible apples, i.e. in tissue that was destined to scald (no DPA treatment), there were no major differences in total phenolic content compared to non-scalding peel. This suggests that the concentration of total phenolics per se does not influence scald development. Duvenage and De Sward (1973) examined the changes in the concentration of the total phenols, flavonols and leucoanthocyanins in the peel of a scald resistant variety, ‘White Winter Pearmain’, and the susceptible variety, ‘Granny Smith’ and concluded that DPA inhibited both the synthesis and oxidation of flavonols during storage. However, the evidence for their conclusions is limited.

Ju et al. (1996) showed in three years of storage experiments with ‘Delicious’ and ‘Ralls’ apple fruit, that simple phenols (such as chlorogenic acid) were positively correlated, flavonoids were not correlated and anthocyanins were negatively correlated, to scald development. They also found no significant changes in the levels of simple phenolics or flavonoids during cold storage. This is contrary to the present results, where there was significant metabolism of phenolics. Ju et al. (1996) concluded that the concentration of simple phenols in the fruit peel at harvest affects tissue browning during scald development. This is also contrasts with the results presented here, where ‘Crofton’ peel had significantly higher concentrations of cinnamic acids both at harvest and during storage, than in the peel of apples which developed scald.

There are some doubts about identification and quantification of phenolics reported by Ju et al. (1996). They conducted spectroscopic assays on gross solvent extracts and separations. The simple phenols were assayed at 313 nm from a water soluble fraction collected from a C18 column. Whilst the ‘flavonoid’ fraction was assayed at 270 nm from the same C18 column washed with methanol. These estimates of the phenolic content of the peel are inherently inaccurate, as they measure all compounds in the extract that absorb at 313 and 270 nm. In addition, referring to the ‘flavonoids’ as only flavonols and procyanidins and the quantification of these relative to the flavan-3-ol, catechin, is inaccurate. They quantified the simple phenolics
using gallic acid as a standard. Gallic acid has never been isolated from apples and its relative response factor is significantly different to the other simple phenolics such as chlorogenic acid. A more accurate and meaningful method of extraction and analysis, such as those described in Section 6.3, is essential to avoid potential mis-interpretation of the data.
6.5 Relation of Phenolics and Superficial Scald

6.5.1 Introduction
The aim of this section was:

- examine the differences in peel phenolics between scalded and non-scalded peel tissue in 'Granny Smith' apples and in the scald resistant 'Crofton'. The non-scalded 'Granny Smith' peel tissue was derived from peel tissue, which did not naturally develop scald, or from fruit treated pre-storage with DPA.

6.5.2 Materials and Methods
The same apples and phenolic extraction procedures were used as in Section 6.3. However in this experiment, after 3 and 6 months storage in air at 0°C, 'Granny Smith' peel showing scald symptoms were physically separated from non-scalded peel and analysed separately. This was compared with DPA treated 'Granny Smith' apples and 'Crofton' apples of the same age.

6.5.3 Results

6.5.3.1 3 months storage at 0°C

Total phenolics Non-scalded 'Granny Smith' apple peel (DPA and no scald) had higher concentrations of total phenolics following storage in air for 3 months, than the scalded 'Granny Smith' and 'Crofton' apples (Figure 6.22). The relative proportions of the various phenolic classes in DPA and no scald 'Granny Smith' treatments were remarkably similar (Figure 6.23). There were no significant differences between the levels of total phenolics in the peel of scalded 'Granny Smith' and 'Crofton' apples after 3 months storage (Figure 6.22), however, the proportions of the various classes of phenolics between scalded and non-scalded peel tissue were significantly different (Figure 6.23). This was mainly due to the presence of benzoyl-β-D-glucose, lower levels of cinnamic acid derivatives and increased procyanidin concentrations in the scalded 'Granny Smith' peel tissue.

Benzoic acid derivatives (excluding benzoyl-β-D-glucose) Within the 'Granny Smith' treatments, the scalded peel had lower levels of total benzoic acid derivatives. There were no differences in the non-scalded peel (DPA and no scald), and contained relatively high...
Figure 6.22  Concentrations of total phenolics (μg.g⁻¹ fresh weight) in 'Granny Smith' and 'Crofton' apple peel after 3 months storage. Scalded 'Granny Smith' peel tissue was analysed separately from peel not showing scald symptoms (no scald). Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.23  Relative proportions of phenolic classes in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald).
concentrations of total benzoic acid derivatives (Figure 6.24A). The concentration of benzoic derivatives in ‘Crofton’ were lower than in ‘Granny Smith’ peel. The concentrations of the benzoic acid derivatives were approximately 3% of total phenolics in all treatments (Figure 6.23). The concentration of Unknown Benzoic 1 showed no significant differences among ‘Crofton’ and any of the ‘Granny Smith’ treatments (Figure 6.24B). However the scalced peel tissue contained a lower concentration of Unknown Benzoic 1 than either DPA or non-scalced peel tissue. The concentrations of Unknown Benzoic 2 were higher in ‘Granny Smith’ than in ‘Crofton’ peel tissue, however there were no significant differences within the ‘Granny Smith’ treatments (Figure 6.24C). Unknown Benzoic 3 was not detected in any of the ‘Granny Smith’ or ‘Crofton’ apple peel samples.

Benzoyl-β-D-glucose Benzoyl-β-D-glucose was only found in scalced ‘Granny Smith’ peel (Figure 6.25) where it contributed 17% of the total phenolics (Figure 6.23).

Cinnamic acid derivatives The levels of total cinnamic acid derivatives in the peel of ‘Crofton’ after 3 months storage were over 4 times greater than in any ‘Granny Smith’ peel (Figure 6.26A). This was reflected in the percentage of the total cinnamic acids contributing to overall total phenolics. In the ‘Granny Smith’ samples, the total cinnamic acids made up only 3-4% of the total phenolics, whilst in ‘Crofton’ peel the cinnamic acid derivatives comprised 22% to the total phenolics (Figure 6.23). Within the ‘Granny Smith’ samples, the concentrations of total cinnamic acid derivatives in the scald affected peel were lower than in the non-scald treatments (Figure 6.26A). The concentrations of individual cinnamic acid derivatives were different for each treatment, however chlorogenic acid dominated the cinnamic acid derivative profile. ‘Crofton’ peel had over 4 times higher concentrations of chlorogenic acid than in ‘Granny Smith’ peel (Figure 6.26B). Caffeic acid was only found in ‘Crofton’ and ‘Granny Smith’ scalced peel (Figure 6.26C), with ‘Crofton’ having more than in ‘Granny Smith’. Coumaric acid was found only in ‘Granny Smith’ peel tissue (Figure 6.26D) and there were no differences between treatments. Ferulic acid was not detected in any apple peel sample.

Flavan-3-ols Scalded ‘Granny Smith’ peel contained higher concentrations of total flavan-3-ols in the peel than ‘Crofton’ and non-scalced ‘Granny Smith’ peels, which were similar (Figure 6.27A). DPA treated ‘Granny Smith’ apples contained the lowest concentrations of total flavan-3-ols (Figure 6.27A). The concentrations of catechin in scalced ‘Granny Smith’ peel tissue were higher than in the non-scalced ‘Granny Smith’ (Figure 6.27B). The lowest concentrations of
Figure 6.24  Concentrations of benzoic acid derivatives (μg.g⁻¹ fresh weight) in 'Granny Smith' and 'Crofton' apple peel after 3 months storage. Scalded 'Granny Smith' peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total benzoic acid derivatives; (B) Unknown benzoic acid 1; (C) Unknown benzoic acid 2. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.25  Concentrations of benzoyl-β-D-glucose (µg.g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.26  Concentrations of cinnamic acid derivatives (μg·g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total cinnamic acid derivatives; (B) Chlorogenic acid; (C) Caffeic acid; (D) Coumaric acid. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.27  Concentrations of flavan-3-ols (μg g\(^{-1}\) fresh weight) in ‘Granny Smith’ and ‘Croton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total flavan-3-ols; (B) Catechin; (C) Epicatechin. Concentrations accompanied by different letters differ significantly at P<0.05
catechin were found in DPA treated ‘Granny Smith’ and the ‘Crofton’ peel. The concentrations of epicatechin were lower in ‘Crofton’ than in the ‘Granny Smith’ samples. Scalded ‘Granny Smith’ peel contained less epicatechin than in apples treated with DPA, which in turn had less epicatechin than non-scaled peel (Figure 6.27C). The concentrations of the Unknown Flavan-3-ol 1 and Unknown Flavan-3-ol 2 were higher in scalded tissue of ‘Granny Smith’ (Figure 6.28A and 6.28B). Unknown Flavan-3-ol 3 and Unknown Flavan-3-ol 4 were only found in ‘Crofton’ peel, which comprised an important fraction of the total concentration of flavan-3-ols in ‘Crofton’ peel (Figure 6.28C and 6.28D).

Flavonols The concentrations of total flavonols were highest in the non-scaled and DPA treated ‘Granny Smith’ peel, whilst the scalded ‘Granny Smith’ peel and ‘Crofton’ peel contained considerably lower concentrations of flavonols (Figure 6.29). These differences were reflected in the proportion of flavonols in the total phenolic profile among treatments, where 22% of the total phenolics in scalded ‘Granny Smith’ peel were flavonols compared to 52% in the non-scaled peel tissue from the same apples (Figure 6.23). Similar trends were observed with individual flavonols. The levels of quercetin rhamnoglucoside, quercetin glucoside, quercetin xyloside, quercetin arabinofuranoside and Unknown Flavonol 3 behaved similarly (Figure 6.30 and 6.31B). The concentration of these individual flavonols in the ‘Granny Smith’ non-scałd treatments (DPA and no scald), were always higher than in ‘Crofton’ and ‘Granny Smith’ scalded peel tissue. Higher concentrations of Unknown Flavonol 1 were detected in ‘Granny Smith’ DPA and ‘Crofton’ peel than in either scalded or non-scaled ‘Granny Smith’ tissue (Figure 6.31A). Quercetin arabinopyranoside was only detected in the ‘Granny Smith’ non-scałd peel (Figure 6.31C). Unknown Flavonol 2 was not detected in either ‘Granny Smith’ or ‘Crofton’ peel.

Procyanidins The concentrations of total procyanidins were similar in all the ‘Granny Smith’ peel samples (Figure 6.32A), and were all higher than in ‘Crofton’ peel. Similar trends were observed in the concentrations of procyanidin B2 and the procyanidin trimer (Figure 6.32B and 6.32C). ‘Granny Smith’ DPA peel contained higher concentrations of procyanidin B2 than in non-scałd peel, which in turn were greater than in the scalded peel tissue. ‘Crofton’ contained less than half the concentrations of procyanidins B2 found in the ‘Granny Smith’ treatments (Figure 6.32B). No differences in the concentrations of procyanidin trimer were found within the ‘Granny Smith’ treatments, but the concentrations of procyanidin trimer were much lower in
Figure 6.28 Concentrations of unknown flavan-3-ols (µg·g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Unknown flavan-3-ol 1; (B) Unknown flavan-3-ol 2; (C) Unknown flavan-3-ol 3; (D) Unknown flavan-3-ol 4. Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.29  Concentrations of total flavonols (µg.g$^{-1}$ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.30  Concentrations of flavonol derivatives (μg.g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Quercetin rhamnoglucoside; (B) Quercetin glucoside; (C) Quercetin xyloside; (D) Quercetin arabinofuranoside
Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.31  Concentrations of flavonol derivatives (µg.g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Unknown flavonol 1; (B) Unknown flavonol 3; (C) Quercetin arabinopyranoside

Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.32 Concentrations of procyanidins (µg·g⁻¹ fresh weight) in 'Granny Smith' and 'Crofton' apple peel after 3 months storage. Scalded 'Granny Smith' peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total procyanidins; (B) Procyanidin B2; (C) Procyanidin trimer; (D) Procyanidin B5. Concentrations accompanied by different letters differ significantly at P<0.05
'Crofton' peel (Figure 6.32C). The concentrations of procyanidin B5 were low and variable, with no differences among the treatments (Figure 6.32D).

**Chalcones**  The concentrations of phloridizin were highest in 'Crofton', and least in 'Granny Smith' scald (Figure 6.33) with intermediate levels in 'Granny Smith' DPA and non-scalded peel. The contribution of phloridizin to the total phenolic profile was very low, contributing only 1 - 2% to the total phenolics across all treatments (Figure 6.23).

**Unknown phenolics**  'Crofton' peel tissue contained large concentrations of unknown phenolic compounds, comprising 8% of the total phenolics (Figure 6.23A). Although unknown phenolics were present in non-scalded and DPA treated 'Granny Smith', they comprised only 2% and 3% of the total phenolics. (Figure 6.23). No unknown phenolics were detected in scalded 'Granny Smith' peel (Figure 6.34A). The Unknown Phenolic 3 and Unknown Phenolic 4 were responsible for the large concentrations of unknowns in 'Crofton' (Figure 6.34C and 6.34D). Unknown Phenolic 2 was only detected in DPA and non scalded 'Granny Smith' peel (Figure 6.34B), whilst Unknown Phenolic 1 was not detected in either 'Granny Smith' or 'Crofton' apple peel samples.

### 6.5.3.2  6 months storage at 0°C

**Total phenolics**  After 6 months air storage at 0°C, the concentration of total phenolics in the non-scalded apple peel was higher (3.5 times greater) than in the scalded peel tissue (Figure 6.35). DPA 'Granny Smith' and 'Crofton' peel had intermediate concentrations of total phenolics. Figure 6.36 describes the contribution of the various classes of phenolics in the peel of 'Granny Smith' and 'Crofton' apples after 6 months storage. The major differences in the relative proportion of the various classes of phenolics in the scalded tissue was the presence of benzoyl-β-D-glucose, and the relatively small contribution of flavonols (Figure 6.36). However the scald resistant 'Crofton' had a high proportion of the cinnamic acid derivatives and a lower proportion of procyanidins. A feature of the non-scalded 'Granny Smith' phenolics profile was the high proportion of flavonols, contributing 62% of the total phenolics.

**Benzoic acid derivatives (excluding benzoyl-β-D-glucose)**  The concentration of total benzoic acid derivatives in the peel of non-scalded 'Granny Smith' treatments (no scald and DPA) were greater than in 'Crofton' (Figure 6.37A). Scalded 'Granny Smith' peel tissue contained lower
Figure 6.33  Concentrations of phoridizin (µg·g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald).

Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.34 Concentrations of unknown phenolics (µg.g⁻¹ fresh weight) in 'Granny Smith' and 'Crofton' apple peel after 3 months storage. Scalded 'Granny Smith' peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total unknown phenolics; (B) Unknown phenolic 2; (C) Unknown phenolic 3; (D) Unknown phenolic 4. Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.35  Concentrations of total phenolics (µg g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.36 Relative proportions of phenolic classes in 'Granny Smith' and 'Crofton' apple peel after 6 months storage. Scalded 'Granny Smith' peel tissue was analysed separately from peel not showing scald symptoms (no scald)
Figure 6.37  Concentrations of benzoic acid derivatives (µg.g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total benzoic acid derivatives; (B) Unknown benzoic acid 1; (C) Unknown benzoic acid 2; (D) Unknown benzoic acid 3.

Concentrations accompanied by different letters differ significantly at P<0.05
concentrations of benzoic acid derivatives and had less than one third the concentration of total benzoic acid derivatives than the other treatments. The percentage of benzoic acid derivatives in the phenolic profile of ‘Granny Smith’ DPA was 4%, whilst in the other treatments, the benzoic acid derivatives contributed approximately 2% to the total phenolics (Figure 6.36). The concentration of the Unknown Benzoic 1 followed a similar trend to the total benzoic acid derivatives and accounted for the majority of all benzoic acid derivatives (Figure 6.37B). The concentrations of Unknown Benzoic 1 were higher in the non-scalded ‘Granny Smith’ treatments (DPA and no scalp) than in ‘Crofton’ which were all greater than in scalded ‘Granny Smith’ peel tissue. ‘Granny Smith’ no scalp and DPA treatment were the only treatments to possess Unknown Benzoic 2 (Figure 6.37C), whilst Unknown Benzoic 3 was only found in low concentrations in ‘Crofton’ peel (Figure 6.37D).

**Benzoyl-β-D-glucose** Benzoyl-β-D-glucose was only present in scalded ‘Granny Smith’ peel tissue (Figure 6.38), and was the predominant phenolic, which contributed over 25% of the total phenolics in the scald phenolic profile (Figure 6.36).

**Cinnamic acid derivatives** The concentration of total cinnamic acid derivatives in the peel of ‘Crofton’ apples after 6 months air storage were over 5 times greater than in any of the ‘Granny Smith’ samples (Figure 6.39). This was reflected in the contribution of cinnamic acids to total phenolics where in ‘Crofton’ peel, 21% of the total phenolics were cinnamic acids, whilst in the ‘Granny Smith’ samples the contribution of cinnamic acids ranged from only 3% -5% (Figure 6.36). Among the ‘Granny Smith’ samples the concentrations of total cinnamic acid derivatives were similar for non-scalded treatments (DPA and non-scalded peel tissue), however these treatments had three times more total cinnamic acids than scalded ‘Granny Smith’ peel. The large differences in total cinnamic acid derivatives were mainly due to the high concentrations of chlorogenic acid in the peel of ‘Crofton’ (435μg.g⁻¹), whereas the highest concentration of chlorogenic acid in ‘Granny Smith’ peel after 6 months storage was only 74μg.g⁻¹ in non-scalded peel (Figure 6.40A). Chlorogenic acid and benzoyl-β-D-glucose had the same HPLC retention time, hence chlorogenic acid could not be detected in scalded tissue. This feature will be discussed in more detail in Section 6.54. Caffeic acid was found exclusively in ‘Crofton’ peel tissue (Figure 6.40A), whereas coumaric acid was only detected in the non-scalded ‘Granny Smith’ treatments (DPA and no scalp) (Figure 6.40B). Ferulic acid was only detected in ‘Crofton’ and scalded ‘Granny Smith’ peel tissue (Figure 6.40D).
Figure 6.38  Concentrations of benzoyl-\(\beta\)-D-glucose (\(\mu\)g.g\(^{-1}\) fresh weight) in 'Granny Smith' and 'Crofton' apple peel after 6 months storage. Scalded 'Granny Smith' peel tissue was analysed separately from peel not showing scald symptoms (no scald). Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.39  Concentrations of cinnamic acid derivatives (µg.g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.40  Concentrations of cinnamic acid derivatives (µg·g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Chlorogenic acid; (B) Caffeic acid; (C) Coumaric acid; (D) Ferulic acid. Concentrations accompanied by different letters differ significantly at P<0.05.
**Flavan-3-ols** The concentrations of total flavan-3-ols in ‘Crofton’ peel after 6 months air storage, were higher than in any of the ‘Granny Smith’ samples (Figure 6.41). Among the ‘Granny Smith’ samples, the non-scalded peel tissue had more total flavan-3-ols than the scald or DPA treated ‘Granny Smith’ peel. Epicatechin and catechin significantly contributed to total flavan-3-ol concentrations. The concentration of catechin was highest in the non-scald ‘Granny Smith’ peel and lowest in the DPA treated ‘Granny Smith’, whilst the levels of catechin in ‘Crofton’ and scalded ‘Granny Smith’ peel tissue were intermediate and similar (Figure 6.41B). In contrast the concentration of epicatechin was lower in the scalded ‘Granny Smith’ peel and higher in the DPA treated fruit (Figure 6.41C). The levels of epicatechin in the non-scalded peel were significantly higher than in ‘Crofton’. In general, the concentrations of individual flavan-3-ols did not follow any common trends and each flavan-3-ol behaved differently in each treatment. The concentration of Unknown Flavan-3-ol 1 were higher in ‘Crofton’ than in any ‘Granny Smith’ treatment and within the ‘Granny Smith’ treatments, scalded peel had more of Unknown Flavan-3-ol 1 than no scald or DPA which had similar concentration (Figure 6.42A). The concentrations of Unknown Flavan-3-ol 2 in ‘Crofton’ and ‘Granny Smith’ scalded peel were similar, and were greater than in ‘Granny Smith’ peel with no scald. Unknown Flavan-3-ol 2 was not detected in DPA treated ‘Granny Smith’ (Figure 6.42B). Whilst Unknown Flavan-3-ol 3 and Unknown Flavan-3-ol 4 were only detected in ‘Crofton’ peel (Figure 6.42C and 6.42D), and significantly contributed to the high levels of total flavan-3-ols in ‘Crofton’.

**Flavonols** The concentrations of total flavonols in the non-scalded ‘Granny Smith’ peel tissue were 11 times higher than in scalded peel from the same apples (Figure 6.43). The concentrations of total flavonols were intermediate in the peel of ‘Crofton’ and DPA treated ‘Granny Smith’. The very large concentrations of flavonols in ‘Granny Smith’ non-scalded tissue significantly contributed to the total phenolic concentration of the peel (Figure 6.36). In non-scalded ‘Granny Smith’ peel, the flavonols comprised 62% of the total phenolics, whilst in scalded ‘Granny Smith’ peel the flavonols contributed only 19% of the total phenolics.

The individual flavonols (quercetin rhamnoglucoside, quercetin glucoside, quercetin xyloside, quercetin arabinofuranoside and Unknown flavonol 3) followed similar trends (Figure 6.44 and 6.45B). Non-scalded ‘Granny Smith’ peel tissue contained higher concentrations of the individual flavonols (quercetin rhamnoglucoside, quercetin glucoside, quercetin xyloside, quercetin arabinofuranoside and Unknown Flavonol 3) followed by ‘Crofton’ and DPA ‘Granny Smith’. The concentrations of quercetin rhamnoglucoside, quercetin glucoside, quercetin
Figure 6.41  Concentrations of flavan-3-ols (μg.g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total flavan-3-ols; (B) Catechin; (C) Epicatechin. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.42  Concentrations of unknown flavan-3-ols (µg g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Unknown flavan-3-ol 1; (B) Unknown flavan-3-ol 2; (C) Unknown flavan-3-ol 3; (D) Unknown flavan-3-ol 4. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.43  Concentrations of total flavonols (μg.g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.44  Concentrations of flavonol derivatives (μg·g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Quercetin rhamnoglucoside; (B) Quercetin glucoside; (C) Quercetin xyloside; (D) Quercetin arabinfuranoside
Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.45  Concentrations of flavonol derivatives (µg g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Unknown flavonol 1; (B) Unknown flavonol 3; (C) Quercetin arabinopyranoside.

Concentrations accompanied by different letters differ significantly at P<0.05.
xyloside, quercetin arabinofuranoside and Unknown Flavonol 3 were all lower in scalded ‘Granny Smith’ peel (Figure 6.44D and 6.45B). The concentration of Unknown Flavonol 1 were similar among ‘Crofton’ and ‘Granny Smith’ no scald and DPA and were more than 3 times higher than in scalded ‘Granny Smith’ peel tissue (Figure 6.45A). Quercetin arabinopyranoside was only detected in ‘Crofton’ peel and its concentration was low (Figure 6.45C). Unknown Flavonol 2 was not detected in either apple variety.

**Procyanidins** The concentrations of total procyanidins in the peel of the non-scaled ‘Granny Smith’ samples (DPA and no scald) were greater than in ‘Crofton’ and scalded ‘Granny Smith’ peel (Figure 6.46A). ‘Granny Smith’ DPA peel had more total procyanidins than non-scaled peel tissue, whilst there was no significant difference between ‘Crofton’ and scalded tissue. These differences were reflected in the trends of the individual procyanidins. For example, the concentrations of procyanidin B2 and procyanidin trimer behaved similarly (Figure 6.46B and 6.46C). The concentrations of procyanidin B2 and procyanidin trimer in scalded ‘Granny Smith’ and ‘Crofton’ peel were similar but lower than in the non-scaled ‘Granny Smith’ treatments. DPA treatment in ‘Granny Smith’ resulted in more procyanidin B5 and procyanidin trimer than non-scaled peel tissue after 6 months storage. Procyanidin B5 was only found in low levels in non-scaled ‘Granny Smith’ peel (Figure 6.46D).

**Chalcones** The concentration of phloridizin in the peel of stored apples was very low and contributed less than 1% of the total phenolics (Figure 6.36). The concentration of phloridizin in the peel of ‘Crofton’ was greater than in any of the ‘Granny Smith’ treatments (Figure 6.47). Phloridizin was not detected in scalded tissue and was present at similar concentrations in the non-scaled ‘Granny Smith’ treatments (DPA and no scald).

**Unknown phenolics** The concentrations of total unknown phenolics were highest in ‘Crofton’ (Figure 6.48A), where they contributed up to 6% of the total phenolics (Figure 6.36). The concentrations of total unknown phenolics were lower in ‘Granny Smith’ peel (Figure 6.48A). The concentrations of total unknowns were similar for scalded and non scalded ‘Granny Smith’ and these were greater than in the peel of DPA treated fruit. Unknown Phenolic 3 was the major unknown phenolic significantly contributing to the unknown phenolic profile of ‘Crofton’ (Figure 6.48C). Within the ‘Granny Smith’ samples, the scalded peel tissue had higher concentrations of Unknown Phenolic 3 than in the peel with no scald symptoms. Unknown Phenolic 3 was not detected in DPA treated fruit. Unknown Phenolic 4 was only detected in
Figure 6.46  Concentrations of procyanidins (μg.g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total procyanidins; (B) Procyanidin B2; (C) Procyanidin trimer; (D) Procyanidin B5. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.47 Concentrations of phoridizin (μg·g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 6 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). Concentrations accompanied by different letters differ significantly at P<0.05.
Figure 6.48  Concentrations of unknown phenolics (μg g⁻¹ fresh weight) in ‘Granny Smith’ and ‘Crofton’ apple peel after 3 months storage. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms (no scald). (A) Total unknown phenolics; (B) Unknown phenolic 2; (C) Unknown phenolic 3; (D) Unknown phenolic 4
Concentrations accompanied by different letters differ significantly at P<0.05
‘Crofton’ peel (Figure 6.48D). Unknown Phenolic 2 was only found in the non-scaled ‘Granny Smith’ samples at similar concentrations (Figure 6.48D), whilst Unknown Phenolic 1 was not detected in any sample.

6.5.4 Discussion

The concentrations of total extractable phenolics in the peel of ‘Granny Smith’ apples, which did not develop scald symptoms (ie DPA treated and non-scaled peel tissue) were greater than in scalded ‘Granny Smith’ peel. By 6 months storage, the concentrations of extractable phenolics in scalded tissue were more than 3.5 times lower than in the non-scaled peel tissue from the same apples. These data are similar to those of Piretti et al. (1994) and Abdallah et al. (1997) who also showed lower concentrations of phenolics in scalded ‘Granny Smith’ apples. The concentration of total extractable phenolics in the peel of non-scaled ‘Granny Smith’ and ‘Crofton’ apples increased between 3 and 6 months storage and may be due to ‘normal’ senescent metabolism and turn-over of phenolics in stored apple peel. However the concentrations of total extractable phenolics from scalded ‘Granny Smith’ tissue decreased from 2,100 μg.g⁻¹ at 3 months to less than 1,000 μg.g⁻¹ after 6 months storage. This suggests that the phenolics in the scalded peel tissue are either metabolised to non-phenolic metabolites or they are no longer extractable in 80% methanol. The degradation of phenolics in scalded peel tissue could be the result of the formation of co-polymers or the coupled oxidation by o-quinones. These o-quinones could be formed from a variety of sources including by polyphenol oxidase (PPO) action. As the brown deposits in the epidermal and hypodermal cells are of phenolic origin (Chapter 3), it is likely that the phenolic substrates have oxidised and polymerised making them insoluble in 80% methanol. Indeed, the ground peel tissue from scalded tissue remained dark after methanol extraction.

Benzoic acid derivatives (excluding benzoyl-β-D-glucose) were a relatively minor component of the phenolic profile comprising less than 4% of the total phenolics. The concentrations of benzoic acid derivatives (except benzoyl-β-D-glucose) were always higher in non-scaled ‘Granny Smith’ samples (no scald and DPA treated) than in ‘Crofton’ or scalded ‘Granny Smith’ peel tissue. There were few differences among the total benzoic acid derivatives after 3 and 6 months storage, except the concentrations in scalded tissue, which declined 3-fold. The exclusive presence of benzoyl-β-D-glucose in scalded ‘Granny Smith’ peel tissue comprising 350 μg.g⁻¹ (17% of the total peel phenolics) after 3 months storage, and 254 μg.g⁻¹ (26% of the
total peel phenolics) after 6 months storage shows that the development of scald is associated with a possible major re-direction of metabolism of benzoic acid derivatives. The question whether the synthesis of benzoyl-β-D-glucose plays a significant role in scald and its possible biosynthetic pathway is further explored in Section 6.8 and 6.9.

‘Crofton’ peel tissue always had more (at least 4 times greater) total cinnamic acids derivatives than ‘Granny Smith’ peel. This was reflected in the proportion of the cinnamic acid derivates in the total phenolic profile, where the cinnamic acid derivatives contributed 21% and 22% of the total phenolics in ‘Crofton’ apple peel stored for 3 and 6 months respectively. However the maximum contribution of the cinnamic acid derivatives to the total phenolics in ‘Granny Smith’ peel was only 4%. The high concentration of total cinnamic acid derivatives in ‘Crofton’ was due mainly to the high concentrations (400 μg.g⁻¹) of chlorogenic acid, which were over 5 times greater than in ‘Granny Smith’. In comparison, the contributions of caffeic, coumaric and ferulic acids to the total cinnamic acid derivatives were minor. There was no detectable chlorogenic acid in scalded ‘Granny Smith’ tissue, however chlorogenic acid and benzoyl-β-D-glucose had similar retention times. The very similar polarity and behaviour of chlorogenic acid and benzoyl-β-D-glucose on C18 HPLC may account for some confusion in the literature. Consequently all quantitative analysis was conducted by identification from the UV spectra provided by the diode array detector. The dominant UV spectra of the scalded peel tissue was of benzoyl-β-D-glucose (Figure 6.6), and no chlorogenic acid could be detected. Indeed no chlorogenic acid was detected when isolating benzoyl-β-D-glucose from scalded peel tissue using semi-preparative HPLC.

The concentrations of total cinnamic acid derivatives remained relatively constant during storage from 3 to 6 months except in ‘Crofton’ peel where the concentrations of total cinnamic acid derivatives increased. However in scalded ‘Granny Smith’ peel, the concentrations of total cinnamic acid derivatives decreased during storage. Abdallah et al. (1997) also showed that the content of total cinnamic acid derivatives, caffeic acid and chlorogenic acid were significantly higher in non-scalded tissue than scalded tissue in ‘Granny Smith’ apple peel, and suggested that the cinnamic acid derivatives may be substrates for PPO during scald development, or may act as antioxidants that reduce the autoxidation of α-farnesene to the conjugated trienes and inhibit scald. However the compartmentation or localisation of phenolics is important in considering the coupled oxidation of α-farnesene and phenolics as antioxidants. Whilst phenolics are localised in the vacuole, the lipid peroxidation reactions thought to contribute to scald
development are located in the tonoplast, plasmalemma, and chloroplast membranes (Chapter 3). In this experiment, the concentrations of total cinnamic acid derivatives (particularly chlorogenic, caffeic and ferulic acid) were significantly lower in scalded peel tissue, and were higher in the scald resistant ‘Crofton’ peel. This suggests that the cinnamic acid derivatives such as chlorogenic acid may act as antioxidants that inhibit scald development. Other antioxidants such as carotenoids are well known free radical quenchers preventing the formation of hydroperoxides in the presence of singlet oxygen (Rajalakshimi and Narasimha, 1995). Carotenoids are also located in apple peel (Knee, 1972; 1988) and may play a role in protecting the peel from scald. However these observations may also be due to other supplementing soluble phenolic and non-phenolic antioxidants or oxidative coupling reactions occurring in ‘Crofton’ peel and not other scald susceptible varieties.

**Phenolic Oxidation**

Coupled oxidation reactions involving phenolics result in the regeneration of the original phenolic and the oxidation of the another phenolic (Figure 6.49). For example, the $o$-quinone of chlorogenic acid are able to co-oxidise catechins into their $o$-quinone form and regenerate chlorogenic acid (Rouet-Mayer et al., 1993). However, the reverse situation is not possible because the reactivity with the reducing compounds is under the control of the redox potentials of the systems involved (Rouet-Mayer et al., 1993). Coupled oxidations have been reported in binary model solutions containing caffeoyl derivatives (chlorogenic acid) and the flavan-3-ols, epicatechin and catechin (Goupy et al., 1995; Richard-Forget et al., 1995; Cheynier et al., 1988; Oszmianski and Lee, 1990), procyanidins (Cheynier et al., 1988; Cheynier and da Silva, 1991), flavonols (Richard-Forget et al., 1993), or the dihydrochalcone, phloretin glucoside (Oszmianski and Lee, 1991).

![Figure 6.49 Coupled oxidation of a PPO phenolic substrate with another phenolic, resulting in the regeneration of the original substrate.](image)

The degraded phenolic may then undergo polymerisation and condensation.
Flavonols such as quercetin are relatively resistant to oxidation, however they have been reported to be involved with a coupled oxidation reaction with chlorogenic acid (Richard-Forget et al., 1993), which may account for the significant decline in quercetin glycosides in scalded tissue. The complete absence of chlorogenic acid and the large decline of flavonols in scalded tissue suggests that the degradation of phenolics is complete. Conversely the concentrations of flavonols and chlorogenic acid in the peel of the non-scalded ‘Granny Smith’ (non-scald and DPA) and in scald resistant ‘Crofton’ suggests that this coupled oxidation reaction with the flavonols is inhibited. Perhaps chlorogenic acid also acts as an antioxidant, preferentially reducing the normal ‘senescent’ oxidation reactions, instead of being diverted to flavonol coupled oxidation reactions. Under ‘normal’ senescent reactions in aging apple peel, the concentration of antioxidants, as highlighted by chlorogenic acid, is sufficient to prevent oxidative damage. However under oxidative stress associated with the induction of scald, the concentration of chlorogenic acid is not sufficient to quench excessive oxidation. This would lead to a diversion of normal phenolic metabolism to the coupled oxidative reactions that would then produce a significant decline in flavonols and perhaps further browning reactions. This diversion to coupled phenolic oxidation is discussed in more detail in Section 6.7.1, where PPO oxidation with a range of phenolic substrates is discussed.

The decline in phenolics in scalded tissue and browning are inextricably linked. However, the browning reactions involved in phenolic oxidation and degradation are complex. The degradation of 4-methylcatechol o-quinone in an acid environment, occurs via a hydroxylation of the o-quinone followed by a coupled oxidation of another molecule or o-quinone leading to the formation of 2-hydroxy 5-methyl-p-benzoquinone and the regeneration of 4-methylcatechol (Richard-Forget et al., 1992a). This same pathway has been observed with chlorogenic acid, but polymerisation reactions dominate (Richard-Forget et al., 1993).

Cheigh et al. (1995) showed that the PPO oxidation products of catechin were all brown pigments of varied intensities which when isolated in the early stages of reaction showed a higher antioxidant activity than those from the later stage. The mechanism of the antioxidant activity of the reaction products is unknown but may be explained by their abilities to donate hydrogen atoms or to scavenge free radicals (Cheigh et al., 1995). The considerable evidence of the role of PPO and POD in the development of scald cannot be overlooked (Lurie et al., 1989a; Du and Bramlage, 1995), and will be examined in Section 6.7. In addition, the metabolism and
Enzymatic oxidation of one mole of caftaric acid requires one half mole of oxygen (Cheynier et al., 1988). Oxidation of catechin can either be enzymatic with one oxygen atom consumed per molecule of catechin oxidised, or proceed via coupled oxidation, again consuming one oxygen atom per molecule of phenolic oxidised as one molecule of caftaric acid is regenerated for each molecule of catechin oxidised (Cheynier et al., 1988). Dimers (or larger oligomers) are then generated by condensation of a hydroquinone with a quinone without further oxygen consumption. The condensation product may be a good substrate for PPO, but has a lower redox potential than the original hydroquinone, and is therefore subject to oxidation by the corresponding quinones (Cheynier et al., 1988). In the same way, each polymerisation step requires one atom of oxygen for the initial enzymatic reaction, eventually followed by one or more coupled oxidations, leading to the formation of one quinone. This quinone then condenses with a hydroquinone to yield a larger oligomer. The oxidation of the latter consumes an additional oxygen atom so that the overall balance equals one oxygen atom per molecule of phenolic incorporated in a polymer (Cheynier et al., 1988).

However, the production of o-quinones does not have to be initiated by enzymatic oxidation. Rigaud et al. (1991) showed that flavonols (catechins and procyanidins) could be non-enzymatically oxidised by a coupled oxidation with enzymatically generated o-quinones, rather than by direct enzymic oxidation. This had also been shown for hydroxycinnamic derivatives, eg caffeic acid (Cilliers and Singleton, 1989; Cilliers and Singleton, 1991). Cilliers and Singleton (1989) and Fulcrand et al. (1994) showed that the non-enzymatic oxidation of caffeic acid was pH dependent, and extremely rapid at pH 8.0 and higher. Even under low temperatures and at low pH, phenolic autoxidation can be appreciable (Cilliers and Singleton, 1989; Fulcrand et al., 1994). Rigaud et al. (1991) suggest that the phenolic acid - flavonol condensation products form more easily than phenolic oligomers, and that the coupled oxidation of flavonols with o-quinones leads to increased discoloration. This reinforces the suggestion that coupled oxidation and condensations of o-quinones and flavonols are the major mechanisms leading to the formation of brown pigments (Rigaud et al., 1991).

The concentrations of total flavonols in non-scalded 'Granny Smith' peel were always higher than in other treatments and was the only peel sample in which total flavonols increased from 3
to 6 months in storage. Scald affected peel tissue always had the lowest levels of total flavonols and these levels declined from 3 to 6 months storage. Flavonols were the largest class of phenolics in the apple peel phenolic profile in both the non-scalked ‘Granny Smith’ samples and in the ‘Crofton’ peel. For example, flavonols contributed 62% of the total phenolic profile in ‘Granny Smith’ peel tissue after 6 months which did not develop scald, whilst in scalded ‘Granny Smith’ peel tissue total flavonols contributed only 22% of the total phenolics. Quercetin rhamnoglucoside, quercetin glucoside, quercetin xyloside, quercetin arabinofuranoside and Unknown Flavonol 3 all behaved similarly among all treatments. Quercetin rhamnoglucoside was the predominant quercetin glycoside detected in the peel of all apple treatments reaching 860 μg g⁻¹ in non-scalked ‘Granny Smith’ peel after 6 months storage that was 25% of the total phenolics in the peel. Piretti et al. (1994) also showed that concentrations of the quercetin glycosides in non-scalked tissue decreased to about 5% of the concentration in unaffected peel tissue. They concluded that in ‘Granny Smith’ apples, oxidative coupling of the o-diphenols in scald affected peel is the most likely explanation of the browning associated with scald. They found no evidence to involve flavonols, or condensation between flavonoid glycosides and gallic acid or polymerisation of flavan-3,4-diols with scald. To account for the decline of flavonols in scalded tissue, Piretti et al. (1994) suggested that the quercetin moiety of flavonol-glycosides was reduced to the corresponding flavan-3,4-diols, which is then readily polymerised to procyanidins (Figure 6.50).

Although knowledge of the biosynthesis of procyanidins is still limited (Porter, 1993), it is probable that procyanidins are formed by the condensation of flavan-3,4-diols and catechins. It would be expected that there would be an increase in the monomeric procyanidins (precursors to polymeric procyanidins) in the scalded tissue, however Piretti et al. (1994) showed that there was a significant decline in the concentration of dimeric procyanidin B2 in scalded tissue, compared to non-scalked peel tissue. In this study, after 3 months storage the concentrations of procyanidins in scalded peel tissue were similar to non-scalked peel. However by 6 months, the concentration of procyanidins in scalded peel tissue significantly decreased whilst remaining high in non-scalked peel tissue. The concentration of total procyanidins decreased by 3-fold in scalded tissue from 3 months to 6 months storage. This suggests that procyanidins are either metabolised in scalded tissue during storage, or they are polymerised into oligomers that are not extracted with 80% methanol and maybe responsible for scald symptoms. Lower concentrations of procyanidins do not directly relate to the development of scald, as the concentration of procyanidins in the scald resistant ‘Crofton’ were always lower than in the non-scalded ‘Granny
'Granny Smith' peel. After 6 months storage, procyanidins contributed 9% of the total phenolics in 'Crofton' peel, but contributed 37% in the peel of DPA treated 'Granny Smith' apples after 6 months storage. Procyanidin trimer was the major procyanidin in apple peel, and together with procyanidin B2, contributed over 95% of the total procyanidins in 'Granny Smith' and 'Crofton'. After 6 months storage, the concentrations of procyanidin B2 and procyanidin trimer in DPA treated fruit were higher than in non-scalded areas of peel in non-DPA treated fruit. Therefore DPA may have a role in preventing the loss of procyanidins in 'Granny Smith' peel during storage.

![Chemical structure diagram]

**Figure 6.50** Proposed pathway of flavonol metabolism and the development of scald symptoms in 'Granny Smith' apple peel

Adapted from Piretti *et al.* (1994, 1996)
Lea (1984) showed that apple procyanidins are themselves not substrates for PPO, but are oxidised by a chlorogenic acid ⇌ chlorogenoquinone redox shuttle. In a model system, the procyanidin was oxidised while the amount of chlorogenic acid remained fairly constant (Lea, 1984). These results were consistent with the in vivo peel observations made in this work, and support a central role for chlorogenic acid in phenolic coupling reactions.

The relative changes in the total flavan-3-ols in all apples and treatments from 3 to 6 months were minor. Although the levels of total flavan-3-ols in scalded peel tissue decreased by more than half from 3 to 6 months, when expressed as a proportion of the total phenolic profile, total flavan-3-ols contributed 22% to the total phenolic profile after 3 months, and 21% after 6 months storage. Hence any the differences in total flavan-3-ols reflected the general differences in phenolic metabolism and turn-over. Catechin and epicatechin contributed the majority of the flavan-3-ols. Concentrations of epicatechin declined during storage from 3 to 6 months while the concentrations of catechin varied during storage. Unknown Flavan-3-ol 3 and Unknown Flavan-3-ol 4 were exclusively detected in ‘Crofton’ peel and the concentration did not change significantly from 3 to 6 months storage. Unfortunately the identity of these unknown phenolics were not elucidated. The presence of these compounds may help explain scald resistance in ‘Crofton’.

The browning associated with the aging / storage of wine may have some similarities to the fate of phenolics in apples storage. Baron et al. (1997) showed that most of the phenolics in wine decrease with aging, and that the sherry-type wines are protected from excessive browning by the partial removal of the flavan-3-ols. They suggested that the flavan-3-ol monomer and dimers are oxidised, and condense into precipitates that are not involved in any further browning of the wine.

If procyanidins are involved in the development of scald symptoms, it may explain why ethanol vapours have been shown to inhibit scald development (Scott et al., 1995a). The mechanism of action of ethanol vapours in preventing scald is unknown. Although ethanol may cause physiological responses such as acting as an antioxidant that inhibits scald development, ethanol treatment may be successful in inhibiting scald symptoms by acetaldehyde cross linking apple peel procyanidins. The oligo procyanidin polymers are then unavailable to further oxidise and exhibit the typical phenolic browning symptoms associated with scald. Acetaldehyde can be generated in situ by oxidation of endogenous and exogenous ethanol and by decarboxylation of
pyruvic acid (Pesis and Ben-Arie, 1986). Tanaka et al. (1994) showed that acetaldehyde is formed from ethanol, presumably from alcohol dehydrogenase, and that during ethanol vapour treatment of persimmons, the water soluble proanthocyanidins become insoluble by condensation with acetaldehyde. Fulcrand et al. (1996) showed that the polymerisation reaction begins with the protonation of acetaldehyde in the acidic medium. This limiting stage yields a carbocation, which suffers a nucleophilic attack by the flavan unit (C-6 or C-8 of the A ring). The ethanol adduct thus formed gives a new carbocation by losing a water molecule. The corresponding species is, in turn, attacked by a second flavan unit to yield a dimer and the process continues, up to at least a hexamer (Fulcrand et al., 1996). (Figure 6.51). This condensation reaction of the water soluble procyanidins and acetaldehyde is non-enzymatic (Fukushima et al., 1991) and is similar to the phenol - formaldehyde reaction used in the plastic industry (Matsu and Itoo, 1982). These large insoluble oligomers may be unable to be further oxidised to produce the characteristic browning associated with scald development.

Figure 6.51  Mechanism of acetaldehyde - induced polymerisation of flavan-3-ols units

Adapted from Fulcrand et al. (1996)
Oh et al. (1980) showed that hydrophobic bonding plays a role in tannin interactions resulting in the relative hydrophobic nature of tannins. However the condensed insoluble polymers are to some degree readily re-solubilised by 1% hydrochloric acid dissolved in methanol (1% HCl - MeOH) (Oshida et al. 1996). No direct work has been undertaken on the condensed precipitated oligomer products as these polymerised tannins are hydrophobic and cannot be extracted (Tanaka et al. 1994). The hydrophobic nature of the precipitated products in scalded tissue may be related to the observation that the water content is lower in scalded hypodermal apple cells than in non-scalded tissue (Golding et al., 1997). In addition, these condensed oligomer products can produce a brown colour, which are no longer extractable with 1% HCl-MeOH, suggesting that the condensed oligomer can undergo further oxidation and is resistant to acid hydrolysis (Oshida et al., 1996).

The concentration of chalcones in the peel of ‘Crofton’ and ‘Granny Smith’ were low contributing a maximum of only 2% of the total phenolics in the peel. ‘Crofton’ had higher levels of phloridizin whilst ‘Granny Smith’ scalded peel tissue had the lowest levels. Non-scalded peel tissue and DPA treated fruit had similar levels of phloridizin and their concentrations were intermediate. The concentrations of phloridizin declined in all treatments from 3 to 6 months, and no phloridizin was detected in scalded tissue.

The concentration of unknown phenolics in ‘Granny Smith’ peel tissue was low and contributed less than 2% of the total phenolics, except after 6 months storage in scald affected peel where the unknown phenolics contributed 6% of the total phenolics. The concentrations of unknown phenolics in the ‘Crofton’ peel were higher contributing up to 8% of the total phenolic profile. Of the unknown phenolics, Unknown Phenolic 3 contributed nearly 80% of the total unknown phenolics in ‘Crofton’ peel, and remained relatively high and constant between 3 and 6 months. Unknown Phenolic 3 was not detected in scalded ‘Granny Smith’ peel tissue after 3 months storage, but after 6 months, there were significant quantities of Unknown Phenolic 3. The structure of these unknown phenolic compounds were not elucidated and more work is required to determine their structure and possible involvement in the development of scald.

Abdallah et al. (1997) showed that wounding inhibited the development of scald in ‘Granny Smith’ apples and also produced an increase in all classes of phenolics. Wounding induces many physiological stress responses. For example, Fleuriet and Macheix (1984) showed that after
wounding, there was a significant increase in enzyme activities in tomato fruit that result in the formation of monomeric units that are then polymerised by a peroxidase enzyme specific for lignification. Perhaps the wounding in ‘Granny Smith’ apple peel induced by Abdallah et al. (1997) had a similar function in wound repair and the preparation of a phytoalexin response to infection or wounding, and in the process inhibited the development of scald symptoms.
6.6 Harvest Maturity and Phenolics After Storage

6.6.1 Introduction

The aim of this section was to:
- compare the phenolic profiles in the peel of early and late harvested ‘Granny Smith’ apples after 6 months storage at 0°C in air

6.6.2 Materials and Methods

‘Granny Smith’ apples were harvested from the same set of trees in a commercial orchard in Bilpin, NSW on March 23 and one month later on 22 April 1993. One hundred apples at each harvest were stored at 0°C for 6 months. After cool storage and one week at 20°C the intensity and incidence of scald were recorded. The phenolics from the scalded and non-scalded peel were extracted and quantified (Section 6.3.2.8).

6.6.3 Results

6.6.3.1 Scald development

After 6 months air storage at 0°C, early harvested fruit developed more scald than later harvested fruit. A prestorage application of DPA completely suppressed any scald symptoms (Table 6.2).

<table>
<thead>
<tr>
<th>Table 6.2</th>
<th>Development of scald in ‘Granny Smith’ apples harvested in March and April with and without a pre-storage DPA dip. Fruit were stored for 6 months at 0°C then held for seven days at 20°C</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td></td>
<td>Granny Smith</td>
</tr>
<tr>
<td>Harvest date</td>
<td>Control</td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
</tr>
<tr>
<td>March (early)</td>
<td>2.8a</td>
</tr>
<tr>
<td>April (late)</td>
<td>0.7b</td>
</tr>
</tbody>
</table>

A scald score of 0 = no scald, 5 = very severe scald
Different letters indicate statistically significant differences (P<0.05)
6.6.3.2  **Total phenolics**

The concentration of total phenolics in the peel of ‘Granny Smith’ apples after 6 months air storage at 0°C was highest in early harvested apples, which did not develop scald (Figure 6.52). There were no differences in the concentrations of total phenolics in the peel of late harvested fruit that did not develop scald and DPA treated fruit of late harvested fruit. However, there were more phenolics in the late harvested DPA treated ‘Granny Smith’ apples than in early harvested fruit treated with DPA. Irrespective of the time of harvest, the concentrations of total phenolics in the peel of scalded apples were always lower than in non-scaled peel tissue. However, the later harvested fruit that developed scald had more total phenolics than early harvested fruit. Figure 6.53 describes the relative contributions of the various phenolics classes to the total phenolic profile in early and late harvested ‘Granny Smith’ apples after 6 months air storage. The relative proportions of the different classes of phenolics between late harvested non-scald treatments (DPA and no scald) were remarkably similar (Figure 6.53), indeed there were no differences in the concentration of total phenolics (Figure 6.52). In all cases, the combined contributions of the chalcones, benzoic acid derivatives, cinnamic acid derivatives and Unknown phenolics contributed less than 14% of the total phenolics for each treatment (Figure 6.53).

6.6.3.3  **Benzoic acid derivatives**

After 6 months storage, the DPA treated fruit had higher levels of benzoic acid derivatives (excluding benzoyl-β-D-glucose) than non-scaled peel tissue which in turn were greater than scalded peel tissue (Figure 6.54A). Harvest time had no significant effect on the concentrations of total benzoic derivatives (excluding benzoyl-β-D-glucose) within treatments. Similar trends were observed in the concentrations of the individual benzoic acid derivatives, particularly Unknown Benzoic 2 (Figure 6.54C). The only obvious trend was that in scalded tissue in later harvested fruit, there were greater concentrations of Unknown Benzoic 1 than in the early harvested fruit which also developed scald (Figure 6.54B). In addition, there were no significant difference among non-scaled treatments. Unknown Benzoic 3 was not detected in any sample.

Benzoyl-β-D-glucose was only detected in scalded tissue. Late harvested fruit contained more benzoyl-β-D-glucose in the peel than in early harvested fruit (Figure 6.55). Benzoyl-β-D-glucose contributed 31% of the total phenolics in early harvested fruit, compared to 18% in the late harvested fruit. (Figure 6.53).
Figure 6.52  Concentrations total phenolics in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.53  Relative proportions of phenolic classes in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early (A, C, E) or late (B, D, F). Scalded ‘Granny Smith’ peel tissue (A, B) was analysed separately from peel not showing scald symptoms (no scald; E, F). Fruit treated with DPA before storage are shown in Figure 6.53 C and D.
Figure 6.54  Concentrations benzoic acid derivatives in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. (A) Total benzoic acid derivatives; (B) Unknown benzoic acid derivative 1; (C) Unknown benzoic acid derivative 2.

Concentrations accompanied by different letters differ significantly at $P<0.05$
Figure 6.55 Concentrations benzoyl-β-D-glucose in 'Granny Smith' apple peel after 6 months storage. Apples were harvested early or late. Scalded 'Granny Smith' peel tissue was analysed separately from peel not showing scald symptoms. Concentrations accompanied by different letters differ significantly at P<0.05
6.6.3.4 Cinnamic acid derivatives

Scalded peel tissue contained lower concentrations of total cinnamic acid derivatives, than the non-scalded samples (Figure 6.56). DPA treated fruit contained high concentrations of cinnamic acid derivatives, but these levels were similar to those in the late harvested fruit that did not develop scald. Later harvested fruit across all treatments, always contained more total cinnamic acid derivatives than earlier harvested fruit. The cinnamic acid derivatives contributed up to 6% of the total phenolics in non-scalded peel tissue (Figure 6.53). However in scalded tissue, the cinnamic acid derivatives contributed only 1% and 3% of total phenolics in late and early harvested fruit, respectively.

The concentration of chlorogenic acid was higher in DPA treated fruit, than in non-scalded peel tissue (Figure 6.56B). The concentration of chlorogenic acid in the peel of late harvested fruit that did not develop scald, was higher than in early harvested fruit that did not develop scald. However, in DPA treated fruit, the earlier harvested fruit had higher concentrations of chlorogenic acid than later harvested fruit. As discussed earlier, chlorogenic acid was not detected in scalded tissue, as the retention times of chlorogenic acid and benzoyl-β-D-glucose were similar. Ferulic acid was only detected in scalded tissue, with higher concentrations in late harvested fruit (Figure 6.56D). Across all treatments the concentrations of coumaric acid were greater in later harvested fruit (Figure 6.56C). The concentrations of coumaric acid in the peel of the non-scalded samples (DPA and no scald) were similar, irrespective of harvest time. No coumaric acid was detected in early harvested peel that developed scald. Caffeic acid was not detected in any apple peel sample after 6 months storage.

6.6.3.5 Flavan-3-ols

The total concentrations of flavan-3-ols in the peel of ‘Granny Smith’ apples stored for 6 months were affected by the timing of harvest. In all cases, the concentration of total flavan-3-ols in late harvested fruit were higher than in early harvested fruit after 6 months storage (Figure 6.57). In most cases, the concentrations of flavan-3-ols in the peel of late harvested fruit were more than twice those in fruit harvested early. Indeed, the flavan-3-ols comprised 17% of the total phenolics in late harvested fruit, but only 7% of the total phenolics in early harvested fruit (Figure 6.53). Between harvest times, DPA and non-scalded peel tissue had similar levels of total flavan-3-ols, whilst the scalded tissue had fewer total flavan-3-ols. The flavan-3-ols contributed over 25% of the total phenolics in later harvested scalded tissue but only 8% of the total phenolic profile in
Figure 6.56  Concentrations cinnamic acid derivatives in 'Granny Smith' apple peel after 6 months storage. Apples were harvested early or late. Scalded 'Granny Smith' peel tissue was analysed separately from peel not showing scald symptoms. (A) Total cinnamic acid derivatives; (B) Chlorogenic acid; (C) Coumaric acid; (D) Ferulic acid.
Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.57  Concentrations total flavan-3-ols in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. Concentrations accompanied by different letters differ significantly at P<0.05
early harvested scalded tissue (Figure 6.53). All flavan-3-ols contributed to the high concentrations in later harvested fruit.

The concentrations of catechin were higher in later harvested fruit than early harvested fruit (Figure 6.58A) and was greatest in the peel of late harvest fruit which had developed scald and lowest in the scalded peel of early harvested fruit. The non-scalded peel generally contained more catechin than the DPA samples. The concentrations of epicatechin behaved similarly to catechin, in that the late harvested fruit always contained higher concentrations than early harvested fruit (Figure 6.58B). The scalded peel tissue contained lower concentrations of epicatechin than any of the non-scalded peel samples. Within each harvest, the DPA treated fruit had higher levels of epicatechin than the non-scalded peel. The concentrations of the Unknown Flavan-3-ol 1 and Unknown Flavan-3-ol 2 were only detected in late harvested fruit, except low concentrations of Unknown Flavan-3-ol 1 in non-scalded early harvest fruit (Figure 6.58C and 6.58D). Unknown Flavan-3-ol 3 and Unknown Flavan-3-ol 4 were not detected in any apple peel sample.

6.6.3.6 Flavonols
The most significant contributor to total phenolics in non-scalded peel tissue were the flavonols. In early harvested fruit, the flavonols comprised 71% of the total phenolics, whilst in the late harvested fruit, the flavonols contributed only 35% of the total phenolics (Figure 6.53). The concentrations of total flavonols were significantly affected by treatment (Figure 6.59). The highest concentrations of flavonols were detected in the early harvested non-scalded tissue, while the lowest levels of flavonols remaining in the peel after 6 months storage were detected in the scalded tissue (Figure 6.59). In non-scalded peel, the early harvested fruit had higher concentrations of flavonols than in later harvested fruit. However, in scalded peel tissue there was no significant difference between early and late harvested fruit. As a percentage of total phenolics, the flavonols in scalded peel tissue contributed over half the total phenolics in early harvested fruit, whilst they only accounted for 18% of the total phenolics in the late harvested fruit.

The concentrations of quercetin rhamnoglucoside, quercetin glucoside, quercetin xyloside, quercetin arabinofuranoside and Unknown Flavonol 3 followed the same trends with treatment and harvest time (Figure 6.60 and 6.61B). All individual flavonol concentrations were higher in early harvested fruit than in later harvested fruit except quercetin glucoside and quercetin
Figure 6.58  Concentrations of flavan-3-ols in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. (A) Catechin; (B) Epicatechin; (C) Unknown flavan-3-ol 1; (D) Unknown flavan-3-ol 2. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.59  Concentrations total flavonols in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.60  Concentrations of flavonols in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. (A) Quercetin rhamnoglucoside; (B) Quercetin glucoside; (C) Quercetin xyloside; (D) Quercetin arabinofuranoside
Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.61  Concentrations of flavonols in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. (A) Unknown flavonol 1; (B) Unknown flavonol 3; (C) Quercetin arabinopyranoside Concentrations accompanied by different letters differ significantly at P<0.05
arabinofuranoside in scalded peel. The early harvested non-scalded peel always had the highest concentrations of the individual flavonols, whilst the scalded peel tissue contained the lowest levels. The levels of the individual flavonols in the late harvested non-scald treatments were always similar, and lower than in early harvested non-scalded peel.

Only Unknown Flavonol 1 and quercetin arabinopyranoside showed differing responses to harvest time and scald development (Figure 6.61A and 6.61C). No differences in the concentrations of Unknown Flavonol 1 between harvest times of the non-scald treatments were observed, although the concentration of Unknown Flavonol 1 was greater in the non-scalded peel (Figure 6.61A). The concentration of Unknown Flavonol 3 was lower in the scalded tissue and higher in later harvested fruit (Figure 6.61B). Quercetin arabinopyranoside was only present in early harvested DPA and non-scalded peel tissue (Figure 6.61C). Unknown Flavonol 2 was not detected in any apple peel sample.

6.6.3.7 Procyanidins
The procyanidins contributed between 15 -35% to the total phenolics in the peel, except in the early harvested scald sample where no procyanidins were detected after 6 months storage. Among treatments, the concentrations of total procyanidins were always higher in the later harvested fruit (Figure 6.62A). DPA treatment at harvest resulted in higher concentrations of total procyanidins. No procyanidins were detected in early harvested scalded tissue. There was a significant interaction between treatment and harvest time. The responses of procyanidin B2 and the procyanidin trimer to treatment and harvest time were similar to the total procyanidins (Figure 6.62B and 6.62C). The concentrations of procyanidin B5 were relatively low (less than 70 μg.g⁻¹) but were higher in non-scalded tissue and not affected by harvest time (Figure 6.62D). Procyanidin B5 was not detected in any scalded tissue and was only present in the late harvested fruit treated with DPA at harvest.

6.6.3.8 Chalcones
The concentrations of chalcones were low and only contributed about 1% of the total phenolics (Figure 6.53), however the concentration of phloridzin in non-scalded peel (no scald and DPA) was significantly higher in the early harvested fruit (Figure 6.63). Within each harvest time there were no differences in the concentrations of phloridzin between DPA and non-scalded peel. Phloridzin was not detected in the scalded peel tissue of early harvested fruit and was relatively low in the later harvested fruit.
Figure 6.62  Concentrations of procyanidins in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. (A) Total procyanidins; (B) Procyanidin B2; (C) Procyanidin trimer; (D) Procyanidin B5 Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.63  Concentrations of phloridizin in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. Concentrations accompanied by different letters differ significantly at P<0.05
Figure 6.64  Concentrations of unknown phenolics in ‘Granny Smith’ apple peel after 6 months storage. Apples were harvested early or late. Scalded ‘Granny Smith’ peel tissue was analysed separately from peel not showing scald symptoms. (A) Total Unknown phenolics; (B) Unknown phenolic 1; (C) Unknown phenolic 2; (D) Unknown phenolic 3
Concentrations accompanied by different letters differ significantly at P<0.05
6.6.3.9 Unknown phenolics

Unknown phenolics contributed less than 1% of the total phenolics (Figure 6.53) and were affected by both treatment and maturity at harvest (Figure 6.64). In the non-scalded peel samples, the later harvested fruit had higher concentrations of unknown phenolics than earlier harvested fruit and the non-scalded peel tissue had higher levels of unknown phenolics than scalded peel tissue. Unknown Phenolic 2 followed the same trends as for the total unknown phenolics (Figure 6.64C). Unknown Phenolic 3 was only detected in the non-scalded peel tissue, and was higher in the earlier harvested fruit (Figure 6.64D). Unknown Phenolic 1 was only detected in late harvested DPA and non-scalded peel (Figure 6.64B). Unknown Phenolic 4 was not detected in any sample.

6.6.4 Discussion

It is well known that fruit maturity at harvest has a significant affect on scald development (Anet, 1972a; Chen et al., 1985), and as expected earlier harvested fruit developed more scald symptoms than later harvested fruit. The concentrations of phenolics in ‘Granny Smith’ peel after 6 months storage were different in the various scald and non-scalded peel samples and harvest fruit maturities. However it is unfortunate that the phenolic samples at harvest are unavailable, which limits interpretation of the data. However there are some significant differences in phenolic concentration after 6 months storage between early and late harvested fruit. As also shown in Section 6.5, non-scalded peel had higher concentrations of total phenolics across both harvest times than scalded peel. The high concentrations of total phenolics in the non-scalded peel tissue was mainly due to the very high concentrations of flavonols, particularly quercetin rhamoglcucoside in non-scalded tissue.

One of the major differences between early and late harvested fruit after 6 months storage at 0°C was the complete absence of procyanidins in scalced peel tissue in early harvested fruit. After 6 months storage the scalced tissue in the late harvested fruit, had 500 µg.g⁻¹ total procyanidins and comprised 30% of the total phenolics, whilst no procyanidins were detected in early harvested fruit which developed scald (Figure 6.53). This might be because the procyanidins in early harvested fruit had completely polymerised and were not extractable in 80% methanol. This was not observed in later harvested fruit because there were still potential antioxidants (such as
cinnamic acid derivatives and flavan-3-ols) that were capable of preventing complete procyanidin polymerisation. The pre-storage application of DPA resulted in an increase in the peel concentrations of total benzoic acid derivatives, cinnamic acid derivatives and procyanidins but had no affect on the concentrations of total flavan-3-ols, chalcones and total unknown phenolics.
6.7 Role of Browning Enzymes in Scald Development

6.7.1 Introduction
The browning of fruit and vegetables is a well known phenomena associated with damage or aging. Most browning is enzymatic, but in some cases non-enzymatic browning occurs. There are several mechanisms of non-enzymatic browning such as Strecker degradation, ascorbic acid degradation and the Maillard reaction (Vamos-Vigyazo, 1981). Peroxidases and polyphenol oxidases are widely distributed in the plant kingdom and have long been associated with the browning of fruit and vegetables. These browning enzymes are of significant economic importance and have been extensively studied and reviewed (Mayer and Harel, 1991; Vamos-Vigyazo, 1981; Rouet-Mayer et al., 1993). In addition, the enzymatic browning in apples and apple products has been extensively studied and reviewed (Richard-Forget et al., 1992a; Nicolas et al., 1994; Goupy et al., 1995; Murata et al., 1995).

6.7.2 Polyphenol oxidase
The role of polyphenol oxidase (PPO) in the development of scald was discussed in Chapter 3. The chemistry, specificity and activity of PPO reactions that may be associated with scald are discussed below.

6.7.3 PPO substrate specificity
Although there are a wide range of phenolics in apples (Table 6.1) only a small number serve as direct substrates for PPO. The specificity of the substrate to PPO oxidation is dependent on the type of phenolic. For example, the caffeic acid derivatives and the monomeric flavan-3-ols are often the best substrates (Stelzig et al., 1972; Janovitz-Klapp et al., 1990b). Procyanidins B2 and C1 have been shown to be oxidised by apple PPO, but their reaction rates are very low. Their maximum rates of oxygen uptake represented only 20 and 7% respectively of the rates for the monomeric flavan-3-ol, (-)-epicatechin (Lea, 1982). However, Cheynier and da Silva (1991) showed that grape seed procyanidins (dimers B1, B2, B5, B7 and the trimers C1, C2, C3 and 5 galloylated procyanidins) were not subject to any direct PPO oxidation, suggesting that any losses of procyanidin in the presence of PPO were due to the adsorption of the procyanidin molecules on proteins in the extract. On the other hand, Cheynier and da Silva (1991) demonstrated that procyanidins could be oxidised by enzymatically generated o-quinones from caffeoyltartaric acid. The coupled oxidation of procyanidin condensation products regenerated the original phenolic from its oxidised o-quinone. However once formed, all condensation
products are rapidly degraded to colourless compounds, either by enzymatic oxidation or by reactions with primary or secondary quinones (Sarni et al., 1995). However, the in vivo degradation and phenolic polymerisation from non-enzymatic coupled oxidation may lead to products which may be intensely brown (Rouet-Mayer et al., 1993). Similarly apple flavonols (quercetin glycosides) are not substrates for apple PPO (Stelzig et al., 1972; Lee, 1992). Stelzig et al. (1972) showed that the aglycone (quercetin) can be very slowly oxidised by ‘Red Delicious’ PPO. This rate of oxidation was over 20 times slower than that of chlorogenic acid. However this oxidation does not result in the formation of coloured compounds (Walker, 1995). The restricted activity of PPO on the glycosides is probably due to steric hindrance of the sugar moiety. Goupy et al. (1995) showed with 11 apple varieties that concentrations of hydroxycinnamic acid derivatives had a positive impact on the PPO oxidation of flavonols, while the high levels of phloretin glucose had a negative impact while the concentration of flavan-3-ols had no impact on flavonol PPO oxidation. This may be explained by the coupled oxidations of flavonol derivatives by o-quinones generated by PPO oxidation of chlorogenic acid (Richard-Forget et al., 1993).

Phloretin glucoside has also been reported to be a poor substrate for PPO (Goodenough et al., 1983), however Raa and Overeem (1968), and Lee (1992) found phloretin glycosides to be good substrates for apple PPO. Furthermore, Raa and Overeem (1968) showed that once the glucose moiety had been hydrolysed, phlorizin is rapidly oxidised via 3-hydroxyphloridzin to the corresponding o-quinone. The o-quinone is a transient intermediate that rapidly undergoes polymerisation reactions to form dark brown products (Raa and Overeem, 1968). The addition of chlorogenic acid, procyanidins or catechin accelerated the enzymatic oxidation and also produced strongly coloured oxidation products (Oleszek et al., 1989; Oszmianski and Lee, 1991). Oszmianski and Lee (1991) and Lee (1992) suggest that the synergistic effects of phloretin glycosides are very important in browning reactions in apple.

6.7.1.4 Apple PPO

PPO has been characterised in apple fruit (Janovitz-Klapp et al., 1989; Janovitz-Klapp et al., 1990b; Trejo-Gonzalez and Soto-Valdez, 1991), and partially purified (Harel et al., 1964; Richard-Forget et al. 1994). Marques et al. (1995) showed that in a native ‘Granny Smith’ pulp extract, there are three immunodetected PPO forms that are closely related and are still partially folded. Marques et al. (1994) suggested there was a latent PPO protein in apple fruit but later
showed that this inactive form was probably the partially unfolded form entering thylakoidal membranes (Marques et al., 1995).

6.7.1.5 Subcellular location of PPO

A comprehensive review and discussion of the subcellular localisation of PPO is covered in Chapter 3. However, it is important to note the subcellular separation of PPO from its substrates. In non-senescent tissue, the phenolic substrates are located in the vacuole, and PPO is a plastid enzyme that is usually found associated with the thylakoid membrane in the chloroplasts (Vaughn and Duke, 1981; Vaughn and Duke, 1984). The activity of the ‘Granny Smith’ pulp PPO was optimum at pH 4.0, which corresponds to the thylakoidal lumen pH where the PPO is localised (Marques et al., 1995).

In ripening and senescent fruit, it is often suggested that readily soluble PPO activity increases during the ripening of apples (Harel et al., 1964; Harel et al., 1966). This would not be surprising as the ripening and senescence processes increase decompartmentalisation, allowing a disruption of the chloroplastic membranes and disintegration of their lamellar structure, thus facilitating the liberation of PPO into the cytoplasm. Marques et al. (1995) suggested that although ‘Granny Smith’ PPO was membrane bound, it contains a hydrophilic protein with only a short hydrophobic anchor, which suggests that it can be solubilised. Barrett et al. (1991) further proposed that when PPO is solubilised from the chloroplast and is released into the cytoplasm, the enzyme remains either soluble or associated with the cell wall. Harel et al. (1964) showed that the addition of ethylene to ripening apples induced a small but significant increase in the activity of the soluble fraction. However, all these reports should be confirmed with modern cytolocalisation techniques as great care is required in subcellular fractionation studies since it is relatively easy to disrupt fragile membranes and contaminate the soluble fractions. This is critical in resolving the role of PPO in scald development, and it remains to be satisfactorily resolved.

6.7.1.6 Physiological role of PPO

Despite extensive studies, the physiological function of PPO is unknown (Vaughn and Duke, 1984; Vaughn et al., 1988; Steffens et al., 1994; Sherman et al., 1995). It is possible that a number of unrelated functions in normal plant growth and development, utilise the ability of PPO to participate in redox reactions and the production of quinones. For example, PPO has been postulated to play a role in buffering of plastid oxygen levels and mediating photosynthetic
electron transport (Tolbert, 1973; Sherman et al., 1995), phenolic metabolism (Vaughn and Duke, 1984), wound healing, and in host defence mechanisms (Beart et al., 1985b). Constabel et al. (1995) demonstrated that PPO and proteinase genes are co-activated systemically and induced by wounding via an octadecanoid signal transduction pathway.

6.7.1.7 PPO activity during storage

The involvement of PPO in the browning of apples and apple products (eg juice, sauces) is well known (Nicolas et al., 1994). However there have been large variations in reported PPO activity in apples (Janovitz-Klapp et al., 1989; Coseteng and Lee, 1987; Wakayama, 1995). This variation is not only related to the age of the fruit, but also to variety and histological localisation. In addition, there are a plethora of extraction and assay procedures that influence the measurement of PPO activity. In general, there is significantly more PPO activity in earlier harvested fruits than in commercially ripe fruits (Mayer and Harel, 1981; Macheix, 1990; Rouet-Mayer et al., 1993). There are significant discrepancies concerning its evolution in the later stages of ripening and development, and during storage (Janovitz-Klapp et al., 1989; Coseteng and Lee, 1987). However at commercial maturity, PPO activity is often at its lowest level, and the level of PPO activity generally remains constant during cold storage (Du and Bramlage, 1995, Lurie et al., 1989a)

PPO activity is dependent on its histological location. Some workers (Harel et al., 1964; Stelzig et al., 1972) found that PPO activity is greatest in the peel, whilst others (Klein, 1987; Prabha and Patwardhan, 1985; Wakayama, 1995) reported that the cortex contained the most activity. Murata et al. (1993) using an immunohistochemical procedure found that the core contained the highest levels of PPO, followed by skin tissue. Such differences may be due not only to the different identification and extraction methods but also to different varieties. For example, Janovitz-Klapp et al. (1989) showed that of the 12 varieties analysed at commercial harvest, seven varieties (’McIntosh’, ‘Fuji’, ‘Gala’, ‘Canada’, ‘Granny Smith’, ‘Estar’ and ‘Carden’) had higher levels of PPO in the cortex than in the peel, four varieties (’Red Delicious’, ‘Florina’, ‘Golden Delicious’ and ‘Jonagold’) had similar levels of PPO in the peel and cortex, while one variety (’Mutsu’) had lower levels in the peel than the cortex.

6.7.1.8 Correlation of PPO with apple browning

Apple browning in relation to PPO has been extensively studied (Amiot et al., 1992; Goupy et al., 1995), however there are no clear and consistent correlations between PPO, phenolic content
and apple browning (Nicholas et al., 1994). This is not surprising due to the many discrepancies in the literature regarding the substrate specificity of PPO, mainly due to the assay procedure, apple variety and age of fruit and subcellular extraction procedures. Klein (1987) concluded that in 22 apple varieties, there were no correlations between PPO activity, phenolic content and the extent of browning. Coseteng and Lee (1987) also showed there was no set pattern of changes in the degree of browning during maturation and cold storage with not all varieties showing the same relationship between browning and PPO activity. In some varieties (‘Classic’, ‘Rhode Island Greening’, ‘McIntosh’ and ‘Cortland’), PPO activity was directly related to the degree of browning, while in other varieties (‘Empire’, ‘Rome’ and ‘Golden Delicious’) the degree of browning was related more to phenolic concentration. Harel et al. (1966) working with 11 apple varieties and Murata et al. (1995) with 5 varieties found that phenolic content was correlated to browning.

Amiot et al. (1992) showed that hydroxycinnamic acid derivatives and the flavan-3-ols were the most important classes of phenolics involved in browning of 11 varieties of apple. Amiot et al. (1992) suggested that most of the soluble brown pigments were derived from hydroxycinnamic acid derivatives (especially chlorogenic acid), whereas the insoluble brown pigments were mainly formed from degraded flavan-3-ols ((-)epicatechin and procyanidin B2). Furthermore, they showed that the susceptibility of apples to browning was strongly correlated to the amounts of oxidised hydroxycinnamic derivatives and flavan-3-ols. Besides these two classes of phenolics, the flavan-3-ols and the dihydrochalcones appeared to have a significant influence on colour development (Goupy et al., 1995). They also found that the degree of browning was related to the amount of degraded phenolics. They were able to predict the amount of oxidised phenolics provided the initial levels of phenolics were known. Due to coupled oxidation, the oxidised amounts within one class of phenolic could be increased or decreased by increasing amounts in the other phenolic classes. Although the hydroxycinnamic derivatives had a major influence on apple browning, the other phenolic classes (flavan-3-ols, procyanidins, dihydrochalcones and flavonols) influenced colour development (Goupy et al., 1995). The rate of the PPO reactions also contributes to the degree of browning (Amiot et al., 1992). Other factors such as ascorbic acid and acidity may also play a role in apple browning (Amiot et al., 1992).

Hence there are no simple correlations as to the extent of browning in epidermal or cortical apple tissue. However, it should be noted that the vast majority of apple browning studies were
conducted on the browning of the cortex and not the peel, where scald is located. These findings clearly show that browning in apples is not directly related to the phenolic content, since the rate of browning is not additive of particular PPO (Lee, 1992). This may help explain the difficulties in finding correlations between total phenolic contents and browning in apples.

6.7.1.9 PPO and scald

Despite the apparent importance of PPO in the scalding process (Bain and Mercer, 1963), the levels of PPO activity have not been satisfactorily described in a range of scald susceptible and resistant varieties. Data presented in Table 6.3 shows there is no clear relationship between scald susceptibility and PPO activity (Janovitz-Klapp et al., 1989). Lurie et al. (1989a) showed that after six months air storage at 0°C, the activity of PPO in scald affected ‘Granny Smith’ peel tissue was nearly 3 times higher than in control green healthy peel tissue.

Table 6.3 Relative PPO activity in the peel and cortex of 12 apple varieties and their scald susceptibility [polarographic assay at pH 4.5]

Adapted from Janovitz-Klapp et al. (1989)

<table>
<thead>
<tr>
<th>Scald Suscep.</th>
<th>Variety</th>
<th>Peel</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>mod</td>
<td>Red Delicious</td>
<td>234</td>
<td>137</td>
</tr>
<tr>
<td>mod</td>
<td>Mutsu</td>
<td>167</td>
<td>74</td>
</tr>
<tr>
<td>slight</td>
<td>Fuji</td>
<td>134</td>
<td>97</td>
</tr>
<tr>
<td>unkn</td>
<td>Cananda</td>
<td>112</td>
<td>121</td>
</tr>
<tr>
<td>mod</td>
<td>McIntosh</td>
<td>108</td>
<td>110</td>
</tr>
<tr>
<td>severe</td>
<td>Granny Smith</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>unkn</td>
<td>unknown scald susceptibility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>slight</td>
<td>slight scald susceptibility</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Scald Suscep.</th>
<th>Variety</th>
<th>Peel</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>slight</td>
<td>Jonagold</td>
<td>100</td>
<td>59</td>
</tr>
<tr>
<td>unkn</td>
<td>Florina</td>
<td>98</td>
<td>55</td>
</tr>
<tr>
<td>slight</td>
<td>Golden Delicious</td>
<td>76</td>
<td>41</td>
</tr>
<tr>
<td>unkn</td>
<td>Charden</td>
<td>72</td>
<td>54</td>
</tr>
<tr>
<td>mod</td>
<td>Gala</td>
<td>69</td>
<td>66</td>
</tr>
<tr>
<td>unkn</td>
<td>Eistar</td>
<td>24</td>
<td>27</td>
</tr>
</tbody>
</table>

Boss et al. (1995) isolated a full length cDNA clone encoding apple PPO and showed PPO mRNA accumulated in wounded tissue and in the peel tissue showing the symptoms of scald. This up-regulation of PPO gene expression in scalded tissue suggests that the browning associated with scald may be due to changes in PPO expression. However this needs to be further examined to determine if this increased expression results in increased PPO activity and contributes to the development of scald symptoms.
6.7.1.10 Peroxidase

Peroxidases (E.C. 1.11.1.7, donor: hydrogen peroxide oxidoreductase) have been implicated in postharvest browning of a wide variety of fruits and vegetables (Burnette, 1977; Vamos-Vigyazo, 1981; Robinson, 1991a, b). Peroxidases can exist in a variety of isoforms and these can be divided into two distinct anionic and cationic groups depending on their isoelectric points (Vamos-Vigyazo, 1981; Robinson, 1991a). Moulding et al. (1987, 1988) isolated and purified five apple POD isozymes from ‘Cox’s Orange Pippin’ apples, and showed that the majority of POD isozymes were found in the soluble fraction of the peel.

It has been suggested that POD isozymes catalyse different oxidative reactions, and might be associated with different physiological processes (Robinson 1991a). This was also suggested by Zapata et al. (1992) in relation to the activity of grape POD isozymes to catalyse the characteristic oxidative pathways of hydroxybenzoic acids. Morales et al. (1993) showed that the basic isozyme appeared to play a role in the metabolic turnover of certain flavonol glycosides (quercetirin) once the aglycones (quercetin) had been released by specific glycosidases. In the xylem cell walls, it is more probable that POD plays a role in the polymerisation of cinnamyl alcohol to lignins (Morales et al., 1993). Morales and Barcelo (1997) showed that the substrate specificity of the basic POD isozyme located in the vacuoles and the cell walls from Vitis vinifera, was relatively plastic for oxidising phenolic substrates found in the two different subcellular locations (vacuole and cell wall). However Chibbar and van Huystee (1984) showed that the different isozymes in peanuts had similar oxidative pathways. Morales and Ros Barcelo (1997) concluded that in Lupinus albus (Fabaceae), Vitis vinifera (Vitaceae), Capsicum annuum (Solonaceae), Lactuca sativa (Asteraceae) and Catharanthus roseus (Apocynaceae), the cell walls contain the complete set of peroxidase isozymes including both basic and acidic POD isozymes, while vacuoles only contain the basic isozyme.

6.7.1.11 POD chemistry

Peroxidases can promote many diverse reactions and therefore have a degree of versatility not exhibited by other enzymes. Four general types of catalytic activity have been associated with peroxidases. These are peroxidatic, oxidatic, catalytic and hydroxylation reactions (Vamos-Vigyazo, 1981). POD can utilise both an oxidising substrate and a reducing substrate. The oxidising substrate is usually hydrogen peroxide or a peroxy radical, ROOH (Robinson, 1991a).
6.7.1.12 POD oxidation products

Peroxidase has low specificity for the hydrogen donor substrate and can catalyse the oxidation of a wide variety of naturally occurring plant phenolics, where the primary oxidation products are o-quinones. Specific examples of POD catalysed reactions have been described on flavanones (Patzlaff and Barz, 1978), aurones (Barz and Hoesel, 1978), p-hydroxybenzoic acids (Zapata et al., 1993), phenolic acids (Cvikrova et al., 1994), chalcones (Wong, 1989) and coumarins (Barz and Hoesel, 1978).

The oxidation of phenolics in model systems by POD has been shown to be extremely complex (Patzlaff and Barz, 1978; Miller and Schreier, 1985; Ryu et al., 1993). In a heterogenous matrix such as fruit, these reactions are likely to be even more complex (Robinson, 1991a). However, Noguchi and Mori (1969) showed that the enzymatic degradation of rutin in the leaves of Fagopyrum vulgare was similar to that found in micro-organisms. Peroxidases catalyse oxidation of the same phenolic compounds as PPO, but POD can also oxidise flavonols (Barz, 1977; Morales et al., 1993) and anthocyanins (Calderon et al., 1992; Lopez-Serrano and Ros Barcelo, 1996), and may contribute to enzymatic browning (Lopez-Serrano and Ros Barcelo, 1996). The main degradative pathways comprise: hydroxylation in the 3’ position, elimination of the B ring, cleavage reactions of the heterocyclic ring and oxidative destruction of the A ring (Barz and Koster, 1981). The diversity of substrates and the wide range of reactions catalysed by POD results in a wide variety of products. For example, in model systems the degradation of the flavanone, naringenin, by POD results in a complex range of reactions with many catabolites, which has important implications to the turnover and degradation of phenolics in fruit.

In the presence of an oxidising substrate, POD can not only degrade the main endogenous substrates of enzymatic browning, but also some phenolics which are poor substrates or even inhibitors of PPO (Richard-Forget and Gauillard, 1997). Richard and Nicholas (1989) showed that the aglycone flavonol, quercetin was a relatively good substrate for ‘Red Delicious’ peel POD but the corresponding glycosides were poorly oxidised. They further showed that the rate of POD oxidation was dependent on the type of glycosylation. They found that glucose (isoquercitrin) and galactose residues (hyperoside) led to a 80-90% decrease in the rates of degradation, whereas when the rhamnosyl residue was present (rutin) the decrease in reaction rates was over 98%. Similar rates were reported for pear, grape and Vicia faba POD isozyme oxidation of flavonols (Richard-Forget and Gauillard, 1997; Morales et al., 1993; Takahama and Egashira, 1991).
Secondary phenolic catabolism pathways generally start with the action of β-glycosidases that yield the corresponding aglycones (Barz and Koster, 1981). Barz and Koster further showed that the aglycone released by enzymatic hydrolysis would undergo an enzymatic oxidation catalysed by POD, which is strictly dependent on the supply of hydrogen peroxide. As the flavonol glycosides are poor substrates for POD (Morales et al., 1993), it would be likely that glycosidases and peroxidases may be coupled in flavonol degradation pathways (Surholt and Hoesel, 1978).

The study of the metabolism of phenolics is significantly complicated by oligo- and polymerisation reactions (Patzlaff and Barz, 1978; Schreier and Miller, 1985). Peroxidase catalysed polymerisation involves the formation of phenoxy radicals, where the unpaired electron is stabilised through the molecule. These radicals can either condense to form a polymer (lignification or polymerisation) or react with H₂O₂ or oxygen to form higher oxidation products, where proteins and carbohydrates may act as a backbone for the polymerisation process (Barz and Koster, 1981). Cell wall and membrane systems seem to be preferred sites of polymerisation reactions, perhaps because of their polysaccharide and / or protein structures serve as a matrix on which the polymers can form (Barz and Koster, 1981). Many of these reactions have been clearly demonstrated in in vitro reactions, but their in vivo functions are unknown.

Although most other plant POD can occur in the absence of H₂O₂ (Vamos-Vigyazo, 1981), Richard and Nicholas (1989) showed that ‘Red Delicious’ apple peel POD was dependent on H₂O₂. This suggests that apple POD is highly efficient in reducing H₂O₂, perhaps suggesting a role in the detoxification of H₂O₂ and possibly coupling of phenolic catabolism with the regulation of vacuolar pools of H₂O₂ (Takahama and Egashira, 1991).

6.7.1.13 Subcellular and histological localisation of POD

POD has been located widely throughout the cell with activity detected in tonoplasts, plasmalemma, mitochondria and microsomes (Abeles et al., 1989; Robinson, 1991a). Cytochemical studies have shown that tomato fruit POD is localised on the inner membrane surface of the tonoplast and the cell walls, and is thought to be only weakly attached to membranes (Thomas et al., 1981). This may also occur in apple hypodermal cells and have a significant role in scald development.
6.7.1.14 Physiological role of POD

Peroxidase has been implicated in many primary and secondary metabolic functions. Cell wall POD are thought to be involved in lignification (formation of $\text{H}_2\text{O}_2$ and dehydration of the monolignonols) (Lagrimini et al., 1993), IAA oxidation (Haard, 1977; Thomas et al., 1981), cross linking cell wall polysaccharides (Fry, 1986), wound healing (Espelie et al., 1986) and pathogen defence (Dowd and Norton, 1995; Appel, 1993). However, the role of vacuolar POD is less clear, and it has been proposed that it is involved in phenolic metabolism (Lagrimini et al., 1993; Morales et al., 1993). POD has also been suggested to play a role in the postharvest storage disorder, ‘Yuzuhada’, which shows excessive sclerid development (Haard, 1977), and the degradation of chlorophyll (Robinson, 1991a), which is a common process accompanying fruit ripening. Abeles et al. (1989) suggested that senescence is a complex process where POD may play a role in both degradative and synthetic processes.

6.7.1.15 Apple POD

There have been few studies devoted to the apple fruit POD. As with PPO studies, the reported activity of POD in apple fruit during storage is generally variable, depending on assay and extraction procedure. Peroxidase activity is higher in the peel than in the cortex of ‘Red Delicious’ (Richard and Nicholas, 1989) and ‘Cox’s Orange Pippin’ apples (Moulding et al., 1987). Moulding et al. (1987) also showed that there was significantly more soluble and ionically bound POD activity in the peel than in the cortex. Peroxidase activity has been found to increase with ripening (Gorin and Heidema, 1976; Vamos-Vigyazo, 1981). Gherghi et al. (1994) further suggests that POD activity is reflected by other respiratory indicators, such as ethylene production. For example both ethylene production and POD activity were inhibited at very low oxygen concentrations (0.5% $\text{O}_2$, 3% $\text{CO}_2$ at 0°C). And increased storage temperature (3°C), increased both ethylene and POD activity (Gherghi et al., 1994).

6.7.1.16 POD and browning

Rouet-Mayer et al. (1993) and Teisson et al. (1979) suggested that although POD are widely distributed, they are not thought to be involved in enzymatic browning following mechanical injury, as the internal level of $\text{H}_2\text{O}_2$ is limited. However their involvement in slow processes such as scald during cold storage is possible. Richard-Forget and Gauillard (1997) recently suggested that ‘Williams’ pear POD may be involved in enzymatic browning. They showed that pear PPO generated $\text{H}_2\text{O}_2$ that was utilised by POD, in the classical POD oxidation mechanism such as a Ping-Pong bi-reactant mechanism. The generation of $\text{H}_2\text{O}_2$, is thought to be due to
semi-quinone radicals which reduce molecular oxygen to then generate $\text{H}_2\text{O}_2$ or via superoxide (Jiang and Miles, 1993). Unlike PPO, all phenolic substrates can be oxidised by POD, but their rates are significantly different (Richard-Forget and Gauilliard, 1997). The relative significance of PPO and POD in the browning of pear flesh are affected by the nature of the oxidised phenolics and therefore by the stability of their corresponding o-quinones. However Richard-Forget and Gauilliard (1997) showed that the involvement of POD needs the presence of PPO activity to be effective. In addition, changes in POD isozymes are often involved in a response to physiological stress, wounding, fungal and viral infection (Robinson, 1991a). The precise chemical reactions catalysed by POD during these complex physiological changes have not been fully elucidated.

6.7.1.17 Ethylene and POD

In general, POD activity has been found to increase during fruit development, ripening and senescence (Thomas et al., 1981; Calderon et al., 1993). However the role of ethylene in regulating POD activity is uncertain, and probably depends on the type of tissue and physiological age of the tissue. In general, exogenous ethylene application has been shown to induce POD activity in alfalfa embryonic culture and in cucumbers and beans (Cvikrova et al., 1994; Abeles et al., 1989). Thomas et al. (1981) showed soluble POD increased dramatically during the early stages of tomato fruit development. Ionically and covalently bound POD were also observed and their activities increased steadily through fruit development (Thomas et al., 1981). This increase in POD activity also correlated with an increase in phenylalanine ammonia-lyase activity and accompanied an increase in phenolic acids, such as ferulic acid and cinnamic acid (Cvikrova et al., 1994). Ke and Saltveit (1988) also showed that ethylene not only induced ionically bound peroxidase activity in Iceberg lettuce, but also increased the flavonoid content which could easily be enzymatically oxidised to brown substances. Tomas-Barberan et al. (1997) showed that there were significant increases in the phenylpropanoid content before browning. However, POD is not always associated with plant tissue senescence. Merzlyak et al. (1993) showed that although some free radical oxidation pathways increase during leaf senescence, POD did not change significantly. Ferrer et al. (1996) showed that POD activity in lettuce leaves were not related to the ethylene concentration.

6.7.1.18 POD and scald

Manganaris and Alston (1993) examined apple POD isozymes from 83 apple varieties and classified them into 19 phenotypic classes, but found no relationship to scald susceptibility. For
example, the scald susceptible ‘Granny Smith’ had similar peroxidase banding patterns to the relatively scald resistant ‘Cox’s Orange Pippin’ (Manganaris and Alston, 1993). This was also demonstrated by Barnes (1993), who examined POD isozyme banding patterns in 12 varieties and showed there were no obvious correlations between the different isozymes. However, these POD isozyme studies were conducted on the leaves, bark, flower buds and roots and not in the fruit peel. Table 6.4 summarises the relative POD activity from the peel of 8 apple varieties (Richard and Nicholas, 1989), and shows there were few correlations between peel POD activity and scald susceptibility.

Table 6.4 Relative POD activity in the peel of 8 apple varieties and their scald susceptibility [spectrophotometric assay at pH 5.8]
Adapted from Richard and Nicholas (1989)

<table>
<thead>
<tr>
<th>Scald Susceptibility</th>
<th>Variety</th>
<th>Relative peel POD activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>moderate</td>
<td>Red Delicious</td>
<td>385</td>
</tr>
<tr>
<td>unknown</td>
<td>Querina</td>
<td>169</td>
</tr>
<tr>
<td>slight</td>
<td>Golden Delicious</td>
<td>153</td>
</tr>
<tr>
<td>moderate</td>
<td>Gala</td>
<td>150</td>
</tr>
<tr>
<td>unknown</td>
<td>Charden</td>
<td>138</td>
</tr>
<tr>
<td><strong>severe</strong></td>
<td><strong>Granny Smith</strong></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td>moderate</td>
<td>McIntosh</td>
<td>85</td>
</tr>
</tbody>
</table>

The browning associated with the development of scald is thought to be mediated by the oxidative enzymes PPO and POD, however our knowledge of these enzymes in scalding peel tissue is incomplete. The aim of this section was:

- measure the activities of the oxidative enzymes, PPO and POD, in the peel tissue of ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apples during storage in air at 0°C.

A pre-storage application of DPA was applied to half the ‘Granny Smith’ and ‘Lady Williams’ apples.
6.7.2 Materials and Methods

6.7.2.1 Apple materials

'Granny Smith', 'Lady Williams' and 'Crofton' apples were harvested from commercial orchards. 'Lady Williams' apples were harvested in May and June in Orange, NSW and Harcourt, Vic. After harvest 'Granny Smith' and 'Lady Williams' fruit were dipped in diphenylamine (3,000 mg.L\(^{-1}\)). All fruit were stored for up to 9 months as described in Section 6.4.2. At monthly intervals, apples were removed from cold storage and allowed to warm to room temperature (20\(^\circ\)C). From each treatment, four apple fruit replicates were sampled for enzyme analysis.

6.7.2.2 Crude enzyme extraction

Enzyme extraction and analysis was conducted modified from Lurie et al. (1989a). Five g of peel was removed using a vegetable peeler, and excess parenchyma tissue was removed. The peel tissue was blended with an ultra-turrux for 1 min in 15mL of 0.1M phosphate buffer, pH 6.2, plus 250mg PVP. The slurry was filtered through two layers of cheesecloth and then spun for 20 min at 12,000g and the supernatant assayed.

6.7.2.3 PPO assay

PPO activity was measured as the change in OD at 420 nm over 3 min in a reaction mixture consisting of 1.5mL crude extract and 1mL catechol solution. Boiled crude extract served as a control. The results were expressed as change in OD.min\(^{-1}\). A PPO standard (Tyrosinase, EC 1.14.18.1, Sigma Chemical Company, St. Louis, USA) was included in each assay to ensure relative PPO activities could be compared.

6.7.2.4 POD assay

POD activity was measured as the change in OD at 470 nm over 1 min in a reaction mixture consisting of 1.0mL crude extract, 1.0mL 30mM peroxide and 1.0mL 20mM guaiacol. Boiled crude extract served as a control. The results were expressed as change in OD.min\(^{-1}\). A POD standard (Type 1 HRP, EC 1.11.1.7, Sigma Chemical Company, St. Louis, USA) was included in each assay to ensure relative POD activities could be compared.
6.7.3 Results

The results of measurements of PPO and POD activities in the peel of ‘Granny Smith’, ‘Lady Williams’ and ‘Crofton’ apples are shown in Figure 6.65 and Figure 6.66. The activities of both POD and PPO during air storage at 0°C were variable. Although DPA treated fruit generally had lower enzyme activities than control fruit, these differences were not always significant (P<0.05). The activities of both PPO and POD in the scald resistant ‘Crofton’ were always lower than in the scald susceptible, ‘Granny Smith’ and ‘Lady Williams’. The activity of PPO was relatively constant during storage of all three apple varieties, whilst POD activity tended to increase during storage at 0°C in both ‘Granny Smith’ and ‘Lady Williams’ peel. The POD activity in ‘Granny Smith’ peel tissue, particularly at the end of the storage period, was higher than in either ‘Lady Williams’ or ‘Crofton’ peel.

6.7.4 Discussion

The relatively low and constant levels of PPO activities during storage is surprising considering the apparent importance of PPO in scald development (Bain and Mercer, 1963). Although PPO activities in ‘Granny Smith’ and ‘Lady Williams’ peel tissue were low and variable over the storage period, they were generally higher than in the peel of the scald resistant ‘Crofton’. Although enzyme standards were used, a measure of the protein content of the peel extract would have helped the interpretation of the enzyme activities, by providing a constant basis for comparison. However, the results show that PPO activity remains relatively constant over the storage period at 0°C.

PPO activity in the earlier harvested ‘Lady Williams’ (Figure 6.65C) was generally higher than in later harvested fruit (Figure 6.65D). This agrees with most of the published reports on apple maturity (Rouet-Mayer et al., 1993). Rouet-Mayer et al. (1993) showed that the overall PPO activity was higher in young green fruits, where high proportions of PPO were present in a bound form. And during ripening, PPO activity generally declined and more PPO was present in soluble forms. However, there are significant discrepancies concerning PPO evolution in the later stages of ripening and development, and during storage. For example, Janovitz-Klapp et al. (1989) found that PPO activity in ‘Granny Smith’ apples one month before and after commercial harvest remained constant whilst in ‘Red Delicious’ apples, PPO activity declined during storage. In addition, Coseteng and Lee (1987) showed that the activity of PPO in both ‘Rhode Island
Figure 6.65 Polyphenol oxidase (PPO) activity (Δ OD.min⁻¹) extracted from apple peel during storage at 0°C. Figure 6.65A ‘Granny Smith’. Figure 6.65B ‘Crofton’. Figure 6.65C ‘Lady Williams’ harvested in May (early). Figure 6.65D ‘Lady Williams’ harvested in June (late). Bars indicate standard error bar of the means (S.E.M.; n = 4); where absent, S.E.M. bars fall within the dimensions of the symbol.
Figure 6.66  Peroxidase (POD) activity (Δ OD.min⁻¹) extracted from apple peel during storage at 0°C. Figure 6.66A ‘Granny Smith’. Figure 6.66B ‘Crofton’. Figure 6.66C ‘Lady Williams’ harvested in May (early). Figure 6.66D ‘Lady Williams’ harvested in June (late). Bars indicate standard error bar of the means (S.E.M.; n = 4); where absent, S.E.M. bars fall within the dimensions of the symbol.
Greening’ and ‘Cortland’ decreased during ripening and remained relatively constant during cold storage.

As with PPO studies, the reported activity of POD in apple fruit during storage is variable, depending on assay and extraction procedures used (Robinson, 1991a). In this study, greater differences in activity were observed in POD than in PPO activity. ‘Granny Smith’ peel tissue at the end of the storage period had relatively high POD activity, whilst the levels in ‘Crofton’ were always low. In other apple studies, POD activity has been found to increase during ripening (Gorin and Heidema, 1976; Vamos-Vigyozo, 1981). Gorin and Heidema (1975) found that POD activity in ‘Golden Delicious’ apples showed two increases in activity during CA storage (3-4°C, 3-4% O₂, 7-8% CO₂). Although no other supporting data were presented, Gorin and Heidema (1975) claimed that POD activity reached a maximum during the climacteric, which subsided then later rose again to signal the start of ‘senescence’. An increase in POD activity was also found different varieties during air storage (Vamos-Vigyozo, 1981). However, Nanos et al. (1994) showed that the POD activity (soluble fraction) in air stored ‘Bartlett’ pears increased well before the respiratory climacteric. Nanos et al. (1994) further showed that storing pears in 0.25% O₂ lowered POD activity, but upon transferring the fruit to air, POD activity increased on the first day and then declined roughly coincident with the delayed climacteric. Gherghi et al. (1994) showed that other respiratory indicators, such as ethylene production reflected the POD activity in ‘Golden Delicious’, Idared ‘,’ Jonathan and Stakkrimson apples. The precise relationship between POD activity, ripening and storage remains unknown.

The low levels of both PPO and POD in the scald resistant ‘Crofton’ suggest that low oxidative enzyme activities may be responsible for resistance to scald. However, other workers (Janovitz-Klapp et al., 1989; Richard and Nicholas, 1989) have shown that the activities of PPO and POD in the peel do not seem to be correlated to scald susceptibility (Tables 6.3 and 6.4). Du and Bramlage (1995) also compared the POD activity of three apple varieties with different scald susceptibilities, and showed that there was no general relationship between scald susceptibility and concentration of peroxidation products. ‘Empire’ is a scald resistant variety and accumulated significantly more TBARS (thiobarbituric acid - reactive substances) in the peel during storage than the scald susceptible ‘Cortland’ and ‘Delicious’. However, ‘Empire’ exhibited lower POD, PPO and catalase activities than that of the other varieties. In addition, there were no differences in TBARS and peroxide concentrations in ‘Cortland’ apples at scald
inducing and non-inducing temperatures, and no sudden increase occurred as ‘Cortland’ apples became liable to scald development on removal from storage.

The postharvest application of DPA lowered the activity of both PPO and POD in ‘Granny Smith’ and ‘Lady Williams’ peel tissue. Lurie et al. (1989a) also showed that after six months air storage at 0°C, PPO and POD activity in the peel tissue of DPA-treated ‘Granny Smith’ fruit were 45% and 30%, respectively of that in the control fruit. This may be an important feature of DPA action, where DPA not only inhibits the oxidation of α-farnesene (Section 5.4, 5.6), but results in the in vivo inhibition of the oxidative enzymes thought to be involved in scald development. DPA may directly interfere with enzyme function, or perhaps lower the selectivity of the enzyme or substrates.

Many compounds have also been reported to inhibit PPO (Walker and McCallion, 1980; Walker, 1995). Phenolics and their quinones are not only substrates for browning, or co-oxidation reactions, but they may also inhibit browning reactions by inhibiting PPO or acting as antioxidants. Walker (1976) and Walker and Wilson (1975) found that the in vitro addition of substituted cinnamic acid derivatives (cinnamic, p-coumaric and ferulic acid), inhibited ‘Granny Smith’ apple PPO. In addition, there is a wide range of results and mechanisms describing the nature and extent of PPO inhibition by the benzoic acid series (Pifferi et al., 1974; Janovitz-Klapp et al., 1990a). Aliphatic alcohols have also been shown to inhibit PPO (cresolase and catecholase) activity (Valero et al., 1990). They showed that the inhibition of PPO is caused more by the hydrophobic chain than the alcohol group, and that an increase in the number of carbon atoms results in a strong increase in the degree of inhibition. Janovitz-Klapp (1990b) showed that compounds containing conjugated double bonds were also effective PPO inhibitors. α-Farnesene is an unsaturated conjugated sesquiterpene that has been associated with scald development (Chapter 5). Perhaps there may be a direct relationship between α-farnesene and PPO activity that relates to scald development.

Lurie et al. (1989a) showed that the activity of PPO in scald affected peel tissue in ‘Granny Smith’ apples stored for six months in air at 0°C, was nearly 3 times higher than in control green healthy peel tissue. Whereas POD activity of scald affected peel tissue was four times lower than in green healthy tissue. However, Du and Bramlage (1995) showed there were no significant changes in POD and PPO activity, or TBARS and peroxide concentrations in the peel between scald-free and scalded ‘Cortland’ apples. Du and Bramlage (1995) further showed the
pre-storage DPA treatment in ‘Delicious’ apples had differential effects on the activity of PPO and POD, and the concentrations of peroxides and TBARS. They concluded that DPA may supplement the endogenous antioxidant concentration in the peel.

High levels of POD activity (Figure 6.66) and its wide substrate specificity suggest that POD may have a wider involvement in scald development than previously proposed. In addition, POD is widely found throughout the cell and could be released by decompartmentation, i.e. degradation of intercellular membranes, by the action of membrane degrading enzymes. It is important not to discount the role other oxidative enzymes, such as lipoxygenase (LOX), and superoxide dismutase (SOD), in the development of scald. For example, LOX is a membrane degrading enzyme which can cause substantial loss of compartmentation (Siedow, 1991). Lurie et al. (1989a) showed that a pre-storage DPA treatment not only reduces scald incidence but also significantly lowers the activity of LOX during cold storage. Du and Bramlage (1994b) showed that the postharvest application of DPA did not alter the activity of another important oxidative enzyme SOD, and showed that SOD was not related to scald development.
6.8 Incubation of Phenolics with Horse Radish Peroxidase

6.8.1 Introduction

6.8.1.1 Benzoyl-β-D-glucose

The presence of high concentrations of benzoyl-β-D-glucose in scalded apple peel raises some interesting questions about its biosynthesis, since benzoyl-β-D-glucose has not previously been reported in apples. The production of compounds containing a benzoyl group in plants is well documented. For example, the biosynthesis of the benzoyl moiety in the alkaloid, cocaine (Leete et al., 1988; Leete and Kim, 1988), is well established and is derived from phenylalanine (Leete, 1983). Leete et al. (1988) further showed that a range of benzoic acid substrates serve as precursors of the benzoyl moiety of cocaine. However the biosynthesis and possible physiological role of benzoyl glucose in apple peel is unknown. It may be derived from the degradation of phenolics or it may be synthesised via a re-directed or new phenolic pathway.

It is proposed that in apple peel benzoic acid is the likely precursor to benzoyl-β-D-glucose and that a glucosyl transferase (GT) esterifies a uridine 5’phosphate (UDP) glucose with benzoic acid.

\[
\text{benzoic acid} + \text{UDP-glucose} \xrightarrow{\text{GT}} \text{benzoyl-β-D-glucose}
\]

UDP glucose should be freely available in mature apple peel and thus the availability of benzoic acid (or its derivatives) or GT activity are likely to regulate the production of benzoyl-β-D-glucose in scalding peel tissue.

6.8.1.2 General phenolic glycosylation

Compartmentation of secondary metabolism at the tissue and cellular level is critical in understanding turnover and degradation of phenolics. The major question concerning the presence of benzoyl-β-D-glucose in scalding apple peel is whether different sites for turnover and / or catabolism exist. Current knowledge of phenolic turnover and catabolism is fragmentary, because most of the degradative pathways, as well as the enzymes involved and their location are unknown (Barz and Koster, 1981). The current theory on the compartmentment of phenolic metabolism involves a multi-enzyme complex located in the endoplasmic reticulum (ER), consisting of both the phenylpropanoid and flavonoid pathways (Hrazdina and Wagner, 1985). Although vacuoles are well known reservoirs for the storage of
phenolics (Macheix et al., 1990), the actual sites of synthesis and possible pathways of turnover and catabolism are unknown. It is thought that the GT is located in the cytoplasm (Hosel, 1981). This location is in keeping with the concept of a multi-enzyme system located in the ER proposed by Hrazdina and Wagner (1985). The glycosylation of flavonoids is considered to be the final step in flavonoid metabolism and is an important modification that increases their water solubility, which is necessary for retention of some flavonoids in the vacuole (Hosel, 1981). Indeed most of the phenolics present in apple fruit are glycosylated, except for the procyanidins and flavan-3-ols.

GTs were briefly discussed in Section 6.2.2.2 whilst Heller and Forkmann (1993) reviewed the various flavonoid GTs in plants. Macheix et al. (1990) concluded that GTs are non-specific and will glycosylate a range of phenolics, whilst Heller and Forkmann (1993) believe that the activity of specific GTs in the formation of various glycosides is a specialised function. For example, McIntosh and Mansell (1990) isolated three isoenzymes that catalyse the formation of F7OG (flavonone-7-O-glucoside). Although two of the isoenzymes could catalyse a range of phenolics, the extensively purified isozyme was highly specific for position 7 glycosylation of flavanones. Further GTs specific for hydroxyl groups in positions 7 (flavonoid 7-O-glucosyltransferase, F7OG), 3’ (F3’OG), 4’ (F4’OG) and 5’ (F5’OG) and carbons 6 and 8 (F6 / 8CG) of the flavonoid structure have been characterised, and some have been purified to apparent homogeneity (Heller and Forkmann, 1993). Using partially purified extracts of GT from ‘Splendour’ and ‘Granny Smith’ apple peel, Lister et al. (1997) showed that the enzyme preparation specifically transferred the glycosyl moiety from sugar donors to the 3-position of the flavonoid nucleus. Only flavonoid 3-glycosides occur in apple peel (Table 6.1).

Earlier literature suggested that all the compounds within a flavonoid class (eg flavonols) are accepted as broad substrates by a particular GT, although at different conversion rates (Hosel, 1981). Indeed, Lister et al. (1997) showed that a GT extract was able to glycosylate, to varying degrees, a range of flavonols and anthocyanins normally found in apple peel. Macheix (1977) and Ju et al. (1995a) further showed that apple UDPG-dependent GTs are a non-specific class of enzymes since they appear to affect the glycosylation of hydroxycinnamic acid derivatives, flavonols, coumarins, anthocyanins and lignin precursors. These observations may be misleading as the methods of enzyme purification and preparation are often non-specific. In addition, the activity of GT depends on the different sugar donors, eg glucose, galactose (Lister et al., 1997). Lancaster et al. (1992) suggest that there are more than ten GTs in apples.
However, Lister et al. (1994) showed that the synthesis of cyanidin glycoside in ‘Splendour’ apples was accompanied by a corresponding increase in quercetin glycoside synthesis, suggesting some GTs may act in unison.

GT activity has been shown to strongly increase in tomato pulp in response to physiological stresses such as wounding (Fleuriet and Macheix, 1984), and also to increase in the peel of ‘Ralls’ and ‘Delicious’ apple peel following ethrel application (Ju et al., 1995b). Since scald symptoms result from a disruption of normal senescence of the peel and can be considered a physiological stress, a general increase in GT activity may be possible. Therefore it is possible that a specialised GT in the hypodermal cells of scalding apple cells is involved in the esterification of benzoic acid and the production of benzoyl glucose during the scalding process. A UDP-glucose and GT have been reported to esterify benzoic acid to produce benzoyl glucose in different plant systems (Hosel, 1980; Schlepphorst and Barz, 1979).

The numerous hydroxycinnamic acid derivatives in apple peel, eg caffeic acid, are not normally present as free acids but as esters. It is possible that the GT involved in the glycosylation of hydroxycinnamic acids may be capable of esterifying benzoic acid. Indeed, in other Rosaceae fruits eg blackberries and raspberries, glucose esters predominate (Schuster and Herrmann, 1985; Macheix et al., 1990). Although the flavonoid GTs may be specific (Heller and Forkmann, 1993; Lister et al., 1997), it may be possible for the GTs responsible for the esterification of the carboxylic acid of the hydroxycinnamic acid derivative and an alcohol group of the organic compound (eg glucose, quinic acid), are relatively non-specific or have low affinity for benzoylation. This may be possible because of the relative simplicity of the esterification of hydroxycinnamic acid derivatives. The flavonoid GT maybe more specific because of its more complex nature and the consequent large steric effects of the flavonoid and sugars.

The presence of benzoyl-β-D-glucose in scalded tissue over the storage life of scalded apples demonstrates that the enzymes for the hydrolysis of glucose conjugates are absent or the catabolism is considerably lower than anabolism. This is surprising considering that the glucose esters of aromatic acids are usually metabolically active and show high turnover rates (Kojima and Uritani, 1973; Moulderez et al., 1978; Schlepphorst and Barz 1979), and the relative ease with which benzoyl-β-D-glucose is hydrolysed to benzoic acid and glucose (Schlepphorst and Barz, 1979). Schlepphorst and Barz (1979) further showed that other oligoglycosyl conjugates (eg malate) were significantly more stable than benzoyl glucose. For example, the addition of
benzoic acid to *Glycine max* cell culture initially produced benzoyl glucose, but eventually produced benzoyl malate. However, Schepphorst and Barz (1979) concluded that the conjugation reactions are governed by the characteristic properties of each species and by the chemical structure of the compounds involved. Therefore the reason benzoyl-β-D-glucose is relatively stable in scalding apple peel is probably specific to scalding tissue in apples.

### 6.8.1.3 Production of benzoyl-β-D-glucose

Knowledge of the mechanisms and especially the enzymology and histochemistry of benzoic acid and its derivatives is limited, particularly in fruit (Macheix *et al.*, 1990). Therefore it is difficult to determine whether the benzoic acid skeleton of benzoyl-β-D-glucose is derived from anabolism or catabolism. Benzoic acid skeletons may be derived or synthesised in several ways, including: (1) direct synthesis (Section 6.8.1.4), (2) natural turnover or metabolism (Section 6.8.1.5) or via (3) enzymatic catabolism (Section 6.8.1.6).

### 6.8.1.4 Direct synthesis of benzoic derivatives

The most important mechanism for the formation of benzoic acids in plants is the side-chain degradation of cinnamic acids (Gross, 1981). This mechanism is similar to the β-oxidation of fatty acids, but the main intermediates are cinnamoyl - coenzyme A (CoA) esters. The removal of the acetate unit yields the corresponding benzoic acid. The consequence of both mechanisms is that the substitution pattern of the individual benzoic acid is determined by their respective C₆-C₃ precursor. This is an important feature in any discussion of the potential source of benzoic acid. The C₆-C₃ precursor of benzoic acid is cinnamic acid, which has not been identified in apple peel (Section 6.2 and Appendix II). Although other cinnamic acid derivatives, such as coumaric and caffeic acid are common in apple peel, complete dehydroxylation to the unsubstituted benzoic ring has not been found. In addition, CoA esters from different sources may also be intermediates in the formation of benzoyl-β-D-glucose. Strictly speaking, benzoic acid is not classified as a phenolic, as phenolics possesses an aromatic ring and at least one attached hydroxy group (Macheix *et al.*, 1990). This latter point is important as the unsubstituted aromatic ring is very difficult to physiologically attain.

Benzoic acid can also be formed directly from the shikimate pathway by aromatization, especially from dehydroshikimic acid, with the consequence that the entire alicyclic C₆-C₃ carbon skeleton is retained (Gross, 1981). This is thought to be the main route for gallic acid and hydrolysable tannin production (Gross, 1981). However hydrolysable tannins have not been
identified in apples (Foo and Porter, 1981). Similar problems of substitution on the phenolic ring are still encountered where benzyol-β-D-glucose could not be produced, as the complete dehydroxylation of the phenolic ring is not known.

6.8.1.5 Phenolic metabolism

In addition to direct synthesis, benzyol glucose maybe derived from general phenolic metabolism. However the regulation of phenolic metabolism in plants is complex (Stafford, 1990). Phenolics are not inert end products, but are further metabolised or may be partially or completely degraded (catabolism). Phenolic metabolism was reviewed by Barz and Koster (1981) and Stafford (1990). The degradation and turnover of phenolics in stored apple peel is significant. For example, while the concentrations of procyanidins remain relatively constant or decline during storage, the concentration of flavonols increased during the first few months in cold storage (Figure 6.16).

Barz and Koster (1981) identified four types of reactions involved in turnover, or further catabolism of phenolics: (1) interconversion reactions involved in biosynthetic sequences, (2) conjugation reactions in which other compounds are attached to the substrate, (3) oxidative polymerisation reactions leading to insoluble high molecular weight polymers and (4) degradation or catabolism reactions. The first two types of reactions are not relevant to the production of benzoic acid derivatives, as the anabolic interversion reactions usually lead to an increase in the degree of substitution and the conjugation reactions only affect the degree or type of conjugation (Barz and Koster, 1981). The oxidative polymerisation reactions themselves could not account for the production of benzoic acid derivatives, but they maybe a consequence of oxidative polymerisation reactions and was discussed in Section 6.5.4.

The further metabolism or turnover of phenolics may involve degradative (catabolic) reactions in which larger molecules are broken down to simpler phenolics. Most degradative processes in plants appear to be integrated into a metabolic system (Barz and Koster, 1981). In such a system, the substrate to be degraded and the intermediates formed may be simultaneously subjected to further degradation, conjugation, interversion and/or polymerisation reactions. In addition, the reaction products are usually very reactive and easily subject to oxidation, substitution and coupling reactions. Therefore, the presence of benzyol-β-D-glucose in scalded tissue illustrates that benzyol-β-D-glucose is probably an end product, rather than an intermediate. It would be logical to link the decline in flavonols with the increase in benzyol-β-
D-glucose in scalding peel tissue. However there is no known mechanisms by which flavonols can be completely catabolised to benzoic acid. De Eds (1959) suggested that m-hydroxy phenylacetic acid could result from the metabolism of quercetin, via 3,4-dihydroxyphenylacetic acid. Although this dehydroxylation stage is theoretically possible, it involves a difficult chemical reaction that has not been reported.

6.8.1.6 Enzymatic catabolism

The major enzyme involved in the catabolism of phenolics in plant cells is POD. Flavonols, phenolic acids, aurones, flavanone and chalcones can be used as substrates by POD (Barz, 1977; Barz and Hoesel, 1978; Patzlaff and Barz, 1978; Schreier and Miller, 1985; Miller and Schreier, 1985). Flavonols are degraded via 2,3-dihydroxyflavanones, where the hydroxyl functions in positions 3 and 4' must be free (Patzlaff and Barz, 1978). Furthermore if the intermediates (2,3-dihydroxyflavanones) are enzymatically degraded, the side chain phenyl rings can be converted to substituted benzoic acids, such as coumaric acid. These acids can be further transformed by hydroxylation, decarboxylation and ring fission reactions (Patzlaff and Barz, 1978). Schreier and Miller (1985) studied the peroxidative transformation of quercetin at pH 5.5. They found more than 20 oxidation products including 2,4,6-trihydroxybenzoic acid and 3,4-dihydroxybenzoic acid. However the production of benzoic acid from substituted benzoic acids has not been reported. Schreiber (1975) isolated benzoic acid from the peroxidative degradation of the unsubstituted 3-hydroflavone, however such unsubstituted flavonoids have not been identified in apples.

Peroxidases are extremely good oxidising agents capable of oxidising a wide range of phenolic substrates (Schreier and Miller, 1984; Miller and Schreier, 1984), and have been implicated in scald (Section 3.5). This experiment aimed to:

- explore the in vitro capabilities of POD to produce benzoyl-β-D-glucose from a range of apple phenolic substrates.
6.8.2 Materials and Methods

Phenolics were extracted from the peel of ‘Crofton’ and ‘Granny Smith’ apples either not treated or treated with DPA (Section 6.4.2). In addition, catechin, quercetin rhamnoglicoside (rutin), chlorogenic acid standards were incubated with Horse Radish Peroxidase (HRP type 1, EC 1.11.1.7) or polyphenol oxidase (PPO, Tyrosinase, EC 1.14.18.1). The phenolic standards, HRP and PPO were obtained from Sigma Chemical Company (St. Louis, USA). The standard assay for the peroxodicatic degradation of the phenolics and apple extracts contained a final volume of 1.5 mL; 400 μl stock substrate (approximately 27 mM in 0.1 M citrate-phosphate buffer (pH 5.5)), 50 μl of aqueous hydrogen peroxide (8 mM.L⁻¹), and 1 mL 0.1 M citrate-phosphate buffer (pH 5.5). Hydrogen peroxide was omitted from the PPO assay. The reaction was started with the addition of 50 μl HRP or PPO (1 mg.mL⁻¹) and maintained at 25°C. The reaction was monitored by HPLC with diode array detection for at least 72 hours. A 20 μL sample from the enzymatic assay was measured on a C18 HPLC column (Section 6.3.2.7). Controls of the apple extract incubations were measured without the addition of HRP.

6.8.3 Results

There was no significant in vitro oxidation of phenolics in apple peel extracts by HRP and benzyol-β-D-glucose was not detected in any sample (Figure 6.67 and 6.68). However standard phenolic substrates were readily oxidised by HRP and PPO (Table 6.5), but no oxidation products were detected at either 254 nm or 280 nm. Rutin was not detected after 6 hours in the PPO incubation. Benzoyl-β-D-glucose was not detected in any sample. The control apple extracts had no residual peroxidatic activity (data not shown).

<table>
<thead>
<tr>
<th>Table 6.5</th>
<th>Incubation of catechin, chlorogenic acid and rutin standards with Horse Radish Peroxidase (HRP) and Polyphenol Oxidase (PPO) for 60 minutes at 25°C in citrate-phosphate buffer (pH 5.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic Standard</td>
<td>Time Zero (area units)</td>
</tr>
<tr>
<td>Catechin</td>
<td>130</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>72</td>
</tr>
<tr>
<td>Rutin</td>
<td>353</td>
</tr>
<tr>
<td>-</td>
<td>data not collected</td>
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Figure 6.67 Relative levels of chlorogenic acid, procyanidin B5, epicatechin, rutin and total phenolics in ‘Granny Smith’ control (a); Granny Smith’ DPA (b); and ‘Crofton’ (c) apple peel extracts incubated with HRP in citrate-phosphate buffer (pH 5.5) at 25°C and followed with C18-HPLC.
Figure 6.68  Relative levels of chlorogenic acid, procyanidin B5, epicatechin, rutin and total phenolics in ‘Granny Smith’ control (a, b); ‘Granny Smith’ DPA(c, d); and ‘Crofton’ (e, f) apple peel extracts incubated with (b, d, f) and without (a, c, e) HRP in citrate-phosphate buffer (pH 5.5) at 25°C and followed with C18-HPLC
The presence of benzoyl-β-D-glucose raises some interesting questions about phenolic metabolism in scalded peel tissue. In these enzyme incubation experiments, HRP did not oxidise the apple peel extracts from either ‘Granny Smith’ or ‘Crofton’ apples and benzoyl-β-D-glucose was not produced in any treatment. Indeed there was no significant oxidation in the apple extracts suggesting there maybe endogenous antioxidants protecting phenolic oxidation. In contrast, the enzymatic oxidation of the phenolic standards was rapid (less than 60 minutes). However no oxidation products were detected at either 254 nm or 280 nm in the standard phenolic oxidation. Miller and Schreier (1984) showed that HRP could rapidly oxidise a quercetin standard solution into over 20 oxidation products. Morales et al. (1993) showed that there was only one UV absorbing oxidation product formed during the course of quercetin oxidation by grape POD. Schreier and Miller (1985) showed that one of the first steps in the peroxidatic transformation of flavonols consists of the attack of the olefinic C-2 / C-3 bond leading to incorporation of oxygen into the flavonoid structure and finally, to the formation of a very complex composition of polar reaction products. Guyot et al. (1996b) isolated eight different products from the oxidation of (+)-catechin by grape PPO and found these oxidation products were mostly dimers which had been observed in other oxidative conditions eg chemical oxidation (Cilliers and Singleton, 1989), and oxidation in the presence of POD (Robinson, 1991a, b). The identified PPO oxidation products of catechin were all isomers of natural procyanidin dimers of the A or the B type (Guyot et al., 1996b), where the colour of the oxidation products depended on the type of dimer produced. Colourless oxidation products are of dehydrodicatechins of the B type which have only one C-C or C-O interflavan link positioned between the B and D rings, whilst the coloured (yellow) compounds corresponded to dehydrodicatechin A and to a structure of the quinone-methide type (Guyot et al., 1996b). Hathway and Seakins (1957) showed that prolonged auto-oxidation of catechin leads to the formation of polymers via a head to tail polymerisation (repeated condensation reactions between the A ring of one unit and the B ring of another) through quinones.

While the reaction products of catechin PPO oxidation have been shown to be dimers and other polymers of catechin with low polarity, the PPO oxidation of chlorogenic acid produced mainly polymers (Pierpoint 1966; Oszmianski and Lee, 1990). However, the enzymatic oxidation of a mixture of catechin and chlorogenic acid produced oxidation products of co-polymers which had higher polarity and were less brown in colour than the oxidation products of catechin or
chlorogenic acid alone (Oszmianski and Lee, 1990). However, when mixed together with other phenolics, catechin and chlorogenic acid can act as reducing agents in oxidising the other phenolics which are not good PPO substrates. Cheynier and Moutounet (1992) showed that following the initial enzymatic oxidation steps, the reactions proceeded via non-oxidative mechanisms, yielding a series of relatively polar compounds. Richard-Forget et al. (1995) and Cheynier and Moutounet (1992) further showed that the secondary co-polymers produced from co-oxidation reactions were able to react with the products of primary enzymatic reaction and hence influence the final colouration.

The complete absence of any detectable oxidation products in the present experiment suggests that the oxidation products were immediately polymerised making them undetectable by HPLC. These preliminary results are inconclusive as no benzyol-β-D-glucose or any oxidation product was detected by the enzymatic oxidation of apple peel extracts or phenolic standards and further work is required. Future studies to explore the production of benzyol-β-D-glucose must include a generalised GT and the use of purified apple peel PPO and POD. However, these results do raise the potential for the direct synthesis of benzyol-β-D-glucose by another route. This would probably be derived from phenylalanine, which is a key amino acid derived from the shikimic acid pathway. Phenylalanine is deaminated by phenylalanine ammonia lyase (PAL) to produce trans-cinnamic acid. The conversion of trans-cinnamate by cinnamate 4-hydroxylase to p-coumaric acid may be disrupted, and / or the removal of acetate from trans-cinnamate may be favoured to produce benzoic acid. The benzoic acid may then be glycosylated to produce benzyol glucose (Figure 6.69).

Barz and Koster (1981) report that a benzoic acid synthetase is associated with chloroplasts. In addition, Loffelhardt and Kindl (1975) showed that benzoic acid can be derived from phenylalanine via cinnamic acid, and occurs on the thylakoid membrane, a process that is apparently not reversible. Loffelhardt and Kindl (1975) further suggested that the production of benzoic acid in the thylakoid membranes of Astilbe chinensis proceeds via a cinnamic acid pool that is different from that of the soluble cinnamic pool. However this work remains incomplete. Therefore if the chloroplasts are still intact and functional in scalding tissue (Chapter 3), there may be a disruption of normal phenolic regulation leading to an over-expression of benzoic acid. The benzoic acid would then be glycosylated with a generalised GT on the endoplasmic reticulum / cytoplasm and benzyol-β-D-glucose sequestered into the vacuole.
Figure 6.69  Proposed pathway of benzoyl-β-D-glucose production in scalded peel tissue

PAL phenylalanine ammonia lyase; C4H cinnamate 4-hydroxylase;
UDP-glucose uridine 5′ phosphate glucose; GT glucosyl transferase
6.9 Discussion

Phenolics have long been associated with scald development in apples (Sando, 1924). Previous studies of apple phenolics have been unreliable because of the lack of specificity of the spectroscopic methods used. The advent of diode array HPLC has enabled the identification and quantification of apple peel phenolics and a re-examination of the role of phenolics in the development of scald.

The metabolism of phenolics in scald affected peel is significantly altered from normal senescence or aging. For example, the presence of benzoyl-\(\beta\)-D-glucose indicated a substantial change in phenolics metabolism. Benzoyl-\(\beta\)-D-glucose has not been previously isolated from apples and its biosynthetic pathway is unknown, but may result from a direct biosynthetic route starting from phenylalanine (Figure 6.69). Further work is required to fully explore other pathways of benzoyl-\(\beta\)-D-glucose production, such as via enzymatic degradation and metabolism of other phenolics.

The concentrations of total extractable phenolics in the peel were not directly related to scald development, as the scald resistant variety ‘Crofton’, contained levels of phenolics that were intermediate between the scald susceptible ‘Granny Smith’ and ‘Lady Williams’ varieties. However, it is possible that individual phenolics may confer either tolerance or susceptibility to scald. Phenolics are well recognised as the substrates for browning reactions, however not all phenolics easily undergo oxidation and polymerisation. For example, quercetin is relatively resistant to oxidation, but it has been reported to be involved with a coupled oxidation reaction with chlorogenic acid (Richard-Forget et al., 1993). Coupled oxidation reactions involving phenolics result in the regeneration of the original phenolic and the oxidation of the another phenolic. However the reverse reaction is not possible because the reactivity with the reducing compounds is controlled by the redox potentials of the reactants (Rouet-Mayer et al., 1993). This may account for the significant decline in quercetin glycosides in scalded tissue.

The complete absence of chlorogenic acid and the large decline of flavonols in scalded tissue suggests that the degradation of phenolics is complete. Conversely, the concentrations of flavonols and chlorogenic acid in the peel of the non-scalded ‘Granny Smith’ (non-scald and DPA) and in scald resistant ‘Crofton’ suggests that this coupled oxidation reaction with the
flavonols is inhibited. Perhaps chlorogenic acid also acts as an antioxidant, preferentially reducing the normal ‘senescent’ oxidation reactions, instead of being diverted to flavonol coupled oxidation reactions.

Although antioxidants have long been thought to be associated with the prevention of scald (Anet, 1974b), no one single antioxidant has been consistently associated with scald (Barden and Bramlage, 1994b). However, it is generally accepted that both lipid and water soluble antioxidants are involved in scald development (Anet, 1974b; Barden and Bramlage, 1994b, c; Meir and Bramlage, 1988). An important mechanism of antioxidant action involves scavenging free radicals at the surface of membranes and within the membranes. α-Tocopherol has been implicated in scald prevention (Anet, 1974b; Meir and Bramlage, 1988; Gallerani et al.; 1990; Barden and Bramlage, 1994b, c), but it is generally accepted that α-tocopherol alone does not play a large role in scald resistance (Barden and Bramlage, 1994c). α-Tocopherol is lipophobic and is thought to be located within membranes, including the tonoplast (Rautenkranz et al., 1994) in which its chromane ring is stabilised by hydrogen bonding with ester carbonyl groups of phospholipids (Terao et al., 1994). Such antioxidants may be conserved by phenolics and may defer the onset of detectable lipid peroxidation. This might be critical in maintaining cellular homeostasis during normal senescence and help prevent the development of scald symptoms.

The very high concentrations of cinnamic acid derivatives, particularly chlorogenic acid, in ‘Crofton’ and to a lesser extent in the non-scalded ‘Granny Smith’ peel tissue suggests that they may play a role in scald development. Ratty et al. (1988) demonstrated that flavonoids interact at the polar surface zone of the phospholipid bilayers and scavenge free radicals. It is likely that quercetin is located near the surface of membranes where aqueous peroxy radicals are easily trapped and are accessible to chain initiating peroxy radicals. Similarly flavan-3-ol monomers such as catechin, which have increased hydrophobicity are also thought to be localised near the membrane surface (Ratty et al., 1988). Perhaps phenolics, especially flavonoids serve as scavengers of aqueous peroxy radicals near the membrane surface, while α-tocopherol mainly acts as a scavenger of chain - propagating lipid peroxy radicals within the membranes. Hence phenolics may conserve the endogenous α-tocopherol content and prevent lipid peroxidation when membranes are exposed to free radicals. Therefore, the continued presence of chlorogenic acid in ‘Crofton’ apples and non-scalded ‘Granny Smith’ apples may be important in providing continued antioxidant protection.
The level of oxidative stress in the non-scalding peel tissue is relatively low and the reactive o-quinones produced are able to be quenched by antioxidants and prevent further oxidation. In non-scalding senescent peel tissue, the concentrations of endogenous antioxidants, such as α-tocopherol, are adequately maintained to prevent oxidative damage by redox coupling of phenolic antioxidants, such as chlorogenic acid. DPA is a well known antioxidant and free radical scavenger and a pre-storage application of DPA may similarly act to directly assist in maintaining the endogenous antioxidant levels to prevent scald. However in scalding peel tissue which is characterised by excessive oxidative stress, the levels of endogenous antioxidants and the ‘re-generative’ phenolics are rapidly depleted which would not be sufficient to quench excessive oxidation. This results in an uncontrolled oxidative stress at the tonoplast and would lead to a diversion to oxidative reactions. These uncoupled phenolic redox reactions may result in numerous oxidation, polymerisation and browning reactions resulting in scald symptoms in the hypodermal and epidermal cells. For example, the polymerisation of procyanidins in scalded tissue would result in a significant decline in procyanidins in the phenolic profile (Figure 6.46), which may then undergo further coupling and oxidation resulting in browning.

Piretti et al. (1994, 1996) suggested there was a link between α-farnesene oxidation and the reduction of the quercetin moiety of flavonol-glycosides (Figure 6.70). It is tempting to directly link the browning associated with scald to α-farnesene oxidation. However, a problem with this hypothesis is that although the coupling of α-farnesene oxidation and flavan-3-ol reduction is mediated by free radicals, the flavan-3,4-diol products expected to result from this reaction have not been identified in fruit. If there is a direct link between α-farnesene oxidation and flavan-3,4-diol production, which leads to the production of polymeric procyanidins, it would be expected that the production of at least the monomeric and dimeric procyanidins (eg procyanidin B2) would increase immediately after the peak of α-farnesene oxidation. Further questions regarding the timing of α-farnesene peak production and its oxidation also need to be considered. The results from Section 5.3 showed that α-farnesene oxidation (as evidenced by 6-methyl hepten-2-one production) occurs at the same time as the rise and subsequent decline in procyanidin concentrations. This supports a direct link between α-farnesene oxidation and quercetin decline in scalded tissue, but does not take into account the changes in other phenolic substrates and the localisation of the browning symptoms.
Figure 6.70  Proposed coupling of α-farnesene oxidation and flavan-3-ol reduction

Adapted from Piretti et al. (1994)
Chapter 7  General Discussion

Superficial scald is a physiological storage disorder of some apple varieties. The cause of scald is unknown, but the current theory proposes that scald is initiated by the oxidation products of a naturally occurring fruit volatile, α-farnesene (Huelin and Coggiola, 1970a,c). These auto-oxidation products of α-farnesene are conjugated trienes, principally conjugated trienols and hydroperoxides, which are thought to cause irreversible membrane damage and mixing of the vacuolar phenolics with PPO in the cytoplasm. This results in electron dense deposits on the tonoplast, which are responsible for the typical brown symptoms in the peel (Bain and Mercer, 1963). A central part of our understanding of scald development begins with the histology of the disorder. The classical work of Bain (1956) and Bain and Mercer (1963) showed that brown (black) deposits of polymerised phenolics or tannins formed on the tonoplast in scalding epidermal and hypodermal cells of the scald susceptible ‘Granny Smith’ apples. In severely affected peel tissue these cells die and the peel collapses giving the typical symptoms of the disorder. In the present study, modern methods of preparing tissue for histological examination were compared, and both EM and MRI were employed. The results confirmed the earlier work of Bain (1956) and Bain and Mercer (1963), and gave some further insights into the nature of scald.

The characteristic symptoms of scald were superimposed on normal senescence and aging during long term storage. The loss of cellular structure in non-scalded hypodermal cells during storage was a consequence of normal aging. The cytoplasm of aging cells contained pieces of plastids and osmophylic bodies, and the vacuole contained aggregates of electron dense material (phenolics) which were finely dispersed throughout the vacuole. TEM observations of scalded peel tissue were difficult due to the fragility of scalded cells. In an effort to reduce these difficulties, different fixation and preparation techniques were employed. These techniques were not available to Bain and Mercer in 1963. The most successful fixation technique used in this study utilised the plunge freezing method that revealed greater cellular and structural detail. The results confirmed that the scald symptoms are restricted to the epidermis and hypodermis. Scald could also be detected by MRI microimaging as the water content is lower in the scalded peel. Presumably, the water in the collapsing hypodermal cells is absorbed by the fleshy underlying parenchyma or is lost to the storage atmosphere. Why the epidermal and hypodermal cells develop scald while the cortical cells do not, remains unknown. This difference maybe related to
the evolutionary origins of the different tissue regions in apples. The epidermis and hypodermis are derived separately from the cortex and are differentiated from each other at a very early stage of fruit development (Gilbert, 1963).

It has generally been accepted that the appearance of brown pigments in scalded cells is an indication that the cells have died. However, the application of the FDA viability test showed that scalding cells were still capable of enzymatically cleaving FDA and retaining fluorescein, suggesting that at least the plasmalemma was still active and functional during scald development. Thus, it appears that scalding cells are still viable, at least during the early stages of scald expression. The use of ESEM showed that the anatomical differences between scalded and non-scalded peel tissue were small, and illustrated the advantages of avoiding fixation and dehydration artefacts produced by other EM techniques. Conventional TEM results confirmed that the electron dense deposits on the tonoplast are responsible for the characteristic scald symptoms. These deposits on the tonoplast were not soluble in 1% caffeine. The application of caffeine before fixation with osmium has been used to precipitate phenolics in the vacuole to aid observation (Flasch, 1955; Mueller and Greenwood, 1978). Therefore, the presence of electron dense deposits on the tonoplast indicates significant oxidation and polymerisation.

The specific browning reactions in scalding peel tissue are unknown, but are likely to result from phenolic oxidation and concomitant polymerisation reactions which may involve oxidative enzymes. It has been suggested that PPO plays a central role in oxidising vacuolar phenolics resulting in electron dense deposits on the tonoplast (Bain and Mercer, 1963). Since PPO is thought to be localised in the chloroplast and is not found in a soluble form in the cytoplasm, scald development should involve disruption of the chloroplasts. However, contrary to the findings of Bain and Mercer (1963), an intact chloroplast was observed in scalded hypodermal cells. In addition, chlorophyll fluorescence was observed in severely scalded tissue suggesting that there are functional chloroplasts in scalding cells. If some chloroplasts are still intact and functional, then PPO may have a more limited role than hitherto proposed. Further questions about the role of PPO in scald development are raised when it is known that PPO cannot oxidise a wide range of common apple phenolics, such as quercetin glycosides.

A strong case for the involvement of POD in scald development is proposed because this enzyme is found in many cellular locations, including the tonoplast, and it can oxidise a wide variety of phenolic substrates, including flavonols. The primary action of POD is to oxidise hydrogen donors at the expense of peroxides. POD is highly specific for H₂O₂ but it accepts a wide range
of hydrogen donors such as phenolics, including hydroxycinnamic acids and flavonoids. Although POD is widely distributed, it is often thought to be involved in enzymatic browning only following mechanical injury, as the internal concentrations of H₂O₂ are limiting. However, involvement of POD in slow processes, such as scald development in long term cool storage might be possible. The subcellular locations of the isozymes of POD and their possible changes during storage have not been widely investigated. A definitive role for POD in scald induced reactions can only be established when the participation of the individual isozymes and their subcellular locations are elucidated. This work should be assisted by the emerging availability of cDNA clones and antibodies that will enable studies on in situ hybridisation and immunocytology (Moerschbacher, 1992). It is envisaged that scald and/or chilling may induce changes in the subcellular location of an isoperoxidase thus bringing POD into contact with 'non-physiological' substrates. The apparently weak substrate specificity of POD may then lead to the metabolism of vacuolar phenolics, particularly flavonols, and the development of scald symptoms. This may also lead to the production of novel POD products, such as benzoyl-β-D-glucose. If, for instance POD became active at the tonoplast, it may produce a range of phenolic oxidation products initiating radical chain reactions on the tonoplast. The result would be the formation of phenolic oxidation products and phenolic co-polymers with other vacuolar or cytoplasmic constituents. Such an 'intracellular polymerisation' would lead to localised browning and cell death as observed in scalded tissue. Similar mechanisms have been suggested for the hypersensitive resistance reaction of wheat to stem rust (Moerschbacher et al., 1990; Tiburzy et al., 1990), where POD reactions result in intracellular lignification that restricts infection by the stem rust fungus, *Puccinia graminis*.

Jiang and Miles (1993) showed that in addition to the well known NADH oxidation pathway, H₂O₂ can be generated by autoxidation and tyrosinase (PPO)-catalysed oxidation of (+)-catechin. This H₂O₂ could then be used as an electron acceptor by POD. Richard-Forget and Gauillard (1997) recently showed that pear PPO generated H₂O₂ that was utilised by POD in classical oxidation reactions. They suggested that both PPO and POD may be involved in enzymatic browning in 'Williams' pears. Similar mechanisms for the oxidation of apple phenolics have not been demonstrated and could be a fertile field of study. Such studies could aid in the elucidation of scald, and also the browning reactions observed in bruised and minimally processed apple and pear products.

The set of intracellular or extracellular conditions that leads to an increase in the steady state concentration of active species and hydroperoxides beyond the capacity of the tissue to scavenge
them is termed oxidative stress (Halliwell, 1991). The formation of active oxygen species (such as singlet oxygen, superoxide, hydroxyl radicals and $\text{H}_2\text{O}_2$) in plant tissue is normal and a fundamental consequence of normal metabolism and senescence (Halliwell, 1991). Active oxygen species are highly toxic and can lead to severe cellular damage, such as lipid peroxidation. Lipid peroxidation is a free radical chain process which occurs during the oxidation of unsaturated fatty acids of lipids such as in membranes such as the tonoplast (Kappus, 1991). This results in disintegration of the membrane, decompartmentation of the cell, and eventually cell death. Under normal conditions, the balance between the formation and consumption of active oxygen species is controlled and in non-scaled senescing cells, the level of oxidative damage is limited. However Brennan and Frankel (1977) found that during the ripening of pears, the levels of peroxides have been found to significantly increase. However, for each source of active oxygen species involved in lipid peroxidation within the cell, there are defence mechanisms to detoxify these potentially hazardous species. A well known example of oxidative metabolism is based on the scavenging of superoxide by SOD. This leads to the formation of $\text{H}_2\text{O}_2$, which in turn can be further degraded to water and oxygen by catalase or is removed by ascorbate and ascorbate peroxidase. Furthermore, the extent of oxidative damage is determined by the presence of natural lipid and water soluble antioxidant systems whereby free radicals generated during senescence are transferred to non-toxic (and non-brown) products or are quenched. For example, $\alpha$-tocopherol and ascorbic acid scavenge free radicals including hydroxyl radicals, while carotenoids quench singlet oxygen.

It is proposed that the effects of oxidative stress on the tonoplast initiates scald, and that chilling may be a causative factor. Although the apple is a temperate species, chilling injury in the form of flesh breakdown in some varieties has been documented (Bramlage and Meir, 1990). Furthermore, it is believed that scald is induced by chilling temperatures (Watkins et al., 1995), however, there is no relation among the varieties that are susceptible to flesh breakdown and those that are susceptible to scald. The differences in susceptibility of the different tissues to chilling is probably due to the differences in metabolism and physiology that impart a range in susceptibility to physiological disorders.

The biochemical and physiological role of chilling injury / stress has yet to be fully explored. It is possible that chilling induces a change in metabolism that significantly increases oxidative stress. This in turn enhances lipid peroxidation and results in increased levels of subcellular decompartmentalisation in scalding cells. It is proposed that peel tissue of varieties that are predisposed to scald is unable to cope with the increase in lipid peroxidation. This may be due to
a significant increase in the levels of peroxidation in scalding tissue, and / or a lowered capacity to cope with oxidative stress. For example, the depletion of the endogenous antioxidants (eg phenolics) results in lipid peroxidation of membranes and uncontrolled uncoupled phenolic redox reactions. This peroxidation of membranes, such as the tonoplast, results in generalised cell disintegration. Alternatively, an increase in oxidative stress may result from an increase in active oxygen species, as a result of increased sensitivity to chilling injury. Chilling could disrupt electron transfer, where oxygen can act as an electron acceptor, leading to the production of superoxide anion and other damaging free radicals.

The current method for estimating the levels of α-farnesene in apple peel involves washing apples with hexane and measuring a specific UV absorption. A direct method of extraction and quantification of α-farnesene by GC determination was developed that avoids the need for large volumes of hexane and interference from other UV absorbing compounds present in hexane extracts. This method is superior to the current spectroscopic method of estimating α-farnesene in hexane washes of apple peel, as it is a direct and accurate measure of α-farnesene not only from the waxy cuticle, but also the epidermal and hypodermal cells where scald develops.

Earlier observations by Huelin and Coggiola (1968; 1970a, b) showed that the principal oxidation products of α-farnesene are conjugated trienes. If these conjugated trienes are responsible for irreversible peroxidative damage to cell membranes, according to the current hypotheses about the causes of scald, then large differences in the concentrations of α-farnesene and its auto-oxidation products among susceptible and resistant varieties would be expected. Indeed, scald susceptible varieties such as ‘Granny Smith’ produce large amounts of α-farnesene, and perhaps the consequent large levels of auto-oxidation products of α-farnesene are responsible for the increase in oxidative stress in scald susceptible varieties. The increase in oxidative stress may overwhelm the natural antioxidant defences (eg phenolics and α-tocopherol), which results in lipid peroxidation in the tonoplast. However a direct link between the oxidation of α-farnesene and scald development has been questioned. The oxidation of α-farnesene may only be a symptom of the increased levels of oxidative stress within scalding peel tissue. In non-scalding peel tissue, where oxidative stress is limited and the oxidation of α-farnesene proceeds slowly, its oxidation product, 6-methyl hepten-2-one is still produced, albeit at low concentrations. This suggests that the oxidation of α-farnesene per se is not directly involved in the initiation of scald.
If scald susceptibility is related to the amount of α-farnesene produced, it should be possible to demonstrate this by applying α-farnesene to apples. In the present study, exogenous application of α-farnesene did not induce scald symptoms in whole apples or apple discs. Similarly, scald did not develop in ‘Crofton’ or DPA treated ‘Granny Smith’ apples that were exposed to high levels of α-farnesene from ‘Granny Smith’ apples. This constant challenge of high concentrations of α-farnesene from ‘Granny Smith’ apples was not enough to overcome the natural or exogenous resistance to scald. However, these negative results should be viewed with caution because exogenous α-farnesene may not penetrate to the sites where scald is initiated. Under normal conditions, the hydrophobic α-farnesene presumably diffuses from the cell into the waxy cuticle or is actively excreted to the cuticle. It is likely that any transport mechanism for α-farnesene is designed to move this non-polar compound out of the tissue and not the reverse.

With this in mind, α-farnesene was applied in vapour and to the underside of peel discs, and scald was not induced by any application method.

A pre-storage application of DPA is well known to inhibit α-farnesene oxidation (Huelin and Coggiola, 1970a). The free radical scavenging abilities of DPA may also help to quench the general increase in oxidative stresses that initiate scald. This would result in a general lowering of the oxidative capacity of the cell, and hence lower 6-methyl hepten-2-one concentrations. The scald resistant ‘Crofton’ still produces 6-methyl hepten-2-one, illustrating that the oxidation α-farnesene still occurs. However, the low concentrations of 6-methyl hepten-2-one in ‘Crofton’ may not be solely due to lower concentrations of α-farnesene naturally produced by ‘Crofton’. The ratio of the concentrations of 6-methyl hepten-2-one to α-farnesene, which would indicate the relative levels of α-farnesene oxidation was significantly lower in ‘Crofton’ apple peel, suggesting that this variety also has a lower capacity to oxidise α-farnesene, and perhaps contain other antioxidant systems.

Scald symptoms often do not develop until late in cold storage and are often only evident after warming to room temperature, a characteristic which is also typical of a chilling injury. Possibly the hydroperoxides that are initiated by oxidative stress, are stable at low storage temperature and the defence and repair processes prevent free radical propagation and further injury. However, when the fruit are warmed to room temperature, the breakdown of hydroperoxides in the tonoplast occurs more rapidly than recovery and repair mechanisms, resulting in rapid phenolic oxidation and polymerisation. It is well known that the application of DPA is only effective in preventing scald symptoms if it is applied at the beginning of cool storage. In these experiments, the timing of DPA application had differential affects on ethylene and α-farnesene production.
However, a delay in treating ‘Granny Smith’ apples with DPA at 0°C had no affect on α-farnesene production. This is contrary to other studies (Watkins et al., 1993; Du and Bramlage, 1994), and suggests the role of DPA in inhibiting scald is not directly related to α-farnesene production. Alternatively the delayed DPA application may be ineffectual in preventing scald as lipid peroxidation in the tonoplast may have proceeded too far for the exogenous application of DPA to act as an effective antioxidant.

A close association between internal ethylene concentration and peel α-farnesene concentrations was observed. At 0°C there was a positive linear relationship between peel α-farnesene and internal ethylene concentrations, in both ‘Croton’ and ‘Granny Smith’ apples. However, the magnitude of this response was significantly different between the varieties, where ‘Granny Smith’ produced significantly more α-farnesene than ‘Croton’. A close relationship between ethylene and α-farnesene production is to be expected, considering the well known regulatory role of ethylene in fruit ripening and volatiles production (Golding et al., 1998). However, following the application of propylene (an ethylene analogue) to preclimacteric fruit, α-farnesene production increased within 1 week of propylene treatment, whilst ethylene production took about 3 weeks to respond. In contrast, in fruit ripened in air, increases in ethylene and α-farnesene production generally coincided. This suggests that the biosynthetic apparatus for α-farnesene production is available in preclimacteric ‘Granny Smith’ apples, but requires ethylene to initiate its production. The production of other fruit volatiles (eg esters) occurs some time later after the ethylene climacteric (Song and Bangerth, 1994). The difference in timing of the increases in α-farnesene and other fruit volatiles is probably related to their inherent differences in biosynthesis. α-Farnesene is derived from isopentenyl diphosphate via the mevalonate or glyceraldehyde pathway, while the majority of the other apple volatiles (esters) arise from branched chain acid moieties and straight chain moieties that are derived from amino acids or fatty acids, respectively.

An holistic approach to peroxidative damage is required to understand the complexity of cellular senescence and the aetiology of scald. For example, Shewfelt and Purvis (1993) proposed that rather than focussing on a single event (eg increase in an active oxygen species, or decrease in activity of a specific enzyme), the overall balance between pro-oxidants and defence reactions must be studied and appreciated. However a narrow focus is evident in most of the published work on scald. Most work has concentrated on remedial measures to prevent scald symptoms with little regard to the mechanisms of scald development. Where the biochemical and physiological mechanisms of scald development have been investigated, the work has often been
limited to re-examining α-farnesene production and oxidation, and other potentially important facets of apple peel physiology have been neglected. For example, the role of phenolics in scald development has not been fully examined in previous studies. Phenolics are important secondary plant metabolites, which can either act as antioxidants that assist in quenching oxidative stress, or they may act as substrates for browning reactions that typify scald symptoms. Previous studies on apple phenolics have been unreliable due to the gross spectroscopic and colorimetric methods used. These methods are inherently inaccurate because they are non-specific and are unable to distinguish between individual apple phenolics. New methods of extraction, separation and quantification were used to show that, in general, the phenolic profiles among varieties were qualitatively similar, but quantitatively distinct. Although total phenolics are not a guide to scald susceptibility or development, individual phenolics may play an important role.

Besides their capacity to scavenge free radicals, phenolics also help to re-generate other antioxidants. Lipid soluble antioxidants such as α-tocopherol mainly act as scavengers of chain propagating lipid peroxy radicals within the tonoplast to prevent oxidative damage. Phenolics are exclusively located in the vacuole and have different degrees of hydrophobicity and hence capabilities to scavenge free radicals within the tonoplast. However Ratty et al. (1988) showed that phenolics generally scavenge aqueous peroxy radicals near the tonoplast surface. Hence phenolics such as chlorogenic acid may conserve endogenous antioxidants (eg α-tocopherol) and prevent lipid peroxidation when the tonoplast is challenged with free radicals. It is proposed that naturally high concentrations of chlorogenic acid may confer scald resistance by enhancing antioxidant and peroxidative protection of the tonoplast.

Some phenolics such as the flavonols (eg quercetin glycosides) are resistant to oxidation, however, they can by oxidised in coupled reactions with other phenolics, such as chlorogenic acid. This may explain the large decrease (two-thirds) in the concentrations of extractable phenolics in scalded ‘Granny Smith’ peel compared to non-scalded peel from the same apples. This decline was mainly due to an 85% decline in the concentration of flavonols (quercetin glycosides) in scalded ‘Granny Smith’ peel tissue. The complete absence of chlorogenic acid and the large decline of flavonols in scalded tissue suggests that the degradation and polymerisation of phenolics was complete. Conversely, the presence of high concentrations of flavonols and chlorogenic acid in the peel of the non-scalded ‘Granny Smith’ (non-scald and DPA treated) and in scald resistant ‘Crofton’ suggests that this coupled oxidation reaction with the flavonols is inhibited. Perhaps chlorogenic acid acts preferentially as an antioxidant, reducing the normal ‘senescent’ oxidation reactions, instead of being diverted to flavonol
coupled oxidation reactions. In non-scalding apple peel, the concentration of antioxidants, highlighted by chlorogenic acid, are sufficient to prevent oxidative damage. However, under oxidative stress associated with the induction of scald, the concentration of chlorogenic acid is not sufficient to quench excessive oxidation. This would lead to a diversion to the coupled oxidative reactions that would then produce a significant decline in flavonols and perhaps further browning reactions.

The three-fold decline in total procyanidins in scalded tissue from 3 to 6 months storage suggests that procyanidins are either metabolised in scalded tissue during storage, or they are polymerised into oligomers that are responsible for scald symptoms. However, lower concentrations of procyanidins did not directly relate to the development of scald, as the concentration of procyanidins in the scald resistant ‘Crofton’ was always lower than in peel of non-scalded ‘Granny Smith’. The fate of the phenolics in scalded tissue has not been elaborated but presumably they undergo extensive oxidation and polymerisation resulting in the electron dense deposits on the tonoplast. The polymerised phenolics are insoluble in 80% methanol resulting in lower concentrations of extractable phenolics. Analysis of phenolic oligomers and polymers is complex but could assist the elucidation of browning reactions responsible for scald symptoms.

A major qualitative difference between scalded and non-scalded tissue was the presence of benzoyl-\(\beta\)-D-glucose in scalded peel tissue. Benzoyl-\(\beta\)-D-glucose has not been previously reported in apples and its function and biosynthesis is unknown. Benzoyl-\(\beta\)-D-glucose is clearly an end-product of the metabolism associated with scald. Phenolics are well known to play a role in mechanical and biological stress, including chilling injury (Abe, 1990; Macheix et al., 1990), perhaps the induced oxidative stress in scalded tissue changes phenolic metabolism and biosynthesis. The normal metabolism and turnover of phenolics in scalded tissue may have been disrupted, such that benzoic acid was produced via a direct route from phenylalanine, and an active general glycosyl transferase (GT) then generated benzoyl-\(\beta\)-D-glucose. GTs are required to facilitate the glycosylation of a UDP-glucose with flavonoids, and are thought to be located in the cytoplasm. The FDA viability test showed that other cytoplasmic enzymes (eg esterases) are active during the early stages of scald development, therefore it is likely that a generalised GT is available to glycosylate benzoic acid. Further work is required to explore alternative pathways of benzoyl-\(\beta\)-D-glucose production such as via enzymatic degradation and metabolism from other phenolics.
The application of modern analytical methods reported in this thesis has confirmed some previous results and posed some new challenges to the current hypothesis on scald development. A revised model is proposed in which general oxidative stress initiates scald by overwhelming a range of defence and antioxidant mechanisms that normally protect the tonoplast from peroxidation. According to this model, oxidative stress exposes the tonoplast to lipid peroxidation. In scald susceptible varieties, this leads to uncontrolled oxidation of phenolics and the typical scald symptoms. The initiation of this oxidative stress only occurs at chilling temperatures. Perhaps high levels of α-farnesene oxidation, whether a symptom or cause, results in compounds which overwhelm the natural antioxidant concentrations. This is consistent with the accepted antioxidant role of DPA and also with the observations that a scald resistant variety, Crofton, which produces only low amounts of α-farnesene but four-fold higher concentrations of chlorogenic acid than the scald susceptible ‘Granny Smith’.

This study has invoked a wider role for phenolics in scald development based on their dual functions as scavengers of active oxygen species and free radicals, and as substrates for browning reactions. Re-examination of published work on the action of PPO together with analysis of the natural occurring peel phenolics casts doubt on the commonly accepted role of PPO in producing the brown pigments associated with scald. It is proposed that POD may play an important role in oxidative stress reactions, phenolic metabolism and browning reactions in scald susceptible apple varieties.

Future work to explore the propositions posed in this thesis should include a wider range of apple varieties. The limited range of varieties used in this thesis showed that there are significant biochemical and physiological differences among and within scald resistant and susceptible varieties. Comparative studies should enable examination of the idea that the difference in oxidative stress is the underlying factor in scald susceptibility. This work should also include a systematic and comprehensive examination of the mechanisms of action of ‘new’ scald control measures (eg ethanol vapours and vegetable oils). This study has confirmed that the development of scald is a complex problem and instead of focussing on a single event (eg α-farnesene oxidation), it is clear that an holistic approach is required to understand the aetiology of superficial scald in apples.
PLEASE NOTE

The greatest amount of care has been taken while scanning the following pages. The best possible results have been obtained.


Barden, C. L. and Bramlage, W. J. (1994c) Relationships of antioxidants in apple peel to changes in α-farnesene and conjugated trienes during storage, and to superficial scald development after storage. Postharvest Biology and Technology 4: 23-33.


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**PLEASE NOTE**

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Nuclear Magnetic Resonance Imaging of Superficial Scald in ‘Granny Smith’ Apples

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Superficial scald is a storage disorder of some apple (Malus domestica Borkh.) cultivars (Bain, 1956). To understand more about this disorder it is important to have a greater understanding of its basic physiology and biochemistry.

1H nuclear magnetic resonance imaging (MRI) is a noninvasive and nondestructive method of examining the physical and chemical composition of complex structures. MRI has been used to examine in vivo changes associated with some apple disorders, e.g., watercore (Wang et al., 1988), but MRI has not been used to examine the development of superficial scald. Initial MRI experiments were conducted to examine the in vivo physical and chemical changes associated with superficial scald.

‘Granny Smith’ apples were stored in air at 0°C for 9 months. After removal and after a simulated shelf life of 7 days at 20°C, the apples were sorted into scalded and nonscalded classes that were used for imaging.

Proton magnetic resonance microscopy was performed with custom designed hardware at 4.7 Tesla for the whole apple imaging experiments. A multilocus spin-echo sequence was used with an 18-ms echo time and an in-plane resolution of 200 µm with a slice thickness of 1.5 mm. The whole apple images (Fig. 1a and 1b) illustrate whole apple T2-weighted multilocus spin-echo images, with a slice thickness of 3 mm. T2 is a sample-dependent parameter that relies on the spin-spin relaxation time due to dephasing of the protons (Callaghan, 1991). Figure 1a is an image of a nonscalded control apple, while in Figure 1b slight scald symptoms are indicated by a diffuse region on the skin on the apple. Proton density images of the same apples were compared (Fig. 1c and 1d). The scalded region of the apple was associated with an irregular outline of the fruit surface, with little visible damage interior to the fruit surface. The changes in proton density suggested a change in water content. This was further investigated with three-dimensional peal imaging.

For the peel imaging experiments, the proton magnetic resonance microscopy was performed at 7 Tesla, with custom designed hardware. A three-dimensional Fourier imaging sequence was used (Callaghan, 1991), with a 12-ms echo time. The longer peel imaging experiments were =40 min in duration, resulting in an in-plane resolution of 35 µm and a slice thickness of 200 µm.

The section chosen to illustrate this imaging work was on the edge of a severely scalded region. Four slices of a 32-slice, three-dimensional image set are shown in Fig. 2. The scald symptoms were restricted to about two-thirds of the apple peel, becoming more severe on the right hand side of the section. The remaining one-third of the apple peel appeared green and healthy. The darker region on the right hand side of the peel showed scald symptoms and indicates a change in water state. The differences were detected ≈200–600 µm below the skin surface. This effect was noticed on ten scalded peel samples and was absent in the eight non-scalded control sections from the same apples. These measurements correlated well with the dimensions of the hypodermal cells where the scald symptoms are localized (Bain, 1956). It is important to note that these apples exhibited short (<50 ms) T2 values at 7 Tesla. Therefore, in imaging sequences that are T2-weighted, the signal-to-noise ratio is low.

To determine whether the changes in water state observed by MRI were due to actual loss of water from the affected areas, the water content of scalded regions was compared to non-scalded regions of peel. The fresh masses of non-scalded and scalded peel sections (5 mm² x 2 mm deep) were measured before freeze drying, and the dry masses were determined from the freeze-dried peel. The water content of the scalded peel tissue (75.6%) was statistically (P < 0.05) lower than that of the non-scalded control (80.0%).

In conclusion, we have shown that scald was detected by NMR and that this change in

Fig. 1. Whole fruit imaging of ‘Granny Smith’ apples showing the development of superficial scald. (a) T2-weighted image of a nonscalded control; (b) T2-weighted image of an apple with slight scald symptoms (S); (c) proton density image of a nonscalded control; (d) proton density image of an apple with slight scald symptoms (S).
water state was due to water loss from scaldfected peel tissue. This finding may help interpret the browning reactions and mechanisms associated with the development of scald. MRI investigations into the relaxation and diffusive behavior in apples developing superficial scald may provide greater insights into the molecular mechanisms inherent in nonscalded and scalded apple tissue.

Literature Cited


Fig. 2. Four slices of a partly scalded 'Granny Smith' peel tissue. NS indicates nonscalded peel tissue, and S indicates scalded peel tissue.
COMPARISON OF THE PHENOLIC PROFILES IN THE PEEL OF SCALDED AND NON SCALDED 'GRANNY SMITH' AND 'CROFTON' APPLES

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Abstract

Superficial scald is a physiological storage disorder of apples, which is characterised by browning of the skin. Scald is thought to be due to an uncontrolled polyphenol oxidase (PPO) system oxidising the vacuolar phenolics. Phenolics have been implicated in numerous physiological processes during the storage of fruit. Therefore, it is important to understand the role of phenolics in long term storage and the development of superficial scald. In these experiments, phenolics were identified and quantified from the peel of pre-climacteric 'Granny Smith' and 'Crofton' apples during air storage at 0°C. The phenolics from both scald affected and apparently healthy green peel tissue from the same control 'Granny Smith' apples were extracted, separated and quantified with diode array HPLC. Analysis of scalded and non scalded peel tissue from the same 'Granny Smith' apples showed there was a significant decline in the concentration of all the phenolics, particularly the flavonols, in scalded tissue. A new phenolic compound, benzoyl-β-D-glucose, not previously identified in apples, was detected in the scalded peel tissue. These changes in phenolic profile may have significant implications concerning the role of phenolics in the storage physiology and the development of scald in apples.

1. Introduction

Superficial scald is a physiological storage disorder of apples which occurs during long term cold storage. It is characterised by browning of the skin and is currently controlled with the post-harvest application of diphenylamine (DPA). The browning is confined to the epidermis and hypodermis of the peel and can be removed with shallow peeling. The disorder affects some important cultivars of apple, such as 'Granny Smith' and 'Lady Williams', whilst other cultivars, e.g. 'Crofton', are resistant.

The current hypothesis concerning the cause of scald is that there is a disruption of cellular membranes which allows mixing of polyphenol oxidase (PPO) and the phenolic substrates (Figure 1). The diphenols located in the vacuoles are oxidised through the action of PPO to their respective o-quinones which then polymerise to form the electron-dense deposits that are a characteristic symptom of scald in the peel of affected tissue (Bain and Mercer, 1963). Phenolics have been implicated in numerous physiological processes that occur during the storage of fruit (Macheix et al., 1990). Therefore, it is important to understand their role in the long term storage and the development of superficial scald.

Much of the early apple literature regarding the identification, separation and quantification of apple fruit phenolics has been inconsistent and unreliable due to uncertainties in their identification and quantification. For example total phenolics have conventionally been measured with the colorimetric assay utilising Folin-Ciocalteu's reagent. This assay is sensitive but not very specific. For example, compounds such as enediols and reductones

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interfere with the quantification (Macheix et al. 1990). However the development of techniques such as diode array HPLC, $^1$H-NMR, $^{13}$C-NMR and FAB-MS, has enabled the accurate identification and quantification of apple phenolics (Macheix et al. 1990).

The objective of this work was to examine the changes in the phenolic profile of scalded and non-scaled peel tissue of the scald susceptible cultivar, 'Granny Smith' and the scald resistant cultivar, 'Crofton', using diode array HPLC.

![Diagram](image)

Figure 1. The uncontrolled polyphenol oxidase (PPO) system thought to be responsible for the browning symptoms associated with superficial scald in apples (adapted from Bain and Mercer, 1963)

2. Materials and Methods

2.1 Sampling

Fruit were harvested from a commercial orchard in the Orange district in New South Wales. 'Granny Smith' apples were collected one month before commercial maturity whilst the 'Crofton' apples were harvested two weeks before commercial maturity. A postharvest diphenylamine (DPA) dip (3.6 g litre$^{-1}$) was applied to half the 'Granny Smith' apples immediately upon collection of the fruit. All fruit were air stored at 0°C. Samples were removed at three and six months of storage and held at 20°C for 7 days. After scald assessment, the apple peel samples were prepared by shallow peeling, care being taken not to remove the underlying fleshy cortex. The DPA-treated 'Granny Smith' and 'Crofton' apples did not develop any scald symptoms. On 'Granny Smith' apples without DPA treatment, the peel was separated into green healthy peel and dark brown scalded peel tissue. The peel was immediately frozen in liquid nitrogen, ground into a fine powder and kept at -80°C until phenolic extraction.
2.2 Extraction of phenolics

Apple peel samples (2 g) were extracted with 80% methanol (10 mL) containing 25 μg.mL⁻¹ internal standard, naringenin. The peel was sonicated for 20 min, then centrifuged at 8,000 rpm for 10 min. The extract was passed through a C18 Sep-Pak (Waters, Millipore) to remove unwanted waxes and chlorophyll.

2.3 Analytical High Performance Liquid Chromatography (HPLC)

Separation and analysis was conducted with a Hewlett Packard Series II 1090 LC with diode array detection. A C18 column (Spherisorb ODS 2.5 micron) was maintained at 40°C during analysis. A solvent program employed 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile as the mobile phase, at a flow rate of 1 mL.min⁻¹. The solvents were constantly sparged with helium to remove any air from the solvents. The column was equilibrated with 10% acetonitrile/TFA and elution with this solvent continued for 3 min following injection of a 20 μL sample. Acetonitrile was then increased from 10% to 20% over 35 min. A second gradient from 20% to 50% acetonitrile over 5 min completed the run. The phenolics were detected using diode array detection (from 200 to 400 nm) but were monitored at 254 nm and 280 nm.

2.4 Peak identification and confirmation

Commercial phenolic standards were used to test the identity of common phenolics in apple peel. Comparison of retention times and UV spectra were used to confirm identifications. Unidentified compounds of interest were further characterised by NMR and MS.

3. Results

The total phenolics content of scalded peel of ‘Granny Smith’ apples after 6 months’ air storage at 0°C was significantly lower than in either DPA treated or non-scalded peel, or in peel from ‘Crofton’ apples of the same storage age (Figure 2). The total phenolics content was up to 4 times greater in the non-scalded peel compared to the scalded peel tissue from the same apples, whereas DPA treated fruit had intermediary levels of total phenolics.

![Figure 2](image)

Figure 2. Concentration of total phenolics in the peel of ‘Granny Smith’ and ‘Crofton’ apples after 6 months air storage at 0°C. Concentrations accompanied by different letters differ significantly at P ≤ 0.05.

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For all classes of phenolics, the non-scaled 'Granny Smith' peel contained a higher concentration than scalded peel after 6 months air storage at 0°C (Figure 3). Flavonols (quercetin glycosides) were a significant contributor (up to 70% in non scalded 'Granny Smith') to the total phenolics content of peel from stored apples. The content of flavan-3-ols (e.g. catechin and epicatechin) behaved similarly to the total flavonols content, with scalded peel tissue having significantly lower levels than non-scalded peel tissue from the same 'Granny Smith' apples.

The DPA-treated apples had phenolics contents intermediate between the scalded and non-scalded peel, except for the hydroxycinnamic acid derivatives. Levels of the total hydroxycinnamic acid derivatives, such as chlorogenic acid, were significantly greater in DPA treated fruit and than in non treated 'Granny Smith' fruit.

The peel of 'Crofton' apples had levels of phenolics similar to those of DPA treated 'Granny Smith' apples, except for the hydroxycinnamic acid derivatives, where the levels were up to three times greater than those in the peel of 'Granny Smith'.

Benzoyl-β-D-glucose was identified and quantified from the peel of scalded 'Granny Smith' apple peel. This 'new' compound was not detected in the non scalded or DPA treated 'Granny Smith' peel or the 'Crofton' peel.

Figure 3. Concentration of different classes of phenolics found in the peel of 'Crofton' apples (first bar in each graph) and 'Granny Smith' apples (bars 2 - 4) after 6 months air storage at 0°C. Concentrations accompanied by different letters differ significantly at $P \leq 0.05$. 

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4. Discussion

The results show that there is a significant decline in the total phenolics content in scalded apple peel tissue of stored 'Granny Smith' apples. This decline in the total phenolics is mainly due to a decline in the flavonols component. This difference has also been shown by Piretti et al. (1994), who suggested that the oxidative coupling of the o-diphenols in scald affected peel tissue is the most likely explanation of the browning associated with scald. Piretti et al. (1996) proposed that there is a reduction of the quercetin aglycone to flavan-3,4-diol followed by a production of polymeric proanthocyanidins. When tissue integrity is damaged there is an interaction of the polymeric proanthocyanidins with the polyphenol oxidases producing the characteristic brown symptoms. Piretti et al. (1994) found no evidence that there was any condensation between flavonols glycosides and gallic acid or polymerisation of flavan-3,4-diols involved with development of scald.

The presence of benzoyl-β-D-glucose in scalded peel tissue is intriguing as this compound has not previously been reported in apples. Heimhuber et al. (1990) identified and quantified benzoyl-β-D-glucose in the fruit of some Vaccinium species including cranberries and red whortleberries, while Horsley and Meinwald (1981) found it in the leaves of black cherry (Prunus serotina). Horsley and Meinwald (1981) suggested that benzoyl-β-D-glucose in black cherry leaves is a potential source of benzoic acid which has allelopathic effects by inhibiting the growth of red maple seedlings. However, its biosynthesis and any possible physiological role in apple peel is unknown and maybe an important area of future research.

References

THE ROLE OF PHENOLICS IN SUPERFICIAL SCALD IN APPLES

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Superficial scald is a long term storage disorder of some important varieties of apple, such as ‘Granny Smith’. Scald is a physiological disorder which is characterized by unsightly browning of the skin that appears during term storage. Superficial scald is confined to the epidermis and hypodermis of the peel and the browning is thought to be due to an uncontrolled polyphenol oxidase (PPO) system. The current theory of scald development involves the production of alpha-farnesene and its auto-oxidation to conjugated dienes. It is thought that these products disrupt the membranes and allow PPO to oxidize the vacuolar phenolics which are deposited as electron dense material on the tonoplast resulting in the brown pigments associated with scald.

Little work has been conducted on the role of the phenolics in the development of scald. Phenolics from both scald affected and apparently healthy green peel tissue from the same control ‘Granny Smith’ apples were extracted, separated and quantified with diode array HPLC. The phenolics of interest were identified with NMR, FTR and MS. The results and implications of the changes in phenolics during storage and their possible role in the development of scald will be discussed.
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ULTRASTRUCTURAL CYTOLOGY OF SUPERFICIAL SCALE IN GRANNY SMITH APPLES

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Abstract. The objective of this work was to re-examine the histology of superficial scald in 'Granny Smith' apples. Ultrastructural changes associated with scald were examined with transmission electron microscopy (TEM) following fixation by conventional chemical fixation and cryofixation (slam and plunge freezing) methods. Examination of tissue fixed by these methods confirmed the previously published TEM observations. The scald symptoms were associated with an electron dense deposit on the tonoplast of scalding cells. However contrary to expectations, the chloroplast membranes in scalded cells appeared to be intact. These observations challenge the conventional wisdom that membrane disruption allows the browning enzyme polyphenol oxidase (PPO), which is localised in the chloroplast, to react with phenolics that leak from the vacuole.

Superficial scald is a cool storage disorder of some important varieties of apple, such as Granny Smith. Scald is a physiological disorder characterised by unsightly browning of the skin that appears during long term storage. There have been relatively few histological investigations into this important postharvest disorder (Bain 1956, Kang and Lee 1987). Bain (1956) demonstrated that the development of scald is due to the progressive browning of the contents of the hypodermal cells. The only electron microscopy (EM) study on superficial scald in apples was conducted by Bain and Mercer (1963). They showed that in Granny Smith apples, the first submicroscopic symptom of scald is the formation of an electron dense material in close association with a normal constituent of the vacuoles of the hypodermal cells. As the disorder becomes more severe, additional material accumulates on the tonoplast and the proplast becomes disorganised. Bain and Mercer (1963) further showed that scald was distinct from normal aging, in that the scald symptoms were superimposed on the regular disorganisation associated with normal senescence.

The classic EM work of Bain and Mercer (1963) was conducted using osmium tetroxide as the fixative and embedded in 'Araldite'. However, since the early 1960's, there have been considerable advances in EM technology, which throw new light onto the interpretation of Bain and Mercer's classical chemical fixative EM work.

Cryofixation, or rapid freezing of the biological sample is a relatively new fixation technique which can physically stabilise cellular components within a few milliseconds, preserving the cell structures in their 'natural' state.

To further examine the development of superficial scald in 'Granny Smith' apples at the sub cellular level, a range of fixation techniques, including chemical and cryofixation (plunge and slam freezing) were used for TEM. This comparison of fixation techniques is essential to determine and interpret fixation artefacts to understand what changes occur in scalding apple cells.

Methods and Materials

Peel sections from scalded and nonscalded peel tissue from the same 6 month old stored Granny Smith apples were fixed with different fixation methods (chemical and cryofixation).

Chemical fixation was conducted using 3% glutaraldehyde in 0.066M cacodylate buffer pH 6.8, overnight. The fixed tissues were then washed in buffer (0.066M...
cacodylate buffer) and postfixed in 1% OsO₄ in the same buffer for 6 hours. Dehydration was conducted in a graded series of methanol, and were embedded in hard grade Spurr resin.

Cryofixation was conducted using two different cryopreparation techniques. The first cryofixation method involved cutting peel tissue sectors (< 0.5mm²) and slam freezing them onto a liquid nitrogen cooled polished sapphire mirror using a LifeCell CF100 cryofixation unit.

In the second cryopreparation technique the peel sectors (< 0.5mm²) were frozen by plunging them into liquid propane cooled to -196°C with liquid nitrogen using a Reichert Jung KF80 cryofixation apparatus.

After cryopreservation all cryofixed specimens were stored in liquid nitrogen until cryosubstitution. The frozen specimens were treated using a custom built unit. The specimens were washed with acetone, 4 times each for 20 minutes and embedded similarly to the chemically fixed tissue. All sections were cut using a Reichert Ultracut E ultramicrotome and stained with ethanolic uranyl acetate and Reynolds lead citrate. The sections were observed in Hitachi H800 and Phillips 400 electron microscopes.

Results
The plunge freeze fixation method showed the greatest amount of detailed internal cell structures. Conventional chemical fixation also gave satisfactory results however subcellular artefacts hindered interpretation. Slam freezing presented special problems for fixing apple tissue. The peel did not fix well and was difficult to cut.

Norscalded 'Granny Smith' hypodermal cells showed typical aging symptoms. There was evidence of a disintegrating cellular structure, the cytoplasm contained pieces of plastids and osmiophilic bodies. The vacuoles contained aggregates of a dense material which was finely dispersed throughout the vacuole.

In the scalded hypodermal cells, the aging symptoms were also present but superimposed on them was a significant deposit of an electron dense material on the tonoplast. In addition to the finely dispersed material in the vacuole there were larger and darker aggregates of the dense material present. This material looked physically similar to the material deposited on the tonoplast.

An interesting feature was the structural integrity of the chloroplasts in the hypodermal layer (Fig. 1). In both scalded and norscalded senescing hypodermal cells, the chloroplasts appeared fragile but still intact. The chloroplast in Figure 1 was in a severely scalded hypodermal cell, with a lack of cellular structure and although there were osmiophilic bodies in the chloroplast, it clearly shows an intact chloroplast structure.

Figure 1. TEM of a scalded hypodermal cell in a 6 month old, air stored 'Granny Smith' apple using conventional chemical fixation, x 17,000. Note the dark electron dense deposit (D) on the tonoplast (T) that is responsible for the browning symptoms, and the relatively 'intact' chloroplast (Ch) showing well defined grana (G). (Cy) cytoplasm, (CW) cell wall, (V) vacuole.

Discussion
Fixation of plant cells is notoriously difficult because of their large vacuoles. These fixation problems are further compounded by the senescent nature of stored Granny Smith tissue. Indeed, Bain and Mercer (1963) could only use a very small proportion of their sections for TEM. However, the fixation and preparation techniques used in this study were superior to those available to Bain and Mercer (1963).

In this study, conventional chemical fixation and the cryofixation methods (slam and plunge freezing) were used on scalded and...
nonscalded Granny Smith apple peel. It is generally thought that the better fixation technique preserves greater structural detail, however this may not be the case, as artefacts in fixation may make interpretation meaningless (Robards 1991). The best results were obtained from the plunge freezing followed by conventional freezing and slam freezing.

The plunge freezing method showed a more detailed internal cell structure and greater structural detail. Obvious fixation artefacts, such as ice crystal formation, only seemed to affect the vacuole but did not significantly obstruct structural details in the sections examined. However, conventional chemical fixation showed typical chemical fixation artefacts. For example dense material congregated in dispersive aggregates in the vacuole. In slam frozen specimens, there was significant structural damage to the cells themselves. In addition there were numerous artefacts in the slammed tissue, which included “chattering” in the cytoplasm.

All fixation methods confirmed the TEM observations of Bain and Mercer (1963), in that the brown symptoms were associated with an electron dense deposit on the tonoplast. It is a common ultrastructural observation that the most susceptible organelle to the onset of senescence or chilling injury is the chloroplast (Butler and Simon 1971 and Marganori et al 1989). Butler and Simon (1971) described the ultrastructural changes in tobacco leaves under normal senescence conditions and Marganori et al (1989) also demonstrated in chilling sensitive mature green tomato fruit cells, that the most sensitive organelle to the onset of senescence or chilling injury is the chloroplast. Indeed, Bain and Mercer (1963) reported that even in lightly scalced hypodermal tissue, the chloroplast membranes became disorganised, dispersing fragments of lamellae, vesicles, starch grains and osmiophillic bodies into the cytoplasm. However in nonscalced peel tissue of the same age, Bain and Mercer (1963) showed that the chloroplasts were slightly swollen but were clearly visible and appeared quite normal. Other ultrastructural changes observed in nonscalded cells due to senescence were in agreement with deBarsy et al (1989). However contrary to Bain and Mercer (1963); the chloroplasts in both scalced and nonscalded hypodermal cells had well defined stroma with distinct structure and appeared ‘normal’ (Fig. 1).

The presence of intact chloroplasts in scalced disorganised cells raises questions about the rate of senescence and injury of the scalcing hypodermal cell and its contents. These apples were stored in air for six months at 0°C, and under these conditions the respiration and sequence of senescence of the cells are dramatically altered. Perhaps the chloroplasts may senesce at a slower rate than other cellular structures. This is unlikely because it has been well established that under normal senescence conditions, the thylakoid membrane is the first subcellular membrane to lose its integrity (Butler and Simon 1971).

The presence of intact and visible chloroplasts in scalced tissue has important implications in the browning reactions involved in scalch development. Bain and Mercer (1963) suggested that the formation of the electron dense materials in scalced ‘Granny Smith’ hypodermal cells was a result of uncontrolled polyphenol oxidase (PPO) activity following the breakdown of the tonoplast. This allowed mixing of the vacuolar contents containing the phenolic substrates with the PPO in the cytoplasm resulting in a dense deposit on the tonoplast (Fig. 2).

Figure 2. Uncontrolled polyphenol oxidase (PPO) system thought to be responsible for the browning symptoms associated with superficial scalch. (adapted from Bain and Mercer, 1963)
This process is simply not senescence as not all the epidermal and hypodermal cells in scald susceptible cultivars undergo this superficial browning. Indeed the incidence and severity of scald is variable within the season, orchard conditions and within the fruit.

PPO is a ubiquitous enzyme that has been implicated in scald development (Bain and Mercer 1963). PPO catalyses the oxidation of phenols to their respective quinones, however despite extensive studies, its function is still unknown (Mayer and Harel 1979). There is now little doubt that PPO is a plastid enzyme localised in a range of plastid types Mayer and Harel (1979), however in most tissues, PPO is localised at the thylakoid membrane within the chloroplast in association with the PSII complex (Lax and Vaughn 1991). Vaughn and Duke (1984) report that PPO can apparently exist free in the cytoplasm of degraded or senescing tissue, such as ripening fruit, but this is thought to be because the membranes in senescing cells are exceptionally fragile and difficult to prepare during fixation (Thompson and Platt-Aloia 1987). Nevertheless, the presence of free PPO in the cytoplasm of senescing cells cannot be totally dismissed.

Phenolics are the primary substrates for PPO activity and are sequestered in the vacuole (Mayer and Harel 1979). The tonoplast in scalding apple cells appears relatively intact although there seemed to be some occasional breaks in the membrane. Indeed, the tonoplast in senescing cells usually remains intact until late into senescence (Woolhouse 1984) and normally, the chloroplast localisation of PPO ensures that the enzyme is physically separated from its phenolic substrates located in the vacuole.

Although the outer chloroplast membrane envelope of the chloroplasts in the scalded hypodermal cells appeared fragile the chloroplasts in the scalded cells seemed to have a regular internal structure, e.g. grana (Fig. 1). If the outer chloroplast envelope is intact there still maybe some passive movement of PPO from the chloroplast into the cytosol resulting in 'free' PPO in the cytoplasm. However if there is no movement of the PPO from the chloroplast, a nonchloroplastic source of PPO would also result in an uncontrolled mixing of the phenolic substrates and PPO, where dense deposits would form on the tonoplast.

PPO from the chloroplast, and there is no other cellular source of PPO then the electron dense deposits in scalded tissue must be generated by another mechanism. Perhaps peroxidases which are found in either a soluble or bound form in the cytoplasm, endoplasmic reticulum, chloroplast, plastid lamellae and cell wall are partly responsible for the browning symptoms (Barz and Koster 1981). Peroxidases can catalyse the oxidation of the major polyphenols in apples e.g. catechins, hydrocinnamic acid derivatives and flavorols (Richard and Nicolas 1989). The primary oxidised products of peroxidases are quinones, which can similarly polymerise (Barz and Koster 1981) and could also form dense complexes on the tonoplast.

Although peroxidases are widely distributed in the peel of apples (Richard and Nicolas 1989) they are often not considered to be involved in browning because it is thought that the internal level of H₂O₂ is limited. However their involvement in slow physiological processes, such as the development of scald is possible. Particularly when the membrane breakdown associated with the breakdown of the chloroplast and vacuolar membrane is thought to be free radical initiated (Anet 1969).

There obviously needs to be more work done on the enzyme localisation and kinetics in the hypodermal cells. This would include comprehensive immunocytochemistry and labelling work particularly over the ripening and storage life of scalding apples. In addition, further characterisation and quantification of the phenolic substrates and oxidised phenolic products will help in the understanding of the nature of the dense deposits on the tonoplast, typical in the scald symptoms.
References


Superficial scald in apples

John Golding

Abstract. Superficial Scald is a major storage disorder of some important local and export cultivars of apples, e.g., Granny Smith and Delicious. It is characterised by unsightly browning of the skin that appears during long term storage. The damage is only superficial and can be removed with shallow peeling. Diphenylamine (DPA) and ethoxyquin are approved postharvest treatments to reduce the incidence of superficial scald. However these chemicals are not accepted by some international health authorities and eventually may be banned in Australia. This would leave the apple industry with no reliable scald control measure. It is therefore imperative that alternative control strategies are developed. The causes of superficial scald have not been established. The research conducted in the late 1960’s still remains the basis of our current knowledge of scald. The primary aim of this project is to develop a better understanding of the physiology and biochemistry of superficial scald. MRI imaging, EM, light and UV microscopy of scald have confirmed much of the previously published work, i.e. that scald is confined to the hypodermis and epidermis of affected apples and rarely affects the underlying cortical tissue. The affected cells eventually become brown and collapse, to give the characteristic scald appearance. A new method for the identification and quantification of α-farnesene has been developed. This has overcome the inherent problems with the previous α-farnesene recovery procedure. It has also allowed the pattern of changes in the accumulation of α-farnesene and its auto-oxidation products to be accurately determined during storage.

Introduction

Superficial scald is a major problem for the long term storage of some important local and export apple and pear cultivars, such as Granny Smith, Delicious and Packham Triumph.

Superficial scald is a physiological disorder that is characterised by browning of the skin which appears during long term storage. This significantly downgrades fruit quality and market return.

Diphenylamine (DPA) and ethoxyquin are currently registered postharvest treatments which effectively control the development of superficial scald. However these amine type antioxidants are already banned in some overseas countries. If they are banned in Australia, this would leave the apple industry with no reliable scald control measure. An estimated 20% of Australian apples would require an alternative treatment to enable marketing.

Our current understanding of superficial scald is limited, and much of the work on which this theory is based is inconclusive and is often contradictory.

The current theory of scald development involves the oxidation of α-farnesene. Alpha-farnesene is a naturally occurring fruit volatile found in apple skin. Huelin and Coggioia (1963) proposed that the oxidation products of α-farnesene produce free radicals which eventually lead to organelle and cellular degradation resulting in cell death and browning of the skin.

The current method for the isolation and quantification of α-farnesene is described by Murray (1969). The method involves washing the surface waxes and cuticle (which contain some α-farnesene) from the surface of apples with hexane. After conducting a urea addition overnight and running the adduct through a florister column the levels of α-farnesene are quantified using UV absorption at 232 nm.

Bain (1956) reported that the development of superficial scald in Granny Smith apples after storage at low temperature is confined to the hypodermis and is characterised by browning of the cell contents. The hypodermis is the layer of cells immediately below the epidermis and is dissimilar to the underlying cortex (fruit flesh). Bain and Mercer (1963) further observed that the browning in the scald affected cells was due to an accumulation of a pigment on the tonoplast. They suggested that this observation was consistent with the breakdown in the metabolism of polyphenols in the cytoplasm and vacuole.

This paper describes the histological development of scald in Granny Smith apples. In addition, a new method for the identification and quantification of α-farnesene and its auto-oxidation products are also briefly described. A thorough understanding of the histology of scald and patterns of biochemical changes (particularly α-farnesene) within apples, will provide the basis on which alternatives to DPA and ethoxyquin can be examined.

Materials and Methods

Immature Granny Smith apples were harvested in April 1992 from a commercial apple orchard in Bilpin, NSW. They were immediately stored in air at 0°C.

The development of scald was followed by removing apples at successive intervals. Scald was allowed to develop for 1 week at 20°C upon removal. To distinguish between normal aging and scald, non scalded tissue from scalded apples was used as the control.
Transversely cut hand sections of peel tissue were examined under light and ultra-violet (UV) excitation using an Olympus BH light microscope.

**Determination of Cell Viability**

A method for determining the viability of the cell utilizes fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison 1970). The FDA technique depends upon the integrity of the plasmalemma and an associated esterase activity. FDA is non-polar and non-fluorescing, and is permeates intact plasmalemma. The esterase from intact cells cleaves the acetate from FDA as it passes into the viable cell. The resultant product, fluorescein, is fluorescent and polar, and is not able to freely cross the intact plasmalemma. Fluorescein accumulates in 'viable' cells.

Transversely cut hand sections of apple tissue were bathed in a 0.1% FDA solution (by dilution from a stock solution of 5 mg/ml of acetone) for 10 minutes at room temperature in darkness. The sections were thoroughly rinsed and examined for fluorescence with UV light.

**Magnetic Resonance Imaging**

Magnetic Resonance Imaging (MRI) is a relatively new and powerful technique for examining the chemical composition of scald development in situ. MRI involves absorption of electromagnetic radiation and a type of nuclear transition creating a characteristic spectra.

Granny Smith apples were stored in air at 5°C for 9 months. Upon removal and simulated shelf life (7 days at 20°C), apples were separated into three classes of scald severity: nil, slight, and severe.

Imaging was conducted at the Centre for Magnetic Resonance at the University of Queensland.

Proton microscopy was performed using a Bruker AMX300 spectrometer interfaced to a 7 Tesla 89mm bore vertical magnet system. The radiofrequency probe was a custom built device with two configurations. The first being a resonator permitting an access diameter of 22 mm, and the second a solenoidal coil with 3 mm access.

Shielded gradients capable of producing 80 G/cm were used for the imaging experiments. These gradients when used in conjunction with mild Z0 compensation produced no temporal or spatial distortion of the required field 0.4 ms after a gradient pulse.

A three-dimensional Fourier imaging sequence was used where TE = 12msec.

Typically 4-8 averages were used with the total sequence time taking about 40 minutes. The in-plane resolution was 90um and the slice thickness was 500um. In the solenoidal experiments the in-plane resolution was 35um and the slice thickness was 300um.

**Apple Volatile Analysis**

Volatile analysis was performed using a headspace - gas chromatograph (HS-GC) technique. The HS-GC system consisted of a Hewlett-Packard 5890 gas chromatograph, equipped with a Hewlett-Packard 1939A headspace sampler.

Samples were separated on a fused silica SE-30 capillary column and detected with a flame ionisation detector. Data was acquired with HP 3385 series II ChemStation software. The components were identified by their mass spectra and comparison of retention times with standards.

**Results and Discussion**

**Non scalded control peel tissue**

A thick wax cuticle covers the epidermis which consists of a single layer of elongated thick walled cells. Underlying this is the hypodermis. The hypodermis is a layer of distinct closely packed cells with small intercellular spaces. It usually consists of 3 - 5 layers of cells. The cells have thick cell walls with thickened corners. Below this begins the larger cells of the outer cortex. These cells merge into the larger cortex cells which comprise the bulk of the apple flesh. These cells have thin walls with large intercellular spaces.

The FDA viability test of the non scalded peel tissue showed that the plasmalemma maintained its integrity, with all intact cells in the tissue sections fluorescing under UV excitation.

**Scalded tissue**

Successive observations of scalded peel tissue indicated that the scald symptoms were of a general browning of the affected cells and were chiefly confined to the hypodermis.

The first symptoms of scald appeared as a slight browning of the outer hypodermal cells. As scald sequentially developed in the apple peel, the browning of the hypodermal cells became more intense and developed throughout the hypodermis. Eventually all the hypodermis and epidermis were scalded. In the later stages of scald, the epidermal cells collapsed to give the peel its characteristic sunken appearance. In very severe cases, scald symptoms appeared in a few cells of the outer cortex underlying the hypodermis.

The FDA viability test in the scalding cells indicated that it took some time for the plasmalemma membrane to lose its integrity, until in scalded cells the plasmalemma was not functional and did not fluoresce.

**MRI Results**

Scald affected apples were significantly different to control (non scalded) apples. A NMR effect was visible approximately 200 - 600 um below the skin surface. This effect was noticed on 10 scalded samples compared with 8 non scalded control apples. It is important to note that these apples exhibited a short (<50ms) T2 values at 7 Tesla. Therefore the microscopy sequences with echo times approaching this value resulted in low signal-to-noise ratio images thus had low spacial resolution.

The results showed that there were significant differences in the water signal produced from scalded and non scalded peel of the same age. This was later found to be due to differences in the relative water content of the peel, with scalded tissue having a lower relative water content than non scalded tissue.

These results are consistent with those of Bain (1956) and Bain and Mercer (1963), in the browning symptoms of superficial scal are due to the progressive browning of the
hypodermal cells. Further examination of scald using transmission electron microscopy has been completed using both chemical and cryostatation. However these results are yet to be completed. This work will complement that of Bain and Mercer (1963). In addition, cell viability and membrane integrity tests using scalded affected tissue are being explored further.

**Headspace Results**

Alpha-farnesene was shown to be readily identified and quantified using HS-GC. The HS-GC method overcomes many of the problems encountered using the current α-farnesene isolation procedure (Murray 1969). Apart from the time consumption and tedious nature of Murray's α-farnesene isolation method there are inherent problems associated with the method. These problems include a lack of reproducibility, the presence of artefacts in the wash and the perennial safety problem in continually handling hexane. The HS-GC technique of α-farnesene isolation overcame these problems and was far more efficient and reliable, as it is a direct measure of the apple headspace volatile profile.

The HS-GC results showed that there were significant differences in volatile profiles between scalded and non-scalded tissue from the same apple.

**Conclusion**

Initial histological examinations with UV and light microscopy of apples naturally affected with superficial scald confirmed much of the previous work, ie superficial scald is confined to the hypodermis and epidermis of scald affected apples and rarely affects the underlying cortical tissue. The affected cells eventually become brown and collapse to give the characteristic scald appearance. The scalded collapsed cells had significantly less water than non-scalded tissue and this was demonstrated with MRI.

Further detailed examinations are required to more fully understand the cytology of the disorder, however this work has provided some valuable histological insights into the disorder.

The successful development of the HS-GC for the isolation and quantification of α-farnesene will ultimately improve the understanding of scald biochemistry and physiology.

This research will help in the development of alternatives to DPA for the control of superficial scald.

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**References**


Proceedings of the
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POSTHARVEST
CONFERENCE

held at
The University of Queensland
Gatton College
Australia

20 September - 24 September 1993
Appendix II

Literature summary of the phenolics in the pulp and peel in mature apple fruit
(Malus domestica)
<table>
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- Burda et al. (1990)
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**(-)-epicatechin**

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<td>210, 40</td>
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**(+)-gallocatechin**

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**(-)-epigallocatechin**

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**Dihydrochalcones**

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<td>phloretic acid</td>
<td>Mazza and Velioglu (1997)</td>
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<td>proanthocyanidin</td>
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<td>Procyanidins (= condensed tannins)</td>
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<td>(-)-epicatechin-4B-6-epicatechin</td>
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<td>procyanidin B2</td>
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peel, flesh; cv Golden Delicious
peel, flesh; cv Empire
peel, flesh; cv Rhode Island Greening
peel, flesh; cv Rhode Island Greening
cider; Tremlett's Bitter cider, Dabinett cider, Vilberie cider
pomace, cv Gala
peel, flesh; cv Golden Delicious
peel, flesh; cv Empire
peel; cv Rhode Island Greening
peel; cv Empire, Gravenstein, McIntosh, Cortland, Spartan, Golden Delicious, Red Delicious, Northern Spy
skin, flesh, cv. Golden Delicious
bulk; cv Empire, Cortland, McIntosh, Golden Delicious, Rome, Rhode Island Greening, Classic Delicious
peel; cv Red Delicious, Spartan, Cortland, Jerseymac, McIntosh, Golden Delicious, Gravenstein, Northern Spy
pomace, cv Gala
bulk; cv Granny Smith
peel, flesh; cv Golden Delicious
peel, flesh; cv Empire

Burda et al. (1990)
Burda et al. (1990)
Burda et al. (1990)
Oleszek et al. (1988)
Mayr et al. (1995)
Lea and Timberlake (1974)
Lu and Foo (1997)
Burda et al. (1990)
Burda et al. (1990)
Dick (1986)
Mayr et al. (1995)
Costeteng and Lee (1987)
McRae et al. (1990)
Lu and Foo (1997)
Lu and Foo (1997)
Foo and Porter (1981)

(1992)
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<td>600,150 qualitative quantitative peel, flesh; cv Rhode Island Greening peel, flesh; cv Golden Delicious</td>
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<td>(-)-epicatechin-(-)-catechin</td>
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<td>procyanidin B3</td>
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<td>Anthocyanins</td>
<td>cyanidin 3-galactoside bulk sample; cv Scugog*</td>
<td>39 qualitative peel; cv Red Delicious (Star Krimson, Star King), Stoke Red, Jonathon, Tremletts Bitter, Cox's Orange Pippin, Ingrid Marie, French Red Delicious</td>
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Burda et al. (1990)  
Oleszek et al. (1989)  
Mayr et al. (1995)  
Mayr et al. (1995)  
Lea and Timberlake (1974)  
Mayr et al. (1995)  
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Mayr et al. (1995)  
Spanos et al. (1990)  
Mayr et al. (1995)  
Oleszek et al. (1989)  
Deilage et al. (1991)  
Spanos et al. (1990)  
Spanos et al. (1990)  
Lea (1978)  
Mazza and Velioglu (1992)  
Timberlake and Bridle (1971)
cyanidin 3-glucoside 27
qualitative

bulk sample; cv. Scugog

peel; cv. Red Delicious (Star Krimson, Star King), Stoke Red, Jonathon, Tremletts Bitter, Cox's Orange Pippin, Ingrid Marie, French Red Delicious

Lin et al. (1989)

Mazza and Veligoğlu (1992)
Timberlake and Bridle (1971)

cyanidin 3-arabinoside 23
qualitative

bulk sample; cv. Scugog

peel; cv. Red Delicious (Star Krimson, Star King), Stoke Red, Jonathon, Tremletts Bitter, Cox's Orange Pippin, Ingrid Marie, French Red Delicious

peel; cv. Starkrimson

Lin et al. (1989)

Mazza and Veligoğlu (1992)
Timberlake and Bridle (1971)

cyanidin 3-xyloside 10
qualitative

bulk sample; cv. Scugog

peel; cv. Red Delicious (Star Krimson, Star King), Stoke Red, Jonathon, Tremletts Bitter, Cox's Orange Pippin, Ingrid Marie, French Red Delicious

Sun and Francis (1967)

Mazza and Veligoğlu (1992)
Timberlake and Bridle (1971)

cyanidin 7-galactoside** qualitative

peel; 74 cultivars

acylated derivatives of:
cyanidin 3-galactoside qualitative

cyanidin 3-glucoside qualitative

cyanidin 3-arabinoside qualitative

peel; cv. Red Delicious (Star Krimson, Star King), Stoke Red, Jonathon, Tremletts Bitter, Cox's Orange Pippin, Ingrid Marie, French Red Delicious

Timberlake and Bridle (1971)
cyanidin 3-xyloside qualitative

French Red Delicious

peel; cv. Red Delicious (Star Krimson, Star King), Stoke Red, Jonathon, Tremletts Bitter, Cox's Orange Pippin, Ingrid Marie, French Red Delicious

Scugog* = red fleshed fruit
** debated cv Timberlake and Bridle (1971)
bulk = flesh + peel combined
*** = only one report with no supporting information
x = dry weight basis

Timberlake and Bridle (1971)
Appendix III

Supporting phenolics data

linearity of phenolic standards response
semi-preparative chromatograms
purity of benzoyl-β-D-glucose
aglycone identification
Linearity of response factors of chlorogenic acid, epicatechin, phloridzin and rutin standards over a range of concentrations (6.25 - 100 μg/ml) at (a) 254 nm and (b) 280 nm. n = 3
Examples of semi-preparative HPLC chromatograms of (a) ‘Crofton’, (b) ‘Granny Smith’ control, (c) ‘Granny Smith’ DPA and (d) ‘Granny Smith’ scalded peel extracts monitored at 280nm. Numbered fractions collected for further NMR and LC/MS analysis are shown. A 540 µl sample was used for each run.
Data: 061-1701.D
Signal: DAD1 A
Peak: 2 at 9.613 min
Date: 4/6/95 7:20:16 AM

The purity factor is within the threshold limit. <-

Purity factor: 996.332 (100% of spectra)
Threshold: 990 (Set by user)
Reference: Peak Apex (integrated) (9.614)
Spectra: 3 (Selection automatic, 9)

Peak spectra
1 DAD1, 9.511 (101 mAU, Up2) Ref= 9.614
2 DAD1, 9.713 (120 mAU, Dn1) Ref= 9.614
3 DAD1, 9.951 (272 mAU, Int) Ref= 9.614

Spectral differences
1 9.511 - 9.951 (2.8 %)
2 9.713 - 9.951 (4.4 %)

Peak signals
1 DAD1 A, Sig=254.4 Ref=450,80 of C:\HPCHEM\1\TMP\HPCHEM\1\DATA\JG0504A\061-1701.D (22.2 mAU)
2 DAD1 B, Sig=280.4 Ref=450,80 of C:\HPCHEM\1\TMP\HPCHEM\1\DATA\JG0504A\061-1701.D (14.5 mAU)

Signal ratios
1 DAD1 A, Sig=254.4 Ref=450,80 / DAD1 B, Sig=280.4 Ref=450,80

Peak purity of unknown scald peak (RT 9.7 min)
HPLC chromatograms from a typical apple extract (A) and rutin (B). C and D are the chromatograms after acid hydrolysis of the apple extract (C) and rutin (D), leaving the flavonol aglycone, quercetin in both cases. E and F are the UV spectra of the products of the acid hydrolysis showing typical quercetin UV spectra.