DEVELOPMENT OF DIAGNOSTIC TOOLS TO PREDICT INCIDENCE OF BITTER PIT DURING APPLE STORAGE

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ABSTRACT

Bitter pit is an important physiological disorder of many apple cultivars where the low uptake and poor distribution of calcium within the cortex of apples pervades. Controlled atmosphere storage and application of 1-MCP (SmartFresh^{SM}) can delay the onset of bitter pit symptoms by delaying maturity and senescence; however, significant losses may occur in long-term stored apples. It is hard to detect internal bitter pit using external examination alone.

Previous studies have focused on improving pre-harvest prediction and curative treatments before harvest. Present prediction models are based on history of orchards, mineral analysis 2-3 weeks before harvest and quality assessments and monitoring over storage time.

This study aimed to identify a greater understanding of the storage potential of fruit based on destructive standard quality assessments, biochemical and molecular analysis, also a non-destructive monitoring method by chlorophyll fluorescence at the point of harvest and monitoring during storage for developing more reliable prediction models to improve storage management. The role of free and conjugated calcium in maintaining cellular integrity and the relationship between biochemical and fluorescence changes and development of bitter pit were investigated.

A diagnostic model based on comparison of changes of ascorbic acid during storage was developed. Another diagnostic model based on changes in the proportion of calcium oxalate content during storage in comparison with harvest was developed to identify samples with higher propensity to bitter pit. Also chlorophyll fluorescence was investigated as a non-destructive method for monitoring fruit during storage and prediction models for detecting changes in the maturity of fruit and developing bitter pit and reduction of fluorescence during storage as an alert to identify incidence of bitter pit were developed. Furthermore, changes in gene expression profiles of a limited number of genes like calmodulin showed the differences in patterns of transcripts between apples suffering from bitter pit and healthy apples.

All the suggested methods have potential of being commercialised and applied practically to improve apple fruit store management. It would be possible to build a multi variate model for predicting the onset of bitter pit development in apple by combination of two or more suggested diagnostic tools.

CONTENTS

ABBREVIATIONS

µL	microlitre
µL L^{-1}	microlitre per litre
µmol	micromoles
µE m^{-2} s^{-1}	microeinsteins per square meter per second
1-MCP	1-Methylcyclopropene
2-MCE	2-mercaptoethanol
AA	Ascorbic Acid
AAS	Atomic Absorption Spectroscopy
ABA	Abscisic Acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
AGPase	ADP-glucose pyrophosphorylase
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AOA	Aminooxyacetic Acid
Area	The area above the fluorescence rise between Fo and Fm
ATP	Adenosine Triphosphate
AVG	Aminoethoxyvinylglycine
B	Boron
bp	base pair
BP	Bitter pit
C_2H_4	Ethylene
CA	Controlled atmosphere
Ca	Calcium
$Ca(No_3)_2$	Calcium nitrate
$CaCl_2$	Calcium chloride
CaM	Calmodulin
cDNA	Complimentary DNA
CO_2	Carbon dioxide
CPRs	The County ploidy ratios

Ctols	Conjugated trienols
CT	Constant Threshold
DAA	Days After Anthesis
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DPA	Diphenylamine
EDTA	Ethylenediaminetetraacetic acid
EELS	Electron Energy Loss Spectroscopy
EMRA	East Malling Research Association
EMRS	East Malling Research Station
EtBr	Ethidium Bromide
EU	European Union
F	Energy lost from photosystem II as fluorescence
FAO	Food and Agriculture Organization
$FeSO_4$	Ferrous sulphate
F_1	Fluorescence Intensity at 50 μs
F_2	Fluorescence Intensity at 150 μs
F_3	Fluorescence Intensity at 300 μs
F_4	Fluorescence Intensity at 2 ms
F_5	Fluorescence Intensity at 30 ms
Fm	Maximum Fluorescence Yield
Fo	Minimum Fluorescence Yield
Fluorescence (arb.)	Fluorescence absorbance (arb. logarithm unit)
FTA	Fruit Texture Analyser
Fv	Variable Fluorescence Yield
Fv/Fm	Maximum Efficiency of Photosystem II
GC	Gas Chromatography
HCl	Hydrogen chloride
HPLC	High Performance Liquid Chromatography
ICP	Inductively Coupled Plasma
ITS	Internal Transcribed Spacer
K	Potassium
KDa	Kilo Dalton

$KMnO_4$	potassium permanganate
KPa	Kilo Pascal
L	Litre
LDA	Linear Discriminant Analysis
LTB	Low Temperature Breakdown
M	Molarity
m	Metre
MeOH	methanol
Mg	Magnesium
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mRNA	messenger RNA
N_2	Nitrogen
NaCl	Sodium chloride
NADP+	Nicotinamide Diphosphate (oxidised)
NADPH2	Nicotinamide Diphosphate (reduced)
$NaHCO_3$	Sodium hydrogen carbonate
NaOAC	Sodium acetate
NaOH	Sodium hydroxide
nL	nanolitre
NRI	Natural Resources Institute
O_2	Oxygen
P	Phosphorus
P	Probability
PAL	Phenyl-alanine Ammonia Lyase
PAGE	Polyacrylamide Gel Electrophoresis
PAs	Polyamines
PCA	Principal Component Analysis
PEA	Plant Efficiency Analyser
PG	Polygalacturonase
pH	measure of activity of hydrogen ion

PME	Pectin Methyl Esterase
ppb	parts per billion
ppm	parts per million
PQC	Produce Quality Centre (EMRA)
PSI	Photosystem I
PSII	Photosystem II
QA	Primary Quinone of Photosystem II
q-PCR	Quantitative (Real Time)- Polymerase Chain Reaction
RC/CS	Reaction Centre Density [Fo×Fv/Fm(F_4-Fo)/(F3-Fo)]
RNA	Ribonucleic acid
RPM	Revolutions Per Minute
rRNA	ribosomal RNA
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
S.D.	Standard Deviation
S.E.	Standard Error
SAM	*s*- adenosyl-methionine (S-AdoMet)
SDS	Sodium Dodecyl Sulphate
SF	Smart Fresh^{SM} (1-MCP)
TBE	Tris Borate EDTA
TSS	Total Soluble Solids
UK	United Kingdom
UV	Ultra Violet
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

CHAPTER 1

INTRODUCTION

Bitter pit is an important physiological disorder of apple that can develop on the tree but is most prevalent during storage. Delaying fruit maturation after harvest through controlled atmosphere storage and application of 1-MCP (SmartFresh^{SM}) can delay the onset of symptoms; however, significant losses may occur in long-term stored apples. It is hard to detect internal bitter pit using external examination alone. Tools that can better predict the incidence of bitter pit developing during storage will help growers designate consignments of fruit for short, medium or long-term storage. Most of the previous studies were focused on improving pre-harvest prediction and treatments before harvest, through late summer pruning of trees to prevent competition between actively growing shoots and fruit for calcium and supplementary orchard sprays of calcium salts. Current predictive methods are based on history of orchards, destructive internal quality assessments and mineral analysis 2-3 weeks before harvest and quality assessments and monitoring over storage time. Developing more reliable prediction models to improve storage management by allowing accurate schedules for the length of storage and thereby reducing the risk of quality losses during storage could help growers to reduce losses. A greater understanding of the storage potential of fruit based on biochemical analysis or molecular diagnostic at the point of harvest would provide growers with a more accurate management tool to classify orchard consignments based on their tendency to maintain fruit quality in storage, allowing for better scheduling of the crop during the storage season.

Landseer Ltd, service provider for 1-MCP (SmartFresh^{SM}) in the UK & Eire, has collaborated in this project with NRI. High risk Bramley orchards were identified from over 90 orchards surveyed across the south east of UK in 2010/11. A subsample of susceptible orchards with fruit that consistently developed bitter pit in storage as well as orchards where fruit remained free from problems were selected. Trials were conducted over two consecutive seasons (2012/13, 2013/14) to evaluate factors affecting incidence of bitter pit in Bramley apples. Apart from standard quality assessments, three types of diagnostic tools were applied in this study for developing prediction models for incidence of bitter pit, including biochemical analysis and molecular diagnostics as destructive methods and chlorophyll fluorescence as non-destructive tool. The schematic research plan (Figure 1.1) shows the research process in this study.

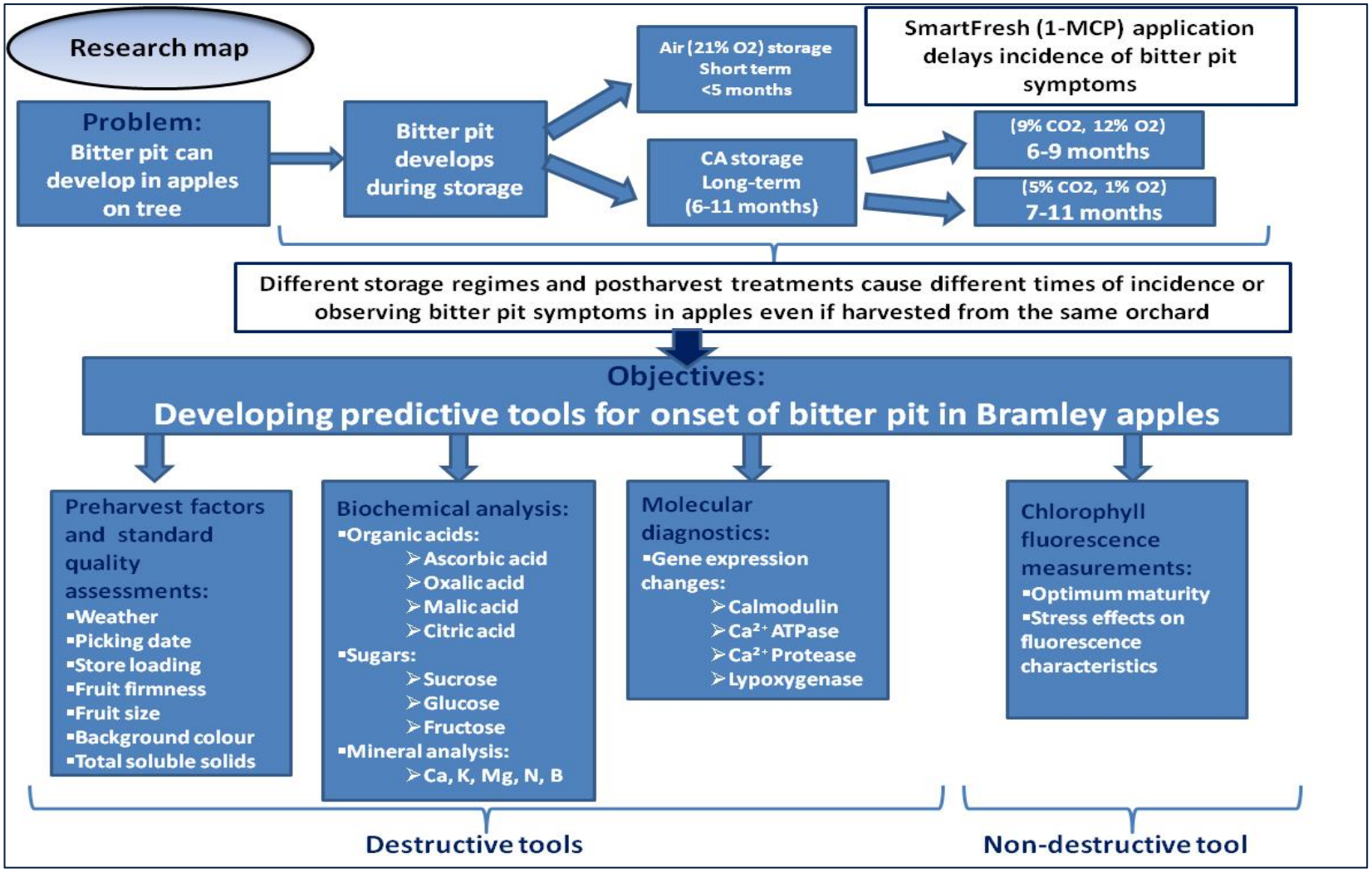


Figure 1.1: Schematic research plan

1.1 Apple cultivars and history:

The cultivated apple belongs to the family Rosaceae: a family of great economic importance which contains 107 genera and 3100 species of trees, shrubs and herbs (Huxley and Griffiths 1992). Many species of the family provide commercially important fruits such as apple (*Malus*), almond, apricot, cherry, peach and plum (*Prunus*), pear (*Pyrus),* blackberry, raspberry and strawberry (*Fragaria*). The genus *Malus* contains 35 species of trees and shrubs that can be found throughout the temperate zone (Mulabagal *et al.*, 2007). The apple tree was perhaps the earliest tree to be cultivated and one of the most widely cultivated fruit trees and its fruits have been improved through selection over thousands of years. It is generally believed that the edible apple originated somewhere in Central Asia (Juniper *et al*., 1999). Bramlage (2001) reported investigations and analysis of DNA composition of wild fruit trees in Kazakhstan that showed they all belonged to the species *Malus sieversii*, but with some genetic sequences common to *Malus domestica* (Petruzzelli *et al.,* 2003).

Morgan and Richard (1993) suggested the European crab-apple, *M. sieversii* and *M. pumila*, as the main maternal wild ancestor of domestic apple. Also Gharghani *et al.* (2009) found that wild species in east and west of Iran respectively belonged to *M. sieversii* (originally from Kazakhstan) and *M. orientalis* (originally from Turkey), as one of the probable minor ancestors of domestic apples.

According to FAO statistics (2014), apple production (2012/13) was 76,378,738 metric tonnes. China with 37.1 million tonnes was the largest apple producer in the world. Turkey and Poland as largest apple producers in Europe produced respectively 2.89 and 2.88 million tonnes. Apple production of UK, as one of the small apple producers in 2012/13, was 202,900 tonnes.

More than 6000 apple cultivars have existed in Britain at some time over the past few hundred years. The first apple variety that was cultivated in Britain was the Old English Pearmain in 1204 (Crawford, 2001). The two most popular and commercial cultivars grown in the UK are Bramley's Seedling and Cox's Orange Pippin. According to Defra horticultural statistics (2014) for the season 2013/14 the area cultivated Bramley was 3450 ha, with production about 83.1 thousand tonnes with a value of £64,000,000, and Cox cultivated area was 1590 ha, with production around 35.1 thousand tonnes with a value of £21,000,000.

1.1.1 Bramley's Seedling:

Bramley's Seedling has very large fruits with greenish-yellow and broad red strips. Bramley's flesh is white, tinged green, sharp, juicy and firm with sharp and tart flavour and is used in the

production of processed apple products (pies, purees, juices). It has a unique specification that retains its tangy taste and the texture during cooking; also it is good for juice and cider (Williams *et al.,* 1977). The Bramley is almost exclusively a British variety and is more widely cultivated in the UK than any other apple; however it is also grown by a few United States farms, and can be found in Canada (Way *et al.,* 1991). In 1809, the first seeds of Bramley were planted by a young girl, Mary Ann Brailsford, from a seed of unknown origin in her garden in Southwell, Nottinghamshire. Her property was subsequently bought by a local butcher, Matthew Bramley, from whom this variety was named (Sanders, 1988). To preserve the true genetic identity of Bramley, meristematic tissue was used to propagate new material and DNA was isolated. Cloning was performed by scientists at the University of Nottingham, because the original tree was suffering from old age and was under attack by fungal diseases and provided a fine example of living history and a genetic bank for the future (Crawford, 2001).

Bramley's Seedling, is triploid that causes sterile forms in plant species (Lacey, 1982). The offspring of triploids, whether derived from selfing or crossing with diploids is weak owing to their aneuploid constitution (Manganaris and Alston, 1997). Bramley's Seedling has sterile pollen. It needs a pollinator, so it is normally grown with two other varieties of apple for pollination (Free, 1966).

1.1.2. Cox's Orange Pippin:

Cox has different cultivars like Queen Cox, Orange and red Pippin, early export, and Red Sport. Cox's Orange Pippin has medium size, greenish-yellow, flushed orange-red fruits. Flesh is deep cream, sweet soft, aromatic (Smith and Stow, 1984). It is a dessert cultivar and good for juice and cider; flowers are susceptible to late spring frosts and disease. Cox was raised from pips of a Ribston Pippin by Richard Cox of Slough in 1825 (Sanders, 1988). Cox has emerged as the most important home-grown dessert apple in the UK's natural season of supply from September to April. Cox is a variety that can be stored for 5-6 months of the year, but not as long as Bramley apples (Neuteboom and Withnall, 1998). It should be noticed that this research is focused on bitter pit in Bramley; however Cox's Orange Pippin as the main English dessert apple is also susceptible to incidence of bitter pit.

1.2 Anatomy of apple fruit:

Apple fruit is a mature, ripened inferior ovary in which the pericarp plus the receptacle tissue become fleshy. Apple is not a true fruit and is not formed from the ovary (Esau, 1977). The fleshy part (hypanthium) completely encloses the true fruit (pericarp) at the middle. Five ovaries

of the apple flower are fused at the base, which along with the receptacle becomes the fruit (Lakso *et al*., 1995). The stem is also called the pedicel or stalk and the opposite end is where the stamen is located, also called the calyx (Pratt, 1988). Figure 1.2 shows cross and longitudinal sections of apple fruit.

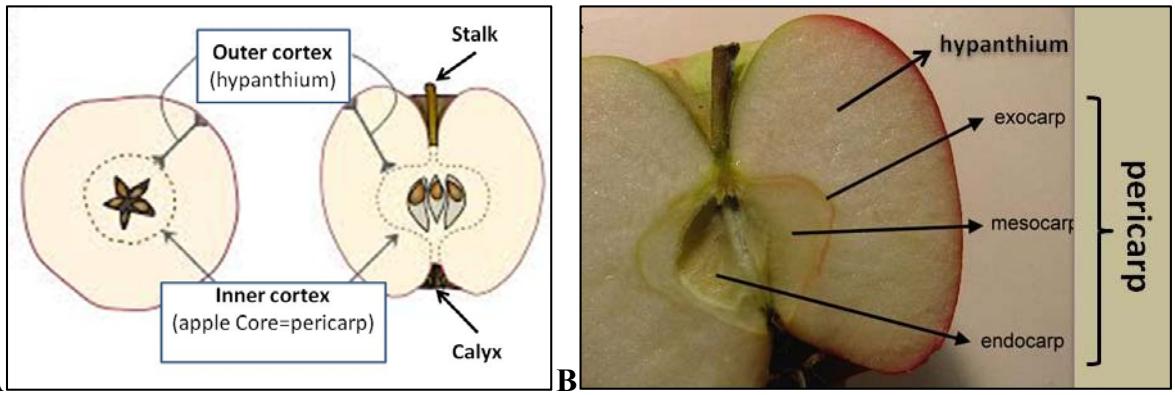


Figure 1.2: A) Cross and longitudinal section of apple fruit and definition between inner/outer cortex and stalk/calyx end of fruit. B) Different parts of apple core (pericarp) and apple flesh (hypanthium). Pictures based on Robinson and Lakso (1995).

Copeland and McDonald (2001) indicated that the core and pericarp of apple consist of two types of tissues: the parenchyma tissue and the cartilaginous tissue made of sclereids. In a study on longitudinal stresses, Horbens *et al.* (2014) found that sclereids have the main role in regulating elements between maintenance of resistance and viscous damping. In mature fleshy parenchyma cells, there are very thin walls with large intercellular spaces which vary greatly in size and are up to 2000 μm in length and 100-200 μm in diameter (Reeve, 1953). The peel section includes: cuticle, epidermis, hypodermis and fleshy parenchyma. The skin contains chloroplasts and anthocyanin (Blanke and Lenz, 1989).

Apple fruit develops over a period of 150 days from anthesis to fully ripe. Janssen *et al*. (2008) monitored changes in chemical compositions, in physical organization, and in cell size during apple fruit development (Figure 1.3). Esau (1977) indicated that rapid wall synthesis occurs during cell extension just before harvest. The earlier stages of fruit development may have a considerable influence on potential storage life. In the parenchyma cells of the edible portion of the mature apple fruit the cell walls are thin and usually consist of middle lamella and primary wall only. During the active growing period the middle lamella seems to be composed mainly of pectic substances and the primary wall of hemicelluloses. Lignin is present in only small amounts and comes mostly from the vascular system (Janssen *et al*. 2008).

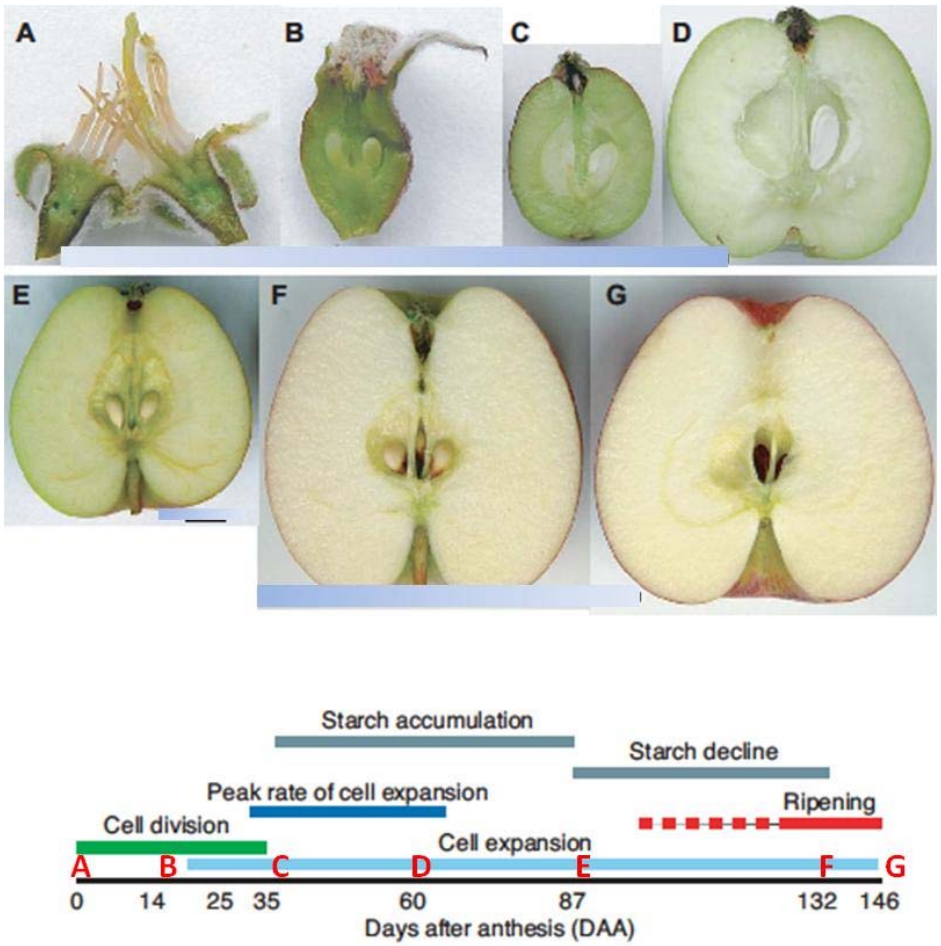


Figure 1.3: Apple fruit development showing the timing of major physiological events. Different stages of development of apple fruit. A: 0 DAA (Days After Anthesis), B: 14 DAA, C: 35 DAA, D: 60 DAA, E: 87 DAA, F: 132 DAA, G: 146 DAA (Source: Janssen *et al*. 2008).

1.3 Physiological disorders of apple:

Ferguson *et al.* (1999) divided apple disorders in two main groups as: 1) disorders pre-determined on the tree, 2) disorders induced by storage conditions. There are many different types of physiological disorders in apple. This study is focused on "bitter pit".

1.3.1 Bitter pit:

Bitter pit is the physiological breakdown of cells under the skin. It is a physiological disorder associated with low levels of calcium in the fruit tissue. It is the most important physiological disorder in apples (Ferguson and Watkins, 1989). Jackson (2005) explained symptoms of bitter pit as deep brown or black lesions or spots varying from 2-10 mm in diameter which disfigure the fruit flesh. The location of pits is usually just below the skin, but in severe cases the pits

may extend throughout the cortex (Figure 1.4), generally concentrated at the calyx end of the fruit as small, water-soaked spots on the skin (Perring, 1985).

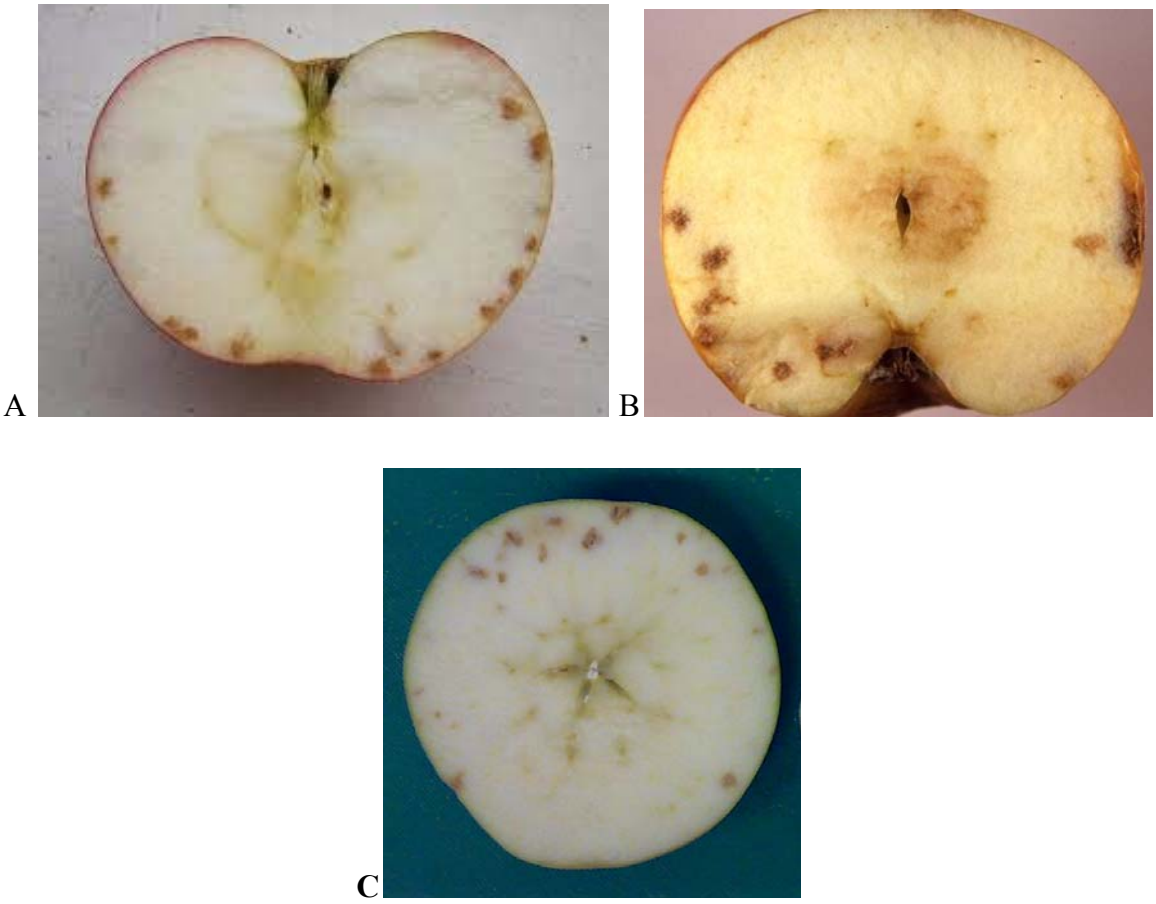


Figure 1.4: Bitter pit symptoms are deep brown or black lesions or spots varying from 2-10 mm in diameter in the fruit flesh. A & B: Pits generally located at the calyx end of the fruit. C: just below the fruit skin.

Bitter pit may not be found at harvest but develops in stored fruit. Bitter pit is much influenced by orchard management and environmental factors (Hewett, 2006). De Freitas *et al*. (2013) observed more bitter pit symptoms during storage in apples located in the shaded part of the tree. Fidler *et al.* (1973) observed that any treatment which delays senescence delays the development of bitter pit. High temperatures, delayed storage, delayed cooling, storage in air instead of CA, water loss all accelerate the development of bitter pit.

Spraying with calcium nitrate ($Ca(NO_3)_2$) and calcium chloride ($CaCl_2$) are advisable (Ferguson and Watkins, 1992). However, calcium nitrate delays ripening and could be more effective to prevent bitter pit, and calcium chloride can be used on early-maturing varieties or for maximising the red colour of fruit in specific cultivars. Prinja (1989) recommended minimum calcium concentration to avoid bitter pit in stored Bramley apple of 5 mg $100g^{-1}$ (fresh weight).

However, this is often difficult to achieve because markets demand Bramley apples of 90 mm and above, the fruit calcium concentration is inversely correlated with mean fruit size (de Freitas *et al*., 2012).

Although pitted fruit contains lower concentrations of calcium than fruit without pit, Ferguson and Watkins (1992) observed the dead cells associated with bitter pit had higher concentration of calcium because calcium leaches into the dead cell spaces. Also they observed these cells still contained starch a long time after starch has disappeared from the healthy cortical cells. Fidler *et al.* (1973) found that pitted areas are low in sucrose but high in glucose and fructose. Citric acid replaces malic acid. Also pitted areas have been found to be relatively high in total nitrogen and protein nitrogen. Tomala and Soska (2004) found that the concentration of calcium in the peel is more closely related to the incidence of bitter pit than the concentration of calcium in either the core or flesh.

Dražeta *et al.* (2004) indicated a dysfunction of the xylem in fruit as the fruit develops, resulting in a reduction of number of the xylem cells consequence on the mineral balance of the fruit leading to incidence of bitter pit. They concluded that any application that delays onset of xylem dysfunction by controlling growth dynamics of the fruit such as a reduction of nitrogen may control occurrence of disorders like bitter pit. Also bitter pit normally is observed near the calyx-end of apple fruit which is also related to the decline of xylem functionality and lower calcium content (Tomala and Soska, 2004). The transport of calcium up through the xylem stops as the fruit approaches maturity and begin to form the abscission layer in preparation for falling off the tree (Ferguson and Watkins, 1989). The cultivars that are more susceptible to bitter pit lose their connection with the xylem earlier than insensitive varieties (Dražeta *et al.* 2004).

Large changes in the concentrations of minerals in the apple cortex occur during storage, often in short intervals of time and the balance of the constituents in every part of fruit changes continuously throughout its storage life (Perring, 1985). Prinja (1989) recommended a 2-3 week delay in harvest for fruits that are critically low in calcium. High concentrations of organic acids together with high potassium and magnesium usually increase with apples susceptible to bitter pit. Fidler *et al.* (1973) suggested that organic anions might also remove calcium from binding sites in cell walls and membranes by chelation (such as citrates) or inactivation (such as oxalates) and thus destroy cellular integrity. Perring (1985) observed that after an initial delay, malic and titratable acid concentrations decline steadily during storage. Citric acid

concentration also fell slightly during bitter pit development (Oke *et al*., 2013). Reduction in acidity and consequent increases in pH during storage are greatest in the peel (since the peel has 5-7 times more cells per unit area than the cortex) and in the outer cortex, which is most likely to be affected by bitter pit and are minimal in the core zones of stored apples (Sharma *et al.,* 2014).

1.4 General physiology of fruit ripening:

Ethylene as a plant hormone has a key role in ripening process; also it is a promoter of aging and senescence (Dilley, 1981; Van Altvorst *et al*., 1995; Schaller, 2012). Ripening of fruit is classified as "climacteric" or "non-climacteric" depending on their respiration and ethylene production rate (Oetiker & Yang, 1995). In climacteric fruits a distinct increase in respiratory rate occurs during ripening (climacteric rise) this is generally associated with elevated ethylene production just before the increase in respiration (Brady, 1987). Dilley (1981) investigated changes in ethylene production during ripening of the climacteric fruits and observed a significant reduction in ethylene production after the climacteric rise (Figure 1.5).

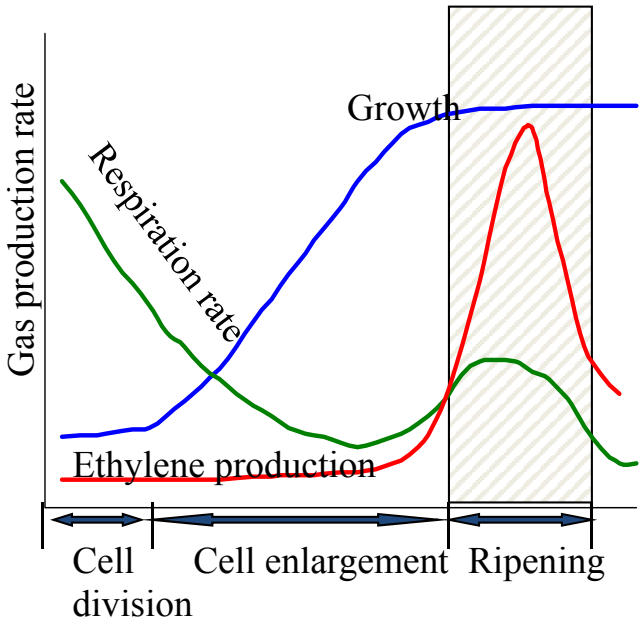


Figure 1.5: Schematic comparison of changes in respiration and ethylene production during different stages of growth and ripening in climacteric fruit (Based on Dilley, 1981).

Rees and Hammond (2002) indicated that in non-climacteric fruits there is no increase in respiration and ethylene production during ripening; even there is a gradual decline in respiration. Also Paliyath *et al.* (2008) observed that climacteric fruits responded to external

ethylene by accelerating ripening; on the other hand non-climacteric fruits showed slight increase in respiration without showing acceleration in the time required for ripening.

McMurchie *et al*. (1972) made a model that described two mechanisms for ethylene (C_2H_4) biosynthesis induction: System (I) and System (II). Immature fruit has a non-autocatalytic system (I), C_2H_4 biosynthetic capability and when the competency to ripen occurs, an autocatalytic system (II), C_2H_4 biosynthetic capability is induced. System (I) is responsible for production of basal levels of ethylene present in vegetative tissues, ethylene stimulates the process and by increasing or decreasing ethylene concentration the process of ripening goes faster or slower, this happens in both climacteric and non-climacterics. System (II) provides for the high rate of ethylene production associated with ripening of fruits and flowers. Oetiker and Yang (1995) and Rees *et al*. (2012) indicated that system (II) ethylene production is autocatalytic and cannot be reversed as a switch that cannot be stopped, even if exogenous sources of ethylene are removed, the process continues in climacteric fruit.

Apples are considered climacteric fruit (Hulme *et al.,* 1963; Galliard, 1968) and have elevated ethylene and respiration production rates during ripening. The rapid rise in ethylene production involves autocatalytic production leading to an exponential rise in ethylene (Paliyath *et al.*, 2008). Klee *et al.* (1991) indicated that although ethylene is always present in the fruit tissues at a very low concentration, young fruitlets are not capable of responding to this endogenous or to exogenous ethylene by initiating ripening. Once a critical point of development has passed ethylene promotes developmental processes that lead to ripening, including further ethylene production (Seymour *et al.,* 2013). Zude *et al.* (2006) showed that apple respiration rate indicates the optimum harvest date at the respiratory minimum before developing the climacteric respiration peak.

1.4.1 Ethylene biosynthesis:

Yang and Hoffman (1984) explained ethylene synthesis in the following process (Figure 1.6): it starts from methionine to S- adenosyl-methionine (S-AdoMet) or (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC). Ripening in apple starts by increases in the activity of enzymes (ACS) and (ACO) for ethylene biosynthesis, then cell wall degradation and finally aroma volatiles accumulation. ACC oxidase is the enzyme required to convert ACC to ethylene (C_2H_4) and increasing respiration (releasing CO_2 and O_2) during this process (Abeles, 2012). Also Saltveit (1999) indicated that aminoethoxyvinylglycine (AVG) and aminooxyacetic

acid (AOA) inhibit the conversion of SAM to ACC, but do not interfere with the conversion of ACC to ethylene.

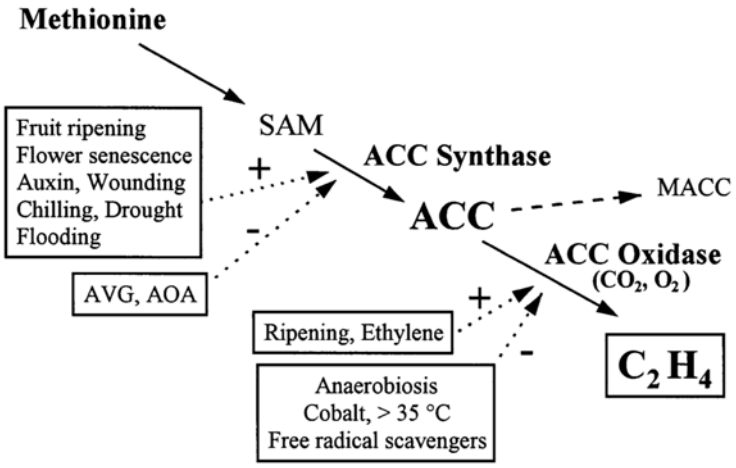


Figure 1.6: The key stages of ethylene biosynthesis. (Sourse: Saltveit, 1999)

Alexander and Grierson (2002) indicated the role of exogenous ethylene that leads to a large increase in ethylene production is related to the enzymes 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO).

Apples also contain ACC malonyl transferase activity and malonylation may regulate the low rate of ethylene synthesis during growth of the fruit, through a conjugation and reversible inactivation process. “Cox’s Orange Pippin” and “Bramley’s Seedling” do not show increase in ethylene synthesis in low temperatures unlike “Golden Delicious” (Jackson, 2005). Alexander and Grierson (2002) have shown that ethylene affects the transcription and translation of many genes related to ripening. Knee (1986) by applying three methods of gas and liquid chromatography and standard assay showed that ACC increased 30-40 fold when ethylene production increased. Rudell *et al.* (2000) found that ethylene production in different parts of apple fruit are different and in all stages of fruit growth it was produced more in the carpel tissue except before the rise in whole fruit internal ethylene concentration, when ethylene production in the skin and carpellary tissue was similar. This indicates that the initial ripening signals originate through the carpels to the rest of the fruit. It is also induced by external factors, such as wounding, viral infection, auxin treatment, chilling injury and drought (Yang and Hofman, 1984).

There is evidence that ethylene synthesis may be inhibited at low O_2 and high CO_2 concentrations and ethylene action may be blocked or modified (Lieberman, 1979; Yang and

Hofman, 1984; Abeles, 2012). Burg and Burg (1967) demonstrated that increasing the concentration level of CO_2 reduced C_2H_4 biosynthesis. Maintenance of ethylene in low concentrations delays fruit softening and other ripening changes, including the development of disorders such as superficial scald and bitter pit (Sharples and Johnson, 1987). Banks *et al*. (1984) demonstrated that reduction of respiration in the controlled atmosphere storage caused suppression of C_2H_4 biosynthesis as one of the primary mechanisms by which controlled atmospheres extend the storage life of apples. Chung *et al.* (2002) applied immunoelectron microscopy to show that ACC-oxidase, whose activity limits ACC, was localised in the cytosol in apple fruit and is a soluble conversion enzyme with a relative molecular mass of 50 kDa and concluded that the final step (conversion of ACC to ethylene, HCN, and CO_2) is catalyzed by ACC oxidase. Dong *et al.* (1992) discovered that removal of CO_2 from the reaction mixture completely abolished the enzyme activity, while 0.5% atmospheric CO_2 gave half-maximal activity. ACC-oxidase reaction occurs in the presence of Fe^{2+}, ascorbate and oxygen. The reaction was determined as:

$$ACC + O_2 + \text{ascorbate}, CO_2, Fe^{2+} \rightarrow C_2H_4 + CO_2 + HCN + \text{dehydroascorbate} + H_2O$$

Theologis (1992) suggested that the putative oxidase of the ethylene metalloprotein receptor might be the protein, a dioxygenase related to ACC oxidase, to inactive gene expression by antisense RNA.

1.4.2 Role of ethylene and other factors in fruit ripening:

Fruit ripening is seen as a process in which the biochemistry and physiology of the organ are altered to influence appearance, texture, flavour, and aroma to make fruit attractive for consumption (Giovanonni, 2004). Apple fruit softening has been associated with the increase in the expression of cell wall hydrolase genes. Fruit softening in apple is associated with an increase in the ripening hormone ethylene. The results of a study conducted by Ley-Yee *et al*. (1990) indicated changes at both the mRNA and protein level which coincide with increasing internal ethylene. Ben-Arie *et al.*, (1982) demonstrated that inhibition of ethylene biosynthesis in apple fruit is related to calcium and spermine (a polyamine involved in cellular metabolism) in different ways: a) as fruit ripened, inhibition by spermine decreased, on the other hand inhibition by calcium increased; b) inhibition by calcium was transitory, whereas that by spermine was persistent; c) at temperatures below 12°C calcium inhibited more than spermine whereas above 12°C it was reversed.

Temperature-dependence of the spermine and calcium effect on ethylene biosynthesis was correlated with specific changes induced by them in the membranes of apple tissue. Bouzayen *et al.* (2010) studied biochemical and physiological changes during fruit ripening and found that they are driven by the coordinated expression of fruit ripening-related genes. These genes encode enzymes that participate directly in biochemical and physiological changes. They also encoded regulatory proteins that participate in the signalling pathways and in the transcriptional machinery that regulate gene expression and set in motion the ripening developmental program.

1.4.3 Methods to control ethylene biosynthesis and perception:

Delaying fruit ripening by reducing ethylene biosynthesis is one of the main achievements of postharvest physiologists. A number of methods have been investigated over the years, with the use of temperature controlled storage being one of the most easily implemented strategies. Unexpectedly storage at too low temperature can accelerate ethylene since ACO protein is accumulated in low temperature to initiate autocatalytic ethylene production and fruit ripening upon re-warming (Lelievre *et al.* 1997). Moreover, extreme changes in the storage atmosphere leading to plant stress can also induce elevated ethylene production. Terai *et al.* (1998) found that the stress of a short term treatment with 80% CO_2 or 100% N_2 activated the ethylene biosynthetic pathway and retained for several days after the treatment. This makes it difficult to inhibit the ripening process when it has been started.

One method is by inhibiting the expression of genes encoding ethylene-biosynthetic enzymes by transformation with the respective antisense genes or lowering the cellular level of ACC by introduction of a bacterial gene encoding ACC deaminase (Kende, 1993).

ACC synthase is a cytoplasmic enzyme. The cellular localization of ACC oxidase is less clear. Lara and Vendrell (2000) observed that the low levels of ACC in immature apple fruit was undetectable through the pre-climacteric period, and concluded that pre-climacteric tissue lacks the capability for the conversion of ACC to ethylene. The action of ethylene in plants is indirectly due to a change in auxin content and distribution. Abscisic acid (ABA) can induce ripening-related ACC synthase in immature tissues. Stearns and Glick (2003) expressed that a mutation in the ethylene binding domain would create a plant that is insensitive to ethylene because of losing the ability to bind ethylene and failiure to ripen.

1.4.3.1 Commercial methods to control ethylene during storage:

1) Potassium permanganate: Ethylene is absorbed by potassium permanganate that is a strong oxidizing agent and produces manganese oxide and potassium hydroxide (Lidster *et al.* 1985):

$3CH_2CH_2 + 2KMnO_4 + H_2O \rightarrow 2MnO_2 + 3CH_3CHO + 2KOH$

Potassium permanganate needs to have a high surface area exposed to the atmosphere; in commercial application it is available with a common carrier being alumina beads (Wills and Warton, 2004). It can absorb ethylene in the atmosphere reducing the overall concentration of exogenous ethylene and lowering the accumulative exposure of fresh produce to ethylene. However, suppression of autocatalytic ethylene production can only be achieved if ethylene concentration in the atmosphere is maintained below 100 nL/L (Knee 1986).

2) Catalytic ethylene scrubbing: these instruments remove the ethylene by passing the atmosphere over metal crystals (Platinum, Nickle, and Palladium) sometimes running at high temperatures (220°C). This method is expensive and needs energy to heat the air and further energy is expended during cooling the hot air exhaust before returning to the store. In another method the store gases pass through a porous heat exchange bed. It is not common commercial practice to use an ethylene scrubber in apple CA store (Wojciechowski and Haber, 1982).

3) SmartFresh^{SM} (1-MCP): 1-methylcylclopropene (1-MCP) is an inhibitor of ethylene, known to reversible bind to ethylene receptors predominantly located on the endoplasmic reticulum within plant cells (Binder *et al.* 2004). This has been shown to delay the post-harvest ripening of climacteric fruit, by blocking access of ethylene to the receptor sites thus delaying the onset of ripening and senescence (Watkins and Nock, 2005). Like ethylene, 1-MCP is a gas and is applied in a sealed chamber or storage room and once released into the store atmosphere is able to penetrate the commodity (Sisler and Serek, 2003). After a short period of time (6 to 24 hours), the store atmosphere is flushed with clean air and the commodity returned to air or a controlled atmosphere (CA) allowed to develop. During storage, new ethylene receptors may be formed (Figure 1.7), and the cells regain sensitivity to ethylene. 1-MCP is a safe product that leaves no detectable residue (Blankenship, 2001). It is known to delay the onset of climacteric ethylene production and respiration and also significantly retarded the activity of pectin degrading enzymes pectin methyl esterase (PME) and polygalacturonase (PG) responsible for loss of cell to cell cohesion and tissue softening during ripening (Luo, 2007). Jouyban (2012) emphasised

on the role of ethylene in mediating the responses to stress and role of 1-MCP in reduction of damages caused by abiotic or biotic stress on fruit during storage.

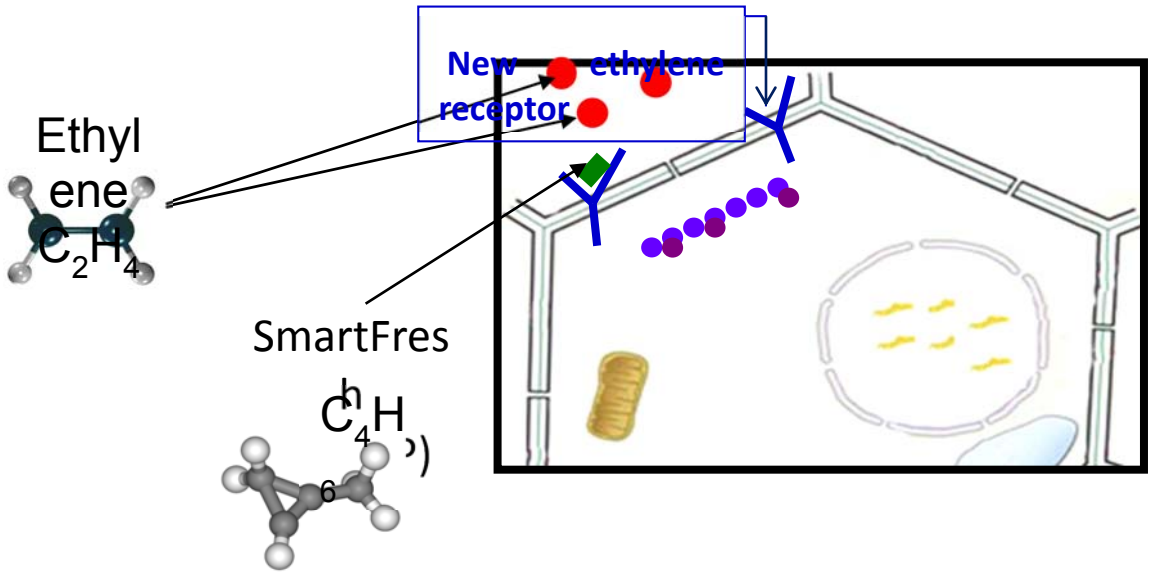


Figure 1.7: Schematic mechanism of binding ethylene receptors by 1-MCP that delays the ripening process, when new ethylene receptors in the fruit naturally developed, the ripening process continues normally (Based on: Blankenship, 2001).

A number of factors influence fruit responses to 1-MCP including concentration during treatment, duration of exposure, fruit maturity at the time of treatment, the interval between harvest and when the treatment is applied and temperature of treatment (Watkins *et al.* 2000). According to Peneau et al. (2006) perception of freshness in apple is related to crispiness, juiciness and aroma. Johnson (2002) indicated that Bramley apples were particularly responsive to 1-MCP. It helps to maintain quality included retention of greenness, firmness and acidity. However he emphasised that treatment with 1-MCP was less effective in Bramley stores when fruit temperature during treatment was at 20°C in comparison with the fruit temperature at 3.5°C. Johnson (2007) reported about dose rate of 1-MCP and there was no consistent effect of dose rate (500 v 1000 nl L^{-1} or 312 v 625 nl L^{-1} of 1-MCP in store free space) on fruit internal ethylene, firmness or scald incidence of 'Bramley' apples. The universal protocol for apple treatment is 625 nl L^{-1} of 1-MCP (based on empty store volume) applied for 24 h with minimal delay between harvest and application (Blankenship, 2001).

Delay in 1-MCP application (more than 7 days after harvest) generally leads to higher internal ethylene concentrations at the time of treatment and lower firmness both at the time of treatment and after storage (Johnson, 2002; Watkins and Nock, 2005). However in some apple cultivars like McIntosh, external CO_2 injury was more prevalent after storage if fruit were treated without delays after harvest for earlier harvests while later harvests were less affected (Nock and

Watkins, 2013). It is important for Bramley apples to delay the establishment of CA conditions after 1-MCP treatment from 10 to 28 days to avoid CO_2 injury in 'Bramley' apples; this delay has no effect on fruit quality (Johnson, 2007).

The blocking of ethylene receptors with 1-MCP has a variety of effects on apple respiration, ethylene production, volatiles production, acids and sugars, chlorophyll degradation, colour changes, and protein and membrane changes, as well as disorders and diseases and responses to biotic and abiotic stress like low-temperature, carbon dioxide and incidence of disease development (Blankenship and Dole, 2003). Apples are composed of a complex mixture of sugars (primarily fructose, glucose, and sucrose), oligosaccharides, and polysaccharides, together with malic and citric acids, polyphenols, amides and other nitrogenous compounds, soluble pectin, vitamins, minerals, water, and a variety of esters (Sapers *et. al.,* 2006). Rudell and Watkins (2011) compared changes in carbohydrates and organic acids in treated and untreated apples at harvest and during 40 weeks storage: 1-MCP decreased the decline in acidity, however the concentration of sugars was similar to untreated samples during storage. There is some evidence that endogenous ethylene production during the ripening process increases the level of phenyl-alanine ammonia-lyase (PAL), a rate-limiting enzyme for anthocyanin formation (Faragher and Brohier, 1984). This could contribute to the delay in changing colour in apples treated with 1-MCP. Treatment with 1-MCP does not control disorders that are initiated prior to harvest including bitter pit, however it can delay incidence of this physiological disorder (Watkins and Nock, 2005).

1.4.4 The use of controlled atmosphere to extend the storage life of fruit:

Low temperature (but higher than 3.5°C that causes chilling injury for Bramley's seedling) has a major role in extending the storage life of perishable fruit (Kader, 2001). However, the storage life of commodities can be extended further by the use of controlled atmosphere (CA) storage by decreasing oxygen and increasing CO_2 thus delaying ripening. In controlled atmosphere (CA) a low oxygen and/or high CO_2 atmosphere is created by natural respiration or artificial means. The effectiveness of CA storage depends on apple variety, maturity and postharvest treatments (Rees *et al.* 2012). In 1929, Bramley for the first time was stored successfully in 8-10% CO_2 with a balance of 11-13% oxygen and this storage regime is recommended still for this cultivar (Bishop, 1996). In air storage, oxygen and CO_2 levels are as ambient (21% O_2, 0.038-0.04% CO_2). Sharples and Stow (1985) and Johnson (1994) recommended storage temperature for Bramley's seedling of 4-4.5°C and CA storage conditions of 10-13% O_2, 8-

10% CO_2 (named as "ventilated CA") for 6 months storage, or 5-6% CO_2, 1-2% O_2, named as "scrubbed CA" for up to 10 months storage. In contrast with this idea, Prinja (1989) declared that there is no advantage in treatment with high CO_2 prior to storage since it leads to a higher risk of CO_2 injury as bronzing of the skin surface and internal browning of the flesh. Also Johanson and Colgan (2003) found that although CO_2 treatment reduces fruit softening over storage, it is injurious to the fruit when stored for long periods. However, increasing CO_2 gradually over 3 weeks reduces CO_2 injury from 15% to 3%. In addition, pre-cooling before storage in the shortest time and removal of CO_2 produced by respiration and adding air to replace consumed oxygen by respiration are very important applications in store management (Bishop, 1996).

Comparison of two commercial CA regimes for Bramley's seedling by Johnson (1994) showed that better control of disorders like bitter pit and scald, also control of firmness is obtained under the (5% CO_2:1% O_2) regime. However, there is a higher risk of rot caused by *Nectria gallegina* in this regime. Also Watkins (2009) showed the storage in low temperature CA conditions causes considerable stresses associated with chilling injury and anoxia caused by low oxygen and high carbon dioxide. Storage of fruit just above the threshold of physiological damage can reduce the development of physiological storage disorders in the fruit. Perring and Pearson (1986) observed that redistribution of minerals in fruit depended on storage temperature, apple variety and pre-harvest treatments. They found that bitter pit development was delayed by storage at lower temperatures possibly by delaying the remobilisation of calcium back to the core. Also Neuwald *et al.* (2014) compared mineral changes during storage and indicated there were no significant changes of the mineral concentrations in the apple fruit during CA-storage.

1.5 Pre-harvest and climate factors:

Fruit remains physiologically active after harvest and during storage. The fruit's behaviour in storage is affected by pre-harvest and post-harvest factors. Pre-harvest factors which may cause disorder development are related to the position of the fruit on the tree, characteristics of the fruiting site, crop load, mineral and carbohydrate nutrition of the developing fruit, water relations, and response to temperatures (Ferguson *et al.,* 1999). An understanding of these factors helps store management to optimize storage quality, and development of methods for predicting disorders risk. Sharples and Johnson (1987), emphasised on significant wastage in Cox and Bramley since both varieties have to be stored at the relatively high temperatures of 3.5-4.0°C and 4-4.5°C respectively to avoid the risk of low temperature breakdown (LTB).

Cuquel *et al.* (2011) showed pre-harvest application of nitrogen fertilizers increased rotting and flesh breakdown, particularly where potassium was also applied. Fruit with high-potassium is also affected by bitter pit and Core Flush.

Factors affecting fruit storage and development of physiological disorders are as follows.

1.5.1 Rootstock:

The former East Malling Research Station (EMRS) now known as East Malling Research pioneered the development of rootstocks for fruit trees and introduced different rootstocks from dwarf to vigorous (Table1.1). A number of researchers (Barritt *et al.*1995; Hirst and Ferree, 1995; and Seleznyova *et al.* 2008) quantified the effect of rootstocks on apple fruit quality. Rootstocks primarily restrict the amount of above ground growth of the scion (fruit tree). Rootstocks are selected on the basis of the type of tree habit (small, medium and large) that is required for a particular location, planting intensity and training system.

M.9 is a dwarfing rootstock was released by East Malling Research Station in the 1920s, most commonly used for commercial rootstocks and is probably the most widely planted of all rootstocks globally. After five years the tree reaches full size and produces large fruit.

M.26 is semi-dwarfing, suitable for most bush and trained tree production. Roots produce suckers, but will not do well on poorly drained sites. It is regarded as producing trees of intermediate vigour between M.9 and MM.106.

MM.106 will produce an apple tree about 3.6 m wide and 3 m tall at maturity. One of the most popular apple rootstocks, developed in collaboration by the East Malling and Merton research stations (John Innes Institute) in the early 20th century. The roots are reasonably vigorous. Each tree produces around 23 Kg fruit as a good sized apple.

Table 1.1: Classification of apple rootstocks by growth.

	Very Small	**Small**	**Medium**	**Large**	**Very Large**
Rootstock	P.22, M.27, G.65	G.11,M.9, G.16, Bud.9	M.26, G.935, G.202, G.30, MM.102, M.7, M.116	MM.106	MM.111, Bud.118, M.25
Height	2m	2.5m	3m	4m	5m

In general, MM.106 rootstocks are commonly found planted in older Bramley orchards, while more modern intensively managed orchards are planted with M.27 rootstock or more recently with M.9 trees which are easier for orchard management and require less pruning and allow for better uptake of pre-harvest spray programmes (Seleznyova *et al.* 2008).

There are several reports about the effects of rootstocks on apple maturity, quality and storage life. Autio (1991) and Barden and Marini (1992) in separate studies showed that rootstock can affect the maturity of fruit which is important for scheduling harvest or long-term storage. Normally fruits on dwarf rootstocks ripen later, with higher calcium contents. Also Webster and Wertheim (2003) worked on the influence of rootstock on the quality of fruit after harvest and storage. They got the same results as Ben (1995) that fruit grown on dwarfing rootstocks like M.9 have higher flesh firmness, increased soluble solids (Brix) and higher calcium contents comparing with fruit grown on vigorous rootstocks such as MM.106 or M.111. However in contrast of higher calcium contents in dwarf rootstocks, Fallahi and Mohan (2000) and Skrzynski (2007), observed that M.9 and M.26 as shorter trees were more susceptible to disorders than bigger trees. There is a competition between shoot growth and fruits for calcium. Hirts and Ferree (1995) measured 30% higher Ca concentration in leaves of dwarf rootstock B.9 comparing with bigger trees on M.26. Vigorous trees often have lower crop load that reduce fruit calcium concentrations (Drake *et al.* 1991).

1.5.2 Orchard management, cropping level and fruit size:

Bitter pit incidence is related to fruit size and normally larger fruits are more susceptible. There is also a relationship between the number of seeds in the fruit and incidence and development of bitter pit. Blazek and Hlusickova (2006) compared different quality parameters in four apple varieties and found that better pollinated fruits contained more seeds and generally had better quality, however high seed counts may accelerate maturity and have negative impact on internal fruit quality in the ripened apple. Broom *et al.* (1998) found a strong relationship between the number of leaves and the amount of calcium in the fruit. They showed as the fruit expands a progressive breakdown of xylem leads to reduce calcium uptake and an imbalance with other minerals in the fruit, which increases the risk of calcium-related disorders.

Ferguson and Watkins (1992) found that movement of minerals into fruit is affected by crop load regardless of final fruit size. Apple fruits from light-cropping trees had lower Ca and higher K and more incidence of bitter pit comparing to heavy-cropped trees. Also Racsko (2006)

indicated that fruits of trees with a heavy-crop load have higher density and less intracellular air space that leads to an increase in Ca concentration.

Soil management also has a key role in nutrient uptake especially calcium. Weibel *et al.* (1998) showed that different soil management practices affected the content of Ca and K concentration in the fruitlets and they found that where the K:Ca ratio in the fruitlet was higher than 5.8 the risk of bitter pit during storage considerably increased. Furthermore irrigation affects incidence of bitter pit. Lopez-Cuevas (2006) observed that incidence of bitter pit was lower on apples from trees that did not receive irrigation compared to irrigated trees. Bitter pit incidence was similar under all crop load levels, foliar calcium applications, foliar N, B, Zn, and Mg applications, nitrogen fertilization rate and potassium fertilization rate did not affect.

1.5.3 Weather and environmental effects:

The interaction between climate and environment and mineral nutrition leads to many storage disorders of apple. Minerals like calcium and boron are taken up by the tree via the mass flow of water as it is drawn up through transpiration (Little and Holmes, 2000). In cold or cloudy weather, demand for water is low therefore the transport of calcium decreases. Some elements like potassium and nitrogen are taken up via active transport through phloem and less affected by weather and environmental changes (Dražeta *et al.,* 2004). Also the time of incidence of weather and environmental stresses is important, for example most of calcium uptake into the fruit occurs in the first weeks of the growing season and any stress at that time could affect the calcium content of fruit (Rees *et al.,* 2012). Also Sharples and Johnson (1987) observed that low temperature breakdown (LTB) during storage occurs less after a warm dry season, and there is an interaction between cool seasons (which causes lower transpiration), late picking and more low temperature breakdown (LTB).

Miller *et al.* (1998) found that acetate ester production was lower (less flavour) for fruit with less than 53% exposure to full sun. The position of fruit on apple trees and high temperatures strongly influences fruit mineral contents that eventually will cause post-harvest disorders like bitter pit (Dražeta *et al.,* 2004). Fruits located in the western or southern part of a tree receive more sunlight and more transpiration occurs therefore they have higher soluble content compared to apples with northern or eastern exposure (Miller *et al.,* 1998). Poor pollination because of lack of bee activity in rainy, cold, or windy weather during bloom leads to poorer fruit quality and increased susceptibility to physiological disorders due to poor seed set (Blazek and Hlusickova, 2006). Sharples and Johnson (1987) found that water stress reduces fruit size

and increases the percentage of total soluble solids which leads to earlier ethylene production. On the other hand fruit grown in the wet season have a more porous skin and are more susceptible to shrivelling during storage.

1.5.4 Harvest date:

Maturity at harvest plays a critical role in postharvest life of fruit. Although early picking of apples helps to maintain texture during storage and handling, they are more susceptible to shrivel, scald and bitter pit. Moreover, apples picked too late may show disorders such as flesh browning and breakdown (Watkins *et al.* 1989; Tong *et al.*1999).

In fruits, starch is the major carbohydrate reserve. It is synthesized from glucose-1-phosphate by the action of AGPase (ADP-glucose pyrophosphorylase) enzyme. Starch degrading enzymes are found in the chloroplast, which convert starch to sugar with fruit ripening. Starch is transformed to glucose-1-phosphate with the action of several enzymes. The glucose-1-phosphate is mobilized into cytoplasm, where sucrose is synthesized. Sucrose is the major sugar that accumulates as the fruit starts to ripen. With the advancement of ripening, sucrose is further converted to glucose and fructose by the enzyme invertase (Paliyath *et al.,* 2008).

The starch iodine test has been used for many years to indicate the onset of harvest when the average starch pattern (black surface area) decreases to two thirds of the maximum coverage (Sharples and Stow, 1985). Baldwin *et al.* (1991) emphasised the need to harvest at optimum fruit maturity since immature fruit produces a limited amount of volatiles and the flavour would never approach that of fruit harvested at more mature stage. Prang *et al.* (2011) observed that minimum incidence of calcium related disorders, bitter pit and breakdown (combined) occurred at the mid-point of the harvest period. Bramley is a particularly unusual variety as it is harvested relatively immature to maintain the firmness and acidity of fruit that is required for processing. In research reported by Jackson (2005) Bramley's Seedling apple was harvested at four different dates, fruit weight almost doubled between the first and the last pickings. Also late picking can severely affect fruit set in the following year.

1.6 Calcium:

Calcium (Ca^{2+}) is one of the most important mineral elements determining fruit quality, cell wall structure and strength, and for plasma membrane structure and integrity (White and Brodley, 2003). The importance of calcium in apple fruit is its role in contributing to the

maintenance of the optimum quality during postharvest storage and fruit ripening. This role is seen directly in the prevention of specific disorders such as bitter pit, and more general quality factors like flesh firmness (Hepler, 2005).

Calcium is a unique macronutrient with fundamental physiological roles in plant structure and signalling. It has a role in cellular signalling via regulation of changes in its cytoplasmic concentration (Atkinson, 2014). Calcium is taken up by root tips and transported to leaves and young fruit by the transpiration stream, exclusively through xylem vessels (Dražeta *et al.,* 2004; Ho and White, 2005). Paiva *et al.* (1998) observed that environmental factors that increase fruit transpiration (such as sunlight exposure or wind) are more effective in increasing fruit calcium uptake than increasing concentrations available to the root system. This was indicated by Atkinson (2014) by increasing free calcium in the xylem sap without significant increase in regulation of shoot calcium delivery. Calcium accumulates mainly in the leaves, since it is immobile in the plant. The amount of calcium in seeds and fruits is relatively low, around 5% of total plant calcium (Terblanche *et al.* 1979; Hepler, 2005). However, calcium cannot be re-distributed from leaves to fruits and it is more difficult to maintain supplies to older fruit as they mature (Picchioni *et al.*, 1998). Water flow through the plant is itself regulated by calcium, both in the apoplast affecting cell wall structure and stomata, and within the symplast which regulates flow across membranes (Hepler, 2005). Poor redistribution of calcium from older tissues to developing ones lead to physiological disorders like bitter pit in apples, the reason for this erratic distribution of calcium within the cell is unknown (Perring, 1985).

Sharples and Johnson (1987) emphasised careful pre-harvest management combining use of calcium sprays (calcium chloride and calcium nitrate) to prevent bitter pit. However, Metzner *et al*. (2008) indicated the importance of balance between calcium sources to xylem sap and ion exchange capacity of stem and their parenchymal tissues. Terblanche *et al.* (1979) indicated the need for balancing tree growth by late-summer pruning to enhance total uptake. They reported that calcium was actively absorbed during the shoot extension period. During the period of rapid shoot extension the calcium reserves from the permanent structure of the trees made a very important contribution (22.8%) to the total calcium content of the new growth (shoots, leaves, and fruit). Choi *et al.* (2011) have suggested that at least a portion of calcium presented in the trees at dormancy was in an exchangeable form and the amount of summer shoot growth should be restricted to redirect calcium into fruit rather than new shoot growth.

Remorini *et al.* (2008) recommended picking at the right time to maximise storage potential of fruit, which also contributes to improved calcium status and hence longer storage life. According to "Defra best practice guide for apple production and storage (2002)", the standard fruit calcium concentration for the satisfactory long-term storage of most apple cultivars is 4.5-5mg/100g, depending on storage regime

Atkinson (1991) explained the mechanism of signalling calcium as a second messenger in plant cell growth and development by relaying signals from receptors on the cell surface to target molecules inside the cell, in the cytoplasm or nucleus. Hepler (2005) indicated two main roles of calcium; one in the cell wall where it has a key role in cross linking acidic pectin residues and the second role in the cellular membrane system, where low calcium increases membrane permeability. Atkinson (1991) also indicated that calcium is involved with signalling potassium to open and close the stomata in plant leaves, so the plant with calcium deficiency is more susceptible to wind, heat or cold stress. One of the main factors in controlling physiological and pathological disorders during storage is related to the amount of calcium taken up by fruit, which is dependent on maturity of the fruit and the changes in structure due to cell enlargement and the increase in intercellular spaces (Conway, 1989). In another study by Conway and Sams (1985), the influence of fruit maturity on calcium uptake was investigated at different harvest times. Fruit picked two weeks later than optimum harvest had three times as much flesh calcium as fruit harvested two weeks earlier than normal harvest time. They also observed that total calcium in the last weeks before harvest is not increased and through future fruit expansion the amount of calcium is diluted.

One of the most important functions of calcium is as a constituent of the cell wall. Cell wall structure is important in keeping quality that maintains cell integrity preventing moisture loss and disorders during maturity, transport and storage (Nelmes and Preston, 1976; Hepler, 2005). Goulao and Oliveira (2008) indicated that environmental growing conditions affect cell wall composition and metabolism, which can potentially affect the dynamics of Ca^{2+} binding to the cell wall and fruit susceptibility to bitter pit.

Calcium infiltration as a post-harvest treatment is an effective method to increase the storage and shelf life of apple (Conway, 1989). Post-harvest changes in membrane lipids of apple fruits infiltrated with calcium were evaluated by Picchioni *et al.* (1998) and reported that during

storage, total phospholipid and acylated steryl glycoside concentrations increased compared to untreated apples. Within the cell wall, phospholipids, galactolipids and free sterols constitute the structure of membranes (Moreau and Preising, 1993). Movement of calcium from the middle lamella and loss of its binding sites occur during apple softening and these processes contribute to changes in tissue structure (Stow, 1988). A loss of cell turgor through an increase in permeability during senescence and decrease of cortical water-soluble Ca^{2+} eventually leads to fruit softening during ripening and pitted fruit have less water soluble Ca^{2+} than sound fruit (Stow, 1993; Pavicic et al., 2004). Trakoontivakron (1987) observed in immature fruits, the forces binding cells are greater than the forces maintaining cell wall integrity and concluded softening is a result of changes in cell to cell cohesion and is not result of the cell walls weakening.

The cell wall of apple cells is a matrix of cellulose, pectin and proteins (Figure 1.8**)** cross linked for extra strength (Keegstra, 2010). Calcium stabilizes and ensures permeability of the cell wall, protecting it from degradation by membrane lipid alteration (Picchioni *et al.*, 1998).

Calcium is necessary for binding phospholipid molecules into cell membranes, determining the size of pores and influencing membrane permeability. Calcium ions are bound to the pectins present in the cell wall (Keegstra, 2010).

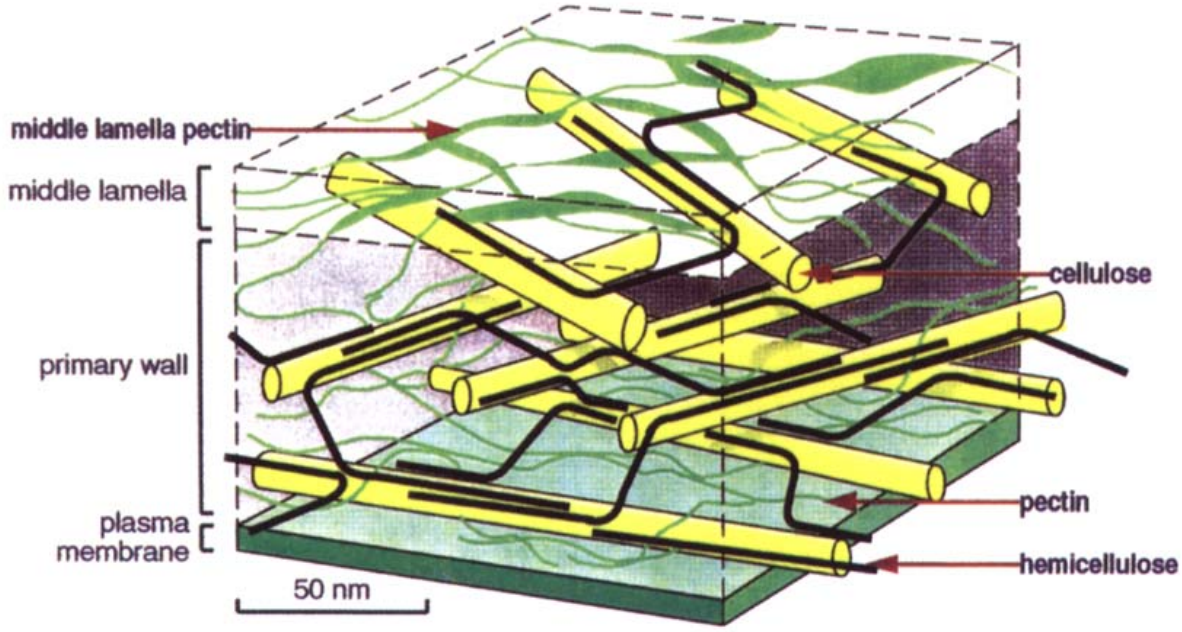


Figure 1.8: The cell wall of apple cells including cellulose, pectin and proteins (Source: Keegstra, 2010)**.**

Tepfer and Taylor (1981) found that the formation of cation cross bridges between pectic acids or other polysaccharides with acid groups may make the cell wall less accessible to enzymes. Val *et al.* (2006) emphasised on the role of fruit protein as a key component of nuclear and cytoplasmic structures also the role of enzymes involved in metabolism during growth, development, maturation and postharvest life. The amount of protein in apples, like most fresh fruits and vegetables, is quite low, representing approximately 0.2% of their fresh weight (Renard and Thibault, 1991).

The distribution and movement of calcium is from core (inner cortex) to outer cortex during growth on the tree as well as storage (Perring, 1985). Movement of Ca from the core to the outer cortical tissues has been monitored during storage by Sharples and Johnson (1987). They observed the rate of movement of calcium into the bitter bit-susceptible outer cortex of apples was similar or slightly faster in controlled atmosphere (CA) than in air stores. Concentration of minerals (apart from Na) is usually higher in the peel and core tissues than in the flesh of apple fruit, probably associated with higher cell numbers in these regions of the fruit. Perring (1998) reported the concentration of calcium in the outer and mid cortical zone nearer the stalk end, which normally shows less pit than similar tissues near the calyx, was slightly higher than calcium concentration near the calyx end of apple fruit. However, Gracia *et al.* (2008) observed that damaged tissues usually have more Ca^{2+} than the surrounding healthy tissues, most in a water-insoluble form. The outward movement of Ca to the outer cortical and calyx end zones were measured and analysed by Terblanche *et al*. (1979) and Perring (1985), who showed that part of the redistribution of calcium could be related to different amounts of dry matter concentration in outer and inner zones. However, Saure (2005) suggested these are based on a hormonal control, mainly executed by gibberellins (GAs) that inhibit calcium translocation.

1.6.1 Balance of calcium and other nutrients:

Calcium, magnesium, potassium and ammonium interact with each other. High concentrations of one will affect the uptake of others (Neuteboom and Withnall, 1998). Movement of calcium into developing fruit is rapid in the early stages of growth, but then often reduces and there may be little or no increase in calcium in the fruit over the later stages of growth. Seed number and fruit set can influence the rate of calcium transport to the apple (Tomala, 1997). By contrast, potassium and magnesium move into the fruit over the whole season, keeping up with fruit growth. This means that at maturity, calcium concentrations have fallen in the flesh, whereas potassium and magnesium concentrations may be steady or increase (Ferguson, 1999). Also

Hepler (2005) reported that unlike calcium, potassium concentration did not show differences in pitted or non-pitted tissues.

De Freitas *et al.* (2009) indicated that more than 40% of the calcium in fruit tissue is in the vacuole and rest is located in the cell wall. Bitter Pit may also involve an abnormal distribution of calcium in the cells, where supply of the free apoplastic calcium affects plasma membrane structure and function and causes an increase in calcium binding to the cell wall and reduces the apoplastic pool of free calcium. However Neuteboom and Withnall, (1998) observed that incidence of disorders related to calcium deficiency is not only related to the concentration of calcium, but also sometimes there is a close correlation between the incidence of these disorders to the ratio of calcium to potassium also ratio of calcium to potassium and magnesium. Keegstra (2010) indicated the capability of calcium in promoting gelling in a pectic solution, unlike magnesium and makes it more resistant to enzymes and significant protection against disorders.

Perring (1985) observed that fruits with low calcium become more susceptible to bitter pit, lenticel blotch pit and cracking as potassium and magnesium concentrations increased. The acidity of fruit increases with increased potassium content. Neuteboom and Withnall (1998) observed when calcium levels are not adequate, high concentrations of potassium and magnesium can increase bitter pit and rots. Burmeister and Dilley (1994) suggested a specific role for calcium and magnesium in bitter pit development. In their trial, extracellular magnesium supplied by infiltration affected the supply of calcium in the apoplast of apple fruit influencing the ability of cells to regulate cytosolic calcium.

De Freitas *et al.* (2013) observed that although total calcium content of fruit increased, fruit susceptibility to bitter pit also increased. When compared fruits with or without bitter pit they found lower ratio Mg/Ca in apples without bitter pit. They found that Ca^{2+} ions were bound in the water-insoluble pectin network in the cell wall, eventually reduced the level of Ca^{2+} available for outer cellular functions. So the combined effect of low Ca^{2+} and high Mg^{2+} concentrations in the cortical tissue lead to higher fruit susceptibility to bitter pit.

1.7 Methods for Predicting Bitter Pit:

For predicting and control of bitter pit, calcium concentrations in the fruit must be adequately assessed. However, because calcium levels are low and variable between fruits it is difficult to measure this mineral directly. Different methods (maturity enhancement; shoot growth; fruit Ca, Mg, and K levels; and fruit Mg infiltration) have been studied.

In general, methods for bitter pit prediction are based on the physiology of bitter pit incidence:

1.7.1 Nutritional Status Methods:

Since incidence of bitter pit is related to the nutritional status of the fruit, the logical approach has been to measure the concentrations of several elements related to bitter pit incidence (i.e., Ca, K, Mg, N, and P). However, this method has not always provided reliable results (Ferguson and Watkins, 1989). Moreover, fruit sampling protocols (e.g. sampling from different parts of fruit tissue) vary between and within the various apple-producing regions, and different laboratories have diverse methods to determine the potential for bitter pit incidence (Marcelle, 1990). Selection of 20 fruit samples followed by pulping and sub sampling of pulp help to ensure a uniform sample.

1.7.2 Maturity Acceleration Methods:

Ferguson and Watkins (1989) used a predictive method of accelerating the maturity of the fruit with the ethylene-releasing compound 2-Chloroethylphosphonic acid (Ethephon) to have earlier appearance of bitter pit symptoms.

1.7.3 Mg infiltration:

In this method calcium concentration is measured indirectly by Mg infiltration. The basis of this method is the antagonism at a cellular level between calcium and magnesium. Cooper and Bangerth (1976) applied this method by immersing apple in a solution rich in magnesium and a vacuum was applied. Magnesium replaces the calcium within the apple and symptoms of calcium deficiency and disorders like bitter pit will appear in the fruit a few days later. Burmeister and Dilley (1994) observed that calcium exchange for magnesium is inversely related to the calcium concentration of the fruit, when there is lower calcium concentration in the fruit, the intensity of the exchange is higher and the more symptoms appear.

1.7.4 Vegetative Growth Methods:

These methods are based on interaction between vegetative growth, calcium distribution among vegetative parts of the tree (shoots and leaves), reproductive (fruit) tissues, and bitter pit incidence. An intense vegetative growth, expressed as shoot growth, indicates that Ca has been diverted to shoots instead of fruit tissues; in this situation higher bitter pit incidence occurs (Fergusen *et al.,* 1999). This observation provides the grower with an early indication that low-calcium related disorders are likely to develop during storage unless additional calcium is supplied to trees (Retamales *et al.,* 1998).

1.8 Chlorophyll fluorescence:

Chlorophyll fluorescence has been developed as a tool for measuring the functioning of photosynthetic organs, including fruits. All the absorbed energy cannot be used for photosynthesis (photochemistry) and the excess is reemitted as light (chlorophyll fluorescence) and heat (Maxwell and Johanson, 2000; Rees *et al.*, 2005). Only 1-2% of total absorbed light re-emitted as chlorophyll fluorescence and the spectrum of fluorescence is different to that of absorbed light. Therefore, fluorescence yield can be quantified by exposing the organ with chlorophyll (leaf or fruit) to light of defined wavelength and measuring the amount of light re-emitted at longer wavelengths (Maxwell and Johanson, 2000).

Stress conditions have similar effects causing biological changes in fruit like ripening or senescence that leads to breakdown of chlorophyll and increasing synthesis of anthocyanins and carotenoids (Huybrechts *et al.*, 2003). The development of devices for measuring fluorescence and their use in monitoring photosynthetic events has shown correlations between rates of photosynthesis and maturity and also physiological disorders during fruit storage (Ross, 2002).

Two separate photochemical steps during photosynthesis are associated with different groups of pigments. These groups are named photosystem I (PSI) and photosystem II (PSII) (Callahan *et al.,* 1986; Parkhill *et al.,* 2001; Schreiber *et al.,* 1986). At least 95% of the chlorophyll fluorescence signal observed under physiological temperatures is derived from chlorophyll molecules associated with photosystem II, which is able to use sunlight to oxidise water to produce oxygen (Schreiber *et al.,* 1994). Damage to PSII is the first symptom of stress and photosynthetic function is maintained by continual repair and rebuilding of PSII, which is an important target of abiotic stresses and can be inhibited when tissues are stressed (Prang *et al.,* 2002; Valcke, 2011). Maxwell and Johanson (2000) worked on the flow of electrons through PSII to estimate photosynthetic performance. Once PSII absorbs light and Q_A (Primary Quinone of Photosystem II) has accepted an electron, it is not able to accept another electron until it has passed the first onto a subsequent electron carrier Q_B (Figure.1.9).

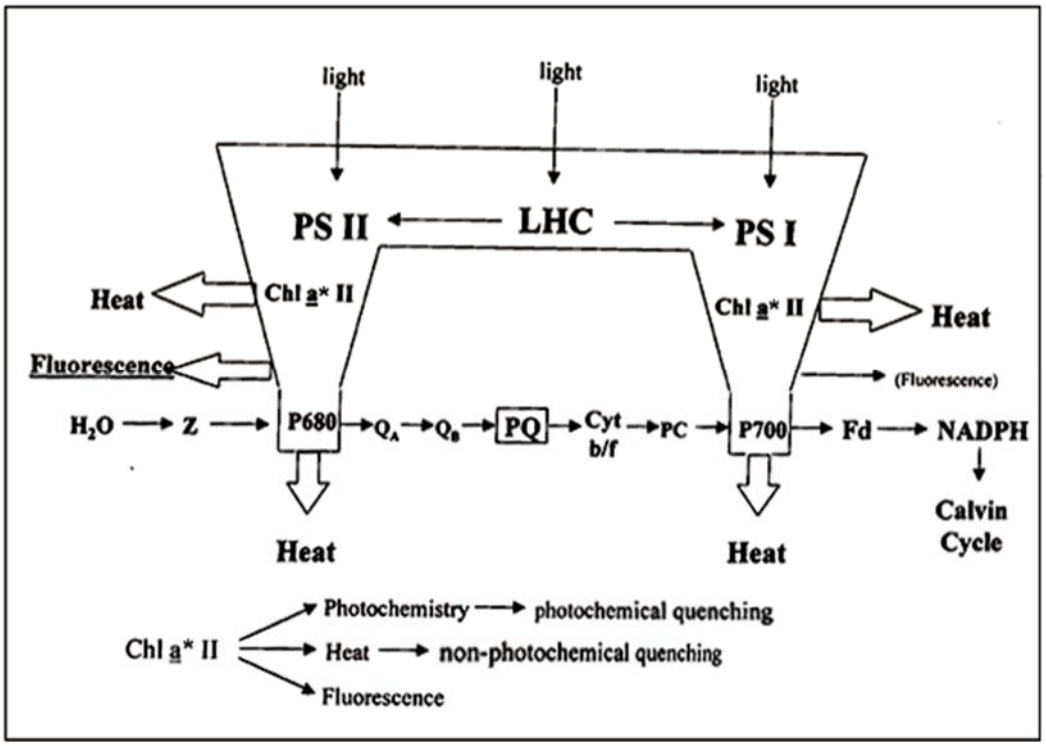
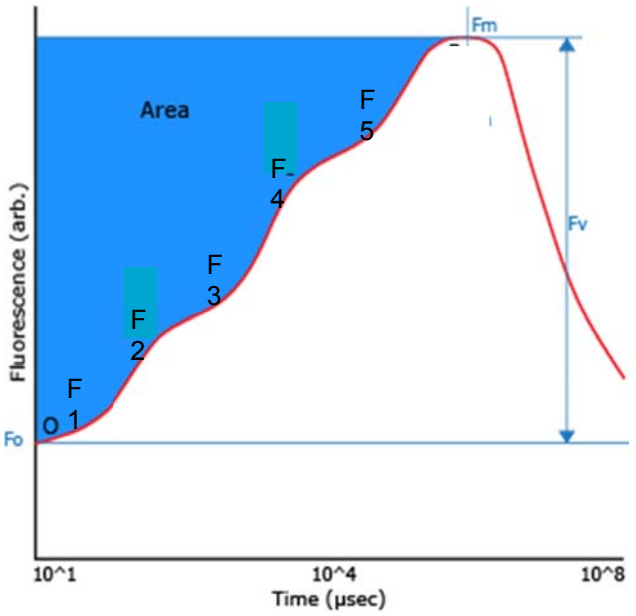


Figure 1.9: Schematic illustration of primary energy conversion in photosynthesis and the process that fluorescence originates from PSII and heat dissipation (Schreiber *et al.,* 1994)

Schreiber *et al.* (1986) measured PSII function after exposure to bright light and observed that dark-adapted samples when exposed to sudden illumination with a high intensity light source, produce a rapid polyphasic rise in chlorophyll fluorescence which was followed by a slow decline in fluorescence intensity to a steady state level of fluorescence.

Toivonen (1992) developed models relating fluorescence to shelf life of different crops and showed that fluorescence measurements unlike surface colour measurements are not affected by the presence of masking pigments. Most of the chlorophyll fluorimeters use focused, high intensity light from red LEDs to induce a fast chlorophyll fluorescence response from a dark adapted sample. Rees *et al.* (2005) found that red light intensity of 2000 $\mu E.m^{-2}.S^{-1}$ showed a relationship with maturity. However, Lichtenthaler *et al.* (2012) measured fluorescence images of green apple during storage with multicolour fluorescence imaging in bands F440 (blue), F520 (green), F690 (red), and F740 (far-red) and got different results in each band. They found the application of multicolour fluorescence imaging of the different fluorescence ratios more reliable and less sensitive to errors as compared to imaging only one single fluorescence band. Zhang *et al.* (2012) emphasised doing analysis of stress–induced changes by fluorescence emission at very early stage of stress. They applied Hyperspectral fluorescence imaging (HSFI) as a combination of both hyperspectral imaging and fluorescence spectroscopy. Figure 1.10

shows a typical fluorescence trace obtained using a fluorimeter which is named the "Kautsky Induction" or "Fast Chlorophyll Fluorescence Induction". It shows an increase in the yield of chlorophyll fluorescence over a time period of around 1s from which several characteristics of the trace like Fo, Fm and Fv can be measured. Kautsky Induction curves must be plotted on a logarithmic axis in order to observe the polyphasic rise to the maximum chlorophyll fluorescence value (Kooten and Snel, 1990; Maxwell and Johanson, 2000).



F_1	Fluorescence Intensity at 50 µs
F_2	Fluorescence Intensity at 150 µs
F_3	Fluorescence Intensity at 300 µs
F_4	Fluorescence Intensity at 2 ms
F_5	Fluorescence Intensity at 30 ms
Fm	Maximum Fluorescence Yield
Fo	Minimum Fluorescence Yield
Fv	Variable Fluorescence Yield
Area	The area above the fluorescence rise between Fo and Fm

Figure 1.10: Typical fluorescence emitted (Kautsky Induction curve) measured by fluorimeter in logarithmic units and main characteristics as Fo, Fm and Fv.
(Based on: Stirbet and Govindjee, 2011)

Fo occurs at time base "0". It is the almost nanoseconds range rise to an origin level of chlorophyll fluorescence upon illumination using a chlorophyll fluorimeter. The "Fm" parameter is the maximum chlorophyll fluorescence value obtained for a continuous light intensity. The "Fv" parameter indicates the variable component of the recording and relates to the maximum capacity for photochemical quenching (Schreiber *et al*., 1986).

The "Fo" parameter represents emission by excited chlorophyll molecules in the antennae structure of Photosystem II (PSII) as an important component as it is able to use sunlight to split water, releasing oxygen (Parkhill *et al.,* 2001). The true Fo level is only observed when the first stable electron acceptor of PSII is fully oxidised. This requires thorough dark adaptation. Fv is

calculated by subtracting the Fo value from the Fm value. Fv/Fm is a parameter widely used to indicate the maximum quantum efficiency of photosystem II. This parameter is widely considered to be a sensitive indication of plant photosynthetic performance with healthy samples typically achieving a maximum Fv/Fm value of approximately 0.85. Values lower than this will be observed if a sample has been exposed to some type of biotic or abiotic stress factor which has reduced the capacity for photochemical quenching of energy within PSII (Strasser *et al*., 2000).

The area above the fluorescence curve between Fo and Fm is proportional to the pool size of the electron acceptors Q_A (Primary Quinone of Photosystem II) on the reducing side of photosystem II. Krause and Weis (1991) indicated the importance of measurement of this area as a very useful parameter as it highlights any change in the shape of the induction kinetic between Fo and Fm which would not be evident from the other parameters e.g. Fo, Fm, Fv/Fm which only express changes of amplitude of the extreme Fo and Fm.

Models of the functioning of the photosynthetic system have been used to relate the fluorescence characteristics to specific physiological aspects of chloroplasts (Krause and Weis, 1991). Hagen *et al.* (2006) showed the chlorophyll fluorescence measurements could be used as a non-destructive estimation of both anthocyanins and total flavonoids in apples. Saquet and Streif (2002) applied chlorophyll fluorescence techniques as a prediction method to detect internal browning disorders caused by low O_2 or high CO_2 during controlled atmosphere storage of different varieties of apples and pears. The breakdown of chlorophyll increases energy dissipation via chlorophyll fluorescence due to a decrease in dissipation via photosynthesis also increases synthesis of anthocyanins and carotenoids (Krause and Weis, 1991; Huybrechts *et al.*, 2003). Since stress factors (cold, drought, nutrient deficiency, etc.) limit the photosynthetic capability of the sample analysing these changes in fluorescence characteristics can be used to monitor samples and screen them effectively for particular types of stress factors which limit the photosynthetic performance of the sample (Dinh *et al.,* 2014; Kalaji and Guo, 2008). Schmitz-Eiberger *et al.* (2002) worked on the relationship between chlorophyll fluorescence characteristics, such as Fm and Fv/Fm and calcium deficiency. They found chlorophyll content dropped when there was shortage of calcium content, while the anti-oxidative capacity increased slightly. The decomposition of chlorophyll in the skin has a relationship with ripening and calcium deficiency. Obaid *et al.* (1996) applied Fv/Fm to predict best harvest time in apple, but they found that Fv/Fm declined very slowly and was not able to determine maturity.

However Rees *et al.* (2005) observed that RC/CS (Reaction Centre/Cross Sectional area) decreased over the ripening time with the same rate of decreasing starch over the same time period and suggested this parameter (RC/CS) for maturity prediction. Rutkowski *et al.* (2008) applied chlorophyll index, normalised difference vegetation index and normalised anthocyanin index for evaluating quality parameters of apple (cv Golden Delicious) for predicting fruit maturity. Ross (2002) found that the ability of tissues to resynthesise PSII, as indicated by Fv/Fm recovery on the removal of stress was a much more reliable indicator of tissue damage. Dinh *et al.* (2014) investigated the effect of the stress caused by low oxygen concentration on chlorophyll fluorescence of apples and found the inverse correlation to oxygen concentration.

Also Moshou *et al.* (2005) and Zude *et al.* (2006) found the correlation between cell wall breakdown and decreasing chlorophyll fluorescence and explained it by parallel metabolic processes of chloroplast degradation and pectin conversion due to fruit maturation and applied this technique for sorting apples in packhouses.

Lotze *et al.* (2006) applied chlorophyll fluorescence imaging by Near-infrared (NIR) spectroscopy as a non-destructive method for pre-harvest detection of bitter bit in apple and observed that pitted fruit displayed lower fluorescence than non-pitted fruit. Although they achieved (+75%) correct classification, misclassification of non-pitted fruit was still too high to be used as a routine commercial tool. The fluorescence of non-pitted fruit did not always vary significantly from that of the pitted fruit. Karoui and Blecker (2011) explained that molecular structure of the samples causes variation of optical pathway of excitation light and fluorescence inside the fruit.

Born *et al.,* 2004 commented that although chlorophyll fluorescence is a helpful non-destructive tool, it should be combined with other quality measurements.

1.9 Molecular diagnostics and transcriptomic analysis:

Although fruit mineral content is strongly related to incidence of bitter pit, there is a need for a more reliable and accurate technique for bitter pit prediction. New molecular and metabolomics approaches may afford the opportunity to deliver a new insight into the changes in cellular metabolism that occur during storage.

The first stage in achieving a reliable molecular diagnostic test for bitter pit is to select a reliable method for extracting high quality RNA. Rapley and Manning (1998) explained how RNA molecules form the bridge between the stable genetic information contained within DNA and

enzymes and proteins that carry out much of the metabolism within the cell. Bustin (2000) emphasised that between different types of RNA species, messenger RNA is a desirable source of material to biologists, since this reflects much of, what ultimately, is translated into enzymes and proteins.

Since ribosomal RNA (rRNA) is synthesized at very high rates in growing tissues, its production is easy to study. Many hundreds of rRNA genes are massed in tandem arrays in the nucleolus of plant cells (Grierson and Covey, 1998). They have a general repeating structure consisting of transcribed regions containing the 18S, 5.8S and 25S rRNAs and non-transcribed intergenic or spacer regions (Kavanagh and Timmis, 1988). Abler and Green (1996) indicated that measuring mRNA concentration levels is a useful tool in determining how the transcriptional machinery of the cell is affected in the presence of external signals, or how cells differ between a healthy state and a diseased state.

Plant development and differentiation involves the selective expression of specific genes. In fact there are different sequences and factors that function to regulate gene expression in different situations (Grierson and Covey, 1988). Bray (1993) emphasised that changes in gene expression are triggered by environmental signals and alterations in the concentration of endogenous hormones. Gong and Yanofsky (2002) showed the molecular mechanism for switching genes on and off involves *cis*-action DNA signals (on the same molecule), that interact with cell or development of specific proteins (*trans*-acting factors) encoded by other genes. The analysis of RNA is based on the methods which have been developed through the application of polymerase chain reaction (PCR) that allow RNA quantification from very small amounts of cellular materials (Ozsolak and Milos, 2011).

In a study conducted by Lay-Yee *et al.* (1990), subtle changes were observed as fruit continued to ripen and internal ethylene concentration increased to 80-100 ppm. Overall, the levels of at least six mRNAs were found to increase, while one mRNA decreased. Analysis of proteins extracted from ripening fruit indicated that the level of at least three proteins increased with ripening.

Korban and Swiader (1994) also found higher fruit flesh calcium (and higher concentration levels of boron with lower concentrations of magnesium and potassium) in pit-resistant compared with pit susceptible seedlings within two seedling families. The significant amount of genetic variation in pit-expression found amongst families suggests that genetic improvement can be made in developing pit-resistant apple cultivars. Tacken *et al*. (2010)

observed that more rapid softening occurs in apples when cold treatment is followed by an ethylene treatment comparing with those apples without cold treatment. They concluded that cell separation could be involved in degradation of pectin by several hydrolytic enzymes, particularly polygalacturonase (PG1), which increases in expression with ethylene following a cold treatment. The increase in activity of PG1 is related to the rate of pectin solubilisation during the ripening process. Paliyath *et al.* (2008) reported that PG1 has a relative molecular mass of 100 kDa. With the advancement of ripening PG2a (43 kDa) and PG2b (45 kDa) isoforms increase, becoming the predominant isoforms in the ripe fruit.

Apple fruit softening has been associated with the increase in the expression of cell wall hydrolases genes. Gray *et al*. (1992) expressed ethylene role to regulate fruit ripening by coordinating the expression of genes responsible for enhancing a rise in the rate of respiration, autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars, and increased activity of cell wall-degrading enzymes. The results of a study conducted by Ley-Yee *et al.* (1990) indicated changes at both the mRNA and protein level which coincide with increasing internal ethylene. Petruzzelli *et al.* (2003) investigated ethylene biosynthesis and the molecular effects of the calcium antagonists and the spatial association of ethylene biosynthesis. Prange *et al.* (2011) showed the effect of ethylene production and increasing size of fruit and Ca distribution on increasing bitter pit incidence especially if the fruit size is larger than 90 mm diameter.

Understanding the tissue and cell specific expression of the ACC synthase and ACC oxidase multigene families during plant development will offer new knowledge of the role of ethylene as a signalling molecule (Theologis, 1992). Any investigation to find the genes responsible for ACC synthase and ACC oxidase helps to prevent and predict bitter pit incidence. Sunako *et al.* (1999) isolated an allele of the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene (*Md-ACS1*) from a genomic library of apple (cv: Golden delicious), the transcript and translated product of which have been identified in ripening apples. DNA gel image and polymerase chain reaction (PCR) analyses of genomic DNAs showed clearly that apple cultivars were either heterozygous for *ACS1-1* and *ACS1-2* or homozygous for each type. However some apple cultivars homozygous for *ACS1-2* allele can also show different storage capability (Wang et al., 2001).

Polyamines (PAs) are organic poly-cations that bind DNA and proteins and stabilise membranes, they are involved in control of flowering and fruit ripening. Diamines (DAs) are

compounds containing two amino groups and PAs are present in the plant cell wall and are associated with pectic polysaccharides (D'Orazi and Bagni, 1987). PAs are adsorbed selectively on plant cell walls, some of them prevent polygalacturonic acid from adopting calcium which leads to less pectin calcium bridges being formed. The mobilization of cytosolic free Ca^{2+} and the cytosolic acidification after treatment with pectic fragments was suppressed (Messiaen and Cutsem, 1999).

Cell wall degradation which is not caused by a fungal or bacterial pathogen releases bound PAs in the apoplastic fluid in addition to Ca^{2+} (Mariani *et al.* 1989). Also Ralet *et al.* (2001) found that pectin methylesterases (PMEs) increased synthesis of deesterified pectins and/or increased gene expression and activity of enzymes that create Ca^{2+} binding sites in the cell and increased Ca^{2+} binding to the cell wall and more susceptibility to bitter pit.

Plant membrane proteomics can also provide valuable information about biological processes. Komatsu *et al.* (2006) suggested correlating the presence of particular proteins with the abundance of different cell membranes. Fruit tissues contain cytoplasmic proteins that are vital to cell processes and cell structure. Val *et al.* (2006) separated polypeptides from apple fruit based on molecular size by using denaturing conditions: polyacrylamide gel electrophoresis (PAGE) bands related to proteins of 42 and 47.5 kDa proteins were related to polygalacturonase and the 40 kDa protein to peroxidase. Also they found a novel 18 kDa protein which was found in bitter pit spots. De Freitasa *et al.* (2010) suggested that this novel protein was an inhibitor of pectin methylesterase. Krawitzky *et al.* (2013) tried to identify this 18 KDa protein. They identified several proteins near or at 18 kDa, with considerable difference found in infectious concentrations between pitted and healthy samples.

1.9.1 Genes related to stress and bitter pit:

There is evidence that bitter pit in apple is under some degree of genetic control. Korban and Swiader (1994) suggested that (external) pit assessed on fruit at harvest was a heritable trait after working on three scab resistant families of apple seedlings derived from controlled crosses, and further suggested that pit expression was controlled by two major genes (Bp-1 and Bp-2) controlling calcium accumulation and distribution within the fruit.

Calmodulin (CaM), an abbreviation for **CAL**cium-**MODUL**ated prote**IN**, is a calcium binding messenger protein (a small acidic protein) expressed in all eukaryotic cells and is one of the best characterised calcium sensors in plants and animals (Stevens, 1983). Watillon *et al.* (1993)

by sequencing calcium/calmodulin-dependent protein kinase expressed in plant cell found that a messenger encoding it was homologous to the mammalian CaM kinase II and this protein could be implicated in signal transduction processes. Yang and Poovaiah (2003) indicated that Ca/Calmodulin in plants and animals have similarities. Although CaM shares a similar structure in plants and animals, within plants CaM is encoded by a multiple gene family. This suggests that there are aspects of Ca^{2+}/CaM mediated signalling that are unique to plants since plants are sessile organisms and must therefore adapt to a changing environment to survive. Hepler (2005) indicated that calcium-dependent protein kinase proteins are part of the serine/threonine protein kinase family and are specifically expressed in plants.

Calmodulin plays a key role in the regulation of intracellular enzymes and physiological processes and acts as an intermediate messenger protein that can interrupt calcium signals by binding calcium ions, thus modifying its interactions with various target proteins (Stevens, 1983). Battey and Venis (1998) found that the apple fruit membranes contain a protein kinase which was sensitive to free calcium and very similar to calmodulin (CaM). They were investigating the role of Ca^{2+}-dependent protein phosphorylation in regulation of apple fruit metabolism and found that calmodulin antagonists cause symptoms similar to those caused by calcium deficiency. Also they found a kinase that showed optimum activity between pH 7 to pH 9. Paliyath and Poovaiah (1984) found that none of the plant regulators (auxin, cytokinin, gibberellin and abscisic acid) had a significant effect on calmodulin-promoted phosphodiesterase activity. They concluded, although abscisic acid and ethylene are known to promote senescence, it is possible that other compounds in an entirely different class affect senescence processes.

Cocucci *et al.* (1983) reported that calmodulin concentration was 40% higher in the pitted apple fruits than in the sound ones. Kim *et al.* (2008) reported that although CaM has no enzymatic activity of its own, the Ca^{2+}/CaM complex can directly modulate the activity of numerous target proteins in the control of a variety of cellular functions or indirectly triggers cellular responses by regulating the expression of genes encoding downstream effectors. However, the physiological functions of calmodulins are still largely unknown in plants. Paliyath and Poovaiah (1984) observed that the inhibition of calmodulin was reduced in extracts from apples stored at 2°C after calcium treatment. Also they found secondary plant products such as papaverine and theophylline also inhibited calmodulin-promoted phosphodiesterase activity.

Xiong *et al.* (2006) showed that a particular calcium binding protein may be localized in a particular compartment as a function of the physiological status of the cell. They have suggested that such multifunctional abilities may have a strong influence on coordination of calcium-dependent events in the plasma membrane and a particular calmodulin in the nucleus. Wang *et al.* (2001) in their experiments on maize gene expression found that there is a relationship between cell metabolism and cell cycle and maturity with calmodulin dependent protein kinase. Nie *et al.* (2012) identified one of the calmodulin binding proteins designated (SR1) that regulates plant defence and ethylene–induced senescence by directly binding the ethylene promoter region and this has an important role in ethylene signalling.

Chin and Means (2000) explained that changes in intracellular Ca^{2+} concentration regulates calmodulin in three distinct ways: initially at the cellular level, secondly, at the molecular level, by promoting different modes of association with many target proteins; thirdly, by directing a variety of conformational states in calmodulin leads to target-specific activation.

Tuteja and Mahajan (2007) observed that calcium signalling is involved in the regulation of cell cycle progression in response to abiotic stress. The regulation of gene expression by cellular calcium is important for plant defence against various stresses.

Kim *et al.* (2008) found that Ca^{2+} and CaM participate in transcriptional regulation in different ways; through binding directly the elevated Ca^{2+} in the nucleus or Ca^{2+} binds CaM directly to promoter sequences and regulates gene expression, which implies that CaM function as a transcription factor. Also they found that Ca^{2+}/CaM complex interacts with transcription factors and modulate either their DNA-binding or transcriptional activity. Also, the Ca^{2+}/CaM complex regulates gene expression by modulating the phosphorylation status of transcription factors. Reddy *et al.* (2011) highlighted the role of CaM as one of the key players in transducing pathogen-induced Ca^{2+} because nearly all signals including hormones and stresses responses cause changes in cellular Ca^{2+}, initially in the cytosol or nucleus. They indicated the specificity of Ca^{2+} signalling should be dependent on the interplay between Ca^{2+} signatures and Ca^{2+} sensing proteins. A study on biotic and abiotic stresses showed a calcium signature due to temporal changes in cytosolic free Ca^{2+}. These calcium signatures are decoded by calcium sensors like calmodulin which has four 'EF' hands that bind to calcium and by binding to its target proteins, relay calcium signalling (Hepler, 2005).

The use of inverse calcium as an indirect selection measure for pit seems attractive given that it shows more consistent genetic variability compared with that found for pit (Voltz *et al.*, 2006). Reddy *et al.* (2011) investigated the effect of multiple stresses on plants to find the type of Ca^{2+} signatures and differences when those evoked by individual stresses. They suggested that Ca^{2+} and Ca^{2+}/CaM regulate transcription factors that play a key role in suppressing inappropriate activation of plant defence and stress adaptation responses both as positive and negative regulators of stress responses.

White and Broadley (2003) reported that increase in free calcium concentration can stimulate the internal cellular activities of CaM. Apart from binding free calcium within the cell, calmodulin can regulate free cellular calcium through actively extruding Ca^{2+} through stimulating Ca^{2+} pumps located in the plasma membrane. It is estimated that CaM can increase Ca^{2+} pumping by up to 10 fold (Clapham, 2007). Schmitz-Eiberger *et al.* (2001) observed that binding calcium with calmodulin boosts the plant natural resistance and immune system in plants and neutralises free radicals (O^{2-}) and converts them into oxygen molecules (O_2). In this case, calcium bound in the cell membranes plays a more important role than the free calcium in the cell plasma.

Watillon *et al.* (1995) described the sequence of the gene corresponding to calmodulin-binding protein kinase cDNA from apple: the 3074 bp sequence presented covers the complete coding region interrupted by four introns, as well as 742 bp of DNA located upstream of the ATG and 361 bp of the 3'untranslated region. Yang *et al.* (2012) observed calmodulin specifically binds to the putative targeting site in a calcium dependent manner. Their study indicated that gene expression levels are differentially regulated mainly by development signals as well as by ethylene. These genes encode calcium/calmodulin-regulated transcription factors. They suggested genes act as ethylene-mediated and calcium- mediated signals, regulating fruit development and ripening.

Fukumoto (2012) indicated to the role of Ca^{2+}/Calmodulin in binding further activates of the plasma membrane by modulating Ca^{2+}–ATPase, leading to enhanced calcium binding. Ca^{2+}–ATPase plays roles in the uptake and transport of calcium from the apoplast to the symplast through all the barriers and as an enzyme in eukaryotes extrudes calcium to the extracellular space in order to restore intracellular calcium to very low levels (Salmi *et al.*, 2011)**.** Burmeister and Dilley (1994) concluded that involving a Ca^{2+}–ATPase interferes with the role of calcium as a second messenger which is related to Ca^{2+}/CaM linked phosphorylation of the enzyme, this activity can increase calcium binding capacity 20-30 fold. In addition, De

Freitas *et al.* (2012) indicated that low levels of water-soluble apoplastic Ca^{2+} increases plasma membrane leakage and high expression of putative vacuolar Ca^{2+} transporters and Ca^{2+}ATPases in fruit tissue.

Ohno *et al.* (1984) suggested that calcium protease arose from the fusion of genes for proteins of completely different function and evolutionary origin. Further, it provides functional insight into cellular regulatory mechanisms mediated by Ca^{2+} through calcium-binding proteins. Anderson *et al.* (2004) indicated that the calcium protease is an alkaline metallo-protease and activity of the extracellular protease is optimal at pH 9 and inhibited by zinc- or calcium-chelators. Emuri *et al*. (1986) studied the gene structure of calcium-dependent protease and noticed the similarity to calmodulin-like calcium-binding domain. They suggested the four calcium-binding regions of calcium-binding proteins like calmodulin arose by two steps of gene duplication. Solomon *et al.* (1999) showed that activating oxidative stress in plant induced a set of cysteine proteases. They concluded the activation of the calcium proteases was instrumental in binding calcium and programmed cell death.

Marcelle (1990) indicated to the influence of the activity of enzymes involved in fruit respiration and storage conditions in development of bitter pit. Lipoxygenase (LOX) activity increases during storage. Wińska-Krysiak and Łata (2010) reported that expression of the genes in fruit depend on the cultivars and sampling time, with a negative correlation between lipoxygenase activity and calcium content, but a positive correlation between K:Ca ratio and lipoxygenase activity. Furthermore, Wińska-Krysiak and Łata (2010) reported a decrease in lipoxygenase activity after storage in apples without symptoms of bitter pit.

1.9.1.1 Summary of key actions of calmodulin and the relationship with bitter pit:

1) Calmodulin as a calcium binding messenger protein expressed in all eukaryotic cells and is one of the best characterised calcium sensors in plants and animals.
2) Calmodulin plays a key role in the regulation of intracellular enzymes and physiological processes and maturity that acts as an intermediate messenger protein that can interrupt calcium signals by binding calcium ions, thus modifying its interactions with target proteins.
3) Calmodulin can regulate free cellular calcium through actively extruding Ca^{2+} through stimulating Ca^{2+} pumps located in the plasma membrane.
4) Calmodulin regulates transcription factors that play a key role in plant defence and stress adaptation responses both as positive and negative regulators of stress responses.

5) There are evidences that calmodulin concentration was 40% higher in the pitted apple fruits than in the sound ones.

AIMS AND OBJECTIVES

The aim of these investigations was to develop further insights into diagnostic methods for predicting or identifying bitter pit formation and calcium distribution and activity in Bramely's Seedling apples. These methods provided data to diagnose incidence of bitter pit in earlier stages to develop a prediction model to aid the identification of consignments of fruits with a high predisposition for developing bitter pit during storage.

The main objectives were:

1) Application of 1-MCP (SmartFresh^{SM}) and investigation on the effects on incidence of bitter pit in order to design an applicable prediction model for 1-MCP treated fruit.

2) Development of prediction models based on biochemical analysis, including measuring organic acids and sugars, and mineral analysis with emphasis on understanding the distribution of calcium in fruit cortex and the development of bitter bit.

3) Measurement of chlorophyll fluorescence as a non-destructive method to find the optimum harvest maturity of fruit to reduce the incidence of bitter pit and investigating changes of chlorophyll fluorescence followed by stress and incidence of bitter pit during storage.

4) Identification of encoding genes in cell wall disassembly especially calmodulin gene, which have a causative role in bitter pit development and quantification by qPCR to develop a diagnostic method of identifying bitter pit in early stages after storage.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample collection:

In order to capture sufficient numbers of apples suffering from bitter pit during storage an extensive sampling of commercial orchards was required at harvest with follow up surveys conducted during the period of CA storage. With a large range of orchards and stores under investigation it was important to select samples where direct comparisons could be made between orchards and store regimes to reduce the variability. Samples were collected in four consecutive harvesting seasons (2010/11), (2011/12), (2012/13) and (2013/14). Access to a large number of orchards in south east of England (Kent) was facilitated by Landseer Ltd, service provider for 1-MCP (SmartFresh^{SM}) in the UK and Eire. Following up monitoring of fruit quality in store was aided by Landseer Ltd and conducted to industry standards (Best Practice Guide 2002).

In 2010/11 season, Bramley apples were collected from 90 commercial orchards in Kent over a five week harvest window between 20th of August until 30th of September 2010. Apples from these orchards were stored on commercial farms in two regimes: 9% CO_2, 12 % O_2 and 5% CO_2 and 1% O_2 kept at 4-4.5°C. In these stores apples were treated with 1 µL L^{-1}1-MCP (SmartFresh^{SM}) for 24 hours when fruit had been cooled to below 10°C. Each storage sample consisted of a net containing of 20 apples randomly selected. For each store, 8 nets of 20 apples were collected and half of them treated with SmartFresh^{SM} (SF), the rest remained untreated. Nets of treated and untreated apples were removed at approximately 2-3 month intervals and used for monitoring of fruit quality during the storage season (Figure 2.1).

A  B 

Figure 2.1: A) collecting samples from orchards (20 apples in each net), B) Treatment with 1-MCP (SmartFresh^{SM}) in commercial store (sample nets were separated from fruit in the bins).

On removal from store, single nets of treated and untreated Bramley apples were transferred to the postharvest laboratory for quality assessments included measuring fruit diameter, fruit colour, ethylene concentration ($\mu L\ L^{-1}$), fruit firmness (N/m^2) and assessed for the presence of external (scald, CO_2 injury, external bitter pit and incidence of disease) and internal physiological disorders (bitter pit, low-temperature breakdown, core flush). Assessments were performed on 10 apples immediately "Ex-store" and followed by further assessments of 10 apples following 7 days at 18°C of shelf life.

Ten apples were cut equatorially and samples of cortex from the inner carpel region, outer cortex and calyx and stalk end) were taken using a cork borer. Discs were immediately frozen (Figure 2.2) by using either dry ice (CO_2) or frozen in liquid then stored at (-80ºC) for molecular and chemical analysis.

In storage season 2010/11, 700 samples were collected from November 2010 to June 2011. In apples showing symptoms of bitter pit, tissue from affected and non-affected region were selected and frozen.

A  B 

C  D 

Figure 2.2: A) slicing apples B) collecting tissues from different parts C) apple with severe bitter pit (BP), D) freezing samples in dry ice in small tubs.

Bitter pit assessment:

Incidence of bitter pit was categorised in three groups as slight, moderate and severe (Figure 2.3) and percentage of incidence of bitter pit was calculated for 10 apples by this formula:

$$\%BP = 100 \times \frac{[(1 \times number\ of\ slight\ BP) + (2 \times number\ of\ moderate\ BP) + (3 \times number\ of\ severe\ BP)\ ]}{30}$$

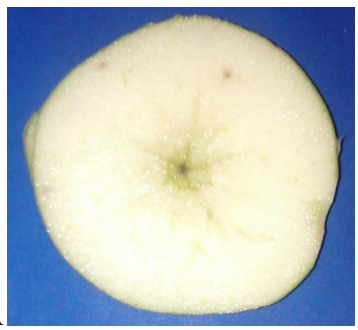
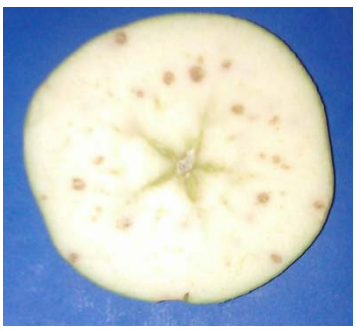
A  B  C 

Figure 2.3: Classifying severity of incidence of bitter pit in three groups: A) slight, B) moderate, C) severe.

Collection of samples continued during the 9-10 months of CA storage. Samples of fruits exhibiting symptoms and symptomless apples were sampled for comparative analysis. From the initial batch of 700 frozen samples a selection of 88 samples showing bitter pit and some symptomless samples were retained for biochemical and transcriptomic analysis (Appendix I). Figure 2.4 schematically shows the process of sample collection in season 2010/11.

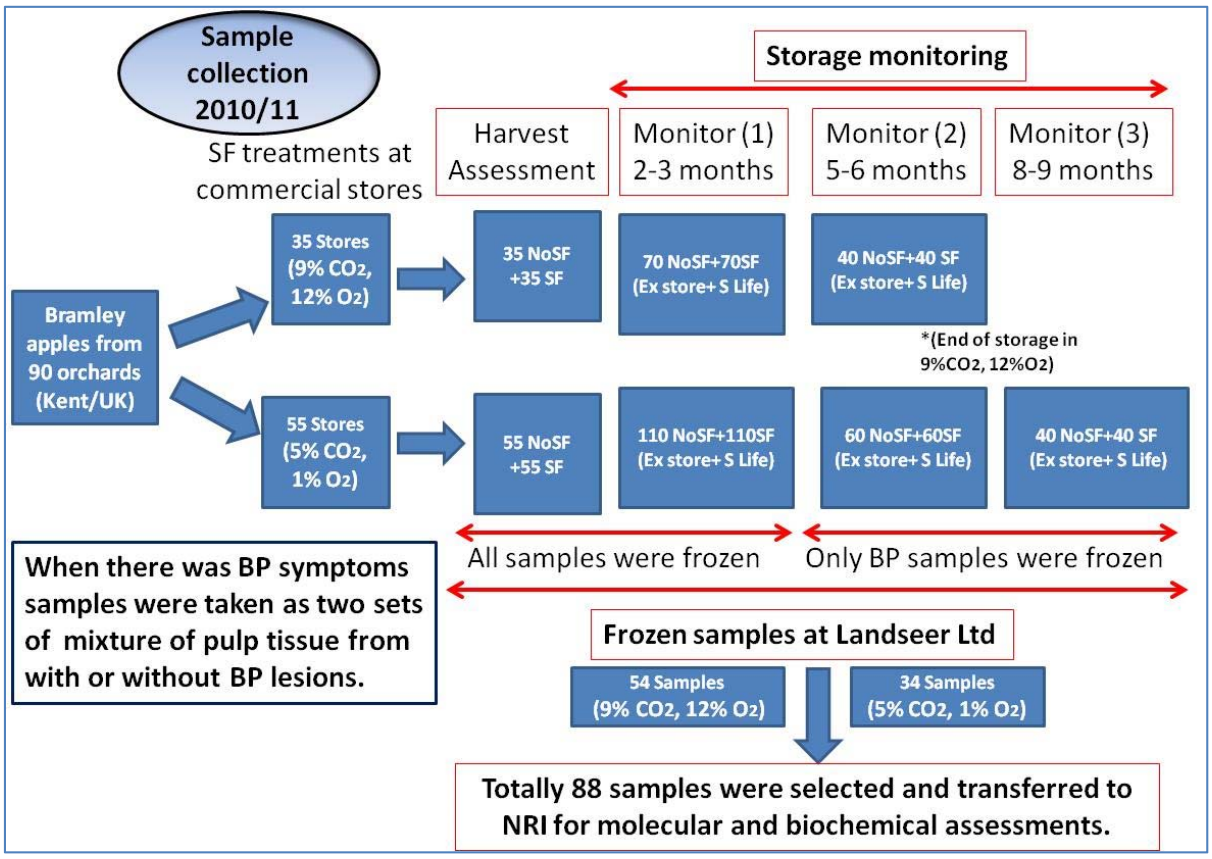


Figure 2.4: Schematic process of sample collection in season 2010/11 and selection of untreated and SF-treated samples with or without bitter pit from two different CA storage regimes. Length of storage regime in 9% CO_2, 12% O_2 was shorter than the regime 5% CO_2, 1% O_2.

In season 2011/12, 60 orchards previously surveyed in 2010/11 were surveyed and samples collected at harvest and during CA storage. A total of 500 samples of apple cortex (+/- SmartFresh^{SM}) were collected frozen. Cortex samples from inner and outer regions of apple fruit taken from the equatorial slice were collected and frozen separately to track changes in the distribution of minerals and transcriptome analysis. All samples were assessed for internal and external quality before being stored at -80°C. Samples (96) of apple juice from apples expressing bitter pit and healthy apples were collected and frozen (-20°C) for organic acids and sugars (HPLC). List of 96 frozen samples is in Appendix II.

Two additional orchards identified as susceptible and resistant to bitter pit development were subject to more detailed study. Bramley apples were harvested over four different dates covering four intervals over a four week period to capture early, optimum and late maturities of fruits. Apples were stored together in air (21% O_2) at the PQC (Produce Quality Centre- East Malling Research) at 4.5°C allowing for direct comparison of orchards under the same storage conditions. Apples were not treated with SmartFresh^{SM} to allow the maximum expression of bitter pit. Apples quality was monitored monthly over a period of four months. A list of samples is detailed in Appendix III. Figure 2.5 schematically shows the process of sample collection in season 2011/12.

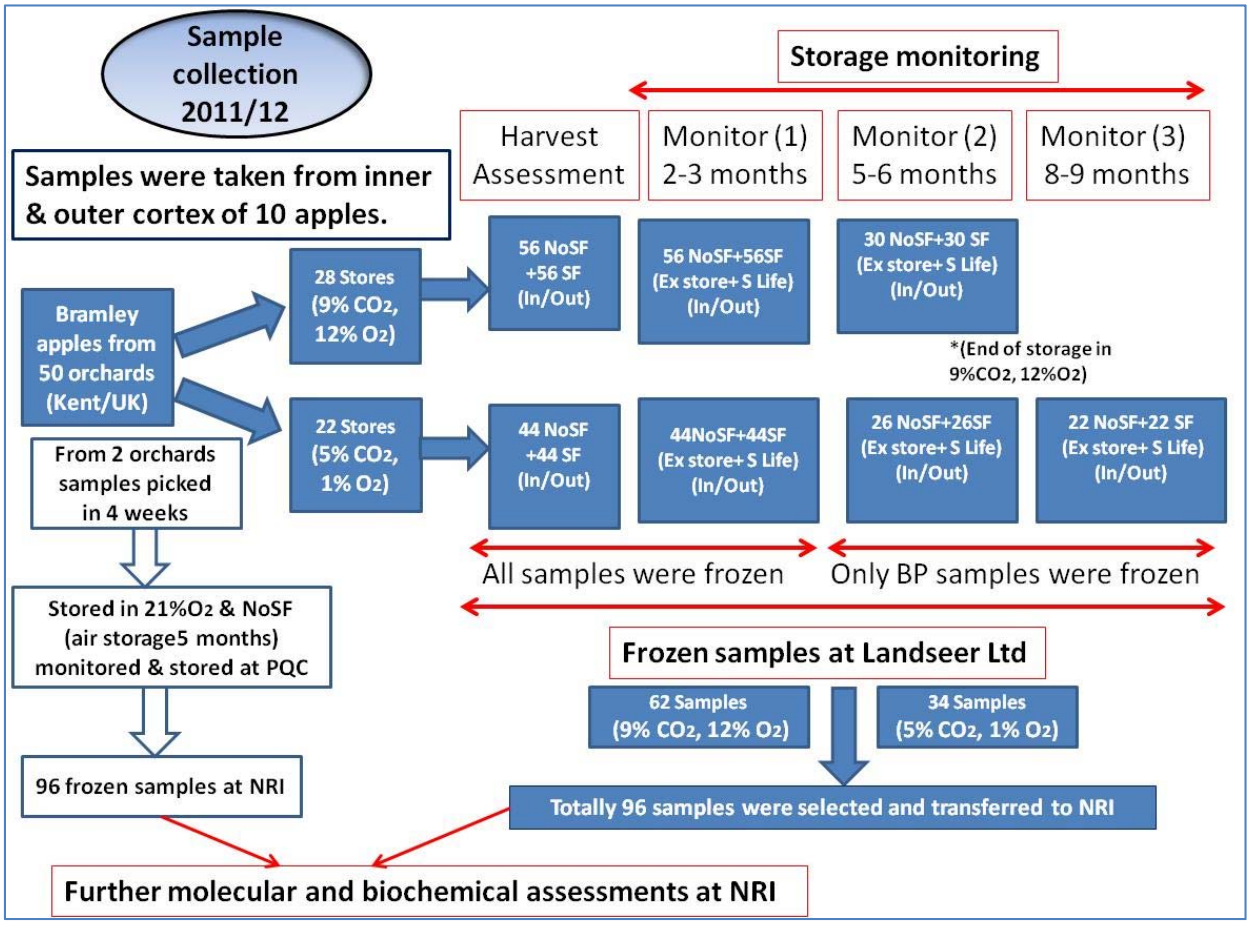


Figure 2.5: Schematic diagram of sample collection in season 2011/12 stored in two different CA storage regimes (9% CO_2, 12% O_2 or 5% CO_2, 1% O_2) and untreated samples of two orchards stored in air (4-4.5°C). Samples were taken from untreated and/or SF-treated samples and from inner and/or outer cortex of 10 apples representing each sample.

Fruit quality measurements included internal ethylene concentration (IEC) by gas chromatography (GC-FID), background colour by colour meter (Minolta), firmness (Lloyd LRX) and total soluble solids (%Brix) by refractometer. Thereafter, samples were snap-frozen in liquid nitrogen and stored in -80°C for future analysis at NRI.

In the season 2012/13 there was a detailed study of four orchards; two that consistently produced fruit with a high propensity to develop bitter pit during storage were selected alongside two orchards where fruit remained free from bitter pit. All samples were harvested on the same day and stored together in the same condition. Samples from the final picking date (commercial harvest date) were treated with SmartFresh^{SM} (1 $\mu L\ L^{-1}$ 1-MCP) in a 360 L cabinet for 24 hours at 4.5°C at the PQC (Produce Quality Centre- East Malling Research).

After treatment, samples were stored in three different storage regimes: in air (4-4.5°C) stored at PQC, additional samples were transferred to two commercial CA stores (5% CO_2, 1%O_2 and 9% CO_2, 12%O_2). Air-stored samples were monitored at monthly intervals (Appendix IV). CA stored samples were monitored in November 2012 and two 3 months intervals until June 2013. Fruits were assessed for external and internal quality as described earlier but with the addition of chlorophyll fluorescence measurements as a non-destructive aid to determine fruit maturity. Chlorophyll fluorescence used to investigate the relationship between bitter pit and changes in chlorophyll fluorescence in the apple peel. Samples of apple cortex were taken from the inner and outer apple cortex and frozen and stored at -80°C for further molecular and biochemical analysis. Figure 2.6 schematically shows the process of sample collection in season 2011/12.

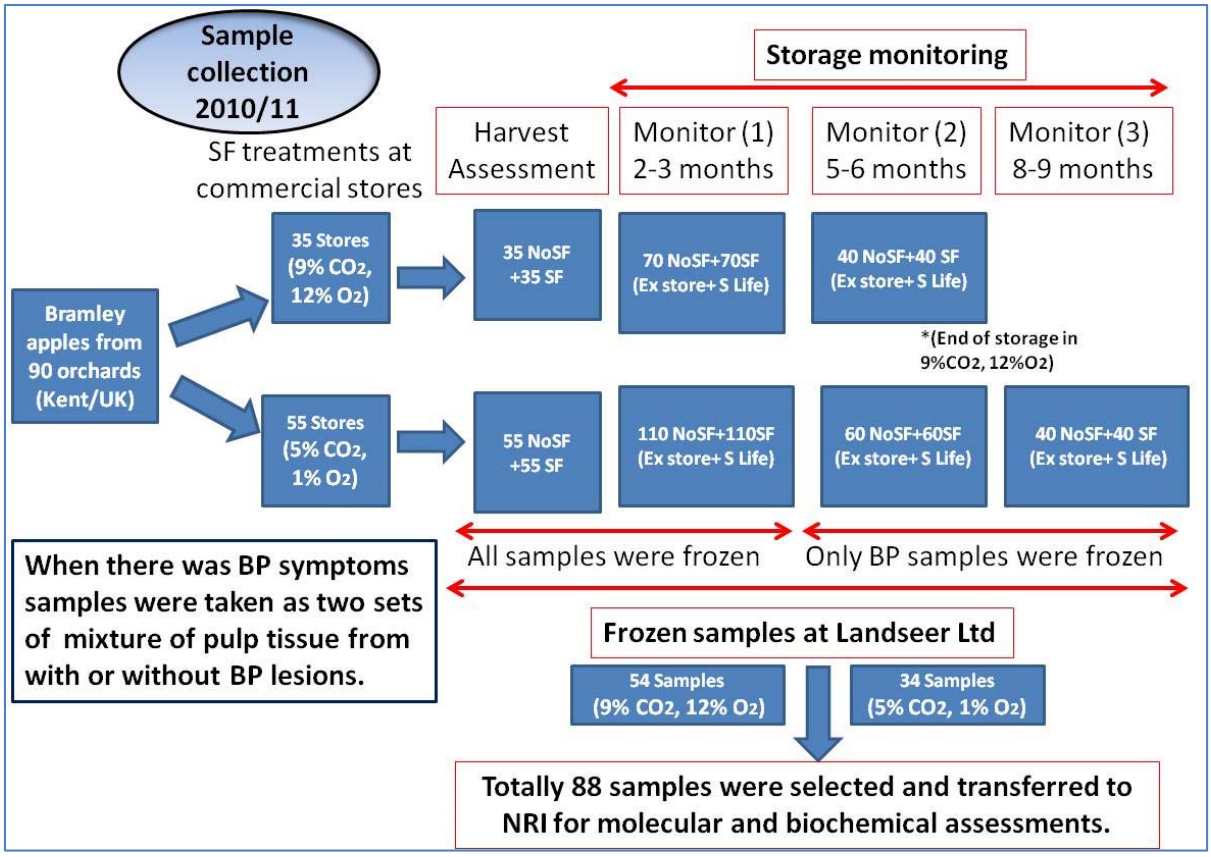


Figure 2.6: Schematic diagram of sample collection in season 2012/13. Samples collected from four selected orchards stored in air and CA regimes as untreated and SF-treated samples and were taken from inner and outer cortex of 10 apples representing each sample.

In the last season 2013/14 two orchards, one susceptible and one resistant, were surveyed and samples were collected. Bramley apples were picked from both orchards on the same day and stored in air (4-4.5°C) at PQC. More extensive monitoring of chlorophyll fluorescence was taken in the final year allowing seasonal changes in chlorophyll fluorescence profiles to be assessed. Moreover, chlorophyll fluorescence assessment was limited to a narrow window; starting one month after harvest then every two weeks for two months. Samples were treated with SmartFresh^{SM} (1 µL L^{-1} 1-MCP) in a 360 L cabinet for 24 hours at 4.5°C at PQC.

Totally five boxes (20 apples) for each treatment was labelled with the dates of assessments. On each assessment date all samples were assessed for chlorophyll fluorescence, then the samples related to that assessment date were assessed for external and internal quality tests then like previous seasons' juice and frozen tissue samples were collected (Appendix V).

Another difference of this trial was collecting samples from calyx/stalk end of apple fruit instead of inner/outer cortex tissues. Cortex samples from the stalk and calyx region were frozen

in liquid nitrogen and stored at -80°C at NRI for biochemical and transcriptomic analysis. Figure 2.7 describes schematically the process of sample collection in season 2013/14.

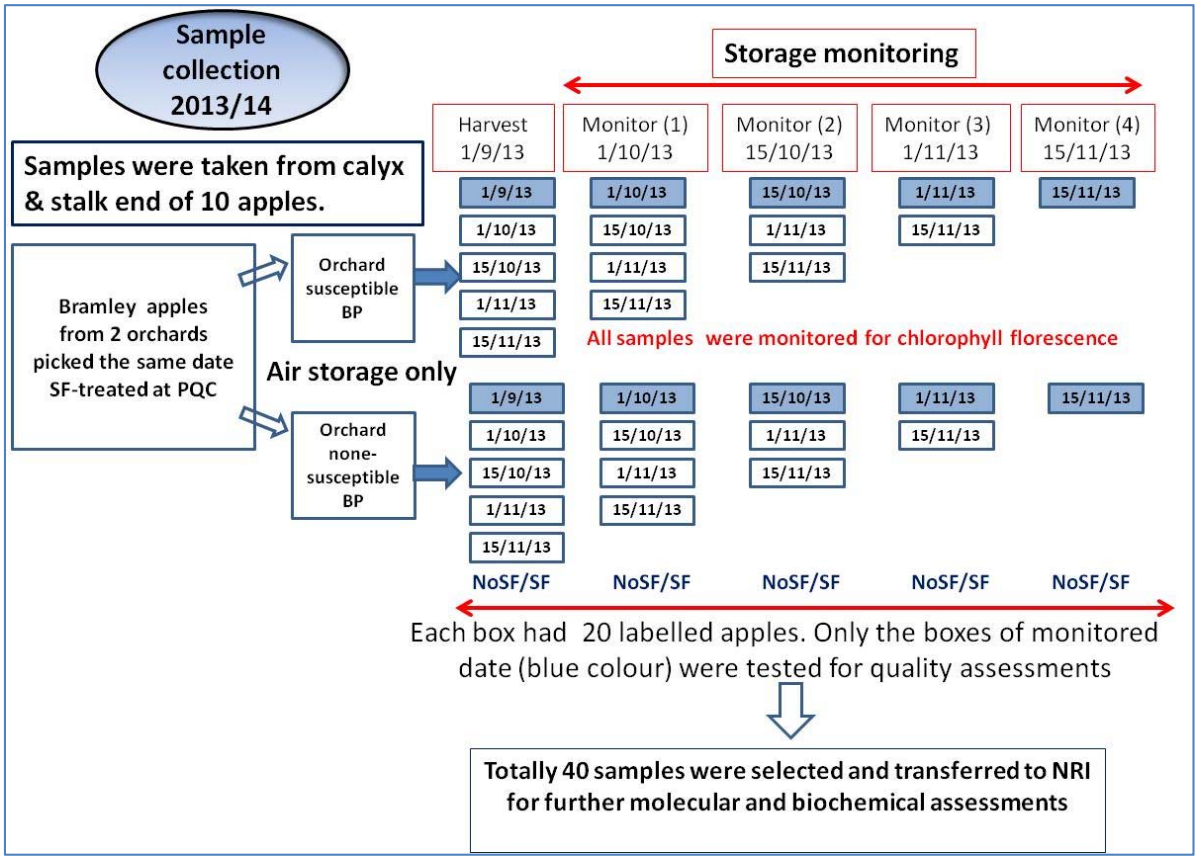


Figure 2.6: Schematic diagram of sample collection in season 2013/14. Samples collected from two selected orchards stored in air regime as untreated and SF-treated samples. The same samples were monitored for chlorophyll fluorescence changes for two months by keeping labelled apples in separate boxes. Samples were taken from calyx and stalk end of 10 apples representing each sample.

2.2 Fruit quality assessments:

2.2.1 Ethylene measurement:

Internal ethylene concentration (IEC) was measured by GC-FID. A 1 mL glass syringe with a 21 gauge hypodermic needle was inserted into the core cavity of each apple (Mousedale and Knee 1981). Apples that had an open calyx were discarded. The end of the needle was bent over to prevent blockage by the tissue. Approximately 0.8 mL of the apples internal atmosphere was withdrawn and the syringe was transferred to a 25 gauge needle and 0.5 mL was injected into the GC. Then injection port heated to 130ºC on a gas chromatograph (ATI-Unicam 610 series). Eluted peaks were integrated using a Delta integrator (Delta Data Systems) and a

concentration of ethylene was calculated in nL L^{-1}. A standard ethylene gas mixture (860 nL L^{-1}) supplied by (BOC, UK) was used to calibrate the GC at the beginning of each run. The limit of detection was 2 nL L^{-1}.

2.2.2 Fruit firmness:

Fruit firmness was measured by the fruit texture analyser in N/m^2. Fruit texture analyser (Lloyd LRX, UK) based at the PQC measuring force as Newton (N/m^2), and the other one at Landseer Ltd (Guss, South Africa) measuring in Kilogram (Kgf/cm^2). All measurments recorded and converted to N/m^2. A slither of apple peel was removed from opposite sides of fruit before measurment.

2.2.3 Background colour:

The background colour of skin of an individual apple fruit was determined by the use of CR400 (Minolta, Japan). The colour parameters measured are lightness (*L**), chromaticity of red/green *(a**), and chromaticity of blue and yellow (*b**). The degree of greenness as *a** value and *b** value which represented the degree of yellow colour (changes from green to yellow colour) of skin of Bramley were measured. The mean of two readings around the equatorial axis of the fruit was calculated and recorded.

2.2.4 Total soluble solids (%Brix):

The amount of total soluble solids was recorded by a digital refractometer (Model AR 200, Reichart ophthalmic instruments, USA). The apple juice was squeezed by using a juicer (Chylofel, France) and juice of all 10 samples mixed and measured by the refractometer as an indication of the percentage of content of water-soluble solids in fruit juice (%Brix). The rest of the apple juice was frozen and stored at -20°C for later measurements of organic acids and sugars by High Performance Liquid Chromatography (HPLC).

2.2.5 Starch:

Another parameter which is measured as a quality assessment in other cultivars of apple is measuring changes in starch especially a few weeks before harvest to find the best maturity of the fruit (Elgar *et al*., 1999). However Bramley's seedling as a cooking apple is picked unripe so starch measurement is not applied.

2.3 Analysis of minerals, organic acids and sugars:

2.3.1 Organic acids and sugars:

The juice samples were thawed on ice before vortexing and 2 mL poured into 2 mL centrifuge tubes and centrifuged at 4000 ×*g* at 4°C for 4 minutes. The supernatant was filtered through 0.45 µl syringe filters (Chromacol Ltd, UK) into glass HPLC vials. This process was separately for acids and sugars. Samples were stored at -80°C freezer. The volume of 10 µL was injected into an Agilent 1200 series HPLC (Agilent, UK) with a Zorbax Carbohydrate Analysis column (Agilent, UK). Both mobile phase and buffer solution had flow rate of 1.0 mL min^{-1}. The mobile phase consisted of 75% of acetonitrile (Fisher Scientific Ltd, UK) and 25% water. The concentration of fructose, glucose and sucrose was determined according to external fructose, glucose and sucrose standards (Sigma-Aldrich, UK).

Organic acids were extracted and analysed using a method described by Nour *et al.* (2010) with some modifications. The extracts were microcentifuged at 9,300*g* for 5 min, then 500 µL was transferred into new 1.5 mL Eppendorf tube and mixed thoroughly, with an equal volume of 1% (11 mg/mL w/v in 1 M K_2HPO_4/H_2O (1/4, v/v)). Citric acid, malic acid, oxalic acid and ascorbic acid (Sigma-Aldrich, UK) were used to make a standard stock solution with acetonitrile, potassium dihydrogen orthophosphate and phosphoric acid (Fisher Scientific Ltd, UK). The mixed standard stock solution contained 1000 mg/L citric acid, 2000 mg/L malic acid, 300 mg/L oxalic and ascorbic acid. Samples were left for 40 min at ambient temperature (19°C), and microcentifuged at 9,300*g* for 5 min. Samples were transferred into HPLC vials. A 5 µL sample was used for HPLC analysis. The organic acids were separated by reversed phase chromatography. Samples were analysed using an Agilent 1100 HPLC (Agilent, UK) with a Luna 5 µm NH2 100 A column (250 mm × 4.6 mm) (Phenomenex, UK) at a flow rate of 1.2 mL min^{-1}. The amount of each organic acid was detected by absorbance and quantified with external calibration graphs.

2.3.2 Mineral analysis:

In seasons 2011/12 and 2012/13 selected frozen samples were sent to an accredited laboratory (Lancrop Laboratories, UK) for mineral analysis.

In 2013 mineral analysis was undertaken in collaboration with School of Science (University of Greenwich/Medway), measured by "Inductivity Coupled Plasma, Optical Emission Spectrometer" (ICP-OES) and "Atomic Absorption Spectrometer (AAS).

Frozen apple samples were ground in liquid nitrogen and 0.5 g of sample was added to 5 mL concentrated nitric acid (HNO_3 70%) and 0.5 mL hydrogen peroxide (H_2O_2) was added. Samples were digested in a microwave accelerated reaction system (CEM MARS 5/UK). The programme as maximum pressure 400 psi, power 1200 W for 20 minutes and maximum temperature 190°C were selected for digesting dissolving and hydrolysing a wide range of materials and preparing samples for analysis by atomic absorption spectrometer (AAS) or inductively coupled plasma optical emission spectroscopy (ICP-OES). The digested samples were diluted to 50mL with deionised water before preceding the analysis.

ICP-OES (PerkinElmer, UK) was calibrated using seven multi-element calibration solutions. ICP-MS standards for calcium, potassium, magnesium and boron (inorganic ventures, US) were prepared by diluting HNO_3 5% in 50 mL plastic volumetric flasks providing a serial dilution from 1000 ppm to 50 ppb, 100 ppb, 200 ppb, 400 ppb, 600 ppb, 800 ppb and 1000 ppb range of standards. A calibration curve was constructed over a range of seven concentrations using ICP-OES software. A threshold of 0.99 is required for a coefficient correlation of calibration curves for each element (Mindak, 2006). Mineral analysis data was subject to correction factors to take into account dilutions. The final mineral content was calculated as mg/100g fresh fruit.

As mentioned before in Chapter 1, it is important to distinguish between free and bound calcium. Since there is no published analytical method to measure free calcium (Ca^{2+}), a novel methodology was developed with Dr. Nazy Zand in School of Science (University of Greenwich) to apply inductively coupled plasma optical emission spectroscopy (ICP-OES) to measure total calcium by mass spectrometry and then quantifying the proportion of calcium bound to oxalic acids as oxalate by atomic absorption spectroscopy (AAS) which would provide information on the level of bound calcium. This method was developed successfully and applied to the samples from season 2013/14 and some selected samples from previous seasons (60 samples).

2.3.2.1 Protocol for calcium oxalate extraction:

The cold oxalate extraction method which was applied by Al-Wahsh and Wu (2012) was modified for measuring total oxalate of apple samples with their total Ca^{2+} measured by ICP-OES.

Frozen apple samples were ground in liquid nitrogen and 1 g of each sample weighed and 5 mL of 2N HCl was added then centrifuged at 4200*g* for 10 minutes. The supernatant was transferred to a 25 ml volumetric flask. This process repeated two more times by adding 2N HCl to

remained pellets in the tubes for extracting rest of oxalate and each time supernatant poured in the 25 ml volumetric flask. The final volume of the collected supernatant from three extractions was diluted to 25 ml with distilled deionised water. Oxalate extractions filtered with 20 mL syringes through 0.45µm filters (Millipore, US).

Before analysing foe extracted oxalate, the Atomic Absorption Spectrometer (AAS) (Thermo Fisher Scientific ICE 3300, US) was calibrated with five calcium calibration solutions. For preparing the calibration solutions a calcium standard (Inorganic Ventures, US) was diluted by 5% HNO_3 in 50 mL plastic volumetric flasks from 1000 ppm to 1 ppm, 2 ppm, 3 ppm, 4 ppm and 5 ppm.

The sample solution was aspirated and transformed into an aerosol; absorption spectrometry assessed the concentration of the calcium in the sample as calcium oxalate according to the calibration with the standard solutions to establish the relation between the measured absorbance and the calcium concentration. The value of calcium extracted from oxalate measured by this technique was subtracted from total calcium to find the amount of free calcium in each sample.

2.4 Chlorophyll fluorescence:

This method was applied only for samples collected in seasons (2012/13) and (2013/14) as a non-destructive method for assessment of harvest maturity and storage quality.
The fluorometer PEA pocket (Hansatech, UK) is a mobile device for measuring chlorophyll fluorescence, which is the re-emitted energy from the sample in the form of red/far-red radiation (Maxwell and Johanson, 2000). Analysis of the fluorescence signal assumes that the plant material starts in a dark state. This device was designed for measuring chlorophyll fluorescence of plant leaves and therefore is provided with leaf-clips which can be attached to the leaves of plant for dark adapting the sample. Since for this project the instrument was used on fruit (apple), it was essential to develop a method for dark adaptation on fruit. In order to prepare a method for dark adaption of the sample, different methods were applied and tested under different lighting conditions, including placing samples in a dark room or covering in a cloth bag to prevent light penetration. The most practical method, which was applied by Rees *et al.* (2005), was modified by holding the fluorometer machine with the attached clip against the surface of the fruit for 10 seconds and then exposing the fruit to the light. The role of the connected clip is to ensure that only a small area of the plant tissue is exposed to the excitation

light and at the correct intensity. Moreover, the difference of measurements in light and dark adapted conditions were achieved by applying the first measurement immediately (after one second) and holding the machine for dark adaption and doing the second measurement after ten seconds (Figure 2.7).

A  B 

Figure 2.7: A) The leaf-clip (the black circle) was attached to the PEA machine to conduct the expositor light just in the spot to the sample. B). For dark adaptation fluorometer was held on the fruit surface for 10 seconds prior to measurement.

The fluorescence data was downloaded from the device (PEA pocket) to the computer and converted by instrument software (PEA plus) into 57 characteristics of fluorescence.

During storage monitoring in season 2012/13 only samples which were assessed for quality assessments were assessed for chlorophyll fluorescence. In season 2013/14 it was decided to monitor the same samples and assess them for chlorophyll fluorescence changes as a non-destructive method and compare the chlorophyll fluorescence changes when bitter pit was observed. Samples of each orchard for each assessment date were kept in separate boxes (crates) and all apples were labelled (1-20) with the dates of assessments. On each assessment date all samples were assessed for chlorophyll fluorescence, then the samples related to that assessment date were assessed for external and internal quality tests and apples were sliced to check for bitter pit symptoms.

2.5 Transcriptomic analysis of genes regulating calcium homeostasis:

The isolation of good quality RNA, free from protein and polysaccharide concentration is essential for down-stream processing of transcriptomic analysis and gene cloning (Cheng *et al*., 1998). However, plant tissue and especially apple fruits are a difficult source from which to isolate high-quality RNA with good yield due to the presence of high amounts of polysaccharides and/or polyphenols that are released during cell division (Gasic *et al.*, 2004).

These compounds form complexes with nucleic acids during tissue extraction. Furthermore, the contaminated RNA is not suitable for cDNA synthesis, reverse transcription PCR amplification, in vitro translation, or gene expression (Rapley and Manning, 1998). A number of RNA extraction protocols were assessed to undertake the best method that yielded high quality RNA.

2.5.1 RNA extraction (method 1):

This protocol was developed by Gasic *et al.* (2004) as one of the most efficient methods for RNA extraction especially from apple fruit.

Diethyl pyrocarbonate (DEPC)–treated water was used for all solutions. The extraction buffer (all chemicals provided from Sigma-Aldrich/UK) composed of 2% CTAB, 2% polyvinylpyrrolidone (PVP) K-30 (soluble), 100 mM Tris HCl [pH 8.0], 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine (free acid) (HRS), 2% β-mercaptoethanol (added just before use) was autoclaved and stored at room temperature.

Approximately 5 g of frozen apple tissue was ground to a fine powder in a pre-chilled autoclaved pestle and mortar under liquid nitrogen. The powdered apple tissue (1g) was added to a 50 mL Oakridge tube containing 10 mL of extraction buffer pre-heated to 60°C in a water bath for 10 minutes. Samples were vortexed twice during incubation. An equal volume of chloroform-isoamylalcohol (24:1) was added and immediately vortexed for 2 minutes. Samples were transferred to pre-labelled, clean, and autoclaved 30 mL Oakridge tubes and vortexed followed by centrifugation at 10,000*g* for 10 min at 4ºC. Supernatant was transferred to a clean Oakridge tube. Re-extracted with an equal volume of Chl:Iaa. Centrifuge at 10,000*g* for 15 min at 4ºC. The supernatant (1.5 mL) was transferred to 2 mL (RNAse-free) microfuge tubes. White inter-phase layer and organic solvent were discarded. Followed by the addition of 0.5 mL of 7.5 M LiCl to each tube and mixed by inversion and stored at 4ºC overnight.

Tubes were centrifuged at 12,000 ×*g* for 30 min at 4ºC. Supernatant was discarded by pipetting and the pellets were air dried. Pellets were washed in 70% ethanol and air-dried for 5 minutes. RNA (pellets) dissolved in 200 µL DEPC-treated water and RNA was stored at -80ºC.

Total RNA was quantified using a spectrophotometer at A260 nm and A280 nm and precipitated in 1/10 volume of 3 M sodium acetate (pH 5.5) and 2.5 volume of 70% ethanol.

This RNA extraction method did not lead itself to high throughput extraction and the quality and concentration of RNA was low (see table 6.1 in chapter 6).

2.5.2 RNA extraction (method 2):

A modified RNA extraction method applied by Sangha *et al.* (2010) was developed as combining the current method and CTAB and RNeasy Mini Kit (Quiagen, Germany).

Approximately 200 mg of frozen apple tissue was ground in liquid nitrogen. CTAB buffer (100mM Tris-HCl [pH8.0], 20mM EDTA [pH8.0], 1.4 M NaCl) (500 mL) was prepared and 1 µL β-mercaptoethanol was added and incubated at 60°C for 10 minutes. Chloroform-isoamylalcohol (24:1) (500 µL) added and vortexed and centrifuged at 13500*g*. Supernatant was transferred to a fresh 1.5 mL micro-centrifuge tube and isopropanol added and stored at -20°C for 1 hour. Samples were transferred to an RNeasy Mini column pink (Quiagen/ Germany). Centrifuged for 15seconds (10000 ×*g*) and the flow-through discarded. To this 700 µL of RW1 buffer (Quiagen) was added to the RNeasy spin column and centrifuged for 15 seconds (10000*g*) and the flow-through discarded. Buffer RPE (500 µL) was added to the RNeasy spin column and centrifuged for 15 seconds (10000*g*) and the flow-through discarded (this stage was repeated another time). RNeasy spin columns were placed in a new 1.5 mL collecting tube and 30 µL RNase-free water was pipetted directly onto the spin column membrane and centrifuged for 4 minutes at 10000*g* to elute RNA.

This method facilitated quicker processing. However because of the limited amount of apple tissue (200 mg) that could be processed in the RNeasy mini spin columns, the quantity and quality of extracted RNA was low.

Since Bramley's seedling apple fruit cortex contains large cells with numerous air spaces, extraction methods that accommodate larger amount of fruit tissue were investigated.

2.5.3 RNA extraction (method 3):

The final method for extracting apple RNA was a method modified by Colgan (2002) originally from Bahloul and Burkard (1993). This method utilised a larger amount of starting material (10 g) and yielded a higher RNA concentration and improved quality was retrieved. However the procedure has more steps than previous methods. All tubes, flasks, funnels, beakers were treated with DEPC water (1% v/v). All plastic materials were treated with 1% [v/v] DEPC solution, as a strong RNAse inhibitor, for at least 12 hours. These materials were autoclaved before being used to remove traces of DEPC (Boztok and Cokuysal, 2006). Glassware was treated with DEPC and autoclaved before use.

These solutions were made up separately with DEPC H_2O (1% v/v), then autoclaved before making up the RNA extraction buffer, 3M Sodium acetate (pH 6), 0.5 M EDTA [pH 8], 4 M NaCl, 10% [w/v] SDS (Sodium dodecyl sulphate), 5 M Potassium acetate [pH 4.8].

2.5.3.1 RNA extraction (method 3-stage 1):

Using a preheated water bath (65°C), 100 mL of RNA extraction buffer was heated to 65 °C in a 250 mL flask for 10 minutes, to which 0.248 g of cysteine hydrochloride was added before adding 10 g of powdered sample. Solutions were incubated for 10 minutes with gently agitation. The solutions were filtered through 2 layers of autoclaved miracloth. Protein precipitation was induced by the addition of 50ml 5M potassium acetate [pH: 4.8]. The flasks were placed on ice box for 40 minutes, with occasional agitation. The solution was decanted into 3 DEPC-treated Oakridge tubes and centrifuged for 30 minutes at 48,000*g*. The supernatant was poured in 250 mL DEPC-treated 500 mL Nalgene bottle with equals volume of cold isopropanol cold (-20°C) followed by storage at freezer -80°C for 1-2 hours to precipitate nucleic acid present in the sample.

Nucleic acids were pelleted from the solution by centrifugation (48000*g*) using DEPC-treated Oakridge tubes for 30 minutes. After centrifuging supernatants were discarded and pellets air dried for 10 minutes. A total of three replicate tubes per sample were used. Nucleic acid pellets of each sample were resuspended in a total volume of 8 mL of DEPC H_2O and stored in a 15 mL Falcon tube and stored at -80°C prior to the purification stage.

2.5.3.2 Final RNA extraction (method 3-stage 2):

Buffers for this stage were prepared separately as: **R6** (2M NaCl, 250 mM MOPS[pH 7]), **QAT** (400 mM NaCl, 50 mM MOPS, 0.15% [v/v] Triton X 100, 15% [v/v] absolute ethanol [pH 7]), **QA (**400 mM NaCl, 50 mM Mops, 15% [v/v] absolute ethanol [pH 7]), **QR** (1.2 M NaCl, 67 mM Mops, 20% [v/v] absolute ethanol [pH 6.7]) and **QRU** (6M Urea+QR).

The samples from the first stage of extraction (contained 8mL DEPC mixed with pellets) were amalgamated by 2mL of R6 buffer and were allowed to thaw on ice. Six Qiagen tip-100 columns (Qiagen, Germany) were placed on 150 mL flasks and 3 mL QAT buffer added to each column to activate columns. After thawing samples were decanted into 10 mL sterile-plastic syringes fitted with 25 gauge hypodermic needles. Samples were passed through the needle (to break up aggregates) on to the columns. Samples in columns were washed 3 times by adding 10 mL of buffer QA. The speed of filtration was increased by adding consistent low pressure behind the solution by suction pump or by applying force manually to the syringe plunger (Figure 2.8).

Figure 2.8: Process of the second stage of RNA extraction by filtering solutions through Qiagen tip-100 columns. Speed of filtering increased by adding consistent low pressure behind the solution by suction pump or manually by air pressure force of a 20 mL syringe.

Columns were placed on top of DEPC-treated glass 30 mL Nalgene tubes. An elution buffer (QRU) was prepared by addition of 6M Urea+QR, 10 mL QRU added to each column. Total RNA eluted through columns, RNA was re-precipitated by adding 10 mL Isopropanol (-20°C) to the tube and kept in the fridge (4°C) for 1 hour.

Tubes were centrifuged for 30 minutes at 11500 ×*g*. Supernatant was discarded and pellets air dried for 10 minutes. Pellets were re-suspended in 200 µL DEPC H_2O and stored in 1.5ml tubes. The concentration of RNA and the ratio of A260/A280 were quantified by NanoDrop 2000 (Thermo Scientific, US), and samples were stored at -80°C.

2.5.3 Quantification of RNA:

The concentration of extracted RNA was measured by spectrophotometer NanoDrop 2000 (Thermo Scientific/US) by placing a 1 µL drop of RNA solution in the sample port of the analyser and measuring as ng/µL. Absorbance at A260 (nucleic acid) and A280 (protein) was estimated alongside the ratio A260/A280. A ratio of 1.7 to 2.0 indicates RNA preparations free of contaminating proteins**.** The concentration and purity of RNA extracted by method-3 was higher than other extraction methods. The quality of the RNA extracted was assessed by running samples on gels (see table 6.2 in chapter 6).

2.5.4 Qualification of RNA by gel electrophoresis:

1.2% [w/v] agarose gel was prepared by the addition of 1.2 g agarose+100ml 0.5×TBE (Tris Borate EDTA) buffer 5% (Brody and Kern, 2004) was poured in 250 mL flask and dissolved by heating in a microwave oven for 2 minutes to dissolve. After cooling to 40°C, the agarose solution was poured into a gel tray until the comb teeth were immersed in the solution. The thickness of gel should be less than 0.5 cm since thick gels may decrease sensitivity.

A 10 µL aliquot of RNA solution was mixed with 5 µL loading dye (2.5%) in 0.25 mL PCR tubes (0.2 mL) and the sample was denatured at 95°C for 10 minutes in a thermocycler in RT-PCR, on removal, samples were placed on ice.

Gels were immersed in a 0.5 x TBE gel buffer and loaded with samples alongside a 1 kb marker (Invitrogen, UK). Gels were run at 110 mA (milliamps) for 20 minutes, allowing sufficient time for the major RNA sub-fragments (18 S, 25 S and 5S) to be separated.

The gel was stained in 10 mg/mL Ethidium Bromide (EtBr) solution for 25 minutes. After a brief (3 minutes) destaining in water, gels were placed in a gel imaging suite (Syngene G: Box, UK) by using the software (Synoptics Group, UK) to capture the image.

In later experiments (from 2012), Ethidium Bromide was substituted with a safer product "RedSafe™" as a nucleic acid staining solution (Intron Biotechnology, UK) for detecting nucleic acid in agarose gels. Gentry *et al.* (2011) highlighted that Ethidium bromide as a mutagen could cause deformation in DNA which has a health risk and may interfere with replication of mitochondrial DNA in some human cell lines. "RedSafe™" was added at a rate of 5 µL to 100 mL agarose solution after removing the flask from microwave and swirl the flask gently to mix the solution and pouring into the gel tray after cooling. After finishing electrophoresis, the gel was placed in a gel imaging suite directly.

2.5.5 cDNA library:

RT-PCR (Reverse transcriptase-PCR) analysis of mRNA as a qualitative method to detect gene expression requires the creation of cDNA (complimentary DNA) transcripts from RNA. For cDNA synthesis 1µg of RNA was used as a starting material, cDNA synthesis was performed using QIAGEN (QuantiTect Reverse Transcription Kit). Genomic DNA removal was done by the addition of 2 µL of gDNAse for samples, then were heated in a PCR block to 42°C, for 3

minutes at 95°C then 4°C. cDNA was stored at -20°C or used directly for down-stream PCR reactions.

2.5.6 Primers:

Primer sets used for gene detection are shown in Table 2.2. In previous studies Colgan (2002) has tested primers Ca^{2+} ATPase, Ca^{2+} Protease, lipoxygenase, calmodulin and the housekeeping primer "ITS1" in apple and fragment were cloned and sequenced. ITS1 (Internal Transcribed Spacer) refers to a piece of non-functional RNA situated between the 18S and 5S regions of ribosomal RNA (rRNA). Pieces are excised and as non-functional maturation by-products rapidly degraded (Kim *et al.,* 2008). Lipoxygenase enzyme is involved in a number of diverse aspects of plant physiology including growth and development. There are more details about Calmodulin and Ca^{2+} ATPase in literature review (chapter 1: 1.9.1).

Table 2.2: Primers which were used for gene detection and amplification.

Primer	Sequence	Melting point
ITS forward	5'CGTCGTCGTCTTCGATAAGTCA3'	66°C
ITS reverse	5'GGATTCTGCAATTCACACCAAGT3'	66°C
Ca^{2+} ATPase forward	5'CCCAAGAACTCGATGATTATG3'	60°C
Ca^{2+} ATPase reverse	5'GCGGCCATGTTTGATTCT3'	54°C
Lipoxygenase forward	5'GGCCGTAAATGACTCTGGAA3'	60°C
Lipoxygenase reverse	5'CCACGCTTAGCTGCCTATTC3'	62°C
Ca^{2+} Protease A forward	5'CCTTTTTCCTGGAAATG3'	48°C
Ca^{2+} Protease A reverse	5'CCATTTGTCCATCTCTCTTGC3'	56°C
Ca^{2+} Protease B forward	5'CCTTTTTCCTGGTGGAAATG3'	58°C
Ca^{2+} Protease B reverse	5'CCATTTGTCCATCTCTCTTGC3'	62°C
Calmodulin A forward	5'AGAGATTTTAGGCAGAGGAGGA3'	64°C
Calmodulin A reverse	5'AATCTAGCTGGCTCGGCTCT3'	62°C
Calmodulin B forward	5'CCGGGTACCCACCTTTTATT3'	60°C
Calmodulin B reverse	5'TGAGGAGGCTCGAAATCAAT3'	56°C
Calmodulin C forward	5'CCGGGTACCCACCTTTTATT3'	60°C
Calmodulin C reverse	5'GTCAGGGTCGACTTTGAGGA3'	62°C
Calmodulin D forward	5'CAAGAAACAAGAAGACTTGCAGA3'	64°C
Calmodulin D reverse	5'CTGGCTCGGCTCTTAATGAC3'	62°C

2.5.6.1 Preparation of primer stocks:

Primers supplied by Invitrogen (UK) as freeze-dried powders were re-suspended using Hypure water (deionised water for nucleotides such as RNA or DNA) to form a stock solution (200 µM), a further dilution to 15 µM was performed to prepare a working primer concentration. For PCR reactions, Platinum PCR Supermix (Invitrogen, UK) was used: 2 µL template (cDNA sample) was mixed in 0.2 mL PCR tube with 12 µL PCR Mix and 1 µL Hypure water and 1 µL of 15 µM forward and reverse primers. Annealing temperatures for each pair of the primer was optimised. A starting point for determining optimum annealing temperature was based on the melting points of the individual primers using the following formula where CGAT refer to the nucleotides cytosine, guanine, arginine and thymine, melting point=[(C/G×4)+(A/T×2)]-5 (Gundry, 2003). A general PCR programme was 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 55°C to 60°C (depending on melting point of primer) for 1 minute and 68°C for 1 minute, with a final extension stage of 68°C for 10 minutes. Distinct PCR products amplified fragments separated by gel electrophoresis were excised from the agarose gel under UV light and frozen at -80°C for later extraction and cloning bands and sequencing. In this study it was decided to focus on quantitative gene expression by qPCR, so the high quality bands which were collected and frozen in this stage were not used for cloning and sequencing and could be used in future work.

2.5.7 Real Time PCR (qPCR):

Quantitative real-time PCR (qPCR) is the method of choice for rapid, easy to use and reliable quantification of mRNA transcription. The qPCR (Eppendorf Mastercycler ep realplex, US) was used to determine the relative expression of calmodulin, Ca ATPase, calcium protease and lipoxygenase genes. Each sample was replicated 3 times on each 96 well qPCR plate. ITS house-keeping primers were tested alongside genes under investigation, control wells containing water (background control) and those where the template was replaced with water (negative control). cDNA (70 ng/µL) were diluted 1:4 to form a working concentration of 17.25 ng/µL. Reagents for qPCR were prepared as follows: 4 µL Template (cDNA) + 1.35 µL Forward primer + 1.35 µL Reverse primer + 5.8 µL Hypure water + 12.5 µL Master Mix (Sybr Green) to form a total volume of 25 µL per sample. Samples were pipetted into individual wells and plates were sealed with cellophane lid and heat sealed before using in qPCR.

The general qPCR thermocycling programme was as follows: at 95°C for 15 minutes (1 cycle), 40 cycles of 95°C for 15 second, 56°C to 59°C (depending on melting point of primers) for 30 second and at 72°C for 30 second; then one cycle of 95°C and 60°C for 15 second (Li *et al.,* 2009). Additional melting point analysis was performed on each reaction to determine the purity of the PCR product and the specificity of the primer sets.

Real-time PCR data expressed as CT threshold values were exported to MS Excel. Data was subject to normalisation using the $2^{-\Delta\Delta CT}$ method revised by Livak and Schmittgen (2001).

2.6 Statistics and experimental design:

Several experiments have been designed for different objectives and the data was analysed using a range of statistical analysis including discriminant analysis, correlation coefficient, Chi square analysis, T-test, analysis of variance (ANOVA) and linear models which were undertaken using software packages R statistics 2.1, XLSTAT 2013 and MS-Excel 2010 and Genstat 13th edition.

Models based on the results have been developed to enable identification of bitter pit and predicting the risk of incidence of bitter pit during storage.

CHAPTER 3

FRUIT QUALITY ASSESSMENTS

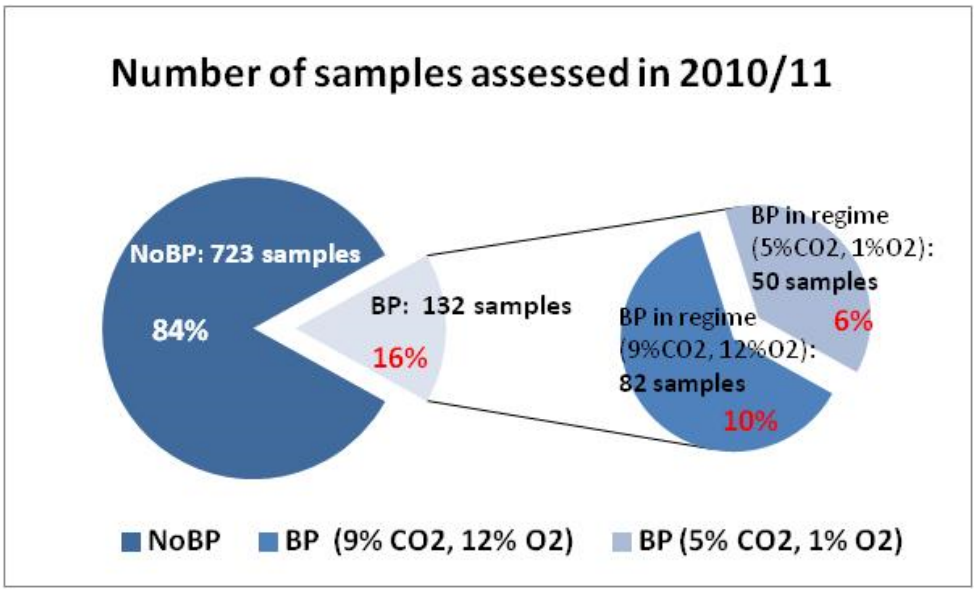
3.1. Introduction:

Bramley's Seedling apple samples were collected during four seasons (2010-13) from selected orchards at harvest and subsequent samples were taken from 1-MCP (SmartFresh^{SM}) treated and untreated samples stored under different storage regimes (9% CO_2, 12% O_2) and (5% CO_2, 1% O_2) or air. The incidence of bitter pit (%) was compared to quality parameters (fruit size, background colour, firmness, % Brix). The main objective of the experiments in this section was to investigate the effects of different storage regimes and treatment with 1-MCP (SmartFresh^{SM}) on incidence of bitter pit and the changes in quality parameters. Furthermore, the relationship between the parameters affecting the quality parameters and incidence of bitter pit was investigated.

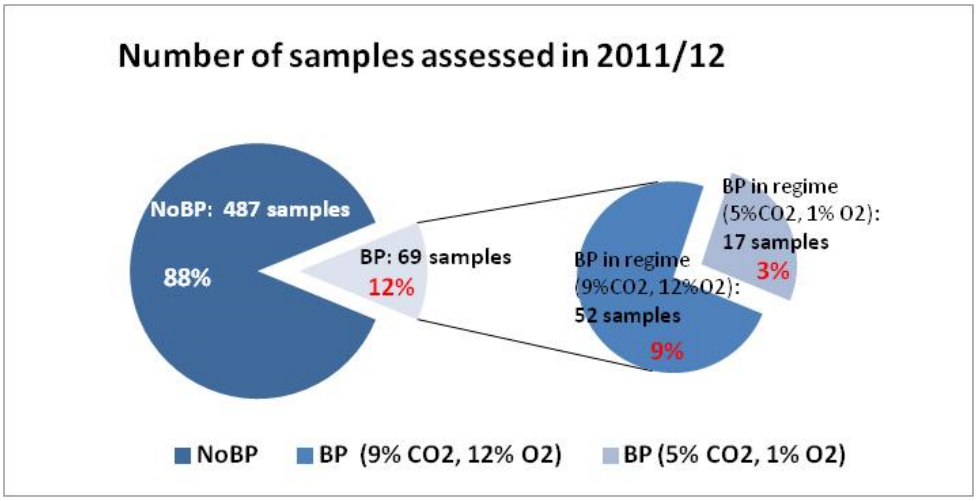
Although this study focused on the assessments of postharvest factors related to bitter pit, it was essential to review the pre-harvest factors and orchard history and the effects of pre-harvest parameters on incidence of bitter pit before analysing the results obtained from quality assessments during postharvest and storage.

3.2. The effect of the days taken to load stores, storage regimes, length of storage and the effect of SmartFresh^{SM} on incidence of bitter pit:

The incidence of bitter pit in Bramley apples from over 90 orchards was used to compare the incidence of the disorder with the time taken to load and seal stores from harvest over two seasons (2010 and 2011). The impact of type of CA regime and the influence of 1-MCP (SmartFresh^{SM}) were also assessed (Figure 3.1).



A



B

Figure 3.1: Number of samples assessed in 2010/11 and 2011/12 with proportion of incidence of bitter pit in CA regimes (9% CO_2, 12% O_2) and (5% CO_2, 1% O_2).

Using Pearson correlation analysis of the data from seasons 2010/11 and 2011/12, there was a significant correlation between a delay in loading stores and incidence of bitter pit in untreated samples in both CA storage regimes (9% CO_2, 12% O_2) and (5% CO_2, 1% O_2) at $P<0.05$ (Two-Tailed test). For the season 2010/11 the correlation coefficient of control (untreated) samples was (R=0.64) for storage regime (9% CO_2, 12% O_2) monitored over six months and was (R=0.65) for control (untreated) samples in storage regime (5% CO_2, 1% O_2) monitored over nine months. However, for 1-MCP (SmartFresh^{SM}) treated fruit the suppression of bitter pit development by this chemical seems to have prevented a correlation between delay in loading stores and incidence of bitter pit (Figure 3.2).

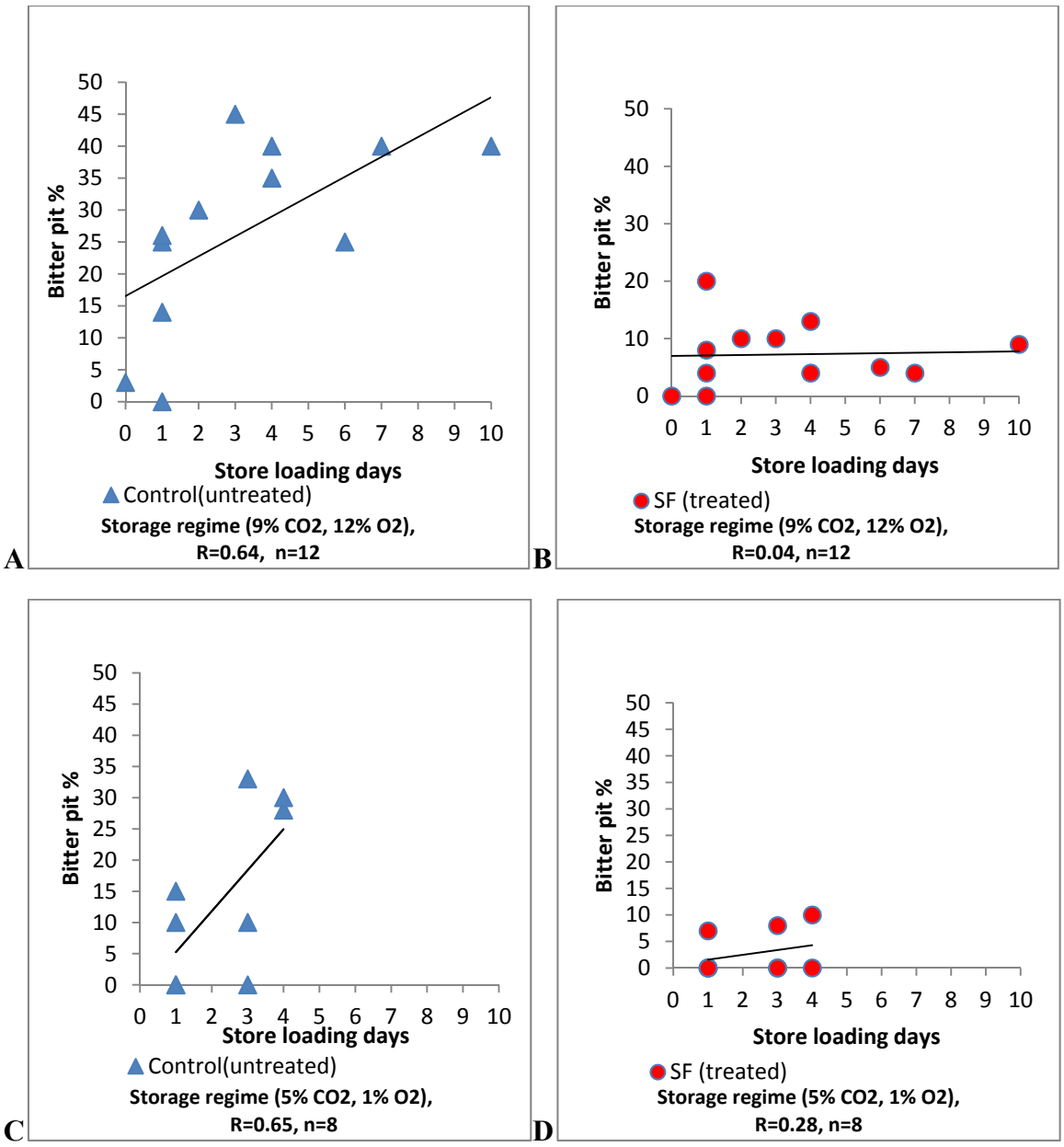


Figure 3.2: Relationship between the delay in loading stores and increasing incidence of bitter pit in Bramley's Seedling apples in 2010/11: A, B) comparison between untreated and SF-treated samples in storage regime (9% CO_2, 12% O_2), monitored for 6 months, and C, D) comparison between untreated and SF-treated samples in storage regime (5% CO_2, 1% O_2) monitored for 9 months. Each data point is the mean of incidence of bitter pit in 10 apples per assessment and the average of 3-4 assessments during storage.

3.3. Picking date and storage regimes:

The effects of different picking dates and storage regimes on incidence of bitter pit in season 2012/13 were investigated. Samples were harvested at three times over a period starting two weeks prior to the commercial harvest window and ending during the commercial Bramley harvest. Samples were stored in the same air store and monitored monthly over a period of four months. Only samples picked at commercial harvest time (P3) were treated with SmartFresh^{SM}.

There was no significant change in the incidence of bitter pit development in Bramley apples harvested at pick 1 (P1) and pick 2 (P2) monitored over the first two month period. However a significant increase of bitter pit was observed between P2 and the commercial harvest (P3). Later picked fruit had more bitter pit after 3 months storage than earlier picked fruit. Treatment with SmartFresh^{SM} suppressed the incidence of bitter pit development (Figure 3.3).

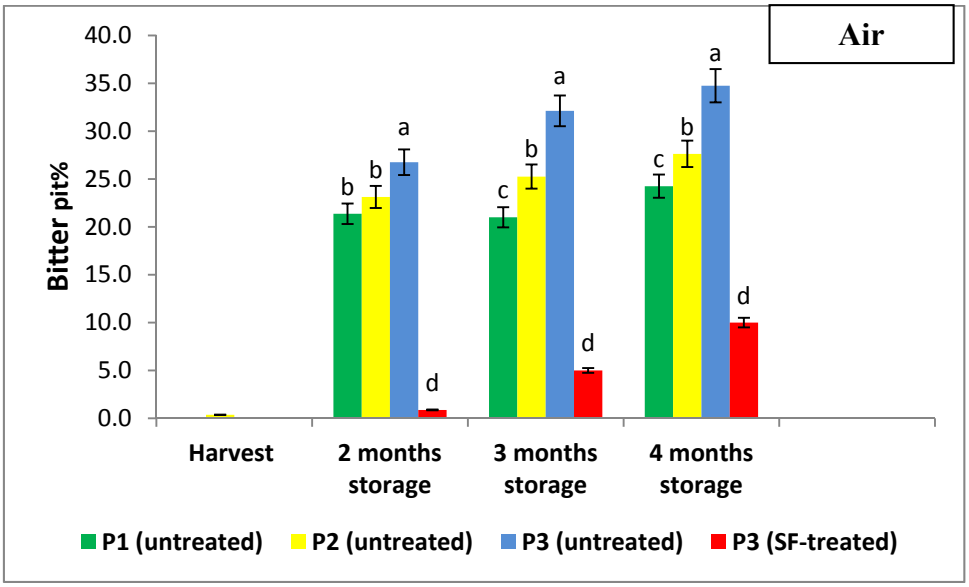


Figure 3.3: Comparison of the effect of different picking dates on incidence of bitter pit. P1 (Pick 1: 13/8/2012), P2 (Pick 2: 20/8/2012) and P3 (Pick 3: 28/8/2012). P3 fruit included +/- 1-MCP (SmartFresh^{SM}) treated. Each data point is the average of data (±SE) collected from four orchards all picked and stored in air then monitored at the same time. Mean values with different letters for the same assessment date were significantly different according to Tukey's test (p<0.05).

Furthermore comparison of different storage regimes showed that although there was a significant difference between incidence of bitter pit in untreated and SF-treated samples in all storage regimes, this difference was decreased in CA regimes in comparison with air regime and the CA regime of (5% CO_2, 1% O_2) reduced the overall incidence of BP comparing with CA regime ((9% CO_2, 12% O_2) for a longer storage time (Figure 3.4).

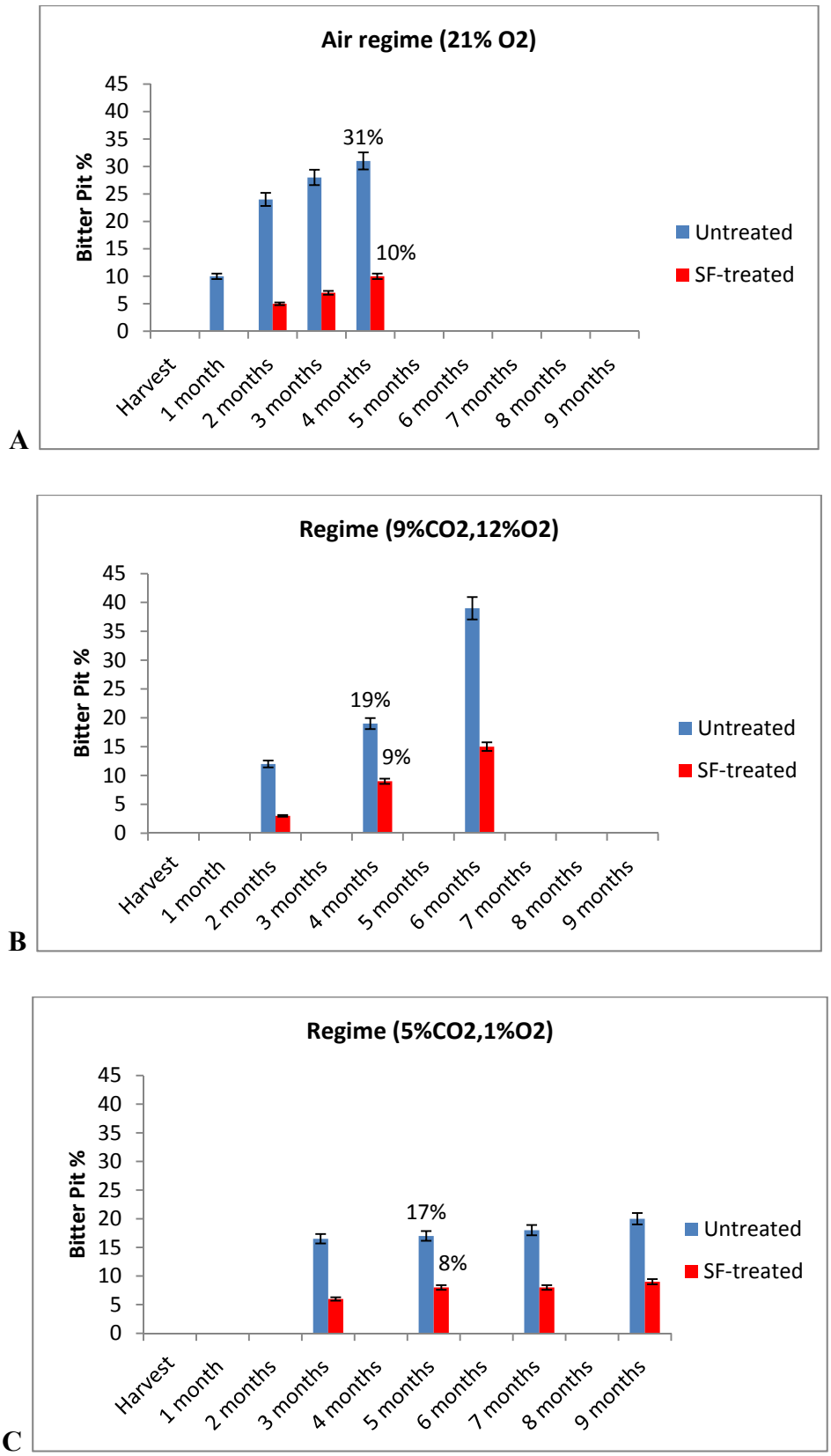


Figure 3.4: Comparison of the effect of different storage regimes: A) air, B) 9% CO_2, 12% O_2 and C) 5% CO_2, 1% O_2 and treatment with SmartFresh^{SM} on incidence of bitter pit. Each data

point is average of results collected from four orchards all picked and stored then monitored at the same time ±SE.

3.4. Fruit firmness (N):

Treatment with SmartFresh^{SM} (SF) delayed the decline in fruit firmness during storage. Changes in fruit firmness were compared in two storage regimes, (9% CO_2, 12% O_2) monitored for 6 months and regime (5% CO_2, 1% O_2) monitored for 9 months (Figure 3.5).

For (5% CO_2, 1% O_2) stored Bramley apples the data is presented as a comparison of the average firmness readings taken over the first 4 months compared to an average between months 4-9. For the shorter stored (9% CO_2, 12% O_2) Bramley apples the comparison was between 0-3 months compared to an average between 3-6 months. In (9% CO_2, 12% O_2) stored Bramley apples a significant drop in firmness between the first and second period of storage was observed in untreated fruit; the decline was halted by treatment with SmartFresh^{SM}, whereas with Bramley apples stored in (5% CO_2, 1% O_2) the firmness decline in untreated and SmartFresh^{SM} treated fruit was not significant and SmartFresh^{SM} treatment had no additional benefit in terms of firmness retention.

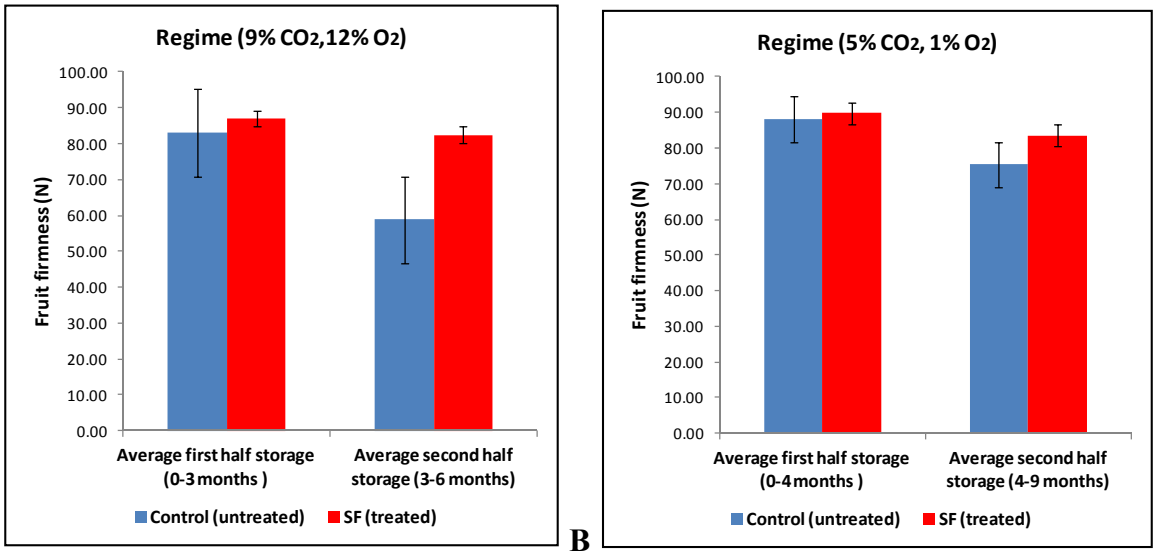


Figure 3.5: Comparison of the effect of treatment with SmartFresh^{SM} in maintaining fruit firmness (N) over storage time in different storage regimes: A) Storage regime (9% CO_2, 12% O_2), monitored for 6 months average firmness compared between (0-3) and (3-6) months storage. B) Storage regime (5% CO_2, 1% O_2) monitored for 9 months average firmness compared between (0-4) and (4-6) months storage. Each data point is the mean of two sets of ten samples ±SE.

There was an inverse correlation between fruit firmness and incidence of bitter pit. As fruits soften they are more prone to senescent disorders such as bitter pit. The correlation coefficient for incidence of bitter pit and decreasing fruit firmness of untreated samples was (R=-0.62) and for SmartFresh^{TM} treated samples (R=-0.59) in storage regime (9% CO_2, 12% O_2).

SmartFresh^{TM} has suppressed firmness decline and bitter pit development in the CA regime of (5% CO_2, 1% O_2) where fruits did not soften as much and had a lower incidence bitter pit. A significant correlation for untreated samples (R=-0.69) was maintained but not so for Smartfresh^{TM} treated fruit where there was even less softening and bitter pit (R=-0.21). The stringent CA regime of (5% CO_2, 1% O_2) reduced the overall incidence of bitter pit and firmness therefore the additional effect of SmartFresh^{SM} (SF) on bitter pit incidence was less (R=-0.21) in this storage regime (Figure 3.6).

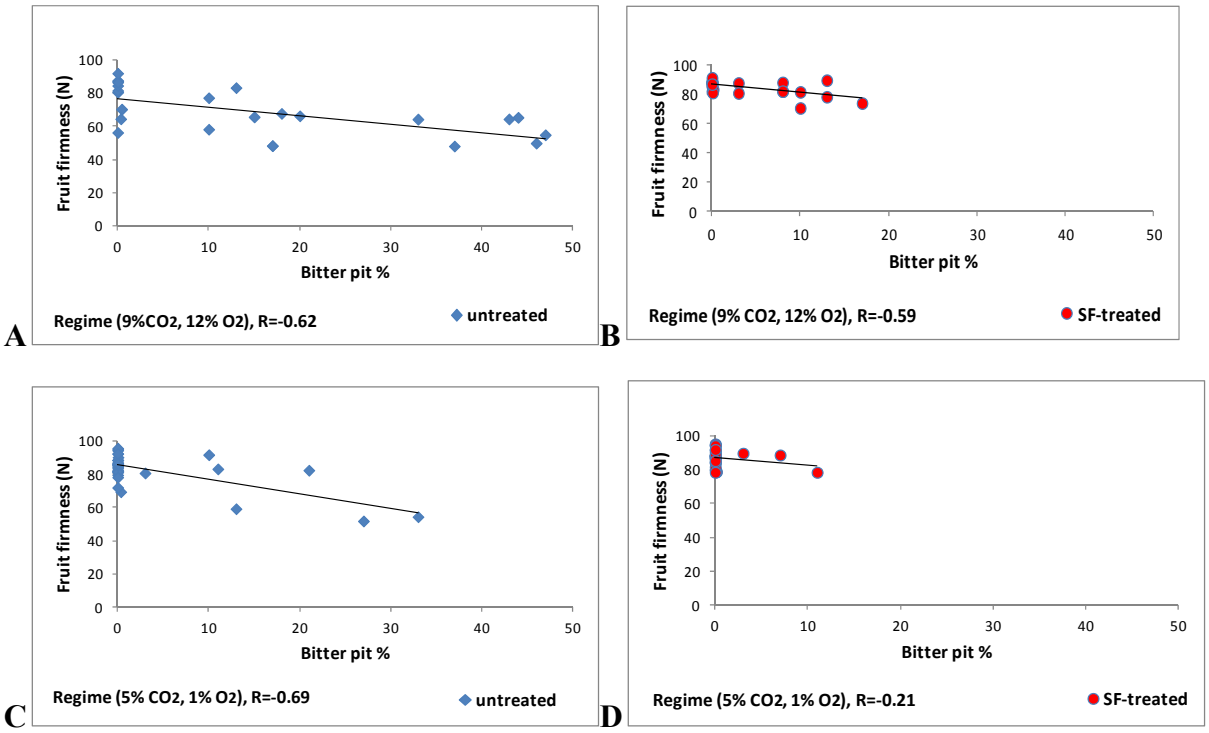


Figure 3.6: Relationship between decreasing fruit firmness and increasing incidence of bitter pit in Bramley's seedling apple in different storage regimes in 2010/11: A, B) comparison of bitter pit between untreated (A) and SF-treated (B) samples in regime 9% CO_2, 12% O_2 for 6 months (n=20). C, D) comparison between untreated and SF-treated samples in storage regime (5% CO_2, 1% O_2) monitored for 9 months (n=15). Each data point is the mean of ten apple samples assessed at different stages in season 2010/11.

The results in season 2012/13 which only focused on untreated samples found a significant inverse correlation between fruit firmness and the incidence of bitter pit in air regime (R=-0.72), which was stronger than that found in controlled atmosphere (CA) regimes; (R=-0.66) in

storage regime (9% CO_2, 12% O_2) and (R=-0.58) in the CA regime of (5% CO_2, 1% O_2) (Figure 3.7).

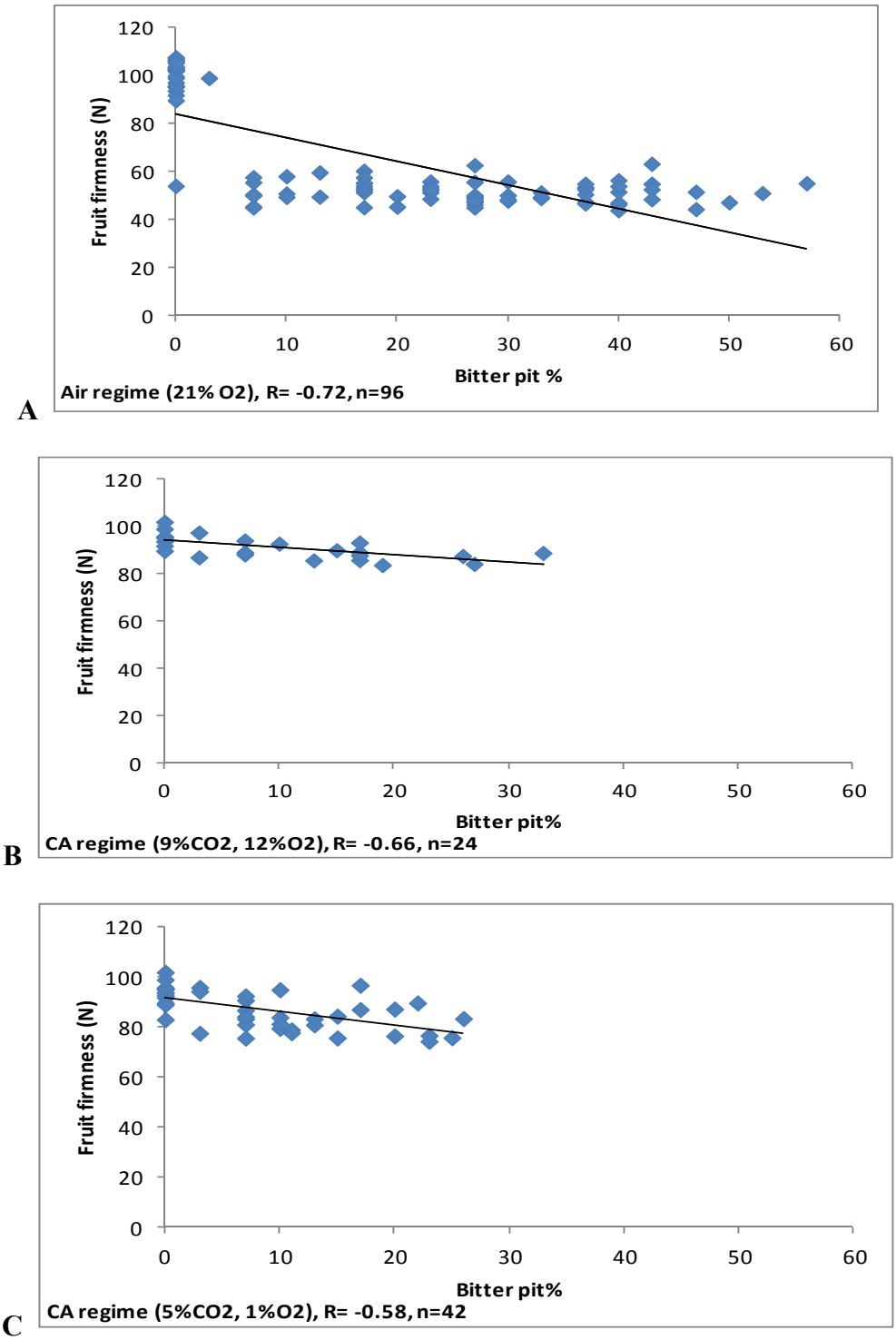


Figure 3.7: Comparison of relationship between fruit firmness and incidence of bitter pit in three different storage regimes: A) air regime (21%O_2), B) CA regime (9%CO_2, 12%O_2) and C) CA regime (5%CO_2, 1%O_2). Data presented is only non-SF treated samples (2012/13).

For the final season (2013/14) there were fewer orchards sampled and less incidence of bitter pit recorded, under these parameters the only correlation with declining firmness and bitter pit was observed in air stored Bramley apples (R=-0.71) (Figure 3.8).

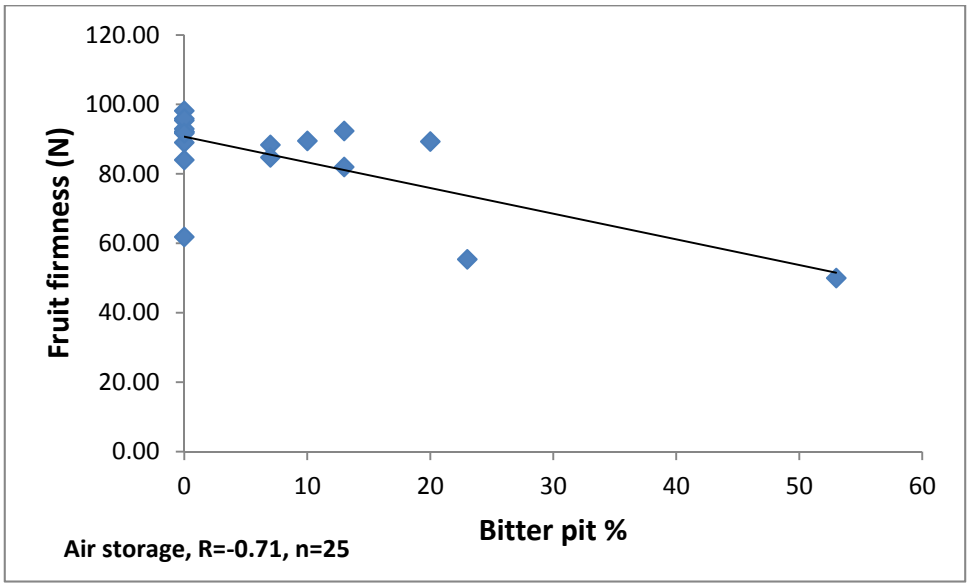


Figure 3.8: Relationship between decreasing fruit firmness and incidence of bitter pit in Bramley's seedling apple samples. Data is combination of untreated and SF-treated fruit collected in season 2013/14 and stored in air for four months from EMR orchard.

3.5 Background colour:

There was a positive correlation (R=0.65) between increasing *b** value (yellow colour) of Bramley's seedling apple fruit skin stored in air and incidence of bitter pit at P<0.01 (Two Tailed test) in season 2011/12 (Figure 3.9).

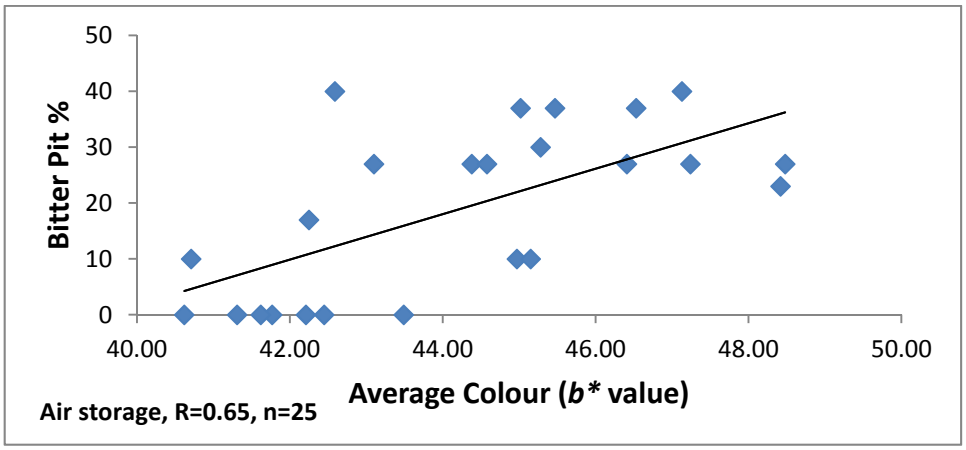


Figure 3.9: Correlation between changing colour of untreated fruit in Bramley's seedling apple from green to yellow (increasing *b** value) and incidence of bitter pit. Each data point is the mean value for a ten fruit sample collected during 2011/12, stored in air and monitored for 4

months. Each data point is the mean of a ten apple sample assessed at different stages from EMR and HOO orchards.

Similar results were obtained in season 2012/13, when results of untreated samples and SF-treated samples were compared (Figure 3.10). In air stored untreated fruit, although the incidence of bitter pit was higher, the spread of data reflecting the incidence of yellowing (increasing *b** value) was greater, a lower correlation (R=0.55) was achieved compared to SmartFresh^{SM} treated fruit where the impact of ethylene suppression reduced the loss of background greenness and delayed the onset of yellowing. This increased the range of *b** value data and resulted in a higher correlation (R=0.65) between bitter pit and changes in skin colour.

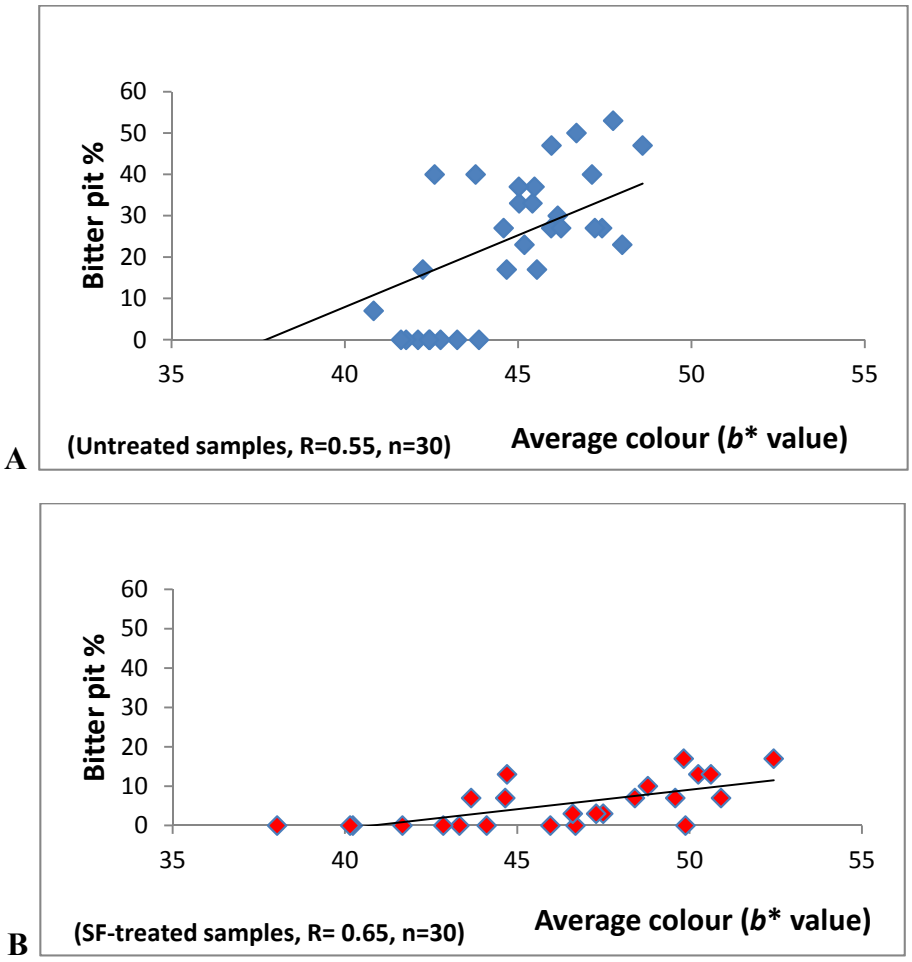


Figure 3.10: Comparison of untreated and SF-treated samples of Bramley's seedling apple for the correlation between changing colour of untreated fruit from green to yellow (increasing *b** value) and incidence of bitter pit. Each data point is the mean value for a ten fruit sample collected during 2012/13 from four orchards and stored in air and monitored for 4 months. Each data point is the mean of a ten apple sample assessed at different stages from EMR and HOO orchards.

In the final year (2013/14) the limited number of samples and observations reduced overall accuracy of prediction. However, in SF-treated samples a correlation of R=0.59 between the incidence of yellowing and bitter pit was observed compared to R=0.43 in untreated samples.

3.6 Total soluble solids (%Brix):

There was no relationship between the amount of soluble solids measured as %Brix and the incidence of bitter pit in untreated Bramley apples stored in air (R=0.39) in season (2011/12) and (R=0.45) in season (2012/13). In the presence of SmartFresh^{SM} the reduction in bitter pit reduced correlation values further.

In season (2013/14) where comparisons between the stalk and calyx region of the fruit were undertaken, the calyx end of the fruit tended to have a higher % Brix than the stalk irrespective of SmartFresh^{SM} treatment (Figure 3.11). The calyx region tended to have a greater incidence of bitter pit. The relationship between changes in % Brix and incidence of bitter pit in untreated and SF-treated samples were compared and there was no relationship between changes of % Brix in calyx or stalk end of fruit and incidence of bitter pit.

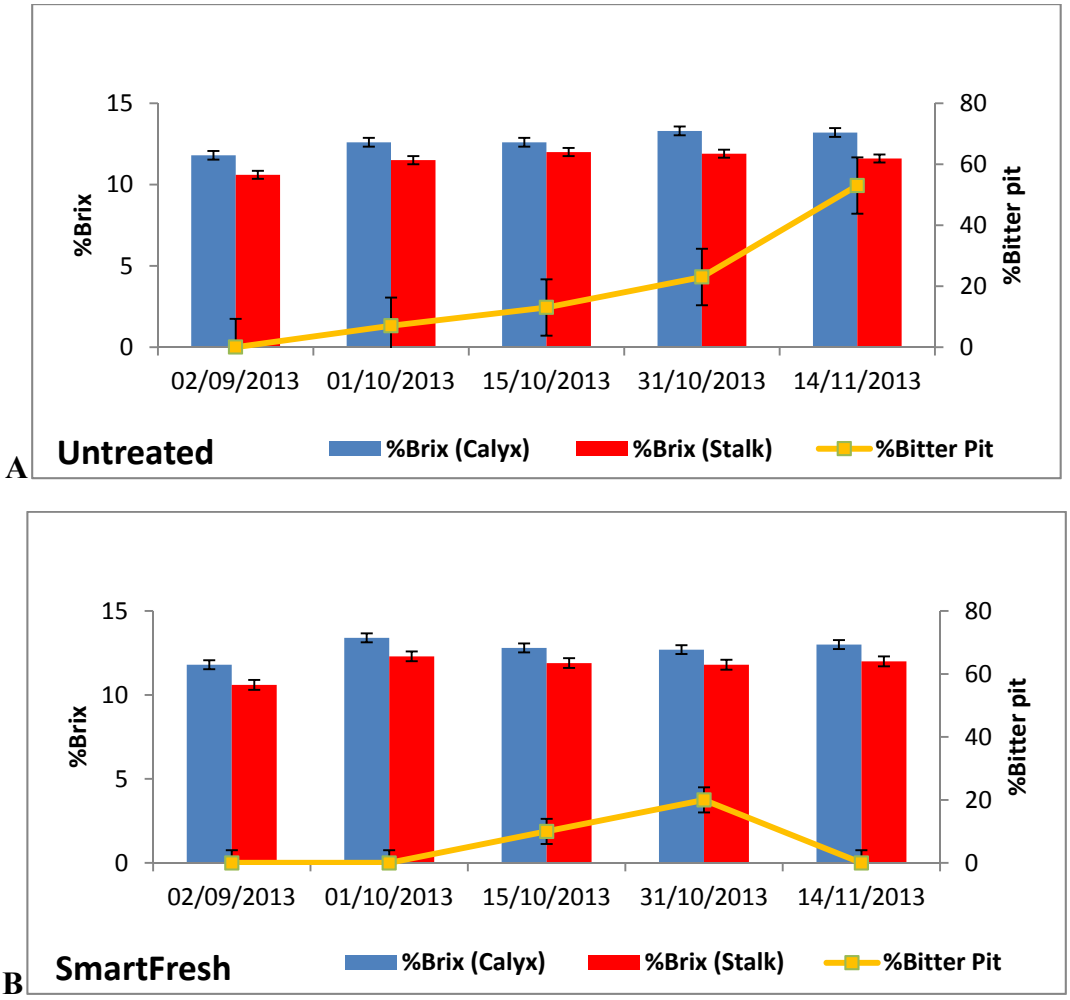


Figure 3.11: Comparison of changes in total soluble solids (%Brix) in calyx and stalk end of Bramley's seedling apple fruit and incidence of bitter pit: A) untreated and B) SmartFresh^{SM} treated samples air stored samples from orchards EMR and HOO in season 2013/14. Each data point is the mean of ten samples ±SE.

3.7. Fruit size:

There was no correlation between increasing fruit size and incidence of bitter pit when averaged across all treatments (R=0.13). However the relationship between increasing fruit size and incidence of bitter pit were different in different storage regimes: air regime (21% O_2) R=0.04, CA regime (9%CO_2, 12%O_2) R=0.17 and CA regime (5%CO_2, 1%O_2) R=0.27. By defining untreated and SF-treated samples the best correlation was related to untreated samples in CA regime (5%CO_2, 1%O_2) in which case R=0.61 for untreated samples and in SF-treated samples R=0.26 (Figure 3.12). These results are related to the orchards which were more susceptible to bitter pit. The orchards with less propensity to bitter pit did not show significant correlation even in untreated samples (untreated sample R=0.16, SF-treated samples R=0.13).

All these results are related to season 2012/13 since samples were collected from four orchards (two orchards more susceptible and two orchards less susceptible to bitter pit stored in different storage regimes). Also in this season samples were picked on three different dates and comparison of these different harvest dates and effect of increasing size and correlation with bitter pit showed better correlation in the last pick: Pick 1 (13/8/2012) R=0.14, pick 2 (20/8/2012) R=0.19, pick 3 (28/8/2012) untreated samples R=0.55, SF-treated samples R=0.31. In season 2013/14 numbers of samples were very limited and were not statistically valid. However untreated samples showed higher correlation between increasing fruit size and incidence of bitter pit.

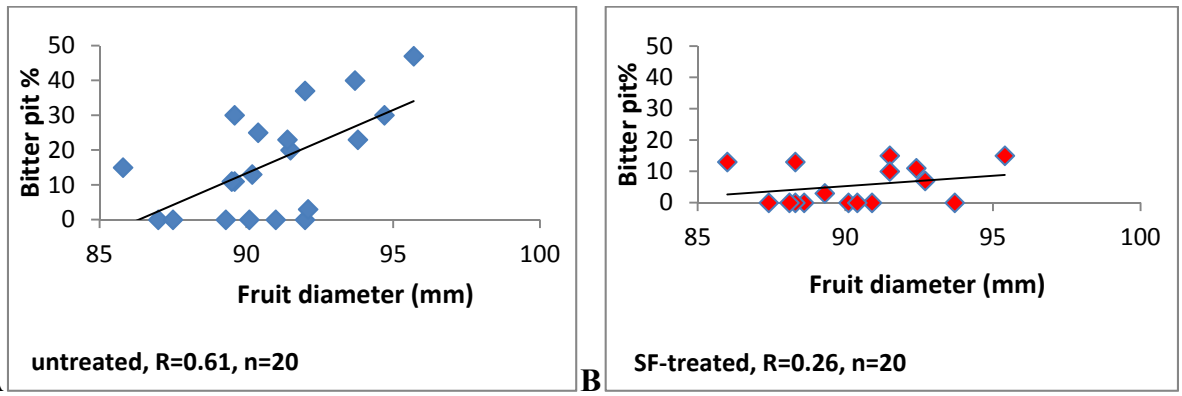


Figure 3.12: Comparison of the relationship between increasing fruit size and incidence of bitter pit in: A) untreated samples and B) SF-treated samples stored in CA regime (5%CO_2, 1%O_2) taken from susceptible orchards to bitter pit collected in season 2012/13.

3. 8. Discussion:

Although this study focused on the assessments of postharvest factors related to bitter pit, it was essential to review the pre-harvest factors and orchard history before analysing the results obtained from quality assessments during postharvest and storage. Previous studies showed that bitter pit starts and develops under the influence of orchard management and environmental factors which affect apple quality (Ferguson *et al*., 1999; Hewett *et al*., 2006; De Freitas *et al*., 2013).

Weather:

Weather patterns during the period of this study varied considerably between seasons. The changes in temperature during spring and summer over four years 2010-13 based on Meteorological Office (2015) records are compared in Appendix V (a & b).

In 2010 the temperature changes during spring and summer were similar to the long-term (30 years) average and relatively normal. However it was the driest spring since 1984 followed by a relatively wet summer especially in August 2010, and in some parts of Kent rain fall was over 100% above the long-term average. Fruit size is often affected by late season rains. A dry spring and wet summer led to swelling of fruit size and a dilution in calcium concentration per unit area and increased incidence of bitter pit (Ferguson, 1999) for Bramley apples in storage season 2010/11. However, in our trials there was no direct relationship between fruit size and bitter pit incidence which may indicate that calcium uptake and fruit calcium were unaffected by weather patterns in 2010/11 (Figure 3.1).

According to Meteorological Office records, 2011 was the warmest spring across the UK since 1910, and was also dry. It was then followed by a cold summer which was the coolest since the summer of 1993 and summer rainfall was similar to 2010. This condition caused a delay in the apple harvest. However, because of the cold summer the average size of fruit was smaller and potentially cell density was higher than the previous year leading to less incidence of bitter pit. The oscillations in climate during the 2012 growing season were particularly high according to Meteorological Office statistics; it was the third warmest March in the UK since 1910, followed by cold weather and April was the coldest since 1989. These unusual fluctuations in temperature during flowering and fruit development affected pollination, calcium uptake and cell expansion of apple fruits. The warm weather in March 2012 resulted in flowers opening two weeks earlier. However, during the pollination period temperatures dropped and affected the efficiency of pollination and led to a protracted pollination period, which causes lower seed number and calcium content (Ferguson *et al*., 1994). The implications of this were observed as a large variation of fruit maturity within orchards that led to an extended harvest period over for Bramley apples. In addition this condition was followed by the cold and wet summer. It was the wettest summer in UK since 1912. This condition caused less transpiration and a reduction in xylem flow; within the tree such condition can result in poorer calcium uptake into the fruit (Atkinson, 1991). Since N and K are transported via active phloem transport system (Terblanche *et al.*, 1979), they were less affected by water/temperature stress. More incidence of bitter bit occurred in Bramley apples stored in season 2012/13.

In 2013 March was colder than any of the winter months and the coldest for the UK since 1962. This caused more than 3 weeks delay in flowering and fruit set. However it was followed by the warmest summer in the UK since 2006. Although commercial harvest started later than in 2012, there was more uniformity in maturity between fruit and less occurrence of bitter pit.

All these changes and oscillations in climate during different seasons affected the results obtained in different seasons. As mentioned before in season 2012/13 a small number of orchards (4) were selected from a wider range of orchards which were monitored for two seasons. However changes in climate affected incidence of bitter pit and sometimes the results were diverse with the expected susceptibility of that specific orchard.

Rootstock:

Rootstock affected fruit size and movement and competition of calcium and other nutrients between actively growing shoots and expanding fruits. Samples in seasons 2010/11 and 2011/12 were collected from 90 orchards with diverse orchard profiles and histories; with fruit collected from trees grafted to different rootstocks (MM.106, M.27 and M.9), and a range of tree ages, soil type, nutrition, irrigation, sprays and picking dates. All these factors affect storage life. As Fallahi and Mohan (2000) indicated, rootstocks impart different susceptibilities to bitter pit. With fruit grown on dwarf M.9 trees more "bitter pit" occurs than fruit grown on MM.106 rootstocks. The diversity observed in samples collected in the first two seasons was considered to be partly related to rootstock, so the four orchards considered in season 2012/13 (two orchards monitored in 2013/14), were selected so that all trees were grafted on the same rootstock (MM.9), were of similar age (10 years) and were located within 15 miles of each other, thereby limiting as many of the orchard variables as possible. However, there were differences in the soil, orchard management and nutrition programmes, which resulted in samples with the range in susceptibility to bitter pit that was needed for this study.

Store management:

The results of this study showed that where a delay in harvesting of fruit or where the time taken to load and seal stores was delayed beyond the recommended 3 day period (Fidler *et al.,* 1973; Johnson, 1994) an increase in bitter pit was recorded. These findings were in accordance with Prinja (1989) who showed that a delay in filling and sealing stores increased the incidence of bitter pit in commercial CA storage regimes. In this study a positive correlation (R=0.64) was found between delay in loading store and incidence of bitter pit for the storage regime (9% CO_2, 12% O_2) monitored for six months and likewise a positive correlation (R=0.65) was found for samples in storage regime (5% CO_2, 1% O_2) monitored for nine months. However this study showed there was no correlation between the delay in loading stores and incidence of bitter pit in samples treated with SmartFresh^{SM} where the incidence of bitter pit development was suppressed and deleterious effects of delayed harvesting and store sealing on fruit quality could

be mitigated by application of SmartFresh^{SM}. This was also reported by Blankenship (2001) and Johnson (2007). This could be related to the delay in onset of climacteric ethylene production due to binding ethylene receptors immediately after application of SmartFresh^{SM}. However, as indicated in previous studies (Watkins and Nock, 2005; Rees *et al*., 2012), delaying the application of SmartFresh^{SM} long-after harvest (more than 7 days) can reduce its efficiency since more receptors have been activated by ethylene and the climacteric production of ethylene has started and cannot be reversed.

Storage regime and SmartFresh^{SM}:

The type of storage regimes under which Bramley's seedling were kept influenced bitter pit development. Although there was significantly lower incidence of bitter pit in SmartFresh^{TM} treated samples compared with untreated samples in all storage regimes, this difference was decreased in CA regimes in comparison with air regimes, and the CA regime of (5% CO_2, 1% O_2) reduced the overall incidence of bitter pit compared with CA regime (9% CO_2, 12% O_2) for a longer storage time. In samples treated with SmartFresh^{SM} development of bitter pit was slower and less severe than untreated samples. However severity and development of incidence of bitter pit in treated samples was related to the storage regime. These results were similar to Watkins *et al.* (2000). It seems in regime (5% CO_2, 1% O_2) unlike other storage regimes for a longer time (more than 6 months) development of bitter pit is very slow even in untreated samples. This is related to the effect of the lower level of oxygen (1% O_2) in combination with high carbon dioxide (5% CO_2) to decrease respiration and reduce ethylene production by reducing the activity of the enzyme ACC oxidases resulting in a reduction in ethylene production (Saltveit, 1999). Although in regime (9% CO_2, 12% O_2) the high level of CO_2 decreases respiration, the oxygen level is higher (12% O_2) than the regime (5% CO2, 1% O2), and the normal practice is that this level of CO_2 (9%) is established by natural respiration of fruit so that ethylene production is not inhibited until establishment of the target atmosphere. In contrast, in newer stores the establishment of the regime (5% CO2, 1% O2) is achieved by scrubbers over a shorter period. Furthermore part of the ethylene could be absorbed by scrubbers (Bishop, 1996). However Kweon *et al.* (2013) reported that when fruit was more mature it was further aggravated by higher level of CO_2 in the storage atmosphere, irrespective of level of O_2. So it seems the main reason of less incidence of bitter pit in number and severity

in the storage regime (5% CO2, 1% O2) is related to a combination of lower level of CO_2 and O_2 for reducing ethylene production.

Picking dates:

A comparison of different picking dates in season 2012/13 showed a correlation between late pick and increasing incidence of bitter pit. This is in contrast with previous studies (Ferguson and Watkins, 1992; Prange *et al.*, 2011) that reported increasing incidence of bitter pit when fruit were picked earlier. Normally it is expected that when fruit is picked later the Ca^{2+} content increases (Remorini *et al.,* 2008). However as Conway (1989) indicated, sometimes when fruit is mature changes in structure such as cell enlargement and the increase in intercellular space can result in the dilution of Ca^{2+} that causes more bitter pit. As mentioned before fluctuations in temperature in spring 2012 led to a large variation of fruit maturity within orchards which could be the reason of this difference and probably the samples which were picked earlier were riper with higher level of Ca^{2+}. Also the incidence of bitter pit where SmartFresh^{SM} was applied was significantly lower than untreated samples that were picked in different dates.

Relationship between fruit quality and bitter pit incidence:

The firmness of samples treated with SmartFresh^{SM} was significantly higher than untreated samples in the storage regime (9% CO_2, 12% O_2) over 6 month's storage. However in storage regime (5% CO_2, 1% O_2) during 9 months storage there was no significant difference in firmness of untreated and SmartFresh^{SM} treated samples, because the (5% CO_2, 1% O_2) regime is very effective at maintaining fruit firmness and control of bitter pit during extended storage. These findings are similar to the results Johnson and Colgan (2003) reported and explained that the role of CO_2 in reducing the progress of fruit maturation is due to its binding to the ethylene receptors that SmartFresh^{SM} inhibits and causes reduction in incidence of bitter pit in Bramley apples stored in both CA storage regimes. However, since fruit was stored in regime (5% CO_2, 1% O_2) for a longer time (9-11 months) treatment with SmartFresh^{SM} helped to delay incidence of bitter pit which was also reported by Watkins and Nock (2005).

There was an inverse correlation between fruit firmness and incidence of bitter pit. Results in different seasons showed greater inverse correlation (R=-0.75) in the air regime in comparison with both CA regimes (R=-0.54). However in SmartFresh^{SM} treated samples stored in regime (5% CO_2, 1% O_2) there was no significant correlation between decreasing fruit firmness and incidence of bitter pit. In regime (9% CO_2, 12% O_2) there was a relationship between decreasing

fruit firmness and increasing incidence of bitter pit in both untreated and SmartFresh^{SM} treated samples. As explained before with respect to the role of storage regimes and treatment with SmartFresh^{SM} they cause reduction in ethylene production and slower maturity of fruit. When bitter pit occurs the pitted fruit have less water soluble Ca^{2+} (Pavicic *et al.*, 2004). When fruit is stored in CA and treated with SmartFresh^{SM}, the delay in ripening causes a reduction in the movement of minerals (especially Ca^{2+}) that causes a delay in fruit softening. However, as Trakoontivaron (1987) indicated softening is the result of changes in cell to cell cohesion and is not a result of cell wall weakening. Since CA storage and SmartFresh^{SM} have a masking role to cover and delay ripening changes, it means that fruit is still firm but bitter pit signs have been started, since the forces binding Ca^{2+} to the cell wall are greater than the forces maintaining cell wall the process of losing water soluble Ca^{2+} through middle lamella starts earlier in the cell and in the pitted cell this loss is greater, so incidence of bitter pit in the cell wall was started earlier than softening of the fruit which is result of changes in cell to cell cohesion. This leads to the condition that still fruit is firm but bitter pit occurred and the correlation between firmness and incidence of bitter pit decreased in CA storage in comparison with air storage.

Changes in background colour occur during storage of Bramley apples and this has been used by Leemans *et al.* (1999) to track changes in the internal quality of apples including the incidence of bitter pit. The results of this study in season 2011/12 showed a positive correlation (R=0.65) between increasing *b** value (background yellow colour) of Bramley apple fruit skin and incidence of bitter pit. Also in season 2012/13 a comparison between untreated samples (R=0.55) and SmartFresh^{SM} treated samples (0.65) showed better correlation between increasing *b** value and incidence of bitter pit. In both seasons samples stored in air regime contributed to this data since background colour measurements were part of assessments at East Malling Research and whereas colour date for samples stored in CA regimes in commercial stores were not available. However the greater correlation between increasing yellow background colour of peel and incidence of bitter pit in SmartFresh^{SM} treated samples could be related to delay in ripening that can keep the background colour greener. Maturity causes changing background colour because of chlorophyll degradation which results in decreasing intensity of green colouration (Łysiak *et al.,* 2014), on the other hand when bitter pit occurs and development starts just under the fruit peel (Ferguson and Watkins, 1989), because of the changes in the cells with bitter pit, background colour starts changing from green to yellow (Rudell *et al.,* 2005). This is the reason that SmartFresh^{SM} treated samples showed better

correlation with bitter pit since in untreated samples some background colour changes were related to ripening of fruit.

Another reason that changes in background colour were less correlated with incidence of bitter pit in samples which were not treated with SmartFresh^{SM} could be related to the greater severity of bitter pit in those samples that bitter pit spreads in different parts of cortex (Perring, 1985), the distance between pitted cells and peel causes peel cells not to be affected by severity of bitter pit. Since in SmartFresh^{SM} treated samples severity of bitter pit is less than untreated samples and most pitted cells are close to the peel, background colour change is more correlated to bitter pit.

Although colour changes can give an indication of internal quality changes, they usually only occur late the storage season and do not provide a reliable predicative method for determining the storage potential of the consignment.

There was little change in the total soluble solids (TSS) or %Brix content of the fruit during storage and these provided a poor parameter to correlate changes in fruit maturity and bitter pit (R=0.39) in season 2011/12, although slightly higher (R=0.45) in season 2012/13. These results were in accordance with Lanauskas *et al.* (2012). Although there are some reports about increasing %Brix during storage (Hayat *et al.,* 2005) this could be more correlated to maturity than disorders like bitter pit. There are some changes in acids and sugars during bitter pit development such as replacement of malic acid by citric acid or decreasing sucrose and increasing glucose and fructose (Fidler *et al.*, 1973; Oke *et al*., 2013). As %Brix represents total soluble solids and does not distinguish between species of sugars or species of acids, there is no significant changes in total soluble solid results.

In season 2013/14 the total soluble solids (%Brix) of each sample from calyx and stalk end of fruit were compared. Total soluble solids (%Brix) in the calyx end of both untreated and SmartFresh^{SM} treated samples were always higher than at the stalk end of fruit although the differences were not significant. This could be related to higher concentration of sorbitol, Yamada *et al.* (2004) indicated an accumulation of sorbitol at the calyx end of apple fruit. However there was no relationship between changes of %Brix in calyx or stalk end of fruit and incidence of bitter pit. Also there was no significant difference between %Brix of untreated and SmartFresh^{SM} treated samples which was similar to Rudell and Watkins (2011) results.

There was not a significant correlation between increasing fruit size and incidence of bitter pit in an overall comparison of all storage regimes. There are many reports about correlation of fruit size and incidence of bitter pit and as De Freitas *et al*. (2012) indicated the fruit calcium is inversely correlated with mean fruit size. However, Ferguson and Watkins (1992) found that movement of minerals into fruit is affected by crop load regardless of final fruit size. It seems the reason that results of this study do not indicate that bitter pit is highly correlated to fruit size, is related to mineral movements and crop load, especially when concerning the weather condition in season 2012/13. As indicated previously because of warm weather in early spring and then cold weather later, the crop load decreased, pollination was affected and there was a large variation of fruit maturity within orchards. In addition a cold and wet summer caused relatively smaller and denser fruit. However, there was a greater correlation between the size of untreated samples and incidence of bitter pit. This was also related to the role of SmartFresh^{SM} in delaying ripening that gave more time to fruit before occurrence of bitter pit and reduced the correlation with fruit size in SmartFresh^{SM} treated fruit.

CHAPTER 4

METABOLIC CHANGES IN BRAMLEY APPLE DURING STORAGE

4.1 Introduction:

This chapter considers the profile of organic acids, sugar content and mineral analysis of Bramley's seedling apple samples collected in three consecutive seasons (2011/12, 2012/13 and 2013/14) from different orchards. Biochemical analysis of organic acids (ascorbic acid, malic acid, oxalic acid and citric acid) and sugars (glucose, fructose and sucrose) and mineral analysis (calcium, potassium, magnesium, nitrogen and boron) were compared to identify how changes in metabolic indicators of fruit ripening (sugars and acids) and mineral content at harvest influenced incidence of bitter pit during storage.

Prediction models based on biochemical analysis including organic acids and sugars were designed and developed including mineral analysis with emphasis on calcium distribution in fruit cortex and the consequences for development of bitter bit.

4.2 Organic acids and sugars:

Analysis of cortex tissue from Bramley apples for ascorbic acid, malic acid, oxalic acid and citric acid and sugars (glucose, fructose and sucrose) was undertaken over three seasons (2011-13) to determine whether changes in selected respiratory metabolites (sugars and acids) during storage could influence the propensity of fruit to develop bitter pit. The effect of CA storage and SmartFresh^{SM} treatment on the organic acid and sugar profiles was investigated. Details of the sampling strategy are listed in Appendix VI (a-c). Samples of Bramley apples stored in air and CA regimes were tested.

In storage season 2011/12 samples of apples from two orchards: one susceptible to bitter pit (A-EMR) and the other less susceptible orchard (B-Hoo) were harvested over successive weeks from mid-August to mid-September. Bramley's were stored in air (4-4.5°C) and sampled at two month intervals during storage. The incidence of bitter pit development between the two orchard sites was significantly different (P<0.05) (Table 4.1).

As expected malic acid was the major organic acid (10-13.5µg/µL) while oxalic, ascorbic and citric acid were much less abundant (0.11-0.29 µg/µL). At harvest ascorbic acid content was

higher (P<0.05) in orchard B (HOO) which had a lower incidence of bitter pit. Citric, malic and oxalic acids remained similar across the different harvest dates.

Between different harvesting dates, H4 (latest pick) showed significantly more incidence of bitter pit than earlier harvest dates. Ascorbic acid was significantly lower in late picked samples. Other acids did not show significant changes between picking dates.

Table 4.1: Comparison of mean of organic acids (oxalic, malic, ascorbic and citric acid) of Bramley apples harvested at different dates (H1: 9/8/2011, H2: 18/8/2011, H3: 25/8/2011 and H4: 9/9/2011) from a susceptible bitter pit orchard (A-EMR) and less susceptible orchard (B-HOO). Apples were stored in air (4-4.5°C) and monitored 3 times during 4 months storage in season 2011/12. LSDs (5%) are for the effects of harvesting dates (H) and orchard (O) n=25.

		Bitter Pit %	Oxalic acid µg/µL	Malic acid µg/µL	Ascorbic acid µg/µL	Citric acid µg/µL
Orchard A (EMR)	Mean H1	6.8	0.24	13.04	0.18	0.16
	Mean H2	12.2	0.25	12.68	0.16	0.14
	Mean H3	8.2	0.22	10.88	0.11	0.14
	Mean H4	22.5	0.29	10.76	0.12	0.13
	Overall means	*12.4*	*0.23*	*11.84*	*0.14*	*0.14*
Orchard B (HOO)	Mean H1	0	0.22	12.52	0.26	0.15
	Mean H2	0	0.23	13.50	0.29	0.12
	Mean H3	5	0.19	10.98	0.21	0.11
	Mean H4	1.8	0.21	11.76	0.24	0.13
	Overall means	*1.7*	*0.21*	*12.20*	*0.25*	*0.13*
Overall means	H1	*3.4*	*0.23*	*12.78*	*0.22*	*0.16*
	H2	*6.1*	*0.24*	*13.08*	*0.23*	*0.13*
	H3	*6.6*	*0.20*	*10.94*	*0.16*	*0.13*
	H4	*12.1*	*0.22*	*11.26*	*0.18*	*0.13*
	df	24	24	24	24	24
	LSD (H)	5.92	0.03	2.08	0.03	0.03
	LSD (O)	4.19	0.02	1.47	0.02	0.02

Additional analysis of sugars (fructose, glucose and sucrose) content showed significant differences (P<0.05) between the two orchards. Glucose concentrations (µg/µL) declined with picking date while the amount of sucrose was higher in later picked fruits. No trend in fructose content was observed. The incidence of bitter pit increased in later picked fruit (Table 4.2).

Table 4.2: Comparison of mean of sugars (fructose, glucose and sucrose) of Bramley apples harvested at different dates (H1: 9/8/2011, H2: 18/8/2011, H3: 25/8/2011 and H4: 9/9/2011) and between two orchards as more susceptible to bitter pit (orchard A-EMR) and less susceptible to bitter pit (orchard B-HOO) stored in air (21% O_2) regime in season 2011/12. LSDs (5%) are for the effects of harvesting dates (H) and orchard (O) n=25.

		Bitter Pit %	Fructose µg/µL	Glucose µg/µL	Sucrose µg/µL
Orchard A (EMR)	Mean H1	6.8	57.72	25.70	15.54
	Mean H2	12.2	62.74	26.08	20.14
	Mean H3	8.2	54.88	20.28	22.88
	Mean H4	22.5	57.46	20.20	22.68
	Overall means	*12.4*	*58.20*	*23.06*	*20.30*
Orchard B (HOO)	Mean H1	0	49.90	27.38	11.30
	Mean H2	0	54.14	28.98	14.46
	Mean H3	5	44.46	21.82	14.02
	Mean H4	1.8	51.82	22.42	16.48
	Overall means	*1.7*	*50.08*	*25.16*	*14.06*
Overall means	H1	*3.4*	*53.80*	*26.54*	*13.42*
	H2	*6.1*	*58.44*	*27.54*	*17.30*
	H3	*6.6*	*55.68*	*21.06*	*18.46*
	H4	*12.1*	*54.64*	*21.32*	*19.58*
	df	24	24	24	24
	LSD (H)	5.92	6.49	2.48	6.99
	LSD (O)	4.19	4.59	1.75	4.94

In storage season 2012/13 the influence of harvest date and storage regime on organic acid and sugar profiles was tested on Bramley apples from four orchards. A comparison of organic acid profiles of apples harvested over a four week period from four orchards followed by air storage (4-4.5°C) are detailed in Table 4.3.

Orchard B (EMR) had a significantly higher (P<0.05) incidence of bitter pit than the other orchards investigated. In contrast to the previous season ascorbic acid content was similar in Bramley apples harvested from the bitter pit susceptible orchard EMR and less susceptible Hoo orchard, and both were lower in ascorbic acid than orchards CAR and NEW, while the incidence of bitter pit in orchards CAR, NEW and HOO were similar (~14%) compared to EMR (21.7%) . Malic acid contents were higher for the earliest picked fruit and then declined in subsequent picks across all four orchards. Malic acid was higher (P<0.05) in Bramley apples from EMR orchard. Overall citric acid content increased with later sampling (T3-T4), but no

orchard effects were observed and no trend in oxalic was found. SmartFresh^{SM}-treated apples had significantly lower (P<0.05) incidence of bitter pit than untreated apples. Oxalic acid and malic acid were significantly higher in SmartFresh^{SM}-treated apples and citric acid was lower and there was no difference in ascorbic acid of untreated and SmartFresh^{SM}-treated apples.

Table 4.3: Comparison of mean of organic acids (oxalic, malic, ascorbic and citric acid) of Bramley apples sampled in four dates from harvest (T0: August 2012) and during storage (T1: November 2012, T2: December 2012, T3: January 2013) from four orchards between untreated and SmartFresh^{SM} treated samples stored in air in season 2012/13. LSDs (5%) are for the effects of SmartFresh^{SM} treatment (SF), effects of orchard (O) and effects of various storage sampling (S) n=25.

		Bitter Pit %	Oxalic acid µg/µL	Malic acid µg/µL	Ascorbic acid µg/µL	Citric acid µg/µL
Orchard (CAR)	NoSF	22.2	0.48	13.24	0.17	0.34
	SF	4.8	0.52	14.34	0.16	0.28
	Overall means	*13.5*	*0.50*	*13.79*	*0.17*	*0.31*
Orchard (EMR)	NoSF	34.3	0.47	15.59	0.14	0.38
	SF	9.0	0.53	17.61	0.15	0.31
	Overall means	*21.7*	*0.50*	*16.60*	*0.15*	*0.35*
Orchard (Hoo)	NoSF	24.9	0.50	14.67	0.15	0.28
	SF	2.8	0.56	16.03	0.15	0.24
	Overall means	*13.9*	*0.53*	*15.35*	*0.15*	*0.26*
Orchard (NEW)	NoSF	24.6	0.48	14.96	0.19	0.27
	SF	4.5	0.53	15.88	0.18	0.30
	Overall means	*14.6*	*0.50*	*15.42*	*0.19*	*0.28*
Mean for sampling date	NoSF	*26.49*	*0.48*	*14.62*	*0.16*	*0.32*
	SF	*5.29*	*0.54*	*15.97*	*0.16*	*0.28*
	Sampling T0	*0*	*0.48*	*18.76*	*0.16*	*0.24*
	Sampling T1	*12.15*	*0.50*	*16.04*	*0.15*	*0.26*
	Sampling T2	*16.31*	*0.52*	*15.38*	*0.16*	*0.34*
	Sampling T3	*19.22*	*0.51*	*14.45*	*0.17*	*0.30*
	df	24	24	24	24	24
	LSD (SF)	2.83	0.01	0.50	0.01	0.01
	LSD (O)	4	0.02	0.70	0.01	0.01
	LSD (Sampling)	3.46	0.02	0.61	0.01	0.01

Sugar profiles (fructose, glucose and sucrose) of Bramley apples in season 2011/12 were assessed in fruit from four orchards (Table 4.4).

Table 4.4: Comparison of mean of sugars (fructose, glucose and sucrose) content of Bramley apples sampled in four dates from harvest (T0: August 2012) and during storage (T1: November 2012, T2: December 2012, T3: January 2013) from four orchards between untreated and SmartFresh^{SM} treated samples stored in air in season 2012/13. LSDs (5%) are for the effects of SmartFresh^{SM} treatment (SF), effects of orchard (O) and effects of various storage sampling (S) n=25.

		Bitter Pit %	Fructose µg/µL	Glucose µg/µL	Sucrose µg/µL
Orchard (CAR)	NoSF	22.2	56.74	21.00	20.74
	SF	4.8	64.80	23.27	27.46
	Overall means	*13.5*	*60.77*	*22.134*	*24.1*
Orchard (EMR)	NoSF	34.3	58.42	20.49	24.54
	SF	9.0	64.88	22.49	28.40
	Overall means	*21.7*	*61.65*	*21.489*	*26.47*
Orchard (Hoo)	NoSF	24.9	61.64	21.91	21.94
	SF	2.8	72.30	26.63	25.56
	Overall means	*13.9*	*66.97*	*24.268*	*23.75*
Orchard (NEW)	NoSF	24.6	58.48	21.79	23.66
	SF	4.5	66.32	26.15	23.62
	Overall means	*14.6*	*62.4*	*23.969*	*23.64*
Mean for sampling date	NoSF	*26.49*	*58.82*	*21.30*	*22.72*
	SF	*5.29*	*67.08*	*24.63*	*26.26*
	Sampling T0	*0*	*64.22*	*12.94*	*37.50*
	Sampling T1	*12.15*	*59.48*	*20.82*	*30.20*
	Sampling T2	*16.31*	*65.46*	*23.90*	*24.30*
	Sampling T3	*19.22*	*63.90*	*24.18*	*18.98*
	df	24	24	24	24
	LSD (SF)	2.83	1.77	0.75	1.41
	LSD (O)	4	2.51	1.06	1.99
	LSD (Sampling)	3.46	2.17	0.92	1.72

Overall, glucose content in apples increased with sampling date, while sucrose content declined, suggesting increased hydrolysis of sucrose to glucose and fructose; however, fructose content remained similar. Comparison of sugar profiles between orchards showed sucrose in orchard

(EMR) with more bitter pit was significantly higher. Sucrose between samplings significantly decreased during storage sampling. Fructose, glucose and sucrose were significantly higher in SmartFresh^{SM}-treated apples.

Bramley apples treated with SmartFresh^{SM} from all four orchards stored in (9%CO_2, 12% O_2) for 6 months had less bitter pit in comparison with untreated apples (Table 4.5).

Table 4.5: Comparison of mean of organic acids (oxalic, malic, ascorbic and citric acid) of Bramley apples sampled in four dates from harvest (T0: August 2012) and during storage (T1: November 2012, T2: January 2013 and T3: February 2013) from four orchards between untreated and SmartFresh^{SM} treated samples stored in CA (9%CO_2, 12% O_2) regime in season 2012/13. LSDs (5%) are for the effects of SmartFresh^{SM} treatment (SF), effects of orchard (O) and effects of various storage sampling (S) n=22.

		Bitter Pit %	Oxalic acid µg/µL	Malic acid µg/µL	Ascorbic acid µg/µL	Citric acid µg/µL
Orchard (CAR)	NoSF	13.5	0.51	14.34	0.15	0.23
	SF	2.5	0.51	14.68	0.15	0.23
	Overall means	*8*	0.51	14.51	0.15	0.23
Orchard (EMR)	NoSF	17.5	0.51	16.20	0.13	0.28
	SF	7.5	0.50	15.82	0.14	0.24
	Overall means	*12.5*	0.50	16.01	0.13	0.26
Orchard (Hoo)	NoSF	6.8	0.58	16.66	0.16	0.26
	SF	7.5	0.56	16.56	0.15	0.27
	Overall means	*7.1*	0.57	16.61	0.15	0.26
Orchard (NEW)	NoSF	21	0.53	17.03	0.18	0.29
	SF	4.2	0.53	16.36	0.18	0.27
	Overall means	*12.6*	0.53	16.69	0.18	0.28
Mean for sampling date	NoSF	*14.7*	0.53	16.05	0.16	0.27
	SF	*5.4*	0.53	15.86	0.15	0.25
	Sampling T0	*0*	0.52	16.84	0.15	0.24
	Sampling T1	*8.5*	0.54	15.28	0.15	0.29
	Sampling T2	*16.1*	0.52	16.77	0.16	0.24
	Sampling T3	*11.2*	0.54	14.94	0.15	0.27
	df	21	21	21	21	21
	LSD (SF)	5.07	0.02	0.72	0.01	0.03
	LSD (O)	7.16	0.03	1.02	0.01	0.04
	LSD (S)	7.16	0.03	1.02	0.01	0.04

The influence of orchard on the incidence of bitter pit was only observed in untreated fruit as SmartFresh^{SM} suppressed the incidence of bitter pit. There was a significant difference in incidence of bitter pit between sampling dates with increase in bitter pit with delayed picking

and increase in fruit maturity. Overall ascorbic acid content in fruit from orchard EMR was lower than the other orchards; these results were similar with the data from air stored fruit.

Analysis of sugar (fructose, glucose and sucrose) content showed a similar effect of increasing fruit maturity on increasing glucose and decreasing sucrose in fruit stored in (9%CO_2, 12% O_2). SmartFresh^{SM} had no effect on the changes in sugar profiles during storage that were already present at harvest (Table 4.6).

Table 4.6: Comparison of mean of sugars (fructose, glucose and sucrose) content of Bramley apples sampled in four dates from harvest (T0: August 2012) and during storage (T1: November 2012, T2: January 2013 and T3: February 2013) from four orchards between untreated and SmartFresh^{SM} treated samples stored in (9%CO_2, 12% O_2) regime in season 2012/13. LSDs (5%) are for the effects of SmartFresh^{SM} treatment (SF), effects of orchard (O) and effects of sampling (S) n=22.

		Bitter Pit %	**Fructose µg/µL**	**Glucose µg/µL**	**Sucrose µg/µL**
Orchard (CAR)	NoSF	13.5	66.76	26.46	24.98
	SF	2.5	68.52	27.80	25.82
	Overall means	*8*	*67.64*	*27.14*	*25.40*
Orchard (EMR)	NoSF	17.5	67.10	24.76	30.02
	SF	7.5	67.70	24.24	29.54
	Overall means	*12.5*	*67.40*	*24.50*	*29.78*
Orchard (Hoo)	NoSF	6.8	72.54	27.82	25.02
	SF	7.5	72.32	26.98	25.58
	Overall means	*7.1*	*72.42*	*27.40*	*25.30*
Orchard (NEW)	NoSF	21	68.18	28.78	24.52
	SF	4.2	68.96	30.74	22.88
	Overall means	*12.6*	*68.56*	*29.76*	*23.70*
Mean for sampling date	NoSF	*14.7*	*68.64*	*26.96*	*26.14*
	SF	*5.4*	*69.38*	*27.44*	*25.96*
	Sampling T0	*0*	*66.08*	*25.30*	*31.88*
	Sampling T1	*8.5*	*71.74*	*28.72*	*21.34*
	Sampling T2	*16.1*	*67.12*	*26.12*	*30.54*
	Sampling T3	*11.2*	*71.06*	*28.68*	*20.40*
	df	21	21	21	21
	LSD (SF)	5.07	2.82	1.37	2.29
	LSD (O)	7.16	3.98	1.94	3.23
	LSD (S)	7.16	3.98	1.94	3.23

Bramley apples from the four orchards stored in (5%CO_2, 1% O_2) had significantly less bitter pit than fruit stored in air and (9% CO_2, 12% O_2) and SmartFresh^{SM} did not reduce the incidence further over the 9 months of storage (Table 4.7).

Overall, the amount of malic acid decreased with each sampling occasion out of store. There was no effect of sampling date on oxalic, citric and ascorbic acid in (5% CO_2, 1% O_2) storage and SmartFresh^{SM} did not affect the proportion of sugars or acids of Bramley apples stored in regime (5% CO_2, 1% O_2).

Table 4.7: Comparison of mean of organic acids (oxalic, malic, ascorbic and citric acid) of Bramley apples sampled in four dates from harvest (T0: August 2012) and during storage (T1: December 2012, T2: February 2013 and T3: April 2013) from four orchards between untreated and SmartFresh^{SM} treated samples stored in (5%CO_2, 1% O_2) regime in season 2012/13. LSDs (5%) are for the effects of SmartFresh^{SM} treatment (SF), effects of orchard (O) and effects of sampling (S) n=39.

		Bitter Pit %	Oxalic acid µg/µL	Malic acid µg/µL	Ascorbic acid µg/µL	Citric acid µg/µL
Orchard (CAR)	NoSF	18.3	0.55	14.32	0.13	0.24
	SF	6.3	0.55	13.85	0.14	0.23
	Overall means	*12.3*	*0.55*	*14.08*	*0.13*	*0.24*
Orchard (EMR)	NoSF	12.8	0.53	14.52	0.15	0.26
	SF	14.5	0.53	14.51	0.15	0.25
	Overall means	*13.7*	*0.53*	*14.51*	*0.15*	*0.25*
Orchard (Hoo)	NoSF	9.5	0.55	15.24	0.16	0.27
	SF	6.7	0.56	14.59	0.16	0.25
	Overall means	*8.1*	*0.56*	*14.91*	*0.16*	*0.26*
Orchard (NEW)	NoSF	16	0.57	15.11	0.15	0.26
	SF	13.8	0.55	14.97	0.15	0.26
	Overall means	*14.9*	*0.56*	*15.04*	*0.15*	*0.26*
Mean for sampling date	NoSF	*14.2*	*0.55*	*14.80*	*0.15*	*0.26*
	SF	*10.3*	*0.55*	*14.48*	*0.15*	*0.25*
	Sampling T0	*8.5*	*0.52*	*16.84*	*0.15*	*0.24*
	Sampling T1	*11.2*	*0.53*	*15.69*	*0.15*	*0.25*
	Sampling T2	*15.9*	*0.56*	*14.74*	*0.15*	*0.26*
	Sampling T3	*9.7*	*0.56*	*13.48*	*0.15*	*0.25*
	df	38	38	38	38	38
	LSD (SF)	6.73	0.02	0.65	0.02	0.02
	LSD (O)	9.51	0.03	0.92	0.02	0.02
	LSD (S)	8.24	0.02	0.79	0.02	0.02

The sucrose content of Bramley apples declined with sampling occasion and glucose increased in line with apples stored in air and (9% CO_2, 12% O_2) storage regimes. SmartFresh^{SM} did not alter the profiles of sugars during storage but did reduce the development of bitter pit (Table 4.8).

Table 4.8: Comparison of mean of sugars (fructose, glucose and sucrose) content of Bramley apples sampled in four dates from harvest (T0: August 2012) and during storage (T1: December 2012, T2: February 2013 and T3: April 2013) from four orchards between untreated and SmartFresh^{SM} treated samples stored in (5%CO_2, 1% O_2) regime in season 2012/13. LSDs (5%) are for the effects of SmartFresh^{SM} treatment (SF), effects of orchard (O) and effects of sampling (S) n=39.

		Bitter Pit %	**Fructose µg/µL**	**Glucose µg/µL**	**Sucrose µg/µL**
Orchard (CAR)	NoSF	18.3	68.32	28.44	16.94
	SF	6.3	69.12	28.84	16.96
	Overall means	*12.3*	*68.70*	*28.64*	*16.96*
Orchard (EMR)	NoSF	12.8	65.28	28.92	14.66
	SF	14.5	65.32	27.98	13.92
	Overall means	*13.7*	*65.30*	*28.44*	*14.30*
Orchard (Hoo)	NoSF	9.5	68.50	30.06	14.64
	SF	6.7	67.18	29.44	13.92
	Overall means	*8.1*	*67.84*	*29.76*	*14.28*
Orchard (NEW)	NoSF	16	68.44	29.22	14.76
	SF	13.8	68.56	29.12	15.74
	Overall means	*14.9*	*68.50*	*29.18*	*15.24*
Mean for sampling date	NoSF	*14.2*	*67.64*	*29.16*	*15.26*
	SF	*10.3*	*67.54*	*28.84*	*15.14*
	Sampling T0	*8.5*	*66.08*	*25.30*	*31.88*
	Sampling T1	*11.2*	*64.88*	*26.42*	*23.00*
	Sampling T2	*15.9*	*69.38*	*30.02*	*13.86*
	Sampling T3	*9.7*	*68.52*	*30.56*	*8.72*
	df	38	38	38	38
	LSD (SF)	6.73	1.40	1.37	2.00
	LSD (O)	9.51	1.99	1.94	2.82
	LSD (S)	8.24	1.72	1.68	2.44

From the initial analysis across seasons and storage regimes it was clear that changes in sucrose, glucose, ascorbic and malic acid content were linked to changes in fruit maturity either at

harvest or related to changes in maturation during storage. The graphs (4.1, 4.2, 4.3 and 4.4) below show storage regimes have impacted on the concentration of these metabolites during storage.

Ascorbic acid content was higher in air stored fruit than CA (9% CO_2, 12% O_2) and (5% CO_2, 1% O_2) and SmartFresh^{SM} had no effect on ascorbic acid (Figure 4.1).

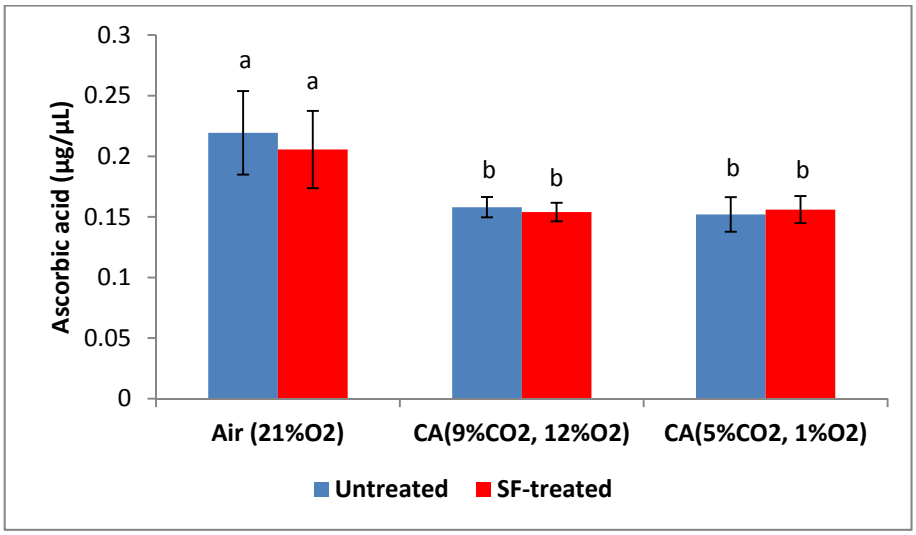


Figure 4.1: Comparison of changes in ascorbic acid contents of untreated and SmartFresh^{TM} treated samples of Bramley apples in different storage regimes: air (21%O_2) and CA regimes (9% CO_2, 12% O_2) and (5% CO_2, 1% O_2). Each data point is the mean of several selected samples from seasons 2011-13, each one consisted of ten apple samples ± SE. Mean values with different letters were significantly different according to Tukey's test (p<0.05).

Fructose content of untreated air-stored Bramley apples were significantly lower than fruit treated with SmartFresh^{SM} moreover, storing fruit in CA alone prevented the loss of fructose seen in air-stored fruit and in these cases the addition of SmartFresh^{SM} did not affect the pool of fructose. (Figure 4.2).

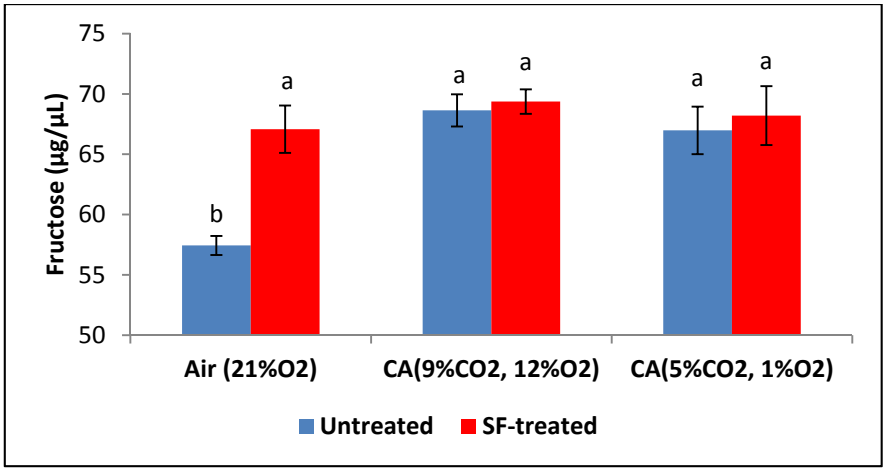


Figure 4.2: Comparison of changes in fructose contents of untreated and SmartFresh^{SM} treated samples of Bramley apples in different storage regimes: air (21%O_2) and CA regimes (9% CO_2, 12% O_2) and (5% CO_2, 1% O_2). Each data point is the mean of several selected samples from seasons 2011-13, each one consisted of ten apple samples ± SE. Mean values with different letters were significantly different according to Tukey's test (p<0.05).

A similar pattern in glucose as for fructose was observed with untreated air-stored Bramley apples having lower glucose than the SmartFresh^{SM} counterparts and CA storage alone prevented the drop in glucose observed in air-stored fruit (Figure 4.3).

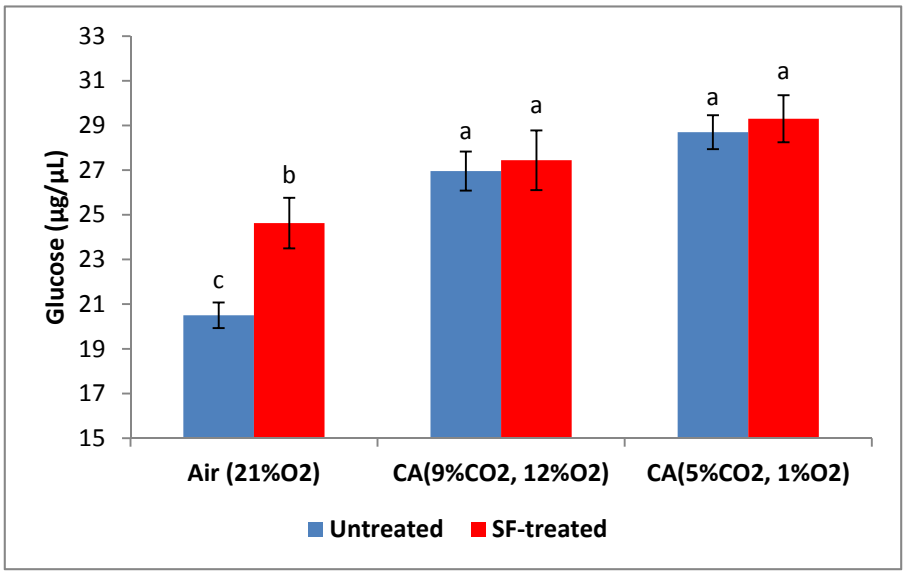


Figure 4.3: Comparison of changes in glucose contents of untreated and SmartFresh^{SM} treated samples of Bramley apples in different storage regimes: air (21%O_2) and CA regimes (9% CO_2, 12% O_2) and (5% CO_2, 1% O_2). Each data point is the mean of several selected samples from seasons 2011-13, each one consisted of ten apple samples ± SE. Mean values with different letters were significantly different according to Tukey's test (p<0.05).

For sucrose, Bramley apples stored in (5% CO_2, 1% O_2) were significantly lower in sucrose than air-stored or CA-stored (9% CO_2, 12% O_2) fruit. SmartFresh^{SM} had a limited effect on sucrose reducing the decline observed in air-stored fruit. However storage time in (5% CO_2, 1% O_2) was longer, also in all storage regimes there was no significant difference in sucrose content of untreated and SmartFresh^{SM} treated samples (Figure 4.4).

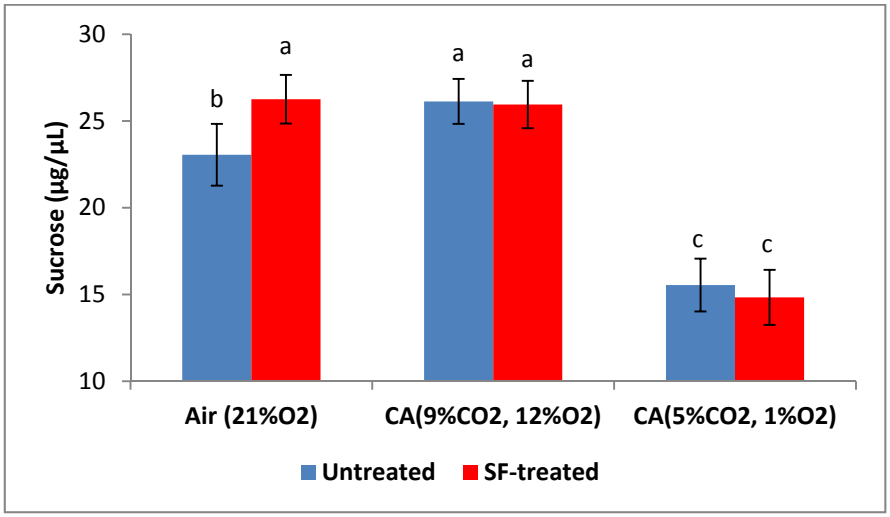


Figure 4.4: Comparison of changes in sucrose contents of untreated and SmartFresh^{SM} treated samples of Bramley apples in different storage regimes: air (21%O_2) and CA regimes (9% CO_2, 12% O_2) and (5% CO_2, 1% O_2). Each data point is the mean of several selected samples from seasons 2011-13, each one consisted of ten apple samples ± SE. Mean values with different letters were significantly different according to Tukey's test (p<0.05).

In the final storage season of 2013/14, a comparison of changes in organic acid profiles of Bramley apples from two orchards (orchard A-EMR and orchard B-Hoo) was undertaken at harvest (September 2013) and subsequently from air-stored samples taken at two-weekly intervals from October -November (2013). Both untreated and SmartFresh^{SM}-treated samples were included in the trial. The incidence of bitter pit in orchard A (EMR) was 19% and followed similar trends observed in previous years, while orchard B (Hoo) showed no sign of bitter pit. SmartFresh^{SM} treatments reduced the incidence (5.4%) of bitter pit in orchard A (Table 4.9).

Cortex samples taken from the stalk and calyx ends of fruit were analysed for organic acids and sugars, however, although no changes in acid profiles were found between the two opposing regions of fruit, changes in ascorbic, oxalic, citric or malic acid content between fruit sampled at harvest and repeat sampled after 2 months of air storage (4-4.5°C) were observed.

Table 4.9: Comparison of mean of organic acids (oxalic, malic, ascorbic and citric acid) of Bramley apples sampled in five dates from harvest (T0: 2/9/2013) and during storage (T1: 1/10/2013, T2: 15/10/2013, T3: 1/11/2013 and T4: 15/11/2013) between two orchards as more susceptible to bitter pit (orchard A-EMR) and less susceptible to bitter pit (orchard B-Hoo) and between untreated and SmartFresh^{SM} treated samples stored in air (21% O_2) regime in season 2013/14. LSDs (5%) are for the effects of SmartFresh^{SM} treatment (SF), effects of orchard (O), effects of sampling (S) and position (P) n=32.

		Bitter Pit %	Oxalic acid µg/µL	Malic acid µg/µL	Ascorbic acid µg/µL	Citric acid µg/µL
Orchard A (EMR)	NoSF	19.2	0.38	18.46	0.32	0.10
	SF	5.4	0.36	17.40	0.30	0.20
	Overall means	*12.3*	*0.37*	*17.92*	*0.31*	*0.15*
Orchard B (Hoo)	NoSF	0	0.32	20.82	0.36	0.22
	SF	0	0.30	18.14	0.31	0.15
	Overall means	*0*	*0.31*	*19.48*	*0.34*	*0.19*
Overall means	NoSF	*9.6*	*0.35*	*19.64*	*0.34*	*0.16*
	SF	*2.7*	*0.33*	*17.78*	*0.31*	*0.17*
	Calyx	*6.2*	*0.35*	*19.24*	*0.33*	*0.15*
	Stalk	*6.2*	*0.34*	*18.16*	*0.31*	*0.18*
	Sampling T0	*0*	*0.36*	*21.32*	*0.37*	*0.11*
	Sampling T1	*1.8*	*0.40*	*22.16*	*0.38*	*0.22*
	Sampling T2	*5*	*0.37*	*18.68*	*0.32*	*0.11*
	Sampling T3	*10.8*	*0.28*	*15.70*	*0.27*	*0.29*
	Sampling T4	*13.3*	*0.29*	*15.66*	*0.27*	*0.11*
	df	31	31	31	31	31
	LSD (SF)	6.34	0.08	3.08	0.05	0.13
	LSD (O)	6.34	0.08	3.08	0.05	0.13
	LSD (S)	10.02	0.12	4.86	0.08	0.20
	LSD (P)	6.34	0.08	3.08	0.05	0.13

4.2.1 Relationship between incidence of bitter pit and organic acids:

The relationships between the incidence of bitter pit in Bramley apples with ascorbic, malic, oxalic and citric acid were compared using correlations for the data collected over three storage seasons. Ascorbic acid was the only organic acid analysed that produced a significant ($P<0.001$, Two-Tailed test) inverse correlation R=-0.67 (2011-12), R=-0.60 (2012-13) and R=-0.59 (2013-14) with the incidence of bitter pit. However, oxalic acid provided a weak correlation with bitter pit over the three seasons tested R=0.35 (20011/12), R= 0.21 (2012/13) and R= 0.37

(2013/14). There was no relationship with citric acid and malic acid content and incidence of bitter pit in any season.

However, changes in untreated samples and samples treated with SmartFresh^{SM} were different. Application of SmartFresh^{SM} to Bramley apple samples reduced the incidence of bitter pit but did not affect ascorbic acid content which varied between 0.11-0.23µg/µL (Table 4.3). Figure.4.5 shows comparison of these changes from orchard (A-EMR and B-Hoo) from harvest (28/8/2012) and four months storage in air (4-4.5°C). SmartFresh^{SM} –treated samples had a delayed development of bitter pit and lowered the overall amount of the ascorbic acid content of fruit while fluctuating during storage showed no significant difference between untreated and SmartFresh^{SM}-treated fruit. However, there was a highly significant inverse correlation (R= -0.97) between incidence of bitter pit and ascorbic acid changes in untreated samples.

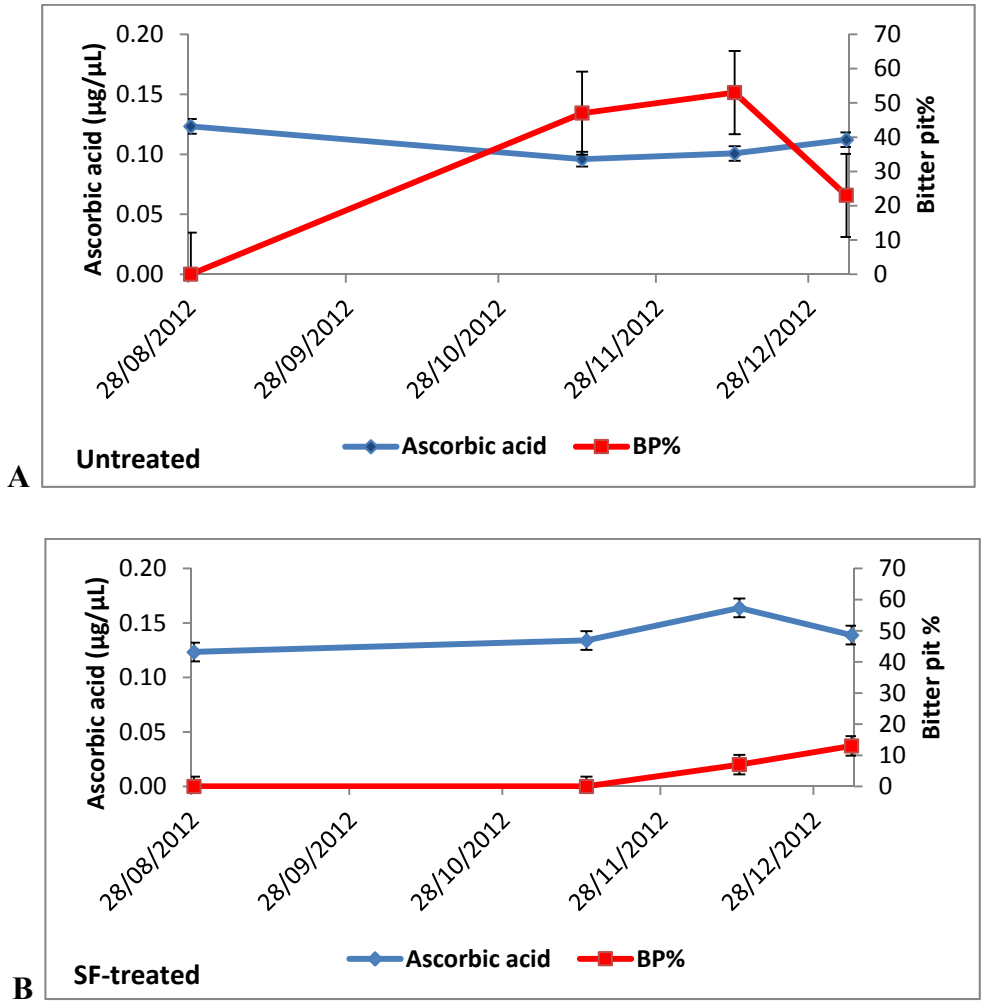


Figure 4.5: Comparison of the relationship between changes in ascorbic acid during air storage (4-4.5°C) in untreated samples (A) and samples treated with SmartFresh^{SM} (B). Each data point is the mean of a ten apples ascorbic acid reading.

In an attempt to determine whether the concentration of ascorbic acid at harvest could be used to help predict the propensity of orchard consignments to develop bitter pit in air-storage the following equation was used to normalise data (Table 4.10).

When the percentage of ascorbic acid content during storage ($\boldsymbol{X_n}$) decreases more than 10% of ascorbic acid content at harvest ($\boldsymbol{X_H}$) , there is more chance of incidence of bitter pit:

$$\boldsymbol{T} = \left(\frac{X_n \times 100}{X_H}\right) \geq 90$$

Table 4.10: Equation for normalising value of ascorbic acid during storage to harvest time and threshold for incidence of bitter pit.

$\boldsymbol{X_n}$	Ascorbic acid during storage
$\boldsymbol{X_H}$	Ascorbic acid at harvest time
$\boldsymbol{T_{acid}} = \left(\frac{X_n \times 100}{X_H}\right) \geq 90$	Threshold of ascorbic acid for incidence of bitter pit (10% less)

Table 4.11 shows the comparison of results of two orchards one generating fruit with a historically high incidence of bitter pit orchard A (EMR) and a commercial planting where fruit were less susceptible orchard B (Hoo) to bitter pit in untreated and SmartFresh^{SM} treated samples.

The formula was derived on percentage of ascorbic acid found during storage compared to the ascorbic acid content present at harvest and the threshold of ascorbic acid for incidence of bitter pit was 10% less than harvest. When the normalised ascorbic acid content is less than 90 % of ascorbic acid at harvest there is chance of incidence of bitter pit.

On occasions the formula based on ascorbic acid data was unable to predict with accuracy the potential for samples to develop bitter pit and generated a false results.

Table 4.11: Comparison of normalised value of ascorbic acid during storage to harvest time in two orchards in storage season 2012/13 in two different storage regimes: air (21%O_2) and CA (5%CO_2, 1%O_2).

Orchard	Date	NoSF/SF	Regime	BP%	Ascorbic acid µg/5µL	T_{acid}	Threshold NoBP: $T_{acid} \geq 90$
EMR	28/08/2012	Harvest	Air	0	0.62	100.0	Harvest
EMR	11/12/2012	NoSF	Air	20	0.52	83.9	(BP) T≤90
EMR	13/02/2013	NoSF	Air	47	0.60	96.7	(BP)T≥90(false+)
EMR	11/04/2013	NoSF	Air	23	0.59	95.2	(BP)T≥90(false+)
EMR	11/12/2012	SF	Air	0	0.66	106.45	(NoBP) T≥90
EMR	13/02/2013	SF	Air	13	0.74	119.7	(BP)T≥90(false+)
EMR	11/04/2013	SF	Air	0	0.68	111.0	(NoBP) T≥90
Hoo	28/08/2012	Harvest	Air	0	0.82	100.0	Harvest
Hoo	11/12/2012	NoSF	Air	0	0.78	95.3	(NoBP) T≥90
Hoo	13/02/2013	NoSF	Air	17	0.66	80.9	(BP) T≤90
Hoo	11/04/2013	NoSF	Air	10	0.64	78.4	(BP) T≤90
Hoo	11/12/2012	SF	Air	0	0.74	90.4	(NoBP) T≥90
Hoo	13/02/2013	SF	Air	7	0.64	77.6	(BP) T≤90
Hoo	11/04/2013	SF	Air	3	0.69	84.2	(BP) T≤90
EMR	28/08/2012	Harvest	CA	0	0.74	100.00	Harvest
EMR	13/11/2012	NoSF	CA	17	0.51	69.36	(BP) T≤90
EMR	13/12/2012	NoSF	CA	27	0.76	102.34	(BP)T≥90(false+)
EMR	04/01/2013	NoSF	CA	47	0.55	74.70	(BP) T≤90
EMR	13/11/2012	SF	CA	0	0.85	115.55	(NoBP) T≥90
EMR	13/12/2012	SF	CA	17	0.64	86.87	(BP) T≤90
EMR	04/01/2013	SF	CA	17	0.74	100.86	(BP)T≥90(false+)
Hoo	28/08/2012	Harvest	CA	0	0.83	100.00	Harvest
Hoo	13/11/2012	NoSF	CA	7	0.54	65.47	(BP) T≤90
Hoo	13/12/2012	NoSF	CA	17	0.74	89.31	(BP) T≤90
Hoo	04/01/2013	NoSF	CA	50	0.77	92.41	(BP)T≥90(false+)
Hoo	13/11/2012	SF	CA	0	0.80	96.68	(NoBP) T≥90
Hoo	13/12/2012	SF	CA	0	0.81	98.19	(NoBP) T≥90
Hoo	04/01/2013	SF	CA	7	0.73	88.47	(BP) T≤90

When $\boldsymbol{T_{acid}} \geq 90$ but there is incidence of bitter pit is named "false positive (+)" or $\boldsymbol{T_{acid}} < 90$ but there is no bitter pit which is named as "false negative (-)". For evaluating the reliability of the equation and prediction tool a number of false positive and negatives were compared with number of correct estimates in a contingency table and there was 25% error in recognising samples with bitter pit by ascorbic acid normalised equation (Table 4.12).

Table 4.12: Contingency table for Chi square test of the threshold to find false∓ and misses in comparison of threshold and incidence of bitter pit.

	No BP	BP	Total
$\boldsymbol{T_{acid}}$ <90	0	11	11
$\boldsymbol{T_{acid}}$ ≥90	7	6	13
Total	7	17	24

	False (numbers)	False (Percentage)
False positive (+): Formula($\boldsymbol{T_{acid}}$ ≥90) & BP(+)	6	35.3%
False negative (-): Formula($\boldsymbol{T_{acid}}$ <90) & BP(-)	0	0%
Total misses	6	25%

	Number of samples	Number of samples	Percentage
Total misses	6	False+: 6	25%
		False -: 0	0%
Total hits	18	Correct BP: 7	29.2%
		Correct NoBP: 11	45.8%

In fruit from orchard A (EMR) the prediction model produced more false positive results of bitter pit in both storage regimes (air and CA) in comparison with the orchard which was less susceptible to bitter pit.

The model may only be linear over a narrow range of bitter pit incidence and where severity is high, changes in ascorbic acid content are limited, and moreover, other factors may also influence severity. As bitter pit forms very localised clusters of damage cells scattered within the apple surrounded by healthy tissue it may be difficult to accurately sample tissue for organic acid or sugar analysis that represents localised changes in tissue health.

In storage season 2013/14 it was decided to take samples for acid measurements from calyx end and stalk end of apple fruit to have a comparison and find out the difference of organic acids in these parts of apple fruit and their correlation with incidence of bitter pit. the relationship between ascorbic acid and bitter pit was higher (Figure 4.6) when the ascorbic acid content of calyx region (R=-0.88) of fruit was compared with bitter pit incidence, while stalk end ascorbic acid content was less well correlated with bitter pit (R=-0.59).

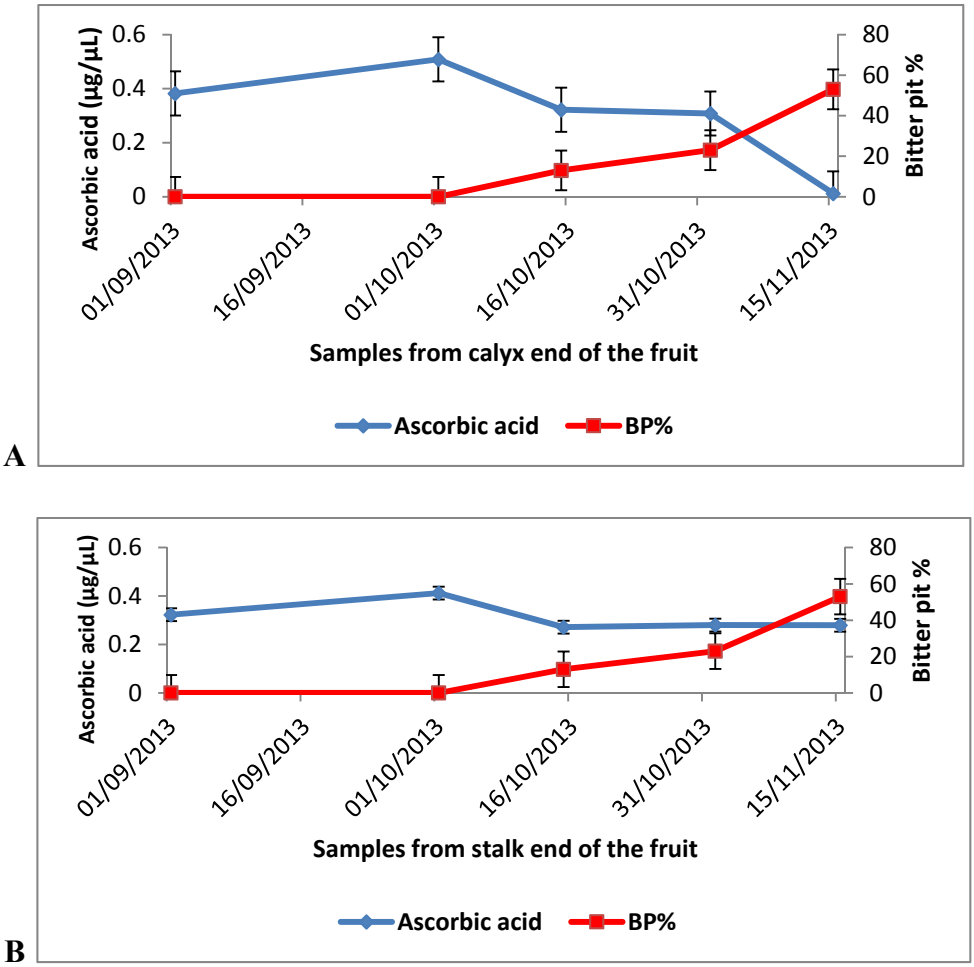


Figure 4.6: Comparison of ascorbic acid changes in samples taken from calyx (A) and stalk (B) end of Bramley's apple fruit and the relationship with incidence of bitter pit in season 2013/14 in the first 10 weeks storage in air (21% O_2).

In season (2013/14) samples collected from orchard B (Hoo) which was less susceptible to bitter pit, did not develop bitter pit during storage so it was impossible to find a correlation with ascorbic acid profiles. Although results showed higher correlation between ascorbic acid from calyx end in comparison with stalk end and better results than previous seasons where samples were taken from whole fruit, this still needs more investigation with a greater number of samples in different orchards and storage regimes.

4.2.2 Relationship between incidence of bitter pit and sugars:

The relationship between the incidence of bitter pit and fructose, glucose and sucrose was investigated in 2011/12 and 2012/13. In the first year (2011/12) no correlation was found between sugars and the incidence of bitter pit. However, in the second year (2012/13) a weak inverse correlation (R=-0.35) was found between sucrose and the incidence of bitter pit at $P<0.01$ (Two-Tailed test), although there was no correlation between glucose and fructose contents and bitter pit. The correlation between sucrose and bitter pit was variable between orchard consignments and where SmartFresh^{SM} was applied the lack of bitter pit reduced the relationship further.

Sucrose concentrations in Bramley apples tend to drop after 3-4 months in air storage (4-4.5°C) at the stage when the incidence of bitter pit increases in untreated fruit.

4.3 Mineral analysis:

A comparison of whole fruit analysis of minerals versus selected analysis of tissue from the intercarpel (inner cortex) regions and outer cortex are presented below.

In the first year (2010/11), samples for mineral analysis were a composite of inner and outer cortex with the skin and seeds removed, following industry standard procedures (Defra Best Practice Guide to Apple Production and Storage 2002).

In most cases calcium content of fruit at harvest in 2010/11, was above the minimum target (4.5 mg/100g) required for long-term storage.

A similar total calcium content was found in untreated tissue samples exhibiting bitter pit symptoms and symptomless apples, removed from 9% CO_2, 12% O_2 (4-4.5°C) after 3 months of storage. The average incidence of bitter pit in samples from untreated fruit was 20% while in the corresponding SmartFresh^{SM}-treated samples it was 5%. As expected the calcium content of fruits sampled at harvest +/- SmartFresh^{SM} were the same (~6.5 mg 100 g^{-1}FW). After 3 months storage the calcium content in untreated fruit was lower (4 mg 100 g^{-1}FW), while calcium content of fruit treated with SmartFresh remained the same as fruit sampled at harvest. In addition samples were taken from symptomless fruits (untreated or treated) after 3 months storage and calcium content analysed , in this case total calcium content was lower (3-4 mg 100 g^{-1}FW), than the counterpart material sampled at harvest (~6.5 mg 100 g^{-1}FW).

As with calcium, a comparison of potassium content in fruit at harvest was made with symptomless and bitter pit affect fruit after 3 months harvest. Fruit potassium content at harvest was (65 mg $100g^{-1}$ FW) below the maximum permissible content (150 mg $100g^{-1}$ FW), suggesting that potassium was not exerting a significant antagonistic effect on calcium in fruit. In fruit showing no signs of bitter pit after storage, the potassium content was similar to fruit at harvest (65 mg $100g^{-1}$ FW) but in fruit with bitter pit higher fruit potassium (120 mg $100g^{-1}$ FW) was recorded.

Multiple regression analysis to consider the influence of minerals on bitter pit showed a significant (*P*-value <0.05) effect for Ca (0.02), N (0.001), K (0.02), Mg (0.03) and N/Ca (0.03). The model indicated a significant relationship (R^2=0.96) between these elements and incidence of bitter pit (Appendix VII).

The relationship between the ratio of N/Ca and (K+Mg)/Ca and incidence of bitter pit were compared. When the calcium/Ca increased the incidence of bitter pit in apples increased with R=0.71 (P<0.05). In addition, when the ratio of calcium antagonists (K+Mg) increased in proportion to Ca there was an increase in bitter pit R=0.58 (P<0.05). The influence of nitrogen alone on bitter pit incidence also showed a significant correlation R=0.86 (P<0.001).

In subsequent years (2011/12 and 2012/13) tissue samples for mineral analysis were collected from the inner cortex and outer cortex of fruit to provide information regarding the re-distribution of minerals in the fruit during storage and how this may relate to the outer cortex and calyx region of apple where the incidence of bitter pit is highest. Mineral analysis results were correlated with the incidence of bitter pit during storage for untreated and SmartFresh^{SM} treated samples. No bitter pit was found at harvest, but averaged 13% in untreated samples after 3 months storage, 10% after 5 months storage and increased during a week's shelf-life to 20%. The calcium content was higher in the inner cortex at harvest compared to the outer cortex and this trend was observed during storage and shelf-life sampling. A small decline in total calcium in the inner cortex was recorded during storage and possibly the result of redistribution to the outer cortex. No relationship between total calcium content and the incidence of bitter pit was observed. No difference in untreated and SmartFresh^{SM} –treated fruit was observed (Figure 4.7)

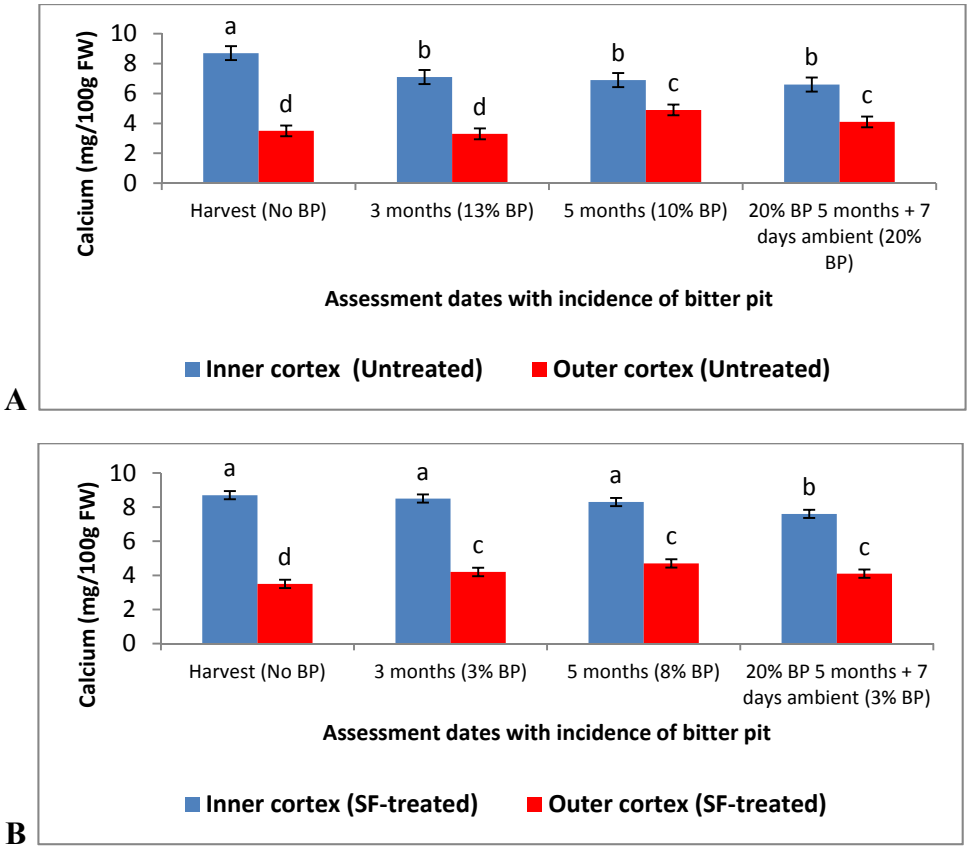


Figure 4.7: Comparison of changes in calcium in inner and outer cortex of A) untreated and B) treated samples with SmartFresh^{SM} of Bramley apples stored at (9% CO_2, 12% O_2), and incidence of bitter pit (BP) in season 2011/12. Each data point is the mean of ten apple samples ± SE. Mean values with different letters were significantly different according to Tukey's test (p<0.05).

While the relationship between total calcium content of Bramley apples and incidence of bitter pit was non-linear, the bioavailability of calcium within tissue may be more important than total calcium. De Freitas *et al* (2013) indicated under some conditions fruit cortical tissue had a higher capacity to strongly bind Ca^{2+} ions in the water-insoluble pectin network in the cell wall matrix, reducing the levels of Ca^{2+} available for other cellular functions. In these conditions, the proportion of freely available Ca^{2+} (non-bound Ca^{2+}) may be more important than total Ca^{2+} concentration in contributing to tissue health. Moreover, Ca^{2+} inactivation by binding to oxalate, phosphate and phytenes can reduce calcium's protective role. As explained in chapter 2 (2.3.2) a method was developed for measuring the proportion of calcium oxalate verses total calcium of apple samples to investigate the relationship of total Ca^{2+} and calcium oxalate in relation to the incidence of bitter pit. Because of limits in time and application of equipment and materials 30 samples from two orchards identified as more susceptible (EMR) and less susceptible (Hoo) to bitter pit, were selected from seasons (2011/12) and (2012/13). Samples were taken from different parts of fruit cortex (Inner/Outer) at harvest and during storage for

measuring total Ca^{2+} and Ca $(COO)_2$ (calcium oxalate). The difference in concentration of minerals (Ca^{2+}, K, Mg & B) sampled from the inner and outer cortex of samples in seasons 2011/12 and 2012/13 (Appendix VIII a) were highly significant (P <0.001). Mineral analysis results were correlated with the incidence of bitter pit during storage for SmartFresh^{SM} treated (SF) and untreated (No SF) Bramley apples. A comparison of mineral analysis profiles between untreated and SmartFresh^{SM}-treated fruit showed that in the majority of cases no difference in mineral profiles were seen between treatments, with the exception that total calcium content in the inner core of apples was lower in SmartFresh^{SM} -treated Bramley apples. Calcium oxalate concentration was similar in both untreated and treated (Table 4.13).

Table 4.13 : Comparison of mean of mineral (Ca^{2+} total, Ca $(COO)_2$, Mg, K and B) concentrations in untreated and SmartFresh^{SM} treated samples and minerals distribution in inner and outer cortex of selected samples collected in seasons 2011/12 and 2012/13.

		Bitter Pit %	Ca^{2+} (total) mg/100g	Ca $(COO)_2$ mg/100g	Mg mg/100g	K mg/100g	B Mg/100g
No SF	Whole fruit	21.1± 7.28					
	Inner cortex		8.1	2.1	5.9	156.6	0.40
	SE		*± 0.80*	*± 0.24*	*± 0.23*	*± 7.95*	*± 0.10*
	Outer cortex		4.0	1.6	4.5	91.5	0.2
	SE		*±0.42*	*±0.17*	*±0.23*	*±9.69*	*±0.07*
SF	Whole fruit	4.25± 1.45					
	Inner cortex		6.2	1.9	5.3	170.6	0.4
	SE		*±0.82*	*±0.38*	*±0.56*	*±11.20*	*±0.12*
	Outer cortex		3.7	1.3	4.5	109.2	0.2
	SE		*±0.05*	*±0.14*	*±0.15*	*±2.96*	*±0.11*
***P* value**	Treatment (NoSF vs SF)	0.002	0.023	0.03	0.09	0.054	0.67
	cortex (Inner vs Outer)		<0.001	<0.001	<0.001	<0.001	0.03
	Treatment: Sample cortex		0.09	0.053	0.11	0.570	0.95

The distribution of calcium was higher in the inner cortex than the outer cortex. Interestingly the inner cortex of SmartFresh^{SM} fruit had lower calcium content than untreated fruit. However, in the outer cortex zone where most bitter pit is observed concentration of calcium between untreated and SmartFresh^{SM} -treated were similar (Figure 4.8).

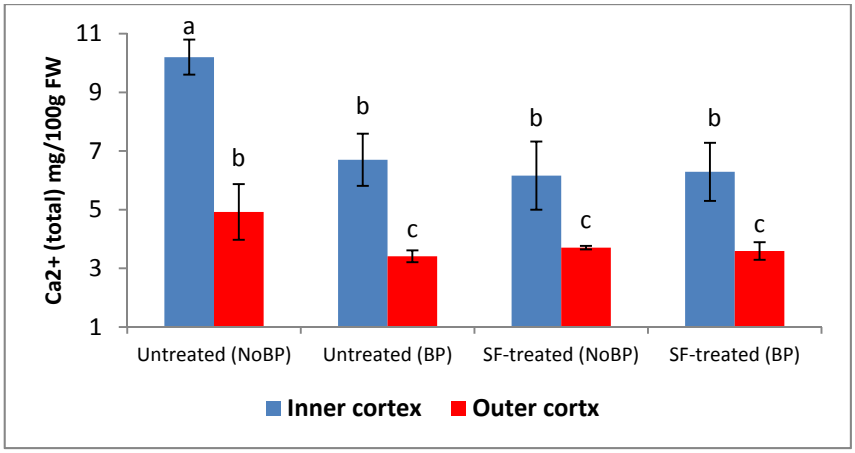


Figure 4.8: Changes in calcium (total) in inner and outer cortex of untreated and treated samples with SmartFresh^{SM} of Bramley apples when there is no symptom of bitter pit (NoBP) and incidence of bitter pit (BP) during 4 months storage in air (21%O_2). Each data point is the mean of several selected samples from seasons 2011/12 and 2012/13 each one consisted of ten apple samples ± SE. Mean values with different letters were significantly different according to Tukey's test (p<0.05).

In season (2011/12) the calcium profile of apple samples from contrasting orchards were analysed for total calcium and calcium oxalate from the inner and outer cortex. Averaged across both orchards the inner cortex samples had higher total calcium, magnesium, potassium and boron content, a reflection in part of the higher cell density of tissues located within the inter-carpel region. Comparison of inner cortex total calcium from both orchards were similar, however, the outer cortex calcium was lower in the susceptible orchard (EMR). While the total amount of calcium oxalate in the inner and outer cortex regions between orchards were similar, the bitter pit prone (EMR) fruit had less total calcium and the proportion of calcium oxalate/total calcium was higher in the fruit from orchard (EMR) outer cortex samples compared to orchard (Hoo) where bitter pit was in lower (Table 4.14) . Moreover, looking at changes in calcium oxalate as a proportion of total calcium, the overall proportion (averaged across orchards) of calcium oxalate increased between the initial sampling at harvest compared with samples taken after 2 months of storage. This suggests that changes in the availability of calcium for membrane stabilisation may over time contribute to bitter pit development.

Table 4.14: Comparison of mean of calcium (total), calcium oxalate and calcium oxalate as a percentage of total calcium in Bramley apples sampled from two orchards classified as more susceptible to bitter pit (orchard A-EMR) and less susceptible to bitter pit (orchard B-Hoo) sampled one time at harvest (T0: September 2011) and during storage (T1: November 2011). Samples were taken from inner and outer cortex of fruit. LSDs (5%) are for the effects of sampling from different parts of cortex (C), orchard (O) and effects of sampling dates (S) n=4.

		Ca^{2+} (total) mg/100g	**$Ca(COO)_2$ mg/100g**	**%Calcium Oxalate**
Orchard A (EMR)	Inner cortex	10.9	2.35	21.3
	Outer cortex	4.9	1.65	35.2
	Overall means	*7.9*	*2*	*28.3*
Orchard B (Hoo)	Inner cortex	10.15	2.45	24.3
	Outer cortex	6.25	1.35	20.1
	Overall means	*8.2*	*1.9*	*22.2*
Overall means	Inner cortex	*10.53*	*2.4*	*22.8*
	Outer cortex	*5.57*	*1.5*	*27.7*
	Sampling T0	*8.3*	*1.55*	*18.6*
	Sampling T1	*7.8*	*2.35*	*31.9*
	df	3	3	3
	LSD (C)	2.77	1.57	13.53
	LSD (O)	2.77	1.57	13.53
	LSD (S)	2.78	1.57	13.53

Analysis of total calcium and calcium oxalate from the same orchards in the subsequent year (2012/13) confirmed the higher amount of calcium in the inner cortex at harvest (T0) and during storage (T1-2). The amount of total calcium in the inner cortex averaged across both orchards declined during storage however, no increase was seen in the outer cortex suggesting either differences were due to sampling error or calcium was redistributed to other parts of the fruit (core, skin or vascular regions).

In 2012/13 the total calcium content of both orchards were similar but lower (~5 mg 100 g^{-1}) than the previous year (~8 mg 100 g^{-1}). Overall the amount of calcium oxalate in the inner cortex were higher than the outer cortex, but the percentage of calcium oxalate in the outer cortex as a proportion of total calcium was higher in this year. Although the incidence of bitter pit was different between orchards, no significant difference in calcium and calcium oxalate of samples taken from each were seen.

Table 4.15 confirms the previous year's observations that the total calcium content of the inner cortex was higher than the outer cortex. The amount of calcium oxalate was higher in the inner cortex, although, the percentage of calcium as calcium oxalate was greater (42.7%) in the outer

cortex compared to the inner cortex (30.4%), No significant change in calcium oxalate was observed during storage.

Table 4.15: Comparison of means of calcium (total), calcium oxalate and % of total calcium in the form of calcium oxalate in Bramley apples sampled from two orchards classified as more susceptible to bitter pit (orchard A-EMR) and less susceptible to bitter pit (orchard B-Hoo) sampled at harvest (T0: September 2012) and during storage (T1: November 2012 and T2: December 2012). Samples were taken from inner and outer cortex of fruit. LSDs (5%) are for the effects of sampling from different parts of cortex (C), sampling (S) and effects of orchard (O) n=6.

		Ca^{2+} (total) mg/100g	**$Ca(COO)_2$ mg/100g**	**%Calcium Oxalate**
Inner cortex	Sampling T0	9.4	2.7	28.8
	Sampling T1	7.65	2.3	30.8
	Sampling T2	5.05	1.6	31.7
	Overall means	*7.37*	*2.2*	*30.4*
Outer cortex	Sampling T0	3.35	1.5	43.3
	Sampling T1	3.05	1.25	40.1
	Sampling T2	3.4	1.5	44.6
	Overall means	*3.27*	*1.42*	*42.7*
Overall means Sampling	T0	*6.38*	*2.1*	*36*
	T1	*5.35*	*1.78*	*35.5*
	T2	*4.23*	*1.55*	*38.1*
Overall means Orchards	A (EMR)	*5.42*	*1.63*	*33.8*
	B (Hoo)	*5.22*	*1.98*	*39.3*
	df	5	5	5
	LSD (C)	1.32	0.48	5.28
	LSD (S)	1.61	0.59	6.47
	LSD (O)	1.32	0.48	5.28

The effect of SmartFresh^{SM} on the incidence of bitter pit in Bramley apples stored in air during storage season 2012/13 and the distribution of total calcium and calcium oxalate in the inner and outer cortex of fruit were compared by averaging data obtained 3 and 4 months after storage in the bitter pit prone orchard (EMR) and in the orchard with less bitter pit (Hoo) in Table 4.16.

Table 4.16: The effect of SmartFresh^{SM} on the incidence of bitter pit in Bramley apples stored in air (4-4.5°C) during storage season 2012/13 and the distribution of total calcium and calcium oxalate in the inner and outer cortex of fruit. Data is the mean of two sampling occasions (November and December 2012).

	Orchard	**Hoo**		**EMR**			
	Cortex	**Untreated**	**SmartFresh**	**Untreated**	**SmartFresh**	**LSD**	**d.f.**
% Bitter pit	Whole Fruit	12	0	50	8.5	11.05	7
Total Calcium (mg/100g)	Inner	5.5	5.2	7.2	7.2	2.38	7
	Outer	3.5	3.7	3.0	3.7		
Ca oxalate (mg/100g)	Inner	2.0	1.8	2.0	2.1	0.34	7
	Outer	1.5	1.5	1.3	1.2		
% Ca oxalate (proportion of total Ca)	Inner	35.5	37.2	27.0	28.5	14.33	7
	Outer	42.7	39.8	42.1	30.3		

SmartFresh^{SM} reduced the incidence of bitter pit in Bramley apples from both orchards, although the incidence was higher in the EMR orchard averaging 50% of untreated fruit during storage.

The inner cortex of apples had higher total fruit calcium ranging from 5.5 mg 100g^{-1} in apples from orchard Hoo, while interestingly EMR fruit with a greater propensity to develop bitter pit had 7.2 mg 100g^{-1}. However, most of the bitter pit is restricted to the outer cortex and fruits from both orchards had similar total calcium content (3-3.7 mg 100g^{-1}). Calcium oxalate content was higher in the inner cortex of Bramley apples, but when evaluated as a proportion of total calcium, the outer cortex tended to have a higher proportion of calcium in the form of calcium oxalate. There was no effect of SmartFresh^{SM} on Bramley apples total calcium content, while there is some suggestion that SmartFresh^{SM} lowered the proportion of calcium oxalate in fruit, it failed to reach significance ($P<0.05$) and this reduction was driven by a lower total calcium in the outer cortex of EMR fruit, rather than higher oxalate.

To try and understand the relationship between calcium and bitter pit, fruit samples were divided into those fruit showing symptoms and the symptomless fruit and a comparison of the incidence of the disorder and calcium and mineral profiles were investigated. In the final season (2013/14), for determining distribution of calcium and calcium oxalate from the stalk and calyx region cortex samples from the calyx/stalk end of the fruit were taken at harvest and then every 15 days during the initial period of air-storage (4-4.5°C) from orchards EMR and Hoo. Results demonstrate that the total calcium content (Table 4.17) in the stalk end was significantly higher than the calyx end and were also higher in the orchard less susceptible to bitter pit (Hoo). There was no significant difference in total calcium content between sampling dates. Calcium oxalate proportion (% calcium in the form of calcium oxalate) in the orchard more susceptible to bitter pit (EMR) was significantly higher. Also calcium oxalate proportion significantly increased between harvest sampling date and 2.5 months after storage.

More detailed analysis of the concentration of minerals sampled from the calyx/stalk end of 30 samples in season (2013/14) can be found in Appendix VIII (b).

Table 4.17: Comparison of means of calcium (total), calcium oxalate and % of total calcium in the form of calcium oxalate in Bramley apples sampled from two orchards as more susceptible to bitter pit (orchard EMR) and less susceptible to bitter pit (orchard HOO) sampled at harvest (T0: September 2013) and during storage (T1: 1/10/2013 and T2: 15/10/2013, T3: 1/11/2013, T4: 15/11/2013). Samples were taken from calyx and stalk end of fruit cortex. LSDs (5%) are for the effects of sampling from different parts of cortex (C), orchard (O), and effects of sampling (S) n=10.

		Ca^{2+} (total) mg/100g	**$Ca(COO)_2$ mg/100g**	**Calcium Oxalate proportion**
Calyx sampling	Means T0	3.77	1.155	30.4
	Means T1	3.83	1.04	27.7
	Means T2	3.68	1.23	33.7
	Means T3	2.93	1.10	38.1
	Means T4	3.26	1.67	52.6
	Overall means	*3.49*	*1.24*	*36.5*
Stalk Sampling	Means T0	4.39	1.07	24.3
	Means T1	5.24	1.18	22.8
	Means T2	4.12	1.22	29.6
	Means T3	3.45	1.34	40
	Means T4	4.47	1.74	45.5
	Overall means	*4.33*	*1.31*	*32.5*
Overall means sampling	T0	*4.08*	*1.11*	*27.4*
	T1	*4.54*	*1.11*	*25.2*
	T2	*3.9*	*1.22*	*31.6*
	T3	*3.19*	*1.22*	*39.2*
	T4	*3.86*	*1.70*	*49.1*
Overall means Orchards	A (EMR)	*3.55*	*1.32*	*39.8*
	B (Hoo)	*4.27*	*1.2*	*29.20*
	df	9	9	9
	LSD (C)	0.78	0.18	9.10
	LSD (S)	1.25	0.29	14.39
	LSD (O)	0.79	0.18	9.1

Mineral analysis results were correlated with the incidence of bitter pit during storage for SmartFresh^{SM} treated (SF) and untreated (No SF) Bramley apples.

In untreated fruit the amount of calcium oxalate as a percentage of total calcium was higher compared to apples treated with SmartFresh^{SM}. In these examples the difference between total

calcium content and calcium oxalate from stalk and calyx were similar (Table 4.18). SmartFresh^{SM} treatment appears to be reducing the inactivation of calcium by conjugation to oxalate; this may be the result of lowering the rate of conversion of ascorbic acid breakdown to oxalic acid.

Table 4.18: Comparison of mean of minerals (Ca^{2+} total, Ca^{2+} oxalate, Mg, K and B) concentrations in untreated and SmartFresh^{SM} treated samples and mineral distribution in calyx and stalk end of selected samples collected in season 2013/14.

		Bitter Pit %	Ca^{2+} (total) mg/100g	Calcium oxalate mg/100g	% Ca^{2+} oxalate	Mg mg/100g	K mg/100g	B mg/100g
NoSF	Whole fruit	9.4± 4.90						
	Calyx end		3.59±0.19	1.24±0.21	34.5	3.55±0.23	102.25±5.06	0.05±0.02
	Stalk end		4.37±0.30	1.29±0.33	29.5	2.97±0.11	112.92±4.18	0.09±0.03
SF	Whole fruit	4.2± 1.77						
	Calyx end		4.84±1.12	0.85±0.15	17.6	3.12±0.16	79.41±13.31	0.01±0.01
	Stalk end		4.31±0.68	0.9±0.19	20.9	2.50±0.18	84.01±13.70	0.02±0.02
***P* value**	Treatment (NoSF vs SF)	0.94	0.06	<0.001	<0.001	0.003	<0.001	0.006
	Sample cortex (Calyx/Stalk)		0.81	0.91	<0.001	<0.001	0.19	0.11
	Treatment: Sample cortex		0.09	0.12	0.06	0.97	0.66	0.38

Multiple regression analysis for the influence of mineral constituents on the incidence of bitter pit showed a significant (P <0.05) effect for Ca^{2+} (total), K and the ratio of (K+Mg)/Ca from calyx end. There was a significant correlation (R^2=0.81) between these elements and incidence of bitter pit (Appendix IX).

The concentration of potassium in the calyx and stalk end of untreated and SmartFresh^{SM} treated samples with or without bitter pit symptoms were compared (Figure 4.9).

Potassium concentrations in the calyx and stalk end of untreated samples were significantly higher than SmartFresh^{SM} treated samples. However in both SmartFresh^{SM}-treated and untreated apples potassium concentration in calyx end was significantly lower than stalk end of apple when there was no bitter pit symptoms. There was no difference between potassium

concentration of calyx and stalk when there was bitter pit whether apples were SmartFresh^{SM} treated or untreated.

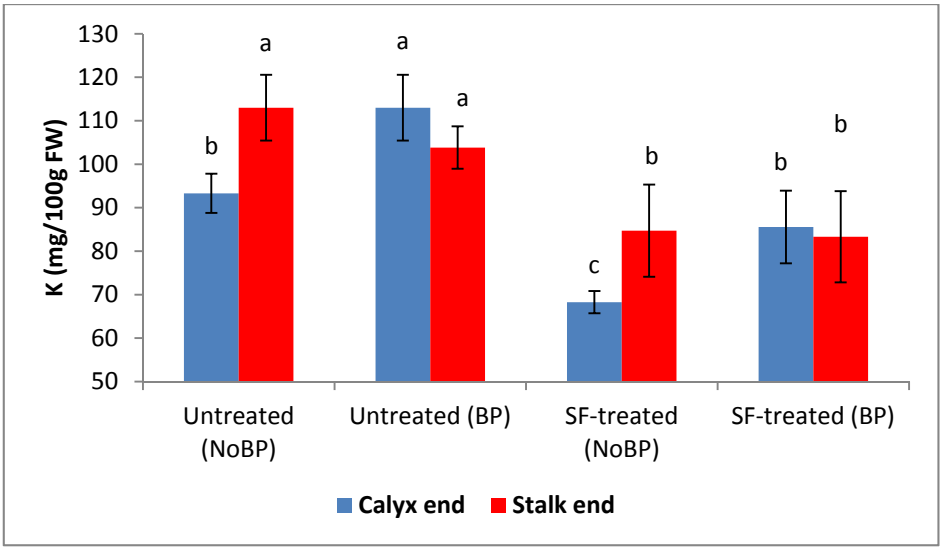


Figure 4.9: Comparison of changes in potassium (K) concentration in calyx and stalk end of untreated and SmartFresh^{SM}-treated Bramley apples when there was no symptom of bitter pit (NoBP) and incidence of bitter pit (BP) during 2.5 months air storage. Each data point is the mean of several selected samples from season (2013/14) each one consisted of ten apple samples ± SE. Mean values with different letters were significantly different according to Tukey's test (p<0.05).

Since there was a significant correlation between incidence of bitter pit and the ratio of (K+Mg/Ca) from the calyx end, the changes in the ratio in calyx and stalk end of untreated and SmartFresh^{SM}-treated samples with or without bitter pit symptoms were compared (Figure 4.10). Only in untreated samples with bitter pit symptoms the ratio of (K+Mg/Ca) in calyx end was significantly higher than stalk end. There was no difference between calyx and stalk end of SmartFresh^{SM}-treated samples with or without bitter pit also in untreated without bitter pit in ratio of (K+Mg/Ca).

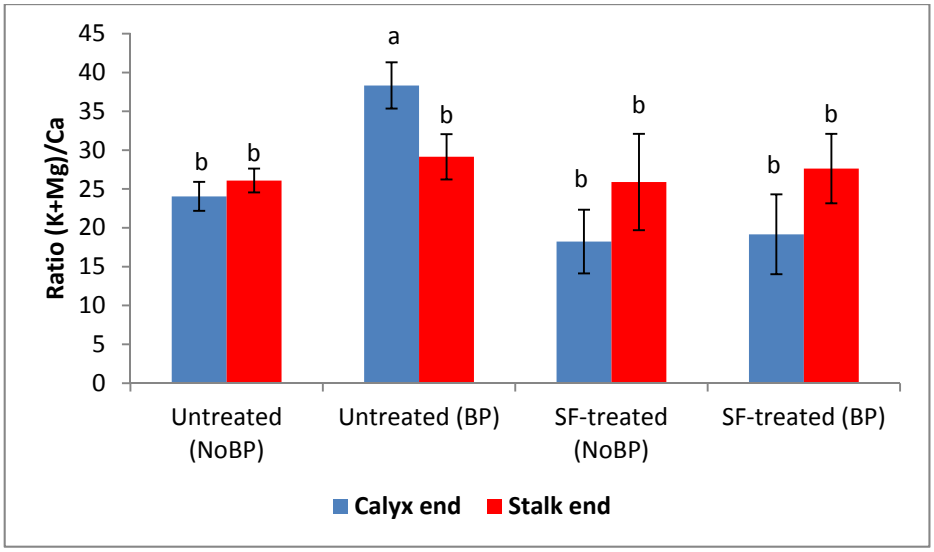


Figure 4.10: Comparison of changes in ratio of (K+Mg)/Ca in calyx and stalk end of untreated and SmartFresh^{SM}-treated Bramley apples when there is no symptom of bitter pit (NoBP) and incidence of bitter pit (BP) during 2.5 months air storage. Each data point is the mean of several selected samples from seasons 2013/14 each one consisted of ten apple samples ± SE. Mean values with different letters were significantly different according to Tukey's test (p<0.05).

Correlations between the incidence of bitter pit and calcium (total), calcium oxalate and calcium oxalate proportion in Bramley apples were compared in two ways as just for incidence of bitter pit and for severity of bitter pit and sampling from different parts of fruit tissue (Table 4.19). Results showed significantly higher correlation with sampling from calyx/stalk end of fruit in comparison with inner/outer cortex.

Table 4.19: Comparison of the correlation with incidence and severity of bitter pit with different positions of sampling from fruit cortex (inner/outer and calyx/stalk end) of calcium (total), calcium oxalate and calcium oxalate proportion in Bramley apples (season 2013/14).

		Ca^{2+} (total)	$Ca(COO)_2$	Calcium Oxalate proportion
Correlation with incidence of bitter pit	**Inner cortex**	-0.43	-0.38	0.03
	Outer cortex	-0.47	-0.49	0.40
	Calyx end	-0.80	-0.76	0.53
	Stalk end	-0.53	-0.56	0.58
Correlation with severity of bitter pit	**Inner cortex**	-0.30	-0.23	0.15
	Outer cortex	-0.42	-0.45	0.43
	Calyx end	-0.69	-0.85	0.91
	Stalk end	-0.69	-0.80	0.94

According to all the obtained results it was decided to focus on calcium as Ca^{2+} (total) and proportion of calcium oxalate, and to consider samples taken separately from the calyx and

stalk end of fruit. Comparison of results of different orchards with different levels of susceptibility to incidence of bitter pit (Figure 4.11) showed that although the proportion of calcium oxalate at harvest time (September 2013) and during storage was correlated with the incidence of bitter pit, there were samples with higher amounts of calcium which showed bitter pit and samples with lower amounts of calcium that did not show bitter pit.

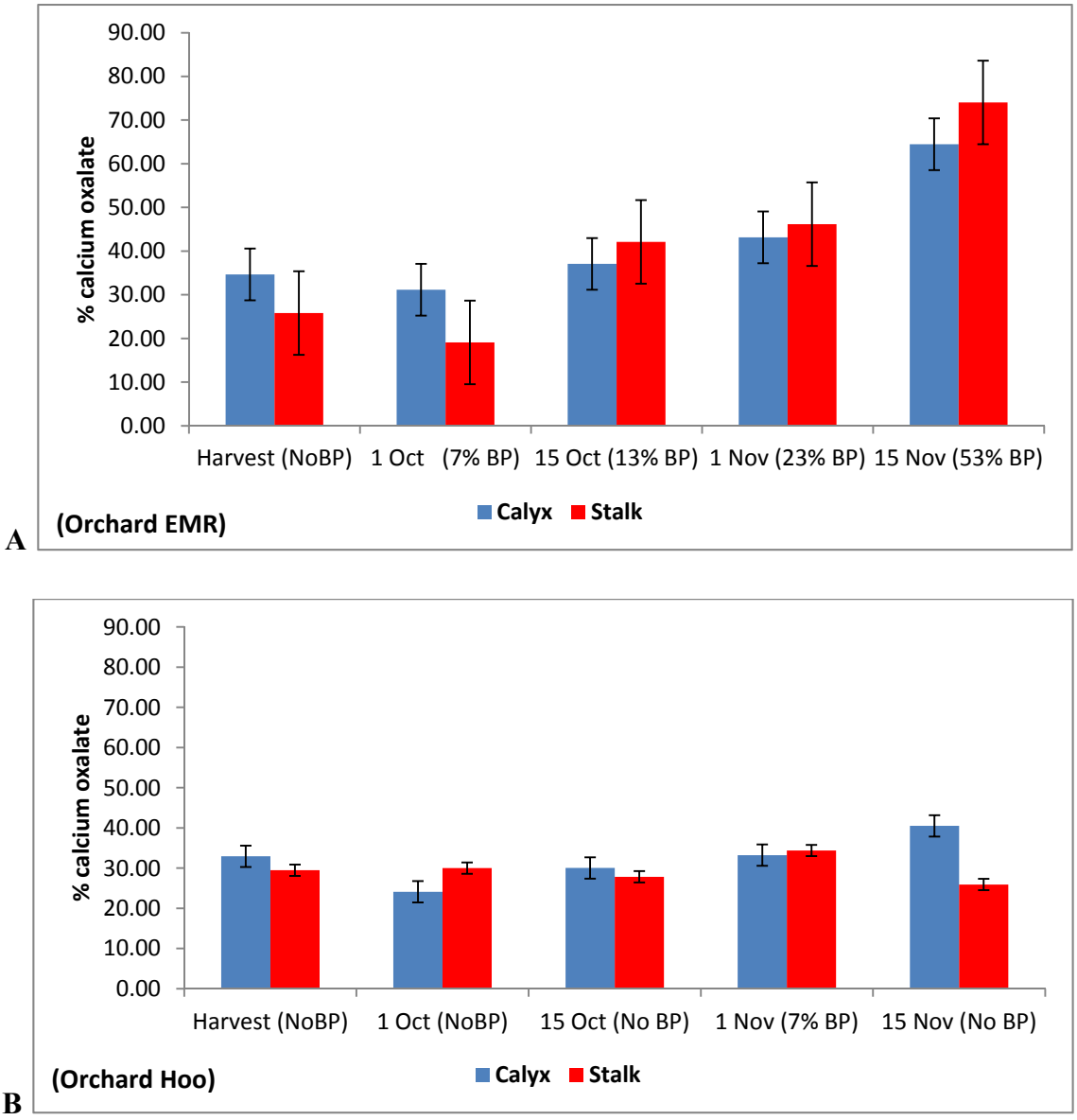


Figure 4.11: Comparison of changes in proportion of calcium oxalate between samples picked from two orchards: A) orchard (EMR) more susceptible to bitter pit (BP) and B) orchard (Hoo) less susceptible to bitter pit. Bramley apples were air stored (4-4.5°C) and sampled from harvest (September) to November15 th (season 2013/14).

For developing a prediction model for incidence of bitter pit based on changes of calcium as Ca^{2+} (total) and proportion of calcium oxalate, it is important to apply equations to normalise

results allowing for the minimum level of total calcium (5mg/100g) and the movement of calcium from inner to outer cortex and from stalk end to calyx end.

Because of the changes in the level of minerals in each season which is related to the differences of environmental factors in each season or even the differences of orchards located in different microclimates, it was decided to find the threshold based on subtracting calcium oxalate from total calcium named as calcium structural (Ca_{St}) during storage in comparison with the harvest time. Threshold is not a fixed range of (Ca_{St}) and is based on two important factors: a) average of (Ca_{St}) which is based on average of structural calcium from inner/outer cortex or stalk/calyx end of apple fruit; b) the difference of structural calcium in different parts of fruit comparing with the threshold of calcium as 5 mg/100g. Table 4.19 shows the equations applied for calculating the threshold (*T*).

Table 4.20: Equations for calculating free calcium in samples taken from different parts of Bramley apple fruit tissue as inner/outer cortex or calyx/stalk end of fruit. When T<50 there is more chance of incidence of bitter pit.

Total Ca: $\boldsymbol{Ca_T}$	Total calcium includes free & bound calcium
Structural Ca: $\boldsymbol{Ca_{St}}$	$\boldsymbol{Ca_{St}} = Ca_T$ - $Ca(COO)_2$
Average Structural Ca: $\overline{\boldsymbol{Ca}}_{\boldsymbol{St}}$ (samples from inner & outer cortex)	$\overline{\boldsymbol{Ca}}_{\boldsymbol{St}} = \frac{Ca_{St}(In) + Ca_{St}(Out)}{2}$
Average Structural Ca: $\overline{\boldsymbol{Ca}}_{\boldsymbol{St}}$ (samples from stalk & calyx end of fruit)	$\overline{\boldsymbol{Ca}}_{\boldsymbol{St}} = \frac{Ca_{St}(Stalk) + Ca_{St}(Calyx)}{2}$
Percentage of $\overline{Ca}_{St}$ to the threshold: $\%\overline{\boldsymbol{Ca}}_{\boldsymbol{St}}$ (threshold is 5mg/100g)	**(A)** $\%\overline{\boldsymbol{Ca}}_{\boldsymbol{St}} = \frac{\overline{Ca}_{St} \times 100}{5}$
Percentage of difference of (In-Out) or (Stalk-Calyx) subtracted from $\overline{Ca}_{St}$ to the threshold (5mg/100g): $\%\overline{\boldsymbol{Ca}}_{\boldsymbol{St}}\boldsymbol{changes}$	**(B)** $\%\overline{\boldsymbol{Ca}}_{\boldsymbol{St}}\boldsymbol{changes} = \frac{\left(\overline{Ca}_{St} - \frac{Ca_{St}(In) - Ca_{St}(Out)}{\overline{Ca}_{St}}\right) \times 100}{5}$
Threshold: $\boldsymbol{T}$ (Less than 50: more chance of bitter pit)	$\boldsymbol{T} = \left(\frac{A+B}{2}\right) - (A - B) > 50$

These formulae were applied for the results of samples collected from different orchards which were as untreated or SmartFresh^{SM}-treated and stored in different storage regimes. Table 4.21 shows the results relating to two orchards in seasons 2011-13. All were air stored (4-4.5°C). Although the values of (Ca_{St}) in each season were different most of the results of comparison of changes of normalised (Ca_{St}) to harvest threshold were correlated to incidence of bitter pit.

However, the results were not always consistent and there were some false positive or negative results. Especially the number of false results when samples were collected from inner and outer cortex were more than samples which were collected from stalk and calyx end of fruit.

Table 4.21: Comparison of total and structural calcium from different parts of fruit cortex (Inner/Outer or Stalk/Calyx) from harvest and during storage and the threshold based on changes from harvest (T) and incidence of bitter pit.

Date	Orchard	Untreated/SF	In/Out or Stalk/Calyx	BP %	Ca_T (Ca total) mg/100g	$Ca(COO)_2$ mg/100g	(Ca_{St}) mg/100g	A	B	T	Threshold NoBP: $T \geq 50$
05/09/2011	EMR	NoSF	In	0	11.91	2.48	9.44				
05/09/2011	EMR	NoSF	Out	0	5.81	1.48	4.34	137.70	122.89	115.48	(NoBP) T≥50
10/11/2011	EMR	NoSF	In	37	9.87	2.15	7.72				
10/11/2011	EMR	NoSF	Out	37	4.00	1.80	2.20	99.20	76.94	65.81	(BP)T≥50(false+)
05/09/2011	Hoo	NoSF	In	0	10.10	1.53	8.58				
05/09/2011	Hoo	NoSF	Out	0	5.42	0.70	4.72	132.95	121.35	115.55	(NoBP) T≥50
10/11/2011	Hoo	NoSF	In	0	10.23	3.43	6.81				
10/11/2011	Hoo	NoSF	Out	0	7.14	1.95	5.19	119.95	114.56	111.87	(NoBP) T≥50
28/08/2012	EMR	NoSF	In	0	9.21	2.10	7.11				
28/08/2012	EMR	NoSF	Out	0	3.00	1.25	1.75	88.60	64.40	52.30	(NoBP) T≥50
13/11/2012	EMR	NoSF	In	47	8.82	2.33	6.50				
13/11/2012	EMR	NoSF	Out	47	2.99	1.15	1.84	83.35	61.01	49.84	(BP) T<50
12/12/2012	EMR	NoSF	In	53	5.62	1.55	4.07				
12/12/2012	EMR	NoSF	Out	53	2.85	1.30	1.55	56.20	38.26	29.30	(BP) T<50
13/11/2012	EMR	SF	In	0	8.07	2.23	5.85				
13/11/2012	EMR	SF	Out	0	3.75	1.25	2.50	83.45	67.42	59.40	(NoBP) T≥50

Date	Orchard	Untreated /SF	In/Out or Stalk/Calyx	BP %	Ca_T mg/100g	$Ca(COO)_2$ mg/100g	(Ca_{St}) mg/100g	A	B	T	Threshold NoBP: $T \geq 50$
28/08/2012	Hoo	NoSF	In	0	9.58	3.33	6.26				
28/08/2012	Hoo	NoSF	Out	0	3.74	1.68	2.07	83.20	63.06	52.98	(NoBP) T≥50
13/11/2012	Hoo	NoSF	In	7	6.48	2.28	4.21				
13/11/2012	Hoo	NoSF	Out	7	3.11	1.30	1.81	60.15	44.22	36.26	(BP) T<50
12/12/2012	Hoo	NoSF	In	17	4.47	1.60	2.87				
12/12/2012	Hoo	NoSF	Out	17	3.90	1.70	2.20	50.70	45.41	42.77	(BP) T<50
03/01/2013	Hoo	NoSF	In	50	4.95	1.35	3.60				
03/01/2013	Hoo	NoSF	Out	50	3.61	1.60	2.01	56.10	44.76	39.09	(BP) T<50
13/11/2012	Hoo	SF	In	0	6.35	1.68	4.68				
13/11/2012	Hoo	SF	Out	0	3.59	1.20	2.39	70.65	57.71	51.24	(NoBP) T≥50
12/12/2012	EMR	SF	In	17	6.29	1.85	4.44				
12/12/2012	EMR	SF	Out	17	3.59	0.98	2.62	70.55	60.20	55.03	(BP)T≥50(false+)

Date	Orchard	Untreated /SF	In/Out or Stalk/Calyx	BP %	Ca_T mg/100g	$Ca(COO)_2$ mg/100g	(Ca_{St}) mg/100g	A	B	T	Threshold NoBP: $T \geq 50$
12/12/2012	Hoo	SF	In	0	4.06	1.95	2.11				
12/12/2012	Hoo	SF	Out	0	3.78	1.75	2.03	41.40	40.63	40.24	(NoBP)T<50 false-
02/09/2013	EMR	NoSF	Calyx	0	4.04	1.40	2.64				
02/09/2013	EMR	NoSF	Stalk	0	4.36	1.13	3.24	58.75	54.70	52.67	(NoBP) T≥50
01/10/2013	EMR	NoSF	Calyx	7	3.21	1.00	2.21				
01/10/2013	EMR	NoSF	Stalk	7	5.63	1.08	4.56	67.65	53.78	46.85	(BP) T<50
15/10/2013	EMR	NoSF	Calyx	13	3.44	1.28	2.17				
15/10/2013	EMR	NoSF	Stalk	13	3.85	1.20	2.65	48.15	44.12	42.11	(BP) T<50
01/11/2013	EMR	NoSF	Calyx	23	2.55	1.10	1.45				
01/11/2013	EMR	NoSF	Stalk	23	2.6	1.20	1.40	28.50	29.20	29.55	(BP) T<50
01/11/2013	EMR	SF	Calyx	20	2.66	0.98	1.69				
01/11/2013	EMR	SF	Stalk	20	2.73	1.18	1.56	32.40	34.00	34.81	(BP) T<50
15/11/2013	EMR	NoSF	Calyx	53	2.87	1.85	1.02				
15/11/2013	EMR	NoSF	Stalk	53	2.96	1.93	1.04	20.55	20.26	20.11	(BP) T<50
02/09/2013	Hoo	NoSF	Calyx	0	3.49	0.91	2.58				
02/09/2013	Hoo	NoSF	Stalk	0	4.41	1.00	3.41	59.90	54.36	51.59	(NoBP) T≥50

Date	Orchard	Untreated /SF	In/Out or Stalk/Calyx	BP %	Ca_T mg/100g	$Ca(COO)_2$ mg/100g	(Ca_{St}) mg/100g	A	B	T	Threshold NoBP: $T \geq 50$
01/10/2013	Hoo	NoSF	Calyx	0	4.46	1.08	3.39				
01/10/2013	Hoo	NoSF	Stalk	0	4.85	1.28	3.58	69.60	68.51	67.96	(NoBP) T≥50
15/10/2013	Hoo	NoSF	Calyx	0	3.91	1.18	2.74				
15/10/2013	Hoo	NoSF	Stalk	0	4.4	1.23	3.18	59.10	56.12	54.63	(NoBP) T≥50
01/11/2013	Hoo	NoSF	Calyx	0	4.61	1.30	3.31				
01/11/2013	Hoo	NoSF	Stalk	0	4.74	1.18	3.57	68.75	67.27	66.52	(NoBP) T≥50
01/11/2013	Hoo	NoSF	Calyx	7	3.31	1.10	2.21				
01/11/2013	Hoo	NoSF	Stalk	7	4.29	1.48	2.82	50.25	45.43	43.03	(BP) T<50
01/11/2013	Hoo	SF	Calyx	0	3.71	1.05	2.66				
01/11/2013	Hoo	SF	Stalk	0	4.28	1.23	3.06	57.15	54.39	53.00	(NoBP) T≥50
15/11/2013	Hoo	NoSF	Calyx	0	3.64	1.48	2.17				
15/11/2013	Hoo	NoSF	Stalk	0	5.98	1.55	4.43	65.95	52.21	45.34	(NoBP)T<50 false-

The threshold of incidence of bitter pit is based on changes of normalised (Ca_{St}) to harvest time and was tested on untreated and SmartFresh^{SM}-treated samples in different storage regimes and in different seasons and orchards and in more than 85% incidence of bitter pit was matched with lower value of threshold (T). comparing to harvest threshold. Table 4.22 shows the contingency table of results in seasons 2012/13 and 2013/14 in different orchards and treatments. Chi square analysis compared thresholds with bitter pit development and a significant correlation P<0.001 was found.

Table 4.22: Contingency table for Chi square test of the threshold to find false∓ and misses in comparison of threshold and incidence of bitter pit.

	No BP	BP	Total
T<50	2	11	13
T≥50	15	2	17
Total	17	13	30

	False (numbers)	False (Percentage)
False positive (+): Formula(T≥50) & BP(+)	2	15%
False negative (-): Formula(T<50) & BP(-)	2	11.8%
Total misses	4	13%

	Number of samples	Number of samples	Percentage
Total misses	4	False+: 2	6.7%
		False -: 2	6.7%
Total hits	26	Correct BP: 11	36.6%
		Correct NoBP: 15	50%

4.4 Discussion

Biochemical analysis on organic acids (ascorbic acid, malic acid, oxalic acid and citric acid) and the content of sugars (glucose, fructose and sucrose) and minerals (calcium, potassium, magnesium, nitrogen and boron) were compared and investigated during three seasons (2011-13) to identify the changes in metabolic indicators of fruit ripening (sugars and acids) and mineral content at harvest and how these influence the incidence of bitter pit during storage. In particular the possibility of using the collected data for developing prediction models for incidence of bitter during storage was considered.

A) Organic acids and sugar content:

SmartFresh^{SM}-treated Bramley apples had the same organic acid profiles (oxalic, malic, ascorbic and citric acid) as untreated fruit in fruit stored under different storage regimes (air or CA). However, a significant difference in sugar content (fructose and glucose) between untreated and SmartFresh^{SM} treated samples in air storage was observed. These results are in contrast with Watkins (2011) who reported SmartFresh^{SM} (1-MCP) application reduced the decline in titratable acidity during storage. In this trial acids were measured individually by HPLC. The lack of effect of 1-MCP on sugar profiles in SmartFresh-treated fruit is in agreement with Watkins (2011).

The relationship between acid and sugar contents and incidence of bitter pit were compared in a series of correlations; metabolite profiles of apple samples showing symptoms of bitter pit were compared with samples that were symptomless. In general as fruits mature on the tree there is an increased propensity to develop bitter pit in store and this is reflected in the increase in sucrose and decrease in malic acid in Bramley apples picked over a 4 week period. These results confirm those reported by Oke *et al.* (2013) however in this study the stronger correlation was reflected by the higher number of apples affected by bitter pit.

There was seasonal variation in the amount of acid and sugars analysed, this may have also been an influence of the time samples were stored in the -80°C with samples from the 2011 season being kept for longer than samples from subsequent years. In 2012 there was a bigger drop in malic acid from samples taken at harvest from those taken from air store compared to CA regimes (9%CO_2, 12%O_2 and 5%CO_2, 1%O_2) even though the final concentrations after prolonged storage were similar. Moreover, ascorbic acid was significantly higher in air storage compared to CA storage. Controlled atmosphere storage suppresses ethylene production and

respiration and lower respiration reduced the utilization of malic acid and prevented the reduction of ascorbic acid. This is consistent with Dong *et al* (1992) who reported that conversion of ACC to ethylene via the action of ACC oxidase requires ascorbate as a co-factor and the pool of ascorbate decreases with ripening. Ascorbate is tightly regulated and is reduced to dehydroascorbic acid before being re-oxidised to ascorbate. The results from this study showed a significantly higher concentration of ascorbic acid in air (21% O_2) regime in comparison with controlled atmosphere regimes.

However, the application of SmartFresh^{SM} while suppressing ethylene and ripening did not alter ascorbic acid content in fruit. Ethylene production in SmartFresh^{SM}-treated fruit is not fully suppressed and it is possible that the ascorbic acid pool is not affected by the degree of ethylene suppression. Rop *et al*. (2010) indicated that ascorbic acid, decreased significantly by increasing ripening of the fruit, also Kevers *et al.* (2011) reported a 75% decrease in ascorbic acid content of apple during long term storage. It seems since storage in air is over a shorter period (4 months) in comparison with CA storage (6-10 months) that this could be the reason for a decrease in ascorbic acid content of fruits stored in CA regimes. The methodology employed to measure ascorbic acid in this study may alter the degree of sensitivity in detecting changes in ascorbic acid identified in other studies.

There was significantly higher concentration of fructose and glucose in air regime in comparison with CA regimes. Interestingly, the rate of sucrose decline was greater in (5% CO_2, 1% O_2) storage than in air or (9% CO_2, 12% O_2) storage even though the rate of ripening was delayed, the extended period of storage and sampling from (5% CO_2, 1% O_2) stored fruit may have contributed to the decline in sucrose.

As indicated by Paliyath *et al.* (2008), in the process of fruit maturation starch is transformed to glucose and fructose, then sucrose. So sucrose is the major sugar which accumulates in the fruit during ripening. The results of this study are consistent with this and show that delay in ripening in CA regimes comparing to air regime causes higher content of glucose and fructose. Likewise the regime (5% CO_2, 1% O_2) delays maturity for a longer time resulting in a lower content of sucrose in fruit.

Bramley apples are fairly unique in that due to their sole use in culinary and processing procedures, fruits are harvested immature before undergoing the climacteric. SmartFresh^{SM} application has a significant effect on extending Bramley apple storage life due to the physiological maturity at the point of application. SmartFresh^{SM} application led to a significant

reduction and a delay in the onset of bitter pit development. This was most likely a consequence of delaying fruit maturity. Part of this delay is clearly seen in air-stored fruit where SmartFresh^{SM} has slowed the breakdown of sucrose during storage leading to higher sucrose content in SmartFresh^{SM}-treated fruit but also led to an increase in glucose and fructose. The pool of reducing sugars (fructose and glucose) is the net result of both the rate of sucrose breakdown and the rate at which sugars are utilised in respiration. It is likely that SmartFresh has reduced the rate of glucose and fructose utilisation by suppressing respiration. Moreover, the higher malic acid content and lower citric acid content would also indicate a reduced rate of respiration in SmartFresh^{SM}-treated Bramley apples. Interestingly, the effect of SmartFresh^{SM} in CA-stored fruit is less obvious as the elevated CO_2 and reduced O_2 has led to a reduction in fruit respiration most likely via suppression of ethylene production. In CA storage no effect of SmartFresh^{SM} was seen on sucrose, glucose or fructose content. Whether, sucrose, fructose and glucose content have a direct role in bitter pit formation or are more related to fruit maturity is not fully understood. It seems higher concentration of oxygen in air regime (21% O_2) and CA regime (9% CO_2, 12% O_2) and this level of CO_2 (9%) establishes during the process of natural respiration of fruit that means until establishment of the desirable atmosphere, ethylene production continues. In contrast the establishment of the regime (5% CO2, 1% O2) is by scrubbers in the shorter period. Increasing respiration of fruit in air and (9% CO_2, 12% O_2) regimes increase maturity and sucrose content.

The relationship between incidence of bitter pit with ascorbic acid, malic acid, oxalic acid and citric acid were compared over the three years of study. A significant inverse correlation ($R \geq -0.6$) to ascorbic acid was found even though changes in malic acid that were greater over the course of storage, were not correlated to bitter pit. The relationship between ascorbic acid and bitter pit is less obvious when SmartFresh^{SM} is applied. The suppression of bitter pit in SmartFresh^{SM} treated fruit and its lack of effect on ascorbic acid content reduces the correlation between ascorbic acid content and bitter pit.

As Loewus (1999) indicated, as the breakdown of ascorbic acid via dehydroascorbic acid leads to the formation of oxalic acid, this is consistent with the relationship between decreasing ascorbic acid and increasing oxalic acid that was observed. When fruit is harvested in a more mature state, it contains generally less ascorbic acid, therefore the fruit has less propensity to accumulate oxalic acid via the breakdown of ascorbic acid with the consequences of less bitter pit formation in fruit. This highlights the importance of determining the optimum stage of maturity for fruit harvesting. Although Wiersum (1979) and Schmitz-Eiberger *et al.* (2001)

indicated that stress stimulates the formation of oxalic acid in cells and the conjugation of calcium by oxalic acid increases the incidence of bitter pit, in this study oxalic acid provided a poor correlation with bitter pit over the three seasons tested (R=0.20-0.35). The type of method employed for oxalic acid determination may influence the overall comparison. Since oxalic acid has been found to have an important role in inactivating calcium and destroying cellular integrity (Fidler *et al*., 1973), methods were developed in this thesis to determine the amount of calcium oxalate present in fruit and see if this could be related to the incidence of bitter pit.

In the first two seasons (2011-12) a study of the relationship between bitter pit incidence and ascorbic acid content of cortex tissue sampled across the equatorial sections of Bramley fruit was undertaken. When sampling and ascorbic acid determination were conducted across the whole fruit the amount of ascorbic acid was variable between samples and the relationship with the incidence of bitter pit provided a poor correlation. Since the symptoms of bitter pit occurs mainly near the calyx end of the apple (Tamala and Soska, 2004) and the xylem sap transfers through the stalk end to calyx end of fruit (Dražeta *et al*., 2004), it was decided to take a more selected sampling strategy in season (2013/14) and samples were collected from calyx end and stalk end of apple fruit for organic acids and sugars analysis. As expected, the calyx region (R=-0.88) had a higher correlation to bitter pit than stalk end of fruit (R=-0.59). These results suggested that for greater accuracy, sampling from the tissue of calyx region can strengthen the relationship between ascorbic acid content and the incidence of bitter pit. These results were observed in the final year of the study and require further investigation over a larger number of samples in different orchards and storage regimes. As ascorbic acid acts as an antioxidant the pool of available ascorbic acid is influenced by the metabolic activity of fruit and by challenges caused by stress; fruits in air storage are subject to greater water loss which may either cause a concentration of its constituents and/or lead to fruit increasing its antioxidant profile to reduce the fruit stress.

In order to determine whether there is a relationship between ascorbic acid content and incidence of bitter pit that could be used as a prediction tool, it is important to compare ascorbic acid changes during storage compared to harvest levels and hence the equation $\boldsymbol{T} = \left(\frac{X_n \times 100}{X_H}\right) \geq 90$ was applied to normalise results. When the percentage of ascorbic acid content during storage ($\boldsymbol{X_n}$) decreases more than 10% of ascorbic acid content at harvest ($\boldsymbol{X_H}$), there is greater chance of incidence of bitter pit. There was a 25% error in recognising samples with bitter pit

by applying this model. However, as indicated above this method needs further refinement in terms of sampling region, intervals of taking samples and timing of monitoring during storage.

A study of the relationship between incidence of bitter pit and sugar content (glucose, fructose and sucrose) compared in different seasons only showed a poor inverse correlation (R=-0.35) to sucrose. However the correlations between changes of sucrose content and incidence of bitter pit were not consistent in different orchards. Also like ascorbic acid results SmartFresh^{SM} treated samples showed weaker correlation than untreated samples between sucrose content changes and incidence of bitter pit. Although the results of this study did not show significant correlation between increasing fructose and incidence of bitter pit, still because of conversion of sucrose to fructose and the weak inverse correlation of sucrose to bitter pit, it is concluded there is a relationship between these sugars (fructose, glucose and sucrose) and incidence of bitter pit as reported in previous studies (Simon, 1978; Mills *et al.,* 1994). However, developing diagnostic models of incidence of bitter pit based on organic acids like ascorbic acid and oxalic acid give more accuracy in evaluation.

B) Mineral analysis:

Traditionally growers have relied on mineral analysis of apples 2-3 weeks before harvest to provide guidance on the suitability for long-term storage with fruit having to reach a minimum of Ca 4.5-5 mg/100g FW, N 50 mg/100g FW, K<120 mg/100g FW, Mg 5 mg/100g FW and B 0.2 mg/100g FW. In addition these ratios are important: K/Ca (<30), (K+Mg)/Ca >22, N/Ca>10 (Defra Best Practice Guide, 2002).

This study focussed on understanding the distribution of minerals within Bramley apples initially looking at overall values in whole fruit and then concentrating on differences between the inner and outer cortex followed by mapping changes between the stalk and calyx end of the fruit to aid our understanding of whether more selective sampling could increase the accuracy in predicting the onset of bitter pit.

In the first year (2010/11), samples were collected from the whole parts of the fruit cortex, and mineral analysis results represented the whole cortex. In year 1, fruits were sent to an accredited laboratory for a full spectrum of mineral analysis. The concentration of calcium, potassium, magnesium and nitrogen were either positively or negatively correlated with the incidence of bitter pit. Interestingly, nitrogen content of fruit was significantly correlated (P<0.001) and

importantly the ratio of nitrogen to calcium appears to be an important factor in affecting the propensity of Bramley apples to develop bitter pit.

There was a significant correlation (R=0.71) between increasing ratio N/Ca and incidence of bitter pit. These findings are in accordance with Hepler (2005) where the ratio of N/Ca was significantly correlated with bitter pit. Dražeta *et al*. (2004) showed that the rate of parenchyma cell expansion in the cortex cells had an influence on calcium distribution within fruit; where expansion occurred too quickly the xylem cells were crushed which led to poor distribution of calcium within the fruit. Higher nitrogen content leads to more rapid fruit expansion. Moreover, higher fruit nitrogen can lead to higher incorporation of nitrogen into the cell walls in the form of amino acids such as proline that may help to stabilise cell wall structure and tissue integrity. This was indicated by Huxham *et al.* (1999), they quantified the level of calcium and nitrogen in the cell walls and middle lamella of apple fruit using electron energy loss spectroscopy (EELS) imaging. They observed firmer fruits had significantly lower levels of the cell wall calcium and higher levels of cell wall nitrogen. It seems the role of nitrogen and the ratio with calcium is more important than potassium and the ratio with calcium and the nitrogen content is associated with higher protein content.

The findings of year 1 also showed a linear correlation (R=0.58) between (K+Mg)/Ca and incidence of bitter pit which was similar to a report by Van der Boon (1980). Potassium, calcium and magnesium concentration decreased during the ripening stages. While the content of all micronutrients as well as phosphorus did not change during ripening stages which concurs with the findings by Rop *et al*. (2010).

In subsequent years (2011-13) mineral analysis was performed at the laboratories of University of Greenwich (Medway), where methods were developed to look at the proportion of total calcium versus the amount of calcium oxalate, due to limited resources, measurements were restricted to Ca^{2+}, K, Mg and B and therefore no data for N_2 is available for years 2-4 (2011-13). Clearly based on year 1 data further work on the ratio of Ca/N is required to aid the prediction of bitter pit.

Although at harvest calcium content was above the minimum target (4.5 mg/100g FW) required for long-term storage, the comparison of changes in calcium content of untreated and SmartFresh^{SM} treated fruit showed that after 3 months storage the overall calcium content of untreated fruit without symptoms of bitter pit was lower than samples with bitter pit symptoms. Whether this decrease reflects redistribution of calcium within fruit or is the result of sampling

errors is unknown. While, SmartFresh^{SM} reduced bitter pit symptoms, it did not affect total calcium content. As calcium and other minerals are not metabolized any apparent changes in calcium total must be through redistribution of calcium from the core to the outer cortex.

Ferguson and Watkins (1992) reported that the higher concentration of calcium in pitted cells was due to calcium leaching into the dead cell space, which can cause misinterpretation of results when comparing calcium data from tissue with pits versus symptomless tissue. From the project results, overall calcium content is not necessarily a good indicator of fruit susceptibility to developing bitter pit as samples with similar calcium content varied between 0-20% incidence of the disorder.

Other minerals such as potassium and magnesium act as antagonists to calcium, from the data it appears that potassium concentration was below (80 mg/100g FW) the threshold of 150 mg/100g FW and the ratio of K/Ca was lower than 30, which suggest that potassium's influence of bitter pit development was low. SmartFresh^{SM} application while delaying ripening also appeared to influence the amount of potassium detected in fruit after 3 months storage. Watkins (2011) reported that SmartFresh application while delaying ripening also affected movement of minerals in fruit, therefore sampling strategy must be taken into consideration when trying to relate the incidence of bitter pit with nutritional profile. While much of the earlier work on nutrition has focussed on the importance of total calcium for plant health the amount of free calcium has a more important role in contributing to tissue health Berridge *et al.* (2003) mentioned overall Ca^{2+} content is not the critical factor affecting cellular function. The dynamic fluxes in free calcium in the cytosol and/or active cellular organelles are translated into changes in metabolism, growth and development. In storage seasons (2011-12) sampling of the inner and outer cortex of Bramley's for mineral analysis confirmed that the inner cortex had a higher concentration of Ca^{2+}, K and Mg compared to the outer cortex and confirms Perring (1985) earlier findings.

Monitoring samples at harvest showed the highest concentration of calcium was present in the inner cortex in tissue located within the inter-carpel regions and could be double that found in the outer cortex. During storage the overall calcium concentration declined in all samples from inner cortex, while a small rise was often seen in the outer cortex. This decline in the inner cortex calcium was more consistent in untreated samples. The calcium content of Bramley apples without bitter pit was significantly higher in the inner cortex than those with bitter pit symptoms. However there was no significant difference between concentrations of calcium in

the inner cortex of samples with different severity of bitter pit. There was no significant decrease in calcium content of SmartFresh^{SM} treated samples during 5 months storage, even when fruit was re-examined after 7 days shelf life at 18°C and confirms earlier work by Watkins and Nock, 2005 who reported that SmartFresh^{SM} reduces the movement of minerals especially Ca^{2+} and in some parts delays incidence of bitter pit development. Little attention has focussed on the different forms of calcium in fruit and the contribution they have on tissue health. Calcium is known to be regulated through conjugation events either through attachment to proteins like calmodulin or by inactivation through binding to phosphates, oxalates or phytenes. There are few reports on the amount or distribution of calcium oxalate in apple and how this influences the storage potential of apple.

Al-Wahsh and Wu (2012) indicated an increase in the propensity for the conjugation of calcium with oxalic acid to form calcium oxalate. The increase in calcium inactivation to form calcium oxalate was considered to be a possible mechanism for bitter pit development. Stow (1988) indicated that the increase in movement of calcium from the middle lamella and loss of its binding sites and increase of cortical water soluble Ca^{2+} was influenced by fruit maturity. Pevicic *et al.* (2004) indicated there was less water soluble Ca^{2+} in pitted fruit compared to healthy tissue. Also De Freitas *et al* (2013) indicated that under some conditions fruit cortical tissue had a higher capacity to strongly bind Ca^{2+} ions in the water-insoluble pectin network in the cell wall matrix, reducing the concentration of Ca^{2+} available for other cellular functions. The onset of softening caused by the breakdown of pectins has been reported to cause a significant loss in available calcium as dissolution of pectin yields temporarily more pectin binding sites for available calcium to bind to, this initial loss in softening has been related to an increase in bitter pit development.

Consideration should be made to the proportion of non-cell wall bound Ca^{2+} rather than total Ca^{2+} in order to determine the potential of fruit consignments to develop bitter pit. It is important to develop new methodologies to quantify the amount of bio-available free Ca^{2+} . In this thesis it was possible to develop new methodologies to distinguish between total calcium and calcium oxalate by the use of inductively coupled plasma (ICP) to measure total calcium by mass spectrometry and then quantifying the proportion of calcium bound to organic acids such as oxalate by atomic absorption spectroscopy (AAS). For the purposes of the study we nominally refer to the proportion of total calcium that is not conjugated to oxalic acid as structural calcium $Ca_{St} = (Ca_T$ - *Ca (COO)₂)* even though the remainder represents a composite of numerous forms of calcium.

Comparison between changes in calcium concentration of untreated and SmartFresh^{SM} treated samples with or without symptoms of bitter pit for two different measurements of calcium as Ca^{2+} (total) and Ca^{2+} (structural) showed a significant difference between inner and outer cortex Moreover, in untreated samples inner and outer cortex calcium (total or structural) concentration decreased significantly when bitter pit occurred. However, there was no significant decrease in concentration of calcium (total or structural) in inner or outer cortex of SmartFresh^{SM} treated samples with or without bitter pit.

More detailed analysis of the calyx and stalk end regions of fruit, confirmed earlier findings that the calyx end of fruit was lower in Ca^{2+} (total) but also Ca^{2+} (structural) across all samples. However in SmartFresh^{SM} treated Bramley apples no difference between calcium content in calyx end of samples was observed even when there was a difference in the incidence of bitter pit. SmartFresh^{SM} treatment appears to be reducing the inactivation of calcium by conjugation to oxalate; this may be the result of lowering the rate of conversion of ascorbic acid breakdown to oxalic acid.

The potassium concentrations in the calyx and stalk end of apples that remained untreated were significantly higher than counterpart samples selected from SmartFresh^{SM} treated fruit. However, the potassium content in the calyx end of fruit was positively correlated with an increase in bitter pit symptoms. There is a propensity for bitter pit to increase in the presence of high concentrations of potassium and magnesium when calcium concentration is low (<4.5 mg/100g FW) (Neuteboom and Withnall, 1998), therefore, the ratio (K+Mg)/Ca may provide more detailed analysis of fruit susceptibility than looking at individual nutrients in isolation.

The results of untreated samples concur with those reported by De Freitas *et al.* (2013), where high concentration of potassium and magnesium in the cortical tissue were found to lead to increased susceptibility to bitter pit. However, the calcium has the strongest correlation to incidence of bitter pit and its distribution between the calyx and stalk regions of fruit.

Changes in the value of Ca^{2+} (total) and Ca^{2+} (structural) content at harvest compared to fruit sampled during storage were correlated with bitter pit. However, the proportion of total and structural calcium did not completely explain the likelihood of disorders developing. In some cases samples with high calcium developed bitter pit and inversely, samples with lower calcium did not develop bitter pit, suggesting that other factors are influencing the development of bitter pit. Attempts were made to normalise results allowing for the minimum level of total calcium

(5mg/100g FW) and the movement of calcium from inner to outer cortex and from stalk end to calyx end.

These equations were applied (details in Table 4.7) as:

A) $\%\overline{\boldsymbol{Ca}}_{\boldsymbol{St}} = \frac{\overline{Ca}_{St} \times 100}{5}$

B) $\%\overline{\boldsymbol{Ca}}_{\boldsymbol{St}}\boldsymbol{changes} = \frac{\left(\overline{Ca}_{St} - \frac{Ca_{St}(In) - Ca_{St}(Out)}{\overline{Ca}_{St}}\right) \times 100}{5}$

Threshold: $\boldsymbol{T} = \left(\frac{A+B}{2}\right) - (A - B) > 50$ (Less than 50: more chance of bitter pit)

Although the values of Ca^{2+} structural (Ca_{St}) in each season were different most of the results of comparison of changes of normalised (Ca_{St}) to harvest threshold were correlated to incidence of bitter pit. An accuracy of more than 85% was achieved in predicting the onset of bitter pit in Bramley apple.

These equations are only based on free (structural) calcium content. The role of other minerals such as K, Mg and N, and their interactions should be considered to refine this prediction model.

As mentioned before these results were obtained from a limited number of samples and orchards and need more investigation with a greater number of samples in different orchards and storage regimes. Also it is important to monitor samples in the shorter intervals (such as every two weeks) for 10-12 weeks after harvest to have a better view of changes in organic acids and sugars and their relationship with incidence of bitter pit.

CHAPTER 5

CHLOROPHYLL FLUORESCENCE

5.1 Introduction:

The objective of this part of the study was to apply chlorophyll fluorescence as an additional non-destructive technique to predict incidence of bitter pit. This method was applied only for samples collected in seasons (2012/13) and (2013/14) for assessment of harvest maturity and storage quality.

The manufacturer (Hansatech Instruments, UK) provided the PEA pocket with red light filter. During monitoring samples a large dataset was collected for analyses. The fluorescence data was converted by instrumental software (PEA plus) into 57 characteristics of fluorescence. There are more details about these characteristics in the literature review (chapter 1: 1.8). These characteristics were calculated from fluorescence signals at different points of the fluorescence rise. Models of the photosynthetic light reactions have been developed to enable calculation of processes or components of the photosynthetic process from the signal.

5.2 Determination of the best characteristics correlated to bitter pit:

The most discriminate characteristics in identifying internal physiological disorders and fruit maturity were determined by applying linear discriminant analysis. The fluorescence data was tested for its ability to categorise three levels of bitter pit as light, moderate and severe. However, the samples for the lower level of bitter pit (light) were in a separate group and samples with moderate and severe bitter pit were not distinct. So, it was possible to use linear discriminant analysis to distinguish between fruit suffering from a low incidence of pit and fruit where severity was greater (Figure 5.1). While samples with slight bitter pit formed a distinct cluster, where the incidence of bitter pit was classed as moderate or severe discriminate analysis was less able to separate severity based on chlorophyll fluorescence profiles.

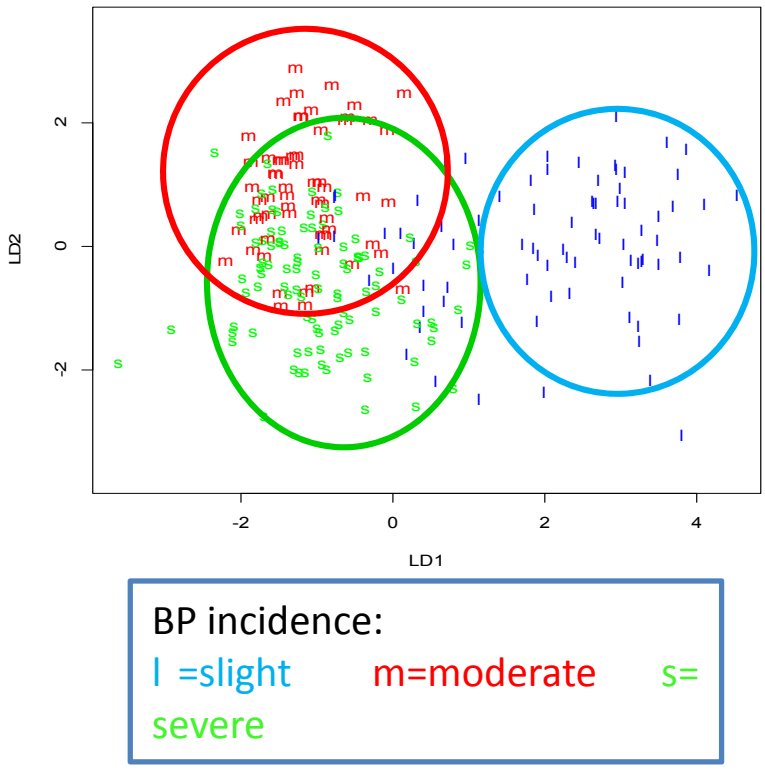


Figure 5.1: Discriminant analysis of bitter pit severity data. Each data point is the mean of a ten apple sample assessed at different stages during storage in season 2012/13.

Ten chlorophyll fluorescence characteristics contributed to LD1 and correlated well (R> ±0.70) with the incidence of bitter pit (Table 5.1). Notably the characteristics that were found to be most useful were direct measurements of fluorescence yield rather than the indirect calculations of physiological characteristics.

Table 5.1: Characteristics with the highest correlation coefficient to incidence of bitter pit.

Fluorescence transients	Correlation coefficient (R)
F4 (Fluorescence Intensity at 2 ms)	-0.83
F5 (Fluorescence Intensity at 30 ms)	-0.83
F3 (Fluorescence Intensity at 300 µs)	-0.81
Fm (Maximum Fluorescence Yield)	-0.81
Fv (Variable Fluorescence Yield)	-0.80
F1 to F3 (Fluorescence Intensity between 50 µs to 300 µs)	-0.78
F1 (Fluorescence Intensity at 50 µs)	-0.77
F2 (Fluorescence Intensity at 150 µs)	-0.76
F1 to F4 (Fluorescence Intensity between 50 µs to 2 ms)	-0.76
Fo (Minimum Fluorescence Yield)	-0.72

Fm (maximum chlorophyll fluorescence yield) was compared in samples suffering from different degrees of bitter pit severity (Figure 5.2). The one-way ANOVA of Fm against bitter pit range codes (slight to very severe) showed P<0.001. The Tukey's test only comes out significant for the "None bitter pit" versus other ranges (between samples without bitter pit and samples with bitter pit) and the changes of Fm between different classes of incidence of bitter pit were not significant.

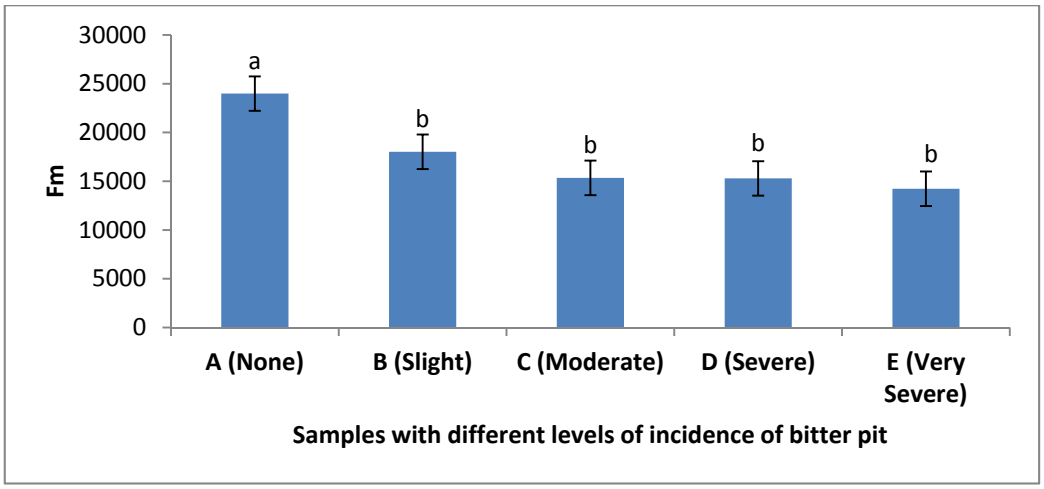


Figure 5.2: Comparison of Fm (Fluorescence maximum) and classes of bitter pit severity in Bramley apples stored at 4.5° C. Each bar is the mean of Fm for the samples assessed at different stages during storage categorised in the same group in season 2012/13. Mean values the same letter are statistically equal according to Tukey's test (P<0.05).

Fm measured from apples treated with 1-MCP (SF-treated) retained a higher level during storage than untreated samples and after four months storage they were significantly higher than untreated samples (Figure 5.3).

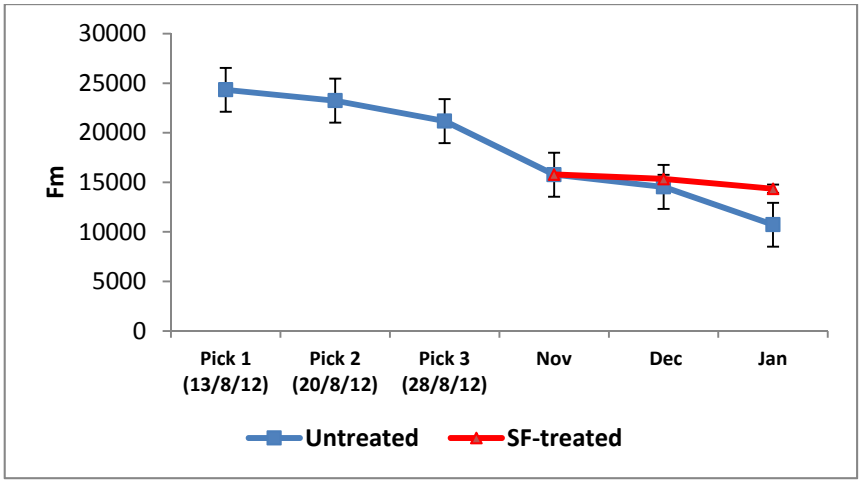


Figure 5.3: Comparison of fluorescence changes in SF-treated and untreated samples during different harvest dates and 5 months of storage in air (21%O_2) in season 2012/13. Each data point is the mean of ten apple samples ± SE.

The changes in fluorescence characteristics (Table 5.1) were compared individually with changes in fruit maturity and the internal quality of fruit. Furthermore in particular the potential of individual characteristics for distinguishing the severity of bitter pit was analysed. Over a range of studies (data not shown) it was found that the characteristics Fo (origin), F1 and F2 were better correlated with bitter pit than other characteristics. Only Fo, F1 and F2 showed significant (P<0.05) difference between samples with or without bitter pit, but the severity of bitter pit could not be estimated by this technique alone (Figure 5.4).

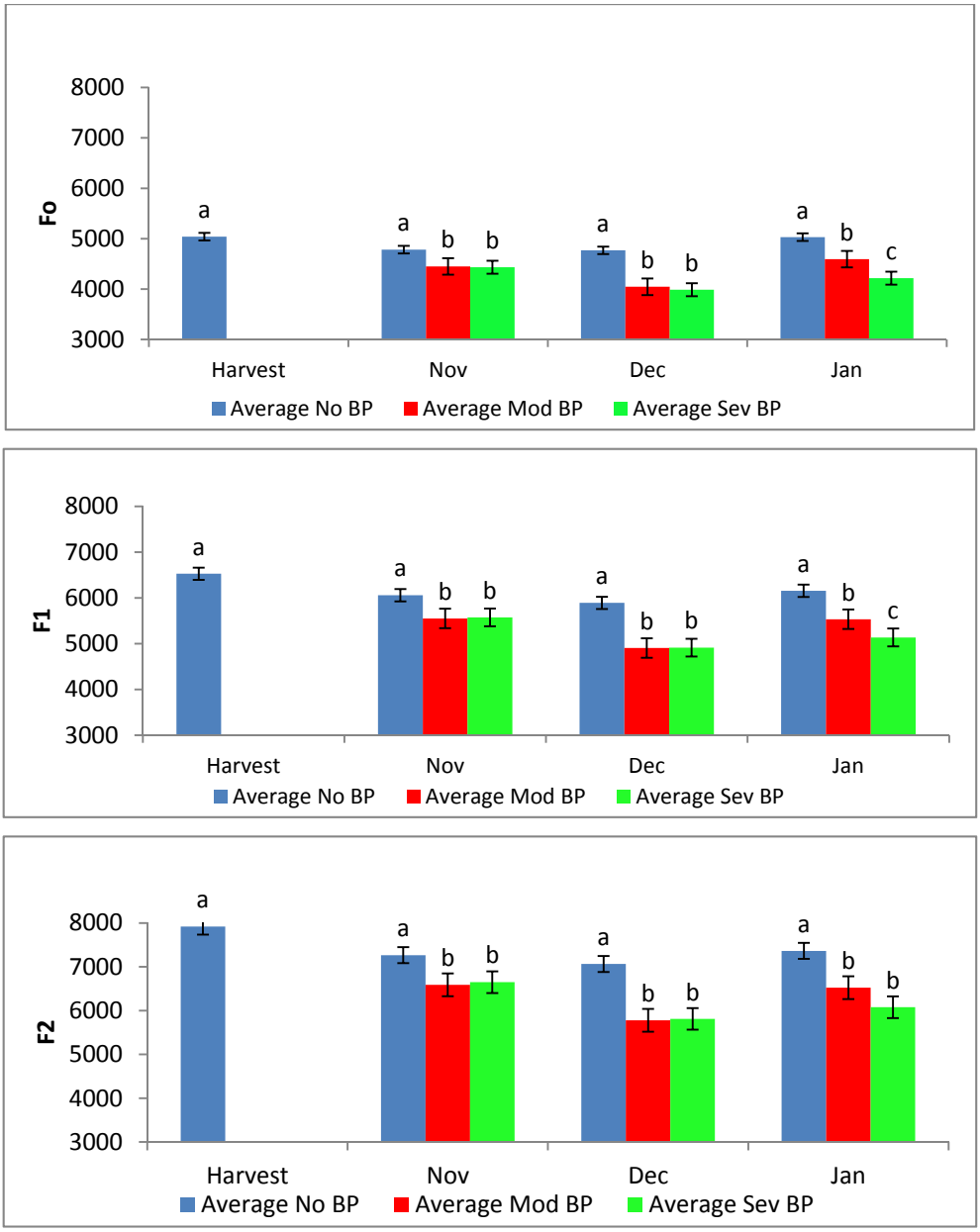


Figure 5.4: Comparison of changes of different characteristics (Fo, F1, F2) for different classes of bitter pit severity stored at air regime (21% O_2). Each data point is the mean of ten apple samples ± SE. Mean values with different letters for the same assessment date were significantly different according to Tukey's test (p<0.05).

The average of three selected characteristics (Fo, F1, F2) was calculated and named as average F: $\left(\overline{F} = \frac{Fo+F1+F2}{3}\right)$. The next step was to find the threshold of $(\overline{F})$ for onset or incidence of bitter pit.

5.3 Determination of the threshold of ($\overline{F}$) for incidence of bitter pit:

The results obtained for ($\overline{F}$) and incidence of bitter pit in samples collected in season 2012/13 were analysed, and the threshold of incidence of bitter pit was determined as 5900 (Figure 5.5). Thus samples with average F ($\overline{F}$) less than 5900 were more susceptible to bitter pit. A contingency table for Chi square analysis (Table 5.2) compared *P* value for $\overline{F}$>5900 was better correlated with bitter pit development and a significant correlation P<0.001 was found.

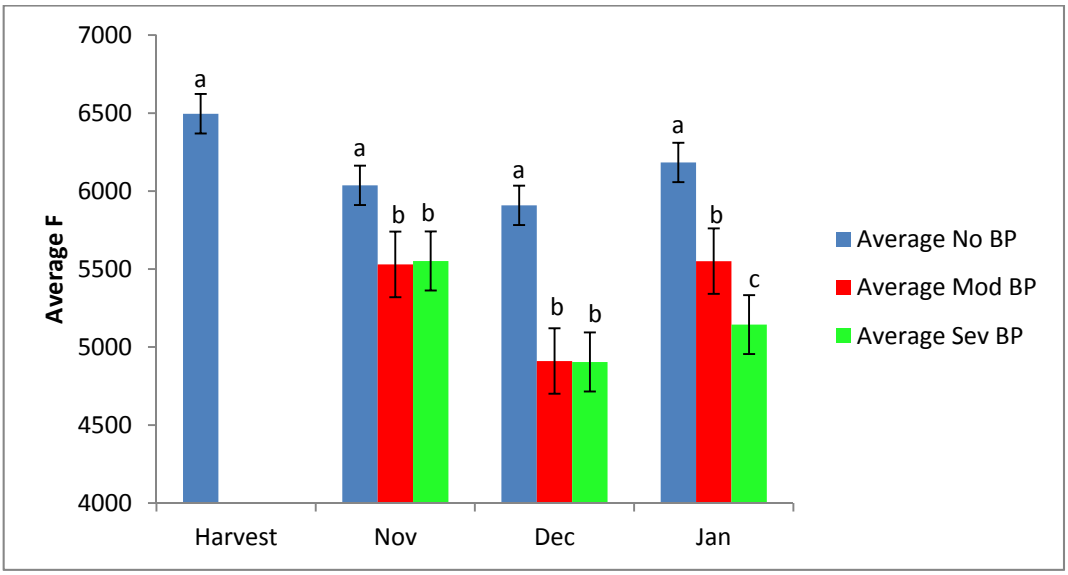


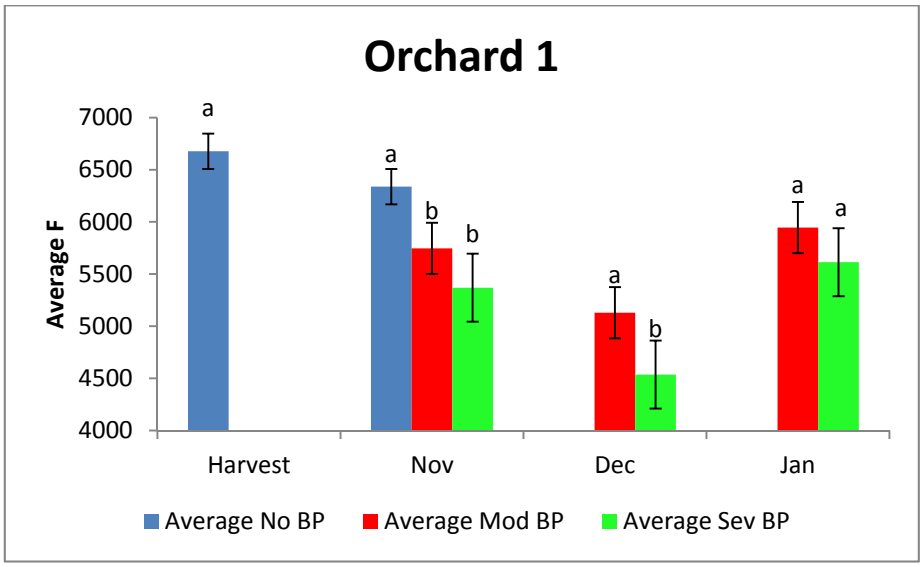
Figure 5.5: Comparison of changes of average of parameters (Fo, F1, F2) named as average F ($\overline{F}$) for different classes (severity) of bitter pit during storage time (Air- storage). Each data point is the mean of ten apple samples ± SE. Mean values the same letter for the same assessment date are statistically equal according to Tukey's test (*P*<0.05).

Table 5.2: Contingency table for chi square test of the threshold of $\overline{F}$<5900 for incidence of bitter pit which was significant *P*<0.001 for each orchard.

Actual:	No BP	BP	Total
$\overline{F}$<5900	3	67	70
$\overline{F}$>5900	24	6	30
Total	27	73	100

The changes of ($\overline{F}$) in samples collected from different orchards predicted bitter pit well in some instances such as orchards 1 and 2. The difference of samples with or without bitter pit during storage time were compared and results showed that when $\overline{F}$<5900, there was a greater

chance of incidence of bitter pit. However this threshold was not always representative of incidence of bitter pit such as, for example, the results for moderate bitter pit in January 2013 (Figure 5.6).



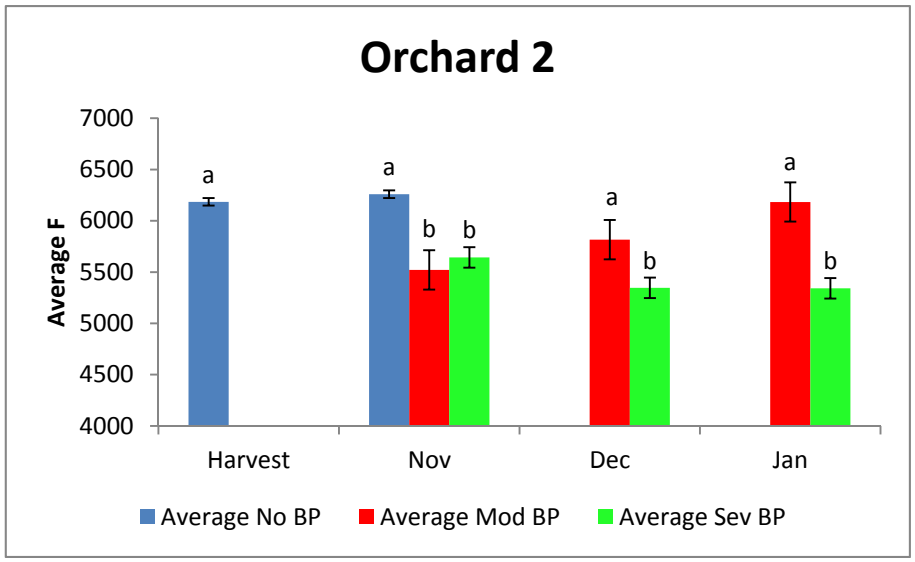


Figure 5.6: Comparison of changes in "average F ($\overline{F}$)" in different orchards and categorised as no BP, moderate BP and severe BP. Samples were untreated and stored in air in season 2012/13 and after December all samples showed bitter pit. Each data point is the mean of ten apple samples ± SE. Mean values with different letters for the same assessment date were significantly different according to Tukey's test (p<0.05).

5.4 Changes of chlorophyll fluorescence in different seasons:

The results obtained in season 2013/14 showed a different level of fluorescence which was lower than season 2012/13. However, it showed the difference between symptomless samples (No BP) and samples with bitter pit (Figure 5.7) and still 95% confidence intervals for different levels of incidence of bitter pit showed that ($\overline{F}$) could be applied for identifying the samples that remained symptomless and the samples with bitter pit symptoms in the early stages of storage, and those samples that showed symptoms of bitter pit development later (Figure 5.8).

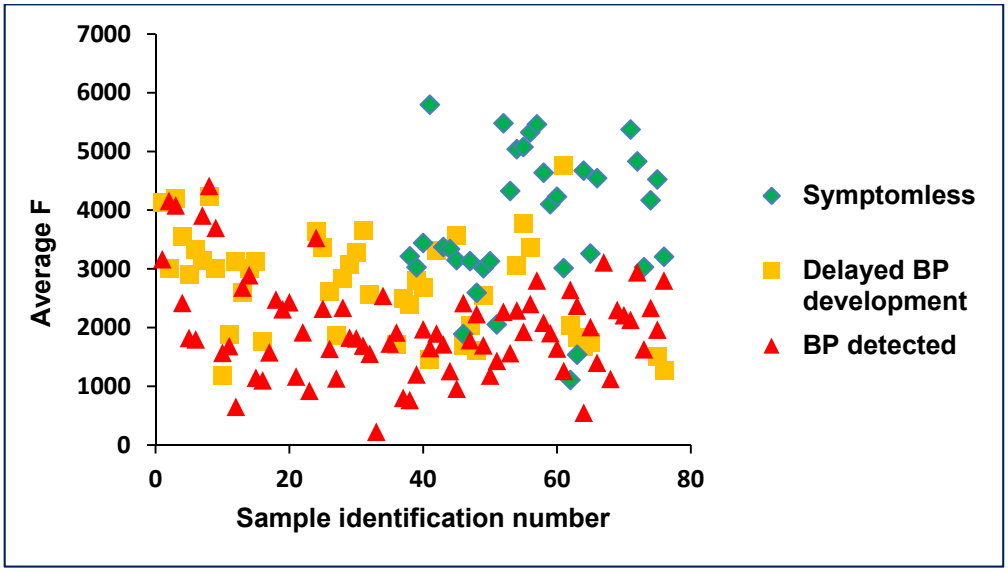


Figure 5.7: Categorising samples with different levels of incidence of bitter pit in three groups as: symptomless (No BP), delayed BP development and BP detected. Each data point is average of 10 samples.

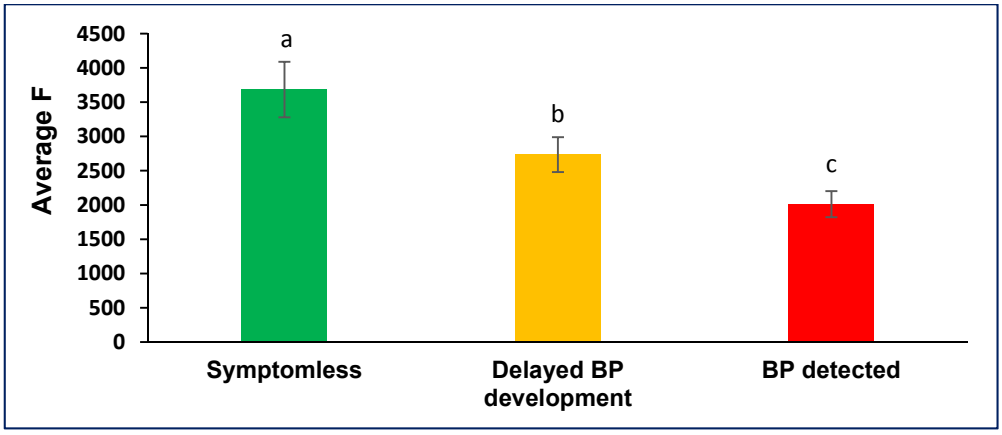


Figure 5.8: Comparison of 95% confidence intervals for different levels of incidence of bitter pit. Each bar is the mean of AvF for the samples categorised in three groups as symptomless (No BP), delayed BP development and BP detected in season 2013/14.

5.5 Final determination of threshold for incidence of bitter pit:

Because of changes in the level of fluorescence in each season which is related to the differences of environmental factors in each season or even the differences of orchards located in different microclimates, it was decided to find the threshold based on changes of $(\overline{F})$ during storage in comparison with the $(\overline{F})$ at harvest time. So, threshold is not a fixed range of $(\overline{F})$ and is calculated as normalised to the average harvest F value $(\overline{F_h})$ and is based on the lowest standard deviation (σ) for the sample (from 10 apples assessed as one sample).

Table 5.3 shows the equations for calculating threshold at harvest time and normalisation of $(\overline{F})$ of samples during storage monitoring.

Table 5.3: Equations for normalising $(\overline{F})$ samples to harvest time and the threshold at harvest.

Average F: $(\overline{\mathrm{F_x}})$	$\left(\overline{\mathrm{F_x}} = \frac{Fo + F1 + F2}{3}\right)$
Normalised $(\overline{\mathrm{F_x}})$ sample to harvest: $(N_{x/h})$	$N_{x/h} = \left(\frac{\overline{\mathrm{F_x}} - \sigma\overline{\mathrm{F_x}}}{\overline{\mathrm{F_h}}}\right) \times 100$
Threshold at harvest: (T_h) (Threshold is 5% less)*	$T_h = \left(\frac{\overline{\mathrm{F_h}} - \sigma\overline{\mathrm{F_h}}}{\overline{\mathrm{F_h}}}\right) \times 95$
More chance of bitter pit	$N_{x/h} < T_h$
Less chance of bitter pit	$N_{x/h} > T_h$

*Different ranges were tested in the equation and 5% less than normalised value of $(\overline{F})$ at harvest was better matched with incidence of bitter pit.

By comparing normalised $(\overline{\mathrm{F_x}})$ of samples during storage to harvest time the calculated figure is used as threshold at harvest. When the result of $N_{x/h} = \left(\frac{\overline{\mathrm{F_x}} - \sigma\overline{\mathrm{F_x}}}{\overline{\mathrm{F_h}}}\right) \times 100$ is lower than threshold there is more chance of bitter pit. Table 6.4 shows an example of comparison of untreated and SF-treated samples collected from one orchard and stored in 21% O_2 (air) regime and monitored for five months in season 2012/13.

Table 5.4: Comparison of normalised ($\overline{F}$) to harvest time and the threshold at harvest time (T_h) and incidence of bitter pit in untreated and SF-treated samples collected from one orchard and stored in 21% O_2 (air) regime and monitored for five months in season 2012/13.

Date	Untreated/SF	BP%	$\overline{F}$	SD (σ)	$N_{x/h}$	Threshold at harvest (T_h)
Harvest (September)	Untreated & SF-treated	0	5952.3	512.3	91.3	**86.8**
November	Untreated	15	5524.3	1570.2	66.8	$N_{x/h}<T_h$ (BP)
December	Untreated	25	4552.6	586.2	67.1	$N_{x/h}<T_h$ (BP)
January	Untreated	38	4822.7	679.1	69.9	$N_{x/h}<T_h$ (BP)
November	SF-treated	0	6121.0	551.8	93.6	$N_{x/h}>T_h$ (NoBP)
December	SF-treated	0	6327.3	1071.3	88.2	$N_{x/h}>T_h$ (NoBP)
January	SF-treated	7	5667.7	908.2	80.4	$N_{x/h}<T_h$ (BP)

Comparison of graphs for untreated and SF-treated samples shows the differences of changes in the value of normalised ($\overline{F}$) to harvest time ($N_{x/h}$). When $N_{x/h}$ is lower than threshold there is incidence of bitter pit (Figure 5.9). Also these graphs show that when the value of ($N_{x/h}$) is higher than threshold there is no sign of bitter pit, but the standard error is lower than threshold, this situation indicates a risk that incidence of bitter pit is likely to happen soon.

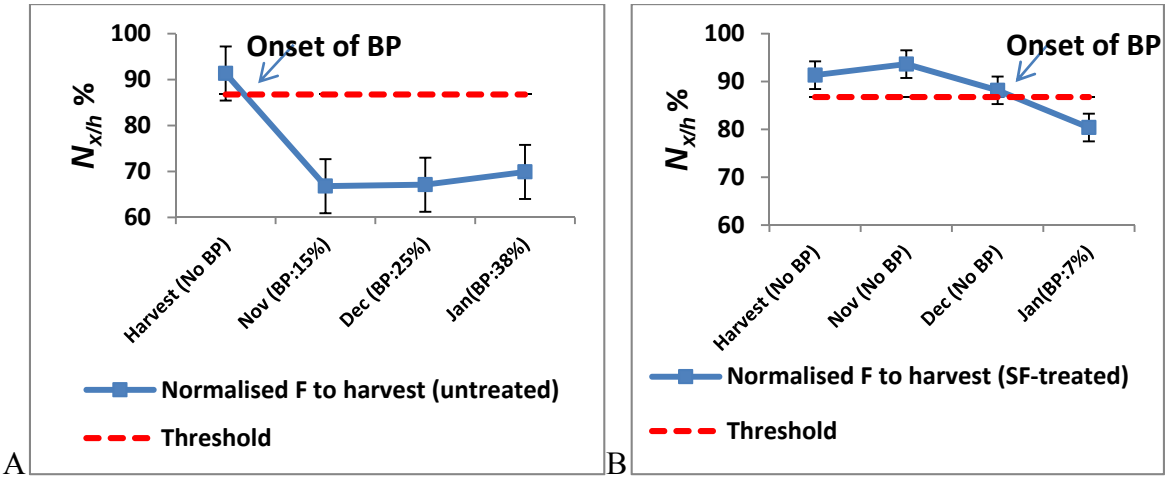


Figure 5.9: Comparison of changes of normalised ($\overline{F}$) to harvest time named as ($N_{x/h}$) and incidence of bitter pit in A) untreated and B) SF-treated samples of the same orchard that stored in 21 % O_2 (air) regime and monitored for five months in season 2012/13.

These formulae were applied to the results collected from samples of different orchards which were untreated or SF-treated and stored in different storage regimes. Tables 5.5 and 5.6 show part of these results related to two orchards in seasons 2012/13 and 2013/4 which were stored in 21% O_2 (air) regime. Although the values of $\overline{F}$ in each season were different most of the results of comparison of changes of $\overline{F}$ normalised to harvest threshold were correlated to incidence of bitter pit. However, these were not always correct and there were some false positive or negative results. In particular the numbers of false results in season 2013/14 were more than 2012/13. This could be related to the different method of assessment that applied in season 2013/14 (details in 2.4 chapter materials & methods) which was based on monitoring the same samples during storage and there was no way to find bitter pit in samples which kept until the last assessment date and bitter pit onset probably was started earlier in them. Also the results of CA regimes which were only assessed in season 2012/13, since the assessments were in longer intervals until end of storage did not correlate well with the formulas. It seems chlorophyll fluorescence assessments are matched with these formulas in the period between second to the third month of storage regardless of the storage regime.

Table 5.5: Summary results of comparison of samples collected from two orchards (more susceptible "EMR" and susceptible "Hoo" to bitter pit) in season 2012/13. Samples were stored in air (21% O_2) and were monitored every month from the second month of storage.

Test date	Sample	$\overline{F}$	SD (σ)	$N_{x/h}$	BP%	Threshold at harvest (T_h)
28/08/2012	**EMR (Harvest)**	**5827.4**	**918.4**	**84.3**	**0**	**80.1**
12/11/2012	EMR (Untreated)	4969.8	719.6	73.5	32	$N_{x/h}<T_h$
12/11/2012	EMR (SF)	5661.5	577.0	87.6	0	$N_{x/h} > T_h$
12/12/2012	EMR (Untreated)	5275.3	1357.9	68.2	40	$N_{x/h}<T_h$
12/12/2012	EMR (SF)	5505.7	975.8	78.6	12	$N_{x/h}<T_h$
03/01/2013	EMR (Untreated)	4525.8	908.7	63.0	35	$N_{x/h}<T_h$
03/01/2013	EMR (SF)	5672.5	869.9	84.0	15	$N_{x/h} > T_h$ (false+)
28/08/2012	**Hoo (Harvest)**	**5952.3**	**512.3**	**91.3**	**0**	**86.8**
12/11/2012	Hoo (Untreated)	5524.3	1570.2	66.8	15	$N_{x/h}<T_h$
12/11/2012	Hoo (SF)	6121.0	551.8	93.6	0	$N_{x/h} > T_h$
12/12/2012	Hoo (Untreated)	4552.6	586.2	67.1	25	$N_{x/h}<T_h$
12/12/2012	Hoo (SF)	6327.3	1071.3	88.2	0	$N_{x/h} > T_h$
03/01/2013	Hoo (Untreated)	4822.7	679.1	69.9	38	$N_{x/h}<T_h$
03/01/2013	Hoo (SF)	5667.7	908.2	80.4	7	$N_{x/h}<T_h$

Table 5.6: Summary results of comparison of samples collected from two orchards (more susceptible "EMR" and susceptible "Hoo" to bitter pit) in season 2013/14. Samples were stored in air (21% O_2) and were monitored every two weeks from the second month of storage.

Test date	Sample	$\overline{F}$	SD (σ)	$N_{x/h}$	BP%	Threshold at harvest (T_h)
02/09/2013	**EMR (Harvest)**	**5199.3**	**698.1**	**86.6**	**0**	**82.3**
01/10/2013	EMR (Untreated)	5705.9	647.0	97.0	0	$N_{x/h} > T_h$
15/10/2013	EMR (Untreated)	4564.6	772.1	73.2	7	$N_{x/h}<T_h$
01/11/2013	EMR (Untreated)	4550.0	794.6	72.5	12	$N_{x/h}<T_h$
15/11/2013	EMR (Untreated)	4427.1	527.0	75.1	27	$N_{x/h}<T_h$
06/09/2013	**EMR (Harvest)**	**5422.1**	**1065.8**	**80.5**	**0**	**76.5**
01/10/2013	EMR (SF)	5008.2	565.3	82.4	0	$N_{x/h} > T_h$
15/10/2013	EMR (SF)	4923.2	781.4	76.8	7	$N_{x/h}<T_h$
01/11/2013	EMR (SF)	4993.7	382.5	85.1	10	$N_{x/h} > T_h$ (false+)
15/11/2013	EMR (SF)	4955.0	542.0	81.4	0	$N_{x/h} > T_h$
02/09/2013	**Hoo (Harvest)**	**4483.1**	**683.2**	**85.0**	**0**	**80.7**
01/10/2013	Hoo (Untreated)	5549.7	876.9	104.6	0	$N_{x/h} > T_h$
15/10/2013	Hoo (Untreated)	4554.6	382.3	93.6	0	$N_{x/h} > T_h$
01/11/2013	Hoo (Untreated)	4405.2	614.1	85.0	0	$N_{x/h} > T_h$
15/11/2013	Hoo (Untreated)	4039.9	607.4	77.3	10	$N_{x/h}<T_h$
06/09/2013	**Hoo (Harvest)**	**4618.7**	**483.0**	**89.7**	**0**	**85.2**
01/10/2013	Hoo (SF)	5078.9	519.2	98.9	0	$N_{x/h} > T_h$
15/10/2013	Hoo (SF)	4428.5	732.5	80.5	0	$N_{x/h}<T_h$ (false-)
01/11/2013	Hoo (SF)	4657.5	455.2	91.1	5	$N_{x/h} > T_h$ (false+)
15/11/2013	Hoo (SF)	4513.3	706.6	82.9	7	$N_{x/h}<T_h$

The threshold of incidence of bitter pit based on changes of ($\overline{F}$) normalised to harvest time was tested on samples in different storage regimes and in different seasons and orchards and in more than 80% incidence of bitter pit matched with lower value of normalised ($\overline{F}$) comparing to harvest threshold ($N_{x/h}$). Table 5.7 shows the contingency table of results in seasons 2012/13 and 2013/14 in different orchards and treatments. Chi square analysis compared thresholds with bitter pit development and a significant correlation P<0.001 was found.

Table 5.7: Contingency table for Chi square test of the threshold to find false$\mp$ and misses in comparison of threshold and incidence of bitter pit.

	No BP	BP	Total
$N_{x/h} < T_h$	7	51	58
$N_{x/h} > T_h$	23	11	34
Total	30	62	92

	False (numbers)	False (Percentage)
False positive (+): Formula($N_{x/h} > T_h$)& BP(+)	11	32%
False negative (-): Formula($N_{x/h} < T_h$)& BP(-)	7	12%
Total misses	18	19.5%

	Number of samples	Number of samples	Percentage
Total misses	18	False+: 11	12%
		False -: 7	8%
Total hits	74	Correct BP: 51	55%
		Correct NoBP: 23	25%

5.6 Discussion

In the first two seasons (2010/11) and (2011/12), this research programme was focused on developing diagnostic tools which were based on destructive methods mainly biochemical and molecular analysis. However, after two seasons it was considered that most destructive methods were time consuming with high cost. The objective was to develop a non-destructive method that was easy to apply and cost efficient. Since the results obtained from colour measurements showed a weak positive correlation (untreated samples R=0.65) between increasing *b** value and incidence of bitter pit and these results were similar to the previous study by Ross (2002) on disorders caused by stress, it was decided to apply chlorophyll fluorescence as a non-destructive tool in the last two seasons of the study alongside destructive methods.

Most of the previous studies on chlorophyll fluorescence were focused on determination of fruit maturity by applying different instruments for measurement; Ross (2002), Rees *et al.* (2005) and Valcke (2011) used the fluorimeter "handy PEA" (Hansatech, UK), Rutkowski *et al.* (2008) used CCM-200 (Optiscience, USA). Each one of these instruments is capable of measuring different fluorescence characteristics for different purposes. In addition Lotze *et al.* (2006) applied an NIR spectrometer (Zeiss-Jena, Germany) and Toivonen *et al*. (2011) used the DA meter (Turoni, Italy) which are not measuring chlorophyll fluorescence but based on measuring absorption/ reflection of specific wavelengths of irradiation. In this study it was decided to use the "Pocket PEA" (Hansatech, UK) as a small and mobile fluorimeter that provides measurement of a range of fluorescence yields at different time points of the fluorescence transient.

Previous studies showed that loss of chlorophyll content is correlated with advancement in maturity (Ross, 2002; Rees et al., 2005; Rutkowski *et al.*, 2008). Also Lotze *et al.* (2006) found that there was a correlation of incidence of bitter pit to the loss of fluorescence yield with the progression of fruit maturity. On the other hand Watkins *et al.* (1989) and Tong *et al.* (1999) emphasised the importance of harvesting apples at the right stage of maturity for improving storage life and delaying incidence of bitter pit. Furthermore determination of maturity is very important for efficiency of 1-MCP treatment (Watkins *et al.* 2000; Mir *et al.,* 2001; Johnson, 2007).

According to the results obtained by previous studies, it was decided to focus on changes of fluorescence characteristics during the last stages of fruit maturity and also during storage, so

measurements started two weeks before commercial harvest time of Bramley apples and continued during storage in different storage regimes as air or CA.

The results showed decreasing chlorophyll fluorescence profiles (Fo to F5, Fm & Fv) of apples that started before commercial harvest and during storage, and indicates loss of chlorophyll. However, the rate of chlorophyll reduction between samples varied and a higher rate of loss in chlorophyll fluorescence was associated with an increased severity of bitter pit.

Different chlorophyll fluorescence characteristics that contributed and correlated well (R> 0.70) with the incidence of bitter pit were selected. Some of the characteristics were investigated in previous studies on fruit stress; characteristics such as Fm (Fluorescence maximum), Fv/Fm, RC/CS (a physiological characteristic calculated from fluorescence yields presented as a calculation of reaction centre density) were applied before by Ross (2002) and Rees *et al.* (2005) to find the best parameter correlated with maturity and stress.

Furthermore, this study showed that the characteristic Fm was able to distinguish samples treated with 1-MCP from untreated fruit. This is consistent with results obtained by Mir *et al*. (2001). In apples treated with 1-MCP, the value of Fm was higher related to slower loss of fluorescence because of lower ethylene production and a delay in ripening of treated fruit.

Although it seems that the characteristic Fm could be applied as a good indicator of maturity, this study indicated that the best characteristics correlated to indicate bitter were found from the first stages of the fluorescence rise as Fo, F1, F2 (fluorescence intensity at 50 µs to 300µs). Our results are similar to Oukarroum *et al.* (2012) who found that thermal stress causes significant changes in the fluorescence characteristics and affected the initial fluorescence (Fo) more than the maximum fluorescence value (F_m). Since bitter pit as a type of stress could affect these changes on characteristics, it was decided to focus on the characteristics Fo, F1 and F2 which were better correlated with bitter pit and the value of the average of three characteristics (Fo, F1, and F2) named as average F:$\left(\overline{F} = \frac{Fo+F1+F2}{3}\right)$, provided the best correlation with incidence of bitter pit. Comparison of results obtained from samples collected from different orchards and stored in different storage regimes (air and CA) showed that $\overline{F}$ was able to distinguish samples without bitter pit from samples with bitter pit symptoms although without distinguishing severity of bitter pit. Chlorophyll fluorescence measurements are based on re-emitted energy from the skin of fruit, and therefore reflect any changes occurring near peel. Bitter pit at first onset occurs under the skin and by increasing severity pits may extend

throughout the cortex (Jackson 2005), so when bitter pit severity increases and affected cells are not close to the skin, the value of chlorophyll fluorescence is not affected as much as severity of internal disorders such as bitter pit.

Results in season 2012/13 showed a threshold of ($\overline{F} \leq 5900$) which was correlated ($P<0.001$) to fruit susceptibility to bitter pit development. This study and the results of season 2012/13 were presented at "V International postharvest unlimited conference" and published in Acta Horticulturae vol. 1079 (2015) (Appendix X).

In season 2013/14 the general level of $\overline{F}$ was lower than season 2012/13 which introduced an inconsistency for the threshold for incidence of bitter pit. Although samples were collected in two seasons at approximately the same date (28/8/2012 and 2/9/2013), the ($\overline{F}$) of the samples from the same orchard in season 2012/13 were higher than season 2013/14. As Gaunter *et al.* (2014) indicated the impact of changes in climate on the value of chlorophyll fluorescence, climate differences during two seasons of fluorescence assessments in our study affected the results obtained. According to Meteorological Office reports for Kent/UK 2012 and 2013 statistics, the average temperature between March 10th and April 10th 2012 was 4°C above the long-term (30 years) average. The warm weather resulted in flowers opening two weeks earlier. This was followed by a period of cold weather with temperatures 3°C below average. These fluctuations continued through April to June 2012. Fluctuations in temperature during flowering and fruit development affected pollination, calcium uptake and cell expansion of fruits. On the other hand until April the year of 2012 was very dry, and then from April to July was very wet. Summer 2012 was the wettest on record for the UK overall since 1912. The implications of these changes were observed through a large variation of fruit maturity within orchards that led to an extended period over which Bramley apples were harvested. Different weather patterns resulted in different chlorophyll concentrations.

The difference between ($\overline{F}$) at harvest in different seasons is related to different parameters including orchard management, environmental factors and climate changes. Because of these impacts and changes in ($\overline{F}$) it was essential to develop a model which is less affected by seasonal factors.

It was decided to apply formulas based on the decrease of ($\overline{F}$) during storage in comparison with the ($\overline{F}$) at harvest. So ($\overline{F}$) normalised by the formula $N_{x/h} = \left(\frac{\overline{F_x} - \sigma\overline{F_x}}{\overline{F_h}}\right) \times 100$ was applied

to samples of 10 apples. The standard deviation is included to account for the higher risk of bitter pit in apple samples of wider variability. The safety threshold at harvest calculated 5% lower:

$$T_h = \left(\frac{\overline{F_h} - \sigma\overline{F_h}}{\overline{F_h}}\right) \times 95.$$

When ($N_{x/h} < T_h$) there is a greater chance of incidence of bitter pit. This threshold was tested with results of both seasons (2012/13 and 2013/14) and was correlated (P<0.001) with the incidence of bitter pit in different seasons and storage conditions also was able to distinct untreated samples from 1-MCP or SF-treated samples. Although the comparison of the thresholds and normalised value $N_{x/h}$ showed a success rate of approximately 80%, there was also 20% misses (12% false positive and 8% false negative) in the results. These are very similar to the results reported by Lotze *et al.* (2006) with their model with chlorophyll fluorescence imaging by Near-infrared (NIR).

Although results indicate that chlorophyll fluorescence can be used to predict the occurrence of bitter pit in samples of fruit sampled during storage it does not have the resolution to predict the incidence of bitter pit development in samples measured at harvest, so a practical strategy is to monitor over the storage time. Rees *et al.* (2005) worked on physiological stress in long term and short term storage by monitoring Fv/Fm changes, which was not successful for predicting damage in fruit kept in the long term storage. Results of this research also showed the chlorophyll profiles that best described fruits propensity to develop bitter pit were obtained by monitoring fruit during second to the fourth month of storage, thereafter the relationship was less apparent and further changes were not seen with prolonged storage. The underlying metabolic imbalances responsible for bitter pit development over long-periods of storage may be very subtle and not always easy to detect externally.

The model works for apples treated with 1-MCP (SmartFresh^{SM}) as well as untreated samples. In SmartFresh^{SM} treated apples chlorophyll fluorescence is more than untreated apples since ripening is delayed and chlorophyll degradation is slower.

Chlorophyll fluorescence is not able to discriminate the severity of bitter pit. The use of chlorophyll fluorescence may be of utility in fruit store management where regular checks on fruit quality are required to predict the rate of fruit maturation and deterioration in the stored crop. As mentioned by Born *et al.* (2004) although chlorophyll fluorescence is a helpful non-

destructive tool, for having a more accurate prediction model it should be combined with other quality measurements. It may help growers to manage their stores of fruit where regular checks on fruit quality are taken routinely during store monitoring and predict the need to market fruit of stores that changes in the chlorophyll fluorescence profiles indicate fruit has a greater risk of bitter pit at the point of storage inspection. According to the results of this study for better store management using a non-destructive tool to predict incidence of internal disorders like bitter pit it is recommended to apply fruit monitoring for chlorophyll fluorescence changes immediately after harvest, filling the store with 100 samples from each cold-store and start regular monitoring of the same samples from the second month of storage every two weeks, comparing the normalised ($\overline{F}$) with the harvest threshold. When the value was close to threshold or less ($N_{x/h} < T_h$) as the warning, it is essential to do destructive quality tests with other samples in the store.

CHAPTER 6

MOLECULAR ANALYSIS

6.1 Introduction:

Molecular diagnostic methods were applied to compare differences between apples suffering from bitter pit and symptomless apples. Expression of a small number of key genes (calmodulin, Ca^{2+}ATPase, Ca^{2+}Protease and lipoxygenase) that regulate calcium and cell wall lipid metabolism, was tested for in samples with different degrees of bitter pit development. The changes in gene expression that could be related to developmental changes in the cell and tissue and the formation of necrotic lesions of bitter pit were investigated and showed that in general there was a differential pattern of expression of transcripts between apples suffering from bitter pit and apples that remained healthy.

6.2 RNA extraction procedures:

Assessment of two methods for high throughput extraction of RNA indicated that the initial CTAB RNA extraction method based on Gasic *et al.* (2004) was less successful due to the low recovery of RNA (Table 6.1). In comparison a method employing Qiagen sepharose columns (Colgan, 2002) provided a higher yield and greater purity of RNA from apple cortex (Table 6.2).

Table 6.1: RNA concentration of samples extracted using method 1 with CTAB- Gasic *et al.* (2004). (+/-: definition between two bands **18S** as small ribosomal subunit and **28S** as large subunit was not clear).

Sample	Smart Fresh (+/-)	RNA Concentration (ng/µL)	A260nm	A280nm	260/280	18S & 28S RNA bands
1) Carpenter	+	149.9	0.073	0.065	1.12	+/-
2) Carpenter	-	129.6	0.064	0.059	1.07	+/-
3) Wheeler	+	122.1	0.060	0.063	0.96	+/-
4) Wheeler	-	121.9	0.060	0.054	1.1	+/-
5) Bardsley	+	134.8	0.087	0.075	1.16	+/-
6) Bardsley	-	110.5	0.054	0.059	0.91	+/-

Table 6.2: RNA concentration of the same samples as in Table 6.1 extracted with Qiagen sepharose columns (method 3). The first series of RNA extraction with high concentration, there was clear distinction of the ***18S*** rRNA (the small ribosomal subunit), and the large subunit ***28S***.

Sample	Smart Fresh (+/-)	RNA Concentration (ng/µL)	A260nm	A280nm	260/280	18S & 28S RNA bands
1) Carpenter	+	814.5	0.407	0.219	1.86	+
2) Carpenter	-	664.6	0.332	0.192	1.73	+
3) Wheeler	+	300.4	0.150	0.119	1.26	+
4) Wheeler	-	337.5	0.169	0.111	1.52	+
5) Bardsley	+	517.9	0.259	0.145	1.79	+
6) Bardsley	-	101.2	0.051	0.036	1.39	+/-

The quality of RNA extracted via the Qiagen method provided clear distinction of the 18S and 28S (ribosomal subunit) RNA when separated by gel electrophoresis (1% Agarose w/v) (Figure 6.1).

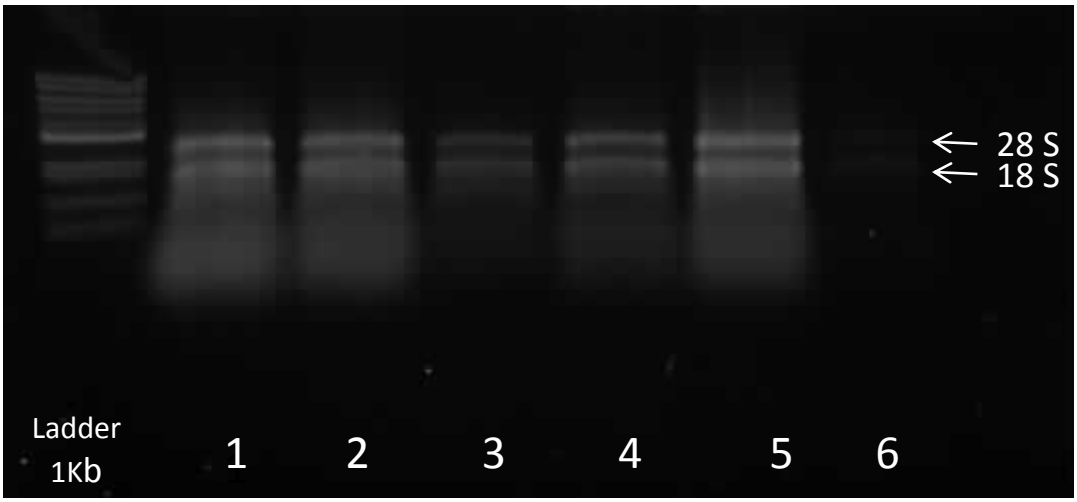


Figure 6.1: rRNA of samples showed two bands (18S and 28S RNA). 1% [w/v] agarose gel was run for 15 min at 100 volts (Brody *et al*., 2004).

RNA of twelve samples related to one orchard (Pitstock) collected during the 2011-12 storage season representing Bramley's seedling apples of different maturities and internal quality was extracted (Table 6.3).

Table 6.3: RNA concentration of samples collected from Bramley's seedling apples treated with or without SmartFresh^{SM} from orchard Pitstock collected during the 2011-12 season. Cortex was sampled from the inner (In) and outer (Out) regions of fruit.

Sample Number	Inner/ Outer	SmartFresh (+/-)	Sampling date	RNA Concentration (ng/µL)	A260nm	A280nm	260/280	18S & 28S RNA bands
88	In	-	6/09/2011	325	8.810	4.220	2.09	+
88	Out	-	6/09/2011	80	2.002	0.960	2.08	+/-
178	In	+	17/11/2011	195.8	4.894	2.343	2.09	+
179	Out	+	17/11/2011	386.5	9.664	4.446	2.07	+
178	In	-	17/11/2011	363.7	9.092	4.436	2.05	+
179	Out	-	17/11/2011	732	18.300	8.886	2.06	+
260	In	-	25/11/2011	580.4	14.509	6.809	2.13	+
261	Out	-	25/11/2011	118.4	2.960	1.402	2.11	+
280	In	-	9/01/2012	531.3	13.283	6.408	2.07	+
281	Out	-	9/01/2012	973.1	24.327	11.418	2.13	+
282	In	-	17/01/2012	485.7	12.143	5.807	2.09	+
283	Out	-	17/01/2012	123.1	3.079	1.454	2.12	+

6.3 Expression of Ca-ATPase and Lipoxygenase genes:

PCR reactions for Ca ATPase and the internal housekeeping gene (ITS-ribosomal RNA) from cDNA samples from Bramley apples were analysed on agarose gel 1% [w/v]. The housekeeping gene (ITS) was amplified in all samples but Ca ATPase amplicons were only present from harvest sampling where no bitter pit was evident. There were weak bands in samples taken from inner cortex with incidence of bitter pit less than 18% (Table 6.4).

Table 6.4: PCR product amplification results of the same samples (orchard Pitstock) in Table 3 (Bands: +: positive, - : negative, +/-: weak positive).

Sample	Inner/Outer	SmartFresh (+/-)	Sampling date	Bitter Pit%	Primer ITS	Primer Ca^{2+}ATPase
88	In	-	6/09/11	0	+	+
89	Out	-	6/09/11	0	+	+
178	In	-	17/11/11	18	+	+/-
179	Out	-	17/11/11	18	+	-
178	In	+	17/11/11	8	+	+/-
179	Out	+	17/11/11	8	+	-
260	In	-	25/11/11	17	+	+/-

261	Out	-	25/11/11	17	+	-
280	In	-	9/01/12	33	+	-
281	Out	-	9/01/12	33	+	-
282	In	-	17/01/12	37	+	-
283	Out	-	17/01/12	37	+	-

Additional isolation of RNA from Bramley apples in a further orchard (Jenner) was conducted on mixed apple cortex samples that had either been treated with SmartFresh^{SM} or remained untreated. Tissues were selected from areas of cortex suffering from bitter pit and from symptomless tissues. PCR products of cDNA samples using primers encoding ITS, Ca-ATPase and lipoxygenase were visualised on agarose gel (1% w/v). The housekeeping gene (ITS-ribosomal RNA) product amplified in all samples. However, Ca^{2+}ATPase amplified only samples without bitter pit incidence. Amplification using primers encoding a lipoxygenase gene produced an amplicon from symptomless apple samples at harvest and during the initial 3 months of storage lipoxygenase genes are not involved in bitter pit formation (Tables 6.5 and 6.6).

Table 6.5: RNA concentration of samples collected from one orchard (Jenner) in season 2010/11.

Sample	Smart Fresh (+/-)	RNA Concentration (ng/µL)	A260nm	A280nm	260/280	18S & 28S RNA
109	-	414.4	8.288	3.949	2.10	+
109	+	134.1	2.682	1.271	2.11	+
323	-	695.4	13.908	6.613	2.10	+
323	+	731.1	14.622	7.029	2.08	+
324	-	57.5	1.150	0.551	2.09	+/-
324	+	144.3	2.887	1.384	2.09	+

Table 6.6: PCR products Ca^{2+}ATPase and lypoxygenase amplification results (orchard-Jenner) in season 2010/11 (Bands: +: positive, - : negative, +/-: weak positive).

Sample	Smart Fresh (+/-)	Sampling date	Bitter Pit%	Primer ITS	Primer Ca^{2+}ATPase	Primer Lypoxygenase
109	-	15/9/10	0	+	+	+
109	+	15/9/10	0	+	+	+
323	-	14/12/10	23	+	-	-
323	+	14/12/10	20	+	-	-
324	-	14/12/10	0	+	+/-	-
324	+	14/12/10	0	+	+	+

***Note:** samples 324 (NoSF & SF) were taken from parts without bitter pit of samples 323 (NoSF & SF), in NoSF there was 23% and in SF 20% BP.

6.4 Analysis of calmodulin expression in apple:

PCR primers used to amplify a 1200 bp (base pair) of a recognised calmodulin gene fragment from Golden Delicious were employed to clone the ortholog calmodulin gene from Bramley apple (calmodulin a & d). In addition two sets of nested primers (calmodulin b & c) were used to amplify a 140 bp fragment for use in qPCR on Bramley's seedling apple cDNA. The PCR products of primer Calmodulin (b) successfully amplified a 140 bp fragment from Bramley's seedling apple cDNA isolated from different samples, and other PCR products from primers (calmodulin a, c & d) failed to amplify (Table 6.7).

Table 6.7: Samples used for PCR amplification for identifying the suitable type of calmodulin. Samples were taken in different dates of storage from one orchard (Popes A) in season 2010/11. (Bands: +: positive, - : negative, +/-: weak positive).

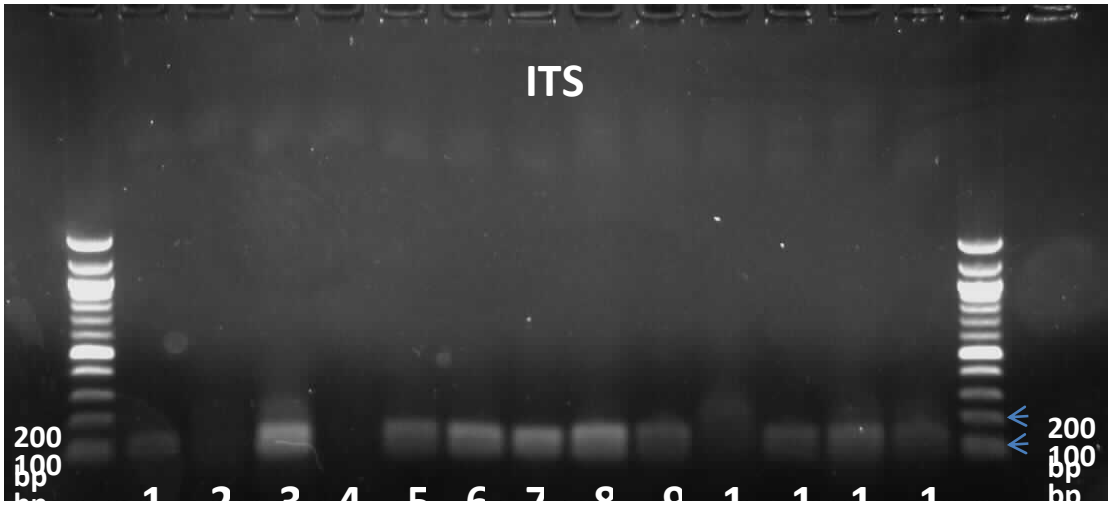
Sample	Smart Fresh (+/-)	Sampling date	BP %	RNA Concentration (ng/µL)	Primer Calmodulin a	Primer Calmodulin b	Primer Calmodulin c	Primer Calmodulin d
34	-	8/09/10	0	87	-	+/-	-	-
209	-	17/11/10	41	196.5	-	+	-	-
209	+	17/11/10	0	537.1	-	+	-	-
260	-	23/11/10	43	76	-	+/-	-	-
260	+	23/11/10	0	98	-	+/-	-	-
261	-	23/11/10	0	548	-	+	-	-
261	+	23/11/10	0	347.3	-	+	-	-

Additional analyses of calmodulin (b) primers on cDNA created from RNA isolated from the inner and outer cortex of Bramley are shown in Table 6.8 and Figure 6.2.

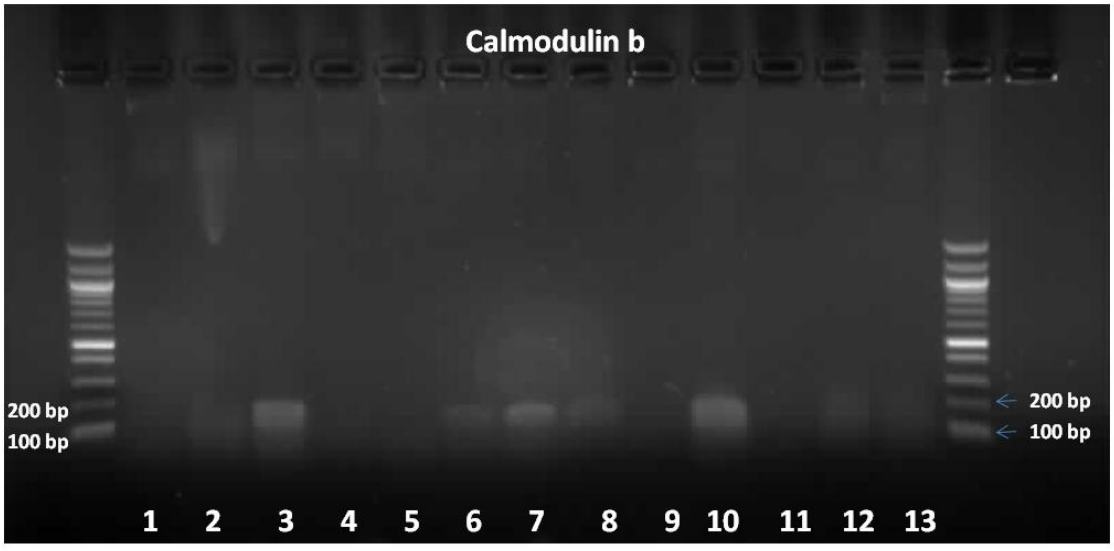
Table 6.8: Twelve samples collected during storage from the same an orchard (Pitstock) in season 2011/12 amplified with primers ITS (housekeeping gene) and "Calmodulin (b)" (Bands: +: positive, - : negative, +/-: weak positive).

Number	Inner/Outer Cortex	NoSF/ SF	Sampling date	Bitter Pit%	RNA Concentration (ng/µL)	Primer ITS	Primer Calmodulin (b)
1	-	-	-	-	-	+/-	-
2	In	-	6/09/11	0	119.1	-	-
3	Out	-	6/09/11	0	156	+	+
4	In	-	17/11/11	18	277.1	-	-
5	Out	-	17/11/11	18	356.5	+	-
6	In	+	17/11/11	8	310.3	+	+/-
7	Out	+	17/11/11	8	536.1	+	+
8	In	-	25/11/11	17	512	+	+/-
9	Out	-	25/11/11	17	332.1	+/-	-
10	In	-	9/01/12	33	111	-	+
11	Out	-	9/01/12	33	912.7	+/-	-
12	In	-	17/01/12	37	139.9	+/-	+/-
13	Out	-	17/01/12	37	119.3	+/-	+/-

***Note:** First sample was water which showed a weak band for primer ITS suggesting contamination.



A



B

Figure 6.2: A) PCR products of ITS (housekeeping gene) amplification, B) PCR products of primer calmodulin b amplification of 12 samples from the same orchard (Pitstock). Sample details are in table 6.8.

The ITS region (housekeeping gene) amplified in most samples, but amplification of calmodulin using primer 'b' resulted in inconsistent products from inner and outer cortex or between samples and no correlation with bitter pit.

"Lipoxygenase" primers designed against a 450 bp fragment which were identified from an ethylene induced Cox cDNA library (Colgan, 2002), were used on cDNA from Bramley.

In Bramley expression of lipoxygenase was detected in selected samples in 2011/12. ITS amplicon was present in most samples; however "Lypoxygenase" and "Calmodulin (b)" expression were not consistently expressed in samples with bitter pit (Table 6.9).

Table 6.9: Fourteen PCR products from samples collected in different times of storage from the same orchard (Carpenter) in season 2011/12, amplified with primers ITS (housekeeping gene), lypoxygenase and calmodulin b. (Bands: +: positive, - : negative, +/-: weak positive).

Sample name	In/Out Cortex	No SF/ SF	Sampling date	RNA Concentration (ng/µL)	260/ 280	BP %	Primer ITS	Primer Lypoxy genase	Primer Calmodulin (b)
47 harvest	In	_	30/08/2011	358.8	1.92	0	+	_	_
48 harvest	Out	_	30/08/2011	458.9	1.9	0	+	+	+/-
267	In	_	25/11/2011	683.4	2.05	13	+	_	+
267	In	+	25/11/2011	451.2	2.02	3	+	_	_
268	Out	_	25/11/2011	708.9	1.9	13	_	_	+
268	Out	+	25/11/2011	311.9	1.8	3	+	+	+/-
291	In	_	27/01/2012	343.5	2.08	10	_	_	+/-
291	In	+	27/01/2012	186.7	2.08	8	+	+	+/-
292	Out	_	27/01/2012	22.4	1.75	10	+	+	+
292	Out	+	27/01/2012	182	2.01	8	+	+	+
333	In	_	03/02/2012	121.5	2.11	20	+	+	+
333	In	+	03/02/2012	266.6	1.51	3	+	+	_
334	Out	-_	03/02/2012	68.2	2.04	20	_	+	+
334	Out	+	03/02/2012	147.4	2.12	3	+	+	+/-

The same samples were used in Real Time PCR with primer calmodulin (b) in two replications.

6.5 Real Time quantitative PCR:

The preliminary experiments showed expression (CT: Constant Threshold) of the housekeeping gene (ITS) was not consistent across all samples, caused by variability in sample loading or inconsistent cDNA synthesis through inaccurate assessment of RNA concentration.

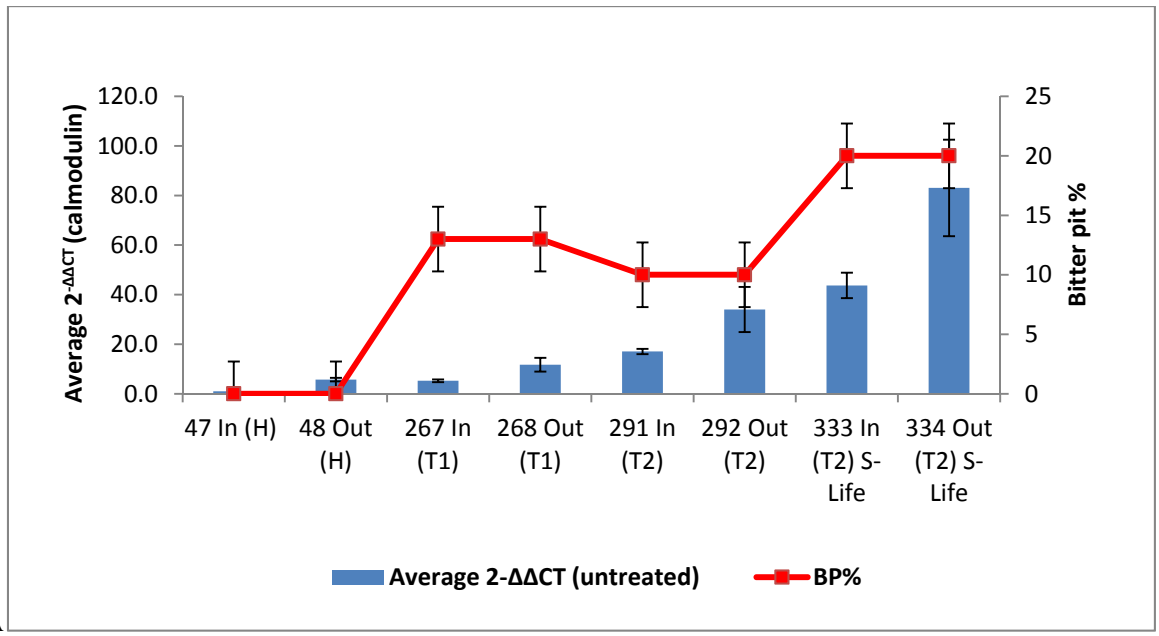
Relative expression of genes based on CT values for ITS and calmodulin primers for samples collected during storage in season 2011/12 are in Appendix XI (a). To determine relative fold differences the CT value for "Calmodulin b" and the housekeeping primer (ITS) data were normalized and expression of genes were compared to the expression pattern of the housekeeping gene ITS using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) algorithm to quantify relative changes in gene expression. The $2^{-\Delta\Delta CT}$ for the samples is described in Table 6.10 and for calmodulin expression in Figure 6.3.

Table 6.10: Average of fold changes in gene expression calmodulin b ($2^{-\Delta\Delta CT}$) of samples from one orchard (Carpenter) season 2011/12 and SE.

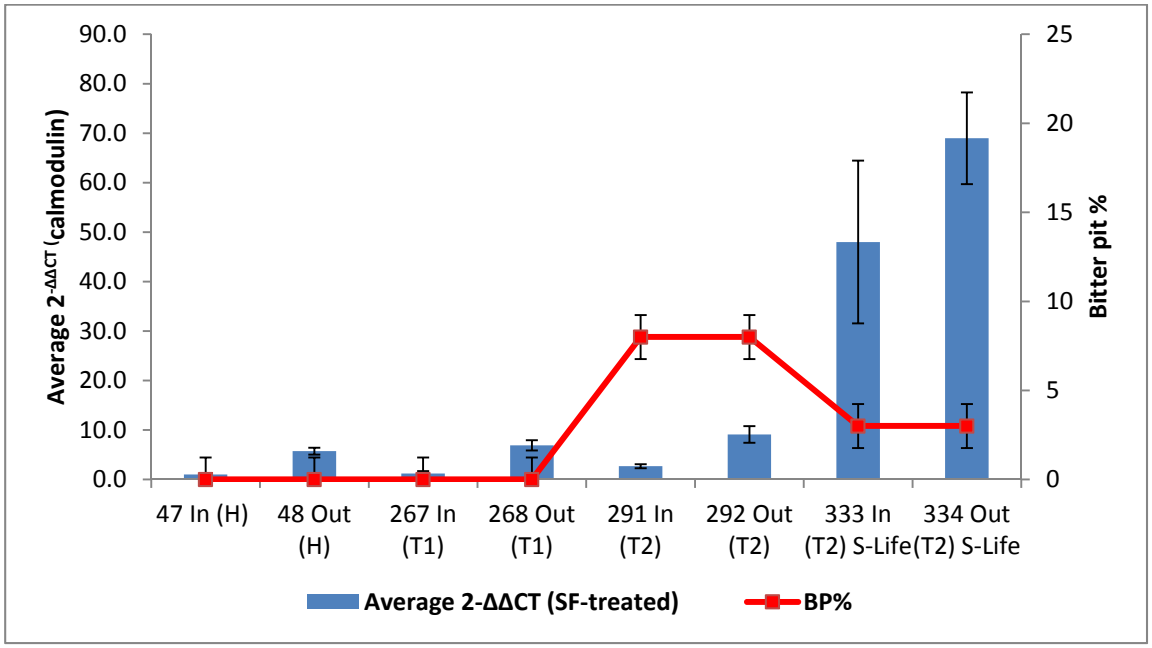
Sample Name	In/Out Cortex	NoSF/ SF	Date	BP%	Average $2^{-\Delta\Delta CT}$	SE
47 (Harvest)	In	_	30/08/2011	0	1.1	0.22
48 (Harvest)	Out	_	30/08/2011	0	5.7	0.70
267	In	_	25/11/2011	13*	5.3	0.56
268	Out	_	25/11/2011	13*	11.7	2.79
267	In	+	25/11/2011	0	1.2	0.49
268	Out	+	25/11/2011	0	6.9	1.02
291	In	_	27/01/2012	10	17.1	1.06
292	Out	_	27/01/2012	10	34.0	9.09
291	In	+	27/01/2012	8	2.7	0.40
292	Out	+	27/01/2012	8	9.1	1.68
333 (S-Life)	In	_	3/02/2012	20	43.7	5.13
334 (S-Life)	Out	_	3/02/2012	20	83.0	19.49
333 (S-Life)	In	+	3/02/2012	3	48.0	16.44
334 (S-Life)	Out	+	3/02/2012	3	68.0	9.27

***Note:** Bitter pit incidence is for the whole apple, the score for inner/outer is similar, but does not mean inner/outer have the same bitter pit incidence.

Expression of calmodulin transcripts generally increased with the length of storage and in some cases higher expression was seen in Bramley samples showing symptoms of bitter pit, and was particularly higher in the outer cortex of fruit. Reduction in ethylene production through the application of SmartFresh^{SM} appeared to suppress the expression of calmodulin transcripts (Table 6.10). The correlation of "calmodulin (b)" expression in untreated samples with bitter pit was R=0.73 and for SmartFresh^{SM} treated samples was not correlated.



A



B

Figure 6.3: Comparison of gene expression (calmodulin b) in Bramley apples from orchard Carpenter (CAR) collected at harvest (H) and during storage: 2 months (T1), 4 months (T2) and 7 days at 18°C (S-Life) in season 2011/12. A) Untreated samples, B) SF-treated samples.

Additional qPCR analysis Ca^{2+}ATPase and "Ca^{2+} Protease A" samples taken from the same orchard (CAR), found expression for housekeeping primer (ITS) and primer "Ca^{2+} Protease A", but no expression observed for Ca^{2+}ATPase. Details are in Appendix XI (b).

Data analysed using the $2^{-\Delta\Delta CT}$ formula is described in Table 6.11 and for "Ca^{2+} Protease A" gene expression in Figure 6.4. Higher gene expression profiles for "Ca^{2+} Protease A" was

observed when the tissue was from outer cortex with symptoms of bitter pit. Samples treated with SmartFresh ^{SM}, where bitter pit was absent had lower level of transcripts.

Table 6.11: Average of fold changes in gene expression "Ca^{2+} Protease A" ($2^{-\Delta\Delta CT}$) of samples from one orchard (CAR) season 2011/12 and SE (only after 2 months storage).

Sample Name	In/Out Cortex	NoSF/ SF	Date	BP%	Average $2^{-\Delta\Delta CT}$	SE
47 (Harvest)	In	_	30/08/2011	0	1.3	0.52
48 (Harvest)	Out	_	30/08/2011	0	0.3	0.13
267	In	_	25/11/2011	13	7.0	1.35
268	Out	_	25/11/2011	13	127.4	39.28
267	In	+	25/11/2011	0	4.6	1.30
268	Out	+	25/11/2011	0	4.3	1.26

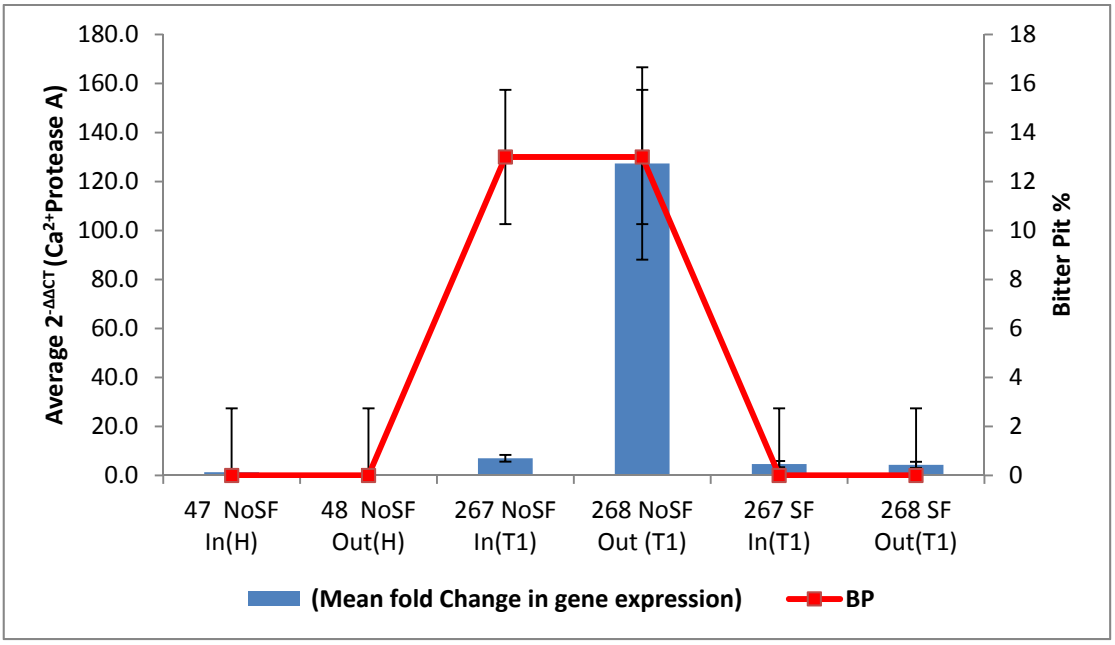


Figure 6.4: Comparison of gene expression "Ca^{2+} Protease A" in different samples of one orchard (Carpenter) collected at harvest (H) and during storage: 2 months (T1), 4 months (T2) in season 2011/12.

In follow-on experiments with samples collected from orchard (Pitstock) in season 2011/12, calmodulin gene expression was low at harvest and in samples taken within the first 2 months of storage even where bitter was present (18%). In later stored fruit where bitter pit had

increased to 33% a 10 fold increase in calmodulin expression was observed. Detailed results of CT values are in Appendix XI (c). The mean fold change in gene expression ($2^{-\Delta\Delta CT}$) for the remaining samples is described in Table 6.12 and for calmodulin expression in Figure 6.5. In this experiment most samples with a higher incidence of bitter pit exhibited higher expression of calmodulin. However, no consistent relationship between increasing gene expression and severity of bitter pit was observed.

Table 6.12: Average of fold changes in gene expression calmodulin b ($2^{-\Delta\Delta CT}$) of samples from one orchard (Pitstock) season 2011/12 and SE.

	In/Out	Date	BP%		
Sample	**Cortex**			**Average $2^{-\Delta\Delta CT}$**	**SE**
89 Harvest	Out	6/09/2011	0	1.1	0.26
178	In	17/11/2011	18	0.1	0.01
179	Out	17/11/2011	18	0.7	0.01
260 (Shelf life)	In	25/11/2011	17	1.1	0.04
261 (Shelf life)	Out	25/11/2011	17	1.4	0.79
280	In	9/01/2012	33	10.7	1.88
280	Out	9/01/2012	33	14.5	2.97

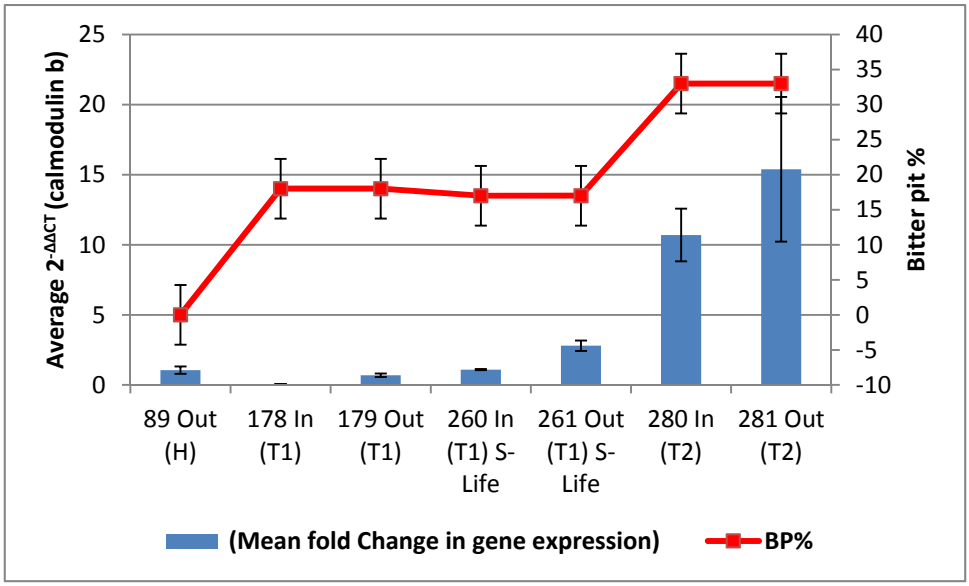


Figure 6.5: Relative abundance of calmodulin transcripts in Bramley apples orchard (Pitstock) collected samples (all untreated) at harvest (H) and during storage: 2 months (T1), 4 months (T2) in season 2011/12.

In season 2013/14, a restricted number of samples from two orchards (EMR and HOO) were collected at harvest and after 2 months air storage (4-4.5°C). Samples were collected from the calyx and stalk end of Bramley apples. Transcript analysis was restricted to the expression of "calmodulin" and "Ca^{2+} Protease".

The results of RNA concentration of samples and PCR product amplification for "calmodulin" and "Ca^{2+} Protease" by gel electrophoresis results were compared (Table 6.13) and showed ITS amplicons were present in most samples. However, amplicons of "Ca^{2+} Protease" and "Calmodulin" were not present in all samples.

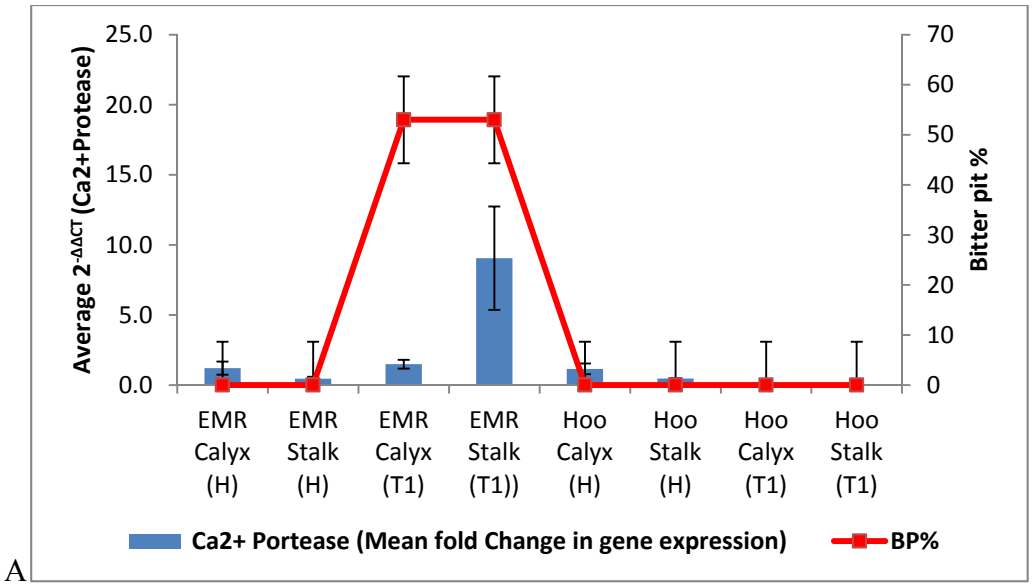
Table 6.13: PCR products of RNA extracted from two orchards (EMR) and (Hoo) in season 2013/14, amplified with primers ITS (housekeeping gene), Ca^{2+} Protease (A) and calmodulin (b). (Bands: +: positive, - : negative, +/-: weak positive).

Sample name	Calyx/ Stalk end	Sampling date	BP %	RNA Concentration (ng/µL)	260 / 280	Primer ITS	Primer Ca^{2+} Protease (A)	Primer Calmodulin (b)
EMR (harvest)	Calyx	2/09/2013	0	133.1	1.48	+/-	-	-
EMR (harvest)	Stalk	2/09/2013	0	243.5	1.57	+	+	-
EMR	Calyx	15/11/2013	53	149.4	2.06	+	+/-	+
EMR	Stalk	15/11/2013	53	129.5	2.02	+/-	+	+
Hoo (harvest)	Calyx	2/09/2013	0	299.8	2.09	+	-	+/-
Hoo (harvest)	Stalk	2/09/2013	0	157.8	2.06	+	+	-
Hoo	Calyx	15/11/2013	0	207.9	2.01	+	+	+
Hoo	Stalk	15/11/2013	0	273.9	2.02	+	+/-	+

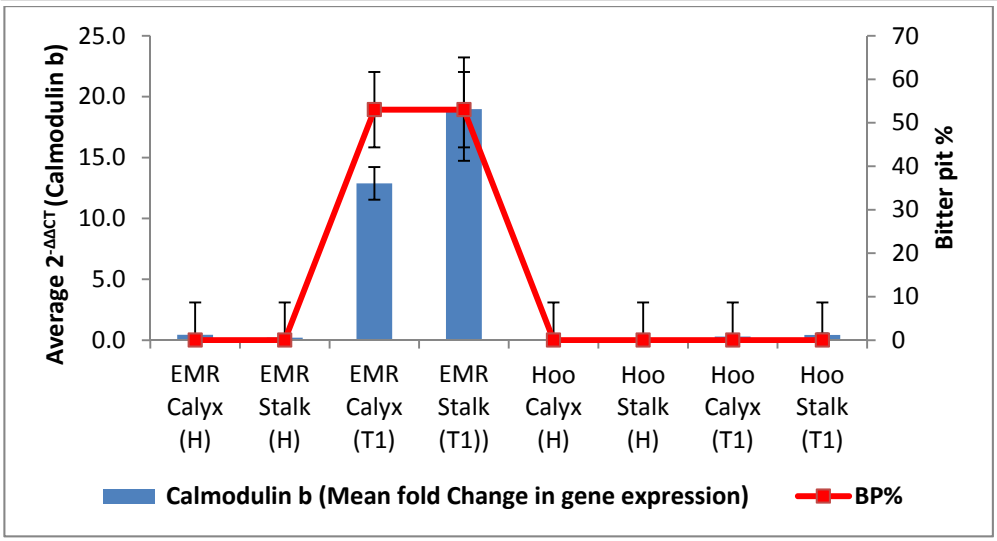
Detailed results of CT values are in Appendix XI (d) The mean fold change in gene expression ($2^{-\Delta\Delta CT}$) for the samples for "Ca^{2+} Protease A" and "Calmodulin (b)" are presented in Table 6.14 and the comparison of gene expressions in Figure 6.6.

Table 6.14: Average of fold changes in gene expression of primers "Ca^{2+} Protease A" and "Calmodulin (b)" ($2^{-\Delta\Delta CT}$) of samples from two orchards (EMR) and (Hoo) season 2013/14 and SE (all samples were untreated).

Sample	Calyx/ Stalk end	Date	BP %	Mean $2^{-\Delta\Delta CT}$ Ca^{2+} Protease	SE	Mean $2^{-\Delta\Delta CT}$ Calmodulin	SE
EMR (harvest)	Calyx	Sept	0	1.2	0.47	0.4	0.06
EMR (harvest)	Stalk	Sept	0	0.5	0.13	0.2	0.01
EMR	Calyx	15 Nov	53	1.5	0.31	12.9	1.34
EMR	Stalk	15 Nov	53	9.1	3.69	19.0	4.25
Hoo (harvest)	Calyx	Sept	0	1.2	0.38	0.0	0.01
Hoo (harvest)	Stalk	Sept	0	0.5	0.03	0.0	0.00
Hoo	Calyx	15 Nov	0	0.1	0.03	0.3	0.14
Hoo	Stalk	15 Nov	0	0.0	0.00	0.4	0.12



A



B

Figure 6.6: Comparison of gene expression A) "Ca^{2+} Protease A" and B) "calmodulin b" in different samples of two orchards (EMR) and (Hoo) collected at harvest (H) and during storage: 2.5 months (T1), air-stored in season 2013-14 (all samples were untreated).

Samples with a higher incidence of bitter pit exhibited in general a greater abundance of "Ca^{2+} Protease" and "calmodulin" transcripts. The stalk end of Bramley apple had higher "Ca^{2+} Protease" and "calmodulin" than the calyx end. Interestingly the calyx end of fruit tends to be more susceptible to bitter pit. The correlation between "calmodulin" gene expression and incidence of bitter pit (R=0.99) was higher than "Ca^{2+} Protease" (R=0.54).

Also in order to have a direct comparison gene expression was compared between tissues from the same apple samples but taken from the tissues with or without bitter pit. This was carried out on samples selected from two orchards in different seasons.

In season 2010/11 RNA samples from orchard (JEN) were investigated for changes in calmodulin gene expression. Detailed results of CT values are in Appendix XI (e). The mean fold changes in gene expression ($2^{-\Delta\Delta CT}$) for the samples for "calmodulin" are presented in Table 6.15 and the comparison of gene expressions in Figure 6.7.

The results showed a greater abundance of calmodulin transcripts in samples with bitter pit in comparison with the tissues where no symptoms were present.

Table 6.15: Average of fold changes in gene expression calmodulin b ($2^{-\Delta\Delta CT}$) of samples from one orchard (Jenner) season 2010/11 as untreated and SF-treated and from tissues with or without bitter pit and SE.

Sample	Date	BP%	Average $2^{-\Delta\Delta CT}$	SE
NoSF NoBP (Harvest)	15 Sept	0	2.7	2.1
NoSF (BP)	15 Dec	23	49.2	18.4
NoSF (NoBP)	15 Dec	0	6.2	5.88
SF (BP)	15 Dec	10	4.8	4.35
SF (NoBP)	15 Dec	0	1.4	1.18

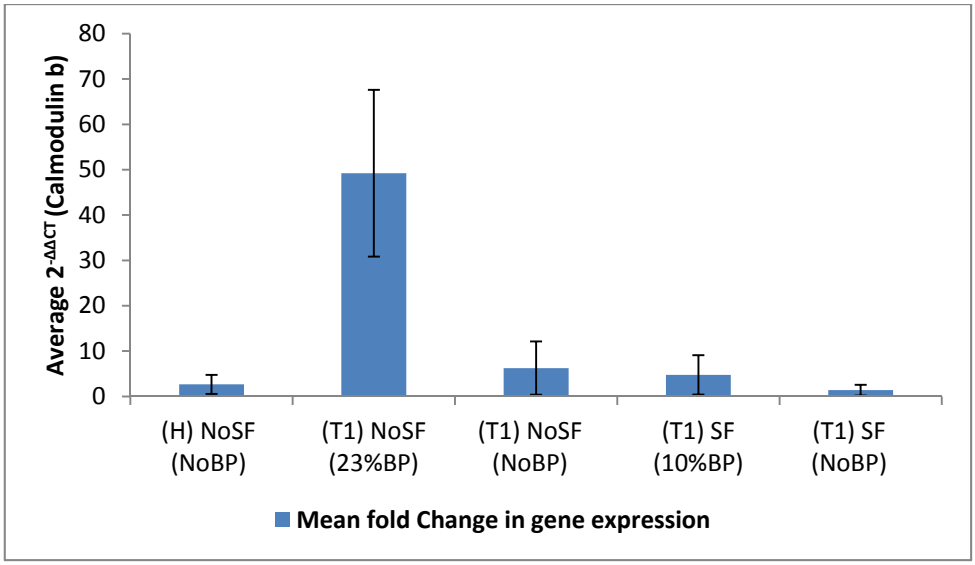


Figure 6.7: Comparison of gene expression (calmodulin b) in untreated and SF-treated samples of one orchard (Jenner) collected at harvest (H) and during storage: 3 months (T1), stored in CA (9% CO_2, 12% O_2) in season 2010-11.

In 2012/13 samples from orchard (Fourey) stored for 10 months in CA regime (5% CO_2, 1% O_2) were analysed for transcript profiles for "Ca^{2+} Protease". Detailed results of CT values are in Appendix XI (f). The mean fold change in gene expression ($2^{-\Delta\Delta CT}$) for the samples for "Ca^{2+} Protease A" are presented in Table 6.16 and the comparison of gene expressions in Figure 6.8. The results found a greater abundance of transcripts for "Ca^{2+} Protease " in samples SF-treated from tissues with bitter pit taken from stalk end of fruit in comparison with the tissues from the same sample taken from tissues without bitter pit symptoms and taken from calyx end of fruit.

Table 6.16: Average of fold changes in gene expression "Ca^{2+} Protease A" ($2^{-\Delta\Delta CT}$) of samples from one orchard (Fourey) season 2012/13 after 10 months CA-storage as SF-treated and from tissues with or without bitter pit and SE.

Sample	Sampling date	BP	Average $2^{-\Delta\Delta CT}$	SE
Calyx	20/08/2013	NoBP	1.4	0.82
Stalk	20/08/2013	NoBP	5.1	1.4
Calyx	20/08/2013	BP	0.6	0.11
Stalk	20/08/2013	BP	30.6	6.68

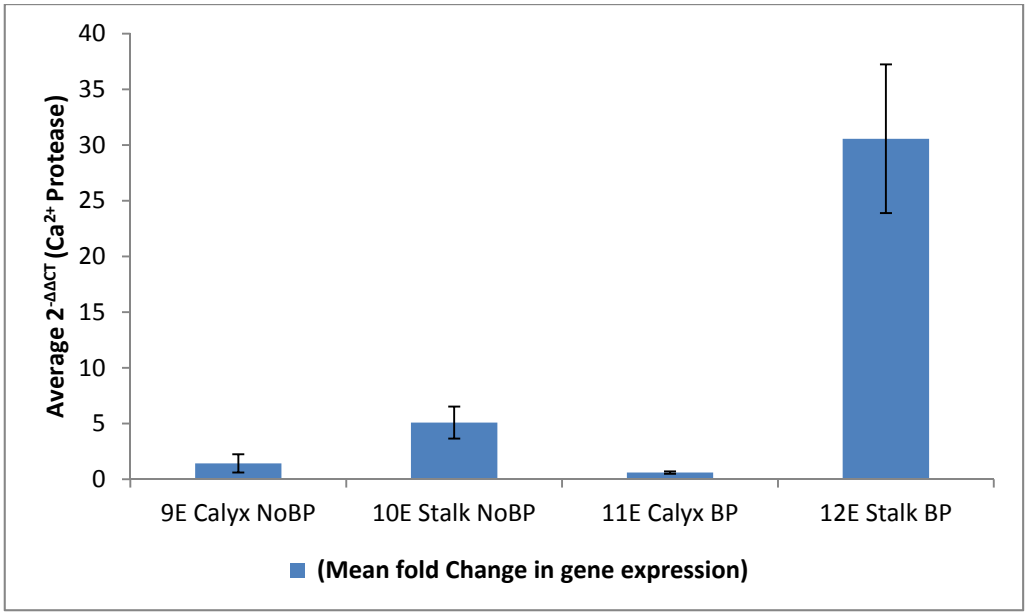


Figure 6.8: Comparison of gene expression "Ca^{2+} Protease A" in samples from one orchard (Fourey) collected after 10 months CA-storage (5% CO_2, 1% O_2) in season 2012/13. Samples were taken from tissues with and without bitter pit symptoms from calyx and stalk end of fruit.

Although the patterns of gene expression of "Ca^{2+} Protease" and "calmodulin" in Bramley showed a general increase in samples that had a higher incidence of bitter pit, the relationship was not linear and therefore correlation was poor which either suggests inconsistent cDNA synthesis or other genes involved in calcium regulation or apoptosis (cell death).

6.6 Discussion

The selective screening of a small number of key genes (calmodulin, Ca^{2+}ATPase, Ca^{2+}Protease and lipoxygenase) that regulate calcium and cell wall lipid metabolism, respectively, were performed in samples with different degrees of bitter pit development. It was anticipated that fold changes in gene expression could be related to developmental changes in the cell and tissue homeostasis that in turn can cause the onset of localised cell death (apoptosis) and the formation of necrotic lesions of bitter pit. It was anticipated that where major changes in the expression of single genes were identified then molecular markers could be developed as a diagnostic tool to predict the onset of bitter pit development or as markers in a "marker assisted breeding programme" to select new varieties less prone to bitter pit. This study focused on the gene expression profiles of a limited number of genes involved in calcium regulation and lipid peroxidation and showed that in general there was a differential pattern of expression of transcripts between apples suffering from bitter pit and apples that remained healthy.

Extraction of a high yielding and high quality RNA is critical for robust analysis of gene expression profile. Although several extraction methods were investigated and finally three methods were developed and applied, there were several problems in this part of the study which ended with inconsistency in quality and concentration of extracted samples. The method which was initially applied was based on CTAB developed by Gasic *et al.* (2004). The second method was based on Sangha *et al.* (2010) and was developed as combining the current method and CTAB and RNeasy Mini Kit. Finally the last method provided better quality and greater yields of RNA which was modified by Colgan (2002) originally from the method applied by Bahloul and Burkard (1993). This method requires more apple tissue (10 g) and although involved more stages in a protracted procedure, produced better quality RNA. Apples (Bramley), which has larger cell structure than most plant tissues with more space between cells (Way *et al*. 1991) requires more starting material and therefore the large sample size does not lend itself to high throughput extraction procedures based on kit-based extraction methods. Furthermore the large amount of polysaccharides and/or polyphenols that are released during cell division (Gasic *et al.*, 2004) interferes with nucleic acid extraction reducing the quality of the final product. Development of a rapid extraction method in future affording faster through put of samples producing higher quality, purity and more abundant RNA will help larger scale analysis of gene expression of genes that may have potential influence on bitter pit development.

Comparison of Ca^{2+}ATPase expression in samples taken in year 1 (2010/11) from cortex taken across the whole fruit against samples taken in 2011/12 from inner and outer cortex showed Ca^{2+}ATPase amplicons were only present in samples without bitter pit symptoms. Burmeister and Dilley (1994) suggested that Ca^{2+}ATPase interferes with the role of calcium as second messenger when calcium is bound by Ca^{2+}/CaM. It seems our results confirm that when there is less free calcium and no symptoms of bitter pit, Ca^{2+}ATPase gene is expressed. This was indicated by De Freitas *et al.* (2012) that low levels of water-soluble Ca^{2+} increases plasma membrane leakage, and high expression of Ca^{2+}ATPases in fruit tissue. The results observed in this project for lipoxygenase genes, are similar to those reported by Lara (2013). However, in some samples with low symptoms of bitter pit lipoxygenase gene expression was observed. Marcelle (1989) also reported that when the expression of lipoxygenase increased, less bitter pit symptoms were observed.

These results indicate that Ca^{2+}ATPase and lipoxygenase genes are more active in healthy tissues with higher content of free calcium or the numbers of transcripts were not easily recoverable in tissue that was undergoing early stages of senescence.

PCR products of primer calmodulin (b) amplification were inconsistent between inner and outer cortex and between samples with different percentages of bitter pit. However, a higher amount of PCR product amplified in samples with more incidence of bitter pit, and calmodulin expression was more prevalent in the outer cortex where more symptoms are present. Calcium binding to calmodulin could explain the increased susceptibility to bitter pit.

Real Time PCR analysis indicated that the expression of the housekeeping gene (ITS) was not consistent across all samples, suggesting variability in either sample loading or inconsistent cDNA synthesis or through low quality of RNA concentration at earlier stages in the extraction process. Results where CT values for ITS gene expression were inconsistent were eliminated and data was analysed using the $2^{-\Delta\Delta CT}$ algorithm to quantify relative changes in gene expression.

There was a relationship between increasing gene expression, calmodulin and the incidence of bitter pit especially in untreated samples when stored longer where the incidence of bitter pit increased alongside a rise in the expression of calmodulin. Wang *et al*. (2001) indicated a relationship between maturity and calmodulin. It seems that SmartFresh^{SM} delays ripening by blocking ethylene receptors and reducing the positive feedback loop that stimulates ACC synthesis and ethylene production. Although there is an indication that reduced ethylene production led to lower calmodulin expression, Smart Fresh's control on bitter pit may be related to its direct role in reducing ethylene-induced apoptosis rather than directly through regulation of calcium activity.

A relationship between Ca^{2+} Protease gene expression and incidence of bitter pit was observed with higher gene expression of "Ca^{2+} Protease A" found from outer cortex which had a higher incidence of bitter pit. Calmodulin and Ca^{2+} Protease showed increasing gene expression where incidence of bitter pit increased. The relationship was weaker in samples collected in the first two years; this may be in some part caused by fruit samples that were taken from affected tissue and adjacent unaffected tissue. In season (2013/14) samples were only taken from apples with or without bitter pit and selected from stalk and calyx end tissues. The results showed higher transcripts of calmodulin, in samples from stalk end than calyx end tissues and higher correlation of calmodulin gene expression and incidence of bitter pit in comparison with "Ca^{2+}

Protease A". Calmodulin expression was higher in samples from untreated or SF-treated from tissues showing signs of bitter pit in comparison with the tissues from the same apple without bitter pit sign. Higher abundance of gene expression of Ca^{2+} Protease and calmodulin in stalk end tissues in comparison with calyx end tissues even when apple does not show bitter pit symptoms is related to binding of free calcium by calmodulin, as indicated by Kim *et al*. (2008), calmodulin has no enzymatic activity of its own, the $Ca^{2+/}$calmodulin complex can modulate the activity of target proteins in the control of the cellular responses by regulating the expression of genes encoding downstream effectors. Since there is more free calcium in the stalk end because of xylem sap movement from stalk to calyx end (Atkinson, 2014), more free calcium is bound by calmodulin leading to increasing Ca^{2+} Protease and calmodulin gene expression.

Although some results showed correlation between the selected genes, especially Ca^{2+} Protease and calmodulin amplification in samples with bitter pit, results were inconsistent and RNA extraction methods should be developed to have more consistent samples and comparisons. It seems with additional data it would be possible to build a multivariate model that can help to predict the onset of bitter pit development in fruit and help growers make informed choice regarding marketing of existing fruit in store and changes to current orchard and store management practices that can help to mitigate some of the bitter pit problems.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

Although this study set out to explore the potential of producing a prediction model of incidence of bitter pit by focusing on the assessments of postharvest factors, it was essential to review weather patterns, preharvest treatments and orchard history as well as comparison of different storage conditions analysing the results obtained from quality assessments during postharvest and storage. These maturity assessments were combined with analysis of sugar and acid profiles at harvest and changes during storage, and changes in the proportion of calcium and calcium oxalate and regulation of selected genes involved in calcium regulation. In addition to these parameters, chlorophyll fluorescence was employed as a non-destructive tool to help to determine the maturity of fruit at harvest and whether subsequent measurements during the early stages of harvest could be used to predict the onset of bitter pit.

7.1 Preharvest factors, storage conditions and quality assessments during storage:

The weather during the period of this study varied considerably between seasons and impacted on maturity, fruit size and density of fruit cells which would have had consequences for the incidence of bitter pit. The fluctuations in temperature during flowering and fruit development affected pollination, calcium uptake and cell expansion of apple fruit. Moreover, warm or cold weather affected pollination and time of flowering between seasons affecting seed set and time taken for fruit to develop and mature on the tree. Changes in temperature during fruit development over the summer months affected transpiration and xylem flow within the tree and calcium uptake into the fruit in different seasons. The implications led to a large variation of fruit maturity and calcium distribution in fruits within orchards that led to an extended period over which Bramley apples were harvested. A big influence on the incidence of bitter pit was the delay in harvesting fruit or where the time taken to load and seal stores became extended. Stricter control on harvesting schedules and store loading and sealing will help to minimise bitter pit development. This pattern is consistent with that presented by Prinja (1989). Furthermore the SmartFresh^{SM} application and storage in controlled atmosphere (CA) storage regimes, especially (5% CO2, 1% O2) suppressed bitter pit by delaying maturity and onset of climacteric ethylene production as reported by Johnson (2007). The type of storage regime influenced the ability to predict the onset of bitter pit; air stored fruit provided the best examples

to predict, but have little commercial significance. However, keeping orchard samples in air will help to identify orchard consignments most-likely to develop bitter pit, even when the main consignment of apples have been stored under CA regimes.

Comparison of different picking dates found a correlation between late pick and increasing incidence of bitter pit which was in contrast with previous studies (Ferguson and Watkins, 1992; Prange *et al.*, 2011) and could be related to the changes in structure (such as cell enlargement) and the increase in intercellular space and leading a dilution of Ca^{2+} predisposing tissue to greater bitter pit development. There was an inverse correlation (R=-0.75) between fruit firmness and incidence of bitter pit in apples stored in air; however the correlation was lower in SmartFresh^{SM} treated fruit and those stored in CA regimes. Since CA storage and SmartFresh^{SM} have a masking role that delay ripening and keep fruit firm, while on the other hand, simultaneously bitter pit is developing. Stow (1998) indicated that the process of losing water soluble Ca^{2+} through middle lamella starts earlier in the pitted cell.

Changes in background colour during storage of Bramley apples correlated to the changes in the incidence of bitter pit. However, the background colour changes in SmartFresh^{SM} treated Bramley apples were better correlated with bitter pit incidence than untreated apples, since in untreated apples some background colour changes are related to ripening of fruit. However, in SmartFresh^{SM} treated samples by delaying ripening, background colour starts changing from green to yellow just because of the changes in the cells with bitter pit. In addition the severity of bitter pit is less than untreated samples and most pitted cells are close to the peel, so background colour changes are more correlated to bitter pit. Background colour changes only occur late in the storage season and therefore are not reliable as predicative method for determining the storage potential of the consignment.

There was little change in the total soluble solids (TSS) of the fruit during storage and this provided a poor parameter to correlate changes in fruit maturity and bitter pit. This is in accordance with Lanauskas *et al.* (2012). It seems that increasing TSS changes little during storage and is more strongly influenced by the dry matter content of fruit related to maturity than disorders such as bitter pit. Also there was no significant difference in TSS between untreated and SmartFresh^{SM} treated samples.

There was not a significant correlation between increasing fruit size and incidence of bitter pit in a general comparison of all storage regimes, which was in contrast with findings of De Freitas *et al*. (2012). However, there was a relationship between fruit size and incidence of bitter pit in

untreated samples collected from orchards which were more susceptible to bitter pit. The orchards with lower propensity to bitter pit did not show significant correlation even in untreated samples. It seems because of mineral movements and crop load that results of this study are not highly correlated to fruit size. Furthermore when SmartFresh^{SM} was used the delay in ripening and incidence of bitter pit masked any effect of fruit size.

As a general conclusion for the effect of preharvest, storage conditions and quality assessments during storage and incidence of bitter pit, all these factors have great impact on accelerating or delaying incidence of bitter pit and all these effects must be considered to have a more accurate and reliable predication model.

Future work:

Future work to look in more detail at weather patterns over the growing period and influence of rootstock, nutrition during fruit growth, orchard management would help to disentangle the complex interactions that influence fruit propensity to develop bitter pit.

This study focused on the effects of different storage regimes and SmartFresh^{SM} treatment without applying these factors in the prediction models directly. Designing a comprehensive multivariate computerised model including all factors and parameters related to preharvest, storage and postharvest treatments is suggested.

7.2 Biochemical analysis:

Biochemical analysis was divided into two parts; measuring organic acids and sugar content as a measure to better predict the metabolic maturity of fruit and mineral analysis to predict the fruit's susceptibility to disorders.

The comparison of different storage regimes showed that there was no significant difference in organic acids of Bramley apples stored in different storage regimes, except that in CA storage regimes ascorbic acid was significantly lower than storage in air (21% O_2). However, there was no significant difference in ascorbic acid content between untreated and SmartFresh^{SM} treated samples. As ascorbic acid acts as an antioxidant, the pool of available ascorbic acid is influenced by the metabolic activity of fruit and by challenges caused by stress, fruits in air store are subject to greater water loss and this may either cause a concentration of its constituents or lead to the fruit increasing its antioxidant profile to reduce the fruit stress. It seems that since storage in air is over a shorter period (4 months) compared to CA storage (6-

10 months) this could explain the decrease in ascorbic acid content of fruits stored in CA regimes.

The relationships between incidence of bitter pit and concentrations of four organic acids ascorbic acid, malic acid, oxalic acid and citric acid compared in different seasons only showed a significant inverse correlation (R=-0.6) to ascorbic acid. When the whole fruit was sampled with a mixture of healthy and bitter pit affected tissues, there was a poor correlation between ascorbic acid. Since bitter pit incidence is normally near the calyx end of the apple fruit and the xylem sap transfers through stalk end to calyx end of the fruit, it is better to collect samples separately from the calyx end and stalk end of apple fruit. Ascorbic acid levels in the calyx region were more strongly correlated (R=-0.88) than from the stalk end of fruit (R=-0.59) to bitter pit incidence.

An equation was applied to compare ascorbic acid levels during storage to harvest levels. The equation $\boldsymbol{T} = \left(\frac{X_n \times 100}{X_H}\right) \geq 90$ was applied to normalise results. When the percentage of ascorbic acid content during storage ($\boldsymbol{X_n}$) decreases more than 10% of ascorbic acid content at harvest ($\boldsymbol{X_H}$), there is more chance of incidence of bitter pit. There was a 25% error in recognising samples with bitter pit when applying this model. However, this method needs refinement by optimising sampling region and sampling times during storage.

The results of this study showed that because of longer storage times under CA regimes compared to air regime, higher content of glucose and fructose was found in the fruit. Also in regime (5% CO_2, 1% O_2) longer storage time causes lower content of sucrose in fruit.

Also the effect of the application of SmartFresh^{SM} in delaying ripening was observed as a significantly higher content of glucose and fructose in SmartFresh^{SM} treated samples than untreated samples stored in air. The reason that there was no difference between untreated and SmartFresh^{SM} treated samples in CA regimes is related to the effect of lower level of oxygen in combination with high carbon dioxide to decrease respiration and reducing ethylene production by reducing the activity of the enzyme ACC oxidase (Saltveit, 1999), which is similar to the effect of SmartFresh^{SM} application.

A study of the relationship between incidence of bitter pit and sugar content (glucose, fructose and sucrose) compared in different seasons only showed a poor inverse correlation (R=-0.35) to sucrose. However the correlation between changes of sucrose content and incidence of bitter pit were not consistent in different orchards. Also similar to the ascorbic acid results,

SmartFresh^{SM} treated samples showed a weaker correlation than untreated samples between sucrose content changes and incidence of bitter pit. Diagnostic models developed to predict incidence of bitter pit based on organic acids such as ascorbic acid and oxalic acid are more accurate in evaluation than relying on sugar content.

When considering calcium, most of the reported literature relates the incidence of bitter pit to the overall total calcium concentration; while it is known that certain forms of calcium such as calcium oxalate are not active within the tissues and that the distribution of minerals is not uniform within apple fruit.

Overall calcium content is not a good indicator of fruit susceptibility to develop bitter pit as samples with similar calcium content varied between 0-20% incidences of the disorder. Sampling of the inner and outer cortex of apples for mineral analysis confirmed that the inner cortex had a higher concentration of Ca^{2+}, K and Mg compared to the outer cortex. Monitoring samples during storage showed the overall calcium concentration declined in all samples from inner cortex. This decline was more consistent in inner cortex and in untreated samples. Calcium content of Bramley apples without bitter pit was significantly higher in inner cortex than those with bitter pit symptoms. However there was no significant difference between concentrations of calcium in inner cortex of samples with different severity of bitter pit.

The increase in calcium inactivation via conjugation to form calcium oxalate was considered to be a possible mechanism for increasing the fruit susceptibility to bitter pit development. New methodologies to distinguish between free and bound calcium, were developed to apply inductively coupled plasma (ICP) to measure total calcium by mass spectrometry and then to quantify the proportion of calcium bound to organic acids such as oxalate by atomic absorption spectroscopy (AAS). The value obtained by subtracting calcium oxalate from total calcium (Ca_T) was named as structural calcium: $Ca_{St} = Ca_T$ - $Ca\ (COO)_2$. Although structural calcium (Ca_{St}) is not representing the amount of free calcium, it still provides an indication of the proportion of calcium not bound by oxalate.

There was a significant difference in content of both Ca^{2+} (total) and Ca^{2+} (structural) in the stalk end in both untreated and SmartFresh^{SM} treated samples. Moreover, a higher potassium content in the calyx end was related to a higher chance of bitter pit symptoms developing.

There was no significant difference of (K+Mg)/Ca in calyx or stalk end of SmartFresh^{SM} treated samples and in untreated samples only the ratio in calyx end of samples with bitter pit were

significantly higher. These showed that high concentration of potassium and magnesium for predicting susceptibility to bitter pit in SmartFresh^{SM} treated samples is not a suitable indicator and the mineral with the highest correlation to incidence of bitter pit especially when samples are treated with SmartFresh^{SM}, is calcium especially when samples are collected separately from the calyx and stalk end of fruit.

This study focused on calcium as Ca^{2+} (total) and calcium oxalate content at harvest and during storage and the relationship with incidence of bitter pit. However, there were samples with higher amounts of calcium which showed bitter pit and samples with lower amounts of calcium that did not show bitter pit. It is important to apply equations to normalise results based on the minimum level of total calcium (5mg/100g FW) and the movement of calcium from inner to outer cortex and from stalk end to calyx end.

Therefore, the equations used (details in Table 4.7) were:

A) $\%\overline{\boldsymbol{Ca}}_{\boldsymbol{St}} = \frac{\overline{Ca}_{St} \times 100}{5}$

B) $\%\overline{\boldsymbol{Ca}}_{\boldsymbol{St}}\boldsymbol{changes} = \frac{\left(\overline{Ca}_{St} - \frac{Ca_{St}(In) - Ca_{St}(Out)}{\overline{Ca}_{St}}\right) \times 100}{5}$

Threshold: $\boldsymbol{T} = \left(\frac{A+B}{2}\right) - (A - B) > 50$ (Less than 50: more chance of bitter pit)

Although the values of Ca^{2+} structural (Ca_{St}) in each season were different most of the results for comparison of changes of normalised $\overline{F}$ to harvest threshold were correlated to incidence of bitter pit in more than 85% incidence of bitter pit matched with lower value of threshold (T).

Future work:

Although a large number of samples were collected in this study the number of samples assessed for biochemical analysis was limited and included samples from different parts of fruit tissue and different intervals of sampling. The models need to be tested on more samples to confirm the obtained results. The suggested equations are only based on free (structural) calcium content. However the role of other minerals like K, Mg and N, and their interactions should be considered to develop a comprehensive prediction model.

7.3 Chlorophyll fluorescence:

Since it was considered that most destructive methods were time consuming with high cost, it was essential to develop a non-destructive method, easy to apply and cost efficient. Most of the previous studies on chlorophyll fluorescence have focused on determination of fruit maturity. Lotze *et al.* (2006) found a correlation of incidence of bitter pit with loss of fluorescence yield, with the progression of fruit maturity.

According to the results obtained by previous studies, it was decided to focus on changes of fluorescence characteristics during the last stages of fruit maturity and storage, so measurements started two weeks before commercial harvest time of Bramley apples and continued during storage in different storage regimes such as air or CA.

The results showed that decreasing chlorophyll fluorescence profiles (Fo to F5, Fm & Fv) of apples started before commercial harvest and during storage. However, the rate of chlorophyll reduction between samples varied and a higher rate of loss of chlorophyll fluorescence was associated with an increased severity of bitter pit; this was also reported by Ross (2002). In this study the results of linear discriminate analysis showed that several fluorescence characteristics can be used to model fruit maturity and the severity of internal bitter pit. However, only samples suffering from a low incidence of bitter pit were distinct from fruit with greater severity of bitter pit.

The chlorophyll fluorescence characteristics which contributed and correlated well ($R > 0.70$) with the incidence of bitter pit were selected from a wider range of chlorophyll fluorescence characteristics. One of the selected characteristics was Fm. Although the results of this study confirmed that there was an inverse correlation ($R=-0.81$) at $P<0.001$ between Fm and incidence of bitter pit, it was just able to distinct symptomless (noBP) samples from samples with bitter pit symptoms without distinguishing severity of bitter pit.

Furthermore, this study showed that characteristic Fm was able to recognise samples treated with SmartFresh^{SM} (1-MCP) from untreated fruit, which is similar to results obtained by Mir *et al*. (2001). In SmartFresh^{SM} treated apples the value of Fm was higher related to slower loss of fluorescence because of lower ethylene production and delay in ripening of treated fruit.

It seems that the characteristic Fm could be applied as a good indicator of maturity. However, this study was more focused on finding the best characteristics correlated to incidence of bitter pit. After analysis of obtained results the best characteristics were found from the first stages of

the fluorescence rise such as Fo, F1, F2 - these relate to the fluorescence intensity at 0, 50 and 300 µs. Our results are similar to those of Oukarroum *et al.* (2012) who found that thermal stress causes significant changes in the fluorescence characteristics resulting in an increase in the initial fluorescence (Fo) and a decrease in the maximum fluorescence values (F_m). Since bitter pit as a type of stress could affect these changes on characteristics Fo, F1 and F2 and they were better correlated with bitter pit, the value of the average of three characteristics (Fo, F1, and F2) was named as average F: $\left(\overline{F} = \frac{Fo+F1+F2}{3}\right)$, and provided the best correlation with incidence of bitter pit. Comparison of results obtained from samples collected from different orchards and stored in different storage regimes (air and CA) showed that $\overline{F}$ was able to distinguish samples without bitter pit from samples with bitter pit symptoms without distinguishing the severity of bitter pit. As chlorophyll fluorescence measurements are based on re-emitted energy from the skin of fruit, they reflect any changes occurring near peel. Bitter pit at first onset appears under the skin and by increasing severity pits may extend throughout the cortex (Jackson 2005), so when bitter pit severity increases and affected cells are not close to the skin, the value of chlorophyll fluorescence is not affected as much as severity of internal disorders such as bitter pit.

Although results in season 2012/13 showed that a threshold of ($\overline{F} \leq 5900$) was correlated ($P<0.001$) to fruit susceptibility to bitter pit development, in season 2013/14 the level of $\overline{F}$ was lower. Although samples were collected in two seasons at approximately the same date (28/8/2012 and 2/9/2013), the ($\overline{F}$) of the samples from the same orchard in season 2012/13 were higher than season 2013/14. The difference between ($\overline{F}$) at harvest in different seasons is related to different parameters including orchard management, environmental factors and climate changes. Because of these impacts and changes in ($\overline{F}$) it was essential to develop a model which is less affected by seasonal factors.

It was decided to apply formulas based on changes of average F ($\overline{F}$) during storage in comparison with the ($\overline{F}$) at harvest time. Normally ($\overline{F}$) decreases during the storage. The value of ($\overline{F}$) at harvest time was therefore normalised using the formula $N_{x/h} = \left(\frac{\overline{F_x} - \sigma\overline{F_x}}{\overline{F_h}}\right) \times 100$. Additionally the standard deviation of ($\overline{F}$) of 10 apples was assessed to show lower ratio for the samples which consist of non-uniform apples in chlorophyll fluorescence and are more susceptible to bitter pit. The threshold at harvest calculated 5% lower: $T_h = \left(\frac{\overline{F_h} - \sigma\overline{F_h}}{\overline{F_h}}\right) \times 95$.

When the value of normalised ($\overline{F}$) is lower than threshold ($N_{x/h} < T_h$) there is more chance of incidence of bitter pit. This threshold was tested with results of both seasons (2012/13 and 2013/14) and was correlated (P<0.001) with the incidence of bitter pit in different seasons and storage conditions. It was also able to distinguish untreated samples from 1-MCP or SF-treated samples. However, the comparison of the thresholds and normalised value $N_{x/h}$ were not always correct and there were some false positive or negative results. Notably there were more false positive (formula showed no bitter pit, but there was bitter pit in the sample). These are very similar to the results that obtained by Lotze *et al.* (2006) with their model with chlorophyll fluorescence imaging by Near-infrared (NIR) and still there is more than 25% error in this model. Although results indicate that chlorophyll fluorescence can be used to predict the occurrence of bitter pit in samples of fruit sampled during storage it does not have the resolution to predict the incidence of bitter pit development in samples measured at harvest. So it is necessary to continue monitoring over the storage time.

For better store management by applying a non-destructive tool to predict incidence of internal disorders like bitter pit it is recommended to apply fruit monitoring for chlorophyll fluorescence changes immediately after harvest, to fill the store with 100 samples from each cold-store and to start regular monitoring of the same samples from the second month of storage every two weeks. The normalised ($\overline{F}$) should be compared with the harvest value and when the value is close to the threshold or less ($N_{x/h} < T_h$) this should be considered as a warning. At this point destructive tests should be applied to other samples in the store to inform decisions about the rest of the fruit in the store.

Future work:

These results of this study were based on two seasons and need more investigations to optimise the position of fruit for doing measurements and the intervals of monitoring in order to test the obtained results and the thresholds in different seasons. There are many different instruments and fluorimeters available in the market that should be compared in terms of potential parameters they are able to assess and the capability for use as part of prediction tools.

7.4 Molecular diagnostics:

This study focused on the gene expression profiles of a limited number of genes involved in calcium regulation and lipid peroxidation and showed there was a differential pattern of expression of transcripts between apples suffering from bitter pit and apples that remained healthy.

Extraction of a high quality RNA with low protein carryover and sufficient yield is the base of all other processes leading to gene expression. Although several extraction methods were investigated and finally three methods developed and applied, there were several problems in this part of the study resulting in inconsistency in quality and concentration of extracted samples and low quality of extracted RNA. All other processes such as cDNA synthesis, PCR product amplification and qPCR are affected by poor quality of RNA.

Comparison of gene expression with the Ca^{2+}ATPase primer in seasons 2010/11 with whole cortex tissue and in 2011/12 with samples from inner and outer cortex sampled tissues showed the PCR product of Ca^{2+}ATPase amplified only for samples without bitter pit incidence. Since Burmeister and Dilley (1994) suggested that Ca^{2+}ATPase interferes with the role of calcium as second messenger when calcium is bound by $Ca^{2+/}$CaM, it seems our results confirm that when there is more free calcium and no symptoms of bitter pit the Ca^{2+}ATPase gene is expressed. Similar results were observed with lypoxygenase genes. This was also reported by Lara (2013). However, in some samples with low symptoms of bitter pit lypoxygenase genes were expressed. It seems Ca^{2+}ATPase and lypoxygenase genes are more active in healthy tissues with high free calcium or the numbers of transcripts were not easily recoverable in tissue that was undergoing early stages of senescence.

Different fragments of calmodulin in apple were used to attempt to clone the calmodulin gene. Only calmodulin (b) successfully amplified a 140 bp fragment from Bramley's seedling apple cDNA isolated from different samples. PCR products of primer calmodulin (b) from samples with more incidence of bitter pit which were taken from outer cortex showed more amplification.

Real Time PCR was applied for quantifying gene expression. Although results were not consistent and the patterns of expression of calmodulin in these samples did not correlate well with the incidence of bitter pit, there was a relationship between increasing gene expression (calmodulin b) and incidence of bitter pit especially in untreated samples when stored longer

where the incidence of bitter pit had risen, in which case the expression of calmodulin was significantly increased. Wang *et al*. (2001) reported a relationship between maturity and calmodulin. It seems since SmartFresh^{SM} delays ripening by interfering with ACC and ethylene production this affects calmodulin gene expression.

There was a higher gene expression of "Ca^{2+} Protease A" when the tissue was from outer cortex and had bitter pit. Calmodulin (b) and "Ca^{2+} Protease A", showed increasing gene expression where incidence of bitter pit increased. Since mixing samples with or without symptoms of bitter pit resulted in a dilution of the tissues with bitter pit, this could be one of the reasons that the molecular results did not show significant difference between samples with or without bitter pit. It was decided in season (2013/14) to use samples only from apples with or without bitter pit, and in this case samples were collected separately from stalk and calyx end tissues. The results after the changes in sampling showed more gene expression in samples from the stalk end than calyx end tissues and higher correlation of calmodulin (b) gene expression and incidence of bitter pit in comparison with "Ca^{2+} Protease A". Also when tissues from the same sample were taken from tissues without signs of bitter pit, there was more gene expression "calmodulin (b)" in samples untreated or SmartFresh^{SM} treated from tissues with bitter pit in comparison with the tissues from the same apple without signs of bitter pit.

It is likely that the reason for higher expression of "Ca^{2+} Protease A" and "calmodulin (b)" in stalk end tissues compared to calyx end tissues even when apple does not show bitter pit symptoms is related to binding of free calcium by calmodulin, as indicated by Kim *et al*. (2008),. Since there is more free calcium in the stalk end because of xylem sap movement from stalk to calyx end (Atkinson, 2014), more free calcium is bound by calmodulin leading to an increase in expression of "Ca^{2+} Protease A" and "calmodulin (b)".

Although some results showed a correlation between expression of the selected genes especially "Ca^{2+} Protease A" and "calmodulin (b)" amplification to bitter pit, results were inconsistent. With additional data it may be possible to build a multivariate model that can help to predict the onset of bitter pit development in fruit and help growers make an informed choice regarding marketing of existing fruit in store and changes to current orchard and store management practices that can help to mitigate some of the bitter pit problems.

Future work:

Improvement of extraction methods for a faster method with higher quality and concentration of RNA that can help to provide a better comparison in gene expression for different degrees of bitter pit development and developing a practical model to predict onset of bitter pit development.

7.5 Overall Conclusions

1) Development of a comprehensive reliable prediction model requires knowledge and data about the history of fruit from preharvest, storage conditions and quality assessments.

2) Application of 1-MCP (SmartFresh^{SM}) delays incidence of bitter pit.

3) In the case of organic acids, changes in ascorbic acid were more correlated to incidence of bitter pit and a diagnostic model based on changes of ascorbic acid during storage in comparison with harvest was developed.

4) The pattern of free calcium distribution is the main factor controlling the incidence of bitter pit. A diagnostic model based on changes in the proportion of calcium oxalate content during storage in comparison with harvest was developed to identify samples with higher propensity to develop bitter pit.

5) Chlorophyll fluorescence as a non-destructive diagnostic tool was applied to compare changes in fruit maturity and reduction of fluorescence during storage as an alarm to identify incidence of bitter pit. This diagnostic method should be combined with other destructive tools to provide a more reliable predictive model for incidence of bitter pit.

6) Changes in gene expression profiles of a limited number of genes such as calmodulin showed the differences in patterns of transcripts between apples suffering from bitter pit and healthy apples. However, molecular diagnostic tools need to be developed to have more consistent results before it is possible to develop an applicable diagnostic tool.

7) It would be possible to build a multi variate model for predicting the onset of bitter pit development in apple by combination of two or more suggested diagnostic tools.

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APPENDICES

Appendix I: Final list of frozen samples in 2010/11 which were used for chemical and molecular analysis (88 samples).

Each sample was taken from different parts of apple cortex of 10 apples. For samples with bitter pit (BP), another sample was collected from parts of symptomless apple cortex (NoBP).

Number of samples	Sample	Grower	Store	Storage regime	Sampling Date	Out of store/ Shelf life	NOS F/ SF	BP%
1	82	AGA	6	9/12	13/09/2010	Out store	-	0
2	82	AGA	6	9/12	13/09/2010	Out store	+	0
3	224 BP	AGA	6	9/12	18/11/2010	Out store	-	17
4	225 NoBP	AGA	6	9/12	18/11/2010	Out store	-	0
5	224	AGA	6	9/12	18/11/2010	Out store	+	0
6	143 BP	AGA	6	9/12	04/02/2011	Out store	-	52
7	144 NoBP	AGA	6	9/12	04/02/2011	Out store	-	0
8	143 BP	AGA	6	9/12	04/02/2011	Out store	+	3
9	144 NoBP	AGA	6	9/12	04/02/2011	Out store	+	0
10	95 BP	AGA	6	9/12	10/02/2011	Shelf life	-	50
11	97 NoBP	AGA	6	9/12	10/02/2011	Shelf life	-	0
12	95 BP	AGA	6	9/12	10/02/2011	Shelf life	+	4
13	97 NoBP	AGA	6	912	10/02/2011	Shelf life	+	0
14	12	C wood	Dit 1	5/1	03/09/2010	Out store	-	0
15	12	C wood	Dit 1	5/1	03/09/2010	Out store	+	0
16	230 BP	C wood	Dit 1	5/1	18/11/2010	Out store	-	60
17	231 NoBP	C wood	Dit 1	5/1	18/11/2010	Out store	-	0
18	230	C wood	Dit 1	5/1	18/11/2010	Out store	+	0
19	94	Langridge	3	9/12	14/09/2010	Out store	-	0
20	94	Langridge	3	9/12	14/09/2010	Out store	+	0
21	336 BP	Langridge	3	9/12	17/12/2010	Shelf life	-	33
22	337 NoBP	Langridge	3	9/12	17/12/2010	Shelf life	-	0
23	336 BP	Langridge	3	9/12	17/12/2010	Shelf life	+	7
24	337 NoBP	Langridge	3	9/12	17/12/2010	Shelf life	+	0
25	146 BP	Langridge	3	9/12	04/02/2011	Out store	-	37
26	149 NoBP	Langridge	3	9/12	04/02/2011	Out store	-	0

27	146	Langridge	3	9/12	04/02/2011	Out store	+	0
28	188	Langridge	3	9/12	09/06/2011	Out store	+	0
29	189	Langridge	3	9/12	09/06/2011	Out store	+	0
No.	**Sample**	**Grower**	**Store**	**Storage regime**	**Sampling Date**	**Out of store/ Shelf life**	**NOS F/ SF**	**BP%**
30	34	Newmafruit	Popes A	9/12	08/09/2010	Out store	-	0
31	34	Newmafruit	Popes A	9/12	08/09/2010	Out store	+	0
32	209 BP	Newmafruit	Popes A	9/12	17/11/2010	Out store	-	42
33	210 NoBP	Newmafruit	Popes A	9/12	17/11/2010	Out store	-	0
34	209	Newmafruit	Popes A	9/12	17/11/2010	Out store	+	0
35	260 BP	Newmafruit	Popes A	9/12	23/11/2010	Shelf life	-	43
36	261 NoBP	Newmafruit	Popes A	9/12	23/11/2010	Shelf life	-	0
37	109	Jenner	Chain 1	9/12	15/09/2010	Out store	-	0
38	109	Jenner	Chain 1	9/12	15/09/2010	Out store	+	0
39	323 BP	Jenner	Chain 1	9/12	14/12/2010	Out store	-	23
40	324 NoBP	Jenner	Chain 1	9/12	14/12/2010	Out store	-	0
41	324 BP	Jenner	Chain 1	9/12	14/12/2010	Out store	+	20
42	324 NoBP	Jenner	Chain 1	9/12	14/12/2010	Out store	+	0
43	17	Newmafruit	Popes B	9/12	05/09/2010	Out store	-	0
44	17	Newmafruit	Popes B	9/12	05/09/2010	Out store	+	0
45	208 BP	Newmafruit	Popes B	9/12	17/11/2010	Out store	-	27
46	209 NoBP	Newmafruit	Popes B	9/12	17/11/2010	Out store	-	0
47	208 BP	Newmafruit	Popes B	9/12	17/11/2010	Out store	+	23
48	209 NoBP	Newmafruit	Popes B	9/12	17/11/2010	Out store	+	0
49	262 BP	Newmafruit	Popes B	9/12	23/11/2010	Shelf life	-	7
50	263 NoBP	Newmafruit	Popes B	9/12	23/11/2010	Shelf life	-	0
51	262 BP	Newmafruit	Popes B	9/12	23/11/2010	Shelf life	+	2
52	263 NoBP	Newmafruit	Popes B	9/12	23/11/2010	Shelf life	+	0
53	24	North Court	11	5/1	06/09/2010	Out store	-	0
54	24	North Court	11	5/1	06/09/2010	Out store	+	0
55	310 BP	North court	11	5/1	10/12/2010	Out store	-	29
56	311 NoBP	North court	11	5/1	10/12/2010	Out store	-	0

57	310 BP	North court	11	5/1	10/12/2010	Out store	+	8
58	311 NoBP	North court	11	5/1	10/12/2010	Out store	+	0
59	338 BP	North court	11	5/1	17/12/2010	Shelf life	-	57
60	339 NoBP	North court	11	5/1	17/12/2010	Shelf life	-	0
61	339	North court	11	5/1	17/12/2010	Shelf life	+	0

No.	Sample	Grower	Store	Storage regime	Sampling Date	Out of store/ Shelf life	NOSF / SF	BP %
62	18	Bardsley	Ware 18	5/1	05/09/2010	Out store	-	0
63	18	Bardsley	Ware 18	5/1	05/09/2010	Out store	+	0
64	13 BP	Bardsley	Ware 18	5/1	17/03/2011	Out store	-	19
65	14 NoBP	Bardsley	Ware 18	5/1	17/03/2011	Out store	-	0
66	13 BP	Bardsley	Ware 18	5/1	17/03/2011	Out store	+	10
67	14 NoBP	Bardsley	Ware 18	5/1	17/03/2011	Out store	+	0
68	62 BP	Bardsley	Ware 18	5/1	24/03/2011	Shelf life	-	7
69	65 NoBP	Bardsley	Ware 18	5/1	24/03/2011	Shelf life	-	0
69	62 BP	Bardsley	Ware 18	5/1	24/03/2011	Shelf life	+	3
69	65 NoBP	Bardsley	Ware 18	5/1	24/03/2011	Shelf life	+	0
70	51	Carpenter	Packhouse 2	9/12	10/09/2010	Out store	-	0
71	51	Carpenter	Packhouse 2	9/12	10/09/2010	Out store	+	0
72	66 BP	Carpenter	Packhouse 2	9/12	10/02/2011	Out store	-	3
73	69 NoBP	Carpenter	Packhouse 2	9/12	10/02/2011	Out store	-	0
74	66	Carpenter	Packhouse 2	9/12	10/02/2011	Out store	+	0
75	11	Wheeler	Bullen 6	9/12	03/09/2010	Out store	-	0
76	11	Wheeler	Bullen 6	9/12	03/09/2010	Out store	+	0
77	172 BP	Wheeler	Bullen 6	9/12	09/06/2011	Out store	+	13
78	174 NoBP	Wheeler	Bullen 6	9/12	09/06/2011	Out store	+	0
79	159 BP	Chapman	8	5/1	09/06/2011	Out store	-	10
80	163 NoBP	Chapman	8	5/1	09/06/2011	Out store	-	0
81	159	Chapman	8	5/1	09/06/2011	Out store	+	0
82	403	Chapman	8	5/1	05/07/2011	Out store	-	16
83	9	Newmafruit	How 21	5/1	03/09/2010	Out store	-	0

84	9	Newmafruit	How 21	5/1	03/09/2010	Out store	+	0
85	207	Newmafruit	How 21	5/1	17/11/2010	Out store	-	0
86	207	Newmafruit	How 21	5/1	17/11/2010	Out store	+	0
87	6	Newmafruit	How 21	5/1	03/02/2011	Out store	-	0
88	6	Newmafruit	How 21	5/1	03/02/2011	Out store	+	0

Appendix II: Final list of frozen samples in 2011/12 which were used for chemical and molecular analysis (96 samples).

Each sample was taken from inner and outer cortex of 10 apples.

No.	Sample	Inner/ Outer cortex	Grower	Store	Storage regime	Sampling Date	Out store/ Shelf life	NOSF/ SF	BP %
1	100	In	Baxter	Ams 7	5/1	19/09/2011	Out store	-	0
2	101	Out	Baxter	Ams 7	5/1	19/09/2011	Out store	+	0
3	293	In	Baxter	Ams 7	5/1	27/01/2012	Out store	-	33
4	293	In	Baxter	Ams 7	5/1	27/01/2012	Out store	+	13
5	294	Out	Baxter	Ams 7	5/1	27/01/2012	Out store	-	33
6	294	Out	Baxter	Ams 7	5/1	27/01/2012	Out store	+	13
7	325	In	Baxter	Ams 7	5/1	03/02/2012	Shelf life	-	27
8	325	In	Baxter	Ams 7	5/1	03/02/2012	Shelf life	+	11
9	326	Out	Baxter	Ams 7	5/1	03/02/2012	Shelf life	-	27
10	326	Out	Baxter	Ams 7	5/1	03/02/2012	Shelf life	+	11
11	335	In	Baxter	Ams 7	5/1	16/02/2012	Out store	-	21
12	335	In	Baxter	Ams 7	5/1	16/02/2012	Out store	+	7
13	336	Out	Baxter	Ams 7	5/1	16/02/2012	Out store	-	21
14	336	Out	Baxter	Ams 7	5/1	16/02/2012	Out store	+	7
15	337	Out	Baxter	Ams 7	5/1	23/02/2012	Shelf life	-	13
16	337	Out	Baxter	Ams 7	5/1	23/02/2012	Shelf life	+	0
17	338	Out	Baxter	Ams 7	5/1	23/02/2012	Shelf life	-	13
18	338	Out	Baxter	Ams 7	5/1	23/02/2012	Shelf life	+	0
19	47	In	Carpenter	70 T	9/12	30/08/2011	Out store	-	0
20	48	Out	Carpenter	70 T	9/12	30/08/2011	Out store	-	0

21	267	In	Carpenter	70 T	9/12	25/11/2011	Out store	-	13
22	267	In	Carpenter	70 T	9/12	25/11/2011	Out store	+	3
23	268	Out	Carpenter	70 T	9/12	25/11/2011	Out store	-	13
24	268	Out	Carpenter	70 T	9/12	25/11/2011	Out store	+	3
25	291	In	Carpenter	70 T	9/12	25/11/2011	Out store	-	10
26	291	In	Carpenter	70 T	9/12	25/11/2011	Out store	+	8
27	292	Out	Carpenter	70 T	9/12	25/11/2011	Out store	-	10
28	292	Out	Carpenter	70 T	9/12	25/11/2011	Out store	+	8
29	333	In	Carpenter	70 T	9/12	25/11/2011	Out store	-	20
30	333	In	Carpenter	70 T	9/12	25/11/2011	Out store	+	3
31	334	Out	Carpenter	70 T	9/12	25/11/2011	Out store	-	20
32	334	Out	Carpenter	70 T	9/12	25/11/2011	Out store	+	3

No.	Sample	Inner/ Outer cortex	Grower	Store	Storage regime	Sampling Date	Out store/ Shelf life	NOSF/ SF	BP %
33	68	In	Goatham	Hall 7	9/12	02/09/2011	Out store	-	0
34	69	Out	Goatham	Hall 7	9/12	02/09/2011	Out store	-	0
35	311	In	Goatham	Hall 7	9/12	27/01/2012	Out store	-	0
36	311	In	Goatham	Hall 7	9/12	27/01/2012	Out store	+	0
37	312	Out	Goatham	Hall 7	9/12	27/01/2012	Out store	-	0
38	312	Out	Goatham	Hall 7	9/12	27/01/2012	Out store	+	0
39	43	In	Goatham	M 26	5/1	28/08/2011	Out store	-	0
40	44	Out	Goatham	M 26	5/1	28/08/2011	Out store	-	0
41	297	In	Goatham	M 26	5/1	27/01/2012	Out store	-	0
42	297	In	Goatham	M 26	5/1	27/01/2012	Out store	+	0
43	298	Out	Goatham	M 26	5/1	27/01/2012	Out store	-	0
44	298	Out	Goatham	M 26	5/1	27/01/2012	Out store	+	0
45	134	In	Jenner	Chain 1	9/12	15/09/2011	Out store	-	0
46	135	Out	Jenner	Chain 1	9/12	15/09/2011	Out store	-	0
47	227	In	Jenner	Chain 1	9/12	15/09/2011	Out store	-	46
48	227	In	Jenner	Chain 1	9/12	15/09/2011	Out store	+	17
49	228	Out	Jenner	Chain 1	9/12	15/09/2011	Out store	-	46
50	228	Out	Jenner	Chain 1	9/12	15/09/2011	Out store	+	17
51	319	In	Jenner	Chain 1	9/12	27/01/2012	Out store	+	10
52	320	Out	Jenner	Chain 1	9/12	27/01/2012	Out store	+	10
53	327	In	Jenner	Chain 1	9/12	27/01/2012	Shelf life	+	43
54	328	Out	Jenner	Chain 1	9/12	27/01/2012	Shelf life	+	43
55	7	In	Newmafruit	How 20	5/1	19/08/2011	Out store	-	0

56	8	Out	Newmafruit	How 20	5/1	19/08/2011	Out store	-	0
57	225	In	Newmafruit	How 20	5/1	19/08/2011	Out store	-	0
58	225	In	Newmafruit	How 20	5/1	19/08/2011	Out store	+	0
59	226	Out	Newmafruit	How 20	5/1	19/08/2011	Out store	-	0
60	226	Out	Newmafruit	How 20	5/1	19/08/2011	Out store	+	0
61	347	In	Newmafruit	How 20	5/1	19/08/2011	Out store	-	0
62	347	In	Newmafruit	How 20	5/1	19/08/2011	Out store	+	0
63	348	Out	Newmafruit	How 20	5/1	19/08/2011	Out store	-	0
64	348	Out	Newmafruit	How 20	5/1	19/08/2011	Out store	+	0

No.	Sample	Inner/ Outer cortex	Grower	Store	Storage regime	Sampling Date	Out store/ Shelf life	NOSF/ SF	BP%
65	88	In	Mansfield	Pitstock 3	9/12	06/09/2011	Out store	-	0
66	89	Out	Mansfield	Pitstock 3	9/12	06/09/2011	Out store	-	0
67	178	In	Mansfield	Pitstock 3	9/12	17/11/2011	Out store	-	18
68	178	In	Mansfield	Pitstock 3	9/12	17/11/2011	Out store	+	8
69	179	In	Mansfield	Pitstock 3	9/12	17/11/2011	Out store	-	18
70	179	Out	Mansfield	Pitstock 3	9/12	17/11/2011	Out store	+	8
71	260	In	Mansfield	Pitstock 3	9/12	25/11/2011	Shelf life	-	17
72	260	In	Mansfield	Pitstock 3	9/12	25/11/2011	Shelf life	+	0
73	261	Out	Mansfield	Pitstock 3	9/12	25/11/2011	Shelf life	-	17
74	261	Out	Mansfield	Pitstock 3	9/12	25/11/2011	Shelf life	+	0
75	280	In	Mansfield	Pitstock 3	9/12	09/01/2012	Out store	-	33
76	280	In	Mansfield	Pitstock 3	9/12	09/01/2012	Out store	+	0
77	281	Out	Mansfield	Pitstock 3	9/12	09/01/2012	Out store	-	33
78	281	Out	Mansfield	Pitstock 3	9/12	09/01/2012	Out store	+	0
79	282	In	Mansfield	Pitstock 3	9/12	17/01/2012	Shelf life	-	37
80	282	In	Mansfield	Pitstock 3	9/12	17/01/2012	Shelf life	+	0
81	283	Out	Mansfield	Pitstock 3	9/12	17/01/2012	Shelf life	-	37
82	283	Out	Mansfield	Pitstock 3	9/12	17/01/2012	Shelf life	+	0
83	49	In	Wheeler	Bullen 6	9/12	30/08/2011	Out store	-	0
84	50	Out	Wheeler	Bullen 6	9/12	30/08/2011	Out store	-	0
85	220	In	Wheeler	Bullen 6	9/12	18/11/2011	Out store	-	43
86	220	In	Wheeler	Bullen 6	9/12	18/11/2011	Out store	+	14
87	221	Out	Wheeler	Bullen 6	9/12	18/11/2011	Out store	-	43

88	221	Out	Wheeler	Bullen 6	9/12	18/11/2011	Out store	+	14
89	257	In	Wheeler	Bullen 6	9/12	25/11/2011	Shelf life	-	10
90	257	In	Wheeler	Bullen 6	9/12	25/11/2011	Shelf life	+	0
91	258	Out	Wheeler	Bullen 6	9/12	25/11/2011	Shelf life	-	10
92	258	Out	Wheeler	Bullen 6	9/12	25/11/2011	Shelf life	+	0
93	NRI 1	In	Wheeler	Bullen 6	9/12	10/04/2012	Shelf life	-	33
94	NRI 1	In	Wheeler	Bullen 6	9/12	10/04/2012	Shelf life	+	7
95	NRI 2	Out	Wheeler	Bullen 6	9/12	10/04/2012	Shelf life	-	33
96	NRI 2	Out	Wheeler	Bullen 6	9/12	10/04/2012	Shelf life	+	7

Appendix III: Final list of frozen samples in 2011/12 which were collected from two orchards (EMR-EE193) and (HOO-Top) for comparing picking dates and air stored (4-4.5°C).

Each sample had inner and outer cortex so the total number of samples was 96.

No.	Sample	Picking Date	Sampling Date	Average firmness (N)	Average Colour (*b* value)	Average Ethylene at Picking (MµL/Sec)	Brix %	BP %
1	EE P1A	09/08/2011	09/08/2011	101.01	44.91	19.5	10.5	0
2	EE P1B	09/08/2011	09/08/2011	101.21	45.32	23.8	10.6	0
3	EE P1A	09/08/2011	10/11/2011	49.72	47.17	NA	11.4	0
4	EE P1B	09/08/2011	10/11/2011	51.88	49.2	NA	11.8	7
5	EE P1A	09/08/2011	10/01/2012	43.54	50.87	NA	10.9	7
6	EE P1B	09/08/2011	10/01/2012	45.99	51.33	NA	11.2	13
7	EE P2A	18/08/2011	18/08/2011	90.13	45.73	13.6	10.9	0
8	EE P2B	18/08/2011	18/08/2011	100.03	45.66	24.1	11	0
9	EE P2A	18/08/2011	10/11/2011	49.23	47.33	NA	12.1	16
10	EE P2B	18/08/2011	10/11/2011	48.25	48.56	NA	12.2	10
11	EE P2A	18/08/2011	10/01/2012	43.35	50.35	NA	11.3	3
12	EE P2B	18/08/2011	10/01/2012	43.74	50.85	NA	11.5	20
13	EE P3A	25/08/2011	25/08/2011	91.21	46.67	45.7	11.8	0
14	EE P3B	25/08/2011	25/08/2011	94.74	46.89	31.4	11.5	0
15	EE P3A	25/08/2011	10/11/2011	49.92	49.72	NA	12.8	7
16	EE P3B	25/08/2011	10/11/2011	50.41	49.72	NA	12.9	16

17	EE P3A	25/08/2011	10/01/2012	44.72	50.68	NA	11.6	0
18	EE P3B	25/08/2011	10/01/2012	41.88	50.08	NA	11.4	10
19	EE P4A	05/09/2011	05/09/2011	87.28	46.86	48.1	12.3	0
20	EE P4B	05/09/2011	05/09/2011	84.63	46.95	56.4	12.1	0
21	EE P4A	05/09/2011	10/11/2011	45.41	49.9	NA	12.5	37
22	EE P4B	05/09/2011	10/11/2011	45.80	48.08	NA	12.1	20
23	EE P4A	05/09/2011	10/01/2012	42.66	51.65	NA	12	20
24	EE P4B	05/09/2011	10/01/2012	41.78	48.82	NA	11.6	13

No.	Sample	Picking Date	Sampling Date	Average firmness (N)	Average Colour (*b* value)	Average Ethylene at Picking (MµL/Sec)	Brix %	BP %
25	Hoo P1A	09/08/2011	09/08/2011	97.38	40.28	13.5	9.9	0
26	Hoo P1B	09/08/2011	09/08/2011	96.30	41.59	14.9	9.6	0
27	Hoo P1A	09/08/2011	10/11/2011	49.33	44.74	NA	11.4	0
28	Hoo P1B	09/08/2011	10/11/2011	46.88	43.54	NA	10.9	0
29	Hoo P1A	09/08/2011	10/01/2012	43.54	46.68	NA	10.7	0
30	Hoo P1B	09/08/2011	10/01/2012	42.86	46	NA	10.7	0
31	Hoo P2A	18/08/2011	18/08/2011	88.75	39.74	17.7	10.1	0
32	Hoo P2B	18/08/2011	18/08/2011	86.69	40.85	22.4	10	0
33	Hoo P2A	18/08/2011	10/11/2011	46.39	44.01	NA	11.1	0
34	Hoo P2B	18/08/2011	10/11/2011	46.68	43.16	NA	10.8	0
35	Hoo P2A	18/08/2011	10/01/2012	42.46	45.83	NA	10.4	0
36	Hoo P2B	18/08/2011	10/01/2012	42.56	46.61	NA	10.7	0
37	Hoo P3A	25/08/2011	25/08/2011	85.32	41.76	25.6	10.6	0
38	Hoo P3B	25/08/2011	25/08/2011	86.89	41.69	27.4	10.5	0
39	Hoo P3A	25/08/2011	10/11/2011	44.82	44	NA	11.7	0
40	Hoo P3B	25/08/2011	10/11/2011	45.41	42.77	NA	11.7	3
41	Hoo P3A	25/08/2011	10/01/2012	39.52	46.62	NA	11	10
42	Hoo P3B	25/08/2011	10/01/2012	40.50	46.57	NA	10.8	7
43	Hoo P4A	05/09/2011	05/09/2011	77.38	42.32	62.8	10.8	0
44	Hoo P4B	05/09/2011	05/09/2011	74.73	42.19	55.1	11.3	0
45	Hoo P4A	05/09/2011	10/11/2011	44.43	44.7	NA	12.1	0
46	Hoo P4B	05/09/2011	10/11/2011	47.17	43.63	NA	11.4	0
47	Hoo P4A	05/09/2011	10/01/2012	40.21	47.49	NA	10.8	7
48	Hoo P4B	05/09/2011	10/01/2012	39.52	46.55	NA	10.3	0

Appendix IV: Final list of frozen samples in 2012/13 which were collected from 4 orchards (EMR-EE193), (HOO-Top), (CAR) and (NEW) at the same time and air-stored in air regime (4-4.5°C).

Each sample had inner and outer cortex so the total number of samples was 256.

Sample	Picking Date	Sampling Date	Ave Size (mm)	Ave firmness (N)	Ave Colour (*b* value)	Ave Ethylene at Picking (MµL/Sec)	Brix %	BP %
EE P1A	13/08/2012	13/08/2012	86.9	107.68	42.12	15.6	9	0
EE P1B	13/08/2012	13/08/2012	84.7	103.37	44.11	26.8	9.1	0
EE P1A	13/08/2012	13/11/2012	79.9	55.80	44.89		11.4	30
EE P1B	13/08/2012	13/11/2012	85.2	55.12	45.41		10.1	57
EE P1A	13/08/2012	12/12/2012	79.8	53.25	46.26		10.4	37
EE P1B	13/08/2012	12/12/2012	84.4	54.92	47.58		10.5	43
EE P1A	13/08/2012	03/01/2013	84.0	46.98	46.71		9.9	40
EE P1B	13/08/2012	03/01/2013	81.4	48.94	79.91		10.6	33
EE P2A	20/08/2012	20/08/2012	92.7	102.29	43.15	17.3	9.9	0
EE P2B	20/08/2012	20/08/2012	94.5	99.05	43.01	28.1	9.5	3
EE P2A	20/08/2012	13/11/2012	90.7	52.57	46.28		11.5	43
EE P2B	20/08/2012	13/11/2012	89.6	53.94	44.99		11.9	17
EE P2A	20/08/2012	12/12/2012	92.5	53.45	46.15		11.4	37
EE P2B	20/08/2012	12/12/2012	93.4	52.66	47.22		11.2	37
EE P2A	20/08/2012	03/01/2013	89.5	53.06	49.04		10.9	23
EE P2B	20/08/2012	03/01/2013	87.3	45.31	48.62		10.7	20
EE PHcA	28/08/2012	28/08/2012	90.1	93.75	42.45	26.4	10.2	0
EE PHcB	28/08/2012	28/08/2012	92.0	91.89	42.11	22.5	10.2	0
EE PHcA	28/08/2012	13/11/2012	89.1	51.58	45.96		11.5	47
EE PHcB	28/08/2012	13/11/2012	86.1	55.21	45.54		12.6	17
EE PHcA	28/08/2012	12/12/2012	86.7	51.00	47.74		11.4	53
EE PHcB	28/08/2012	12/12/2012	83.5	50.11	45.95		11.5	27
EE PHcA	28/08/2012	03/01/2013	90.2	48.64	48.00		11.3	23
EE PHcB	28/08/2012	03/01/2013	88.5	44.33	48.59		11.5	47
EE HsfA	28/08/2012	28/08/2012						
EE HsfB	28/08/2012	28/08/2012						
EE HsfA	28/08/2012	13/11/2012	86.0	89.34	38.03		12.9	0
EE HsfB	28/08/2012	13/11/2012	91.2	89.15	40.23		11.9	0
EE HsfA	28/08/2012	12/12/2012	91.3	80.61	50.91		12	7

EE HsfB	28/08/2012	12/12/2012	86.2	82.87	49.83		12.1	17
EE HsfA	28/08/2012	03/01/2013	88.7	73.26	50.25		12.3	13
EE HsfB	28/08/2012	03/01/2013	85.8	73.16	52.44		11.9	17

Sample	Picking Date	Sampling Date	Ave Size (mm)	Ave firmness (N)	Ave Colour (*b* value)	Ave Ethylene at Picking (MµL/Sec)	Brix %	BP %
Hoo P1A	13/08/2012	13/08/2012	84.5	105.52	42.09	181.5	9	0
Hoo P1B	13/08/2012	13/08/2012	81.5	107.58	43.49	23.6	9.4	0
Hoo P1A	13/08/2012	13/11/2012	81.7	60.31	42.49		11.9	17
Hoo P1B	13/08/2012	13/11/2012	82.8	62.67	43.86		12.5	27
Hoo P1A	13/08/2012	12/12/2012	80.8	55.51	44.27		10.6	7
Hoo P1B	13/08/2012	12/12/2012	85.3	55.31	45.99		10.7	17
Hoo P1A	13/08/2012	03/01/2013	84.5	49.43	45.79		10.8	27
Hoo P1B	13/08/2012	03/01/2013	82.4	50.11	47.18		10.8	30
Hoo P2A	20/08/2012	20/08/2012	88.0	102.58	42.28	34.2	9.8	0
Hoo P2B	20/08/2012	20/08/2012	87.4	102.97	42.01	30.8	9.6	0
Hoo P2A	20/08/2012	13/11/2012	89.4	59.63	43.18		12.2	13
Hoo P2B	20/08/2012	13/11/2012	88.5	63.26	45.24		12.1	43
Hoo P2A	20/08/2012	12/12/2012	86.2	56.39	45.42		11.6	40
Hoo P2B	20/08/2012	12/12/2012	87.9	55.70	46.28		11.5	27
Hoo P2A	20/08/2012	03/01/2013	88.9	49.72	45.75		11	20
Hoo P2B	20/08/2012	03/01/2013	87.8	51.29	46.98		10.8	23
Hoo HcA	28/08/2012	28/08/2012	89.2	95.42	43.24	29.2	10.2	0
Hoo HcB	28/08/2012	28/08/2012	90.5	101.99	43.87	44.85	10.3	0
Hoo HcA	28/08/2012	13/11/2012	91.0	55.80	45.18		13.5	23
Hoo HcB	28/08/2012	13/11/2012	91.7	57.67	40.83		12.5	7
Hoo HcA	28/08/2012	12/12/2012	90.7	51.29	45.03		11.4	33
Hoo HcB	28/08/2012	12/12/2012	83.1	51.39	44.67		11.9	17
Hoo HcA	28/08/2012	03/01/2013	89.9	47.17	47.42		11	27
Hoo HcB	28/08/2012	03/01/2013	89.8	47.17	46.68		10.7	50
Hoo HsfA	28/08/2012	28/08/2012						
Hoo HsfB	28/08/2012	28/08/2012						
Hoo HsfA	28/08/2012	13/11/2012	86.9	95.91	43.32		13.4	0
Hoo HsfB	28/08/2012	13/11/2012	89.6	92.28	42.85		12.8	0
Hoo HsfA	28/08/2012	12/12/2012	87.5	81.20	45.96		12	0

Hoo HsfB	28/08/2012	12/12/2012	89.7	85.52	47.49		11.8	3
Hoo HsfA	28/08/2012	03/01/2013	90.1	77.57	48.41		11.7	7
Hoo HsfB	28/08/2012	03/01/2013	87.5	69.24	49.58		11.8	7

Sample	Picking Date	Sampling Date	Ave Size (mm)	Ave firmness (N)	Ave Colour (b value)	Ave Ethylene at Picking (MµL/Sec)	Brix %	BP %
Car P1A	13/08/2012	13/08/2012	81.5	106.60	39.91	293.7	9	0
Car P1B	13/08/2012	13/08/2012	81.6	103.17	40.99	103.7	9	0
Car P1A	13/08/2012	13/11/2012	83.0	52.96	43.50		12.4	17
Car P1B	13/08/2012	13/11/2012	81.2	49.53	43.35		11.8	13
Car P1A	13/08/2012	12/12/2012	80.3	52.37	44.74		10.5	17
Car P1B	13/08/2012	12/12/2012	81.0	50.41	45.51		10.2	7
Car P1A	13/08/2012	03/01/2013	77.6	45.41	44.29		9.8	7
Car P1B	13/08/2012	03/01/2013	82.0	45.01	44.31		9.9	7
Car P2A	20/08/2012	20/08/2012	88.2	97.19	42.56	22.8	9.4	0
Car P2B	20/08/2012	20/08/2012	86.8	99.74	41.81	27	9.2	0
Car P2A	20/08/2012	13/11/2012	88.1	50.21	43.81		11.2	7
Car P2B	20/08/2012	13/11/2012	87.9	54.92	44.50		12.2	37
Car P2A	20/08/2012	12/12/2012	86.5	50.02	45.17		11	7
Car P2B	20/08/2012	12/12/2012	79.7	48.45	45.61		11.8	43
Car P2A	20/08/2012	03/01/2013	87.7	45.11	45.71		10	17
Car P2B	20/08/2012	03/01/2013	86.4	46.29	48.26		10.4	40
Car HcA	28/08/2012	28/08/2012	91.0	89.73	42.76	18.9	9.6	0
Car HcB	28/08/2012	28/08/2012	89.3	95.42	42.43	50.9	9.5	0
Car HcA	28/08/2012	13/11/2012	89.7	51.58	43.77		12.5	40
Car HcB	28/08/2012	13/11/2012	89.6	53.94	34.93		12.4	23
Car HcA	28/08/2012	12/12/2012	86.5	47.86	46.14		11	30
Car HcB	28/08/2012	12/12/2012	90.3	49.43	45.41		11	33
Car HcA	28/08/2012	03/01/2013	90.4	46.09	46.24		10.3	27
Car HcB	28/08/2012	03/01/2013	93.3	45.01	47.21		10.4	27
Car HsfA	28/08/2012	28/08/2012						
Car HsfB	28/08/2012	28/08/2012						
Car HsfA	28/08/2012	13/11/2012	88.8	92.28	40.15		12.2	0

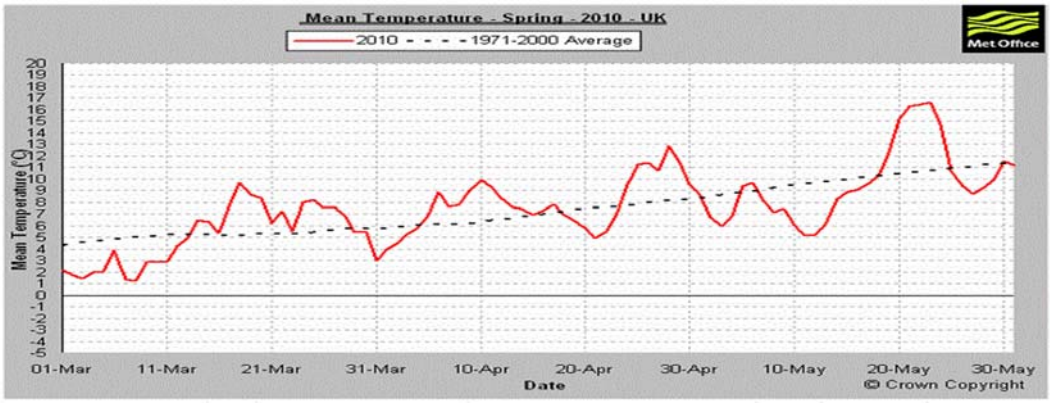
Car HsfB	28/08/2012	13/11/2012	90.7	86.01	41.67		12.1	0
Car HsfA	28/08/2012	12/12/2012	90.9	79.73	46.69		11.4	0
Car HsfB	28/08/2012	12/12/2012	90.7	74.93	47.29		11.6	3
Car HsfA	28/08/2012	03/01/2013	89.4	67.96	44.70		11.2	13
Car HsfB	28/08/2012	03/01/2013	87.6	73.55	50.62		11.2	13

Sample	Picking Date	Sampling Date	Ave Size (mm)	Ave firmness (N)	Ave Colour (b value)	Ave Ethylene at Picking (MµL/Sec)	Brix %	BP %
New P1A	13/08/2012	13/08/2012	87.5	106.41	41.31	19.4	9	0
New P1B	13/08/2012	13/08/2012	86.0	106.90	40.62	20.8	9.2	0
New P1A	13/08/2012	13/11/2012	88.9	54.04	43.49		12.8	0
New P1B	13/08/2012	13/11/2012	85.4	58.06	40.71		12.4	10
New P1A	13/08/2012	12/12/2012	90.7	48.45	45.28		10.7	30
New P1B	13/08/2012	12/12/2012	87.6	50.80	44.97		10.5	10
New P1A	13/08/2012	03/01/2013	84.1	49.23	48.48		10.8	27
New P1B	13/08/2012	03/01/2013	84.3	52.37	48.42		10.4	23
New P2A	20/08/2012	20/08/2012	92.1	103.95	42.21	42.5	9.5	0
New P2B	20/08/2012	20/08/2012	90.7	105.62	42.45	16.1	9.7	0
New P2A	20/08/2012	13/11/2012	89.7	55.61	43.10		12.1	27
New P2B	20/08/2012	13/11/2012	92.8	49.43	45.15		12.3	10
New P2A	20/08/2012	12/12/2012	91.7	48.94	46.41		11.2	27
New P2B	20/08/2012	12/12/2012	94.0	49.62	44.38		10.9	27
New P2A	20/08/2012	03/01/2013	91.9	47.47	46.53		10.5	37
New P2B	20/08/2012	03/01/2013	93.8	47.37	47.24		10.4	27
New HcA	28/08/2012	28/08/2012	98.1	95.91	41.77	515.5	9.7	0
New HcB	28/08/2012	28/08/2012	97.9	99.05	41.62	433.3	9.6	0
New HcA	28/08/2012	13/11/2012	94.2	53.94	42.59		12.5	40
New HcB	28/08/2012	13/11/2012	98.3	57.57	42.25		12.4	17
New HcA	28/08/2012	12/12/2012	95.5	48.15	44.58		11.4	27
New HcB	28/08/2012	12/12/2012	97.3	50.51	45.47		11.2	37
New HcA	28/08/2012	03/01/2013	95.1	46.68	45.02		10.2	37
New HcB	28/08/2012	03/01/2013	94.7	43.84	47.13		10.5	40
New HsfA	28/08/2012	28/08/2012						

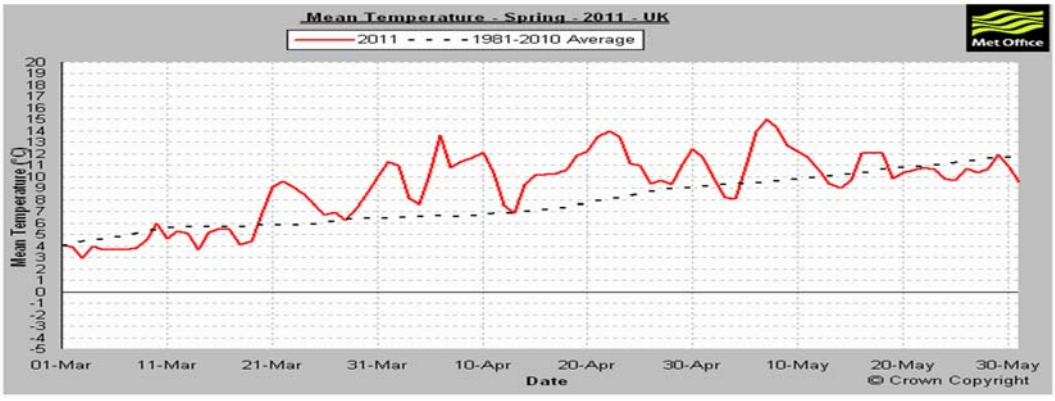
New HsfB	28/08/2012	28/08/2012						
New HsfA	28/08/2012	13/11/2012	96.1	91.79	44.11		13.4	0
New HsfB	28/08/2012	13/11/2012	94.0	89.34	43.66		12.9	7
New HsfA	28/08/2012	12/12/2012	93.0	81.30	44.65		11.8	7
New HsfB	28/08/2012	12/12/2012	95.0	79.93	46.61		11.8	3
New HsfA	28/08/2012	03/01/2013	94.2	70.81	49.88		10.9	0
New HsfB	28/08/2012	03/01/2013	94.1	66.49	48.79		11.7	10

Appendix V (a): Comparison of temperature changes in spring (March, April and May) 2010 to 2013: A) 2010, B) 2011, C) 2012 and D) 2013. (Source: Met Office, 2015)

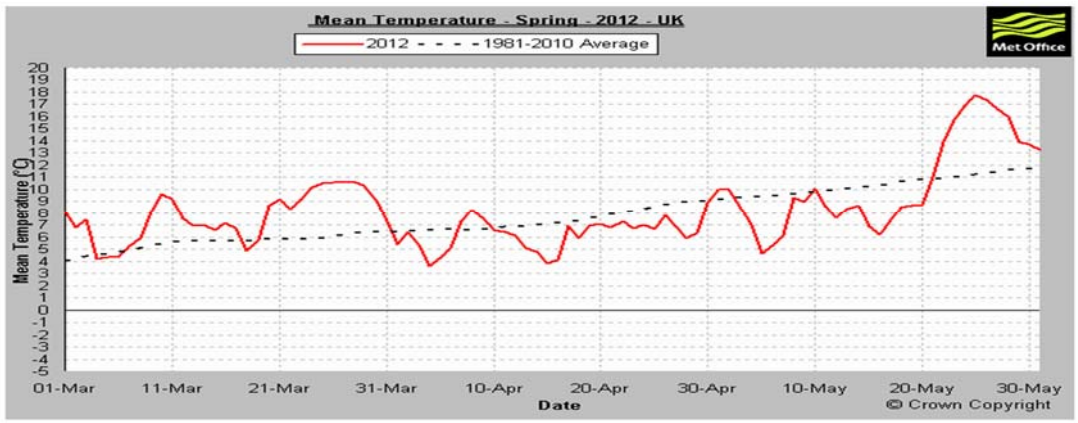
A



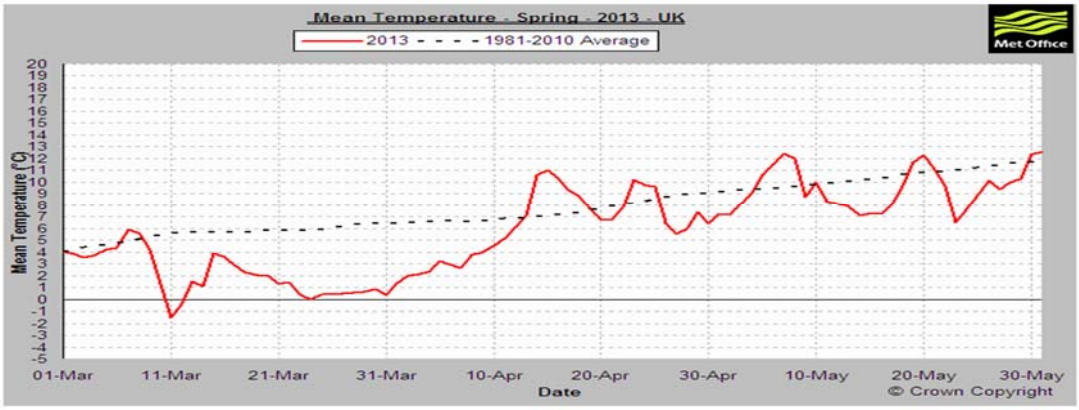
B



C

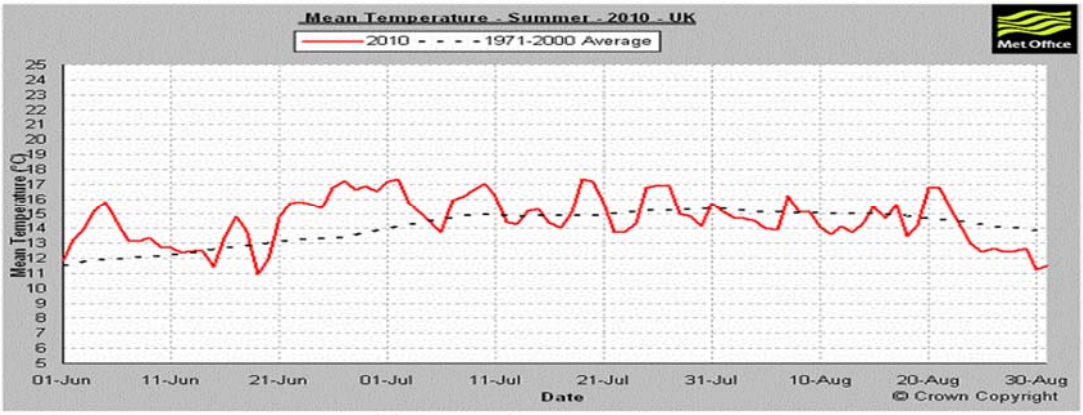


D

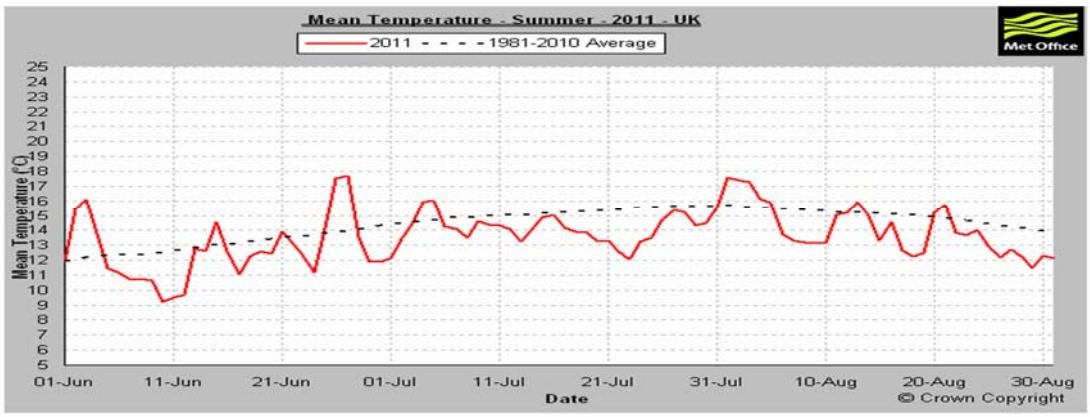


Appendix V (b): Comparison of temperature changes in summer (Jun, July and August) 2010 to 2013: A) 2010, B) 2011, C) 2012 and D) 2013. (Source: Met Office, 2015)

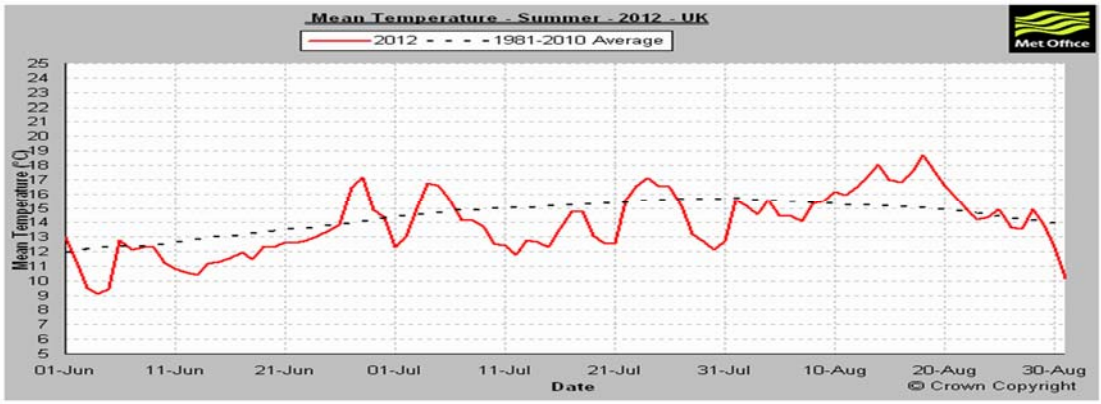
A



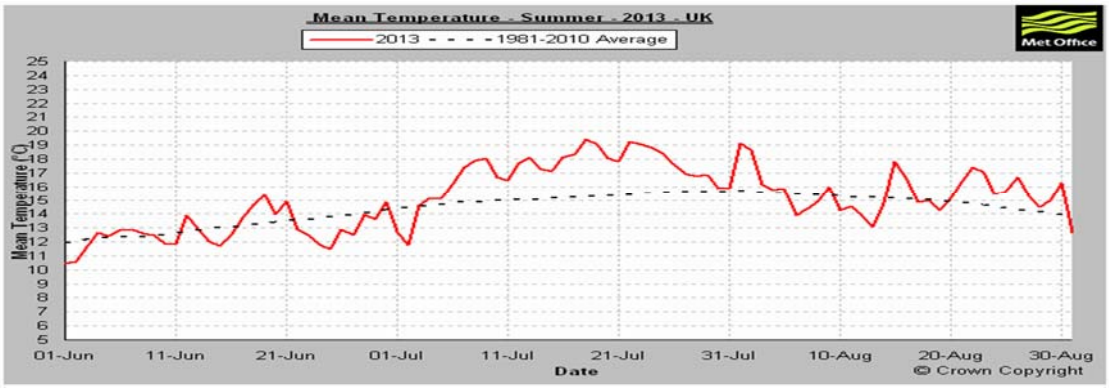
B



C



D



Appendix VI (a): Organic acid and sugars content of samples in (2011/12) air stored.

Each sample was taken from 10 apples.

		Acids (µg/µL)				Sugars (µg/µL)		
Orchard	BP%	Oxalic acid	malic acid	Ascorbic acid	citric acid	Fructose	Glucose	Sucrose
EMR	0	0.24	14.77	0.21	0.13	58.31	27.87	16.38
	16	0.28	15.87	0.20	0.16	71.80	30.75	27.38
	7	0.23	12.73	0.12	0.19	59.62	20.01	33.44
	37	0.23	11.25	0.10	0.11	59.88	19.02	28.64
	7	0.26	16.30	0.20	0.19	66.04	28.13	24.47
	10	0.29	15.36	0.17	0.16	73.39	28.40	27.03

	16	0.26	13.11	0.11	0.15	64.43	21.93	30.67
	20	0.23	11.38	0.10	0.15	57.81	17.94	27.48
	7	0.21	10.71	0.17	0.16	51.28	22.59	10.50
	3	0.21	9.14	0.12	0.11	50.47	21.58	11.67
	0	0.16	8.24	0.11	0.10	43.91	18.54	11.55
	20	0.22	9.59	0.12	0.13	54.72	20.95	19.38
	13	0.23	10.43	0.15	0.16	55.21	24.23	10.81
	20	0.23	10.33	0.15	0.11	55.32	23.63	14.45
	10	0.22	9.43	0.11	0.12	51.53	20.66	15.88
	13	0.23	10.83	0.14	0.15	57.45	22.90	15.24
Hoo	0	0.21	13.20	0.26	0.13	50.89	26.54	16.41
	0	0.22	13.93	0.29	0.12	52.61	27.85	17.98
	0	0.15	8.75	0.17	0.07	34.31	17.17	13.31
	0	0.21	12.63	0.25	0.15	51.26	23.24	18.37
	0	0.21	13.08	0.28	0.15	48.54	28.08	12.72
	0	0.25	15.00	0.30	0.10	56.09	29.68	19.63
	3	0.20	12.45	0.23	0.11	48.48	23.15	20.37
	0	0.22	12.31	0.19	0.12	52.60	22.29	24.02
	0	0.24	12.46	0.29	0.18	50.67	28.61	7.74
	0	0.22	12.14	0.29	0.13	52.52	28.91	8.53
	10	0.19	10.95	0.21	0.12	45.57	22.51	11.18
	7	0.23	10.88	0.24	0.12	54.15	23.57	11.03
	0	0.22	11.32	0.22	0.15	49.53	26.30	8.32
	0	0.23	12.93	0.29	0.14	55.32	29.52	11.70
	7	0.20	11.81	0.24	0.15	49.52	24.49	11.21
	0	0.20	11.24	0.26	0.13	49.23	20.59	12.50

Appendix VI (b): Organic acid content of samples in (2012/13) CA storage (9%CO_2, 12%O_2) and (5%CO_2, 1%O_2).

Each sample was taken from 10 apples.

Regime	**(9%CO_2, 12%O_2)**				**(5%CO_2, 1%O_2)**			
Acid	**NoSF**	**BP%**	**SF**	**BP%**	**NoSF**	**BP%**	**SF**	**BP%**
Oxalic acid (µg/µL)	0.22	54	0.25	8	0.22	0	0.23	0
	0.22	0	0.22	0	0.24	33	0.26	13
	0.21	43	0.24	13	0.24	0	0.24	0
	0.23	0	0.24	0	0.26	10	0.24	0

	0.23	43	0.26	14	0.22	0	0.26	0
	0.23	46	0.23	17	0.26	0	0.21	0
	0.22	23	0.23	0	0.27	3	0.25	0
	0.24	0	0.22	0	0.27	0	0.30	0
	0.25	13	0.23	3	0.23	11	0.26	0
	0.26	10	0.28	8	0.25	0	0.21	0
Malic acid (µg/µL)	11.99	54	13.17	8	13.05	0	16.23	0
	13.54	0	14.21	0	12.30	33	13.31	13
	15.98	43	14.54	13	13.36	0	11.66	0
	13.19	0	14.40	0	18.33	10	15.93	0
	17.59	43	17.27	14	13.60	0	14.67	0
	10.88	46	12.14	17	17.11	0	16.20	0
	11.64	23	9.77	0	16.08	3	12.20	0
	11.42	0	10.43	0	12.48	0	13.42	0
	14.07	13	14.33	3	14.37	11	12.46	0
	11.80	10	12.35	8	12.48	0	13.29	0
Ascorbic acid (µg/µL)	0.10	54	0.10	8	0.15	0	0.13	0
	0.16	0	0.17	0	0.17	33	0.17	13
	0.18	43	0.13	13	0.19	0	0.17	0
	0.14	0	0.15	0	0.21	10	0.21	0
	0.21	43	0.18	14	0.16	0	0.18	0
	0.17	46	0.17	17	0.28	0	0.19	0
	0.18	23	0.15	0	0.23	3	0.17	0
	0.16	0	0.14	0	0.13	0	0.16	0
	0.19	13	0.21	3	0.19	11	0.20	0
	0.16	10	0.16	8	0.20	0	0.21	0
Citric acid (µg/µL)	0.18	54	0.16	8	0.06	0	0.14	0
	0.07	0	0.10	0	0.15	33	0.15	13
	0.22	43	0.19	13	0.10	0	0.13	0
	0.12	0	0.13	0	0.14	10	0.13	0
	0.18	43	0.18	14	0.13	0	0.13	0
	0.09	46	0.11	17	0.15	0	0.15	0
	0.12	23	0.10	0	0.17	3	0.11	0
	0.10	0	0.09	0	0.13	0	0.14	0
	0.13	13	0.13	3	0.14	11	0.11	0
	0.08	10	0.13	8	0.15	0	0.14	0

Appendix VI (c): Organic acids content of samples collected from 2 orchards (EMR) and (Hoo) in (2013/14) air stored for 3 months.

Each sample was taken from tissue of calyx/stalk end of 10 apples.

	Calyx/Stalk	Date	BP%	Oxalic Acid (µg/µL)	Malic acid (µg/µL)	Ascorbic acid (µg/µL)	Citric acid (µg/µL)
EMR (NoSF)	Calyx	02/09/2013	0	0.37	22.11	0.38	0.00
	Stalk	02/09/2013	0	0.42	18.67	0.32	0.09
	Calyx	01/10/2013	7	0.58	29.42	0.51	0.21
	Stalk	01/10/2013	7	0.48	23.85	0.41	0.26
	Calyx	15/10/2013	13	0.30	18.62	0.32	0.00
	Stalk	15/10/2013	13	0.43	15.69	0.27	0.11
	Calyx	01/11/2013	23	0.42	20.14	0.35	0.12
	Stalk	01/11/2013	23	0.44	19.16	0.33	0.11
	Calyx	15/11/2013	53	0.02	0.70	0.01	0.00
	Stalk	15/11/2013	53	0.36	16.16	0.28	0.10
EMR (SF)	Calyx	01/10/2013	0	0.41	20.11	0.35	0.20
	Stalk	01/10/2013	0	0.20	20.05	0.35	0.26
	Calyx	15/10/2013	7	0.43	18.17	0.31	0.09
	Stalk	15/10/2013	7	0.48	18.19	0.31	0.11
	Calyx	01/11/2013	20	0.37	16.67	0.29	1.02
	Stalk	01/11/2013	20	0.05	4.92	0.08	0.00
	Calyx	15/11/2013	0	0.40	16.81	0.29	0.08
	Stalk	15/11/2013	0	0.49	18.33	0.32	0.11
Hoo (NoSF)	Calyx	02/09/2013	0	0.26	21.42	0.37	0.14
	Stalk	02/09/2013	0	0.37	23.07	0.40	0.20
	Calyx	01/10/2013	0	0.39	22.06	0.38	0.25
	Stalk	01/10/2013	0	0.37	18.44	0.32	0.11
	Calyx	15/10/2013	0	0.38	21.30	0.37	0.14
	Stalk	15/10/2013	0	0.30	21.18	0.37	0.20
	Calyx	01/11/2013	0	0.42	23.04	0.40	0.12
	Stalk	01/11/2013	0	0.19	20.03	0.35	0.84
	Calyx	15/11/2013	0	0.22	17.99	0.31	0.08
	Stalk	15/11/2013	0	0.34	19.59	0.34	0.13
Hoo (SF)	Calyx	01/10/2013	0	0.39	20.50	0.35	0.26
	Stalk	01/10/2013	0	0.41	22.93	0.40	0.23
	Calyx	15/10/2013	0	0.27	16.83	0.29	0.09
	Stalk	15/10/2013	0	0.40	19.41	0.34	0.12
	Calyx	01/11/2013	0	0.36	18.48	0.32	0.08
	Stalk	01/11/2013	0	0.02	3.12	0.05	0.00
	Calyx	15/11/2013	0	0.32	16.92	0.29	0.08
	Stalk	15/11/2013	0	0.16	18.77	0.32	0.32

Appendix VII: Multiple regression analysis for the influence of mineral constituents Ca^{2+}, P, N, K, Mg, B, Zn on the incidence of bitter pit of selected samples.

Samples were collected from CA storage (9%CO_2, 12%O_2) in season 2011/12.

Regression Statistics	
Multiple R	0.98
R Square	0.96
Adjusted R Square	0.91
Standard Error	2.09
Observations	16

ANOVA

	df	***SS***	***MS***	***F***	***Significance F***
Regression	9	663.58	73.73	16.90	0.001
Residual	6	26.17	4.36		
Total	15	689.75			

	Coefficients	***Standard Error***	***t Stat***	***P-value***	***Lower 95%***
Intercept	-10.63	16.74	-0.64	0.549	-51.58
Ca^{2+}	-8.08	2.63	-3.07	0.022	-14.52
P	-1.63	0.88	-1.87	0.111	-3.78
N	1.77	0.28	6.40	0.001	1.09
K	-0.37	0.12	-2.99	0.024	-0.67
Mg	5.99	2.13	2.82	0.030	0.79
B	-18.98	59.01	-0.32	0.759	-163.38
Zn	24.82	71.69	0.35	0.741	-150.61
K+Mg/Ca	1.55	0.80	1.93	0.103	-0.42
N/Ca	-3.87	1.38	-2.82	0.030	-7.24

Appendix VIII (a): List of samples in 2011-12 air stored which were used for mineral analysis (30 samples).

Each sample was taken from inner/outer cortex of 10 apples.

Sample	SF/ NoSF	In/ Out	Sampling Date	BP%	Ca^{2+} (total) mg/ 100g	Ca $(COO)_2$ mg/ 100g	Mg mg/ 100g	K mg/ 100g	B Mg/ 100g
EMR	-	In	05/09/2011	0	11.91	2.48	6.59	132.80	0.09
EMR	-	Out	05/09/2011	0	5.81	1.48	3.52	75.87	0.00
EMR	-	In	10/11/2011	37	9.87	2.15	6.36	143.80	0.04
EMR	-	Out	10/11/2011	37	4.00	1.20	4.95	82.59	0.00
Hoo	-	In	05/09/2011	0	10.10	1.53	6.82	146.20	0.07
Hoo	-	Out	05/09/2011	0	5.42	0.70	4.70	89.94	0.01
Hoo	-	In	10/11/2011	0	5.80	3.43	0.00	0.00	0.00
Hoo	-	Out	10/11/2011	0	7.14	1.95	5.58	83.83	0.04
EMR	-	In	28/08/2012	0	9.21	2.10	6.30	156.90	0.26
EMR	-	Out	28/08/2012	0	3.00	1.25	4.12	107.30	0.09
EMR	-	In	13/11/2012	47	8.82	2.33	6.41	208.30	0.33
EMR	-	Out	13/11/2012	47	2.99	1.15	5.00	124.60	0.10
EMR	-	In	12/12/2012	53	5.62	1.55	5.00	181.30	0.31
EMR	-	Out	12/12/2012	53	2.85	1.30	4.99	116.00	0.09
EMR	+	In	13/11/2012	0	8.07	2.23	5.92	188.20	0.18
EMR	+	Out	13/11/2012	0	3.75	1.25	4.04	101.80	0.02
EMR	+	In	12/12/2012	17	6.29	1.85	6.42	190.50	0.20
EMR	+	Out	12/12/2012	17	3.59	0.98	4.62	107.70	0.04
Hoo	-	In	28/08/2012	0	9.58	3.33	5.99	135.50	0.82
Hoo	-	Out	28/08/2012	0	3.74	1.68	3.87	109.10	0.55
Hoo	-	In	13/11/2012	7	6.48	2.28	5.32	174.90	0.93
Hoo	-	Out	13/11/2012	7	3.11	1.30	3.47	95.86	0.47
Hoo	-	In	12/12/2012	17	4.47	1.60	4.74	128.60	0.56
Hoo	-	Out	12/12/2012	17	3.90	1.70	4.57	108.50	0.47
Hoo	-	In	03/01/2013	50	4.95	1.35	5.19	157.40	0.61
Hoo	-	Out	03/01/2013	50	3.61	1.60	5.28	11.79	0.40
Hoo	-	In	13/11/2012	0	6.35	2.48	4.78	159.00	0.66

Hoo	-	Out	13/11/2012	0	3.59	1.55	4.70	111.90	0.38
Hoo	-	In	12/12/2012	0	4.06	1.95	3.93	144.80	0.52
Hoo	-	Out	12/12/2012	0	3.78	1.75	4.54	115.60	0.44

Appendix VIII (b): List of samples in 2011-12 air stored which were used for mineral analysis (30 samples).

Each sample was taken from tissue of calyx/stalk end of 10 apples.

Sample	SF/ NoSF	Calyx/ Stalk	Sampling Date	BP%	Ca^{2+} (total) mg/ 100g	$Ca(COO)_2$ mg/ 100g	Mg mg/ 100g	K mg/ 100g	B Mg/ 100 g
EMR	NoSF	Calyx	02/09/2013	0	4.04	1.40	3.38	102.50	0.00
EMR	NoSF	Stalk	02/09/2013	0	4.36	1.13	2.90	120.30	0.00
EMR	NoSF	Calyx	01/10/2013	7	3.21	1.00	2.96	111.00	0.00
EMR	NoSF	Stalk	01/10/2013	7	5.63	1.08	2.55	110.60	0.00
EMR	NoSF	Calyx	15/10/2013	13	3.44	1.28	5.48	139.00	0.04
EMR	NoSF	Stalk	15/10/2013	13	2.85	1.20	2.56	99.27	0.00
EMR	NoSF	Calyx	01/11/2013	23	2.55	1.10	3.55	118.40	0.00
EMR	NoSF	Stalk	01/11/2013	23	2.60	1.20	2.44	93.54	0.00
EMR	SF	Calyx	01/11/2013	20	2.66	0.98	3.39	113.90	0.00
EMR	SF	Stalk	01/11/2013	20	2.73	1.18	2.39	109.80	0.00

EMR	NoSF	Calyx	15/11/2013	53	2.87	1.85	3.94	98.64	0.00
EMR	NoSF	Stalk	15/11/2013	53	2.60	1.93	3.12	96.26	0.00
Hoo	NoSF	Calyx	02/09/2013	0	3.49	1.15	2.80	84.55	0.02
Hoo	NoSF	Stalk	02/09/2013	0	4.41	1.30	3.34	119.10	0.14
Hoo	NoSF	Calyx	01/10/2013	0	4.46	1.08	3.99	103.30	0.06
Hoo	NoSF	Stalk	01/10/2013	0	4.25	1.28	2.92	98.75	0.11
Hoo	NoSF	Calyx	15/10/2013	0	3.91	1.18	3.44	98.39	0.05
Hoo	NoSF	Stalk	15/10/2013	0	4.40	1.23	3.35	131.60	0.14
Hoo	NoSF	Calyx	01/11/2013	0	4.61	1.30	3.23	95.59	0.14
Hoo	NoSF	Stalk	01/11/2013	0	4.74	1.18	3.04	124.00	0.17
Hoo	NoSF	Calyx	01/11/2013	7	3.31	1.10	3.36	97.92	0.16
Hoo	NoSF	Stalk	01/11/2013	7	4.29	1.48	3.53	119.50	0.19
Hoo	SF	Calyx	01/11/2013	0	3.71	1.05	3.41	86.82	0.04
Hoo	SF	Stalk	01/11/2013	0	4.28	1.23	2.99	105.30	0.09
Hoo	NoSF	Calyx	15/11/2013	0	3.64	1.48	2.87	75.46	0.05
Hoo	NoSF	Stalk	15/11/2013	0	5.98	1.55	2.91	129.20	0.27

Fouray *	SF	Calyx	20/08/201 3	0	5.16	1.05	2.84	59.73	0.00
Fouray *	SF	Stalk	20/08/201 3	0	6.04	1.68	2.46	64.10	0.00
Fouray *	SF	Calyx	20/08/201 3	50	7.81	2.33	2.84	57.19	0.00
Fouray *	SF	Stalk	20/08/201 3	50	4.17	1.30	2.16	56.84	0.00

*****Samples (Fouray) were stored in CA (5%CO2, 1%O2) and samples collected after 10 months storage to compare pitted tissue with symptomless tissue.

Appendix IX: Multiple regression analysis for the influence of mineral constituents Ca^{2+} total, Ca^{2+} oxalate, Mg, K and B on the incidence of bitter pit of selected samples and incidence of bitter pit.

Samples were collected during air storage (4-4.5°C) in season 2013/14.

Regression Statistics	
Multiple R	0.89
R Square	0.81
Adjusted R Square	0.59
Standard Error	9.69
Observations	15

ANOVA

	df	***SS***	***MS***	***F***	***Significance F***
Regression	6	3559.48	593.25	6.31	0.01

Residual	9	845.52	93.95	
Total	15	4405		

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*
Ca^{2+} (total)	18.92	8.57	2.21	0.048	-0.47
Mg	10.68	6.54	1.63	0.136	-4.11
K	-1.25	0.38	-3.31	0.009	-2.11
B	-39.35	57.35	-0.69	0.509	-169.10
(K+Mg)/Ca	2.34	0.73	3.19	0.011	0.68

Appendix X: The study on chlorophyll flurescence and the results of season 2012/13 were presented at "V International postharvest unlimited conference" and published in Acta Horticulture vol. 1079 (2015) p: 235-242

Diagnosing Bitter Pit in Apple during Storage by Chlorophyll Fluorescence as a Non-Destructive Tool

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Keywords: 'Bramley's Seedling' apple, CA storage, 1-MCP, bitter pit, chlorophyll fluorescence characteristics

Abstract

Bitter pit is an important physiological disorder of apple that can develop on the tree but is most prevalent during storage. Delaying fruit maturation after harvest through controlled atmosphere storage and application of 1-MCP (Smartfresh^{SM}) can delay the onset of symptoms; however, significant losses may occur in long-term stored apples. It is hard to detect internal bitter pit using external examination alone. Current predictive methods are based on destructive internal quality assessments and mineral analysis. A non-destructive method to detect and predict the propensity of fruit to develop bitter pit at harvest and during the early stages of storage would improve store management practices. High risk 'Bramley' orchards were

identified from over 100 orchards surveyed across the south east of UK in 2010/11. A subsample of susceptible orchards with fruit that consistently developed bitter pit in storage as well as orchards where fruit remained free from problems were selected. Trials were conducted over two consecutive seasons (2012/13 and 2013/14) to evaluate changes of chlorophyll fluorescence in SmartFresh^{TM} treated and untreated 'Bramley' apples during storage. The relationship between chlorophyll fluorescence profiles and bitter pit incidence were similar in the presence or absence of SmartFresh^{SM}. In the early stages of storage, the chlorophyll fluorescence profiles were able to distinguish between SmartFresh^{SM} treated and untreated samples and correlated well with fruit maturity, rate of ethylene production and onset of bitter pit; however, they were less discriminatory in determining the severity of bitter pit in long-term storage. The most discriminant chlorophyll fluorescence characteristics correlated to bitter pit were identified. Models have been developed to predict the likely incidence and development of bitter pit during storage, although they are not capable of estimating severity of the disorder.

INTRODUCTION

'Bramley's Seedling' apple is a triploid cultivar used specifically for cooking and processing and is susceptible to incidence of bitter pit (Saure, 1996) which is a physiological disorder associated with low concentrations of calcium in fruit cortex. It is one of the most important physiological disorders in apples (Jackson, 2005). Bitter pit is caused by the breakdown of cellular integrity forming internal pits varying 2-10 mm in diameter which develop as corky lesions in the cortex which are not always externally visible (Ferguson and Watkins, 1989). It would be advantageous to apply a non-destructive technique to aid estimation of the optimum maturity for harvesting 'Bramley' apples and to monitor quality changes during storage to predict incidence of bitter pit.

The development of point source chlorophyll fluorescence techniques for monitoring photosynthetic events has been used to correlate rates of photosynthesis and maturity and physiological disorders during fruit storage (DeEll and Toivonen, 2000; Ross, 2002).

A number of chlorophyll parameters have been used to correlate photosynthetic activity and physiological developments. "Fo" is the initial fluorescence level, detected within nanoseconds depending on the rate of illumination and response of the fluorimeter detector. The "Fm" parameter is the maximum chlorophyll fluorescence value obtained for a continuous light intensity. The "Fv" parameter indicates the variable component of the recording and relates to the maximum capacity for photochemical quenching (Schreiber et al., 1986). The

ratio of Fv/Fm is a parameter widely used to indicate the maximum quantum efficiency of photosystem II. It is widely considered to provide a sensitive indication of plant photosynthetic performance with healthy samples typically achieving a maximum Fv/Fm value of approximately 0.85. Plants under biotic or abiotic stress have a reduced capacity for photochemical quenching within PSII and typically have Fv/Fm ratio lower than 0.85 (Strasser et al., 2000).

Models describing photosynthetic system function of the fluorescence characteristics to specific physiological aspects of chloroplasts have been developed (Krause and Weis, 1991; Strasser et al., 2000). Kalaji and Guo (2008) found changes in fluorescence characteristics were an indicator of plant stress factors that limited photosynthetic performance. For example, increased energy dissipation via chlorophyll fluorescence increases is caused by a decrease in dissipation via photosynthesis. Schmitz-Eiberger et al. (2001) identified a relationship between the chlorophyll fluorescence parameters Fm and Fv/Fm and calcium deficiency; chlorophyll content dropped under low calcium conditions, while the anti-oxidative capacity increased. The decomposition of chlorophyll in the skin has a relationship with ripening and calcium deficiency. Obaid et al. (1996) applied Fv/Fm to predict maturity in apple. However Rees et al. (2005) observed that Fv/Fm was not a robust indicator of physiological damage during long term and short term storage. They found RC/CS (Reaction Centre Density) decreased during fruit maturation and correlated well with the decline in starch. Ross (2002) found that the ability of tissues to re-synthesise PSII, as indicated by Fv/Fm recovery on the removal of stress was a more reliable indicator of tissue damage. Chlorophyll fluorescence imaging has been used as a non-destructive method to predict with up to 75% accuracy the incidence of bitter pit in apple (Lotze et al., 2006; Valcke, 2011).

The main objective of this research was to develop a non-destructive tool by measuring chlorophyll fluorescence to optimise harvest maturity of fruit and to predict the incidence of bitter pit during storage.

MATERIALS AND METHODS

To identify high risk orchards that were susceptible to bitter pit, over 100 orchards of ‘Bramley’s Seedling’ in South East of England were sampled over a five-week period from the end of August until the end of September 2010. Apples were stored in two storage regimes: (9% CO_2, 12% O_2) and (5% CO_2 and 1% O_2) and kept at 4-4.5°C. Apples were either treated

at harvest with 1 µl L^{-1} 1-MCP (SmartFresh™, SF) or left untreated.

Sample collections continued in season (2011/12) from 40 orchards, and in the last two seasons (2012/13) and (2013/14), two orchards that had consistently produced fruit with a high propensity to develop bitter pit in store were selected alongside two orchards where fruit remained free from disorders. In addition to samples stored in commercial controlled atmosphere (CA) stores, samples were stored in air (21% O_2) at the Produce Quality Centre (PQC), East Malling Research allowing for direct comparison of orchards under similar storage condition and to achieve maximum bitter pit potential of fruit. In season (2012/13) samples were harvested over four different dates covering a four-week period to capture early, optimum and late harvesting of fruits.

In each assessment 10 SF and non-SF treated fruit were assessed for chlorophyll fluorescence measurements using a PEA pocket (Hansatech Instruments, UK). All samples were dark adapted for fluorescence measurements. Hansatech software calculated fluorescence characteristics from the fluorescence yield recorded at the start of the rise (F0), after 50 µs (F1), 150 µs (F2), 300 µs (F3), 2 ms (F4), 30 ms (F5), maximum fluorescence yield (Fm), and the area above the fluorescence rise between Fo and Fm. Also destructive assessments applied by measuring fruit firmness (kgf/cm^2) (Lloyd, UK) and assessments of external and internal physiological disorders. Incidence of bitter pit was categorised in three groups as slight, moderate and severe (Fig. 1).

RESULTS AND DISCUSSION

Results showed that development of bitter pit was delayed when fruit was stored in control atmosphere with lower oxygen and treated with 1-MCP (Fig. 2). The storage regimes for ‘Bramley’s Seedling’ influenced bitter pit development; storage in 5% CO_2, 1% O_2 reduced the incidence of bitter pit over comparable samples stored in 9% CO_2, 12% O_2. 1-MCP application delays ripening and delays the development of bitter pit in ‘Bramley’ during storage and was most likely the result of slowing ethylene production and maturation of fruit during storage; these results are consistent with Watkins et al. (2000).

The results of linear discriminant analysis showed that several fluorescence characteristics can be used to model fruit maturity and the severity of internal bitter pit. It was possible to use linear discriminant analysis to distinguish between fruit suffering from a low incidence of pit and fruit where severity was greater (Fig. 3). Ten chlorophyll fluorescence

characteristics contributed to LD1 and correlating well (R>0.70) with the incidence of bitter pit were selected (Table 1).

Fm (maximum chlorophyll fluorescence yield) was compared in samples suffering from different degrees of bitter pit severity (Fig. 4). There was an inverse correlation (R= -0.81) at P<0.001 between Fm and incidence of bitter pit. Fm measured from apples treated with 1-MCP was higher than untreated samples at harvest and was maintained during storage (Fig. 5). The changes in fluorescence characteristics (Table 1) were compared individually with changes in fruit maturity and the internal quality of fruit, and in particular as to whether individual characteristics could distinguish the severity of bitter pit. The characteristics Fo (origin), F1 and F2 were better correlated with bitter pit, but the severity of bitter pit could not be estimated by this technique alone (Fig. 7).

The average of three selected parameters (Fo, F1, F2) was calculated and named as AvF (Average F). The threshold of incidence of bitter pit based on AvF was determined as 5900 (Fig. 6). Thus samples with AvF less than 5900 were more susceptible to bitter pit. Chi square analysis (Table 2) compared thresholds between 5800 to 6000 and the 5900 with bitter pit development and a significant correlation P<0.001 was found. However, the Chi square P value for >5900 was better correlated with bitter pit development.

CONCLUSIONS

The incidence of bitter pit was related to loss of fluorescence yield with the progression of fruit maturity where loss of chlorophyll content is correlated with advancement in maturity. The results showed decreasing chlorophyll fluorescence profiles (Fo to F5, Fm and Fv) of 'Bramley's Seedling' during storage. However, the rate of chlorophyll reduction between samples varied and with a higher rate of loss in chlorophyll fluorescence was associated with an increased severity of bitter pit. Results of this research found the chlorophyll profiles that best described fruits propensity to develop bitter pit were obtained by monitoring fruit during the first two months of storage, thereafter the relationship was less apparent. The underlying metabolic imbalances responsible for bitter pit development over long-periods of storage may be very subtle and not always easy to detect externally. The results confirmed the relationship between Fm and incidence of bitter pit however; AvF characteristic provided a stronger correlation with incidence of bitter pit. A threshold fluorescence AvF profile of (≤5900) was used to predict fruits' susceptibility to bitter pit, where the AvF value lower than 5900, fruits were more susceptible to bitter pit development. The AvF threshold was correlated (P<0.001)

with the incidence of bitter pit. However, these results were based on data of two seasons and further assessments are required over additional seasons to validate the model. Additional work to compare chlorophyll fluorescence parameters and bitter pit development in other cultivars are required. While results indicate that chlorophyll fluorescence can be used to predict the occurrence of bitter pit in samples of fruit sampled during storage it does not have the resolution to predict the incidence of bitter pit development in samples measured at harvest. Also it is not able to discriminate the severity of bitter pit. The use of chlorophyll fluorescence may be of utility in fruit store management where regular checks on fruit quality are required to predict the rate of fruit maturation and deterioration in the stored crop.

ACKNOWLEDGEMENTS

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Tables

Table 1. Ten fluorescence characteristics with highest correlation coefficient to incidence of bitter pit.

Fluorescence transients	Correlation coefficient (R)
F4 (fluorescence intensity at 2 ms)	-0.83
F5 (fluorescence intensity at 30 ms)	-0.83
F3 (fluorescence intensity at 300 μs)	-0.81
Fm (maximum fluorescence yield)	-0.81
Fv (variable fluorescence yield)	-0.80
F1 to F3 (fluorescence intensity between 50 to 300 μs)	-0.78
F1 (fluorescence intensity at 50 μs)	-0.77

F2 (fluorescence intensity at 150 µs)	-0.76
F1 to F4 (fluorescence intensity between 50 to 2 ms)	-0.76
Fo (minimum fluorescence yield)	-0.72

Table 2. Contingency table for chi square test of the threshold of average F<5900 for incidence of bitter pit (BP) which was significant P<0.001 for each orchard.

Actual	No BP	BP	Total
<5900	3	67	70
>5900	24	6	30
Total	27	73	100
Expected			
18.9	51.1		
8.1	21.9		
Chi square P value			
For >5900	$0.008\times(10^{-11})$		
For >5800	$2.3\times(10^{-11})$		
For >6000	$0.83\times(10^{-11})$		

Figures

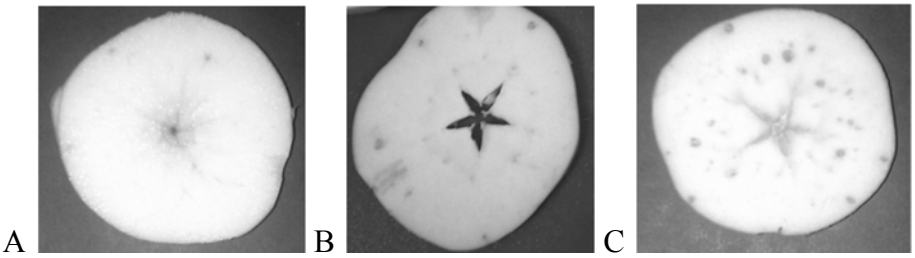
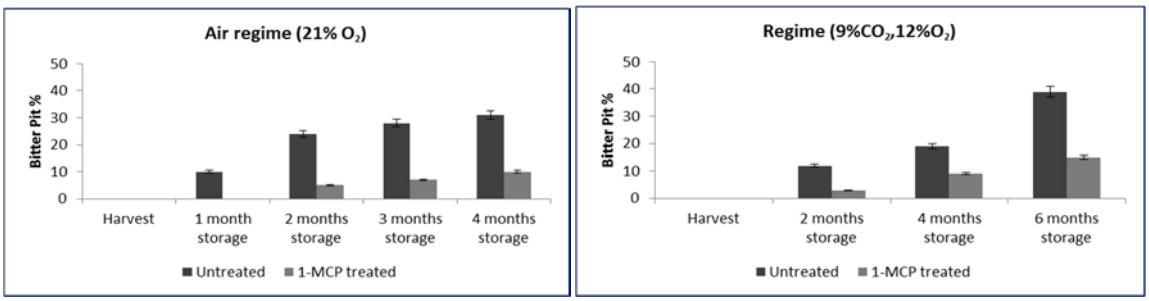


Fig.1. Classifying severity of incidence of bitter pit in three groups: A) slight, B) moderate, C) severe.



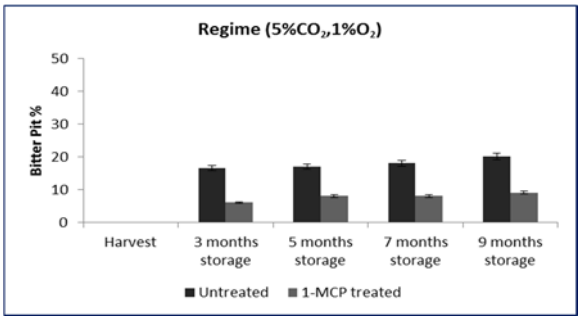


Fig.2. Comparison of incidence of bitter pit in different storage regimes between untreated samples and treated samples with 1-MCP. Each data point is the mean of 10 samples ± SE.

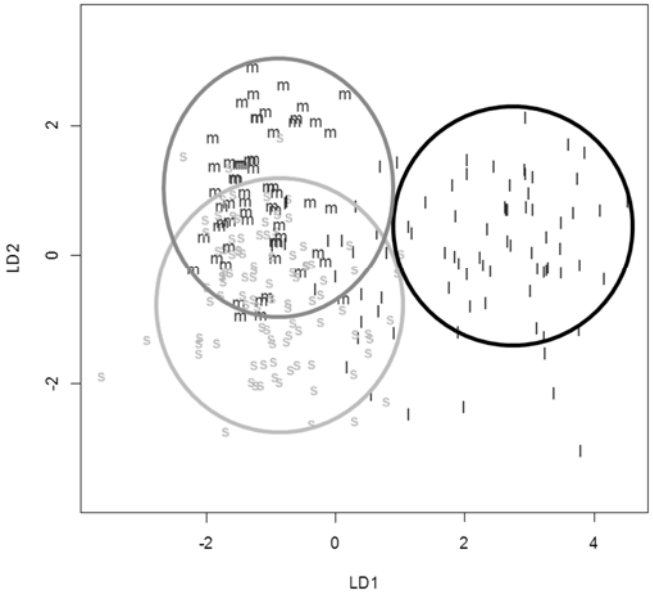


Fig.3. Discriminant analysis bitter pit severity data, while samples with slight bitter pit (l) formed a distinct cluster, where the incidence of bitter pit was classed as moderate (m) or severe (s) discriminate analysis was less able to separate severity based on chlorophyll fluorescence profiles. Each data point is the mean of a ten apple sample assessed at different stages during storage in season 2012/13.

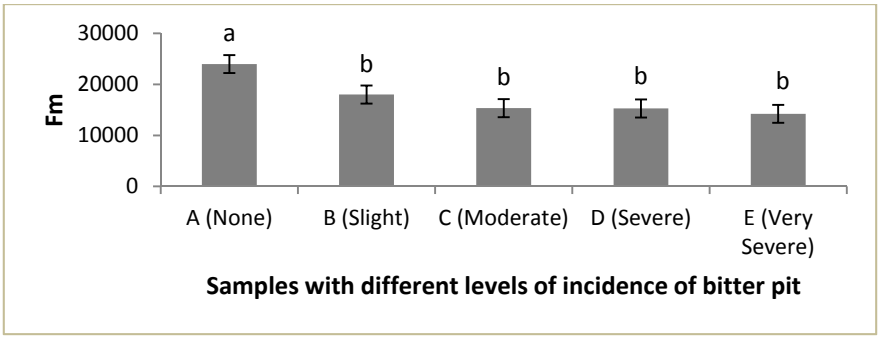


Fig.4. The relationship between Fm (fluorescence maximum) and classes of bitter pit severity in 'Bramley's' stored at 4.5°C. There was a significant inverse correlation (R=-0.81) between incidence of bitter pit (classified in 4 groups as slight, moderate, severe and very severe) and Fm. Each bar is the mean of Fm for the samples assessed at different stages during storage categorised in the same group in season 2012/13. Mean values the same letter are statistically equal according to Tukey's test ($P<0.05$).

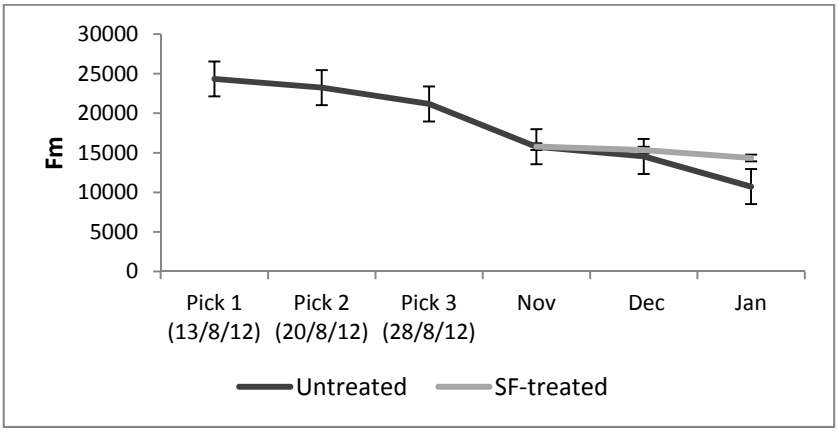


Fig.5. Comparison of fluorescence changes in SF-treated and untreated samples during different harvest dates and 5 months of storage in air (21%O_2) in season 2012/13. Each data point is the mean of ten apple samples ± SE.

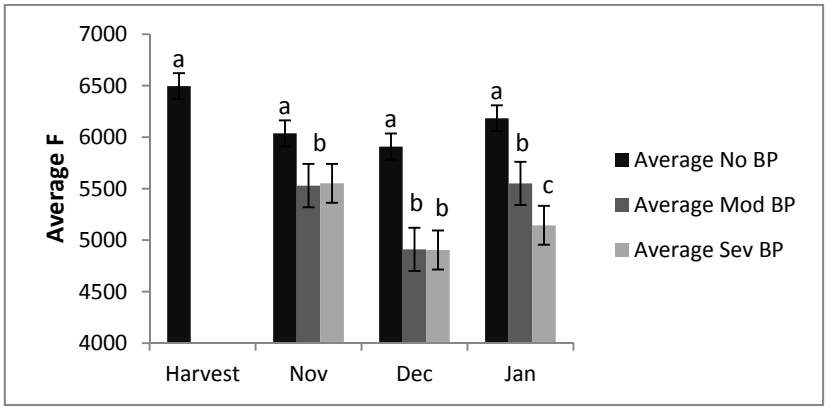


Fig.6. Comparison of changes of average of parameters (Fo, F1, F2) named as average F ($\overline{AvF}$) for different classes (severity) of bitter pit during storage time (air regime 21% O_2). Each data point is the mean of ten apple samples ± SE. Mean values the same letter are statistically equal according to Tukey's test ($P<0.05$).

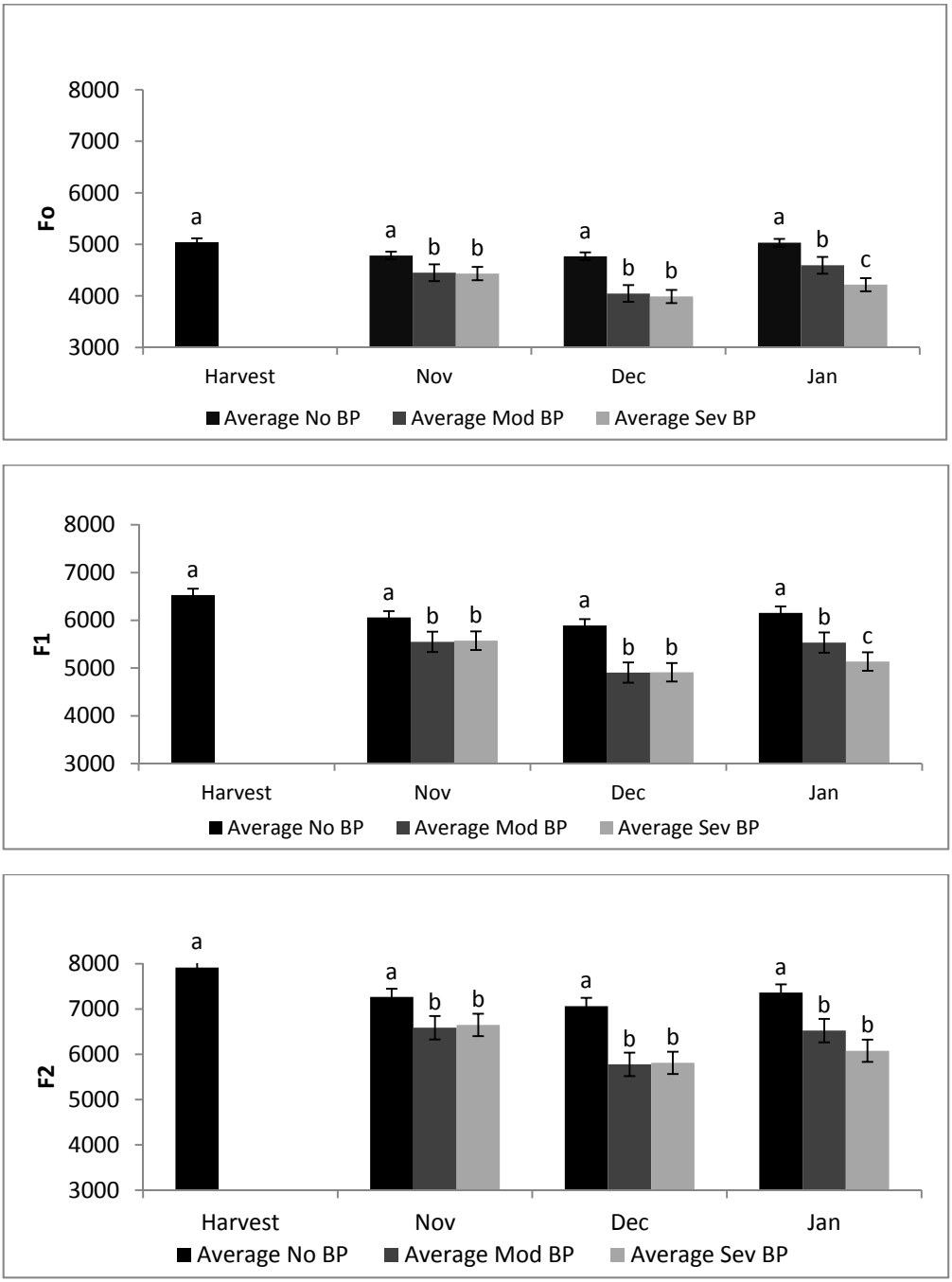


Fig.7. Comparison of changes of different characteristics (Fo, F1, F2) for different classes of bitter pit severity stored at air regime (21% O_2). Each data point is the mean of ten

apple samples ± SE. Mean values with different letters for the same assessment date were significantly different according to Tukey's test ($p<0.05$).

Appendix XI (a): Results of CT value for housekeeping primer (ITS) and primer "Calmodulin b"

Samples collected at different times of storage from one orchard (Carpenter) in season 2011/12 ($2^{-\Delta\Delta CT}$ was applied to analyze the relative changes in gene expression).

Sample	In/Out Cortex	Smart Fresh +/-	BP %	CT (ITS)	CT (Calmodulin b)	$2^{-\Delta\Delta CT}$
47 (Harvest)	In	-	0	23.21	29.58	0.80
48 (Harvest)	In	-	0	23.63	29.36	1.25
47 (Harvest)	Out	-	0	25.07	28.81	4.96
48 (Harvest)	Out	-	0	25.44	28.79	6.49
267	In	+	13	25.93	29.75	4.69
267	In	+	13	26.16	29.67	5.82
268	Out	+	3	26	28.89	8.93
268	Out	+	3	26.54	28.73	18.12
291	In	-	10	26.39	28.26	36.25
291	In	-	10	25.27	27.32	16.00
291	In	+	8	24.77	29.63	2.28
291	In	+	8	24.92	29.35	3.07
292*	Out	-	10	31.02	31.64	43.11
292*	Out	-	10	30.38	31.79	24.93
291	Out	+	8	22.55	25.17	10.78
291	Out	+	8	22.36	25.52	7.41
333 (S-life)	In	-	20	23.58	24.02	48.84
333 (S-life)	In	-	20	23.44	24.22	38.59
333 (S-life)	In	+	3	24.62	25.69	31.56
333 (S-life)	In	+	3	25.73	25.77	64.45
334 (S-life)	Out	-	20	26.21	25.58	102.54
334 (S-life)	Out	-	20	26.38	26.44	63.56
334 (S-life)	Out	+	3	26.24	26	78.25
334 (S-life)	Out	+	3	25.93	26.08	69.71

***Note:** Samples with (*) showed high level of CT value for housekeeping gene (ITS) so were eliminated from comparison. Table of average of remaining samples and comparisons are in table 6.10 and figure 6.3.

Appendix XI (b): Results of CT value for housekeeping primer (ITS) and primer "Ca^{2+} Port A" for samples collected at different times of storage from one orchard (CAR) in season 2011/12 ($2^{-\Delta\Delta CT}$ was applied to analyze the relative changes in gene expression). Table of average of samples and comparisons are in Table 6.11.

Sample	Date	In/Out	Smart Fresh +/-	BP %	CT (ITS)	CT (Ca^{2+} Prot A)	$2^{-\Delta\Delta CT}$
47 (Harvest)	30/08/2011	In	-	0	23.7	28.31	0.29
47 (Harvest)	30/08/2011	In	-	0	26.22	28.2	1.80
47 (Harvest)	30/08/2011	In	-	0	26.28	28.18	1.91
48 (Harvest)	30/08/2011	Out	-	0	25.87	29.67	0.51
48 (Harvest)	30/08/2011	Out	-	0	23.35	29.54	0.10
48 (Harvest)	30/08/2011	Out	-	0	24.37	29.76	0.17
267	25/11/2011	In	-	13	25.59	25.57	7.21
267	25/11/2011	In	-	13	24.88	25.82	3.71
267	25/11/2011	In	-	13	25.8	27.04	3.01
268	25/11/2011	Out	-	13	23.91	28.18	0.37
268	25/11/2011	Out	-	13	24.17	24.91	4.26
268	25/11/2011	Out	-	13	24	27.63	0.57
267	25/11/2011	In	+	0	23.89	20.53	73.01
267	25/11/2011	In	+	0	24.54	19.7	203.66
267	25/11/2011	In	+	0	23.89	20	105.42
268	25/11/2011	Out	+	0	25.08	24.83	8.46
268	25/11/2011	Out	+	0	25.17	24.95	8.28
268	25/11/2011	Out	+	0	25.15	25.87	4.32

Appendix XI (c): Results of CT value for housekeeping primer (ITS) and primer "Calmodulin (b)" for samples collected at different times of storage from one orchard (Pitstock) in season 2011/12 ($2^{-\Delta\Delta CT}$ was applied to analyze the relative changes in gene expression).

Sample	Date	In/Out	BP%	CT (ITS)	CT (Calmodulin b)	$2^{-\Delta\Delta CT}$
89 (Harvest)	6/09/2011	Out	0	21.04	25.74	1.38
89 (Harvest)	6/09/2011	Out	0	20.55	26.16	0.73
178	17/11/2011	In	18	22.74	32.09	0.05
178	17/11/2011	In	18	22.74	31.9	0.06
179	17/11/2011	Out	18	22.93	28.99	0.54
179	17/11/2011	Out	18	22.78	28.19	0.84
260 (S-Life)	25/11/2011	In	17	21.97	26.94	1.14
260 (S-Life)	25/11/2011	In	17	21.64	26.73	1.05
261 (S-Life)	25/11/2011	Out	17	23.32	26.78	3.25
261 (S-Life)	25/11/2011	Out	17	22.49	26.42	2.35
280	9/01/2012	In	33	20.58	22.67	8.40
280	9/01/2012	In	33	20.67	22.13	12.99
281	9/01/2012	Out	33	23.04	23.76	21.07
281	9/01/2012	Out	33	22.65	24.63	9.06
282 *	17/01/2012	In	37	27.05	35.11	0.13
282 *	17/01/2012	In	37	27.75	35.49	0.17
283 *	17/01/2012	Out	37	27.04	35.76	0.08
283 *	17/01/2012	Out	37	27.65	35.63	0.14

***Note:** Samples with (*) showed high level of CT value for housekeeping gene (ITS) so were eliminated from comparison. Table of average of remaining samples and comparisons are in Table 6.12.

Appendix XI (d): Results of CT value for housekeeping primer (ITS) and primers "Calmodulin (b)" and "Ca^{2+} Port A" for samples collected at different times of storage from two orchards (EMR) and (Hoo) in season 2013/14 ($2^{-\Delta\Delta CT}$ was applied to analyze the relative changes in gene expression).

Sample	Date	Calyx /Stalk	BP %	CT ITS	CT Ca prot A	Ca prot A($2^{-\Delta\Delta CT}$)	CT Cal (B)	Cal b ($2^{-\Delta\Delta CT}$)
EMR	(Harvest)	Calyx	0	17.89	25.73	1.15	26.86	0.52
EMR	(Harvest)	Calyx	0	17.86	24.87	2.04	26.93	0.49
EMR	(Harvest)	Calyx	0	17.45	26.72	0.43	27.13	0.32
EMR	(Harvest)	Stalk	0	17.61	26.83	0.44	27.82	0.22
EMR	(Harvest)	Stalk	0	17.43	25.99	0.70	27.91	0.18
EMR	(Harvest)	Stalk	0	17.43	27.52	0.24	27.8	0.20
EMR	15 Nov	Calyx	53	19.89	28.14	0.86	24.02	15.03
EMR	15 Nov	Calyx	53	19.79	26.99	1.79	24.11	13.18
EMR	15 Nov	Calyx	53	19.54	26.72	1.82	24.2	10.41
EMR	15 Nov	Stalk	53	21.85	27.76	4.38	25.14	26.91
EMR	15 Nov	Stalk	53	21.07	26.42	6.45	25.48	12.38
EMR	15 Nov	Stalk	53	21.01	25.02	16.34	24.91	17.63
Hoo	(Harvest)	Calyx	0	20.47	23.08	1.78	28.77	0.03
Hoo	(Harvest)	Calyx	0	18.8	23.37	0.46	29.19	0.01
Hoo	(Harvest)	Calyx	0	19.16	22.31	1.23	28.96	0.01
Hoo	(Harvest)	Stalk	0	19.66	24.38	0.41	29.54	0.01
Hoo	(Harvest)	Stalk	0	19.42	23.99	0.46	29.46	0.01
Hoo	(Harvest)	Stalk	0	19.26	23.63	0.53	29.47	0.01
Hoo *	15 Nov	Calyx	0	15.62	27.41	0.00	24.46	0.02
Hoo*	15 Nov	Calyx	0	20.32	27.91	0.06	25.23	0.36
Hoo	15 Nov	Calyx	0	20.09	26.78	0.11	24.56	0.49
Hoo	15 Nov	Stalk	0	20.59	29.4	0.02	24.62	0.67
Hoo	15 Nov	Stalk	0	19.47	28.91	0.02	24.67	0.30
Hoo	15 Nov	Stalk	0	19.88	28.65	0.02	24.94	0.33

***Note:** Samples with (*) showed high level of CT value for housekeeping gene (ITS) so were eliminated from comparison. Table of average of remaining samples and comparisons are in Table 6.14.

Appendix XI (e): Results of CT value for housekeeping primer (ITS) and primers "Calmodulin (b)" for samples collected at different times of storage and from tissues with and without symptoms of bitter pit from one orchard (JEN) in season 2010/11 ($2^{-\Delta\Delta CT}$ was applied to analyze the relative changes in gene expression).

Sample	Date	Smart Fresh +/-	NoBP/ BP	CT (ITS)	CT (Calmodulin)	$2^{-\Delta\Delta CT}$
109*	19/09/2010	-	NoBP	3.53	24.68	0.00
109	19/09/2010	-	NoBP	19.21	24.21	1.06
109	19/09/2010	-	NoBP	19.78	24.94	0.95
109*	19/09/2010	+	NoBP	27.09	29.39	6.87
109	19/09/2010	+	NoBP	18.42	23.92	0.75
109	19/09/2010	+	NoBP	19.4	25.85	0.39
323	14/12/2010	-	BP	21.36	25.25	56.02
323	14/12/2010	-	BP	20.23	26.06	47.15
323	14/12/2010	-	BP	21.31	23.79	44.43
324*	14/12/2010	-	NoBP	24.53	25.44	18.00
324	14/12/2010	-	NoBP	18.14	24.23	0.50
324	14/12/2010	-	NoBP	19.27	26.42	0.24
323*	14/12/2010	+	BP	24.27	25.6	13.45
323	14/12/2010	+	BP	18.3	24.1	0.61
323	14/12/2010	+	BP	19.3	26.51	0.23
324*	14/12/2010	+	NoBP	24.45	27.62	3.76
324	14/12/2010	+	NoBP	18.26	25.52	0.22
324	14/12/2010	+	NoBP	19.23	26.57	0.21

***Note:** Samples with (*) showed the value out of range of CT value for housekeeping gene (ITS) so they were eliminated from comparison. Table of average of remaining samples and comparisons are in Table 6.15.

Appendix XI (f): Results of CT value for housekeeping primer (ITS) and primers "Ca^{2+} Port A" for samples collected after 10 months CA storage (5%CO_2, 1%O_2) and from tissues with and without symptoms of bitter pit from one orchard (Fouray) in season 2012/13 ($2^{-\Delta\Delta CT}$ was applied to analyze the relative changes in gene expression). Table of average of samples and comparisons are in Table 6.16.

Sample	Date	Calyx/ Stalk	NoBP/ BP	CT (ITS)	CT (Ca^{2+} Protease)	$2^{-\Delta\Delta CT}$
9E	20/08/2014	Calyx	NoBP	31.94	39.27	0.42
9E	20/08/2014	Calyx	NoBP	31.42	35.9	3.05
9E	20/08/2014	Calyx	NoBP	30.99	37.45	0.77
10E	20/08/2014	Stalk	NoBP	29.81	32.91	7.94
10E	20/08/2014	Stalk	NoBP	28.64	32.94	3.46
10E	20/08/2014	Stalk	NoBP	28.54	32.69	3.84
11E	20/08/2014	Calyx	BP	25.34	31.76	0.79
11E	20/08/2014	Calyx	BP	25.77	32.78	0.53
11E	20/08/2014	Calyx	BP	25.13	32.36	0.45
12E	20/08/2014	Stalk	BP	32.33	35.91	5.69
12E	20/08/2014	Stalk	BP	34.23	34.36	62.25
12E	20/08/2014	Stalk	BP	31.71	33.23	23.75