Enhancement of aroma and flavour volatiles in apple juice

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Abstract

Aroma typical to apples develops during ripening and is comprised of a large range of volatile chemical compounds from several chemical classes. Previous research has established that exposing apples to hypoxic conditions induces changes in volatile concentrations; acetaldehyde and ethanol accumulate to high concentrations and after return to aerobic conditions ethyl esters increase and non-ethyl esters decrease. The present study investigated the effect of short term-hypoxic treatments on the enhancement of ethyl esters and decrease in non-ethyl esters with respect to: organoleptic changes in apple aroma induced by exposure to hypoxia; the influence of temperature and time at 0 °C before treatment on the magnitude of enhancement of ethyl esters after exposure to hypoxia; the effect of cultivars and ripeness stage on types and quantities of ethyl esters enhanced after exposure to hypoxia. Brief periods of hypoxia at ambient temperatures have potential for disinfestation treatments or as pre-treatments to maintain fruit quality during extended storage.

Volatile compounds were extracted from 20 mL aliquots of apple juice with an equal volume of diethyl ether:*n*-pentane (2:1 v/v), vigorously stirred for 3-5 seconds, frozen at -18 °C to separate solvent and aqueous phases, concentrated with a fast stream of oxygen free nitrogen (200 $mL \cdot min^{-1}$) to 200 µL and analysed by gas chromatography. Apple juice could be held in ice or air up to 256 minutes without loss of volatile compounds. Loss in solvent washes was 76.5% for octyl acetate and recoveries during concentration of the solvent extract ranged from 2.5% for ethyl acetate to 86.4% for *trans*-2-hexenal. Solvent extraction was simpler, faster, extracted more compounds, and had better reproducibility than dynamic headspace extracts obtained using Tenax® traps.

Nine cultivars of apples, Cox's Orange Pippin, Fuji, Golden Delicious, Granny Smith, Pacific Rose, Red Delicious, Royal Gala, Splendour and Southern Snap were exposed to 100% carbon dioxide for 24 h at 20°C. Apples exposed to hypoxia had concentrations of acetaldehdye, ethanol, ethyl acetate and ethyl esters consistently enhanced while concentrations of acetate esters and aldehydes were depressed. Maximum ethyl ester enhancement occurs within 2 to 3 d after removal from hypoxia. Exposure to hypoxia for 24 h at 20 °C did not affect rates of softening or induce physiological damage. Cultivars varied considerably in response to hypoxic treatment with Cox's Orange Pippin and Golden Delicious having the least and Fuji and Red Delicious the greatest enhancement in ethyl esters. Fruit exposed to hypoxia had larger odour unit scores than control fruit suggesting that such changes in volatile concentration may affect the aroma and/or flavour.

Fuji and Royal Gala apples were exposed to 100% CO_2 for 24 h, at 10, 15, 20 or 25 °C and maintained at treatment temperature for up to 14 d. Carbon dioxide and ethylene production and firmness were proportional to temperature but were unaffected by exposure to hypoxia. Ethyl esters were enhanced at all temperatures at differential rates according to cultivar. Apples treated and maintained at 10 °C had the greatest overall enhancement of ethyl esters and the

least decrease in other esters compared to apples at 15, 20 or 25 °C. This enhancement in volatiles persisted for up to 10 d after removal from hypoxia. Best maintenance of apple quality after treatment with hypoxia is at low temperatures suggesting that apples treated with hypoxia and maintained below 15 °C would have enhanced volatile concentration.

Noncooled Fuji and Royal Gala apples at preclimacteric to postclimacteric ripeness stages were exposed to 100% CO_2 for 24 h at 20 °C for up to 14 d. A batch of the same fruit were placed at 0 °C, removed to 20 °C and exposed to hypoxia at monthly intervals for up to 5 months. Exposure to hypoxia decreased carbon dioxide production in Fuji apples at the preclimacteric and rising climacteric stages and at the climacteric. Respiration rate, ethylene production and volatile concentration of RG apples were not affected by exposure to hypoxia at any stage of ripeness or period at 0 °C. After exposure to hypoxia Fuji apples had enhanced ethyl esters at the preclimacteric and rising climacteric stages and after being at 0 °C for up to 5 months. Volatile concentrations were lower in apples maintained at 0 °C compared to noncooled apples. Apples at 0 °C had the greatest enhancement of ethyl esters after hypoxia suggesting that exposure to low temperatures did not just slow volatile biosynthesis but had an additional effect on volatile biosynthesis.

Apple aroma consists of mainly low molecular weight esters produced by esterification of alcohol's by alcohol acyl CoA transferase (AAT) where acyl CoA's are substrates. Increased esterification activity in apples returned to air, following a hypoxic treatment, is due possibly to enhanced AAT activity or to competitive inhibition of other alcohols by ethanol. Concentrations of acetate and ethyl esters from skin disks of Cox's Orange Pippin, Fuji, Golden Delicious, Granny Smith, Pacific Rose, Red Delicious, Royal Gala, Splendour and Southern Snap apples exposed to 100% CO_2 for 24 h at 20 °C, were compared to disks from control fruit, after addition of C_2 to C_6 alcohols, either individually, or as a mixture in equimolar amounts to the disks. Ethanol added as an individual alcohol induced high ethyl acetate concentrations, but when added as part of a mixture, little ethyl acetate was produced indicating substrate preference was for longer chain alcohols. Apple cultivars had four patterns of change in ester production after exposure to hypoxia: increased acetate and ethyl esters; increased acetate esters and decreased or no change in ethyl esters; no change or decreased acetate esters and increased ethyl esters; no change or decreased acetate esters and decreased or no change in ethyl esters, implying that AAT activity is affected differentially by hypoxia. Hypoxia induces changes in capacity to produce esters which last up to 7 d indicating that pre-storage treatments using hypoxia has the potential to change the aroma profile of apples.

Juice of Fuji and Royal Gala apples exposed to a brief period of hypoxia (100% CO_2 for 24 h at 20 °C) and ripened at 20 °C for up to 8 d, was analysed by taste panels using quantitative descriptive analysis. Hypoxia induced large increases in ethyl esters including ethyl butanoate and ethyl-2-methyl butanoate in Fuji apples but not in Royal Gala apples. There was no difference in average panellist scores for sensory characteristics for Fuji and Royal Gala apples at any sampling time.

The lack of difference may have been due to large variation between panellist's assessment of sensory characteristics and/or inability to assess aroma, flavour and sweetness independently. A number of individual volatiles correlated with aroma in juice from apples exposed to hypoxia, including hexan-1-ol, butyl acetate, 2 methyl butyl acetate and propyl butanoate for Fuji; and ethanol, ethyl acetate, propyl acetate and propyl butanoate for Royal Gala. Multivariate analysis indicated that panellists associated increased ethyl esters with off flavour rather than more intense apple aroma. This could have been due to juice from apples exposed to hypoxia having a different apple-like character than control fruit which did not fit the definition of apple aroma used to train panellists.

The enhanced ethyl ester concentrations in fruit exposed to hypoxia are probably due to large increases in ethanol concentration that competitively inhibited formation of non-ethyl esters. Golden Delicious and RG did not have enhanced concentration of ethyl esters and/or decreases in acetate ester concentration even though fermentation volatiles were enhanced to high concentrations and ethyl acetate increased to concentrations similar to those found in fruit which had enhanced ethyl esters. The mechanism producing ethyl acetate and ethyl esters in GD and RG was probably different from that in CO, FU, PR, RD, SP, SS cultivars. Therefore, after exposure to hypoxia, additional factors influence changes in volatile concentration other than the increased pool of substrate available for esterification.

A possible mechanism by which hypoxia affects ester biosynthesis is that under hypoxic conditions cytoplasmic pH falls below the optimum of 7 to 8, inducing increased ADH activity and synthesis and producing large increases in ethanol concentration. Ester biosynthesis is suppressed during hypoxia leading to increased alcohol and aldehyde concentrations creating a pool of substrates that could be rapidly utilised by AAT on return to aerobic conditions. It is possible that AAT activity or concentration changes are induced by hypoxic conditions. The different capacity of apple cultivars to esterify alcohols from control and hypoxic treated fruit may be due to changes in substrate specificity of either, or both, newly induced ADH and AAT.

Exposure to hypoxia consistently caused increases in ethyl esters in several apple cultivars. The practical uses for treatments where apples are exposed to hypoxia for 24 h include: disinfestation treatments, manufacture of apple juice concentrates, enhancement of aroma in apples maintained in long term air or controlled atmosphere storage and as a tool for examining volatile biosynthesis.

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I dedicate this thesis to my son Alexander for whom this all seems worthwhile.

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List of Abbreviations

-p	without addition of precursor
+p	with addition of precursor
AAT	Alcohol acylCoA transferase
ACP	Anaerobic compensation point
ADH	Alcohol dehydrogenase
ATP	Adenosine triphosphate
C	Control
CA	Controlled atmosphere
C_2H_4	Ethylene
CoA	Coenzyme A
CO_2	Carbon dioxide
CO	Cox's Orange Pippin
d	Day
FID	Flame ionisation detector
FU	Fuji
GC-MS	Gas chromatography-mass spectroscopy
GD	Golden Delicious
GLC	Gas liquid chromatograph
GS	Granny Smith
h	Hour
LDH	Lactate dehydrogenase
LOX	Lipoxygenase
N_2	Nitrogen
NADH	Nicotinamide adenine dinucleotide-reduced
O_2	Oxygen
PDH	Pyruvate dehydrogenase
PR	Pacific Rose
QDA	Quantitative descriptive analysis
RD	Red Delicious
RG	Royal Gala
RH	Relative humidity
SP	Splendour
SPME	Solid phase microextraction
SS	Southern Snap
TCA	Tricarboxylic acid

Chapter One

Introduction

1.1 General Overview

Apples are the major horticultural crop grown in New Zealand representing 62% of fruit exported worth an estimated $398 million (fob) in 1996 (Anon, 1997a). The majority (93%) of New Zealand apples are exported as fresh fruit to markets in Europe, North America and the Asia/Pacific region. Fruit which are considered unacceptable for fresh export are processed into apple juice, juice concentrates and essences for local and international markets. While the value of export sales of fresh apples have increased by 60% until 1996, there has also been over a 100% increase in sales of apple juice concentrates, from $8.6 million in 1993/4 to $37 million in 1995/6 (Anon, 1997a). Plantings of apples have continued to increase from 13,500 ha in 1993/4 to 15,500 ha in 1997/8 which is expected to increase the crop available for processing from 143,000 tonnes in 1993 to 213,000 tonnes in 1998 (Frucor Processors, pers. comm.). Processed apple products from New Zealand represent about 4% of the annual world consumption but face increasing competition from apple processors in South America, South Africa and Eastern Europe (Frucor Processors pers. comm.). To continue to be successful in the world market New Zealand apple juice processors will need to be able to provide apple juice concentrates and essences to meet individual client specifications.

The volume of fresh apple exports from New Zealand is minor (2%) when compared to total world production, but represents a significant proportion of world trade (7%) (Steele, 1995). New Zealand apples are considered to be of premium quality in overseas markets and the New Zealand apple industry is regarded as innovative and forward thinking with the release of several new internationally important apple cultivars in the last decade. Consumers are increasingly demanding that stored apples more closely match the appearance, taste and texture of freshly harvested apples. This represents a particular challenge for New Zealand apple exporters owing to long distances to principal markets in Europe and North America. Controlled atmosphere (CA) storage has attracted considerable interest as a supplement to the more traditional air storage for better maintenance of fruit quality. One of the drawbacks of CA and long term air storage is the loss of acceptable apple flavour and aroma (Bangerth and Streif, 1987).

Until the late 1970's most research on aroma and characteristic flavours of apple fruit concentrated on identifying volatiles produced by ripening fruit (Tressl et al., 1975). Improvements in methods for separation and identification of volatile compounds, often in only trace amounts of a few parts per million, have continued until the present day. These new methods have allowed researchers to examine in more detail biosynthetic pathways and control mechanisms in the synthesis and subsequent accumulation and release of volatiles from apples. This is resulting in a greater understanding of how biochemical and environmental

factors influence aroma and flavour of apple fruit. Increased interest in non-chemical pre-treatments to preserve or improve apple fruit quality, and as disinfestation treatments, has highlighted deficiencies in our knowledge of factors which affect postharvest apple flavour development. The research reported in this thesis outlines experiments conducted to examine the response of a wide range of apple cultivars to a brief hypoxic treatment with respect to subsequent aroma/flavour volatile biosynthesis. The following literature review summarises knowledge of the composition of apple volatiles, how they are synthesised, their measurement and how postharvest factors and exposure to hypoxia together affect volatile concentration.

1.2 Apple Volatiles

Fruit aroma is a complex mixture of a large number of volatile compounds which contribute to the overall sensory quality of fruit specific to species and variety (Sanz et al., 1997). Over 300 volatile compounds have been measured in the aroma profile of apples. These compounds include alcohols, aldehydes, carboxylic esters, ketones and ethers (Dimick and Hoskin, 1983). Very few of these chemicals are considered to be 'character impact' compounds. These are compounds that have very low aroma thresholds but potent aroma characteristics considered to be typical of apple aroma/flavour (*e.g.* ethyl-2-methyl butanoate (Flath et al., 1967)), contribute to the intensity of an aroma (*e.g.* *trans*-2-hexenal) or are related to aroma quality (*e.g.* ethanol) (Dürr and Schobinger, 1981).

1.2.1 Volatile Compounds Identified in Apple Aroma

Extensive lists of volatiles extracted from apples and apple essences can be found in reviews by Dimick and Hoskin (1983) and Paillard (1990). While there is a great range of chemical compounds in the volatile profile of apples the majority are esters (78-92%) and alcohols (6-16%), the remainder are aldehydes, ketones and ethers (Paillard, 1990). The most abundant compounds are even numbered carbon chains including combinations of acetic, butanoic and hexanoic acids with ethyl, butyl and hexyl alcohols (Paillard, 1990). Higher molecular weight volatiles, often with one or two hydrophobic aliphatic chains, are likely to be trapped by skin waxes and are generally not found in the headspace (Paillard, 1990). Charm response analysis has indicated that a general description of apple aroma from processed apples would include the volatiles β-damascenone, butyl-, isoamyl- and hexyl hexanoates, ethyl-, propyl- and hexyl butanoates (Cunningham et al., 1986). Cultivar specific aroma has been proposed to result from changing proportions of hexan-1-ol, butyl acetate, hexyl acetate, butyl propionate, hexyl butanoate, propyl butanoate, butyl 2-methyl butanoate, hexyl 2-methyl butanoate, hexyl hexanoate, 4-methoxyllylbenzene, and α-farnesene (Dirinck and Schamp, 1989).

1.2.2 Apple Aroma in Different Cultivars

Most aroma compounds, in variable proportions, are always present in volatile emissions from a large range of apple cultivars but there is no key characteristic compound for any given cultivar (Cunningham et al., 1986; Paillard, 1990).

Notwithstanding this, there are large sensory differences in flavour and aroma of apple cultivars (Cunningham et al., 1986; Poll, 1981). A Danish taste panel assessed pasteurised apple juice from 18 apple cultivars after 6 months storage at 10 °C for aroma and taste according to Danish bias (Poll, 1981). Panellists indicated that cultivars with strong aroma and 'characteristic' apple taste are preferred over juices with weak aroma and 'uncharacteristic' apple taste.

Comparison of aroma profiles reported in the literature between different apple cultivars or for an individual cultivar is difficult, as the same method of aroma extraction has not been used and often the fruit tested are not at the same stage of ripeness or have not had a similar storage history (Brackmann and Streif, 1994). Different methods of volatile extraction selectively remove more of one class of chemical compounds than another. Absorbent polymers used in traps for extracting volatiles from the sample headspace preferentially extract esters over alcohols, resulting in under-estimation of alcohol concentrations. Methods which use direct extraction from homogenised samples preferentially extract alcohols and aldehydes (Table 1.1) (Fernandez and Diaz-Marta, 1997). Many extraction methods do not measure low molecular weight volatiles, such as ethanol, methanol, and ethyl acetate which have high vapour pressures. Volatile profiles without low molecular weight compounds do not represent accurately the volatile compounds emanating from fruit, as low molecular weight volatiles could potentially contribute to overall aroma.

Table 1.1 Percentage of esters, alcohols and other volatile compounds for apple cultivars.

Cultivar	Esters	Alcohols	Others	Method[1]	Fruit History	Reference
Bisbee Delicious	70.3	13.6	16.1	Purge & Trap	At harvest	Mattheis et al., 1991b
	92.5	7.4	0.1	Purge & Trap	42 wk, 1 °C	Mattheis et al., 1995
Delicious	50.4	4.0	45.6	Purge & Trap	35 wk, CA	Mattheis et al., 1991b
Fuji	81.1	6.2	12.7	Purge & Trap	At harvest	Kakiuchi et al., 1986
	11.1	75.7	13.2	Distillation	At harvest	Kakiuchi et al., 1986
Golden Delicious	96.4	1.0	2.6	Purge & Trap	At harvest	Kakiuchi et al., 1986
	29.3	48.1	22.6	Distillation	At harvest	Kakiuchi et al., 1986
	21.6	78.4	0.0	Headspace	30 wk, CA	Zerbini et al., 1996
Hatsuaki	90.6	1.5	7.9	Purge & Trap	At harvest	Kakiuchi et al., 1986
	33.0	53.3	13.7	Distillation	At harvest	Kakiuchi et al., 1986
Jonathan	93.0	3.9	3.1	Purge & Trap	At harvest	Kakiuchi et al., 1986
	16.3	69.7	14.0	Distillation	At harvest	Kakiuchi et al., 1986
Mutsu	85.2	1.6	13.2	Purge & Trap	At harvest	Kakiuchi et al., 1986
	17.4	68.8	13.8	Distillation	At harvest	Kakiuchi et al., 1986
Rome	13.8	86.2	0.0	Purge & Trap	63 wk, 0 °C	Fellman et al., 1993a
Royal Gala	29.7	69.7	0.6	Distillation	Supermarket	Young et al., 1996
Summerred	84.9	7.0	8.1	Purge & Trap	2 wk, 20 °C	Fan et al., 1997

[1] Extraction method.

Cultivars have been categorised according to type and quantity of esters or alcohols predominant in their volatile profile (Dirinck and Schamp, 1989; Paillard, 1990), aroma production pattern (Dirinck and Schamp, 1989), skin colour (Paillard, 1979), concentration of specific ester compounds (Dirinck and Schamp, 1989) or C_6 aldehydes (Paillard, 1990). Ester types of cultivars have been further categorised according to types of esters: acetate ester types (Calville blanc, Golden Delicious), butanoate ester types (Belle de Boskoop, Canada Blanc, Richared), propanoate ester types (Reinette du Mans, Richared, Starking),

and ethanolic ester types (Starking) (Paillard, 1990). Yellow skinned cultivars have been reported to produce mainly acetic acid esters and red skinned cultivars mostly butyric acid esters (Paillard, 1979). High concentrations of hexyl acetate and butyl acetate were considered to characterise Cox's Orange Pippin, Elstar, Golden Delicious, Jonagold and Jublié Delbar, while Granny Smith, Nico, Paulared, and Summerred were characterised by high concentrations of ethyl butanoate and hexan-1-ol; Boskoop and Jacques Lebel were characterised by α-farnesene and hexyl 2-methyl butanoate (Dirinck and Schamp, 1989). Concentration of C_6 aldehydes for Cox's Orange Pippin and Jonathan apples was four to five times that of Golden Delicious for hexanal and 100 fold more for *trans*-2-hexenal (Paillard, 1990). Apple cultivars also differ in concentrations of specific volatiles such as 4-methoxyllylbenzene (a spice-like aroma compound, according to an English sensory panel) which can constitute up to 0.27% of headspace volatiles in some cultivars (Williams et al., 1977).

1.3 Biogenesis of Volatiles

As volatiles are comprised of at least 8 chemical classes there are several pathways involved in volatile synthesis. These have not been fully described but appear to be common to different fruits. Flavour and aroma characteristics of apples develop during ripening after harvest (Tressl and Drawert, 1973; Tressl et al., 1975). Volatiles important for aroma and flavour are synthesised from amino acids, membrane lipids and carbohydrates (Sanz et al., 1997). In apple aroma the majority of volatiles are esters, the formation of which is dependent on availability of C_2 to C_8 acids and alcohol (De Pooter et al., 1981; Knee and Hatfield, 1981; Paillard, 1979). The location within cells of the sites of volatile biosynthesis are not known, although lipoxygenase occurs at a membrane site in tomatoes (Riley et al., 1996).

1.3.1 Fatty Acids

Fatty acids are the major precursors of aroma volatiles in most fruit (Sanz et al., 1997). The biosynthetic pathways involved include β-oxidation, hydroxyacid cleavage (leading to lactones) and lipoxygenase to form aldehydes, ketones, acids, alcohols, lactones and esters from lipids (Heath and Reineccius, 1986). Aroma volatiles in intact fruit are formed via the β-oxidation biosynthetic pathway whereas when fruit tissue is disrupted volatiles are formed via the lipoxygenase pathway (Schreier, 1984).

As apples ripen, rates of lipid synthesis and membrane fluidity increase (Bartley, 1985) as does lipoxygenase activity in disrupted tissue of riper fruit (Wooltorton et al., 1965). The pulp of Cox's Orange Pippin apples contains mostly phospho-, galacto-, steryl lipids which are composed mostly of linoleic and linolenic fatty acids (about 50% and 10 to 25% of total lipid, respectively) (Galliard, 1968). The proportion of linolenic acid in lipids of post-climacteric apples is lower than in pre-climacteric apples. Lower linolenic acid concentrations are associated with plastid structures, and result from decreased concentrations of monogalactosyl diglyceride, digalactosyl diglyceride and phosphatidal glycerol and not a change to the fatty acid distribution of individual lipids (Galliard, 1968). Decreases in

chlorophyll concentration were observed to occur with decreases in lipids, which agrees with the observation that during apple ripening chloroplasts breakdown (Galliard, 1968). Chloroplast breakdown could therefore provide the major proportion of linoleic and linolenic fatty acids for volatile biosynthesis.

1.3.2 β-Oxidation

From studies using radio-labelled substrates and precursors with pears (Jennings, 1967), bananas (Tressl and Drawert, 1973), and apples (Bartley et al., 1985; Paillard, 1979) it has been established that β-oxidation of fatty acids is the primary biosynthetic process providing alcohols and acyl CoAs for ester formation (Figure 1.1) (Sanz et al., 1997). The β-oxidation pathway is assumed to be the same as that for animals and micro-organisms and its location may be in peroxisomes (Gerhardt, 1983). Substrate feeding experiments with Golden Delicious apples using C_1 to C_6 aldehydes, or C_2 to C_6 carboxylic acid vapours (De Pooter et al., 1983; De Pooter et al., 1987), induced increases in esters typical of those expected for β-oxidation of the added compounds (De Pooter et al., 1981, 1982). Similar experiments with Cox's Orange Pippin apples, using methyl esters of short chain C_4 to C_8 fatty acids, resulted in esters with an alkyl group of C_{n-2}, C_{n-4} confirming the presence in whole fruit of an active β-oxidation pathway (Bartley et al., 1985). Perdeuterated linoleic acid fed to Red Delicious apples produced only C_6 metabolites, implying that saturated ester volatiles arise by β-oxidation, rather than peroxidation, of fatty acid precursors (Rowan et al., 1997). Substrates for ester biosynthesis may also be formed via α-oxidation (Tressl and Drawert, 1973; Rowan et al., 1997). Feeding dueterated *cis*-3-hexenal and *trans*-2-hexenal to Red Delicious apples resulted in a range of labelled volatiles, including ethyl pentanoate and pentyl acetate which could only result from α-oxidation (Rowan et al., 1997).

Fatty acid acyl-CoA derivatives are converted to shorter chain acyl-CoA's by losing two carbons in every round of the β-oxidation cycle, using the following enzymes in order: acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, acetyl-CoA acetyltransferase or thiolase (Sanz et al., 1997). These steps require the electron acceptors, FAD and NAD, as well as free coenzyme A for the scission step. The acyl-CoA's produced in this process are reduced by acyl CoA reductase to aldehyde which in turn is reduced to alcohol by alcohol dehydrogenase which is utilised by the ester forming enzyme, alcohol acylCoA transferase (AAT), to produce esters (Bartley et al., 1985). It has been proposed that varietal differences in volatile composition of apples depends on the specific activities of β-oxidation enzymes (Bartley et al.,1985), that will influence for example, the rate of transformation of butanoate to acetate (Paillard, 1979).

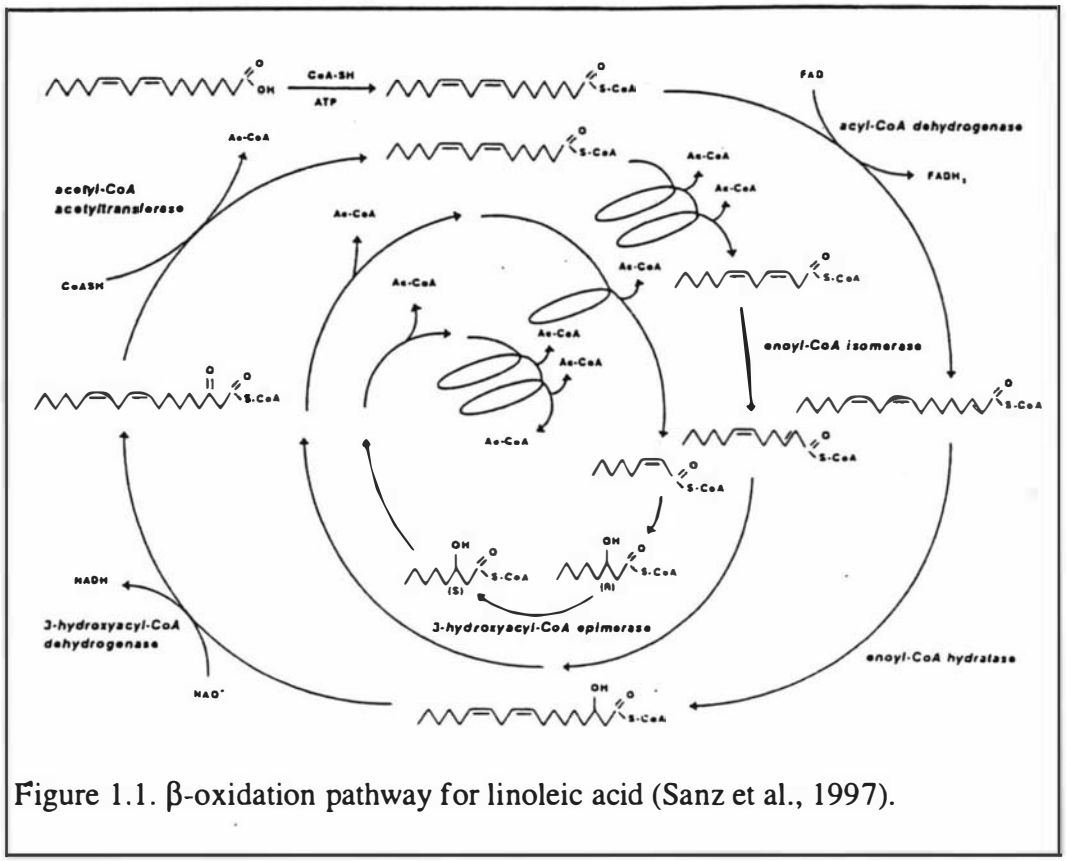

Figure 1.1. β-oxidation pathway for linoleic acid (Sanz et al., 1997).

1.3.3 Lipoxygenase (LOX)

When fruit are homogenised, linoleic and linolenic acid are oxidised to various C_6 and C_9 aldehydes (Drawert, 1975; Galliard and Matthew, 1977; Lea, 1995). These volatiles reach maximum concentration in the first 10 to 30 minutes after homogenisation (Drawert et al., 1986). Such C_6 aldehydes are responsible for the 'green' odour notes in plant aroma (Hatanaka, 1993). In intact fruit, enzymes in the LOX biosynthetic pathway and their substrates have different subcellular locations, preventing formation of volatile compounds (Sanz et al., 1997). During ripening cell walls and membranes may become more permeable, allowing the LOX pathway to become active without tissue disruption (Sanz et al., 1997). Lipoxygenase activity of Schone van Boskoop apples is greatest during the climacteric peak. Golden Delicious apples metabolise linolenic acid more readily than linoleic acid (Kim and Grosch, 1979). Golden Delicious apples treated with hexanal and hexanoic acid vapours had increased hexyl, butyl and ethyl esters (De Pooter et al., 1983). Therefore, the LOX biosynthetic pathway has the potential to provide substrates for ester production (De Pooter et al., 1983). If the LOX biosynthetic pathway was active during ripening, it would act as an alternative to β-oxidation of fatty acids. Ester formation, β-oxidation activity and LOX are suppressed by low O_2 concentration explaining why storage in CA conditions reduces volatile production in fruit. If the LOX pathway is active during apple ripening, it could account for the high hexan-1-ol production in apple disks incubated with unsaturated linoleic acid as compared to saturated stearic acid (Sanz et al., 1997). An important LOX enzyme is a membrane-bound enzyme, hydroperoxide lyase, which cleaves fatty acid hydroperoxides to

aldehydes and oxoacids (Sanz et al., 1997). Hydroperoxide lyase is divided into 9-, 13- and non-specific types which determine aldehyde composition in many plants (Sanz et al., 1997). Depending on the plant and isozymes present, oxygen can be incorporated preferentially at C-9 (tomato), C-13 (grape and apple), or at either C-9 or C-13 in a non-specific manner to convert the *cis (Z)* double bond to the *trans (E)* configuration (Feys et al., 1982; Sanz et al., 1997). Aldehydes produced by LOX are reduced by alcohol dehydrogenase (ADH) to produce alcohols for ester biosynthesis.

1.3.4 Amino Acids

Branched chain alcohols, carbonyls and esters are produced by metabolism of the amino acids valine, leucine, iso-leucine, alanine and aspartic acid (Heath and Reineccius, 1986). Volatiles are formed from amino acids in intact fruit or can also be transformed after transamination to volatiles through several metabolic pathways after cell disruption (Sanz et al., 1997). Varying concentrations of free amino acids could account for different concentrations of branched chain volatiles in fruit, for example, during ripening of banana fruit, L-leucine and L-valine increased three fold while other amino acids remained constant (Tressl and Drawert, 1973). Radio labelled amino acids are converted to branched chain alcohols and esters in post-climacteric banana slices (Table 1.2). The formation of volatiles from amino acids may involve enzymes from three biosynthetic pathways: aminotransferase, decarboxylase and ADH (Figure 1.2; Sanz et al., 1997). Iso-leucine is considered to be the biosynthetic precursor of 2-methyl butanoic acid and its esters in apples (Paillard, 1990). Deuterated iso-leucine was metabolised by Red Delicious apples to 2-methyl butan-1-ol and to 2-methyl butyl and 2-methyl-2-butenyl esters, whereas Granny Smith apples produced ethyl-2-methyl butanoate almost exclusively (Rowan et al., 1996). Different ratios of amino acid conversion to volatiles, in particular the differential rates of metabolism of leucine and iso-leucine, occur in Braeburn, Granny Smith, Fuji, Red Delicious, and Royal Gala apples (Rowan et al., 1997). This suggests that different enzyme activity and selectivity, rather than substrate availability of the amino acid degradation pathway, determines the concentration of branched chain esters for each cultivar. Presence of labelled butyl and hexyl acetate indicates that amino acids provide substrates for acetate esters via β-oxidation (Rowan et al., 1997).

Table 1.2. Volatile compounds produced by fruit tissue after addition of selected amino acid precursors.

Amino acid	Volatile	Tissue	Reference
L-leucine	3-methyl-1-butan-1-ol	banana	Tressl and Drawert, 1973
	3-methyl butyl esters		
	3-methyl butanoates		
	2-ketoisocaproate		
L-valine	2-methyl-1-propan-1-ol	banana	Tressl and Drawert, 1973
	2-methylpropyl acetate	apple	Hansen and Poll, 1993
	2-methylpropionic acid		
L-phenylalanine	phenolic esters	banana	Tressl and Drawert, 1973
	2-phenylethanol	apple	Hansen and Poll, 1993
L-isoleucine	2-methyl butan-1-ol	apple	Hansen and Poll, 1993
	2-methyl butyl esters	apple	Rowan et al., 1997

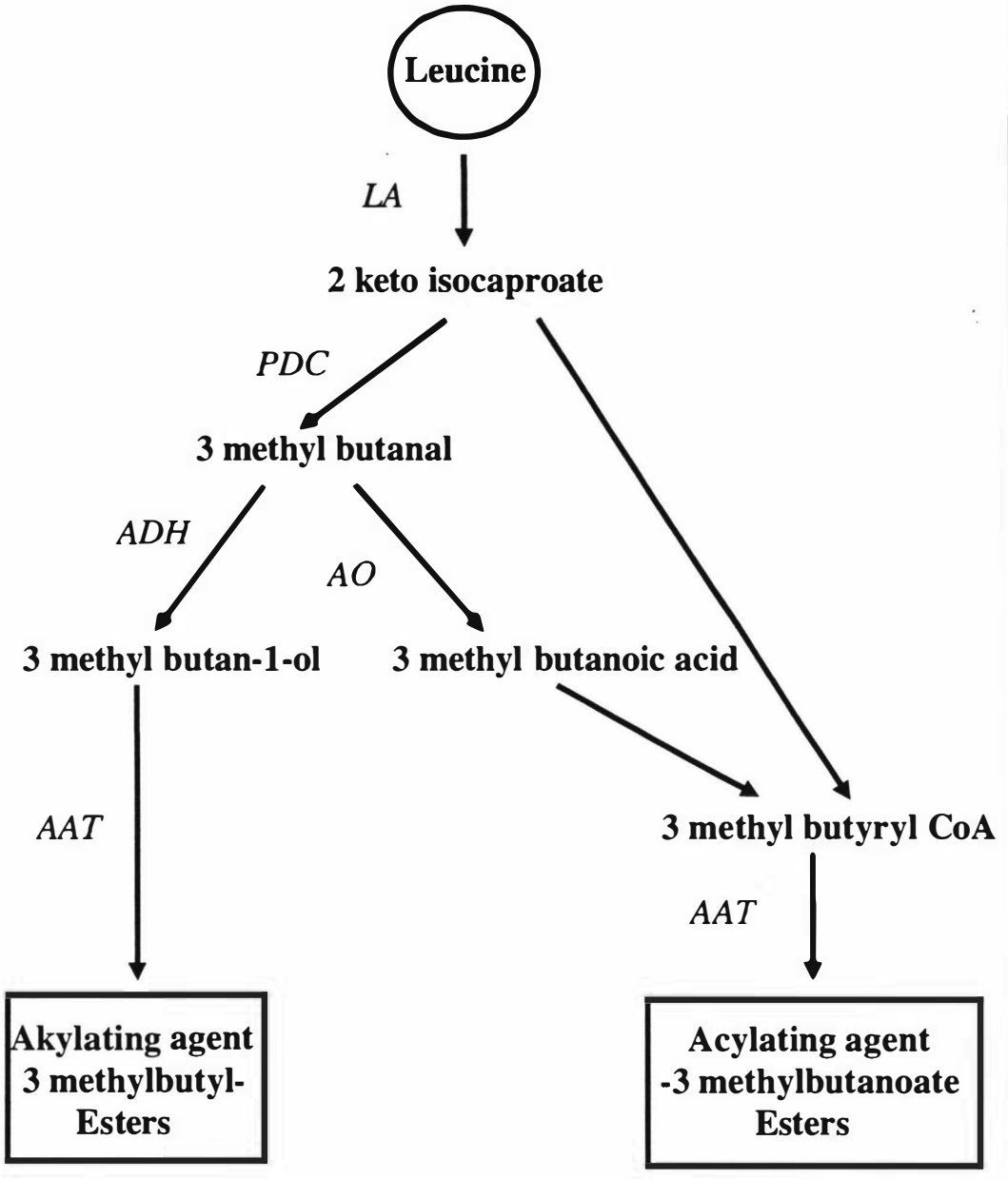

Figure 1.2 Pathway for formation of volatiles from leucine for banana (adapted from Ampun, 1997 and Sanz et al., 1997). *LA* = leucine aminotransferase, *PDC* = pyruvate decarboxylase, *ADH* = alcohol dehydrogenase, *AO* = aldehyde oxidase, *AAT* = alcohol acyl CoA transferase.

1.3.5 Aldehydes

Conversion of aldehydes to alcohols involves ADH which catalyses oxidation of alcohols and reduction of aldehydes with NAD and NADH as cofactors (Bartley and Hindley, 1980). Alcohol dehydrogenase from Cox's Orange Pippin apples has optimal activity at pH 5.5 to 6.0 when reducing acetaldehyde and pH 7.0 to 10.0 when oxidising ethanol (Bartley and Hindley, 1980). Alcohol

dehydrogenase is thought to be located in the cytoplasm and has a 13-fold higher affinity for acetaldehyde than ethanol in apples (Bartley and Hindley, 1980) and is the preferred substrate in grapes (Molina et al., 1987). Although the enzyme in apples has activity over a wide range of aldehyde compounds, substrate affinity for compounds longer than C_2 carbon chain aldehydes and alcohols is greater for straight chain than for branched chain compounds (Table 1.3). Grape ADH has broad substrate specificity but preferentially reduces aldehydes rather than oxidising alcohols (Molina et al., 1987).

Table 1.3. Relative activity of ADH to acetaldehyde for aldehydes and to ethanol for alcohols of Cox's Orange Pippin apples and Carignane grapes (adapted from Bartley and Hindley, 1980; Molina et al., 1987).

Aldehyde	% Activity of ADH		Alcohol	% Activity of ADH	
	Apple	Grape		Apple	Grape
Acetaldehyde	100.0[a]	100.0	Methanol	0.0	
Propanal	30.1		Ethanol	100.0[b]	100.0
Butanal	37.2		Propan-1-ol	44.7	31.0
2-Methylpropanal	4.3		Propan-2-ol	8.5	
Pentanal	31.1		Butan-1-ol	45.0	64.0
Hexanal	5.7	63.0	2-Methylpropan-1-ol	0.0	
trans-2-hexenal	7.2	6.3	Pentan-1-ol	19.1	
			Hexan-1-ol	18.3	
			trans-2-Hexen-1-ol	121.3	117.0

[a] 0.21 µmol NADH oxidised min^{-1} g^{-1} tissue; [b] 0.03 µmol NAD reduced min^{-1} g^{-1} tissue.

1.3.6 Esters

Esters form the largest group of volatile compounds produced by fruit, but the ester biosynthetic pathway is not well understood. Few studies have investigated detailed biochemical aspects of ester formation in contrast to β-oxidation or LOX breakdown of fatty acids. Ester production in fruit tissue is due to activity of AAT which uses acyl-CoA's, alcohols and carboxylic acids in an oxygen dependent reaction (Drawert and Berger, 1983) and is considered to be the most active in epidermis (Berger et al., 1992). Banana tissue slices produce the corresponding acetate esters when alcohols are added with different acyl CoA's (Gilliver and Nursten, 1976). There are few similarities between substrate specificity of AAT enzymes from different fruits. Optimal temperature for maximum activity is about 30°C, pH range is 7 to 8.5, sulfydryl groups are essential for activity and activity is linked to lipid metabolism in micro-organisms (Sanz et al., 1997). Ester forming enzymes in yeasts are localised in the cell membrane (Yoshioka and Hashimoto, 1981) while in banana AAT may be localised in the cytoplasm (Harada et al., 1985). Strawberry AAT acts on a wide range of alcohols and activity was greater with straight-chain alcohols than with branched chain alcohols of the same carbon number (Pérez et al., 1993). Acetyl CoA and hexan-1-ol are the preferred substrates of strawberry AAT (Pérez et al., 1993). Banana fruit tissue produces esters when supplied with acetate, butanoate, hexanoate, octanoate and decanoate or alcohols (Tressl and Drawert, 1973). Strawberry fruit produce alcohols and esters when supplied with C_2 to C_6 straight and branched chain aldehydes (Yamashita et al., 1976). In apples atmospheres containing low molecular weight alcohols ('Precursor Atmosphere') have enhanced concentration of esters with the corresponding

alcohol moiety (Berger, 1995). Therefore, the mixture of esters produced in different fruits depends on the activity and substrate specificity of AAT.

Acetyl-CoA, the most abundant acyl-CoA in cells, is synthesised within mitochondria via glycolysis and is an end product of β-oxidation. The specific location of acetyl CoA within the cell is in mitochondria, cytosol and glyoxysomes (Mathews and van Holde, 1996). Membranes are thought to be impermeable to acetyl CoA which must be transported across membranes from the cytoplasm (Mathews and van Holde, 1996). Substrate specificity of AAT differs from fruit to fruit and esterification of straight-chain alcohols is preferred over branched-chain alcohols (Olias et al., 1995; Rowan et al., 1996). Such differences in preference for acyl CoA's and alcohols may determine concentration of different esters in fruit aroma profiles. Jonagold apples exposed to hexanal vapours synthesised hexan-1-ol in preference to related volatiles, such as hexyl acetate, butyl hexanoate and hexyl hexanoate, while Golden Delicious apples had a greater capacity to convert hexanal to hexan-1-ol than Jonagold apples (Song et al., 1996). In yeast AAT activity is inhibited *in vitro* by synthesis of unsaturated fatty acids (Mauricio et al., 1993). The relationship between lipid synthesis and ester synthesis is not known for apples. Apples kept in low oxygen storage conditions, which inhibit fatty acid degradation, have a reduced pool of alcohol precursors (Brackmann et al., 1993). Activity of AAT increases with advancing maturity and is suppressed by atmospheres containing 0.5% and 1% O_2 (Fellman et al., 1993a). In addition to AAT, the enzyme esterase which break esters down to alcohols and carboxylic acids may have some synthetic capacity as well as its ability to hydrolyse esters (Bartley and Stevens, 1981; Sanz et al., 1997).

Ester synthesis in apple tissue may be the sum of ester formation by AAT, reverse reaction of ester hydrolysis and ester hydrolysis (Knee and Hatfield, 1981). Apple juice has high concentrations of alcohols and esters; for example, Cox's Orange Pippin apple juice contains large amounts of hexan-1-ol, butan-1-ol, and pentan-1-ol and hexyl acetate, butyl acetate and pentyl acetate (Goodenough, 1983). The high concentration of alcohols may result from esterase activity which increases during the climacteric (Goodenough, 1983).

Substrate concentrations can change the composition of volatiles emanating from Red Delicious apple disks. High ethanol concentrations promote formation of C_8 compounds or longer acyl moieties, whereas low ethanol concentrations increase short chain acyl moiety (Berger and Drawert, 1984). This rise in esters is temporary reaching a maximum within 12 to 24 hours. Maximum production of esters was achieved using butan-1-ol and pentan-1-ol, the least using methanol and ethanol (Berger and Drawert, 1984). Addition of butan-1-ol increased butyl acetate concentrations and butanoate esters of all alcohol moieties indicating that alcohols were being converted to butyl CoA.

Ester biosynthesis is considered to be limited by alcohol concentration (Berger et al., 1992; Gilliver and Nursten, 1976). Ethyl and hexyl ester synthesis was stimulated by ethanol and hexan-1-ol at the expense of butyl esters, indicating that ester formation in apple fruit is a competitive reaction (Kollmannsberger and

after application of hexanal vapours to intact fruit (Song et al., 1996). Increases in hexyl acetate concentration took about 5 h and increases in esters with hexyl acyl moiety took up to 24 h (Song et al., 1996). This would suggest that hexanal is being incorporated into the fruit, first as an alcohol, then as acetate ester, and is further metabolised into acyl CoA compounds. This implies that substrate availability rather than enzyme activity limits volatile production (Knee and Hatfield, 1981; Song et al., 1996).

1.4 Effect of Hypoxia on Volatile Concentration

1.4.1 Hypoxic treatments before storage

Exposure of fruit to hypoxic atmospheres for several days have been investigated as an alternative non-chemical insect disinfestation treatment to the fumigant methyl bromide (Lay-Yee and Whiting, 1996; Whiting et al., 1996). Methyl bromide is a greenhouse gas and its use is to be phased out by the year 2001 in the USA and by 2010 in the rest of the world (Anon, 1995; 1997b). Warm fumigation temperatures generally allow decreased methyl bromide concentrations and/or decreased fumigant exposure time to achieve insect kill, as the target insect has increased metabolic activity and rate of fumigant uptake (Paull and Armstrong, 1994). A disinfestation treatment using hypoxic atmospheres at warm temperatures (>20 °C) will kill insects faster than hypoxic atmospheres at low (<5 °C) temperatures (Ke and Kader, 1992). The effect of exposure to hypoxia on apple quality at warm temperatures has been investigated by Ampun (1997) who determined that exposure to hypoxia induces substantial qualitative and quantitative changes in concentration of volatiles thought to be important in apple aroma. The type of compounds enhanced included low odour threshold volatiles such as ethyl-2-methyl butanoate and ethyl butanoate which are of commercial significance to the apple juice processing industry (Frucor Processors, pers. comm.). Enhanced amounts of specific volatiles could be added to juice concentrates from cultivars which lack these compounds. For example, ethyl-2-methyl butanoate may be added to juices from Golden Delicious apples which contain relatively low concentrations of this compound. It may also be possible to isolate specific compounds for use as fragrances for perfumes and food additives (Ampun, 1997).

In this review the term hypoxia is used to describe atmospheres containing various concentrations of oxygen where apple fruit are predominantly respiring anaerobically. Respiration is the oxidative breakdown of sugars and acids to CO_2 and water. This process provides energy to form adenosine triphosphate (ATP) and other compounds which are used for maintenance of cellular integrity and for synthesis or breakdown of cell components during ripening (Blanke, 1991; Kader, 1987; Wills et al., 1997). Respiration rate is often used as a guide to potential storage life of a fruit, high respiration rates indicate a short storage life through rapid ripening (Kader, 1992; Wills et al., 1997). Respiration is used to describe metabolism in a wide range of oxygen concentrations. Fruit held in air (about 20% O_2) is considered to be in normoxic conditions where aerobic respiration occurs. Anoxic conditions are those were total absence of O_2 prevents mitochondrial activity (Ricard et al., 1994) whereas, hypoxic conditions are those

mitochondrial activity (Ricard et al., 1994) whereas, hypoxic conditions are those where the O_2 partial pressure limits mitochondrial activity (Pradet and Bomsel, 1978). True anoxic conditions are difficult to achieve in practice therefore most experimental conditions reported as being anoxic are redefined as 'deep hypoxia' (Roberts et al., 1992). Hypoxic treatments applied to apples would be considered as deep hypoxia.

1.4.2 Effect on general fruit quality

Fruit maintained at low temperatures or in CA retain their quality for longer than fruit stored in air (Kader, 1986). Hypoxia generally reduces softening, CO_2 and ethylene production in apples (Table 1.4) due to inhibitory effects that high concentrations of CO_2, acetaldehyde, ethanol or low O_2 concentrations have on enzyme systems within fruit. Fruit exposed to hypoxic atmospheres ranging from 10% to 100% CO_2, 50% to 100% N_2 and 0% to 17% O_2 in temperatures from -1.1 to 32 °C and duration's of a few hours to 42 days will depending on the fruit: have enhanced maintenance of quality during storage (Eaves et al., 1968; Pesis and Avissar, 1989; Pesis et al., 1988; Pesis et al., 1994); be acclimatised to low oxygen storage conditions (Little et al., 1982); have tolerance to non-chemical disinfestation treatments (Ke et al., 1991a, b; Yahia and Vazquez-Moreno, 1993); and have enhanced aroma/flavour volatile concentrations in fruit (Ampun, 1997; Dourtoglou et al., 1994; Pesis, 1994; Shaw et al., 1991,1992). Golden Delicious apples, peaches and nectarines after a brief exposure to hypoxia , 86 to 95% CO_2 or 97% N_2 for 1 d, were rated by panellists as better flavoured than control fruit after 2 weeks or 5 days at 20°C (Lurie and Pesis, 1992; Pesis et al., 1994).

Table 1.4. Effects of hypoxic and high carbon dioxide treatments prior to storage on quality attributes of apples.

Cultivar	Treatment	Duration (d)	Temp (°C)	Effect of treatment[b] Firm.	Flavour	Resp[c]	Ethylene	Reference
Braeburn	100% CO_2	1	20	Decr.[d]		Incr.	Decr.	Ampun, 1997
Cox's Orange Pippin	100% N_2	3-10	3.5	Incr.[e]		Incr.		Fidler and North, 1971
	15-30% CO_2, 6% O_2[a]	15	3.5	Incr.	Decr.		Decr.	Stow, 1988
	100% CO_2	1	20	Incr.		Decr.	Incr.	Ampun, 1997
Golden Delicious	10-30% CO_2, 10% O_2[a]	10	-1	Incr.	Incr.		Decr.	Couey and Olsen, 1975
	14 or 18% CO_2, 6% O_2[a]	10	0	Incr.				Lau and Looney, 1978
	15% CO_2, 18% O_2[a]	20	0	Incr.				Hribar et al., 1994
	20% CO_2, 17% O_2	4	20				Decr.	Gorny and Kader, 1996
	99.75% N_2	4	20				Decr.	Gorny and Kader, 1996
	95% CO_2	1-2	20	Incr.	Incr.	Incr.	Incr.	Pesis et al., 1994
Granny Smith	99.75-100% N_2	3-35	0-10	Incr.	Decr.[f]	Decr.	Decr.	Ke et al., 1991b
	99.5% N_2, 0.5% O_2[a]	3-9	-0.5	Incr.	Decr.[f]			Little et al., 1982
	100% CO_2	1	20	Decr.		Incr.		Ampun, 1997
McIntosh	100% N_2[a]	7	3.5	Incr.				Eaves et al., 1968
	12% CO_2, 5% O_2[a]	14	0	Incr.	Incr.	Decr.	Decr.	Bramlage et al., 1977
	100% N_2[a]	1.5-4.5	20					Dilley et al., 1963
Pacific Rose™	100% CO_2	1	20			Incr.		Ampun, 1997
Red Delicious	100% N_2[a]	7	0	Incr.	Incr.			Eaves et al., 1968
	100% CO_2	1	20	Incr.		Incr,	Incr.	Ampun, 1997
Royal Gala	100% CO_2	1	20				Incr.	Ampun, 1997
Yellow Newton	99.75-100% $_{N2}$	3-35	0-10	Incr.	Decr.[f]	Decr.	Decr.	Ke et al., 1991b
Wagener	100% N_2[a]	7	0	Incr.	Incr.			Eaves et al., 1968

[a] Treatment before CA storage, [b] compared to air stored controls, [c] carbon dioxide production, [d] Decr. = decrease, [e] Incr. = increase, [f] usually an alcoholic off-flavour.

1.4.3 Respiration and Hypoxia

Low temperature refrigeration is the most common storage technology used to preserve quality of horticultural produce; controlled or modified atmosphere storage (CA or MA) further supplements refrigeration to extend storage life and maintain quality (Kader, 1986). Once O_2 concentration in air around fruit is lowered to less than 10% their respiration rate is reduced in proportion to O_2 concentration, but a concentration of about 1 to 3% O_2, depending on the fruit, is required to induce anaerobic respiration. Under hypoxic conditions the glycolytic pathway replaces the tricarboxylic acid cycle (TCA) as the main source of energy provided by plant tissue. Pyruvic acid is decarboxylated to form acetaldehyde, CO_2 and ethanol (Kader, 1986).

In aerobic conditions pyruvate ($C_3H_4O_3$), via pyruvate dehydrogenase (PDH), is converted to acetyl CoA in mitochondria where it enters the TCA cycle (Figure 1.3). Oxygen is utilised by oxidative phosphorylation, where electrons flow down a potential gradient maintained by protons being pumped across the inner mitochondrial membrane resulting in production of ATP. This process generates 18 moles of ATP for every mole of pyruvate; by contrast anaerobic reactions generate only 2 moles of ATP per mole of pyruvate (Mathews and van Holde, 1996; Pradet and Bomsel, 1978).

Carbohydrate metabolism produces a large array of organic compounds. Central in these reactions is the conversion of starch and sugars to pyruvate which is involved in the metabolism of many compounds. Under anaerobic conditions oxidation of pyruvate in mitochondria is greatly reduced and accumulation of pyruvate and acetyl CoA activate the fermentation biosynthetic pathway (Figure 1.3). Pyruvate is converted to acetaldehyde and CO_2, by the enzyme pyruvate decarboxylase (PDC), acetaldehyde is reduced by NADH to ethanol by alcohol dehydrogenase (ADH) (Mathews and van Holde, 1996). Under anaerobic conditions most fruit produce ethanol hence the large increases in ethanol when fruit are anaerobic.

Fermentation may be regulated by two mechanisms: molecular control of PDC and ADH by increased concentration or production of new isozymes; and metabolic control by feedback mechanisms of products and co-factors inhibiting enzyme function (Ke et al., 1995; Perata and Alpi, 1993). While the induction of PDC, ADH and their isozymes (Chen and Chase, 1993; Longhurst et al., 1990; Pradet et al., 1985; Sachs et al., 1985) occurs in hypoxic conditions, the concentration of these enzymes does not correlate with enzyme activity unless enzyme concentration is very low (Ke at al., 1995; Roberts et al., 1989). This implies that regulators of metabolic control of anaerobic enzymes include changes in pH, substrate concentration, cofactors and inhibitors.

One theory of induction of fermentation in fruits and vegetables is thought to involve reduction in cellular pH that selectively activates PDC and ADH. When O_2 concentration reaches low levels, NADH rises increasing the activity of lactate dehydrogenase; the subsequent increase in lactate concentration lowers cytoplasm pH which in turn activates PDC, suggesting a metabolic pH regulation

of fermentation (Davies et al., 1974; Davies 1980). A decrease in cytosolic pH, along with transient lactate fermentation, has been reported in avocado (Hess et al., 1993), 'Bartlet' pears (Nanos and Kader, 1993), and in tomato root cultures (Rivoal and Hanson, 1994).

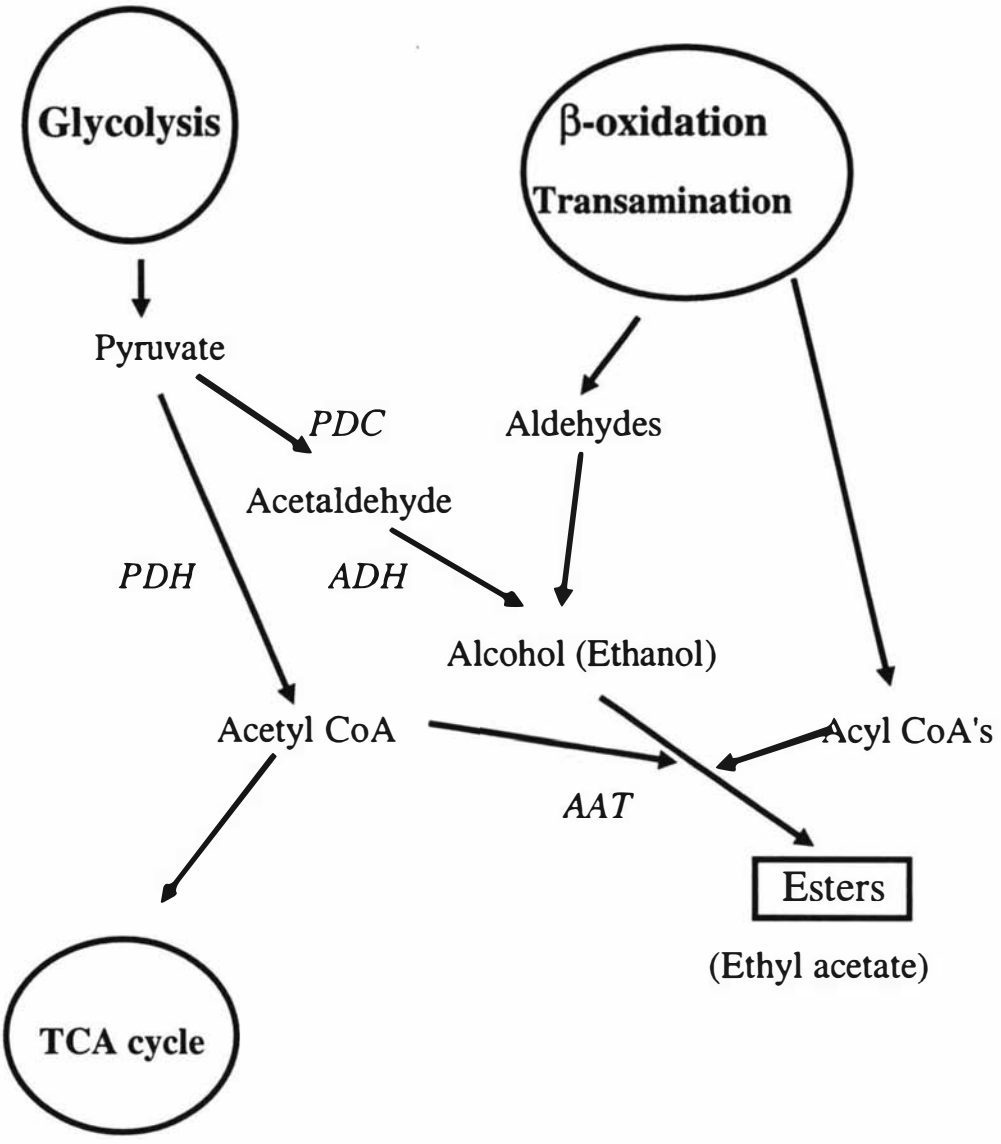

Figure 1.3. Anaerobic biosynthetic pathway, the formation of acetaldehyde, ethanol and esters (adapted from Mathews and van Holde, 1996). Highlighted text represents compounds which accumulate under hypoxic conditions. *PDH* = pyruvate dehydrogenase, *PDC* = pyruvate decarboxylase, *ADH* = alcohol dehydrogenase, *AAT* = alcohol acyl CoA transferase.

However, not all plants produce lactic acid before a rise in ethanol is noted (Andreev and Vartapetian, 1992). A decrease in pH need not be due to lactate but could involve: inhibited proton pumping at low ATP concentration and proton release by ATP hydrolysis (Chervin et al., 1996; Saint-Ges et al., 1991); release of malic acid into the cytoplasm from the vacuole (Bufler and Bangerth, 1982);

high CO_2 concentrations could decrease cytoplasm pH to initiate PDC (Blanke, 1991).

Based on the above Ke et al. (1993, 1995) proposed that the mode of action of low O_2 on avocado fruit (Figure 1.4) results from low O_2 reducing NADH flux through the electron transport system in the mitochondria. The pH of the cytoplasm decreases reducing PDH activity and pyruvic acid concentration rises. Pyruvate dehydrogenase and LDH activities increase and new ADH isozyme is induced. Increased PDC activity results in an increase in acetaldehyde, which along with an increase in NADH, promotes the activity of ADH, resulting in ethanol accumulation. The higher NADH and pyruvate also activate LDH increasing lactate production. Therefore, ethanol and acetaldehyde accumulate in anaerobic fruit and if endogenous ADH and new ADH isozymes are induced by anaerobiosis, ADH activity could remain high after release of hypoxic conditions. This has yet to be confirmed by other researchers.

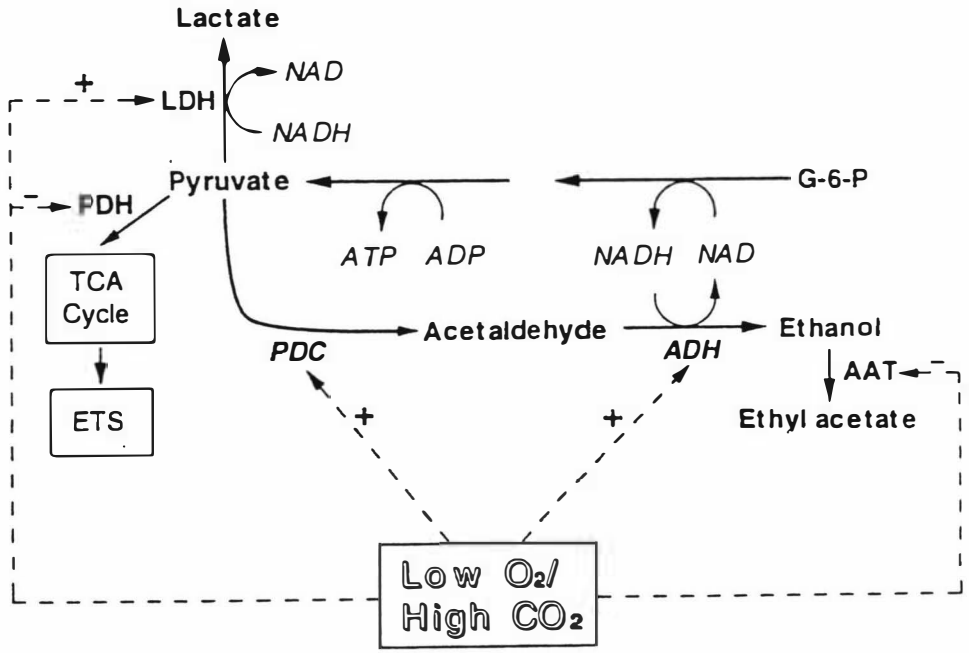

Figure 1.4. Proposed mode of action of hypoxia (low O_2 and/or high CO_2) on fermentative metabolism of fruit (Ke et al., 1993). Symbols and abbreviations: - = decrease or inhibition, + = increase or activation, LDH = lactate dehydrogenase, PDH = pyruvate dehydrogenase, PDC = pyruvate decarboxylase, ADH = alcohol dehydrogenase, AAT = alcohol acyl CoA transferase, G-6-P = glucose-6-phosphate, ETS = electron transport system.

Hypoxic conditions consistently enhance acetaldehyde and ethanol concentration in a wide range of fruits. Under hypoxic conditions acetaldehyde and ethanol can reach concentrations of several hundred parts per million (Knee, 1991) with ethanol accumulation as high as 47 $(\mu L \cdot L^{-1}) \cdot kg^{-1} \cdot d^{-1}$ at 0 °C (Knee, 1991). When returned to air acetaldehyde and ethanol concentrations reduce to initial values over 1 to 2 weeks (Ampun, 1997; Salviet and Ballinger, 1983a,b). Fruit metabolism may be affected by acetaldehyde and ethanol that stimulate or inhibit various biochemical pathways involved in ripening. Application of acetaldehyde vapours to apples (Fidler, 1968), blueberries (Paz et al., 1981), feijoa (Pesis, 1994), oranges (Pesis and Avissar, 1989; Shaw et al., 1990, 1991), peaches (Pesis, 1994) and pears (Janes and Frenkel, 1978) induced an ethylene-like stimulation of ripening by enhancing ethylene production and directly stimulating CO_2 production.

Decrease in acetaldehyde and ethanol after removal from hypoxia is associated with a several fold enhancement in ethyl esters and some alcohols, as well as decreases in non-ethyl esters and aldehydes (Ampun, 1997; Mattheis et al., 1991a). Increased ethyl ester concentration after removal from hypoxia may be due to enhanced ethanol concentrations (Mattheis et al., 1991a) as exogenous application of ethanol vapour increases concentration of ethyl esters and reduces non-ethyl esters (Berger et al., 1992). Exposure of apples to hypoxia for 18 and 24 h enhanced ethyl esters more than in fruit exposed for 6 h, 12 h or 48 h and degree of enhancement of ethyl ester concentration was the same regardless of whether CO_2 or N_2 was used to generate hypoxic conditions (Ampun, 1997). Oxygen concentrations of $< 5\%$ O_2 was required to induce enhancement of ethyl esters; the lower the O_2 concentration the greater the response to hypoxia (Ampun, 1997). When O_2 concentrations were maintained at 20% and CO_2 concentrations ranged from 10 to 80%, only fruit exposed to CO_2 concentrations greater than 20% had enhanced ethyl esters after treatment; the higher the CO_2 concentrations the greater the change in volatile concentration (Ampun, 1997). It is unknown whether this increase in ethyl esters is due to the increase in ethanol during hypoxia or new isozymes of AAT and ADH are induced in response to hypoxia.

1.4.4 Pre-treatment Factors

1.4.4.1 Ripeness and Maturity

Ripening is a process of physical, metabolic and biochemical changes initiated by ethylene, either on or off the tree, and includes loss of background green colour, softening of fruit tissue and development of characteristic aroma and flavour (Wills et al., 1997). Typical flavour compounds of apples are only produced after ripening has been initiated by ethylene (Tressl et al., 1975). Apples are classified as having a climacteric ripening pattern which is a rapid increase in production of ethylene and/or respiration rate to a maximum after which the rate declines (Wills et al., 1997). Different stages of ripeness and maturity can be defined by their production of ethylene and/or carbon dioxide. Physiologically immature pre-climacteric apples have low aminocyclopropane-1-carboxylic acid (ACC) concentration and ACC synthase (ACS) activity and fail to ripen normally while

physiologically mature pre-climacteric apples have increased ACS activity, are accumulating ACC and can be induced to ripen normally by exposure to exogenous ethylene (Lelièvre et al., 1997). Fruit harvested at this stage of maturity have the greatest postharvest storage life potential. Peak climacteric apples are those which have reached their maximum respiration rate and often maximum ethylene production while post-climacteric apples would be considered 'ripe' having moderate ethylene production and low firmness.

There is a consistent correlation between the climacteric and volatile production, where typical aroma/flavour volatiles increase in concentration during climacteric ripening reaching maxima at the climacteric peak (Mattheis et al., 1991b; Sapers et al., 1977; Song and Bangerth, 1994, 1996; Yahia et al., 1990b). Apples harvested physiologically immature produce very low concentrations of volatiles while overripe post-climacteric fruit produce low and declining concentrations of volatiles (Brown et al., 1966; Hansen et al., 1992b; Song and Bangerth 1996; Vanoli et al., 1995). Early harvested fruit have reduced, but the same pattern of, volatile production as later harvested fruit. Volatile production follows ethylene production during ripening in some cultivars (Brown et al., 1966) but not in others (Hansen et al., 1992b). Increased ethylene production and respiration may be needed to provide precursors for increased volatile synthesis (Song and Bangerth, 1996). There is little evidence that there is a direct relationship between ethylene production and volatile production, but such a relationship has not been investigated fully.

Specific volatiles in intact fruit appear to have five patterns of production as the fruit ripen which may relate to patterns of substrate production/availability (Brown et al., 1966; Mattheis et al., 1991b; Sapers et al., 1977; Yahia et al., 1990b). The patterns are: a continuous decline; steady stable levels; a transient rise in production followed by a return to previous levels; a steady rise in production followed by a rapid rise as the fruit become fully mature; and production once the fruit are fully mature. Volatile production in apples ripened at warm temperatures after harvest or during coolstorage is, in general, sigmoidal with volatile production declining as the fruit become overripe (Dirinck et al., 1989; Hansen et al., 1992b; Vanoli et al., 1995). Propyl acetate production of Jonagold apples increased for 3 weeks at 20 °C after removal from 2 °C then decreased (Hansen et al., 1992a). By contrast, butyl and hexyl acetate production declined steadily over time at 20 °C. As propan-1-ol is thought to be produced by α-oxidation and butan-1-ol and hexan-1-ol arise from β-oxidation, differences in acetate ester production indicate that the metabolic origin of alcohols affects the pattern of ester production (Hansen et al., 1992a). To establish if substrate production/availability affects rates of volatile production rates of β-oxidation, transamination and AAT activity would require measurement.

1.4.4.2 Temperature

Ester and alcohol production of apples increases during ripening, with volatile concentrations increasing as temperature increases to a limit, as volatile production is reduced at temperatures above 32 °C. Jonathan apples stored at

temperatures from -1 to 10 °C for up to 12 weeks, had increasing ester and alcohol concentrations and rates of production as temperature increased (Wills and McGlasson, 1971). Red Delicious apples had maximum ester production at 22 °C; production decreased at 32 °C and was inhibited at 46 °C (Guadagni et al., 1971) indicating that heat treatment may temporarily inhibit or destroy volatile producing enzymes. A heat treatment of 38 °C for 4 days reduced volatile production in Golden Delicious apples compared to fruit at 22 °C (Fallik et al., 1997). However, characterisation of the relationship of volatile production with temperature over the range of -1 to 45 °C has not been done. Therefore, how apples may respond to hypoxic conditions at temperatures other than 20 °C is not known.

Apples removed to 20 °C after low temperature storage produce greater concentrations of volatiles and reach maximum production earlier than freshly harvested apples, in a cultivar specific manner. After harvest Cox's Orange Pippin apples took 18 d to reach maximum concentration of butyl acetate and hexyl acetate and 27 d for butan-1-ol and hexan-1-ol (Hatfield and Patterson, 1975). On return to 20 °C after 3 and a half months at 3.3 °C, maximum butyl acetate concentration, which was about twice the concentration of freshly harvested fruit, was reached after 4 days, while butan-1-ol took 15 days to reach a maximum concentration that was about 5 times that of freshly harvested fruit (Hatfield and Patterson, 1975). This may be a result of accumulation of volatile precursors while fruit were at low temperatures.

Storage temperature affects volatile concentration and pattern of concentration change during storage in a cultivar specific manner. Total volatiles of Cortland and McIntosh apples at 20 °C had the same pattern of change in volatile concentration over time, a rapid rise to a peak before declining to original concentrations after about 40 d (Yahia et al., 1990b; Yahia et al., 1991). Cortland apples at 3.3 and 0 °C had the same pattern of total volatile change as at 20 °C but took about 100 d to return to original total volatile concentrations. In Cortland apples at 3.3 °C, maximum total volatile concentration was about 60 % that of 20 °C fruit, while fruit at 0 °C had about 25% greater volatile concentration than 20 °C fruit (Yahia et al., 1990b; Yahia et al., 1991). By contrast, McIntosh apples at 3.3 and 0 °C had a different pattern of volatile change, a slow steady increase in concentration but at only half the concentration of fruit at 20 °C (Yahia et al., 1990b; Yahia et al., 1991).

Specific volatiles have different patterns of concentration depending on temperature and cultivar. Hexanal concentrations of Cortland and McIntosh apples had the same pattern of change, a rapid rise to a peak before declining to original concentrations, irrespective of temperature (Yahia et al., 1990b; Yahia et al., 1991). Ethyl butanoate concentrations for Cortland and McIntosh apples at 20 °C rose to a peak before decreasing to original levels, while in fruit at 0 and 3.3 °C, ethyl butanoate concentrations rose steadily with no peak (Yahia et al., 1990b; Yahia et al., 1991).

1.4.4.3 Duration of storage

Low temperature storage in air for periods of longer than 3 months reduce volatile production and concentration of apples (Ampun, 1997). These decreases in volatile production are detectable by sensory panellists after 6 and 8 months of storage (Plotto et al., 1997). Maximum concentration of Red Delicious volatiles was reached after 2 to 4 months at 1 °C and declined for longer storage periods (Guadagni et al., 1971). Golden Delicious apples after 5 months at 1 °C had reduced concentrations of volatiles compared to apples stored for 3 months (Streif and Bangerth, 1988). Total volatile concentration of Golden Delicious apples after 3 months at 1 °C was about 50% greater than after 8 months (Brackmann et al., 1993). Butyl acetate and hexyl acetate concentration of Golden Delicious apples increased to a maximum after 2.5 months at 4 °C then decreased after 3.5 months (Bachmann, 1983). Ester concentrations in Law Rome and 262 Rome apples were lower after 6 months at 0.5 °C than in freshly harvested fruit and in fruit stored for 3 months at 0.5 °C (Fellman et al., 1993b). The reduction in ester concentration was associated with reduced ester biosynthesis where AAT activity was decreased after 6 months at 0.5 °C compared to freshly harvested fruit and fruit stored for 3 months at 0.5 °C (Fellman et al., 1993b). Reduced ester concentration may also be due to reduced availability of substrates for esterification although this requires measurement.

When hypoxia is applied before CA or low temperature storage or at warm temperatures (>20°C), high concentrations of ethanol and ethyl acetate can persist throughout the storage period and shelf life of a fruit (Mattheis et al., 1991a). This residual effect of hypoxic treatment may cause off-flavours (Ke at al., 1991b). Exposing apples to hypoxia for 24 h, after removal from different periods of CA storage, enhanced acetaldehyde, ethanol and ethyl esters while decreasing concentrations of non-ethyl esters (Ampun,1997). The longer the duration of CA storage the less the enhancement of ethyl esters induced by exposure to hypoxia (Ampun, 1997). This suggests that apples stored in CA have a reduced capacity to produce esters. This suggests that the capacity of apples to produce esters decreases possibly due to decreased AAT activity (Fellman et al., 1993b) or by reduced availability of substrates (Knee and Hatfield, 1981).

1.5 Sensory Analysis

1.5.1 Descriptive analysis

While it is relatively straight forward to measure concentration changes of volatile compounds in fruit or juice, predicting how such changes in concentration may affect perception of aroma or flavour is not simple. Volatile compounds can be measured using objective analytical techniques, most commonly GLC, but these instruments are relatively insensitive compared to the human sense of smell. Typical limits of GLC detectors are about 10^{-12} molar compared to 10^{-18} molar for smell. This indicates that despite efforts to concentrate volatile extracts for analysis, some volatile compounds contributing to odour may not be measured by GLC. To determine accurately what changes in

volatile concentration may have on perception of aroma requires sensory analysis. Descriptive analysis is a sensory analysis method often used in postharvest research where flavour or aroma quality is measured. It describes the perceived quantitative and qualitative characteristics of a product (Gilbert and Heymann, 1995). Several techniques using descriptive analysis have been developed, including flavour profiling, texture profiling and quantitative descriptive analysis (QDA, Gilbert and Heymann, 1995; Zook and Pearce, 1988). All techniques recruit, screen, select and train potential panellists. The number of panellists and length of training vary. Following training, products are evaluated in controlled environments using experimental procedures specific for each technique. Data collected can be analysed by several different univariate and multivariate statistical techniques. Descriptive analysis requires considerable time to recruit, screen and train panellists and once trained, the panel must be maintained over a lengthy period of time which may require additional training sessions (Anon, 1981). This technique is time consuming, requires special environmentally controlled facilities and make the tests expensive.

In descriptive analysis, panellists judge products through individual assessment of each attribute independently of one another. Collective examination of assessments of each attribute allows a profile to be constructed for each product. By pooling panellist responses, information about the ability of panellists to collectively identify and quantify attributes is obtained, but information about relationships between attributes is not. Descriptive analysis is limited by the individuals ability to adequately describe the product. Once words are found to describe attributes, agreement between panellists may be difficult to obtain as to their interpretation and meaning. Individuals may also vary widely in their sensitivity to an attribute which will be reflected in scoring differences. When products are similar, variance in assessment by panellists between products becomes important, hence the need for panellist training, but changes in panellist evaluation from session to session are never removed entirely.

1.5.2 Aroma

The human sense of smell, that is qualitatively more diverse than the sense of taste, is characterised by an almost infinite number of odour qualities. There are more than 17000 chemical compounds that are potential odourants. The sense of smell, which is much more sensitive than taste, can detect certain volatile compounds at concentrations as low as 10^{-18} molar (Thomson, 1986). Sensitivity to a particular compound usually diminishes quickly over time, often to the point of complete insensitivity. Once the stimulus has been withdrawn, recovery is usually rapid. The most widely accepted mechanism by which aroma volatiles act on olfactory cells is that when molecules are adsorbed and desorbed from cell surfaces, they leave a momentary breach in the receptor cell membrane through which Ca^{2+} ions leak from the nerve cells. The number of aroma volatile molecules required to desorb simultaneously from a receptor site, in order to breach the membrane, varies according to the compound and may explain the observed differences in threshold values (Table 1.5) (Thomson, 1986).

Table 1.5. Aroma threshold values of volatile compounds in apples.

Compound	Threshold	Reference
Aldehydes	$\mu L \cdot L^{-1}$	
acetaldehdye	0.015-0.12	Flath et al., 1967; Mulders, 1973
hexanal	0.005	Paillard, 1990
trans-2-hexenal	0.001-0.017	Flath et al., 1967; Hatanaka, 1993
Alcohols		
ethanol	100-900	Teranishi et al., 1987; Mulders, 1973
propan-1-ol	9-40	Flath et al., 1967; Mulders, 1973
butan-1-ol	0.5	Flath et al., 1967
pentan-1-ol		
hexan-1-ol	0.15-0.5	Flath et al., 1967
2 methyl butan-1-ol	0.25	Buttery, 1973
Esters		
ethyl acetate	0.005-13.5	Mulders, 1973; Takeoka et al., 1996; Teranishi et al., 1987
propyl acetate	2.0	Takeoka et al., 1996
butyl acetate	0.066	Takeoka et al., 1996; Teranishi et al., 1987
pentyl acetate	0.005-0.043	Takeoka et al., 1996; Teranishi et al., 1987
hexyl acetate	0.002-0.115	Takeoka et al., 1996; Teranishi et al., 1987
ethyl butanoate	0.001	Takeoka et al., 1995; Teranishi et al., 1987
ethyl-2-methyl butanoate	0.000006-0.0001	Flath et al., 1967; Takeoka et al., 1995; Teranishi et al., 1987
ethyl propionate	0.01	Takeoka et al., 1995; Teranishi et al., 1987
ethyl pentanoate	0.0015-0.005	Takeoka et al., 1995; Teranishi et al., 1987
ethyl hexanoate	0.001	Takeoka et al., 1995; Teranishi et al., 1987
propyl butanoate	0.018	Teranishi et al., 1987
2 methyl butyl acetate	0.005-0.011	Teranishi et al., 1987

1.5.2.1 Volatile compounds important for apple aroma

At present, is it difficult to ascertain the relative contribution of each volatile component to a characteristic or a desirable aroma (Sanz et al., 1997). The odour characteristic of each compound is difficult to identify and quantify when concentrated (Takeoka and Full, 1997). Sensory characteristics of apples and apple products have been correlated with 24 volatile compounds mostly alcohols, esters and aldehydes (Table 1.6). It is generally agreed that ethanol concentration does not correlate with aroma (Panasiuk et al., 1980). The character of aroma for a given variety is thought to result from the proportion of each volatile compound in apples and not the concentration of individual volatiles (Grosch, 1994; Paillard, 1975). Sensory description of volatile compounds changes when assessed as a mixture (Rizzolo et al., 1989). Although the sensory characteristics of individual compounds have been described (Dimick and Hoskin, 1983), there has been little progress in determining what mixtures of volatile compounds impart 'typical' or expected apple aroma or flavour to apples. However, the effect of quantitative changes in volatiles of apples on aroma intensity and description has been investigated for Royal Gala apple aroma (Young et al., 1996). The volatiles 2 methyl butyl acetate, butyl acetate, hexyl acetate and butan-1-ol are important contributors to Royal Gala flavour (Young et al., 1996). Increasing concentration and ratios of hexyl acetate, 2 methyl butyl acetate and butan-1-ol to one another increased intensities of red apple aroma and red apple flavour (Young et al., 1996). This suggests that relationships between volatiles is

complex as changes in the ratio of volatile compounds to one another can affect the description of the sensory characteristic and intensity of descriptor.

1.5.2.2. Odour units

Odour units have been used extensively in applied flavour research (Frijters, 1979). Odour units are defined as the ratio of the concentration of a volatile component in an extract and the threshold concentration of that volatile in water (Frijters, 1979). A list of odour threshold values for volatile compounds found in apples is presented in Table 1.5. It is an arbitrary scale for which the base unit is a particular threshold concentration. Odour units rely on the following psychophysical assumptions: the relative contribution of a volatile to the intensity of a mixture is linearly proportional to the number of odour units and that two components of a mixture having equal numbers of odour units will be perceived with equal intensity. Neither of these assumptions is supported by psychophysical evidence where odour intensity is a power function of concentration. By definition the odour unit of a mixture is the sum of odour units of individual compounds (Laing and Panhuber, 1979). The relative contribution of a component in a mixture can be described as a percentage of the total odour unit numbers. However, odour units do not describe odour quality of a mixture or provide any information about the relationship between compound concentration and perception above threshold concentration.

Table 1.6 Apple volatile compounds and their sensory descriptions in aroma emanating from intact apples or apple juice.

Compound	Sensory description	Cultivar	Reference
Aldehydes			
Acetaldehyde	green/sharp	Golden Delicious	Rizzolo et al., 1989
trans-2-hexenal	green/sharp	Golden Delicious	Rizzolo et al., 1989
	overall intensity	McIntosh	Panasiuk et al., 1980
	green apple	Delicious	Flath et al., 1969
	harmonious, fruity	Many	Duerr, 1979
Hexanal	green/sharp, earthy	Golden Delicious	Rizzolo et al., 1989
	overall intensity	McIntosh	Panasiuk et al., 1980
	good, green apple	Delicious	Flath et al., 1969
	grass like	Many	Duerr, 1979
Alcohols			
Butan-1-ol	overall flavour, aroma, sweet aroma	Royal Gala, Golden Delicious	Young et al., 1996 Rizzolo et al., 1989
Hexan-1-ol	earthy, unpleasant	Golden Delicious	Rizzolo et al., 1989
trans-2-hexenol	harmonious, fruity	Many	Duerr, 1979
Esters			
Butyl acetate	red apple aroma	Royal Gala	Young et al., 1996
	'Cox' like aroma	Cox's Orange Pippin	Williams and Knee, 1977
	harmonious	Many	Duerr, 1979
	nail polish	Gala	Plotto, 1998
Pentyl acetate	banana like	Cox's Orange Pippin	Williams and Knee, 1977
	apple, fruity	Golden Delicious	Rizzolo et al., 1989
	Gala	Gala	Plotto, 1998
Hexyl acetate	red apple aroma characteristic apple	Royal Gala	Young et al., 1996
	'Cox' like aroma	Cox's Orange Pippin	Williams and Knee, 1977

Table 1.6, continued	ripe Golden Delicious	Golden Delicious	Rizzolo et al., 1989
	sweet fruity, apple		
	Gala, ripe, pear		Plotto, 1998
2 methyl butyl acetate	overall aroma, characteristic apple	Royal Gala	Young et al., 1996
	solvent	Gala	Plotto, 1998
	banana like	Cox's Orange Pippin	Williams and Knee, 1977
Ethyl butanoate	fruity, estery	Golden Delicious	Rizzolo et al., 1989
	harmonious, fruity	Many	Deurr, 1979
ethyl-2-methyl butanoate	fruity	Golden Delicious	Rizzolo et al., 1989
	apple like	Delicious	Flath et al., 1967
	sweet strawberry	Gala	Plotto, 1998
4-methoxyallyl benzene	spicy, aniseed	Many	Williams et al., 1977
methyl-2-methyl butanoate	sweet fruity	Gala	Plotto, 1998
propyl-2-methyl butanoate	very sweet, strawberry	Gala	Plotto, 1998
butyl-2-methyl butanoate	fruity, apple	Gala	Plotto, 1998
hexyl-2-methyl butanoate	apple, grapefruit	Gala	Plotto, 1998
butyl hexanoate	green apple	Gala	Plotto, 1998
hexyl propanoate	apple	Gala	Plotto, 1998
6-methyl-5-hepten-2-one	fruity, tape	Gala	Plotto, 1998
butyl butanoate	rotten apple, cheesy	Gala	Plotto, 1998
butyl propanoate	fruity, apple	Gala	Plotto, 1998
hexyl butanoate	apple	Gala	Plotto, 1998
hexyl hexanoate	apple	Gala	Plotto, 1998

1.5.2.3 Odour interactions effects on odour perception

It is difficult to estimate the importance and contribution of individual aroma components of a mixture. Attempts have been made to obtain an answer to this question by correlating sensory responses from panellists with gas chromatographic data (Peppard, 1994; Togari et al., 1995). The relative importance of each component identified is estimated from odour thresholds and peak area of chromatograms. Such an approach depends on the presence of compounds whose odour resembles the overall aroma of the product or is similar to an odour characteristic of the product. Odour threshold is assumed to provide a measure of perceived intensity of a component at all concentrations, including supra-threshold concentrations, but odour intensity follows Steven's psychophysical power function: $s = kc^n$; which relates perceived intensity s to stimulus concentration c, n being the slope or intensity exponent and k a constant (intercept on ordinate in log-log plot) (Land, 1979; Laing and Panhuber, 1979). Odour intensity can vary widely between odour compounds, between different concentrations of the same odour and does not account for interactions between odours that may result in odours being altered or masked (Williams, 1994).

The odour threshold of a single compound in water does not always provide an indication of its contribution to overall aroma when it is part of a mixture in an

aqueous solution. Threshold concentrations are limited in usefulness as a measure of perceived aroma, as the absolute threshold does not always reflect the intensity of an odour at supra-threshold concentrations and the intensity of an odour can be varied systematically by varying its concentration. The perceived intensity of a binary mixture is less than the sum of intensities of component compounds, but is more than the average of the two at about 60% of the sum of single components (Laing and Panhuber, 1979). As only 4 individual odourants can be identified accurately and consistently in a mixture, humans therefore have a physiological limitation in their ability to discriminate and identify odours in mixtures (Laing, 1994). This suggests that only four or five volatile compounds are important in the perception of apple aroma. The difficulty is identifying which compounds are important.

Complex aromas of fruit often do not resemble any of the major components (Laing, 1994). The intensity of an odour mixture is almost always less than the sum of perceived intensities of the components (Lawless, 1986). When odours are mixed, one or more of the following effects may be perceived: odour quality of a mixture may differ from individual components; some components may not be detected due to neutralisation or masking by other components; odour intensity of a mixture may be greater, equal to or less than the sum of intensities of individual components; and detection threshold of the mixture may differ from that of individual components. Such effects are reported to be dependent on odourants, concentration and mixing ratios (Lawless, 1986; Laing and Panhuber, 1979). The most common result of mixing odours is that the mixture will have a different threshold and/or intensity to its individual components by: odour enhancement (in mixtures containing subthreshold concentrations of components); odour synergism; enhancement or suppression (concentrations at or just above threshold); odour suppression (supratheshold concentrations); and odour qualities of components do not blend into a new or novel odour quality (Laing and Panhuber, 1979).

More recently the aroma perception of an odourant mixture has been related to the effects of spatial and temporal filtering of odourants as they interact with the olfactory receptor cells (Laing, 1994). Spatial filtering occurs when an odourant reaches the olfactory cells where it stimulates widely distributed cells producing a pattern of responses characteristic for the odourant. When the response patterns for two odours overlap, they compete for receptor sites, changing the pattern of response. Temporal filtering is where different odourants differ in the time they take to reach and stimulate receptor cells, the difference being in the order of hundreds of milliseconds. This suggests that one odourant will be identified before another. A 'fast odourant' may reduce or block perception of a 'slower odourant', suppressing its intensity and altering the characteristic pattern of response (Laing, 1994). As yet there is no information on the speed at which apple volatiles are perceived by olfactory receptor cells. Until the interaction between spatial and temporal filtering for the mixture of apple volatiles has been determined, the effect of changes in volatile concentration on perception of aroma cannot be assessed with confidence.

1.6 Methods of Volatile Analysis

Volatile constituents are distributed throughout the fruit matrix; isolating very low concentrations of volatile compounds from the mix of sugars, carbohydrates, lipids, proteins and water requires selective techniques. Volatile compounds are represented by a large number of chemical classes, and while there are several hundred compounds of interest in fruit, not all contribute equally to aroma or flavour (Takeoka and Full, 1997). Concentration of important compounds may be very low and difficult to quantify by GLC. The human nose has a theoretical odour detection limit of about 10^{-18} moles which is about 10^{6} more sensitive than the best analytical detectors presently available (Heath and Reineccius, 1986). Chemical processes occurring in the sample between the time of collection and analysis can alter volatile concentration. These processes include photo-decomposition, adsorption, evaporation, microbial action and chemical reaction (Takeoka and Full , 1997). No single method of volatile extraction provides a volatile profile that is truly representative of the sample being analysed (Jakobsen, 1997; Jennings and Filsoof, 1977).

1.6.1 Treatment of samples before extraction of volatile compounds

Methods used for analysis of volatiles involve intact tissue or disrupted tissue (Jakobsen, 1997). Before volatiles from fruit juice can be analysed, grinding, homogenisation, centrifugation, filtration or pressing are required to extract compounds of interest (Maarse and Belz, 1981). During grinding or homogenisation, cells are disrupted allowing enzymes to react with substrates which are not normally in contact, forming artifacts by chemical or biochemical reactions. To inhibit enzyme activity, homogenisation in the presence of methanol is usually recommended for apple fruit (Drawert et al., 1966). Thus it is very difficult to know what exists *in situ*, although biting into fresh fruit and homogenisation to produce juice, may both result in humans perceiving aroma volatiles that can be detected using physical methods.

1.6.2 Extraction of volatiles

Volatiles can be extracted from the headspace (vapour phase) surrounding fruit or fruit juice or directly from the fruit (Jakobsen, 1997). Compounds contributing to the odour of a fruit are present at very low concentrations requiring volatiles to be collected from large volumes of headspace air (Maarse and Belz, 1981). Thus accumulation of sufficient volatile concentration is time consuming limiting sample numbers.

1.6.3 Headspace methods

In headspace sampling there are two major techniques: static and dynamic. In the static method the vapour phase is in equilibrium with the aqueous phase, while in the dynamic method vapour equilibrium continuously alters, the carrier gas circulating over the surface of the sample or throughout the sample (Salinas

Fernandez and Alonso Diaz-Marta, 1997). Extracting volatiles from the headspace of intact fruit avoids confounding effects of fruit wounding and artefact formation when the fruit is homogenised. The same fruit can be monitored repeatedly and ethylene and CO_2 production can be measured simultaneously. However the only volatiles measured are those that emanate from the fruit and volatile concentration may be underestimated as they are dependent on the vapour pressure partition co-efficient between fruit and air. Volatile concentrations in the headspace of apple juice are greater than from intact apples (Poll 1985, Sapers et al., 1977) hence small changes that occur during ripening in volatile concentration may not be detected.

1.6.3.1 Direct injection

Headspace analysis is limited by samples with low volumes. Direct injections of headspace gas is limited to 10 mL or less requiring volatiles to exceed concentrations of 10^{-7} g·L^{-1} for GLC and 10^{-5} g·L^{-1} for mass spectrometry (Heath and Reineccius, 1986).

1.6.3.2 Headspace concentration

Concentration of trace amounts of volatiles in the headspace can be achieved using porous polymer traps which are washed with an organic solvent giving a dilute solvent extract. Sodium chloride, is often added to aqueous samples to enhance partitioning of volatiles to the headspace, to inhibit micro-organisms growth and enzyme activity (Murray, 1977). Water is the most abundant compound emanating from fruit making the headspace above fruit or juice mostly water (Heath and Reineccius, 1986). An absorbent such as activated charcoal which has many of the same properties as porous polymers strongly adsorbs non-polar substances and weakly retains water vapour. Activated carbon can adsorb volatiles to an amount equal to its weight but once saturated, compounds with greater affinity displace more weakly adsorbed compounds. Therefore, overloading of the adsorbent can result in atypical volatile profiles. Activated carbon has the advantage over synthetic adsorbents in that it has a very large adsorption capacity, but the probability of artifacts is high as pure forms of activated carbon are difficult to achieve.

Tenax® polymers have become the adsorbent of choice for trapping volatiles over recent years. This polymer has excellent recovery of adsorbed volatiles, is thermally stable when compared to other synthetic polymers and can be used to store samples for long periods (Maga, 1990). These advantages outweigh the poor adsorption capacity of Tenax® relative to activated carbon. Assuming that the Tenax® trap behaves as a GC column, the passage of a previously adsorbed compound through the trap results in its complete removal. The volume of gas required for the adsorbed compound to emerge is known as breakthrough volume; it varies with parameters such as adsorption temperature, volatile concentration and carrier gas flow rate (Salinas Fernandez and Alonso Diaz-Marta, 1997). Although breakthrough volumes of headspace samples are low for Tenax®, after 2.5 h purging apple juice with N_2, total peak area in the first trap of 3 in series was 89.2% of the total volatiles trapped with the 3 traps (Ampun,

1997). Therefore, using a trapping duration of 2 to 4 h, Tenax® has sufficient capacity to adsorb volatile compounds from apple juice without significant losses due to breakthrough. Tenax® retains alcohols poorly, with retention volumes for methanol, ethanol, propan-1-ol, pentan-1-ol and hexan-1-ol being 0.148, 1.8, 8.4, 2.1 and 1.8 L of wine headspace per gram of Tenax® (Salinas Fernandez and Alonso Diaz-Marta, 1997). In contrast, esters have high retention volumes being 35.4, 192, 846 and 117 L of wine headspace per gram of Tenax® for ethyl acetate, propyl acetate, butyl acetate and ethyl propionate, respectively (Salinas Fernandez and Alonso Diaz-Marta, 1997). Tenax® recovers high boiling point compounds poorly as they have low volatility (Heath and Reineccius, 1986). Despite these disadvantages, headspace adsorption methods have excellent reproducibility (Poll and Hansen, 1990); as an example coefficients of variation were 5 to 22% in a dynamic purging system and less than 10% for static headspace (Olafsdottir et al., 1985) and so this technique has become the method of choice for quantitative volatile studies.

Solid phase micro-extraction (SPME) is a new technique for extraction of volatiles (Arthur and Pawliszyn, 1990) involving absorbtion of volatiles onto chemically modified fused silica fibres. There is no need for solvents as this solid support, or fibre, is placed directly into the injection port of a GLC and thermally desorbed. Extraction times are reduced to a few minutes. Sampling using SPME is a single batch process making quantitative adsorption difficult (Yang and Peppard, 1994). Results obtained using SPME depend on experimental conditions and sample matrix, as changes in experimental conditions affect adsorption distribution of volatiles influencing sensitivity and reproducibility of the samples being analysed (Yang and Peppard, 1994). When adsorption duration is standardised, quantitative analysis using SPME can be conducted (Song et al., 1997). Volatiles from samples of cola using dynamic headspace with Tenax® traps were greater in concentration and number of volatiles than samples extracted using SPME (Elmore et al., 1997). Therefore Tenax® traps are still the method of choice when volatiles in trace concentrations are required to be measured (Elmore et al., 1997).

1.6.4 Choice of solvent

Most volatiles have substantial solubility in organic solvents such as diethyl ether and pentane (Heath and Reineccius, 1986). When extracting volatiles directly from samples, polarity of the solvent must match that of the volatile of interest (Maarse and Belz, 1981) and the solvent have a low boiling point (Sugisawa, 1981). The distribution coefficient between solvent and sample should favour the solvent, thereby requiring small volumes of solvent. A comprehensive list of organic solvents used to extract volatiles from food is given by Weurman (1969). Diethyl ether has been used extensively as it is non-selective and extraction efficiency is high; by contrast, hydrocarbon solvents are non-selective, but have low extraction efficiency. Pentane and fluorocarbons have low affinity for ethanol (Williams and Tucknott, 1973). Low molecular weight alcohols (C_1-C_5) are removed from aqueous samples more completely and efficiently by ether than by iso-pentane (Heath and Reineccius, 1986). Extraction of volatiles with

solvents has the potential to introduce impurities from the solvent (Heath and Reineccius, 1986). Solvent impurities may arise from solvent synthesis, unclean glassware or be intentional, such as antioxidants added to diethyl ether. As impurities cannot be removed completely, solvent blanks should always be run to determine impurities (Heath and Reineccius, 1986) and to allow corrections to volatile concentrations (Maarse and Belz, 1981). Impurities must be kept as low as possible as they can accumulate on GLC injectors and slowly degrade to produce volatiles, leading to tailing peaks and ghost peaks (Takeoka and Full, 1997).

1.6.5 Concentration of solvent extracts

Solvent extraction produces a dilute solution of volatiles which requires concentration for analysis by GLC (Heath and Reineccius, 1986). The major problems of concentration is loss of volatile compounds by evaporation and the increase in artefact concentration.

1.6.5.1 Evaporation

Evaporation of the solvent relies on a substantial boiling point difference between volatile compounds and the solvent. This requires the solvent to have a low boiling point, be inert, thermally stable and have high purity (Heath and Reineccius, 1986). During evaporation volatiles may be lost by co-distillation which is not the same for all compounds. Therefore, quantitative results from solvent extracts may be erroneous, even with the use of multiple internal standards, unless corrected for individual losses. Aroma isolates contain small amounts of water requiring removal using dessicants or freezing the solvent before concentration. During concentration in air oxidation of volatiles may occur; this can be minimised by evaporating in an inert atmosphere (Heath and Reineccius, 1986).

1.6.6 Use of disks for studying aroma biosynthesis

Applying precursors to intact apples or tissue disks has been used in an attempt to elucidate the biosynthetic pathways for ester biosynthesis (Berger and Drawert, 1984; Knee and Hatfield, 1981; Paillard, 1979). Disks of cortical or skin tissue have the advantages of: allowing easier ingress of precursors so that these are metabolised quickly reducing incubation times; allowing selective examination of different fruit tissue; and requiring smaller less expensive equipment and less fruit. The disadvantages of disks are that: disks require a buffer system to keep the fruit cells alive which may interact with precursors forming artefacts; may form volatiles from secondary reactions, such as hexanal and *trans*-2-hexenal from fatty acids (Tressl and Drawert, 1973) and wound hormones like methyl jasmonate (Fan et al., 1997); are susceptible to microbial contamination; and disks may release volatiles differently (i.e. not through the skin), than intact fruit preventing results from experiments with disks from being quantitative. Although Red Delicious apple disks and intact fruit metabolised alcohols in the same way (Berger and Drawert, 1984) because of the above disadvantages, most recent researchers use intact fruit (Rowan et al., 1996,1997; Song et al., 1996)

1.7 Objectives

Flavour typical to apples develops during ripening (Tressl et al., 1975) and may be associated with ethylene production and metabolic activity (Song and Bangerth, 1996). The greatest concentrations of volatiles are produced at the climacteric peak in ethylene production (Mattheis et al., 1991b; Sapers et al., 1977, Song and Bangerth, 1994; Yahia et al., 1990a). However, it is unknown whether the enzymes involved in volatile biosynthesis are induced during the climacteric or are constitutive. If some or all enzyme systems involved in volatile biosynthesis are induced during ripening, then volatile changes after exposure to hypoxia could differ according to stage of ripeness. If enzymes are constitutive then it is likely that volatile changes would be similar at all stage of ripeness assuming precursors were not limiting. Changes in volatile concentration after exposure to hypoxia of apples at different stages of ripeness has not been determined. Storage at low temperatures is a commonly used method for slowing ripening to allow transport over long distances to markets or for delaying marketing of fruit to achieve higher returns (Wills et al., 1997). Volatile production is considered to be proportional to temperature, the higher the temperature, the greater the production of volatiles (Wills and McGlasson, 1971; Guadagni et al., 1971; Fallik et al., 1997). Temperature may also affect production of specific volatiles with some compounds only produced at certain temperatures by affecting rates substrate supply and volatile biosynthesis. If this is so then the different biosynthetic pathways producing volatiles may be active at different rates according to temperature. It is currently not known if the activity of enzymes used in volatile synthesis are thermally labile or if storage at low temperatures reduces or enhances apple capacity to produce volatile compounds after exposure to hypoxia. If exposure to hypoxia changes the volatile biosynthetic pathway then hypoxic conditions imposed at different temperatures could change the type of volatile compounds which increase after hypoxia.

Exposing apples to hypoxic conditions induces changes in volatile concentrations; acetaldehyde and ethanol accumulate to high concentrations and after return to aerobic conditions ethyl esters are enhanced and non-ethyl esters are decreased (Ampun, 1997; Mattheis et al., 1991a). Differences in proportions of volatile compounds exist between cultivars (Dirinck and Schamp, 1989; Paillard, 1990) as does degree of ethyl ester enhancement (Ampun, 1997). Possible reasons for these differences might include separate iso-forms of AAT and ADH in each cultivar with their own substrate specificity for substrates or alcohol precursors being available in varying concentrations; hypoxia may induce increased activity or synthesis of AAT and ADH depending on culitvar.

Therefore there were four objectives of this study:

1) To determine the mode of action of hypoxia on enhancement of ethyl esters and decrease in non-ethyl esters by utilising the hypotheses of competitive inhibition by enhanced ethanol concentrations of non-ethyl esters and hypoxia induced new ester forming isozymes of AAT.

2) To determine the influence of environmental conditions on the magnitude of enhancement of ethyl esters after exposure to hypoxia, specifically examining treatment temperature and time at 0 °C before treatment.

3) To examine the effect of physiological factors on efficacy of treatment with hypoxia in relation to cultivar, ripeness as phase of the climacteric, and non-cooled versus cooled apples.

4) To determine organoleptic changes in apple aroma induced by exposure to hypoxia.

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Chapter Two

General Materials and Methods

Qualitative and quantitative changes in fruit aroma volatiles were studied using apple (*Malus domestica* Borkh) cultivars Cox's Orange Pippin (CO), Fuji (FU), Golden Delicious (GD), Granny Smith (GS), Pacific Rose™ (Sciros, PR), Red Delicious (RD), Royal Gala (RG) and Southern Snap™ (Sciglo, SS), harvested during February to May, from commercial orchards in Hawkes Bay, New Zealand. Splendour apples were sourced from the Massey University Fruit Crops Unit, Palmerston North. Measurements common to different experiments are outlined below. Detailed descriptions of materials and methods for specific experiments are outlined in appropriate chapters following.

2.1 Fruit Selection and Supply

Apples were obtained through ENZA™ New Zealand (International), a division of the New Zealand Apple and Pear Marketing Board. In all years, apples were picked at mid-commercial harvest, as determined by ENZA, graded to export standard and packed to count 100-125 (individual fruit weight was in the range 148-188 g fresh weight at packing) and transported to Massey University by road, giving up to 3 d from harvest to receipt. Upon receipt fruit were either placed into coolstore at 0°C or placed at 20 °C depending on timing of treatments.

2.2 Temperature Control

After treatments, fruit were placed in a room where temperature and relative humidity (RH) were maintained at 20 °C ± 3 °C and 60% ± 5% using an air conditioning unit (Toshiba model RAS-30SKH(W), Toshiba Corporation, Japan). A coolstore at 0 °C ± 1 °C and RH 80% was used for long term storage. Where required, fruit were stored in temperature controlled rooms at 10 °C ± 1 °C, 15 °C ± 1 °C and 25 °C ± 1 °C, RH 85%.

2.3 Carbon Dioxide Treatment

Fruit were placed into 24.1 L perspex chambers connected to a manifold leading from a high pressure cylinder of pure carbon dioxide (CO_2; BOC Gases New Zealand Ltd., New Zealand). Carbon dioxide was humidified by bubbling through water before passing into the manifold. During treatment, chambers were purged continuously for 24 h at 20 °C ± 3 °C. Outlets from each chamber were joined and the outflow was bubbled through a jar of water to create enough back pressure to maintain constant CO_2 flowrates for all chambers. Initial CO_2 flowrate in each chamber was 2.5 $L \cdot min^{-1}$ (6.2 air changes per h) for 2-3 h after which the flowrate was reduced to about 1 $L \cdot min^{-1}$ (2.5 air changes per h). Excess gas was vented to outside air. The CO_2 and O_2 levels within chambers were monitored every few minutes initially and every few hours throughout each experiment and before terminating the treatment, by taking a 1 mL gas sample for analysis by gas chromatography (GC). Within 4 h of starting to purge, O_2 level in

chambers had declined to < 0.5% and remained below 0.5% throughout CO_2 treatment.

2.4 Fruit Juice

Before juicing, fruit were weighed, CO_2 and ethylene production measured and firmness recorded using a penetrometer. Apples were cut longitudinally into two pieces then each of these was cut into four pieces which were homogenised in a domestic juicer (Kenwood Centrifuga, model JE500, Kenwood Appliances, NZ). In 1994, juice was collected in a beaker on ice before solvent extraction. In subsequent years, juice was collected and left at ambient (20 °C ± 3 °C) for at least 30 minutes (section 3.14.3) before solvent extraction.

2.5 Measurements of Quality

2.5.1 Firmness

Firmness was measured on pared surfaces at opposite sides of each fruit at the equator using a hand held Effigi penetrometer (model FT327) with a 11.1 mm (5/8") head (Harker et al., 1996). The mean of the two measurements was multiplied by 9.81 to convert kilograms force to Newtons.

2.5.2 Total Soluble Solids

Total soluble solids levels were measured using a hand-held, non-temperature compensated Atago N20 refractometer on juice squeezed from pieces of flesh cut from each side of apple fruit or juice produced by a domestic juicer.

2.5.3 Titratable Acidity

Titratable acidity of apple juice was determined by titration against 0.1 N sodium hydroxide, using a Mettler DL21 Autotitrator equipped with a Mettler DG111 pH electrode, to the endpoint of malic acid (pH 7.2) for 1 mL of apple juice diluted in 49 mL of distilled water. Sodium hydroxide concentration was checked by titrating against a 0.1 N hydrochloric acid solution (Convol solution, BDH chemicals) to an endpoint of pH 7. A sample of distilled water used to dilute the fruit juice was titrated to pH 7.2. The volume of sodium hydroxide used to neutralise the distilled water was subtracted from the subsequent volume of sodium hydroxide used to neutralise the juice. Initial pH of apple juice was recorded before titration commenced. Percentage equivalent malic acid was calculated as described in section A1.1, Appendix 1.

2.5.4 Weight Loss

Fruit were weighed at all sampling times on a Mettler top pan electronic balance (PE3600) to two decimal places. Differences in weight were used to calculate weight loss as a percentage according to section A1.2, Appendix 1.

2.6 Respiration and Ethylene Production

2.6.1 Internal atmosphere

Direct sampling of internal atmospheres of apples was conducted as described by Dadzie (1992) and Banks (1983). Fruit were submerged in water at 20 °C where a 1 mL disposable hypodermic syringe fitted with a stainless steel canula (20 gauge, 38 mm long, Luer fitting) occluded by the head of an insect mounting pin (to prevent blockage) was inserted through the calyx into the core cavity. Dead air space in the syringe was replaced with water. Once the syringe was in the core cavity it was withdrawn slightly to allow gas to move past the pin head before about 1.2 mL of internal atmosphere was withdrawn slowly. The syringe was removed from the canula and while under water a rubber septum (the plunger tip of another plastic syringe) was used to cap the end of the sample syringe. The gas sample in the syringe was transferred to a fresh syringe while under water. Excess water was wiped off the needle and syringe with a paper tissue before the sample was injected into a GC for analysis of CO_2 or ethylene (C_2H_4) (as described in Section 2.8).

2.6.2 Carbon Dioxide Production and Ethylene Production

Four groups each of three apples randomly selected from treatments, were weighed and placed into 1.6 L or 1.8 L glass preserving jars at 20 °C ± 3 °C. Each jar was fitted with a lid equipped with a septum (a rubber bung from a Vacutainer™ blood collection tube). Jars were sealed and the time noted. A pair of empty jars was sealed at the same time as blanks representing CO_2 and C_2H_4 concentrations in surrounding air when jars were sealed. In experiments on ripeness, jars were placed under water at 20 °C to guarantee an air tight seal. Individual jars were sampled by taking 1 mL of gas using a plastic syringe (1 mL Graduated Monoject® syringe with detachable needle, 25 gauge x 5/8", Sherwood Medical, MO, USA) 15-30 min after sealing which was then analysed for CO_2 or C_2H_4 by GC. No more than four jars were sampled at any one time to reduce the delay between sampling and analysis to a minimum. Quantification of CO_2 or C_2H_4 in gas samples was achieved by comparison with authentic standards of known composition, provided as certified mixtures by BOC Gases, New Zealand Ltd. Carbon dioxide and ethylene production were calculated according to Banks et al. (1995); the procedure used is outlined in Appendix 1, sections A1.3 and A1.4.

2.6.3 Headspace Volatiles

Acetaldehyde, ethyl acetate and ethanol were collected from the headspace of apple juice at 30 °C. Samples of 30 mL of apple juice were placed into 50 mL glass Erlenmeyer flasks which were sealed with Suba-Seal® (No. 33) rubber stoppers and placed into a water bath at 30 °C. Flasks were equilibrated for at least 15 min before a 1 mL gas sample was taken from the flask headspace and injected into a GLC (Section 2.8). Quantification of the gases was by comparison with authentic gases made to a concentration of 100 $\mu L \cdot L^{-1}$ in a 1.8 L

glass preserving jar (Section A1.5, Appendix 1). Experiments on skin disks used a combined headspace standard by adding liquid volumes of compounds to a sealed 1.8 L glass jar according to Section A1.6, Appendix 1.

2.7 Aroma Volatiles

2.7.1 Solvent Extraction

Volatile compounds were extracted from apple juice using a diethyl ether:*n*-pentane solvent mixture (2:1 v/v, Analar BDH; Larsen and Poll, 1990). Two 10 mL aliquots of juice were each placed into separate 20 mL scintillation vials (Wheaton Scientific, NJ, USA) fitted with a metal foil liner cap. The internal standard (IS), consisting of 0.2 µL octyl acetate (Aldrich Chemical Company, WI, USA), was added to each juice aliquot before an equal volume of solvent mixture was added. Vials were capped tightly and mixed with a Vortex stirrer for 3-5 s before storage at -18 °C until the aqueous phase was frozen. The unfrozen solvent phase was decanted into a clean 20 mL scintillation vial and the aqueous phase was discarded. The diethyl ether:*n*-pentane solvent extract was dried from 20 mL to about 200 µL (100 fold concentration) and placed into a glass 250µL flat bottom glass insert (Sun International Trading Cat. no. 200 232) in a 1.5 mL glass screw top autosampler vial (Sun International Trading Cat. no. 200 250) suitable for a Hewlett Packard 5890 series II plus GLC autosampler (Hewlett Packard 7673 Controller and Injector and Model 185968 100 sample carousel). Vials were sealed with a plastic septum (Sun International Trading Cat. no. 200 368) before placement in the carousel. One µL samples were injected into the GLC after which the syringe was washed 8 times with clean diethyl ether:*n*-pentane between each injection (Section 2.8). Quantification of volatiles in the solvent extract was by comparison with authentic compounds made to a concentration of 200 $\mu L \cdot L^{-1}$ in the solvent mixture (Figure 2.1).

Each year selected solvent extracts and a mixture of authentic standards were analysed by GC-mass spectrometry at HortResearch, Palmerston North using the same capillary column as used at Massey University. Retention times of peaks identified by mass spectrometry were used to confirm the identity of volatile compounds in the standard and GLC traces of samples analysed at Massey University.

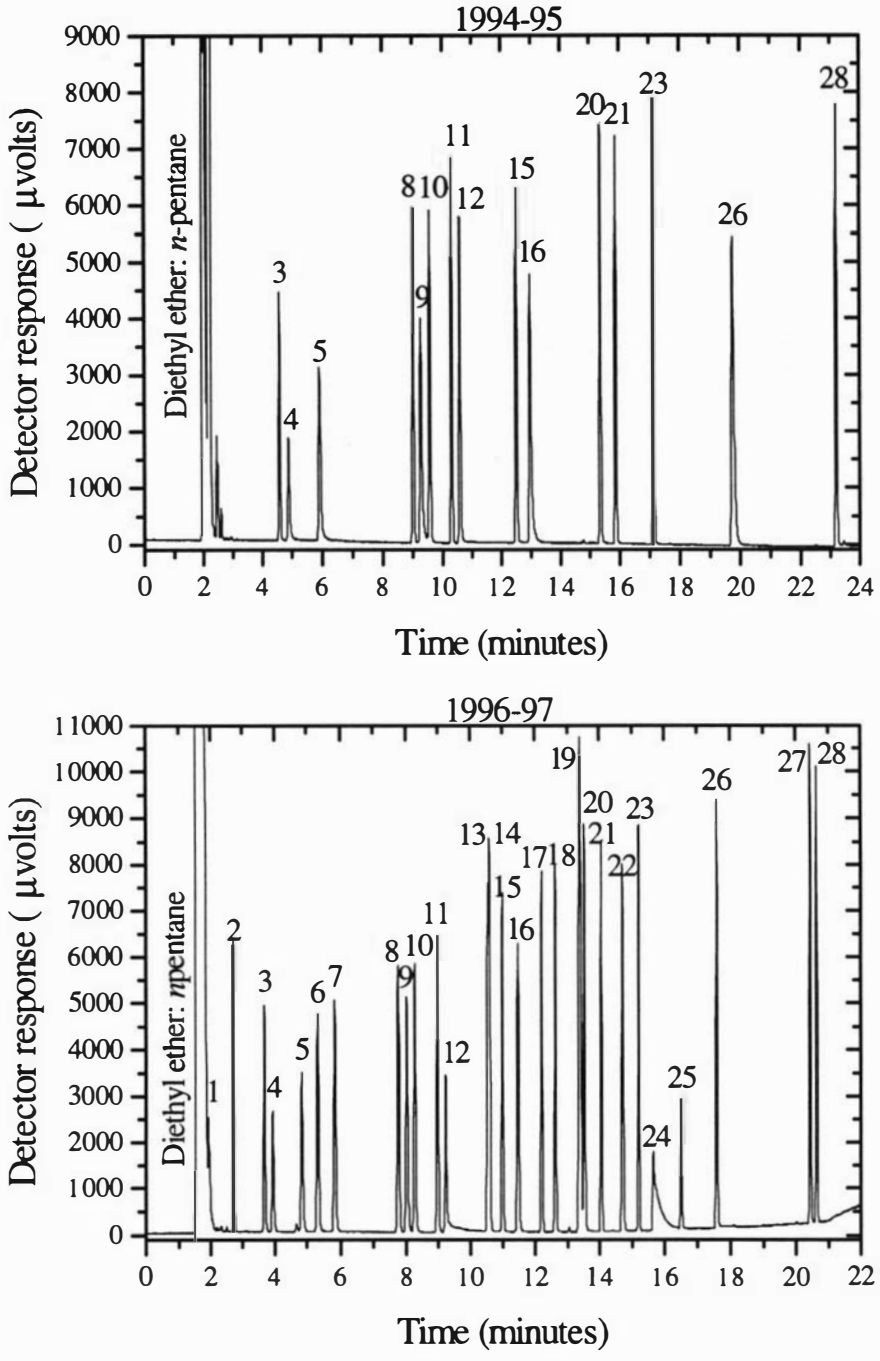

Figure 2.1. Gas chromatographic traces of volatile standard mixtures in diethyl ether:*n*-pentane in 1994-95 and 1996-97. Volatile compounds were: 1 = acetaldehyde, 2 = acetone, 3 = ethyl acetate, 4 = methanol, 5 = ethanol, 6 = ethyl propionate, 7 = propyl acetate, 8 = ethyl butanoate, 9 = propan-1-ol, 10 = ethyl-2-methyl butanoate, 11 = butyl acetate, 12 = hexanal, 13 = 2-methyl butyl acetate, 14 = propyl butanoate, 15 = ethyl pentanoate, 16 = butan-1-ol, 17 = pentyl acetate, 18 = methyl hexanoate, 19 = 2&3 methyl butan-1-ol, 20 = *trans*-2-hexenal , 21 = ethyl hexanoate, 22 = pentanol, 23 = hexyl acetate, 24 = octanal, 25 = 4-methyl pentan-1-ol, 26 = hexan-1-ol, 27 = 6-methyl-5-hepten-2-ol, 28 = octyl acetate.

2.7.2 Purge and Trap

The purge and trap technique involves traps that contain porous polymer beads which selectively absorb organic compounds from air. Compounds trapped on polymer beads are washed off using organic solvents. The volume of solvent is then reduced using nitrogen to concentrate volatile compounds sufficiently for analysis by GLC. This technique involves preparation of traps containing an absorbant polymer Tenax®, use of the traps and elution of volatile compounds in preparation for GLC.

2.7.2.1 Tenax® trap preparation

Glass tubes 3 mm i.d. x 300 mm were washed with diethyl ether and oven-dried before a small piece of silane treated glass wool (dimethyl dichlorosilane-treated, Alltech cat no. 4037) was inserted about 50 mm from one end. Four hundred mg of 60/80 mesh Tenax® GR (2,6-diphenyl-p-phenylene oxide and activated charcoal, Alltech cat no. 4937) was poured into a glass tube vibrated by a Vortex stirrer to ensure tight packing of beads. Another small piece of silane-treated glass wool was used to plug the glass tube. Before use, columns were rinsed four times with about 4 mL diethyl ether, forced through with a low flow rate of O_2-free N_2 (about 50 mL·min^{-1}). Following washing, columns were dried at ambient while being flushed with O_2-free N_2. Columns were preconditioned and re–conditioned by heating to 220°C to 230°C for at least 2 to 3 h while being constantly purged by O_2–free N_2 at about 20 mL·min^{-1}. After conditioning columns were cooled to ambient, capped and sealed with parafilm (American National Can, USA).

2.7.2.2 Elution and Concentration of Samples on Tenax® traps

Volatile compounds were washed off Tenax® columns by repeatedly eluting, 1 mL at a time, with 4 mL of diethyl ether:pentane (2:1 v/v) solvent mixture. Solvent was pushed through the column with a low flow rate of O_2-free N_2 and collected in a 4.5 mL autosampler vial. Columns were re–conditioned as described in Section 2.7.2.1. Solvent eluted from Tenax® traps had 0.5 µL of octyl acetate added, and were then concentrated to about 200 µL using O_2-free N_2 before analysis by GLC.

2.7.3 Calculation of volatile concentration in juice samples

Volatile concentration in juice samples was calculated from the concentration measured in solvent extracts corrected for losses in the solvent extraction process (Section A1.8, Appendix 1).

2.7.4 Calculation of headspace volatile concentration

The vapour pressure of a solute in solution depends on its molar concentration in the liquid. This relationship is linear over a wide range solute concentrations in a liquid (Nawar, 1966) and can be used to estimate solute concentration from

headspace concentration. Thus it was possible to calculate concentration of volatiles from their levels in headspace samples of juice using appropriate air-water partition co-efficients (Section A1.9, Appendix 1; Buttery et al., 1969).

2.8 Gas Chromatography

Gas chromatography was used to measure CO_2, C_2H_4, acetaldehyde, methanol, ethyl acetate, ethanol, propan-1-ol, propyl acetate, butan-1-ol and butyl acetate in the headspace over juice or skin disks and aroma volatiles of apple juice from solvent extracts. Details of extraction and collection of samples are outlined above.

2.8.1 Carbon dioxide

One mL samples of gas were measured using a thermal conductivity detector at 60 °C and 90 mA current in a Shimadzu GC 8A GC equipped with an Alltech CTR I column (Alltech Cat. no. 8700) at 30 °C with hydrogen carrier gas at a flow rate of 30 $mL \cdot min^{-1}$. The Alltech CTR I column is a dual column, outer column 1.83 m x 6.35 mm packed with an activated molecular sieve, the inner column 1.83 m x 3.18 mm packed with porapak mixture.

2.8.2 Ethylene

One mL samples of gas were measured using a flame ionization detector (FID) at 150 °C, column at 110 °C and injector at 190 °C in a Shimadzu GC 4B-PTF gas chromatograph equipped with a F-1 grade, 80/100 mesh activated alumina in a 1.83 m x 3.18 mm column (Alltech Cat. no 80072). Nitrogen at a flowrate of 30 $mL \cdot min^{-1}$ was used as the carrier gas. The flame was maintained with hydrogen at 30 $mL \cdot min^{-1}$ and air at 300 $mL \cdot min^{-1}$.

2.8.3 Headspace volatiles (acetaldehyde, ethyl acetate, ethanol)

One mL samples of gas were measured using a FID at 180 °C, column at 45 °C and injector at 110 °C in a PYE UNICAM gas chromatograph fitted with a 10% Carbowax 20M coating on 80/100 Chromosorb WAW support in a 1.83 m x 3.18 mm stainless steel column (Supelco Cat. no 1-2212). Nitrogen at a flowrate of 30 $mL \cdot min^{-1}$ was used as the carrier gas. The flame was maintained with hydrogen at 30 $mL \cdot min^{-1}$ and air at 300 $mL \cdot min^{-1}$.

2.8.4 Aroma volatiles and Headspace Volatiles for Skin Disks

One µL of solvent extract was measured by capillary gas chromatography using a Hewlett Packard 5890 Series II Plus gas chromatograph equipped with a FID connected to an IBM-compatible personal computer equipped with Hewlett Packard ChemStation software (version B.02.04). The capillary column was a J&W 30 m x 0.32 mm (i.d.) fused silica, DBWAX, 0.5 µm film thickness (Alltech Cat. no 93526). Injector and detector temperatures were 150 °C and 250 °C, respectively. In 1994-96 the oven temperature was held at 40 °C for 5

minutes, then programmed to 190 °C at 5 °C·minute^{-1} and held at 190 °C for 10 minutes making a total run time of 45 minutes. In 1996 the temperature programme was changed: oven temperature was held at 40 °C for 5 minutes, then programmed to 120 °C at 5 °C·minute^{-1} then to 190 °C at 20 °C·minute^{-1} with no holding time making a total run time of 24.5 minutes. Hydrogen was used as carrier gas with a linear flow rate of 30 cm·s^{-1}. Split injection mode was used with a split flow rate of 100 mL·min^{-1}, split ratio of 15:1. Septum purge flow rate was 5-6 mL·min^{-1}. Air and hydrogen flow rates to the detector were 400 and 30 mL·min^{-1}, respectively. For headspace analysis one mL of headspace gas (air) over skin disks was used. The column was held at 70 °C for 6 minutes with carrier gas and detector gas flowrates as for solvent samples. The FID detector used had a linear response over the range of volatile concentrations found in solvent extracts (Figure 2.2).

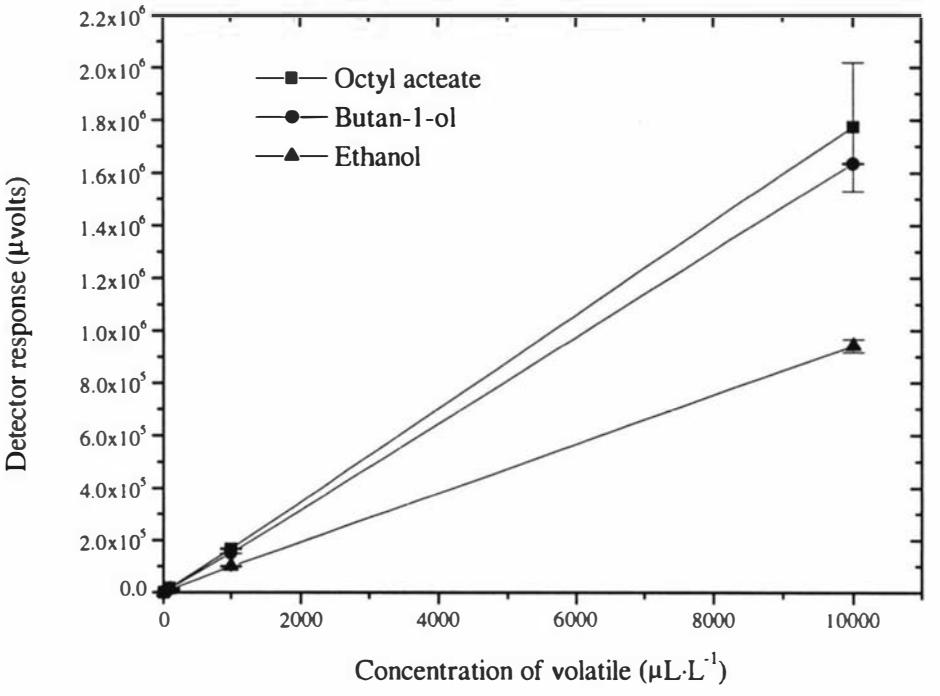

Figure 2.2. Linear relationship between peak area and concentration of selected volatiles in diethyl ether:*n*-pentane (2:1 v/v). Regression values were: octyl acetate y = 177.37·x, r^2 = 0.99996, P<0.001; butan-1-ol y = 163.41·x, r^2 = 0.99991, P<0.001; ethanol y = 94.22·x, r^2 = 0.99996, P<0.001

2.9 Data analysis

Details of data analysis techniques used are described in each chapter in the experimental descriptions.

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The following chapter contains data gathered by Dr Wirut Ampun as part of his PhD dissertation 'Enhancement of aroma volatile compounds in apples'. This text is highlighted thus to indicate that the data presented is NOT the authors own work. It is included in this paper draft for completeness as Dr Ampun's PhD was a parallel study on a similar topic to this thesis.

Chapter Three

Solvent extraction: A simple technique for extracting volatile compounds from apple juice.

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Additional index words. Diethyl ether; pentane; solvent extraction; aroma volatiles.

3.1 Abstract

Volatile compounds can be effectively extracted from 20 mL aliquots of apple juice with an equal volume of diethyl ether:*n*-pentane (2:1 v/v), vigorous stirring for 3-5 seconds, freezing at -18 °C to separate solvent and aqueous phases, concentration with a fast stream of oxygen free nitrogen (200 $mL \cdot min^{-1}$) to 200 µL and analysis by gas chromatography. Apple juice could be held on ice or in air at 20 °C up to 256 minutes without loss of volatile compounds. Loss in volatiles for solvent washes was 76.5% for octyl acetate and recoveries during concentration of the solvent extract ranged from 2.5% for ethyl acetate to 86.4% for *trans*-2-hexenal. Solvent extraction compared to dynamic headspace using Tenax® was simpler, faster, extracted more compounds, and had better reproducibility than dynamic headspace extracts.

3.2 Introduction

Aroma volatiles of apples comprise about eight chemical classes but compounds responsible for a particular aroma/flavour attribute are not easily identified (Erikkson, 1979). Some compounds present in low concentrations ($<0.1\ \mu L \cdot L^{-1}$) may be of critical importance for a given flavour attribute (Berger, 1991). Perceived aroma of a product is an integrated response to several compounds and variations of the relative ratios and/or concentrations of these compounds may change aroma in a manner that is unpredictable (Jennings, 1977; Maarse, 1991). At present there is no definitive list of aroma volatiles that characterises apple aroma or flavour despite decades of research. Composition changes of volatiles are frequently induced during extraction and preparation of juice samples to a form suitable for gas liquid chromatography (GLC) analysis (Maga, 1990). As the human olfactory system can detect many volatiles at much lower concentrations than can be detected using a GLC, samples must be concentrated prior to GLC analysis (Heath and Reineccius, 1986).

No current sample preparation procedure for GLC analysis can be considered ideal for extracting volatiles from fruit juice. Extraction methods are chosen depending on the sample composition and compounds of interest (Bemelmans, 1979;1981; Jennings, 1977; Maarse, 1991; Maga, 1990; Teranishi and Kint, 1993; Weurman, 1969). Most procedures use distillation to separate volatile fractions from a non-volatile food matrix, and a favourable distribution co-efficient of volatility between the extractant and sample. The method used for extraction influences the composition of volatiles detected (Fischer et al., 1995; Kakiuchi et al., 1986; Larsen and Poll, 1990).

Dynamic headspace, referred to as purge and trap, is frequently used to extract volatile compounds from samples (Maga, 1990). This procedure uses air or an inert gas (such as nitrogen (N_2) or helium) to flush over or pass through a sample for a specific time, and volatile compounds are trapped onto absorbent materials (Bemelmans, 1981). In recent years Tenax® has become the adsorbent of choice for studies on volatile compounds (Maga, 1990). Tenax® is reported to have excellent recovery of adsorbed volatiles, is thermally stable when compared to other synthetic polymers and can be used to store samples for long periods, though it has poor adsorptive capacity relative to other methods and adsorbents (Maga, 1990). To improve extraction of volatiles, adaptations of this purge and trap technique have been developed and have included a 'falling film sampler' (Rivier et al., 1990) and 'solid phase micro extraction' (SPME) (Arthur and Pawliszyn, 1990; Song et al., 1996; Yang and Peppard, 1994). Thermal desorption, washing with an organic solvent or simultaneous distillation and adsorption are commonly used to remove volatiles from adsorbent traps (Cole, 1980; Schreier, 1984; Sugisawa, 1981). The purge and trap technique is limited by low affinity of absorbent materials for alcohols (Maga, 1990; Sugisawa, 1981) so volatiles trapped on an adsorbent column may not reflect the true aroma of a sample (Bemelmans, 1981; Maarse, 1991).

Aroma volatile compounds are non-polar or slightly polar, making them more soluble in organic solvents than in water (Bemelmans, 1981; Maga, 1990; Wuerman, 1969). Most volatile compounds can be effectively separated from juice samples by direct contact with a suitable solvent. The ideal solvent extraction method for volatiles should remove the major part of all compounds of interest from the aqueous sample, be easily separated from water, concentrate without loss of volatile compounds, and be non-toxic (Larsen, 1993). This may be either as liquid-liquid extraction or combined with distillation and is limited to homogenised and aqueous materials (Maga, 1990). Most organic solvents are non-polar, are selected for their extraction capacity and selectivity for all volatile compounds of interest (Larsen, 1993), and should also have low boiling points to allow easy concentration by evaporation (Weurman, 1969). A wide range of organic solvents has been investigated (Weurman, 1969). Diethyl ether has a high extraction efficiency for volatile compounds, especially C_1-C_5 alcohols (Sugisawa, 1981). Pentane has also been used in many studies as it is non-polar and has a low affinity for ethanol (Sugisawa, 1981). Solvent mixtures of *n*-pentane:methylene chloride (2:1) for apple juice (Drawert et al., 1966) and

diethyl ether:*n*-pentane (2:1) for strawberries, raspberries and apples (Larsen and Poll, 1990; Pyysalo et al., 1979) have also been used.

In experiments involving volatile analysis of large numbers of samples within a limited time, a technique with short extraction times is required. Dynamic headspace extraction requires at least one hour (Sugisawa and Hirose, 1981), solvent extraction involving distillation requires at least 30 minutes (Schultz et al., 1977). A liquid-liquid solvent extraction reported for pulp and juice of strawberries, apricots, oranges and apples takes less than 5 minutes (Larsen and Poll, 1990; Larsen, 1993). This method may have the potential to be a useful extraction technique for large numbers of samples. This study investigated some factors influencing extraction and isolation of volatile compounds from apple juice by solvent extraction and compared the extraction efficiency to that of dynamic headspace.

3.3 Materials and Methods

3.3.1 Preparation of juice

Apples were equilibrated to 20 °C before juicing. Each batch of apple juice was prepared by homogenising several apples with a domestic centrifugal juicer (Kenwood Centrifuga, model JE500, Kenwood Appliances, NZ). In some experiments juice was filtered through 2 layers of cheesecloth and placed into an ice bath or in other experiments left standing at room temperature (20 °C ± 3 °C) before volatiles were extracted.

3.3.2 Solvent extraction

Volatile compounds were extracted from apple juice using a diethyl ether:*n*-pentane solvent mixture (2:1 v/v, Analar BDH; Larsen and Poll, 1990). Two 10 mL aliquots of juice were each placed into separate 20 mL scintillation vials (Wheaton Scientific, NJ, USA) fitted with a metal foil liner cap. The internal standard (IS) consisted of either 10 µL of 2000 $\mu L \cdot L^{-1}$ octyl acetate in the solvent mixture or 0.4 µL octyl acetate (Aldrich Chemical Company, WI, USA) added to juice before an equal volume of solvent mixture. Vials were capped tightly and mixed with a Vortex stirrer for 3-5 seconds before storage at -18 °C until the water phase was frozen. The unfrozen solvent phase was decanted from the water phase which was discarded. If necessary the solvent phase was centrifuged at 500-1000 rpm for 5-10 minutes (Gallenkamp Junior Centrifuge, CF430, England) to remove particulate matter.

3.3.3 Dynamic headspace

Traps were prepared using 400 mg of 60/80 mesh Tenax GC® (Alltech, , USA), packed in a clean oven-dried glass tube (3 mm i.d. and 300 mm length) and was secured in place with small pieces of silanized glass wool at each end. The column was preconditioned by rinsing with about 4 mL of solvent mixture at room temperature (about 20 °C), heated to 280 to 300 °C for at least 2 hours, and

purged with oxygen-free N_2 (about 20 mL·min^{-1}). Columns were capped and stored in a cool dark place until use. One hundred mL of juice was placed into a 250 mL glass Dreschel gas washing bottle equipped with a glass sintered head. About 0.5 g NaCl and an IS of 10µL of 25000 µL·L^{-1} octyl acetate in the solvent mixture was added to juice. Volatile compounds were collected with a Tenax® trap by purging juice with oxygen-free N_2 (50 mL·min^{-1}) for 2 hours at 20°C. Volatiles were washed off traps using 4 mL of solvent mixture with the aid of oxygen-free N_2.

3.3.4 Concentration of solvent extract

Solvent extracts were concentrated to about 200 - 250 µL (80 - 100 fold) at room temperature using a fast (200 mL·min^{-1}) stream of oxygen-free N_2 (Larsen, 1993). The concentrated solvent was transferred to a 250 µL glass, flat bottom insert (Sun International Trading cat no. 200 232) for a 1.5 mL glass autosampler vial (Sun International Trading cat no. 200 250) using a clean Pasteur pipette. The vials were capped with a plastic septum (Sun International Trading cat no. 200 368) and solvent extracts analysed by GLC.

3.3.5 Gas chromatography

One µL of concentrated solvent sample was analysed using a FID fitted to a GLC (Hewlett Packard, HP5890 Series II Plus, Hewlett Packard, , USA) equipped with a capillary column (DB-WAX, 0.32 mm i.d. x 30 m, 0.5 µm film thickness, J&W Scientific, USA). Temperature programme of the column was 40 °C for 5 min, increasing to 130 °C at 5 °C·min^{-1}, to 190 °C at 20 °C·min^{-1}, holding at 190 °C for 5 min. Injector and detector temperatures were 150 °C and 250 °C, respectively. The injector was set to a split ratio of 15:1. Hydrogen carrier gas flow rate was 1.2 mL·min^{-1} (linear velocity 30 cm·min^{-1}) set on constant flow mode with initial pressure of 29 kPa at 40 °C. Flow rates of hydrogen and air for the detector were 30 and 400 mL·min^{-1}, respectively.

3.3.6 Mass spectroscopy

Electron impact GC-MS were recorded on a VG70-250S double focusing magnetic sector mass spectrometer with ionization potential of 70 eV, mass spectral interface 180 °C with 1 s scans and 0.2 s delay with GC conditions as above. Aroma volatiles were identified by computer library matching of the mass spectra (NIST and Wiley databases; Rowan et al., 1996).

3.3.7 Volatile identification and analysis

Compounds of interest were identified by comparison of retention times with those of authentic compounds. These were confirmed by mass spectroscopy of selected solvent extracts. Concentrations of volatiles were calculated from external standards (200 µL·L^{-1}, in diethyl ether:*n*-pentane, 2:1 v/v) used for calibration. Volatile concentrations were normalised against the octyl acetate internal standard and were expressed as µmol·L^{-1}.

3.3.8 Experiments

Testing of the extraction procedure was conducted in a stepwise manner rather than as a multi-factorial experiment. This allowed examination of different conditions to identify the most suitable conditions for volatile extraction.

3.3.8.1 Experiment (I a): Juice holding time at 20 °C or at 0 °C in an ice bath

Apple juice from controlled atmosphere (CA) stored FU apples (20 weeks after harvest, 2% CO_2 + 2% O_2, 0.5 °C, 92-95% RH) was used in a 2 x 5 factorial completely randomised design (CRD) experiment where juice was held at 20 °C or in an ice bath. Holding times were: 0, 0.5, 1, 1.5 and 2 hours after which volatiles were extracted using solvent extraction.

3.3.8.2 Experiment (Ib): Juice holding time at 20 °C before placement into ice before solvent extraction

Apple juice from freshly harvested RG apples (7 d at 0 °C) was used in a CRD in which four replicates of juice were sampled at 0, 2, 4, 8, 16, 32, 64, 128 and 256 minutes after juicing, placed into an ice bath until the 256 minute sampling when volatiles were extracted using solvent extraction. In this experiment, all samples were analysed 256 minutes after homogenisation, with this total time being broken up into a period at 20 °C in air and a subsequent period at 0 °C on ice.

3.3.8.3 Experiment (Ic): Juice holding time at 20 °C before immediate solvent extraction

Apple juice from freshly harvested FU apples (7 d at 0 °C) was used to repeat experiment (Ib) except that volatiles were extracted immediately at each sampling time by solvent extraction.

3.3.8.4 Experiment II(a): Volumes of juice and solvent to extract

Five volumes, 10, 20, 30, 40 and 50 mL, of RD apple juice, spiked with 1 $\mu L \cdot L^{-1}$ of 4-methyl-1-pentanol, four replicates of each, were analysed by solvent extraction with equal volumes of solvent mixture and concentrated to about 200 µL before analysis by GLC. This resulted in solvent extract concentrated 50, 100, 150, 200 and 250 fold.

3.3.8.5 Experiment II(b): Ratio of solvent volume to juice volume

Juice from three groups of 10 RD apples was left to stand in air at 20 °C for 30 minutes. An internal standard of 4-methyl-1-pentanol (1 $\mu L \cdot L^{-1}$) was added to the juice. Five ratios of solvent volume to juice volume were: 0.5, 1, 2, 4 and 8 with a constant juice volume of 20 mL. Solvent extracts were concentrated to about 200 µL before analysis by GLC.

3.3.8.6 Experiment II(c): Ratio of juice volume to solvent volume

Juice from three groups of 10 RD apples was left to stand for 30 min in air at 20 °C. An internal standard of 4-methyl-1-pentanol (1 $\mu L \cdot L^{-1}$) was added to the juice. Five ratios of juice volume to solvent volume were: 0.5, 1, 2, 4 and 8 with a constant solvent volume of 20 mL. Solvent extracts were concentrated to about 200 µL before analysis by GLC.

3.3.8.7 Experiment III: Recovery of volatiles

Four replicates of SP apple juice spiked with octyl acetate at 20 $\mu L \cdot L^{-1}$, volume 20 mL, were washed with an equal volume of solvent mixture and frozen at -18 °C five times. An additional four replicates of juice were washed with an equal volume of solvent mixture five times without freezing the juice-solvent mixture. Solvent extracts were concentrated to about 200 µL before analysis by GLC.

3.3.8.8 Experiment IV: Concentration factor for solvent extracts

Solvent extracts (2 replicates) of RD apple juice obtained by solvent extraction were concentrated from 20 mL to 0.25, 0.5, 0.75 and 1.0 mL (80, 40, 27, 20 fold concentration) before analysis by GLC.

3.3.8.9 Experiment V: Losses due to concentration

Volatile concentration in model solutions of 20 mL solvent mixtures containing 2 $\mu L \cdot L^{-1}$ of individual volatile compounds (Table 1) dried to 200 µL, using O_2 free N_2 at 200 $mL \cdot min^{-1}$ was determined by GLC.

3.3.8.10 Experiment VI: Volatile identification, artefacts and solvent contamination in GLC analysis

Volatiles were identified by retention time and quantified using external standards at 200 $\mu L \cdot L^{-1}$ in the solvent mixture (Table 3.1). Mass spectroscopy was used on selected volatile extracts to authenticate volatile retention times and to identify contaminants in the solvent mixture and co-eluting compounds.

3.3.8.11 Experiment VII: Solvent extraction versus dynamic headspace

Solvent extracts from 12 replicates of RD apple juice were compared to volatile extracts obtained by dynamic headspace.

Results were analysed using the SAS statistical package (SAS Institute Inc., Cary, USA) and graphed using the Origin graphics package v 5.0 (Microcal Software Inc., USA).

3.4 Results and Discussion

Analyses were undertaken for selected volatile compounds representative of the broad range of compounds found in solvent extracts, from very volatile compounds such as ethanol and ethyl acetate, to less volatile compounds such as hexan-1-ol and hexyl acetate, and including the internal standard octyl acetate (Table 2.1).

Table 3.1. Molecular weight, density and drying recovery of volatiles used as external standards.

Compound	Molecular weight ($g \cdot mol^{-1}$)	Density ($g \cdot mL^{-1}$)	Drying recovery[1,2]	Boiling points[3] (°C)
Acetaldehyde	44.05	0.783	ND	21
Ethyl acetate	88.12	0.900	0.0254	77
Methanol	32.04	0.791	0.0000	65
Ethanol	46.07	0.790	0.0803	78
Ethyl propionate	102.13	0.890	0.6513	99
Propyl acetate	102.13	0.888	0.6125	102
Ethyl butanoate	116.16	0.880	0.8431	120
Propan-1-ol	60.10	0.800	0.4556	97
Ethyl-2-methyl butanoate	130.19	0.870	0.8423	133
Butyl acetate	116.16	0.880	0.8355	125
Hexanal	100.16	0.810	0.6536	131
2-Methyl butyl acetate	130.19	0.876	0.5670	142
Propyl butanoate	130.19	0.870	ND	143
Ethyl pentanoate	130.19	0.875	0.6482	
Butan-1-ol	74.12	0.810	0.5971	117
Pentyl acetate	130.19	0.876	0.6934	149
Methyl hexanoate	130.19	0.880	ND	151
2&3-Methyl butan-1-ol	88.15	0.815	0.6492	130
trans-2-hexenal	98.15	0.850	0.8635	146
Ethyl hexanoate	144.22	0.870	0.6085	168
Pentan-1-ol	88.15	0.810	0.5590	135
Hexyl acetate	144.22	0.878	0.8305	169
Hexan-1-ol	102.18	0.820	0.6772	156
6-Methyl-5-hepten-2-ol	126.20	0.850	ND	128
Octyl acetate	172.27	0.871	0.8313	210

[1]Drying recovery is the proportion remaining using a flow rate of 200 $mL \cdot min^{-1}$ of oxygen free N_2. [2] ND loss due to drying was not determined. [3] Boiling point at 1 Atm.

3.4.1 Experiment I(a) The effect of varying juice holding time in air at 20 °C or in an ice bath

Juice in air at 20 °C and in an ice bath had little change in individual volatile concentration 30 to 60 minutes after juicing (Table 3.2), although there was a tendency for juice on ice to have lower concentrations of volatiles than juice in air. Significant losses of butan-1-ol occurred after 60 minutes incubation. Butan-1-ol, hexan-1-ol and *trans*-2-hexenal concentrations were up to 20 % lower in juice stored on ice than juice left in air at 20 °C after 60 minutes holding time (Table 3.2) although these changes were not statistically significant, with time or treatment.

Table 3.2. Concentrations of volatile compounds extracted from FU apple juice held in air at 20 °C or on ice for up to 120 minutes.

Compounds	Treatment	Time (minutes) 0	30	60	90	120	P T	t	Txt
				$\mu mol \cdot L^{-1}$					
Butyl acetate	20 °C	31.1	40.4	40.4	45.4	36.8			
	0 °C	31.1	38.7	40.2	34.6	41.1	ns	ns	ns
Hexyl acetate	20 °C	13.9	15.1	15.3	15.8	14.2			
	0 °C	13.9	15.8	16.5	14.5	13.2	ns	ns	ns
trans-2-hexenal	20 °C	51.8	52.2	56.5	57.1	54.2			
	0 °C	51.8	52.2	53.1	45.9	48.2	ns	ns	ns
Butan-1-ol	20 °C	120.9	136.3	148.9	152.8	143.3			
	0 °C	120.9	136.3	137.0	118.1	115.1	*	ns	ns
Hexan-1-ol	20 °C	25.4	28.7	33.1	33.0	32.4			
	0 °C	25.4	29.2	30.5	26.2	25.9	ns	ns	ns

Data were mean concentration of 5 replicates, t = levels of significance among time, T = levels of significance between treatments, Txt = treatment x time interaction, Levels of significance at P = 0.05 (*) or non significant (ns).

3.4.2 Experiment I(b): Juice holding time at 20 °C before placement into ice prior to solvent extraction

There was no difference in volatile concentration in apple juice held in air at 20 °C or on ice for different periods for up to 256 minutes (Figure 3.1). This differs from previous findings that reported apple juice to have reduced volatile concentrations over time possibly due to esterase activity in extracts (Drawert et al., 1969). Production of secondary aromas occurs during and after homogenisation of apple tissue as enzymes such as lipoxygenase oxidise lipids from cell membranes to form hexanal and *trans*-2-hexenal (Yahia, 1994). These volatiles were present at high concentrations at the start of the experiment and were probably formed during homogenisation. Juice was aerated during grinding of apple tissue which would have favoured formation of secondary aromas. In this experiment, time in air and time on ice were confounding factors. Times presented in Figure 3.1 are combinations of time in air and time on ice, e.g. 32 minutes is 32 minutes in air and 224 minutes on ice making a total time of 256 minutes before solvent extraction. There was no difference in volatile concentration in samples from time 0 (256 minutes on ice) and time 256 (0 time on ice) (Figure 3.1). This experiment was repeated in Experiment I(c) where volatiles were extracted immediately at each sampling time.

3.4.3 Experiment I(c): Juice holding time at 20 °C before immediate solvent extraction

There was no difference in volatile concentration in apple juice held in air at 20 °C up to 256 minutes (Figure 3.2). Volatile concentration was very variable, 50 to 67% of mean values during the first 30 minutes after juicing which reduced to

20 to 30% for times longer than 30 minutes which may reflect chemical reaction occurring after cellular disruption. These very high variations in volatile concentration in the first 30 minutes may explain high volatile concentration variation reported where apple volatiles are extracted and analysed within the first few minutes of homogenisation (Fellman et al., 1993). There was little variation in volatile concentration in juice samples held longer than 30 minutes with a slight downward trend through 256 minutes (Figure 3.2). Holding juice for at least 30 minutes before solvent extraction of volatiles may be useful in reducing sample to sample variability.

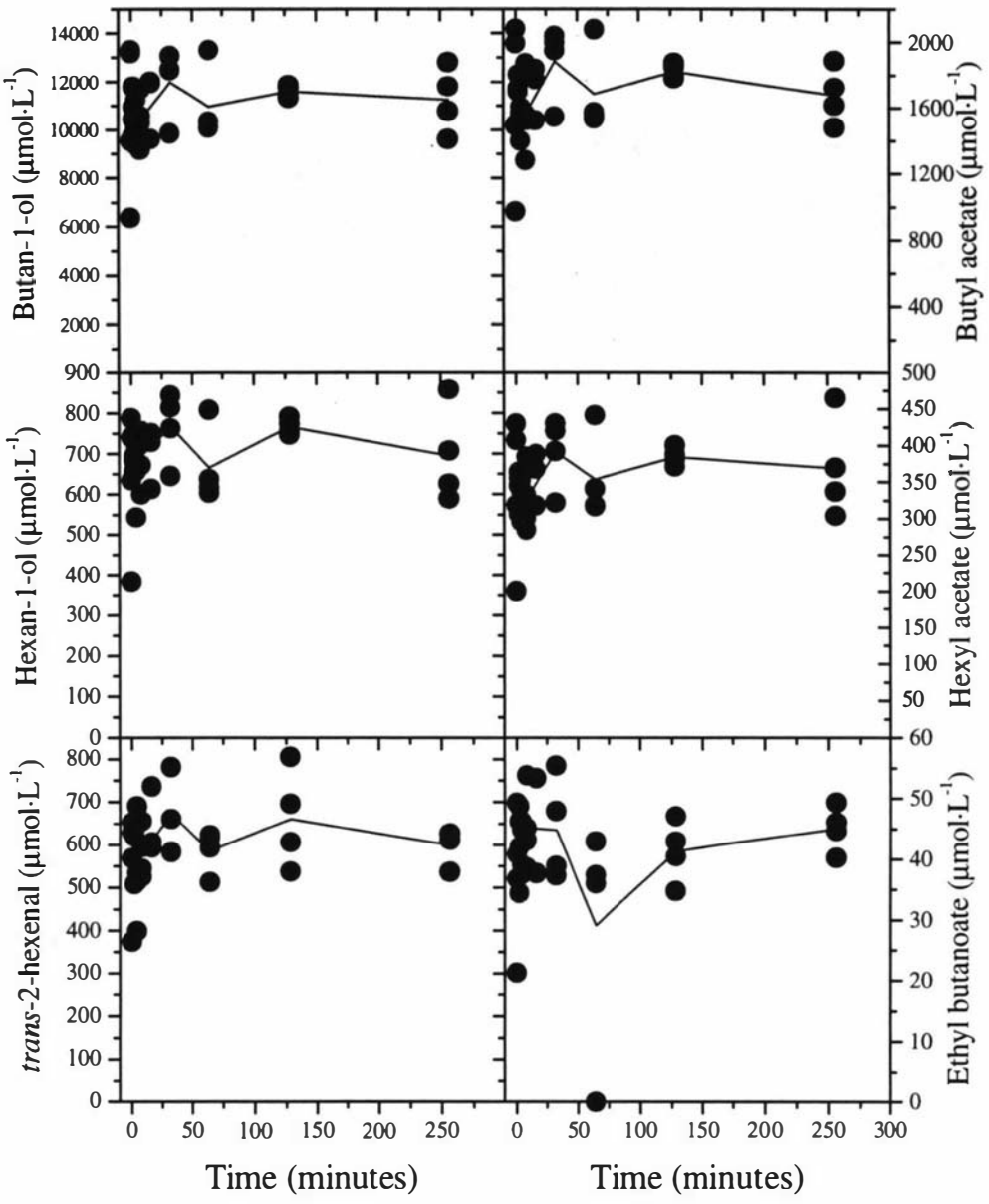

Figure 3.1. Scatter plots of selected volatiles from RG apple juice at 20 °C held in air and sampled after 0, 2, 4, 8, 16, 32, 64, 128 and 256 minutes then held on ice until 256 minutes after juicing, before solvent extraction. Lines represent the average volatile concentration at each sampling time.

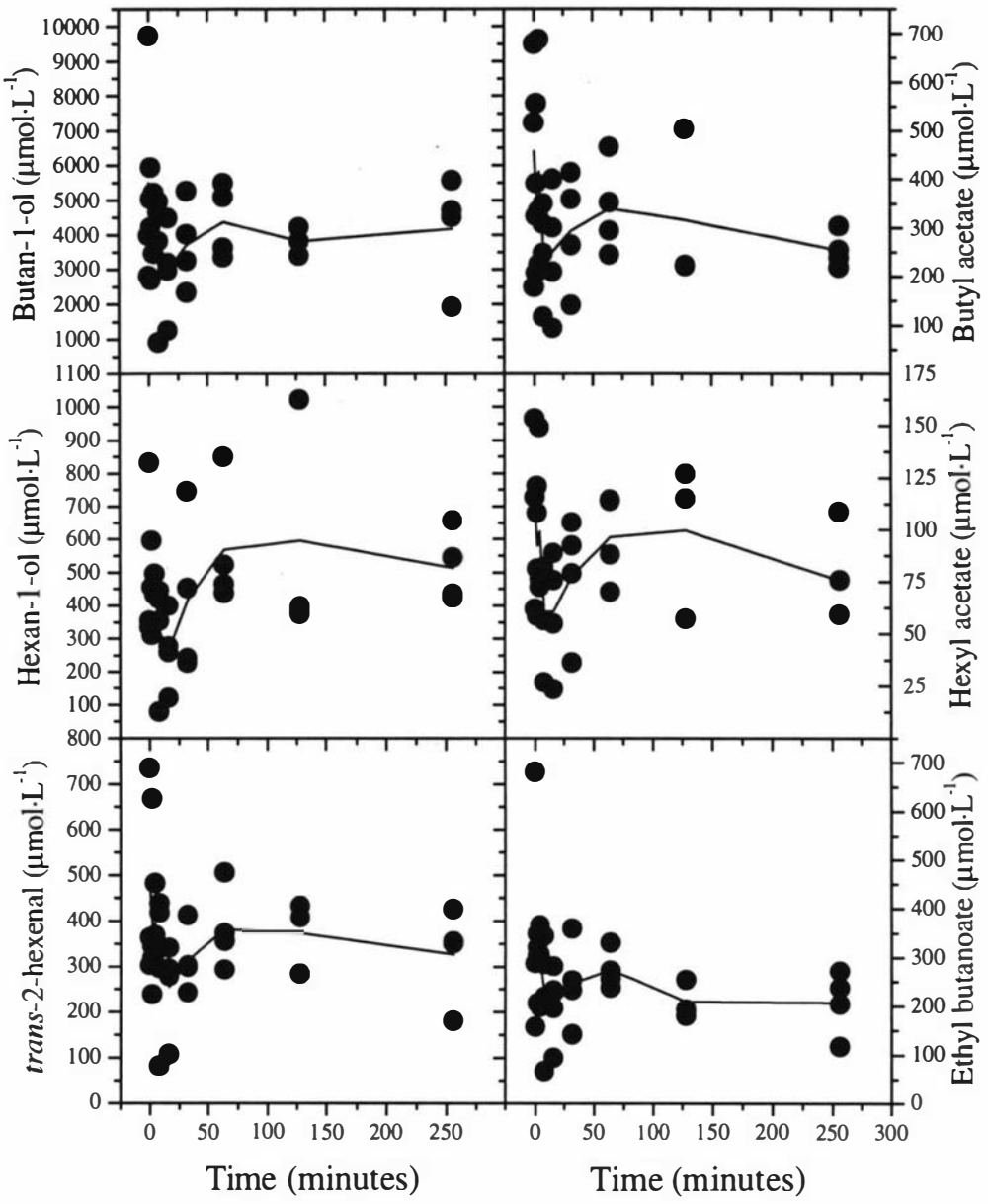

Figure 3.2. Scatter plots of selected volatiles from FU apple juice at 20 °C, extracted after 0, 2, 4, 8, 16, 32, 64, 128 and 256 minutes in air before solvent extraction. Lines represent the average volatile concentration at each sampling time.

3.4.4 Experiment II(a): Volume of juice to extract

Greater volumes of juice had greater concentrations of volatiles in the solvent extract (Figure 3.3). This relationship was not linear for all volatiles suggesting that the diethyl ether:*n*-pentane mixture became saturated with some volatile compounds during extraction from larger volumes. For example, the solvent

mixture may be saturated with hexyl acetate at about 140 $\mu mol \cdot L^{-1}$ and 8500 $\mu mol \cdot L^{-1}$ of butan-1-ol (Figure 3.3). The decline in volatile concentration at high juice volumes may also have been due to evaporation occurring during concentration of solvent extracts. Large volumes of solvent take longer to reduce to 200 µL, increasing the probability that some volatiles present would volatilise with the solvent. Solvent mixture volumes of about 20 mL or 50 mL took about 40 and 100 minutes, respectively, to reduce to 200 µL at 200 $mL \cdot min^{-1}$ O_2-free N_2 at ambient temperatures (15 to 25 °C depending on time of year). Under conditions of this study juice volumes above 40 mL were not suitable for solvent extraction as volatile concentration in solvent extracts from these volumes are no longer in the linear part of the curve (Figure 3.3).

3.4.5 Experiment II(b): Ratio of solvent volume to juice volume

Concentrations of volatiles, selected as representative of alcohols, esters and aldehydes in general, in the solvent extract were similar over a wide range of solvent to juice volume ratios (Figure 3.4a). As juice volume was constant the absolute concentration of volatiles should be similar in each solvent extract when concentrated to 200 µL. Concentrating solvent extracts by 100 to 150 fold (from 20 to 30 mL to 200 µL of solvent mixture) appeared to be a good compromise between having sufficient volatile concentration to analyse by GLC and acceptable losses during extraction.

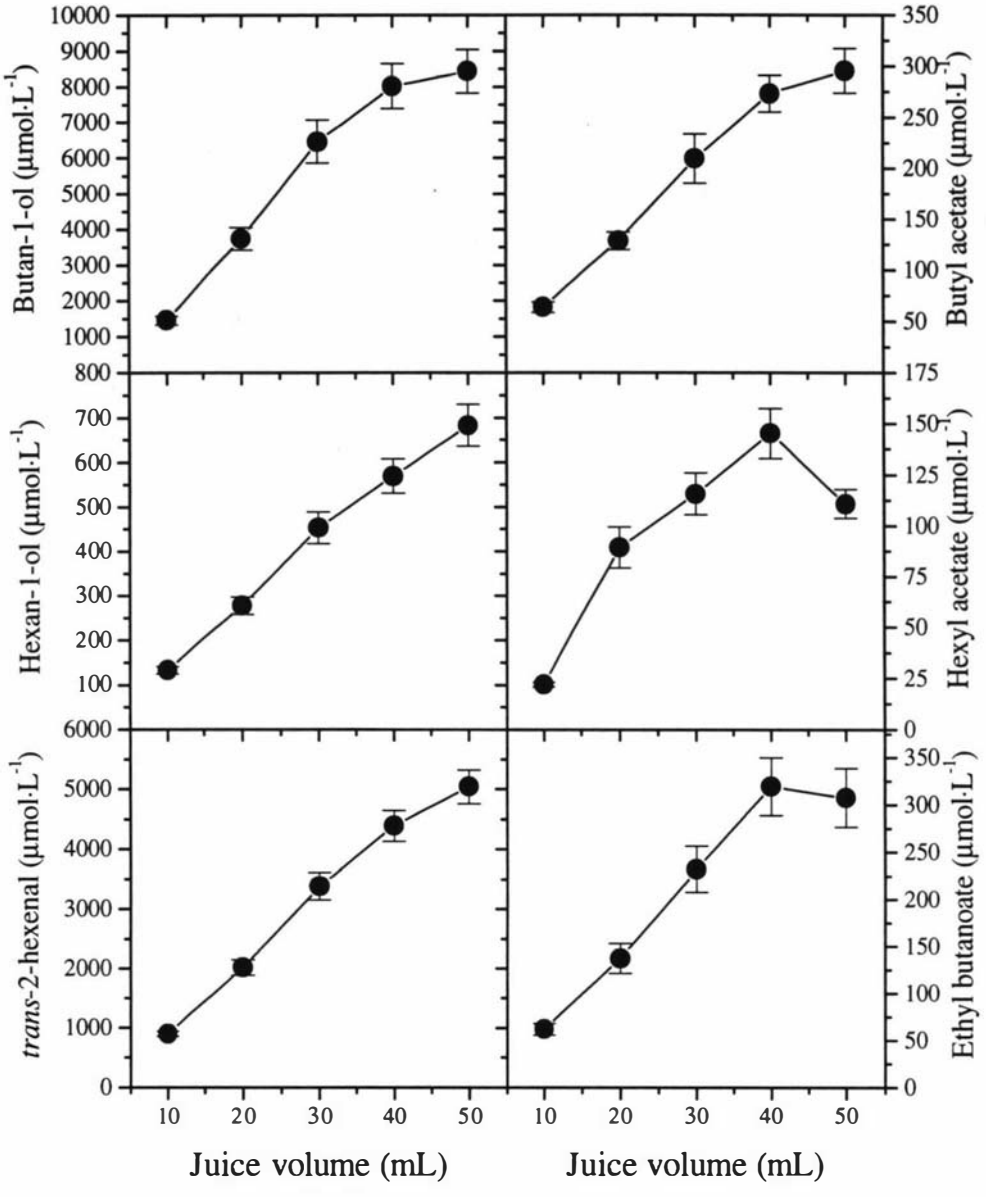

Figure 3.3. Relationship between RD apple juice volume and volatile concentration analysed by solvent extraction.

3.4.6 Experiment II(c): Ratio of juice volume to solvent volume

Concentration of most volatiles in solvent extracts tended to increase linearly over ratios of 0.5:1 to 2:1 juice to solvent (Figure 3.4b). At ratios above 2:1 there was a reduced increase in volatile concentration suggesting either that the solvent mixture may be becoming saturated with respect to individual volatiles or there was increased loss of volatiles during concentration. Ratios of 0.5:1 to 2:1 were considered the most suitable for volatile extraction as these were in the most

linear part of the curve.

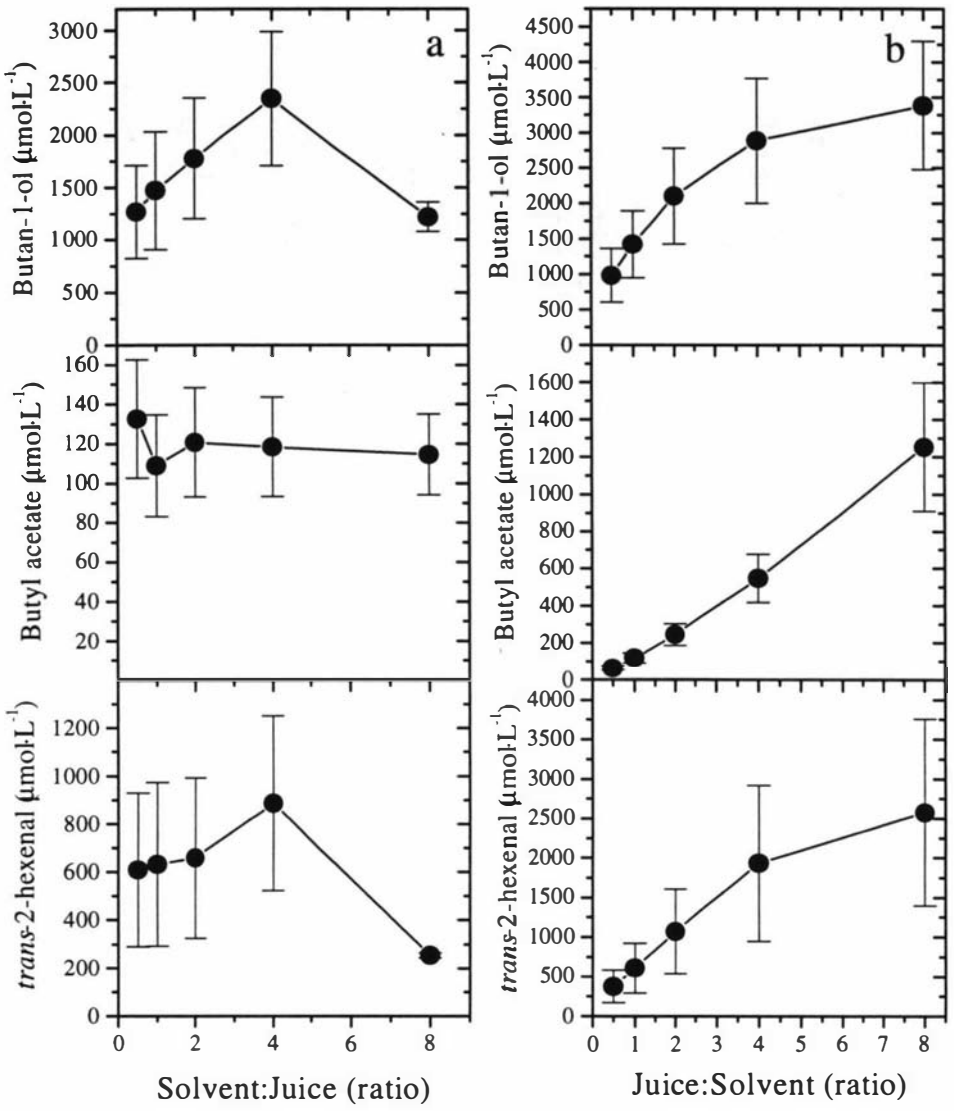

Figure 3.4. Volatile concentration in different ratios of (a) solvent:juice and (b) juice:solvent extracts.

3.4.7 Experiment III: Recovery of volatiles

About 76 % of octyl acetate was recovered from juice with the first solvent wash when juice and solvent were frozen compared with 61 % without freezing (Table 3.3). A second solvent wash extracted 8 and 11 % of octyl acetate for fresh and frozen juice, respectively. After five washes about 92 % of octyl acetate in frozen samples and 70 % of octyl acetate in fresh samples was extracted. By the third solvent wash octyl acetate concentration was similar to the solvent blank indicating that little octyl acetate remained in juice. The octyl acetate unaccounted for could have remained in juice bound to macromolecules or been lost by evaporation during concentration. Freezing the juice-solvent mixture increased partitioning of octyl acetate from juice to organic solvents as well as

making phase separation easy compared to separating two liquid layers. Using these results 76.5% of octyl acetate was assumed to be removed by one solvent extraction and was used to correct octyl acetate concentration in samples.

Table 3.3. Percentage extraction of octyl acetate for each wash from SP apple juice using solvent extraction.

	Frozen juice			Fresh juice		
Wash	Extracted	SE	Total	Extracted	SE	Total
1	76.5	5.0	76.5	60.8	2.6	60.8
2	11.1	1.6	87.6	8.2	1.4	69.0
3	1.7	0.3	89.3	0.1	0.1	69.1
4	1.1	0.1	90.4	0.0	0.0	69.1
5	1.3	0.3	91.7	0.0	0.0	69.1

3.4.8 Experiment IV: Concentration factor for solvent extracts

Concentration of volatile compounds was proportional to concentrated extract volume with a concentrated solvent extract volume of 0.25 mL, unadjusted for octyl acetate IS, having the greatest concentration of volatile compounds. Concentration of solvent extract to at least 0.5 mL was required to allow detection of hexanal and ethyl hexanoate (Table 3.4). A 40 fold concentration (500 µL) of the solvent extract could be considered adequate for analysis of most compounds of interest. This may be acceptable for an aromatic cultivar like fully ripe Red Delicious apples (Dirinck and Schamp, 1989) but would not be in low volatile cultivars like Granny Smith or from less mature fruit (Brackmann and Streif, 1994; Kakiuchi et al., 1986). Concentrating to 80 fold or greater is more likely to ensure that most volatile compounds of interest are concentrated sufficiently for analysis.

Table 3.4. Concentrations of selected volatile compounds of RD apple juice solvent extracts concentrated from 20 mL by 20 to 80 fold.

Compound	Concentrated volume (mL)			
	0.25	0.5	0.75	1.0
		$\mu L \cdot L^{-1}$		
ethyl butanoate	68.2	68.0	80.1	79.7
propan-1-ol	154.1	147.6	189.8	190.5
E2MB[1]	22.8	22.4	27.6	26.7
butyl acetate	39.6	35.6	46.1	44.7
hexanal	12.3	11.3	ND[2]	ND
butan-1-ol	293.5	322.9	331.6	321.4
trans-2-hexenal	25.4	23.8	32.3	28.5
ethyl hexanoate	10.1	9.5	10.4	ND
hexyl acetate	15.8	15.5	18.0	15.3
hexan-1-ol	62.7	61.7	62.5	64.7

[1]E2MB = ethyl-2-methyl butanoate. [2]ND = not detected.

3.4.9 Experiment V: Losses during concentration

All volatiles examined were lost to some extent during concentration with 200 $mL \cdot min^{-1}$ O_2-free N_2 (Table 3.1). This may be due to losses caused by evaporation of solvent during concentration as these compounds have boiling points similar to the solvent mixture (Guichard, 1988; Williams et al., 1978). Compounds with higher boiling points had comparable concentrations over all volumes (Table 3.1). Methanol was lost completely from the solvent mixture and only 8.0 % ethanol and 2.5 % ethyl acetate remained after solvent concentration. Thus very volatile compounds could not be assessed accurately by a method that requires a long period of solvent concentration and should be assessed by direct analysis of headspace samples. Percentage recovery of other volatiles ranged from 45-86% while that of the IS was 83.13 %. Assessment of losses during concentration was done using model solvent solutions; values obtained were used to adjust measured volatile concentration to account for volatile losses before IS adjustment.

3.4.10 Experiment VI: Volatile identification, artefacts and solvent contamination in GLC analysis

Thirty-two compounds were identified either as possible contaminants in the solvent mixture or as products formed by reaction with the solvent mixture (Table 3.5). Ethanol, ethyl acetate, and methanol were present in the solvent mixture; ethanol (about 500 $\mu L \cdot L^{-1}$) was introduced to prevent the diethyl ether from forming peroxides.

All of the 16 compounds found here have been reported as apple volatiles; these may have been produced as secondary aroma products or by reaction of cell components with enzymes in the apple juice (Table 3.5). The compounds 2 methyl butyl acetate, ethyl benzene and ethyl butanoate and toluene co-eluted as indicated by mass spectroscopy. Ethyl benzene or toluene were not considered a problem as apple juice does not contain these compounds (Gilbert, 1994).

Table 3.5. List of compounds identified by GC-MS of volatile extracts in a diethyl ether:*n*-pentane solvent mixture.

Possible contaminants	Aroma compounds, precursors, and substrates.
1,2 propanediol	2 Ethyl hexyl acetate
1,2,4 trimethyl benzene	2 Hexen-1-ol
1,3 butanediol	2 methyl butanoic acid
1,3,5 trimethyl benzene	5 Hexen-1-ol
2 butanone	Butan-2-ol
2 dimethyl cyclohexane	Butanal
2 methyl octane	*cis*-3-hexenal
2,3 butanediol	Ethyl-2-butenoate
3 methyl nonane	Octanol
3 methyl octane	Pentan-2-ol
3 octen-1-ol	Propanoic acid
3,6 dimethyl decane	trans-Linalool oxide
Alkanes	Ethanol
Benzaldehyde	Ethyl acetate
Butyl hydroxy toluene (antioxidant)	Tetradecane
Decane	Tridecane
Dodecane	
Ethyl benzene	
Ethyl enyl benzene	
Ethyl-3-methyl benzene	
Heptadecane	
Hexadecane	
Methanol	
Nonane	
Octadecane	
Pentadecane	
Pentyl cyclohexane	
Propyl cyclohexane	
Toluene	
Trimethylbenzene	
Undecane	
Xylene, ρ Xylene	

3.4.11 Experiment VII: Solvent extraction versus dynamic headspace

Samples obtained by solvent extraction had greater total peak area and more peaks than those obtained using dynamic headspace (Table 3.6). Concentrations of most alcohols and *trans*-2-hexenal were significantly higher in solvent extraction samples, whereas higher concentrations of esters and hexanal were obtained by dynamic headspace reflecting the relative affinity of Tenax® for some compounds. Tenax® is known to have poor adsorption and hence retentive capacity for alcohols (Maga, 1990). In contrast esters have high retention volumes with ethyl acetate, propyl acetate, butyl acetate and ethyl propionate having retention volumes of 35.4, 192, 846 and 1 17 L of headspace per gram of Tenax® respectively (Salinas Fernandez and Alonso Diaz-Marta, 1997). High boiling point compounds have low volatility and these are poorly recovered on Tenax® (Heath and Reineccius, 1986). Therefore, low alcohol concentrations

and greater ester concentrations in solvent extracts obtained using Tenax® traps was due to inherent characteristics of Tenax®.

Table 3.6. Concentrations and coefficient of variation of some volatile compounds from Red Delicious apple juice using solvent extraction and dynamic headspace.

Compounds	Concentration (μmol·L^{-1})			C.V.[3] (%)	
	Headspace[1]	Extraction[2]	P[4]	Headspace	Extraction
Alcohols					
propan-1-ol	409.7	3462.6	***	25.6	9.1
butan-1-ol	872.1	4972.6	***	31.5	7.2
hexan-1-ol	157.6	760.8	***	33.0	5.3
Aldehydes					
hexanal	499.5	265.2	***	21.5	9.9
trans-2-hexenal	276.9	511.5	***	38.9	12.5
Esters					
ethyl butanoate	2118.3	1287.1	***	23.4	5.4
ethyl-2-methyl butanoate	613.1	368.3	***	24.7	7.8
ethyl hexanoate	136.3	131.7	ns	27.7	11.1
butyl acetate	738.3	511.7	***	23.6	6.5
hexyl acetate	194.9	174.9	ns	26.5	6.4
Total area (10^5 count)	3.67	6.20	***	19.7	5.8
Number of peaks	67	108	***	19.1	13.6

[1]Headspace = dynamic headspace. [2]Extraction = solvent extraction. [3]C.V. = coefficient of variation. [4]*,**,*** significant at P = 0.05, 0.01, 0.001 respectively, ns = not significant

The 'salting out' effect (Bemelmans, 1981; Nelson and Hoff, 1968) partially explains the higher concentrations of esters and hexanal in dynamic headspace samples compared to solvent extraction. Sodium chloride as well as other salts, such as $CaCl_2$ and Na_2SO_4, are thought to reduce solvent-solute interaction by binding to water molecules increasing volatile concentration in the headspace (Bemelmans, 1981; Buttery et al., 1987; Jennings, 1965). The effect of salts on volatile concentration in the headspace varies from one volatile compound to another and is different over a range of volatile concentrations (Nawar, 1966). Addition of NaCl does not to affect alcohol concentration in the headspace (Jennings and Filsoof, 1977) but NaCl inhibits formation of *trans*-2-hexenal but not hexanal in tomatoes (Buttery et al., 1987). This may explain the reduced concentration of *trans*-2-hexenal in dynamic headspace samples compared to solvent extraction samples.

3.4.12 Solvent extraction

The diethyl ether:*n*-pentane solvent removes most volatile compounds of interest and is easily separated from water meeting two requirements of the ideal solvent for volatile extraction (Larsen, 1993). However, large volatile losses can occur in the concentration step and the method is potentially hazardous requiring all steps to be conducted in a fumehood. Separating the organic and aqueous phases was made simple by freezing the juice-solvent mixture thus removing azeotropic particles formed by *n*-pentane and water (Larsen, 1993) accounting for the greater recoveries of octyl acetate from frozen than from unfrozen juice-solvent mixtures (Table 3.3).

3.4.13 Overall Discussion

Holding juice in air for up to 4 hours did not affect estimates of volatile concentration using solvent extraction. Juice held on ice before extraction had reduced volatile concentrations of some compounds but these differences were not significant. These results indicate that enzyme inactivation by cold or addition of methanol may not be required after homogenisation as proposed by Drawert et al. (1966).

During concentration the rate of evaporation differed among volatiles assessed in this study. The loss due to evaporation of volatiles of interest was different to octyl acetate. Therefore, to account for volatile loss during concentration of the solvent mixture it is recommended that a correction be made for each volatile compound on its measured concentration along with the IS correction. Very volatile compounds (methanol, ethanol, ethyl acetate) were almost completely lost during concentration. The importance of these volatiles to aroma profiles is unknown as they have been mostly ignored by researchers whose methods remove these compounds from aroma extracts.

The chemical used as IS in solvent extraction should have similar losses to compounds of interest during the extraction process. The ideal IS is one that is not present in the sample and can be separated easily by GLC from other volatiles in the sample. Suitable volatile compounds with low and moderate boiling points would appear on the GLC trace in crowded regions limiting compounds that can be used. The IS, octyl acetate, used in this study had similar drying recoveries to high boiling point volatiles and greater drying recoveries than low boiling point volatiles. To improve the accuracy of estimation of volatile losses during extraction, three IS compounds should be used to represent very volatile compounds, compounds with low solubilities in the solvent mixture, such as C_3 to C_6 alcohols, and high boiling point compounds. Such compounds could be the decane series of hydrocarbons; decane has a retention time of about 6 minutes, undecane about 13 minutes and tridecane about 16 minutes in the GLC temperature programme used in this study.

The recoveries of model solutions of volatile compounds in the solvent mixture ranged from 45 to 85 % were similar to those found by Larsen (1993). These values were in broad agreement with those of Bemelmans (1979) for 2 mL model solutions in diethyl ether of ethyl formate and butanal having 22.0 and 37.9% recovery when dried to 100 µL. Recovery of individual volatile compounds, apart from octyl acetate, with successive solvent washes was not investigated and it is possible that other compounds may have greater or lesser wash recoveries than octyl acetate. Having estimates of wash recoveries for individual volatile concentrations would allow more accurate estimates of volatile concentration in samples.

Dynamic headspace and solvent extraction are not directly comparable as they measure volatile compounds in headspace air and aqueous solution. The use of Tenax® traps for dynamic headspace preferentially retains esters while solvent extraction preferentially retains alcohols.

Dynamic headspace can be used on either intact samples or homogenised samples where solvent extraction is limited to homogenised samples. Homogenised samples have aromas produced by secondary reactions not present in intact samples. Secondary aromas can be avoided using dynamic headspace methods on intact samples but cannot be avoided with solvent extraction. This makes dynamic headspace methods more suitable for investigating such aspects of volatile production as the effect of adding precursors on quantity and type of volatile production.

Solvent extraction could be considered to be a more quantitative method than dynamic headspace as solvent extraction removes most volatiles present in contrast to dynamic headspace which extracts volatiles according to the rate at which they partition from the sample to the gas phase. The extent of sample purging in dynamic headspace is limited to the retention volume of Tenax® which can be very low for some compounds (Salinas Fernandez and Alonso Diaz-Marta, 1997). To obtain sufficient volatile concentrations in solvent washes from Tenax® traps, periods of more than 1 h may be required for sample collection in dynamic headspace methods. A purging time of 1 h will not give an exhaustive removal of volatiles from the sample. With dynamic headspace volatile concentrations have to be expressed as a concentration in a given volume of gas purged from the sample rather than as an absolute concentration. Therefore, under dynamic headspace methods the total absolute concentration of volatiles in a sample cannot be accurately measured.

Dynamic headspace volatile extracts are likely to have less contaminants from the solvent and sample, as cell constituents like lipids, waxes, carotenoids, and chlorophylls are not extracted along with volatile compounds. Less solvent is used to elute Tenax® traps than is used in solvent extraction; less evaporation of solvent is required for dynamic headspace samples. In dynamic headspace samples possible contaminants from the solvent are at a concentration less than the GLC detector limits, given the same solvent.

Solvent extraction had similar recoveries of volatiles with 3 or more carbon atoms compared to dynamic headspace. Neither method is suited to extracting and analysing volatiles with less than 3 carbons as they evaporate leaving negligible concentrations; these can be better measured by direct injection of headspace samples into a GLC. Both solvent extraction and dynamic headspace have good reproducibility with solvent extraction having lower coefficients of variation than dynamic headspace. Many more samples can be processed at a time with solvent extraction than for dynamic headspace. Purging of apple juice samples took 2 h whereas solvent extracts were prepared for freezing in less than 5 minutes. Furthermore, solvent extractions can be processed in batches using inexpensive equipment compared to the expensive glass or teflon apparatus required to hold samples for dynamic headspace. Increasing the number of samples that can be processed at one time allows for more replication within treatments, leading to better assessment of variability of treatment effects.

3.5 Conclusion

Based on this study volatile compounds in apple juice can be estimated by extraction from 20 mL of apple juice with 20 mL diethyl ether:*n*-pentane (2:1 v/v), freezing at -18 °C, decanting the aqueous phase and concentrating 100 fold to 200 µL using 200 mL·min^{-1} O_2-free N_2. This gave the most appropriate ratio of solvent to juice with a concentration of volatiles sufficient for analysis by GLC.

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Chapter Four

Hypoxic treatments alter volatile concentration of apple cultivars.

Additional index words. Solvent extraction; aroma; odour units

4.1 Abstract

Brief periods of hypoxia at ambient temperatures (20 °C) have potential for disinfestation treatments or as pre-treatments to maintain fruit quality during extended storage. Nine cultivars of apples, Cox's Orange Pippin, Fuji, Golden Delicious, Granny Smith, Pacific Rose, Red Delicious, Royal Gala, Splendour and Southern Snap were exposed to hypoxic conditions, 100% carbon dioxide for 24 h at 20°C. Volatile composition was analysed on removal from hypoxia and during storage at 20°C for one week. Apples exposed to hypoxia had concentrations of acetaldehdye, ethanol, ethyl acetate and ethyl esters consistently enhanced while concentrations of acetate esters and aldehydes were depressed. Cultivars varied considerably in response to hypoxic treatment with Cox's Orange Pippin and Golden Delicious having the least and Fuji and Red Delicious the greatest enhancement in ethyl esters. Fruit exposed to hypoxia had larger odour unit scores than control fruit suggesting that such changes in volatile concentration may affect the aroma and/or flavour. The enhanced ethyl ester concentrations in fruit exposed to hypoxia may be due to large increases in ethanol concentration that competitively inhibited formation of non-ethyl esters. In addition, there may have been a change in ester forming enzyme activity and/or substrate specificity of the volatile biosynthetic pathway. This study has shown that short-term hypoxic treatments have the potential to change the aroma/flavour of apples.

4.2 Introduction

Typical apple aroma is comprised of more than 300 volatile compounds (Paillard, 1990) emanating from the fruit (Sanz et al. 1997) including alcohols, aldehydes, esters, ketones and ethers (Dimick and Hoskin, 1983) where most are esters (78-92%) and alcohols (6-16%). Although most aroma compounds are present in volatile emissions from apples, only about 13 compounds define characteristic apple aroma or taste (Cunningham et al., 1986; Paillard, 1990). Sensory differences in apple juice and aroma are apparent to sensory panellists where cultivars with strong aroma and 'typical' apple taste are preferred (Poll, 1981).

Aroma compounds are thought to be synthesised from amino acids, membrane lipids and carbohydrates (Sanz et al., 1997). Fatty acids supply straight chain alcohols and acyl CoA's via β-oxidation occurring in peroxisomes (Gerhardt, 1983). Branched chain volatiles are formed by metabolism of amino acids in particular iso-leucine, leucine and valine (Heath and Reineccius, 1986). Acetyl CoA is probably synthesised mainly from pyruvate in mitochondria, as it is the

substrate of the tricarboxylic acid cycle (Mathews and van Holde, 1996). The final step in formation of alcohols from fatty acids and amino acids is reduction of aldehydes to alcohols catalysed by alcohol dehydrogenase (ADH, EC 1.1.1.1) (Sanz et al., 1997). Esters are produced by combining alcohols and CoA derivatives of carboxylic acids in an oxygen dependent reaction catalysed by alcohol acyl CoA transferase (AAT, EC 2.3.1.84) (Harada et al., 1985). As acetyl CoA is the most abundant CoA present in fruit tissue the majority of esters are acetate esters.

Hypoxic environments are those where there is insufficient oxygen (O_2) in the surrounding atmosphere to support aerobic metabolism (Ricard et al., 1994). Such conditions induce anaerobic respiration where acetaldehyde and ethanol accumulate (Kader, 1986). Brief periods of hypoxia reduce postharvest decay (Ke et al., 1991; Pesis and Avissar, 1989), help maintain general fruit quality (Pesis, 1994), increase volatile concentration in citrus and feijoa fruit (Pesis et al., 1991; Shaw et al., 1991), and have been evaluated as potential disinfestation treatments. Such treatments use <2% O_2 and up to 100% carbon dioxide (CO_2) or nitrogen (N_2) atmospheres for 1 to 14 days at about 20 °C (Gaunce et al., 1982, Hallman, 1994). Low O_2 conditions are considered beneficial for apple storage as fruit maintains colour, firmness and juiciness longer than when stored in air. These attributes are used to define apple fruit quality for storage and marketing, but consumers require fruit with acceptable flavour and aroma. Apples maintained in hypoxic conditions for long periods (controlled atmosphere (CA) storage) had reduced volatile production with poor flavour and aroma compared to fruit stored in air (Patterson et al., 1974; Yahia et al., 1990).

Sensory panel analysis indicates that fruit treated with hypoxia increase in flavour. Apples treated with 10% to 15% CO_2 before CA storage were rated by taste panellists as having better flavour and texture than untreated fruit stored in CA only (Tietjen and Hudson, 1984). Peaches and nectarines exposed to 86% CO_2 or 97% N_2 for 1 d at 20 °C were preferred over untreated fruit after 7 d at 20 °C by a panel of 15 tasters (Lurie and Pesis, 1992). Feijoa fruit treated with 98% N_2 + 2% O_2 for 24 h at 20 °C were rated by a sensory panel as sweeter than control fruit after 7 d at 20 °C (Pesis, 1994). Golden Delicious apples exposed to >95% CO_2 for 24 or 48 h at 20 °C had better flavour than untreated control fruit after 2 weeks at 20 °C, with 24 h treatment being the most preferred (Ampunpong, 1991; Pesis et al., 1994; Shusiri, 1992). Such improvement in flavour may be due to an increase in concentration of aroma/flavour volatiles induced by exposure to hypoxia (Pesis, 1994). Apart from Pesis et al. (1994) the effect of short term exposure to hypoxic conditions on apple cultivars has not been determined. It is likely that exposure of apples to a brief period of hypoxia will increase the concentration of aroma and flavour volatiles thus improving flavour acceptability to consumers.

Should hypoxic treatments become acceptable for disinfestation the consequences of such a treatment on apple quality needs to be explored fully. If brief periods of hypoxia are to be recommended as disinfestation treatments it would be desirable for one set of treatment conditions to have the same efficacy

selections. Therefore quantitative and qualitative changes in volatiles of a range of commercial cultivars were analysed after exposure to a brief period of hypoxia.

4.3 Materials and Methods

4.3.1 Fruit supply

Nine cultivars of apples (*Malus domestica* Borkh.) were assessed for their response to hypoxia. In 1996 fruit was harvested from Hawkes Bay, New Zealand at mid commercial harvest, as determined by the New Zealand Apple and Pear Marketing Board, on 13 Feb. for Cox's Orange Pippin (CO), 1 Apr. for Fuji (F), 10 Apr. for Golden Delicious (GD), Granny Smith (GS), Sciros (PR, Pacific Rose™), Red Delicious (RD), 23 Feb. for Royal Gala (RG), and Sciglo (SS, Southern Snap™). Fruit were graded to export standard, packed to count 100-125 (148 to 188 g fresh weight), transported unrefrigerated to Massey University, Palmerston North, by road, and placed at 0 °C ± 1 °C within 3 days of harvest. Splendour (SP) apples were harvested from the Fruit Crops Unit, Massey University (mid-May 1996), graded to 125 count and placed at 0 °C ± 1 °C. Before treatment fruit were removed from coolstore and equilibrated to 20 °C ± 3 °C overnight. Fruit were divided into two groups, one was exposed to hypoxia (T), the other maintained in air as controls (C) with 4 replicates of 3 apples each for each treatment.

4.3.2 Hypoxic treatment

Fruit were placed into 24.1 L perspex chambers connected to a manifold, in parallel, leading from a high pressure cylinder of pure CO_2 (BOC Gases New Zealand Ltd., New Zealand). Carbon dioxide was humidified by bubbling through water before passing into the manifold. During treatment chambers were purged continuously with CO_2 for 24 hours at 20 °C ± 3 °C. Outlets from each chamber were joined and the outflow bubbled through water to create sufficient backpressure to minimise CO_2 flowrate differences between chambers. Initial CO_2 flowrate in each chamber was 2.5 $L \cdot min^{-1}$ for 2-3 hours (6.2 air changes h^{-1}) after which it was reduced to 1 $L \cdot min^{-1}$ (2.5 air changes h^{-1}). Excess gas was vented to outside air. The CO_2 and O_2 content of chambers were monitored by gas chromatography, every few minutes initially and every few hours thereafter. A concentration of <0.5% O_2 was reached within 4 h of commencing purging and remained constant until chamber opening.

4.3.3 Fruit assessment

Groups of 3 fruit were assessed for CO_2 and ethylene production, firmness, weight loss, volatile concentration of juice and fermentation volatiles in the juice headspace the day before treatment (d -1), on removal from treatment (d 0) and 1, 3, 5, and 7 d after treatment during ripening at 20 °C ± 3 °C.

4.3.4 Firmness

Firmness was measured using a hand held Effigi penetrometer (model FT327) with a 11.1mm measuring head on pared surfaces on opposing sides of fruit at the equator (Harker et al., 1996). The average of the two measurements was multiplied by 9.81 to convert kilograms force to Newtons.

4.3.5 Carbon dioxide and ethylene production

Each replicate of three apples, from each treatment, was weighed and depending on size of fruit placed into 1.6 L or 1.8 L glass preserving jars. One mL of gas was removed from each jar 15-30 minutes after sealing using a plastic syringe (1 cc Graduated Monoject® syringe with detachable needle, 25 gauge x 5/8", Sherwood Medical, MO, USA). Carbon dioxide was analysed using a thermal conductivity detector at 60 °C and 90 mA current in a Shimadzu 8A gas chromatograph (GC) equipped with an Alltech CTR I column (Alltech cat. no. 8700) at 30 °C with hydrogen at a flow rate of 30 mL·min^{-1} as carrier gas. Ethylene was measured using a flame ionization detector at 150 °C, column 110 °C and injector 190 °C, in a Shimadzu 4B-PTF GC equipped with a F-1 grade, 80/100 mesh activated alumina 1.83 m x 3.18 mm column (Alltech cat. no 80072). Nitrogen at a flowrate of 30 mL·min^{-1} was the carrier gas. The flame was maintained with hydrogen at 30 mL·min^{-1} and air at 300 mL·min^{-1}. Carbon dioxide and ethylene production were calculated according to Banks et al. (1995).

4.3.6 Juice preparation

Each group of three apples from each treatment were ground in a domestic juicer (Kenwood Centrifuga, model JE500, Kenwood Appliances, NZ). Juice was collected and left at ambient temperature (20 °C ± 3 °C) for at least 30 minutes before volatile extraction and analysis.

4.3.7 Headspace volatiles

Acetaldehyde, ethyl acetate and ethanol in the headspace of apple juice were analysed. Thirty mL of apple juice was placed into 50 mL glass Erlenymer flasks sealed with Suba-Seal® (No. 33) rubber stoppers, maintained at 30 °C in a water bath. After at least 15 minutes a one mL gas sample, taken from the flask headspace, was measured using a flame ionization detector at 180 °C, column 45 °C and injector 110 °C in a PYE UNICAM GC fitted with a 1.83 m x 3.18 mm stainless steel column (Supelco cat. no 1-2212) containing a 10% Carbowax 20M coating on 80/100 Chromosorb WAW support. Nitrogen at a flowrate of 30 mL·min^{-1} was the carrier gas. The flame was maintained with hydrogen at 30 mL·min^{-1} and air at 300 mL·min^{-1}.

4.3.8 Juice volatiles

Volatile compounds were extracted from apple juice using a diethyl ether:*n*-pentane solvent mixture (2:1 v/v, Analar BDH) (Larsen and Poll, 1990). Two 10

4.3.8 Juice volatiles

Volatile compounds were extracted from apple juice using a diethyl ether:*n*-pentane solvent mixture (2:1 v/v, Analar BDH) (Larsen and Poll, 1990). Two 10 mL aliquots of juice were each placed into separate 20 mL scintillation vials (Wheaton Scientific, NJ, USA) fitted with a metal foil liner cap. The internal standard (IS) consisted of 0.2 µL octyl acetate (Aldrich Chemical Company, WI, USA) added to each juice aliquot before 10 mL of solvent mixture. Vials were capped tightly and mixed with a vortex stirrer for 3-5 seconds before storage at -18 °C until the aqueous phase was frozen. The unfrozen solvent phase was decanted from the aqueous phase that was discarded. The diethyl ether:*n*-pentane solvent extract was dried from 20 mL to about 200 µL (about 100 fold concentration) and placed into a glass 250µL flat bottom glass insert (Sun International Trading cat. no. 200 232) in a 1.5 mL glass screw top autosampler vial (Sun International Trading cat. no. 200 250) suitable for a Hewlett Packard 5890 series II plus GLC autosampler (Hewlett Packard 7673 Controller and Injector and Model 185968 100 sample carousel). Vials were sealed with a plastic septum (Sun International Trading cat. no. 200 368) before placement in the carousel. One µL samples were injected into the GLC. Quantification of volatiles in the solvent extract was by comparison with authentic compounds made to a concentration of 200 $\mu L \cdot L^{-1}$ in the solvent mixture. Odour unit values were calculated according to Teranishi et al. (1987) using published aroma threshold values (Flath et al., 1967; Takeoka et al., 1995, 1996; Teranishi et al., 1987).

One µL of solvent extract was measured by capillary gas chromatography using a Hewlett Packard 5890 Series II Plus GC connected to an IBM compatible personal computer equipped with Hewlett Packard ChemStation software (version B.02.04). The capillary column was a J&W 30 m x 0.32 mm (i.d.) fused silica, DBWAX, 0.5 µm film thickness (Alltech cat. no 93526). Injector and detector temperatures were 150 °C and 250 °C respectively. Oven temperature was held at 40 °C for 5 minutes, then programmed to 120 °C at 5 $°C \cdot minute^{-1}$ then to 190 °C at 20 $°C \cdot minute^{-1}$ with no holding time making a total run time of 24.5 minutes. Hydrogen was used as the carrier gas with a linear flow rate of 30 $cm \cdot s^{-1}$. A split injection mode was used with a split flow rate of 100 $mL \cdot min^{-1}$ and split ratio of 15:1. Septum purge flow rate was 5-6 $mL \cdot min^{-1}$. Air and hydrogen flow rates to the detector were 400 and 30 $mL \cdot min^{-1}$, respectively.

4.3.9 Data analysis

Each experiment on each cultivar was conducted as a completely random design with sampling during storage at 20 °C as repeated measures. Means and standard errors of the means for each cultivar and treatment for headspace and juice volatiles were graphed using an Origin graphics package v. 5 (Microcal Software Inc., USA). Data were subjected to analysis of variance using SAS v. 6.12 (SAS Institute Inc., Cary, USA). Significant main effect means were separated by Duncan's multiple range test at the 5% level of significance. Odour units were calculated as the sum of ratios of the concentration of a volatile component in an

extract and the average threshold concentration of that volatile in water (Table 1.5; Frijters, 1979).

4.4 Results

4.4.1 Carbon dioxide production

Over all cultivars CO_2 production in control fruit averaged 134.5 $nmol \cdot kg^{-1} \cdot s^{-1}$ compared to 119.8 $nmol \cdot kg^{-1} \cdot s^{-1}$ for fruit exposed to hypoxia but apart from SP there was no significant effect of treatment on respiration rate (Table 4.1). Production of CO_2 was the greatest for CO fruit and the least for PR fruit. Golden Delicious and RG fruit had a slight but non-significant increase in CO_2 production while CO, FU, GS, PR, RD, SP and SS had a slight but non-significant decrease in CO_2 production 3 d after removal from hypoxia (Table 4.1).

4.4.2 Ethylene production

After exposure to hypoxia CO, FU, PR, RG and SP fruit had reduced ethylene production compared to control fruit (Table 4.1). Golden Delicious fruit had increased ethylene production, while there was no change in ethylene production following treatment for GS, RD and SS fruit (Table 4.1). Ethylene production was greatest for GD and RG fruit and the least for FU, PR, and SP fruit.

4.4.3 Firmness

Apart from SP apples, fruit firmness at 3 days was the same for control and hypoxic treated apples (Table 4.1). Control SP apples were firmer than SP fruit exposed to hypoxia.

Table 4.1 Carbon dioxide, ethylene production and firmness for apple cultivars stored in air for 3 d at 20 °C, in control fruit and in fruit after exposure to hypoxia for 24 h. Mean of 4 replicates.

	Carbon dioxide		Ethylene		Firmness	
Cultivar	C[1]	T	C	T	C	T
	$nmol \cdot kg^{-1} \cdot s^{-1}$		$nmol \cdot kg^{-1} \cdot s^{-1}$		N	
CO[2]	190.5a	158.8a	1.0a	0.5b	42.3a	43.7a
FU	143.1a	136.6a	0.3a	0.2b	70.5a	68.9a
GD	144.7a	153.8a	1.4a	1.7b	41.1a	43.9a
GS	135.2a	129.9a	0.7a	0.6a	70.0a	71.1a
PR	89.4a	45.6a	0.1a	0.0b	72.2a	72.2a
RD	112.1a	98.2a	0.5a	0.5a	61.7a	61.2a
RG	120.3a	136.3a	1.7a	1.4b	50.2a	52.4a
SP	128.8a	86.3b	0.2a	0.0b	75.5a	70.3b
SS	146.1a	132.5a	0.7a	0.5a	70.0a	70.2a

[1] Treatment: C = control, T = exposed to hypoxia. [2] Mean separation in a row for a given cultivar by Duncan's multiple range test, values followed by a letter in common are not significantly different at 5%.

4.4.4 Disorders

No physiological disorders or CO_2 injury, external or internal, was observed for any fruit exposed to hypoxia (Data not shown).

4.4.5 Anaerobic volatiles

Acetaldehdye, ethyl acetate and ethanol concentrations were greater in fruit exposed to hypoxia than in control fruit at removal from treatment and through 7 d at 20 °C. On removal from hypoxia, the increase in acetaldehyde concentrations varied from cultivar to cultivar with GS fruit having the least increase and RG the greatest (Table 4.2). By contrast CO and FU fruit had the greatest concentration of ethanol, RD and SS the lowest. Ethyl acetate concentrations were low on removal from hypoxia (typically less than 10 $\mu mol \cdot L^{-1}$) but 3 d after removal from hypoxia had increased to 86.5 $\mu mol \cdot L^{-1}$ for CO fruit and remained very low 1.0 $\mu mol \cdot L^{-1}$ in GS fruit (Tables 4.2 and 4.3). Acetaldehyde, ethyl acetate and ethanol concentrations in control fruit varied greatly from cultivar to cultivar. There appeared to be no relationship between control concentrations and magnitude of enhancement after hypoxia of acetaldehyde, ethanol and ethyl acetate. At d 0 acetaldehyde was detected in control fruit of CO, RG and SS, ethanol was not detected in GD and PR but was present in (highest concentration to lowest) CO, RG, GS, FU, SS, RD and SP and ethyl acetate was not detected PR but was present in (highest concentration to lowest) CO, SS, RD, RG, SP, GS, GD and FU (Table 4.2). At d 3 after treatment acetaldehyde was only present in CO and RG at the same concentration, ethanol was not found in FU, GS, SP and SS and was present in (highest concentration to lowest concentration) CO, RG, PR, RD and GD, and ethyl acetate was not detected in FU but was present in (highest concentration to lowest concentration) PR, RD, RG, SS, GD, SP, CO and GS (Table 4.3). Acetaldehyde concentrations of treated fruit remained high 3 d after treatment, increased in CO, GS and SP fruit and decreased in FU, GD, PR, RD, RG and SS fruit. In fruit exposed to hypoxia ethanol concentrations continued to be high at 3 d after treatment increased in GD, RD and SS fruit (Table 4.3). In treated GS fruit ethanol concentration at d 3 had decreased to levels less than in control fruit at d 0.

Table 4.2a. Concentration of volatiles ($\mu mol \cdot L^{-1}$) for apples stored in air and on removal from hypoxia (d 0). Mean of four replicates.

Cultivar	CO		FU		GD		GS		PR	
Treatment	C[1]	T	C	T	C	T	C	T	C	T
Alcohols										
Ethanol	5621a[3]	18100b	1486a	17623b	ND[4]	14511b	1699a	7432b	ND	6879b
Propan-1-ol	36a	39a	28a	91b	61a	142b	ND	28b	ND	22a
Butan-1-ol	1044a	1795b	975a	853a	1407a	2820b	43a	20b	260a	186a
Pentan-1-ol	7a	17b	15a	15a	10a	26b	ND	ND	6a	5a
Hexan-1-ol	117a	259b	130a	122a	149a	313b	31a	25a	47a	35a
2MB[2]	11a	31a	240a	354a	43a	130b	51a	41a	33a	50a
Acetate esters										
Ethyl acetate	3a	8b	1a	9b	1a	5b	1a	3b	ND	4b
Propyl acetate	43a	48a	ND	ND	20a	19a	ND	ND	8a	8a
Butyl acetate	543a	383b	93a	46b	452a	332b	ND	ND	55a	39a
Pentyl acetate	12a	6a	ND	3a	7a	7a	ND	ND	ND	ND
Hexyl acetate	120a	91b	23a	14a	74a	60a	11a	8a	17a	16a
2MBA[2]	22a	17a	73a	36b	33a	31a	ND	ND	23a	17a
Ethyl esters										
Ethyl propionate	ND	ND	4a	8a	4a	10a	7a	4a	ND	6a
Ethyl butanoate	ND	11b	10a	36b	11a	22b	13a	12a	12a	22b
Ethyl pentanoate	ND	2a	13a	12a	15a	29b	15a	11a	15a	15a
Ethyl hexanoate	ND	ND	ND	6b	ND	6b	ND	ND	ND	9b
E2MB[2]	ND	ND	ND	ND	ND	2a	ND	ND	ND	ND
Aldehydes										
Acetaldehyde	85a	291b	ND	279b	ND	421b	ND	56b	ND	127b
Hexanal	75a	51a	29a	25a	92a	91a	41a	16a	28a	13a
trans-2-Hexenal	476a	346a	156a	160a	166a	179a	283a	215a	88a	98a

[1] C = control, T = exposed to hypoxia. [2] 2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = Ethyl-2-methyl butanoate. [3] Mean separation in a row for a given cultivar by Duncan's multiple range test, values followed by a letter in common are not significantly different at 5%. [4] ND = not detected, concentration below 0.1 $\mu L \cdot L^{-1}$.

Table 4.2b. Concentration of volatiles ($\mu mol \cdot L^{-1}$) for apples stored in air and on removal from hypoxia (d 0). Mean of four replicates.

Cultivar	RD		RG		SP		SS	
Treatment	C[1]	T	C	T	C	T	C	T
Alcohols								
Ethanol	215a[3]	4738b	4477a	9240b	87a	15682b	969a	4738b
Propan-1-ol	19a	91b	66a	106b	34a	74a	10a	94b
Butan-1-ol	324a	488a	2546a	2622a	670a	835a	1326a	1464a
Pentan-1-ol	ND[4]	6b	17a	21a	16a	12a	25a	18a
Hexan-1-ol	44a	64b	180a	232a	94a	96a	187a	214a
2MB[2]	28a	50a	52a	86a	111a	79a	76a	101a
Acetate esters								
Ethyl acetate	1a	7b	1a	2a	1a	6b	2a	10b
Propyl acetate	16a	31a	48a	47a	ND	ND	40a	33a
Butyl acetate	83a	87a	404a	188b	74a	63a	427a	228b
Pentyl acetate	ND	ND	2a	ND	ND	ND	17a	8a
Hexyl acetate	23a	28a	101a	37b	20a	21a	106a	79a
2MBA[2]	28a	25a	32a	14b	32a	6a	58a	44a
Ethyl esters								
Ethyl propionate	ND	9a	ND	ND	3a	7a	ND	4a
Ethyl butanoate	3a	36b	ND	ND	13a	42b	8a	29b
Ethyl pentanoate	13a	13a	6.a	2a	13a	14a	19a	15a
Ethyl hexanoate	ND	15b	ND	ND	ND	ND	2a	12a
E2MB[2]	ND	ND	ND	ND	ND	ND	ND	ND
Aldehydes								
Acetaldehyde	ND	226b	50a	557b	ND	222b	19a	336b
Hexanal	ND	ND	72a	68a	19a	0b	85a	61a
trans-2-hexenal	124a	128a	114a	128a	200a	147b	363a	220a

[1] C = control, T = exposed to hypoxia. [2] 2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = Ethyl-2-methyl butanoate. [3] Mean separation in a row for a given cultivar by Duncan's multiple range test, values followed by a letter in common are not significantly different at 5%. [4] ND = not detected, concentration below 0.1 $\mu L \cdot L^{-1}$.

Table 4.3a. Concentration of volatiles ($\mu mol \cdot L^{-1}$) for apples stored in air and on removal from hypoxia (d 3). Mean of four replicates.

Cultivar	CO		FU		GD		GS		PR	
Treatment	C[1]	T	C	T	C	T	C	T	C	T
Alcohols										
Ethanol	3589a[3]	17947b	ND[4]	4604b	4a	26892b	ND	292a	30a	6381b
Propan-1-ol	ND	34b	183a	173a	149a	151a	69a	60a	38a	55a
Butan-1-ol	1306a	2177a	857a	838a	1630a	2326a	60a	110a	253a	347a
Pentan-1-ol	16a	16a	11a	13a	14a	15a	9a	2a	9a	12a
Hexan-1-ol	119a	143a	111a	80a	236a	229a	53a	68a	52a	57a
2MB[2]	21a	27a	297a	272a	79a	89a	224a	82a	70a	45a
Acetate esters										
Ethyl acetate	1a	87b	ND	9b	1a	46b	1a	1a	2a	27b
Propyl acetate	14a	14a	ND	ND	ND	ND	ND	3a	ND	6a
Butyl acetate	482a	448a	63a	36b	390a	336a	1a	ND	44a	39a
Pentyl acetate	13a	ND	ND	ND	5a	2a	ND	ND	ND	2a
Hexyl acetate	98a	57a	18a	10b	111a	50b	10a	10a	15a	12a
2MBA[2]	25a	15a	74a	23b	47a	26b	ND	ND	44a	9a
Ethyl esters										
Ethyl propionate	5a	ND	6a	128b	7a	ND	8a	52b	ND	121b
Ethyl butanoate	11a	25b	13a	207b	13a	17a	12a	74a	9a	238b
Ethyl pentanoate	17a	15a	11a	13a	14a	13a	14a	13a	13a	15a
Ethyl hexanoate	2a	ND	ND	35b	ND	ND	ND	5a	ND	24b
E2MB[2]	ND	ND	ND	52b	ND	ND	1a	7a	ND	21b
Aldehydes										
Acetaldehyde	11a	346b	ND	149b	ND	130b	ND	71a	ND	35b
Hexanal	47a	58a	71a	71a	207a	118b	75a	71a	69a	50a
trans-2-Hexenal	341a	306a	173a	181a	232a	155a	389a	338a	174a	111b

[1] C = control, T = hypoxic treated. [2] 2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = Ethyl-2-methyl butanoate. [3] Mean separation in a row for a given cultivar by Duncan's multiple range test, values followed by a letter in common are not significantly different at 5%. [4] ND = not detected, concentration below 0.1 $\mu L \cdot L^{-1}$.

Table 4.3b. Concentration of volatiles ($\mu mol \cdot L^{-1}$) for apples stored in air and on removal from hypoxia (d 3). Mean of four replicates.

Cultivar	RD		RG		SP		SS	
Treatment	C[1]	T	C	T	C	T	C	T
Alcohols								
Ethanol	22a[3]	9448b	184a	1693a	ND[4]	10130b	ND	24120b
Propan-1-ol	106a	135a	136a	196a	139a	72a	66a	107a
Butan-1-ol	365a	469a	3384a	4640a	1174a	624a	1416a	1697a
Pentan-1-ol	11a	13a	26a	28a	24a	11a	16a	27b
Hexan-1-ol	53a	70a	236a	281a	217a	60b	142a	234b
2MB[2]	112a	67b	106a	116a	169a	59b	71a	78a
Acetate esters								
Ethyl acetate	2a	26b	1a	8a	1a	16b	1a	38b
Propyl acetate	31a	3b	3a	35a	5a	ND	ND	ND
Butyl acetate	72a	63a	516a	534a	128a	37b	273a	164a
Pentyl acetate	2a	3a	9a	11a	4a	ND	8a	7a
Hexyl acetate	30a	19b	137a	118a	57a	10b	58a	40a
2MBA[2]	111a	29b	62a	49a	59a	0b	59a	26b
Ethyl esters								
Ethyl propionate	3a	216b	ND	10a	14a	151b	10a	98b
Ethyl butanoate	10a	353b	11a	15a	15a	244b	10a	346b
Ethyl pentanoate	19a	23a	17a	19a	17a	13a	20a	21a
Ethyl hexanoate	2a	65b	7a	2a	ND	27b	2a	24b
E2MB[2]	ND	49b	ND	ND	ND	16b	ND	17b
Aldehydes								
Acetaldehyde	ND	118b	11a	28a	ND	265b	ND	182b
Hexanal	28a	67a	161a	158a	97a	41b	107a	70a
trans-2-Hexenal	170a	175a	196a	211a	348a	171b	265a	192a

[1] C = control, T = hypoxic treated. [2] 2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = Ethyl-2-methyl butanoate.[3] Mean separation in a row for a given cultivar by Duncan's multiple range test, values followed by a letter in common are not significantly different at 5%. [4] ND = not detected, concentration below 0.1 $\mu L \cdot L^{-1}$.

4.4.6 Alcohols

In general, concentrations of alcohols were greater in apples on removal from hypoxia than in control fruit but cultivar specific enhancement of particular alcohols occurred (Table 4.2). Golden Delicious fruit had enhanced concentrations of propan-1-ol, butan-1-ol, pentan-1-ol, hexan-1-ol and 2 methyl butan-1-ol on removal from hypoxia. Butan-1-ol, pentan-1-ol and hexan-1-ol concentrations were enhanced in CO fruit. Propan-1-ol, pentan-1-ol and hexan-1-ol concentrations were increased in RD fruit. Granny Smith fruit had increased concentrations of propan-1-ol and butan-1-ol. Propan-1-ol was enhanced in FU, RG and SS fruit. In contrast no enhancement of alcohols was noted for PR and SP fruit. By 3 d after removal from hypoxia there were few differences in alcohol concentration between treatments although propan-1-ol concentration was increased in CO fruit, while pentan-1-ol and hexan-1-ol concentrations were enhanced in SS fruit (Table 4.3). Significant decreases in 2 methyl butan-1-ol were noted for RD and SP fruit and hexan-1-ol decreased in SP fruit.

Ethanol concentrations decreased during ripening from d 0 to d 3 for control fruit in CO, GS, FU, RD, RG, SP and SS, tended to increase for PR and were unchanged for GD apples (Tables 4.2 and 4.3). Propan-1-ol concentrations tended to increase in FU, GD, GS, PR, RD, RG, SP and SS fruit while propan-1-ol concentrations tended to decrease for CO fruit. Butan-1-ol concentrations tended to increase for CO, GD, RG and SP fruit, tended to decrease for FU and were unchanged for GS, PR, RD and SS fruit. Pentan-1-ol concentrations tended to increase in CO, GD, GS, PR, RD, RG and SP fruit while tending to decrease in FU and SS. Hexan-1-ol concentrations tended to increase in GD, GS, RG and SP fruit, tended to decrease in SS fruit and were unchanged in CO, FU, PR and RD fruit. Concentration of 2 methyl butan-1-ol tended to increase for CO, GD, GS, PR, RD, RG and SP and were unchanged in FU and SS fruit (Tables 4.2 and 4.3).

4.4.7 Hexanal and trans-2-Hexenal

In general, concentrations of hexanal and *trans*-2-hexenal in hypoxic treated fruit were unaffected by exposure to hypoxia when compared to control apples, both at removal from hypoxia and after 3 d at 20 °C (Tables 4.2 and 4.3). After removal from hypoxia and after 3 d at 20 °C, concentrations of hexanal and *trans*-2-hexenal had decreased in SP fruit compared to control fruit. After 3 d at 20 °C, concentration of hexanal in GD and *trans*-2-hexenal in PR had decreased in fruit exposed to hypoxia more than control fruit. Hexanal and *trans*-2-hexenal concentrations from d 0 to d 3 for control tended to increase during ripening in FU, GD, GS, PR, RD, RG and SP while tending to decrease in CO and SS apples (Tables 4.2 and 4.3).

4.4.8 Acetate esters

On removal from hypoxia CO, FU, GD, RG and SS fruit had decreased concentrations of butyl acetate, CO and RG fruit hexyl acetate and FU and RG fruit 2 methyl butyl acetate compared to control fruit (Table 4.2). There was no change in concentration of acetate esters for GS, PR, RD and SP fruit. Acetate ester concentrations in fruit exposed to hypoxia continued to decrease during storage in air (Table 4.3). After 3 d at 20°C, FU, GD, RD, SP and SS fruit had decreased concentrations of butyl-, hexyl- and 2 methyl butyl acetate in hypoxic treated compared with control fruit with similar

concentrations in CO and RG fruit. The decreases in acetate ester concentrations induced by hypoxia were less than the increases in ethyl ester concentrations; for example, the greatest decrease of butyl acetate was 3.4 fold in SP fruit exposed to hypoxia compared to control fruit whereas ethyl butanoate increased by 34.5 fold.

Ethyl acetate concentrations in control fruit in all cultivars for control fruit were unchanged during ripening from d 0 to d 3 (Tables 4.2 and 4.3). Propyl acetate concentrations tended to decrease in CO, GD, PR, RG and SS fruit, tended to increase for RD fruit and were undetectable in FU, GS and SP apples. Butyl acetate concentrations tended to increase for RG and SP fruit, tended to decrease for CO, FU, GD, RD, and SS fruit and were unchanged for GS and PR fruit. Pentyl acetate concentrations tended to decrease for SS fruit and were unchanged in CO, GD, RD, RG and SP fruit and not detected in GS, FU and PR. Hexyl acetate concentrations tended to increase in GD, RG and SP fruit, tended to decrease in CO, FU and SS fruit and were unchanged in GS, PR and RD fruit. Concentrations of 2 methyl butyl acetate tended to increase for GD, PR, RD, RG and SP and was unchanged in CO, FU and SS fruit but undetected in GS (Tables 4.2 and 4.3).

4.4.9 Ethyl esters

Hypoxic treatment enhanced ethyl ester concentrations at d 0 at 20 °C in CO, FU, GD, PR, RD, SP and SS fruit but had no effect on GS and RG fruit (Table 4.2). Ethyl butanoate was enhanced in CO, FU, GD, PR, RD, SP and SS fruit and ethyl hexanoate was enhanced in FU, GD, PR and RD fruit. Ethyl pentanoate was enhanced only in GD fruit on removal from hypoxic treatment.

After 3 d at 20 °C concentrations of ethyl esters in control and treated fruit were greater and a different pattern of ethyl ester enhancement was apparent than in d 0 fruit (Table 4.3). Ethyl butanoate, ethyl hexanoate and ethyl-2-methyl butanoate were present in increased concentrations in FU, PR, RD, SP and SS fruit while only ethyl butanoate was increased in CO fruit. Ethyl esters were no longer enhanced in GD fruit and ethyl pentanoate concentrations in hypoxic treated fruit were similar to concentrations in controls. In general, control fruit tended to have greater concentrations and more ethyl esters after 3 d at 20 °C than fruit at d 0 (Tables 4.2 and 4.3). Depending on cultivar and ester, the proportional increases due to exposure to hypoxia in ethyl esters was generally large, for example at d 3 up to 86.5 fold for ethyl acetate in CO fruit, 34.5 fold for ethyl butanoate in SS fruit and up to 65 fold for ethyl propionate in RD fruit (Table 4.3). Ethyl propionate at d 0 was present in FU, GD, GS and SP fruit but after 3 d was present in CO, FU, GD, GS, RD, SP and SS apples. At d 0 ethyl butanoate and ethyl pentanoate were in FU, GD, GS, PR, RD, SP and SS fruit but were present in all cultivars after 3 d at 20 °C. Ethyl hexanoate only appeared in volatile extracts of CO, RD, RG and SS fruit after 3 d at 20 °C. In control, fruit ethyl-2-methyl butanoate was not detected except for trace amounts in GS fruit both at removal and after 3 d at 20 °C (Tables 4.2 and 4.3). At d 0 control CO fruit had no ethyl esters while RG fruit had only ethyl pentanoate but after 3 d there was a greater number of ethyl esters for both cultivars.

4.4.10 Volatile changes after hypoxic treatment

Hypoxic treatment consistently enhanced concentrations of acetaldehyde, ethanol and ethyl acetate in all cultivars examined both on removal from hypoxia and during ripening at 20 °C (Tables 4.2 and 4.3). The general effect of a brief exposure to hypoxia was for ethyl esters to be enhanced and acetate esters to be decreased depending on specific esters and cultivars (Figure 4.1, Table 4.4). Cox's Orange Pippin is an example of a cultivar that did not increase ethyl esters after hypoxia, and RD is one that had an increase in ethyl esters (Figure 4.1) where a maximum occurred 3 d after removal from hypoxia and declined slowly thereafter (Figure 4.1). Decreases in acetate esters were associated with increases in their corresponding alcohols. Acetaldehyde showed the greatest enhancement 1 d after removal from hypoxia. There was no significant difference in hexanal and *trans*-2-hexenal concentrations between control and treated fruit.

Cultivars could be divided in 4 groups depending on the changes in acetate and ethyl esters after 3 d at 20 °C (Table 4.4).

Table 4.4 Apple cultivars classified according to their changes in ethyl and acetate esters after exposure to hypoxia, 100% CO_2 for 24 h at 20 °C, d 3 after removal from hypoxia.

	No change in acetate esters	Decrease in acetate esters
Enhanced ethyl esters	PR, SS	FU, RD, SP
No change in ethyl esters	CO, GS, RG	GD

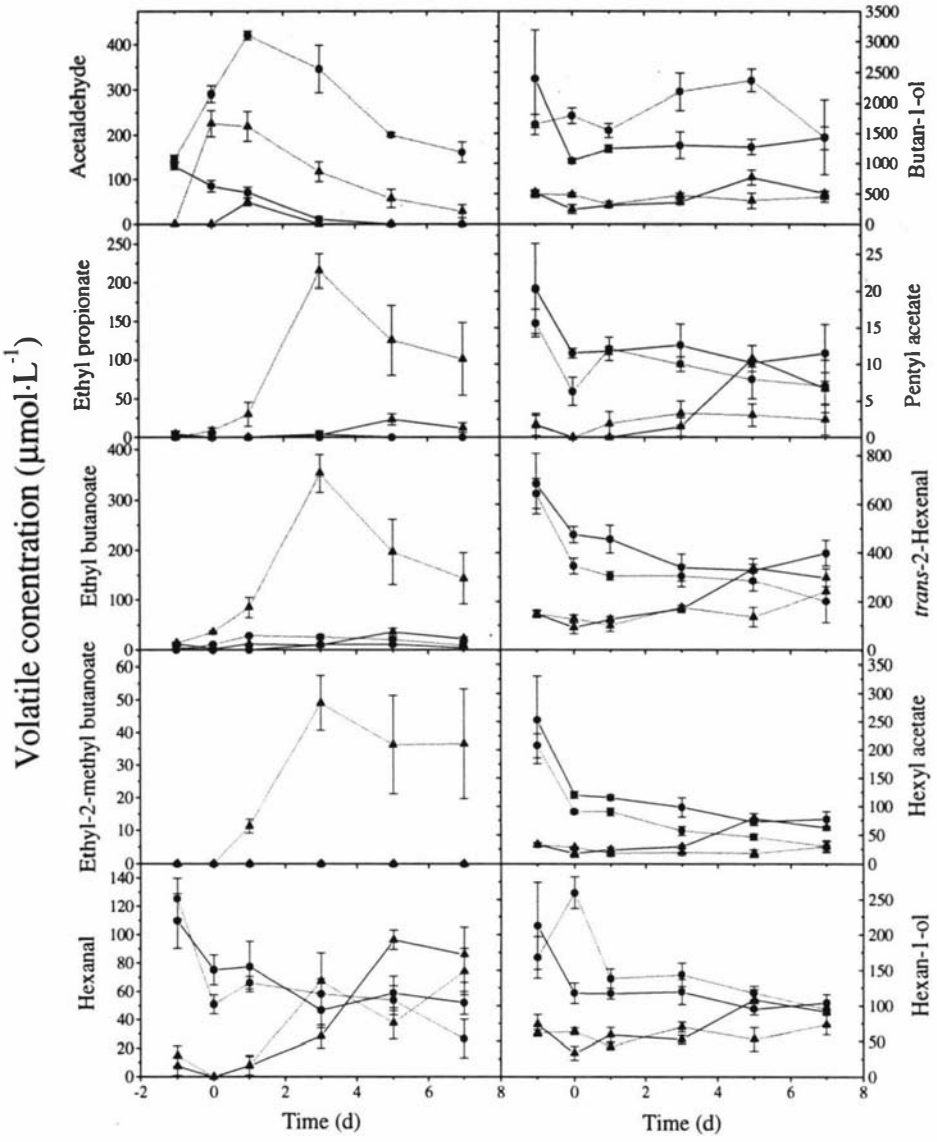

Figure 4.1 Concentration of selected aroma volatiles extracted from juice of CO (●) and RD (▲) apples exposed to a 24 h hypoxic treatment (·····) or air (—) and maintained at 20 °C. Aroma volatiles shown represent compounds thought to have important sensory characteristics in apple (Rizzolo et al., 1989). Average and standard errors of 4 replicates.

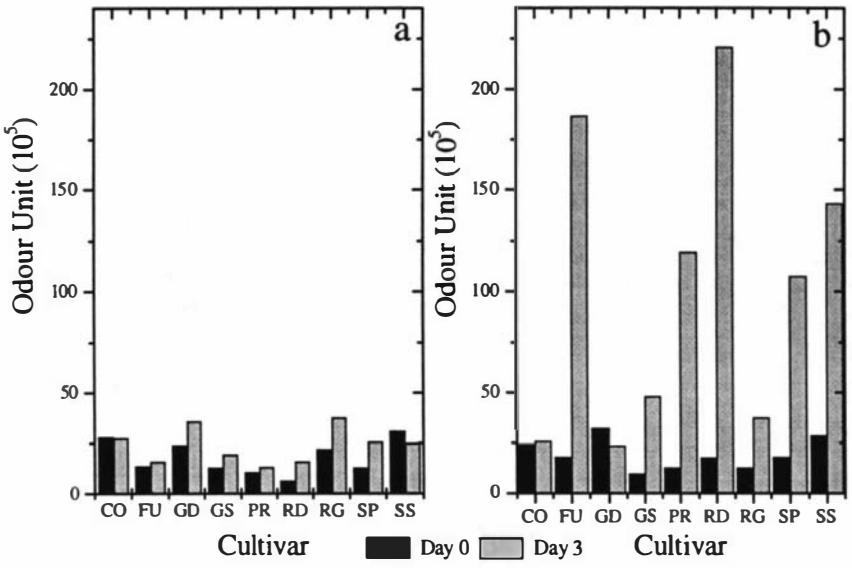

Figure 4.2. Odour unit values for apple juice from (a) control and (b) apples exposed to hypoxia, on removal from treatment (day 0) and after 3 d at 20 °C.

4.4.11 Odour units

Odour unit values, based on volatile concentrations presented in Tables 4.2 and 4.3, indicate that those cultivars which had enhanced ethyl esters and decreased acetate esters had the highest odour unit values (Figure 4.2). Golden Delicious apples had no change in ethyl esters but a decrease in acetate esters and this was reflected in a decrease in odour units. Apart from CO and GD, apples treated with hypoxia would have greatly enhanced aroma in apples exposed to hypoxia according to their aroma unit values (Figure 4.2). The hypoxic treatment increased concentration of volatiles affecting aroma intensity with the variation among cultivars ranging from a 1.5 fold decrease in GD to a ten fold increase in RD (Figure 4.2).

4.5 Discussion

4.5.1 Fruit ripening

Exposure of a number of commercial apple cultivars to a brief period of hypoxia using high CO_2 concentrations, reduced ethylene production but did not affect carbon dioxide production or fruit firmness during 7 d at 20 °C. This was similar to results for Braeburn, GS, and RD cultivars reported by Ampun (1997). An exception was the cultivar SP that, when exposed to hypoxia, had decreased CO_2 and ethylene production and fruit firmness compared to control fruit. This suggests that brief exposure to hypoxia will generally have little effect on ripening of apples.

Ethylene production was decreased in several cultivars tested and was not related to CO_2 production or increased firmness. Similar effects on CO_2 and ethylene production after treatment with a wide range of CO_2 concentrations and exposure times have been

reported for peaches, nectarines (Lurie and Pesis, 1992; Pesis, 1994), pears (Wang and Mellenthin, 1975), strawberry (Ke at al., 1991; Larsen and Watkins, 1995a, b; Ueda and Bai, 1993;) and apples (Chaves and Tomás, 1984; Couey and Olsen, 1975; Hribar et al., 1994; Solomos, 1993; Stow, 1988). The general reduction in ethylene production and unchanged CO_2 production, for cultivars other than GD, was in contrast to the effect of high CO_2 treatment reported previously for GD where high CO_2 induced an increase in CO_2 and ethylene production and enhanced softening (Pesis et al., 1994). The trend of CO_2 and ethylene production of GD apples reported here, although non-significant, was similar to those of Pesis et al. (1994).

Softening of many types of fruit is significantly retarded by high CO_2 and low O_2 treatments (Hribar et al., 1994; Larsen and Watkins, 1995a, b; Lurie and Pesis, 1992; Pesis, 1994). Apart from SP fruit 3 d after hypoxia firmness of cultivars, was unaffected over the 7 d of the experiment and would probably remain so in long term air stored fruit. Although SP apples exposed to hypoxia were softer after 3 d at 20 °C, firmness was not different from control fruit at d -1, 0, 5 and 7 (data not shown).

Concentrations of volatiles increase as apples ripen reaching a peak at the climacteric maximum (Mattheis et al., 1991; Song and Bangerth, 1994, 1996; Yahia et al., 1990). After 3 d ripening control fruit had generally greater concentrations of volatiles than fruit at d 0 but the increase in volatile concentration was not related to ethylene production. All types of volatiles increased with changes in specific volatiles depending on cultivar. While alcohols and acetate esters were present at d 0, ethyl esters ethyl hexanoate and ethyl-2-methyl butanoate only appeared during ripening indicating that certain ethyl esters are synthesised as ripening progresses. Increased internal ethylene concentration may be a prerequisite for enhanced volatile production in apples (Song and Bangerth, 1996) as increased ester biosynthesis in Bisbee Delicious apples coincided with an increase in the climacteric rise of internal ethylene (Mattheis et al., 1991). The cultivars CO, GD and RG had ethylene production of 1 $nmol \cdot kg^{-1} \cdot s^{-1}$ or greater after 3 d at 20 °C, at which time butyl acetate concentrations were greater than 300 $\mu mol \cdot L^{-1}$ and hexyl acetate concentrations greater than 95 $\mu mol \cdot L^{-1}$. In contrast FU, GS, PR, RD, SP and SS fruit had had less than 1 $nmol \cdot kg^{-1} \cdot s^{-1}$ ethylene production and butyl acetate concentrations less than 300 $\mu mol \cdot L^{-1}$ and hexyl acetate concentrations less than 95 $\mu mol \cdot L^{-1}$. These data suggest that rather than ester biosynthesis being proportional to ethylene production rate (Bangerth et al., 1998) high concentrations of acetate esters in apples requires ethylene production to exceed 1 $nmol \cdot kg^{-1} \cdot s^{-1}$.

4.5.2 Anaerobic volatiles

Endogenous ethanol concentrations were high in control CO, FU and RG apples during normal ripening compared to other cultivars in agreement with previous results (Fidler and North, 1971). Exposure of apples to hypoxia induced large increases in ethanol concentration but with differences between cultivars. Cox's Orange Pippin had the highest endogenous concentration of ethanol amongst cultivars, but there was only a 3 fold increase induced by hypoxia. Ethanol was not detected in control fruit of PR and GD but had increased to 6,879 and 14,510 $\mu mol \cdot L^{-1}$, respectively, on removal from hypoxia. Such increases made ethanol the most abundant alcohol in the fruit tissue. In contrast, maximum concentration of the next most abundant alcohol butan-1-ol ranged

from 20 $\mu mol \cdot L^{-1}$ in GS to 2820 $\mu mol \cdot L^{-1}$ in GD apples after treatment. Concentrations of ethanol and acetaldehyde induced by hypoxia may be related to the specific O_2 concentration at which anaerobic respiration begins for each cultivar, the anaerobic compensation point (ACP) (Yearsley, 1996). The ACP for Rome apples is less than 0.5% O_2 which is lower than for GD and Delicious that produce less ethanol in hypoxic conditions (Fellman et al., 1993). Marshall McIntosh, RedMax McIntosh and Red Chief Delicious apples had oxygen thresholds for ethanol accumulation of 1.9%, 0.5% and 0.1% with ethanol concentrations of 8200, 6295 and 2822 $\mu mol \cdot kg^{-1}$ after 6 days at 2.2 °C, respectively (Blanpied and Jozwiak, 1993). This suggests that the degree of ethanol enhancement after hypoxia will depend on the ACP of the cultivar; those cultivars with the highest ACP will produce the greatest amount of ethanol following hypoxia.

Acetaldehyde and ethanol concentrations although declining remained high for several days after treatment (Figure 3.1) suggesting that production of fermentation volatiles declines slowly to pre-treatment levels in fruit returned to air. Similar enhancement in ethanol concentration has been noted in blueberries (Saltveit and Ballinger, 1983a), grapes (Saltveit and Ballinger, 1983b) and oranges (Pesis and Avissar, 1989) following hypoxic treatments. The persistence of high ethanol concentrations in hypoxic treated fruit probably provides a large pool of substrate for rapid and sustained production of ethyl esters, even though fruit had been returned to an aerobic environment.

Apples appear to tolerate a brief exposure to hypoxia at 20 °C, as no physical or physiological damage was observed following treatment. Apple cultivars may vary in their ability both to metabolise ethanol and lose ethanol through evaporation from the fruit. Ethyl acetate concentrations were low on removal from hypoxia but increased to a peak between 3 to 5 days after removal from hypoxia confirming previous results (Ampun, 1997). This suggests that tolerance to hypoxia may involve metabolising ethanol to ethyl acetate. Ethyl acetate has a low solubility in water (Buttery et al., 1969) and evaporates readily from the fruit unlike ethanol which being completely soluble in water can accumulate to very high concentrations which may be phytotoxic.

4.5.3 Changes in alcohols, esters and aldehydes

Hypoxic treatment consistently enhanced concentrations of the anaerobic volatiles acetaldehyde and ethanol, in all cultivars examined, both on removal from hypoxia and during ripening at 20 °C (Tables 4.2 and 4.3). The general effect of a brief exposure to hypoxia was for some or all ethyl esters to be enhanced and acetate esters to be decreased (Figure 4.1, Table 4.5). In general, enhancement of ethyl esters reached a maximum 3 d after removal from hypoxia and declined slowly thereafter (Figure 4.1). For acetaldehdye greatest enhancement was 1 d after removal from hypoxia.

Oxygen is considered to be an essential co-factor for esterification of alcohols in fruit tissue (Drawert and Berger, 1983; Harada et al., 1985) by supplying NADH from aerobic respiration (Molina et al., 1987). In the absence of O_2, esterification reactions stop resulting in an increase in all types alcohols. On return to aerobic conditions, these alcohols are metabolised either to esters (Yamashita et al., 1976) or to shorter chain compounds before esterification (Rowan et al., 1997) or they evaporate from the fruit tissue. Therefore, after removal from hypoxic conditions, there should be an increase in concentration of a wide range of esters in the apple aroma. However, only ethyl esters

increased after hypoxic treatment and most cultivars continued to produce ethanol at high concentrations for several days after hypoxic treatment. This indicates that the fermentative biosynthetic pathway may be active for some time after return to normal aerobic conditions. Therefore, in fruit exposed to hypoxia, ethyl ester production has the potential to be enhanced for some time after removal to air.

Branched chain volatiles are thought to arise primarily from amino acids (Sanz et al., 1997). Iso-leucine is metabolised to 2-methyl butan-1-ol, 2-methyl butyl acetate and ethyl-2-methyl butanoate (Rowan et al., 1996). In general 2-methyl butyl type compounds followed the same pattern of response to hypoxic treatments as straight chain compounds (Tables 4.2 and 4.3). The decrease in 2-methyl butyl acetate was significant after 3 d at 20 °C in FU, RD, SP, SS which coincided with significant increases in ethyl-2-methyl butanoate. Cultivars differed widely in concentrations of compounds with 2-methyl butyl moieties, an example being ethyl-2-methyl butanoate which was detected in hypoxic treated FU, GS, PR, RD, SP and SS apples, but not in CO, GD and RG fruit. This may indicate that in hypoxic treated fruit, 2-methyl butyl compounds are converted to 2-methyl butyl CoA rather than being esterified with acetyl CoA to the acetate ester form. The reduction of 2-methyl butyl acetate may also have been due to competitive inhibition of ethanol with 2-methyl butanol.

Production of hexanal and *trans*-2-hexenal is an O_2 dependent process occurring by degradation of linoleic and linolenic acids via the lipoxygenase pathway (Sanz et al., 1997) and could be expected to be inhibited by hypoxia. That these compounds showed, in general, little change or only a minor reduction in concentration after exposure to hypoxia, suggests that this treatment had little if any effect on the lipoxygenase biosynthetic pathway.

Cultivars which had the most significant increases in ethyl esters after hypoxic treatment (FU, PR, RD, SP and SS), also tended to have the most significant decreases in acetate esters (Table 4.5). The same trends were found for CO, GD and RG fruit although they were non-significant for these cultivars. In GS there was a tendency for ethyl esters to increase with little change in acetate esters. This result supports the hypothesis that increases in ethyl ester concentrations induced by hypoxia because increased ethanol is available for esterification and most available acyl CoA's are then used in the production of ethyl esters. Similar increases in ethyl esters have been noted for Delicious apples after hypoxia for 30 d at 1 °C (Mattheis et al., 1991), grapes after carbonic maceration (Tesnière et al., 1989) and oranges after 24 h of hypoxia (Shaw et al., 1990).

4.5.4 Mode of action of hypoxia

It is suggested that changes in volatile concentration following hypoxic treatment could be due to two effects on the volatile biosynthetic pathway working either independently of one another or in combination. First the large increase in ethanol concentration may competitively inhibit formation of non-ethyl esters (Ke et al., 1994). Second hypoxic conditions induce new isozymes of enzymes that use ethanol as the preferred substrate for formation of ester compounds. High concentrations of ethanol, induced by hypoxia, competitively use available acyl CoA's resulting in large concentration increases of ethyl esters and decreased non-ethyl ester concentrations. Therefore, high ethanol concentrations may be competitively inhibiting formation of non-ethyl ester volatiles.

That hypoxic treatments induce large increases in ethyl esters, decreases in non-ethyl esters and increased concentration of C_3+ alcohols on removal from hypoxia support this hypothesis. Similar increases in ethyl esters and decreases in acetate esters have been shown for Delicious apples exposed to hypoxia for long periods at low temperatures (Mattheis et al., 1991).

Individual apple cultivars had one of four patterns of response to hypoxia in relation to their ability to further metabolise ethanol to esters. These patterns were independent of ethanol concentration after hypoxia; for example, SS apples had a very large increase in ethanol concentration and very large increases in ethyl ester concentrations whereas CO fruit had a very large increase in ethanol concentration but little change in concentration of ethyl esters (Table 4.3).

Table 4.5. Trends of increase or decrease in volatile concentration 3 d after hypoxic treatment.

Volatile	CO	FU	GD	GS	PR	RD	RG	SP	SS	Overall
Acetate esters										
Ethyl acetate	+***	+***	+***	NC	+**	+**	+*	+***	+***	+***
Propyl acetate	NC	ND	ND	+	+	−**	+	−	ND	NC
Butyl acetate	−	+*	−	−	−	−	NC	−*	−	−
Pentyl acetate	+	ND	−	ND	+	+	+	−	−	−
Hexyl acetate	−	−*	−**	NC	−	−*	−	−*	−	−***
2MBA	−	−**	−*	ND	−*	−***	−	−**	−**	−***
Ethyl esters										
Ethyl propionate	−	+***	−	+*	+***	+***	+	+**	+**	+***
Ethyl butanoate	+*	+***	+	+	+***	+***	+	+***	+**	+***
Ethyl pentanoate	+	+	−	−	+	+	+	−	+	NC
Ethyl hexanoate	−	+***	ND	+	+*	+*	−	+***	+**	+***
E2MB	ND	+***	ND	+	+***	+**	ND	+***	+**	+***

Trends: + = increase, − = decrease, NC = no change, ND = not detected.
Levels of significance: * = $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The patterns of response to hypoxia relate changes in concentration of ethyl esters to concentration changes in acetate esters. After exposure to hypoxia, CO, FU, RD and SP apples had increased concentration of ethyl esters and decreased acetate esters compared to control fruit. As the proportion of ethanol to other alcohols is high it is likely that ethanol competitively inhibits production of other esters. Non-ethyl esters continue to be produced, as C_3+ alcohols increase in concentration, presumably by not being used as substrates for ester biosynthesis. Alcohols other than ethanol, may be preferred as substrates in the ester biosynthetic pathway, but as their concentrations relative to ethanol are so low, their corresponding esters are at low concentrations.

After exposure to hypoxia, PR and SS apples had increased concentrations of ethyl esters but no change in acetate ester concentration compared to control fruit. This suggests that ethanol is not competitively inhibiting non-ethyl ester production in these cultivars. It is possible that the ester forming biosynthetic pathway may have a preference for C_3+ alcohols, but as concentration of ethanol induced after hypoxia is so great, increased ethyl ester synthesis occurs at the expense of other esters.

Exposing GD and RG apples to hypoxia did not change ethyl ester concentration but did result in decreased acetate esters compared to control fruit. In these cultivars the ester

forming biosynthetic pathway may have a low affinity for ethanol. Even though high concentrations of ethanol are present, esterification of C_3+ alcohols to non-ethyl ester forms occurs preferentially. Although not measured in this study, in GD apples butyl and hexyl esters, *i.e.* butyl butanoate, hexyl butanoate, hexyl hexanoate, were produced preferentially to ethyl and acetate esters (Song and Bangerth, 1996).

Exposure to hypoxia of GS apples resulted in no change in ester concentration compared to control fruit. In this cultivar ester concentration was low indicating that ester production occurs only slowly under normal equilibrium conditions.

Hypoxic conditions may induce changes in enzyme concentration and/or substrate specificity of enzymes in the biosynthetic pathways which produce volatile compounds. New isozymes of ADH are produced in response to hypoxia in grapes, tomato and avocado (Tesnière et al., 1993; Chen and Chase, 1993; Kanellis et al., 1991). If new isozymes of ADH are induced in apple fruit after hypoxia, then increased ADH activity may explain the continued production and accumulation of ethanol, that occurs in some cultivars, for several days after return to aerobic conditions.

Different fruits have different AAT isozymes each with their own preference for specific alcohols and acyl CoA's (Olias et al., 1995). Between cultivars, differences may exist in substrate specificities of AAT for alcohols or acyl CoA's as has been found in strawberry (Sanz et al., 1997). Exposure to hypoxia induces changes in AAT activity (Fellman et al., 1993) and it is possible that, by analogy with ADH, such a treatment may induce new isozymes of AAT with different substrate specificities for alcohols or acyl CoA's. It is also possible that a period of hypoxia induces changes in the concentration of acyl CoA's by influencing the β-oxidation biosynthetic pathway in which long carbon chain acyl CoA's are synthesised (Sanz et al., 1997). However, little is known of the factors that affect acyl CoA concentrations and synthesis in fruit and this would be a most interesting and valuable topic for further research.

Treatments using hypoxia consistently enhance volatile biosynthesis over a wide range of cultivars. Apple cultivars can be classified according to their capacity to produce ethyl esters after hypoxic treatment which follow the 'ester type' and 'alcohol type' classifications proposed by Palliard (1990). Golden Delicious, GS and RG apples would be examples of 'alcohol' types and CO, FU, PR, RD SP and SS examples of 'ester' types. Those cultivars which produce high concentrations of ethyl esters during ripening tend to produce very high concentrations of ethyl esters after hypoxic treatment. The reason for this is not known but it is clear that in the cultivars assessed in this study, appropriate ester biosynthetic pathways are present and can be induced to greater activity by exposure to hypoxia. As such these responses to hypoxic treatments may be a useful tool to explore the mechanisms for differences in types of esters between cultivars. This study has identified some cultivars that produce high ethyl ester concentrations after hypoxic treatment and some which do not. Volatile metabolism of specific cultivars after hypoxic treatment could be investigated using precursors for volatile biosynthesis to determine changes in biochemical pathways induced by hypoxia.

4.5.5 Putative effects on aroma

Volatiles considered important as characteristic for apple aroma (Dürr and Schobinger, 1981; Rizzolo et al., 1989) were either enhanced or suppressed after exposure to hypoxia (Figure 4.1). The relative contributions to the aroma of apples by ethyl and acetate esters are different; ethyl esters contribute fruity apple like characteristics while acetate esters contribute a more fruity/solvent like overtones to apple aroma (Rizzolo et al., 1989). Estimates of odour units indicate that apples exposed to hypoxia would in general have a more intense aroma, due mostly to the increase in ethyl esters known to have very low odour threshold values.

Odour unit values estimate apple aroma and should indicate treatment effects on sensory characteristics (Teranishi et al., 1987). Odour units are an arbitrary scale defined by the number of times by which the odour threshold concentration is exceeded. The contribution of a volatile to the aroma intensity of a mixture is proportional to the number of odour units (Laing and Panhuber, 1979). Volatile compounds with very low aroma thresholds at high concentrations may not have more intense aroma but a different aroma which could be considered an off flavour. For this reason apples exposed to hypoxia require evaluation by a sensory panel to determine if the increase in ethyl esters and decrease in acetate ester concentration changes sensory perception of apple aroma.

4.6 Conclusions

A brief period of hypoxia, induced by exposure to 100% carbon dioxide for 24 h at 20 °C, enhanced ethyl esters and anaerobic volatiles while suppressing acetate esters and aldehydes for a wide range of apple cultivars. Cultivars were ranked from greatest to least for enhancement in ethyl esters following 3 d at 20 °C after removal from hypoxia: RD, SS, SP, FU, PR, GS, RG, CO, GD. Enhancement of ethyl esters may be due to competitive inhibition by ethanol with other alcohols in the ester biosynthetic pathway or to a change in the enzymes which produce volatile compounds.

While the mechanism of volatile enhancement and suppression after hypoxic treatment is unknown, any postharvest treatment using brief periods of hypoxia at ambient temperatures, such as disinfestation, have the potential to change the aroma and flavour of apples albeit transiently. Such results highlight the need for careful selection of cultivars when using a hypoxic treatment if these aroma/flavour changes are likely to change concentration of aroma volatiles. The results presented in this study indicate that the enhancement of volatiles induced by hypoxic treatment are transitory lasting up to 7 d in some cases. The long term effect of hypoxic treatment before or during air or controlled atmosphere low temperature storage requires investigation.

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Chapter Five

Ester production of apples following a brief period of hypoxia.

Additional index words. Alcohol acyl CoA transferase; cultivars; precursors; volatiles; aroma.

5.1 Abstract

Apple aroma consists of mainly low molecular weight esters produced by esterification of alcohol's by alcohol acyl CoA transferase (AAT) where acyl CoA's are substrates. Apples exposed to hypoxic conditions for brief periods enhance fermentation volatiles and ethyl esters while decreasing acetate esters. Such treatments increased esterification activity in fruit returned to air, possibly due to enhanced AAT activity or by competitive inhibition of other alcohols by ethanol. Experiments to measure esterification activity were undertaken using 9 apple cultivars. Short periods of hypoxia (100% CO_2 for 24 h at 20 °C) enhanced ethanol and ethyl acetate concentrations which quickly dissipated on return of fruit to air. Concentration of acetate and ethyl esters from skin disks of fruit exposed to hypoxia were compared to disks from control fruit, after addition of C_2 to C_6 alcohols, either individually or as a mixture in equimolar amounts to the disks. Ethanol added as an individual alcohol induced high ethyl acetate concentrations, but when added as part of a mixture, little ethyl acetate was produced indicating substrate preference was for longer chain alcohols. Apple cultivars had the following 4 patterns of change in ester production after exposure to hypoxia: increased acetate and ethyl esters, increased acetate esters and decreased or no change ethyl esters, no change or decreased acetate esters and increased ethyl esters, no change or decreased acetate esters and decreased or no change ethyl esters. This implies that AAT activity is affected differentially by hypoxia. It is suggested that AAT is located in mitochondria and peroxisomes at the sites of acyl CoA production. Hypoxia induces changes in capacity to produce esters which last up to 7 d indicating that pre-storage treatments using brief periods of hypoxia have the potential to change the aroma profile of apples.

5.2 Introduction

Brief periods of hypoxic conditions, where oxygen concentration does not support aerobic metabolism (Ricard et al., 1994), utilising atmospheres of up to 100% carbon dioxide (CO_2) or nitrogen (N_2) for 1 to 2 d at about 20°C are potential disinfestation treatments (Gaunce et al., 1982; Morgan and Gaunce, 1975; Soderstrom et al., 1990). In addition to killing insects, postharvest application of hypoxia reduces incidence of postharvest decay (Ke et al., 1991; Pesis and Avissar, 1989), improves general fruit quality (Pesis and Avissar, 1989; Pesis, 1994) and increases volatile concentration in oranges and feijoa (Pesis et al., 1991; Shaw et al., 1990; Shaw et al., 1991). Apple fruit exposed to hypoxia for long periods have increased ethyl ester and decreased non-ethyl ester concentrations (Mattheis et al., 1991a). These changes in concentration could be important to flavour perception, as ethyl esters are thought to confer fruity or apple-like characters to the overall aroma of apple fruit (Flath et al., 1967). The consequences of a

brief period of hypoxia on apple quality, in particular volatile production, have not been explored fully and the potential exists that such treatments evaluated for disinfestation purposes may affect aroma/flavour and hence acceptability of the fruit to consumers.

Enhanced ethyl ester and decreased acetate ester concentrations were found in 9 apple cultivars exposed to hypoxia (100% CO_2 for 24 hours at 20°C, Chapter 4). The consistent enhancement of ethyl esters after hypoxia probably resulted from the very large increase in ethanol concentration induced by the treatment. High ethanol concentrations, up to 10 fold greater than the next most abundant alcohol, butan-1-ol, could be competitively inhibiting formation of esters from other alcohols. Increases in ethyl ester concentrations continued during 7 d at 20 °C in air after hypoxic treatment (Chapter 4) indicating that exposure to hypoxia may induce persistent changes to volatile biosynthesis.

Apple aroma consists of alcohols, aldehydes, esters, ketones and ethers where most are C_3 to C_{12} esters and alcohols (Dimick and Hoskin, 1983; Sanz et al., 1997). Alcohols and acyl CoA's are synthesised from amino acids and membrane lipids primarily by β-oxidation and by the action of alcohol dehydrogenase (Sanz et al., 1997). Whole fruit or fruit disks supplied with volatile precursors have been used to explore the biosynthetic pathway of aroma volatiles in banana (Gilliver and Nursten, 1976; Harada et al., 1985), strawberry (Drawert and Berger, 1983; Pérez et al., 1993; Ke et al., 1994; Sanz et al., 1997) and grape (Molina et al., 1986; 1987). Addition of acetic acid and butan-1-ol to disks of apple cortical tissue resulted in enhanced concentration of butyl acetate (Paillard, 1975). Intact Red Delicious apples exposed to ethanol vapours and aged tissue disks supplied with a range of straight chain alcohols produced increased concentrations of many different esters after a 24 hour incubation period (Berger and Drawert, 1984). Exposure to alcohol vapours has been developed as 'Precursor atmosphere' technology to enhance apple tissue ester concentrations before processing (Berger et al., 1992). Applying C_2 to C_6 alcohols as volatile precursors successfully enhanced ester concentrations in long term controlled atmosphere stored apples (Harb et al., 1994).

A wide range of alcohols are simultaneously available to the ester biosynthetic pathway in apple tissue. The enzyme alcohol acyl CoA transferase (AAT, EC 2.3.1.84) converts these alcohols to esters using acyl CoA's in an oxygen dependent reaction (Harada et al., 1985) which is most active in apple skin (Berger, 1990). Normal synthesis of esters, regarded as a balance with ester hydrolysis (Bartley and Stevens, 1981; Goodenough, 1983), occurs slowly, is limited by alcohol substrate availability (Knee and Hatfield, 1981) and is most active during the climacteric (Mattheis et al., 1991b).

Exposure of apples to various environmental treatments, for example, low oxygen (Mattheis et al., 1991a) and high temperatures (Fallik et al., 1997) may induce quantitative and qualitative differences in ester concentration indicating that changes in environment may alter the ester biosynthetic pathway. Concentration of esters formed by AAT is proportional to the concentration of alcohol, assuming acyl CoA concentrations are not limiting (Olias et al., 1995). Acetyl CoA is thought to be the most abundant acyl CoA in fruit tissue which is the reason for high concentrations of acetate esters (Knee and Hatfield, 1981). Specific alcohols added to apple tissue induce large increases in corresponding acetate ester concentrations and small increases in corresponding esters with other acyl moieties (Berger and Drawert, 1984). Therefore, adding alcohols

individually to apple tissue in high concentrations should saturate the ester biosynthetic pathway to produce mostly acetate esters of the added alcohol and depress the concentration of esters from other alcohols. Changes in the spectrum of esters produced or ester concentrations after hypoxia might be caused by several independent or combined processes including: a general increase in the substrate ethanol, an increase in AAT activity or production/activity of new isozymes of AAT which have substrate specificity for ethanol. Therefore, it is possible that apples exposed to hypoxia have an enhanced capacity for biosynthesis of all esters compared to control fruit but ester production is limited by substrate availability in control and treated fruit. After addition of precursors apples exposed to hypoxia should have greater ester production and concentrations than untreated control apples.

To determine if apples exposed to hypoxia have enhanced capacity to produce specific esters, individual C_2 to C_6 alcohols were added to skin disks from control and hypoxic treated apples. Use of individual alcohols should allow differences to be determined in the relative ability of apple tissue to utilise different alcohols after exposure to hypoxia. Differences in increases between control and hypoxic treated fruit in ester concentrations after addition of individual alcohols may not accurately reflect the substrate preference of the ester biosynthetic pathway. A very high concentration of one specific alcohol should competitively inhibit production of esters from other alcohols. Use of individual alcohols to determine alcohol preference of the ester biosynthetic pathway does not account for the fact that a wide range of alcohols are simultaneously available for ester formation. To remove competitive inhibition effects when using alcohol precursors, an equimolar mixture of C_2 to C_6 alcohols was added to disks from control and hypoxic treated apples. By examining changes in concentration of specific volatiles related to the alcohols added as precursors, changes in substrate preference for specific alcohols could be identified for the ester forming biosynthetic pathway in fruit exposed to hypoxia compared control apples. To determine the duration of changes in the volatile biosynthetic pathway induced by exposure to hypoxia, a mixture of C_2 to C_6 alcohols was added to apple disks from apples exposed to hypoxia 18 h and 1 week after treatment.

5.3 Materials and Methods

5.3.1 Fruit Supply

Nine cultivars of apples (*Malus domestica* Borkh.) were harvested in 1997 at mid commercial harvest: 10 Feb. for Cox's Orange Pippin (CO), 3 Apr. for Fuji (FU), 12 Apr. for Golden Delicious (GD), Granny Smith (GS), Sciros (PR, Pacific Rose™), 19 Apr. for Red Delicious (RD), 25 Feb. for Royal Gala (RG), Sciglo (SS, Southern Snap™), from Hawke's Bay, New Zealand. Fruit were graded to export standard, packed to count 100-125 (about 148 to 188 g fresh weight on packing), transported to Massey University by road and placed at 0 °C ± 1 °C within 3 days of harvest. Splendour apples (SP) were harvested from the Fruit Crops Unit, Massey University (mid-May 1997), graded to 125 count and placed at 0 °C ± 1 °C.

5.3.2 Hypoxic Treatment

Fruit were removed from coolstore and equilibrated at 20 °C ± 3 °C, treated with 1000 ppm ethylene for 1 h at 20 °C ± 3 °C, then divided into two groups: one hypoxic treated, the other stored in air as controls. Controls were left in cartons in the laboratory. For details of hypoxic treatment refer to section 4.3.2.

5.3.3 Trapping of Volatiles

After hypoxic treatment apples were left in air for 18 hours to allow the anaerobic volatiles, acetaldehyde and ethanol, to dissipate before addition of alcohol precursors. Skin disks (14 mm dia. about 0.5 mm thick) were cut from 10 fruit per treatment from the equator using a cork borer. No attempt was made to obtain disks of uniform colour. Five disks were randomly assigned from each fruit to make a group of 50 disks (7 to 10 g fresh weight) which were placed into 250 ml glass Drechsel gas washing bottles containing 100 mLs of McIllvanes buffer (citric acid and Na_2HPO_4 adjusted to an osmotic strength of 0.5 M using KCl, pH 5.8) which had been aerated for 1 hour. A range of C_2 to C_6 alcohols, putative precursors of aroma volatiles, were added either individually, concentration 3 mmol each, or as a mixture, concentration 1 mmol each giving a 5 mmol solution. Disks were left standing in buffer for 30 minutes, then aerated for 12 hours at a air flow rate of 45 $mL \cdot min^{-1}$. A bottle containing buffer only was used as a blank.

Aroma volatiles were purged from the disks and buffer by passing 5 L of air through the Drechsel bottles and Tenax® traps (400 mg Tenax®-GR, 60/80 mesh, in 3 mm i.d. x 300 mm length glass tubes plugged with silanized glass wool). As each trap had variable resistance to air flow the time of trapping varied from 1 to 4 h. Traps were washed 4 times with 1 mL of diethyl ether:*n*-pentane (2:1 v/v) to desorb volatiles. Octyl acetate, 0.5 µL, was added to the solvent before concentrating with oxygen-free N_2 at a flowrate of 200 $mL \cdot min^{-1}$. This allowed correction for volatile losses during subsequent drying with N_2. The diethyl ether:*n*-pentane extract was dried from 4 mL to about 200 µL (20 fold concentration) and placed into a 250µL flat bottom glass insert (Sun International Trading cat. no. 200 232) in a 1.5 mL glass screw top autosampler vial (Sun International Trading cat. no. 200 250) suitable for a Hewlett Packard 5890 series II plus GLC autosampler (Hewlett Packard 7673 Controller and Injector and Model 185968 100 sample carousel). Vials were sealed with a plastic septum (Sun International Trading cat. no. 200 368) before placement in the carousel. One µL samples were injected into the GLC, the syringe was washed 8 times with clean diethyl ether:*n*-pentane between each injection.

5.3.4 Headspace Analysis

After purging, disks were separated from buffer using a sieve and excess moisture was removed from disks before weighing and placement into 50 mL Erlenmeyer flasks sealed using SUBA-SEAL® (no. 33) rubber septa. Flasks were immersed in a 30 °C water bath (Grant SX, 0-100 °C, UK) for 15 minutes (the time taken for volatiles emanating from disks to reach equilibrium) then a 1 mL headspace sample was removed from each flask and analysed for ethanol and ethyl acetate by GLC.

5.3.5 Gas Chromatography

One μL of solvent extract was analysed by capillary gas chromatography using a Hewlett Packard 5890 Series II Plus gas chromatograph with a FID detector, connected to an IBM compatible personal computer equipped with Hewlett Packard ChemStation software (version B.02.04). The capillary column was a J&W 30 m x 0.32 mm (i.d.) fused silica, DBWAX, 0.5 μm film thickness (Alltech cat. no 93526). Injector and detector temperatures were 150 °C and 250 °C, respectively. The oven temperature programme was: 40 °C for 5 minutes, then to 120 °C at 5 °C·minute^{-1}, then to 190 °C at 20 °C·minute^{-1} with no holding time making a total run time of 24.5 minutes. Hydrogen was the carrier gas with linear velocity 30 cm·s^{-1}. Split injection mode was used with a split flow rate of 100 mL·min^{-1}, split ratio of 15:1. Septum purge flow rate was 5-6 mL·min^{-1}. Air and hydrogen flow rates to the detector were 400 and 30 mL·min^{-1}, respectively. Quantification of volatiles in the solvent extract was by comparison with authentic compounds made to a concentration of 200 μL·L^{-1} in the solvent mixture.

One mL of gas from over skin disks was used for headspace analysis. The column was held at 70 °C for 6 minutes with carrier gas and detector gas flowrates as for solvent samples. Quantification of headspace volatiles was by comparison with authentic compounds made to a concentration of 100 μL·L^{-1} in air.

5.3.6 Experiment I. Addition of Individual Precursors

Red Delicious apples stored in air for 3 months at 0 °C ± 1 °C, RH 80%, were used in an experiment conducted as a completely random design using four treatment combinations. After equilibration to 20 °C, fruit were divided into two groups, one was exposed to hypoxia (T) for 24 h at 20 °C, the other was kept in air as a control (C). Disks were then taken from apples of each group and further divided into two groups, one had precursors added (+p) the other without precursors added (-p). Treatment combinations were: disks from control fruit maintained in air, plus or minus precursor (C+p, C-p); and disks from fruit exposed to hypoxia, plus or minus precursor (T+p, T-p). Immediately after disks were placed into bottles, 3 mmol each of ethanol, propan-1-ol, butan-1-ol, pentan-1-ol or hexan-1-ol was added individually to bottles. Each bottle had 5 disks from each of 10 apples for each treatment giving 5 pairs of bottles for air stored fruit (C-p, C+p) and 5 pairs of bottles for fruit exposed to hypoxia (T-p, T+p), making 20 bottles in total. There was no replication of precursor treatments within an experiment. Each experiment was conducted 3 times and the results pooled for data analysis. Blanks consisting of buffer only were used to check for contaminants in the air stream purging bottles.

5.2.7 Experiment II. Addition of Precursor Mixtures

Experiments were conducted on 9 apple cultivars each stored in air for 2 to 3 weeks at 0 °C ± 1 °C, RH 80%, in a completely random design using the same treatment combinations as described above (Section 4.2.6). Immediately after disks were placed into bottles, a mixture of C_2 to C_6 alcohols consisting of 1 mmol each of ethanol, propan-1-ol, butan-1-ol, pentan-1-ol, and hexan-1-ol, giving a mixture concentration of 5 mmol, were added bottles containing CO, FU, GD, GS, PR, RD, RG, SP or SS apple

disks. Each bottle had 5 disks from each of 10 apples for each treatment. There were 3 replicates of C-p, C+p, T-p and T+p disks giving 3 pairs of bottles for air stored fruit (C-p, C+p) and 3 pairs of bottles for fruit exposed to hypoxia (T-p, T+p), making 12 bottles in total for each cultivar. Blanks consisting of buffer only were used to check for contaminants in the air stream purging bottles.

5.2.8 Experiment III. Addition of Precursors One Week After Hypoxic Treatment

Disks were taken from PR apples stored in air for 2 weeks at 0 °C ± 1 °C, RH 80% before exposure to hypoxia. Disks were taken 18 hours (fresh) and 1 week at 20 °C after exposure to hypoxia and used in a completely random design using the same treatment combinations and precursor mixture as described above (Section 5.2.7). Blanks consisting of buffer only were used to check for contaminants in the air stream purging bottles.

5.2.9 Results Presentation and Data Analysis

Means and standard errors of volatile concentrations were expressed as quantity of volatile purged from disks (μmol) per mass of tissue (kg) per volume (L) of air passed through each trap, for each cultivar and treatment for headspace and purged volatiles. Results are presented for acetate esters of each alcohol precursor added to disks as these esters can be expected to be present in apple tissue in the highest concentration (Knee and Hatfield, 1981; Berger and Drawert, 1984). To examine the effect of precursor addition, volatile concentrations from C+p disks were compared to volatile concentrations from C-p disks and volatile concentrations from T+p disks were compared to volatile concentrations from T-p disks. To determine the effect of hypoxia, volatile concentrations in C-p disks were compared to T-p disks. The net change in volatile concentration due to the hypoxic treatment was calculated by subtracting the volatile concentration in C-p disks from C+p disks and volatile concentration in T-p disks from T+p disks. Data were subjected to analysis of variance using SAS v. 6.12 (SAS Institute Inc., Cary, USA) and were graphed using an Origin graphics package v. 5.0 (Microcal Software Inc., USA).

5.4 Results

No acetate esters were detected after addition of alcohols to buffer without disks (data not shown). Therefore, increases in acetate esters after addition of alcohol precursors (Figure 5.1) were considered to come from disks and not by chemical reaction with the buffer solution. No detectable esters were produced when dilute alcohol solutions were incubated with acetyl CoA (Gilliver and Nursten, 1976).

Exposure of apples to a brief period to hypoxia (T-p) enhanced ethanol concentration to twice that for C-p disks (Figure 5.1). Such increases in ethanol persisted for at least 7 d after removal from hypoxia (Chapter 4; Ampun, 1997). The concentration of alcohols added to disks was expected to greatly exceed endogenous ethanol concentrations induced by hypoxia; achieving this would reduce possible confounding effects of competitive inhibition of ester formation by enhanced ethanol concentrations. That disks from fruit exposed to hypoxia had higher ethanol concentrations than control fruit

indicates that ethanol was not removed sufficiently from the fruit before applying alcohol precursors.

5.4.1 Addition of individual precursors

5.4.1.1 Alcohols

Air purged from C and T disks 12 h after addition of C_2 to C_6 alcohols had increased concentrations of C_2 to C_6 alcohols compared to controls (-p disks) (Figure 5.1). In -p disks, ethanol concentration tended to be greater in T disks than C disks. This difference in ethanol concentration was due to endogenous ethanol produced during exposure to hypoxia. There was no difference in concentration of other alcohols between C and T disks (Figure 5.1). Alcohol concentrations, in extracts, ranged from about 1000 $\mu mol \cdot kg^{-1} \cdot L^{-1}$ for ethanol and propan-1-ol to about 4000 $\mu mol \cdot kg^{-1} \cdot L^{-1}$ for hexan-1-ol. The increasing values for larger molecular weight alcohols may reflect the poor trapping efficiency of Tenax® for low boiling point alcohols rather actual tissue alcohol concentration (Fernandez and Diaz-Marta, 1997).

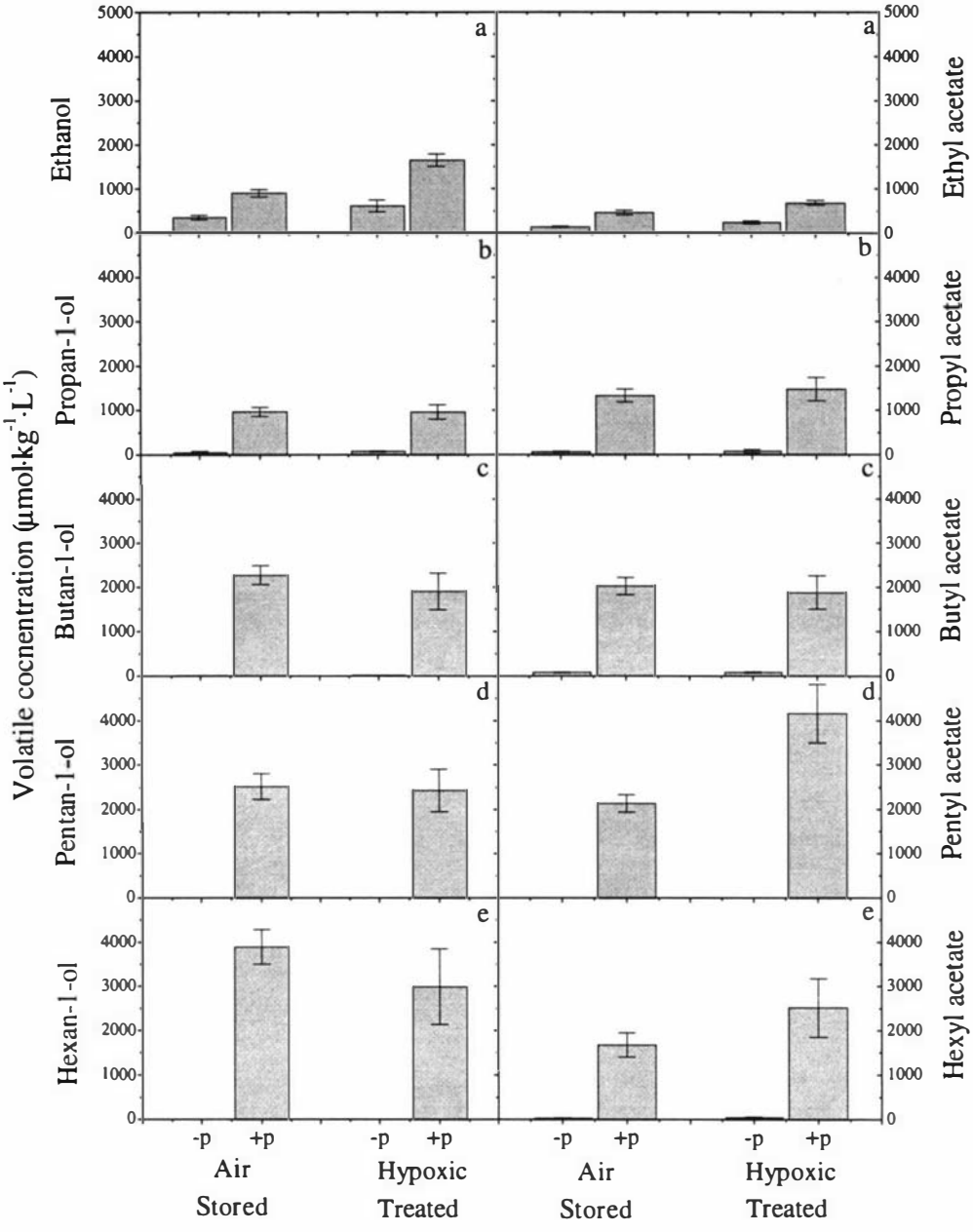

Figure 5.1 Concentrations of alcohols and acetate esters purged from skin disks of air stored and hypoxic treated, 100% CO_2 for 24 hours at 20 °C, RD apples with (+p) and without (-p) addition of 3 mmol of individual alcohols: a) ethanol, b) propan-1-ol, c) butan-1-ol, d) pentan-1-ol, e) hexan-1-ol. Mean and standard errors of 3 replicates.

5.4.1.2 Acetate esters

Addition of individual alcohols increased concentration of corresponding acetate esters in C+p and T+p disks (Figure 5.1). In C+p disks acetate ester concentration was similar to alcohol concentration. Pentyl acetate concentration increased from 2131.0 $\mu mol \cdot kg^{-1} \cdot L^{-1}$ in C+p disks to 4157.6 $\mu mol \cdot kg^{-1} \cdot L^{-1}$ in T+p disks after addition of pentan-1-ol ($P < 0.05$, Figure 5.1). There was no difference in acetate ester concentrations in C+p disks

when compared with T+p disks after addition of ethanol, propan-1-ol, butan-1-ol and hexan-1-ol.

5.4.1.3 Ethyl esters

Concentrations of ethyl esters were very much less than acetate esters from both C+p and T+p disks (Figure 5.2). Addition of ethanol to C disks resulted in non-significant increased ethyl ester concentrations, with the exception of ethyl pentanoate. There was no significant difference in ethyl ester concentrations in T disks compared to C disks regardless of whether ethanol was added or not.

Increases or decreases in ethyl ester concentrations of C+p and T+p disks were variable and followed no set pattern (Figure 5.2). In C+p disks compared to C-p disks addition of butan-1-ol and pentan-1-ol decreased ethyl propionate; propan-1-ol and pentan-1-ol decreased ethyl butanoate; and propan-1-ol increased ethyl pentanoate concentration. In T+p disks compared to T-p disks addition of propan-1-ol increased ethyl propionate and ethyl pentanoate and decreased ethyl butanoate; pentan-1-ol decreased ethyl butanoate; hexan-1-ol decreased ethyl propionate and ethyl butanoate. Ethyl hexanoate concentration was the same for C-p and C+p disks as well as T-p and T+p disks regardless of alcohol added. Disks from T fruit had the same ethyl ester concentrations as C disks after addition of alcohols, except for a decrease in ethyl butanoate after addition of pentan-1-ol ($P< 0.05$) and hexan-1-ol ($P < 0.01$).

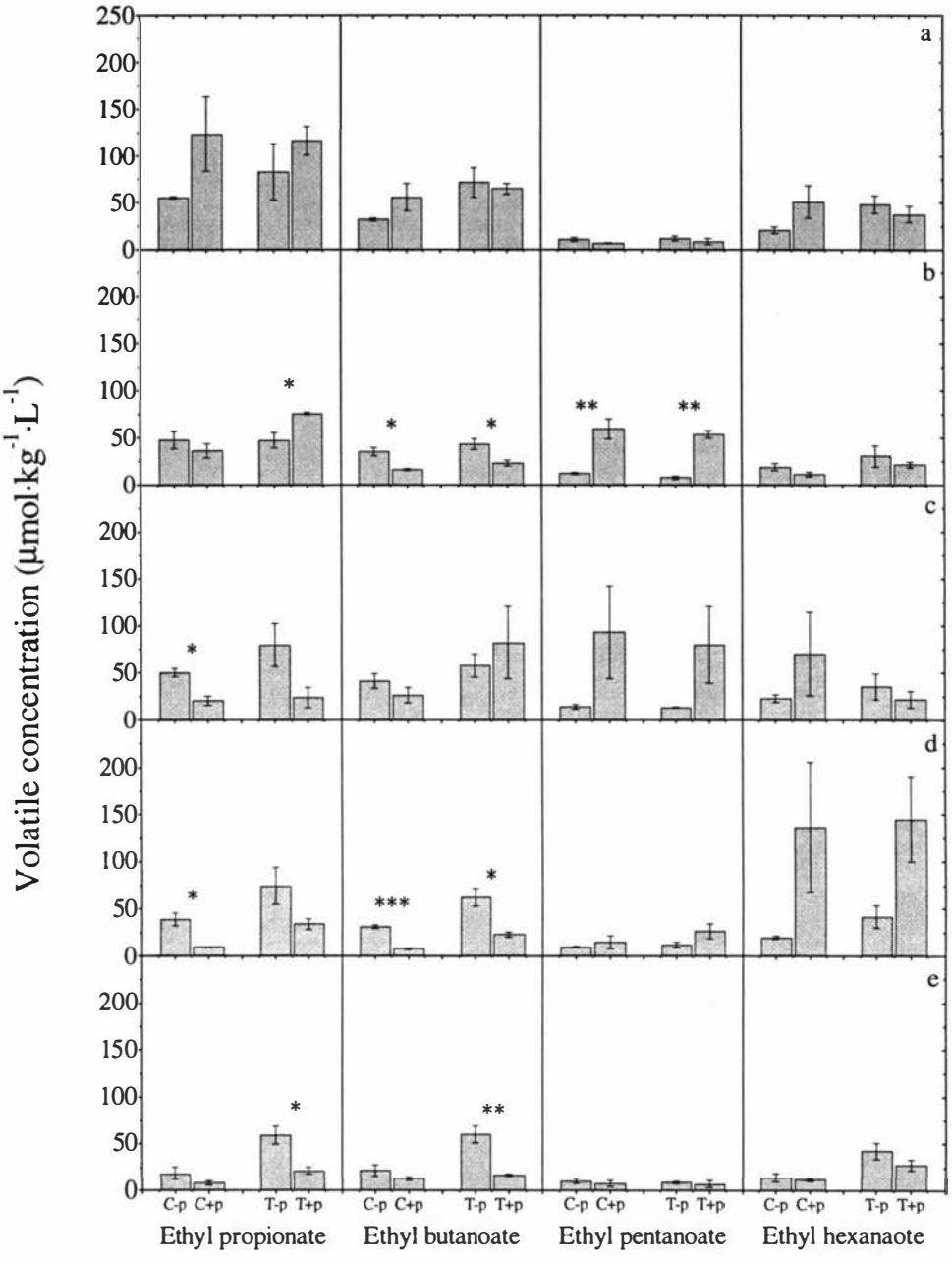

Figure 5.2 Concentrations of ethyl esters purged from skin disks of control (C) and hypoxic treated (T, 100% CO_2 for 24 hours at 20 °C), RD apples with (+p) and without (-p) addition of 3 mmol of individual alcohols: a) ethanol, b) propan-1-ol, c) butan-1-ol, d) pentan-1-ol, e) hexan-1-ol. Mean and standard errors of 3 replicates. Significant differences within a treatment between precursor treatments are * $P > 0.05$, ** $P > 0.01$, *** $P > 0.001$.

5.4.2 Addition of precursor mixtures

5.4.2.1 Alcohols

In general, ethanol and butan-1-ol concentrations increased after hypoxia while concentrations of propan-1-ol, pentan-1-ol and hexan-1-ol were unchanged (Table 5.1) following addition of a mixture of C2 to C6 alcohols to disks from apples exposed to hypoxia compared to fruit stored in air. After hypoxia cultivars produced different amounts of alcohols even though high saturating concentration of exogenous alcohols were added as a mixture. Butan-1-ol and pentan-1-ol were increased the most in PR fruit while disks from SS fruit had a decrease in propan-1-ol and pentan-1-ol (Table 5.1).

Table 5.1. Trend for change in mean volatile concentration after addition of a mixture of C_2 to C_6 alcohols to C disks compared to T disks.

Volatile	CO	FU	GD	GS	PR	RD	RG	SP	SS	Overall
Alcohols										
Ethanol	–	+**	+	+	+	+	NC	+	+	+***
Propan-1-ol	NC	NC	NC	+	+	NC	–	–	–*	NC
Butan-1-ol	NC	+	NC	+	+**	+	–	–	NC	+*
Pentan-1-ol	–	+	–	–	+*	+	NC	–	–**	NC
Hexan-1-ol	NC	+	+	–	+	NC	+	NC	–	NC
Acetate esters										
Ethyl acetate	+	–	–	+*	NC	–	–	–	+	+
Propyl acetate	+**	+***	+	+	+	+	+	–	+	+***
Butyl acetate	+	+***	+	+	+	+***	+**	+	+***	+***
Pentyl acetate	+	+***	+	+	–	+*	+	+	+**	+***
Hexyl acetate	+	+**	+	+	–	+	+	+	+*	+***
Ethyl esters										
Ethyl propionate	NC	+*	NC	+	+	+*	NC	NC	+	+**
Ethyl butanoate	NC	+*	NC	+*	+	+**	NC	+	+	+***
Ethyl pentanoate	NC	+*	NC	+*	NC	NC	–	NC	–	NC
Ethyl hexanoate	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

Trends, greater than 10 $\mu mol \cdot L^{-1}$: + = increase, – = decrease, NC = no change.
Levels of significance: **= $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

5.4.2.2 Acetate esters

Addition of an equimolar mixture of C_2 to C_6 alcohols to disks from fruit exposed to hypoxia and air stored fruit induced consistent increases in acetate ester concentrations, except for ethyl acetate for all cultivars examined (Figure 5.3).

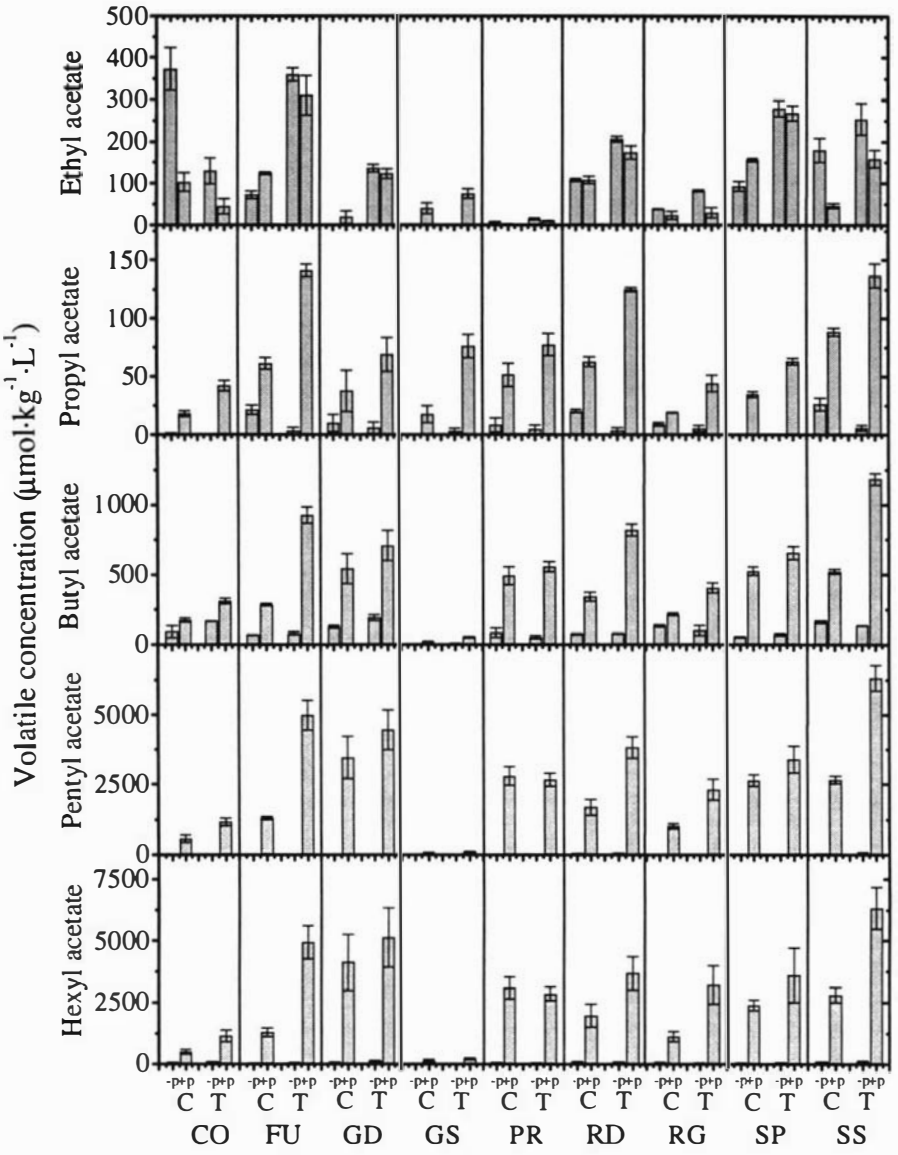

Figure 5.3 Concentrations of acetate esters purged from skin disks of air stored (C) and hypoxic treated (T, 100% CO_2 for 24 h at 20 °C), CO, FU, GD, GS, PR, RD, RG, SP and SS apples with (+p) and without (-p) addition of a mixture of 1 mmol each of ethanol, propan-1-ol, butan-1-ol, pentan-1-ol and hexan-1-ol. Mean and standard errors of 3 replicates.

Ethyl acetate concentration in C-p disks varied from 0 $\mu mol \cdot kg^{-1} \cdot L^{-1}$ in GD and GS fruit to about 375 $\mu mol \cdot kg^{-1} \cdot L^{-1}$ in CO fruit (Figure 5.3). In T-p disks ethyl acetate concentrations were enhanced compared to C-p disks in FU, GD, PR, RD, RG and SP fruit, decreased in CO fruit, and were unchanged in GS and SS fruit. After addition of alcohols ethyl acetate concentrations were enhanced in C+p disks for FU, GS and SP, decreased in CO and SS; and were unchanged in GD, PR, RD and RG. When C+p and T+p disks were compared, concentrations of ethyl acetate were enhanced in FU, GD, RD, SP and SS, decreased in CO, and unchanged in GS, PR and RG fruit.

Significant net increases occurred in concentrations of all acetate esters except for ethyl acetate for Fuji T+p disks compared to C+p disks (Table 5.2). Disks from SS had increased butyl acetate, pentyl acetate and hexyl acetate concentrations, while RD fruit had increases in butyl acetate and pentyl acetate concentration. Only propyl acetate increased in CO fruit and butyl acetate in RG fruit. Other differences in acetate ester concentration between C+p and T+p disks of GD, GS, PR and SP fruit, although large, were not significant.

Among cultivars four trends of change were identified for acetate esters when C+p disks were compared with T+p disks where all acetate esters increased in CO, GS and SS fruit; ethyl acetate decreased while other acetate esters increased in FU, GD, RD and RG fruit; ethyl acetate was unchanged , propyl acetate and butyl acetate increased and pentyl acetate and hexyl acetate decreased in PR fruit; and ethyl acetate, propyl acetate decreased while other acetate esters increased in SP fruit (Table 5.1).

5.4.2.3 Ethyl esters

Concentrations of ethyl esters were very much less than acetate esters from disks of both control and fruit exposed to hypoxia after addition of a mixture of alcohols (Figure 5.4). Disks from fruit exposed to hypoxia but without alcohols added (T-p) compared to C-p disks had enhanced ethyl propionate concentrations in GS, RD and SS fruit; ethyl butanoate in FU, GD, GS, RD and SS; ethyl hexanoate in FU and GD fruit; and decreases in ethyl pentanoate concentration in FU and PR fruit. Comparing C+p with C-p disks addition of alcohols enhanced ethyl propionate concentration in FU, GS, PR, RD and SS fruit; ethyl butanoate in FU, GS, PR, RD and SS fruit; ethyl pentanoate in FU, PR, RD, RG, SP and SS fruit; and ethyl hexanoate in all cultivars. Comparing T+p with T-p disks addition of alcohols enhanced ethyl propionate in FU, GS, PR, RD and SS fruit; ethyl butanoate in FU, GS, PR, RD, SP and SS fruit; ethyl pentanoate in FU, GS, PR, RD, SP and SS; and ethyl hexanoate in all cultivars.

The cultivar response to hypoxia and addition of C_2 to C_6 alcohols varied with 6 trends occurring in pattern of change of ethyl ester concentrations relative to control fruit receiving no alcohol mixture (Table 5.1). These 6 trends of change comparing C+p and T+p disks were: enhanced ethyl propionate, ethyl butanoate and ethyl pentanoate concentration in FU and GS; enhanced ethyl propionate and ethyl butanoate in PR and RD; no change in any ethyl ester concentration in CO and GD; decreased ethyl pentanoate in RG; increased ethyl butanoate in SP; increased ethyl propionate and ethyl butanoate and decreased ethyl pentanoate in SS fruit (Table 5.1).

Net change in ethyl propionate concentration was enhanced in T+p disks from FU and RD fruit and decreased in T+p disks from RG fruit compared to C+p disks (Table 5.2). Net change in ethyl butanoate concentration was enhanced in T+p disks compared to C+p disks from FU, GS and RD fruit. Net change in ethyl pentanoate concentrations were enhanced in T+p disks compared to C+p disks from FU and GS.

Table 5.2 Net volatile concentration for esters purged from disks of air stored (C) and hypoxic treated (T) apples after addition of a mixture, 1 mmol each, of ethanol, propan-1-ol, butan-1-ol, pentan-1-ol and hexan-1-ol. Means of three replicates. Net volatile concentration is the difference in ester concentration between disks without alcohols added subtracted from disks with alcohols added.

Ester	Ethyl acetate		Propyl acetate		Butyl acetate		Pentyl acetate		Hexyl acetate	
Cv.	C[1]	T	C	T	C	T	C	T	C	T
					$\mu mol \cdot kg^{-1} \cdot L^{-1}$					
CO	-271	-85	17	42*	82	147	572	1152	456	1061
FU	52	-57	40	137***	216	845***	1307	4976***	1260	4882*
GD	18	-13	27	63	418	518	3443	4452	4064	5005
GS	39	74	17	73	18	46	54	104	115	209
PR	-4	-4	43	73	406	503	2778	2648	3045	2814
RD	1	-32	42	121	268	741***	1643	3790*	1901	3624
RG	-15	-53	10	39	83	305**	1012	2294	1059	3177
SP	64	-12	74	63	472	583	2615	3387	2351	3554
SS	-134	-96	62	131	362	1053***	2625	6274**	2720	6209*

Ester	Ethyl propionate		Ethyl butanoate		Ethyl pentanoate		Ethyl hexanoate	
Cv.	C[1]	T	C	T	C	T	C	T
				$\mu mol \cdot kg^{-1} \cdot L^{-1}$				
CO	4	0	-4	-23	7	5	26	21
FU	30	92*	15	75*	33	61*	163	175
GD	-1	-21	-4	-42	7	4	138	74
GS	54	160	48	252*	10	49*	39	57
PR	21	55	59	83	17	24	137	109
RD	43	78*	36	111**	29	31	99	86
RG	0	-4*	4	-1	17	5	81	85
SP	-27	5	-4	34	26	38	99	90
SS	97	60	134	93	27	17	102	65

[1] C = stored in air, T = exposed to hypoxia, 100% CO_2 for 24 h at 20 °C.

[2] Diff = difference between treated and untreated disks. * = significant at $P < 0.05$, ** = significant at $P < 0.01$, *** = significant at $P < 0.001$.

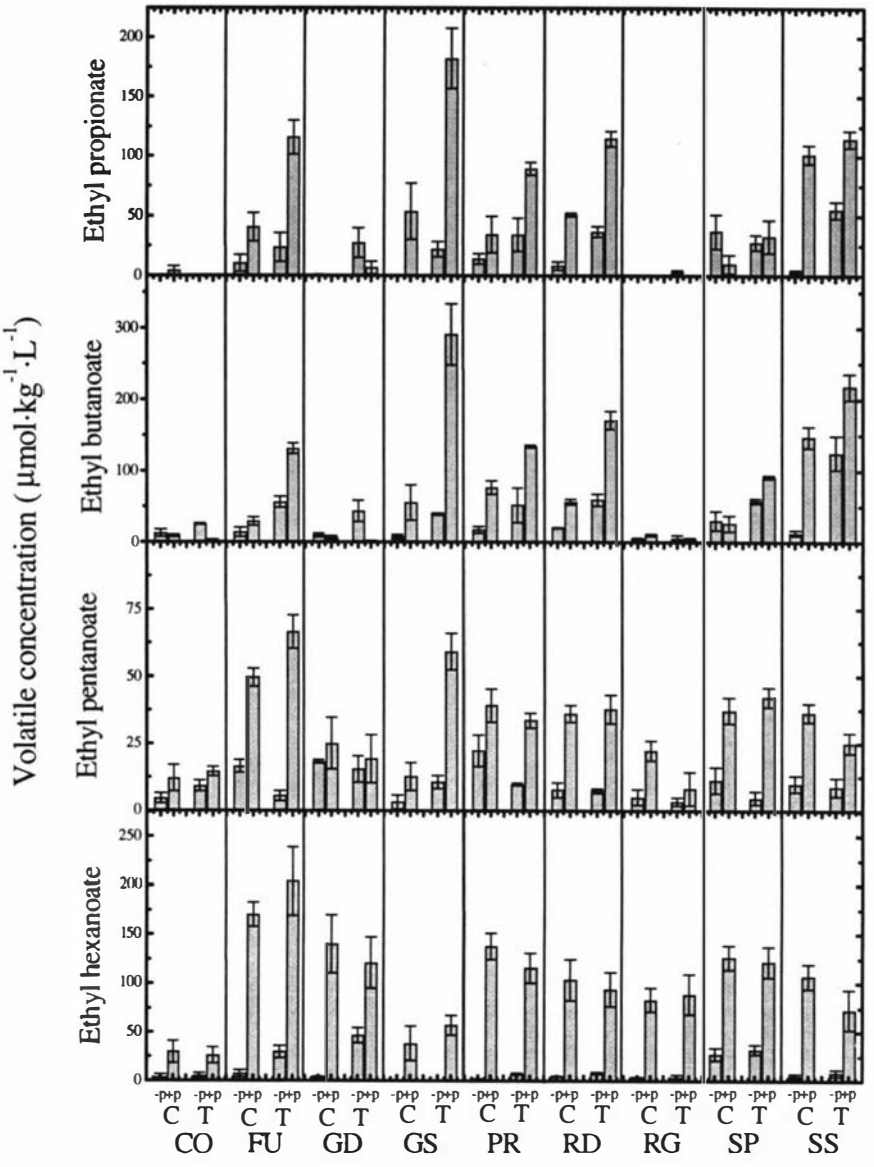

Figure 5.4 Concentrations of ethyl esters purged from skin disks of air stored (C) and hypoxic treated (T, 100% CO_2 for 24 h at 20 °C), CO, FU, GD, GS, PR, RD, RG, SP and SS apples with (+p) and without (-p) addition of a mixture of 1 mmol each of ethanol, propan-1-ol, butan-1-ol, pentan-1-ol and hexan-1-ol. Mean and standard errors of 3 replicates.

5.4.3 Addition of precursors 7 d after treatment

Alcohol concentrations were the same after 7 d as they were 18 h after exposure to hypoxia (Figure 5.5). Ethanol concentration was twice as high in T-p disks than C-p disks 18 h after removal from hypoxia but there was no difference in ethanol concentration between C-p and T-p disks 7 d after removal from hypoxia (Figure 5.5b). Acetate ester concentrations from C+p and T+p disks 7 d after hypoxic treatment were much lower ($P > 0.001$) than after 18 h (Figure 5.6). There was no difference in acetate ester concentration in C+p and T+p disks after 18 h or 7 d (Figure 5.6).

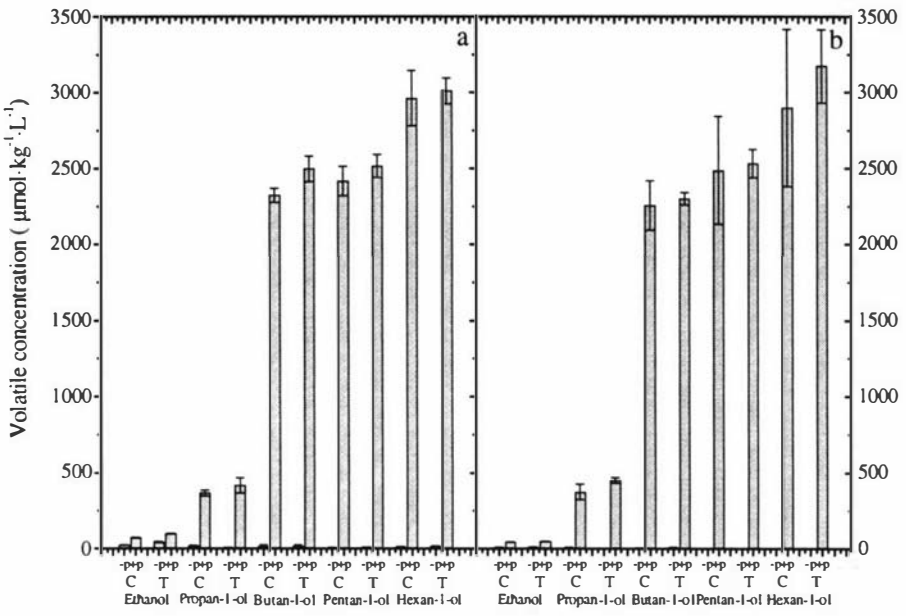

Figure 5.5 Concentrations of alcohols purged from skin disks of air stored (C) and hypoxic treated (T) PR apples with (+p) and without (-p) addition of a mixture of C_2 to C_6 alcohols, a) 18 h and b) 7 d after removal from hypoxia. Mean and standard errors of 3 replicates.

Ethyl ester concentrations were not significantly different in C-p and T-p disks 18 h and 7 d after removal from hypoxia (Figure 5.7). Addition of alcohols enhanced ethyl ester concentrations in T+p disks compared to T-p disks 18 h and 7 d after removal from hypoxia (Figure 5.7). In contrast, addition of alcohols enhanced only ethyl butanoate and ethyl hexanoate in C+p disks compared to C-p disks 18 h and 7 d after removal from hypoxia (Figure 5.7). Ethyl ester concentrations were, in general, lower in disks from fruit 7 d after removal from hypoxia than disks after 18 h. Ethyl propionate, ethyl butanoate and ethyl hexanoate concentrations were greater from T+p disks 18 h after removal from hypoxia than C+p and T+p disks after 7 d (Figure 5.7).

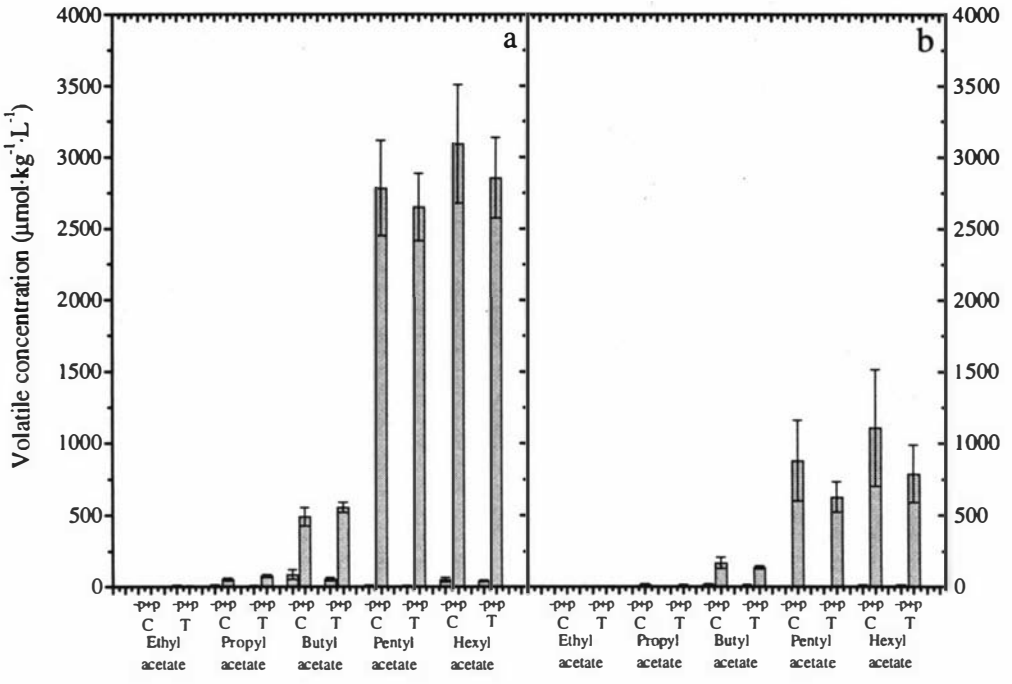

Figure 5.6 Concentrations of acetate esters purged from skin disks of air stored (C) and hypoxic treated (T) PR apples with (+p) and without (-p) addition of a mixture of C_2 to C_6 alcohols, a) 18 h and b) 7 d after removal from hypoxia. Mean and standard errors of 3 replicates.

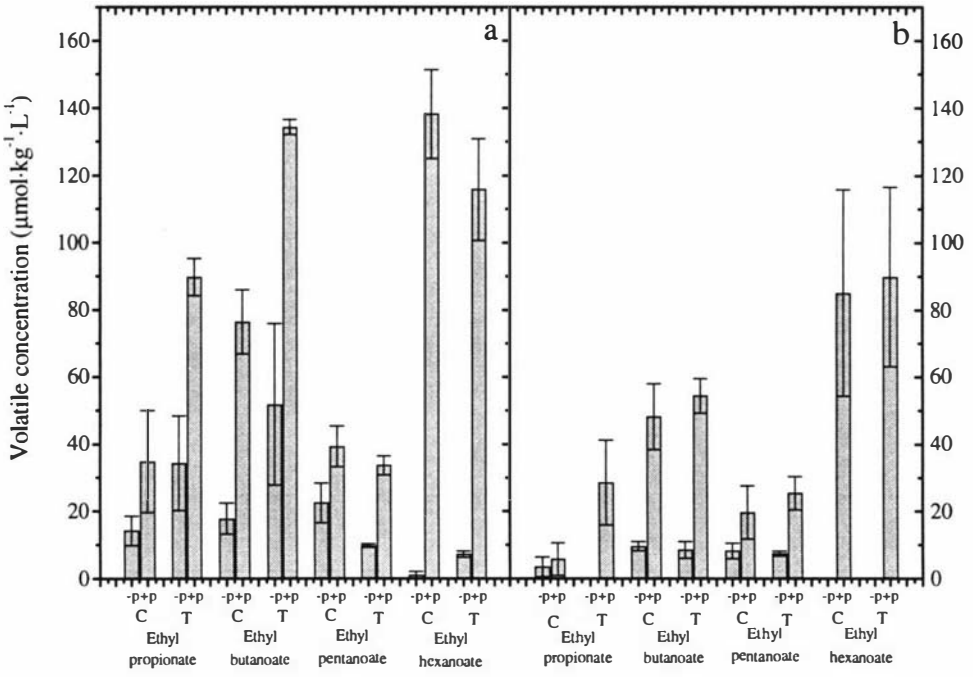

Figure 5.7 Concentrations of ethyl esters purged from skin disks of air stored (C) and hypoxic treated (T) PR apples with (+p) and without (-p) addition of a mixture of C_2 to C_6 alcohols, a) 18 h and b) 7 d after removal from hypoxia. Mean and standard errors of 3 replicates.

5.5 Discussion

Apples exposed to hypoxia had enhanced concentrations of ethanol and ethyl acetate in agreement with previous research (Ampun, 1997; Chapter 4). Exogenous alcohols added to disks from control and hypoxic treated apples induced increases in acetate esters of the corresponding alcohol moiety to many times their normal concentration. In contrast ethyl esters had only minor increases in concentration after addition of exogenous alcohols suggesting that alkylation is the preferred process for metabolism of C_2 to C_6 alcohols to esters in apple disks. The greatest increases in ester concentration after addition of C_2 to C_6 alcohols were for C_6 and longer carbon chain esters indicating that ester biosynthesis in apples favours esterification of carbon chain alcohols longer than C_3 (Bartley et al., 1985; Berger and Drawert, 1984). This preference would account for the predominance of acetate esters in volatile extracts after incubation with alcohols. That acetate esters are produced in the greatest concentration after addition of alcohols indicates that AAT and acetyl CoA are not limiting ester biosynthesis; acetate esters increase regardless of alcohol(s) added. The capacity for high rates of acetate ester production exists in apple tissue, but does not normally occur because availability of a specific alcohol precursor is limited. Previous research in apples has indicated that ester production is proportional to alcohol concentration and acyl CoA concentrations are not rate limiting (Bartley et al., 1985; Berger, 1990; Berger and Drawert, 1984; Harb et al., 1994; Knee and Hatfield, 1981; Mattheis et al., 1991a). However, the possibility exists that alcohols added to apple tissue could be converted to acyl CoA's and used to form esters (Berger and Drawert, 1984; Song et al., 1996). Differences in aroma composition between apple cultivars may, in part, be due to differences in acyl CoA concentration, as well as differences in acyl CoA preference of the ester biosynthetic pathway (Paillard, 1979). For example, banana fruit slices produce different esters depending on the acyl CoA moiety; addition of butyl CoA lead to a 5 fold increase in isobutyl and isopentyl butanoate and ethyl butanoate while addition of acetyl CoA lead to a doubling of isobutyl and butyl acetate (Gilliver and Nursten, 1976). Concentration changes of acyl CoA's in fruit tissue during ripening and after postharvest treatments have not been determined. A better understanding of ester synthesis could be achieved if endogenous concentrations of acyl CoA's were quantified during ripening and following exposure to various pre-storage stress treatments such as hypoxia, that are known to affect volatile production.

When alcohols were added individually to RD disks from apples exposed to hypoxia there was an enhanced capacity to esterify pentan-1-ol to pentyl acetate compared to disks from control fruit thereby representing a change in substrate preference. That pentyl acetate increased more than other acetate esters suggests that hypoxia may have made pentan-1-ol the preferred substrate for esterification, possibly by inducting an AAT isozyme specific to pentan-1-ol, or by promoting esterification rates of pentan-1-ol over those for other alcohols. The reason for this apparent selective effect on pentan-1-ol synthesis to pentyl acetate, as reported previously by Berger and Drawert (1984), is not known. In contrast when a mixture of C_2 to C_6 alcohols were added to disks from RD apples, and other cultivars, exposed to hypoxia there was a general enhancement of acetate ester concentrations compared to disks from control fruit. This suggests that hypoxia increased the capacity of apple tissue to synthesise acetate esters but did not change specificity for substrate alcohols in the ester biosynthetic pathway. Such contradictory results may be due to the different age of fruit used in each experiment, 2

to 3 weeks at 0 °C for alcohol mixtures and 3 months at 0 °C for individual alcohols. Activity of AAT has been reported to decline after 3 months at 0 °C (Fellman et al., 1993) and it is also possible that substrate specificity of AAT changes to that for pentan-1-ol during storage at 0 °C. To determine this further research is required to examine if AAT substrate specificity changes during ripening and storage.

Addition of butan-1-ol and hexan-1-ol individually to RD disks did not result in a corresponding increase in ethanol or butan-1-ol (Figure 5.1). This contrasts with increases in short carbon chain alcohols found in intact GD apples after application of butan-1-ol or hexan-1-ol which was attributed to β-oxidation of these alcohols (Harb et al., 1994). This may reflect cultivar differences in substrate specificity for alcohols or fruit age as the GD apples were freshly harvested unlike the RD apples used here. That cultivars vary in their enhancement of ethanol and other alcohols concentrations following hypoxia (Chapter 4, Tables 4.2 and 4.3) suggests that cultivars differ both qualitatively and quantatively in their capacity to produce alcohols. Addition of alcohols to apple disks has allowed these differences to be quantified in both control and fruit exposed to hypoxia.

The variable enhancement of ethyl acetate concentration amongst cultivars briefly exposed to hypoxia also occurred in apples stored for long periods in low O_2 CA storage (Fellman et al., 1993). This suggests that ethyl acetate is synthesised to concentrations specific for individual apple cultivars regardless of duration of hypoxic conditions. That differences between cultivars exist in ethyl acetate concentration compared to other acetate esters after exposure to hypoxia, suggests that ethanol may be esterified by another enzyme(s). Hypoxic treatment could induce a new AAT isozyme that specifically produces ethyl acetate in FU, GD, RD, SP and SS fruit but not in CO, GS, PR and RG fruit. Although AAT differs between fruit types (Harada et al., 1985; Pérez et al., 1993; Yamashita et al., 1976) it has not been determined if concentration or activity AAT or induction of new AAT isozyme(s) occurs during or after removal of apples from hypoxia. Detailed enzymatic analysis is required to determine if changes in AAT and esterification capacity exist between apple tissue exposed to air and to hypoxia for short periods.

An alternative explanation for variability in enhancement of ethyl acetate concentration between cultivars after exposure to hypoxia is that ethyl acetate can be produced by two separate biosynthetic pathways. In yeast, used for fermentation of wine, it has been proposed that ethyl acetate synthesis occurs either by ester biosynthesis catalysed by AAT or by the reverse reaction of unspecified esterases (Mauricio et al., 1993). Hence, cultivar variability in ethyl acetate concentration after different treatments could be explained by differential rates of ethyl acetate formation from each biosynthetic pathway (Mauricio et al., 1993). There may be at least five ester hydrolases in CO apple peel (Bartley and Stevens, 1981) that, while not specific for ethyl acetate, could possibly contribute to ethyl ester formation. The number and type of esterases may vary from cultivar to cultivar and differences in esterase activity may be partially responsible for differences in volatile concentrations between cultivars.

Differences in ethyl ester concentrations occurred depending on whether alcohols were added as individuals or mixtures. In RD apples exposed to hypoxia, incubation of disks with individual alcohols induced a decrease in ethyl propionate and ethyl butanoate and

an increase as ethyl pentanoate and ethyl hexanoate, whereas a mixture of alcohols induced all ethyl esters to increase in fruit relative to control fruit. While other cultivars had similar increases in ethyl esters after addition of a mixture of alcohols only ethyl hexanoate increased in C+p and T+p disks compared to C-p and T-p disks in CO, GD and RG apples. This suggests that following hypoxia these cultivars may have an AAT that poorly utilises ethanol as a substrate for esters compared to other alcohols. These results may also indicate that the same range of acyl moieties for butyl or hexyl esters in preference to ethyl esters were enhanced by addition of their respective alcohols for these cultivars after exposure to hypoxia. In addition to possible changes in alcohol preference of AAT, exposure to hypoxia may affect acyl CoA proportions in apple tissue leading to production of a different range of esters, potentially changing the aroma or flavour of hypoxic treated fruit.

That ethyl esters with acyl moieties specific to added alcohols were not formed consistently, indicates that these alcohols were not readily converted to their respective acyl CoA's. The ethyl esters formed were probably derived from pre-existing acyl CoA's, concentrations of which may have been affected by addition of specific alcohols but not by hypoxic treatment. Lack of enhancement of ethyl esters specific to an added alcohol contrasts with experiments using GD apples where propionate and butanoate esters increased after apples were dipped in propionic and butyric acid solutions, respectively (De Pooter et al., 1982). This indicates that alcohols are not readily converted to acyl CoA's whereas organic acids are. To determine differences in the capacity of various cultivars to synthesis esters of acyl moieties other than acetyl CoA it would be useful to evaluate combinations of alcohols and acyl CoA's as substrates for AAT.

The apple cultivars evaluated here can be divided into 4 groups based on alcohol and acyl CoA moiety's concentration changes, after addition of alcohols to disks from fruit exposed to hypoxia compared to those from control fruit. Ethyl esters were assumed to represent other esters with the same acyl CoA moieties. Fuji, RD and SS fruit are in a group where both acetate and ethyl esters increased more in disks from hypoxic treated fruit than from control fruit. This suggests that in these cultivars the AAT enzyme and acyl CoA's were not limiting. The increase in acetate esters and decrease in ethyl esters in CO and RG fruit suggests that a selective increase in ester forming capacity for acetate esters occurred after exposure to hypoxia. In these cultivars exposure to hypoxia may have induced a new isozyme of AAT that does not prefer ethanol as a substrate but readily uses acetyl CoA. Unchanged or decreased acetate esters and increased ethyl esters occurred in GS and PR fruit after exposure to hypoxia suggesting that this treatment causes a selective increase in ester formation based on acyl CoA's other than acetyl CoA. As there is a lack of information on concentrations of acyl CoA's in fruit tissue, measurements of acyl CoA flux during and after hypoxia would be required to establish if apple cultivars differ in their production of esters due to different acyl CoA availability for ester formation. Unchanged or decreased acetate and ethyl esters in GD and SP fruit suggests that hypoxia induced no change in AAT activity. If such responses to hypoxia described above were due to changes in one enzyme then the differential effects observed in acetate and non-acetate ester formation would be due to a change in AAT activity and limiting acyl CoA concentrations. As this is not true for FU, RD and SS fruit it is possible that there may be two isozymes of AAT involved in ester formation allowing a more selective effect of hypoxia on apple cultivars resulting in the

different patterns of ester formation observed after fruit were exposed to hypoxia. There may be more than one isozyme of AAT in apples due to it being located in two places in the cell, close to or in mitochondria, the main site of acetyl CoA synthesis (Mathews and van Holde, 1996) and/or in the peroxisomes, sites of lipid β-oxidation (Gerhardt, 1983). Because of its large size CoA has to be actively transported across membranes, for example, the carnitine cycle transports fatty acyl-CoA's across mitochondrial membranes (Mathews and van Holde, 1996), and is unlikely to be present outside organelles where its derivatives are synthesised, it is possible that AAT is located in mitochondria and peroxisomes within a cell.

All types of esters were produced by control fruit that had no alcohols added, indicating that fruit were ripe at the commencement of experiments, as many esters are produced only after several days ripening (Fellman et al., 1993). Activity of AAT follows ripening closely, peaking at the climacteric maximum and reducing thereafter (Fellman and Mattheis, 1995). In experiment III PR fruit maintained at 20 °C for 7 d had probably passed their climacteric maximum and were becoming overripe, hence the reduced ester concentrations compared to fresh apple disks. Irrespective of age of fruit and treatment after addition of alcohols, ethyl ester concentrations were between 15 to 90% higher than in control fruit. Exposure to hypoxia has the potential to induce changes to the ester biosynthetic pathway that may last at least 7 d at 20 °C and possibly several weeks at low temperatures. Exposing fruit to hypoxia for a brief period at a 20 °C affects ester concentration within 24 h indicting that the ester biosynthetic pathway is fully active. In contrast fruit stored for long periods at low temperatures in hypoxic conditions (CA) take 6 to 9 days to increase AAT activity on return to air (Fellman et al., 1993); presumably this is the time required for fruit to ripen sufficiently to produce esters.

5.6 Conclusions

Adding C_2 to C_6 alcohols to apple disks resulted in large increases in acetate ester and variable increases in ethyl ester concentrations. This indicates that under normal conditions apple tissue has a high capacity to produce esters, the production of which is limited by alcohol availability. Apples exposed to hypoxia had, in general, an enhanced capacity to produce acetate esters and some ethyl esters. There were 4 patterns of enhanced capacity to form esters in fruit exposed to hypoxia compared to fruit maintained in air: increase in acetate and ethyl esters; increase in acetate esters and decrease or no change in ethyl esters; no change or decrease in acetate esters and increase in ethyl esters; and no change or decrease in acetate esters and decrease or no change in ethyl esters. Such results suggest that AAT activity may be affected differentially by hypoxia in different apple cultivars. It is suggested that AAT may be located in both mitochondria and peroxisomes as these are thought to be sites of acyl CoA production. Hypoxic conditions may affect the AAT's at these two locations differentially. Changes induced by hypoxia persist for at least 7 d indicating that pre-storage treatments using brief periods of hypoxia have the potential to change the aroma profile of apples. Therefore, any prestorage treatments that utilise high concentrations of CO_2 and N_2 to generate low O_2 environments could result in apple fruit with flavour and/or aroma different to that of fruit maintained in air.

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Chapter Six

Sensory characteristics of apple juice from apples exposed to hypoxia.

Additional Index Words. aroma; flavour; solvent extraction; gas chromatography; volatiles

6.1 Abstract

Juice of Fuji and Royal Gala apples exposed to a brief period of hypoxia (100% CO_2 for 24 h at 20 °C) and ripened at 20 °C for up to 8 d, was analysed by quantitative descriptive analysis. Panellist assessments were correlated with the chemical composition of volatiles analysed by gas chromatography. Hypoxia induced large increases in ethyl esters including ethyl butanoate and ethyl-2-methyl butanoate in Fuji apples but not Royal Gala apples. Hypoxia did not change odour units in Royal Gala juice but they were increased in Fuji apples 2 d after removal from hypoxia. There was no difference in average panellist scores for sensory characteristics for Fuji and Royal Gala apples at any sampling time. The lack of difference may have been due to large variation between panellists assessment of sensory characteristics and/or inability to assess aroma, flavour and sweetness independently. A number of individual volatiles correlated with aroma in juice from apples exposed to hypoxia: hexan-1-ol, butyl acetate, 2 methyl butyl acetate and propyl butanoate for Fuji; and ethanol, ethyl acetate, propyl acetate and propyl butanoate for Royal Gala. Multivariate analysis using principal components indicated that panellists associated increased ethyl esters with off flavour rather than more intense apple aroma. This could have been due to juice from apples exposed to hypoxia having a different apple-like character than control fruit which did not fit the definition of apple aroma used to train panellists. A different method of assessing apple juice by taste panellists is proposed to measure nuances in apple aroma between treatments.

6.2 Introduction

Typical apple aroma and flavour is influenced by more than 300 volatile compounds, synthesised within fruit, consisting mostly of alcohols (6-16%), esters (78-92%), aldehydes (2-3%) and traces of ketones and ethers (Dimick and Hoskin, 1983; Paillard, 1990; Sanz et al., 1997). About 20 to 30 of these compounds may define characteristic apple aroma or taste (Cunningham et al., 1986; Paillard, 1990). Volatile compounds may contribute to apple juice aroma in several ways by contributing strongly to the typical odour; causing off-flavours; enhancing intensity of an aroma; not contributing directly to aroma but enhancing perception of other volatiles; making no contribution (Dürr and Schobinger, 1981). Small differences in concentration of volatile compounds can be apparent to sensory panellists where cultivars with strong aroma and 'typical' apple taste are often preferred (Poll, 1981).

Anaerobic respiration is induced by hypoxic conditions, where oxygen (O_2) concentration is < 5%, resulting in acetaldehyde and ethanol accumulation (Kader, 1986). Pre-storage treatment with brief periods of hypoxia reduce postharvest decay (Ke et al., 1991; Pesis and Avissar, 1989), maintain general fruit quality (Pesis, 1994), and

increase volatile concentration in citrus and feijoa fruit (Pesis et al., 1991; Shaw et al., 1991). Such treatments using <2% O_2 and up to 100% carbon dioxide (CO_2) or nitrogen (N_2) atmospheres for 1 to 14 days at about 20 °C have been evaluated as potential disinfestation treatments (Gaunce et al., 1982; Hallman, 1994). In addition long term storage in low O_2 conditions are considered beneficial for maintaining apple quality by maintaining colour, firmness and juiciness for longer than apples stored in air. While these fruit attributes define apple fruit quality for marketers, consumers also require fruit with acceptable flavour and aroma. Hypoxic conditions used for long term controlled atmosphere (CA) storage of apples result in fruit with poor flavour, aroma and reduced volatile production compared to fruit stored in air (Patterson et al., 1974; Yahia et al., 1990).

Informal sensory panels have reported that fruits exposed to hypoxia have better flavour than untreated fruit. Pre-treatment of apples with 10% to 15% CO_2 before CA storage improved flavour and texture compared to untreated CA stored fruit (Tietjen and Hudson, 1984). Peaches and nectarines exposed to 86% CO_2 or 97% N_2 for 1 d at 20 °C had improved flavour acceptability after 7 d at 20 °C compared to untreated fruit (Lurie and Pesis, 1992). Perception of sweetness was increased in feijoa fruit treated with 98% N_2 + 2% O_2 for 24 h at 20 °C, after 7 d at 20 °C, compared to air stored fruit (Pesis, 1994). Treating Golden Delicious apples with greater than 95% CO_2 for 24 to 48 h at 20 °C had more flavour than untreated fruit after 2 weeks ripening at 20 °C, with treatment for 24 h the most preferred (Ampunpong, 1991; Pesis et al., 1994; Shusiri, 1992). Such improvements in flavour could be due to changes in aroma/flavour volatile concentration induced by hypoxic treatments (Pesis, 1994). Apples exposed to an atmosphere of 100% CO_2 for 24 h at 20 °C had consistently enhanced acetaldehyde, ethyl acetate and ethanol concentrations during ripening at 20 °C after treatment (Ampun, 1997, Chapter 4). The increase in ethanol concentration was followed by an increase in concentration of ethyl esters including compounds considered important for aroma such as ethyl butanoate, ethyl-2-methyl butanoate and ethyl hexanoate (Dimick and Hoskin, 1983; Flath et al., 1967). However, these increases were accompanied by decreases in acetate esters, considered to have fruity characteristics, and aldehydes such as *trans*-2-hexenal, considered to have apple green/grassy characteristics (Dürr and Schobinger, 1981). While these were general changes in volatile concentration, specific cultivars had greater changes than others. For example, ethyl esters in Royal Gala apples show little change while increasing dramatically in Red Delicious apples after exposure to hypoxia (Ampun, 1997, Chapter 4).

The hypothesis was that exposure of apples to a brief period of hypoxia would increase the concentration of aroma and flavour volatiles resulting in a stronger more intense apple aroma and flavour. To determine aroma and flavour changes Fuji and Royal Gala apples were exposed to hypoxia and ripened at 20 °C. Sensory characteristics of juice from these fruit was assessed by a trained sensory panel both before exposure to hypoxia and after ripening. Changes in perception in aroma and flavour were correlated with volatile concentrations to identify what changes in concentration of volatile compounds influenced apple aroma and flavour the most.

6.3 Materials and Methods

6.3.1 Fruit supply

Fruit of Fuji (FU) and Royal Gala (RG) apple (*Malus domestica* Borkh.) cultivars were assessed for their response to hypoxia. Cultivars were harvested at mid commercial harvest: 1 Apr. 1996 for FU, and 23 Feb. 1996 for RG as determined by the New Zealand Apple and Pear Marketing Board, in the Hawke's Bay region of New Zealand. Fruit were graded to export standard, packed to count 125 (148g average fresh weight), transported unrefrigerated to Massey University by road where the fruit was placed at 0 °C ± 1 °C within 3 days of harvest. FU apples were maintained at 0 °C for 29 d and RG apples 21 d before treatment. Fruit were removed from coolstore and equilibrated to 20 °C ± 3 °C overnight prior to exposure to hypoxia. Fruit were divided into two groups, one was exposed to hypoxia, the other stored in air. For each treatment, there were 2 replicates each of 20 apples.

6.3.2 Hypoxic treatment

Refer to details in Section 4.3.2.

6.3.3 Fruit assessment

Fruit were assessed the day before treatment (d -1) and 2, 5, and 8 d after treatment during ripening at 20 °C ± 3 °C for: CO_2 and ethylene production, firmness, mass, volatile concentration of juice and fermentation volatiles in the juice headspace. This allowed differences in volatile concentration due to fruit ripening to be determined.

6.3.4 Firmness

Refer to Section 4.3.4.

6.3.5 Carbon dioxide and ethylene production

Refer to Section 4.3.5.

6.3.6 Juice preparation

Refer to Section 4.3.6.

6.3.7 Headspace volatiles

Refer to Section 4.3.7.

6.3.8 Juice volatiles

Refer to Section 4.3.8.

6.3.9 Taste panel

Apple juice was analysed by quantitative descriptive analysis (QDA) using 4 to 10 trained panellists at any one session. Panellists were screened for suitability by testing their ability to taste and discriminate characteristics of apple juice. Screening procedures were conducted according to the published guidelines (Anon., 1981) and involved triangle and ranking tests. Descriptors of apple taste characteristics were collated and by panel discussion when consensus was reached about the definition of each descriptor (Table 6.1).

Table 6.1 Apple juice descriptors.

Descriptor	Definition
Natural Apple Aroma	the aroma characteristic of fresh apple juice, evaluated after 3 quick sniffs.
Natural Apple Flavour	the flavour characteristic of fresh apple juice, evaluated after drinking the sample and holding on the tongue for 3 seconds.
Sweetness	the taste characteristic of sucrose in water, evaluated on the tip of the tongue usually at the beginning of the perception process.
Acidic Flavour	the taste of acid in solution (citric acid, malic acid) or any sourness associated with apple juice, perceived on the sides of the tongue, during the later stages of the perception process.
Woody/Grassy Flavour	fresh saw dust, tea, green wood, tree resin, wet wood.
Off Flavour	the combination of flavour and aroma characteristic of fermented fruit.

Panellists were presented with samples of single strength undiluted apple juice from apples exposed to hypoxia or stored in air and a reference standard at all tasting sessions. This allowed panellists to have a consistent reference when tasting samples. The reference consisted of 97 % reconstituted apple juice from concentrate diluted 25:10 v/v with water, 3% apple essence v/v (Frucor Processors Limited, Hastings, New Zealand), 2 % sucrose by weight, 0.1 % citric acid by weight and 100 $\mu L \cdot L^{-1}$ ethyl acetate as an off-flavour character. Total soluble solids of the reference ranged from 13.3 to 13.8 °Brix. Nominal intensities of flavour characteristics in the reference were marked as specific points on a 10 cm line scale (Figure 6.1). These intensities were chosen during training and represented midpoints of the intensities of taste characteristics that panellists could be expected to assess.

Three experiments were conducted using FU or RG apples. Fruit were randomly assigned to one of four air tight chambers. Two chambers were sealed and flushed with 100% carbon dioxide for 24 h at 20 °C, fruit in the other chambers were left open to air. Four samples were assessed at each tasting session, about the maximum number of samples that can be tasted before panellist fatigue. Samples consisted of two juice samples from apples exposed to hypoxia and from apples stored in air plus a reference sample used to test consistency of panellist assessment of the reference. Panellists were asked to mark on a 100 mm line where they thought the intensity of a sample fell for each descriptor (Figure 6.1). Distance along the line from the left anchor point was used as the measurement for a descriptor. Samples were presented in randomised order for each tasting session.

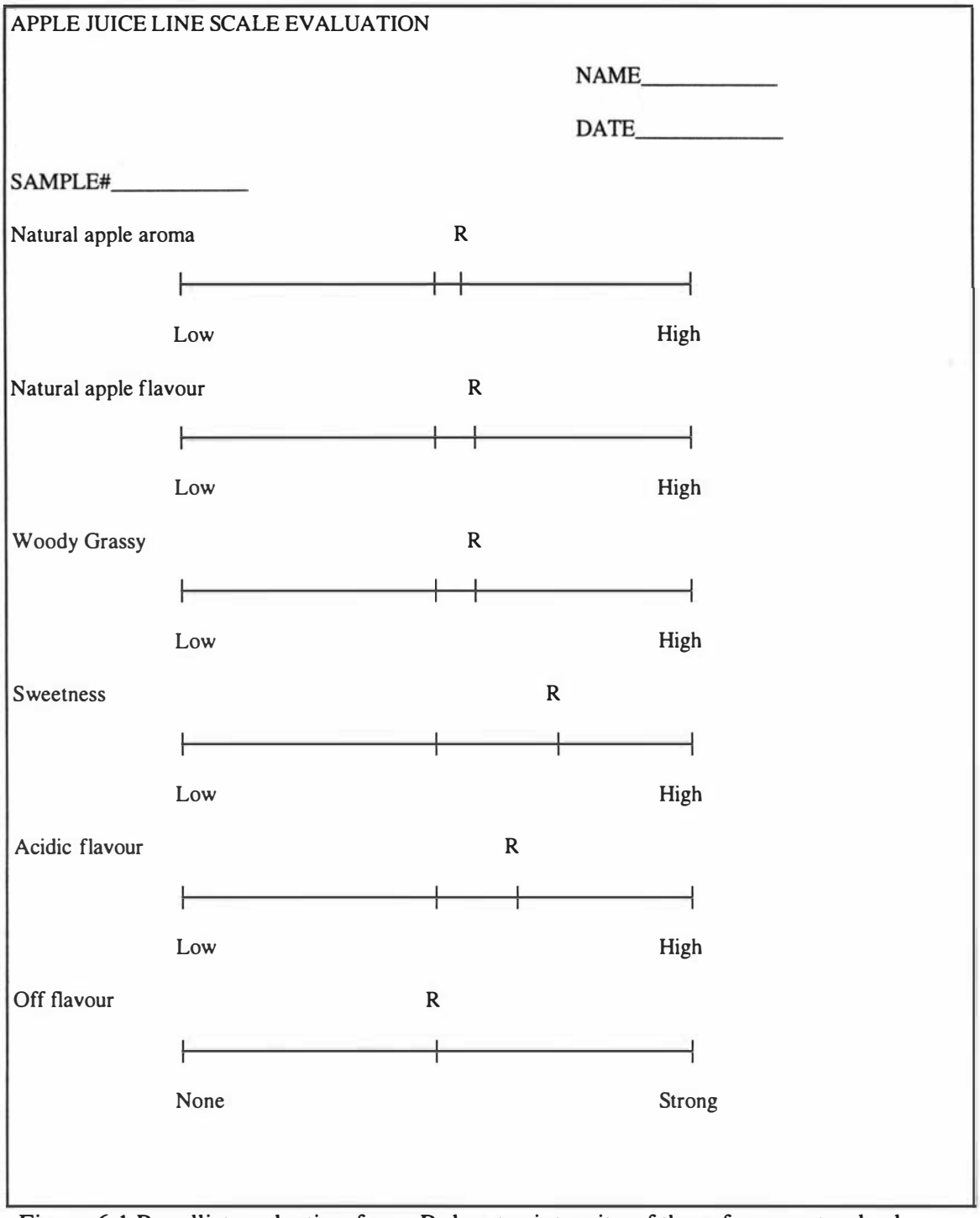

Figure 6.1 Panellist evaluation form. R denotes intensity of the reference standard.

6.3.10 Data analysis

Each experiment on each cultivar was conducted as a split plot design because one factor, sampling over time, is nested in replicates; in this sense it was a single factor design with repeated measures. Each experiment was repeated three times for each cultivar. Experimental results for each cultivar were pooled for data analysis. Means and standard errors of the means for each cultivar and treatment for headspace and juice volatiles were graphed using an Origin graphics package v. 5 (Microcal Software Inc.,

CORR and FACTOR procedures of SAS v. 6.12 (SAS Institute Inc., Cary, USA). Odour units were calculated as the sum of ratios of the concentration of a volatile component in an extract and the average threshold concentration of that volatile in water (Table 1.5; Frijters, 1979).

6.4 Results

6.4.1 Sensory scores

There was no difference in scores for sensory characteristics for FU or RG apples during ripening in juice from apples exposed to hypoxia or stored in air (Figure 6.2). Juice from RG apples were rated as having more aroma, sweetness and wood/grassy character than FU juice. There was no difference in scores for flavour, acidic flavour and off flavour.

6.4.2 Volatile concentration in juice

Exposure to hypoxia induced increased concentration of the fermentation volatiles acetaldehyde, ethanol and ethyl acetate from FU and RG apples (Figure 6.3). Changes in volatile concentrations were similar to those in previous experiments (Chapter 4). Concentrations of the ethyl esters, ethyl butanoate, ethyl propionate and ethyl-2-methyl butanoate, were enhanced to high concentrations by hypoxia in FU apples but were unchanged in RG apples (Figure 6.3). Concentrations of acetate esters, alcohols and aldehydes although different between cultivars, were not different for fruit exposed to hypoxia and untreated controls. Therefore exposure to hypoxia affected volatile concentration in a manner consistent with previous experiments.

6.4.3 Odour units

Odour unit values of RG apple juice from apples exposed to hypoxia were not different in juice from untreated fruit (Figure 6.4). There was an increase in odour units for FU apples from 2 d after removal from hypoxia but this was not reflected in sensory panel scores. Because of higher aroma volatile concentrations odour unit values for control RG apple juice should be greater than for FU juice, this was the case and was in general agreement with the sensory panel scores (Figure 6.2). Therefore odour units would predict that large increases in ethyl esters should result in more intense aroma in treated FU apples at least for short time after hypoxic treatment.

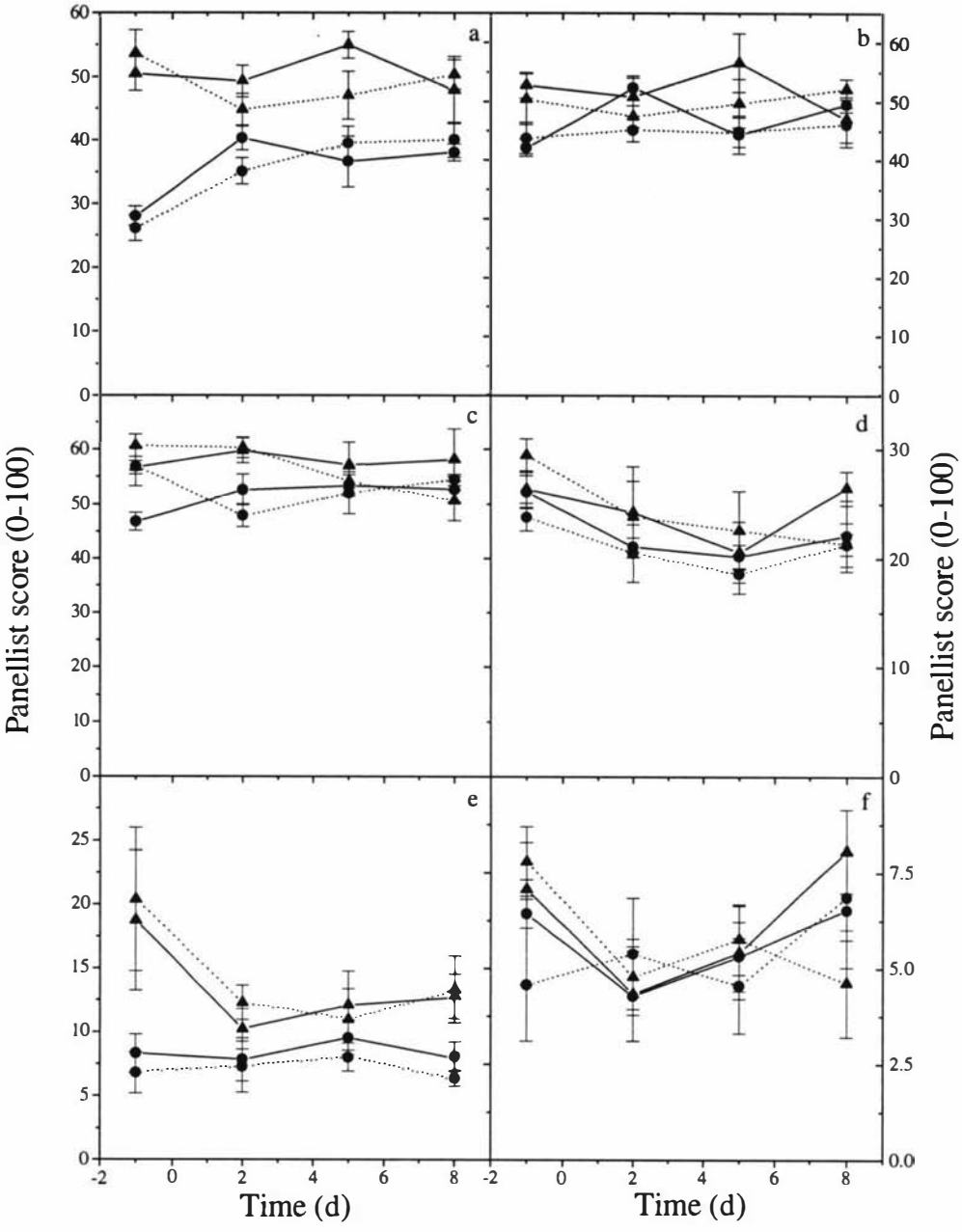

Figure 6.2 Sensory characteristic scores of: a) natural apple aroma, b) natural apple flavour, c) sweetness, d) acidic flavour, e) woody/grassy, f) off flavour, for FU (●) and RG (▲) apple juice from apples exposed to hypoxic conditions (— untreated, ····· treated), generated using an atmosphere of 100% CO_2 for 24 hours at 20 °C, before treatment (d -1), on removal from treatment (d 0) and during subsequent storage at 20 °C for up to 8 d. Means and standard errors of 4 to 10 panellists.

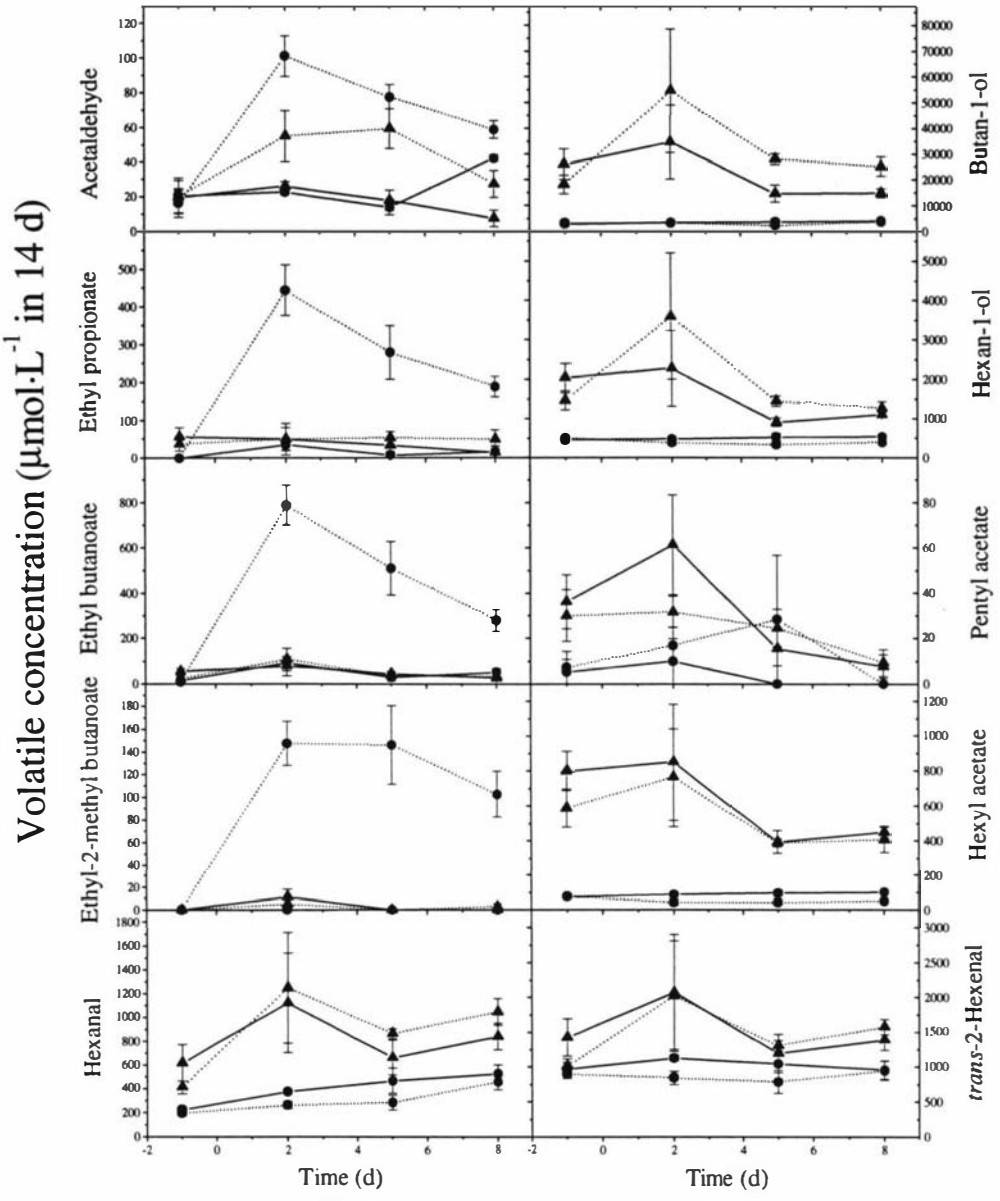

Figure 6.3 Concentration of selected volatile compounds, reported to have important apple aroma characteristics, of juice from FU (●) and RG (▲) apples after exposure to hypoxia for 24 hours at 20 °C (—) untreated and (·····) treated, d -1 was the day before treatment, d 0 on removal from treatment and subsequent storage at 20 °C for up to 8 d. Means and standard errors of six replicates.

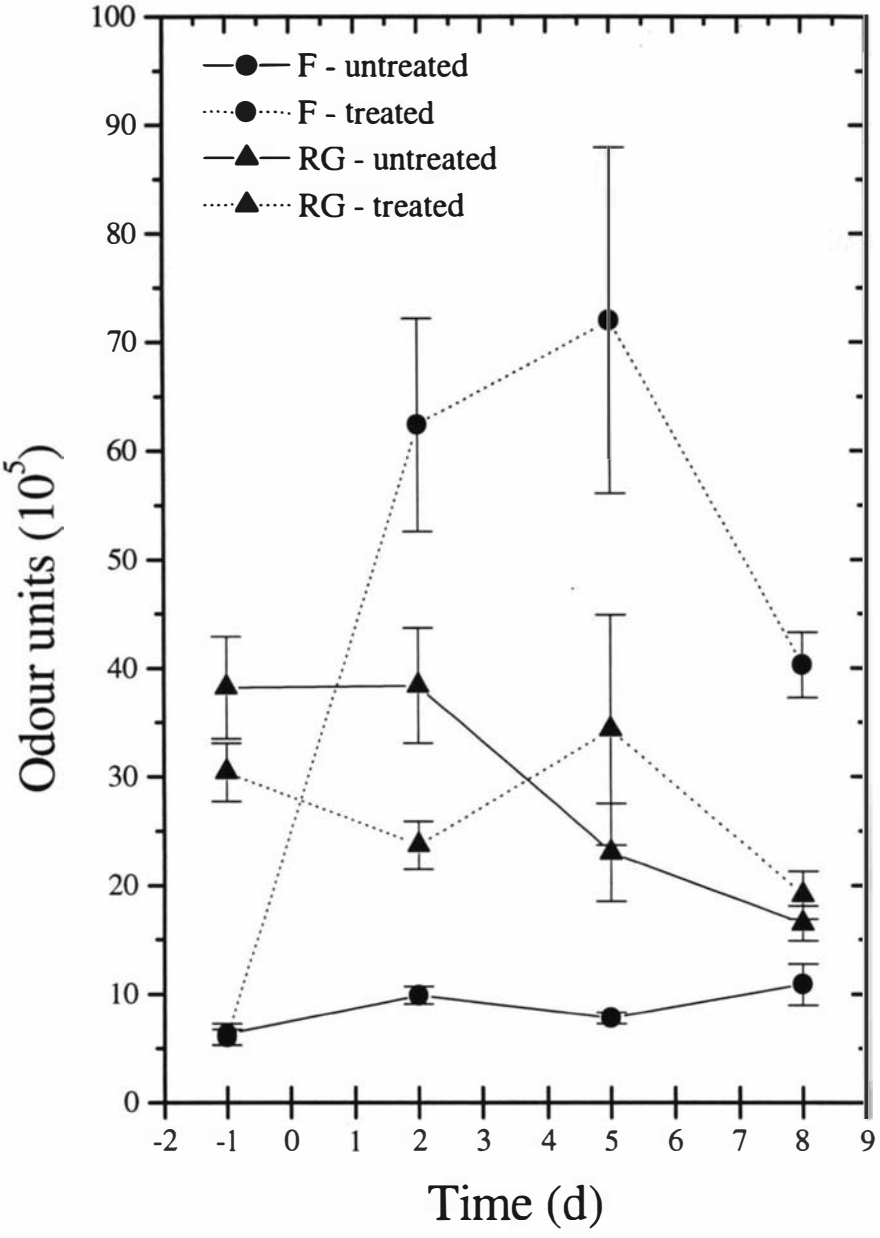

Figure 6.4 Odour unit values for FU and RG apple juice from untreated and hypoxic treated apples before and after removal from hypoxia (d 0) and after storage at 20 °C for up to 8 d at 20 °C.

6.4.4 Consistency between panellists

Differences in average sensory scores between panellists was large, suggesting that panellists were inconsistent in their assessment of treated and untreated juice. For example, when comparing aroma of control and treated FU juice, 2 panellists thought treated juice had more aroma, 3 panellists thought control juice had more aroma and 2

panellists could not distinguish a difference in aroma (Figure 6.5). Panellists were slightly more consistent with RG apple juice where 2 panellists thought treated juice had less aroma and 5 panellists could not distinguish a difference (Figure 6.5). These differences in the ability of panellists to discriminate sensory characteristics between treatments may explain why there was no difference between treatments when sensory scores were averaged.

If sensory characters are correlated, the above result indicates that such characters were not assessed independently of each other. For untreated FU and RG juice there were significant correlations between aroma, flavour and sweetness implying these characteristics were considered the same by panellists (Table 6.2). In treated juice, aroma and flavour were correlated indicating there had been a change in the nature of these sensory characters. Other correlations noted highlight differences in sensory characters between FU and RG apples. In FU untreated juice, sweetness correlates with acidic flavour, but these are not correlated in treated juice (Table 6.2). In contrast, off flavour correlates with aroma in untreated RG juice, while in treated juice sweetness and woody correlate with off-flavour. This implies there has been a change in how panellists are assessing sensory characteristics between untreated juice and treated juice.

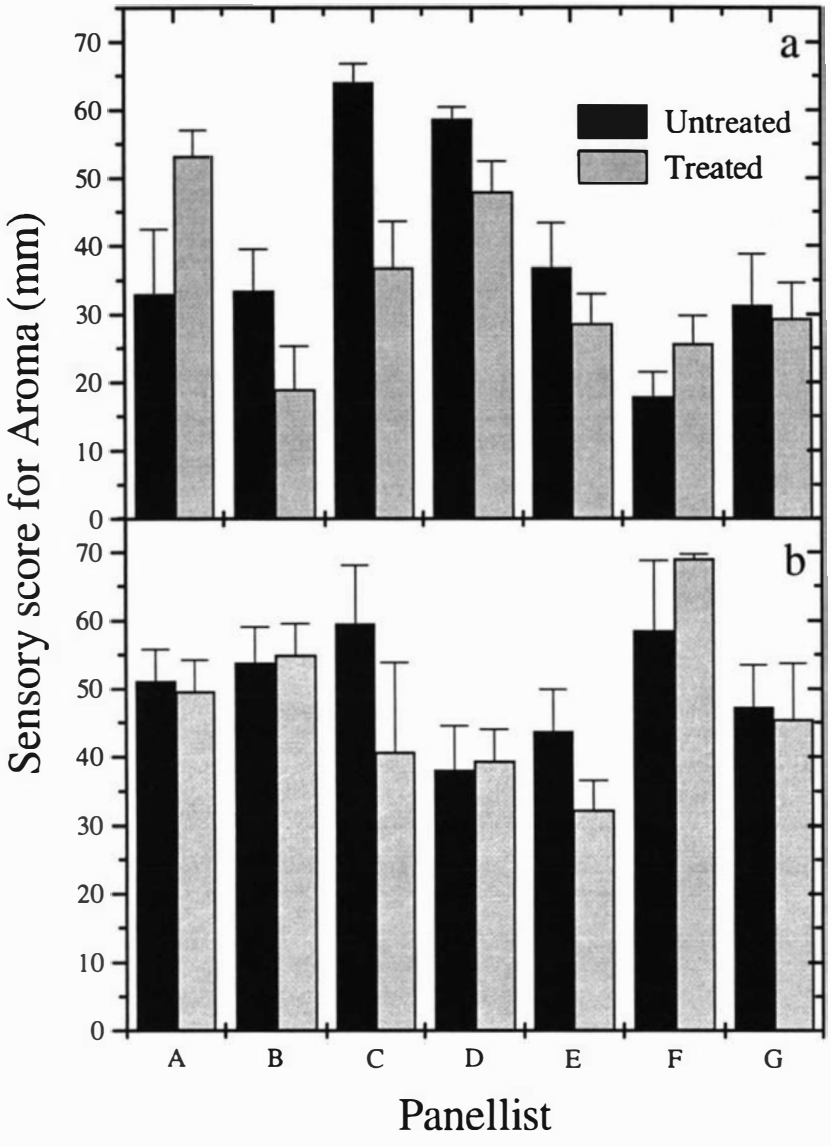

Figure 6.5 Sensory scores of aroma, as distance from the origin, for individual panellists for juice of (a) FU and (b) RG apples from untreated controls and from fruit exposed to hypoxia (100% CO_2, < 0.5% O_2 for 24 hours at 20 °C), maintained at 20 °C for up to 8 d. Average and standard errors of 24 assessments (2 replicates x 4 sample times x 3 experiment repeats).

Table 6.2 Correlation coefficients of average sensory scores collected from seven panellists by QDA, evaluating FU and RG apple juice, from apples exposed to hypoxia (100% CO_2,<0.5% O_2 for 24 hours at 20 °C) and stored in air at 20 °C for up to 8 d.

Fuji						
Character	Aroma	Flavour	Sweet	Acidic	Woody	Off [1]
			Untreated			
Aroma	x					
Flavour	0.71**	x				
Sweet	0.15	0.41	x			
Acidic	0.01	0.07	-0.47*	x		
Woody	0.26	-0.03	-0.25	0.29	x	
Off	0.25	-0.12	-0.40	0.25	0.46	x
			Treated			
Aroma	x					
Flavour	0.63**	x				
Sweet	0.19	0.07	x			
Acidic	0.20	0.51*	-0.14	x		
Woody	0.09	0.03	0.15	-0.24	x	
Off	0.08	-0.21	0.08	0.25	-0.15	x

Royal Gala						
Character	Aroma	Flavour	Sweet	Acidic	Woody	Off
			Untreated			
Aroma	x					
Flavour	0.78***	x				
Sweet	0.62**	0.67**	x			
Acidic	-0.03	-0.28	0.00	x		
Woody	0.08	0.00	0.38	0.34	x	
Off	-0.56*	-0.66**	-0.46	0.38	0.10	x
			Treated			
Aroma	x					
Flavour	0.69**	x				
Sweet	0.30	0.38	x			
Acidic	0.40	-0.08	0.02	x		
Woody	0.54*	0.24	0.38	0.44	x	
Off	0.44	0.16	0.48*	0.27	0.70**	x

Significance levels between attributes for a given treatment: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. [1] Off = off flavour.

Table 6.3 Correlation coefficients of individual average volatile concentration with corresponding average sensory scores for Aroma and Flavour of FU and RG apple juice from apples exposed to hypoxia (100% CO_2,<0.5% O_2 for 24 hours at 20 °C) and stored in air at 20 °C for up to 8 d.

Sensory characteristic	Aroma				Flavour			
Cultivar	F		RG		F		RG	
Treatment	U[1]	T	U	T	U	T	U	T
Alcohols								
Ethanol	0.12	-0.37	0.17	-0.66**	0.06	-0.21	0.07	-0.25
Propan-1-ol	-0.21	0.10	-0.40	-0.27	-0.26	0.02	-0.62**	-0.10
Butan-1-ol	-0.22	-0.16	-0.12	-0.30	-0.39	0.00	-0.34	-0.23
Pentan-1-ol	-0.02	0.09	-0.05	-0.35	-0.13	0.00	-0.16	-0.24
Hexan-1-ol	-0.22	-0.06	0.01	-0.29	0.04	-0.15	-0.23	-0.26
2MB[3]	-0.27	-0.23	-0.33	-0.26	-0.40	-0.17	-0.58**	-0.21
Acetate esters								
Ethyl acetate	-0.06	-0.23	0.42	-0.69**	0.37	-0.02	0.36	-0.16
Propyl acetate	0.17	-0.29	-0.27	-0.38*	0.19	-0.35	-0.37	0.01
Butyl acetate	-0.12	-0.11	-0.13	-0.24	-0.33	0.06	-0.34	-0.19
Pentyl acetate	0.24	0.49*	0.05	-0.08	0.14	0.16	-0.11	0.20
Hexyl acetate	-0.18	0.14	0.02	-0.22	0.20	-0.02	0.23	-0.20
2MBA[3]	-0.08	-0.20	-0.37	0.01	-0.31	-0.06	-0.62**	-0.14
Ethyl esters								
Ethyl propionate	0.07	-0.32	-0.04	0.38	0.06	-0.23	0.06	0.30
Ethyl butanoate	-0.06	-0.45	-0.03	-0.11	0.28	-0.26	-0.20	-0.05
Ethyl pentanoate	-0.25	0.13	0.09	0.00	-0.48*	0.24	-0.15	-0.10
Ethyl hexanoate	0.05	-0.22	0.04	0.17	0.12	-0.13	-0.21	0.19
E2MB	ND[2]	-0.28	-0.13	0.30	ND	-0.26	-0.16	0.09
Aldehydes								
Acetaldehyde	-0.29	-0.43	0.42	-0.43	-0.03	-0.13	0.33	-0.21
Hexanal	-0.20	-0.06	-0.20	-0.14	-0.40	-0.07	-0.41	-0.15
trans-2-hexenal	-0.10	-0.22	-0.13	-0.11	-0.23	-0.20	-0.37	-0.15

Between treatment within a cultivar and sensory characteristics siginificance levels: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

[1]U = untreated T = hypoxic treated. [2]ND = Not detected. [3]2MB = 2 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = ethyl-2-methyl butanoate.

6.4.5 Correlation between sensory characteristics and volatile concentration

In untreated FU juice there were no correlations of volatiles with aroma, but in treated juice there was a positive correlation with pentyl acetate (Table 6.3). Untreated RG juice aroma was not correlated with volatiles, and treated juice was negatively correlated with ethanol, ethyl acetate and propyl acetate (Table 6.3). Negative correlations indicate that in treated juice changes in volatile concentration of increased ethyl esters and decreased non-ethyl esters decreased the perception of aroma.

Untreated FU juice had a negative correlation of ethyl pentanoate with flavour but treated FU juice had no correlations with any volatile measured (Table 6.3). Flavour of untreated RG juice was negatively correlated with propan-1-ol, 2 methyl butan-1-ol and 2 methyl butyl acetate while flavour of treated juice was not correlated to any volatile concentration (Table 6.3). These results imply that exposure to hypoxia decreases perception of flavour which was associated with decrease in concentration of non-ethyl esters.

The multivariate analysis technique of principal components indicates the relationship between groups of variables and identifies which variables are correlated and contribute most to variance in the data. In principal component analysis of volatile concentration and sensory characters, off flavour appears to be associated with ethanol, acetaldehyde, ethyl butanoate, ethyl-2-methyl butanoate, ethyl hexanoate and ethyl propionate (Figure 6.6). Woody/grassy was associated with aldehydes, branched chain alcohols, butan-1-ol and non ethyl esters. Aroma is associated with acetate esters and propan-1-ol which were decreased by treatment. The ester, 2 methyl butyl acetate, was not grouped with other volatiles but may be associated with sweetness and aroma. Acidic flavour was associated with ethyl acetate and pentan-1-ol. Flavour was not associated with any volatiles. The increase in ethyl esters induced by exposure to hypoxia was associated with off flavour and not aroma. Compounds, such as acetate esters, which have fruity characters, were associated with aroma but these were perceived to have been decreased by hypoxic treatment.

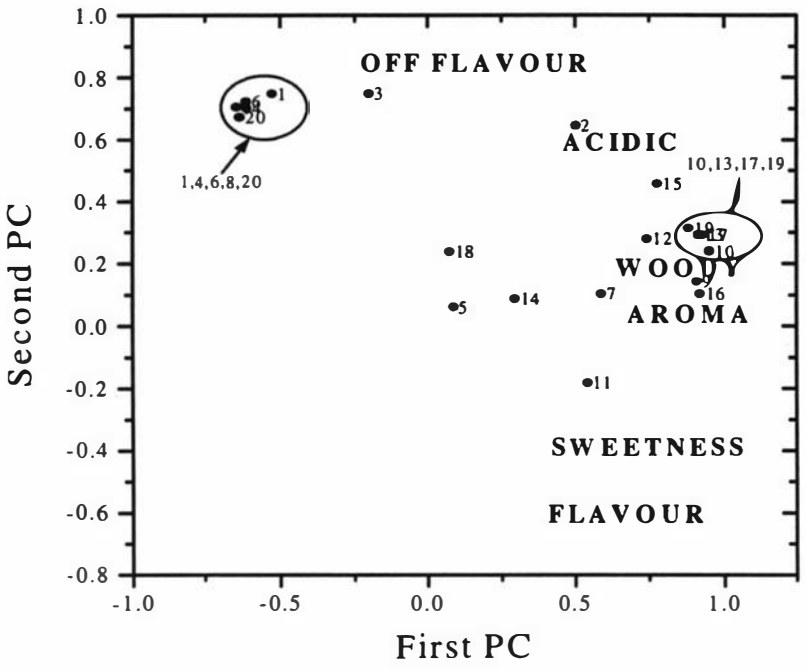

Figure 6.6 Scatter plot of volatile compounds and sensory characteristics of FU and RG apple juice from apples exposed to hypoxia for 24 hours at 20 °C based on the first and second principal component scores extracted from GC analysis and taste panel scores obtained by QDA. Volatile compounds were: 1 = acetaldehyde, 2 = ethyl acetate, 3 = ethanol, 4 = ethyl propionate, 5 = propyl acetate, 6 = ethyl butanoate, 7 = propan-1-ol, 8 = ethyl-2-methyl butanoate, 9 = butyl acetate, 10 = hexanal, 11 = 2 methyl butyl acetate, 12 = ethyl pentanoate, 13 = butan-1-ol, 14 = pentyl acetate, 15 = pentan-1-ol, 16 = hexyl acetate, 17 = hexan-1-ol, 18 = 2 methyl butan-1-ol, 19 = *trans*-2-hexenal, 20 = ethyl hexanoate.

6.5 Discussion

Exposure to hypoxia induced large changes in the volatile composition of FU apple juice which did not translate to changes in sensory perception of apple aroma or apple flavour of FU juice as the fruit ripened at 20 °C. By contrast RG apples had little change in volatile concentration after exposure to hypoxia and, as expected, had no change the sensory perception of apple aroma and apple flavour compared to control fruit. Similar lack of correlation between volatiles and sensory attributes has also been noted with Golden Delicious and York Imperial apples (Watada et al., 1981), although hexyl acetate, 2 methyl butyl acetate and butan-1-ol have been related to red apple aroma and red apple flavour

for RG apples (Young et al., 1996) and over-ripeness has been correlated with all types of esters in non-cooled McIntosh apples (Panasiuk et al., 1980). The volatiles enhanced by hypoxia in FU apples were ethyl esters which are reported to have fruity and apple like odour when smelled individually (Flath et al., 1967). Some of these ethyl esters, such as ethyl-2-methyl butanoate, have very low odour thresholds which some authors claim makes these compounds very important in apple aroma (Flath et al., 1967; Williams, 1979). In contrast to taste panel results, odour unit values for FU apples indicated that from 2 d after removal from hypoxia there should have been an increase in the intensity of apple aroma. Royal Gala odour units indicated that there was no difference in aroma between control and apples exposed to hypoxia.

The lack of difference in aroma percieved by panellists when some volatiles thought to be important for apple aroma were significantly enhanced may be related to the limitations of the odour unit concept and of the QDA sensory technique. Consideration must also be given to the relationship between volatile concentration and description of sensory character and how changes to a mixture of volatiles may affect perception of apple aroma.

The relative odour intensity of a component has been defined as the threshold concentration representing one odour unit. From the number of odour units for a particular compound, the relative contribution to overall aroma intensity can be estimated (Guadagni et al., 1966). An underlying assumption of this method is that compounds with equal odour units have the same odour intensity; however olfactory intensity for many compounds is a power function of concentration and the exponent of the power function differs for different compounds (Land 1979; Mulders, 1973). The odour unit concept also assumes that compounds present resemble the overall aroma of the product and that odour units provide a measure of perceived intensity of a component at suprathreshold concentrations, *i.e* there are no additive or synergistic effects of one compound on another (Laing and Panhuber, 1979). The relationship between volatile concentration and perception of intensity may be sigmodial where the concentration range of a volatile over which changes in concentration can readily perceived is small (Marin et al., 1991). Below the threshold concentration the volatile is not perceived and above the saturation concentration, volatile concentration changes make no difference to the perception of the volatile.

These assumptions are not often met as aroma intensity can vary widely between compounds and different concentrations of the same compound. Interactions between odours that may mask or alter aroma characteristics of compounds are not considered (Laing and Panhuber, 1979). In the results presented here ethyl-2-methyl butanoate is a good example of a compound that is probably at suprathreshold concentrations in FU apples exposed to hypoxia and may have been perceived by panellists as having a different aroma character. Some compounds may normally be present at suprathreshold concentrations (e.g. butyl acetate) and therefore contribute to aroma differently to what is assumed in the odour unit calculation. The pyschophysical evidence indicates that the aroma/flavour intensity of a mixture does not necessarily equal the summed intensities of individual components (Frijters, 1979). The fact that odour unit

values at each sampling time did not match panellists perception of aroma, indicates that this concept does not accurately indicate the effect that exposure to hypoxia may have on apple aroma. Use of odour units is losing favour among researchers examining odour and its perception; currently much more use is being made of multivariate statistical techniques that examine the correlations of sensory characters with volatile concentrations (Dijksterhuis, 1995b; Peppard, 1994; Togari et al., 1995; Williams et al., 1988).

A common multivariate method used to indicate relationships between sensory characters and chemical composition is principal component analysis (PCA) which is used to examine correlations between attributes and the chemical composition (Williams, 1986). Principal components analysis has two main functions by indicating relationships among groups of variables and by showing relationships between objects (Piggott and Sharman, 1986). Linear combinations of variables are identified which account for the maximum possible proportion of variance in the original data. The relationship between physical stimulus and the perceived intensity of that stimulus is not linear but logarithmic or exponential, therefore linear approximations may not always give the correct results in the analysis of sensory data (Dijksterhuis, 1995b). When analysed by PCA, FU and RG apple juice had a clear association of off flavour with most of the volatiles enhanced by exposure to hypoxia. This suggests that the enhanced concentration of ethyl esters was perceived, not as more intense apple aroma, but as a different aroma/flavour characteristic associated with off flavour. Sweetness and aroma were associated with 2 methyl butyl acetate which is in agreement with results for RG apples (Young et al., 1996).

The taste panel technique QDA is commonly used to provide data which can be statistically analysed readily and is claimed to have the following advantages: precise and reliable information specific to the product being examined; generates quantitative data that can be used for statistical analysis and gives a measure of variability in sensory response; it is repeatable over time and panellists have less bias (Zook and Pearce, 1988). The disadvantages are that panellists require intensive training making such panels expensive to conduct. Other problems with a method such as QDA relate to panellist performance and their ability to describe and discriminate between sensory characteristics. The rating for one attribute may influence the criteria for assessing another; for example good appearance leads to expectations of good flavour (Williams, 1994). The sensory characters aroma, flavour and sweetness reported here were highly correlated in control FU and RG apple juice. This would suggest that the panellists were not assessing these sensory characters independently. Possible reasons for this may have been inadequate panellist training or definition of sensory characteristics. However, panellists were presented at each tasting session with a 'reference' that had the same sensory characteristics and served as a reminder of intensities. In juice from apples exposed to hypoxia, aroma and flavour were still correlated, but flavour was no longer correlated with sweetness indicating that there had been a change in the nature of sensory characters. Attaching different meanings to the same attribute by different panellists is a well recognised problem (Dijksterhuis, 1995a); this may be a reason for the variability in sensory scores for aroma between panellists (Figure 6.5).

Unlike alcohols and acetate esters, ethyl esters did not correlate with apple aroma for juice from FU or RG apples exposed to hypoxia. This result was surprising as ethyl esters had the greatest proportional changes in concentration after exposure to hypoxia and are thought to be important for apple aroma (Dimiick and Hoskin, 1983). It suggests that ethyl esters may not be important in characterising apple aroma by the panellists used in this study. Ethyl esters could have been at suprathreshold concentrations that will suppress perception of aroma. Further taste panel assessments may be required where juice from apples exposed to hypoxia is diluted so that volatile concentrations match those of untreated fruit. When dealing with trace volatile compounds, care must be taken to ensure that GLC measurements do relate to the compound identified (Williams et al., 1988). For example, 2 methyl butyl acetate and propyl butanoate have retention times which are very close and as these two compounds were correlated with FU aroma in fruit exposed to hypoxia, it is possible that the values for propyl butanoate may well be confounded with those for 2 methyl butyl acetate.

Juice from FU and RG apples exposed to hypoxia had significant negative correlations with some volatiles while juice from control fruit did not. This would suggest that aroma of juice from apples exposed to hypoxia had changed in character compared to juice from control fruit. The change in character may be a suppression of 'fruity apple-like' aroma due to some ester concentrations being enhanced to suprathreshold levels. It has been recognised that the perceived intensity of a mixture is less than the sum of its components due to masking or suppression (Lawless, 1986) and spatial and temporal effects of when compounds interact with olfactory cells (Laing, 1994).

Although QDA is a widely recommended taste panel method, its use here has not provided a definitive answer to what effects exposure to hypoxia has on apple aroma and flavour. To determine the effect that changing proportions of volatile concentrations in juice has on sensory perception of apple aroma and flavour, a different taste panel procedure be employed. One possible procedure would be to ask each panellist to write down their own descriptors of apple aroma and flavour for each sample and to indicate their relative intensity. By using this method training times and costs would be reduced to one session explaining and practising process. Descriptors would be scrutinised for differences in perception of juice samples by panellists; changes or differences would then indicate how juice from fruit exposed to hypoxia and control fruit are different. For example, juice from apples exposed to hypoxia may be described as flat with a strong after taste and control fruit with intense apple flavour but with no after taste. In both examples the juice still tastes of 'apple' but is a different variation of apple flavour. It is such differences that panellists may have been rated when evaluating juice samples, but because the apple aroma/flavour of juice from apples exposed to hypoxia did not meet the rigid definition established in the training for QDA, it was marked as off flavour. Such a method as described above would help to better understand what changes treatments such as exposure to hypoxia may have on changing sensory characteristics of apple juice.

6.6 Conclusions

Exposure to hypoxia has no effect on apple aroma or flavour as perceived by a trained panel, despite very large increases in concentration of ethyl esters, some of which are claimed to be character impact compounds and therefore important for apple aroma. The reasons for this may have been due to the taste panel method used not measuring nuances in apple aroma and to panellists being unable to adequately distinguish between sensory characteristics. Enhanced concentrations of ethyl esters were perceived as off flavour by panellists rather than as a more intense aroma/flavour, possibly due to masking or suppressive effects in the volatile mixture. A more definitive answer to how juice from apples exposed to hypoxia is different from control fruit may require a different sensory analysis procedure.

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Chapter Seven

Temperature of hypoxic treatment alters volatile composition of 'Fuji' and 'Royal Gala' apple juice.

Additional Index Words. aroma; flavour; solvent extraction

7.1 Abstract

Exposure to hypoxia induces changes in volatile concentration potentially affecting aroma quality but there is little information on how the temperature of hypoxic treatment may affect volatile concentration. Fuji and Royal Gala apples were exposed to hypoxia, 100% CO_2 for 24 h, at 10, 15, 20 or 25 °C and maintained at treatment temperature for up to 14 d. Carbon dioxide and ethylene production and firmness were proportional to temperature but were unaffected by exposure to hypoxia. Ethyl esters were enhanced at all temperatures at differential rates according to cultivar. Four patterns of total volatile concentration change with temperature after exposure to hypoxia were identified which may be related to changes in rates of volatilisation and volatile production. Apples treated and maintained at 10 °C had the greatest overall enhancement of ethyl esters and least decrease in other esters compared to apples treated and maintained at 15, 20 and 25 °C. This enhancement in volatiles persisted for up to 10 d after removal from hypoxia. Maintenance of apple quality after treatment with hypoxia was better at low temperatures suggesting that apples treated with hypoxia and maintained below 15 °C would have enhanced volatile concentration compared with untreated apples and apples maintained above 15 °C.

7.2 Introduction

Apple aroma contains more than 300 volatile compounds consisting mainly of esters, alcohols and aldehydes, whose production is influenced by temperature. Ester and alcohol concentrations and production increased as temperature increased in Jonathan apples stored at temperatures from -1 to 10 °C for up to 12 weeks (Wills and McGlasson, 1971). Ester concentration was maximum at 22 °C for Red Delicious apples; at 32 °C ester production was decreased while 46 °C inhibited aroma production (Guadagni et al., 1971). Heat treatment of Golden Delicious apples of 38 °C for 4 days reduced volatile production compared to fruit at 22 °C (Fallik et al., 1997).

Exposure of fruit to hypoxic atmospheres for up to several days has been investigated as a non-chemical insect disinfestation treatment (Lay-Yee and Whiting, 1996; Whiting et al., 1996). Hypoxic atmospheres have faster insect kill at warm temperatures (20 °C or above) than at low (<5 °C) temperatures as the target insect has increased metabolic activity and rate of fumigant uptake (Ke and Kader, 1992; Paull and Armstrong, 1994). Exposure to hypoxia induces substantial qualitative and quantitative changes in volatile concentration (Ampun, 1997; Chapter 4) and generally reduces softening, CO_2 and ethylene production in apples, probably due to the inhibitory effects that high concentrations of CO_2,

acetaldehyde, ethanol or low O_2 concentrations have on enzyme systems within fruit. Treatments using hypoxic atmospheres up to 100% CO_2 or N_2 at -1.1 °C to 32 °C for a few hours up to 6 weeks have enhanced maintenance of quality during long term storage (Eaves et al., 1968), acclimatised fruit to low oxygen storage conditions (Little et al., 1982), quantified fruit tolerance to non-chemical disinfestation treatments (Ke et al., 1991, Yahia and Vazquez-Moreno, 1993) and enhanced aroma/flavour volatile concentration in fruit (Ampun, 1997; Dourtoglou et al., 1994, Shaw et al., 1992).

Little is known as to how volatile concentrations in apples may be affected by the temperature of a hypoxic treatment. At 20 °C there is a large increase in acetaldehyde, ethanol, ethyl esters and some alcohols while there is a corresponding decrease in acetate esters and no change in aldehyde concentration (Ampun, 1997; Chapter 4). If lowering fruit temperature slows fruit metabolism, then volatile production should also be reduced. After exposure to hypoxia, increases in acetaldehyde, ethanol and ethyl esters should be decreased at reduced temperatures while raising fruit temperature should increase volatile concentration. If hypoxic treatments are to be used as disinfestation treatments, consideration must be given as to when such treatments may be applied in the coolchain. Apple ripening is proportional to temperature, the higher the temperature the faster the ripening (Wills et al., 1997). To maximise preservation of postharvest quality it is recommended that apples be cooled to low temperatures as soon as possible after harvest, packing and grading. By exposing apples to hypoxia at temperatures of 20 °C and above, unacceptable losses in quality parameters such as firmness and background colour may occur. As an example, for Cox's Orange Pippin apples 1 d at 20 °C is estimated to be the equivalent of 9 days at 3 °C for loss in background colour (Dixon and Hewett, 1998). To determine if exposing apples to hypoxia induces different changes in volatile concentration, Fuji and Royal Gala apples were exposed to hypoxic conditions and subsequently stored at treatment temperatures ranging from 10 to 25 °C.

7.3 Materials and Methods

7.3.1 Fruit supply

Fuji and Royal Gala apple (*Malus domestica* Borkh.) cultivars were assessed for their response to hypoxia. In 1995 cultivars were harvested at mid commercial harvest, 7 May for Fuji (FU) and 31 Mar. for Royal Gala (RG), as determined by the New Zealand Apple and Pear Marketing Board, from the Hawke's Bay region of New Zealand. Fruit were graded to export standard, packed to count 125 (148g average fresh weight), transported unrefrigerated to Massey University by road where the fruit was placed at 0 °C ± 1 °C within 3 days of harvest. Before treatment fruit were removed from coolstore and equilibrated to 20 °C ± 3 °C overnight. Fruit were divided into two groups, one was exposed to hypoxia, the other was maintained in air as control. For each treatment and sample time there were 4 replicates each of 3 apples.

7.3.2 Hypoxic treatment

Refer to Section 4.3.2.

7.3.3 Fruit assessment

Before exposure to hypoxia Fuji apples for treatment at 10 °C and 25 °C were maintained at 0 °C ± 1 °C for 52 d and 82 d for treatments at 15 °C and 20 °C. Royal Gala apples treated at 10 °C and 25 °C were kept at 0 °C ± 1 °C for 75 d and 109 d for treatments at 15 °C and 20 °C. Fruit were removed from 0 °C and allowed to equilibrate to treatment temperature overnight. Fruit were assessed the day before treatment (d -1), on removal from hypoxia (d 0) and at 2 d intervals following treatment during ripening at treatment temperatures for CO_2 and ethylene, firmness, mass, volatile concentration of juice and fermentation volatiles in the juice headspace. This allowed for differences in volatile concentration due to fruit ripening to be accounted for in the data analysis.

7.3.4 Firmness

Refer to Section 4.3.4.

7.3.5 Carbon dioxide and ethylene production

Refer to Section 4.3.5.

7.3.6 Juice preparation

Refer to Section 4.3.6.

7.3.7 Headspace volatiles

Refer to Section 4.3.7.

7.3.8 Juice volatiles

Refer to Section 4.3.8.

7.3.9 Data analysis

Each experiment was conducted as a completely random design with sampling during storage as repeated measures. Means and standard errors of the means for each cultivar and treatment temperature for headspace and juice volatiles were graphed using an Origin graphics package v. 5 (Microcal Software Inc., USA). Data were subjected to analysis of variance using SAS v. 6.12 (SAS Institute Inc., Cry, USA). Significant main mean effects were separated by Duncan's multiple range test at the 5% level of significance.

7.4 Results

7.4.1 CO_2 and ethylene production, firmness

At each temperature FU and RG fruit had similar rates of CO_2 production (Figure 7.1). For both cultivars CO_2 production was proportional to temperature, increasing by about 50 $nmol \cdot kg^{-1} \cdot s^{-1}$ per 5 °C (Figure 7.1). Carbon dioxide production remained unchanged for 14 d at 10 and 15 °C but slowly declined for 20 and 25 °C fruit. Exposure to hypoxia did not affect carbon dioxide production for FU and RG fruit except for RG fruit exposed to hypoxia and stored at 25 °C that had enhanced CO_2 production from 4 to 8 days.

In FU and RG fruit at 15, 20 and 25 °C exposed to hypoxia ethylene production was initially decreased 2 d after removal before it rose at 4 d to be similar to untreated control fruit (Figure 7.2). For FU and RG fruit at 10 °C exposed to hypoxia ethylene production was decreased only at 4 d. Rate of ethylene production increased with temperature to a maximum at 20 °C, but declined at 25 °C to similar rates as 15 °C (Figure 7.2). Pattern of ethylene production was different between cultivars; in FU fruit ethylene production steadily increased during 14 d at 10 and 15 °C but declined at 20 and 25 °C while in RG fruit ethylene production increased to a peak then declined. The higher the temperature the sooner maximum ethylene production occurred (Figure 7.2).

There was no difference in firmness of FU or RG apples after exposure to hypoxia at any temperature or sampling time (Figure 7.3).

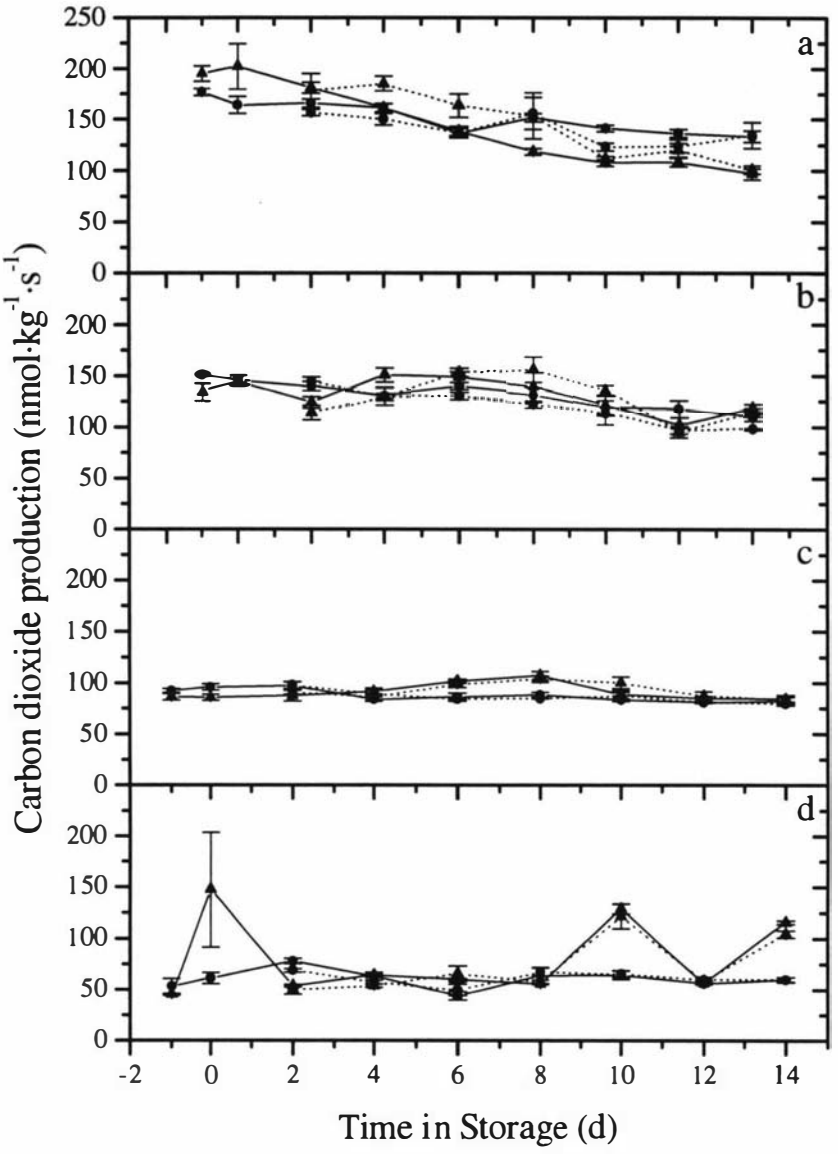

Figure 7.1 Carbon dioxide production of FU (●) and RG (▲) apples stored in air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h at a) 25 °C, b) 20 °C, c) 15 °C and d) 10 °C and maintained at treatment temperature for up to 14 d. D -1 is the day before treatment, d 0 is on removal from treatment. Average and standard errors of 4 replicates.

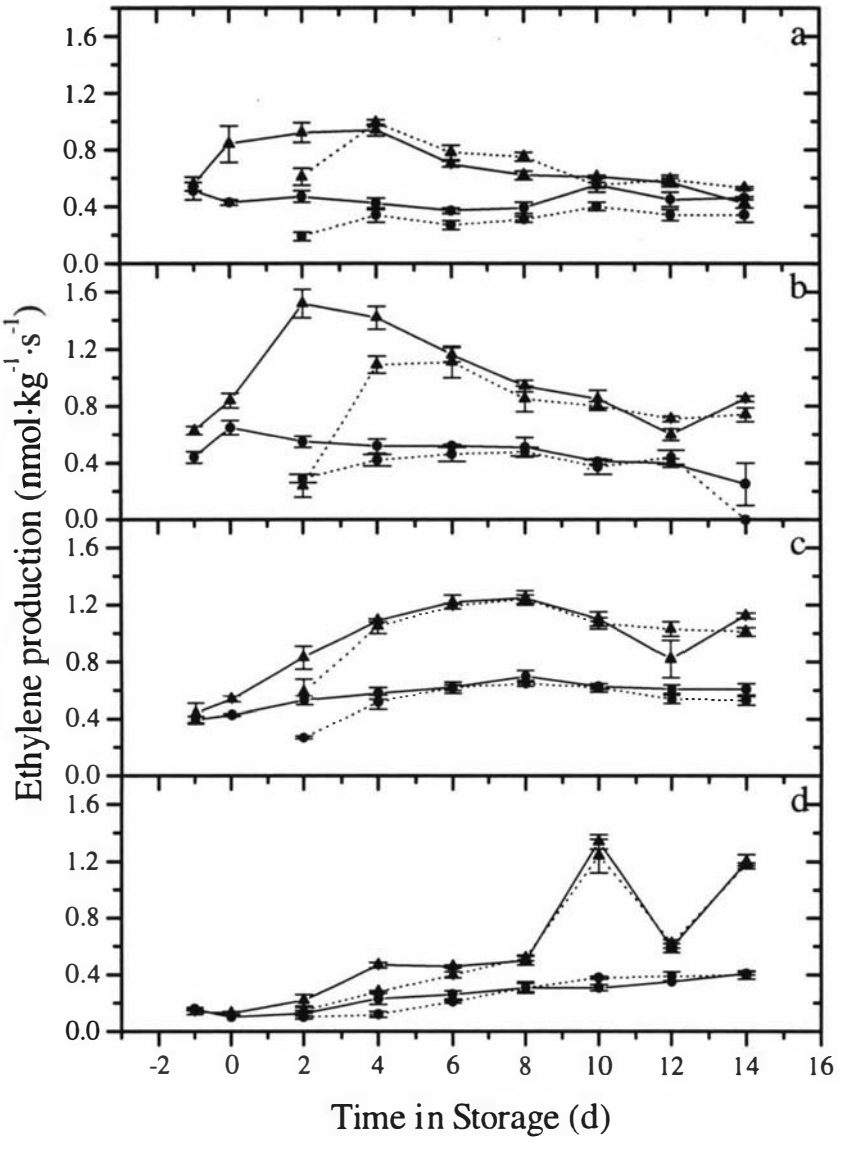

Figure 7.2 Ethylene production of FU (●) and RG (▲) apples stored in air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h at a) 25 °C, b) 20 °C, c) 15 °C and d) 10 °C and maintained at treatment temperature for up to 14 d. D -1 is the day before treatment, d 0 is on removal from treatment. Average and standard errors of 4 replicates.

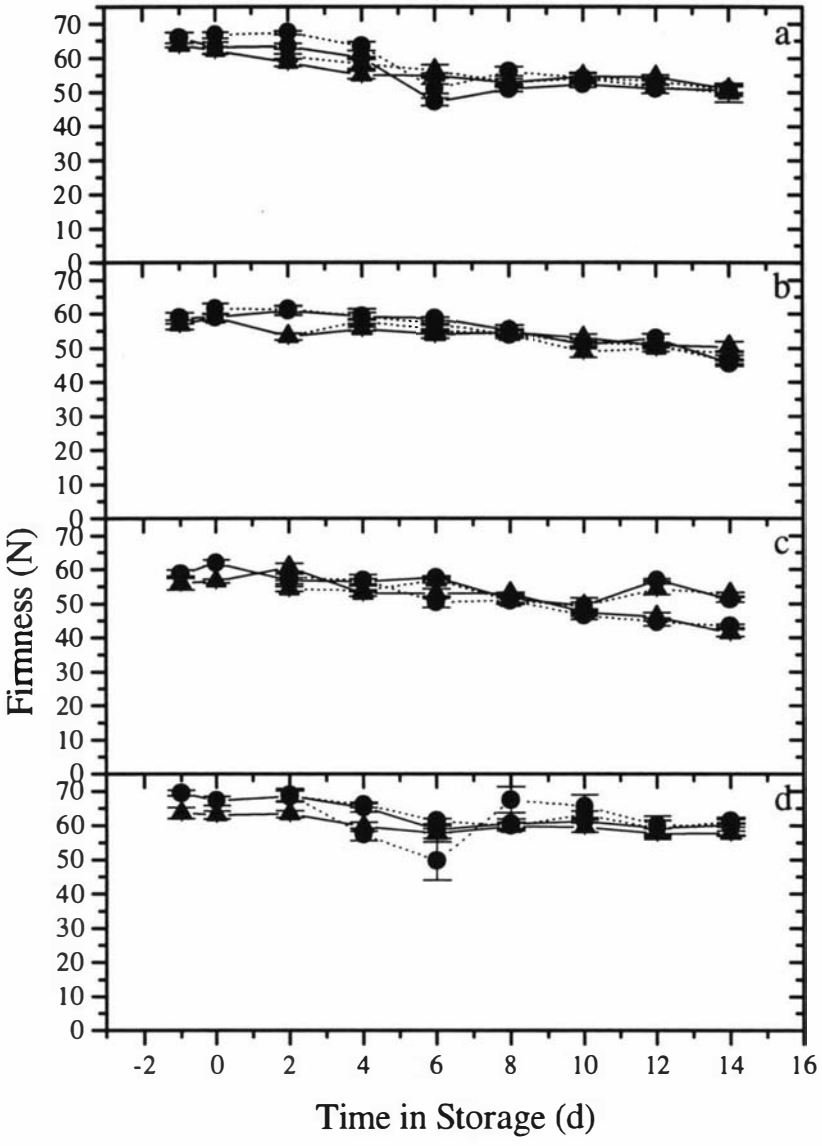

Figure 7.3 Firmness of FU (●) and RG (▲) apples stored in air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h at a) 25 °C, b) 20 °C, c) 15 °C and d) 10 °C and maintained at treatment temperature for up to 14 d. D -1 is the day before treatment, d 0 is on removal from treatment. Average and standard errors of 4 replicates.

7.4.2 Hypoxic effects on ester concentration

Detailed changes in concentration of ethyl butanoate and butyl acetate are presented as general examples of ethyl esters, which increase after exposure to hypoxia, and acetate esters which decrease after exposure to hypoxia (Figure 7.4). In FU fruit ethyl butanoate concentration was enhanced after exposure to hypoxia reaching a maximum concentration 2 to 4 d after removal from hypoxia and declining to concentrations similar to controls after 10 to 14 d. The magnitude of the enhancement in ethyl butanoate concentration after exposure to

hypoxia in FU fruit increased 39.5, 16, 18.3 and 8 fold from 10, 15, 20 and 25 °C, respectively, as temperature increased (Figure 7.4a). This contrasted with control fruit where ethyl butanoate concentrations appeared to increase slightly as temperature increased (Figure 7.4a). Only in RG fruit exposed to hypoxia at 20 °C were ethyl butanoate concentrations were enhanced from 0 to 4 d (Figure 7.4b); there was no increase RG fruit exposed to hypoxia at 10, 15 and 25 °C. Control FU fruit had about the same ethyl butanoate concentrations as RG fruit at 10 °C, but were 2 to 3 times that of control RG fruit at 15, 20 and 25 °C. There were different patterns of RG ethyl butanoate concentration at 15 and 20 °C than at 10 and 25 °C. The fruit treated at 15 and 20 °C may have been riper than fruit at 10 and 25 °C as they had 4 weeks longer at 0 °C before treatment.

The highest concentration of butyl acetate in FU and RG fruit occurred at 10 °C and the lowest at 25 °C (Figures 7.4c and 7.4d). As fruit ripened at each temperature butyl acetate concentration declined. Exposure to hypoxia reduced butyl acetate concentration in FU fruit by about 50% in fruit treated at 10 and 15 °C (Figure 7.4c). Decrease in butyl acetate concentration was less and returned to control concentrations earlier as temperature increased being 4d, 6 d and 12 d at 25, 20 and 15 °C respectively, and with no recovery at 10 °C (Figure 7.4c). Butyl acetate concentration after exposure to hypoxia was unchanged compared to control fruit for RG fruit at 10 °C (Figure 7.4d). At 15 °C butyl acetate was initially less than control concentrations but was similar from 4 d. At 20 °C butyl acetate was initially less than control fruit rose to concentration of control before decreasing from 6 d through 14 d. At 25 °C butyl acetate was decreased initially on 0 d and 2 d then increased slightly from 4 d.

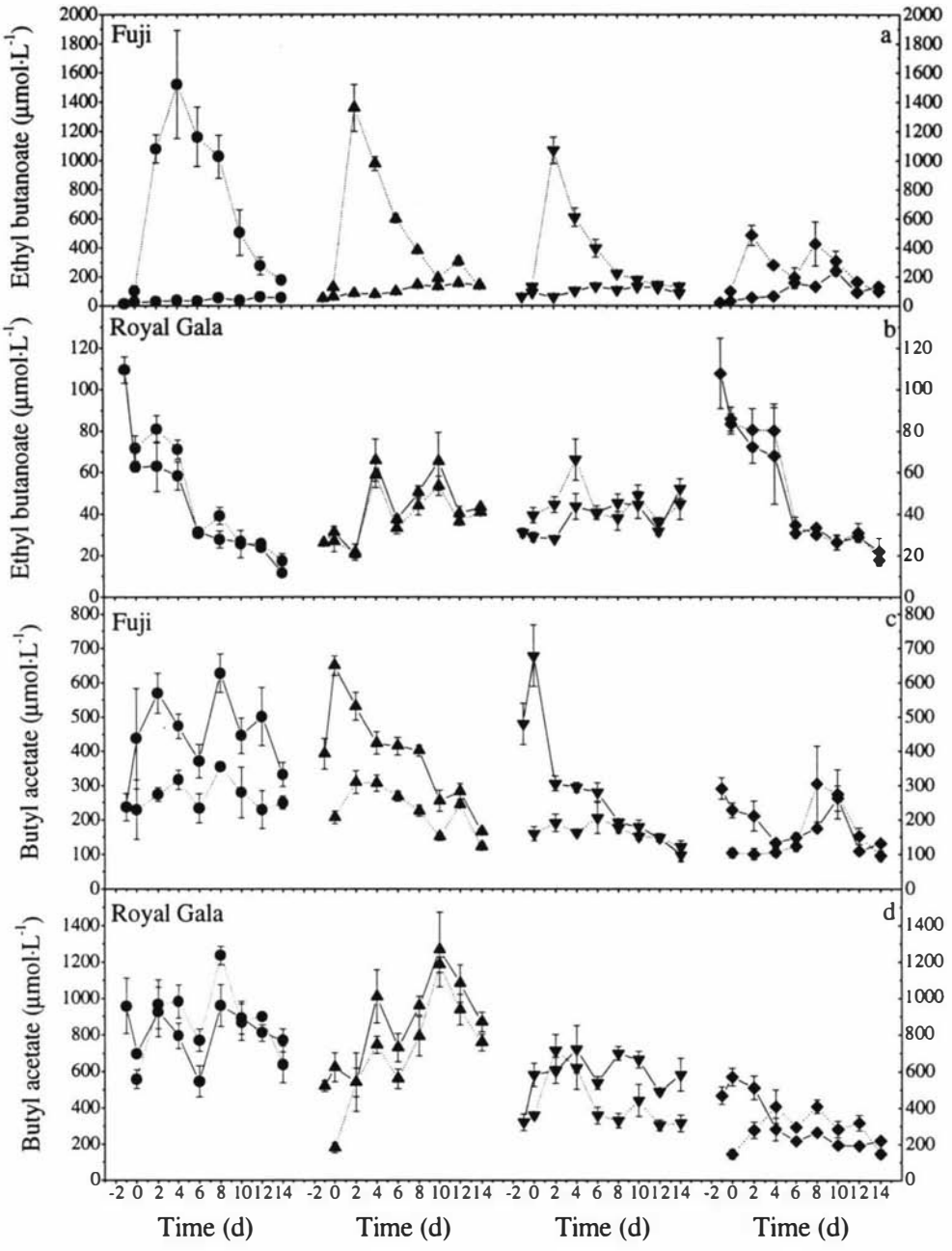

Figure 7.4 Ethyl butanoate and butyl acetate concentration in apple juice from a), c) FU and b), d) RG apples treated with air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h and maintained at treatment temperature at 10 °C (●), 15 °C (▲), 20 °C (▼) and 25 °C (◆) for up to 14 d. D -1 is the day before treatment, d 0 is on removal from treatment. Average and standard errors of 4 replicates.

7.4.3 Total volatile concentration over 14 d ripening

Total volatile production (Figure 7.5) was calculated as area under the curve of plots of volatile concentration with time, for volatiles regarded as having important sensory characteristics (Rizzolo et al., 1989). Control FU apples had 6 patterns of change in total volatile concentration with temperature and control

RG fruit had 5 patterns of change (Figure 7.5). Four of these patterns were common to control fruit of both cultivars. These patterns of change in control FU and RG apples over temperatures ranging from 10 to 25 °C were: no change in concentration of hexan-1-ol for FU and acetaldehyde, ethyl propionate and ethyl butanoate for RG; same hexyl acetate concentration for both FU and RG fruit at 10 and 15 °C then decreases at 20 to 25 °C; increase in pentyl acetate concentration for FU and butan-1-ol, hexan-1-ol and *trans*-2-hexenal concentration for RG; from 10 to 15 °C, the same at 15 and 20 °C then decreasing at 25 °C to the same concentration for FU at 10 °C of increase in concentration from 10 to 15 °C and decreasing concentration at 20 and 25 °C of butan-1-ol for FU and hexanal and pentyl acetate for RG. For FU fruit total volatile concentration of acetaldehyde, ethyl propionate, ethyl-2-methyl butanoate, *trans*-2-hexenal and hexanal increased with temperature while ethyl butanoate concentration increased from 10 to 15 °C and remained the same at 20 and 25 °C. No ethyl-2-methyl butanoate was detected in RG fruit.

In general, pattern of total volatile concentration with temperature was the same in hypoxic treated fruit as control fruit. In FU fruit there were 6 patterns of enhancement or decrease in total volatile concentration with temperature while there were 5 patterns for RG fruit (Figure 7.5). Two of these patterns were common to both cultivars. These patterns of enhancement or decrease in total volatile concentration compared to control fruit in FU or RG apples exposed to hypoxia were: no change in total volatile concentration of butan-1-ol, hexan-1-ol, hexanal and pentyl acetate for FU and ethyl propionate, ethyl butanoate, hexanal and pentyl acetate for RG; no change in concentration at 10 and 25 °C and a decrease in concentration at 15 and 20 °C of *trans*-2-hexenal for FU and hexan-1-ol and hexyl acetate for RG. For FU fruit exposed to hypoxia compared to control fruit total volatile concentration: was enhanced having the same pattern of increase with temperature as control fruit but the magnitude of enhancement decreased as temperature rose for ethyl propionate and ethyl-2-methyl butanoate; magnitude of enhancement of ethyl butanoate decreased with temperature; hexyl acetate was less than control concentrations at 10, 15 and 20 °C and was not different at 25 °C, the amount of decrease being less as temperatures increased; acetaldehyde was enhanced at all temperatures but the magnitude of enhancement declined from 10 to 15 °C, was the same for 15 and 20°C before rising at 25 °C. Total volatile concentration of RG fruit exposed to hypoxia compared to control fruit had: no difference in butan-1-ol concentration at 10, 15 and 20 °C and an increase in concentration at 25 °C; a decrease in *trans*-2-hexenal concentration at all temperatures. Ethyl-2-methyl butanoate was not detected in control or hypoxic treated RG apples.

Compared with RG, FU fruit had greater total concentrations of acetaldehyde, ethyl propionate, pentyl acetate, ethyl butanoate, ethyl-2-methyl butanoate and hexanal but lower concentrations of butan-1-ol and hexyl acetate (Figure 7.5). The concentration of *trans*-2-hexenal and hexan-1-ol were similar between cultivars. Exposure to hypoxia induced changes in total volatile concentration according to cultivar. Fuji fruit exposed to hypoxia had increased concentrations of acetaldehyde, ethyl propionate, ethyl butanoate and ethyl-2-methyl butanoate

and no change in concentration of butan-1-ol, hexan-1-ol, pentyl acetate, hexanal and *trans*-2-hexenal but decreased concentration of hexyl acetate. In contrast RG fruit exposed to hypoxia had increased concentration of acetaldehyde, no change in concentration of ethyl propionate, butan-1-ol, pentyl acetate, ethyl butanoate, hexanal and hexan-1-ol; decreased concentrations of *trans*-2-hexenal and hexyl acetate; and ethyl-2-methyl butanoate was not detected.

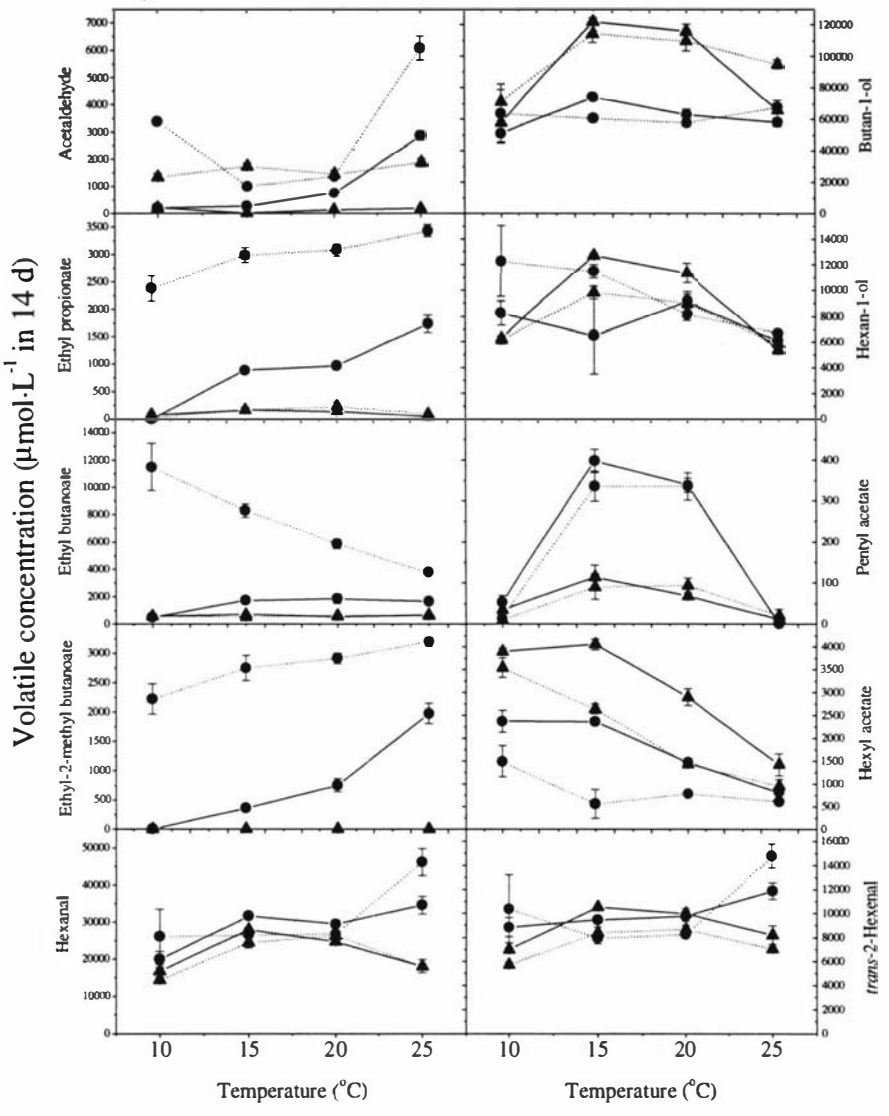

Figure 7.5 Total volatile concentration over 14 d, as area under the curve, of selected volatiles from FU (●) and RG (▲) apples stored in air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h at 25, 20, 15 and 10 °C and maintained at treatment temperature for up to 14 d. D -1 is the day before treatment, d 0 is on removal from treatment. Average and standard errors of 4 replicates.

7.4.4 Volatile changes after exposure to hypoxia

Concentrations of all volatiles were analysed after removal from hypoxia for 2 d and 10 d as this was when enhancement of ethyl esters was the greatest and after volatile concentrations had returned to control concentrations, respectively (Tables 7.1 and 7.2). While the effect of temperature varied for specific volatiles, generally volatile concentration was lower as temperatures increased.

At 2 d after removal from hypoxia FU and RG fruit produced enhanced concentration of the fermentation volatiles acetaldehyde, ethanol and ethyl acetate at all temperatures. Fuji apples had consistent enhancements of ethyl propionate, ethyl butanoate, ethyl hexanoate and ethyl-2-methyl butanoate at 10 °C to 25 °C. Production of ethyl hexanoate and ethyl-2-methyl butanoate only occurred in fruit exposed to hypoxia. Concentration of specific ethyl esters varied with temperature, for example, ethyl propionate concentrations at 15 to 25 °C were similar but lower than at 10 °C (Table 7.1), while ethyl butanoate concentrations at 10 to 20 °C were similar with concentration at 25 °C the lowest. In contrast to FU fruit, RG fruit had no enhancement of ethyl ester or decrease in acetate ester concentration after exposure to hypoxia at 10, 15 and 20 °C. Royal Gala fruit at 25 °C had decreases in butyl acetate, hexyl acetate, 2 methyl butyl acetate, hexanal and *trans*-2-hexenal (Table 7.1).

Ethyl esters were enhanced in FU fruit exposed to hypoxia after 10 d at 10 °C in contrast to fruit at 20 and 25 °C where there was no difference between control and hypoxic treated fruit. Ten days after hypoxic treatment FU fruit at 10 °C had increased concentrations of ethyl propionate, ethyl butanoate, ethyl hexanoate, ethyl-2-methyl butanoate and some alcohols (propan-1-ol, pentan-1-ol, 2 methyl butan-1-ol) and acetaldehyde. Fuji fruit at 15 °C 10 d after exposure to hypoxia had increased concentrations of ethanol, ethyl propionate, ethyl-2-methyl butanoate and acetaldehyde with decreased concentrations of butyl acetate and hexyl acetate. Royal Gala fruit at 10 and 15 °C 10 d after exposure to hypoxia had increased concentrations of ethanol and acetaldehyde. No volatile enhancement occurred in either cultivar at 20 and 25 °C 10 d after treatment.

The acetate esters, butyl acetate, hexyl acetate and 2 methyl butyl acetate tended to decrease with increasing temperature in control fruit. No consistent changes occurred in alcohol concentrations with temperature. Detectable concentrations of some volatiles were found only at specific temperatures; for example, 2 methyl butan-1-ol at 25 °C in FU fruit 2 d after treatment, ethyl propionate at 10 °C, ethyl pentanoate at 10 and 25 °C and pentyl acetate at 10 and 15 °C in RG fruit 2 d after treatment (Table 7.1).

Table 7.1. Concentration of volatiles in juice from Fuji and Royal Gala apples stored in air and 2 d after removal from 100% CO_2. Mean of 4 replicates.

Temperature	10		15		20		25	
Treatment	C[1]	T	C	T	C	T	C	T
				Fuji				
Alcohols				µmol·L^{-1}				
Ethanol	0[3]	**7954**	80	**2633**	80	**4793**	1126	**21110**
Propan-1-ol	194	**289**	484	618	785	790	1186	772
Butan-1-ol	2999	3169	**5215**	4120	4346	4936	3728	2457
Pentan-1-ol	ND	ND	55	62	49	83	73	42
Hexan-1-ol	621	**911**	1006	1420	833	**1348**	698	461
2MB[2]	ND	ND	ND	ND	ND	ND	85	**1293**
Acetate esters								
Ethyl acetate	15	**148**	10	**64**	11	**53**	15	**161**
Propyl acetate	33	31	87	98	81	61	59	33
Butyl acetate	**569**	274	**531**	309	**306**	191	211	100
Pentyl acetate	**21**	ND	29	28	ND	**34**	ND	ND
Hexyl acetate	**187**	99	**190**	131	**124**	69	**85**	30
2MBA[2]	**519**	239	**387**	90	**138**	59	**236**	73
Ethyl esters								
Ethyl propionate	ND	**199**	50	**388**	43	**384**	ND	**364**
Ethyl butanoate	28	**1077**	89	**1427**	61	**1123**	58	**486**
Ethyl pentanoate	**37**	ND	65	56	67	57	ND	ND
Ethyl hexanoate	ND	**543**	ND	**420**	ND	**207**	ND	**78**
E2MB[2]	ND	**145**	ND	**280**	ND	**316**	ND	**203**
Aldehydes								
Acetaldehyde	0	**295**	7	**69**	7	**111**	94	**649**
Hexanal	136	117	231	230	140	209	194	140
trans-2-Hexenal	**794**	528	823	769	783	696	785	524
				Royal Gala				
Alcohols				µmol·L^{-1}				
Ethanol	131	**3005**	0	**3719**	0	**3211**	65	**11548**
Propan-1-ol	55	**101**	88	108	168	**329**	331	318
Butan-1-ol	3931	4152	4300	4477	7159	**10671**	6169	6182
Pentan-1-ol	**40**	ND	42	42	72	**111**	78	70
Hexan-1-ol	548	578	714	630	1188	1393	857	690
2MB[2]	129	170	ND	**165**	**154**	ND	**594**	422
Acetate esters								
Ethyl acetate	8	**85**	9	**81**	9	**61**	10	**96**
Propyl acetate	35	45	16	19	ND	ND	54	29
Butyl acetate	926	969	556	556	623	736	**510**	277
Pentyl acetate	**17**	ND	13	15	ND	ND	ND	ND
Hexyl acetate	306	335	226	202	279	225	**174**	74
2MBA[2]	25	37	25	24	51	39	**54**	ND
Ethyl esters								
Ethyl propionate	32	35	ND	ND	ND	ND	ND	ND
Ethyl butanoate	63	81	21	21	28	**45**	72	81
Ethyl pentanoate	26	32	ND	ND	ND	ND	38	36
Ethyl hexanoate	ND	ND	ND	9	ND	ND	ND	ND
E2MB[2]	ND	ND	ND	ND	ND	ND	ND	ND
Aldehydes								
Acetaldehyde	0	**30**	0	**76**	0	**63**	0	**67**
Hexanal	117	131	142	140	162	178	**173**	102
trans-2-Hexenal	501	501	529	552	669	609	**713**	333

[1]C = stored in air, T = exposed to hypoxia. [2]2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = ethyl-2-methyl butanoate. [3]Numbers in bold in a row for a given temperature are significantly greater at $P = 0.05$. [4]ND = not detected, concentration below 0.1 µL·L^{-1}.

Table 7.2. Concentration of volatiles in juice from Fuji and Royal Gala apples stored in air and 10 d after removal from 100% CO_2. Mean of 4 replicates.

Temperature	10		15		20		25	
Treatment	C[1]	T	C	T	C	T	C	T
				Fuji				
Alcohols				µmol·L^{-1}				
Ethanol	5874[3]	4231	322	**704**	704	**1487**	5985	4879
Propan-1-ol	ND	**598**	1330	1263	3344	2926	10795	10686
Butan-1-ol	3632	3488	4568	3516	3753	4339	6517	7887
Pentan-1-ol	ND	**51**	51	48	79	87	134	165
Hexan-1-ol	751	905	790	618	451	417	599	780
2MB[2]	ND	**1603**	3513	2503	ND	ND	ND	**180**
Acetate esters								
Ethyl acetate	24	25	13	14	14	14	**44**	29
Propyl acetate	52	45	89	82	164	127	286	268
Butyl acetate	445	280	**255**	151	178	151	262	275
Pentyl acetate	ND	ND	30	28	36	37	ND	ND
Hexyl acetate	186	107	**90**	44	51	39	66	68
2MBA[2]	418	247	110	48	51	47	724	619
Ethyl esters								
Ethyl propionate	ND	**129**	59	**110**	130	165	318	384
Ethyl butanoate	38	**503**	141	199	137	188	240	309
Ethyl pentanoate	ND	ND	63	46	78	41	60	35
Ethyl hexanoate	ND	**39**	14	14	23	18	ND	**22**
E2MB[2]	ND	**129**	45	**107**	110	153	380	381
Aldehydes								
Acetaldehyde	9	**47**	28	**57**	71	71	**296**	162
Hexanal	125	147	200	158	210	222	424	646
trans-2-Hexenal	483	595	448	421	667	663	1276	1839
				Royal Gala				
Alcohols				µmol·L^{-1}				
Ethanol	0	**518**	0	**440**	1765	222	415	1035
Propan-1-ol	119	126	580	678	**1110**	471	1020	1080
Butan-1-ol	3887	4081	12359	13182	9424	7958	3422	6089
Pentan-1-ol	ND	ND	85	88	66	**89**	ND	**60**
Hexan-1-ol	471	467	1431	1172	837	708	162	277
2MB[2]	217	184	270	239	311	196	216	221
Acetate esters								
Ethyl acetate	5	19	5	**15**	12	7	6	8
Propyl acetate	25	22	86	89	101	68	49	44
Butyl acetate	894	869	1308	1222	684	454	194	282
Pentyl acetate	ND	ND	13	ND	ND	27	ND	ND
Hexyl acetate	302	253	355	269	139	95	31	39
2MBA[2]	39	28	94	110	**102**	59	73	65
Ethyl esters								
Ethyl propionate	ND	ND	**31**	ND	ND	**45**	ND	ND
Ethyl butanoate	27	26	65	54	44	49	26	26
Ethyl pentanoate	ND	ND	63	53	51	38	ND	ND
Ethyl hexanoate	ND	ND	ND	ND	ND	ND	ND	ND
E2MB[2]	ND	ND	ND	ND	ND	ND	ND	ND
Aldehydes								
Acetaldehyde	0	**44**	0	**24**	14	5	0	15
Hexanal	94	85	239	258	194	218	76	97
trans-2-Hexenal	346	280	894	903	753	727	357	392

[1]C = stored in air, T = exposed to hypoxia. [2]2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = ethyl-2-methyl butanoate. [3]Numbers in bold in a row for a given temperature are significantly greater at $P = 0.05$. [4]ND = not detected, concentration below 0.1 µL·L^{-1}.

7.5 Discussion

In general enhancement of ethyl ester concentration in hypoxic treated fruit decreased as temperature increased. Control volatile concentration increased with temperature in agreement with previous researchers, where volatile production of apples increased with temperature to maximum production at about 20 to 25 °C (Guadagni et al., 1971; Wills and McGlasson, 1971). In RD apples maintained at 10, 19 or 28 °C while exposed to an atmosphere of 130 mM ethanol per kg of apple, ethyl ester accumulation was decreased at 28 °C but enhanced at 10 °C compared to control fruit kept at 19 °C (Berger et al., 1992). The respiration rate of apples increases as temperature increases (Fidler and North , 1971) which might suggest that rates of volatile production from fruit would rise with temperature. Such an assumption may not always be true for all volatiles, as precursors which arise from β-oxidation or transamination may have different temperature optima. Differential changes in specific volatile concentrations at each temperature could also reflect differences in rates of volatilisation (Carelli et al., 1991; Jennings, 1965) rather than changes in rates of volatile biosynthesis (Wills and McGlasson, 1969). This may explain the different patterns of total volatile concentration change over 14 d for each temperature. Volatile concentration increase with temperature for certain compounds (acetaldehyde, ethyl propionate and ethyl-2-methyl butanoate) suggesting that rate of biosynthesis for these compounds increases faster than rates of volatilisation from the liquid to the gas phase. Volatile concentration might decrease with temperature for other compounds (ethyl butanoate and hexyl acetate) where volatile biosynthesis is insufficient to compensate for increased rates of volatilisation. That no change occurs in volatile concentration of hexanal and *trans*-2-hexenal as temperature increases may be because volatile biosynthesis increase at the same rate as increases in volatilisation. A peak in butan-1-ol, hexan-1-ol and pentyl acetate concentration at 15 °C or 20 °C suggests these temperatures may be about optimum for biosynthesis. To better define temperature effects on volatile biosynthesis the relationship of volatile biosynthetic rate with temperature, requires characterisation.

Some researchers have proposed that volatile production and ethylene production are correlated (Bangerth et al., 1998; Song and Bangerth, 1996). Ethylene production rose to a peak at 20 °C for FU and RG apples before declining at 25 °C. This pattern was not observed for the volatiles analysed in this study and suggests that volatile concentration is related to an indirect effect of ethylene on co-ordinating metabolism rather than being influenced specifically by ethylene.

In general, exposing FU and RG apples to hypoxia at each temperature enhanced ethyl esters, fermentation volatiles and decreased acetate esters in agreement with previous results (Chapter 4; Ampun, 1997). Specific changes in volatile concentrations depended on temperature and cultivar, with volatile concentrations following the same patterns of change with temperature as control fruit. The greatest increase in overall concentration of volatiles after exposure to hypoxia was in fruit treated and maintained at 10 °C and this increase lasted for up to 10 d. This would suggest that although the temperature optimum for the ester forming enzyme, alcohol acylCoA transferase, has been determined as being about 30 °C (Sanz et al., 1997) other factors, such as increased volatile evaporation due to

greater rates of volatilisation at warmer temperatures, and limited synthesis of acyl CoA's and alcohols from fatty acids at low temperatures (Sanz et al., 1997), may limit volatile concentration in apple tissue. In banana fruit different biosynthetic pathways for volatiles appear to have different thermal sensitivities resulting in different mixtures of volatile compounds being produced depending on temperature (Mattei, 1975). One example of an enzyme system that may have differential temperature effects on volatile concentration is the fermentation pathway in which ethanol is produced by reduction of acetaldehyde by alcohol dehydrogenase. Alcohol dehydrogenase of Cox's Orange Pippin apples has different substrate specificities for aldehydes (Bartley and Hindley, 1980) and could potentially have different temperature optima for each aldehyde. In grapes and blueberries increases in ethanol concentration were proportional to temperature showing an increasing exponential relationship similar to the Arrhenius function (Saltveit and Ballinger, 1983a; 1983b). It has been postulated that ethanol accumulation at different temperatures could depend on specific anaerobic respiration rates which differ among fruit and cultivars (Ke et al., 1991). The fact that ethanol concentrations in juice from control and FU apples 10 d after exposure to hypoxia were very much greater than in juice from RG apples would suggest that in FU apples the anaerobic respiration pathway remains more active than in RG apples after hypoxia has been removed.

Other evidence for differential temperature effects on volatile concentration is the presence or absence of specific volatiles at some temperatures but not others. In FU apples 2 methyl butan-1-ol was only present 2 d after exposure to hypoxia in fruit maintained at 25 °C. Ethyl propionate was found only in RG apples 2 d after exposure to hypoxia at 10 °C. Pentyl acetate 2 d after exposure to hypoxia was absent in FU apples at 25 °C and in RG apples at 20 and 25 °C. Such differences in volatile composition at different temperatures suggest that rates of synthesis of some volatiles are very temperature dependent. However, temperature optima and substrate specificity of the enzymes involved in volatile formation have not been determined in apple tissue.

7.6 Conclusions

Apples maintained at increasingly warmer temperatures have reduced enhancement of volatile concentrations which may be due to increased rates of volatilisation at higher temperatures and altered rates of production. Exposing FU and RG apples at 10 °C to hypoxia results in apples with greater volatile concentrations than comparable apples maintained at 15, 20 or 25 °C. At 10 °C the decrease in acetate esters was the least while increase in ethyl esters was only slightly less than at warmer temperatures. For maintenance of apple quality, treatment with hypoxia at lower temperatures is desirable. These results suggest that apples treated with hypoxia and maintained below 15 °C would have enhanced volatile concentration.

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Chapter Eight

Coolstorage and stage of ripeness before exposure to hypoxia alters volatile composition of 'Fuji' and 'Royal Gala' apple juice.

Additional Index Words. aroma; flavour; solvent extraction

8.1 Abstract

Apple fruit develop aroma during ripening with maximum aroma volatile production occurring at or after the climacteric. Storage at low temperatures is a commonly used technique to slow ripening of apples but it can affect concentrations and patterns of aroma volatile production. Exposing apples to a brief period of hypoxia is a potential disinfestation treatment which results in enhancement of ethyl esters and decreases in non-ethyl esters. These changes in volatile composition may be affected by ripeness and duration at 0 °C. Noncooled Fuji and Royal Gala apples at preclimacteric to postclimacteric stage of devlopment were exposed to hypoxia and ripened at 20 °C for up to 14 d. A batch of the same fruit were placed at 0 °C, removed to 20 °C and exposed to hypoxia at monthly intervals for up to 5 months. Royal Gala ripened more rapidly than Fuji apples with definitive peak in ethylene production. Exposure to hypoxia decreased carbon dioxide production in Fuji apples at the preclimacteric and rising climacteric stages and at the climacteric peak. Respiration rate, ethylene production and volatile concentration of RG apples were not affected by exposure to hypoxia at any stage of ripeness or period at 0 °C. After exposure to hypoxia Fuji apples had enhanced ethyl esters at the preclimacteric and rising climacteric stages and after being at 0 °C for up to 5 months. Volatile concentrations were lower in apples maintained at 0 °C compared to noncooled apples. That ethyl esters were not enhanced by hypoxia at the climacteric and postclimacteric stages suggested that the volatile biosynthetic pathways were operating at full capacity in untreated fruit. Apples at 0 °C had a greater enhancement of ethyl esters after hypoxia than apples maintained continuously at 20 °C suggesting that exposure to low temperatures did not simply slow volatile biosynthesis due to slowing of the rate of ripening but had an additional effect on volatile biosynthesis. Any prestorage treatment involving brief exposure to hypoxia will induce the greatest enhancement of ethyl esters and least decrease in non-ethyl esters when applied to preclimacteric fruit.

8.2 Introduction

Postharvest ripening in apples is a process involving physical, metabolic and biochemical changes initiated/coordinated by ethylene which includes loss of background colour, softening and development of aroma and flavour (Tressl et al., 1975; Wills et al., 1997). Apples are classed as climacteric fruit where carbon dioxide production increases to a maximum after which the rate declines (Wills et al., 1997). Stage of ripeness can be defined by the rate of associated ethylene production during the respiratory climacteric. Volatile production and the climacteric are correlated where typical aroma/flavour volatiles increase in concentration reaching a maximum at the climacteric peak (Mattheis et al., 1991b;

Sapers et al., 1977; Song and Bangerth, 1994, 1996; Yahia et al., 1990). Apples harvested preclimacteric produce very low concentrations of volatiles while postclimacteric fruit have declining concentrations of volatiles (Brown et al., 1966; Hansen et al., 1992; Song and Bangerth 1996; Vanoli et al., 1995). Early harvested physiologically mature fruit have the same pattern of volatile production as later harvested fruit but at reduced concentrations. During ripening specific volatiles can have one of five patterns of volatile production: continuous decline, steady stable levels, a transient rising climacteric in production followed by a return to previous levels, a steady rising climacteric in production followed by a rapid rising climacteric as fruit pass through the climacteric, and increase once the fruit are postclimacteric (Brown et al., 1966; Mattheis et al., 1991b; Sapers et al., 1977; Yahia et al., 1990).

Apples placed at 20 °C, after storage at 0 °C have, in general, volatile production rising to a peak before declining (Dirinck et al., 1989; Hansen et al., 1992; Vanoli et al., 1995). Storage at low temperatures can affect the pattern of volatile concentration of fruit subsequently removed to 20 °C. For example, total volatile concentration of Cortland and McIntosh apples at 20 °C rose rapidly after harvest. McIntosh apples at 0 °C and 3.3 °C had a slow steady rise in total volatile concentration to about half the concentration at 20 °C (Yahia et al., 1990; Yahia et al., 1991). Total volatile concentration of Cortland apples at 0 and 3.3 °C was about half that at 20 °C, but at the climacteric peak fruit at 0 °C produced about 25% more volatiles than at 3.3 °C (Yahia et al., 1990; Yahia et al., 1991). Specific volatiles had different patterns of production at 0 and 3.3 °C compared to 20 °C. Hexanal concentrations in Cortland apples at 0 °C had the same pattern of volatile production as at 20 °C, but in McIntosh apples, hexanal concentration increased slowly instead of rising to a maximum at the climacteric peak (Yahia et al., 1990). Ethyl butanoate concentrations in Cortland apples at 0 and 3.3 °C rose slowly during storage, but in McIntosh apples ethyl butanoate concentration increased to similar concentrations as at 20 °C (Yahia et al., 1990; Yahia et al., 1991). Ethyl-2-methyl butanoate was not produced at 0 °C in either Cortland or McIntosh apples but was present at low concentrations at 3.3 °C in both cultivars (Yahia et al., 1990; Yahia et al., 1991).

Apples removed to 20 °C after storage at 0 °C for different times produce greater concentrations of volatiles and reached maximum production more quickly than those maintained continuously at 20 °C. After harvest Cox's Orange Pippin apples at 20 °C took 18 d to reach maximum butyl acetate and hexyl acetate concentrations and 27 d for butan-1-ol and hexan-1-ol (Hatfield and Patterson, 1975). On removal to 20 °C after 3.5 months at 3.3 °C it took 4 days for butyl acetate concentration to reach about twice the concentration of noncooled fruit and took 15 days for butan-1-ol to reach a concentration of about 5 times that of noncooled fruit (Hatfield and Patterson, 1975).

Low temperature storage for longer than 3 months may reduce volatile production from apples (Ampun, 1997). After 6 months low temperature storage decreases in aroma associated with reduced volatile concentration are detectable by taste panellists (Plotto et al., 1997). Volatile concentrations were greater after 3 months than 5 months in Golden Delicious apples at 1 °C (Streif and Bangerth, 1988) with

total volatile concentration of Golden Delicious apples at 1 °C being about 50% greater after 3 months than after 8 months at 1 °C (Brackmann et al., 1993). Butyl acetate and hexyl acetate concentrations increased during storage up to 2.5 months at 4 °C before decreasing after 3.5 months in Golden Delicious apples (Bachmann, 1983). Fresh Law Rome and '262' Rome apples at harvest and after 0.5 °C for 3 months had greater ester concentrations than after 6 months at 0.5 °C (Fellman et al., 1993). Reduced ester concentration was associated with reduced ester biosynthesis (Fellman et al., 1993).

Hypoxic conditions applied before controlled atmosphere and/or low temperature storage or at temperatures greater than 20°C induces enhanced concentrations of ethanol and ethyl acetate in apples which may continue to be high throughout storage and shelf life (Mattheis et al., 1991a). This residual effect of hypoxic treatment may cause alcoholic off-flavours (Ke at al., 1991). Exposing Granny Smith and Fuji apples to hypoxia for 24 h at 20 °C after up to 27 weeks at 0.5 °C enhanced acetaldehyde and ethanol and ethyl esters while decreasing concentrations of non-ethyl esters (Ampun,1997). Hypoxia induced enhancements of total ethyl ester concentration decreased by about 40% in Granny Smith apples from 6 weeks to 27 weeks at 0.5 °C (Ampun, 1997). In contrast ethyl esters in Fuji apples were enhanced by hypoxia to approximately the same degree regardless of storage period to 27 weeks (Ampun, 1997).

Brief periods of hypoxia have been investigated as potential disinfestation treatments (Hallman, 1994). Hypoxia may be applied as a disinfestation treatment before or after storage at 0 °C prior to or after transport to market. Low temperature storage will slow apple ripening, therefore if volatile production at different stages of ripeness is affected differentially by exposure to hypoxia, the confounding effects of ripeness and low temperature storage need to be separated. To determine the response of apples to hypoxia at different stages of ripeness and after cold storage Fuji and Royal Gala apples which were noncooled were compared to fruit from the same batch exposed to hypoxia after storage at 0 °C.

8.3 Materials and Methods

8.3.1 Fruit supply

Fuji (FU) and Royal Gala (RG) apple (*Malus domestica* Borkh.) cultivars were assessed for their response to hypoxia. Cultivars were harvested at mid commercial harvest: 7 May 1994 for F, 16 Mar. 1994 for RG as determined by the New Zealand Apple and Pear Marketing Board, in the Hawke's Bay region of New Zealand. Fruit were graded to export standard, packed to count 125 (148g average fresh weight), transported unrefrigerated to Massey University by road, within 3 days of harvest. Upon receipt the fruit was divided in 2 lots. One was maintained at 20 °C ± 3 °C and the other placed at 0 °C ± 1 °C. Fruit at 0 °C were equilibrated to 20 °C ± 3 °C overnight before treatment. Each lot of fruit was then divided into two groups, one was exposed to hypoxia, the other stored in air as controls. For each treatment and sampling time there were 4 replicates of 3 apples each.

8.3.2 Hypoxic treatment

Refer to Section 4.3.2.

8.3.3 Fruit assessment

Fuji fruit were maintained at 20 °C ± 3 °C for up to 54 d and RG fruit for 32 d. On arrival at Massey University FU and RG apples had average internal ethylene concentrations of 0 $\mu L \cdot L^{-1}$ and 0.41 $\mu L \cdot L^{-1}$, respectively. Fuji fruit were exposed to hypoxia at different stages of ripeness or phase of the climacteric based on ethylene production rates. Fuji fruit were exposed to hypoxia after 2 (preclimacteric, 0.02 C_2H_4 $nmol \cdot kg^{-1} \cdot s^{-1}$), 10 (rising climacteric, 0.1 C_2H_4 $nmol \cdot kg^{-1} \cdot s^{-1}$), 28 (at the climacteric peak, 0.29 C_2H_4 $nmol \cdot kg^{-1} \cdot s^{-1}$) and 40 (postclimacteric, 0.29 C_2H_4 $nmol \cdot kg^{-1} \cdot s^{-1}$) d at 20 °C ± 3 °C, RG fruit after 2 (preclimacteric, 0.53 C_2H_4 $nmol \cdot kg^{-1} \cdot s^{-1}$), 6 (rising climacteric, 2.6 C_2H_4 $nmol \cdot kg^{-1} \cdot s^{-1}$), 10 (at the climacteric peak, 4.0 C_2H_4 $nmol \cdot kg^{-1} \cdot s^{-1}$) and 20 (postclimacteric, 0.86 C_2H_4 $nmol \cdot kg^{-1} \cdot s^{-1}$) d at 20 °C ± 1 °C. Fuji fruit were maintained at 0 °C ± 1 °C for 56, 89, 121 or 155 d, and RG were maintained at 0 °C ± 1 °C for 30, 69, 107, 141 or 173 d before removal and equilibrating to 20 °C ± 3 °C before exposure to hypoxia. Fruit were assessed the day before treatment (d -1), on removal from hypoxia (d 0) and 2, 4, 6, 8, 10, 12 and 14 d after treatment during ripening at 20 °C ± 3 °C for CO_2, for ethylene, firmness, mass, volatile concentration of juice and fermentation volatiles in the juice headspace. These sampling times allowed for differences in volatile concentration due to fruit ripening to be accounted for in the data analysis.

8.3.4 Firmness

Refer to Section 4.3.4.

8.3.5 Carbon dioxide and ethylene production

Refer to Section 4.3.5.

8.3.6 Juice preparation

Refer to Section 4.3.6.

8.3.7 Headspace volatiles

Refer to Section 4.3.7.

8.3.8 Juice volatiles

Refer to Section 4.3.8.

8.3.9 Data analysis

Each experiment was conducted as a completely randomised design with sampling during storage as repeated measures. Means and standard errors of the means for

each cultivar and treatment time for headspace and juice volatiles were graphed using an Origin graphics package v. 5 (Microcal Software Inc., USA). Data were subjected to analysis of variance using SAS v. 6.12 (SAS Institute Inc., Cry, USA). Significant main mean effects were separated by Duncan's multiple range test at the 5% level of significance.

8.4 Results

8.4.1 Carbon dioxide and ethylene production, firmness

Carbon dioxide production of noncooled control FU apples depended on stage of ripeness. Peak CO_2 production of preclimacteric and rising climacteric FU apples were similar but tended to decrease in riper fruit (Figure 8.1a). Compared with controls CO_2 production of hypoxic treated FU apples 8 d after removal from hypoxia in preclimacteric fruit decreased, was decreased for rising climacteric fruit generally, at the climacteric peak CO_2 production decreased from 2 d to 4 d and remained unchanged in postclimacteric fruit. Peak CO_2 production of FU fruit from 0 °C was about half that of noncooled FU fruit (Figures 8.1 a and 8.1b). After removal from 0 °C CO_2 production was the same as for fruit maintained at 20 °C regardless of storage period (Figure 8.1b). Exposure to hypoxia decreased CO_2 production in noncooled FU apples but had no effect on FU apples maintained at 0 °C.

In contrast CO_2 production of noncooled RG fruit declined steadily with increasing ripeness (Figure 8.1c) and did not change with duration at 20 °C (Figure 8.1d). The rate of CO_2 production of RG apples from 0 °C was about half that of noncooled fruit even after 14 d at 20 °C. Exposure to hypoxia had no effect on CO_2 production of RG apples maintained at 20 °C or from 0 °C.

In noncooled control FU apples rate of ethylene production rose in preclimacteric and rising climacteric fruit to a steady state at the climacteric peak and postclimacteric stages of ripeness (Figure 8.2a). Ethylene production of preclimacteric fruit exposed to hypoxia was the same as control fruit. Exposure to hypoxia at rising climacteric, at the climacteric peak and postclimacteric ripeness stages reduced ethylene production for 2 d after removal after which ethylene production increased to be the same as control fruit. Control FU fruit from 0 °C had a transitory increase in ethylene production from 2d to 6d after removal that increased with increasing storage period (Figure 8.2b). Ethylene production rates at 20 °C from fruit maintained at 0 °C for 4 and 5 months were about twice that of noncooled apples. Exposure of 0 °C FU apples to hypoxia reduced ethylene production for 2 d after removal after which it increased to be the same as control fruit (Figure 8.2b).

Ethylene production of RG apples was related to ripeness stage rising to a maximum at the climacteric peak at 10 d after which it declined (Figure 8.2c). Slightly enhanced ethylene production occurred at all ripeness stages, 6d to 8d after exposure to hypoxia (Figure 8.2c). Ethylene production of RG fruit maintained at 0 °C was about half that of noncooled RG fruit (Figures 8.2c and 8.2d) with peak production being 2.5 $nmol \cdot kg^{-1} \cdot s^{-1}$ and 4.0 $nmol \cdot kg^{-1} \cdot s^{-1}$, respectively. Ethylene

production increased in control RG fruit maintained at 0 °C when removed to 20 °C, to peak 2 d to 6 d after removal before declining (Figure 8.2d). In fruit from 0 °C ethylene production was less than control fruit for 2 d after removal from hypoxia after which ethylene production rates increased to control rates (Figure 8.2d).

There was no effect of hypoxia on firmness of noncooled or fruit maintained at 0 °C FU or RG apples, except that RG preclimacteric fruit after exposure to hypoxia were firmer than control fruit for 4 d (Figure 8.3). Firmness decreased in both cultivars with increasing ripeness and storage duration.

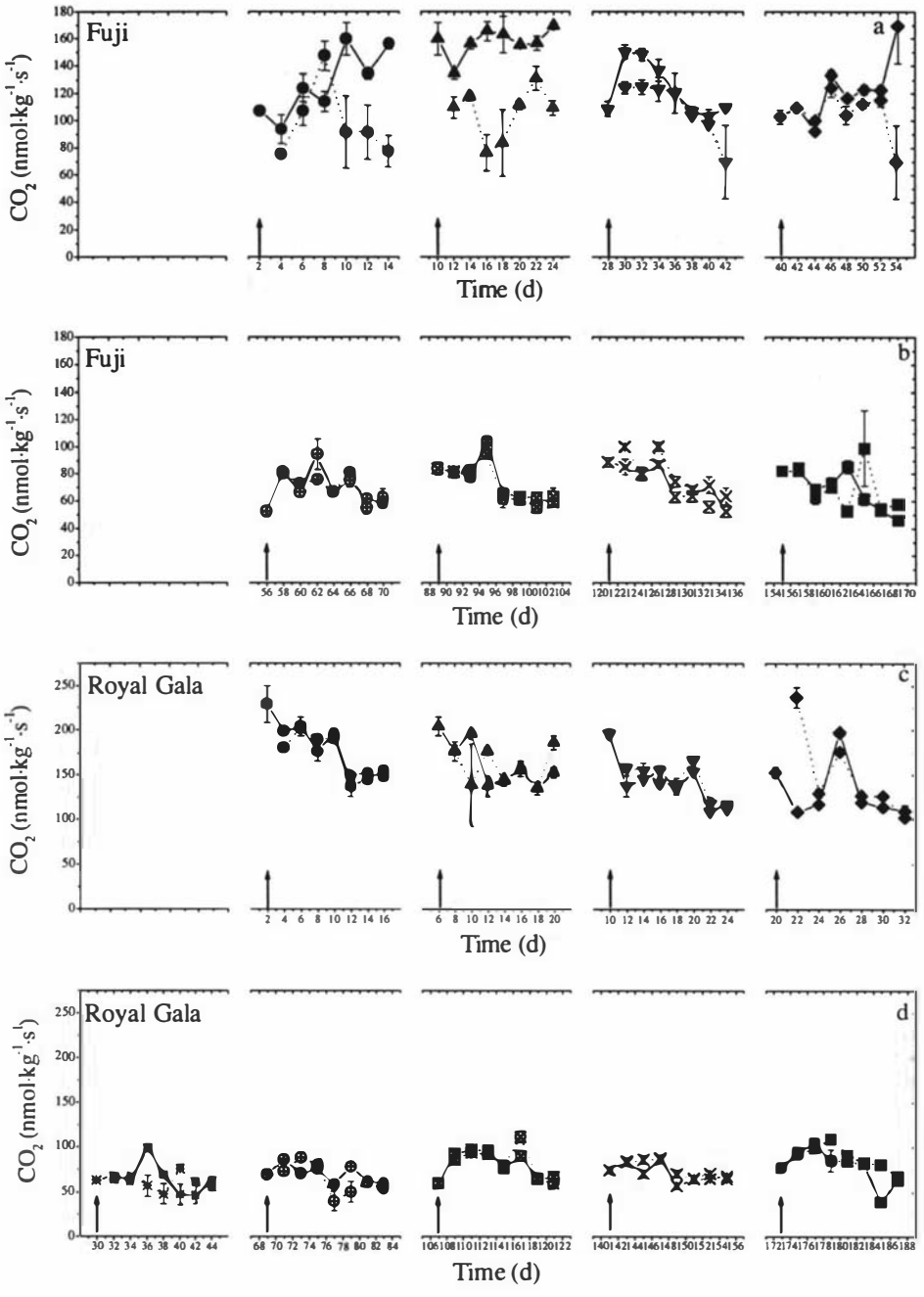

Figure 8.1 Carbon dioxide production from FU (a and b) and RG (c and d) apples treated with air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h at 20 °C. Graphs a) and c) are for apples maintained at 20 °C and treated preclimacteric (●), rising climacteric (▲), at the climacteric peak (▼) and postclimacteric (◆) based on ethylene production. Graphs b) and d) are for apples at 0 °C for 1 month (✳), 2 months (⊕), 3 months (⊗), 4 months (×) or 5 months (■) and then maintained for up to 14 d at 20 °C. Arrows indicate removal from hypoxia. Average and standard errors of 4 replicates.

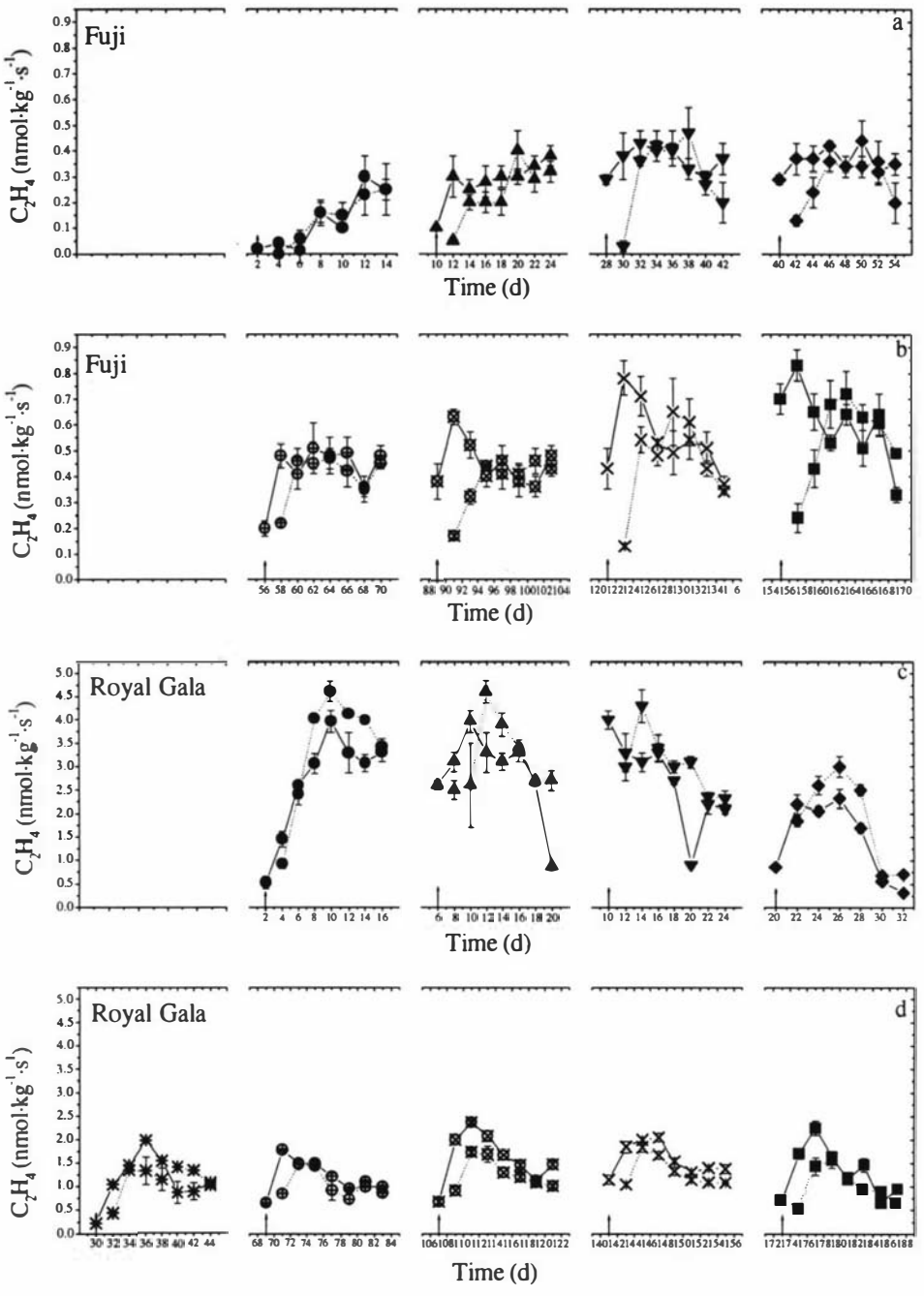

Figure 8.2 Ethylene production from FU (a and b) and RG (c and d) apples treated with air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h at 20 °C. Graphs a) and c) are for apples maintained at 20 °C and treated preclimacteric (●), rising climacteric (▲), at the climacteric peak (▼) and postclimacteric (◆) based on ethylene production. Graphs b) and d) are for apples at 0 °C for 1 month (✳), 2 months (⊕), 3 months (⊗), 4 months (×) or 5 months (■) and then maintained for up to 14 d at 20 °C. Arrows indicate removal from hypoxia. Average and standard errors of 4 replicates.

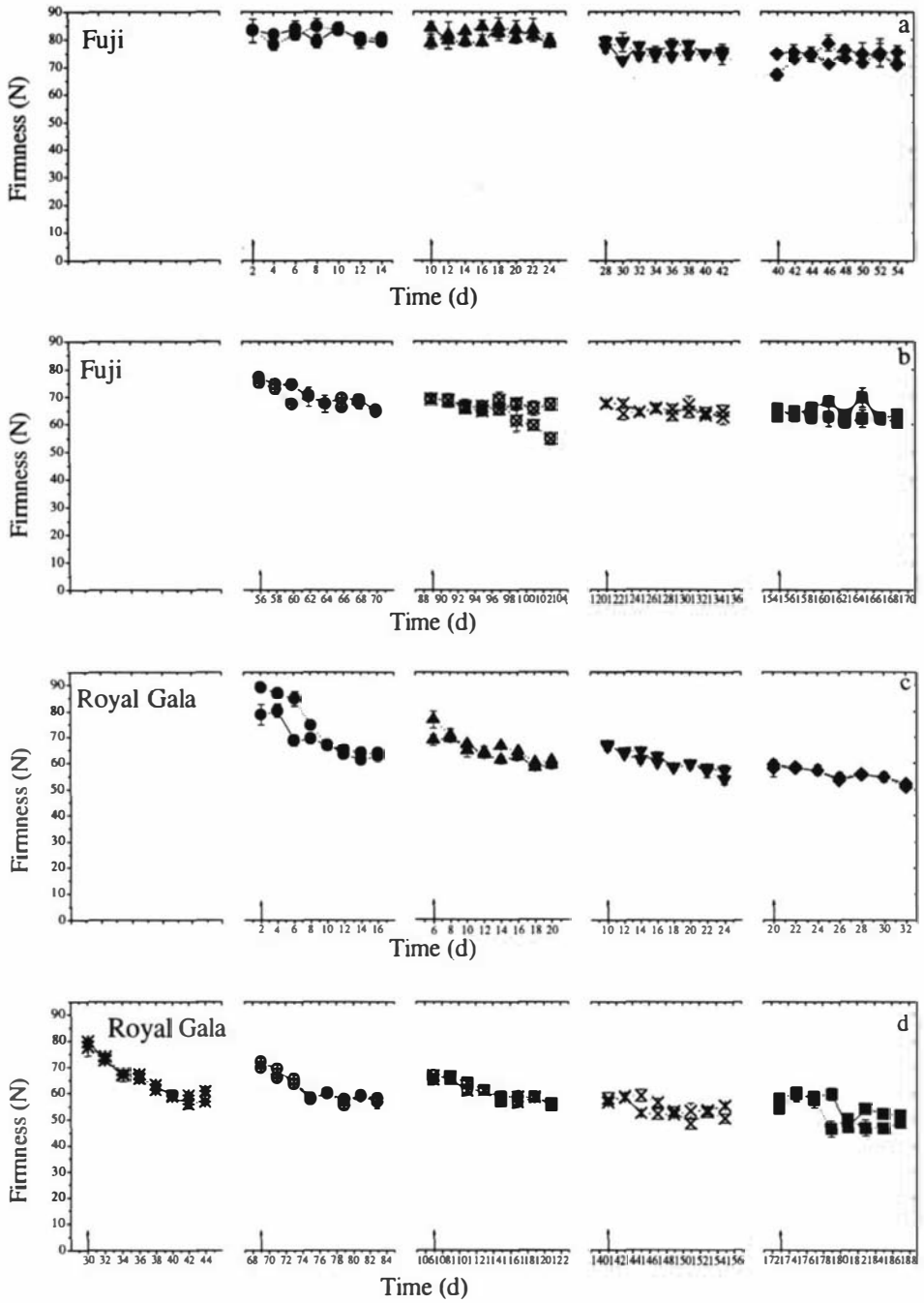

Figure 8.3 Firmness from FU (a and b) and RG (c and d) apples treated with air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h at 20 °C. Graphs a) and c) are for apples maintained at 20 °C and treated preclimacteric (●), rising climacteric (▲), at the climacteric peak (▼) and postclimacteric (◆) based on ethylene production. Graphs b) and d) are for apples at 0 °C for 1 month (∗), 2 months (⊕), 3 months (⊗), 4 months (×) or 5 months (■) and then maintained for up to 14 d at 20 °C. Arrows indicate removal from hypoxia. Average and standard errors of 4 replicates.

8.4.2 Hypoxic effects on ester concentration

Detailed changes in concentration of ethyl butanoate and butyl acetate are presented in Figures 8.4 and 8.5 as representative examples of ethyl esters and acetate esters but 20 volatile compounds were measured, details of these are presented in Tables 8.1, 8.2, 8.3 and 8.4. In noncooled control FU apples ethyl butanoate concentration increased from low concentrations in preclimacteric fruit to peak at the rising climacteric and at the climacteric peak stages before declining in postclimacteric fruit (Figure 8.4a). Enhancement of ethyl butanoate concentration occurred following exposure to hypoxia but depended on stage of ripeness of noncooled fruit. Exposure to hypoxia induced immediate increases in ethyl butanoate concentration which lasted for 8 to 10 d for preclimacteric and rising climacteric fruit but concentrations were unchanged compared to control fruit at the climacteric peak. Postclimacteric fruit had increased ethyl butanoate concentration 8 d to 10 d after removal from hypoxia. Ethyl butanoate concentrations decreased in RG control apples with increasing ripeness (Figure 8.4b) and exposure to hypoxia induced slight decreases in concentration up to the climacteric peak stage.

Butyl acetate concentration in noncooled control FU apples increased with increasing ripeness in a pattern similar to ethylene production (Figure 8.4c). Apart from a slight decrease in rising climacteric fruit and an increase at 8d in fruit at the climacteric peak, exposure to hypoxia did not affect butyl acetate concentration. Noncooled control RG had similar butyl acetate concentrations at the rising climacteric, at the climacteric peak and postclimacteric stages (Figure 8.4d). Butyl acetate concentration in preclimacteric RG fruit increased over 14 d to similar concentrations of other ripeness stages. Butyl acetate concentration was not changed by exposure to hypoxia at any stage.

Ethyl butanoate concentration in control FU apples increased with increasing time at 0 °C (Figure 8.5a). Concentrations of ethyl butanoate in control FU after different times at 0 °C were similar to those of control FU apples at different stages of the climacteric. After 56 d at 0 °C FU fruit had similar ethyl butanoate concentrations to preclimacteric fruit, after 89 d at 0 °C fruit were similar to rising climacteric fruit, after 121 d at 0 °C FU fruit were similar to at the climacteric peak fruit and after 155 d at 0 °C FU fruit were similar to postclimacteric fruit (Figures 8.4a and 8.5a). Concentrations of ethyl butanoate in control FU fruit maintained at 0 °C were similar to noncooled fruit. Exposure to hypoxia induced large increases in ethyl butanoate concentration; the magnitude of this enhancement decreased with increasing storage duration, probably because of the increasing ethyl butanoate concentrations that had occurred in ripening control fruit. Increase in ethyl butanoate concentration, as integrated area under the curve, after hypoxia at 2 months was 13.3 fold, 3 months 8.2 fold, 4 months 2.3 fold and 5 months 5.4 fold. Fruit maintained at 0 °C after treatment with hypoxia had about twice the increase in ethyl butanoate as hypoxic treated noncooled fruit. Ethyl butanoate concentration in RG control fruit was similar regardless of time at 0 °C and there was no influence of hypoxia on ethyl butanoate concentration (Figure 8.5b). Ethyl butanoate concentrations in RG fruit maintained at 0 °C were similar to those of noncooled RG fruit (Figures 8.4b and 8.5b).

Control FU apples from 0 °C had similar butyl acetate concentrations regardless of time at 0 °C (Figure 8.5c). After exposure to hypoxia butyl acetate concentration decreased by about 50% compared to control fruit stored for 89 d or longer at 0 °C. This contrasts with fruit maintained at 20 °C fruit where butyl acetate concentration did not change after hypoxia. In control RG apples butyl acetate concentrations were greater in 107 and 141 d removals than 30, 69 and 173 d removals from 0 °C (Figure 8.5d). Butyl acetate concentration of control RG fruit maintained at 0 °C was about 50% that of control RG fruit maintained at 20 °C (Figures 8.4d and 8.5d). There was no change in butyl acetate concentration of RG apples from 0 °C after exposure to hypoxia (Figure 8.5d).

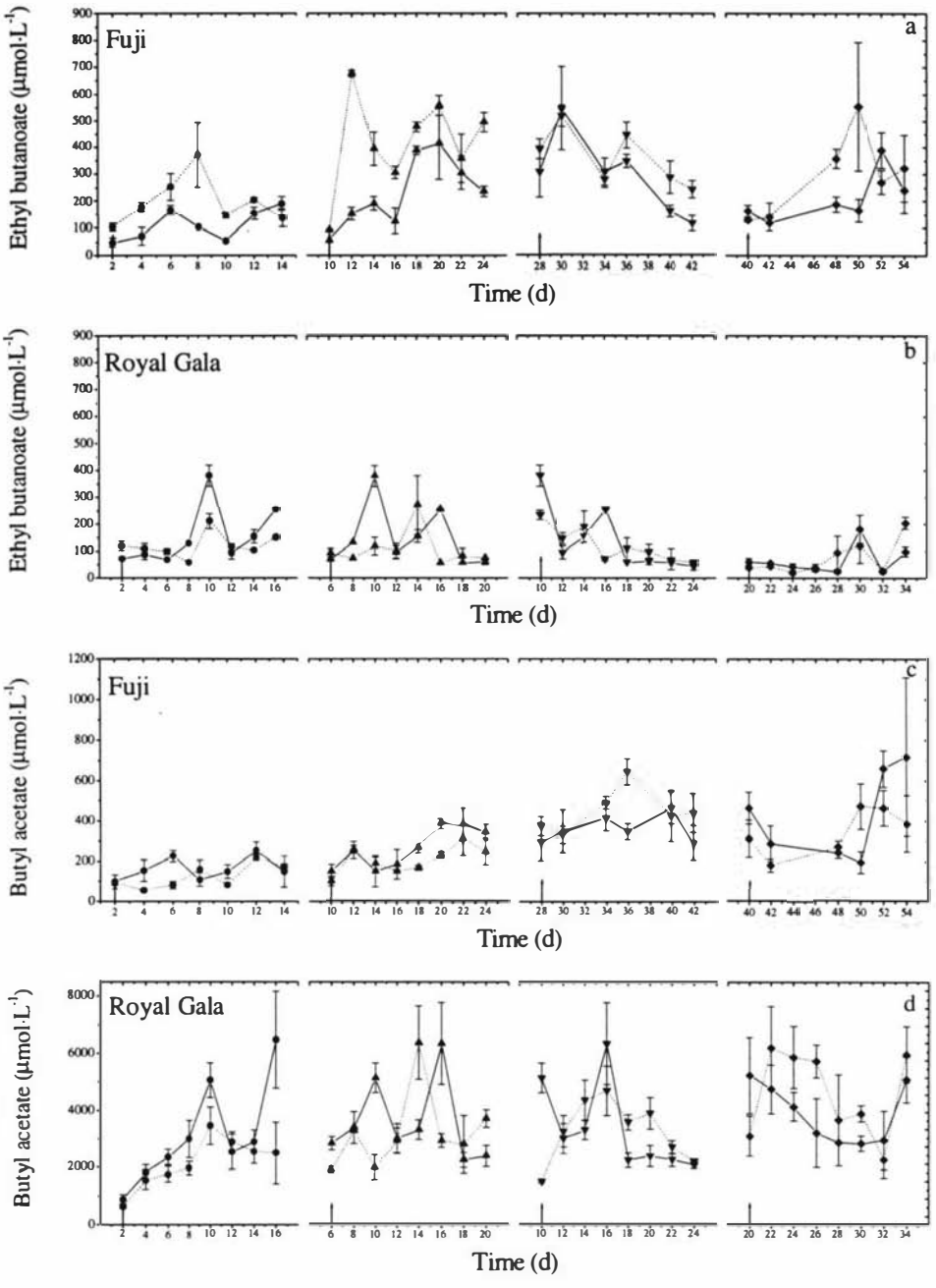

Figure 8.4 Ethyl butanoate and butyl acetate concentration in apple juice from FU (a and c) and RG (b and d) apples treated with in air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h, at 20 °C, preclimacteric (●), rising climacteric (▲), at the climacteric peak (▼) and postclimacteric (◆) stages based on ethylene production and then maintained for up to 14 d at 20 °C. Arrows indicate removal from hypoxia. Average and standard errors of 4 replicates.

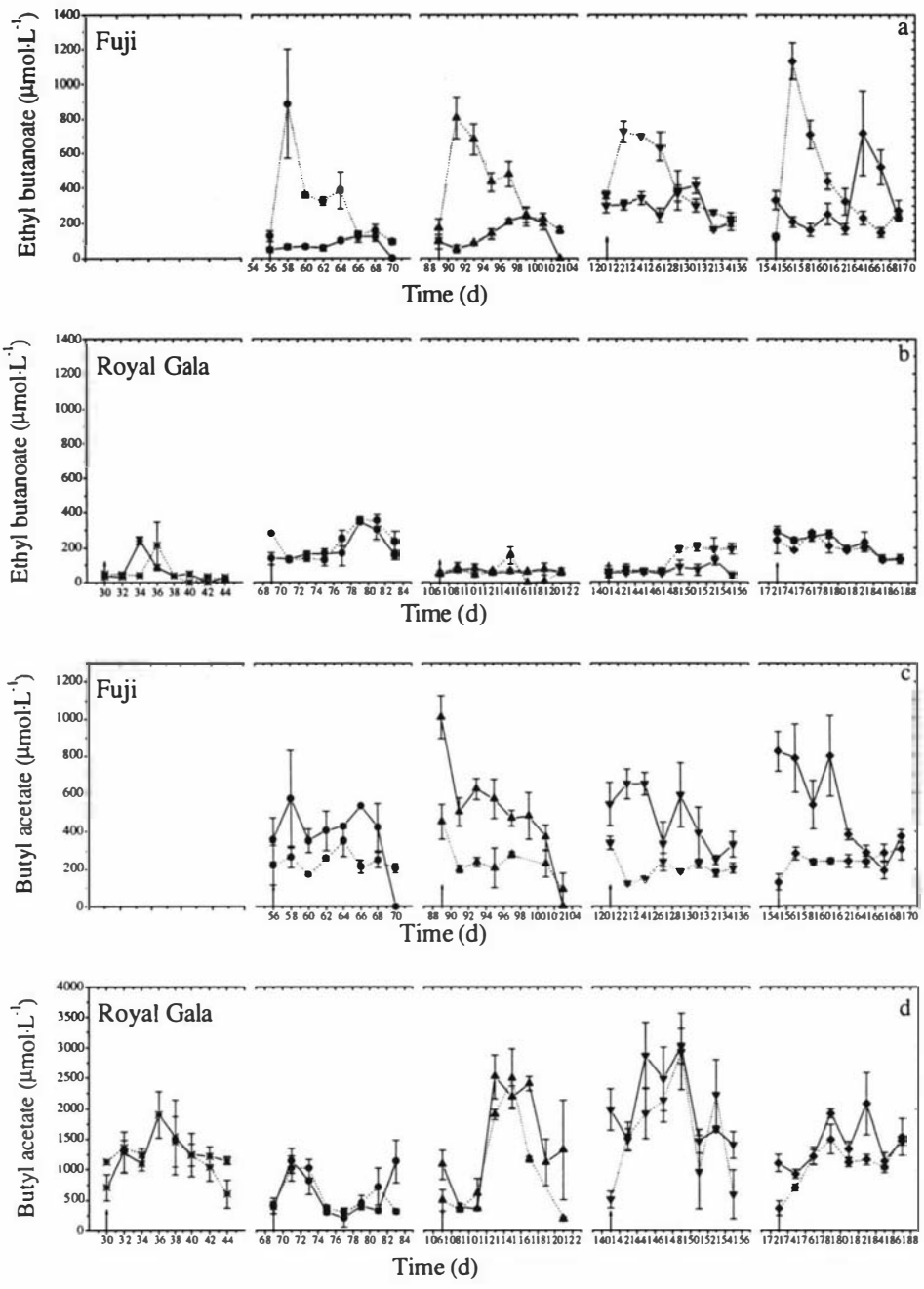

Figure 8.5 Ethyl butanoate and butyl acetate concentration in apple juice from FU (a and c) and RG (b and d) apples treated with air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h at 20 °C, after storage at 0 °C for 1 month (✳), 2 months (●), 3 months (▲), 4 months (▼) and 5 months (◆), then maintained for up to 14 d at 20 °C. Arrows indicate removal from hypoxia. Average and standard errors of 4 replicates.

8.4.3 Total volatile concentration over 14 d ripening

There were 4 patterns of change in total volatile concentration, calculated as the integrated area under the curve of volatile concentration over 14 d, with increasing ripeness for noncooled control FU apples (Figure 8.6). The patterns were: no change in total volatile concentration for hexan-1-ol, hexyl acetate, *trans*-2-hexenal and pentyl acetate with ripening; ethyl butanoate, ethyl propionate rose to maximum concentrations at the climacteric peak before declining in postclimacteric fruit; an increase in total concentration to reach the same concentration in fruit at the climacteric peak and postclimacteric fruit for acetaldehyde, ethyl-2-methyl butanoate and hexanal; an increase in concentration only in postclimacteric fruit for butan-1-ol. Exposure to hypoxia enhanced concentration of ethyl-2-methyl butanoate, hexanal, ethyl butanoate, ethyl propionate, pentyl acetate and acetaldehyde but did not affect concentration of butan-1-ol, *trans*-2-hexenal, hexyl acetate and hexan-1-ol.

Untreated RG apples maintained at 20 °C had 4 patterns of change in total volatile concentration with increasing ripeness, none of which were similar to control FU fruit (Figure 8.6). The patterns were: acetaldehyde increased with ripeness; ethyl propionate and ethyl-2-methyl butanoate were not detected; a decrease in *trans*-2-hexenal as the fruit progressed through to the postclimacteric stage; pentyl acetate increased in preclimacteric fruit through to fruit at the climacteric peak before declining in postclimacteric fruit; similar concentration for preclimacteric and rising climacteric fruit before decreasing for fruit at the climacteric peak and postclimacteric fruit for ethyl butanoate, butan-1-ol, hexanal, hexan-1-ol and hexyl acetate. Exposure to hypoxia induced increased acetaldehyde at stages of the climacteric, decreased hexan-1-ol, hexanal, hexyl acetate at the preclimacteric and rising climacteric stages but had no effect at the climacteric peak and postclimacteric stages as production in both control and hypoxic treated fruit declined. Hypoxia did not effect butan-1-ol, ethyl butanoate, pentyl acetate and *trans*-2-hexenal concentrations.

There were 4 patterns of change for control FU apples at 0 °C in total volatile concentration during storage (Figure 8.7). These patterns were: an increase in concentration with increasing time at 0 °C for ethyl propionate, ethyl butanoate and pentyl acetate; no change occurred for hexyl acetate, hexan-1-ol and butan-1-ol; *trans*-2-hexenal decreased with time at 0 °C; hexanal increased to a maximum after 3 months at 0 °C before decreasing at 4 and 5 months; and acetaldehyde and ethyl-2-methyl butanoate had similar concentrations for up to 4 months before increasing after 5 months at 0 °C. Exposure to hypoxia induced increases in of acetaldehyde, ethyl propionate, ethyl butanoate and ethyl-2-methyl butanoate but a decrease for hexanal, *trans*-2-hexenal, pentyl acetate and hexyl acetate. Hypoxia did not affect butan-1-ol and hexan-1-ol total concentration. As time at 0 °C increased for FU apples the enhancement of ethyl butanoate and ethyl propionate following hypoxia decreased; after 5 months there was no difference between treatments. The opposite pattern was observed for pentyl acetate and *trans*-2-hexenal where there was no difference between untreated and hypoxic treated fruit after 2 months at 0 °C, the decreases became greater with increasing duration at 0 °C.

Royal Gala control fruit from 0 °C had 3 patterns of change in total volatile concentration with increasing duration at 0 °C (Figure 8.7), two being the same as for control FU fruit at 0 °C. The patterns for RG were: no change in concentration of *trans*-2-hexenal, ethyl butanoate, acetaldehyde and ethyl-2-methyl butanoate; concentrations of hexan-1-ol, hexanal, hexyl acetate, butan-1-ol and ethyl propionate at 1 month were similar for 2 months then increased to a maximum at 4 months after which they declined; pentyl acetate was decreased after 2 months at 0 °C but then increased steadily until after 5 months at 0 °C it was the same as in fruit after 1 month at 0 °C.

Royal Gala apples exposed to hypoxia had enhanced acetaldehyde concentration but the treatment had no effect on butan-1-ol, pentyl acetate, ethyl propionate, ethyl butanoate, *trans*-2-hexenal, ethyl-2-methyl butanoate, hexanal and hexan-1-ol. After 1 month at 0 °C hexyl acetate concentration was less in fruit exposed to hypoxia than in control fruit

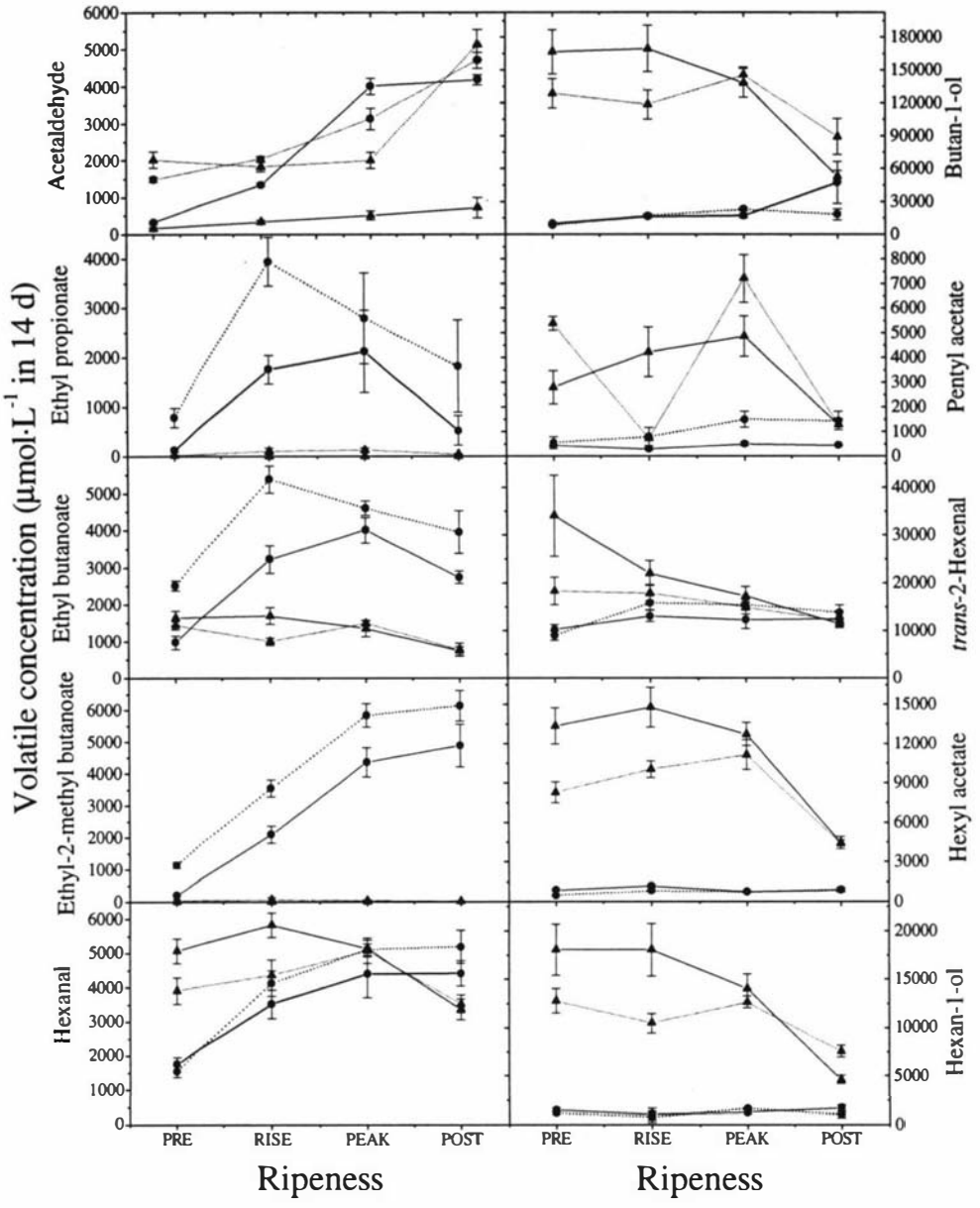

Figure 8.6 Total volatile concentration over 14 d, as area under the curve, of selected volatiles in juice from FU (●) and RG (▲) apples treated with air (—) or exposed to hypoxia (····), 100% CO_2 for 24 h, at 20 °C, at 4 ripeness stages of preclimacteric (PRE), rising climacteric (RISE), at the climacteric peak (PEAK) and postclimacteric (POST), based on ethylene production. Average and standard errors of 4 replicates.

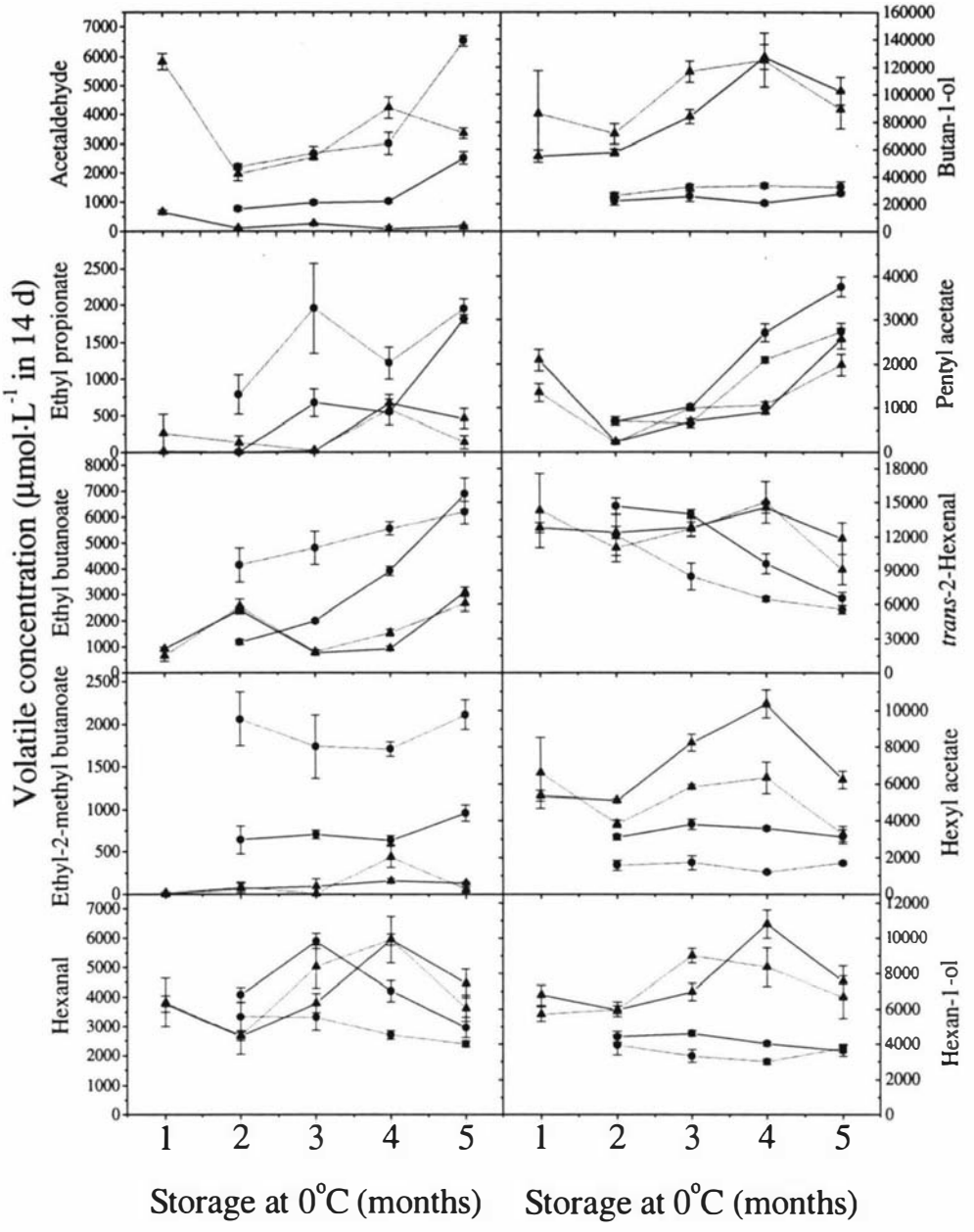

Figure 8.7 Total volatile concentration over 14 d, as area under the curve, of selected volatiles in juice from FU (●) and RG (▲) apples treated with air (—) or exposed to hypoxia (····), 100% CO_2 for 24 h at 20 °C, after transfer from 0 °C to 20 °C for 14 d. Average and standard errors of 4 replicates.

8.4.4 Volatile changes after exposure to hypoxia

Concentration of individual volatiles from FU and RG apples measured 2 d and 8 d after removal from hypoxia for fruit maintained at 20 °C or 0 °C are presented in Tables 8.1, 8.2, 8.3 and 8.4; data is for 2 d after hypoxia the time of maximum volatile enhancement and after 8 d, when the magnitude of the enhancement has declined.

8.4.4.1 Fruit at 20 °C continuously

For control FU and RG apples ethanol concentration was greatest at the climacteric peak compared with other stages at 2 d after removal from hypoxia (Table 8.1). In both FU and RG apples ethanol, ethyl acetate and acetaldehyde increased in fruit exposed to hypoxia compared to control fruit (Table 8.1), with ethanol and ethyl acetate concentrations still enhanced after 8 d at 20 °C (Table 8.2). For FU fruit the absolute enhancement of ethanol and ethyl acetate concentration at 2 d after removal from hypoxia was similar at all stages of ripeness. In FU fruit 8 d after hypoxia the concentrations of ethanol and ethyl acetate remained enhanced in fruit which were preclimacteric, rising climacteric and post climacteric. While the enhancement in concentration of ethanol and ethyl acetate were the same at 2 d after hypoxia the absolute increases were greater in fruit at the climacteric peak and postclimacteric than preclimacteric and rising climacteric fruit (Table 8.1). In RG apples 2 d after exposure to hypoxia the greatest enhancement in ethanol was for preclimacteric fruit and the enhancement decreased as the fruit ripened (Table 8.1). Enhancement of ethyl acetate concentration in RG fruit 2 d after hypoxia was similar for all stages of ripeness. By 8 d after exposure to hypoxia postclimacteric RG fruit had the greatest enhancement of ethanol and ethyl acetate.

Ethyl ester concentrations were generally enhanced in preclimacteric and rising climacteric FU apples at 2 d and 8 d after removal from hypoxia (Tables 8.1 and 8.2) with the greatest increase after 2 d being in rising climacteric fruit. After 8 d some ethyl esters in FU fruit remained enhanced in preclimacteric and rising climacteric fruit but were lower than controls for postclimacteric fruit. Royal Gala apples had no consistent pattern of change in volatile concentration after exposure to hypoxia (Tables 8.1 and 8.2). Ethyl ester concentrations were related to ripeness stage reaching maxima at rising climacteric and at the climacteric peak stages with the lowest concentrations in postclimacteric fruit. Royal Gala apples produced ethyl-2-methyl butanoate only at 2 d after exposure to hypoxia.

In general, acetate ester concentrations in FU and RG apples were unaffected by exposure to hypoxia at 2 d but were greater in control FU apples after 8 d (Tables 8.1 and 8.2). Acetate esters were present in RG apples at greater concentrations than in FU fruit. Concentrations of acetate esters tended to increase with increasing ripeness to a maximum at the climacteric peak after which they declined. Acetate esters in FU apples were similar 2 and 8 d after removal from hypoxia while RG apples had greater concentrations at 8 d than at 2d.

Table 8.1. Volatiles in juice from noncooled Fuji and Royal Gala apples 2 d at 20 °C after removal from 100% CO_2 at different ripeness stages. Mean of 4 replicates.

Climacteric stage	Preclimacteric		Rising		Peak		Postclimacteric	
Treatment	C[1]	T	C	T	C	T	C	T
				Fuji				
Alcohols				µmol·L^{-1}				
Ethanol	370	**11178**	512	**11011**	12970	**27447**	16802	**26859**
Propan-1-ol	303	314	1094	2003	3198	2934	2954	2527
Butan-1-ol	784	306	1108	1546	1544	1443	826	959
Pentan-1-ol	17	14	20	41	31	73	50	26
Hexan-1-ol	112	65	180	253	100	126	51	38
2MB[2]	ND	**73**	**50**	ND	19	16	33	52
Acetate esters								
Ethyl acetate	13	**263**	15	**259**	170	**359**	181	**290**
Propyl acetate	ND	**18**	196	287	661	220	3.0	164
Butyl acetate	152	55	252	256	345	326	288	180
Pentyl acetate	38	69	43	39	ND	**68**	31	244
Hexyl acetate	63	21	95	78	72	52	33	48
2MBA[2]	233	49	382	215	522	265	263	177
Ethyl esters								
Ethyl propionate	19	85	34	**821**	564	1	39	238
Ethyl butanoate	70	**179**	153	**678**	546	519	119	141
Ethyl pentanoate	39	32	51	**107**	31	27	41	43
Ethyl hexanoate	15	**38**	20	**116**	28	69	12	19
E2MB[2]	ND	**72**	47	**372**	392	398	244	215
Aldehydes								
Acetaldehyde	13	**146**	31	130	164	**287**	209	**330**
Hexanal	86	60	262	479	426	304	231	199
trans-2-Hexenal	754	510	996	2012	1226	1041	580	493
				Royal Gala				
Alcohols				µmol·L^{-1}				
Ethanol	289	**31967**	948	**11266**	1271	**12473**	892	**6525**
Propan-1-ol	453	851	1425	1030	1009	1598	1049	1384
Butan-1-ol	10645	11744	13991	11805	8119	10970	4830	7098
Pentan-1-ol	104	168	96	71	**43**	ND	26	41
Hexan-1-ol	1169	1287	1640	1297	967	967	442	634
2MB[2]	174	321	**235**	ND	ND	**261**	ND	**149**
Acetate esters								
Ethyl acetate	5	**305**	9	**259**	11	**362**	ND	**441**
Propyl acetate	82	108	223	238	265	296	275	336
Butyl acetate	1823	1564	2993	2864	2542	2847	1665	2141
Pentyl acetate	**82**	ND	85	63	50	**900**	62	64
Hexyl acetate	616	373	925	783	863	857	464	553
2MBA[2]	537	447	638	475	**662**	80	547	610
Ethyl esters								
Ethyl propionate	**7.0**	ND	4	27	ND	**34**	ND	ND
Ethyl butanoate	88	108	131	73	96	147	56	44
Ethyl pentanoate	122	148	93	92	75	73	73	77
Ethyl hexanoate	45	51	44	31	11	36	36	29
E2MB[2]	ND	**59**	ND	ND	ND	**17**	ND	**21**
Aldehydes								
Acetaldehyde	6	**135**	16	**98**	ND	**95**	67	**319**
Hexanal	288	368	375	270	356	309	273	252
trans-2-Hexenal	2690	2868	2138	1839	**1555**	ND	1041	990

[1]C = stored in air, T = exposed to hypoxia. [2]2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = ethyl-2-methyl butanoate. [3]Numbers in bold in a row for a given ripeness are significantly greater at $P = 0.05$. [4]ND = not detected, concentration below 0.1 µL·L^{-1}.

Table 8.2. Volatiles in juice from noncooled Fuji and Royal Gala apples 8 d at 20 °C after removal from 100% CO_2 at different ripeness stages. Mean of 4 replicates.

Climacteric stage	Preclimacteric		Rising		Peak		Postclimacteric	
Treatment	C[1]	T	C	T	C	T	C	T
				Fuji				
Alcohols				µmol·L^{-1}				
Ethanol	555	**2779**	2965	**5668**	7481	9782	2301	**16725**
Propan-1-ol	416	305	**2381**	1545	4845	6091	101	113
Butan-1-ol	647	535	1273	1178	1790	2322	4483	4375
Pentan-1-ol	11	10	27	29	34	**117**	35	37
Hexan-1-ol	63	87	173	153	131	170	400	402
2MB[2]	23	**71**	26	32	ND	**39**	**83**	58
Acetate esters								
Ethyl acetate	16	**99**	83	**212**	183	**395**	11	**271**
Propyl acetate	**82**	15	**408**	136	**410**	ND	19	42
Butyl acetate	148	83	**265**	166	344	**639**	**939**	697
Pentyl acetate	25	12	16	16	56	62	**135**	129
Hexyl acetate	64	27	**84**	59	77	74	**415**	290
2MBA[2]	184	85	534	254	**580**	379	57	32
Ethyl esters								
Ethyl propionate	12	26	166	206	ND	ND	1	28
Ethyl butanoate	55	**145**	388	**477**	349	448	**240**	186
Ethyl pentanoate	28	24	24	27	72	94	**40**	31
Ethyl hexanoate	8	8	18	**31**	26	37	**12**	ND
E2MB[2]	13	**56**	191	**254**	425	640	**16**	ND
Aldehydes								
Acetaldehyde	24	**63**	123	156	242	316	29	**132**
Hexanal	104	101	366	263	471	502	**202**	121
trans-2-Hexenal	504	520	1211	945	1259	1490	**455**	264
				Royal Gala				
Alcohols				µmol·L^{-1}				
Ethanol	ND	**353**	ND	**374**	2070	1041	1489	**3064**
Propan-1-ol	1553	1444	1605	2781	1131	1712	819	1218
Butan-1-ol	2043	14191	9951	18861	5542	**10123**	3220	4680
Pentan-1-ol	125	82	49	94	33	40	20	25
Hexan-1-ol	2350	1334	972	1556	543	767	259	444
2MB[2]	ND	**116**	380	355	ND	**185**	ND	**77**
Acetate esters								
Ethyl acetate	ND	**79**	1	**41**	15	**101**	ND	**112**
Propyl acetate	305	288	322	701	247	381	219	264
Butyl acetate	5052	3458	2906	6495	1652	**3223**	1051	1307
Pentyl acetate	83	954	664	91	72	**458**	42	47
Hexyl acetate	1664	954	941	1833	538	**803**	230	362
2MBA[2]	949	443	509	1275	520	644	470	452
Ethyl esters								
Ethyl propionate	ND	ND	ND	ND	ND	**10**	ND	**12**
Ethyl butanoate	**378**	210	155	192	59	107	24	95
Ethyl pentanoate	45	35	107	164	77	97	45	47
Ethyl hexanoate	ND	**45**	40	69	**33**	ND	23	12
E2MB[2]	ND	ND	ND	ND	ND	ND	ND	**5**
Aldehydes								
Acetaldehyde	ND	**25**	ND	**12**	**81**	34	60	66
Hexanal	477	396	373	719	249	307	217	289
trans-2-Hexenal	2542	2074	1901	3010	991	1263	744	849

[1]C = stored in air, T = exposed to hypoxia. [2]2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = ethyl-2-methyl butanoate. [3]Numbers in bold in a row for a given ripeness are significantly greater at $P = 0.05$. [4]ND = not detected, concentration below 0.1 µL·L^{-1}.

8.4.4.2 Fruit maintained at 0 °C

In general, ethanol and ethyl acetate increased by similar amounts in FU and RG apples 2 d after exposure to hypoxia regardless of time at 0 °C (Tables 8.3 and 8.4). Ethanol and ethyl acetate enhancement in FU apples was maintained for 8 d after removal from hypoxia the enhancement tending to be greater the longer the time at 0 °C. In contrast RG had enhanced ethyl acetate, but not acetaldehyde and ethanol concentrations 8 d after removal from hypoxia.

Ethyl ester concentrations of FU apples 2 d after hypoxic treatment were generally enhanced by similar amounts regardless of time at 0 °C. After 8 d ethyl-2-methyl butanoate concentration in FU apples remained enhanced at all storage periods unlike ethyl butanoate concentrations which were enhanced for 2 and 3 months but not after 4 and 5 months at 0 °C. Exposure of RG fruit to hypoxia did not influence ethyl ester concentration 2 d after exposure to hypoxia in fruit at 0 °C for up 4 months; after 5 months ethyl ester concentration in fruit exposed to hypoxia was decreased relative to controls. Although ethyl-2-methyl butanoate concentration was detected in RG apples after 3 months for 2 d fruit and after 4 months for 8 d fruit it was not enhanced by hypoxia.

In general, hypoxia tended to decrease acetate ester concentrations of FU apples 2 d after exposure. After 8 d acetate ester concentration tended to decrease after 3 months storage. In RG apples, hypoxia had no consisitent affect on acetate ester concentration at 2d or 8 d after exposure.

Table 8.3. Volatiles in juice from Fuji and Royal Gala apples 2 d after removal from 100% CO_2 after storage at 0 °C for different months. Mean of 4 replicates.

Storage (month)	2		3		4		5		1		2		3		4		5	
Treatment	C[1]	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
	Fuji												Royal Gala					
Alcohols	µmol·L^{-1}												µmol·L^{-1}					
Ethanol	5926	**18126**	372	**8189**	4756	**20100**	1048	**4976**	1037	**13572**	3578	4764	3660	**15685**	1276	**9572**	2301	**16725**
Propan-1-ol	264	587	608	592	323	308	576	513	88	313	1036	260	93	243	126	222	101	113
Butan-1-ol	2886	2232	2522	2353	**2460**	1044	3066	2163	4769	5076	3224	**6054**	5100	8429	6426	9284	4483	4375
Pentan-1-ol	347	82	37	42	26	23	37	39	39	40	32	46	986	852	56	**77**	35	37
Hexan-1-ol	556	506	408	452	404	285	378	476	711	642	359	560	597	950	654	912	400	402
2MB[2]	160	100	70	**227**	**79**	35	99	46	92	96	ND	ND	ND	**20**	ND	**118**	**83**	58
Acetate esters																		
Ethyl acetate	17	**307**	12	**192**	27	**359**	23	**324**	ND[4]	**222**	27	**194**	5	**298**	13	**214**	11	**271**
Propyl acetate	42	109	186	98	59	49	22	75	35	96	27	48	ND	**2**	ND	**52**	19	42
Butyl acetate	576	267	626	239	**653**	123	792	**286**	1287	1366	819	1019	392	347	1484	1546	**939**	697
Pentyl acetate	54	69	79	38	148	134	239	232	118	187	20	14	53	52	41	85	135	129
Hexyl acetate	**375**[3]	130	**376**	129	**458**	96	455	210	551	445	326	326	623	563	706	635	**415**	290
2MBA[2]	**401**	92	230	84	213	25	302	39	84	61	208	112	92	78	475	106	57	32
Ethyl esters																		
Ethyl propionate	1	**489**	2	300	19	204	121	276	ND	**16**	1	11	ND	**1**	82	54	28	1
Ethyl butanoate	67	**886**	84	**683**	310	**728**	208	**1131**	32	47	160	140	67	85	55	75	**240**	186
Ethyl pentanoate	82	89	43	41	**49**	25	**47**	12	115	136	33	20	59	58	138	47	**40**	31
Ethyl hexanoate	24	**345**	7	109	6	**283**	17	**342**	ND	**12**	14	12	22	22	ND	**13**	**12**	ND
E2MB[2]	2	**286**	12	**242**	7	**141**	43	**271**	ND	ND	ND	ND	ND	**13**	21	15	**16**	ND
Aldehydes																		
Acetaldehyde	38	**247**	24	**185**	63	**385**	181	**1335**	28	94	46	75	21	**81**	23	**93**	29	**132**
Hexanal	289	211	401	266	**193**	120	199	147	236	216	219	191	308	230	423	**240**	**202**	121
trans-2-Hexenal	1518	1217	1090	769	**479**	308	505	366	1078	897	846	757	972	707	**947**	563	**455**	264

[1]C = stored in air, T = exposed to hypoxia. [2]2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = ethyl-2-methyl butanoate. [3]Numbers in bold in a row for a given storage duration are significantly greater at $P = 0.05$. [4]ND = not detected, concentration below 0.1 µL·L^{-1}.

Table 8.4. Volatiles in juice from Fuji and Royal Gala apples 8 d after removal from 100% CO_2 after storage at 0 °C for different months. Mean of 4 replicates.

Storage (month)	2		3		4		5		1		2		3		4		5	
Treatment	C[1]	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
	Fuji								Royal Gala									
Alcohols	µmol·L^{-1}								µmol·L^{-1}									
Ethanol	687	**2552**	4824	7638	1077	**6191**	5815	**10240**	389	276	795	**4202**	786	1080	2814	4221	260	**3858**
Propan-1-ol	1429	2034	**1777**	1278	**1994**	936	**1376**	779	508	362	331	543	ND	**879**	481	**1173**	383	446
Butan-1-ol	2063	2750	23236	2188	3119	1703	2108	1938	3631	4627	3108	6365	7923	**13189**	11381	14842	7551	7327
Pentan-1-ol	34	52	30	40	47	29	30	34	29	30	599	534	41	**78**	38	81	57	45
Hexan-1-ol	342	464	271	268	337	185	212	194	467	498	407	525	589	**889**	757	879	547	372
2MB[2]	ND	ND	ND	**45**	88	35	72	50	116	133	ND	ND	ND	**39**	ND	**178**	**93**	65
Acetate esters																		
Ethyl acetate	16	**68**	49	**152**	26	**134**	57	**184**	9	35	ND	**6**	4	**35**	22	**94**	5	**113**
Propyl acetate	ND	**6**	3	145	357	69	177	51	113	130	**4**	ND	**1**	ND	ND	**161**	42	81
Butyl acetate	428	353	**471**	278	594	185	**383**	245	1535	1456	210	299	2195	2493	3025	2934	1346	1128
Pentyl acetate	51	92	77	72	241	153	207	183	75	79	10	**30**	62	**105**	90	95	161	155
Hexyl acetate	152	242	**224**	110	**252**	7	**149**	83	501	532	379	337	728	706	849	670	**378**	220
2MBA[2]	349	436	**498**	157	**543**	141	31	39	269	220	205	233	279	288	345	305	143	100
Ethyl esters																		
Ethyl propionate	0	23	173	266	277	78	149	114	ND	ND	**4**	ND	**2**	ND	91	106	**23**	ND
Ethyl butanoate	102	**387**	208	**479**	388	371	168	324	38	38	169	254	63	153	86	190	194	184
Ethyl pentanoate	67	113	47	49	61	41	43	41	68	71	32	25	77	110	58	60	41	39
Ethyl hexanoate	16	43	11	**52**	15	35	23	28	19	29	ND	ND	27	**45**	17	13	ND	**7**
E2MB[2]	38	**228**	51	**231**	62	**134**	65	**159**	ND	ND	ND	ND	ND	ND	27	10	16	14
Aldehydes																		
Acetaldehyde	48	74	124	171	65	**183**	133	230	33	31	21	21	21	21	31	54	24	**66**
Hexanal	385	401	**545**	340	435	221	232	169	389	382	165	235	373	483	547	667	285	282
trans-2-Hexenal	1109	1294	**1068**	721	988	465	443	348	950	1335	726	993	1228	1675	1343	1603	760	719

[1]C = stored in air, T = exposed to hypoxia. [2]2MB = 2&3 Methyl butan-1-ol, 2MBA = 2 Methyl butyl acetate, E2MB = ethyl-2-methyl butanoate. [3]Numbers in bold in a row for a given storage duration are significantly greater at $P = 0.05$. [4]ND = not detected, concentration below 0.1 µL·L^{-1}.

8.5 Discussion

8.5.1 Carbon dioxide and ethylene production and firmness

Fuji and RG apples had different patterns of carbon dioxide and ethylene production during ripening indicating that these cultivars ripen at different rates. There was a clear respiratory climacteric in FU apples maintained at 20 °C after harvest but there was no corresponding peak in ethylene production (Jobling and McGlasson, 1995). In contrast, noncooled RG apples at 20 °C had a clear peak in ethylene production but no respiratory climacteric. Respiration in FU apples was decreased by hypoxia applied at the preclimacteric and rising climacteric stages and at the climacteric peak but there was no effect on carbon dioxide production of RG apples. Such effects have been observed previously where exposure of preclimacteric fruits to high carbon dioxide concentration had no effect or caused only slight reduction in respiration, while a significant reduction was observed for climacteric fruits producing ethylene (Kubo et al., 1989). In FU apples hypoxia may suppress respiration by inhibiting ethylene production through reduced *in vivo* ACC synthase activity (Gorney and Kader, 1997). Once ripening has commenced, suppressive effects of hypoxia on ripening are not as great as for preclimacteric fruit (Solomos, 1993). It is also possible that hypoxic conditions might reduce activity of the TCA cycle by inhibiting mitochondrial activity and divert pyruvate to the fermentation biosynthetic pathway (Mathooko, 1996); exposure to hypoxia could possibly induce pyruvate decarboxylase and alcohol dehydrogenase that would compete with pyruvate dehydrogenase for pyruvate. Once fruit are postclimacteric, both the TCA and fermentation pathways would be fully active with hypoxia having no effect on respiration. Carbon dioxide production was unaffected in FU and RG apples maintained at 0 °C before exposure to hypoxia. This suggests that for FU and RG that these fruit are equivalent in ripeness to preclimacteric or postclimacteric apples.

The pattern of ethylene production was different for FU and RG apples. Each cultivar had different changes in their pattern of ethylene production after exposure to hypoxia. In preclimacteric FU apples maintained at 20 °C, ethylene production was unaffected by hypoxia, whereas in fruit at later stages of the climacteric, and after being held at 0 °C, ethylene production was initially decreased before rising to rates similar to control fruit. Such general effects of high carbon dioxide and low oxygen on ethylene production have been observed previously for apples (Chaves and Tomás, 1984; Oetiker and Yang, 1995). Exposure of RG apples to hypoxia induced slightly enhanced ethylene production at all stages of ripeness. This contrasts with ethylene production of Golden Delicious apples exposed to hypoxia where treated fruit had greatly increased ethylene production after treatment (Pesis et al., 1994). Untreated FU apples maintained at 0 °C had ethylene production rates similar or greater than those for fruit at the climacteric peak and postclimacteric stages suggesting that these fruit had ripened during coolstorage. This implies that for control FU apples maintained at 0 °C their volatile concentrations should be similar to that of fruit at the climacteric peak and postclimacteric fruit maintained at 20 °C continuously as they are at the same ripeness. A possible reason for increased ethylene

production in FU apples at 0 °C is that low temperatures may induce ACC synthase synthesis and enhanced ethylene production in fruit transferred from 0 to 20 °C, in a manner similar to Granny Smith apples (Jobling et al., 1991). Ethylene production rates for control RG fruit after removal from 0 °C was about half that of apples maintained at 20 °C after harvest which suggests that low temperature inhibition of ethylene synthesis was maintained even when fruit were returned to 20 °C. For RG apples low temperatures do not seem necessary for induction of ACC and ethylene synthesis but low temperatures may have inhibited ACC oxidase activity which reduced ethylene production (Jobling et al., 1991).

Firmness of FU and RG apples was unaffected by exposure to hypoxia in noncooled or fruit maintained at 0 °C. As noncooled apples ripened they softened at different rates with RG apples softening faster than FU apples. The different rates of softening may reflect different rates of ethylene production from these two cultivars where ethylene production of RG apples was about 9 times greater that of FU apples.

8.5.2 Aroma concentration in apples maintained at 20 °C continuously and at 0 °C

Concentration of FU and RG apple aroma volatiles developed during ripening with volatile concentration increasing as the respiratory climacteric progressed in agreement with previous research (Bachmann, 1983; Brown et al., 1966; Fan et al., 1997; Rhodes, 1980; Song and Bangerth, 1994; Vanoli et al., 1995). Ester and alcohol concentrations rose and peaked with the respiratory climacteric in FU apples or with ethylene production in RG apples. Volatile concentrations reached a maximum during the climacteric but not all compounds reach maximum concentrations at the same time (Hansen et al., 1992). For example, in untreated FU apples, highest concentrations of butan-1-ol occurred at the climacteric peak, hexyl acetate at the rising climacteric stage and acetaldehyde at the postclimacteric stage (Table 8.1). Untreated RG apples had the highest concentrations of butan-1-ol and hexyl acetate at the rising climacteric stage and acetaldehyde peaked in postclimacteric fruit (Table 8.1). Concentrations of different volatile types found in the juice changed as the apples ripened. Such a result has been noted with RG apples where alcohol and aldehyde production was lowest for the most mature fruit even though ester production was unaffected by ripeness (Mattheis et al., 1998). Ester forming activity is thought to be sensitive to stage of harvest maturity with acetate ester concentrations increasing about 2 fold from onset of the climacteric to the postclimacteric stage (Fellman and Mattheis, 1995). Late harvested Jonagold apples had increased concentrations of esters with C_2 to C_4 acid moieties compared to early harvested fruit (Girard and Lau, 1995). Late harvested Starkspur Golden apples had higher concentration of ethyl esters, butanoate esters and alcohols and lower concentrations of aldehydes and acetate esters than early harvested fruit (Vanoli et al., 1995). Such changes in ester forming activity were associated with increasing alcohol acylCoA transferase (AAT) activity as apples pass through the climacteric (Fellman and Mattheis, 1995).

The patterns of volatile production at different stages of the climacteric may be due to availability of substrates for volatile synthesis rather than *de novo* induction of enzymes required for volatile synthesis. That the concentration of ethanol and acetaldehyde increase during ripening in control fruit at 0 °C and fruit at 20 °C continuously suggests that the fermentation pathway is active and may be associated with physical changes in the fruit as they ripen such as restriction of gas movement by blockage of air spaces induced by cell wall breakdown. Aerobic production of ethanol has been noted in some apple cultivars (Knee, 1971). Butanal added to preclimacteric Golden Delicious apples was converted to butan-1-ol and butyl esters, suggesting that low substrate availability limited ester formation rather than absence or low activity of enzymes (Song and Bangerth, 1994). Ethyl-2-methyl butanoate and 2 methyl butan-1-ol were absent in untreated preclimacteric FU apples 2 d after removal from treatment and increased as fruit ripened while ethyl-2-methyl butanoate was induced by hypoxia in RG apples. Golden Delicious apples contain different proportions of fatty acids at different stages of the climacteric, the concentration of which increase as the climacteric advances and correlate closely with aroma production (Song and Bangerth, 1994). This suggests that different metabolic processes and/or reactions are consecutively operating as fruit progress through the climacteric.

Aroma production of FU and RG apples studied here did not correlate with ethylene production which agrees with similar findings for many apple cultivars (Brackmann and Streif, 1994) but did follow the climacteric with maximum volatile concentration at the climacteric peak. This is in contrast to Golden Delicious apples for which total volatile production was temporally related to the climacteric rise in CO_2 and ethylene production (Song and Bangerth, 1996). Apples picked physiologically immature went through a climacteric and produced aroma volatiles after 36 d at 20 °C while preclimacteric physiologically mature fruit entered the climacteric after only 3 d at 20 °C (Song and Bangerth, 1996). Therefore the increase in ethylene production found during the climacteric may be required for initiation of aroma volatile production. This is likely to be a general effect of metabolism increase and not a specific effect of ethylene as both straight and branched chain volatiles increase at the same time even though their precursors have different metabolic origins (Song and Bangerth, 1996).

Fuji and RG apples at 20 °C after being at 0 °C, had a reduced metabolic rate, ethylene production and volatile concentrations compared with apples maintained at 20 °C after harvest. Keeping apples at 0 °C also reduced variation in volatile concentrations and carbon dioxide production between replicates compared to fruit maintained at 20 °C from harvest, an effect that has been noted for a number of cultivars even after 3 days at 0 °C (Brown et al., 1966). The reasons for these differences in fruit maintained at 20 °C or 0 °C are not known but suggest that maintaining FU and RG apples at 0 °C affects fruit metabolism more than merely by slowing the rate of ripening.

There was a tendency for total volatile concentrations of specific compounds to decrease with increasing duration at 0 °C in FU apples while in RG apples they

increased for up to 4 months before decreasing at 5 months. Such differences in volatile concentration between cultivars in relation to length of time at 0 °C have been observed previously for Law Rome apples which had increasing concentration of total acetate esters after 3 months at 0.5 °C while 262 Rome apples had decreasing concentration (Fellman and Mattheis, 1995). Golden Delicious apples at 4 °C for 3.5 months had less volatiles than apples stored for less than 3.5 months (Bachmann, 1983). These differences were associated with decreased AAT activity as time at 0 °C increased (Fellman and Mattheis, 1995). While the sum of volatiles increase or decrease with storage duration, different types of volatiles have different trends of change. In FU apples, acetate and ethyl ester concentrations continue to increase during 5 months at 0 °C, unlike alcohol and aldehyde concentrations that tend to decrease. In RG apples the effect of low temperature on volatile concentration had the same general pattern as in FU apples. In FU and RG apples at 0 °C, the activity of biosynthetic pathways providing substrates for ester biosynthesis, such as β-oxidation and transamination, may be reduced more than AAT activity. This suggests that ester biosynthesis *per se* could be unaffected by time at 0 °C but a steady decline in alcohol and aldehyde biosynthesis and/or concentration would lead to a steady decline in substrates required for ester formation.

8.5.3 Effect of hypoxia

In agreement with previous findings (Ampun, 1997) exposing FU apples to hypoxia resulted in enhancement of ethyl esters whereas the same treatment did not alter acetate and ethyl esters in RG fruit. Royal Gala apples may lack the necessary enzymes for synthesising ethyl esters but contain enzymes for synthesising non-ethyl esters as concentrations of substrates for ester formation appear not to be limiting. As there were no changes in acetate and ethyl ester concentrations of RG apples the following discussion concentrates on effects of hypoxia on FU apples. In general, the greatest increase in ethyl esters occurred 2 d after exposure to hypoxia when ethylene production was decreased; this suggests that ethylene production has no direct effect on ester biosynthesis. The magnitude of ethyl ester enhancement and decrease in non-ethyl esters in FU was greater in fruit from 0 °C than it was from fruit maintained at 20 °C after harvest. This suggests that in FU apples, low temperatures not only slow ripening rate, but also induce changes in the capacity of fruit to produce esters. It is possible that at 0 °C there is a change in activity of AAT, fermentation enzymes (PDC and ADH) or in precursor biosynthetic pathways, such as, β-oxidation.

At different phases of the climacteric FU apples have different capacities to produce esters as the greatest enhancement of ethyl esters, induced by hypoxia, took place in fruit just prior to the climacteric and the least enhancement occurred in fruit at the climacteric peak and in postclimacteric fruit. At these more advanced stages of ripeness of untreated FU apples, it is probable that activity of ester forming enzymes was higher and/or the biosynthetic pathways for precursors were more active than in more immature fruit. Untreated preclimacteric fruit produce low concentrations of ethyl esters possibly due to a lack of ethanol. Exposure to hypoxia at this stage induces large increases in

ethanol, acetaldehyde and ethyl esters indicating that the enzymes and co-factors for volatile biosynthesis are present and active. At later developmental stages the volatile biosynthetic pathways might be saturated, or operate at maximum capacity; adding more precursors, such as a large increase in ethanol concentration induced by hypoxia, would not effect volatile production as other factors, such as acyl CoA's, may be limiting.

Exposure to hypoxia induces significant increases in ethyl esters depending on stage of ripeness but irrespective of duration of time at 0°C. Therefore preclimacteric FU apples or those maintained at 0 °C then treated with hypoxia could be expected to have altered aroma while RG apples treated at any phase of the climacteric will have unchanged aroma.

8.6 Conclusions

The magnitude of enhancement of ethyl esters in preclimacteric apples exposed to a 24 h hypoxic treatment is more affected by stage of ripeness than by duration at 0 °C. Once apples have reached the climacteric, enhancement of ethyl esters induced by hypoxia is much reduced. Fuji apples maintained at 0 °C had greater enhancement of ethyl esters after exposure to hypoxia than fruit maintained at 20 °C after harvest but the reason for this is not known. These results suggest that when hypoxia is used as a disinfestation treatment, it will have the greatest effect on production of volatiles when applied to preclimacteric fruit or after fruit has been at 0 °C. Exposure to hypoxia for RG apples does not affect concentration of aroma volatiles, suggesting that the volatile biosynthetic pathway in this cultivar is different from that in FU apples.

8.7 References

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Chapter Nine

General Discussion

This thesis forms the second of two parallel PhD studies investigating the enhancement of aroma volatiles in apples after exposure to a brief period of hypoxia. The first study was by Dr. Wirut Ampun and was completed in 1997. In his thesis Dr. Ampun reported on experiments investigating the efficacy of Tenax® traps and solvent extraction for extracting volatile compounds from apples, the effects on volatile enhancement after using different concentrations of carbon dioxide, nitrogen or an ethylene treatment, duration of exposure to hypoxia, differential responses of apples to hypoxia after storage in air or controlled atmospheres, and responses of Braeburn, Cox's Orange Pippin, Granny Smith, Fuji, Pacific Rose, Red Delicious and Royal Gala apple cultivars to hypoxia. Therefore these aspects of treating apples with hypoxia are not reported in this thesis but the interested reader is encouraged to refer to Dr. Ampun's thesis.

In this thesis compromises were made with respect to the method used to extract and analyse volatile compounds from apples and a selective number of volatile compounds have been reported in the results. The solvent extraction method is not the perfect method for volatile extraction (Chapter 3) but was suitable for batchwise processing of large numbers of samples which allowed time for a reasonable number of sample replicates to be analysed. Solvent extraction is also a relatively cheap and simple method which does not require expensive equipment. The volatiles measured were selected according to their abundance in apple juice, reported importance to apple aroma (Dimick and Hoskin, 1983; Cunningham et al., 1986; Rizzolo et al., 1989) and their relationship to one another in the ester biosynthetic pathway (Dimick and Hoskin, 1983; Sanz et al., 1997). The types of volatile compounds measured in solvent extracts were analysed by GLC-Mass Spectroscopy at the beginning of each apple season to confirm their identity and to allow authentic compounds to be used as external standards. The twenty volatile compounds that are referred to in this study are the fermentation volatiles, C_3 to C_6 alcohols and their acetate esters, ethyl esters to ethyl hexanoate, aldehydes and one branched chain alcohol and its esters. Therefore, a number of volatile compounds which may be important to apple aroma were not measured, and in experiments where alcohol precursors were added to tissue disks, the full range of volatiles that may have been enhanced or suppressed were not measured, i.e. after addition of butan-1-ol only butyl acetate was measured but butyl propionate, butyl butanoate and butyl hexanoate were not.

Twenty volatile compounds were measured in each sample analysed. For a small experiment with 8 sampling times, 4 replicates, 2 treatments and at least 2 cultivars, this involved at least 2560 measurements to analyse. These large numbers of measurements presented a challenge for presentation and analysis of

results. Multivariate analysis, such as Principal Components, was suggested as one method for condensing what could be very large numbers of graphs; for example, twenty compounds could each be plotted against time, or presented in large cumbersome tables. The use of multivariate analysis was rejected as many volatile compounds are highly correlated with one another, meaning that there was nothing new reported in the analysis, and most volatile compounds do not have linear relationships to the various treatments investigated, the latter being an essential prerequisite for most types of multivariate analysis. A selection of volatile compounds assumed to be representative of a group of similar compounds was used for analysis of treatment effects. For example, ethyl esters were used to represent the acyl CoA moiety of butyl- and hexyl- compounds; other compounds were selected as having important sensory characteristics or illustrated aspects of the ester biosynthetic pathway. By presenting data in this way, an attempt has been made illustrate the effect that exposing apples to a brief period of hypoxia has on composition of volatile compounds found in apple fruit.

9.1 Background

Hypoxic conditions induced by high concentrations of CO_2 or N_2, induced fermentative metabolism thereby increasing endogenous concentrations of acetaldehyde and ethanol in a range of fruit; reduced fruit softening, as well as CO_2 and ethylene production (Ampun, 1997; Pesis et al., 1994; Ke at al., 1991). Apples, oranges and grapes exposed to hypoxia for a brief period (24 h) had increased concentrations of esters synthesised from ethanol and decreased concentrations of esters synthesised from other alcohols (Ampun, 1997; Pesis and Avissar, 1989; Shaw et al., 1991; Tesnière et al., 1989). These changes in volatile concentration have been associated with better flavoured fruit in informal taste tests (Ampunpong, 1991; Pesis et al., 1994; Shusiri, 1992). Although many chemical compounds, from a large number of chemical classes emanate from apples, esters, alcohols and aldehydes are present in the greatest concentrations (Paillard, 1990). However, only a few are thought to be important in the sensory perception of aroma (Cunningham et al., 1986; Paillard, 1990; Plotto, 1998). The description of aroma characteristics varies greatly for individual compounds which do not necessarily correlate with odour thresholds (Plotto, 1998). People can smell compounds at concentrations much lower than can be measured by GLC techniques (Heath and Reineccius, 1986). There are volatile compounds which remain to be identified which may be important in defining apple aroma (Cunningham et al., 1986; Plotto, 1998). The distinctive aroma of each cultivar is thought to be related to different proportions of each volatile compound rather than a few 'key' character impact compounds (Grosch, 1994; Paillard, 1975). To date about 13 compounds have been positively identified as contributing to apple aroma character (Cunningham et al., 1986; Plotto, 1998). It is very difficult to assess how a change in concentration of one or several compounds will affect perception of aroma due to synergistic, antagonistic and neutralisation effects (Plotto, 1998; Young et al., 1996). Exposing apples to a brief period of hypoxia changes volatile concentration, altering their proportions and may affect the perception of aroma and flavour (Ampun, 1997).

Apple cultivars have different proportions of volatile compounds allowing classification as 'ester' or 'alcohol' types according to concentration of their principal volatile chemical class (Paillard, 1990). These cultivars may have different changes in volatile concentration after exposure to hypoxia due to possible differences in enzyme substrate specificity or to different rates of biosynthesis of major precursors to volatile compounds (Ampun, 1997). If the biosynthetic pathways for volatile synthesis are similar between apple cultivars, then exposure to hypoxia should induce similar changes in volatile concentration in a wide range of apple cultivars. Some apple cultivars produce low concentrations of ethyl esters during ripening (Ampun, 1997; Chapter 4), suggesting that such cultivars may not have the same relative increase in ethyl esters after exposure to hypoxia as cultivars which produce high concentrations of ethyl esters.

The general hypothesis of the research presented in this thesis is that exposure to hypoxia induces a large increase in ethyl esters and decreases in non-ethyl esters due to a large increase in ethanol concentration from anaerobic respiration competitively inhibiting non-ethyl ester production. By examining changes in volatile concentrations of apples at different stages of ripeness, at different temperatures, after different duration at 0 °C and from a wide range of apple cultivars, after exposure to hypoxia, it may be possible to elucidate the mechanisms for volatile change. In addition providing substrates for the formation of volatiles to untreated apples and apples exposed to hypoxia should allow inferences to be made as to the biochemical nature of changes in volatile biosynthesis. Such experiments should increase our understanding of how volatiles are synthesised in apples and how changes in the environment around apples may influence volatile composition.

9.1.1 Conceptual model of volatile biosynthesis

The conceptual model of ester biosynthesis has been derived from the literature and is used for interpreting volatile changes in experiments reported in this thesis (Figure 9.1). Biosynthesis of the aldehydes hexanal and *trans*-2-hexenal was considered to come from oxidation of linoleic and linolenic fatty acids during homogenisation of apple fruit (Sanz et al., 1997). These are commonly referred to as secondary aroma compounds as they are not normally synthesised by intact fruit (Sanz et al., 1997). Results presented in this thesis do not contradict this conceptual model or add new pathways and branch points but highlight the importance of ADH and AAT to ester formation.

The model of ester biosynthesis used is based on the fermentation pathway and has ADH and AAT as key enzymes involved in ester formation. The primary sources of substrates for ester formation are lipids, proteins and carbohydrates. Lipids provide the main source of straight chain volatile precursors greater than three carbons in length and produce most of the acyl CoA compounds; proteins are the main source of branched chain alcohols and acyl CoA's and carbohydrates supply ethanol and acetyl CoA (Sanz et al., 1997). The biosynthetic pathways leading to acyl CoA's, aldehydes and alcohols are assumed to occur in mitochondria (Mathews and van Holde, 1996) with the initial breakdown

occurring in other organelles, peroxisomes for β-oxidation of fatty acids (Gerhardt, 1983), secondary lysosomes for transamination of proteins with gylcolysis of carbohydrates taking place in the cytosol (Mathews and van Holde, 1996).

The start of the basic pathway for ester formation was assumed to be conversion of aldehyde to alcohol by ADH (Mathews and van Holde, 1996). Alcohol dehydrogenase is best known as an enzyme which reduces acetaldehyde to ethanol but it also has significant activity with a wide range of other aldehydes (Bartley and Hindley, 1980; Molina et al., 1987). Under hypoxic conditions the greatest activity of this pathway is for acetaldehyde to be formed following reduction of pyruvate by PDC, then reduced to ethanol by ADH (Mathews and van Holde, 1996). Ethanol is then the major product of this pathway and ester production decreases until aerobic conditions are restored. Alcohols formed by the action of ADH are combined with acyl CoA's to form esters via AAT (Drawert and Berger, 1983; Harada et al., 1985). The CoA released by this reaction is then recycled with acids, by PDH and enzymes in the β-oxidation pathway, to form more acyl CoA compounds (Mathews and van Holde, 1996).

Esters could be hydrolysed to alcohol and acids by esterases and ester biosynthesis is a balance between synthesis and degradation (Goodenough, 1983). Therefore ester biosynthesis is a reversible reaction, as is conversion of aldehyde to alcohols but acyl CoA's are irreversibly used and require resynthesis.

Previous research has suggested that availability of alcohols limits the rate of ester biosynthesis and acyl CoA's are not limiting (Berger et al., 1992; Knee and Hatfield, 1981). The model presented in Figure 9.1 illustrates that acyl CoA's can arise from at least 3 different biosynthetic pathways, β-oxidation, transamination and the PDH complex, while alcohols are only formed by the action of ADH and esterases, and esters by action of AAT. Acetyl CoA will be the most abundant acyl CoA under aerobic conditions as this compound is the substrate of the tricarboxylic acid cycle that is of central importance to generation of cellular ATP (Mathews and van Holde, 1996).

Based on the conceptual model outlined in Figure 9.1 concentration of alcohols will be highly correlated with formation of esters with the respective alcohol moiety and the type of esters with the greatest concentration will be acetate esters as acetyl CoA is assumed to be present in the greatest concentration of acyl CoA's.

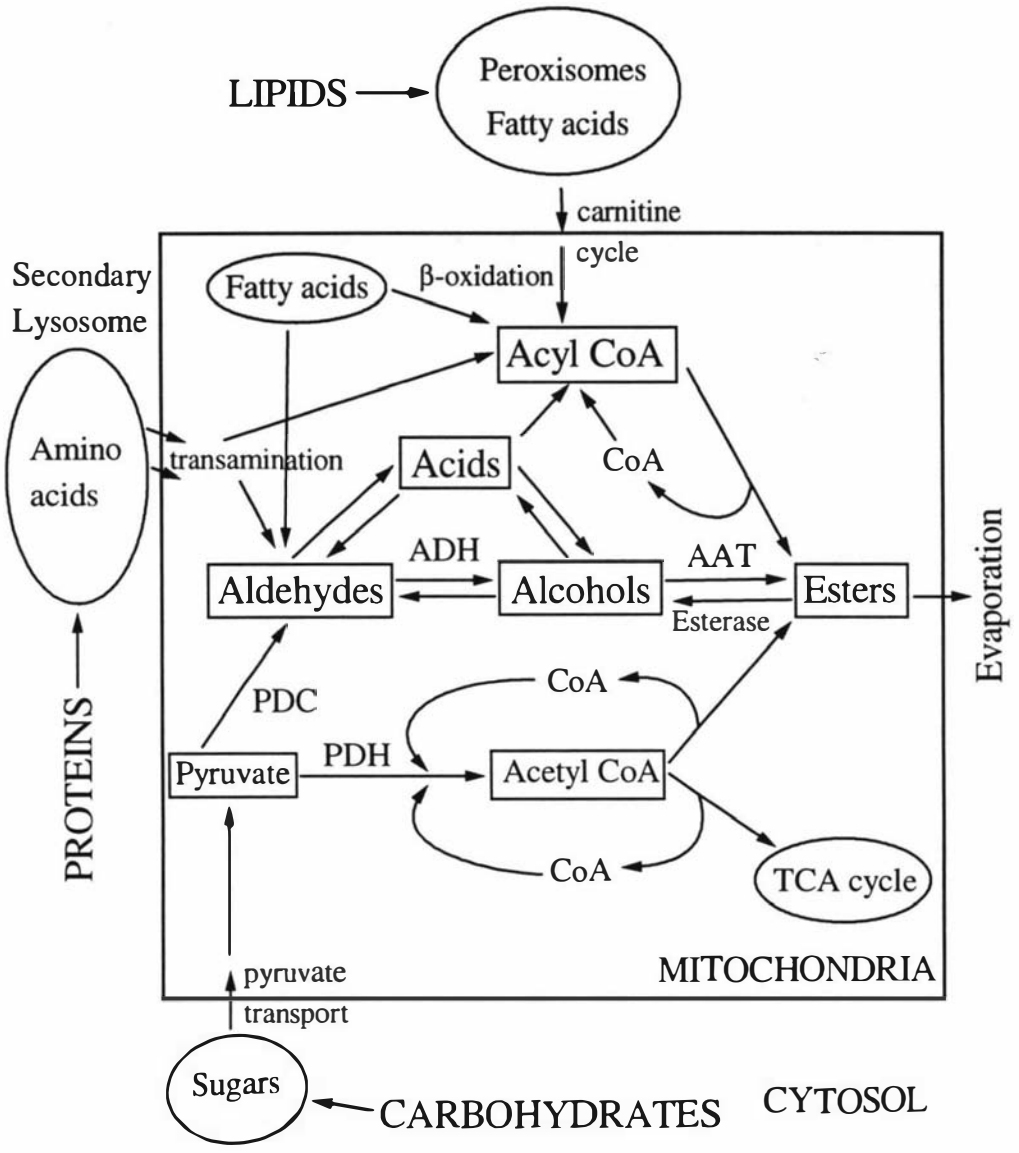

Figure 9.1 Conceptual model of ester biosynthesis in apples (adapted from Ampun, 1997; Mathew and van Holde, 1996, Sanz et al., 1997). ADH = alcohol dehydrogenase, AAT = alcohol acyl CoA transferase, PDC = pyruvate decarboxylase, PDH = pyruvate dehydrogenase complex, CoA = coenzyme A.

9.2 Mechanism of hypoxic effect on volatile biosynthesis

9.2.1 Hypothesis of competitive inhibition

In all of the experiments reported in this thesis, exposure of apple fruit to 100% CO_2 for 24 h at temperatures from 10 to 25 °C consistently induced enhanced concentrations of acetaldehyde and ethanol (Chapters 4, 7 and 8). On return to air ethyl acetate concentrations were enhanced 2 to 3 d after removal from hypoxia. This enhancement in fermentation volatiles occurred in all cultivars examined. On return of the cultivars CO, FU, PR, RD, SP, SS to air, ethyl esters other than ethyl acetate also increased and acetate esters decreased. These results formed the basis of the hypothesis that for apples the large increase in ethanol provided increased substrate for ester formation which in turn competitively inhibited formation of non-ethyl esters for a 7 to 14 d period after which ethanol decreased to control levels. Such changes in volatile concentration typical for FU and RG apples in the present experiments are illustrated in Figure 9.2.

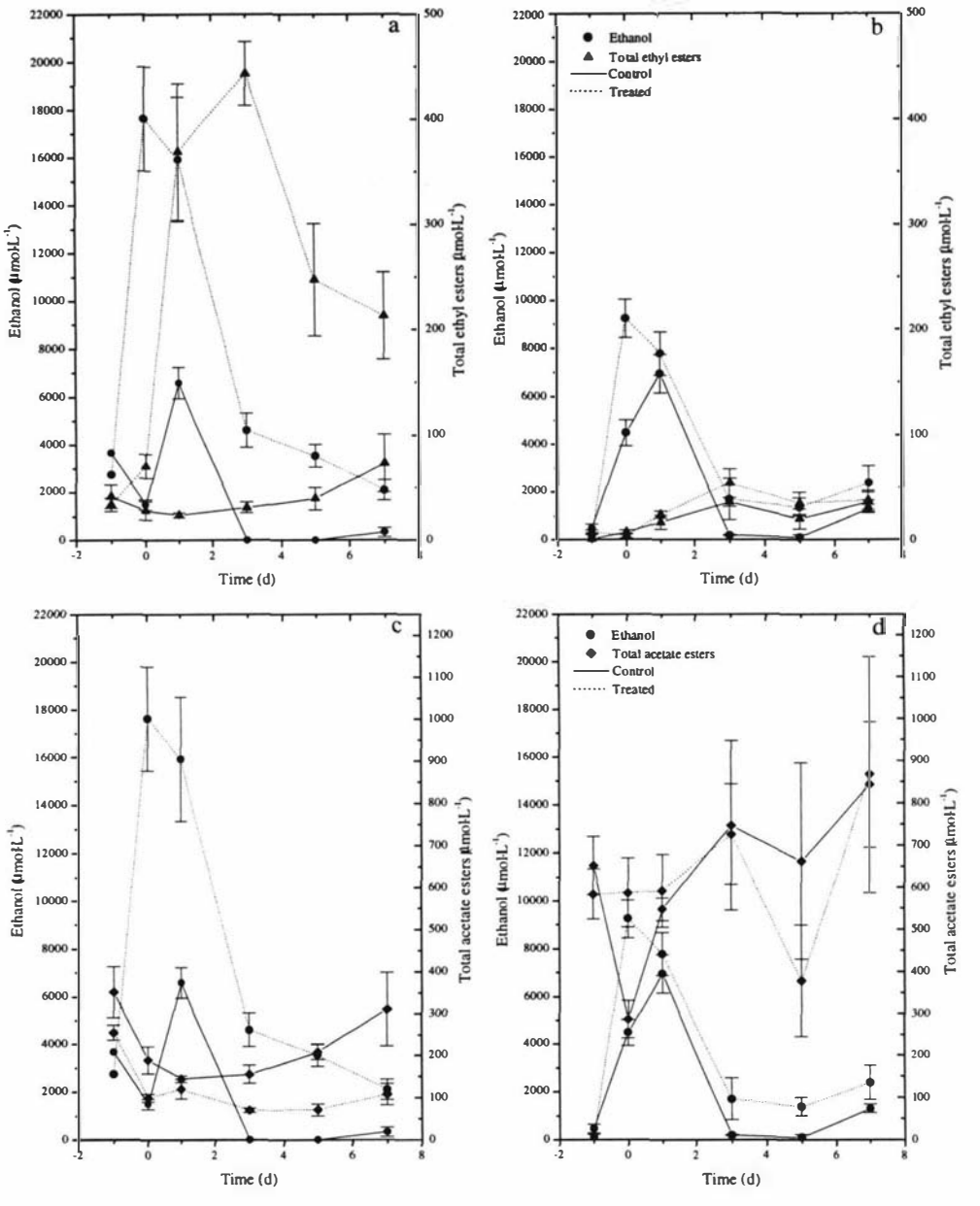

Figure 9.2 Ethanol, total ethyl ester and total acetate concentration in apple juice from a) and c) FU, b) and d) RG apples treated with air (—) or exposed to hypoxia (·····), 100% CO_2 for 24 h, at 20 °C and then maintained at 20 °C for up to 7 d. D 0 represents removal from hypoxia. Means and standard errors of 4 replicates. Data reworked from Chapter 4.

Golden Delicious and RG did not have enhanced ethyl esters and/or decreases in acetate esters even though fermentation volatiles were enhanced to high concentrations and ethyl acetate increased to concentrations similar to those found in fruit which had enhanced ethyl esters (Tables 4.2, 4.3, 7.1 and 8.1). The

mechanism producing ethyl acetate and ethyl esters in GD and RG was probably different from that in CO, FU, PR, RD, SP and SS cultivars, as after exposure to hypoxia, ethyl acetate increased but other ethyl esters did not. Therefore, after exposure to hypoxia, additional factors must influence changes in volatile concentration other than the increased pool of substrate available for esterification. This may be occurring in all cultivars and not just in GD and RG fruit. Thus the simple model that postulates competitive inhibition as the explanation for increases in ethyl esters following hypoxic treatment only partially explains volatile changes after exposure to hypoxia and requires modification.

9.2.2 Cultivar differences

The apple cultivars examined in Chapter 4 had 4 patterns of increase or decrease in esters after exposure to hypoxia: increase in ethyl esters and decrease in acetate esters (CO, FU, RD, SP), no change in ethyl esters and decrease in acetate esters (GD, RG), increase in ethyl esters and no change in acetate esters (PR, SS), and no change in ester concentration (GS). Such patterns suggest that the ester forming pathway had a substrate preference for certain kinds of alcohols. The enzymes involved which may have different substrate preferences could be ADH or AAT. If these enzymes have a preferences for producing esters with specific alcohol moieties then some cultivars could produce non-ethyl esters preferentially to ethyl esters.

Fuji is an example of a cultivar that has enhanced ethyl esters after exposure to hypoxia, while RG is an example of a cultivar in which ethyl esters are not enhanced after exposure to hypoxia. The difference in response to hypoxia by FU and RG apples suggests that the enzymes involved in the biosynthetic pathway of ester formation differ from cultivar to cultivar. After FU apples are exposed to hypoxia the proportions of alcohols change greatly (Table 9.1). In control FU fruit butan-1-ol and 2 methyl butan-1-ol comprise 79% of all alcohols, whereas after exposure to hypoxia, ethanol comprises 76.2% of all alcohols. This is not reflected in changes to the proportion of acetate esters where in control FU apples butyl acetate, hexyl acetate and 2 methyl butyl acetate are the only acetate esters present but after exposure to hypoxia ethyl acetate comprises 11.1% of the acetate esters present. This increase in ethyl acetate appears to be at the expense of 2 methyl butyl acetate, as both butyl acetate and hexyl acetate are relatively unchanged by hypoxic treatment. Since ethyl-2-methyl butanoate increases to very high concentrations in FU apples after exposure to hypoxia, 2 methyl butan-1-ol may be converted to 2 methyl butyl CoA rather than being esterified to an acetate ester. In contrast RG apples have 82.3% of alcohols as butan-1-ol in control fruit and this drops to 65.5% in treated fruit with ethanol increasing from 5% in controls to 25.4% of total alcohols (Table 9.1). In control RG apples butyl acetate and hexyl acetate comprise 89.3% of acetate esters; this drops slightly to 85.9% in treated fruit, while ethyl acetate increased marginally from 0.2 to 1.5% of total acetate esters after treatment. The lack of change in proportions of acetate esters suggests that degree of esterification of alcohols to acetate esters also depends on the substrate specificity in the ester biosynthetic pathway of each cultivar as well as alcohol concentration. Differences in the proportions of esters

produced by different cultivars may be related to changes in ability of apple tissues to synthesise and metabolise ethanol during ripening.

Table 9.1 Percentage of alcohols and acetate esters of total alcohols or total acetate esters in apple juice from FU and RG apples 3 d at 20 °C after treatment with air (control) or exposed to hypoxia (treated), 100% CO_2 for 24 h, at 20 °C. Percentages calculated from Table 4.3.

Cultivar	FU		RG	
Volatile	percentage			
Alcohol	control	treated	control	treated
Ethanol	0.0	76.2	5.0	25.4
Propan-1-ol	12.5	2.9	3.2	2.9
Butan-1-ol	58.5	14.5	82.3	65.5
Pentan-1-ol	0.8	0.2	0.7	0.4
Hexan-1-ol	7.7	1.4	6.1	4.1
2 methyl butan-1-ol	20.5	4.8	2.7	1.7
Acetate ester				
Ethyl acetate	0	11.1	0.2	1.5
Propyl acetate	0	0	0.8	4.5
Butyl acetate	41.0	46.1	69.1	70.0
Pentyl acetate	0	0	1.0	1.4
Hexyl acetate	11.6	13.0	20.2	15.9
2 methyl butyl acetate	47.4	29.8	8.7	6.7

9.2.3 Ripening effects

Exposure to hypoxia decreased respiration in FU at the preclimacteric, rising climacteric and at the climacteric peak stages which may be due to pyruvate being used increasingly by PDC rather than by PDH, resulting in reduced supply of acetyl CoA. There was increasing endogenous ethanol concentrations in control FU apples with phase of the climacteric but in control RG increases in ethanol concentration ceased at the rising climacteric stage as the fruit ripened (Table 8.1). After exposure to hypoxia FU fruit had the same absolute ethanol enhancement at all phases of the climacteric while RG fruit had increasing enhancement with increasing ripeness. If there was a diversion of pyruvate to the fermentation pathway, respiration rate should be decreased for preclimacteric RG apples but this did not occur.

As apples ripened endogenous ethanol and ethyl esters concentration increased even though fruit are aerobic, indicating that the fermentation pathway becomes active during ripening, possibly due to physical changes in the fruit, such as blockage of intercellular spaces as cell walls soften (Knee, 1991). Therefore, exposing apples to hypoxia stimulates production of acetaldehyde and ethanol but does not induce it *per se* as the fermentation pathway is already active, albeit at very low levels. Concentrations of ethanol in apples in aerobic conditions at the climacteric peak and postclimacteric can be quite high, equivalent to ethanol concentration in apples exposed to hypoxia. For example, ethanol concentration in FU apples 2 d after removal from hypoxia at the climacteric peak and postclimacteric had ethanol concentrations similar to those in control preclimacteric and rising climacteric (Table 8.1). This ethanol may provide

substrate for ethyl ester synthesis in cultivars which normally produce ethyl esters during ripening. Conversion of ethanol to ethyl esters in aerobic fruit could help to ensure that ethanol does not build up to toxic concentrations; ethyl esters evaporate more readily than ethanol due to their low boiling points and poor solubility in water. Should ADH activity be increased following hypoxia, ethanol production would be enhanced over that of controls, once fruit are returned to aerobic conditions. This increased activity may be why ethyl esters in apples exposed to hypoxia remain above those of control fruit for up to 10 d while ethanol decreases to slightly above that in control fruit (Figures 7.4a, 8.4a, 8.5a).

Apples at the early phases of the climacteric have low ethyl ester concentrations presumably due low endogenous ethanol concentration. After exposure to hypoxia ethanol and ethyl esters increase in preclimacteric fruit indicating that the ester biosynthetic pathway is present. Exposure to hypoxia at later phases of the climacteric no longer enhances ethyl esters suggesting that some factor is limiting ethyl ester biosynthesis. One reason may be that the ester biosynthetic pathway is operating at maximum capacity already at these stages. Activity of AAT increases as the climacteric progresses (Fellman et al., 1993) but it is not known how activity of AAT in postclimacteric apples or activity of ADH and β-oxidation rates change during ripening. If enzyme activity increased during ripening then availability of substrates could be limiting rates of ester formation.

9.2.4 Substrates limit ester production

Addition of alcohols to skin disks increased acetate esters to high concentrations without increasing esters of the respective acyl CoA moieties (Table 5.1, Figures 5.3 and 5.4). Therefore alkylation of alcohols is the preferred process of the ester biosynthetic pathway. This supports the assertion by Knee and Hatfield (1981) that for apples in air, ester synthesis is limited by the concentration of alcohols and not by a lack of enzyme, enzyme activity or low concentration of acyl CoA's. In general, ethyl esters increased after treatment with hypoxia and decreased as ethanol declined. The reduction in ethyl esters continued even though ethanol remained higher than in control fruit and acetate esters did not return to control levels (Table 4.3). This suggests that some other factor or factors were limiting ethyl ester production. Although no factors that may limit ethyl ester production have been identified they could include limited supply of acyl CoA, change in proportion of ethanol to other alcohols so competitive inhibition effects were reduced or production of new isozymes of AAT with substrate preference to alcohols other than ethanol. These factors may have been related to changes in the volatile biosynthetic pathway induced as the fruit continued to ripen. Ethyl esters increased the most after exposure to hypoxia when ethylene production was decreased which suggests that ethylene does not directly effect ester biosynthesis.

9.2.5 Ethylene production and volatile concentration

There was no consistent relationship of ethylene production with volatile production, although ethylene production and volatile concentration in control fruit was greatest at 20 °C. Ethylene is thought to have a general effect on volatile biosynthesis, as all types of volatile compounds increase even though their substrates are of different metabolic origins (Song and Bangerth, 1996). Biosynthesis of esters in RD apples may require continous exposure to high concentrations of ethylene (Fan et al., 1998). Maximum volatile concentration correlated with the climacteric peak in FU and RG fruit (Table 8.1) suggesting that ethylene may drive the overall rate of volatile synthesis (Fan et al., 1998). Ethylene production rates of greater than 1 $nmol \cdot kg^{-1} \cdot s^{-1}$ may be required for cultivars to have high concentrations of acetate esters; for example, at the climacteric peak control FU apples had a maximum ethylene production rate of 0.5 $nmol \cdot kg^{-1} \cdot s^{-1}$ and 1769.7 total acetate ester concentration while control RG apples had a maximum ethylene production rate of 4.0 $nmol \cdot kg^{-1} \cdot s^{-1}$ and 4959.4 total acetate ester concentration (Figure 8.2; Table 8.1). However, such concentrations may simply reflect metabolic rate which is greater in RG, at about 225 $nmolCO_2 \cdot kg^{-1} \cdot s^{-1}$, than in FU apples at about 170 $nmolCO_2 \cdot kg^{-1} \cdot s^{-1}$ (Figure 8.1).

9.2.6 Time taken for ethyl ester concentration to increase

Intact fruit had a slower increase in ester concentration than did disks. In CO, FU, PR, RD, SP and SS cultivars ethyl esters increased to a peak 2 to 3 d after exposure to hypoxia (Table 4.3, Figure 4.1). This increase in ethyl esters lagged behind the increase in ethanol by one day and coincided with decreased ethylene production. However, when alcohols were added to disks of apple tissue, acetate esters increased to high concentrations within 12 h (Figure 4.3, Berger and Drawert, 1984) and esters of other acyl CoA moieties within 48 h (Song et al., 1996). This suggests that in tissue disks added alcohols may have reached the metabolic sites of ester biosynthesis more readily than in they would in intact fruit; and/or by excising tissue from apples a 'wounding' effect may have been created which stimulated the respiratory mechanisms thereby priming the apple tissue to produce esters once alcohols were added. This implies that in intact fruit enhanced synthesis of esters either takes time to be induced or that tissue concentrations need to increase to specific/threshold levels before they are detectable either in the headspace or apple juice.

If ethanol is produced in mitochondria during hypoxia, and has to reach a different site/organelle for esterification, then ethyl ester production would lag behind that of ethanol production. To determine if ethyl esters increase immediately after ethanol concentration rises would require measurement of esters at every few minutes but at regular intervals after treatment.

Another possibility for the lag in ethyl ester rise after the rise in ethanol is that ethyl ester concentration, apart from ethyl acetate, is only induced once ethanol concentrations reach certain levels. For example, when ethanol was added to intact RD apples at concentrations greater than 10 $mmol \cdot 100g^{-1}$, there was an

increase in ethyl esters with acyl moieties C_8 and longer while ethanol at less than 10 mmol·100g^{-1} increased ethyl esters with acyl moieties less than C_8 (Berger and Drawert, 1984). This suggests that differences in ethyl ester concentrations between cultivars could be related to ethanol concentrations reached during ripening. Apples exposed to hypoxia had the greatest absolute concentrations of ethyl acetate, ethyl propionate and ethyl butanoate (Tables 4.3, 7.1, 8.1 and 8.3) implying that exposure to hypoxia induced ethanol less than 10 mmol·100g^{-1} and thus only stimulated production of ethyl esters with acyl moieties of less than C_8. The mechanism by which selective enhancement of ethyl esters according to ethanol concentration occurs is not known.

9.2.7 Substrate specificity of AAT

That apple cultivars differ in the proportions of volatile compounds produced after exposure to hypoxia may be due to possible differences in substrate specificities of enzymes in the ester biosynthetic pathway and consequently the ability of individual cultivars to synthesise ethanol. Ester production appears to be limited by substrate concentration, either alcohols or acyl CoA's; as the fruit ripen this limitation may be by increased by AAT activity depleting the pool of volatile precursors. Increased concentration of ethyl esters in CO, FU, PR, RD, SP and SS apples could be due to induction of new isozymes of AAT specific to ethanol, while no new isozymes of AAT are induced in GD, GS and RG apples after exposure to hypoxia. Therefore the new hypothesis is that, after exposure to hypoxia the great increase in ethanol concentration provides increased substrate for ethyl ester formation which in turn competitively inhibits formation of non-ethyl esters in apple cultivars which have either constitutive or hypoxia induced forms of AAT with substrate specificities for ethanol.

Having at least two enzymes, ADH and AAT, which can differ in substrate specificity and activity, suggests that there could be an almost infinite variation in rates of production of different types of esters resulting in greatly variable concentrations of esters from cultivar to cultivar. Esters in apples are predominantly even numbered (Bartley et al., 1985) implying that β-oxidation provides most of the substrates for ester formation either as acyl CoA's or aldehydes. The aldehydes are then further metabolised to alcohols by ADH, providing the alcohol moiety of esters (Gilliver and Nursten, 1976). New isozymes of ADH and/or AAT induced by hypoxia may have specificity for ethanol and acetyl CoA. New isozymes of AAT need not be located in mitochondria, as suggested in Figure 8.1, but could be in alternative sites of β-oxidation, such as peroxisomes (Gerhardt, 1983). At present AAT is assumed to have one form and AAT extracted from fruits has not been examined for the presence of different isozymes or even cellular location of AAT. To advance understanding of ester biosynthesis during fruit ripening and after exposure to hypoxia identification of AAT isozymes is required.

If there are several isozymes of AAT then these could have substrate specificity for particular alcohols and/or acyl CoA's. The differences in substrate specificity may explain the variations in aroma composition among cultivars. Should several isozymes of AAT exist, it may be possible to identify the genes

responsible for the different forms of AAT and which could be isolated and used to create new cultivars with specific aroma characteristics. Such characteristics could include an ethanol specific AAT, so that ethanol could be removed rapidly in aerobic conditions, after exposure to hypoxia or cultivars could be developed to produce specific esters giving apples with unique aroma characteristics, for example, apples which smell like bananas. If increased concentrations of AAT are induced by hypoxia then the mechanism by which this occurs could be used to switch on genes producing extra AAT after storage at low oxygen concentrations, which currently reduces the capacity of apples to produce esters.

9.2.8 Acyl CoA and ester concentration

If the biosynthesis of the ester substrates, alcohol and acyl CoA's, occurs only in mitochondria, then while β-oxidation is active, acyl CoA's other than acetyl CoA should be present in significant concentrations. But if acyl CoA's are transported into mitochondria via the carnitine cycle (Mathews and van Holde, 1996), then they might be transported at different rates into the mitochondria which would change the availability of acyl CoA compounds for esterification in this organelle. In addition, there are a small number of acyl CoA types (e.g. acetyl CoA, butyl CoA and hexyl CoA) produced depending on the original chain length of fatty acids undergoing β-oxidation. For example, if linolenic acid was the fatty acid undergoing β-oxidation, then chain lengths would then be even numbered of C_{18} and less with the majority of acyl CoA's being butyl CoA and hexyl CoA (Bartley et al., 1985). That β-oxidation can occur at more than place in the cell (Gerhardt, 1983) suggests that there are separate pools of mitochondrial, peroxisomal and cytosolic CoA. The CoA in each of these cellular locations could differ in concentration and rates of recycling between cultivars, which in turn may influence what predominant forms of esters occur in each cultivar. The degradative pathway of β-oxidation is considered to provide many of the precursors for ester biosynthesis (Sanz et al., 1997). Therefore, measurement of β-oxidation pathway activity and concentration of acyl CoA's in whole cells and in cellular compartments would be helpful in identifying the flux of substrate availability for ester biosynthesis.

Acetate esters are the most common esters in volatile profiles from apples as acetyl CoA is likely to be present in the greatest abundance of acyl CoA's present in apples. Little is known about metabolism of CoA supply, but acetyl CoA supply must be strictly regulated as it is the substrate for the TCA cycle and is of central importance for cellular function (Mathews and van Holde, 1996). Alcohols are not as readily converted to acyl CoA's as they are to esters (Song et al., 1996). Acyl CoA's were assumed to be used by whatever alcohol was being esterified, i.e. the substrate specificity is for alcohols and not acyl CoA. It is unknown if acyl CoA's accumulate in apples after exposure to hypoxia. By measuring acyl CoA concentration changes from control fruit and fruit exposed to hypoxia, inferences could be made as to whether acyl CoA concentrations limit ester biosynthesis. Cultivar differences in quantities of esters produced during ripening or after exposure to hypoxia could be due to differences in concentration of acyl CoA compounds, as well as substrate specificity of the ester

biosynthetic pathway for alcohols and acyl CoA's. The changes occurring in acyl CoA concentration in apple tissue during ripening and storage at low temperatures are not known. That apples exposed to hypoxia continued to have an increased capacity for ester synthesis but decreased ester concentration suggests that acyl CoA concentration compared to control fruit for 7 d (Figure 5.6) has become limiting.

That there was a change in the substrate preference of apple tissue and its capacity to produce esters after exposure to hypoxia was demonstrated by the experiments where alcohols were added to skin disks. Exposure to hypoxia induced a substrate preference change in RD apples disks as they had increased capacity to esterify pentan-1-ol when added as an individual alcohol (Figure 5.1). Addition of a mixture of alcohols to nine cultivars indicated that there was an enhanced capacity to produce acetate esters but no preference for specific alcohols (Figure 5.3). The reason for this is not known, but may have been due to the age of RD apples; those used in the experiment with individual alcohols had been at 0 °C for 3 months whilst those used where alcohols were added as a mixture had been at 0 °C for 2 to 3 weeks. Time at 0 °C could induce change in substrate specificity for ester biosynthesis. To establish if this was so, it will be necessary to extract AAT and possible isozymes and to measure substrate specificity of AAT in apples maintained at 0°C for different periods of time.

The two most intensively studied cultivars in this thesis were FU and RG. Fuji was representative of cultivars RD, PR, SP and SS, which after hypoxia had enhanced ethyl esters and decreased acetate esters, while RG represented cultivars such as GD which have no enhancement of ethyl esters. If such differences in substrate specificity, due to possible production of new isozymes of AAT do exist, this may help to explain cultivar differences in ester production after exposure to hypoxia. However, changes in volatile concentration after hypoxia in FU and RG were not identical to changes in CO and GS apples. Measurement of AAT activity or different substrate specificities will be necessary to establish the difference in capacity to form esters between FU and RG apples. Therefore, there would a requirement to assess the effect of combinations of alcohols and acyl CoA's on ester production for each cultivar.

By taking the information presented in the paragraphs above a possible mechanism by which hypoxia affects ester biosynthesis can be postulated. Under hypoxic conditions ADH activity and synthesis increase producing large increases in ethanol concentration (Ke et al., 1994). Activity of AAT, and hence ester biosynthesis, is suppressed during hypoxia (Fellman et al., 1993) by decreases in cytoplasmic pH below the optimum (7 to 8) for AAT activity (Ke et al., 1994, Harada et al., 1985). In most plant tissues, cytoplasmic pH is about 7, and this is reduced by 0.2 to 0.8 units under low O_2 and high CO_2 conditions (Hess et al., 1993; Lange and Kader, 1997). Activity of ADH from CO apples is optimal at pH 5.5 to 6 for conversion of acetaldehyde to ethanol, but for conversion of ethanol to acetaldehyde, ADH activity is optimal at pH 7 to 10 (Bartley and Hindley, 1980). If cytoplasmic pH in apples is reduced to below pH 7 by 100% CO_2 treatments, ADH activity is likely to be promoted and AAT activity inhibited. Such changes are probably induced by hypoxia providing a

large pool of aldehydes which can then be used as acyl CoA derivatives for esterification. As AAT activity is very low, it is possible that acyl CoA's begin to accumulate, creating a pool of substrates that could be utilised rapidly once fruit are returned to aerobic conditions. Hypoxic conditions induce new isozymes of ADH in other fruit (Tesnière et al., 1993; Chen and Chase, 1993) as well as suppressing other enzymes (Kanellis et al., 1991). Short periods of hypoxia increased ADH concentration and induced the ADH isozyme, ADH2, in tomato, avocado and grapes (Chen and Chase, 1993; Kanellis et al., 1991, Tesnière et al., 1993). While not demonstrated in apples, this induction occurs within 3 h in avocados (Kanellis et al., 1991). It is likely that AAT activity or concentration changes are induced in apples exposed to hypoxic conditions (Fellman et al., 1993). The different capacity of apple cultivars to esterify alcohols from control and hypoxic treated fruit may be due to changes in substrate specificity of either or both newly induced ADH (Speirs et al., 1998) and AAT. Once apples are returned to aerobic conditions, the high concentrations of ethanol and acyl CoA's are used to produce ethyl esters. Further research is required to establish if ADH or AAT are different in apple cultivars exposed to hypoxia. Such experiments could use enzyme extracts and incubation with different substrate combinations and the mRNA's and protein changes that occur following hypoxia could be determined.

9.2.9 Esterases

Ester concentrations measured in apple fruit result from the difference of production by AAT and degradation by esterase (Bartley and Stevens, 1981). Esterase activity could potentially be AAT acting in reverse or could be a different set of enzymes which have both synthetic and degradative capabilities (Tesnière, et al., 1989). While esterases have been reported in apple tissue, only those from CO apples have been characterised (Bartley and Stevens, 1981). To establish how much ester biosynthesis has been enhanced after hypoxia, esterase activity requires measurement as well as ester synthesis via AAT.

9.2.10 Cellular location of ester biosynthesis

Mitochondria contain all the enzymes necessary for biosynthesis of esters and their substrates (Mathews and van Holde, 1996) and could be candidates for key sites of ester formation. Environmental conditions, such as low temperatures and high CO_2/low O_2 conditions, which affect mitochondrial activity, are likely to affect ester biosynthesis. After prolonged exposure to low O_2 or high CO_2, mitochondria slowly lose their synthetic function, i.e. oxidation of ADP to ATP (Lange and Kader, 1993; Mathooko et al., 1995). Mitochondria from apples maintained in atmospheres of 6 and 12% CO_2 and 3% O_2 for up to 16 weeks at 0 °C had reduced capacity to oxidise succinate or NADH which was not reversed once the fruit had been returned to air (Shipway and Bramlage, 1973). In a similar manner mitochondria could slowly lose their ability to produce esters. Such changes in mitochondrial function could explain why apple tissue loses the ability to produce esters when stored in controlled atmospheres and why senescent fruit have a diminished capacity to produce esters. Measurement of

mitochondrial ester synthesis during ripening, at low temperatures in air or in controlled atmospheres would be required to confirm or disprove this suggestion.

9.2.11 Environmental effects on ester production

The environment in which apples are maintained effects the concentration and production of compounds associated with apple aroma (Knee, 1991). The effect on volatile concentration is either physical, related to solubility and volatilisation of volatile compounds and rates of biosynthesis; or physiological with increased induction of activity in specific biochemical pathways and accumulation of substrates for volatile biosynthesis promoting or inhibiting formation of aroma compounds as fruit tissue ripens. A decrease in mitochondrial function could be the reason why respiration of FU and RG is less in apples at 0 °C than at 20 °C (Figure 8.1). Extended exposure to 0 °C would also reduce rates of β-oxidation and transamination, decreasing substrate concentrations and hence volatiles. The pathways involved in ester biosynthesis may be affected at different temperatures. This may be indicated by presence or absence of specific volatiles, for example, if branched chain esters are absent then transamination of amino acids has been affected. To determine if this is happening at different temperatures more volatile compounds would need to be measured than have been reported in this thesis.

Maximum volatile production in control fruit occurred at 20 to 25 °C which coincided with peak ethylene production rates. Magnitude of enhancement in ethyl esters after removal from hypoxia was greatest at 10 °C and decreased as temperature increased. This was an unexpected result and may be due to physical effects such as decreased volatile solubility in the cytoplasm and/or increased rates of volatilisation at higher temperatures. As temperature increases volatile concentrations will decrease if rate of volatile production is less than the rate of evaporation. That apples at 10 °C maintain enhancement in ethyl esters after hypoxia longer than at 20 or 25 °C suggests that exposing apples to hypoxia at temperatures below 10 °C could result in greater enhancement in ethyl esters than reported here. The relationship of volatile production and degree of enhancement in ethyl esters requires characterisation for temperatures in the range of 0 to 35 °C. Defining this relationship would allow sensible decisions to be made as to the best temperature for enhancement of volatiles and maintenance of apple quality. Exposure to apples to temperature above 25 °C to 40 °C, such as those used in heat shock treatments, severely depressed volatile production (Fallik et al., 1997) and it is likely that exposure to hypoxia at temperatures above 25 °C would have no effect on volatile production.

Control FU and RG apples maintained at 0 °C then removed to 20 °C had less variable carbon dioxide, ethylene and volatile concentrations than fruit held at 20 °C continuously. The reason for this 'evening out' of fruit metabolic activity could be due to an affect on ethylene production as exposure of GS apples to 0 °C for short periods activates ACC synthase, ACC oxidase and ethylene (Jobling et al., 1991). Another less likely possibility is that such low temperatures could result in different genes being expressed, possible development of low grade

chilling injury affecting cell membranes or differential temperature optima of different enzymes.

9.3 Measurement of volatiles

One of the difficulties of aroma research is that there is no standardisation of units. Each researcher appears to use their own set of units without regard to compatibility with other researchers work (Table 9.2). This can make comparing results from one publication to another very difficult and inferences about volatile biochemistry almost impossible. For this reason units of $mol \cdot L^{-1}$ of juice for measurements of volatiles in juice and $mol \cdot kg^{-1} \cdot L^{-1}$ of air for headspace concentration with Tenax® traps have been utilised in this study. By expressing volatile concentration as moles, concentrations of different compounds can be compared directly, whereas if mass is used as the unit of volatile concentration, different compounds cannot be compared until the molecular weights of each have been allowed for.

Table 9.2 Units used for expressing concentrations of volatile compounds emanating from apple fruit.

Units	Reference
$\mu mol \cdot kg^{-1} \cdot h^{-1}$	Vanoli et al., 1995
$\mu g \cdot kg^{-1} \cdot L^{-1}$	Hansen et al., 1992
$\mu g \cdot kg^{-1} \cdot 6L^{-1}$	Dirinck and Schamp, 1989
$\mu g \cdot kg^{-1} \cdot h^{-1}$	Bangerth et al., 1998; Fan et al., 1997; Miller et al., 1998; Song and Bangerth, 1996
$\mu L \cdot kg^{-1} \cdot h^{-1}$	Mattheis et al., 1998
$\mu g \cdot 100g^{-1}$	Berger and Drawert 1984; Drawert 1975
$nmol \cdot g^{-1}$	Knee and Hatfield, 1981
ppm ($\mu L \cdot L^{-1}$)	Bachmann, 1983; Pesis, 1994; Shaw et al., 1991
peak area	Girard and Lau, 1995

In this thesis the following three methods have been used for determination of aroma volatiles: direct injection of headspace samples, concentrated extracts of headspace from Tenax® traps and solvent extraction of juice using diethyl ether and pentane. Solvent extraction has the advantages of complete removal of all volatile compounds from a sample; it is an inexpensive, rapid and straight forward method removing alcohols and aldehydes with similar efficiency to esters. The disadvantages of solvent extraction is the high degree of concentration required that may lead to excessive losses of some compounds, presence of contaminants and a lack of extraction of long carbon chain compounds. Using Tenax® traps volatiles emanating from intact or homogenised samples can be extracted; however longer carbon chain compounds are trapped at the expense of shorter carbon chain compounds, esters are selectively extracted over alcohols and aldehydes, and the technique can be used to measure rates of volatile production. Solvent extraction is best suited for quantitative extraction of volatile compounds from ethyl propionate to hexyl esters, propan-1-ol to hexan-1-ol and aldehydes. The Tenax® traps are best suited to measuring rates of volatile production over the same range of compounds while direct headspace analysis is best suited to acetaldehdye, methanol, ethanol and ethyl acetate.

The method used to extract volatile compounds defines what compounds are detected. Both solvent extraction and Tenax® traps are inefficient in extracting low molecular weight volatile compounds such as ethanol, methanol and ethyl acetate. If low molecular weight volatile compounds are not measured, this is equivalent to discarding them from the analysis of volatiles without establishing if these compounds are important to apple aroma. Such lack of measurement of low boiling point compounds such as acetaldehyde, ethanol and propionate esters occurs frequently in the literature on volatile compounds emanating from apples (Young et al., 1996; Plotto, 1998; Berger and Drawert, 1984; Song and Bangerth, 1996). Ethanol and acetaldehyde have been implicated as being important in fruit aroma (Dürr and Schobinger, 1981). By not measuring these compounds, assessment of whether the fruit have an active anaerobic respiration, indicated by high production of acetaldehyde and ethanol indicating that there could be production of ethyl esters. Any attempt to correlate organoleptic characters of apple aroma with volatile concentrations that does not include the entire range of volatile compounds, must therefore be less comprehensive and incomplete than if these compounds were included.

The fact that so many volatile compounds have high air-water partition coefficients (Buttery et al., 1969; Nawar, 1966; Plotto, 1998) indicates that many volatile compounds present in solution in apple tissue readily move from the liquid to the gas phase. Ethanol appears to be a special case, where concentration of this alcohol can be high in tissue but appear as low concentrations in the headspace. This is because ethanol has a very low air-water partition coefficient, in the order of 0.0002, i.e. if 1 $\mu L \cdot L^{-1}$ is measured in the gas phase then there is 5000 $\mu L \cdot L^{-1}$ in the liquid phase, which means that there is very much more ethanol present in solution in fruit tissue than in the headspace. Therefore, measuring ethanol in the headspace of fruit juice or fruit tissue is an insensitive method of assessing ethanol concentration in fruit tissue as the detection limit of current FID detectors for ethanol is too high to determine accurately if it ethanol is present in aerobic conditions. Ethanol can be assayed by chemical reaction from fruit tissue rather than in the gaseous phase (Saltveit and Ballinger, 1983).

9.4 Sensory impact of changes in volatile concentration

Enhanced concentration of ethyl esters and decreased concentration of acetate esters of apples induced by exposure to hypoxia would appear at an intuitive level to affect perception of aroma. However, despite large changes in volatile concentrations after exposure to hypoxia, sensory panellists were unable to detect a difference between juice from untreated control fruit and juice from fruit exposed to hypoxia (Figure 6.2). Odour unit values for FU apples 2 d after exposure to hypoxia, indicated that there should have been a difference in the intensity of apple aroma (Figure 6.4). At first this result appears to be puzzling, suggesting that something was wrong with the way samples were prepared or the sensory analysis was conducted incorrectly. In Chapter 6 it was suggested that the inability to detect a difference in aroma between juice from control and treated apples was due to poor panellist performance, possibly due to inadequate training, and by extending tasting sessions over 8 weeks. The same reference standard was used at each tasting session and this was consistently assessed to

apples was due to poor panellist performance, possibly due to inadequate training, and by extending tasting sessions over 8 weeks. The same reference standard was used at each tasting session and this was consistently assessed to have the same qualities by each panellist at each session suggesting that it was the very extended time over which tasting sessions were held which may have affected how panellists rated the apple juice. Ideally with QDA, all samples should be assessed in the same session (Gilbert and Heymann, 1995) or contrasting effects can occur (Meilgaard et al., 1991), as panellists are known to contrast samples in the current session with those they remember the most vividly from the previous sessions. This was not possible in the current research as it would not have been practicable to achieve proper replication of treatments or to evaluate the effect of ripening after treatment. In hindsight QDA was probably not the correct method of sensory analysis to use for these experiments.

Even though all apple cultivars have apple-like aroma the composition of volatile compounds in their aroma profiles is very varied. Although fruit to fruit variation in headspace volatile concentration can be as high as 20% (Poll and Hansen, 1990), all fruit will have an 'apple' aroma. This suggests that those volatile compounds which are common to all cultivars, and differ in concentration the least from fruit to fruit, are the most important in characterising the base level of apple aroma. Candidates for such compounds would be some acetate esters and aldehydes. For Gala apples, the acetate esters butyl acetate, hexyl acetate and 2 methyl butyl acetate and the aldehyde hexanal had the greatest aroma intensity and were common to all samples (Plotto, 1998). By contrast, differences in concentration of pentyl acetate and hexyl-2-methyl butanoate were associated with the greatest differences in aroma intensity of Gala apples (Plotto, 1998). Volatile compounds present in very low concentration, even though they may be potentially very potent odourants, may be only adding nuances, such as fruity or grassy characters, to the apple aroma. Therefore, changes in the composition of apple aroma may not be detected easily using sensory techniques, making it very difficult to determine if different treatments induce sensory changes in apple aroma.

For Royal Gala apples the sum of butyl acetate, hexyl acetate, 2 methyl butyl acetate, hexanal and ethyl-2-methyl butanoate was in broad agreement with the panellist aroma score but there was no correlation of these volatiles with panellist aroma score for FU apples (Figure 9.3). The concentration of volatiles for RG apples was strikingly similar to that of odour units (Figure 6.4). This suggests that butyl acetate, hexyl acetate, 2 methyl butyl acetate, hexanal and ethyl-2-methyl butanoate define the underlying aroma of RG apples in agreement with Plotto (1998); other apple cultivars may have different combinations of compounds that define their base aroma. In control FU apples acetate esters were present in much lower concentrations than in control RG apples (Table 4.3) and ethyl esters were at similar concentrations but panellist aroma scores for FU apples were only lower by about 20%. This suggests that there are some compounds not detected in this study that are important for defining FU apple aroma.

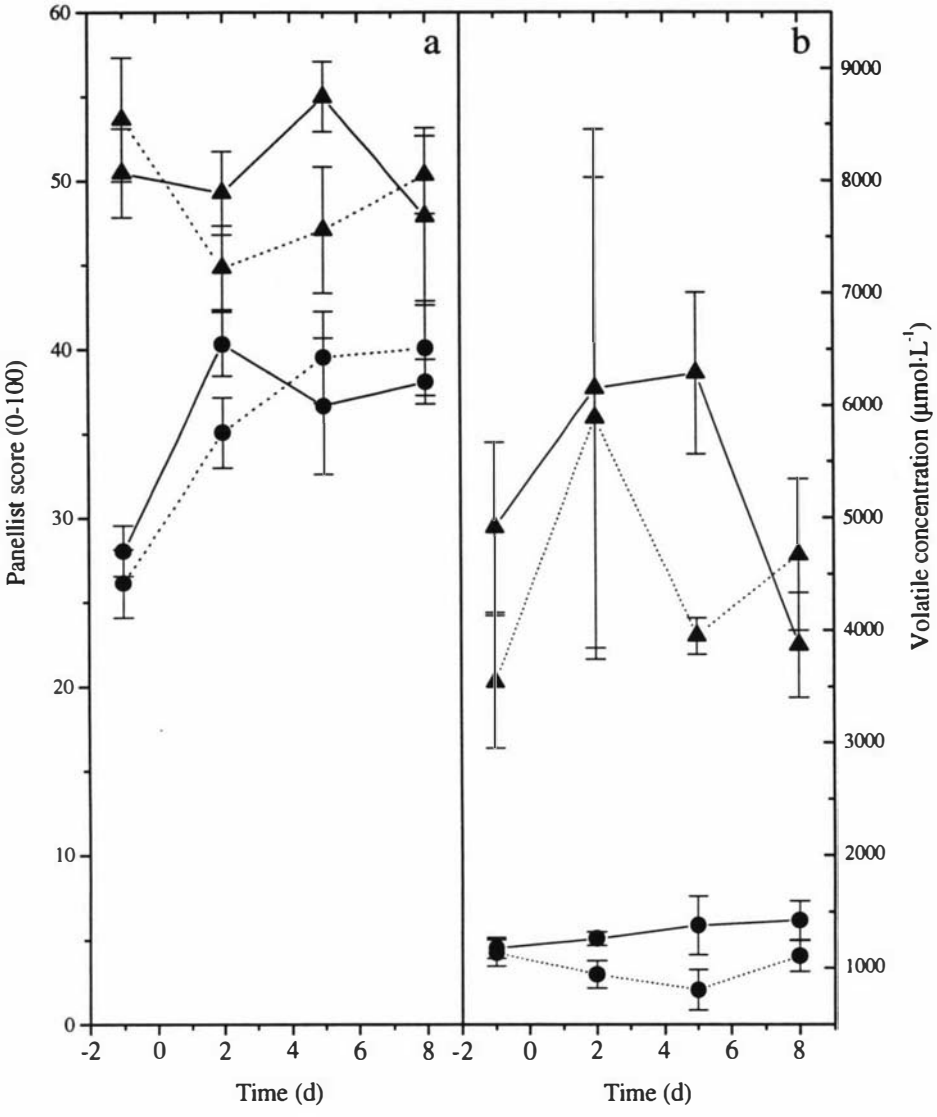

Figure 9.3. Comparison of (a) average sensory score for aroma and (b) sum of the volatiles butyl acetate, hexyl acetate, 2 methyl butyl acetate, ethyl-2-methyl butanoate and hexanal for FU (●) and RG (▲) apple juice from apples exposed to hypoxic conditions (— untreated, ····· treated), generated using an atmosphere of 100% CO_2 for 24 h at 20 °C, before treatment (d-1), on removal from treatment (d 0) and during subsequent storage at 20 °C for up to 8 d. Sensory scores are the means and standard errors of 4 to 10 panellists, volatile concentration is the means and standard errors of 4 replicates.

Other explanations of why panellists failed to detect a difference in aroma between juice from untreated control fruit and fruit exposed to hypoxia include: that the very high ethyl ester concentrations were perceived as off flavour or they changed the character of apple aroma; high concentrations of ethyl esters may saturate olfactory receptors, therefore above a specified concentration no change in aroma may be detected and concentrations required to achieve saturation may

not be much higher than was already present in apple tissue; and high concentration of a small number of compounds may effectively block olfactory receptors preventing minor compounds from being recognised. The apple aroma in juice from treated fruit may still have been recognisably 'apple' but it varied from control fruit. The sensory character of apple aroma had been rigidly defined for panellists at the beginning of the process; therefore any deviations from the defined aroma would be considered as an 'atypical aroma' and classed as off flavour (Figure 5.6). Such a result would appear to be a limitation of the QDA sensory analysis method in evaluating samples like apples which may change over time but still have a recognisable sensory character.

Other methods of sensory analysis need to be examined for their suitability to analyse changes in aroma in time series experiments or those involving a relatively large number of replicates. To overcome the possibility that suprathreshold concentrations of aroma compounds are being perceived as having different aroma characteristics, it would be necessary to analyse by taste panel juice from apples exposed to hypoxia diluted with untreated juice, e.g. 50% treated juice: 50% untreated juice, 25% treated juice: 75% untreated juice. This would dilute concentrations of aroma compounds without significantly altering the character of the apple juice being assessed. Another suggestion would be to ask panellists to provide as comprehensive a range of descriptors of the apple juice aroma as they are able. Differences in descriptors between untreated and treated apple juice would then indicate how changes in apple juice were perceived.

9.5 Implications of research

The results of research presented in this thesis suggest that there are practical uses for treatments where apples are exposed to hypoxia for 24 h. However, duration of treatment with hypoxia may not be limited to 24 h for all apple cultivars or types of fruit. The areas where exposure to hypoxia may be used could be in the manufacture of apple juice concentrates, enhancement of aroma in apples maintained in long term air or controlled atmosphere storage and as a tool for examining volatile biosynthesis.

Disinfestation treatments which use hypoxia for 24 h, at 20 °C will not adversely affect the quality of apple cultivars, CO, FU, GD, GS, PR, RD, RG, SP and SS and will enhance ethyl ester concentrations in CO, FU, GS, PR, RD, SP and SS apple cultivars. Exposure to hypoxia for 24 to 96 h, at 20 °C, may also be possible as disinfestation treatments as there was no negative effect on GD apple quality once these fruit were returned to air (Ampunpong, 1991).

Increases in ethyl esters after exposure to hypoxia present opportunities for apple juice processors in that:

a) increased volatile concentrations allows greater yields of aroma concentrates from the same volume of fruit;

b) ethyl esters are induced to high concentrations in cultivars where they are normally at low concentrations thereby enhancing specific characteristics of apple aroma;

c) increasing concentrations of ethyl esters to very high levels in some cultivars may allow small volumes of treated apples to be blended with untreated apples, which lack ethyl esters at desired concentrations, to raise ethyl ester concentrations to acceptable levels. By using small volumes of treated apples decreased acetate ester concentrations will affect overall acetate ester concentration in the blended mixture.

A treatment which could be used by apple juice manufacturers to enhance ethyl esters in apple juice is outlined below. Maximum enhancement of ethyl esters in apple juice could be obtained by exposing fresh, physiologically mature apples or apples maintained at 0 °C for up to 5 months to 100% carbon dioxide for 24 h at 10 to 15 °C, followed by juicing and processing after 2 to 3 days at ambient temperatures. The following cultivars were ranked in order of most enhancement to least enhancement of ethyl esters: RD, SP, PR, FU, SS, GS and CO; RG and GD had no enhancement of ethyl esters. Applying hypoxic conditions to enhance ethyl esters to RG and GD apples is unlikely to be effective.

Exposure to hypoxia could be useful as a method for ameliorating loss of volatile compounds important for apple aroma in apples maintained at low temperatures in air or in controlled atmosphere conditions for long periods although Ampun (1997) discovered that the magnitude of enhancement of ethyl esters decreases as time at 0 °C increased. Combinations of 'precursor atmospheres' (Berger et al., 1992) and exposure to hypoxia may restore aroma to apples stored for long periods through a combination of increasing the capacity of apple tissue to synthesise esters and by supplying the necessary substrates to enhance acetate ester synthesis.

Exposure to hypoxia changes volatile biosynthesis for short periods, presenting an opportunity for hypoxia to be used to help identify the mechanisms by which aroma compounds are produced. If mitochondria are the main organelles producing esters, then reasons why enhancement or suppression of volatile production occur may be explained in relation to changes in synthetic capacity of mitochondria. Investigation of whether such changes occur is worthy of further research.

9.6 Conclusion and future work

Exposure of apples to hypoxia generated by an atmosphere of 100% CO_2 for 24 h at 20 °C will enhance the concentration of ethyl esters and decrease non-ethyl esters for up to 10 d after removal from hypoxia and storage at 20 °C. Maximum ethyl ester enhancement occurs within 2 to 3 d after removal from hypoxia. Exposure to hypoxia for 24 h at 20 °C has no other effect on apple quality; it does not change rates of softening or induce physiological damage. Magnitude of enhancement of ethyl esters is cultivar dependent with the cultivars RD, SS, SP, PR and FU having the greatest enhancement in ethyl esters, GS and CO moderate enhancement, GD and RG little or no enhancement. Two cultivars were intensively investigated, FU representing cultivars with enhanced ethyl esters after exposure to hypoxia and RG representing cultivars without ethyl ester

enhancement. For FU apples degree of ethyl ester enhancement depended on: phase of the climacteric where maximum enhancement was found in preclimacteric and rising climacteric apples; and temperature with treatment at 10 °C resulting in apples with the greatest enhancement in ethyl esters compared to fruit at 15, 20 and 25 °C. Royal Gala apples had no response to hypoxia therefore further conclusions about the effect of hypoxia cannot be made. As FU apples did have an increase in ethyl esters after hypoxia, inferences about mechanisms of enhancement of their synthesis can be made. For FU apples time at 0 °C up to 5 months did not affect the magnitude of enhancement of ethyl esters. Although there were large increases in ethyl ester concentration and decreases in acetate esters in FU apples, a sensory panel could not detect a difference in aroma or flavour in juice between untreated control apples or apples exposed to hypoxia, despite odour unit values up to 10 fold greater for treated apples.

The initial effect of hypoxia induced by high CO_2 on volatile biosynthesis may be to decrease cytoplasmic pH. The decrease in pH will increase ADH activity and synthesis, suppresses AAT activity and thus allow an increase in acetaldehyde, ethanol, acyl CoA's and other alcohols. During hypoxia new isozymes of ADH and AAT, with different substrate specificities to constitutive enzymes, may be expressed, once the fruit are returned to aerobic conditions. When returned to normal oxygen levels, the increased amounts of alcohols and acyl CoA's could be used to produce esters over a few days. This biosynthetic activity possibly occurs in mitochondria so that any environmental factors that affect mitochondrial activity also affect volatile biosynthesis.

This study is complementary to that of Ampun (1997) and has increased our knowledge of volatile production from apples after exposure to hypoxia with respect to cultivars, treatment temperature, time at 0 °C, non-cooled versus cooled apples, stages of ripeness, biosynthetic capacity to form esters and sensory changes in treated apple juice. Significant gains in additional knowledge about the effect of hypoxia will be achieved by investigating aspects of the biochemistry of volatile biosynthesis in relation to fruit physiological status.

Suggestions for further research using FU and RG apples as model systems are:

(1) Clearly establish the role of ethylene in volatile biosynthesis.

(2) Mode of action of hypoxic treatment.

a) measure ADH and AAT activity before and after exposure to hypoxia.

b) identify if new isozymes of ADH and AAT are produced by hypoxic treated fruit.

c) analyse which mRNA's are activated by exposure to hypoxia and determine which genes are related to volatile biosynthesis.

d) measure concentration and production of acyl CoA compounds in untreated apples and apples exposed to hypoxia.

e) determine if competitive inhibition of esterification of ethanol is the reason for decreases in acetate esters by using skin disks and adding different mixtures of C_2 to C_6 alcohols.

f) measure substrate specificity of ADH and AAT in untreated and hypoxic treated fruit for alcohols and acyl CoA's.

g) measure ester degrading activity of AAT and esterases from apple fruit and substrate specificity of esterase for esters.

h) measure β-oxidation and transamination activity.

(3) Determine cultivar differences in concentration, activity and substrate specificity of AAT and ADH, and rates of β-oxidation and transamination.

(4) Establish the cellular location of volatile biosynthesis by extracting mitochondria and/or peroxisomes and testing these for ester forming capacity.

(5) Measure mitochondrial activity of apples exposed to hypoxia. If mitochondria are sites of ester and fermentation volatile formation, then a decrease in mitochondrial activity decreases may the cause of loss of aroma biosynthetic capacity in fruit maintained in air or controlled atmospheres at low temperatures.

(6) Determine the influence of preharvest factors on volatile concentration and production, such factors could include degree of pigmentation (Miller et al., 1998) and fertiliser treatments (Somogyi et al., 1964).

(7) Explore aspects of sensory analysis:

a) determine the relationship between concentration of ethyl esters and sensory character of individual volatile compounds.

b) examine synergistic and antagonistic effects on perception of aroma of mixtures of aroma compounds.

c) conduct sensory tests on apple juice from treated apples after dilution to determine if the increase in ethyl ester concentration was so great it was perceived as an off odour/flavour.

d) use a sensory method which will better determine if enhanced ethyl ester concentrations affects sensory perception of aroma.

e) identify compounds most important to the sensory perception of apple aroma.

(8) To determine accurately volatile concentrations within and emanating from apples by comparing concentrations obtained by several methods of volatile extraction and measurement.

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Appendix 1

Calculations of chemical concentrations.

A1.1 Percentage equivalent malic acid

$$\%\text{malic acid} = N.\frac{M_{wgt_{malic}}}{2}.V_t.\frac{100}{V_j}$$

where: N = normality of the sodium hydroxide solution.
$M_{wgt_{malic}}$ = molecular weight of malic acid.
V_t = volume of titrant.
V_j = volume of juice.

A1.2 Weight loss

$$\text{Moisture loss } (\%) = \frac{\text{Initial F.W. (g) - Final F.W. (g)}}{\text{Final F.W. (g)}} \text{ x } 100$$

where: Initial F.W. = fruit fresh weight before start of treatment

Final F.W. = fruit fresh weight at a particular sampling time.

A1.3 Carbon dioxide production

1) Fruit volume = fruit weight / fruit density (g x $ml \cdot g^{-1}$).

2) Gas density = density of CO_2 at 0 °C x $\frac{273.15}{(273.15 + \text{temperature})}$.

3) Volume of (mLs) CO_2 = $\frac{(\text{final } CO_2 - \text{initial } CO_2)}{100}$ x (jar volume - fruit volume).

Where: initial CO_2 = % CO_2 when jars were sealed
final CO_2 = % CO_2 after jars were sealed for x time
jar volume = volume of container (mL).

4) mg $CO_2 \cdot kg^{-1} \cdot h^{-1}$ = $\frac{(\text{mLs } CO_2 \text{ x gas density})}{\frac{\text{fruit weight}}{1000} \text{ x } \frac{\text{time}}{60}}$.

5) nmol $CO_2 \cdot kg^{-1} \cdot s^{-1}$ =

$$\text{mg } CO2.kg^{-1}.h^{-1} \times \left(\frac{1\,g}{1000\,mg} \times \frac{1\,mol}{44\,g} \times \frac{1\,h}{3600\,s} \times \frac{1\,nmol}{1 \times 10^{-9}\,mol} \right).$$

Factor = 6.313

A1.4 Ethylene production

1) 1) Fruit volume = fruit weight / fruit density (g x $ml \cdot g^{-1}$).

2) µL C_2H_4 produced = final C_2H_4 - initial C_2H_4.

Where: initial C_2H_4 = ethylene concentration when jars were sealed

final C_2H_4 = ethylene concentration after x time.

3) µL $C_2H_4 \cdot kg^{-1} \cdot h^{-1}$ = $\dfrac{\mu L\, C_2H_4}{\dfrac{\text{fruit weight}}{1000} \times \dfrac{\text{time}}{60}}$.

4) nmol $C_2H_4 \cdot kg^{-1} \cdot s^{-1}$ =

$$\mu L\, C_2H_4.kg^{-1}.h^{-1} \times \left(\frac{1\,m^3}{10^9\,\mu L} \times 101325\,Pa \times \frac{1\,mol\,K}{8.3143\,m^3Pa} \times \frac{1}{273.15 + temp} \times \frac{1\,h}{3600\,s} \times \frac{1 \times 10^9\,nmol}{1\,mol} \right)$$

Factor = 1.155×10^{-2}

Example calculations:

final CO_2 = 0.465 %
initial CO_2 = 0.050 %
fruit weight = 448.38 g
fruit density = 1.138952 $mL \cdot g^{-1}$
density of CO_2 at 0 °C = 1.976 $g \cdot mL^{-1}$
time elapsed = 41 minutes
jar volume = 1808.21 mL
initial C_2H_4 = 0 $\mu L \cdot L^{-1}$
final C_2H_4 = 52.962 $\mu L \cdot L^{-1}$
temperature = 20 °C

1) Fruit volume = 448.38 x 1.138952 g x $mL \cdot g^{-1}$
= 510.68 mL

2) Gas density $= 1.976 \times \dfrac{273.15}{273.15 + 20}$
= 1.8412 $g \cdot mL^{-1}$

3) mL CO_2 produced $= \dfrac{0.465 - 0.050}{100} \times (1808.21 - 510.68)$
= 5.385 mL

$$4)\ \text{mg } CO_2 \text{ produced} = \frac{5.385 \times 1.8412}{0.44838 \times \frac{41}{60}}$$

$$= 32.36 \text{ mg } CO_2 \cdot kg^{-1} \cdot h^{-1}$$

$$5)\ \text{nmol } CO_2^* \cdot kg^{-1} \cdot s^{-1} = 32.36 \times 6.31313$$
$$= 204.29 \text{ nmol } CO_2 \cdot kg^{-1} \cdot s^{-1}$$

$$6)\ \mu L\ C_2H_4 = 56.962 - 0$$

$$= 56.962\ \mu L$$

$$7)\ \mu L\ C_2H_4 \cdot kg^{-1} \cdot h^{-1} = \frac{56.962}{0.44838 \times \frac{41}{60}}$$

$$= 86.81\ \mu L\ C_2H_4 \cdot kg^{-1} \cdot h^{-1}$$

$$8)\ \text{nmol } C_2H_4^* \cdot kg^{-1} \cdot s^{-1} = 86.81 \times 1.15479 \times 10^{-2}$$

$$= 1.002 \text{ nmol } C_2H_4 \cdot kg^{-1} \cdot s^{-1}$$

* assuming 1 standard atmosphere (101325 Pa)

A1.5 Headspace standards of acetaldehyde, ethanol and ethyl acetate

1) 5000 $\mu L \cdot L^{-1}$ gaseous stock standards of each gas were prepared individually using sealed glass preserving jars, approximately 1.8 L, whose volumes were measured accurately, each containing a magnetic stirrer bar.

2) 100 mL of air from each jar was removed using a 60 mL plastic syringe (Omnifix, B. Braun Melsungen AG, Germany).

3) The appropriate amount of redistilled acetaldehyde, ethyl acetate or ethanol kept at 0 °C was added to a jar through a rubber septum in the jar lid using a Hamilton glass microsyringe.

4) Compounds were brought to room temperature and thoroughly mixed by the use of a magnetic stirrer.

5) Air was added to each jar to bring the equivalent volume of gas in the jars to 2 L and the jar atmosphere allowed to mix for a further period at room temperature.

Calculation of volume of liquid to add to jars for stock standards was:

1) Number of moles of gas in the jar, assuming all compounds behave as perfect gases:

$$n = \frac{p_{tot} V}{R\,(T + 273.15)}$$

where: n = absolute amount of gas in sample (mol)

p_{tot} = total pressure in system (Pa), assumed to be 101325

R = gas constant (8.3143 $m^3 \cdot Pa \cdot mol^{-1} \cdot K^{-1}$)

T = temperature (°C)

V = volume (m^3)

2) Moles of standard in jar: n_{gas} = mole fraction x n

3) Mass standard: $M = n_{gas}$ x molecular mass of gas

4) Volume standard at 0 °C: $V = \frac{M}{\text{density of standard at 0 °C}}$.

Table A1.1. Calculation of headspace stock standards (5000 $\mu L \cdot L^{-1}$).

	Ethanol	Ethyl acetate	Acetaldehyde
Molecular mass ($g \cdot mol^{-1}$)	46.07	88.106	44.053
Density ($kg \cdot m^{-3}$)	806.25	902.00	834.00
Jar Volume (mL)	1824.4	1822.2	1826.5
Moles of gas in jar* (mol)	8.3575×10^{-2}	8.3474×10^{-2}	8.3761×10^{-2}
Moles of standard in jar** (mol)	4.1788×10^{-4}	4.1737×10^{-4}	4.1835×10^{-4}
Mass of standard (kg)	1.9251×10^{-5}	3.6773×10^{-5}	1.8430×10^{-5}
Volume of standard (µL)	23.88	40.77	22.10

* Pressure is 111653 for 2 L of air at standard pressure compressed into a 1.8 L jar at 20 °C.
** mole fraction of 5000 $\mu L \cdot L^{-1}$ is 5×10^{-3} $mol \cdot mol^{-1}$.

From stock solutions a combined standard of 100 $\mu L \cdot L^{-1}$ ethanol, ethyl acetate and acetaldehyde was prepared in a sealed 1.8 L jar as follows:
1) Volumes of 40 mL of 5000 $\mu L \cdot L^{-1}$ stock standards were transferred to the combined standard jar using separate 60 mL plastic syringes.
2) About 80 mL of air, depending on individual jar volumes, was added to pressurise the jar by bringing the total compressed volume to 2 L and the mixture was stirred using a magnetic stirrer.
3) One mL samples of this gas mixture were used to calibrate the gas chromatograph for headspace volatiles.

A1.6 Headspace standards for skin disks

Headspace standards for experiments using skin disks were prepared as in 1.6 using the volumes of liquids at 0°C presented in Table A1.2.

Table A1.2. Volumes of headspace standards required for 100 $\mu L \cdot L^{-1}$.

Chemical	Molecular weight ($g \cdot mol^{-1}$)	Density ($kg \cdot m^3$)	Volume to add (μL)[1]
Acetaldehyde	44.05	783.4	0.44
Methanol	32.04	791.4	0.33
Ethanol	46.07	790.0	0.48
Ethyl acetate	88.12	900.0	0.82
Propan-1-ol	60.10	800.0	0.63
Propyl acetate	102.13	887.8	0.96
Butan-1-ol	74.12	809.8	0.76
Butyl acetate	116.16	880.0	1.10

[1] Jar volume = 1821.96 mL.

A1.7 Calculation of volatile concentration in juice samples

1) Correct for losses of the internal standard (IS) during the solvent extraction process.

$$\text{Corrected IS} = \frac{\text{measured IS}}{\text{wash recovery x drying recovery}}$$

$$\text{Concentration correction} = \frac{\text{nominal IS}}{\text{corrected IS}}$$

2) Correct for drying losses and sample volatile concentration relative to IS.

$$\text{Sample concentration (SC)} = \frac{\text{sample measurement x concentration correction}}{\text{drying recovery}}$$

3) Express sample concentration as moles of volatile as a liquid per litre of juice.

$$\text{Volatile concentration} = \frac{\text{SC x D}}{\text{MW}}$$

where:
SC = sample concentration ($\mu L \cdot L^{-1}$)
D = volatile density ($g \cdot L^{-1}$)
MW = molecular weight ($g \cdot mol^{-1}$)
units: $\mu L \cdot L^{-1}$ x $g \cdot L^{-1}$ x $mol \cdot g^{-1}$ = $\mu mol \cdot L^{-1}$

Example calculation:
Volatile: Butyl acetate (BA)
IS measured concentration: 79.60 $\mu L \cdot L^{-1}$
IS nominal concentration: 50 $\mu L \cdot L^{-1}$
Wash recovery of IS: 76.51 %
Relative molar mass: 116.16 $g \cdot mol^{-1}$
sample concentration: 37.54 $\mu L \cdot L^{-1}$
drying recovery of IS: 83.13 %
density: 880 $g \cdot L^{-1}$
drying recovery of BA: 83.55 %

$$\text{1) Internal standard} = \frac{79.6}{0.7651 \text{ x } 0.8313}$$

$= 125.15\ \mu L \cdot L^{-1}$

2) IS correction $= \frac{50}{125.15}$

$= 0.400$

3) Sample concentration $= \frac{37.54 \times 0.4000}{0.8355}$

$= 17.95\ \mu L \cdot L^{-1}$

4) $\mu mol \cdot L^{-1}$ Butyl acetate $= \frac{17.95 \times 880}{116.16}$

$= \frac{12733.6}{116.16}$

$= 109.62\ \mu mol \cdot L^{-1}$

A1.8 Calculation of headspace volatile concentration

A1.8.1 Headspace

1) Free air volume of container:

Air volume = container volume - juice or tissue volume

2) Number of moles of air:

$$n_{air} = \frac{P_{tot} V}{R(T + 273.15)}$$

where: n_{air} = absolute amount of gas in contianer (mol)

P_{tot} = total pressure in the system (Pa)

V = volume of air (m^3)

R = gas constant (8.3143 $m^3 \cdot Pa \cdot mol^{-1} \cdot K^{-1}$)

T = temperature (°C)

3) Number of moles of a volatile as a gas:

mole fraction = $\mu L \cdot L^{-1}$ ($mol \cdot mol^{-1}$)

$n_{volatile}$ = mole fraction x n_{air} (mol)

Moles of gas in headspace $= \frac{n_{volatile}}{V}$ (mol·L^{-1})

Tenax® traps used the same formulae but used an air volume of 5000 mL as this was the total volume of gas passed through each trap (Chapter 4).

A1.8.2 Juice concentration

1) Concentration of volatile in liquid:

$$C_{liquid} = \frac{C_{gas}}{\text{Partition coefficient}}$$

where: C_{liquid} = concentration in liquid phase
C_{gas} = concentration in gas phase

Example calculation:
Volatile: Ethanol
Ethanol concentration: 20 µL·L^{-1} Container volume: 55.51 mL
Juice volume: 30 mL Temperature: 20 °C
Relative molar mass: 46.07 g·mol^{-1} Partition co-efficient: 2 x 10^{-4}

1) Air volume = 55.15 - 30

= 25.15 mL

2) $n_{air} = \frac{101325 \times 2.515 \times 10^{-5}}{8.3143 \times 293.15}$

$= \frac{2.548}{2437.34}$

= 1.045 x 10^{-3} mol

3) $n_{volatile}$ = 2 x 10^{-5} x 1.045 x 10^{-3}

= 2.09 x 10^{-8} mol

4) $Ethanol_{gas} = \frac{2.09 \times 10^{-8}}{2.515 \times 10^{-2}}$

= 0.831 µmol·L^{-1}

5) $$\text{Ethanol}_{liquid} = \frac{0.831}{2 \times 10^{-4}}$$

$$= 4155\ \mu\text{mol}\cdot\text{L}^{-1}$$

Appendix 2

Disk and buffer system.

A2.1 Determining isotonic strength of solutions for apple disks

Isotonic strength of solutions for use with apple disks was determined using a gravimetric method (Tian, 1991). Four replicates of 10 disks from Splendour and Golden Delicious apples, 10 mm diameter about 1 mm thick, in petri dishes were incubated in mannitol solutions up to 4 hours. Mannitol concentrations were 0.2, 0.4, 0.6, 0.8 M. Disk weights were accurately measured before and after incubation on a 4 decimal place electronic balance (Mettler PE360). Results are expressed as increase or decrease in tissue weight (Figure A2.1). Splendour apples had no weight gain or loss at 0.6M, Golden Delicious apples had no weight gain or loss at about 0.4 M mannitol. An osmotic strength of 0.5 M was considered to represent the average isotonic strength of most apple cultivars and was used in experiments with apple skin disks.

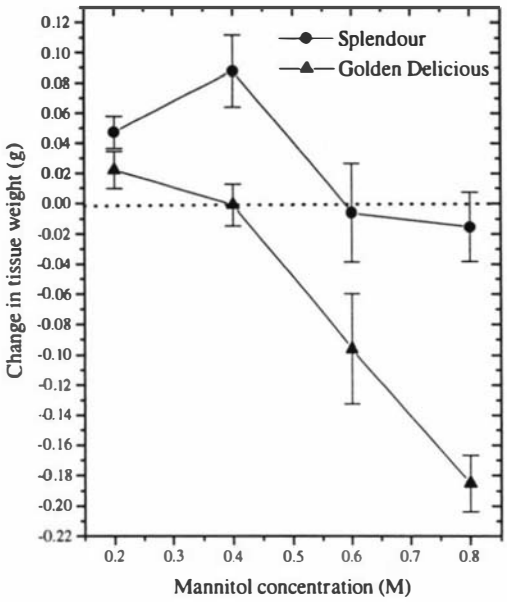

Figure A2.1 Change in tissue weight after 4 hours of incubation in mannitol solutions of various osmotic strength. Vertical bars represent standard errors of the mean.

A2.2 Comparison of buffers using KCl or Mannitol for osmotic adjustment

Volatile emissions were compared from Red Delicious apple disks treated with C_2-C_6 alcohol precursors incubated in a citric acid–disodium hydrogen orthophosphate buffer using either KCl or mannitol for osmotic adjustment (Figure A2.2). Volatiles were collected using adsorbent traps (Section 2.7.2). There was no difference in volatile emissions for low boiling point acetate esters, C_2 to C_4, and more higher boiling point acetate esters

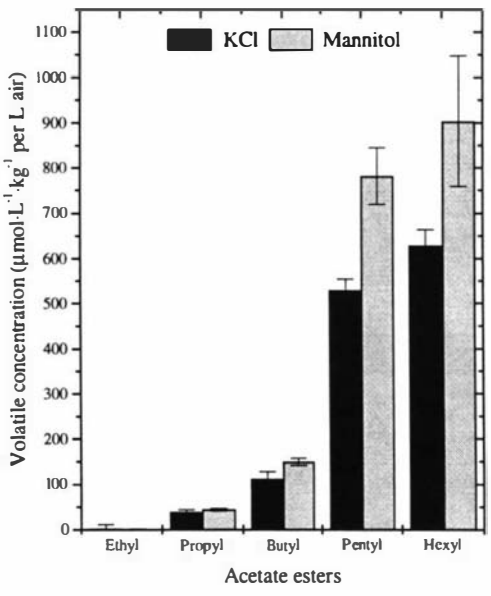

Figure A2.2 Acetate ester emmission from RD apple disks after addition of C_2 to C_6 alcohols and incubation in a citric acid-dihydrogen phosphate buffer osmotic strength 0.5M.

were purged from disks in mannitol compared to KCl. This may have been due to a 'salting out' effect of KCl (Bemelmans, 1981) decreasing the solubility of esters from the buffer solution resulting in lower equilibrium concentrations and greater losses in ester during purging before volatile trapping.

A2.3 Optimal pH of buffer for maximum ester production

Ten Albany Beauty skin disks, 10 mm diameter about 2 mm thick, were incubated in 100 mLs of a citric acid--dihydrogen orthophosphate buffer (McIlvaine buffer, Dawson et al., 1969) ionic strength 0.5 M using KCl with 10.93 mmol butan-1-ol up to 24 hours. Volatiles were collected by purging air through adsorbent traps for periods of 80 to 127 minutes. Butyl acetate production was assessed for pH values of 4.5, 5.5, 6.5 and 7.5 (Figure A2.3). Disks at pH 5.5 had the greatest concentration of butyl acetate. This pH is similar to the pH 5.8 McIlvaine buffer used by Berger and Drawert (1984). A pH of 5.8 was used for experiments using apple skin disks.

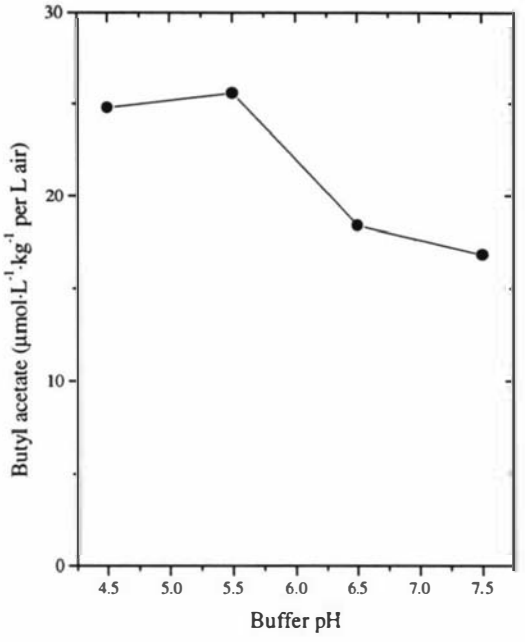

Figure A2.3 Butyl acetate production of Albany Beauty apple skin disks.

A2.4 Production of butyl acetate over time after addition of precursor

Three replicates of 50 Albany Beauty apple skin disks, 10 mm diameter about 1 mm thick, were incubated in 100 mLs of McIlvaine buffer osmotic strength 0.5 M pH 5.8 after addition of 5 mmol butan-1-ol for up to 48 hours. Volatiles were collected by purging air through adsorbent traps (Chapter 5) for 30 minutes. Butyl acetate concentration had increased by 3 to 5 h after addition of butan-1-ol and continued to be greater than initial measurements 24 h later (Figure A2.4). By 48 hours after addition of butan-1-ol, butyl acetate

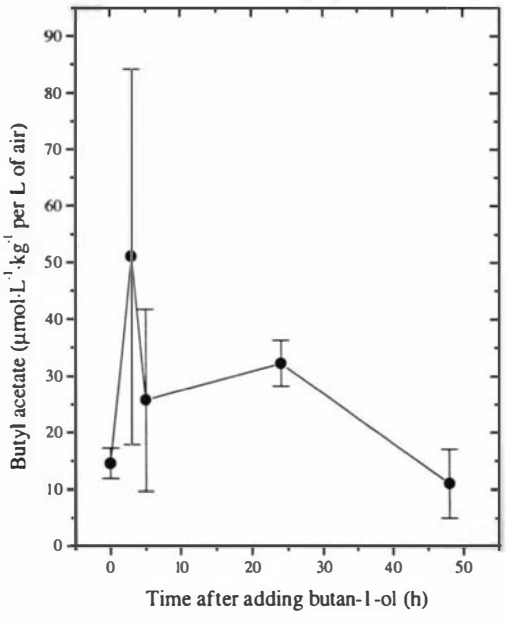

Figure A2.4 Production of butyl acetate from Albany Beauty apples after addition of butan-1-ol.

concentration was similar to initial measurements but the buffer solution was discoloured possibly indicating presence of microbial organisms. These results were similar to those found by other researchers (Berger and Drawert, 1984). Based on this data volatiles were trapped emanating from skin disks after 12 hours of incubation in all subsequent work.

A2.5 Volume of air to trap

Three replicates of 50 Albany Beauty apple skin disks, 10 mm diameter about 1 mm thick, were incubated in 100 mLs of McIlvaine buffer osmotic strength 0.5 M pH 5.8 after addition of 5 mmol butan-1-ol for 12 hours. Volatiles were collected by purging 1, 2.5, 5, 7.5 or 10 L of air through adsorbent traps. Each adsorbent trap has a different resistance to air flow therefore the flowrate of air through each trap was measured and used to calculate the duration of trapping. Traps purged with 2.5 to 10 L of air had accumulated similar amounts of butyl acetate concentrations (Figure A2.5). One L of air trapped had less than 25% of the butyl acetate trapped with greater volumes. Variability in butyl acetate concentration amongst traps reduced as the volume of air passed through increased. This may indicate that the adsorbent traps had become saturated with respect to butyl acetate at volumes above 2.5 L. A volume of 5 L was selected as there was low variability in butyl acetate concentration.

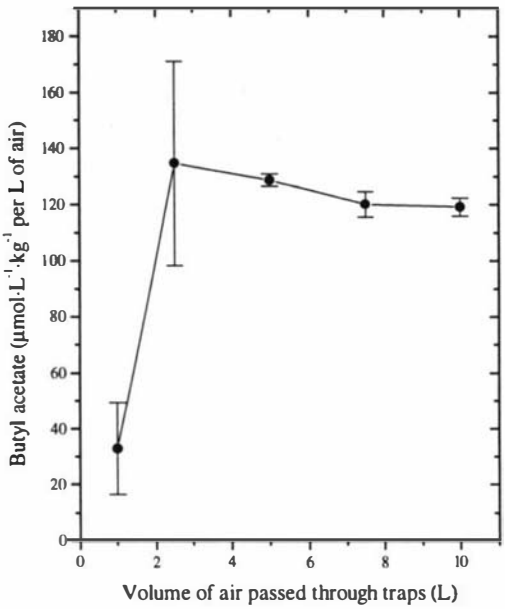

Figure A2.5 Butyl acetate production of Albany Beauty skin disks after incubation with butan-1-ol and different volumes of air passing through adsorbent traps.

A2.6 Concentration of precursor

Duplicate samples of 50 Oratia Beauty apple skin disks, 10 mm diameter about 1 mm thick, were incubated in 100 mLs of McIlvaine buffer osmotic strength 0.5 M pH 5.8 after addition of 0, 0.9, 2.3, 4.6, 6.8 or 9.1 mmol butan-1-ol for 12 hours. Volatiles were collected by purging 5 L of air through adsorbent traps. Butyl acetate concentration increased with increase in butan-1-ol concentration, reaching a maximum at 6.8 mmol butan-1-ol (Figure A2.6). Butan-1-ol concentrations above 6.8

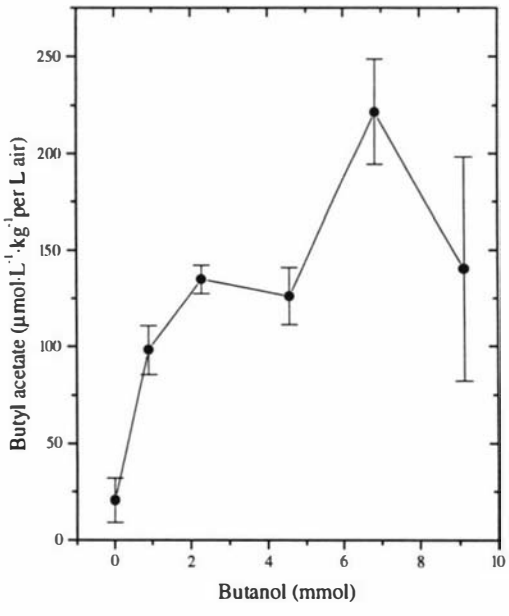

Figure A2.6 Butyl acetate production after addition of butan-1-ol.

mmol resulted in the same butyl acetate concentration. Concentrations of alcohol precursors used in experiments on skin disks were in the concentration range of 3 to 5 mmol .

A2.7 Concentration of volatiles in skin disks and buffer

It is important to determine in experiments feeding precursors to tissue disks if the precursors got into the tissue to be metabolised. Six replicates of 50 RD apple skin disks, 10 mm diameter about 1 mm thick, were incubated in 100 mLs of McIlvaine buffer osmotic strength 0.5 M pH 5.8 after addition of 5 mmol of a mixture of 1 mmol C_2 to C_6 alcohols for 12 hours. After purging disks were separated from the buffer using a sieve and ground to a fine paste using a high speed homogeniser (Polytron PT 10–35, Kinematica GmbH, Littan–Luzern, Switzerland), the volatiles were extracted by solvent extraction (Section 2.7). There was no difference in alcohol concentration of skin disks and buffer (Figure A2.7a) but concentrations in both were very high indicating that the skin disks had absorbed significant quantities of alcohols. Skin disks had greater concentration of acetate esters than buffer (Figure A2.7b). This would indicate that alcohols added to the disks (Chapter 5) were metabolised into their corresponding acetate esters.

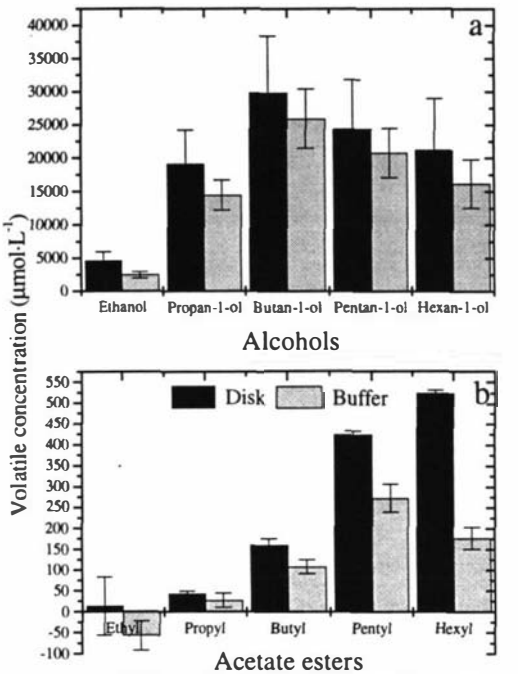

Figure A2.7 Concentration of a) alcohols and b) acetate esters in buffer or RD apple s in dis s after incubation with C_2 to C_6 alcohols.

A2.8 References

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Appendix 3

Aroma profiles of individual apple cultivars stored in air and exposed to hypoxia.

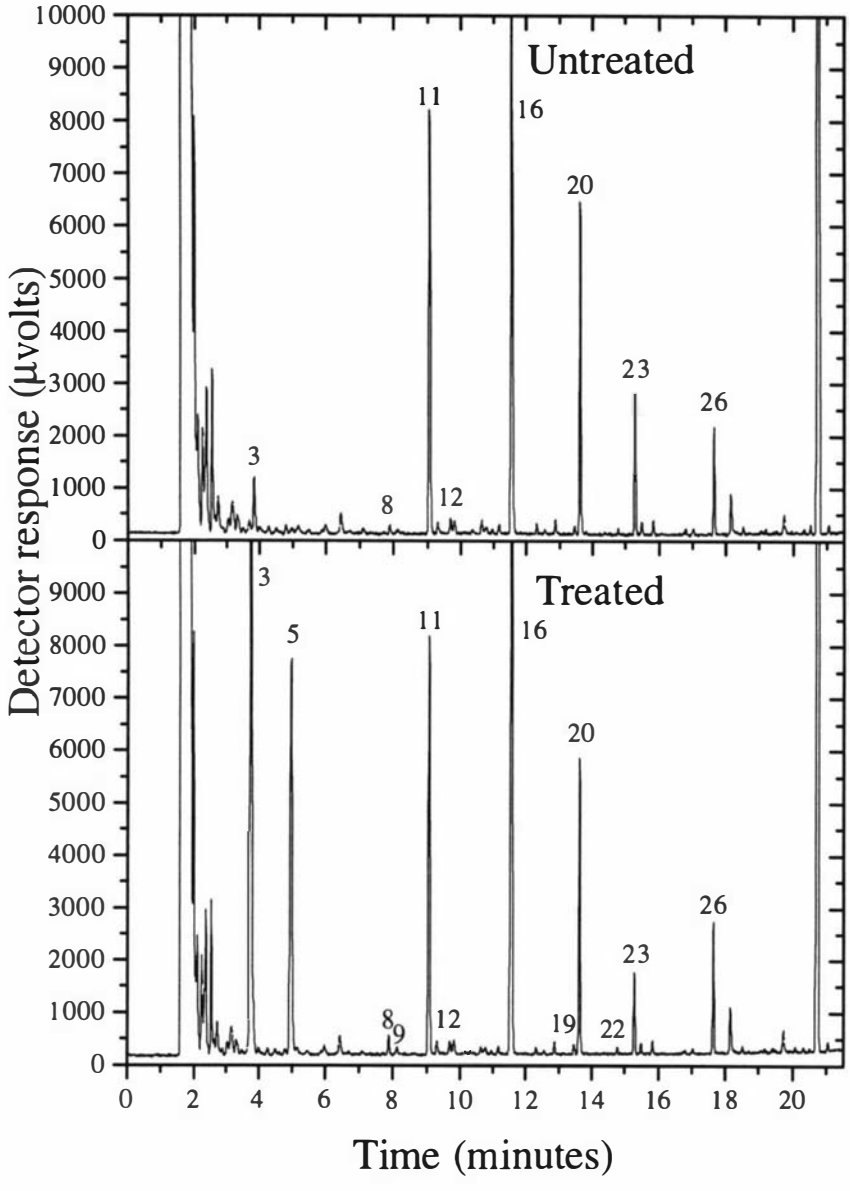

Figure A3.1 Aroma profiles of **Cox's Orange Pippin** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).

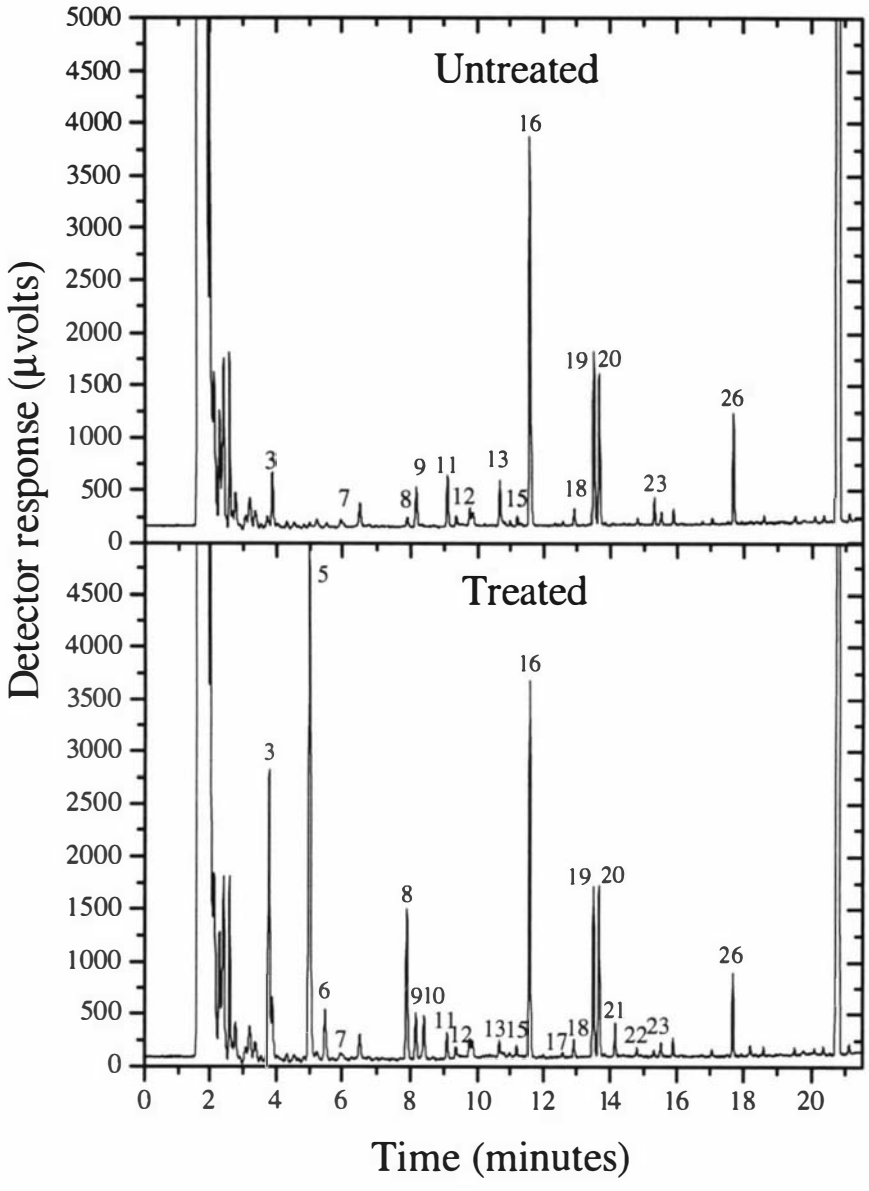

Figure A3.2 Aroma profiles of **Fuji** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).

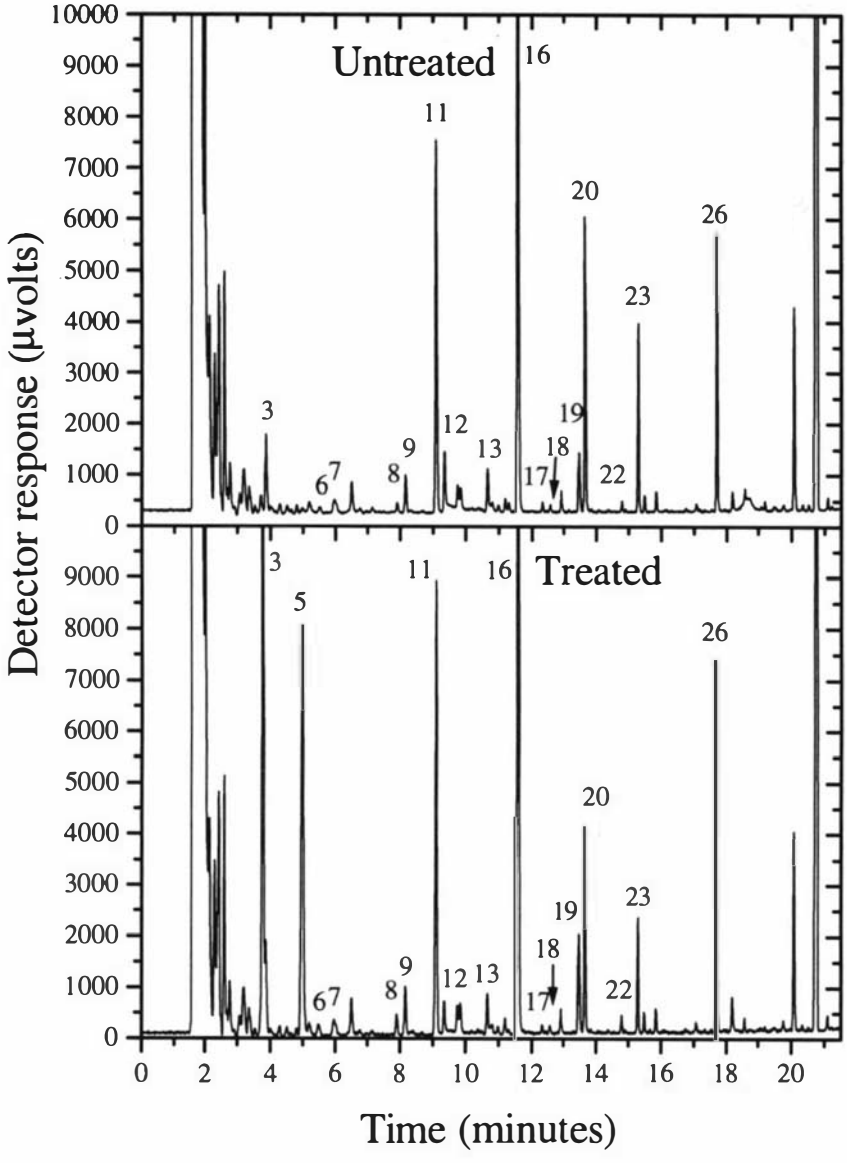

Figure A3.3 Aroma profiles of **Golden Delicious** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).

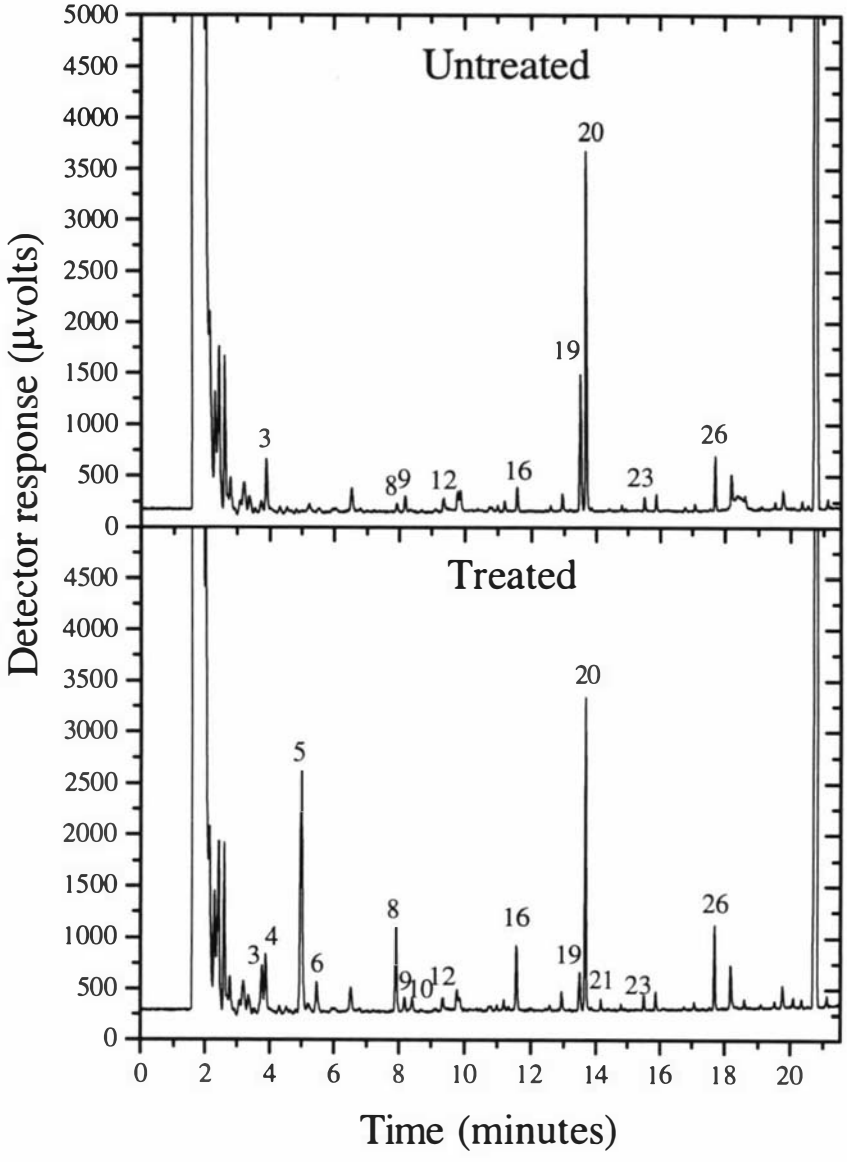

Figure A3.4 Aroma profiles of **Granny Smith** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).

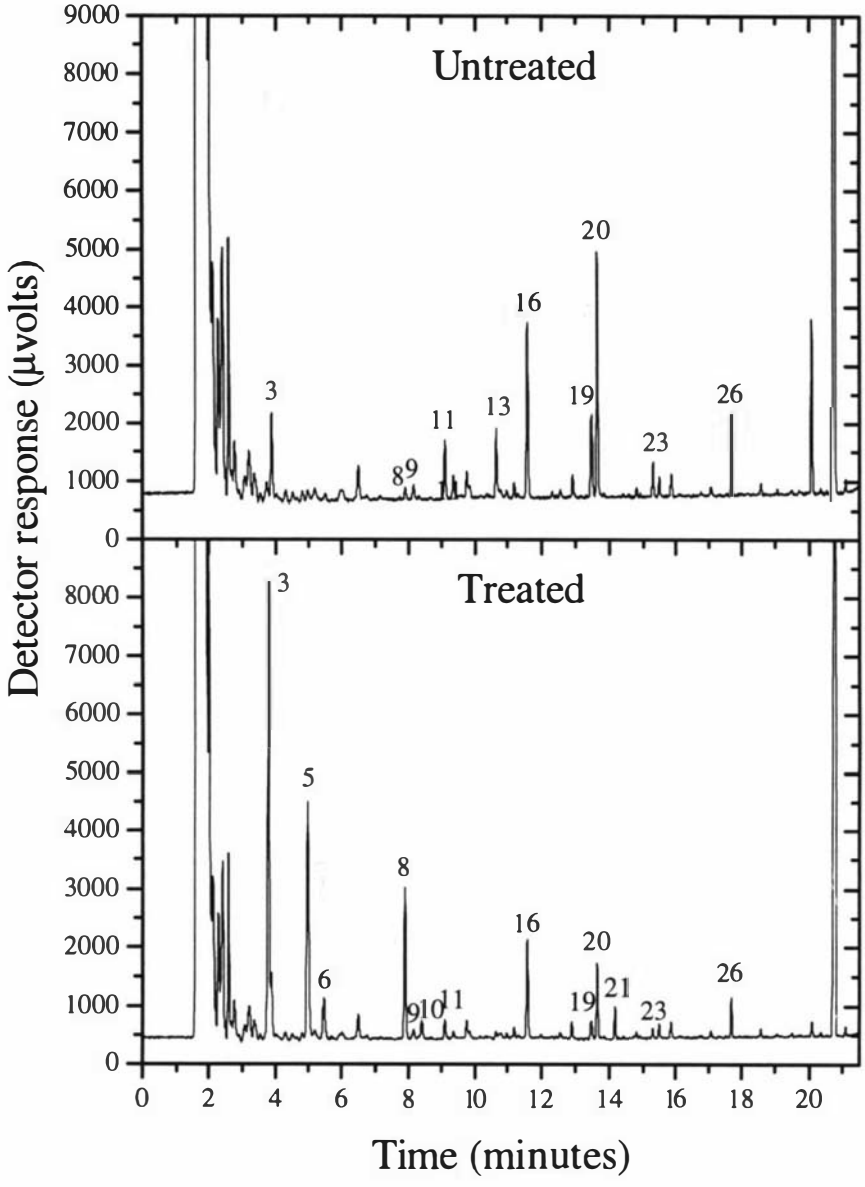

Figure A3.5 Aroma profiles of **Pacific Rose** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).

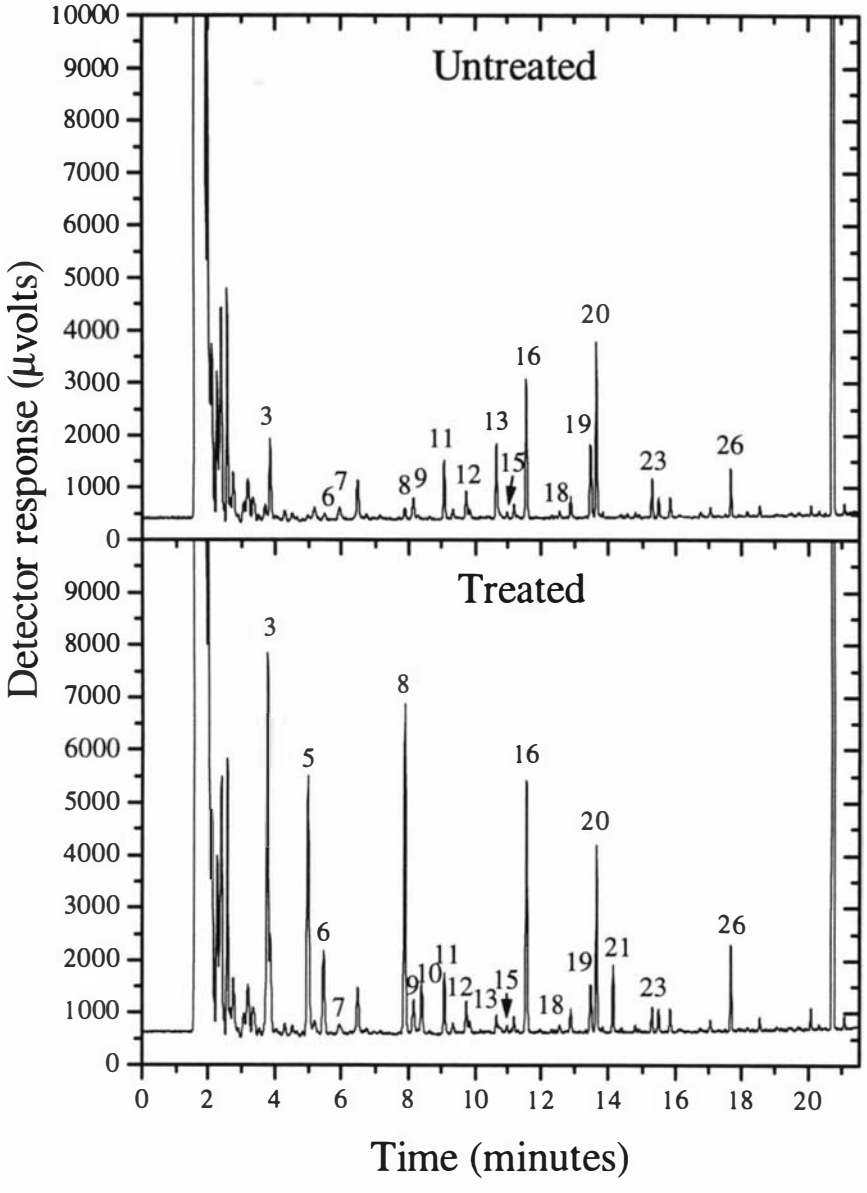

Figure A3.6 Aroma profiles of **Red Delicious** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).

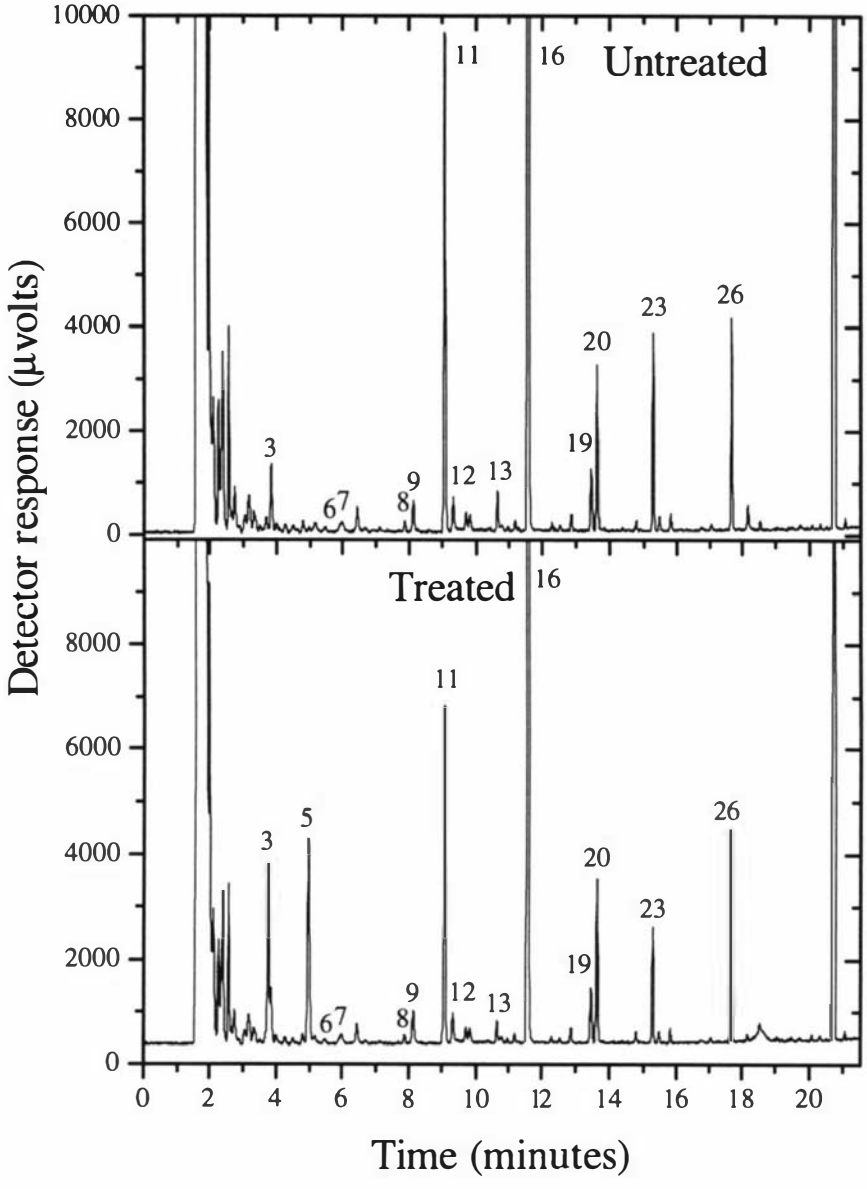

Figure A3.7 Aroma profiles of **Royal Gala** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).

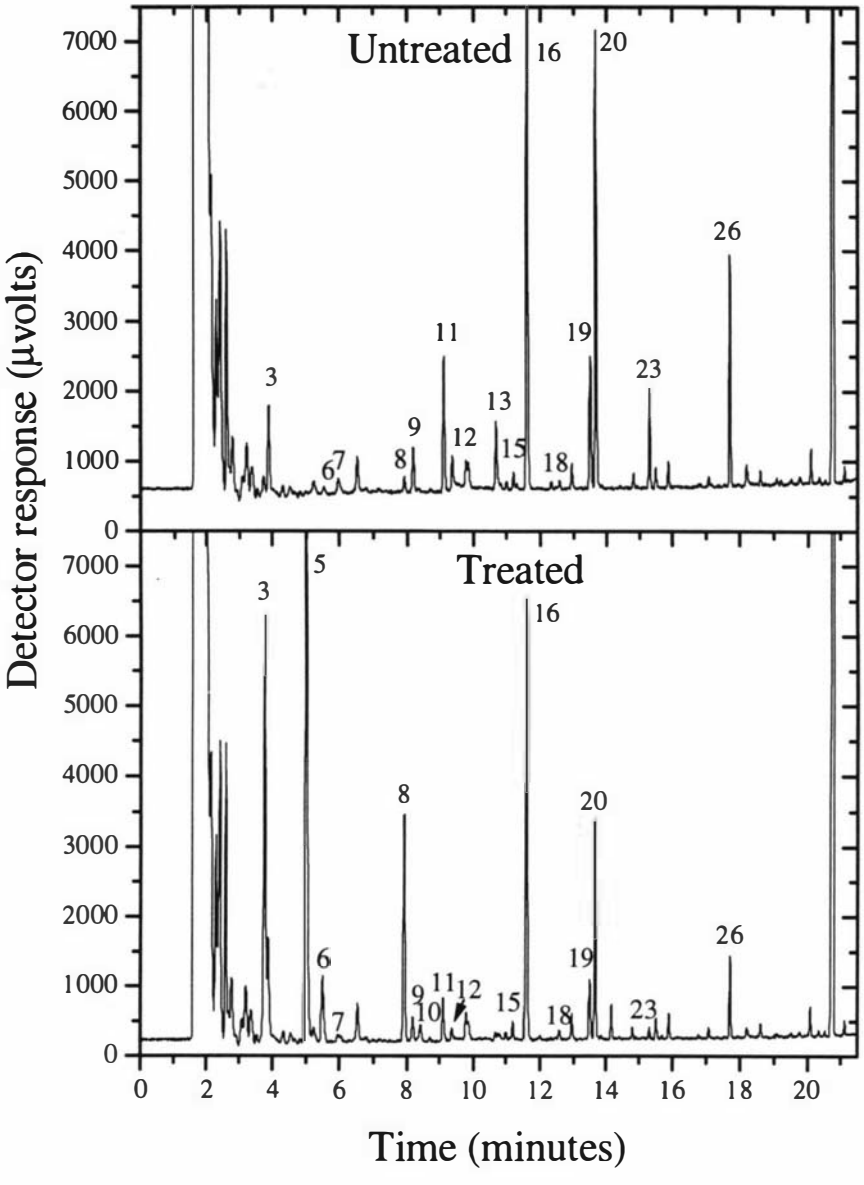

Figure A3.8 Aroma profiles of **Splendour** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).

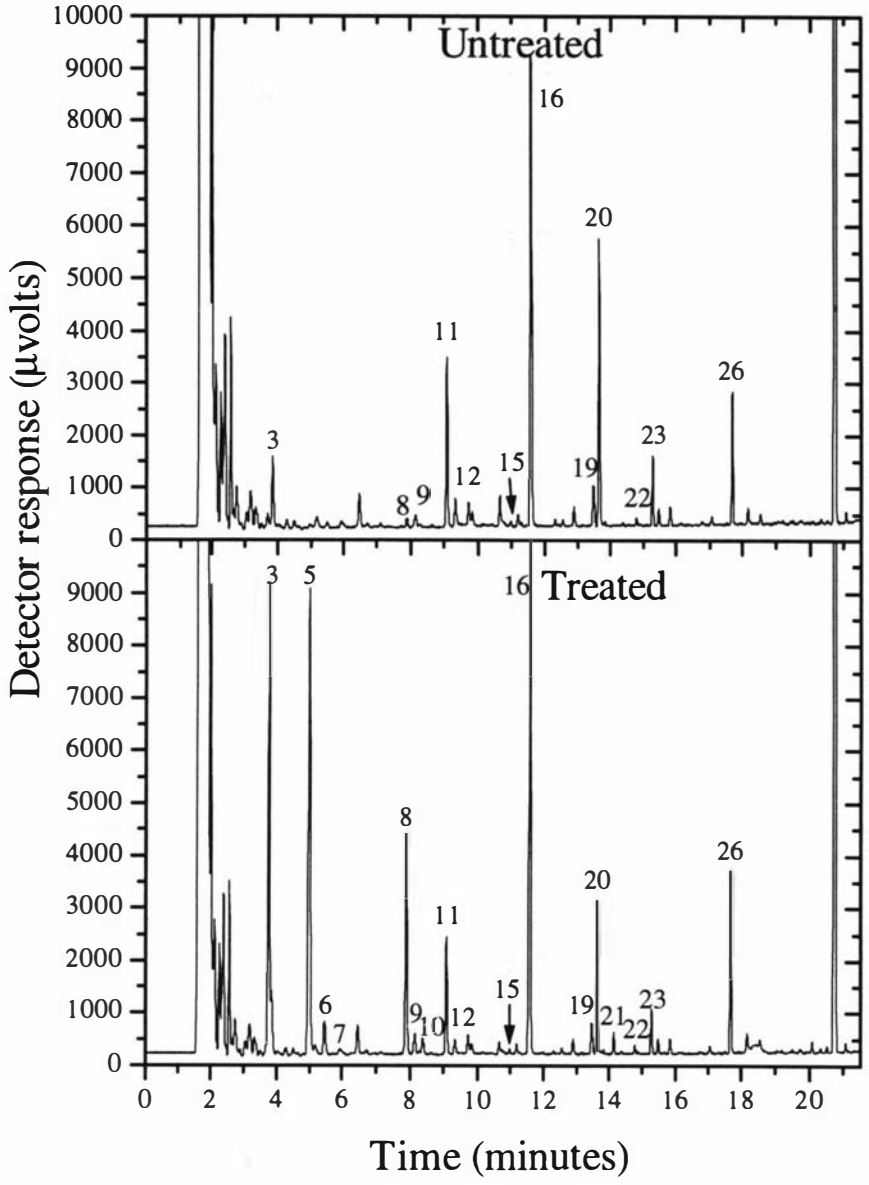

Figure A3.9 Aroma profiles of **Southern Snap** apples untreated and hypoxic treated (100% CO_2 for 24 hours at 20 °C) after 3 days ripening at 20 °C after treatment. Volatiles were extracted by solvent extraction (Chapter 2, Section 2.7) and analysed by GLC (Chapter 2, Section 2.8.4).