Enhancement of Aroma Volatile Compounds in Apples

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ABSTRACT

Apple aroma is a complex of volatile compounds produced by fruit. It is one of the most important quality factors giving a characteristic flavour to fruit and its products. The present study investigated the effect of short-term hypoxic concentrations and duration of CO_2 and/or N_2 treatments at 20°C on changes in production of important aroma volatile compounds after treatment during shelf-life conditions at 20°C and 70% RH, in some commercial apple cultivars at harvest and following storage in air or controlled-atmospheres (CA).

Different methods of extracting and analysing aroma volatiles from apples were compared in order to optimise a process for subsequent experiments. Juice from homogenised apple fruit could be held in an ice bath for up to 60 minutes after homogenisation without loss of major volatile compounds. Octyl acetate concentrations between 50 and 5000 ppm in the final concentrated sample was a suitable internal standard for GLC analysis; as an external standard, a mixture of authentic volatile compounds of known concentration provided accurate quantification of aroma volatiles from juice samples. Volatiles were obtained from apple juice either by a dynamic headspace (purge and trap) technique using a Tenax® trap or by direct extraction with a diethyl ether:n-pentane (2:1, v/v) mixture. The solvent extraction technique was simpler and faster than the purge and trap method, taking 5 seconds rather than 1 hour; it was also more efficient, resulting in 1.7 fold more total volatiles with better reproducibility.

Application of hypoxic gas atmosphere to apples (100% CO_2, 100% N_2 or < 2.5% O_2) demonstrated consistently enhanced headspace acetaldehyde, ethanol and ethyl acetate in 'Granny Smith', 'Braeburn' and 'Red Delicious' cultivars. A marked increased production of ethanol in response to hypoxic treatments was always followed by a several fold enhancement in both number and concentration of ethyl esters, including the aroma-impact volatiles ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate in juice from all cultivars. The increased concentration of these compounds

was accompanied by decreased production of other esters requiring the same acyl group for their synthesis, such as propyl butanoate, butyl acetate and hexyl acetate, and some alcohols and aldehydes, including hexan-1-ol, 2/3-methyl butan-1-ol and *trans*-2-hexenal. These results confirmed the hypothesis of the competitive nature of the acyl esterifying system in apples. Exposing apples to 100% CO_2 for 18 - 24 hours at 20°C achieved optimum enhancement; a longer time (48 hours) did not further increase important aroma-impact ethyl esters but caused a marked reduction in non-ethyl esters, while a shorter period of exposure (6 - 12 hours) had minimal effect. Apples previously stored (0°C) for 6 months or longer before exposure to hypoxic treatments did not have important volatile ethyl esters enhanced. Application of 1000 ppm ethylene for 24 hours did not increase volatile production in apples compared with non-treated fruit.

Poststorage production of most aroma volatile compounds from 'Granny Smith' and 'Fuji' apples stored in a controlled-atmosphere (CA) of 2% CO_2 + 2% O_2 at 0.5°C was depressed after 10 - 14 weeks storage, although 2-methyl butan-1-ol was enhanced and there was no effect on *trans*-2-hexenal. Poststorage treatment with 100% CO_2 (24 hours at 20°C) consistently and markedly enhanced headspace acetaldehyde, headspace ethanol, headspace ethyl acetate and aroma impact compounds, ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate in fruit from both refrigerated air (RA) and CA storage regimes. The hypoxic treatment had no effect on production of butan-1-ol and hexan-1-ol in CA, or hexanal in RA 'Granny Smith', propan-1-ol and butan-1-ol in either RA or CA, or hexanal and *trans*-2-hexenal in RA 'Fuji' apples. Hypoxic treatments reduced production of butyl and hexyl acetate in 'Fuji' apples, and 2-methyl butan-1-ol in both cultivars. The ability to sustain enhanced production of ethyl esters in CO_2 treated RA or CA 'Granny Smith' or CA 'Fuji' apples decreased as time of storage progressed, probably due to shortage of acyl CoA substrates for volatile synthesis. CA retarded flesh softening and acid loss in both cultivars.

Examination of volatile production from 6 commercial apple cultivars established that esters, alcohols and hydrocarbons accounted for 70% - 88% of the total number of volatiles found and compounds with straight chain and even numbered carbons predominated in the composition of esters and alcohols. Differences among apple

cultivars were associated with number and concentration of volatile compounds produced. Treatment with 100% CO_2 (24 hours at 20°C) consistently enhanced production of headspace volatiles in all cultivars. Production of aroma impact ethyl esters following hypoxic treatments consistently occurred with 'Red Delicious', 'Braeburn', 'Granny Smith' and 'Pacific Rose', with less of a response in 'Cox's Orange Pippin' and none in 'Royal Gala' apples. Non ethyl esters and alcohols, other than ethanol and propan-1-ol, were reduced after CO_2 treatment in all cultivars, except for butan-1-ol in 'Cox's Orange Pippin' apples. Hexanal and *trans*-2-hexenal were either reduced or not affected following CO_2 treatment, depending on cultivar. The magnitude of enhancement and the degree of reduction in production of aroma volatile compounds following hypoxic treatment varied among cultivars; the higher the magnitude of enhancement of ethyl acetate, the greater the degree of reduction in other acetate esters, supporting the concept of a competitive interaction in the acyl esterification in apples.

Hypoxic treatments consistently caused profound increases in aroma volatiles in most apples cultivars studied. Such a treatment has potential to increase the strength and value of commercially important flavour fractions in apple juice and aqueous essence and enhance aroma in fresh apples.

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Chapter One

GENERAL INTRODUCTION

1.1 GENERAL BACKGROUND

Apples (*Malus domestica* Borkh.) are amongst the most extensively cultivated and widely consumed of temperate fruit, perhaps taking second place only to grapes (Lea, 1995). The annual world production is estimated to be in the order of 42 million metric tonnes (Willis, 1993), of which at least five million tonnes are processed into juice (Lea, 1995). The world major apple producers are the EEC (22%), the former USSR (15%), USA (11%) and China (11%). New Zealand produces less than 1% of the world pipfruit production, but it has a larger share of international market for export supply, ie. about 5% (Willis, 1993).

In New Zealand, horticultural exports represented 7.1% of the total export (Anon., 1995b). Fresh apples are New Zealand's leading horticultural export accounting for 33.1% of total horticultural exports for the year ending 30 June 1996 (**Fig. 1.1**). In terms of fresh fruits, fresh apples account for 52% of the total fresh fruit exports and about 96% of the total pipfruit, ie. apples, pears and nashi pears (Mirams, 1996). Of the total New Zealand apple production, about 45% of apples are either processed or consumed locally. It has been estimated that over 30% of the total apple production is processed, mainly into apple juice concentrates and essence, with a smaller part processed into other products such as slices, sauce and pie filling (Willis, 1993).

During the last five decades there have been substantial improvements in cultural practices, fruit preservation technologies, and cultivar selection of apples. A wide range of new apple cultivars are produced worldwide (Yahia, 1994) and many of them can be stored fresh for up to a year (Meheriuk, 1993). In New Zealand several new cultivars, such as 'Pacific Rose'™ and 'Southern Snap'™, have been developed and released to growers for commercial production (Anon., 1995c). This development has been made in order to meet the demand for new products by consumers (Steele, 1995) and to retain New Zealand's competitive edge in apple varieties in the world market (Anon., 1995a,

Willis, 1993). Although much has been achieved, improvement on apple flavour (taste and odour) has largely been neglected. Alston (1992) stated that plant breeders rarely aimed specifically to improve flavour, rather they usually planned to make overall improvement to a crop and expected to maintain flavour standards rather than improve them. Despite a great deal of research having been done on apple flavour, mainly on apple aroma, during the last 20 years and an increasing interest in the subject, apple flavour is still not well understood (Berger, 1991; Dimick and Hoskin, 1983; Paillard, 1990; Williams, 1979; Yahia, 1994).

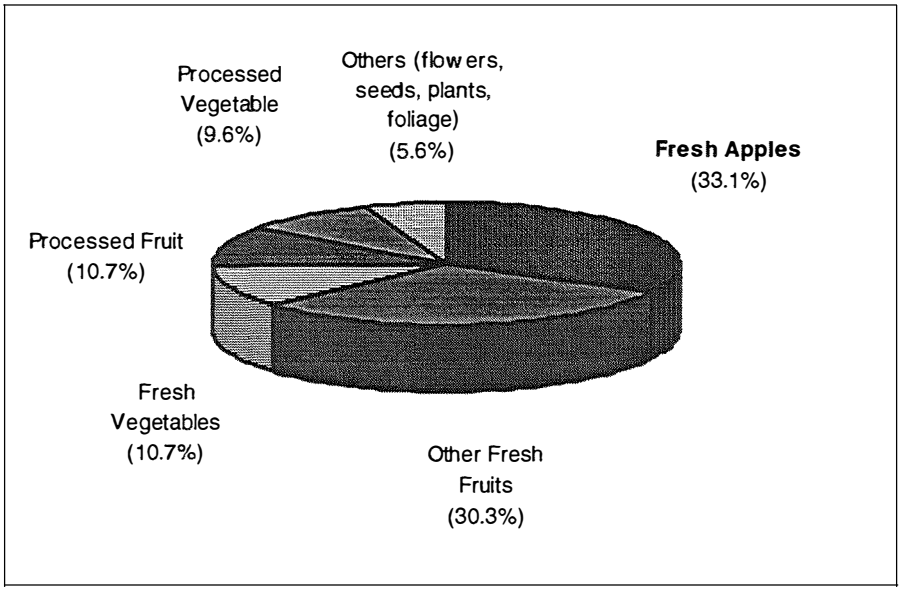


Fig. 1.1 Distribution of New Zealand horticultural exports for the year ending 30 June 1996 (derived from Mirams, 1996)

Flavour is complex in nature. It is a complex mixture of organic and inorganic compounds and it is determined by numerous sensations acting simultaneously in the brain (Lawless and Lee, 1993; Thomson, 1986). The components of flavour are interrelated and are difficult to differentiate, nevertheless, they can be divided into two major parts, namely taste and odour (Williams *et al.*, 1977a). Taste is the perception of chemical components by taste buds on the human tongue. Odour is perceived by receptors in the mucous layer of the ceiling of the nose (Thomson, 1986). Volatility of a

compound is an important factor contributing to odour perception; however, there are also several physico-chemical parameters, such as structural feature, molecular weight and partition coefficient, that allow a compound to possess odour (Boelens and van Gemert, 1986). It is still not known why a compound is odorous nor is the mechanism by which we perceive it well understood (Acree, 1993). Several quality factors, such as texture, of a product, can also affect flavour perception (Thomson, 1986). All factors affecting fruit during growth, development, maturation, ripening, and senescence, as well as the postharvest storage environment, can affect development and metabolism of flavour formation of fruit (Paillard, 1981).

Because the fundamental nature of flavour perception is still not known, an objective means of quantifying flavour has not been fully developed. Thomson (1986) suggested that flavour is a psychological phenomenon, not a physico-chemical phenomenon, and it can only be evaluated or measured by sensory methods. However, any sensory method is mainly subjective and it can be very expensive and time consuming to conduct. Thus, the search for further understanding of human flavour perception in relation to sensory response to flavour may be needed to develop a more objective technique of measurement.

This review presents a summary of the components of apple flavour, with an emphasis on aroma volatile compounds, how they are formed and how they are affected by various preharvest and postharvest factors and treatments.

1.2 APPLE FLAVOUR

Flavour of apple and apple juice appears to be a result of a delicate balance of sweet, sour, and astringent taste and aroma of a number of volatile compounds (Dürr and Schobinger, 1981; Kingston, 1992; Yahia, 1994). Although flavour is mainly a function of taste and odour, other qualities, such as texture, contribute to the overall perception (deMan, 1990). Several attempts have been made to identify and understand apple flavour and to evaluate it objectively using sensory analysis (Watada *et al.*, 1980; Williams *et al.*, 1977a). The sensory attributes associated with flavour and taste of apples have been reported to comprise of sweetness, sourness, astringency, floral-fruitiness,

mustiness, juiciness, crispness, hardness, starchiness, and mealiness (Watada *et al.*, 1981). The importance of flavour, especially aroma, to the quality of apple and apple juice has not been clearly defined (Lea, 1995; Yahia, 1994). Poll (1981) reported that aroma intensity of apple cultivars had a strong influence on the overall judgement of apple juice quality, in that the stronger the fruit aroma, the higher the overall fruit quality of 18 varieties tested. von Sydow *et al.* (1974) suggested that taste-odour interactions can occur at two different levels: in the food itself (physical or chemical interaction) and in the brain (a sensory or perceptual interaction). They found that addition of sucrose to apple juice slightly changed the headspace composition but did not affect the 'sniffing' odour. However, it did reduce the astringent-bitter taste and unpleasant odour ('vinegar' or 'green'), while it increased pleasantness ('sweet' odour) and overall acceptability. von Sydow *et al.* (1974) concluded that sucrose appeared to enhance desirable aroma by reducing the harsh taste on a psychological level rather than acting upon the chemical constituents and thus modifying the vapour composition.

1.2.1 Apple Taste

Taste is generally considered to consist of the 4 basic attributes, sweetness, sourness, bitterness, and saltiness (Williams, 1979). Sweetness and sourness, and to a lesser extent astringency, are major contributors to the taste of fresh apple and apple juice (Poll, 1981; Watada *et al.*, 1980) and apple taste is mainly associated with balance between sugar and acid content (Poll, 1993). As sugars and acids in apples are easily measured and their synthesis and metabolism are fairly well understood, the influence of these components with respect to apple flavour will be briefly discussed.

The sourness or acidity of apples is due mainly to malic acid, and citric acid in some cultivars (Ulrich, 1970). Titratable acid (TA) is an indicator of the acid content and thus the degree of sourness of apple and apple juice (Boylston *et al.*, 1994; Poll, 1993). Poll (1993) suggested that titratable acid was possibly the most important quality factor of apple juice; a juice with a given sugar concentration and a low acid concentration was flat and tasteless, while a very high acid juice was sour and unpleasant to taste. The TA has been used as a maturity index of apples in conjunction with other indices, including

total soluble solids, firmness, and starch index (Kingston, 1992). TA of apples varies among cultivars, season, orchards, fertiliser application, and harvest date (Lau, 1988; Poll, 1983; Smith, 1984).

Sweetness of apples is mainly caused by the three sugars, fructose (quantitatively the main contributor), sucrose, and glucose (Fourie *et al.*, 1991). Sugar content can be measured directly by chemical means, but as sugar is the major component of soluble solids in apples, it is easier to measure total soluble solids (TSS) of extracted juice using a refractometer (Wills *et al.*, 1989). TSS can be used as an indicator for sweetness of fruit (Boylston *et al.*, 1994; Kingston, 1992). The TSS content of apples tends to increase as apples ripen and it is also useful as one of the harvest maturity indices (Blanpied, 1974). The TSS content of apples varies among cultivars, position of fruit on tree, season, and growing location (Kingston, 1992; Shaw and Rowe, 1982).

The TSS/TA ratio has been reported to be a better indicator than either TSS or TA alone in determining the perceived sweetness or tartness of apples or apple juice (Boylston *et al.*, 1994; Poll, 1981). In addition, it has also been reported that the TSS/TA ratio of USA apples correlates very well with days from full bloom, and as such is a commonly used guide to assist in determining picking date (Hammett *et al.*, 1977). Although sugars and acids contribute to sweetness and sourness, respectively, they are not equal in their effect on the palate; the relative sweetness and sourness thresholds, and the synergistic effect of their interaction, contribute more to the flavour character of fruit than mere acidity or sweetness alone (Williams, 1979).

Polyphenols are major contributors to astringency and bitterness of apples and apple juice (Poll, 1981; Williams, 1979). However, the amount of phenolic compounds present in ripe fruit are normally thought to be too low to contribute significantly to such astringency. Poll (1981) reported that polyphenol concentration did influence taste of apple juice and found a relatively good relationship between bitter-astringent taste and polyphenol concentration; apple juice with a high phenol content of above 900 $mg \cdot l^{-1}$ was bitter-astringent while that with a low phenol content (260 $mg \cdot l^{-1}$) was found to have 'a watery (thin) taste'.

1.2.2 Apple Aroma

Although taste and texture are crucially important to its perception, it is the presence of trace amounts of volatile compounds which is responsible for odour that gives much of the character to fruits and their processed products (Williams, 1979). Detection of volatile compounds at the part per million level or lower has become possible since the introduction of gas-liquid chromatography (GLC) in 1952 (Maga, 1990) and associated sensitive detectors, such as the flame ionisation detector (FID) (Maarse, 1991). Chromatographic systems have evolved tremendously since then and column technology has advanced to very high level of separation power (Westendorf, 1985). Combining a GLC, having a capillary column, with a modern mass spectrometer (GC-MS) or with a Fourier transform infrared spectrometer (GC-FTIR), or with GC-FTIR-MS, each one linked to a powerful computer, is a typical method used nowadays for determining major fruit flavour impact compounds (Maarse, 1991). Recently multidimensional GLC, where the second chiral column is used for the enantio-differentiation of isomeric compounds, has been introduced commercially for the authenticity control and/or adulteration of flavour compounds (Mosandl *et al.*, 1995; Rouseff and Leahy, 1995). As a result of such developments the number of volatile compounds identified has increased and in 1991 up to 356 compounds had been reported in apples and apple products (Maarse, 1991).

Williams *et al.* (1977a) were among the first scientists to measure flavour quality in apple juice; they analysed aroma by GLC and they sub-divided a precise combination of compounds which defined a specific odour. Using 'Ellison's Orange' apples, their taste panellists commented on the odour of a number of GLC isolated peaks, indicating fruity, apple, pear-drop, sugary, pineapple, estery, sharp, cooked apple, strong apple peel, green, green apple, and aniseed odours. The odour descriptions were those definitive of apples and/or apple juice. Numerous compounds undoubtedly contributed to each descriptor and it is most unlikely that one single compound is the sole agent producing the specific odour of apples (Berger, 1991; Dimick and Hoskin, 1983). When each apple volatile compound is isolated and its sensory description is defined (**Table**

1.1), only a few of such compounds are described as having 'apple' odour (Paillard, 1990; Williams *et al.*, 1977a).

Table 1.1 Some apple volatile compounds and their odour characteristics (from deMan, 1990; Dimick and Hoskin, 1983; Dürr, 1979; Dürr and Schobinger, 1981; Paillard, 1990; Williams *et al.*, 1977a).

Compound	Sensory Characteristic
Esters	
methyl butanoate	fruity
methyl hexanoate	sweet, fruity
2-and/or 3-methylbutyl acetate	sweet-fruity, banana, pear-like
ethyl acetate	fruity, solvent-like
ethyl propanoate	ethereal, fruity-rum-like odour
ethyl butanoate	fruity, banana, pineapple, sweet, ester-like
ethyl isobutanoate	sweet, fruity odour
ethyl pentanoate	floral, fruity
ethyl hexanoate	fruity, fresh, sweet, winey, ester-like
ethyl octanoate	estery, wax-like
ethyl nonanoate	cognac
ethyl 2-methylbutanoate	powerful, green-fruity, apple-like, pungent
ethyl 2-(*E*)-octanoate	fruity
ethyl 4-(*Z*)-decanoate	green
ethyl 3-hydroxy hexanoate	pungent
ethyl 3-hydroxy octanoate	fruity
propyl butanoate	pineapple, apricot
butyl acetate	pungent, very diffusive, ethereal-fruity,
butyl propanoate	fruity, pineapple
butyl butanoate	pear, pineapple
butyl pentanoate	apple, raspberry
butyl hexanoate	pineapple
pentyl acetate	fruity, banana oil, pineapple
hexyl acetate	fruity, floral, sweet
hexyl hexanoate	green
hexyl 2-methylbutanoate	sickly sweet
heptyl acetate	fruity, green, slight floral odour
Alcohols	
ethanol	sweet-ethereal, mild, alcoholic
n-propanol	alcoholic-nauseating, sweet odour
n-butanol	mild 'fusel'-like odour
isobutanol	mild, chemical, sweet, harsh when dilute
2/3-methylbutanol	pleasant (when highly dilute), fruity-winey, chocolate
n-hexanol	very penetrating, fatty-green grassy odour
cis-3-hexanol	powerful and intensely green, grassy, apple
trans-2-hexenol	powerful fruity-green, fruity, slightly caramel-like
n-octanol	soapy
Aldehydes	
acetaldehyde	pungent, ethereal, diluted coffee or wine
propanal	penetrating, suffocating odour, diluted roasted coffee
n-butanal	penetrating, pungent-irritating odour, diluted fruity
n-hexanal	very powerful penetrating, grassy, green apple

trans-2-hexenal	powerful, green apple, green fruity, pungent, vegetable
cis-3-hexanal	green, grassy
benzaldehyde	almond-like
Acids	
formic acid	pungent, acid
acetic acid	acid, sour, vinegary, sharp
propionic acid	acid, sour, pungent, rancid, cheesy
butanoic acid	acid, sweaty, rancid
hexanoic acid	sweaty, buttery, goaty
octanoic acid	rancid,
decanoic acid	soapy
Miscellaneous	
γ-hexalactone	sweet, powerful, warm herbaceous, coumarin-taste
γ-undecalactone	sweet, oily-fruity, peach-like taste
2-pentanone	floral
β-damascenone	fruity, perfumery, sweet
4-methoxyallyl benzene	spice-like, aniseed
cis/trans-linalool oxide	powerful sweet-woody, penetrating odour

Dürr and Röthlin (1981) developed a synthetic apple essence that was composed of a few commercially available chemicals as a reproducible standard for sensory analysis. This synthetic essence consisted of water (5g), 1-butanol (30mg), 2-methylbutanol (50ng), 1-hexanol (30mg), ethyl butanoate (5mg), pentyl acetate (50mg), hexanal (3mg), *trans*-2-hexenal (100mg), benzaldehyde (0.1mg), ethyl 2-methyl butanoate (5mg), and ethanol added up to 50 g total. The mixture was aged for 24 hours at 20°C and then stored at 5°C. When used as sensory standard, this essence was diluted 1:749 with double distilled water. This synthetic mixture was not recognised as being different from natural apple essence by 20 sensory test panels who had 6 years experience in quality evaluation of apple essence. This study clearly demonstrated that the characteristic aroma of apples and/or apple products is the integration of several compounds (Dirinck *et al.*, 1989; Dürr and Röthlin, 1981).

1.2.2.1 Characterisation of Apple Aroma

Studies of aroma volatiles in the last three decades have been directed towards assessing the sensory impact of individual volatile compounds found in fruit (Maga, 1990; Teranishi *et al.*, 1987). In early studies, this was done by splitting (eg. 50:1 ratio

splitter) the effluent from the GLC at the oven with a larger portion of the effluent going to a human sniffer and the smaller portion through the FID. Various concentrations of odorous compounds could be determined for their odour intensity and description, and simultaneously quantified. From this combination of a human subjective sniffer and objective physical measurement it was possible to estimate the odour threshold for a range of flavour volatiles (Guadagni *et al.*, 1966). Odour threshold has been defined as the minimum physical intensity detection where the subject is not required to identify the stimulus but just to detect the existence of the stimulus (Teranishi *et al.*, 1987). Flath *et al.*(1967) found that ethyl 2-methylbutanoate, hexanal, and (*E*)-2-hexenal had odour thresholds of 0.0001, 0.005, and 0.017 ppm(v/v), respectively. These are the main volatile compounds responsible for the odour of the 'Delicious' apple essence.

Although splitting the GLC for sniffing paved the way for some understanding of the sensory significance of volatile components, this method has some physical drawbacks. For example, sniffing the GLC effluent is both uncomfortable and inaccurate because of the irritating effect of the hot dry carrier gas and the lingering presence of strong odours in the environment of the GLC output (Acree *et al.*, 1976). Some of these problems have been overcome by the development of a 'sniffer' in which the GLC effluent is mixed with a large volume of humidified air flow at the rate of 250 $ml \cdot min^{-1}$ (Acree *et al.*, 1976). Undoubtedly, many complex mixtures have been separated and their individual components characterised and quantified for the threshold values by sniffing column effluents. Maga (1990) has given a word of caution that the long-term health implications associated with continued inhalation of numerous relatively pure unknown compounds with various structures should be considered. The practice of sniffing, although potentially harmful, seems to be especially useful in attempting to isolate compounds or areas on chromatograms where objectionable aroma of fruit appears (Williams, 1979).

As apples and apple products contain numerous volatile constituents, it is important to have a method which enables odour determination of those constituents that significantly contribute to flavour of a fresh or processed product (Guadagni *et al.*, 1966). Practically, only one method has been proposed in this respect by Guadagni *et al.* (1966), the so-called 'odour unit'. The 'odour unit' has been defined as the

concentration of the compound in the food or vegetable oil under study divided by its threshold concentration in water. The larger the value of the odour unit for a given compound, the greater the probability of a compound's odour being detected. This technique gives a quantitative number for comparing the relative odour contribution of different compounds of a product. Compounds in concentrations in excess of their threshold values (**Table 1.2**) are considered to make contributions to flavour, whereas those below their thresholds are thought to have little or no effect (Land, 1979). For a given mixture of compounds, the odour unit can be calculated by the following additive relationship (Guadagni *et al.*, 1966).

$$U_m = \frac{C_1}{T_1} + \frac{C_2}{T_2} + \frac{C_3}{T_3} + \cdots + \frac{C_n}{T_n}$$

where U_m = odour unit of a mixture
C = concentration of a component, and
T = threshold of a corresponding component.

The additive relationship is valid only with values that are near to threshold concentrations (Teranishi *et al.*, 1987). Several additional terms have been suggested by other workers, such as 'aroma value', 'odour unit', and 'odour value', all of which are essentially the ratio between the concentration of a compound in a product and its odour threshold value (Maarse, 1991). Pesis (1994) applied the odour unit concept, called a 'relative contribution value', to explain the increase in sensory aroma quality of fruits after treatments with acetaldehyde or short-term anaerobic conditions. Buttery (1993) also used odour unit to describe the aroma of fresh tomato and tomato paste; this made it possible to make a synthetic mixture with compounds of about the same concentrations as those present in samples. However, accuracy of this method is limited by the accuracy of the threshold measurement and the sensitivity of equipment to measure very low concentration of compounds that have high impact characteristics (Teranishi *et al.*, 1987). Furthermore, odour unit does not predict the nature of the aroma impact of a compound in a mixture because the perception of a mixture of aroma compounds is a complicated human response that currently can not be predicted from the knowledge of separated compounds (Acree, 1993).

Table 1.2 Some odour thresholds of volatiles identified from apple fruit.

Compound	Threshold) (ppm in water)	Reference
Esters		
methyl butanoate	0.01-0.09	Kollmannsberger and Berger (1992)
methyl hexanoate	0.07	Kollmannsberger and Berger (1992)
methyl octanoate	0.27-0.87	Kollmannsberger and Berger (1992)
methyl 2-methylbutanoate	0.0001-0.0003	Kollmannsberger and Berger (1992)
2- methylbutanoate	0.011	Teranishi *et al.* (1987)
3- methylbutanoate	0.002-0.04	Teranishi *et al.* (1987)
ethyl acetate	5	Flath *et al.* (1967)
ethyl propanoate	0.01-0.04	Kollmannsberger and Berger (1992)
ethyl butanoate	0.001-0.007	Teranishi *et al.* (1987)
ethyl isobutanoate	0.0001	Teranishi *et al.* (1987)
ethyl pentanoate	0.005	Flath *et al.* (1967)
ethyl hexanoate	0.001-0.003	Paillard (1990)
ethyl heptanoate	0.002	Teranishi *et al.* (1987)
ethyl octanoate	0.08	Kollmannsberger and Berger (1992)
ethyl 2-methylbutanoate	0.0001	Flath *et al.* (1967)
propyl propanoate	0.057	Teranishi *et al.* (1987)
propyl butanoate	0.018	Teranishi *et al.* (1987)
propyl 2-methylbutanoate	0.0001-0.0004	Kollmannsberger and Berger (1992)
butyl acetate	0.04-0.09	Kollmannsberger and Berger (1992)
butyl propanoate	0.025	Flath *et al.* (1967)
butyl butanoate	0.1	Kollmannsberger and Berger (1992)
butyl hexanoate	0.7	Kollmannsberger and Berger (1992)
butyl 2-methylbutanoate	0.02	Kollmannsberger and Berger (1992)
n-amyl acetate	0.005	Teranishi et al. (1987)
pentyl acetate	0.005	Teranishi *et al.* (1987)
pentyl butanoate	0.21	Kollmannsberger and Berger (1992)
pentyl 2-methylbutanoate	0.01-0.04	Kollmannsberger and Berger (1992)
hexyl acetate	0.002	Flath *et al.* (1967)
hexyl propanoate	0.008	Teranishi *et al.* (1987)
hexyl butanoate	0.25	Kollmannsberger and Berger (1992)
hexyl isobutanoate	0.013	Teranishi *et al.* (1987)
hexyl 2-methylbutanoate	0.02	Kollmannsberger and Berger (1992)
heptyl acetate	0.1	Kollmannsberger and Berger (1992)
heptyl propanoate	0.004	Teranishi *et al.* (1987)
heptyl isobutanoate	0.012	Teranishi *et al.* (1987)
octyl acetate	0.012	Teranishi *et al.* (1987)
Aldehydes		
acetaldehyde	0.015	Flath *et al.* (1967)
hexanal	0.005	Paillard (1990)
trans-2-hexenal	0.017	Flath *et al.* (1967)
2-methylbutanal	0.0002	Buttery (1993)

Alcohols		
ethanol	100	Flath *et al.* (1967)
propanol	9	Flath *et al.* (1967)
butanol	0.5	Flath *et al.* (1967)
hexanol	0.15-0.5	Flath *et al.* (1967);
heptanol	0.094	Paillard (1990)
octanol	0.077	Paillard (1990)
nonanol	0.034	Paillard (1990)
2-methylbutanol	0.25	Buttery (1993)
Ketones		
butanone	37	Paillard (1990)
pentan-2-one	11	Paillard (1990)
heptan-2-one	5	Paillard (1990)
Acids		
butanoic acid	6.8	Paillard (1990)
hexanoic acid	3.7	Paillard (1990)
Miscellaneous		
β-damascenone	0.5	Teranishi *et al.* (1987)
4-methoxyallyl benzene	0.035	Williams *et al.* (1977a)
1,3,5,-(*E,Z*)-undecatriene	0.0002	Kollmannsberger and Berger (1992)

Cunningham *et al.* (1986) determined volatile compounds responsible for the aroma in 40 apple cultivars. They used the aroma value or odour unit concept, but estimated relative odour threshold values of compounds in a flavour extract by using GLC sniffing and termed as 'charm analysis'. 'Charm analysis' was defined as 'a qualitative and quantitative bioassay of odour activity in gas chromatography effluents by measuring the dilution value over the entire time the compounds eluted' and 'Charm' was 'the ratio of the amount of an odour active compound to its detection threshold in gas chromatography effluents' (Cunningham *et al.*, 1986). The "charm values" were calculated on the basis of sensory responses maintained during GLC sniffing of dilutions of the original extracts, where charm values are directly proportional to odour units (Acree, 1993; Cunningham *et al.*, 1986). Cunningham and colleagues (1986) ranked the most intense odour-activity (Charm) of volatile compounds detected from apple juice of 40 cultivars (**Table 1.3**).

A simpler method, involving stepwise dilution of an aroma extract until no more odorous compounds are observed in the GLC effluent, has also been used. The strongest dilution at which the substance can still be smelled is its flavour dilution (FD) factor. Thus, an FD factor of 50 means that the concentration of an odorous compound in the extract is 50 times that of its threshold value as perceived by GLC sniffing, therefore FD factors are proportional to aroma value or odour unit (Maarse, 1991).

Table 1.3 Fifteen most intense aroma volatile compounds in processed apple juice (after Cunningham *et al.*, 1986).

Rank	%Charm	Compound
1	11.8	β-damascenone
2	10.1	unknown
3	9.4	hexyl hexanoate and butyl octanoate
4	7.5	ethyl butanoate
5	6.7	butyl hexanoate, hexyl butanoate, benzyl acetone, benzothiazol
6	5.4	unknown
7	4.4	6-methyl-hept-5-en-2-one
8	3.8	unknown
9	2.7	unknown
10	2.5	propyl butanoate and ethyl pentanoate
11	2.2	unknown
12	2.0	isoamyl hexanoate
13	1.4	propyl propanoate and butyl acetate
14	1.0	1-octene-3-one and isopentyl propanoate
15	0.9	ethyl 2-methylbutanoate

Although the odour unit concept is frequently used, it has been subjected to criticism (Frijters, 1979; Rothe, 1975). The odour unit can be calculated only if both the concentration and threshold value of a compound is known. Therefore the major flavour compounds can only be selected after identification and quantitative assessment of a large number of constituents. This is obviously very laborious and costly. Moreover, it does not guarantee that all important flavour compounds are taken into consideration (Maarse, 1991), because very low odour threshold compounds with extremely low concentrations may not be detected by GLC (Berger, 1991). Frijters (1979) criticised the concept and made it clear that it was inconsistent in some respects with current psychophysical views; it did not make much sense to order the compound according to odour unit since the odour did not necessarily correspond to the order of ranking of

perceived intensities. In addition, compounds within a mixture showed different concentration/perceived intensity behaviour, for example 50 times the threshold concentration of compound X does not yield the same aroma intensity effect as 50 times the odour threshold concentration of compound Y. Laing and Panhuber (1979) suggested that when odorous compounds were mixed, the odour quality of the mixture may differ from that of the individual compounds or some compounds may not be detected (perceived) due to a masking effect. The intensity of a mixture may be greater (synergism), equal to, or less (depression) than the sum of the intensities of the individual compounds. Moreover, the detection threshold of a mixture may differ from that of the individual compound (Laing and Panhuber, 1979; Land, 1979). Thus, the major drawback of the odour unit concept is the assumption that the perceived intensities of all odorous substances increase at the same rate with concentration (Land, 1979; Rothe, 1975). However, so far no better concept has been suggested (Maarse, 1991), and Frijters (1979) admitted that 'although this concept does not have precise validity, it can give some guidance in a particular investigation'.

1.2.2.2 Character-Impact Compounds

According to Dürr and Schobinger (1981), the contribution of a volatile compound to fruit and fruit products can be divided into 7 categories, including that which

(a) contributes strongly to the typical odour, a so-called 'character impact compound' such as citral in lemon;

(b) contributes in part to the typical aroma, termed as a 'desirable compound', such as hexanal in apple juice aroma;

(c) contributes to the intensity of an aroma, such as *trans*-2-hexenal in apple juice aroma;

(d) does not contribute directly to the aroma but which is related to aroma quality, such as ethanol and isobutanol in apple juice;

(e) does not contribute at all due to the concentration being far below sensory threshold;

(f) acts as a precursor for off-flavour components, such as limonene in orange juice;

(g) is responsible for an off-flavour or after taste, the so-called 'undesirable or off-flavour' compounds, such as α-terpineol in orange juice.

A considerable number of individual volatile compounds have been reported to be 'major contributors' or 'character-impact' compounds to the aroma of apples and apple products (**Table 1.4**). These compounds have been identified and sensorily characterised by panel evaluation using capillary GLC sniffing (Paillard, 1990). The large number of these character-impact compounds reported are possibly due to several factors. The large number of apple cultivars, (it has been estimated that there are more than 20,000 varieties world-wide (Berger, 1991)), makes it difficult to define only one or a few character-impact compounds to apples in general as each cultivar has its own flavour character. Investigators also differ greatly in the way of determining the sensory significance of different volatile compounds (Dürr, 1994; Yahia, 1994). In addition, there is a frequent lack of separation between 'primary' and 'secondary' volatiles (Berger, 1991). 'Primary' volatile compounds are those produced by controlled enzymatic reactions in intact tissue, while 'secondary' volatiles are formed by various uncontrolled enzymatic reactions when plant tissues are disrupted, such as by homogenisation, cutting, chewing, or heating (Roberts and Acree, 1995; Schreier, 1984). Although primary volatiles are important to the aroma and flavour of fresh fruit, secondary volatiles also play a significant role in the flavour of the juice or fresh fruit during chewing (Paillard, 1990). Several volatile compounds listed in **Table 1.4**, such as (*E*)-2-hexenal, (*E*)-2-hexenol, (*Z*)-3-hexenal, and (*Z*)-3-hexenol, are secondary volatiles (Drawert *et al.*, 1968; Hatanaka, 1993) that contribute significantly to aroma of apple juice and essence (Dürr and Schobinger 1981; Flath *et al.*, 1967).

Guadagni *et al.* (1966) and Flath *et al.* (1967) found that ethyl 2-methyl butanoate, which had a very low odour threshold (**Table 1.2**), had an intense apple odour characteristic of ripe 'Delicious' fruit. They also reported that (*E*)-2-hexenal and hexanal were associated with a green apple odour, which contributed to the aroma of 'Delicious' apples and apple juice essence. Hexyl 2-methylbutanoate also has a typical

apple-like aroma and increased levels of this volatile improve quality of 'Golden Delicious' apples (Paillard, 1990). Butyl acetate and hexyl acetate have been reported to be the principle aroma compounds synthesised by apples during ripening (Bartley *et al.*, 1985). Other esters, such as ethyl butanoate, which has a low olfactory threshold, also has a rather fruity odour (Nursten and Woolfe, 1972). 4-Methoxyallyl benzene was identified in the aroma of several cultivars, especially those related to 'Ellison's Orange' apples, giving them a recognisable aniseed-like or a spicy-note character (Williams *et al.*, 1977b). Kollmannsberger and Berger (1992) found that by using GLC sniffing of serially diluted extracts, the key contributions of ethyl 2-methylbutanoate, propyl 2-methylbutanoate, hexyl acetate, ethyl butanoate, ethyl hexanoate, hexyl hexanoate and 1,3,5-(*E,Z*)-undecatriene to 'Red Delicious' apple aroma was confirmed. By employing the 'charm analysis' technique, Cunningham *et al.*, (1986) reported that β-damascenone, 3 carboxylic acid esters, and at least 2 unknown compounds had the highest flavour significance for juice of 40 cultivars of apples.

Table 1.4 Volatile 'character-impact' compounds in apple fruit and its products.

Compound	Reference
2-methylbutyl acetate	Young *et al.* (1996)
ethyl butanoate	Cunningham *et al.* (1986)
ethyl 2-methylbutanoate	Flath *et al.* (1967)
ethyl hexanoate	Kollmannsberger and Berger (1992)
ethyl 3-hydroxy-5-(*Z*)-octenoate	Kollmannsberger and Berger (1992)
propyl 2-methylbutanoate	Kollmannsberger and Berger (1992)
butyl acetate	Bartley *et al.* (1985)
butyl hexanoate	Cunningham *et al.* (1986)
hexyl acetate	Bartley *et al.* (1985)
hexyl butanoate	Cunningham *et al.* (1986)
hexyl 2-methylbutanoate	Berger (1991)
hexyl hexanoate	Kollmannsberger and Berger (1992)
(*E*)-2-hexenyl acetate	Berger (1991)
hexanal	Flath *et al.* (1967)
(*E*)-2-hexenal	Flath *et al.* (1967)
(*Z*)-3-hexenal	Dürr and Schobinger (1981)
butan-1-ol	Young *et al.* (1996)
hexan-1-ol	Berger (1991)
(*E*)-hexenol	Dürr and Schobinger (1981)
4-methoxyallyl benzene	Williams *et al.* (1977b)
β-damascenone	Cunningham *et al.* (1986)
1,3,5-(*E,Z*)-undecatriene	Kollmannsberger and Berger (1992)
unknown	Berger (1991), Cunningham *et al.* (1986)

Dürr and Schobinger (1981) evaluated the contribution of 30 volatile compounds to the sensory quality of commercial apple juice and essence. Based on their contribution to sensory quality, they classified the volatiles into 4 groups, as important, desirable, undesirable, and contribution to the aroma intensity of apple juice and essence (**Table 1.5**).

Table 1.5 Volatile compounds contributing to apple juice aroma (after Dürr and Schobinger, 1981).

Important	Desirable	Undesirable	Aroma Intensity
trans-2-hexenal	hexanal	ethanol	*trans*-2-hexenal
cis-3-hexenal	benzaldehyde	isobutanol	*cis*-3-hexenal
trans-2-hexenol	propyl butanoate	2-methylbutanol	isobutanol
cis-3-hexenol	pentyl acetate	3-methylbutanol	isobutyl acetate
ethyl butanoate	2-pentanone	β-phenyl ethanol	
ethyl 2-methylbutanoate	isobutyl acetate		

1.3 BIOGENESIS OF APPLE FLAVOUR

The typical flavour of fruit is not present during early stages of fruit formation but develops entirely during a rather brief period of fruit ripening (Nursten, 1970). Ripening is characterised by some physiological and metabolic processes influencing the biogenesis of volatiles, such as ethylene, respiration climacteric rise, protein synthesis, increase in enzymatic activities and permeability of cell membranes (Brady, 1987; Marangoni *et al.*, 1996). During growth and development, fruits synthesise high molecular structure compounds, such as proteins, polysaccharides, lipids and flavonoids (Hulme and Rhodes, 1971; Yamaki, 1995). During the pre-climacteric stage, fruits produce low levels of ethylene, a ripening hormone derived from methionine (Yang and Hoffman, 1984). During ripening, ethylene, as low as 0.1 ppm, may induce biochemical, physical, and chemical changes, such as the increase in synthesis and activities of ripening related enzymes (Oetiker and Yang, 1995; Pratt and Goeschl, 1969). The increased activity and synthesis of various enzymes, such as amylase, lipase, protease and esterase, results in accumulation of metabolites such as sugars, fatty acids, amino acids, alcohols,

some of which can act as substrates for production of volatile compounds (Pratt and Goeschl, 1969; Schreier, 1984).

The volatiles of fruit may be considered to originate from the basic parents (**Fig. 1.2**) of carbohydrates (especially the mono- and di-saccharides), proteins and lipids (ie. the triglycerides and their derivatives), as well as vitamins (Salunkhe and Do, 1976; Tressl *et al.*, 1975). A number of representative aroma compounds derived from these base materials are listed in **Table 1.6**.

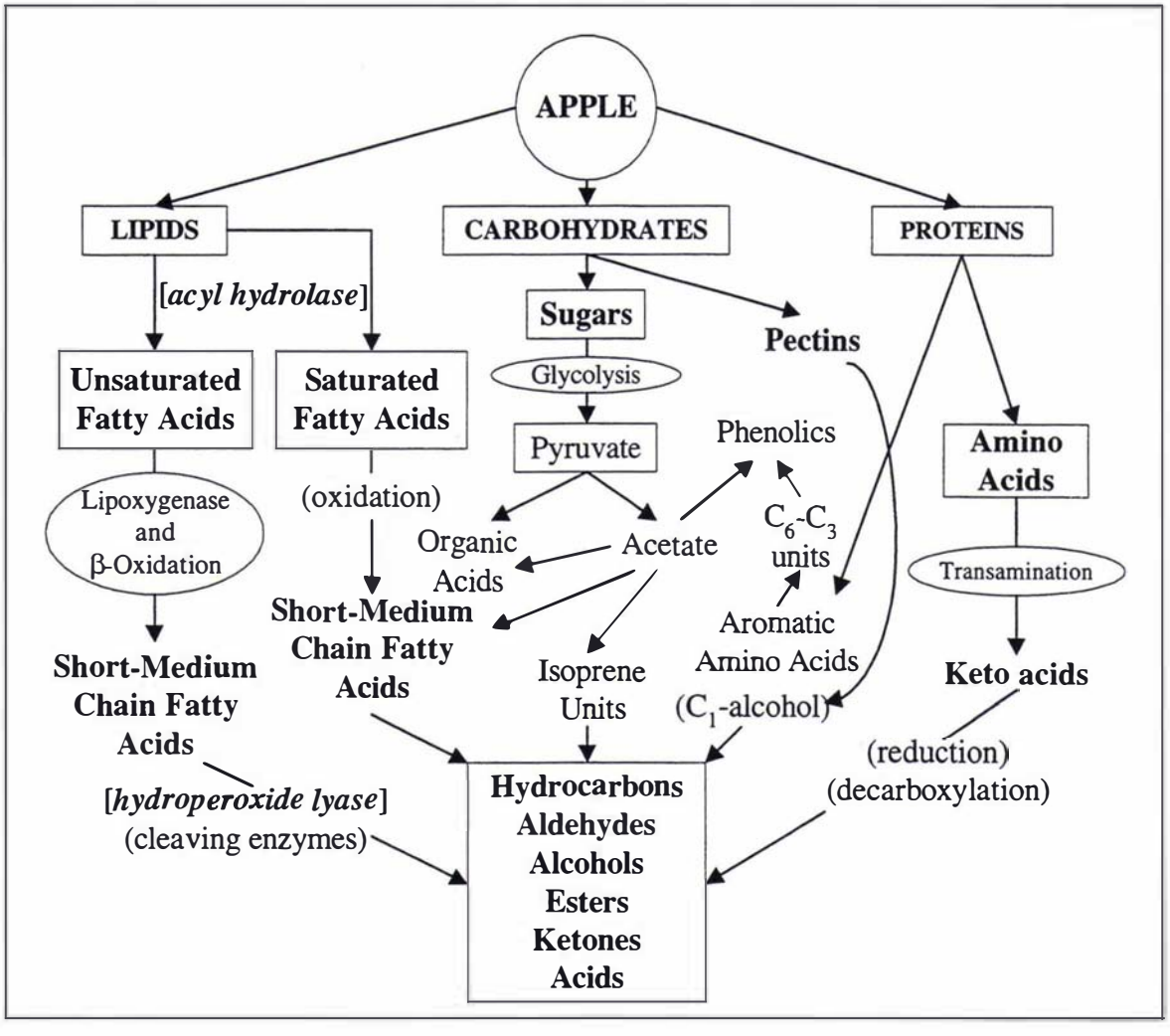


Fig. 1.2 Some possible pathways for the biosynthesis of aroma volatile compounds in apples (derived from Salunkhe and Do, 1976; Schreier, 1984; Yahia, 1994).

Table 1.6 Human nutrients in relation to aroma volatile production in food (after Salunkhe and Do, 1976).

Nutrients	Aroma Compound
Carbohydrates	
Glucose, Fructose, Sucrose	***Organic acids***: pyruvic, acetic, propionic, butyric, hexanoic, octanoic ***Esters***: acetates, propanoates, butanoates, acetoacetates, hexanoates, octanoates ***Alcohols***: methanol, ethanol, propanol, butanol, hexanol, octanol *Aldehydes:* acetaldehyde, propanal, butanal, hexanal, octanal ***Terpenes***: monoterpene, linalool, limonene, α-pinene, citronellal, citral, geranial
Amino Acids	
Alanine	pyruvic acid, acetaldehyde, ethanol
Valine	isopropanal, isopropanol, α-keto-isobutyric acid
Leucine	3-methylbutanal, 3-methylbutanol, α-keto-isocarproic acid
Isoleucine	2-methylbutanal, 2-methylbutanol
Phenylalanine	benzaldehyde, phenylacetaldehyde, cinnamaldehyde, hydrocinnamaldehyde, ρ-hydroxybenzaldehyde, ρ-hydroxy-phenylacetaldehyde, ρ-hydroxy cinnamaldehyde
Serine, Threonine, Glycine, Cystine/Cysteine	pyruvic acid, thiazoles, glyoxal
Fatty Acids	
Linoleic/Linolenic acids	*trans*-2-*trans*-4-decadienal, hexanal, *trans*-2-octanal, *trans*-2-pentanal, *trans*-2-hexenal, hexanol, *cis*-3-hexenal, *cis*-3-hexenol, *trans*-2-*trans*-4-heptadienal, propanal
Organic Acids	
Citric acid, Oxaloacetic acid, Malic acid, Lactic acid	Glyoxylic acids, glyoxal, pyruvic acid, acetaldehyde
Vitamins	
β-Carotene	β-Ionone
Xanthophylls	β-Damascenone
Thiamine	Thiazoles

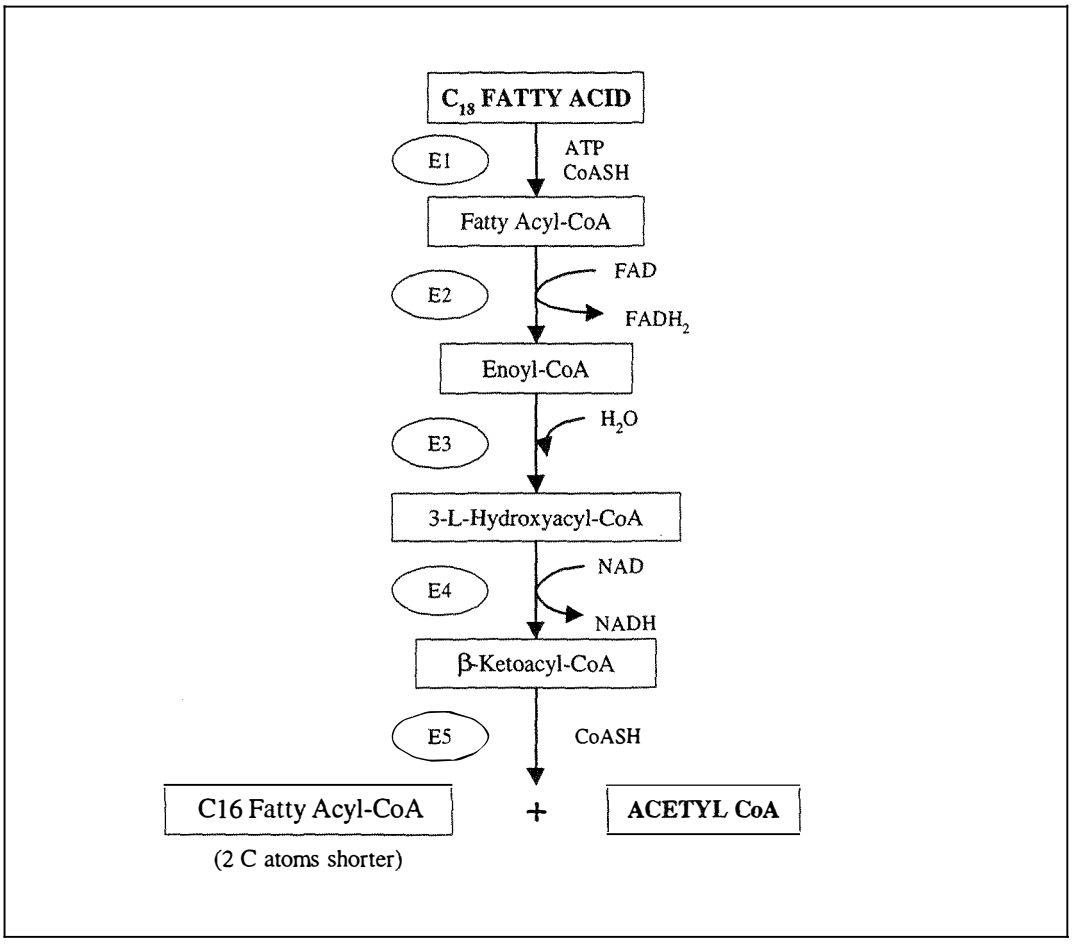
1.3.1 The Primary Volatiles

Precursors of both alcohol and carboxylic acid moieties, which are the immediate precursors of most esters in intact fruit, are thought to originate mainly from fatty acids and partly from amino acids (Hansen *et al.*, 1992a; Schreier, 1984; Tressl and Drawert,

1973). Most fruits produce a variety of fatty acids ranging from C_1 to C_{20} and trace amounts of primary and secondary alcohols (Heath and Reineccius, 1986). Galliard (1968) studied the identity, composition and concentration of individual lipids in apple pulp in both the pre- and post-climacteric stages of ripening. The fatty acids of each acyl lipid was similar at both stages. Linoleic acid was found to be the main fatty acid component of the major acyl lipids, while linolenic acid was the predominant acid in mono- and di-galactosyl diglycerides. The decreased level of linoleic acid found during the post-climacteric stage was thought to be due to the decreased concentration of galactosyl diglycerides. Meigh *et al.* (1967) reported that both saturated and unsaturated free fatty acids and esters of fatty acids accumulated in apple peel about 120 days after fruitset. However, after 150 days from fruitset during fruit maturation, breakdown of free fatty acids occurred rapidly, at a faster rate than their synthesis. This declined level of free fatty acids was thought to be due to esterification. The rapid accumulation of free and esterified fatty acids in apple peel coincided with the respiratory climacteric rise of apple fruit and these fatty acids subsequently decreased during the postclimacteric phase (Meigh *et al.*, 1967). Song and Bangerth (1994) found that concentrations of palmitic, stearic, oleic, and linoleic acids were much lower in early harvested fruit compared to late harvested 'Golden Delicious' apples. Bartley (1985) reported that the rate of degradation of phospholipids increased during ripening of 'Cox's Orange Pippin' apples, suggesting that these long-chain fatty acids could supply free fatty acids and low molecular weight alcohols for the synthesis of volatile compounds in apples.

Studies using ^{14}C-radiolabelled fatty acids in banana (Tressl and Drawert, 1973), or feeding experiments in strawberries (Yamashita *et al.*, 1977), and apples (Bartley *et al.*, 1985; De Pooter *et al.*, 1983) during fruit ripening, found that some fatty acids converted into aldehydes, alcohols, esters, and ketones. Paillard (1979) studied the biosynthesis of volatiles in apples by supplying substrates to discs of aged tissues. Alcohols were formed via β-oxidation from aliphatic acids (C_3 to C_{18}) having either the same or higher number of carbon atoms. Fatty acids with an even carbon number gave rise to butanol and hexanol, while odd numbered carbon acids generated propanol and pentanol. In addition, esters were found to be synthesised from the corresponding acids and alcohols. In the β-oxidation process (**Fig. 1.3**), a fatty acid, such as linoleic acid, is

metabolised, two carbons at a time, to shorter chain CoA derivatives which may react with alcohols to yield esters. During this process, isomerisation may occur to yield *trans-* and *cis-* isomers (Drawert, 1975; Heath and Reineccius, 1986). Although it was thought that the enzyme complex catalysing β-oxidation is situated in the mitochondria (Galliard, 1980; Tressl *et al.*, 1975), it is now believed that the site of fatty-acyl-CoA oxidising activity is located in the peroxisomes, ie. glyoxysomes, of most higher plant cells (Gerhardt, 1983).



Abbreviations : E1 = Acyl CoA synthase, E2 = Acyl CoA dehydrogenase, E3 = Enoyl CoA hydratase, E4 = 3-L-Hydroxyacyl CoA dehydrogenase, E5 = β-Ketoacyl CoA thiolase, ATP = Adenosine triphosphate, CoASH = Coenzyme A, FAD = Flavin adenine dinucleotide, NAD = nicotinamide adenine dinucleotide

Fig. 1.3 Reaction sequence in each round of β-oxidation of fatty acid (after Stryer, 1988).

Oxidation of linoleic and linolenic acids produces a large number of volatiles, acids and ketones as well as other intermediates in the oxidation process which are readily converted to alcohols, aldehydes and esters by other enzyme systems in fruit (Heath and Reineccius, 1986; Schreier, 1984). Both α- and β-oxidation pathways have been demonstrated to exist, thereby providing a wide range of volatiles for further conversion to flavour compounds (Tressl *et al.*, 1975). Thus fatty acid metabolism seems to be the most important source of primary odour volatiles in pome fruits (Tressl and Drawert, 1973).

Amino acids are known to generate aliphatic and branched chain alcohols, acids, carbonyls and esters which are important to fruit flavour (Tressl *et al.*, 1975). Valine, leucine, isoleucine, alanine and aspartic acids can be converted to short chain carbonyls by tomato extracts (Heath and Reineccius, 1986). Radioactive labelling has shown that leucine (Myers *et al.*, 1970) and valine (Tressl and Drawert, 1973) are transformed into branched chain flavour compounds in banana. Infiltration of L-isoleucine solution into the central cavity of intact 'Golden Delicious' apples increased several branched chain esters and alcohols (Hansen and Poll, 1993). The conversion of these amino acids into volatiles is thought to proceed via transamination to 3-keto acids which are then oxidised and decarboxylated to the corresponding 'activated' aldehydes and subsequently converted to alcohols and esters (Tressl *et al.*, 1975).

Among volatile compounds identified in apple aroma, esters form the largest chemical category, followed by alcohols and then aldehydes, ketones, hydrocarbons and terpenes (Paillard, 1990).

Alcohols : C_1 to C_6 alcohols and those containing an even number of carbon atoms up to C_{12} are believed to be present in most fruits (Nursten, 1970). Alcohols represent 6% to 16% of the total headspace volatiles of intact apples, depending on cultivar (Paillard, 1990); on the other hand, the proportion of alcohols in apple juice (using a vacuum distillation method of extraction) range from 48.3% to 75.5% (Kakiuchi *et al.*, 1986). This suggests that concentration of alcohols not only depends on cultivar, but also on the method used for determination. When compared with other classes of compounds; such as esters, aldehydes, and ketones, low molecular weight alcohols are less volatile in very dilute solution while higher molecular weight alcohols, such as octanol, are more volatile

than methanol (Buttery *et al.*, 1971; King, 1983). The origin of methanol is thought to be from demethylation of pectic substances in ripening apples (Berger and Drawert, 1984; Knee, 1978). Ethanol is formed from anaerobic fermentation through pyruvic acid and acetaldehyde (Nursten, 1970). Higher alcohols such as propanol, butanol and octanol are believed to form via β-oxidation from even and odd carbon numbered fatty acids (Paillard, 1979). Branched chain alcohols, such as 2/3-methylbutanol and 2-methyl-1-propanol, are derived by transamination from the amino acids L-leucine, L-valine, and L-isoleucine (Schreier, 1984; Tressl and Drawert, 1973). Glycine and alanine may also give rise to methanol and ethanol production (Nursten, 1970).

Aldehydes **:** Straight chain or branched chain aliphatic aldehydes identified among apple volatiles generally accompany corresponding alcohols (Paillard, 1990). Acetaldehyde, often considered as a fermentative product, is found when fruit undergo a physiological alteration such as stress or during ripening and senescence (Nichols and Patterson, 1987). Acetaldehyde is normally derived from pyruvic acid the reaction being catalysed by pyruvate decarboxylase (PDC) (Ke *et al.*, 1993a), although it may also be produced following lipid peroxidation of membrane fatty acids (Kimmerer and Kozlowski, 1982). Other aldehydes are believed to form mainly following β-oxidation of fatty acids (De Pooter *et al.*, 1987; Drawert, 1975). Amino acids are also possible processors of a limited number of aldehydes via α-oxo acids (Nursten, 1970). Benzaldehyde, a volatile constituent of many fruits including apples, is probably formed by β-oxidation from cinnamic acids via benzoyl-S-CoA (Schreier, 1984).

Aldehydes are found in large amount in extracts, but they also exist in much smaller quantities in emissions from whole fruit (Paillard, 1990). Aldehydes are believed to be the immediate precursors of alcohols, with alcohol dehydrogenase (ADH) responsible for the conversion (Bartley and Hindley, 1980). In the presence of NAD, the ADH can catalyse conversion of alcohols to aldehydes and *vice versa* (Eriksson, 1979). Aldehydes can also be converted into the corresponding acids, such as butanal to butanoic acid, (De Pooter *et al.*, 1983) by aldehyde oxidase (Eriksson, 1979).

Esters **:** Esters are particularly well represented in analysis of volatiles emitted by apples where they may account for 81 - 96% of total headspace vapour of intact apples, but only 11 - 33% in juice (using an extraction distillation method) of the same material

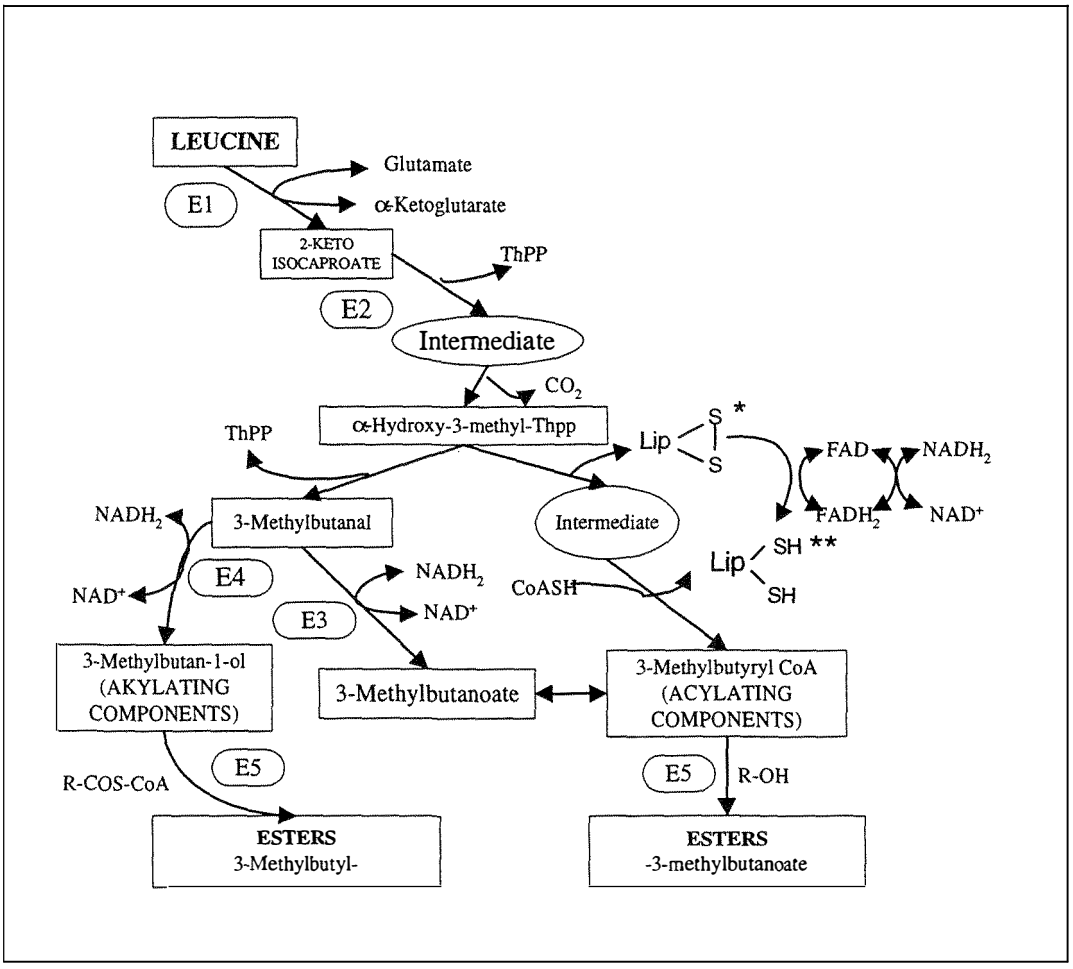
which depends on cultivars (Kakiuchi *et al.*, 1986, Paillard, 1990). This is probably due to several factors involved such as vapour pressure, solubility in aqueous medium or waxes and molecular weight of volatile compounds. Vapour pressures of the lower esters (up to C_9) at room temperature are relatively high; 1mm Hg for pentyl butanoate, 8mm Hg for butyl acetate, and 78mm Hg for ethyl acetate (King, 1983; Paillard, 1990). However, vapour pressure is not the only factor in volatility. Solubility of esters in lipids and skin waxes increases for longer aliphatic chains (> C_9), and thus they are less volatile from intact fruit than from juice, while in dilute aqueous solutions ester volatility increases for chain length of C_9 and longer (Buttery *et al.*, 1971; Paillard, 1990). The higher molecular weight esters (> C_{11}) are normally found in extracts and only trace amounts are found in emissions from intact fruit; this reduced volatility may prevent liberation from fruit in detectable quantities (Paillard, 1990).

Early studies in the formation of volatile esters were made during fermentation of fruit with brewer's yeast under hypoxic conditions (Nursten, 1970). Although esters are qualitatively and quantitatively one of the most important class of volatile compounds in fruit, including apples, there are relatively few reports on the biochemical aspects of ester formation (Fellman and Mattheis 1995; Hansen *et al.*, 1992b). Biosynthesis of fruit esters has been studied by adding radio-labelled and non-labelled precursor substrates, such as carboxylic acids, amino acids, alcohols, and aldehydes to tissue discs of intact fruits.

In banana, Tressl and Drawert (1973) showed that [^{14}C]-leucine was converted into 3-methyl-1-butanol, 3-methyl butyl esters, 3-methyl butanoates and 2-ketoisocaproate (**Fig. 1.4**), while [^{14}C]-valine was converted into 2-methyl-1-propanol, 2-methyl propyl acetate, 2-methyl propionic acid, and 2-keto isopentanoic acid. Feeding L-isoleucine solution to intact 'Golden Delicious' apples induced the increased production of various esters such as ethyl 2/3-methyl butanoate, 2/3-methyl butyl acetate, 2/3-methyl propanoate, 2-methyl but-2-enyl acetate, 2/3-methyl butanoate, and 2/3-methyl butyl 2/3 methyl butanoate (Hansen and Poll, 1993).

Amino acids, such as L-isoleucine, are thought to be transaminated and oxidatively decarboxylated to form a carboxylic acid, such as 2-methyl butanoic acid (Tressl *et al.*, 1975), which can be dehydrogenated to yield *trans*-2-methylbut-2-enoic

acid. The acid is then reduced to the corresponding alcohol, ie. 2-methylbut-2-enol, via aldehyde for incorporation into an ester (Hansen and Poll, 1993; Mazelis, 1980).



Abbreviations and Symbols

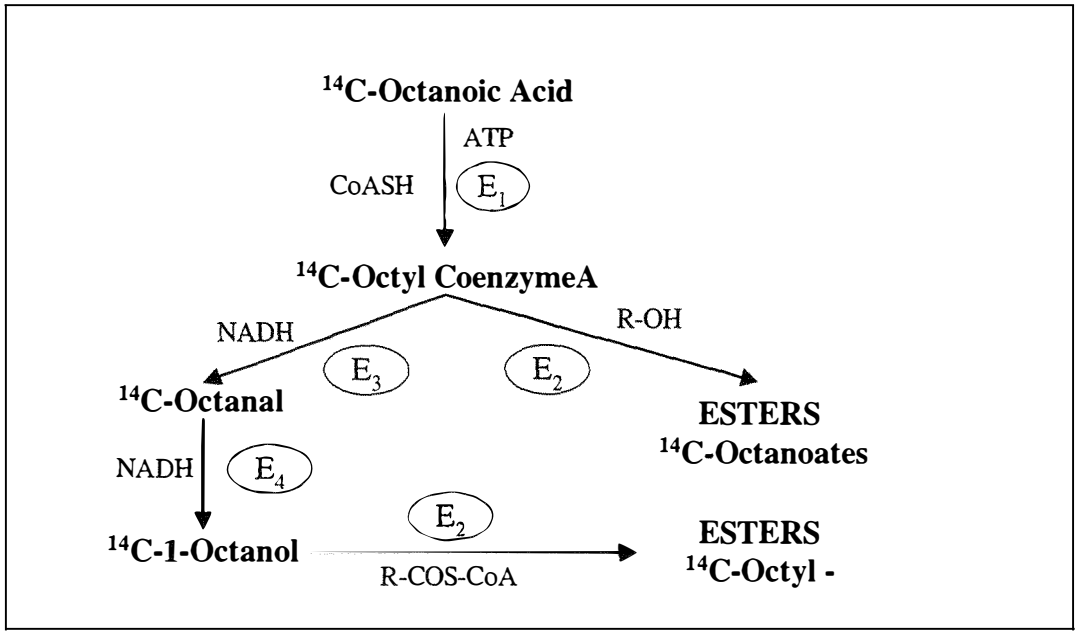
E1 = L-leucine aminotransferase, E2 = pyruvate decarboxylase, E3 = aldehyde dehydrogenase, E4 = alcohol dehydrogenase, E5 = alcohol acyl transferase (AAT), ThPP = thiamine pyrophosphate, FAD = Flavin adenine dinucleotide, $FADH_2$ = reduced FAD, NAD^+ = oxidised nicotinamide adenine dinucleotide, $NADH_2$ = reduced NAD, CoASH = coenzyme A, * = oxidised lipoic acid, ** = reduced lipoic acid

Fig. 1.4 A tentative biosynthesis pathway of aroma compounds derived from amino acids in banana as illustrated by leucine (after Drawert, 1975).

Radio-labelled fatty acids (C_2 to C_{10}) convert into the corresponding alcohols esters and ketones in banana slices; for example [2-^{14}C] hexanoic acid is converted to 1-hexanol, hexyl acetate, butyl hexanoate, 3-methyl butyl hexanoate, hexyl hexanoate and 2-pentanone (Tressl and Drawert, 1973). The [^{14}C] C_4 to C_{16} acids were metabolised into lower acids (C_1 to C_{12}) by α- and β-oxidation processes. The possible pathway for

the transformation ^{14}C-labelled fatty acid substrates as represented by octanoic acid is illustrated in **Fig. 1.5** (Tressl *et al.*, 1975).

Yamashita *et al.* (1977) reported that supplied fatty acids (C_2 to C_6) were readily converted into the corresponding esters in ripe strawberries, with an esterification efficiency in the range of 90 - 100% for acetic, propionic, isobutyric, n-butyric and n-caproic acids.



E_1 = Acyl thiokinase, E_2 = Acyl-CoA-alcohol-transacylase (AAT), E_3 = Acyl-CoA reductase, E_4 = Alcohol-NAD-oxydoreductase

Fig. 1.5 Possible reaction pathways for conversion of octanoic acid into esters (after Tressl *et al.*, 1975).

Paillard (1979) found that adding C_4 to C_{18} fatty acids to incubated apple tissue discs induced production of the corresponding alcohols, which were thought to result from β-oxidation of aliphatic acids. Esters were synthesised from the corresponding acid and alcohol. Application of propionic acid to 'Golden Delicious' apples increased the production of propanal, an intermediate in the transformation into propanol. The content of individual propyl esters, namely propyl acetate, propyl propanoate, propyl butanoate, butyl propanoate, 3-methyl butyl propanoate, pentyl propanoate and hexyl propanoate, increased to reach a maximum 7 - 9 days after application and then diminished. During

this time transformation of other esters, such as hexyl acetate, butyl acetate, and hexyl butanoate, decreased but they did increase strongly when propionic acid was withdrawn (De Pooter *et al.*, 1981). When vapours of C_2 to C_6 carboxylic acids were supplied to intact 'Golden Delicious' apples, the result was the formation of corresponding alcohols, esters, and smaller carboxylic acids (De Pooter *et al.*, 1983). On the other hand, fatty acid (C_2 to C_4) vapours had a negligible effect on aldehyde production of pre-climacteric or cold stored 'Golden Delicious' apples, but they generated an almost 10 fold production of esters (De Pooter *et al.*, 1987). Bartley *et al.* (1985) found that application of methyl esters of short-chain fatty acid (C_4 to C_8) to 'Cox's Orange Pippin' apples resulted in their conversion into esters with an alkyl group (C_{n-2}; C_{n-4}) confirming the presence in whole fruit of an active β-oxidation pathway for fatty acids.

Gilliver and Nursten (1976) studied the role of the acyl moiety in the biosynthesis of volatile esters in banana tissue slices. When tissue slices were incubated with alcohol and a source of acyl moiety there was a dramatic increase in headspace ester concentration; for example incubation of ethanol with butyryl-CoA resulted in a substantial increase in ethyl butanoate concentration. Gilliver and Nursten (1976) suggested that acyl-CoA or acyl carrier protein (ACP) (both were formed from the corresponding CoA compound) could act as an acylating agent in ester formation.

Application of aldehydes and alcohols to other fruit has also been used in studying the biosynthesis of esters. In strawberries, pentanal was reduced to 1-pentanol and converted into pentyl esters, such as pentyl acetate and this transformation efficiency increased with maturity (Yamashita *et al.*, 1977). Incubation of strawberries with acetaldehyde, propanal, 2-methyl propanal, 3-methyl butanal, and hexanal resulted in reduction into their corresponding alcohols, which in turn were converted to their acetate, propanoate, n-butanoate, isopentanoate and n-hexanoate esters during incubation. The simultaneous reaction of isobutanoic, n-pentanoic, and isohexanoic acids with aldehyde resulted in the formation of esters of these acids (Yamashita *et al.*, 1977). Vapours of C_3 to C_6 aldehydes supplied to 'Golden Delicious' apples were either transformed into the corresponding alcohols and esterified with carboxylic acids, or to a small degree, they were oxidised into acids (De Pooter *et al.*, 1983).

Knee and Hatfield (1981) studied the interconversion of short-chain aliphatic alcohols, aldehydes and esters in 'Cox's Orange Pippin' apples where alcohols were supplied as vapours to fruit slices at 20°C or to whole fruit during storage at 3°C. When a range of n-alkan-1-ols was supplied to apple cortex tissues, acetate ester formation increased sharply with carbon number up to C_5, the rate of formation being similar for hexan-1-ol and pentan-1-ol. Acetate ester formation from 2-methyl propanol was slower than from n-butanol and 2-methyl butanol. Formation in the peel tissue was more rapid than in the cortex but it declined rapidly after 10 min of incubation. It was suggested that the esterifying system had a relatively high specificity for longer chain carbon alcohols and its activity was not greatly diminished with branched-chain alcohols. When supplying vapours of C_2 to C_8 straight chain alcohols to intact 'Cox's Orange Pippin' apples, Bartley *et al.* (1985) found that the major product for each alcohol supplied was the corresponding acetate ester. Butanol, pentanol, and hexanol were more readily esterified than the other alcohols which reflected the substrate of the ester forming enzyme (Bartley *et al.*, 1985).

Similar studies on postharvest application of alcohols to 'Red Delicious' apples were reported by Berger and Drawert (1984) and Berger *et al.* (1992). When ethanol vapour was applied to intact apples at ambient temperature, it caused an increased production of ethyl esters, including ethyl 2-methylbutanoate, but a decrease in concentration of some butyl and hexyl esters. This indicates that esterification of the acyl moieties, especially C_4 and longer, is a competitive reaction (Berger *et al.*, 1992). Higher concentrations of ethanol favour esterification of medium-chain acyl moieties (C_8 and longer), such as ethyl octanoate and ethyl decanoate, whereas compounds with a short-chain moieties, such as ethyl acetate and ethyl butanoate, increase concentration more rapidly at low ethanol concentrations and begin to decrease at higher concentrations. Similar findings have been reported when methanol was supplied to apple tissue discs (Berger and Drawert, 1984). When a range of C_1-to C_6-alkan-1-ols were supplied, the increase in concentration of the corresponding acetate, propanoate, butanoate, pentanoate, methyl butanoate, hexanoate and octanoate esters was observed. The maximum esterification was achieved with butanol and pentanol, the former being converted into butyl esters and the latter almost completely converted into pentyl esters

(Berger *et al.*, 1992). These results are in agreement with those previously reported by Knee and Hatfield (1981).

These various studies of supplying the fruit with precursors as described above, have shown that esters are formed from carboxylic acids, which are converted into acyl-CoA derivatives, and their alcohols by an ester forming enzyme. The ester forming enzyme, alcohol acyltransferase (AAT), has been partially purified and characterised from banana (Harada *et al.* 1985) and strawberry fruits (Pérez *et al.*, 1993). The enzyme from banana fruit is very labile at pH lower than 7.0, but relatively stable at pH 7.5 ~ 8.5. Its molecular weight has been estimated to be about 40 KDa by gel filtration and its K_m value for acetyl-CoA and isoamyl alcohol is 50 μM and 0.4 mM, respectively (Harada *et al.* 1985). The AAT enzyme from strawberries is reported to have activity at a pH optimum of 8.0 at 35°C and the apparent molecular weight following gel filtration is 70 KDa. The enzyme is shown to have the maximum activity when using acetyl-CoA and hexyl alcohol as substrates (Pérez *et al.*, 1993), and oxygen is apparently required for the enzyme reaction (Fellman and Mattheis, 1995). Thus the possible pathways involved in ester formation in fruit may be summarised as **Fig. 1.6**. There are 2 main factors that could determine volatile ester composition in fruit: (a) availability of the substrates, acyl-CoAs and alcohols, and (b) the inherited properties of the AAT enzyme which will determine substrate specificity (Berger *et al.*, 1992; Oliás *et al.*, 1995).

Other Compounds A few ketones have been reported to be present in apples and apple products including acetone, butanone, heptanone, octanone and β-ionone but they are thought to of minor importance in the aroma of apples (Boylston *et al.*, 1994; Girard and Lau, 1995; Mattheis *et al.*, 1991a, 1991b; 1995; Paillard, 1990).

Short chain free fatty acids have been identified in apples, apple juice and essence, and some of these compounds are thought to derive from hydrolysis of esters (Paillard, 1990). Some of these free fatty acids reported are acetic acid, propionic acid, butanoic acid, hexanoic acid and octanoic acid (Dimick and Hoskin, 1983; Girard and Lau, 1995; Mattheis *et al.*, 1991b) and they are believed to make little or no contribution at all to the aroma of fruit (Nursten, 1970).

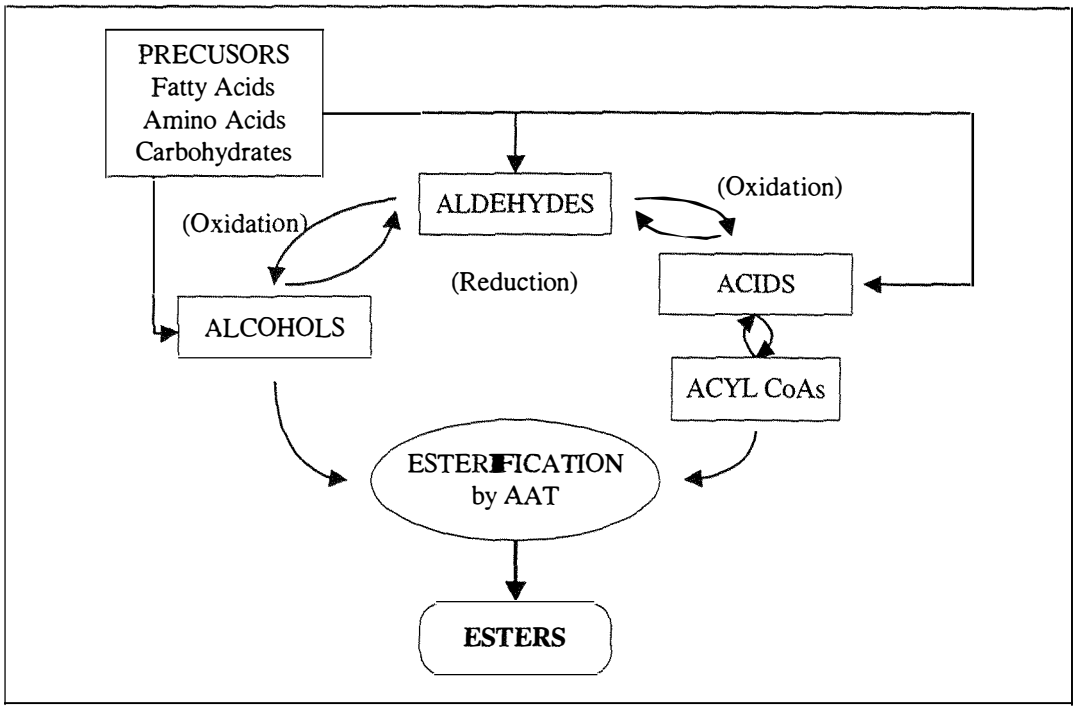


Fig. 1.6 Possible biochemical pathways leading to the formation of volatile carboxylic esters in fruits (derived from Eriksson, 1979; Oliás *et al.*, 1995).

Terpenes are reported to be rare in apples; they are represented by a few hydrocarbons in oxygenated form by alcohols, such as linalool, linalool oxide, α-pinene and geraniol (Paillard, 1990). Terpenes are major contributors to aroma of some fruits such as citrus (Nursten, 1970; Shaw *et al.* 1990), apricots and mangoes (Chassagne and Crouzet, 1995).

The hydrocarbon, ethylene - the ripening hormone (Burg and Burg, 1967), present in large amounts in ripening fruit is thought to make no significant contribution to the aroma of apples (Schreier, 1984). Other hydrocarbons such as nonane, decane, benzene, ethyl benzene, cyclohexane, tridecane and undecane are considered to be contaminants from solvents used in the extraction process (Dirinck *et al.*, 1977; Flath *et al.*, 1967). However, other aromatic hydrocarbons have been identified in apples, such as benzyl acetate, benzaldehyde and acetophane (Paillard, 1990). An important hydrocarbon, α-farnesene, has been studied in detail because of its involvement in superficial scald formation in apples and pears (Barden and Bramlage, 1994; Huelin and Coggiola, 1968, 1970). The compound is found in both apple extracts (Girard and Lau,

1995) and in headspace vapour (Boylston *et al.*, 1994). Some of these hydrocarbons may contribute to aroma of apples to some degree and it has been suggested that some may be responsible for a 'dried leaves' note odour (Williams, 1979).

Miscellaneous compounds, such as γ-hexalactone, methyl eugenol, methyl chavicol and benzothiazole are reported to be present in apples and apple products and their contribution to aroma has not been investigated (Dimick and Hoskin, 1983; Yahia, 1994). A compound, 4-methoxyallylbenzene or estragole (Willaert *et al.*, 1983) has been identified as a compound contributing to the 'spice-like' or 'aniseed-like' note in the aroma of 'Ellison's Orange' apples and a number of apple cultivars (Williams *et al.*, 1977b). β-Damascenone, which is believed to derive from xanthophylls, is reported to be an important aroma impact compound in most apple juice and essence (Roberts and Acree, 1995), and hence is probably a product resulting from processing.

1.3.2 The Secondary Volatiles

The 'secondary volatiles' are compounds formed from non-volatile precursors, such as linoleic acid, during disruption of fruit tissue by the action of enzymes and, to a smaller extent, by heating during processing (Schreier, 1986). Berger (1991) stated that the flavour profile of apples and apple juice can change rapidly after disintegration of cells due to several reactions: (a) lipoxygenase-induced degradation of unsaturated C_{18} fatty acids, leading to intensely aromatic C_6 or C_9 cleavage products within minutes; (b) enzymatic hydrolysis of carboxylic acid esters, leading to a loss of fruity odour components; (c) modification of monoterpenes and their precursors, leading to rearranged and new products; and (d) formation of aromatic acid esters from activated precursors. Drawert *et al.* (1986) identified *cis*-3-hexenal, *trans*-3-hexenal, *cis*-3-hexenol and phenyl ethyl alcohol in apple homogenates. These C_6 volatile compounds are found in low levels in headspace of intact apples, but are abundant in apple juice and essence (Paillard, 1990). They are formed by the enzymatic oxidation of linoleic and linolenic acids after the fruit is crushed and exposed to oxygen (Hatanaka, 1993) with maximum production during the first 30 minutes after maceration of 'Cox's Orange Pippin' apple peel (Drawert *et al.*, 1986). Cold storage of such apple peel for 24 hours at 6°C favoured formation of these secondary aldehydes, in contrast with those stored at

ambient temperature (Drawert *et al.*, 1986). Feys *et al.* (1980b) reported that reducing the time between homogenisation at room temperature and distillation of apples at low temperature to 10 minutes could not prevent formation of significant amounts of hexanal and *trans*-2-hexenal. Addition of 0.1% linoleic acid and 0.1% linolenic acid to apples during homogenisation resulted in a 3-fold and 4-fold increase in n-hexanal and *trans*-2-hexenal, respectively, over control (Feys *et al.*, 1980b). Production of secondary volatiles from fatty acids is common in damaged plant tissues, such as leaves and fruits (Galliard and Matthew, 1977a; Hatanaka, 1993; 1996), and they play an important role in the overall flavour in a range of plant products, including tea (Hatanaka, 1996), apple juice and essence (Dürr and Schobinger, 1981; Flath *et al.*, 1967).

The formation of secondary volatiles, such as hexanal and *trans*-2-hexenal follows hydroperoxidation of linoleic and linolenic acids by the lipoxygenase enzyme (Galliard *et al.*, 1977; Gatfield, 1988). However, enzymatic oxidative degradation of lipids is preceded by the action of acyl hydrolase, which liberates free fatty acids (Galliard, 1980). The lipoxygenase [linoleate : oxygen oxidoreductase] of apple has been partially purified; it is membrane-bound, has a pH optimum at 6.0 and converts linoleic and linolenic acids into hydroperoxides (Kim and Grosch, 1979). These hydroperoxides are relatively unstable being cleaved rapidly by a hydroperoxide lyase enzyme (**Fig. 1.7**) to form volatile carbonyl compounds (Eriksson, 1979; Galliard and Matthew, 1977a; 1977b) which are cytotoxic to proteins and membrane structures of cells (Schreier, 1984). In leaves, the lipoxygenase and hydroperoxide lyase enzyme systems are bound to the thylakoid membrane of chloroplasts (Hatanaka, 1993; 1996). Lipoxygenases from different sources (ie. species of plants) differ considerable in their pH optima, substrate specificities and especially in the isomeric structure of their reaction products (Gatfield, 1988; Schrödter, 1990). Thus, depending on the type of lipoxygenase and plant tissue, the reaction produces either 9- or 13-hydroperoxides or a mixture of both (Schreier, 1984). The enzymatic cleavage of the 13-hydroperoxides leads to formation of hexanal and *cis*-3-hexenal, where the latter may undergo isomerisation by isomerase enzyme to give *trans*-2-hexenal (Gatfield, 1988). The volatile products from the 9-hydroperoxides are *cis*-3-nonenal *trans*-2-nonadienal and *cis*-6-nonadienal, respectively (Eriksson, 1979).

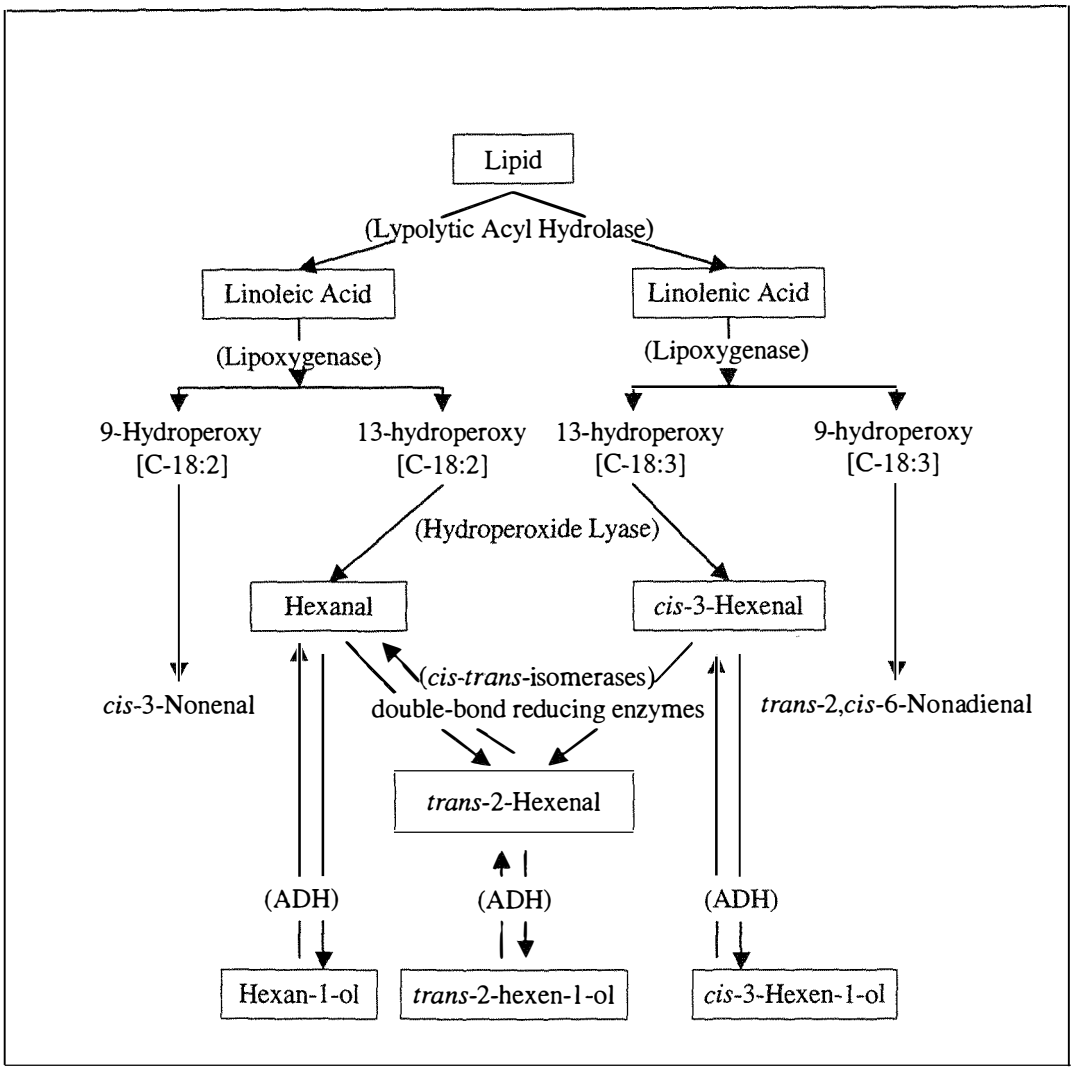


Fig. 1.7 Possible biochemical pathways for the formation of C_6-aldehydes and alcohols from fruit lipid (after Eriksson, 1979).

Lipoxygenase activity has been found to increase just before the respiration climacteric and ethylene production during ripening of apples (Meigh *et al.*, 1967). The increased lipoxygenase activity is found in the peel of apples and this has been suggested to be responsible for the decrease of linoleic and linolenic fatty acids during ripening, both on and off the tree (Meigh and Hulme, 1965).

The double bond position of the C_6 aldehyde and alcohol secondary volatiles has been found to have some impact on their olfactory characteristics. The fruity and sweet character is found with aldehydes and alcohols having a double bond at the C-2

position, a high green and fresh note is found with *cis*-3-hexenol, and a strong spicy and grassy green odour is associated with *cis*-3-hexenal. Compounds having a double bond at C-4, such as 4-hexenal give an impression of spicy and vegetable-like green smell, while those with a double bond at C-5, such as 5-hexenol and 5-hexenal, have strong oily-fatty, insect-like green and herbal odours (Hatanaka, 1993).

Investigations on the biogenesis of apple volatiles have shown that some of the volatile compounds are 'primary', which are considered to be direct metabolites produced by intracellular biogenetic pathways and these volatiles are emitted by the whole, intact fruit. These compounds comprise the odour from the fruit and the first impression given by the smell. Their quality and quantity depend on genetic factors and are influenced by ripening and storage, which will be discussed later. On the other hand, some of the volatile compounds, such as n-hexanal and *trans*-2-hexenal, are 'secondary' products, which formed very quickly during disruption of cell structure due to enzymatic reactions in the present of oxygen. These compounds are produced in very low concentrations or are absent in intact fruit. It has been suggested by some authors that it is necessary to distinguish between the 'primary' and 'secondary' volatile compounds (Berger, 1991; Paillard, 1990). For example, hexanal and *trans*-2-hexenal should not be cited as 'impact' compounds of apple aroma, as the 'green' note of these compounds can at best be regarded as important to apple juice and essence in terms of a habituation of consumer's sense of smell (Berger, 1991). Nonetheless, these compounds may be important during cutting or chewing of fresh apples.

1.3.3 Influence of Fruit Structure

Studies on the location of the aroma formation in whole apple fruit, have shown that the peel portion generally produced more volatiles than the cortex (Paillard, 1990; Williams and Knee, 1977). Guadagni *et al.* (1971) demonstrated the prominent role of the epidermal tissue in flavour formation of 'Red Delicious' apples. Apple peel stored for 2 days at 22° C produced more volatiles than the flesh or intact unpeeled apples with the largest production being esters, including ethyl acetate, ethyl propanoate, ethyl 2-methyl propanoate, ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate, butyl acetate and hexyl acetate. Removal of the oily-wax coating on the skin did not affect production of

those esters which indicated some other source of precursors. Fellman and Mattheis (1995) found that ethyl propanoate, ethyl 2-methyl butanoate, hexyl propanoate, and hexyl butanoate were synthesised only in the peel of 'Rome' apples, whereas ethyl acetate, butyl acetate, 2-methyl butyl acetate and hexyl acetate were formed in both the peel and flesh portions, but propyl acetate was synthesised only in the cortex tissues.

Knee and Hatfield (1981) investigated the esterification of supplied volatile precursors (C_2 - C_6 alcohols) in 'Cox's Orange Pippin' apples. Esterification was more rapid initially in the peel tissue but it quickly declined when compared with the cortex tissues. Bartley *et al.* (1985) found that after application of C_2 to C_8 alcohols the formation of the acetate esters; ethyl acetate, propyl acetate, pentyl acetate, hexyl acetate, heptyl acetate and octyl acetate; was much higher in the peel and outer cortex than in the inner cortex tissues, while there were no differences in butyl acetate production among peel, outer, or inner cortex. Bioconversion of some volatiles from fruit parts and substrates in fruit tissues are summarised in **Table 1.7** (Berger, 1991).

Table 1.7 Formation of volatiles by apple tissue in relation to substrates and fruit parts (after Berger, 1991).

Substrate	Product	Tissue	Reference
Carboxylic acids			
C_2 - C_4	aldehydes	fruit	De Pooter *et al.* (1987)
C_2 - C_6	esters and alcohols	epidermis	De Pooter *et al.* (1983)
C_4 and C_6	esters	cortex	Paillard (1979)
C_4 - C_{12}	2-ketones	epidermis	Paillard (1979)
C_4 - C_{12}	alcohols	peel	Paillard (1979)
Propionic acid	propyl esters	fruit	De Pooter *et al.* (1981)
	propionates	fruit	De Pooter *et al.* (1981)
Methyl esters			
C_2 to C_8	esters	fruit	Bartley *et al.* (1985)
Butyl acetate	butanol	peel	Williams and Knee (1977)
Butanol	butyl esters	fruit	Williams and Knee (1977)
Alcohols			
C_1 to C_6	esters	peel and fruit	Berger and Drawert (1984)
C_2 to C_8	esters	fruit	Bartley *et al.* (1985)
Aldehydes(C_3 to C_6)	alcohols and esters	fruit	De Pooter *et al.* (1983)

The 'secondary' aroma volatiles, such as hexanal and *trans*-2-hexenal, were released in high amounts from peel, outer cortex and core tissues after crushing and disruption of cells of 'Golden Delicious' apples (Paillard, 1981). This was probably due to the action of lipoxygenase which has high activity in the peel and core tissues compared with the other parts of apples (Feys *et al.*, 1980a). It has been suggested that the prominent volatile production in the peel of apples is due to the high enzymatic activity in that tissue. Lipid metabolism is pronounced in the epidermis and subepidermal layers of waxy skinned fruit and may generate free fatty acids. These may contribute to the metabolic changes that occur in the disrupted fruit tissues (Paillard, 1990).

1.4 FACTORS AFFECTING AROMA VOLATILE FORMATION

Both the internal factors (such as genetic differences and metabolic activity) and external factors (such as pre- and postharvest handling treatments) are known to influence aroma volatile formation in fruits, including apples (Paillard, 1981; Yahia, 1994).

1.4.1 Cultivars

Both qualitative and quantitative differences in aroma volatiles produced occur between apple cultivars. A comparison of aroma volatile production from several cultivars of apples has shown that all major constituents, chiefly esters are always present but in different proportions (Paillard, 1981). Brown *et al.* (1966) found that 'Starkrimson' and 'Royal Red Delicious' apples had a very similar pattern of volatiles produced, indicating the common origin of the two sports, but the amount of several volatiles produced were greater in the former cultivar. Similarly, 'Ruby' ('Starking Delicious' x 'Gallia Beauty') had volatile pattern resembling that from 'Delicious' suggesting the dominance of 'Delicious' volatiles in the make-up of 'Ruby'.

Poll (1981) reported that large differences in sensory aroma and taste were observed among 18 cultivars of apples. For example; a sensory panel indicated that 'McIntosh' had a strong persistent good fruit aroma with fine taste; 'Spartan' had a

strong, slightly perfume-like fruit aroma, good and slightly sweet taste; 'Cox's Orange Pippin' had a spicy 'special' fruit aroma; and 'Bramley' had very weak fruit aroma with some off-aroma and a very sour taste. Brackmann and Streif (1994) examined 28 apple cultivars and found that 'Gravenstein', 'Jonagold', and 'Golden Delicious' were among the cultivars which produced highest volatile production, while 'Granny Smith', 'Fuji' and 'Boskoop' produced the lowest levels. The high volatile production of 'Gravenstein' may not reflect the best aroma quality, since there were more alcohols than esters in the volatile composition. It was suggested that the low level of volatile production in 'Fuji' and 'Granny Smith' may be related to the low metabolic activity during shelf-life, ie. low ethylene production and respiration rate (Brackmann and Streif, 1994). Kakiuchi *et al.* (1986) reported that the total amounts of volatile compounds in juice of 5 apple cultivars were highest in 'Hatsuaki', followed by 'Kogyoku' ('Jonathan'), 'Golden Delicious', 'Mutzu', and 'Fuji'. The corresponding values were 8.94, 9.42, 5.96, 3.71 and 2.27 ppm, respectively.

'McIntosh' apples were found to produce higher amounts of ethyl butanoate, propyl butanoate, ethyl 2-methyl butanoate, propyl 2-methyl butanoate, ethyl propanoate, butyl pentanoate, and butyl hexanoate than 'Cortland' apples, but the latter produced more *trans*-2-hexenal than the former (Yahia *et al.*, 1990a). Schreier *et al.* (1978a) found that the C_6-secondary aroma compounds differed between cultivars; 'Fünfjahresplan' fruit formed more C_6 aroma compounds than did 'Jonathan' apples, regardless of its ripeness.

Differences in proportions of volatile compounds between cultivars may be explained by their biogenesis which has been studied by supplying precursors to fruit tissues. Yellow-skin cultivars of apples, such as 'Golden Delicious', produce mainly acetate esters, while red-skin cultivars, such as 'Richared' and 'Starking', produce chiefly butanoate esters (Paillard, 1979). Such differences may be dependent on different reaction rates in the β-oxidation of fatty acids, ie. on transformation of butanoate into acetate. Paillard (1979) found that butanoate and ethanol applied exogenously to tissue discs of 'Golden Delicious' apples were converted rapidly and completely into ethyl acetate, while in 'Richared' and 'Starking' they were transformed mainly into ethyl butanoate and partly into ethyl acetate. Paillard (1981) reported that exogenously applied

octanoate was transformed into alcohols and esters by peel tissue of 'Starking' apples, which has normal waxy cuticle, while it was converted into alcohols and large amount of ketones, but not esters, in 'Canada Gris' which has a corky coating.

Dirinck and Schamp (1989) classified apple cultivars into 'ester' and 'alcohol' types according to their aroma volatile production pattern. The 'ester' types are characterised by a high concentration of acetate esters, such as hexyl and butyl acetate, whereas the 'alcohol' type is characterised by cultivars producing high levels of hexanol and branched chain alcohols and esters (Brackmann *et al.*, 1993). Further classification has also been made, in that 'Red Delicious', 'Golden Delicious', 'Jonagold', and 'Jubilé' are closely related and characterised by high concentrations of butyl and hexyl acetate. 'Elstar' and 'Cox's Orange Pippin' have similar aroma patterns to each other, while 'Nico', 'Granny Smith', 'Paulared' and 'Summered' are characterised by high content of ethyl butanoate and hexanol, and 'Boskoop' and 'Jacques Lebel' are characterised by high concentration of α-famesene and hexyl 2-methyl butanoate (Dirinck and Schamp, 1989). Thus, there are qualitative and quantitative differences among apple cultivars.

1.4.2 Cultural Practice

Growth and development of apples, as well as future ripening and aroma development are influenced by some cultural practices. Somogyi *et al.* (1964) studied the effect of fertiliser applications on volatile production in several apple cultivars. Apples from high nitrogen treatments produced higher concentration of volatiles than fruit from low nitrogen treatments. However, when nitrogen was applied alone there was a lower volatile production from fruit of 'Delicious', 'Rome Beauty', and 'Spartan' than in fruit from those trees with complete fertiliser (N, P, K) application. Volatile production in 'Cox's Orange Pippin' was higher in fruit from trees receiving a soil application of $Ca(NO_3)_2$ than apples from a no nitrogen treatment. Fellman (1994) reported that a nitrogen fertiliser rate of 0.1 to 1.3 lb/tree or time of application (at full bloom, postharvest, or split) did not affect butyl acetate production in 'Redspur Delicious' apples. However, fruit from trees receiving a split nitrogen application had higher 2-methyl butyl acetate than other treatments and the split rate of 1.3 lb/tree gave the highest concentration of this compound. Phosphorus fertiliser increased production of 3

volatile esters in freshly harvested 'Golden Delicious' apples when compared with fruit from low phosphorus applications (Brown *et al.*, 1968). On the other hand, increased phosphorus supply to 4-year old sand-cultured 'McIntosh', seemed to decrease the amount of ethyl butanoate, ethyl propanoate, ethyl acetate, acetaldehyde, hexanal, and ethanol evolving from fruit after cold store compared with those fruit from trees receiving normal phosphorus levels. Increased potassium levels had little or no effect on volatile production from apples (Forsyth and Webster, 1971).

1.4.3 Growing Location and Seasonal Variation

The amount of volatiles produced by apples may also be influenced by growing location. Brown *et al.* (1968) reported that the amount of volatiles produced by both 'Starkrimson' and 'Royal Red Delicious' apples from a Watsonville plot in California were generally greater than similar samples of comparable maturity from a Sebastopol orchard. Temperatures during the growing season and harvest were usually warmer at Sebastopol, suggesting that the cooler climate of Watsonville may favour development of a higher potential for the production of volatiles by apples than those grown in a warmer district. Kollmannsberger and Berger (1992) reported that the main volatiles of fully ripe 'Red Delicious' apples from different geographic origins were shown to be different. Ethyl esters dominated in the spectrum of apples from South Africa, whereas the same cultivar from New Zealand was dominated by butyl and hexyl esters. However, the authors did not indicate the storage history of the apples used for this comparison.

Seasonal variation in volatile production from apples has also been reported. Concentrations of ethyl butanoate and butyl acetate in 'Golden Delicious' apples at a comparable maturity have been found to be high in one season but low in the next and the opposite has been observed with butyl butanoate (Grevers and Doesburg, 1965). Hansen *et al.* (1992a) found that the optimal picking date with respect to the volatile production of 'Jonagold' apples, varied from year to year, with a large variation in total volatile production occurring between years over 3 consecutive seasons.

1.4.4 Growth Regulators

Diaminozide (Butanedioic acid 2,2-dimethyl hydrazide) is a growth regulator which has been used in apples to inhibit ethylene production, delay ripening and reduce pre-harvest fruit drop and loss of firmness after harvest; its use is now prohibited as the breakdown products are thought to be carcinogenic (Yahia, 1994). Postharvest application of 1000 ppm diaminozide delayed production of *trans*-2-hexenal, methyl hexanoate, butyl hexanoate, hexyl hexanoate, and hexyl 2-methyl butanoate at the same time as it delayed ripening of 'McIntosh' and 'Cortland' apples. It was speculated that diaminozide might have a specific and selective effect on the formation of C_6 aroma compounds (Yahia *et al.*, 1990b). This phenomenon is probably due to the indirect effect of diaminozide on volatile production, presumably by delaying the onset of climacteric and associated events, such as ethylene production, thereby decreasing a general metabolic activity of fruit that may result in a decreased availability of precursors for volatile biosynthesis (Song and Bangerth, 1996). A sensory panel evaluated 'McIntosh' apples treated with 2000 ppm diaminozide as being poorer in quality than untreated fruit, although a lower level of application (500 ppm) had no adverse effect on flavour (Murphy *et al.*, 1971).

Pre-harvest treatment with aminoethoxyvinylglycine (AVG), an effective inhibitor of ethylene biosynthesis and fruit ripening (Yang and Hoffman, 1984), had an adverse effect on aroma production at 20°C of 'Golden Delicious' apples after 3, 5, 7, and 9 months in low pressure storage when compared with untreated fruit (Bangerth and Streif, 1987). Similarly, pre-harvest treatment of 'Bartlett' pears with AVG combined with storage treatment reduced subsequent production of the volatiles methyl, ethyl, and hexyl acetate (Romani *et al.*, 1983). It has been suggested that AVG or low pressure storage probably reduced the sensitivity of fruit to ethylene (Bangerth and Streif, 1987), thus retarding general metabolic events such as autocatalytic ethylene production and respiratory activity, which provide substrates and/or precursors for aroma volatile formation, and hence resulting in a reduction of volatile production (Song and Bangerth, 1994; Song and Bangerth, 1996).

Ethylene is known to influence flavour development of apples during ripening, where it stimulates ripening and changes in acids, sugars, and aromatic volatiles, either directly or indirectly. (Jeffery *et al.*, 1984; Paillard, 1981). Ethylene regulates fruit ripening by coordinating expression of genes that are responsible for a variety of processes, including a rise in respiration, autocatalytic ethylene production, and change in colour, texture and flavour (Jeffery *et al.*, 1984; Oetiker and Yang, 1995). Ethylene is biosynthesised from S-adenosyl methionine via 1-amino cyclopropane-1-carboxylic acid (ACC), catalysed by ACC synthase and ACC oxidase (Kende, 1993). Both ACC synthase and ACC oxidase are encoded by multigene families and both are limiting in preclimacteric fruit but are greatly induced during fruit ripening. (Kende, 1993; Oetiker and Yang, 1995). Studies using ethylene inhibitors, such as 2,5-norbornadiene and AVG, pulse ethylene treatment and antisense transgenic fruit, (reviewed by Oetiker and Yang, 1995), have shown that ethylene synthesis plays a key role in regulating fruit maturation and ripening. Ethylene triggers the respiration climacteric in the apples by stimulating synthesis of RNA and proteins, and the onset of ripening is accompanied by increased synthesis of enzymes required for normal ripening (Brady, 1987; Oetiker and Yang, 1995). During ripening increased activity of hydrolytic enzymes such as lipase, acid phosphatase, chlorophyllase and transaminase results in the accumulation of substrates, including fatty acids and amino acids, for the biosynthesis of aroma volatiles in fruit (Paillard, 1990). Ethylene treatment of early harvested 'Golden Delicious' stimulated the production of free fatty acids as well as aroma volatiles of the fruit. The production of branched chain aroma compounds, suggested that ethylene treatment also affected amino acid metabolism (Song and Bangerth, 1994).

Ethylene may be indirectly involved in aroma volatile production through its effect on increasing a general metabolic activity of fruit such as respiratory activity and associated events which subsequently generates precursors and/or substrates for volatile biosynthesis (Song and Bangerth, 1996). However, so far there seems to be no evidence that ethylene is directly involved in aroma volatile biosynthesis in fruit.

1.4.5 Maturity and Postharvest Ripening

Production of volatiles in fruit such as apples, pears, and bananas is related to stage of maturity. In apples, volatile production is initiated during the climacteric rise in respiration, reaching a maximum during the postclimacteric ripening phase and decreasing as fruit senesce (Fellman and Mattheis, 1995; Paillard, 1990; Vanoli *et al.*, 1995). Aldehydes such as butanal, pentanal, *trans*-2-hexenal, and heptanal were the dominant volatiles detectable in intact immature 'Golden Delicious' (De Pooter *et al.*, 1987) and 'Bisbee Delicious' (Mattheis *et al.*, 1991b) apples, whereas maturing and ripening fruit produced primarily esters and aliphatic alcohols (Flath *et al.*, 1967; Schreier, 1984). Unripe 'McIntosh' apples contained low levels of all volatiles giving a grassy and green aroma note; hexanal was correlated with ripe fruit aroma, but overripe fruit aroma was associated with ethyl propanoate, ethyl butanoate, and ethyl 2-methyl butanoate (Panasiuk *et al.*, 1980).

By trapping volatile compounds on Porapak Q (purge and trap technique), generated from sequential samples of air surrounding ripening 'Bartlett' pears during 16 days at 25°C, Tressl *et al.* (1975) found that α-farnesene and ester formation (butyl acetate and hexyl acetate) were produced out of phase with each other in a cyclic manner. They suggested that one pathway could be activated, while another pathway was inhibited and the cycle might reverse itself, indicating that the biosynthesis of volatile compounds in fruit was a dynamic system.

Yahia *et al.* (1990b) studied aroma volatile production of 'McIntosh' and 'Cortland' apples during maturation and ripening on the tree. Of those volatiles monitored, only 12 were produced on the tree and those considered to be the most odour-active, such as *trans*-2-hexenal, ethyl butanoate, ethyl 2-methyl butanoate, and hexyl hexanoate were not produced until later in ripening following the autocatalytic production of ethylene. Hexyl acetate and methyl butanoate concentrations were high in immature apples and decreased gradually during ripening. Volatile production of 'Bisbee Delicious' apples harvested at weekly interval during the 8-week transition period from pre- to post-climacteric stages was studied by Mattheis *et al.* (1991b). As apple development progressed, concentrations of butanal, pentanal, *trans*-2-hexenal and

heptanal declined, while most alcohols were present throughout the same period; 2-methylbutanol was present only in samples from late harvest and 2-methyl butyl acetate appeared before the onset of ethylene production. Production of acetate esters preceded the increased ethylene levels, while many other esters were detected near the end of harvest period as fruit entered the climacteric stage which coincided with increased internal ethylene concentrations. During the preclimacteric stage, number and concentration of esters was negligible and a large number of low molecular weight aldehydes were detected; however, the aldehydes were absent as fruit approached physiological maturity and entered the ripening phase. As 2-methyl butyl acetate was detected several weeks prior to increased ethylene production, it was suggested that this compound could be useful as an indicator of fruit maturity for scheduling commercial harvest (Mattheis *et al.*, 1991b).

Date of harvest or the number of days after full bloom, which are often used as one of the harvest indices, may have a substantial influence on aroma volatile production of apples after harvest (Bachmann, 1983; Brown *et al.*, 1966). When 'Golden Delicious' apples were harvested about 4 weeks prior to commercial harvest, fruit produced very low amounts of esters and alcohols (Song and Bangerth, 1994) and the evolution of total volatiles by such immature apples after storage was negligible even after 44 days of ripening (Brown *et al.*, 1966). Onset of volatile production was delayed in early picked 'Jonagold' apples and the concentrations produced were much lower when compared with late picked fruit (Hansen *et al.*, 1992a).

Vanoli *et al.* (1995) studied volatile production in 'Starkspur Golden' apples harvested at 158, 172, and 181 days after full bloom (AFB). Apples harvested 158 (commercial harvest) and 172 days AFB took 33 days of postharvest ripening to develop maximum production of volatiles, while those harvested 181 days AFB required only 10 days. Late harvested apples had high butanoate esters and alcohols, while commercial harvested fruit produced lowest amount of aldehydes, alcohols and esters. Apples picked 172 days AFB had the highest amount of most volatiles which reached maximum production after 14 days of ripening; such fruit had low amounts of butanoates and alcohols, but were high in acetate esters, such as pentyl and hexyl acetate. In addition, butyl and hexyl acetate reached their maximum when butanol and hexanol concentrations

were also maximal, especially in apples harvested 158 and 172 days AFB. A greater ester production in late harvested apples, relative to early harvested fruit, may increase flavour or aroma quality because esters are the major contributors giving a typical aroma characteristic to the fruit (Dimick and Hoskin, 1983; Williams, 1979).

When 'Jonagold' apples were harvested 3 times at fortnightly intervals starting approximately 2 weeks prior to commercial harvest and stored at 0°C in regular air (RA) and controlled atmosphere (CA) storage, the late harvested fruit produced 14% more volatile compounds than did first harvest fruit. The increases were due mainly to straight chain C_3 to C_6 acetate esters and alcohols and some hydrocarbons (Girard and Lau, 1995). Apples from optimum and late harvests normally developed aroma volatiles, at ambient temperature, immediately or shortly after harvest and reached a considerably higher maximum than that from early picked fruit (Bachmann, 1983; Dirinck *et al.*, 1989). Dirinck and Schamp (1989) reported that the optimum harvest date of 'Golden Delicious' apples corresponded with a concentration of butyl acetate of 0.4 $\mu g \cdot kg^{-1} \cdot 6L^{-1}$ in the headspace. They suggested that the optimum harvest date could be predicted by regressing log butyl acetate (after 2 days ripening) against picking date.

1.4.6 Postharvest Temperature

The lowest possible temperature that fruit can tolerate is generally used to delay fruit ripening and senescence, retain fruit quality and prolong storage life of apples after harvest. Low temperature reduces fruit metabolism in general and this applies to development of aroma volatiles in particular (Yahia, 1994).

A study with 'Jonathan' apples stored between -1°C and 10°C showed that an increase in emission of acetate esters, such as butyl acetate, isopentyl acetate and hexyl acetate, corresponded with an increase of storage temperature (Wills and McGlasson, 1970; 1971). Volatile production of 'Calville Blanc' apples stored at 4°C was compared with that from fruit at 15°C; while metabolic changes in the fruit were greatly retarded at the lower temperature, total volatile emissions were still marked and reached a maximum during ripening as indicated by the loss of green colour. At 4°C, relatively high molecular weight volatiles, such as butyl and hexyl acetate, were decreased, while the lower

molecular compounds, such as ethyl acetate, were increased as compared with 15°C. However, when the fruit became yellowish, a pronounce loss of flavour was observed and very low volatile production occurred (Paillard, 1990).

Volatile emissions from 'Golden Delicious' apples stored at 3°C were generally lower than fruit stored at 6° or 10°C during 1 to 4 months. Similarly, 'Cox's Orange Pippin' volatile production was lower at 3°C than at 6°C during the same period of storage. However, during ripening at 15°C, apples previously stored at the lower temperature generally emanated higher amounts of volatiles than fruit that had been stored at 6°C, except for those stored for 1 month, where production of volatiles was similar (Grevers and Doesburg, 1965). Ripening of 'James Grieve', 'Cox's Orange Pippin' and 'Golden Delicious' at 18°C stimulated an increase in concentration of volatile compounds compared with that occurring at 4°C (Bachmann, 1983). High temperatures during ripening have been reported to affect volatile output of 'Red Delicious' apples; ripening at 46°C inhibited aroma production, at 32°C the rate of ester production was increased, and 22°C yielded the maximum amount of esters (Guadagni *et al.*, 1971). It was suggested that the optimum temperature for ester production would be between 20° - 30°C, while higher temperatures could cause a partial inactivation of the enzyme system responsible for volatile ester synthesis (Guadagni *et al.*, 1971).

Apples removed from short-term (< 3 months) cold storage had increased volatile production compared with fruit stored at ambient temperatures. Stimulation of ester and alcohol synthesis was observed upon transferring 'Red Delicious' apples from cold storage for 7 days at 5°C to 23°C. Total ethyl esters increased from 3.7 to 45.7 $\mu mol \cdot 100g^{-1}$ and hexyl esters from 1.7 to 19.9 $\mu mol \cdot 100g^{-1}$; most of the esters that increased were known to be those contributing to apple aroma, such as ethyl butanoate, ethyl hexanoate, ethyl 2-methyl butanoate, and ethyl 4-(*Z*)-decanoate (Kollmannsberger and Berger, 1992). Ester production of fresh 'Rome' apples increased when fruit was allowed to stand at 23°C for 6 days after removal from air storage at 0° - 1°C for three months (Fellman and Mattheis, 1995).

Freezing of strawberries decreased concentration of most aroma substances, but amounts of 2,5-dimethyl-4-methoxy-3(2H)-furanone, which gives the characteristic wild

strawberry aroma was higher in frozen fruit than in freshly harvested (Schreier, 1980). However, the reason for such an increase in this ketone is not known.

When single strength juice from 5 apple cultivars ('McIntosh', 'Filippa', 'Mutzu', 'Ingrid Marie' and 'James Grieve'), and 4-fold concentrates from 2 cultivars ('McIntosh' and 'Filippa') were stored for up to 12 months at 3°C and 20°C, no difference in sensory quality of juice and essence was found during storage at 3°C. However, storage at 20°C gave juice with less aroma after 6 months and this became more pronounced after 12 months storage. In addition, 20°C storage gave apple juice with a cooked-like aroma, which has been found to be associated with hydroxy methyl furfural (HMF) found in both the juice and essence (Poll and Flink, 1983). The level of HMF in juices was also dependent on the degree of ripeness of the apples, with the highest level found in 'eating ripe' fruit and the lowest in 'unripe' samples (Poll, 1985). On storing 'McIntosh' apple juice for 1 year at 3°, 10°, 20°, and 30°C, increasing storage temperature resulted in a marked reduction in fruit aroma scores and an increase in cooked-apple aroma scores. Storage at 30°C caused an 87% reduction of total ester and aldehyde concentration, while total alcohols decreased by about 33% (Poll, 1983).

Heating apple juice during processing increased β-damascenone, an unusually potent aroma compound which has a fruity odour (Roberts and Acree, 1995). Distillation of apple juice for brandy enhanced the concentration of ethyl esters and many higher alcohols, as well as β-damascenone. The increased amounts of ethyl esters was thought to occur during fermentation of apple juice (Schreier *et al.*, 1978a).

1.4.7 Water Loss

During air storage of 'Jonathan' apples at -1°C in air at different humidities, increased rate of water loss increased the evolution of acetate esters such as hexyl, isopentyl and butyl acetate, but caused a decrease of n-hexanol, isopentyl alcohol, and n-butanol. It has been suggested that water loss enhanced the production of acetate esters as well as providing a 'carrier' for their removal from the fruit (Wills, 1968). Loss of volatiles as influenced by increasing the rate of water loss has also been reported to occur in an order, ie. hexyl acetate and pentyl acetate, then hexanol, butyl acetate,

isopentanol, and butanol last. This differential effect is thought to be due to the ease of evaporation of the esters from apples (Wills and McGlasson, 1970; Wills and Scott, 1972).

1.4.8 Controlled Atmosphere Storage

Controlled atmosphere (CA) storage, in which the concentrations of O_2, CO_2 and/or ethylene in the storage atmosphere are precisely controlled, is used extensively to prolong storage life of apples. It is well known that CA storage decreases the rate of ethylene synthesis and action, and hence delays ripening, but it can also suppress aroma volatile production (Girard and Lau, 1995; Knee, 1991a; Smock, 1979). It has long been known that apples ripened after CA storage do not develop full characteristic aromas. Grevers and Doesburg (1965) reported that the amount of volatile constituents of 'Golden Delicious' apples decreased as storage time increased. Guadagni *et al.* (1971) found that 'Delicious' apples from commercial CA store were defective in their ester production after transfer to air compared with air stored fruit. 'Cox's Orange Pippin' apples lost their capacity to ripen and to develop aroma in air at 20°C after they had been stored in 2% O_2 at 3.5°C for 5 months when compared with fruit stored in air or 5% CO_2 + 19% O_2 (Patterson *et al.*, 1974). As the esterifying enzyme AAT was present in fruit, low ester production was thought to be due to the lack of alcohol precursors (Knee and Hatfield, 1981; Williams and Knee, 1977).

Willaert *et al.* (1983) stored 'Golden Delicious' apples for various times (3 to 9 months) at 5°C in 1 - 2% CO_2 + 1 - 2% O_2 and then allowed them to ripen at 20°C. A significant decrease in flavour and reduced ester production was noted after long-term CA storage (> 6 months). Short-term CA storage, ie. less than 6 months, has been suggested in order to retain fruit aroma quality (Dirinck *et al.*, 1989). Lidster *et al.* (1983) showed that short-term storage of 'McIntosh' apples for 90 days in an atmosphere of 1.5% CO_2 + 1% O_2 at 2.8°C did not inhibit regeneration of ethyl butanoate and hexanal, but did suppress methyl acetate, ethyl acetate and ethyl propanoate on transferring fruit to air at 20°C. Long-term storage (320 days) of apples under these conditions resulted in complete loss of the main headspace volatiles and

blocked their formation after return to air (Lidster *et al.*, 1983; Streif and Bangerth, 1988; Yahia *et al.*, 1991). The reduced capacity of aroma production during shelf-life after low O_2 CA storage has been found in apple cultivars producing mainly ester compounds with straight chain carbon atoms, such as 'Golden Delicious' and 'Cox's Orange Pippin' apples. However, high CO_2 CA (> 3%) storage suppressed both straight chain and branched chain ester production of apples (Brackmann *et al.*, 1993).

Streif and Bangerth (1988) studied CA storage of 'Golden Delicious' in 11 combinations of O_2 (1 - 21 %) and CO_2 (0.8 - 9%) concentrations at 1°C for 3 - 9 months. Decreasing O_2 concentrations to 3% had little effect on volatile production, but a further decrease to 1% significantly reduced it. At O_2 concentrations of 3%, higher CO_2 (>3%) became decisive in diminishing volatile production. There was neither a simple additive or synergistic, nor a linear relationship between changes in storage atmosphere composition and aroma volatile production, although increasing CO_2 and decreasing O_2 concentrations reduced aroma volatile production. It has been suggested that high CO_2 in a storage atmosphere may interfere with carboxylic acid metabolism and alcohol dehydrogenase activity, thus leading to a deterioration of the aroma quality (De Pooter *et al.*, 1987).

Hansen *et al.* (1990) reported the effects of various concentrations of O_2 (1 to 21%) in CA storage at 2°C on the production curves of esters during regeneration of individual aroma volatiles of 'Jonagold' apples on transferring to air at 20°C. Oxygen concentrations of 1% and 2% reduced production of most acetate and branched chain esters as compared with air storage (21% O_2); intermediate O_2 concentrations (3 to 5%) gave the highest production of branched chain esters such as 2/3-methyl butyl esters and 2-methyl-2-butenyl esters. All straight chain esters were produced in a positive correlation with O_2 concentrations during storage (Hansen *et al.* 1992b). Hansen *et al.* (1990) suggested that the lack of volatile production in apples stored in low O_2 concentrations for a long period may also be due to lack of O_2 needed for the processes underlying the ester synthesis, such as the catabolism of precursors, and these processes could a have different affinity to O_2 and/or sensitivity to ethylene resulting in different patterns of emanation of esters according to the nature of the precursors (Hansen *et al.*

1992a). Fellman *et al.* (1993b) reported that the activity of alcohol acyltransferase (AAT) enzyme in apples is apparently sensitive to low oxygen atmosphere.

A comprehensive study on the effect of CA storage on the volatile production of 'McIntosh' and 'Cortland' apples was reported by Yahia and colleagues (Yahia, 1989; 1991; Yahia *et al.*, 1990a; 1991). Apples stored in air and in CA for 5 months at 0°C produced similar amounts of some volatiles; such as hexanal, *trans*-2-hexenal, hexyl acetate, methyl butanoate, and butyl 2-methyl butanoate while ethyl 2-methyl butanoate was produced only in fruit kept in air at 20°C. However, apples ripened after 5 months of CA storage produced less total volatiles than fruit before storage or fruit stored in air (Yahia *et al.*, 1990a). Although all CA treatments reduced some flavour volatiles and there was no difference in aldehydes and acetates produced between conventional and low ethylene CA (LCA), butanoates, 2-methyl butanoates, pentanoates and hexanoates were either severely or completely suppressed under LCA conditions, somehow reduced by the low ethylene levels (Yahia, 1989; Yahia *et al.*, 1991). On the other hand, it has been reported that the presence of ethylene in CA storage atmosphere had little or no effect on aroma volatile production in 'Golden Delicious' apples (Brackmann, 1989).

Controlled atmosphere storage does significantly reduce or alter normal metabolism of fruit. It is possible that reduction in volatiles reported may be due to the general inhibition of ripening under CA conditions (Streif and Bangerth, 1988). Decreased biosynthesis and/or degradation of fatty acids has also been proposed to be the cause of suppression in volatile ester production in apples under CA storage (Brackmann *et al.*, 1993; Harb *et al.*, 1994; Knee, 1991a). Amino acids, the putative precursors of some esters, are known to decrease in concentration during ripening and remain relatively constant at low levels in storage (Ackermann *et al.*, 1992). Decreased concentrations of these precursors could result in a reduced availability of substrates for ester synthesis, which may also be an important factor in aroma regeneration of fruit stored in long-term CA (Fellman *et al.*, 1993a).

Aroma volatile production of long-term CA-stored apples may be partially improved by increasing O_2 levels above 2% during storage at a time relatively close to marketing; such a process can retain flesh firmness as well as increasing aroma (Smith, 1984). However, Streif and Bangerth (1988) reported that after 9 months CA storage, a

3-week treatment at 1°C in air was not effective in recovering the aroma production of 'Golden Delicious' apples. Exposing 9-month CA-stored 'McIntosh' apples to air at 20°C (for 3 to 4 weeks) could increase production of some volatiles, while a 3 to 4 week treatment with 100% O_2, air at 3.3°C, was not effective in recovering aroma volatile production (Yahia, 1991). Brackmann *et al.* (1993) reported that a slight recovery of aroma production of 8-month CA-stored 'Golden Delicious' apples was achieved by exposing fruit to air at 1°C for 14 days following CA storage.

CA is a well-established apple storage technique which is known to affect aroma production of fruit both qualitatively and quantitatively, compared to refrigerated-air storage. Reduced O_2 concentrations and/or elevated CO_2 concentrations used in CA storage decrease the production of volatile compounds responsible for the aroma quality of apples. The reduced production of volatile compounds occurs even after removing the fruit to normal atmosphere, and this is known as the 'residual' effect of CA storage. Although the phenomenon is well described, the biochemical basis for this effect has yet to be explained.

1.4.9 Other Treatments

Enzyme treatment of apple pulp during the juice extraction process influences the formation and distribution of C_6 aroma compounds in juice. Pectolytic preparations lead to preservation of C_6-aldehydes, whereas application of cellulolytic enzymes strongly reduced the aldehydes to corresponding alcohols. Treating apple pulp with pectolytic and cellulolytic preparations for 26 hours or longer caused considerable hydrolysis of esters, resulting decreased aroma quality of apple juice (Schreier *et al.*, 1978b).

Increasing holding time of 'Mutzu' apple pulp (for 0, 15, 30, 60, 240, and 480 hours) before juicing decreased C_6 aldehydes and acetate esters and after 8 hours 75 - 90% of these volatiles were lost compared with initial concentrations (Poll, 1988). Concentration of some butanoate esters, such as ethyl butanoate and ethyl 2-methyl butanoate, decreased during the first 60 minutes of pulp holding, but increased after this to the same or higher levels than were initially present, while other butanoate and hexanoate esters changed very little. Ethanol also increased during the pulp holding

period and this could be responsible for the increase in ethyl butanoate and ethyl 2-methyl butanoate, as ethanol is thought to be the immediate precursor of ethyl esters (Berger and Drawert, 1984; Poll, 1988).

Short-term anaerobiosis has been reported to alter production of various aroma volatile in fruits (Mattheis *et al.*, 1991a; Pesis *et al.*, 1991; Shaw *et al.*, 1990; 1991; 1992); the effect of such conditions will be discussed later (**Section 1.5.4**).

1.5 METABOLISM UNDER HYPOXIA AND ANOXIA IN FRUIT

Fruit tissues require molecular oxygen (O_2) for many biosynthetic and/or degradative processes (Brady, 1987; Powrie and Skura, 1991; Rhode, 1980), with a major one being mitochondrial respiration (Ricard *et al.*, 1994). Supply of O_2 to fruit tissue depends on its concentration and its diffusion rate in the surrounding medium (Knee, 1991b). Under some natural conditions, where oxygen is abundant and in spite of the high affinity of cytochrome C oxidase for O_2, the supply of O_2 to cells may not meet respiratory demand, thus resulting in hypoxia (Blanke, 1991). Since the diffusion of O_2 is about 100 times slower in water than in air (Thompson and Greenway, 1991), hypoxia is often found in tissue surrounded by a layer of water (Boersig *et al.*, 1988). In bulky organs, such as apples, concentration gradients of O_2 and CO_2 will exist, so that tissues experience internal concentrations that differ from external concentrations and partial hypoxia can be established (Banks, 1985; Blanke, 1991; Knee, 1991a; Yearsley *et al.*, 1996). Some endogenous and exogenous factors known to affect diffusion of O_2 and/or CO_2 gas in apple fruit tissue include skin resistance and porosity (Cameron and Yang, 1982; Park *et al.*, 1993), intercellular space (Blanke, 1995), accumulation of epicuticlar wax (Park *et al.*, 1993), respiration rate (Blanke, 1995; Ke and Kader, 1992a; Ke *et al.*, 1994a), stage of maturity (Andrich *et al.*, 1990), temperature (Dilley *et al.*, 1964) and gas composition in the storage atmosphere (Kader, 1995). Some of these factors, if not all, influence the tolerance of apples to hypoxia and they may vary with cultivar and growing condition (Blanpied and Jozwiak, 1993; Park *et al.*, 1993).

The term 'hypoxia' loosely applies to any oxygen partial pressure less than 21 kPa, whereas the term 'anoxia' means a total absence of oxygen, and 'normoxia' denotes

the normal partial pressure of oxygen in air, ie. 21 kPa (Chen and Solomos, 1996; Chervin *et al.*, 1996). Hypoxia and anoxia at the cellular level can be defined in terms of respiratory metabolism (Pradet and Bomsel, 1978). The cell may be considered to be under 'hypoxic' condition when the oxygen partial pressure limits the production of energy-rich ATP by mitochondria. The cell is under 'anoxia' when the production of energy-rich bonds by oxidative phosphorylation becomes negligible in comparison to those generated by the fermentation process. Recently, Ricard *et al.* (1994) suggested that hypoxic metabolism is a concurrent activity of both aerobic metabolism and some degree of fermentation. The metabolism of an organ may be heterogenous under hypoxic conditions, since the outer layer of tissues receive more oxygen and are, therefore, less hypoxic than the core of the organ. The term 'anoxia' or 'deep hypoxia' (Roberts *et al.*, 1992) should be restricted to situations where fermentation could be considered to be the only source of ATP as mitochondrial respiration has declined to almost zero.

1.5.1 Anaerobic Metabolism

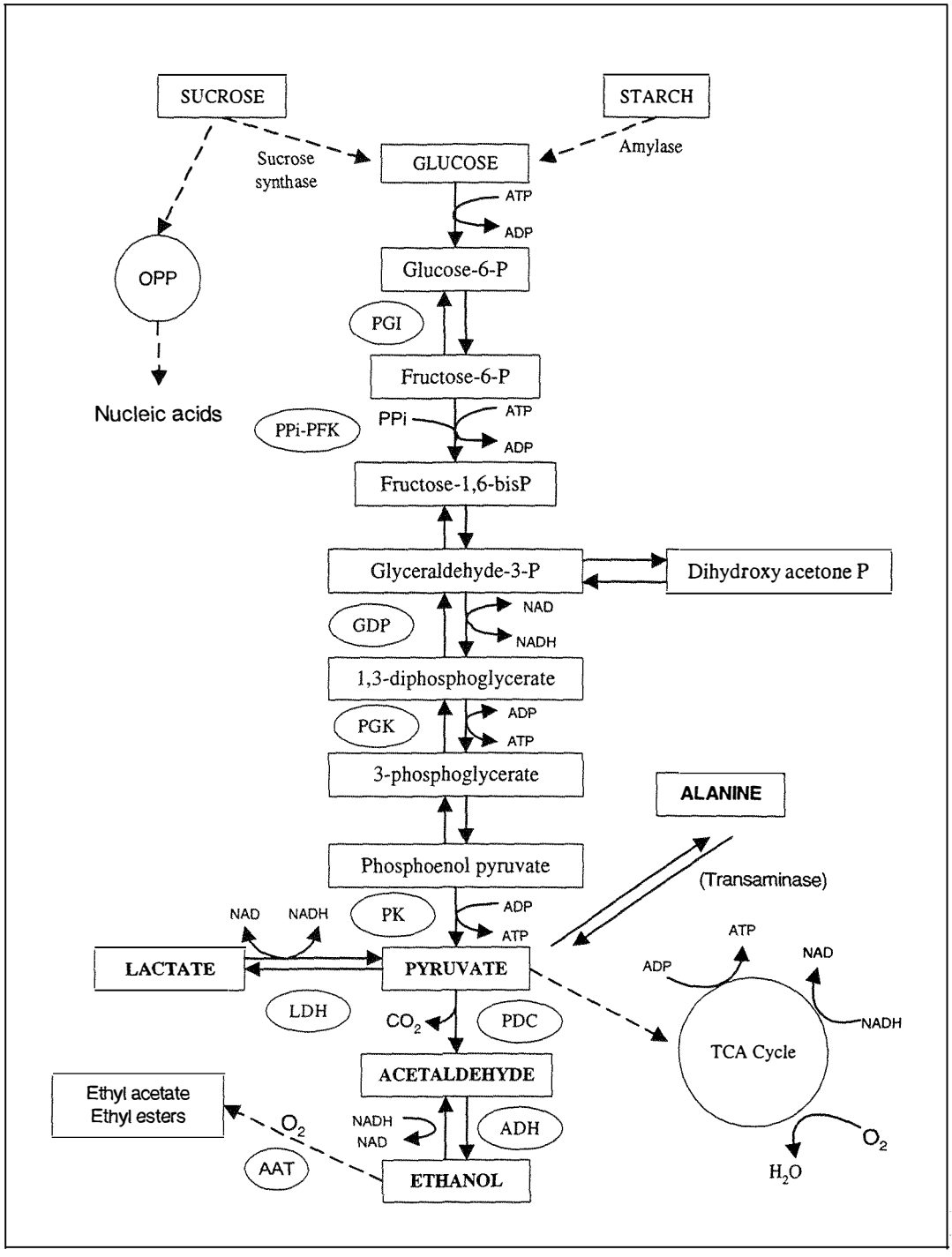
Glycolysis (**Fig. 1.8**) is a ubiquitous pathway that operates under both aerobic and anaerobic conditions (Chervin *et al.*, 1996; Kato-Noguchi and Watada, 1996; Kennedy *et al.*, 1992). Breakdown of the respiratory substrates in glycolysis is accelerated when a cell is deprived of O_2 leading to an increase in CO_2 production. Conversely, when the same cell is transferred from anaerobic conditions into a medium containing O_2, the rate of carbon utilisation is reduced.

The microatmospheric O_2 concentration at which anaerobic respiration of a fruit tissue commences is known as the extinction point; this is the lowest O_2 concentration at which ethanol production ceases (Powrie and Skura, 1991). Above this point, sufficient oxygen is transported to the mitochondria for aerobic respiration. Since ethanol is known to form in tissue in some fruit under aerobic conditions, Boersig *et al.* (1988) have suggested the term 'Anaerobic Compensation Point (ACP)', as an indicator of anaerobic respiratory transition; it is defined as the lowest O_2 concentration at which CO_2 evolution is minimum. Recently, Beaudry (1993) has suggested the term 'RQ Breakpoint', which is

defined as the O_2 partial pressure at which the respiratory quotient (RQ) begins to increase as O_2 level decreases.

The switching between aerobic and anaerobic respiration of plant cell is thought to be due to allosteric inhibitors, such as citric acid and ATP, and an activator, such as inorganic phosphate (P_i), on phosphofructokinase (PFK). The enzyme, PFK, catalyses the non-equilibrium reaction which converts fructose-6-phosphate into fructose-1,6-bisphospate. During aerobic respiration, these two inhibitors, citric acid and ATP are synthesised continuously by the TCA cycle leading to a suppression or regulation of glycolysis. However, under anaerobic condition, the TCA cycle is thought to be switched off, citric acid is no longer synthesised and the concentration of ATP is reduced, thus the blocking of PFK by citric acid and ATP is removed. This stimulates an intensive phosphorylation of fructose-6-phosphate as the amount of the activator (P_i) is increased under anoxia through activation of PFK (Vartapetian, 1978).

Under anaerobic metabolism, ethanol, lactate, and alanine are the major products of fermentation in plant tissues; they are all derived from pyruvate, the end product of glycolysis (Chervin *et al.*, 1996; Ricard *et al.*, 1994). Other major components are succinate and γ-aminobutyrate, which are derived from oxaloacetate following the TCA cycle (Ricard *et al.*, 1994; Smith and ap Rees, 1979; Zemlianukhin and Ivanov, 1978). Sucrose, which is presumed to be the major form of storage carbohydrate, is metabolised via glycolysis (**Fig. 1.8**) to produce pyruvate. Pyruvate is either reduced to lactic acid by lactate dehydrogenase (LDH) with NADH, or decarboxylated to acetaldehyde by pyruvate decarboxylase (PDC) with the formation of CO_2 (Davies, 1980; Davies *et al.*, 1974). Acetaldehyde is reduced by alcohol dehydrogenase (ADH) in the presence of NADH to form ethanol and NAD^+. This generated NAD^+ can re-enter the glycolysis pathway as an oxidising agent to maintain the glycolytic process (Kennedy *et al.*, 1992). Sucrose is also metabolised via the oxidative pentose phosphate pathway to produce intermediates for nucleic acid synthesis and reducing equivalent, ie. NADPH (Rumpho and Kennedy, 1983). Lipids are synthesised and accumulated under anaerobic condition and this could serve as a mechanism for the re-oxidation of NADPH produced by the oxidative pentose phosphate pathway. As such, lipids could serve as 'alternative electron acceptors' (Kennedy *et al.*, 1992).



Abbreviations : AAT = Alcohol acyltransferase, ADH = Alcohol dehydrogenase, PGI = Glucose phosphate isomerase, PPi-PFK = Pyrophosphate-dependent phosphofructokinase, GDP = Glyceraldehyde-3-phosphate dehydrogenase, PGK = Phosphoglycero kinase, PK = Pyruvate kinase, LDH = Lactate dehydrogenase, PDC = Pyruvate decarboxylase, OPP = Oxidative pentose pathway

Fig. 1.8 Glycolysis and alcoholic fermentation pathways (derived from Chervin *et al.*, 1996; Kennedy *et al.*, 1992; Perata and Alpi, 1993; Stryer, 1988).

Effect of Carbon Dioxide Gas Carbon dioxide is very active cell metabolite, but its specific regulating effect is difficult to differentiate because of its involvement in a range of metabolic events including the process of carboxylation and photosynthetic fixation (Blanke, 1991). Exposure of apple fruit tissue to high concentrations of CO_2 (10% or higher) can result in accumulation of acetaldehyde and ethanol (Patterson and Nichols, 1988). Under such condition, other metabolites, such as succinate, alanine and γ-aminobutyrate, are also known to accumulate (Zemlianukhin and Ivanov, 1978). This indicates that the hypoxic level of CO_2 gas affects the metabolism of both glycolysis (Kerbel *et al.*, 1988) and the TCA cycle (Monning, 1983; Shipway and Bramlage, 1973). This suggests that there may be a similarity between the hypoxic levels of CO_2 and O_2 in regulating the cellular respiration system and other fruit metabolism (Ke *et al.*, 1993a).

Several internal and external factors that influence metabolic changes in response to hypoxic and/or anoxic level of CO_2 and O_2 have been investigated in plant tissues. These factors, in turn, govern the tolerance of plant tissues to such conditions (Ke and Kader, 1992a; Knee, 1991a).

1.5.2 Factors Influencing Fermentative Metabolism

As O_2 concentration is reduced to less than 5%, or CO_2 concentration is increased to more than 20% in atmosphere, depending on commodity and temperature, generally more acetaldehyde and ethanol accumulate (Ke *et al.*, 1993b). Gran and Beaudry (1993) reported that in mature apples at 0.1°C, O_2 concentrations for transition to accumulation of ethanol were below 2% depending on cultivar being 0.69%, 0.79%, and 1.87%O_2 for 'Red Delicious', 'Law Rome', and 'McIntosh'; respectively. 'Delicious' apples at 0.5°C began to accumulate acetaldehyde and ethanol in the atmospheres containing 12% CO_2 with 0% O_2 (88% N_2) and in 1% O_2 (99% N_2) without CO_2 (Patterson and Nichols, 1988). In both mature-green 'Bartlett' pears (Kader, 1989) and ripe strawberries (Li and Kader, 1989) at 2°C or 5°C, concentrations of 0.25% O_2 and 20% CO_2, respectively, caused the transition to anaerobic respiration with subsequent ethanol accumulation.

The critical values of O_2 and CO_2 necessary to stimulate anaerobic respiration increase with temperature. Gran and Beaudry (1993) studied the lower O_2 concentration

limit in modified atmosphere (MA) packaging by measuring fruit RQ and headspace ethanol production in the package. The O_2 concentrations in MA packages at which headspace ethanol began to accumulate increased from 0.74% to 3.95% and from 1.97% to 6.51% for 'Redmax McIntosh' and 'Marshall McIntosh', respectively, as temperature increased from 3°C to 25°C. Similar but smaller increases were found for 'Red Delicious' and 'Golden Delicious' over the same temperature range, perhaps indicating greater skin permeance for these cultivars compared with 'McIntosh' strains (Gran and Beaudry, 1993). High temperatures accelerate chemical reactions which then require more O_2 for respiration to remain aerobic than when exposed to low temperatures (Blanke, 1991). In 'Bartlett' pears at 0.25% O_2, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities and accumulation of acetaldehyde, ethanol and ethyl acetate were higher at 20°C than at 5°C (Ke *et al.*, 1994b). Transferring fruit from a low to a higher temperature increased production of acetaldehyde and ethanol. Removal of apples from 0° - 2°C to 20°C stimulated production of acetaldehyde and ethanol, primarily due to partial anaerobiosis resulting from a temporary depletion of O_2 after temperature equilibration (Blanpied and Jozwiak, 1993; Dilley *et al.*, 1964).

The ethanol concentrations of blueberries (Saltveit and Ballinger, 1983a) and grapes (Saltveit and Ballinger, 1983b) increased linearly with time (over 1 to 4 hours) and exponentially with temperature (0°C to 30°C) during exposure to an atmosphere of 100% N_2 or 100% CO_2. Application of very high concentration of CO_2 (99%), N_2 (98%), or CO_2 + N_2 (49% + 50%) with 1% O_2 for 24 hours at 20°C, caused accumulation of anaerobic volatiles in 'Valencia' oranges, with CO_2 + N_2 or CO_2 producing higher acetaldehyde and ethanol concentrations than N_2 treatment (Pesis and Avissar, 1989). However, in feijoa, higher production of acetaldehyde and ethanol occurred following CO_2 + N_2 or N_2 treatments than with CO_2 treatment (Pesis *et al.*, 1991). In persimmon, Pesis and Ben-Arie (1984) found that treatment with 100% CO_2 for 96 hours at 20°C produced higher acetaldehyde than 100% N_2, while ethanol contents were similar. A mixture of 80% CO_2 + 20% N_2 caused the highest acetaldehyde accumulation with less ethanol than did 100% N_2 or other combinations. It is possible that CO_2 depressed conversion of acetaldehyde to ethanol (Pesis and Ben-Arie, 1984) or that atmospheric CO_2 was fixed into malate by dark CO_2 fixation, involving phosphoenol

pyruvate (PEP) carboxylase and malate dehydrogenase, leading to acetaldehyde accumulation (Pesis and Ben-Arie, 1986).

In strawberries at 5°C, 50% CO_2 in air resulted in high acetaldehyde accumulation, and increasing CO_2 from 50% to 80% enhanced acetaldehyde but not ethanol production (Ke *et al.*, 1993c). The combination of 0.25% O_2 + 80% CO_2 atmosphere appeared to have an additive effect on ethanol accumulation in avocados (Ke *et al.*, 1995) and pears (Ke *et al.*, 1994a). Patterson and Nichols (1988) reported an increase in acetaldehyde concentration of apples during a week in air at 20°C following storage in 0% O_2 (100% N_2) at 0.1°C for 4 - 14 weeks. These authors suggested that the increase was a function of reversible ADH activity during ethanol oxidation, which produced acetaldehyde faster than it could diffuse away or be oxidised.

The sensitivity of fruit to low O_2 and/or high CO_2 stress is also related to its stage of maturity, with mature fruit being more sensitive to hypoxic stress than less mature ones (Zemlianukhin and Ivanov, 1978). Boersig *et al.* (1988) studied the aerobic-anaerobic respiratory transition in pears and found that the ACP of intact pear fruit at 25°C shifted towards a higher O_2 concentration with physiological age and the potential for anaerobic respiration increased as fruit aged during storage. 'Bartlett' pears at the mature-green stage (2 days after harvest) and after 3 months at -1°C had ACPs at O_2 concentrations just below 1.3% and 5%, with corresponding O_2 diffusion coefficients being 4.4 and 2.5 x 10^{-16} $cm^2 \cdot s^{-1}$, respectively (Boersig *et al.*, 1988). In air apples (Dilley *et al.*, 1964; Blanpied and Jozwiak, 1993), peaches and pears (Ke *et al.*, 1991a), and oranges (Davis, 1970) accumulated more acetaldehyde and ethanol in mature fruit than in immature fruit.

The degree to which ethanol and acetaldehyde will be accumulated in fruit under hypoxia also depends on fruit resistance to gas diffusion. Differences in the degree of ethanol accumulation in the same apple cultivar under hypoxic conditions is thought to be associated with differences in resistance to gas diffusion in fruit (Park *et al.*, 1993). Ke and Kader (1992a) studied factors influencing tolerance of fruits (apples, Asian pears, cherries, nectarines and plums) to low-oxygen atmospheres. They found that relatively high temperatures, high respiration rates and greater resistance to gas diffusion enhanced

accumulation of anaerobic volatiles, while relatively high O_2 concentration and higher total soluble solids content reduced them.

Induction of fermentative metabolism by hypoxic concentrations of O_2 and/or CO_2 results in the production of anaerobic volatiles, acetaldehyde, ethanol and ethyl acetate. The degree to which these volatiles begin to accumulate depends on the gas concentration in the atmosphere, commodity and temperature. Different fruits exhibit widely different thresholds that are both temperature- and duration-dependent (Beaudry, 1993). The build up of these anaerobic volatiles in fruit stored under hypoxia is thought to be a cause of, or has been linked to, tissue damage and/or development of off flavour (Beaudry, 1993; Blanpied and Jozwiak, 1993; Ke *et al.*, 1993c). However, to date there is no direct evidence to confirm this hypothesis.

1.5.3 Molecular and Metabolic Control of Fermentative Metabolism

There are two mechanisms that regulate anaerobic metabolism; these are referred to as 'molecular control'- also known as 'coarse control', and 'metabolic control'- also called 'fine control' (Pradet and Bomsel, 1978). Coarse control operates at the molecular or gene level and determines the nature and/or concentration of various enzymes in the cell. This process requires synthesis of mRNA and proteins and/or activation of precursors. The fine control regulates, almost instantaneously, the reactivity between enzymes and substrates present in plant tissue under low O_2 and/or high CO_2 stresses (Ke *et al.*, 1993a; Pradet and Bomsel, 1978; Webster *et al.*, 1991).

Early studies in anaerobic metabolism focused predominantly on 3 main functional groups of induced enzymes. These were (1) enzymes metabolising sucrose and starch (such as sucrose synthase and α-amylase), (2) glycolytic enzymes (such as glucose phosphate isomerase, fructose 1,6-bisphosphate aldolase, and glyceraldehyde-3-phosphate dehydrogenase), and (3) enzymes of ethanol fermentation, such as PDC and ADH (Ricard *et al.*, 1994).

The induction of ADH, PDC, and/or LDH enzymes under low O_2 and/or high CO_2 stresses in plant tissue is viewed as one reason for the accumulation of fermentative end products (Kennedy *et al.*, 1992; Chervin *et al.*, 1996). Activity of PDC and ADH increased in sweet potato roots (Chang *et al.*, 1983) and carnation flowers stressed by

low O_2 and/or high CO_2 levels (Chen and Solomos, 1996). However, in avocado (Ke *et al.*, 1995) and mango (Yahia and Vazques-Moreno, 1993), the total extractable ADH activity did not change in response to low O_2 and high CO_2 stresses, but a new isozyme appears under hypoxic condition (Kanellis *et al.*, 1991). Redirecting protein synthesis under anaerobic condition (Kanellis *et al.*, 1993; Sachs *et al.*, 1985), ie. new mRNA synthesis and *de novo* synthesis of corresponding enzyme proteins resulting from transcription and translation, induced production of PDC, ADH, and LDH (Good and Crosby, 1989; Ricard *et al.*, 1994). The increase in hypoxically induced ADH in barley is reported to be due to the induction of the alcohol dehydrogenase1 (adh1) gene (Good and Crosby, 1989). In pears, an increase in the activity of ADH is mainly due to the induction of new ADH isozyme (Nanos *et al.*, 1992).

It is now thought that regulation of pH in the cytosol is a major determinant of plant tissue tolerance during anoxia (Ricard *et al.*, 1994). Earlier, Davies (1980) had proposed that metabolic control under anoxia involved tight regulation of pH status preventing cytosolic acidosis. In this hypothesis, the relative rate of synthesis of lactate and ethanol depended on the cytosolic pH. Under anaerobic conditions, pyruvate was initially converted to lactate, causing the cytosolic pH to decrease, with the activity of LDH inhibited and PDC activity stimulated, leading to predominance of ethanol synthesis (Davies *et al.*, 1974). However, changes in cytosolic pH have been found to be much faster than the increase in levels of lactate and closely following the decrease of ATP, suggesting that decrease of ATP is the main cause for rapid acidification of the cytosol (Ricard *et al.*, 1994). Ricard *et al.* (1994) have suggested that acidification may result from both inhibition of proton pumping at low ATP concentration and proton release through hydrolysis. The intercellular pH may also be a signal to the translation of enzymes such as sucrose synthase and ADH (Webster *et al.*, 1991). Although a decrease in cytosolic pH is a common phenomenon in response to low O_2 and/or high CO_2 stresses, not all plants undergo such a pH change, examples including rice and barnyard grass (Kennedy *et al.*, 1992). It seems that either an increase in fermentative enzymes such as ADH, LDH, and PDC, or a decrease in cytosolic pH can be a unifying mechanism for the induction of fermentative metabolism.

Mitochondria have always been assumed to be generally inoperative during anaerobiosis because of the requirement of O_2 as a terminal electron acceptor (Kennedy *et al.*, 1992). Low O_2 and high CO_2 may influence the activity of respiratory enzymes in mitochondria, such as cytochrome oxidase, depending on the concentration and duration of exposure (Rahman *et al.*, 1993). It has long been recognised that succinate accumulated during anoxia (Zemlianukhin and Ivanov, 1978). High concentration of CO_2 (10% or more) depressed succinate dehydrogenase (SDH) activity in apple mitochondria (Shipway and Bramlage, 1973) and tissue (Hulme, 1956; Knee, 1973), causing succinate to accumulate (Monning, 1983). On the other hand, hypoxic conditions activated glutamate decarboxylase (GDC) resulting in accumulation of γ-aminobutyrate (GABA) and a decrease in the concentrations of malate and glutamate (Ke *et al.*, 1993c).

Under low O_2 levels, close to 0%, the cytochrome oxidase (K_m < 1 μM) in the electron transport system is substantially depressed (Chervin *et al.*, 1996; Kennedy *et al.*, 1987; Knee, 1991a) and at elevated levels of CO_2 the cyanide-resistant respiration (the alternative pathway) may be transiently induced (Palet *et al.*, 1992). In situations where the cytochrome pathway is restricted, the alternative respiration pathway may have a function when increased demand for ATP exists (Wagner and Krab, 1995). Under high CO_2 with normal O_2 levels, ie. 40 - 80% CO_2 + 20% O_2, the alternative pathway of electron transport is enhanced through the induction and/or activation of the alternative oxidase, while that through the cytochrome pathway may be inhibited by suppression of cytochrome oxidase activity (Lange and Kader, 1993). The resultant induction of the alternative pathway produced about 8 times more ATP than fermentation with less substrate consumption and less disturbance to normal metabolism as the TCA cycle was maintained (Kader, 1995).

Pear fruit discs maintained at 0.25% O_2 (balanced with N_2) at 20°C have a lower cytosolic pH (about 0.4 pH unit) and lower a ATP/ADP ratio than discs maintained in air, indicating a lower energy charge (Nanos and Kader, 1993). In avocado, 20% O_2 + 80% CO_2 and 0.25% O_2 + 80% CO_2 (balanced with N_2) caused a greater reduction in cytosolic pH than 0.25% O_2 (balanced with N_2) or air controls and 0.25% O_2 + 80% CO_2 dramatically reduced ATP level (Hess *et al.*, 1993). Hess *et al.* (1993) also reported that a decrease in pH under low O_2 and/or high CO_2 stress in avocado caused a reduction

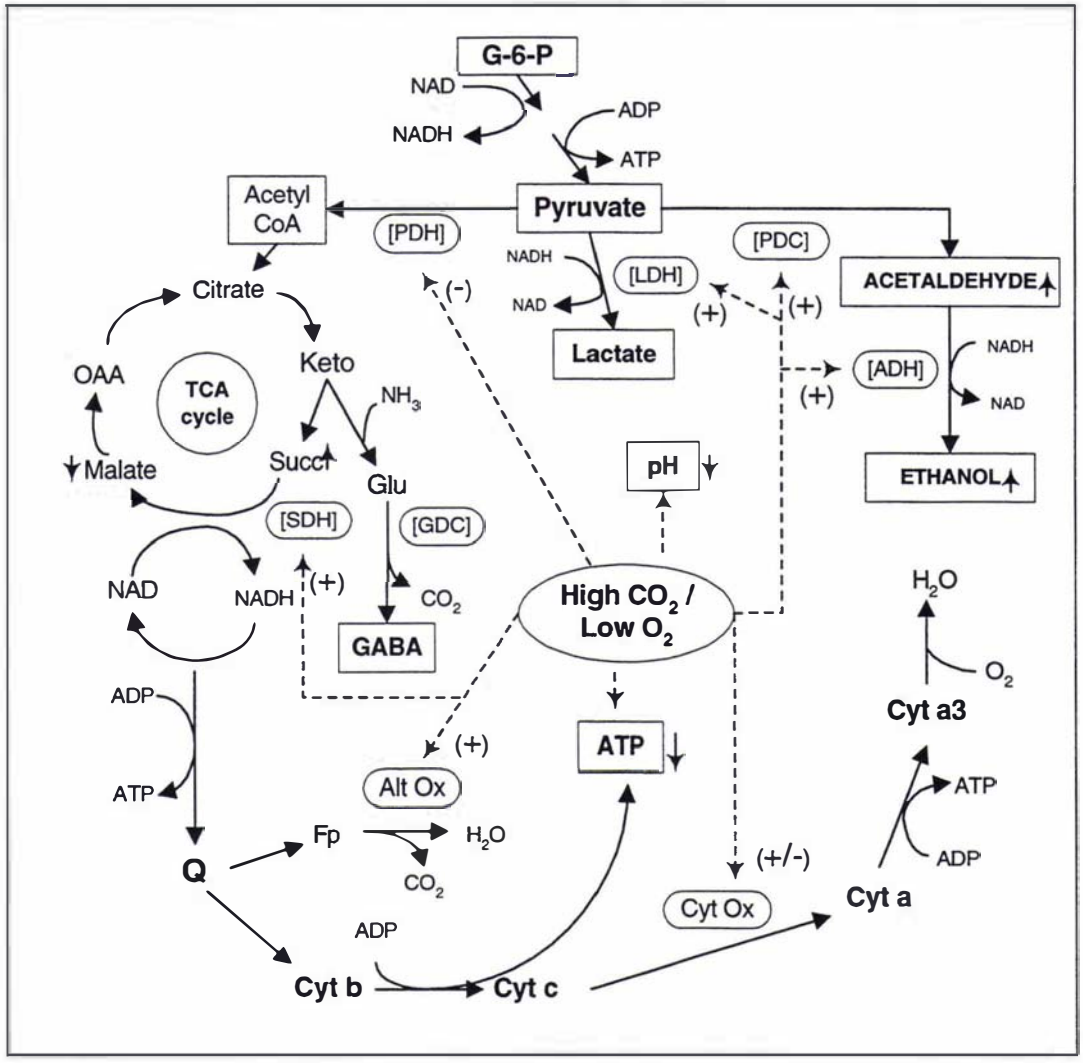
(about 15%) in ATP:phosphofructokinase (PFK) and PPi:phosphofructokinase (PFP) became inactive.

Pyruvate decarboxylase (PDC) is probably the most important enzyme in anaerobic metabolism, catalysing decarboxylation of pyruvate, yielding CO_2 and acetaldehyde - the immediate precursor of ethanol (Kennedy *et al.*, 1992). PDC of 'Golden Delicious' apples had a pH optimum between 6.1 to 6.4 (Bufler and Bangerth, 1982). Under hypoxic/anoxic conditions, PDC can be activated several ways. Apart from its activation by a decrease in cytosolic pH, it can be activated by an increase in pyruvate concentration through conformational change, as PDC is an allosteric enzyme (Hubner *et al.*, 1978). PDC is also activated by a high NADH/NAD ratio (Ke *et al.*, 1995), which can occur when the electron transport pathway is inhibited (John and Greenway, 1976). LDH is also an allosteric enzyme which is activated by an increase in NADH or pyruvate at low concentration (Betsche, 1981). ATP, NAD, and high cytosolic pH inhibit activity of LDH, while fructose-6-phosphate diminishes ATP inhibition of LDH (Betsche, 1981; Oba *et al.*, 1977).

Some effects of hypoxic conditions, caused by low O_2 and/or high CO_2, on some important respiratory enzymes, substrates, products, and cofactors are summarised in **Table 1.8**. A proposed mode of action of stress levels of O_2 and/or CO_2 on the electron transport system and on organic acid metabolism of fruits and vegetables has been developed by Kader and his colleagues and is shown in **Fig. 1.9** (Kader, 1995; Ke *et al.*, 1993b; 1995).

The hypoxic stress of O_2 and/or CO_2 reduces NADH supply to the cytochrome electron transport system (**Fig. 1.9**) and oxidative phosphorylation is greatly reduced. The PDH activity is reduced, hence acetyl CoA entry into the TCA cycle is decreased. Under such conditions, PDC, ADH and/or LDH may be activated by a decrease in cytosolic pH and/or induced by transcriptional or translational control, resulting in an increased concentration of pyruvate, fructose-6-phosphate and NADH; and decreased ATP and NAD levels. Thus, pyruvate is directed to the fermentative pathway causing the accumulation of acetaldehyde, ethanol, and/or lactate. Induction of fermentative metabolism allows glycolysis to function, NADH can be utilised and a small amount of

ATP can be produced through substrate phosphorylation to permit the plant tissue to temporarily survive (Chervin *et al.*, 1996; Kader, 1995; Ke *et al.*, 1993b).



Abbreviations and symbols:

[ADH] = alcohol dehydrogenase, [Alt Ox] = alternative oxidase, [Cyt Ox] = cytochrome oxidase, [GDC] = glutamate decarboxylase, [PDC] = pyruvate decarboxylase, [PDH] = pyruvate dehydrogenase, [SDH] = succinate dehydrogenase, [LDH] = lactate dehydrogenase Cyt = cytochrome, F_p = Flavoproteins, GABA = γ-aminobutyrate, Glu = glutamate, G-6-P = glucose-6-phosphate, Keto = α-ketoglutamate, OAA = oxaloacetate, Q = Ubiquinone, Succ = succinate, TCA = tricarboxylic acid, + = induction/activation, – = reduction/inhibition, +/– = either inhibition or activation, ↑ = content increase, ↓ = content decrease

Fig. 1.9 Proposed mode of action of stress levels of low O_2 and/or CO_2 on electron transport system and organic acid metabolism in fruits and vegetables (derived from Kader, 1995; Ke *et al.*, 1993b; Ke *et al.*, 1995).

Table 1.8 Biochemical and physiological basis for effects of stresses low O_2 and/or CO_2 on respiratory metabolism of fruits (after Ke *et al.*, 1993a).

Parameter	General Effect of	
	Low O_2	High CO_2
shift to fermentative metabolism	+ (<1%)	+ (>20%)
pH	–	–
ATP	–	–
NADH	+	+
pyruvate	+	+
ATP:phosphofructokinase (PFK)	–, 0, or +	–, 0, or +
PPi:phosphofructokinase (PFP)	–, 0, or +	–, 0, or +
cytochrome oxidase (Cyt Ox)	–, or 0	–, 0, or +
pyruvate dehydrogenase (PDH)	–	–
pyruvate decarboxylase (PDC)	+ or 0	+ or 0
alcohol dehydrogenase (ADH)	–	+ or 0
alcohol acyltransferase (AAT)	+ or 0	–
lactate dehydrogenase (LDH)	+ or 0	+ or 0
succinate dehydrogenase (SDH)	+	–
acetaldehyde	+	+
ethanol	+	+
ethyl acetate	+ or 0	+ or 0
glutamate	+	+
lactate	+	+
succinate	+ or 0	+ or 0

Symbols – : decrease or inhibit; 0 : no effect; + : increase or activate

Under hypoxia ADH concentration is greatly induced and its activity in catalysing acetaldehyde into ethanol is markedly increased (Ricard *et al.*, 1994). However, under hypoxia, together with low temperature, at which concentration of O_2 is probably well above ACP of a fruit, such as is used in commercial CA storage, induction of ADH is believed to be depressed (Chervin *et al.*, 1996) and the production of ethanol is negligible. For instance, storage of 'McIntosh' apples in 1.5% O_2 + 1.5% CO_2 at 2.8°C for 40 - 210 days severely suppressed acetaldehyde and ethanol production during storage as compared with RA storage (Lidster *et al.*, 1983). ADH is also responsible for catalysing other aldehydes into alcohols, such as butanal and 2-methyl butanal into butanol and 2-methyl butanol, respectively (Eriksson, 1979). These aldehydes and alcohols are important compounds contributing to aroma of fruit and their products,

including apples (Dürr and Schobinger, 1981; Young *et al.*, 1996). Alcohols are one of the substrates essential for the biosynthesis of esters in fruit (Oliás *et al.*, 1995) and volatile esters are the main contributor of the fruity odour in many ripening fruit and their products (Paillard, 1990). However, studies on production of alcohols, other than ethanol, in relation to the metabolism of ADH under hypoxia/anoxia and/or on removal to air after such conditions are lacking.

Alcohol acyltransferase (AAT) is the only ester forming enzyme that has been studied in any detail; it is believed to be responsible for catalysing alcohols and acyl CoAs into corresponding esters in ripening fruits (Fellman and Mattheis, 1995; Oliás *et al.*, 1995). This enzyme has been partially purified and characterised recently from apples (Fellman, 1994) and strawberries (Pérez *et al.*, 1993). Fellman and Mattheis (1995) reported that the ester forming activity of AAT in 'Rome' apples stored for 3 - 6 months in 1.0% O_2 + 1.0% CO_2 at 0 - 1°C was initially depressed, but recovered to levels approaching that of air-stored control apples after 6 days in air. They suggested that AAT was suppressed by low O_2 and was reactivated on exposure of fruit to ambient O_2, since the enzyme activity was not shown to be greater than that observed in RA fruit.

Exposure of fruits, such as oranges (Shaw *et al.*, 1990) and feijoas (Pesis *et al.*, 1991) to short-term anaerobic condition (in 99% N_2, 24 hours at 20°C) resulted in accumulation of acetaldehyde, ethanol and several ethyl esters on return to air after treatment. The total volatile compounds obtained in the essence of the treated oranges was almost twice from control fruit (Shaw *et al.*, 1990).

However, there is very little research reporting the effect of hypoxia on AAT and its activity in fruits, including apples, and there is virtually no information on the effect of anoxia on this enzyme. Thus, it is not known how hypoxia and/or anoxia affects AAT and its activity in fruit; presumably the enzyme is suppressed in the absence of O_2 or its activity falls off with time under low O_2 atmospheres, such as in CA storage, being reactivated and/or induced on removal of fruit to normoxia.

1.5.4 Effect of Hypoxia/Anoxia on Volatile Production and Composition

There has been an increased interest in recent years in studying the potential of using hypoxic atmosphere for postharvest insect disinfestation in horticultural produce to

meet quarantine requirements for exports (Ke and Kader, 1992b; Ke *et al.*, 1993b; Lay-Yee and Whiting, 1996; Lidster *et al.*, 1984; Whiting *et al.*, 1991; 1992; 1996; Yahia, 1993). Fumigation with ethylene dibromide or methyl bromide used to be very common for postharvest disinfestation. However, ethylene dibromide was removed from the list of approved chemicals in 1984 and methyl bromide use is being more restricted (Ke and Kader, 1992b). Thus, alternative methods need to be developed and hypoxic conditions at low or high temperature could potentially be one of these (Lay-Yee and Whiting, 1996; Lidster *et al.*, 1984; Whiting *et al.*, 1996).

Some postharvest insect pests of fruit can be effectively controlled by hypoxic atmospheres of low O_2 and/or high CO_2 (**Table 1.9**). The treatment has advantages of leaving no chemical residues in fresh produce and being safe to the operators and the environment. However, the time required to achieve 100% mortality of insects (**Table 1.9**) varies with insect species, stage of their development, concentration of O_2 and/or CO_2 used and temperature (Ke and Kader, 1992b). Therefore, it is becoming increasingly important to understand the specific product response and the biochemical and/or physiological processes underlying this response, in order to derive optimal conditions to achieve total insect mortality yet retaining optimal product quality.

Table 1.9 Gas composition, temperature and time required to kill certain insects in some fruit (after Ke and Kader, 1992b).

Produce	Insect	Stage	Temp. (°C)	Gas Composition(%) O_2	CO_2	N_2	Time for 100% Mortality
Apple	brownheaded leafrollers	1st instar	0.5	3	3	94	32 days
	apple maggot	egg	0	0		100	24 days
		egg	20	0		100	7 days
	apple rust mite	egg	2.8	1	1	98	160 days
	European red mite	egg	2.8	1	1	98	160 days
		egg	7.5	1	1.5	97.5	14 days
	codling moth	larvae	0	2	1	97	91 days
		larvae	27	1	95	4	2 days
	lightbrown apple moth	1st instar	2	3	3	94	90 days
		1st instar	20	0.4	5	94.6	20 hours
Peach	codling moth	egg	25	0.5	10	89.5	2 - 3 days
		egg	25	8.4	60	31.6	2 - 3 days
Orange	Caribean fruit fly	egg	15.6	2	30	68	10 days
Strawberry	Western flower thrips	adult	2.5	1	90	9	1 - 2 days

Under hypoxia, decreasing O_2 levels to near 0% and/or increasing CO_2 above 10% with increasing temperature, is known to cause accumulation of fermentative volatiles, such as acetaldehyde, ethanol and may be other volatiles, in fruit (Ke and Kader, 1992a; Ke *at al.*, 1995; Lurie and Pesis, 1992). Accumulation of these volatiles has been linked to the development of tissue damage and off-flavour in some fruits and vegetables stored under hypoxia (Ke and Saltveit, 1989; Ke *et al.*, 1993a). However, it is not known whether accumulation of these volatiles *per se* in response to hypoxia is the cause of such phenomena or whether the effect is indirect by influencing other metabolic processes; this has yet to be elucidated.

Treatment of 'Red Delicious' apples with 0% O_2 for 14 days at 0.5°C resulted in higher acetaldehyde and ethanol production than in fruit treated with 0.5% or 1% O_2, and ethanol, but not acetaldehyde, increased linearly with duration of exposure (Patterson and Nichols, 1988). In 'Bartlett' pears treated with low O_2 (0.25% to 1%) for 10 days at 5° or 10°C, only 0.25% O_2 increased acetaldehyde and ethanol accumulation, but at the higher temperature both compounds were increased compared with controls and with the 5°C treatments (Ke *et al.*, 1990).

The effect of high CO_2 concentrations above 20% in inducing fermentative volatile accumulation in plants is well known (Kader, 1986; Kimmerer and MacDonald, 1987). Exposure of mature-green 'Bartlett' pears to 20% CO_2 in air for 10 days at 0° to 10°C had no effect on acetaldehyde and ethanol production; these volatiles only began to accumulate at 50% CO_2, with greater production occurring at the higher temperature and longer exposure time (Ke *et al.*, 1990). 'Delicious' apples stored under various CO_2 concentrations (0 - 12%) in combination with 0% - 1% O_2 at 5°C for 14 weeks, produced significantly less acetaldehyde and ethanol at 12% CO_2 + 0% O_2 than at 0% CO_2 + 0% O_2. This lead to a suggestion that CO_2 inhibited ethanol accumulation in the absence of O_2, and increasing CO_2 concentrations markedly reduced the rate of ethanol accumulation (Patterson and Nichols, 1988). On the other hand, ethanol production in persimmons during treatments with 80% CO_2 + 20% N_2, 50% CO_2 + 50% N_2, 30% CO_2 + 70% N_2, or 100% N_2 for 48 hours at 20°C was similar, and the CO_2 inhibition effect was not observed; however, 80% CO_2 induced highest acetaldehyde accumulation, while 100% N_2 caused the lowest (Pesis and Ben-Arie, 1984). Feijoa exposed to 49% CO_2 +

49% N_2 + 2% O_2 for 24 hours at 20°C also accumulated more acetaldehyde, ethanol and ethyl acetate on transfer to air than fruit exposed to 98% N_2 + 2% O_2 (Pesis, 1994).

A short-term exposure of strawberries to 20% CO_2 in air at 1°C for 12 - 48 hours increased acetaldehyde, ethanol and ethyl acetate compared with air controls (Ueda and Bai, 1993). Similar findings have been reported by Ke *et al.*(1991a), where 20% to 80% CO_2 and increasing temperature from 0° to 5°C induced higher production of fermentative volatiles in strawberries. Ke *et al.* (1993b) reported that CO_2 levels above 50% in air enhanced acetaldehyde production compared with 20% CO_2 in air, 0.25% O_2 + 99.75% N_2 or air controls, while the 0.25% O_2 treatment caused highest ethyl acetate accumulation in strawberries compared to the other treatments. The effect of low O_2 and high CO_2 levels on anaerobic volatiles production in strawberries is additive and accentuated by high temperature (Li and Kader, 1989).

The relationship between exposure time (1 to 4 hours) and temperature (0° to 30°C) on ethanol accumulation in blueberries (Saltveit and Ballinger, 1983a) and grapes (Saltveit and Ballinger, 1983b) during treatment with 100% N_2 or 100% CO_2 has been investigated. In both blueberries and grapes, ethanol accumulation increased linearly with time of treatment, but increased in a curvilinear manner with temperature. There were no differences in ethanol production between CO_2 or N_2 treatments. Similarly, in persimmons treated with 100% CO_2 or 100% N_2 at 20°C for 96 hours, ethanol production increased linearly with time of exposure and the amount of ethanol in fruit tissue was similar for both treatments (Pesis and Ben-Arie, 1984).

Ethyl acetate, another fermentative volatile, has been found to accumulate in various fruits on transfer to air after treatment with high CO_2 and/or low O_2 concentrations. Formation of ethyl acetate is believed to proceed via esterification of ethanol and acetyl CoA, the reaction is catalysed by ester forming enzyme(s) in the presence of O_2 (Bartley *et al.*, 1985; Knee and Hatfield, 1981). Ke *et al.* (1993c) showed that after exposure to 0.25% O_2 in N_2, strawberries produced more ethyl acetate than did fruit exposed to 50% CO_2 in air. On the other hand, feijoas (Pesis *et al.*, 1991) and mandarins (Shaw *et al.*, 1992) had higher ethyl acetate contents after treatment with 99% CO_2 + 1% O_2 than after treatment with 98% N_2 + 2% O_2.

When fruit are transferred from low O_2 and/or high CO_2 atmospheres to air, fermentative volatiles generally decreased with time (Larsen and Watkins, 1995a). In 100% CO_2 or N_2 treated blueberries, the rapid ethanol loss was linear during 6 days in air at 0°C, and after that a slower decrease occurred to a level similar to that of control after 12 days (Saltveit and Ballinger, 1983a). The higher the ethanol produced by fruit in response to anaerobic treatment, the longer the time required to lose the volatile on transfer to air, while the lower the ethanol, the shorter the time required (Saltveit and Ballinger, 1983b). It has been suggested that after hypoxic or anoxic treatments, the decrease in fermentative volatiles which occurs when fruit are transferred to air is due mainly to diffusion of compounds out of the fruit (Kimmerer and Kozlowski, 1982), although they may also be metabolised into other compounds within fruit tissues (Cossins, 1978; Fidler and North, 1971).

Ethanol content of mangoes treated with 25% to 75% CO_2, either in air or with 3% O_2, for 21 days at 12°C remained high, even after 5 days in air at 20°C (Bender *et al.*, 1994). Similar observation were reported for oranges after treatment with 99% CO_2 or 98% N_2 (Pesis and Avissar, 1989). It is possible that these high levels could be due to slow loss of fermentative volatiles after treatment or alternatively they could be due to respiration via the anaerobic pathway continuing to operate even though the fruit is no longer under hypoxia or anoxia (Pesis and Avissar, 1989). Nichols and Patterson (1987) suggested that although aerobic respiration increased upon returning to air, PDC was still present, and thus a continued conversion of pyruvate into acetaldehyde might still occur, which could account for the continued accumulation of ethanol after removal from anaerobic conditions. Another possibility is that because ethanol and acetaldehyde are soluble with water (Lide and Frederikse, 1995), the slow loss could have been due also to the retention of the volatiles in cell sap with a slow release occurring.

Increases in fermentative volatiles immediately or 1 - 2 days after anaerobic treatment have been reported in several fruits. These fermentative volatiles such as acetaldehyde and ethanol are probably induced and accumulated under hypoxic and/or anoxic conditions (Chen and Solomos, 1996; Chervin *et al.*, 1996). Acetaldehyde increased in apples on removal from a 0% O_2 atmosphere (Patterson and Nichols, 1988). Acetaldehyde, ethanol and ethyl acetate contents of oranges (Shaw *et al.*, 1991) and

mandarins (Shaw *et al.*, 1992) increased to a maximum immediately or 1 - 2 days after hypoxic treatment depending on the particular volatiles. Patterson and Nichols (1988) suggested that the increase in acetaldehyde in apples on removal to air is a function of reversible ADH activity during ethanol oxidation, where the process appears to produce acetaldehyde faster than diffusion and oxidation of acetaldehyde can eliminate it.

Acetaldehyde, ethanol and ethyl acetate, which are minor flavour compounds (Nursten, 1970) are commonly found to accumulate naturally during ripening and senescence of fruit (Eaks, 1980; Janes and Frenkel, 1978; Nichols and Patterson, 1987). However, accumulation of these volatiles, either naturally or hypoxically induced, has been related to off-flavour characteristics in many fruits (Fidler and North, 1971; Ke and Kader, 1992a; Ueda and Bai, 1993). Fidler and North (1971) suggested that an ethanol concentration of 350 ppm could be detected by taste in 'Cox's Orange Pippin' apples, while concentration of more than 1000 ppm was reported to be necessary to cause off-flavour in 'Yellow Newtown' apples (Ke *et al.*, 1991a). In strawberries, concentrations of acetaldehyde, ethanol, ethyl acetate and methanol of 8.1, 23, 63, and 0.66 ppm, respectively, have been correlated to slight off-flavour scores (Ke *et al.*, 1991b). Larsen and Watkins (1995b) reported that off-flavour of strawberries was related to increases in ethyl acetate and ethanol, but not acetaldehyde, and a similar finding has been reported by Ueda and Bai (1993). Ethyl acetate has been regarded as an indicator compound in anaerobic off-flavour of strawberries (Larsen and Watkins, 1995b). Ethanol may not be so important in off-flavour development, as its odour threshold is quite high (100 ppm in water (Teranishi, 1970)), and its contribution to off-flavour is likely to be greater at high concentration (Larsen, 1994).

However, questions have been raised as to whether accumulation of acetaldehyde and ethanol is directly associated with off-flavour of fruits after being subjected to anaerobic conditions. Richardson and Kosittrakun (1995) reported that accumulation of large amounts of acetaldehyde and ethanol in various fruits, including apples and pears, after exposure to N_2 atmospheres (0.3 - 4.3% O_2) at 0°C and 20°C for 10 days had no correlation with off-flavour upon transfer to air at 20°C. Moreover, reversibility of off-flavour was observed without much change of the two compounds in the tissue, which casts doubt on any connection between them. It has been suggested that the mode of

reversibility may involve conversion of the offending compounds to esters or other modifications via oxidations during shelf-life of the fruit in air (Richardson and Kosittrakun. 1995). Postharvest application of acetaldehyde or ethanol to pears, blueberries and tomatoes leads to enhancement of fruit flavour, and it has been suggested that these compounds may be important in development of the sensory quality of these fruit (Paz *et al.*, 1981; Richardson and Kosittrakun. 1995). Similar findings on enhancement of fruit flavour by exogenously applied acetaldehyde have been reported for peaches (Pesis, 1994), feijoas (Pesis *et al.*, 1991) and oranges (Shaw *et al.*, 1991).

A short-term application of anaerobic conditions, such as 95% CO_2 or N_2 for 24 hours at 20°C, lead to endogenous accumulation of acetaldehyde and ethanol which could perhaps influence constituents and aroma of fruits during subsequent shelf-life or storage (Pesis and Avissar, 1989). After subjecting peaches and nectarines to 99% CO_2 (+ 1% O_2) or 98% N_2 (+ 2% O_2) for 24 hours at 20°C the concentrations of acetaldehyde and ethanol were similar in both treated and non-treated fruit; the majority of a sensory panel preferred treated fruit, while the off-flavour was noticed only in control fruit (Lurie and Pesis, 1992). Pesis *et al.* (1991) found that exposing feijoas to 99% CO_2 (+ 1% O_2), 98% N_2 (+ 2% O_2), or 49% CO_2 + 50% N_2 (+ 1% O_2) for 24 hours at 20°C enhanced sensory quality of the fruit 7-13 days after treatment. The treatments increased ethyl acetate and ethyl butanoate production which was thought to make a strong contribution to flavour enhancement (Pesis, 1994). Larsen and Watkins (1995a) suggested that increases in ethyl butanoate and ethyl hexanoate, both compounds having pleasant, fruity flavours and low odour thresholds, could have contributed to a pleasant flavour that masked any off-flavour of strawberries which might develop after treatment with 20% CO_2 for 12 days at 0°C.

Exposing strawberries to 20% CO_2 in air for 12 - 48 hours at 1°C had little effect on methyl acetate and methyl butanoate esters, but it increased ethyl acetate and ethyl butanoate production (Ueda and Bai, 1993). Larsen and Watkins (1995a) reported that treatment of strawberries with 20% CO_2 in air for 12 days at 0°C caused substantial increases in acetaldehyde, ethanol, ethyl acetate, ethyl butanoate, ethyl hexanoate and acetic acid from 4 days onwards during the treatment compared with the air control. Concentrations of *trans*-2-hexenal, γ-decalactone and butanoic acid showed very little

change or were unaffected. On the other hand, hexyl acetate, methyl hexanoate, 2-methyl propanoic acid, 2-methyl butanoic acid and hexanoic acid decreased in treated fruit. On removal to air at 0°C, acetic acid and ethyl butanoate continued to increase, while ethyl acetate, ethyl hexanoate and ethanol generally decreased. Storage of strawberries in atmospheres of reduced O_2 (0.25%) in N_2 and/or elevated CO_2 (50%) for 7 days at 5°C enhanced production of acetaldehyde, ethanol, ethyl acetate, and ethyl butanoate. The 0.25% O_2 treatment caused the greatest accumulation of ethyl acetate and ethyl butanoate, while 21% O_2 + 50% CO_2 induced the highest acetaldehyde concentration. In contrast, the concentration of isopropyl acetate, propyl acetate, and butyl acetate in the treated fruit decreased to lower levels than that of control (Ke *et al.*, 1994b).

Coatings with commercial polysaccharide-based or shellac-based water soluble coatings increased internal CO_2 (up to 10%) and decreased internal O_2 (to 6%) of oranges stored at 21°C; this resulted in changes in several aroma volatile compounds. Coated oranges had higher ethanol, ethyl acetate, ethyl butanoate, and α-pinene, and lower hexanol, valencene and α-terpineol contents than uncoated fruit (Baldwin *et al.*, 1995).

A short-term exposure (24 hours) to hypoxic atmospheres at 20° to 25°C was found to alter volatile profiles of orange juice (Shaw *et al.*, 1991), orange aqueous essence and essence oil (Shaw *et al.*, 1990), as well as mandarin and tangelo juice (Shaw *et al.*, 1992). Shaw *et al.* (1991) found that after treatment with 95% CO_2 in air or 98% N_2 (+ 1% O_2) atmospheres for 8 - 24 hours at 20°C, acetaldehyde, ethanol, ethyl acetate, and ethyl butanoate from 'Pineapple' and 'Valencia' oranges showed the most significant increases, rising to a maximum immediately or 1 - 2 days, depending on the compound, on removal to air at 20°C. Methanol, methyl butanoate, and hexanal showed very little change. This effect of hypoxic atmosphere treatment inducing changes of certain volatiles was suggested to have potential for increasing the amount of certain volatile flavour compounds in blended orange-mandarin juice products (Shaw *et al.*, 1992).

The effect of anaerobic treatments on aqueous essence and essence oil obtained from treated oranges was studied by Shaw *et al.* (1990). After exposure of 'Pineapple' and 'Valecia' oranges to 99% N_2 (+ 1% O_2) for 24 hours at 20° or 25°C, certain alcohols, esters, and aldehydes recovered in the aqueous essence and essence oil flavour

fractions were almost twice that found in untreated fruit. Concentrations of ethanol, ethyl acetate, and ethyl butanoate in the aqueous essence almost doubled, while 1,1-dioxyethane also increased, but this acetal was considered to be an artefact formed from ethanol and acetaldehyde during processing in an acidic juice medium. Concentrations of terpinen-4-ol and α-terpineol alcohols, methyl butanoate, and ethyl-3-hydroxy hexanoate esters were unaffected by the treatment. In control fruit, concentrations of most volatile compounds in 'Valencia' aqueous essence were higher than in 'Pineapple' oranges. In addition, the flavour of aqueous essence and essence oil from treated 'Valencia' were found to be significantly different from comparable flavour fractions from control fruit when added to a bland juice base and evaluated (Shaw *et al.*, 1990).

Mattheis *et al.* (1991a) studied the effect of anaerobic atmospheres on volatile production of 'Delicious' apples previously stored in CA for 4 - 5 months. The concentrations of acetaldehyde and ethanol increased during 30 days storage in 0.05% O_2 at 1°C and declined on return to air for 7 days at 20°C. Upon transfer to air for ripening, several ethyl esters, which had not been detected in CA control or initial samples, were detected in anaerobic apples. These compounds included ethyl acetate (the most prevalent), ethyl propanoate, ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate, ethyl heptanoate, and ethyl octanoate. Decreases were measured for some other esters, aldehydes, and alcohols as well as some ketones, including butyl acetate, hexyl acetate, 2-methyl butyl acetate, butyl butanoate, hexyl butanoate, benzaldehyde, hexanal, heptanal, octanal, hexan-1-ol and 6-methyl-5-hepten-2-one. Anaerobic conditions caused a 9- and 12-fold increase in total volatiles and total esters, respectively; while the sum of all aldehydes, alcohols (excluded ethanol and acetaldehyde) and ketones were comparable to those found in control fruit 1 day after removal to air at 20°C (Mattheis *et al.*, 1991a).

The biosynthesis of carboxylic esters in fruits is believed to proceed via esterification of acyl CoA intermediates of the β-oxidation pathway with alcohols (Berger *et al.*, 1992; De Pooter *et al.*, 1981). The enzyme, alcohol acyltransferase (AAT) plays a major role in the biosynthesis of volatile esters, as it catalyses the transfer of an acyl moiety from acyl CoA onto the corresponding alcohol (Fellman and Mattheis, 1995; Oliás *et al.*, 1995). Thus, alcohols, acyl CoAs and AAT are three main factors that

influence the biosynthesis of carboxylic esters in fruits (Gilliver and Nursten, 1976; Oliás *et al.*, 1995).

Exogenous applied alcohols (Berger and Drawert, 1984; Knee and Hatfield, 1981), aldehydes (De Pooter *et al.*, 1983; Yamashita *et al.*, 1977), or carboxylic acids (Bartley *et al.*, 1985; De Pooter *et al.*, 1987) are metabolised into a variety of corresponding esters in apples, bananas, and strawberries. Bartley *et al.* (1985) found that supplying ethanol to apples enhanced ethyl acetate as a major ester. Supplying ethanol vapour (0.5 $ml \cdot l^{-1} \cdot kg^{-1}$) at ambient temperature resulted in a more than 3-fold increase in the sum of ethyl esters, but decreased hexyl and butyl esters, indicating the competitive reactions for the acyl moiety (of C_4 and longer) needed for esterification (Bartley *et al.*, 1985; Berger and Drawert, 1984). Increases in the production of ethyl esters and decreases in other acyl esters of low O_2 and/or high CO_2 treated apples (Mattheis *et al.*, 1991a), strawberries (Ke *et al.*, 1994b; Larsen and Watkins, 1995a) and oranges (Shaw *et al.*, 1992) could possible be due to a similar competitive process of esterification with increased ethanol concentrations in response to hypoxic treatment. Berger *et al.* (1992) suggested that exogenously applied substrates in fruits appear to be involved in highly competitive reactions, as an internal excess of one alkyl moiety always results in a decrease of esters not containing that particular alkyl moiety. High concentrations of ethanol supplied to apples favoured esterification of medium-chain acyl moieties (C_8 or longer) with ethanol, whereas lower concentrations favoured the short chain moieties (Berger and Drawert, 1984). The ethanol concentration in the gas phase that saturated the esterifying system was reached for short-chain fatty acids at about 50 mM $EtOH \cdot kg^{-1}$ fruit ($\approx$ 2300 ppm), whereas at higher concentrations only the acyl moiety of fatty acid chains greater than C_5 were affected. Acetylation of the exogenous ethanol substrate operated rapidly with a K_m of about 20 $mM \cdot kg^{-1}$ fruit (Berger *et al.*, 1992).

While increases in volatile ethyl esters have been observed following substrate feeding experiments (De Pooter *et al.*, 1987) and from anaerobic treatments of apples (Mattheis *et al.*, 1991a), reduced concentrations of aldehydes and alcohols, other than acetaldehyde and ethanol, have also been observed. The mechanism by which such a reduction in aldehydes and alcohols occurs has not been investigated. Most aldehydes are

thought to be derived from oxidation of fatty acids through a number of cycles of the β-oxidation pathway. Short-chain acyl CoA is reduced by acyl CoA reductase to aldehyde which in turn is reduced to alcohol by ADH (Bartley and Hindley, 1980). Such acyl derivatives are required for esterification (Gilliver and Nursten, 1976; Oliás *et al.*, 1995), although it has been reported that acetyl CoA concentration is not a limiting factor for ester synthesis by apples in CA conditions (Bartley *et al.*, 1985). However, the high concentration of ethanol in hypoxically treated fruit may compete with other alcohols for the acyl CoAs needed for esterification (Eriksson, 1979). Hence, it is possible that the competition for acyl CoAs may cause a reduction in the concentration of acyl CoAs needed for the conversion to aldehydes; this could lead to a lowering of the concentration of aldehydes and subsequently alcohols, other than acetaldehyde and ethanol in treated fruit.

Carboxylic acids are also important precursors of aldehydes and alcohols in apples (De Pooter *et al.*, 1987), being reduced to aldehydes and alcohols (De Pooter *et al.*, 1983). The formation of esters are also dependent on the availability of acids (Knee and Hatfield, 1981; Oliás *et al.*, 1995). A decrease in aldehydes and alcohols also may be due to competition for acids in the esterification process; this may reduce the pool of acids available for conversion into aldehydes and alcohols.

To date, there have been only a few investigations of the beneficial effect of short-term application of hypoxic or anoxic atmospheres at normal temperature (20°C) as a possible means of enhancing aroma volatiles and quality of fruits. Changes in volatile compounds during and after subjecting fruit to such conditions have been reported for example in oranges, mandarins (Shaw *et al.*, 1991; 1992) and strawberries (Larsen and Watkins, 1995a). The effect of a fairly long term (30 days) anaerobic treatment (low O_2) at low temperature (1°C) on volatile production of apples has been reported by Mattheis *et al.* (1991a). There are numerous studies on the detrimental effect of hypoxic/anoxic atmosphere application in various fruits, mainly focussed on particular aspects of quality deterioration, such as physiological injury and development of off-flavours. The volatile compounds which have been extensively monitored are acetaldehyde, ethanol and ethyl acetate, as these compounds are thought to be closely related to the development of tissue injury (Smagula *et al.*, 1968) and off-flavour (Ke *et al.*, 1991a; Ueda and Bai,

1993) in fruit following treatment with low O_2 and/or high CO_2 conditions. However, the reversibility of off-flavours without much change in fermentative volatiles of apples, pears, berries and plums after having been subjected to a short-time anaerobic conditions have cast doubt on the importance of these compounds to off-flavour perception (Richardson and Kosittrakun, 1995). Moreover, the application of short-term anaerobic treatments and acetaldehyde or ethanol vapours in several fruits have been reported to improve rather than decrease flavour during shelf-life (Paz *et al.*, 1981; Pesis and Avissar, 1989).

Application of volatile precursors, such as alcohols, aldehydes and carboxylic acids in the vapour phase, enhanced aroma volatile compounds in apples (Berger and Drawert, 1984; De Pooter *et al.*, 1987) and it has been suggested that such treatments can be employed to improve aroma volatiles in apples and apple products (Berger and Drawert, 1984). Using this knowledge, a technique to enhance fruit ester production, the so-called 'Precursor Atmosphere (PA) technology', was developed and has been extensively investigated in some fruits, such as apples, banana and strawberries in Germany (Berger, 1990; Berger, 1995; Berger *et al.*, 1992; Kollmannsberger and Berger, 1992). 'PA-technology' is a short-time (24 - 48 hours at 20°C) storage biotechnology that uses intact or mature fruit tissue as a biocatalyst for production of aroma by exposing fruit tissue to controlled vapour of specific precursor substrates, such as alcohols or aldehydes (Berger *et al.*, 1992). The precursor atmosphere (PA) technology has been used for *in vivo* enrichment of apple aroma to compensate for lack of volatiles in fruit obtained from long-term CA storage atmosphere or during processing (Berger *et al.*, 1992). It has been reported that New Zealand grown 'Red Delicious' apples contain less ethyl esters than those from South Africa and the use of PA technology could compensate for the lack of such volatiles (Kollmannsberger and Berger, 1992).

In Europe and USA, 'natural' aroma compounds are compounds isolated by 'physical, enzymatic, or microbial processes or traditional food preparation processes solely or almost solely from the food stuff concerned' (Berger, 1995; Knights, 1986). 'Synthetic' compounds derived from petro-chemistry or other natural chemicals which are added for flavouring purpose are considered 'artificial' and/or 'natural identical'

compounds (Berger, 1995) The Flavour and Extract Manufacturer association (FEMA) and the Food and Drug Administration (FDA) have published lists of 'Generally Recognised As Safe' (GRAS) materials. The most recent GRAS list contains about 3800 entries of extracts, essential oils, and natural and artificial compounds and also includes maximum dosage levels anticipated for various categories of food and beverages (Smith and Ford, 1993). These listed GRAS compounds will present no greater hazard to health than the food supply itself, providing they are present at no greater level than in food (Knight, 1986; Smith and Ford, 1993). Authentication of fruit flavour as natural is important in assuring compliance with labelling regulation (Rouseff and Leahy, 1995) and all GRAS materials are exempted from the requirements for regular food additives (Berger, 1995).

Although PA technology enhances various aroma volatile compounds, addition of chemical compounds to fruit in order to enhance or improve aroma in fruit and their products would likely to be restricted by legislation. Moreover, increasing consumer demand and awareness of natural high quality food products, which includes fruit juice (Gunter and Fairchild, 1985; Lea, 1995), suggests that there may be considerable potential for utilising natural flavour fractions to enhance or improve flavour and aroma quality in apple juice (Lea, 1995, Shaw *et al.*, 1991). An economical, rapid method for enhancing the levels of important flavour impact volatile compounds in apples prior to juice extraction would increase the use and value of the aqueous essence from fruit. Furthermore, such a method could improve aroma quality of fresh apples prior to marketing, especially with fruit from long-term CA storage which is well-known to lack flavour and aroma volatiles. The hypoxic/anoxic methods indicated previously have the advantage of being relatively 'clean and green', especially if N_2 is used rather than CO_2, as the latter may contribute to the greenhouse effect. There has been no extensive monitoring of important volatile compounds, other than the fermentative volatiles, after the exposure of apple to a short-term hypoxic/anoxic treatment. In addition, responses of different cultivars to such a treatment, the effects of varying concentrations of high CO_2 and/or N_2 in the atmosphere, time of exposure, storage conditions and duration, and other factors, such as growing location, fruit size, and cultural practice, have not been investigated. Therefore, it is worth exploring the effect of a short-term high CO_2 and/or

N_2 atmosphere treatment at ambient temperature on changes of selected important volatile compounds for its potential to increase certain aroma impact compounds in apple fruit and juice, as well as its effects on fruit physiology and quality.

1.6 OBJECTIVES

The aim of the present study is to investigate the effect of short-term hypoxic concentrations and duration of CO_2 and/or N_2 treatments at 20°C on changes in aroma volatile production, physiology and quality of apples after treatment during shelf life conditions at 20°C and 70% RH.

Chapter Two

GENERAL MATERIALS AND METHODS

2.1 FRUIT SUPPLY

Freshly harvested commercially mature apples (*Malus domestica* Borkh.), cultivars 'Royal Gala', 'Red Delicious', 'Braeburn' and 'Granny Smith', used for the 1993 experimental season, were harvested from Fruit Crops Unit, Massey University, Palmerston North. Fruit sizes varied depending on cultivar used and details are given in **Chapter Six**. In 1994, cool stored apples (1 - 8 months at 0.5° ± 0.5°C, 92 - 95% RH), Class I local fancy grade, 125 count (average weight of 148 ± 5 g) from Central Hawkes Bay were obtained through ENZA™ New Zealand (International), a division of the New Zealand Apple and Pear Marketing Board. Cultivars used were 'Braeburn', 'Cox's Orange Pippin', 'Fuji', 'Granny Smith', 'Pacific Rose' (GS#2085), and 'Red Delicious'. In 1995, Class I local fancy grade 'Red Delicious' apples (125 count), were obtained from a local grower. After harvest in 1993 and 1995, and after removal from cool store in 1994 apples were transported to the Department of Plant Science, Massey University, Palmerston North where treatments were applied after 24 hours equilibration at 20°C or placed at 0.5° ± 0.5°C, 92 - 95% RH until required. Details of storage duration of apples prior to application of treatments are given in **Chapter Four** and **Chapter Five**.

Prior to applying treatments, apples of uniform size and colour were subjected to a randomisation procedure. For a particular experiment, apples were generally divided according to the number of treatments and the treatments were randomly allocated. In each treatment, fruit were further divided according to the number of replications and the replications were randomly assigned. For each replication, apples to be taken for analysis at each sampling time (6 - 10 intervals) were also randomly allocated. Thus, each fruit was individually labelled according to its replication, treatment, and sampling date. Therefore, at a particular sampling date (day after treatment) the fruit numbers to be used for analysis in a replication and a treatment were known, and were taken

accordingly. Details of treatments, replications and fruit sampling time after treatment for each experiment are described in appropriate chapters. After this allocation process, the fruit was left at 20° ± 2°C and 70% RH for a minimum of 12 hours to equilibrate. Fresh weight of each apple was measured prior to application of any treatment.

2.2 CARBON DIOXIDE AND NITROGEN GAS TREATMENTS

Gas treatments were applied to fruit in a 27-litre, gas-tight Perspex chamber which was closed with a gas-tight lid (**Plate 2.1**). Fruit were exposed to gases using a flow-through system which was created by connecting polyethylene tubes (∅ 0.6 cm) for applying and removing CO_2 or N_2 gas to the respective inlet and outlet ports of each chamber. The 100% food grade CO_2 or 100% N_2 gas (New Zealand Industrial Gas, NZIG®) was applied through the system from a compressed gas cylinder via a pressure reduction valve (needle valve), a manometer, and a manifold. The gas was humidified by passing through water before entering the chambers.

Chambers were flushed with CO_2 or N_2 gas at a flow rate of 5 $l \cdot min^{-1}$ during the first 1 - 2 hours of the gas treatment or until the concentration of gas in the chambers reached 98 - 100%. The zero time for the high CO_2 or N_2 gas treatments began at this stage. Flow rate was then reduced to approximately 500 $ml \cdot min^{-1} \cdot chamber^{-1}$ and was maintained at this rate until the chambers were opened; then excess gas was expelled through the outlet ports to open air outside the laboratory. Concentration of CO_2 gas in the chambers was monitored regularly by taking a 1 ml sample of headspace gas with an air-tight tuberculin syringe (1 cc Graduated Monoject®, with detachable needle, 25 Gauge x 5/8", Sherwood Medical, MO, USA) and injecting it into a Shimadzu GC-8A gas liquid chromatograph (GLC). The GLC was equipped with a thermal conductivity detector (TCD) and a Hewlett Packard 3392A integrator. The column used was 6-foot (1.83 m) AllTech® Concentric CTR1 which contained an outer column (∅ 1/4" or 6.35 mm) packed with Activated Molecular Sieve and an inner column (∅ 1/8" or 3.18 mm) packed with a mixture of porous polymers. Temperature settings for the column, injector, and detector were 30°, 60°, and 60°C, respectively. The electrical current and

attenuation of the detector were 90 mA and 8x, respectively. Hydrogen carrier gas flow rate was 60 $ml \cdot min^{-1}$. Concentrations of CO_2, N_2 and O_2 were calculated based on a gas standard of 9.96% CO_2, 19.8% O_2 and 70.24% N_2.

Control fruit was left untreated within open-lidded chambers, the atmospheres of which were also monitored.

After the CO_2 or N_2 gas treatments, apples were removed from the chambers, placed on trays and stored in 18 kg apple cardboard cartons for further analysis. All treatments, storage, and analyses were made at 20° ± 2°C and 70% RH.

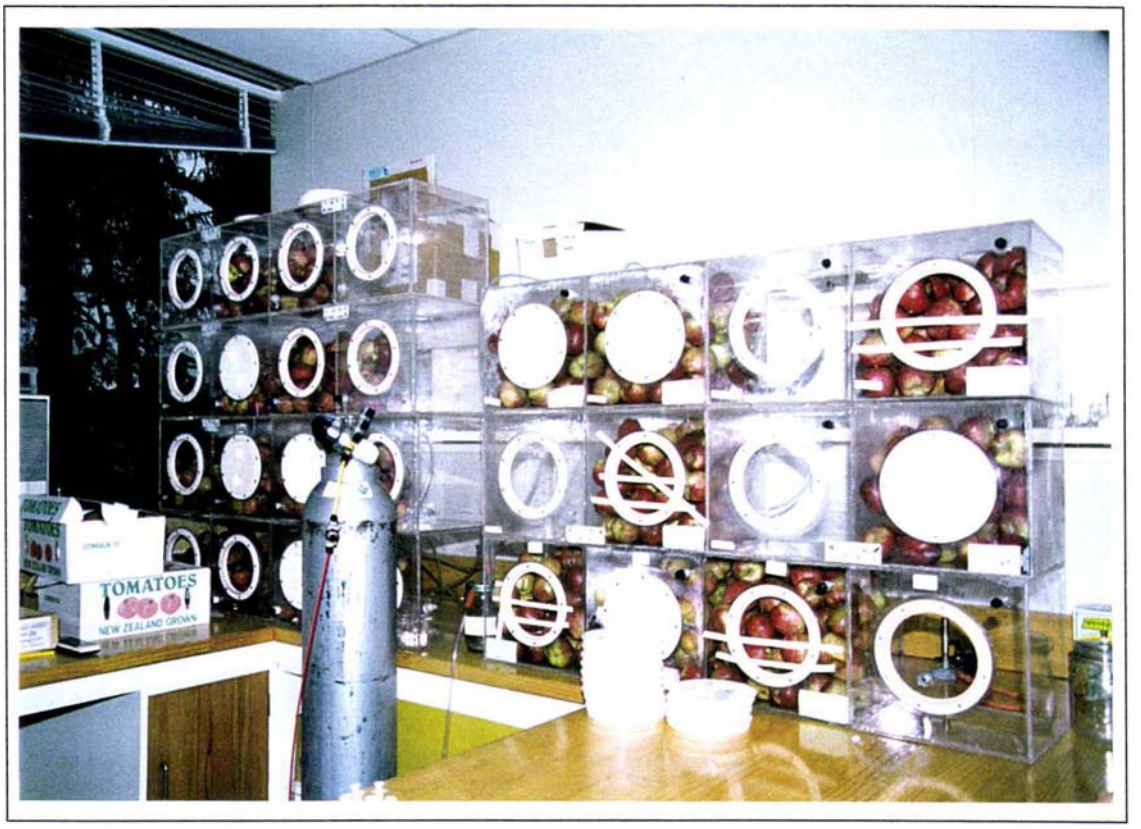


Plate 2.1 Perspex chambers used for exposing apples to gas treatments.

Fruit samples were taken according to a randomised sequence determined prior to treatment. Sampling intervals were -1, 0, 1, 3, 5,... days after treatment, where

-1 day was the time of starting the CO_2 gas application,

0 day was immediately after removal from treatment chambers,

1 day was 24 hours after treatment, and so on.

2.3 FRUIT ANALYSIS

Changes in physiological and physical properties of control and treated apples were determined by analysing the following attributes:

2.3.1 Fruit Moisture Loss

Fruit mass(g) of each apple was determined by weighing on a Mettler® PE 3600 electronic balance at each sampling interval. Fruit moisture loss was then calculated based on initial fresh weight of the respective apple fruit and the result was expressed as per cent moisture loss (**Appendix 1, Section 1.1**).

2.3.2 Fruit Volume

Apple fruit volume was determined by the water displacement technique at room temperature (Baumann and Henze, 1983). This method involved weighing the fruit under water. A 1500 ml Pyrex beaker was 3/4-filled with water and placed on a Mettler® PE 3600 electronic balance which was then tared. A swing arm lampstand was modified by replacing the bulb with a rubber bung which had 3 blunt-tip needles attached. Each fruit sample was forced just under the water surface by the needle tips. The weight was then recorded; it was assumed that 1 g of water was equivalent to 1 cc of fruit volume.

2.3.3 Fruit Respiration and Ethylene Production Rates

A static system was used to determine fruit respiration and ethylene production rates. Each individual fruit was placed inside an air-tight, Agee® glass jar of known volume, equipped with a lid through which a Vacutainer® silicone rubber septum (Becton Dickinson and Company, USA) port was attached. The jar was sealed for a period of 15 minutes to 1 hour, depending on the CO_2 production of the fruit sample at 20°C. Fruit which had lower CO_2 production rates were kept in the jars for longer periods than those which had higher production rates. Concentration of CO_2 in jars when taking gas samples was generally 0.2 - 0.4%. Two empty jars were also employed as blanks under

the same atmospheric condition. A 1 ml gas sample, drawn from the headspace through a rubber septum port using an air tight tuberculin Monoject® syringe was injected onto a GLC to determine concentrations of CO_2 and ethylene in the jar. Results obtained were compared with the respective external standards. The standard gas mixture used for calibration comprised of CO_2, O_2, and C_2H_4 gases with the respective concentrations of 9.96%, 19.8% and 9.7 ppm.

2.3.3.1 Fruit Respiration Rate

The 1 ml headspace gas sample was determined for CO_2 concentration using a Shimadzu GC-8A GLC as described in **Section 2.3**. The Hewlett Packard integrator parameters were set as follows **:** zero baseline 10%, attenuation 3x, chart speed 0.5 $cm \cdot min^{-1}$, peak width 0.04 min, threshold -1, area reject 0, and stop time 2.25 min. Based on a gas standard of 9.96% CO_2, respiration rate was calculated according to the method outlined in **Appendix 1**, **Section 1.2** and expressed as nmol $CO_2 \cdot kg^{-1} \cdot s^{-1}$ (Banks *et al.*, 1995).

2.3.3.2 Ethylene Production Rate

The 1 ml headspace gas sample was analysed for ethylene concentration on a Shimadzu GC-4B PTF Gas Chromatograph equipped with a flame ionisation detector (FID) and a Hewlett Packard 3390A integrator. The Alltech® nickel column used was 1/8" o.d. x 6' in length and packed with 80/100 mesh activated alumina. Temperature settings for the column, injector, and detector were at 110°, 150°, and 155°C, respectively. Flow rates of oxygen-free nitrogen carrier gas, hydrogen and dry air (NZIG®) were 30, 30, and 300 $ml \cdot min^{-1}$ respectively. The integrator parameters were set as follows**:** zero baseline 20%, attenuation 3x, chart speed 0.5 $cm \cdot min^{-1}$, peak width 0.04 min, threshold 3, area rejected 0, and stop time 1.25 min. Based on gas a standard of 9.7 ppm ethylene, the ethylene production rate was calculated according to the method outlined in **Appendix 1**, **Section 1.3** and expressed as nmol $C_2H_4 \cdot kg^{-1} \cdot s^{-1}$ (Banks *et al.*, 1995).

2.3.4 Fruit Skin Colour

Skin colour of each apple was measured on opposite sides of the equator using a Minolta Chromameter® CR 200, which automatically generated lightness (%) and hue angle (°) values. Calibration of the instrument was made against a standard green plate (CR-A47 G) for each period of measurement. Calibration and measurements were performed in the same room condition. Lightness and hue angle values were averaged for the two readings of each fruit. The L*C*H° colour system for absolute measurement of colour with the light source C (= CIE standard illuminant C conditions) and 0° viewing angle was employed for all measurements.

2.3.5 Flesh Firmness

Flesh firmness of each fruit sample was measured on pared surfaces on opposite sides of the equator using a hand-held Effigi® penetrometer (R. Bryce, Fruit Tester FT327, 0 ~ 12.5 kg (0 ~ 28 lbs), Italy) equipped with an 11.1 mm diameter plunger head. The mean of the 2 readings (kg) from each fruit sample was converted into Newtons by multiplying with a correction factor of 9.81.

After the above measurements were completed, the fruit sample was diced quickly on a chopping board. The cut pieces, including peel, cortex, core, and seeds, were juiced with a kitchen centrifuge juicer. Extracted apple juice was collected and filtered through 3 layers of cheese cloth into a beaker which was immediately placed into an ice bath. A portion of juice was transferred into a 20 ml Wheaton® liquid scintillation glass vial equipped with a metal foil liner cap (Wheaton, USA) and frozen at -20°C for later determination of titratable acids and juice pH. The rest of the juice was used immediately for determination of soluble solids and for extraction of the aroma volatile compounds and headspace volatiles.

2.3.6 Soluble Solids

A few drops of filtered apple juice were taken for determining soluble solids content using a hand-held refractometer (Atago® ATC-1: Automatic Temperature Compensation, Brix 0 ~ 32%) calibrated against distilled water, ie. 0%. The prism surface and light plate cover were thoroughly washed with distilled water and dried with a piece of soft, clean tissue paper after each reading. The result was expressed as per cent total soluble solids of the juice sample.

2.3.7 Titratable Acids and Juice pH

Determination of both titratable acids and pH of fruit juice samples was made on a Mettler® DL21 Automatic Titrator equipped with a combination universal glass electrode for acid/base titration (Mettler® DG111, 0 - 14 pH, 0 - 70°C, 3M KCl). The frozen juice sample was quickly thawed to ambient temperature in the laboratory by using an electric fan blowing over the sample vials. A 1 ml aliquot juice sample was taken with a graduated auto-pipette, mixed with 40 ml of distilled water and titrated against 0.1 N sodium hydroxide solution to pH 7.1 end point. Blank samples of 40 ml distilled water were also measured. The pH of each juice sample was automatically measured by the titrator at the beginning of the titration process. The titrant was freshly prepared from a 1 N NaOH stock solution and standardised against a 1 N hydrochloric acid BDH® standard solution before analysing each lot of samples.

Prior to performing the titration, the titrator was configured and the configuration parameters are listed in **Appendix 1, Section 1.4**.

Results given by the titrator were the volumes of NaOH required to neutralise the acids in the juice sample, after subtracting the blank value. After further calculation the results were expressed as per cent malic acid equivalents (**Appendix 1, Section 1.4**).

2.4 ANALYSIS OF VOLATILE COMPOUNDS OF APPLES

2.4.1 Headspace Volatiles

The headspace volatiles acetaldehyde, ethanol and ethyl acetate from the juice samples were determined by the following procedure:

A 30 ml sample of freshly extracted and filtered apple juice was placed in a 50 ml Erlenmeyer flask and immediately sealed with a SUBA-SEAL® (No. 33) rubber septum. The flask was immersed in a 30°C water bath (Grant SX, 0 - 100°C, UK) for at least 30 min (Davis and Chace, 1969) where the water temperature was constantly and evenly controlled by a heating coil equipped with a thermostat and a built-in centrifugal pump for water circulation. A headspace gas sample was taken through the rubber septum using an air tight syringe for the determination of the volatiles on a GLC at room temperature. The GLC was calibrated using a standard gas mixture containing 100 $\mu l \cdot l^{-1}$ of each compound concerned. The standard gas mixture was thoroughly stirred on a magnetic stirrer (Chilton® MM80 , Stirrer Hotplate) before taking the gas mixture for calibration.

In 1993, a 100 µl sample of the headspace gas was taken with a 100 µl air-tight glass syringe (Hamilton, Gastight® Microsyringe, No. 1701) fitted with a removable needle (22s gauge, 0.15 mm i.d. x 5.08 cm, 22° bevel point). A Varian 3400 Series Gas Chromatograph equipped with a flame ionisation detector (FID) and a Hewlett Packard 3393A integrator was used for the analysis. The column used was a 0.32 mm i.d. x 30 m in length Alltech Econocap® Carbowax 20M (0.25 µm film thickness) capillary column. Gas flow settings for the oxygen-free nitrogen carrier gas, hydrogen, and dry air were 10, 30, and 300 $ml \cdot min^{-1}$, respectively. Temperature settings for the column (oven), injector, and detector were 40°, 150°, and 300°C, respectively. The respective attenuation and range of detection were set at 1x and 12. The hold time for detection was 5 min. Parameter settings for the integrator were as follows : zero baseline 10%, attenuation 3x, chart speed 0.5 $cm \cdot min^{-1}$, area reject 0, threshold 2, and peak width 0.16 min.

In 1994 and 1995, 1 ml headspace gas samples were drawn from the flasks with an air-tight tuberculin Monoject® syringe and injected onto a Pye Unicam GCD Chromatograph. The GLC was equipped with a flame ionisation detector (FID) and a Rikadenki Chart recorder. The column used was an Alltech® stainless steel of 3.715 mm o.d. x 1.82 m in length, packed with 10% Carbowax 20M on Chromsorb W AW 80/100 mesh. Flow rates of the oxygen-free nitrogen carrier gas, hydrogen, and dry air were 30, 30, and 300 $ml \cdot min^{-1}$, respectively. Temperature settings for the column, injector, and detector were 40°, 110°, and 180°C, respectively. The range and hold time of detection were set at 1 and 5 min. Chart speed and the attenuator of the recorder were set at 0.5 $cm \cdot min^{-1}$ and 0.2V, respectively. Peak area or peak height obtained from the chromatogram was calculated and expressed in terms of $\mu l \cdot l^{-1}$ by comparing with the average of the standard gas mixture for the individual volatile.

2.4.1.1 Preparation of the Standard Headspace Volatile Gas Mixture for Calibration

The 100 $\mu l \cdot l^{-1}$ standard gas mixture of acetaldehyde, ethanol, and ethyl acetate was prepared in an air-washed Agee® jar (approximately 2 litres in volume) equipped with a lid and a magnetic stirrer rod placed inside. The air-tight lid was fitted with a silicone rubber septum (Vacutainer® rubber stopper). Two hundred cc of the air inside the jar was evacuated with an Omnifix® syringe (50/60 ml Luer, Braun Melsungen AG, W.Germany), then 40 cc of each of the 5000 $\mu l \cdot l^{-1}$ stock standard gases were injected into the jar. The procedure for preparing the stock standard gases is described in **Appendix 1, Section 1.5**. The stock standard gas mixture was thoroughly mixed by stirring constantly on a Chilton® MM80 magnetic stirrer. The gas mixture was made up to 2000 cc by injecting air into the jar. The standard gas mixture was prepared and maintained at room temperature.

2.4.2 Determination of the Aroma Volatile Compounds in Apple Juice

2.4.2.1 The Purge and Trap Technique

In 1993, the purge and trap technique (Cole, 1980; Dirinck *et al.*, 1977) was used for extracting volatile compounds from apple juice samples. This technique involved the preparation of a Tenax® trap column, the purge and trap procedure, and elution of volatile compounds for GLC analysis.

***Tenax Trap Column Preparation*:** A glass tube of 3 mm i.d. x 300 mm in length was cleaned and oven dried. A small piece of silane treated glass wool (Dimethyl dichlorosilane DMS treated, Alltech®) was inserted into the tube approximately 5 cm from one end. Four hundred mg of porous polymers 60/80 mesh Tenax® GC (2,6-diphenyl-p-phenylene oxide, Alltech®) was poured into the glass tube while vibrating on a Vortex mixer to ensure that the packing particles were packed tightly in the tube. A small piece of silane treated glass wool was plugged into the other end of the tube to secure the packing material in place. The column was further vibrated for a few seconds and the glass wool plug was adjusted.

The Tenax® column was then rinsed with approximately 4 ml of diethyl ether (Analar, BDH) by delivering approximately 1 ml of the solvent at a time into the column with a Pasteur pipette. The solvent was forced through the packing material by a low flow rate of oxygen-free nitrogen gas (approx. 50 $ml \cdot min^{-1}$) from a compressed gas cylinder. After 4 washes, the column was dried by passing oxygen-free nitrogen gas through at room temperature.

After drying, the column was (pre-) conditioned by the following procedure. The column was placed between aluminium blocks that contained grooves to hold the column in place. The column along with the aluminium blocks was placed on a hot plate (Chilton® MM80, Stirrer Hot Plate). Oxygen-free nitrogen gas was continuously passing through the column via a piece of silicone rubber tube at a flow rate of 20 $ml \cdot min^{-1}$. The column was heated to 280° - 300°C for at least 2 hours. After heating, the column was cooled to room temperature while the nitrogen gas was still flowing through. The

column was then capped with 1/4" GC End Caps (Alltech®) at both ends, sealed with a piece of Parafilm® M (American National Can, USA) and kept under nitrogen until used.

***Extraction and Trapping of the Volatile Compounds*:** Two hundred ml of ice-chilled, filtered apple juice was placed into a 250 ml Dreschel gas washing bottle fitted with a sintered head (Quickfit® MF 29/3/250). Approximately 0.5 g sodium chloride (NaCl) salt was added to increase the partial pressures of volatile compounds in the juice sample (Jennings, 1965). One µl of octyl acetate (Analar, BDH) was added to the juice as an internal standard. The Tenax® column was connected onto the outlet of the Dreschel bottle with a piece of silicone rubber tube. Oxygen-free nitrogen gas was continuously passed through the juice at a flow rate of approximately 50 $ml \cdot min^{-1}$ from a compressed gas cylinder via a pressure reduction valve and a piece of silicone rubber tube. The nitrogen gas, after mixing with the juice, was passed through a Tenax® column for 2 hours at room temperature. The column was then disconnected, capped with the GC End Caps (AllTech®) and sealed with Parafilm® M and stored at -20°C until required.

***Elution and Concentration of Sample for GLC Analysis*:** The trapped volatile compounds collected on the Tenax® GC were eluted repeatedly with diethyl ether (Cole, 1980) delivered, approximately 1 ml at a time, into the column from a Pasteur pipette. The solvent was pressurised and washed through the Tenax® with the aid of a low flow rate of nitrogen gas (approx. 20 $ml \cdot min^{-1}$) supplied from a cylinder. Approximately 4 ml of eluted diethyl ether along with the dissolved volatile compounds was collected into a 4.5 ml autosampler vial equipped with an open-hole screw cap and a 12 mm polytetra-fluoroethylene (PTFE) disc (Autosampler vial for Waters, Alltech®).

The Tenax® column was then subjected to drying and conditioning as previously described prior to reuse.

The eluted diethyl ether sample was concentrated to 200 µl by gently blowing a stream of oxygen-free nitrogen gas via a Pasteur pipette tip over the extracted solvent in the vial. Toluene, 10 µl of a 1000 $\mu l \cdot l^{-1}$ solution in diethyl ether, was added to the concentrated extract as an external standard. The vial was capped, sealed with Parafilm® M and stored at -20°C until required for GLC.

***Determination of Volatile Compounds on a GLC*:** One µl of the concentrated extract was withdrawn from the sample vial with an air-tight syringe (Hamilton Gastight® Microsyringe, 10µl) and injected onto a Varian 3400 Series Gas Chromatograph for qualitative and quantitative measurement of the volatile compounds. The GLC was equipped with a flame ionisation detector (FID) and a Hewlett Packard 3393A integrator. The column used was a 0.25 mm i.d. x 30 m Alltech Econocap® capillary column coated with 0.25 µm thickness of Carbowax 20M. Column temperature was programmed with the initial temperature of 40°C for 5 min, followed by a linear increase of 5°C $\cdot min^{-1}$ to 190°C and holding at this final temperature for 10 min. The injector was set on a split mode (3:1 ratio) at a temperature of 250°C. The FID temperature was 300°C and the detection range and attenuation were 11 and 8x, respectively. The baseline was set on the auto-zero baseline mode. Initially, oxygen-free nitrogen gas was used as carrier gas for the 'Royal Gala' aroma volatile compounds analysis but it was later changed to hydrogen gas which produced sharper, well separated peaks on a chromatogram (H. Young, 1993, personal communication). Flow rates of the carrier gas, hydrogen, and air were 30, 30, and 300 $ml \cdot min^{-1}$, respectively. The linear flow velocity of the carrier gas was 32 $cm \cdot s^{-1}$. Total run time of a sample was 45 min. Setting parameters of the integrator were: zero baseline 10%, attenuation 0, chart speed 0.5 $cm \cdot min^{-1}$, threshold 1, peak width 0.04 min, area rejected 0, and stop time 45 min.

Samples were sent to HortResearch Laboratory, Crown Research Institute, Palmerston North for definitive peak identification by Mass Spectrometry. Peaks obtained from the chromatogram of a sample were then matched with reference to retention time of known compounds. Peak area of each peak of interest were taken and normalised against the octyl acetate internal standard (**Appendix 1**, **Section 1.6**). The corrected peak area was used when presenting data. The toluene external standard could not be used, since it eluted at the same retention time as ethyl butanoate on this Carbowax 20M column.

2.4.2.2 The Direct Solvent Extraction Technique

In 1994 and 1995, a quick and simple extraction method for analysis of aroma compounds in fruit products (Larsen and Poll, 1990) was modified for extracting the

volatile compounds from apple juice samples. This technique was adopted because it was faster and required less juice for extraction than the purge and trap method.

***Extraction and Concentration Procedure*:** A mixture of 2:1 v/v of diethyl ether and n-pentane (Analar, BDH) was prepared at room temperature under a fume cabinet.

A 20 ml sample of ice-chilled, filtered apple juice was placed into a 50 ml Wheaton® liquid scintillation vial fitted with a metal foil liner cap. Ten µl of 2000 $\mu l \cdot l^{-1}$ octyl acetate in the ether:pentane mixture was injected into the juice with a SGE microsyringe (SGE Series II guided plunger, 10µl, Alltech®) fitted with a detachable bevel tip needle (0.11 i.d. x 50 mm, Alltech®). Twenty ml of ether:pentane mixture was added, the vial was capped tightly and the juice sample and the solvent in the vial were thoroughly mixed on a Vortex mixer for 5 - 10 seconds.

The vial was stored at -20°C until the water phase was frozen. The unfrozen solvent was then decanted into a new Wheaton® liquid scintillation vial and the frozen portion of juice was discarded. Some gummy precipitate or suspended cell debris in the solvent phase was experienced occasionally, especially with juice obtained from apples that had been stored for a lengthy period (> 5 months). This was removed by centrifuging the vial and its contents at 500 - 1000 rpm for 5 - 10 min (Gallenkamp Junior Centrifuge, CF430, England) to precipitate extraneous particles before collecting the extracted solvent into a vial that was capped tightly; the solvent was either concentrated immediately or stored at -20°C.

The extracted solvent was concentrated with a stream of oxygen-free nitrogen gas as previously described (**Section *2.4.2.1***) except that the initial volume of the solvent of 20 ml was concentrated to 0.25 ml (ie. 80 fold concentration).

With a Pasteur pipette, the concentrated extract was immediately transferred into a 250 µl glass, flat bottom insert that fitted in a 1.5 ml standard mouth autosampler glass vial (Alltech®). The vial was then capped tightly with an open-hole polypropylene cap fitted with a 12 mm white TFE disc (Alltech®). The concentrated extract in the vial was used for analysis of the volatile compounds on a GLC.

Preparation of the External Standard Mixture: A standard mixture of 15 volatile compounds in ether:pentane, with the concentration of 200 $\mu l \cdot l^{-1}$ each, was prepared in a 50 ml volumetric flask. The standard mixture was prepared at room temperature in a fume cabinet.

More than 350 volatile compounds have been found in apples and apple products (Maarse, 1991) but less than 30 contribute in a dominant way to aroma of apples and apple products (Cunningham *et al.*, 1986; Dürr and Schobinger, 1981). Therefore it was decided to select 15 compounds for studying in the 1994 and 1995 experiments (**Table 2.1**).

Table 2.1 Apple volatile compounds chosen for study in 1994 - 1995 experiments.

Headspace	Alcohols	Aldehydes	Esters
acetaldehyde	methanol	hexanal	ethyl acetate
ethanol	ethanol	*trans*-2-hexenal	ethyl butanoate
ethyl acetate	propan-1-ol		ethyl 2-methyl butanoate
	butan-1-ol		ethyl pentanoate
	hexan-1-ol		ethyl hexanoate
			butyl acetate
			hexyl acetate

Firstly, acetaldehyde, ethanol and ethyl acetate were selected because they are byproducts of fermentative metabolism (Ke *et al.*, 1993a; Kennedy *et al.*, 1992) and treatments used in this study involved different degrees of hypoxia known to induce fermentation. Secondly, most of the other volatiles chosen have been reported to be important contributors to the aroma of apple juice and fruit (see **Table 1.4**, **Chapter One**). Thirdly, some volatile compounds, such as methanol and ethanol, are thought to be 'undesirable' compounds contributing to off-flavours of apple juice aroma (Dürr and Schobinger, 1981; Lea, 1995). Finally, some of these compounds, such as propan-1-ol, butan-1-ol, *trans*-2-hexenal, ethanol and ethyl acetate were quantitatively major volatiles found in juice of hypoxically treated or non-treated fruit compared to other compounds and these compounds may also contribute to aroma of apple juice. The choice of the

above volatiles does not infer that other compounds not reported, do not contribute to apple aroma and flavour.

Five µl of each compound was taken by a SGE 10 µl microsyringe and injected through a silicone rubber septum that fitted in the cap into the flask that contained some of the ether:pentane mixture. After each delivery, the syringe was rinsed and washed repeatedly with ether:pentane and dried in air prior to taking an aliquot of the next compound. Standard concentrations were then achieved by making up the volume with ether:pentane. The flask was re-capped with a new cap and sealed with a piece of Parafilm® M. The standard mixture was stored at -20°C for later use.

Volatile compounds selected for making up the external standard mixture had been individually tested for their retention times on the same column used for analysing samples. Results from the analyses of the standard mixture were used to create a calibration table.

***Determination of Volatile Compounds on a GLC*:** One µl of the concentrated extract sample was used for determining the volatile compounds on a Hewlett Packard HP5890 Series II Plus Gas Chromatograph equipped with an automatic sampler unit, a personal computer (Silicon, USA), and a Hewlett Packard Deskjet 500 printer. After programming the operation parameters, the analysis procedures were entirely controlled by the HP ChemStation® (version A. 03.34) software from the computer.

The column used was a DB-WAX 0.32 mm i.d. x 30 m in length capillary column (widebore, 0.5 micrometer film thickness, 20° - 250°C, P/N 123 - 703, J and W Scientific, USA). Temperature of the column was programmed with the initial temperature of 40°C for 5 min, increasing to 130°C at a rate of 5°C·min^{-1}, then increasing to 190°C at 20°C·min^{-1} before being held at this final temperature for 5 min. Maximum temperature of the oven was set at 320°C and time for temperature equilibration was 1 min.

The injector port was fitted with an 11 mm Hewlett Packard red septum and temperature of the injector was set at 150°C. The injection was set on a split mode with a split ratio of 15 : 1. Injection of sample was programmed, in that, before injecting a sample, the syringe was washed with ether:pentane solvent 3 times, and washed with

sample once. Then, it drew the sample and held it for 4 seconds (the viscosity delay time). After that, it reduced the sample to 1 µl before injecting it into the injector port with the fast injection mode (a patent method of introducing sample to a heated inlet without the negative effect of needle fractionation). After injecting the sample, the syringe was again washed with ether:pentane solvent 5 times.

The flame ionisation temperature was 250°C.

The hydrogen carrier gas was set at the constant flow mode with the initial pressure of 29 kPa at 40°C oven temperature. Flow rate of the carrier gas was 1.16 $ml \cdot min^{-1}$, where the flow velocity was 30.3 $cm \cdot s^{-1}$. Flow rates of hydrogen gas and dry air for the detector were 30 and 400 $ml \cdot min^{-1}$, respectively.

The signal plot information was configured as follows: attenuation 0, offset 10%, and time 35 min. The integration events were programmed as follows: area rejected 100, peak width 0.04, threshold -1, and shoulder detection mode on. Detected signals were automatically stored into computer memory during the analysis.

The Hewlett Packard HP7673 automatic sampler unit was made up of 3 modules ie. the controller, injector, and tray modules. The HP18594B controller and communication module supplied power and communication interface for the injector and tray. The HP18593B injector was equipped with a Hamilton® HP autosampler microsyringe (glass syringe, 10 µl, 23 gauge cone tip, fix needle, No. 80377), a syringe carriage, a cooling fan, an electronic assembly (HP 7673 GC/SFC Injector) and a 5-position turret. The turret rotated the sample vial, solvent and waste bottles into position under the syringe. The HP 18596B tray module contained 4 removable tray quadrants, each held 25 vials, and a robotics arm. The arm and the gripper mechanism in the centre of the tray loaded a sample vial into the injector turret and returned to the tray after each injection.

Allocation of the sample vials was systematically randomised on the sample tray. The 200 $\mu l \cdot l^{-1}$ mixture of standard compounds were allocated at every 5 sample vials, at the beginning and at the end of each lot of samples to be analysed. After loading the vials onto the tray, a sequence of samples and standards to be analysed was created and saved as a file on the computer. The analysis was started from the HP ChemStation® on the computer.

After each batch was analysed, the average of the respective peaks of the standard compounds were used as reference peaks for calibration. Raw data of each sample was integrated and peaks were identified based on retention time. Concentrations of the volatile compounds were calculated based on the external standard used for calibration. Results of the calculated data (in ppm), as well as the chromatogram of each sample was printed onto the screen of the computer, printer, or stored in a digital form in the computer. Data obtained were further normalised against the octyl acetate internal standard and expressed in terms of $\mu mol \cdot l^{-1}$ (**Appendix 1, Section 1.7**).

2.5 DATA ANALYSIS

The experimental design for each experiment conducted in the present study is described separately in **Chapter Three** to **Chapter Six**. Means and standard errors (S.E.) for each attribute determined were calculated and graphed or tabulated. Data, otherwise stated, were subjected to analysis of variance using a SAS package (SAS® Institute Inc., Cary, USA). Means and pooled standard error of each attribute were calculated and significant main effects were separated by Duncan's multiple range test at 5% level of significance.

Chapter Three

ANALYSIS OF AROMA VOLATILE COMPOUNDS IN APPLES

3.1 INTRODUCTION

Flavour has been defined by the British Standard Institute as 'the combination of taste and odour. It may be influenced by sensations of pain, heat and cold, and by tactile sensations' (Thomson, 1986). However, odour or aroma is believed to be the most important characteristic of flavour (Heath and Reineccius, 1986). Because the importance of flavour perception is recognised, the search for methods to identify and measure flavour objectively began early in the 19th century, and gave birth to flavour chemistry (Maarse, 1991).

The objective measurement of flavour quality occurs by using a chemical and/or physical method for measuring the amount of a substance responsible for a particular flavour attribute (Jennings, 1977). However, the identification of volatile flavour components is very difficult to achieve for several reasons.

Firstly, aroma volatiles comprise a large number of chemical classes (Eriksson, 1979) and the compound responsible for a particular attribute is not easy to identify. In addition, some minor compounds are not well resolved from larger constituents or may not be detected in the analysis, yet be of critical important to a given flavour attribute (Berger, 1991). In many cases, aroma of a product is due to an integrated response to at least several compounds, and variations of the relative ratios and/or amount of these compounds may change the aroma response in a manner that is usually unpredictable (Jennings, 1977). Furthermore, synergism and antagonism can exist between compounds that elicit aroma or flavour response (Maarse, 1991).

Secondly, aroma volatile compounds are generally minor constituents with exceedingly low concentrations in a given product, when compared with other constituents such as water, carbohydrate and acids, and this may cause problems with

extraction and identification. The presence of proteins may hinder isolation of volatile compounds as flavour compounds may bind to them. The presence of lipids in the sample may lower the volatile vapour pressure and may also interfere with the analysis (Heath and Reineccius, 1986).

Thirdly, composition changes are frequently induced in the extraction and preparation of samples in a suitable form for analysis by gas chromatography. Moreover, not all compounds are stable in the conditions used for preparation and analysis, and the chromatogram may not accurately reflect the composition of the material injected (Maga, 1990). Most procedures used involve distillation, extraction, evaporation or adsorption, all of which may cause qualitative changes in concentrated samples, so that they no longer reflect the true composition of the starting material (Jennings, 1977).

Finally, laboratory instruments, such as the gas liquid chromatograph (GLC), are not as sensitive to many odours as is the human olfactory system (Heath and Reineccius, 1986). The estimated minimum sample size requirement for identification of compounds with flame ionisation detection (FID) and mass spectrometry (MS) are 10^{-12} and 10^{-11} $g \cdot l^{-1}$, respectively (Teranishi and Kint, 1993). On the other hand, odour thresholds as low as 10^{-15} $g \cdot l^{-1}$, for compounds such as vanillin, have been reported (Bemelmans, 1979). It has been estimated that as few as 8 molecules of a powerful odorant compound are required for triggering one human olfactory neuron and that only 40 molecules can produce an identifiable sensation (Flath *et al.*, 1981). Flath *et al.* (1981) assumed that if only 1 of 1000 molecules inhaled reached a receptor site, the other 999 being adsorbed or never reaching an olfactory region, then 40000 molecules or about 10^{-19} moles can be detected by the nose at least theoretically. This surpasses even the most sensitive analytical abilities.

Jennings (1977) stated that there is no single sample preparation procedure that can be accepted as ideally satisfactory, as one or another may be superior depending on the sample composition and the compounds of interest. Furthermore, the parameters for the analysis on GLC must be carefully selected in relation to sample composition, its stability under the conditions of analysis, and the goal for which resolution or capacity is required. There are two main groups of methods available for isolating volatiles directly from food products. The first group is based on the solubility of the compounds

concerned, which includes various distillation procedures to separate the volatile fraction from the non-volatile food matrix. The other group is based on a favourable distribution coefficient or volatility of the volatiles between an extractant and the food. Many procedures have been developed using these two principles (Bemelmans, 1979).

3.1.1 Preparation of Raw Samples

Prior to analysing aroma volatile compounds in a product, it is necessary to decide whether destructive or non-destructive sampling is to be used. Non-destructive sampling allows analysis of the same sample several times during developmental process, such as the changes in volatile composition that occur during ripening (Ito *et al.*, 1990; Mattheis *et al.*, 1991b) and/or storage (Dirinck and Schamp, 1989; Hansen *et al.*, 1992a, 1992b; Mattheis *et al.*, 1995; Streif and Bangerth, 1988). The results of such a method should be expressed as the amount of aroma volatile compounds released during analysis rather than the total concentration of the compounds in the product. In addition, results could be related to the pure odour of the produce rather than the flavour perceived during eating (Schreier, 1984). Taking sample from the headspace above the product is the only technique that can be used for non-destructive analysis.

The destructive method for extracting aroma volatile compounds is widely used and usually involves disruption of tissue by homogenising, juicing, or grinding (Maga, 1990). Relatively large samples can be homogenised and/or juiced at any one time; it is a simple matter to then take subsamples for subsequent analysis.

When fruit is disrupted during the process of destructive extraction, a series of rapid enzyme induced changes occur in aroma composition (Berger, 1991; Drawert *et al.*, 1986; Hatanaka, 1993). This includes the generation of intensely aromatic unsaturated and saturated C_6 or C_9 aldehydes and alcohols by lipoxygenase-induced degradation of the unsaturated C_{18} fatty acids, linoleic and linolenic acid (Hatanaka, 1993; 1996). These secondary volatile compounds are generated generally within 10 to 30 minutes of crushing (Drawert *et al.*, 1986). Simultaneously, enzymatic hydrolysis of some of the original fruit esters (eg. butyl butanoate) to their parent alcohols (eg. butanol) and acids (eg. butanoic acid) by esterases leads to a loss of fruity smelling constituents (Bartley and Stevens, 1981; Berger, 1991). Formation of some aromatic

acid esters from activated precursors also occur during this process (Berger, 1991). These changes may be desirable if the purpose of the study is to find out about the compounds responsible for the flavour perceived during eating of a product; the process of chewing or grinding fruit tissue is somewhat akin to homogenisation and the enzymic reactions are likely to be similar. In addition, these enzymatic changes may be important in the fruit juice industry, as C_6 aldehydes, such as hexanal and *trans*-2-hexenal are desirable constituents in fruit juice where they contribute to the green, fresh apple aroma note (Dürr and Schobinger, 1981; Flath *et al.*, 1967).

If one is interested in determining the volatile responsible for the characteristic odour of a particular product, then enzymatic changes may be undesirable. The suppression of these secondary compounds by inactivation of enzymes during extraction is important in work on genuine constituents. A common method used for enzyme inactivation is to blend the sample with methanol (Berger *et al.*, 1992; Schreier *et al.*, 1978a, 1978b; Tressl and Drawert, 1973). Although total inactivation of enzymes may be achieved by this method, it dilutes the sample, decreases polarity of the juice or aqueous sample slurry, and may well interfere with the later method of analysis (Heath and Reineccius, 1986). Calcium chloride ($CaCl_2$) and sodium chloride (NaCl) have been employed for inhibition of enzymatic activity during or after sample preparation (Berger *et al.*, 1992; Buttery *et al.*, 1987; Geduspan and Peng, 1986; Murray and Whitfield, 1975; Sapers *et al.*, 1977). However, these may behave like other salts added to the juice sample, influencing solubility and hence the distribution of some aroma volatile compounds between the headspace and the sample (Buttery *et al.*, 1987; Geduspan and Peng, 1986; Jennings, 1965; Nawar, 1966; Nelson and Hoff, 1968). Preparation of samples at sub-zero temperatures under nitrogen (Weurman, 1969) or carbon dioxide atmospheres (Bemelmans, 1981) have also been suggested for inhibiting enzymatic activity that may occur during extraction.

Thermal processes may also be employed for enzymatic inactivation of juice products in commercial practice which rapidly pass through a high temperature short-time heat exchanger (Lea, 1995). Poll and Flink (1983) and Poll (1988) used a microwave oven to pasteurise apple juice to an internal temperature of 90°C; juice was then sequentially cooled, and stored until required for analysis. Such thermal processes

can generate an additional range of 'process' volatile compounds, such as furfural, and hydroxy methyl furfural (HMF) from the Maillard reaction or sugar degradation (Lea, 1995; Poll, 1983) giving juice a more cooked, less fruit-like aroma (Poll, 1983; Poll and Flink, 1983). Another compound known to be induced by heating is β-damascenone, which is believed to be derived from xanthophylls; Roberts and Acree (1995) have suggested that it may be the most important odour-active volatile with a fruity note in processed apple products. Losses of some aroma volatile compounds can be expected during the heating process.

After extraction fruit juice samples are normally filtered through cheese cloth (Bemelmans, 1981) and immediately used for further extraction and analysis. However, in practice, samples may stand for some time before the actual extraction takes place. Changes due to enzymatic reactions and evaporative loss of the highly volatile compounds are inevitable during this standing time. Panasiuk *et al.* (1980) investigated the use of nitrogen atmosphere, dry ice, open and closed collecting containers, quick and slow freezing and thawing of 'McIntosh' apple juice in an attempt to minimise changes of aroma compounds. They found that juice collected in flask immersed in an ice bath, then frozen at -18°C and then thawed in a refrigerator for further analysis caused only a slight change in a gas chromatographic peaks compared with fresh juice.

Forms of fruit sample used, whether pulp or juice, may give different results in analysis of volatile compounds. Larsen and Poll (1990) compared analysis of juice and pulp of raspberry and strawberry and found higher amounts of C_6 aldehydes in the pulp fraction while the other volatile compounds were found to be comparable with those in serum (clear juice) fraction. However, they found that ionones, which are important aroma compounds in raspberry, could be extracted in much higher amount from pulp than from the serum. Radford *et al.* (1974) compared the distribution of volatile compounds between pulp and serum of oranges, apples, and mangoes; they found that esters and alcohols of oranges were found mainly in the serum, while hydrocarbons were mainly associated with pulp. In mangoes and apples, the majority of volatile compounds were in the serum. These studies suggested that the analysis of the apple aroma volatile compounds should be conducted on a clear, filtered juice sample for satisfactory analysis.

3.1.2 Extraction of Aroma Volatile Compounds

Procedures for extraction and concentration of aroma volatile compounds have been reviewed by several authors including Weurman (1969), Bemelmans (1979, 1981), Sugisawa (1981), Jennings (1981), Cronin (1982), Schreier (1984), Heath and Reineccius (1986), Maga (1990), Maarse (1991), and Teranishi and Kint (1993). Several techniques that have been employed are outlined in **Table 3.1**. The method used for isolation influence the resulting composition of the volatile compounds extracted (Fischer *et al.*, 1995; Kakiuchi *et al.*, 1986; Klein *et al.*, 1990; Larsen and Poll, 1990). The techniques for extraction which have been used increasingly in recent years are adsorption on porous polymers and simultaneous distillation; steam distillation extraction is generally avoided as it known to produce thermal decomposition artefacts (Rouseff and Leahy 1995; Schreier, 1984).

Table 3.1 Techniques for extraction and concentration of aroma volatile compounds (after Schreier, 1984; Sugisawa, 1981).

Distillation	Extraction	Others
CO_2 Distillation	CO_2 Extraction	Adsorption
Flash Distillation	-supercritical CO_2	-charcoal
-atmospheric	Solvent Extraction	-porous polymers
-reduced pressure	-simultaneous distillation-extraction	-Chromsorb®
-combination		-Porapak®
Steam Distillation		-Tenax®
-atmospheric pressure		-silica gel
-reduced pressure		-fused silica optic fibres
Vacuum Distillation		-others
-vacuum degassing		Gas Entrainment
-fractional distillation		-open
-high vacuum sublimation		-closed system
-molecular distillation		Freeze Concentration
		Lyophilization
		Zone Melting

3.1.2.1 Extraction of Aroma Compounds by Headspace Technique

The headspace technique is a logical choice for studying aroma compounds as it reveals the identity and concentration of compounds in the vapour phase that are directly responsible for the odour of the product under investigation. This method is rapid and efficient, and the likelihood of artefact formation is minimal (Maarse, 1991). In addition, this type of analysis may be made on intact fruit as well as homogenised or juice materials (Bartley *et al.*, 1985; Baldwin *et al.*, 1995; Forsyth and Webster, 1971; Ingham *et al.*, 1995; Larsen and Watkins, 1995b). The headspace procedure is of 2 main types, either static or dynamic headspace analysis.

***(a) The Static Headspace Procedure*:** This technique is probably the simplest method of obtaining the aroma volatile samples involving direct sampling of gas vapour over the product (usually referred to as 'headspace') for analysis. Samples, such as juice (mainly), fruit tissues or whole fruit, are placed in a gas-tight glass container, fitted with channelled rubber stoppers, and maintained in a constant temperature bath for a predetermined time. The headspace sample is then taken by a syringe and injected onto a GLC for analysis. Davis and Chace (1969) used the static headspace technique to determine ethanol concentration in orange juice; they found that the volume of headspace per volume of juice (ratio ranged from 5 to 600) in container, temperature of the bath (24° to 50°C) and headspace sampling after 1, 4 and 7 hours were not critical in determining the concentration. However, increasing bath temperature increased the detecting response in a curvilinear manner, thus it was necessary to maintain a constant bath temperature during the analysis of samples. This method has produced satisfactory results in some cases (Ingham, *et al.*, 1995). The problem is that some (or all) of the aroma compounds produced in very low amounts cannot be detected by GLC which may not be sensitive enough. The volatile compounds present in the headspace at concentrations exceeding 10^{-7} g·l^{-1} will be detected by GLC and only those exceeding 10^{-5} g·l^{-1} will be adequate for mass spectrometry (Schaefer, 1981a). In addition, the direct headspace injections are generally limited to 10 ml or less (Schaefer, 1981a; 1981b). It is also difficult and often impossible to relate concentration in the vapour

phase to that in the product, except in liquid samples (Maarse, 1991). Additional problems with this technique include condensation of volatile compounds inside the sample syringe, absorption in the septum of sampling jars, and irreproducibility of injection size due to syringe leaks or partial vacuum in the sample bottle (Heath and Reineccius, 1986). Furthermore, the headspace usually contains water vapour that can have a detrimental effect on the gas chromatographic stationary phase (Maga, 1990).

Despite the problems of the static headspace sampling, the technique has found substantial application in aroma volatile research of apples used by Sapers *et al.* (1977) and Panasiuk *et al.* (1980) on apple juice; Brown *et al.* (1966) and Bachmann (1983) on whole fruit; Guadagni *et al.* (1971) on apple peels and apple essence; Knee and Hatfield (1976) on apple parts; and Bartley and Stevens (1981), Bartley *et al.* (1985) and Knee and Hatfield (1981) on studies of the metabolism of some aroma volatile compounds in apples. The method is relatively simple, rapid and convenient in terms of sample preparation and analysis on a GLC. Thus, the static headspace may be the method of preference provided that analysis of trace amounts of volatile compounds is not of interest.

***(b) The Dynamic Headspace Procedure*:** For analysis of trace amounts of aroma volatile compounds, it is necessary to use a large volume of headspace which is subsequently concentrated. The most commonly used technique to obtain higher amounts of aroma compounds is the dynamic headspace or purge and trap technique (Maga, 1990). The principle of this procedure is that air or inert gas (N_2 or He) is flushed over or passed through the sample for a specified time. During this period of collection, a partial or total equilibrium of aroma compounds between the sample and the surrounding atmosphere occurs. The aroma compounds are then trapped from the atmosphere after passing over or through the sample (Bemelmans, 1981). The common ways of trapping are liquid (solvents), cryogenic, and solid phase adsorption on porous polymers, such as charcoal, and synthetic porous polymers (Schaefer, 1981a; 1981b). Cryogenic methods are based on the principle that vapour pressures diminish with decreasing temperature. Vapour is passed through a cryogenic trap that is kept at a temperature low enough to condense the volatile compounds, while N_2, O_2 and CO_2 pass

through. Ice formation is a major problem as it would eventually block the trap and limit the volume that can be sampled. Solvent trap methods are based on the fact that most volatiles are dissolved in organic solvents. The vapour is passed through the solvent trap, which generally operates at room temperature, and volatiles are accumulated in the solvent (Bemelmans, 1981). Several improvements of the technique over the years, either on the procedure or the trapping, have been made. A recent development to improve the extraction of aroma compounds in aqueous samples with this technique has been proposed by Rivier *et al.*(1990) using the so-called 'falling film sampler'. The technique is basically a dynamic headspace method using adsorbent for trapping volatiles with a modification in the sample introduction procedure. The liquid sample is introduced into the top of a vertical (falling film) tube with the aid of a constant flow N_2 gas. The sample falls down in a film of 0.2 - 0.3 mm thickness along the side of the tube into a bottle fitted with an adsorbent trap. Vapour of the falling sample is trapped and the excess sample is collected at the bottom of the bottle which is re-introduced back to the falling film tube for repeating extraction. This technique has been found to increase the extraction and recovery of highly polar compounds such as volatile aliphatic acids.

The purge and trap technique has been used extensively for investigating aroma volatile compounds in apple juice (Poll, 1988; Poll and Hansen, 1990) and apple fruit during ripening and storage (Fellman and Mattheis, 1995; Hansen *et al.*, 1992a, 1992b; Mattheis *et al.*, 1995; Vanoli *et al.*, 1995). Various types of adsorbent are available and those commonly used are activated charcoal (Oliás *et al.*, 1995; Rizzolo *et al.*, 1989; Vanoli *et al.*, 1995), Porapak® (Brockhoff *et al.*, 1993; Hansen *et al.*, 1992a, 1992b), Tenax® (Boylston *et al.*, 1994; di Pentima *et al.*, 1995; Fellman and Mattheis, 1995; Fellman *et al.*, 1993a), and Chromsorb® (Murray and Whitfield, 1975). The major advantages of these porous polymers are their ability to retain most organic compounds even at ambient temperatures and their general lack of affinity to water. In addition, they have low susceptibility to both oxidative and thermal decomposition (Maga, 1990; Schreier, 1984). However, some of these polymers show a low affinity for low molecular weight alcohols and some other organic compounds, so that low proportions of these compounds are adsorbed and a large proportion 'breaking through' the trap (Weurman, 1969).

Various types of adsorbent, which are normally used in the aroma research, have been studied and their physical properties, specific absorption and desorption characteristics compared (Buckholz *et al.*, 1980; Cole, 1980; Jennings and Filsoof, 1977; Schaefer, 1981a; 1981b; Williams *et al.*, 1978). Activated carbon strongly absorbs non-polar substances, yet only weakly adsorbs water vapour and it has a very large adsorption capacity far exceeding that of synthetic polymers (Maga, 1990). Tenax® and Porapak® give superior recovery of adsorbed volatile compounds and are relatively stable at high temperature compared with Chromsorb®. However, losses of highly volatile compounds through the trap (Buckholz *et al.*, 1980) and poor retention of low molecular weight alcohols have been reported (Jennings and Filsoof, 1977). High boiling point compounds (**Appendix 2, Section 2.1**) have also been found to show poor recovery on both Tenax® and Porapak® traps, thought to be due to the low volatility of those compounds (Maga, 1990).

The major advantages of the dynamic headspace technique are that the apparatus used is simple, the adsorbed volatile compounds can be stored prior to analysis, and they can be directly transported in a stable form which makes on-site sampling easy. The adsorbed volatile compounds can be desorbed easily either by heat or by a solvent. However, the major concern about this technique is that the human nose characterises headspace aroma in an equilibrium state. When large volumes of a gas are passed through a headspace, such equilibrium conditions are no longer present. Therefore, the composite volatiles trapped in one of the adsorbent columns may not necessary reflect the true aroma of the sample (Bemelmans, 1981; Maga, 1990).

3.1.2.1.1 Desorption and Concentration

The common techniques used for obtaining aroma volatile compounds from adsorption traps are thermal desorption, washing with an organic solvent, and simultaneous distillation and adsorption (Schreier, 1984; Schultz *et al.*, 1967; 1977; Sugisawa, 1981). A detailed study of the methods used for desorption from the adsorbent charcoal has been reported by Sugisawa and Hirose (1981). The simultaneous distillation and adsorption method has been developed for small quantities (up to 1 ppm) of volatile compounds by Schultz *et al.* (1977).

(a) Thermal Desorption : The thermal desorption technique has been widely employed in apple aroma research. This method is usually used in conjunction with cryofocusing (Fellman *et al.*, 1993a; 1993b; Mattheis *et al.*, 1995). The principle of this technique is that the adsorption trap is quickly heated to 150° - 200°C within 10 - 20 seconds (either by electricity, microwave, or laser), the desorbed volatile compounds are transferred onto a gas chromatographic column through an injection needle by flushing with a carrier gas via a 3-way valve. Alternatively, volatile compounds are condensed in a 'cryogenic trap' or cold trap, which is achieved by the use of liquid nitrogen, while the bulk of the carrier gas being N_2, O_2, or CO_2, passes through the cold trap. After that the trap is heated and the compounds are transferred onto a gas chromatographic column for separation (Farwell *et al.*, 1979). This technique minimises the possibility of chemical reaction between individual compounds.

***(b) The Solvent Desorption Technique*:** This technique involves the use of a small volume of solvent added to the trap which is then pressurised with an inert gas (N_2 or He) to elute the solvent for analysis (Cole, 1980). A concentration step may be required if a larger amount of solvent is used to elute volatile compounds from the trap. Concentration of the eluent can be done by distillation (Buttery *et al.*, 1987), or evaporation using a stream of inert gas blowing onto the surface of the eluent (Boylston *et al.*, 1994; Larsen and Watkins, 1995a). The major disadvantages of this technique are loss of low boiling point compounds due to evaporation, presence of contaminants from solvent impurities, and the presence of a large solvent peak on the GLC which may obscure some rapidly eluting compounds (Sugisawa and Hirose, 1981).

Solvents that are generally used include diethyl ether (Hansen *et al.*, 1992a, 1992b), n-pentane, a combination of ether and pentane (Larsen and Watkins, 1995a; Schultz *et al.*, 1977), carbon disulfide (Oliás *et al.*, 1995), dichloromethane (Vanoli *et al.*, 1995), methylene chloride (Rizzolo *et al.*, 1989), and hexane (Boylston *et al*, 1994).

A recent innovation for studying aroma compounds has been developed by Arthur and Pawliszyn (1990); it involves microextraction of volatiles onto fused silica optic fibres with subsequent thermal desorption - so-called 'solid phase microextraction' (SPME). This technique was claimed to offer several advantages over the conventional

solid phase extraction technique, as solvents are not required for desorption of volatiles and extraction time can be reduced to a few minutes compared with the conventional purge and trap method which generally required more than 30 min of extraction (Sugisawa and Hirose, 1981). This technique has been used to extract aroma compounds in several products, including fruit juice, by Yang and Peppard (1994) who discussed the advantages and limitations of the technique. The advantages over the conventional purge and trap technique are simplicity, short time requirement (2 - 10 minutes) for extraction and no solvent use. In addition, it can apply to aroma analysis of solid, liquid, or gaseous samples, particularly for quick screening of volatile composition. However, SPME is a single-batch process, so that quantitative adsorption is often very difficult and results obtained using this technique strongly depend on experimental conditions and sample matrix. Any changes in experimental conditions that affect the adsorption distribution will be reflected in the sensitivity and reproducibility of the analytical method. An external calibration method for SPME is generally not suitable for quantification, because a synthetic matrix can hardly match that of an authentic sample (Yang and Peppard, 1994).

3.1.2.2 Extraction of Aroma Compounds Using Extraction Technique

Aroma volatile compounds can be effectively extracted because of differences in solubility between compounds in the sample and the specific individual solvent (Maga, 1990). This method is based on favourable distribution coefficients of the volatile compounds between the solvent and the aqueous sample (Bemelmans, 1981). This technique of extraction may be either as a direct liquid-liquid extraction or as a combined distillation technique, such as continuous liquid-liquid extraction. The method is limited to use on homogenised and aqueous materials only. During the extraction process, an equilibrium is obtained between concentrations of aroma compounds in the solvent and the aqueous phase. The simplest technique is by shaking the sample solution in a water-solvent system with the solvent being separated from water either by a separation funnel, freezing, rotary evaporator, or simultaneous liquid extraction techniques. This method can separate a large number of important aroma compounds from a sample (Maga, 1990).

Most organic solvents used for this type of extraction are non-polar, as the majority of the aroma volatile compounds are relatively non-polar (Weurman, 1969). A number of investigations on volatile compounds of apples and apple products have been carried out using solvent extraction either with individual solvents or a combination of solvents (Berger *et al*, 1992; Larsen and Poll, 1990; Schreier *et al.*, 1978a; 1978b; Schultz *et al.*, 1967). Solvents are normally selected on the basis of their extraction capacity, selectivity and boiling point and solvents which are generally employed for the aroma research have been listed in detail by Weurman (1969).

The major problem with solvent extraction involves the presence of solvent impurities in concentrated samples. Impurities may be byproducts of solvent synthesis or antioxidants added to prevent peroxide formation in the solvents such as diethyl ether. Although there are several methods to remove impurities from solvents, a solvent blank in the form that is suitable for analysis, ie. after concentration, should always be run to determine the effect of impurities (Heath and Reineccius, 1986; Martin and Nishijima, 1977). An additional problem is that compounds which may interfere with aroma analysis, such as lipids, waxes, carotenoids, and chlorophylls, may also be removed during extraction. Furthermore, most solvents used have boiling points near those of low molecular weight volatile compounds, and therefore partial loss of separated volatile compounds during the removal of excess solvent still remains a problem. (Bemelmans, 1981; Maarse, 1991; Maga, 1990; Schreier, 1984; Sugisawa, 1981).

Traditionally, the solvent used was in a liquid state. However, recent developments have involved compound extraction using liquefied gases such as carbon dioxide, freon, butane, and supercritical carbon dioxide (Maarse, 1991; Maga, 1990). The application of liquid carbon dioxide for apple aroma extraction has been reported by Schultz *et al.* (1967). They found that it gave an extract analytically similar to that obtained by ether.

The supercritical carbon dioxide method has been used increasingly for extraction because it has moderate critical parameters (7.38 MPa and 31.04°C) and high volatility; it is chemically inactive, non inflammable, easily available and relatively inexpensive; it produces no toxic waste and does not leave contaminating residues (Hawthorne *et al.*, 1988). Substances with high solubility in carbon dioxide can be separated at low

temperature and pressure, ca. 5 - 12 MPa and 0° - 60°C. (Kerrola, 1995; Moyler, 1986). Applications of supercritical carbon dioxide to volatile research on plant products have been reported by Hawthorne *et al.* (1988), Morin *et al.* (1987) and Nykänen *et al.* (1990) and applications to aroma volatile research have been reviewed by Flament *et al.* (1987), Kerrola (1995), Krukonis (1985) and Maarse (1991). This method is very efficient because of its low viscosity and high diffusivity; there is also less likely to be artefacts formed as cold temperatures are used during extraction. However, the major limitation is that highly sophisticated apparatus capable of withstanding very high pressure is necessary (Maga, 1990; Maarse, 1991; Morin *et al.*, 1987).

3.1.2.2.1 Solvent Selection

Several factors are involved in selecting a solvent for extraction of aroma compounds from a sample. The initial consideration is type of compounds of interest and the form of sample, whether it is solid, liquid or gas, to be extracted. The solvent used should extract the major part of all aroma compounds and it should be easily separated from water after extraction. During the concentration process, it should be reduced easily without losing the aroma compounds (Bemelmans, 1981; Schultz *et al.*, 1967; Williams and Tucknott, 1973).

The most commonly used solvent for extraction of aroma compounds in apples and apple products is diethyl ether (35°C b.p.) due to its high extraction capacity for both polar and non polar compounds, and its slightly polar property (Schreier, 1984; Schultz *et al.*, 1967; Somogyi *et al.*, 1964; Williams and Tucknott, 1973). Other non-polar solvents for extraction of distillate, essence, or juice are n-pentane (36°C b.p.), hexane (69°C b.p.), isopentane (28°C b.p.), fluorocarbons (various b.p.), dichloromethane (40.5°C b.p.) and methyl chloride(-24°C b.p.) (Cunningham *et al.*, 1986; Flath *et al.*, 1967; Horvat and Chapman, 1990; Schreier *et al.*, 1978a, 1978b; Schultz *et al.*, 1967; Williams and Tucknott, 1973; Yahia *et al.*, 1990a, 1990b). These non-polar solvents have been recommended for extraction of alcoholic samples and when methanol is used for enzyme inactivation of fresh samples, as alcohols have limited solubility in these solvents (Bemelmans, 1981; Weurman, 1969; Williams and Tucknott, 1973).

Based on their extraction properties, a mixture of solvents has been widely used to obtain the highest possible recovery of aroma compounds from a sample. Commonly used solvent mixtures in apple aroma research include diethyl ether and n-pentane of 2:1 v/v ratio (Larsen and Poll, 1990), diethyl ether and isopentane of 1:1 v/v ratio (Flath *et al.*, 1967), n-pentane and dichloromethane of 2:1 v/v ratio (Kollmannsberger and Berger, 1992; Schreier *et al.*, 1978a; 1978b), and n-pentane and methylene chloride of 2:1 v/v ratio (Berger and Drawert, 1984). Some differences in results were obtained among these solvents and/or their combinations; they are likely to be due to differences in experimental conditions and the capacity of solvents to extract certain volatiles. Just because there are differences does not mean that any of these mixtures is not as good as, or better than, individual solvents.

Solvents such as n-pentane and dichloromethane, including ethanol and n-propanol, may form azeotropic mixtures with water (Aylward and Findlay, 1966; Lide and Frederikse, 1995). Thus, water present in an extract obtained by use of these solvents may evaporate together with the solvent during the concentration process, as well as some volatile compounds. Most solvents used are highly volatile and flammable, and some solvents, such as dichloromethane, are suspected to be cancer causing agents (Lide and Frederikse, 1995). Therefore, diethyl ether and/or n-pentane are the preferred solvents for aroma volatile extraction.

3.1.2.2.2 Concentration of Solvent Extracts

After extraction, relatively large amounts of solvent are obtained especially with liquid-liquid extraction procedures. (Desorption of the headspace traps with solvents may also result in relatively large volumes of dilute aqueous solution.) To obtain a suitably concentrated extract for analysis, a concentration step is required. Several concentration methods have been used, including distillation, zone melting, freeze concentration, adsorption extraction, and evaporation (Bemelmans, 1979; 1981; Kepner *et al.*, 1969; Poll and Hansen, 1990; Sugisawa, 1981). The principle of concentration is based on the fact that most solvents are more volatile than aroma compounds, therefore, the solvents are more liable to evaporate than the aroma compounds. However, certain low molecular weight and very low boiling point volatile compounds (**Appendix 2.1**),

such as acetaldehyde (20.2°C b.p.), methyl acetate (57.3°C b.p.), methanol (64.6°C b.p.), ethyl acetate (77.2°C b.p.) and ethanol (78.52°C b.p.), have boiling points close to most solvents and are likely to be totally or partially lost during the concentration process (Bemelmans, 1979). Some of these compounds, however, can be easily analysed by the static headspace technique without using solvents.

The common concentration methods used in apple aroma research are boiling with slow evaporation using a Vigruex distillation or similar column (Buttery *et al.*, 1987; Schreier *et al.*, 1978a; 1978b), using a rotary evaporator (Cunningham *et al.*, 1986; Yahia *et al.*, 1990a; 1990b), or evaporating under a stream of oxygen-free nitrogen gas (Boylston *et al.*, 1994; Cole, 1980; Larsen and Poll, 1990; Poll and Hansen, 1990).

3.1.3 Separation and Identification of Aroma Compounds

Separation of volatile compounds, often present in trace amounts, is achieved mostly by using a high resolution GLC equipped with a glass capillary column, which is considered to be a pre-requisite (Heath and Reineccius, 1986; Jennings, 1981; Schreier, 1984; Teranishi, 1970). As aroma volatile compounds are composed of several chemical classes over a range of molecular weights (**Appendix 2, Section 2.1**), it may sometimes be appropriate to pre-separate the chemical compounds of interest in a sample prior to analysis. There are several pre-separation methods that can be applied, such as derivitization, separation according to molecular size, fractionation into chemical classes, and separation into acidic, basic, and neutral fractions (Schreier, 1984).

Identification of compounds is normally based on GLC retention times, and most often in combination with mass spectrometry (MS). The use of other spectroscopic techniques such as Gas Chromatography-Infrared Spectroscopy (GC-IR), Nuclear Magnetic Resonance Spectroscopy (NMR), and tandem mass spectrometry (MS-MS), are often employed for identification purposes. The combination of several techniques gives a more accurate identification of the compounds of interest than one technique alone (Maarse, 1991; Maga, 1990). Methods used for the separation and identification of the aroma volatile compounds have been published by a number of authors, including Busch and Kroha (1985), Cronin (1982), Flath (1981), Heath and Reineccius (1986),

Jennings (1981), Maarse (1991); Maarse and Belz (1981), Maga (1990), Mussinan (1993), Rothe (1988), Schreier (1984), Takeoka *et al.* (1985), Teranishi (1981) and Westendorf (1985).

Current instrumental techniques focus on the use of combined systems - so-called 'multi-hyphenated systems', which are usually fully automated. They are simpler, faster, and more efficient than traditional techniques (Maarse, 1991; Mussinan, 1993; Widmer, 1990). Examples of these multi-hyphenated systems include : High Resolution Gas Chromatography with Fourier Transform Infrared Spectrometry and Mass Spectrometry (HRGC-FTIR-MS); automated Purge and Trap Gas Chromatography with Mass Selective Detection and Flame Ionisation Detection (PT-GC-MSD-FID); coupling of ^{13}C Isotope Ratio Mass Spectrometry with Gas Chromatography and a Combustion interface (GC-C-IRMS) (Coleman III, 1992; Idstein and Schreier, 1985; Maarse, 1991; Martin, 1995; Morin *et al.*, 1987; Mosandl *et al.*, 1995; Widmer, 1990). Equipment for such analyses were not available in our laboratory; the main technique used for volatile analysis was GLC.

3.1.4 Quantification of Aroma Compounds

Results from the analysis of aroma compounds can be expressed as the amount isolated from a sample or the sample concentration during the time of analysis. When a static headspace method is used for determination of volatiles, concentrations of compounds present in the vapour and liquid phases during the process is normally determined. When dynamic headspace analysis of intact fruit is conducted, the results may be expressed as amounts of the aroma compounds produced during the period of collection. With most solvent extraction methods involving several extraction steps (assuming extraction of volatile compounds from a sample is believed to be complete), then the amounts present in the sample should be the same as the amounts present in the original starting material (Boelens and van Gemert, 1986).

To minimise variations due to extraction and chromatographic procedures, a known concentration of internal and/or external standard should be added to samples. The standards used are normally volatile compounds not naturally occurring in the sample and having retention times within the range of the aroma compounds of interest.

If there is a wide range between low and high molecular weight compounds being determined, more than one internal standard may be required. The internal standard is normally added to the sample prior to the extraction process, thus it allows variations in efficiency of the extraction process for aroma compounds to be taken into account (Larsen and Watkins, 1995a; Poll and Hansen, 1990). External standards are utilised to minimise variations due to the gas chromatographic conditions. Authentic chemical compounds of known concentrations that are analysed along with a batch of samples may be used as external standards for quantification. The external standard may also be added to the concentrated sample or onto the headspace adsorbent traps before desorption of the aroma compounds from the trap. (Mattheis *et al.*, 1995; Oliás *et al.*, 1995; Poll and Hansen, 1990). Calculations are then made relative to the internal and/or external standards used, which are normally satisfactory for comparison within a study. However, if a comparison between studies is to be made, then knowledge of differences in the detection response for the volatile compounds, the standards, and several other conditions involved is required (Poll and Hansen, 1990).

3.2 OBJECTIVE

The objectives of the present study were :

1.) to determine the GLC detection response to a concentration range of octyl acetate, an internal standard,

2.) to investigate some factors influencing recovery of aroma volatiles of apple juice obtained using the dynamic headspace (with Tenax® adsorbent traps) or direct solvent (diethyl ether and n-pentane, 2:1 ratio v/v, mixture) extraction methods,

3.) to compare efficiency of extraction between the dynamic headspace and direct solvent extraction techniques on the recovery of some aroma volatile compounds in apple juice.

3.3 MATERIALS AND METHODS

Experiments **3.3.1** to **3.3.7** were conducted during 14 - 16 October 1995 at 20°C and 70% RH. Cold stored 'Red Delicious' apples (0.5° ± 0.5°C, 90 - 92% RH, stored since April 1995) were used. Experiment **3.3.8** was conducted on 28 September 1994 on CA stored 'Fuji' apples (20 weeks after harvest, 2% CO_2 + 2% O_2, 0.5° ± 0.5°C, 92 - 95% RH).

In order to evaluate the methods used for analysis of aroma compounds in apple juice in this study, the following experiments were carried out:

3.3.1 Test of Linear Responses of Internal Standards

The aim of this test was to determine whether the concentrations of octyl acetate internal standard used had a linear relationship with the gas chromatographic detection responses.

A series of low and high concentration ranges of pure octyl acetate were prepared in a mixture of diethyl ether and n-pentane of 2:1 v/v ratio. The low concentration range was 0 - 600 ppm with a 50 ppm interval, and the high range was 0 - 5500 ppm with 500 ppm interval. There were 3 replicates for each concentration at both the low and high ranges. Samples (1 µl) from each concentration range were randomly injected onto a Hewlett Packard GLC and the detection responses (peak area) were plotted against the concentrations of octyl acetate used. Regression analysis of data was performed using the SAS package.

3.3.2 Test of Breakthrough of Volatile Compounds Through Tenax® Trap

The purpose of this test was to qualitatively examine the retention capacity of the Tenax® adsorbent trap for apple volatile compounds.

The synthetic porous polymer Tenax®-GR (2,6-diphenyl-p-phenylene oxide with 30% co-precipitated graphitised carbon, Alltech) of 250 mg per trap was prepared and pre-conditioned as previously described (**Chapter Two, Section *2.4.2.1***). Three Tenax® traps were employed in a series by connecting the traps together with small pieces of

silicone rubber tube. One hundred ml of freshly extracted and filtered 'Red Delicious' apple juice was transferred into a Dreschel gas washing bottle equipped with a glass sintered head. Approximately 0.5 g of sodium chloride salt and 10 µl of 25000 ppm octyl acetate in a diethyl ether and n-pentane mixture (2:1 v/v ratio) internal standard were added to the juice. The trap series was connected to the outlet of the bottle with a piece of silicone rubber tube (**Fig. 3.1**). and it was replicated 3 times. The juice was purged with oxygen-free nitrogen gas (NZIG®) from a compressed gas cylinder with a flow rate of 25 ml·min^{-1} for 2.5 hours at 20°C. The volatile compounds were desorbed from each trap with approximately 4 ml of diethyl ether and n-pentane mixture, and the eluent concentrated with a slow flow of oxygen-free nitrogen gas stream to 250 µl; 1 µl was used for analysis on a GLC.

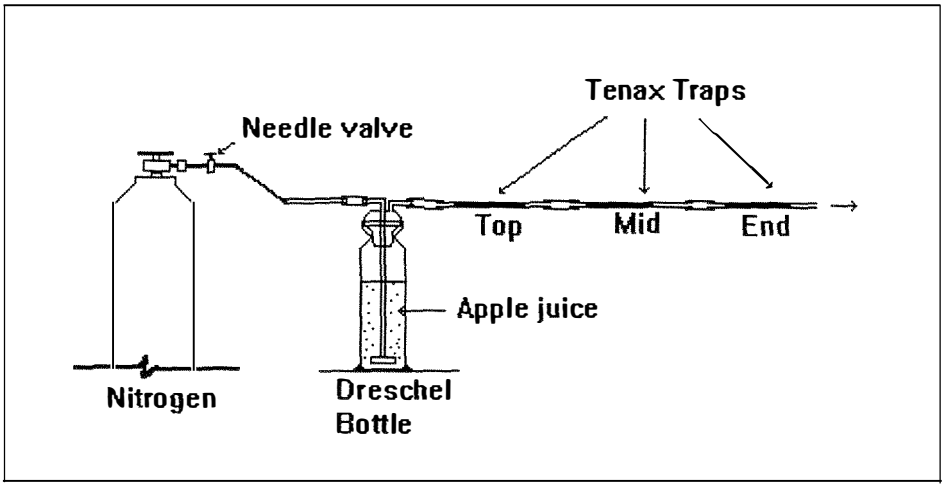


Fig. 3.1 Diagram illustrating the collection of volatile compounds in 'Red Delicious' apple juice by the purge and trap technique with 3 Tenax® traps in series.

3.3.3 Test of Washing Time of Tenax® Trap

The purpose of this experiment was to investigate the number of times that the Tenax® trap had to be washed with solvent to remove all the volatile compounds from the adsorbent.

Sample preparation and purge and trap procedures were as described in **3.3.2** above, except that only one trap per sample was used, and there were 3 replicates. The volatile compounds were washed off the Tenax® trap with approximately 1.5 ml of diethyl ether and n-pentane mixture at a time. Each trap was washed 3 times and the eluent of each wash was collected separately. The 3 eluent samples obtained from each trap was concentrated to 0.25 ml and 1 µl was analysed by GLC.

3.3.4 A Comparison on the Duration of Trapping

The objective of this experiment was to study the effect of the duration used for the purge and trap process on the concentration of some aroma volatile compounds extracted from apple juice.

In order to obtain a batch of approximately 1200 ml apple juice, several 'Red Delicious' apples were homogenised with a kitchen type centrifuge juicer. The juice was collected in a beaker immersed in an ice bath. All treatments and replicates used this batch of juice. A 50 ml aliquot of freshly extracted and filtered juice was used, about 0.25 g NaCl salt and 10 µl of 25000 ppm octyl acetate internal standard were added. The purge and trap durations were 0.5, 1, 2, 4, and 6 h. with 4 replicates each. The aroma volatile compounds were desorbed from the Tenax® trap with diethyl ether and n-pentane mixture, concentrated and analysed on a GLC as described in **3.3.2**. Blank samples were also prepared by using an equivalent amount, ie. about 4 ml, of the diethyl ether and n-pentane mixture with added octyl acetate internal standard (10 µl), concentrated to the volume (250 µl) and analysed for contaminants. Quantification was performed by comparing the values obtained against the authentic mixture of external standard chemical compounds and normalising against the octyl acetate internal standard. Results were expressed as $\mu mol \cdot l^{-1}$. Results for individual volatile compounds were subjected to analysis of variance using the SAS package.

3.3.5 Comparison of the Dynamic Headspace and Direct Solvent Extraction Procedures

The aim of this experiment was to compare the recovery of some aroma volatile compounds isolated from apple juice obtained using the dynamic headspace (the purge and trap) and direct solvent extraction techniques.

A batch of approximately 600 ml of apple juice was prepared as described above (**3.3.4**). The juice was divided equally into 2 portions for extraction of aroma compounds with the purge and trap and solvent extraction procedures, respectively. A 20 ml aliquot of apple juice was used for both procedures and there were 12 replicates each. The purge and trap procedure used was the same as described in **3.3.2**, and it was run for a period of 2 hours. The direct solvent extraction procedure has already been described in detail (**Chapter Two**, ***Section 2.4.2.2***). Individual samples were concentrated, analysed and quantified as described above (**3.3.4**). The results of individual compounds recovered by using the two techniques were compared by a t-test procedure with the SAS package.

3.3.6 Test of Concentrated Volume of Extracts for GLC Analysis

The purpose of this test was to determine the volume of concentrated extracts needed to give satisfactory concentrations of aroma compounds of interest for detection.

The solvent samples, with 2 replicates each, were obtained from extracting 20 ml 'Red Delicious' apple juice using the direct solvent extraction technique described in **3.3.5**. The solvent extracts were concentrated to 0.25, 0.5, 0.75, and 1 ml in 10-ml graduated glass centrifuges tubes with a low-flow of nitrogen gas stream. The extracts (1 μl) were analysed by GLC.

3.3.7 Effect of Juice Volume Used for Extraction

The purpose of this experiment was to study the effect of varying volumes of juice sample with fixed volume (20 ml) of solvent mixture, as in **3.3.5**, used for extraction, on the recovery of some aroma compounds by the direct solvent extraction technique.

The experiment was designed as a completely randomised design with volume of juice sample used as treatment. Five different volumes of apple juice, ie. 5, 10, 20, 30, and 40 ml, were employed for the extraction process. There were 5 replicates, where aliquots were taken 5 times from the same batch of juice. Each sample had 10 µl of 2000 ppm octyl acetate internal standard added before being extracted with 20 ml of diethyl ether and n-pentane (2:1 v/v ratio) solvent mixture. Blank samples were also employed using 20 ml solvent mixture with added octyl acetate internal standard. Extracted and blank samples were concentrated, analysed, and quantified as previously described in **3.3.4**. Results of individual volatile compounds were statistically analysed using the SAS package.

3.3.8 Effect of Juice Holding Time and Conditions

The aim of this experiment was to study the effect of varying juice holding times under 2 conditions, at room temperature (20°C) or in an ice bath, prior to extraction of the aroma compounds from juice of CA-stored 'Fuji' apples.

The experiment was designed as a 2 x 5 factorial, with juice holding condition and holding time as main factors. A batch of approximately 1200 ml juice was prepared from CA-stored 'Fuji' apples on the 28 September 1994. The juice was equally divided into 2 portions, where one was left at room temperature, and the other was transferred into an Erlenmeyer flask immersed in an ice bath. Five 20-ml aliquot juice samples from each portion was taken (10 µl of 2000 ppm octyl acetate internal standard was added) for aroma compound extraction for each time interval after 0, 0.5, 1, 1.5, and 2 h. At 0 h, the sample was taken immediately after filtering the juice through cheese cloth. The volatile compounds were extracted using the direct solvent extraction procedure as previously described (**Chapter Two**, ***Section 2.4.2.2***). Extracts were concentrated, analysed, and quantified as described in **3.3.4**. Results of individual volatile compounds were statistically analysed using the SAS package.

The analysis of solvent samples from all trials were made on the Hewlett Packard HP5890 Series II Plus Gas Chromatograph equipped with a flame ionisation detector (FID). Parameter settings for gas chromatographic conditions were as previously

described (**Chapter Two**, ***Section 2.4.2.2***) and identification of the compounds was based on retention times.

3.4 RESULTS AND DISCUSSION

3.4.1 Test of Linear Responses of Internal Standards

The relationship between octyl acetate concentrations and gas chromatographic response (peak areas) was linearly positive and statistically significant for both the low ($P < 0.001$, $r^2 = 0.96$) and high ($P < 0.001$, $r^2 = 0.95$) concentration ranges (**Fig. 3.2**). These results indicated that the concentration range of octyl acetate in this test could be satisfactorily used as an internal standard for the study of aroma volatile compounds in apple juice. Variation in detector response increased with increasing concentrations in both the low and high concentration ranges. The high concentration range also showed a higher variability in the detector response where the coefficient of variation (c.v.) was 14.66% compare with 12.39% in the low range. Therefore, it is suggested that concentrations of octyl acetate higher than 4500 ppm should not be used as an internal standard. The lower concentration range would be more applicable since it shows less variation and better correlation with the detector responses.

The use of an internal standard is based on the assumption that volatile compounds of interest behave similarly, having linear detection responses in the same manner as the internal standard used. If they behave differently, more than one internal standard may be required (Buttery *et al*, 1987). Although octyl acetate concentrations are linearly correlated with detector response, erroneous results may still occur when using this compound as an internal standard. Endogenous volatile compounds may not be extracted from a sample in the same ratio as the internal standard and some compounds may have different slopes with detection response.

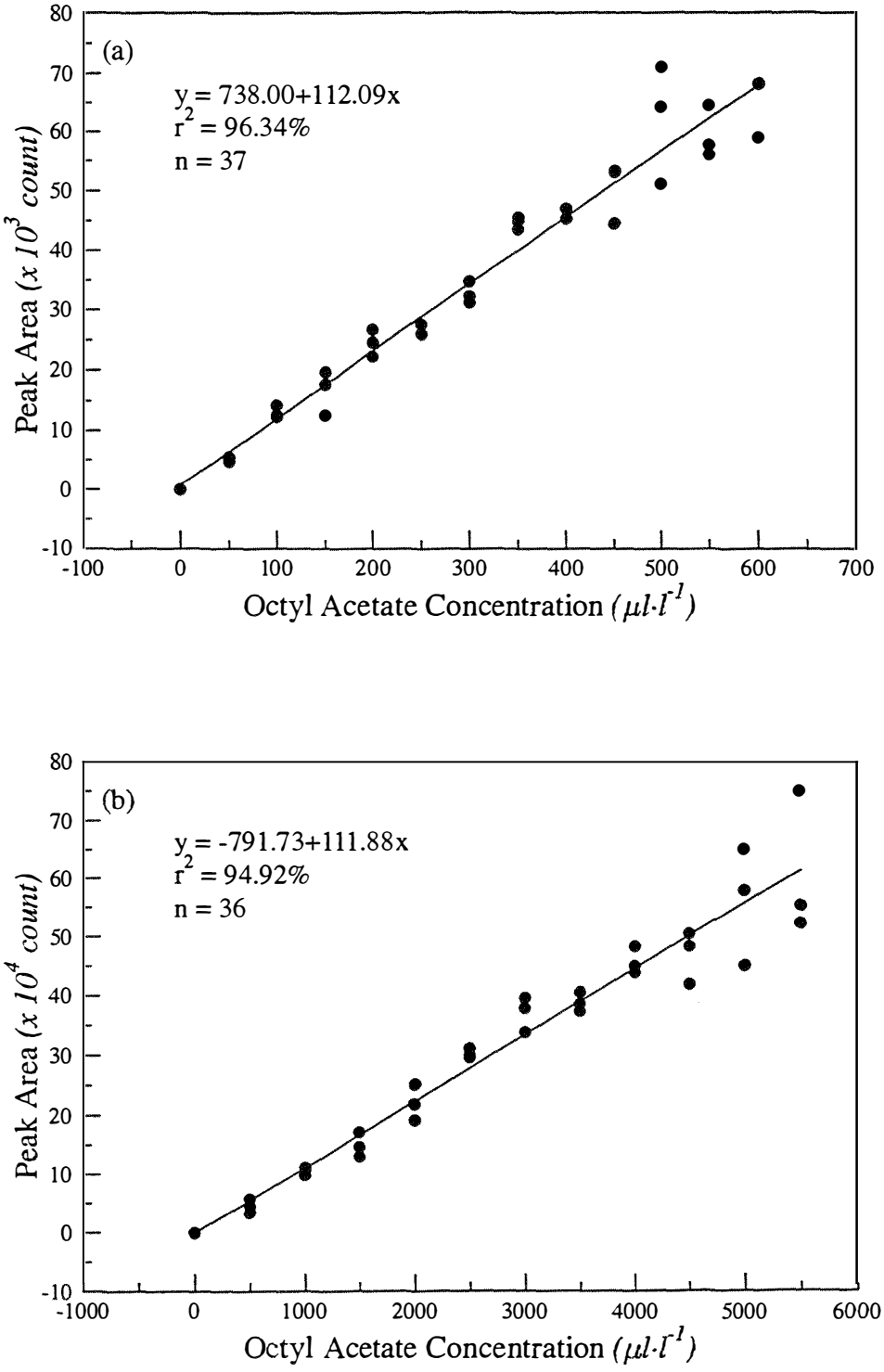


Fig. 3.2 Linear relationship between peak area and concentration of octyl acetate internal standard at (a) low and (b) high concentration range.

Schreier (1980) experimented with a model solution using 2 internal standards, and found that only those compounds that have the similar physical properties to the internal standards were in agreement. Guichard (1988) investigated the effect of adding known amounts of 12 authentic volatile compounds from apricot to a juice sample and compared this with an extraction technique using only one internal standard. The quantitative results indicated large differences between the two techniques, where some compounds were over-estimated when using only one internal standard in the extraction and quantification processes. Furthermore, Guichard (1988) reported that examination the variation among slopes of the linear response curves of different compounds, indicated differences among the extraction coefficients of those compounds. Most of the esters had slopes close to 1, meaning that they had a similar behaviour as the ester chosen as internal standard, while alcohols and ketones had slopes greater than 1, which suggested that the concentrations calculated using a single internal standard were greater than the actual concentrations (ie. over-estimated) calculated by adding known amounts of pure standards of the respective compounds (Guichard, 1988).

The adsorption of some volatile compounds on non-volatile compounds, such as sugars and protein, present in the sample may also cause erroneous results when using an internal standard for quantification. The retention can vary considerably depending on the polarity of the compounds. (Buttery *et al.*, 1987; Heath and Reineccius, 1986; Pangaborn and Szczesniak, 1974). Guichard (1988) found that aliphatic esters and ketones were less retained in slurries of apricots, while benzaldehyde, acetophene, alcohols, and lactones were more retained in the samples as they were more polar than the esters and ketones. Wientjes (1968) reported that quantities of alcohols determined in the headspace of an aqueous solution decreased with increasing fructose concentrations. Hexan-1-ol and propan-1-ol were reported to be retained on sugars and maltodextrines, thought to be due to hydrogen binding. In addition, octyl acetate has been reported to occur in apples naturally (Dimick and Hoskin, 1983); if this does occur in all cultivars tested, this could contribute to errors in quantifying volatile compounds in apple juice. However, juice samples without added octyl acetate internal standard were also extracted, concentrated and analysed; octyl acetate was not detected at the sensitivity of the GLC used for analysis in the present study.

3.4.2 Test of Breakthrough of Volatile Compounds Through Tenax® Trap

The majority of volatile compounds were retained on the first (Top) Tenax® GR traps (89.2%) through which the headspace flowed (**Table 3.2),** while relatively much smaller portions of 8.1% and 2.7% were collected in the second (Mid) and third (End) traps, respectively. Volatile compounds retained in the first trap ranged from low molecular weight and low boiling point, such as ethyl acetate (MW = 88, b.p. = 77°C) to relatively high molecular weight and high boiling point compounds, including octyl acetate (MW = 172.27, b.p. = 210°C). Volatile peaks detected from the first trap were relatively much taller than peaks obtained from the second and third traps, suggesting greater concentrations of volatiles being trapped in the first trap. On the other hand, fewer and relatively smaller peaks were obtained from the second (up to ethyl pentanoate, MW = 130.19, b.p. = 146°C) and the third traps (up to ethanol, MW = 46.07, b.p. = 78°C), compared with the first trap. The small amounts of volatile compounds found in the solvent concentrate (Blank) which were clustered near to the solvent peak were due to impurities (**Fig. 3.3**).

Table 3.2 Total peak area (excluding solvent and octyl acetate internal standard peaks) of volatile compounds recovered from a series of 3 Tenax® GR traps using a dynamic headspace extraction method of 'Red Delicious' apple juice.

Trap Position	Total Peak Area (count)	Per Cent[1]
Top	46334	89.2%
Mid	4212	8.1%
End	1424	2.7%

[1] percent of the grand total of total peak area of the 3 traps

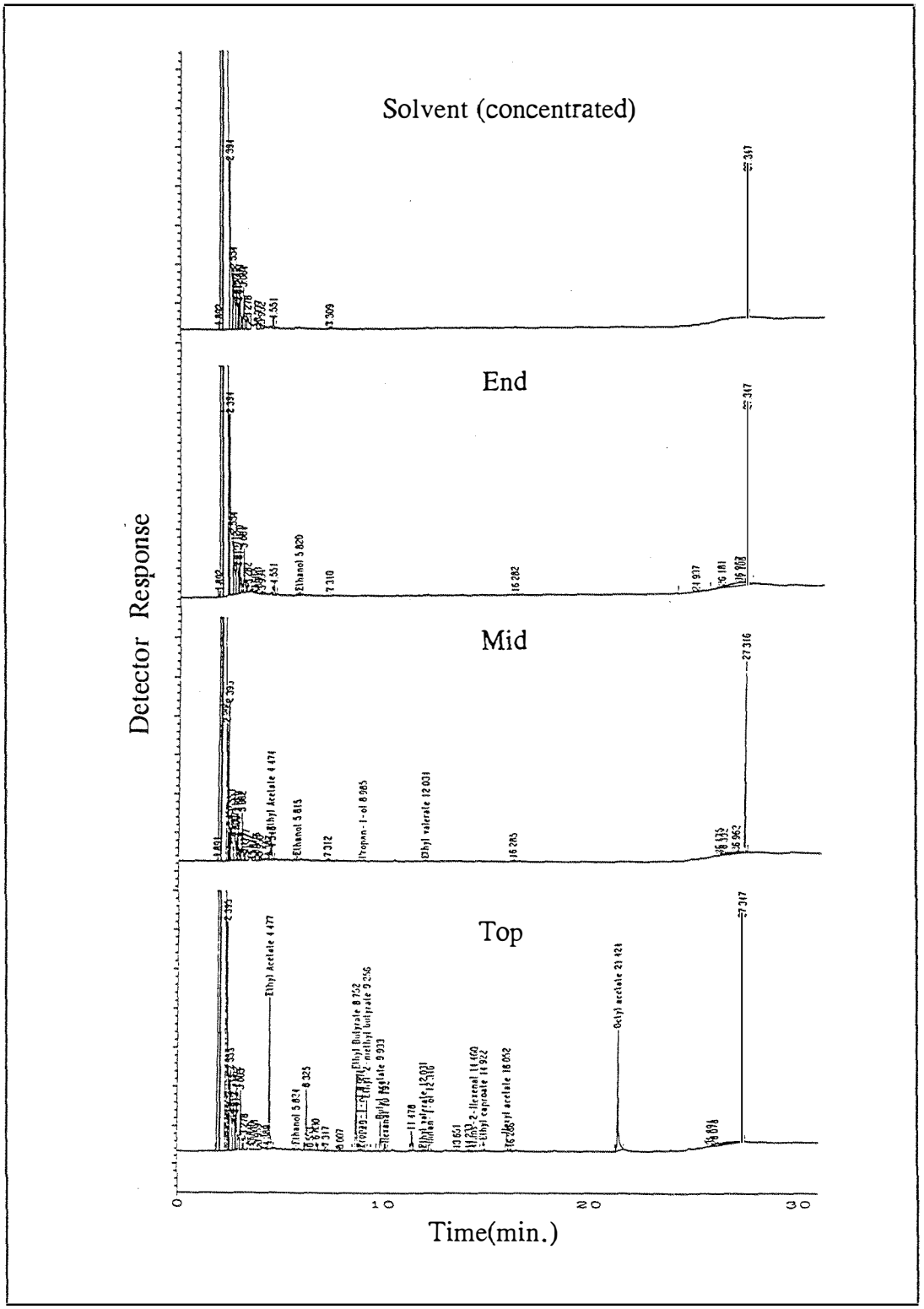


Fig. 3.3 Gas chromatographic profiles in relation to breakthrough of some volatile compounds through a series of 3 Tenax® GR traps using a dynamic headspace extraction of 'Red Delicious' apple juice (Top = 1st trap connected to the outlet of Dreschel gas washing bottle, Mid = 2nd trap connected to the 1st trap, and End = 3rd trap connected to the 2nd trap).

Buckholz *et al.* (1980) found that some low molecular weight peanut volatile compounds reached an early adsorption-desorption equilibrium and breakthrough into the second and third traps occurred after 15 minutes of collection. The adsorbent is a gas chromatographic packing material, therefore compounds with high volatility, by analogy with their gas chromatographic properties, have low-breakthrough volumes, ie. they reach the adsorption-desorption equilibrium quickly (Schaefer, 1981a; 1981b). This would lead to a comparatively low concentration of some of these low molecular weight compounds being retained by the adsorbent trap. The first trap collected most of the volatile compounds. Only small amounts of low molecular weight compounds reaching the second trap and almost none in the third trap after 2.5 hours of collection. In addition, the less volatile compounds, ie. those having molecular weight greater than hexyl acetate (MW = 144.12, retention time = 16.05 min) were poorly recovered as shown on the chromatogram where the peaks were very small and/or absent (**Fig. 3.3**). This is probably due to several factors such as type, quantity and dimension of the traps used, nitrogen gas flow rate and collection time (Buckholz *et al.*, 1980; Murray, 1977; Williams *et al.*, 1978).

The adsorbent used in this study was Tenax® GR which has a slightly higher retention for several low molecular weight compounds than other Tenax® adsorbents (Alltech, 1993). The dimension (diameter and length) of the trap column and quantity of adsorbent used is critical for reasonable recoveries of all volatile compounds. The smaller the quantity of adsorbent used the greater the chance of losing low boiling point compounds when collecting a large volume of headspace. The larger the diameter of the collection tubes, especially with small quantities of adsorbent, the greater the chance of the gas stream channelling, resulting in insufficient contact between the volatile compounds and the polymer (Williams *et al.*, 1978). The amount of Tenax® used in this study was 250 mg per trap and it was prepared in a glass tube of 3 mm (Ø i.d.) x 300 mm which gave the trap length of approximately 10 cm in the tube. This ensured that more than enough adsorbent was present for the volatile compounds to be collected without saturation or without being displaced by one having greater affinity for the polymer (Schaefer, 1981a; 1981b). Breakthrough of the low molecular weight volatile compounds has been reported following use of small quantities of adsorbent (30 - 40

mg) with the trap diameters ranging from 2.5 to 6.0 mm (Buckholz *et al.*, 1980; Murray, 1977; Williams *et al.*, 1978).

As traps were connected in series, this created a fairly high resistance to gas flow due to high back pressure during collection of aroma compounds. This led to diminished flow rate of the nitrogen gas passing through the juice sample and thus reduced the volume of volatile compounds collected. Hence, some compounds, which were produced in low concentrations and/or high molecular weight compounds (**Appendix 2, Section 2.1**), may not be detected on a GLC because of the low amount of volatiles being trapped. The quantities of volatile compounds recovered was reported to be related directly to the volume of headspace gas collected, especially with alcohols, and the rate of transfer of the volatiles to the vapour phase for high boiling point compounds (Williams *et al.*, 1978). The low flow rate purge and the increased duration of collection was reported to increase recovery of high boiling point compounds (Buckholz *et al.*, 1980).

3.4.3 Test of Washing Time of Tenax® Trap

Most of the volatile compounds were eluted off with the first wash of approximately 1.5 ml of diethyl ether and n-pentane (2:1 v/v ratio) solvent mixture (**Table 3.3**). Only small amounts were eluted in the second wash and almost none were found in the third wash. Peaks of the highly volatile compounds in the third wash was probably due to solvent impurities (**Fig. 3.4**).

Table 3.3 Total peak area (excluding solvent and octyl acetate internal standard peaks) of volatile compounds recovered from the first, second and third wash with diethyl ether and n-pentane solvent mixture of Tenax® GR trap using a dynamic headspace extraction method of 'Red Delicious' apple juice.

Wash Time	Total Peak Area (count)	Per Cent[1]
first	126842	95.2%
second	4842	3.6%
third	1558	1.2%

[1] per cent of the grand total of total peak area of the three traps

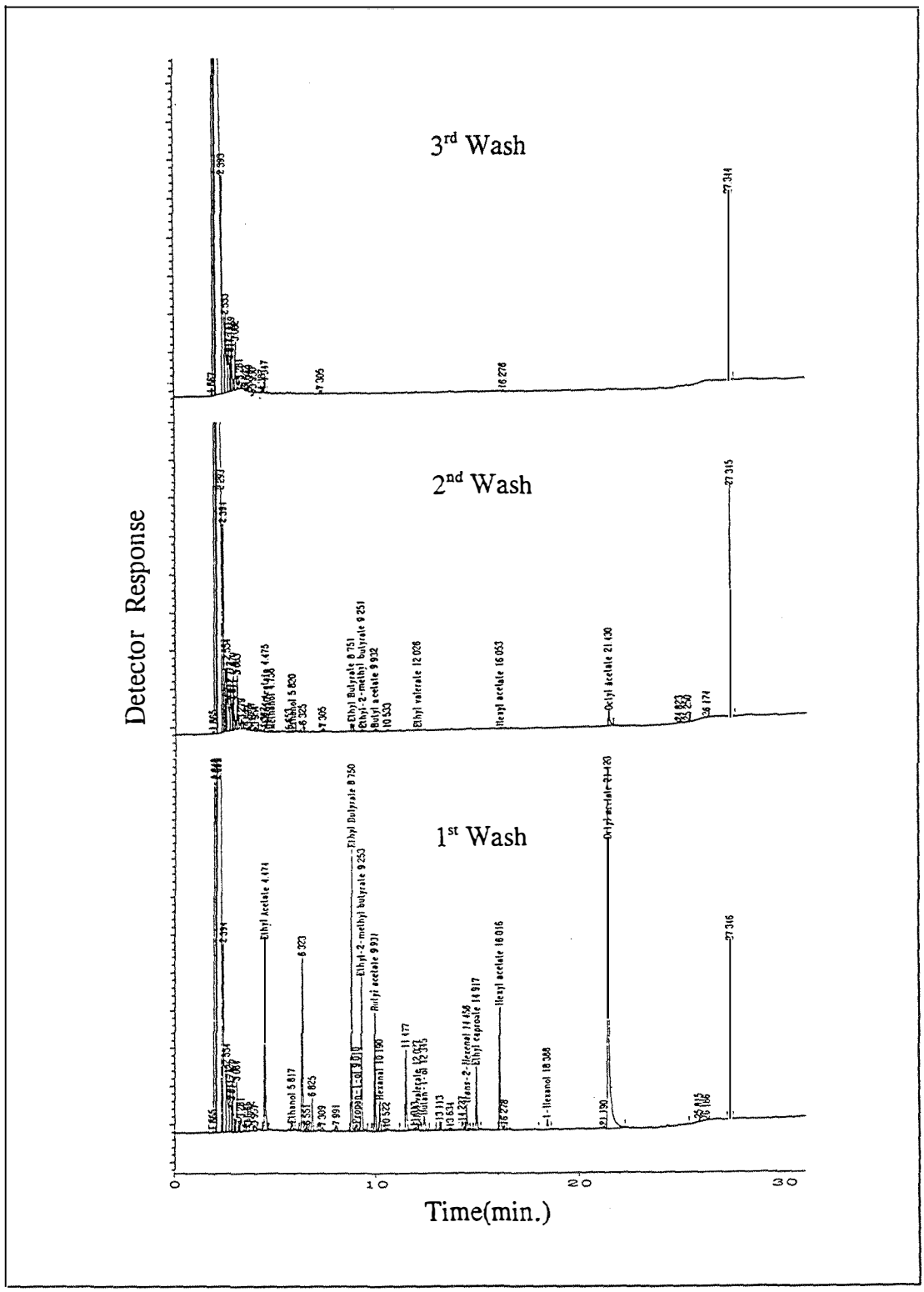


Fig. 3.4 Gas chromatographic profiles of 3 consecutive washes of a Tenax® GR trap with diethyl ether and n-pentane solvent mixture for desorption of volatile compounds of 'Red Delicious' apple juice.

These results suggest that two 1.5-ml washes with the solvent mixture are sufficient for eluting more than 98% of volatile compounds from the Tenax® trap. This means that solvent volume is minimised as is the time required for the desorption and concentration processes. Thus, loss of lower molecular weight compounds due to evaporation during the concentration process and the introduction of impurities from the solvent used could also be reduced using this recommended process (Cole, 1980).

3.4.4 A Comparison on the Duration of Trapping

In general, maximum recovery of all compounds tested occurred after 1 h trapping time (**Fig. 3.5** to **Fig. 3.8**) while minimum recovery occurred after 0.5 h trapping. However, 4 general patterns of recovery occurred for the different compounds in relation to trapping time.

In the first group, comprising propan-1-ol (**Fig. 3.5**c), ethyl hexanoate (**Fig. 3.6**c), and hexyl acetate (**Fig. 3.7**c) there was little difference among relative amounts recovered with increased trapping time. In the second group, the relative amounts recovered were significantly highest after 1 h of trapping time with the longer trapping time being the same as the 0.5 h trapping; this group includes ethyl acetate (**Fig. 3.6**a), ethyl 2-methyl butanoate (**Fig. 3.7**a), and ethanol (**Fig. 3.5**a). The third group comprising, butan-1-ol (**Fig. 3.5**b), ethyl butanoate (**Fig. 3.6**b), butyl acetate (**Fig. 3.7**b), and hexanal (**Fig. 3.8**b) had low recoveries after 0.5 h of trapping, reached a peak at 1 hour and was more or less constant thereafter. The final group which had an increase in the relative amounts recovered with increasing time of trapping involved hexan-1-ol (**Fig. 3.8**a) and *trans*-2-hexenal (**Fig. 3.8**c).

The low recovery of most volatile compounds following the shortest trapping time probably occurred because those compounds had not reached the adsorption-desorption equilibrium in the adsorbent trap (Buckholz *et al.*, 1980; Schaefer, 1981a; 1981b). Sugisawa (1981) reported that trapping of apple volatile compounds required at least 60 minutes. Compounds such as ethyl hexanoate, hexyl acetate and propan-1-ol (group 1) may have high rate of transfer to the vapour phase (Williams *et al.*, 1978)

and/or are less retained in the juice sample (Guichard, 1988), thus they reach the particular adsorption-desorption equilibrium quickly after 0.5 h of trapping.

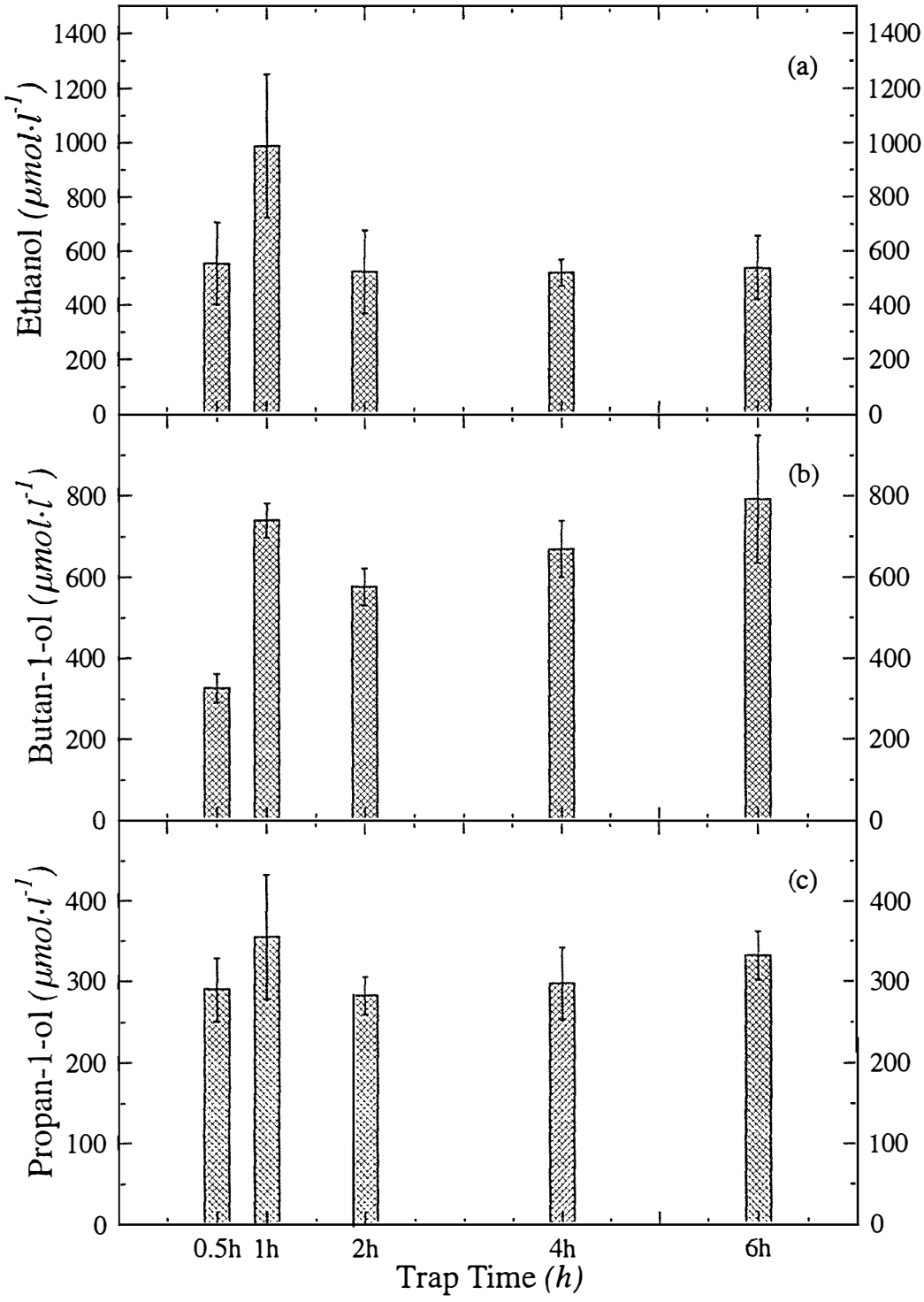


Fig. 3.5 Concentration of (a) ethanol, (b) butan-1-ol and (c) propan-1-ol of 'Red Delicious' apple juice at different trapping time on Tenax® GR at 20°C using a dynamic headspace method.

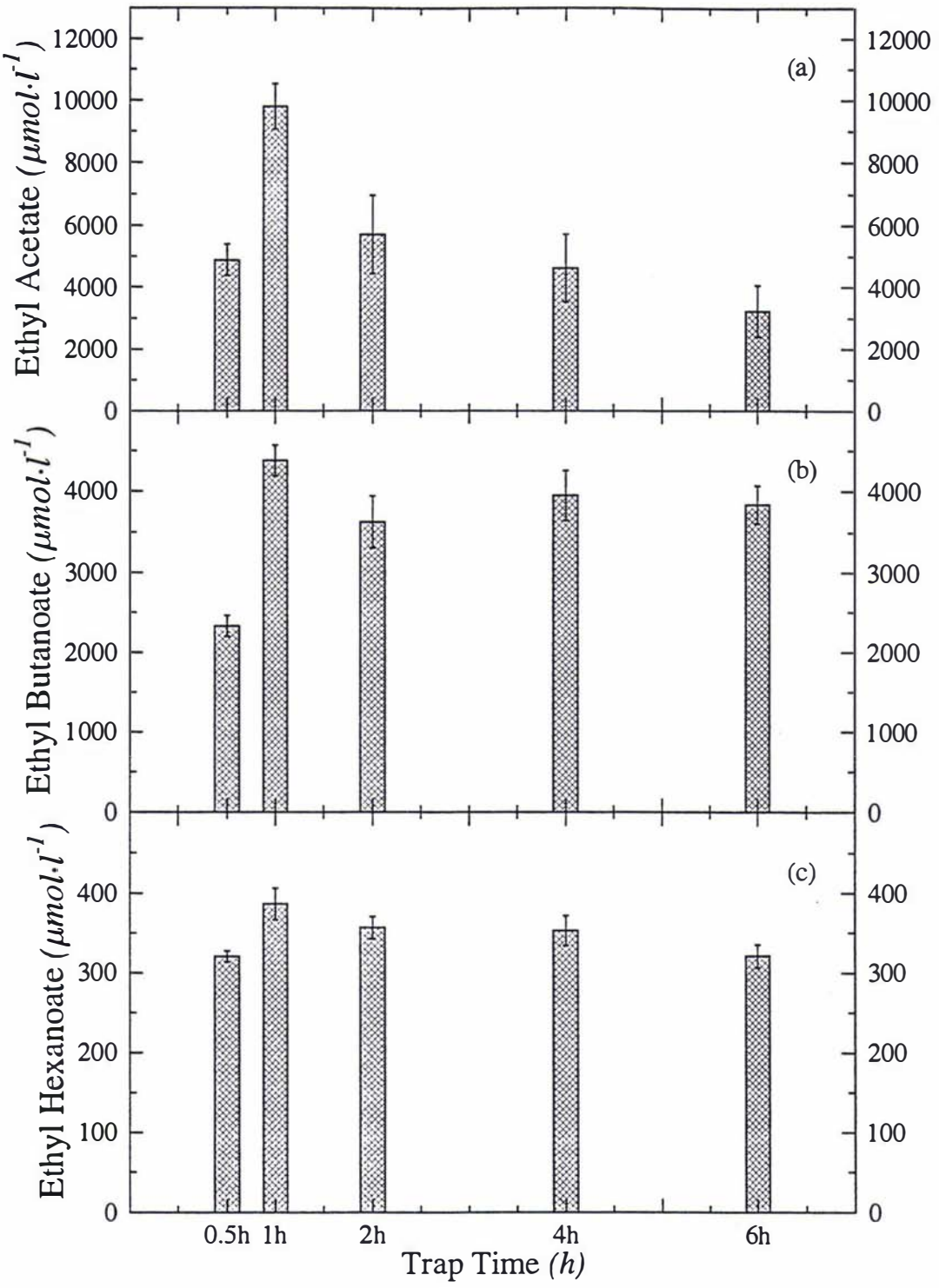


Fig. 3.6 Concentration of (a) ethyl acetate, (b) ethyl butanoate and (c) ethyl hexanoate of 'Red Delicious' apple juice at different trapping time on Tenax® GR at 20°C using a dynamic headspace method.

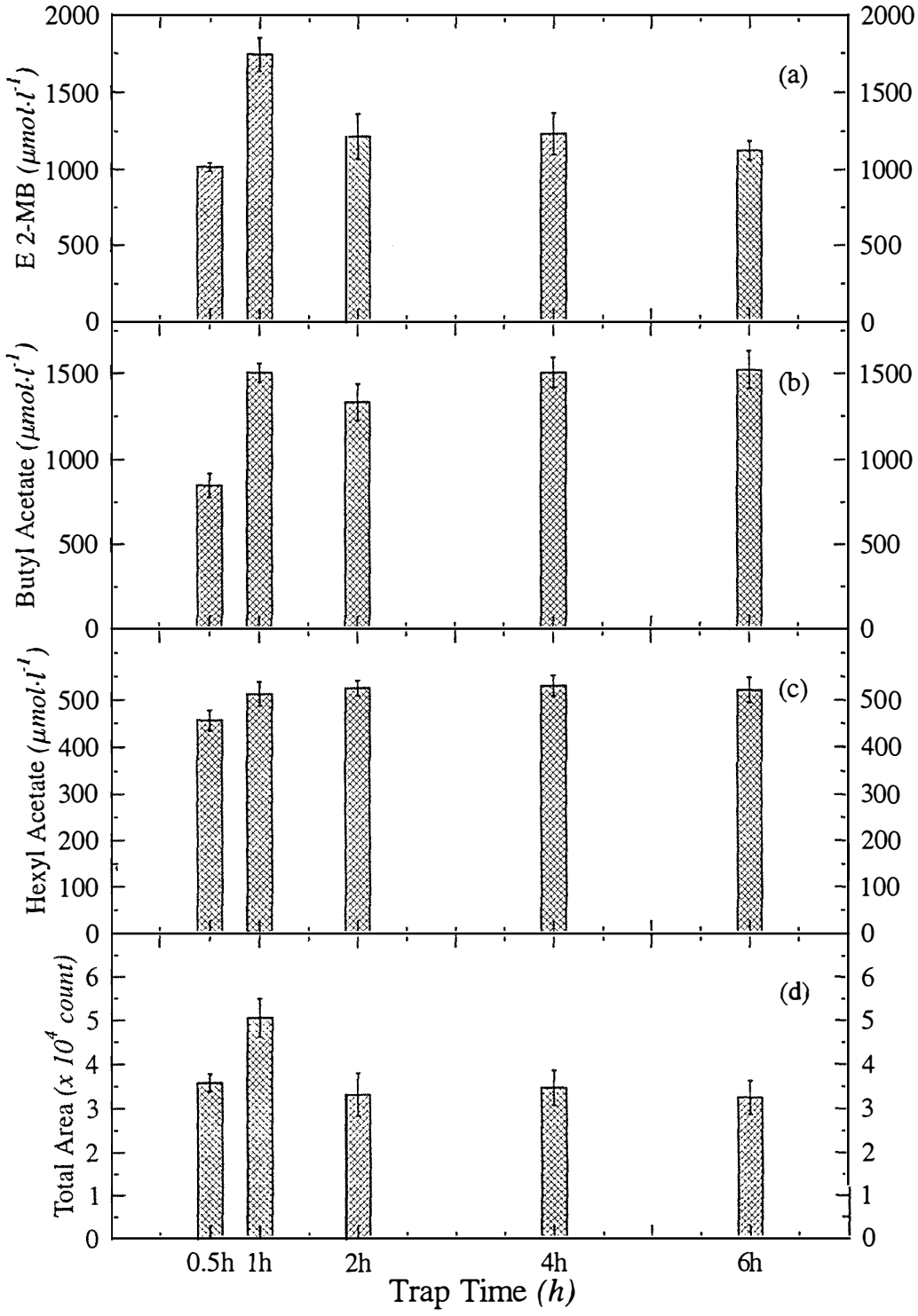


Fig. 3.7 Concentration of (a) ethyl 2-methyl butanoate, (b) butyl acetate, (c) hexyl acetate and (d) total area of 'Red Delicious' apple juice at different trapping time on Tenax® GR at 20°C using a dynamic headspace method.

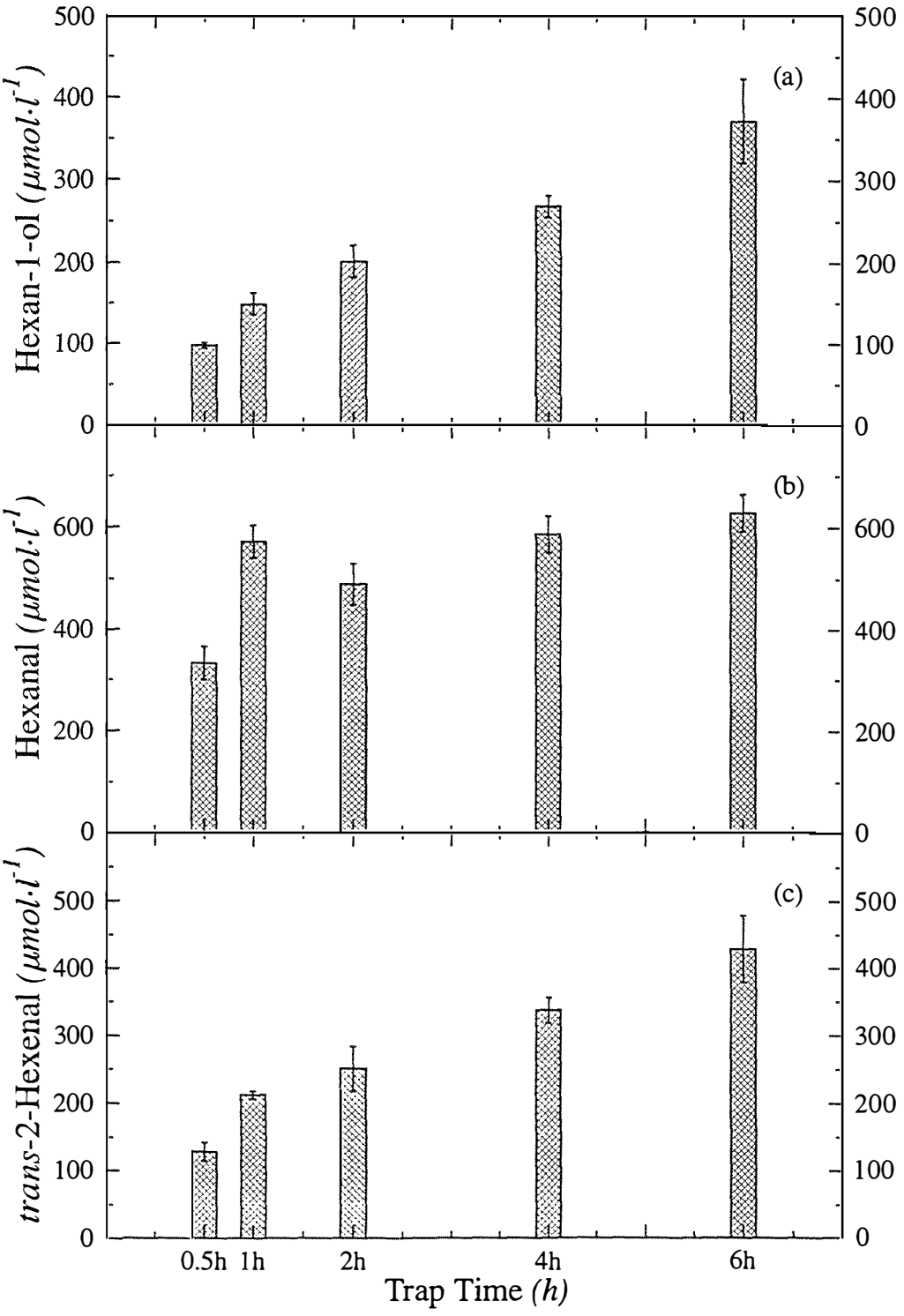


Fig. 3.8 Concentration of (a) hexan-1-ol, (b) hexanal and (c) *trans*-2-hexenal of 'Red Delicious' apple juice at different time of trapping on Tenax® GR at 20°C using a dynamic headspace method.

The low molecular weight compounds, such as ethyl acetate, ethanol, and ethyl 2-methyl butanoate (group 2), may have low breakthrough volumes (Schaefer, 1981a; 1981b), and once they reach the particular adsorption-desorption equilibrium they virtually breakthrough the trap and are lost (Buckholz *et al.*, 1980; Schaefer, 1981a; 1981b). These compounds also show a decrease in concentration with increased trapping time, especially ethyl acetate (**Fig. 3.6**a), which is probably due to displacement by other compounds having higher concentration and larger breakthrough volumes (Schaefer, 1981a, 1981b). The initial low recoveries (after 0.5 h) of some compounds, such as butan-1-ol, butyl acetate and hexanal (group 3) suggest that they may be partially retained in the juice sample before reaching the particular adsorption-desorption equilibrium (after 1 h). After 1 h of trapping, concentrations of these compounds are fairly constant, which may indicate that they have reached maximum amounts that can be adsorbed by the Tenax® GR adsorbent trap and/or have reached maximum concentrations in the juice sample (Schaefer, 1981a; 1981b). The pattern of the total area in relation to trapping time is probably influenced by ethyl acetate (**Fig. 3.6**a), ethanol (**Fig. 3.5**a) and to the lesser extent ethyl 2-methyl butanoate (**Fig. 3.7**a) as they are found in a greater concentrations in the sample than other compounds. It is probable that other compounds, which have not been identified, may behave similarly, contributing to the total area pattern(**Fig. 3.7**d).

Hexan-1-ol (**Fig. 3.8**a, $P < 0.001$, $r^2 = 0.80$) and *trans*-2-hexenal (**Fig. 3.8**c, $P < 0.001$, $r^2 = 0.79$) are found to increase linearly with trapping time (group 4). This is more likely to be due to the slow rate of transfer of these compounds to the vapour phase than to their equilibrium status which control the concentrations and ratios of components (Williams *et al.*, 1978). In the case of *trans*-2-hexenal, it is unlikely that the enzymatic formation of the compound would contribute to this phenomena, as sodium chloride salt was added to the juice where it is known to inhibit enzymes (Berger *et al.*, 1992; Buttery *et al.*, 1987). Furthermore, the formation of this compound would have reached its maximum within a few minutes (10 - 30 min) during the juice preparation process due to the reaction of lipoxygenase on C_{18} linoleic and linolenic fatty acid (Drawert *et al.*, 1986) and the use of oxygen-free nitrogen gas for purging would replace most, if not all, of the oxygen present in the juice and the headspace atmosphere.

There is considerable variation between results obtained from particular treatments. High coefficients of variation (c.v. ≥ 20%) of the individual volatile compounds were found mainly in the low boiling point compounds and alcohols. These compounds are ethyl acetate (c.v. = 30.95%), propan-1-ol (c.v. = 30.45%), butan-1-ol (c.v. = 27.20%), and ethanol, which has an exceptionally high coefficient of variation (c.v. = 50.06%). Other compounds tested show less variation (c.v. < 15%). Several factors may have contributed to the variations experienced. Firstly, Tenax® GR is known to have low affinity for low molecular weight volatile compounds and alcohols, especially ethanol (Jennings and Filsoof, 1977; Sugisawa, 1981; Williams *et al.*, 1978). Secondly, differences in the purging flow rate of the nitrogen gas may be affected by differences in the trap dimensions. It was observed that not all Tenax® traps used had exactly the same diameter, ranging from 2.5 mm to 3.4 mm. Williams *et al.* (1978) pointed out that minor fluctuations in flow rate, temperature, and pressure required to get gas to flow through the trap can greatly affect reproducibility of the dynamic headspace system. Finally, some highly volatile compounds may breakthrough during the collection process (Buckholz *et al.*, 1980) and some may be lost partially or entirely together with the solvent (Cole, 1980; Weurman, 1969). In addition, variation in flow rate of the nitrogen gas stream used during evaporation of excess solvent in the concentration step, and slight differences in residual concentrate volume may contribute to such variations, especially with samples having highly volatile compounds.

3.4.5 Comparison of the Dynamic Headspace and Direct Solvent Extraction Procedures

The direct solvent extraction technique resulted in a greater total peak area, incorporating all compounds extracted from the juice sample, than that obtained using the purge and trap method (**Table 3.4**). Comparison of chromatograms obtained from both systems indicated that the direct solvent extraction technique resulted in many peaks representing compounds with a wide range of boiling points (**Fig. 3.9**b) such as ethyl acetate (77°C b.p.), hexan-1-ol (156.5 °C b.p.), hexyl hexanoate (246°C b.p.), acetic acid (117°C b.p.), and 2-methyl butanoic acid (177°C b.p.). These peaks include volatile and non-volatile slightly non-polar compounds, such as propanoic acid and

2-methyl butanoic acid (Larsen and Poll, 1990; Maga, 1990). This contrasts with chromatograms following the purge and trap method where the only compounds extracted were volatile chemicals from the juice sample (**Fig. 3.9**a) (Schaefer, 1981a; 1981b; Sugisawa, 1981).

The concentrations of most alcohols and *trans*-2-hexenal were significantly higher when the direct solvent extraction method was used, whereas much higher concentrations of most esters and hexanal were obtained by the purge and trap technique. However, there was no difference between concentrations of ethyl hexanoate and hexyl acetate in relation to the extraction techniques used.

The 'salting out' effect (Bemelmans, 1981; Nelson and Hoff, 1968) is probably the major reason for the significantly higher concentration of most esters and hexanal found in the purge and trap method than in the direct solvent extraction method. Sodium chloride (NaCl) salt was added to the apple juice sample to insolubilise volatile compounds before extraction by the purge and trap method, but it was not added to the juice prior to extraction using direct solvent extraction. Sodium chloride, along with other salts, such as $CaCl_2$ and Na_2SO_4, is thought to bind to water molecules, giving a reduced solvent-solute interaction, and thus an increase in the air to water partition coefficient. Therefore, it enhances concentration of some volatile compounds in the headspace (Bemelmans, 1981; Buttery *et al.*, 1987; Jennings, 1965; Nelson and Hoff, 1968). However, the effect of salt on releasing the volatile compounds in the headspace may vary from one compound to another and be different for different concentrations (Narwar, 1966; Weurman, 1969). Jennings and Filsoof (1977) found that addition of sodium chloride to a 100-ppm model solution appeared to have no effect on alcohol concentration in the headspace. On the other hand, sodium chloride has been found to partially inhibit the formation of (*E*)-2-hexenal, but not hexanal in tomatoes (Buttery *et al.*, 1987). This may be the reason for the reduced amount of *trans*-2-hexenal in the purge and trap technique compared with that obtained using the direct solvent extraction method. Furthermore, the presence of specific volatile compounds in the sample may also affect the concentration of the other volatile compounds in the headspace (Narwar, 1966). For example, high ethanol concentration in rum and beer was reported to depress the vapour pressure of other volatile compounds in the mixture (Narwar, 1966).

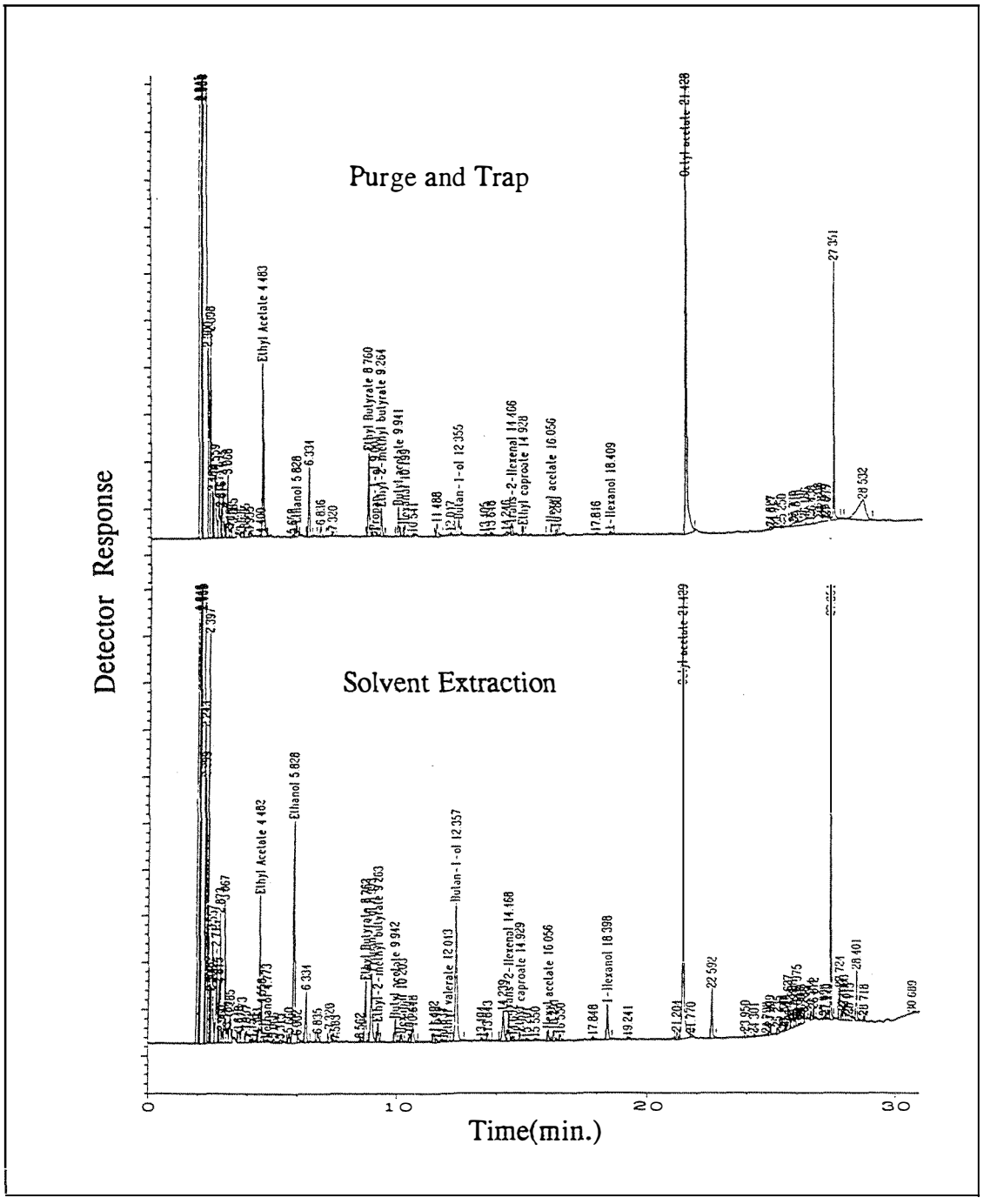


Fig. 3.9 Gas chromatographic profiles of volatile compounds from 'Red Delicious' apple juice using (a) purge and trap and (b) direct solvent extraction methods.

Table 3.4 Concentrations and coefficients of variation of some volatile compounds of 'Red Delicious' apple juice using the dynamic headspace and direct solvent extraction methods.

	Concentration ($\mu mol \cdot l^{-1}$)			C.V. (%)	
Compounds	Headspace	Extraction	(*P*)	Headspace	Extraction
Alcohols					
ethanol	408.1[b]	20026.4[a]	* * *	62.9	22.9
propan-1-ol	409.7[b]	3462.6[a]	* * *	25.6	9.1
butan-1-ol	872.1[b]	4972.6[a]	* * *	31.5	7.2
hexan-1-ol	157.6[b]	760.8[a]	* * *	33.0	5.3
Aldehydes					
hexanal	499.5[a]	265.2[b]	* * *	21.5	9.9
trans-2-hexenal	276.9[b]	511.5[a]	* * *	38.9	12.5
Esters					
ethyl acetate	7155.7[a]	5173.7[b]	*	33.8	8.5
ethyl butanoate	2118.3[a]	1287.1[b]	* * *	23.4	5.4
ethyl 2-methyl butanoate	613.1[a]	368.3[b]	* * *	24.7	7.8
ethyl hexanoate	136.3[a]	131.7[a]	ns	27.7	11.1
butyl acetate	738.3[a]	511.7[b]	* * *	23.6	6.5
hexyl acetate	194.9[a]	174.9[a]	ns	26.5	6.4
total area (x 10^5 count)	3.67[b]	6.20[a]	* * *	19.4	5.8
total number of peaks	67	108	* * *	19.1	13.6

Headspace = dynamic headspace (syn. purge and trap) method; Extraction = direct solvent extraction method; C.V. = coefficient of variation
Levels of significance at $P = 0.05$ (*), 0.01 (* *), 0.001 (* * *), or non significant (ns)

The solubility of volatile compounds in water is probably another factor that governs the amount recovered from the sample (Maga, 1990). Most alcohols, especially low molecular weight alcohols, are more soluble in water than corresponding esters and aldehydes (Lide and Frederikse, 1995); thus alcohols are likely to be retained in the juice sample (Jennings and Filsoof, 1977). Apart from that, it has been reported by several investigators that Tenax® has a low affinity for low molecular weight alcohols (Jennings and Filsoof, 1977; Murray, 1977; Sugisawa and Hirose, 1981; Williams *et al.*, 1978). Thus, amounts of low molecular weight alcohols such as methanol and ethanol being extracted from juice and adsorbed onto Tenax® would be relatively low, which could lead to under-estimation of their concentrations present in juice samples.

As most aroma volatile compounds are non-polar or slightly polar, they are more soluble in organic solvents than in water (Bemelmans, 1981; Maga, 1990; Weurman, 1969). Therefore, most volatile compounds can be effectively separated from juice samples by a direct contact with the solvent. However, certain volatile compounds also

have limited solubilities in some solvents; once the solvent becomes saturated such compounds can no longer enter into the solvent phase. This phenomenon will be discussed later in this chapter.

Apart from the ability to extract a large number of compounds in a comparatively short extraction time, the direct solvent extraction method is preferred because of a better reproducibility than the purge and trap method for most compounds studied. Coefficients of variation are much higher in the purge and trap than in the direct solvent extraction method for all compounds (**Table 3.4**). In addition, the range and mean of the recovery percentages of the octyl acetate internal standard is higher in the direct solvent extraction (range = 27 - 54%, mean = 39%) than in the purge and trap (range = 15 - 42%, mean = 27%) methods.

3.4.6 Test of Concentrated Volume of Extracts for GLC Analysis

A preliminary study on the concentrate volume of the solvent extract for gas chromatographic analysis was conducted on 31 January 1994 using cold stored (1° ± 1°C, 90 - 92% RH) 'Granny Smith' apples which had been stored since May 1993. The extraction was made on 20 ml juice samples using 20 ml diethyl ether and n-pentane mixture (2:1 ratio, v/v) for direct solvent extraction. The extracted solvent was concentrated to 0.2 (100 fold), 0.5 (40 fold) and 1.0 ml (20 fold), with 2 replicates each, by a stream of oxygen-free nitrogen gas. One µl from each concentrate was analysed on a Varian 3400 Series Gas Chromatograph as previously described (**Chapter Two, *Section 2.4.2.1***). The gas chromatographic profiles (**Appendix 2, Section 2.2**) demonstrated that the 0.2 ml concentrate (100 fold) gave the most satisfactory detection. This indicated that some trace compounds were not sufficiently concentrated to be detected by the GLC when concentrated to 0.5 and 1.0 ml.

A concentrated solvent extract volume of 0.25 ml from 'Red Delicious' apple juice resulted in a greater total peak area (unadjusted for octyl acetate internal standard), incorporating all compounds, than that obtained using other volumes (**Table 3.5**). The chromatograms obtained from the 0.25 and 0.5 ml concentrates, show more larger peaks

than those from the larger (0.75 and 1 ml) volume concentrates (**Fig. 3.10** and **Fig. 3.11**).

Table 3.5 Total peak area (excluding solvent and octyl acetate internal standard peaks) of volatile compounds of 'Red Delicious' apple juice recovered from 4 different concentrated volumes of extracted solvent (diethyl ether : n-pentane, 2:1 ratio, v/v) using a direct solvent extraction method.

Concentrated Volume (ml)	Fold	Total Peak Area[1] (count)
0.25	80	110341
0.50	40	72926
0.75	26	52388
1.00	20	43897

[1] unadjusted for octyl acetate internal standard

As expected, recoveries of unadjusted concentrations of all volatile compounds tested generally decreased with increasing volume of concentrates from 0.25 ml to 1.0 ml (**Table 3.6**). However, once they were adjusted for octyl acetate internal standard they showed a different picture (**Table 3.6**).

Table 3.6 Concentration of volatile compounds (unadjusted and adjusted) of 20 ml 'Red Delicious' apple juice recovered from 4 different concentrated volumes of solvent extracts using the direct solvent extraction method.

Compound	Unadjusted[1] ($\mu l \cdot l^{-1}$)				Adjusted[1] ($\mu l \cdot l^{-1}$)			
	Concentrated Volume (ml)				Concentrated Volume (ml)			
	0.25	0.5	0.75	1.0	0.25	0.5	0.75	1.0
ethyl acetate	194.3	105.6	82.8	69.3	273.8	265.1	366.1	376.3
ethanol	407.2	241.6	214.7	202.7	573.8	606.6	949.1	1110.9
ethyl butanoate	48.4	27.0	18.1	14.7	68.2	68.0	80.1	79.7
propan-1-ol	109.3	58.8	42.8	35.1	154.1	147.6	189.8	190.5
E 2-MB	16.2	8.9	6.3	4.9	22.8	22.4	27.6	26.7
butyl acetate	28.1	14.2	10.4	8.2	39.6	35.6	46.1	44.7
hexanal	8.7	4.5	n.d.	n.d.	12.3	11.3	n.d.	n.d.
butan-1-ol	208.2	102.7	75.0	59.2	293.5	322.9	331.6	321.4
trans-2-hexenal	18.1	10.2	7.3	5.3	25.4	23.8	32.3	28.5
ethyl hexanoate	7.1	3.8	2.4	n.d.	10.1	9.5	10.4	n.d.
hexyl acetate	11.2	5.5	4.1	2.8	15.8	15.5	18.0	15.3
hexan-1-ol	44.5	21.6	14.1	11.9	62.7	61.7	62.5	64.7

[1] unadjusted or adjusted for octyl acetate internal standard; E 2-MB = ethyl 2-methyl butanoate, n.d. = not detected

Adjusted concentration of some compounds, such as ethyl acetate, ethanol, ethyl butanoate and propan-1-ol, in the larger volume of concentrates (ie. 0.75 and 1.0 ml) were higher than in the smaller volumes, ie. 0.25 and 0.5 ml (**Table 3.6**). This is likely to have been due to partial loss of these volatile compounds by evaporation during the concentration process because of their low boiling points (**Appendix 2, Section 2.1**) and the closeness to that of the solvent used (Guichard, 1988; Williams *et al.*, 1978). Concentrations (adjusted) of compounds which having higher boiling points and larger molecular weights than those compounds above, were comparable and included ethyl 2-methyl butanoate, butan-1-ol, hexyl acetate and hexan-1-ol for all volumes tested. This suggests that they are not prone to losses from evaporation because their boiling points are much higher than that of the solvent used.

Volumes of concentrate greater than 0.5 ml may dilute the sample to such an extent that some volatile compounds, such as hexanal and ethyl hexanoate, are present in such low concentrations in the juice sample that they may not be detected by the GLC (**Table 3.6**). From these results, it seems that concentrating 20 ml of solvent extract to 0.5 ml (40 fold) is an adequate volume for analysis as most peaks of interest were present (**Fig. 3.11**). This may be true for a very high aroma producing cultivar like a fully ripe 'Red Delicious' (Dirinck and Schamp, 1989; Guadagni *et al.*, 1971). However, production of volatile compounds in apples not only depends on maturity, but is also cultivar dependent (Dirinck and Schamp, 1989; Mattheis *et al.*; 1991b; Paillard, 1990; Yahia, 1994). Thus, a sample 0.25 ml is likely to be a more reliable volume of concentrate for samples obtained from less mature fruit or from low volatile producing cultivars such as 'Granny Smith' and 'Fuji' (Brackmann and Streif, 1994; Kakiuchi *et al.*, 1986); this would ensure that most compounds are sufficiently concentrated for detection. In addition, the 0.25 ml volume of concentrate is preferred as it is a convenient size to handle on the GLC autosampler.

The chromatogram of the blank solvent concentrate (**Fig. 3.10**) indicated that substantial impurities do occur in the solvents with some volatile compounds of interest found as impurities in the solvent, including methanol, ethanol, ethyl butanoate and ethyl pentanoate (valerate). The concentrations of these impurities are quantified and subtracted from the corresponding compounds found in authentic juice samples.

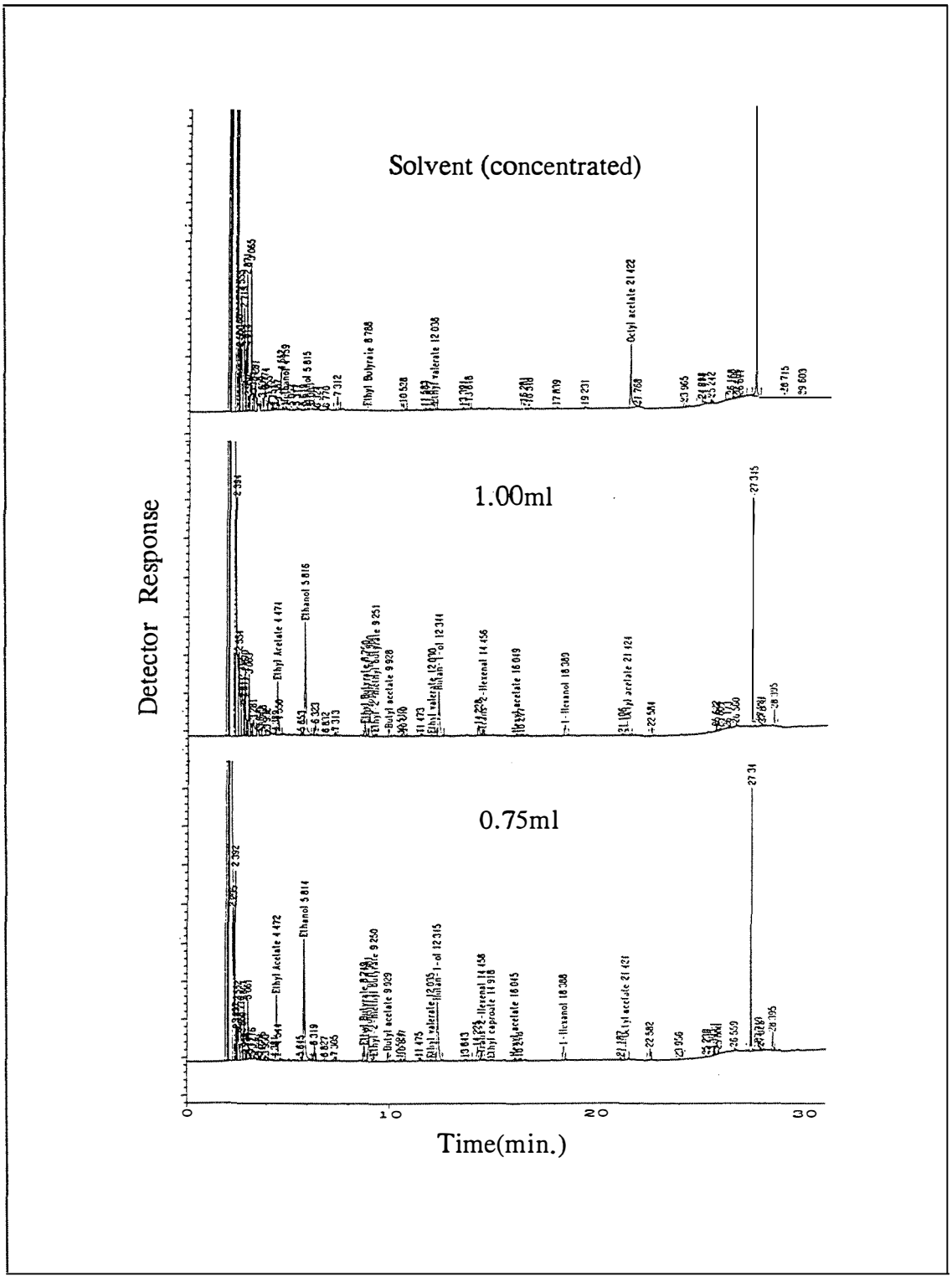


Fig. 3.10 Gas chromatographic profiles of volatile compounds in 'Red Delicious' apple juice in relation to concentrated volumes of solvent extracts of 1.0, 0.75 and 0.25 ml of solvent (blank) using the direct solvent extraction methods.

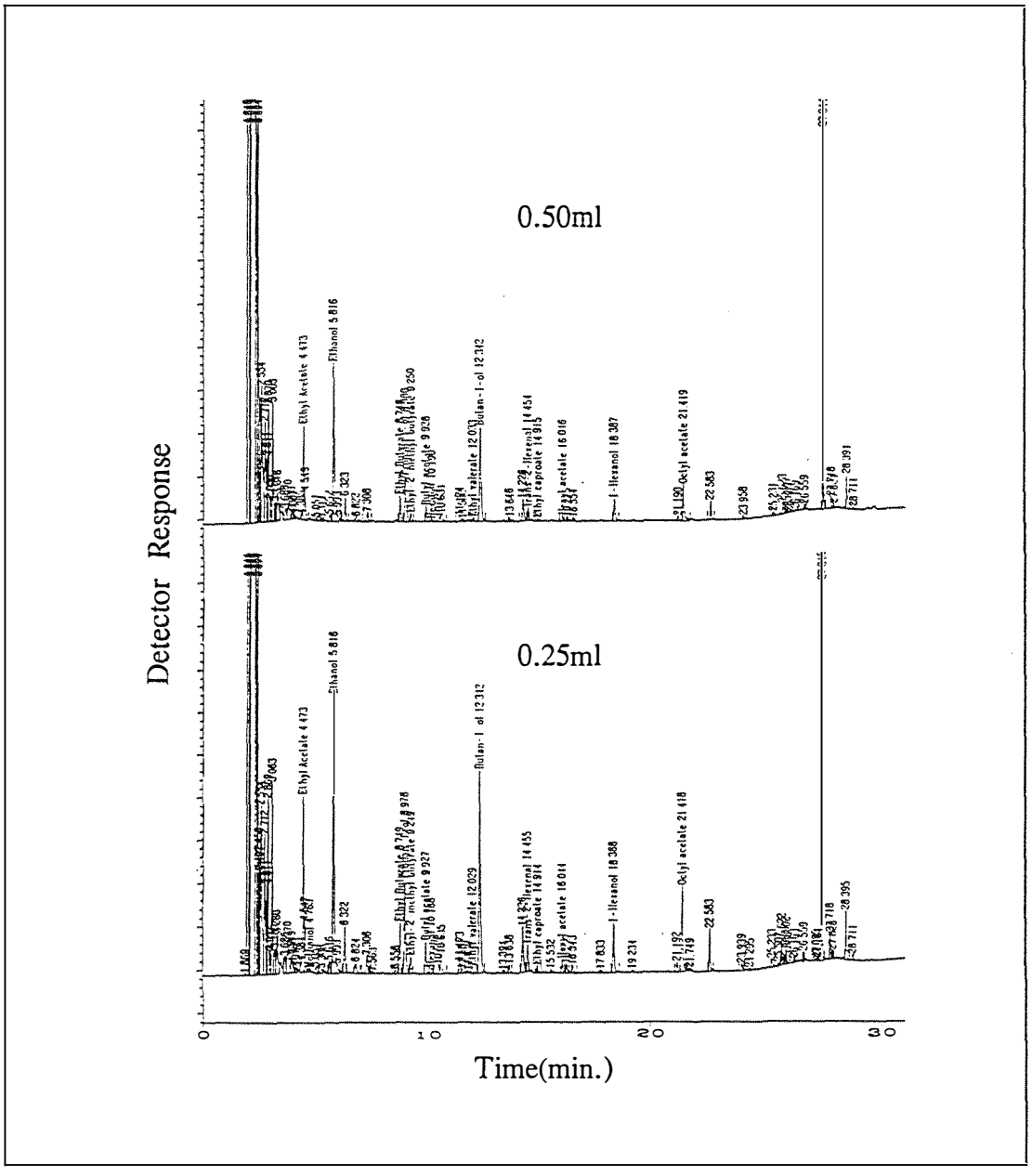


Fig. 3.11 Gas chromatographic profiles of volatile compounds in 'Red Delicious' apple juice in relation to concentrated volumes of solvent extracts of 0.25 and 0.5 ml using the direct solvent extraction methods.

3.4.7 Effect of Juice Volume Used for Extraction

A preliminary study on the amounts of apple juice used for the direct solvent extraction technique was conducted on 31 January 1994 using 'Granny Smith' apples, cold stored (1° ± 1°C and 90 - 92% RH) since May 1993. There were 4 volumes of juice

tested, ie. 10, 20, 30, and 40 ml with 2 replicates each. An equal volume ratio (1:1 v/v) of juice to the diethyl ether and n-pentane solvent mixture (2:1 ratio, v/v) was used. The extract obtained was concentrated to 0.20 ml and 1 µl was used for analysis on a Varian 3400 Series Gas Chromatograph as previously described (**Chapter Two**, ***Section 2.4.2.1***). The gas chromatographic profiles (**Appendix 2, Section 2.3**) obtained were visually assessed; they indicated that a large number of compounds can be detected when using 40 ml of juice, with peaks becoming fewer and smaller as less juice was used. Only a small number of compounds were detected when using 10 ml of juice for extraction. The time required for evaporating excess solvent during the concentration process was substantially longer the larger the amount solvent used. The possibility of losing some highly volatile compounds due to evaporation and increasing concentrations of impurities in the sample was greater with the larger amount of solvent used. Therefore, the 20:20 ml volume ratio of juice to solvent was selected for additional studies, as it gave a sufficient concentration of volatile compounds for GLC detection with a reasonable amount of solvent and required a comparatively short time for the concentration process.

A further study was conducted to examine the effect of the amount of juice sample used for extraction with a fixed volume (20 ml) of solvent on the recovery of the aroma volatile compounds in 'Red Delicious' apple juice. Results were similar to those previously found for 'Granny Smith' apple juice (**Appendix, Section 2 2.4**).

The concentrations of most esters, aldehydes, and some alcohols (**Fig. 3.12** to **Fig. 3.14**) had positive linear relationships ($P < 0.001$; $r^2 > 0.85$) with the juice volumes used, except that of ethanol, propan-1-ol, and total area (**Fig. 3.15**). The positive linear relationship as such is expected, as the concentration of a compound should increase in proportion to the volume of juice used. While a quadratic response curve may explain the relationship slightly more accurately for some compounds, such as ethyl butanoate, ethyl 2-methyl butanoate, butyl acetate, hexyl acetate and *trans*-2-hexenal, the linear response still holds true and is highly significant.

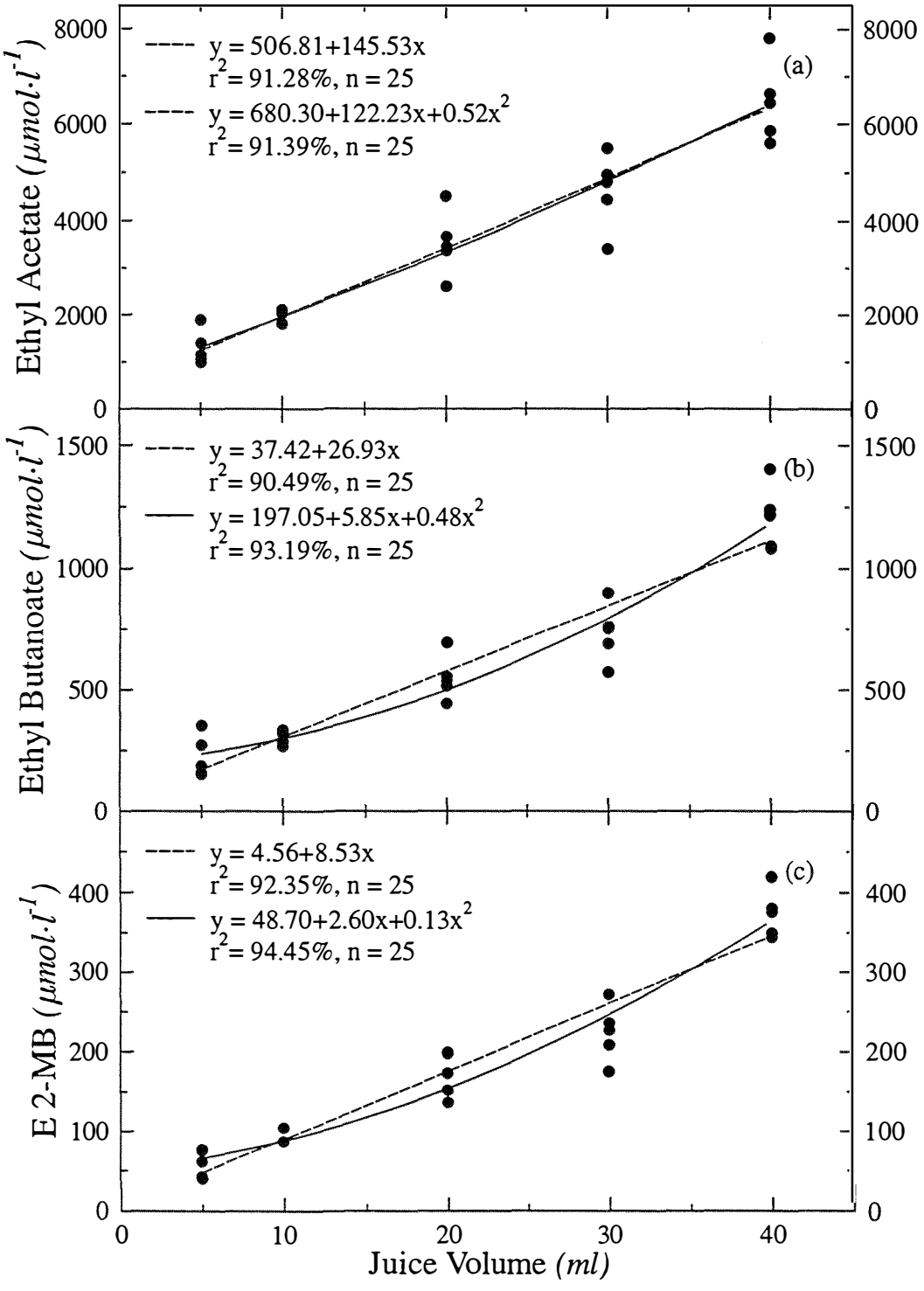


Fig. 3.12 Relationship between juice volume and concentration of (a) ethyl acetate, (b) ethyl butanoate and (c) ethyl 2-methyl butanoate in 'Red Delicious' apple juice.

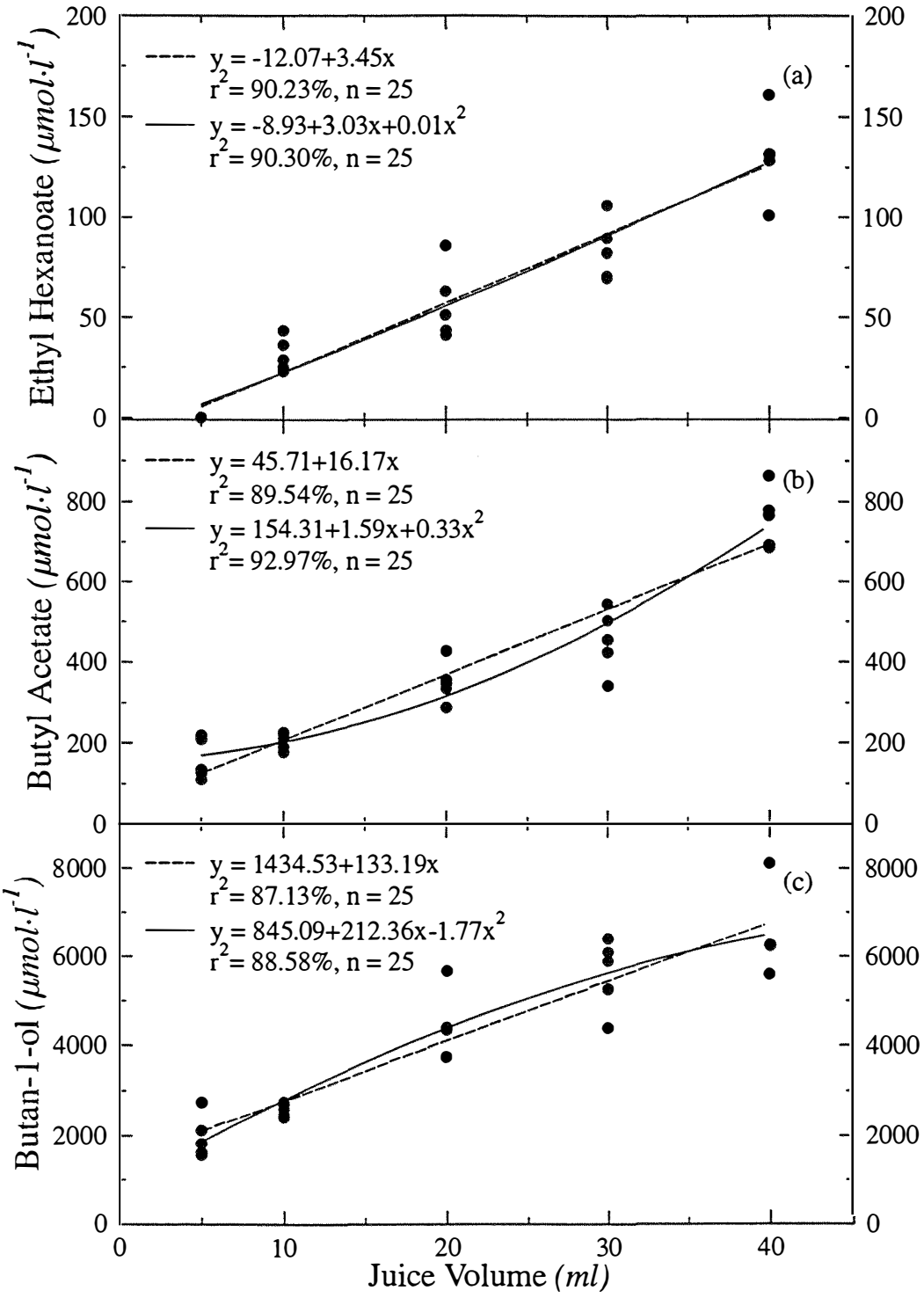


Fig. 3.13 Relationship between juice volume and concentration of (a) ethyl hexanoate, (b) butyl acetate and (c) butan-1-ol in 'Red Delicious' apple juice.

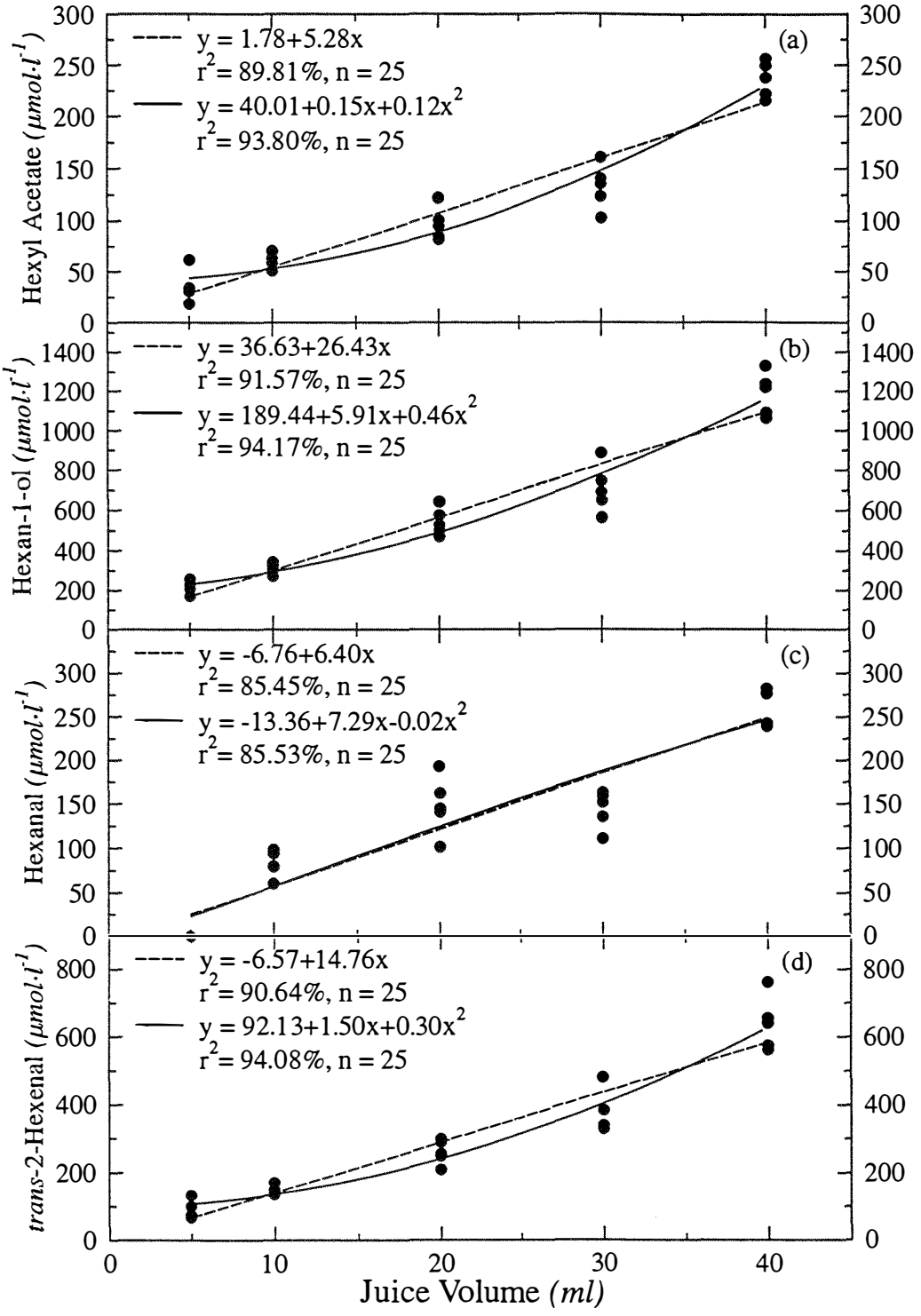


Fig. 3.14 Relationship between juice volume and concentration of (a) hexyl acetate, (b) hexan-1-ol, (c) hexanal and (d) *trans*-2-hexenal in 'Red Delicious' apple juice.

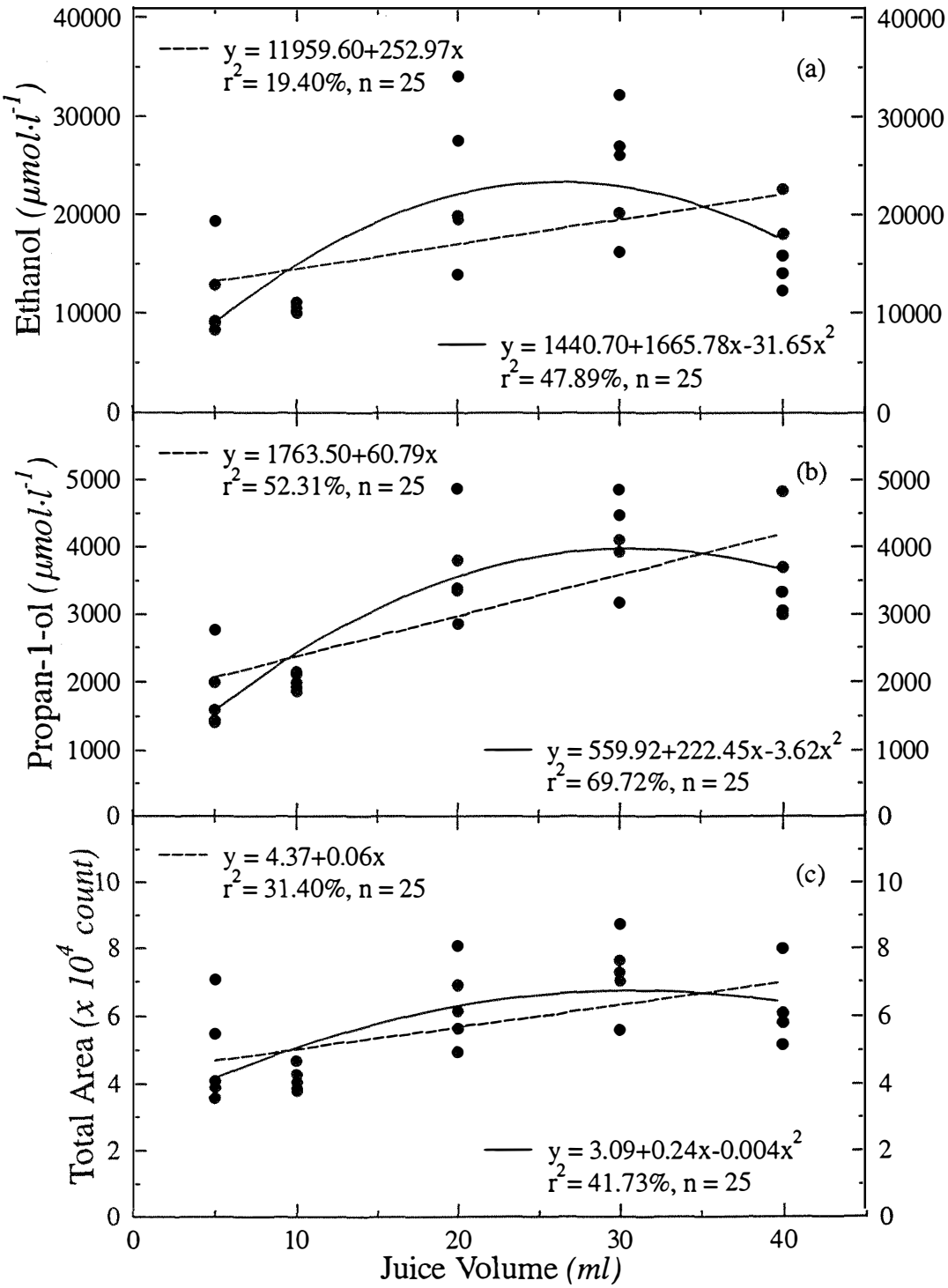


Fig. 3.15 Relationship between juice volume and concentration of (a) ethanol, (b) propan-1-ol and (c) total area in 'Red Delicious' apple juice.

The concentrations of the ethanol, propan-1-ol, and the total area (**Fig. 3.15**) suggested a quadratic relationship with juice volume used. The scatter plots illustrated that the concentrations reach a maximum at approximately 20 ml of juice sample used. This type of response is more likely to occur because the solvent becomes saturated with the compounds. Ethanol and propan-1-ol are miscible in diethyl ether (Lide and Frederikse, 1995), but methanol, ethanol and probably propan-1-ol have very limited solubility in n-pentane (Heath and Reineccius, 1986; Schultz *et al.*, 1977; Williams and Tucknott, 1973). Since the mixture of these two solvents was used, it may affect the solubility of these compounds. The other less likely possibility is that ethanol and propan-1-ol are at their maximum concentrations in the juice, therefore, no matter how large the volume of juice is used the concentrations extracted are still the same.

The total area (**Fig. 3.15**) also shows a response curve similar to that of ethanol and propan-1-ol. This is probably due to the influence of ethanol and propan-1-ol, which were present in high concentrations in the juice sample relative to other aroma volatile compounds. In addition, there may be some other unidentified compounds, which behave similarly.

The smallest volume of juice (5 ml) tested is not practical for routine tests, as the concentration of compounds such as hexanal and ethyl hexanoate are not sufficient to be detected by GLC. A 20 ml juice sample, 1:1 (v/v) with diethyl ether and n-pentane mixture is probably the most suitable ratio for extraction for studying of apple aroma volatile compounds using the direct solvent extraction method.

3.4.8 Effect of Juice Holding Time and Conditions

Changes in some of the aroma volatile compounds from juice of the controlled atmosphere-stored 'Fuji' apple juice held in air at 20°C or in an ice bath for a period of 2 hours after extraction are shown in **Fig. 3.16** to **Fig. 3.18**. There are some indications that small changes in the concentrations of individual volatile compounds did occur during the holding period of 30 to 60 minutes, both in air at 20°C and in an ice bath. When these occurred they were generally after 60 or 90 minutes. Thereafter, there are some slight fluctuations and variations in both conditions under study. However, these

slight changes are not statistically significant, either with time or condition of holding for the compounds studied.

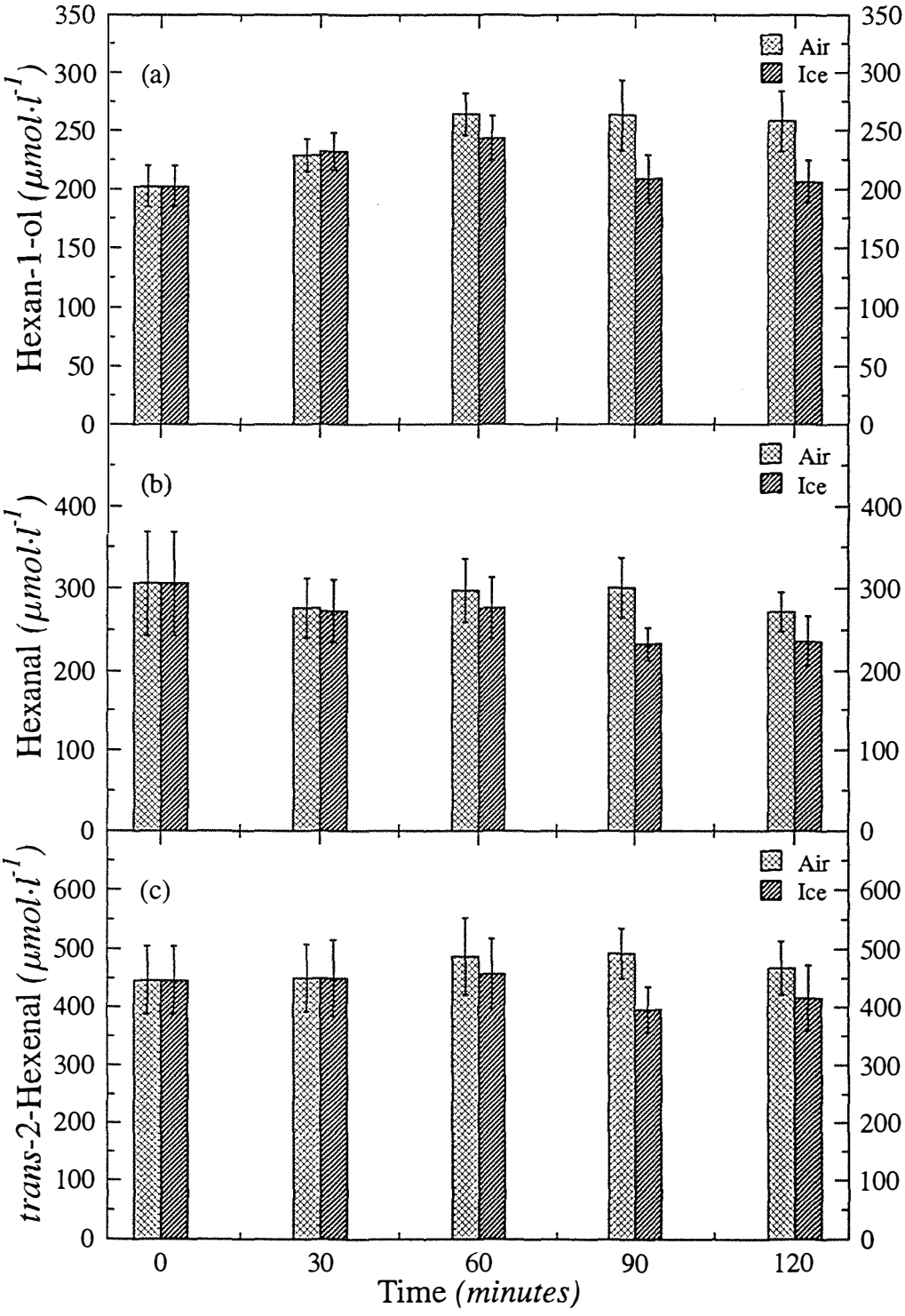


Fig. 3.16 Concentration of (a) hexan-1-ol, (b) hexanal and *trans*-2-hexenal in 'Fuji' apple juice during 2 hours of holding at room temperature (20°C) or in ice bath.

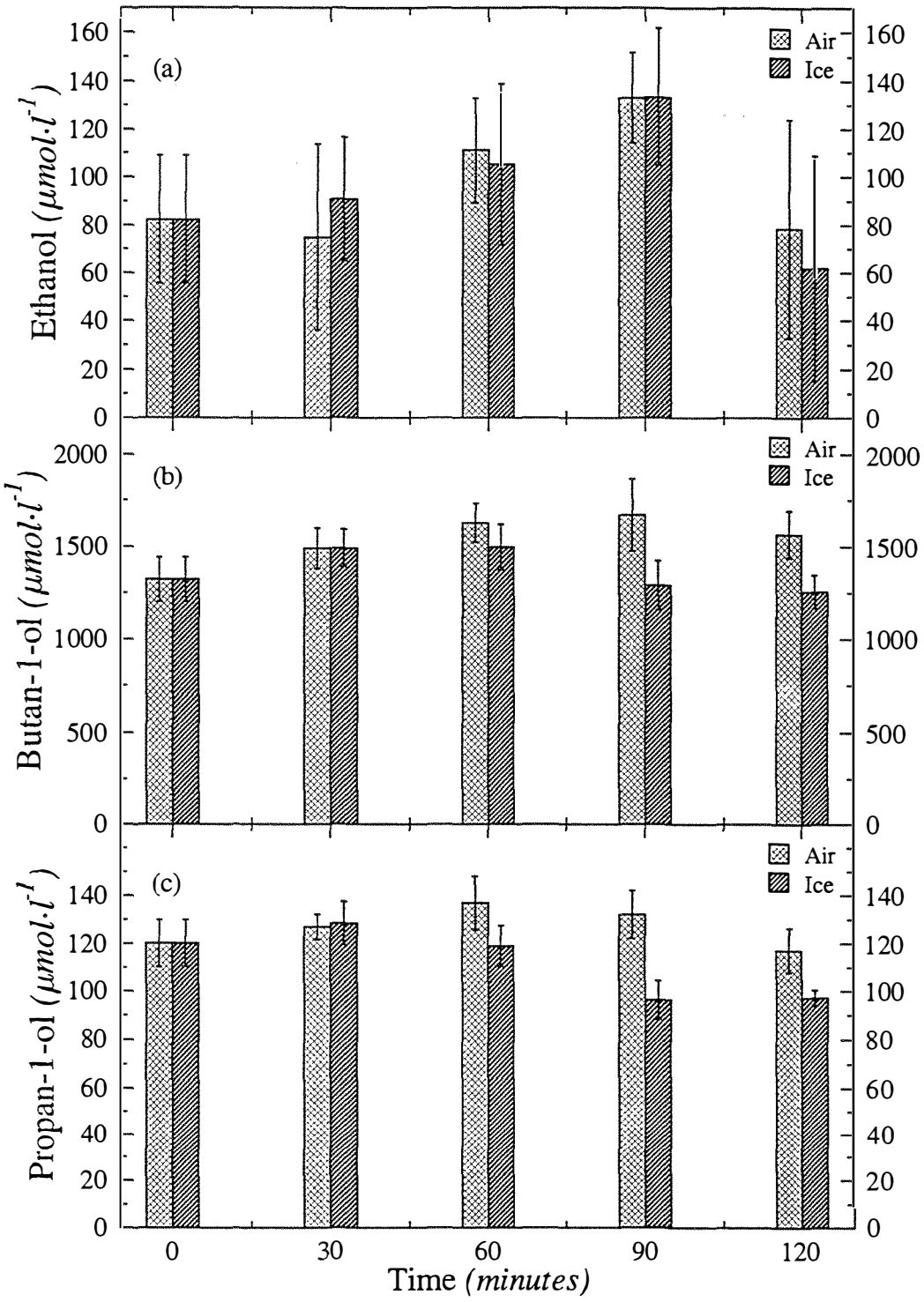


Fig. 3.17 Concentration of (a) ethanol, (b) butan-1-ol and (c) propan-1-ol in 'Fuji' apple juice during 2 hours of holding at room temperature (20°C) or in ice bath.

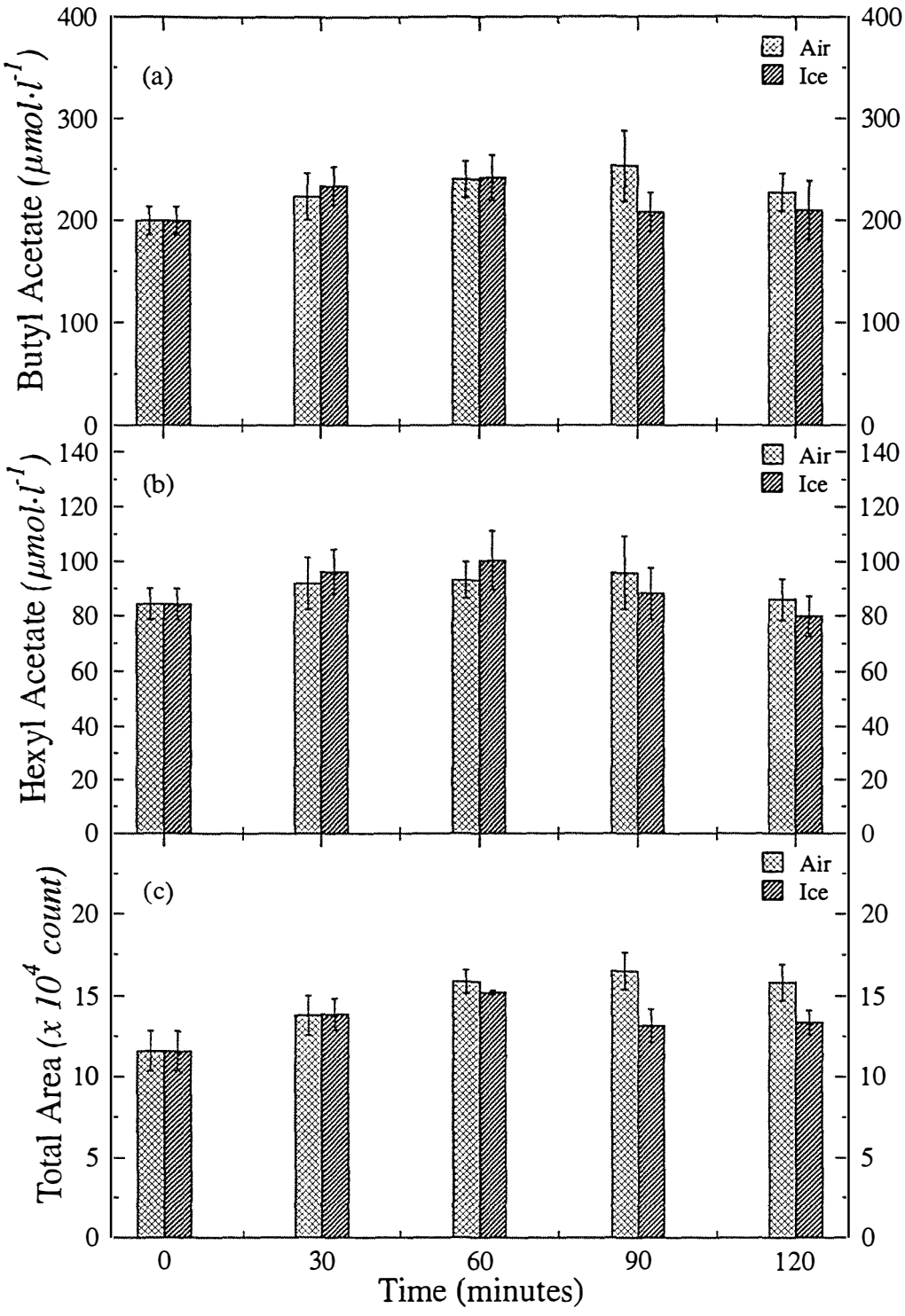


Fig. 3.18 Concentration of (a) butyl acetate, (b) hexyl acetate and (c) total area in 'Fuji' apple juice during 2 hours of holding at room temperature (20°C) or in ice bath.

Therefore, it is suggested that apple juice can be held for at least 60 minutes either in air at 20°C or in an ice bath prior to taking aliquots for extraction of aroma volatile compounds. Notwithstanding the above results, some other highly volatile compounds, such as ethyl acetate, ethyl butanoate, or ethyl 2-methyl butanoate which were not detected in this experiment, could be lost by evaporation at 20°C. Hence, as a safety precaution, an ice bath should still be used before extraction to reduce potential losses of aroma volatile compounds, either from evaporation or enzymatic conversion (Sapers *et al.*, 1977; Maarse, 1991; Yahia, 1994).

3.5 CONCLUSION

A study of aroma volatile compounds in apple juice requires the use of standards, either internal and/or external standards, for quantification purposes. The concentration range from 100 ppm to 5000 ppm octyl acetate in the concentrated solvent extract sample would be appropriate to use as an internal standard, because this range gives a positive linear relationship with the GLC detection response and gives a reasonably low coefficient of variation. The lower concentration range of 0 - 600 ppm octyl acetate is recommended because it has lower variation (c.v. = 12.4%) than the higher concentration range of 0 - 5500 ppm (c.v. = 14.7%). Preferably more than one internal standard should be used to cover the range of volatile compounds under investigation, as not all compounds can be recovered from the sample in the same ratio as the internal standard used. The compound to be selected as internal standard should not be found naturally in the sample. A mixture of authentic volatile compounds should also be used as an external standard to provide accuracy in quantifying the volatile compounds of interest.

On extraction of the apple juice, the apple juice can be held either in air at 20°C, or in an ice bath for at least 60 minutes without losing any of the major volatile compounds. However, holding the juice in an ice bath before taking aliquots for extraction of volatile compounds may be preferable as a precaution for reducing losses of some volatile compounds due to evaporation or enzymatic conversion.

The extracted solvent, obtained either by desorption from the Tenax® trap or from direct solvent extraction, should be concentrated to 250 µl or a smaller volume for gas chromatographic analysis. A larger volume of concentrates may not have sufficient concentrations to allow detection of some volatile compounds. In addition, this volume of concentrate is a convenient size to handle on GLC autosampler.

The extraction of the volatile compounds with the purge and trap technique using Tenax® GR adsorbent polymers may result in some breakthrough of highly volatile compounds through the trap. Trapping time for apple juice volatile compounds is suggested to be at least 1 hour. After extraction, the Tenax® trap should be washed with diethyl ether and n-pentane mixture (2:1 ratio, v/v) at least twice with approximately 1.5 ml of solvent each time for a complete desorption of volatile compounds from the trap.

To extract volatile compounds using the direct solvent extraction method it is suggested that an equal volume ratio of 20:20 ml of apple juice to diethyl ether and n-pentane mixture (2:1 v/v ratio) be used.

In comparing the direct solvent extraction and purge and trap methods for extraction of volatile compounds in apple juice, the former is preferred as it is simpler, faster, and more efficient. The direct solvent extraction method has the advantage that all volatile compounds can be easily extracted from the apple juice sample within a comparatively short time. The reproducibility of most compounds recovered is higher than that of the purge and trap technique. However, the major disadvantage is that it requires a comparatively large volume of solvent for extraction. This can introduce more impurities into the sample and increase the time required for concentrating the extract. In addition, some non-volatile compounds and waxes are also extracted by this method, which may interfere with the gas chromatographic analysis.

The purge and trap method may have advantages as it isolated only volatile compounds from the juice sample. The direct solvent extraction method extracted both volatile and non-volatile compounds, and the latter may interfere with compounds of interest during the GLC separation process. The purge and trap method requires a comparatively smaller volume of solvent for desorption, so that contamination of impurities is much less and the time required for solvent concentration is shorter than the

direct solvent extraction method. In addition, the technique can be used for a non-destructive study of aroma volatile compounds from the headspace of whole apples. However, several factors may affect the recovery of the volatile compounds by using this technique; these include trap dimension, quantity of adsorbent used, purge and trap time and pressure, and more importantly, the retention capacity of some volatile compounds of the Tenax® adsorbent. In addition, the technique requires a comparative long time for the processes of extraction and reconditioning of the trap after desorption of the volatile compounds.

Chapter Four

AROMA VOLATILE PRODUCTION, PHYSIOLOGY AND QUALITY ATTRIBUTES OF APPLES FOLLOWING TREATMENT WITH HYPOXIC GAS ATMOSPHERES

4.1 INTRODUCTION

Exposing harvested apples to optimal concentrations of low O_2 and/or high CO_2 atmosphere that do not cause severe physiological stress can be beneficial in prolonging fruit shelf life (Kader, 1995; Stow, 1995). Conversely, exceedingly low O_2 and/or high CO_2 concentrations can result in fruit tissue damage and/or development of off-flavours (Ke *et al.*, 1991b; 1993b). Thus, such atmospheres can be either beneficial or detrimental to fruit, depending on concentration, duration of exposure, temperature, and inherited characteristics of the fruit cultivar, such as skin permeance (Gran and Beaudry, 1993; Ke *et al.*, 1990; Park *et al.*, 1993). The effects of long-term exposure (ie. more than 3 months) to low O_2 and/or high CO_2 at low temperatures on postharvest physiology and quality attributes of apples have been extensively investigated (Bramlage *et al.*, 1977; Drake, 1993; Lambrinos *et al.*, 1995; Lau and Looney, 1982; Smock, 1979; Stow, 1995). The advantageous effects of long-term controlled atmospheres (CA) include suppression of respiration rates and ethylene production; retardation of fruit softening and colour change; retention of acids, sugars, and flavour; and reduction of some physiological disorders and decay and these result in extension of apple storage life. Short-term exposures, such as 1 - 20 days, to very low O_2 (< 2%) or very high CO_2 (> 20%) concentrations at low (0° - 10°C) or high (> 20°C) temperature have also been extensively investigated, especially in relation to potential benefits for insect disinfestation (Ke and Kader, 1992b; Lay-Yee and Whiting, 1996; Lidster *et al.*, 1984; Whiting *et al.*, 1991; 1992; 1996), control of microbial growth (Chen *et al.*, 1981; Daniels *et al.*, 1985), minimising some physiological disorders such as scald and core

flush following CA storage (Little *et al.*, 1982) and enhancement of fruit flavours (Larsen and Watkins, 1995a; Pesis, 1994; Pesis *et al.*, 1991). However, extended exposure of fruits to stress levels of low O_2 and/or high CO_2 atmospheres for periods longer than their tolerance limits may result in detrimental effects such as development of off-flavours (Ke *et al.*, 1990; 1991a; 1991b; Larsen and Watkins, 1995b; Ueda and Bai, 1993; Yahia, 1993), tissue browning (Bramlage *et al.*, 1977; Drake and Kupferman, 1993; Little and Peggie, 1987; Meheriuk, 1977; Smock, 1977), failure to ripen or abnormal (uneven) ripening on removal to air (Kader, 1986; Smilanick and Fouse, 1989) and enhancement of softening and decay (Nanos and Mitchell, 1991; Yahia, 1993).

Treatment with low O_2 (below 8%) atmospheres reduced respiration rates and ethylene production of apples (Ke *et al.*, 1991a; Knee, 1991a; Solomos, 1993), pears (Kader, 1989; Ke *et al.*, 1990) and strawberries (Ke *et al.*, 1991b; Li and Kader, 1989). However, respiration rates and ethylene production of commercially mature peaches and nectarines (Lurie and Pesis, 1992) and mature green banana (Wills *et al.*, 1982) were not different from control after treatment with 2% O_2 at 20°C. The mechanism by which different fruit had such different responses to hypoxia is not known (Blanke, 1991).

Increasing CO_2 concentrations (> 10%) can reduce, promote, or have no effect on respiration rates and ethylene production in fruits, depending on type, cultivar, stage of maturity, CO_2 concentration, temperature, and duration of exposure to such conditions (Kader, 1995; Kubo *et al.*, 1990; Mathooko, 1996). Respiration rates and ethylene production of apples (Bramlage *et al.*, 1977; Chaves and Tomás; 1984) and pears (Kerbel *et al.*, 1988; Ke *et al.*, 1990) were suppressed by exposure to 10 - 20% CO_2, but enhanced by 60% and 95% CO_2 in cucumber fruit (Mathooko *et al.*, 1995a; 1995b) and 'Golden Delicious' apples (Pesis *et al.*, 1994), respectively.

Very low O_2 (< 3%) or high CO_2 (> 20%) atmospheres can induce a shift from aerobic to anaerobic respiration in fruit leading to accumulation of acetaldehyde, ethanol and ethyl acetate (Boersig *et al.*, 1988; Kader, 1995; Ke *et al.*, 1993b; Vartapetian *et al.*, 1978). Under hypoxia, glycolysis leads to acetaldehyde and ethanol biosynthesis via pyruvate by the action of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) with concomitant oxidation of NADH (Chervin *et al.*, 1996; Kennedy *et al.*, 1992). Lowering O_2 or increasing CO_2 concentrations, extending exposure times and

high temperatures can result in greater production of these volatiles (Ke *et al.*, 1990; Gran and Beaudry, 1993). These fermentative volatiles (acetaldehyde and ethanol) are also found to accumulate naturally during ripening and senescence in various fruits (Janes and Frenkel, 1978; Nichols and Patterson, 1987; Bender *et al.*, 1994) and have been related to off-flavour development in apples, pears, plums (Ke *et al.*, 1991a) and strawberries (Ke *et al.*, 1991b; Larsen and Watkins, 1995b; Ueda and Bai; 1993). On the other hand, high amounts of acetaldehyde, ethanol and ethyl acetate in apples, pears and berries, which were present on returning to air after treatment with low O_2 (0.3 - 4.0%), had no correlation with off-flavours (Richardson and Kosittrakun, 1995). Moreover, application of acetaldehyde, ethanol or anaerobic conditions to pears, blueberries, tomatoes (Paz *et al.*, 1981), peaches (Pesis 1994), feijoa (Pesis *et al.*, 1991), oranges, mandarins (Shaw *et al.*, 1990; 1991; 1992) and strawberries (Larsen and Watkins, 1995a) lead to enhancement of fruit flavour.

Many attempts have been made to increase apple aroma volatiles artificially by addition of aldehydes, alcohols, and carboxylic acids which increased volatile esters in the fruit (Bartley *et al.*, 1985; Berger and Drawert, 1984; De Pooter *et al.*, 1981; 1983; 1987). Precursor atmosphere (PA) technology is the short-time exposure of intact mature fruit to vapour of specific volatile precursors added in a controlled manner to a reaction vessel, resulting in enhanced production of specific volatiles (Berger, 1990; Berger *et al.*, 1992; Kollmannsberger and Berger, 1992). The apples are biocatalysts for the production of specific esters. Volatile enhancement can be produced by short-term application of very low O_2 (0.05 - 1.5%) or high CO_2 (95%) atmospheres in various fruits, including apples (Mattheis *et al.*, 1991a), strawberries (Ke *et al.*, 1994b; Larsen and Watkins, 1995a), feijoa (Pesis *et al.*, 1991) and citrus (Pesis and Avissar, 1989; Shaw *et al.*, 1990; 1991; 1992).

Some of the above studies focused mainly on the detrimental effect of hypoxic atmospheres on fresh fruit, with the intention of determining fruit tolerance limits to such atmospheres (Ke and Kader, 1992a, 1992b; Ke *et al.*, 1990; Ke *et al.*, 1991a; 1991b; 1994b). Experimental conditions usually involved low temperatures (0° - 10°C) with low O_2 concentrations of 0.05% - 2% and/or high CO_2 of 10% - 50% (Ke *et al.*, 1991a; 1991b; 1993b; 1994b). Short-term anaerobic treatments with 99% N_2 or 98% CO_2 at

room temperature (20°C) have been studied in other fruits such as feijoas, oranges, peaches and nectarines (Pesis, 1994; Pesis and Avissar, 1989; Pesis *et al.*, 1991) as a possible means to enhance flavour of fresh fruit. Volatile compounds monitored in these studies were mainly fermentative volatiles and a few aroma volatiles such as ethyl acetate and ethyl butanoate. Shaw *et al.* (1990; 1991; 1992) were an exception, studying a range of volatile compounds in juice and essence of hypoxically treated oranges and mandarins. Mattheis *et al.* (1991a) studied a range of volatile compounds produced in fresh apples on return to air after they had been stored in low O_2 (0.05%) for 30 days at 1°C to assess the consequence of accidental anaerobiosis which could occur under CA storage.

Although flavour enhancement in many fruits seems possible by using hypoxic atmospheres, there have been no reports of the effects of short-term anaerobic conditions as a means for enhancing aroma volatile compounds in apples processed into juice. Such hypoxic treatments may provide an economic, rapid, natural and environmentally friendly method for enhancing some important flavour impact volatile compounds prior to juice extraction that would increase the use and value of aqueous essence from fruit. Therefore, it was of interest to study whether short-term hypoxic atmosphere treatments, with varying times and gas concentrations at 20°C could enhance aroma volatile compounds in juice of treated apples.

4.2 OBJECTIVES

The objectives of this study were to investigate the effect of applying CO_2, O_2, and N_2 gases in different combinations and for different times on fruit physiology including quality, volatile compounds in headspace and in juice from a number of important commercial apple cultivars.

4.3 MATERIALS AND METHODS

4.3.1 Materials and Treatments

Cold stored apples, Local Fancy Grade Class 1, cvs. 'Granny Smith', 'Braeburn' and 'Red Delicious', were used (**Table 4.1**).

Table 4.1 History of apple cultivars used for experiments

Experiment	Size (g)	Time				Source
		Harvest (Approx)	Arrival	Removal	Storage	
1. Effects of CO_2, N_2 and C_2H_4 on 'Granny Smith' (1993)	174g	25/5/93	26/5/93	29/6/93	34days[1]	F.C.U.[4]
2. Effects of CO_2 exposure time on 'Braeburn' (1994)	148g	2nd week of April	5/5/94	12/7/94	4mth[2]	NZAPMB[5] (ENZA®);
3. Effects of CO_2 exposure time on 'Red Delicious' (1994)	148g	1st week of April	15/4/94	11/8/94	4mth[2]	(as in 2)
4. Effects of low O_2 concentrations on 'Red Delicious' (1995)	148g	3rd week of April	5/5/95	9/5/95	2wks[2]	Local Packhouse, via F.C.U.
5. Effects of low O_2 concentrations on 'Red Delicious' (1994)	148g	1st week of April	15/4/94	8/11/94	8mths[3]	(as in 2)
6. Effects of CO_2 concentrations on 'Red Delicious' (1994)	148g	1st week of April	15/4/94	13/9/94	6mths[3]	(as in 2)

[1] Department of Plant Science cold store (1° ± 1°C, 90 - 92% RH), [2] Fruit Crop Unit cold store (0.5° ± 0.5°C, 92 - 95% RH), [3] Apples were stored in commercial cold store at NZAPMB (0.5° ± 0.5°C, 92 - 95% RH) prior to arrival at Fruit Crop Unit, [4] F.C.U.: Fruit Crop Unit, Massey University, Palmerston North, [5] NZAPMB.: NZ Apple and Pear Marketing Board (ENZA®), Hawkes Bay Region,

For each experiment, apples were transferred from cool store to ambient temperatures in the laboratory, where fruit from all cartons were initially assessed. Damaged, misshaped, and non-uniform fruit were eliminated and good fruit with similar size and colour were selected. Apples were then divided into groups according to the total number of replicates for a particular experiment, and fruit in each group were individually numbered and labelled. Treatments, replicates and fruit to be taken for analysis at each sampling interval were randomly allocated. After that, apples were transferred to 20°C to equilibrate overnight and treatments were applied the following day.

Experiment 1: An experiment evaluating the effect of CO_2, N_2 and C_2H_4 on 'Granny Smith' apples and apple juice was conducted on 30 June 1993. It comprised 4 treatments (**Table 4.2**), each with 4 replicates and 50 fruit per replicate. Twenty fruit were analysed before the application of treatment, ie. day -1, giving 820 fruit in total. Apples were placed in 27-litre Perspex chambers, where a continuous flow of humidified food grade CO_2 or oxygen-free N_2 (New Zealand Industrial Gases, NZIG®) was applied from compressed gas cylinders at 2.5 $ml \cdot min^{-1} \cdot chamber^{-1}$ during the first 3 hours and then at 500 $ml \cdot min^{-1} \cdot chamber^{-1}$, for 24 hours at 20°C. The gas treatment was deemed to start when gas concentration in the chamber reached the desired level of 98 - 99% for the 100% CO_2 treatment, at which time the flow rate of the applied gas was reduced to 500 $ml \cdot min^{-1} \cdot chamber^{-1}$. This procedure was used for all gas treatments in most experiments. Details of the treatment system and method of application for CO_2 or N_2 was described in **Chapter Two, *Section 2.2***. For the ethylene (1000 ppm) treatment, 27 ml of 100% ethylene gas (Ethylene C.P., Matheson, USA) was taken from a compressed ethylene cylinder by an air tight, 50-ml syringe (Omnifix® 50/60 ml, B. Braun Melsungen AG, Germany), and injected into the gas-tight treatment chamber via a rubber septum port. Apples were held under the static C_2H_4 atmosphere for 24 hours at 20°C. Concentrations of CO_2, O_2, and C_2H_4 were monitored and analysed by injecting 1ml headspace gas samples from chambers onto a Gas Liquid Chromatograph (GLC) (**Table 4.2**). Control fruit were left untreated at 20°C and 70% RH. On the same day but before treatments were applied, 4 replicates, each of 5 apples were taken for analysis (day-1). After treatment, apples were removed from chambers and stored in cardboard cartons at 20°C

and 70% RH for 15 days. Samples were taken for analysis -1, 0, 1, 3, 5, ... ,15 days after treatment; where -1 day was before treatment, 0 day was immediately after treatment, 1 day was 24 hours after, and so on. These procedures for checking atmospheres in chambers, treatment of control fruit, storing and subsequent analysis were the same for ***Experiment 2*** - ***Experiment 6***.

Table 4.2 Concentration of O_2, CO_2 and C_2H_4 in chambers during gas application for 24 hours at 20°C on 'Granny Smith' apples (***Experiment 1***).

Treatment	O_2(%)	CO_2(%)	C_2H_4(ppm)
100% CO_2	0.2	98.7	1.5
100% N_2	0.2	0.3	0.6
1000 ppm C_2H_4	10.2	11.1	1050
Control	20.6	0.2	n.d.*

* n.d. : not detected, Data were means from 4 replicates of 5 sampling time taken during the 24 hours exposure time

Experiment 2: As severe fruit damage of 'Braeburn' apples occurred in 1993 (**Chapter Six**) following 100% CO_2 treatment for 24 hours at 20°C, the effects of varying time of exposure to CO_2 for this cultivar was investigated on 13 July 1994. There were 5 treatments (**Table 4.3**), each consisting of 6 replicates (5 fruit per replicate); in a replicate, a single fruit was used for analysis at each sampling interval (5 sampling times). Six fruit were analysed before the application of treatments, ie. at day-1, giving 156 fruit in total. A continuous flow of humidified food grade CO_2 gas was applied from a compressed gas cylinder to treatment chambers at 2.5 $l \cdot min^{-1} \cdot chamber^{-1}$ for the first 3 hours and then reduced to 500 $ml \cdot min^{-1} \cdot chamber^{-1}$ for a period of 6, 12, 18 or 24 hours at 20°C.

Experiment 3: The effect of CO_2 exposure time on 'Red Delicious' apples was conducted on 12 August 1994. There were 4 treatments (**Table 4.3**), each consisting of 7 replicates (5 fruit per replicate); in a replicate a single fruit was used for analysis at each sampling time (5 sampling times). Seven fruit were analysed before the application of treatment (ie. at day -1), giving 147 fruit in total. The flow rate of CO_2 applied was as for 'Braeburn' (***Experiment 2***) above, but exposure times were 12, 24 and 48 hours at 20°C.

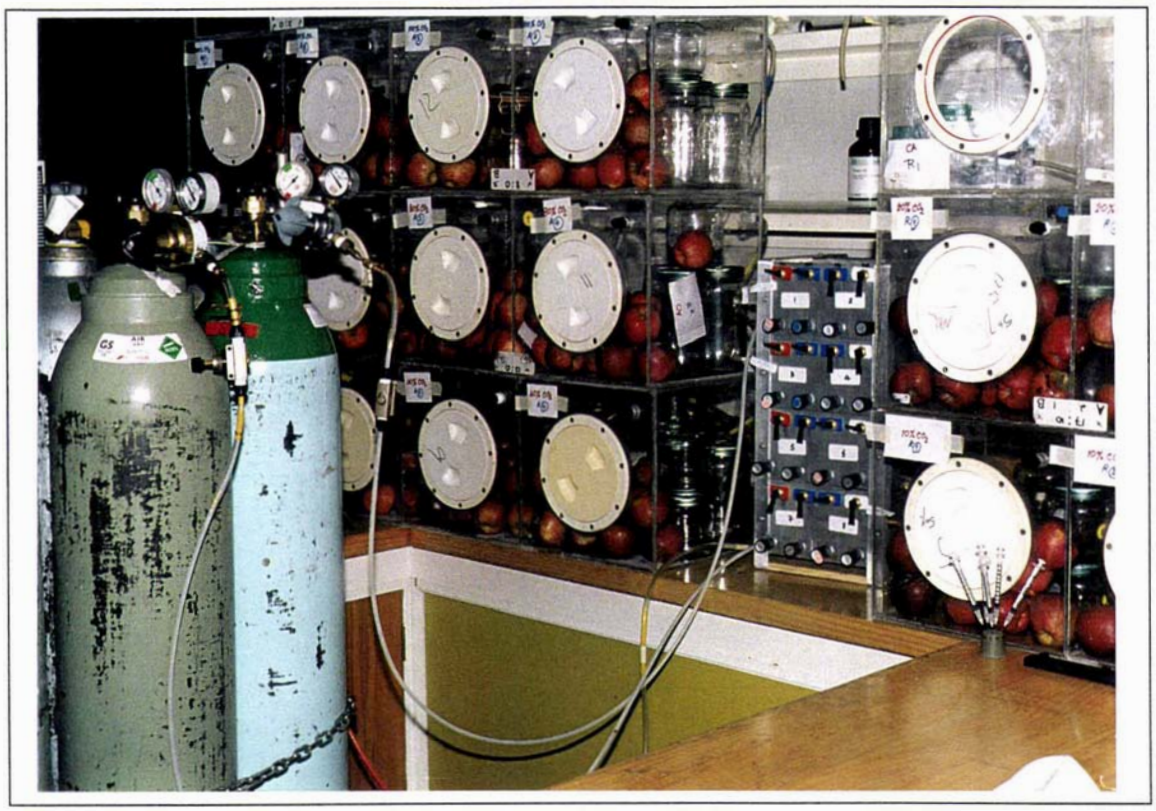


Plate 4.1 Treatment chambers and a gas flow control panel used for treatment of apples with varying concentrations of CO_2 or N_2 at 20°C.

Experiment 4: The effect of low O_2 concentrations on 'Red Delicious' apples was conducted on 8 November 1994. There were 7 treatments (**Table 4.3**), each consisting of 4 replicates and 15 fruit per replicate. Because of the difficulty in obtaining enough juice for extraction of volatile compounds from these apples (juice became thick, viscous and full of cell debris) 3 fruit were bulked for juicing in a replicate at each sampling time (5 sampling times). Twelve fruit were analysed before the application of treatments (at day -1), giving 432 fruit in total. Apples were treated with 100% CO_2, 100% N_2 or a specific gas mixture of N_2 with air at 500 $ml \cdot min^{-1} \cdot chamber^{-1}$ for 24 hours at 20°C.

Experiment 5: The effect of low O_2 concentrations on 'Red Delicious' apples was conducted on 9 May 1995; this was a modification of the 1994 trial (***Experiment 4***). Earlier results (***Experiment 1*** and ***Experiment 4***) had indicated that there were no

differences in major volatile compounds in apple juice or fruit quality attributes between the 100% CO_2 and 100% N_2 (0% O_2) treatments. Therefore, it was decided to use 100% CO_2 as the 0% O_2 treatment for this particular experiment as the 100% CO_2 (24 hours at 20°C) treatment was the standard treatment for most experiments studied herein.

For ***Experiment 5***, there were 5 treatments (**Table 4.3**), each consisting of 4 replicates and 15 fruit per replicate. In a replicate, 3 fruit were bulked for juicing at each sampling time (5 sampling times). Twelve fruit were analysed before the application of treatments (ie. at day -1), giving 312 fruit in total. Apples were treated with a continuous flow of humidified air (control), 100% CO_2 or a specific gas mixture of N_2 with air at 500 $ml \cdot min^{-1} \cdot chamber^{-1}$ for 24 hours at 20°C. Mixtures of gas were achieved by combining oxygen-free N_2 with dry air via a flow control panel (**Plate 4.1**), mixing and then bubbled through water before entering the chambers.

Experiment 6: The effect of CO_2 concentrations in 20% O_2 on 'Red Delicious' apples was conducted on 14 September 1994. There were 6 treatments (**Table 4.3**), each consisting of 4 replicates and 15 fruit per replicate. In a replicate, 3 fruit were bulked for juicing at each sampling time (5 sampling times). Twelve fruit were analysed before the application of treatment (ie. at day -1), giving 372 fruit in total. A ready mixed gas of 80% CO_2 + 20% O_2 (NZIG®) and dry air were used to achieve the desired concentration of CO_2 in a specific treatment (**Table 4.3**). Apples were treated with a continuous flow of humidified 100% CO_2 or a particular mixture of CO_2 + 20% O_2 at 500 $ml \cdot min^{-1} \cdot chamber^{-1}$ for 24 hours at 20°C.

4.3.2 Instrumental Analysis

Carbon dioxide and O_2 concentrations in treatment atmospheres were analysed as described in **Chapter Two**, ***Section 2.2***. Methods for determining fruit respiration rate, ethylene production, skin colour, flesh firmness, soluble solids, titratable acids and juice pH, and the method used to prepare juice and for analysing headspace volatiles have been described in **Chapter Two.**

Table 4.3 Concentration of O_2 and CO_2 in treatment chambers during treatment of apples at 20°C.

Experiment/Treatment	O_2 (%)	CO_2 (%)
2. Time of CO_2 Exposure on 'Braeburn'.		
6 hours	0.2	99.6
12 hours	0.2	99.7
18 hours	0.1	99.7
24 hours	0.1	99.8
Control	20.6	0.1
3. Time of CO_2 Exposure on 'Red Delicious'.		
12 hours	0.1	99.7
24 hours	0.1	99.8
48 hours	0.1	99.8
Control	20.6	0.2
4. Low O_2 Concentrations on 'Red Delicious' (1994).		
0% O_2 (100% CO_2)	0.1	99.8
0% O_2 (100% N_2)	0.1	0.5
1.25% O_2 + 98.75% N_2	1.2	0.5
2.5% O_2 + 97.5% N_2	2.4	0.3
5% O_2 + 95% N_2	4.8	0.2
10% O_2 + 90% N_2	9.7	0.3
Control	20.7	0.1
5. Low O_2 Concentrations on 'Red Delicious' (1995).		
0% O_2 (100% CO_2)	0.1	99.8
2.5% O_2 + 97.5% N_2	2.3	0.1
5% O_2 + 95% N_2	5.3	0.1
10% O_2 + 90% N_2	11.2	0.1
Control	20.3	0.1
6. CO_2 Concentrations in 20% O_2 on 'Red Delicious'.		
10% CO_2 + 20% O_2 + 70% N_2	20.3	10.1
20% CO_2 + 20% O_2 + 60% N_2	19.9	19.9
40% CO_2 + 20% O_2 + 40% N_2	19.9	39.4
80% CO_2 + 20% O_2 + 0% N_2	19.7	79.8
100% CO_2	0.1	99.8
Control	20.6	0.2

Data were means from 4 replicates of 5 sampling times taken during the exposure time

The Purge and Trap technique (**Chapter Two**, ***Section 2.4.2.1***) was used to determine aroma volatile compounds in ***Experiment 1*** and the Direct Solvent Extraction technique (**Chapter Two**, ***Section 2.4.2.2***) was used for other experiments. Details of sample preparation, analysis and calculation of aroma volatile compounds were described

in **Chapter Two**, ***Section 2.4.2***. Fifteen selected volatile compounds (**Chapter Two**, ***Section 2.4.2.2***) and 3 headspace volatiles were monitored for the 1994 and 1995 experiments. Typical chromatograms of headspace volatiles and aroma volatile compounds in juice from control and treated apples are shown in **Appendix 3**.

4.3.3 Data Analysis

All measurements of TSS, TA, pH, headspace volatiles and aroma volatiles were from juice. Each experiment was designed as a factorial (Day x Treatment) with replication nested in treatments. Means and standard errors of the means of each treatment for respiration rate, ethylene production, weight loss, flesh firmness, headspace volatiles and selected volatile compounds were graphed using a GLE (Graphics Language Editor) package (Pugmire and Mundt, 1994).

Data were subjected to analysis of variance using a SAS package (SAS Institute Inc., Cary, USA). Means and pooled standard error for each experiment were calculated. Significant main effect means were separated by Duncan's multiple range test at the 5% level of significance and interactions between these factors were determined by least squares means (lsmeans) at 1% or 5% levels.

4.4 RESULTS

4.4.1 Effect of 100% CO_2, 100% N_2, and 1000 ppm C_2H_4 on 'Granny Smith' Apples *(Experiment 1)*

Respiration rate (**Fig. 4.1**a) of 'Granny Smith' apples increased immediately after treatment with 1000 ppm C_2H_4, but decreased rapidly to a level similar to that of control fruit 1 day after treatment. Respiration rates of fruit treated with 100% CO_2 or 100% N_2 did not differ from controls. During day 1 to day 15 at 20°C fruit respiration rates in all treatments were comparable and fluctuated slightly or remained relatively constant.

Ethylene (**Fig. 4.1**b) production was suppressed by anaerobic conditions 1 day after treatment with the greatest suppression occurring for up to 3 days following the 100% CO_2 treatment. However, ethylene production was higher ($P < 0.05$) than control

at day 5 in the 100% CO_2 treated fruit and during day 5 - day 11 in 100% N_2 treated fruit. The high ethylene production in the 1000 ppm ethylene treatment at day 0 was probably due to excess ethylene diffusing out of the fruit.

Cumulative weight loss of 'Granny Smith' apples was highest in control ($P < 0.05$) and lowest in 100% N_2, while 100% CO_2 or 1000 ppm C_2H_4 was intermediate. Generally, weight loss increased linearly ($r^2 = 0.98$) with time at 20°C with an average loss rate of 0.35% per day (**Fig. 4.1**c). Flesh firmness (**Fig. 4.1**d) was not affected by treatments and it decreased linearly ($r^2 = 0.84$) at an average rate of 1.18 Newton per day.

Headspace volatiles increased several fold in fruit exposed to anaerobic conditions induced by 100% CO_2 or 100% N_2, compared with control fruit or those exposed to 1000 ppm C_2H_4 (**Fig. 4.2**). Peak production of acetaldehyde, ethanol and ethyl acetate from fruit treated with 100% CO_2 occurred at day 0, on removal from treatment, thereafter declining to the same level as control or C_2H_4 treated fruit after 7 - 9 days. In juice from fruit treated with 100% N_2, acetaldehyde peaked 3 days after treatment, ethanol between 0 and 3 days and ethyl acetate at 0 days, after which concentrations of all headspace volatiles declined to control levels after 7 - 9 days. Hypoxic treatments of 100% CO_2 and 100% N_2 had the same effect in enhancing ethyl acetate, acetaldehyde and ethanol and the mean concentrations at their maximum were 7, 30, and 150 fold, respectively, more than control or 1000 ppm C_2H_4 fruit.

Both 100% CO_2 and 100% N_2 treatments induced major increases in selected ethyl esters in 'Granny Smith' apples after treatment compared with control or 1000 ppm C_2H_4 treated fruit, where the latter was not different from control. Peak production of ethyl 2-methyl butanoate from fruit treated with 100% CO_2 and 100% N_2 occurred between day 3 and day 7, thereafter declining to the same levels as control. In fruit treated with 100% CO_2 and 100% N_2, ethyl acetate and ethyl hexanoate peaked at 1 day, after which concentrations declined to control levels after 5 and 7 days, respectively. Production of ethyl 2-methyl butanoate, ethyl hexanoate and ethyl acetate from control and C_2H_4 treated fruit were at low levels throughout 15 days at 20°C; the CO_2 and N_2 treatment enhanced peak concentrations 9, 11 and 30 fold respectively compared with control fruit (**Fig. 4.3**).

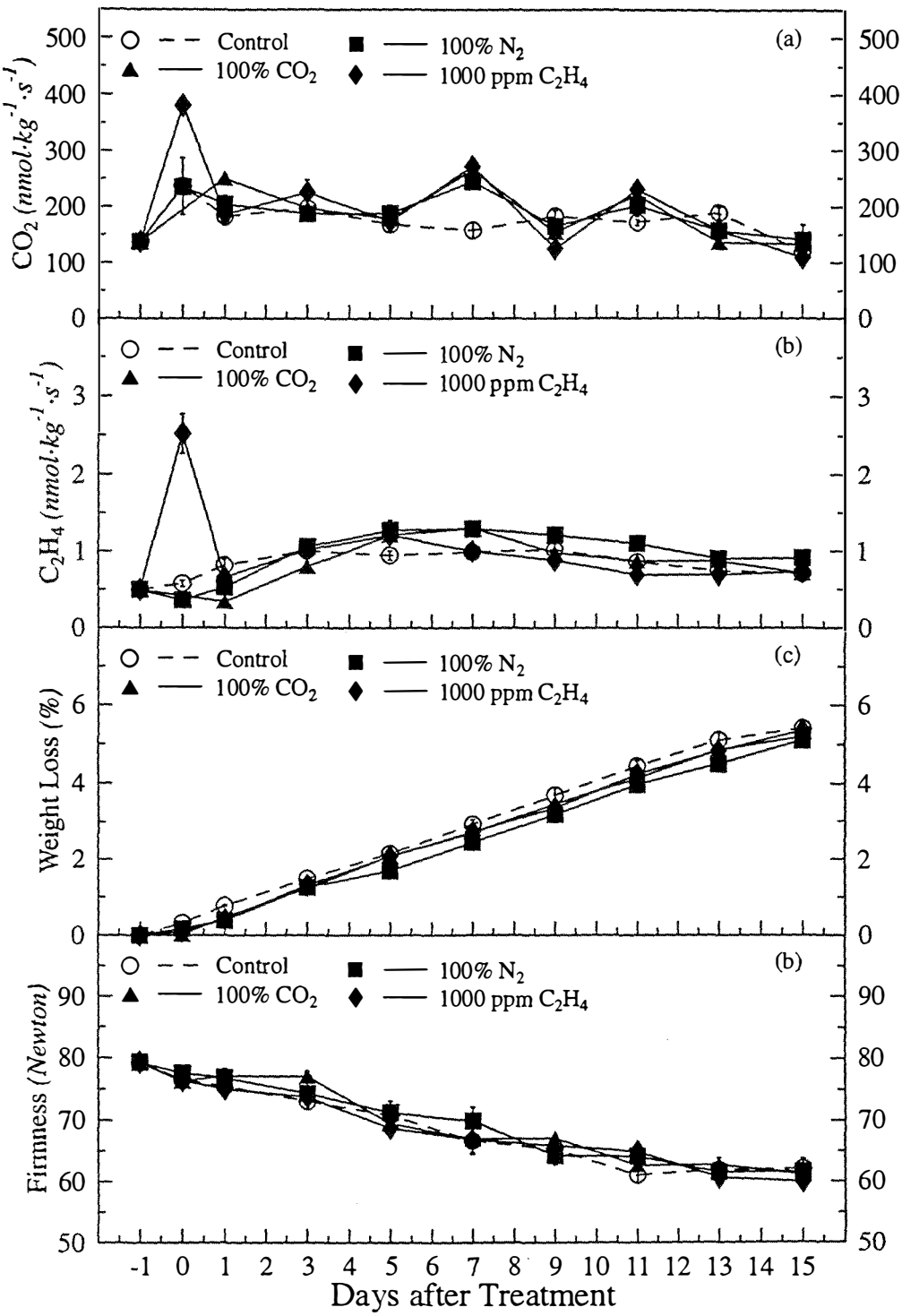


Fig. 4.1 Fruit (a) respiration rates, (b) ethylene production, (c) weight loss and (d) flesh firmness of 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

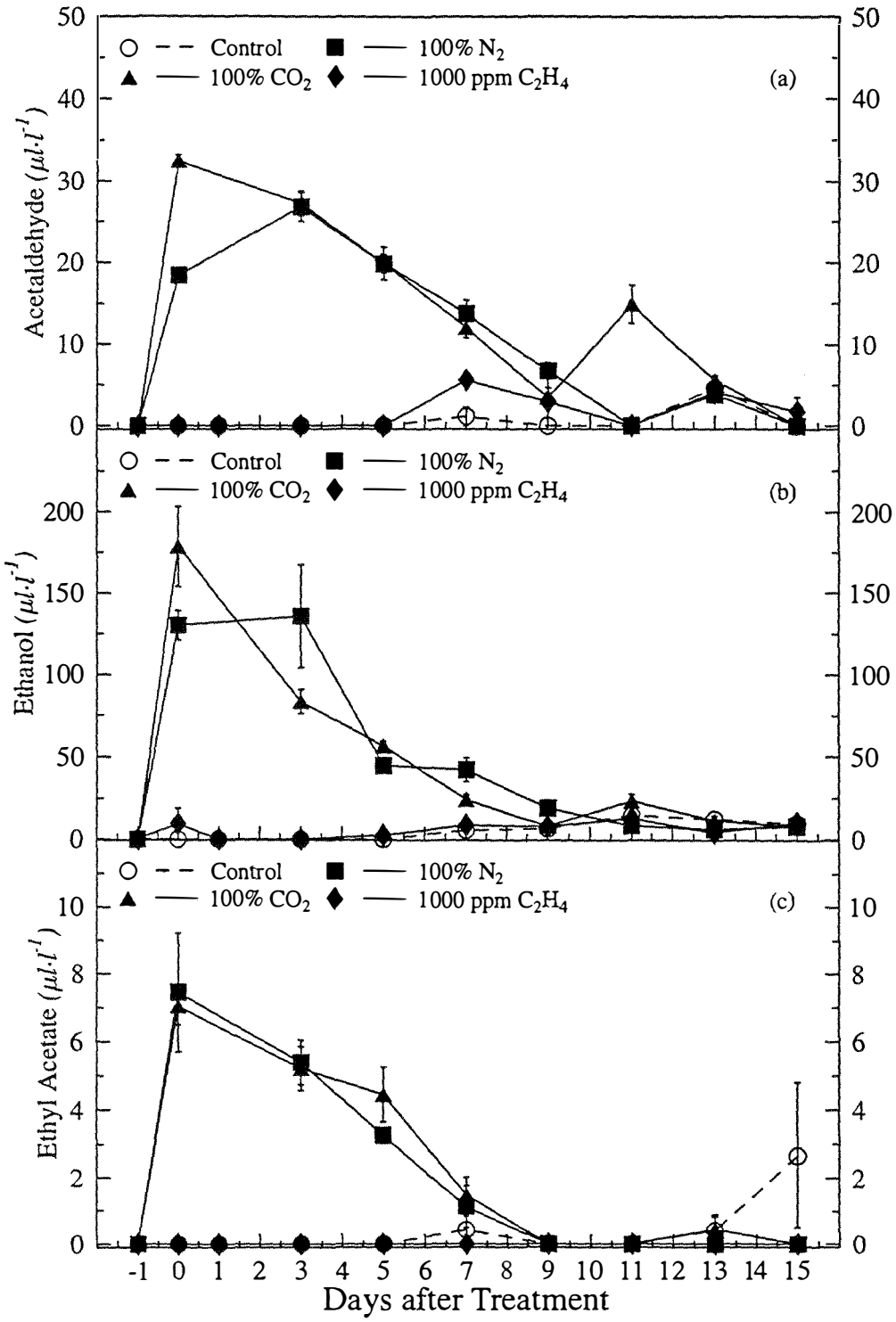


Fig. 4.2 Concentrations of headspace (a) acetaldehyde, (b) ethanol and (c) ethyl acetate from juice of 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

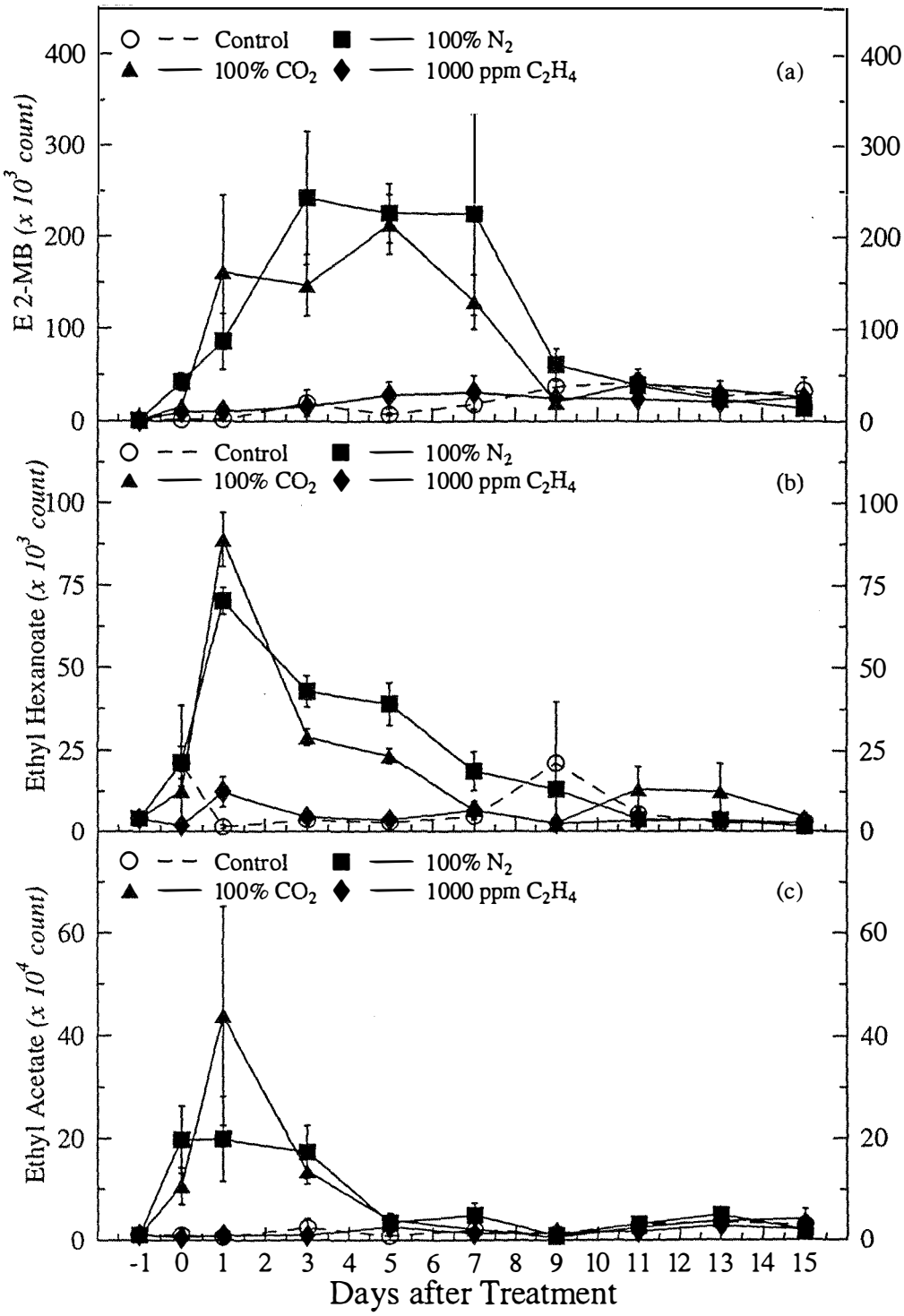


Fig. 4.3 Peak areas of (a) ethyl 2-methyl butanoate, (b) ethyl hexanoate and (c) ethyl acetate in juice from 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

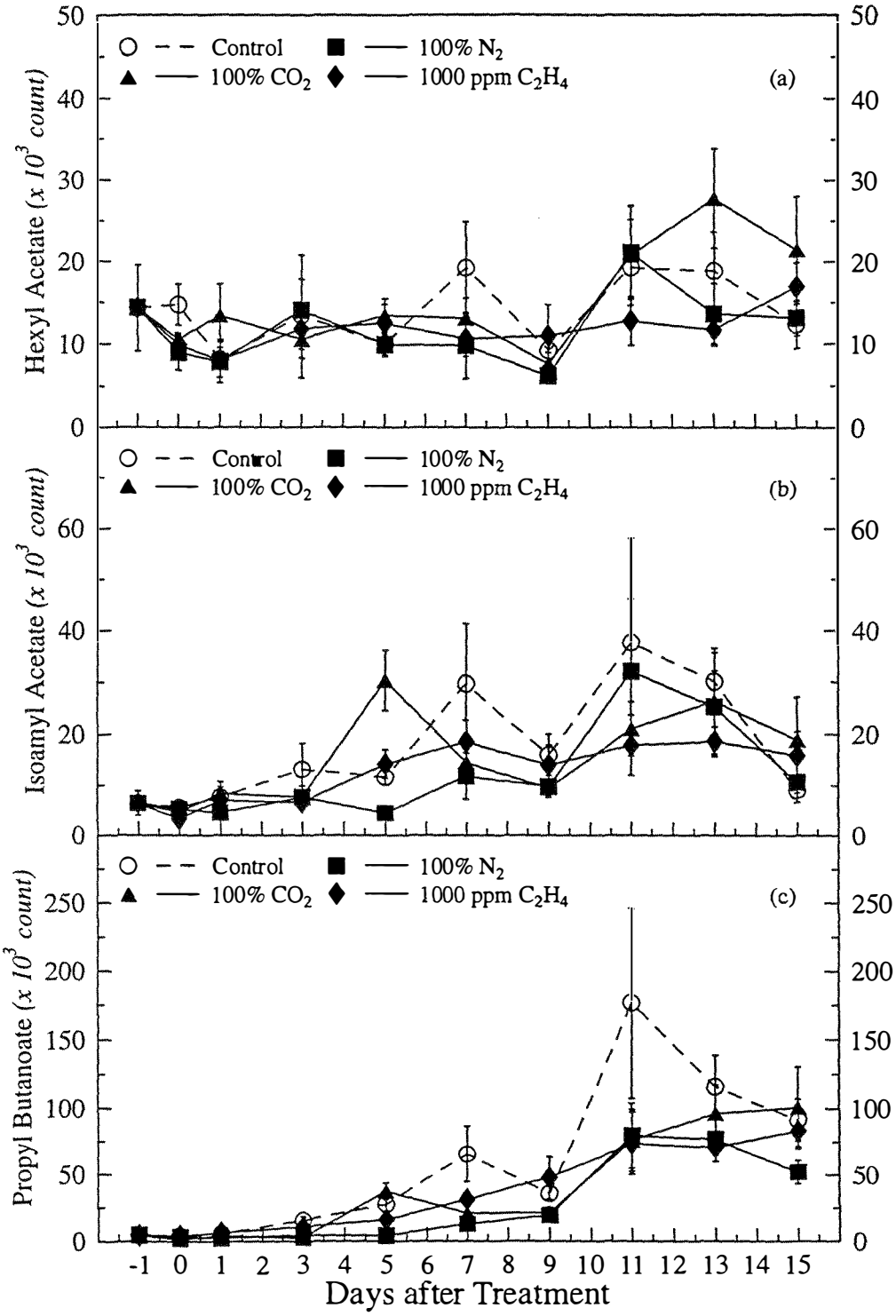


Fig. 4.4 Peak areas of (a) hexyl acetate, (b) isoamyl acetate and (c) propyl butanoate in juice from 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

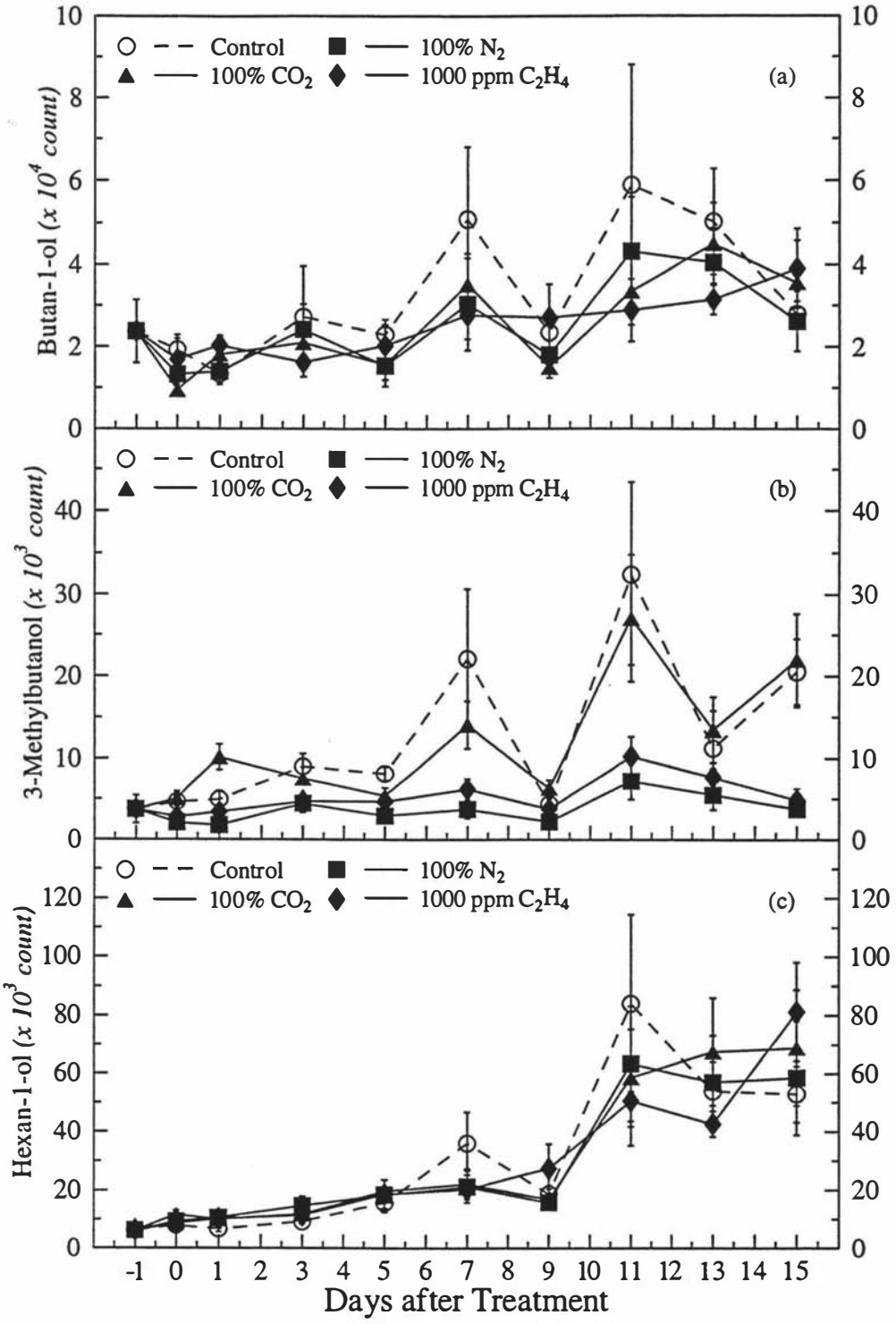


Fig. 4.5 Peak areas of (a) butan-1-ol, (b) 3-methyl butan-1-ol and (c) hexan-1-ol in juice from 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

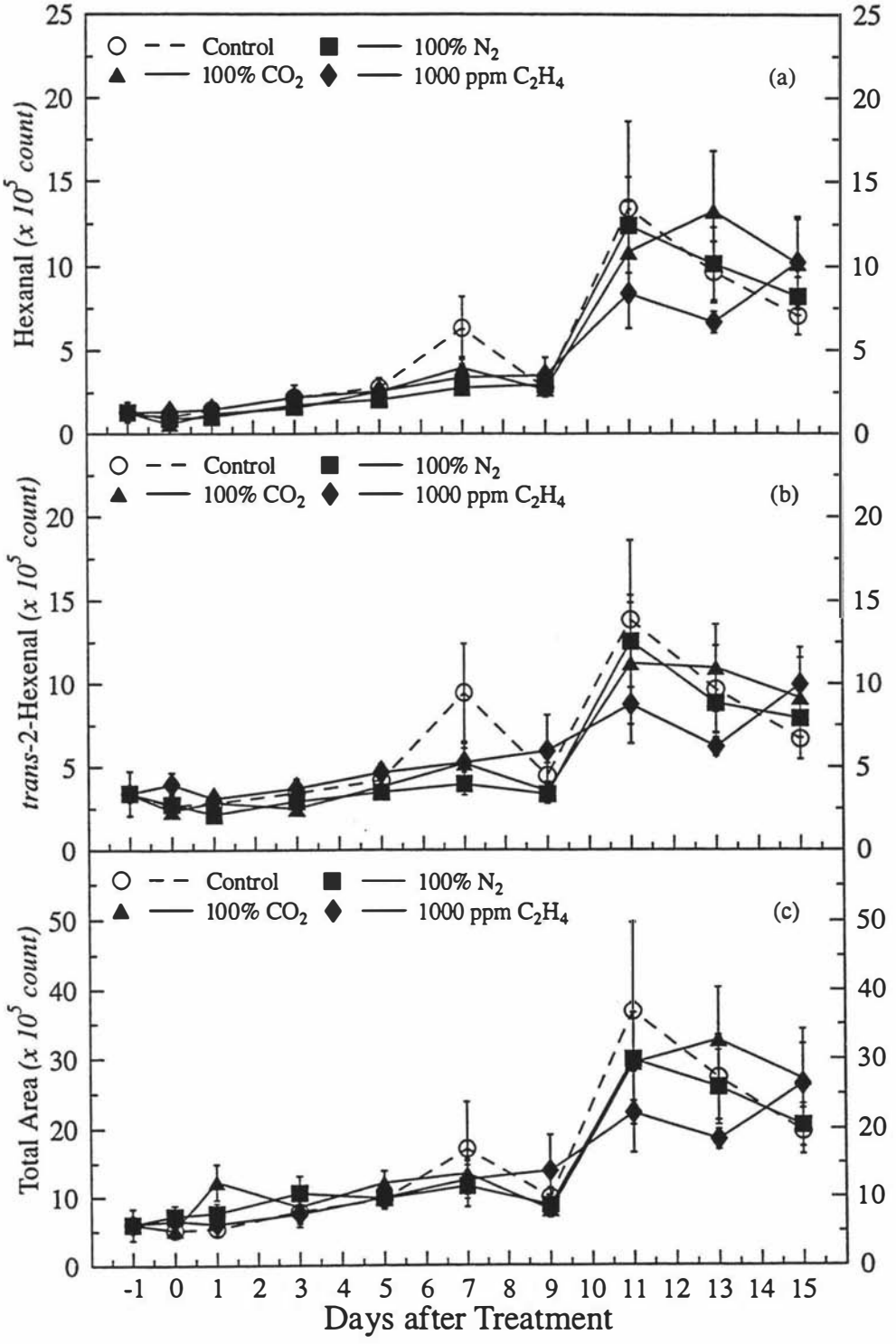


Fig. 4.6 Peak areas of (a) hexanal, (b) *trans*-2-hexenal and (c) total area in juice from 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

Treatments affected production of other esters differently. Isoamyl acetate concentration (**Fig. 4.4**b) was lowest following 100% N_2 ($P < 0.05$), while the concentration from the other treatments was similar to control. Treated fruits produced less ($P < 0.05$) propyl butanoate (**Fig. 4.4**c) than did control fruit and those fruit treated with 100% N_2 had the lowest amount. Hexyl acetate was not affected by the treatments used (**Fig. 4.4**a).

Concentration of 3-methyl butan-1-ol (**Fig. 4.5**b) in apple was decreased by 1000 ppm C_2H_4 and 100% N_2 treatments ($P < 0.05$) but not by 100% CO_2, while butan-1-ol (**Fig. 4.5**a) and hexan-1-ol (**Fig. 4.5**c), hexanal and *trans*-2-hexenal were not affected by the treatments (**Fig. 4.6**). However, hexanal and trans-2-hexenal concentration in C_2H_2 treated fruit were reduced during day 11 - 15 after treatment. Overall total peak area of volatile production, excluding ethanol and solvent peak areas, (**Fig. 4.6**c) was unaffected by treatments.

Table 4.4 Fruit skin colour in 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

	Lightness (%)				Hue Angle (°)			
Day	Ctrl	CO_2	N_2	C_2H_4	Ctrl	CO_2	N_2	C_2H_4
-1	62.2	62.2	62.2	62.2	111.9	111.9	111.9	111.9
0	61.9	61.1	61.9	61.6	111.2	112.1	111.7	111.5
1	62.2	61.9	62.0	62.3	111.8	112.1	111.6	111.6
3	62.6	62.6	64.3	63.8	111.4	111.1	110.8	110.7
5	64.4	64.2	62.3	63.9	110.5	110.1	111.2	110.9
7	64.7	64.1	63.5	65.0	109.7	110.5	110.1	110.1
9	65.3	65.9	65.1	66.3	109.0	108.9	109.7	108.4
11	67.6	66.3	66.2	66.0	106.9	107.8	107.7	107.3
13	68.4	68.2	67.2	66.8	105.8	106.4	106.6	107.4
15	68.6	68.0	68.6	67.9	106.1	106.4	105.9	106.2
c.v.	1.80%				0.88%			
Day	***				***			
Treat	ns				ns			
Day x Treat	ns				ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns)
Treatments : Ctrl = Control, CO_2 = 100% CO_2, N_2 = 100% N_2, C_2H_4 = 1000 ppm C_2H_4

Production of some volatiles such as propyl butanoate, butan-1-ol, 3-methyl butan-1-ol and hexan-1-ol from control fruit increased after 11 days at 20°C. This coincided with fruit yellowing in all treatments, indicating that they were fully ripe.

Treatments had no effect on fruit skin colour measured either as skin lightness or hue angle (**Table 4.4**). Lightness increased linearly (r^2 = 0.74) at an average rate of 0.42% per day, while hue angle decreased (loss of green colour) in a linear manner (r^2 = 0.78) at an average rate of 0.38° per day after treatment. No treatment effects were found on TSS, TA (**Table 4.5**) or TSS:TA ratio (**Table 4.6**). A slight increase in TSS occurred during storage, but TA decreased linearly (r^2 = 0.69) at an average rate of 0.008% per day, while TSS:TA ratio increased linearly (r^2 = 0.69) at 0.44 units per day. Juice pH (**Table 4.6**) increased in a quadratic manner (r^2 = 0.70) and was slightly higher ($P < 0.05$) in 100% CO_2 treated fruit than from other treatments which were not different from control.

Table 4.5 Total soluble solids (TSS) and titratable acids (TA) in juice from 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

	TSS (%)				TA (%)			
Day	Ctrl	CO_2	N_2	C_2H_4	Ctrl	CO_2	N_2	C_2H_4
-1	13.0	13.0	13.0	13.0	0.59	0.59	0.59	0.59
0	13.3	13.0	13.0	13.0	0.62	0.63	0.61	0.60
1	13.2	12.8	13.1	13.1	0.59	0.59	0.58	0.58
3	13.2	13.1	13.0	13.0	0.57	0.56	0.56	0.54
5	13.2	13.2	13.4	13.1	0.56	0.54	0.57	0.57
7	13.2	12.8	13.3	13.2	0.57	0.54	0.55	0.53
9	13.3	12.8	13.1	13.4	0.52	0.48	0.52	0.51
11	13.5	12.9	13.4	13.2	0.49	0.49	0.44	0.52
13	13.6	13.1	13.4	13.5	0.50	0.46	0.49	0.49
15	13.5	13.2	13.6	13.4	0.48	0.45	0.49	0.47
c.v.	2.68%				5.01%			
Day	**				***			
Treat	ns				ns			
Day x Treat	ns				ns			

Levels of Significance at P = 0.05 (*), 0.01 (**), or 0.001 (***), non significant (ns)
Treatments : Ctrl = Control, CO_2 = 100% CO_2, N_2 = 100% N_2, C_2H_4 = 1000 ppm C_2H_4

Table 4.6 TSS:TA ratio and pH in juice from 'Granny Smith' apples (previously stored for 34 days at 0°C) after treatment with 100% CO_2, 100% N_2 or 1000 ppm C_2H_4 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 1***).

	TSS:TA Ratio				Juice pH			
Day	Ctrl	CO_2	N_2	C_2H_4	Ctrl	CO_2	N_2	C_2H_4
-1	22.1	22.1	22.1	22.1	3.62	3.62	3.62	3.62
0	21.5	20.6	21.5	21.5	3.59	3.62	3.59	3.60
1	22.6	21.6	22.5	22.7	3.61	3.61	3.60	3.59
3	23.1	23.4	23.0	24.2	3.60	3.62	3.61	3.62
5	23.5	24.4	23.7	23.0	3.63	3.66	3.64	3.62
7	23.3	23.6	24.1	24.7	3.63	3.65	3.61	3.63
9	25.7	26.5	25.3	26.4	3.63	3.66	3.64	3.64
11	26.9	26.4	30.7	25.4	3.65	3.68	3.68	3.67
13	27.2	28.6	27.1	27.5	3.67	3.70	3.66	3.66
15	28.4	29.0	27.8	28.4	3.69	3.73	3.70	3.72
c.v.	6.04%				0.45%			
Day	***				***			
Treat	ns				*			
Day x Treat	ns				ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns)
Treatments : Ctrl = Control, CO_2 = 100% CO_2, N_2 = 100% N_2, C_2H_4 = 1000 ppm C_2H_4

4.4.2 Effect of Exposure Time to 100% CO_2 on 'Braeburn' and 'Red Delicious' Apples (*Experiment 2* and *Experiment 3*)

Exposing apples to 100% CO_2 affected respiration rates after treatment differently between cultivars; regardless of exposure time. CO_2 production of 'Braeburn' (**Fig. 4.7**a) was increased over control ($P < 0.05$) 1 day after treatment but there was no consistent effect of treatment in 'Red Delicious' apples (**Fig. 4.8**a). The sharp drop in 'Red Delicious' respiration rate at day 3 was caused by a power cut which resulted in a decrease in storage temperature prior to measurement. Apart from the increase in CO_2 production at day 1 in the 100% CO_2 treated 'Braeburn' and the decrease at day 3 of the 24 hour treated 'Red Delicious' apples, generally fruit respiration rate of both apple cultivars in all treatments remained relatively constant for 7 days at 20°C.

Ethylene production of both cultivars was significantly suppressed by 100% CO_2 ($P < 0.05$) at 1 day after treatment (**Fig. 4.7**b and **Fig. 4.8**b). The residual effect of

suppression was greater with a longer exposure time to 100% CO_2 in both cultivars. Ethylene production of both 100% CO_2 treated and control 'Braeburn' apples generally increased gradually during 7 days at 20°C. In 'Red Delicious' apples, ethylene production in control fruit decreased gradually for 7 days at 20°C, while that in 100% CO_2 treated fruit decreased sharply at day 1, then increased at day 3 or day 5 and slightly declined thereafter.

Cumulative weight loss of both cultivars (**Fig. 4.7**c and **Fig. 4.8**c) was slightly increased by the treatments, the longer the exposure time to 100% CO_2, greater the loss ($P < 0.05$). Generally, weight loss increased linearly with time at 20°C at 0.24% ($r^2 = 0.94$) and 0.30% ($r^2 = 0.95$) per day for 'Braeburn' and 'Red Delicious', respectively.

'Braeburn' apples exposed to 100% CO_2 for 12 to 24 hours, but not 6 hours, retained higher flesh firmness than control ($P < 0.05$) (**Fig. 4.7**d). Treatments had no significant effect on flesh firmness in 'Red Delicious' apples (**Fig. 4.8**d).

Treatments enhanced production of headspace volatiles from both 'Braeburn' and 'Red Delicious' apples (**Table 4.7**). Headspace concentrations of acetaldehyde and ethanol from 'Braeburn' (**Fig. 4.9**) and 'Red Delicious' (**Fig. 4.10**) increased immediately after treatment with 100% CO_2, regardless of exposure time, while headspace ethyl acetate increased to peak 1 day later. In 'Braeburn', headspace gases (**Fig. 4.9**) increased in proportion to time of exposure; mean concentrations of ethanol 1 day after exposure to 100% CO_2 for 6, 12, 18 and 24 hours were about 11, 22, 33, and 54 fold, respectively, greater than controls. This difference between treatments generally persisted during 7 days at 20°C, except for 6 hours exposure; acetaldehyde and ethanol gradually declined and ethyl acetate remained relatively constant. Headspace volatiles from 'Red Delicious' (**Fig. 4.10**) also increased in proportion to exposure of 12, 24 and 48 hours of 100% CO_2, with mean concentrations of acetaldehyde at day 1 being 11, 17, and 24 fold, ethanol 39, 85, and 149 fold, and ethyl acetate 46, 87, and 115 fold, respectively, greater than in controls. Enhanced concentrations of headspace volatiles persisted for 7 days at 20°C, remaining relatively constant for acetaldehyde and declining gradually for ethanol and ethyl acetate.

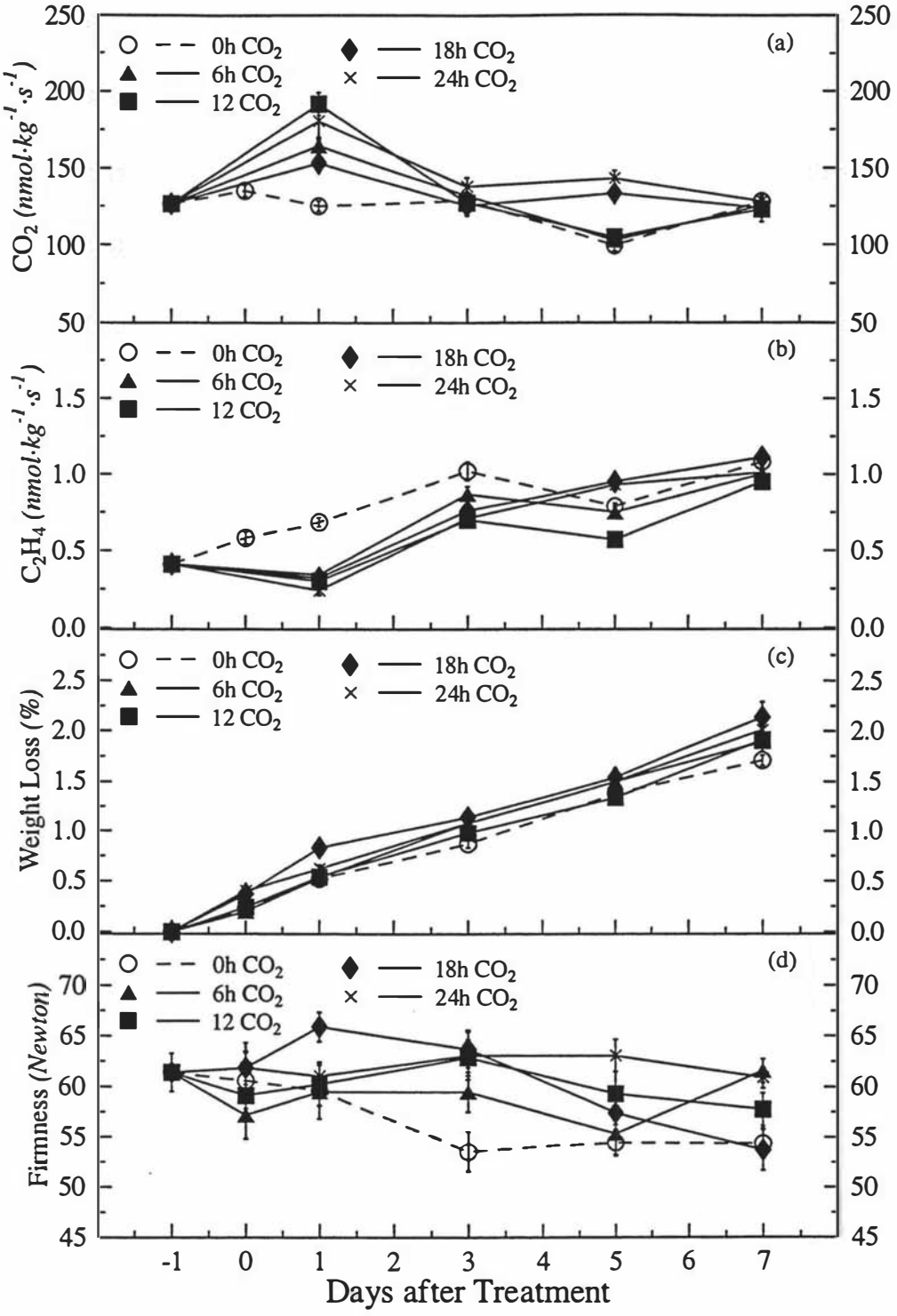


Fig. 4.7 Fruit (a) respiration rate, (b) ethylene production, (c) weight loss, and (d) flesh firmness in 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

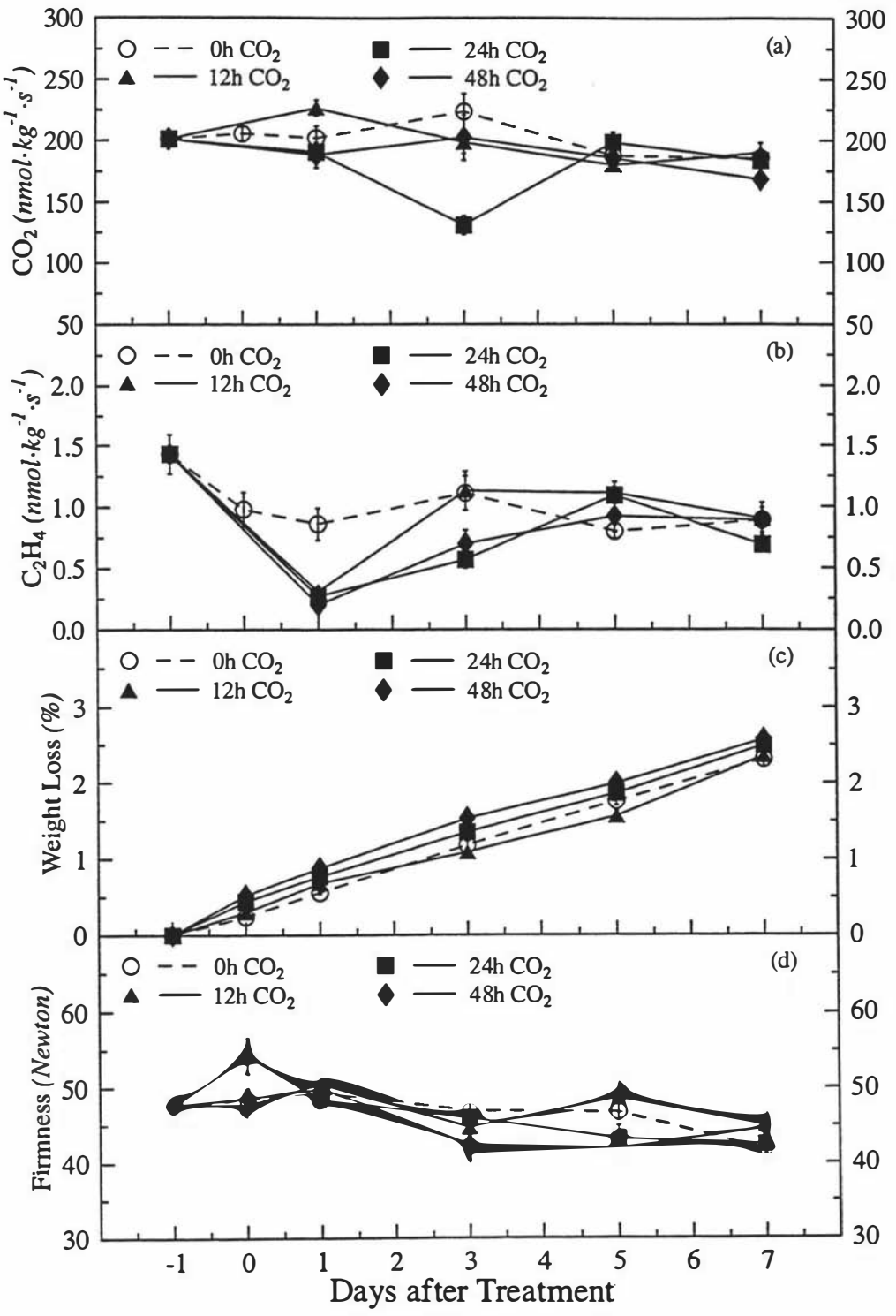


Fig. 4.8 Fruit (a) respiration rate, (b) ethylene production, (c) weight loss, and (d) flesh firmness in 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

Treatments enhanced production of selected ethyl esters in both 'Braeburn' (**Fig. 4.11** and **Fig. 4.12**) and 'Red Delicious' (**Fig. 4.13** and **Fig. 4.14**), the longer the exposure time to CO_2 the greater the enhancement (**Table 4.7**). In 'Braeburn' apples, 18 and 24 hours exposure had similar effects in enhancing ethyl esters, and the concentrations of ethyl 2-methyl butanoate (day 5), ethyl acetate(day 3), ethyl butanoate (day 3), and ethyl hexanoate (day 1) following the 24 hour treatment were about 4, 9, 14, and 75 fold greater than control, respectively (**Fig. 4.11** and **Fig. 4.12**a). Enhanced concentration of these esters from treated apples persisted for 7 days at 20°C, although they generally declined after the peak. Enhancement of these esters from the 6 hour treatment was comparatively less than other treatments.

Table 4.7 Concentration at peak production of selected volatile compounds in juice from 'Braeburn' and 'Red Delicious' apples (both previously stored for 4 months at 0°C) after treatment with 100% CO_2 for different times at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2*** and ***Experiment 3***).

cv. 'Braeburn'	Treatment / Concentration				
Compound	Ctrl	6h	12h	18h	24h
Headspace Volatiles ($\mu l \cdot l^{-1}$)					
Acetaldehyde	4.0	23.9	13.7	19.3	22.3
Ethanol	8.1	22.4	43.2	72.9	103.5
Ethyl acetate	28.4	33.2	77.7	79.9	115.8
Aroma Volatiles ($\mu mol \cdot l^{-1}$)					
Ethyl butanoate	147.1	491.5	727.6	823.2	957.1
Ethyl 2-methyl butanoate	108.6	182.0	392.2	382.6	436.7
Ethyl hexanoate	22.2	128.0	212.5	294.9	349.5
Ethyl acetate	849.1	2091.1	3247.5	4538.1	5301.8
Ethanol	631.4	1595.0	2275.6	4483.7	7127.6

cv. 'Red Delicious'	Treatment / Concentration			
Compound	Ctrl	12h	24h	48h
Headspace Volatiles ($\mu l \cdot l^{-1}$)				
Acetaldehyde	11.2	27.3	34.0	47.2
Ethanol	9.4	70.5	151.0	237.9
Ethyl acetate	6.4	40.8	76.3	102.6
Aroma Volatiles ($\mu mol \cdot l^{-1}$)				
Ethyl butanoate	242.8	943.6	1020.3	921.5
Ethyl 2-methyl butanoate	130.8	212.5	397.6	284.0
Ethyl hexanoate	78.6	603.1	570.7	705.0
Ethyl acetate	801.3	3786.4	7114.0	9210.5
Ethanol	729.8	13097.4	27173.3	49258.4

data were mean concentrations of 4 replicates

In 'Red Delicious' apples, 24 and 48 hours exposure to CO_2 exerted similar effects in stimulating production of ethyl esters, which peaked 1 to 5 days after treatment. Peak concentrations of ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate (**Fig. 4.13**) and ethyl acetate (**Fig. 4.14**a) from fruit exposed for 24 hours were about 4, 8, 25 and 75 fold higher than controls, respectively. This enhancement of selected ethyl esters in 24 or 48 hour treated fruit generally persisted for 7 days at 20°C. In general, enhancement of ethyl esters from the 12 hour treatment was comparatively less than from 24 hour and 48 hour treatments and concentrations generally decreased after reaching their peaks to levels similar to controls 7 days after treatment. Appreciable concentrations of ethyl esters were also produced by both 'Braeburn' and 'Red Delicious' control fruit after 3 - 7 days at 20°C. Ethyl pentanoate production in all treatments was variable without any obvious pattern of changes, particularly in 'Braeburn' apples (**Fig. 4.12**b).

Treatments with 100% CO_2 significantly ($P < 0.05$) depressed emission of butyl and hexyl acetate in juice from both cultivars. The degree of suppression was greater ($P < 0.05$) the longer the exposure time of fruit to CO_2 and this effect was more pronounced in 'Red Delicious' than in 'Braeburn'. Concentrations of butyl acetate (**Fig. 4.12**c) and hexyl acetate (**Fig. 4.15**a) in juice of 'Braeburn' apples immediately after 24 hours CO_2 exposure were approximately 2.5 fold lower than in controls. In 'Red Delicious', reduction in butyl acetate (**Fig. 4.14**c) and hexyl acetate concentrations (**Fig. 4.16**a) immediately after 48 hours exposure were about 11 and 7 fold less and after 24 hour exposure 5 and 3 fold less, respectively, than controls.

Butyl acetate from control 'Braeburn' apples increased slightly at day 0 and then declined gradually, but following 6 hours CO_2 treatment concentrations remained relatively constant for 7 days. After 12 hours CO_2 treatment, butyl acetate decreased at day 3, then increased slightly, while following 18 and 24 hours of treatment concentration decreased markedly at day 0, increased slightly at day 1, again decreased slightly at day 3 and remained approximately constant thereafter (**Fig. 4.12**c). In 'Red Delicious' apples, butyl acetate from control fruit generally decreased from day 0 to day 3 and remained approximately constant thereafter. Butyl acetate from all CO_2 treated

fruit decreased sharply at day 0 and remained more or less constant thereafter (**Fig. 4.14**c).

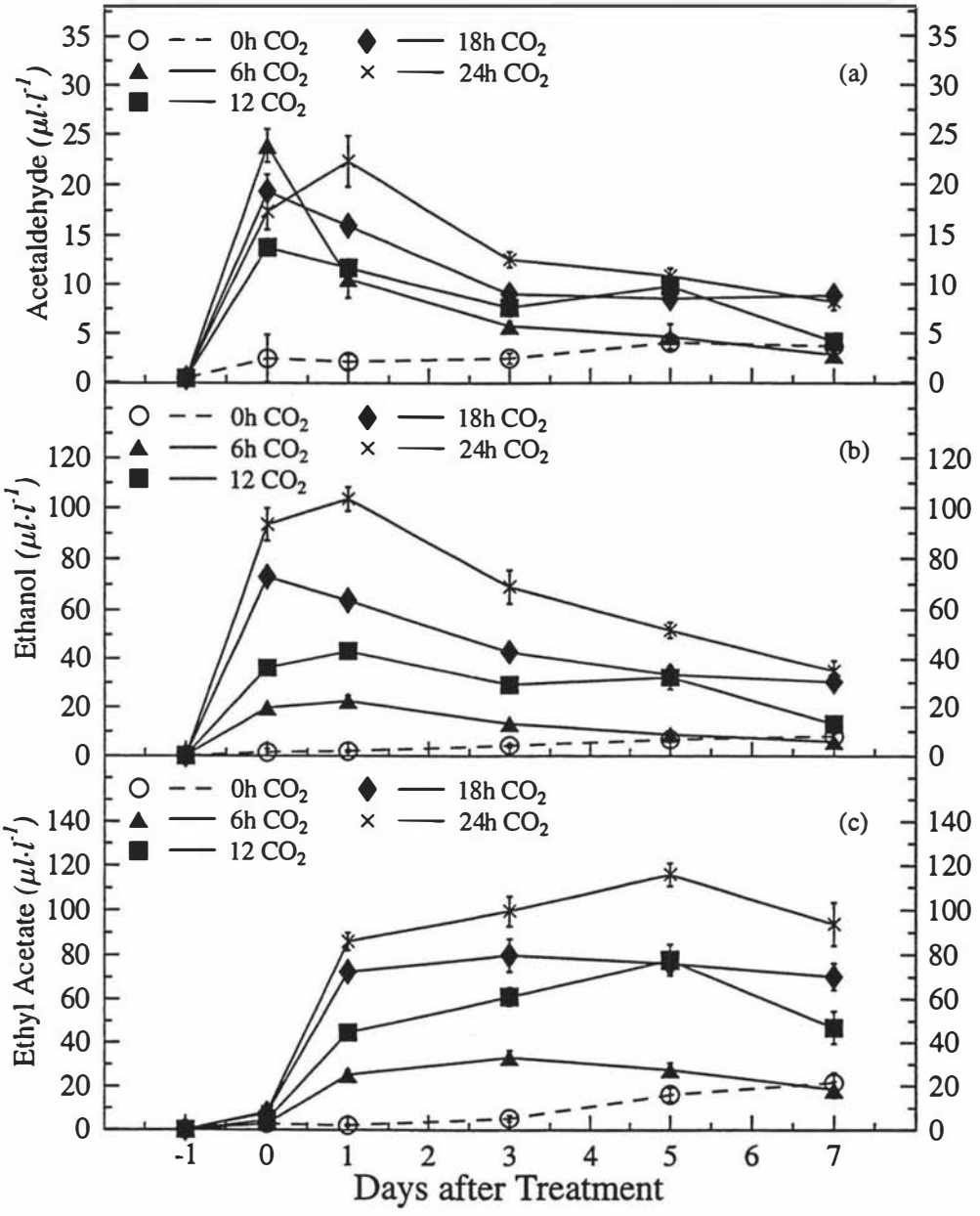


Fig. 4.9 Concentrations of headspace (a) acetaldehyde, (b) ethanol and (c) ethyl acetate from juice of 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

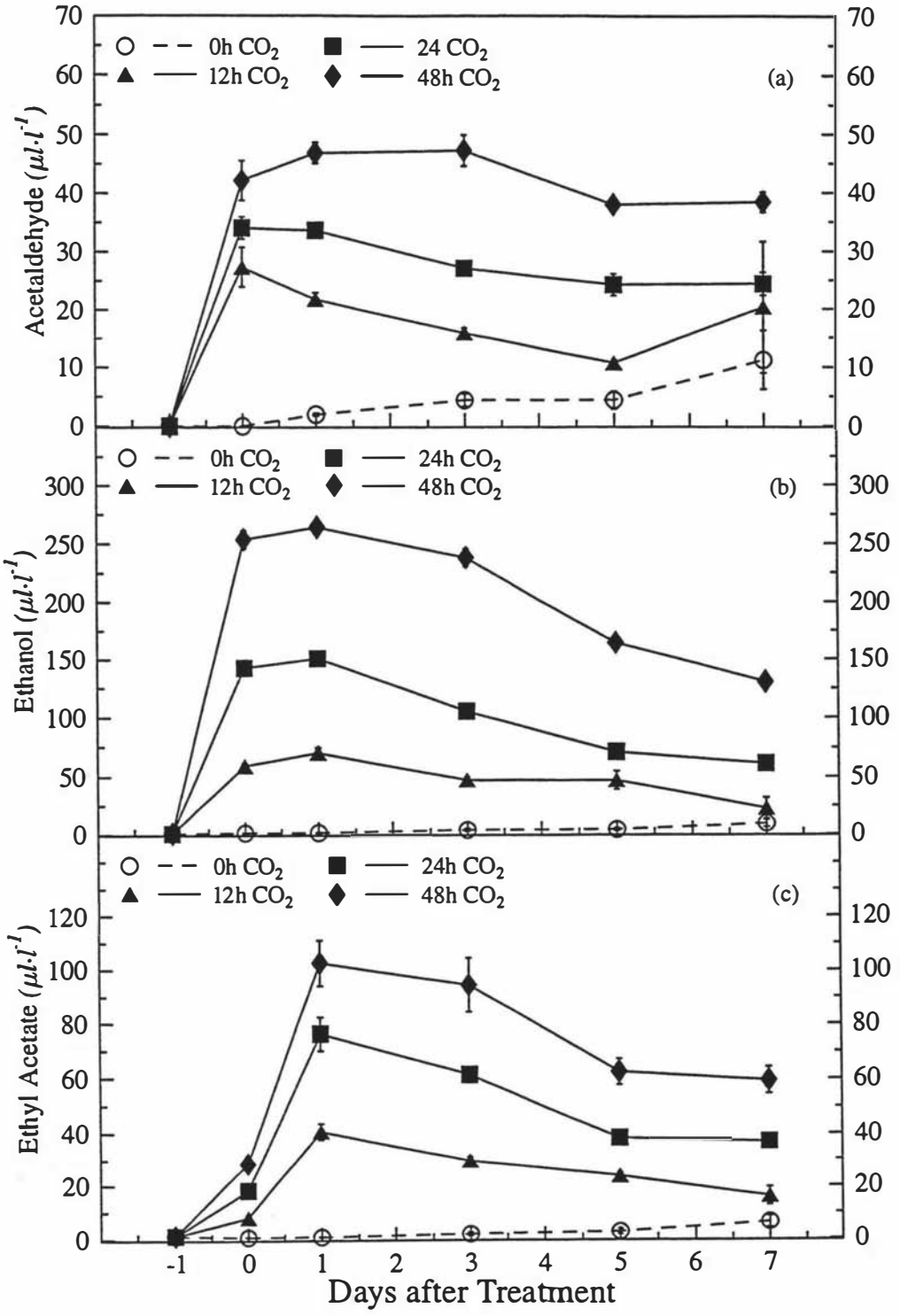


Fig. 4.10 Concentrations of headspace (a) acetaldehyde, (b) ethanol and (c) ethyl acetate from juice of 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

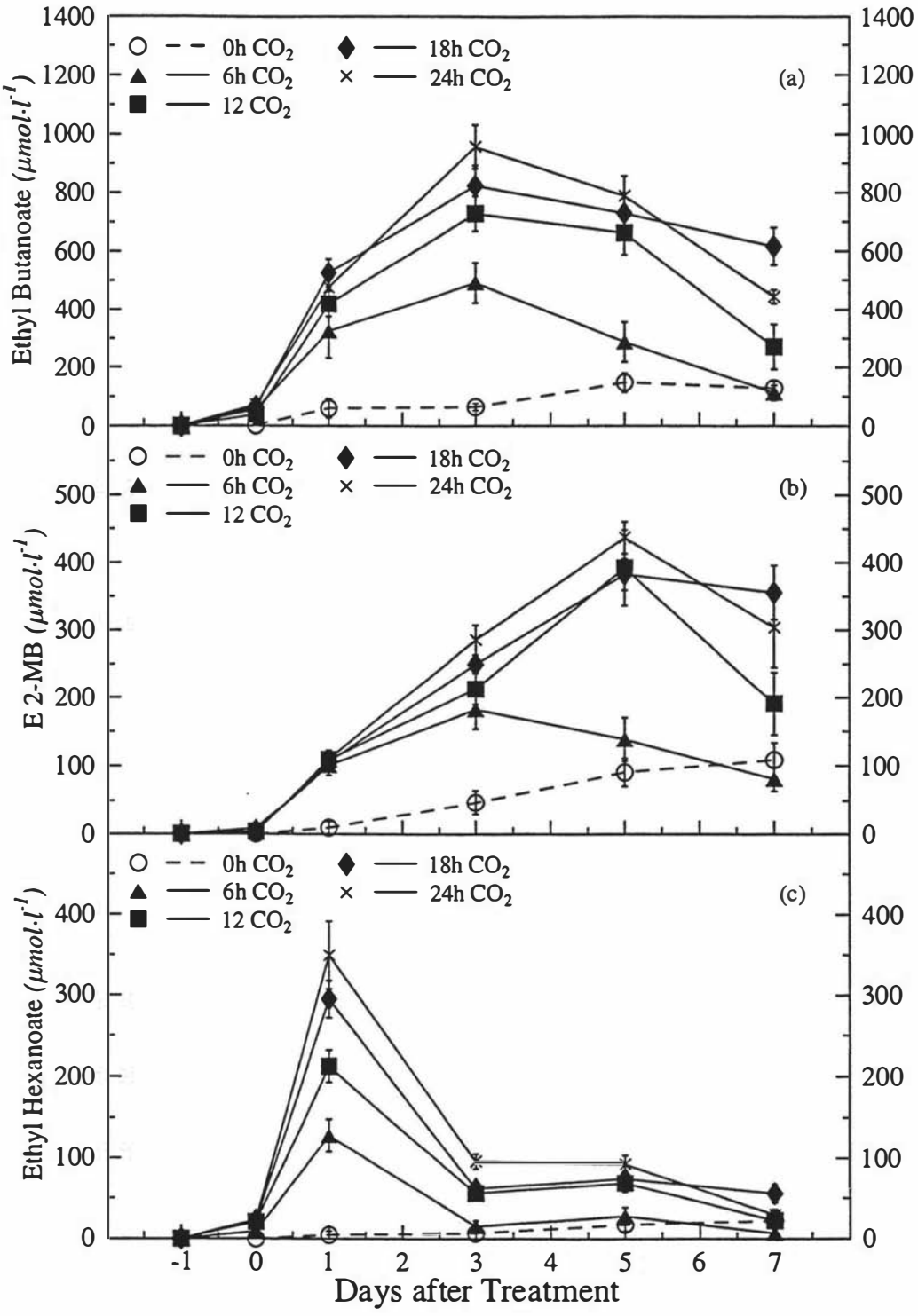


Fig. 4.11 Concentrations of (a) ethyl butanoate, (b) ethyl 2-methyl butanoate and (c) ethyl hexanoate in juice from 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

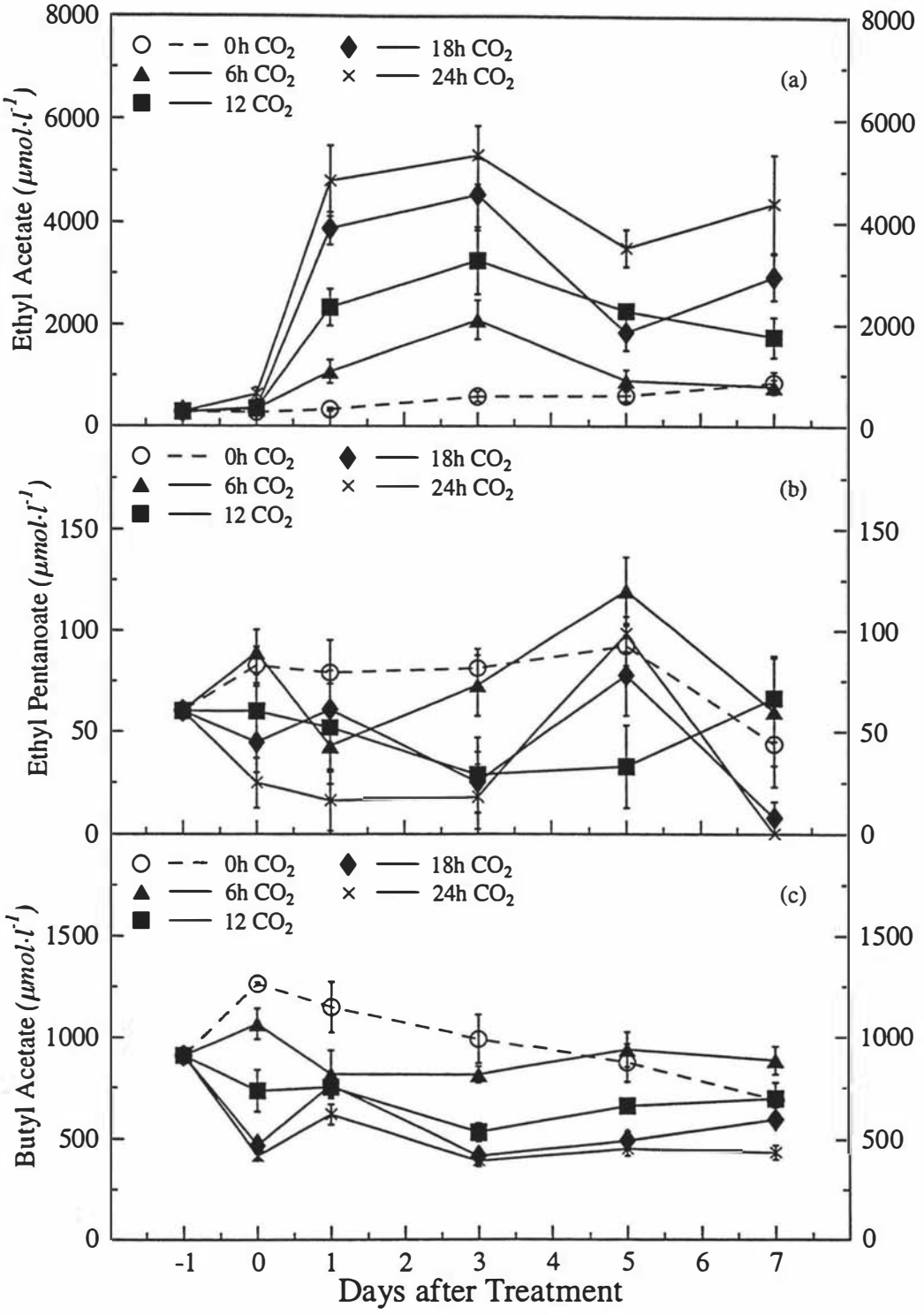


Fig. 4.12 Concentrations of (a) ethyl acetate, (b) ethyl pentanoate and (c) butyl acetate in juice from 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

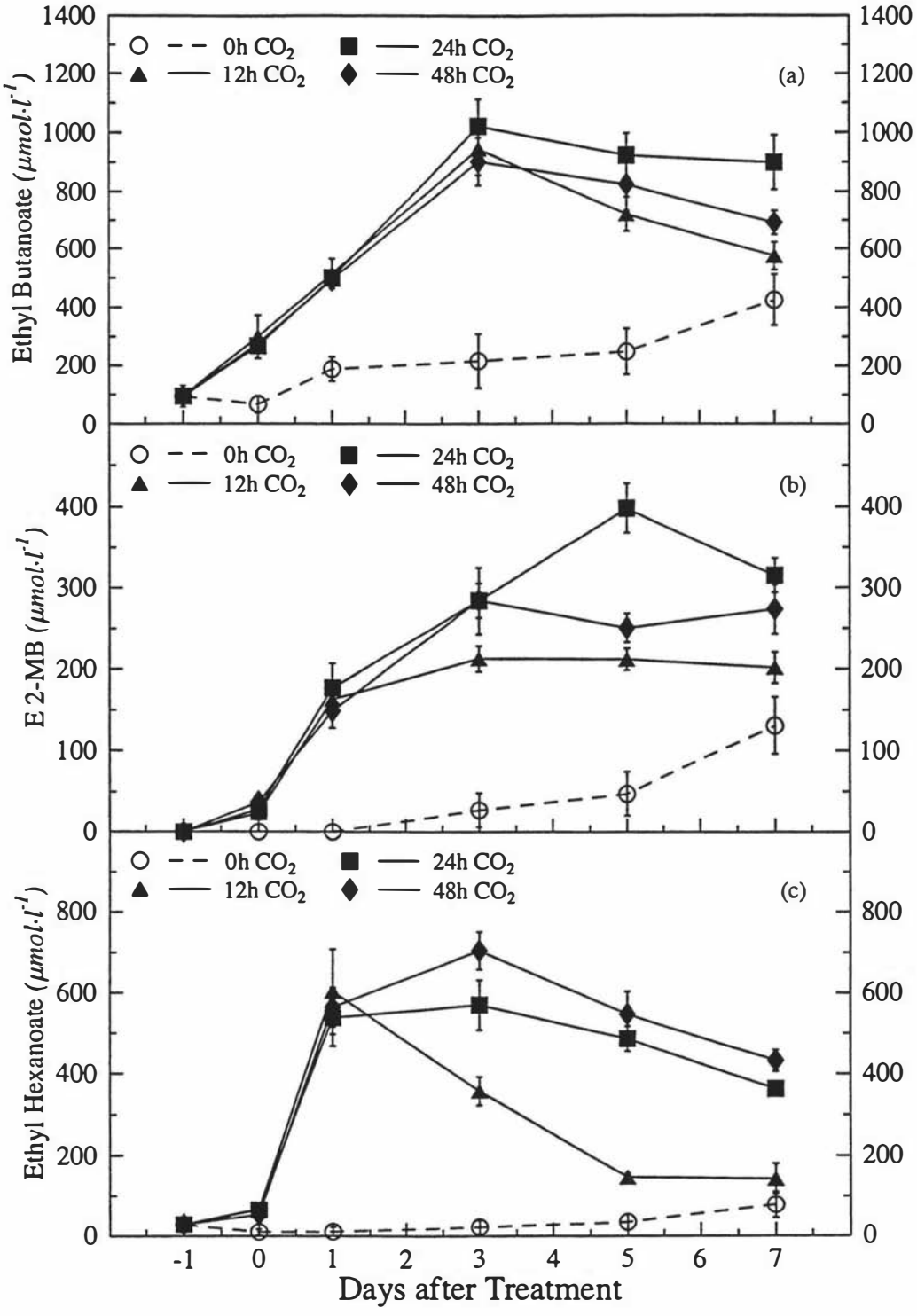


Fig. 4.13 Concentrations of (a) ethyl butanoate, (b) ethyl 2-methyl butanoate and (c) ethyl hexanoate in juice from 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

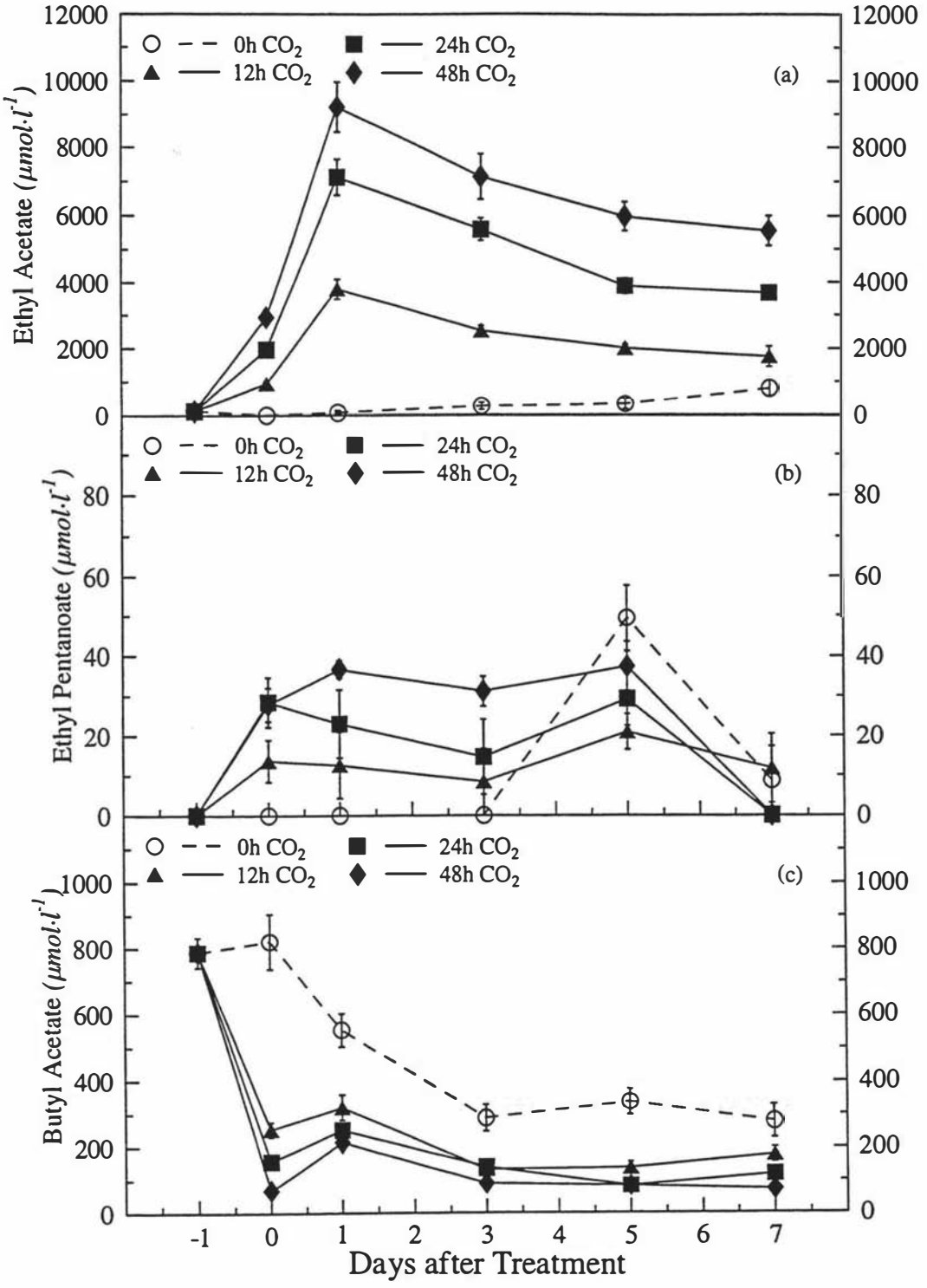


Fig. 4.14 Concentrations of (a) ethyl acetate, (b) ethyl pentanoate and (c) butyl acetate in juice from 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 0, 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

In control 'Braeburn' fruit, hexyl acetate concentration remained relatively constant during 7 days, while in 'Red Delicious' concentration fell during the first 3 days remaining constant thereafter. Treatment with CO_2 generally depressed hexyl acetate, the longer the exposure time the greater the reduction (**Fig. 4.15**a and **Fig. 4.16**a).

Both *trans*-2-hexenal and hexanal were affected slightly by CO_2 treatments. Subjecting 'Braeburn' apples for 12 to 24 hours, but not 6 hours exposure, slightly decreased ($P < 0.05$) *trans*-2-hexenal (**Fig. 4.15**b) and hexanal (**Fig. 4.15**c) immediately after treatment, but overall production of these aldehydes were similar to control. In 'Red Delicious' apples, both aldehydes (**Fig. 4.16**b and **Fig. 4.16**c) were reduced by treatments used ($P < 0.05$) and the 48 hours exposure caused the greatest reduction. Both *trans*-2-hexenal and hexanal from all treatments of 'Braeburn' apples generally increased during 7 days (**Fig. 4.15**b and **Fig. 4.15**c). These aldehydes from 'Red Delicious' apples (**Fig. 4.16**b and **Fig. 4.16**c) also increased after treatment, except that the increase was less and occurred later, the longer the exposure to CO_2.

Production of methanol from 'Braeburn' apples was unaffected by treatment and there were no obvious pattern of change during 7 days shelf-life (**Fig. 4.17**a). In 'Red Delicious' apples, methanol was not detected in this experiment (**Fig. 4.19**a). Concentration of ethanol from all CO_2 treated apples was substantially enhanced after treatment, compared with control for both cultivars, with a positive relationship between exposure time to CO_2 and ethanol concentration for both cultivars. Peak production occurred at day 0 or day 1, depending on treatment (**Fig. 4.17**b and **Fig. 4.19**b). Ethanol was present in much higher concentrations in 'Red Delicious' than in 'Braeburn'.

In general, propan-1-ol concentration from 'Braeburn' apples of all treatments increased moderately during 7 days at 20°C with concentration in juice from 12, 18 and 24 hours CO_2 treatment generally being slightly higher than controls (**Fig. 4.17**c). In 'Red Delicious' apples, propan-1-ol was markedly enhanced ($P < 0.001$) at day 0 and day 1, especially following the 24 and 48 hour treatments, but after 5 days there was little difference from controls (**Fig. 4.19**c).

Concentrations of butan-1-ol and hexan-1-ol in juice from 'Braeburn' apples were not affected by the treatments, and they declined slowly during 7 days (**Fig. 4.18**a

and **Fig. 4.18**b). In contrast, butan-1-ol in CO_2 treated 'Red Delicious' was markedly depressed ($P < 0.001$) compared with control fruit (**Fig. 4.20**a).

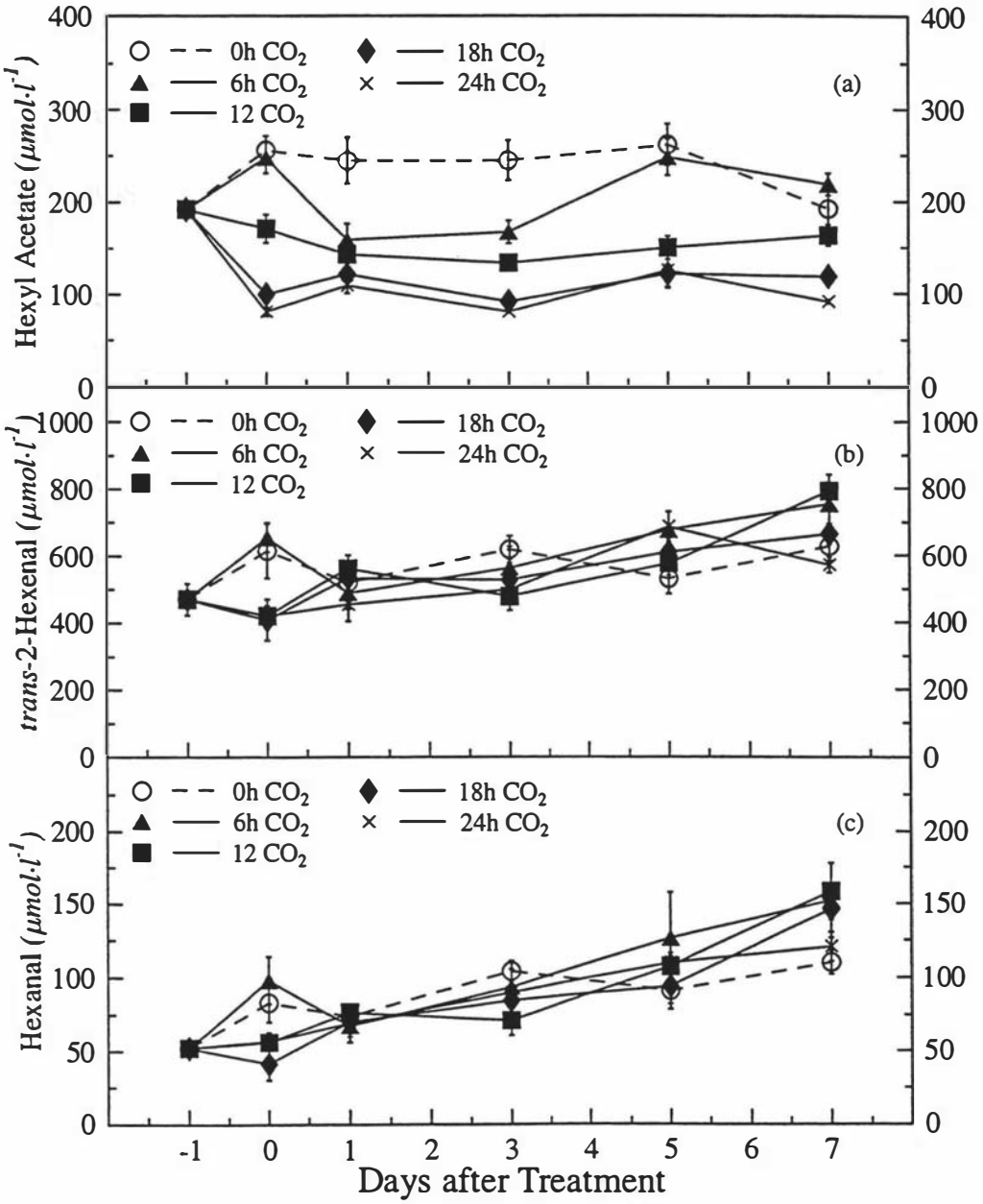


Fig. 4.15 Concentrations of (a) hexyl acetate, (b) *trans*-2-hexenal and (c) hexanal in juice from 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

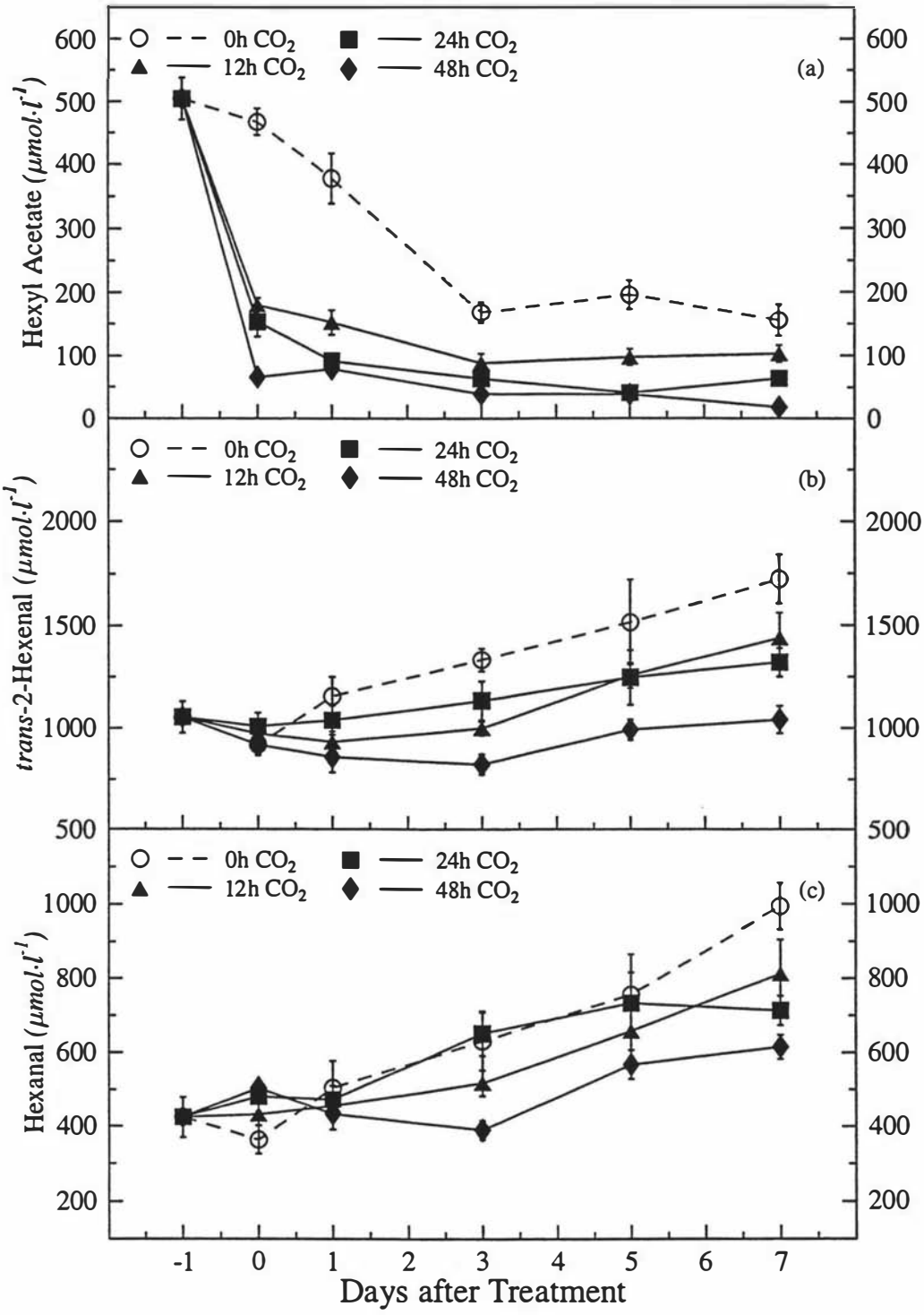


Fig. 4.16 Concentrations of (a) hexyl acetate, (b) *trans*-2-hexenal and (c) hexanal in juice from 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO2 for 0, 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

Hexan-1-ol in CO_2 treated 'Red Delicious' apples was slightly enhanced at day 0 after treatment but production was depressed compared with controls from day 1 through day 7 (**Fig. 4.20**b).

Total volatile production, indicated as total peak area (excluding solvent and internal standard peaks), of treated apples significantly exceeded controls ($P < 0.05$) in both 'Braeburn' (**Fig. 4.18**c) and 'Red Delicious' (**Fig. 4.20**c) cultivars; this reflected a higher production of some volatile compounds, in particular ethanol, from treated apples. The pattern of production was similar to that of ethanol, especially in 'Red Delicious'; because it was present in such high concentrations in this cultivar it dominated the total area curve.

Concentrations of most volatiles monitored such as headspace volatiles, ethyl esters, alcohols, and aldehydes, as well as total peak area from 'Red Delicious' apples exposure to 24 hour CO_2 were relatively greater than from 'Braeburn', except for butyl acetate, hexyl acetate, ethyl 2-methyl butanoate and methanol which were greater in the latter cultivar.

Exposing 'Braeburn' apples to varying durations of 100% CO_2 had no significant effects on fruit quality attributes, including skin colour (**Table 4.8**), juice TSS, TA (**Table 4.9**), TSS:TA ratio and juice pH (**Table 4.10**). In general, fruit skin colour and juice pH increased slightly with time after treatment, while juice TSS, TA and TSS:TA ratio fluctuated slightly without any obvious patterns during 7 days at 20°C. However, exposure of 'Red Delicious' apples to CO_2 for 24 or 48 hours slightly lowered ($P < 0.05$) TA content (**Table 4.12**), and increased ($P < 0.05$) TSS:TA ratio and juice pH (**Table 4.13**) compared to control, while fruit skin colour (**Table 4.11**) was unaffected. Fruit skin colour and juice TSS of 'Red Delicious' apples in all treatments generally did not change after treatment, while juice TA slightly decreased and juice pH and TSS:TA ratio increased slightly with time.

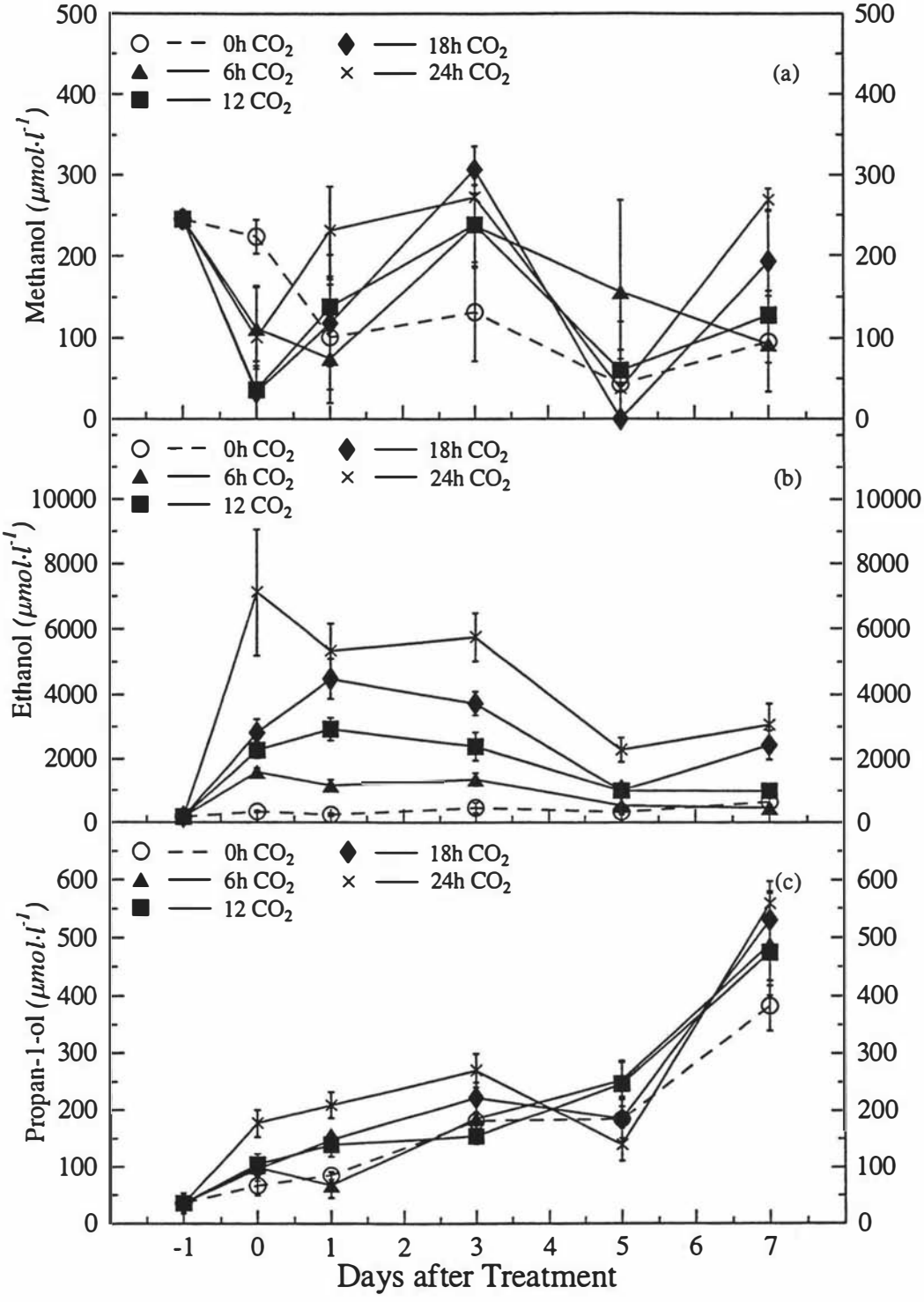


Fig. 4.17 Concentrations of (a) methanol, (b) ethanol and (c) propan-1-ol in juice from 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

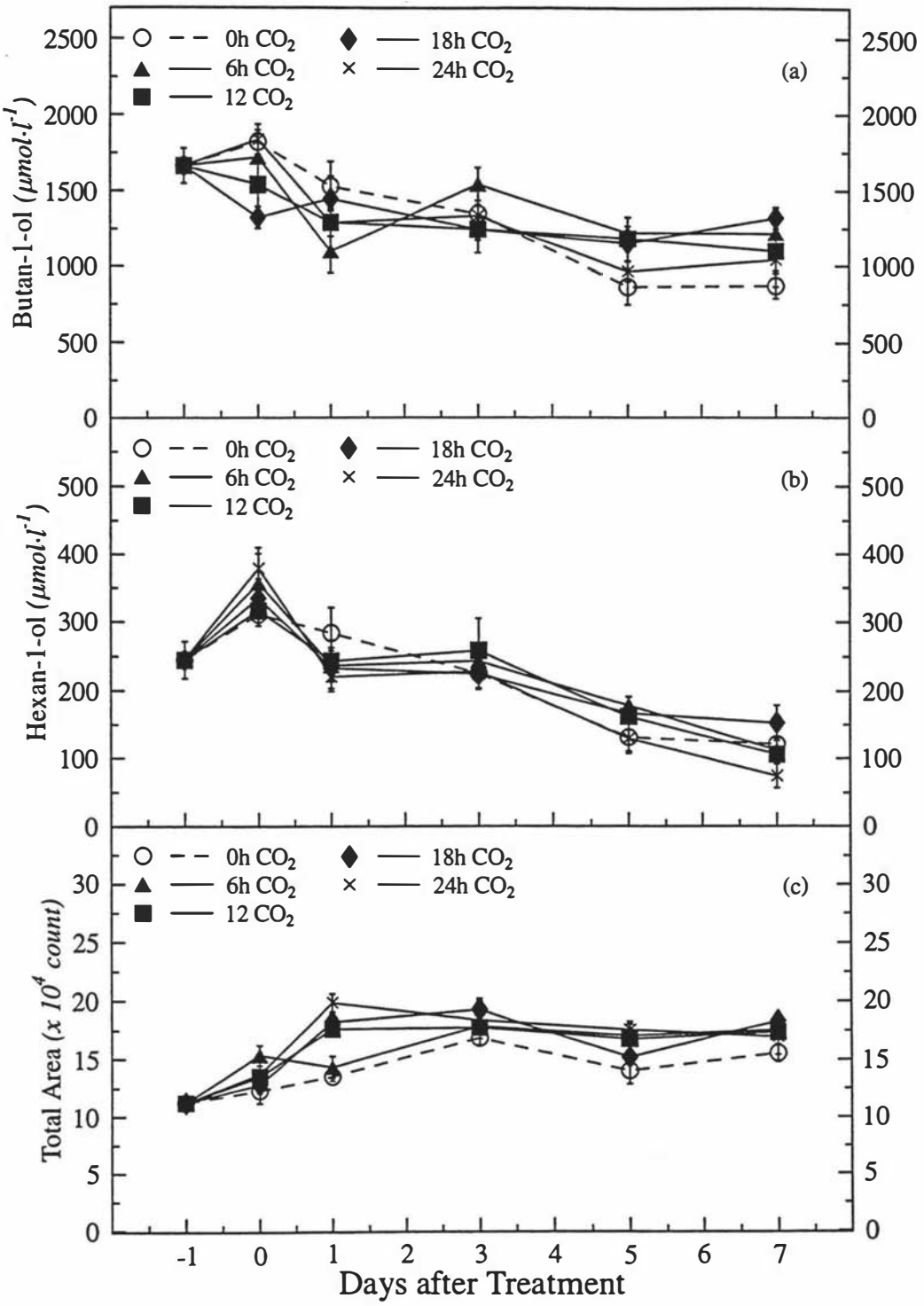


Fig. 4.18 Concentrations of (a) butan-1-ol, (b) hexan-1-ol and (c) total peak area in juice from 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

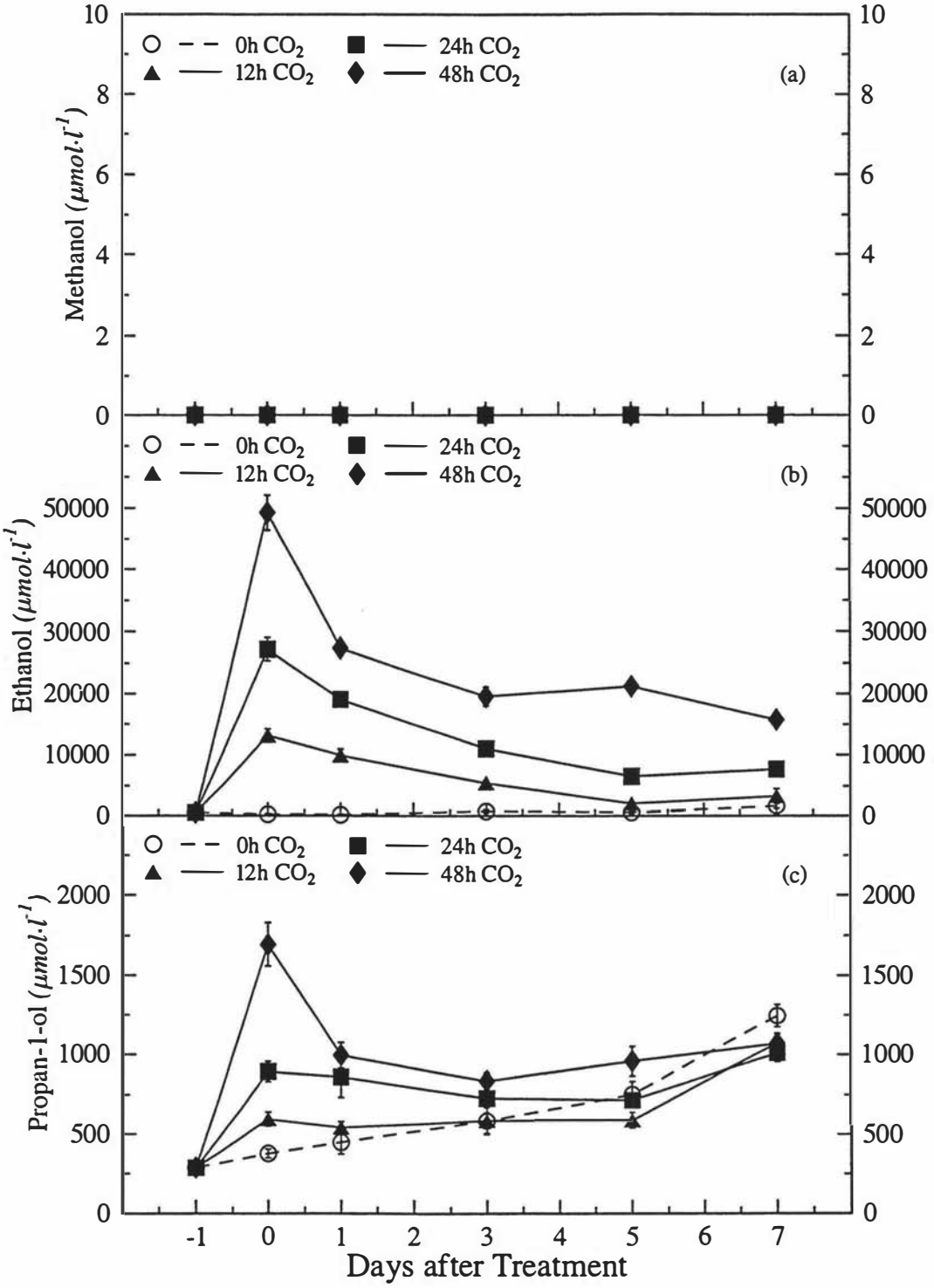


Fig. 4.19 Concentrations of (a) methanol, (b) ethanol and (c) propan-1-ol in juice from 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

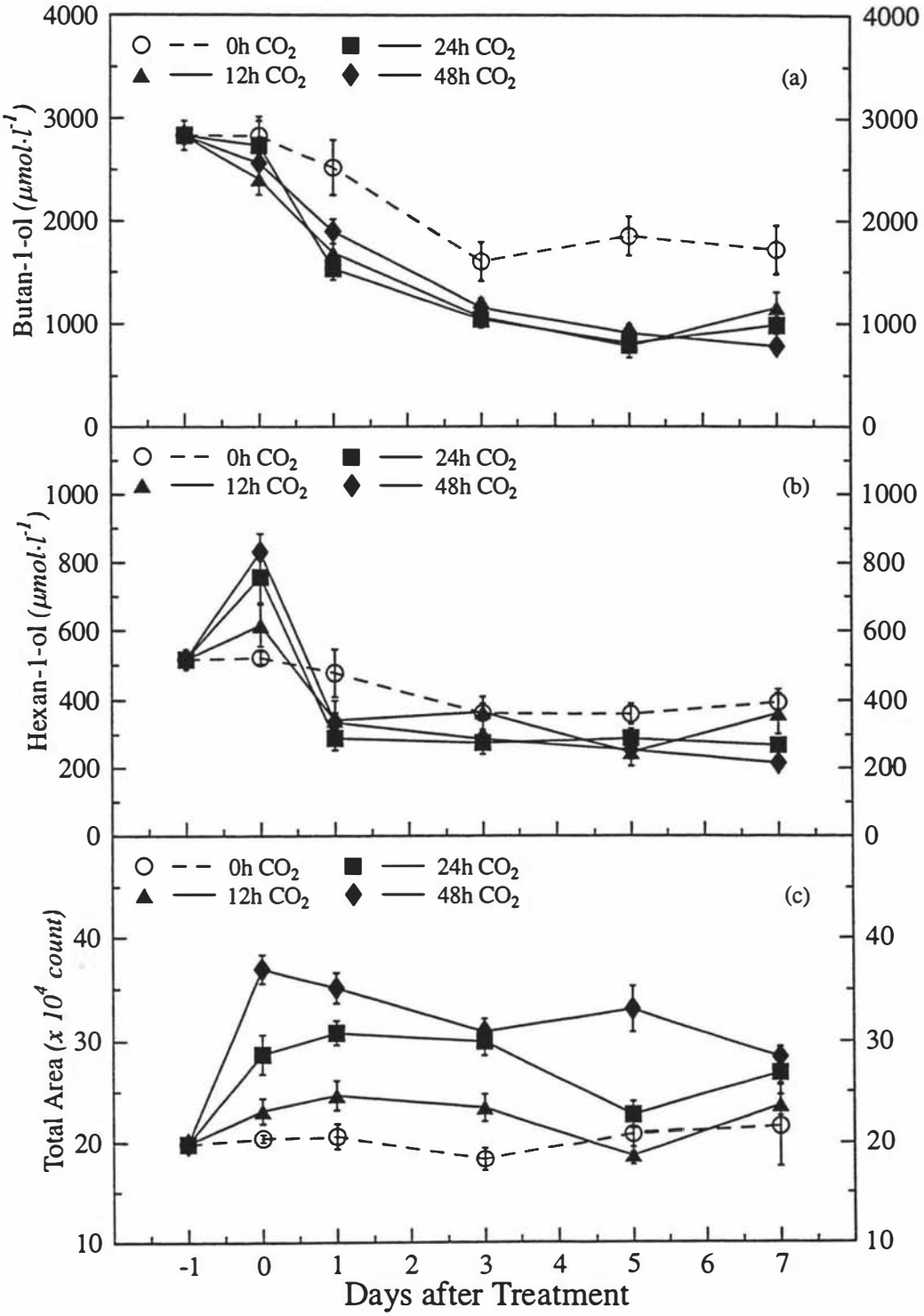


Fig. 4.20 Concentrations of (a) butan-1-ol, (b) hexan-1-ol and (c) total peak area in juice from 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

Table 4.8 Fruit skin colour in 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

	Lightness (%)					Hue Angle (°)				
Day	Ctrl	6h	12h	18h	24h	Ctrl	6h	12h	18h	24h
-1	54.6	54.6	54.6	54.6	54.6	63.7	63.7	63.7	63.7	63.7
0	58.2	56.9	53.5	55.4	57.3	70.8	65.1	61.0	66.3	68.6
1	55.3	57.4	58.8	57.6	58.5	64.2	69.2	72.3	72.4	71.5
3	58.2	56.9	58.1	57.0	57.9	73.5	66.1	68.2	70.6	69.2
5	58.2	56.4	59.6	56.5	58.8	71.7	66.3	70.8	64.2	70.9
7	59.2	58.7	58.1	56.8	57.8	71.2	70.0	71.0	67.4	68.2
c.v.			4.90%					9.87%		
Day			***					**		
Treat			ns					ns		
Day x Treat			ns					ns		

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

Table 4.9 Total soluble solids (TSS) and titratable acids (TA) in juice from 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

	TSS (%)					TA (%)				
Day	Ctrl	6h	12h	18h	24h	Ctrl	6h	12h	18h	24h
-1	12.1	12.1	12.1	12.1	12.1	0.40	0.40	0.40	0.40	0.40
0	11.5	11.6	11.8	12.3	11.3	0.39	0.38	0.36	0.42	0.37
1	11.8	11.6	11.6	11.3	11.3	0.40	0.40	0.40	0.43	0.39
3	11.4	11.3	11.9	11.1	11.3	0.41	0.39	0.39	0.38	0.39
5	11.7	11.9	11.7	12.2	11.8	0.36	0.34	0.35	0.36	0.40
7	11.7	11.6	12.2	11.1	11.4	0.39	0.36	0.39	0.33	0.37
c.v.			6.07 %					11.43 %		
Day			**					**		
Treat			ns					ns		
Day x Treat			ns					ns		

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

Table 4.10 TSS:TA ratio and pH in juice from 'Braeburn' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 6, 12, 18 or 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 2***).

	TSS:TA Ratio					Juice pH				
Day	Ctrl	6h	12h	18h	24h	Ctrl	6h	12h	18h	24h
-1	30.9	30.9	30.9	30.9	30.9	3.09	3.09	3.09	3.09	3.09
0	30.2	30.7	32.3	29.4	31.2	3.11	3.15	3.14	3.09	3.15
1	29.7	29.2	28.9	26.6	29.6	3.10	3.11	3.11	3.11	3.15
3	27.8	29.9	31.4	29.4	29.7	3.15	3.17	3.16	3.17	3.18
5	33.2	35.1	33.0	34.7	29.8	3.10	3.13	3.12	3.12	3.09
7	30.0	33.1	31.2	34.4	31.4	3.11	3.14	3.12	3.18	3.12
c.v.			13.46 %					1.58 %		
Day			**					***		
Treat			ns					ns		
Day x Treat			ns					ns		

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

Table 4.11 Fruit skin colour in 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

	Lightness (%)				Hue Angle (°)			
Day	Ctrl	12h	24h	48h	Ctrl	12h	24h	48h
-1	39.7	39.7	39.7	39.7	28.3	28.3	28.3	28.3
0	37.9	67.9	40.3	39.4	26.4	28.9	29.3	28.6
1	40.2	38.0	40.8	39.2	28.5	26.2	32.9	30.1
3	41.2	39.0	40.8	37.7	31.9	29.1	32.8	26.3
5	39.2	37.7	39.0	41.4	27.8	27.3	27.4	32.1
7	39.4	38.1	36.3	37.2	29.2	25.7	21.9	26.5
c.v.		8.59%				23.28%		
Day		ns				ns		
Treat		ns				ns		
Day x Treat		ns				ns		

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

Table 4.12 Total soluble solids (TSS) and titratable acids (TA) in juice from 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 12, 24 or 48 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

	TSS (%)				TA (%)			
Day	Ctrl	12h	24h	48h	Ctrl	12h	24h	48h
-1	14.6	14.6	14.6	14.6	0.19	0.19	0.19	0.19
0	15.5	14.0	15.4	13.9	0.19	0.16	0.18	0.15
1	15.4	15.4	14.5	14.8	0.18	0.20	0.18	0.18
3	14.8	14.8	14.2	16.0	0.19	0.18	0.17	0.17
5	15.3	14.3	14.3	15.0	0.18	0.18	0.16	0.17
7	14.1	14.7	15.1	14.4	0.15	0.19	0.16	0.17
c.v.	10.69%				15.76%			
Day	ns				*			
Treat	ns				*			
Day x Treat	ns				ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

Table 4.13 TSS:TA ratio and pH in juice from 'Red Delicious' apples (previously stored for 4 months at 0°C) after treatment with 100% CO_2 for 12, 24 or 48 hours at 20° and during subsequent storage at 20°C and 70% RH (***Experiment 3***).

	TSS:TA Ratio				Juice pH			
Day	Ctrl	12h	24h	48h	Ctrl	12h	24h	48h
-1	78.2	78.2	78.2	78.2	3.25	3.25	3.25	3.25
0	83.6	85.9	86.3	96.5	3.24	3.30	3.37	3.44
1	86.9	78.0	79.5	82.2	3.28	3.30	3.28	3.29
3	79.5	85.0	86.0	96.0	3.31	3.32	3.33	3.39
5	85.3	78.4	92.7	91.0	3.33	3.30	3.48	3.48
7	92.8	79.3	94.1	88.1	3.52	3.44	3.51	3.48
c.v.	13.62%				1.98%			
Day	*				***			
Treat	*				***			
Day x Treat	ns				***			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

4.4.3 Effect of Low O_2 Concentrations on 'Red Delicious' Apples (*Experiment 4* and *Experiment 5*)

Freshly harvested 'Red Delicious' apples were used for the 1995 trial and fruit which had been in cold store at 0°C were employed in 1994 (**Table 4.1**).

In freshly harvested apples, low O_2 concentrations (2.5% - 10%) significantly decreased CO_2 production immediately after treatment ($P < 0.001$) but this effect disappeared after 1 day (**Fig. 4.21**a). Stored apples treated with 0% to 2.5% O_2 had lower overall respiration rates ($P < 0.05$) than that of control (**Fig. 4.22**a). Treatment with 100% CO_2 had no effect on fruit respiration rate after treatment, regardless of fruit age.

Regardless of fruit age ethylene production (**Fig. 4.21**b and **Fig. 4.22**b) was reduced ($P < 0.05$) immediately after treatment by application of 0% and 2.5% O_2, or 100% CO_2, where the latter exerted a greater suppressive effect ($P < 0.001$), but there was no difference from controls after 3 days.

Treatments had no influence on weight loss of freshly harvested apples (**Fig. 4.21**c), although it was slightly higher ($P < 0.05$) in untreated stored fruit (**Fig. 4.22**c). Weight loss increased linearly with time with average rates of 0.20% ($r^2 = 0.74$) and 0.25% per day ($r^2 = 0.98$) for the 1995 (freshly harvested fruit) and 1994 (8 months cold stored fruit) experiments, respectively.

Freshly harvested apples treated with 0% to 5% O_2 were slightly firmer ($P < 0.05$) than controls and retention of firmness at 20°C was greater with 0% O_2 (100% CO_2) treatment (**Fig. 4.21**d). Firmness generally decreased gradually with time, except that of 100% CO_2 treated fruit where it remained relatively constant. Generally all treated 8 month cold stored apples tended to be slightly softer than controls after low O_2 treatment and decreased gradually with time at 20°C (**Fig. 4.22**d).

Headspace volatiles from freshly harvested apples (**Fig. 4.23**) increased immediately after treatment with 0% O_2 (100% CO_2) and this difference persisted for 7 days at 20°C. Concentrations of acetaldehyde, ethyl acetate, and ethanol at their peaks were about 16, 38, and 70 fold greater than control, respectively. Treatments with 2.5% O_2 also stimulated accumulation of these volatiles, to a lesser degree than with 0% O_2 ($P < 0.001$), with concentrations of the respective volatiles being approximately 2, 9, and 18

fold higher than control. There was no stimulation following treatment with 5% and 10% O_2.

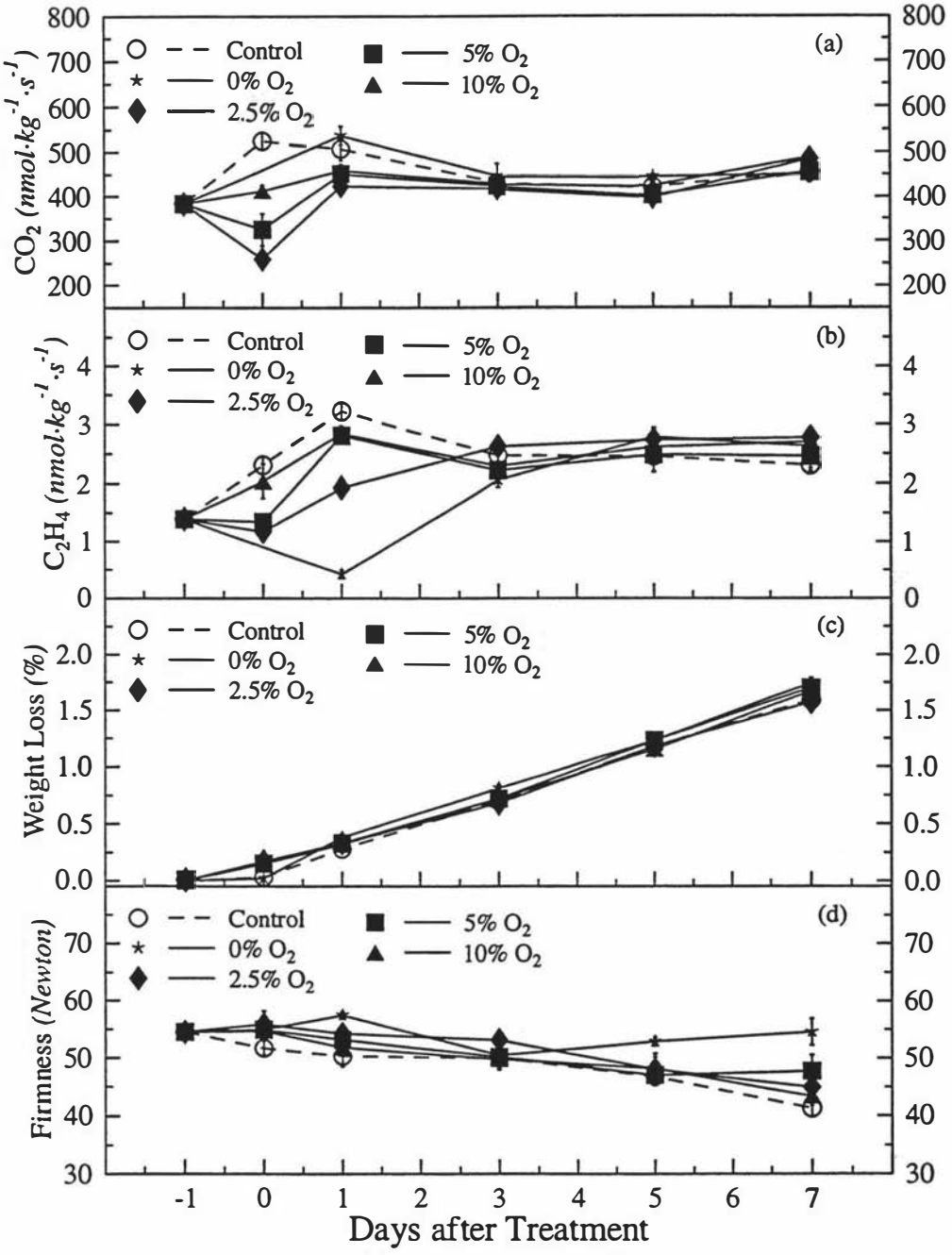


Fig. 4.21 Fruit (a) respiration rate, (b) ethylene production, (c) weight loss and (d) flesh firmness (d) in freshly harvested 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

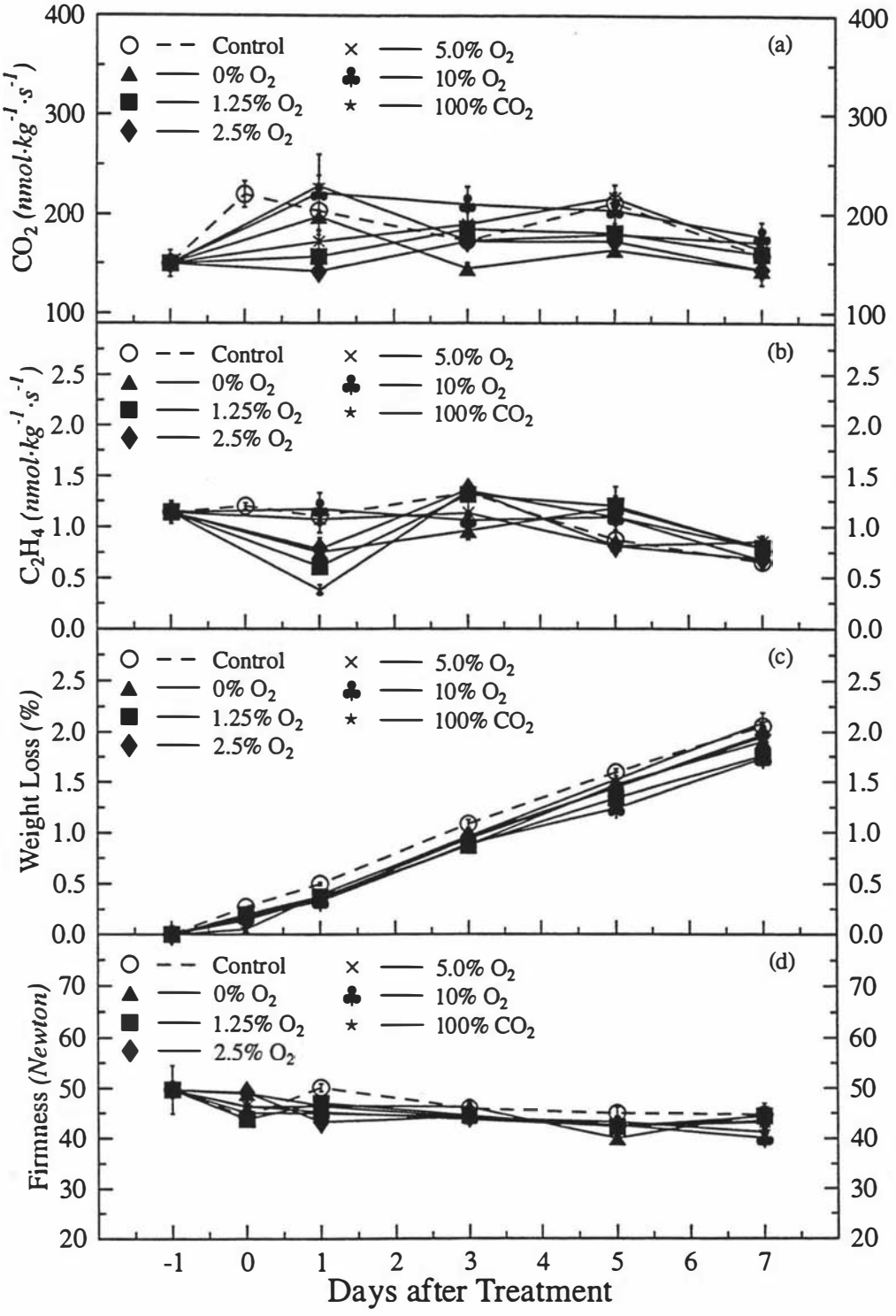


Fig. 4.22 Fruit (a) respiration rate, (b) ethylene production, (c) weight loss and (d) flesh firmness in 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

Headspace acetaldehyde and ethanol from freshly harvested apples peaked immediately, and ethyl acetate peaked between day 1 and day 3, after removal from the 0% O_2 (100% CO_2) treatment and the concentrations declined thereafter. Exposure to 5% and 10% O_2 had no effect on these headspace gases (**Fig. 4.23**).

Concentrations of headspace ethyl acetate, ethanol, and acetaldehyde from apples stored for 8 months were 6, 12, and 28 ppm, respectively, at the beginning of experiment (day -1) and they progressively increased with time of storage at 20°C (**Fig. 4.24**). In marked contrast these volatiles were not detected in freshly harvested apples at the beginning of the trial and only low levels were observed in control fruit after 3 days at 20°C (**Fig. 4.23**). Overall production of acetaldehyde after treatment was comparable in all treatments (**Fig. 4.24**a), but ethanol (**Fig. 4.24**b) and ethyl acetate (**Fig. 4.24**c) were enhanced by 0%, 1.25% and 2.5% O_2 or 100% CO_2, but not with 5% or 10% O_2 treatments. The highest enhancement of headspace ethyl acetate, about 4.5 fold over control, was observed in the 100% CO_2 treatment.

All ethyl esters from freshly harvested 'Red Delicious' apples, except ethyl pentanoate (**Fig. 4.26**b), were greatly enhanced by 0% O_2; enhancement was less following the 2.5% O_2 treatment, while ethyl esters from 5% or 10% O_2 treatments were not different from control. At peak production, concentrations of ethyl acetate (**Fig. 4.26**a) and ethyl butanoate (**Fig. 4.25**a) following 0% O_2 treatment were enhanced about 12 and 15 fold, respectively, and those of 2.5% O_2 increased approximately 3 and 8 fold, respectively, over control. Concentrations of ethyl 2-methyl butanoate and ethyl hexanoate from fruit exposed to 0% or 2.5% O_2 were 465.20 or 174.97 $\mu mol \cdot l^{-1}$ and 321.65 or 36.35 $\mu mol \cdot l^{-1}$, respectively, but these esters were not detected in control fruit (**Table 4.14**).

Concentrations of ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate and ethyl acetate from 0% O_2 (100% CO_2) treated fruit increased markedly to peak between day 1 and day 5 after treatment, depending on compound, and decreased thereafter, with concentrations substantially greater than other treatments and control. Concentrations of these ethyl esters from fruit exposed to 2.5% O_2, peaked at day 1, then decreased to the same levels as control or 5% and 10% O_2 treated fruit 3 or 5 days after treatment and remained at low levels thereafter. Concentrations of these ethyl esters from 5% and 10%

O_2 treated fruit were similar to control fruit and remained at low levels or were not detected (**Fig. 4.25** and **Fig. 4.26**a).

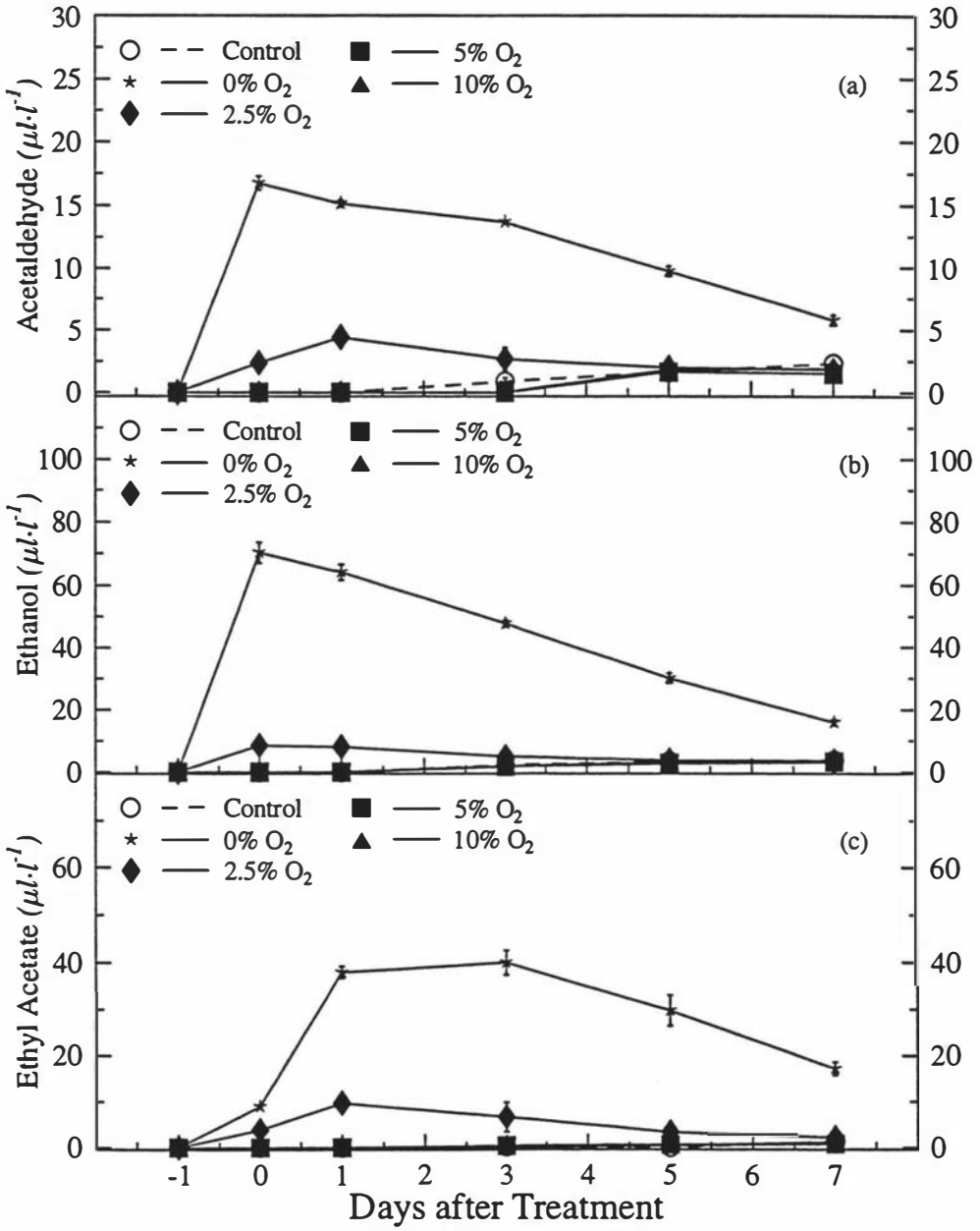


Fig. 4.23 Concentrations of headspace (a) acetaldehyde, (b) ethanol and (c) ethyl acetate in juice from freshly harvest 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

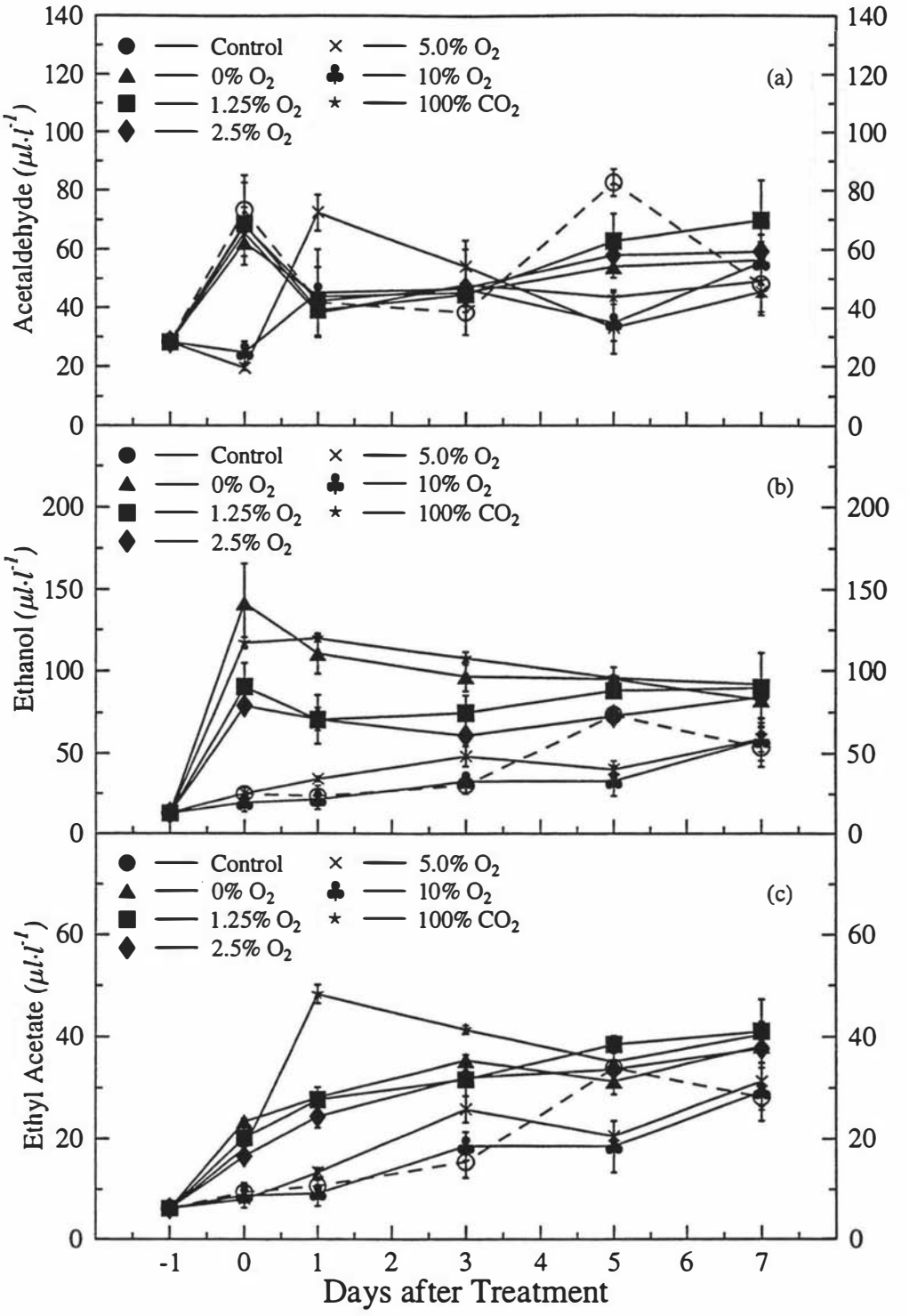


Fig. 4.24 Concentrations of headspace (a) acetaldehyde, (b) ethanol and (c) ethyl acetate in juice from 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

High concentrations of ethyl 2-methyl butanoate, ethyl hexanoate, ethyl butanoate and ethyl acetate of 77, 162, 568 and 568 $\mu mol \cdot l^{-1}$, respectively, were found at the beginning of the experiment on 8 month cold stored 'Red Delicious' apples, where they increased steadily with time at 20°C. There were few differences in production of ethyl butanoate and ethyl 2-methyl butanoate between treatments (**Fig. 4.27**a and **Fig. 4.27**b). There was a tendency for both esters to be less immediately after treatment (day 0) and at day 3 and day 5 for 0, 1.25 and 2.5% O_2 and 100% CO_2 (**Fig. 4.27**a and **Fig. 4.27**b).

Ethyl hexanoate was enhanced 2.5 - 3.5 fold at day 1 in 8 month cold stored apples exposed to 0%, 1.25%, 2.5% O_2 and 100% CO_2, while 5% and 10% O_2 treatments were the same as controls (**Fig. 4.27**c).

Ethyl acetate in 8 month cold stored apples treated with 0%, 1.25%, 2.5% O_2 and 100% CO_2 was enhanced ($P < 0.001$) 1 - 3 fold, depending on treatments, at 0 and 3 days after treatment compared with controls (**Fig. 4.28**a).

Overall production of ethyl pentanoate following all treatments of freshly harvested fruit was not different from controls , where it generally increased slightly during 7 days at 20°C (**Fig. 4.26**b). There was a tendency for ethyl pentanoate to decrease following 0, 1.25 and 2.5% O_2 and 100% CO_2 treatments in 8 month cold stored apples (**Fig. 4.28**b).

In freshly harvested 'Red Delicious' apples, 0% O_2 (100% CO_2) and 2.5% O_2 treatments effectively enhanced headspace volatiles and selected ethyl volatiles during 7 days at 20°C, compared with 5%, 10% O_2 or control fruit (**Table 4.14**). However, in 8 month cold stored 'Red Delicious' apples, concentrations of only a few compounds, such as ethyl hexanoate, ethanol and headspace ethanol from 0%, 1.25%, 2.5% O_2 or 100% CO_2 treatments were greater than 5%, 10% O_2 or control treatments. At their highest concentrations (during 7 days at 20°C), some compounds such as ethyl butanoate, ethyl hexanoate and headspace ethanol from control fruit of 8 month old apples were similar to the respective compounds from 0% O_2 (100% CO_2) treated freshly harvested fruit (**Table 4.14**).

Table 4.14 Peak concentrations of headspace volatiles and selected ethyl volatiles during 7 days at 20°C in juice from freshly harvested or 8 month cold stored 'Red Delicious' apples after treatment with various low O_2 or 100% CO_2 atmospheres at 20°C.

Compound	Concentration				
	Experiment 5 (Freshly Harvested 'Red Delicious' Apples)				
	Ctrl	0% O_2	2.5% O_2	5% O_2	10% O_2
Hspace Volatiles[1]					
Acetaldehyde	2.3^{c}	16.8^{a}	4.2^{b}	1.6^{c}	1.9^{c}
Ethanol	3.7^{c}	70.3^{a}	8.6^{b}	3.2^{c}	3.4^{c}
Ethyl Acetate	1.3^{c}	40.0^{a}	9.7^{b}	0.7^{c}	1.8^{c}
Ethyl Volatiles[2]					
Ethyl butanoate	104.1^{c}	1232.2^{a}	310.4^{b}	97.6^{c}	106.0^{c}
E 2-MB	n.d.	465.2^{a}	175.0^{b}	n.d.	n.d.
Ethyl Hexanoate	n.d.	321.7^{a}	36.4^{b}	n.d.	n.d.
Ethyl acetate	829.3^{c}	11063.8^{a}	3146.3^{b}	647.5^{c}	788.5^{c}
Ethanol	503.8^{c}	32345.1^{a}	5250.4^{b}	416.4^{c}	486.3^{c}

	Experiment 4 (8 Month Cold Stored 'Red Delicious' Apples)						
	Ctrl	0% O_2	1.25% O_2	2.5% O_2	5% O_2	10% O_2	100% CO_2
Hspace Volatiles[1]							
Acetaldehyde	82.5^{a}	62.3^{a}	69.7^{a}	68.0^{a}	72.4^{a}	55.4^{a}	65.6^{a}
Ethanol	73.4^{c}	141.5^{a}	90.3^{c}	84.0^{c}	58.8^{c}	58.3^{c}	119.6^{b}
Ethyl Acetate	33.9^{b}	38.1^{b}	41.0^{b}	37.7^{b}	31.2^{b}	29.2^{b}	48.3^{a}
Ethyl Volatiles[2]							
Ethyl butanoate	1068.8^{b}	596.7^{a}	1536.1^{a}	1276.3^{ab}	1257.5^{ab}	1087.0^{b}	1269.3^{ab}
E 2-MB	250.9^{b}	252.4^{ab}	349.3^{a}	274.7^{ab}	262.5^{ab}	203.7^{bc}	304.4^{ab}
Ethyl Hexanoate	321.8^{c}	798.4^{a}	773.8^{a}	548.2^{b}	318.6^{c}	237.0^{c}	782.0^{a}
Ethyl acetate	3045.6^{d}	3551.1^{cd}	3593.1^{ad}	2762.3^{d}	3260.9^{ad}	2463.6^{bd}	3833.0^{a}
Ethanol	7984.8^{c}	17276.9^{a}	13126.9^{b}	9552.4^{c}	9519.5^{c}	7288.7^{cd}	16158.0^{ab}

0% O_2 was 100% CO_2 treatment for ***Experiment 5***, [1] Hspace Volatiles = Headspace Volatiles in $\mu l \cdot l^{-1}$, [2] Ethyl Volatiles in $\mu mol \cdot l^{-1}$, E 2-MB = Ethyl 2-methyl butanoate, n.d. = not detected, All data were mean concentration from 4 replicates, and those different letters among treatments (row) are significantly different at the 5% level.

Regardless of fruit age, butyl acetate (**Fig. 4.26**c and **Fig. 4.28**c) and hexyl acetate (**Fig. 4.29**a and **Fig. 4.30**a) from hypoxically treated apples were significantly reduced ($P < 0.05$) by 100% CO_2, 0% or 1.25% O_2 treatments compared with control fruit. Butyl acetate and hexyl acetate in freshly harvested fruit generally decreased initially at day 0 and increased thereafter, except in 0% O_2 (100% CO_2) where it remained relatively constant; in 8 month stored fruit these compounds decreased with time at 20°C.

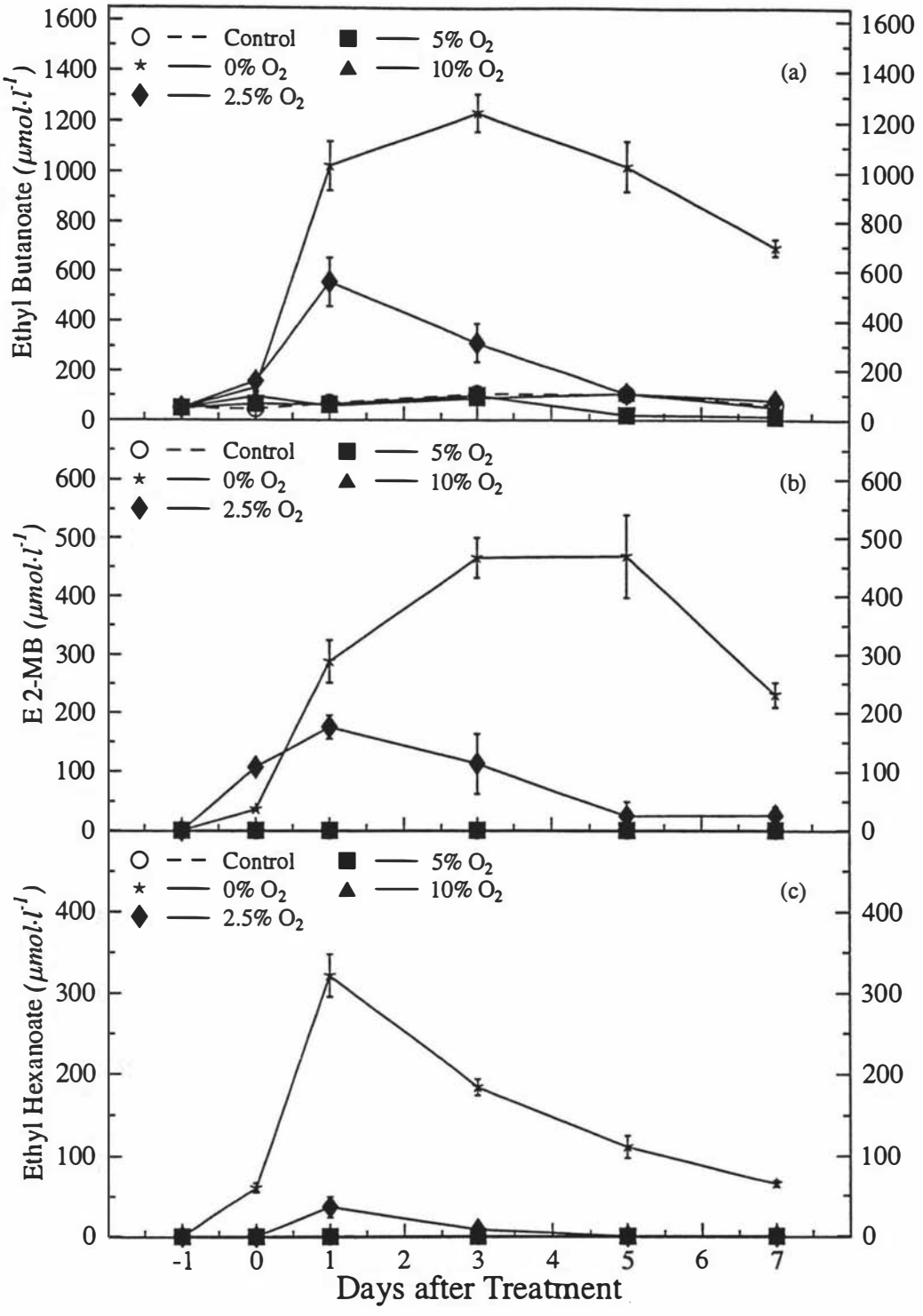


Fig. 4.25 Concentrations of (a) ethyl butanoate, (b) ethyl 2-methyl butanoate and (c) ethyl hexanoate in juice from freshly harvested 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

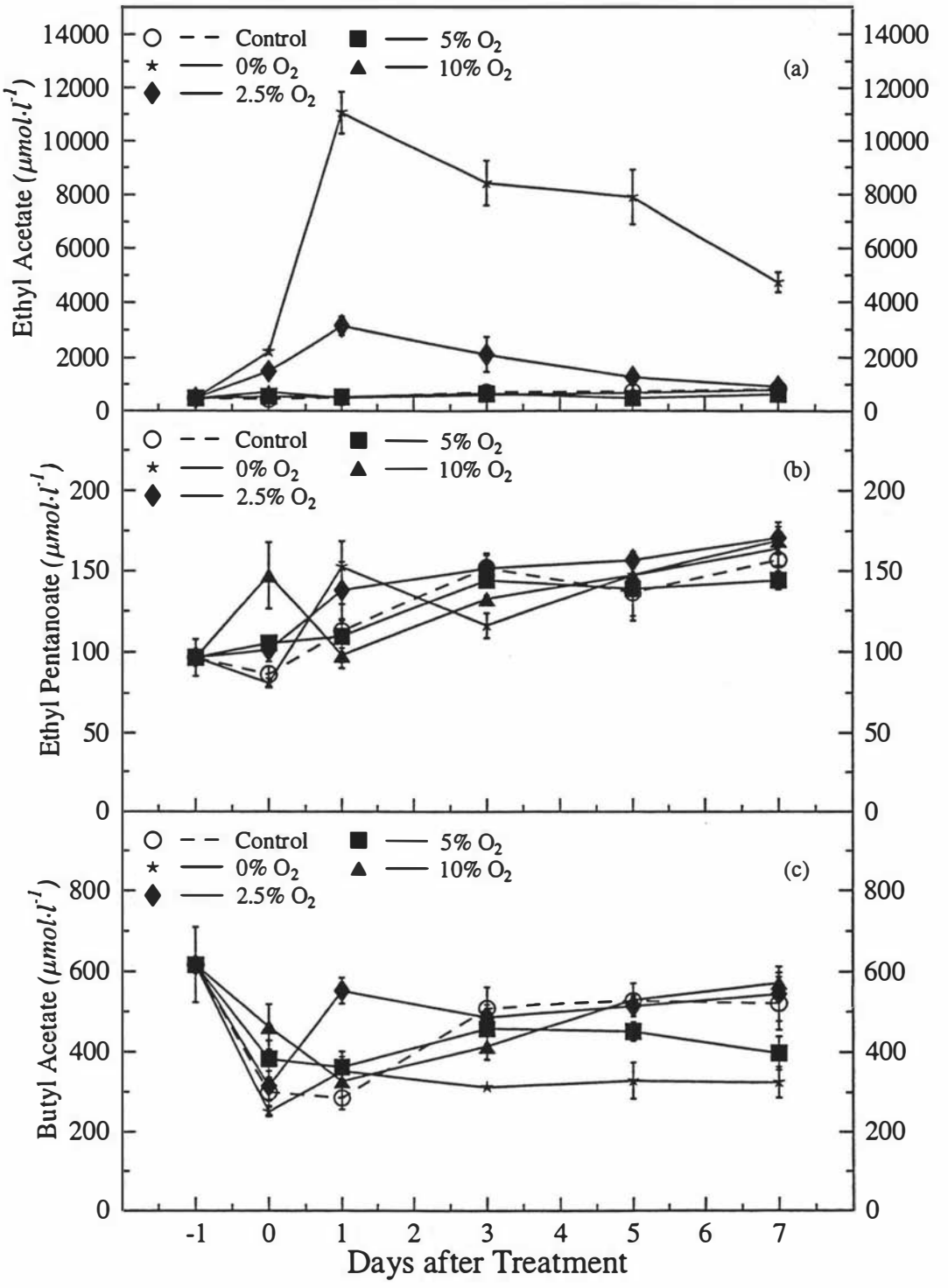


Fig. 4.26 Concentrations of (a) ethyl acetate, (b) ethyl pentanoate and (c) butyl acetate in juice from freshly harvest 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

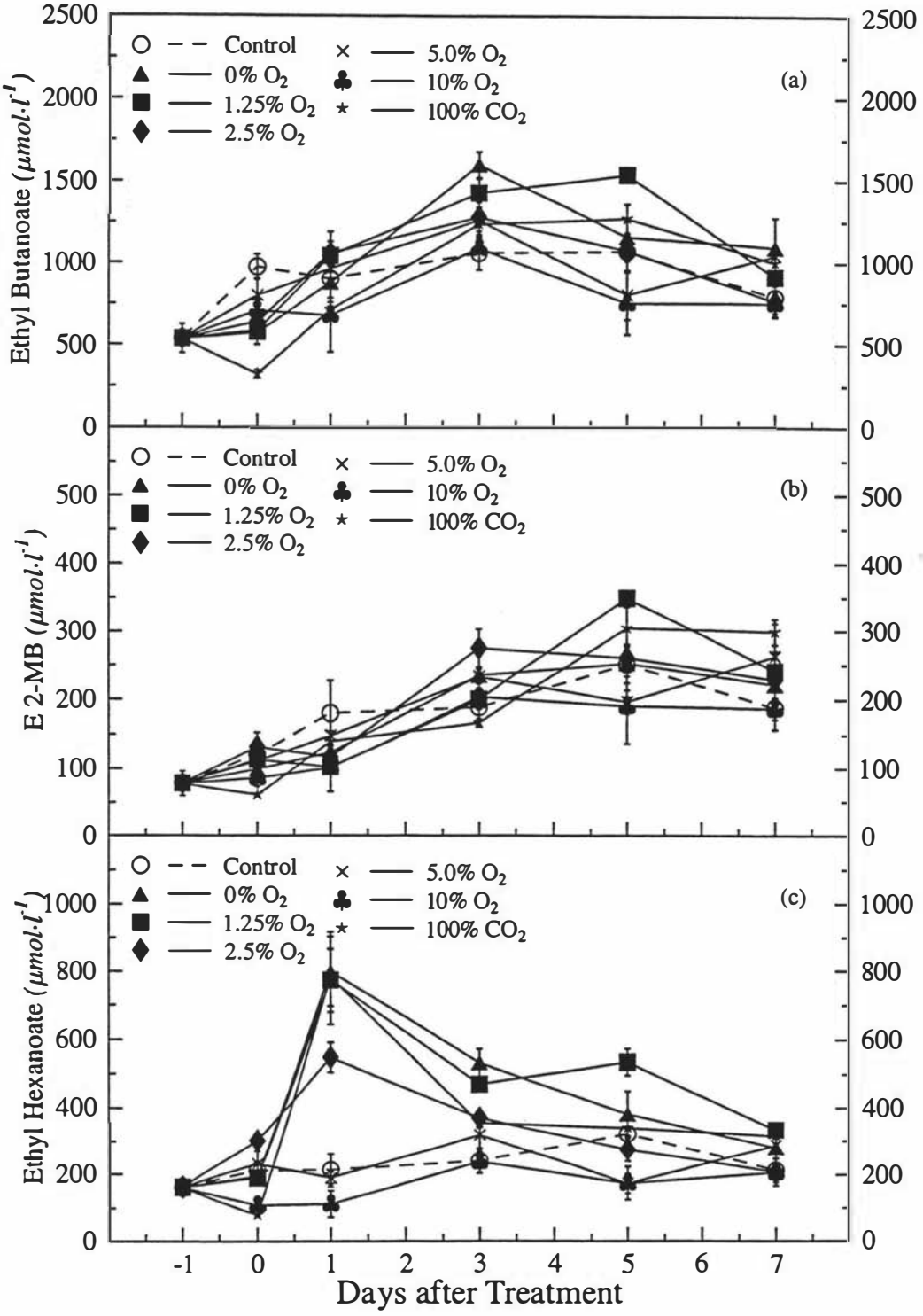


Fig. 4.27 Concentrations of (a) ethyl butanoate, (b) ethyl 2-methyl butanoate and (c) ethyl hexanoate in juice from 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

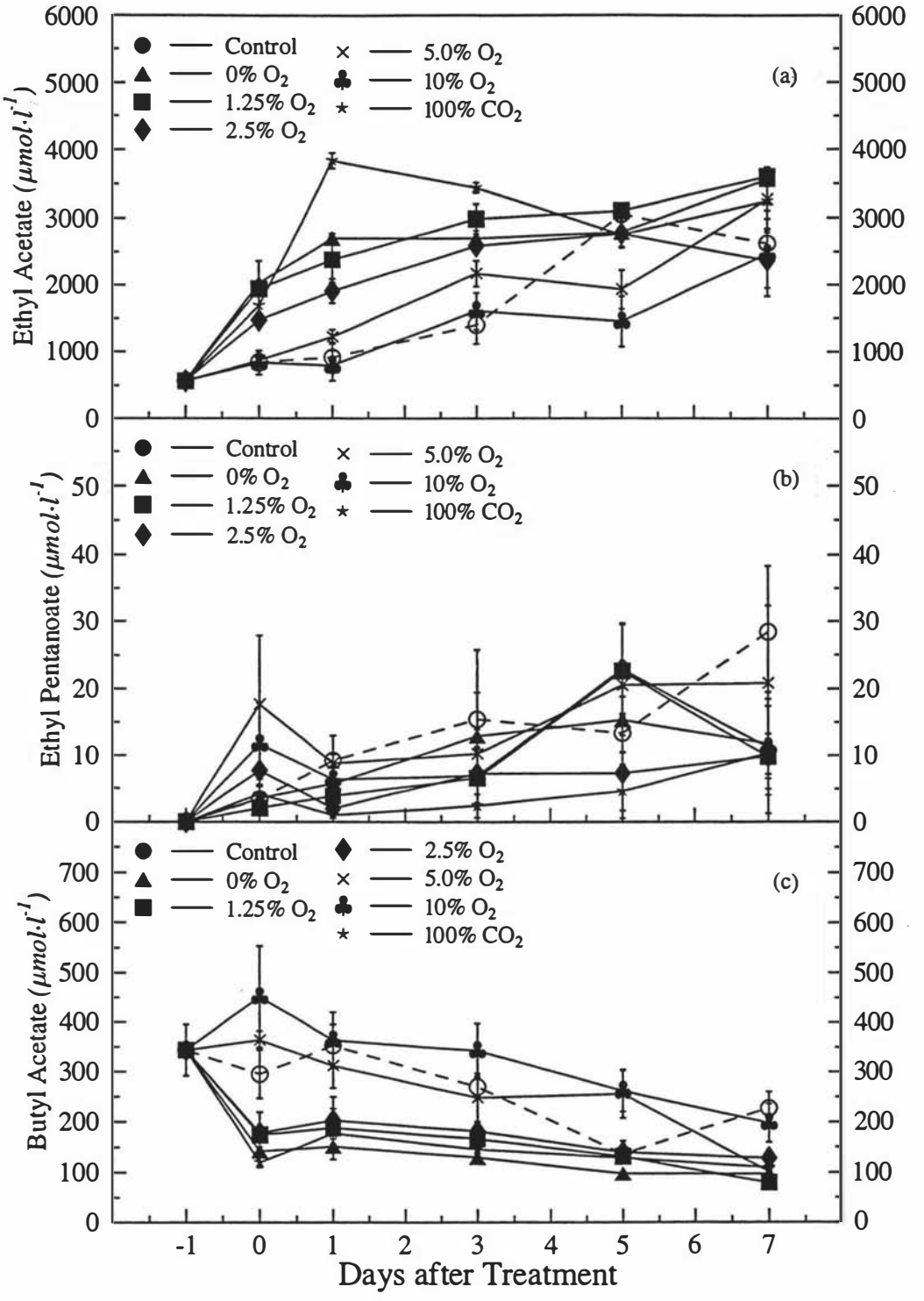


Fig. 4.28 Concentrations of (a) ethyl acetate, (b) ethyl pentanoate and (c) butyl acetate in juice from 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

Aldehydes, other than acetaldehyde, were either unaffected or only slightly affected by low O_2 treatments. Hexanal production (**Fig. 4.29**c and **Fig. 4.30**c) was unaffected by treatments, while *trans*-2-hexenal from freshly harvested apples (**Fig. 4.29**b) was reduced ($P < 0.05$) by treatments used, particularly at day 0 and day 1 after treatment (**Fig. 4.30**b). Both aldehydes, either from freshly harvested or from old fruit, generally increased with time at 20°C, where *trans*-2-hexenal was relatively higher in fresh fruit than in old fruit, and hexanal was similar.

Ethanol from 0% O_2 (100% CO_2) treated freshly harvested apples increased substantially (**Table 4.14**), peaking at day 1, where the 2.5% O_2 treatment induced a peak at day 0, after which there was a gradual decrease; other treatments did not differ from controls (**Fig. 4.31**b). The 0%, 1.25% O_2, 100% CO_2 treatments induced a marked ethanol enhancement ($P < 0.001$) compared with 5% or 10% O_2 and control treatments in 8 month old apples. In general, ethanol was negatively related to O_2 concentration, increasing immediately after treatment and remaining relatively constant thereafter (**Fig. 4.33**b).

Apart from ethanol, the effect of low O_2 treatment on alcohol production was relatively minor in both freshly harvested and old fruit. Low O_2 treatments slightly increased methanol (**Fig. 4.31**a), propan-1-ol (**Fig. 4.31**c), butan-1-ol (**Fig. 4.32**a) and hexan-1-ol (**Fig. 4.32**b) in freshly harvested fruit at day 0 and/or day 1 at 20°C. In old apples, methanol (**Fig. 4.33**a) was not detected, while propan-1-ol (**Fig. 4.33**c) and hexan-1-ol (**Fig. 4.34**b), but not butan-1-ol (**Fig. 4.34**a), were increased by low O_2 treatments at day 0 or day 1 and increasing or decreasing thereafter.

Total peak area from 0% O_2 (100% CO_2) treated freshly harvested apples was markedly greater ($P < 0.001$) than other treatments and controls, persisting from day 3 through day 7 at 20°C. Total peak area from other low O_2 treatments was slightly enhanced or comparable to control (**Fig. 4.32**c). Only a slight enhancement of total peak area was observed by low O_2 treatments in old apples (**Fig. 4.34**c).

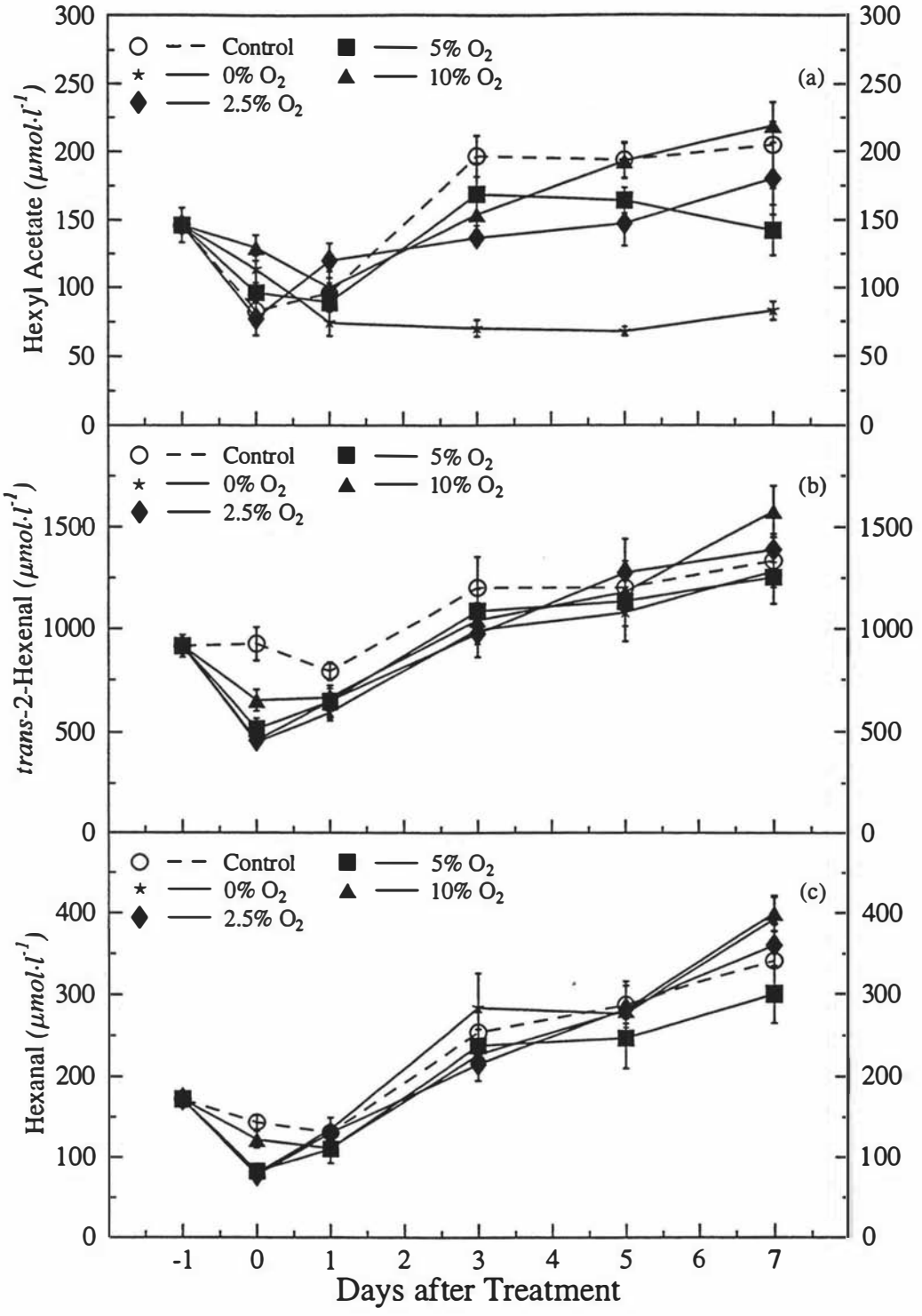


Fig. 4.29 Concentrations of (a) hexyl acetate, (b) *trans*-2-hexenal and (c) hexanal in juice from freshly harvested 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

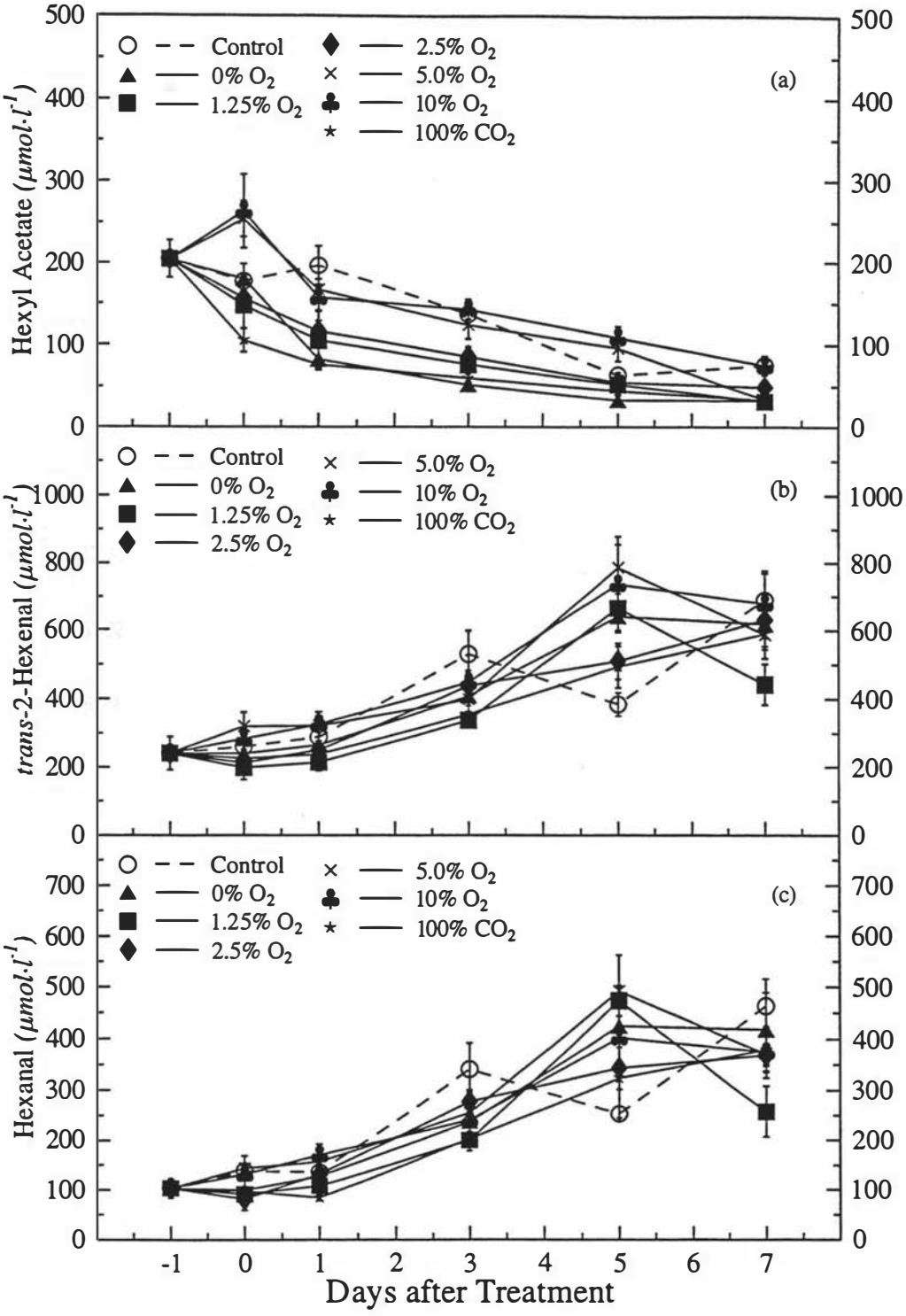


Fig. 4.30 Concentrations of (a) hexyl acetate, (b) *trans*-2-hexenal and (c) hexanal in 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

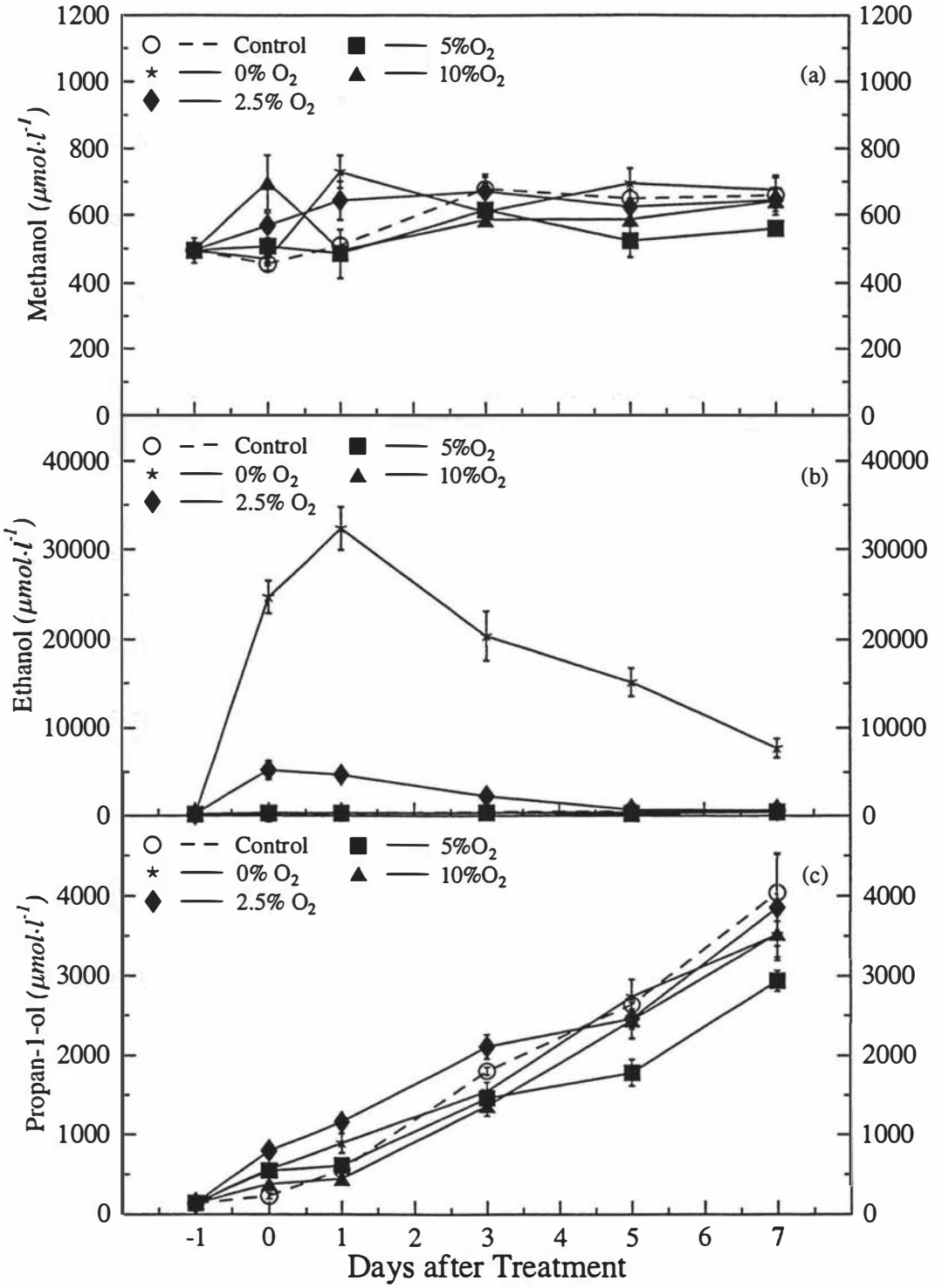


Fig. 4.31 Concentrations of (a) methanol, (b) ethanol and (c) propan-1-ol in freshly harvested 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

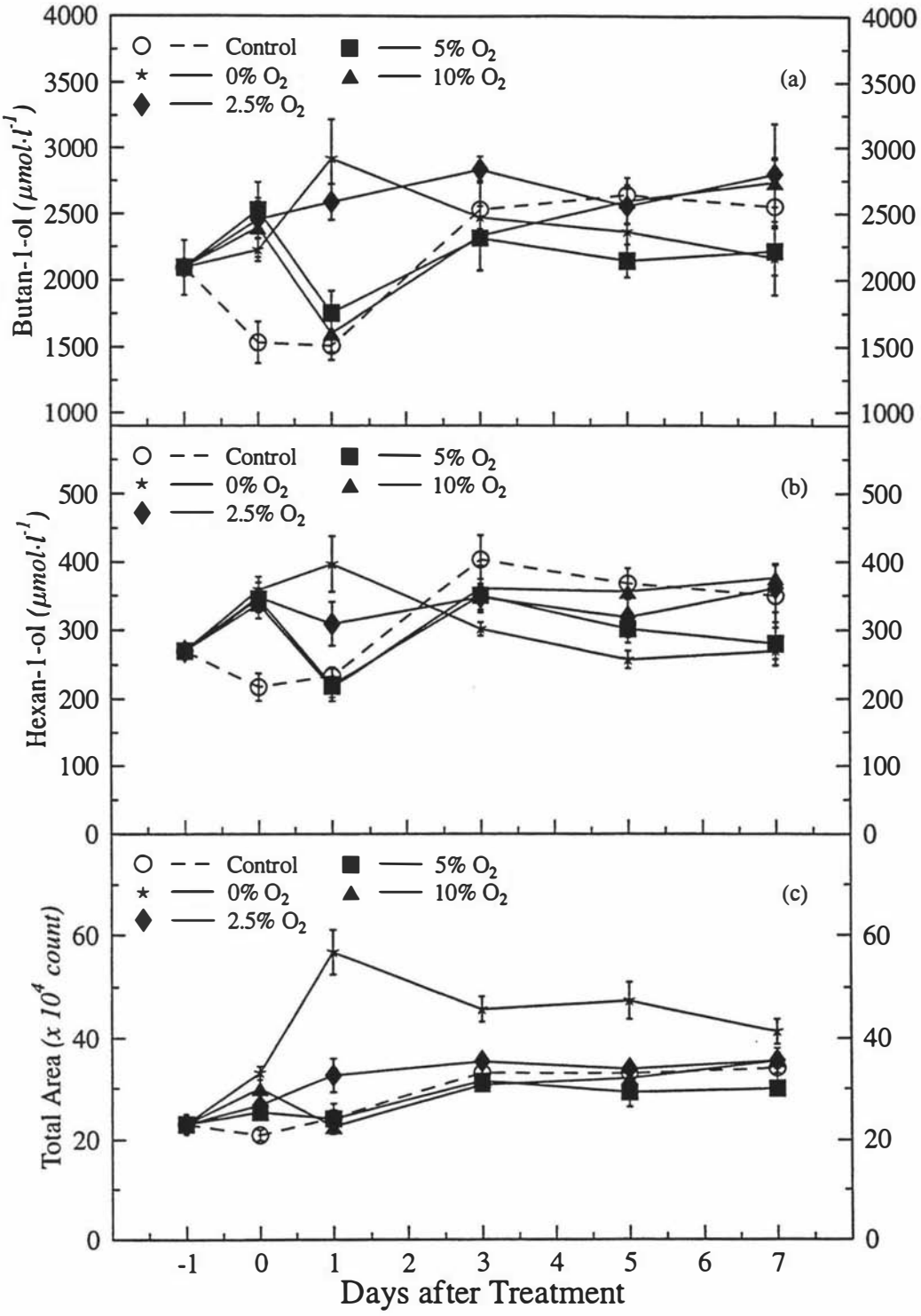


Fig. 4.32 Concentrations of (a) butan-1-ol, (b) hexan-1-ol and (c) total peak area in freshly harvested 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

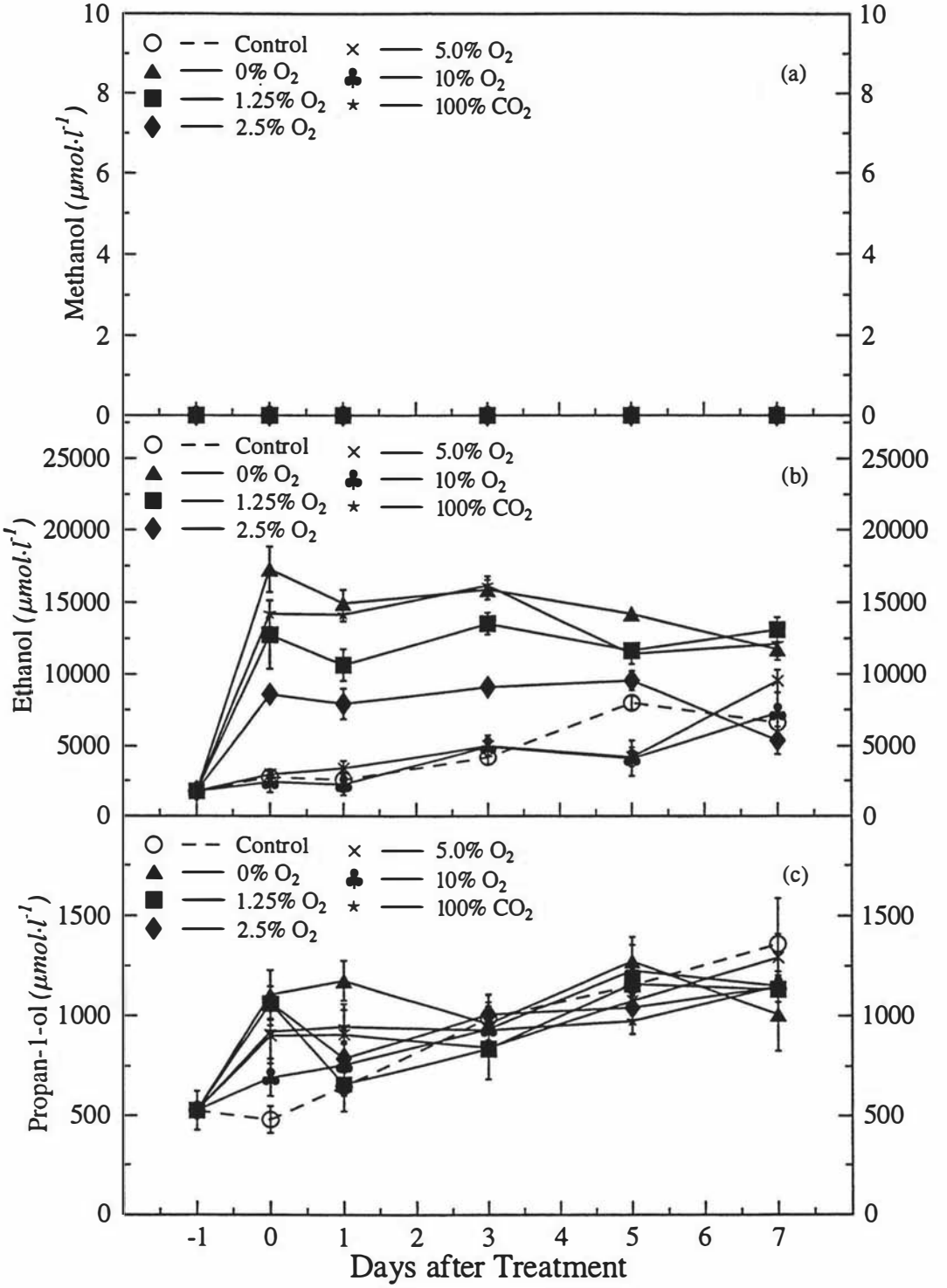


Fig. 4.33 Concentrations of (a) methanol, (b) ethanol and (c) propan-1-ol in 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

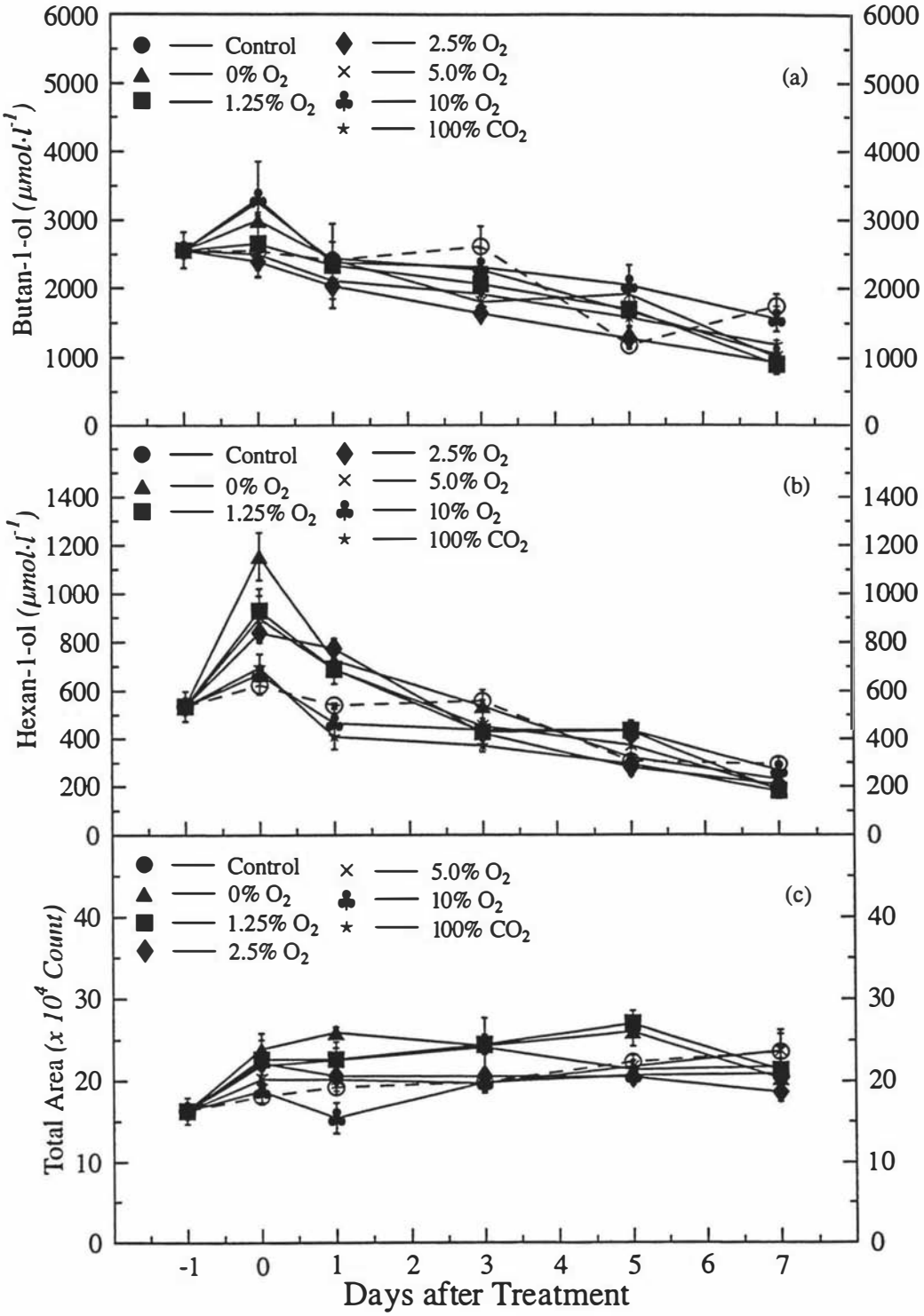


Fig. 4.34 Concentrations of (a) butan-1-ol, (b) hexan-1-ol and (c) total peak area in 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

Low O_2 treatments did not affect fruit skin colour (**Table 4.15**), TSS or TA (**Table 4.16**) of freshly harvested apples, but 10% O_2 slightly decreased ($P < 0.05$) TSS:TA ratio and juice pH (**Table 4.17**). Quality attributes monitored in cold stored apples were unaffected by treatment (**Table 4.18** to **Table 4.20**). Although significant changes of juice TSS, TA, TSS:TA ratio, or pH occurred during storage, they were variable and did not show any obvious trends or patterns with treatment.

Table 4.15 Fruit skin colour in freshly harvested 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

	Lightness (%)					Hue Angle (°)				
Day	Ctrl	0%[1]	2.5%	5%	10%	Ctrl	0%	2.5%	5%	10%
-1	35.4	35.4	35.4	35.4	35.4	21.4	21.4	21.4	21.4	21.4
3	35.1	33.9	33.4	33.6	33.6	22.2	19.3	19.4	20.7	20.2
7	35.3	35.8	34.9	35.6	34.1	23.2	23.6	22.7	23.6	21.8
c.v.			5.88%					12.33%		
Day			ns					*		
Treat			ns					ns		
Day x Treat			ns					ns		

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns)
[1] 100% CO_2 was used as 0% O_2 treatment; Ctrl = control

Table 4.16 Total soluble solids (TSS) and titratable acids (TA) in juice from freshly harvested 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

	TSS (%)					TA (%)				
Day	Ctrl	0%[1]	2.5%	5%	10%	Ctrl	0%	2.5%	5%	10%
-1	12.0	12.0	12.0	12.0	12.0	0.22	0.22	0.22	0.22	0.22
0	12.5	12.5	12.5	12.5	12.4	0.23	0.21	0.24	0.23	0.27
1	12.2	12.4	12.4	12.5	12.4	0.24	0.21	0.20	0.23	0.23
3	12.5	12.5	12.4	12.5	12.2	0.22	0.20	0.23	0.22	0.24
5	12.8	12.5	12.4	12.5	12.7	0.21	0.23	0.25	0.22	0.26
7	12.5	13.0	12.5	12.4	12.6	0.22	0.19	0.22	0.23	0.20
c.v.			2.72%					11.46%		
Day			***					**		
Treat			ns					ns		
Day x Treat			ns					*		

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns)
[1] 100% CO_2 was used as 0% O_2 treatment; Ctrl = control

Table 4.17 TSS:TA ratio and pH in juice from freshly harvested 'Red Delicious' apples after treatment with 0%, 2.5%, 5% or 10% O_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 5***).

	TSS:TA Ratio					Juice pH				
Day	Ctrl	0%[1]	2.5%	5%	10%	Ctrl	0%	2.5%	5%	10%
-1	53.9	53.9	53.9	53.9	53.9	3.68	3.68	3.68	3.68	3.68
0	53.8	53.9	52.3	55.8	46.7	3.62	3.64	3.62	3.62	3.62
1	51.3	59.8	62.4	56.1	53.4	3.62	3.53	3.55	3.55	3.47
3	75.4	61.1	54.4	57.8	53.1	356	3.56	3.55	3.54	3.53
5	61.4	54.9	51.6	57.9	49.0	3.52	3.58	3.50	3.57	3.56
7	59.6	64.1	58.6	56.3	64.8	3.62	3.63	3.61	3.60	3.60
c.v.			12.14%					1.03%		
Day			**					***		
Treat			*					*		
Day x Treat			**					*		

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns)
[1] 100% CO_2 was used as 0% O_2 treatment; Ctrl = control

Table 4.18 Fruit skin colour in 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

	Lightness (%)						
Day	Ctrl	0% O_2	1.25% O_2	2.5% O_2	5% O_2	10% O_2	100% CO_2
-1	41.8	41.8	41.8	41.8	41.8	41.8	41.8
0	39.6	41.6	42.9	42.1	36.9	39.5	42.0
1	39.0	40.9	37.1	40.3	42.5	39.5	41.6
3	43.1	37.1	41.5	39.9	40.1	38.1	42.0
5	39.7	41.4	38.7	39.8	39.7	42.9	39.0
7	40.0	40.6	42.1	43.3	40.0	40.4	38.5
c.v.				6.93%			
Day				ns			
Treat				ns			
Day x Treat				ns			
Day	Hue Angle (°)						
-1	36.2	36.2	36.2	36.2	36.2	36.2	36.2
0	31.5	35.0	39.9	36.6	28.0	33.8	40.0
1	30.5	36.2	28.1	32.1	38.3	32.4	38.2
3	38.6	29.4	36.7	32.7	30.9	29.0	38.8
5	32.3	37.2	29.8	32.8	32.1	38.9	32.9
7	31.8	35.6	37.0	40.5	33.0	34.7	31.5
c.v.				15.99%			
Day				ns			
Treat				ns			
Day x Treat				ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

Table 4.19 Total soluble solids (TSS) and titratable acids (TA) in juice from 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

	TSS (%)						
Day	Ctrl	0% O_2	1.25% O_2	2.5% O_2	5% O_2	10% O_2	100% CO_2
-1	13.5	13.5	13.5	14.5	13.5	13.5	13.5
0	14.0	14.8	14.1	14.4	14.3	13.8	14.5
1	14.7	13.9	14.7	14.1	13.6	14.9	14.2
3	14.1	14.7	14.1	14.2	14.2	14.2	12.9
5	13.7	13.3	13.6	14.3	14.9	14.4	13.9
7	14.0	13.8	13.6	12.8	13.6	13.4	14.1
c.v.				5.94%			
Day				***			
Treat				ns			
Day x Treat				ns			
Day				TA (%)			
-1	0.16	0.16	0.16	0.16	0.16	0.16	0.16
0	0.14	0.14	0.16	0.17	0.16	0.16	0.15
1	0.17	0.15	0.16	0.15	0.17	0.16	0.17
3	0.16	0.15	0.16	0.16	0.15	0.16	0.17
5	0.14	0.15	0.14	0.14	0.16	0.17	0.15
7	0.15	0.16	0.15	0.15	0.16	0.15	0.14
c.v.				10.81%			
Day				**			
Treat				ns			
Day x Treat				ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

Table 4.20 TSS:TA ratio and pH in juice from 'Red Delicious' apples (previously stored for 8 months at 0°C) after treatment with 0%, 1.25%, 2.5%, 5%, 10% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 4***).

	TSS:TA Ratio						
Day	Ctrl	0% O_2	1.25% O_2	2.5% O_2	5% O_2	10% O_2	100% CO_2
-1	83.4	83.4	83.4	83.4	83.4	83.4	83.4
0	101.3	105.8	87.8	85.6	94.2	89.7	99.6
1	87.8	92.0	94.3	96.7	84.2	96.0	85.4
3	91.2	101.6	91.3	92.4	94.4	89.1	81.3
5	101.7	91.2	99.7	104.4	93.2	86.5	93.7
7	96.7	92.8	92.6	89.7	87.1	92.2	99.3
c.v.				11.83%			
Day				***			
Treat				ns			
Day x Treat				ns			
Day	Juice pH						
-1	4.00	4.00	4.00	4.00	4.00	4.00	4.00
0	4.00	4.08	3.93	3.93	3.94	3.94	4.06
1	3.95	3.98	3.99	3.94	3.93	3.98	3.94
3	4.11	4.08	3.95	4.04	4.02	4.03	3.97
5	4.12	4.08	4.11	4.11	4.03	3.99	4.05
7	4.10	4.06	3.98	3.99	3.98	4.06	4.03
c.v.				1.64%			
Treat				***			
Day				ns			
Day x Treat				ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns); Ctrl = control

4.4.4 Effect of CO_2 Concentrations in 20% O_2 on 'Red Delicious' Apples (*Experiment 6*)

Exposure of 'Red Delicious' apples, stored for 6 months at 0.5°C, to CO_2 concentrations from 40% to 80% plus 20% O_2 or 100% CO_2, but not to 10% or 20% CO_2 plus 20% O_2, increased respiration rates 1 day after treatment ($P < 0.05$) after which it decreased to the same level as controls at day 3 and decreased gradually thereafter (**Fig. 4.35**a).

Ethylene production was depressed by all CO_2 treatments ($P < 0.05$) 1 day after treatment and 100% CO_2 exerted the greatest depressive effect, which lasted for at least 3 days (**Fig. 4.35**b).

Cumulative weight loss of apples was highest in 100% CO_2 treated fruit and slightly lower ($P < 0.05$) than control in other treatments (**Fig. 4.35**c). In general, weight loss of apples increased linearly ($r^2 = 0.99$) at average rate of 0.25% per day.

Treatments had no effect on flesh firmness and there was a linear loss of apple firmness during storage ($r^2 = 0.32$, $P < 0.001$) at 0.5 Newtons per day (**Fig. 4.35**d).

Carbon dioxide concentrations of 40% and 80% stimulated the highest production of headspace acetaldehyde ($P < 0.001$), about 3 fold more than control, immediately after treatment, while 10% and 20% CO_2 were slightly greater than control (**Fig. 4.36**a); 100% CO_2 was the same as control. Enhancement of headspace acetaldehyde lasted for at least 2 days before decreasing to the same level as control at day 3 and remaining constant thereafter.

Exposing apples to 20% to 100% CO_2, but not to 10% CO_2 (in 20% O_2), caused headspace ethanol (**Fig. 4.36**b) and ethyl acetate (**Fig. 4.36**c) to accumulate compared to control ($P < 0.05$) with 100% CO_2 inducing the highest production ($P < 0.001$). Headspace ethanol and ethyl acetate production generally increased as CO_2 concentration increased. It should be noted that relatively high concentrations of headspace ethyl acetate (16 ppm), ethanol (30 ppm), and acetaldehyde (47 ppm), were detected in control fruit at the beginning of the experiment (day -1) and they increased with time at 20°C (**Fig. 4.36**).

Headspace ethanol from 100% CO_2 treated apples increased immediately after treatment and decreased gradually thereafter, with concentration remaining higher than

other treatments for 7 days (**Fig. 4.36**b). Headspace ethanol from 80% CO_2 + 20% O_2 treated fruit increased to a peak at day 3 after treatment and decreased slightly thereafter, while headspace ethanol from 40% and 20% CO_2 treated fruit peaked at day 1 before decreasing gradually. There was only a slight increase of ethanol in 10% CO_2 treated fruit at day 1.

Headspace ethyl acetate from 80% CO_2 + 20% O_2 treated fruit increased sharply at day 0 before decreasing to control levels at day 5, while the concentration from 100% CO_2 treated fruit increased markedly to day 1, then continued to increase gradually over 7 days. Headspace ethyl acetate from 40% CO_2 increased at day 1 after treatment, then decreased to the same levels as control at day 5, while the concentrations from 20% CO_2 were slightly above and from 10% CO_2 slightly below controls throughout 7 days at 20°C. (**Fig. 4.36**c).

Concentrations of ethyl 2-methyl butanoate, ethyl hexanoate, ethyl butanoate, and ethyl acetate from control apples detected at the beginning of the experiment (day -1) were 124, 359, 984, and 1797 $\mu mol \cdot l^{-1}$, respectively, and they either increased or remained constant during storage at 20°C. High CO_2 concentrations, from 40% to 100%, seemed to reduce overall production of some ethyl esters after treatment of apples stored for 6 months at 0°C before treatment. Total production of ethyl butanoate (**Fig. 4.37**a), ethyl 2-methyl butanoate (**Fig. 4.37**b) after treatment in 80% and 100% CO_2 treated fruit, and ethyl hexanoate (**Fig. 4.37**c) in 40% and 80% CO_2 treated fruit were significantly lower than control ($P < 0.05$). Ethyl butanoate or ethyl 2-methyl butanoate was decreased during 0 - 1 days or after 0 day, respectively, after CO_2 treatments. Only ethyl acetate production (**Fig. 4.38**a) was enhanced by high CO_2 (40% to 100%). Ethyl pentanoate (**Fig. 4.38**b) was variable and only detected in some fruit.

Application of 40% to 100% CO_2 to apples reduced production ($P < 0.05$) of butyl acetate (**Fig. 4.38**c) and hexyl acetate (**Fig. 4.39**a) in treated fruit after treatment compared to control, the higher the concentration of CO_2 the greater the reduction. Concentrations of butyl acetate ($r^2 = 0.38$, $P < 0.001$) and hexyl acetate ($r^2 = 0.51$, $P < 0.001$) generally decreased with time at 20°C in a quadratic manner.

Treatment with 80% CO_2 + 20% O_2 also caused a lower production of hexanal (**Fig. 4.39**c) than in control ($P < 0.05$), while treatments had no significant effects on

trans-2-hexenal (**Fig. 4.39**b). In general, *trans*-2-hexenal and hexanal from all treatments increased throughout day 7 at 20°C (**Fig. 4.39**b).

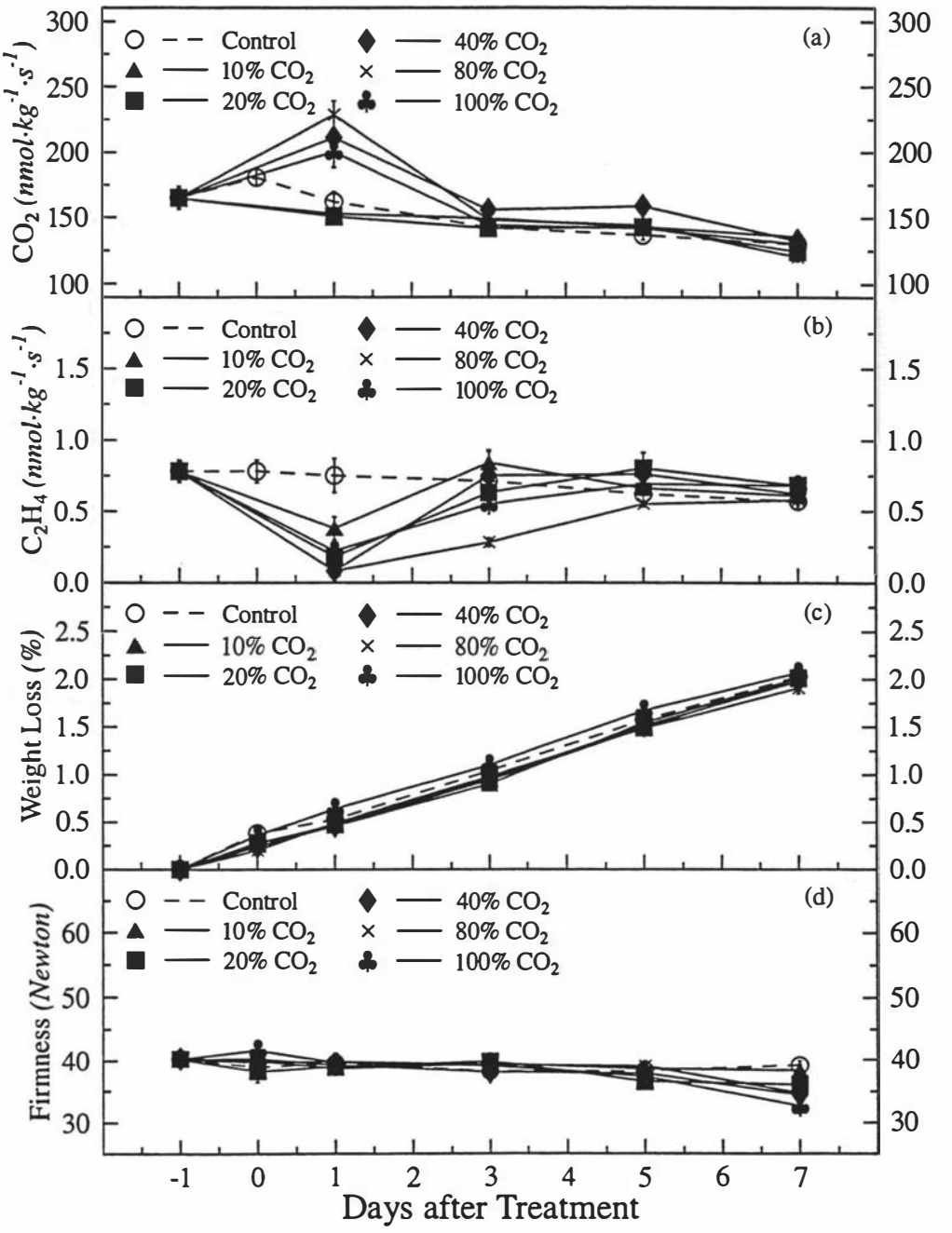


Fig. 4.35 Fruit (a) respiration rate, (b) ethylene production, (c) weight loss and (d) flesh firmness in 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2, or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

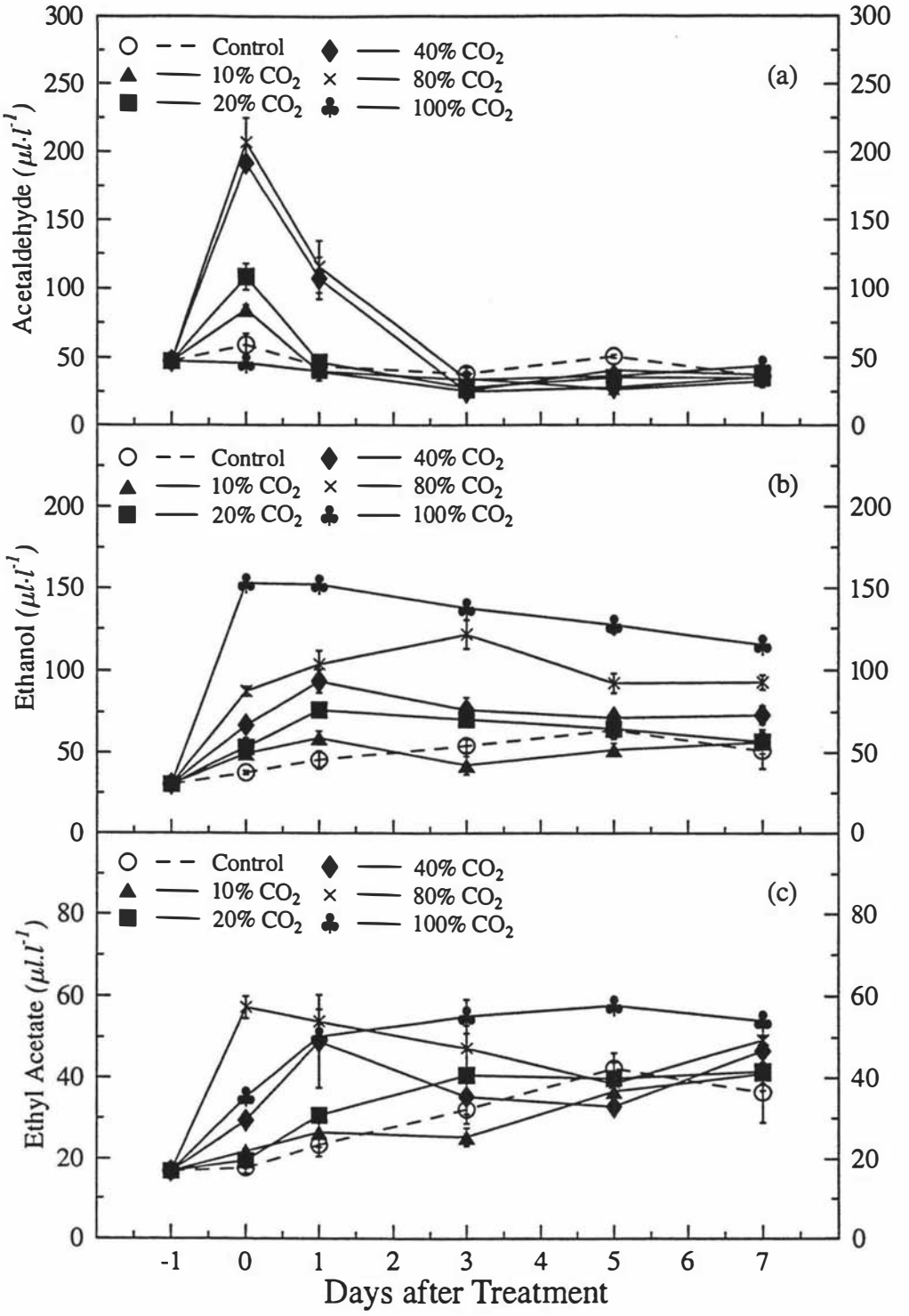


Fig. 4.36 Concentrations of (a) headspace acetaldehyde, (b) ethanol and (c) ethyl acetate in juice from 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 with 20% O_2, or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

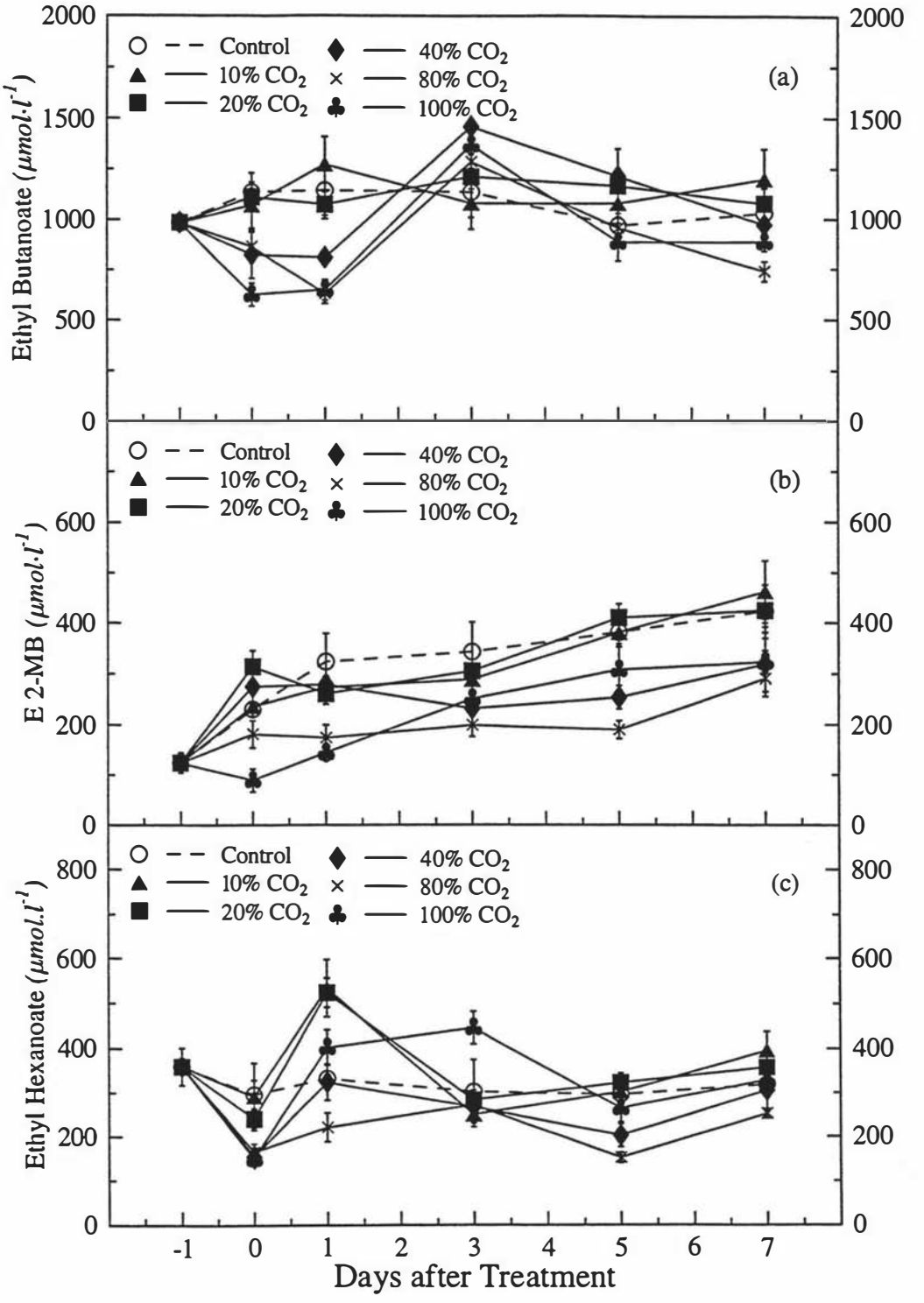


Fig. 4.37 Concentrations of (a) ethyl butanoate, (b) ethyl 2-methyl butanoate and (c) ethyl hexanoate in juice from 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

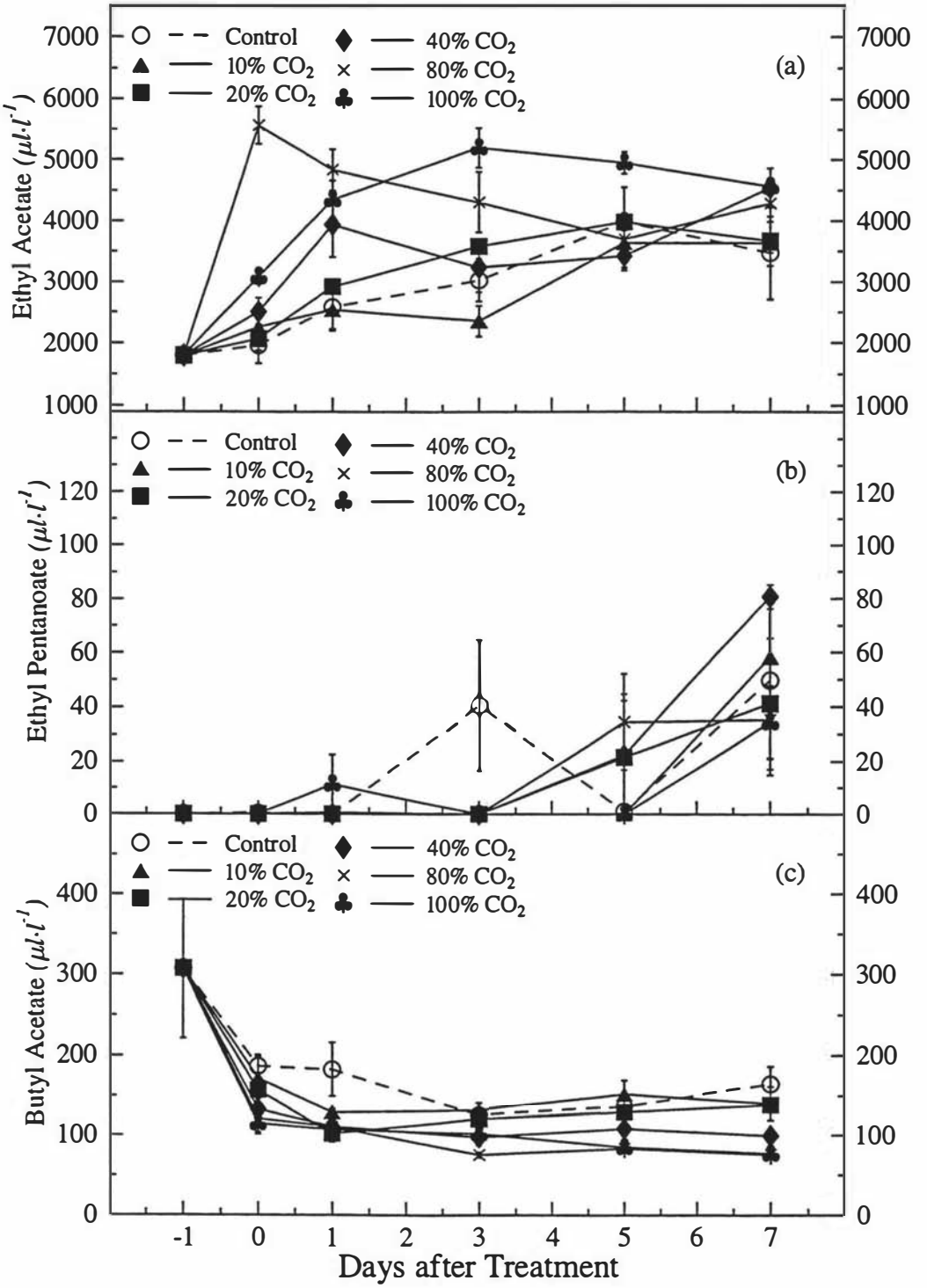


Fig. 4.38 Concentrations of (a) ethyl acetate, (b) ethyl pentanoate and (c) butyl acetate in juice from 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

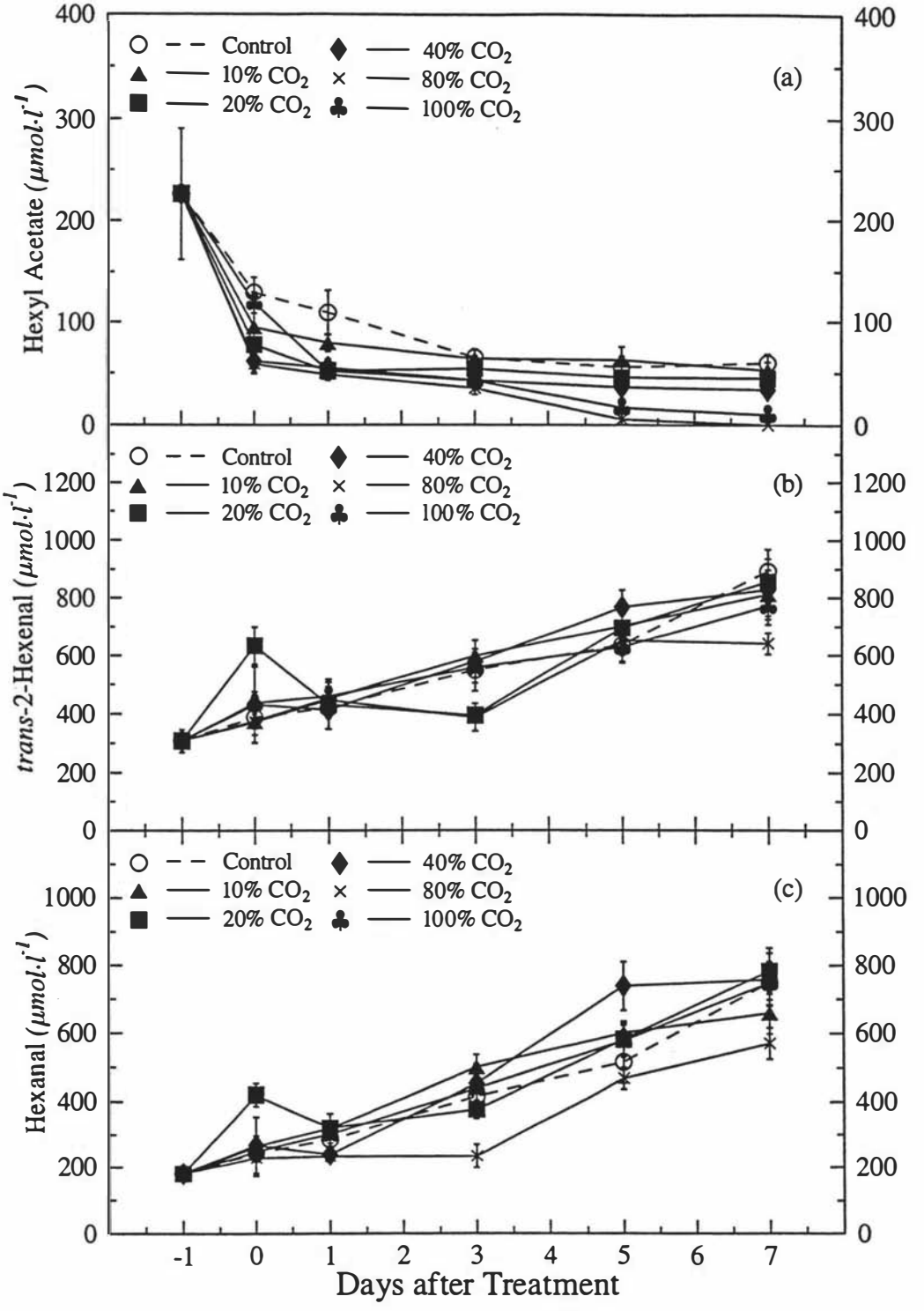


Fig. 4.39 Concentrations of (a) hexyl acetate, (b) *trans*-2-hexenal and (c) hexanal in juice from 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

Methanol concentration was variable from all treatments and were only detected occasionally from some fruit (**Fig. 4.40**a).

Production of ethanol from 100% CO_2 treated apples increased immediately after treatment and decreased slightly over 7 days, although the concentration remained substantially greater ($P < 0.001$) than other treatments. Ethanol from 80% CO_2 + 20% O_2 treated fruit increased to a peak at 3 days before decreasing, while ethanol from the 40% CO_2 exposure peaked at day 1 and decreased slightly thereafter, with concentrations from both treatments being greater ($P < 0.001$) than control. Ethanol from control fruit at day -1 was about 4650 $\mu mol \cdot l^{-1}$ and remained similar to concentrations from 10% CO_2 and 20% CO_2 treated fruit during 7 days (**Fig. 4.40**b).

Apples treated with high CO_2 from 10% to 80% had lower ($P < 0.05$) propan-1-ol production than in control, particularly 5 to 7 days after treatment, while 100% CO_2 increased this compound for 3 days (**Fig. 4.40**c).

Treatments (10 - 100% CO_2) slightly decreased butan-1-ol 0 to 1 day after treatment but there was no consistent difference from control during 7 days (**Fig. 4.41**a). A similar result was obtained with hexan-1-ol, where CO_2 treatments generally depressed production(**Fig. 4.41**b).

Total peak area (excluding solvent and internal standard peaks) from juice of treated and control apples were similar, indicating comparable total volatiles produced by these fruit, where total peak area remained relatively constant throughout 7 days at 20°C (**Fig. 4.41**c).

Treatments had no significant effects on most fruit quality attributes monitored, which included fruit skin colour (**Table 4.21**), TSS, TA (**Table 4.22**), TSS:TA ratio or pH (**Table 4.23**). Significant changes in some attributes of fruit composition occurred during storage. Fruit skin lightness did not change during 7 days after treatment, but fruit skin hue angle increased slightly with time at 20°C (**Table 4.21**). TSS decreased slightly in all treatments, while TA, TSS:TA ratio and pH increased slightly during 7 days at 20°C (**Table 4.22** and **Table 4.23**).

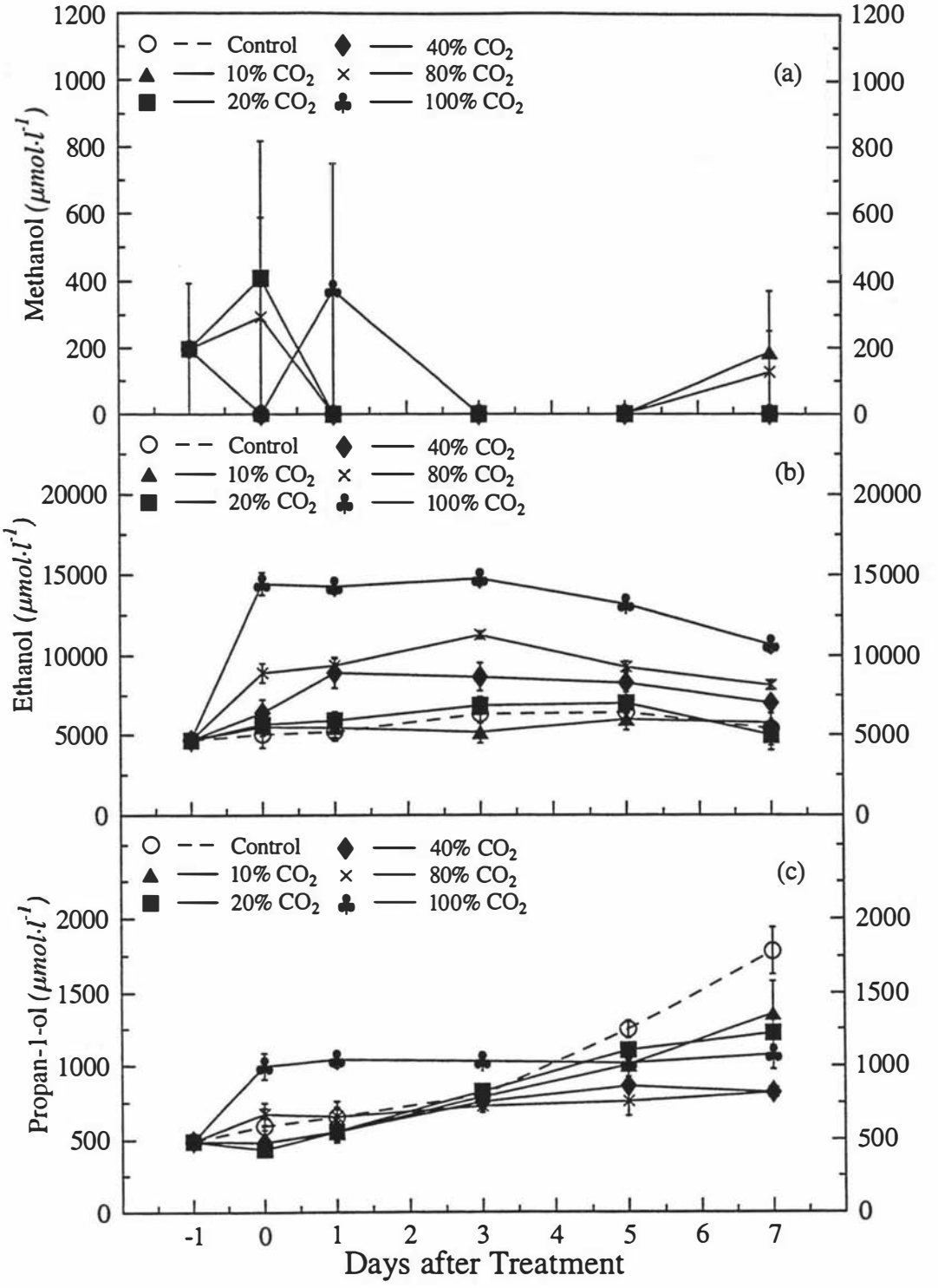


Fig. 4.40 Concentrations of (a) methanol, (b) ethanol and (c) propan-1-ol in juice from 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

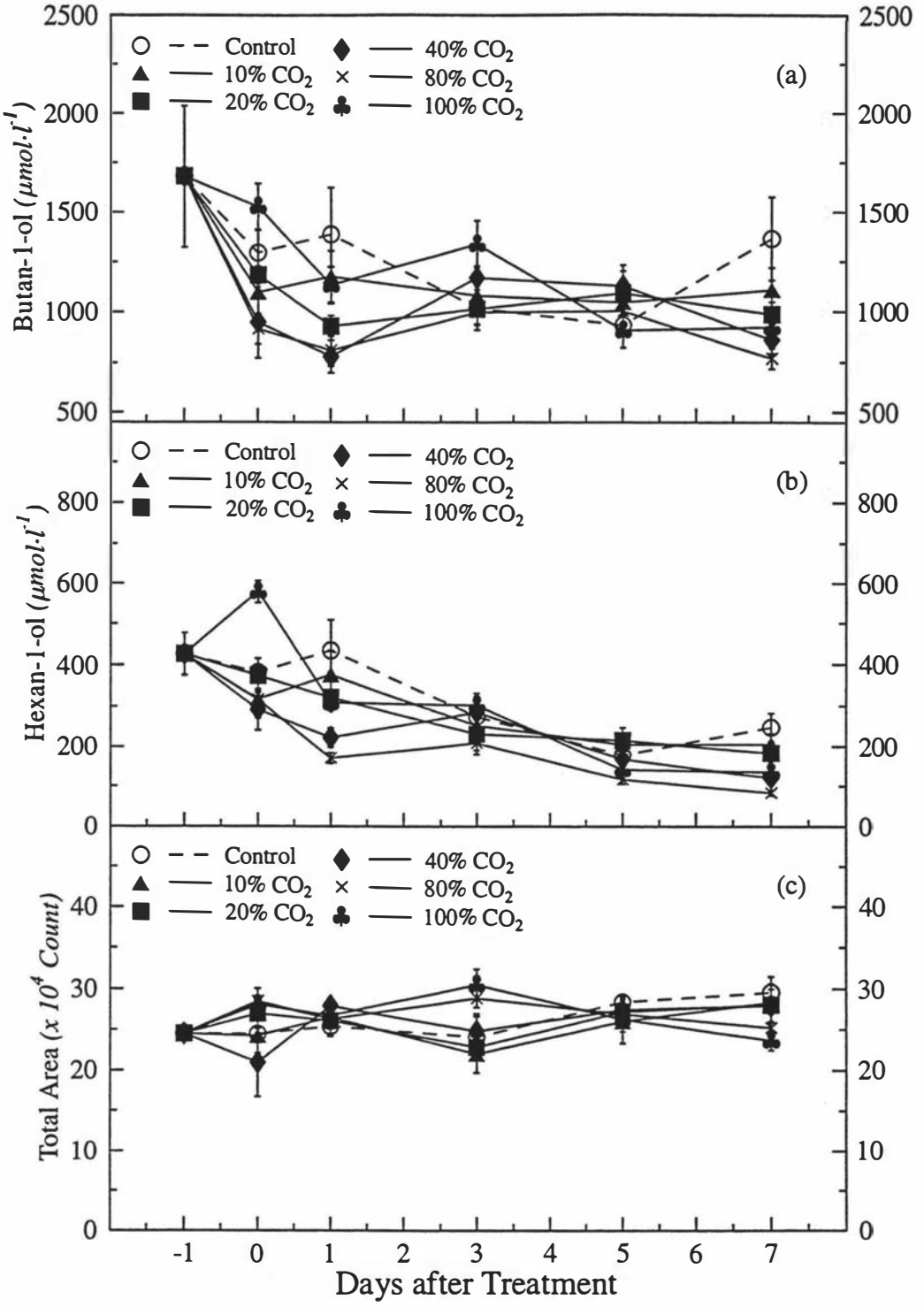


Fig. 4.41 Concentrations of (a) butan-1-ol, (b) hexan-1-ol and (c) total peak area in juice from 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

Table 4.21 Fruit skin colour in 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

	Lightness (%)					
Day	Control	10%	20%	40%	80%	100%
-1	39.8	39.8	39.8	39.8	39.8	39.8
0	39.5	41.1	37.3	40.3	41.5	40.2
1	39.9	38.9	40.5	40.6	41.3	39.4
3	42.7	38.8	38.9	39.5	38.6	40.9
5	42.2	40.5	41.0	40.4	38.8	41.9
7	42.1	40.0	38.2	38.6	38.9	41.1
c.v.			7.74%			
Day			ns			
Treat			ns			
Day x Treat			ns			
	Hue Angle (°)					
-1	27.6	27.6	27.6	27.6	27.6	27.6
0	26.0	28.9	25.4	30.3	32.1	31.3
1	31.0	29.1	32.2	32.4	36.3	32.9
3	34.9	27.4	26.7	30.8	31.0	33.0
5	34.3	30.5	32.5	37.1	30.3	36.7
7	34.4	30.9	28.7	30.1	31.6	35.5
c.v.			16.28%			
Day			**			
Treat			ns			
Day x Treat			ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns)

Table 4.22 Total soluble solids (TSS) and titratable acids (TA) in juice from 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

	TSS (%)					
Day	Control	10%	20%	40%	80%	100%
-1	12.8	12.8	12.8	12.8	12.8	12.8
0	12.7	12.7	12.6	12.6	12.4	12.7
1	12.8	12.8	12.7	12.2	12.2	12.4
3	12.8	12.3	12.7	12.6	12.2	12.1
5	12.2	12.4	12.5	12.2	12.3	12.2
7	12.5	12.5	12.6	11.9	12.2	12.2
c.v.			3.55%			
Day			***			
Treat			ns			
Day x Treat			ns			
	TA (%)					
-1	0.18	0.18	0.18	0.18	0.18	0.18
0	0.18	0.17	0.16	0.18	0.16	0.17
1	0.18	0.16	0.16	0.18	0.17	0.16
3	0.17	0.16	0.17	0.15	0.16	0.15
5	0.15	0.15	0.16	0.15	0.16	0.15
7	0.16	0.17	0.15	0.14	0.14	0.15
c.v.			10.11%			
Day			***			
Treat			ns			
Day x Treat			ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns)

Table 4.23 TSS:TA ratio and pH in juice from 'Red Delicious' apples (previously stored for 6 months at 0°C) after treatment with 10%, 20%, 40%, 80% CO_2 in 20% O_2 or 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH (***Experiment 6***).

	TSS:TA Ratio					
Day	Control	10%	20%	40%	80%	100%
-1	73.7	73.7	73.7	73.7	73.7	73.7
0	73.3	76.7	82.0	69.3	80.8	76.3
1	71.6	83.6	79.8	66.6	74.2	80.2
3	81.0	77.1	77.1	86.0	78.5	81.8
5	84.7	83.1	78.3	80.0	79.5	80.0
7	77.7	77.4	86.9	84.6	87.4	81.0
c.v.			10.66%			
Day			* *			
Treat			ns			
Day x Treat			ns			
	Juice pH					
-1	3.86	3.86	3.86	3.86	3.86	3.86
0	3.76	3.82	3.82	3.76	3.85	3.80
1	3.75	3.76	3.79	3.75	3.77	3.80
3	3.81	3.85	3.81	3.87	3.77	3.82
5	3.89	3.90	3.82	3.90	3.87	3.91
7	3.98	3.92	3.95	3.91	3.88	3.89
c.v.			1.71%			
Day			* * *			
Treat			ns			
Day x Treat			ns			

Levels of Significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), non significant (ns)

4.5 DISCUSSION

Hypoxic conditions achieved by high concentrations of CO_2, N_2 or low O_2 consistently enhanced headspace acetaldehyde, ethanol and ethyl acetate from juice of 'Braeburn', 'Granny Smith' and 'Red Delicious' apples after treatment. Endogenous enhancement of ethanol, which followed a similar pattern, occurred in response to hypoxia and accumulated in fruit tissue to high levels. This large increase of ethanol was followed by the production of substantial amounts of volatile ethyl acetate and several ethyl esters including ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate in juice from hypoxically treated apples in all cultivars studied. Enhancement of these aroma-impact ethyl esters in apple juice consistently occurred during 1 to 5 days at 20°C after hypoxic treatments. In contrast, such conditions consistently decreased esters (other than ethyl esters) such as butyl acetate, hexyl acetate and propyl butanoate in juice from the treated apples, generally occurring during the same time as the increase in ethyl esters. Production of some alcohols (other than ethanol), such as butan-1-ol, and aldehydes (except acetaldehyde), such as *trans*-2-hexenal, in juice from hypoxically treated apples also appeared to decrease slightly or moderately after treatment. Hypoxic conditions were less effective in enhancing headspace acetaldehyde, ethanol and ethyl acetate and some important ethyl esters from juice of apples stored for more than 6 months at 0°C than from freshly harvested fruit, although other esters, such as butyl acetate and hexyl acetate, did decrease after treatment.

In fruits such as apples, pears and strawberries, aroma develops during the normal ripening process (Mattheis *et al.*, 1991b; Song and Bangerth, 1996; Tressl *et al.*, 1975). An increase in ester synthesis in 'Bisbee Delicious' apples coincided with fruit ripening, indicated by an increase in climacteric rise of internal ethylene (Mattheis *et al.*, 1991b). Yamashita *et al.* (1977) found that ester synthesis in strawberries was absent in immature fruit until ripening began.

It is generally accepted that ethylene is required for normal ripening of fruit (Yang, 1985) and at the onset of ripening there is an increase in internal concentration and in the rate of production of ethylene (Brady, 1987; Lieberman, 1979). During ripening, activities of hydrolase enzymes such as amylase, lipase, chlorophyllase and

protease increase (Brady, 1987) leading to degradation of macromolecules including lipids, carbohydrates and proteins and accumulation of metabolites, precursors and substrates (free fatty acids, amino acids, sugars) for volatile synthesis (Bartley, 1985; Paillard, 1981; 1990). Song and Bangerth (1996) suggested that increased internal ethylene production, respiratory activity and more general metabolic activity is a prerequisite for the stimulation of aroma volatile production of apples, where increased metabolic activity could be essential for providing precursors for volatile synthesis. In spite of the possible involvement of ethylene on volatile production of fruit, there appears to be no evidence that ethylene directly induces production of volatile compounds. Application of exogenous ethylene is known to hasten ripening and senescence in various horticultural products (Kader, 1985; Watada, 1986). Ethylene may indirectly influence volatile production through its effects on enhancing senescence; some fruit such as apples, pears and mangoes produce appreciable amounts of acetaldehyde and ethanol (Bender *et al.*, 1994; Janes and Frenkel, 1978; Nichols and Patterson, 1987) which may act as precursors or substrates for production of important flavour impact compounds during ripening and senescence. This will be discussed in **Chapter Six**.

It is generally believed that fruit volatile esters are synthesised enzymatically by coupling the respective acid (or acyl CoA) and alcohol moieties (Salunkhe and Do, 1976), a reaction catalysed by alcohol acyltransferase (AAT) (Fellman and Mattheis, 1995; Oliás *et al.*, 1995). Precursors of straight-chain alcohols and acyl CoAs for ester synthesis are thought to be derived from the oxidation of long-chain fatty acids, such as linoleic and linolenic acids, through a number of cycles of the β-oxidation pathway, which results in short-chain acyl CoAs (Bartley *et al.*, 1985). The short-chain acyl CoAs are either substrates for esterification with alcohols or reduced by acyl CoA reductase to aldehydes which are reduced in turn to alcohols by ADH (Bartley *et al.*, 1985; Drawert, 1975). Branched-chain acyl CoAs and alcohols are thought to be derived from transamination of amino acids, such as leucine and isoleucine, via keto acid metabolism (Tressl *et al.*, 1975). Thus ethyl butanoate is believed to form from esterification of ethanol and butyl CoA, which is derived from fatty acids, whereas ethyl 2-methyl butanoate is derived from ethanol and 2-methyl butyl CoA, which is derived from isoleucine; the reactions are catalysed by AAT, the only known esterifying enzyme (Oliás

et al., 1995). The dramatic enhancement of ethyl esters in juice from treated apples in the present study seemed to be an indirect effect of hypoxic conditions which resulted in the accumulation of ethanol, which was subsequently esterified into ethyl esters.

Stress levels of low O_2 and/or high CO_2 atmospheres are known to stimulate production of acetaldehyde, ethanol and ethyl acetate in various fruits, including apples (Patterson and Nichols, 1988), pears (Ke *et al.*, 1990), strawberries (Ueda and Bai, 1993), oranges (Shaw *et al.*, 1991), nectarines and peaches (Lurie and Pesis, 1992). Production of these volatiles in fruit under hypoxia appears to be time, temperature and fruit age dependent, in which production increases with increasing temperature and time of exposure, as well as advancing fruit age (Boersig *et al.*, 1988; Gran and Beaudry, 1993; Ke *et al.*, 1993a; 1993b).

Under hypoxic atmospheres, pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and/or lactate dehydrogenase (LDH) are induced by a decrease in cytosolic pH, by an increase in levels of pyruvate and NADH, and by a decrease in ATP and NAD levels, which direct pyruvate to the fermentation pathway and result in production of acetaldehyde and ethanol (Chen and Solomos, 1996; Chervin *et al.*, 1996; Kennedy *et al.*, 1992; Ricard *et al.*, 1994). Accumulation of ethanol produced in response to hypoxia led to substantial amounts of ethyl esters in the treated fruit. Apples can metabolise exogenously applied ethanol to acetaldehyde and a variety of ethyl esters (Berger and Drawert, 1984) with ethyl acetate being a major ester produced following ethanol treatment (Bartley *et al.*, 1985).

The time of peaking of enhanced headspace and ethyl volatiles from juice after treatments varied slightly during 0 - 5 days at 20°C, depending on compounds and experiment. Headspace acetaldehyde and ethanol peaked 0 - 1 day after removal from hypoxic conditions, while ethyl acetate peaked 1 - 3 days after treatment. Under hypoxia, acetaldehyde induced through the fermentative pathway can be readily and rapidly reduced into ethanol by ADH (Chervin *et al.*, 1996; Perata and Alpi, 1993). These processes lead to an immediately high production of both volatile compounds in apples upon removal to air at 20°C after treatment, peaking after 0 - 1 day and decreasing thereafter.

Upon removing the treated apples to air, a lag period occurred before an increase in ethyl esters was noted. Increases in ethyl acetate and ethyl hexanoate, which occurred immediately after treatment were slight, relative to ethanol, but such increases became marked and rapid at day 1, peaking after 1 - 3 days at 20°C. Increases in ethyl butanoate and ethyl 2-methyl butanoate followed a similar pattern, but peaked 3 - 5 days after treatment. The reason for such a lag phase could possibly due to residual suppression of ester forming enzymes by hypoxia and it may require time to (re)-activate the existing enzymes to become operative (Fellman and Mattheis, 1995; Fellman *et al.*, 1993a; 1993b). Fellman *et al.* (1993a; 1993b) found that AAT activity was undetectable in apples immediately after removal from 0.5% O_2 storage conditions to air, but its activity resumed shortly after.

The reason for the late peaking time (3 - 5 days after treatment) of ethyl butanoate and ethyl 2-methyl butanoate, relative to ethyl acetate and ethyl hexanoate, is not known. However, it is possible that the hypoxic treatment leads to the depletion of O_2 in fruit tissue which may suppress some enzymes and/or reactions requiring O_2 for liberating substrates for esterification (Stryer, 1988). It is probable that there are time and O_2 requiring steps for the process of generating the acyl CoAs, butyl and 2-methyl butyl CoAs, from precursors (fatty acids and amino acids) for ethyl ester synthesis. If this is so, generation of these acyl CoAs would take some time upon removal to air after treatment, and hence delay peaking time of the corresponding ethyl esters. On the other hand, acetyl CoA and hexyl CoA may be readily available for esterification with ethanol, thus ethyl acetate and ethyl hexanoate would peak earlier than ethyl butanoate and ethyl 2-methyl butanoate.

The increase in fruit ethanol and the number and amount of ethyl esters occurring following hypoxic treatments of apples generally lasted only 5 - 7 days at 20°C. In addition to direct diffusion out of the fruit (Kimmerer and Kozlowski, 1982; Pesis and Ben-Arie, 1986), ethanol was probably dissipated as a result of conversion to esters and acetaldehyde that are sufficiently volatile to evaporate out of the fruit to the surrounding atmosphere (Cossins, 1978; Knee and Hatfield, 1981). Therefore, in order to increase some important character-impact volatile compounds in fresh apples, in juice or aqueous essence and to obtain benefits in a commercial scale production system, apples should be

processed 1 - 5 days after treatment with hypoxic atmospheres. For instance, if ethyl 2-methyl butanoate or ethyl butanoate is important, fruit should be processed between 3 - 5 days after treatment, or if ethyl hexanoate is important apples should be processed 1 day after treatment.

Apples exposed to 100% CO_2 for 6 - 12 hours produced more headspace and ethyl volatiles than controls, but less than the 18 - 48 hour treatments. Concentrations of some ethyl esters such as ethyl butanoate and ethyl 2-methyl butanoate were slightly greater following 18 - 24 hours treatment than after 48 hour treatment. On the other hand, enhancement of ethanol, ethyl acetate and ethyl hexanoate was positively related to time of CO_2 application with the highest concentrations occurring following the 48 hour treatment.

A 6 - 12 hour exposure time to hypoxic conditions was not long enough to allow ethanol to accumulate to concentrations high enough to lead to substantiative enhancement of ethyl esters (Berger and Drawert, 1984; Knee and Hatfield, 1981). On the other hand, the comparatively lower production of ethyl butanoate and ethyl 2-methyl butanoate following 48 hours treatment than from 24 hours could possibly be due to a shift in ethyl esterification to the higher molecular weight ethyl esters (higher than ethyl butanoate) as ethyl hexanoate was also enhanced in a positive manner with time of exposure. In a study on exogenously applied ethanol (concentrations 0 - 300,000 $\mu mol \cdot l^{-1}$) to tissue discs or whole fruit of 'Red Delicious' apples, Berger and Drawert (1984) found that increasing ethanol concentrations favoured esterification of medium-chain acyl moieties (C_6 and longer), while compounds with short-chain acyl moieties ($< C_6$) increased in concentrations more rapidly at lower ethanol concentration ranges and began to decrease at high ethanol concentrations. Berger *et al.* (1992) also found that ethanol concentrations (in gas phase) of less than 50,000 $\mu mol \cdot l^{-1}$ saturated esterification of short-chain acyl moieties and the higher concentrations reduced this process.

Although ethyl hexanoate from the 48 hour CO_2 treatment was greater than from 24 hours, the magnitude of enhancement was relatively small compared to the increase in ethanol substrate from the corresponding treatment. It seems that other factors may be involved; it is possible that limited availability of hexyl CoA for esterification (Gilliver

and Nursten, 1976; Oliás et al., 1995) is responsible for the relatively slight enhancement observed.

Ethyl acetate is a relatively low molecular weight ethyl ester, that like ethanol increased in response to time of exposure to CO_2; in these experiments concentration was not reduced as reported by Berger *et al.* (1992). The reason for this phenomenon could be that ethanol and acetyl CoA substrates are not limiting for esterification (Bartley *et al.*, 1985; Knee and Hatfield, 1981). Acetyl CoA may be derived from at least 3 routes including β-oxidation of fatty acids (Bartley *et al.*, 1985), from pyruvate as a result of pyruvate dehydrogenase (PDH) activity (Ke *et al.*, 1993a; Kennedy *et al.*, 1992) and oxidation of acetaldehyde (Eriksson, 1979; Reazin *et al.*, 1970). Because of this, acetyl CoA is not likely to be limited; with large amounts of ethanol produced in response to hypoxic treatments, the increased production of ethyl acetate will be directly proportional to ethanol which in turn is related to time of CO_2 application.

Carbon dioxide stimulates acetaldehyde and ethanol production in plant tissues even in the presence of high concentrations of O_2 (Vartapetian *et al.*, 1978; Wager, 1974; Zemlianukhin and Ivanov, 1978) and these volatiles, particularly ethanol, are believed to be partly metabolised into ethyl esters and other compounds (Berger and Drawert, 1984; Cossins, 1978). Carbon dioxide concentrations of more than 20% in air or in 20% O_2 stimulated acetaldehyde, ethanol and ethyl acetate in fruits, including apples, pears and strawberries (Ke *et al.*, 1991a; Larsen and Watkins, 1995b; Ueda and Bai, 1993).

Although N_2 is an inert gas, the 100% N_2 treatment had a similar effect on inducing volatile production in apples as did the 100% CO_2 treatment. This stimulation of volatiles in response to 100% N_2 is probably directly due to the effect of low O_2 concentrations rather than high N_2 on fruit metabolism. Oxygen concentrations lower than 2.5% (in N_2) were necessary to achieve an enhancement of important aroma-impact esters of freshly harvested apples. Results indicated that 2.5% O_2 (in N_2) moderately enhanced production of several ethyl esters and slightly decreased butyl acetate and hexyl acetate in freshly harvested apples, while 5% O_2 (in N_2) treatment did not differ from control fruit. The greatest response was achieved with 0% O_2 (100% CO_2). High concentrations of CO_2 were more effective than high N_2 for inducing headspace volatiles,

even in old fruit. For example, peak concentrations of acetaldehyde, ethanol and ethyl acetate obtained from the 80% CO_2 (plus 20% O_2) treatment were 206, 121 and 57 $\mu l \cdot l^{-1}$, respectively (**Fig. 4.36**), while from the 90% N_2 (plus 10% O_2) were 55, 58 and 29 $\mu l \cdot l^{-1}$, respectively (**Fig. 4.24**). Even at higher concentration of N_2 of 97% (plus 2.5% O_2), concentrations of the respective compounds above were 68, 84 and 38 $\mu l \cdot l^{-1}$ (**Fig. 4.24**). In oranges, a 99% CO_2 + 1% O_2 (24 hours at 20°C) treatment induced greater production of acetaldehyde and ethanol after treatment than did 49% CO_2 + 50% N_2 + 1% O_2 (Pesis and Avissar, 1989). It was suggested that CO_2 affected the accumulation of volatiles differently from N_2 (under the same O_2 concentration), as the CO_2 molecule itself may become involved in acetaldehyde formation, probably entering into metabolism by phosphoenol pyruvate carboxylase into the C-4 of malate, which is then decarboxylated into acetaldehyde and in turn ethanol (Pesis and Avissar, 1989; Pesis and Ben-Arie, 1986). Thus, CO_2 gas may be a more practical choice for enhancing volatile compounds in apples than N_2 gas.

Apples which had been stored for more than 6 months at 0°C had high TSS (≈ 13% - 14%) and pH (≈ 3.9 - 4.1), and low TA (≈ 0.14% - 0.17%) and flesh firmness (≈ 40 - 50 Newtons), indicating that they were over-ripe. An attempt was made to investigate the tendency of changes in the production of some volatile compounds of apples which had been stored for various time by compiling data (as mean concentrations during 7 days at 20°C after removal from cool store) in control fruit from 5 experiments (**Table 4.24**). Headspace acetaldehyde, ethanol, ethyl acetate, volatile ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate, ethyl acetate and ethanol increased to a maximum at 6 months of cool storage and decreased thereafter. Butyl acetate, hexyl acetate, hexanal and *trans*-2-hexenal were high initially, peaking at 4 months and decreasing thereafter. Propan-1-ol and methanol were high in freshly harvested fruit, decreasing or disappearing thereafter, while butan-1-ol and hexan-1-ol fluctuating throughout storage (**Table 4.24**).

It has been suggested that production of acetaldehyde and ethanol during fruit ripening occurs as a result of tissue disorganisation causing blockage of intercellular spaces (Lidster *et al.*, 1983; Smagula and Bramlage, 1977), thus restricting gas exchange between fruit tissue and the outer atmosphere (Dadzie, 1992) and resulting in partial

anaerobiosis in fruit tissue (Blanke, 1991). It has also been found that the anaerobic compensation points of apple and pear fruit gradually increase to higher O_2 concentrations with prolonged storage suggesting a decrease in skin permeance as fruit became less permeable to gases with advancing physiological age (Boersig *et al.*, 1988; Dadzie, 1992). Any restriction of gas exchange in fruit tissue could result in partial anaerobiosis (Blanke, 1991; Yearsley *et al.*, 1996), which may in turn induce production of acetaldehyde and ethanol as fruit age. Removal of fruit, such as apples (0° - 2°C) and oranges (0° - 5°C), from cold storage to 20°C stimulated production of acetaldehyde and ethanol (Blanpied and Jozwaik, 1993; Eaks, 1980) probably because the increased temperature accelerated chemical reaction rates and thus increased O_2 requirement of fruit tissue; any blockage of lenticels would lead to a temporal depletion of O_2 and partial anaerobiosis (Dilley *et al.*, 1964). Therefore, the degree of accumulation of these fermentative volatiles would be higher with prolonged cold storage of apples, relative to freshly harvested fruit.

Table 4.24 Mean concentration of volatile compounds from juice of control apples during 7 days at 20°C in relation to fruit age.

Compound	Cultivar (time in storage at 0°C) / Concentration				
	Red Delicious (2wk)	Braeburn (4mth)	Red Delicious (4mth)	Red Delicious (6mth)	Red Delicious (8mth)
Headspace Volatiles ($\mu l \cdot l^{-1}$)					
Acetaldehyde	0.8	2.5	3.6	45.9	52.0
Ethanol	1.5	3.7	3.6	47.2	36.3
Ethyl acetate	0.3	9.9	2.3	28.1	17.3
Aroma Volatiles ($\mu mol \cdot l^{-1}$)					
Ethyl acetate	610	486	264	2803	1565
Ethyl butanoate	72	69	206	1065	888
Ethyl 2-methyl butanoate	n.d.	44	34	304	168
Ethyl hexanoate	n.d.	9	31	317	227
Butyl acetate	460	970	510	184	271
Hexyl acetate	153	231	312	108	142
Hexanal	222	86	612	398	239
trans-2-Hexenal	1061	562	1283	532	399
Methanol	574	137	n.d.	32	n.d.
Ethanol	349	360	638	5487	4306
Propan-1-ol	1569	158	616	928	859
Butan-1-ol	2143	1334	2222	1280	2175
Hexan-1-ol	307	217	439	234	476

Data were means from 4 to 7 replicates, depending on experiment (see **Materials and Treatments, *Section 4.3.1***), wk = week, mth = month

Increased production of ethyl esters such as ethyl acetate, ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate, with increasing time in storage probably results from esterification of ethanol with acyl CoAs by apples (Knee and Hatfield, 1981, Nursten, 1970); as ethanol production increased, production of ethyl esters also increased and the opposite was true (**Table 4.24**). A decreased production of these ethyl esters after 8 months storage could be partly due to a reduced availability of acyl CoAs, such as acetyl CoA, butyl CoA, hexyl CoA and 2-methyl butyl CoA for esterification of ethanol (Gilliver and Nursten, 1976). Production of other esters, such as butyl acetate and hexyl acetate increased and peaked about 4 months of storage, which could be associated with the time of fruit ripening that may occur slowly during cool storage (Paillard, 1990; Yahia, 1994). Production of these esters decreased after 6 months storage, while production of butan-1-ol and hexan-1-ol fluctuated throughout storage, hence it seems likely that such decreases in butyl acetate and hexyl acetate may be due to the lack of acetyl CoA for esterification of the corresponding alcohols (Gilliver and Nursten, 1976; Oliás *et al.*, 1995). Another possibility for such a decrease in production of these esters may be an increased production of ethanol, which could favour esterification of low molecular weight esters such as ethyl acetate, causing a competition for acetyl CoA for esterification with higher molecular weight alcohols, butan-1-ol and hexan-1-ol, and in turn decreasing production of butyl and hexyl acetate (Berger and Drawert, 1984; Knee and Hatfield, 1981). A reduced capacity of fruit to synthesise aroma volatile compounds would have occurred in apples stored for more than 6 months as a result of natural senescence (Brady, 1987; Paillard, 1981; Yahia, 1989) perhaps the most important factor responsible for decreased production of aroma volatiles after a long term cool storage of apples.

Although fruit ripening is slow during cool storage, Paillard (1990) reported that total emission of total volatiles from 'Golden Delicious' and 'Caville Blanc' apples stored at 0° and 4°C, respectively, was marked and reached a peak after 20 weeks which corresponded to the time of fruit ripening during storage. However, storage of these apples for 32 weeks (8 months) caused a reduced production of volatiles which was suggested to be due to exhaustion of substrates and reduced capacity to synthesise aroma volatiles (Paillard, 1990).

The degree of enhancement of ethyl esters production following hypoxic treatments in apples that had been stored for more than 6 months at 0°C was obviously less than that from freshly harvested fruit (detailed discussion on this issue is given in **Chapter Seven**). Freshly harvested apples may lack the required alcohol substrates for normal volatile production (Knee and Hatfield, 1981; Mattheis *et al.*, 1991b). The induction of large amounts of ethanol by high CO_2 or N_2 treatments allowed *in situ* esterification to proceed resulting in a substantial increase in ethyl ester production. Despite substantial ethanol accumulation in response to hypoxic treatments, only a marginal enhancement in production of some important ethyl esters occurred after prolonged storage of apples. The high concentrations of ethyl esters already present in control fruit on removal from cool store presumably resulted from esterification of ethanol in fruit during storage (Berger and Drawert, 1984, Berger *et al.*, 1992), indicating that the enzyme system is operative.

Another possible reason for the lack of response to hypoxic treatments in old apples, could be due to a reduced capacity of the fruit to synthesise aroma volatile compounds, although the enzyme systems are operative (Brady, 1987; Grierson, 1987; Tressl *et al.*, 1975). Ester biosynthetic capacity of 'Rome' apples was reduced substantially (about 50%) after 6 months in refrigerated-air (RA) storage (Fellman *et al.*, 1993b). The availability of alcohol and acyl CoA substrates needed for esterification may be another important factor (Oliás *et al.*, 1995) affecting production of esters in apples which had been stored for more than 6 months. It has been suggested that availability of acetyl CoA is adequate for esterification in a long-term (> 6 months) CA stored apples, as it may be derived in different ways as discussed earlier (Bartley *et al.*, 1985; Knee and Hatfield, 1981). However, reduced availability of acetyl CoA and other acyl CoAs could occur as a consequence of exhaustion of precursors as fruit age resulting in decreased ester production (Paillard, 1990). Amino acids, known to be precursors of branched-chain volatiles, decreased during ripening and senescence of apples (Ackermann *et al.*, 1992) and synthesis and/or degradation of fatty acids also decreased after long-term storage of apples (Harb *et al.*, 1994). This aspect will be discussed in detail in **Chapter Five**.

The large quantity of ethanol produced by the fruit in response to hypoxic conditions is sufficient to displace other alcohols in reactions with ester forming enzymes; in addition concentration of other major alcohols such as butan-1-ol and hexan-1-ol all decreased following hypoxia. This in turn led to reduced production of propyl butanoate, butyl acetate and hexyl acetate in hypoxically treated apples. A decrease in butyl and hexyl esters emanating from fruit following treatment with ethanol or anaerobic conditions has previously been observed in apples (Berger and Drawert, 1984; Mattheis *et al.*, 1991a) and strawberries (Ke *et al.*, 1994b; Larsen and Watkins, 1995a). This highlights the competitive demand for acyl moieties required for esterification, as excess of one alkyl moiety always results in a decrease in esters not containing that moiety (Bartley *et al.*, 1985; Berger *et al.*, 1992; De Pooter *et al.*, 1987).

Another consequence of hypoxic treatments was the decrease in C_6 - aldehydes (hexanal and *trans*-2-hexenal) and alcohols (other than ethanol) such as butan-1-ol, hexan-1-ol and 3-methyl butan-1-ol following treatment. In apples, hexanal and *trans*-2-hexenal are believed to be derived from linoleic and linolenic acids by the reaction of lipoxygenase during the disruption of fruit tissues (Feys *et al.*, 1980b; Hatanaka, 1996; Riley *et al.*, 1996). Straight-chain alcohols (C_3 or higher) and branched-chain alcohols are thought to derive from fatty acid and amino acid metabolism, respectively (Bartley, 1985; Drawert, 1975). High CO_2 and/or low O_2 atmospheres have been thought to suppress synthesis and/or degradation of fatty acids and amino acids in apples (Brackmann *et al.*, 1993). It has also been suggested that high CO_2 may operate through its effect on polyamine biosynthesis, maintaining putrescine and spermadine concentration in tissue, thereby inhibiting lipid oxidation (Philosoph-Hadas *et al.*, 1993). Thus reduced production of these aldehydes and alcohols may be partly due to the suppressive effect of hypoxic conditions on fatty acid and/or amino acid metabolism, and hence reducing availability of precursors and/or substrates for their synthesis.

Competition on acyl CoAs moieties for esterification could possibly account for decreased production of some alcohols in hypoxically treated apples. Straight-chain alcohols are supposed to derive from short-chain fatty acyl CoAs via aldehyde intermediates and branched-chain alcohols are believed to derive from keto acid metabolism of amino acids via branched-chain aldehydes (Paillard, 1990; Tressl *et al.*,

1975). For example, ethanol in fruit tissue may react rapidly with butyl CoA using ester forming enzymes to produce ethyl butanoate (Berger *et al.*, 1992); this would decrease the quantity of butyl CoA to be converted into butanal and subsequently butan-1-ol. Aldehydes could be converted into acyl CoAs via their respective acids (De Pooter *et al.*, 1983); butanal could be converted to butanoate and then into butyl CoA, which subsequently serves as substrate for esterification of ethanol into ethyl butanoate (Drawert, 1975). This conversion of aldehydes into acyl CoAs (instead of alcohols) may be another reason for the reduced production of some alcohols in treated apples.

The residual suppressive effect of hypoxic conditions may only be brief, as some of these volatiles, such as *trans*-2-hexenal, return to levels comparable to control fruit 3 - 5 days after treatment. However, increasing exposure time (> 24 hours) to hypoxic conditions seemed to exert an increasingly suppressive effect on production of these volatiles.

Accumulation of acetaldehyde, ethanol and ethyl acetate, either naturally produced during ripening and senescence or hypoxically induced, has been linked to off-flavour development in many fruit, including apples, pears (Fidler and North, 1971; Ke *et al.*, 1991a) and strawberries (Ke *et al.*, 1993b; Larsen and Watkins, 1995b; Ueda and Bai, 1993). The connection between these volatiles and off-flavours in fruit seems to be concentration dependent, however the association between them is still unclear. Ke *et al.* (1991a) reported that ethanol concentrations of more than 1000 $\mu l \cdot l^{-1}$ caused off-flavour in 'Granny Smith' and 'Yellow Newtown' apples and only 200 $\mu l \cdot l^{-1}$ was needed to induce the same effect in '20th Century' pears after treatment with low O_2 (0.02% - 0.25% in N_2) atmospheres. In hypoxically treated strawberries, Ke *et al.* (1991b) reported that the maximum concentrations of acetaldehyde, ethanol and ethyl acetate not causing off-flavours were 8.1, 23 and 63 $\mu l \cdot l^{-1}$, respectively. However, Larsen and Watkins (1995b) reported that off-flavour scores were correlated highly with ethyl acetate and slightly with ethanol, but not with acetaldehyde, where 5 - 10 $\mu l \cdot kg^{-1}$ ethyl acetate was acceptable for hypoxically treated strawberry fruit and higher concentration rendered the fruit unacceptable. On the other hand, Richardson and Kosittrakun (1995) found no correlation between these fermentative volatiles and off-flavours in hypoxically treated 'Golden Delicious' or 'Granny Smith' apples, blueberries and plums after

treatment, where concentration of acetaldehyde and ethanol were very high and changes were slight. They suggested that these volatiles could not account for off-flavour development in these fruits.

In spite of their possible connection with off-flavour development in fruit, application of ethanol, acetaldehyde or anaerobic conditions enhanced flavour of some fruit such as apples, pears, blueberries (Paz *et al.*, 1981; Richardson and Kosittrakun, 1995), peaches (Pesis, 1994), feijoa (Pesis *et al.*, 1991), oranges, mandarins (Shaw *et al.*, 1990; 1991; 1992) and bananas (Esguerra *et al.*, 1993). Several ethyl esters were enhanced by hypoxic treatments in the present study. Of these ethyl esters, at least 3 compounds, ethyl butanoate (Cunningham *et al.*, 1986), ethyl 2-methyl butanoate (Flath *et al.*, 1967) and ethyl hexanoate (Kollmannsberger and Berger, 1992) (which have odour threshold values of 0.001, 0.0001 and 0.001 ppm in water, respectively) were believed to be 'aroma-impact' compounds mainly responsible for aroma of apples and apple juice (Dürr and Röthlin, 1981). These compounds are generally found in very low concentrations or are not detectable in freshly harvested apples (Yahia *et al.*, 1990b) but are detectable in fully ripe fruit (Sapers *et al.*, 1977). Having such low odour thresholds and being substantially enhanced in hypoxically treated or acetaldehyde and ethanol treated fruit, these ethyl esters could be responsible both for enhancement of flavour and aroma, and for masking the unpleasant odours of other volatiles (Larsen and Watkins, 1995a).

Respiration rate of 'Braeburn' apples increased 1 day after treatment with 100% CO_2 at 20°C, while in 'Red Delicious' and 'Granny Smith' it was comparable to control. Treatments with combinations of 40% - 80% CO_2 in 20% O_2 also caused respiration rate to increase in 'Red Delicious' apples. Low O_2 treatments reduced respiration rate of 'Red Delicious' apples 1 day after treatment. The increase or decrease in respiration rate could possibly be related to the effects of high CO_2 or low O_2 on stimulation or inhibition, either directly or indirectly, of mitochondrial activity (Knee, 1991a; Kubo *et al.*, 1990; Mathooko *et al.*, 1995a; 1995b; Pesis *et al.*, 1993; 1994; Rahman *et al.*, 1993; 1995). Treatment with 1000 ppm C_2H_4 to 'Granny Smith' apples increased CO_2 production immediately after treatment. The effect of applied ethylene on stimulating respiration rate of fruit is well known (Lieberman, 1979; Pratt and Goeschl, 1969), and

may to be due to switching the flow of electrons in the respiratory electron transfer chain from the conventional cytochrome system to an alternative cyanide-resistant oxidase (Solomos and Laties, 1975; Lieberman, 1979).

Ethylene production of both high CO_2 and low O_2 treated fruit was generally retarded following treatments. Low O_2 is known to suppress ethylene production and action (Burg and Burg, 1967) by inhibiting the activities of ACC synthase and ACC oxidase (Kader, 1995; Mathooko, 1996). The inhibiting effect of high CO_2 may be due to competition with ethylene for the same active site on the ethylene receptor (Burg and Burg, 1967) or through inhibition of ACC synthase activity via suppression of the ACC synthase gene expression at the transcriptional level (Mathooko, 1996). Suppression of ethylene production after anaerobiosis in fruit may also be due in part to ethanol accumulation and/or ADH activity, as both were reported to inhibit ethylene production and action by inhibiting the conversion of ACC into ethylene in fruit tissue (Botondi *et al.*, 1993; Massantini *et al.*, 1995; Saltveit, 1989; Saltveit and Mencarelli, 1988).

Treatments used generally had negligible or no effects on fruit quality attributes, including fruit weight loss, skin colour, flesh firmness, TSS, TA, TSS:TA ratio or pH. There were no injury symptoms on apple fruit tissue observed in any treatment used in the present study, except for 'Braeburn' apples in 1993 experiments; this problem will be discussed in **Chapter Six**.

4.6 CONCLUSION

Application of high CO_2 (100%) and low O_2 (< 2.5%) at 20°C consistently stimulated accumulation of headspace volatiles in juice from 'Granny Smith', 'Braeburn', and 'Red Delicious' apples immediately after treatment. A substantial production of ethanol in response to treatments was followed by an increase in both number and concentration of aroma-impact volatile ethyl esters including ethyl butanoate, ethyl 2-methyl butanoate, and ethyl hexanoate with a concomitant decrease in non-ethyl esters, some alcohols, and/or C_6 - aldehydes. This finding supported the hypothesis of the competitive nature of acyl esterification.

Oxygen concentrations of 2.5% or higher had little effect on enhancing volatile ethyl ester production in treated apples. Treatments with 100% CO_2 and 100% N_2 (0% O_2) generated similar effects on volatile production from treated apples. To obtain optimum enhancement, time of exposure to 100% CO_2 was 18 - 24 hours at 20°C; a shorter duration exerted a lesser effect and a longer period (48 hours) did not further enhance important ethyl esters while causing a severe suppression on some volatile compounds, including *trans*-2-hexenal and hexyl acetate.

Exogenous application of 1000 ppm C_2H_4 for 24 hours at 20°C did not enhance volatile production in juice from the treated 'Granny Smith' apples compared with control.

There was very little effect of treatments on fruit quality attributes, such as weight loss, flesh firmness, skin colour, TSS, TA, TSS:TA ratio or pH after treatment and during subsequent storage at 20°C.

Volatile ethyl esters from 'Red Delicious' apples previously stored at 0°C for 6 months or longer were not enhanced by hypoxic treatments, which may have been due to a gradual loss of precursors and/or activity of the esterifying enzyme system which declined as a consequence of natural senescence.

From a practical point of view, there would be no benefit obtained by applying hypoxic treatments to old fruit (> 6 months cool storage) to enhance some important character-impact volatile compounds in fruit or juice. However, a more extensive study is needed to obtain quantitative and qualitative information on changes in base levels of volatiles present in fruit stored for various times and the magnitude of the response of such fruit following hypoxic treatment. Such information will be essential before this information can be used by the apple processing industry.

Chapter Five

AROMA VOLATILE PRODUCTION OF 'GRANNY SMITH' AND 'FUJI' APPLES MAINTAINED IN AIR AND CONTROLLED ATMOSPHERE PRIOR TO A 24h HYPOXIC TREATMENT WITH CARBON DIOXIDE

5.1 INTRODUCTION

Ripening of apples after harvest is characterised by simultaneous changes in both physiological and metabolic processes (Brady, 1987; Paillard, 1990). Refrigerated-air (RA) and controlled-atmosphere (CA) storage are widely adopted to slow down ripening and aging, and to extend shelf-life of apples (Meheriuk, 1993; Smock, 1979). CA storage, in which concentrations of O_2 and/or CO_2 in the atmosphere are closely regulated, has been used extensively to prolong storage life of apples (Smock, 1979, Yahia *et al.*, 1990a). The physiological and biochemical basis for the beneficial affect of CA on apples is still not fully understood (Kader, 1980; 1986; Yahia, 1994), but CA is known to slow the ripening process, including reduction of ethylene biosynthesis and action, respiration rate, flesh softening, colour change and loss of acids, as well as minimising or delaying onset of storage disorders (Knee, 1991a; Smock, 1979). Therefore, CA and RA storage can prolong the normal ripening pattern of apples.

Specific concentrations of O_2 and CO_2 mixtures and temperature of CA conditions, as well as time of harvest, have been recommended for many apple cultivars in order to maintain fruit quality, prolong storage life and minimise wastage (Drake and Kupferman, 1993; Lau, 1990; Meheriuk, 1993). Until recently, the effect of storage conditions on subsequent production of aroma volatile compounds has largely been neglected. However over the last few years, there have been an increasing number of reports on the effects of harvest maturity and storage conditions in relation to the nature, occurrence and concentration of volatile compounds responsible for perception of aroma

and flavour in apples (Brackmann *et al.*, 1993; Girard and Lau, 1995; Hansen *et al.*, 1992a; Song and Bangerth, 1996; Streif and Bangerth, 1988; Yahia *et al.*, 1990a).

CA conditions were reported to depress aroma volatile production in apples during subsequent ripening (Patterson *et al.*, 1974; Willaert *et al.*, 1983; Williams and Knee, 1977; Yahia, 1989). The ability of 'Red Delicious' apple peel to produce esters was arrested on fruit removed from commercial CA storage (Guadagni *et al.*, 1971). Patterson *et al.* (1974) reported a 75% reduction in production of some volatile esters in 'Cox's Orange Pippin' apples after 5 months storage in 2% O_2 with or without 5% CO_2. The higher the CO_2 and the lower the O_2 levels and the longer the apples were stored in CA, the greater the suppression of aroma volatile production (Hansen *et al.*, 1992b; Lidster *et al.*, 1983; Streif and Bangerth, 1988; Yahia *et al.*, 1990a).

Low O_2 concentrations have been found to exert variable effects on post storage production of different esters in CA stored apples. Hansen *et al.* (1990) reported that storage atmospheres containing 1% or 2% O_2 (in N_2) significantly depressed post storage production of propyl acetate, butyl acetate and 2-methyl-2-butenyl acetate in 'Jonagold' apples after 3 months storage, while 4% O_2 had little effect, compared with apples stored in 21% O_2. Oxygen concentrations of 3 - 5% (in N_2) were found to enhance production of ethyl esters containing a methyl-branched-alcohol moiety such as 2/3-methyl butyl acetate and 2-methyl but-2-enyl acetate after 6 month CA storage of 'Jonagold' apples (Hansen *et al.*, 1992b). Streif and Bangerth (1988) investigated the effects of specific mixtures of CO_2 (0.8, 3, 6 and 9%) and O_2 (1, 3, 12 and 21%) concentrations in storage atmospheres on post storage volatile production of 'Golden Delicious' apples after 3, 5, 7 and 9 months in CA. They found that increasing CO_2 and decreasing O_2 concentrations depressed volatile production; decreasing O_2 to 3% had little effect but a further decrease to 1% significantly reduced volatile production. Furthermore, above 3% O_2, higher CO_2 concentrations (> 3%) became decisive in reducing volatile production. Harb *et al.* (1994) also found that both 3% CO_2 + 3% O_2 and 3% CO_2 + 1% O_2 significantly decreased volatile production of 'Golden Delicious' apples after 8 month storage (at 1°C), while 1% CO_2 + 3% O_2 had little effect. Thus it seems that the reduced production of volatiles in a long-term CA stored apples (> 6

months) is influenced by concentration of gas composition with the threshold for O_2 being less than 3% and for CO_2 being more than 3% in the storage atmosphere.

Production of volatiles during ripening after storage was quantitatively lower in early picked apples than in fruit harvested at intermediate or optimum maturity (Dirinck *et al.*, 1989; Fellman *et al.*, 1993b; Girard and Lau, 1995; Mattheis *et al.*, 1995; Song and Bangerth, 1996). It may be important to identify pre-climacteric windows that includes information concerning the aroma volatile compounds to ensure optimum quality of apples stored for long period of time (Fellman *et al.*, 1993b).

Production of alcohols, aldehydes, and esters during subsequent ripening of 6-month CA stored 'Delicious' apples was less than in RA stored fruit (Mattheis *et al.*, 1995). Volatile compounds such as ethyl butanoate, propyl butanoate, butyl acetate, propyl acetate, propan-1-ol, butan-1-ol, and hexan-1-ol were produced in smaller amounts from CA fruit than from RA fruit after 6 months storage (Brackmann *et al.*, 1993; Girard and Lau, 1995; Fellman *et al.*, 1993a; Mattheis *et al.*, 1995; Yahia *et al.*, 1990b). However, not all volatile components were suppressed by CA storage; 2-methyl-2-butenyl acetate (Hansen *et al.*, 1990; 1992b), methyl 2-methyl butanoate, ethyl 2-methyl butanoate, butyl pentanoate (Yahia *et al.*, 1990a), 2-methyl-1-butyl acetate, butyl-2-methyl butanoate and hexanal (Fellman *et al.*, 1993a) were produced in larger amounts during ripening of apples from CA than from RA storage. Some compounds, such as *trans*-2-hexenal and methyl butanoate were not affected by storage regime (Girard and Lau, 1995; Yahia, 1989).

Several studies have been conducted in an attempt to improve aroma development of CA stored apples with only a partial improvement achieved. Raising O_2 concentrations from 1% to 3% or above immediately prior to marketing was suggested (Smith, 1984) but this technique did not increase volatile compounds in 6-month CA stored 'Delicious' apples (Mattheis *et al.*, 1995). A partial recovery of volatile production of 8-month CA stored 'Golden Delicious' apples was obtained by exposing the fruit to air at 1°C for 14 days following CA storage. However exposing 9-month CA stored 'McIntosh' apples to air at 3.3°C or 100% O_2 did not effectively increase volatile production of the fruit, while storage of fruit at 20°C (after 9-month CA storage) increased some volatile compounds (Yahia, 1991). Illumination of apples during storage

(2 - 2.5% O_2) increased butyl and hexyl acetate in 'Cox's Orange Pippin' apples (Knee *et al.*, 1979), but was not effective in restoring aroma volatile production of the 9-month CA stored 'McIntosh' apples (Yahia, 1991). Precursor atmosphere (PA) storage, in which intact fruit are exposed to atmospheres containing specific aroma volatile precursors for 24 - 48 hours at 20°C, substantially enhanced a number of esters in several fruits, including apples and banana (Berger *et al.*, 1992). It has been suggested that this technique could be used to overcome the lack of aroma in CA stored and of thermally processed products by treating the fruit before processing (Berger, 1990; 1995; Berger *et al.*, 1992). Although such a technique did improve volatile production, its effect is only transient lasting a relatively short time and it probably has a limited practical application. Increasing consumer awareness and preference for high quality, 'natural' products, as well as the implementation of legislation for food safety standards (Berger, 1995; Fronza *et al.*, 1996; Knights, 1986; Lea, 1995) may make it difficult to commercially add 'artificial' chemicals to enhance aroma in apples and its products.

Enhancement of flavour and aroma of fruit and their products, as well as some important aroma compounds, induced following a short-term hypoxia of nitrogen or CO_2 atmospheres was previously reported for strawberries (Larsen and Watkins, 1995a; Berger, 1995), feijoa, peaches (Pesis, 1994; Pesis *et al.*, 1991), citrus (Shaw *et al.*, 1990; 1991; 1992) and other berries (Richardson and Kosittrakun, 1995). Moreover, it has been demonstrated in this study (**Chapter Four**) that application of short-term 100% CO_2 (24 hours at 20°C) treatment to apples dramatically increased production of several volatile components in juice of several cultivars. Some important character impact compounds, such as ethyl butanoate, and ethyl 2-methyl butanoate, which have odour thresholds in water of 0.001 and 0.0001 ppm, respectively (Teranishi *et al.*, 1987), were substantially increased in juice of the treated apples. Therefore, it was of interest to test whether or not an hypoxic technique applied to CA and/or RA stored apples could enhance aroma volatile compounds in juice of fruit that were known to have such volatiles depressed by storage conditions.

5.2 OBJECTIVES

The objective of this study were :

(1) to study changes in some important volatile compounds in juice of RA and CA stored 'Granny Smith' and 'Fuji' apples during and after storage for up to 27 weeks after harvest.

(2) to study the effect of 100% CO_2 (24 hours at 20°C) treatment on production of some aroma volatile compounds of apple juice in relation to storage time and conditions.

5.3 MATERIALS AND METHODS

5.3.1 Materials and Treatments

Refrigerated-air (RA) and controlled-atmosphere (CA) stored apples of a Fancy Grade Class 1 'Granny Smith' and 'Fuji' cultivars were obtained from the New Zealand Apple and Pear Marketing Board (NZAPMB, ENZA®), Hastings. Apples were harvested during the 2nd ('Fuji') and 3rd ('Granny Smith') week of April 1994, respectively. Apples were stored in a commercial RA at 0.5° ± 0.5°C and 92 - 95% RH, or CA storage of 2% O_2 + 2% CO_2 at 0.5°C and 95% RH at Hastings (L. Frampton, personal communication). Fruit was removed from store at intervals and transported overnight to the Department of Plant Science, Massey University, Palmerston North by a courier land transport and post storage treatments were generally conducted the following day (**Table 5.1**). The initial sampling was made after apples had been in storage for 6 and 7 weeks and only RA stored fruit were used for treatment and analysis at this sampling time. The average fruit size of RA stored 'Granny Smith' was 185 g (100 count) and that of CA stored was 163 g (113 count) and that of both RA and CA stored 'Fuji' apples was 148 g (125 count).

Table 5.1 Time after harvest and time during post storage data collection at 20°C (70% RH) of 'Granny Smith' and 'Fuji' apples.

cv. 'Granny Smith'		cv. 'Fuji'	
Time after Harvest (week)	Post Storage Date	Time after Harvest (week)	Post Storage Date
6*	9/6 - 19/6/1994	7*	9/6 - 19/6/1994
10	1/7 - 11/7/1994	10	23/6 - 3/7/1994
14*	30/7 - 9/8/1994	14*	22/7 - 1/8/1994
18	26/8 - 5/9/1994	19	21/8 - 31/8/1994
23*	30/9 - 10/10/1994	23*	23/9 - 3/10/1994
27*	28/10 - 7/11/1994	27*	21/10 - 31/10/1994

* analysed for volatile compounds

On arrival at Palmerston North, RA and CA stored apples were removed from cartons and separately placed on laboratory tables for initial assessment. Damaged, misshaped, and non-uniform fruit were eliminated and good fruit with similar size and colour were selected. Treatments and replicates were then randomly allocated and fruit was individually numbered and labelled. After that, the apples were transferred into a 20°C room for temperature equilibration, generally overnight, and treatments were applied the following day.

Cultivars were assigned as separate experiments. For each cultivar at each time after harvest, the experiment comprised 2 storage conditions, namely RA and CA storage and each storage condition had 2 treatments, ie. 100% CO_2 gas for 24 hours at 20°C and control. Each treatment consisting of 7 replicates and in each replicate, a single fruit was used for analysis at each post storage sampling time (6 sampling times). Seven fruit from each storage condition were analysed just before the application of treatment (day -1), giving 182 fruit in total.

Detailed methods for 100% CO_2 gas application and for monitoring concentrations of CO_2 and O_2 in treatment atmosphere have been previously described in **Chapter Two**. Control fruit were left untreated in chambers at 20°C and 70% RH. After treatment, apples were removed from treatment chambers and stored in cardboard cartons for 9 days at 20°C and 70% RH. Fruit samples were taken for analysis at -1, 0,

1, 3, 5, 7, and 9 days after treatment, where day -1 was just before treatment, day 0 was immediately after treatment, day 1 was 24 hours after treatment, and so on.

5.3.2 Fruit Analysis

Respiration rate, ethylene production, and skin colour was measured on individual fruit, after which flesh firmness was determined. Then fruit were diced and all parts, including skin, cortex, core and seeds were used immediately for juice preparation. Apple juice obtained was used directly for analysis of headspace volatile compounds, aroma volatile compounds, and total soluble solids (TSS). A portion of juice was kept frozen at -20°C for further determination of titratable acids (TA) and juice pH (methods were described in **Chapter Two**).

The direct solvent extraction technique was used to determine aroma volatile compounds at 6, 14, 23, and 27 weeks after harvest and for each time after harvest at day -1, day 1, day 3 and day 7 following the CO_2 treatment. Details of sample preparation, analysis, identification and quantification of the aroma volatile compounds were described in **Chapter Two**. All measurements of TSS, TA, pH, headspace and aroma volatiles were made from juice.

5.3.3 Data Analysis

The experiment was designed as a factorial (time after harvest x storage condition x treatment) with replication nested in treatment. Each cultivar was designated as a separate experiment.

Means and standard errors (S.E.) of each attribute for each post storage period of 7 - 9 days at 20°C and 70% RH were calculated. Data of volatile compounds, respiration rate, ethylene production for each replicate at each post storage period were calculated for total production, in terms of area under curve, by integration using an Origin® Graphics package (Microcal ™ Software Inc., Northampton, USA). Means and standard errors of the area under curve data at each post storage period were calculated

and graphed or tabulated. Fruit weight loss rate data for each replicate at each post storage period were calculated by means of linear regression analysis. Means and standard errors of fruit weight loss rate were calculated and tabulated. Means of flesh firmness, TSS, TA, TSS:TA ratio, juice pH, skin lightness, and skin hue angle of each replicate at each post storage period were calculated and tabulated.

Data on fruit quality attributes, as means, rates, or area under curve were subjected to analysis of variance using a SAS package (SAS Institute Inc., Cary, USA). Means and pooled standard errors of each attribute were calculated. Significant main effect were separated by Duncan's multiple range test at the 5% level of significance. In the present study, data on fruit weight loss rate, skin lightness, skin hue angle, TSS, TSS:TA ratio, juice pH, respiration rate, and ethylene production have not been presented (see **Appendix 4**).

5.4 RESULTS

Controlled-atmosphere (CA) storage clearly exerted a residual effect on retention of flesh firmness and juice titratable acids during post storage for 9 days at 20°C and 70% RH in both 'Granny Smith' and 'Fuji' apples, as compared with RA storage (**Table 5.2**). In general, flesh firmness and TA decreased with time after storage, but reduction of both attributes was much slower in CA than in RA stored apples. While both RA and CA stored apples treated with 100% CO_2 for 24 hours at 20°C on removal from storage were slightly firmer than control, particularly 6 - 14 weeks after harvest, TA was similar for both treatments. 'Fuji' were slightly firmer and contained much less TA than 'Granny Smith' apples (**Table 5.2**).

5.4.1 Headspace Volatile Compounds

During 9 days post storage at 20°C, headspace volatile compounds, comprising acetaldehyde, ethanol and ethyl acetate, from CA stored 'Granny Smith' apples were either not detected or detected in a negligible concentrations; those from RA stored fruit

were found in low concentrations at 23 or 27 weeks after harvest (**Fig. 5.1**, **Fig. 5.3** and **Fig. 5.5**). Headspace volatiles in both RA and CA stored 'Fuji' apples gradually increased during the post storage period, with concentrations slightly higher in RA than in CA stored fruit (**Fig. 5.2**, **Fig. 5.4** and **Fig. 5.6**).

Table 5.2 Flesh firmness and titratable acidity after 9 days at 20°C and 70% RH of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

	cv. 'Granny Smith'							
Week after Harvest	Firmness (Newton)				TA (%)			
	RA		CA		RA		CA	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
6	60.0	63.8	-	-	0.59	0.57	-	-
10	56.9	58.3	67.4	68.6	0.60	0.62	0.62	0.61
14	57.3	58.3	68.3	69.3	0.56	0.56	0.57	0.57
18	53.2	54.1	65.3	65.6	0.55	0.55	0.61	0.60
23	51.3	50.9	64.8	65.3	0.52	0.53	0.54	0.53
27	45.9	45.0	59.4	60.5	0.45	0.45	0.53	0.50
c.v.	2.23 %				5.33 %			
Time	***				***			
Cond.	***				***			
Treat.	**				ns			
	cv. 'Fuji'							
	Firmness (Newton)				TA (%)			
7	60.9	65.4	-	-	0.24	0.24	-	-
10	59.9	60.9	66.9	70.1	0.22	0.21	0.24	0.23
14	56.5	59.9	66.6	67.3	0.20	0.19	0.24	0.23
19	52.6	53.0	65.6	65.7	0.16	0.16	0.22	0.21
23	49.2	49.2	64.0	63.9	0.13	0.13	0.20	0.21
27	47.6	48.8	63.2	63.9	0.11	0.11	0.19	0.19
c.v.	3.56 %				7.25 %			
Time	***				***			
Cond.	***				***			
Treat.	***				*			

Abbreviations : Ctrl = control, CO_2 = 100% CO_2 for 24h at 20°C, c.v. = coefficient of variation, Time = time after harvest, Cond. = storage conditions, RA = refrigerated air storage, CA = controlled atmosphere storage, Treat. = treatments
Levels of significance at $P = 0.05$ (*), 0.01 (**), or 0.001 (***), or non significant (ns).

Treatment with 100% CO_2 for 24 hours at 20°C stimulated a marked increase in headspace volatiles of both cultivars with response being greater in 'Granny Smith' than in 'Fuji'; these peaked immediately or 1 day after treatment and declined thereafter in both RA and CA stored apples (**Fig. 5.1** to **Fig. 5.6**). Generally headspace acetaldehyde of the CO_2 treated fruit from RA 'Granny Smith' apples (**Fig. 5.1**) decreased steadily from the peak at 0 or 1 day after hypoxic treatment; production was less from CA than RA fruit especially after 23 and 27 weeks storage. Peak acetaldehyde production in the CO_2 treated RA stored 'Fuji' apples was transient and occurred on removal from treatment, subsequent reduction tended to be faster as time after harvest increased. Interestingly, peak acetaldehyde production in RA control fruit increased with length of storage (**Fig. 5.2**).

Headspace ethanol of CO_2 treated RA stored fruit of both cultivars reached their peaks at 0 or 1 day after removal from treatments and decreased slowly as time after harvest progressed (**Fig. 5.3** and **Fig. 5.4**). The magnitude of response between cultivars was the same initially, but it was greater in 'Granny Smith' than in 'Fuji' as time after harvest increased.

Generally, headspace ethyl acetate of the CO_2 treated fruit from both RA and CA stored 'Granny Smith' and 'Fuji' apples peaked 1 day after treatments, then decreased to levels similar to controls after 7 or 9 days at 20°C; production was less from CA than from RA fruit throughout 27 weeks storage (**Fig. 5.5** and **Fig. 5.6**). The magnitude of response was greater in 'Fuji' than in 'Granny Smith' apples through 27 weeks after harvest.

To examine the volatile production capacity and overall concentration produced during post storage of apples removed from RA or CA storage at different times after harvest and then treated with air or 100% CO_2, concentrations of volatiles monitored (for each replicate and treatment) were plotted against time (days) after treatment. The plotted curve of a volatile (for each replicate) was integrated (as area under curve) to obtain a total concentration of volatile produced during post storage (days) and it was expressed as concentration x day ($\mu l \cdot l^{-1} \cdot$day or $\mu mol \cdot l^{-1} \cdot$day). Mean and S.E. of the total concentration of each volatile in each treatment was calculated and plotted against time (weeks) after harvest. This approach provided an overall picture for a volatile compound

produced by apples at different times after harvest, from different storage conditions and in response to hypoxic treatment imposed.

Total concentration of headspace volatiles during post storage for 9 days (as area under curve) was undetectable or negligible in control CA stored 'Granny Smith' apples throughout 27 weeks after harvest, and remained constant at a low level for 'Fuji' (**Fig. 5.7** to **Fig. 5.9**). The total concentration in control RA stored apples of both cultivars was low but they tended to increase after 23 to 27 weeks in cool store.

Treatment with 100% CO_2 markedly enhanced total concentration of headspace volatiles over control in both RA and CA stored fruit of both cultivars. Headspace acetaldehyde of the CO_2 treated RA and CA stored fruit of both cultivars was generally increased as time after harvest increased, while headspace ethanol was more or less constant, except in CO_2 treated RA stored 'Granny Smith' apples which increased continuously with time after harvest (**Fig. 5.7** and **Fig. 5.8**). Total concentrations of headspace ethyl acetate during 9 days post storage from CO_2 treated RA and CA stored 'Granny Smith' apples generally decreased with time after harvest. On the other hand, ethyl acetate production in CO_2 treated RA stored 'Fuji' apples was higher than for 'Granny Smith', increasing with time of storage until 23 weeks and decreasing thereafter. Total concentration of headspace ethyl acetate in CO_2 treated CA stored 'Fuji' apples fluctuated during 14 weeks storage (**Fig. 5.9**).

Total concentration of headspace volatiles in CO_2 treated fruit of RA stored apples of both cultivars was generally greater than in CA stored fruit and the difference between the two storage regimes was obvious within 10 weeks of cool storage. Headspace acetaldehyde and ethanol from CO_2 treated 'Granny Smith' apples were higher than from 'Fuji', however, ethyl acetate production was lower in the former than the latter. Appreciable concentration of headspace volatiles were found in control fruit of both RA and CA stored 'Fuji' apples and they were always higher in RA stored fruit, while there were present in negligible amounts in 'Granny Smith' apples throughout 27 weeks in store (**Fig. 5.7** to **Fig. 5.9**).

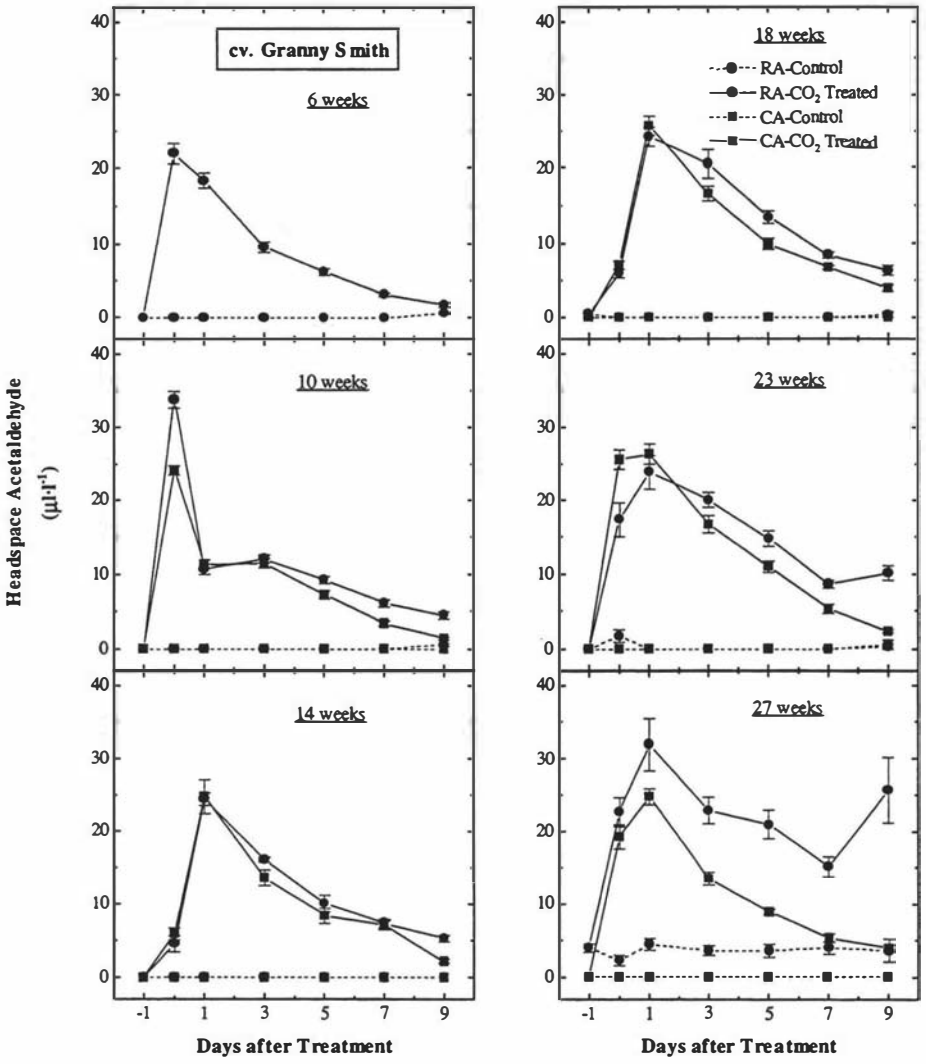


Fig. 5.1 Headspace acetaldehyde concentrations from juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

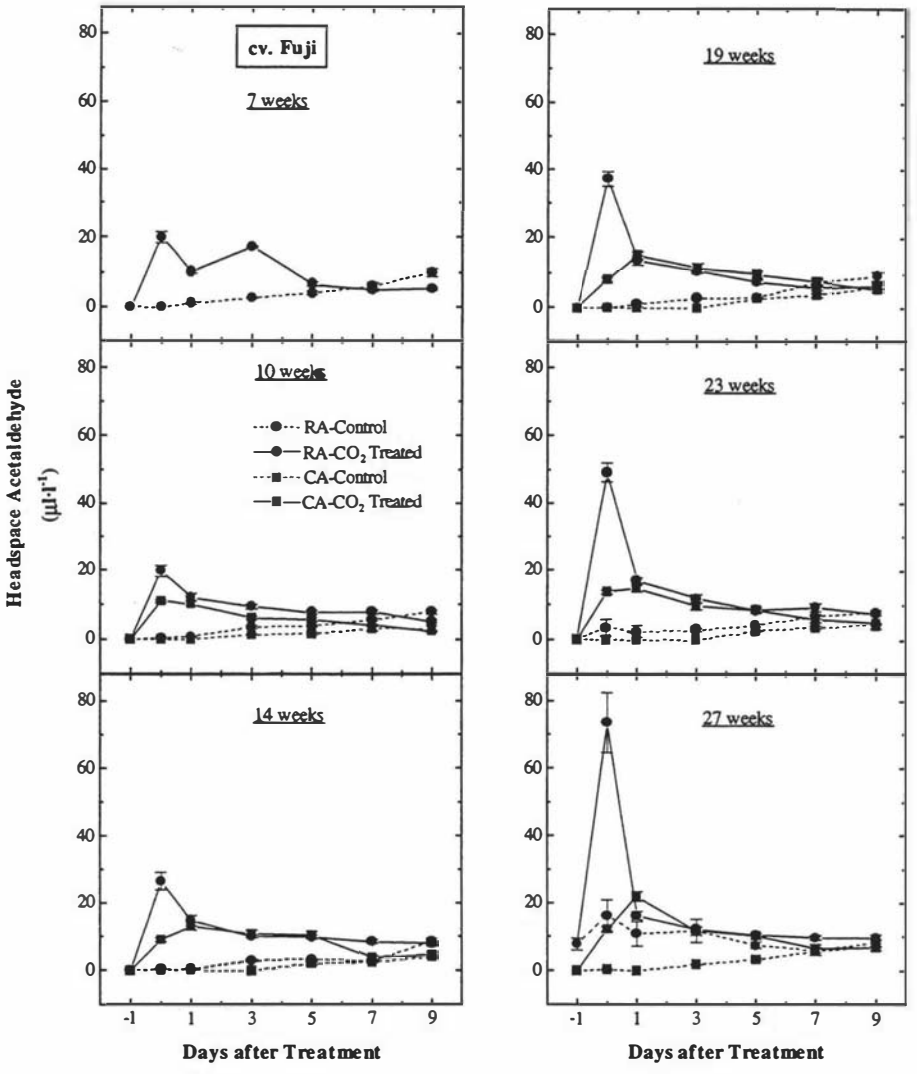


Fig. 5.2 Headspace acetaldehyde concentrations from juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

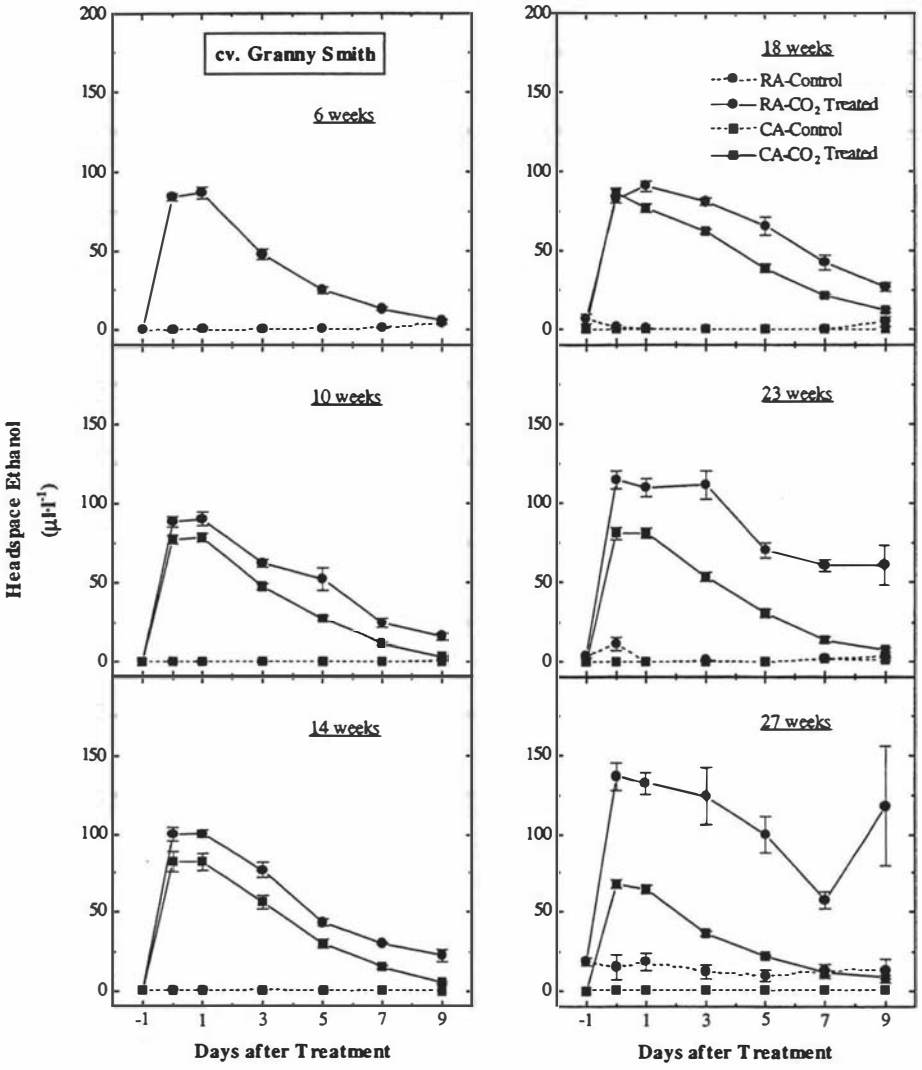


Fig. 5.3 Headspace ethanol concentrations from juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

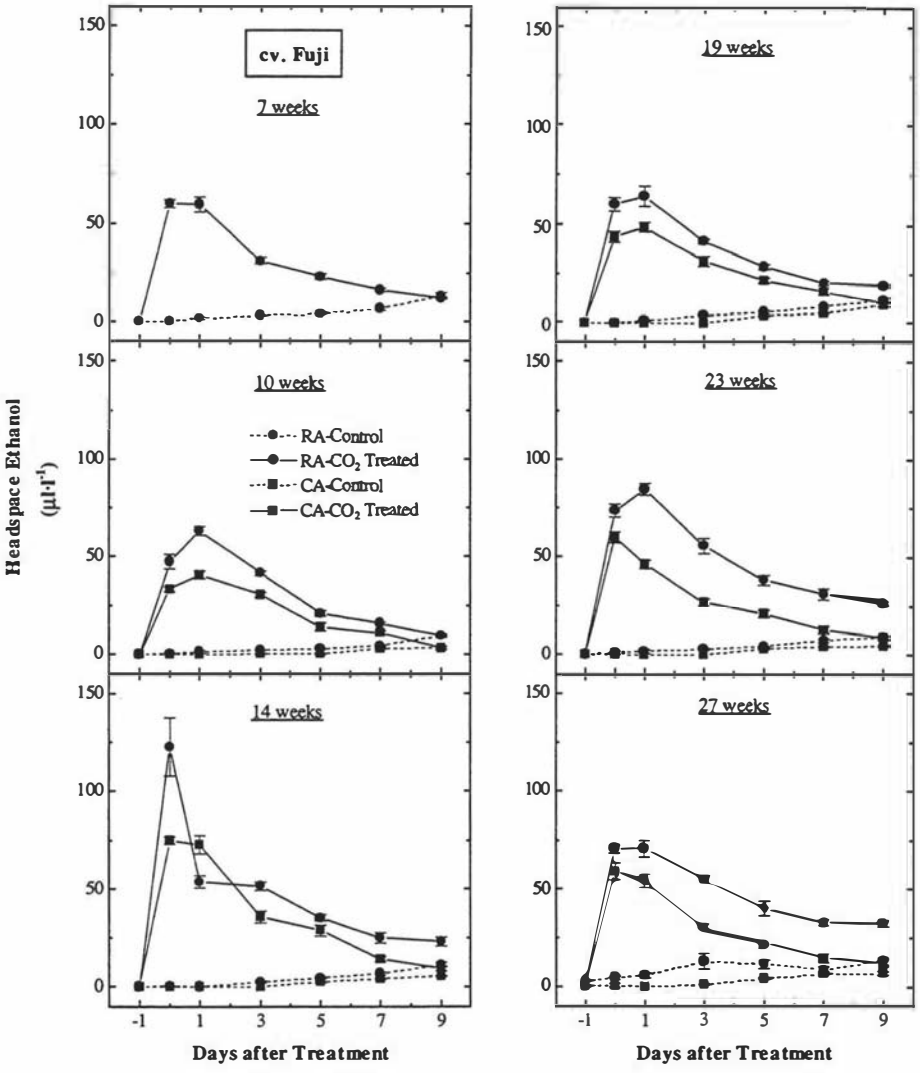


Fig. 5.4 Headspace ethanol concentrations from juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

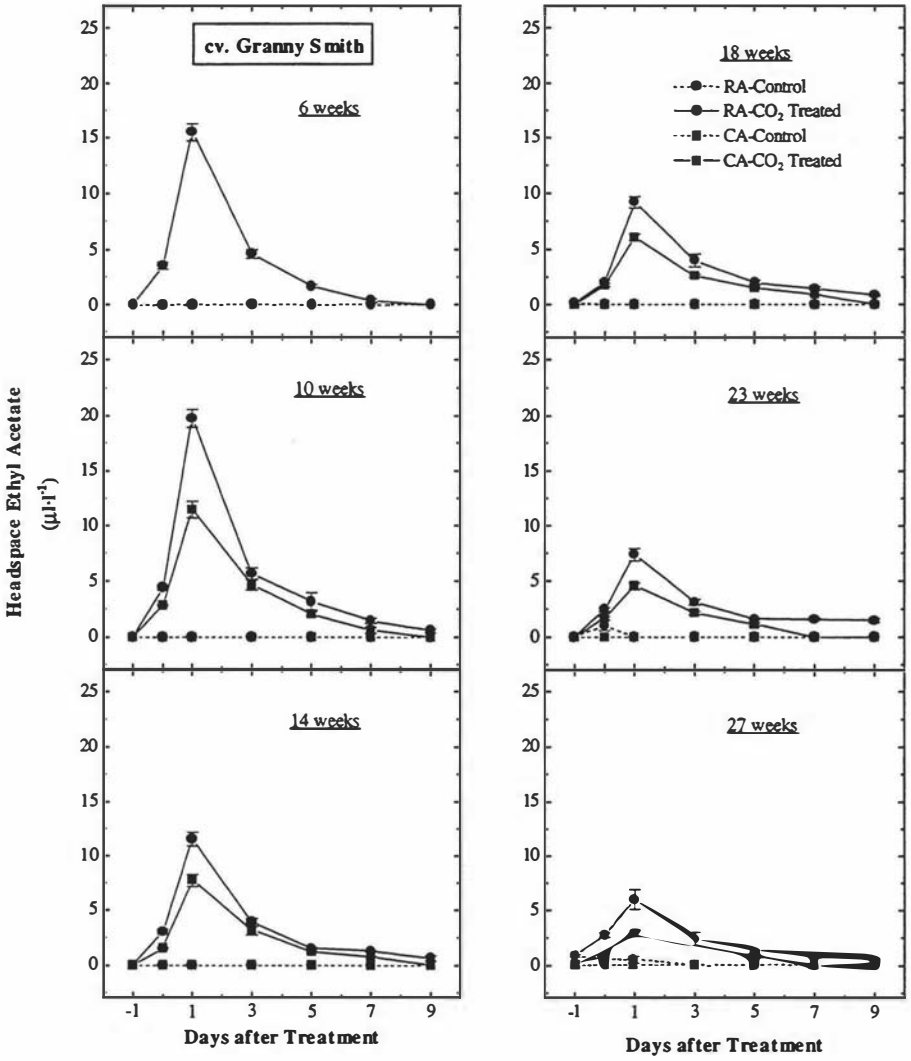


Fig. 5.5 Headspace ethyl acetate concentrations from juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

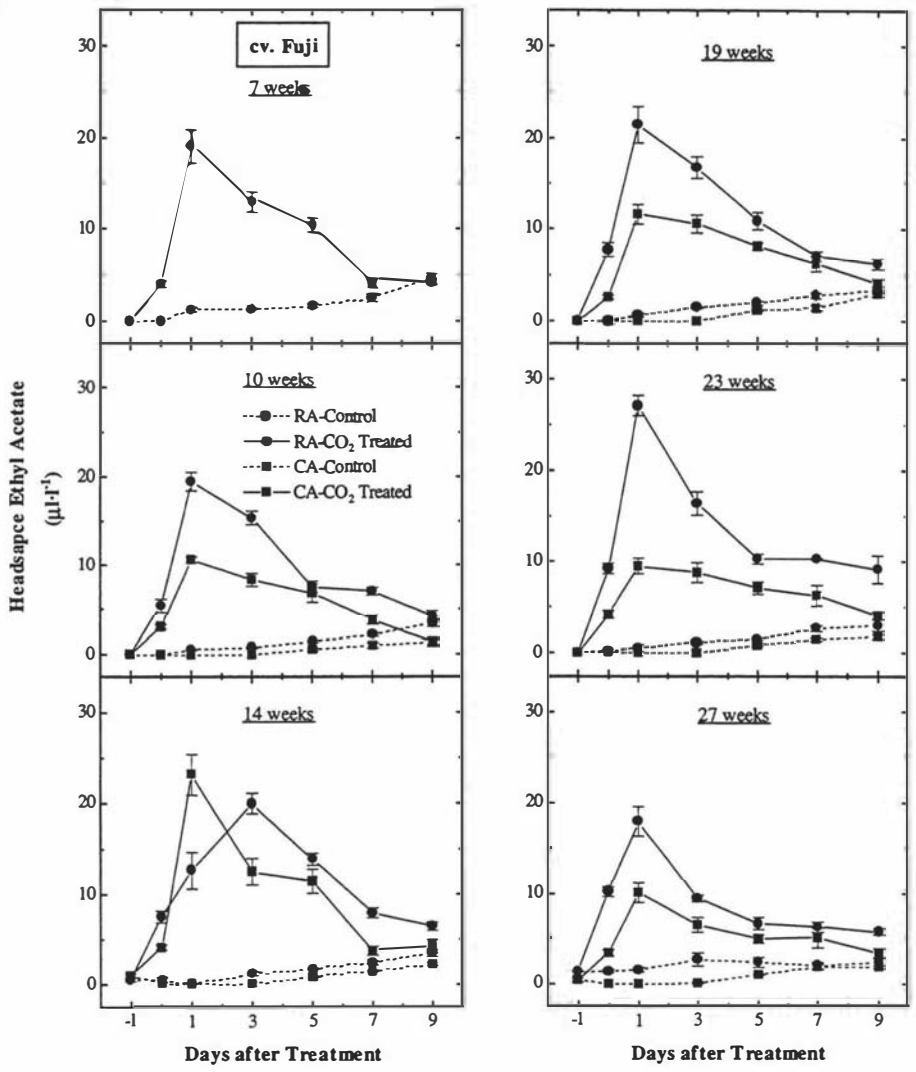


Fig. 5.6 Headspace ethyl acetate concentrations from juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

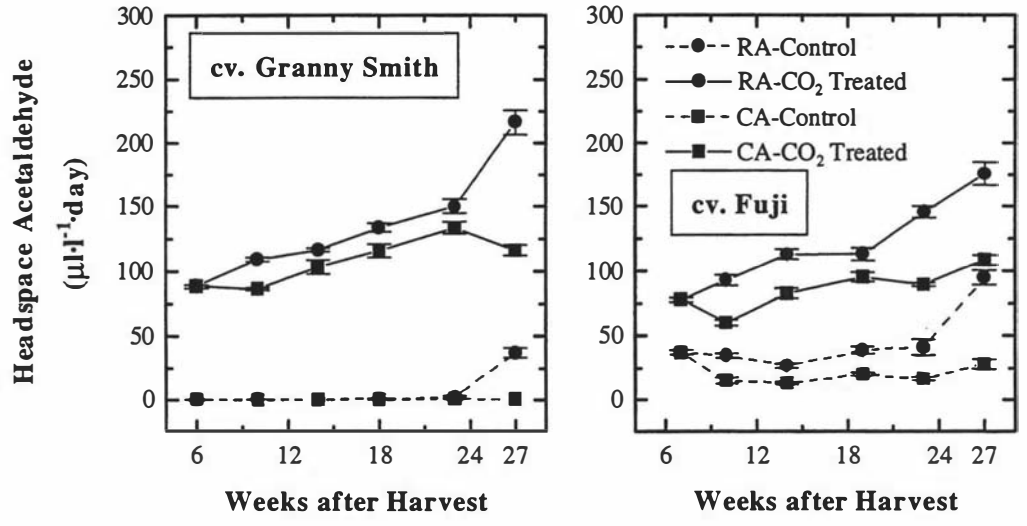


Fig. 5.7 Total headspace acetaldehyde concentration during post storage for 9 days at 20°C (as of area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

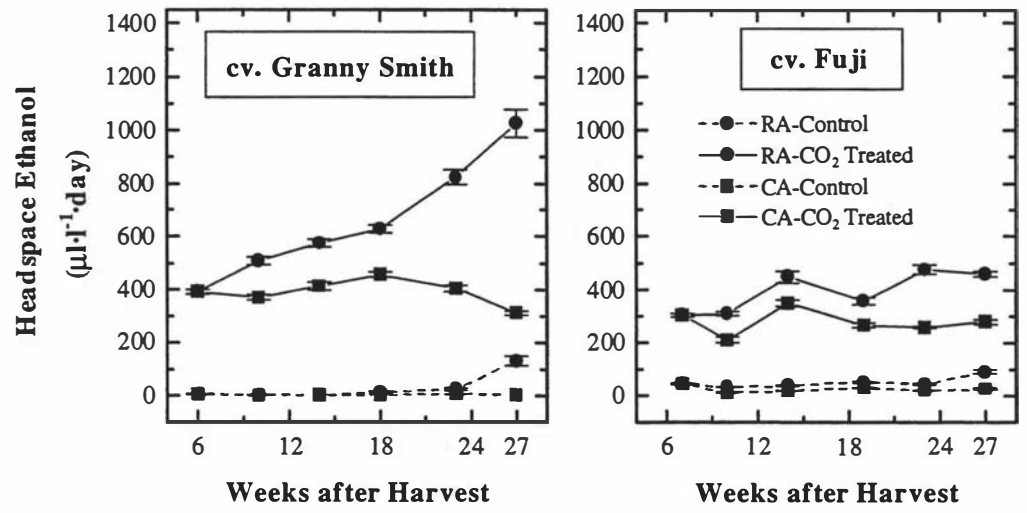


Fig. 5.8 Total headspace ethanol concentration during post storage for 9 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

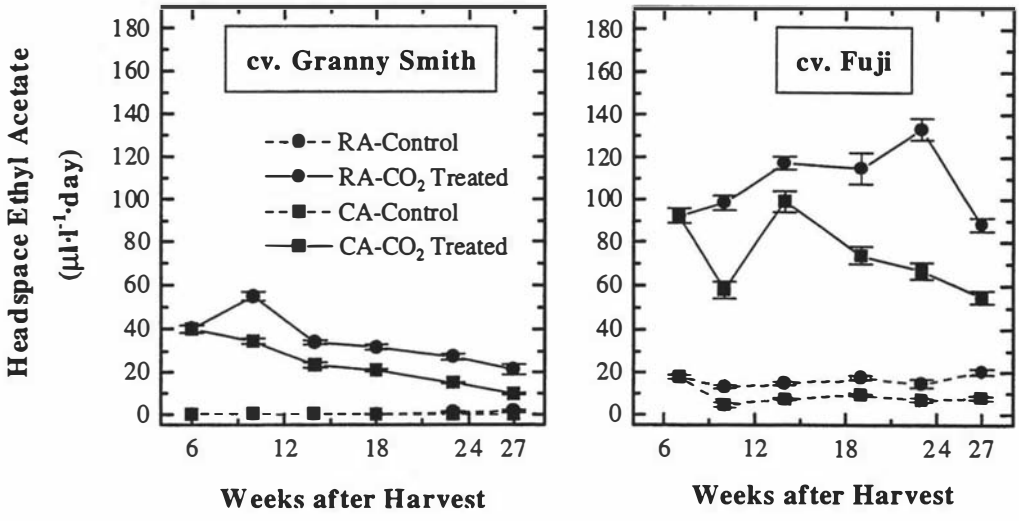


Fig. 5.9 Total headspace ethyl acetate concentration during post storage for 9 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

5.4.2 Volatile Esters

Production of ethyl esters by RA and CA stored fruit of both apple cultivars either control or hypoxically treated, varied in terms of pattern of production and in the magnitude of production over time after harvest. However, treatment with 100% CO_2 markedly and consistently enhanced production of ethyl esters over control in both RA and CA stored fruit of both cultivars (**Fig. 5.10** to **Fig. 5.17**).

Ethyl acetate of control RA stored 'Granny Smith' and 'Fuji' at 6 and 7 weeks after harvest, respectively (initial), increased gradually during post storage for 7 days at 20°C (**Fig. 5.10** and **Fig. 5.11**). Negligible concentrations of ethyl acetate from control fruit of both RA and CA stored 'Granny Smith' apples occurred post storage subsequent to removal from cool store, while in control fruit of 'Fuji' apples there was a slight increase. Ethyl acetate from CO_2 treated fruit increased immediately after treatment in both RA and CA stored apples of both cultivars, where it generally peaked at day 1 after treatment and decreased thereafter. Treated CA stored fruit always produced lower

concentrations of ethyl acetate than treated RA stored apples in both cultivars (**Fig. 5.10** and **Fig. 5.11**).

Ethyl butanoate concentration of control RA stored 'Granny Smith' and 'Fuji' apples at 6 and 7 weeks after harvest (initial), respectively, was initially high and decreased during 7 days post storage at 20°C (**Fig. 5.12** and **Fig. 5.13**). The concentration tended to increase again at 23 and 27 weeks after harvest in control RA stored apples of both cultivars, while in CA stored apples ethyl butanoate concentration remained low or was not detected. Treatment with 100% CO_2 enhanced peak production of ethyl butanoate to between 300 and 400 $\mu mol \cdot l^{-1}$ in both RA and CA stored apples. Ethyl butanoate concentration of CO_2 treated RA and CA stored 'Granny Smith' apples generally increased to a peak 3 days after treatment remaining more or less constant thereafter, while in 'Fuji' apples concentration reached a peak at 1 or 3 days after treatment and decreased thereafter (**Fig. 5.12** and **Fig. 5.13**). Concentrations of ethyl butanoate in CO_2 treated RA and CA stored 'Granny Smith' were similar , while they were always higher in treated RA than CA stored 'Fuji' fruit throughout the 27 weeks of storage.

Ethyl 2-methyl butanoate was not detected during post storage at 20°C in control CA stored 'Granny Smith' apples throughout storage, but it did occur in RA stored fruit 27 weeks after harvest (**Fig. 5.14**). In control fruit of 'Fuji' apples, ethyl 2-methyl butanoate concentration increased after 3 days of post storage at 20°C in both RA and CA stored fruit with the highest concentration being in juice of RA fruit (**Fig. 5.15**). Treatment with 100% CO_2 markedly increased ethyl 2-methyl butanoate concentration over control both in RA and CA stored apples of both cultivars. The concentration increased immediately after treatment and stayed approximately constant at a high level or decreased after reaching its peak. It was interesting to note that ethyl 2-methyl butanoate in CO_2 treated CA stored 'Granny Smith' apples became higher than that in RA stored fruit at 23 and 27 weeks after harvest (**Fig. 5.14**). In contrast the concentration in CO_2 treated CA stored 'Fuji' apples was always lower than in RA stored fruit (**Fig. 5.15**).

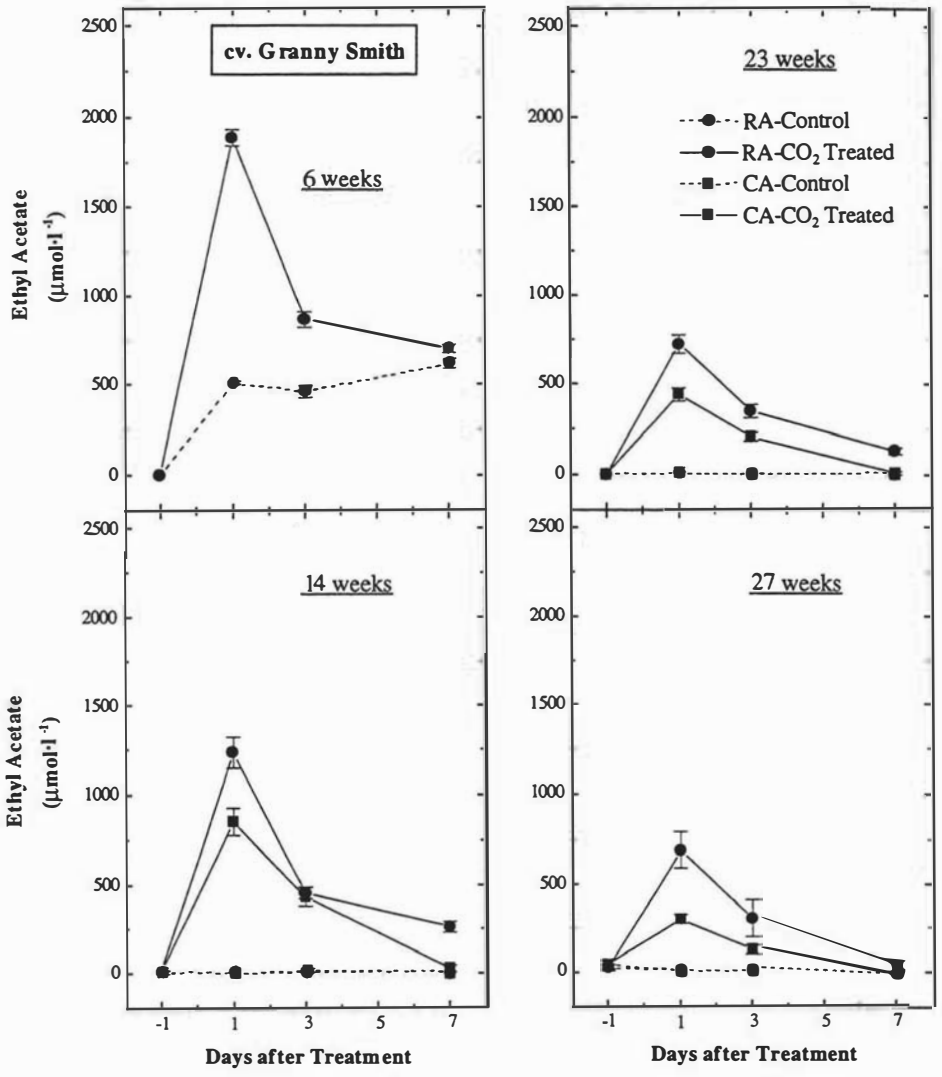


Fig. 5.10 Ethyl acetate concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

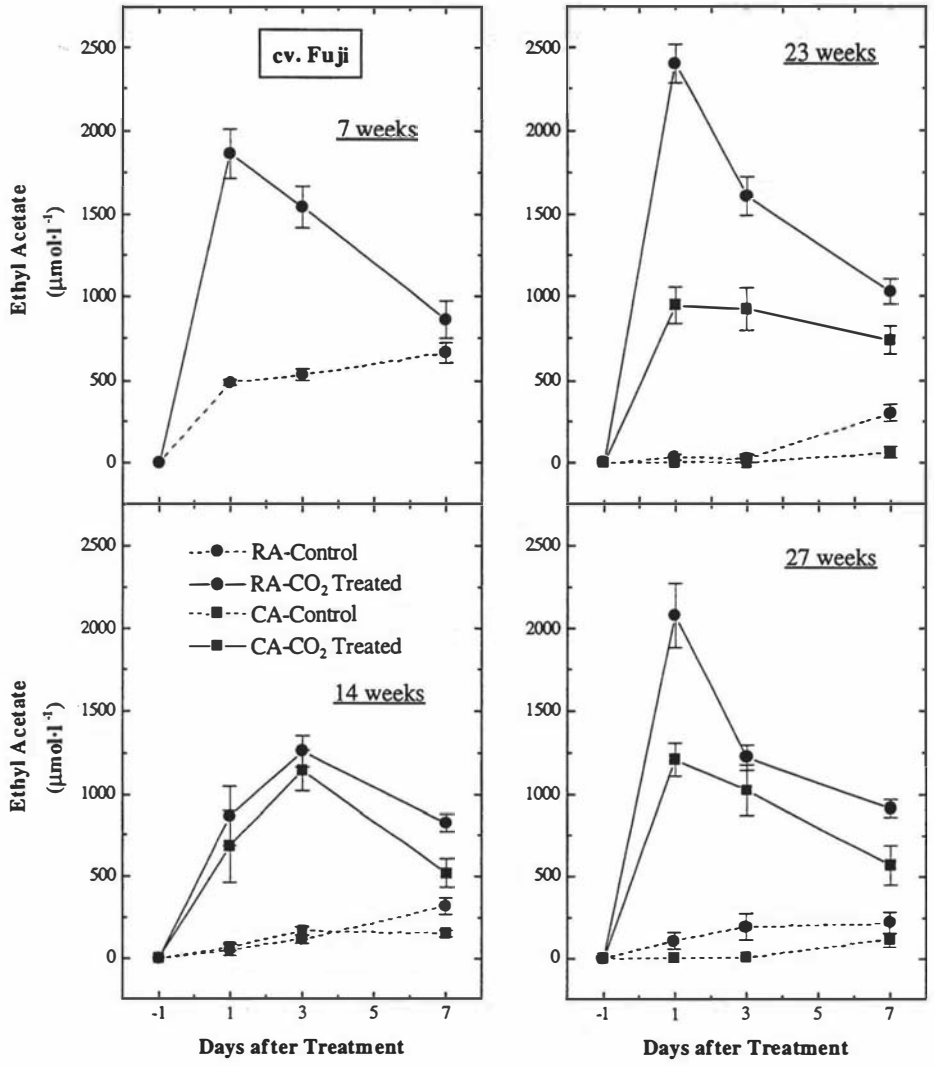


Fig. 5.11 Ethyl acetate concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

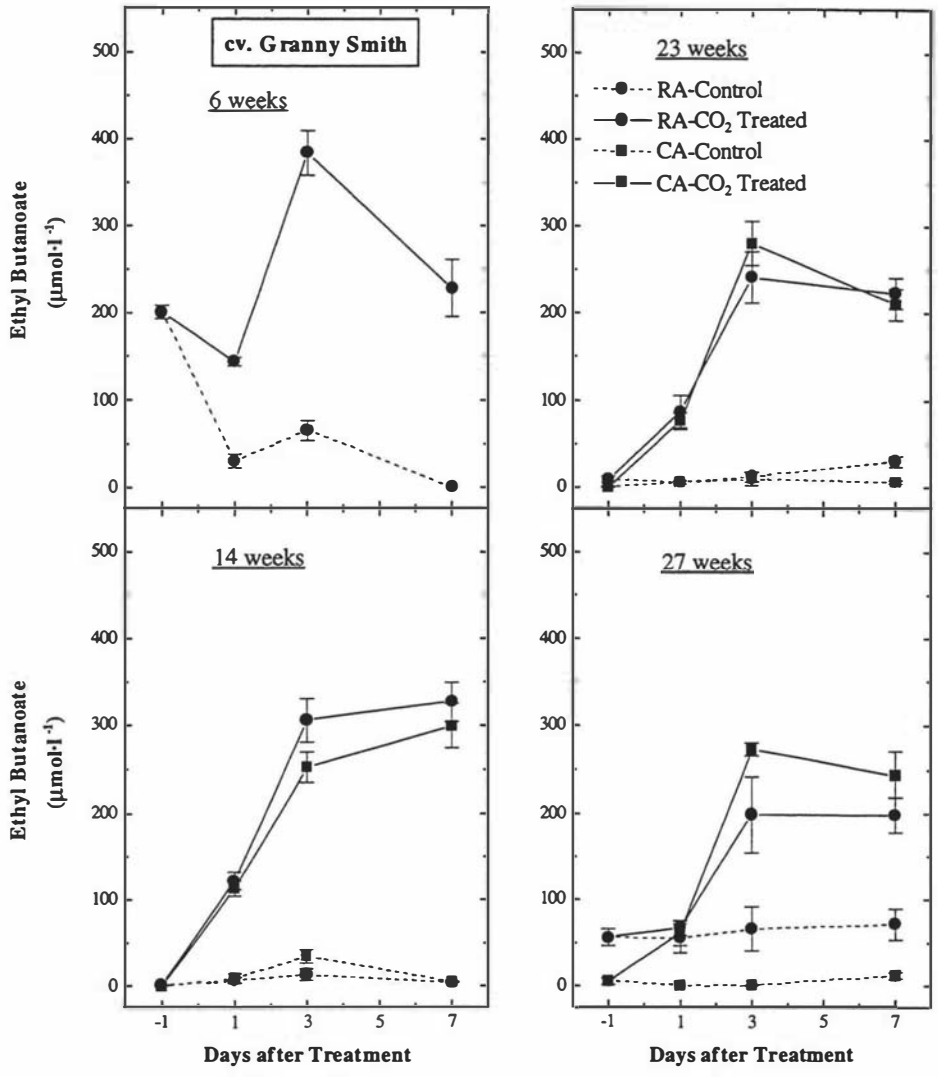


Fig. 5.12 Ethyl butanoate concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

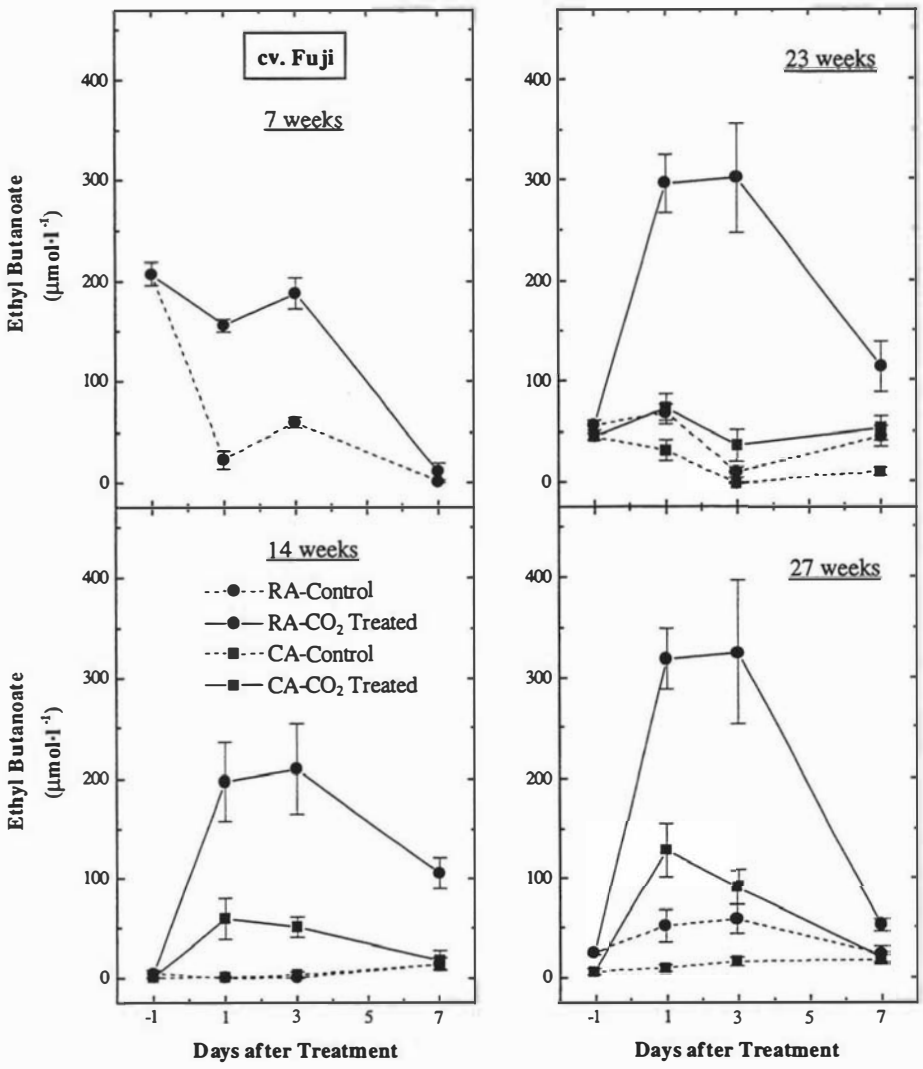


Fig. 5.13 Ethyl butanoate concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

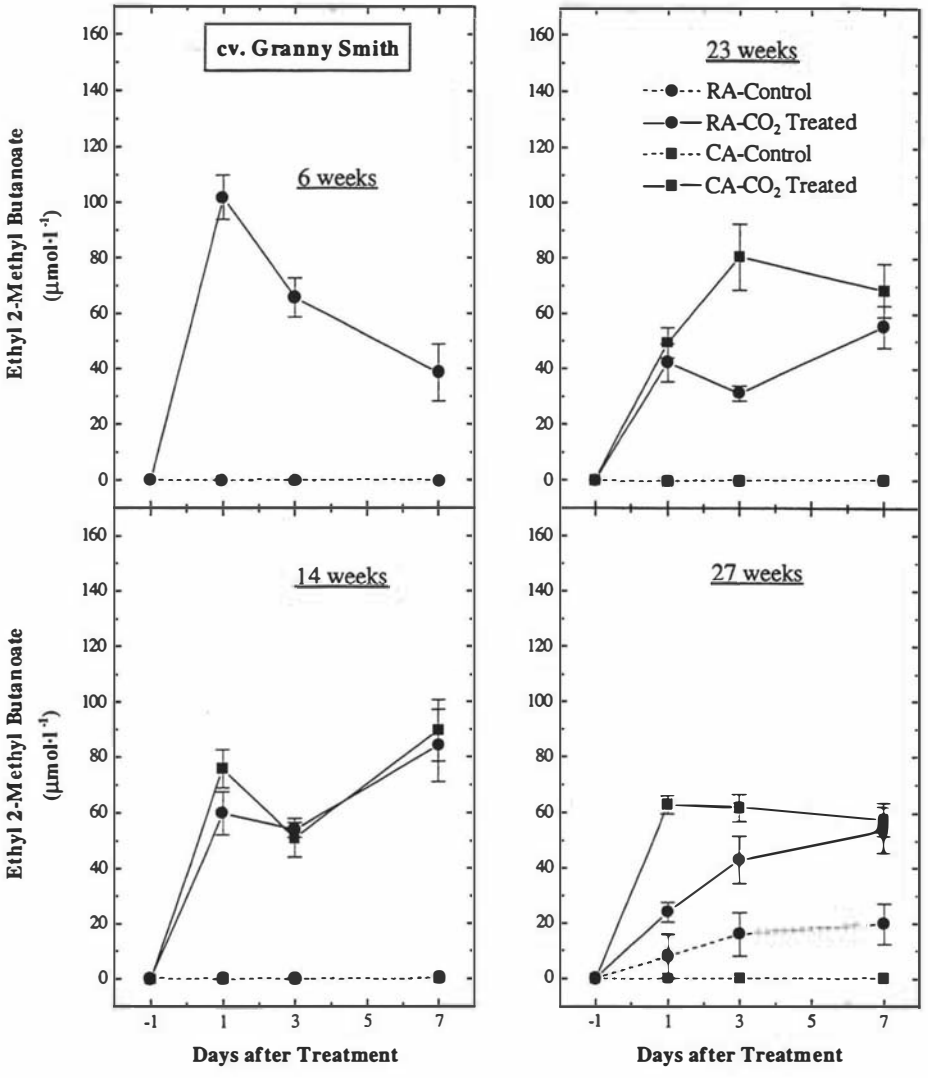


Fig. 5.14 Ethyl 2-methyl butanoate concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

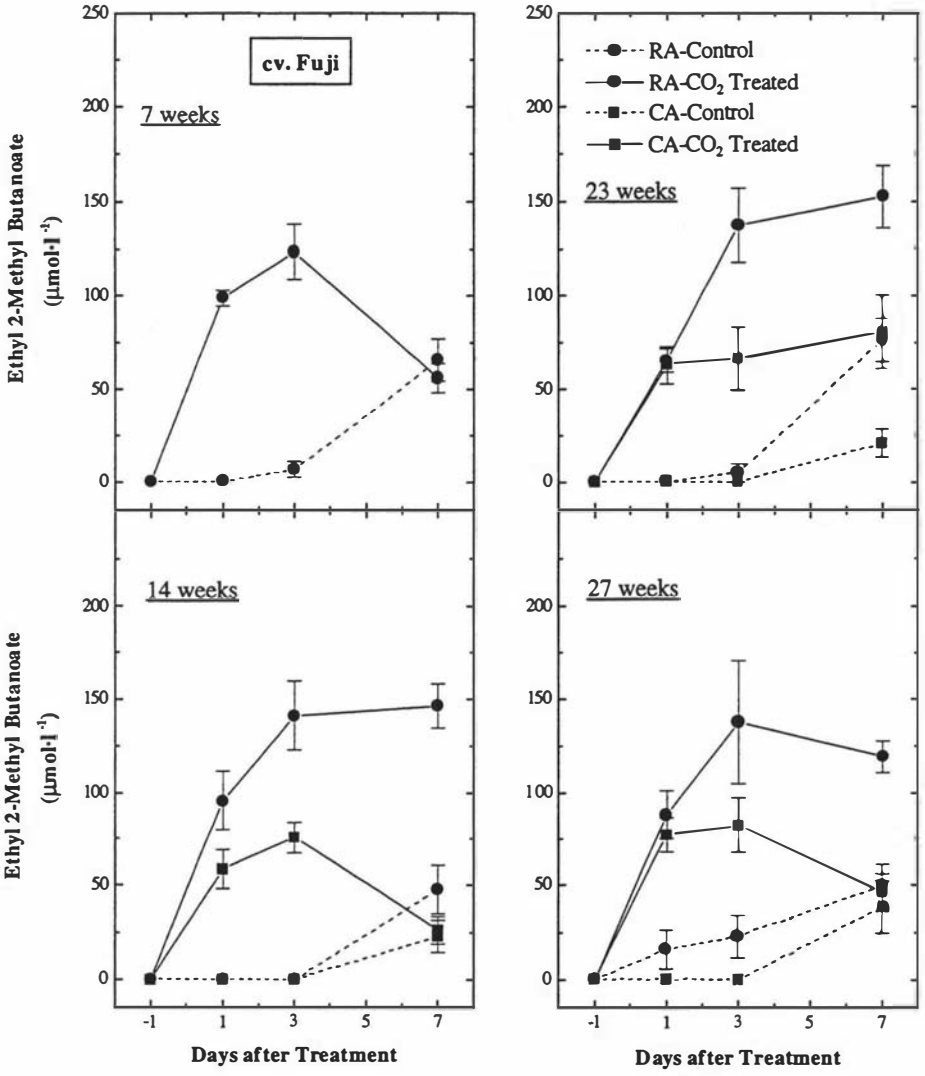


Fig. 5.15 Ethyl 2-methyl butanoate concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

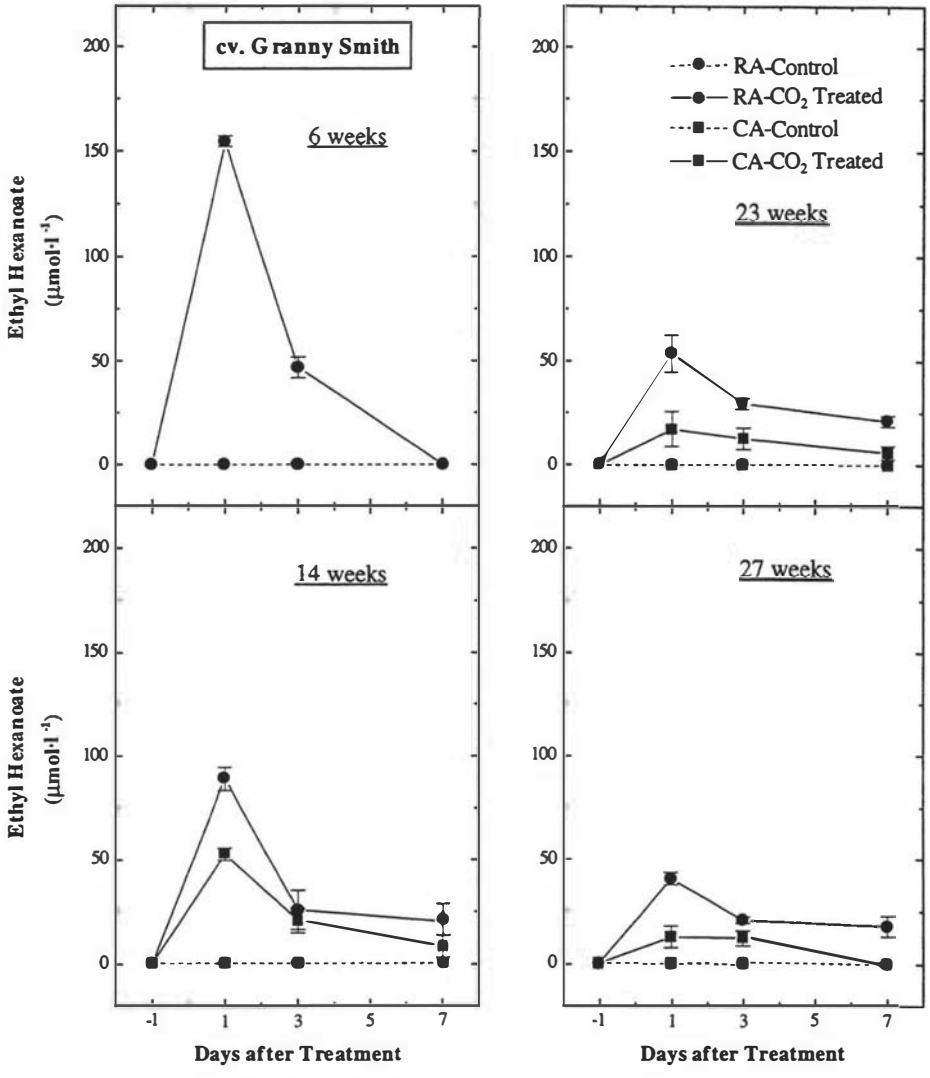


Fig. 5.16 Ethyl hexanoate concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

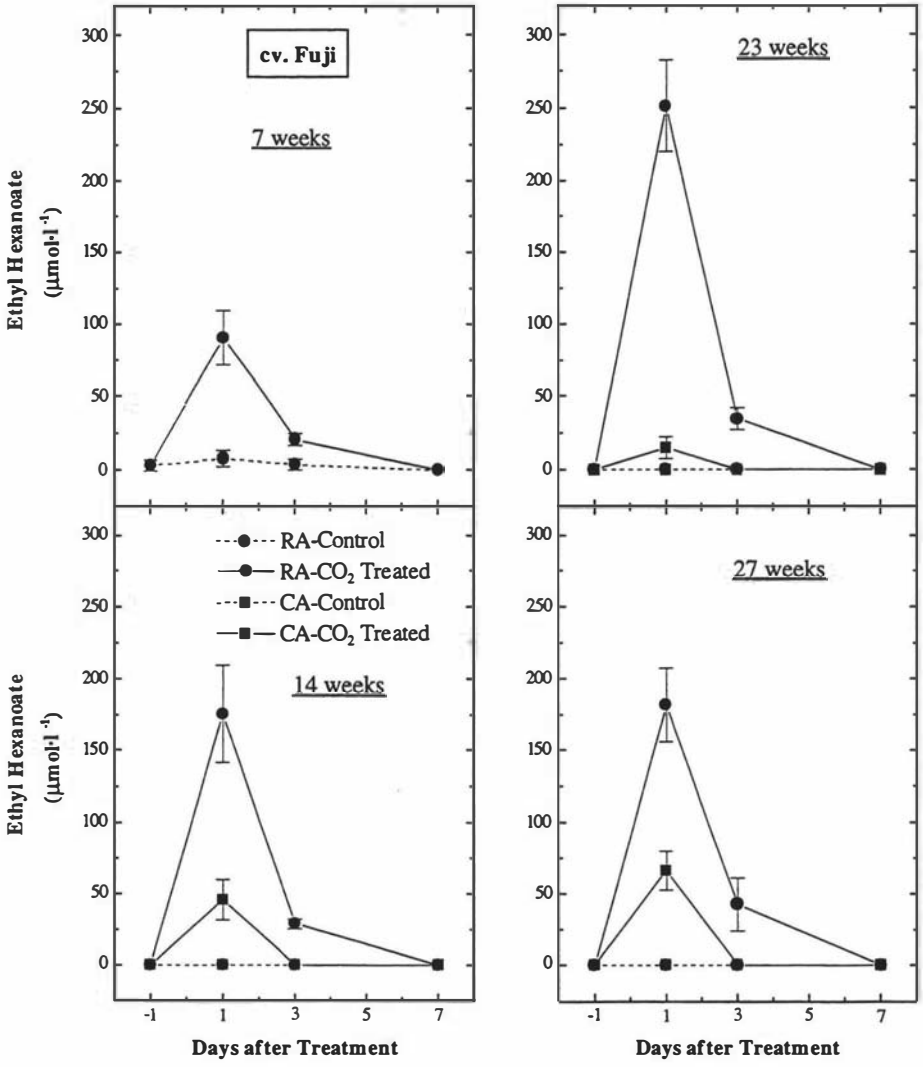


Fig. 5.17 Ethyl hexanoate concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

Ethyl hexanoate concentration of control fruit of RA and CA stored 'Granny Smith' (**Fig. 5.16**) and 'Fuji' (**Fig. 5.17**) apples during post storage at 20°C was negligible or not detected. Treatment with 100% CO_2 dramatically enhanced ethyl hexanoate during post storage in both RA and CA stored fruit of both cultivars. In

general, ethyl hexanoate concentration increased to a peak 1 day after treatment and rapidly decreased thereafter. The concentration of ethyl hexanoate in CO_2 treated CA stored fruit was always lower than in RA stored apples. From 14 to 27 weeks after harvest the magnitude of the ethyl hexanoate induced in response to CO_2 treatment was greater in 'Fuji' than in 'Granny Smith' apples (**Fig. 5.16** and **Fig. 5.17**).

Examining total concentration of ethyl esters (**Fig. 5.18** to **Fig. 5.21**), values obtained by integrating response during post storage for 7 days at 20°C over a period of 27 weeks after harvest, indicated that production of individual ethyl esters varied between cultivars, storage conditions, and treatments.

Total concentrations of ethyl esters from control RA and CA stored apples of both 'Granny Smith' and 'Fuji' were generally low, except at first removal (6 or 7 weeks after harvest) for both ethyl acetate (**Fig. 5.18**) and ethyl butanoate (**Fig. 5.19**). Total concentrations of ethyl esters from control CA stored fruit of both cultivars remained low or decreased slowly with time after harvest, while from RA stored fruit they increased slowly after 23 weeks storage. Ethyl hexanoate from control fruit of both RA and CA stored 'Granny Smith' and 'Fuji' apples was negligible or not detected throughout 27 weeks (**Fig. 5.21**).

Total concentration of ethyl esters (**Fig. 5.18** to **Fig. 5.21**) was markedly enhanced by CO_2 treatment in both cultivars and at all removal times from either RA or CA cool stores, although quantitative differences did exist. Ethyl esters from 100% CO_2 treated RA stored fruit of both cultivars were generally greater than from CA stored apples throughout storage. An exception to this trend was that of ethyl butanoate (**Fig. 5.19**) and ethyl 2-methyl butanoate (**Fig. 5.20**) in 'Granny Smith' apples; total concentration of ethyl butanoate was similar for both RA and CA fruit, while ethyl 2-methyl butanoate from CA stored fruit was greater than from RA stored fruit, especially after 23 and 27 weeks in cool store. The ability to produce ethyl esters after 100% CO_2 treatment by both RA and CA stored 'Granny Smith' apples decreased continuously with time after harvest, except with ethyl 2-methyl butanoate (**Fig. 5.20**) of CA stored fruit, which remained more or less constant throughout 27 weeks. Total concentration of ethyl esters from 100% CO_2 treated RA stored 'Fuji' generally increased initially then stayed constant or decreased slightly during 27 weeks after harvest; ethyl esters from treated

CA stored fruit initially decreased 14 weeks after harvest, then remained constant or increased slightly towards the end of the 27-week period. Total concentration of ethyl hexanoate from the CO_2 treated CA stored apples of both cultivars decreased continuously to 23 weeks after which there was a slight increase at 27 weeks after harvest for 'Fuji' (**Fig. 5.21**). Relatively, CO_2 treated 'Fuji' apples seemed to have a greater ability to produce ethyl acetate (**Fig. 5.18**), and ethyl 2-methyl butanoate (**Fig. 5.20**) than treated 'Granny Smith' apples, whereas 'Granny Smith' produced relatively more ethyl butanoate (**Fig. 5.19**) than 'Fuji'.

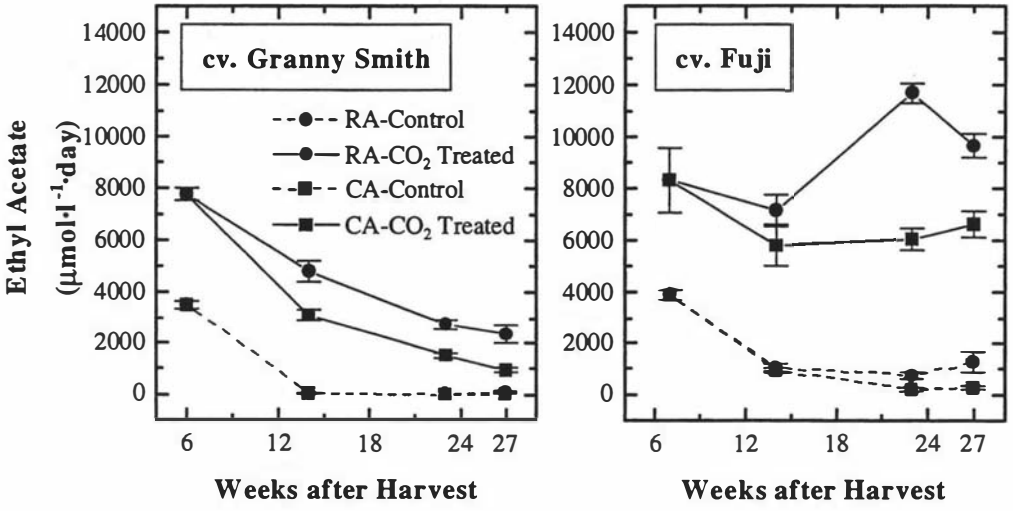


Fig. 5.18 Total ethyl acetate concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

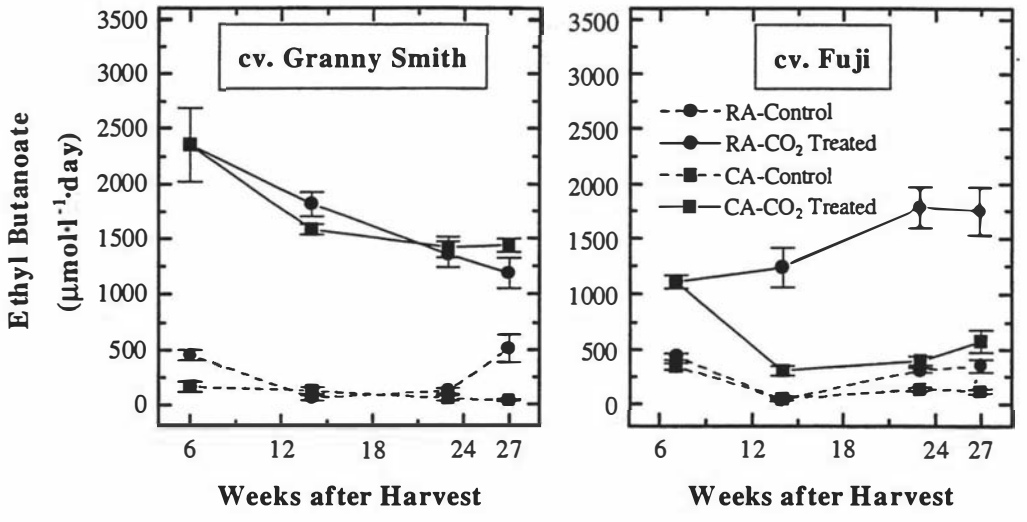


Fig. 5.19 Total ethyl butanoate concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

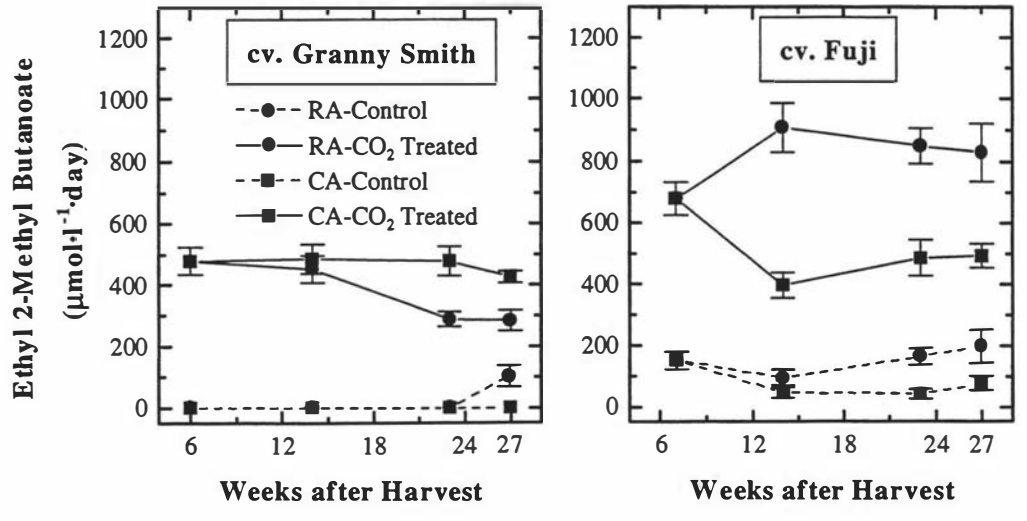


Fig. 5.20 Total ethyl 2-methyl butanoate concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

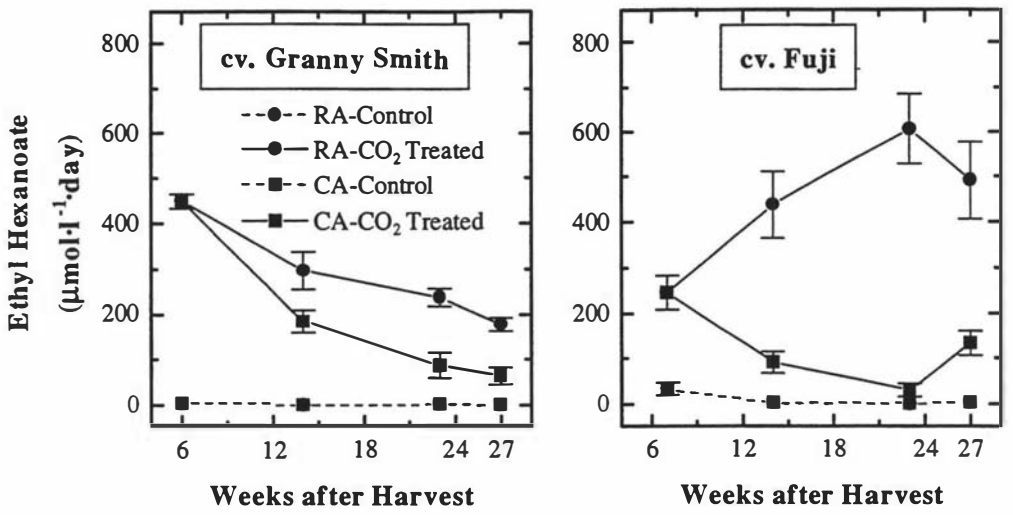


Fig. 5.21 Total ethyl hexanoate concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

Butyl acetate and hexyl acetate were found in 'Fuji' apples, but not in 'Granny Smith' apples. Butyl acetate (**Fig. 5.22**) and hexyl acetate (**Fig. 5.23**) concentrations of the RA stored 'Fuji' apples generally decreased continuously during post storage for 7 days at 20°C throughout a period of 27 weeks after harvest, while concentration from CA stored fruit generally decreased slightly or stayed constant. Butyl acetate and hexyl acetate concentrations from RA stored fruit were higher than from CA stored apples and those from control fruit were usually greater than those from CO_2 treated apples.

Treatment with 100% CO_2 reduced production of butyl acetate (**Fig. 5.22**) and hexyl acetate (**Fig. 5.23**) from both RA and CA stored apples. This depressive effect of CO_2 treatment became less pronounced with advancing time after harvest.

Total concentration during post storage for 7 days at 20°C of butyl acetate (**Fig. 5.24**) and hexyl acetate (**Fig. 5.25**) over a period of 27 weeks after harvest indicated that the production of both compounds in RA stored 'Fuji' fruit increased during storage to a peak at 23 and 14 weeks, respectively, and decreased thereafter. Total concentration of these compounds in CO_2 treated fruit of RA stored apples followed a similar pattern as those of the respective control fruit but the amount produced was less.

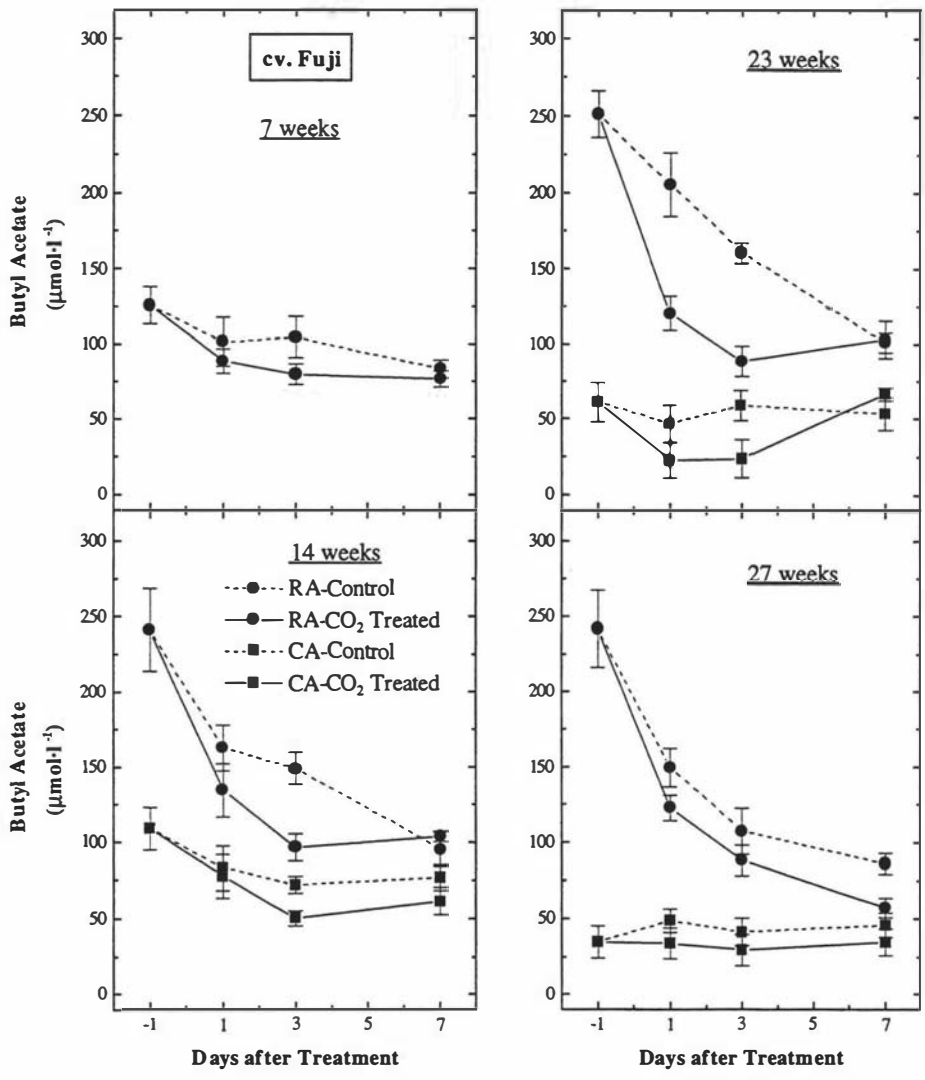


Fig. 5.22 Butyl acetate concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

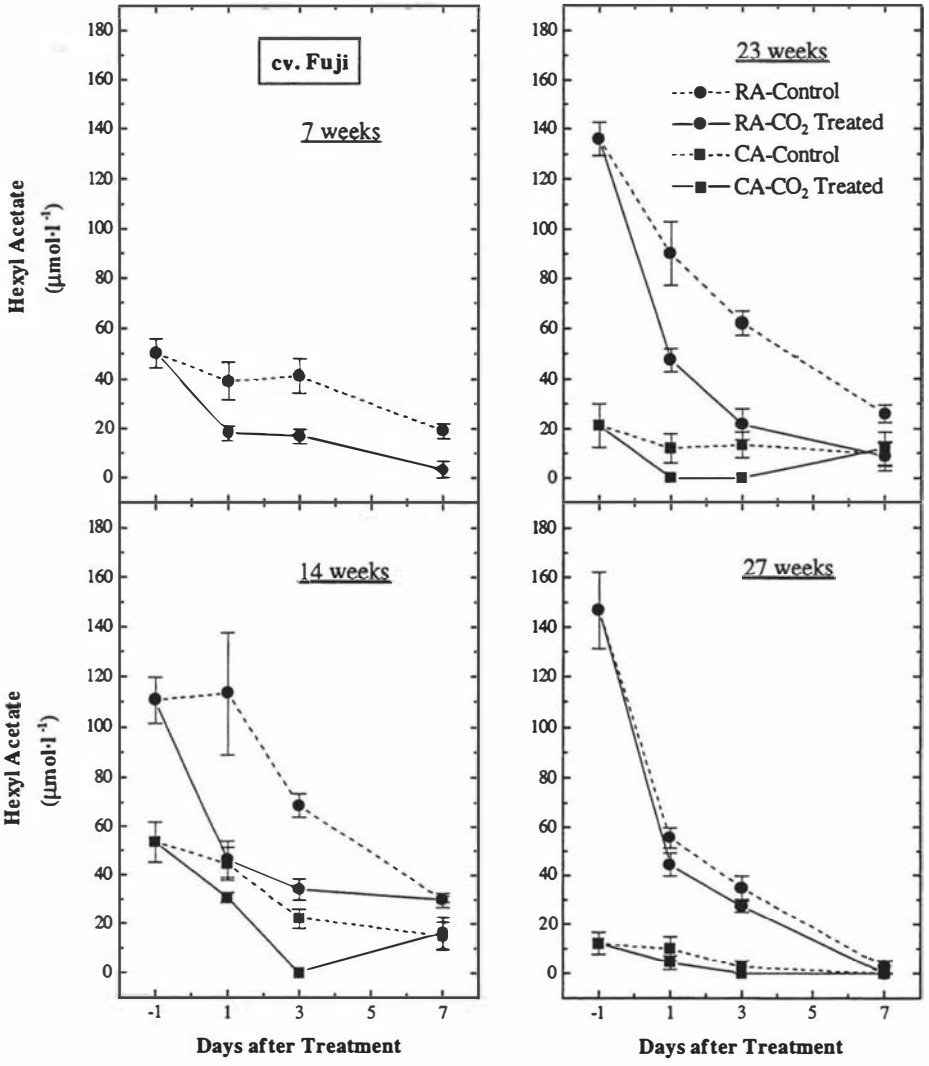


Fig. 5.23 Hexyl acetate concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

CA storage diminished the ability of apples to produce both butyl and hexyl acetate, as the total concentration decreased continuously throughout a period of 27 weeks after harvest. In addition, treatment with 100% CO_2 further depressed the total concentration of both compounds. The depressive effect of CO_2 treatment on the total

concentration of both compounds was greater in RA than in CA stored 'Fuji' apples (**Fig. 5.24** and **Fig. 5.25**).

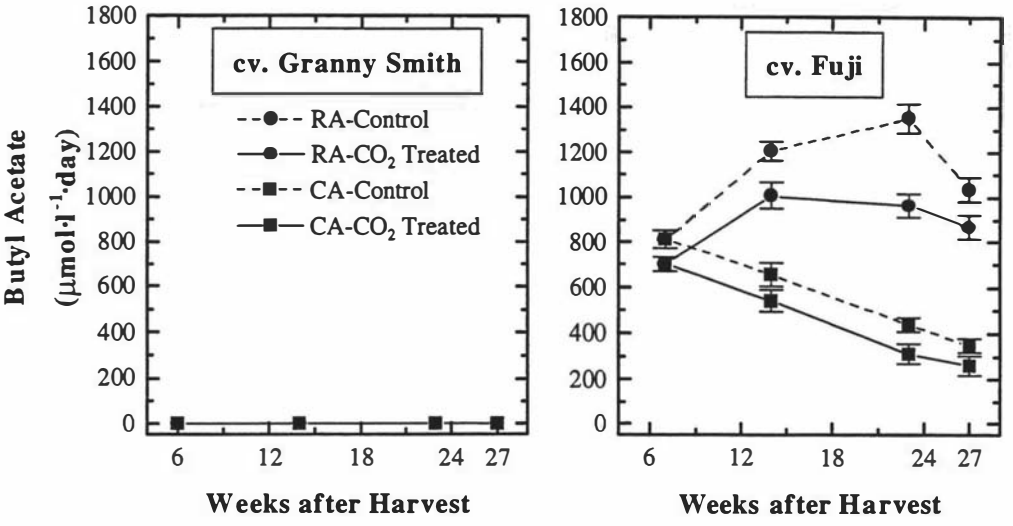


Fig. 5.24 Total butyl acetate concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA and CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

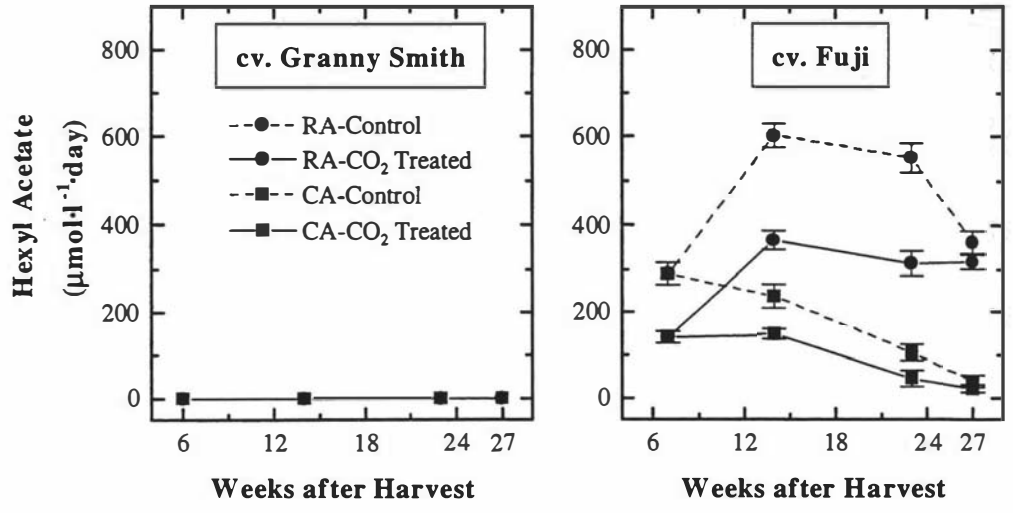


Fig. 5.25 Total hexyl acetate concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

5.4.3 Volatile Alcohols

Concentration of volatile alcohols from apples varied markedly in relation to storage conditions, time after harvest and response to 100% CO_2 treatment, as well as between cultivars.

Ethanol from control RA stored 'Granny Smith' (**Fig. 5.26**) or 'Fuji' (**Fig. 5.27**) apples at 6 or 7 weeks after harvest (initial), respectively, was initially high and increased slowly in 'Fuji' apples or remained constant in 'Granny Smith' during 7 days at 20°C. Ethanol in RA stored 'Granny Smith' apples during 14 and 23 weeks after harvest was low but an appreciable amount was detected at 27 weeks after harvest. However, ethanol from CA stored 'Granny Smith' apples was negligible throughout 27 weeks storage (**Fig. 5.26**). Both RA and CA stored control fruit of 'Fuji' apples always produced a low concentration of ethanol (**Fig. 5.27**).

Treatment with 100% CO_2 increased ethanol concentration immediately after treatment in RA and CA stored apples of both cultivars. The concentration peaked after 1 - 3 days and decreased thereafter, being generally higher in treated RA stored than CA stored apples (**Fig. 5.26** and **Fig. 5.27**).

Total concentration of ethanol in control fruit of both RA and CA stored apples of both cultivars decreased slightly from 6 to 14 weeks of storage after which it was more or less constant at low levels throughout 27 weeks after harvest, except for RA stored apples where ethanol increased again at 27 weeks (**Fig. 5.28**).

Total concentration of ethanol in CO_2 treated RA and CA stored apples of both cultivars was markedly greater than that from control fruit, being higher in 'Granny Smith' than in 'Fuji'. High CO_2 treated RA stored 'Granny Smith' apples produced a fairly constant amount of ethanol for up to 23 weeks, but increased sharply at 27 weeks after harvest, while that in 'Fuji' initially decreased at 14 weeks and increased thereafter. Total concentration of ethanol in the CO_2 treated CA stored 'Granny Smith' apples generally decreased slowly, while that in 'Fuji' decreased sharply to 14 weeks and then gradually increased with storage time (**Fig. 5.28**).

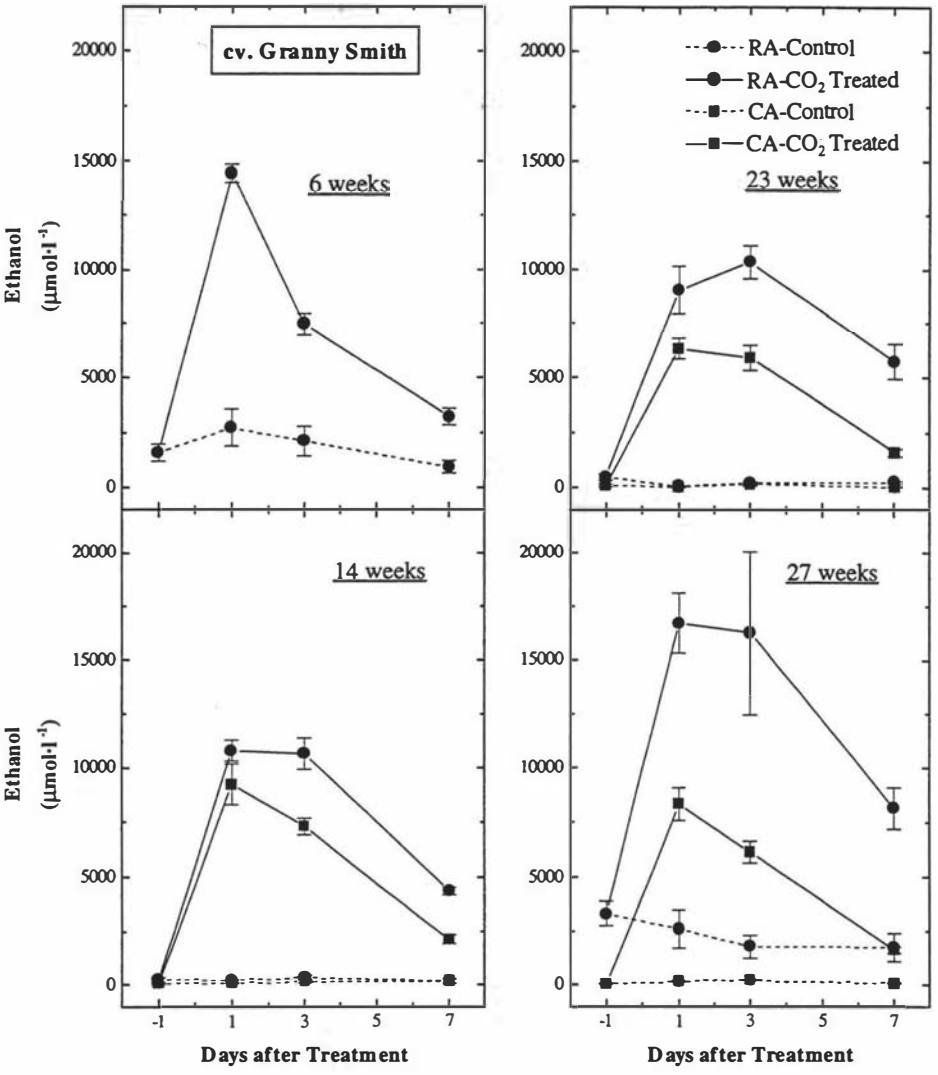


Fig. 5.26 Ethanol concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

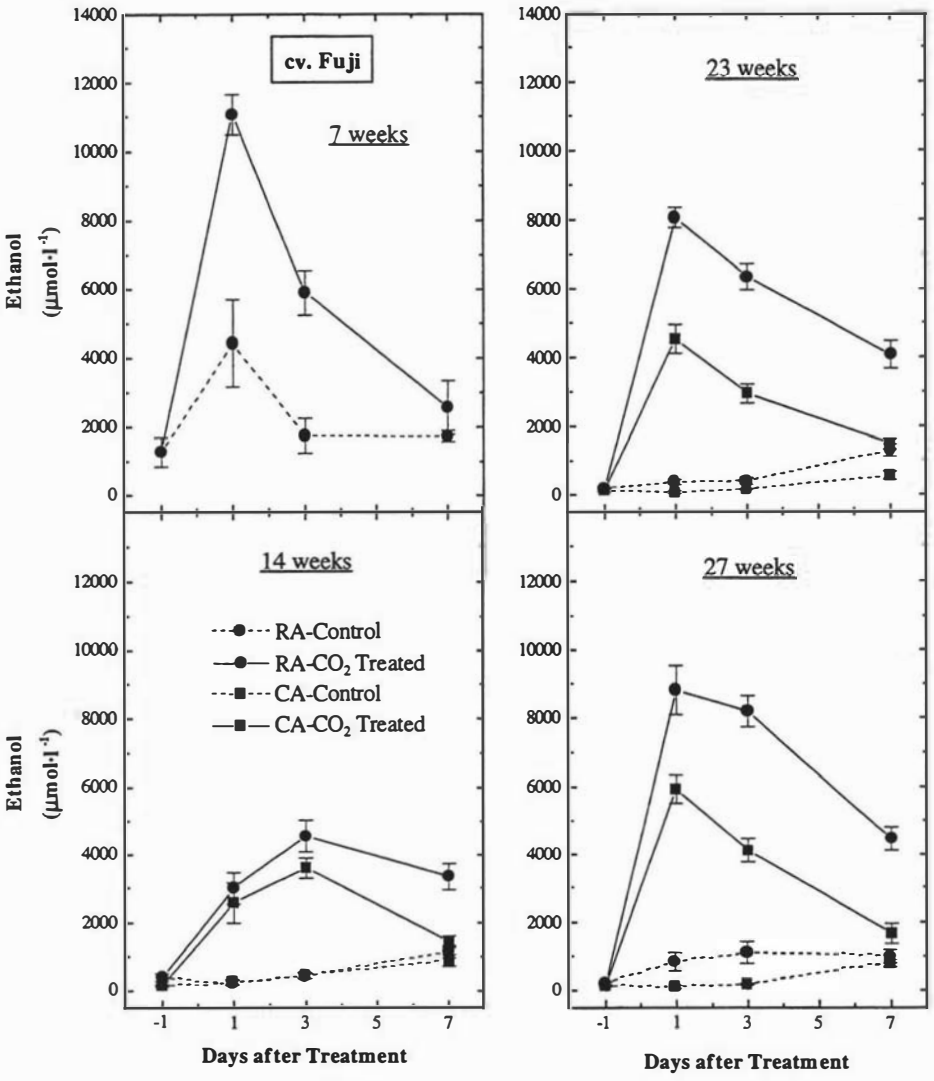


Fig. 5.27 Ethanol concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

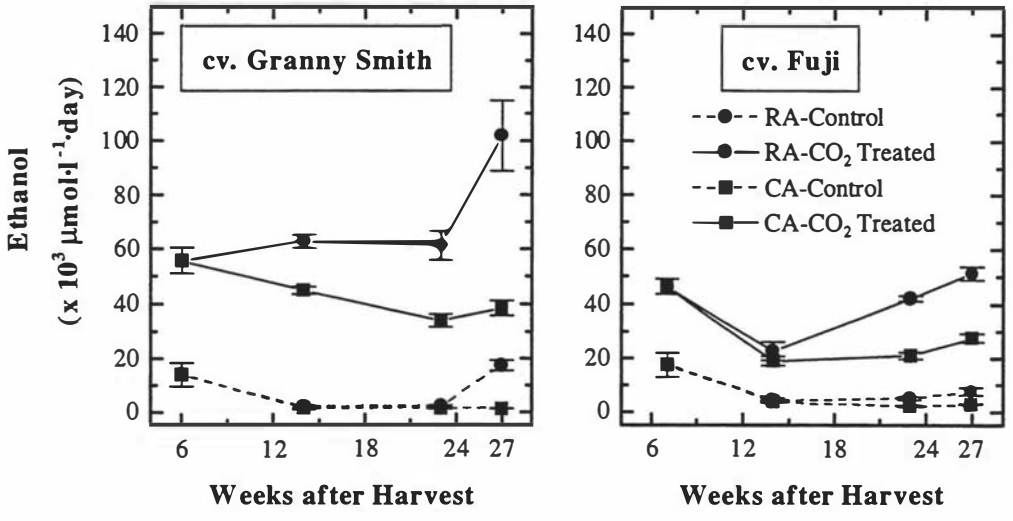


Fig. 5.28 Total ethanol concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

Propan-1-ol concentration in RA and CA stored 'Granny Smith' (**Fig. 5.29**) and 'Fuji' (**Fig. 5.30**) apples, from either control or CO_2 treatments, generally increased in an almost linear manner during 7 days at 20°C at each time of removal from cool storage. Propan-1-ol from RA stored 'Granny Smith' apples was suppressed by the CO_2 treatment with the degree of suppression being less with time in cool store. In contrast, propan-1-ol from CA stored 'Granny Smith' apples was enhanced by CO_2 treatment (**Fig. 5.29**). In both RA and CA stored 'Fuji' apples, however, CO_2 treatment did not have any marked effect on propan-1-ol during post storage at 20°C (**Fig. 5.30**). Propan-1-ol concentration from RA stored fruit of both apple cultivars was generally higher than from CA stored fruit.

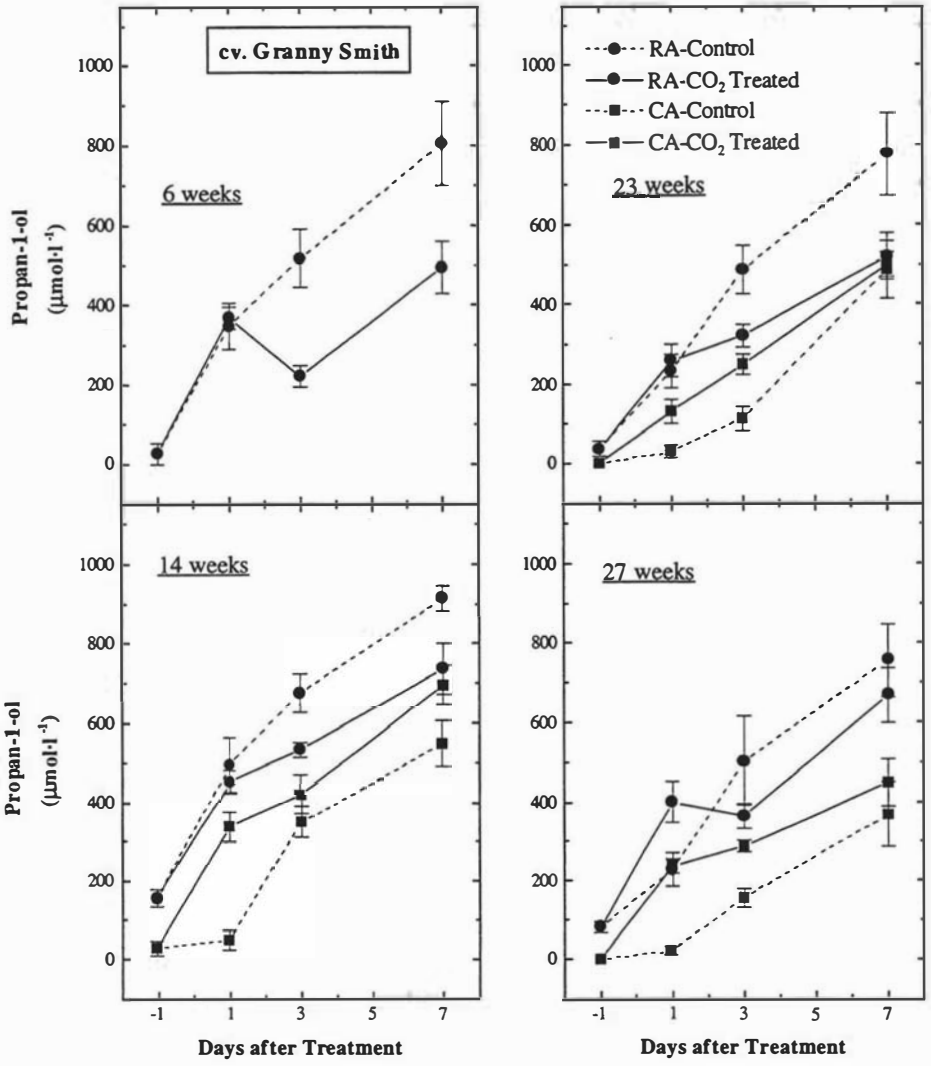


Fig. 5.29 Propan-1-ol concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

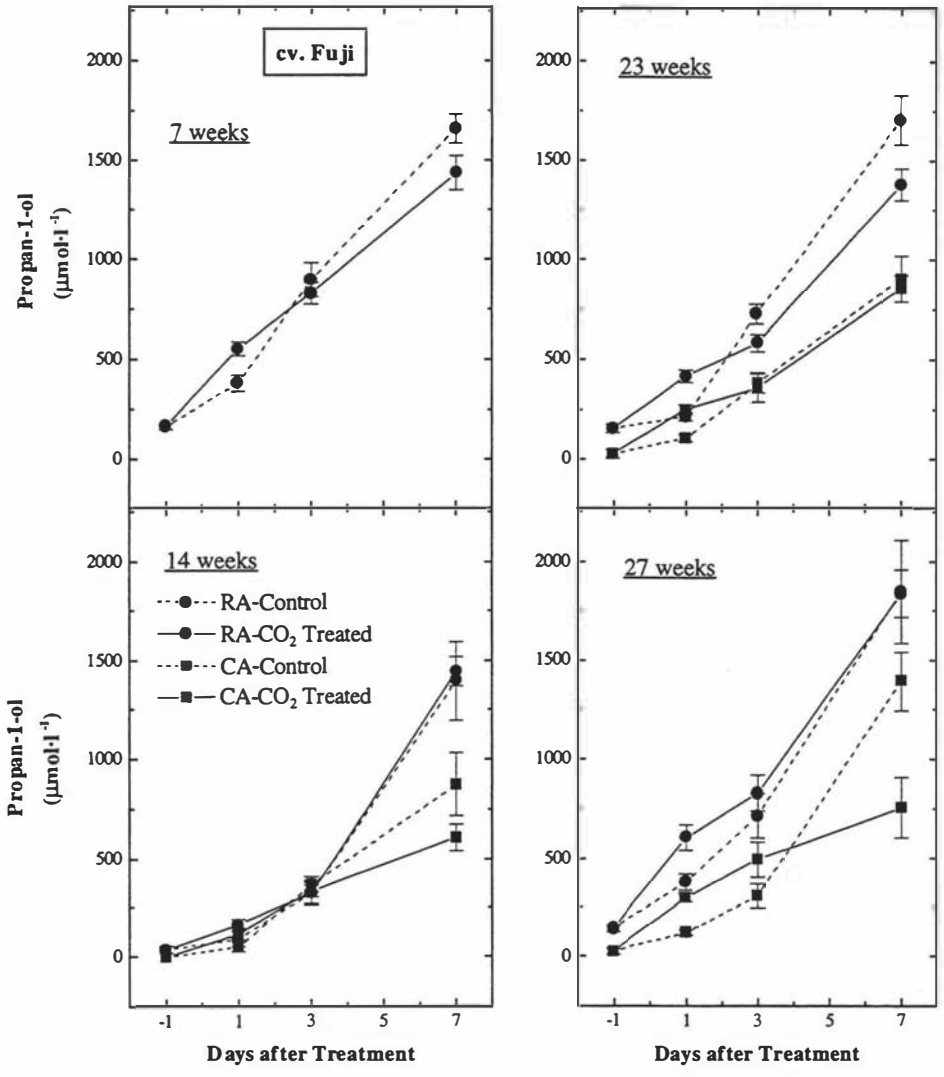


Fig. 5.30 Propan-1-ol concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

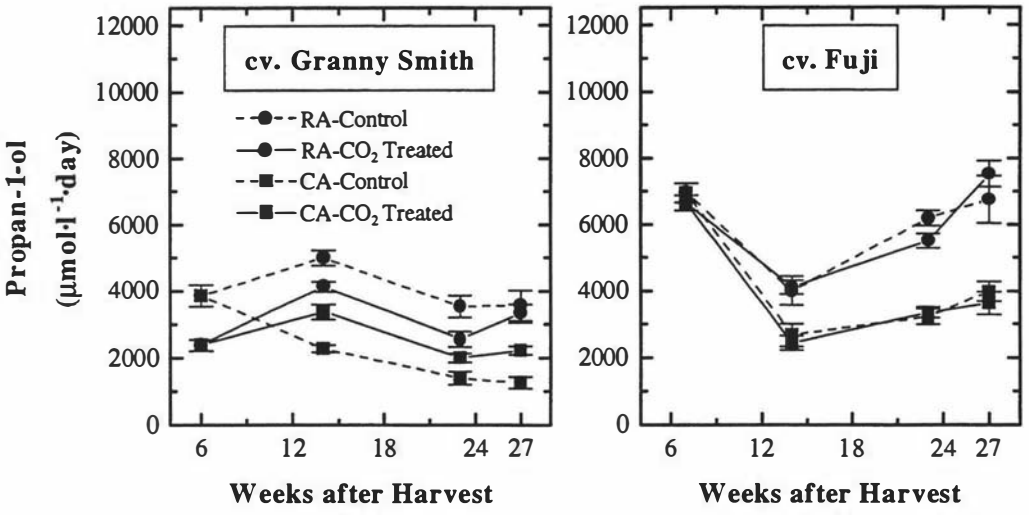


Fig. 5.31 Total propan-1-ol concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

Total concentration of propan-1-ol (as area under curve) in control RA stored 'Granny Smith' apples increased to a peak at 14 weeks after harvest and decreased thereafter (**Fig. 5.31**), whereas in CA stored fruit it decreased continuously throughout storage. Propan-1-ol in CO_2 treated RA stored 'Granny Smith' followed a similar pattern to that of control RA stored apples, except that the total concentration was generally lower and it increased slightly to a level similar to controls at 27 weeks after harvest. Treatment with CO_2 enhanced total concentration of propan-1-ol in CA stored 'Granny Smith' apples over control and it had a similar pattern to that of RA stored fruit but the amount was lower. In 'Fuji' apples propan-1-ol from control and CO_2 treated fruit was similar. However, total concentration was markedly depressed by CA storage as compared with that from RA storage. The production pattern was similar in all treatments, in which it was initially decreased at 14 weeks after harvest and then increased thereafter (**Fig. 5.31**).

Butan-1-ol concentration during post storage at 20°C in control RA stored 'Granny Smith' apples after 6 or 14 weeks storage increased to a peak after 1 to 3 days and decreased thereafter. However, the concentration tended to increase continuously as length of storage increased. Butan-1-ol from CO_2 treated RA stored, and from CA

stored apples, either control or treated, generally increased continuously during post storage at 20°C throughout 27 weeks after harvest (**Fig. 5.32**).

Butan-1-ol concentration from CO_2 treated RA stored 'Granny Smith' apples was initially depressed 1 - 3 days after treatment, but the concentration exceeded butan-1-ol from control at day 7. The suppressive effect of CO_2 treatment as such became less pronounced as time after harvest progressed (**Fig. 5.32**).

Butan-1-ol concentration from RA stored 'Fuji' apples was initially high on removal from cool store; it decreased at day 1 and remained approximately constant thereafter (**Fig. 5.33**). Butan-1-ol from CA stored 'Fuji' apples changed very little during post storage throughout a period of 27 weeks after harvest (**Fig. 5.33**).

Treatment with CO_2 did not have any dramatic effect on butan-1-ol concentration in RA or CA stored 'Fuji' apples, except that in RA stored apples at 23 weeks after harvest where the concentration was lower in treated fruit than control (**Fig. 5.33**).

Total concentration of butan-1-ol (as area under curve) in RA stored fruit of 'Granny Smith' apples increased continuously, while remaining at a lower but constant concentration from CA stored fruit throughout storage (**Fig. 5.34**). Butan-1-ol from RA stored 'Fuji' apples remained constant to 14 weeks and increased markedly thereafter. Butan-1-ol from CA stored fruit was initially depressed after 14 weeks in storage being less than 50% that from RA fruit in both cultivars; the level of production remained approximately constant thereafter. In general, treatment with CO_2 had a minimal negative effect on total concentration of butan-1-ol regardless of storage condition or cultivar, and the total concentration was much higher in 'Fuji' than in 'Granny Smith' apples (**Fig. 5.34**).

Hexan-1-ol concentration during post storage at 20°C of 'Granny Smith' apples (**Fig. 5.35**) generally increased to a peak at day 3 and then it stayed constant, slightly decreased, or increased depended on treatments, storage conditions and time after harvest. The concentration in CA stored apples was generally lower than in RA stored fruit. There was no consistent effect of CO_2 treatment on hexan-1-ol in 'Granny Smith' apples, except in RA stored fruit at 14 weeks after harvest where the concentration in CO_2 treated fruit was lower than in controls (**Fig. 5.35**).

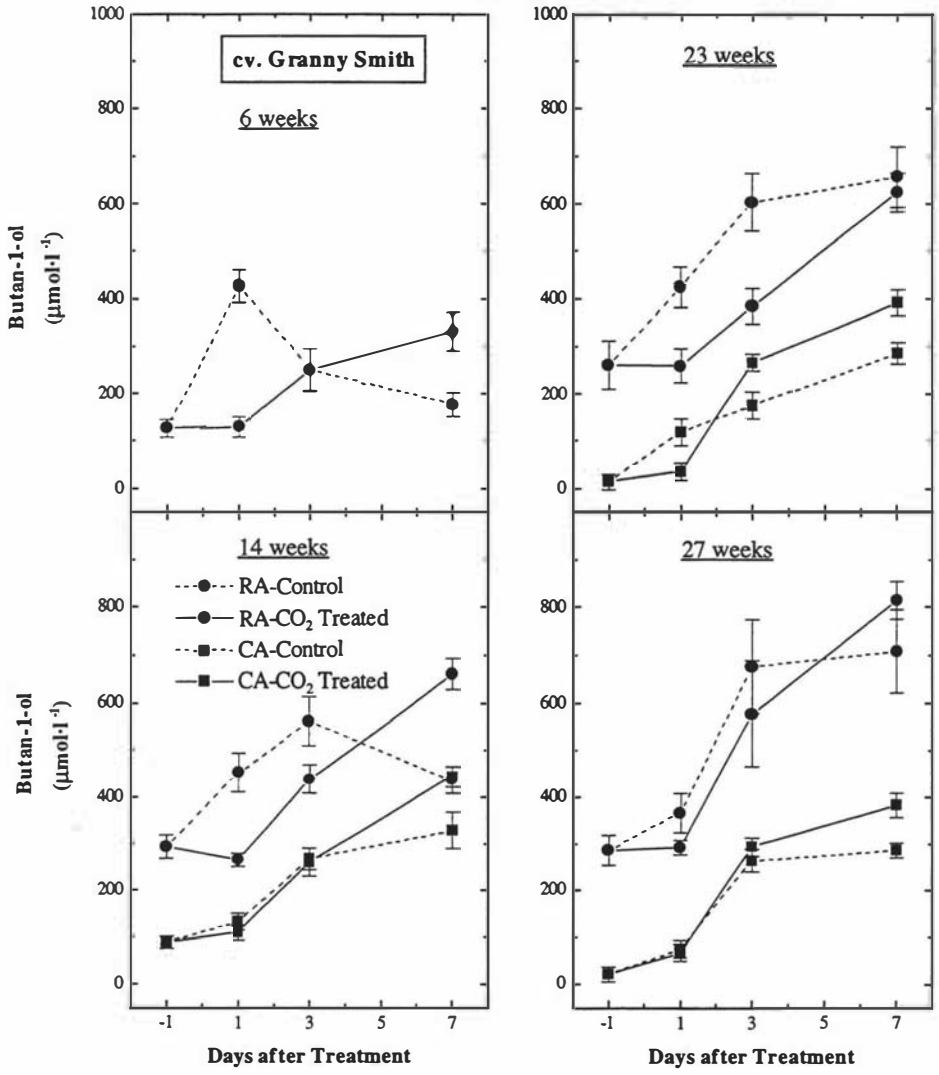


Fig. 5.32 Butan-1-ol concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

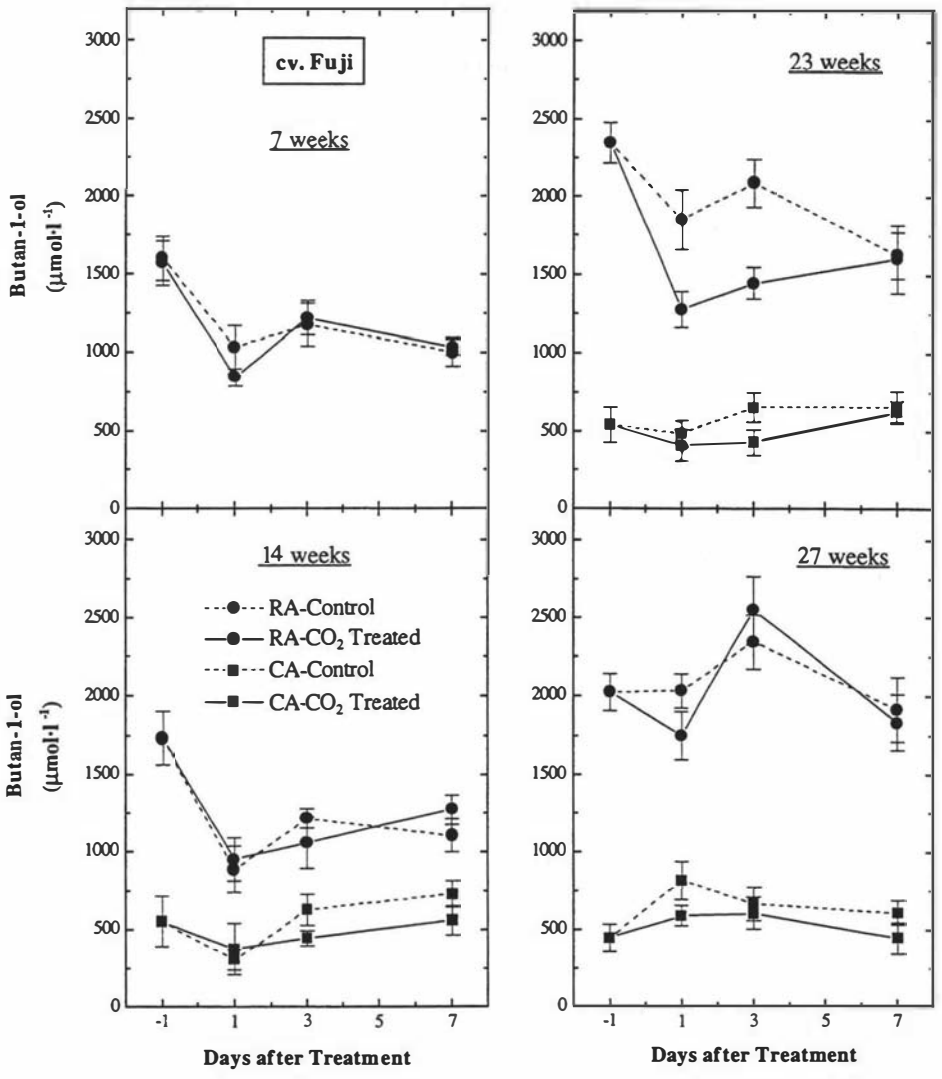


Fig. 5.33 Butan-1-ol concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

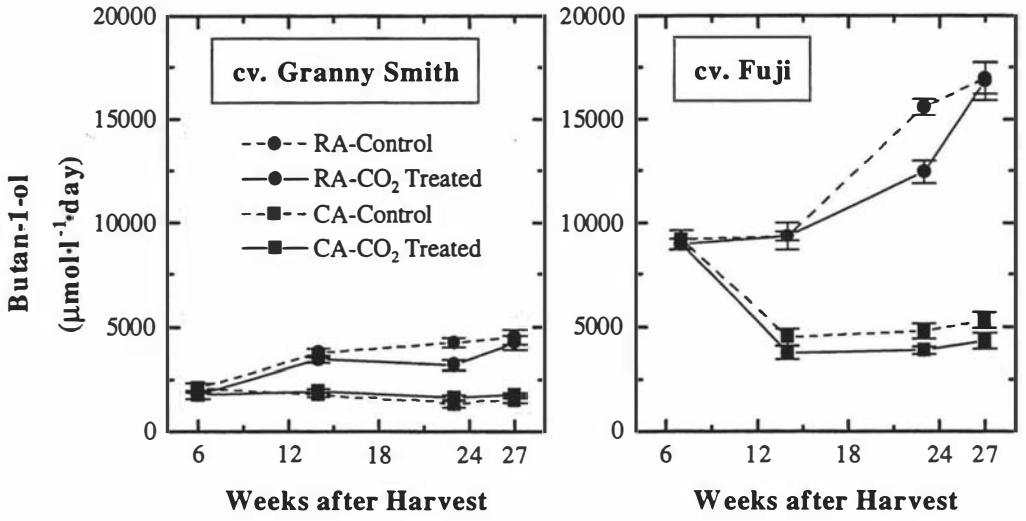


Fig. 5.34 Total butan-1-ol concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air to 100% CO_2 for 24 hours at 20°C.

Hexan-1-ol concentration from 'Fuji' apples (**Fig. 5.36**), in general, was initially high decreasing gradually during post storage for 7 days at 20°C. Hexan-1-ol from CA stored apples was always lower than from RA stored fruit. Treatment with CO_2 decreased hexan-1-ol in RA stored 'Fuji' apples during 14 - 23 weeks, but not at 6 or 27 weeks after harvest. Concentration from CO_2 treated CA stored 'Fuji' apples during post storage at 20°C was generally lower than from control fruit throughout a period of 27 weeks after harvest (**Fig. 5.36**).

Total concentration of hexan-1-ol (as area under curve) from control RA stored 'Granny Smith' apples increased slightly at 14 weeks but stayed approximately constant thereafter. Hexan-1-ol from CO_2 treated fruit decreased at 14 weeks after harvest and then increased to a level similar to that of control at about 23 weeks. In fact, total concentration of hexan-1-ol from all RA stored apples at 27 weeks was comparable to that at 6 weeks (initial) after harvest (**Fig. 5.37**).

In 'Fuji' apples, total concentration of hexan-1-ol in RA stored fruit increased through 23 weeks in cool store, but dropped sharply in control fruit at 27 weeks, while

that in CO_2 treated fruit was less than in control, but stayed approximately constant throughout (**Fig. 5.37**).

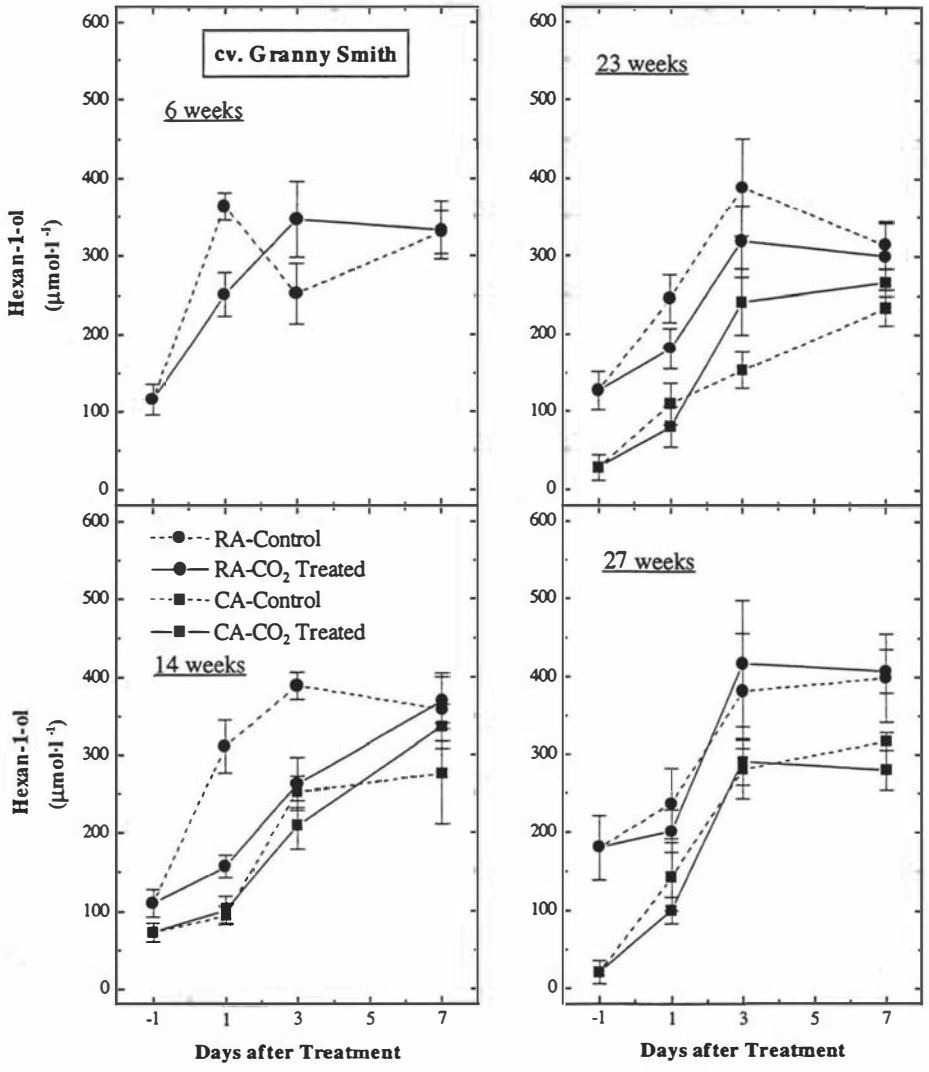


Fig. 5.35 Hexan-1-ol concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

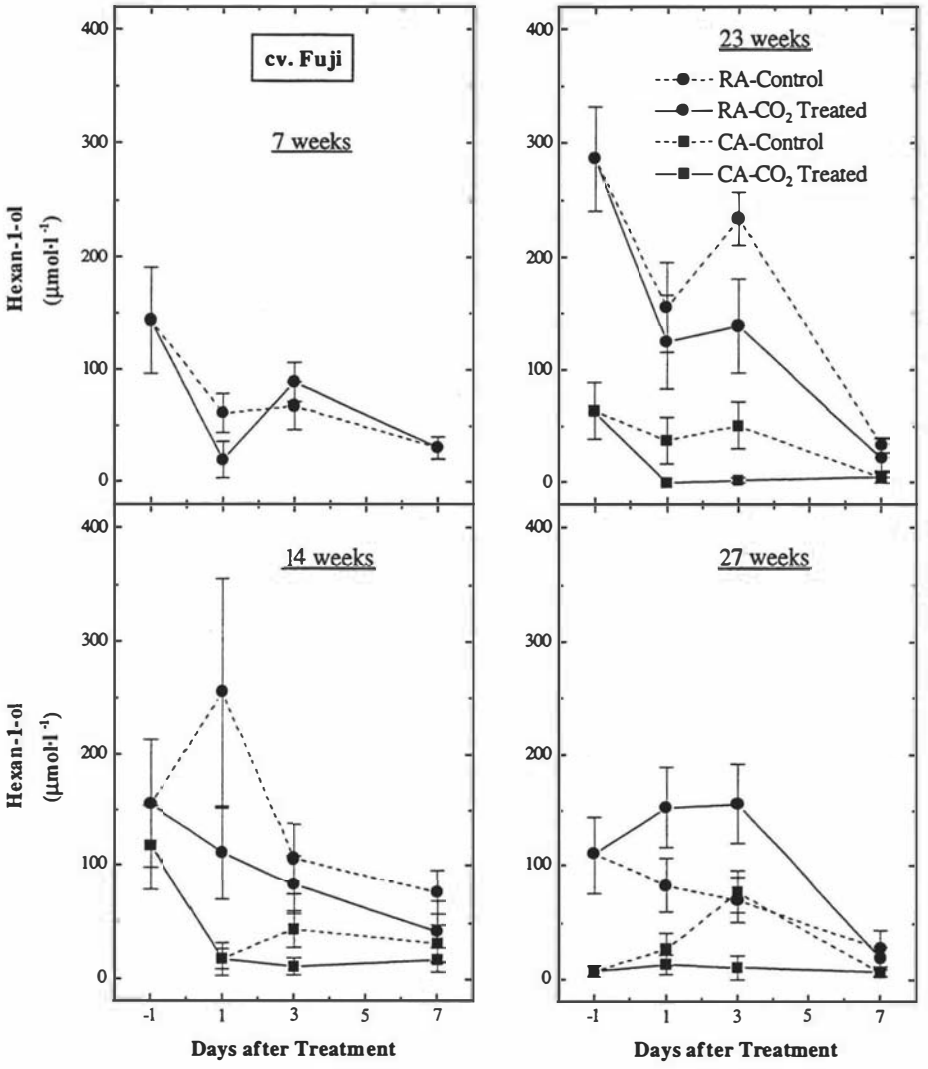


Fig. 5.36 Hexan-1-ol concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

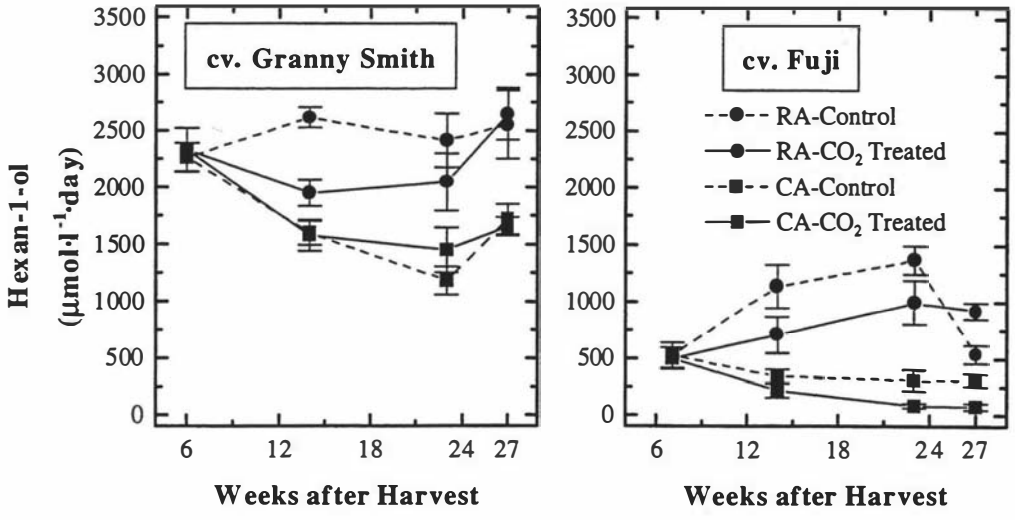


Fig. 5.37 Total hexan-1-ol concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

CA storage depressed total concentration of hexan-1-ol in both cultivars. Hexan-1-ol generally decreased gradually as time after harvest increased. Treatment with CO_2 depressed the total concentration in RA stored fruit of both apple cultivars and in CA stored 'Fuji', but not in CA stored 'Granny Smith' apples (**Fig. 5.37**).

In this study, the branched chain alcohols 2-methyl butan-1-ol and 3-methyl butan-1-ol eluted at exactly the same retention time and hence were not separated by the DB-WAX column used. Therefore, although results are presented for 2-methyl butan-1-ol it must be recognised that it represents both alcohols.

Concentration of 2-methyl butan-1-ol from control RA stored 'Granny Smith' apples generally stayed constant or increased slightly during post storage for 7 days at 20°C. In CA stored fruit this compound increased continuously and the magnitude of the increase became greater with advancing time after harvest. Treatment with CO_2 decreased the concentration 2-methyl butan-1-ol in both RA and CA stored 'Granny Smith' apples (**Fig. 5.38**).

Concentration of 2-methyl butan-1-ol from 'Fuji' apples (**Fig. 5.39**) generally increased during post storage at 20°C; this increase was greatest at 27 weeks after

harvest, particularly in control fruit. The concentration from control RA and CA stored fruit was comparable. Treatment with CO_2 slightly decreased the concentration in 'Fuji', both RA and CA stored apples (**Fig. 5.39**).

Total concentration of 2-methyl butan-1-ol (as area under curve during post storage for 7 days at 20°C) in control RA stored 'Granny Smith' apples increased to a peak at 14 weeks and decreased gradually thereafter. In CA stored fruit it increased continuously and became higher than in RA stored fruit 23 - 27 weeks after harvest (**Fig. 5.40**). Treatment with CO_2 reduced total concentration of 2-methyl butan-1-ol in both RA and CA stored fruit.

Total concentration of 2-methyl butan-1-ol in control RA and CA stored 'Fuji' apples was similar and it was fairly constant, except there was a slight increase at 27 weeks after harvest in CA stored apples (**Fig. 5.40**). Total concentration in CO_2 treated CA stored apples remained approximately constant, while in RA stored fruit it decreased gradually, but increased again slightly to a level similar to that in CA stored fruit at 27 weeks after harvest. Treatment with CO_2 slightly depressed 2-methyl butan-1-ol in both RA and CA stored 'Fuji' apples and it was more pronounced in RA stored fruit. Overall CA storage enhanced total concentration of 2-methyl butan-1-ol in both apple cultivars, particularly in 'Granny Smith' apples (**Fig. 5.40**).

5.4.4 Volatile C_6 Aldehydes

The secondary volatile compounds evaluated in this study were hexanal and *trans*-2-hexenal, these C_6 aldehydes are produced in the presence of O_2 by enzymatic reactions on fatty acids, linolenic and linolenic acids, during the disruption of fruit tissues.

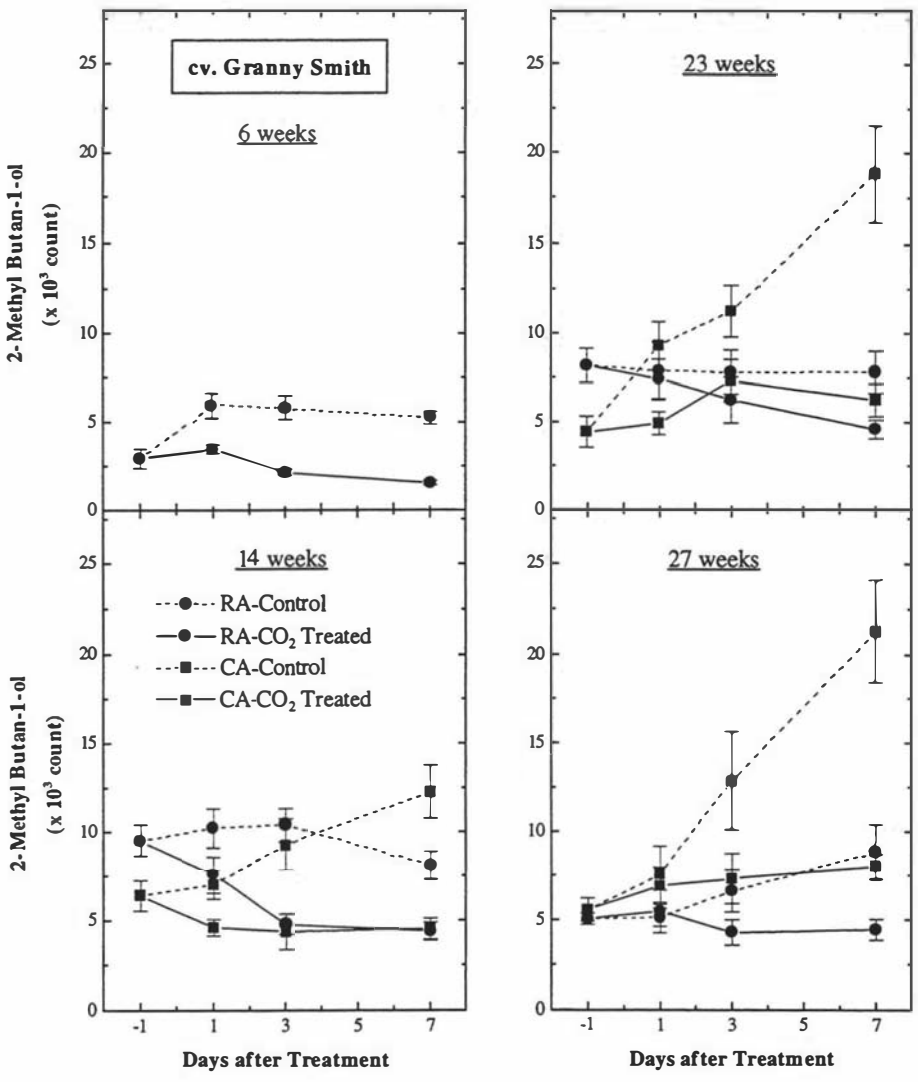


Fig. 5.38 2-Methyl butan-1-ol concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

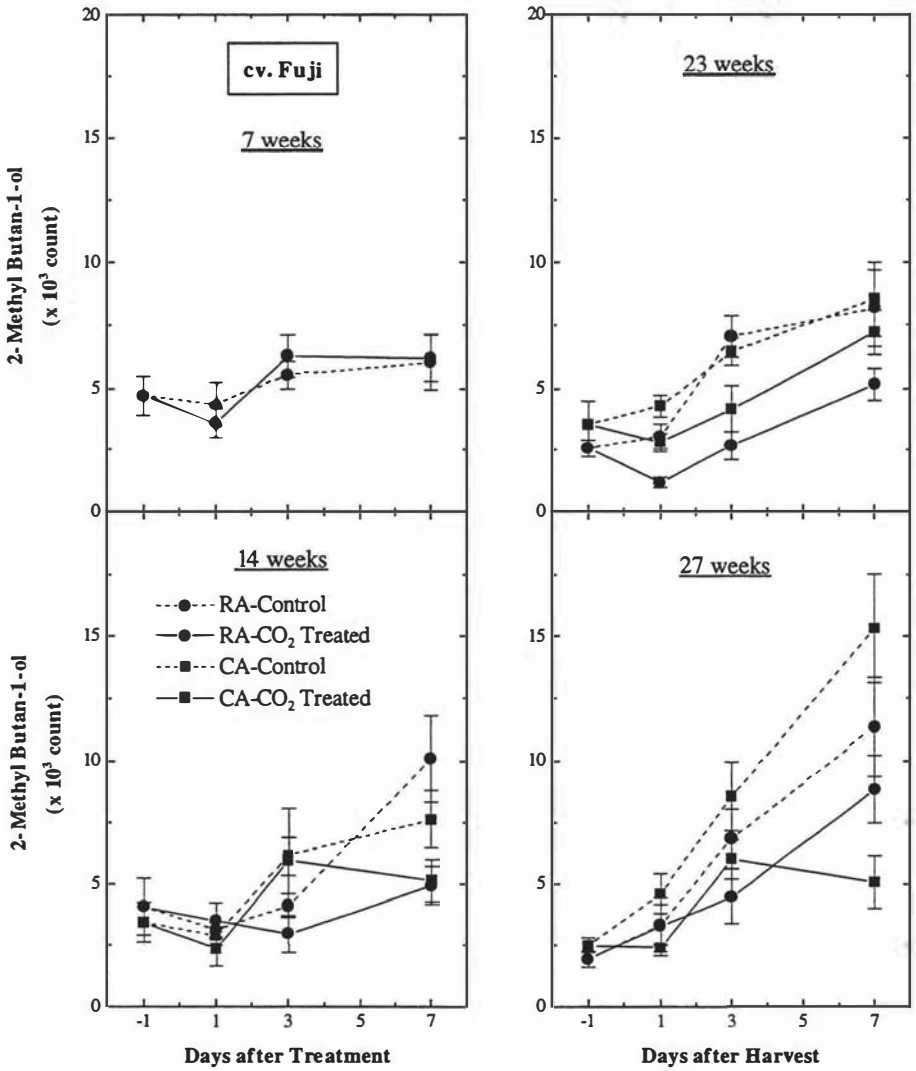


Fig. 5.39 2-Methyl butan-1-ol concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

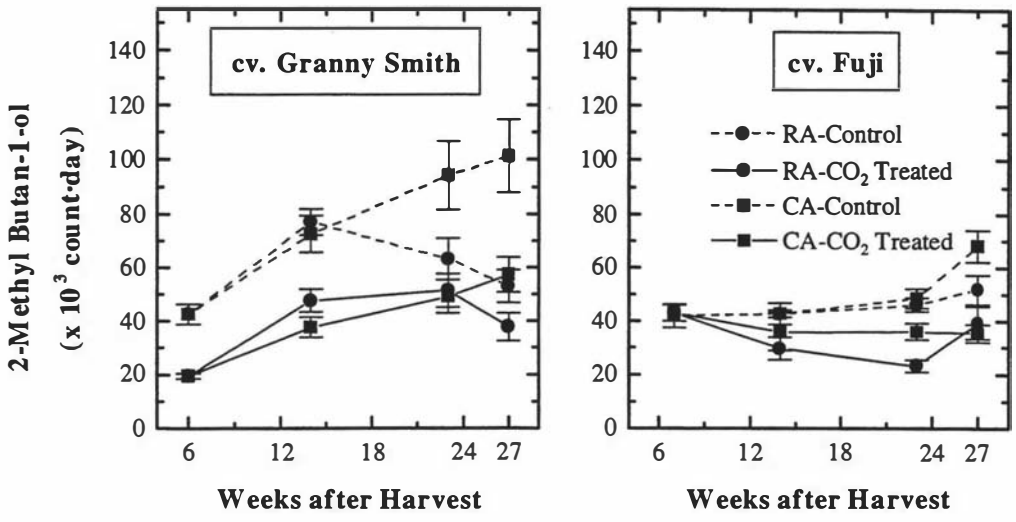


Fig. 5.40 Total concentration of 2-methyl butan-1-ol during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

Hexanal concentrations from both RA and CA stored 'Granny Smith' apples generally increased during post storage for 7 days at 20°C throughout a period of 27 weeks storage (**Fig. 5.41**). Treatment with 100% CO_2 reduced hexanal in RA stored apples at day 1 but the concentration became similar to or higher than in control at day 7; however the treatment did not affect the concentration at 27 weeks after harvest. Hexanal in both control and CO_2 treated CA stored 'Granny Smith' apples during post storage was similar, except at 14 weeks after harvest, in which the concentration in treated fruit was lower than in control (**Fig. 5.41**).

Hexanal concentration in 'Fuji' apples generally increased during post storage at 20°C (**Fig. 5.42**). Treatment with CO_2 decreased hexanal in RA stored apples only at day 1 of the initial sampling (7 weeks after harvest), while it reduced the concentration in CA stored fruit after day 3 at all other times of removal from cool store (**Fig. 5.42**).

Hexanal concentration in RA and CA stored fruit of 'Granny Smith' apples was comparable, except at 23 weeks after harvest where it was higher in RA stored fruit (**Fig. 5.41**). In 'Fuji apples the concentration in RA stored fruit was slightly higher than

in CA stored apples, both in control and CO_2 treated, throughout a period of 27 weeks after harvest (**Fig. 5.42**).

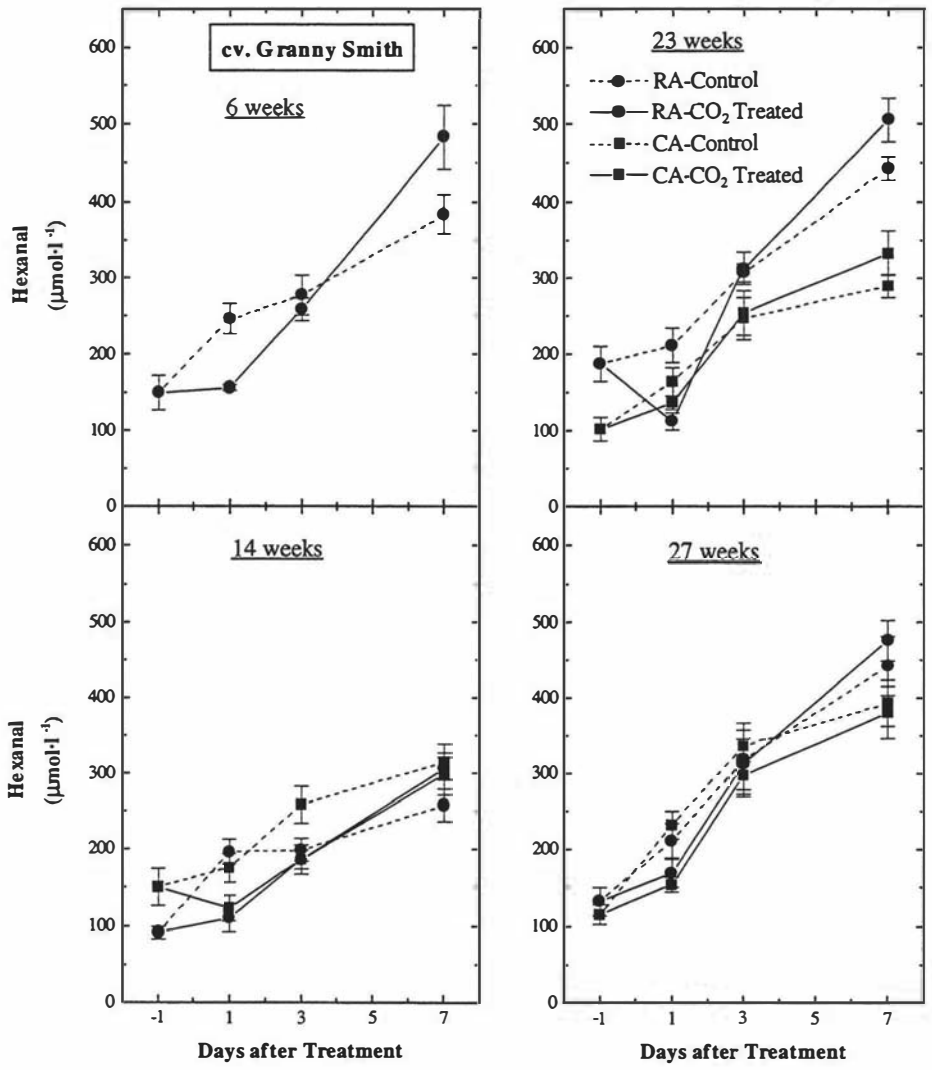


Fig. 5.41 Hexanal concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

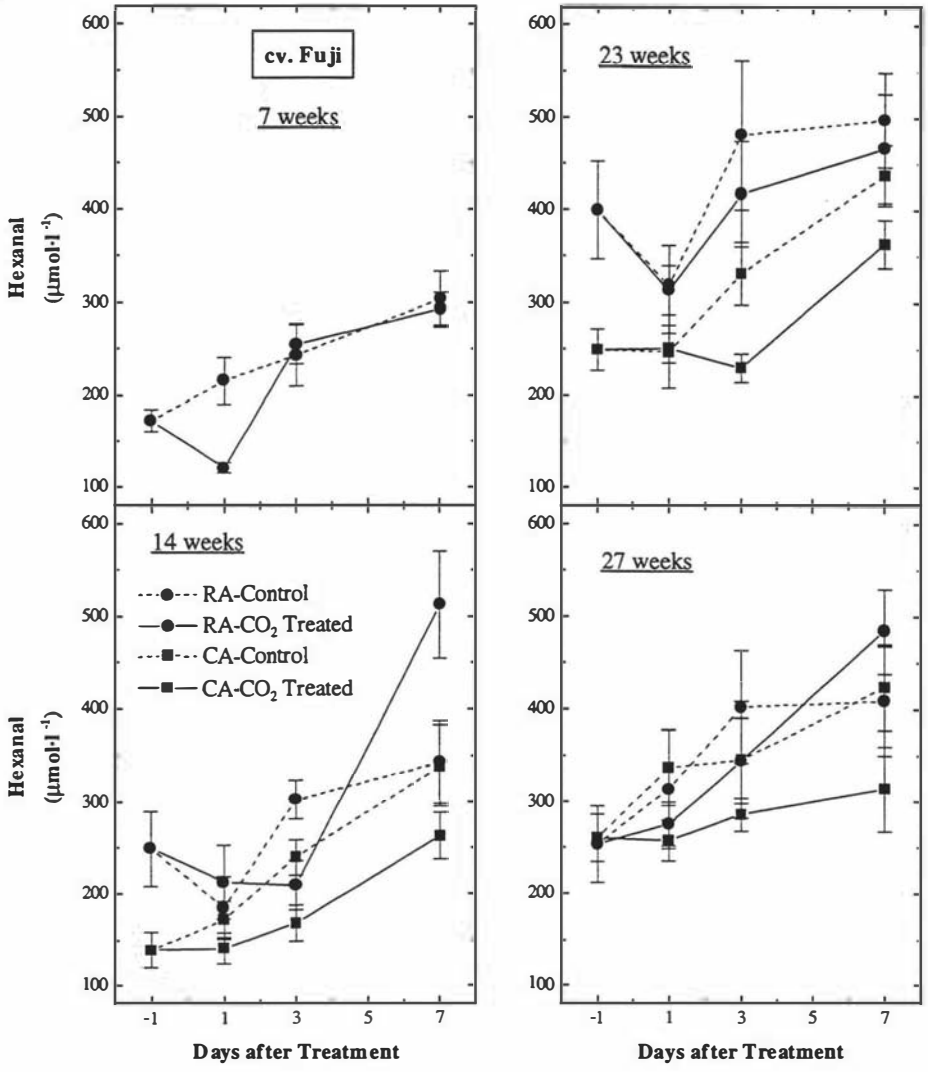


Fig. 5.42 Hexanal concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

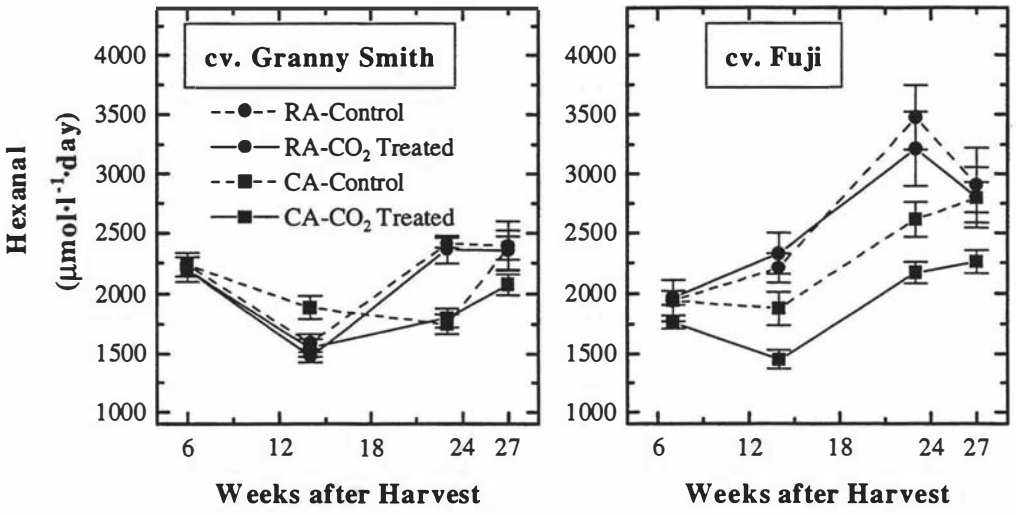


Fig. 5.43 Total hexanal concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

Total concentration of hexanal (as area under curve) in control and CO_2 treated 'Granny Smith' apples was similar throughout 27 weeks after harvest. Total concentration decreased at 14 weeks and then increased again to a level similar to that at 6 weeks after harvest (**Fig. 5.43**). Hexanal in control CA stored apples decreased gradually through 23 weeks and then increased at 27 weeks, while that in CO_2 treated CA stored apples decreased at 14 weeks and increased moderately thereafter. The total concentration of hexanal in CO_2 treated CA stored 'Granny Smith' was slightly lower than in control apples, particularly at 14 and 27 weeks after harvest.

In 'Fuji' apples (**Fig. 5.43**) total concentration of hexanal in control and CO_2 treated RA stored apples was comparable, increasing to peak at 23 weeks after harvest and decreasing slightly thereafter. Hexanal in control CA stored fruit was approximately constant initially to about 14 weeks and then it increased gradually towards the end of 27 week period. Treatment with CO_2 slightly depressed hexanal in CA stored fruit and the degree of depression was relatively constant throughout storage (**Fig. 5.43**).

The total concentration of hexanal in 'Fuji was greater than in 'Granny Smith' apples, particularly during 23 - 27 weeks after harvest, but hexanal in CO_2 treated CA stored fruit were comparable between the two cultivars (**Fig. 5.43**).

Concentration of *trans*-2-hexenal in 'Granny Smith' apples (**Fig. 5.44**) generally increased during post storage for 7 days at 20°C. Treatment with CO_2 decreased *trans*-2-hexenal, particularly from day 1 to day 3 in both RA and CA stored fruit. The effect of CO_2 treatment in depressing *trans*-2-hexenal became less pronounced as time after harvest progressed, except at 27 weeks after harvest where its concentration in control CA stored 'Granny Smith' apples had increased markedly (**Fig. 5.44**).

In 'Fuji' apples (**Fig. 5.45**) *trans*-2-hexenal concentration in RA stored apples was initially high, decreasing by day 1 to 3 remaining approximately constant or slightly increasing thereafter. However, as length of time in storage increased the concentration tended to increase steadily during post storage at 20°C. Concentration of *trans*-2-hexenal in CA stored apples generally increased during post storage at 20°C. Treatment with CO_2 depressed *trans*-2-hexenal in CA stored 'Fuji' apples, but not in RA stored fruit (**Fig. 5.45**).

Total concentration of *trans*-2-hexenal (as area under curve) was similar in both RA and CA stored 'Granny Smith' apples decreasing steadily with time after harvest, except for a slight increase in control CA stored apples at 27 weeks (**Fig. 5.46**). Treatment with CO_2 after harvest depressed *trans*-2-hexenal in both RA and CA stored 'Granny Smith' apples by about 20% compared with control but this depression became less as storage time increased.

In 'Fuji' apples, total concentration of *trans*-2-hexenal in control fruit of both RA and CA stored was comparable throughout 27 weeks after harvest. The total concentration in RA stored apples was not affected by 100% CO_2 treatment, but in CA stored fruit, this treatment depressed *trans*-2-hexenal throughout 27 weeks after harvest (**Fig. 5.46**).

Total concentration of *trans*-2-hexenal was not depressed by CA storage condition during a period of 27 weeks after harvest, either in control 'Granny Smith' or 'Fuji' apples.

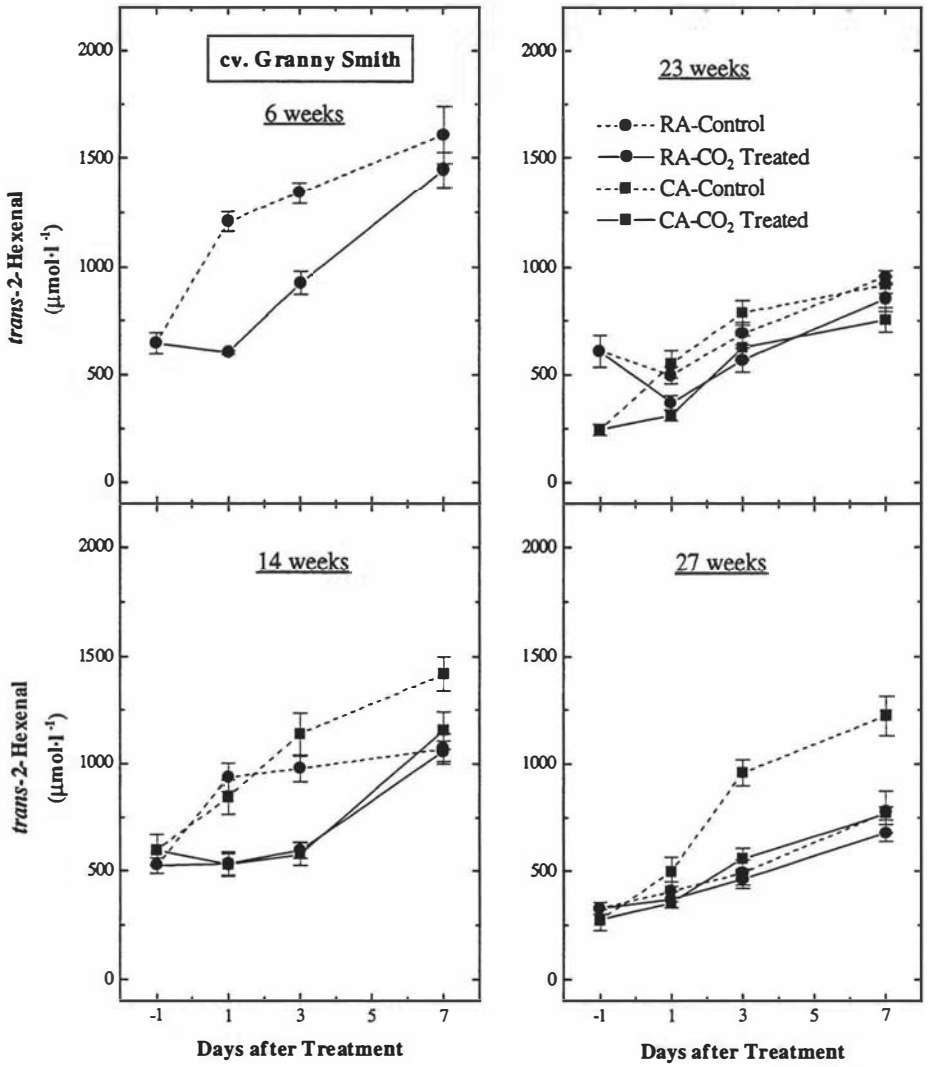


Fig. 5.44 *trans*-2-Hexenal concentrations in juice of 'Granny Smith' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

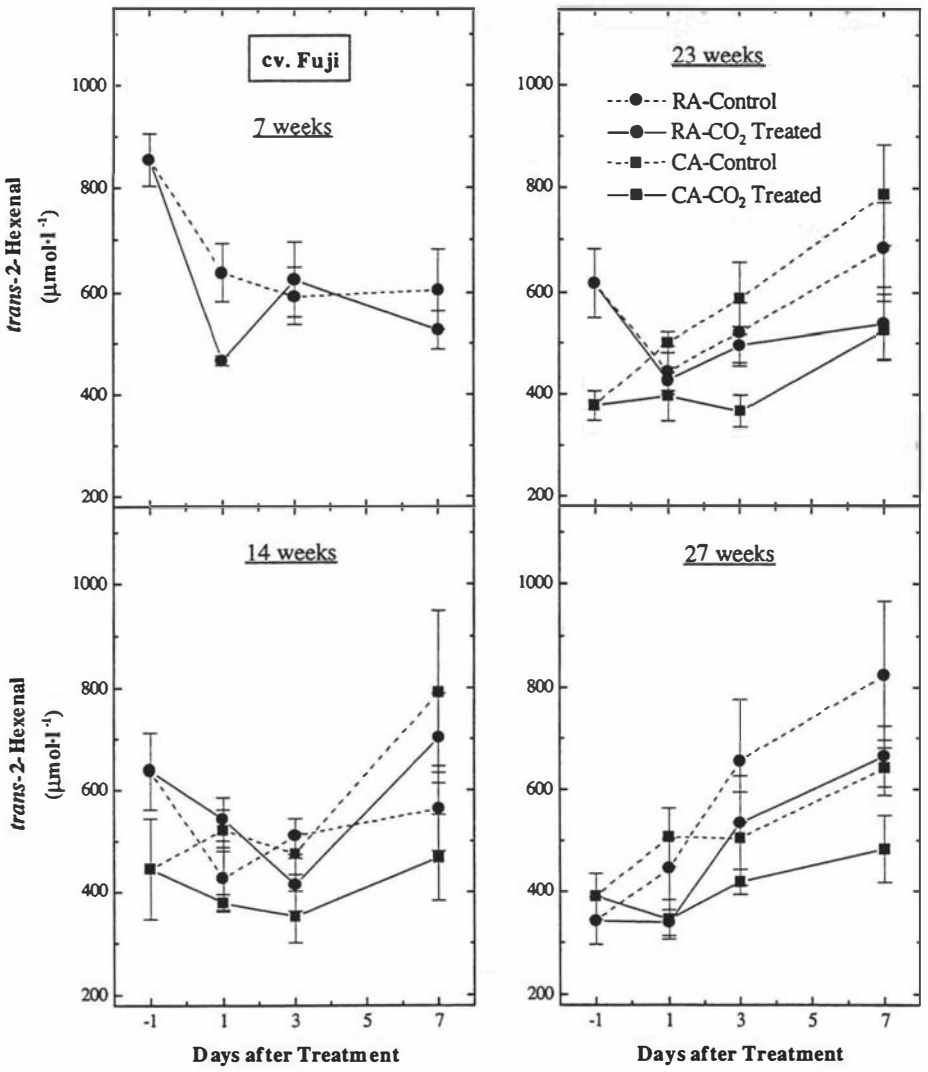


Fig. 5.45 *trans*-2-Hexenal concentrations in juice of 'Fuji' apples removed from RA and CA storage at various times after harvest, following treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

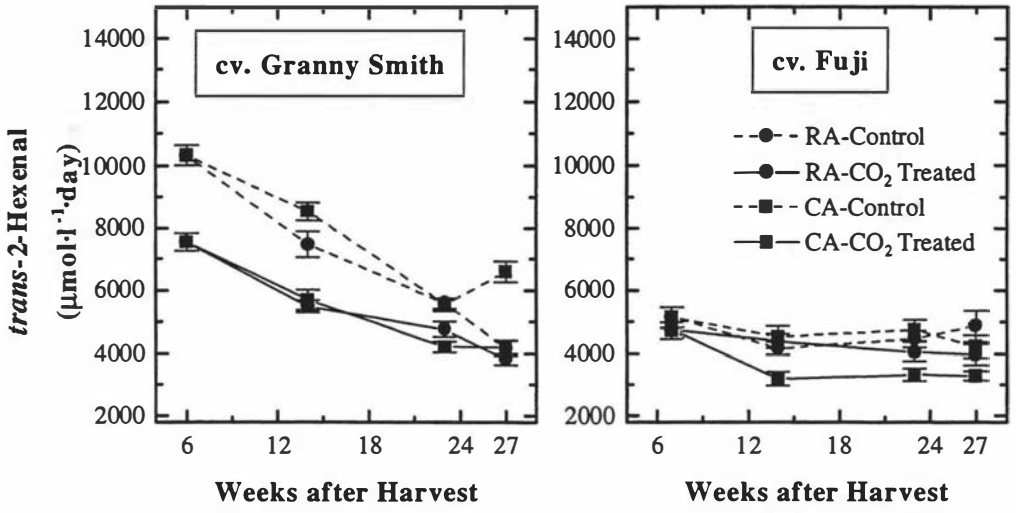


Fig. 5.46 Total *trans*-2-hexenal concentration during post storage for 7 days at 20°C (as area under curve) from juice of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

This study indicates that not all volatile compounds were depressed by CA storage. In fact, CA storage enhanced 2-methyl butan-1-ol production moderately in 'Granny Smith' and slightly in 'Fuji' apples (**Table 5.3**). Production of hexanal in 'Granny Smith' and *trans*-2-hexenal in both apple cultivars were not affected by CA. However, most straight chain alcohols and esters were either slightly or moderately depressed by CA storage. The residual suppressive effect of CA on volatile compounds was more pronounced in 'Fuji' than those in 'Granny Smith' apples (**Table 5.3**).

Treatment with 100% CO_2 increased production of most headspace volatile compounds, ethyl esters and ethanol of both apple cultivars, and propan-1-ol of CA stored fruit of 'Granny Smith' apples (**Table 5.4**). The treatment had a marginal effect on juice butan-1-ol and hexan-1-ol production in CA stored 'Granny Smith' apples. Treatment did not affect production of hexanal and *trans*-2-hexenal in RA stored or propan-1-ol and butan-1-ol in either RA or CA stored 'Fuji' apples. Production of butyl and hexyl acetate in 'Fuji' apples, and 2-methyl butan-1-ol in both cultivars were

depressed by 100% CO_2 treatment. The treatment severely depressed *trans*-2-hexenal production in CA stored fruit of both apple cultivars (**Table 5.4**).

Table 5.3 Summary of the residual effect of CA storage, relative to RA storage, on production of post storage volatile compounds in control fruit of 'Granny Smith' and 'Fuji' apples during a period of 27 weeks after harvest.

Volatile Compound	cv. 'Granny Smith'	cv. 'Fuji'
Headspace Volatiles		
acetaldehyde	–	– –
ethanol	–	– –
ethyl acetate	–	– –
Esters		
ethyl acetate	–	– –
ethyl butanoate	–	– –
ethyl 2-methyl butanoate	–	– –
ethyl hexanoate	–	– –
butyl acetate	n.d.	– –
hexyl acetate	n.d.	– – –
Alcohols		
methanol	n.d.	n.d.
ethanol	–	–
propan-1-ol	– – –	– – –
butan-1-ol	–	– – –
hexan-1-ol	– – –	– – –
2-methyl butan-1-ol	+ +	+
Aldehydes		
hexanal	0	–
trans-2-hexenal	0	0

+ + + markedly enhanced, + + moderately enhanced, + slightly enhanced; – – – severely depressed, – – moderately depressed, – slightly depressed; 0 no effect; n.d. = not detected,

The ability of apples to produce volatile components, in terms of volatile production during post storage at 20°C, during a period of 27 weeks after harvest may be increased, decreased, or remain approximately constant; variations will occur depending on compounds being monitored, between cultivars, storage conditions and treatments used (**Table 5.5**). For instance, the ability of both cultivars to produce *trans*-2-hexenal, either in control or CO_2 treated, RA or CA stored fruit, decreased as time after harvest progressed and the degree of reduction was greater in 'Granny Smith' than in 'Fuji' apples. Butan-1-ol production in both control and CO_2 treated RA stored fruit

of both apple cultivars increased with time after harvest, while in CA stored 'Granny Smith' apples it decreased slightly or stayed approximately constant and in CA stored 'Fuji' apples decreased moderately (**Table 5.5**).

Table 5.4 Summary of the effect of 100% CO_2 treatment (24 hours at 20°C) on post storage production (7 - 9 days at 20°C and 70% RH) of volatile compounds in juice of RA and CA stored 'Granny Smith' and 'Fuji' apples.

	cv. 'Granny Smith'		cv. 'Fuji'	
Volatile Compound	RA	CA	RA	CA
Headspace Volatile				
acetaldehyde	+++	+++	+++	+++
ethanol	+++	+++	+++	+++
ethyl acetate	+++	+++	+++	+++
Esters				
ethyl acetate	+++	+++	+++	+++
ethyl butanoate	+++	+++	+++	++
ethyl 2-methyl butanoate	+++	+++	+++	+++
ethyl hexanoate	+++	+++	+++	++
butyl acetate	n.d.	n.d.	−−	−
hexyl acetate	n.d.	n.d.	−−−	−
Alcohols				
methanol	n.d.	n.d.	n.d.	n.d.
ethanol	+++	+++	+++	+++
propan-1-ol	−−	++	0	0
butan-1-ol	−	0	0	0
hexan-1-ol	−	0	−	−
2-methyl butan-1-ol	−−	−−−	−−	−
Aldehydes				
hexanal	0	−	0	−−
trans-2-hexenal	−−	−−−	0	−−−

+++ markedly enhanced, ++ moderately enhanced, + slightly enhanced; −−− severely depressed, −− moderately depressed, − slightly depressed; 0 no effect; n.d. not detected

Another example is provided by ethyl 2-methyl butanoate in which production from control RA stored fruit of both cultivars increased slightly with advancing time after harvest, while that from CA stored 'Granny Smith' was not detected and that from 'Fuji' apples was slightly decreased. Although the 100% CO_2 treatment dramatically enhanced production of ethyl 2-methyl butanoate in both apple cultivars, the production in CO_2 treated RA stored 'Granny Smith' decreased moderately and in contrast, that in 'Fuji' apples increased moderately with time after harvest. Furthermore, the production

in treated CA stored 'Granny Smith' apples stayed approximately constant at a high level, whereas that in 'Fuji' decreased moderately during a period of 27 weeks after harvest (**Table 5.5**).

Table 5.5 Summary of production pattern of volatile compounds during a period of 27 weeks after harvest in 'Granny Smith' and 'Fuji' apples in relation to storage conditions and treatments.

Volatile Compound	cv. 'Granny Smith'				cv. 'Fuji'			
	RA		CA		RA		CA	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
Headspace Volatiles								
acetaldehyde	+	+ + +	n.d.	+ +	+ +	+ + +	0	+
ethanol	+	+ + +	n.d.	–	+	+ +	0	0
ethyl acetate	+	– – –	n.d.	– – –	0	+ +	0	– –
Esters								
ethyl acetate	– –	– – –	– –	– – –	–	+ +	–	–
ethyl butanoate	+	– –	–	– –	+	+ + +	–	– – –
ethyl 2-methyl butanoate	+	– –	n.d.	0	+	+ +	–	– –
ethyl hexanoate	n.d.	– – –	n.d.	– – –	–	+ +	–	– –
butyl acetate	n.d.	n.d.	n.d.	n.d.	+ +	+	– –	– –
hexyl acetate	n.d.	n.d.	n.d.	n.d.	+ +	+	– –	– –
Alcohols								
methanol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ethanol	+	+ +	–	–	– –	+	–	– –
propan-1-ol	0	+	– – –	+	0	0	– –	– –
butan-1-ol	+ + +	+ + +	–	0	+ + +	+ + +	– –	– –
hexan-1-ol	+	–	– –	– –	+ +	+ +	–	– –
2-methyl butan-1-ol	+	+	+ + +	+ +	+	–	+	0
Aldehydes								
hexanal	0	0	0	0	+ +	+ +	+ +	+
trans-2-hexenal	– – –	– – –	– – –	– – –	–	–	– –	– –

RA refrigerated-air, CA controlled-atmosphere, Ctrl control, CO_2 100% CO_2 (24 hours, 20°C), + + + increased markedly, + + increased moderately, + increased slightly, – – – decreased markedly, – – decreased moderately, – decreased slightly, 0 stayed approximately constant, n.d. not detected.

5.4.5 Disorders

The incidence of apple disorders, such as core flush, superficial scald, and CO_2 injury in relation to storage conditions and/or 100% CO_2 treatment was recorded, particularly during the latter period of 27 weeks after harvest.

Core flush occurred in RA stored fruit of both 'Granny Smith' and 'Fuji' apples, but not in CA stored fruit. About 90% of RA stored 'Granny Smith' at 23 and 27 weeks and about 23% of RA stored 'Fuji' apples at 27 weeks after harvest were found to have core flush.

Superficial scald was observed only in CA stored 'Granny Smith' apples at 27 weeks after harvest where 25% of fruit were affected.

Carbon dioxide injury (**Plate 5.1**) was found mainly in the 100% CO_2 treated fruit of RA stored 'Granny Smith' apples from 18 to 27 weeks after harvest. About 38% of the total number of fruit in this treatment during this period sustained slight to moderate CO_2 injury.

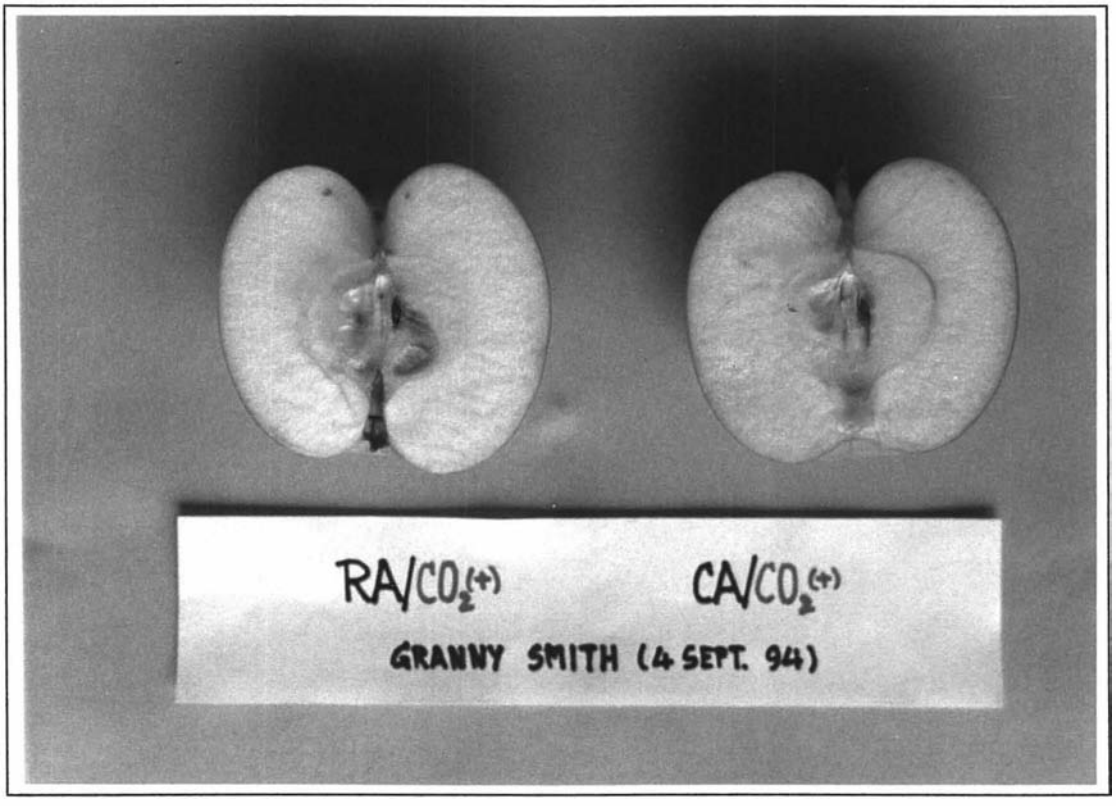


Plate 5.1 Carbon dioxide injury (moderate) of RA stored 'Granny Smith' apples (at 18 weeks after harvest) after treatment with 100% CO_2 for 24 hours at 20°C.

5.5 DISCUSSION

The 'CA residual effect' had been defined as 'the suppression effect which lasts during post storage ripening of the fruit after CA storage and is clearly due to the action of CA' (Yahia *et al.*, 1990a). The residual effect of 2% O_2 + 2% CO_2 CA storage at 0.5°C for a period of 27 weeks after harvest was the delay of ripening during post storage for 9 days at 20°C and 70% RH, manifested as retention of flesh firmness and maintenance of titratable acids in both 'Granny Smith' and 'Fuji' apples in the present study. Such an effect of CA probably involved suppression of ethylene synthesis and action by the fruit and its response to ethylene (Kader, 1980; Knee, 1991a). Knee (1991a) speculated that the low oxygen levels used in CA were directly involved in the retardation of ripening, in which respiration, ethylene synthesis, fruit softening and chlorophyll degradation were regulated by a common oxygen requiring step. However, the mode of action of CA is still not fully understood.

Exposure of fruits to high CO_2 atmosphere, either after harvest (Lurie and Pesis, 1992) or prior to CA storage (Lau, 1983; Meheriuk, 1977), has an added beneficial effect on delaying fruit ripening and softening. The effect of CO_2 treatment was thought to slow down fruit metabolic processes such as respiration and ethylene biosynthesis, thereby delaying ripening and softening of fruit (Blanke, 1991). In this study, a short term treatment with 100% CO_2 (24 hours at 20°C), had only a marginal beneficial effect on flesh firmness in RA and CA stored apples of both cultivars. The CO_2 treated apples were slightly firmer than control fruit removed during 6 - 14 weeks of cool storage, however after 14 weeks of storage such a beneficial effect was not observed.

This residual effect of CA, relative to RA, depressed post storage production of most of the 17 volatile compounds from control fruit of both 'Granny Smith' and 'Fuji' apples evaluated in this study. Exceptions were *trans*-2-hexenal from both cultivars and hexanal from 'Granny Smith' apples which were unaffected by CA conditions. As the biosynthetic pathway of these secondary volatile compounds is different from the other esters and alcohols (Hatanaka, 1996; Schreier, 1984; 1986), this issue will be discussed separately. In contrast, production of 2-methyl butan-1-ol, a branched chain alcohol, was

enhanced by CA conditions in both cultivars, where the degree of enhancement was greater in 'Granny Smith' than 'Fuji' apples (**Table 5.3**).

It is generally accepted that most straight chain aroma volatile compounds are derived from metabolism of free fatty acids, especially linoleic and linolenic acids (Schreier, 1984; Tressl *et al.*, 1975). Alcohols are derived from oxidation of long chain fatty acids, thought to proceed via an intermediate reduction of the acids into the corresponding aldehydes, through a number of β-oxidation cycles (Bartley *et al.*, 1985). Alcohols with even numbered carbon atoms, such as butan-1-ol, and with odd numbered carbon atoms, such as propan-1-ol, are thought to originate from fatty acids containing even and odd carbon numbers, respectively (Paillard, 1990). Branched chain volatile compounds are believed to be derived from metabolism of amino acids, such as L-leucine, L-isoleucine and L-valine, via transamination and subsequent oxidative decarboxylation of the acids into methyl branched aliphatic aldehydes, alcohols and acids (Drawert, 1975; Schreier, 1984; 1986; Tressl *et al.*, 1975). Biosynthesis of esters from the respective acids (probably via acyl CoA) and alcohol moieties is believed to be catalysed by alcohol acyltransferase (AAT) - the only known ester forming enzyme (Eriksson, 1979; Fellman and Mattheis, 1995; Oliás *et al.*, 1995).

The depressive effect of low O_2 (< 3%) CA on volatile compounds in apples was reported to exert its effect mainly on straight chain compounds (Harb *et al.*, 1994; Mattheis *et al.*, 1995). However, high CO_2 (> 3%) CA conditions were reported to suppress both straight and branched chain volatile compounds (Brackmann *et al.*, 1993). Several reasons for such phenomena have been suggested, including loss of substrates and/or enzymes after long term storage (Fellman *et al.*, 1993b; Patterson *et al.*, 1974; Yahia *et al.*, 1990a), lack of alcohol production (Knee and Hatfield, 1981; Hansen *et al.*, 1992b) and inhibition of respiration and ethylene biosynthesis and/or action (Bangerth and Streif, 1987; Song and Bangerth, 1996; Streif and Bangerth, 1988). Furthermore, CA conditions affected carboxylic acid metabolism as well as alcohol dehydrogenase activity, especially under high CO_2 conditions (De Pooter *et al.*, 1987). CA interference with degradation and/or biosynthesis of lipids and reduction of amino acid metabolism was also proposed to be responsible for reduction in aroma volatiles in apples

(Brackmann *et al.*, 1993; Harb *et al.*, 1994). Nevertheless, the reason for the depression of aroma volatile production after CA storage remains unclear.

The enhancement of a branched chain alcohol, 2-methyl butan-1-ol, during post storage ripening in CA stored 'Granny Smith' and 'Fuji' apples was interesting. Both 2-methyl butan-1-ol and its acetate esters are important contributors to the aroma and flavour of 'Royal Gala' apples (Young *et al.*, 1996). This branched chain volatile is believed to be derived from L-isoleucine (Tressl *et al.*, 1975). Hansen and Poll (1993) reported that infiltration of L-isoleucine solution into core tissue of intact 'Golden Delicious' apples stimulated production of 2/3-methyl butyl branched chain volatile alcohols and esters, in which 2/3-methyl butanol was the most prevalent alcohol formed. In the present study, 2-methyl butan-1-ol production increased with time after harvest, especially in CA stored 'Granny Smith' apples. It is possible that the CA condition of 2% O_2 + 2% CO_2 favours synthesis of amino acids, presumably L-isoleucine, and/or conversion of the acid into substrates for volatile synthesis, thereby increasing production of 2-methyl butan-1-ol. This may also explain the increase in ethyl 2-methyl butanoate production in 'McIntosh' and 'Cortland' apples that occurred during ripening after CA storage (Yahia, 1989).

Apple cultivars have been classified into two types, the 'ester-type' and the 'alcohol-type' according to their volatile composition (Dirinck *et al.*, 1989). Ester type cultivars produce mainly straight chain esters, primarily derived from fatty acids, while 'alcohol-type' cultivars produce mainly alcohols, either straight chain or branched chain, with the latter predominating (Brackmann *et al.*, 1993; Dirinck *et al.*, 1989). Butyl acetate and hexyl acetate are believed to be derived from esterification of the alcohols, butan-1-ol and hexan-1-ol, with acetyl CoA respectively (Bartley *et al.*, 1985; Knee and Hatfield, 1981). Appreciable concentrations of butan-1-ol and hexan-1-ol were found in both fruit cultivars with hexan-1-ol in 'Granny Smith' being much greater than in 'Fuji'. Production of large amounts of ethyl acetate in CO_2 treated 'Granny Smith' apples indicated that acetyl CoA was available for acetate ester synthesis. Thus, the lack of butyl acetate and hexyl acetate production in 'Granny Smith' apples is unlikely to be due to lack of substrates for esterification, unless there is a degree of specificity of ester forming enzyme(s), or perhaps different isoforms of the enzymes exist. By measuring the

in vitro activity of partially purified alcohol acyltransferase (ATT) extracted from strawberries or banana, Oliás *et al.* (1995) found that their AAT had preferences for acetyl CoA and hexan-1-ol or acetyl CoA and butan-1-ol for esterification, respectively. Such a study has indicated that ester forming enzymes from different fruit vary in their substrate specificity for esterification. However, it is not known whether or not different types or isoforms of AAT are present in fruit. Thus, it seems likely that the lack of some volatile compounds such as butyl acetate and hexyl acetate, or the presence of high amounts of 2-methyl butan-1-ol in 'Granny Smith' as compared with 'Fuji' apples could be due to cultivar characteristics (Paillard, 1990), perhaps indicating that different inherited properties of enzyme system may be involved. Differences between cultivars may reflect differences in enzyme or substrate specificity.

To examine the specific effect of CA storage conditions on selected volatile compounds such as headspace acetaldehyde, ethanol, ethyl butanoate and ethyl 2-methyl butanoate from control fruit of both cultivars, the area under the curve following each removal from cool store over 7 - 9 days was re-plotted without the CO_2 treatments. This allows direct observation of the timing and magnitude of the effect of CA on production of these volatiles.

Headspace acetaldehyde, headspace ethanol (**Fig. 5.47**), ethyl butanoate and ethyl 2-methyl butanoate (**Fig. 5.48**) from control RA stored of both 'Granny Smith' and 'Fuji' apples increased after 14 weeks storage. In general, the longer the time they had been in cool store (0.5°C), the greater the amounts produced during post storage at 20°C. Increases in total concentrations of headspace acetaldehyde, ethanol, ethyl butanoate and ethyl 2-methyl butanoate in RA stored apples during post storage at 20°C was quite pronounced at 27 weeks after harvest (**Fig. 5.47** and **Fig. 5.48**). The magnitude of the increase in headspace ethanol was relatively higher in 'Granny Smith' than in 'Fuji' apples, while the increases in headspace acetaldehyde and ethyl 2-methyl butanoate were relatively greater in 'Fuji' than in 'Granny Smith' apples. However, the increase in ethyl butanoate from RA stored apples after 27 weeks storage was similar for both cultivars. It has been suggested that 'Granny Smith' apples belong to 'alcohol-type' cultivars (alcohols predominating), while 'Fuji' belongs to the 'ester-type' cultivars where esters predominate in its volatile profile (Dirinck *et al.*, 1989; Paillard, 1990). The

high concentration of ethanol and low concentration of ethyl 2-methyl butanoate in 'Granny Smith' apples, relative to 'Fuji', must reflect differences in metabolic processes between the two cultivars. On the other hand, these compounds from both apple cultivars were moderately or markedly depressed by CA conditions as time in storage increased. The residual suppressive effect of CA on some volatiles such as acetaldehyde and ethanol occurred as early as 10 weeks storage. It is also possible that depression of these volatiles may have occurred even earlier, however CA stored fruit samples were unavailable for analysis during the early storage period. Most studies reported that a decrease in volatile production of apples occurred after 3 - 6 months CA storage (Fellman and Mattheis, 1995; Fellman *et al.*, 1993a; 1993b; Hansen *et al.*, 1990; Streif and Bangerth, 1988; Willaert *et al.*, 1983). As apples were generally taken from CA stores after 3 or 5 months storage for determination of volatiles, these studies may not have determined the time at which the residual depressive effect of CA on volatiles first occurred in fruit.

Previous results (**Chapter Four**) indicated that untreated 'Red Delicious' apples which had been stored for more than 6 months, produced very high concentrations of ethyl esters during post storage at 20°C where the amounts reached levels comparable to those in freshly harvested fruit exposed to hypoxic conditions. However, such an effect was not observed in 'Granny Smith' and 'Fuji' cultivars, even in fruit that had been in RA storage for 6 months. 'Red Delicious' are 'ester-type' apples (Dirinck and Schamp, 1989) and are one of the highest volatile producing cultivars, quantitatively and qualitatively (Brackmann and Streif, 1994; Kollmannsberger and Berger, 1992). The high volatile production characteristic of 'Red Delicious' apples suggests that it is rich in precursors and/or substrates available for volatile synthesis, as well as having high activity of the enzymes involved (Knee and Hatfield, 1981; Paillard, 1990). On the other hand, 'Granny Smith' and 'Fuji' apples were found to be quantitatively low in volatile production among several commercial cultivars (Brackmann and Streif, 1994; Kakiuchi *et al.*, 1986); it is possible that a relatively low availability of precursors and/or capacity of enzymes results in the relatively low volatile production. The enhanced production of ethyl volatiles induced by CO_2 treatment in these cultivars probably occurred because the treatment induced production of appropriate precursors, ie. ethanol.

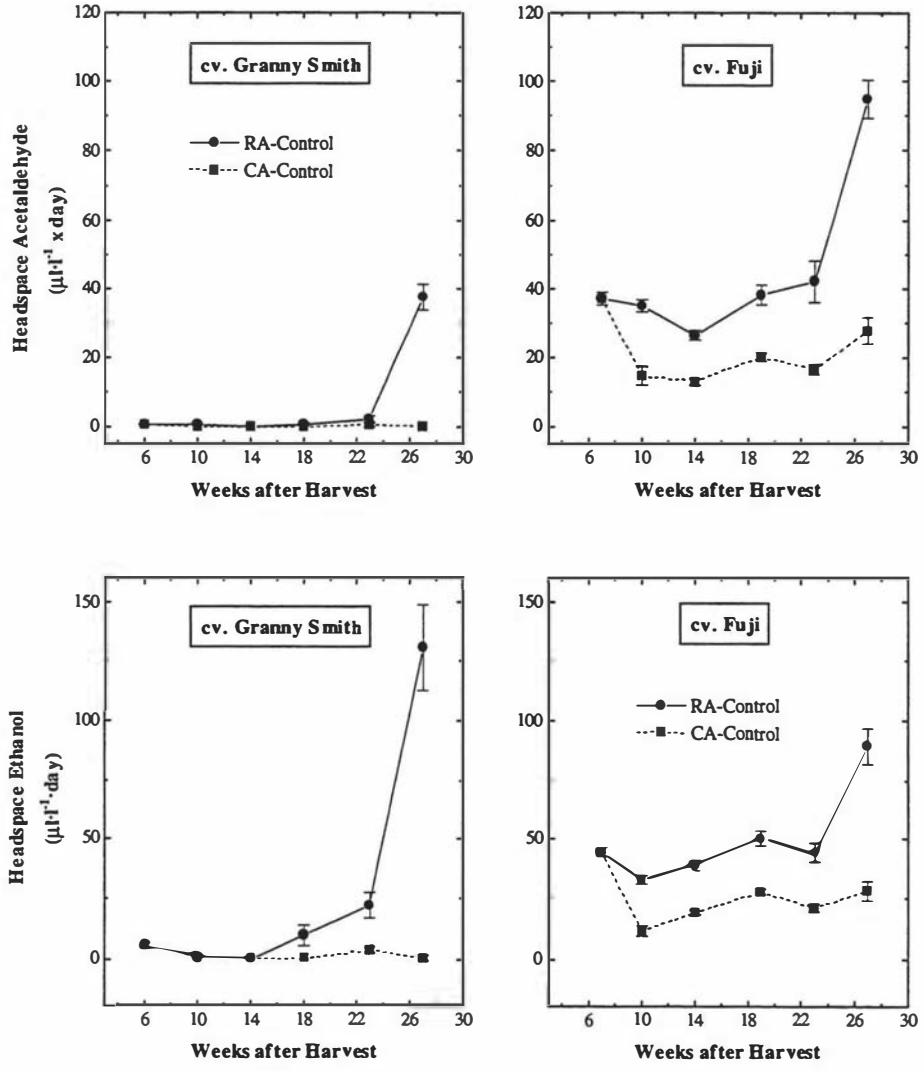


Fig. 5.47 Total concentration during post storage for 9 days at 20°C (as area under curve) of headspace acetaldehyde and ethanol from juice of control fruit of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air for 24 hours at 20°C.

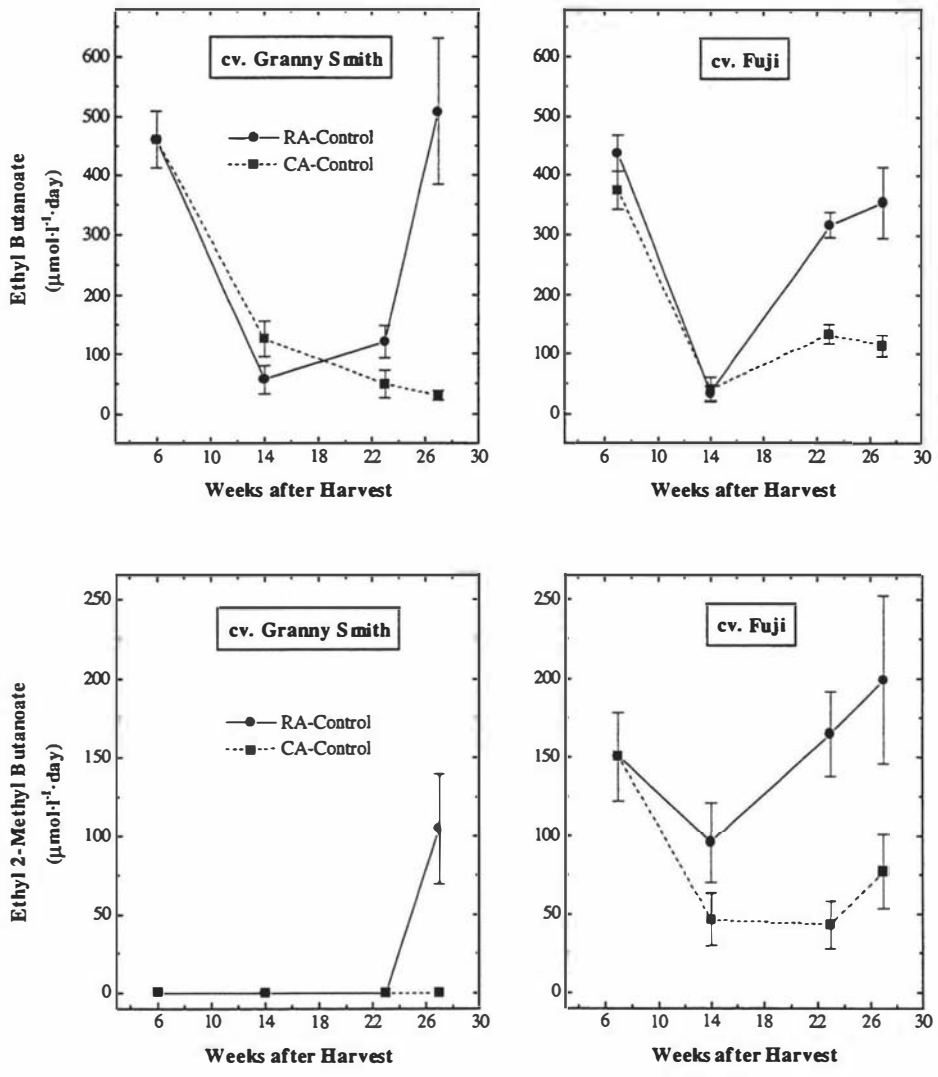


Fig. 5.48 Total concentration during post storage for 7 days at 20°C (as area under curve) of ethyl butanoate and ethyl 2-methyl butanoate from juice of control fruit of 'Granny Smith and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air for 24 hours at 20°C.

In this study headspace acetaldehyde, ethanol and some ethyl esters, such as ethyl acetate and ethyl 2-methyl butanoate, tended to increase as time after harvest progressed in control fruit of RA stored apples, particularly 'Fuji'. Increase in acetaldehyde and

ethanol could, in part, be associated with natural senescence (Jane and Frenkel, 1978; Nursten, 1970). Removal of fruit from cold storage to air at 20°C (Bender *et al.*, 1994; Blanpied and Jozwaik, 1993; Eaks, 1980) and a decrease in fruit skin permeance of apples with time in storage (Dadzie, 1992) could lead to partial anaerobiosis in fruit tissue, resulting in production of acetaldehyde and ethanol, as previously discussed (**Chapter Four**). Such phenomenon may indirectly contribute to the increased production of these volatiles in control RA stored apples as time after harvest progressed. The ethanol produced as a result of anaerobiosis could be metabolised further into ethyl esters, including ethyl acetate, ethyl butanoate and ethyl 2-methyl butanoate (Berger and Drawert, 1984).

It was noted that production of ethanol (**Fig. 5.28**) and some ethyl esters such as ethyl acetate (**Fig. 5.18**) and ethyl butanoate (**Fig. 5.19**) at 6 or 7 weeks after harvest (initial sampling) in control fruit of both cultivars was appreciably high. This particular lot of apples had been transported from Hawkes Bay to Palmerston North by non-refrigerated truck; for unknown reasons it took 4 days for them to be delivered and it is unlikely that they had been in a cool store in the interim. On arrival at the laboratory they were placed at 0°C for 24 hours prior to removing to 20°C for conducting experiments. This repeated cooling and warming of the apples could possibly have contributed to the high production of these volatiles noted above. Temperature-induced phase transition in cellular membranes, caused by rewarming fruit tissue, could lead to metabolic aberrations resulting in accumulation of metabolites, including ethanol and acetaldehyde (Eaks, 1980; Marangoni *et al.*, 1996) which act as precursors for these volatiles.

Post storage treatment with 100% CO_2 for 24 hours at 20°C dramatically stimulated production of juice headspace volatiles, ethanol and ethyl esters in both RA and CA stored fruit of 'Granny Smith' and 'Fuji' apples (**Table 5.4**). The large amount of ethanol produced in response to a hypoxic atmosphere of CO_2 would undoubtedly allow production of substantial amounts of several ethyl esters. In addition, this large amount of ethanol produced was sufficient to displace other alcohols in reactions with ester forming enzyme(s). This was evident in the reduced production of butyl and hexyl acetate, suggesting the competitive nature of acyl esterification (Berger and Drawert, 1984; Knee and Hatfield, 1981). The treatment decreased hexan-1-ol, 2-methyl butanol

and *trans*-2-hexenal in fruit of both cultivars, as well as some alcohols in RA stored 'Granny Smith' apples. This phenomenon has been observed previously in other cultivars, such as 'Red Delicious' and 'Braeburn' (**Chapter Four**).

Production of volatile ethyl esters in 100% CO_2 treated apples, in both RA and CA stored 'Granny Smith' apples decreased with time after harvest, except for ethyl 2-methyl butanoate (**Fig. 5.20**) from CA stored fruit which stayed approximately constant with a slight decrease at 27 weeks. Post storage production of ethyl esters in treated RA stored 'Fuji' apples generally increased with time after harvest, whereas they generally decreased in CA stored fruit (**Table 5.5**). As mention earlier, esters are synthesised enzymatically by coupling the respective alcohol and acid (or acyl CoA) moieties (Salunkhe and Do, 1976). Thus, the main factors determining ester production are (a) availability of the alcohols and acids (or acyl CoAs) substrates, and (b) the inherited properties (substrate specificity) of the ester forming enzyme - the alcohol acyltransferase (AAT) (Oliás *et al.*, 1995). Knee and Hatfield (1981) showed that CA stored apples were able to produce esters from externally supplied alcohols, indicating that the requisite enzymes and co-factors were present, thus impaired aroma from apples after long-term CA storage was due probably to lack of alcohols. In the present study, one alcohol, ethanol, was not limiting for ethyl ester synthesis in CO_2 treated fruit of CA stored apples, and the decrease in ethanol production with time after harvest was not as pronounced as that of ethyl esters. Thus, it is likely that the decrease in ethyl ester production in CO_2 treated fruit of RA and CA stored 'Granny Smith', and in CA stored 'Fuji' apples is directly associated with the shortage of acids or acyl CoAs or probably due to lack of appropriate enzymes, isoenzymes and/or co-factors required for esterification to proceed.

The acids and/or acyl CoAs needed for ester synthesis are believed to be derived from oxidation of long chain fatty acids through a number of β-oxidation cycles which generate short chain acyl CoAs and acetyl CoA with each cycle of the β-oxidation (Bartley *et al.*, 1985). Short chain acyl CoAs generated are either enzymatically incorporated with alcohols producing esters or reduced by acyl CoA reductase into aldehydes, which are reduced in turn to alcohols (Paillard, 1990). Aldehydes could be oxidised into the corresponding acids (De Pooter *et al.*, 1983) and acids could be further

converted into their acyl CoA derivatives in the presence of ATP and/or other co-factors, such as inorganic ions (Gilliver and Nursten, 1976). It had previously been indicated that acyl CoAs were not limiting for ester synthesis in long-term CA stored apples (Knee and Hatfield, 1981). However, it is possible that CA conditions may interfere with normal metabolic processes for liberating acids from their precursors, probably on the enzymes involved, resulting in a reduced production of acids or acyl CoAs for esterification.

The enhanced important ethyl esters, ethyl butanoate (**Fig. 5.19**) and ethyl hexanoate (**Fig. 5.21**) in CA stored apples of both cultivars following CO_2 treatment decreased continuously with time of storage. The initial total concentrations (as area under the curve) of ethyl butanoate and ethyl hexanoate were 2353 and 449 $\mu mol \cdot l^{-1} \cdot day$ for 'Granny Smith' or 1105 and 244 $\mu mol \cdot l^{-1} \cdot day$ for 'Fuji' apples, respectively. However, their respective amounts at 23 weeks storage were 1416 and 84 $\mu mol \cdot l^{-1} \cdot day$, or 84 and 29 $\mu mol \cdot l^{-1} \cdot day$. Such a decline in response may continue and could reach a point at which hypoxic treatments no longer enhanced these esters. It seems that enhancement of some important ethyl esters in CA stored apples by CO_2 treatment can be achieved from fruit at harvest and those in cool store for 27 weeks; beyond this time such a treatment may have negligible or no effect.

The continued decrease in total concentrations of ethyl esters with time after harvest, could be related to reduced fatty acid metabolism, or to the availability of free fatty acid precursors (Bangerth, 1996, personal communication). It has been suggested previously that the reduced aroma volatile compounds in long-term CA stored apples was associated with reduced biosynthesis and/or degradation of free fatty acids (Brackmann *et al.*, 1993; Harb *et al.*, 1994). Harb *et al.* (1994) found that $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ fatty acids of 'Golden Delicious' apples were reduced by more than 50% after 8 months CA storage, compared with freshly harvested fruit. In RA stored 'Granny Smith' apples, production of ethyl esters in CO_2 treated fruit decreased continuously, while in 'Fuji' apples they generally increased. It is probable that 'Granny Smith' apples have a limited pool of free fatty acid precursors to provide acyl CoAs for esterification. This cultivar was categorised as an 'alcohol-type' cultivar, one of those which produce most of their volatile precursors from amino acids (Brackmann *et al.*, 1993). This is

probably a reason for the decreased production of ethyl esters, and a relatively constant production of ethyl 2-methyl butanoate occurred with time of storage in CO_2 treated 'Granny Smith' apples. 'Fuji' apples were classified as an 'ester-type' cultivar where the precursors for ester synthesis originate mainly from fatty acids (Dirinck *et al.*, 1989); if this is so then it implies that fatty acids were not limiting in this cultivar. In ripening apples, an increased rate of lipid turnover could generate free fatty acids for oxidation, thus resulting in an increased quantity of alcohol and acyl CoA substrates for ester synthesis (Bartley, 1985; Bartley *et al.*, 1985). This could possibly explain the increase in ethyl esters production such as ethyl esters, butyl and hexyl acetate, in RA stored fruit of both control and CO_2 treated 'Fuji' apples.

Concentrations of ethyl esters such as ethyl acetate, ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate (**Fig. 5.10** to **Fig. 5.17**) at day -1 from RA and CA stored apples of both cultivars, for CO_2 treated and control fruit, were negligible or not detected. These esters were observed after 1 day at 20°C and coincided with increased ethanol concentrations, particularly in the CO_2 treatment. Ethanol is supposed to be the alcohol substrate for esterification with acyl CoAs to produce ethyl esters, as previously discussed. The lack of ethyl ester formation at day -1 could be partly due to the lack of ethanol for esterification, although small amounts of ethanol were measured in control RA fruit. It is likely that concentration of ethanol at day -1 was not high enough to compete with other alcohols, such as butan-1-ol and hexan-1-ol, for esterification with acyl CoAs (Berger and Drawert, 1984; Mattheis *et al.*, 1991a), as butyl acetate (**Fig. 5.22**) and hexyl acetate (**Fig. 5.23**) were found in high concentrations. High amounts of butyl and hexyl acetate also suggest that acyl CoAs, such as acetyl CoA, are not limiting for esterification. Other probable reasons for the lack of ethyl ester formation at day -1 could be associated with substrate specificity of the ester forming enzyme(s) (Gilliver and Nursten, 1976; Oliás *et al.*, 1995) and/or the suppressive effect of low temperature and CA on the activity of these enzymes (Fellman *et al.*, 1993a). Ester forming enzymes of 'Red Delicious' and 'Cox's Orange Pippin' have been found to favour esterification of the supplied medium chain alcohols, such as butan-1-ol, pentan-1-ol and hexan-1-ol, relative to methanol and ethanol (Berger and Drawert, 1984; Knee and Hatfield, 1981). On removal of 'Rome' apples from cool stores (after 3 - 6 months), the activity of AAT

from RA fruit was low, and from CA fruit was undetectable at day 0, but the activity was increased after 1 day at 20°C (Fellman *et al.*, 1993a). Thus, the lack of ethyl ester formation at day -1 is probably related to the competitive nature of acyl esterification, and the ester forming enzymes involved, either due to substrate specificity or the suppressive effect of storage conditions on the enzymes.

The important aroma impact compound of apples, ethyl 2-methyl butanoate, is generally undetected in untreated RA and CA 'Granny Smith' apples, except in control RA fruit at 27 weeks storage (**Fig. 5.14**). Treatment with 100% CO_2 markedly enhanced this ester in RA and CA apples in both cultivars (**Fig. 5.14** and **Fig. 5.15**). Such an enhancement of this compound in apples is probably due to CO_2 induced ethanol substrade required for ethyl 2-methyl butanoate formation, as previously discussed. Production of ethyl 2-methyl butanoate in control RA 'Granny Smith' apples was observed at 27 weeks storage (**Fig. 5.14**) and appreciable amounts of ethanol from fruit of the respective treatment was also found during the same period (**Fig. 5.26**). Moreover, CO_2 treatment stimulated a marked increase in ethanol concentrations which was followed by a dramatic increase in the amount of ethyl 2-methyl butanoate. Thus, the effect of CO_2 on enhancing this particular ester in 'Granny Smith' apples is believed to be due to the increased ethanol in response to hypoxia which in turn is metabolised into ethyl 2-methyl butanoate and other ethyl esters. However, synthesis of esters requires specific substrates, enzymes and/or co-factors for the reaction to proceed (Fellman and Mattheis, 1995; Gillivar and Nursten, 1976; Schreier, 1984) and CO_2 treatment enhanced ethyl 2-methyl butanoate which is not normally detected in 'Granny Smith' apples, thus it seems that the effect of hypoxic conditions may not merely stimulate an accumulation of ethanol substrate for esterification. It is probable that CO_2 treatment stimulates and/or induces specific enzymes, isoenzymes and co-factors required for generation of 2-methyl butyl CoA and/or for catalysing the reaction between ethanol and 2-methyl butyl CoA to produce ethyl 2-methyl butanoate. It has been reported that hypoxic treatments caused marked alterations in the profile of proteins, stability of mRNA species and gene expression in fruit (Kanellis *et al.*, 1991). Moreover, hypoxic treatments (0 - 3% O_2) induced the appearance of new protein enzymes or isoenzymes in avocado fruit (Kanellis *et al.*, 1991; 1993). However, it is not known

whether or not specific enzymes or isoenzymes required for ethyl 2-methyl butanoate synthesis in apples are induced following CO_2 treatment in the present study.

Ethyl 2-methyl butanoate is supposed to form from esterification of ethyl alcohol and 2-methyl butyl CoA moieties, with the methyl branched chain acyl CoA group being derived from the metabolism of an amino acid, probably L-isoleucine (Hansen and Poll, 1993; Tressl *et al.*, 1975). Production of ethyl 2-methyl butanoate in CO_2 treated fruit of CA stored 'Granny Smith' apples (**Fig. 5.20**) did not decrease as did other ethyl esters and only showed a slight decrease at 27 weeks after harvest. This indicated that the acyl CoA for esterification of this ester was not deficient. It is likely that esterification of this compound was at maximum throughout storage as production was approximately constant. Production of 2-methyl butan-1-ol in control fruit of CA stored 'Granny Smith' apples also increased with time after harvest (**Fig. 5.40**). This suggests that the appropriate amino acid precursor was available and the metabolic pathway necessary to provide an acyl CoA moiety for esterification was fully operative, thereby production of ethyl 2-methyl butanoate was at its maximum when high amounts of ethanol were present.

In control fruit of RA stored 'Granny Smith' apples, production of 2-methyl butan-1-ol increased to peak at 14 weeks after harvest and decreased thereafter (**Fig. 5.40**). This alcohol is believed to be derived from 2-methyl butyl CoA (from L-isoleucine) (Drawert, 1975) and reduced production of 2-methyl butan-1-ol reflects the decrease in 2-methyl butyl CoA and hence ethyl 2-methyl butanoate. It is also possible that there is a decrease in the amino acid precursor, L-isoleucine, with time after harvest. It has been reported that concentrations of amino acids of apples remained constant in storage and decreased in concentrations during ripening (Ackermann *et al.*, 1992), although details on changes of individual amino acids during long-term CA storage has not been determined.

The ester forming enzyme, alcohol acyltransferase, is thought to be one of the important factors governing ester production in ripening fruit, including apples (Fellman *et al.*, 1993a; Oliás *et al.*, 1995). Bartley *et al.* (1985) found that the enzymes required to catalyse ester synthesis were present in 'Cox's Orange Pippin' apples at all times during 17 weeks of CA storage (2% O_2 + 1% CO_2) as indicated by feeding experiments with

methyl octanoate vapour as a precursor to the fruit. They found that supplying apples stored for 17 weeks in CA with methyl octanoate, induced substantially more production of butyl acetate, hexyl acetate, propyl and butyl butanoate than when given to non treated fruit (Bartley *et al.*, 1985). Supplying vapour of C_2 to C_6 straight chain and branched chain alcohols at ambient temperature to 'Golden Delicious' apples, previously stored in ultra-low oxygen (ULO) CA for 5 months, stimulated ester production in the fruit suggesting that the ester forming enzyme was fully operative (Harb *et al.*, 1994).

It is possible that the decreased production of ethyl 2-methyl butanoate in CO_2 treated fruit of CA stored 'Granny Smith' apples at 27 weeks after harvest was due to a decrease in alcohol acyltransferase activity, although the enzyme is operative, since ethanol and probably 2-methyl butyl CoA moieties necessary for esterification did not decrease during 27 weeks storage. Fellman *et al.* (1993b) found that the activity of alcohol acyl transferase of 'Rome' apples decreased after 3 to 6 months of both RA and CA storage. A decrease in ethyl ester production in CO_2 treated RA stored 'Fuji' apples or in butyl and hexyl acetate in control RA stored 'Fuji' apples could be partly due to a reduced capacity of the AAT as well as a shortage of acyl CoAs, such as acetyl CoA, butyl CoA and hexyl CoA, necessary for esterification.

Another consequence of CA storage, as compared with RA, was the decrease in production of most alcohols, except 2-methyl butan-1-ol (**Table 5.3**). In the present study, most straight chain alcohols, except ethanol, are thought to be derived from fatty acid metabolism. Thus, any decrease in alcohols could probably be associated with the residual effect of CA on the synthesis and/or degradation of fatty acids, as earlier discussed. Post storage treatment with 100% CO_2 was shown to have a variable effect on alcohol production depending on cultivar (**Table 5.4**). For example, following high CO_2 treatment 2-methyl butan-1-ol production in both cultivars decreased, butan-1-ol was marginally affected, but propan-1-ol in CA stored 'Granny Smith' apples was increased (**Table 5.4** and **Fig. 5.31**). Decrease in production of alcohols following treatment with 100% CO_2 was possibly due to competition for acyl CoAs with ester synthesis. Alcohols such as hexan-1-ol and 2-methyl butan-1-ol are derived from acyl CoAs, hexyl CoA and 2-methyl butyl CoA, respectively (Bartley *et al.*, 1985; Tressl *et al.*, 1975). The large quantity of ethanol produced in response to CO_2 treatment may

compete for acyl CoAs for esterification, thus decreasing the amount of acyl CoAs available for conversion into alcohols. If this is so, it would result in reduced production of alcohols such as butan-1-ol, hexan-1-ol and 2-methyl butan-1-ol following the CO_2 treatment. Another possibility is that the aldehydes could be oxidised into the respective acids (De Pooter *et al.*, 1983) and subsequently converted to acyl CoAs (Gilliver and Nursten, 1976) to meet the requirement for ethyl esterification. Competition for alcohol dehydrogenase for catalysing aldehydes into alcohols could occur due to high production of acetaldehyde in CO_2 treated apples. However, this possibility seemed to be less likely because no substantial amount of aldehydes, such as propanal and 2-methyl butanal, were measured from control or CO_2 treated fruit.

Butan-1-ol is generally thought to be derived from even numbered carbon atom fatty acids via β-oxidation (Bartley *et al.*, 1985; Paillard, 1990). Thus, it is most likely to be affected by CA and high CO_2 treatments as discussed above. However, it was observed that butan-1-ol production in both apple cultivars was marginally affected by CO_2 treatment, ie. was slightly reduced in 'Fuji' or was comparable to control in 'Granny Smith' apples (**Fig. 5.34**). Thus, it seemed that butan-1-ol could, perhaps, be derived from a source other than from β-oxidation of fatty acids. Butan-1-ol could be derived from carbohydrate during fermentation through the amino acid biosynthetic pathway into a keto acid intermediate and subsequently transformed into the alcohol (Reazin *et al.*, 1970), and this issue will be discussed shortly. Fellman (1996, personal communication) suggested that butan-1-ol (via butanal) could also be derived from the oxidative reaction of lipoxygenase on fatty acids during maceration of fruit tissue. If this was so, the aldehyde in juice could be quickly reduced into the corresponding alcohol, ie. butan-1-ol, by alcohol dehydrogenase (Eriksson, 1975). Perhaps, some of the butan-1-ol derived from this process could contribute to total butan-1-ol present and thus be reflected in the marginal difference in production between control and CO_2 treated apples. Similarly, if true this mechanism could explain the less pronounced effect of CO_2 treatment on hexan-1-ol production of CA stored 'Granny Smith' apples. However, 'Fuji' apples are an 'ester-type' cultivar in which butan-1-ol and hexan-1-ol are thought to be derived mainly from fatty acid metabolism (Brackmann *et al.*, 1993), and thus production of these alcohols in 'Fuji' are more likely to be affected by CA and/or high CO_2 treatment.

Production of propan-1-ol in CO_2 treated CA stored 'Granny Smith' apples was increased, while the treatment had no effect on propan-1-ol production in either RA or CA stored 'Fuji' apples (**Fig. 5.31**). Increased production of this alcohol following high CO_2 treatment has previously been observed in other cultivars, such as 'Red Delicious' and 'Braeburn' (**Chapter Four**). Propan-1-ol is thought to be derived from the odd numbered carbon atom fatty acids via β-oxidation pathway (Paillard, 1990). If so, the production of this alcohol should be decreased as a result of competition for the acyl CoA moiety, propyl CoA, which is needed for esterification with ethanol to form ethyl propanoate (Knee and Hatfield, 1981).

Increased production of propan-1-ol may be directly involved with fermentative metabolism induced by the CO_2 treatment used. Propan-1-ol, butan-1-ol, 2-methyl butan-1-ol and other higher alcohols (fusel oils) are produced during the main alcoholic fermentation of wine, cider, beer and whisky (Dürr, 1986; Meilgaard and Peppard, 1986; Montedoro and Bertuccioli, 1986; ter Heide, 1986). These alcohols can be derived from carbohydrates via biosynthesis of amino acids (Heath and Reineccius, 1986; Reazin *et al.*, 1970; Webb, 1967). The mechanism by which carbohydrates are transformed into higher alcohols is thought to follow the glycolytic pathway to pyruvic acid. Pyruvic acid is either reduced to ethanol, oxidised to acetate or combined with another molecule to form acetolactic acid. Acetolactic acid is then transformed into a keto acid via the pathway of amino acid biosynthesis (isoleucine, leucine or valine) and the keto acid (such as α-keto butyric acid) is reduced to alcohols such as propan-1-ol (Heath and Reineccius, 1986; Reazin *et al.*, 1970). The conversion of carbohydrates or amino acids into propan-1-ol above may occur in CO_2 treated apples due to this fermentation process.

Propan-1-ol could also be derived from carbohydrate metabolism (Salunkhe and Do, 1976) via oxo-acids through decarboxylation and reduction into the alcohol (Nursten, 1970). In addition, it was suggested that atmospheric CO_2 could be metabolised by dark CO_2 fixation into carboxylic acid and amino acid fractions (Pesis and Ben-Arie, 1986), in which carboxylic acids could be subsequently reduced into aldehydes and alcohols (De Pooter *et al.*, 1983) and amino acids may reduce into aldehydes and alcohols via keto acids intermediates (Drawert, 1975; Reazin *et al.*, 1970).

It had been reported that increased CO_2 concentrations in the CA storage atmosphere markedly increased impact volatile compounds, such as mesifurane, furaneol and 4-decanolide, in strawberries cv. 'Red Gauntlet' (Berger, 1995). However, the author did not indicate the condition and concentration of CO_2 used, but suggested that a pathway, different from the normal metabolic pathway existing for these compounds, may be triggered and become operative. Therefore, it is not known how high concentration of CO_2 treatment increased volatile compounds other than acetaldehyde ethanol and ethyl esters, and further investigation is required to elucidate the mechanism and/or mode of action involved in this phenomenon.

Hexanal and *trans*-2-hexenal are the character impact compounds in apple juice responsible for the grassy, green-apple like aroma; they have odour thresholds in water of 0.005 and 0.017 ppm (v/v), respectively (Flath *et al.*, 1967). These aldehydes are usually produced in very low concentrations in intact apples, but found in large quantities in juice (Berger, 1991; Paillard, 1990) as they are formed enzymatically during disruption of tissues (Hatanaka, 1996; Riley *et al.*, 1996). These compounds are believed to form from linoleic and linolenic acids of disrupted tissues via lipoxygenase activity (Galliard and Matthew, 1977a; 1977b; Hatanaka, 1993; 1996). Changes in the C_6 aldehyde concentration in juice from treated apples after different times of storage could be important to aroma of apple juice (Dürr and Schobinger, 1981). Therefore, concentration or production of these volatiles in juice from apples after different times in storage, storage conditions and hypoxic treatment were examined.

During a period of 27 weeks after harvest, hexanal production in 'Granny Smith' apples was slightly decreased initially but increased again, with production at 6 weeks and 27 weeks after harvest being comparable, while in 'Fuji' apples production generally increased (**Fig. 5.43**). These results suggest that the lipoxygenase enzyme system was fully functional in both apple cultivars. Data also indicated that availability of substrates such as linoleic or linolenic acids in 'Granny Smith' apples remained approximately constant, and in 'Fuji' was increased, with time after harvest. The different production curves between the two cultivars, suggested differences in the metabolic pool of fatty acids and/or lipoxygenases. However, it is not known whether concentrations of these fatty acids vary among cultivars of apples, although synthesis of hexanal and *trans*-2-

hexenal is cultivar dependent (Drawert *et al.*, 1986). Lipoxygenases from different species differ considerably in their pH optima and substrate specificity (Gatfield, 1988). It has been reported there are two types of lipoxygenase: (a) that present in soy bean seeds and tea leaves, oxygenates specifically at C-13 of α-linolenic or linoleic acids, and (b) that in potato tubers which oxygenates at C-9 of these fatty acids (Hatanaka, 1996). Lipoxygenases have been found to occur in most plant tissues including cotyledons, leaves, roots, stems and fruits and they may be in soluble form or as a membrane associated form (Hatanaka, 1993; 1996; Riley *et al.*, 1996). It seems that no comprehensive study has been undertaken of lipoxygenases in apple cultivars, where it is conceivable that different types or isoenzymes exist (Hatanaka, 1996).

Another important secondary volatile component, *trans*-2-hexenal, is believed to be derived from the action of *cis-trans*-isomerases (the double-bond reducing enzymes) on *cis*-3-hexenal from linolenic acid or on hexanal from linoleic acid (**Fig. 1.4 Chapter One**). Post storage production of *trans*-2-hexenal in 'Granny Smith' apples was generally decreased, while that in 'Fuji' was approximately constant or slightly decreased (**Fig. 5.46**). It was likely that *trans*-2-hexenal is mainly derived from hexanal, as *cis*-3-hexenal as was not detected (from GC-MS identification) in the present study. If this is so, then isomerase activity would have stayed more or less constant or slightly decreased in 'Fuji' apples, whereas it may have decreased continuously with time after harvest in 'Granny Smith' apples. It is unlikely that the decrease in *trans*-2-hexenal production was due to the action of alcohol dehydrogenase, which leads to production of *trans*-2-hexenol (since the compound was not detected) or conversion via hexanal into hexan-1-ol, as this alcohol did not increase. Therefore, production of *trans*-2-hexenal probably depended on the activity of *cis-trans*-isomerases which presumably vary between cultivars.

Compared with RA storage, CA had no residual effect on hexanal production in 'Granny Smith' apples, but slightly depressed production in 'Fuji' apples (**Fig. 5.46**). Production of *trans*-2-hexenal in both cultivars was unaffected by CA storage. Production of these aldehydes in ULOCA stored 'Cortland' and 'McIntosh' apple were unaffected by CA conditions (Yahia, 1989). CA seemed to exert its residual effect on production of hexanal in 'Fuji' apples, presumably on lipoxygenases, but not in 'Granny

Smith' apples. In addition, treatment with 100% CO_2 further depressed production of hexanal in CA stored 'Fuji' apples but did not affect the production in 'Granny Smith'. This may indicate that there are differences in the nature of lipoxygenases, perhaps, types or isoenzymes, between the two cultivars.

The increase in *trans*-2-hexenal production in control fruit of CA stored 'Granny Smith' apples at 27 weeks after harvest is worth mentioning (**Fig. 5.46**), as this phenomenon coincided with development of the superficial scald disorder; it is possible that these two events may be related. This relationship has been found in some apple cultivars prone to this disorder in Idaho and Washington (Fellman, 1996; personal communication). The oxidative products of α-farnesene, conjugated trienes, are believed to be the actual cause of scald symptoms (Emongor *et al.*, 1994; Huelin and Coggiola, 1968; 1970) and the severity of scald is proportional to the extent of α-farnesene oxidation (Barden and Bramlage, 1994; Chen *et al.*, 1993). Low O_2 (1% or below) in combination with safe CO_2 levels (< 6%), and ethylene scrubbed (< 6 ppm) storage atmospheres at - 0.5°C reduced the incidence of scald in CA stored (8 - 10 months) 'Granny Smith' apples (Little and Peggie, 1987). Exposure of apples to an initial low O_2 stress (0.5% or below) for 9 days at - 0.5°C before CA storage (1% CO_2 + 1.5% O_2 at 0.5°C) gave additional benefits in scald control (Little *et al.*, 1982). Hyper low O_2 (0.5%) and an elevated CO_2 stress (2.5%) inhibited the accumulation of α-farnesene and conjugated trienes in peel tissue of CA stored 'd'Anjou' pears (Chen *et al.*, 1993). The reduction in concentrations of α-farnesene and conjugated trienes was thought to be due to CA suppression of certain enzymes required for α-farnesene synthesis and low O_2 reduced oxidative reactions of α-farnesene into the conjugated trienes (Chen *et al.*, 1993; Emongor *et al.*, 1994). It is not known whether *trans*-2-hexenal increased prior to the appearance of scald symptoms, at the same time, or later. It is likely that the compound increased before symptoms appeared as it is the product of lipoxygenase activity. Feys *et al.* (1980b) reported that lipoxygenase activity in peel of 'Boskoop' apples increased with time in storage which suggested disorganisation of lipoprotein membrane structures. They further speculated that lipoxygenase oxidation of polyunsaturated acids may in some way be involved in the mechanism of scald induction in apples. If this was so, *trans*-2-hexenal concentration or production, normally produced

in low concentrations in intact apples (Mattheis *et al.*, 1991b), may well increase prior to development of superficial scald symptoms. Monitoring *trans*-2-hexenal concentrations in storage atmospheres may provide a useful indicator for predicting superficial scald development or other disorders associated with disorganisation of fruit tissue of stored apples. Further research is needed to elucidate this phenomenon.

A dramatic increase in skin resistance to gas diffusion with time after storage of 'Granny Smith' apples has been reported (Dadzie, 1992). It was suggested that this was related to an increase in development of the oil fraction in fruit cuticles leading to blockage of lenticels, thereby impeding gas exchange, modifying the internal atmosphere of the fruit with consequent potential for triggering development of physiological disorders, such as core flush (Dadzie, 1992). In the present study, RA stored 'Granny Smith' apples became very greasy and slippery to touch after about 18 weeks of storage which coincided with the time when CO_2 injury was observed in the core tissue of some fruit following the 100% CO_2 treatment. The injured core tissue appeared to be dry and subsequently became cavities or pockets (**Plate 5.1**) surrounded by patches of discoloured tissues. Thus the symptom is likely to be a CO_2 induced cavity or internal CO_2 injury (Smock, 1977). It is conceivable that the incidence of injury may, in part, be related to the increase in fruit skin resistance during storage.

However, the mechanism by which tissue is injured as a result of hypoxia is still unknown. Acetaldehyde was reported to be toxic to plant tissue, perhaps inactivating enzymes with uncharged amino groups (Chervin *et al.*, 1996; Perata and Alpi, 1993). It has been shown that acetaldehyde accumulated after high CO_2 treatment, and that its production increased with time after harvest (**Fig. 5.7**); these factors along with an increase in fruit skin resistance with storage, would expose fruit tissue to acetaldehyde for long periods of time, and probably an increased level of inherent susceptibility to injury of fruit tissue with time in storage, hence lead to post hypoxic injury of apples. In contrast to the above arguments, postharvest treatments with acetaldehyde vapour (1000 - 5000 ppm) caused no visible injury to peaches, oranges, feijoas or nectarines (Lurie and Pesis, 1992; Pesis, 1994; Pesis and Avissar, 1989; Pesis *et al*, 1991). An alternative reason for injury may be restriction of normal metabolism which occurs under hypoxic conditions resulting in accumulation of free radicals (Knee, 1991a).

5.6 CONCLUSION

'Granny Smith' and Fuji' apples stored in CA conditions, relative to those in RA, had depressed post storage production of those headspace volatiles, aroma volatile esters and alcohols mainly derived from β-oxidation of fatty acids. CA had no effect on production of *trans*-2-hexenal in either cultivar or hexanal in 'Granny Smith' apples. CA enhanced production of 2-methyl butan-1-ol, particularly in 'Granny Smith' apples. Thus, CA affected the production of aroma volatile compounds in juice of the two apple cultivars differently. This may reflect differences in the origin of the volatile precursors in 'alcohol type' (Granny Smith) and 'ester type' (Fuji) cultivars.

Post storage treatment with 100% CO_2 for 24 hours at 20°C consistently and dramatically increased production of juice headspace acetaldehyde, ethanol, and ethyl acetate over control in both RA and CA stored fruit of both apple cultivars. The large accumulation of ethanol that occurred after this treatment, was followed by increased production of several ethyl esters, including the key aroma impact compounds ethyl butanoate, ethyl 2-methyl butanoate, and ethyl hexanoate. The treatment had no effect on production of hexanal, *trans*-2-hexenal, propan-1-ol and butan-1-ol from RA stored fruit, or butan-1-ol and hexan-1-ol from CA stored 'Granny Smith' apples. High CO_2 treatment decreased production of butyl and hexyl acetate in 'Fuji' apples and reduced production of 2-methyl butan-1-ol in both apple cultivars. The decreased production of volatile compounds, other than those containing the ethyl group, following the CO_2 treatment was probably due to the competitive nature of acyl esterification and/or the effect of the treatment on enzyme systems involved in the generation of substrates from precursors.

Production of headspace volatiles and some ethyl esters, such as ethyl 2-methyl butanoate, in control fruit of RA stored apples, particularly in 'Fuji', tended to increase gradually as storage time progressed. It is likely that this increase was due to natural ripening. Hexyl acetate or butyl acetate production in control fruit of RA stored 'Fuji' apples (neither compound was detected in 'Granny Smith') increased to a peak at about 14 or 23 weeks after harvest and decreased thereafter. Such a decrease could possibly be due to a decreased availability of substrate and/or precursors, as well as a reduced

activity of the ester forming enzyme(s) associated with the natural senescence process. Production of other volatiles in control fruit of RA and CA stored apples were varied, where some were increased, some stayed approximately constant and some decreased, possibly due to differences in their substrates and/or precursors, as well as the metabolic processes involved.

The ability to sustain enhanced production of ethyl esters in CO_2 treated fruit of RA or CA stored 'Granny Smith', and CA stored 'Fuji' apples decreased as time after harvest progressed. Such a decrease was likely to be due to shortage of acyl CoA moieties for esterification, as ethanol was not in short supply. This probably was related to the reduced availability of precursors such as amino acids and fatty acids, caused either by a decrease in their synthesis or by their degradation. On the other hand, production of ethyl esters and non ethyl esters in control fruit of RA stored 'Fuji' apples generally increased with time up to 23 weeks after harvest thereafter decreasing slightly. This increase probably indicated that acyl CoA moieties were not limiting esterification and also suggested the plentiful availability of precursors in this cultivar under RA storage. The slight decrease in ester production late in storage possibly related to the reduced activity of ester forming enzyme(s) which occurs as natural senescence progresses.

In the present study, CA retarded flesh softening and acid loss in both 'Granny Smith' and 'Fuji' apples as compared with RA storage, indicating that CA exerted its effect by delaying ripening of the fruit. Post storage treatment with 100% CO_2 for 24 hours at 20°C had a marginal effect on flesh firmness and titratable acids, in RA or CA stored fruit of both cultivars. However, this treatment caused CO_2 injury in core tissue of 'Granny Smith' apples stored in RA for more than 18 weeks with 38% of fruit in this treatment being affected. This injury was not observed in CA stored 'Granny Smith' or in RA and CA stored 'Fuji' apples which probably reflected differences in their skin permeance and/or sensitivity to hypoxia.

Chapter Six

EFFECT OF A SHORT-TERM HYPOXIC CO_2 TREATMENT ON AROMA VOLATILE COMPOUNDS, PHYSIOLOGY AND QUALITY ATTRIBUTES OF SELECTED APPLES CULTIVARS

6.1 INTRODUCTION

Diversification of the number of cultivars offered to customers is one of the most important strategies of the quality apple industry worldwide (Anon., 1995a; Dirinck *et al.*, 1989). A range of cultivars are produced for both export and for local consumption in New Zealand (**Table 6.1**). Recently new cultivars such as 'Pacific Rose' (GS 2085) and 'Southern Snap' (GS 330) have been released to growers on a commercial scale following their introduction in 1991 (Anon., 1995c). This is intended to retain New Zealand's competitive edge in apple cultivars and to achieve sustainable competitive advantage in international markets (Anon., 1995c). Moreover, this meets the changing demand of consumer tastes that are moving away from standard cultivars such as 'Granny Smith', 'Red Delicious' and 'Golden Delicious' with a greater demand for new cultivars such as 'Royal Gala', 'Braeburn', 'Fuji' and 'Pacific Rose' which are perceived to be superior in quality (Anon., 1995c; Boylston *et al.*, 1994; Steele, 1995). Such changes are also reflected in the changing pattern of export percentage of apple cultivars in New Zealand, where export percentage of common cultivars have decreased and that of new cultivars have increased over recent years (**Table 6.1**).

Table 6.1 Percentage of export of some apple cultivars in New Zealand during 1990 - 1995 (derived from L. Frampton, personal communication).

Cultivar	Export (%)		
	1990	1993	1995
Braeburn	12	22	32
Fuji	1	7	8
Royal Gala	11	17	21
Cox's Orange Pippin	9	7	8
Gala	4	4	3
Splendour	1	1	0
Granny Smith	29	20	11
Red Delicious	23	16	11
Golden Delicious	3	2	1
Sturmer Pippin	3	2	1
Other	3	2	4

There is no question that apple cultivars differ markedly in their flavour which is genetically controlled (Paillard, 1981; Thomson, 1986; Yahia, 1994). Aroma is one of the most important factors governing the characteristic flavour of an apple cultivar (Paillard, 1990; Williams, 1979). The characteristic fruity, apple-like aroma of apples is due to volatile compounds, primarily esters, aldehydes and alcohols (Cunningham *et al.*, 1986; Dürr and Schobinger; 1981; Flath *et al.*, 1967; Willaert *et al.*, 1983). Even-numbered carbon chain volatile compounds are usually found in large quantities, and for esters these include acetate, butanoate and hexanoate with ethyl, butyl and hexyl moieties (Paillard, 1990). Apple cultivars are known to differ in the type and amount of volatile compounds they contain, especially esters (Brackmann and Streif, 1994; Cunningham *et al.*, 1986; Kakiuchi *et al.*, 1986). For instance, ethyl butanoate and ethyl 2-methyl butanoate were reported to be higher in 'McIntosh' than in 'Cortland' apples (Yahia *et al.* 1991), and pentanoate esters are absent in both cultivars (Yahia, 1989). As apples differ in their volatile composition, attempts have been made to classify apple cultivars into 'ester' and 'alcohol' groups depending on the type of volatile production pattern (Dirinck and Schamp, 1989). The 'ester' group cultivars produce high concentrations of butyl acetate and hexyl acetate, and most of their volatile precursors originate from fatty acids. The 'alcohol' cultivars contain high concentration of hexan-1-ol and branched-

chain alcohols, their volatile precursors being derived mainly from amino acids (Brackmann *et al.*, 1993; Dirinck and Schamp, 1989; Dirinck *et al.*, 1989). Cultivars had also been categorised in relation to the type of esters, such as acetate, butanoate and propanoate, predominating in the volatile profile (Paillard, 1990). Other compounds such as hexanal and *trans*-2-hexenal were also reported to be quantitatively different in juice and essence among apple cultivars (Drawert *et al.*, 1986). Thus this quantitative difference in composition of volatile compounds contribute to the distinctive, diverse flavour and aroma of apple cultivars.

Different cultivars also differ in their suitability for juice preparation and consumers may demand juice from certain types of apples (Poll, 1981). For example, 'Cox's Orange Pippin' apple juice is preferred in the Canadian market and New Zealand is the sole supplier of this product in the world (Steele, 1995). Generally, apple juice is derived from second-grade or 'cull' fruit which is unsuitable for the fresh fruit market, although in parts of central Europe fruit may be grown specifically for juice production (Lea, 1995; Poll, 1981). It has been estimated that over 30% of apple production in New Zealand is processed mainly into juice and essence (Willis, 1993). The greatest volume of apple juice is usually processed into 70° Brix apple concentrate; the aroma is recovered as an 'essence' where it may be traded as a separate product or used during reconstitution of the concentrate (Lea, 1995). A few volatile compounds such as ethyl butanoate, ethyl 2-methyl butanoate and *trans*-2-hexenal are considered to be critically important contributors to aroma of apple juice and essence (Dürr and Schobinger; 1981; Flath *et al.*, 1967). Enhancement of these character-impact volatile compounds in apple juice and essence, using a simple, rapid and economic treatment would increase value and use of the product. In **Chapter Four**, it was demonstrated that a short-term application of a hypoxic CO_2 atmosphere (24 hours at 20°C) led to substantial increases in several aroma-impact volatile compounds in 'Braeburn', 'Granny Smith' and 'Red Delicious' apple juice. Such a treatment had a negligible or no effect on other fruit quality attributes, such as flesh firmness, skin colour, TSS and TA, after treatment. However, as apple cultivars are genetically and physiologically different (Paillard, 1990; Salunkhe and Do, 1976) they may respond differently to such a treatment, in terms of aroma volatile production and fruit quality attributes. There has been no extensive

investigation of the effect of such a treatment on the volatile compounds in apple cultivars previously reported.

6.2 OBJECTIVES

The objectives of this study were:

(1) to study the differences in volatile compounds among the 6 commercial apple cultivars, 'Royal Gala', 'Red Delicious', 'Braeburn', 'Granny Smith', 'Cox's Orange Pippin' and 'Pacific Rose';

(2) to study the effect of a short-term application of 100% CO_2 (24 hours at 20°C) on aroma volatile production among the selected cultivars; and

(3) to study the influence of this treatment on physiological and quality attributes of the apple cultivars.

6.3 MATERIALS AND METHODS

6.3.1 Materials and Treatments

Freshly harvested 'Royal Gala', 'Red Delicious', 'Braeburn', 'Granny Smith', and cold stored 'Cox's Orange Pippin' and 'Pacific Rose' apples were used (**Table 6.2**).

Table 6.2 History of apple cultivars used for experiments.

Cultivar	Average Size(g)	Time of Harvest	Time of Treatment	Storage	Source
1993 Experiment					
Royal Gala	150	9/3/93	10/3/93	–	FCU
Red Delicious	142	6/4/93	7/4/93	–	FCU
Braeburn	148	28/4/93	29/4/93	–	FCU
Granny Smith	173	25/5/93	26/5/93	–	FCU
1994 Experiment					
Cox's Orange Pippin	149	1stwk Mar.	22/3/94	3wk[1]	NZAPMB
Pacific Rose	180*	3rdwk Apr.	19/5/94	4wk[1]	NZAPMB

* size range 115 - 238g, [1] Fruit Crop Unit cold store (0.5° ± 0.5°C, 92 - 95% RH), FCU = Fruit Crop Unit, Massey University, Palmerston North, NZAPMB = New Zealand Apple and Pear Marketing Board (ENZA®), Hastings, Hawkes Bay, wk = week

For each experiment, apples (either freshly harvested or removed from cold store) from all cartons were placed on a laboratory table for initial assessment. Damaged, misshaped and non-uniform fruit were discarded and good fruit with similar size and colour were selected. 'Pacific Rose' apples were from young trees, thus fruit size was variable and the number of fruit available for selection was limited. Apples were then divided into groups according to the number of replicates of a specific experiment; fruit in each group was individually numbered and labelled. Treatments, replicates and fruit number to be taken for analysis at each sampling interval, were randomly allocated. Apples were then transferred to 20°C for temperature equilibration, generally overnight, and treatments were applied the following day.

***The 1993 Experiments*:** There were 4 experiments conducted in 1993 (**Table 6.2**). Freshly harvested apples of 'Royal Gala', 'Red Delicious', 'Braeburn' and 'Granny Smith' were used in separate cultivar experiments. Each experiment comprised 2 treatments; 100% CO_2 for 24 hours at 20°C and control; each consisted of 4 replicates. In each replicate, 5 fruit were used for analysis and for juicing at each sampling interval (9 sampling times). Twenty fruit were analysed just before the application of treatment (day -1); giving 380 fruit in total. Apples of the 100% CO_2 treatment were transferred into 27-litre Perspex chambers, where humidified 100% CO_2 gas was applied for 24 hours at 20°C. Details of the gas treatment system and method of application for CO_2 was described in **Chapter Two**. Control fruit were left untreated at 20°C and 70% RH. After treatment apples were removed from treatment chambers and maintained at 20°C. Fruit samples were taken for analysis at -1, 0, 1, 3, ..., 15 days after treatment, where day -1 was just before treatment, day 0 was immediately after treatment, day 1 was 24 hours after and so on.

***The 1994 Experiments*:** There were 2 experiments conducted in 1994 using cold stored 'Cox's Orange Pippin' and 'Pacific Rose' apples which had been stored at 0°C for 3 - 4 weeks prior to the experiment (**Table 6.2**). An experiment comprised 2 treatments, as in 1993, but each consisted of 10 replicates. In a replicate, a single fruit was used for analysis and for juicing at each sampling interval (9 sampling times). Ten fruit were

analysed just before the application of treatment (day -1); giving 190 fruit in total. Application of treatments and fruit sampling were as described above.

6.3.2 Fruit Analysis

Fruit respiration rate, ethylene production and skin colour were measured on individual fruit, after which flesh firmness was determined. Fruit were then diced and all parts, including skin, cortex, core and seed, were used immediately for juice preparation. Apple juice obtained was used directly for analysis of headspace volatiles (1994 experiment only), aroma volatile compounds and TSS. A portion of juice was kept frozen for further determination of TA and pH.

Methods for determination of fruit respiration, ethylene production, skin colour, flesh firmness and juice pH, and the methods used to prepare juice and to analyse headspace volatiles of apple juice, were described in **Chapter Two**. The purge and trap technique was used to determine aroma volatile compounds in the 1993 experiments, and the direct solvent extraction technique was used for the 1994 experiments. Details of sample preparation, analysis, and quantification of volatile compounds were described in **Chapter Two**.

6.3.3 Data Analysis

The experiment was designed as a factorial (Day x Treatment) with replication nested in treatment. Means and standard error of the means of each treatment for respiration rate, ethylene production, headspace volatiles and selected aroma volatile compounds were graphed using an Origin® Graphic Package (Microcal™ Software Inc., Northampton, USA). Means of each treatment for flesh firmness, fruit weight loss, skin colour, TSS, TA, TSS:TA ratio and pH were tabulated. All data of TSS, TA, pH, headspace volatiles and aroma volatile compounds were from apple juice.

Data were subjected to analysis of variance using an SAS package (SAS Institute Inc., Cary, USA). Means and a pooled standard error for each experiment were calculated. Significant main effect means were separated by Duncan's multiple range test

at the 5% level of significance and the interaction between these factors were determined by least squares means (lsmeans) at 1% or 5% levels.

6.4 RESULTS

6.4.1 Fruit Respiration Rate and Ethylene Production

***Fruit Respiration Rate*:** Respiration rate of control and 100% CO_2 treated apples within a cultivar generally followed similar patterns of change during storage for 2 weeks at 20°C (**Fig. 6.1**). Respiration of 'Royal Gala' was not affected by CO_2 treatment. Treated 'Red Delicious', 'Granny Smith' and 'Pacific Rose' apples had slightly higher overall CO_2 production than did controls ($P < 0.05$). Respiration rate of CO_2 treated 'Cox's Orange Pippin' apples was slightly lower than control ($P < 0.05$) between 3 and 7 days, but it was higher from 11 days after treatment. 'Braeburn' was different from the other cultivars in that the CO_2 treatment resulted in a major enhancement of respiration rate. This may have been directly related to fruit injury caused by the treatment, which will be described latter.

A sudden surge in CO_2 production, such as that in 'Red Delicious' at day 0 and day 3 of control and CO_2 treated apples, respectively, was suspected to result from contamination from breathing during the process of enclosing apples into respiration jars. For subsequent measurements an electric fan was used to flush out the jar containing fruit; in addition every effort was made to avoid breathing into jars before closing them. This was subsequently used as a standard practice.

Respiration rate of both control and CO_2 treated 'Royal Gala' apples was high initially, then rapidly declined at day 7, remaining constant thereafter. 'Red Delicious' showed a climacteric rise in respiration rate at day 9, peaking at day 11 and decreasing thereafter. Respiration rate of control 'Braeburn' apples was initially constant for 7 days and thereafter increased slowly through 15 days at 20°C. In contrast, CO_2 production of the treated 'Braeburn' increased markedly and peaked 1 day after treatment, then decreased to day 5 and remaining constant thereafter, where the magnitude of

production was much greater than in control. Respiration rate of 'Granny Smith' apples was variable; increasing or decreasing slightly at day 1 after treatment, peaking at day 5, decreasing at day 7, then remaining constant during day 7 - 13 and increasing again thereafter. The CO_2 production of both control and CO_2 treated 'Cox's Orange Pippin' apples decreased gradually through 15 days storage. Neither control nor CO_2 treated 'Pacific Rose' apples showed any apparent pattern of change in respiration rate over time and there was little difference between treatments (**Fig. 6.1**).

***Fruit Ethylene Production*:** Ethylene production of the 6 apple cultivars during 15 days storage at 20°C followed a similar pattern of changes, in which it increased or was not detected initially, rising substantially to a maximum and thereafter remaining constant or decreasing slightly (**Fig. 6.2**).

Application of 100% CO_2 for 24 hours caused higher overall ethylene production than that of control ($P < 0.05$) in 'Royal Gala', 'Red Delicious' and 'Cox's Orange Pippin', but lower ($P < 0.05$) in 'Braeburn', while similar to that of control in 'Granny Smith' and 'Pacific Rose' apples (**Fig. 6.2**).

'Royal Gala' apples showed a climacteric rise in ethylene production at day 5 for CO_2 treated fruit, 2 days before that of control, while the peak concentration was similar for both control and treated fruit. Ethylene production of 'Red Delicious' apples remained low for 3 days, then it increased and peaked at day 11 in CO_2 treated fruit and at day 13 in controls, decreasing thereafter. The peak production of ethylene in CO_2 treated fruit was greater ($P < 0.05$) than in controls (**Fig. 6.2**).

In 'Braeburn' apples ethylene concentration was initially low or not detected, but it increased slowly after day 5 through 15 days at 20°C. Ethylene production in CO_2 treated 'Granny Smith' was initially depressed through day 3, relative to controls; then it increased to a peak at day 5 and decreased thereafter for both treatments. In 'Cox's Orange Pippin' apples ethylene production increased continuously to a peak at day 11. Concentration of ethylene in CO_2 treated fruit was initially lower, but then higher than controls ($P < 0.001$) after 7 days. The drop of ethylene production at day 13 was due to a power cut the night prior to analysis which caused the air conditioning to turn off and decreased storage temperature (**Fig. 6.2**).

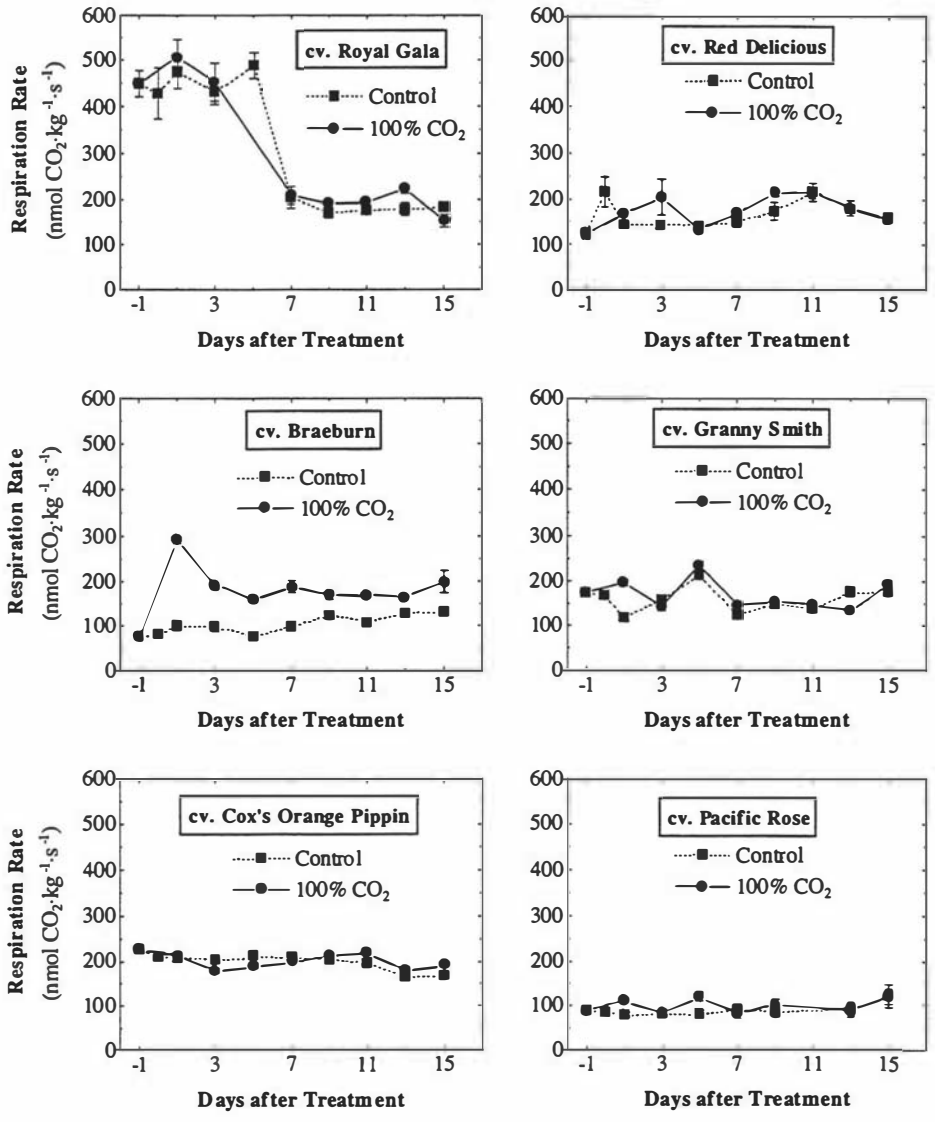


Fig. 6.1 Respiration rate of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

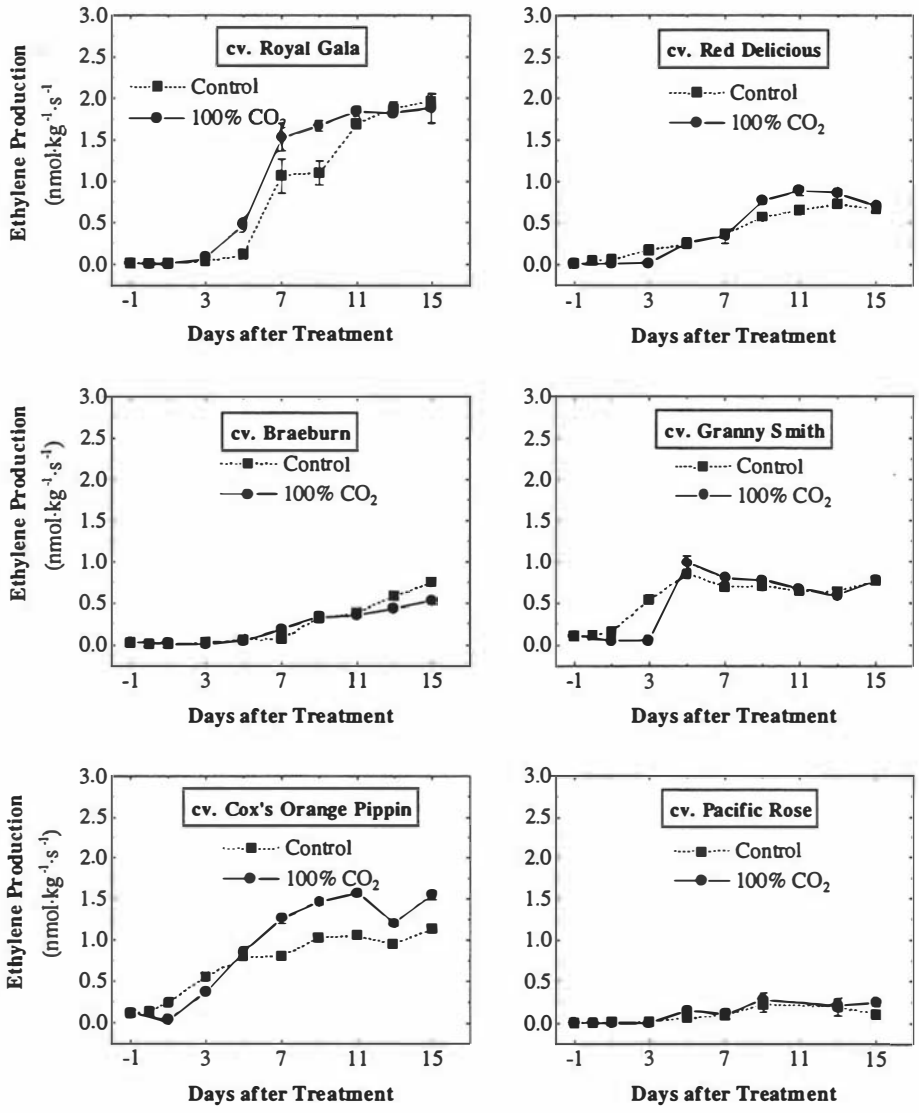


Fig. 6.2 Ethylene production of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

Ethylene production in CO_2 treated 'Pacific Rose' apples was not detected during the first 3 days; production increased after day 3 and reached a peak at day 9, decreasing thereafter, with production was similar in both control and treated fruit (**Fig. 6.2**).

6.4.2 Volatile Production

Examples of volatile chromatograms from single strength juice of 'Red Delicious' apples analysed by the purge and trap technique (1993 experiment) and by the direct solvent extraction technique (1994 experiment) are shown in **Fig. 6.3** and **Fig. 6.4**, respectively. Typical chromatograms for the other 5 cultivars studied are shown in **Appendix 5**. Detailed method of analysis for both techniques were described and the advantages or disadvantages of each were discussed in **Chapter Three**.

Almost all of the peaks detected using the purge and trap technique of extraction were of low to medium molecular weight volatile compounds between C_2 and C_8, up to 19.6 min, the retention time of the octyl acetate internal standard (**Fig. 6.3**). Peaks obtained from using the direct solvent extraction technique covered a larger range of compounds between C_2 and C_{16}, eluting over a long period of time (30 min) and after the internal standard (**Fig. 6.4**).

Some peaks in chromatograms obtained from the 100% CO_2 treatment were larger and some were smaller than those obtained from controls; this phenomenon was observed in all cultivars and from both methods used for extraction (**Appendix 5**). For example, chromatograms from 'Red Delicious' apple volatiles obtained by using the purge and trap technique (**Fig. 6.3**), gave peaks with retention time of 6.9 min (butyl acetate), 8.5 min (isoamyl acetate) and 13.5 min (hexyl acetate) from control juice that were relatively taller than the respective peaks from the 100% CO_2 treatment. On the other hand, peaks at 2.9 min (ethyl acetate), 6.3 min (ethyl 2-methyl butanoate) and 12.3 min (ethyl hexanoate) from CO_2 treated fruit were markedly taller than the respective peaks from the control treatment.

The majority of volatile compounds found in apples were esters, alcohols and hydrocarbons, while the other classes of volatiles such as aldehydes, ketones, acids and others were also present in lesser numbers (**Table 6.3**). 'Red Delicious' apples produced the highest number of esters, 'Royal Gala' had the highest number of alcohols, while 'Granny Smith' possessed the highest number of hydrocarbons. The number of aldehydes detected were comparable among these cultivars, while only one ketone was found in 'Royal Gala', 'Braeburn' and 'Cox's Orange Pippin' apples. Total number of volatile

compounds found in 'Red Delicious' apples was the same as in 'Braeburn' and these were the highest among the cultivars studied (**Table 6.3**).

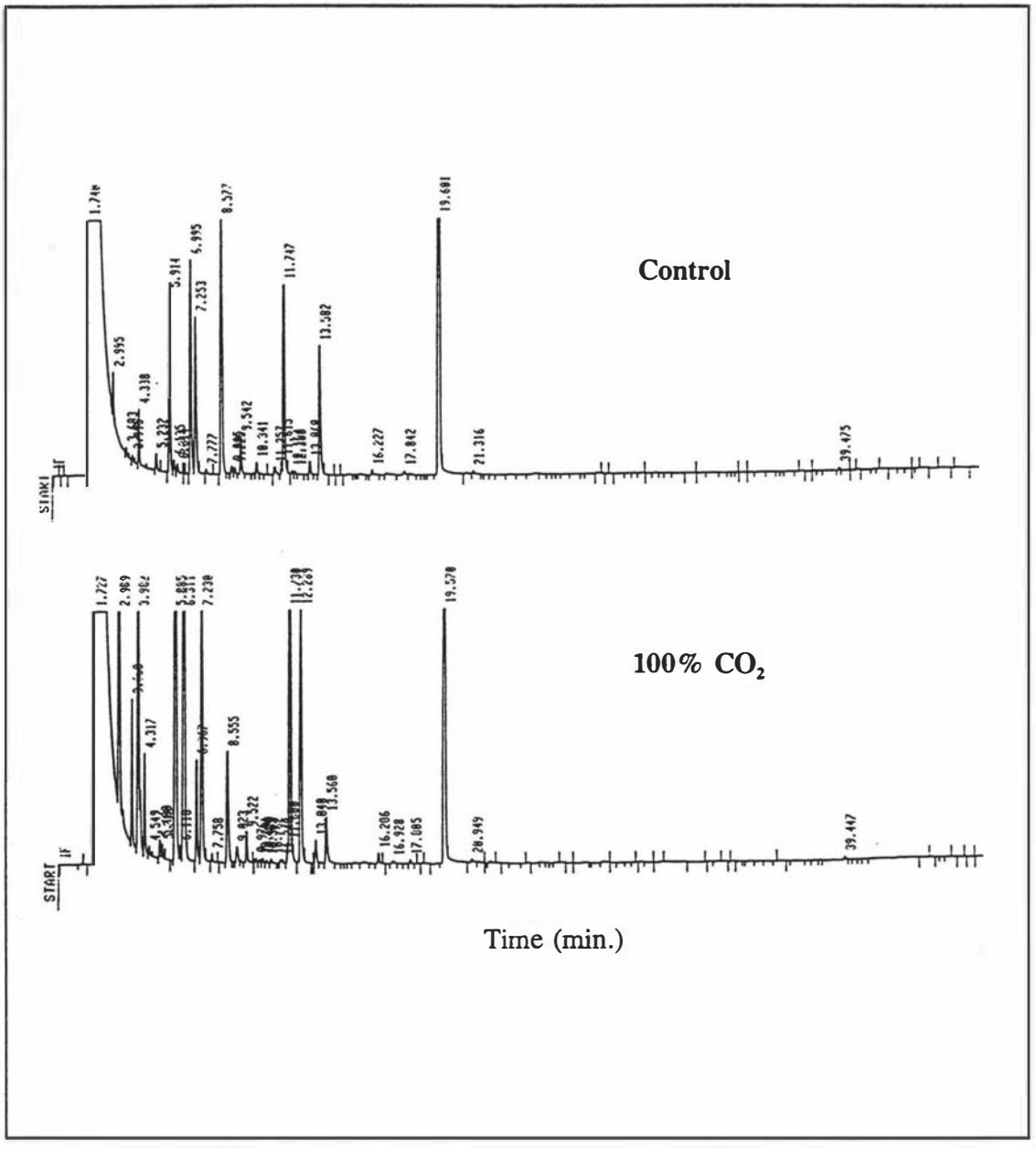


Fig. 6.3 Typical chromatograms of volatile compounds analysed by the purge and trap technique from juice of control and 100% CO_2 treated (24 hours at 20°C) 'Red Delicious' apples (1993 Experiment).

Diethyl ether solvent (retention time 1.7 min), ethyl acetate (2.9), ethanol (3.6), ethyl propanoate (3.9), ethyl butanoate + toluene (5.9), ethyl 2-methyl butanoate (6.3), butyl acetate (6.9), hexanal (7.2), isoamyl acetate (8.5), butan-1-ol (9.5), *trans*-2-hexenal (11.7), ethyl hexanoate (12.2), ethenyl benzene (13.0), hexyl acetate (13.5), hexan-1-ol (16.2), and octyl acetate internal standard (19.6 min).

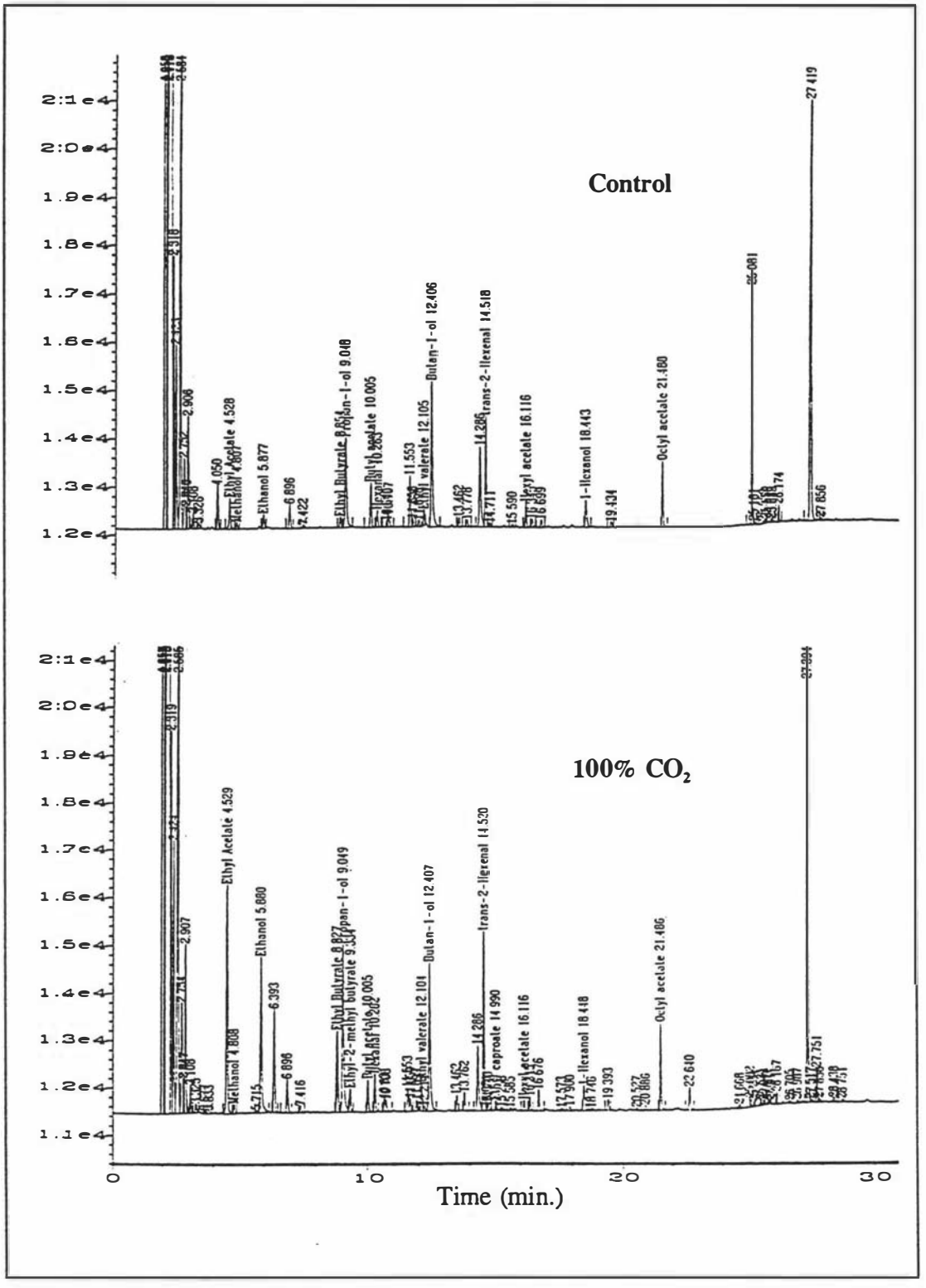


Fig. 6.4 Typical chromatograms of volatile compounds analysed by the direct solvent extraction technique from juice of control and 100% CO_2 treated (24 hours at 20°C) 'Red Delicious' apples (1994 Experiment).

Volatile compounds (both in control and 100% CO_2 treatments) such as ethyl acetate, hexyl acetate, ethanol, propan-1-ol, butan-1-ol, hexan-1-ol, acetaldehyde, hexanal, *trans*-2-hexenal and acetic acid were always present. Some important character-impact compounds, such as ethyl 2-methyl butanoate and ethyl hexanoate, were not found in 'Royal Gala' or 'Cox's Orange Pippin' apples. Butyl acetate, another character-impact compound, was not detected in 'Granny Smith', but found in all the other cultivars. Hexyl hexanoate was identified only in 'Red Delicious' and 'Braeburn', while 2-methyl butyl acetate was not found in 'Granny Smith' or 'Cox's Orange Pippin' apples. Butanal, 2-hexen-1-ol, 5-hexen-1-ol and octanol were found only in 'Royal Gala' apples. A ketone, 3-hydroxy butanone (acetoin), was found only in 'Braeburn', while another ketone, 2-butanone, was detected in 'Royal Gala' and 'Cox's Orange Pippin' apples. Other compounds such as hydrocarbons, acids, and others were also present in some cultivars but absent in the other cultivars (**Table 6.3**). Although some common compounds were always present in apples, different cultivars produced different numbers of volatiles among chemical classes, where some were detected in some cultivars but were not found in the other cultivars.

In the 1993 experiments, some compounds such as, ethanol, could not be quantified as it was present in the diethyl ether solvent, added by the manufacturer (BDH) for preventing peroxide formation. Toluene (1000 ppm), another internal standard, co-eluted with ethyl butanoate on the AllTech Econo Cap® capillary column used. In addition, only a few major volatile compounds were identified unequivocally by mass spectrometry due to low concentration of compounds in the concentrated samples. Thus, for the compounds present in these cultivars, obtained from the 1994 and 1995 experiments, only data for those which were analysed by the direct solvent extraction technique and identified by mass spectrometry are presented (**Table 6.3**).

Table 6.3 Volatile compounds detected in juice of 'Royal Gala' (RG), 'Red Delicious' (RD), 'Braeburn' (Bb), 'Granny Smith' (GS), 'Cox's Orange Pippin' (COP) and 'Pacific Rose' (PR) apples analysed by the direct solvent extraction technique and identified by mass spectrometry.

Compound	RG	RD	Bb	GS	COP[1]	PR
Esters						
methyl hexanoate	–	+	–	–	–	–
2-methyl butyl acetate	+	+	+	–	–	+
ethyl acetate	+	+	+	+	+	+
ethyl propanoate	–	+	+	+	–	+
ethyl butanoate	–	+	+	+	–	+
ethyl hexanoate	–	+	+	+	–	+
ethyl 2-methyl butanoate	–	+	+	+	–	+
ethyl 3-hydroxy butanoate	–	+	+	+	n.a.	+
ethyl 3-hydroxy hexanoate	–	+	+	–	n.a.	+
propyl acetate	+	+	+	+	–	+
propyl butanoate	–	+	–	+	–	–
butyl acetate	+	+	+	–	+	+
amyl acetate	+	+	+	–	–	+
isoamyl acetate	+	+	+	+	–	–
hexyl acetate	+	+	+	+	+	+
hexyl hexanoate	–	+	+	–	n.a.	–
octyl propanoate	+	+	–	+	n.a.	–
(Total Esters)	(8)	(17)	(14)	(11)	(3)	(12)
Alcohols						
methanol[2]	+	+	+	+	+	+
ethanol	+	+	+	+	+	+
propan-1-ol	+	+	+	+	+	+
butan-1-ol	+	+	+	+	+	+
pentan-1-ol	+	+	+	+	–	+
hexan-1-ol	+	+	+	+	+	+
2-hexen-1-ol	+	–	–	–	–	–
5-hexen-1-ol	+	–	–	–	–	–
octanol	+	–	–	–	n.a.	–
3-octen-1-ol	–	–	–	+	n.a.	–
2-methyl propan-1-ol	+	+	+	–	+	–
2-methyl butan-1-ol	+	+	+	+	–	+
6-methyl 5 hepten-2-ol	+	+	+	–	–	+
3-(methyl thio) propan-1-ol	+	–	–	–	n.a.	–
1,3 dichloro 2-propanol	+	–	–	–	n.a.	–
1,2 propanediol	–	+	+	+	n.a.	–
1,3 butanediol	–	–	–	–	n.a.	+
2,3 butanediol	+	+	+	–	n.a.	–
(Total Alcohols)	(15)	(11)	(12)	(9)	(6)	(9)

Table 6.3 (Continued)

Compound	RG	RD	Bb	GS	COP[1]	PR
Aldehydes						
acetaldehyde	+	+	+	+	+	+
butanal	+	–	–	–	–	–
hexanal	+	+	+	+	+	+
trans-2-hexenal	+	+	+	+	+	+
(Total Aldehydes)	(4)	(3)	(3)	(3)	(3)	(3)
Ketones						
2-butanone	+	–	–	–	+	–
3-hydroxy 2-butanone (acetoin)	–	–	+	–	–	–
(Total Ketones)	(1)	(–)	(1)	(–)	(1)	(–)
Hydrocarbons						
propyl cyclohexane	–	–	–	+	–	–
pentyl cyclohexane	–	+	–	–	–	–
nonane	–	+	–	+	–	–
3-methyl nonane	–	–	–	+	–	–
decane	–	+	+	+	+	–
undecane	–	+	+	+	–	–
tridecane	–	+	+	+	–	+
tetradecane	–	–	+	+	+	+
pentadecane	–	+	+	+	n.a.	+
hexadecane	+	+	+	–	n.a.	+
heptadecane	–	+	–	+	n.a.	+
dodecane	–	+	+	+	–	–
3,6 dimethyl decane	–	–	+	–	–	–
1-nonene	–	+	–	–	–	–
toluene	+	-	–	+	+	–
xylene (dimethyl benzene)	+	+	+	+	–	+
ρ-xylene	+	+	+	+	–	+
1-ethyl 3-methyl benzene	–	–	+	–	–	+
1,2,3 trimethyl benzene	–	–	+	–	–	–
1,2,4 trimethyl benzene	–	–	+	+	–	+
1,3,5 trimethyl benzene	–	–	–	+	–	–
ethyl benzene	–	–	+	+	–	+
ethenyl benzene	–	–	–	+	–	–
α-farnesene	+	+	+	+	n.a.	+
(Total Hydrocarbons)	(5)	(13)	(15)	(18)	(3)	(11)
Acids and others						
acetic acid	+	+	+	+	+	+
propanoic acid	–	+	–	–	n.a.	–
2-methyl butanoic acid	+	+	–	–	n.a.	–
estragole	+	–	–	–	n.a.	–
trans-linalool oxide	–	–	+	+	–	–
(Total Acids and others)	(3)	(3)	(2)	(2)	(1)	(1)
Total Volatile Compounds	**36**	**47**	**47**	**43**	**17**	**36**

[1] Volatile peaks of 'Cox's Orange Pippin' were identified up to retention time of 21.3 min. of octyl acetate internal standard; [2] identified by retention time; + = detected and identified; – = not detected; n.a. = GC-MS identification was not available

***Headspace Volatiles*:** Headspace acetaldehyde, ethanol and ethyl acetate production of apples were analysed only in 'Cox's Orange Pippin' and 'Pacific Rose' from the 1994 experiments (**Fig. 6.5**). Exposing apples to 100% CO_2 for 24 hours at 20°C stimulated substantial accumulation of headspace acetaldehyde and ethanol in both cultivars immediately after treatment. Peak concentrations of acetaldehyde in 'Pacific Rose' and 'Cox's Orange Pippin' were 13 and 40 $\mu l \cdot l^{-1}$, respectively, while those of ethanol were 68 and 425 $\mu l \cdot l^{-1}$, respectively. Headspace ethyl acetate peaked 1 and 3 days after treatment in 'Pacific Rose' and 'Cox's Orange Pippin' apples, respectively, with peaked concentrations being 35 and 121 $\mu l \cdot l^{-1}$. After reaching peak production, these headspace volatiles decreased; reaching concentrations similar to controls between day 9 and day 13 after treatment. These volatiles were not detected in control 'Cox's Orange Pippin' apples throughout the experimental period, while in 'Pacific Rose' they were detected at low levels after 5 days at 20°C. 'Cox's Orange Pippin' apples had a relatively greater ability to produce headspace volatiles than 'Pacific Rose' after CO_2 treatment (**Fig. 6.5**).

***Volatile Esters*:** Treatment with 100% CO_2 induced a substantial enhancement of ethyl acetate in all apple cultivars. Concentration of ethyl acetate generally increased and peaked at 1 - 3 days after CO_2 treatment and thereafter decreased to a level similar to controls at 11 or 13 days, except for 'Granny Smith' apples which decreased to the control level at 5 days at 20°C. Concentration of ethyl acetate in control fruit was low throughout the 2 weeks at 20°C (**Fig. 6.6**).

Among 4 apple cultivars of the 1993 experiments, the highest to the lowest production of ethyl acetate (area count) following CO_2 treatment was in the order of 'Red Delicious', 'Braeburn', 'Royal Gala' and 'Granny Smith', respectively. The ability to produce ethyl acetate after CO_2 treatment of 'Cox's Orange Pippin' apples was relatively greater than 'Pacific Rose' (**Fig. 6.6**), and the production pattern was similar to that of the headspace result (**Fig. 6.5**).

Ethyl butanoate was monitored only in 'Cox's Orange Pippin' and Pacific Rose' apples in 1994, and not in the other cultivars in 1993 because it co-eluted with the toluene internal standard. Ethyl butanoate was enhanced by CO_2 treatment, peaking at 3 or 5 days after treatment for 'Pacific Rose' and 'Cox's Orange Pippin' apples, respectively, thereafter decreasing to a level similar to controls at 5 or 9 days of storage

(**Fig. 6.7**). Ethyl butanoate in untreated 'Cox's Orange Pippin' was low throughout, while it increased in 'Pacific Rose' apples during exposure to 20°C over 15 days. 'Pacific Rose' apples produced more ethyl butanoate than did 'Cox's Orange Pippin' apples.

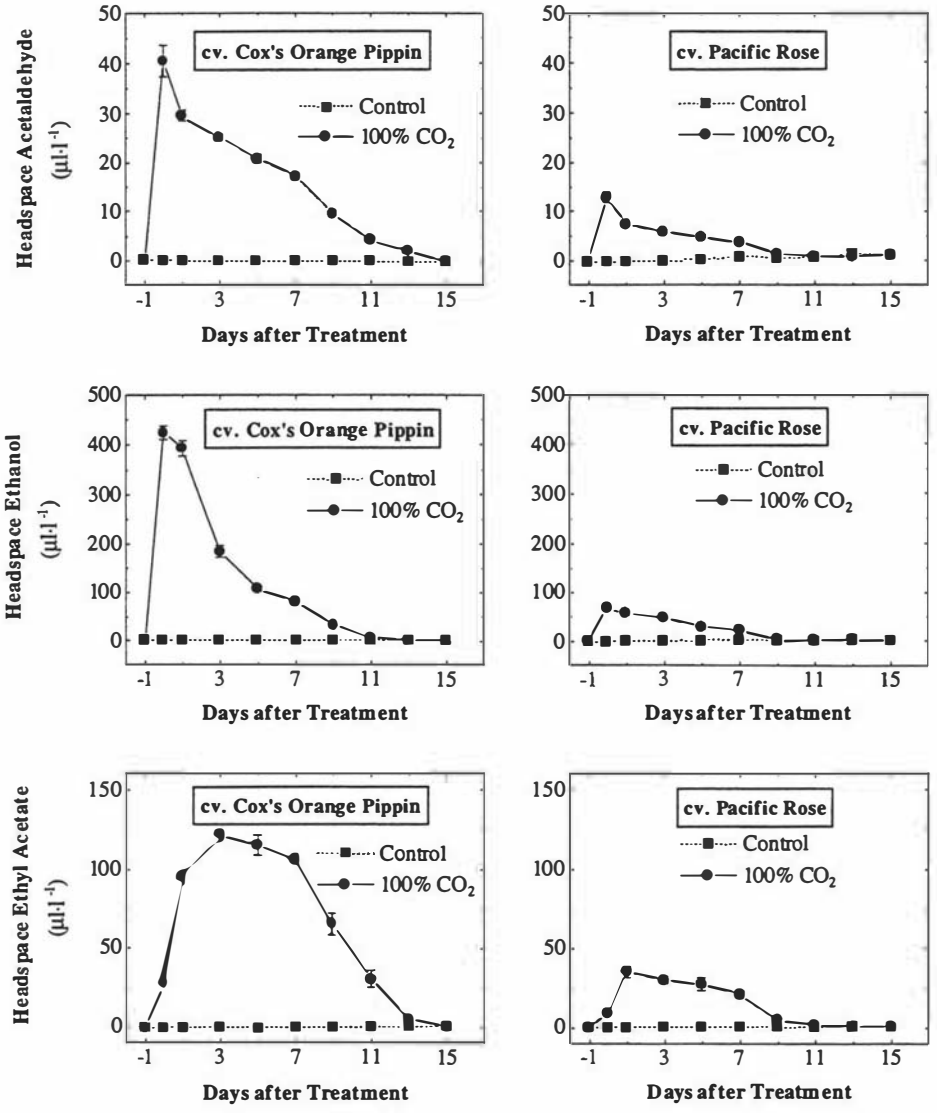


Fig. 6.5 Headspace acetaldehyde, ethanol and ethyl acetate of 'Cox's Orange Pippin' and 'Pacific Rose' apples after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

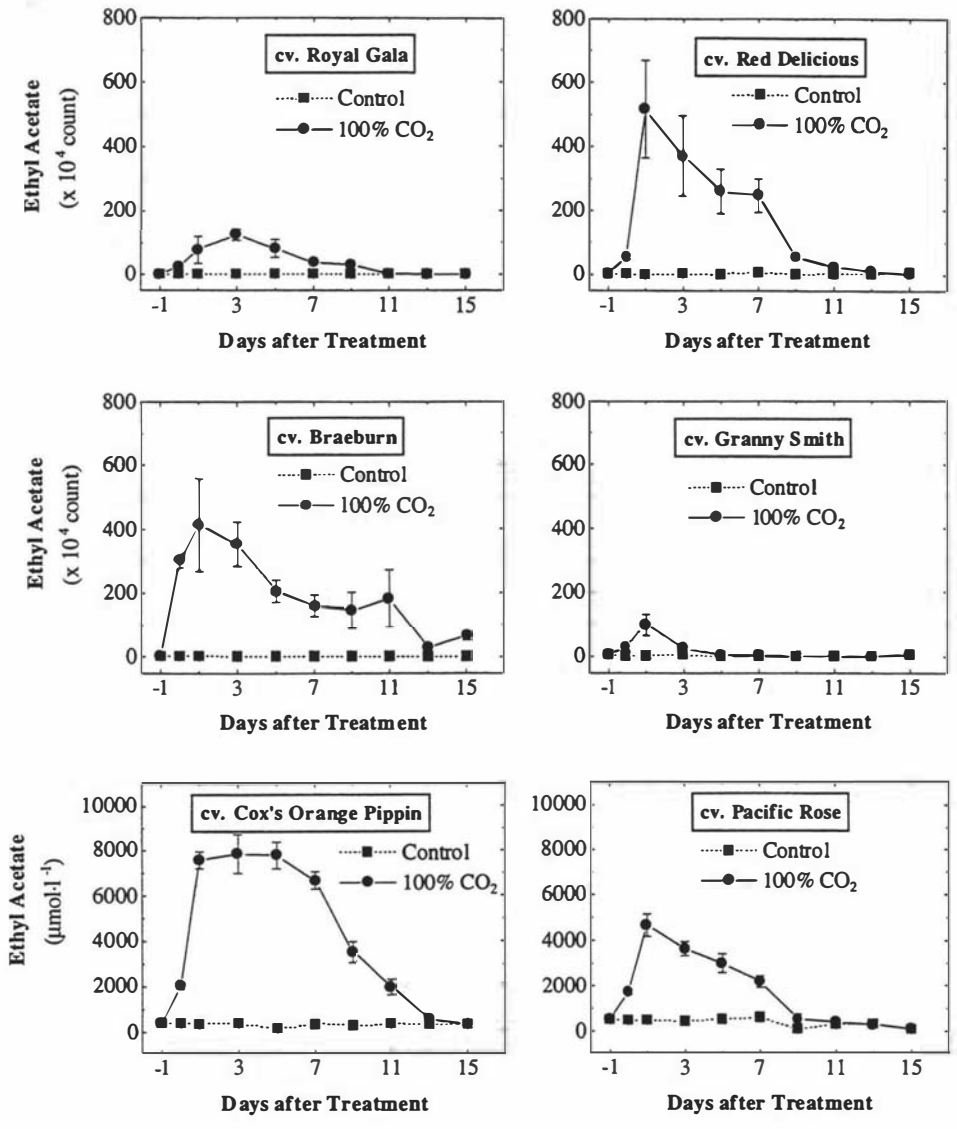


Fig. 6.6 Ethyl acetate of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

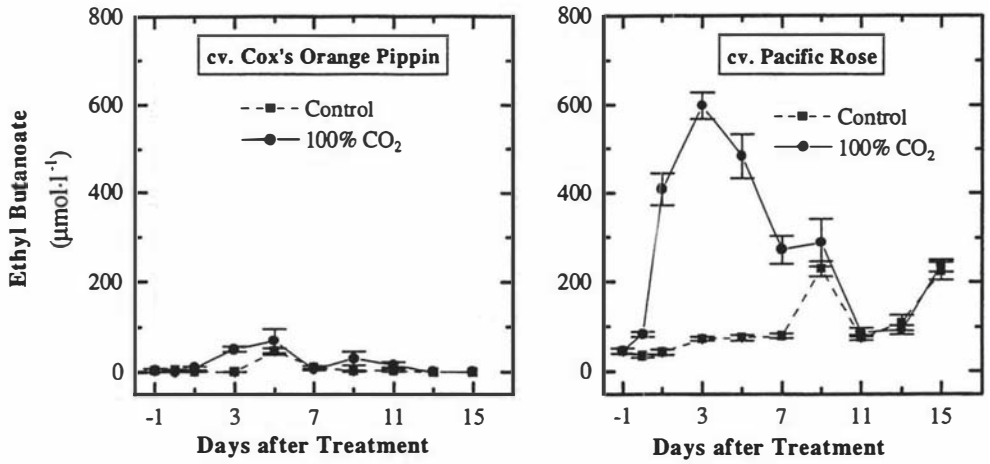


Fig. 6.7 Ethyl butanoate of 'Cox's Orange Pippin' and 'Pacific Rose' apples after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

Ethyl acetate was the only ethyl ester found in the CO_2 treated and control 'Royal Gala' apples (**Table 6.3**). However, CO_2 treatment induced a several fold enhancement over control of other ethyl esters such as ethyl 2-methyl butanoate (**Fig. 6.8**) and ethyl hexanoate (**Fig. 6.9**) in 'Red Delicious', 'Braeburn', 'Granny Smith' and 'Pacific Rose', but not in 'Royal Gala' or 'Cox's Orange Pippin' apples, where these esters were not detected.

Concentration of ethyl 2-methyl butanoate increased markedly and peaked at 1, 3 or 5 days after CO_2 treatment for 'Red Delicious', 'Pacific Rose' and 'Granny Smith' apples, respectively; thereafter decreasing to a level similar to controls at 9 or 11 days at 20°C (**Fig. 6.8**). In 'Braeburn' apples, ethyl 2-methyl butanoate was also enhanced by the CO_2 treatment, but it was not as pronounced as in 'Red Delicious' or 'Granny Smith', where the concentration continued to be greater than in control fruit through 15 days at 20°C. In control fruit, ethyl 2-methyl butanoate concentration was low; increasing slightly in 'Red Delicious', 'Granny Smith' and 'Pacific Rose', but not in 'Braeburn' where it remained approximately constant during 2 weeks at 20°C (**Fig. 6.8**).

Ethyl hexanoate concentration generally increased markedly and peaked at 1 day after CO_2 treatment in 'Red Delicious', 'Braeburn', 'Granny Smith' and 'Pacific Rose'

apples, then it decreased rapidly to a level comparable to controls at 3, 7 or 11 days, depending on cultivars (**Fig. 6.9**).

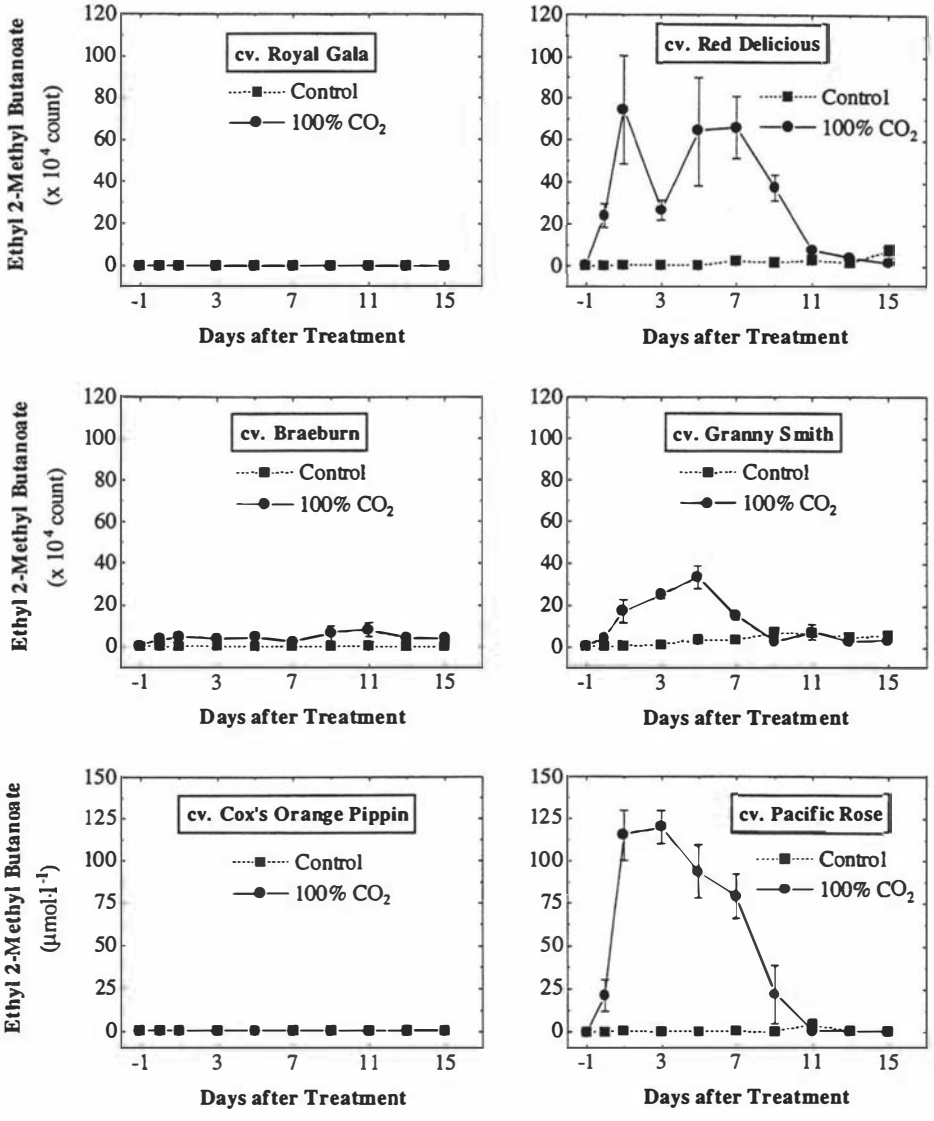


Fig. 6.8 Ethyl 2-methyl butanoate in 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

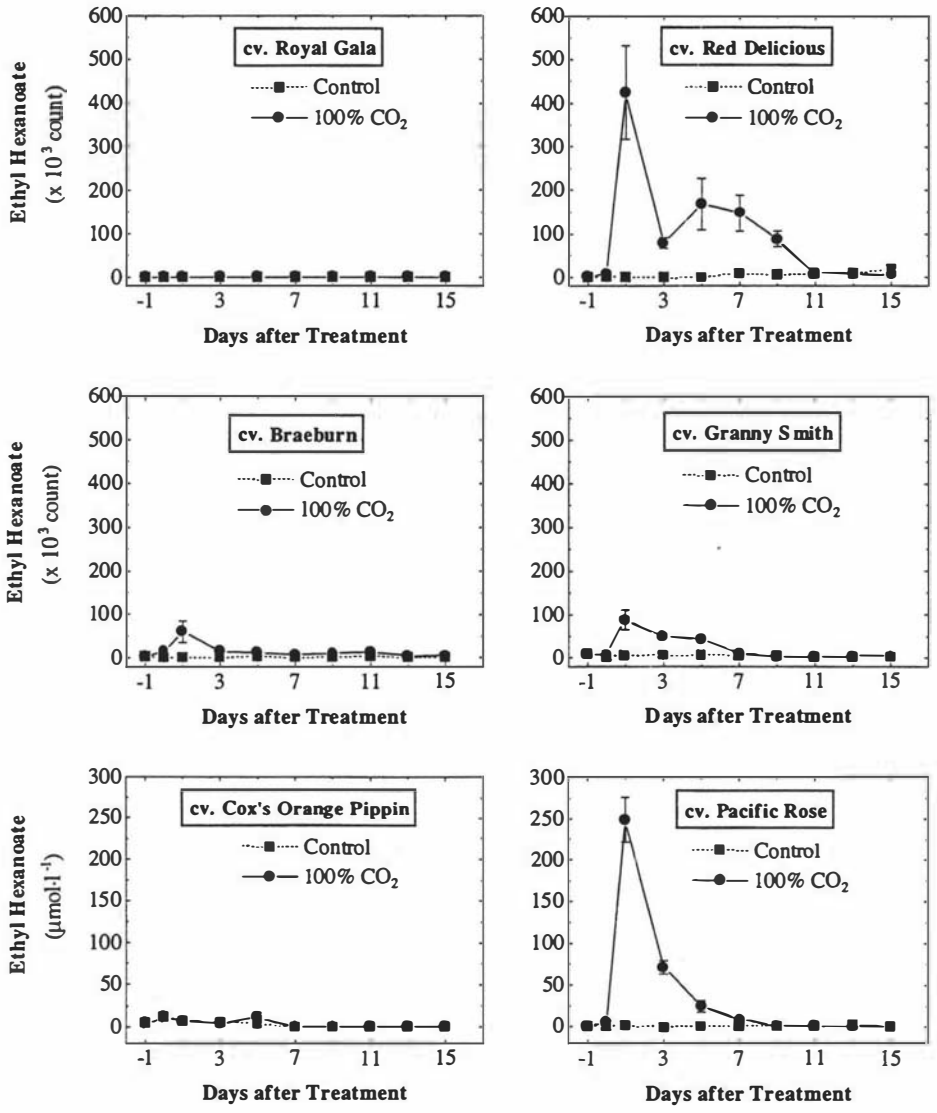


Fig. 6.9 Ethyl hexanoate in 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

Ethyl hexanoate concentration in both control and CO_2 treated 'Cox's Orange Pippin' apples was comparable at low level and it was variable, where it was detected only between -1 and 5 days at 20°C. In control fruit, ethyl hexanoate was generally

negligible or not detected, except in 'Red Delicious' where it tended to increase slightly between 7 and 15 days at 20°C. 'Red Delicious' and 'Pacific Rose' produced relatively greater ethyl hexanoate than the other cultivars after CO_2 treatment (**Fig. 6.9**).

Concentration of butyl acetate followed a similar pattern as hexyl acetate in all cultivars studied, except in 'Granny Smith' apples where butyl acetate was not detected and hexyl acetate was present in a very low concentration (**Fig. 6.10** and **Fig. 6.11**).

Treatment with 100% CO_2 generally depressed production of both butyl acetate and hexyl acetate in all apple cultivars, compared with controls. Butyl acetate and hexyl acetate concentration in CO_2 treated 'Royal Gala' followed a similar pattern to that in control fruit, while concentrations in 'Red Delicious', 'Braeburn' and 'Pacific 'Rose' remained constantly low for 7 days after treatment; before increasing slowly thereafter. Both butyl acetate and hexyl acetate in 'Cox's Orange Pippin' apples remained approximately constant initially from -1 to 1 day after treatment, peaked at 7 days for control fruit and after which they declined; concentrations from CO_2 treated fruit increased moderately through storage but at a lower concentration than control (**Fig. 6.10** and **Fig. 6.11**).

Isoamyl acetate was found in apple cultivars other than 'Cox's Orange Pippin' or 'Pacific Rose' in 1993 (**Fig. 6.12**). Isoamyl acetate concentration in all cultivars was generally depressed by the CO_2 treatment, the degree of depression varying depending on cultivar. 'Red Delicious' and 'Royal Gala' produced the greatest amount of isoamyl acetate.

***Volatile Alcohols*:** Methanol and propan-1-ol were not identified by mass spectrometry in the 1993 experiments due to low concentrations in the concentrated samples, and ethanol could not be quantified because of the ethanol contamination in the solvent used. Thus data for these alcohols for the 1993 experiments were not available, and they are shown only for 'Cox's Orange Pippin' and 'Pacific Rose' apples from 1994.

Methanol concentration in 'Cox's Orange Pippin' apples was slightly enhanced by CO_2 treatment between 1 and 3 days after treatment, but concentration was similar to controls thereafter, while it was comparable in both control and CO_2 treated 'Pacific

Rose' apples. Methanol concentration in 'Cox's Orange Pippin' peaked at day 5, while in 'Pacific Rose' it fluctuated throughout (**Fig. 6.13**).

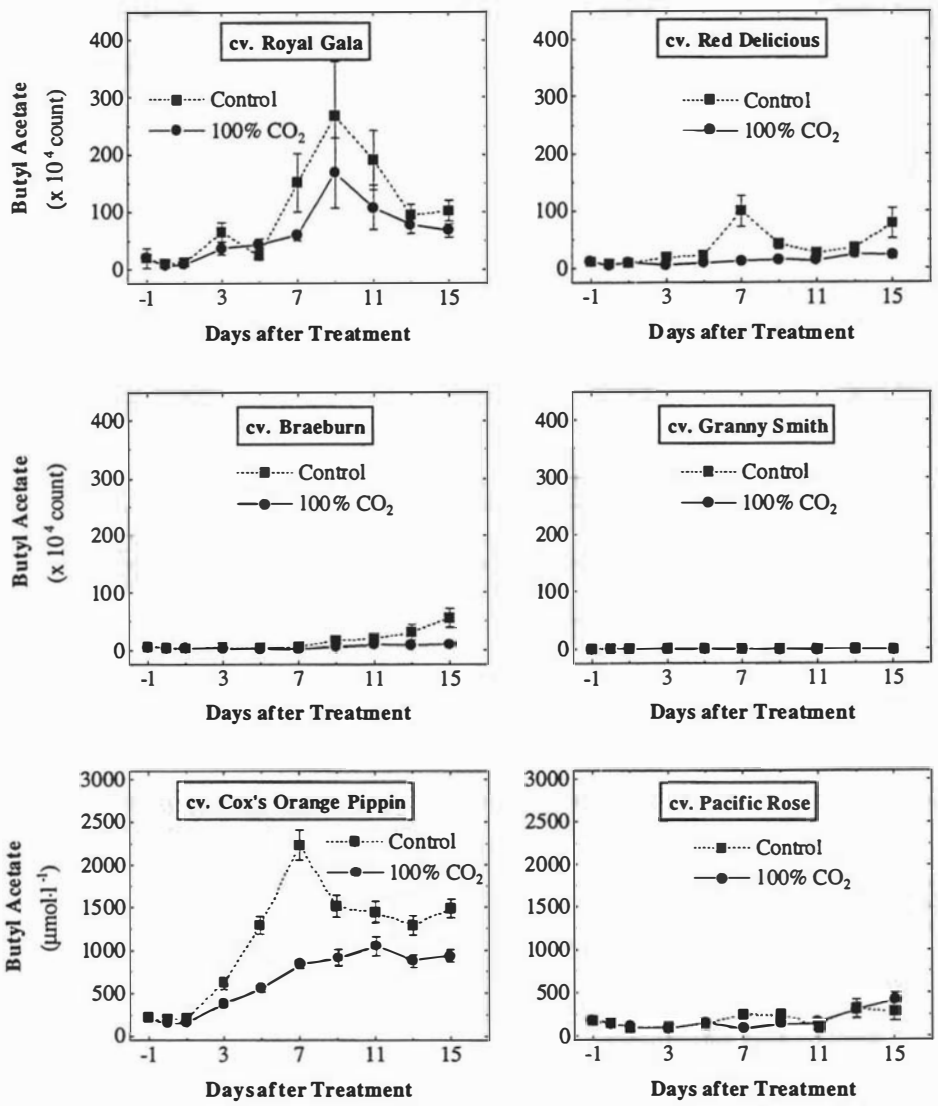


Fig. 6.10 Butyl acetate of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

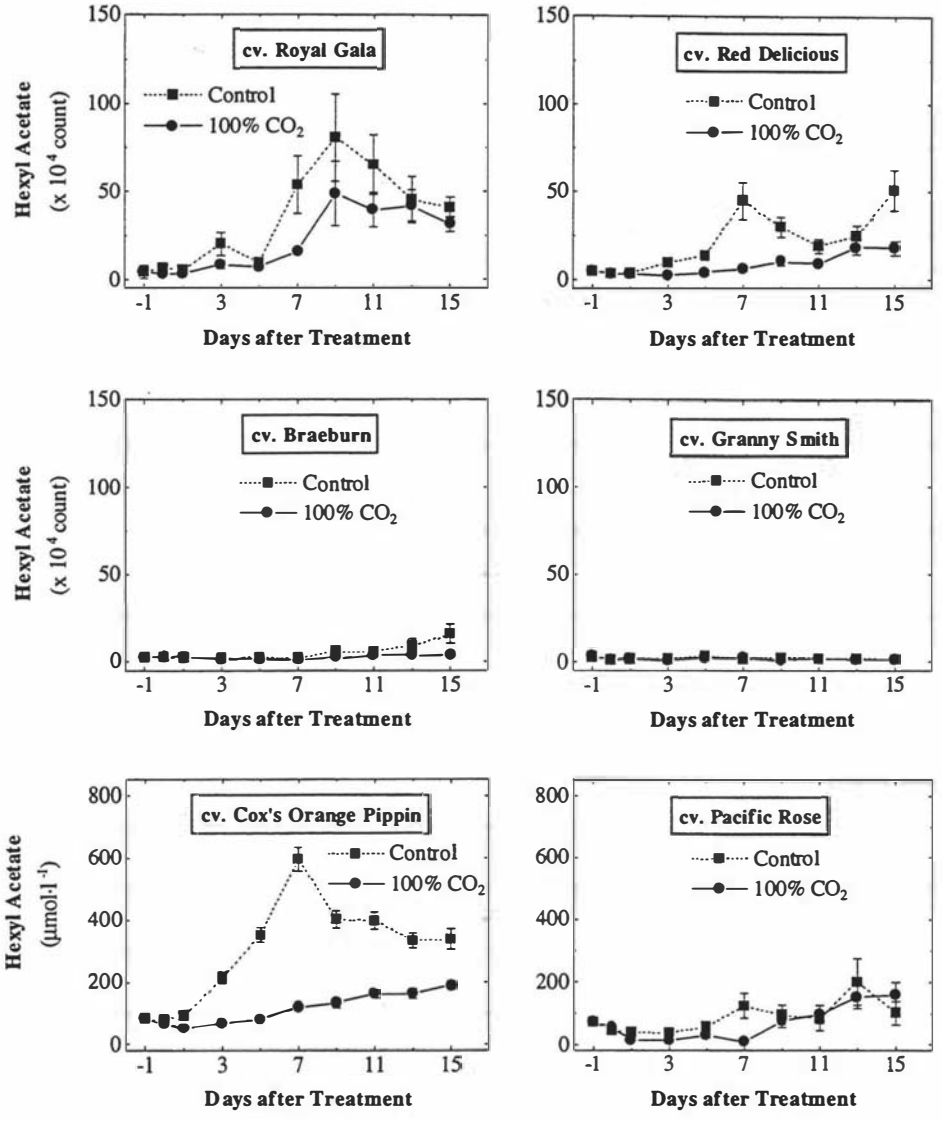


Fig. 6.11 Hexyl acetate of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

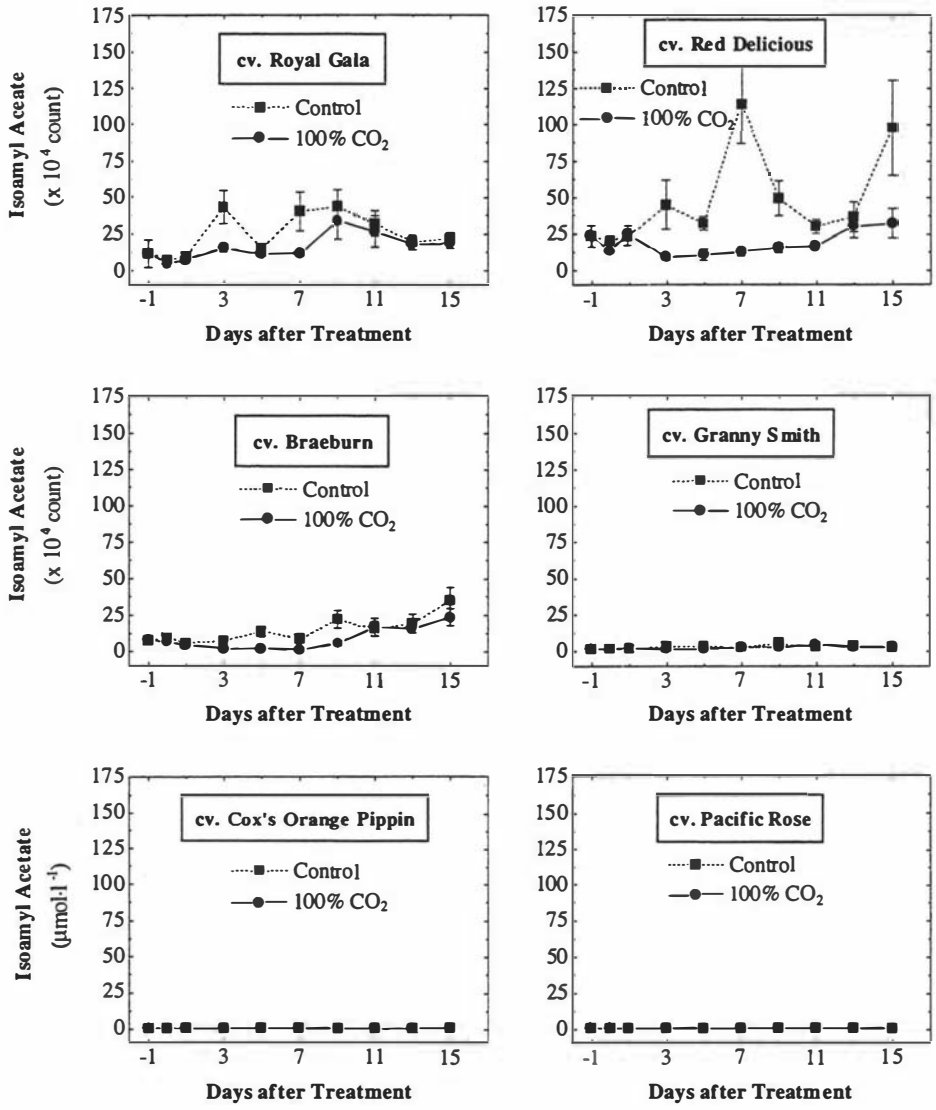


Fig. 6.12 Isoamyl acetate of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

Ethanol concentration was substantially enhanced by the CO_2 treatment, which followed a similar pattern to headspace ethanol (**Fig. 6.5**); peaking at 0 and 1 day and decreasing to a level similar to controls at 13 and 9 days after treatment for 'Pacific

Rose' and 'Cox's Orange Pippin', respectively. Ethanol concentration in control fruit of both cultivars fluctuated at a low level over 15 days at 20°C (**Fig. 6.13**).

Propan-1-ol concentration of 'Pacific Rose' was enhanced by the CO_2 treatment between 0 and 5 days after treatment before decreasing to the same level as in control fruit after 7 days at 20°C (**Fig. 6.13**). Propan-1-ol concentration in CO_2 treated 'Cox's Orange Pippin' was also enhanced between 5 and 9 days, compared with controls, declining to a level comparable to control at 11 days after treatment. In control fruit, propan-1-ol in 'Pacific Rose' generally increased gradually over 15 days at 20°C, while in 'Cox's Orange Pippin' apples very little propan-1-ol was detected. Propan-1-ol production in 'Pacific Rose' was relatively greater than in 'Cox's Orange Pippin' apples (**Fig. 6.13**)

Treatment with 100% CO_2 slightly reduced butan-1-ol concentrations in 'Royal Gala', 'Red Delicious', 'Braeburn', 'Granny Smith' and 'Cox's Orange Pippin' apples. On the other hand, CO_2 treatment enhanced butan-1-ol in 'Pacific Rose' between 1 and 5 days after treatment, compared with control, however it was comparable thereafter (**Fig. 6.14**).

Concentrations of butan-1-ol in 'Royal Gala' and 'Cox's Orange Pippin', in both control and CO_2 treated fruit, generally increased and peaked at 7 - 9 days, then decreased; in the other cultivars it increased slightly or remained approximately constant over 15 days at 20°C (**Fig. 6.14**).

Hexan-1-ol concentration in control fruit of all apple cultivars followed a similar pattern to that of butan-1-ol for the same cultivar. Treatment with 100% CO_2 significantly depressed hexan-1-ol ($P < 0.001$) in 'Cox's Orange Pippin' and slightly reduced it in 'Royal Gala', 'Red Delicious', 'Braeburn' and 'Granny Smith', but not in 'Pacific Rose' apples, where hexan-1-ol was comparable in both treatments (**Fig. 6.15**).

***Volatile C_6 Aldehydes*:** Hexanal and *trans*-2-hexenal were the major aldehydes found in juice from all apple cultivars. Hexanal and *trans*-2-hexenal concentrations in the CO_2 treated 'Cox's Orange Pippin' apples were significantly lower ($P < 0.001$) than in control fruit through 15 days at 20°C. Hexanal and *trans*-2-hexenal concentrations in the other apple cultivars were slightly lower following CO_2 treatment than in controls, particularly

between 0 and 7 days after treatment, however, the overall concentrations were not different. Concentrations of hexanal and *trans*-2-hexenal, in both control and CO_2 treated fruit, generally fluctuated or increased over 15 days at 20°C, depending on cultivar (**Fig. 6.16** and **Fig. 6.17**).

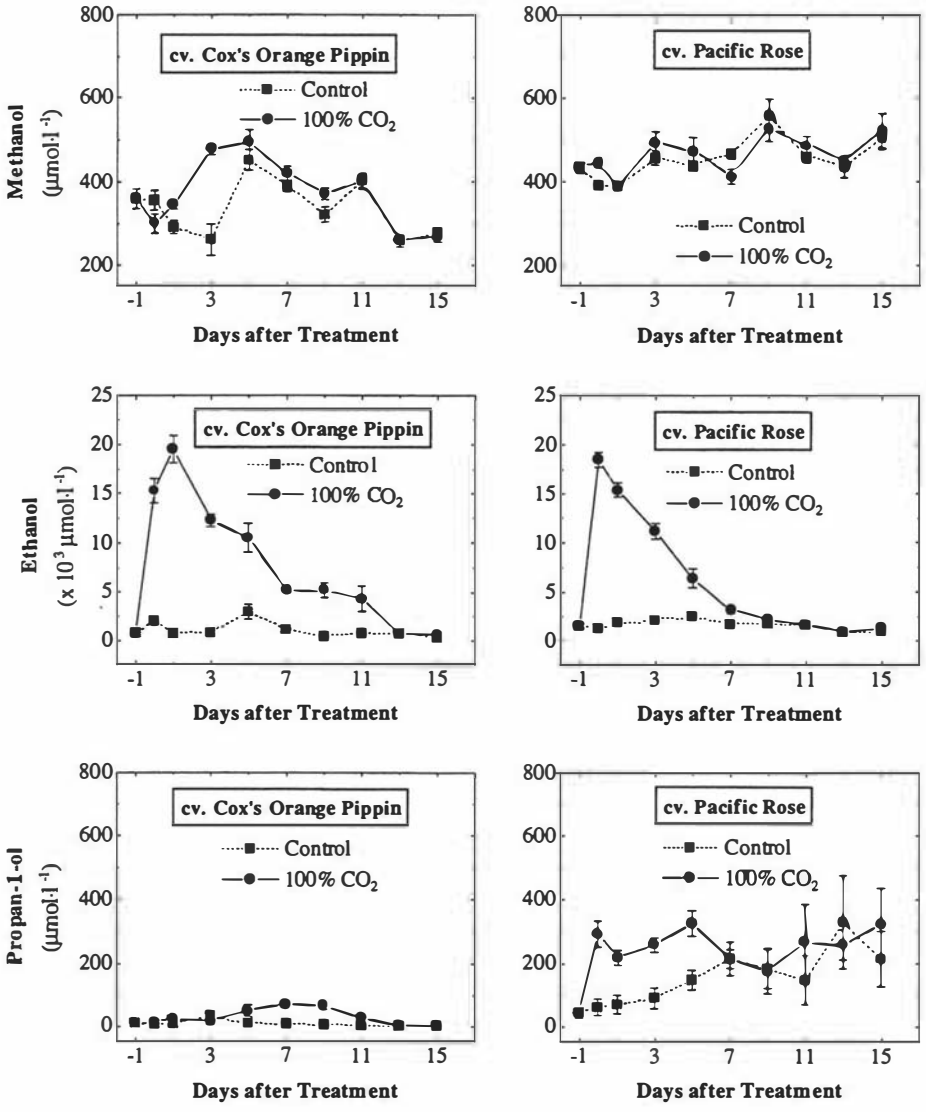


Fig. 6.13 Methanol, ethanol and propan-1-ol of 'Cox's Orange Pippin' and 'Pacific Rose' apples after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

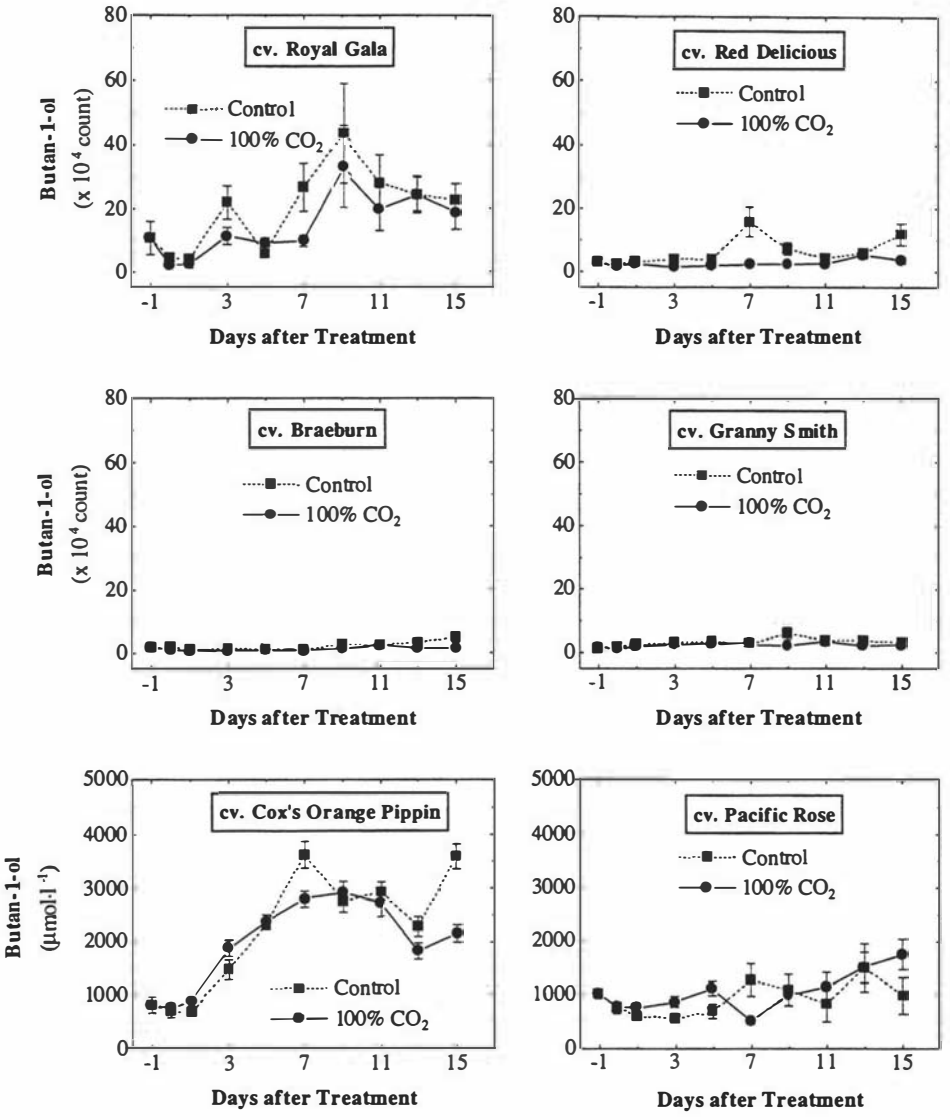


Fig. 6.14 Butan-1-ol of 6 apples cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

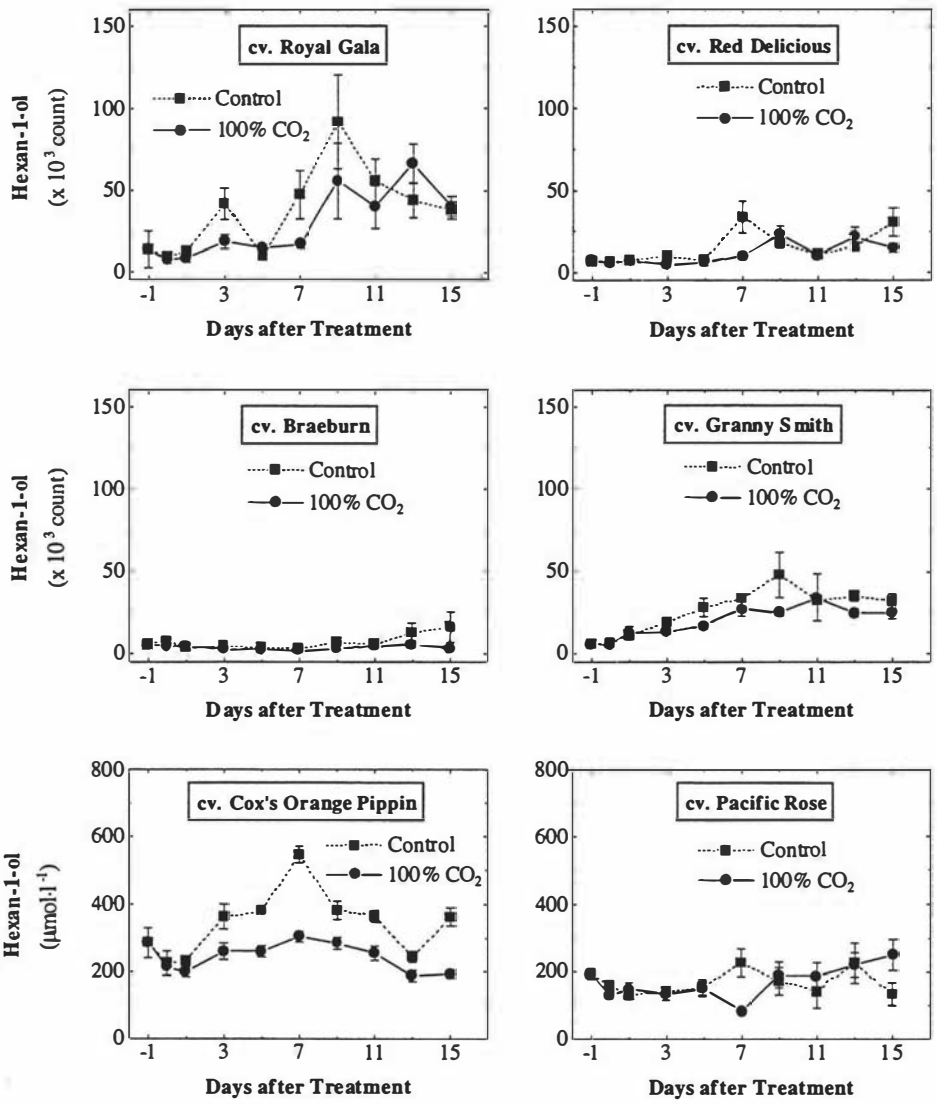


Fig. 6.15 Hexan-1-ol of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

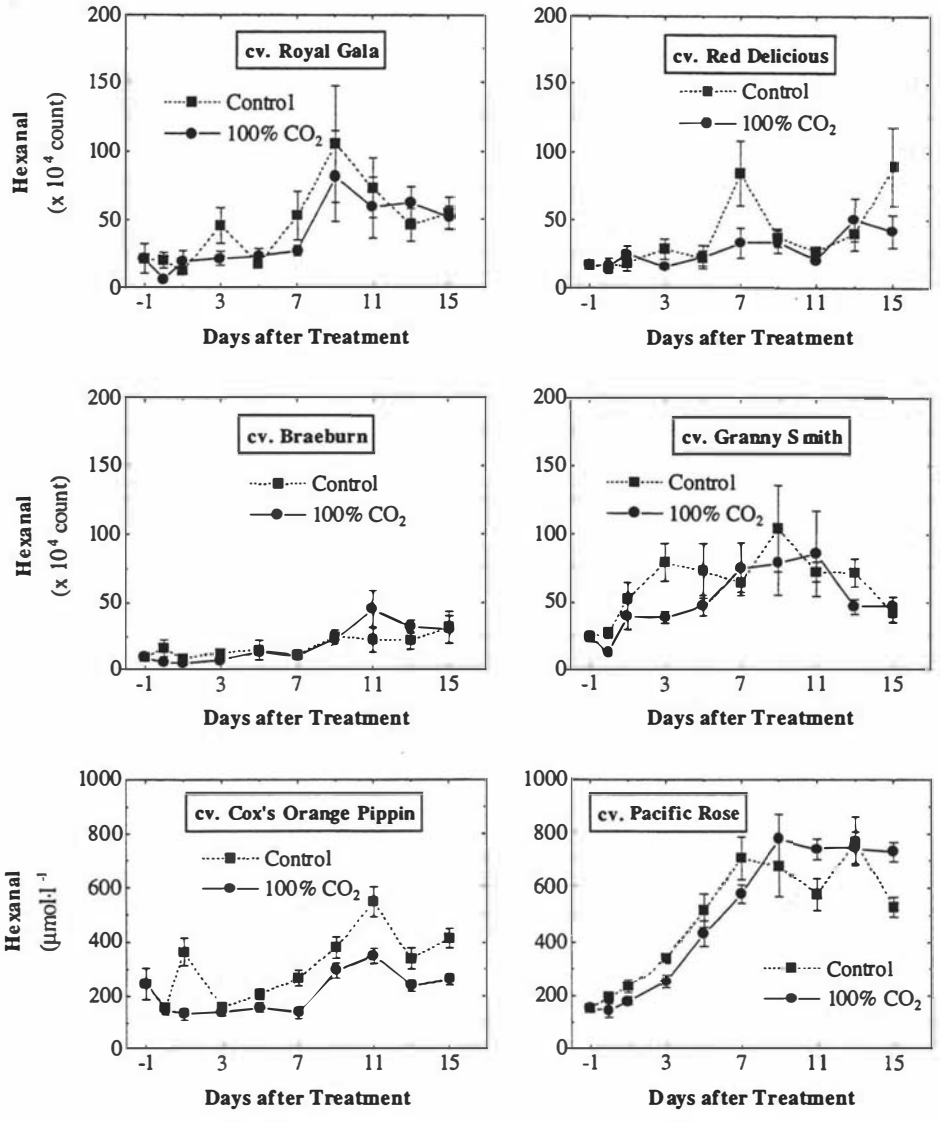


Fig. 6.16 Hexanal of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

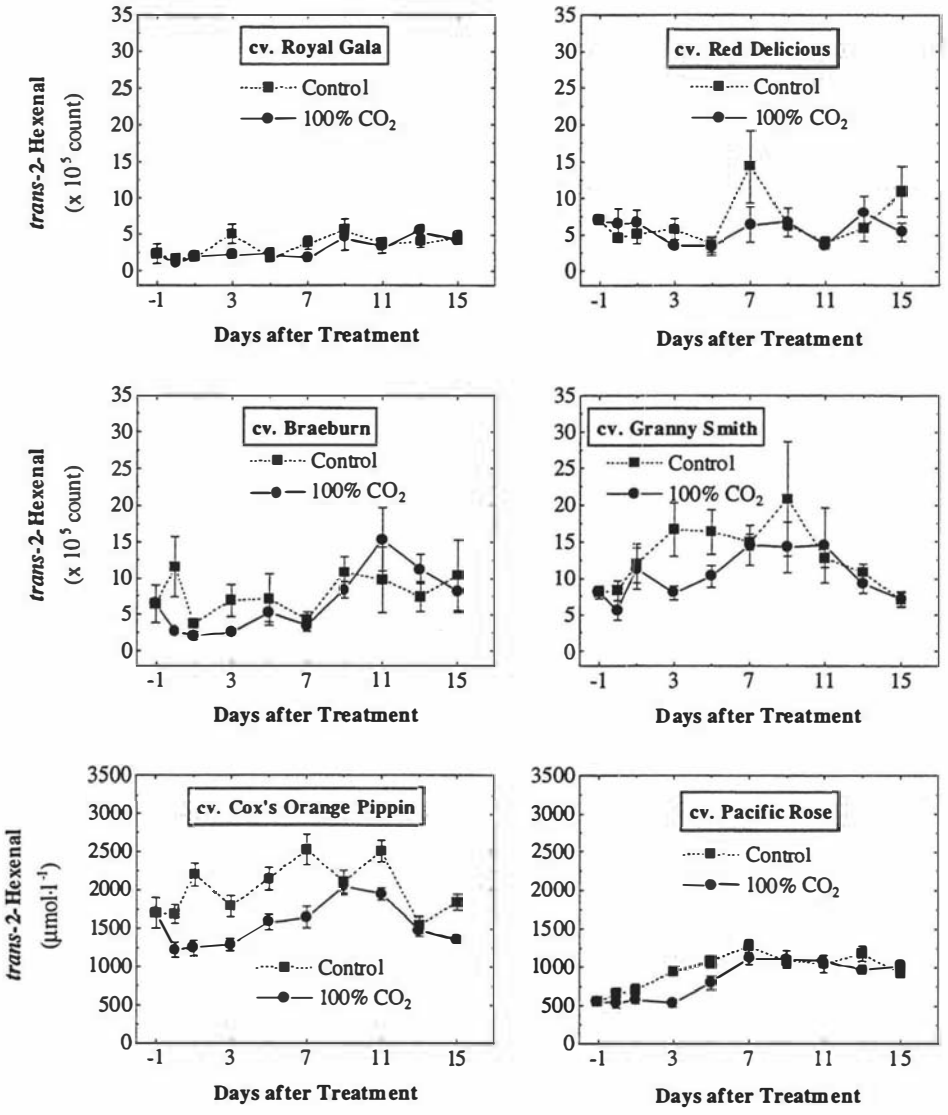


Fig. 6.17 Concentration of *trans*-2-hexenal of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

A summary of the effects of the 100% CO_2 treatment on selected volatile compounds in 6 apple cultivars showed that acetaldehyde, ethanol and ethyl acetate concentrations were greatly enhanced in all cultivars (**Table 6.4**). Ethyl esters were also

substantially enhanced in 'Red Delicious', 'Granny Smith', and 'Pacific Rose'. Ethyl butanoate was only moderately enhanced in 'Cox's Orange Pippin', while ethyl esters other than ethyl acetate were not detected in 'Cox's Orange Pippin' or 'Royal Gala' apples after CO_2 treatment. Propan-1-ol concentration was either slightly or greatly increased in the CO_2 treated apples, depending on cultivar (**Table 6.4**).

Non ethyl esters, in particular butyl acetate, hexyl acetate and isoamyl acetate, were reduced in CO_2 treated fruit, although the degree of reduction was cultivar dependent. Butan-1-ol, hexan-1-ol, hexanal and *trans*-2-hexenal concentrations were generally reduced slightly, moderately or not affected depending on the cultivar (**Table 6.4**).

Table 6.4 Summary of selected volatile compounds in 6 apple cultivars enhanced, reduced or not affected by 100% CO_2 treatment (24 hours at 20°C).

Compound	RG	RD	Bb	GS	COP	PR
ethyl acetate	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
ethyl propanoate	nd	+ + +	+ + +	+ + +	nd	+ + +
ethyl butanoate[1]	nd	+ + +	+ + +	+ + +	+	+ + +
ethyl 2-methyl butanoate	nd	+ + +	+ + +	+ + +	nd	+ + +
ethyl hexanoate	nd	+ + +	+ + +	+ + +	nd	+ + +
butyl acetate	–	– –	– – –	nd	– – –	–
hexyl acetate	–	– –	– –	–	– – –	– –
isoamyl acetate	–	– –	– –	–	nd	nd
methanol[1]	0	0	0	0	+	0
ethanol[1]	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
propan-1-ol[1]	+	+ +	+	+	+ +	+ +
butan-1-ol	–	– –	–	–	0	+
hexan-1-ol	–	–	–	–	– –	0
acetaldehyde[1]	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
hexanal	–	0	–	–	– –	–
trans-2-hexenal	–	0	–	–	– – –	–

Abbreviations and Symbols: RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, COP = Cox's Orange Pippin, PR = Pacific Rose, nd = not detected, slightly enhanced/reduced (+/–), moderately enhanced/reduced (+ +/– –), greatly enhanced/reduced (+ + +/– – –), or comparable to control (0); [1] some data obtained from other experiments in the 1994 and 1995 experiments for RG, RD, Bb, and GS at similar fruit age.

A summary of the relative comparison of the selected volatile compounds measured indicates that concentration of volatiles produced by apples were quantitatively

different among cultivars studied (**Table 6.5**). Of 4 apple cultivars studied in 1993, 'Red Delicious' and 'Royal Gala' produced higher concentrations of some esters, butan-1-ol and hexan-1-ol than did 'Granny Smith' and 'Braeburn'. However, 'Granny Smith' produced the highest concentration of hexanal and *trans*-2-hexenal, of all cultivars in 1993. In 1994, 'Pacific Rose' produced higher concentrations of ethyl esters (except ethyl acetate), methanol, propan-1-ol, and hexanal than did 'Cox's Orange Pippin', which in turn produced more ethyl acetate, butyl acetate, ethanol, butan-1-ol, hexan-1-ol and *trans*-2-hexenal than did 'Pacific Rose' (**Table 6.5**).

Table 6.5 Summary of the relative comparison among the selected volatile compounds in 4 apple cultivars in the 1993 experiments and between 2 cultivars in the 1994 experiments over a period of 15 days at 20°C.

	1993 Experiment*				1994 Experiment*	
Compound	RG	RD	Bb	GS	COP	PR
ethyl acetate	3	2	1	4	H	L
ethyl 2-methyl butanoate	nd	1	3	2	L	H
ethyl hexanoate	nd	1	3	2	L	H
ethyl butanoate	–	–	–	–	L	H
butyl acetate	1	2	3	nd	H	L
hexyl acetate	1	2	3	4	H	L
isoamyl acetate	2	1	3	4	–	–
methanol	–	–	–	–	L	H
ethanol	–	–	–	–	H	L
propan-1-ol	–	–	–	–	L	H
butan-1-ol	1	2	4	3	H	L
hexan-1-ol	1	3	4	2	H	L
acetaldehyde	–	–	–	–	H	L
hexanal	2	3	4	1	L	H
trans-2-hexenal	4	3	2	1	H	L

Abbreviations and Symbols: RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, COP = Cox's Orange Pippin, PR = Pacific Rose, * quantification in peak area (count) for 1993 or in $\mu mol \cdot l^{-1}$ for 1994 experiments, Rank : 1 = highest, 4 = lowest, H = higher, L = lower, – = not available, nd = not detected; Data obtained by integration of concentration of each compound from the 100% CO_2 treatment over 15 days at 20°C before ranking.

6.4.3 Fruit Quality Attributes

Fruit quality attributes of apple cultivars measured were fruit weight loss, firmness, skin colour, TSS, TA, TSS:TA ratio and pH, as well as fruit injury caused by the 100% CO_2 treatment.

Cumulative weight loss of apples generally increased linearly with time over 15 days at 20°C and 70% RH. Treatment with 100% CO_2 had no effect on fruit weight loss of any cultivar. The highest to the lowest fruit weight loss rate (per cent per day) in the 6 apple cultivars were in the order of 'Pacific Rose', 'Cox's Orange Pippin', 'Braeburn', 'Granny Smith', 'Red Delicious' and 'Royal Gala', respectively (**Table 6.6**).

Flesh firmness of apples generally decreased with time in storage at 20°C, but the general pattern of change was different among cultivars and 'Pacific Rose' did not follow the general pattern. Apple cultivars lost firmness linearly with time and the highest to the lowest rate of firmness loss (Newtons per day) was in the order of 'Cox's Orange Pippin', 'Red Delicious' 'Braeburn', 'Granny Smith' and 'Royal Gala', respectively (**Table 6.7**). Flesh firmness of 'Granny Smith' apples increased between 3 and 5 days, as did control fruit of 'Braeburn' which increased from 0 to 3 days, before decreasing thereafter. 'Pacific Rose' apples showed very high variation in flesh firmness, possibly due to variation in fruit size and it appeared to have no obvious pattern of change over 15 days storage. (**Table 6.7**).

Treatment with 100% CO_2 exerted different effects on firmness of apple cultivars. CO_2 treated 'Red Delicious', 'Cox's Orange Pippin', and 'Pacific Rose' apples was slightly firmer than control fruit ($P < 0.05$), but there was no treatment effect in 'Royal Gala'. However, CO_2 treated 'Braeburn' and 'Granny Smith' apples was markedly or slightly softer than controls ($P < 0.05$), respectively, where in this case of 'Braeburn' the softening was presumable directly associated with fruit injury that occurred following CO_2 treatment (**Table 6.7**).

Exposure of apples to 100% CO_2 did not affect fruit skin colour, measured as either lightness or hue angle, in any cultivar. Skin lightness and hue angle of apples generally fluctuated, increased or decreased slightly, depending on cultivar (**Table 6.8**).

Table 6.6 Fruit weight loss in 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

Day after Treat.	Cultivar / weight Loss (%)											
	RG		RD		Bb		GS		COP		PR	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
-1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0	0.3	0.1	0.3	0.2	0.5	0.2	0.2	0.1	0.4	0.4	0.3	0.1
1	0.4	0.3	0.5	0.5	0.9	0.5	0.5	0.3	0.9	0.8	0.7	0.5
3	1.1	0.9	1.0	1.0	1.5	1.4	1.1	0.9	1.4	1.5	1.4	1.1
5	1.4	1.4	1.4	1.5	2.0	2.0	1.7	1.6	2.1	2.0	2.0	1.7
7	1.9	1.8	1.8	1.9	2.5	2.8	2.1	2.1	2.6	2.8	2.7	2.4
9	2.5	2.2	2.1	2.5	3.0	3.4	2.6	2.7	3.3	3.4	2.8	3.2
11	2.7	2.9	2.9	2.9	3.5	3.5	3.1	3.2	3.9	3.7	3.5	3.4
13	3.2	3.3	3.2	3.5	4.0	4.0	3.5	3.6	4.6	4.5	5.1	4.3
15	3.7	3.7	3.7	3.9	4.9	4.9	4.0	4.1	4.8	4.9	4.7	5.0
c.v.	8.1%		9.9%		11.4%		5.2%		14.6%		20.2%	
Day	* * *		* * *		* * *		* * *		* * *		* * *	
Treat	ns		ns		ns		ns		ns		ns	
D x T	*		ns		*		*		ns		* *	
Rate	0.23		0.24		0.29		0.26		0.31		0.32	
r^2	0.99		0.98		0.96		0.99		0.95		0.92	

Abbreviations and Symbols: RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, COP = Cox's Orange Pippin, PR = Pacific Rose, Ctrl = control, CO_2 = 100% CO_2 24 hours at 20°C, c.v. = coefficient of variation, Treat = treatment, D x T = day x treatment interaction, Rate = weight loss rate in per cent per day, r^2 = coefficient of determination of linear regression analysis; Levels of significance at $P = 0.05$ (*), 0.01 (* *), 0.001 (* * *), non significant (ns).

CO_2 treatment had no effect on TSS, TA, TSS:TA ratio or pH in 'Red Delicious' and 'Cox's Orange Pippin' apples (**Table 6.9** and **Table 6.10**). However, CO_2 treated fruit of 'Royal Gala', 'Granny Smith', and 'Pacific Rose' had slightly lower TA contents than in controls ($P < 0.05$). TSS:TA ratio was slightly higher ($P < 0.05$) in CO_2 treated 'Granny Smith' and 'Pacific Rose' apples than in control fruit. Juice pH was significantly greater in CO_2 treated 'Granny Smith' than in controls ($P < 0.001$).

In 'Braeburn' apples, the TSS, TA, TSS:TA ratio and pH were severely affected by the 100% CO_2 treatment (**Table 6.9** and **Table 6.10**). TSS content was slightly lower ($P < 0.05$), and TA was greatly reduced ($P < 0.001$), resulting in a significantly higher ($P < 0.001$) TSS:TA ratio and pH of the treated apples compared to controls. This

appeared to be directly related to fruit injury (**Plate 6.1**) which resulted from the hypoxic CO_2 treatment in this cultivar in 1993.

Although changes in fruit quality attributes occurred during the 15 days storage at 20°C, only a few attributes in some cultivars had obvious patterns of change (**Table 6.9** and **Table 6.10**). TSS increased linearly with time of storage in 'Royal Gala' and 'Red Delicious' apples, while other cultivars showed no apparent pattern. TA decreased, while TSS:TA ratio increased in a linear manner during storage in 'Royal Gala' and 'Cox's Orange Pippin' apples. Juice pH of 'Royal Gala', 'Granny Smith' and 'Cox's Orange Pippin' apples also increased linearly with time at 20°C.

Table 6.7 Flesh firmness of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

Day after Treat	Cultivar / Firmness (Newton)											
	RG		RD		Bb		GS		COP		PR	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
-1	96.7	96.7	91.3	91.3	84.5	84.5	79.6	79.6	76.9	76.9	64.0	64.0
0	97.8	90.2	88.6	91.0	88.2	81.7	78.9	77.9	78.5	73.5	66.5	65.3
1	95.6	92.5	83.0	85.4	89.2	90.1	77.4	76.6	76.1	78.1	66.3	64.9
3	91.1	93.0	80.1	85.7	89.7	82.3	79.7	79.7	72.1	69.4	67.1	68.1
5	90.2	90.6	81.3	84.4	87.9	78.2	83.7	82.2	62.2	71.4	63.4	68.1
7	81.7	81.0	77.8	82.4	86.9	77.1	83.1	80.1	57.0	64.5	62.9	69.6
9	77.1	76.8	75.4	78.7	86.3	77.5	79.5	77.1	52.1	59.1	62.8	64.5
11	70.2	66.6	71.6	74.1	83.7	74.4	78.6	75.4	53.3	57.6	64.6	69.6
13	68.8	66.1	70.7	70.5	81.6	72.7	74.4	73.1	52.1	55.4	66.1	67.4
15	68.8	66.6	69.6	67.7	73.6	64.6	74.6	72.1	52.2	55.3	65.8	66.8
c.v.	5.2%		3.4%		3.4%		3.3%		8.2%		8.6%	
Day	* * *		* * *		* * *		* * *		* * *		ns	
Treat	ns		*		* * *		ns		* * *		*	
D x T	ns		ns		* * *		ns		* * *		ns	
Rate	-0.20		-1.34		-1.02		-0.36		-1.72		0.07	
r^2	0.87		0.87		0.56		0.30		0.79		0.004	

Abbreviations and Symbols: RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, COP = Cox's Orange Pippin, PR = Pacific Rose, Ctrl = control, CO_2 = 100% CO_2 24 hours at 20°C, c.v. = coefficient of variation, Treat = treatment, D x T = day x treatment interaction, Rate = firmness loss rate in Newton per day, r^2 = coefficient of determination of linear regression analysis; Levels of significance at $P = 0.05$ (*), 0.01 (* *), 0.001 (* * *), non significant (ns).

Table 6.8 Fruit skin lightness and hue angle of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C.

Day after Treat	Cultivar / Skin Lightness (%)											
	RG		RD		Bb		GS		COP		PR	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
-1	55	55	46	46	56	56	61	61	57	57	47	47
0	52	54	49	47	56	56	62	61	58	59	49	50
1	54	54	47	48	56	55	62	62	57	57	47	49
3	51	52	49	49	56	55	61	62	60	59	53	52
5	56	56	46	47	56	56	62	62	63	60	53	51
7	56	55	44	48	56	55	64	64	61	60	53	54
9	55	57	47	47	55	54	65	65	63	62	50	52
11	56	57	48	47	57	56	66	65	63	62	51	52
13	55	55	47	49	57	56	66	68	63	65	53	52
15	56	56	48	47	56	55	67	68	64	65	52	54
c.v.	4.6%		4.5%		2.5%		1.7%		6.9%		8.6%	
Day	* *		ns		*		* * *		* * *		* * *	
Treat	ns		ns		ns		ns		ns		ns	
D x T	ns		ns		ns		ns		ns		ns	
Rate	0.14		0.02		0.06		0.41		0.44		0.31	
r^2	0.09		0.003		0.05		0.81		0.26		0.12	
	Hue Angle (°)											
-1	48	48	43	43	80	80	113	113	83	83	31	31
0	42	44	49	49	78	79	112	111	86	88	38	42
1	46	46	46	49	76	76	112	112	87	88	37	39
3	41	42	51	53	78	77	111	111	86	85	49	43
5	52	51	44	47	78	81	111	111	94	84	50	43
7	53	52	42	49	79	83	111	111	88	86	49	55
9	51	54	50	49	79	78	110	111	88	85	44	48
11	53	52	48	49	79	80	109	108	86	82	45	49
13	51	51	46	51	80	79	109	108	84	86	49	47
15	53	51	49	47	78	76	107	107	82	84	46	51
c.v.	10.4%		9.8%		4.5%		1.0%		11.1%		22.7%	
Day	* *		*		ns		* * *		ns		* * *	
Treat	ns		ns		ns		ns		ns		ns	
D x T	ns		ns		ns		ns		ns		ns	
Rate	0.40		0.04		0.03		-0.32		-0.13		0.84	
r^2	0.16		0.009		0.003		0.71		0.005		0.16	

Abbreviations and Symbols: RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, COP = Cox's Orange Pippin, PR = Pacific Rose, Ctrl = control, CO_2 = 100% CO_2 24 hours at 20°C, c.v. = coefficient of variation, Treat = treatment, D x T = day x treatment interaction, Rate = per cent or degree per day, r^2 = coefficient of determination of linear regression analysis; Levels of significance at $P = 0.05$ (*), 0.01 (* *), 0.001 (* * *), non significant (ns).

Table 6.9 TSS and TA of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20°C and during subsequent storage at 20°C and 70% RH.

Day after Treat	Cultivar / TSS (%)											
	RG		RD		Bb		GS		COP		PR	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
-1	10.9	10.9	13.3	13.3	11.2	11.2	13.1	13.1	11.3	11.3	12.7	12.7
0	11.2	11.7	13.2	13.3	11.7	11.3	13.0	12.8	11.6	11.4	13.1	12.9
1	11.5	11.7	13.5	13.4	11.8	11.4	12.6	12.8	12.1	11.3	13.4	12.7
3	12.3	12.0	14.3	13.8	12.2	11.9	13.3	13.0	12.2	11.4	13.6	13.4
5	12.5	12.3	14.5	14.7	12.2	11.3	13.4	13.0	11.7	11.8	13.3	13.1
7	12.5	12.5	14.8	14.9	12.4	11.4	13.1	13.1	12.2	12.0	13.5	13.6
9	12.7	12.6	15.1	15.3	12.8	11.6	13.6	12.9	11.7	12.3	13.1	13.4
11	12.7	12.3	15.4	15.6	12.4	11.5	13.1	13.3	12.2	12.0	13.6	13.4
13	13.1	13.2	15.5	15.8	12.6	11.6	13.0	13.0	12.1	12.7	14.3	13.6
15	13.4	13.5	15.7	15.8	12.5	11.9	13.1	13.1	12.5	11.8	13.8	13.5
c.v.	3.3%		2.3%		3.8%		2.4%		5.2%		6.9%	
Day	* * *		* * *		* * *		*		* * *		* *	
Treat	ns		ns		*		ns		ns		ns	
D x T	ns		ns		ns		ns		* * *		ns	
Rate	0.12		0.15		0.04		0.01		0.05		0.05	
r^2	0.72		0.80		0.16		0.03		0.16		0.09	
	TA(%)											
-1	0.47	0.47	0.16	0.16	0.34	0.34	0.56	0.56	0.76	0.76	0.23	0.23
0	0.46	0.29	0.17	0.19	0.31	0.31	0.57	0.44	0.79	0.73	0.22	0.20
1	0.43	0.45	0.21	0.20	0.33	0.28	0.59	0.59	0.79	0.77	0.24	0.20
3	0.42	0.40	0.17	0.23	0.28	0.22	0.66	0.62	0.70	0.70	0.23	0.23
5	0.39	0.37	0.20	0.19	0.33	0.21	0.65	0.59	0.69	0.61	0.24	0.22
7	0.39	0.35	0.22	0.22	0.33	0.18	0.69	0.69	0.63	0.65	0.22	0.23
9	0.27	0.22	0.18	0.21	0.35	0.23	0.49	0.52	0.59	0.61	0.21	0.21
11	0.21	0.22	0.26	0.14	0.33	0.21	0.57	0.46	0.59	0.55	0.23	0.22
13	0.20	0.26	0.27	0.23	0.31	0.17	0.59	0.53	0.58	0.55	0.21	0.21
15	0.21	0.18	0.23	0.25	0.32	0.19	0.53	0.43	0.53	0.51	0.21	0.21
c.v.	12.3%		14.5*		13.9%		14.4%		9.6%		13.5%	
Day	* * *		* * *		* * *		* * *		*		ns	
Treat	*		ns		* * *		*		ns		*	
D x T	* * *		ns		* * *		ns		ns		ns	
Rate	-0.02		0.004		-0.003		-0.004		0.02		-0.001	
r^2	0.68		0.31		0.08		0.04		0.63		0.02	

Abbreviations and Symbols: RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, COP = Cox's Orange Pippin, PR = Pacific Rose, Ctrl = control, CO_2 = 100% CO_2 24 hours at 20°C, c.v. = coefficient of variation, Treat = treatment, D x T = day x treatment interaction, Rate = per cent per day, r^2 = coefficient of determination of linear regression analysis; Levels of significance at P = 0.05 (*), 0.01 (* *), 0.001 (* * *), non significant (ns).

Table 6.10 TSS:TA ratio and pH of 6 apple cultivars after treatment with 100% CO_2 for 24 hours at 20C and during subsequent storage at 20C and 70% RH.

Day after Treat	Cultivar / TSS:TA Ratio											
	RG		RD		Bb		GS		COP		PR	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
-1	23.1	23.1	82.9	82.9	33.0	33.0	23.6	23.6	15.0	15.0	57.0	57.0
0	24.7	40.9	78.0	71.1	37.5	36.6	22.8	30.0	14.9	15.8	60.2	64.1
1	27.1	26.0	65.7	71.4	36.9	41.6	21.7	22.1	15.4	14.8	55.0	64.7
3	29.1	30.3	88.5	66.4	43.4	54.4	20.7	21.1	17.6	16.5	58.9	58.5
5	32.6	33.2	75.1	77.3	38.2	54.1	21.1	23.0	14.1	19.5	57.2	62.1
7	32.2	35.7	69.3	69.1	38.6	63.9	19.2	19.0	19.6	18.4	63.2	59.6
9	48.1	60.4	88.9	75.5	35.2	54.2	28.0	25.1	19.9	20.3	62.4	56.7
11	63.5	58.3	60.4	66.3	38.5	53.9	23.4	29.6	20.9	21.8	61.0	61.5
13	66.7	54.2	57.8	70.1	41.3	73.0	22.2	25.1	21.0	23.4	68.1	67.8
15	69.9	79.4	65.8	64.3	40.4	65.5	24.6	30.3	23.9	23.0	67.3	66.8
c.v.	23.7%		14.5%		14.7%		15.8%		10.7%		12.3%	
Day	* * *		*		* * *		* * *		* * *		* * *	
Treat	ns		ns		* * *		*		ns		*	
D x T	ns		ns		* * *		ns		*		ns	
Rate	2.56		-0.63		0.85		0.16		0.54		0.52	
r^2	0.62		0.09		0.15		0.05		0.67		0.12	
	pH											
-1	3.84	3.84	3.97	3.97	3.65	3.65	3.55	3.55	3.35	3.35	–	–
0	3.83	3.95	4.03	4.01	3.69	3.70	3.54	3.60	3.33	3.36	–	–
1	3.84	3.83	4.00	4.03	3.68	3.76	3.53	3.57	3.34	3.36	–	–
3	3.84	3.85	4.05	4.01	3.71	3.85	3.52	3.53	3.36	3.38	–	–
5	3.83	3.86	4.02	4.05	3.69	3.90	3.53	3.54	3.40	3.39	–	–
7	3.88	3.92	4.02	4.02	3.66	3.95	3.53	3.54	3.37	3.37	–	–
9	3.93	3.98	4.06	4.06	3.67	3.87	3.64	3.60	3.36	3.38	–	–
11	3.99	3.98	4.03	4.05	3.68	3.90	3.61	3.65	3.37	3.38	–	–
13	3.98	3.96	4.01	4.06	3.71	3.99	3.61	3.64	3.47	3.46	–	–
15	3.99	4.03	4.03	4.05	3.70	3.95	3.65	3.68	3.47	3.46	–	–
c.v.	1.0%		0.8%		1.1%		0.7%		0.9%		–	
Day	* * *		* * *		* * *		* * *		* * *		–	
Treat	ns		ns		* * *		* * *		ns		–	
D x T	ns		ns		* * *		ns		ns		–	
Rate	0.01		0.002		0.008		0.007		0.006		–	
r^2	0.58		0.16		0.14		0.53		0.47		–	

Abbreviations and Symbols: RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, COP = Cox's Orange Pippin, PR = Pacific Rose, Ctrl = control, CO_2 = 100% CO_2 24 hours at 20°C, c.v. = coefficient of variation, Treat = treatment, D x T = day x treatment interaction, Rate = unit per day, r^2 = coefficient of determination of linear regression analysis; – = not determined, Levels of significance at $P = 0.05$ (*), 0.01 (* *), 0.001 (* * *), non significant (ns).

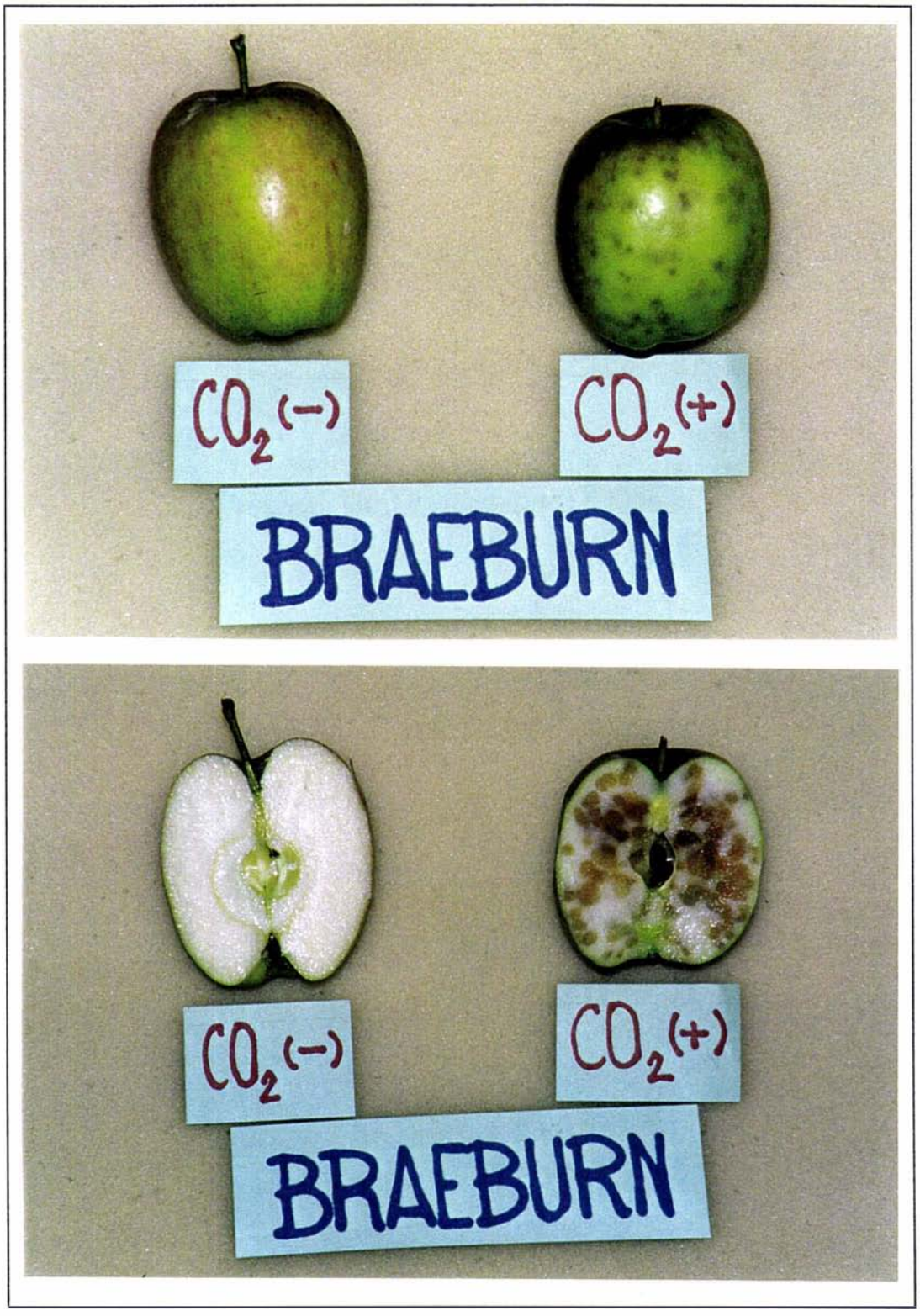


Plate 6.1 Fruit injury caused by 100% CO_2 treatment (24 hours at 20°C) in 'Braeburn' apples.

***'Braeburn' Fruit Injury*:** Severe fruit injury development as a result from 100% CO_2 treatment occurred in 'Braeburn' apples in the 1993 experiment, while there was no injury found in this cultivar in the 1994 experiment (**Chapter Four**) nor in the other cultivars studied. The injury symptom appeared immediately on removal of 'Braeburn' apples from 100% CO_2 (24 hours at 20°C) atmosphere and it was noticeable both on the skin and in flesh or core tissues (**Plate 6.1**).

The symptom on the skin occurred as scattered light brown discoloured, irregular spots about 2 - 4 mm in diameter without any obvious pattern. These spots became dark brown within 24 hours and slightly sunken after 5 days in air. Injury symptom in the flesh and core tissues initially appeared as light brown, water-soaked spots, but they were quite noticeable through the skin and there was no pattern or particular location in these affected tissues. Some of these brown spots in the flesh tissue became dry at the centre, creating small cavities after 7 days at 20°C, but the majority remained dark brown and did not spread further into adjacent unaffected tissue through 15 days storage (**Plate 6.1**).

6.5 DISCUSSION

Ripening of climacteric fruit is usually associated with a climacteric rise in ethylene production and respiration rate, where autocatalytic ethylene production is essential for the ripening process; it triggers the respiration climacteric and ripening proceeds (Brady, 1987; Oetiker and Yang, 1995; Pratt and Geoschl, 1969). The increase in ethylene production and respiration rate associated with ripening has been investigated frequently (Mattheis *et al.*, 1991b; Yang and Hoffman, 1984) and has been used as a benchmark in establishing the maturity of climacteric fruit such as apples and pears (Lieberman, 1979). During ripening, the activity of several hydrolytic enzymes increase which lead to biosynthesis and/or degradation of macro-molecules including carbohydrates, proteins and lipids resulting in accumulation of metabolites, precursors and substrates, such as free fatty acids, amino acids and sugars used for volatile production (Paillard, 1981; Tressl *et al.*, 1975). The rate of degradation of phospholipids into free fatty acids increased during ripening in 'Cox's Orange Pippin' apples (Bartley,

1985) which were further oxidised into straight-chain aroma volatile compounds in fruit (Bartley *et al.*, 1985). A several fold increase in transaminase activity was noted in mitochondria of apples at the climacteric (Hulme *et al.*, 1967), where this reaction was involved in biosynthesis of amino acid, which could be further converted into branched-chain volatile compounds in apples (Tressl *et al.*, 1975).

Apple and pear aromas generally develop during ripening in which the total volatile production increases and reaches a maximum coincident with the climacteric, production decreases as fruit senesce (Dirinck *et al.*, 1989; Paillard, 1990). In the present study, increases in some volatile compounds such as butyl acetate, hexyl acetate, butan-1-ol, hexan-1-ol and hexanal in control apples during 15 days storage at 20°C seemed to coincide with an increase in ethylene production of the fruit. In general, as ethylene production increased these volatile compounds in control fruit also temporally increased. However, these volatiles reached their maximum concentrations slightly before, at the same time or slightly after ethylene concentration attained its peaks in 'Cox's Orange Pippin', 'Braeburn' and 'Royal Gala', respectively. Previous studies also indicated that alcohols, such as butan-1-ol and hexan-1-ol, and esters, including propyl acetate, butyl acetate, hexyl acetate and butyl butanoate increased to peaks at the climacteric (Paillard, 1990). Changes in total volatiles and total esters followed similar patterns in different cultivars (Brown *et al.*, 1966; Dirinck and Schamp, 1989; Song and Bangerth, 1996). Dirinck and Schamp (1989) found that an increase in butyl acetate concentration in 'Golden Delicious' apples corresponded with an increase in internal ethylene concentration. At the climacteric rise of internal ethylene in 'Bisbee Delicious' apples, Mattheis *et al.* (1991b) found that butanal and pentanal disappeared from the headspace samples with a concomitant appearance of 2-methyl butan-1-ol, butyl acetate, 2-methyl butyl acetate, butyl propanoate, pentyl acetate, butyl butanoate and hexyl 2-methyl butanoate. Song and Bangerth (1996) found that production trends of straight-chain butyl acetate and hexyl acetate, and branched-chain 2-methyl butyl acetate (but not ethyl 2-methyl butanoate), as well as total volatiles, reaching maximum levels at approximately the same time as a peak production of internal ethylene. Results from the present study and those reported suggested that production of aroma volatile compounds is in some way related to climacteric rise in ethylene production or ripening of apples. However it is

likely that this association is indirect, as exogenous application of ethylene (1000 ppm for 24 hours at 20°C) did not increase aroma volatile compounds over controls (**Chapter Four**).

Ethylene may be indirectly involved with aroma volatile production through its effects on triggering ripening of fruit leading to an increase in general metabolic activity which subsequently provides precursors and/or substrates for aroma volatile synthesis. Song and Bangerth (1996) suggested that a more general (autocatalytic ethylene production, respiration and subsequent metabolism) and not a specific increase in metabolic activity is a pre-requisite for the stimulation of aroma volatile production in climacteric fruit.

The coincidence of an increase in some volatile compounds such as butyl acetate, hexyl acetate and 2-methyl butyl acetate with an increase in ethylene production leads to a suggestion that production of these volatile compounds may used as a harvest index to obtain optimum maturity of apples (Dirinck *et al.*, 1989; Mattheis *et al.*, 1991b; Song and Bangerth, 1996). However, this clearly can not be applied universally for every apple cultivar, because butyl acetate was not detected in 'Granny Smith' apples in the present study. Furthermore, different compounds were suggested for different cultivars, such as butyl acetate for 'Golden Delicious' (Dirinck and Schamp, 1989) and 2-methyl butyl acetate for 'Bisbee Delicious' apples (Mattheis *et al.*, 1991b). Analysis of volatile compounds requires sensitive and sophisticated instruments, it is a time consuming and expensive process, compared with determining other harvest indices such as ethylene production, starch index, firmness, colour, TSS or TA. At present, the practical implication of using a certain aroma volatile compound as a benchmark for establishing apple fruit maturity is limited, particularly for apple growers.

While esters, alcohols, aldehydes and hydrocarbons were present as aroma volatile compounds in 6 apple cultivars, there were quantitative and qualitative differences between cultivars. Esters and alcohols with straight-chain and even-numbered carbon atoms were found to predominate in the volatile fraction, while those with branched-chain and odd-numbered carbons were less dominant. Previous studies with other commercial apple cultivars such as 'Red Delicious' (Kollmannsberger and Berger, 1992), 'Delicious' (Mattheis *et al.*, 1991b), 'Jonagold' (Girard and Lau, 1995),

'Starkspur Golden' (Vanoli *et al.*, 1995), 'Golden Delicious', 'Jonathan' and 'Fuji' (Kakiuchi *et al.*, 1986) indicated a similar relationship between these types of volatile compounds. It has been suggested that this is a common characteristic of aroma volatiles produced in most commercial apple cultivars (Brown *et al.*, 1968; Dimick and Hoskin, 1983; Paillard, 1990).

The greatest number of esters were produced by 'Red Delicious', 'Braeburn' and 'Pacific Rose'; 'Royal Gala', 'Braeburn', 'Cox's Orange Pippin' and 'Red Delicious' had a high number of alcohols, while in 'Granny Smith' and 'Red Delicious' hydrocarbons predominated. These 3 classes of volatiles contributed 70% - 88% of the total number detected in the cultivars studied, while 12% - 30% were aldehydes, ketones, acids and others. 'Red Delicious' had the highest number of esters, accounting for 36% of the total number of volatiles found, while 'Royal Gala' contained the highest number of alcohols, which made up to 41% of the total number found in fruit. 'Granny Smith' contained the highest number of hydrocarbons contributing about 41% of the total number detected. 'Red Delicious', 'Braeburn' and 'Granny Smith' produced the largest number of volatile compounds detected. The amounts of total esters and total volatile compounds estimated by the purge and trap technique in 5 apple cultivars grown in Japan were reported to be the highest in 'Hatsuaki' and the lowest in 'Fuji', while 'Golden Delicious', 'Jonathan' and 'Mutsu' were intermediate (Kakiuchi *et al.*, 1986). Brackmann and Streif (1994) examined 28 apple cultivars and found that 'Gravenstein', 'Golden Delicious' and 'Jonagold' were among those cultivars producing the highest amount of volatiles; 'Granny Smith' and 'Fuji' were among the lowest, while the other cultivars were intermediate.

The highest total volatile production may not necessarily reflect the highest aroma for a given apple cultivar, as it depends on the proportion of chemical classes of volatile compounds produced by the fruit. Although it is generally accepted that the aroma of apples is due to a combination of several volatile compounds (Berger, 1991; Maarse, 1991), there have been few studies reported on specific volatiles or more particularly combinations of volatiles, that contribute to specific and characteristic apple flavour. Young *et al.* (1996) found that 2-methyl butyl acetate, butan-1-ol and hexyl acetate had the greatest sensory contribution to the aroma of 'Royal Gala' apples.

Williams (1979) suggested that combinations of 4-methoxyallylbenzene, butan-1-ol, hexan-1-ol, pentyl acetate, butyl hexanoate, hexyl butanoate and hexyl 2-methyl butanoate made important contribution to the aroma of 'Cox's Orange Pippin' apples. Esters are the most important contributors to aroma intensity and quality and they are responsible for the fruity note of apple and pear aroma (Dimick and Hoskin, 1983; Dürr and Schobinger, 1981). Alcohols, hydrocarbons, aldehydes (except hexanal and *trans*-2-hexenal) and others also contribute to aroma of apples to some degree (Williams *et al.*, 1977a; 1977b), but are of minor importance compared with esters (Cunningham *et al.*, 1986; Teranishi *et al.*, 1987). Within the ester group, ethyl esters are known to have the most potent odour, having relatively low odour thresholds in water (Teranishi *et al.*, 1987) and a number of compounds in this group, such as ethyl 2-methyl butanoate, ethyl butanoate, and ethyl hexanoate are reported to be aroma impact compounds in apples and apple products (Berger, 1991; Dimick and Hoskin, 1983; Flath *et al.*, 1967). Brackmann and Streif (1994) found that 'Gravenstein' and 'Golden Delicious' apples produced the highest volatiles among 28 apple cultivars, but the former had a poorer aroma than the latter. It was suggested that this was because 'Gravenstein' produced higher total alcohols than esters, while 'Golden Delicious' had more esters than other compounds in the volatile composition (Brackmann and Streif, 1994).

In the present study, 'Red Delicious' and 'Braeburn' contained more esters than 'Royal Gala' and 'Granny Smith' (1993 experiments) and 'Pacific Rose' had more esters than 'Cox's Orange Pippin' apples (1994 experiments), suggesting that cultivars having higher esters may possess a stronger aroma than those having relatively less esters because esters are mainly responsible for the fruity, sweet, scented and floral odour of fruit (Dimick and Hoskin, 1983; Nursten, 1970; Williams, 1979). 'Red Delicious', 'Royal Gala' and 'Granny Smith' apples had the highest volatiles among the cultivars studied, 'Red Delicious' had the most esters, while alcohols and hydrocarbons predominated in 'Royal Gala' and 'Granny Smith', respectively. This suggests that 'Red Delicious' apples have a relatively stronger aroma than the other cultivars. Moreover, 'Red Delicious' also produced the highest concentrations of ethyl esters, such as ethyl 2-methyl butanoate and ethyl hexanoate relative to the other cultivars studied. Thus, it seems that the aroma

intensity and/or quality of an apple cultivar is influenced by the proportion of chemical classes of volatile compounds produced.

Although some individual volatile compounds were found in most apple cultivars, some others were found only in a few cultivars. Hexyl acetate was detected in all apple cultivars, with 'Royal Gala' producing relatively higher concentration than 'Red Delicious', 'Braeburn' or 'Granny Smith' (1993 experiments), or 'Cox's Orange Pippin' having more than 'Pacific Rose' (1994 experiments). Butyl acetate was found in all cultivars, except 'Granny Smith' apples. Butyl acetate and hexyl acetate are thought to be character-impact aroma compounds in some apple cultivars such as 'Cox's Orange Pippin' and 'Red Delicious' (Bartley *et al.*, 1985; Kollmannsberger and Berger, 1992; Patterson *et al.*, 1974), where they have been shown to be responsible for a fruity, sweet and floral note (Dimick and Hoskin, 1983; Dürr and Röthlin, 1981). Ethyl 2-methyl butanoate, a ripe apple-like aroma compound of 'Red Delicious' apples, was generally found in low concentrations in 'Red Delicious', 'Braeburn' and 'Granny Smith' but was not detected in control 'Royal Gala', 'Cox's Orange Pippin' or 'Pacific Rose' apples. This compound and other ethyl esters such as ethyl propanoate, ethyl butanoate and ethyl hexanoate are important contributors to the aroma quality of apples and apple products (Dürr and Schobinger, 1981), and they were greatly enhanced in some CO_2 treated apple cultivars; this issue will be discussed later. Other esters such as methyl hexanoate, hexyl hexanoate, isoamyl acetate and 2-methyl butyl acetate were found in some cultivars but not in the others. For instance, methyl hexanoate was detected only in 'Red Delicious', while hexyl hexanoate was found in 'Red Delicious' and 'Braeburn', but not in other cultivars. Similarly, the other chemical classes of volatile compounds, alcohols, aldehydes, hydrocarbons and ketones varied in both number and amount among the apple cultivars studied.

It has been reported that some individual esters such as propyl butanoate, ethyl 2-methyl butanoate, and ethyl acetate concentrations were higher in 'McIntosh' than in 'Cortland' apples, while some esters such as butyl pentanoate and pentyl pentanoate were absent in these cultivars (Yahia, 1989). Concentrations of the aroma impact C_6 aldehydes of apple juice and essence, hexanal and *trans*-2-hexenal (Flath *et al.*, 1967) were found to vary among cultivars in the present study, as well as those reported by

Drawert *et al.* (1986) and Schreier *et al.* (1978a; 1978b). The estragole, 4-methoxyallyl benzene, a compound that contributes to the spice-like or aniseed-like characteristics of many apple cultivars, particularly 'Cox's Orange Pippin' and related cultivars (Williams *et al.*, 1977b), was detected only in 'Royal Gala', but not in 'Cox's Orange Pippin' due to a problem with mass spectrometric identification.

Differences in the composition of the individual volatile compounds may play a major role in giving a characteristic aroma to each apple cultivar. Thus, differences in the composition of volatile compounds are a major determinant contributing to differences in flavour of apples and their products (Acree, 1993; Dürr and Schobinger, 1981). Apple cultivars that had high ester production such as 'McIntosh', 'Red Delicious' and 'Spartan' (Dirinck and Schamp, 1989; Yahia, 1989) were reported to give a strong, persistent, good aroma in processed apple juice, while 'Bramley' and 'Pederstrup', which had low volatile production, had a very weak fruity aroma, an unpleasant off-aroma, a very sour taste and were astringent (Poll, 1981).

A common hydrocarbon, α-farnesene, was detected in all cultivars, except 'Cox's Orange Pippin', evaluated in this study. This compound may be characteristic of pome fruit, being present in pears and quinces (Paillard, 1990) and it is an important volatile involved in the development of superficial scald disorder in apples and pears (Emongor *et al.*, 1994; Huelin and Coggiola, 1968). It is possible that this compound may contribute to the 'dried leaves' note in apple aroma (Williams, 1979). Aromatic hydrocarbons detected in large numbers in this study are known to be present in various apple cultivars (Dimick and Hoskin, 1983; Paillard, 1990) and probably contribute to aroma of apples to some degree (Williams, 1979). Some aromatic hydrocarbons detected in apple cultivars, such as nonane, benzene and ethyl benzene, may be pollutants derived either from solvents or from air (Dirinck *et al.*, 1977; Martin and Nishijima, 1977; Weurman, 1969) and their presence should be treated with caution (Paillard, 1990). In the present study, hydrocarbons detected were not consistent across cultivars, and because methods used for extraction were constant, the compounds monitored are probably derived from the fruit.

Apple cultivars have been classified into 2 types depending on whether they produced predominantly esters or alcohols (Dirinck and Schamp, 1989; Dirinck *et al.*,

1989). The 'ester' type cultivars produce a high concentration and numbers of esters, particularly acetate esters, while the 'alcohol' type produce high levels of alcohols, particularly hexan-1-ol (Dirinck *et al.*, 1989). In addition, it has been suggested that the ester type cultivars produce most of their volatile precursors from fatty acids, while the precursors of the alcohol type cultivars originated mainly from amino acids (Brackmann *et al.*, 1993). According to this logic, 'Red Delicious', 'Braeburn', 'Cox's Orange Pippin' and 'Pacific Rose' could be classified as ester type cultivars because they produced large numbers of straight-chain volatile esters and alcohols which are thought to be derived from fatty acid metabolism (Bartley *et al.*, 1985). 'Granny Smith' and 'Royal Gala', which had a large number of alcohols and were low in number of esters, would be regarded as alcohol types. This confirms previous suggestions that cultivars such as 'Delicious', 'Golden Delicious', 'Red Delicious' and 'Cox's Orange Pippin' were ester types, while 'Granny Smith' was an alcohol type cultivar (Brackmann *et al.*, 1993; Dirinck *et al.*, 1989).

In apples and pears, it is generally believed that straight-chain aldehydes, alcohols and esters are derived from β-oxidation of fatty aids, while branched-chained volatiles originate from amino acid metabolism (Bartley *et al.*, 1985; Drawert, 1975; Tressl *et al.*, 1975). Synthesis of both straight-chain and branched-chain esters occurs by coupling of alcohols and short-chain acyl CoAs, and the reaction is catalysed by the ester forming enzyme - alcohol acyltransferase (AAT) (Fellman and Mattheis, 1995; Knee and Hatfield, 1981; Oliás *et al.*, 1995). If this is the case, the production curve for esters should follow a similar pattern to that of alcohols. Results from regression analyses between butyl acetate and butan-1-ol or hexyl acetate and hexan-1-ol (**Table 6.11**) indicate that butyl acetate and hexyl acetate were positively associated with their respective alcohols, except for hexyl acetate in 'Granny Smith' apples. This result confirms the involvement of alcohols in ester production of apples.

However, alcohols are not the only determinant for ester production in apples; other important factors, acyl CoAs and AAT, also influence ester synthesis (Fellman *et al.*, 1993a; Gilliver and Nursten, 1976). Availability of acyl CoAs such as acetyl CoA, butyl CoA, hexyl CoA and 2-methyl butyl CoA can vary among apple cultivars; a detailed discussion was presented in **Chapter Five**. In 'Granny Smith' apples,

appreciable concentration of butan-1-ol was measured and acetyl CoA was unlikely to be deficient, as ethyl acetate was enhanced substantially after CO_2 treatment. Thus, the absence of butyl acetate in 'Granny Smith' apples, and probably some other esters in some cultivars, could be directly related to the absence or inactivity of the ester forming enzyme(s). It is possible that ester forming enzyme(s) from different cultivars may have different isoforms or types. Gilliver and Nursten (1976) found that incubation of banana slices (var. Giant Cavendish) with acetyl CoA led to a marginal increase in acetate esters, but incubation with butyl CoA resulted in 5 fold increase in isobutyl and isopentyl butanoate. When banana slices were incubated with a range of alcohols and acyl CoAs, the greatest ester formation was achieved with butyl CoA and butan-1-ol, isobutan-1-ol, and isopentan-1-ol (Gilliver and Nursten, 1976). Oliás *et al.* (1995) found that partially purified AAT from strawberries and banana (var. Cavendish) preferred esterification of acetyl CoA and hexan-1-ol, and acetyl CoA and butan-1-ol, respectively. Thus, AAT within a species (banana) or different species of fruit probably has different substrate preferences (or specificity) for esterification. It seems that factors which determine differences in volatile ester composition in fruit include alcohols, acyl CoAs and the inherited properties of the ester forming enzymes (Oliás *et al.*, 1995; Pérez *et al.*, 1993).

Table 6.11 Coefficient of determinations (r^2) of linear regression analyses between concentrations of butyl acetate and butan-1-ol, or hexyl acetate and hexan-1-ol from juice of 100% CO_2 treated (24 hours at 20°C) and control apple cultivars during 15 days storage at 20°C and 70% RH.

	Butyl Acetate v. Butan-1-ol		Hexyl acetate v. Hexan-1-ol	
Cultivar	100% CO_2	Control	100% CO_2	Control
Royal Gala	0.89 (* * *)	0.91 (* * *)	0.88 (* * *)	0.85 (* * *)
Red Delicious	0.89 (* * *)	0.96 (* * *)	0.58 (* * *)	0.76 (* * *)
Braeburn	0.62 (* * *)	0.82 (* * *)	0.57 (* * *)	0.64 (* * *)
Granny Smith	–[1]	–	0.06 (ns)	0.04 (ns)
Cox's Orange Pippin	0.75 (* * *)	0.90 (* * *)	0.09 (*)	0.59 (* * *)
Pacific Rose	0.58 (* * *)	0.55 (* * *)	0.78 (* * *)	0.76 (* * *)

Levels of Significance at $P = 0.05$ (*), 0.01 (* *), 0.001 (* * *), non significant (ns), [1] butyl acetate not detected in 'Granny Smith' apples.

Availability of precursors could also be one of the major factors responsible for differences in aroma volatile production between apple cultivars. As precursors of aroma volatile compounds are derived from macro-molecules such as lipids, proteins and carbohydrates (Schreier, 1984; 1986), availability of these precursors is presumably dependent on the metabolic reactions involved in degradation of these macromolecules and/or synthesis of new compounds (Song and Bangerth, 1994). It is possible that these processes could be different among apple cultivars. If this is true, concentrations and types of precursors, as well as the enzyme system involved, could vary. This in turn would influence the differences in amounts and numbers of aroma volatile compounds produced in different apple cultivars. Paillard (1990) found that the rate of transformation of C_4 fatty acid into acetyl CoA was much higher in 'Golden Delicious' apples than in 'Starking' or 'Richared', and the former was found to produce mainly acetate esters, while the latter had high levels of butanoate esters. Therefore, differences in volatile production of different cultivars seem to be associated with differences in the availability of precursors and/or substrates and the inherited properties of the enzyme system involved, and it is presumably genetically controlled.

Apple cultivars exposed to 100% CO_2 for 24 hours generally responded in a similar manner; fruit from CO_2 treatments always contained enhanced concentrations of aroma volatiles compared with control fruit. Hypoxic treatment caused a substantial increase in headspace acetaldehyde, ethanol and ethyl acetate in all apple cultivars within 24 hours after treatment. Production of headspace ethanol followed closely to that of acetaldehyde (**Table 6.12**) as expected, because it is derived from a single step decarboxylation of acetaldehyde, where the reaction is catalysed by alcohol dehydrogenase (Perata and Alpi, 1993; Ricard *et al.*, 1994). Treatment with 100% CO_2 also led to a large increase in ethyl acetate and several other ethyl esters such as ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate. However, CO_2 did not enhance production of ethyl esters in 'Royal Gala' or 'Cox's Orange Pippin', except for ethyl acetate in the former and ethyl acetate and ethyl butanoate in the latter.

Increased production of ethyl esters after CO_2 treatment is probably directly associated with an increase in ethanol concentration in response to hypoxia. This phenomenon has been reported in other apple cultivars (Ke *et al.*, 1991a; Mattheis *et al.*,

1991a), oranges (Shaw *et al.*, 1990; 1991; 1992) and feijoa (Pesis, 1994). As stated earlier, formation of esters in apples and pears is thought to proceed via the reaction of ester forming enzyme(s) on alcohols and acyl CoAs (Bartley *et al.*, 1985; Knee and Hatfield, 1981). It has been suggested that there is a deficiency of availability of alcohols available for ester synthesis in pre-climacteric apples (Hansen *et al.*, 1992a; Mattheis *et al.*, 1991b) and in long-term CA stored apples (Bartley *et al.*, 1985; Knee and Hatfield, 1981). Treatment with 100% CO_2 substantially enhanced concentrations of ethanol after treatment and thus this alcohol would not be lacking for esterification. Acyl CoAs, particularly acetyl CoA, have been assumed to be readily available in apples (Knee and Hatfield, 1981) as they could be derived from several metabolic routes such as β-oxidation of fatty acids (Bartley *et al.*, 1985), reaction on pyruvate by pyruvate dehydrogenase (Ke *et al.*, 1993a) and oxidation of acetaldehyde (Mattheis *et al.*, 1991a; Reazin *et al.*, 1970). If this is true, production of ethyl esters should follow closely that of ethanol. An attempt had been made to examine this relationship in 'Cox's Orange Pippin' and 'Pacific Rose' apples, but not in the other cultivars as ethanol could not be determined due to the ethanol contamination in solvent used for extraction.

A relationship between ethyl acetate, ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate, or the sum of ethyl esters and ethanol exists in 'Pacific Rose', while in 'Cox's Orange Pippin' apples only ethyl acetate and the sum of ethyl esters (mainly ethyl acetate) have some relationship with ethanol (**Table 6.12**). This suggests that ethanol is involved, to some degree, in ethyl ester synthesis. However, the relationship between concentration of these ethyl esters and ethanol was not robust, suggesting that factors other than ethanol, influence the synthesis of ethyl esters.

The increase in ethanol concentration after CO_2 treatment, generally occurred immediately on removal to air after treatment, peaking after 0 or 1 day before decreasing. Increases in concentration of ethyl esters generally occurred 1 to 3 days after treatment before declining. Thus, there is a 1 to 2 day lag period in ethyl ester production following CO_2 treatment, relative to ethanol. This lag period is probably due to the lack of O_2 in fruit tissue immediately after treatment; after O_2 equilibration takes place, concentration of ethyl esters increased markedly. This phenomenon suggests that ester forming enzyme(s) required O_2 for esterification (Fellman *et al.*, 1993a). In addition,

ethanol is supposed to be a substrate for ethyl ester synthesis (Berger and Drawert, 1984; Knee and Hatfield, 1981), thus the increased production of ethyl esters depends on ethanol accumulation before esterification can proceed. This lag phase in ethyl ester formation could be a major reason for the relatively low r^2 of regression analyses.

Table 6.12 Coefficient of determination (r^2) of linear regression analyses between concentrations of selected ethyl esters and ethanol in 100% CO_2 treated (24 hours at 20°C) or control 'Cox's Orange Pippin' and 'Pacific Rose' apples during 15 days storage at 20°C and 70% RH.

	cv. Cox's Orange Pippin		cv. Pacific Rose	
Parameter	100% CO_2	Control	100% CO_2	Control
Hspace EtOH v. Hspace AA	0.81 (* * *)	–[1]	0.91 (* * *)	0.06 (*)
Hspace EtOH v EtOH	0.73 (* * *)	–	0.84 (* * *)	0.30 (* * *)
Hspace EA v. EA	0.90(* * *)	–	0.93 (* * *)	0.00 (ns)
Hspace EA v. Hspace EtOH	0.13 (* * *)	–	0.50 (* * *)	0.23 (* * *)
EA v. EtOH	0.31 (* * *)	0.03 (ns)	0.48 (* * *)	0.05 (*)
EB v. EtOH	0.02 (ns)	0.42 (* * *)	0.14 (* * *)	0.02 (ns)
E2MB v. EtOH	–[2]	–	0.60 (* * *)	0.09 (* * *)
EH v. EtOH	–	–	0.30 (* * *)	0.09 (* * *)
Sum of ethyl esters v. EtOH	0.34 (* * *)	0.002 (ns)	0.45 (* * *)	0.04 (*)

Abbreviations and Symbols: Hspace = headspace, AA = acetaldehyde, EtOH = ethanol, EA = ethyl acetate, EB = ethyl butanoate, E2MB = ethyl 2-methyl butanoate, EH = ethyl hexanoate, sum of ethyl esters = EA + EB + E2MB + EH, [1] headspace volatile in control fruit were not detected, [2] E2MB and EH were not detected in both control and CO_2 treated 'Cox's Orange Pippin' apples.
Levels of Significance at $P = 0.05$ (*), 001 (* *), 0.001 (* * *), or non significant (ns).

Another factor could possibly be related to exhaustion of acyl CoAs for ester synthesis, particularly hexyl CoA, but not acetyl CoA. In 'Pacific Rose' apples, a decrease in concentration of ethyl acetate to the level similar to controls occurred at the same time as that of ethanol concentration. This suggests that acetyl CoA was not lacking. On the other hand, ethyl hexanoate increased and peaked 1 day after treatment and decreased rapidly to the control level at 7 days, while ethanol decreased moderately to the control level after 9 days. This more rapid decrease in ethyl hexanoate, relative to ethanol, probably suggests that hexyl CoA could be exhausted and not available for esterification with ethanol. Such a change, along with the lag phase of the increase in ethyl esters, probably affects the linear relationship between ethyl hexanoate and ethanol concentration, which is reflected in a relatively low coefficient of determination of the

regression analyses. These reasons may also explain the low r^2 for the other ethyl esters in this analysis (**Table 6.12**).

In 'Cox's Orange Pippin' apples, ethyl butanoate was found only sporadically and ethyl 2-methyl butanoate and ethyl hexanoate were not detected in control or after CO_2 treatment. This could possibly be related to the lack of acyl CoAs, butyl CoA and hexyl CoA, for esterification (Gilliver and Nursten, 1976) and/or metabolism involved in liberating these acyl moieties (Paillard, 1990). Some acyl CoAs can be deficient due to the slow rate of transformation of longer chain acyl CoAs into shorter chains. Paillard (1990) found that exogenously applied C_4 fatty acids were rapidly and almost completely transformed into acetyl CoA in 'Golden Delicious' apples, with a concomitant increase in acetate esters, but only a slight conversion into acetyl CoA occurred in 'Starking' apples where they were mainly incorporated into butanoate esters. In the present study, it was found that production of ethyl acetate in CO_2 treated 'Cox's Orange Pippin' was much greater than in 'Pacific Rose'. Similarly, butyl acetate and hexyl acetate concentrations in control 'Royal Gala' apples were greater than in 'Cox's Orange Pippin', where both cultivars produced very little or no ethyl butanoate, ethyl hexanoate and ethyl 2-methyl butanoate. It seems that the slow rate of transformation of long chain acyl CoAs into short chain acyl CoAs for esterification may be partly responsible for the lack of some aroma volatiles in apple cultivars.

Another possibility for the lack of some ethyl esters, other than ethyl acetate, in 'Royal Gala' and 'Cox's Orange Pippin' apples could be due to substrate specificity of the ester forming enzyme(s), which may prefer esterification of butan-1-ol or hexan-1-ol rather than ethanol with acetyl CoA, resulting in a greater production of butyl acetate and hexyl acetate in these two cultivars than in the other cultivars, but reduced or nil amounts of other esters such as ethyl 2-methyl butanoate and ethyl hexanoate (Berger and Drawert, 1984; Oliás *et al.*, 1995).

Treatment with 100% CO_2 also enhanced production of propan-1-ol in 'Cox's Orange Pippin' and 'Pacific Rose' apples. Enhancement of propan-1-ol concentration probably results from fermentation (Heath and Reineccius, 1986; Reazin *et al.*, 1970), in which sugars and/or amino acids are converted into keto acids via an amino acid

biosynthesis pathway and subsequently reduced into propan-1-ol and fusel oils, as previously discussed (**Chapter Four**).

Table 6.13 Ratios between total concentration of some volatile compounds (as area under curve during 15 days storage at 20°C and 70% RH) of 100% CO_2 treated and control fruit of 6 apple cultivars.

Compound	Cultivar					
	COP	PR	RG	RD	Bb	GS
headspace acetaldehyde	244.4[1]	8.5	–[2]	–	–	–
headspace ethanol	1839.9[1]	29.9	–	–	–	–
headspace ethyl acetate	1051.6[1]	71.5	–	–	–	–
ethyl butanoate	2.7	2.8	–	–	–	–
ethyl 2-methyl butanoate	nd	121.2	nd	17.9	48.2	3.8
ethyl hexanoate	nd	142.4	nd	20.7	7.7	5.1
ethyl acetate	17.4	4.9	109.2	72.6	239.1	10.1
methanol	1.1	1.0	–	–	–	–
ethanol	7.2	3.6	–	–	–	–
propan-1-ol	4.0	1.5	–	–	–	–

Abbreviations and Symbols: COP = Cox's Orange Pippin, PR = Pacific Rose, RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, nd = not detected, [1] not detected in control fruit and data were total concentrations in $\mu mol \cdot l^{-1} \cdot day$ [2] not analysed

Although 100% CO_2 treatment enhanced concentrations of several volatile compounds in apples, the magnitude of enhancement of these compounds varied among cultivars studied (**Table 6.13**). The magnitude of enhancement can be illustrated by integrating the area under the curve for selected aroma volatile production during 15 days storage at 20°C. The magnitude of enhancement in headspace volatile concentrations between 'Cox's Orange Pippin' and 'Pacific Rose' could not be compared by this approach, as these volatiles were not detected in control fruit of the former. Nevertheless, differences in headspace volatile concentrations between these two cultivars were obvious where 'Cox's Orange Pippin' produced substantially more volatiles than 'Pacific Rose' (**Fig. 6.5**). It was interesting that the magnitude of enhancement of ethyl butanoate between 'Cox's Orange Pippin' and 'Pacific Rose' was comparable, although the actual concentration was substantially greater in 'Pacific Rose' than in 'Cox's Orange Pippin' apples (**Fig. 6.7**). The magnitude of enhancement of ethyl 2-methyl butanoate and ethyl hexanoate was highest in 'Pacific Rose', ethyl acetate was

in 'Braeburn', while the lowest for these compounds was in 'Granny Smith' apples. The magnitude of enhancement of ethanol and propan-1-ol was greater in 'Cox's Orange Pippin' than in 'Pacific Rose' (**Table 6.13**).

Differences in the magnitude of enhancement of volatile compounds among apple cultivars after CO_2 treatment probably suggest that different cultivars have different abilities to produce aroma volatile compounds. This may reflect involvement of different enzymes or isoenzymes. It is possible that the higher the magnitude of enhancement of a volatile compound, the greater the capacity of the enzyme system involved in producing that compound. However, the highest magnitude of enhancement of a compound in a cultivar does not mean that the actual concentration produced is also the highest. The magnitude of enhancement of a compound depends on the concentration of both control and CO_2 treated fruit; if the concentration of a compound in control fruit is already high, the ratio obtained may well be small even if a substantial enhancement is induced by the CO_2 treatment. For example, the magnitude of enhancement of ethyl 2-methyl butanoate in 'Red Delicious' was 18 and in 'Braeburn' it was 47 (x 10^4 area count·day), where total concentrations (as area under the curve) in control and CO_2 treated fruit in the former were 30.8 and 555.2 (x 10^4 area count·day), and that of the latter were 1.5 and 70.8 (x 10^4 area count·day), respectively.

Increase in production of ethyl esters in response to CO_2 treatment is thought to be directly associated with a substantial increase in ethanol concentration which in turn is sufficient to displace other alcohols reacting with the ester forming enzyme(s). In the present study, several non ethyl esters, alcohols, other than ethanol and propan-1-ol, and hexanal and *trans*-2-hexenal were slightly or markedly decreased after CO_2 treatment in all apple cultivars. Previous results indicated a similar pattern (**Chapter Four** and **Chapter Five**). Increase in ethyl esters, with a concomitant decrease in other esters, alcohols and aldehydes in fruit after treatment with hypoxic atmosphere or after storage under anaerobic conditions has previously been reported (Ke *et al.*, 1994b; Larsen and Watkins, 1995a; 1995b; Mattheis *et al.*, 1991a; Shaw *et al.*, 1990; 1991; 1992; Ueda and Bai, 1993). It has also been noted in fruit following application of volatile precursors such as ethanol, other alcohols, aldehydes, and short-chain carboxylic acids (Bartley *et al.*, 1985; Berger and Drawert, 1984; Berger *et al.*, 1992; De Pooter *et al.*, 1983; 1987;

Gilliver and Nursten, 1967; Knee and Hatfield, 1981). This phenomenon is thought to be due to the competitive nature of the acyl esterifying system, as an internal excess of one alkyl moiety always resulted in a decrease of esters not containing that alkyl moiety (Berger and Drawert, 1984; Berger *et al.*, 1992). A decrease in alcohols, other than ethanol and propan-1-ol, in CO_2 treated fruit probably results from competition for acyl moieties for generating alcohols. Fatty acids and amino acids provide both acyl CoAs and alcohols for esterification (Bartley *et al.*, 1985; Tressl *et al.*, 1975). Alcohols, other than methanol and ethanol, are thought to be derived from reduction of acyl moieties, via aldehyde intermediates, into alcohols. For example, butyl CoA and 2-methyl butyl CoA can be reduced into butan-1-ol and 2-methyl butan-1-ol, respectively; at the same time these acyl CoAs can act as substrates for esterification with alcohols, such as ethanol, generating ethyl butanoate and ethyl 2-methyl butanoate, respectively (Bartley *et al.*, 1985; Drawert, 1975). An excess concentration of ethanol in response to hypoxia would encourage ethyl esterification; this would lead to reduction in acyl moieties for esterification of the other esters, as well as for conversion into other alcohols. Such competitiveness for acyl moieties may be the main reason for the reduction in non ethyl esters and alcohols not containing an ethyl moiety in the present study.

Another consequence of CO_2 treatment was a reduction in the C_6 aldehydes, hexanal and *trans*-2-hexenal in juice of the treated apples, compared with controls. Hexanal and *trans*-2-hexenal are formed from unsaturated linoleic and linolenic fatty acids during the disruption of plant tissues in the presence of O_2 via the lipoxygenase enzyme system (Hatanaka, 1996; Riley *et al.*, 1996). A decrease in concentrations of these compounds is probably due to the effect of CO_2 reducing the capacity of the enzyme system involved and/or decreasing the availability of precursors for the production of hexanal and *trans*-2-hexenal; a detailed discussion of this was given in **Chapter Five**.

Reduction of volatile compounds, other than those containing an ethyl moiety and propan-1-ol, in apples after CO_2 treatment varied among apple cultivars and compounds (**Table 6.14**). The overall degree of reduction, in terms of percentage reduction, of volatile compounds (as sum or ratio) were among the highest in 'Red Delicious', 'Braeburn' and 'Cox's Orange Pippin' after CO_2 treatment, while 'Pacific

Rose' was the lowest, and 'Royal Gala' or 'Granny Smith' were intermediate. The degree of reduction in acetate esters was relatively lower in 'Granny Smith' and 'Pacific Rose' than in other cultivars, while reduction in the alcohols, butan-1-ol and hexan-1-ol, was lowest in 'Pacific Rose', but was the highest in 'Red Delicious' and 'Braeburn'. Percentage reduction in hexanal and *trans*-2-hexenal was greatest in 'Cox's Orange Pippin' and was relatively the lowest in 'Braeburn' and 'Pacific Rose' apples.

The reduction in butyl acetate and hexyl acetate seemed to be associated with reductions in butan-1-ol and hexan-1-ol, respectively; the greater the reduction in these alcohols, the greater the reduction in the respective esters, although the opposite was true in 'Pacific Rose' apples. This probably indicated the involvement of alcohols in esterification. An exception was observed between butan-1-ol and butyl acetate in 'Cox's Orange Pippin' apples, where a relatively high reduction in butyl acetate was not associated with a large reduction in butan-1-ol (**Table 6.14**).

A relatively low reduction in acetate esters in 'Granny Smith' and 'Pacific Rose' seemed to coincide with a relatively low magnitude of enhancement in ethyl acetate after CO_2 treatment for these cultivars (**Table 6.13**). On the other hand, the relatively high percentage of reduction in butyl acetate, hexyl acetate and isoamyl acetate in 'Cox's Orange Pippin', 'Royal Gala', 'Red Delicious' and 'Braeburn' appears to be related to the relatively high magnitude of enhancement of ethyl acetate in these cultivars. Such a conformity and contrast in the degree of reduction and the magnitude of enhancement of these selected volatile compounds is undoubtedly a reflection of the competitive nature of acyl esterification of aroma volatile production in apples. In the case of butan-1-ol in 'Cox's Orange Pippin', which did not comply with this phenomenon, it is possible that this alcohol is derived from a metabolic pathway other than β-oxidation of fatty acids (Bartley *et al.*, 1985; Paillard, 1990). Butan-1-ol could be derived from butanal as a result of lipoxygenase activity that occurs during tissue disruption (Fellman, 1996, personal communication) and/or from fermentation of sugars, via an amino acid biosynthesis pathway (Reazin *et al.*, 1970), as previously discussed (**Chapter Four** and **Chapter Five**). This is probably the reason for a relatively low degree of reduction of butan-1-ol in 'Cox's Orange Pippin'; however the relatively high degree of reduction of

butyl acetate may be because of the reduced acetyl CoA available for esterification with butan-1-ol due to competition with ethanol.

Table 6.14 Percentage of reduction in total concentrations of selected volatile compounds (as area under curve during 15 days storage at 20°C) from 100% CO_2 treated fruit, relative to control, in 6 apple cultivars.

	Cultivar / Reduction (%)					
Compound	COP	PR	RG	RD	Bb	GS
butyl acetate	44.0	11.9	37.6	66.8	68.9	–
hexyl acetate	65.3	28.7	39.7	61.9	53.5	21.9
isoamyl acetate	–	–	40.1	52.9	33.1	25.4
butan-1-ol	8.0	9.4	27.6	57.3	41.6	29.3
hexan-1-ol	30.0	2.8	24.5	23.4	50.4	26.4
hexanal	33.5	0.5	17.5	25.9	*	19.8
trans-2-hexenal	23.0	13.4	16.1	17.0	11.0	21.0
Sum	203.8	66.7	163.0	252.3	225.3	118.4
Ratio	0.20	0.06	0.16	0.24	0.24	0.11

Abbreviations and Symbols: COP = Cox's Orange Pippin, PR = Pacific Rose, RG = Royal Gala, RD = Red Delicious, Bb = Braeburn, GS = Granny Smith, Sum = sum of percentage reduction excluding isoamyl acetate, ratio = ratio among sum of percentage of reduction, – = not detected or not determined, * hexanal in 'Braeburn' increased by 10.9%.

'Braeburn' apples sustained excessive damage following the 100% CO_2 treatment in 1993. Most important ethyl esters were substantially enhanced in response to CO_2 treatment, but other esters and alcohols were severely depressed. In 1994 (**Chapter Four**), concentrations of several enhanced ethyl esters in 'Braeburn' apples were comparable to those in 'Red Delicious' and no injury symptoms occurred after CO_2 treatment. Although some ethyl esters were enhanced in damaged 'Braeburn', actual concentrations of these esters, including ethyl 2-methyl butanoate and ethyl hexanoate, were substantially lower, relative to 'Red Delicious'. This suggests that the esterifying enzyme system in 'Braeburn' was only partially operative, presumably in undamaged tissue.

Fruit injury caused by CO_2 treatment of 'Braeburn' apples also led to a continuously high respiration rate, low ethylene production, and a reduction in most fruit quality attributes, including loss of appearance and firmness, low TSS and TA and a high pH resulting in such fruit becoming totally unacceptable.

The injury of 'Braeburn' that developed after CO_2 treatment seems to resemble the 'Braeburn browning disorder (BBD)' a serious but sporadic disorder found in this cultivar; its incidence and severity is season dependent (Banks, 1995; Kupferman, 1994). The mechanism which leads to the development of BBD is unknown, but it is thought to be related to the inherent susceptibility of fruit tissue (Banks, 1995). Both pre- and post harvest factors such as light crop load, cool temperature during fruit development, low fruit calcium, high magnesium and potassium contents, fruit maturity at harvest (mid season fruit) and high humidity during storage aggravate tissue susceptibility to BBD development (Banks, 1995; Kupferman, 1994; Tough *et al.*, 1996). A recent study at the Department of Plant Science, Massey University (Banks, 1995) suggested that increase in internal CO_2 in fruit, chilling temperature and high humidity during storage could trigger BBD development.

In 1993 susceptibility of 'Braeburn' fruit tissue was clearly high, relative to 1994, the 100% CO_2 treatment created an extreme modified atmosphere composition in fruit imposing severe physiological stress to susceptible tissue. This may have caused physiological damage to cell membranes or cell death, resulting in ion leakage and cellular decompartmentation (Marangoni *et al.*, 1996). On removal to air after treatment, browning of damaged tissue may rapidly develop due to oxidation of phenolic compounds by the reaction of polyphenol oxidases in the presence of O_2 (Amiot *et al.*, 1992; Coseteng and Lee, 1987; Martinez and Whitaker, 1995).

Since injury symptoms occurred after CO_2 treatment in one year, not in the other, it is obvious that incidence of 'Braeburn' injury is seasonal, probably related to variation in tissue susceptibility (Banks, 1995). Because symptoms are very similar to, if not the same as BBD, then a short-term high CO_2 treatment could possibly be used as a quick, simple and inexpensive method for determining tissue susceptibility for development of BBD, and perhaps for screening fruit prior to storage or export.

Fruit respiration rate after CO_2 treatment generally followed a similar pattern to controls and only a slight increase in respiration rate over control was observed in 'Red Delicious', 'Granny Smith' and 'Pacific Rose', while respiration rate in 'Cox's Orange Pippin' and 'Royal Gala' was similar to controls. An exception was in 'Braeburn', where respiration rate was greatly enhanced after CO_2 treatment which is probably directly

associated with fruit injury of this cultivar (Kader, 1986). Enhancement of respiration rate in 'Golden Delicious' apples following 24 - 48 hours 95% CO_2 treatment at 20°C has previously been reported, and it was suggested to result from activation of enzymes involved in respiration or as a response to stress (Pesis *et al.*, 1993; 1994). Such an increase in respiration rate could be due to switching the flow of electrons in the respiratory electron transfer chain from the conventional cytochrome system to an alternative cyanide-resistant oxidase (Janes *et al.*, 1979; 1981; Solomos and Laties, 1975). Induction of the alternative pathway is generally found in stress conditions, including wounding of tissues, chilling, osmotic stress, atmospheric stress and drought (Janes *et al.*, 1979; Wagner and Krab, 1995).

Ethylene production of 'Royal Gala', 'Cox's Orange Pippin' and 'Red Delicious' apples was enhanced 3 - 5 days after CO_2 treatment; in contrast it was depressed between 0 and 3 days after treatment in 'Granny Smith', 'Cox's Orange Pippin' and 'Red Delicious', while in 'Pacific Rose' there was no difference from controls. Different responses between CO_2 and ethylene production have been reported in various horticultural products (Kubo *et al.*, 1990; Mathooko *et al.*, 1995a; 1995b). A decrease in ethylene from day 0 to day 3 following CO_2 treatment may have been caused by competition between CO_2 and ethylene for the same active site on the ethylene receptor (Burg and Burg, 1967) or through inhibition of ACC synthase gene expression at the transcriptional level (Mathooko, 1996). Kubo *et al.* (1990) demonstrated that a 60% CO_2 + 20% O_2 treatment at 25°C suppressed ethylene production during treatment, but caused a dramatic increase on removal to air. Fernandez-Maculet *et al.* (1993) reported that ACC oxidase from apple fruit had requirements for CO_2 in addition to Fe^{2+} and ascorbate for its activity *in vitro* and *in vivo*. Tian *et al.* (1994) found that ethylene forming enzyme activity was stimulated by CO_2 in both Japanese pear and 'Granny Smith' apple fruit discs where it increased with increasing CO_2 concentration from 5% to 30%. They suggested that the stimulating effect of CO_2 on EFE activity could be caused by a CO_2-EFE-ACC or EFE-ACC-CO_2 complex which enhanced conversion of ACC to ethylene (Tian *et al.*, 1994). Pesis *et al.* (1994) suggested that CO_2 treatment may increase cellular CO_2 concentration leading to accumulation of ACC and resulting in an increase in ethylene following removal from CO_2 treatment. This perhaps, may be a

reason for a higher ethylene production in CO_2 treated apples after 3 or 5 days in air at 20°C found in the present study.

Flesh firmness of 'Red Delicious' and 'Cox's Orange Pippin' apples was significantly higher in CO_2 treated fruit than in controls, while firmness in 'Royal Gala', 'Pacific Rose' and 'Granny Smith' was similar to controls. Retention of flesh firmness after CO_2 treatment is known (Couey and Olsen, 1975; Meheriuk, 1977; Pesis *et al.*, 1993), and is probably associated with the inhibitory effect of CO_2 on ethylene synthesis and action (Burg and Burg, 1967). Alternatively ethanol accumulation after CO_2 treatment may have suppressed ethylene action (Saltveit, 1989). A prominent increase in flesh firmness occurred in 'Granny Smith' after 3 and 5 days at 20°C. Such an increase may have been due to partial desiccation of tissue, resulting in a decrease in cell turgor and a subsequent increase in cell cohesiveness of the tissue (Bourne, 1986; Hatfield and Knee, 1988).

Treatment with 100% CO_2 generally had little, if any, effect on fruit weight loss, skin colour, TSS, TA, TSS:TA ratio or pH of the treated apples, except in 'Braeburn' fruit damaged by the CO_2 treatment.

6.6 CONCLUSION

Volatile compounds detected in 6 commercial apple cultivars comprised mainly esters, alcohols and hydrocarbons, which accounted for 70% - 88% of the total number found, the remainder being aldehydes, ketones and acids. Compounds with straight-chain and even numbered carbons predominated in the composition of esters and alcohols detected in apples volatiles.

Differences in volatile production among apple cultivars were associated with number and concentration of chemical compounds produced. Treatment with 100% CO_2 for 24 hours at 20°C consistently enhanced production of headspace volatiles in all cultivars. Production of aroma impact ethyl esters such as ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate occurred consistently in response to hypoxic CO_2 treatments with 'Red Delicious', 'Braeburn', 'Granny Smith' and 'Pacific Rose', with less of a response in 'Cox's Orange Pippin' and none in 'Royal Gala' apples. Ethanol,

and to the lesser extent propan-1-ol, were also markedly enhanced in apples following CO_2 treatment.

Non ethyl esters and alcohols, other than ethanol and propan-1-ol, were consistently reduced after CO_2 treatment in all cultivars, except for butan-1-ol in 'Cox's Orange Pippin' apples. This phenomenon may be due to competition for the acyl CoAs needed for esterification and alcohol production. Hexanal and *trans*-2-hexenal were either reduced or not affected after CO_2 treatment, depending on cultivar, and this was probably due to the effects of CO_2 on enzyme systems associated with generation of these compounds.

The magnitude of enhancement and the degree of reduction in aroma volatile compounds after CO_2 treatment varied among cultivars. The higher the magnitude of enhancement of ethyl acetate concentration following CO_2 treatment, the greater the degree of reduction in other acetate esters, including butyl acetate, hexyl acetate and isoamyl acetate; supporting the concept of competitive interaction in the acyl esterification of apples.

Treatment with 100% CO_2 had little or no effect on apple quality attributes such as weight loss, skin colour, TSS, TA, pH or injury symptom during 15 days at 20°C, except in 'Braeburn' apples where severe fruit damage occurred making this fruit unacceptable.

Therefore, it is suggested that the apple cultivars, 'Red Delicious', 'Granny Smith' and 'Pacific Rose' can be treated with 100% CO_2 in order to obtained a substantial enhancement of some aroma impact compounds. Application of high CO_2 to 'Royal Gala' and 'Cox's Orange Pippin' apples is not recommended, as it did not enhance production of aroma impact compounds, except ethyl acetate, while it caused a reduction in other important compounds such as butyl acetate, hexyl acetate and *trans*-2-hexenal. Although aroma impact compounds can be effectively enhanced substantially, CO_2 treatment should not be applied to 'Braeburn' apples, as excessive damage to fruit could result in some seasons causing a severe impairment to fruit quality.

Chapter Seven

GENERAL DISCUSSION

7.1 POSSIBLE MODE OF ACTION OF HYPOXIC TREATMENTS ON VOLATILE PRODUCTION

Aroma volatile compounds of apples can be consistently enhanced by short-term hypoxic treatments of CO_2 and/or N_2 (low O_2). Hypoxic treatments at 20°C substantially enhanced headspace acetaldehyde, ethanol and ethyl acetate in all commercial apple cultivars studied, regardless of whether they were freshly harvested or had been stored in refrigerated-air and/or in a controlled-atmosphere. Hypoxic treatments also markedly enhanced several other important aroma volatile compounds in apples, including ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate.

Enhancement of acetaldehyde and ethanol occurred immediately after treatment, while increases in ethyl acetate and other ethyl esters appeared 1 day after apples were removed to air at 20°C. In general, acetaldehyde and ethanol increased to their peak concentrations 0 - 1 day after hypoxic treatments, while ethyl esters reached their maximum production between 1 and 5 days after treatment, depending on compounds, cultivars and storage duration, and thereafter decreased during post storage at 20°C. Concentrations of CO_2 of more than 40% and/or O_2 of lower than 2.5% (N_2 > 97.5%) at 20°C were required to cause a marked enhancement of headspace acetaldehyde, ethanol and ethyl acetate, volatile ethanol, propan-1-ol, and ethyl esters. Treatment with 100% CO_2 or 100% N_2 (24 hours at 20°C) was equally effective in enhancing these apple volatiles. The optimum duration of hypoxic treatment to achieve a dramatic enhancement of ethyl esters by 100% CO_2 was between 18 and 24 hours, whereas only a slight or moderate enhancement was obtained with a shorter time (6 or 12 hours), and a longer

period (48 hours) did not further increase important aroma volatiles, except ethanol and ethyl acetate.

On the other hand, hypoxic treatments decreased, either slightly or moderately, production of aldehydes, other than acetaldehyde, alcohols, except ethanol, propan-1-ol and methanol, and other esters such as butyl acetate and hexyl acetate, not containing an ethyl moiety. Production of the aroma impact C_6 aldehydes, hexanal and *trans*-2-hexenal, of apple juice was either decreased or not affected by hypoxic treatments used, depending on cultivar and storage regime. Hypoxic treatments did not affect, or affected in a minor way, if any, fruit quality attributes, including skin colour, flesh firmness, weight loss, TSS, TA and pH or physical injury in most apple cultivars studied. An exception was observed with 'Braeburn' apples in 1993, where severe fruit damage occurred following CO_2 treatment, this did not recur in 1994. This injury may be associated with the 'Braeburn browning disorder (BBD)'; this cultivar is prone to such a disorder, possibly due to the inherent susceptibility of fruit tissue to injury thought to be seasonally dependent (Banks, 1995).

The flavour of a fruit is an integrated response composed primarily of the sensation of aroma and taste (Acree, 1993; Cronin, 1982). The odour or aroma is the single most important contributor to the characteristic flavour of most fruit (Dimick and Hoskin, 1983; Dürr and Schobinger, 1981). Aroma is the subjective sensation produced by smelling (Nursten, 1970). It is perceived when a fruit or its products taken into the mouth releases volatile compounds, normally in gaseous or vapour forms, which travel to odour receptor sites on the olfactory epithelium high up in the nasal cavity; the resulting interaction triggers a specific response in the brain (Acree, 1993; Land, 1979; Thomson, 1986). Characteristic acute sensitivity for a particular compound usually diminishes very quickly after the onset of stimulation, often to the point of complete insensitivity, but once the stimulation has been withdrawn, recovery is usually rapid (Thomson, 1986). The perception of flavour and/or aroma involves highly complex physiological and psychological processes (Acree, 1993; Boelens and van Gemert, 1986; Cronin, 1982; Frijters, 1979; Laing and Panhuber, 1979). Nursten (1970) stated that to understand the aroma of a fruit it is necessary to know (a) the nature of the volatile constituents present; (b) the quality of the aroma of each, if any; (c) the quantity of each

present; and (d) the intensity of the aroma of each. Furthermore, it is also important to know how the pattern of the significant constituents change in kind and quantity during development, maturation, ripening, storage and processing, and how each arises and is in turn metabolised (Nursten, 1970). However, because of the complex nature of aroma, the full picture or understanding is not yet complete for any fruit, including apples (Acree, 1993; Buttery, 1993; Mussinan, 1993; Yahia, 1994).

Aroma volatiles, esters and alcohols, are the principle aroma compounds synthesised by apples during ripening (Paillard, 1990; Song and Bangerth, 1996; Tressl *et al.*, 1975). Primary volatiles, which are produced by intact whole apples, are thought to be derived from enzymatically controlled lipid, amino acid and sugar metabolism (Salunkhe and Do, 1976; Schreier, 1984; 1986). Fatty acids are the most important source of volatile compounds produced in apples and pears (Drawert, 1975; Paillard, 1981; Tressl *et al.*, 1975). Long chain fatty acids with even- and odd-numbered carbon atoms are believed to be precursors of the respective even- and odd-numbered carbon aldehydes and alcohols, which are derived through a number of β-oxidation cycles (see **Chapter One**) (Bartley *et al.*, 1985; Paillard, 1990; Schreier, 1984). Short chain acyl CoAs of fatty acids are enzymatically reduced into aldehydes, which are reduced in turn to straight chain alcohols (Bartley *et al.*, 1985; Eriksson, 1979; Nursten, 1970). Branched-chain aldehydes and alcohols such as 2/3-methyl propanal, 2/3-methyl butanal, 2/3-methyl propan-1-ol and 2/3-methyl butan-1-ol are derived from appropriate amino acids, L-leucine, L-isoleucine and L-valine, via transamination and subsequent oxidative decarboxylation reactions (see **Chapter One**) (Nursten, 1970; Eriksson, 1979; Tressl *et al.*, 1975). Methanol is thought to be partly derived from methoxy groups of pectin, while ethanol is formed by reactions analogous to anaerobic fermentation, via pyruvic acid and acetaldehyde (Berger and Drawert, 1984; Knee and Hatfield, 1981; Nursten, 1970). Apart from fatty acid metabolism, propan-1-ol, butan-1-ol and fusel alcohols may be derived from fermentation of sugars, via the pathway of amino acid biosynthesis (Heath and Reineccius, 1986; Reazin *at al.*, 1970).

Biosynthesis of both straight and branched chain esters such as propyl butanoate, and ethyl 2-methyl butanoate is believed to occur via coupling of the corresponding alcohols, propan-1-ol and ethanol, with short chain acyl CoAs, butyl CoA and 2-methyl

butyl CoA, respectively, with reactions catalysed by the ester forming enzyme(s) in the presence of O_2 (Berger, 1990; 1991; Berger and Drawert, 1984; Brackmann *et al.*, 1993, Knee and Hatfield, 1981). Both straight and branched chain volatiles, particularly esters, are believed to be the most important compounds responsible for the typical characteristic aroma of apples (Acree, 1993; Cunningham *et al.*, 1986; Dürr and Schobinger, 1981; Flath *et al.*, 1967; Young *et al.*, 1996).

Secondary volatiles, hexanal and *trans*-2-hexenal, which are formed during the disruption of fruit tissue, are believed to originate from oxidative reaction of free linoleic and linolenic fatty acids and the process is catalysed by lipoxygenases (see **Chapter One**) (Hatanaka, 1993; 1996; Riley *et al.*, 1996). It has been demonstrated that these aldehydes are important to the aroma of apple products such as juice and essence, responsible for the fresh, green apple odour (Dürr and Schobinger, 1981; Flath *et al.*, 1967). They may be also of importance during cutting and chewing of fresh apples.

Production of specific esters in apples increased corresponding to supplied vapour of specific short chain alcohols, aldehydes and/or carboxylic acids (Bartley *et al.*, 1985; Berger and Drawert, 1984; Berger *et al*, 1992; De Pooter *et al.*, 1981; 1983; 1987; Knee and Hatfield, 1981). Results from these studies above also indicated that the lack of aroma volatile production in apples harvested prior to attaining the climacteric ripening or after a long-term CA storage was due to the deficiency of alcohols, but not acyl CoAs, needed for esterification (Bartley *et al.*, 1985; Hansen *et al.*, 1992a; 1992b; Mattheis *et al.*, 1995). The present study has demonstrated that production of important aroma compounds, ethyl butanoate, ethyl 2-methyl butanoate, ethyl propanoate, ethyl hexanoate and ethyl 3-hydroxy butanoate can be consistently enhanced by short-term hypoxic treatments. Moreover, ethyl 2-methyl butanoate - a commercially important aroma impact ester of 'Red Delicious' apples (Flath *et al.*, 1967) - was dramatically enhanced by hypoxic treatments in 'Granny Smith', where it is generally not produced. Short-term hypoxic treatments have also been reported to enhance aroma and/or flavour of many fruit including strawberries (Larsen and Watkins, 1995a), feijoas, peaches (Pesis, 1994; Pesis and Avissar, 1989), mandarins and oranges (Shaw *et al.*, 1990; 1991; 1992). Such an enhancement of aroma from hypoxically treated fruit is probably due to

an increased production of ethyl esters, which are known to be the most potent odour esters as a group (Teranishi *et al.*, 1987).

Increased production of important ethyl esters following hypoxic treatments is undoubtedly due to an increase in ethanol concentration in response to hypoxia. Ethanol is enzymatically coupling with acyl CoAs, such as 2-methyl butyl CoA, into ethyl esters, including ethyl 2-methyl butanoate, by reaction of ester forming enzyme(s) (Bartley *et al.*, 1985; Knee and Hatfield, 1981). Ethyl esters such as ethyl acetate and ethyl butanoate share acyl CoAs, acetyl CoA and butyl CoA, with other esters, such as hexyl acetate and propyl butanoate, as one of their substrates. The dramatic increase in ethanol used a lot of acyl CoAs to produce ethyl esters, which may have competed with other alcohols for the same acyl CoAs and limited the availability of these substrates for biosynthesis of non-ethyl esters. Alcohols other than ethanol also decreased, which is probably due to the limited availability of acyl CoAs to be reduced into their corresponding alcohols. Acyl CoAs are thought to be plentiful for esterification (Bartley *et al.*, 1985; Harb *et al.*, 1994; Knee and Hatfield, 1981), but may be limiting for synthesis of some volatiles in hypoxically treated apples. It has been reported that low O_2 treatments on apples and strawberries caused accumulation of ethyl esters, but decreased concentrations of non-ethyl esters requiring the same acyl groups for their synthesis, alcohols and aldehydes other than ethanol and acetaldehyde (Larsen and Watkins, 1995a; Mattheis *et al.*, 1991a). Results suggested that ethanol competed with other alcohols for acyl groups in esterification reactions, as well as limiting acyl CoAs to be reduced into alcohols higher than ethanol. This concept is further supported by observations that applying ethanol vapour to apples caused increases in ethyl esters but reduced concentrations of non-ethyl esters and alcohols other than ethanol (Berger and Drawert, 1984; Knee and Hatfield, 1981). Thus hypoxic treatments may have altered normal metabolism of volatile formation of apples; and the treatment itself may provide an alternative approach to study aroma volatile synthesis in fruit.

A possible mode of action by which hypoxic treatments enhance and/or reduce aroma volatile aldehydes, alcohols and esters in apples may be summarised in **Fig. 7.1**. Under hypoxia induced by very low O_2, mitochondrial respiration is generally reduced (Chervin *et al.*, 1996; Perata and Alpi, 1993) and decreases in cytosolic pH, ATP and

pyruvate dehydrogenase (PDH) activity occur; these occur concomitantly with increases in activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Chervin *et al.*, 1996; Ke *et al.*, 1993a; Kennedy *et al.*, 1992; Perata and Alpi, 1993). This phenomenon results in a subsequent accumulation of pyruvate (Kennedy *et al.*, 1992; Perata and Alpi, 1993; Ricard *et al.*, 1994) and a switch to fermentative metabolism allowing both re-oxidation of NADH produced by the glycolytic pathway and an increase in ATP production (Chervin *et al.*, 1996; Perata and Alpi, 1993).

Pyruvate is decarboxylated to acetaldehyde by PDC, which in turn is rapidly reduced by ADH causing ethanol to accumulate (Chen and Solomos, 1996; Davies, 1980; Ke *et al.*, 1994b). On removal of fruit from hypoxic conditions to air, pyruvate may be oxidised to acetate or combined with another molecule of pyruvate to form acetolactate (Reazin *et al.*, 1970). Acetolactate may be transformed via the 'valine - isoleucine biosynthetic pathway' to keto acids and subsequently converted into propan-1-ol and other fusel alcohols (Heath and Reineccius, 1986; Reazin *et al.*, 1970).

After removing fruit to air, O_2 concentrations in fruit tissues and the activity of alcohol acyl transferase (AAT) increase. AAT is the only known ester forming enzyme and its activity is suppressed under hypoxic or CA conditions (Fellman *et al.*, 1993a; 1993b). The large increase in ethanol accumulation in response to hypoxia stimulates production of ethyl acetate and other ethyl esters by coupling ethanol with acetyl CoA and ethanol with other acyl CoAs, respectively, where the reaction is catalysed by AAT and O_2 is required for the reaction (Bartley *et al.*, 1985; Berger and Drawert, 1984; Fellman and Mattheis, 1995; Oliás *et al.*, 1995). Both straight chain and branched chained acyl CoAs, such as butyl CoA and 2-methyl butyl CoA, either serve as substrates for esterification with alcohols, or are reduced to aldehydes by acyl CoA reductase (this enzyme has yet not been characterised) which in turn are reduced to alcohols by ADH activity (Bartley *et al.*, 1985; De Pooter *et al.*, 1987; Eriksson, 1979; Gilliver and Nursten, 1976; Paillard, 1990).

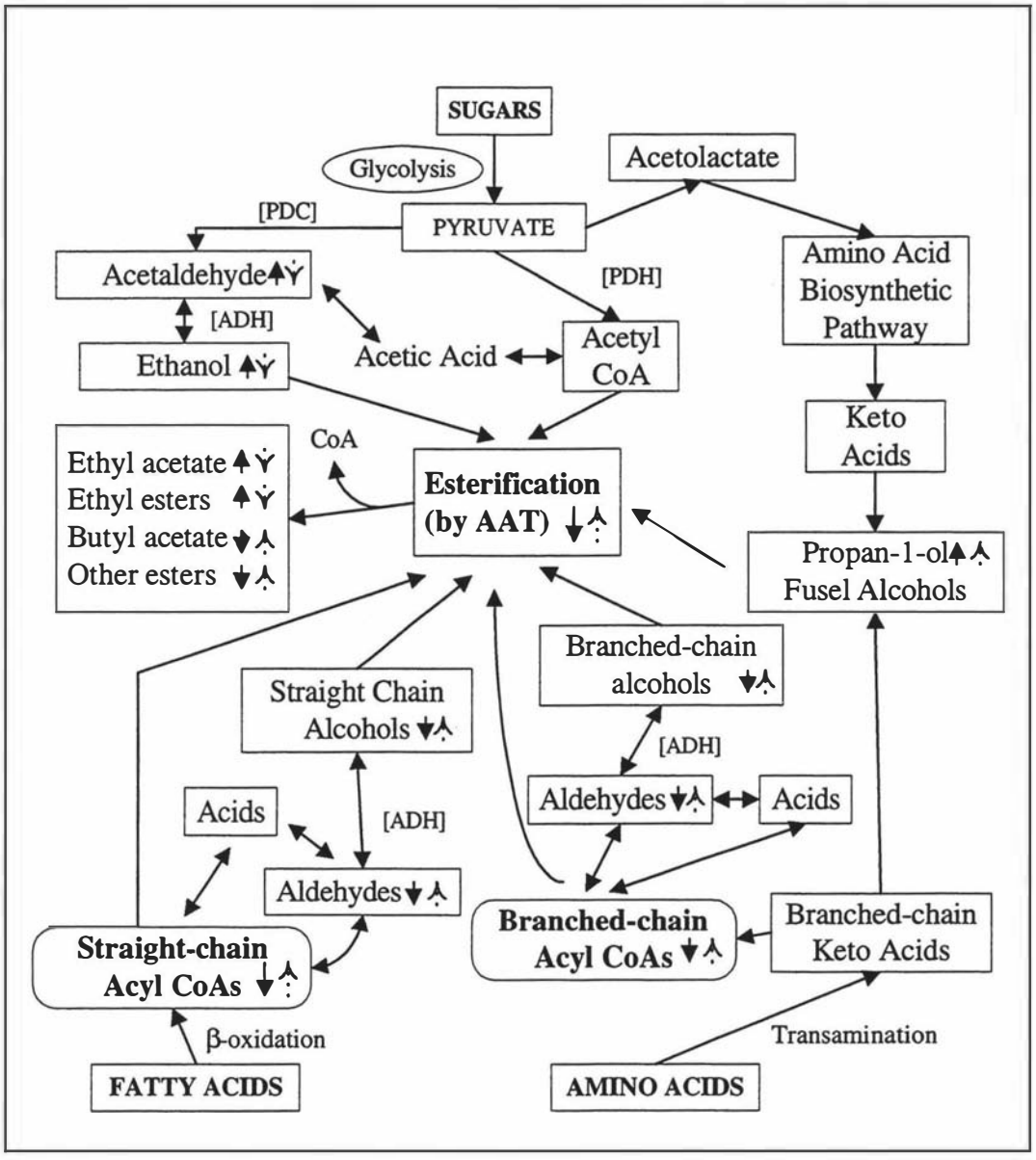


Fig. 7.1 A possible pathway of aroma volatile biosynthesis during hypoxic treatments and after removal to air in apples (after Drawert, 1975; Ke *et al.*, 1994b; Oliás *et al*, 1995; Reazin *et al.*, 1970; Tressl *et al.*, 1975).

Abbreviations and Symbols: AAT = alcohol acyltransferase, ADH = alcohol dehydrogenase, PDC = pyruvate decarboxylase, PDH = pyruvate dehydrogenase, ↑ ↓ = increase or decrease under hypoxic condition, ↑ ↓= increase or decrease under aerobic condition.

The large quantity of ethanol accumulated following hypoxic treatments is sufficient to displace other alcohols in their reaction with acyl CoAs by ester forming enzyme(s); this causes a decrease in other esters such as butyl acetate and hexyl acetate (Berger and Drawert, 1984; Berger *et al.*, 1992; De Pooter *et al.*, 1983; 1987; Knee and

Hatfield, 1981; Mattheis *et al.*, 1991a). Aldehydes, other than acetaldehyde, and alcohols, except methanol, ethanol and propan-1-ol, are also decreased because of the competition for acyl CoAs between the esterification with ethanol and the reduction into aldehydes and/or alcohols (Berger and Drawert, 1984; Berger *et al.*, 1992; Knee and Hatfield, 1981). While ethanol concentration remains high, with the availability of acyl CoAs, production of ethyl esters continues to predominate until the ethanol substrate is exhausted; at this time ethyl esters decrease, while other esters and alcohols increase. Therefore, the increase in ethanol and ethyl esters, and the decrease in other esters not containing an ethyl moiety, as well as other alcohols, following hypoxic treatment is suggested to result from the competitive nature of acyl esterification of apples.

7.2 INFLUENCE OF STORAGE CONDITIONS ON VOLATILE PRODUCTION IN APPLES

The typical aroma of apples, as well as other fruits, is not present during the early stages of fruit development, but occurs entirely at a rather brief ripening phase. Aroma development appears to occur during or after the climacteric rise in ethylene and respiration (Mattheis *et al.*, 1991b; Song and Bangerth, 1996; Yahia *et al.*, 1990a). As ripening begins, metabolism of fruit changes where minute quantities of lipids, fatty acids, proteins, amino acids and sugars, are enzymatically converted to aroma volatile compounds and other constituents (Brady, 1987; Heath and Reineccius, 1986; Paillard, 1990).

The rate of aroma volatile production generally reaches a maximum during the climacteric ripening period (Brown *et al.*, 1966; Sapers *et al.*, 1977; Song and Bangerth, 1996). However, not all volatile compounds produced by apples reach their maximum production at the same time. Aldehydes, such as butanal, pentanal and *trans*-2-hexenal, were detected in high concentrations in immature intact fruit and their concentrations decreased or disappeared as internal ethylene began to increase (De Pooter *et al.*, 1987; Mattheis *et al.*, 1991b). Most esters, such as propyl acetate, butyl acetate and butyl butanoate, and alcohols, including 2-propan-1-ol, butan-1-ol and 2-methyl butan-1-ol

increased and peaked approximately at or shortly after the climacteric (Mattheis *et al.*, 1991b; Song and Bangerth, 1996; Yahia *et al.*, 1990a). Some esters, such as hexyl acetate, hexyl hexanoate and 2-methyl butyl acetate were detected several weeks prior to the increase in ethylene production of 'Delicious' apples (Mattheis *et al.*, 1991b). Detection of these esters prior to a rise in ethylene climacteric probably indicates that ester forming enzyme(s) are functional prior to attainment of physiological maturity of the fruit. Volatile compounds such as acetaldehyde, ethanol, ethyl propanoate, ethyl 2-methyl butanoate and other ethyl esters increased steadily during the post-climacteric phase and after cold storage (0° - 2°C) in apples (Patterson and Nichols, 1988; Sapers *et al.*, 1977; Yahia *et al.*, 1990a). Differences in aroma volatile production pattern from producing mainly aldehydes to esters and alcohols during the final stage of fruit development suggest that there are different metabolic processes and/or reactions being consecutively operated, liberating substrates and/or precursors for aroma volatile synthesis. As fruit age, or after prolonged cold storage, volatile production from apples was weak; such fruit exhibited a pronounced loss of flavour (Paillard, 1990), which may be due to changes in metabolism involved in volatile synthesis and the reduced capacity of an esterifying enzyme system.

The magnitude of enhancement of aroma volatile compounds following 100% CO_2 treatment of apples appeared to be affected by fruit age (**Table 7.1**). In freshly harvested 'Red Delicious' apples, CO_2 treatment substantially enhanced several volatile compounds, where the magnitude of enhancement was in the order of 9 - 74 fold over control, depending on compound. Some aroma impact volatile compounds, including ethyl 2-methyl butanoate and ethyl hexanoate, were not detected in freshly harvested control apples, but were enhanced by CO_2 treatment with the mean concentrations during 7 days storage at 20°C of 239 and 121 $\mu mol \cdot l^{-1}$, respectively (**Table 7.1**).

In contrast, the magnitude of enhancement of these volatile compounds following CO_2 treatment in fruit stored (0° - 1°C) for 8 months was minimal or non-existent (**Table 7.1**). The magnitude of enhancement obtained for headspace ethanol and volatile ethanol was over 2 fold, but less than 2 fold for headspace ethyl acetate, volatile ethyl acetate and ethyl hexanoate, relative to controls. Concentrations of aroma volatiles such

as ethyl butanoate, ethyl 2-methyl butanoate and headspace acetaldehyde were similar to controls (**Table 7.1**).

It is interesting to note that, concentrations of some volatile compounds, such as headspace ethanol, headspace ethyl acetate and hexanal in freshly harvested apples treated with CO_2 were similar to those in control 8-month old fruit. More importantly control values of 8-month old fruit was higher (much in some cases) than the enhanced values for freshly harvested CO_2 treated fruit, for ethyl butanoate and ethyl hexanoate (**Table 7.1**).

Table 7.1 Mean concentration, degree of enhancement (ratio) or percentage of reduction of selected volatile compounds during 7 days post storage at 20°C (70% RH) from juice of control and 100% CO_2 treated (24 hours at 20°C) freshly harvested and after 8-month cold stored (0°C) 'Red Delicious' apples.

Compound	Concentration					
	Freshly Harvested			After 8-month Cold Storage		
	Ctrl	CO_2	Increase[3] Decrease[3]	Ctrl	CO_2	Increase[3] Decrease[3]
Headspace Volatiles[1]						
Acetaldehyde	0.8[b]	10.2[a]	+12.8	52.0[z]	45.4[y]	-12%
Ethanol	1.5[b]	38.0[a]	+25.3	36.3[z]	90.6[y]	+2.5
Ethyl acetate	0.3[b]	22.3[a]	+74.3	17.3[z]	31.6[y]	+31.6
Other Volatiles[2]						
Ethyl acetate	611[b]	5686[a]	+9.3	1565[z]	2587[y]	+1.6
Ethyl butanoate	72[b]	668[a]	+9.3	888[y]	826[y]	-7%
E 2-MB	n.d.	239	–[4]	168[y]	169[y]	+1.0
Ethyl hexanoate	n.d.	121	–	227[z]	339[y]	+1.5
Butyl acetate	460[a]	367[b]	-20%	271[y]	173[z]	-36%
Hexyl acetate	153[a]	93[b]	-39%	142[y]	89[z]	-37%
Methanol	574[a]	612[a]	+1.1	n.d.	n.d.	–
Ethanol	349[b]	16600[a]	+47.6	4306[z]	11654[y]	+2.7
Propan-1-ol	1569[a]	1566[a]	+1.0	859[y]	905[y]	+1.1
Butan-1-ol	2142[a]	2365[a]	+1.1	2175[y]	1991[y]	-8%
Hexan-1-ol	307[a]	309[a]	+1.0	477[y]	420[y]	-12%
Hexanal	221[a]	220[a]	+1.0	239[y]	193[z]	-19%
trans-2-Hexenal	1061[a]	810[b]	-17%	399[y]	351[y]	-12%

Data were mean concentrations of 4 replicates during 7 days at 20°C, Values followed by different letters between treatments (within rows for each harvest date) were significantly different at 5% level, [1] Concentration in $\mu l \cdot l^{-1}$, [2] Concentration in $\mu mol \cdot l^{-1}$, [3] Increase was the ratio (fold) between concentrations from CO_2 treated and control fruit, and Decrease was percentage of reduction in concentration in CO_2 treated fruit, relative to control, [4] could not be calculated. Ctrl = control, CO_2 = 100% CO_2 for 24 hours at 20°C, E 2-MB = ethyl 2-methyl butanoate, n.d. = not detected

The degree of reduction of some volatile compounds, such as butyl acetate, hexanal, butan-1-ol and hexan-1-ol increased with fruit age. Concentrations of some volatile compounds following the CO_2 treatment were relatively higher in 8-month old fruit than in freshly harvested apples; these included the headspace volatiles, ethyl butanoate, ethyl hexanoate and hexan-1-ol. On the other hand, concentrations of ethyl acetate, ethyl 2-methyl butanoate, butyl acetate, ethanol and propan-1-ol in CO_2 treated 8-month old apples were lower than in freshly harvested CO_2 treated fruit. Concentrations of hexyl acetate, butan-1-ol and hexanal in CO_2 treated apples were little affected by fruit age. It seems that concentrations of low molecular weight alcohols such as methanol, ethanol and propan-1-ol in CO_2 treated fruit decreased, while medium molecular weight alcohols, including butan-1-ol, were unaffected and higher alcohols, such as hexan-1-ol, increased as fruit aged.

Low production of ethyl esters in control fruit of freshly harvested apples appears to be due to a lack of the alcohol, ethanol, which is required for esterification. Treatment with CO_2 stimulates a large accumulation of ethanol in fruit tissues, which in turn serves as a substrate for ester synthesis with other acyl CoAs, resulting in substantial increases in ethyl esters such as ethyl acetate, ethyl butanoate and ethyl 2-methyl butanoate (**Fig 7.1**). This phenomenon also suggests that acyl CoA substrates such as acetyl CoA, butyl CoA, and 2-methyl butyl CoA are not limiting for esterification and the ester forming enzyme(s) are fully functional.

As fruit aged, concentration of ethanol, as well as acetaldehyde, increased markedly in control fruit and this was accompanied by a rise in concentrations of low molecular weight ethyl esters. Such increases in acetaldehyde, ethanol and ethyl esters in the latter period of ripening and after cold storage (0°C, 3 - 6 months) has been observed in apples and pears (Janes and Frenkel, 1978; Nichols and Patterson, 1987; Sapers *et al.*, 1977); it has been suggested that this is a normal phenomenon in fruit during the latter period of ripening and senescence (Nursten, 1970; Paz *et al.*, 1981). Such an increase in ethanol and acetaldehyde suggests that fermentation-directed metabolism, where ethanol is subsequently metabolised into ethyl esters, is a naturally occurring phenomenon which can occur in air in aging fruit that had not been subjected to exogenously applied hypoxic conditions. This increase in endogenous ethanol and acetaldehyde in air stored fruit

could be associated with a decrease in fruit skin permeance (Dadzie, 1992) or with increased flesh tissue disintegration by cell wall hydrolytic enzymes (Bartley and Knee, 1982; Brady, 1987), either or both of which could result in restriction of gas movement (Blanke, 1991; Dadzie, 1992) causing partial anaerobiosis in apple fruit tissue (Bramlage *et al.*, 1977; Patterson and Nichols, 1988), and consequent acetaldehyde and ethanol accumulation. As the length of time in storage increases, restriction to gas exchange may be aggravated as fruit become increasingly senescent. Hence, anaerobic metabolism may be increased, particularly in the inner cortex tissue (Blanke, 1991), and may reach a point at which ethanol accumulation becomes as high as that found in CO_2 treated freshly harvested fruit. This phenomenon was true with headspace ethanol (**Table 7.1**). This large ethanol accumulation in old apples is high enough to be esterified with other acyl CoAs into ethyl esters including ethyl butanoate, and ethyl 2-methyl butanoate.

It has been suggested that alcohol substrate availability limited the extent of esterification in early harvested 'Delicious', 'Golden Delicious' or 'Jonagold' apples (Hansen *et al.*, 1992a; Mattheis *et al.*, 1991b; Song and Bangerth, 1994) and exogenously supplied aldehydes or alcohols to fruit dramatically increased subsequent production of the corresponding alcohols and/or esters (Bartley *et al.*, 1985; Knee and Hatfield, 1981; Song and Bangerth, 1994). Alcohols, other than ethanol, such as butan-1-ol and hexan-1-ol increased during climacteric ripening in apples (Mattheis *et al.*, 1991b; Paillard, 1990), suggesting an increased metabolism and availability of precursors, fatty acids and amino acids, for the generation of alcohols, as well as acyl CoAs, for subsequent ester biosynthesis (Drawert, 1975; Schreier, 1984; Tressl *et al.*, 1975). Concentrations of C_{16} - C_{18} fatty acids, particularly linoleic and linolenic acids, increased markedly during ripening of 'Golden Delicious' apples (Song and Bangerth, 1994). Although amino acids are putative precursors for branched-chain alcohols and esters (Drawert, 1975; Hansen and Poll, 1993; Tressl *et al.*, 1975), there is a lack of information on quantitative changes of individual amino acids in apples in relation to fruit maturation, ripening and senescence or as affected by pre- and postharvest conditions. Presumably, degradation of amino acids and fatty acids occur as fruit ripen, providing substrates for ester synthesis and hence increasing both straight chain and branched chain volatile compounds in apples as fruit ripening proceeds.

Although CO_2 treatment enhanced ethanol concentration nearly 3 fold in 8-month old apples compared with control, important aroma impact volatile compounds such as ethyl butanoate and ethyl 2-methyl butanoate were not enhanced, while a slight enhancement was achieved with ethyl acetate and ethyl hexanoate. In fact, the extent of enhancement of ethyl acetate and ethyl 2-methyl butanoate in CO_2 treated 8-month cold stored apples was decreased, relative to those in freshly harvested CO_2 treated fruit. Acetate esters such as butyl acetate and hexyl acetate also decreased in 8-month old control apples relative to freshly harvested control fruit, while butan-1-ol remained approximately constant and hexan-1-ol was relatively increased (**Table 7.1**). This phenomenon suggests that acyl CoAs, including acetyl CoA and 2-methyl butyl CoA, may become deficient as fruit age, although quantitative changes of the individual acyl CoAs and their metabolism in apples awaits elucidation.

Despite the indication that ester forming enzyme(s) were operative in 8-month old apples, a reduced capacity of the enzyme system could occur, due to a general deterioration as fruit aged (Brady, 1987; Paillard, 1990). If this was so it may be partly responsible for the reduced capacity of ester synthesis, as suggested by the low magnitude of enhancement in ethyl esters, relative to that in freshly harvested fruit. In 'Rome' apples, the *in vitro* activity of AAT from 3-month refrigerated-air stored apples was comparable to that of freshly harvested fruit, but it was substantially decreased after fruit had been stored for 6 months (Fellman *et al.*, 1993b).

Production of some ethyl esters such as ethyl butanoate and ethyl hexanoate in 8-month old control fruit was greater than in freshly harvested CO_2 treated apples (**Table 7.1**), and is probably associated with the enzyme system involved in synthesis of these esters. Fruit ester forming enzyme(s) are known to possess inherent properties, such as substrate specificity (Berger and Drawert, 1984; Gilliver and Nursten, 1976; Oliás *et al.*, 1995), and as fruit age with concomitant ethanol accumulation, the ester forming enzyme(s) may favour esterification to these ethyl esters. Another possibility is that specific new enzymes, isoenzymes or co-factors are activated and/or induced (Kanellis *et al.*, 1991), which encourage esterification of these ethyl esters. At the same time, a reduced activity of ester forming enzyme(s) having substrate specificity for esterification of high molecular weight alcohols, such as butan-1-ol and hexan-1-ol could have

occurred. Together these processes might result in an increase in ethyl esters and a decrease in butyl and hexyl acetate production as fruit age.

Changes in metabolism could be another factor influencing the reduced production of some volatiles as fruit aged, in both control and CO_2 treated apples. Decreased production of low molecular weight alcohols, such as propan-1-ol and an increased production of higher molecular weight alcohols, such as hexan-1-ol, indicates a reduced capacity in metabolism, probably β-oxidation, which reduces longer chain fatty acids into shorter chain fatty acids. This in turn, would reduce availability of low molecular weight alcohols and acyl CoAs needed for low molecular weight volatile synthesis, but increase relatively high molecular weight substrates. That this might occur is suggested by the increased concentrations of hexan-1-ol and ethyl hexanoate, and decreased butyl acetate and propan-1-ol in 8-month old apples, relative to freshly harvested fruit.

Exhaustion of precursors and/or substrates as fruit aged is possibly another factor effecting a reduced magnitude of enhancement of important volatile compounds in 8-month old apples. After long-term refrigerated air storage (> 6 months at 0° - 2°C) concentrations of fatty acids, particularly $C_{18:3}$ fatty acids, decreased in apples (Paillard, 1990). Such reduced concentrations of free fatty acids could result in a subsequent lack of availability of the substrates, alcohols and acyl CoAs needed for ester synthesis. This may also be responsible for reduced production of *trans*-2-hexenal (of more than 50%) in juice of old apples, relative to freshly harvested fruit (**Table 7.1**), as in the presence of O_2 the $C_{18:3}$ fatty acid is a putative precursor of *trans*-2-hexenal catalysed by lipoxygenase during tissue disintegration (Hatanaka, 1993; 1996; Schreier, 1984). Furthermore, a reduced capacity of lipoxygenase could also account for the decreased production of *trans*-2-hexenal as fruit aged. It has been reported that *in vitro* lipoxygenase activity decreased by more than 50% in fully ripe (red) tomatoes, compared with mature-green or breaker (pink) fruit (Riley *et al.*, 1996).

Therefore it seems that as fruit aged, several metabolic systems could be acting consecutively or independently, resulting in the relatively small enhancement of volatile ethyl esters and/or ethanol, and the decrease and/or increase of some volatile

compounds, both in control and CO_2 treated 8-month old cold stored apples, relative to similar treatments in freshly harvested fruit.

Apple cultivars are known to differ both quantitatively and qualitatively in their volatile production (**Chapter Six**), which may be associated with differences in their general metabolism, availability of precursors and/or substrate pools and the capacity of the enzyme systems involved in volatile synthesis (Brackmann *et al.*, 1993; Gilliver and Nursten, 1976; Song and Bangerth, 1996). Brackmann and Streif (1994) found that not only was ethylene production and respiration rate of 'Granny Smith' and 'Fuji' apples relatively lower than 'Red Delicious', but so was the total volatile production.

Post storage production of some ethyl esters such as ethyl acetate, ethyl butanoate and ethyl 2-methyl butanoate in control 'Granny Smith' and 'Fuji' apples increased markedly during the latter period of 6-month refrigerated-air storage at 0°C (**Chapter Five**). These ethyl esters did not increase to the concentration found in 8-month old CO_2 treated 'Red Delicious' apples, even though they tended to increase with time in storage. This phenomenon may reflect differences in fruit age and the inherited properties of cultivars which reflect differences in their general metabolism. 'Granny Smith' and 'Fuji' apples are well known for their ability to store well, even in air (Fleming, 1990; Jobling *et al.*, 1993; Kupferman, 1994), which suggests that the deterioration rate of these apples is relatively slow. Results from the present study confirmed that respiration rate and ethylene production of these two cultivars were relatively low, compared with 'Red Delicious' apples (Brackmann and Streif, 1994). Low respiration rate and ethylene production retard ripening rate and/or deterioration of these cultivars and may be the reason for their ability to store well. Within a given fruit species, a high respiration rate and/or ethylene production is commonly associated with a rapid rate of deterioration in quality and hence short storage life and *vice versa* (Blanke, 1991; Jobling *et al.*, 1993; Kader, 1986). If this is so, rate of ripening and senescence of 'Granny Smith' and 'Fuji' would be slower, relative to 'Red Delicious' apples with a consequent delay in ethanol production, a natural post-ripening and senescence volatile (Janes and Frenkel, 1978; Nursten, 1970), and subsequent production of ethyl esters.

Thus, it seems that a relatively low metabolic rate, which would result in a relatively slow ripening rate, may lead to relatively less ethanol accumulation occurring

in 'Granny Smith' and 'Fuji' relative to 'Red Delicious' apples after 6 months storage. If this was associated with a relatively low availability of precursors and/or substrates, as well as low capacity of appropriate enzyme systems, then this may be the reason for the relatively lower ethyl ester production in control 'Granny Smith' and 'Fuji' than in 'Red Delicious' apples after a similar length of storage time.

Controlled-atmosphere storage is well known to cause a reduction in aroma of apples stored for more than 6 months (Fellman and Mattheis, 1995; Fellman *et al.*, 1993a, 1993b; Mattheis *et al.*, 1995; Song and Bangerth, 1996; Streif and Bangerth, 1988). The same phenomenon also occurred in control 'Granny Smith' and 'Fuji' apples, with production of some volatile compounds such as acetaldehyde, ethanol, ethyl butanoate and ethyl 2-methyl butanoate from CA stored fruit depressed even after 10 - 14 weeks storage, compared with RA fruit. Post storage treatment with 100% CO_2 consistently and markedly enhanced aroma impact volatile ethyl esters in both RA and CA stored apples. Such a response can be effectively achieved in both apple cultivars, even after 6 months storage (**Chapter Five**). However, there was a steady reduction of these enhanced ethyl esters with increasing time in CA storage, and a point could be reached eventually that such an enhancement could not be achieved by hypoxic treatments. Such a decrease in these enhanced ethyl esters is likely to be due to a reduced availability of acyl CoAs needed for ester synthesis, although it may also relate to a reduced activity of the ester forming enzyme(s) (Fellman and Mattheis, 1995; Fellman *et al.*, 1993a; 1993b). Knee and Hatfield (1981) suggested that apples under CA storage may lack capacity for alcohol synthesis, but were able to generate acetyl CoA to esterify alcohols in excess (Bartley *et al.*, 1985; Knee and Hatfield, 1981). Because post storage ethanol concentrations were high and did not decrease with time in storage, while ethyl esters such as ethyl butanoate and ethyl hexanoate decreased continuously following CO_2 treatments (see **Chapter Five**), it is suggested that acyl CoAs could be a limiting factor for ethyl ester synthesis in CA stored apples. A similar phenomenon was observed in production of butyl acetate and hexyl acetate from CA stored 'Fuji' apples, which decreased continuously with relatively greater rates than in their corresponding alcohols, butan-1-ol and hexan-1-ol (**Chapter Five**). The rate of reduction of hexyl acetate concentrations in CA stored 'Fuji' between 6 and 14 weeks was relatively less

than that during 14 - 27 weeks storage, while a decrease in hexan-1-ol during the latter period (14 - 27 weeks) was slight (**Chapter Five**); this suggests that both a reduced availability of acetyl CoA and a reduced activity of ester forming enzyme(s) were responsible for such reduction. Fellman and Mattheis (1995) demonstrated that the *in vitro* activity of AAT of 'Rome' apples decreased markedly between 3 and 6 months CA storage, compared with freshly harvested fruit. Apart from its effect on depressing AAT activity, it seems that the effect of CA on reducing the aroma of apples lies further back in the pathway that generates substrates for volatile production, perhaps involving a general suppression of lipid and/or amino acid metabolism of the fruit, as the majority of both substrates, alcohols and acyl CoAs, required for ester synthesis in apples are derived from these precursors (Paillard, 1990; Tressl *et al.*, 1975).

The present study shows that the magnitude of enhancement of aroma volatile compounds induced in apples by hypoxic treatments is affected by fruit age, cultivars and storage regimes. However, other factors such as temperature (lower or higher than 20°C) during and/or following treatments, fruit size, fruit parts, fruit position on tree, seasonal variation and growing region can probably influence the degree of enhancement and/or reduction of aroma volatile compounds in apples after treatment with a short-term hypoxic atmosphere. Detailed responses are not known and await further investigation.

7.3 POSSIBLE AROMA ENHANCEMENT OF HYPOXICALLY TREATED APPLES

Aroma is a complex mixture of trace concentrations of volatile compounds responsible for odour that give much of the character to fruit. Esters, aldehydes and alcohols are major volatile compounds found in apples and they are believed to be the key odour constituents, especially esters, that provide desirable characteristic fruity aroma notes to the fruit (Acree, 1993; Dimick and Hoskin, 1983; Dürr and Schobinger, 1981). The origins and metabolism of these chemical classes of volatiles (esters, alcohols and aldehydes) in fruits have been investigated in some detail and they are the compounds reported in most studies involving aroma of apple fruit and its products

(Bartley *et al.*, 1985; Berger and Drawert, 1984; De Pooter *et al.*, 1981; 1983; 1987; Fellman and Mattheis, 1995; Fellman *et al.*, 1993a; 1993b; Knee and Hatfield, 1981; Song and Bangerth, 1996; Willaert *et al.*, 1983; Yahia, 1989; Young *et al.*, 1996). Other chemical classes of volatile compounds such as ketones, terpenes, hydrocarbons and acids are present in minor proportions in the volatile profile of apples, but their metabolic origins are less well known and they are thought to have low, if any, aroma value (Nursten, 1970; Williams, 1979; Yahia, 1994). There are exceptions for some compounds in these chemical classes, where their aroma significance and/or metabolic origins have been studied in some detail. For example, β-damascenone believed to be derived from xanthophylls (Roberts and Acree, 1995), is a potent fruity odoriferous compound, regarded as an important aroma compound of processed apple juice (Cunningham *et al.*, 1986). Another example is 4-methoxyallyl benzene, which has been shown to be responsible for a spicy aroma note in several apple cultivars and it is thought to be derived from sugars via shikimic acid and phenylalanine (Williams *et al.*, 1977b). Williams (1979) speculated that some volatile compounds, which are generally found as a minor portion of total volatile composition, may well make some contribution to the aroma of apples; some ketones and hydrocarbons probably contribute to 'dried leaves' notes and a terpene, linalool-5-oxide, gives the 'scented' character to apple fruit. Thus, minor volatile compounds could also be important contributors to specific aroma notes to apples.

Apple fruit and their products such as apple juice and apple aqueous essence, contain several hundred volatile compounds (Dimick and Hoskin, 1983; Maarse, 1991; Paillard, 1990). Therefore, it is essential that highly accurate and sensitive methods are available to enable clear and consistent separation and quantification of the volatile constituents contributing significantly to the aroma of a fruit or its products (Acree, 1993; Buttery, 1993). An often used technique for quantifying and concomitantly characterising volatile compounds which contribute significantly to the aroma of a product is gas liquid chromatography-olfactometry (GC-O) - synonym 'GLC sniffing' (Acree, 1993; Cunningham *et al.*, 1986; Flath *et al.*, 1967; Guadagni *et al.*, 1966; Kollmannsberger and Berger, 1992; Young *et al.*, 1996). GC-O is an analytical technique that combines high resolution GLC to separate volatile compounds extracted from a

product with olfactometry. An olfactometer combines separated chemicals (measured concentrations) with purified air and delivers them to a human 'sniffer'. The sniffer smells the air through the nose and records any perception (Acree, 1993; Marin *et al.*, 1991). Quantitative analysis of volatile compounds is very important to the understanding of the effect of an odour compound in a product because concentration is directly associated with the intensity of an odour to be perceived. For example, if a compound is present at a concentration well below its odour threshold (the minimum physical intensity for detection), that compound is unlikely to contribute to the odour of that product, and perhaps can be ignored. On the other hand, if a compound is present in a concentration that greatly exceeds its odour threshold value, then it is likely to contribute to the odour of that product (Acree, 1993; Buttery; 1993; Guadagni *et al.*, 1966; Teranishi *et al.*, 1987).

The relative contribution of a compound to the odour (or aroma) can be calculated from a ratio between concentration of that compound and its odour threshold concentration (Guadagni *et al.*, 1966) giving the so-called 'odour unit', 'aroma unit', 'aroma value' or 'relative contribution value' (Buttery, 1993; Guadagni *et al.*, 1966; Maarse, 1991; Pesis, 1994; Teranishi *et al.*, 1987). The probability of an odorous compound being detected should be greater, the larger the value of the odour unit for that compound (Teranishi *et al.*, 1987). Odour unit can be useful in pinpointing those aroma compounds that are important and those that can be disregarded. If the odour unit is greater than 1, the compound is present above its threshold concentration, and should contribute to the aroma. If the odour unit is less than 1, the compound is below its threshold value, and probably does not contribute significantly to the total odour (Acree, 1993).

To determine the odour unit of a compound, the concentration of that compound in a sample and its odour threshold concentration must be known (Guadagni *et al.*, 1966). As most fruit and vegetables contain 70% - 90% water, the threshold value of a compound is generally determined by using that chemical (usually synthetic) in a water medium, based on the assumption that the volatility of the compound in aqueous products is approximately the same as it would be in water (Buttery, 1993). Guadagni *et al.* (1966) indicated that the threshold of a mixture of odorous compounds is related to

the thresholds of all the individual compounds by a simple additive law. This additive relationship has been found to be valid only with values near threshold concentrations of those compounds in a mixture (Guadagni *et al.*, 1966; Teranishi *et al.*, 1987).

Base on the concept of odour unit, synthetic mixtures were prepared according to concentration of volatiles found in sliced fresh tomatoes (10 compounds) or tomato paste (7 compounds), which had odour units greater than 1; sensory panel studies found such synthetic mixtures to have aromas very similar to sliced fresh tomatoes or commercial tomato paste (Buttery, 1993), supporting the validity of the quantitative analysis of aroma volatile compounds. This also indicates that the aroma of a product is due to a combination of several volatile compounds presence.

In the present study, a number of volatile compounds were enhanced by hypoxic treatment. An attempt was made to predict the relative contribution of these volatiles to the aroma of apple juice, based on the odour unit concept (**Table 7.2**). Data presented here are by no means conclusive, as several other compounds that were not quantified may also contribute to the aroma of apple juice. The number of compounds and calculated odour units were also limited by the information on odour threshold concentrations available from the literature.

Odour threshold concentrations in water differ greatly among the selected volatile compounds, ranging from 10^{-4} ppm (ethyl 2-methyl butanoate) to 10^{2} ppm for ethanol and acetic acid. Treatment with 100% CO_2 for 24 hours at 20°C increased, decreased or had no effect on concentrations of aroma volatile compounds in juice of 'Red Delicious' apples 3 days at 20°C following treatment, depending on compounds (**Table 7.2**). The reason for selecting volatile data for calculation at 3 days after CO_2 treatment was that the majority of the enhanced aroma volatile compounds such as ethyl butanoate and ethyl 2-methyl butanoate generally reached their peak concentrations at this time, before decreasing thereafter.

Table 7.2 Concentrations of selected aroma volatile compounds in juice from control and 100% CO_2 treated (24 hours at 20°C) freshly harvested 'Red Delicious' apples 3 days at 20°C after treatment, the magnit de of enhancement or degree reduction, and relative contribution (odour unit) to the aroma of these compounds.

Compound	Concentration (ppm)		increase[1]/ decrease[1]	Threshold[3] (ppm)	Odour Unit[5]	
	Ctrl	CO_2			Ctrl	CO_2
E 2-MB	n.d.	69.7	–[2]	0.0001	0	697000
Ethyl hexanoate	n.d.	30.4	–	0.001	0	30400
Ethyl propanoate	n.d.	122.7	–	0.01	0	12270
E 3-(OH)B	n.d.	29.1	–	n.a.		
Hexyl hexanoate	n.d.	2.8	–	n.a.		
Ethyl acetate	78.1	8330.0	+ 107	5	15	1666
Ethanol	24.7	1191.0	+ 48	100	0.25	11
Acetaldehyde	0.9	13.7	+ 16	0.015	57	913
Ethyl butanoate	22.3	171.6	+ 7	0.001	22300	171600
Acetic acid	2.1	12.6	+ 6	100	0.02	0.13
Pentan-1-ol	1.5	4.2	+ 2	4.5	0.33	0.9
Hexanal	30.6	34.1	+ 1	0.005	6120	6820
Butan-1-ol	231.4	226.0	- 2%	0.5	462	452
Decane	8.5	8.0	- 6%	n.a.		
Tridecane	12.6	11.7	- 7%	n.a.		
Dodecane	12.9	11.7	- 8%	n.a.		
Methanol	27.4	24.6	- 10%	100[4]	0.27	0.25
Propan-1-ol	134.9	115.8	- 14%	9	15	13
Propyl butanoate	14.5	12.3	- 15%	0.018	805	683
trans-2-Hexenal	139.3	115.4	-17 %	0.017	8194	6788
2-Methyl propan-1-ol	17.9	14.8	- 17%	n.a.		
Amyl acetate	16.2	13.1	- 19%	0.005	3240	2620
Xylene	28.0	22.5	- 19%	n.a.		
Hexan-1-ol	48.1	37.8	- 21%	0.5	96	76
Butyl acetate	66.9	41.2	- 38%	0.066	1013	624
2/3-Methyl butan-1-ol	140.3	84.3	- 40%	0.25	561	337
Propyl acetate	38.8	19.6	- 50%	n.a.		
Hexyl acetate	32.3	11.6	- 64%	0.002	16150	5800
2-Methyl butyl acetate	82.6	21.8	- 73%	0.005	16520	4360
α-Farnesene	140.6	8.7	- 94%	n.a.		
Sum of Odour Unit			(+12.5)[6]		**75549**	**942434**

Data were mean of 4 replicates, [1] Increase was the ratio (fold) between concentrations from CO_2 and control treatments, and decrease was percentage of reduction from CO_2 treatment relative to control, [2] Could not be calculated, [3] Odour threshold concentration in water (after Brennand *et al.*, 1989; Buttery 1993; Kollmannsberger and Berger, 1992; Teranishi *et al.*, 1987), [4] Odour threshold concentration in air (after Shaw, 1986), [5] Odour unit = compound concentration ÷ odour threshold concentration, [6] Ratio (fold) between sum of odour unit from CO_2 and control treatments, n.a. = not available, n.d. = not detected, E 2-MB = ethyl 2-methyl butanoate, E 3-(OH)B = ethyl 3-hydroxy butanoate

Table 7.3 The magnitude of enhancement and the degree of reduction in concentrations of selected volatile compounds in juice from 100% CO_2 treated (24 hours at 20°C) fruit of 5 apple cultivars, relative to control fruit 3 days after treatment at 20°C.

Compound	Cultivar / Enhancement (fold) or Reduction (%)[1]				
	RD	GS	Fuji	PR	RG
Ethyl 2-methyl butanoate	+++[2]	+++	+19.4	+++	n.d.
Ethyl hexanoate	+++	+++	+6.0	+++	n.d.
Ethyl propanoate	+++	+++	+++	+++	n.d.
Ethyl 3-hydroxy butanoate	+++	+++	+++	+++	n.d.
Hexyl hexanoate	+++	n.d.	n.d.	n.d.	n.d.
Ethyl acetate	+106.7	+++	+2.9	+8.6	+12.0
Ethanol	+48.2	+42.9	+4.3	+5.4	+5.4
Acetaldehyde	+15.2	+++	+2.7	+++	+++
Ethyl butanoate	+7.7	+4.4	+1.9	+4.9	n.d.
Acetic acid	+6.0	+3.0	+1.1	n.d.	n.d.
Pentan-1-ol	+2.8	n.d.	+2.1	n.d.	n.d.
2-Methyl butanoic acid	n.d.	n.d.	n.d.	n.d.	+1.2
2-Butanone	n.d.	n.d.	n.d.	n.d.	+1.2
6-Methyl 5-hepten-2-ol	n.d.	+1.2	n.d.	n.d.	n.d.
1,3-Butanediol	n.d.	+1.02	n.d.	n.d.	n.d.
Decane	-5.9%	n.d.	-28.8%	n.d.	n.d.
Tridecane	-7.1%	-4.6%	-2.2%	-16.7%	n.d.
Tetradecane	n.d.	-28.1%	-2.4%	n.d.	n.d.
Pentadecane	n.d.	+1.03	n.d.	n.d.	n.d.
Dodecane	-8.5%	n.d.	+1.1	n.d.	n.d.
Xylene	-19.6%	-7.1%	n.d.	+1.6	n.d.
ρ-Xylene	n.d.	n.d.	-8.8%	1.0	+1.2
Hexanal	+1.1	-4.8%	+1.1	+1.6	+1.3
Butan-1-ol	-2.3%	-25.5%	+1.04	-25.3%	+1.8
Methanol	-10.2%	n.d.	-12.8%	+1.1	n.d.
Propan-1-ol	-14.2%	+1.3	-7.3%	+2.8	+1.1
Propyl butanoate	-15.2%	n.d.	n.d.	n.d.	n.d.
trans-2-Hexenal	-17.2%	-36.9%	+1.1	-42.1%	+1.1
2-Methyl propan-1-ol	-17.3%	n.d.	n.d.	n.d.	+1.3
Amyl acetate	-19.1%	-7.4%	n.d.	n.d.	+1.2
Hexan-1-ol	-21.4%	-33.2%	+1.3	-5.7%	-4.8%
Butyl acetate	-38.4%	n.d.	-23.4%	-10.1%	-9.2%
2/3 Methyl butan-1-ol	-39.9%	-50.0%	-0.3%	-29.3%	-9.2%
Propyl acetate	-49.5%	-10.7%	-1.5%	n.d.	-35.8%
Hexyl acetate	-64.1%	n.d.	-49.4%	-36.5%	-25.9%
2/3 Methyl butyl acetate	-73.6%	-26.7%	-38.9%	-58.9%	-33.5%
α-Farnesene	-93.8%	---[3]	-98.3%	n.d.	n.d.

[1] Increase was the ratio (fold) between concentrations from CO_2 and control treatments, and decrease was percentage of reduction from CO_2 treatment relative to control (Details for replication, concentration of compounds and calculation are presented in **Table 7.2** and **Appendix 6**), [2] Enhanced markedly by CO_2 treatment and not detected in control, [3] Decreased markedly to non-detectable concentration by CO_2 treatment, RD = Red Delicious, GS = Granny Smith, PR = Pacific Rose, RG = Royal Gala, n.d. = not detected or not determined

The magnitude of enhancement and the degree of reduction in concentrations of selected volatile compounds from CO_2 treated 'Granny Smith', 'Fuji', 'Pacific Rose' and 'Royal Gala' apples relative to control fruit (**Table 7.3**) were obtained from using the same calculation method as provided for 'Red Delicious' (**Table 7.2**) and details are given in **Appendix 6**. The majority of enhanced volatile compounds were ethyl esters, ethanol and acetaldehyde for most cultivars, except 'Royal Gala' where only ethanol, acetaldehyde and ethyl acetate were the major enhanced volatiles; ethyl esters other than ethyl acetate were not detected. In general, the magnitude of enhancement of volatile compounds among 5 apple cultivars following hypoxic treatments, from the highest to lowest, were in the order of 'Red Delicious', 'Pacific Rose', 'Granny Smith', 'Fuji' and 'Royal Gala'. Ethyl esters possess the most potent odours among esters as a group; their low odour threshold values (Teranishi *et al.*, 1987), indicate that small concentrations of these esters provide a major contribution to aroma. Most of these ethyl esters were not detected or detected in low concentrations in control fruit. The character impact volatile, ethyl 2-methyl butanoate, which possesses an apple-like aroma had the lowest odour threshold concentration among compounds monitored (Flath *et al.*, 1967); was not detected in control fruit of most cultivars, except 'Fuji', but was greatly enhanced by CO_2 treatment in most cultivars other than 'Royal Gala', providing the highest relative contribution (odour unit) to the aroma. Other ethyl esters, ethyl butanoate, ethyl hexanoate, ethyl propanoate and ethyl 3-hydroxy butanoate have been shown to be important volatile compounds contributing to a fruity and/or sweet aroma note of apples (Cunningham *et al.*, 1986; Kollmannsberger and Berger, 1992). These esters were also greatly enhanced by CO_2 treatment in 'Red Delicious', 'Granny Smith', 'Pacific Rose' and 'Fuji', but not in 'Royal' Gala', and consequently their odour units were also markedly increased (**Table 7.2** and **Appendix 6**).

Ethyl acetate and ethanol were markedly enhanced by CO_2 treatment in all cultivars, however, their relative contribution to the aroma was not so obvious, particularly ethanol, due to their high odour threshold concentrations. Both compounds have the potential to cause off-aroma development in CO_2 treated apples and apple juice, if they reach high enough concentrations (**Chapter Four**). Hexyl hexanoate, another important aroma volatile, has an apple peel-like aroma (Cunningham *et al.*, 1986); it was

found only in CO_2 treated 'Red Delicious' apples. This compound could have some contribution to the aroma, but may be to a lesser degree, compared with ethyl 2-methyl butanoate, as its concentration was quite low; however its odour threshold value is not available (**Table 7.2**).

It is worth noting that acetic acid concentration was also enhanced 3 and 6 fold following CO_2 treatment in 'Granny Smith' and 'Red Delicious', though it was unlikely to make any contribution to aroma as its odour unit was less than 1. An increase in acetic acid after CO_2 treatment (20% at 0°C) was found in strawberries (Larsen and Watkins, 1995a). Such an increase was probably due to conversion of acetaldehyde (Mattheis *et al.*, 1991a) or oxidation of pyruvate (Reazin *et al.*, 1970) into acetic acid, which could be further converted into acetyl CoA (Gilliver and Nursten, 1976) and can serve as substrate for acetate ester biosynthesis.

Concentrations of several aroma volatile compounds were decreased 2% to 98% relative to controls 3 days after CO_2 treatment, depending on compound and cultivar (**Table 7.3**). In general, the greater the overall magnitude of enhancement of ethyl volatiles, the greater the degree of reduction in non-ethyl volatile compounds. Important aroma volatile compounds such as *trans*-2-hexenal and hexan-1-ol, which contribute a green, fresh apple-like aroma (Berger, 1991; Flath *et al.*, 1967) either decreased, unaffected or increased, depending on cultivar; while butyl acetate, hexyl acetate and 2-methyl butyl acetate, which possess a sweet, perfumy, floral and fruity odour (Cunningham *et al.*, 1986; Dimick and Hoskin, 1983; Young *et al.*, 1996), decreased by 39% - 73%, depending on compound and cultivar. The hydrocarbon, α-farnesene, was dramatically suppressed by CO_2 treatment and this compound will be discussed separately later. Such decreases of these important aroma volatile compounds may have some effect on the potency of the fruity, sweet, perfumy or floral odour of the CO_2 treated apple juice. However, such an effect may not be dramatic because their concentrations largely exceeded their threshold concentrations (**Table 7.2** and **Appendix 6**). Thus, these important aroma compounds which decreased following CO_2 treatment may still have contributed meaningfully to aroma, as they had relatively high odour unit values and it is unlikely to reduce specific aroma of the fruit.

Hexanal and butan-1-ol are important aroma compounds of apples and apple juice (Dürr and Schobinger, 1981; Young *et al.*, 1996) and were unaffected by the hypoxic treatment in 'Red Delicious', 'Fuji' and 'Royal Gala', while they decreased in the others; their odour unit values confirm their relative contribution to the aroma of apples, particularly hexanal. Some volatile compounds, including pentan-1-ol and methanol may not contribute at all to the aroma because concentrations present were below threshold values, where the threshold concentration in air of methanol is 100 ppm (Shaw, 1986). Hydrocarbons such as decane, dodecane and tridecane were decreased, unaffected or increased by CO_2 treatment, depending on cultivar, and since there is no information on their odour characteristics and/or odour threshold values it is not possible to determine their contribution to aroma.

Differences in the magnitude of enhancement and the degree of reduction in selected volatile compounds among 5 apple cultivars were obvious. Important aroma impact ethyl esters such as ethyl 2-methyl butanoate and ethyl butanoate were consistently and markedly enhanced following CO_2 treatment in most cultivars, except 'Royal Gala'. Important secondary aldehydes, hexanal and *trans*-2-hexenal, were either increased, unaffected or decreased, depending on cultivar. Important non-ethyl esters including butyl acetate, hexyl acetate and 2/3 methyl butyl acetate decreased slightly or markedly, depending on cultivar. Such differences suggest that different cultivars may have different metabolic processes for volatile synthesis, which may be associated with enzyme systems involving in formation of esters and other volatiles, generation of substrates from precursors, degradation and/or synthesis of precursors and/or availability of appropriate enzymes, isoenzymes, co-factors, substrates and/or precursor pools required for volatile production. Detailed discussion for this issue has been given in **Chapter Six**, and these differences are presumably genetically controlled.

While some important aroma volatile compounds such as butyl acetate, hexyl acetate and 2/3 methyl butyl acetate were reduced by CO_2 treatment, concentrations of these compounds were well above their odour thresholds values, hence their contribution to aroma are unlikely to be affected by such reduction. For example, the degree of reduction in hexyl acetate from CO_2 treated 'Red Delicious', 'Fuji' and 'Pacific Rose' were 64%, 49% and 36% (**Table 7.3**), yet their respective concentrations were 5800,

1590 and 2000 times above their odour threshold values (see **Table 7.2** and **Appendix 6**). Moreover, CO_2 treatment substantially enhanced a number of low odour threshold compounds in most cultivars, except 'Royal Gala', which are generally produced in low concentrations or are absent in control fruit. Thus, it is likely that the aroma of apple juice would also be enhanced markedly following CO_2 treatment and this was indicated by a 5, 5, 6 and 12 fold increase in the sum of odour unit values between CO_2 and control treatments for 'Fuji', 'Granny Smith', 'Pacific Rose' and 'Red Delicious', respectively (see **Table 7.2** and **Appendix 6**). An exception was found with 'Royal Gala', where CO_2 treatment caused a 33% reduction in the sum of odour unit values (see **Appendix 6**), and thus hypoxic treatments should not be applied to this cultivar. The 10 most important aroma volatile compounds which contributed to the aroma in juice from control and CO_2 treated fruit of 5 apple cultivars can be ranked based on their calculated odour unit values (**Table 7.4**).

On the basis of average concentrations, odour threshold data from the literature and sniffing-GLC of serially diluted extracts by sensory panels, Kollmannsberger and Berger (1992) found that the key contributors to the aroma of 'Red Delicious' apples were ethyl 2-methyl butanoate, propyl 2-methyl butanoate, hexyl acetate, ethyl hexanoate, ethyl butanoate, hexyl hexanoate and 1,3,5-(E,Z) undecatriene. Young *et al.* (1996) found that 2-methyl butyl acetate, butyl acetate, hexyl acetate and butan-1-ol were the four most important contributors to the aroma of 'Royal Gala' apples, as indicated by gas chromatography-olfactometry (GCO) analysis of volatile distillates. Mattheis *et al.* (1991b) suggested that 2-methyl butyl acetate was the major aroma volatile compound in 'Bisbee Delicious' apples. In an earlier study, sensory panels found that ethyl 2-methyl butanoate, hexanal and *trans*-2-hexenal were aroma impact compounds having apple-like aromas of 'Delicious' apple essence (Flath *et al.*, 1967). Therefore, the key volatile compounds contributing to the aroma of 5 apple cultivars apples obtained by a quantitative approach in the present study (**Table 7.4**) were in agreement with other sensory studies.

Table 7.4 Ten most important volatile compounds which contribute to the aroma in juice from control and 100% CO_2 treated (24 hours at 20°C) fruit of 5 apple cultivars 3 days after treatment at 20°C.

	Cultivar / Treatment / Compound				
Rank[1]	**Red Delicious**	**Granny Smith**	**Fuji**	**Pacific Rose**	**Royal Gala**
			Control		
1	Ethyl butanoate	Ethyl butanoate	Ethyl butanoate	Ethyl butanoate	Hexyl acetate
2	2/3 MBA	*trans*-2-hexenal	E 2-MB	Hexanal	Hexanal
3	Hexyl acetate	Hexanal	Hexanal	*trans*-2-hexenal	*trans*-2-hexenal
4	*trans*-2-hexenal	2/3 MBA	2/3 MBA	2/3 MBA	2/3 MBA
5	Hexanal	Amyl acetate	*trans*-2-hexenal	Hexyl acetate	Butyl acetate
6	Amyl acetate	2/3 MBOH	Hexyl acetate	2/3 MBOH	Amyl acetate
7	Butyl acetate	Hexan-1-ol	Ethyl hexanoate	Butan-1-ol	Butan-1-ol
8	Propyl butanoate	Butan-1-ol	2/3 MBOH	Hexan-1-ol	2/3 MBOH
9	2/3 MBOH	Propan-1-ol	Butan-1-ol	Butyl acetate	Hexan-1-ol
10	Butan-1-ol	Propyl acetate	Butyl acetate	Ethyl acetate	Ethyl acetate
			100% CO_2		
1	E 2-MB	E 2-MB	E 2-MB	E 2-MB	Hexyl acetate
2	Ethyl butanoate	Ethyl butanoate	Ethyl butanoate	Ethyl butanoate	Hexanal
3	Ethyl hexanoate	Ethyl hexanoate	Hexanal	Ethyl hexanoate	*trans*-2-hexenal
4	EP	EP	*trans*-2-hexenal	Hexanal	2/3 MBA
5	Hexanal	*trans*-2-hexenal	Ethyl hexanoate	EP	Amyl acetate
6	*trans*-2-hexenal	Hexanal	2/3 MBA	*trans*-2-hexenal	Butyl acetate
7	Hexyl acetate	2/3 MBA	EP	Hexyl acetate	Butan-1-ol
8	2/3 MBA	Acetaldehyde	Hexyl acetate	2/3 MBA	Acetaldehyde
9	Amyl acetate	Amyl acetate	Acetaldehyde	2/3 MBOH	2/3 MBOH
10	Ethyl acetate	2/3 MBOH	2/3 MBOH	Acetaldehyde	Hexan-1-ol

[1] Ranking based on odour unit values of volatile compounds monitored (see **Table 7.2** and **Appendix 6**), 2/3 MBA = 2/3 methyl butyl acetate, 2/3 MBOH = 2/3 methyl butan-1-ol, E 2-MB = ethyl 2-methyl butanoate, EP = ethyl propanoate.

Although the odour unit concept has merits in determining particular volatile compounds most likely to contribute to the aroma of a product and is still the most commonly used method to assess the aroma activity (potency) of chemical(s) in a mixture (Acree, 1993; Acree and Barnard, 1994; Buttery, 1993; Cunningham *et al.*, 1986, Kollmannsberger and Berger, 1992; Pesis, 1994; Young *et al.*, 1996), there are cogent arguments about the psychophysical meaning and validity of this concept. Frijters (1979) criticised the technique indicating that different odorous compounds may not necessarily have equal perceived intensity and the detection threshold of a compound in a mixture may differ from that of individual components. The odour quality of a mixture

may be greater (synergism), equal to, or less (suppression) than the sum of intensities of the individual compounds and the detection threshold of a mixture may differ from that of individual compounds (Frijters, 1979; Land, 1979; Laing and Panhuber, 1979; Maarse, 1991). Laing and Panhuber (1979) indicated that results commonly obtained from mixtures with different concentrations of odorous compounds were: (a) synergism (odour enhancement) occurred when a mixture containing subthreshold quantities of compounds, (b) synergism, addition and suppression obtained from a mixture with concentrations at or just above their thresholds, and (c) odour suppression was the most common resulted from a mixture containing suprathreshold concentrations of volatile compounds.

Odour units measure aroma activity in the same way that biological activity is usually measured [activity = activity coefficient x concentration], where activity is linearly related to concentration or some function of concentration (Acree, 1993; Frijters, 1979). Theoretically, the activity coefficient of a given compound is constant (Laing and Panhuber, 1979), but concentration has a sigmoid relationship with response behaviour (Acree, 1993; Marin *et al.*, 1991). This implies that at concentrations below the minimum threshold concentration and above the point of maximum response, the activity (response behaviour) is not linear and that at very high concentrations (above the maximum response), the response curve usually flattens off because of either adaptation, inhibition, suppression, or in terms of behaviour avoidance (Acree, 1993; Lawless, 1986; Laing and Panhuber, 1986; Marin *et al.*, 1991). Rothe *et al.* (1994) found that three methyl ketones, 2-heptanone, 2-nonanone and 2-undecanone (key aroma compounds of blue cheese), either individually or in combination, had a positive hedonic effect for flavour quality at concentrations 2 - 5 times their threshold concentrations, but over-dosage (> 5 times) decreased flavour quality. Lawless (1986) reported that suppression or partial masking among aroma compounds was observed in a mixture of lavender oil and pyridine and it was suggested that this was due to counteraction of one odour compound in the presence of a second.

Shaw *et al.* (1990) found that sensory panels could not find differences in aroma between aqueous essence or essence oil prepared from 99% N_2 treated (24 hours at 20°C) and control 'Valencia' or 'Pineapple' oranges, although concentrations of a

number of volatile compounds were double in treated fruit, relative to controls. However, when an equal volume of essence or oil from the two treatments was added to bland juice base, flavour (aroma) differences were noted by sensory panels (Shaw *et al.*, 1990). This suggested that concentrations of the enhanced aroma volatile compounds in samples obtained from N_2 treated fruit exceeded the point of maximum response (Marin *et al.*, 1991), and thus sensory panels were unable to distinguish the differences in the aroma from controls. Although taste panels were not carried out, a similar event could have occurred in juice from the present study; substantial enhancement of both high and/or low odour threshold aroma compounds by the CO_2 treatment, in excess of their optimal or maximal response, may mean that a taste panel could not detect significant differences between treated and untreated samples, even though major quantitative and qualitative differences were detected by GLC.

Thus, odour units do not in themselves predict the nature of the aroma impact of a compound in a mixture. The perception of a mixture of odorous compounds, as usually found in natural products, is a complicated human response that currently can not be predicted from knowledge of separate compounds (Acree, 1993). Nevertheless, use of the odour unit concept can effectively eliminate compounds that are unlikely to contribute to the aroma of a product under study and focus attention on those compounds having high odour activity or odour units (Buttery, 1993). Therefore, odour unit values may provide a simple and a logical approach to understanding the aroma and/or flavour of apples and apple products.

It is interesting to note that α-farnesene concentration was reduced by 94%, 98% and to undetectable concentrations at 3 days following CO_2 treatment in 'Red Delicious', 'Fuji' and 'Granny Smith', respectively (**Table 7.3**). This volatile compound may contribute to the aroma of apples to some degree, suspected to be responsible for a 'dried leaves' note odour (Williams, 1979). This volatile is believed to be derived from farnesyl pyrophosphate via farnesol in the skin of apples and it has been implicated in superficial scald development through its oxidation products, conjugated trienes (Barden and Bramlage, 1994; Emongor *et al.*, 1994; Huelin and Coggiola, 1968). Low O_2 (1% or below) and/or high CO_2 (> 5%) in storage atmospheres can reduce incidence of scald disorder in apples (Chen *et al.*, 1993; Emongor *et al.*, 1994; Little and Peggie, 1987)

thought to be due to reduced oxidation of α-farnesene (Chen *et al.*, 1993; Emongor *et al.*, 1994). The reason for the dramatic reduction in α-farnesene concentration after CO_2 treatment is not known. However, it seems likely that hypoxic CO_2 treatment directly suppresses metabolism involving biosynthesis of α-farnesene from its precursors, either farnesyl pyrophosphate or farnesol. Because of this, the CO_2 treatment may have potential as a control measure to prevent superficial scald in either air or CA stored apples, although further study on the mode of action of CO_2 on scald development and/or its practical implication is required.

According to Paillard (1990), the first analysis of volatile compounds in apples was published in 1920 by Power and Chestnut, indicating the presence of acetaldehyde, pentyl formate, pentyl acetate, pentyl hexanoate, methanol and ethanol. In these early studies, much emphasis was placed on the identification and characterisation of volatile compounds, while other studies dealt with total volatile organic compounds by means of chemical reactions, using a variety of techniques such as separation by paper chromatography, column chromatography and spectrophotometry (Maga, 1990; Paillard, 1990). Analytical progress was made possible following introduction of GLC in 1952 (Maga, 1990) where the detection of volatiles at concentrations of parts per billion or lower became possible (Berger, 1991; Teranishi and Kint, 1993). The number of identified volatile compounds increased regularly with technical advances, especially with capillary column separation and combined uses of GLC - MS and/or GLC - IR (Maarse, 1991; Maga, 1990; Martin, 1995; Mussinan, 1993). Thirty volatile compounds of 'McIntosh' apples were reported in 1964, including esters, alcohols, aldehydes, ketones and acids (MacGregor *et al.*, 1964) and 56 compounds were identified in 'Delicious' apple essence in 1967 (Flath *et al.*, 1967). Nursten (1970) listed 159 volatiles, Dimick and Hoskin (1983) tabulated 266 compounds, while 356 volatile compounds were represented from apples and its products in 1991 (Maarse, 1991).

In more recent publications during the past 3 decades, usually far fewer volatile compounds have been considered and studies of apple aroma have focused on other aspects. Recent aspects of study include comparison of cultivars (Brackmann and Streif, 1994; Dirinck *et al.*, 1989; Kakiuchi *et al.*, 1986), changes during physiological maturation (Hansen *et al.*, 1992a; Mattheis *et al.*, 1991b; Vanoli *et al.*, 1995; Yahia *et*

al., 1990a), changes during air and/or CA storage (Bartley *et al.*, 1985; Fellman *et al.*, 1993a; 1993b; Streif and Bangerth, 1988; Willaert *et al.*, 1983; Yahia, 1989), biosynthesis and metabolic origins (Bartley *et al.*, 1985; Berger and Drawert, 1984; De Pooter *et al.*, 1981; 1983; 1987; Fellman and Mattheis, 1995; Fellman *et al.*, 1993a; 1993b; Knee and Hatfield, 1981) and organoleptic significance of volatile constituents (Dürr and Röthlin, 1981; Dürr and Schobinger, 1981; Flath *et al*, 1967; Kollmannsberger and Berger, 1992; Williams, 1979; Young *et al.*, 1996).

A range of studies, including the present work, have shown that loss of aroma and/or some important volatile compounds occurred in apples stored for a long period under CA conditions and in processed products of apples (Berger, 1995; Brackmann *et al.*, 1993; Hansen *et al.*, 1992b; Song and Bangerth, 1996; Willaert *et al.*, 1983). The mechanism causing such a loss of volatile compounds in CA stored apples is still not fully understood. Some investigations have searched for methods of improving flavour and aroma volatiles by manipulation of O_2 concentrations in storage atmosphere prior to marketing, by increasing storage temperature, by illumination during storage and by exposing fruit to cold air on removal from CA storage (Knee *et al.*, 1979; Smith, 1984; Streif and Bangerth, 1988; Yahia, 1991). However, only partial improvement of aroma volatiles was achieved by these methods and results obtained were inconsistent. Recently, Eccher-Zerbini *et al.* (1996) reported that water scrubbing the atmosphere in a CA store enhanced sensory aroma of 'Golden Delicious' apples as well as increasing concentrations of volatile compounds such as 2/3-methyl butyl acetate, *trans*-2-hexenol, hexyl acetate, pentyl acetate and butyl butanoate during 30 weeks storage and/or during post storage ripening, compared with conventional CA. However, such enhancement of volatiles and aroma may have resulted from an acceleration of the ripening process caused by water scrubbing since fruit from this treatment had higher TSS, less TA, as well as being softer and yellowier than normal CA stored fruit (Eccher-Zerbini *et al.*, 1996), and it is well known that aroma volatile production is associated with ripening of fruit (Paillard, 1990; Song and Bangerth, 1996; Tressl *et al.*, 1975).

Enhancement of aroma by increasing the concentration of volatile compounds is a fairly new concept for improving organoleptic quality of fruit and their products, and the practical implication of this approach has been studied recently (Berger *et al.*, 1992;

Pesis, 1994). Techniques used for enhancing aroma generally involve living fruit tissues or whole fruit, which are supplied with, or stimulated to induce, substrates and/or precursors for aroma volatile synthesis within the fruit tissues at normal temperature (20°C). Currently there are two major methods being investigated. First is a the technique called 'Precursor Atmosphere (PA) Technology' (see **Chapter One**). With this system a range of volatile precursors and/or substrates, such as volatile carboxylic acids, aldehydes and/or alcohols, are supplied to fruit which act as biocatalysts and these compounds are subsequently metabolised into aroma volatiles by the fruit (Berger, 1991; 1995; Berger *et al.*, 1992). This technique arose following several studies on the metabolism of aroma volatile formation in fruit, particularly esters, which involved adding a range of precursors to fruit or fruit slices (Bartley *et al.*, 1985; Berger and Drawert, 1984; De Pooter *et al*, 1981; 1983; 1987; Knee and Hatfield, 1981). The PA technology has effectively enhanced a wide range of aroma volatile compounds in several fruits, including apples (Berger *et al.*, 1992; Kollmannsberger and Berger, 1992) and strawberries (Berger, 1995), and the nature and proportion of individual volatiles can be partly controlled by adjusting the type and concentration of the supplied chemical precursors (Berger *et al.*, 1992). However, this method may be difficult to implement commercially because of food safety legislation, such as GRAS (**Chapter One**).

Second, a more simple, inexpensive and rather environmentally friendly technique for enhancing aroma volatiles in fruit was demonstrated by Pesis and Avissar (1989), using a short-term hypoxic treatments (24 hours at 20°C) with high concentrations (95% - 98%) of N_2 and/or CO_2 gas. This technique enhanced aroma and/or volatile compounds in several fruits, including feijoas (Pesis *et al.*, 1991; peaches (Pesis, 1994), 'Shamouti' and 'Valencia' oranges (Pesis and Avissar, 1989), 'Hamlin' and 'Pineapple' oranges (Shaw *et al.*, 1990; 1991), mandarins and mandarin hybrids (Shaw *et al.*, 1992) and strawberries (Larsen and Watkins, 1995a). The present study has demonstrated that a range of low odour threshold aroma volatile compounds, known to be important contributors to aroma, can be effectively and substantially enhanced following a short-term hypoxic treatments (24 hours at 20°C) with CO_2 and/or N_2 gas in a range of commercially important apple cultivars and in fruit from different storage regimes. These enhanced aroma volatile compounds such as ethyl 2-methyl butanoate and ethyl

butanoate would be of commercial significance, particular for apples designated for processing into juice or aqueous essence. These specific volatile compounds can be consistently and substantially enhanced in juice of the treated fruit; thus for a given quantity of apples, this could increase prices received. Alternatively it would allow these enhanced volatiles to be added to juice and/or concentrates of other cultivars which lack the required compounds. For example 'Royal Gala' and 'Cox's Orange Pippin' apples generally lack ethyl 2-methyl butanoate and this commercially important impact compound can be effectively enhanced by hypoxic treatments in a range of other cultivars, including 'Red Delicious', 'Granny Smith', 'Fuji' and 'Pacific Rose'. With the advancement of processing technology, it may be possible to isolate and capture these enhanced volatiles and trade them separately as 'fragrance', which can be used as food additives or as perfume in a wide range of products, such as toiletry. These choices would create opportunity for diversifying new products as well as adding value to process grade apples.

For the fresh fruit industry, potential for enhancing aroma by hypoxic treatments may be limited because the increased production of enhanced aroma volatile compounds was transient and generally lasted for only 3 - 9 days at 20°C after treatment, depending on cultivar and compound.

Hypoxic treatments also have a good potential for use as quarantine procedures for some insect pests in apples, as an alternative to chemical fumigants such as methyl bromide (Ahumada *et al.*, 1996; Lay-Yee and Whiting, 1996). Effectiveness of hypoxic treatments for insect disinfestation depends on concentration of gases, time of exposure and temperature, and more importantly on the tolerance limit of fruit to such conditions (Ke and Kader, 1992b; Lay-Yee and Whiting, 1996; Yahia and Vazquez-Moreno, 1993). Apples can tolerate 100% CO_2 atmosphere at 20°C for at least 24 hours, or up to 48 hours in the case of 'Red Delicious', without any physical injury, except for 'Braeburn' in 1993 (**Chapter Six**), however the effect of temperature during treatment has not been investigated. It has been reported that apple codling moths (Whiting *et al.*, 1992), light brown apple moths (Whiting *et al.*, 1991) and San Jose scale (Ke and Kader, 1992b) can be effectively killed by hypoxic atmospheres of high CO_2 (5% - 95%) and/or low O_2 (0.4% - 1%) at 20° - 30°C within 1 - 2 days.

As an alternative to radiolabelling or suppling precursors (Bartley *et al.*, 1985; Berger and Drawert, 1984; De Pooter *et al.*, 1983; 1987; Tressl and Drawert, 1973), hypoxic treatments allow manipulation of the metabolic system of fruit, hence providing another approach to understanding the biosynthetic pathway of volatile compounds from apples. In addition, the present study has provided new information on volatile profiles of New Zealand grown apples, particularly the new cultivars 'Pacific Rose' and 'Royal Gala'; and has contributed further understanding on the effect of short-term hypoxic treatments on changes in production of a range of important volatile compounds in apples.

7.4 CONCLUSION AND SUGGESTION FOR FURTHER STUDY

The mode of action of hypoxic atmospheres of CO_2 and/or N_2 gas on the enhancement of aroma volatile compounds in apples following treatment probably occurs through its induction of fermentative metabolism which causes accumulation of substrates for a subsequent aroma volatile biosynthesis. Hypoxic treatments stimulate a large increase in ethanol accumulation under hypoxia; this in turn is enzymatically metabolised by coupling with acyl CoAs into ethyl esters, where the reaction is catalysed by the ester forming enzyme(s) in the presence of O_2 upon removing apples into air. These enhanced ethyl esters, which are characteristically low in their odour threshold concentrations and are important contributors to the aroma, are markedly enhanced following the hypoxic treatments. This phenomenon results in a significant enhancement of the aroma of hypoxically treated apples which is indicated by a several fold increase in the sum of the relative contribution to the aroma values (odour units), compared with control.

A dramatic increase in ethyl esters causes production of other esters, not containing the ethyl moiety, and alcohols, other than ethanol, to decrease slightly or markedly, probably due to competition for acyl CoAs for aroma volatile synthesis. Such a decrease in important esters, such as butyl acetate, amyl acetate, hexyl acetate and 2-methyl butyl acetate, and alcohols, including hexan-1-ol and 2/3 methyl butan-1-ol

following hypoxic treatments is less likely to have a marked effect in reducing their contribution to aroma of hypoxically treated apples because their concentrations still greatly exceeded their odour saturation concentrations.

A range of important aroma volatile compounds can be effectively and markedly enhanced by a short-term hypoxic treatment in freshly harvested apples. However, enhancement of these important aroma compounds, particularly ethyl esters, was minimal and/or became non existent as fruit aged. This phenomenon is probably due to a high production of (background) ethyl esters, resulting from the large quantity of ethanol accumulated as a natural ripening and senescence progresses in apples. The reduced activity of the ester forming enzyme(s) and/or a deficiency of acyl CoA substrates resulting from a change in metabolism, the reduced capacity of enzyme systems involved and/or exhaustion of precursors as fruit aged, either independently or together probably account for the relative ineffectiveness of hypoxic treatments in enhancing important volatiles in aging fruit.

While the present study has broadened the knowledge and understanding of aroma volatile production in apples in relation to hypoxic treatments, cultivars, storage regimes and fruit age, there are several other important aspects concerning the metabolism and factors that may influence aroma volatile production that have not yet been fully investigated or understood. Further studies are needed in order to gain a more in depth understanding in these areas, some of which are suggested as follows:

(1) To date, the alcohol acyltransferase (AAT) is the only known ester forming enzyme(s) in fruit and studies reported on this enzyme in apples were mainly made with partially purified or crude extracts (Fellman, 1994; Fellman and Mattheis, 1995; Fellman *et al.*, 1993a; 1993b). Purification of the AAT to homogeneity has not yet been achieved, nor has characterisation of the enzyme. It is not known whether only one enzyme is responsible for ester synthesis in fruit, or whether other types or isoforms of this enzyme exist, either within or between different apple cultivars. Molecular, genetic and biochemical aspects of the enzyme(s) in relation to apple fruit development, maturation, ripening and senescence as affected by pre- and post harvest conditions await further investigation.

(2) Acyl CoAs and alcohols are known to be substrates for ester biosynthesis in fruit, but most studies on the metabolism of ester formation have focused on alcohol substrates (Bartley *et al.*, 1985; Berger and Drawert, 1984; De Pooter *et al.*, 1981; 1983; 1987; Harb *et al.*, 1994; Knee and Hatfield, 1981), with only a few investigating acyl CoAs (Gilliver and Nursten, 1976; Oliás *et al.*, 1995), despite their importance in ester synthesis. Until now, no method for direct quantification, identification and/or characterisation of individual acyl CoAs in apples has been established. Information on the metabolism involved in the liberation of acyl CoAs from precursors, the enzyme system involved and the quantitative changes of individual acyl CoAs during fruit development, maturation, ripening, and senescence as influenced by pre- and post-harvest conditions has not been determined.

(3) Fatty acids, amino acids and sugars are putative precursors of most volatile compounds in fruit, including apples (Drawert, 1975; Paillard, 1990; Salunkhe and Do, 1976; Schreier, 1984; Tressl *et al.*, 1975). A few studies have been made recently to find a relationship between changes in individual fatty acid concentrations and aroma volatiles produced in apples during and after CA storage (Brackmann *et al.*, 1993; Harb *et al.*, 1994; Song and Bangerth, 1994). Information on concentrations and changes in individual amino acids and/or sugars, as well as metabolic pathways involved in generating specific substrates in relation to aroma volatile biosynthesis and production in apples awaits generation.

(4) Apart from cultivars, storage regimes and fruit age, factors such as temperature during and after hypoxic treatments, fruit size, growing conditions and seasonal variations, all of which may influence effectiveness of hypoxic treatments for enhancing aroma volatile production in apples have yet to be established. Alternative techniques, which are potentially simpler, cheaper, more practical and/or equally effective to hypoxic treatments for enhancing volatile production, such as submerging fruit under water, need investigation. The feasibility of practically utilising the hypoxic treatment technique to enhance volatiles in apples in commercial operations needs to be demonstrated. Moreover, since the human nose and mind control the final decision as to acceptability or non-acceptability of a specific food aroma, the sensory significance of these enhanced volatile compounds following hypoxic treatments must be determined.

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APPENDIX 1

Calculations of Fruit Attributes

1.1 Fruit Moisture Loss

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

Where : Initial F.W. = fruit fresh weight at day -1
Final F.W. = fruit fresh weight at a particular sampling date

1.2 Fruit Respiration Rate

Respiration rate of each fruit sample was determined by the amount of CO_2 gas evolved per unit mass and time. A series of calculation were performed. The percentage of measured CO_2 gas was first calculated in terms of ml $CO_2 \cdot kg^{-1} \cdot h^{-1}$.

$$\text{Respiration Rate} = \frac{\text{(Jar Vol (cc) - Fruit Vol (cc)) x } (\Delta \% CO_2 / 100)}{\text{Fruit F.W. (kg) x Time (h)}}$$

Where : $\Delta\%$ CO_2 = CO_2 concentration (%) in jar containing fruit after a time (15 minutes to 1 hour) subtracted from CO_2 concentration in a blank jar.

Normally millitres of CO_2 gas are converted to milligrams to remove the effect of temperature on gas volume. Thus, the temperature correction was calculated. As 1 mole of gas is equal to 22.4 litres at 0°C at 1 atmosphere, its volume (V_1) at the temperature of the produce can be calculated with the following equation :

$$V_1 = 22.4 \times (1 + \frac{\text{Fruit Temperature °C}}{273° K})$$

Thus, the volume of 1 mole of CO_2 gas at 20°C would be :

$$V_1 = 22.4 \times (1 + \frac{20}{273})$$

$$= 24.04 \text{ litres}$$

The volume of gas per gram was then calculated by dividing the corrected volume by the molecular weight of the gas (CO_2 = 44) :

$$= 24.04/44 = 0.546\ l \cdot g^{-1} = 546ml/1000\ mg$$

Therefore, ml CO_2 gas could be converted to milligram (as mg $CO_2 \cdot kg^{-1} \cdot h^{-1}$) by the following equation :

$$\text{Respiration Rate} = \frac{1000mg \times CO_2 \text{ of sample } (ml \cdot kg^{-1} \cdot h^{-1})}{546\ ml}$$

A further calculation was made to express the rate of transfer of CO_2 gas in an absolute unit (Banks *et al.*, 1995); $mg \cdot kg^{-1} \cdot h^{-1}$ was converted into $nmol \cdot kg^{-1} \cdot s^{-1}$ using the following equation :

$$\text{Respiration Rate} = \frac{y\ mg}{kg \cdot h} \times \left(\frac{1g}{1000mg} \times \frac{1mol}{44g} \times \frac{1h}{3600s} \right) \times \frac{1nmol}{1 \times 10^{-9}\ mol}$$

where y = respiration rate in $mg \cdot kg^{-1} \cdot h^{-1}$ unit

The terms in parentheses can be computed to give a convertion factor (ie. = $2.778 \times 10^{-7}/MW$) for ease of calculation.

For example, assuming a fruit has a rate of CO_2 production of 30 $mg \cdot kg^{-1} \cdot h^{-1}$ at 20°C, this would be equated to 189.4 $nmol \cdot kg^{-1} \cdot s^{-1}$, calculated as follow :

$$= \frac{30mg}{kg \cdot h} \times \frac{1g}{1000mg} \times \frac{1mol}{44g} \times \frac{1h}{3600s} \times \frac{1nmol}{1 \times 10^{-9}\ mol}$$

$$= 189.4\ nmol \cdot kg^{-1} \cdot s^{-1}$$

1.3 Fruit Ethylene Production

The amount of measured ethylene (ppm) in jars containing each fruit sample was converted to $\mu l \cdot kg^{-1} \cdot h^{-1}$ unit with the following equation :

$$\text{Ethylene} = \frac{(\text{Jar Vol (cc)} - \text{Fruit Vol (cc)}) \times \Delta C_2H_4 \text{ (ppm)}}{\text{Fruit F.W.(kg)} \times \text{Time (h)} \times 1000}$$

Where ΔC_2H_4 (ppm) = ethylene concentration (ppm) in jar containing fruit after a time (15 minutes to 1 hour) subtracted by ethylene concentration in blank jar.

By assuming the atmospheric pressure during ethylene determination of sample was equal to 1 atmosphere (= 101325 Pa), then the conversion of the $\mu l \cdot kg^{-1} \cdot h^{-1}$ into $nmol \cdot kg^{-1} \cdot s^{-1}$ unit was computed with the following equation (Banks *et al.*, 1995) :

$$C_2H_4 = \frac{y\mu l}{kg \cdot h} x \left(\frac{1m^3}{10^9 \mu l} x 101325Pa \; x \; \frac{1mol \cdot K}{8.3143m^3 \cdot Pa} x \frac{1}{(273.15+T)K} x \frac{1h}{3600s}\right) x \frac{10^9 nmol}{1mol}$$

Where y = ethylene production rate in $\mu l \cdot kg^{-1} \cdot h^{-1}$

For ease of calculation, the terms in parentheses can be computed to give an appropriate correction factor :

$$\text{Correction Factor} = 3.414 \times 10^{-4} P_{tot}/(T+273.15)$$

Where P_{tot} = total pressure in Pascal, and
T = temperature in °C.

For example, assuming that a fruit produces ethylene at the rate of 120 $\mu l \cdot kg^{-1} \cdot h^{-1}$ at 20°C and 1 standard atmosphere (101325 Pa), this can be expressed in terms of $nmol \cdot kg^{-1} \cdot s^{-1}$ with the following equation :

$$= \frac{120\mu l}{kg \cdot h} x \frac{1m^3}{10^9 \mu l} x 101325Pa \; x \; \frac{1mol \cdot K}{8.3143m^3 \cdot Pa} x \frac{1}{(273.15+20)K} x \frac{1h}{3600s} x \frac{10^9 nmol}{1mol}$$

$$= 1.39 \; nmol \cdot kg^{-1} \cdot s^{-1}$$

1.4 Titratable Acids

The Mettler® DL21 Automatic Titrator used for determination of titratable acids of apple juice sample was configured prior to performing titration. The parameter settings were as follows:

1.) Titration Type; parameter 1014; where
- 1 : tendency positive special mode
- 0 : ΔV_{min} = 1% of V minimum increment
- 1 : pH measured signal
- 4 : positive end point titration mode

2.) Control and measured value acquisition; parameter 2111; where
- 2 : 1% of burette volume maximum increment, ΔV_{max}
- 1 : 4 mV potential change, set ΔE
- 1 : 6...60s time condition (t_{min}...t_{max}, Δt)
- 1 : 1 mV/s equilibrium, drift with 1.5s t_{min}

3.) Predispensing; parameter 0; (ie. no predispensed volume of titrant)
4.) Burette volume; maximum volume 30 ml
5.) End point 7.1
6.) Stirring time 5 seconds before predispensing
7.) Result, calibration and representation; parameter 910; where
- 9 : ml result unit with constant 1
- 1 : initial pH or mV and ml additional data
- 0 : no curve representation

8.) System; parameter 1; ie. dot matrix printer
9.) Series titration with sampler changer; parameter 0; ie. series with sample data input
A : Rinsing time 1 second for pump at connection for RINSE
B : Dosing 1; parameter 0; ie. dosing time for every increment for pump at connection RINSE
C : Dosing 2; dosing time 1 second for pump at connection DOSE.
Abbreviations :
- t_{min} minimum waiting time
- t_{max} maximum waiting time
- Δt waiting time between two increment
- ml millitre
- V volume

The volume (ml) of titrant required to neutralise the acids in a sample was calculated and the result was expressed as per cent malic acid equivalent derived from the following equation :

$$\text{Titratable Acid (\%)} = \frac{\text{ml NaOH x Normality of NaOH x 67.05}}{\text{ml of juice x 10}}$$

where 67.05 was the molecular weight of malic acid divided by 2 (ie. 134.1/2).

1.5 Preparation and Calculation of the Standard Headspace Volatile Mixture for Calibration

1.5.1 Preparation of 5000 $\mu l \cdot l^{-1}$ Standard Gas Mixture

Redistilled acetaldehyde, ethanol and ethyl acetate (HPLC Grade) were kept 0°C along with 3 air-tight, glass microsyringes (Hamilton Gastight® Microsyringe) used for taking the chemicals separately. Three air-washed Agee® jars of known volume

(approximately 2 litres) with lids each fitted with a silicone rubber septum (Vacutainer® rubber stopper) were prepared. A stirrer rod was also placed inside each jar for mixing the gas.

The 5000 $\mu l \cdot l^{-1}$ stock standard gas was prepared separately for each chemical at 0°C using the following procedure :

1.) evacuated 100 ml of air from the jar with a 50/60 ml Omnifix® syringe,
2.) withdraw an aliquot volume of the compound with a Hamilton glass microsyringe and inject into the jar through the rubber septum port on the lid,
3.) let the liquid volatilise,
4.) refill the jar with air back to its original volume and move the jar to a normal room temperature,
5.) inject volume of air for making up to the final volume of 2000 ml.

Calculation of the amount of ethanol required for making up a 5000 $\mu l \cdot l^{-1}$ stock standard gas in 2 litres at 20°C would be as follows :

The density of ethanol (C_2H_5OH, MW = 46.07) at 20°C is 0.7894 $g \cdot ml^{-1}$ (liquid phase). As the density of a compound is higher at lower temperature, thus a correction factor for temperature at 0°C must be used, ie.

$$\text{Correction Factor} = (1 + \frac{t°C}{273°K})$$

$$\text{Correction Factor} = (1 + \frac{20}{273}) = 1.07326$$

Therefore, the density of liquid ethanol at 0°C = 0.7894 x 1.07326 = 0.8476 $g \cdot ml^{-1}$

Ethanol used for the standard was in the gas phase and as 1 mole of gas at 20°C = 22.4 litres, the density of ethanol in gas phase can be calculated. As the volume of gas is less at lower temperature, thus temperature correction is applied.

Volume of 1 mole of gas at 0°C = 22.4/1.07326 = 20.871 litres, and

the density of ethanol in gas phase at 0°C = 0.8723/20.871

= 0.040594 $g \cdot l^{-1}$

Thus, the density of 1µl of ethanol gas at 0°C = 4.0594 x 10^{-8} $g \cdot \mu l^{-1}$.

To make a 10000 $\mu l \cdot l^{-1}$ concentration in 2 litres, the amount of liquid ethanol required can be computed as follow :

Mass required = density of ethanol gas at 0°C x MW x Vol x Concentration

= 4.0594 x 10^{-8} $g \cdot \mu l^{-1}$ x 46.06 x 2 litres x 10000 $\mu l \cdot l^{-1}$

= 0.0374 g

Thus, the volume of liquid ethanol required at 20°C can be computed as follow :

Volume = Mass/Density

= 0.0374/0.7894

= 0.04738 ml (or = 47.38 µl)

Therefore, it required 47.38 µl of liquid ethanol in 2 litres to make the 10000 $\mu l \cdot l^{-1}$ stock standard ethanol gas.

For making up a stock standard ethanol gas of 5000 $\mu l \cdot l^{-1}$ in 2 litres, half of the volume above is required, ie. 23.69 µl of liquid ethanol.

Using similar calculations as above, the volume of liqiud required for making 5000 $\mu l \cdot l^{-1}$ stock standard gases of acetaldehyde, ethanol and ethyl acetate in 2 litres would be :

acetaldehyde (CH_3CHO, MW = 44.05, d_{20} = 0.7834) = 22.65 µl

ethanol (C_2H_5OH, MW = 46.07, d_{20} = 0.7894) = 23.69 µl

ethyl acetate ($CH_3COOC_2H_5$, MW = 88.1, d_{20} = 0.902) = 45.30 µl

1.5.2 Preparation of 100 $\mu l \cdot l^{-1}$ Standard Gas Mixture for GLC Calibration

Forty ml of each of 5000 $\mu l \cdot l^{-1}$ of the stock standard gas was mixed in an approximately 2 litres Agee® jar equipped with a magnetic stirrer and a lid fitted with a silicone rubber septum. Air was injected for making up a final volume of 2000 cc. After that it was stirred continuously on a magnetic stirrer. The preparation was made at room temperature. The volume of each stock gas used was calculated by the following equation :

$$N_1V_1 = N_2V_2$$

N_1 = concentration of stock gas (5000 $\mu l \cdot l^{-1}$)
N_2 = concentration required (100 $\mu l \cdot l^{-1}$)
V_1 = volume of stock gas required (cc)
V_2 = total volume to be made (2000 cc)

$$5000\ \mu l \cdot l^{-1} \times V_1 = 100\ \mu l \cdot l^{-1} \times 2000\ cc$$

$$V_1 = 40\ cc$$

1.6 Calculation of Peak Area for Volatile Compounds in the 1993 Experimental Season

1.6.1 The Concentration of Octyl Acetate Internal Standard in the Concentrated Extract

As 1 µl of octyl acetate was added to a 200 ml juice sample during the volatile extraction process, its concentration in the juice was 5 $\mu l \cdot l^{-1}$. It was assumed that all volatile compounds in the juice were trapped in the Tenax® column and there were no losses. The trapped volatile compounds were eluted with diethyl ether solvent and concentrated to 0.20 ml (ie. = 1000 fold). Therefore, concentration of octyl acetate in the concentrated extract would be = 5 $\mu l \cdot l^{-1}$ x 1000 = 5000 $\mu l \cdot l^{-1}$.

Since the samples were analysed on a GLC over a period of time for each experiment, and the detecting efficiency of the GLC varied from run to run, an average peak area (count) internal standard for each experiment was determined. The average peak area was assigned to be equivalent to 5000 $\mu l \cdot l^{-1}$, ie. the total concentration of octyl acetate in the concentrated extract and the amount per unit area were computed. A correction factor was computed for each run to normalise the peaks of interest as follows:

$$\text{Correction Factor} = \frac{\text{(amount / area of sample octyl acetate)}}{\text{(amount / area of mean octyl acetate)}}$$

For example, knowing that the amount of octyl acetate in concentrated extract is 5000 $\mu l \cdot l^{-1}$, if the mean peak area of octyl acetate is 897122 area count, and the peak area of octyl acetate in a sample is 888100 area count, the correction factor would be :

$$\text{Correction Factor} = (5000/888100)/(5000/987122)$$

$$= 1.115$$

If the peak area of the sample for hexan-1-ol is 1341 area count, and ethyl acetate is 9136 area count, then the corrected peak area of the compounds would be :

Hexan-1-ol = 1341 x 1.115 = 1490 area count, and for

Ethyl acetate = 9136 x 1.115 = 10154 area count.

1.7 Calculation of the Amount of Volatile Compounds in the 1994 and 1995 Experimental Season

As 10 µl of 2000 $\mu l \cdot l^{-1}$ octyl acetate in ether:pentane (2:1 v/v) was added to 20 ml (= 20000 µl) juice sample during the extraction process, the concentration of the compound in juice would be :

$$N_1V_1 = N_2V_2$$

$$N_1 \times 20000\ \mu l = 2000\ \mu l \cdot l^{-1} \times 10\ \mu l$$

$$N_1 = 1\ \mu l \cdot l^{-1}$$

The extracted solvent was concentrated to 0.25 ml (ie. = 80 fold). Therefore, the concentration of octyl acetate in the concentrated extract would be :

$$1\ \mu l \cdot l^{-1} \times 80 = 80\ \mu l \cdot l^{-1}$$

Theoretically, if no losses occurred, the concentration of octyl acetate internal standard in the concentrated extract sample would be 80 $\mu l \cdot l^{-1}$. Thus, the integrated concentration of octyl acetate obtained from a run of sample was used to compute the correction factor, where

$$\text{Correction Factor} = 80\ \mu l \cdot l^{-1} / \mu l \cdot l^{-1} \text{ octyl acetate of sample}$$

The correction factor obtained was multiplied to the amount in $\mu l \cdot l^{-1}$ of other compounds of interest to obtain the corrected values.

For example, if the concentration of octyl acetate internal standard of a sample after integration is 63.34 $\mu l \cdot l^{-1}$ and the ethyl hexanoate is 14.55 $\mu l \cdot l^{-1}$,

$$\text{Correction Factor} = 80/63.34 = 1.2244$$

Therefore, the corrected concentration of ethyl hexanoate would be :

$$= 14.55 \times 1.2244 = 17.81\ \mu l \cdot l^{-1}$$

The corrected values of the volatile compounds were further calculated and expressed in terms of $\mu mol \cdot l^{-1}$.

For example, the concentration of ethyl hexanoate is 17.81 $\mu l \cdot l^{-1}$, the molecular weight is 144.21 $g \cdot mol^{-1}$, the density at 20°C is 0.873 $g \cdot ml^{-1}$, the computation is as follows :

As 17.81 µl = 0.01781 ml, the mass of the compound would be : 0.01781 x 0.873 = 0.01555 g, and

$$N = M/MW$$

where, N = number of mole
M = mass (g)
MW = molecular weight ($g \cdot mol^{-1}$)

Then, $N = 0.01555/144.21$

$= 1.078 \times 10^{-4}\ mol \cdot l^{-1}$

As 1 mole is 10^6 μmole, thus

$= (1.078 \times 10^{-4}) \times 10^6$

$= 107.82\ \mu mol \cdot l^{-1}$

All the steps involved above can be derived into an equation as follows :

$$\text{Concentration } (\mu mol \cdot l^{-1}) = ((y/1000) \times \text{density})/MW) \times 1000000$$

where y = corrected value in $\mu l \cdot l^{-1}$ of a compound from sample
density = density ($g \cdot ml^{-1}$) of the compound at 20°C
MW = molecular weight ($g \cdot mol^{-1}$) of the compound

Note : An equivalent volume of ether:pentane mixture (20 ml) with the internal standard (10 μl) added was also concentrated and determined as the 'blank' baseline. The concentration of contaminants found in the concentrated solvent was determined and the average value of each of such compound was used for correcting the concentration of volatile compounds of interest from the samples.

APPENDIX 2

Volatile Compounds and Volatile Chromatograms

2.1 Formula and Physical Properties of some Volatile Compounds Found in Apple Juice

Formula and some physical properties of volatile compounds detected in apple juice in this study.

Compound	Formula	M.W. (g/mol)	b.p. (°C)	Solubility H_2O	Solubility Ether
Esters					
methyl hexanoate	$CH_3(CH_2)_4CO_2CH_3$	130.19	151	1	4
2-methyl butyl acetate	$CH_3CO_2CH_2CH_2CH(CH_3)_2$	130.19	142	5	na
ethyl acetate	$CH_3CO_2C_2H_5$	88.11	77	3	5
ethyl propanoate	$C_2H_5CO_2C_2H_5$	102.13	99	2	5
ethyl butanoate	$CH_3CH_2CH_2CO_2C_2H_5$	116.16	120	2	3
ethyl hexanoate	$CH_3(CH_2)_4CO_2C_2H_5$	144.21	168	1	4
ethyl 2-methyl butanoate	$C_2H_5CH(CH_3)CO_2C_2H_5$	130.16	133	1	na
ethyl 3-hydroxy butanoate	$CH_3CH(OH)CH_2CO_2C_2H_5$	132.16	170	3	na
ethyl 3-hydroxy hexanoate	$CH_3(CH_2)_3CH(OH)\ CO_2C_2H_5$	160.22	195	na	na
propyl acetate	$CH_3CO_2CH_2CH_2CH_3$	102.13	102	2	5
propyl butanoate	$CH_3CH_2CH_2CO_2CH_2CH_2CH_3$	130.19	142.7	na	na
butyl acetate	$CH_3CO_2(CH_2)_3CH_3$	116.16	125	2	5
amyl acetate	$CH_3CO_2(CH_2)_4CH_3$	130.19	149	3	5
isoamyl acetate	$CH_3CO_2(CH_2)_2CH(CH_3)_2$	130.19	142	1	na
hexyl acetate	$CH_3CO_2(CH_2)_5CH_3$	144.21	169	1	4
hexyl hexanoate	$CH_3(CH_2)_4CO_2(CH_2)_5CH_3$	200.33	246	1	3
octyl propanoate	$CH_3CO_2(CH_2)_7CH_3$	186.30	228	1	2
Alcohols					
methanol	CH_3OH	32.04	64.6	5	5
ethanol	C_2H_5OH	46.07	78	5	5
propanol	$CH_3CH_2CH_2OH$	60.16	97	5	5
butanol	$CH_3(CH_2)_3OH$	74.12	117	3	5
pentanol	$CH_3(CH_2)_4OH$	88.15	135	1	5
hexanol	$CH_3(CH_2)_5OH$	102.18	156	2	5
2-hexen-1-ol	$CH_3CH_2CH_2CH{:}CHCH_2OH$	100.16	159	na	na
5-hexen-1-ol	$H_2C{:}CH(CH_2)_4OH$	100.16	na	na	na
octanol	$CH_3(CH_2)_7OH$	130.23	196	1	5
3-octen-1-ol	$CH_3(CH_2)_4CH(OH)C_2H_5$	130.23	175	na	na
2-methyl propanol	$(CH_3)_2CHCH_2OH$	74.12	108	na	na
2-methyl butanol	$C_2H_5CH(CH_3)CH_2OH$	88.15	130	1	5
6-methyl 5-hepten-2-ol	$(CH_3)_2C{:}CHCH_2CH_2OH(OH)CH_3$	128.22	na	na	na
3-(methylthio)-propan-1-ol	$CH_3S(CH_2)_3OH$	106.19	na	na	na
1,3-dichloro 2-propan-1-ol	$ClCH_2CH(OH)CH_2Cl$	128.99	176	4	5
1,2 propanediol	$CH_3CH(OH)CH_2OH$	76.10	189	5	3
1,3 butanediol	$CH_3CH(OH)CH_2CH_2OH$	90.12	203	3	1
2,3 butanediol	$CH_3CH(OH)CH(OH)CH_3$	90.12	180	5	3

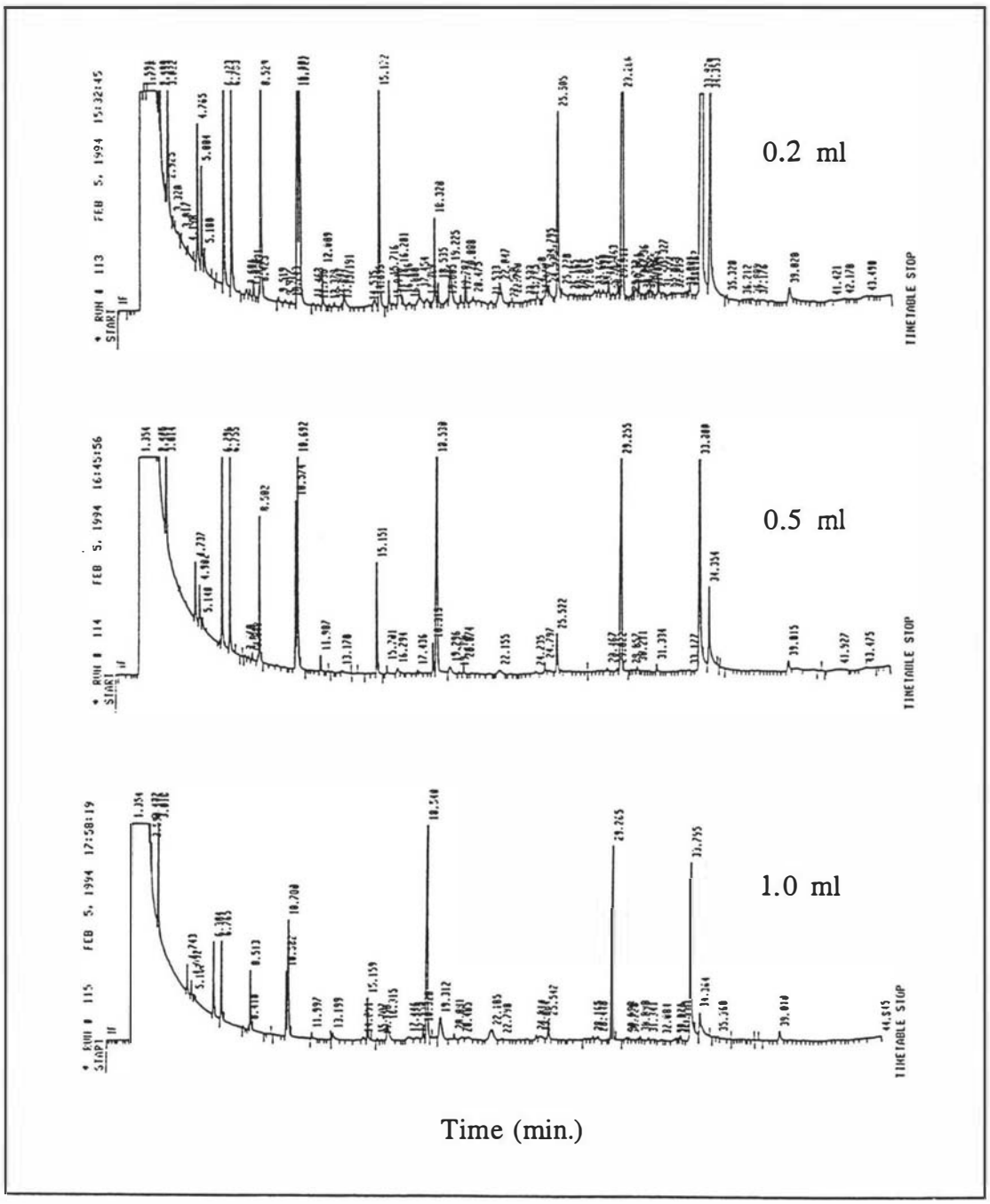
(Continued)

Compound	Formula	M.W. (g/mol)	b.p. (°C)	Solubility H_2O	Solubility Ether
Aldehydes					
acetaldehyde	CH_3CHO	44.05	21	5	5
butanal	$CH_3CH_2CH_2CHO$	72.12	75.7	3	5
hexanal	$CH_3(CH_2)_4CHO$	100.16	131	2	4
trans-2-hexenal	$CH_3CH_2CH_2CH{:}CHCHO$	98.15	146	1	na
Ketones					
2-butanone	$CH_3CH_2COCH_3$	72.15	79.6	4	5
3-hydroxy 2-butanone	$CH_3COCH(OH)CH_3$	88.11	143	5	2
Acids					
acetic acid	CH_3COOH	60.05	117	5	5
propanoic acid	CH_3CH_2COOH	74.08	141	5	3
2-methyl butanoic acid	$CH_3CH_2CH(CH_3)COOH$	102.13	177	2	5
Hydrocarbons					
propyl cyclohexane	$C_6H_{11}CH_2CH_2CH_3$	126.24	156.7	1	3
pentyl cyclohexane	$C_{11}H_{22}$	154.30	202.8	1	3
nonane	$CH_3(CH_2)_7CH_3$	128.26	151	1	4
3-methyl nonane	$C_{10}H_{22}$	142.28	167.9	na	4
decane	$CH_3(CH_2)_8CH_3$	142.29	174.2	1	3
undecane	$CH_3(CH_2)_9CH_3$	156.31	196	1	5
dodecane	$CH_3(CH_2)_{10}CH_3$	170.34	216.2	1	3
tridecane	$CH_3(CH_2)_{11}CH_3$	184.37	234	1	4
tetradecane	$CH_3(CH_2)_{12}CH_3$	198.40	253	1	4
pentadecane	$CH_3(CH_2)_{13}CH_3$	212.42	270	1	4
hexadecane	$CH_3(CH_2)_{14}CH_3$	226.45	287	1	5
heptadecane	$CH_3(CH_2)_{15}CH_3$	240.48	302	1	3
3,6 dimethyl decane	na	na	na	na	na
1-nonene	$CH_3(CH_2)_6CH{:}CH_2$	126.24	146	na	na
toluene	$C_6H_5CH_3$	92.15	110.6	1	5
xylene	$CH_3C_6H_4CH_3$	106.17	138	1	5
ρ-xylene	C_6H_4-1,4-$(CH_3)_2$	106.17	138	1	5
1-ethyl 3-methyl benzene	C_9H_{12}	120.19	161.3	1	4
1,2,3 trimethyl benzene	$C_6H_3(CH_3)_3$	120.20	176.1	1	5
1,2,4 trimethyl benzene	$C_6H_3(CH_3)_3$	120.20	168	1	5
1,3,5 trimethyl benzene	$C_6H_3(CH_3)_3$	120.20	164.7	1	5
ethylbenzene	$C_6H_5C_2H_5$	106.17	136	1	5
ethenyl benzene	$C_6H_5CH{:}CH_2$	104.16	145.2	1	3
α-farnesene	$C_{15}H_{24}$	204.34	130*	1	3
Others					
estragole	$H_2C{:}CHCH_2CH_2C_2H_4OCH_3$	148.21	216	na	na
trans-linalool oxide	$C_{10}H_{18}O_2$	170	na	na	na

Formula and physical properties of volatile compounds were after Lide and Frederikse (1995)
Abbreviations : M.W. = molecular weight, b.p. = boiling point (at 760 mm Hg), Ether = diethyl ether
Solubility : 1 = insoluble, 2 = slight, 3 = soluble, 4 = very soluble, 5 = miscible
* at 12 mm Hg, na = not available

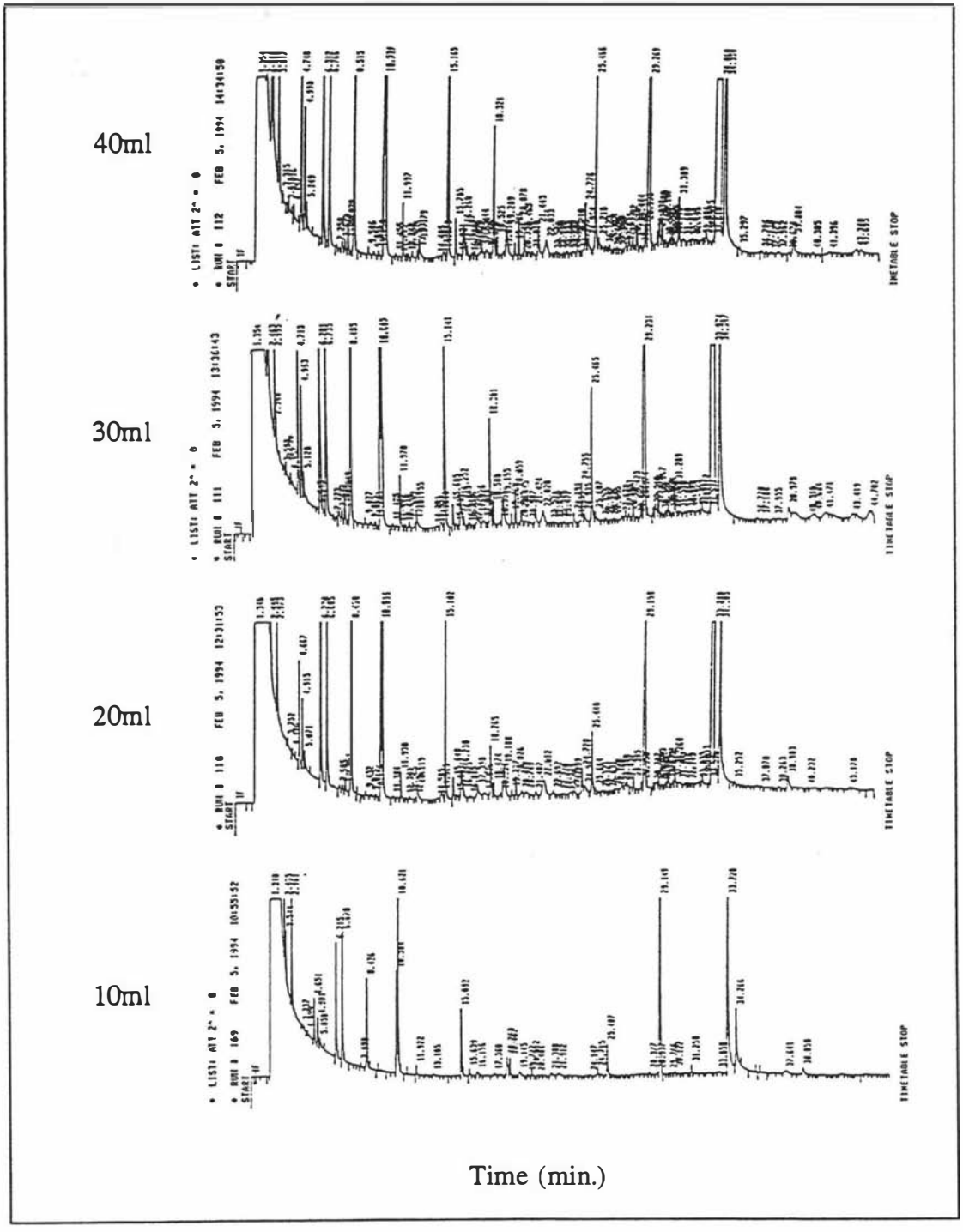
2.2 Preliminary Test of Concentrate Volume Used for GLC Analysis

Gas chromatographic profiles of 'Granny Smith' apple juice volatile compounds in relation to the volume of concentrated solvent extracts of 0.2, 0.5 and 1.0 ml (extracting 20 ml juice sample with 20 ml diethyl ether and n-pentane (2:1 ratio v/v) solvent mixture) using the direct solvent extraction method and 1 µl used for GLC analysis.



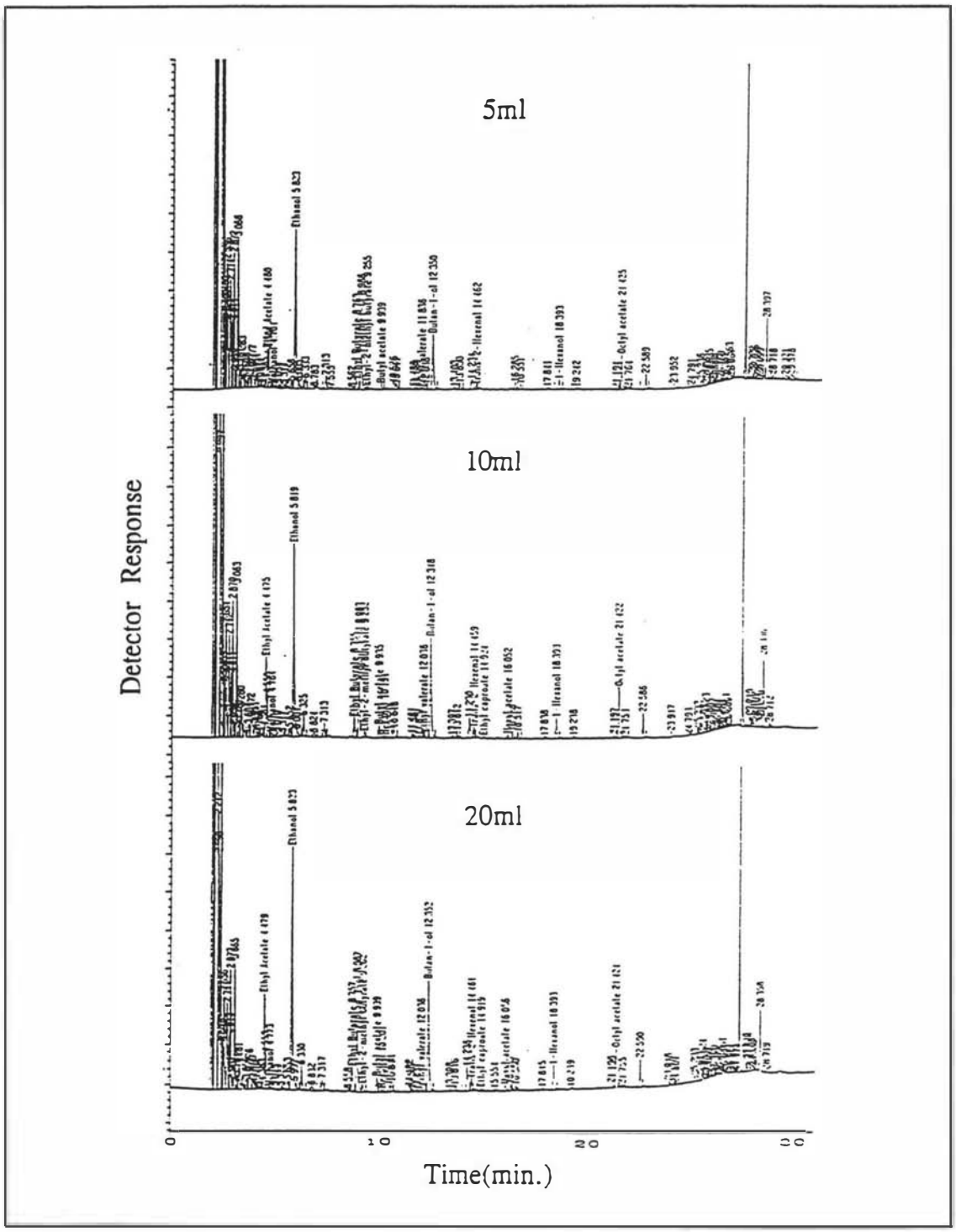
2.3 Preliminary Test of Juice Volume Used for Extraction

Gas chromatographic profiles of 'Granny Smith' apple juice volatile compounds in relation to juice volume of 10, 20, 30, and 40 ml extracted with equal volume (1:1 ratio v/v) of diethyl ether and n-pentane (2:1 ratio v/v) solvent mixture using the direct solvent extraction method. The extracted solvent was concentrated to 0.2 ml and 1 µl of concentrate was used for GLC analysis.



2.4 Test of Juice Volume Used for Extraction

Gas chromatographic profiles of 'Red Delicious' apple juice volatile compounds in relation to the volume of juice volume of 5, 10, 20, 30 and 40 ml extracted with fixed volume (20 ml) of diethyl ether and n-pentane (2:1 ratio v/v) solvent mixture using the direct solvent extraction method. The extracted solvent was concentrated to 0.25 ml and 1 µl of the concentrate was used for GLC analysis.



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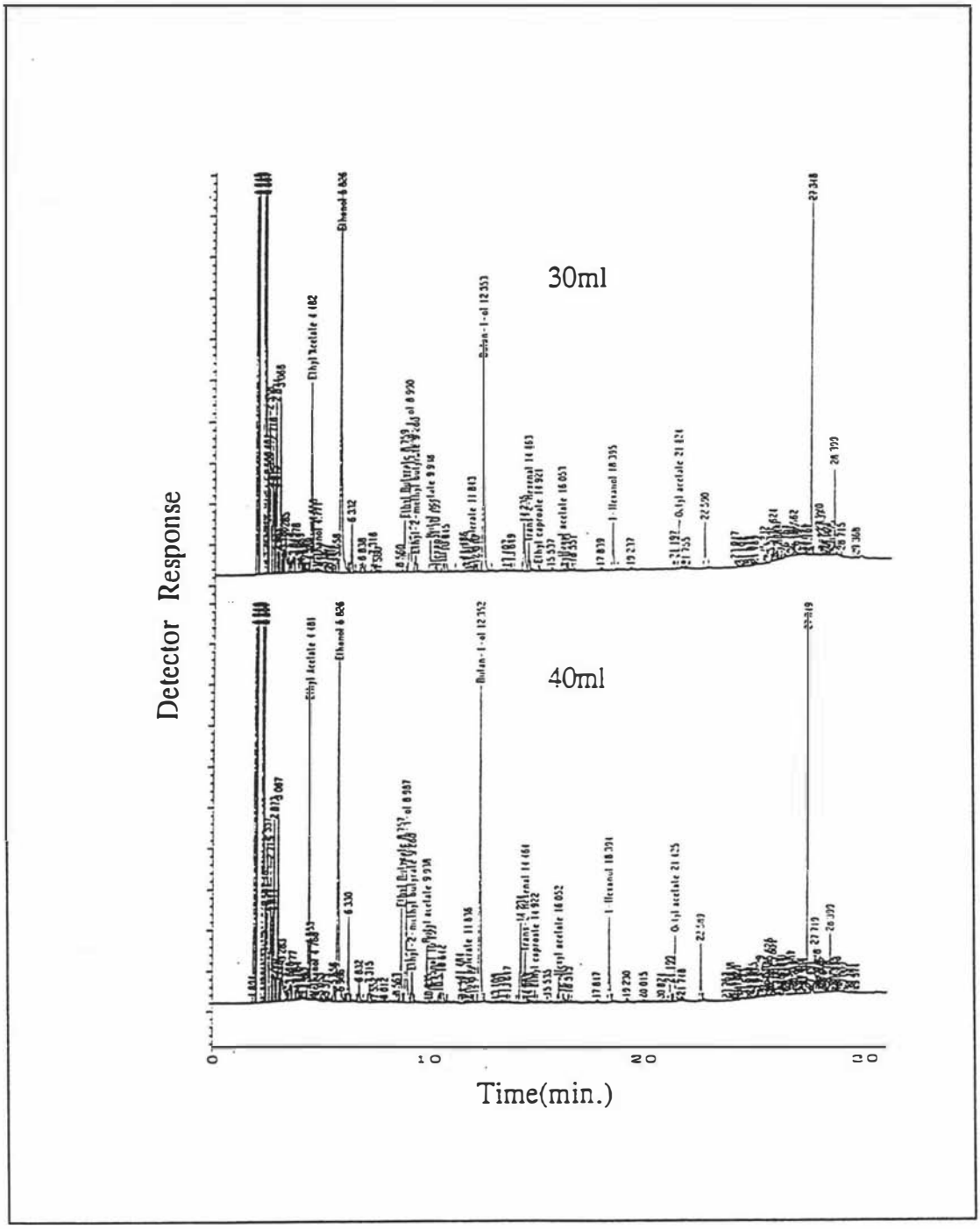
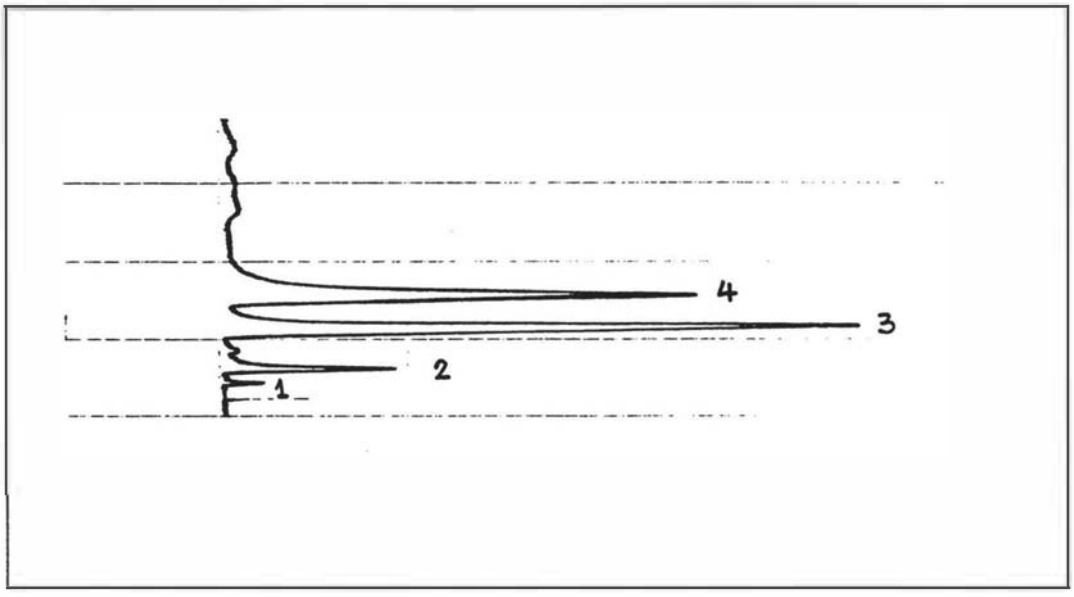


APPENDIX 3

Volatile Chromatograms

3.1 Chromatogram of Headspace Volatiles from Apple Juice

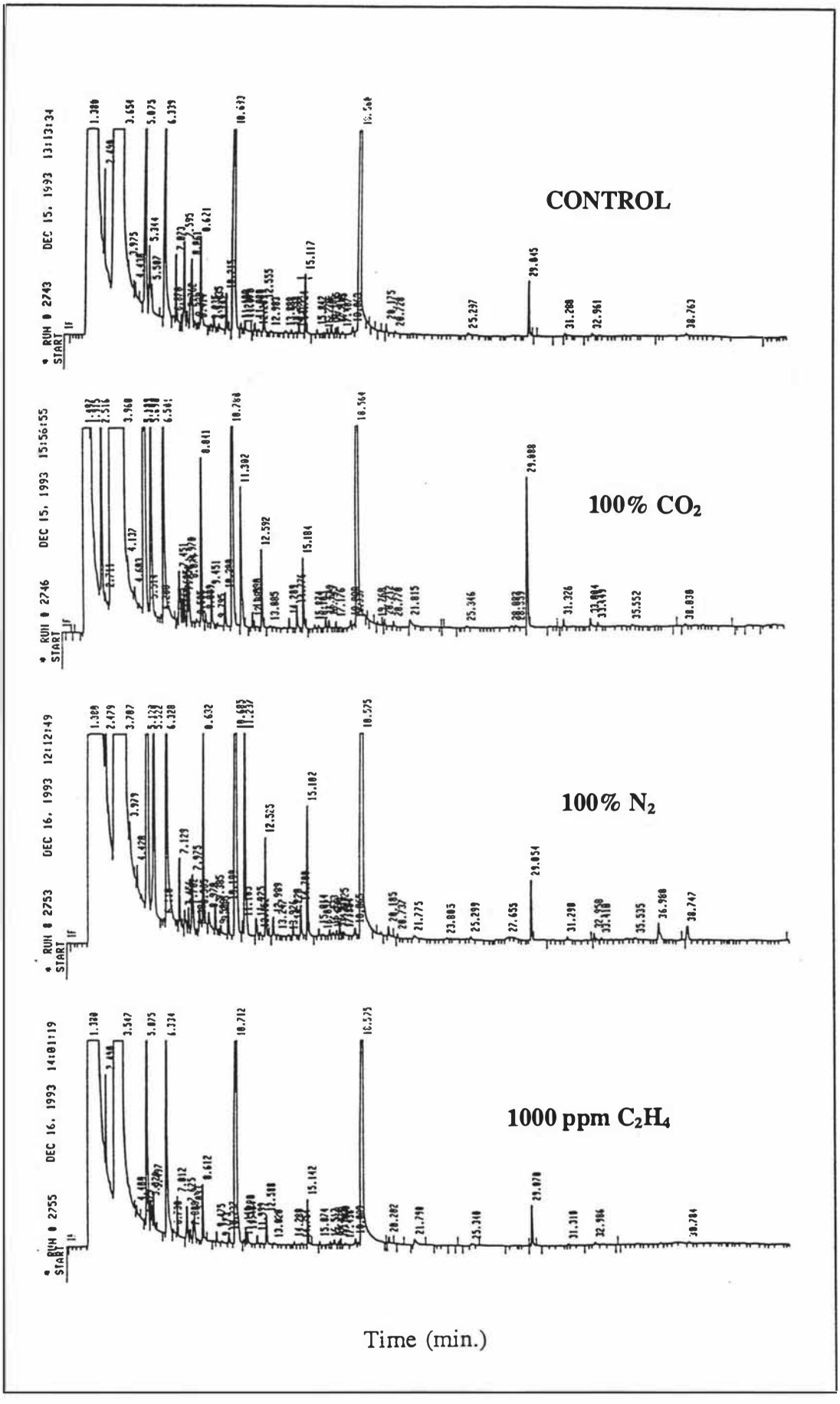
A typical chromatogram of headspace acetaldehyde, ethanol and ethyl acetate from juice of 'Red Delicious' apples obtained by using the static headspace method. Thirty ml of apple juice was placed in an Erlenmeyer flask equipped with a Suba Seal® rubber septum and incubated in a water bath at 30°C for 30 minutes to 1 hour. One ml of headspace vapour was injected onto a Pye Unicam GLC for analysis.



Peak 1 = Air peak, Peak 2 = Acetaldehyde, Peak 3 = Ethyl acetate, Peak 4 = Ethanol

3.2 Chromatograms of Volatiles from 'Granny Smith' Apple Juice

Typical chromatograms of volatile compounds in juice from 'Granny Smith' apples 3 days after treatment with 100% CO_2, 100% N_2, or 1000 ppm C_2H_4 for 24 hours at 20°C obtained by using the dynamic headspace (purge and trap) method. Volatiles were extracted from 200 ml apple juice (1µl of pure octyl acetate added as internal standard) on to a Tenax® TA adsorbent trap by a low flow rate of oxygen-free N_2 gas (about 50 ml·min^{-1}) for 2 hours at 20°C. The adsorbed volatiles were desorbed using diethyl ether (about 4 ml) with a low flow rate of oxygen-free N_2 gas (about 20 ml·min^{-1}). The extracted solvent was concentrated to 200 µl using a low flow rate of oxygen-free N_2 gas (about 20 ml·min^{-1}) and 10 µl of 1000 ppm toluene in diethyl ether was added as an external standard. One µl of concentrated sample was injected onto a Varian GLC for analysis.


CONTROL
100% CO_2
100% N_2
1000 ppm C_2H_4
Time (min.)

Retention Time (min)	Compound
1.38	diethyl ether (solvent)
2.5	ethyl acetate
3.7	ethanol *
5.1	toluene/ethyl butanoate * *
5.5	ethyl 2-methyl butanoate
6.3	hexanal
7.2	isoamyl acetate
7.5	propyl butanoate
8.7	butan-1-ol
10.2	3-methyl butan-1-ol
10.7	*trans*-2-hexenal
11.2	ethyl hexanoate
12.5	hexyl acetate
15.1	hexan-1-ol
18.5	octyl acetate (internal standard)

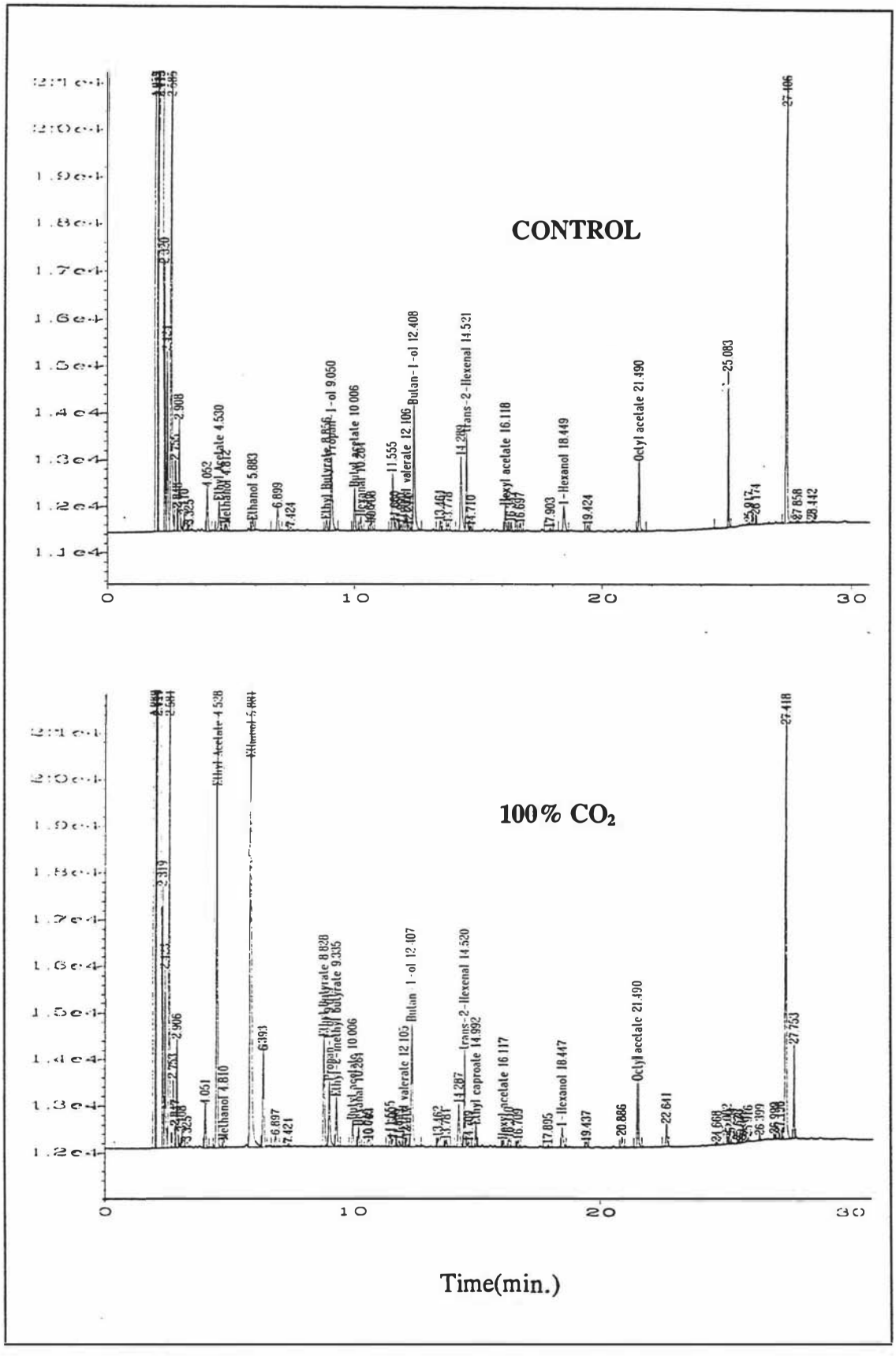
* ethanol was added by manufacturer to prevent peroxide formation in this batch of solvent,

* * toluene (added external standard) eluted at the same retention time as ethyl butanoate.

Note : butyl acetate was not detected in 'Granny Smith' apples and for 1993 experiments 15 volatile compounds of known concentrations had not been employed for external standards.

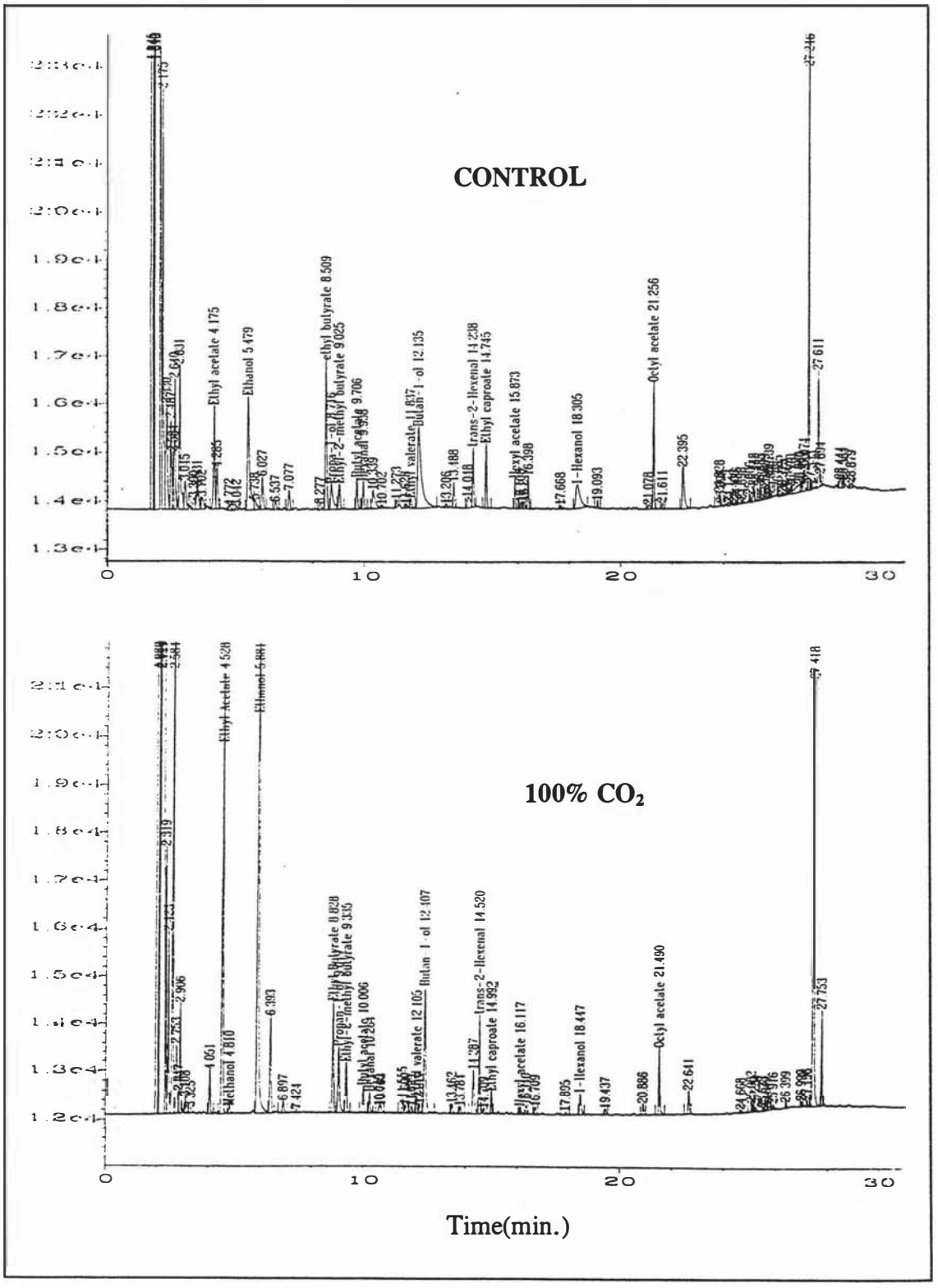
3.3 Chromatograms of Volatiles in Freshly Harvested 'Red Delicious' Apple Juice.

Typical chromatograms of volatile compounds in juice from freshly harvested 'Red Delicious' apple (at day 3) after treatment with 100% CO_2 for 24 hours at 20°C obtained by using the direct solvent extraction method. Volatiles were extracted from 20 ml apple juice (10 µl of 2000 ppm octyl acetate added as internal standard) with 20 ml of diethyl ether and n-pentane mixture (2:1 ratio, v/v). The extracted solvent was concentrated to 250 µl using a low flow rate of oxygen-free N_2 gas (about 20 $ml \cdot min^{-1}$) and 1 µl of the concentrated sample was injected onto the Hewlett Packard HP5890 Series II Plus GLC for analysis.


CONTROL
100% CO_2
Ethanol 5.883
Ethyl Acetate 4.530
Methanol 4.810
Butan-1-ol 12.408
Hexyl acetate 16.118
1-Hexanol 18.449
Octyl acetate 21.490
25.083
27.486
Ethyl Acetate 4.528
Methanol 5.884
Butan-1-ol 12.407
Hexyl acetate 16.117
1-Hexanol 18.447
Octyl acetate 21.490
27.418
0
10
20
30
Time(min.)

3.4 Chromatograms of Volatiles in 8 Months Old 'Red Delicious' Apple Juice

Typical chromatograms of volatile compounds in juice from 'Red Delicious' apple (previously stored for 8 months at 0°C) at day 3 after treatment with 100% CO_2 for 24 hours at 20°C obtained by using the direct solvent extraction method, described in 3.3. One µl of concentrated sample was injected onto the HP5890 GLC for analysis.

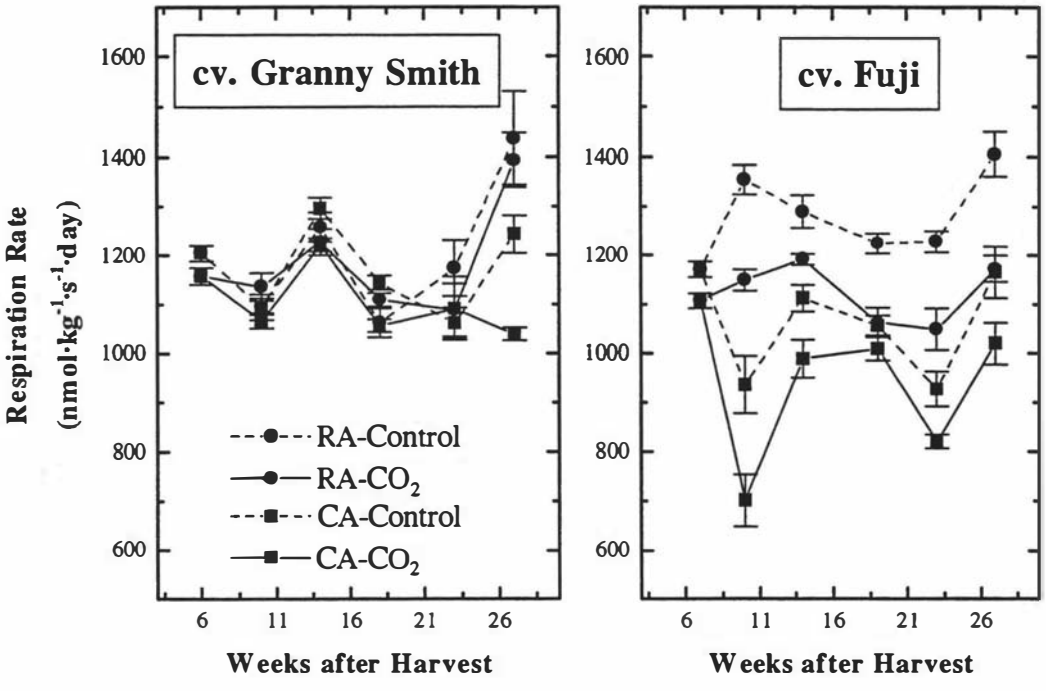


APPENDIX 4

Fruit Physiology and Quality Attributes of 'Granny Smith' and 'Fuji' Apples Maintained in Air or Controlled Atmospheres Prior to a 24h Treatment with Carbon Dioxide

4.1 Fruit Respiration Rate and Ethylene Production

Respiration rate and ethylene production of 'Granny Smith' apples were determined during post storage for 9 days at 20°C on fruit removal from RA and CA storage at different times after harvest and after treatment with air or 100% CO_2 for 24 hours at 20°C. To examine the effect of treatments on the overall respiration rate and ethylene production, data obtained during post storage were integrated (as area under curve) and plotted against time after harvest.

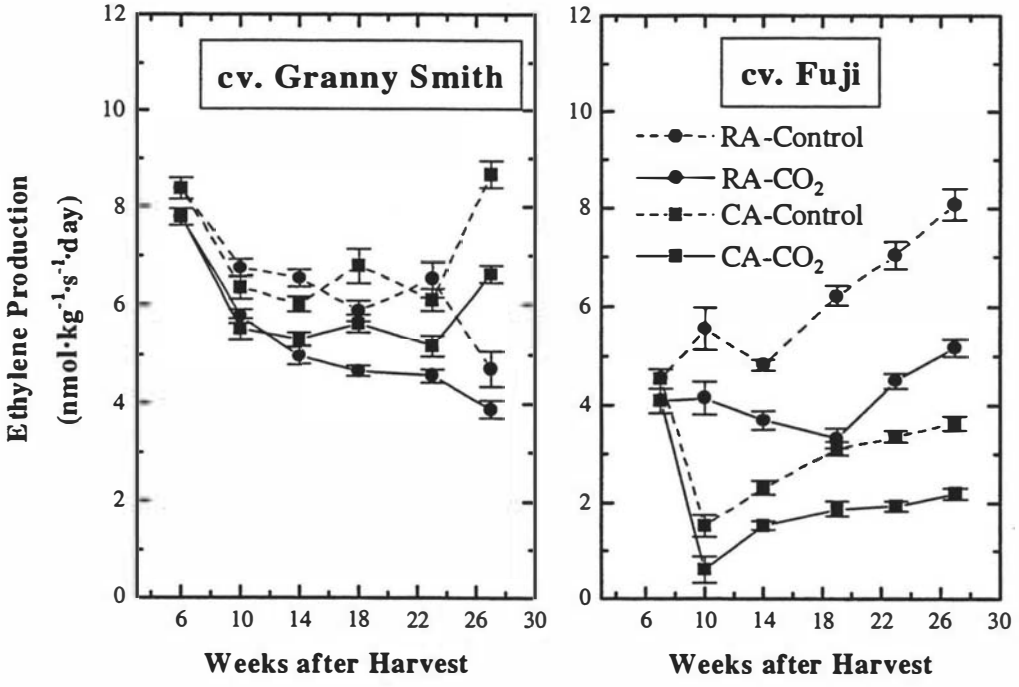


Appendix 4.1 Respiration rate during post storage for 9 days at 20°C (as area under curve) of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

In 'Granny Smith' apples, respiration rate was not affected, either by storage conditions or CO_2 treatment, except in CO_2 treated CA fruit after 27 weeks storage where respiration rate was markedly depressed compared to control fruit. In general, respiration rate fluctuated up to 23 weeks of storage and then increased, except in CO_2 treated CA fruit where it decreased, at 27 weeks after harvest (**Appendix 4.1**).

In 'Fuji' apples, respiration rate was markedly depressed ($P < 0.001$) by both CA conditions and CO_2 treatment throughout storage compared to controls. The residual depressive effect of CA and CO_2 treatments was pronounced after 10 weeks storage. In general, respiration rate in control RA fruit increased slightly, while in CO_2 treated RA fruit remained relatively constant throughout storage. Respiration rate of both control and CO_2 treated CA fruit fluctuated throughout storage, where respiration rate at 27 weeks was approximately the same as 6 weeks after harvest (**Appendix 4.1**).

Post storage production of C_2H_4 in 'Granny Smith' apples was markedly depressed by CO_2 treatment, but not by CA conditions. In fact, C_2H_4 production was comparable for control RA and control CA fruit for up to 23 weeks of storage, but production in RA fruit was markedly less ($P < 0.001$) than in CA fruit after 27 weeks storage. Ethylene production in RA fruit generally decreased continuously throughout storage, while in CA fruit it decreased at 10 weeks, then remained approximately constant through 23 weeks and increased markedly thereafter (**Appendix 4.2**).



Appendix 4.2 Ethylene production during post storage for 9 days at 20°C (as area under curve) of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treated with air or 100% CO_2 for 24 hours at 20°C.

In 'Fuji' apples, C_2H_4 was substantially depressed by both CO_2 and CA treatments throughout storage, where the depressive effect of CO_2 became greater with increasing time of storage. In general, C_2H_4 production in control RA fruit increased continuously throughout storage, while in CO_2 treated RA fruit it remained more or less constant for up to 23 weeks and increased slightly thereafter. Ethylene production in CA fruit, both control and CO_2 treated, decreased markedly at 10 weeks storage, and increased continuously thereafter (**Appendix 4.2**).

4.2 Fruit Quality Attributes

Fruit weight loss rate during post storage for 9 days at 20°C in RA stored 'Granny Smith' increased with time after harvest, while in CA fruit it remained approximately constant throughout storage. CO_2 treatment did not affect rate of fruit weight loss, but CA conditions significantly retained it ($P < 0.001$), compared to RA. Neither CA or CO_2 treatments affected rate of weight loss in 'Fuji' apples which remained approximately constant throughout storage. Fruit weight loss rate was relatively higher in 'Fuji' than in Granny Smith' apples (**Appendix 4.3**).

Juice pH which increased slightly with time in storage was not affected by CO_2 treatment in either cultivars. CA conditions, relative to RA, did not affect juice pH in 'Granny Smith' but reduced its increase in 'Fuji' apples. Juice pH in 'Fuji' was slightly higher than in 'Granny Smith' apples (**Appendix 4.3**).

Fruit skin colour (lightness and hue angle) of both cultivars were only marginally affected, if at all, by CA or CO_2 treatments used throughout storage. Skin colour fluctuated with time in storage, where differences found were rather due to variations among individual samples, since no obvious pattern was observed (**Appendix 4.4**).

Juice TSS and TSS:TA ratio was not affected by CO_2 treatment, either in 'Granny Smith' or 'Fuji' apples. Juice TSS in both cultivars fluctuated slightly with time of storage, while TSS:TA ratio increased, particularly in 'Fuji' apples. TSS:TA ratio was lower in CA fruit compared to control, particularly in 'Fuji' apples. Juice TSS from both cultivars was comparable, while TSS:TA ratio was higher in 'Fuji' apples. (**Appendix 4.5**).

Appendix 4.3 Fruit weight loss rate and juice pH during 9 days at 20°C of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treatment with air or 100% CO_2 for 24 hours at 20°C.

Week after Harvest	Fruit Weight Loss Rate (% per day)							
	cv. 'Granny Smith'				cv. 'Fuji'			
	RA		CA		RA		CA	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
6(7)#	0.23	0.22	-	-	0.36	0.37	-	-
10	0.23	0.27	0.21	0.20	0.37	0.38	0.35	0.35
14	0.25	0.23	0.23	0.26	0.38	0.39	0.34	0.32
18(19)#	0.26	0.26	0.20	0.22	0.38	0.37	0.37	0.36
23	0.26	0.28	0.22	0.21	0.39	0.39	0.33	0.34
27	0.26	0.29	0.21	0.22	0.39	0.37	0.37	0.38
c.v.	8.96%				8.80%			
Time	**				ns			
Cond.	***				***			
Treat.	ns				ns			
	Juice pH							
6(7)#	3.37	3.37	-	-	3.74	3.73	-	-
10	3.33	3.33	3.32	3.33	3.73	3.75	3.64	3.64
14	3.35	3.35	3.34	3.35	3.81	3.81	3.77	3.77
18(19)#	3.36	3.37	3.34	3.35	3.94	3.93	3.74	3.78
23	3.38	3.41	3.35	3.36	4.00	3.98	3.79	3.77
27	3.38	3.37	3.38	3.37	4.07	4.06	3.76	3.77
c.v.	0.41%				0.95%			
Time	***				***			
Cond.	ns				***			
Treat.	ns				ns			

# time after harvest for 'Fuji' that different from 'Granny Smith' apples indicated in bracket,
c.v. = coefficient of variation, Time = time after harvest, Cond. = storage conditions : RA = refrigerated air, CA = controlled-atmosphere, Treat. treatments : Ctrl = control, CO_2 = 100% CO_2 for 24 hours at 20°C. Levels of significance at P = 0.05 (*), 0.01 (* *), 0.001 (* * *), or non significant (ns).

Appendix 4.4 Fruit skin colour (lightness and hue angle) during 9 days at 20°C of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treatment with air or 100% CO_2 for 24 hours at 20°C.

Week after Harvest	Skin Lightness (%)							
	cv. 'Granny Smith'				cv. 'Fuji'			
	RA		CA		RA		CA	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
6(7)#	63.9	63.6	-	-	55.3	54.6	-	-
10	62.8	62.9	62.6	62.7	54.1	54.5	53.0	53.9
14	64.9	64.1	64.9	64.0	56.7	56.4	55.0	55.0
18(19)#	64.6	64.9	63.4	63.3	55.9	55.6	56.0	55.4
23	63.9	62.7	65.8	63.5	55.8	55.6	55.3	54.7
27	63.1	63.4	64.5	64.4	56.4	55.5	55.4	54.2
c.v.	1.72%				2.13%			
Time	* * *				* * *			
Cond.	ns				* * *			
Treat.	ns				ns			
	Skin Hue Angle (°)							
6(7)#	112.1	112.0	-	-	65.3	66.8	-	-
10	112.5	112.4	113.0	113.2	65.0	66.5	62.4	65.2
14	111.6	111.9	111.8	112.4	69.4	66.1	66.3	67.5
18(19)#	111.9	111.7	112.9	113.1	65.5	65.6	67.4	67.0
23	111.6	112.2	111.4	112.5	65.2	65.7	67.3	67.4
27	110.7	110.6	111.8	112.3	67.4	67.3	68.1	68.1
c.v.	0.52%				3.73%			
Time	* * *				* * *			
Cond.	* * *				ns			
Treat.	*				ns			

# time after harvest for 'Fuji' that different from 'Granny Smith' apples indicated in bracket,
c.v. = coefficient of variation, Time = time after harvest, Cond. = storage conditions : RA = refrigerated air, CA = controlled-atmosphere, Treat. treatments : Ctrl = control, CO_2 = 100% CO_2 for 24 hours at 20°C. Levels of significance at P = 0.05 (*), 0.01 (* *), 0.001 (* * *), or non significant (ns).

Appendix 4.5 TSS and TSS:TA ratio during 9 days at 20°C of 'Granny Smith' and 'Fuji' apples removed from RA or CA storage at different times after harvest and treatment with air or 100% CO_2 for 24 hours at 20°C.

	TSS (%)							
Week after Harvest	cv. 'Granny Smith'				cv. 'Fuji'			
	RA		CA		RA		CA	
	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2	Ctrl	CO_2
6(7)#	11.8	11.4	-	-	11.5	11.4	-	-
10	12.8	12.7	11.3	11.2	12.1	11.9	12.1	12.1
14	12.3	12.2	11.9	11.9	11.4	11.4	11.6	11.5
18(19)#	11.7	11.7	11.2	11.3	11.3	11.2	11.4	11.7
23	11.5	11.5	11.9	11.9	11.4	11.0	11.3	11.5
27	11.0	11.2	11.0	11.0	10.6	10.7	11.7	11.6
c.v.	2.71%				2.51%			
Time	***				***			
Cond.	***				***			
Treat.	ns				ns			
	TSS:TA Ratio							
6(7)#	20.4	20.3	-	-	48.4	49.2	-	-
10	21.5	20.9	18.7	18.8	57.3	58.8	52.5	52.5
14	22.5	22.3	21.1	21.1	59.3	61.0	49.9	49.8
18(19)#	21.5	21.6	18.6	18.9	72.5	71.0	53.1	55.5
23	22.5	22.0	22.6	22.8	92.4	88.3	57.8	55.2
27	24.8	25.0	21.0	22.4	103.0	101.4	62.4	63.0
c.v.	6.47%				10.71%			
Time	***				***			
Cond.	***				***			
Treat.	ns				ns			

# time after harvest for 'Fuji' that different from 'Granny Smith' apples indicated in bracket,
c.v. = coefficient of variation, Time = time after harvest, Cond. = storage conditions : RA = refrigerated air, CA = controlled-atmosphere, Treat. treatments : Ctrl = control, CO_2 = 100% CO_2 for 24 hours at 20°C. Levels of significance at P = 0.05 (*), 0.01 (* *), 0.001 (* * *), or non significant (ns).

APPENDIX 5

Chromatograms of Volatile Compounds from 5 Apple Cultivars

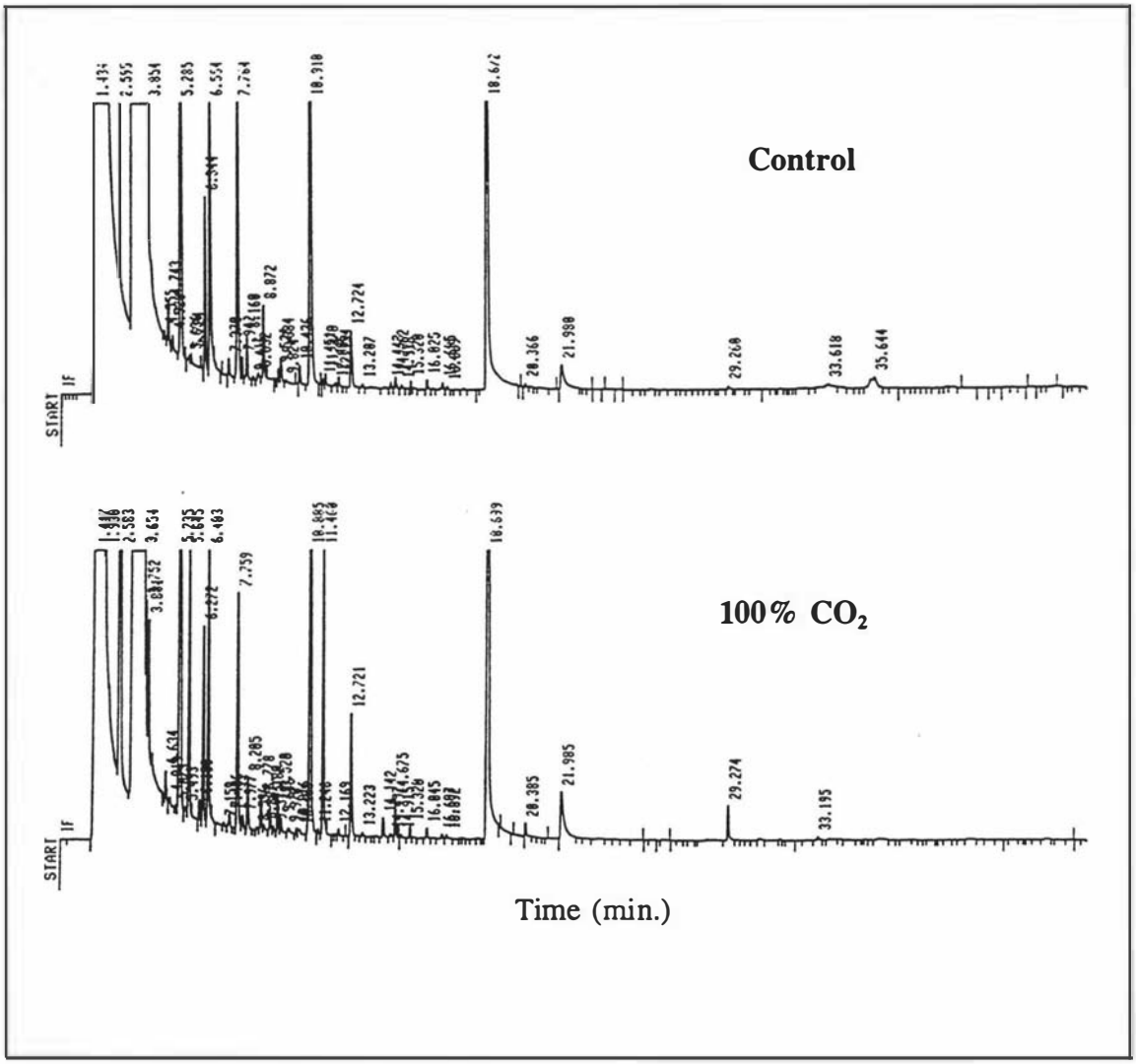
5.1 Typical Volatile Chromatograms from 5 Apple Cultivars

Typical chromatograms of volatile compounds from a single strength juice of 5 apple cultivars, both from control and 100% CO_2 treated fruit, analysed by the purge and trap technique (1993 experiments) and by the direct solvent extraction technique (1994 experiments) are shown in **Appendix 5.1 - Appendix 5.8**. Detailed methods for sample preparation, extraction, determination, identification and quantification of volatile compounds for both techniques were described in **Chapter Two**. A detailed study of the two techniques of extraction and the advantages or disadvantages of each were discussed in **Chapter Three**.

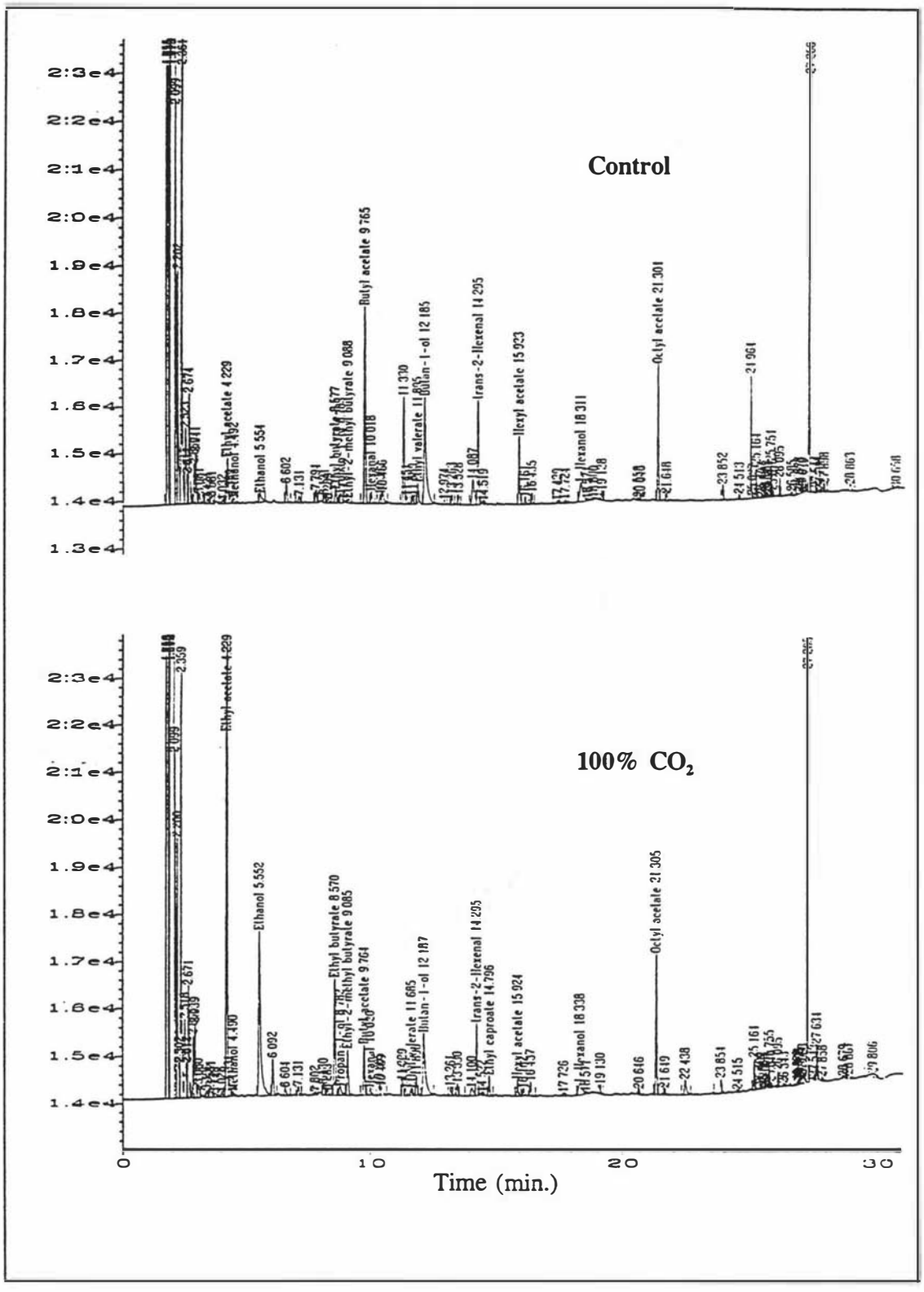
Some identified peaks (by mass spectrometry) and their retention times (min) from chromatograms obtained by using the purge and trap technique for extraction were as follows:

Compound	Retention Time (min)		
	Braeburn	Granny Smith	Royal Gala
diethyl ether	1.4	1.4	1.7
ethyl acetate	2.5	2.5	3.0
ethanol	3.7	3.7	3.7
ethyl propanoate	3.9	4.0	–
ethyl butanoate + toluene*	5.2	5.1	5.9
ethyl 2-methyl butanoate	5.6	5.6	–
butyl acetate	6.3	–	7.0
hexanal	6.5	6.4	7.3
isoamyl acetate	7.7	7.6	8.6
propyl butanoate	–	8.0	–
butan-1-ol	8.8	8.7	9.6
3-methyl butan-1-ol	–	10.3	–
trans-2-hexenal	10.9	10.8	11.8
ethyl hexanoate	11.5	11.3	–
hexyl acetate	12.7	12.6	13.6
hexan-1-ol	15.3	15.2	16.2
octyl acetate (internal standard)	18.6	18.6	19.6

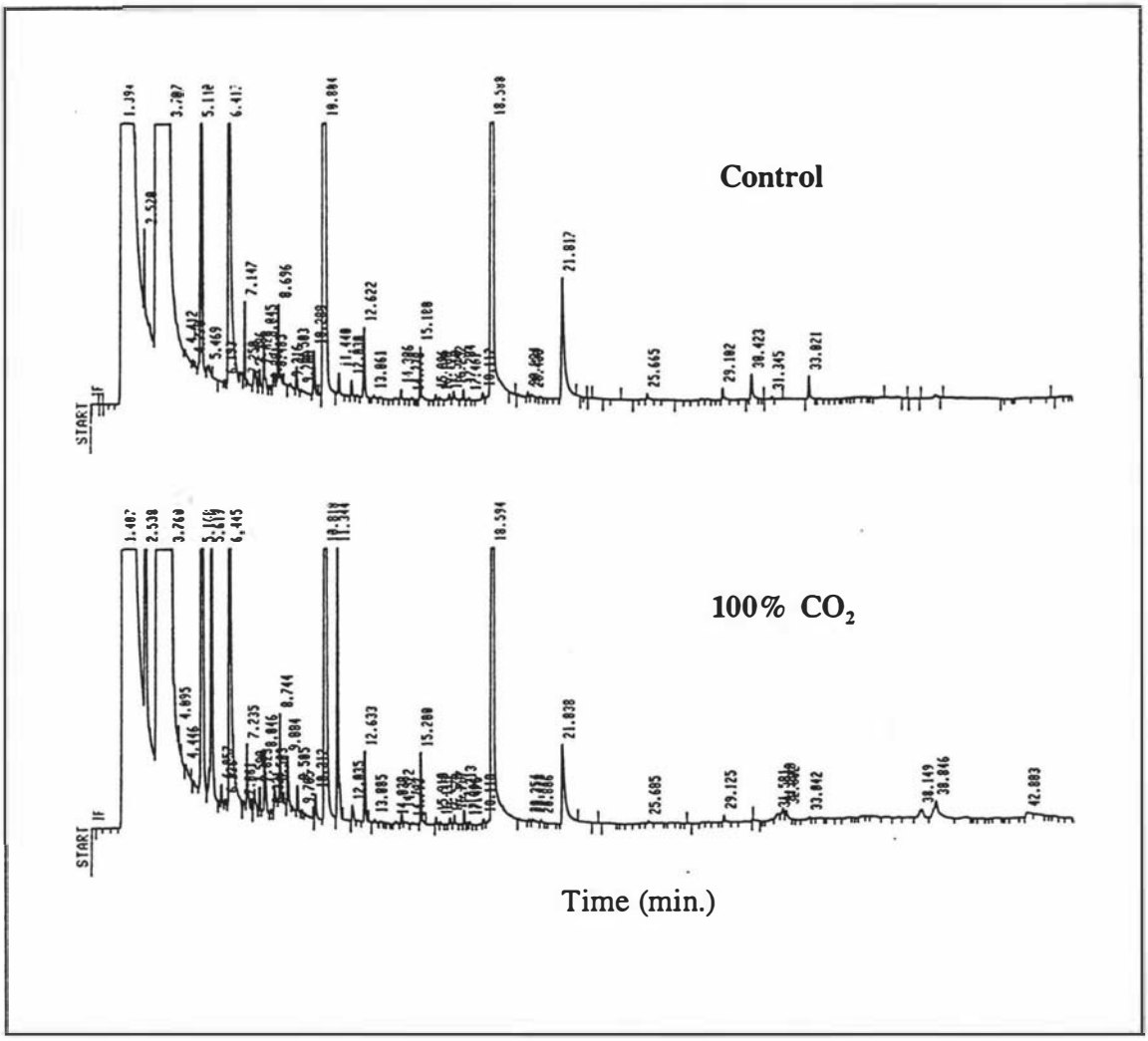
* ethyl butanoate and toluene eluted at the same retention time, – = not detected or identified.



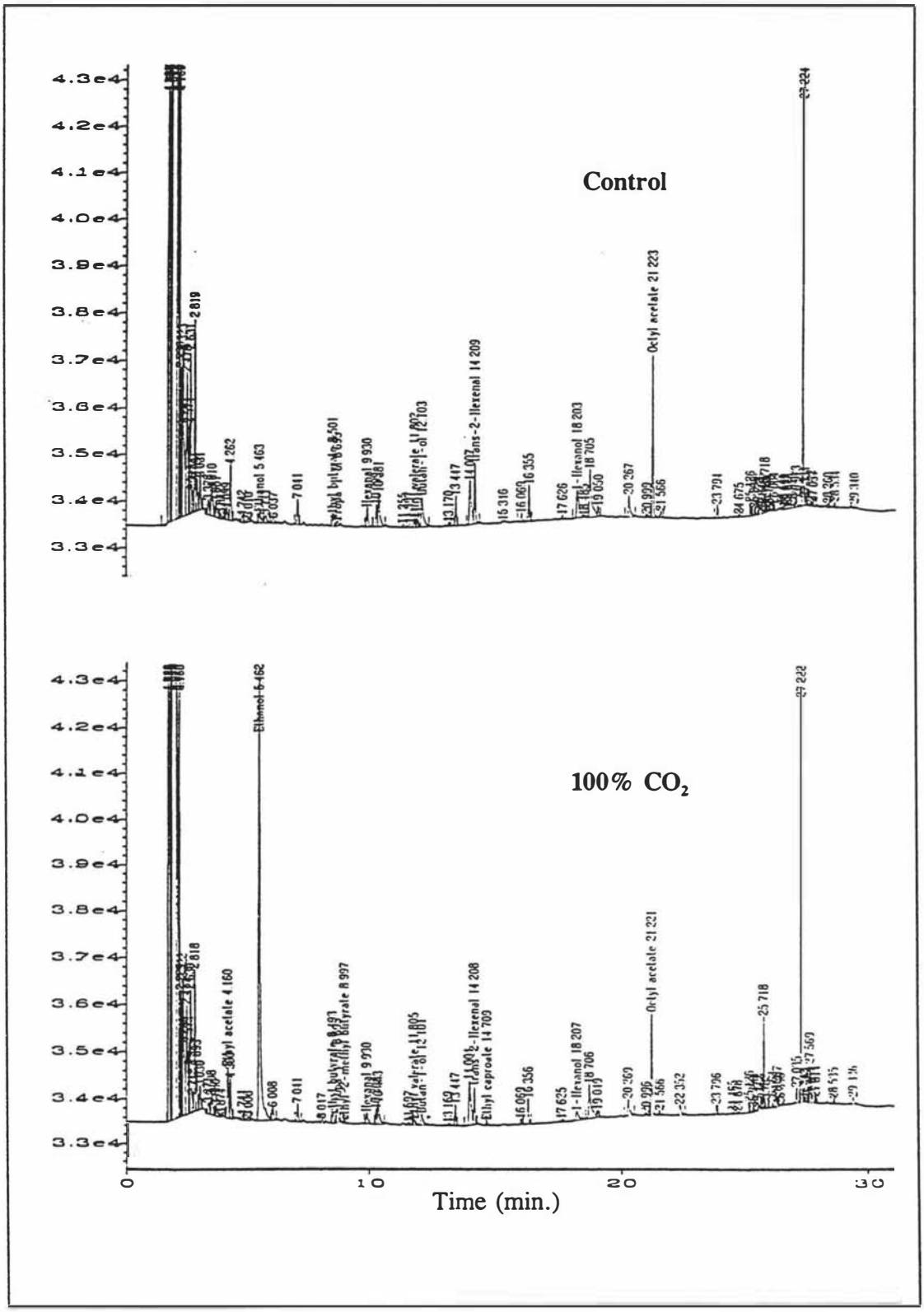
Appendix 5.1 Chromatograms of volatile compounds analysed by the purge and trap technique from juice of control and 100% CO_2 treated (24 hours at 20°C) 'Braeburn' apples (1993 experiment).



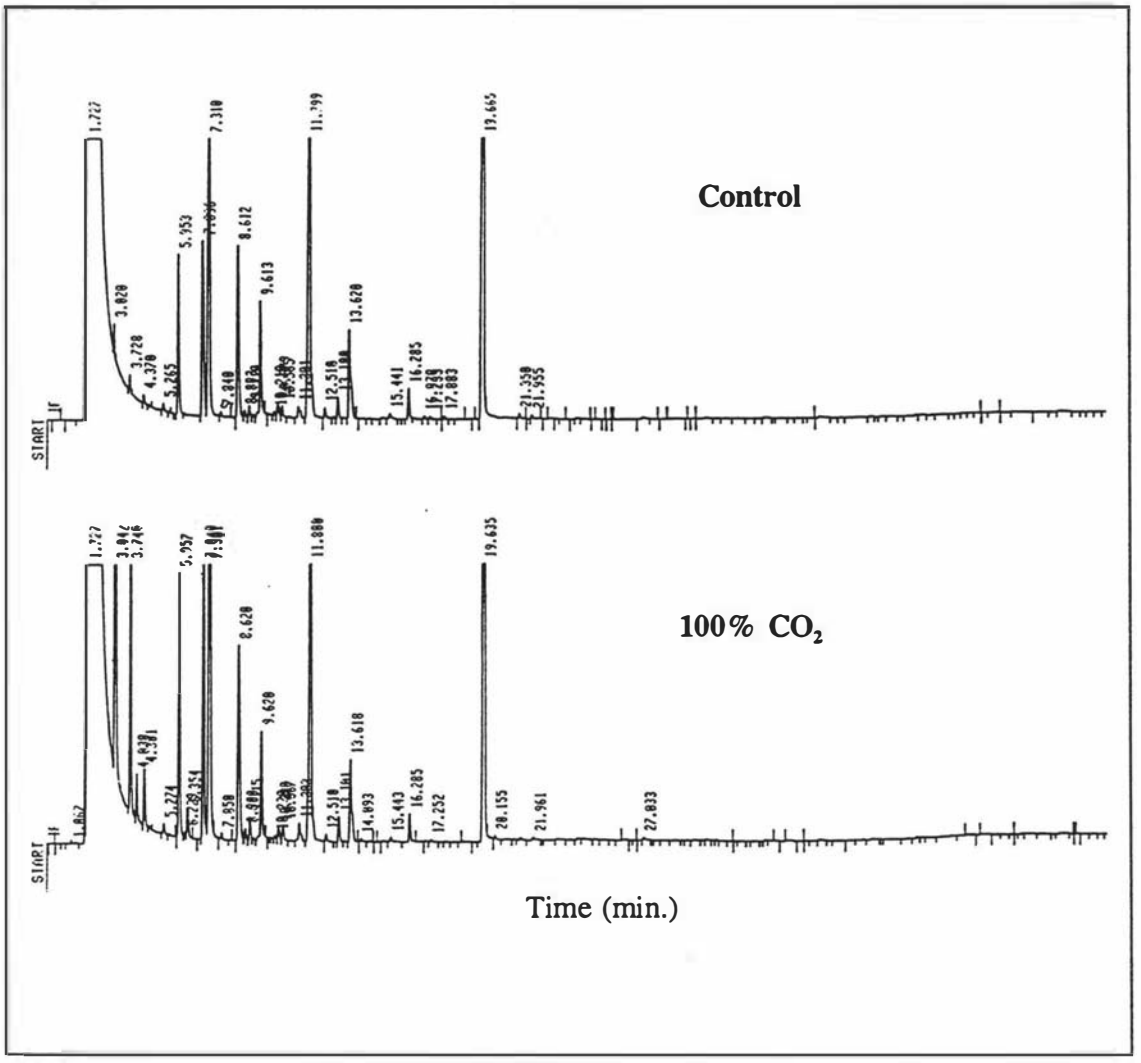
Appendix 5.2 Chromatograms of volatile compounds analysed by the direct solvent extraction technique in juice from control and 100% CO_2 treated (24 hours at 20°C) 'Braeburn' apples (1994 experiment).



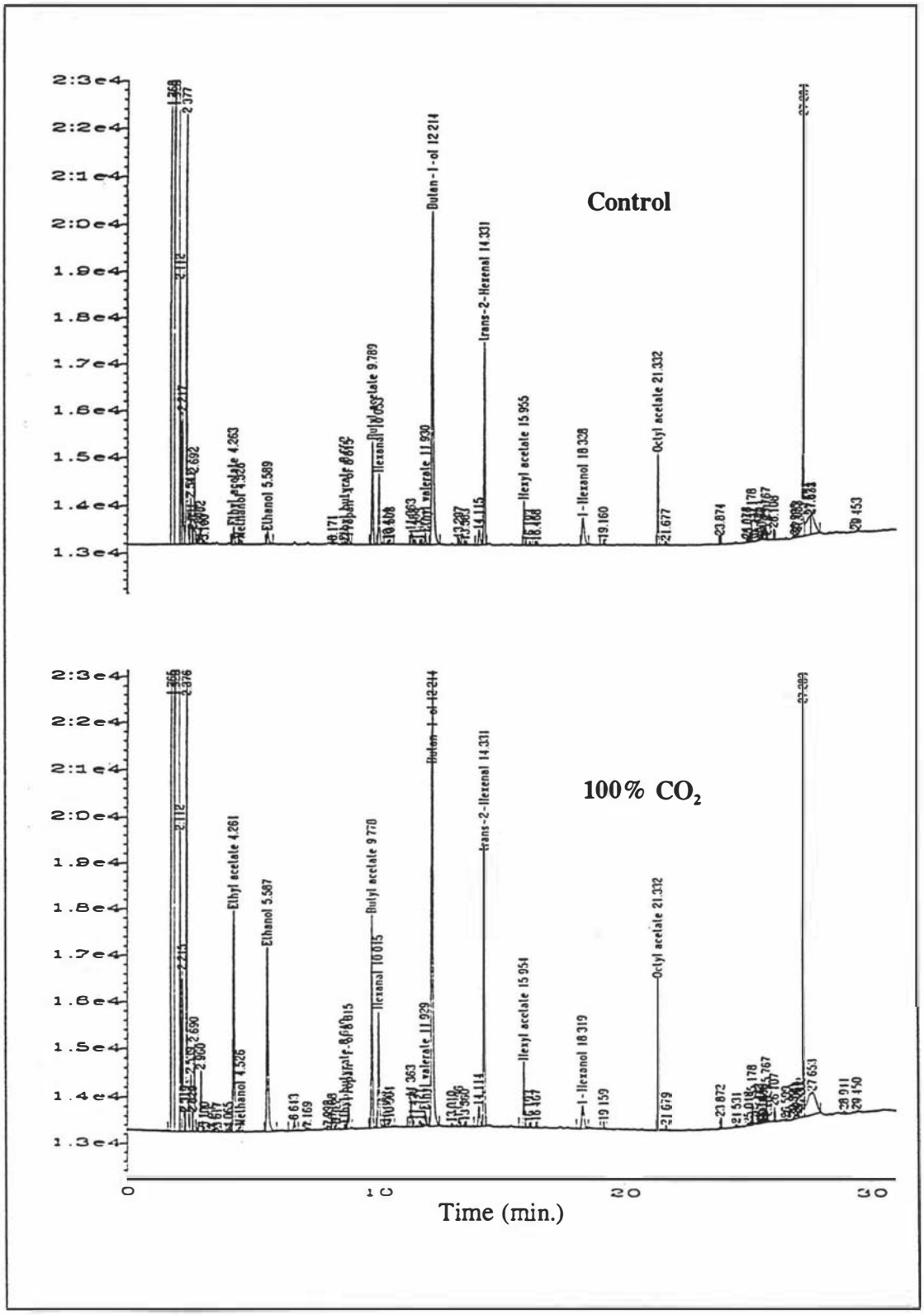
Appendix 5.3 Chromatograms of volatile compounds analysed by the purge and trap technique from juice of control and 100% CO_2 treated (24 hours at 20°C) 'Granny Smith' apples (1993 experiment).



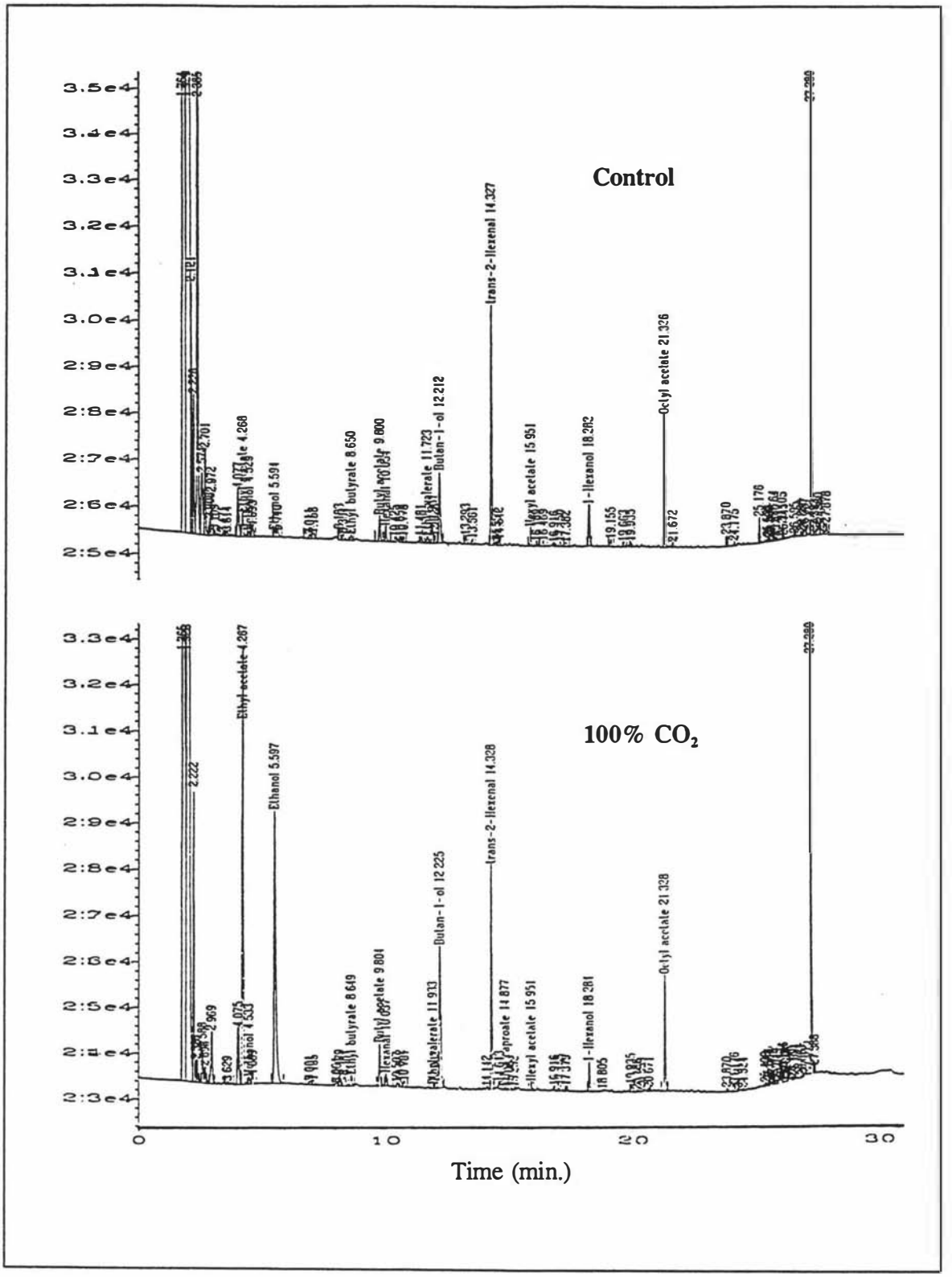
Appendix 5.4 Chromatograms of volatile compounds analysed by the direct solvent extraction technique from juice of control and 100% CO_2 treated (24 hours at 20°C) 'Granny Smith' apples (1994 experiment).



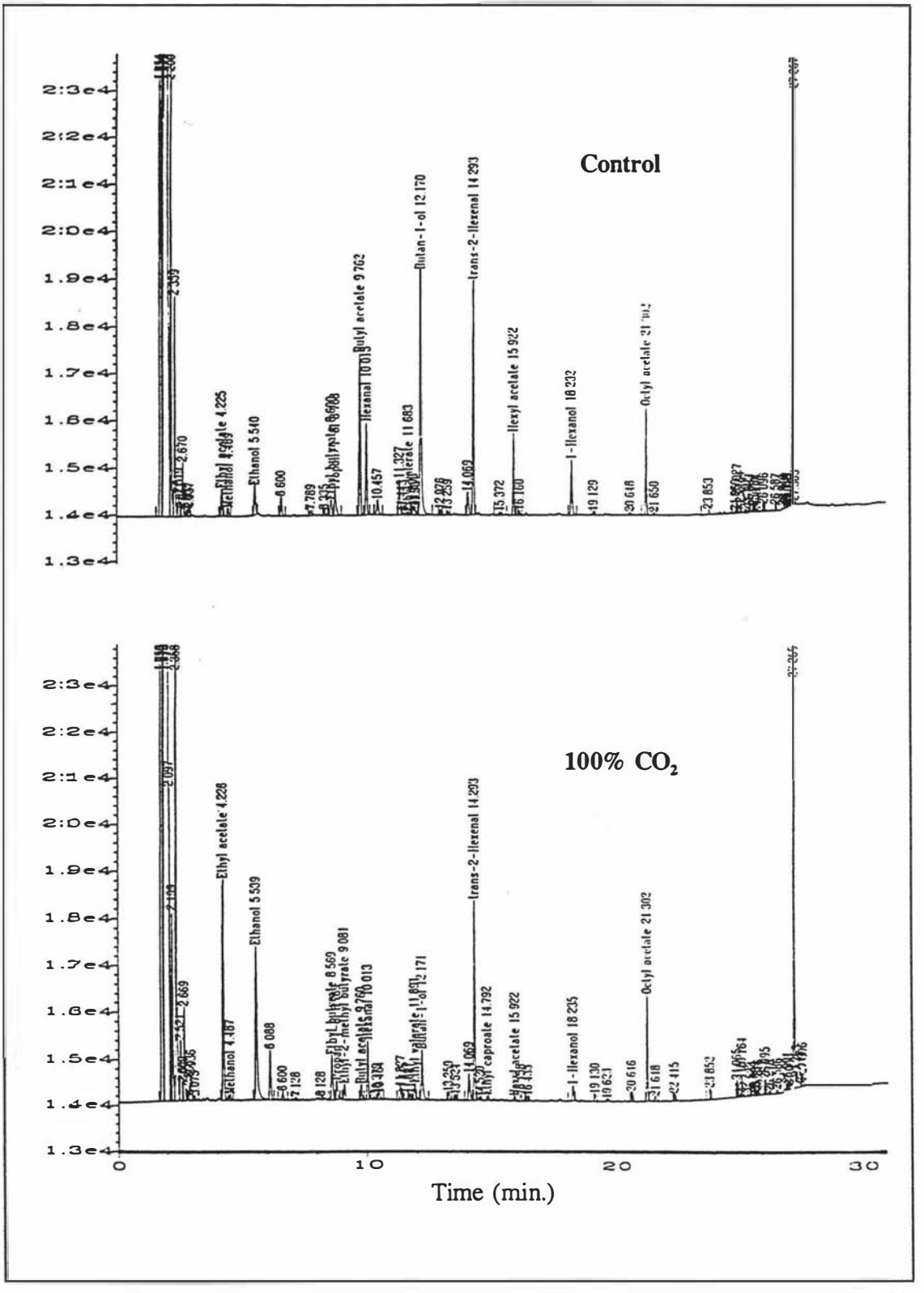
Appendix 5.5 Chromatograms of volatile compounds analysed by the purge and trap technique from juice of control and 100% CO_2 treated (24 hours at 20°C) 'Royal Gala' apples (1993 experiment).



Appendix 5.6 Chromatograms of volatile compounds analysed by the direct solvent extraction technique from juice of control and CO_2 treated (24 hours at 20°C) 'Royal Gala' apples (1994 experiment).



Appendix 5.7 Chromatograms of volatile compounds analysed by the direct solvent extraction technique from juice of control and 100% CO_2 treated (24 hours at 20°C) 'Cox's Orange Pippin' apples (1994 experiment).



Appendix 5.8 Chromatograms of volatile compounds analysed by the direct solvent extraction technique from juice of control and 100% CO_2 treated (24 hours at 20°C) 'Pacific Rose' apples (1994 experiment).

APPENDIX 6

Calculation of Odour Unit

6.1 Odour Unit of Selected Volatiles from 'Royal Gala' Apples

Concentrations of selected volatile compounds in juice from control and 100% CO_2 treated (24 hours at 20°C) 'Royal Gala' apples which had been stored for 4 weeks at 0.5°C, the magnitude of enhancement or degree of reduction, and relative contribution (odour unit) to the aroma of these compounds 3 days after treatment at 20°C.

	Concentration (ppm)		increase[1]	Threshold[3]	Odour unit[4]	
Compound	Ctrl	CO_2	decrease[1]	(ppm)	Ctrl	CO_2
Acetaldehyde	n.d.	12.15	–[2]	0.015		810
Ethyl acetate	27.70	331.23	+11.9	5	5	66
Ethanol	83.56	448.32	+5.4	100	0.8	5
Butan-1-ol	365.71	432.86	+1.8	0.5	731	866
Hexanal	57.68	76.43	+1.3	0.005	11536	15286
2-MPOH	5.70	7.13	+1.3	n.a.		
Amyl acetate	7.21	8.62	+1.2	0.005	1442	1724
2-MB acid	4.77	5.74	+1.2	1.6	3	4
2-Butanone	16.62	19.43	+1.2	37	0.5	0.5
ρ-Xylene	14.54	16.78	+1.5	n.a.		
Toluene	5.14	5.92	+1.2	n.a.		
Propan-1-ol	18.07	19.85	+1.1	9	2	2
trans-2-Hexenal	167.85	177.50	+1.1	0.017	9874	10441
Hexan-1-ol	39.70	37.78	-4.8%	0.5	79	76
Butyl acetate	118.50	107.56	-9.2%	0.066	1796	1630
2/3-MBOH	22.75	20.65	-9.2%	0.25	91	83
Hexyl acetate	41.36	30.63	-25.9%	0.002	20680	15315
2/3-MBA	19.87	13.21	-33.5%	0.005	3974	2642
Propyl acetate	6.56	4.21	-35.8%	n.a.		
Sum of odour unit			**(-33.0%)[5]**		**50214**	**33649**

Data were means of 6 replicates, [1] increase was the ratio (fold) between concentrations from CO_2 and control treatments, and decrease was percentage of reduction from CO_2 treatment relative to control, [2] could not be calculated, [3] Odour threshold concentration in water (after Brennand *et al.*, 1989; Buttery, 1993; Flath *et al.*, 1967; Kollmannsberger and Berger, 1992; Paillard, 1990; Teranishi *et al.*, 1987), [4] Odour unit = compound concentration ÷ odour threshold concentration, [5] difference in sum of odour unit between treatments, 2/3-MBA = 2/3-methyl butyl acetate, 2-MB acid = 2-methyl butanoic acid, 2/3-MBOH = 2/3-methyl butan-1-ol, 2-MPOH = 2-methyl propan-1-ol, Ctrl = control, CO_2 = 100% CO_2 treatment for 24 hours at 20°C, n.a. = not available, n.d. = not detected.

6.2 Odour Unit of Selected Volatiles from 'Fuji' Apples

Concentrations of selected volatile compounds in juice from control and 100% CO_2 treated (24 hours at 20°C) 'Fuji' apples which had been stored for 6 weeks at 0.5°C, the magnitude of enhancement or degree of reduction, and relative contribution (odour unit) to the aroma of these compounds 3 days after treatment at 20°C.

Compound	Concentration (ppm)		increase[1] decrease[1]	Threshold[3] (ppm)	Odour unit[5]	
	Ctrl	CO_2			Ctrl	CO_2
E 3-(OH)B	n.d.	3.02	–[2]	n.a.		
Ethyl propanoate	n.d.	20.36	–	0.01		2036
E 2-MB	0.96	18.63	+19.4	0.0001	9600	186300
Ethyl hexanoate	0.60	3.61	+6.0	0.001	600	3610
Ethanol	80.40	345.10	+4.3	100	0.8	3.5
Ethyl acetate	51.92	150.50	+2.9	5	10.4	30
Acetaldehyde	2.70	7.20	+2.7	0.015	179	478
Pentan-1-ol	0.67	1.40	+2.1	4.5	0.2	0.3
Ethyl butanoate	18.30	35.30	+1.9	0.001	18340	35250
Hexan-1-ol	9.30	12.10	+1.3	0.5	18	24
Acetic acid	3.34	3.64	+1.1	100	0.03	0.04
Dodecane	7.02	7.71	+1.1	n.a.		
trans-2-Hexenal	68.80	72.50	+1.1	0.017	4047	4266
Hexanal	29.20	30.60	+1.1	0.005	5848	6114
Butan-1-ol	107.90	111.70	+1.1	0.5	216	223
2/3-Methyl butan-1-ol	70.90	70.70	-0.3%	0.25	284	283
Propyl acetate	7.50	7.40	-1.5%	n.a.		
Tridecane	5.10	5.00	-2.2%	n.a.		
Tetradecane	5.50	5.40	-2.4%	n.a.		
Propan-1-ol	67.30	62.40	-7.3%	9	8	7
ρ-Xylene	18.10	16.50	-8.8%	n.a.		
Methanol	15.30	13.40	-12.8%	100[4]	0.15	0.13
Undecane	10.30	8.90	-13.5%	n.a.		
Butyl acetate	13.80	10.60	-23.4%	0.066	209	160
Decane	5.40	3.90	-28.8%	n.a.		
2/3-MBA	20.30	12.40	-38.9%	0.005	4056	2476
Hexyl acetate	6.30	3.20	-49.4%	0.002	3140	1590
α-Farnesene	239.80	4.00	-98.3%	n.a.		
Sum of odour unit			**(+5.2)[6]**		**46557**	**242851**

Data were means of 7 replicates, [1] increase was the ratio (fold) between concentrations from CO_2 and control treatments, and decrease was percentage of reduction from CO_2 treatment relative to control, [2] could not be calculated, [3] Odour threshold concentration in water (after Brennand *et al.*, 1989; Buttery, 1993; Flath *et al.*, 1967; Kollmannsberger and Berger, 1992; Paillard, 1990; Teranishi *et al.*, 1987), [4] threshold concentration in air (after Shaw, 1986), [5] Odour unit = compound concentration ÷ odour threshold concentration, [6] difference in sum of odour unit between treatments, E 2-MB = ethyl 2-methyl butanoate, E-3-(OH)B = ethyl 3-hydroxy butanoate, 2/3-MBA = 2/3-methyl butyl acetate, Ctrl = control, CO_2 = 100% CO_2 treatment for 24 hours at 20°C, n.a. = not available, n.d. = not detected.

6.3 Odour Unit of Selected Volatiles from 'Pacific Rose' Apples

Concentrations of selected volatile compounds in juice from control and 100% CO_2 treated (24 hours at 20°C) 'Pacific Rose' apples which had been stored for 2 weeks at 0.5°C, the magnitude of enhancement or degree of reduction, and relative contribution (odour unit) to the aroma of these compounds 3 days after treatment at 20°C.

Compound	Concentration (ppm)		increase[1] decrease[1]	Threshold[3] (ppm)	Odour unit[5]	
	Ctrl	CO_2				
E 2-MB	n.d.	18.00	–[2]	0.0001		180000
Ethyl hexanoate	n.d.	11.80	–	0.001		11800
Ethyl propanoate	n.d.	41.90	–	0.01		4190
Acetaldehyde	n.d.	5.85	–	0.015		390
E 3-(OH)B	n.d.	6.00	–	n.a.		
Acetic acid	n.d.	5.60	–	100		0.1
Ethyl acetate	41.40	354.40	+8.6	5	8.3	71
Ethanol	121.80	656.90	+5.4	100	1.2	6.6
Ethyl butanoate	17.90	87.30	+4.9	0.001	17900	87300
Propan-1-ol	6.80	19.20	+2.8	4.5	1.5	4.3
Butan-1-ol	49.40	78.30	+1.6	0.5	99	157
Xylene	5.70	8.90	+1.6	n.a.		
Methanol	17.70	19.90	+1.2	100[4]	0.2	0.2
ρ-Xylene	3.80	3.80	1.0	n.a.		
Hexan-1-ol	17.50	16.50	-5.7%	0.5	35	33
Butyl Acetate	11.90	10.70	-10.1%	0.5	24	21
Tridecane	4.20	3.50	-16.7%	n.a.		
Hexanal	40.70	30.40	-25.3%	0.005	8140	6080
2/3-Methyl butan-1-ol	37.60	26.60	-29.3%	0.066	570	403
Hexyl acetate	6.30	4.00	-36.5%	0.002	3150	2000
trans-2-Hexenal	108.80	63.00	-42.1%	0.017	6400	3706
2/3-MBA	20.90	8.60	-58.9%	0.005	4180	1720
Sum of odour unit			**(+6.5)[6]**		**45636**	**297882**

Data were means of 10 replicates, [1] increase was the ratio (fold) between concentrations from CO_2 and control treatments, and decrease was percentage of reduction from CO_2 treatment relative to control, [2] could not be calculated, [3] Odour threshold concentration in water (after Brennand *et al.*, 1989; Buttery, 1993; Flath *et al.*, 1967; Kollmannsberger and Berger, 1992; Paillard, 1990; Teranishi *et al.*, 1987), [4] threshold concentration in air (after Shaw, 1986), [5] Odour unit = compound concentration ÷ odour threshold concentration, [6] difference in sum of odour unit between treatments, E 2-MB = ethyl 2-methyl butanoate, E-3-(OH)B = ethyl 3-hydroxy butanoate, 2/3-MBA = 2/3-methyl butyl acetate, Ctrl = control, CO_2 = 100% CO_2 treatment for 24 hours at 20°C, n.a. = not available, n.d. = not detected.

6.4 Odour Unit of Selected Volatiles from 'Granny Smith' Apples

Concentrations of selected volatile compounds in juice from control and 100% CO_2 treated (24 hours at 20°C) 'Granny Smith' apples which had been stored for 3 months at 0.5°C, the magnitude of enhancement or degree of reduction, and relative contribution (odour unit) to the aroma of these compounds 3 days after treatment at 20°C.

	Concentration (ppm)		increase[1]	Threshold[3]	Odour unit[4]	
Compound	Ctrl	CO_2	decrease[1]	(ppm)	Ctrl	CO_2
E 2-MB	n.d.	7.54	–[2]	0.0001		75400
Ethyl hexanoate	n.d.	6.96	–	0.001		6960
Ethyl propanoate	n.d.	60.35	–	0.01		6035
E-3-(OH)B	n.d.	45.85	–	n.a.		
Ethyl acetate	n.d.	49.16	–	5		10
Acetaldehyde	n.d.	21.01	–	0.015		1401
Ethanol	15.45	662.38	+42.9	100	0.2	6.6
Ethyl butanoate	11.00	48.51	+4.4	0.001	11000	48510
Acetic acid	8.84	26.28	+3.0	100	0.1	.3
Propan-1-ol	21.28	41.90	+1.3	9	3.5	4.7
6-M 5-hep-2-ol	5.13	5.98	+1.2	n.a.		
Pentadecane	5.32	5.46	+1.0	n.a.		
1,3-Butanediol	7.84	8.00	+1.0	n.a.		
Tridecane	6.30	6.01	-4.6%	n.a.		
Hexanal	23.34	22.22	-4.8%	0.005	4668	4444
Xylene	11.13	10.30	-7.1%	n.a.		
Amyl acetate	4.62	4.28	-7.4%	0.005	924	856
Propyl acetate	22.06	19.69	-10.7%	n.a.		
Butan-1-ol	48.61	36.22	-25.5%	0.5	97	72
2/3-MBA	8.97	7.15	-26.7%	0.005	1952	1430
Tetradecane	6.12	4.40	-28.1%	n.a.		
Hexan-1-ol	49.51	33.08	-33.2%	0.5	99	66
trans-2-Hexenal	137.55	86.75	-36.9%	0.017	8091	5103
2/3-Methyl butan-1-ol	142.01	71.01	-50.0%	0.25	568	284
α-Farnesene	10.50	n.d.	–	n.a.		
Sum of odour unit			**(+5.5)[5]**		**27403**	**150583**

Data were means of 6 replicates, [1] increase was the ratio (fold) between concentrations from CO_2 and control treatments, and decrease was percentage of reduction from CO_2 treatment relative to control, [2] could not be calculated, [3] Odour threshold concentration in water (after Brennand *et al.*, 1989; Buttery, 1993; Flath *et al.*, 1967; Kollmannsberger and Berger, 1992; Paillard, 1990; Teranishi *et al.*, 1987), [4] Odour unit = compound concentration ÷ odour threshold concentration, [5] difference in sum of odour unit between treatments, E 2-MB = ethyl 2-methyl butanoate, E-3-(OH)B = ethyl 3-hydroxy butanoate, 2/3-MBA = 2-methyl butyl acetate, 6-M 5-hep-2-ol = 6-methyl 5-hepten-2-ol, Ctrl = control, CO_2 = 100% CO_2 treatment for 24 hours at 20°C, n.a. = not available, n.d. = not detected.