

**EFFECTS OF TEMPERATURE REGIMES UNDER
LOW LIGHT CONDITIONS ON GROWTH RATE AND
PHYTOCHEMICAL COMPOSITION OF
LETTUCE AND CARROT PLANTS**

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A thesis submitted for the degree of Doctor Philosophy



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January 2014

Abstract

Environmental conditions such as temperature have a large impact on the growth and development of plants. Lettuce and carrot contain phytochemicals (secondary metabolites) with high nutritional value. The temperature conditions that the plant is grown in may affect the content of phytochemicals, which in turn affects the quality of crops. In view of climate change this may be important for the supply of human food resources for a growing population, if the increase of temperature systematically affects food quality.

The objective of this study was to determine the effects of different growth temperatures on plant growth parameters and nutritionally relevant phytochemicals in different varieties of lettuce and carrots.

Plants of two varieties each of carrots and lettuce were grown in controlled environments at different day/night temperatures: 12/8, 17/13, 22/18, 27/23 and 32/28 °C for lettuce and 12/8, 17/13, 22/18 and 27/23 °C for carrots. Each temperature treatment was applied at a separate time and the plants were harvested after having produced 10-12 leaves (lettuce) and 6-8 leaves (carrots). The light was constant 150 $\mu\text{mol}/\text{m}^2/\text{s}$ with an 11-hr-light/13-hr-dark cycle, approximately corresponding to winter outdoor conditions in Southern Europe.

Growth parameters such as number of leaves, plant height, leaf area of lettuce, fresh and dry weight were recorded. The sugar levels, nitrate concentration, phenolic compounds, carotenoids and chlorophyll pigments were determined in leaves and root of both species, in addition to polyacetylene compounds in carrot roots.

The results showed that temperature had a highly significant effect on growth parameters. The lowest temperature (12/8 °C) produced the highest dry matter content of both shoots and roots; the biomass of both species peaked at 17/13 °C and the leaf area of lettuce was greatest at 22/18 °C. The rate of leaf production was more rapid at higher temperatures than below 18 °C. Lettuce varieties responded more positively to high temperature than the carrots. Carrot root growth was inhibited with increasing temperatures above (22/18 °C).

Concentrations of phytochemicals were also affected significantly by growth temperature. Polyacetylenes in carrot roots increased by at least 50 % when grown at the lowest temperature of 12/8 °C. The accumulation of phenolic compounds in both carrots and lettuce leaves was associated with both low and high temperatures over the range tested, while the levels at 22/18 °C were only 3-50 % of the highest values. High temperatures (17, 22, 27 °C) also more than doubled nitrate contents, particularly in lettuce. In contrast there were higher levels of sugar in plants grown at low temperature compared with high temperature. Temperatures above 22 °C increased accumulation of chlorophylls in lettuce leaves. Contents of carotenoids (lutein, α -carotene and β -carotene) were relatively unaffected by temperature, although contents of β -carotene and lutein in lettuce leaves were slightly higher at 22/18 °C than other regimes. Growth temperature had a greater effect on the composition of carrots and lettuce than variety (genotype).

The directions of the effects on composition were as expected from the physiological mechanisms involved. However for most of the measured compounds, the present study is the first to determine magnitudes of the effects. Such information could be useful to growers as it may help them to make decisions about variety choice or fertilizer application rates dependent on the temperature regimes experienced or applied during growth.

These effects could become increasingly important aspects as climate change and global warming proceeds with potentially substantial implications for the links between diet and human health.

ACKNOWLEDGEMENTS

This research project was financially supported by the Higher Education Ministry of Libya. I would like to show my gratitude to both my supervisors, Dr Kirsten Brandt and Dr Stephen Wilcockson for their helpful advice during the course of this project.

Many thanks to technician team: Wendy Bal, Fiona Maclachlan, Peter Shotton, Chris Bulman, Brian Brown and Steven Hall for their support, help with laboratory work and health and safety. Also I would like to thank all the staff members in the School of Agriculture, Food and Rural Development for their cooperation.

Finally, but not least my sincere thanks to my husband, my mother and brothers for their encouragement, support and endless prayers.

Abbreviations used

Abbreviation Formatting	Description
NO ₃	Nitrate
Temp	Temperature
Var	Variety
Chla	Chlorophyll a
Chlb	Chlorophyll b
Abs	Absorption
Car	Carotenoids
Cx+c	Total carotenoids
FaDOH	Falcarindiol
FaDOAc	Falcarinol 3- acetate
FaOH	Falcarinol
α- Carotene	Alpha carotene
β-Carotene	Beta carotene
Lut	Lutein
DW	Dry weight
FW	Fresh weight
CP	Carrot variety Parmex
CLF	Carrot variety Little Finger
LD	Lettuce variety Dixter
LE	Lettuce variety Exbury
hr	Hours
HPLC	High Performance Liquid Chromatography
RT	Retention time
GLM	General Linear Model
SEM	Standard Error of Mean
L A	Leaf area
Con	Concentration
H ₂ O	Water
TFA	Trifluoroacetic acid
CAN	Acetonitrile
3-CQA	Chlorogenic acid (3-Caffeoylquinic acid)
C3G	Cyanidin 3-glucoside
NEDD	N-(1-Naphthyl) ethylenediamine dihydrochloride

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CHAPTER 1 . INTRODUCTION AND LITERATURE
REVIEW

1. Introduction

Temperature is an environmental factor that has large effects on growth, development, yield and quality of food crops. Currently, relatively little is known about the extent to which temperature influences the phytochemical composition and concentration of plant tissues. However, many researchers have previously shown the major importance of phytochemicals in the human diet. Some phytochemicals have beneficial health effects and may help to resist diseases such as some forms of cancers, whilst others may actually encourage disease.

Clearly therefore, the effects of temperature on growth yield and phytochemical content of important vegetable crops such as lettuce and carrot, which have high nutritional value and are widely consumed, may have very important implications. This is becoming more significant in view of a) global warming – temperature changes and stresses will undoubtedly influence plant composition - and b) the increasing interest in the link between diet and human health. Appropriate investigations to improve our knowledge and understanding of the inter-relationships should make it possible to identify the impact of increasing temperature on the functionality of these foods.

The research programme reported in this thesis aims to investigate the effects of different temperature regimes, on a) The growth and development of lettuce and carrot in growth chambers, b) Concentrations of different components such as sugar content, chlorophyll, nitrate, carotenoids, phenolic acids, and polyacetylene compound levels. These are important characteristics in terms of food quality, and may be manipulated to advantage by growing plants at appropriate and desirable temperatures.

Carrot and lettuce were chosen for this project, because both are widely cultivated and consumed around the world, and have high nutritional value. On a more practical note, these two species were easy to manage as test plants by growing them in modules in growth chambers over relatively short periods of time.

1.1 Plant growth and development

The growth environment of plants, particularly the combination of solar radiation, water supply and temperature affects their survival, development, productivity and quality (Stringer and Dennis, 2000). The combination of biotic (associated with living-organisms' functions, processes and interactions) and abiotic (physical) factors (e.g. temperature, water supply) affect plants from the molecular to the whole-plant level. Therefore, an understanding of the different balances of these different types of factors is necessary to allow a precise analysis of the plant condition in different growth environments (Rojdestvenski *et al.*, 1999; Hirt and Shinozaki, 2003; Souza and Cardoso, 2003; Pinheiro and Chaves, 2011; Vítolo *et al.*, 2012)

The climatic conditions play a significant role on plant growth, yield, maturation and quality, (Chormova, 2010). Moreover, Khoo *et al.* (2011) have reported that the growing conditions, maturity stage, carotenoid concentration and food processing are considered to be the primary factors affecting the compositional characteristics of vegetables and fruits.

Temperature, light intensity, CO₂, humidity, water and mineral supply are abiotic factors and are the main environmental factors that can affect a plant's development. At sub-optimal levels, stress occurs with negative impacts on the plant, (Galindo *et al.*, 2007). Most of the development processes of plants and their rates, are controlled primarily by temperature, while other environmental factors have less marked effects (Fitter and Hay, 2002). Moreover responses to environmental changes differ between the cultivated species, especially between C₃ and C₄ plants, (Vitollo *et al.*, 2012).

Vegetables are an important part of the human diet and increasing their consumption is highly recommended. They are a major source of dietary fibre and micronutrients, including, the antioxidant compounds, such as carotenoids and polyphenols: 70-90 % of carotenoids are provided by fruits and vegetables in the diet (Granado-Lorencio *et al.*, 2007; Singh *et al.*, 2012). In their raw state, alliums (onions and garlic), green vegetables, carrots and tomatoes are known to be protective against cancer, (Barbosa-Filho *et al.*, 2008). However, there are large variations in antioxidant contents (phytochemical) between the different fruits and vegetables, which reflect species differences in response to the environmental conditions under which the crop is produced (Hayashi *et al.*, 2012).

The World Health Organization (WHO, 2003) recommends that at least five portions of fruit and vegetables are eaten per person day (80 g is a typical portion, resulting in $5 \times 80 = 400$ g per day and 2800 g per week). However the consumption rate in England is still below the required levels (Doyle and Hosfield, 2001), at about 3 portions a day. This represents a low daily intake according to UK government recommendations with an increased risk factor of contracting certain diseases as a consequence (Block, 1992; Sanders, 2007). However, Figure 1.1 below shows an average household's fruit and vegetables consumption in the UK during the period (1990-2010). According to (UK household purchased quantities of food and drink, 2011), it shows an increasing rate of fruit consumption from 1990-1998, increasing and decreasing until 2004 then increasing to the highest consumption level (1313g/person/week) in 2006 before decreasing again until 2010. Vegetables also show variable consumption levels during the ten years while 1990 -1992 had the highest consumption rate (~1200 g/person/week). One of the top ten factors affecting the global mortality rate is the low consumption of fruit and vegetables (WHO, 2003).

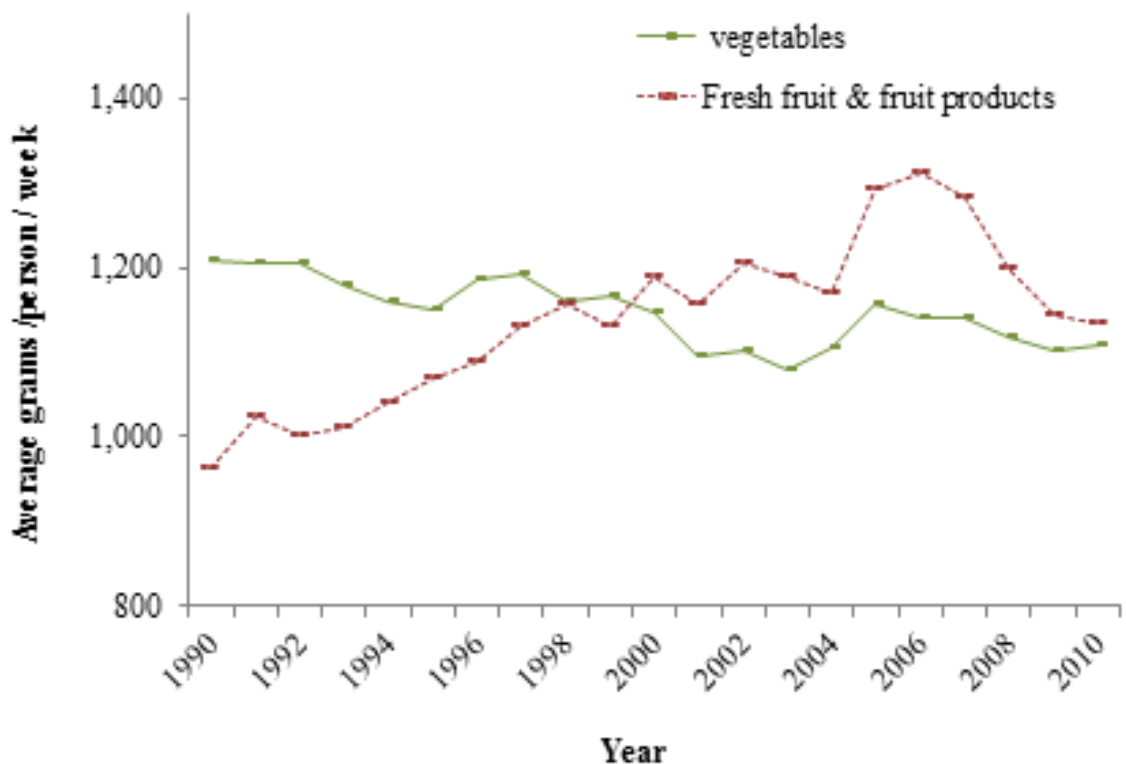


Figure 1.1 Average household consumption of fruit and vegetables 1990-2010 in the UK, UK household purchased quantities of food and drink (2011).

1.1.1 Carrot (*Daucus carota* L)

The carrot (*Daucus carota* L.) storage root vegetable belongs to the Umbelliferae group. In many countries it is considered a primary vegetable in a person's diet all-year-round (Nicolle *et al.*, 2004; Gajewski *et al.*, 2010; Singh *et al.*, 2012). It has a pleasant flavor and contains a high concentration of minerals, vitamins and fibre which provide health benefits (Alasalvar *et al.*, 2001). Over the past few decades the consumption of carrots has been increasing in the U.S (Lucier and Lin, 2007) and the UK (Wright, 2005) relative to most other commonly consumed vegetables. Annual carrot production equates to approximately 28 million tonnes globally (FAOSTAT, 2009).

Carrot crops are grown in temperate and subtropical regions (Macko and Grzebelus, 2008). They are direct seeded (not transplanted) and establishment is sensitive to both low or high temperatures which can cause poor stands and low yields (Nascimento *et al.*, 2008). Carrots have a deeper root system than almost all other vegetables (Thorup-Kristensen, 2006) and require deep fertile soil with good water holding capacity, free of stones and loose consistency for optimum development (Munro and Small, 1997).

There are different sizes and shapes of carrot roots such as cylindrical or tapering roots. The Nantes, conical Chantenays, Autumn King and Berlicum varieties are the main types in the UK, (Stringer and Dennis, 2000). The common varieties of carrot available in the United States are Emperor (most common), Nantes, Chantenay and Danvers (Lucier and Lin, 2007). They can be eaten raw or processed into a range of foods, including, cooked, canned, frozen and dehydrated products (Kreutzmann *et al.*, 2008).

Unlike some other vegetables, an important feature of carrots is the possibility of being harvested at different times during the growth cycle to achieve high prices, depending on the size and conditions of the market. The crop may be harvested early when roots are small although it is often harvested when it has grown to its full size and may be stored for long periods prior to consumption (Martin *et al.*, 2009).

In general, concentrations of bioactive compounds found in the storage roots of carrots, which are the basic content for both human nutrition and taste, are mostly affected by growing conditions and crop agronomy (Singh *et al.*, 2012). The carrot contains a number of mineral elements and other micronutrients (Nicolle *et al.*, 2004).

The phytochemicals include anthocyanin, phenolic acids and carotenoids, which are responsible for various colours in carrot varieties such as orange, yellow, red, and

purple and white. However, the colour of some plants is determined by the concentration of phytochemicals.

High concentrations of α - and β -carotene present in carrots gives the characteristic orange colour, while increased lycopene gives the red colour. Yellow carrots contain increased lutein concentrations while white carrots have lower concentrations of all carotenoids, as observed by (Poudyal *et al.*, 2010). The genotype, plant development (growth stages) and growth temperature are the main influences that determine the colour of the carrot (Nicolle *et al.*, 2004).

However, Nicolle *et al.* (2004) conclude that there are high correlations between the carrot root colour and carotenoid levels, with high carotenoid concentration correlated (associated) with the dark orange carrot roots.

Krinsky and Johnson (2005) have reported that the β -carotene concentration of raw carrots and cooked carrots are 18.3 and 8.0 mg/100 g fresh weight respectively. This provides an indication of the effect of food processing on carotenoid concentrations.

1.1.2 Lettuce (*Lactuca sativa* L)

Lettuce is an important worldwide, dietary, leafy vegetable that is primarily cultivated and consumed as a fresh product or in salad mixes. It is an important source of phytonutrients, (Liu *et al.*, 2007; Khoo *et al.*, 2011; Cruz *et al.*, 2012). Lettuce is an annual herbaceous plant belonging to the Compositae (Asteraceae family). It is abundant and there are many types that probably originated in Mediterranean areas. Now it is commercially distributed throughout the world. Within the European Union, lettuce is the fourth most important vegetable crop in a human's diet (Boo *et al.*, 2011; Baslam and Goicoechea, 2012), but fifth in the United States (Lucier and Jerardo, 2005). There are different types of lettuce cultivars such as head romaine (cos) and leaf (loose), with diverse colours, shapes and textures, (Hedges and Lister, 2005; Koike *et al.*, 2006). Specific types of lettuce grown in the UK are called round, cos, iceberg, baby leaf, little gem and coloured lettuce. Green leafy vegetables are a good source of vitamins and minerals, (Raju *et al.*, 2007) and lettuce particularly so. In addition to these nutrients, the phenolic antioxidants are one of the most important components present in lettuce (Romani *et al.*, 2002). Whereas the most important pigments which most lettuce cultivars contain are β -carotene, lutein, zeaxanthin, green cultivars contain chlorophyll, and red cultivars anthocyanin, (Hedges and Lister, 2005).

It has been shown by (DuPont *et al.*, 2000; Manach *et al.*, 2004) that the phenolic components in lettuce are probably affected by genotype, crop agronomy and environmental factors. Since lettuce is shallow rooting, irrigation is required for improvement of growth and development (Baslam and Goicoechea, 2012). Lettuce is a cool temperature crop, growing best within a range between 10-20 °C, (Joy and Roger, 2003) although Kimball *et al.* (1967) report that the optimum temperature ranges are 17-28 °C day temperature and 2-11 °C night temperature. However, lettuce seeds fail to germinate at 25 °C, as they are small-seeded and hence they rapidly dehydrate, (Fountain and Bewley, 1976). According to FAOSTAT, (2010) the production of lettuce and chicory was 133,900 tonnes in the United Kingdom and 4,105,580 tonnes in the United States of America respectively in 2010.

1.2 Abiotic environmental stress of plants

Temperature (low or high), drought, light, and salinity are among the common environmental factors that cause abiotic stresses, (Rao *et al.*, 2006; Toivonen and Hodges, 2011). Abiotic stresses are considered to be major factors that negatively affect plant growth and productivity at different stages of growth (Levitt, 1972; Haferkamp, 1987; Gao *et al.*, 2007; Hodges and Tolvonen, 2008). Moreover, prolonged exposure to stress can cause plant death, (Rao *et al.*, 2006). According to Cramer *et al.* (2011) it is the sub-optimal environmental conditions that cause abiotic stresses which can cause a reduction of plant growth and yield

Plant growth, productivity and many morphological, physiological and molecular processes respond and adapt to abiotic stresses through various mechanisms (Cao *et al.*, 2011; Sanghera *et al.*, 2011). For example, plants exposed to abiotic stress may stimulate some genes to initiate a metabolic process and hence increase protein levels to provide protection against these stresses, (Sanghera *et al.*, 2011). Various plant species differ in their response to abiotic stress which may have more damaging effects at certain plant growth stages (Jones *et al.*, 1989) .

1.2.1 Effect of temperature on plant growth and development

Temperature is one of the major ecological abiotic factors that limits the geographical distribution of plants (Berry and Bjorkman, 1980; Kopsell, 2010) and has an effect on plant growth, development and function (Morison and Lawlor, 1999) with important implications for agricultural crop production. Furthermore, the genetic adaption of plants to different temperature regimes is considered a key factor to growth and survival of a species in their environment, (Criddle *et al.*, 1997). For each plant species there are defined minimum (below 0 °C), optimum and maximum (above 40 °C) temperatures for growth (Figure 1.2). Therefore the temperate and tropical plant classification is based on the response to different temperatures. On this basis the overall physiology component processes can be varied in response to temperature and thus determine the net photosynthesis (Fitter and Hay, 2002; Qin *et al.*, 2007).

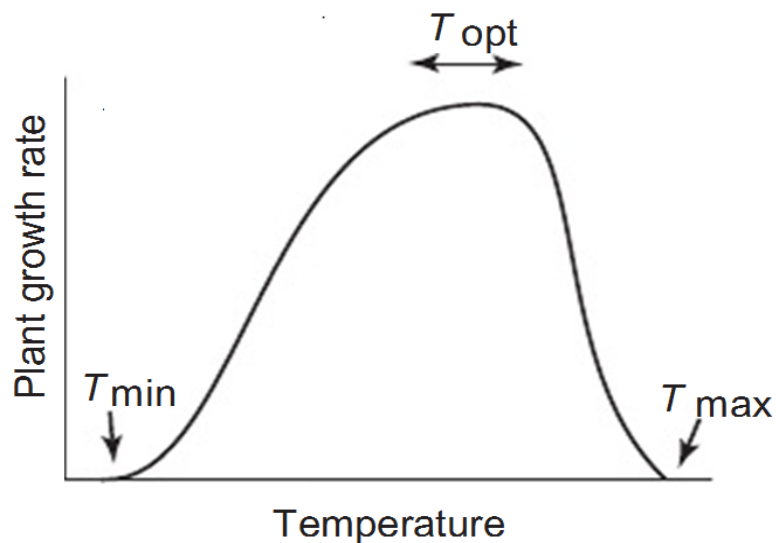


Figure 1.2 Plant growth rate responses to minimum (T_{min}), optimum (T_{opt}) and maximum temperatures (T_{max}), (Fitter and Hay, 2002).

Temperature is considered to have the most important influence on sensory and chemical aspects of crop quality although light seems to have the main effect on morphological growth parameters such as total leaf and root dry weight, leaf area, length and diameter of roots, additionally to light intensity effects, (Albayrak and Çamas, 2007; Seljasen *et al.*, 2012). However, whatever the pattern and timing of plant

development, the temperature parameter may be key for these processes, (Fitter and Hay, 2002).

To achieve high productivity and good quality crops the whole plants require optimal day and night temperatures, (Kimball *et al.*, 1967) whilst sub-optimal temperatures restrict the processes of plant growth and development to some extent, (Vítolo *et al.*, 2012). The temperature range of plant growth is from 0 to 40 °C (Figure 1.2), and within this range the performance of most physiological processes behaves normally, while very high and very low temperatures ranges can cause injury effects, (Went, 1953). The main functions that are essential to plant growth and development are photosynthesis, respiration and transpiration (Llorach *et al.*, 2008). Photosynthesis is strongly affected by temperature regimes and typically is near to maximal within a range of moderate temperatures (15-30 °C) for most temperate species and plants (Atkinson *et al.*, 2010). Due to the effect of enzymatically catalysed reactions of photosynthesis processes (Lambers *et al.*, 2008), there is an instant reduction in net CO₂ exchange in light, caused by the exposure to high (Seemann *et al.*, 1984) or low temperatures (Berry and Bjorkman, 1980). The change of net photosynthesis with temperature has been summarized by (Fitter and Hay, 2002). Below 0 °C and above 40 °C gross photosynthesis stops, at high temperatures due to the increasing rate of respiration, and at intermediate temperatures < 20 °C respiration rates are slightly lower than at higher temperatures. Different plant species respond differently to the environmental variables, however many may differ in their ability to resist temperature extremes during their different growth stages. During each stage of growth and development a plant has its own specific optimum temperature range that differs with different species, as well as with a plant's age, (Agrios, 2005).

1.2.1.1 The influence of low temperature on plants

Plant growth processes such as photosynthesis, water transport, survival, cell division and yield are affected by low temperature. Therefore low temperature is an abiotic factor that affects plant growth and productivity, (Gray *et al.*, 1997; Badea and Basu, 2009; Sanghera *et al.*, 2011). Plant metabolism rates, growth and development slow down at low temperatures (Fitter and Hay, 2002). Two stress conditions of low growth temperature are chilling (< 20 °C) and freezing (< 0 °C) and are classified under cold stress. This can have in some cases significant negative effects on growth and

development of plants and thus plays a key, limiting role in plant productivity and distribution (Chinnusamy *et al.*, 2007; Badea and Basu, 2009). In contrast, some crop genotypes exhibit cold tolerance and have the ability to survive and perform considerably better than some other less tolerant genotypes under low temperature conditions, (Sanghera *et al.*, 2011).

At low temperatures in the range 1-12 °C, several plant species are either injured or killed, whilst other species appear to be more tolerant to low temperatures and can survive below 0 °C (Lyons, 1973).

Agrios (2005) shows that plants affected by low temperature, deteriorate or/and may at times stop growing when the temperature becomes near to, or in, the freezing range. On the other hand, the ability of plants to withstand temperature differs between growth stages: generally, young seedlings are less able to resist cold temperatures than older plants. Moreover, different organs of the same plant type may have a different ability to resist low temperatures, (Badea and Basu, 2009). The response of plants to cold stress has different phenotypic symptoms as described by (Yadav, 2010; Sanghera *et al.*, 2011). These symptoms are poor germination or growth retardation, stunted growth of seedlings, chlorosis (yellowing leaves) and wilting. However, the primary injury effect of cold stress occurs in the cellular membranes and this damage leads to dehydration. Protection of the plant membrane's stability is a common defence mechanism and the first reaction to exposure to cold stress is via changing of membrane lipid composition by the increase of fatty acid unsaturation rate, (Badea and Basu, 2009; Sanghera *et al.*, 2011). In addition to causing membrane injury, cold stress damages the chloroplasts and decreases photosynthesis, (Sanghera *et al.*, 2011) but the extent of the adverse effects depends upon the intensity and duration of the stresses, (Jan *et al.*, 2009).

It has been demonstrated that (Chinnusamy *et al.*, 2007) plant metabolic reactions are affected both directly and indirectly by low temperature. The membrane fluidity, water and nutrient absorption and DNA formation represent the direct effects, these reduce the rates of biochemical reactions immediately or by an indirect effect through the genetic change over the much longer term. One way of responding to cold stress is the expression of genes that represent increasing a number of metabolites, some of which are known to act as protection factors against damage caused by cold stress, (Sanghera *et al.*, 2011).

1.2.1.2 Effects of high temperature on plant growth

The other abiotic stress that belongs to extreme temperatures and has an unfavourable effect on plant growth and which is often associated with deficiency of water is high temperature. This is due to the increased demand of evaporation through transpiration. In general, growth occurs within the temperature range 0-40 °C, although the optimum growth occurs between 15-30 °C. However, when the temperature rises above the maximum, plants deteriorate more rapidly than when the temperature is lower than the minimum, (Agrios, 2005). Shahrzad and Bitsch (1996) and Schwarz *et al.* (2010) show that the metabolic rates of a plant increase exponentially with temperature above 25–30 °C. The complex process resulting from high temperature depends on duration, intensity and the range of change temperature. However, photosynthesis, respiration, membrane stability, hormone levels, and thus metabolites (primary and secondary) are possibly adversely affected, (Wahid *et al.*, 2007). Heat stress effects on photosynthetic reactions are the most sensitive processes (thermosensitive) of a plant function, which may cause growth limitations due to extremely high temperatures, whereas the damage to photosynthetic electron transport is one of the primary consequences caused by high temperature stress (Seemann *et al.*, 1984; Taub *et al.*, 2000).

Carrots grown in high temperature (mainly in the summer) may establish poorly and show limited growth and development (Nascimento *et al.*, 2008). Although the growth of lettuce root is inhibited and the root diameter increased when grown at high temperature e.g. 38 °C, this is due to the limitation of enzymes metabolism activity (Qin *et al.*, 2007).

The length of the germination period and germination percentage for carrot genotypes differs when grown in high temperatures (Nascimento *et al.*, 2008). The germination period decreased as temperatures increase up to 20 °C, whilst germination percentage decreased with an increase of temperature for most seed cultivars of carrot grown at 35 °C, (Pereira *et al.*, 2007). Both the genotypes and environmental conditions control performance of lettuce germination in high temperatures when the temperature increases above 28 °C, (Sung *et al.*, 1998). This difference in ability of lettuce to grow in high temperatures led to two groups being identified: thermotolerant and thermosensitive, (Kozarewa *et al.*, 2006).

1.3 Phytochemicals

Phytochemicals are natural chemical bioactive compounds present in fruit and vegetables. They make an important nutritional contribution to the human diet, providing health benefits (McCann *et al.*, 2005). The levels of phytochemicals in various vegetables and fruits change over pre and post-harvest stages, between raw and processed states and according to the colour of vegetables:- dark coloured vegetables contain higher levels compared with paler coloured vegetables (Tiwari and Cummins, 2011). Also several plant foods have very low levels of phytochemicals or none at all. On the basis of their chemical structure, function and source, phytochemicals have been classified in many fruit and vegetables, (Tiwari and Cummins, 2011). In excess of more than 100 different phytochemicals have been found in plant food. Among these compounds are phenols and polyphenol, Bioflavonoids, Carotenoids, Flavonoids and Isoflavones distributed in a wide range of food plants (Figure 1.3). They are characterised by their numerous health benefits (Hark and Deen, 2005), due to the phytochemicals' functions that act as antioxidants, (Lv *et al.*, 2012), that are reported to have potential health effects such as reducing cancer risk (McCann *et al.*, 2005). Many researchers agree that the concentration of phytochemicals has strong correlations with various factors including genotype, environmental conditions, agricultural practices, food processes and storage conditions, (Tiwari and Cummins, 2011).

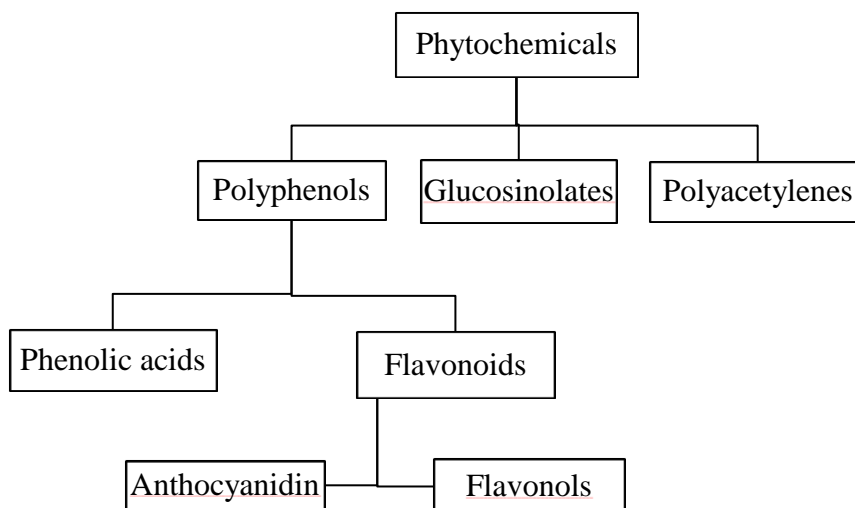


Figure 1.3 Main classification of phytochemicals according to (Tiwari and Cummins, 2011).

1.3.1 Carotenoids

A very important group of pigments in fruit and vegetable-based products are called carotenoids, (Knockaert *et al.*, 2012). Carotenoids are widely distributed with a significantly structural range and various functions. More than 600 carotenoids have been isolated and characterised from natural sources, with around 40 found in the normal human diet. The simple identification of carotenoids are lipid-soluble (insoluble in water) pigments present in all photosynthetic organisms: these are considered bioactive substances in foods for their antioxidant activities, (Paiva and Russell, 1999; Kopsell and Kopsell, 2006; Marinova and Ribarova, 2007).

The central location that extends the conjugated double-bond system is considered to be the important characteristic which constitutes the light-absorbing chromosphere. This gives the colour of carotenoids as well as providing the visible absorption spectrum that is determined through their identification and quantification, (Hendry and Houghton, 1996; Rodriguez-Amaya and Kimura, 2004). Carotenes act as a precursor for vitamin A, which is the most important antioxidant known to play a role in the prevention of certain diseases, (Singh *et al.*, 2012). The absorption spectrum (λ) of carotenoids ranging from 400 -500 nm, and the number of conjugated double bonds determines the absorption, (Berg *et al.*, 2000). Carotenoids are classified into two main groups: the first is called carotenes (hydrocarbons) such as α - and β - carotene and lycopene, while the second group is xanthophylls (oxocarotenoids) that contain an oxygen atom in their molecule structure such as lutein, zeaxanthin (Rodic *et al.*, 2012). Due to the carotenoid pigments and anthocyanin present in the carrot, these create different colours such as orange, red, purple, black and white, produced in roots during maturation and are present in high concentrations in carrots, (Kjellenberg *et al.*, 2012; Knockaert *et al.*, 2012; Singh *et al.*, 2012). Numerous studies have been focused on carotenoids due to their important biological functions for humans and also as a natural pigment (Pinheiro-Sant'Ana *et al.*, 1998). There is a wide abundance of carotenoid pigments in many fruits and vegetables in diverse forms involved in photobiology, photochemistry and photomedicine (Dutta *et al.*, 2005). Many epidemiological studies have shown clear correlation between the rich sources of carotenoids provided by vegetables and fruit in fresh, frozen and canned food products and cardiovascular diseases and incidence of cancer, (Mueller, 1997; Rickman *et al.*, 2007). The quality and the quantity of carotenoid composition differ. The leafy and non-leafy green vegetables are known to

contain major carotenoids such as lutein, β -carotene, violaxanthin, and neoxanthin, in addition to small concentrations of other carotene like α -carotene, zeaxanthin (Rodriguez-Amaya and Kimura, 2004).

Hendry and Houghton (1996) considered the pigments to be quite large molecules, however the molecular weights of most common pigments range from 200, 300, 400, 500 to 800 Daltons for anthraquinones, anthocyanidins, betalaines, carotenoids and chlorophylls respectively. As previously mentioned carotenes concentration is mainly influenced by genotype/cultivar, production area, and other environmental factors, biotic (e.g. oxidation) and abiotic (e.g. temperature). Due to the structure of carotenoids and lipophilic features, during their exposure to biotic or abiotic factors the molecules of carotenoids, oxidation and isomerization degradation may occur that affects the biochemistry and bioavailability of carotenoids, (Baranska and Schulz, 2005; Kopsell and Kopsell, 2006). Within the plants, the process of biosynthesis of carotenoids occurs as per the following sequence: - phytoene \rightarrow phytofluene \rightarrow ζ -carotene \rightarrow neurosporene \rightarrow lycopene \rightarrow γ -carotene \rightarrow β -carotene as observed by (Paiva and Russell, 1999). The carotenes have a similar chemical structure in plants, bacteria and protists but the synthetic make-up of carotene is different during the primary steps between different organisms (Kim and DellaPenna, 2006).

Table 1.1 Main carotenoids identified in carrot and lettuce (Nicolle *et al.*, 2004; Larsen and Christensen, 2005; Kopsell and Kopsell, 2006).

Food plant	Carotenoids identified
Carrot	All-trans β -carotene (highest concentration), all- <i>trans</i> α -carotene, lutein and lycopene.
Lettuce	All- <i>trans</i> β -carotene, lactucaxanthin, all-translutein, 9- <i>cis</i> lutein, 9'- <i>cis</i> lutein, 13- <i>cis</i> lutein, all- <i>trans</i> and <i>cis</i> lutein epoxide, neolutein, all- <i>trans</i> neoxanthin, 9'- <i>cis</i> neoxanthin, violaxanthin, all- <i>trans</i> zeaxanthin, 9- <i>cis</i> zeaxanthin and 13- <i>cis</i> zeaxanthin.

1.3.1.1 Beta Carotene (β -Car)

α - and β - carotene shown in Figure 1.4 are the main carotenoid compounds contained in fruits and vegetables, and most abundant in carrot roots that are responsible for the orange colour (Gajewski *et al.*, 2010). β -carotene is located in the chromoplasts (surrounded by a double bilayer membrane) of the plant cells (surrounded by a cell membrane and a cell wall), where it is often associated with proteins and/or residual membranes, as found in chromoplasts of carrot (Knockaert *et al.*, 2012). Due to the ability of β -Carotene to convert into a vitamin A, it is considered to be one of the most important compounds that has health benefits, (Krinsky and Johnson, 2005).

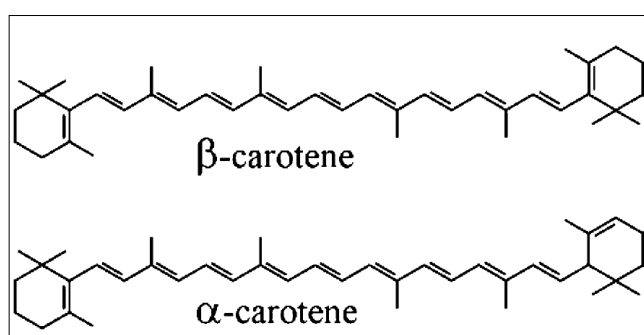


Figure 1.4 Chemical structures of β - carotene and α - carotene. (Baranska *et al.*, 2005).

The other derivatives of β -carotene are present in many vegetables and fruits, but are usually found at low rates compared to *trans*-isomer (Khoo *et al.*, 2011). They are *Cis*-isomers (9, 13 and 15-*Cis*- β -carotene) (O'Sullivan *et al.*, 2010) and exist in two forms either naturally present in the matrix of food, or generated as solubilised substance as a result of diverse food processing. There is a high correlation between the high levels of β - carotene in blood and the low rate of some types of cancer spreading as shown in several Epidemiological studies,(Christensen and Brandt, 2006). There were highly correlated values between the intake of α and β - carotene and carrot consumption in North America and European countries (Christensen and Kreutzmann, 2007).

Observations by (Mueller, 1997) indicate that the β -carotene concentration in lettuce 1.29 mg/100g FW and for large, medium and young size carrots was 9.02, 6.50 and 4.65 mg/100g FW respectively. In contrast (Ben-Amotz and Fishler, 1998) found that lettuce contained 104 μ g/g DW of β - carotene among 198 μ g/g DW of total carotenoids while

carrot contained 1030 $\mu\text{g/g}$ DW of β - carotene among a total 1608 $\mu\text{g/g}$ DW of other carotenoids.

1.3.1.2 Lutein

Lutein (3*R*, 3'*R*- β , ϵ -carotene-3, 3'-diol) belongs to xanthophyll carotenoids (hydroxyl-carotenoid) and a rich source is the photosynthetic apparatus of higher plants (Dall'Osto *et al.*, 2006). The lutein in chemical terms is a Dihydroxy derivative of Alpha-carotene (β -ring and ϵ -ring) mainly playing roles in light harvesting complex II (LHCII), structure and function, while the synthesis process of lutein occurs by the sequence of four enzymatic reactions of lycopene, (Kim and DellaPenna, 2006).

Kijlstra *et al.* (2012) have described the chemical structure of lutein as a long carbon chain with a single followed by double (carbon-carbon bonds) connected with methyl groups. Furthermore cyclic hexenyl with hydroxyl groups are both ends of this carbon chain, whereas the site of the double bonds in the hexenyl ring and the site of methyl groups on this chain are considered to be the difference between the lutein and zeaxanthin in terms of chemical structure. Two significant functions of lutein in plants and humans are present, the first function acts as a filter of blue light energy and as an antioxidant that is caused by reactive oxygen species (ROS), that were described by (Alves-Rodrigues and Shao, 2004). Lutein is the most common of carotenoids present in green vegetables, leaves, (Kayser, 2012) egg yolks (Johnson, 2002) and flowers, whereas lutein accounts for about 45 % of carotenoids in leaves, (Rodriguez-Amaya, 2001). The lutein appears in food supplements in two forms, either as free lutein (green leafy vegetables) or a mixture of lutein diesters with saturated high fatty acids as in flowers (Maci, 2011; Rodic *et al.*, 2012). All-trans-lutein, cis-lutein, epoxy-lutein, and lutein associated to proteins represent the different forms of lutein found in vegetables and fruits according to (Calvo, 2005). Lutein is either a yellow colour compound (Calvo, 2005) or orange depending on the concentration (Kijlstra *et al.*, 2012). The main dietary source of lutein is peas in the UK and Republic of Ireland (O'Neill *et al.*, 2001).

1.3.2 Chlorophylls structure and function

Chlorophyll is originally a Greek word composed of two sections green (*chloros*) and leaves (*phyllos*) (Scheer, 2006). Chlorophylls are almost the only natural green plant pigment and definitely the only ones in great abundance. Photosynthetic activity in organelles such as chloroplasts mainly depends on chlorophylls (mostly) and carotenoids (as accessory pigment). Therefore the chlorophylls act as an absorbent for light energy for the transfer of energy (Hendry and Houghton, 1996; Liu *et al.*, 2012). Photosynthesis efficiency is affected by chlorophyll concentrations (Liang *et al.*, 2013). Li *et al.* (2012) have reported five different forms of chlorophylls and their discovery through the centuries until the current time. In the 19th century the following chlorophylls were identified: a, b and c (Govindjee and Krogmann, 2004), more than 70 years later, in 1943 chlorophyll d was identified (Manning and Strain, 1943). A new one was reported in 2010 called chlorophyll f, whose absorption corresponds to the red wavelength spectrum with a maximum absorption at 706 nm, (Chen *et al.*, 2010). All these chlorophylls types have different absorption properties. In the higher plants, more than 17 enzymes are required for the process of chlorophyll biosynthesis to occur (Tripathy and Pattanayak, 2012).

Generally, the chlorophylls contain the single Mg atom present in a central position and at C₁₇ is a connection with the long-chain esterifying alcohol, with a spectral range between 330-800 nm (Scheer, 2006). Figure 1.5, shows the chlorophyll structure Chlorophyll b structure is similar to chlorophyll a, except for the presence of formyl group (-CHO) instead of methyl group (-CH₃) on ring B of chlorophyll a, (Nobel, 2009). In most organisms that contain both chlorophyll a and b, the ratio between them is 3:1 (Nobel, 2009).

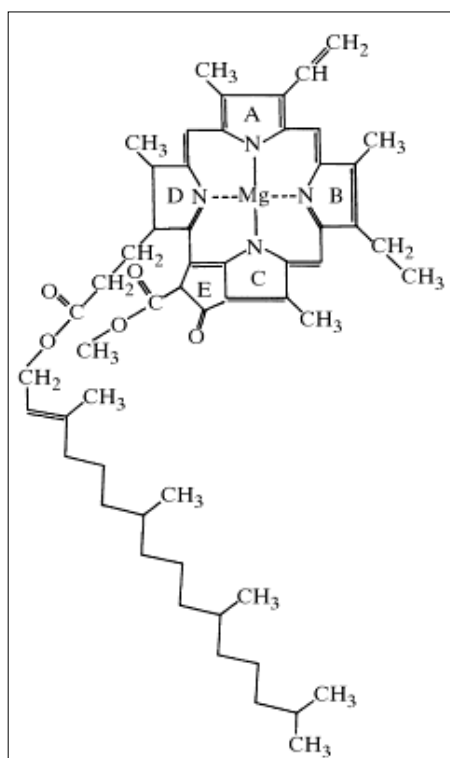


Figure 1.5 The chemical structure of chlorophyll a, (Nobel, 2009)

The chlorophyll pigments found in plants such as chlorophyll a (Chl a) and bacteriochlorophyll (BChl) (Tamiaki *et al.*, 2007), are from different groups of macrocyclic tetrapyrrole pigments, and play a major role in light-harvesting of photosynthesis (Scheer, 2006). Chlorophyll a is considered the most widespread and major green pigment in plants with a complex structure and is extremely reactive and sensitive (Woodward, 1961).

Light, temperature, oxygen conditions and chemical degradation are considered the most influential factors on the sensitive structure of chlorophyll, especially during the extraction process, (Pocock *et al.*, 2004). Due to the acquisition of chlorophylls and their unique optical properties (structure is relatively rare in nature and is likely to have a high photodynamic property) photosynthetic organisms have been adapt to different light environments (Scheer, 2006; Tsuchiya *et al.*, 2012).

1.3.3 Sugar content in plants

Sugars are organic compounds in plants resulting from photosynthesis, which play an important role in respiration by providing the energy. Sugars are condensed to store energy in the form of starch, whilst the energy is transported in sucrose form and sugars play a key role in cell wall structure, (Harborne, 1998). In addition to these functions, sugars perform a regulatory role in numerous mechanisms of plant growth and development (Rosa *et al.*, 2009). They aid control of plant metabolism and different stress responses during the whole growth stages from embryogenesis to senescence, involving a number of sugar signals that are generated depending on the surrounding environmental conditions (Rolland *et al.*, 2006).

Physiologically, the production and consumption of sugars are performed by plants, whereas the environmental abiotic stresses are some of the main factors that have an influence on soluble sugars due to their sensitivity. However, the soluble sugar levels increase in a plant with low temperature conditions (Rosa *et al.*, 2009). According to molecular size the sugars are divided into three groups: monosaccharides such as glucose and fructose, oligosaccharide and polysaccharides (Harborne, 1998). The main sugars present in carrot root are sucrose, glucose, and fructose in different ratios. The highest level for fresh carrot was for sucrose at 353 g/kg DM then glucose at 120 g/kg DM with the lowest value for fructose at 113 g/kg DM (Svanberg *et al.*, 1997). Gaweda (2007) observed that sucrose formed about 80 % of the total sugar of root vegetables and for carrot roots, they consisted of 50.60, 14.02 and 8.22 mg/kg FM for sucrose, glucose, and fructose. In contrast, for leafy vegetables including lettuce, fructose contributed the highest concentration (about 55 %) of total soluble sugars. In a lettuce plant sugar content changes during the late stages of growth and development as plants age and continues to increase. Furthermore, sugar levels increase from winter to summer when grown in greenhouses (Gent, 2012). This may be due to the light intensity during the growth of plants.

1.3.4 Plant nitrate accumulation

The consumption of vegetables is considered to be one of the most important sources through which the body obtains nitrate, (Rousta *et al.*, 2010). A total of 70-90 % nitrate intake is supplied by vegetables, (Food Standards Agency, 2001).

Vegetables accumulate nitrate in different parts of a plant to different extents, the leaves have greater levels (lettuce and spinach) of nitrate accumulated than either seeds or tubers. Moreover the concentrations of nitrate accumulation are affected by several abiotic and biotic factors, (Alexander *et al.*, 2008), among which are the growing conditions, season, temperature, light and fertilizer use (Dich *et al.*, 1996). Carrot roots have been classified as vegetables that contain low nitrate levels (200- 500 mg/kg fresh weight) according to (Santamaria, 2006).

Nitrate is one component of the human diet that is potentially harmful. Whilst it is considered a relatively non-toxic compound in its common form, the products resulting from its metabolism could be the cause of a number of health effects (Santamaria, 2006). Therefore, the European Commission adopted EC Regulation No. 1822/2005 which determined the maximum level of nitrate permitted in lettuce and spinach (Table 1.2) and this value depends on the season and harvest time. These levels should not be exceeded because they may cause possible health risks to humans.

Table 1.2 Maximum levels of nitrate according to European Commission (EC) regulation No. 1822/2005.

Vegetables	Harvest period	Maximum level (mg NO ₃ ⁻ /Kg)
Fresh spinach (<i>Spinacia oleracea</i>)	1 October to 31 March	3,000
	1 April to 31 September	2,500
Preserved, deep-frozen or frozen spinach		2,000
Fresh lettuce (<i>Lactuca sativa</i> L.) (protected and open-grown lettuce) Excluding “iceberg” type lettuce	1 October to 31 March	
	- grown under cover	4,500
	- grown in open air	4,000
	1 April to 30 September	
	- grown under cover	3,500
	-grown in open air	2,500
Lettuce (Iceberg type)	Lettuce grown under cover	2,500
	Lettuce grown in the open air	2,000

The data shown in Table 1.2 indicates that the vegetables grown during the winter season have potentially higher levels of nitrate than those grown during the summer. This could be due to exposure to sufficient light intensity in summer, as demonstrated by (Byrne *et al.*, 2004). In higher plants, the most important inorganic nitrogen components that the root system absorbs are nitrate (Ruiz *et al.*, 2000) and ammonium; (Lastra *et al.*, 2009) describes the part of the nitrogen cycle that plays a role in the function and nutrition of plants, (Alexander *et al.*, 2008).

The sufficient supply of nitrogen (N) is necessary for the formation of proteins, amino acids and nucleic acids upon which plant growth and development depends (Ruiz *et al.*, 2000). Whereas nitrate is assimilated by the plants in a sequential process by the reduction of the nitrate (NO_3) to nitrite (NO_2), it is then converted from NO_2 to ammonium (NH_4), before finally being incorporated into organic compounds (Migge and Becker, 1996; Sivasankar and Oaks, 1996). The observations by Frota and Tucker (1972) noted that an increase of air and root temperature lead to an increase of nitrate and ammonium absorption in lettuce. In addition Demsar *et al.* (2004) and Gent (2012) have demonstrated the agricultural application and use of nitrogenous fertiliser and low light conditions are associated with the accumulations of high concentrations of nitrate in lettuce.

There is a broad and diverse distribution of nitrate in a number of the important vegetables in the human diet. Nitrate concentration represents a side benefit to human health in terms of biological activity and its role in the immune system, (Bryan and Grinsven, 2013).

The detriments of nitrate concentration on human health are usually accompanied by toxic effects e.g. the formation of carcinogenic nitroso compounds, as one of metabolic products, (Bryan and Grinsven, 2013; Gorenjak and Cencic, 2013).

In humans it is known that the nitrite reacts with haemoglobin in the formation of methaemoglobin and nitrate. Therefore, this form of methaemoglobin can impede the delivery of oxygen to the tissues of the body, which can lead to death, (Bryan and Grinsven, 2013; Gorenjak and Cencic, 2013; Weitzberg and Lundberg, 2013).

1.3.5 Phenolic acids

The polyphenols are one of the major groups of plant metabolites and have many essential functions in different species of plant (Pandey and Rizvi, 2009). The phenolic acids are one of the four polyphenol categories in addition to flavonoids, lignans and stilbenes that are classified on the basis of chemical structure (Spencer *et al.*, 2008). Polyphenols have been identified and estimated at around 8000 polyphenolics as the main part of the human diet (Pandey and Rizvi, 2009). The benzoic and cinnamic acid derivatives are primary structures of phenolic acids distributed and dispersed throughout seeds, leaves and roots of plants. They are considered mainly for their many vital functions such as photosynthesis and structural components (Mendoza *et al.*, 2011) whereas the hydroxycinnamic acids are more abundant and contain a less complex structure than hydroxybenzoic acids, (Figure 1.6) (Manach *et al.*, 2004).

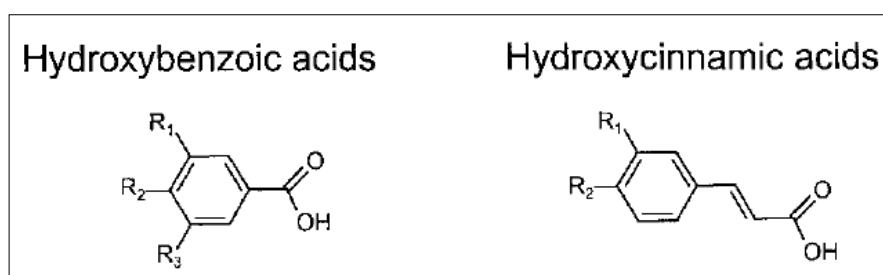


Figure 1.6 Chemical structure of two main phenolic acid classes (Manach *et al.*, 2004).

Naturally, the occurrence of phenolic compounds is in three forms that are ethers, mixtures and free acid, (Shahrzad and Bitsch, 1996; Marques and Farah, 2009), however, in plant food most of the phenolic acids are found in bound form. The caffeic, ferulic and p-coumaric acids that belong to the hydroxycinnamic group occur by way of the simple ester with quinic acid or glucose in food (Mattila and Hellstrom, 2007). Phenolic compounds have been found in vegetables especially in high concentration levels and in plants generally. For example in the UK the two main sources of phenolic acids present in lettuce are cichoric acid (dicafeoyltartaric) and caffeoylmalic acid with (5-15 mg) and (> 3 mg) per 100g respectively, as indicated by Clifford (2000).

They are considered secondary metabolites and most important antioxidants: thus they are necessary components in humans' nutritional diet (Jacobo-Velazquez and Cisneros-Zevallos, 2009; You *et al.*, 2011). Therefore, many research topics have shown the

relationship between phenolics and their ability to reduce the risk of some types of cancers and cardiovascular disease associated with the consumption of fruit and vegetables. Thus protection against some diseases and improvement of human health possibly depends on high levels of phenolics in plant food (Du *et al.*, 2012).

Temperature during growth is considered to be a major factor affecting phenolic acid concentration, in addition. But different growth stages and maturation of plants also have an effect (Ellnain-Wojtaszek *et al.*, 2001; Wang and Zheng, 2001). It has been concluded that increase in night temperature along with a constant day temperature can lead to a significant increase in the phenolic acid levels, as well as increasing flavonols, and anthocyanins in strawberry fruit (Wang and Zheng, 2001). However, the different stages of growth lead to a change of phenolic compounds: whereas at the ripening stage the phenolic acid decreases, in contrast, the concentration of anthocyanins increases, (Manach *et al.*, 2004; Spencer *et al.*, 2008).

1.3.5.1 Chlorogenic acid (CGA)

Chlorogenic acid is formed by a combination of caffeic and quinic acid which is present in high concentrations in numerous fruits, vegetables and coffee. It is one of the main antioxidant dietary polyphenols in the human diet. Chlorogenic acid is structured according to its identity, number and position of the acyl residue and can be divided into four sub-groups which are: mono-esters group (most common) of caffeic acid, p-coumaric acid and ferulic acid, di-esters (diCQA), tri-esters (triCQA) and the single tetra-ester group of caffeic acid, mixed di-esters group of caffeic and ferulic acid, and the fourth group is mixed esters, (Clifford, 2000). Chlorogenic acid or (3-O-caffeoylquinic acid/ 5-O-caffeoylquinic acid) is a phenolic acid abundant in carrots and has been identified in addition to other phenolics, (Kreutzmann *et al.*, 2008).

1.3.5.2 Caffeic acid

Caffeic acid is a natural essential phenolic compound in plants (Lin and Yan, 2012). It belongs to hydroxycinnamic acid derivatives found commonly in the plant kingdom. However, it is the most abundant of phenolic acids in fruits either in the free (seldom) or esterified form, that represents 75-100 % of hydroxycinnamic quantity present in fruit Manach *et al.* (2004), while coffee and beans are commonly considered the main

sources of caffeic acid production (Lin and Yan, 2012). In addition, Llorach *et al.* (2008) have shown that the caffeic acid derivatives are major phenolics found in green varieties of lettuce. Like other phenolics, the previous studies demonstrated that there are various biological activities and properties of caffeic acid which are represented in antioxidant activity (RiceEvans *et al.*, 1996), anti-virus (Ikeda *et al.*, 2011) and anti-cancer (Rajendra *et al.*, 2011). It is reported that caffeic acid has the ability to reduce the risk of chronic cardiovascular diseases (Park, 2009). In plants, caffeic acid is formed from p-coumaric acid and cinnamic acid through shikimic acid metabolism (Katsuragi *et al.*, 2010).

1.3.6 Polyacetylene compounds

The aliphatic C₁₇ -polyacetylenes are bioactive compounds belonging to the Apiaceae family, which has cytotoxicity against cancer cells, as many epidemiological studies have indicated (Kobæk-Larsen *et al.*, 2005; Christensen and Brandt, 2006). It may also exhibit antiallergenic effects (Wang *et al.*, 2001), and some polyacetylenes have anti-inflammatory activities (Resch *et al.*, 2001; Zidorn *et al.*, 2005). Additionally for beneficial effects on human health, polyacetylenes are characterised by several features which are of great importance for the plant due to their antifungal activities, resisting infection and pathogens (Mercier *et al.*, 1993; Baranska and Schulz, 2005). Some polyacetylenes e.g. falcarinol are classified as substances that have toxic properties when present at high concentration levels, and can become an undesirable substance in food (Baranska and Schulz, 2005; Christensen and Brandt, 2006). In higher plants, especially the widespread families such as Apiaceae, Araliaceae, and Asteraceae, different polyacetylenes exceeding 1400 compounds have been isolated. The three common components that have been found in carrot root are called falcarinol, falcarindiol and falcarindiol-3-acetate, (Rawson *et al.*, 2012), (Figure 1.7). They have also been found in other food plants such as celery, and parsley (Christensen and Brandt, 2006). In the carrot root, the falcarinol is considered the most abundant bioactive of polyacetylenes (Matsunaga *et al.*, 1990; Hansen *et al.*, 2003; Kobæk-Larsen *et al.*, 2005). Moreover, the concentration of polyacetylenes in the different parts of carrots has been investigated and there is a different ability for the accumulation in upper, lower, xylem and phloem parts of the root. The distribution of Falcarindiol

shows the highest concentration and maximum accumulation in the upper and phloem parts, with a smaller amount in the lower and xylem part. Falcarinol has a similar amount in a whole carrot, whilst the concentration of Falcarindiol 3-acetate was higher in the upper part than the lower part of the carrot root (Czepa and Hofmann, 2004; Baranska *et al.*, 2005). The unsaturated fatty acids are considered to be a biosynthesis of most of the polyacetylenes (Christensen and Brandt, 2006).

One of the main difficulties with polyacetylene compounds studies is that they are sensitive to light and heat accounting for their unstable properties, which could hamper any research investigations of these compounds (Baranska and Schulz, 2005).

A diverse range of other factors could have also an effect on polyacetylenes levels in carrots, including cultivar (genotypes) (Baranska *et al.*, 2005) location, climate, water stress, storage and processing (Hansen *et al.*, 2003; Kjeldsen *et al.*, 2003; Christensen and Kreutzmann, 2007).

Moreover, Kjellenberg *et al.* (2012) have demonstrated that there is a positive correlation between the falcarinol ratio, root size and sucrose ratio for both fresh and stored carrots, while the correlation was the inverse between polyacetylenes of stored carrots and the total sugar.

Polyacetylenes have been considered in many studies and indicate that they are responsible for the bitter taste of both fresh and stored carrot roots, whereas the bitter off-taste is mostly influenced by falcarindiol among other compounds (Czepa and Hofmann, 2003; Czepa and Hofmann, 2004).

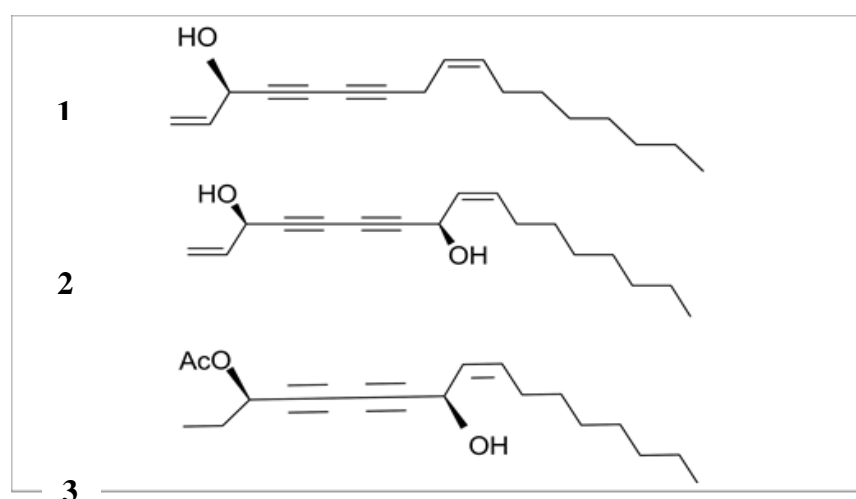


Figure 1.7 Chemical structure of the main polyacetylenes, (1) falcarinol (FaOH), (2) falcarindiol (FaDOH), (3) falcarindiol -3-acetate (FaDOAc) present in carrot roots.

1.4 Aims and objectives

Very little is known about the effects of environmental factors on phenolic acid concentrations and other phytochemicals, although temperature, plant growth stage and degree of maturity may all be important. Increased knowledge and understanding is required for a number of reasons. There is an urgent need to further exploit the potential benefits of dietary vegetable phytochemicals, to promote human health. Climate change will influence future ambient conditions and therefore the composition of major plant foods and indications about the extent of this response would be very useful. Vegetable growers need to be aware how the production environment may be manipulated to advantage by growing plants at appropriate and desirable temperatures to influence crop quality

The research programme aimed to investigate these issues and the objectives were to determine the effects of different growth temperature regimes on

- a) Growth and development of lettuce and carrot in growth chambers.
- b) Concentrations of different components including sugars, chlorophyll, nitrate, carotenoids and phenolic acids in both species and polyacetylene compounds levels in carrot roots.

CHAPTER 2 . MATERIAL AND METHODS

2.1 Preliminary experiments

2.1.1 Growth chamber test

For this study, two different types of cabinets were available to grow the carrot and lettuce plants: Fisons (Fi-totron 600) growth chamber (tungsten and fluorescent light) and Sanyo (MLR-351) growth chamber with fluorescent light. The two cabinets operated in the same temperature ranges (22/18 °C) and provided the same overall PAR (150 $\mu\text{mol}/\text{m}^2/\text{s}$). Each growth chamber contained two trays, one tray for each species. Each tray contained 12 plants: - two varieties of carrot (6 of Parmex, 6 of Little Finger) and two varieties of lettuce (6 of Ashbrook, 6 of Dixter). Both trays were placed in the middle shelf to ensure uniform illumination. The lettuce varieties were harvested after 5 weeks and the carrot varieties after 8 weeks.

Preliminary tests were done to determine which was the best one to use.

In the Fisons growth cabinet the seedlings were taller and thinner than the plants in the Sanyo growth cabinet, mainly because of differences in light quality. This difference in light quality influenced the size of the leaves and roots and biomass of the plants as shown in Figures 2.1 and 2.2 respectively.

The combination of tungsten and fluorescent light in the Fisons growth chamber was less favourable for growth than in the Sanyo growth chamber which had fluorescent lighting only providing better light quality.



Figure 2.1 Effect of different cabinets Fisons and Sanyo on carrot growth after 8 weeks at 22/18 °C. The lengths of the roots in both chambers were more or less the same about 3 cm.

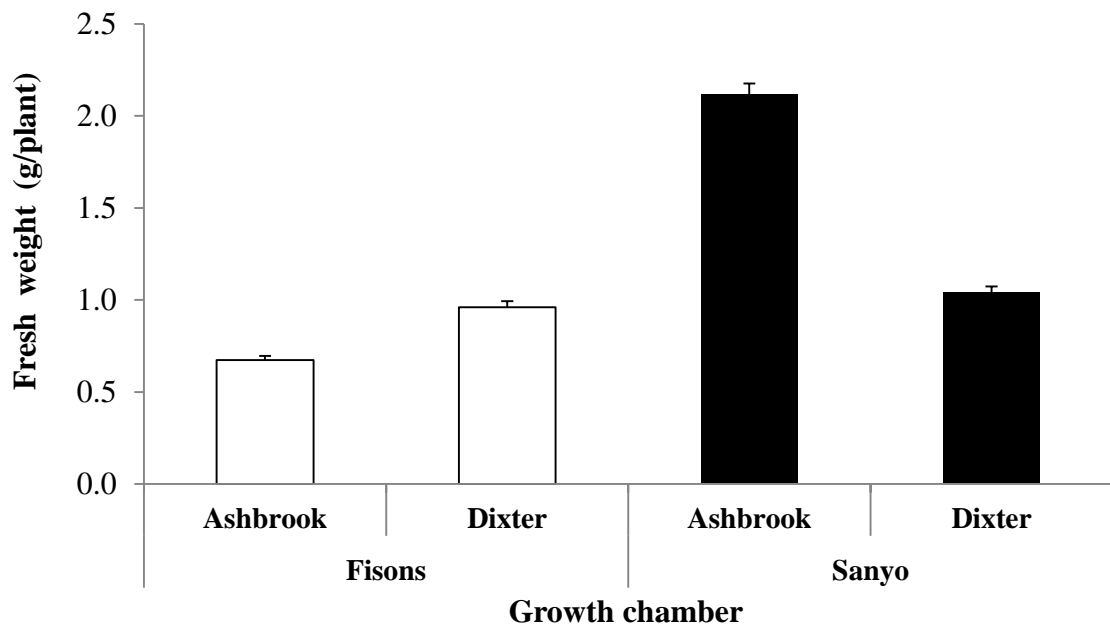


Figure 2.2 Effect of the different type of cabinets Fisons and Sanyo on a fresh weight of lettuce varieties (Ashbrook and Dexter) grown after 5 weeks at 22/18 °C. The values represent the means, the error bars indicate the SEM, (n=2).

2.1.2 Screening lettuce varieties

During the preliminary experiments, six varieties of lettuce were tested: Ashbrook, Exbury, Osterley, Wentworth, Bridgemere and Dexter, all grown at 22/18 °C for 6 weeks (Sanyo growth chamber). The growth chamber was set to an 11-hr-light/13-hr-dark cycle. Levington Seed and Modular plus sand (F2+Sand) compost was used, this is a specialist and general purpose peat-based compost for all plant raising requirements (Low conductivity - 300 - 360 μ s, Standard pH - 5.3 - 5.7, Levington, 2010).

Two trays, each containing 12 plants of two varieties, were used for this test. The plants were watered by system A (section 2.1.3).

The number of leaves and plant height were recorded weekly until the harvest time (after 5 weeks). At the harvest time the fresh weight per plant was also recorded.

Dexter and Exbury were chosen because they grew most rapidly under those conditions as shown by data on fresh weight, height and leaf number in Figures 2.2, 2.3 and 2.6.

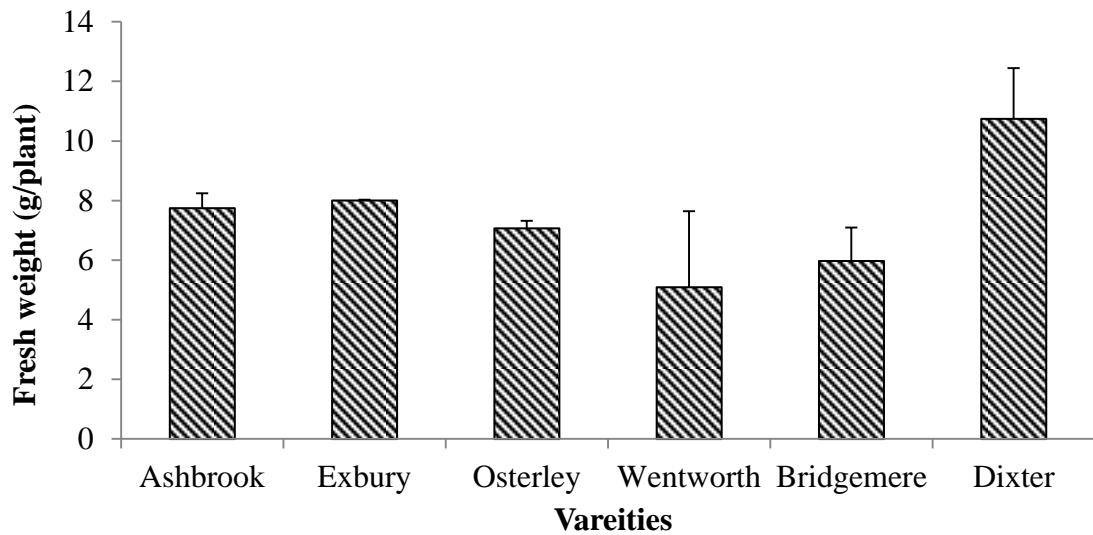


Figure 2.3 Fresh leaf weight of different lettuce varieties grown at 22/18 °C (Sanyo growth chamber). Values are the mean, the error bars indicate the SEM, (n=2).

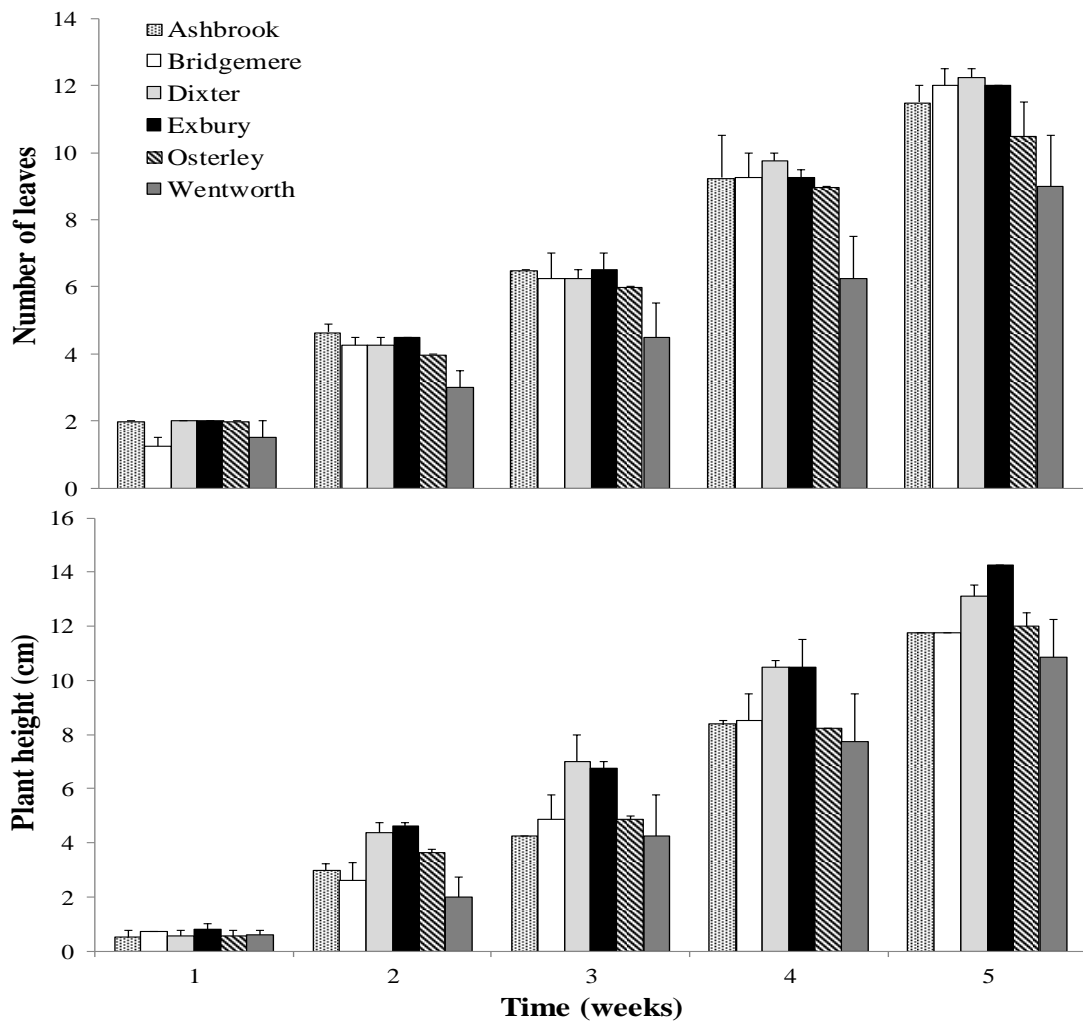


Figure 2.4 Numbers of leaves per plant and height of different lettuce varieties grown at 22/18 °C (Sanyo growth chamber). Values are the mean, the error bars indicate the SEM, (n=2).

2.1.3 Watering system

In the course of the first experimental year, plants were watered by two different systems in the same experiment (section 2.1.2). Two trays were used, containing two plants each of six varieties of lettuce: Ashbrook, Exbury, Osterley, Wentworth, Bridgemere and Dixter:

System A: the tray was put on a water-holding mat inside a tray (direct absorption of water) and system B: the ends of the capillary mat hung over into the water reservoir, Figure 2.5.

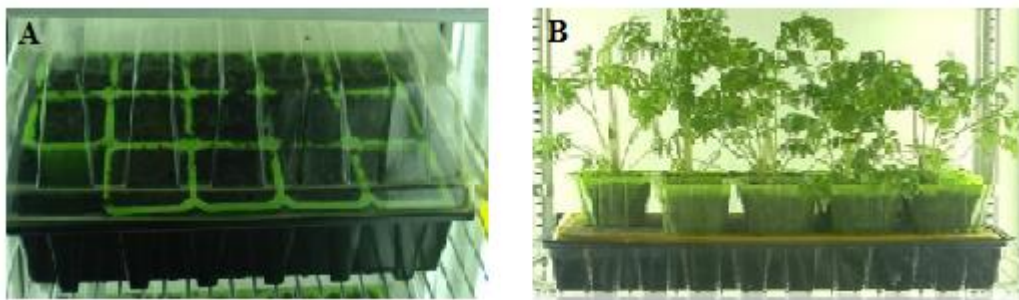


Figure 2.5 Water systems (A) and (B) tested during the preliminary experiment (section 2.1.2) at (22/18 °C), in the Sanyo growth chamber.

The system capacity to provide sufficient water for the plants without suffering water stress was taken into consideration. During the testing period, the water quantity (ml/week/ tray) was recorded every week in each system, (Figure 2.5, and 2.6).

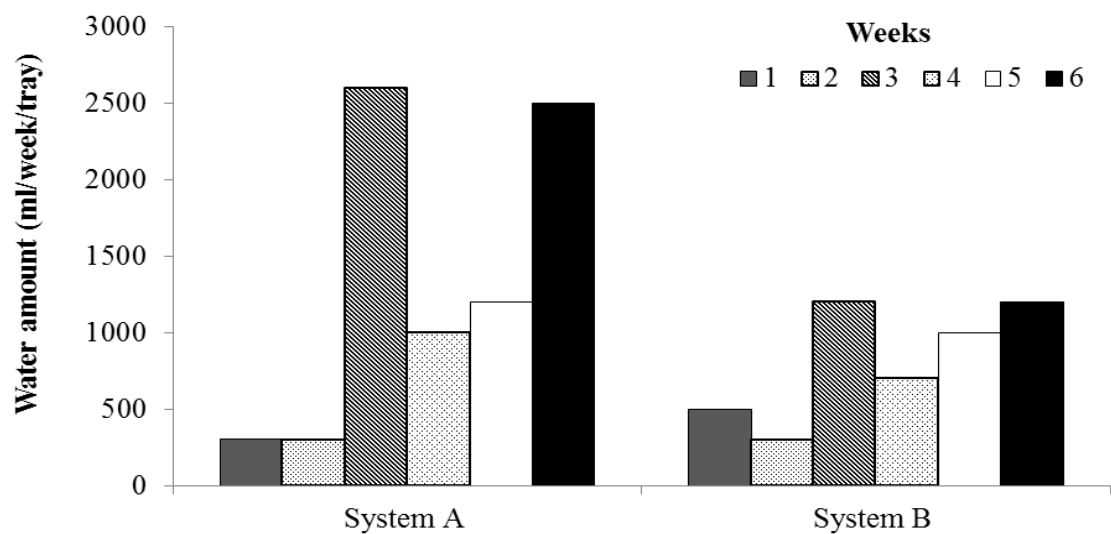


Figure 2.6 Amount of water supplied to plants in two different watering systems at 22/18 °C.

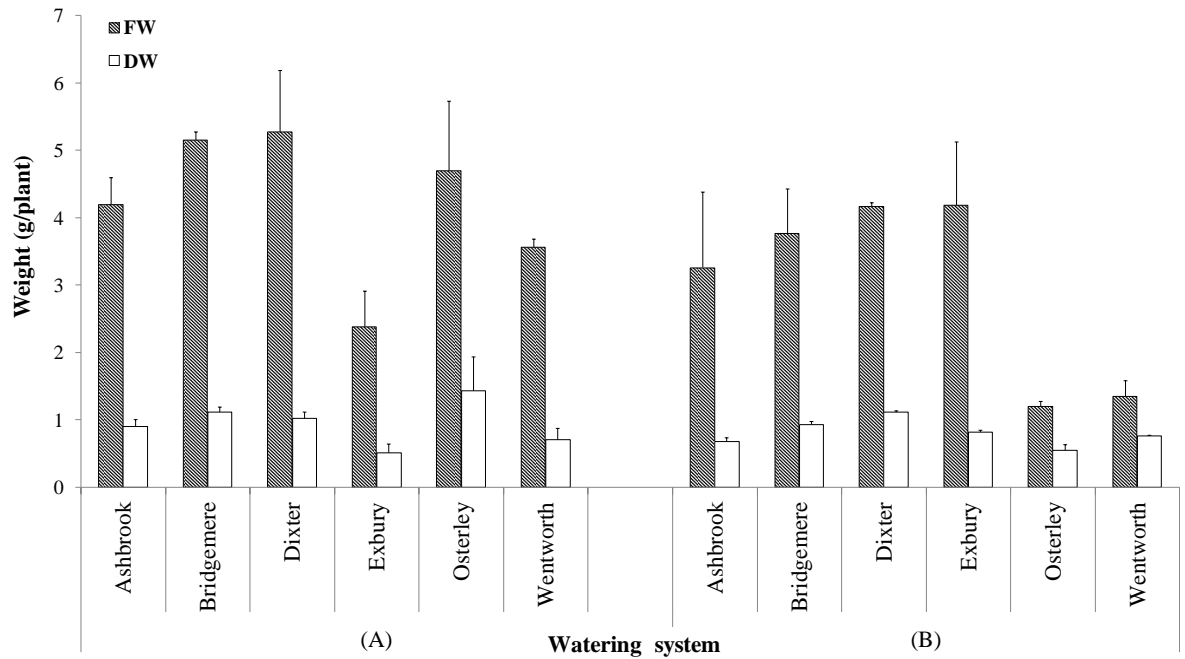


Figure 2.7 Effect of fresh plant (FW) and dry weight (DW) per plant of different lettuce varieties grown after a 6 week period at 22/18 °C within two watering systems (A and B). Values are the mean, error bars indicate the SEM, (n = 2).

ANOVA (GLM) was done on data to compare efficacy of the systems. There was a significant effect ($P < 0.05$) of the watering systems on fresh weight of plants, but no significant effect on dry weight of plants. There seems to be more difference in the efficacy of both systems for some varieties than for others, including Exbury and Dixter. The results showed that the plants grew better with system A than B (Figure 2.7). This was because the direct absorption method supplied water more efficiently to meet plant requirements even when the rate of water demand increased, whereas system B was less efficient with some pots experiencing drought. Also, the type of mat did not allow for effective distribution of the water in the whole pots in the tray. In conclusion, system A was chosen.

2.1.4 Light and temperature test in the growth chamber

A Photometer light meter (Gossen Profisix) was used to ensure the light supply was stable and the amount of light reaching each shelf was similar. To avoid any effect of shadow and to ensure uniform illumination of plants inside the cabinet, the trays were moved to different shelves twice a week throughout the growing period, until the time of harvest.

However, the light levels were recorded weekly in for the three shelves (for 6 weeks during the preliminary experiment (section 2.1.2).

The readings were only slightly different from one shelf to another and the average reading was in the range of $150\mu\text{mol}/\text{m}^2/\text{s}$ (preliminary work on a borrowed photometer went missing). The results were checked with a current student using the same chamber (Mr Othman Qadir) and his light levels measurements for the three shelves were 140, 150 and $160\mu\text{mol}/\text{m}^2/\text{s}$, so this confirmed the average ($150\mu\text{mol}/\text{m}^2/\text{s}$) was correct and the differences less than 8 %.

The temperature regime was checked for consistency and uniformity using thermometers on each shelf in the cabinet at different times during the preliminary experiment (for 5 weeks).

The average reading at the temperature regime (22/ 18 °C) was between 21.7 to 22 °C, there was no significant change in the adjusted regime.

2.2 Experimental set up

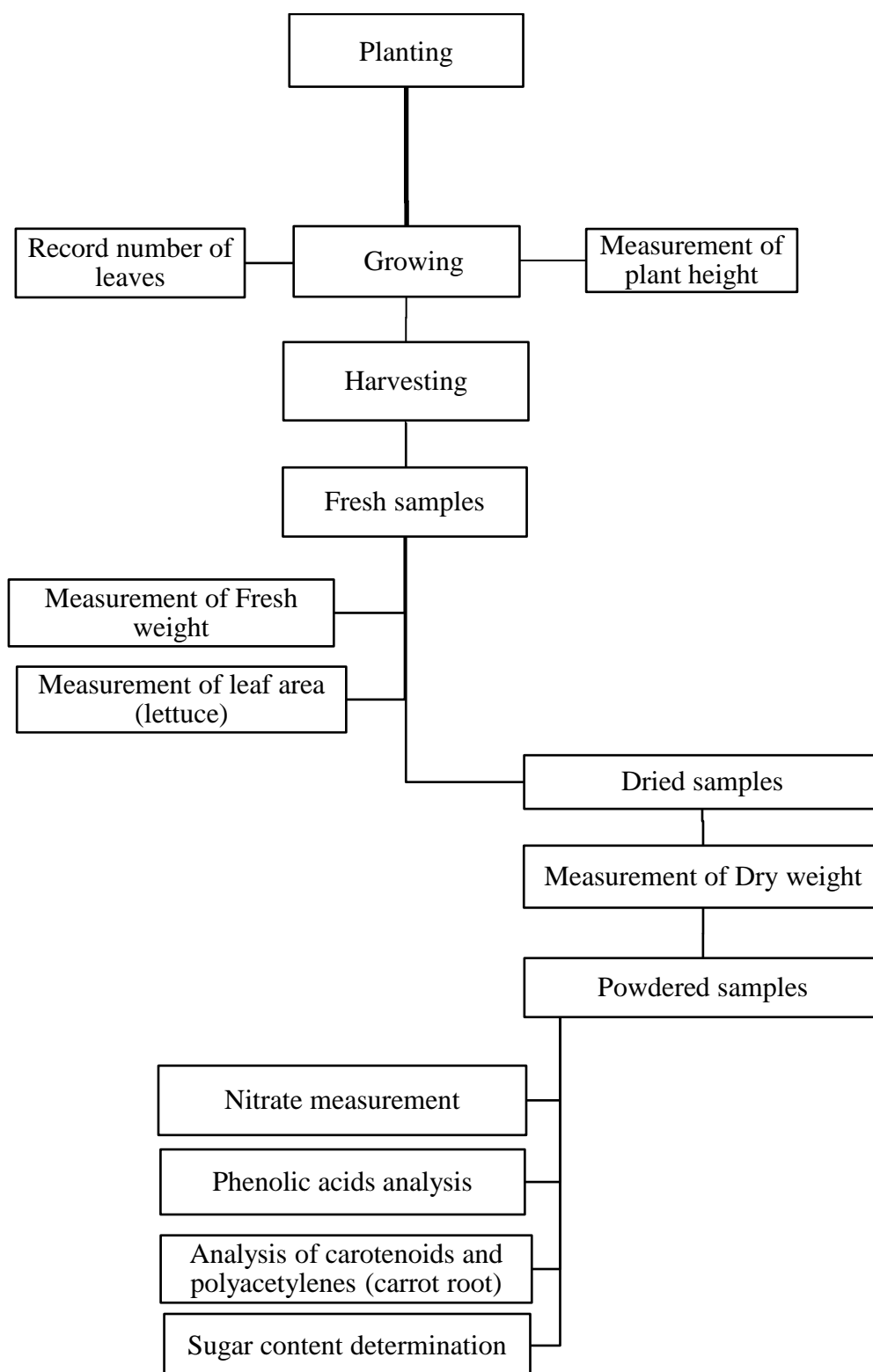


Figure 2.8 Overview of experimental design beginning with planting, growing, harvesting and analysis stages.

2.2.1 Planting and growth parameters measurement

2.3.1.1 Location, Design of experiments

All experiments were done in the School of Agriculture, Food and Rural Development, Newcastle University, in growth chamber with different temperature regimes to determine which conditions are optimal for plant growth and composition. Three replications were used for each plant variety and temperature treatment.

2.2.1.2 Plant materials and growth conditions

Certified seeds of two varieties of lettuce (*Lactuca sativa* L.), Exbury and Dixter and two varieties of carrot (*Daucus carota* L.), Parmex and Little Finger (Nickys Nursery Ltd, UK) were selected based on their suitability for growth chamber experiments, including tolerance of low light conditions and for carrot, a miniature growth habit.

A growth chamber, Sanyo (MLR-351 H) was purchased from Sanyo Electric Co., Ltd, with fluorescent light (photoperiod with a photosynthetically active photon flux: $150\mu\text{mol}/\text{m}^2/\text{s}$). The growth chamber was set to an 11-hr-light/13-hr-dark cycle with 4°C day/night temperature difference. Plants were grown at different temperatures ranges 12/8, 17/13, 22/18, 27/23 and 32/28 °C for lettuce (5 times at the 5 different temperature regimes), and 12/8, 17/13, 22/18 and 27/23 °C for carrots (4 times at the 4 different temperature regimes). Levington Seed and Modular plus sand (F2+Sand) compost was used, this is a specialist and general purpose peat-based compost for all plant raising requirements (Low conductivity - 300 - 360 μs , Standard pH - 5.3 - 5.7, Levington, 2010), (N 150, P 200 and N 150). The plants were grown in a modular tray with 15 individual square (5cm×6.5cm) cells within a watering tray and on a capillary matting base (direct absorption of water) were purchased from VACAPOT & VACAPAK (H. Smith Plastics Ltd, Essex, UK). Cell 15 was cut off and used to check the water level and apply water. Before sowing the compost was weighed and mixed with half the amount (volume: weight) of water (approx. 600 mL). 3-5 seeds per cell were sown 10 mm deep. The water reservoir was filled with 1L water for each tray. The plastic lid of the propagator was placed over the tray (to increase humidity until germination started). After germination, unwanted seedlings were removed from the

soil carefully with tweezers to leave suitable space for one plant per module. The experiments were performed with each treatment temperature separately and both the carrot and lettuce varieties were grown concurrently in the growth chamber.

To avoid any effect of shadow and to ensure uniform illumination of plants inside the cabinet, the trays were moved to different shelves twice a week throughout the growing period, until the time of harvest.

Due to the limitations of space, a growth chamber contained three trays of each species each with seven plants. The plants were subjected to regime conditions until harvest.

2.2.1.3 Plant growth parameters recorded and strategy of harvest

Records of plant growth included number of leaves, length of shoot, which was measured on a weekly basis (using a ruler). The amount of water used was also recorded each week.

The period from sowing until harvest ranged from 6 to 14 weeks depending on temperature, being shortest for the highest temperature regime with the fastest growth rate and longest for the lowest. Irrespective of temperature regime and rate of plant growth, harvest took place when lettuce plants had between 10-12 leaves and carrots 6-8 leaves. This specific stage or number of leaves for harvest was determined because the plants had reached an appropriate size to be marketable as baby vegetables (Figure 3.2). Fresh and dry weight of leaves and root were measured after harvest for each plant individually, and leaf area for lettuce varieties only.

2.2.1.4 Biomass determination

Seven plants per treatment were harvested and divided into roots and shoots. For both varieties of carrot and lettuce the roots were washed carefully in flowing tap water and dried using soft tissue. All leaves and the root for each plant were placed in small plastic bags separately.

After measuring fresh weight of roots and shoots by a digital measuring device (Adam Equipment PW124 Lab Balance), the samples were kept in a freezer for 2-3 days at (-80 °C) before transfer to freeze-drying in a Lyolab G freezer-drier for a week. The samples

were dried at -20 °C until the pressure reached ≤ 0.06 m bar, then the temperature was allowed to reach room temperature for nearly 24 hours and the dried samples were weighed to record their dry weights.

2.2.1.5 Storage conditions

After freeze drying and dry matter determination, the samples were milled using a 1093 Cyclotec Sample Mill. All 7 plants for each variety in each tray (which are separate samples within the treatment) were milled together and the dry material put in separate, new plastic bags from which the air was expelled. These were transferred to the freezer (-20 °C) and kept in the dark in a black bag to minimize degradation until analysis.

2.2.1.6 Measuring leaf area

The method was as described by (Davidson, 2010). Fresh leaves were harvested then were directly separated and flattened onto a background with a grid of known dimensions (42 cm x 39 cm). Photographs were taken of each plant separately by using a vertically positioned digital camera, mounted 40cm above the surface without macro focus and no flash to avoid shadows. The images were downloaded and saved to computer, the background or an object of known dimensions or form was used for scale reference when analysing image. The *ImageJ 1.38x* software (Figure 2.9) was downloaded to computer from the link: <http://rsb.info.nih.gov/ij/download.html>, (section 7.1.3.1 Appendixes).

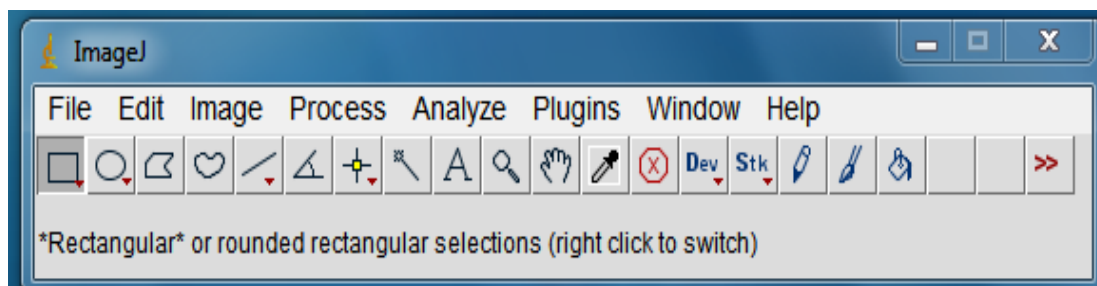


Figure 2.9 ImageJ 1.38x software

2.2.2 Phytochemicals analysis

Different methods and modifications were investigated, to take account of the number of samples and phytochemicals to be assessed in this project and to determine the minimum quantities per sample required for the assays.

2.2.2.1 Sugar content analysis (total soluble solids)

For estimates of sugar content of freeze dried carrot and lettuce samples, 1 mL ultra – pure water was added to 100 mg of samples, vortex mixed for a few minutes then the concentrated extracts were measured by hand with a refractometer (Bellingham and Stanley Eclipse refractometer, 0-30 BRIX°). The reading of each sample was repeated twice for confirmation. Between each reading the refractometer was rinsed with distilled water and dried.

The afore mentioned method was done by calculating the average values of each sugar (glucose, fructose and sucrose) found in the lettuce and carrot samples in order to prepare a standard for estimating the total sugar content.

For carrot, the concentration of standard solution was prepared as follows: 1.92 g, 1.13 g and 6.95g / 100 mL for glucose, fructose and sucrose respectively (the values of standard for each sugar were estimated according to data published by (Gaweda, 2007) in carrot root), according to the standard curve that provided the conversion of BRIX° unit to sugar gram per 100 mL: therefore one BRIX corresponded to 1.1075g/ 100 mL (Figure 2.10 A).

For lettuce, the concentrations of sugars were proportionally distributed as glucose 2.9 g, fructose 5.4 g and sucrose 1.7g / 100 mL (the values of standard for each sugar were estimated according to data published by (Gaweda, 2007) in lettuce leaf). The standard curve provided the conversion of BRIX° unit to sugar gram per 100 mL, therefore one BRIX corresponded to 1.1125g /100 mL (Figure 2.10 B).

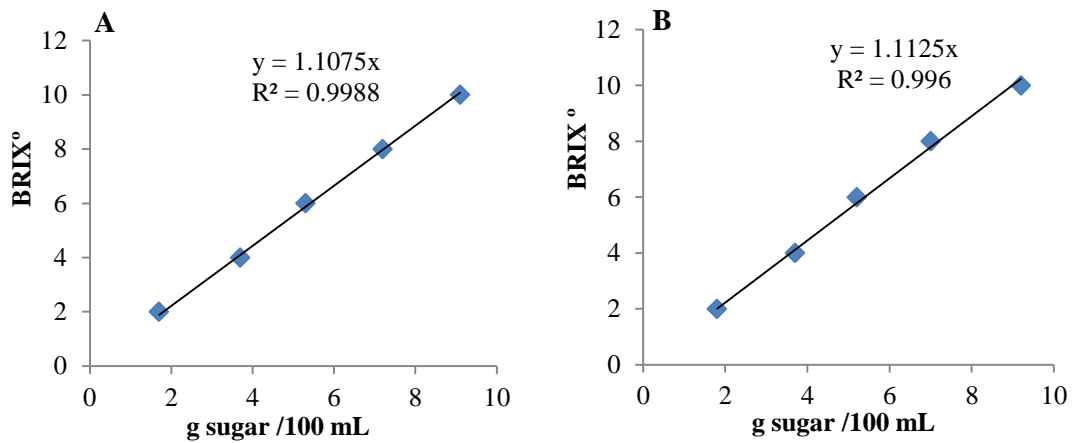


Figure 2.10 Standard curves for the sugar determination of carrot (A) and lettuce (B).

Non-sugar content: however, the residue (non-sugar or insoluble solids) mg/100g FW was calculated by subtracting the sugar content (mg sugar /100g FW) from total dry matter (g dry weight /100g FW) values.

2.2.2.2 Measurement of Nitrate (spectrophotometer)

The method was adapted from the method devised by (Miranda *et al.*, 2001): the principle of this assay is reduction of nitrate by vanadium (III) combined with detection by the acidic Griess reaction.

2.2.2.2.1 Developments and modifications of method

The method chosen for nitrate analysis (section 2.3.6.2) was based on preliminary tests with the duration of incubation adjusted and with different sample sizes. The results of tests for the determination of appropriate durations and sample sizes and for nitrate analysis (absorption at 540 nm) are shown in Figures 2.11 and 2.12 respectively.

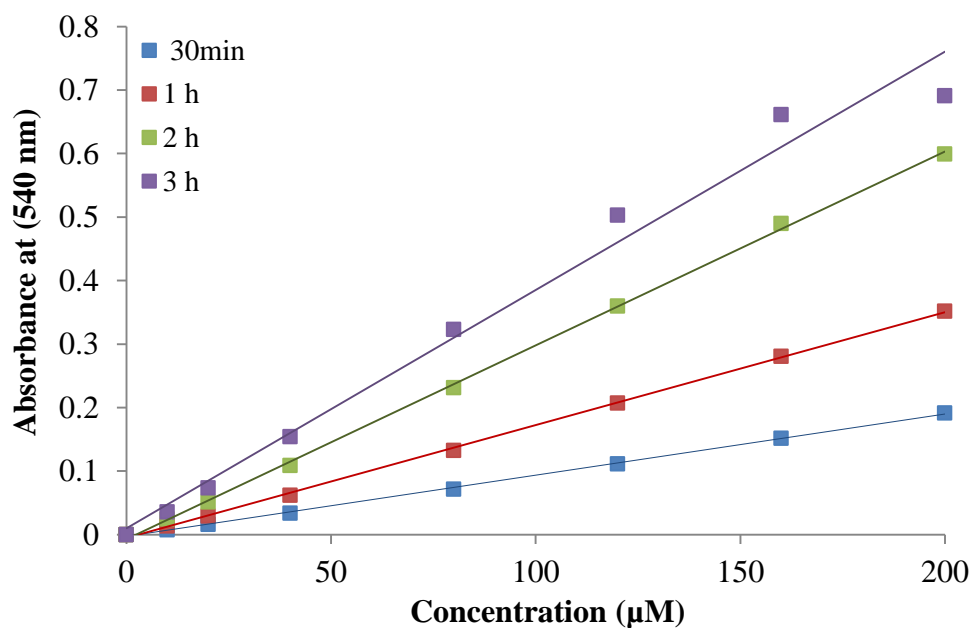


Figure 2.11 The effects of nitrate concentration and duration of incubation i.e. half an hour, one hour, two hours and three hours on the standard absorbance curve of nitrate measurement.

10, 50 and 100 mg of either carrot root or lettuce leaf were mixed with 3 and 5 mL of water. Absorbance values (Figure 2.14) were in accord with the range of maximum standard absorption in Figure 2.13. Absorbance values for lettuce leaves were more than double that for carrot root samples at all concentrations, but the carrot root samples were still within the range of the standard and thus acceptable (Figure 2.12).

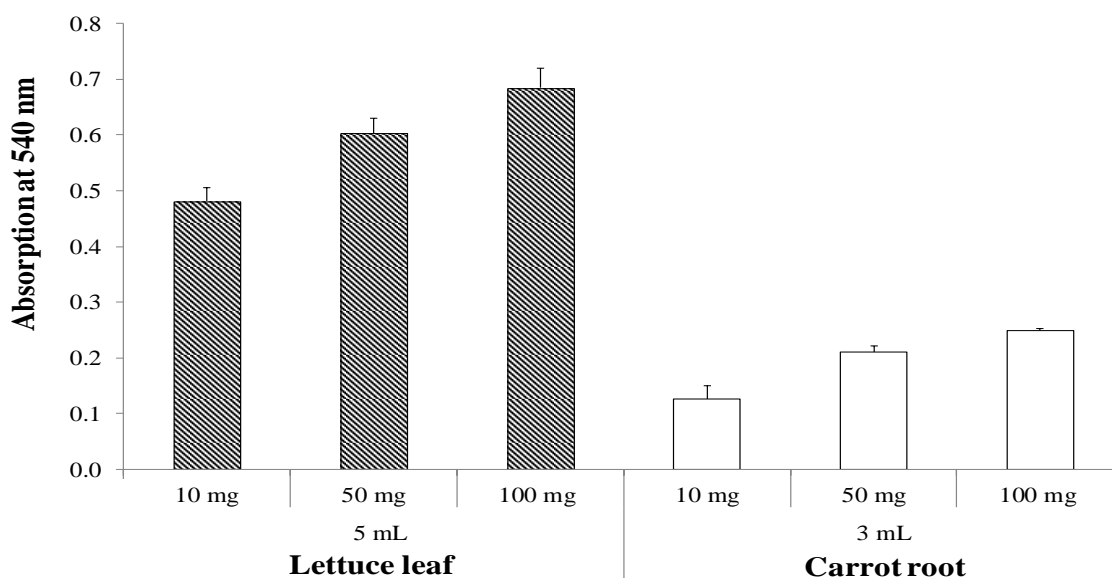


Figure 2.12 The effects of sample size (mg dry weight) and quantity of water diluent on nitrate absorption of lettuce leaf and carrot root. Values represent the means (7 replications of each sample), error bars indicate the SEM.

Following these tests, 10-20 mg dry weight of samples (both shoot and root) were mixed with 5-50 mL of water, to keep the concentration of samples within range of the standard (0-0.7). The optimal duration (incubation time of standard and samples) for absorbance reading was between 1-2 hours to reach the maximum (0.7). The absorption of samples was read at least 3 times (at 30, 60 and 120 min), to make sure the absorption of samples was in the range of standard absorption.

2.2.2.2.2 Final method used for main experiments

Preparation of stock solutions: Saturated V(III) solution was prepared quickly because it reacts with air, by dissolving 400 mg of VCl_3 in 50 mL of 1 M HCl and the excess solid was removed by using filter paper. Secondly, 0.1 % NEDD solution was prepared by dissolving 0.1 g N-(1-Naphthyl) ethylenediamine dihydrochloride in 100 mL of H_2O , which required heating and stirring. Finally, 2 % sulphanilamide solution was prepared, where 2 g of sulphanilamide was dissolved in 100 mL of 5% HCl requiring heating and stirring. All the stock solutions were stored in the dark at $4^{\circ}C$ and were stable for at least a few weeks.

Procedure for analysis: The analyses were performed at room temperature with some modification as follows. Vanadium cocktail was prepared daily and only enough volume for a day (e.g. 50 mL). In a 100 mL beaker, 5 mL of saturated V(III) was added, 0.33 mL of 2% sulphanilamide, 0.66 mL of 0.1% NEDD and 40 mL of H_2O , then it was purged with N_2 (5-10 min) and kept in a closed container. Nitrate stock solution (10 mM) was prepared by dissolving 0.101 g of KNO_3 in 100 mL of ultra-pure water and stored in the dark at $4^{\circ}C$. 200 μM nitrate standards were prepared daily by pipetting 2mL of 10 mM stock solution into 100 mL volumetric flask, and then making up to 100 mL using sample matrix (H_2O). Nitrate standard curve solutions were prepared in concentrations of 0-200 μM by diluting 200 μM nitrate standard with water to 10 ml final volume. 10-20 mg of each sample was weighed accurately into a screw glass-top tube then 5-50 mL pure ultra-water was added depending on which plant part (leaf or root) was tested and adjusted. The solution was vortex mixed for 10 min then transferred to a centrifuge and centrifuged for 10 min at 4000 g. The clear supernatant was filtered using Whatman no 1 filter paper. 100 μL of sample and standard (0-200 μM) were pipetted into a 96-well plate (PS, Flat bottom Greiner Bio-One Ltd), then 100

μ L of vanadium cocktail solution were added. The microplate was shaken for a few minutes by putting the plate in a spectrophotometer (Molecular Devices-UV plate reader, Spectra max plus 384), and then incubated at room temperature for 1-2 hours. Finally the absorbance was read at 540 nm after 30 minutes, 1 hour and 2 hour to obtain optimal absorption data (Figure 2.11).

2.2.2.3 Extraction of carotenes, chlorophylls and polyacetylenes

The chlorophylls and carotenoids were measured using two different methods:

Method (A): The chlorophylls and carotenoids were measured spectrophotometrically in fresh plant material as described by Wellburn (1994).

Method (B): Was based on (Rashed, 2009), separating carotenoid, chlorophyll and polyacetylene compounds by HPLC.

2.2.2.3.1 Chlorophylls measurement spectrophotometrically (method A)

The use of fresh material for the analysis of chlorophyll is an important aspect for consideration with common methods for chlorophyll measurement. Thus, in accordance with (Wellburn, 1994), three fresh leaf discs per sample (carrot and lettuce from the main experiments) were cut from three randomly selected plants (for each variety and tray) and weighed (0.1-0.2 g). Then the samples were transferred into separate 15 mL Falcon tubes and placed in a freezer overnight. Each leaf disc was extracted by grinding with a pestle using 5 mL 80 % acetone (Sigma Aldrich Co, UK). The acetone extract was then decanted into a Falcon tube, thoroughly mixed, then left in the fridge overnight. The Falcon tubes were centrifuged for 5min at 4000 rpm, then the supernatant was decanted into another tube and the pellet resuspended in 2 mL acetone before being placed in the fridge again for 1h. After centrifugation, the acetone extract was decanted into the same tube as the first extract. The total volume of acetone in the tube was measured and the supernatant was transferred into quartz cuvettes. The determination of photosynthetic pigment content was performed under low light condition to avoid any degradation of pigments. Absorbance on the spectrophotometer

(UV-2401 PC, SHIMADZU) was read at wavelengths 663, 645 and 470 nm against a blank of 80 % acetone as reference.

Chlorophyll a, chlorophyll b and carotenoids concentration were calculated using the following equations:

$$\text{Chla} = 12.25 * A_{663.2} - 2.79 * A_{646.8} \dots \dots \dots (1)$$

$$\text{Chlb} = 21.5 * A_{646.8} - 5.1 * A_{663.2} \dots \dots \dots (2)$$

$$\text{Carotenoids (C}_x + c) = (1000 * A_{470} - 1.82 * C_a - 85.02 * C_b) / 198 \dots \dots \dots (3)$$

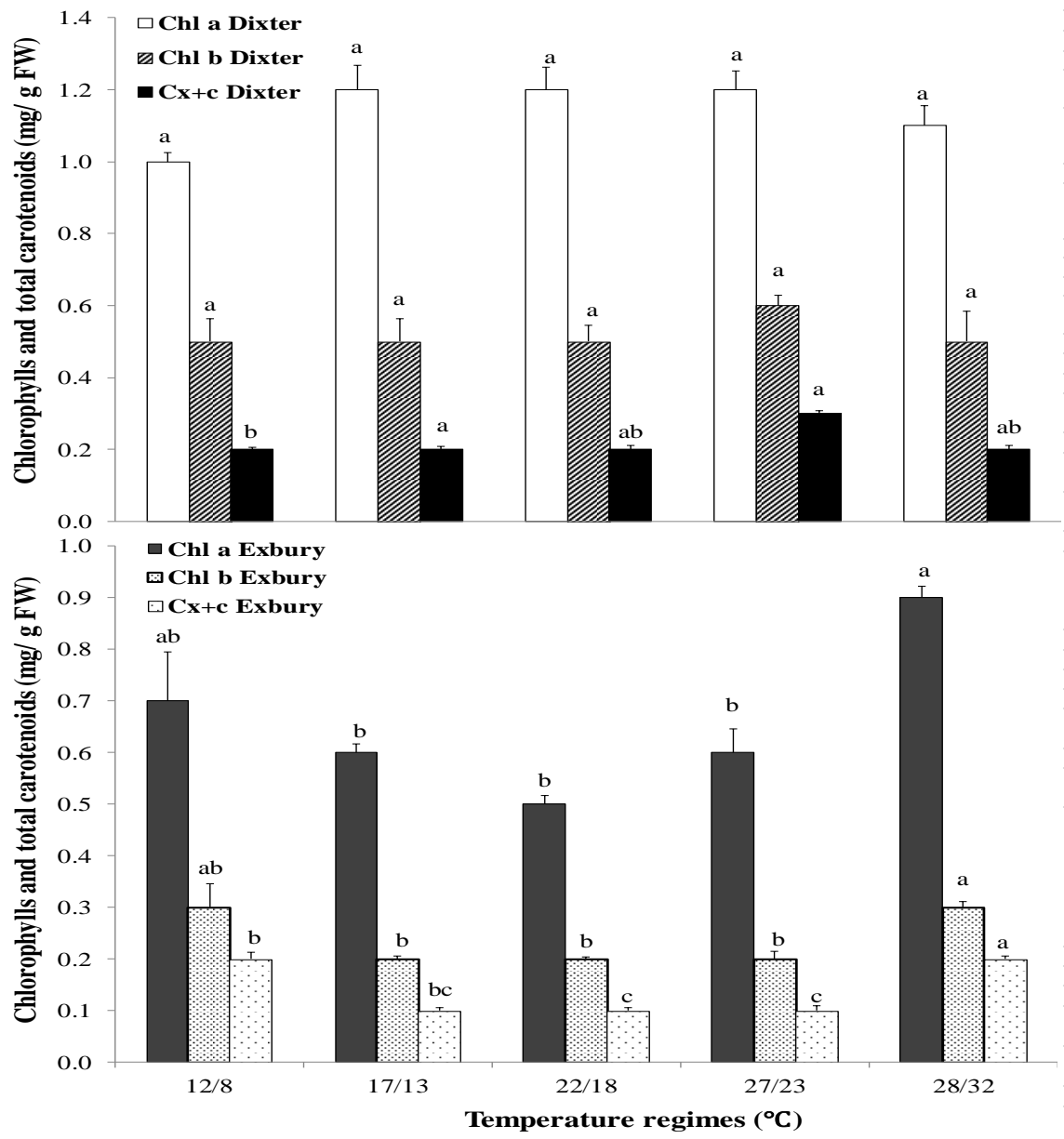


Figure 2.13 The effect of lettuce variety (Dexter, Exbury) and temperature on chlorophylls (chl a and b) and total carotenoids (C_x + c), (measured spectrophotometrically). Means with different letters within a chlorophyll type are significantly different (P < 0.05). Error bars indicate SEM, (n = 3).

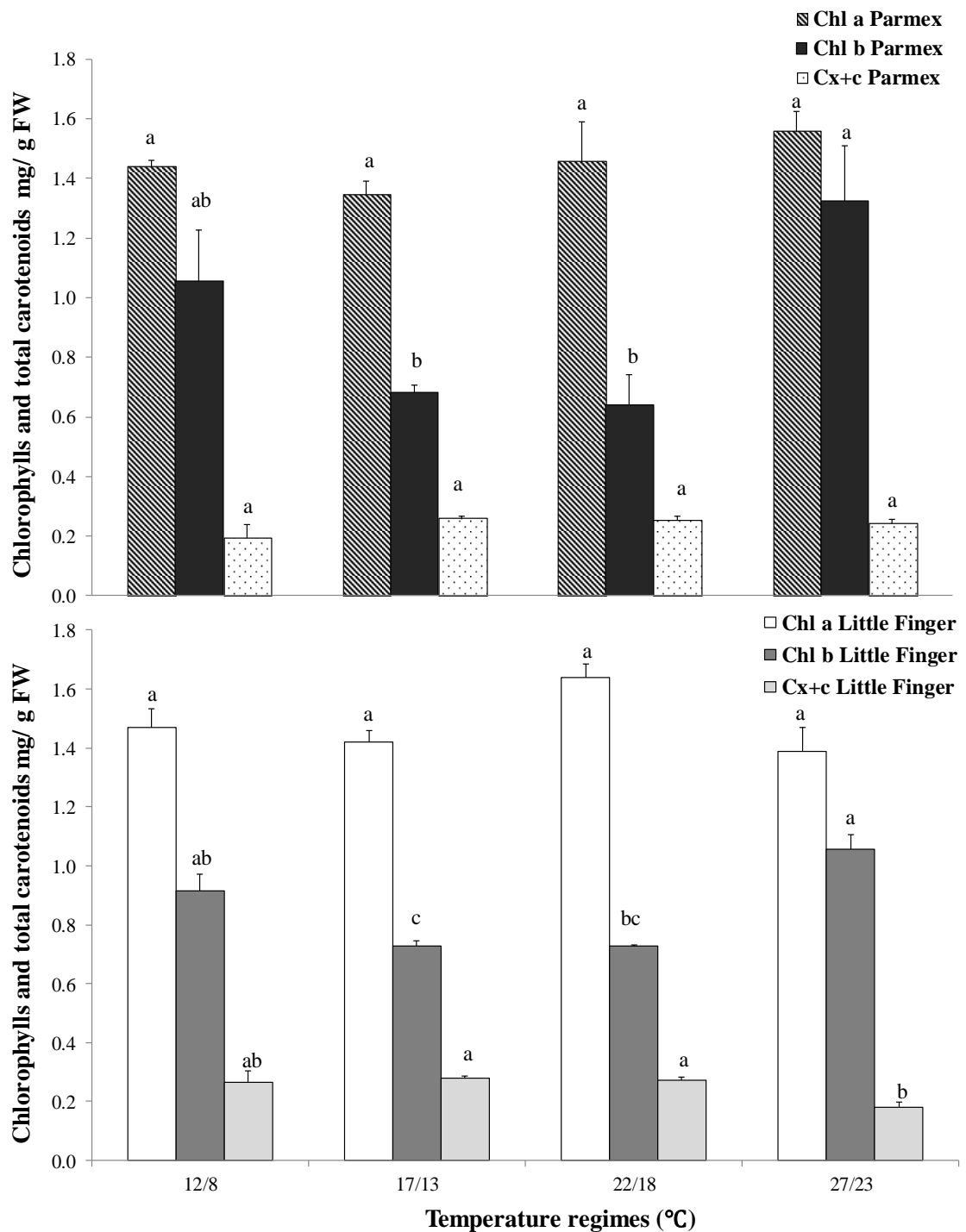


Figure 2.14 The effect of carrot variety (Parmex and Little Finger) and temperature on chlorophylls (chl a and b) and total carotenoids ($C_x + C_c$), (measured spectrophotometrically). Means with different letters within a chlorophyll type are significantly different ($P < 0.05$).

Due mainly to the lack of detailed knowledge (the limited range of absorbance in the spectrophotometer and/or limits absorption) and the saturation of the samples (the concentration of fresh samples may well have exceeded the absorbance of the machine used) even after ten times dilution, the results showed that temperature had no effect on Chlorophyll concentration, (Figures 2.13 and 2.14). Therefore, it was decided to use more reliable and accurate methods such as HPLC, as mentioned in (section 2.3.6.2).

2.2.2.3.2 Development and modifications of the HPLC method (B)

In the original method by Rashed (2009), 2 g of dry plant material were extracted with ethyl acetate for three days. Each time they were left one day in the refrigerator before centrifugation. The supernatants were combined and filtered then the samples were directly analysed by HPLC.

Because of the large amount of plant material used (2 g) for the analysis of carotenoid, chlorophyll and polyacetylenes and the long extraction time (3 days) for this method and also the number of components to be investigated in this study, it was necessary to test a smaller amount of plant material in proportion to the amount available from the experiments. The extraction was tested in a shorter time than the time used in the original reference.

The modifications were as follows:- The amount of plant material was reduced from 2 g to 70 mg through the testing of different quantities, including matching the amount of solvent required (g plant material in mL solvent): this ensured successful methods and results. Furthermore, the duration of extraction of total carotenoids and polyacetylenes was tested by using a small amount for the analysis and reducing the time of extraction from 3 days to 1 hour on the second day: the results were similar (no effect on number of peaks or time absorption) for both methods (extraction duration for 3 days and 2 days (1 hour next day)).

The average peak areas of each of the compounds and their standard deviation were used to calculate the relative variability (%), (standard deviation of peak area divided by the mean of peak area * 100). The values in Figure (2.15) are broadly similar with each quantity.

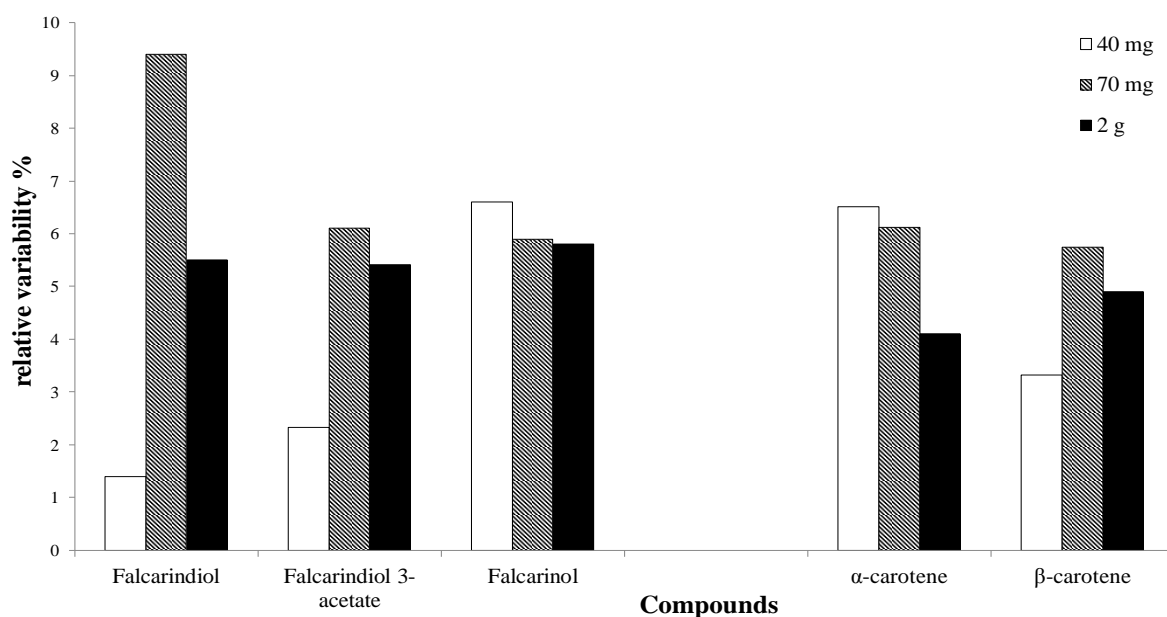


Figure 2.15 Tests undertaken on different quantities of samples (dry material) and extraction time (40 and 70 mg for 2 days and 2 g for 3 days) for relative variability percentage of polyacetylenes (falcarindiol, falcarindiol 3-acetate and falcarinol) and carotenes (α and β -carotenes) in carrot root which were analysed by HPLC.

The preliminary work on a tested large quantity (2 g) went missing; However Figure 2.15 includes results of analyses of 2 g samples that were done by another student (Mrs Huda Saleh).

Clearly both quantities (plant dry material) were satisfactory as identified and described in the section 2.2.2.3.3. However, 70 mg powdered dry plant material was chosen as the appropriate amount for analysis for each experiment.

2.2.2.3.3 Final method used for main experiments

70 mg of freeze dried sample was homogenized with 1mL of ethyl acetate in 10 mL centrifuge tubes, samples were then vortexed for a few minutes, covered to keep them dark and placed in the refrigerator at 4 °C overnight. The next day the samples were centrifuged for 10 minutes at a speed of 4000 g and the supernatant put into a new screw-top test tube. 0.25 mL of ethyl acetate was added to the residue tube and mixed. Again, the samples were covered and put in the refrigerator for 1 hour. After that the samples were centrifuged and the supernatant transferred to screw-top test tube for extraction, and 0.1 mL of ethyl acetate was added to the residue tube and kept for 1 hour again. Samples were then centrifuged and the supernatant removed. In total, the extraction procedure was carried out three times with the supernatant being placed into the same screw-top test tubes. They were then filtered using 1mL syringe and filtered (0.2 µm). The extracts were combined and directly analyzed by high performance liquid chromatography (HPLC).

2.2.2.3.4 UV-Visible Spectroscopy

UV-Visible spectra were obtained with a UV-2401 PC, SHIMADZU double beam scanning spectrophotometer (ranging between 200 to 800 nm) at room temperature. The standards were measured on the same day the HPLC analysis was done. Baseline spectra were taken for each solvent prior to measurement, and every experiment was performed under low light conditions, in a laboratory with the lights turned off and all windows closed and covered with curtains, (Figure 2. 16 and 2. 17).

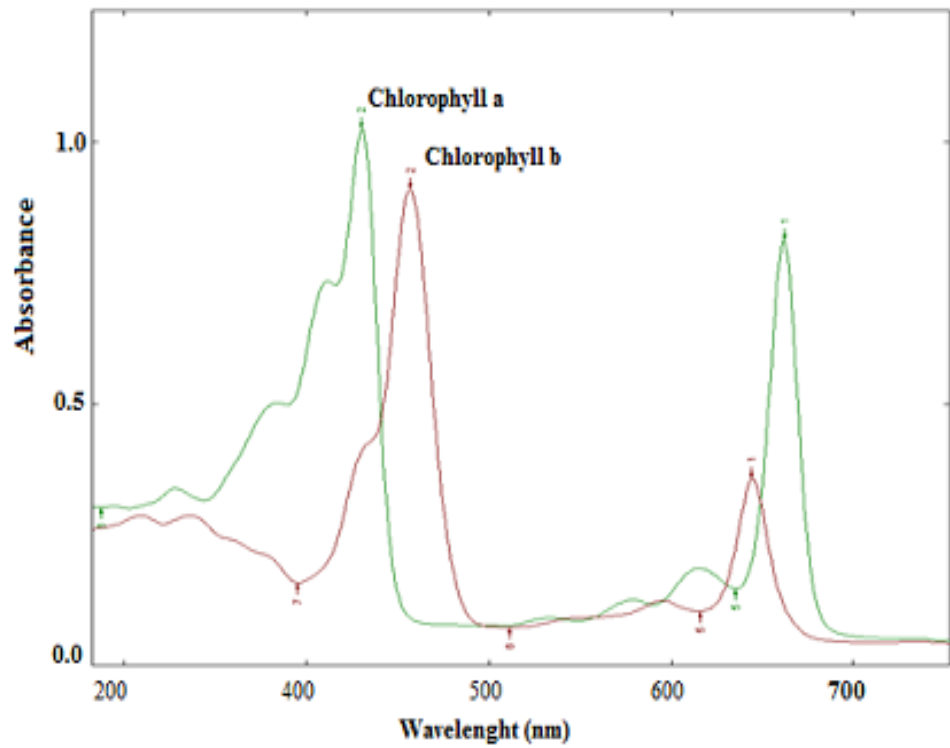


Figure 2.16 Visible spectra of chlorophylls a and b standard

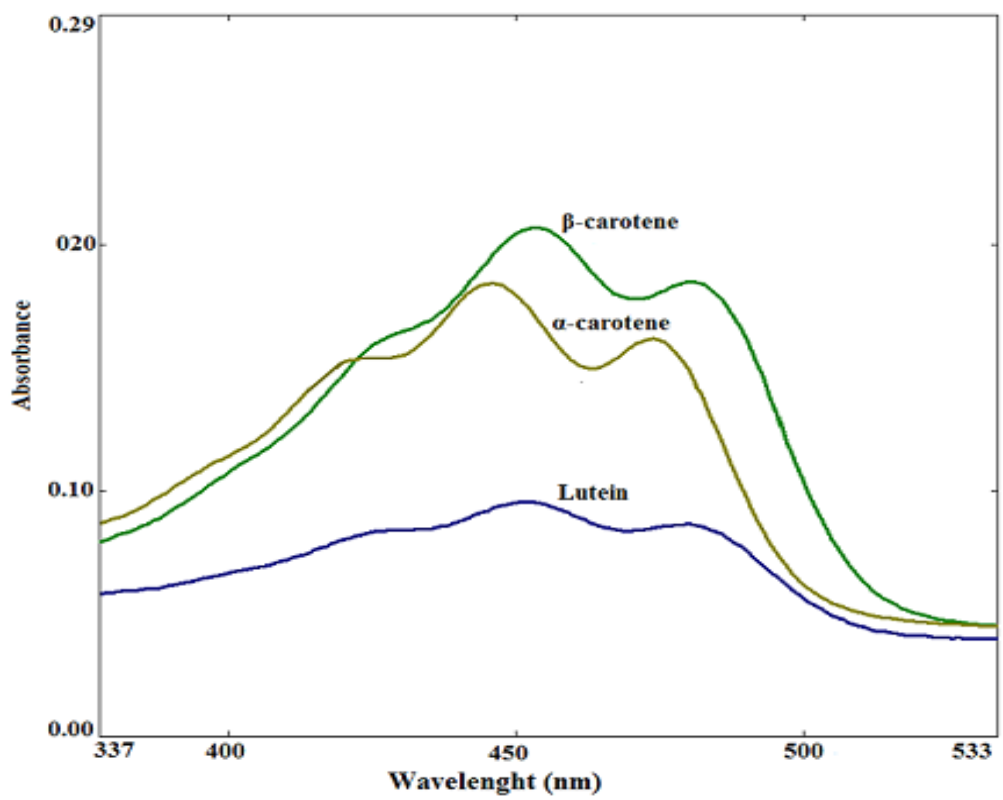


Figure 2.17 Visible spectra of lutein, α -carotene and β -carotene standard

2.2.2.3.5 HPLC Analysis of carotenes, chlorophylls and polyacetylenes

Both the carotene and polyacetylene analysis was carried out on the same HPLC. The column was a HyperClone Reverse phase C18 (250 × 4.6 mm, 5 μ m), and column oven set at 40 °C. The flow rate was 1 mL/min, injection volume 20 μ l and detection at 205 nm of polyacetylenes, and the detection of carotenoids was at 450 nm.

The mobile phase was, pure water solvent (A), methanol solvent (B), and ethyl acetate solvent (C) (Fisher scientific, UK). The solvent gradient ratios are shown in Figure 2.18.

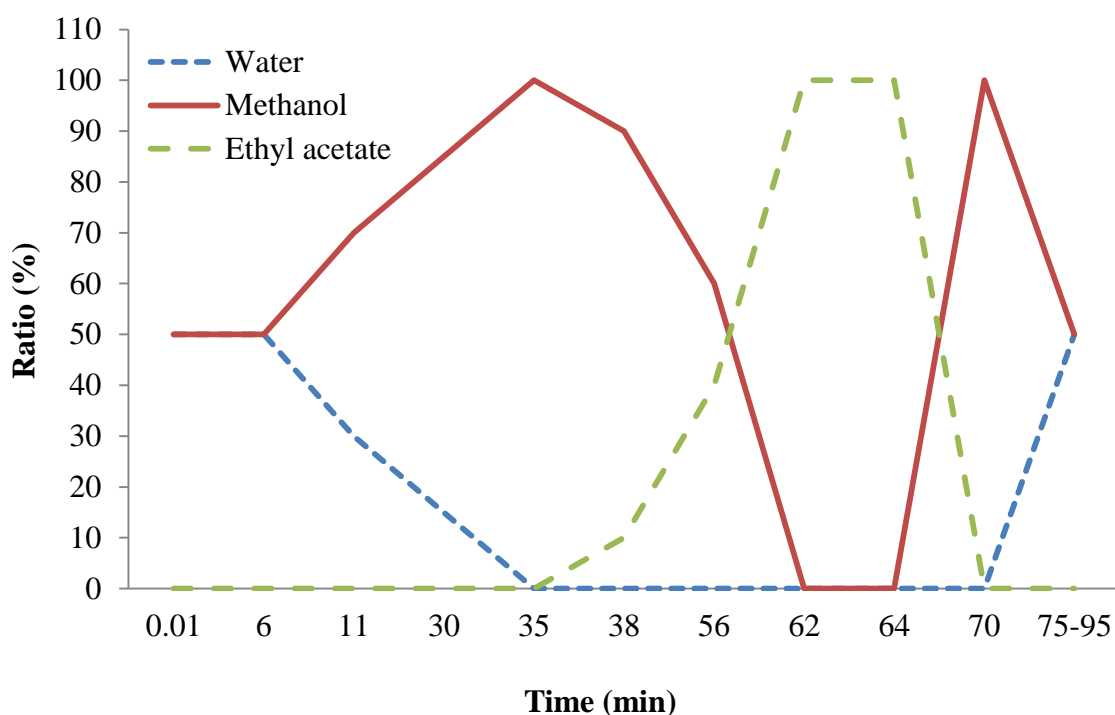


Figure 2.18 Percentages and ratio of solvents added at specific time points.

2.2.2.3.6 Identification and quantification

Peak area and retention times of carotenoids were quantified by comparison with corresponding authentic standards, which were checked with photodiode array detector (DAD) on HPLC. α -carotene, β -carotene, lutein, chlorophyll a from alga and chlorophyll b from spinach were purchased from Sigma-Aldrich Ltd. The retention times of the identified carotenoids at 450 nm were as follows: Lutein 38.25 min, chlorophyll b 40.52 min, chlorophyll a 41.85 min, α -carotene 45.47 min, β -carotene

45.79 min and *Cis*- β -carotene 46.23 min, (Figure 3.25 and 3.26, Table 7.5 Appendix). In addition, the peak area of polyacetylenes was quantified by comparison with falcarindiol, falcarinol 3- acetate and falcarinol standards isolated from carrots by students (Ahlam Rashed and Huda Saleh) in Newcastle University, and the retention time of polyacetylenes compounds at 205 nm, falcarindiol 19.02 min, falcarinol 3- acetate 23.14 min and falcarinol 26.5 min. The accurate carotenoid concentrations of the standards were determined spectrophotometrically using corresponding extinction coefficients Table 2.1, (Alpha values (α) were used in the calculations).

The Polyacetylene compounds: Falcarinol, falcarinol 3-acetate and falcarindiol authentic standards were isolated from carrot root and were calculated as falcarinol equivalents at the three UV maxima $\lambda = 256$ nm ($\epsilon = 445$ /M), 242 nm ($\epsilon = 739$ /M) and 230 nm ($\epsilon = 986$ /M) in methanol based on (Hansen and Boll, 1986).

Table 2.1 Accurate specific (α) and millimolar (ϵ mM) extinction coefficients for chlorophyll a, b (Chl), lutein (Lut) and α , β - carotene (α , β - car) in different solvents

Pigments	Solvent	Wavelengths (nm)	Extinction coefficients		References
			(ϵ) l/mM/cm	(α) (E $\frac{1\%}{1\text{cm}}$)	
Chl a	80% Acetone	663	76.79	840	(Pocock <i>et al.</i> , 2004)
Chl b	80% Acetone	645	47.04	518	(Pocock <i>et al.</i> , 2004)
Lut	Methanol	442	149.60	2629	(Craft and Soares, 1992)
α-Car	Hexane	445	145	2710	(Rodriguez-Amaya, 2001)
β-Car	Ethanol	450	135.80	2529	(Craft and Soares, 1992)

2.2.2.4 Extraction of phenolic acids

Three different methods were used to investigate the phenolic compounds using HPLC.

2.3.2.4.1 Development and modifications of method

Three methods were tested for phenolics analysis to determine the clearest separation of peaks and the characteristics of phenolics, including absorption spectra: all separations were done on the same HPLC and by the same column HyperClone Reverse –phase C18 (250 × 4.6mm, 5µm):

Method A based on (Rashed, 2009) (solvent extraction 40 % acetonitrile adjusted to pH 3 with phosphoric acid, analysis duration 70 min), (Table 2.2).

Method B based on (Mpofu *et al.*, 2006) with some modification by Lucas Patzek, (solvent extraction 80 % methanol, 2M NaOH was added for 4 hrs for hydrolysis reaction, analysis duration 42 min), (Table 2.2).

Method C according to (Almuairfi *et al.*, 2010) (solvent extraction 70 % v/v methanol. The samples were completely dried with nitrogen gas, the residues were re-suspended in the original volume taken with water, analysis duration 65 min) as described in methods (Table 2.2 and section 2.2.2.4.2).

Table 2.2 Shows the three methods used to test phenolic compounds

Method A (40% ACN)			Method B (80 % MeOH)			Method C (70 % MeOH)		
HPLC Analysis of phenolic acids								
Flow rate: 0.1 mL/min, oven temperature: 40°C			Flow rate: 1.5 mL/min, oven temperature 25°C			Flow rate: 0.1 mL/min, oven temperature: 25°C		
Solvent gradient (%)								
Time (min)	A (2% (v/v) acetic acid in H2O)	B (acetonitrile)	Time (min)	A (2% (v/v) acetic acid in H2O)	B (acetonitrile)	Time (min)	A (0.1% (v/v) TFA in H2O)	B (0.1% (v/v) TFA in acetonitrile (%))
0	100	0	0	85	15	0	100	0
30	85	15	10	80	20	5	100	0
50	50	50	16	77	23	15	83	17
55	0	100	24	73	27	17	83	17
60	100	0	28	73	27	22	75	25
70	Stop	Stop	30	0	100	30	65	35
			35	0	100	35	50	50
			37	85	15	40	0	100
			42	85	15	50	0	100
			42	Stop	Stop	55	100	0
						65	100	0
						65	Stop	Stop

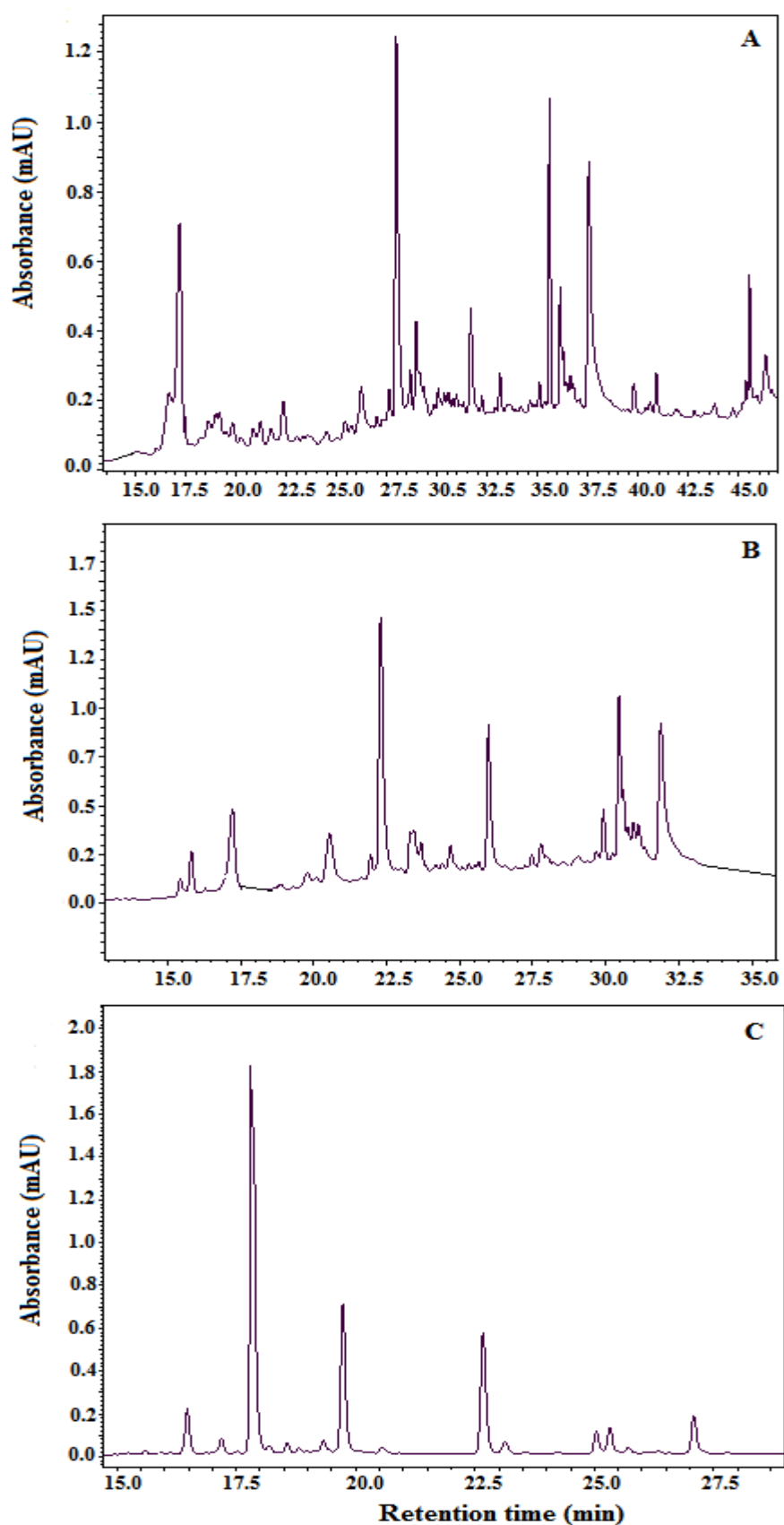


Figure 2.19 HPLC absorbance of phenolic compounds was recorded at 320 nm from the carrot root in each of the three methods A (40% ACN), B (80 % MeOH) and C (70 % MeOH).

As Figure 2.19 shows, methods A and B were not as successful as method C, which gave clearer separation and the greatest number of peaks (according to UV spectra of the phenolics). It also gave the clearest separation of peaks and the flattest baseline. For these reasons, method C was chosen.

2.2.2.4.2 Final method used for main experiments

The method (C) was chosen according to (Bennett *et al.*, 2004) with some modifications by (Almuairfi *et al.*, 2010). 40 mg freeze dried powder was weighed into a 10 mL screw glass-top tube, and the heating block was pre –set to 70 °C to start the extractions. To each sample 950 µL of 70 % v/v HPLC-grade methanol in ultra –pure water was added, the tubes sealed and vortex mixed for 10 seconds. The tubes were opened and 50 µL of 0.1 mg/mL Naringin (naringenin 7- O-retinoside) purchased from Sigma was added as the internal standard (IS), then the tubes sealed and vortex mixed. Samples were extracted for 20 min at 70 °C with vortex mixing every 5min to optimize extraction. The samples were then centrifuged (4000 g, 4 °C, 20 min) and the supernatants processed as follows: 600 µL of the supernatant was transferred to a new micro tube using a Gilson pipette. The samples were completely dried with nitrogen gas using a concentrator system in combination with the heating block set at 50 °C. The residues were re-suspended in the original volume taken with ultra-pure water (Nanopure- Diamond ultra-water system). The samples were vortex mixed for 5-10 min and left to stand at room temperature for 10 min. Then the samples were vortex mixed again and the whole sample taken up into a 1mL syringe and filtered (0.2 µm) into screw top HPLC vials and sealed with standard HPLC vial caps and PTFE inserts, (Supplier VWR International Ltd, UK). The samples were stored at -20 °C in a freezer (not analysed immediately) prior to analysis.

2.2.3.4.3 HPLC analyses

The HPLC column used was a HyperClone Reverse –phase C18 (250 ×4.6mm, 5µm), and column oven was set at 25 °C. The PDA was set to collect all data from 200-600 nm. The detection of the separated compounds was monitored at 280 and 320 nm, with

a flow rate 0.1mL/min, 20µL of each sample was injected into an HPLC (Shimadzu Corporation, Kyoto, Japan), system equipped with a Shimadzu 2 LC-10AD pump, SiL-10A system Auto sampler (controller), SPD-M 10A photodiode array UV-VIS detector, CTO-10AD column oven and CLASSVP.

0.1% v/v trifluoroacetic acid (TFA) in ultra-pure water (solvent A) and 0.1% v/v trifluoroacetic acid in HPLC- grade acetonitrile ACN (solvent B), and the solvent gradient was: (H₂O+ 0.1% TFA): (ACN+0.1% TFA), 0 min (100:0), 5 min (100:0), 15 min (83:17), 17 min (83:17), 22 min (75:25), 30 min (65:35), 35 min (50:50), 40 min (0:100), 50 min (0:100), 55 min (100:0) and 65 min (100:0).

2.2.3.4.4 Quantification of phenolic compounds

A diode-array detector was set at 320 nm and 520 nm to detect the phenolic acids and anthocyanin, respectively, in extracted lettuce and carrot samples. Table 2.3 shows the different standards that were tested for the identification, where all the phenolic acids represent compounds that have been reported to be present in lettuce and/or carrots (Tackenberg, 2007; Kreutzmann *et al.*, 2008; Bumgarner *et al.*, 2012). The quantification of the concentration of unknown phenolics was carried out as chlorogenic acid standard equivalents for those phenolic acids which could not be identified individually, but where the peaks had absorption spectra characteristic of caffeic acid derivatives (a broad peak near 325 nm with a shoulder near 295 nm), Figure 3.44.

For lettuce the anthocyanin concentration (Figure 3.40) was expressed in mg of cyanidin 3-O-glucoside equivalents at 520 nm as in (McCann *et al.*, 2005), (Figure 7.7 Appendix). For peaks with absorption spectra resembling quercetin or kaempferol derivatives (peaks near 354 nm or 255 nm) the concentrations were calculated as rutin equivalents.

Some phenolic compounds were found where the retention time and absorption spectra resembled literature description of compounds identified in carrot root such as *Cis*-5-caffeoylquinic acid (Samuoliene and Duchovskis, 2006; Tackenberg, 2007; Simoes *et al.*, 2011), where however the identity could not be confirmed, as it was not possible to find a commercial standard.

Table 2.3 List of standards used to compare and identify phenolic compounds. All standards were prepared as stock concentration of 0.1 mg/mL in 70 % Methanol.

Scientific name	Common name	CAS No	Sourced
3-Caffeoylquinic acid	Chlorogenic acid	327-97-9	Chemstrong scientific Co Ltd
4-Caffeoylquinic acid	Cryptochlorogenic acid	905-99-7	Biopurify Phytochemicals Ltd
5-Caffeoylquinic acid	Neochlorogenic acid	906-33-2	Biopurify Phytochemicals Ltd
1,3-Dicaffeoylquinic acid	Cynarin, Cynarine	19870-46-3	Biopurify Phytochemicals Ltd
3,5-Dicaffeoylquinic acid	Isochlorogenic acid A	2450-53-5	Biopurify Phytochemicals Ltd
3,4-Dicaffeoylquinic acid	Isochlorogenic acid B	14534-61-3	Biopurify Phytochemicals Ltd
4,5-Dicaffeoylquinic acid	Isochlorogenic acid C	32451-88-0	Biopurify Phytochemicals Ltd
Dicaffeoyltartaric acid	Cichoric acid	70831-56-0	Biopurify Phytochemicals Ltd
Caffeoyltartaric acid	Caftaric acid	67879-58-7	Biopurify Phytochemicals Ltd
3,4-Dihydroxycinnamic	Caffeic acid	331-39-5	Sigma-Aldrich Company Ltd
4-Hydroxycinnamic acid	P-coumaric acid	501-98-4	Sigma-Aldrich Company Ltd
Quercetin-3-O-rutinoside	Rutin	153-18-4	Sigma-Aldrich Company Ltd
Cyanidin 3-O-glucoside	Kuromanin chloride	7084-24-4	Sigma-Aldrich Company Ltd

2.3 Statistical analysis

All data were subjected to statistical analysis using Minitab 16, ANOVA- general linear model (GLM). Statistically the different experiment variables and their interactions were determined. Thereafter the whole data sets were tested for normality distribution of residual data using the Anderson-Darling test. The $P < 0.05$ was considered a significance level. Where the residuals were not normally distributed ($P < 0.05$) the data were either logarithmically or square root transformed. Tukey's test was used for pair wise comparisons between the temperature regimes' effects.

Leaf production rate was calculated as the slope of the linear relationship between leaf number recorded and weeks for each replication of each experiment. Then the gradient values were analysed using Minitab 16, ANOVA (GLM).

All experiments were carried out using triplicate samples of each variety. The results show the mean \pm standard error of mean (SEM). All the results were expressed as milligram per 100 gram fresh weight (FW). Analysis of values on a fresh weight basis is consistent with the inclusion and consumption of these vegetables in the fresh rather than dried state in human nutrition. However, some compounds were also analysed as milligram per g dry weight (DW).

The residue (non-sugar or insoluble solids) mg/100g FW was calculated by subtracting the sugar content (mg sugar /100g FW) from dry matter (g dry weight /100g FW) values.

CHAPTER 3 . RESULTS

3.1 Growth rate parameters

3.1.1 Effect of temperature on rate of leaf production and harvest period

There was a large effect of temperature regime on growth of lettuce and carrot varieties, the rate of leaf production and consequently harvest time (Figures 3.1, 3.3, 3.4, 3.5, and 3.6). Low temperature regimes resulted in slow growth and leaf production and thus a longer period until harvest as plants were harvested when they had produced 10-12 leaves (lettuce) or 6-8 leaves (carrot). In contrast, high temperature encouraged rapid growth.

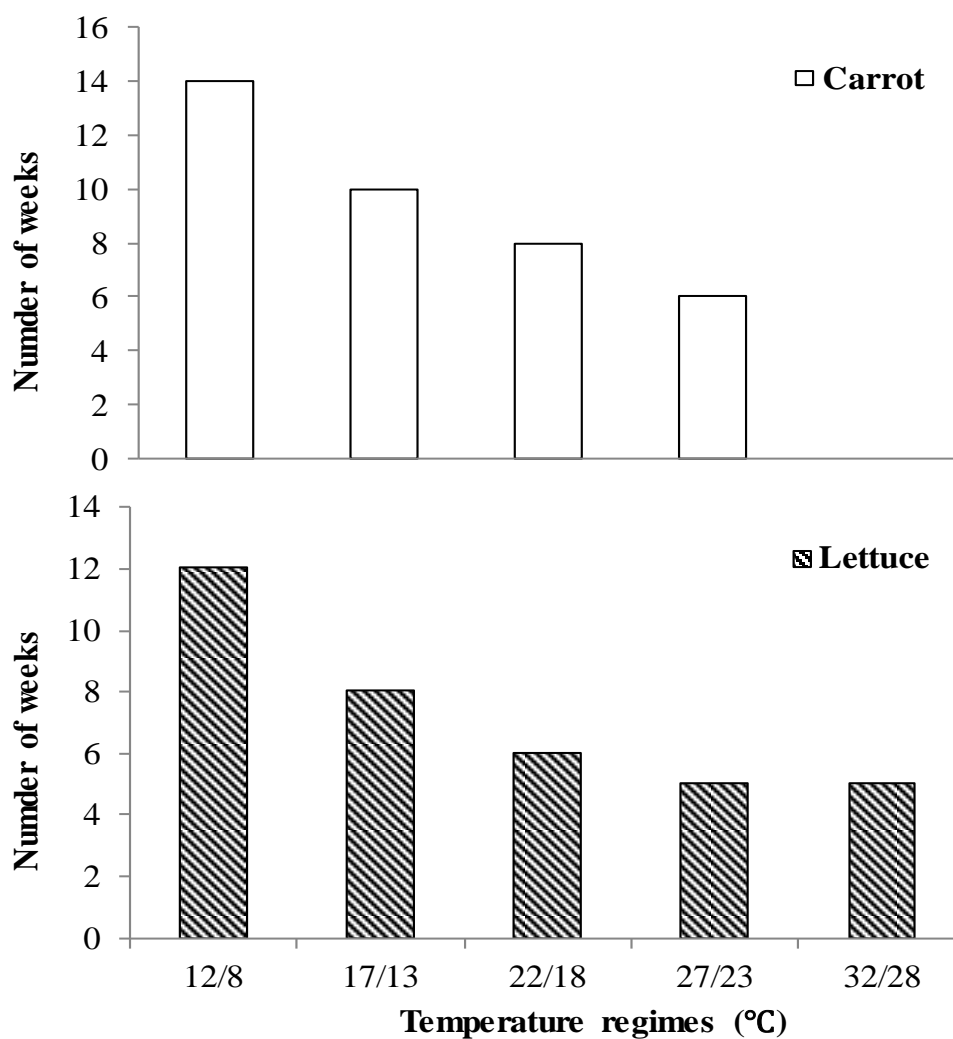


Figure 3.1 Effects of different temperatures on weeks to harvest of lettuce and carrot.

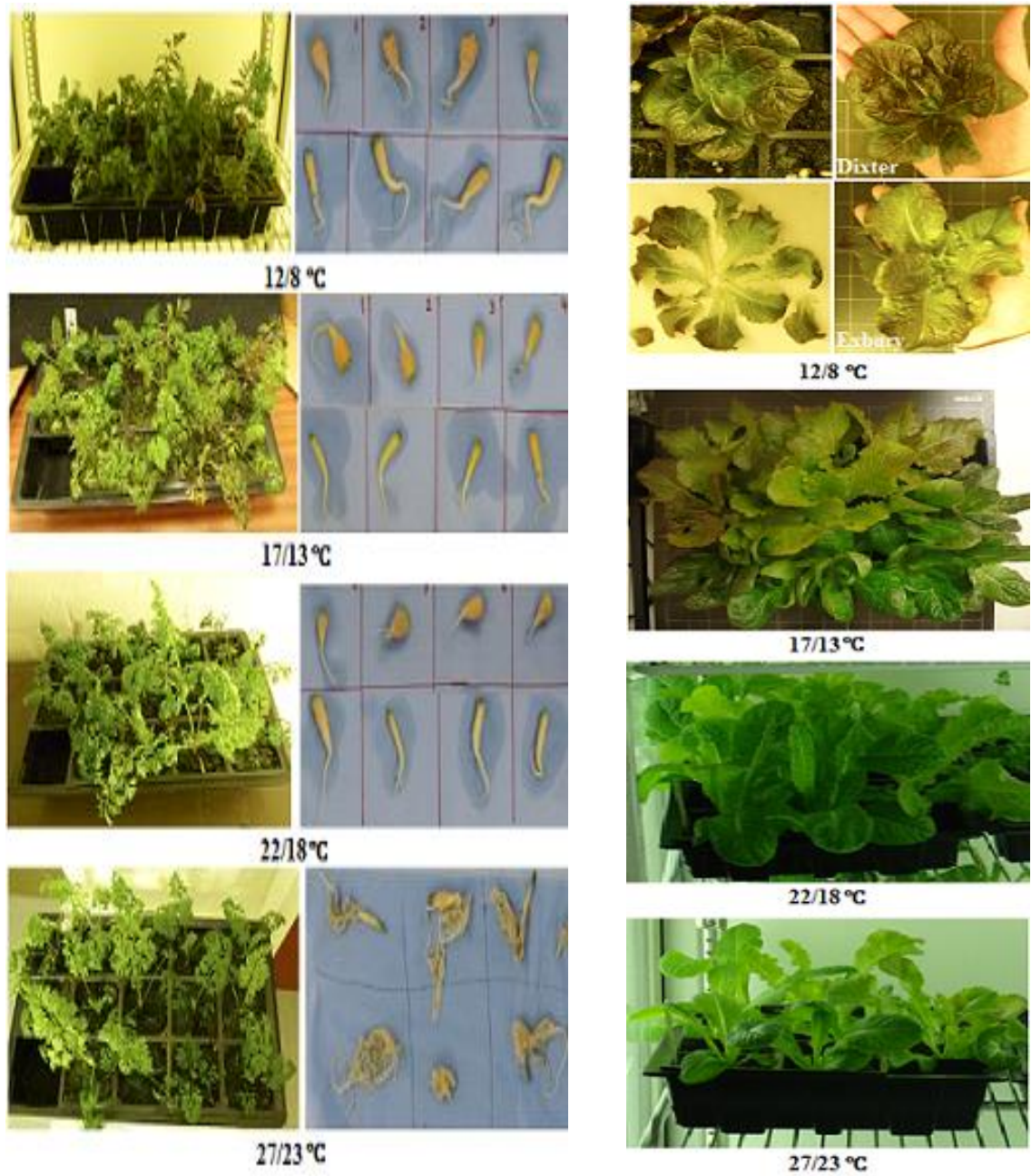


Figure 3.2 The effect of temperature regimes on shoot and root growth of carrot varieties (Parmex and Little Finger) (left side) and lettuce varieties (Dixter and Exbury) (right side) at harvest stage. The carrot root length was approximately 3-5 cm.

3.1.2 Leaf production rate

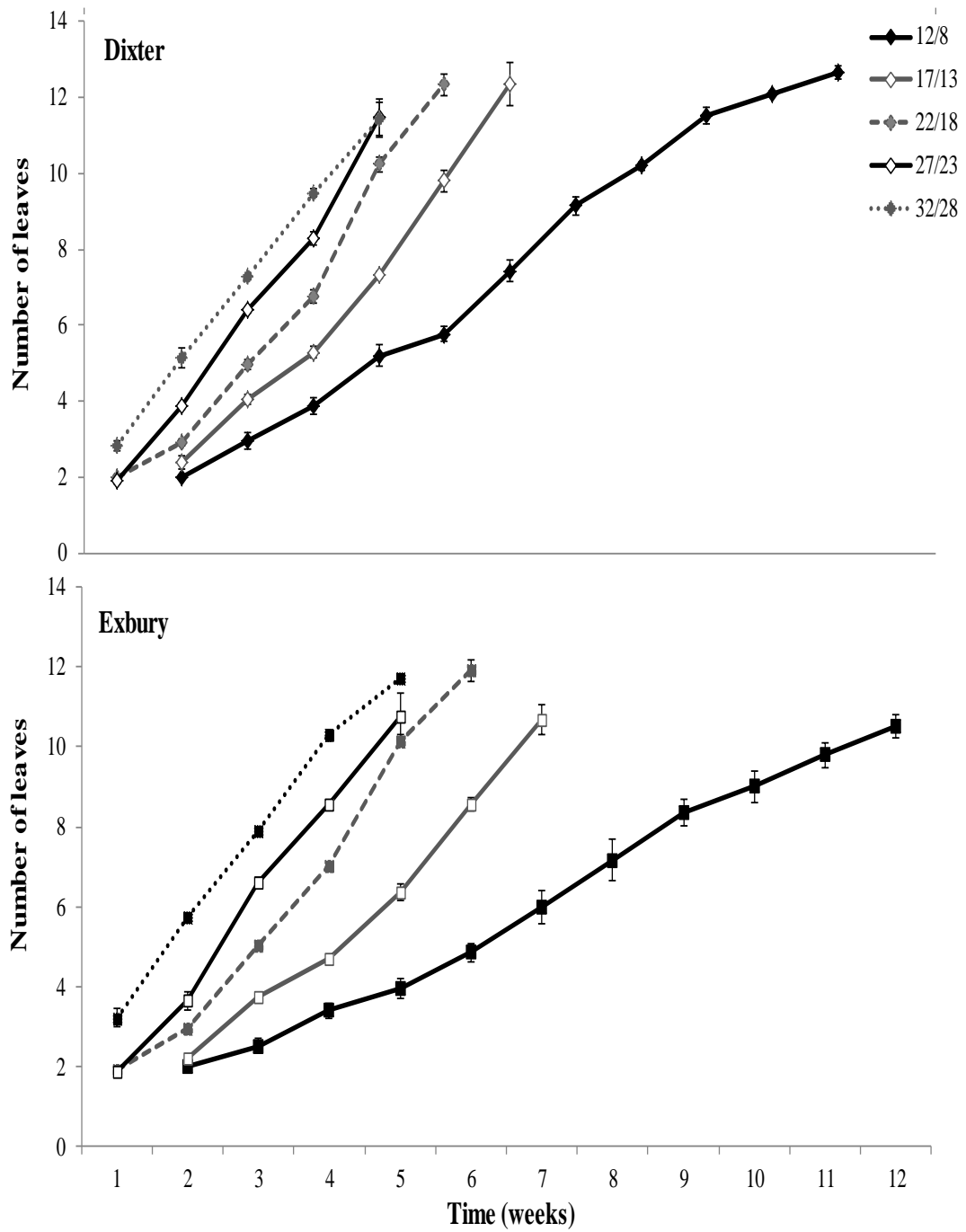


Figure 3.3 Effect of temperature regimes on leaf production rate of lettuce varieties (Dexter and Exbury).

There was as a significant effect of varieties ($P < 0.05$), and a highly significant effect of temperature regime ($P < 0.001$) on the rate of leaf production in both varieties of lettuce, at the low temperature (12/8 °C) both varieties had the lowest rate of leaf production, but no significant interaction effects were found between the varieties and temperature, (Figure 3.4).

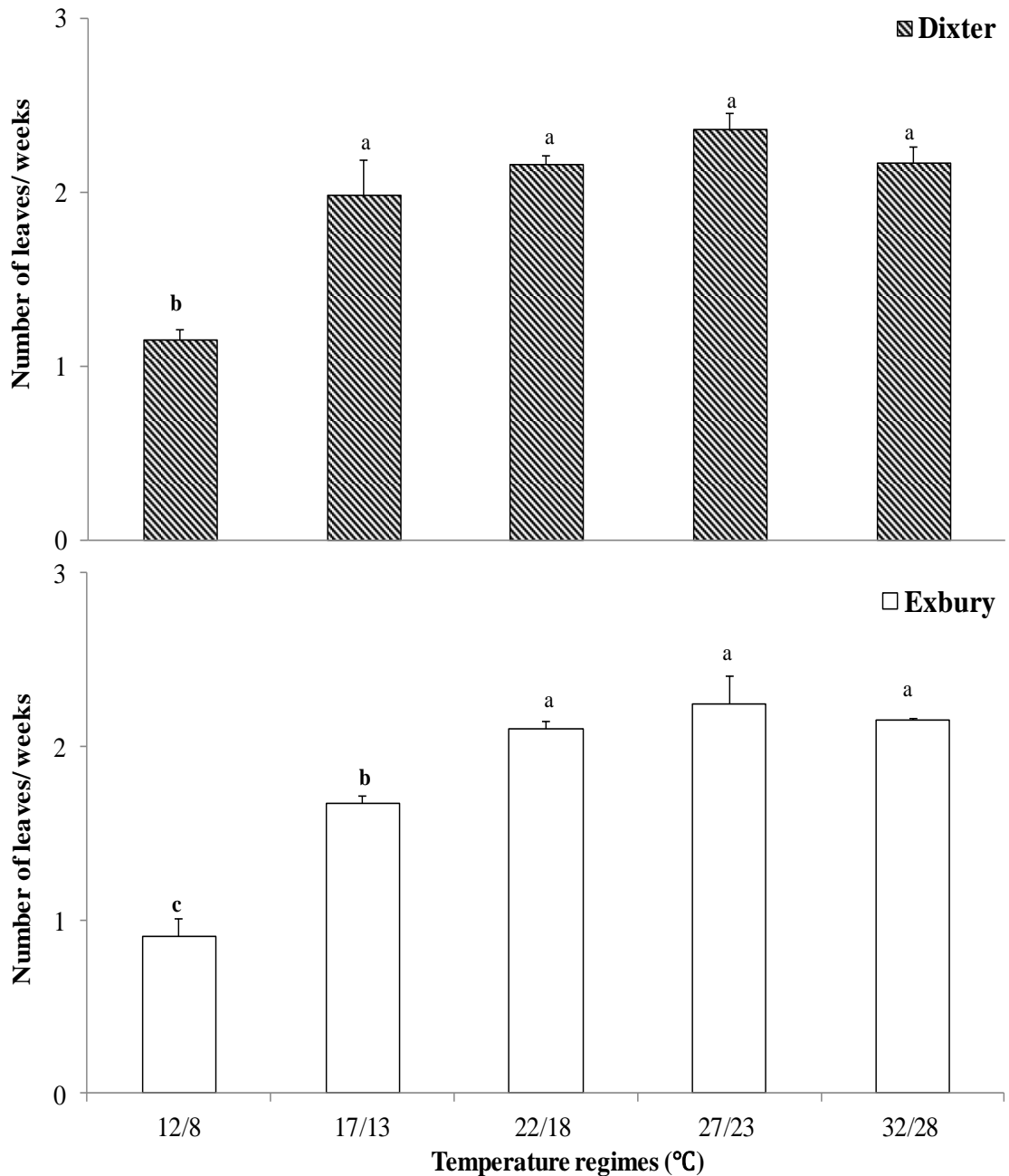


Figure 3.4 The effect of lettuce variety (Dixter and Exbury) and different growth temperature on the average rate of leaf production until harvest. Means with different letters differ significantly at ($P < 0.05$), error bars indicate Standard Error of Mean, ($n = 3$).

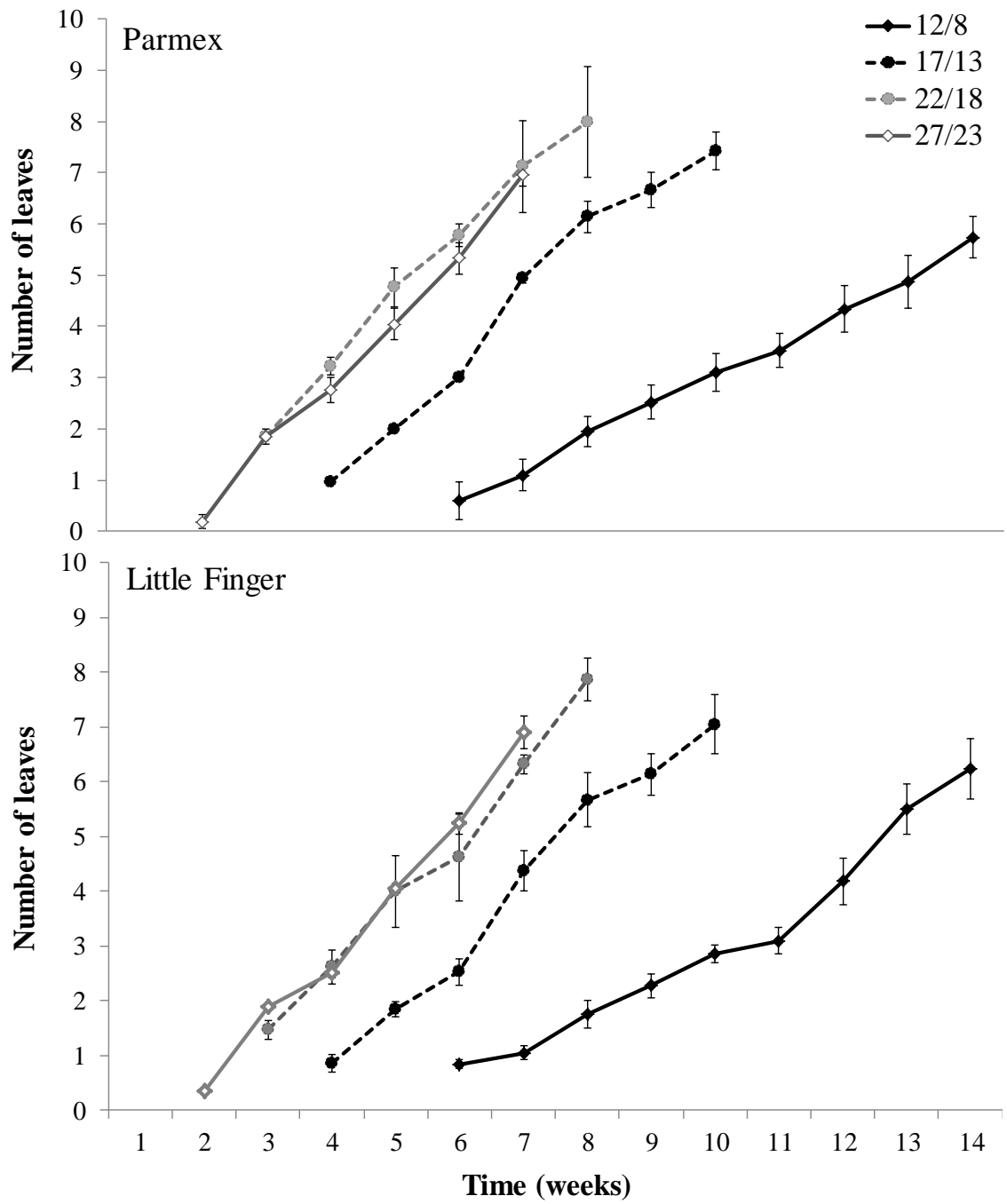


Figure 3.5 Effect of temperature regimes on leaf production rate of carrot (Parmex and Little Finger).

For carrot, there was no significant effect of variety, but there was a significant effect of temperature regime ($P < 0.001$) on the rate of leaf production, at the low temperature regime (12/8 °C) both varieties had the lowest rate of leaf production. No significant interaction effects were found between the varieties and temperature, (Figure 3.6).

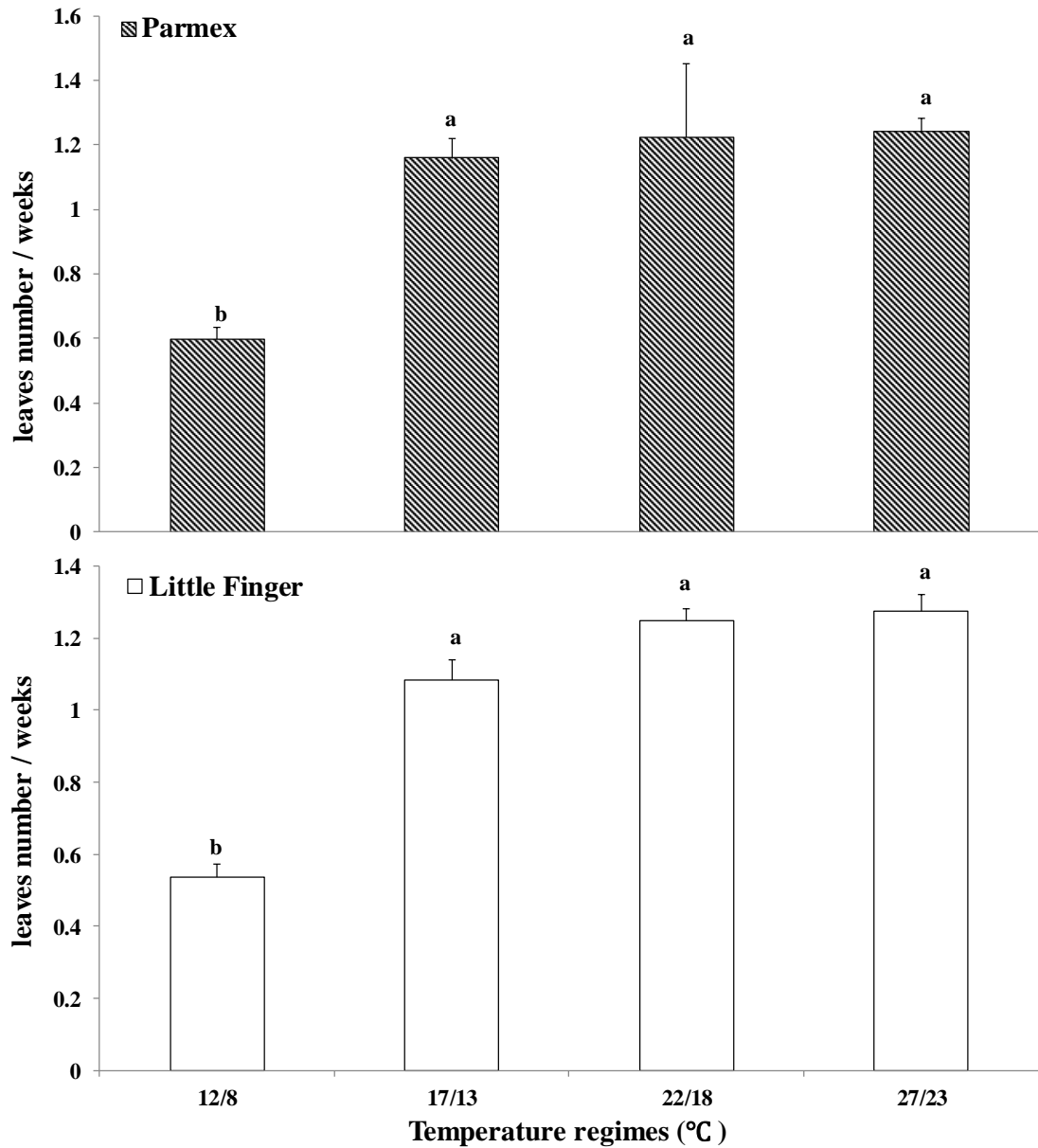


Figure 3.6 The effect of carrot variety (Parmex and Little Finger) and different growth temperatures on the average rate of leaf production until harvest. Means with different letters differ significantly at ($P < 0.05$), error bars indicate Standard Error of Mean (SEM), ($n = 3$).

3.1.3 Plant height (growth rate)

There were consistent and large effects of temperature regimes on plant height with both varieties of lettuce and of carrot showing a similar response (Figure 3.7).

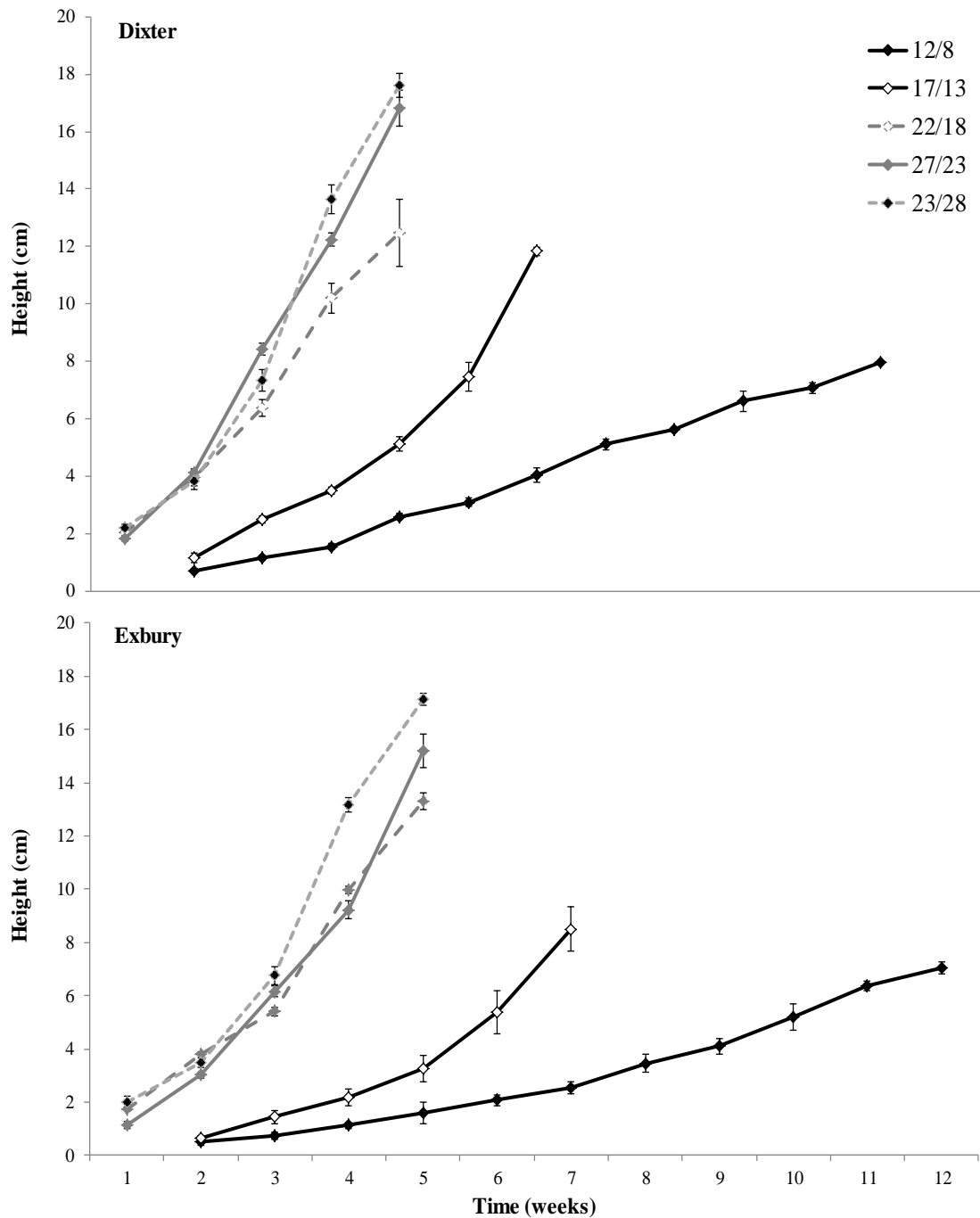


Figure 3.7 Effect of temperature regimes on height of lettuce (Dexter and Exbury).

For lettuce, there was a significant effect of variety ($P < 0.01$) and a highly significant effect of temperature regime ($P < 0.001$) on plant height, an plant height increased with increasing temperature, at the high temperature regime (32/28 °C) both varieties had the highest plant height. No significant interactions were found between the varieties and temperature, (Figure 3.8).

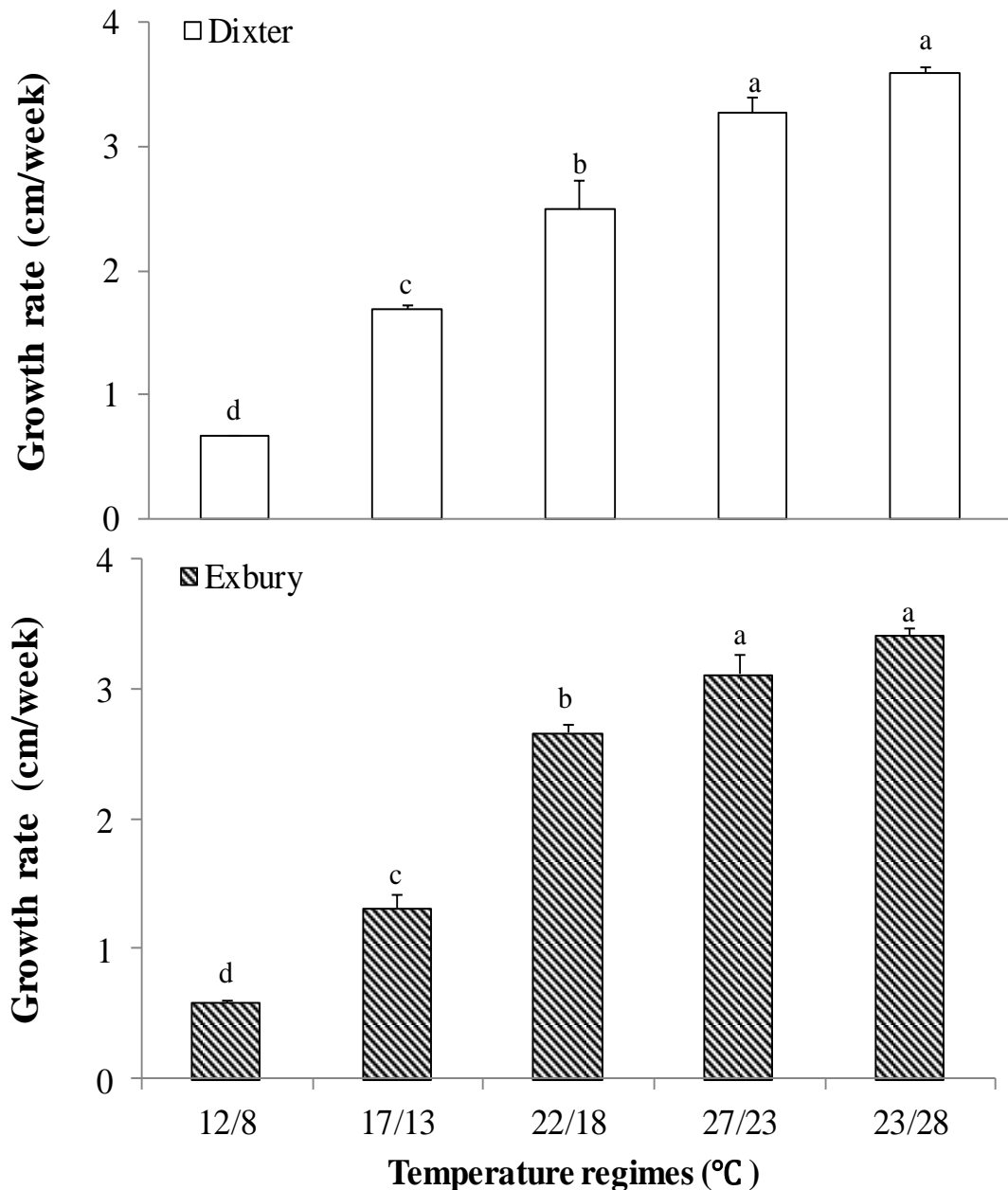


Figure 3.8 The effect of lettuce variety (Dexter and Exbury) and different growth temperature regimes on growth rate (as height change) until harvest (when the plants had 10-12 leaves). Means with different letters differ significantly at ($P < 0.05$), error bars indicate Standard Error of Mean, ($n = 3$).

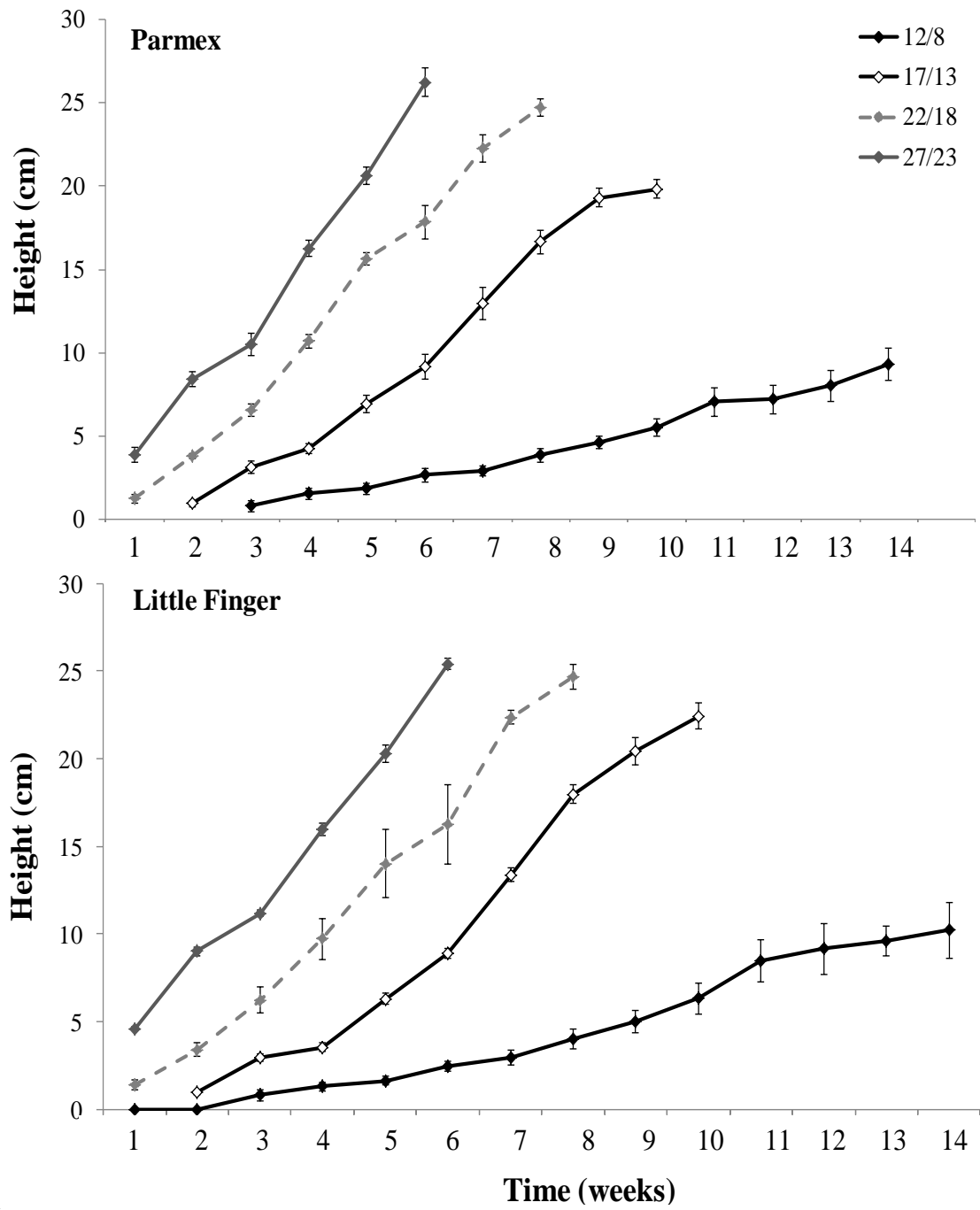


Figure 3.9 Effect of temperature regimes on height of carrot (Parmex and Little Finger).

There was no significant effect of variety but a highly significant effect of temperature on height ($P < 0.001$); the plant height increased with the increasing temperature, moreover, no significant interaction was found between the varieties and temperature, (Figure 3.10).

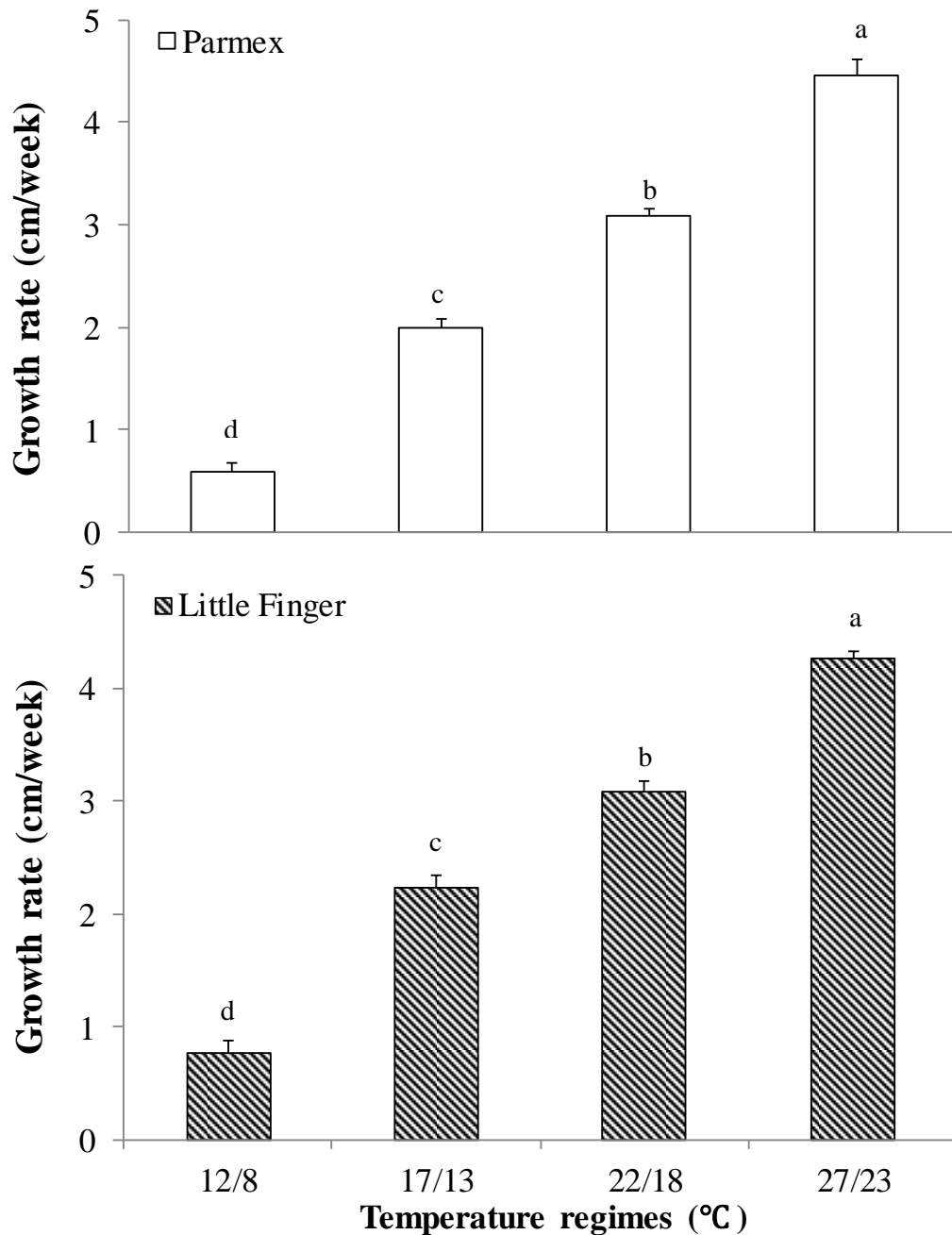


Figure 3.10 The effect of carrot variety (Parmex and Little Finger) and different growth temperature regimes on growth rate (as height change) until harvest (when the plants had 6 - 8 leaves). Means with different letters differ significantly at ($P < 0.05$), error bars indicate Standard Error of Mean, (n=3).

3.1.4 Leaf area

There was a significant effect of varieties ($P < 0.001$) and a highly significant effect of temperature on leaf area ($P < 0.001$) of lettuce varieties. The highest leaf area was at a moderate temperature regime (22/18 °C) of both varieties. As well as a significant interaction effect between variety and temperature ($P < 0.05$), for the variety Dexter there was a gradual increase in leaf area at (22/18 °C) which began to decrease at temperatures above this regime. Exbury had smaller leaf area at the lower and higher temperature regimes and larger at the moderate temperature regime (22/18 °C), (Figure 3.11).

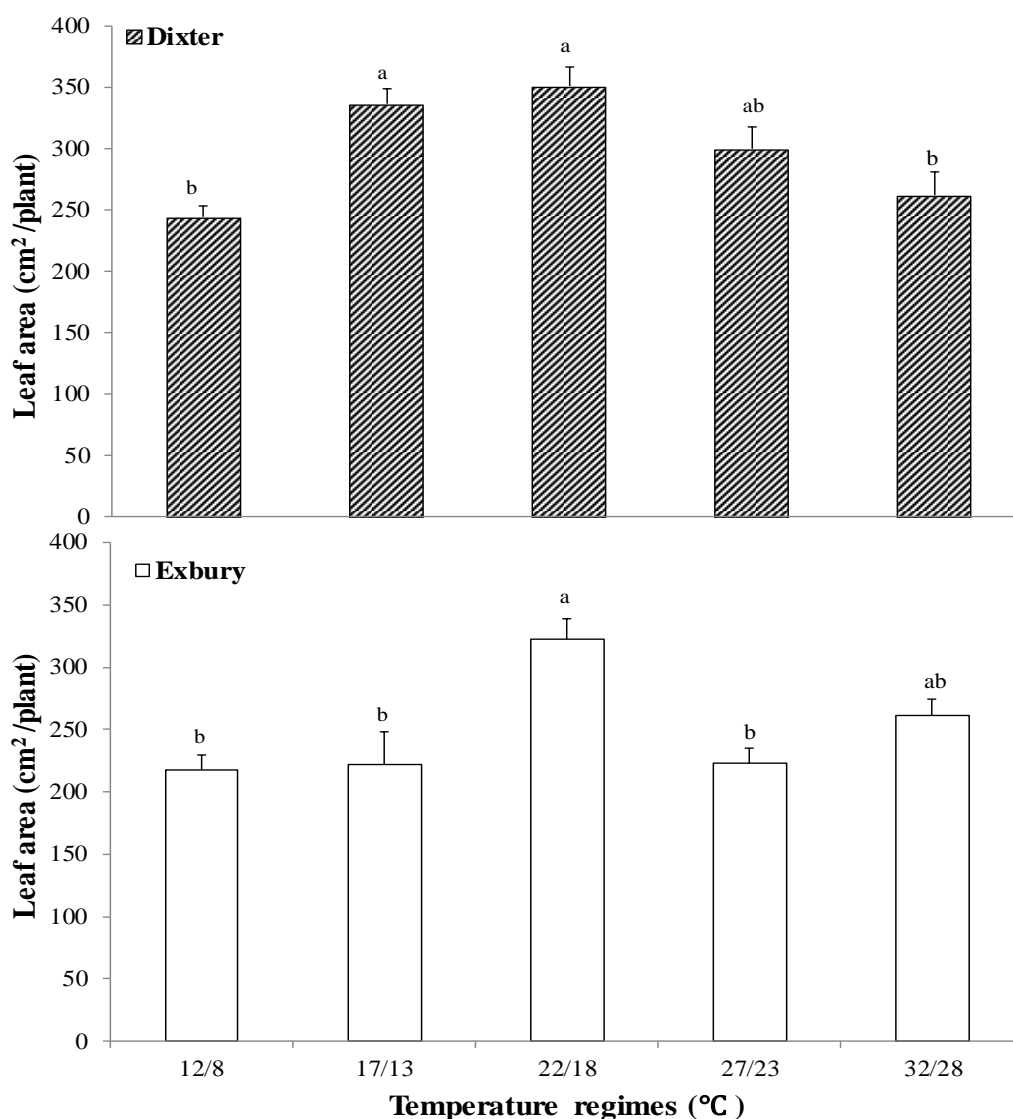


Figure 3.11 The effect of lettuce variety (Dexter and Exbury) and temperature regimes on leaf area. Means with different letters are significantly different ($P < 0.05$), error bars indicate Standard Error of Mean, ($n = 3$).

3.1.5 Fresh and dry weight (plant biomass)

There were highly significant effects of variety ($P < 0.001$) and temperature ($P < 0.001$) on fresh weight of lettuce leaves and roots of both varieties. Furthermore a significant interaction ($P < 0.01$) was found between variety and temperature. Whilst the fresh weight of Exbury was similar at all temperature regimes, Dexter produced the highest fresh weight at 17/13 °C, and generally growth of this variety was better when grown at lower temperature regimes (Figure 3.12).

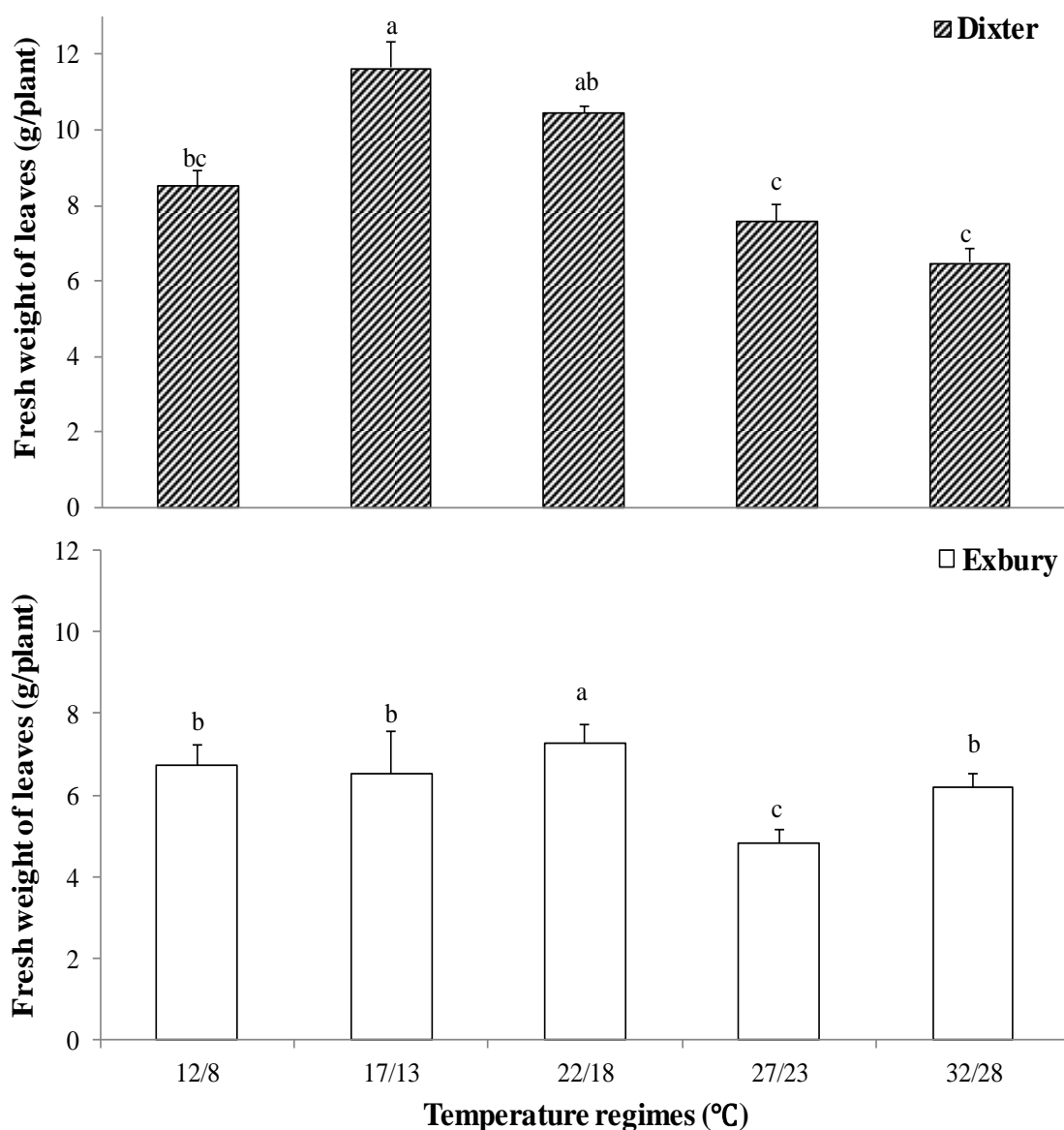


Figure 3.12 The effect of lettuce variety (Dexter and Exbury) and temperature regimes on leaf fresh weight per plant. Means with different letters are significantly different ($P < 0.05$), error bars indicate Standard Error of Mean, ($n = 3$).

Also there were highly significant effects of variety ($P < 0.001$) and temperature ($P < 0.001$) on dry weight of lettuce leaves and roots of both varieties and a significant interaction ($P < 0.001$) was found between variety and temperature. Again varieties differed in their response: Dixter had the highest DW at the lowest regimes and decreased gradually as temperature increased, while Exbury had a significantly lower DW at a temperature regime of (27/ 23 °C) than other regimes, (Figure 3.13).

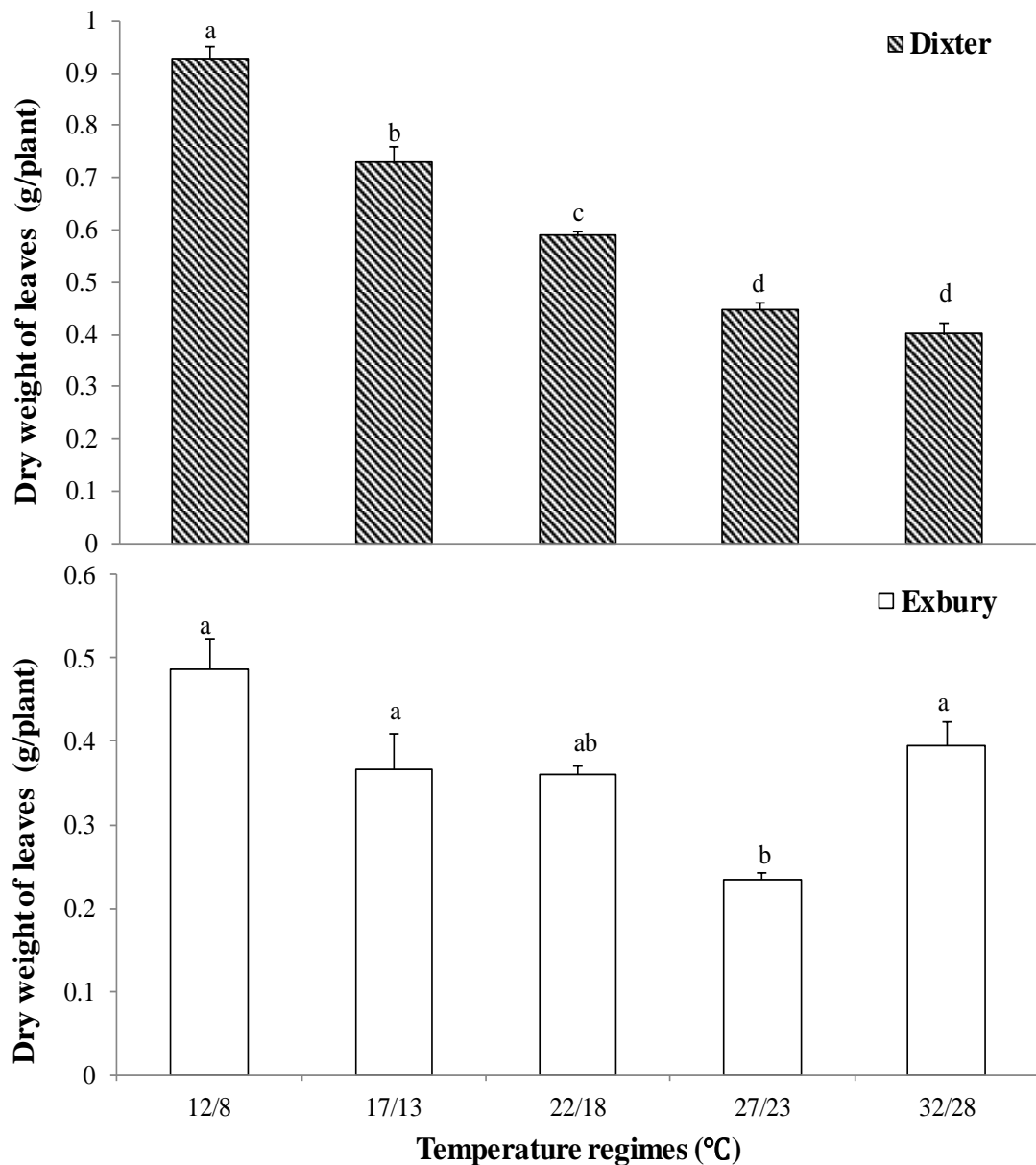


Figure 3.13 The effect of lettuce variety (Dixter and Exbury) and temperature regime on leaf dry weight. Means with different letters are significantly different ($P < 0.05$), error bars indicate Standard Error of Mean, ($n = 3$).

For carrot, there were significant effects of variety ($P < 0.001$) and temperature regime ($P < 0.001$) on fresh weight of carrot root. Increasing temperature to 27 °C inhibited carrot root growth. Significant interaction ($P < 0.001$) was found between variety and temperature, Parmex had higher FW than Little Finger at all temperature regimes with comparable response, (Figure 3.14). There were also significant effects of temperature on fresh weight of carrot leaves (Figure 7.7 Appendix).

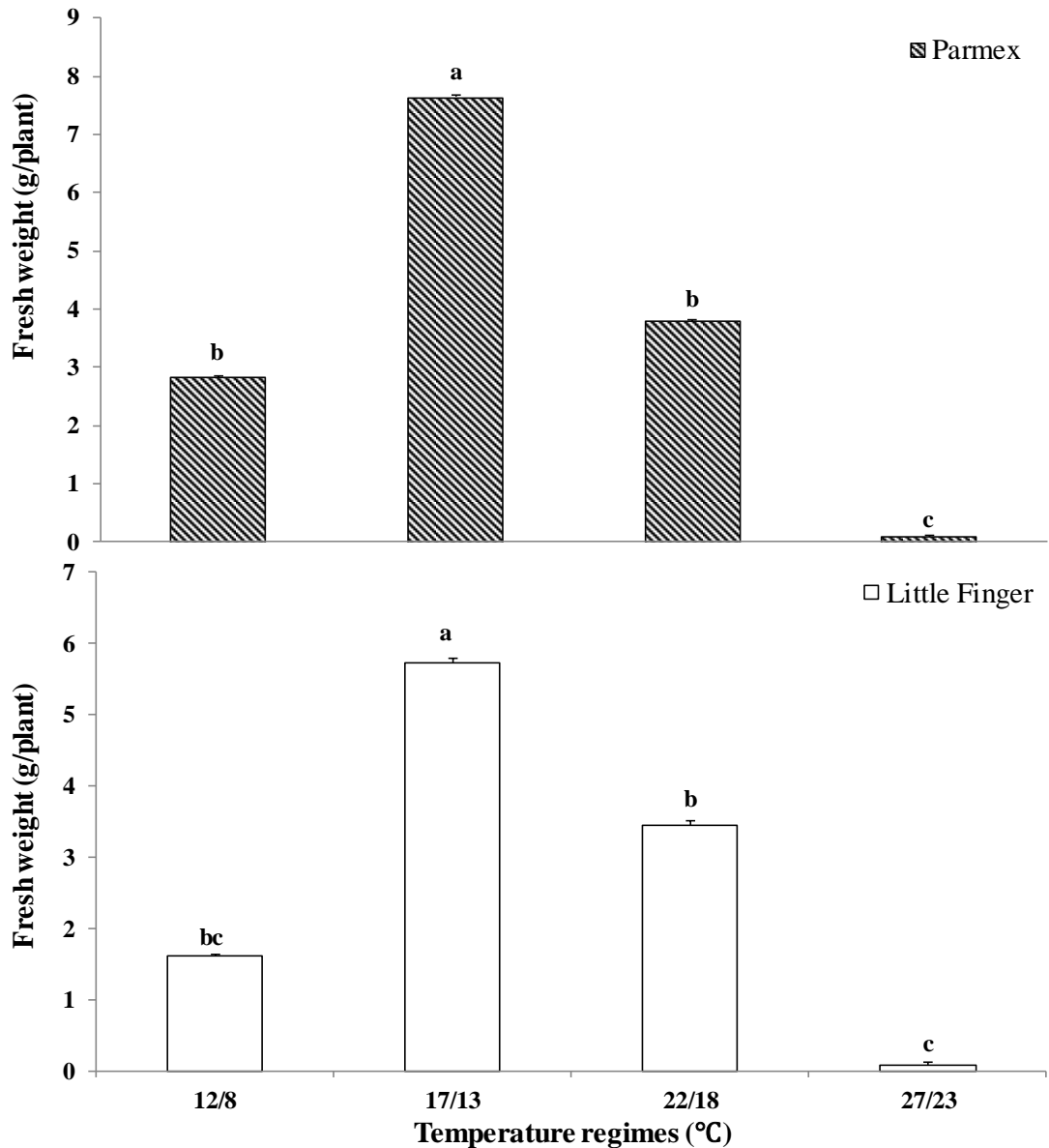


Figure 3.14 The effect of carrot variety (Parmex and Little Finger) and temperature regimes on root fresh weight. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, (n=3).

There were also highly significant effects of variety ($P < 0.001$), temperature regimes ($P < 0.001$) on dry weight of carrot root and a significant interaction ($P < 0.001$) between variety and temperature (Figure 3.15). At 17/13 °C both carrot varieties had the highest dry weight of roots. There were also significant effects of temperature on dry weight of carrot leaves (Figure 7.23 Appendix).

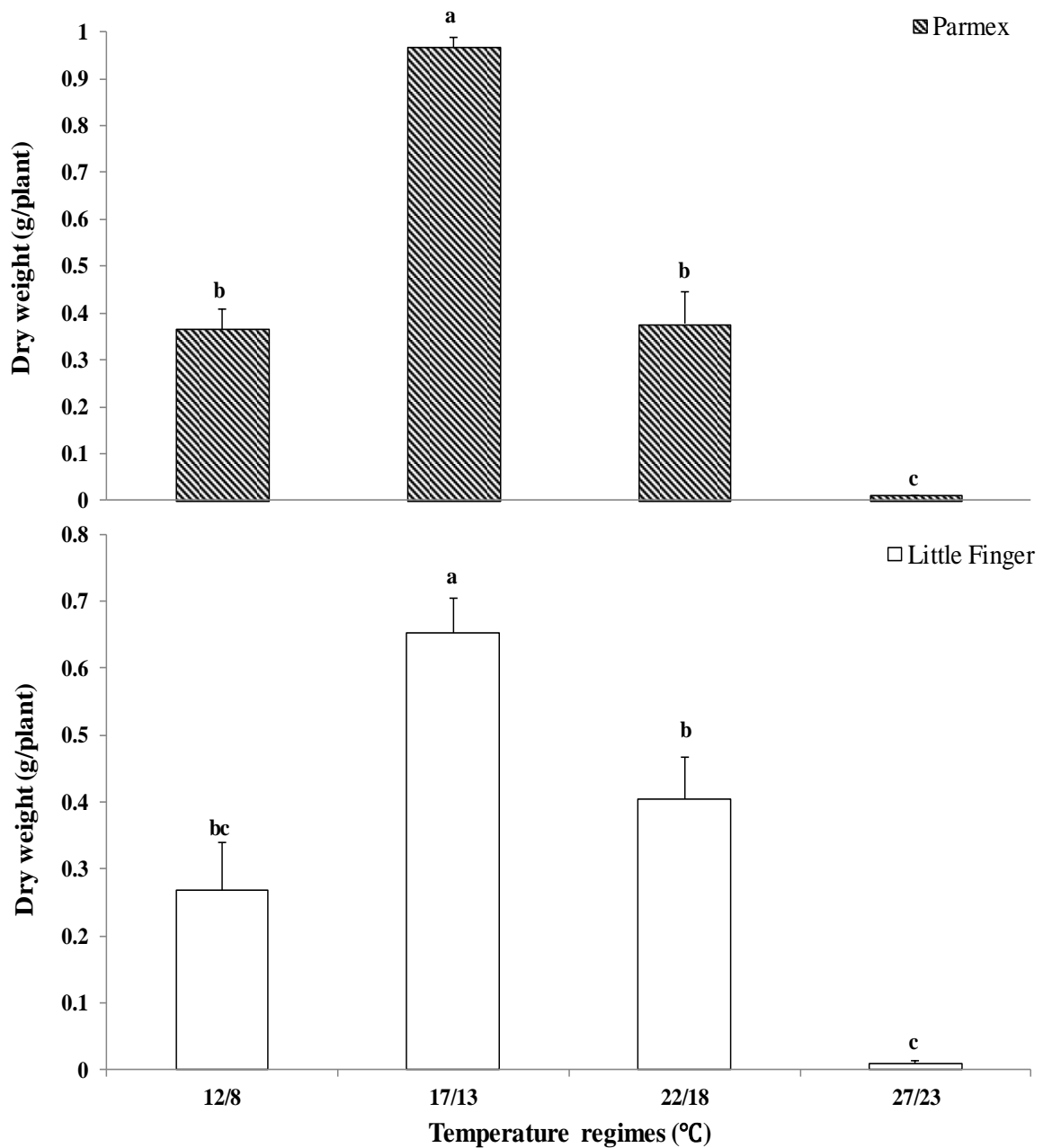


Figure 3.15 The effect of carrot variety (Parmex and Little Finger) and temperature regimes on root dry weight. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n=3$).

3.1.6 Dry matter (%)

There were highly significant effects of variety ($P < 0.001$) and temperature regimes ($P < 0.001$) on dry matter % leaves and root of lettuce varieties.

The highest DM % was at a low temperature regime (12/8 °C) of both varieties (Dixter and Exbury). A highly significant interaction effect ($P < 0.001$) was found between variety and temperature. At low temperature (12/8 °C) Dixter had higher DM % than Exbury, but with other regimes the DM % was broadly similar, (Figure 3.16).

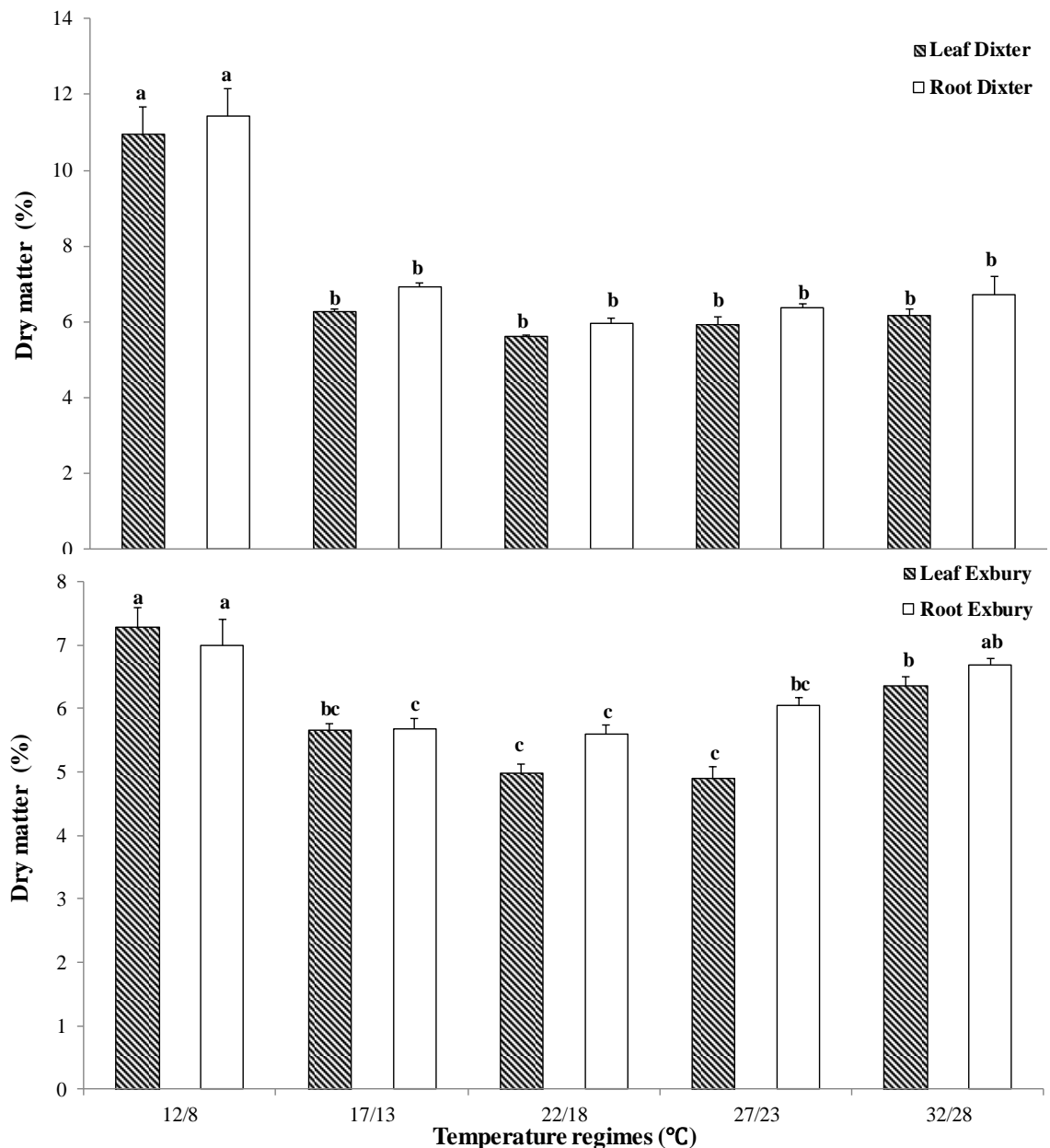


Figure 3.16 The effect of lettuce variety (Dixter and Exbury) and temperature regimes on dry matter content of leaves and root. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

For carrot, there were no significant effects of variety on leaf dry matter percentage, but there was a significant effect on the root ($P < 0.05$). Temperature regimes had significant effects ($P < 0.001$ and $P < 0.05$) on dry matter percentage of leaf and root respectively. No significant interaction for leaf and root was found between variety and temperature, (Figure 3.17).

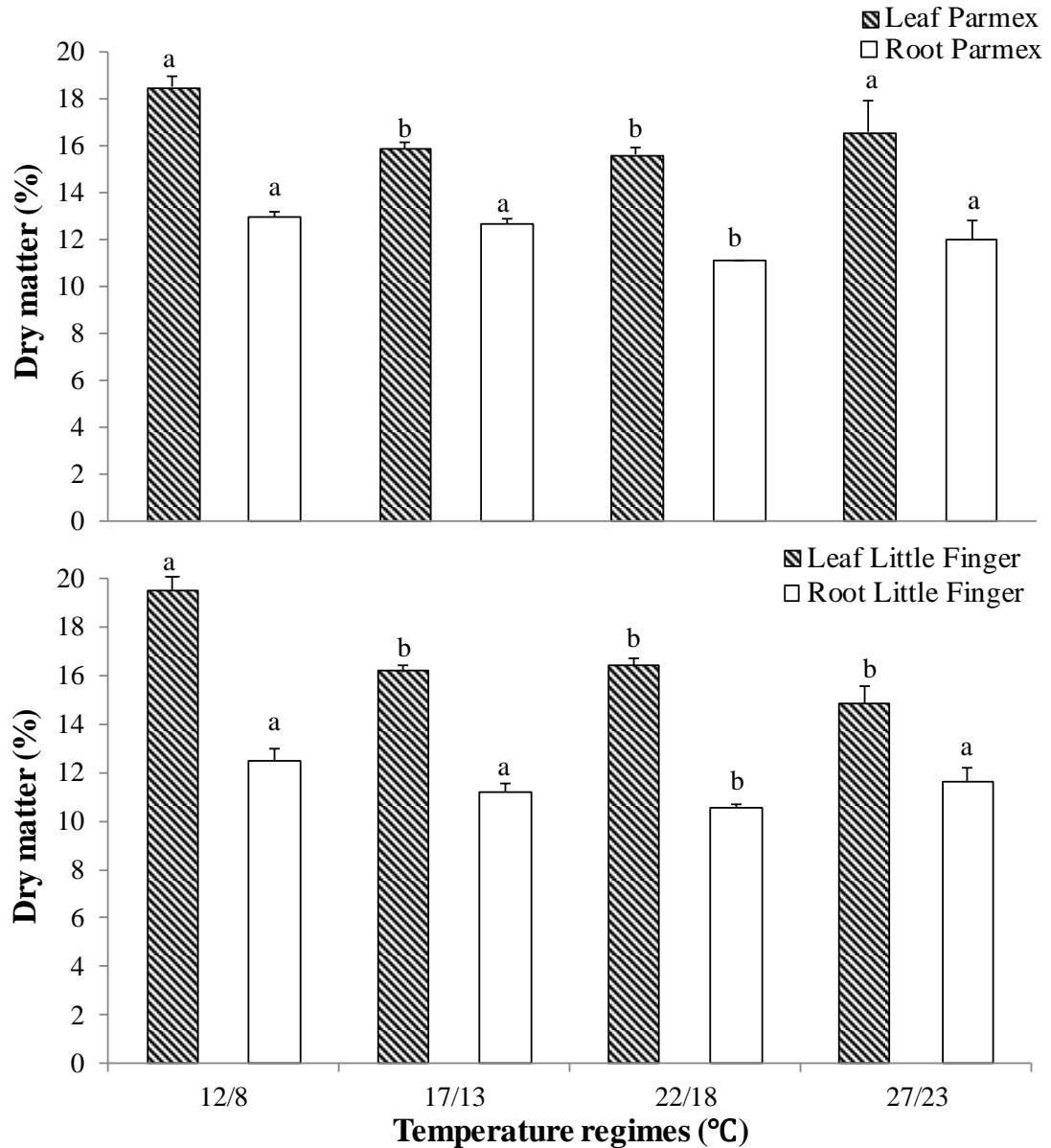


Figure 3.17 The effect of carrot variety (Parmex and Little Finger) and temperature regime on dry matter content of leaves and root. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

3.1.7 Root /shoot dry weight

There was a significant effect of variety ($P < 0.001$) and highly significant effect of temperature ($P < 0.001$) on root/shoot dry weight ratio of lettuce. The root /shoot ratio was greater at the lowest temperature (12/8 °C) than higher temperature regimes: there was also a significant interaction ($P < 0.001$) between variety and temperature. Dexter had a higher ratio than Exbury at all temperature regimes (Figure 3.18).

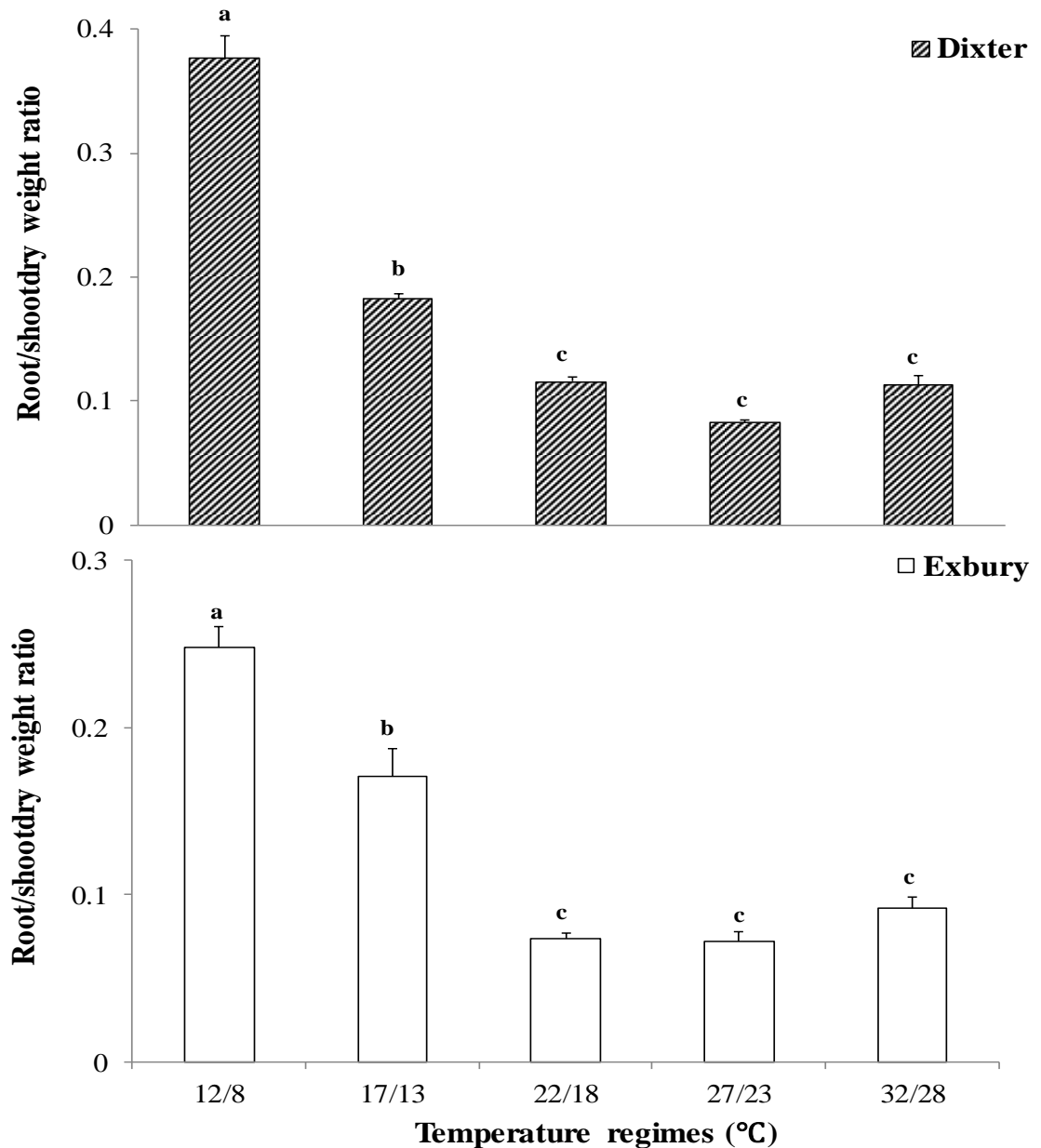


Figure 3.18 The effect of lettuce variety (Dexter and Exbury) and temperature regimes on root/shoot dry weight ratio. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

For carrot, there were a significant effect of variety ($P < 0.01$) and temperature regimes ($P < 0.001$) on root/shoot dry weight ratio of variety, the highest root/ shoot ratio was at 17/13 °C, and at high temperature, the growth of the root was inhibited. Significant interaction ($P < 0.01$) between variety and temperature, Parmex had a higher root/shoot dry weight ratio than Little Finger at all temperature regimes, (Figure 3.19).

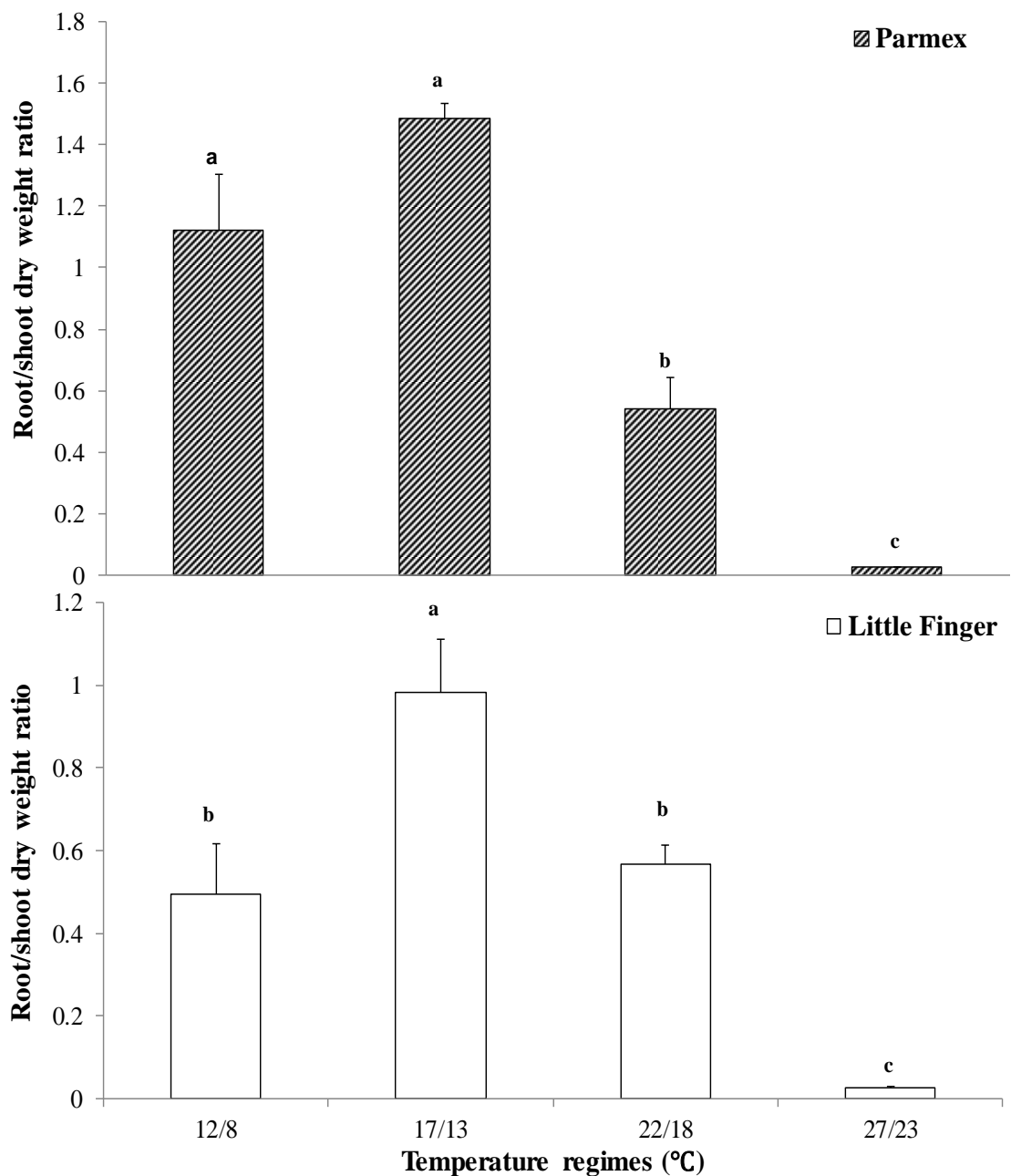


Figure 3.19 The effect of carrot variety (Parmex and Little Finger) and temperature regimes on root/shoot dry weight ratio. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

3.2 Phytochemicals

3.2.1 Sugar content (soluble solids)

There were significant effects of variety ($P < 0.01$) on sugar content of both leaf and root: lettuce Dixter had higher sugar content than Exbury. Temperature regimes had a highly significant effect ($P < 0.001$) on shoot and root sugar contents of both lettuce varieties. The highest content of sugar was at low temperature (12/8 °C) and it decreased as temperature increased (Figure 3.20). Moreover a significant interaction ($P < 0.001$) was found between variety and temperature regime of both leaf and root of lettuce variety.

The non-sugar content (insoluble solids) of lettuce varied between the varieties and plant parts. There were no significant effects of variety on leaf and root, but significant effects of temperature regimes ($P < 0.001$) on both leaf and root. No significant interaction effects were found between the varieties and temperature for leaf, but there was significant effect of a root ($P < 0.05$), (Figure 3.20).

For carrot, there were no significant effects of variety on sugar content of leaf but there were significant effects ($P < 0.001$) on root.

There were highly significant effects ($P < 0.001$) of temperature on sugar content of both leaf and root of carrot varieties. No significant interaction effects were found between the varieties and temperature regimes of leaf or of root Figure 3. 21.

There were no significant effects of variety on either leaf or root non-sugar content (insoluble solids) of carrot varieties, but significant effects of temperature regimes ($P < 0.01$) on both leaf and root contents. No significant interaction effects were found between the varieties and temperature for leaf but there was a significant interaction for root, Figure 3. 21.

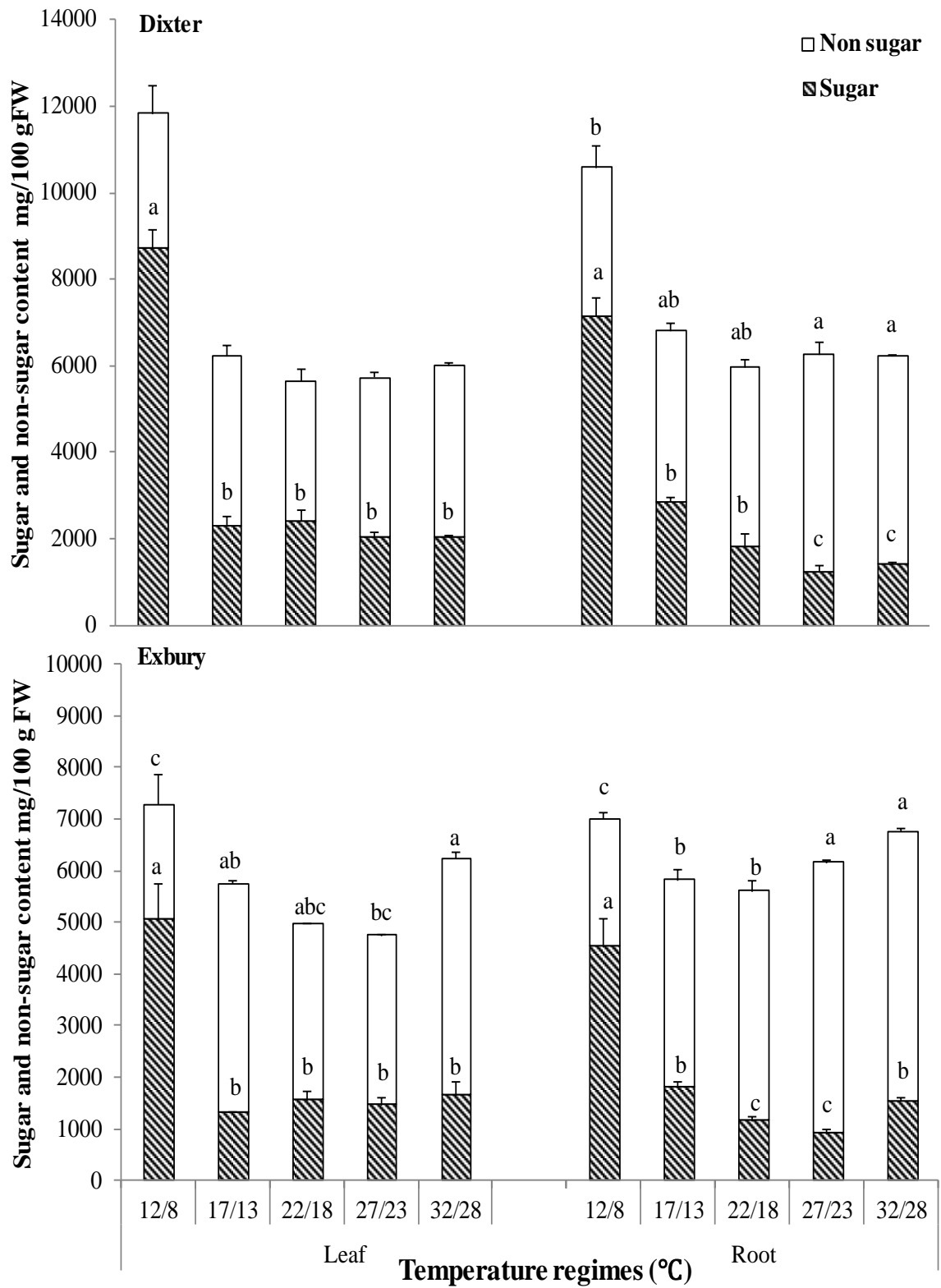


Figure 3.20 The effect of lettuce variety (Dexter and Exbury) and temperature on sugar and non-sugar content of leaves and root. Means with different letters are significantly different ($P < 0.05$). Error bars indicated SEM, (n=3).

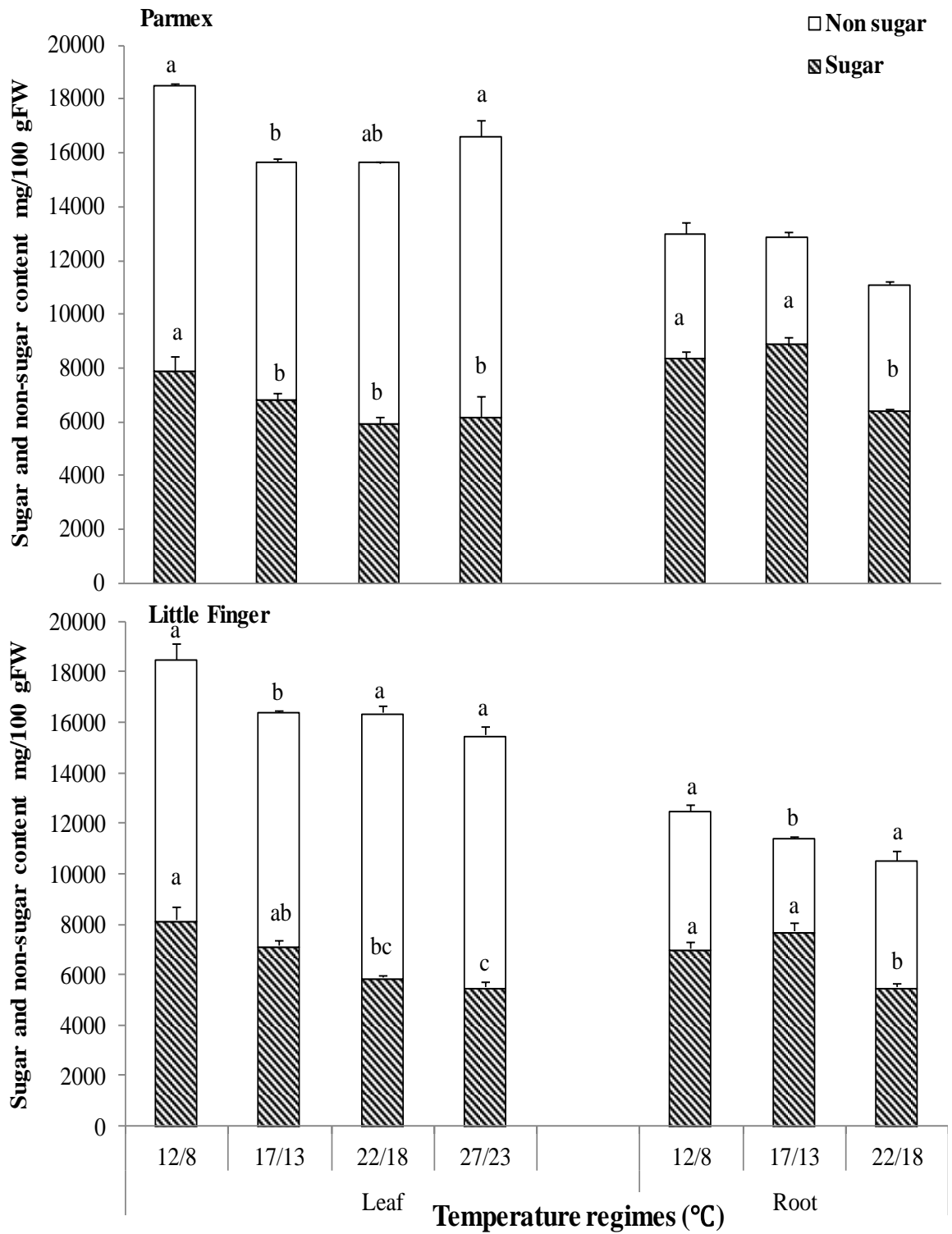


Figure 3.21 The effect of carrot variety (Parmex and Little Finger) and temperature regime on dry matter content of leaves and root. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean.

3.2.2 Nitrate concentration

There were highly significant effects of variety ($P < 0.001$) and temperature ($P < 0.001$) on nitrate concentration of lettuce leaves and root. The nitrate concentration increased gradually with temperature increase, then seemed to decrease at highest temperature regimes (32/28 °C). Furthermore there were highly significant interactions ($P < 0.001$ for leaf and $P < 0.05$ for root) between variety and temperature regimes. Lettuce Exbury had higher concentrations than Dexter in both shoots and root at all temperature regimes, (Figure 3.22).

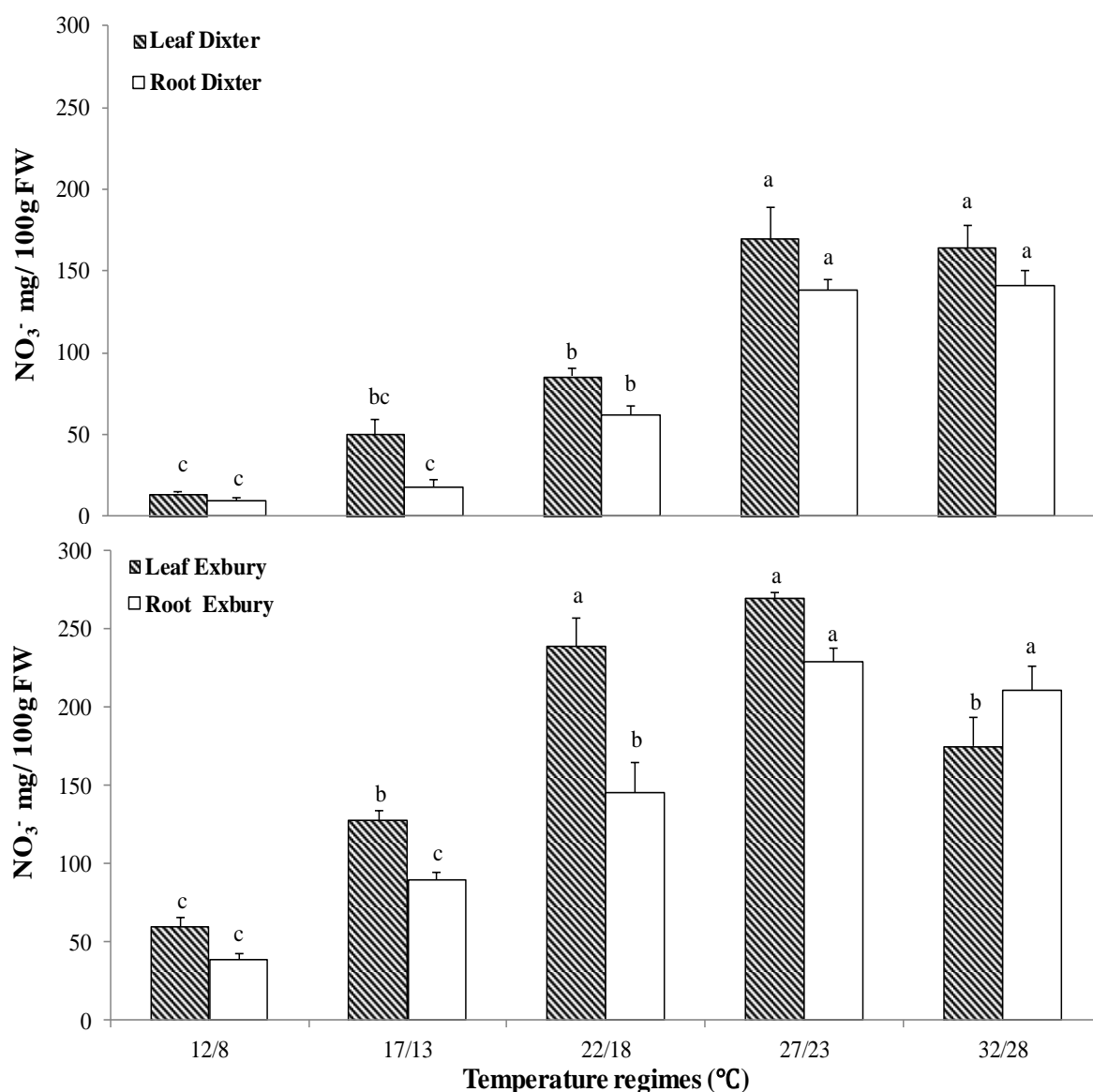


Figure 3.22 The effect of lettuce variety (Dexter and Exbury) and the temperature regime on nitrate concentration of leaves and root. Means with different letters within a plant fraction are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n= 3$).

For the carrots, there were no significant effects of variety on nitrate concentration of leaves and root, but there were significant effects of temperature on nitrate concentration and greater on leaves ($P < 0.001$) than on the roots ($P < 0.01$). In addition, there was a highly significant interaction ($P < 0.01$) between variety and temperature regimes for leaves, but not for carrot root (Figure 3.23 and 3.24). Parmex leaves had higher concentrations than Little Finger at 22/18 °C compared with lower temperature, while in other regimes Little Finger had higher levels than Parmex in leaves and roots. Very large increase in leaf nitrate concentration of both Parmex and Little Finger between 22/18 °C and 27/23 °C

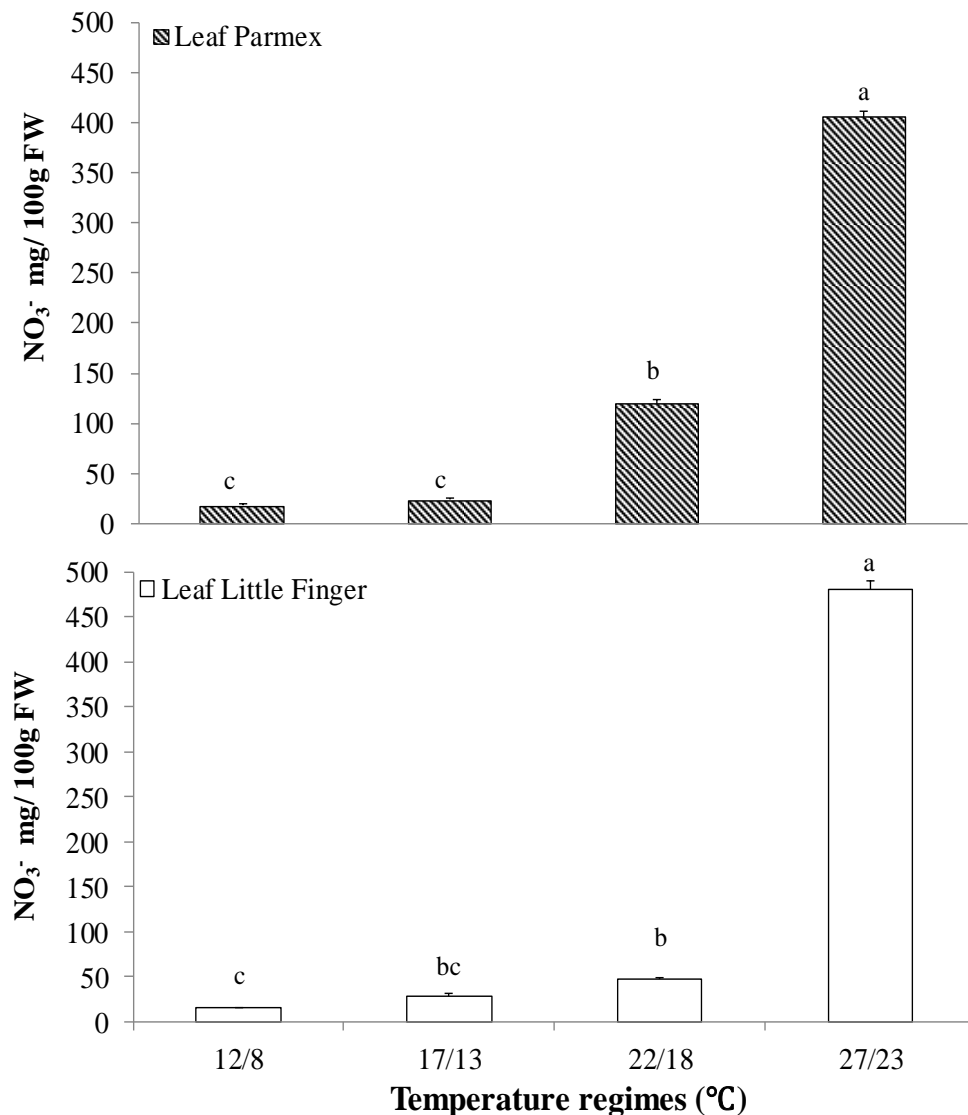


Figure 3.23 The effect of carrot variety (Parmex and Little Finger) and temperature regimes on nitrate concentration of leaves. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n=3$).

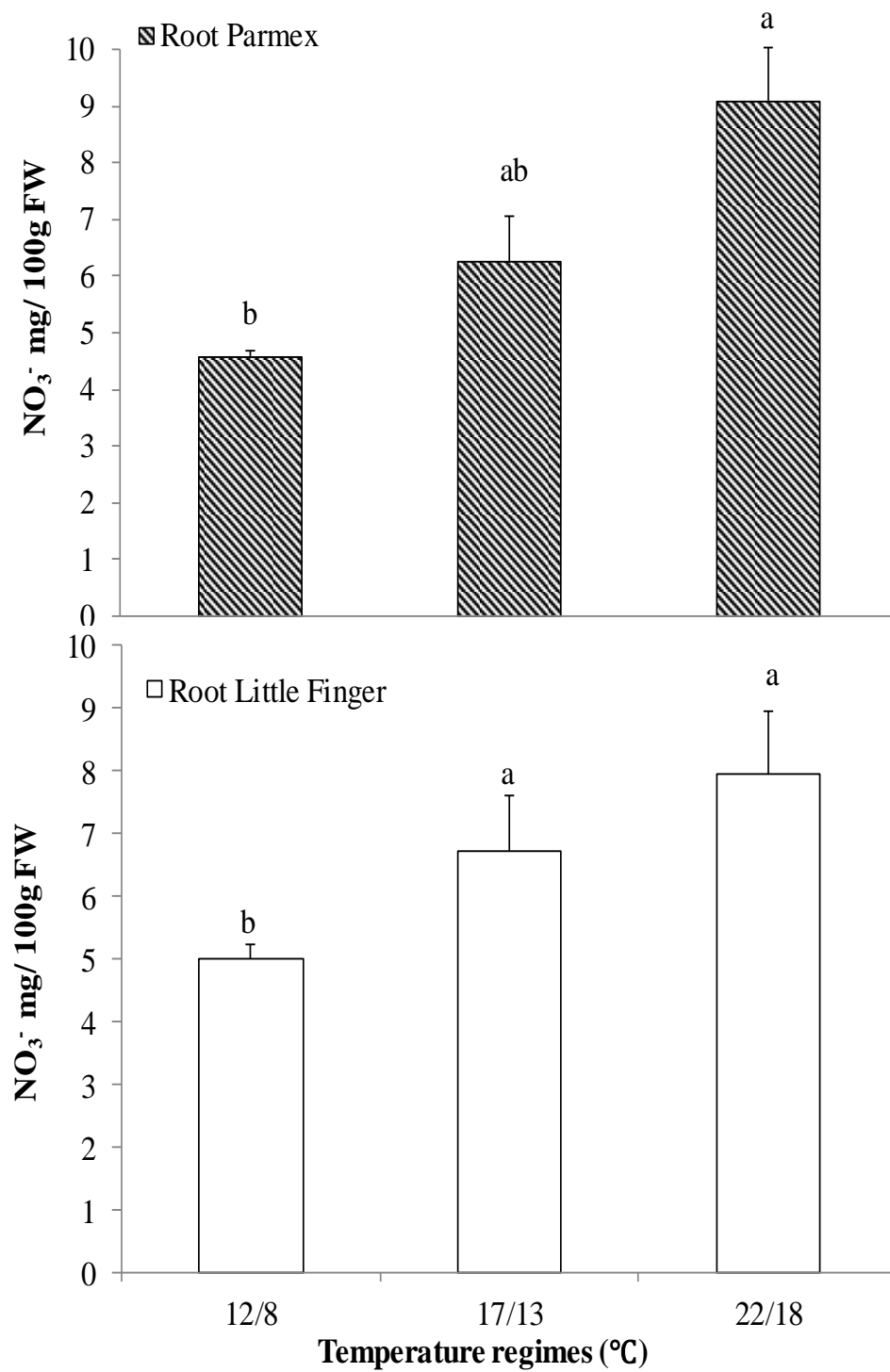


Figure 3.24 The effect of carrot variety (Parmex and Little Finger) and temperature regimes on nitrate concentration of root. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n=3$).

3.2.4 Chlorophylls and carotenoids (HPLC analysis)

The main chlorophylls and carotenoids were separated and identified in lettuce leaf and carrot root (Figure 3.25 and 3.26).

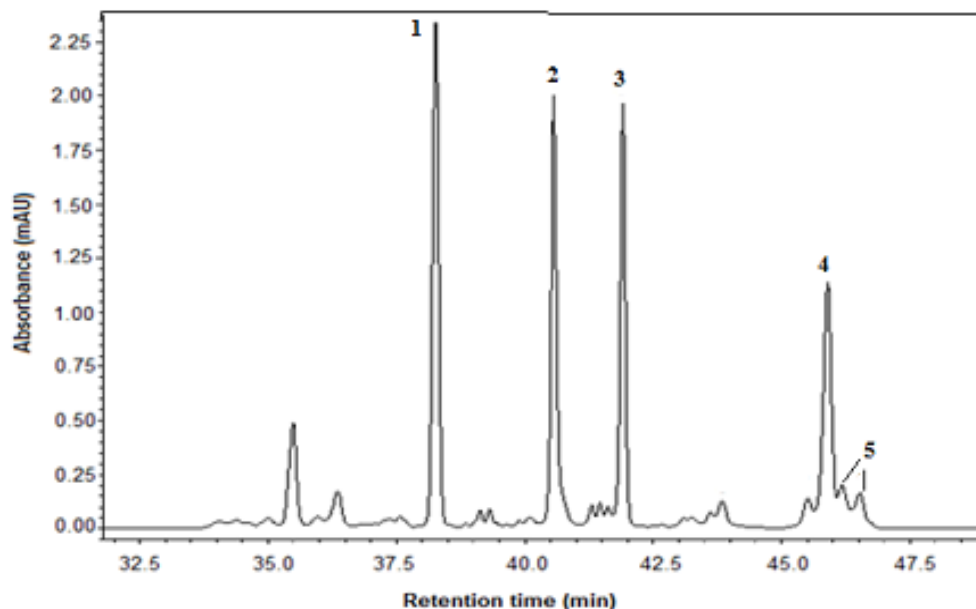


Figure 3.25 HPLC chromatogram recorded at 450 nm with diode array detector of chlorophylls and carotenoid compounds in lettuce leaf (Dixter) : 1, lutein; 2, chlorophyll b; 3, chlorophyll a; 4, β -carotene; 4 and 5, *Cis* β -carotene.

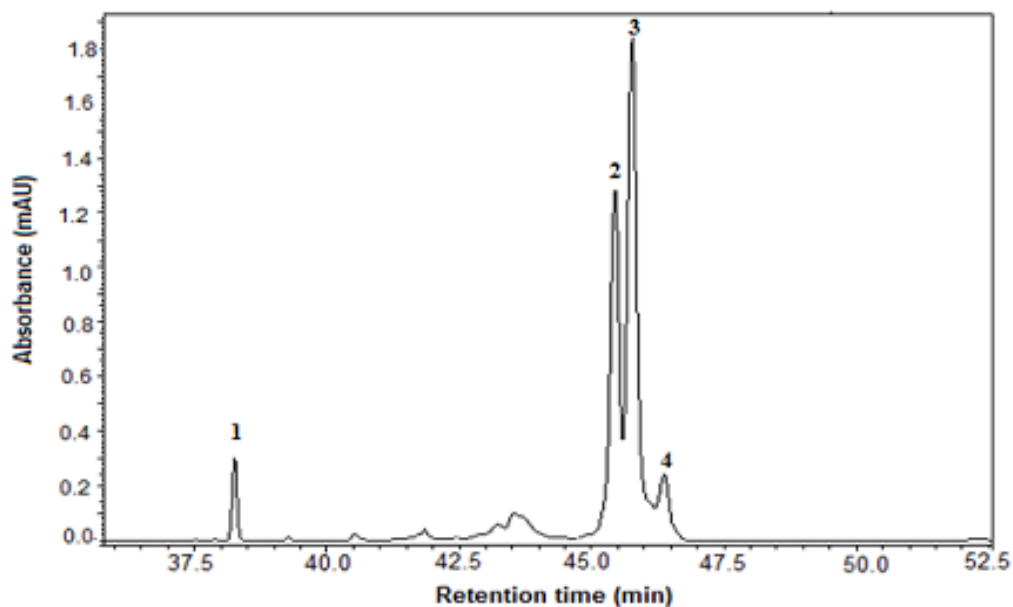


Figure 3.26 HPLC chromatogram recorded at 450 nm with diode array detector of carotenoid compounds in carrot root: 1, lutein; 2, α -carotene; 3, β -carotene; 4, *Cis* β -carotene.

3.2.4.1 Chlorophylls

There were significant effects of variety ($P < 0.001$) and of temperature ($P < 0.001$) on leaf chlorophylls (a and b) of lettuce varieties. Moreover a significant interaction ($P < 0.01$) was found between variety and temperature regime (Figure 3.27). Chlorophylls increased with increasing temperature. Dexter had higher concentrations than Exbury. At the highest temperature (32/28 °C) Exbury responded in different way to Dexter as chlorophylls a and b decreased.

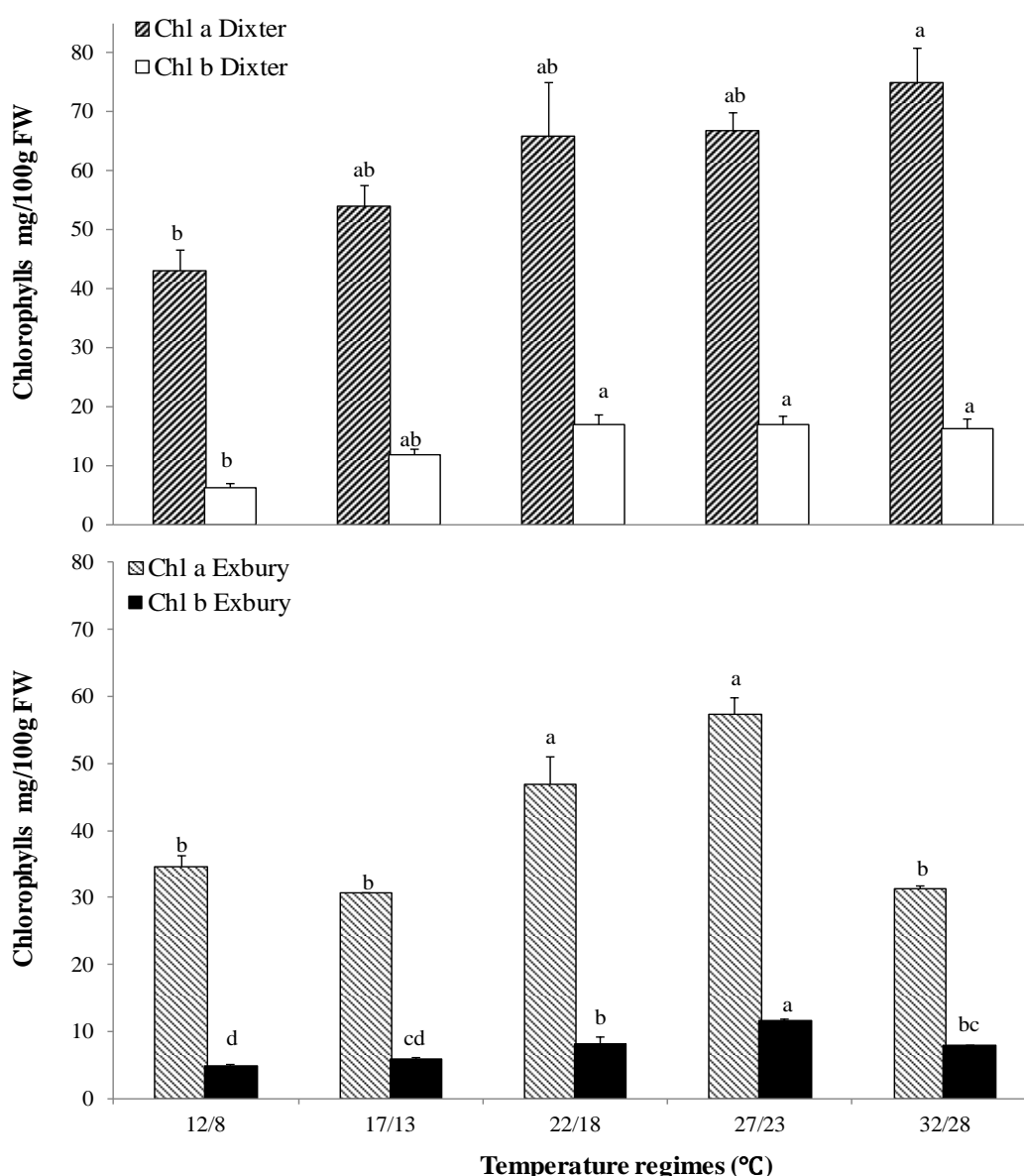


Figure 3.27 The effect of lettuce variety (Dexter and Exbury) and temperature on leaf chlorophylls a (Chl a) and b (Chl b). Means with different letters within a chlorophyll type are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

For carrot, there were significant effects of variety ($P < 0.001$) and of temperature ($P < 0.05$) and ($P < 0.001$) on leaf chlorophylls a and b respectively. There were no significant interactions between variety and temperature, for chl a and for chl b (Figure 3.28). The highest concentration of chlorophylls were recorded at moderate temperatures (17 and 22 °C) and decreased at the highest temperatures. The chlorophylls of Little Finger tended to increase with increasing temperature up to the highest concentration at 22/18 °C, and then decreased at 27/23 °C.

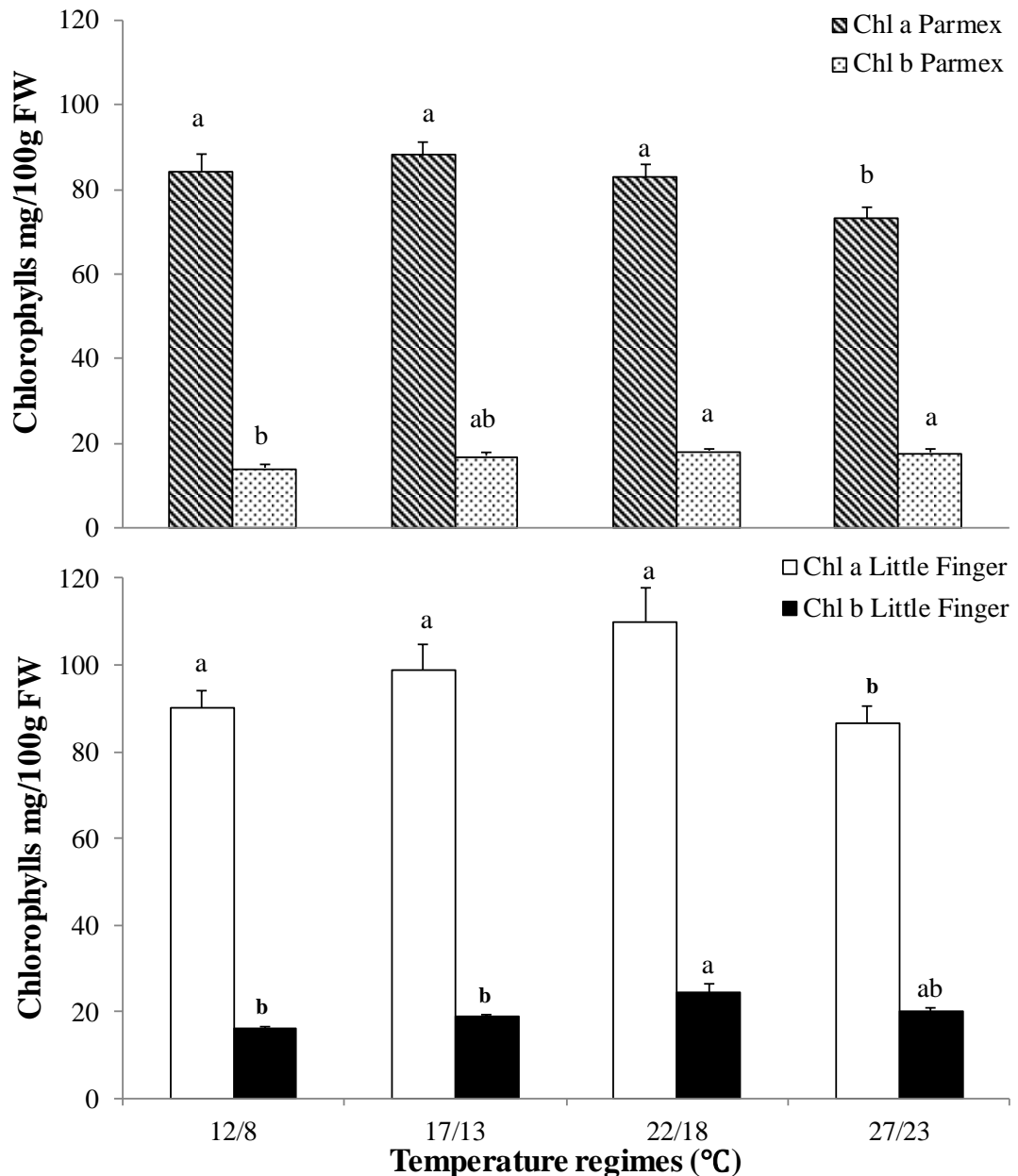


Figure 3.28 The effect of carrot variety (Parmex and Little Finger) and temperature on leaf chlorophylls a (Chl a) and b (Chl b). Means with different letters for each chlorophyll type are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

3.2.4.2 Carotenoids

There were highly significant effects of variety ($P < 0.001$) and of temperature ($P < 0.001$) on carotenoids (lutein, β -carotene and *Cis* β -carotene) of lettuce leaves, as well as highly significant interaction ($P < 0.001$) for each of the carotenoids between variety and temperature regime (Figure 3.29). At 22/18 °C Dixter had the highest concentrations of lutein, β -carotene and *Cis* β -carotene but the concentrations were similar at all the other temperature regimes. In contrast, Exbury had the highest concentrations of each at 22 and 27 °C than other regimes.

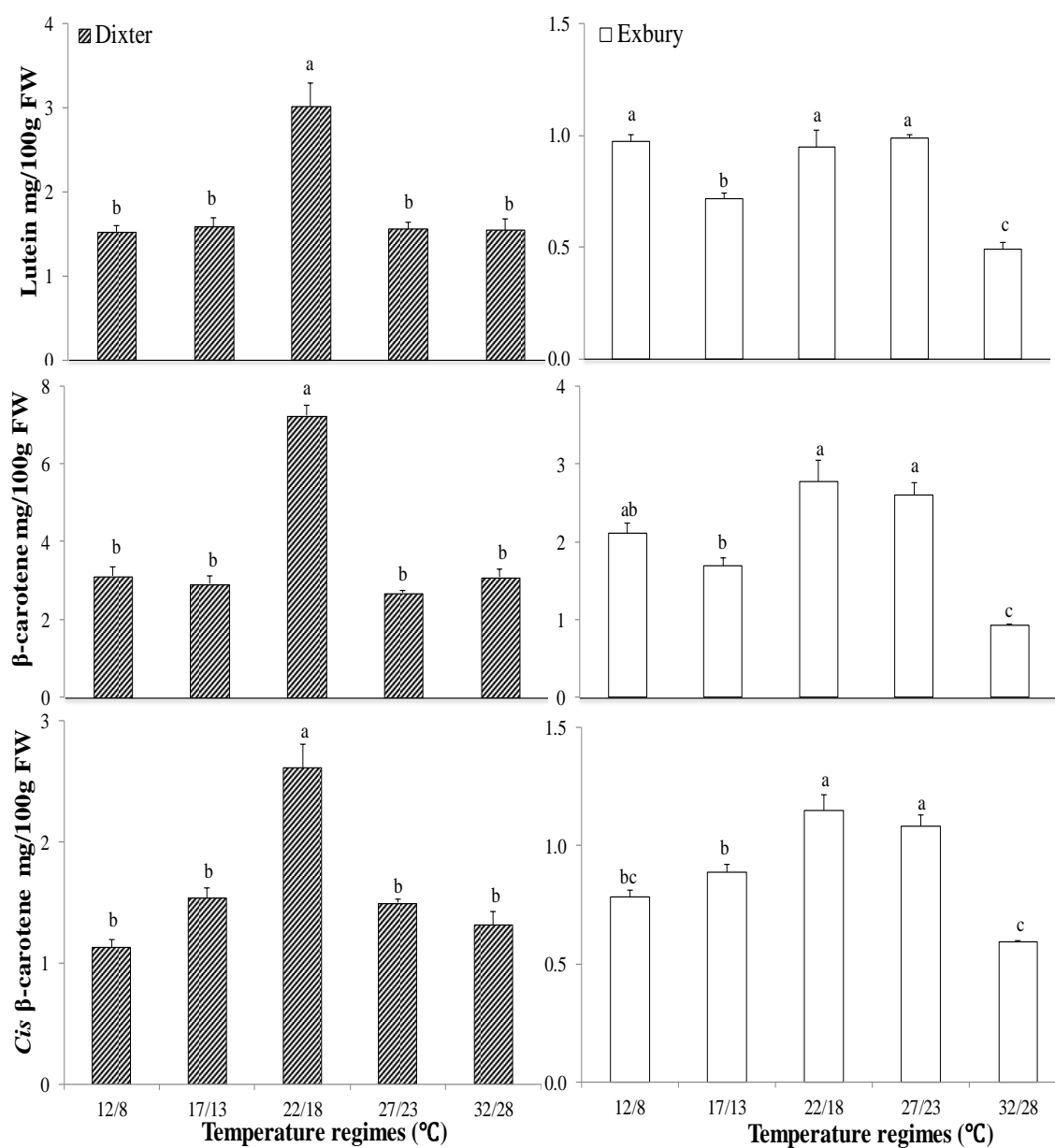


Figure 3.29 The effect of lettuce variety (Dixter and Exbury) and temperature on leaf lutein, β -carotene and *Cis* β -carotene concentrations. Means with different letters are significantly different ($P < 0.05$). Error bars indicate SEM, ($n = 3$).

For carrot leaf, no significant effect of variety was observed for lutein, but there was significant effect of variety ($P < 0.05$ and $P < 0.05$) on β -carotene and *Cis* β -carotene respectively.

There was a highly significant effect ($P < 0.001$) of temperature regimes on lutein, β , and *Cis* β -carotene. The highest concentration of lutein was achieved at low temperature (12/8 °C) and decreased gradually with increasing temperature of both varieties. β -carotene accumulated its highest concentration in moderate temperature at 22/18 °C, Little Finger at this regime had greater concentration than Parmex.

Although there was no significant interaction between variety and temperature regimes on lutein, but a significant interaction ($P < 0.01$) and ($P < 0.05$) of β , and *Cis*-carotene respectively was found between variety and temperature regimes (Figure 3.30).

For carrot root, there was a highly significant effect ($P < 0.001$) of variety and temperature ($P < 0.001$) on lutein, α -carotene, β -carotene and *Cis* β -carotene respectively. The lutein concentration increased gradually with temperature increase. Parmex had a higher concentration of lutein than Little Finger. The α , β -carotene and *Cis* β -carotene responded similarly to temperature regimes and had highest concentrations at 17/13 °C.

A highly significant interaction ($P < 0.001$) between variety and temperature regimes, Parmex had a higher concentration of α , β , and *Cis* -carotene than Little Finger (Figure 3.31).

In contrast for carrot leaf, the lowest concentration of lutein was achieved at low temperature and increased gradually with increasing temperature.

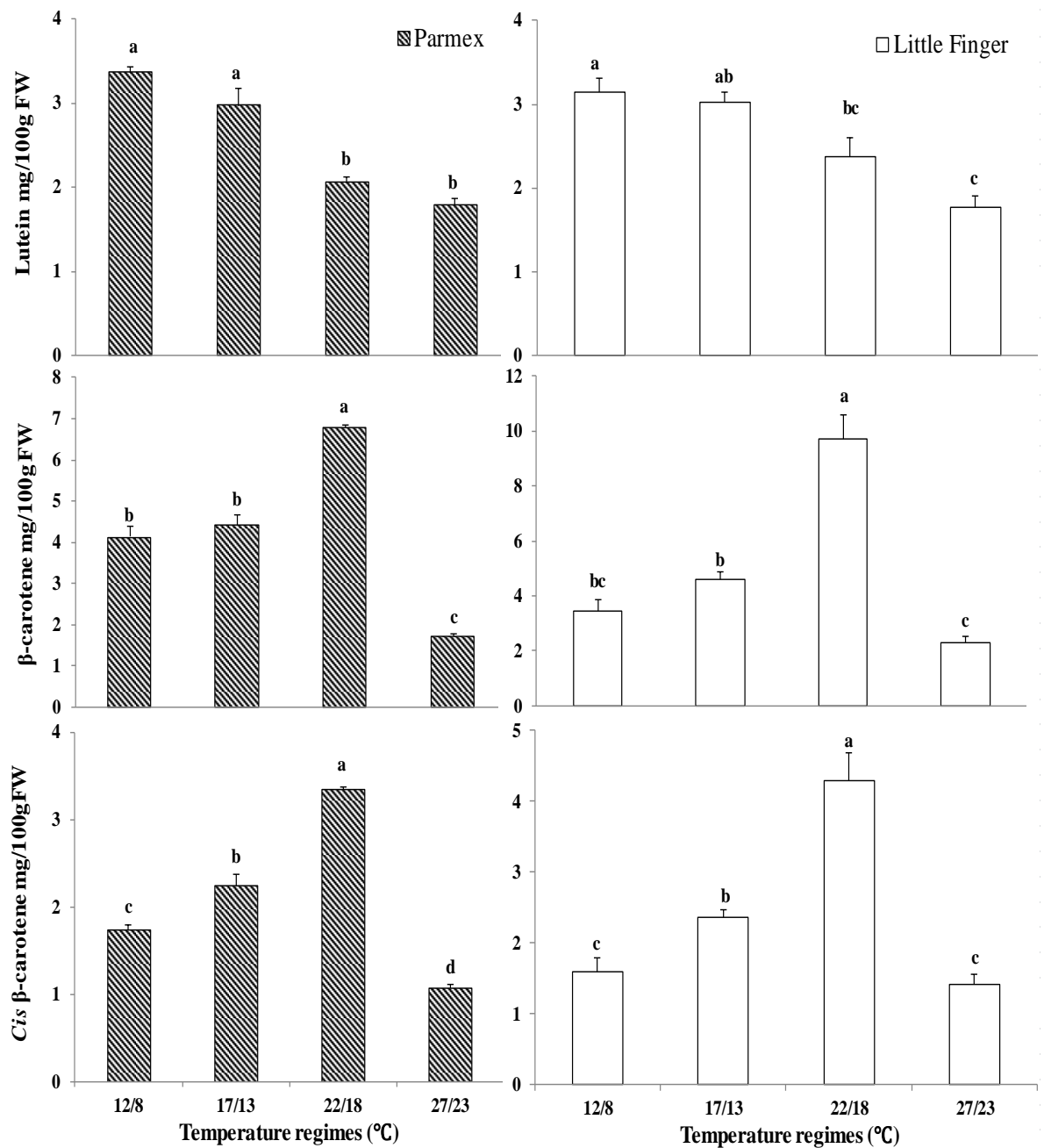


Figure 3.30 The effect of carrot variety (Parmex and Little Finger) and temperature on leaf lutein, β -carotene and *Cis* β -carotene. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

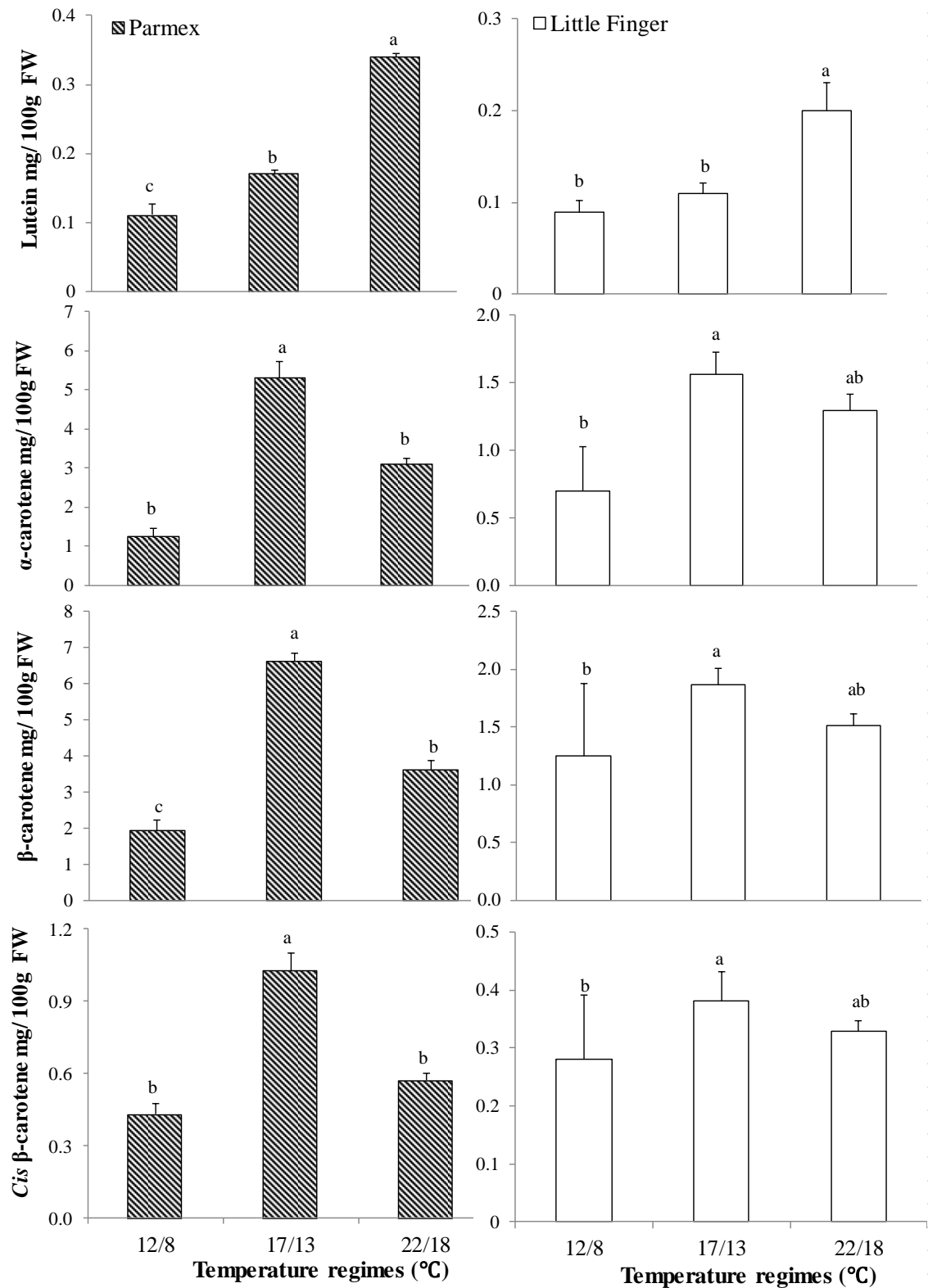


Figure 3.31 The effect of carrot variety (Parmex and Little Finger) and temperature on root lutein, α -carotene, β -carotene and *Cis* β -carotene. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

3.2.5 Polyacetylene compounds of carrot root

The three main compounds found and identified in carrot root were falcarindiol at 19.1 min; falcarinol 3-acetate at 23.14 min and falcarinol at 26 min respectively, (Figure 3.32 and 3.33).

Whilst there was no significant effect of variety on falcarindiol, it was significant for falcarindiol 3-acetate ($P < 0.05$) and falcarinol ($P < 0.001$) respectively.

Temperature significantly ($P < 0.01$, 0.01 and 0.001) affected polyacetylene compound concentrations of falcarindiol, falcarindiol 3-acetate and falcarinol respectively, which were higher at low temperatures and decreased with increasing temperature.

Moreover, no significant interaction was found between variety and temperature regime of falcarindiol and falcarindiol 3-acetate respectively, but it was significant ($P < 0.01$) for falcarinol (Figure 3.34).

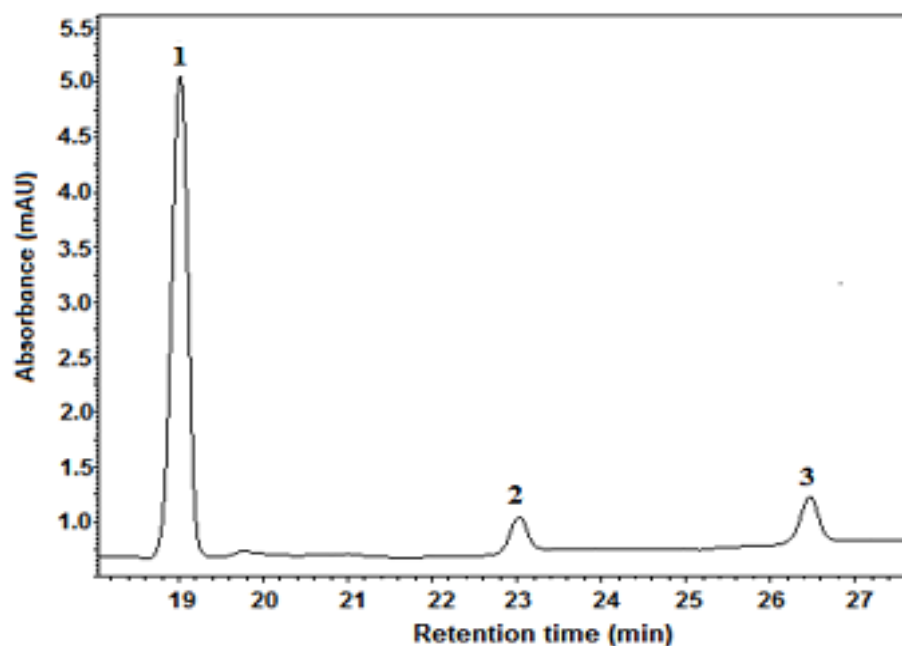


Figure 3.32 HPLC chromatogram recorded at 205 nm with diode array detector of polyacetylene compounds in carrot Parmex root: 1, falcarindiol (FaDOH, $R_t = 19.1$ min; λ_{\max} 233, 245, 259 nm); 2, falcarinol 3-acetate (FaDOAc) ($R_t = 23.14$ min; λ_{\max} 234, 246, 260 nm) and 3, falcarinol (FaOH) ($R_t = 26$ min; λ_{\max} 231, 244, 256 nm) respectively.

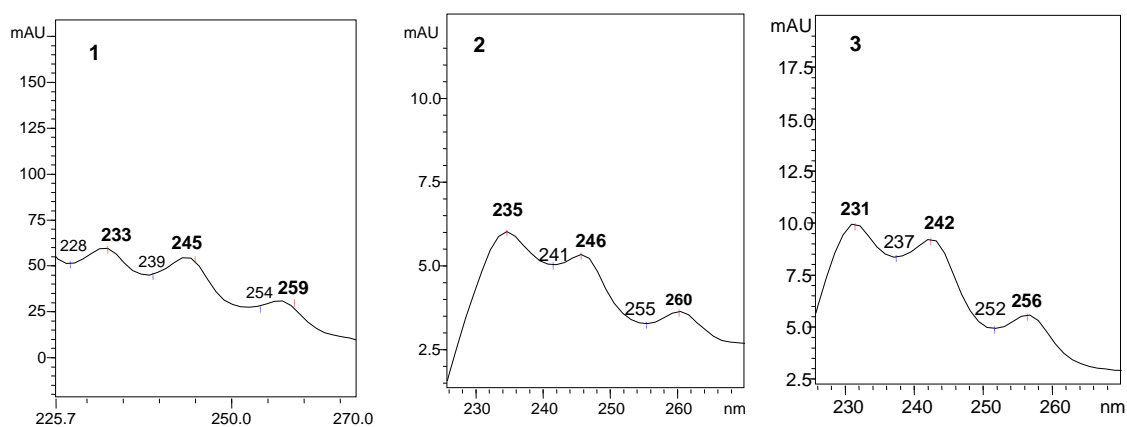


Figure 3.33 UV spectra characteristics of 1, falcarindiol (FaDOH); 2, falcarinol 3-acetate (FaDOAc) and 3, falcarinol (FaOH) in carrot root (Parmex variety) collected at 205 nm.

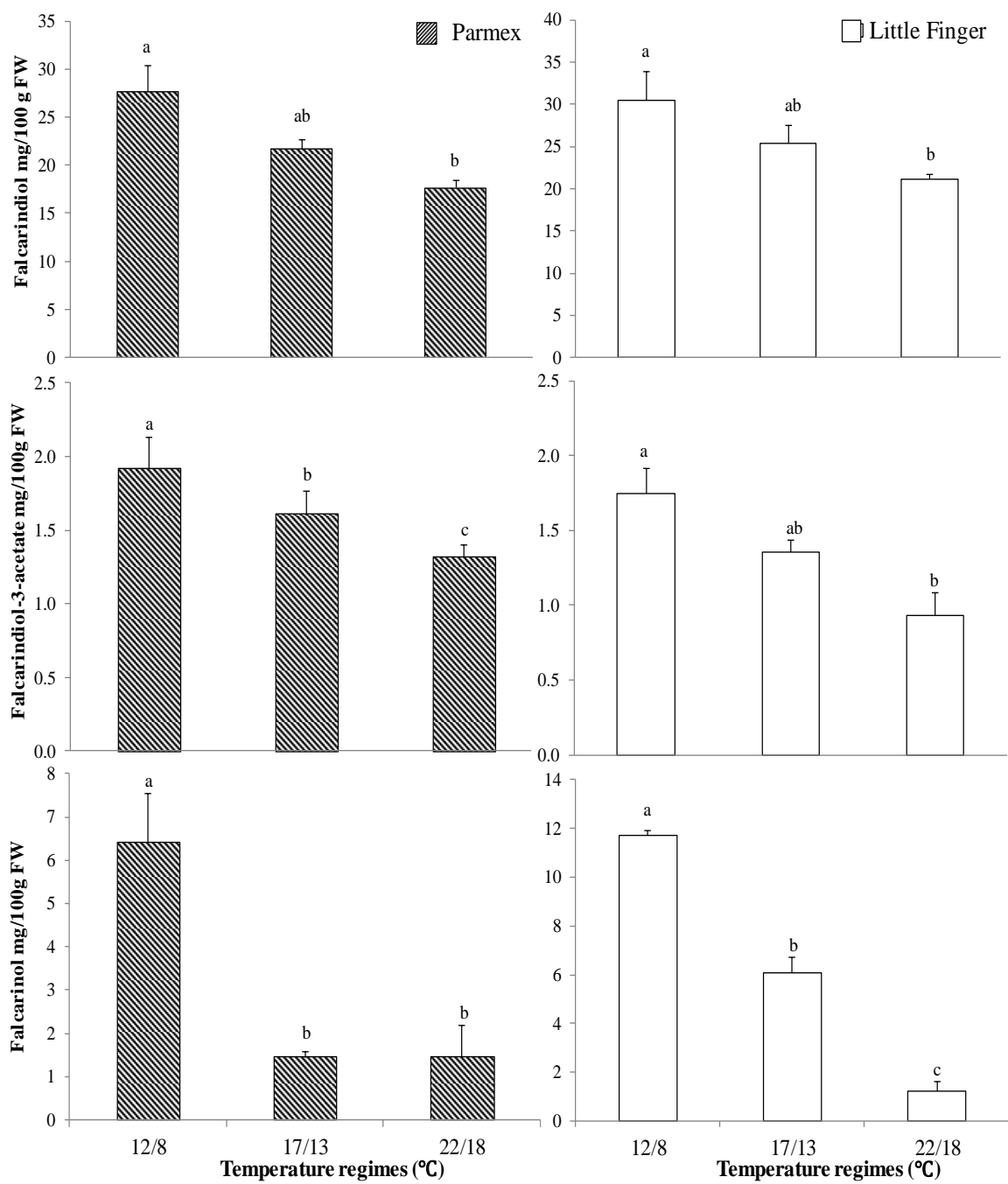


Figure 3.34 The effect of carrot variety (Parmex and Little Finger) and temperature on root falcarindiol, falcariniol-3-acetate and falcarinol concentrations. Means with different letters are significantly different ($P < 0.05$). Error bars indicate SEM, ($n = 3$).

3.2.6 Phenolic compounds

3.2.6.1 Phenolic acids in Lettuce:

The three main phenolic acids - caftaric acid (Caffeoyltartaric acid), chlorogenic acid (3-caffeoylquinic acid) and cichoric acid (Dicafeoyltartaric acid) were abundant in high concentrations in lettuce leaves indicated by peaks 1, 2 and 6 respectively, (Figure 3.35).

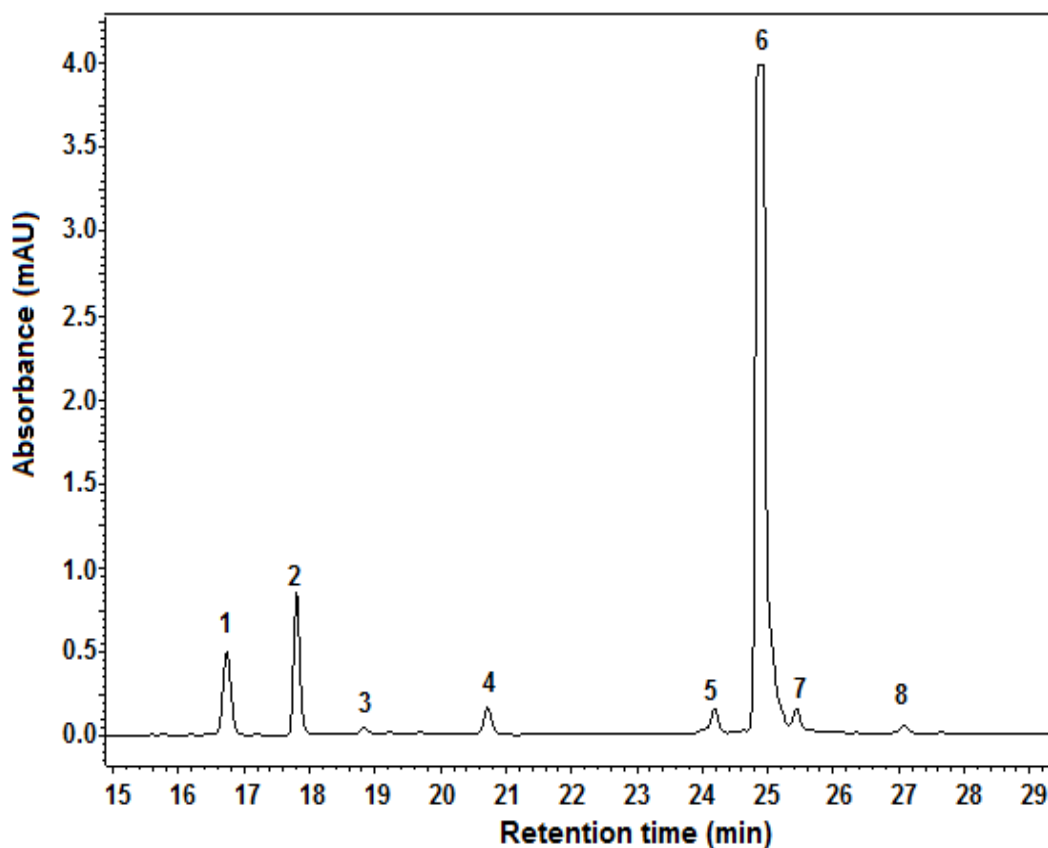


Figure 3.35 Typical HPLC-DAD chromatogram of phenolic acids of lettuce leaf extract recorded at $\lambda = 320$ nm. Peaks are 1, caftaric acid; 2, chlorogenic acid (3-CQA); 3,4 unknown phenolic acids; 5, 8, quercetin derivatives; 6, cichoric acid, 7, internal standard (naringenin).

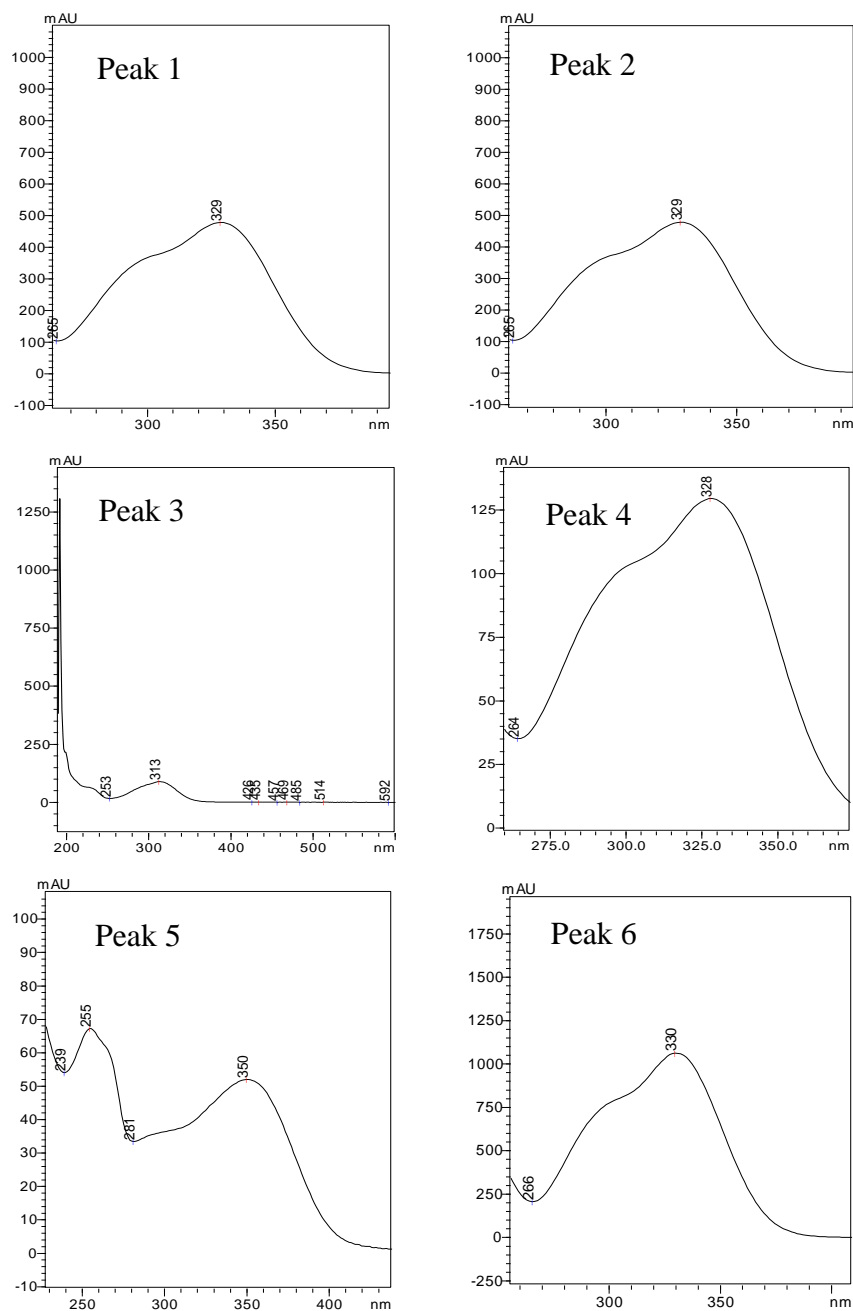


Figure 3.36 UV spectra characteristics of main phenolic peaks 1, 2, 3, 4, 5, and 6 in lettuce leaf (as shown in Figure 3.35 when detected at 320 nm).

There was a highly significant effect ($P < 0.001$) of variety and temperature ($P < 0.001$) on caftaric acid, chlorogenic acid and cichoric acid levels (Figure 3.37). Phenolic concentration was affected significantly by temperature: at the lowest temperature 12/8 °C concentrations were significantly higher than at higher temperatures as it decreased

gradually until 27/23 °C. Thereafter there appeared to be a significant increase once again. There was a highly significant interaction ($P < 0.001$) between variety and temperature. Dexter had the highest concentration of the three phenolic acids (caftaric, chlorogenic and cichoric acid) at low temperature (12/8 °C). In contrast Exbury had the highest concentration of the three main phenolic acids (caftaric, chlorogenic and cichoric acid) at high temperature (32/28 °C), the concentration for both varieties was lower at the moderate temperature (22 and 27 °C), (Figure 3.37).

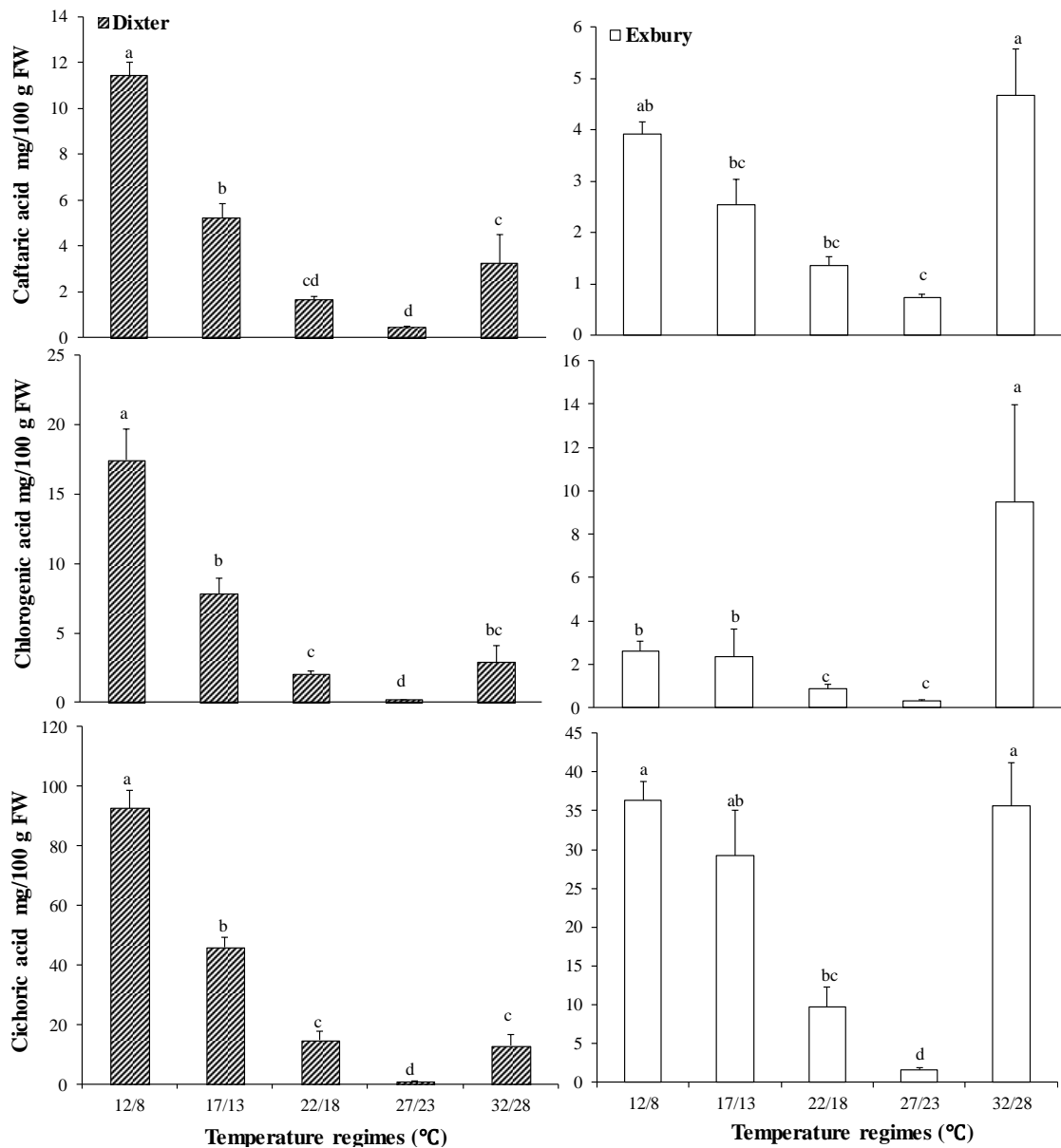


Figure 3.37 The effect of lettuce variety (Dexter and Exbury) and temperature regimes on leaf concentrations of caftaric acid, chlorogenic acid (3-CQA) and cichoric acid. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

3.2.6.2 Quercetin derivatives in lettuce

Quercetin derivatives in lettuce leaves (Peaks 5 and 8, Figure 3. 35) were also recorded at 320 nm. A highly significant effect ($P < 0.001$) of variety on quercetin derivatives, a significant effect of temperature ($P < 0.001$), as well as significant interaction ($P < 0.001$) was found between variety and temperature for lettuce (Figure 3.38).

At the lowest temperature (12/8 °C) Dexter had higher concentrations of quercetin derivatives than Exbury, while at the other temperature regimes, both varieties showed broadly similar responses.

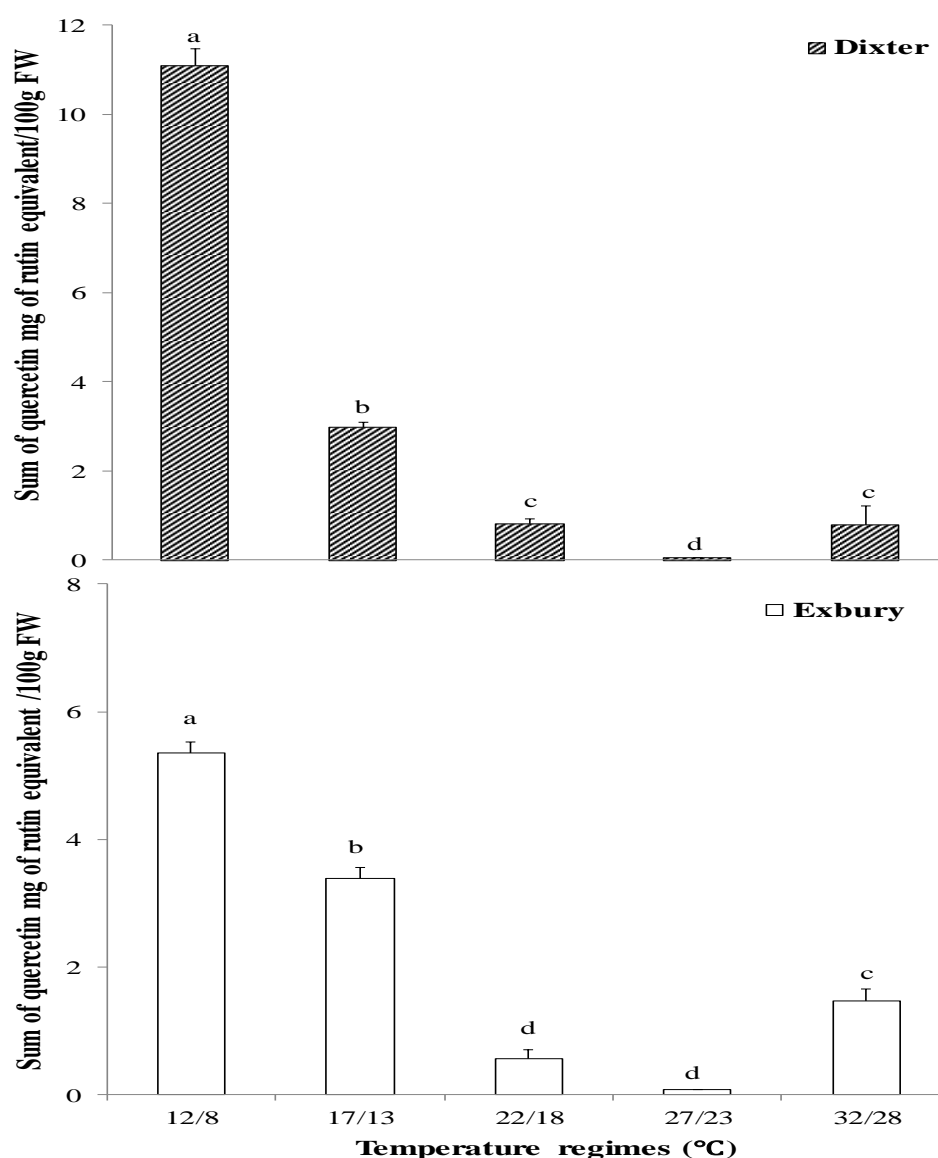


Figure 3.38 The effect of lettuce variety (Dexter and Exbury) and temperature regimes on the sum of leaf quercetin derivatives (peaks 5 and 8, from Figure 3. 35). Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

3.2.6.3 Anthocyanin in lettuce leaves

The anthocyanin in lettuce leaves was recorded at 520 nm at 23.5 min (Figure 3.40).

There were significant effects of variety ($P < 0.001$) and temperature regime ($P < 0.001$) on anthocyanin of lettuce leaf. A significant interaction ($P < 0.001$) was found between variety and temperature (Figure 3.41). Clearly, anthocyanin levels in lettuce leaves were highest at low temperature. Differences between varieties were easily seen: Dexter was much darker green and red (attributed to the high accumulation of anthocyanin) than Exbury, which was lighter green and had less anthocyanin (Figure 3.39).

Anthocyanin accumulated in highest concentration at low temperature (12/8 °C) of both varieties and decreased gradually as temperature increased. Dexter had much higher anthocyanin than Exbury at (12/8 °C), by the order of threefold.











				
Dexter	Dexter	Dexter	Dexter	Dexter
				
Exbury	Exbury	Exbury	Exbury	Exbury
At 12/8 °C	At 17/13 °C	At 22/18 °C	At 27/23 °C	At 32/28 °C

Figure 3.39 The effect of temperature regimes on the accumulation of anthocyanin (red colour) in lettuce leaves of the varieties (Dexter and Exbury).

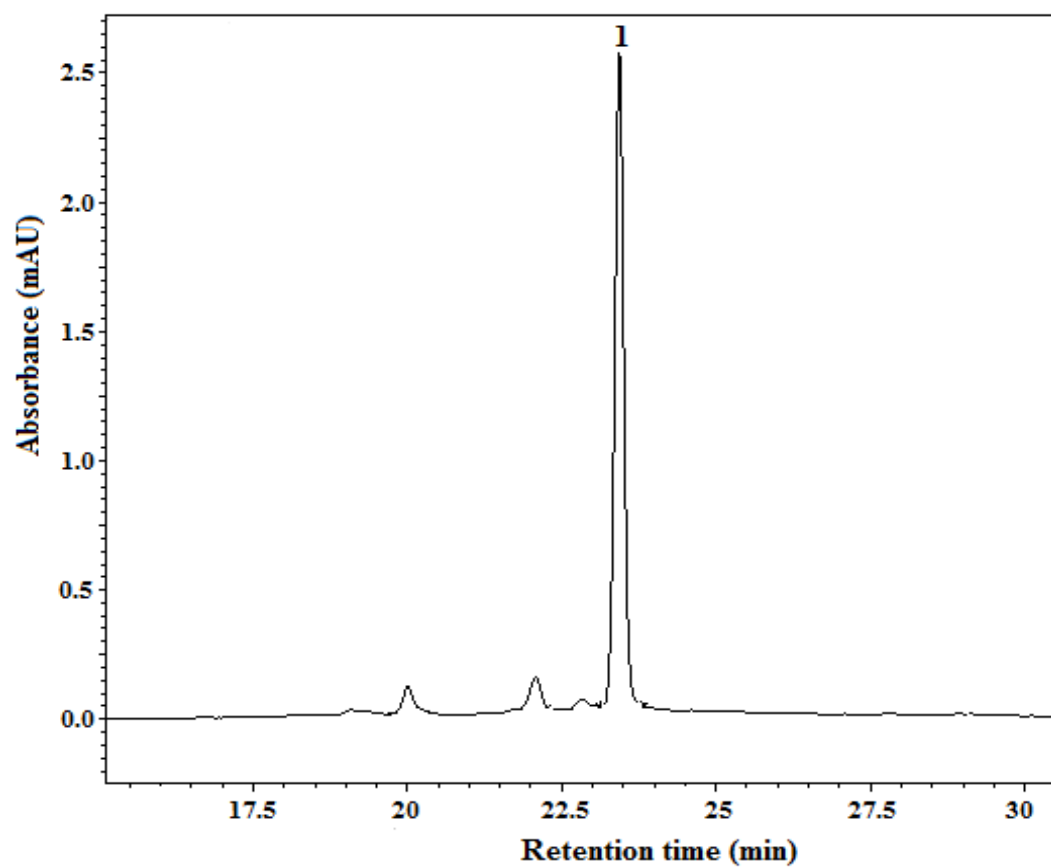


Figure 3.40 Typical HPLC-DAD chromatogram of anthocyanin of lettuce leaf extract recorded at $\lambda = 520$ nm, (peak 1 with λ max 521 nm at 23.5 min).

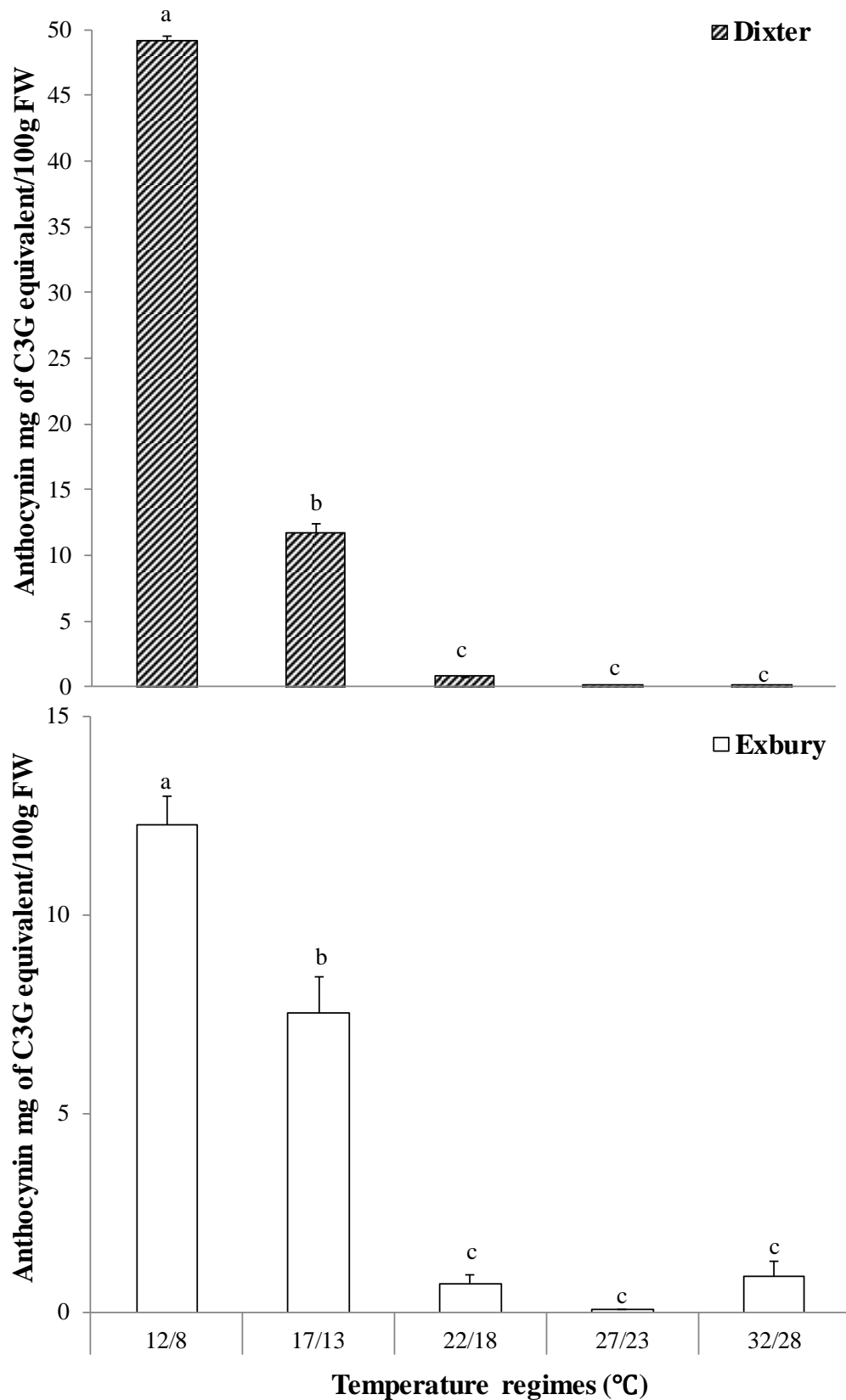


Figure 3.41 The effect of lettuce variety (Dexter and Exbury) and temperature regimes on leaf anthocyanin concentration. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

3.2.6.4 Comparisons with standards of phenolic acids in carrot:

For both carrot and lettuce leaf, the retention time and UV spectra of chlorogenic acid (3-caffeoylquinic acid, 3-CQA) CAS number 327-97-9, was similar to an authentic standard. There is still confusion as regards (3-CQA) as, in numerous publications, it is named either 3-CQA or (5-caffeoylquinic acid, 5-CQA) CAS number 906-33-2, as demonstrated by (Frota and Tucker, 1972).

Much of the literature (Samuoliene and Duchovskis, 2006; Kreutzmann *et al.*, 2008; Du *et al.*, 2012) has identified 5-CAQ as the main chlorogenic acid in carrots. By contrast, during the present study, both chlorogenic acids (3 and 5-CQA) were tested, but only 3-CQA was found at a similar time and in the UV spectra in samples (leaf and root). For carrot chlorogenic acid (3-CQA or 3-caffeoylquinic acid) with λ max 298, 327 nm at 17.7 min was matched to the standard (peak 2) in leaf (Figure 3.42) and in root (Figure 3.43 and 3.44).

The other phenolics were unknown and did not match the whole standard (Caffeic acid, 4-Caffeoylquinic acid, 5-Caffeoylquinic acid (5-CQA), 1,3-Dicaffeoylquinic acid, 3,5-Dicaffeoylquinic acid, 3,4-Dicaffeoylquinic acid and 4,5-Dicaffeoylquinic acid) that were tested or to any published in previous literature (section 2.2.3.4.4).

Therefore, all phenolic peaks were calculated as total phenolic acids (the sum of all individual peaks (PK) as mg of chlorogenic acid equivalent / 100g FW). Clearly, the UV spectra characteristics of unknown peaks in the leaf and root of the carrot (Figure 3.44) were matched to UV spectra of caffeic acid derivatives as shown in many publications (section 2.2.3.4.4).

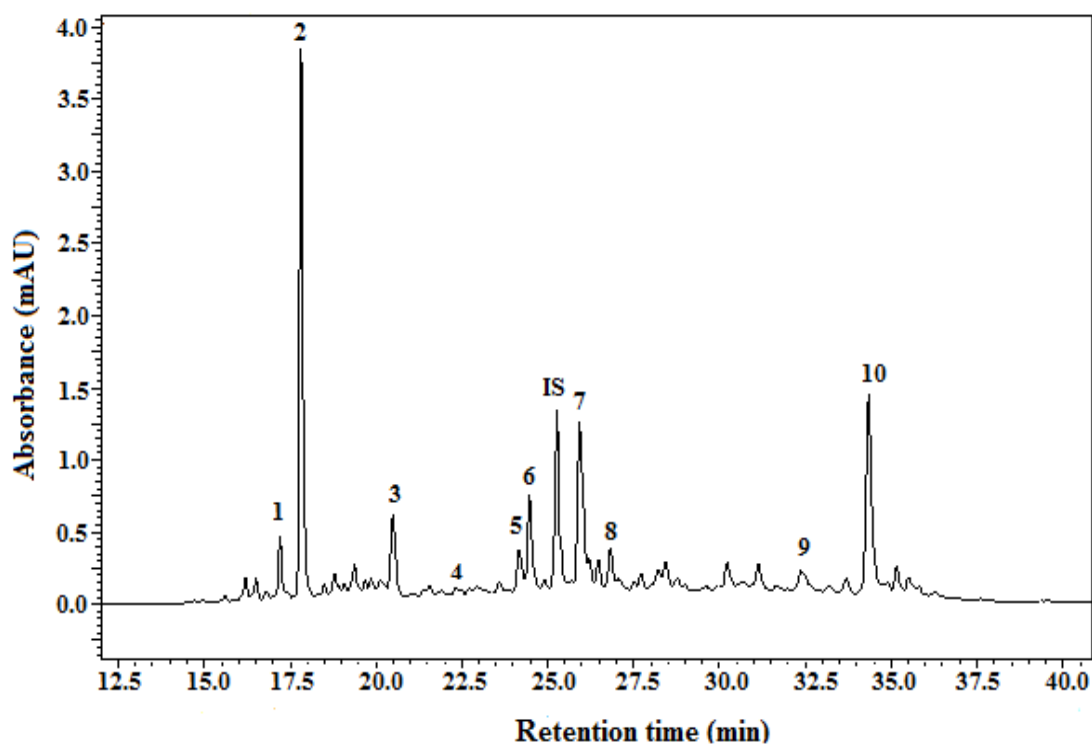


Figure 3.42 Typical HPLC-DAD chromatogram of phenolic acids of carrot leaf extracts recorded at $\lambda = 320$ nm. Peaks: 2 is chlorogenic acid (3-CQA), other peaks are unknown phenolic acids. , IS is internal standard (naringenin).

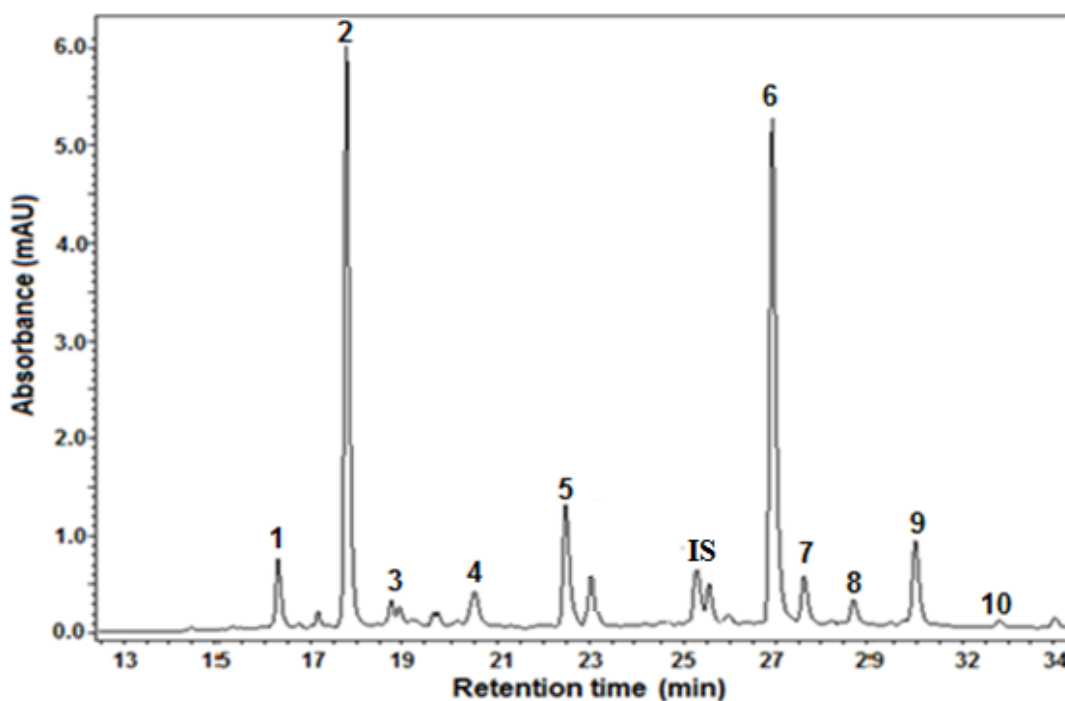


Figure 3.43 Typical HPLC-DAD chromatogram of phenolic acids of carrot root extracts recorded at $\lambda = 320$ nm. Peaks are: 2 is chlorogenic acid (3-CQA), other peaks are unknown phenolic acids, IS is internal standard (naringenin).

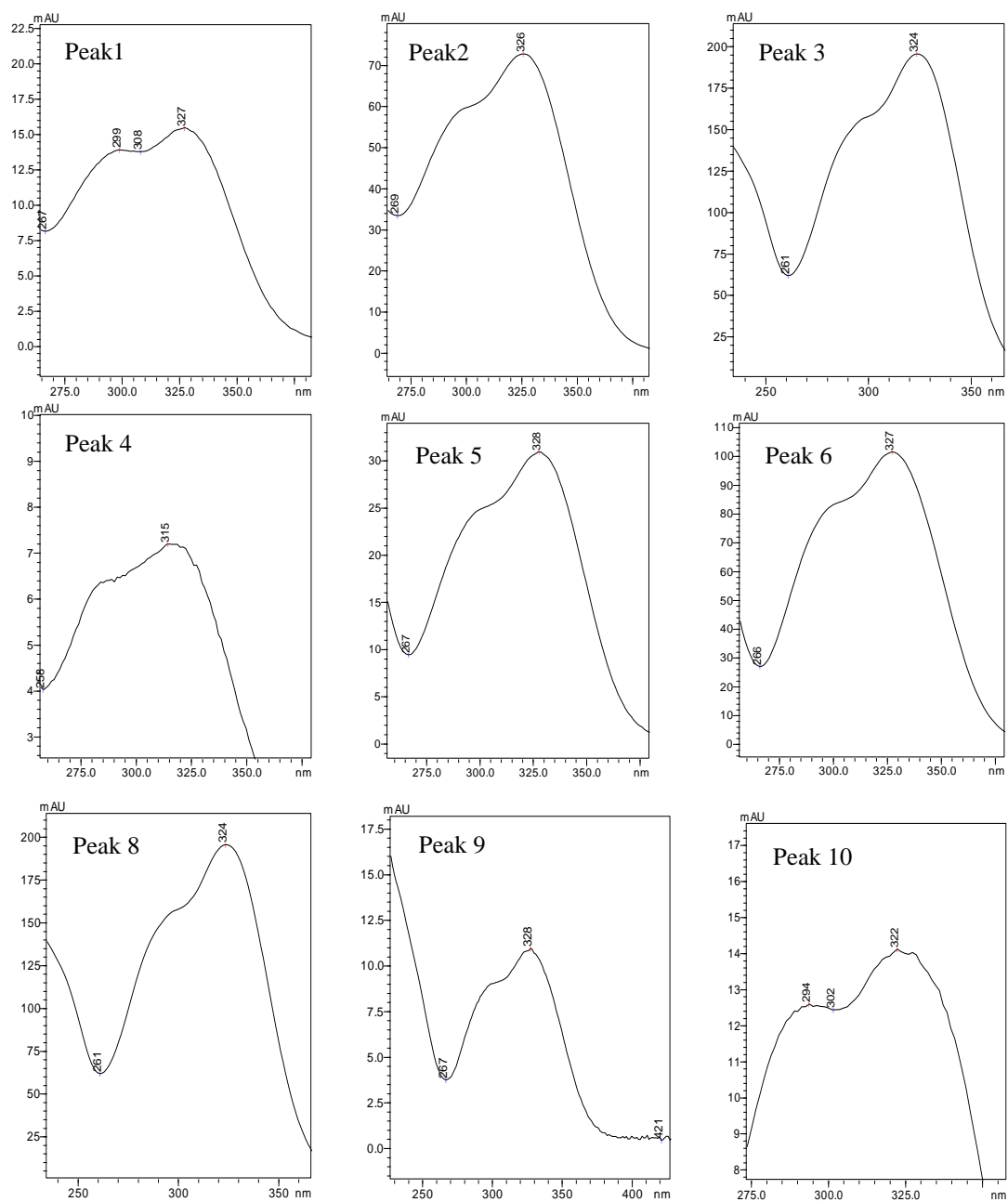


Figure 3.44 UV spectra characteristics of main phenolic peaks 1, 2, 3, 4, 5, 6, 8, 9 and 10 in carrot (as shown in Figure 3.43 when detected at 320 nm).

There were no significant effects or interactions of variety or temperature on chlorogenic acid content of carrot roots, (Figure 3.45).

For carrot leaf, there was a significant effect ($P < 0.01$) of variety, a highly significant effect of temperature ($P < 0.001$) on chlorogenic acid level and a significant interaction ($P < 0.01$) between the varieties and temperature, (Figure 3.45).

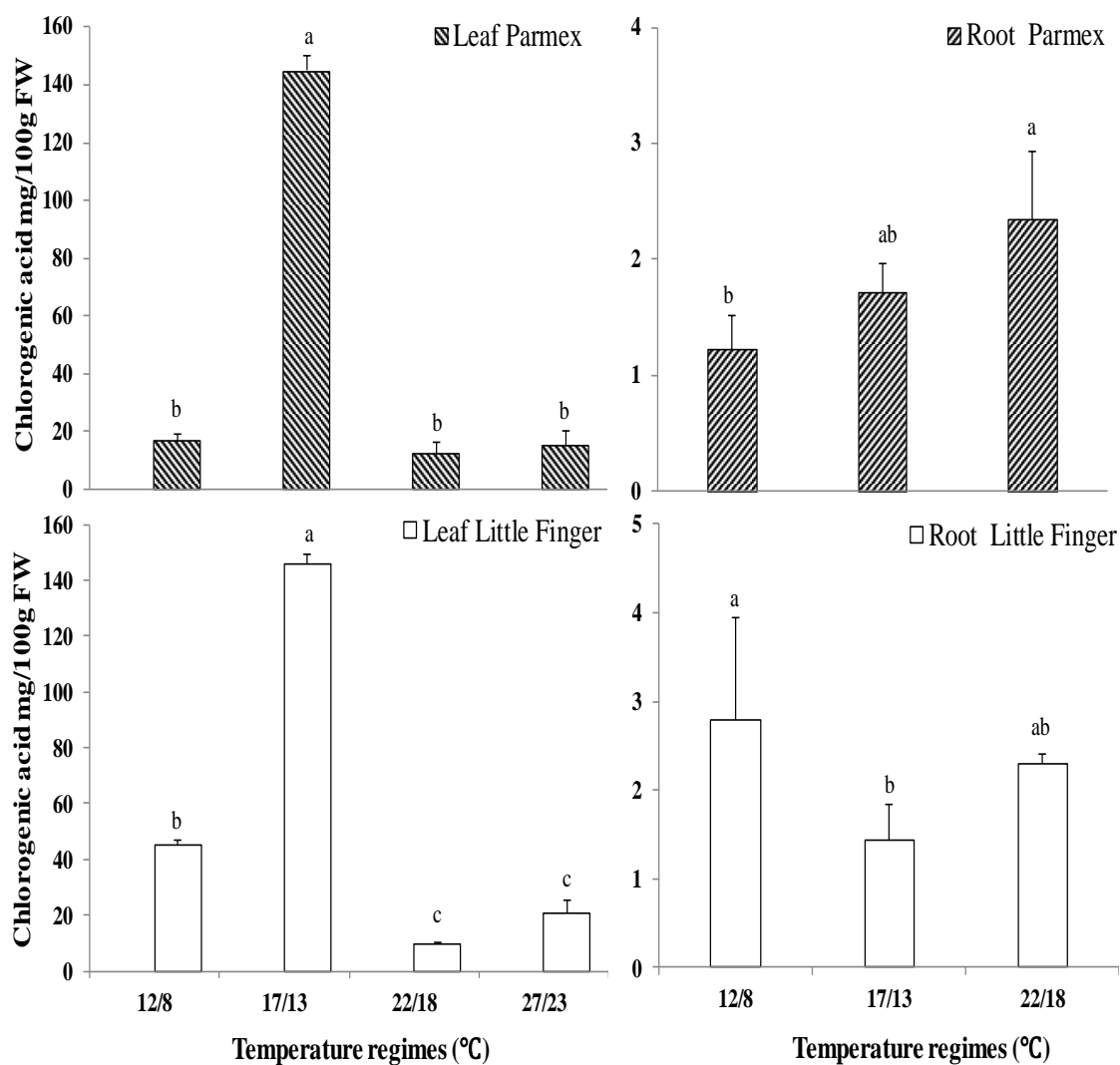


Figure 3.45 The effect of carrot variety (Parmex and Little Finger) and temperature regimes on chlorogenic acid concentration in the leaves and roots. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

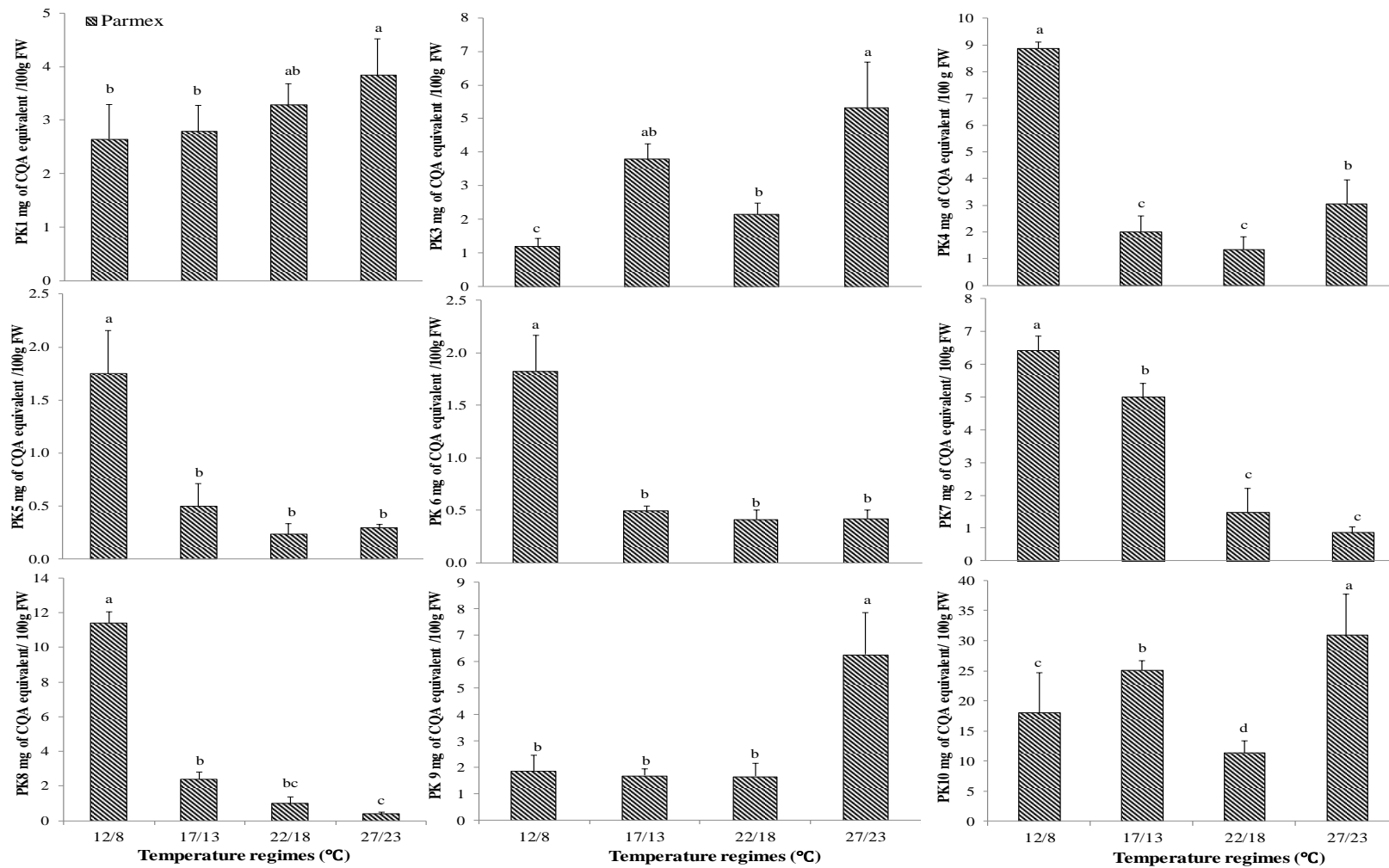


Figure 3.46 The effect of carrot variety (Parmex) and temperature regimes on individual phenolic acids concentration in the leaf (as shown in Figure 3.42). Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

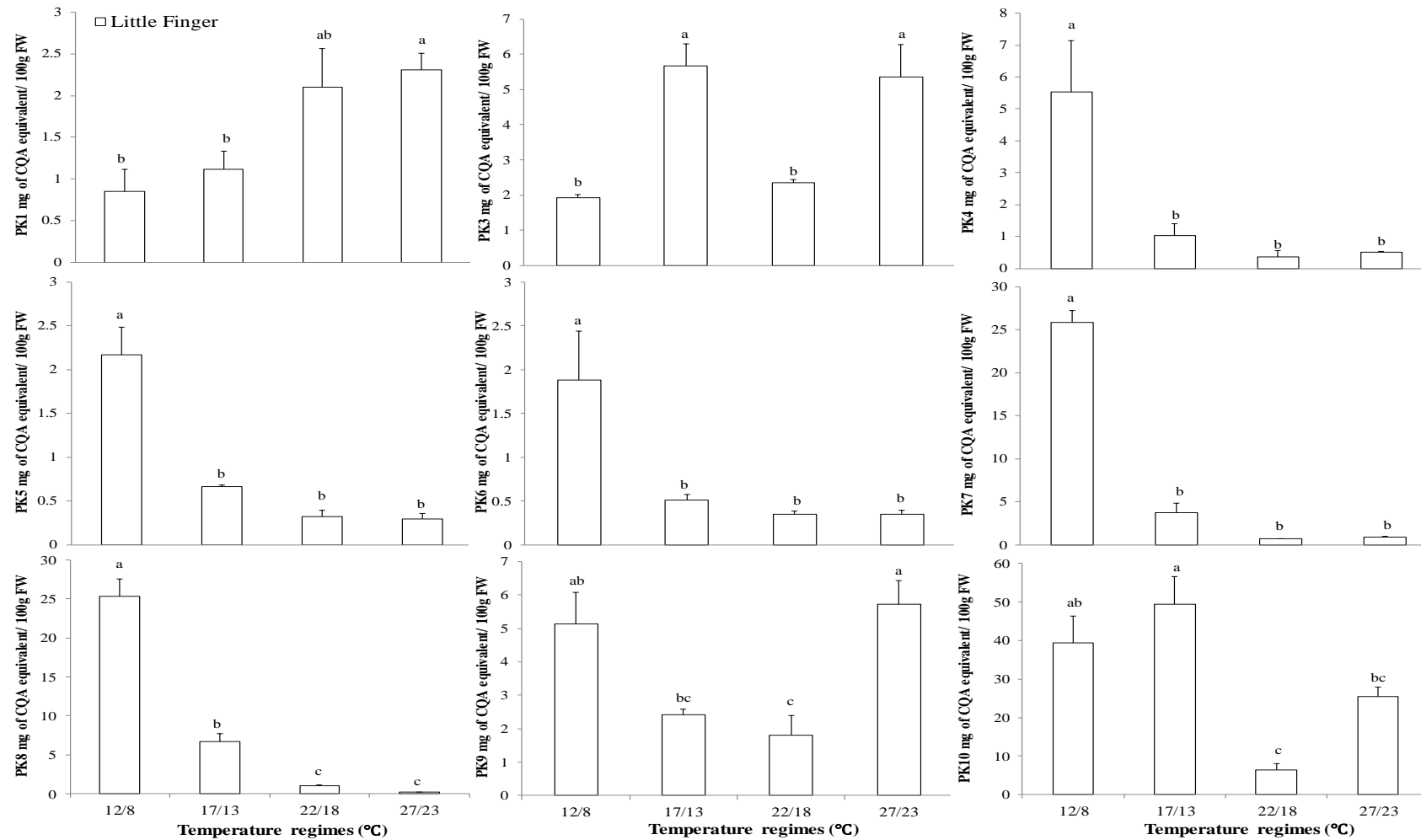


Figure 3.47 The effect of carrot variety (Little Finger) and temperature regimes on individual phenolic acids concentration in the leaf (shown in Figure 3.42). Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

There was a highly significant effect of temperature ($P < 0.001$) on the sum of phenolic peaks in carrot leaves (10 peaks in Figure 3.35, based on 3-CQA equivalent), as well as a significant interaction ($P < 0.01$) between the varieties and temperature: the highest concentration of summed peaks was at 17/ 13 °C in both varieties and at two low temperatures Little Finger had a higher concentration than Parmex, but not at the two high temperatures (Figure 3. 48).

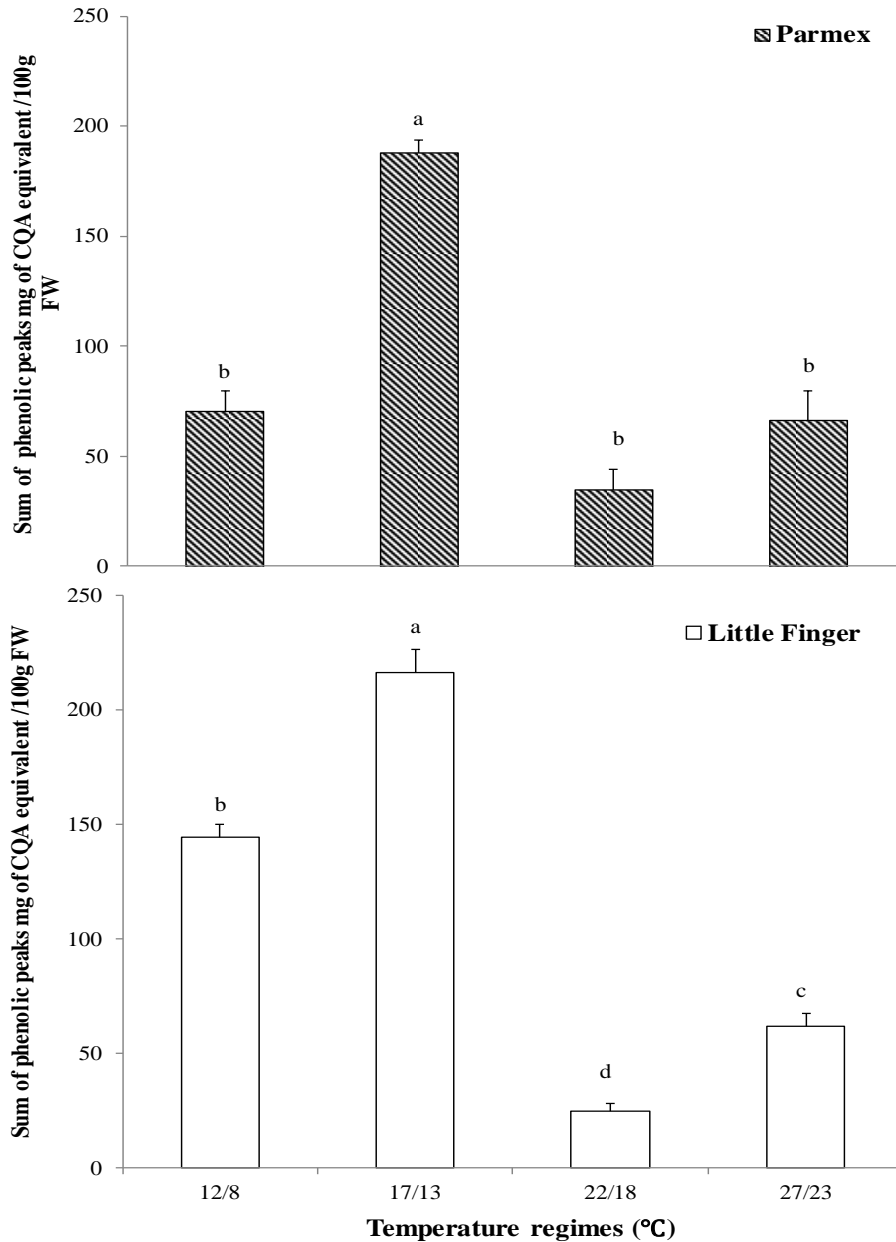


Figure 3.48 The effect of carrot variety (Parmex and Little Finger) and temperature regimes on the sum of phenolic compounds (the sum of 10 individual peaks mg of 3-CQA equivalent) in the leaves. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

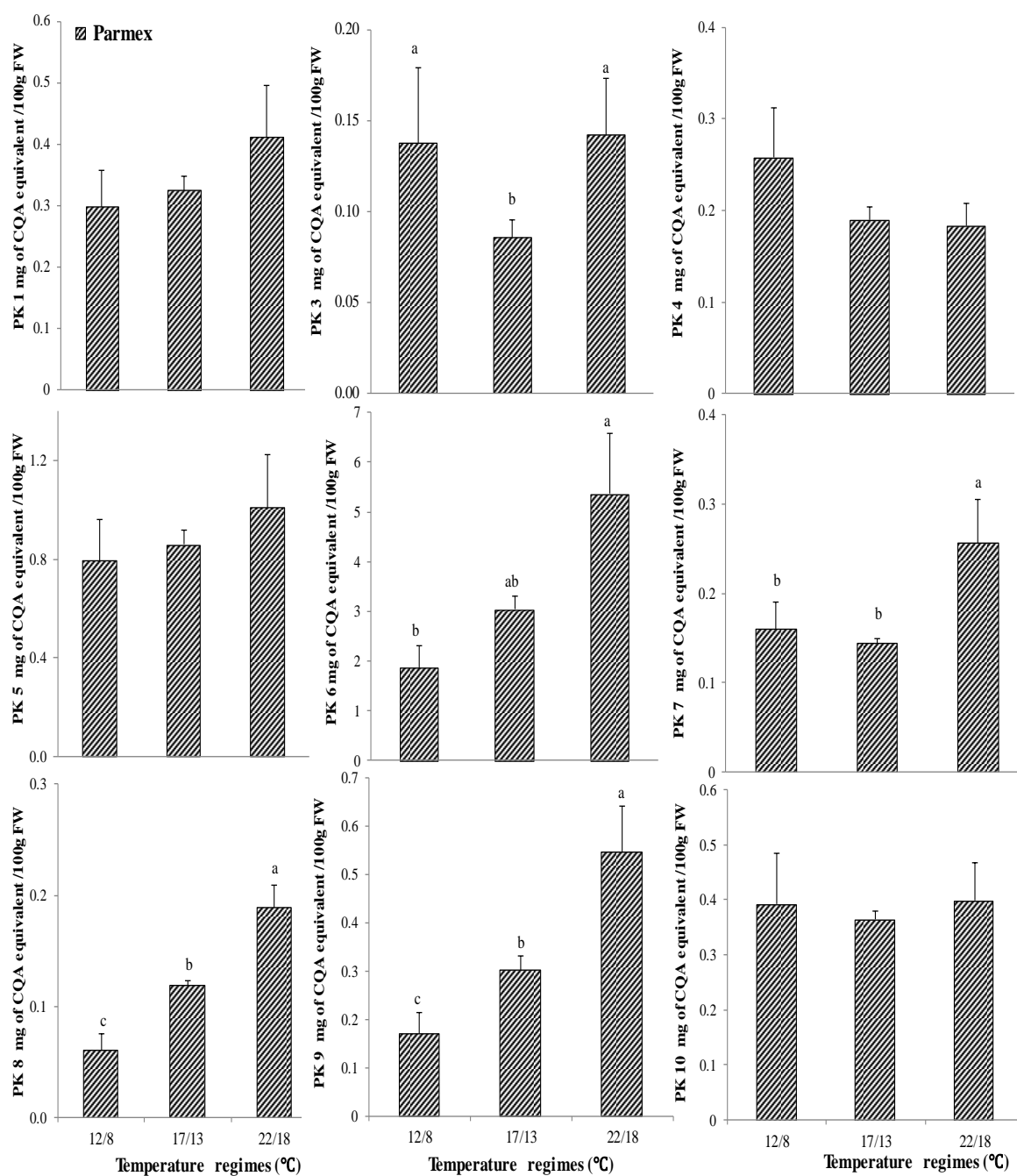


Figure 3.49 The effect of carrot variety (Parmex) and temperature regimes on individual phenolic acid concentrations in the roots (shown in Figure 3.43). Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

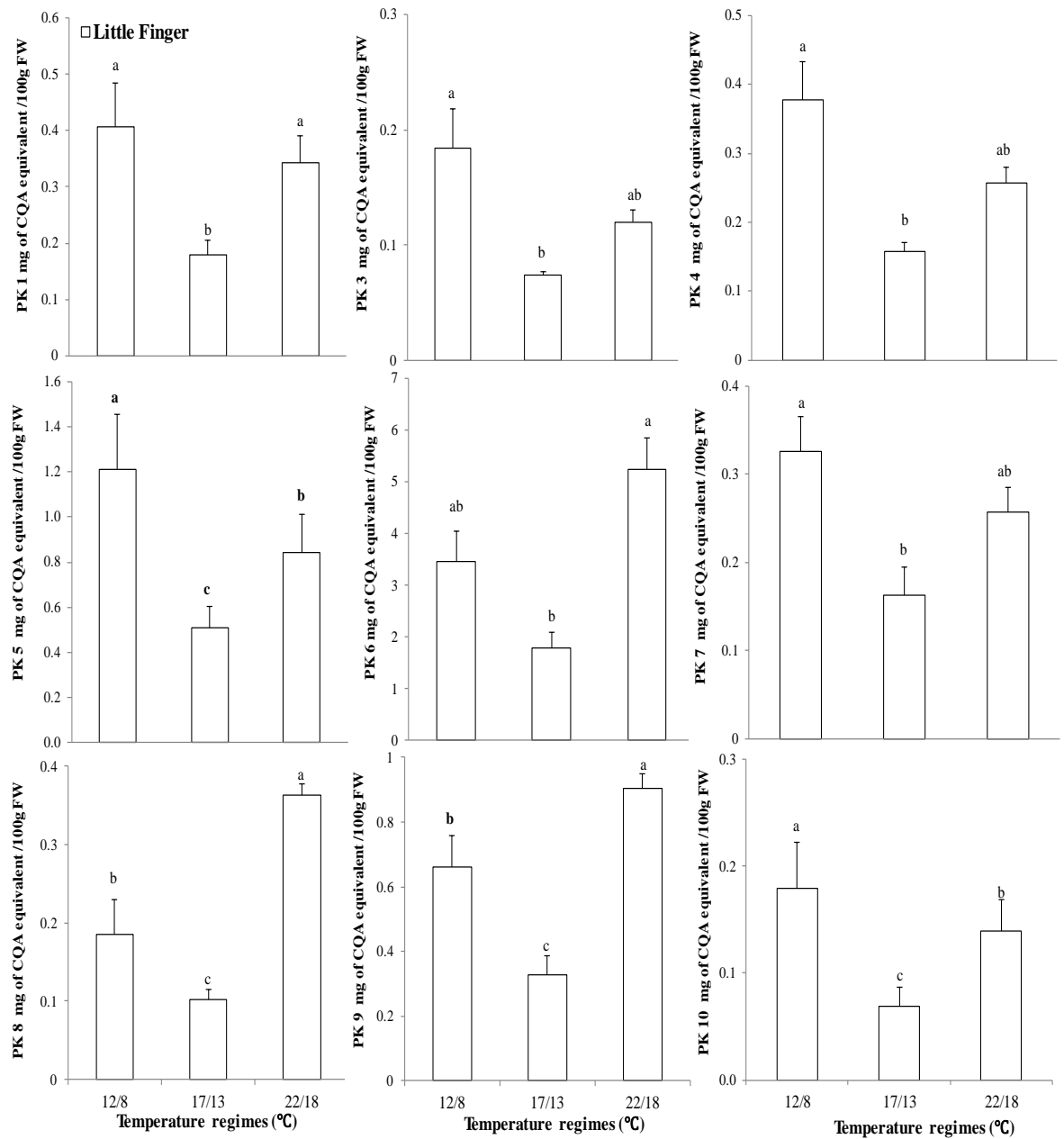


Figure 3.50 The effect of carrot variety (Little Finger) and temperature regimes on individual phenolic acid concentrations in the roots. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

There was no significant effect of variety at each temperature on carrot root but a significant effect of temperature ($P < 0.05$) on sum of all phenolics (all 10 peaks in Figure 3.36), 3-CQA and other unknown compounds based on 3-CQA equivalent) level. No significant interaction was found between the varieties and temperature, (Figure 3.51). Additionally it was observed that the carrot leaves had much higher concentrations than roots.

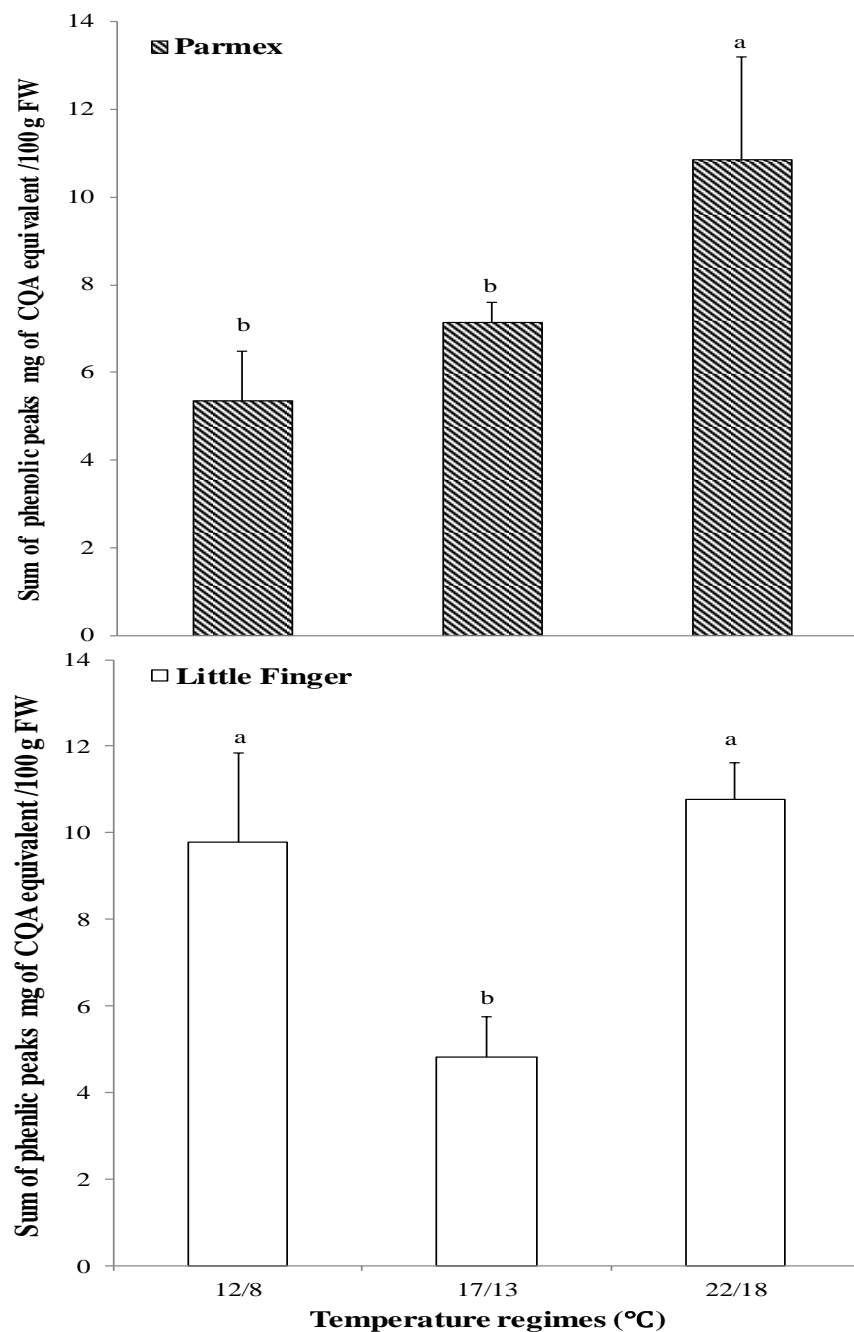


Figure 3.51 The effect of carrot variety (Parmex and Little Finger) and temperature regimes on the sum of phenolics (the sum of all individual phenolics (10 peaks) include 3-CQA) in the roots. Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

Table 3.1 Analysis of Variance (ANOVA) for the phenolic peaks of leaf and root of carrot varieties grown at different temperatures

Chlorogenic acid and unknown phenolic peaks (mg/100g FW) of carrot variety (leaf and root)											
	Pk 1	Pk 2 (3-CQA)	Pk 3	Pk 4	Pk 5	Pk 6	Pk 7	Pk 8	Pk 9	Pk 10	Sum of all peaks
<u>ANOVA P-values (Leaf)</u>											
Varieties	0.000	0.007	0.209	0.001	0.236	0.916	0.000	0.000	0.176	0.028	0.002
Temperature	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Var * Temp	0.929	0.005	0.588	0.275	0.750	0.988	0.000	0.000	0.210	0.010	0.001
<u>ANOVA P-values (Root)</u>											
Varieties	0.456	0.372	0.837	0.074	0.804	0.877	0.031	0.000	0.000	0.000	0.558
Temperature	0.090	0.417	0.018	0.003	0.147	0.001	0.010	0.000	0.000	0.374	0.012
Var * Temp	0.098	0.234	0.374	0.098	0.081	0.103	0.042	0.001	0.007	0.713	0.080

The highlighted P-value indicates significant differences ($P < 0.05$), (n=3).

CHAPTER 4 . DISCUSSION

4.1 Effect of temperature on plant growth parameters

4.1.1 Effect of temperature on plant growth rate and time to harvest

Throughout the current study, growth of lettuce proceeded over a wider range of temperatures than the carrot. Under the highest temperature regime (27/23 °C) the growth of carrot was almost entirely suppressed. This concurs with many previous reports which have considered the responses of different plant species and genotypes subject to different growth temperatures throughout the whole of the growth cycle. For example, (Nascimento *et al.*, 2008) observed that under high temperature treatments, the success of carrot germination is dependent on genotype and the extent of the vigour of seeds (although effects of temperature on seed germination were not tested in the experiments reported here). The time to reach harvestable stage was affected by temperature regime: rapid growth and achievement of the appropriate stage of growth or maturity of plants is encouraged by higher temperatures whilst plants grown under lowest temperature regimes take a longer time to reach harvest on account of slower growth and delayed ripening/maturity. This observation was also reported by (Reddy and Hodges, 2000), who stated that early growth of lettuce is determined by temperature, and the production rate increases with increasing temperature. At low temperature slow growth is associated with a slow rate of production of leaves and more limited plant height but increases gradually as temperature regimes increase. This reflects the effect of temperature on the photosynthesis mechanism the rate of which rises with increasing temperature. Consequently, as demonstrated by (Berry and Bjorkman, 1980) temperature is a major environmental factor to which the photosynthesis process in plants is particularly sensitive.

4.1.2 Growth and plant biomass production

It is well-known that plant morphology and growth parameters are affected by growth conditions and, in the present study, plant biomass (leaf and root) were affected significantly by temperature. According to (Tackenberg, 2007), basic plant growth rate and production is the result of effects of growth conditions such as temperature. Growth

(weeks to harvest) was slowest and biomass at harvest highest at lower rather than higher temperature regimes, since in this experiment the harvest time was defined by the number of leaves, not by the weight of the plant. Although there were clear variety differences in response to temperature regimes for leaf fresh weight, (Dixter had highest leaf (FW) at 17/13 °C, while Exbury had highest leaf (FW) at moderate temperature (22/18 °C) this was mainly due to differences in moisture content.

Both lettuce varieties had highest (DW) at lowest temperature at 12/8 °C. Clearly, the rate of dry matter accumulation is the primary variable affected by temperature in many species, including lettuce (Lopes *et al.*, 2004). However it may also influence the dry matter content of fresh plant material and have an effect on product quality e.g. texture and storage potential of horticultural crops.

The temperature regimes of 12 °C and 17 °C resulted in the highest total biomass of lettuce. This could be due to the adaptation of this species to lower optimal temperatures as a 'cool' crop that performs better at lower rather than at higher temperature. This is especially the case with carrots in which root growth is more severely affected than leaves. Similar results regarding the effects of high temperature on different species were observed (Bewley, 1997). Moreover, within a species there is varietal difference: the total fresh and dry mass of lettuce Dixter (leaf and root) was higher than that of Exbury over the whole of the treatment range due to the differing response of genotypes to temperature regimes.

Since the plants were grown under constant light and similar watering regimes, the significant variations in dry matter were obviously effects of the temperature regimes. Lopes *et al.* (2004) made a similar observation. In addition to the temperature effect, light conditions have an important impact on the dry matter content, due to the strong relationship between the dry matter accumulation and the amount of photosynthetically active radiation of plants (Lopes *et al.*, 2004). Current results concur with (Koontz and Prince, 1986) who concluded that the dry matter of the lettuce plant is higher when exposed to irradiance ($< 500 \mu\text{mol} / \text{m}^2 / \text{s}$) for a longer period than when exposed to irradiance ($> 500 \mu\text{mol} / \text{m}^2 / \text{s}$) for shorter periods. This explains the effect of time until harvesting and that differences are dependent on the temperature regime even with a similar light condition ($150 \mu\text{mol} / \text{m}^2 / \text{s}$), whereas a plant which is grown slowly (longer period) has the highest dry matter in this study specifically that of lettuce. By contrast, at a high temperature, the dry matter content is affected negatively due to an increase in

maintenance respiration (Straten *et al.*, 2010), thus, plants grown at a high temperature have a less dry matter.

4.1.3 Leaf area

The rate of leaf production increased with increasing day temperature regimes which concurs with the conclusion provided by (Wurr *et al.*, 1996): this is consistent with lettuce being a vegetable that matures more quickly and reaches a marketable stage when grown at a high temperature. The temperature response of the leaf area of lettuce was affected by variety: Dexter being more vigorous than Exbury. Temperature had a significant effect on the average leaf area of lettuce varieties for all temperatures, moderate temperature (22/18 °C) gave the highest leaf area, with average of varieties increasing by 45.9 % from 12/8 to 22/18 °C and then decreasing by 22.2 % from 22/18 to 32/28 °C. This could be due to the effects of the lowest and highest regimes on photosynthetic efficiency and hence on the leaf area: 22/18 °C was the optimum regime for lettuce growth.

Al-Hamdani and Todd (1990) observed slow growth, a large leaf area, lower respiration and the lowest net photosynthesis in alfalfa plants grown at 21 °C than those grown at 34 °C, but different light conditions were imposed (850 and 520 $\mu\text{mol}/\text{m}^2/\text{s}$). This is similar to the current results, even with much reduced light condition (150 $\mu\text{mol}/\text{m}^2/\text{s}$).

4.2 Phytochemicals

Clearly, growth temperature regimes effects on morphology and physiological processes lead to impacts on the quality and nutritional value of plant products (Moretti *et al.*, 2010). This is reflected in the large changes in the phytochemical concentrations in this study. However, the response of plants to growth temperature conditions and, thus, the accumulation of phytochemicals differs, as has been reported in previous studies of environmental stress influences (Taub *et al.*, 2000; Moretti *et al.*, 2010; Sanghera *et al.*, 2011).

4.2.1 Sugar content

Accumulation of sugar in both shoots and roots of lettuce and carrot was highest at a low temperature and decreased with increasing temperatures. According to (Pietrini *et al.*, 2002), at low temperatures respiration rates slow down and there is less enzyme activity, which tends to reduce energy demand and leads to a high accumulation of sugar instead of nitrate. By contrast, at a high temperature, increasing respiration (more energy requirement) leads to a decrease in the sugar content with a different response between the genotypes, as a result of the associated active nitrogen metabolism process that utilizes sugars, (Champigny, 1995).

This offers an explanation for current results whereby, the mechanism of sugar production results in high levels in response to the effect of low temperatures, leading to the a reduction of nitrate accumulation associated with an increase in the rate of nitrogen metabolism. Similar observations were made in lettuce leaves by (Zhou *et al.*, 2013) with the same light intensity but a different light quality.

Additionally, the greater concentration of sugar is associated with slow growth and development of plants, due to the main effect of low temperatures on sugar accumulation. This contrasts with higher temperature regimes that lead to more rapid growth and less sugar. Therefore the physiological process (the photosynthesis and respiration alterations caused by temperature) is a possible reason, since dark respiration slows down under low temperature conditions, which causes reduction of carbohydrate consumption (Khayat *et al.*, 1988). Thus carbohydrate levels are influenced by the maturation extent of leaves and shoots (Jiao *et al.*, 1989) in rose plants.

Although effects on sugar content were variable dependent on temperature regimes in the recent study, this was not the case for the content of non-sugar materials (plant residue) of both lettuce and carrot plants, where there are no reported studies to compare with.

4.2.2 Nitrate concentration

Previous research has shown that light (Lillo, 2004; Santamaria, 2006) and temperature (Hill, 1991; Gorenjak and Cencic, 2013) are both important for nitrate accumulation in

plants, they are considered to be the main factors affecting of both uptake and reduction of nitrate in plants.

Nevertheless, in the present study, growth temperature regimes were observed to be a significantly influential factor on nitrate accumulation, while the light was constant for all temperature treatments. Nitrate accumulation in both the leaves and roots of carrots and lettuces increased significantly with temperature. However, concentrations differed between roots and leaves and between different varieties of the same plant species.

It is well known that the enzyme reductase rapidly can convert nitrate to amino acids in the plant, and that its conversion requires important factors that are: light, nutrients, water and appropriate temperature, (Weitzberg and Lundberg, 2013). However, if the plant is exposed to different stresses, the conversion of nitrate to proteins becomes restricted, thereby resulting the accumulation of nitrate (Hill, 1991).

Other studies have demonstrated that lettuce which is grown in winter (low temperature and low conditions) has higher nitrate concentration than those grown in summer (high temperature and high light conditions) due to differences in light intensity thus affecting nitrate reductase enzyme activity leading to variation of nitrate uptake in plant tissues, (more light result in more nitrate being converted into protein) (Leyva *et al.*, 1995). Light energy also stimulates the photosynthetic electron transport to provide the required electrons that are responsible for reduction of nitrate to nitrite, (McCashin, 2000).

However, in complete contrast, in this study nitrate concentration increased with temperature. This is probably due to the fact that light inputs was constant at all temperatures and relatively lower ($150 \mu\text{mol} / \text{m}^2 / \text{s}$) compared with other reports. According to (Brown *et al.*, 1993) the increase in accumulation of nitrate in plants grown at high temperature is high due to the rapid transpiration of water that occurs at faster rate than the nitrate can be metabolised.

Some studies have raised concerns about links between the nitrate concentration in the diet and some health issues such as cancer in the digestive tract or methaemoglobinaemia in infants. This may be caused by toxic products such as nitrite resulting from the conversion of ingested nitrate in saliva and the gastrointestinal tract, (Lundberg *et al.*, 1994; Bryan and Grinsven, 2013).

However, nitrate concentration of leafy salads and vegetables is a feature related to the European Commission's adopted EC Regulation No. 1822/2005 associated with health requirements of consumers. This specifies the maximum level of nitrate permitted in lettuce and spinach (Table 1.2), Exbury variety had the highest concentration of NO_3

(2690 mg/Kg) at 27/23 °C. This level exceeds the maximum permitted level in lettuce during summer (for crops grown in the open air and under cover) and could therefore have implications for human health. Clearly, there are differential effects of temperature and also variety on leaf nitrogen concentrations which are significant for both producers and consumers.

Carrot root growth was inhibited at a high temperature (27/23 °C), however, it was not possible to measure nitrate concentration in the carrot root at this temperature regime, since the amount of root material (of the tap root) was too small, therefore the nitrate concentrations was investigated in the leaves only. Whereas leaves had the highest accumulation of nitrate under this temperature regime (27/23 °C), so this may also have been the case for the roots, if it had been possible to measure the content in the very small roots that were formed at this regime.

Nitrate accumulation reacts in the opposite way to sugar and increases with an increase in the temperature, as concluded by (Zhang *et al.*, 1997). However, most of the earlier studies show a strong correlation between low light intensity and high accumulation of nitrate in both the field and greenhouse.

In the present study, the growth temperature played a significant role in nitrate accumulation. This observation will thus be useful for growers when growing plants at different temperatures (seasonal differences between summer and winter) and when using fertilizers which lead to an increase in the nitrate level (Brown *et al.*, 1993; Byrne *et al.*, 2004; Bumgarner *et al.*, 2012). Thus nitrogen fertiliser recommendations and determination of the appropriate fertilizer dose to be applied should take account of the prevailing temperature regime, taking into account both light and temperature changes between seasons. On other hand climate change is predicted to increase temperature without changing the light conditions.

Different studies used different experimental design have achieved very different results, however, it is well known that the nitrate concentration can be controlled (reduced) during winter growing in greenhouses by using either a low temperature or with supplementary light or both to develop a strategy of growth, (Ioslovich and Seginer, 2002; Demsar *et al.*, 2004; Lillo, 2004; Xu and Huang, 2011).

4.2.3 Chlorophyll pigments

Many researchers consider that HPLC measurement to be the more accurate quantification technique for chlorophyll pigments and the results of this study was based on this. Also the chlorophylls were measured spectrophotometrically (fresh material), but it was not possible to use the results of the spectrophotometric method because of an error in the procedure used.

According to the observation by (Al-Hamdani and Todd, 1990) chlorophyll a and b increased with increasing temperatures. However, as expected the results showed that the high temperature regimes increased chlorophyll levels of both lettuce and carrot leaves. Similarly, Al-Hamdani and Todd (1990) reported that chlorophylls a and b of Alfalfa grown at 12, 21 and 34 °C under high light conditions increased with an increase in temperatures. Although lettuce varieties respond differently to temperature regimes in terms of chlorophyll accumulation, in the current study, chlorophylls a and b of lettuce increased as the temperature increased. As consideration the chlorophylls are the primary pigments of photosynthesis in plants, and its concentration can affect different factors. Through the present study with constant light conditions, the concentration appears low at the lowest regime and increases with an increase in temperatures due to the greater activity of photosynthesis as observed by (Fitter and Hay, 2002; Qin *et al.*, 2007; Moretti *et al.*, 2010), which in turn increases the chlorophyll concentration. This observation disagrees with the results reported by (Gazula *et al.*, 2005) who observed that lettuce grown at 20 °C has a higher concentration of chlorophyll b than lettuce grown at 30 °C due to pigment degradation at high temperatures, the likely reason for this disagreement is the genotypes variation to temperature response and high light conditions ($600 \mu\text{mol} / \text{m}^2 / \text{s}$) which is considered high for the light conditions when compared to the current study which is ($150 \mu\text{mol} / \text{m}^2 / \text{s}$).

The observation for both chlorophylls a and b concentration of carrot leaves in present study are higher at a moderate temperature 22/18 °C than at a lower temperature and slightly decreased at 27/23 °C (above optimal). This suggests that the optimal temperature regime is 22/18 °C. Consequently, an increase in photosynthesis activity is associated with an increase in chlorophyll pigment concentrations associated with increasing temperatures (up to 32 °C in the present study) and the ability of plants to

adapt up to this range. However, it could be that had the temperature range under test been extended above 32 °C there may have been a decrease or/ and inhibition of pigmentation. According to (Berry and Bjorkman, 1980) however, high temperature (> 20 °C) has a negative effect on the photosynthetic apparatus (Photosystem II and CO₂ fixation) through disruption of the functional integrity, due to the high sensitivity of photosynthesis to high temperatures .

As reported by (Nobel, 1999) that the normal ratio of chl a: chl b is (3:1), while the present results shows an increase of chlorophyll a / chlorophyll b ratio, which even exceeded the normal range which is considered to be 4:1.

However, this is most likely in response to the low light condition in this study. A similar observation was reported by (Beneragama and Goto, 2010), who observed increased chlorophyll a/b ratios in *Euglena* in low light conditions.

4.2.4 Carotenoid composition

In the present study temperature regimes had a highly significant effect on carotenoids' concentration. The concentrations of lutein, β -carotene and *Cis* β -carotene in the variety Dixter were similar over all temperature regimes except at the moderate temperature (22/18 °C) where it was highest. The variety Exbury also exhibited significant effect of temperature on carotenoid concentration but the concentrations of lutein, β -carotene and *Cis* β -carotene were broadly similar at all temperature regimes. This could be a varietal response to the temperature effect. Generally, these results concur with those reported by (Mou, 2005), that carotenoids levels in autumn (where the temperature close to 17/13 °C in present study) are lower than in the summer (where the temperature close to 22/18 and 27/23 °C in present study, taking into account the differing light conditions), in addition to the genetic variation that is significant for lettuce varieties. Regarding carrots, however, (Simon and Wolff, 1987) reported that the variation of carotenes' concentration is likely to be caused by genetic and environment factors but the genotypes are considered the main influence on carrot roots. In the present study, the concentrations of lutein, α -carotene and β -carotene were significantly affected by temperature which is consistent with the results for carrot roots, as reported by Nicolle *et al.* (2004). Carotenoid levels of carrot leaves were also significantly affected by temperature. There is a different response of carotenoids to

temperature regimes and a greater temperature effect on lutein concentration in both carrot leaves and roots, although they respond in different directions. Whereas the lutein in the carrot root increases with an increase in temperature, the concentration in the leaves decreases with an increase in temperature. In this study, it was evident that the effect of temperature on accumulation of lutein in leaves is opposite to that of chlorophyll pigments. Therefore, these results disagree with other studies e.g. Mou (2005) who concluded that both carotenoids and chlorophyll concentrations were both affected in the same direction. The low light conditions in the present study are probably a reason of this disagreement. This observation needs further research to clarify understanding.

The greater β -carotene of carrot root at 17/13 °C could be due to the more prolific growth of roots, dry matter accumulation with advancing maturity and increasingly dark orange colour at this temperature, especially in the Parmex variety (Figure 3.31): this is supported by (Samuoliene and Duchovskis, 2006) who indicated that the carotenes' concentrations increase with the age and size of carrot roots. However, carrot leaves have about twice the concentration of carotenoid levels than the roots.

4.2.5 Polyacetylene compounds of carrot root

Concentration of the three polyacetylene compounds (falcarindiol, falcarinol 3-acetate and falcarinol) varied considerably between the temperature regimes. At the lowest temperature (12/8 °C) both carrot varieties exhibited the highest concentration of polyacetylenes in the roots: on average the concentrations in both varieties decreased by 45.3 % with an increase in temperature (12-22 °C). The influence of temperature on polyacetylenes accumulation is considered to be one of the different defence mechanisms of plants against environmental stresses, including temperature and also pests and diseases. Many studies have reported the antifungal activity and antibacterial properties of polyacetylenes (Mercier *et al.*, 1993; Baranska and Schulz, 2005; Christensen and Brandt, 2006).

Falcarindiol is the most abundant polyacetylene in both varieties of carrot root, as reported by (Czepa and Hofmann, 2003; Czepa and Hofmann, 2004; Christensen and Kreutzmann, 2007; Kjellenberg *et al.*, 2010) and also reported by (Kreutzmann *et al.*, 2008) in the carrot peel. On the other hand, in other studies, Falcarindiol was present at

a lower concentration than Falcarinol (Kreutzmann *et al.*, 2008). Falcarinol differed markedly between the two varieties of carrots, which may reflect the ability of different genotypes to accumulate polyacetylenes, both during growth and in the post-harvest phase which is apparent in the effects of root size and storage conditions as reported by (Kidmose *et al.*, 2004). Many previous studies have shown the sensitivity of polyacetylenes to several different factors, but no reports have highlighted the effects of growth temperature on the polyacetylene concentration in carrots. Information about the accumulation of these bioactive compounds at different temperatures is still new and the present study demonstrates that the low growth temperature regime enhances increased levels of polyacetylenes. This might be due to the role of polyacetylene compounds as a defence against sub-optimal temperature stresses and thus the synthesis effects depend on temperature. Further research is needed to confirm this observation in the absence of clear investigations of growth temperature influences and the associated physiological processes.

4.2.6 Phenolic compounds

4.2.6.1 Phenolic acids

Present results confirm chlorogenic acid, (3-CQA), as the main phenolic acid in carrot root and leaf, and also its presence in lettuce leaves. Other major phenolic acids have been identified by other groups of researchers. However, most of the phenolic standards were tested, which had previously been reported for carrots, were not present in the samples analysed in present study. Therefore, the results reported in the previous literature were probably not correct. However of all of the alternative compounds, the standards were impossible to purchase for phenolic acids. So there is full agreement in all of the literature that these peaks are phenolic acids, it's just not certain which phenolic acids they are.

Regarding lettuce leaves, the current study which identified chlorogenic acid named as (3-CQA) as the main phenolic acid does not concur with (Romani *et al.*, 2002) who reported that the name of main caffeic derivative was (5-CQA).

Many studies have shown the high accumulation of phenolics under the different stresses. Therefore, the current results show similar observations that have previously

been reported (Ellnain-Wojtaszek *et al.*, 2001; Wang and Zheng, 2001; Gliszczynska-Swiglo *et al.*, 2007; Oh *et al.*, 2009): exposure of plants to stresses, increases the action of enzymes that lead to an increase in the production of phenolic acids.

The temperature regimes (22/18 and 27/23 °C) that are considered an the moderate range (least stressful) to growth and development of both species, however, the plants at these regimes had the lowest concentration of phenolic compounds. Clearly, the stress temperature regimes (12/8 and 32/28 °C) particularly lower temperature (12/8 °C) affected the biosynthesis of phenolic compounds (phenolic acids and quercetin) and thus resulted in the accumulation of higher levels in the lettuce and carrot leaves. Similarly, (Dixon and Paiva, 1995) reported during the exposure of plants to diverse stresses such as temperature and/or light conditions, as a result it is likely antioxidants act for the defence through accumulation in response to stress. This reflects on plants which adapt to the surrounding conditions. These defence mechanisms of genetic activity were also reported in lettuce by Oh *et al.* (2009).

Likewise, carrot shoots have a higher concentration of phenolic acids than roots and both parts vary in response to temperatures.

In addition, that the least stressful temperature for carrot is lower than for lettuce, and this corresponds with the temperature where the concentration of phenolic acids is lowest.

The present study provides strong evidence that contributes further to previous reports about accumulation of phenolic compounds under stress. Due to the important role of some enzymes (such as phenylalanine ammonia-lyase (PAL)) and activation resulting from stresses, accumulation of phenolics increases to levels according to the extent of the stress, (Rosa *et al.*, 2001; Oh *et al.*, 2009; Boo *et al.*, 2011).

4.2.6.2 Anthocyanin of lettuce

High concentrations of anthocyanin in lettuce grown at lower temperatures (12/8 and 17/13 °C) can be easily observed by the red pigmentation of the leaves, which is not apparent at higher temperatures (27/23 and 32/28 °C).

Boo *et al.* (2011) also reported higher concentrations of anthocyanin in lettuce grown at relatively low temperatures (13/10 °C and 20/13 °C) compared with higher temperature

(25/20 and 30/25 °C) that had lower concentration, with the same light conditions as the present study. Therefore, the temperature regimes result in significant alterations of plant metabolism which cause different anthocyanin accumulation in lettuce tissue. However, increased occurrence of both activities of PAL (phenylalanine ammonia-lyase) and PPO (polyphenol oxidase) more quickly at low temperature than at high temperature and increased the anthocyanin accumulation (Boo *et al.*, 2011).

Zhang *et al.* (1997) reported a high accumulation and production of anthocyanin in strawberry cells grown at a lower temperature (15 and 20 °C) than that of higher regimes (30 °C) associated with a longer period of growth, due to the different biosynthesis of anthocyanin. Thus, the physiological mechanisms (response) were different (during various growth temperatures). This concurs with the present study which identified greater levels of anthocyanin in lettuce grown slowly at low temperature regimes even with low light conditions.

By contrast, high temperature suppression of anthocyanin accumulation, could arise because the unstable state, genetic mechanisms of anthocyanin and short periods of growth at high temperatures as concluded by (He *et al.*, 2010). As well as a high temperature is the main influence on anthocyanin accumulation. Two mechanisms were reported by (Chao and Yang, 1996; Shaked-Sachray *et al.*, 2002), one is that the synthesis decreases and the other is an increased rate of degradation, both reducing the concentrations at high temperatures.

The physiological aspects of anthocyanin accumulation at low temperature conditions have been reported by many researchers who are aware of this protective mechanism (Leyva *et al.*, 1995) being an important key of plant growth. This acts as light screening to protect the plants against the temperature stresses and the photo induced oxidative reaction inhibited through the photosynthetic apparatus, whereby anthocyanin molecules provide this protection during low temperatures associated with slow rate of photosynthesis and slow enzyme activity (Chalker-Scott, 1999; Pietrini *et al.*, 2002; Bumgarner *et al.*, 2012). These explanations support the results of the present study as regards low temperature regimes.

The varieties significantly vary in their response to low temperature regimes and thus lead to a variation in anthocyanin levels which could be due to the different genetics and responsiveness. These results provide additional firm confirmation of high accumulations of anthocyanin associated with low temperature regimes and that high

temperatures (especially the moderate 22/18 °C) lead to the inhibition of pigment synthesis, as has been reported in many investigations.

In contradiction to the present experiment actually proves that (He *et al.*, 2010) hypothesis is incorrect, and that the anthocyanin can be formed also at high temperatures (the Dixter and Exbury dramatically had slightly increasing of anthocyanin at the highest regime (32/28 °C) than lower moderate regimes), probably when there is stress that requires protection (as mentioned with phenolic acids). However, further researches are required to support this observation.

4.2.7 Effects of temperature on plant composition

Temperature played significant effects on growth rate and the different stages of development of plants.

Practically, this study points out the importance to growers of knowledge and understanding about the different response of varieties to temperature. This may help their decisions about choice of varieties to be grown under different environmental conditions in order to achieve the best yields and quality and hence optimise their returns. On other hand it was clear that some the important photochemical compounds either increased or decreased according to temperature i.e. the optimum was different for different compounds which may mean that compromises may be needed. The present study has shown how the temperature regimes affect two species of vegetable plants, and may be considered as a starting point for further studies focusing on the link between production environment and the effects of these important phytochemicals that relate to diet and human health and effects of climate change that lead to increasing of temperature. The role of some as beneficial antioxidants and others that may have potential toxic effects is still an area of great debate: potential inhibition against diseases such as some cancers by producing plants for human consumption that have high levels of beneficial phytochemicals is an important goal. Just as important is the avoidance of producing potentially harmful levels of phytochemicals for consumption. It is essential however to confirm precisely which are beneficial and which are harmful and in what amounts.

Most previously published work in this area has investigated effects of temperature on individual phytochemicals, in most cases focusing on the types of stress or other

environmental influences (e.g light stress, water stress) that are particularly relevant for this type of compounds.

The present study has investigated one specific environmental factor - temperature - across a wide range of different phytochemicals in two plant species. For phytochemicals present in both carrots and lettuce, most temperature effects show the same trends for both species, although the temperature optimum for carrot growth is lower than for lettuce.

The present study also confirms that responses to temperature differ between different groups of phytochemicals which have different functions within the plant. Clearly therefore, no single temperature regime can optimize the concentration of all the potentially health-promoting compounds in vegetables. Conclusions based on single-compound investigations are thus likely to contradict comparable outcome focusing on other types of compounds.

This highlights the need for more research to determine which phytochemicals are more or less important for human health, as a necessary basis for any successful implementation of the horticultural/agronomic studies.

CHAPTER 5 . CONCLUSION AND FUTURE WORK

5.1 Conclusion

The results of this study confirm what is well known - that growth temperature regimes have a significant effect on plant morphology, resulting in a variation of growth parameters. The temperature stresses that were imposed had very clear effects on growth. As temperature increased plants grew more rapidly with leaf growth being more positively affected than root growth. On the other hand, low temperatures resulted in slow growth and increased root biomass (especially the roots of carrots). The lettuce plants were able to grow in a wide range of temperature regimes, whilst carrot root growth was more affected than that of shoot growth and at 27/22°C growth became inhibited. Plants grown in a higher temperature regime were significantly taller and had a greater leaf production than in lower regimes. There was a very consistent effect of temperature regimes on dry matter content: the lowest temperature (12/8 °C) produced the highest dry matter content of both shoots and roots.

Clearly growth temperature regimes have an effect on the physiological processes of plants and involve components, which either respond to or defend against extreme temperatures by means of large alterations in phytochemical concentrations.

Highly significant effects of the temperature regimes on the lettuce and carrot varieties were observed. At the lowest temperature, the sugar content increased in both shoot and roots. Growth temperature plays an important role in nitrate accumulation of plants; however it can be concluded that increasing temperatures will increase nitrate under low light conditions. Consequently, the response of sugar and nitrate concentrations to temperature was in the opposite direction.

There was a marked effect of the three temperature regimes for the polyacetylenes in carrot roots which decreased by 45 % at the lowest temperature. Falcarindiol was at the highest level followed by intermediate levels of Falcarinol and Falcarindiol-3-acetate at the lowest level in all temperature regimes. The chlorophylls and carotenoids which are directly involved in photosynthesis were higher at moderate temperatures than in the lowest temperature regimes.

Temperature had a significant effect on total phenolic compounds. The accumulation of phenolic compounds in both carrots and lettuce leaves is associated with both low and high temperature treatments, and the levels at 22/18 °C are only 3-50 % of the highest values, whilst the total phenolics of carrot roots are at consistent levels.

Accordingly, it can be noted that growth temperatures, particularly stress regimes, enhance (stimulate) high accumulation of phytochemicals and thus increase the nutritional value of main food plants which, in turn, may help to compensate for the generally below recommended rate of vegetable consumption which is apparent in some populations. To summarize, these effects could become increasingly important aspects as climate change and global warming proceeds with potentially substantial implications for the links between diet and human health.

5.2 Future work and recommendation

Further investigations are required to test the performance and composition of plants under different temperature and light levels with more replications of each treatment to confirm these observations. Continuation of this line of research with various different types of vegetables and different genotypes and how they are affected by different temperature treatments should also be pursued. In terms of nutritional value, it would be interesting to investigate the levels of phytochemicals of plants grown at different temperatures and compare them with the changes in levels that may occur as a result of postharvest storage processes and assess the extent to which such changes in concentrations may be achieved by manipulating environmental conditions in the field, greenhouse or store. It should then be possible to assess the potential benefits or dangers to humans of the changes in nutritional value brought about by such modifications. Furthermore, for commercial vegetable production, detailed knowledge about the interactions between changing environmental temperatures and water and nutrient supply throughout growth that impact on crop nutritional value may help to design and optimise management strategies to control crop product quality future basic research is recommended. For example, it would be useful to investigate and identify the main phenolic compounds present in carrot roots by using new techniques with confirmation of their names and chemical structure.

CHAPTER 6 . REFERENCES

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CHAPTER 7 . APPENDIX

7.1 Materials and methods

7.1.1 Experimental overview



Figure 7.1 Growth chamber model Sanyo (MLR-351) and plant trays (replication).



Figure.7.2 Processing of carrot and lettuce variety samples at harvest time: sample collection, storage conditions and milled samples.

7.1.2 Growth parameters

7.1.2.1 Leaf area analysis

The analysis was done as following: each image file was opened in image J program Figure 7.3.

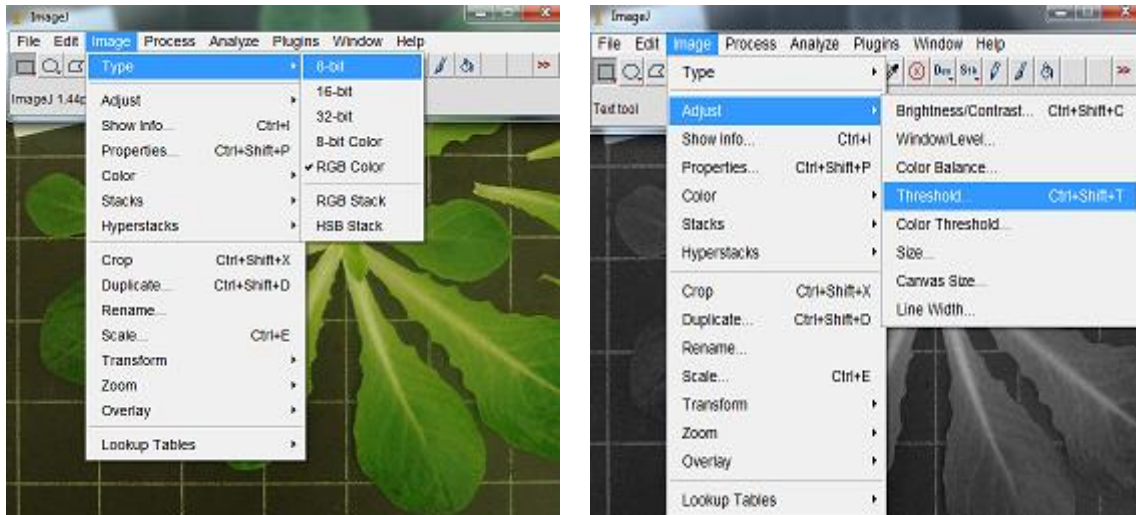


Figure.7.3 Show the steps of measurement of lettuce leaf area

The image menu was opened and the “type” selected. Then “8-bit” was selected, then again from image menu the “adjust” tools were selected, then “threshold”. The sliding bars were adjusted until the whole leaf area was all red and the background was white. Then a black and white image appeared. The scale on image were set from toolbar, the straight-line tool was selected then a line drawn across the area of known dimensions (which is = 3 cm). For each plant whole leaves were analysed at one time, by selection of the box or polygon tool from the toolbar and drawing a box or outline around the whole leaf, a single click placed a new line in the shape and double click to close the shape. Also a specific leaf can be measured by selection of required leaf. From the analyse menu “set measurements”, were selected and the area box was ticked. Analyse menu again opened “analyse particles” were selected, then a dialogue box were opened, the minimum size of an object where it say “size (cm²) were selected and clicking OK, then a new box page were appearing with the results.

7.1.3 Phytochemicals analysis

7.1.3.1 Nitrate measurement

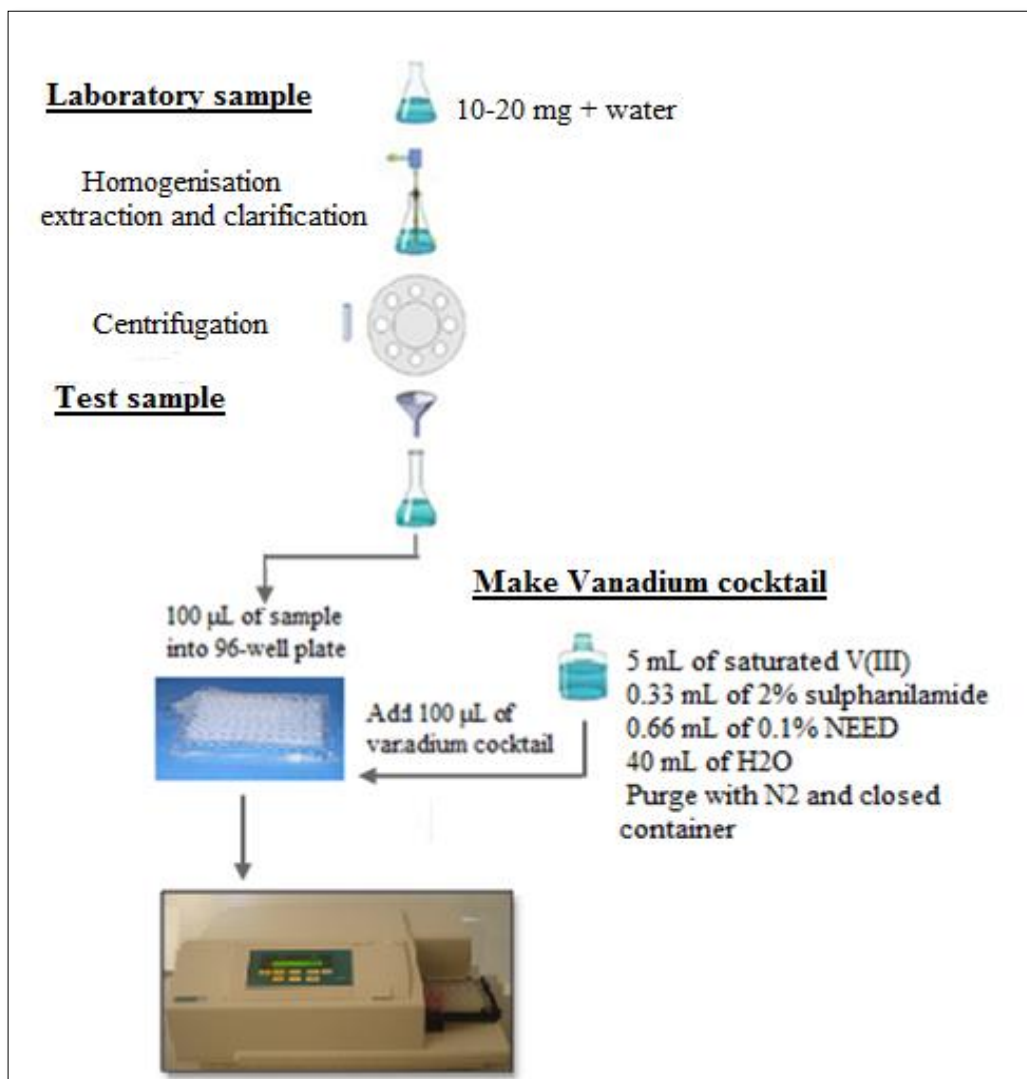


Figure 7.4 Flow chart of the nitrate analytical procedure

7.2 Results

7.2.1 Growth rate

7.2.1.1 Plant biomass

Table 7.1 Analysis of Variance (ANOVA) for the weight of lettuce variety root grown in different temperature regime, (n=3).

<u>Variety</u>	<u>Weight of root (g/plant)</u>	
	<u>Fresh weight</u>	<u>Dry weight</u>
<u>Dixter</u>		
12/8	3.057 ± 0.086 a	0.326 ± 0.027 a
17/13	1.928 ± 0.102 b	0.133 ± 0.007 b
22/18	1.144 ± 0.021 c	0.068 ± 0.003 c
27/23	0.579 ± 0.019 d	0.037 ± 0.001 c
32/28	0.673 ± 0.052 d	0.045 ± 0.003 c
<u>Exbury</u>		
12/8	1.722 ± 0.103 a	0.121 ± 0.013 a
17/13	1.084 ± 0.113 b	0.061 ± 0.005 b
22/18	0.476 ± 0.038 c	0.026 ± 0.002 c
27/23	0.277 ± 0.018 c	0.017 ± 0.001 c
32/28	0.550 ± 0.076 c	0.037 ± 0.005 bc
<u>ANOVA P-Values</u>		
Varieties	0.000	0.000
Temperature	0.000	0.000
Varieties* temperature	0.000	0.000

The values are Mean ± standard error of means followed by different letters are significantly different, the highlight P-value indicated significant differences (P< 0.05).

Table 7.2 Analysis of Variance (ANOVA) for the weight of carrot variety leaf grown in different temperature regimes, (n=3).

-	Weight of leaf (g/plant)	
	<u>Fresh weight</u>	<u>Dry weight</u>
<u>Parmex</u>		
12/8	1.816 ± 0.191 b	0.334 ± 0.028 b
17/13	4.140 ± 0.179 a	0.657 ± 0.033 a
22/18	4.281 ± 0.191 a	0.668 ± 0.022 a
27/23	2.535 ± 0.422 b	0.427 ± 0.026 b
<u>Little Finger</u>		
12/8	2.229 ± 0.089 b	0.436 ± 0.031 b
17/13	4.508 ± 0.264 a	0.730 ± 0.053 a
22/18	4.070 ± 0.357 a	0.667 ± 0.057 a
27/23	2.563 ± 0.143 b	0.380 ± 0.030 b
<u>ANOVA P-Values</u>		
Varieties	0.437	0.251
Temperature	0.000	0.000
Varieties* temperature	0.634	0.199

The values are Mean ± standard error of means followed by different letters are significantly different, the highlight P-value indicated significant differences (P< 0.05).

Table 7.3 Analysis of Variance (ANOVA) for the total plant weight (fresh and dry) of lettuce varieties grown in different temperature regime, (n=3).

<u>Variety</u>	Total plant weight(g)	
	Fresh weight	Dry weight
<u>Dixter</u>		
12/8	11.57 ± 0.43 a	1.27 ± 0.05 a
17/13	13.57 ± 0.68 a	0.86 ± 0.04 b
22/18	11.59 ± 0.21 a	0.66 ± 0.01 c
27/23	8.17 ± 0.45 b	0.48 ± 0.01 d
32/28	7.16 ± 0.43 b	0.44 ± 0.02 d
<u>Exbury</u>		
12/8	8.44 ± 0.63 a	0.61 ± 0.05 a
17/13	7.61 ± 0.98 ab	0.43 ± 0.05 b
22/18	7.77 ± 0.46 ab	0.39 ± 0.01 bc
27/23	5.11 ± 0.34 b	0.25 ± 0.01 c
32/28	6.76 ± 0.41 ab	0.43 ± 0.03 b
<u>ANOVA P-Values</u>		
Varieties	0.000	0.000
Temperature	0.000	0.000
Varieties* temperature	0.001	0.000

The values are Mean ± standard error of means (SEM) followed by different letters are significantly different; the highlight P-value indicated significant differences (P< 0.05).

Table 7.4 Analysis of Variance (ANOVA) for the total plant weight (fresh and dry) of carrot varieties grown in different temperature regime, (n=3).

<u>Variety</u>	Total plant weight(g)	
	Fresh weight	Dry weight
<u>Parmex</u>		
12/8	4.64 ± 0.26 c	0.70 ± 0.04 c
17/13	11.78 ± 0.38 a	1.62 ± 0.05 a
22/18	7.52 ± 0.54 b	1.03 ± 0.07 b
27/23	2.62 ± 0.43 d	0.44 ± 0.03 d
<u>Little Finger</u>		
12/8	3.97 ± 0.55 c	0.66 ± 0.10 c
17/13	10.81 ± 0.56 a	1.43 ± 0.06 a
22/18	7.69 ± 0.87 b	1.05 ± 0.12 b
27/23	2.65 ± 0.16 c	0.39 ± 0.03 c
<u>ANOVA P-Values</u>		
Varieties	0.309	0.154
Temperature	0.000	0.000
Varieties* temperature	0.588	0.363

The values are Mean ± standard error of means followed by different letters are significantly different, the highlight P-value indicated significant differences (P< 0.05).

7.2.2 Chlorophylls and carotenoids

Table 7.5 Carotenoids of lettuce leaves with the HPLC specific UV spectrum

Peak	Retention time (RT)	symbol	compound	HPLC- DAD UV spectrum λ_{\max} (nm)
1	38.2	Lut	Lutein	421sh,443,471
2	40.5	Chl b	Chlorophyll b	436sh, 463,470
3	41.8	Chl a	Chlorophyll a	338,385,418,432
4	45.4	α -car	α -carotene	424sh, 444, 472
5	45.7	β -car	β -carotene	422,450,474
6	46.3	Cis β -car	Cis β -carotene	421,446,469

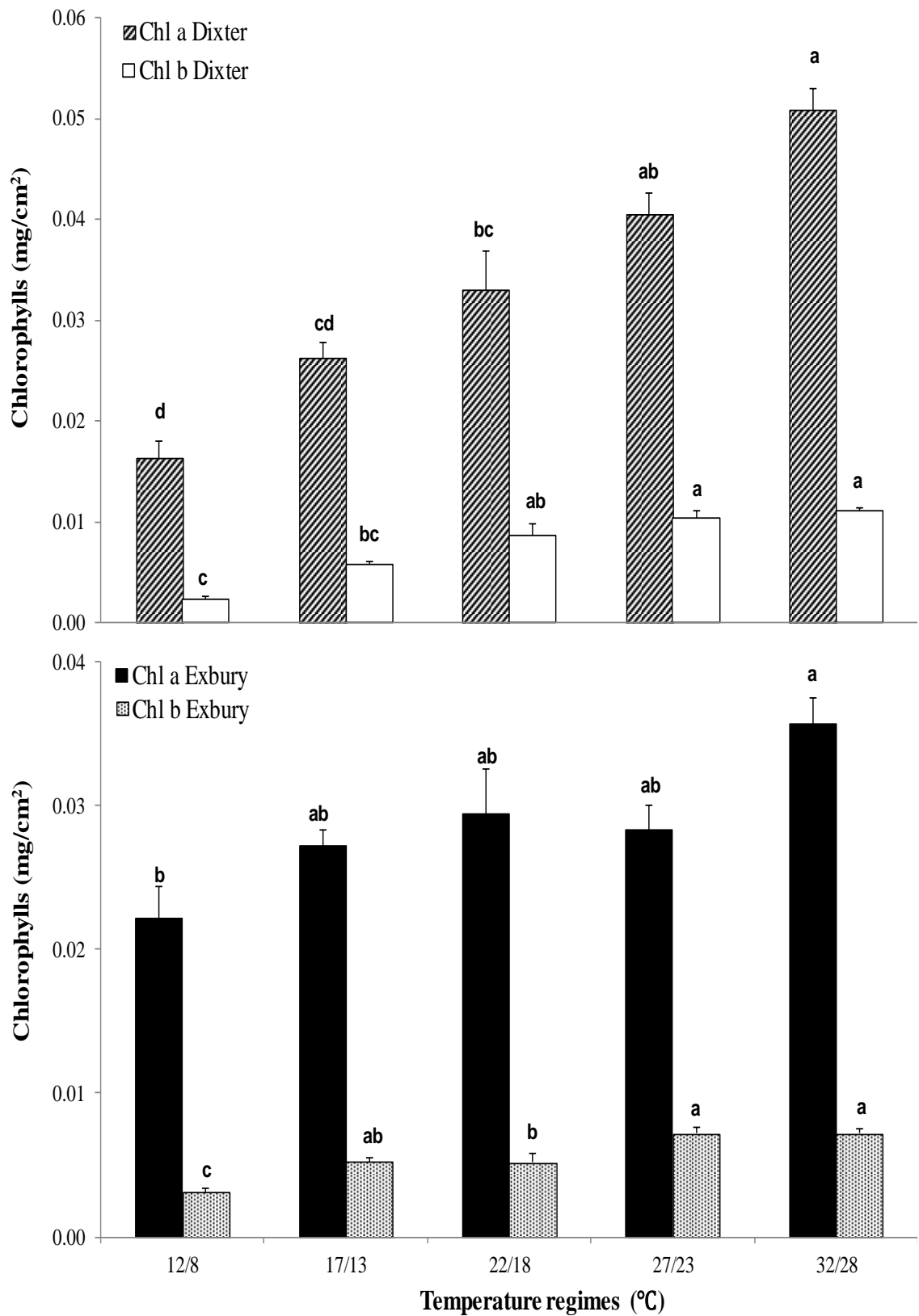


Figure 7.5 The effect of lettuce leaf variety (Dexter and Exbury) and temperature on chlorophyll a and b (mg/cm²). Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

Table 7.6 Analysis of Variance (ANOVA) for the sum chlorophylls (a and b) and carotenoids (Lutein, β -carotene and *Cis* β -carotene) of lettuce leaf varieties grown in different temperature regime, (n=3).

Sum of chlorophylls and carotenoids of lettuce leaf (mg/ 100g FW)		
Variety	Sum of chlorophylls (a + b)	sum of carotenoids (Lut, β -car and <i>Cis</i> β -car)
<u>Dixter</u>		
12/8	49.34 \pm 4.17 b	5.76 \pm 0.35 b
17/13	65.61 \pm 4.46 ab	6.03 \pm 0.38 b
22/18	82.52 \pm 7.69 a	12.98 \pm 0.67 a
27/23	83.61 \pm 4.37 a	5.72 \pm 0.18 b
32/28	91.13 \pm 7.61 a	5.93 \pm 0.45 b
<u>Exbury</u>		
12/8	39.44 \pm 1.95 c	3.87 \pm 0.18 ab
17/13	36.53 \pm 0.11 c	3.30 \pm 0.15 b
22/18	55.02 \pm 5.17 b	4.87 \pm 0.41 a
27/23	68.91 \pm 2.73 a	4.68 \pm 0.22 a
32/28	39.19 \pm 0.52 c	2.01 \pm 0.05 c
<u>ANOVA P-Values</u>		
Variety	0.000	0.000
Temperature	0.000	0.000
Variety *Temperature	0.002	0.000

The values are Mean \pm standard error of means within followed by different letters are significantly different, the highlight P-value indicated significant differences (P< 0.05).

Table 7.7 Analysis of Variance (ANOVA) for the sum chlorophylls (a + b) and carotenoids (Lut, α -car, β -car and *Cis* β -car) of carrot leaf and root variety grown in different temperature regime, (n=3).

Sum of chlorophylls and carotenoids of carrot (mg/100 g FW)			
	Leaf		Root
	sum of chlorophylls (a + b)	sum of carotenoids (Lut, β -car and <i>Cis</i> β -car)	sum of carotenoids (Lut, α -car, β -car and <i>Cis</i> β -car)
<u>Variety</u>			
<u>Parmex</u>			
12/8	98.30 \pm 4.96 a	9.22 \pm 0.33 b	3.72 \pm 0.58 b
17/13	105.00 \pm 3.95 a	9.63 \pm 0.58 b	11.43 \pm 0.65 a
22/18	100.90 \pm 3.53 a	12.16 \pm 0.11 a	7.63 \pm 0.42 ab
27/23	90.90 \pm 3.57 a	4.58 \pm 0.04 c	
<u>Little Finger</u>			
12/8	106.10 \pm 4.76 a	8.19 \pm 0.77 bc	2.33 \pm 1.07 a
17/13	112.90 \pm 6.79 a	9.99 \pm 0.47 b	3.43 \pm 0.19 a
22/18	134.40 \pm 9.85 a	16.36 \pm 1.41 a	3.34 \pm 0.21 a
27/23	106.40 \pm 4.91 a	5.47 \pm 0.53 c	
<u>ANOVA P-values</u>			
Varieties	0.000	0.028	0.000
Temperature	0.012	0.000	0.001
Var * Temp	0.082	0.008	0.006

The values are Mean \pm standard error of means within followed by different letters are significantly different, the highlight P-value indicated significant differences (P< 0.05).

7.2.3 Polyacetylene compounds (HPLC)

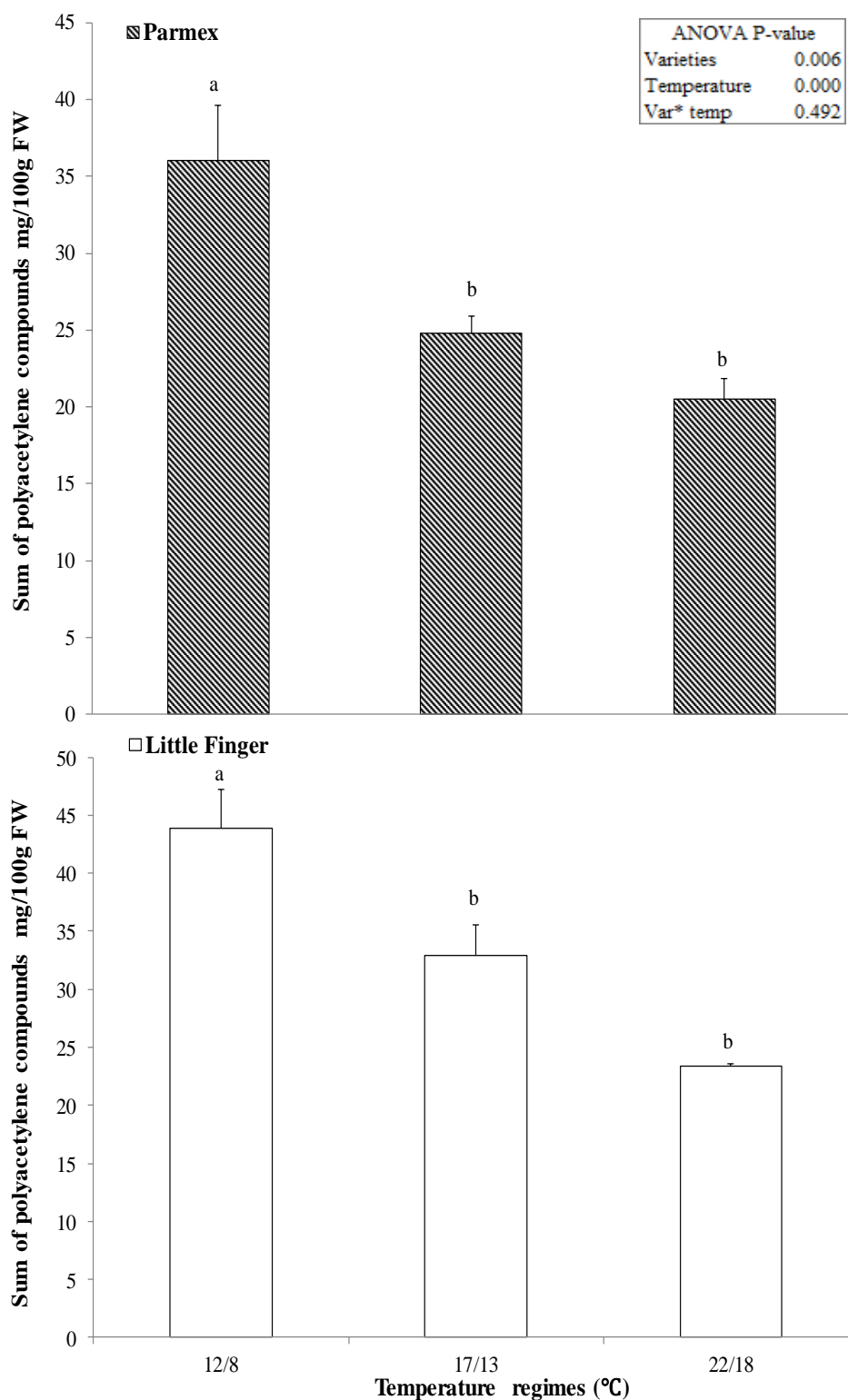


Figure 7. 6 The effect of temperature regimes and carrot variety (Parmex and Little Finger) and temperature on total polyacetylene compounds (FaDOH, FaDOAc and FaOH). Means with different letters are significantly different ($P < 0.05$). Error bars indicate Standard Error of Mean, ($n = 3$).

7.2.4 Phenolic compounds

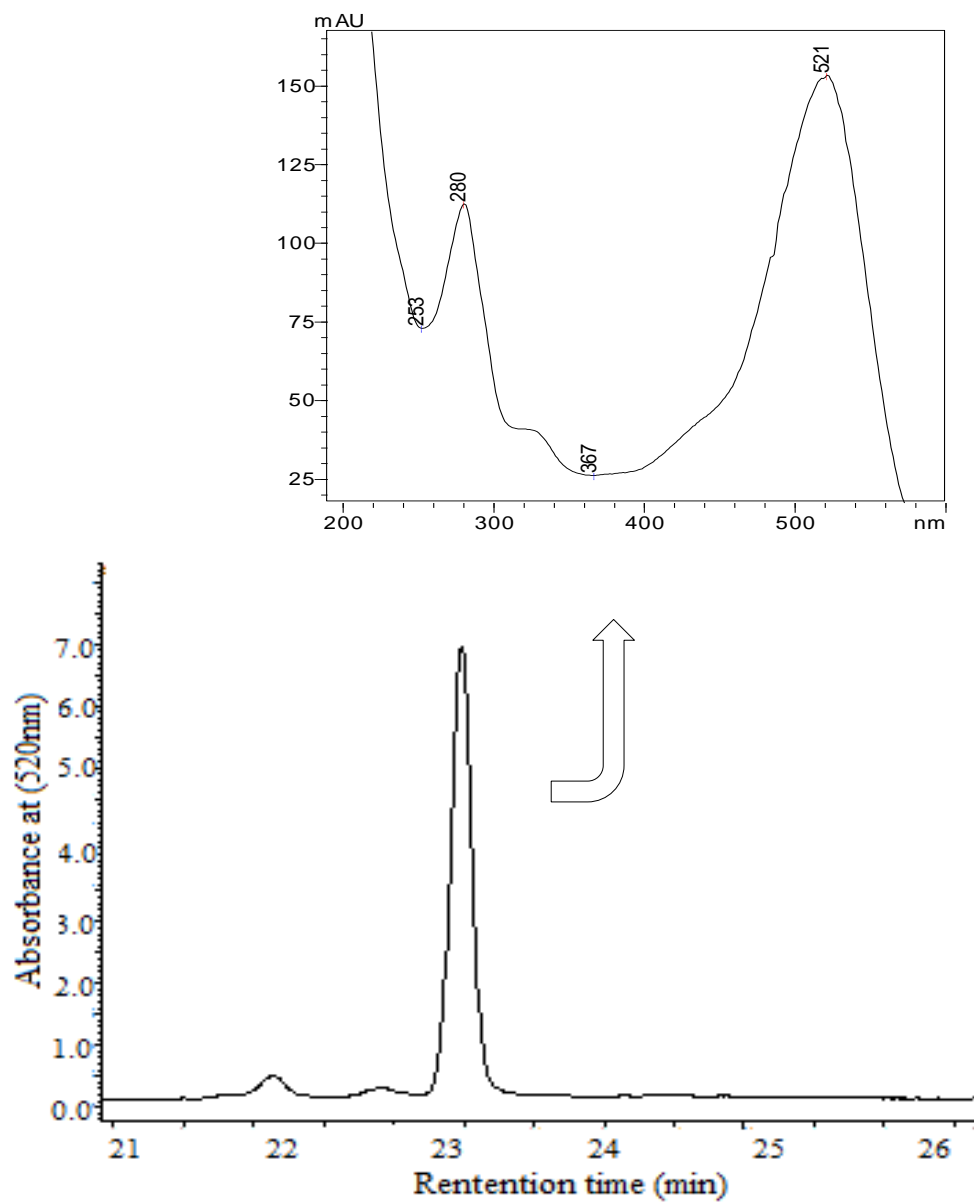


Figure 7.7 HPLC chromatogram recorded at 520 nm with diode array detector of Anthocyanin in Lettuce leaf grown (at 12/8 °C).

Table 7.8 Analysis of Variance (ANOVA) for the phenolic peaks of carrot leaf variety grown in different temperature regime, (n=3).

Chlorogenic acid and unknown phenolic peaks (mg/100g FW) of carrot leaf											
	Pk 1	Pk 2 (3-CQA)	Pk 3	Pk 4	Pk 5	Pk 6	Pk 7	Pk 8	Pk 9	Pk 10	Sum of phenolic (all peaks)
<u>Parmex</u>											
12/8	2.65 ± 0.64 a	16.25 ± 2.87 b	1.20 ± 0.23 b	8.88 ± 0.23 a	1.75 ± 0.41 a	1.82 ± 0.35 a	6.42 ± 0.44 a	11.40 ± 0.63 a	1.86 ± 0.60 a	17.93 ± 6.71 a	70.16 ± 9.70 b
17/13	2.79 ± 0.49 a	144.81 ± 5.22 a	3.81 ± 0.44 ab	2.01 ± 0.60 b	0.50 ± 0.22 b	0.49 ± 0.04 b	5.00 ± 0.43 a	2.37 ± 0.45 b	1.69 ± 0.23 a	25.09 ± 1.63 a	188.00 ± 5.38 a
22/18	3.28 ± 0.39 a	11.84 ± 4.77 b	2.16 ± 0.33 ab	1.33 ± 0.51 b	0.23 ± 0.10 b	0.41 ± 0.09 b	1.47 ± 0.75 b	0.96 ± 0.42 bc	1.65 ± 0.49 a	11.31 ± 1.99 a	34.65 ± 9.16 b
27/23	3.84 ± 0.68 a	14.87 ± 3.99 b	5.31 ± 1.37 a	3.04 ± 0.92 b	0.29 ± 0.03 b	0.42 ± 0.08 b	0.87 ± 0.17 b	0.38 ± 0.13 c	6.25 ± 1.60 a	30.84 ± 6.95 a	66.03 ± 13.91 b
<u>Little Finger</u>											
12/8	0.85 ± 0.27 b	45.05 ± 2.06 b	1.93 ± 0.10 b	5.54 ± 1.60 a	2.17 ± 0.31 a	1.88 ± 0.56 a	25.85 ± 1.32 a	25.35 ± 2.23 a	5.14 ± 0.93ab	39.33 ± 7.17 ab	144.46 ± 5.52 b
17/13	1.12 ± 0.22 b	145.55 ± 3.53 a	5.68 ± 0.61 a	1.04 ± 0.36 b	0.66 ± 0.01 b	0.51 ± 0.06 b	3.74 ± 1.14 b	6.74 ± 0.95 b	2.42 ± 0.17bc	49.49 ± 7.15 a	216.04 ± 10.23a
22/18	2.10 ± 0.47 ab	9.68 ± 0.74 c	2.35 ± 0.09 b	0.37 ± 0.19 b	0.32 ± 0.08 b	0.34 ± 0.04 b	0.68 ± 0.06 b	1.00 ± 0.15 c	1.81 ± 0.58 c	6.31 ± 1.67 c	24.97 ± 2.97 d
27/23	2.31 ± 0.20 a	20.82 ± 3.75 c	5.36 ± 0.92 a	0.50 ± 0.03 b	0.30 ± 0.06 b	0.35 ± 0.05 b	0.87 ± 0.08 b	0.20 ± 0.05 c	5.73 ± 0.71 a	25.43 ± 2.54 bc	61.87 ± 5.65 c
<u>ANOVA P-values</u>											
Varieties	0.000	0.007	0.209	0.001	0.236	0.916	0.000	0.000	0.176	0.028	0.002
Temperature	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Var*Temp	0.929	0.005	0.588	0.275	0.750	0.988	0.000	0.000	0.210	0.010	0.001

The values are Mean ± standard error of mean followed by different letters are significantly different, the highlight P-value indicated significant differences (P< 0.05), (n = 3).

Table 7.9 Analysis of Variance (ANOVA) for the phenolic peaks of carrot root variety grown in different temperature regime, (n=3).

Chlorogenic acid and unknown phenolic peaks (mg/100g FW) of carrot root											
	Pk 1	Pk 2 (3-CQA)	Pk 3	Pk 4	Pk 5	Pk 6	Pk 7	Pk 8	Pk 9	Pk 10	Sum of phenolics (10 peaks)
<u>Parmex</u>											
12/8	0.30 ± 0.06 a	1.22 ± 0.31 a	0.14 ± 0.04 a	0.26 ± 0.06 a	0.80 ± 0.17 a	1.86 ± 0.45 b	0.16 ± 0.03 a	0.06 ± 0.02 c	0.17 ± 0.05 b	0.39 ± 0.01 a	5.34 ± 1.13 a
17/13	0.33 ± 0.02 a	1.71 ± 0.25 a	0.09 ± 0.01 a	0.19 ± 0.01 a	0.86 ± 0.06 a	3.04 ± 0.28 ab	0.14 ± 0.01 a	0.12 ± 0.01 b	0.30 ± 0.03 b	0.36 ± 0.02 a	7.14 ± 0.47 a
22/18	0.41 ± 0.09 a	2.34 ± 0.60 a	0.14 ± 0.03 a	0.18 ± 0.02 a	1.01 ± 0.21 a	5.36 ± 1.22 a	0.26 ± 0.05 a	0.19 ± 0.02 a	0.54 ± 0.01 a	0.40 ± 0.07 a	10.83 ± 2.36 a
<u>Little Finger</u>											
12/8	0.41 ± 0.08 a	2.79 ± 1.16 a	0.18 ± 0.03 a	0.38 ± 0.05 a	1.21 ± 0.24 a	3.47 ± 0.57 ab	0.33 ± 0.04 a	0.19 ± 0.04 b	0.66 ± 0.10 a	0.18 ± 0.04 a	9.78 ± 2.07 a
17/13	0.18 ± 0.03 a	1.44 ± 0.40 a	0.07 ± 0.00 b	0.16 ± 0.01 b	0.51 ± 0.09 a	1.80 ± 0.30 b	0.16 ± 0.03 b	0.10 ± 0.01 b	0.33 ± 0.06 b	0.07 ± 0.02 a	4.81 ± 0.93 a
22/18	0.34 ± 0.05 a	2.30 ± 0.11 a	0.12 ± 0.01 ab	0.26 ± 0.02ab	0.84 ± 0.17 a	5.24 ± 0.60 a	0.26 ± 0.03ab	0.36 ± 0.01 a	0.91 ± 0.04 a	0.14 ± 0.03 a	10.76 ± 0.86 a
<u>ANOVA P-values</u>											
Varieties	0.456	0.372	0.837	0.074	0.804	0.877	0.031	0.000	0.000	0.000	0.558
Temperature	0.090	0.417	0.018	0.003	0.147	0.001	0.010	0.000	0.000	0.374	0.012
Var*Temp	0.098	0.234	0.374	0.098	0.081	0.103	0.042	0.001	0.007	0.713	0.080

The values are Mean ± standard error of mean followed by different letters are significantly different, the highlight P-value indicated significant differences (P< 0.05), (n = 3).

Table 7.10 show the conversion factors of main standards used

Standard, name	Concentration, mg/ml	Area	nm
Lutein	0.000737	200239	450
α -carotene	0.000344	26717	450
β -carotene	0.00193	746815	450
Chlorophyll a	0.01238	111736	450
Chlorophyll b	0.00809	397733	450
Falcarinol	0.5412	19211412	205
Falcarindiol	0.2177	12929874	205
Falcarindiol-3-acetate	0.1230	5975803	205
Caftaric acid	0.1	7563305	320
Chlorogenic acid	0.1	8588202	320
Cichoric acid	0.1	9523060	320

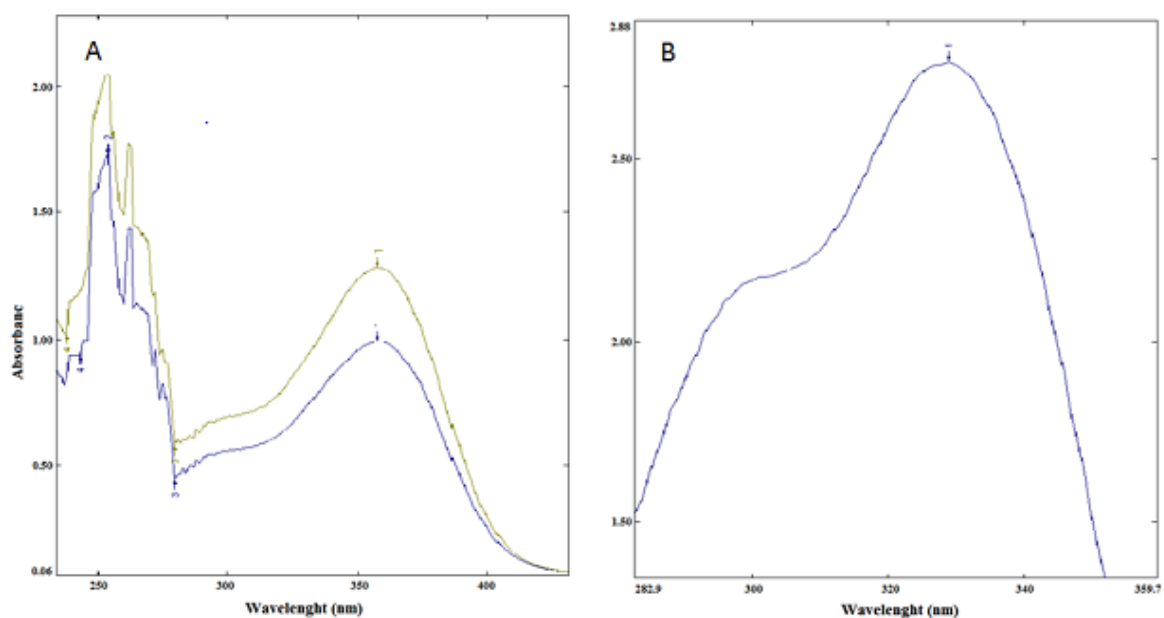


Figure 7. 8 Visible spectra of Rutin (A) and chlorogenic acid (B) standards