THE BIOCHEMICAL BASIS OF COLOUR AS AN AESTHETIC QUALITY IN *CITRUS SINENSIS*

By

Renate Oberholster

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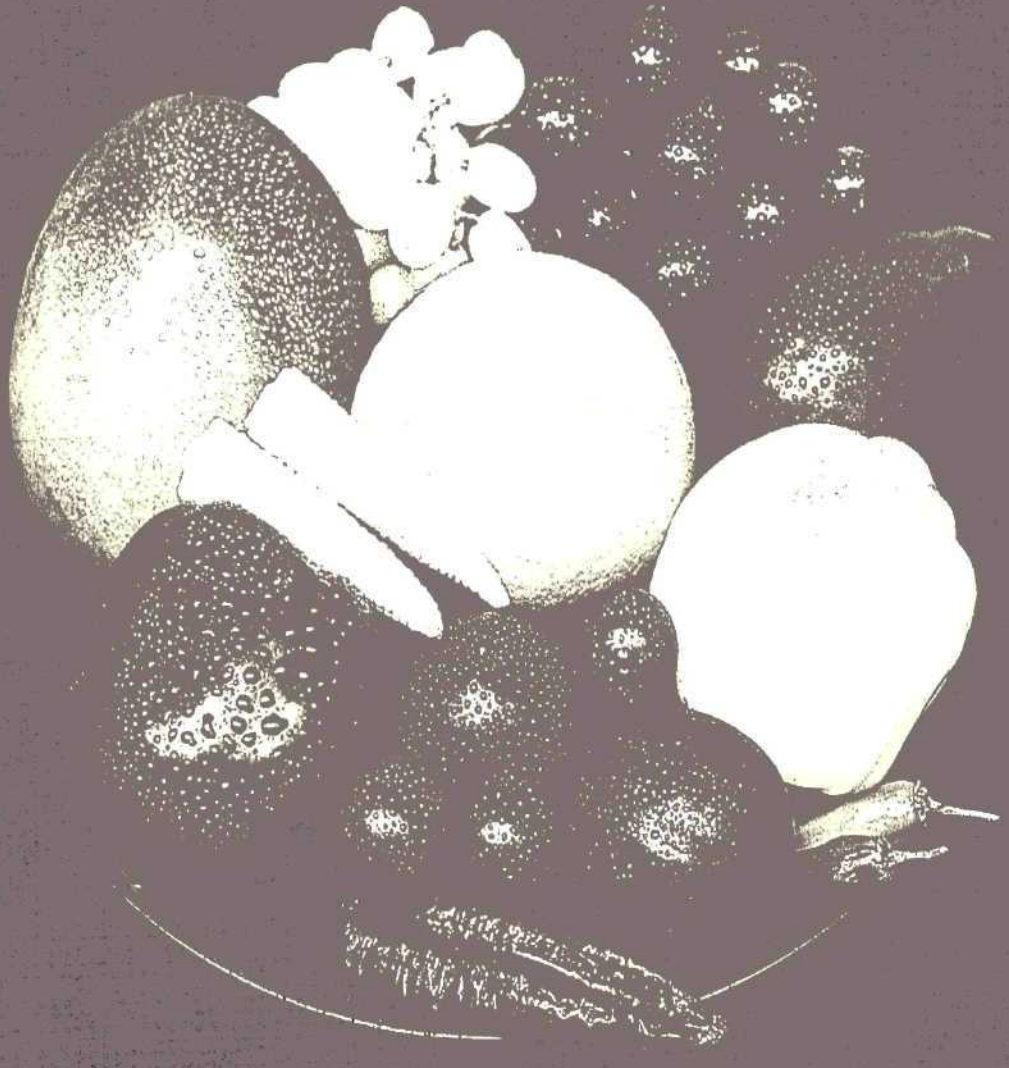

THE VALUE OF FRUIT COLOUR

ABSTRACT

The development of fruit colour in the sweet orange (*Citrus sinensis*) is a cultivar characteristic affected by climate and environment. Although external colour is not always an indication of internal quality or maturity, it is probably the most important factor determining consumer acceptance. In the present investigation, efforts were made to determine the biochemical basis of colour as an aesthetic quality in *C. sinensis* 'Navel' and 'Valencia'. Furthermore, the changes in pigment content and composition during the period of colour development were recorded. Finally, in an attempt to manipulate citrus colour, the effect of dehydrating agents (alcohols), plant hormones, micro-nutrients and low temperature on flavedo carotenoid content was determined.

Saponification of the two major colour-imparting components resolved by thin layer chromatography, followed by reversed-phase high performance liquid chromatography revealed that the principal colour-imparting pigments in *C. sinensis* flavedo are the yellow-coloured xanthophyll 9-*Z*-violaxanthin and the red C_{30} apocarotenoid β-citraurin. Both pigments occur in the flavedo in esterified form. Identification of the chromophores was based on co-chromatography and online spectral analysis. The colour quality of mature fruit was dependant on the content and relative amounts of 9-*Z*-violaxanthin and β-citraurin. Quantitative results revealed that increased colour intensity of citrus flavedo was associated with a decline in the 9-*Z*-violaxanthin : β-citraurin ratio from greater than 50 to below 10, and an increase in 9-*Z*-violaxanthin and β-citraurin content. Measurement of the mass and ratio of these pigments can be used to accurately colour-grade orange fruit for local and export markets. These parameters will also aid in the evaluation of colour manipulatory techniques.

Visual colour break in *C. sinensis* appears to be associated with a minimum in total pigment as well as total carotenoid content. The period prior to colour break is characterised by a reduction in chlorophylls, carotenes and free xanthophylls usually

associated with photosynthetic activity. Following colour break, a massive increase in xanthophyll acyl esters (particularly 9-*Z*-violaxanthin) is observed.

Efforts were made to manipulate carotenoid content of citrus flavedo in vivo, with a view to manipulate fruit colour commercially. It was shown that the micro-nutrients tungsten and molybdenum, and the plant hormones abscisic acid and jasmonic acid increased carotenoid content of flavedo discs; whereas the plant hormone gibberellic acid decreased carotenoid levels. The dehydrating agents ethanol and butanol increased carotenoid content in whole fruit flavedo and flavedo discs. Optimum concentrations were shown to be 20-30% (v/v) for ethanol and 5-10% (v/v) for butanol.

DECLARATION

I hereby declare that the research work reported in this dissertation is the result of my own investigation, except where acknowledged.

Signed: _____

Renate Oberholster

I certify that the above statement is correct.

Signed: _____

Prof. A.K. Cowan

Supervisor

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CHAPTER 1

GENERAL INTRODUCTION

Plant pigments in horticultural crops, including fruits, vegetables and ornamentals, have been intensively studied because of their essential role in visual appeal. Visual colour of flowers and fruit is produced by a surprisingly small number of pigments. Most red and blue plant pigments belong to the class of water-soluble anthocyanins. Many red, orange and yellow-coloured fruit such as citrus, owe their colour to the presence of oil-soluble carotenoids. More recently attention has shifted to the nutritional benefits afforded by pigments, particularly carotenoids (Bartley and Scolnik, 1995), but horticulturists still consider colour as a major criterion for determining both grade and quality of produce. In citrus, flavedo colour is likely the most important external quality parameter used to determine consumer acceptance, although it is not always an indication of internal quality. This demonstrates that consumers buy fruit primarily on the basis of eye appeal.

Visual expression of citrus colour is a cultivar characteristic affected by climate and environment that can, to some extent, be manipulated by cultural practice (Goldschmidt, 1988). The South African citrus industry may incur losses amounting to millions of Rand during production seasons that are not conducive to desirable colour development. Even more important are unquantifiable losses due to a reduction in market share, lost market opportunity, damage to brand names and increased degreening costs. Competition between growers to secure and maintain niche markets has therefore fuelled efforts to produce quality fruit of uniformly and consistently good colour.

But what is colour? The phenomenon known as 'colour' involves an interaction between the light incident on an object, the spectral reflectance characteristics of that object, and the spectral sensitivity of the human eye (Voss and Hale, 1998). The basic attributes of colour perception are hue, lightness and chroma. 'Hue' or 'tint' refers to the perception of colour as red, orange, yellow, green, blue, purple or an intermediate of these. 'Lightness' indicates the relative lightness or darkness of colour and ranges

from black through grey to white. 'Chroma' indicates the degree of departure from grey towards pure chromatic colour, and can be referred to as the purity, vividness, intensity or saturation of colour (Macguire,1992; Lancaster *et al.*, 1997; Voss and Hale, 1998).

Careful colour evaluation is desirable for botanical and horticultural description, plant breeding and quality management of horticultural produce. Communicating perceptions of colour entails not only the evaluation and description of colour by an individual, but also the visualisation of described colours by another (Voss, 1992). Colour of horticultural products such as fruit, vegetables and flowers can be measured in a number of ways and the use of colour charts has long played an important role in colour measurement. Very detailed charts such as the 'Royal Horticultural Society Colour Chart' are used for biological description, whereas less detailed charts are available for selected produce e.g. the 'Outspan Citrus Colour Chart'. In this chart, only different colour grades of citrus, from green through orange, are illustrated. Although colour charts afford easy, nondestructive colour measurement, colour evaluation utilising this method is subjective, and depends on an individual's ability to perceive and distinguish between colours. Furthermore, factors such as the size of the surface being viewed, the background or 'surround', presence or absence of gloss, direction of illumination and viewing, and nature of the light source may all affect the perception of colour (Voss and Hale, 1998).

The increased availability of rather costly, portable colour-measuring instruments (such as the Hunter and Minolta colorimeter, and Colortron reflectometer), makes possible a more objective notation of colour (Reeves *et al.*, 1997). These instruments express any given colour as a point in a three-dimensional space, where lightness is measured directly, but some computation is required to yield explicit measures of hue and chroma (McGuire, 1992). Although colorimeters provide a fairly undemanding and nondestructive measurement of colour, they afford very little information on pigment content and composition. Lancaster and co-workers (1997) report that there is no unique linear combination of pigments that give rise to a unique point in the colour space, in a wide variety of fruits and vegetables. In simple systems, where one pigment predominates, or where only one pigment is present, linear relationships

between pigment content and colour may be significant. Colorimeters are therefore convenient for descriptive studies, but care should be taken not to use these measurements as descriptors for pigment change.

Any manipulation studies that endeavour to improve colour need to be evaluated by examining pigment content and composition, as it is after all the plant pigments that afford visual colour. Therefore, any visible manipulation of plant colour has to be preceded by a change in pigment concentration and/or composition. Following extraction of pigments, total pigment content can be determined very accurately with the use of a spectrophotometer and the concentration and identity of individual pigments can be determined using high performance liquid chromatography (HPLC). In order to improve understanding and visualisation of the role of pigments in colour expression, total and individual pigment concentration may be related to colour chart readings. In this way, one can direct manipulation of pigments towards desirable visual colour.

1.1 HORTICULTURE OF CITRUS

The term Citrus originated from the greek word 'Kedros', used to describe trees such as cedar, pine and cypress. As the smell of citron leaves and fruit was reminiscent of that of cedar, the name citrus was applied to the citron (Spiegel-Roy and Goldschmidt, 1996). The genus Citrus belongs to the family Rutaceae and is believed to have originated in the tropical and subtropical regions of southeast Asia, from where it was dispersed across the world through natural spread and cultivation. Modern molecular biochemical techniques indicate that all known citrus species originate from three groups *viz*: citron (*Citrus medica* L.), mandarin or tangerine (*C. reticulata* Blanco) and pummelo (*C. grandis* Osbeck). It is believed that the sweet orange (*C. sinensis* Osbeck) originated as a natural hybrid between pummelo and mandarin grown in village gardens in China. Citron and pummelo are cultivated on a small scale, whereas mandarin is commercially produced. Other commercially important citrus species include grapefruit (*C. paradisi* Macf.), lemon (*C. limon* (L.) Burm.f.), sour orange (*C. aurantium* L.) and lime (*C. aurantifolia* Christm). Citrus is a widely cultivated and variable genus and a number of commercially available cultivars are the

result of inter-species crosses and classical plant breeding practices (Spiegel-Roy and Goldschmidt, 1996).

The sweet orange is considered the most important citrus group in terms of production area and market value. Cultivars are usually classed as early, mid-season or late, depending on the season of maturity (Jackson, 1990). The 'Washington Navel' is the standard early or winter orange and is distinguished by the development of a 'navel' at the stylar end of the fruit (in fact an aborted secondary fruit). Worldwide distribution of Navel orange started after budwood was sent from Bahia, Brazil, to the US Department of Agriculture in 1870 (Spiegel-Roy and Goldschmidt, 1996). 'Valencia' is regarded as a late maturing cultivar and is also known for its late hanging quality. Mature fruit can remain on the tree for four to five months before appreciable deterioration of quality sets in. The exact origin of 'Valencia' is unknown, but it is thought to be an old variety of the Mediterranean region. 'Valencia' was introduced in the USA in 1873, from where it was spread to other citrus producing regions (Bowman, 1956).

1.2 COLOUR DEVELOPMENT

1.2.1 Chromoplast development

Colour break in citrus peel is defined as the stage during which natural colour conversion from dark green to an ultimate yellow or orange, has progressed to the extent that a tinge of yellow or orange is apparent (Wardowski *et al.*, 1986). Colour-break coincides with the conversion of chloroplasts to chromoplasts (Eilati *et al.*, 1975), in which pigments associated with photosynthetic activity such as chlorophyll *a* and *b*, α- and β-carotene, lutein and the unesterified xanthophylls: *E*-neoxanthin, *E*-violaxanthin and *E*-antheraxanthin, decline (Gross, 1987). The typical chloroplast carotenoid pattern is replaced by a complex chromoplast carotenoid pattern that is characteristic of a specific fruit. Chromoplasts can be defined as plastids containing pigments other than chlorophyll, usually carotenoids (Raven *et al.*, 1986)

Chromoplasts can be subclassified into 4 groups (Thomson and Whatley, 1980; Marano *et al.*, 1993; Vishnevetsky *et al.* 1999), depending on the form of carotenoid storage *viz:*

- globular, in which the carotenoids are concentrated in plastoglobuli,
- tubular/fibrillar, characterised by the presence of numerous tubules,
- membranous, contain multiple arrays of membranes,
- crystalline, carotenoids are organized in the form of crystals.

Globular chromoplasts contain lens-shaped or spheroidal plastoglobules and are the most common type. These are also considered to be the oldest and most primitive in evolutionary terms. Plastoglobules have been proposed to consist of a monolayer of polar lipids and proteins covering the surface, with apolar components buried in the interior (Vishnevetsky *et al.*, 1999). Thomson (1966) described the ultrastructural changes associated with the development of globular chromoplasts in 'Valencia' orange. During colour development, there was a co-ordinated disassembly of the internal membrane system of the chloroplast, and large osmiophilic globules were formed. It was originally suggested that these globules arose due to breakdown of granal membranes and the synthesis of new carotenoids, but more recent studies using tomato (*Lycopersicon esculentum*) and pepper (*Capsicum annuum*) mutants that retain chloroplast thylakoids during chromoplast differentiation, indicate that the new set of chromoplast substructures is formed *de novo* (Camara *et al.*,1995). Membranes are always associated with the large globules, suggesting that carotenoid synthesis takes place in association with chromoplast membrane formation. During regreening, fully coloured citrus peel transforms to a yellow-green colour, a process that is usually associated with a rise in temperature in early summer. Ultrastructural studies of regreening Valencia flavedo indicate that chromoplasts are retransformed into chloroplasts (Thomson *et al.*, 1966). The osmiophilic globules are reduced in size and number and the grana network system is evidently built up from small vesicles which pinch off from the inner plastid membrane. Regreening involves the *de novo* synthesis of chlorophyll and protein complexes of the photosynthetic apparatus, along with the reassembly of thylakoid structure and re-acquisition of photosynthetic activity (Mayfield and Huff, 1986; Goldschmidt, 1988).

Evidence from comparative biochemical and ultrastructural studies indicates that the shape of chromoplasts is governed by their lipid-to-protein ratio (Deruère *et al.*,1994).

A high proportion of lipid induces the formation of globular structures, whereas a high proportion of protein leads to the formation of membranous or threadlike structures. During chromoplast development in pepper, 95% of newly synthesized carotenoids accumulate in specific lipoprotein fibrils. In addition to carotenoids, purified fibrils have been shown to contain galactolipids, phospholipids, and a single 32-kD protein, designated fibrillin (Deruère *et al.*, 1994). These authors have therefore proposed a model for fibril architecture and suggest that carotenoids accumulate and are surrounded by a layer of polar lipids, which in turn are surrounded by an outer layer of fibrillin protein. Vishnevetsky *et al.* (1999) propose an alternative model in which polar lipids on the fibril surface are protected by carotenoid-associated proteins, whose hydrophobic regions are embedded in the inner carotenoid core.

Both models indicate that carotenoid-associated proteins play a vital role in the accumulation and sequestration of carotenoids in plants. In chromoplasts, special plastid-lipid-associated proteins are involved in the sequestration of carotenoids. Two chromoplast-specific proteins *ChrA* and *ChrB* (58- and 35-kD respectively) were identified in pepper and shown to accumulate during ripening (Newman *et al.*,1989). Similarly, a carotenoid-associated 35-kD protein (*ChrC)* accumulated in cucumber corollas during chromoplast development (Smirra *et al.*, 1993; Vainstein *et al.*, 1994). It was later shown that *ChrB* is identical to fibrillin in peppers (Pozueta-Romero *et al.*, 1997) and that the predicted amino acid sequence of *ChrC* (in cucumber) shares significant homology with fibrillin (Vishnevetsky *et al.*, 1999). Furthermore, a cDNA (*CitPAP*) homologous to *ChrC* was isolated from Satsuma mandarin (Moriguchi *et al.*, 1998). Pozueta-Romera and co-workers (1997) isolated a cDNA corresponding to a single nuclear gene, *PAP* (plastid-lipid-associated protein), that encodes fibrillin. It was revealed that this gene is expressed at a low level in every organ of the plant and that the amount of corresponding transcript and protein dramatically increased in the later stages of fruit development. Based on homology with the *PAP* gene, carotenoid-associated genes of other plants that contain fibrillar and globular chromoplasts have been discovered. These findings demonstrate the existence of a group of homologous and highly conserved genes coding for carotenoid-associated proteins that aid in carotenoid sequestration during chromoplast development (Vishnevetsky *et al.*, 1999)

Deruère *et al.* (1994) found that the type of carotenoids accumulated in chromoplasts also play a role in determining chromoplast structure. Fibril reconstitution assays showed that cyclic carotenoids (eg. zeaxanthin, zeaxanthin diester, capsanthin and capsanthin diester) facilitated fibril assembly, whereas the acyclic carotenoid lycopene inhibited this process. This was further confirmed by the application of 2-(4-chlorophenylthio) triethylamine hydrochloride (CPTA) to intact pepper fruits (a compound that inhibits cyclization of lycopene and leads to the accumulation of lycopene (Benedict *et al.*, 1985). Noticeable structural differences were observed in chromoplasts, resulting in the formation of lycopene crystals with the absence of fibrils. Crystalline chromoplasts are characteristic of tomato, a fruit that accumulates large amounts of lycopene (Mohr, 1979). Chromoplasts containing lycopene crystals are also characteristic of the pulp of 'Ruby Red' grapefruit (Gross, 1987).

1.2.2 Regulation of colour development

The transformation of chloroplasts to chromoplasts and the resultant colour change seems to be under environmental, nutritional, hormonal and genetic control, and the control mechanisms appear to be strongly interrelated (Goldschmidt, 1988).

In general, rind colour of citrus fruit is more brilliant the closer the trees are cultivated to their climatic limit in terms of winter temperature. Citrus fruit grown in tropical areas, often remain green long after internal maturation has been reached, whereas brilliantly coloured fruit are produced in areas with a distinct seasonal cold period (Reuther and Rios-Castaño, 1969). It should also be noted that the occurrence of regreening in Valencia orange has been closely correlated with a rise in average air temperature during the summer months (Coggins *et al.*, 1981, El-Zeftawi, 1977). The optimal conditions for rapid loss of chlorophyll and the accumulation of carotenoids is a day (air) temperature of 20 °C, night temperature of 7 °C and soil temperature of 12 °C (Young and Erickson,1961). Colour break usually occurs in autumn or early winter, and is accompanied by a sudden drop of temperature which is referred to colloquially as a 'cold snap'. An effect of temperature on the level of specific carotenoids has also been noted. In tomato (Goodwin and Jamikorn, 1952), Redblush grapefruit (Meredith and Young, 1971) and bittermelon (Lan Huong Tran and Raymundo, 1999),

temperatures above 35 °C inhibited lycopene accumulation whereas the accumulation of β-carotene was not significantly affected by high temperature. Stewart and Wheaton (1971) also reported reduced accumulation of β-citraurin in the flavedo of citrus at temperatures above 30 °C.

Although other factors are involved in colour development, these seem less important than changes in temperature. For example, light effects have been noted. Fruit located near the outside of the canopy usually experience colour-break earlier than fruit on the inside and these can therefore be harvested earlier, but only if internally mature (Krajewski, 1998). Similarly, de Vries and Bester (1996) observed that fruit near the outside and in the top of the tree canopy matured earlier in terms of colour, total soluble solids (TSS), and TSS/titratable acidity (TA) ratio. Relative humidity is also implicated in the regulation of colour development as uniformly brightly coloured fruit is usually produced in areas typified by a dry climate (Stewart, 1977). A possible reason for this is that growing regions with low humidity are usually characterised by greater day-night temperature extremes.

Nutrition status of orchards impacts on colour development and it is known that excessive nitrogen in the soil delays degreening and promotes regreening (Huff, 1984). The form in which nitrogen is provided also plays a role. Trees supplied with an ammonium-nitrate nitrogen produce better coloured fruit than trees fertilized with nitrate alone (Collado *et al.*, 1996). Huff (1983; 1984) investigated the effect of nutritional status of the plant on chloroplast/chromoplast transformation in a series of *in vitro* experiments using citrus flavedo discs. It was found that high nitrogen favoured the development of chloroplasts (regreening), whereas high sucrose in the medium led to the development of chromoplasts (degreening). The effect of other nutrients on the progression of colour development is less marked and also less well-documented. Potassium was reported to have both promotive (Trudel and Ozbun, 1970; 1971), and inhibitory (Wardowski *et al.*, 1986) effects on fruit colour.

The likelihood that sugars play a role in plastid transformation and hence colour development is strengthened by the observation that colour break and colour

development in citrus is accompanied by an increase in sugar levels in the juice and flavedo (Tadeo *et al.*,1987). Furthermore, the late season citrus cultivar 'Hernandina' remains green until late in the season and displays sugar accumulation at a much reduced rate when compared to 'Washington Navel' (Tadeo *et al.*, 1987), a variety in which development of maturity and colour is rapid. Reducing sugars are believed to play an important role in a plants resistance to chilling injury (Purvis *et al.*, 1979) and it was reported that reducing sugar level increased in the flavedo of 'Marsh' grapefruit when trees were exposed to cold hardening temperatures and decreased at dehardening temperatures (Purvis and Grierson,1982; Purvis and Rice, 1983). Invertase activity was shown to parallel the levels of reducing sugars in grapefruit flavedo (Purvis and Rice, 1983). In addition, a fivefold increase in invertase activity was reported in pepper fruit during chromoplast differentiation (Camara *et al.*, 1995).

Considerable attention has also been given to the effect of exogenously applied plant growth regulators and plant hormones on degreening and regreening of citrus fruit peel. Gibberellin (GA) delayed the loss of green pigmentation and accumulation of carotenoids in 'Navel' (Coggins and Hield, 1962; Lewis and Coggins, 1964) and 'Shamouti' oranges (Goldschmidt and Eilati, 1970), and enhanced regreening of fully coloured 'Valencia' fruit, although no regreening was observed in 'Navel' (Coggins and Lewis, 1962). Similarly, application of cytokinin resulted in delayed colour development of 'Valencia' (Rasmussen *et al.*, 1973). No effect of auxin on peel colour was noted, although the shelf-life of fruit treated with 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and naphthaleneacetic acid (NAA) was increased and processes associated with senescence were delayed (Kefford and Chandler, 1970). Exogenous abscisic acid (ABA) treatment did not have consistent effects on citrus fruit colouration, but application of ABA accelerated fibril synthesis and enhanced the formation of chromoplasts in pepper, whereas GA and indole-3-acetic acid (IAA) delayed this process (Deruère *et al.*, 1994). Ethylene treatment is reported to result in rapid degreening and colour development in numerous citrus cultivars, including 'Temple' (Stewart and Wheaton, 1971), 'Valencia' (Wheaton and Stewart, 1973) and Satsuma mandarin (Le Roux *et al.*, 1997). Ethylene appears to enhance colouration by influencing the *de novo* synthesis of chlorophyllase (Trebitsh

et al., 1993), the chlorophyll degrading enzyme, and by inducing carotenoid synthesis (Stewart and Wheaton, 1971; 1972). An increase in ABA content (Brisker *et al.*,1976), free amino acids, respiration rates, reducing sugars, and appearance of phenylalanine ammonia lyase activity were also reported following ethylene treatment of citrus fruit (Goldschmidt *et al.*, 1977).

Far less is known about the role of endogenous plant hormones in chromoplast formation and colour development, although correlative evidence has been established in some cases. In Satsuma mandarin peel, GA-like activity decreased rapidly once the fruit were fully expanded and remained at a low level until fruit maturity (Kuroaka *et al.,* 1977). It was also noted that an increase in GA activity in the flavedo of 'Valencia' oranges preceded an increase in chlorophyll content during regreening (Rasmussen, 1973). Furthermore, greater amounts of GA-like substance were found in rough-peeled than in smooth-peeled 'Shamouti' orange (El-Otmani *et al.*, 1995; Erner *et al.*, 1976). Rough-peel disorder occurs in 'Shamouti' orange grown under marginal conditions and results in fruit with excessively rough peel, greater acid content and delayed peel colouring, which is indicative of a delay in maturity. This disorder can be overcome in part by pre-harvest application of the growth retardants daminozide and chlormequat, that act as GA inhibitors (Monselise and Goren, 1978).

Richardson and Cowan (1995) described the change in ABA content of citrus flavedo in relation to colour development. ABA content increased throughout the course of colour change and reached a maximum at time of colour-break. A decline in ABA content occurred concomitantly with full expression of colour, probably due to enhanced catabolism and formation of ABA conjugates. These results are in accordance with a previous study (Harris and Dugger, 1986), in which it was shown that ABA levels remained fairly stable during chromoplast development, but that levels of the ABA conjugate increased approximately 12-fold in flavedo tissue. Aung *et al.* (1991) also reported an increase in ABA conjugates in the peel of mature, coloured lemon. These results are not surprising considering that the biosynthetic route to ABA is now accepted to occur predominantly via the metabolism of epoxy-carotenoids and in particular the 9-*Z*-xanthophylls, neoxanthin and violaxanthin (Li and Walton, 1990; Rock and Zeevaart, 1991; Cowan and Richardson, 1997; Schwartz *et al.*, 1997).

The role of endogenous ethylene in ripening and colour development of citrus deserves some attention, as citrus is known to be a non-climacteric fruit (Baldwin, 1993). Gas chromatographic analysis has revealed measurable, although relatively small amounts of ethylene in both the internal atmosphere of, and emanations from, mature orange fruit (Apelbaum *et al.*, 1976, and references therein), but it has been difficult to establish a correlation between ethylene evolution and colour development (Goldschmidt, 1988). Apelbaum *et al.* (1976) showed that by reducing the level of endogenous ethylene in intact orange fruit, using subatmospheric pressure, the rate of chlorophyll destruction or colour change remained unchanged. By comparison, Purvis and Barmore (1981) reported that hypobaric storage greatly reduced chlorophyll destruction in citrus. It seems, however, that these authors did not allow for the maintenance of a sufficiently high O_2 concentration during hypobaric storage, which is known to delay the degreening process (Goldschmidt *et al.*, 1993). The use of ethylene antagonists, such as norbornadiene and silver nitrate, counteracted the degreening process by 55-60%, suggesting that endogenous ethylene is involved in the process of colour development in citrus (Goldschmidt *et al.*, 1993). In fact, ethylene induced changes in the level of a number of specific mRNAs in citrus fruit that are regulated both by ethylene treatment and maturation (Alonso *et al.*, 1995). It is therefore possible that gene expression is activated by an increase in sensitivity to basal ethylene levels or by the low increase of ethylene concentration during fruit maturation.

1.3 CAROTENOID BIOSYNTHESIS

An understanding of the biosynthesis and interrelationship of carotenoids is deemed essential before any attempt at colour manipulation can be undertaken. The biosynthesis of carotenoids, including biochemical and molecular aspects, has been extensively reviewed by a number of authors (Goodwin, 1961; Bartley *et al.*, 1994; McGarvey and Croteau, 1995; Chappell, 1995; Cunningham and Gantt, 1998). Current knowledge of the molecular biology of carotenoids is derived primarily from the study of the pathway in specific organisms, including the photosynthetic prokaryotes *Rhodobacter* and *Synechococcus,* bacteria of the genus *Erwinia*, the fungi *Neurospora* and *Phycomyces*, and the higher plants corn *(Zea mays)*, tomato, daffodil *(Narcissus pseudonarcissus)* and pepper (Bartley *et al.*, 1994).

Carotenoids can be broadly divided into carotenes (hydrocarbons) and their oxygenated derivatives, the xanthophylls. They are generally C_{40} terpenoid compounds formed by the condensation of eight isoprene units (Bartley and Scolnik, 1995). The linkage order at the center of the molecule is reversed, so that the molecule as a whole is symmetrical (Gross, 1987). A series of conjugated double bonds termed the chromophore is responsible for the absorption of light in the visible region of the spectrum (MacKinney, 1961)

1.3.1 Source of isopentenyl diphosphate (IDP)

The central metabolite and building block of all isoprenoid compounds is the 5-carbon compound isopentenyl diphosphate (IDP). A modular assembly process that produces compounds of 5, 10, 15, 20 or more carbons (in multiples of 5) allows the biosynthesis of the many and varied isoprenoids (Cunningham and Gantt, 1998). The 'classical' acetate/mevalonate route to IDP proceeds from acetyl-CoA via 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonic acid (MVA). The reduction of HMG-CoA to produce MVA is catalysed by the enzyme HMG-CoA reductase (HMGR) and is purported to be an irreversible, rate-determining step in mammalian systems (Chappell, 1995). HMGR has been localised in the cytosol of plants. Emerging evidence points to the existence of a non-mevalonic acid pathway for the generation of IDP in plastids (Lichtenthaler *et al.*, 1997). The non-mevalonate pathway, as derived from ^{13}C-incorporation studies, is involved in the biosynthesis of all essential chloroplast isoprenoids e.g. phytol, carotenoids and plastoquinone (Rohmer, 1999). This pathway begins with the condensation of pyruvate (C_2) and glyceraldehyde-3-phosphate (C_3) to yield 1-deoxyxylulose-5-phosphate (DOXP), which is transformed in several steps to IDP (Lichtenthaler *et al.*,1997). Bouvier and co-workers (1998b) recently described the characterization of two cDNAs, CapTKT1 and CapTKT2, from pepper, that encode transketolases with distinct specificities. CapTKT1 is primarily involved in plastidial pentose phosphate and glycolytic cycle integration, whereas CapTKT2 initiates the synthesis of isoprenoids in plastids by catalysing the formation of DOXP. CapTKT1 is almost constitutively expressed during chloroplast-to-chromoplast transformation, while CapTKT2 is overexpressed during this period and more than likely provides IDP necessary for increased carotenoid synthesis. In

addition, DOXP is involved in thiamine and pyridoxine synthesis and CapTKT2 plays a key role in this pathway. This may explain the observation that the thiamine-biosynthesis gene *c-thi1*, is strongly induced during natural and ethylene-induced ripening in citrus fruit (Jakob-Wilk *et al.*, 1997).

1.3.2 Assembly of the C40 backbone

Cloned plant genes for enzymes involved in carotenogenesis, as discussed in section 1.3.2 to 1.3.4, are shown in Table 1.1 and the carotenoid biosynthetic pathway is illustrated in Fig.1.1.

Table 1.1 Cloned plant genes of the carotenoid biosynthetic pathway

Gene	Plant	Reference
Geranylgeranyl diphosphate synthase	Arabidopsis, lupine, pepper	Bartley and Scolnik, 1994; 1995; Bouvier *et al.*, 1998a.
Phytoene synthase	Pepper, tomato, arabidopsis, maize, daffodil, rice	Giuliano *et al.*,1993; Bartley and Scolnik, 1995; Bouvier *et al*, 1998a.
Phytoene desaturase	Tomato, arabidopsis, maize, daffodil, pepper, rice, soybean, wild tobacco	Giuliano *et al.*, 1993; Bartley and Scolnik, 1995; Bouvier *et al.*, 1998a.
Lycopene cyclase	Arabidopsis, tomato, tobacco, pepper	Bartley and Scolnik, 1995; Bouvier *et al.*, 1998a.
Zeaxanthin epoxidase	Pepper, tobacco	Bouvier *et al.*, 1998a; Cunningham and Gantt, 1998.
Capsanthin-capsorubin synthase	Pepper	Bouvier *et al.*, 1994; 1998a.

The reversible isomerization of IDP to its allylic isomer dimethylallyl diphosphate (DMADP) is carried out by the soluble enzyme IDP isomerase. Geranylgeranyl diphosphate (GGDP) is formed by the sequential addition of three molecules of IDP

to one molecule of DMADP. These three successive condensation reactions are carried out by GGDP synthase. In addition to its role in carotenoid biosynthesis, GGDP is a precursor of plant compounds (e.g. GA_3), and its synthesis is therefore more than likely subject to complex regulation (Bartley and Scolnik, 1995). In the first dedicated step of carotenoid biosynthesis, phytoene synthase (*PSY*) catalyses the two-step condensation of two molecules of GGPP into phytoene via prephytoene pyrophosphate. *PSY* was found to be peripherally associated with plastid membranes.

1.3.3 Desaturation and cyclization

Phytoene, the first lipophilic compound in the pathway, undergoes a series of four desaturation reactions that transforms the colourless compound into yellow, orange and red carotenoids. Phytoene desaturase (*PDS*) and ζ-carotene desaturase (*ZDS*) are responsible for the formation of phytofluene, ζ-carotene, neurosporene, and lycopene, and are membrane-associated enzymes (Cunningham and Gantt, 1998). Cyclization of lycopene leads to the formation of α- and β-carotene. β-Cyclase (*LCYB*) is responsible for the formation of β-rings, whereas ε-rings are formed by ε-cyclase (*LCYE*). β-Carotene, with two β-rings, serves as the precursor to several other carotenoids. α-Carotene, with one β- and one ε-ring, is the immediate precursor to lutein, the predominant carotenoid in photosynthetic membranes of many green plants, and formed in an apparently futile pathway (Pogson *et al.*, 1998). Carotenoids with two ε-rings are not commonly found in plants. Lactucaxanthin is one of the rare examples of a carotenoid with two ε-rings, and accumulates in lettuce (Cunningham and Gantt, 1998).

1.3.4 Xanthophyll formation

Xanthophylls are hydroxy, epoxy, furanoxy and oxy derivatives of the carotenes formed in the later stages of the pathway (Bartley and Scolnik, 1995). Hydroxylases transform α- and β-carotene into lutein and zeaxanthin (via β-cryptoxanthin) respectively. The epoxidation of zeaxanthin to form violaxanthin (via antheraxanthin) and de-epoxidation of violaxanthin to regenerate zeaxanthin is known as the xanthophyll cycle. These reactions are carried out by zeaxanthin epoxidase (*ZEP*) and violaxanthin de-epoxidase (*VDE*) (Cunningham and Gantt, 1998).

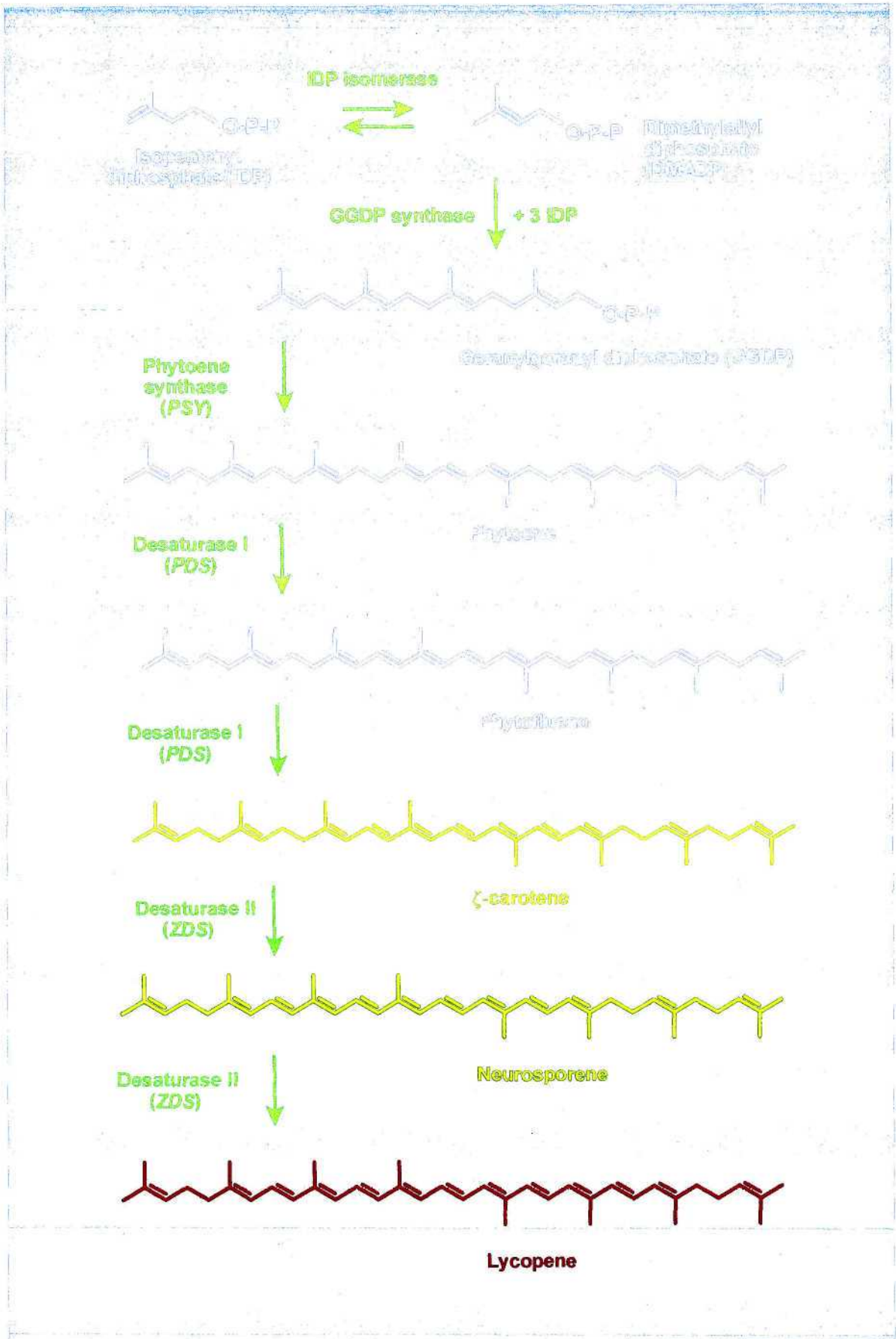


Figure 1.1 The carotenoid biosynthetic pathway (adapted from Gross, 1987).

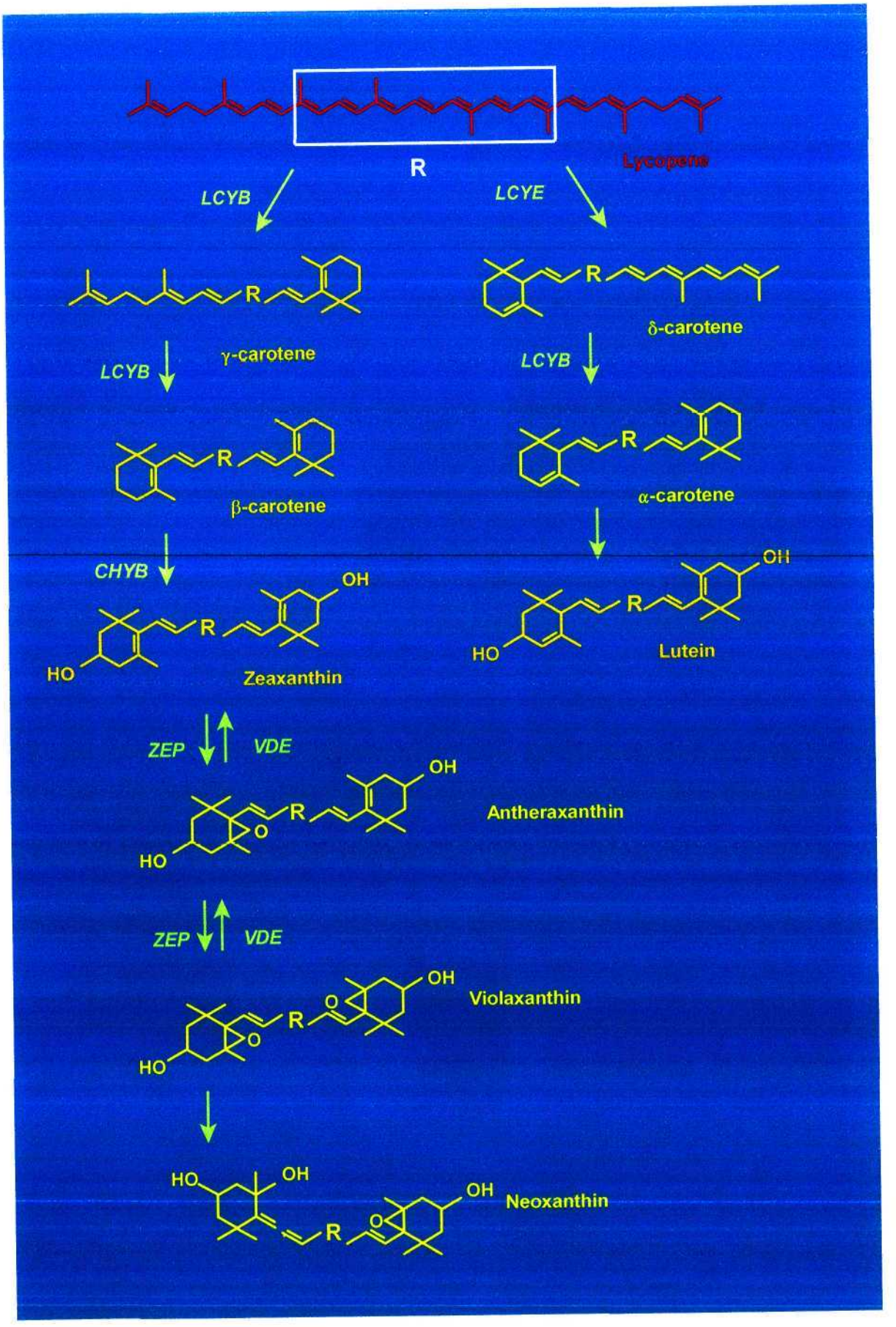


Figure 1.1 (cont.) The carotenoid biosynthetic pathway (adapted from Gross, 1987).

Bouvier *et al.* (1994) isolated and characterized capsanthin-capsorubin synthase, the enzyme responsible for the conversion of the 5,6-epoxycarotenoids, antheraxanthin and violaxanthin, into the ketocarotenoids capsanthin and capsorubin. These ketocarotenoids are characteristic of pepper fruit and responsible for the bright red colour. It was later shown that oxidative stress induced the expression of multiple carotenogenic gene mRNAs that give rise to capsanthin (Bouvier *et al.*, 1998a).

1.3.5 Carotenoid catabolism

Apocarotenoids, carotenoids with fewer than 40 carbon atoms, are formed from the cleavage of carotenoids. Citrus is a particularly rich source of apocarotenoids (Table 1.2), including β-citraurin and β-citraurin epoxide (Molnar and Szabolcs, 1980). Other apocarotenoids include β-ionone, retinal and the plant growth hormone, ABA. Cowan and Richardson (1993a; 1993b;1997) developed a cell-free system from citrus to study the conversion of epoxycarotenoids to ABA and successfully demonstrated the *in vitro* conversion of 9-*Z*-neoxanthin to ABA via xanthoxin (XAN), XAN-acid, and 1'4'-*E*-ABAdiol. Cleavage of 9-*Z*-neoxanthin is regulated by the activity of an inducible enzyme, probably a dioxygenase with a high turnover rate (Parry, 1993). Attempts to define the characteristics of the cleavage enzyme using inhibitors of lipoxygenase activity suggest that the *in vivo* reaction is catalysed by a non-heme oxygenase with lipoxygenase-like properties (Creelman *et al.*, 1992). The terminal step in ABA formation is thought to be mediated by a molybdenum-containing oxidase enzyme (likely a molybdo-aldehyde oxidase), indicating a molybdenum requirement (Walker-Simmons *et al.*, 1989; Richardson and Cowan, 1996; Lee and Milborrow, 1997)

1.3.6 Carotenoid conjugation

Xanthophylls and apocarotenoids in chromoplasts are usually found in conjugated form, either through the process of glucosylation or esterification. Esterified xanthophylls have been described in flower petals (Kleinig and Nietsche, 1968), citrus (Eilati *et al.*, 1972), and peppers (Camara and Moneger, 1978). The physiological significance of these catabolites may be attached to the fact that acylation increases the lipophilic nature of xanthophylls and thereby makes possible their accumulation in plastoglobules. Further, esterified carotenoids are more stable than the unesterified

forms and esterification with saturated fatty acids is responsible for the increased stability (Minguez-Mosquera and Hornero-Mendez, 1994).

1.4 CITRUS CAROTENOIDS

By 1973, more than 110 different carotenoids had been reported to occur in citrus (Stewart and Wheaton, 1973). Zechmeister and Tuzson are reported (MacKinney, 1961) to have made the first systematic attempt to isolate individual pigments in the orange in 1931. They isolated cryptoxanthin, lutein, zeaxanthin, β-citraurin and what was probably violaxanthin. Curl (and co-workers) reported some 74 carotenoids in several varieties of citrus between 1953 to 1967 (Curl, 1953; 1956; 1960; 1962a; 1962b; 1967; Curl and Bailey, 1954; 1955; 1956; 1957; 1959; 1961). Pigment identification methods included countercurrent distribution, chromatography, spectrophotometry and reaction with hydrochloric acid. The credibility of these methods was later questioned (Stewart and Wheaton, 1973b) and it was subsequently shown that trollixanthin and trolliflor were in fact identical to all-*E*-neoxanthin from chromatographic, visible (Vis) and infra-red (IR) spectrometry, nuclear magnetic resonance (NMR) spectrometry, mass spectrometry (MS), optical rotary dispersion (ORD) and circular dichroism (CD) data (Buchecker and Liaaen-Jensen, 1975). It is also likely that many of the reported carotenoids are the result of artifacts formed during extraction. This was shown to be the case for reticulataxanthin and citranaxanthin, formed artifactually from β-citraurin and β-apo-8'-carotenal during the saponification of citrus carotenoids in the presence of small amounts of acetone (Stewart and Wheaton, 1973a). The analysis of crystalline material using NMR, infrared and high resolution mass spectrometry should therefore be the recognised standard in the absolute identification of carotenoids. A list of carotenoids identified in citrus flavedo utilising modern biochemical techniques, is shown in Table 1.2.

Table 1.2 Carotenoids identified in citrus flavedo using modern biochemical techniques

Carotenoid	Citrus species / cultivar	Identification method	Reference
Colourless Carotenoids			
Phytofluene	mandarin	MS	Farin *et al.*,1983
ζ-Carotene	Sinton citrangequat	UV-vis, TLC	Yokoyama and White, 1966
	mandarin	MS	Farin *et al.*,1983
Neurosporene	Sinton citrangequat	UV-vis, TLC	Yokoyama and White, 1966
Carotenes			
Lycopene	mandarin	MS	Farin *et al.*,1983
δ-Carotene	mandarin	MS	Farin *et al.*,1983
α-Carotene	mandarin	MS	Farin *et al.*,1983
β-Carotene	Sinton citrangequat	UV-vis, TLC	Yokoyama and White, 1966
	mandarin	MS	Farin *et al.*,1983
xanthophylls			
Lutein	mandarin	MS	Farin *et al.*,1983
Mutatoxanthin	mandarin	MS	Farin *et al.*,1983
β-Cryptoxanthin	Sinton citrangequat	UV-vis, TLC	Yokoyama and White, 1966
	Valencia	UV-vis, MS, CD	Molnar and Szabolcs, 1980
	mandarin	MS	Farin *et al.*,1983
Cryptoxanthin 5,6-epoxide	mandarin	MS	Farin *et al.*,1983
Zeaxanthin	mandarin	MS	Farin *et al.*,1983
Antheraxanthin	Valencia	UV-vis, MS, CD	Molnar and Szabolcs, 1980
	mandarin	MS	Farin *et al.*,1983

Carotenoid	Citrus species / cultivar	Identification method	Reference
9-*Z*-Violaxanthin	Valencia	UV-vis, MS, CD	Molnar and Szabolcs, 1980
all-*E*-Violaxanthin	Valencia	UV-vis, MS, CD	Molnar and Szabolcs, 1980
Di-*Z*-Violaxanthin	Valencia	UV-vis, MS, CD	Molnar and Szabolcs, 1980
E-Neoxanthin	mandarin	MS	Farin *et al.*,1983
9-*Z*-Neoxanthin	mandarin	MS	Farin *et al.*,1983
Apocarotenoids			
β-Citraurin	Sinton citrangequat	UV-vis, TLC	Yokoyama and White, 1966
	Valencia	UV-vis, MS, CD	Molnar and Szabolcs, 1980
	mandarin	MS	Farin *et al.*,1983
β-Citraurin epoxide	Valencia	UV-vis, MS, CD	Molnar and Szabolcs, 1980
β-Citraurinene	Robinson (Orlando tangelo*clementine mandarin)	UV-vis, TLC, HPLC, IR, MS, NMR,	Leuenberger and Stewart, 1976a
β-Citraurol	Robinson (Orlando tangelo*clementine mandarin)	UV-vis, TLC, HPLC, IR, MS, NMR,	Leuenberger and Stewart, 1976b
β-Apo-8'-carotenal	Sinton citrangequat	UV-vis, IR, TLC	Yokoyama and White, 1966
β-Apo-10'-carotenal	Sinton citrangequat	UV-vis, IR, TLC	Yokoyama and White, 1966
Apo-8'-violaxanthal	Valencia	UV-vis, CD, MS,	Molnar and Szabolcs, 1980
Apo-10'-violaxanthal	Valencia	UV-vis, CD, MS,	Molnar and Szabolcs, 1980
Apo-12'-violaxanthal	Valencia	UV-vis, CD, MS,	Molnar and Szabolcs, 1980

1.5 OBJECTIVES

The bulk of available literature pertaining to citrus carotenoids and colour development was written in the period 1970-1985, with little recent interest in the subject. An understanding of pigments involved in colour development and colour regulatory aspects in *Citrus sinensis* (and other carotenogenic fruit) is however considered essential to any manipulation of this characteristic. The objectives of the current study were therefore:

1. To provide a biochemical basis for the question: 'What is good colour';
2. To determine the changes in flavedo pigment content and composition during colour development;
3. To establish the effect of a variety of plant growth regulators, dehydrating agents and metal ions on carotenoid content and composition with a view to manipulate the process of colour development.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

2.1.1 Authentic carotenoids

Carotenoid standards were kindly supplied by Dr. Peter Molnár, Dept. Medical Chemistry, Pécs University, Hungary and were prepared as described by Molnár and Szabolcs (1979).

2.1.2 Growth regulators

(±)-Z,E-abscisic acid (ABA), gibberellic acid (GA_3), indole butyric acid (IBA) and jasmonic acid (JA) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

2.1.3 General chemicals

Potassium molybdate (K_2MoO_4) and potassium tungstate (K_2WO_4) were obtained from Aldrich Chemical Co. (Milwaukee, USA). Cobaltous sulphate ($CoSO_4$) and nickel sulphate ($NiSO_4$) were purchased from Saarchem (Krugersdorp, South Africa). Diethyldithiocarbamate (DDC) and butylated hydroxy toluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Tween-20 and potassium hydroxide (KOH) were purchased from BDH Laboratory Supplies (Poole, UK).

2.1.4 Solvents

HPLC grade solvents (acetonitrile, methanol, ethyl acetate, methylene chloride and hexane) were purchased from Burdick and Jackson (Allied Signal Inc., Muskegon, MI, USA). Ethanol, propan-(1)-ol, butan-(2)-ol and acetone were of analytical grade and were purchased from BDH Laboratory Supplies (Poole, UK). Triethylamine (TEA) was purchased from Sigma Chemical Co. (St Louis, Mo, USA).

2.2 CHROMATOGRAPHIC MEDIA

Thin layer plates of silica gel, type 60 (20 x 20 cm, 0.25 mm thickness), were purchased from Merck (Darmstadt, Germany).

2.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) SYSTEM

The HPLC system comprised of a 5µm Vydac 201TP54 C_{18} (250 × 4.6 mm i.d.) column (VYDAC, Hesperia, CA, USA), a SpectraSYSTEM P2000 pump, UV3000 rapid-scanning detector and PC1000 software (Thermo Separations Products, Fremont, CA, USA).

2.4 PLANT MATERIAL

Fruit was harvested from ten-year old *C. sinensis* Navel and Valencia trees on rough lemon rootstocks from Orangewood farm, located in the Albert falls region (29°25'S and 30°22'E), KwaZulu-Natal midlands, South Africa. Fruit from ten 'Navel' and ten 'Valencia' trees (in a row) were harvested, constituting a simple randomised design. Annual rainfall in this region has averaged 983.7 mm over a 90 year period (Anon, 2000). The period from May to August is considered to be ecologically dry, with the remaining months being ecologically humid. Mean maximum and minimum temperatures in January are 28 °C and 17 °C respectively, with the July maximum and minimum being 22 °C and 5 °C. Mean maximum and minimum temperatures experienced in 1996 are illustrated in Fig. 2.1

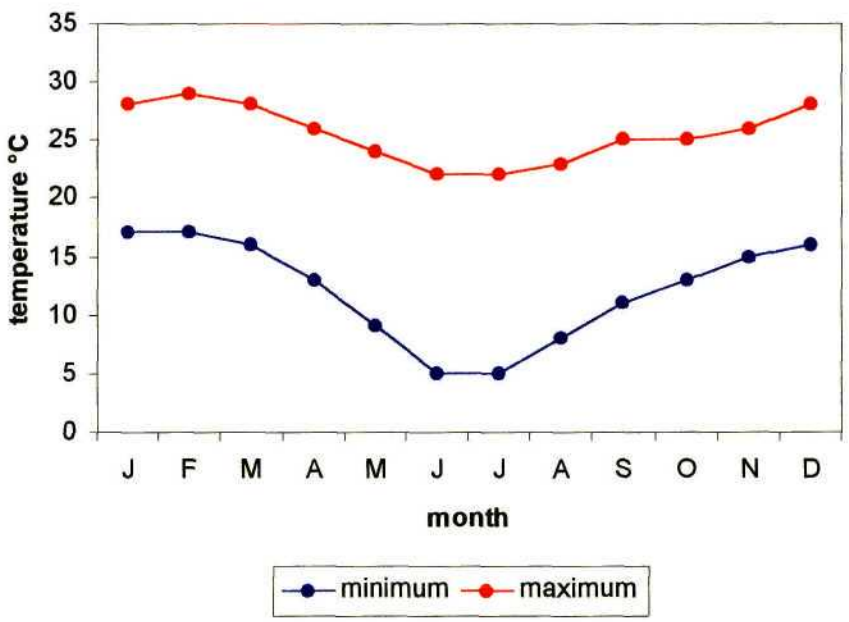


Figure 2.1 Minimum and maximum temperatures experienced at the study site in 1996. (Source CCWR, Pietermaritzburg)

Trees were cultivated in a Hutton form soil which is characterised by an orthic A horizon overlying a red apedal B horizon. Typically these soils are medium to heavy textured (with a clay content of 35-55%) and form in well-drained oxidising environments. They generally have weakly structured, acidic topsoils (MacVicar *et al.*, 1984).

All trees were subjected to orchard management practices commonly employed in this area. No cover crop was planted and the orchard was irrigated through a micro-jet system.

2.5 APPLICATION OF CHEMICALS

2.5.1 Post-harvest application of chemicals to intact fruit

Fruit were harvested at different stages of colour development and treated with dehydrating agents. Intact fruit were surface sterilised by immersion in 1% (v/v) sodium hypochlorite for 20 min followed by two changes of distilled water and subsequently dipped in aqueous solutions of methanol, ethanol, propanol and butanol at 40°C for 4 min. (The hot water/fungicidal bath in most citrus packhouses operates at 40 °C and fruit spend approximately 4 min in this solution. It is envisaged that any successful treatments may be incorporated in the hot water bath). Nine fruit were included in each treatment. Treated fruit were allowed to dry and wrapped in polyethylene film or waxed. Control fruit were dipped in water at 40 °C for 4 min, and untreated fruit were also included in the experiment. Fruit was incubated in cardboard boxes at room temperature (in darkness) to simulate packhouse conditions. Carotenoid content of the flavedo of three fruit was determined spectrophotometrically after 0, 1, 2 and 4 weeks.

2.5.2 *In vitro* treatment of flavedo discs

Sections, approximately 3 mm thick to ensure that the flavedo remained intact, were cut from the peel of surface sterilised fruit with a razor blade and submerged in a shallow tray containing distilled water. Discs were cut with a sterilised 10-mm-diameter cork borer, floated on distilled water and 8 discs were distributed randomly in petri dishes containing 8 mL of bathing solution. Treatments were replicated three times.

All treatments (Table 2.1) were made up in 10 mM phosphate buffer, pH 7.4. Discs were incubated at 22 °C in a Labcon incubator. 4 discs from each petri dish were removed at 0, 24 and 96 h, and total carotenoid content determined spectrophotometrically.

Table 2.1 Summary of treatments applied to *Citrus sinensis* flavedo discs

Treatment	Concentration/time
Plant growth regulators IAA, GA_3, ABA, JA	0.1 mM and 0.1 µM
Dehydrating agents* ethanol and butanol	2.5, 10 and 20% (v/v)
Micro-nutrients Co/Ni, Mo, W	1 µM and 10 µM
Temperature** 4 °C	2, 6, 10 h

* incubated for 2 h in dehydrating agent and transferred to phosphate buffer (pH 7.4)
** incubated for 2, 6 and 10 h at 4 °C, transferred to 22 °C for remainder of time period

The effect of a short low temperature treatment on subsequent colour development was investigated as colour break is usually correlated with a sudden decrease in temperature. As the mean minimum during winter in the study area varies between 3 and 5 °C, a temperature of 4°C was chosen.

2.6 VISUAL DETERMINATION OF FLAVEDO COLOUR

Visual colour of whole fruit was determined by comparing the flavedo colour with Outspan blemish standards chart no. 19 (colour) which is used for prescribing citrus export and local market grading regulations in South Africa (Fig 2.2). Fruit are rated on a scale of 1 (orange, fully coloured) to 8 (green, unmarketable), according to the criteria used by the Southern African Citrus Growers' Association.

Figure 2.2 The Outspan Citrus Colour Chart (Chart no. 19; Colour Prints for Blemish standards)

2.7 PIGMENT EXTRACTION

The high degree of unsaturation in carotenoids renders the compounds heat and light sensitive (Gross, 1986) and therefore all extraction and purification procedures were carried out at low temperature and under reduced light intensity to avoid photo-oxidation and isomerization of the compounds of interest.

The outer layer of the flavedo was grated from the fruit with a Moulinex™ hand grater and finely homogenised to a powder in liquid nitrogen using a mortar and pestle. Flavedo discs were sliced finely with a razor blade and crushed to a powder in liquid nitrogen. For TLC separation, a larger amount of tissue was needed and flavedo was grated and freeze-dried for a minimum of 48 hrs. 100 µg DDC in 3 g silica gel was added to the bottom of the flasks to minimise oxidation. Dried samples were milled in a Janke & Kunkel benchtop mill and stored in dry form at -20 °C in darkness under N_2.

A number of solvents were evaluated for the extraction of carotenoids from citrus flavedo. 1 g of fresh tissue was homogenised (2 x 1 min bursts), in 10 ml solvent (Table 2.2) containing BHT (100 mg L^{-1}) and DDC (200 mg L^{-1}) as antioxidants, with PVP (Polyclar SB100, 1 g per 10 g fresh weight) using a Janke & Kunkel Ultra-Turrax T25 top-drive tissue homogenizer.

Table 2.2 Summary of solvent systems for carotenoid extraction from *C. sinensis* flavedo

Solvent
Acetone/hexane (2:1, v/v)*
Methanol (MeOH)/chloroform (2:1, v/v)*
95 % Aqueous ethanol (EtOH) (v/v)
Methanol (MeOH)/ethyl acetate (EtoAc) (50:50, v/v)

*carotenoids partitioned with equal volume water and hexane

Where necessary (Table 2.2), carotenoids were partitioned into hexane by the addition of equal volumes of water and hexane. Homogenates were centrifuged for 5 min at 3800 rpm in a Hermle 2510 centrifuge. The carotenoid-containing hexane fraction or supernatant was removed and the extraction process repeated to ensure complete extraction of carotenoids. Extracts were pooled and subject to spectrophotometric or HPLC analysis. Very dilute extracts were concentrated *in vacuo* at 35 °C, using a Bibby RE 100 rotary evaporator. The efficiency of carotenoid extraction for a given sample subject to HPLC analysis is illustrated in Table 2.3

Table 2.3 Comparative efficiency of different solvent systems in carotenoid extraction

Extraction method	carotenoid content (µg β-carotene/g fw)
Acetone/hexane	85.15 ± 0.22
Methanol/chloroform	88.43 ± 0.37
95% Ethanol	92.18 ± 0.13
Methanol/ethyl acetate	94.6 ± 0.14

The stability of carotenoid extracts were tested by leaving extracts at room temperature, in darkness, for approximately 12 h. HPLC analysis revealed no difference in carotenoid concentration and composition of these extracts when compared to extracts analysed immediately, indicating that carotenoid extracts are fairly stable. This is likely due to the fact that carotenoids accumulate in citrus peel in esterified form (Philip, 1973a; 1973b).

2.8 THIN LAYER CHROMATOGRAPHY (TLC)

Concentrated crude extracts (approximately 100 µg) were resuspended in 500 µL methanol/ethyl acetate (50:50; v/v), loaded onto silica gel plates (Type 60) and developed to 15 cm in a closed tank using the solvent system hexane/ethyl acetate/ethanol/acetone (95:3:2:2; v/v) at 2 - 4 °C in darkness (Molnár and Szabolcs, 1980). The plate was allowed to dry and the carotenoid-containing zones removed

and eluted with acetone through small glass funnels plugged with glass wool. Rf values of standards used in the system are illustrated in Table 2.4

Table 2.4 Rf values of authentic carotenoid standards subject to TLC analysis utilising the solvent system hexane/ethyl acetate/ethanol/acetone (95:3:2:2)

Component	Rf value
Antheraxanthin, violaxanthin and neoxanthin	0
Lutein	0.04
Chlorophyll *b*	0.047
β-citraurin	0.057
9-Z-Neoxanthin acyl ester	0.1
Chlorophyll *a*	0.113
β-Citraurin acyl ester	0.33

2.9 SAPONIFICATION

Following extraction, pigments were resuspended in 8 mL of methanol to which was added 2mL KOH (1M). Extracts were vortexed, and left to stand for approximately 12 h in complete darkness at room temperature. After removal of the methanol fraction using a rotary evaporator (35 °C), 3 mL water and 5 mL diethyl ether was added and thoroughly vortexed. The highly coloured ether fraction was removed and a further 5 mL ether added to the aqueous phase, vortexed and removed, until the ether fraction remained clear. Combined ether fractions were pooled and reduced to dryness .

2.10 SPECTROPHOTOMETRY

Plant tissue was extracted in 95% (v/v) aqueous ethanol and the carotenoid concentration determined spectrophotometrically at 470, 649 and 664 nm in a Beckman DU-65 spectrophotometer as described by Lichtenthaler, 1987. Absorbance values were computed to calculate the concentrations of chlorophyll *a* (C_a), chlorophyll *b* (C_b), total chlorophylls (C_{a+b}) and total carotenoids (C_{x+c}) using the following equations:

$$C_a = 13.36A_{664} - 5.19A_{649}$$

$$C_b = 27.43A_{649} - 8.12A_{664}$$

$$C_{a+b} = 5.24A_{664} + 22.24A_{649}$$

$$C_{x+c} = \frac{1000A_{470} - 2.13C_a - 97.64C_b}{209}$$

Equations were not recalculated against pure chlorophyll *a* and *b* (as described by Lichtenthaler (1987)), as it was reported by Wellburn (1994) that chlorophyll *a*, *b* and total carotenoid content of mixed extracts could be accurately determined with a range of modern spectrophotometers using the equations published by Lichtenthaler (1987). Pigment concentrations thus obtained were expressed as micrograms per millilitre plant extract solution or converted to micrograms per gram dry or fresh weight.

2.11 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Dry crude and saponified extracts were resuspended in methanol/acetonitrile (9:1, v/v):ethyl acetate (50:50; v/v). Extracts were filtered using a 0.2 µm syringe filter and individual carotenoids separated by reversed-phase HPLC on a 5 µm Vydac C_{18} column, eluted isocratically at 26 °C with methanol/acetonitrile (9:1; v/v) containing 0.1% (w/v) BHT and 0.05 % (v/v) TEA at a flow rate of 1 mL min^{-1}. Compounds of interest were detected at 460 nm and quantified by peak integration using a UV3000 rapid-scanning detector in the range 370 to 550 nm after calibration with authentic standards. Identification was achieved with the use of PC1000 software that allowed for online comparison of absorption spectra of unknown compounds with authentic carotenoid standards. Maxima and retention times of carotenoids eluted in the system described above are illustrated in Table 2.5. The absorption spectra of the carotene and xanthophyll standards are shown in Fig. 2.3, 2.4 and 2.5 and that of β-citraurin, an apo-carotenoid, is depicted in Fig. 2.6.

Table 2.5 Retention times and spectral characteristics of chlorophylls and carotenoids eluted isocratically with methanol/acetonitrile (9:1, v/v) at 1 mL min^{-1} in the reversed-phase HPLC system described in section 2.12.

Component	Retention time	λ max
E-Neoxanthin	3.266	434, 462, 414
9-*Z*-Neoxanthin	3.595	436, 464, 413
E-Violaxanthin	3.975	438, 468, 415
9-*Z*-Violaxanthin	4.057	435, 464, 412
Antheraxanthin	4.668	444, 471
Chlorophyll *b*	5.228	467
Lutein	5.459	444, 471
Zeaxanthin	5.657	449, 476
β-Citraurin	6.201	457
Chlorophyll *a*	6.977	433, 421
α-Carotene	18.373	445, 473
E-β-Carotene	20.649	451, 476
9-*Z*-β-Carotene	23.717	445, 467
Lycopene	29.292	470, 501, 445

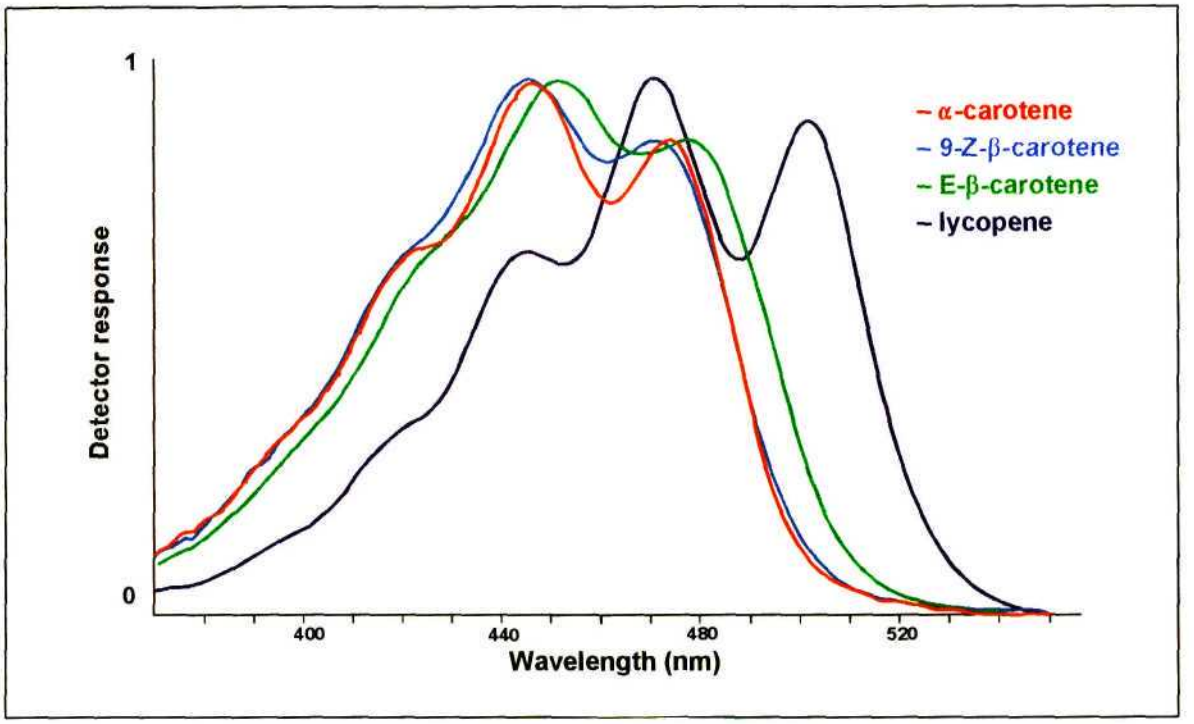


Figure 2.3 Absorption spectra of authentic carotenes α-carotene, 9-*Z*-β-carotene, *E*-β-carotene and lycopene

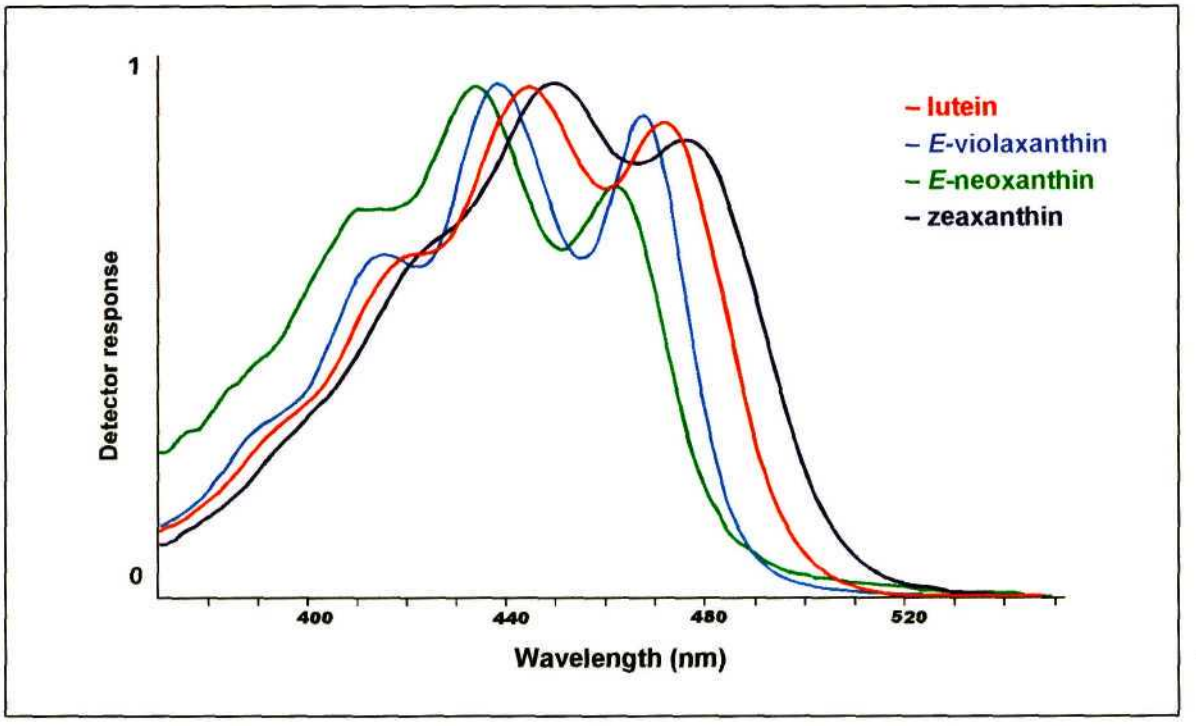


Figure 2.4 Absorption spectra of authentic xanthophylls lutein, *E*-violaxanthin, *E*- neoxanthin and zeaxanthin

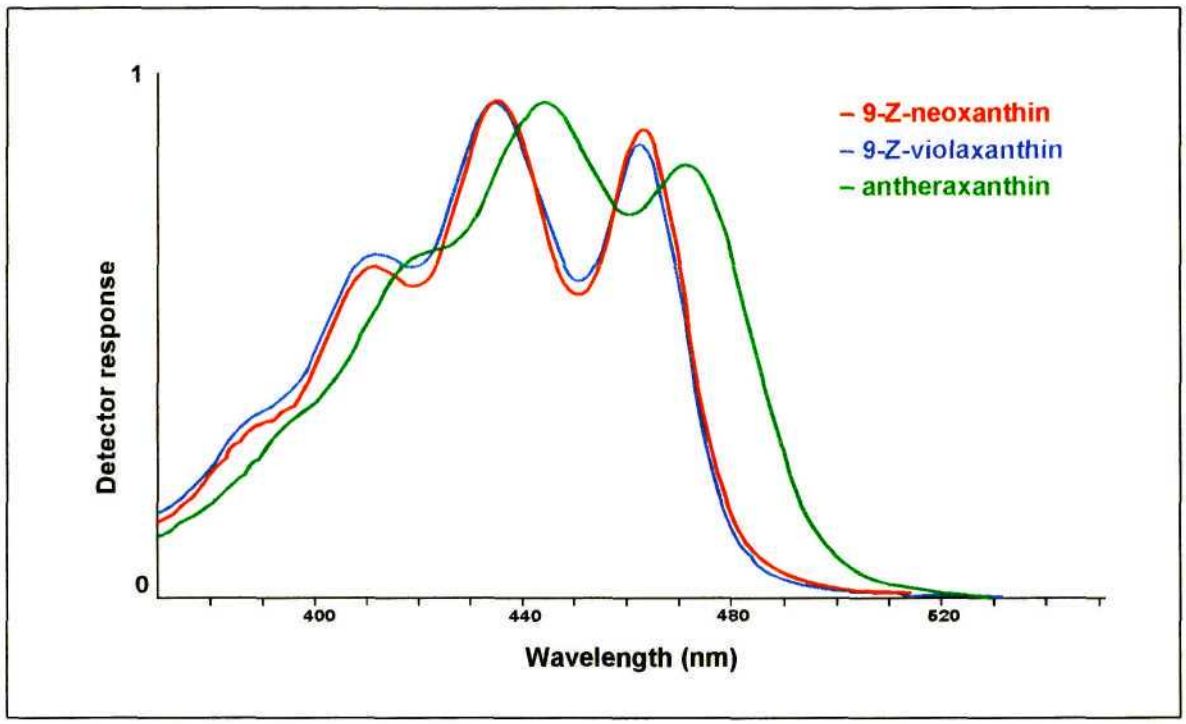


Figure 2.5 Absorption spectra of authentic xanthophylls 9-Z-neoxanthin, 9-Z-violaxanthin and antheraxanthin

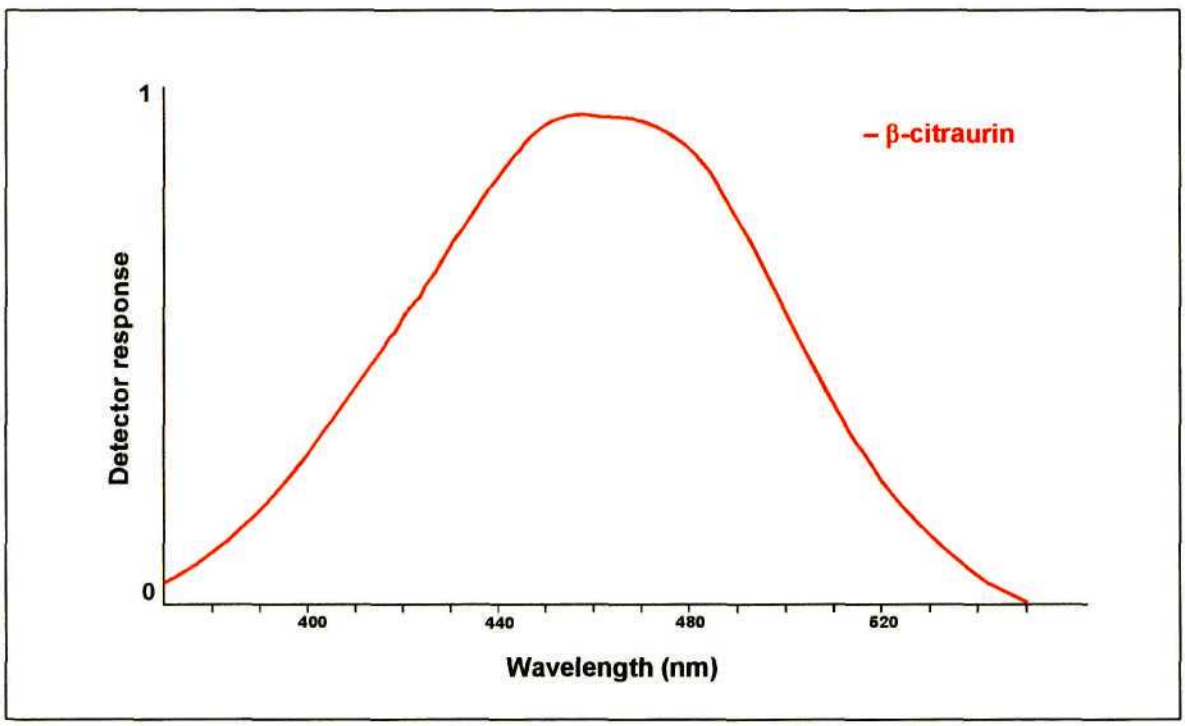


Figure 2.6 Absorption spectrum of authentic β-citraurin

CHAPTER 3

A BIOCHEMICAL BASIS OF COLOUR AS AN AESTHETIC QUALITY IN *CITRUS SINENSIS*

3.1 INTRODUCTION

Visual colour of a plant organ results mainly from the plant pigments; chlorophyll and carotenoids in the chloro- and chromoplasts, and phenolic pigments in the vacuole. The expression of pigment colour can also be affected by a number of physical characteristics such as surface topography, presence of cuticular waxes, epidermal hairs and even the shape and orientation of cells in the epidermis and sub-epidermal tissues (Lancaster *et al.*, 1997). Although total carotenoid content is important in visual colour perception, the presence or absence of certain pigments often play a more significant role. This phenomenon is noticeable in peppers (*C. annuum*), where lutein, neoxanthin and violaxanthin are characteristic pigments of yellow-fruited varieties, whereas red-fruited varieties accumulate the characteristic paprika ketones, capsanthin and capsorubin (Davies *et al.*, 1970).

In citrus, violaxanthin and its *Z*-isomers (9-*Z*, 13-*Z* and di-*Z*-) have been shown to predominate in the flavedo (Molnár and Szabolcs, 1980). Violaxanthin is typically a yellow pigment and therefore unlikely to be solely responsible for good quality colour of mature orange fruit. In fact, the presence of the red and orange pigments, β-citraurin and β-cryptoxanthin, have been reported to impart an intensive reddish tinge to a mandarin hybrid (Farin *et al.*,1983), 'Dancy' tangerine (Gross, 1981) and 'Minneola', 'Temple', 'Robinson' and 'Osceola' (Stewart and Wheaton, 1973b). In the present investigation efforts were made to determine the biochemical basis of flavedo colour as an aesthetic quality using 'Navel' and 'Valencia' fruit. Typically mature 'Navel' fruit are brightly coloured whereas 'Valencia' are recalcitrant with respect to colour development and therefore ideal for comparative purposes.

3.2 RESULTS

To address the question: 'What does good colour constitute?', carotenoid content and composition of 'Navel' and 'Valencia' fruit of similar maturity, but differing in colour grade/quality were examined.

3.2.1 Identification of colour-imparting carotenoids

Crude pigment extracts were prepared from flavedo of 'Navel' and 'Valencia' fruit of a range of colour grades (colour grades 7, 5, 3 and 1 on the Outspan colour chart). Separation of extracts on thin layers of silica gel revealed, in addition to numerous minor pigment-containing zones, two intensely coloured bands at Rf 0.1 (yellow-orange, zone A) and Rf 0.33 (orange-red, zone B) (Fig. 3.1) in fruit of colour grades 3 and 1. These bands were eluted from the gel and further analysed by reversed-phase HPLC (rHPLC). The chromatographic profiles are illustrated in Fig. 3.2 and 3.3. Since identical results were obtained for flavedo of both 'Navel' and 'Valencia' fruit, and for ease of data presentation, only chromatograms for 'Valencia' are shown. The results show that TLC zones A and B were each resolved into three major components and that the retention time of the components in zone A was very similar to the components in zone B. Online spectral analysis of components A1, A2 and A3 (Fig. 3.2) produced results consistent with a chromophore similar to 9-*Z*-violaxanthin (λ_{max} nm: 435) whereas B1, B2 and B3 (Fig. 3.3) produced spectra with a single maxima at 457 nm typical of β-citraurin. Confirmation of the identity of these chromophores as 9-*Z*-violaxanthin and β-citraurin was achieved by saponification of zones A and B from TLC, prior to reversed-phase HPLC analysis. The results in Fig. 3.4 and 3.5 show that after saponification, zones A and B yielded single peaks which co-chromatographed with authentic standards of 9-*Z*-violaxanthin and β-citraurin respectively. In addition, comparison of spectral characteristics with those of authentic standards showed >99% similarity between zone A and 9-*Z*-violaxanthin and zone B and β-citraurin. Furthermore, these results indicate that both 9-*Z*-violaxanthin and β-citraurin accumulate in orange flavedo in an esterified form and that esterification is responsible for the differences in retention times noted in Fig.3.2 and 3.3.

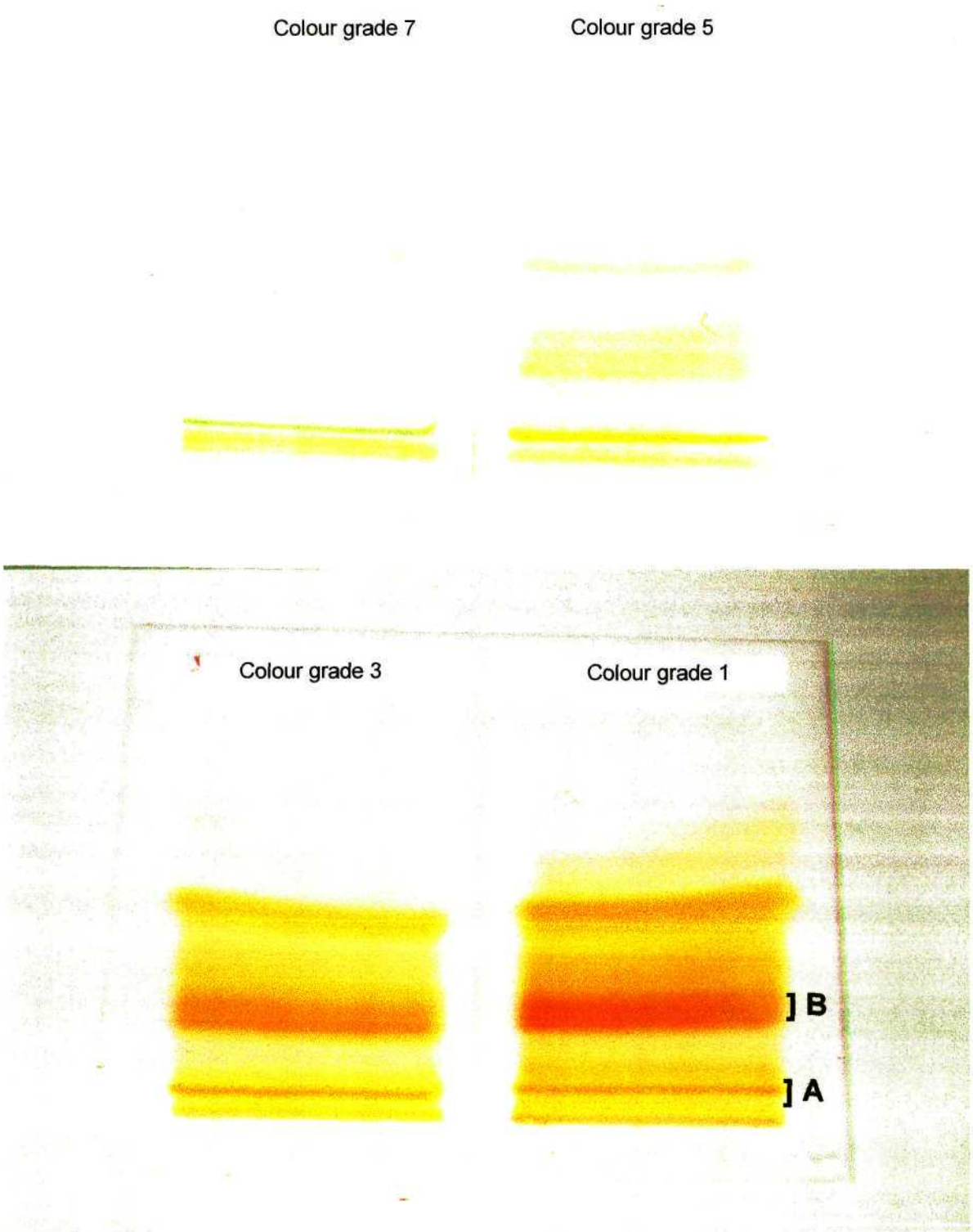


Figure 3.1 TLC separation of citrus flavedo pigments extracted with MeOH/EtoAc (50:50; v/v) indicating two colour-imparting zones A and B. TLC system utilised was hexane/ethyl acetate/ethanol/acetone (95:3:2:2, v/v). Fruit were of four different colour grades (grade 1, 3, 5 and 7) on the Outspan colour chart.

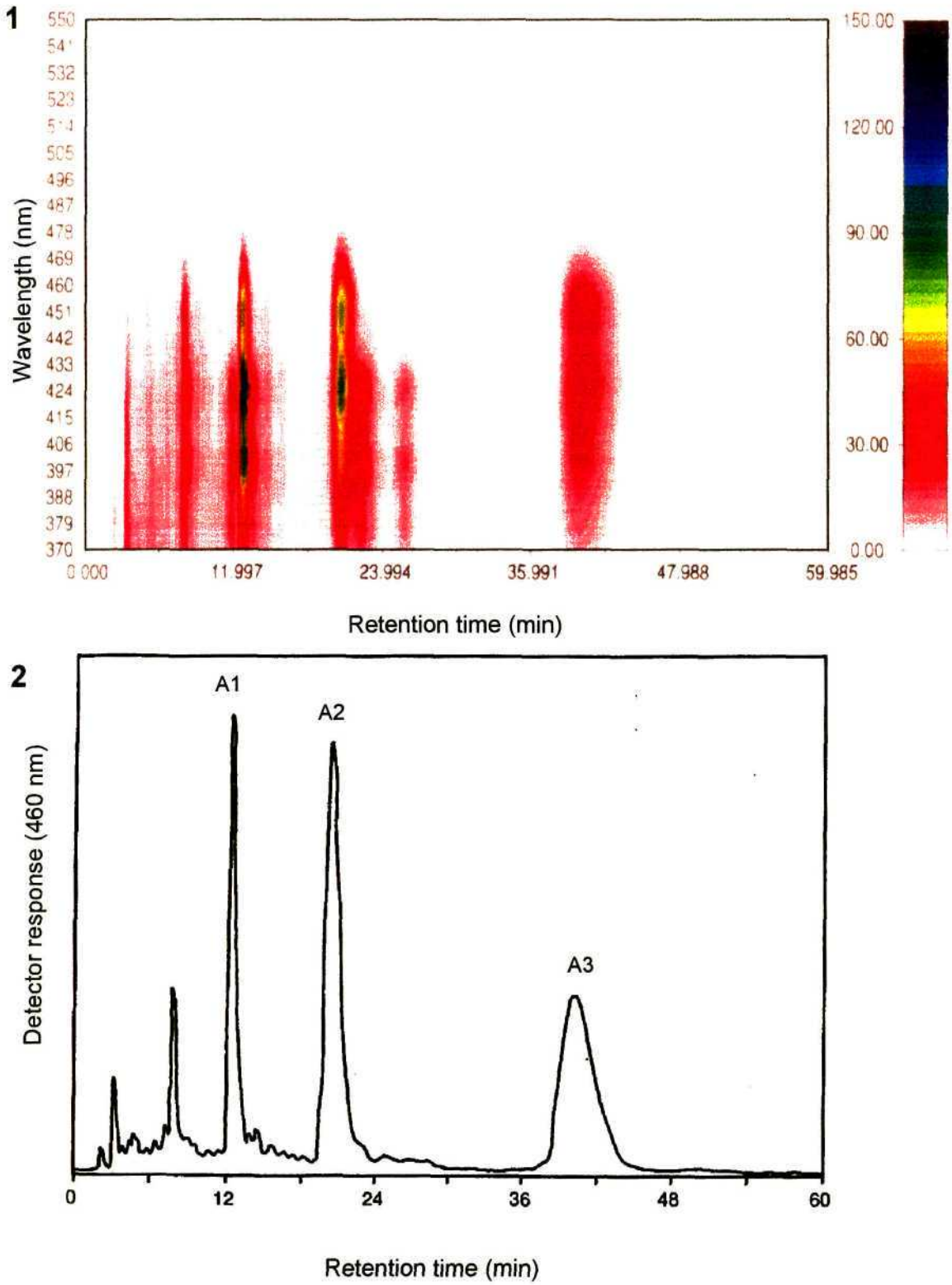


Figure 3.2 Reversed-phase HPLC (1) isoplot and (2) chromatogram at 460 nm of unsaponified zone A from TLC separated crude pigment extracts of Valencia orange flavedo (colour grade 1).

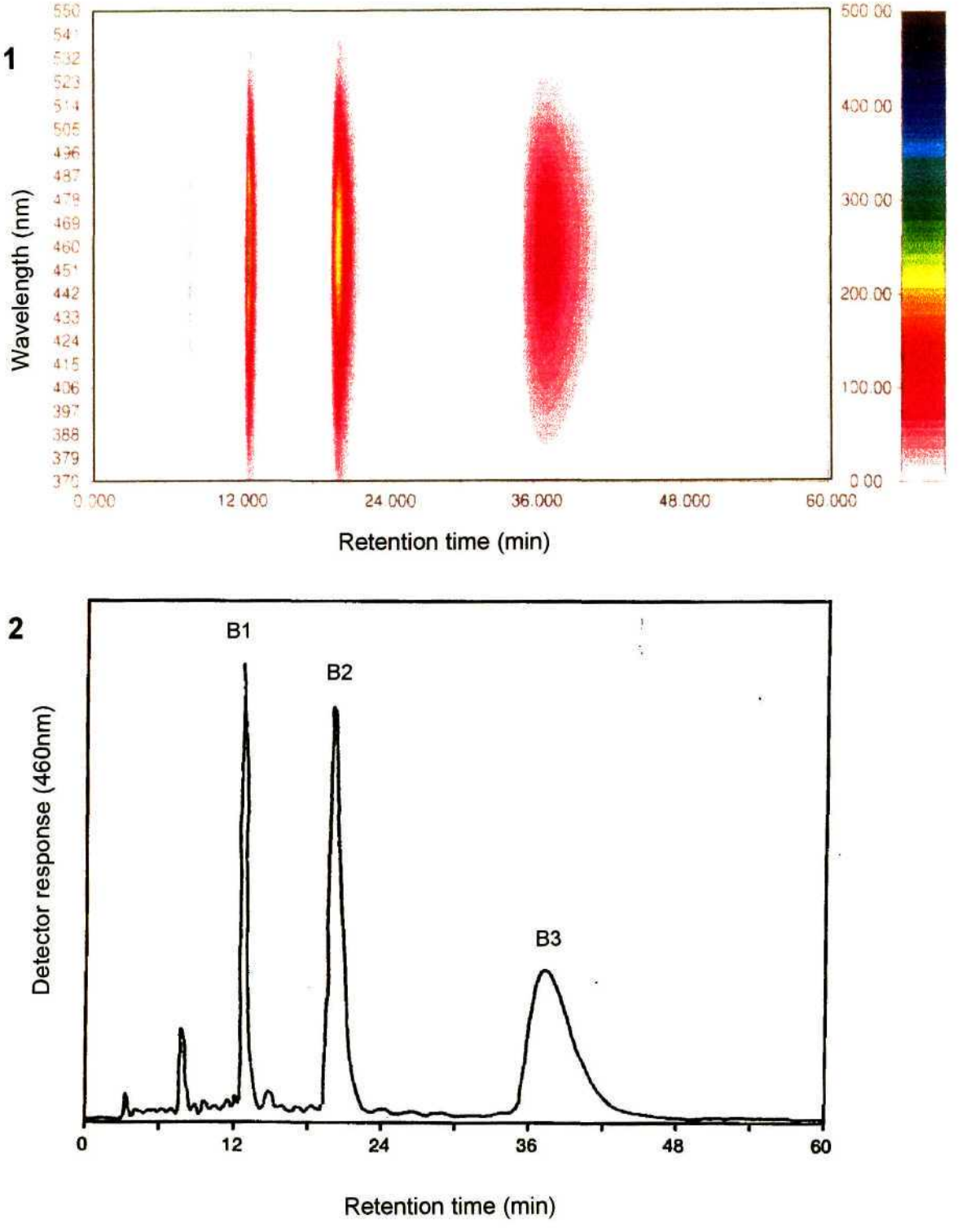


Figure 3.3 Reversed-phase HPLC (1) isoplot and (2) chromatogram at 460 nm of unsaponified zone B from TLC separated crude pigment extracts of Valencia orange flavedo (colour grade 1).

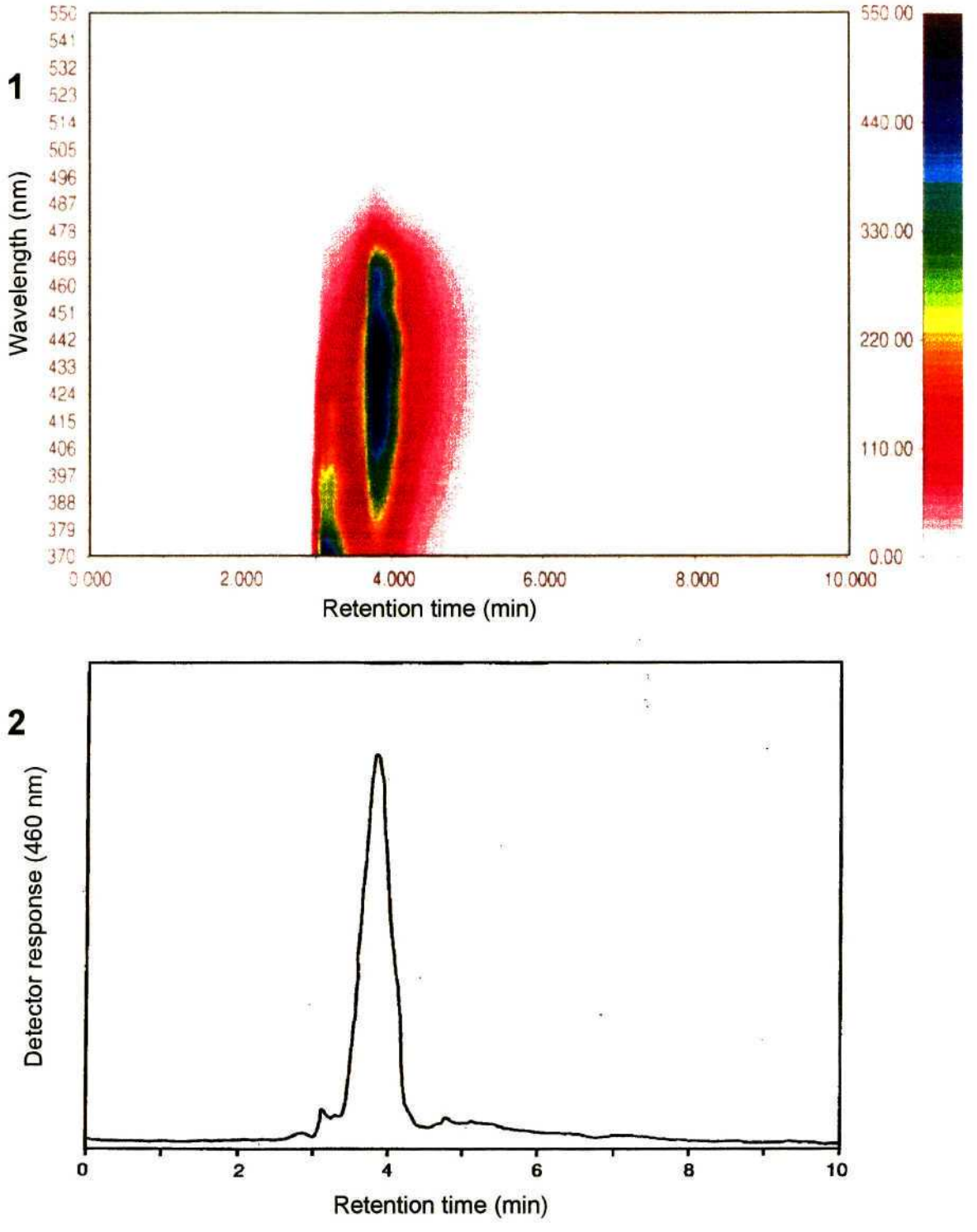


Figure 3.4 Reversed-phase HPLC (1) isoplot and (2) chromatogram at 460 nm of saponified zone A.

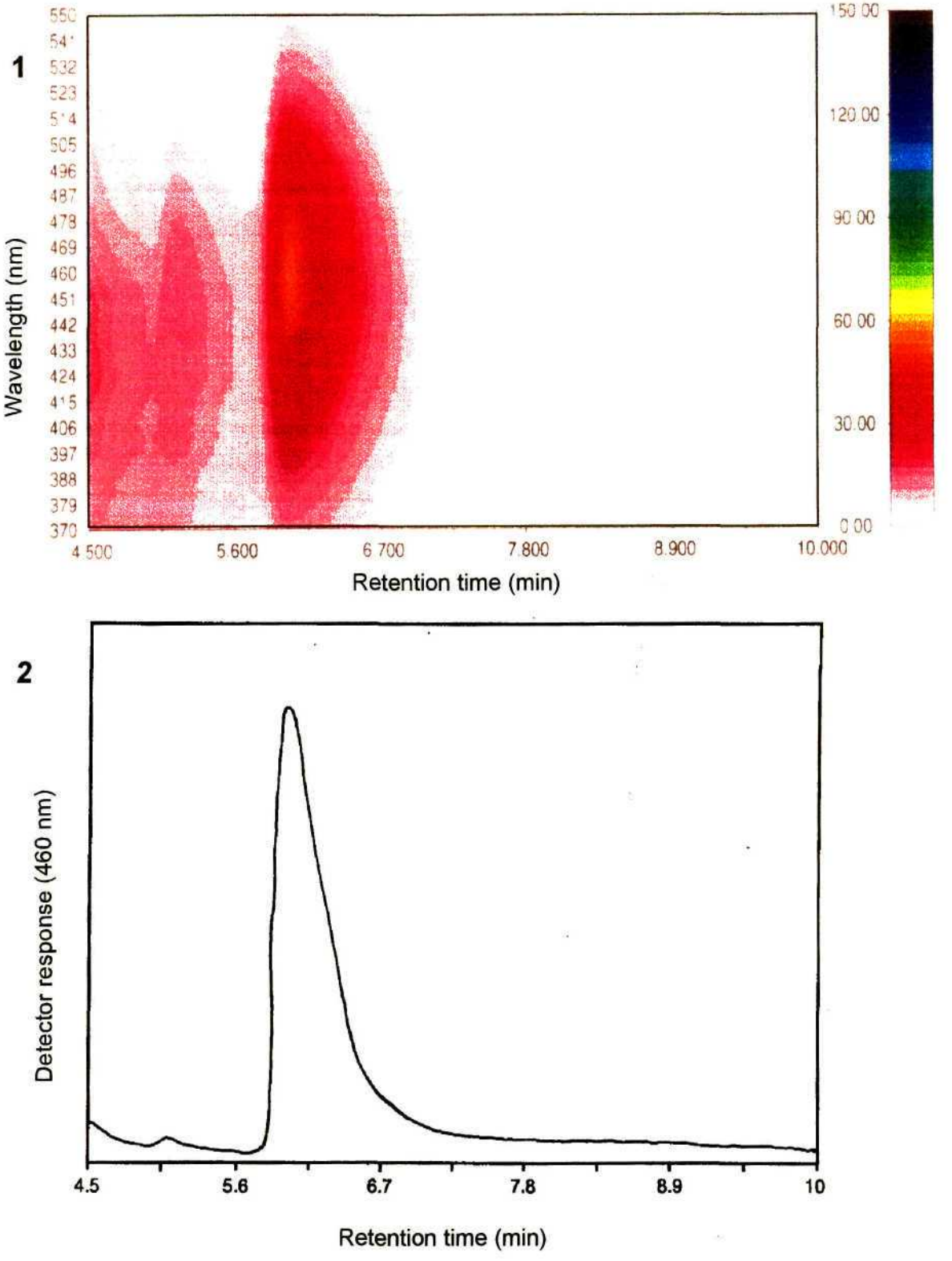


Figure 3.5 Reversed-phase HPLC (1) isoplot and (2) chromatogram at 460 nm of saponified zone B.

β-citraurin and 9-*Z*-violaxanthin acyl esters occur in close association when crude flavedo extracts are analysed by HPLC (Fig. 3.6), complicating quantification of these compounds. Saponification of crude extracts prior to HPLC analysis ensures that these two major colour-imparting pigments are well resolved for quantification purposes (Fig. 3.7). Therefore, accurate quantification of the chromophores of these acyl esters is possible without prior separation by thin layer chromatography. Additionally, the absorption spectrum of each pigment is unaffected by saponification and is identical to the absorption spectra of the esterified compound. This indicates that esterification does not affect the colour of 9-*Z*-violaxanthin and β-citraurin.

3.2.2 Biochemical basis of flavedo colour

9-*Z*-Violaxanthin is the most abundant carotenoid in citrus flavedo (Molnár and Szabolcs, 1980; Gross, 1987). As a yellow pigment, it contributes mainly to the background colour of orange fruit flavedo. β-Citraurin, a C_{30} apocarotenal, is a red-orange pigment and is responsible for the bright orange colour of tangerine flavedo (Farin *et al.*, 1983). In order to demonstrate a similar role for β-citraurin in flavedo colour of *C. sinensis*, 9-*Z*-violaxanthin and β-citraurin levels of different colour grades of mature 'Valencia' and 'Navel' fruit were determined by HPLC after saponification. The results are illustrated in Fig. 3.8. No fruit of Grade 7 or 8 were available at the time of harvest and very brightly coloured 'Navel' fruit (more orange than Grade 1) were designated Grade 0. Poorly coloured fruit (Grade 5-6) had high levels of chlorophyll (data not shown), low levels of 9-*Z*-violaxanthin and almost undetectable amounts of β-citraurin. Fruit of average colour (Grade 3-4) had relatively low levels of both colour-imparting pigments. Fruit of good colour (Grade 0-2) contained increased levels of 9-*Z*-violaxanthin and β-citraurin. Thus, an increase in the colour grade (i.e. from 6 to 1) was associated with massive accumulation of 9-*Z*-violaxanthin concomitant with a less dramatic increase in β-citraurin in both 'Navel' and 'Valencia' flavedo. More importantly however, the increase in colour grade occurred as a result of a decline in the 9-*Z*-violaxanthin : β-citraurin ratio from >50 to <10. For Grade 1 fruit, the β-citraurin levels of flavedo of 'Navel' and 'Valencia' were similar but 9-*Z*-violaxanthin levels were higher in flavedo of 'Valencia'.

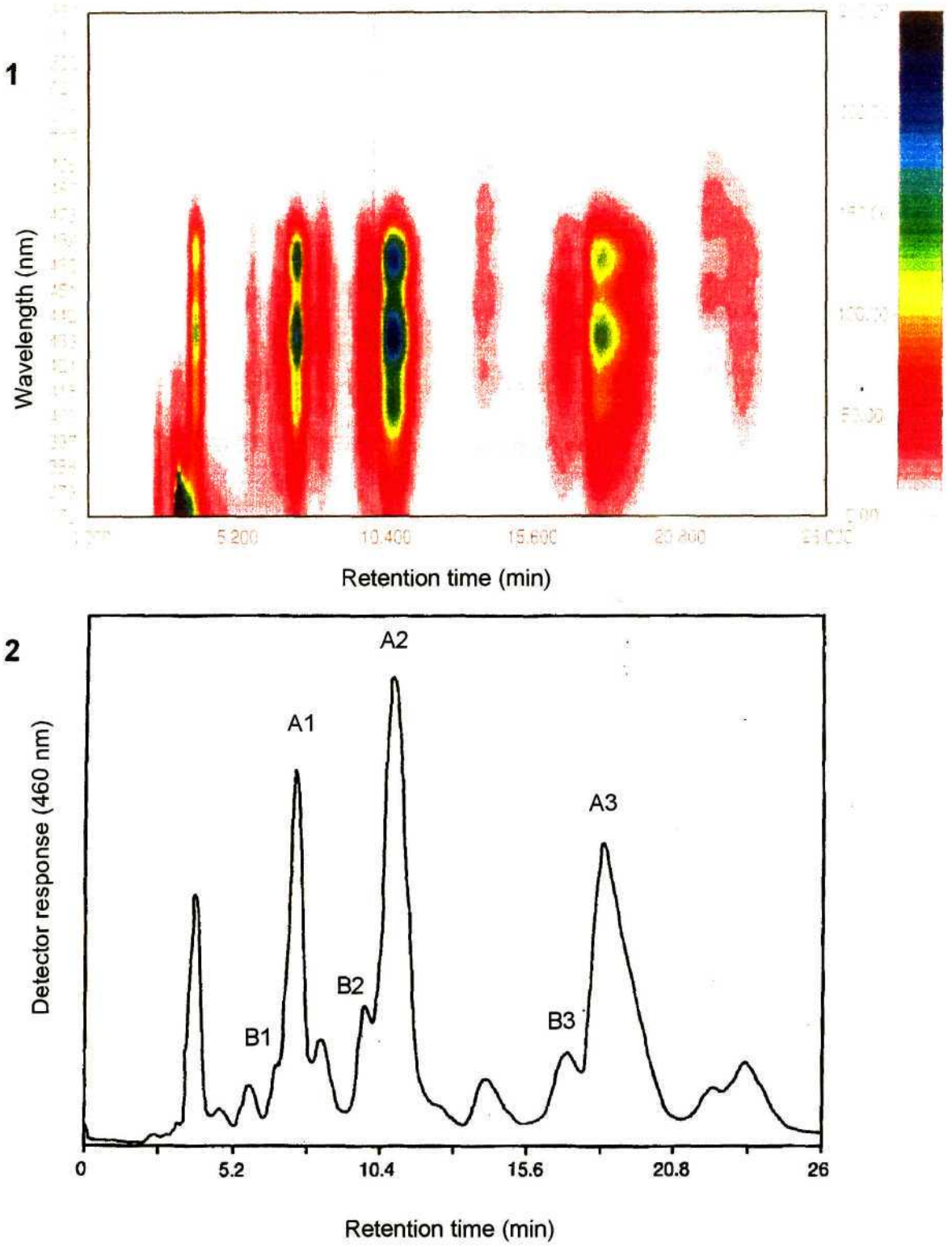


Figure 3.6 Reversed-phase HPLC (1) isoplot and (2) chromatogram at 460 nm of unsaponified extracts of mature Valencia flavedo (colour grade 1). Peaks: A, 9-Z-violaxanthin; B, β-citraurin

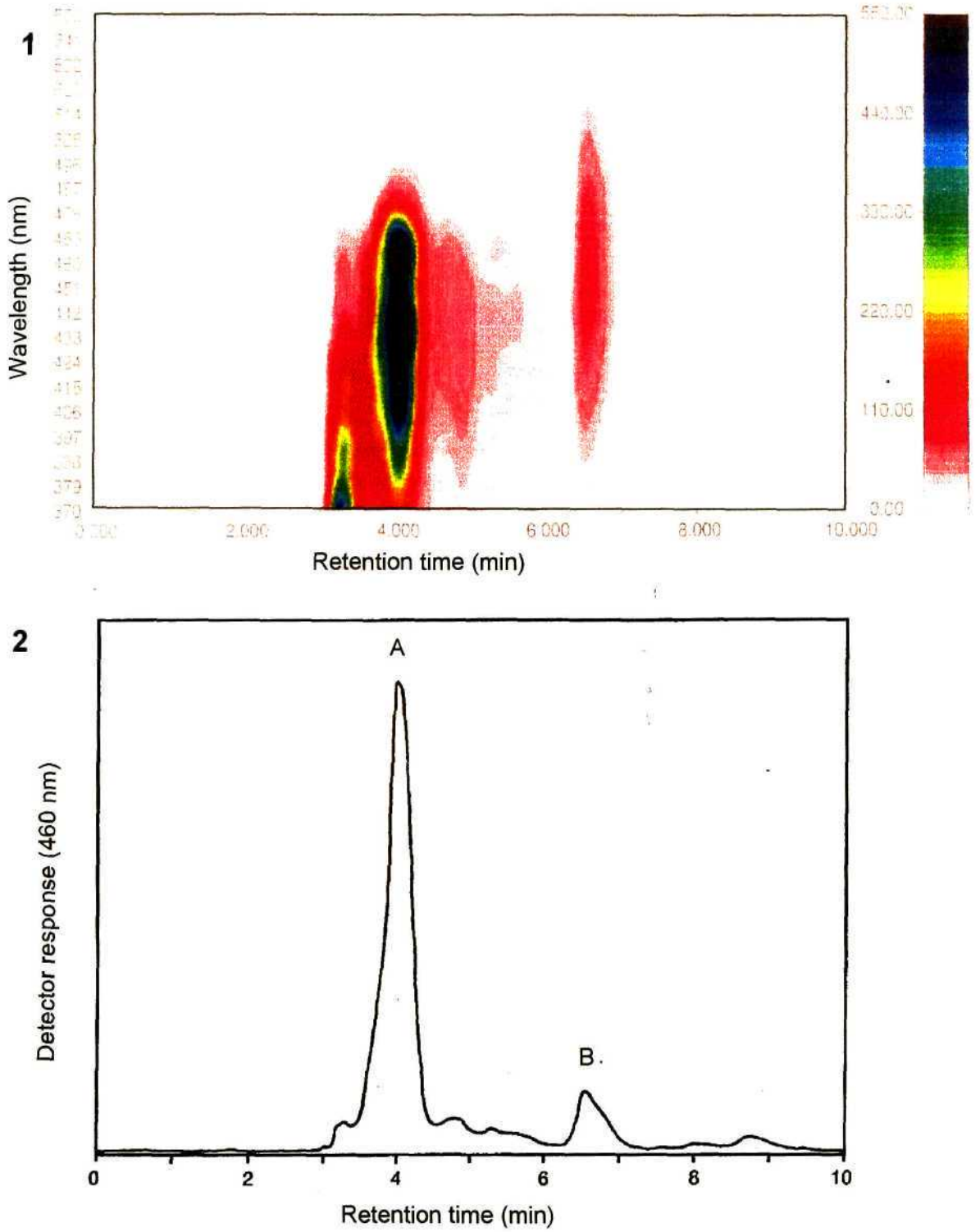
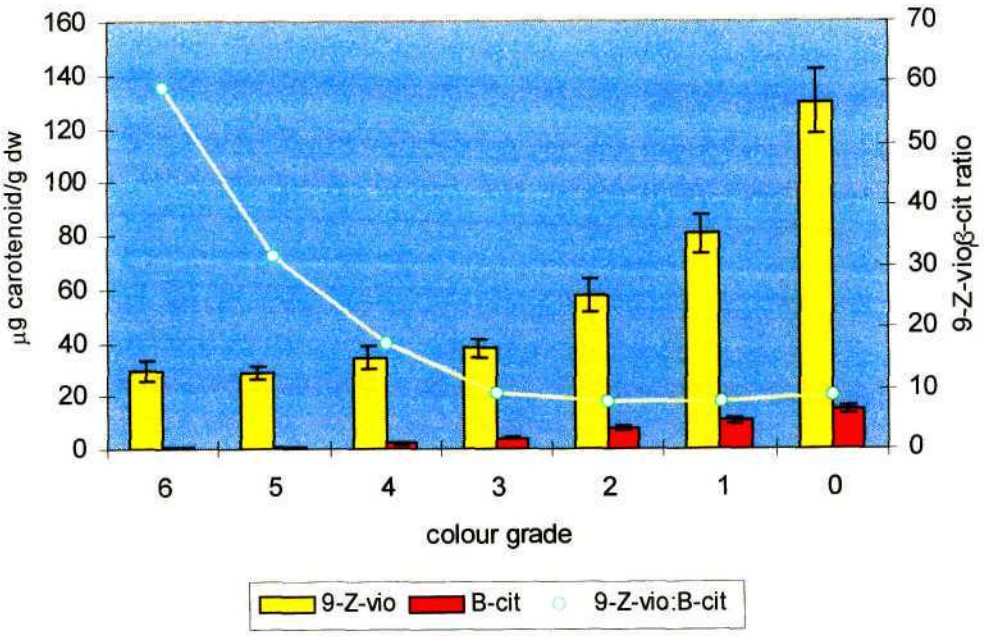


Figure 3.7 Reversed-phase HPLC (1) isoplot and (2) chromatogram at 460 nm of saponified extracts of mature Valencia flavedo (colour grade 1). Peaks: A, 9-Z-violaxanthin; B, β-citraurin

1



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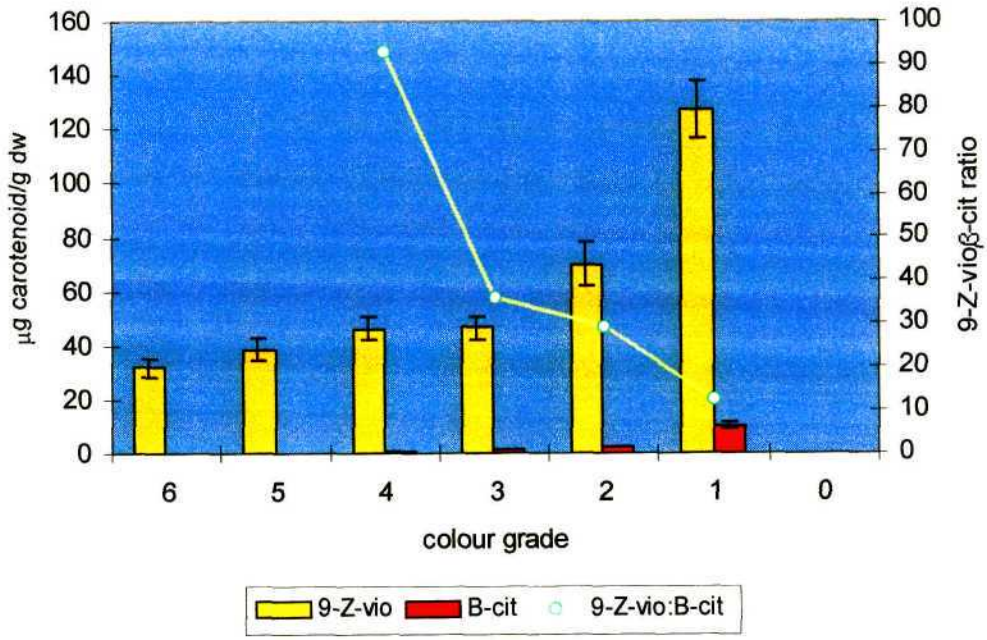


Figure 3.8 Quantification of 9-*Z*-violaxanthin and β-citraurin, and the 9-*Z*-violaxanthin : β-citraurin ratio in flavedo of mature Navel (1) and Valencia (2) fruit colour-graded using the Outspan blemish standards chart (no. 19; colour). Colour grades: 6, poor; to 1, fully/brightly coloured.

3.3 SUMMARY

1. 9-*Z*-Violaxanthin and β-citraurin were identified as two important colour-imparting pigments in the flavedo of *C. sinensis* 'Navel' and 'Valencia'.
2. Both these pigments occur in the flavedo in esterified form.
3. 9-*Z*-Violaxanthin and β-citraurin can be quantified in citrus flavedo by saponification of crude pigment extracts prior to reversed-phase HPLC analysis.
4. Well-coloured fruit display high levels of 9-*Z*-violaxanthin and β-citraurin and also have decreased 9-*Z*-violaxanthin : β-citraurin ratios (<10).

CHAPTER 4

COLOUR DEVELOPMENT AND THE MANIPULATION THEREOF IN *CITRUS SINENSIS*

4.1 INTRODUCTION

During ripening, most carotenogenic fruit undergo an alteration in peel colour from green to a characteristic yellow, orange or red. Colour development involves a change in both hue and chroma of peel colour. In citrus fruit hue changes from green through yellow to orange and the chroma also increases, as orange colour becomes more intense. This period of colour change is characterised by alterations in the pigment content and composition of fruit peel. Gross (1987) distinguishes between two different fruit ripening patterns with regard to carotenoid content. In fruits that synthesize large amounts of carotenoids, carotenoid content initially decreases to a minimum level, after which massive carotenoid accumulation takes place. The minimum in carotenoid content is correlated with the visual phenomenon of colour break and has been reported for 'Cherry' tomato (Laval-Martin *et al.*, 1975), orange (Eilati *et al.*, 1975), Satsuma mandarin (Gross, 1987), Clementine (Farin *et al.*, 1983), 'Dancy' tangerine (Gross, 1981), a mandarin hybrid (Farin *et al.*, 1983), kumquat (Huyskens *et al.*, 1985), persimmon (Gross, 1987), peach (Lessertois and Monéger, 1978) and banana (Gross 1987). Fruit in which the final peel colour is imparted by anthocyanins, such as the sweet cherry (Okombi *et al.*, 1986), red currant and strawberry (Gross, 1987) or fruit in which the yellow colour becomes apparent due to 'unmasking' of carotenoids as chlorophyll degrades, e.g. muskmelon and yellow cherry (Gross, 1987), display a continuous decrease in carotenoid content during fruit ripening.

An understanding of the pigments and regulational factors involved in colour development may afford manipulation of the process, to ultimately improve fruit colour. A number of cultural methods can be employed to enhance citrus fruit colouration, but the effects are usually marginal (Krajewski, 1998). The only commercially utilised method of carotenoid manipulation is that of ethylene degreening. Ethylene is applied pre- or post-harvest (either as ethylene gas or as Ethrel® spray/dip), and brings about colour improvement through the degradation of chlorophyll and accumulation of

carotenoids. The term 'degreening' is therefore misleading, as the development of good colour hinges not only on removal of green hue, but also the evolution of bright orange hue and chroma. Although widely utilised, ethylene degreening may lead to certain post-harvest problems that are usually associated with accelerated maturation. These include advanced decay, loss of fruit buttons and shortened shelf-life (Krajewski, 1998). Thus there is scope for the development of colour manipulation techniques that produce excellent colour with reduced post-harvest decay problems.

The process of colour development in *C. sinensis* 'Navel' and 'Valencia' was investigated by monitoring changes in pigment content and composition during fruit development and maturation. Furthermore, the effect of a number of dehydrating agents (alcohols), plant hormones, micro-nutrients and environmental stimuli on carotenoid content and therefore colour development were investigated.

4.2 RESULTS

4.2.1. Pigment identification

In order to ascertain the pigments involved in colour development of *Citrus sinensis,* flavedo pigment content of three distinct colour stages of 'Navel' and 'Valencia' fruit, *viz:* green, yellow and orange were determined with the use of rHPLC. These colour stages will be referred to as stage 1, 2 and 3 respectively. As identical results with regards to pigment composition were obtained in both 'Navel' and 'Valencia' fruit, only results for 'Navel' are presented. The chromatograms as well as a visual representation of the corresponding colour stage are illustrated in Fig. 4.1.

The principal pigments identified in green, immature flavedo (stage 1) were the xanthophylls *E*-neoxanthin, *E*-violaxanthin, *E*-antheraxanthin, lutein, zeaxanthin; chlorophyll *a* and *b*, and α- and *E*-β-carotene. This pigment distribution pattern is similar to that observed in foliar tissue and the very bright green colour of the flavedo can be attributed to high levels of the green pigments, chlorophyll *a* and *b*. Yellow-coloured fruit (stage 2) had just reached colour-break and visually the fruit displayed a pale lime-yellow colour. rHPLC of flavedo extracts of stage 2 fruit reveal a much reduced total pigment level; the free xanthophylls and carotenes present in immature,

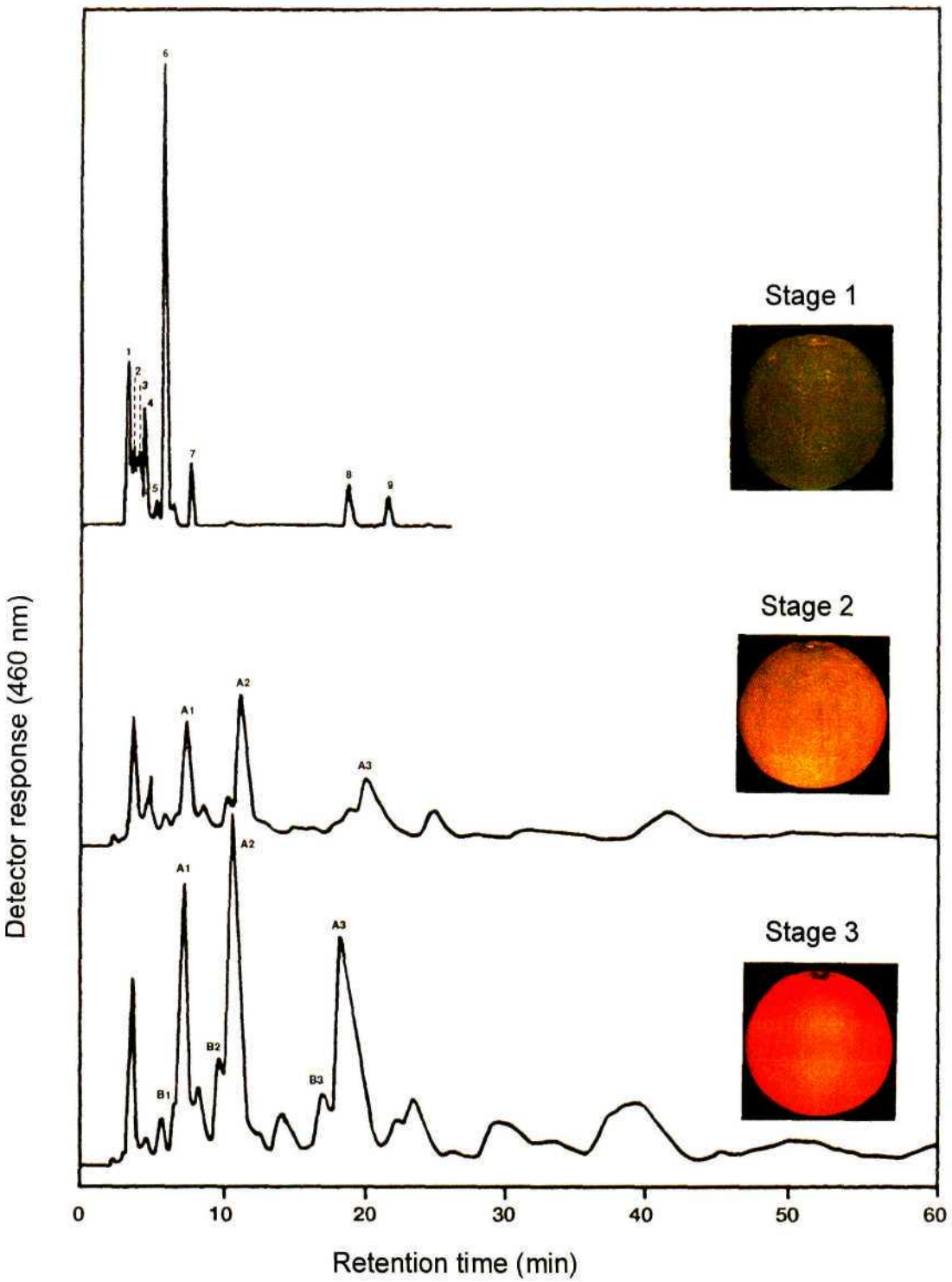


Figure 4.1 Reversed-phase HPLC chromatograms of crude pigment extracts of three distinct colour stages of Navel flavedo. Stage 1, green, immature; stage 2, colour break; stage 3, fully coloured. Peaks: 1, *E*-neoxanthin; 2, *E*-violaxanthin; 3, antheraxanthin; 4, lutein; 5, zeaxanthin; 6, chlorophyll *b*; 7, chlorophyll *a*; 8, α-carotene; 9, β-carotene; A1-3, 9-*Z*-violaxanthin acyl esters; B1-3, β-citraurin acyl esters

green flavedo have reached almost imperceptible levels, while chlorophyll *a* and *b* are present at low levels. The emergence of the three principal 9-Z-violaxanthin acyl esters discussed in Chapter 3 can be noted in the chromatogram, but no β-citraurin can be identified in crude extracts at this stage. Brightly coloured, orange flavedo (stage 3) is characterised by an increase in the three principal 9-Z-violaxanthin acyl esters. The β-citraurin acyl esters discussed in Chapter 3 are also identifiable in the chromatogram (also refer to Fig. 3.6).

4.2.2 Pigment changes during colour development

An analysis of the pigments present in different colour stages of citrus fruit therefore indicate that a decrease in the green chlorophylls and an increase in the yellow and red carotenoids are responsible for the visual change from green through yellow to orange.

In order to examine the role of these individual pigments in colour development more closely, the changes in individual colour-imparting pigments were determined over the full colour developmental period. Similar trends were observed during the 1995/6 period and only results for 1996 are presented.

4.2.2.1 Chlorophyll

The changes in total chlorophyll content of 'Navel' and 'Valencia' flavedo during colour development were determined by rHPLC and are illustrated in Fig. 4.2. In both 'Navel' and 'Valencia' chlorophyll content initially remains at a steady state level, after which a rapid degradation of chlorophyll occurs. Chlorophyll degradation in 'Navel' commences at approximately 180-190 days after full bloom (DAFB), whereas chlorophyll levels in 'Valencia' remain steady until 250 DAFB. Rapid chlorophyll degradation can be visually correlated with the initial loss of dark green flavedo colour and concomitant yellowing of the peel that precedes colour break in citrus. The approximate time of visual colour break is indicated in Fig. 4.2. Colour break occurs ± 210 DAFB in Navel, and ± 270 DAFB in 'Valencia' and chlorophyll levels are below 50 µg/g fresh weight at the time of colour break. It is interesting to note that all processes regarding the degradation of chlorophyll take place approximately 8 weeks later in 'Valencia' than it does in 'Navel'; this includes initial degradation, time of colour

break and time of attaining minimum chlorophyll levels. No regreening was observed in either cultivar.

4.2.2.2 Total carotenoids

In Chapter 3 it was shown that total carotenoid concentration contributes to final colour of citrus. The changes observed in total carotenoid content in 'Navel' and 'Valencia' during colour development were therefore monitored and the results are illustrated in Fig. 4.3. In both 'Navel' and 'Valencia' carotenoid levels initially decrease to a minimum level whereafter massive carotenoid accumulation occurs. Minimum carotenoid levels were 39.9 ± 5.8 µg β-carotene equivalents (eqvs)/g fresh weight (fw) and 39.1 ± 8.1 µg β-carotene eqvs/g fw for 'Navel' and 'Valencia' respectively, and in both cases the minimum in carotenoid level was correlated with visual observations of colour break. Total carotenoids increased almost linearly in flavedo of both cultivars after colour break and this time frame corresponds to the change in visual colour from yellow-green to bright yellow to an eventual bright orange. Carotenoid levels increase for approximately 12 weeks after colour break. Total carotenoid content was found to be higher in fully coloured 'Valencia' peel when compared to 'Navel'; 206 ± 16.2 µg/g fw as opposed to 164 ± 10.1 µg/g fw. Similar to results obtained with chlorophyll levels (section 4.2.2.1) all aspects of colour development with regards to total carotenoid content occurred 8 weeks later in 'Valencia' when compared to 'Navel'.

4.2.2.3 9-Z-Violaxanthin acyl esters

In Chapter 3 it was established that 9-*Z*-violaxanthin and β-citraurin acyl esters are the principal pigments involved in visual colour of citrus flavedo. Changes that occurred in the levels of three main 9-*Z*-violaxanthin acyl esters (A1, A2 and A3) are illustrated in Fig. 4.4. β-Citraurin cannot be readily identified in crude extracts and saponification prior to rHPLC is required to quantify changes in the levels of this red pigment (section 3.2.2). The accumulation of 9-*Z*-violaxanthin acyl esters commences 3-4 weeks before colour break and levels increase rapidly after colour break. At the time of colour break the concentration of the individual compounds are comparable in 'Navel' and 'Valencia', whereas higher levels of all three compounds are detected in 'Valencia' at full colour development when compared to 'Navel'. As with total carotenoid level, the level of 9-*Z*-violaxanthin acyl esters increase for approximately 12 weeks after colour break.

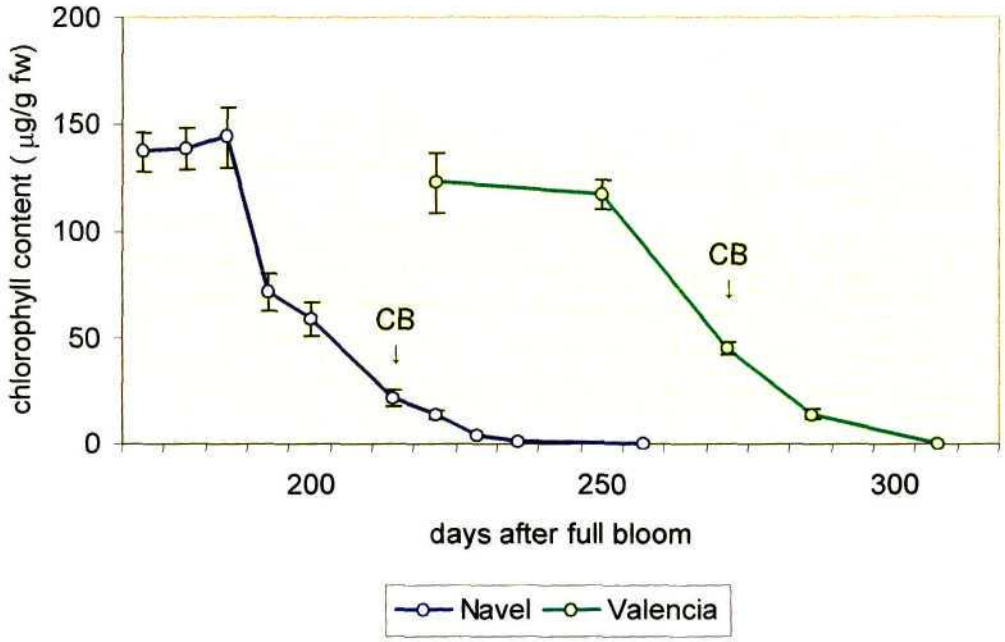


Figure 4.2 Total chlorophyll content (µg/g fw) in the flavedo of Navel and Valencia during the course of colour development. The time of visual colour break (CB) is indicated. Values represent the mean ± SE of 4 replicate fruits.

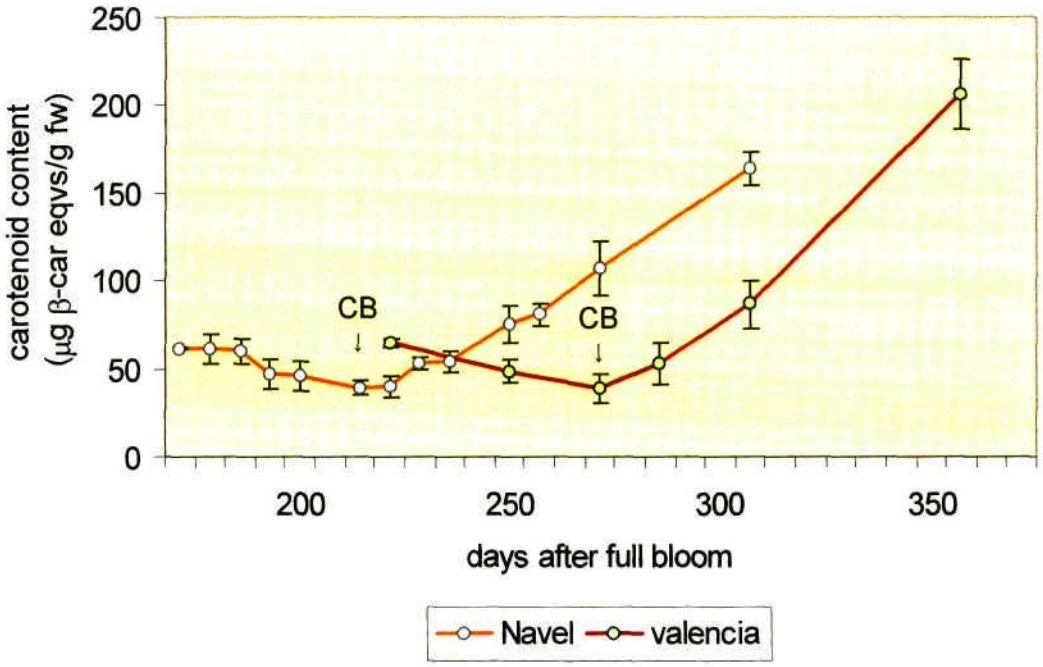


Figure 4.3 Total carotenoid content (µg/g fw) in the flavedo of Navel and Valencia during the course of colour development. The time of visual colour break (CB) is indicated. Values represent the mean ± SE of 4 replicate fruits.

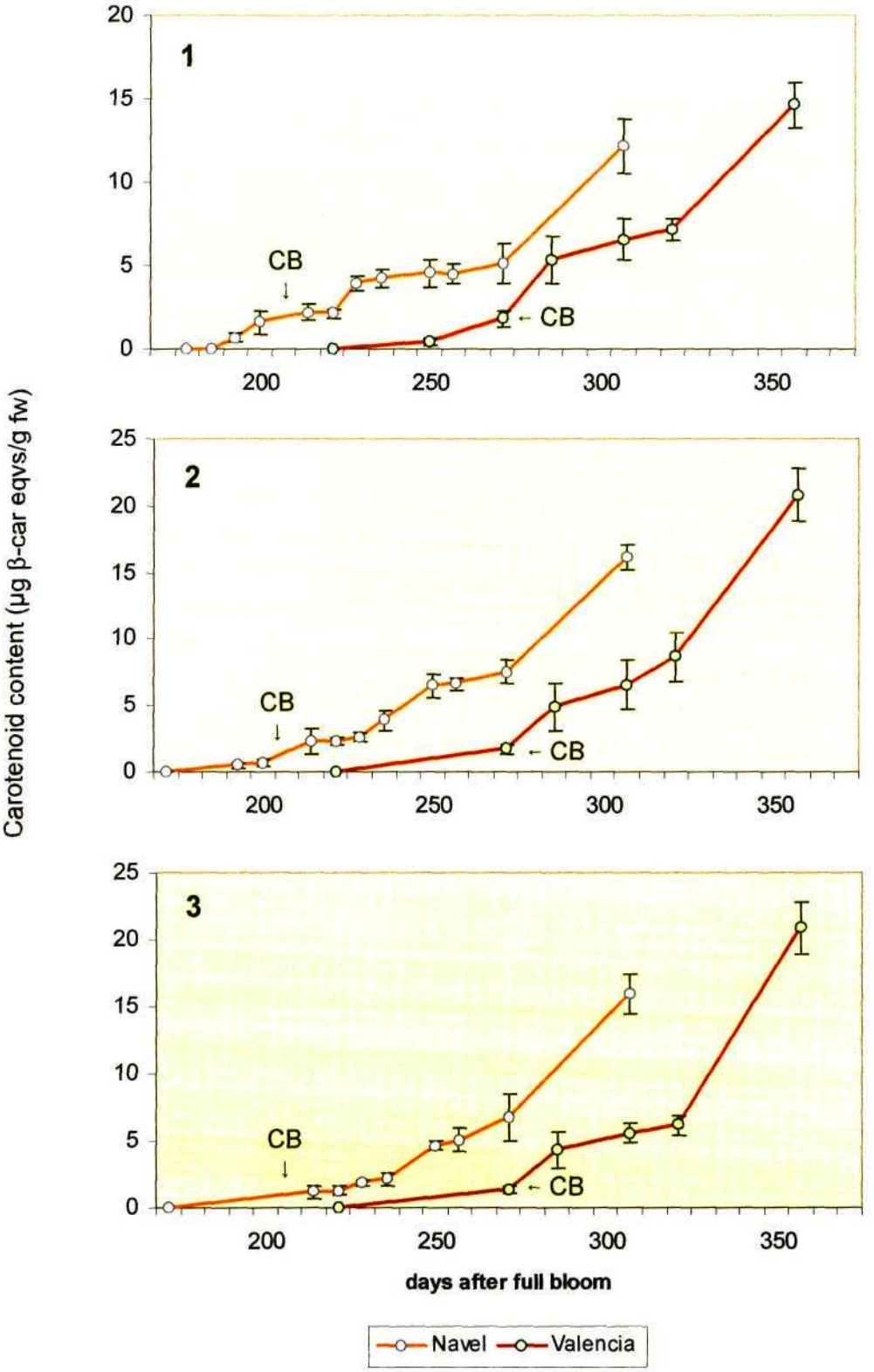


Figure 4.4 Content (µg/g fw) of 9-*Z*-violaxanthin acyl esters designated A1 (1), A2 (2) and A3 (3) (Chapter 3) in the flavedo of Navel and Valencia. The time of visual colour break (CB) is indicated. Values represent the mean ± SE of 4 replicate fruits.

4.2.3 Manipulation of colour development

The effect of a number of alcohols, plant hormones, micronutrients and low temperature on total carotenoid content of flavedo discs and where indicated, whole fruit, was examined. Knowledge of agents that elicit a change in carotenoid content may prove useful in the manipulation of fruit colour commercially.

4.2.3.1 Alcohol treatment

Ethanol is known to enhance fruit ripening and lycopene accumulation in tomatoes at low concentrations, although high concentrations inhibited these responses (Bieulieu and Saltveit, 1997). As part of a pilot study to determine the effect of alcohol treatment on visual colour development of citrus, whole 'Navel' fruit harvested both before and after colour break, were exposed to aqueous solutions of methanol, ethanol, propan-(1)-ol and butan-(2)-ol at a concentration of 10% (v/v) and incubated at room temperature for two weeks. Fruit colour was rated visually with the use of the Outspan colour chart. This pilot study revealed that alcohol treatment had no effect on visual colour development of fruit harvested before colour break had occurred (data not shown). However, fruit harvested after colour break exhibited an improvement in colour of 1 to 2 colour grades following treatment with ethanol and butan-(2)-ol. Methanol and propan-(1)-ol treatment had no visible effect on flavedo colour of 'Navel'.

As the effect of alcohol on flavedo colour is likely concentration dependant, whole 'Navel' fruit were exposed to aqueous solutions of ethanol and butan-(1)-ol at concentrations of 5, 10, 20 and 30% (v/v) (40 °C) and incubated for four weeks at room temperature. Total carotenoid content was determined spectrophotometrically at 0, 1, 2 and 4 weeks and the results are illustrated in Fig. 4.5 and 4.6. As shown in Fig. 4.5, 30% ethanol induced a rapid accumulation of carotenoids within 7 days, and carotenoid levels were maintained at a higher level than that of untreated fruit for the duration of the experiment. At concentrations of 5 and 10%, ethanol was less effective in increasing flavedo carotenoid content. At 5%, butan-(2)-ol induced rapid accumulation of carotenoids and levels were sustained for the 28 day duration of the experiment (Fig. 4.6). At higher concentrations butanol was much less effective and routinely caused rind damage to the fruit, particularly in the navel area. The effect of ethanol and butan-(2)-ol treatment on visual colour of whole fruit and typical rind damage observed with butanol treatment is illustrated photographically in Fig. 4.7.

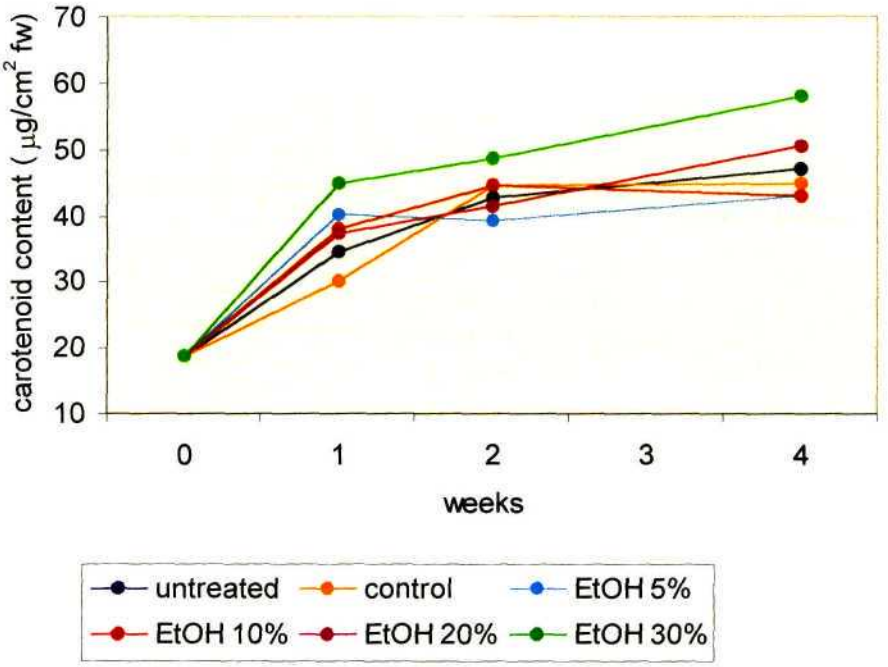


Figure 4.5 Response of detached Valencia fruit to varying concentrations of ethanol (EtOH) applied for 4 min at 40 °C. Fruit were harvested and treated after colour break and carotenoid content determined spectrophotometrically. SE (diff) = 2.9 at 0 weeks, 5.3 at 1 week, 3.6 at 2 weeks and 5.7 at 4 weeks.

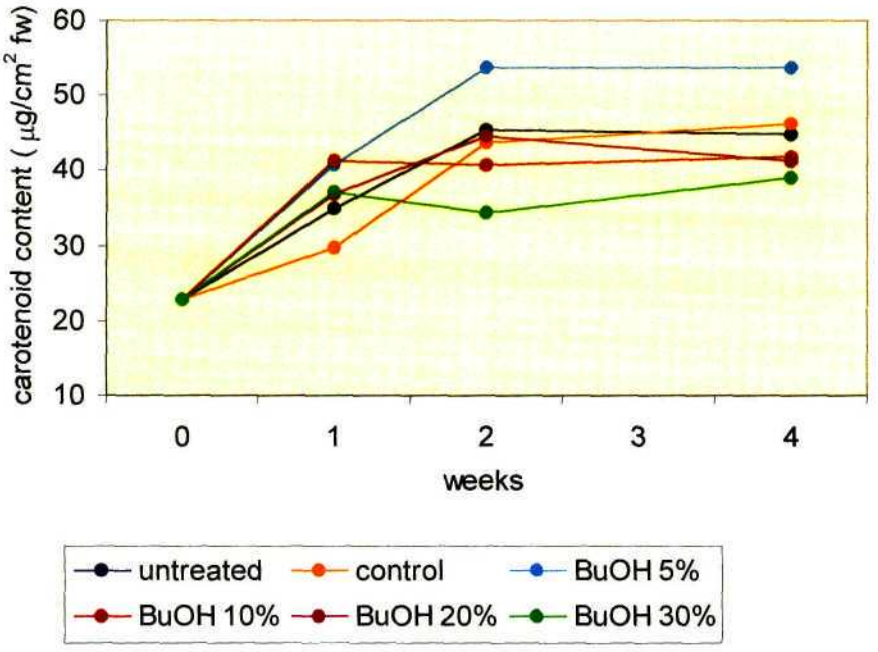
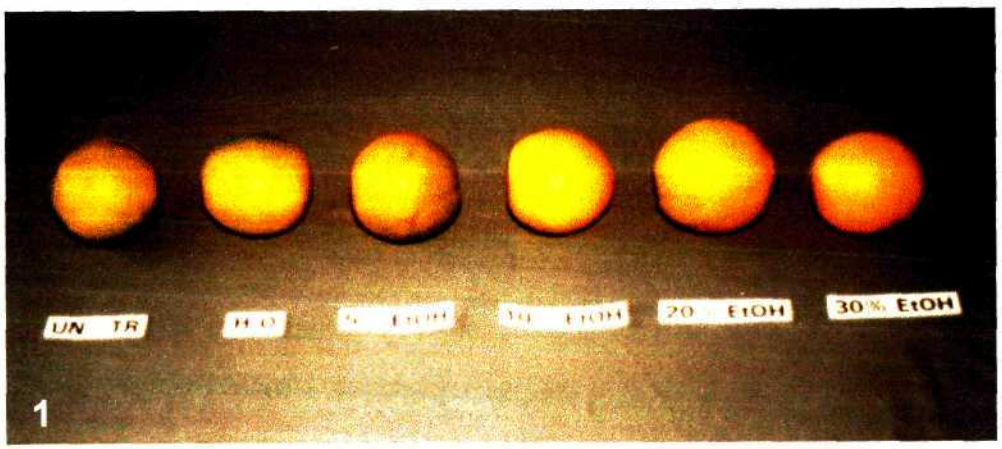
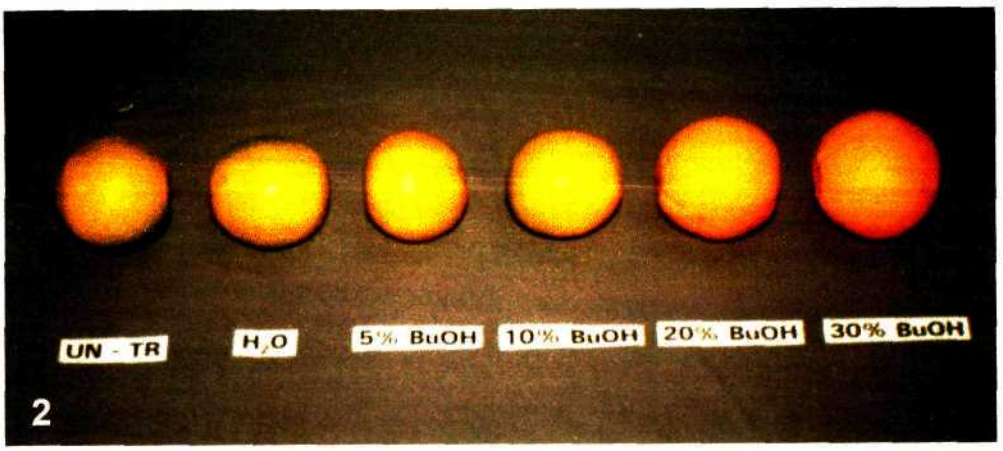


Figure 4.6 Response of detached Valencia fruit to varying concentrations of butanol (BuOH) applied for 4 min at 40 °C. Fruit were harvested and treated after colour break and carotenoid content determined spectrophotometrically. SE (diff) = 2.9 at 0 weeks, 5.6 at 1 week, 5.9 at 2 weeks and 5.1 at 4 weeks.





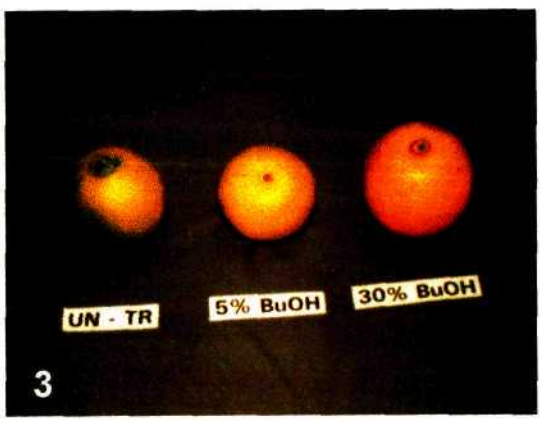


Figure 4.7 The effect of varying concentrations of (1) ethanol (EtOH) and (2) butanol (BuOH) 4 weeks after treatment on visual colour of Navel flavedo. Fruit were harvested after colour break and incubated in aqueous solutions of alcohol (40 °C) for 4 min. (3)Typical rind damage observed with butanol treatment.

Treatment of whole fruit is often cumbersome and the use of a flavedo disc system to study the effect of various treatments on carotenoid content was therefore evaluated. Huff (1983, 1984) showed that flavedo discs could be used successfully to determine the effect of nitrogen and sucrose treatment on de- and regreening of citrus peel. The effect of ethanol and butan-(2)-ol treatment on carotenoid content (expressed as percentage change) is demonstrated in Fig. 4.8. Discs were treated for 2 h, removed from the alcohol and incubated in phosphate buffer for the remainder of the 96 h time period to eliminate extreme dehydration and tissue damage associated with continuous alcohol exposure. Ethanol at 10 and 20% (v/v) resulted in significantly higher carotenoid levels when compared to control discs after 24 h. After 96 h, discs treated with all concentrations of ethanol displayed higher carotenoid levels than control discs. Thus, ethanol concentrations as high as 20% (v/v) did not inhibit carotenoid accumulation in flavedo discs. Discs treated with butanol at 2.5 and 10% (v/v) exhibited significantly higher carotenoid levels after 96 h when compared to control discs. Higher concentrations of butanol (20%; v/v), however, caused a reduction in carotenoid content. These results are in accordance with those obtained in whole fruit and indicate that high concentrations of ethanol (30%; v/v) and lower concentrations of butanol (5-10%; v/v) increase carotenoid accumulation, whereas high concentrations of butanol cause a reduction in carotenoid content. The result further illustrates that flavedo discs are an effective system for the evaluation of treatments that may affect carotenoid accumulation and consequently colour development.

4.2.3.2 Plant hormones

The role of plant hormones in colour development has been partially established through correlation of endogenous hormone levels with flavedo colour development (Monselise and Goren, 1978); and through exogenous application of hormones to citrus peel (Lewis and Coggins, 1964; Rasmussen *et al.*, 1973; Wheaton and Stewart, 1973). The effect of indolebutyric acid (IBA), gibberellic acid (GA_3), abscisic acid (ABA) and jasmonic acid (JA) on total carotenoid content and therefore resultant colour change of flavedo peel discs was investigated and the results are illustrated in Fig. 4.9. Exposure of discs to IBA at 0.1 mM resulted in a significant reduction in carotenoid content after 24 h when compared to discs exposed to distilled water (control).

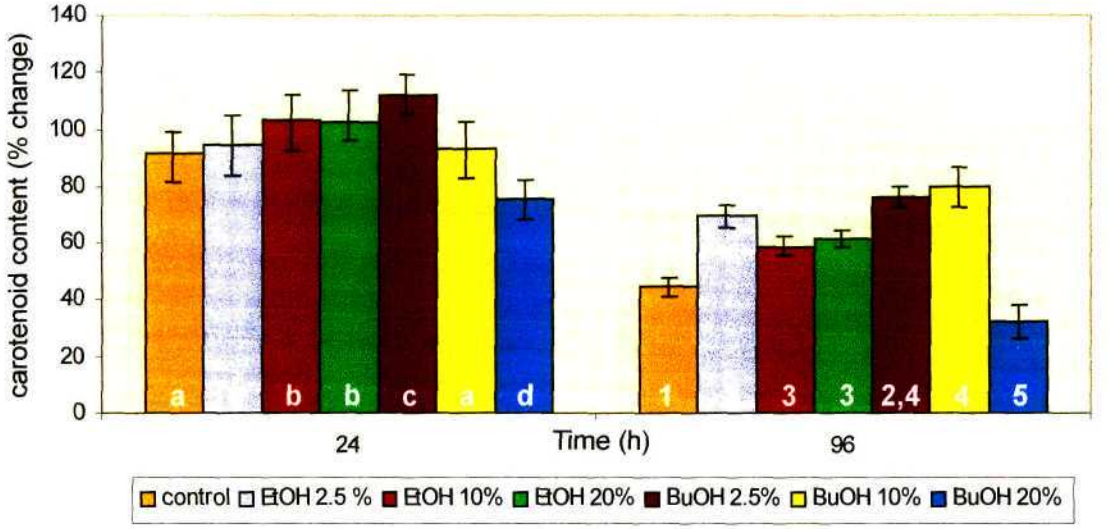


Figure 4.8 Percentage change in carotenoid content of 'Valencia' flavedo discs treated with varying concentrations of ethanol (EtOH) and butanol (BuOH). Carotenoid content was determined spectrophotometrically at 0, 24 and 96 h. 0 h (100%) represents a carotenoid content of 11.72 ± 0.37 $\mu g/cm^2$. Values are means of 12 discs ± SE. Bars of different letters (24 h) and numbers (36 h) are significantly (95%) different. $LSD_{0.05}$ = 7.75 at 24 h; 6.97 at 96 h.

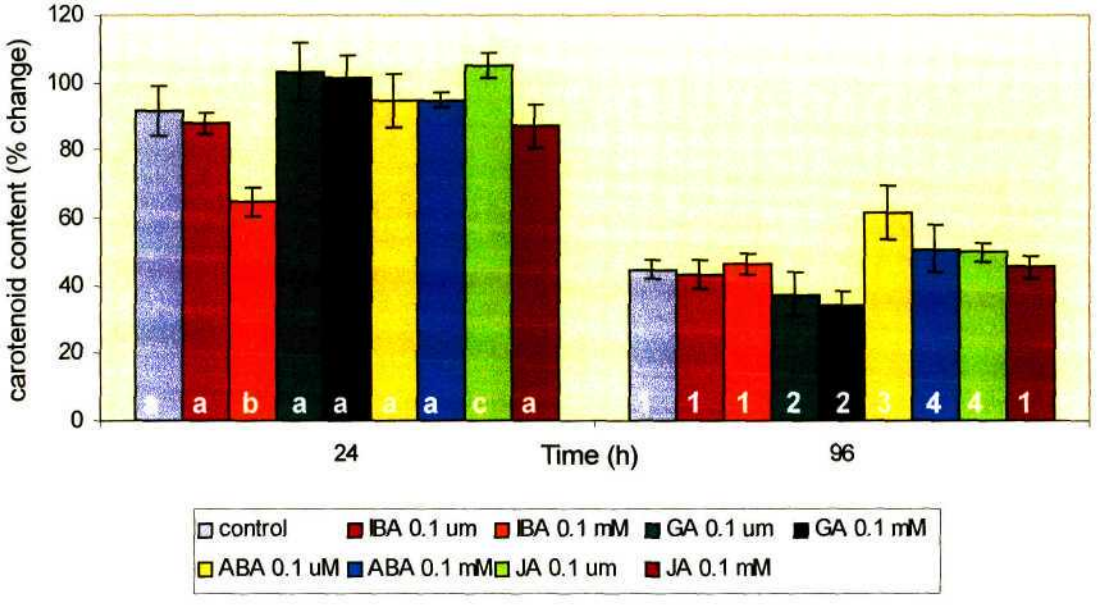


Figure 4.9 Percentage change in carotenoid content of 'Valencia' flavedo discs treated with varying concentrations of indolebutyric acid (IBA), gibberellic acid (GA),abscisic acid (ABA) and jasmonic acid (JA). Carotenoid content was determined spectrophotometrically at 0, 24 and 96 h. 0 h (100%) represents a carotenoid content of 11.72 ± 0.37 $\mu g/cm^2$. Values are means of 12 discs ± SE. Bars of different letters (24 h) and numbers (96 h) are significantly (95%) different. $LSD_{0.05}$ = 11.26 at 24 h; 6.03 at 96 h.

JA at 0.1 µM resulted in carotenoid levels higher than those reported at the start of the experiment after 24 h, indicating that JA not only delays carotenoid loss, but enhances carotenoid accumulation. No differences between ABA-treated discs and controls are noted at 24 h, however, after 96 h, discs treated with ABA (0.1 µM) display significantly higher carotenoid levels than control discs. Discs treated with GA3 have significantly lower carotenoid levels after 96 h.

4.2.3.3 Micro-nutrients

Nutrient status of citrus trees have been reported to impact on colour development. Trees fertilised with high levels of nitrogen often grow excessively vegetative and are characterised by poorly coloured fruit. Huff (1983) showed that high nitrogen levels in a flavedo disc system are inhibitory to degreening, carotenoid accumulation and therefore colour development. Limited information is however available on the effect of micro-nutrients on carotenoid accumulation and colour development. The effect of cobalt (Co), nickel (Ni), molybdenum (Mo) and tungsten (W) on carotenoid content was therefore investigated and the results are illustrated in Fig. 4.10. No significant differences were noted after treatment with a combination of Co and Ni after 24 or 96 h. Disc exposure to Mo and W resulted in no significant differences after 24 h, but after 96 h the carotenoid level of discs treated with Mo (10 µM) and W (1 and 10 µM) were significantly higher than that of the control discs.

4.2.3.4 Temperature

Brilliantly coloured citrus fruit are usually associated with production areas that exhibit a distinct seasonal cold period (Reuther and Rios-Castaño, 1969) and it is known that colour development is usually associated with a sudden drop in temperature. The effect of a short, low temperature period (4 °C), followed by incubation at higher temperatures (22 °C) on carotenoid content of citrus flavedo discs was investigated and results are illustrated in Fig. 4.11. A low temperature period of 2, 6 and 10 h resulted in a significant increase in carotenoid content after 24 h. The differences are even more pronounced after 96 h. After 24 h, carotenoid content is higher than that reported at the start of the experiment, indicating that a low temperature period enhances carotenoid accumulation in flavedo discs, rather than merely delay degradation. No differences were noted between treatments, suggesting that the duration of the treatment is of secondary importance.

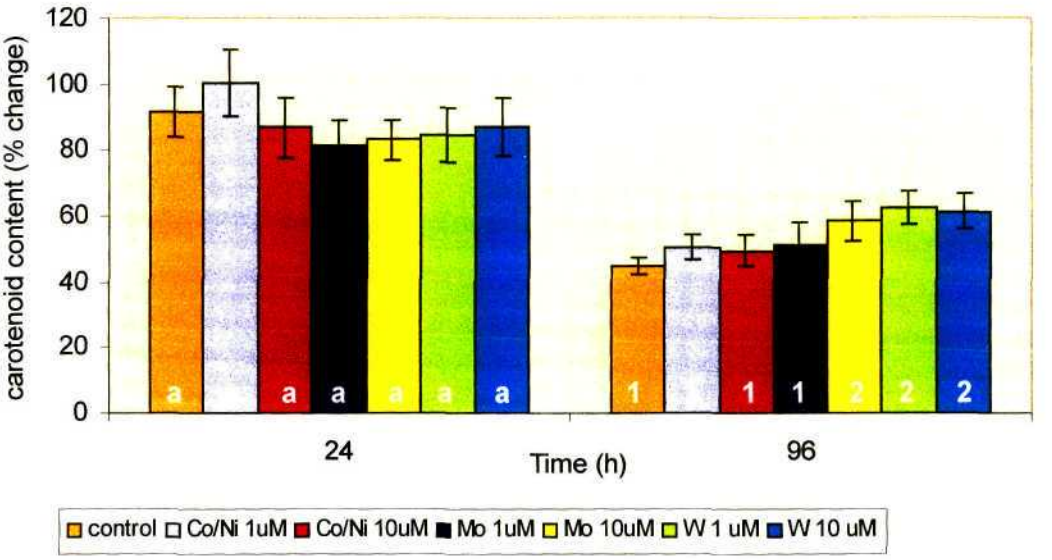


Figure 4.10 Percentage change in carotenoid content of 'Valencia' flavedo discs treated with varying concentrations of cobalt and nickel (Co/Ni), molybdenum (Mo) and tungsten (W). Carotenoid content was determined spectrophotometrically at 0, 24 and 96 h. 0 h (100%) represents a carotenoid content of 11.72 ± 0.37 μg/cm^2. Values are means of 12 discs ± SE. Bars of different letters (24 h) and numbers (96 h) are significantly (95%) different. $LSD_{0.05}$ = 12.01 at 24 h; 8.59 at 96 h.

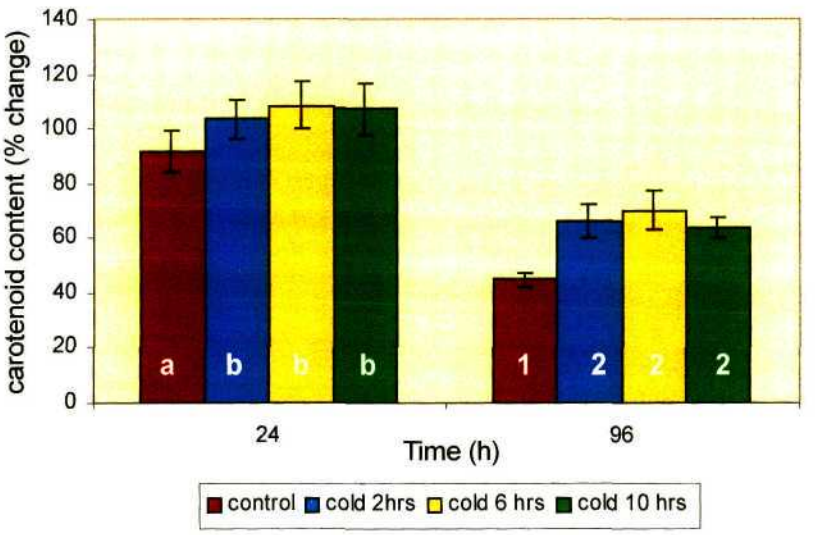


Figure 4.11 Percentage change in carotenoid content of 'Valencia' flavedo discs exposed to a temperature of 4 °C for a time period of 2, 6 and 10 h, followed by incubation at 22 °C. Carotenoid content was determined spectrophotometrically at 0, 24 and 96 h. 0 h (100%) represents a carotenoid content of 11.72 ± 0.37 μg/cm^2. Values are means of 12 discs ± SE. Bars of different letters (24 h) and numbers (96 h) are significantly (95%) different. $LSD_{0.05}$ = 8.27 at 24 h; 6.69 at 96 h.

4.3 SUMMARY

1. The signature pigments of green, immature *C. sinensis* flavedo are *E*-neoxanthin, *E*-violaxanthin, antheraxanthin, lutein, zeaxanthin, chlorophyll *a* and *b*, α- and *E*-β-carotene.
2. Colour break in citrus coincides with a minimum in carotenoid levels.
3. After colour break the total level of carotenoids and that of 9-Z-violaxanthin and β-citraurin acyl esters increase dramatically.
4. Carotenoid manipulatory treatments have to be applied post-colour-break in order to be effective.
5. The application of ethanol and butanol after colour break increased carotenoid content of whole fruit and flavedo discs. Optimum concentrations are 20-30 % (v/v) for ethanol and 5 % (v/v) for butanol.
6. Exogenous application of GA_3 reduced carotenoid content of flavedo discs, whereas JA and ABA increased carotenoid levels.
7. The micro-nutrients Mo and W increased carotenoid content of flavedo discs.
8. A short, low temperature period (4 °C), followed by higher temperatures (22 °C) increased carotenoid content of flavedo discs.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION

Colour expression in citrus is a cultivar characteristic affected by climate and environment and can to some extent be manipulated by cultural practice (Goldschmidt, 1988). Although visual colour is not always an indication of internal maturity, it is probably the single most important external quality parameter determining consumer acceptance. Therefore, competition between growers to secure niche markets has fuelled efforts to produce quality fruit that is uniformly of good colour.

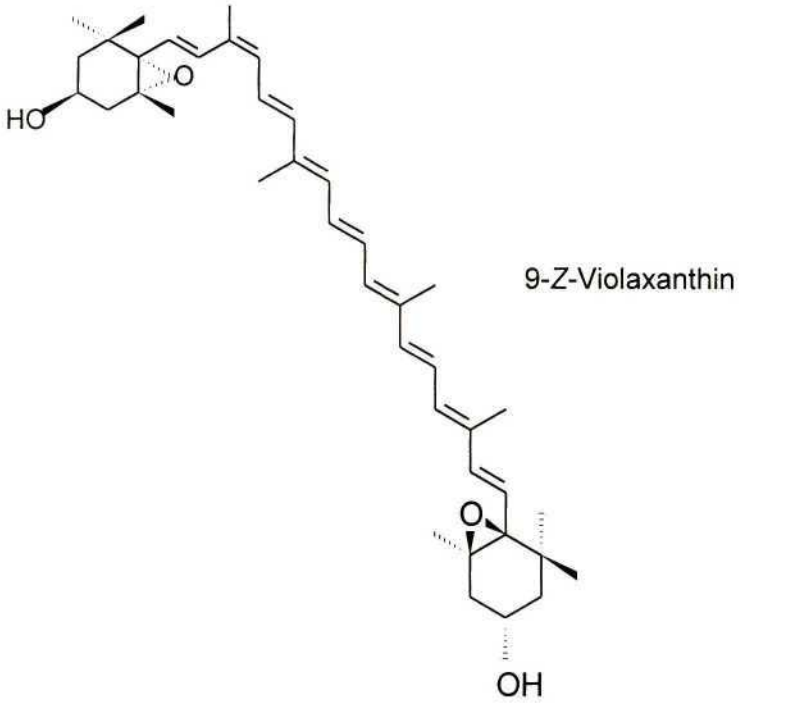
Visual fruit colour in the South African citrus industry is routinely measured with the use of the Outspan Colour Chart, which prescribes citrus export and local market grading regulations (Fig. 2.2). Colour charts afford an easy, nondestructive method of colour measurement, but it should be noted that colour evaluation is subjective, and that external factors such as background light quality may influence the perceived colour. Portable colour-measuring instruments make possible a more objective notation of colour, although it may be difficult for an individual to visualise colour expressed as a point in a three-dimensional space. Furthermore, these instruments are costly, which may preclude their use in a packhouse environment. An intrinsic problem of both colour-measurement techniques employed by the citrus industry, is that neither method provides information on the pigment content and composition of citrus flavedo. As it is after all the pigments that afford visual colour, it was reasoned that a basic knowledge and understanding of the pigments involved in colour development are essential before any attempt at colour manipulation is undertaken. In an endeavour to address these issues, the present study provides the biochemical basis for colour as an aesthetic, visual quality in *C. sinensis*. It is shown that an increase in 9-*Z*-violaxanthin and β-citraurin content, concomitant with a decline in the 9-*Z*-violaxanthin : β-citraurin ratio is associated with increased intensity of flavedo colour and can be used to assess colour of mature fruit as a quality parameter. Furthermore, the process of colour development as a function of pigment change over time was determined. It was illustrated that colour break in *C. sinensis* coincides with

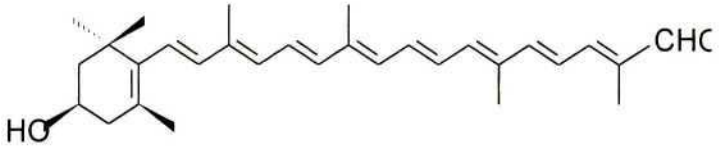
a minimum in carotenoid and total pigment content. The period prior to colour break is characterised by a decline in chloroplastic pigments, whereafter a massive increase in xanthophyll acyl esters, particularly 9-*Z*-violaxanthin, occurs. Finally, in an attempt to manipulate carotenoid content and resultant colour development, the effect of dehydrating agents (e.g. alcohols), plant hormones, micro-nutrients and low temperature on flavedo carotenoid content was examined. It was shown that the micro-nutrients Mo and W, and the plant hormones ABA and JA may be utilised as pre-harvest sprays to improve flavedo colour. Furthermore, post-harvest application of the alcohols ethanol and butanol can improve visual fruit colour. The possible commercial application of the abovementioned manipulatory techniques should be investigated in more detail.

5.1.2 A biochemical basis of colour as an aesthetic quality in *Citrus sinensis*

The distribution pattern of carotenoids in citrus varies greatly and is responsible for distinctive, varietal peel colour. Pale-coloured citrus fruit such as white grapefruit, lemon and pummelo generally accumulate large amounts of the colourless carotenoid precursors phytoene and phytofluene (Gross, 1983,1987). Although the coloured carotenoid fraction is very low, these fruit nevertheless display a typical citrus accumulation pattern with respect to xanthophyll accumulation. 9-*Z*-, 13-*Z*- and di-*Z*-violaxanthin were shown to be the predominant xanthophylls. The combination of predominantly colourless and yellow pigments is responsible for the pale colour of the flavedo. Lycopene and β-carotene accumulate in the flavedo of pink and red grapefruit and pummelo and are responsible for the pink-orange tinge of the flavedo (Gross, 1983). The present study indicates that the principal colour-imparting pigments in *C. sinensis* 'Navel' and 'Valencia' flavedo are the yellow-coloured xanthophyll 9-*Z*-violaxanthin and the red C_{30} apocarotenal β-citraurin. The structures of these pigments are illustrated in Fig. 5.1.

It was further shown that both pigments accumulate in the flavedo of 'Navel' and 'Valencia' in esterified form. Saponification prior to rHPLC analysis ensures that the two major colour-imparting pigments are well resolved for quantification purposes. The absorption spectrum of the pigments were unaffected by saponification, indicating that





β-Citraurin

Figure 5.1 The structure of 9-*Z*-violaxanthin and β-citraurin, the principal colour-imparting carotenoids in *C. sinensis* flavedo

esterification does not alter the visual properties, and therefore perceived colour, of carotenoids. While the identity of the esters was not determined in the present study, β-citraurin myristate has been isolated from the peel of 'Marsh Seedless' grapefruit (Philip, 1973a) and the laurate esters of β-cryptoxanthin, 9-*Z*-violaxanthin and β-citraurin have been detected in flavedo of 'Valencia' orange (Philip, 1973b). Physiological significance may be attributed to the fact that esterification increases the lipophilic character of the carotenoids, making possible their accumulation in

chromoplast-localised globuli (Eilati *et al.*, 1972). Acylation has also been shown to increase the stability of pigments (Camara and Moneger, 1978).

9-*Z*-Violaxanthin is the most abundant carotenoid in *C. sinensis* flavedo (Molnár and Szabolcs, 1980; Gross, 1987). As a yellow pigment it contributes mainly to the background colour of orange fruit flavedo. β-citraurin has been reported to occur at low concentrations (1% of total carotenoids) in 'Valencia' flavedo (Molnár and Szabolcs, 1980). However, elevated concentrations have been reported in the flavedo of brightly coloured mandarin (8%) (Gross, 1981), 'Dancy' tangerine (8%) (Gross, 1981) and a mandarin hybrid (26%) (Farin et al., 1983). The present study (Oberholster *et al.*, 2000, Appendix A) indicates that good colour development in citrus flavedo occurs with an increase in the concentration of both colour-imparting carotenoids concomitant with a decrease in 9-Z-violaxanthin : β-citraurin ratio from >50 to <10. Comparison of the pigment content and composition of fruit of different colour grades depicted in Fig. 2.2 (Outspan colour chart) allows one to correlate pigment levels with visual colour. Poorly coloured fruit (grade 5-6) display high levels of chlorophyll, low levels of 9-*Z*-violaxanthin and almost undetectable levels of β-citraurin. Visually the fruit appear greenish as a result of the chlorophyll present, while the pale yellow background colour is brought about by the yellow pigment 9-*Z*-violaxanthin. Fruit of average colour (grade 3-4) contain relatively low levels of both colour-imparting pigments. Green colour was receding due to reduced levels of chlorophyll, while the yellow background colour was becoming more intense. Fruit of good colour (grade 1-2) contained high levels of 9-*Z*-violaxanthin and β-citraurin and, furthermore, exhibited a decline in 9-*Z*-violaxanthin : β-citraurin ratio when compared to fruit of an inferior colour grade. Visually, these changes can be correlated to a change in hue from yellow-orange to a deep orange, concomitant with an increase in the intensity of the colour. It is tempting to suggest therefore that the amount of 9-*Z*-violaxanthin and β-citraurin in citrus flavedo specify chroma (colour intensity) whereas the 9-*Z*-violaxanthin : β-citraurin ratio is responsible for hue or shade perceived. This can be compared to the addition of small amounts of red paint (β-citraurin) to yellow paint (9-Z-violaxanthin), during which hue would change from yellow to orange.

To manipulate carotenogenesis *in vivo* to improve flavedo colour and aesthetic quality of orange fruits, knowledge of the major colour-imparting pigments in flavedo was initially required. Clearly, *in vivo* manipulation of carotenoid content for improved colour rests on an understanding of the biochemistry of the pigments concerned. Nevertheless, the biosynthetic origin of both 9-*Z*-violaxanthin and β-citraurin remain unresolved. It is assumed that 9-*Z*-violaxanthin arises due to isomerization of *E*-violaxanthin *in vivo*. However, 9-*Z*-β-carotene and other *Z*-isomers of β-carotene have been shown to occur in plant tissue (Young and Britton, 1993), and the biosynthetic pathway may therefore occur from 9-*Z*-β-carotene to 9-*Z*-violaxanthin via a series of *Z*-isomers. β-Citraurin, a C_{30} apocarotenoid, is believed to be a degradation product of either zeaxanthin (Yokoyama and White, 1966) or β-cryptoxanthin, the mono-hydroxy derivative of β-carotene (Gross, 1981), although other xanthophylls e.g. *E*-neoxanthin, *E*-violaxanthin and *E*-antheraxanthin could also serve as parent pigments. β-Citraurin may be formed through asymmetric degradation of a C_{10} fragment from one side of the C_{40} parent carotenoid. A similar mechanism is thought to be responsible for the exocentric cleavage of β-carotene in the formation of retinal (van Vliet *et al.*, 1996) and the plant hormone abscisic acid (Schwartz *et al.*, 1997). Taylor and Davies (1974) reported the existence of a group of C_{30} carotenes isolated from bacteria, these include analogs of phytoene, phytofluene, ζ-carotene and neurosporene. Their existence may point to the existence of a separate C_{30} biosynthetic pathway, but these compounds have never been isolated from plants. Xanthophyll cleavage is therefore accepted as the more likely biosynthetic origin of apocarotenoids.

Citrus flavedo is considered an ideal system in which to study the expression of genes/proteins involved in plant stress responses (Sanchez-Ballesta *et al.*, 1999). Richardson and Cowan (1995) showed that the levels of the plant stress hormone ABA reached a maximum coincident with the onset of colour break, and that a subsequent decline in levels of ABA correlated with expression of full colour. These authors further showed that an enzyme system prepared from orange fruit flavedo converted 9-*Z*-neoxanthin to xanthoxal (the aldehyde product of xanthophyll cleavage) and ABA (Cowan and Richardson, 1997). Similar findings on the biosynthetic origin of ABA via dioxygenase-mediated cleavage of 9-*Z*-xanthophylls, have been reported in other plant

tissues in response to stress (Schwartz *et al.*, 1997). The pathway for formation of xanthoxal from 9-*Z*-xanthophylls is now well established and the enzyme responsible for xanthophyll cleavage has been cloned (Cutler and Krochko, 1999). Furthermore, expression of the mRNA for the xanthophyll cleavage enzyme increases in response to water deficit, a stimulus known to induce ABA accumulation (Qin and Zeevaart, 1999). Since the development of colour in *C. sinensis* is exacerbated by low temperature, a cold stress may be the stimulus required for synthesis and accumulation of β-citraurin via dioxygenase-mediated cleavage of the parent xanthophyll. Parry and Horgan (1991) showed that lipoxygenase cleaved 9-*Z*-neoxanthin and *E*-violaxanthin into apocarotenoids in *in vitro* assays. The majority of these compounds were volatile (C_9 to C_{13}) compounds, many of which are important aroma constituents, but others range in size up to C_{30}, e.g. apo-8'-violaxanthal which has been extracted from citrus peel (Molnár and Szabolcs, 1980). Further investigations into citrus colour development should therefore concentrate on cloning and characterization of the proposed dioxygenase enzyme. As a logical first step, a product-precursor relationship between the parent xanthophyll and β-citraurin needs to be established. It might also be possible to clone and characterize the dioxygenase enzyme if mutants deficient in β-citraurin were identified.

5.1.3 Colour development in *Citrus sinensis* and the manipulation thereof

Visual colour break in *C. sinensis* 'Navel' and 'Valencia' appears to be associated with a minimum in total pigment and total carotenoid content of flavedo tissue. The pigment minimum observed at colour break has been shown to occur in a variety of citrus *viz*: Satsuma mandarin (Gross, 1987), Clementine (Farin *et al.*, 1983), 'Dancy' tangerine (Gross, 1981), a mandarin hybrid (Farin *et al.*, 1983) and kumquat (Huyskens *et al.*, 1985). Green, immature flavedo displays a pigment pattern similar to that observed in leaf tissue and the period before colour break is characterised by a decline in all pigments present. Colour break is followed by a massive accumulation of xanthophyll acyl esters, in particular 9-*Z*-violaxanthin acyl esters. Colour break in *C. sinensis* coincides with the conversion of chloroplasts to chromoplasts (Eilati *et al.*, 1975) and the conversion and resultant functional change accounts for the loss of photosynthetic, chloroplastic pigments followed by an increase of chromoplastic pigments.

A variety of treatments, including dehydrating agents (alcohols), plant hormones, micro-nutrients and low temperature were evaluated for their efficacy in increasing carotenoid content and hence improving fruit colour. It was concluded that any attempts at colour manipulation must be carried out post-colour-break. Fruit that had not attained a visual colour rating of at least colour stage 6 on the Outspan colour chart did not respond favourably to any colour manipulation treatment. Krajewski (1998) noted that ethylene degreening should only be considered once colour has broken, and stated that fruit of colour grade 8 do not respond to treatment with this plant growth regulator.

The most promising method of pre-harvest colour manipulation was that of micro-nutrient treatment, followed by plant hormone application. It was observed that W- and Mo-treated flavedo discs had significantly higher carotenoid content than untreated discs 96 h after treatment, with W being the more effective treatment (also effective at lower concentrations than Mo). The terminal step in ABA formation from 9-*Z*-neoxanthin is mediated by a molybdo-aldehyde oxidase (Mendel, 1997), indicating a molybdenum requirement for xanthophyll and ABA metabolism (Richardson and Cowan, 1996). It may be expected that an increase in carotenoid level is responsible for sustaining higher conversion rates to ABA. Conversely, W, as the element below Mo in group VIb of the periodic table could substitute for Mo and was shown to be a specific inhibitor of the aldehyde oxidase involved in ABA biosynthesis (Lee and Milborrow, 1997). Such an inhibition would lead to an accumulation of carotenoids. The effect of Mo and W on carotenoid synthesis and/or accumulation needs to be investigated in more detail, but it is envisaged that these micro-nutrients could be applied as a pre-harvest spray to ensure rapid and uniform colour development.

The role of plant hormones in citrus colour development has been partially established through exogenous application of hormones to citrus peel. GA_3 application resulted in a delay in flavedo colour development of 'Navel' and 'Valencia' (Coggins and Hield, 1962) and furthermore enhanced regreening in fully coloured 'Valencia' fruit (Coggins and Lewis, 1962). Ethylene application has been shown to increase carotenoid content and enhance colour development of citrus flavedo in a number of citrus

species (Stewart and Wheaton, 1972; 1973; Le Roux *et al.*, 1997). Commercially, ethylene application pre- or post-harvest is employed to hasten colour development, although problems such as enhanced post-harvest decay is often associated with the use of ethylene (Krajewski, 1998). GA is applied to control creasing (a physiological disorder leading to creased rind) in soft citrus and may retard fruit colour development if applied within two months of harvest (Krajewski, 1998). GA may also be employed to lengthen the harvesting season of grapefruit and 'Navel' oranges. In the present study, both ABA and JA enhanced carotenoid content of flavedo discs, whereas GA reduced carotenoid levels. The observation that treatment with Mo resulted in increased carotenoid content may therefore be an indirect response to increased ABA levels. Pérez *et al.* (1993) reported that methyl jasmonate vapour promoted β-carotene synthesis and chlorophyll degradation in 'Golden Delicious' apple peel. The application of ABA and/or JA may therefore prove to be beneficial in improving flavedo colour in *C. sinensis*. It is however possible that senescence could be accelerated and that enhanced post-harvest decay may be experienced with application of these plant hormones.

Dehydrating agents such as ethanol and butanol warrant further investigation as a post-harvest method of colour improvement. Ethanol and butanol increased carotenoid content in whole fruit flavedo and flavedo discs and the optimum concentrations were shown to be 20-30% (v/v) for ethanol and 5-10% (v/v) for butanol. Fruit ripening in citrus is associated with an increase in lipophilic pigments (Eilati *et al.*, 1972) and concomitant dehydration, a process enhanced by alcohol application. Loss of water through dehydration treatments may therefore facilitate carotenoid accumulation in chromoplast-localised globuli. Citrus colour development is enhanced by environmental stresses such as low temperature (Young and Erickson, 1961) and drought conditions (Peng and Rabe, 1996), hence dehydration stress may play a role in acceleration of colour expression. It should be noted that plant stress response is usually associated with an increase in ABA (Raven *et al.*, 1986). Ethanol application has been shown to enhance fruit sensory quality (e.g. an increase in sugar content, increase in sugar : acid ratio and flavour changes judged to be acceptable), in blueberries, tomatoes, grapes and pears (Paz *et al.*, 1981). Ethanol was also shown

to enhance fruit ripening and lycopene accumulation in tomatoes at low concentrations, although high concentrations inhibited these responses (Bieulieu and Saltveit, 1997). This trend was observed in whole fruit and flavedo discs of citrus treated with butanol, where high concentrations of butanol (>10%; v/v) inhibited carotenoid accumulation. Of the two alcohols evaluated, ethanol was the more likely choice as a commercial enhancer of citrus flavedo colour. Butanol-treated fruit routinely exhibited rind damage whereas ethanol concentrations as high as 30% (v/v) did not damage fruit peel. Furthermore, food-grade ethanol is safe for human consumption and can be considered environmentally sound. Ethanol application could be incorporated into the hot water bath (40-45C°) of a packhouse system without any adjustment of equipment. Additional benefits are the fungicidal effects obtained from ethanol application. It was shown that ethanol solutions heated to 45°C effectively controlled post-harvest green mould (*Penicillium digitatum*) in lemon fruit without injury to the flavedo (Smilanick *et al.*, 1995).

In addition, a short low temperature treatment, followed by incubation at higher temperatures resulted in increased carotenoid content of flavedo discs. While this result supports the idea that low temperature stress enhances colour development, it also indicates that regulation of post-harvest storage temperature may play an important role in colour development. Fruit stored or shipped at a constant temperature of 4.5 °C showed little improvement in fruit colour, whereas fruit exposed to shipping temperatures of 11 °C displayed a consistent improvement in fruit colour (Krajewski, 1998). A short low temperature period followed by shipping at slightly elevated temperatures may prove to be effective in improving flavedo colour.

5.2 CONCLUSIONS AND FUTURE PROSPECTS

This study shows that brilliant orange rind colour in *C. sinensis* can be related to the presence of the yellow xanthophyll 9-*Z*-violaxanthin and the red apocarotenal β-citraurin. Furthermore, the ratio of 9-*Z*-violaxanthin : β-citraurin was shown to be critical for pleasing, visual colour perception. 9-*Z*-violaxanthin and β-citraurin content, as well as 9-*Z*-violaxanthin : β-citraurin ratio in the flavedo of citrus fruit can therefore be used to quantify visual colour and these parameters can aid in the evaluation of colour and genetic manipulatory techniques. Further studies aimed at improvement of citrus fruit colour should concentrate on delineating the biosynthetic pathway of the colour-imparting carotenoids, and cloning and characterisation of the genes and enzymes involved. Carotenoid manipulation studies should aim at increasing the level of the colour-imparting carotenoids and decreasing the ratio of 9-*Z*-violaxanthin:β-citraurin in citrus flavedo, thereby improving visual colour.

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APPENDIX A

PAPER(S) IN PRESS

Biochemical Basis of Color as an Aesthetic Quality in *Citrus sinensis*

Renate Oberholster and A. Keith Cowan

Research Centre for Plant Growth and Development, School of Agricultural Sciences and Agribusiness, University of Natal—Pietermaritzburg, Private Bag X01, 3209, Scottsville, South Africa

Péter Molnár and Gy. Tóth

Department of Medical Chemistry, Faculty of Medicine, Pécs University, Szigeti út 12, P.O. Box 99, H-7601, Hungary

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Renate Oberholster and A. Keith Cowan*

Research Centre for Plant Growth and Development, School of Agricultural Sciences and Agribusiness, University of Natal–Pietermaritzburg, Private Bag X01, 3209, Scottsville, South Africa

Péter Molnár and Gy. Tóth

Department of Medical Chemistry, Faculty of Medicine, Pécs University, Szigeti út 12, P.O. Box 99, H-7601, Hungary

The biochemical basis of color as an aesthetic quality in mature fruit of navel and Valencia orange (*Citrus sinensis*) was determined. Saponification of the two major color-imparting components resolved by thin-layer chromatography, followed by reversed-phase high-performance liquid chromatography, revealed that these comprised acyl esters of (9*Z*)-violaxanthin and β-citraurin. Identification of the chromophores was based on cochromatography and online spectral analysis. The color quality of flavedo of mature fruit was dependent on the content and relative amounts of (9*Z*)-violaxanthin and β-citraurin. Quantitative results revealed that increased color intensity was associated with a decline in the (9*Z*)-violaxanthin:β-citraurin ratio from greater than 50 to below 10, an increase in flavedo (9*Z*)-violaxanthin and β-citraurin content, and that measurement of the mass and ratio of these carotenoids can be used to accurately color-grade orange fruit for local and export markets.

Keywords: *Citrus sinensis; carotenoids; color; β-citraurin; (9Z)-violaxanthin*

INTRODUCTION

Plant pigments in vegetables, fruits, and ornamental crops have been studied intensively because of their vital role in visual appeal. Although attention has shifted to the nutritional benefits afforded by plant pigments, in particular carotenoids (Bartley and Scolnik, 1995; King et al., 1997), color is used by horticulturalists as a major criterion for determining both grade and quality of fruit. In citrus, flavedo color is probably the most important external quality parameter used in determining consumer acceptance. However, it is not usually an indication of internal quality. Nevertheless, visual expression of color is a cultivar characteristic affected by climate and environment that can, to some extent, be manipulated by cultural practice (Goldschmidt, 1988). Competition between growers to secure niche markets has fueled efforts to produce quality fruit that is uniformly of good color.

The development of color in citrus occurs concomitantly with the transformation of photosynthetically active chloroplasts to carotenoid-containing chromoplasts (Thomson, 1966; Gross, 1987; Gross et al., 1983). Carotenoids of the orange (*Citrus sinensis* (*C. sinensis*)) are probably the most studied pigments in citrus, and the flavedo of fully mature, colored fruit is one of the richest sources of these pigments in plants. The carotenoid content and composition of orange flavedo has been described in detail (Curl, 1965, 1967; Curl and Bailey, 1956). More recently, Molnár and Szabolcs (1980) reported on the identification of β-citraurin epoxide (3-hydroxy-5,6-epoxy-5,6-dihydro-8'-apo-β-caroten-8'-al) and several isomers of violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-caroten-3,3'-diol) in flavedo of the "Valencia" orange. These authors also provided quantitative data on the spectrum of carotenoids in orange flavedo and demonstrated that violaxanthin and its *Z*-isomers (9*Z*-, 13*Z*-, and di-*Z*-) predominate. Violaxanthin is typically a yellow pigment and therefore unlikely to be solely responsible for good quality color of the flavedo of the mature orange fruit. In fact, Gross (1981) showed that although violaxanthin comprised 52.8% of the total carotenoid content of the "Dancy" tangerine, it was the red β-citraurin (3-hydroxy-8'-apo-β-caroten-8'-al) and the orange β-cryptoxanthin (β,β-caroten-3-ol) that were responsible for the orange-reddish color of tangerine fruit. The structures of these carotenoids are illustrated in Figure 1.

In the present investigation efforts were made to determine the biochemical basis of flavedo color as an aesthetic quality in orange using navel and Valencia fruit. Typically mature navel fruit are brightly colored, whereas Valencia are recalcitrant with respect to color development and therefore ideal for comparative purposes. Identification of the major color-imparting carotenoids is described and differences in the relative amounts of (9*Z*)-violaxanthin and β-citraurin used to assess color of mature fruit as a quality parameter. It is demonstrated that an increase in (9*Z*)-violaxanthin and β-citraurin content, concomitant with a decline in the (9*Z*)-violaxanthin:β-citraurin ratio, is associated with increased intensity of flavedo color. Results are discussed in terms of manipulation of carotenogenesis in vivo to enhance flavedo color and the aesthetic appeal of orange fruits.

* To whom correspondence should be addressed. Telephone: +27 33 260 5472. Fax: +27 33 260 5073. E-mail: cowan@nu.ac.za..

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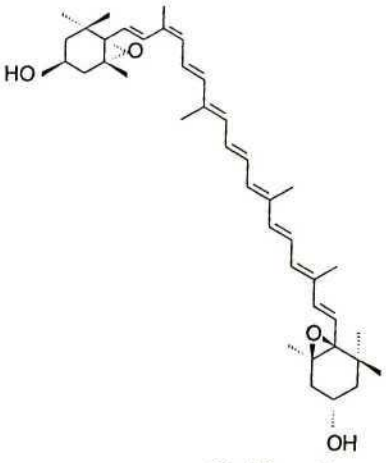
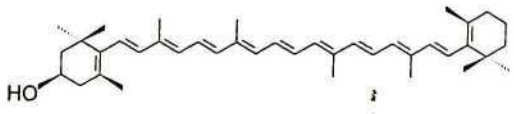
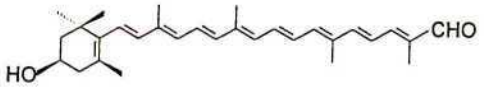


9Z-violaxanthin



β-cryptoxanthin



β-citraurin

Figure 1. Structures of (9Z)-violaxanthin, β-cryptoxanthin, and β-citraurin, the major color-imparting carotenoids in citrus flavedo.

MATERIALS AND METHODS

Chemicals and Reagents. HPLC grade methanol, acetonitrile, ethyl acetate, and hexane were obtained from Burdick and Jackson (AlliedSignal Inc., Muskegon, MI). All other solvents were of analytical grade and obtained from BDH Laboratory Supplies (Poole, U.K.). Butylated hydroxytoluene (BHT), diethyldithiocarbamate (DDC), and triethylamine (TEA) were from Sigma Chemical Co. (St Louis, MO). (9Z)-Violaxanthin and β-citraurin were prepared as described previously (Molnár and Szabolcs, 1979).

Plant Material and Growing Conditions. Fruits of *C. sinensis* "navel" and "Valencia" were harvested from 10 year old trees on rough lemon rootstocks in the Albert Falls region, KwaZulu Natal midlands, South Africa. All trees were subjected to the cultural practices commonly used in citrus orchards in this region. Harvested fruit was surface-sterilized by immersion in 1% sodium hypochlorite for 20 min followed by several changes of distilled water. Fruit were graded according to industry standards for visual color by comparing flavedo color with the Outspan blemish-standards chart (no.19, color) that is used to prescribe citrus for export and assigned a rating on a scale of 1 (orange, fully colored) to 8 (green, unmarketable).

Carotenoid Extraction and Analysis. All steps for the extraction and identification of carotenoids were carried out under low temperature and light intensity to avoid photooxidation and isomerization of the compounds of interest. The outer layer of the flavedo (peel) was grated from the fruit, finely crushed in liquid nitrogen using a mortar and pestle, and homogenized (2 by 1 min bursts) in methanol/ethyl acetate (50:50, v/v) containing BHT (100 mg L^{-1}) and DDC (200 mg L^{-1}) as antioxidants, with PVP (Polyclar SB100, 1 g/(10 g of fresh weight)) using an Ultra-Turrax top-drive tissue homogenizer. Homogenates were centrifuged for 5 min, and the pellet was homogenized in further methanol/ethyl acetate (50:50, v/v). The combined supernatant was reduced to dryness in vacuo at 35 °C using a rotary evaporator, and the extracts were either analyzed immediately or stored under nitrogen at −20 °C.

Concentrated crude extracts were resuspended in organic solvent and partially purified by thin-layer chromatography (TLC) on layers (20 × 20 cm, 0.25 mm thickness) of silica gel (Merck, Type 60) developed to 15 cm in a closed tank containing hexane/ethyl acetate/ethanol/acetone (95:3:2:2, v/v) at 2−4 °C in darkness. Carotenoid-containing zones were scraped from the plate into small glass funnels plugged with glass wool and the pigments eluted from the gel with acetone and concentrated under a stream of nitrogen. Where specified, saponification was carried out by resuspending pigment samples in 8 mL of methanol to which was added 2 mL of KOH (1 M), the mixture vortexed and allowed to stand for approximately 12 h in complete darkness at room temperature. After removal of the methanol, 3 mL of water was added and the carotenoids were partitioned into an equal volume of diethyl ether (repeated three times). Combined ether fractions were pooled and concentrated under nitrogen.

Crude and saponified extracts were filtered using a 0.2 μm syringe filter and the individual carotenoids separated by reversed-phase HPLC on a 5 μm Vydac 201TP54 (VYDAC, Hesperia, CA) C18 column (250 × 4.6 mm i.d.) eluted isocratically at 26 °C with methanol/acetonitrile (9:1, v/v) containing 0.1% (w/v) BHT and 0.05% (v/v) TEA at a flow rate of 1 mL min^{-1}, using a SpectraSYSTEM P2000 pump (Thermo Separations Products, Fremont, CA). Compounds of interest were detected at 460 nm and quantified by peak integration using a UV3000 rapid-scanning detector (Thermo Separations Products, Fremont, CA) in the range 370−550 nm calibrated using authentic standards. Identification was achieved with the use of PC1000 software (Thermo Separations Products) that allowed for online comparison of absorption spectra of unknown compounds with authentic (9Z)-violaxanthin and β-citraurin.

RESULTS AND DISCUSSION

Identification of Color-Imparting Carotenoids. The carotenoids present in flavedo of mature orange fruit have been identified by physicochemical methods (Molnár and Szabolcs, 1980). To gain insight into the major color-imparting carotenoids in orange fruit flavedo, we initially examined crude pigment extracts prepared from brightly colored (Outspan color chart, Grade 1) Valencia and navel fruit. Separation of extracts on thin layers of silica gel revealed, in addition to numerous minor pigment-containing zones, two intensely colored bands at R_f 0.1 (yellow-orange, TLC zone A) and R_f 0.33 (orange-red, TLC zone B). These were eluted from the gel and further analyzed by reversed-phase HPLC, and the chromatographic profiles are illustrated in Figure 2. Since the identical results were obtained for flavedo of both Valencia and navel fruits, and for ease of data presentation, only chromatograms for Valencia are shown. The results show that TLC zones A and B were each resolved into three major components and the retention time of the components in zone A was similar to the components in zone B. Online spectral analysis of components A1, A2, and A3 (Figure 2A) produced results consistent with a chromophore similar to (9Z)-violaxanthin (λ_{max}, nm: 435), whereas components B1, B2, and B3 in Figure 2B produced spectra with a single maxima at 457 nm typical of β-citraurin (Figure 2C). Confirmation of the identity of these chromophores as (9Z)-violaxanthin and β-citraurin was achieved by saponification of zones A and B from TLC, prior to reversed-phase HPLC analy-

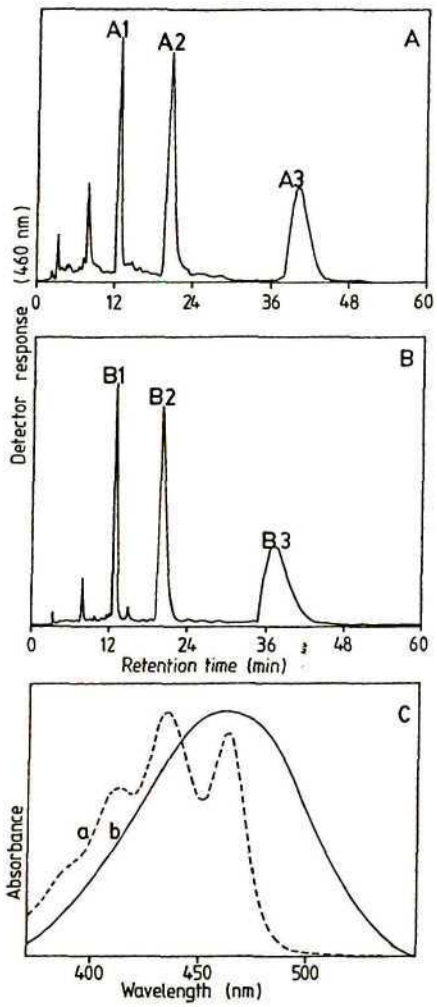


Figure 2. Reversed-phase HPLC chromatograms of unsaponified zone A (A) and zone B (B) from TLC-separated crude pigment extracts of flavedo of color-grade 1 Valencia orange. (C) Absorption spectra of A1, A2, and A3 (a) and B1, B2, and B3 (b).

sis. Zones A and B yielded single peaks which cochromatographed with authentic (9*Z*)-violaxanthin and *β*-citraurin, respectively. In addition, comparison of spectral characteristics with those of authentic standards showed >99% similarity between zone A and (9*Z*)-violaxanthin and zone B and *β*-citraurin. Furthermore, these results indicated that both (9*Z*)-violaxanthin and *β*-citraurin accumulate in orange flavedo in an esterified form and that esterification is responsible for the differences in retention time noted in Figure 2.

Although *β*-citraurin and (9*Z*)-violaxanthin acyl esters occur in close association when crude extracts are analyzed by HPLC (Figure 3A), saponification prior to HPLC analysis ensures that these two major color-imparting pigments are well-resolved for quantification purposes (Figure 3B). This result indicates that accurate quantification of the chromophores of these acyl esters is possible without prior separation by thin-layer chromatography. Additionally, the absorption spectrum of each pigment is unaffected by saponification (data not shown). While the identity of the esters was not determined in the present study, *β*-citraurin myristate has been isolated from the peel of Marsh seedless grapefruit (Philip, 1973a), and the laurate esters of *β*-cryptoxanthin, (9*Z*)-violaxanthin and *β*-citraurin have been detected in flavedo of Valencia orange (Philip,

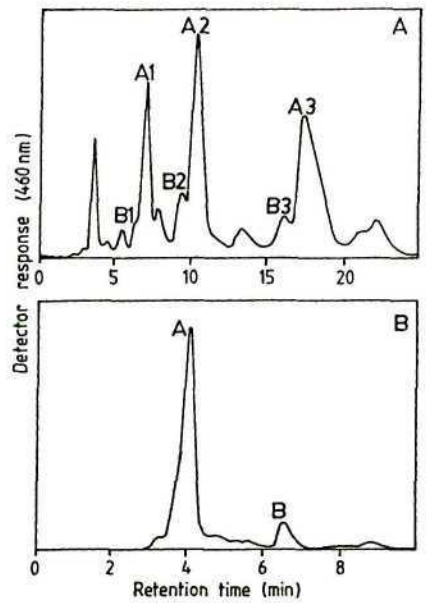


Figure 3. Reversed-phase HPLC chromatograms of unsaponified (A) and saponified (B) pigment extracts of flavedo of mature color-grade 1 Valencia orange fruit. Peaks: A, (9*Z*)-violaxanthin; B, *β*-citraurin.

1973b). The physiological significance of esterification may be attributed to the fact that it increases the lipophilic character of the carotenoids, making possible their accumulation in chromoplast-localized plastoglobuli (Eilati et al., 1972). Acylation has also been shown to increase the stability of these pigments (Camara and Moneger, 1978).

Biochemical Basis of Flavedo Color. (9*Z*)-Violaxanthin is the most abundant carotenoid in citrus flavedo (Molnár and Szabolcs, 1980; Gross, 1987). As a yellow pigment, it contributes mainly to the background color of orange fruit flavedo. *β*-Citraurin, a C-30 apocarotenal, is a red-orange pigment and is responsible for the bright orange color of tangerine flavedo (Farin et al., 1983). To demonstrate a similar role for *β*-citraurin in flavedo color of *C. sinensis*, (9*Z*)-violaxanthin and *β*-citraurin levels of different color grades of mature Valencia and navel fruit were determined after saponification, by HPLC, and the results are shown in Figure 4. No fruit of grade 7 or 8 were available at the time of harvest, and very brightly colored navel fruit (more orange than grade 1) were designated grade 0. Poorly colored fruit (grades 5–6) had high levels of chlorophyll (data not shown), low levels of (9*Z*)-violaxanthin, and almost undetectable amounts of *β*-citraurin. Fruit of average color (grades 3–4) had relatively low levels of both color-imparting pigments. Fruit of good color (grades 0–2) contained increased levels of (9*Z*)-violaxanthin and *β*-citraurin. Thus, an increase in the color-grade (i.e. from 6 to 1) was associated with massive accumulation of (9*Z*)-violaxanthin concomitant with a less dramatic increase in *β*-citraurin in both navel and Valencia flavedo. In addition, the increase in color grade was associated with a decline in the (9*Z*)-violaxanthin:*β*-citraurin ratio from >50 to <10. For grade 1 fruit, the *β*-citraurin levels of flavedo of navel and Valencia fruits were similar, but (9*Z*)-violaxanthin levels were higher in flavedo of Valencia. While this might be a factor in the apparent recalcitrancy of color development in Valencia, it clearly indicates that the flavedo content of, and relative level of, (9*Z*)-violaxanthin and *β*-citraurin is crucial for visual color appeal. It is tempting to

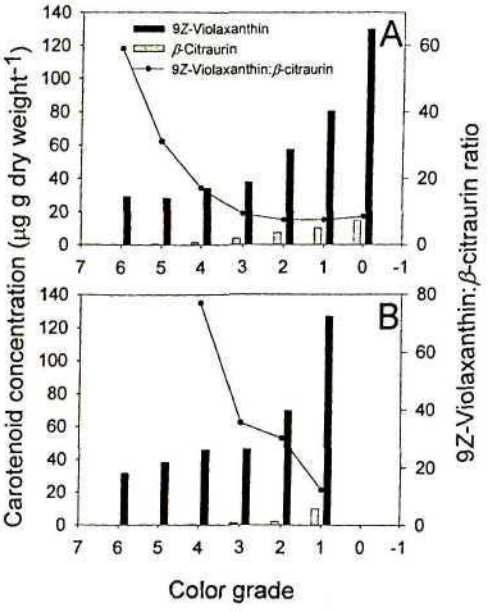


Figure 4. Quantification of (9Z)-violaxanthin and β-citraurin, and the (9Z)-violxanthin:β-citraurin ratio in flavedo of mature navel (A) and Valencia (B) fruit color-graded using the Outspan blemish standards chart (no. 19; color). Color grades: 6, poor; to 1, fully/brightly colored.

suggest, therefore, that the amount of (9Z)-violaxanthin and β-citraurin specify chroma (color intensity), whereas the (9Z)-violaxanthin:β-citraurin ratio is responsible for hue, in the quality assessment of fruit using a colorimeter (Voss, 1992; Reeves et al., 1997).

The study described in this paper was carried out with a view to manipulating carotenogenesis in vivo to improve flavedo color and aesthetic quality of orange fruits. As a first step, the identity of, and relationship between, the major color-imparting pigments in flavedo was determined. Clearly, in vivo manipulation of carotenoid content for improved color rests on an understanding of the biochemistry of the pigments concerned. However, the biosynthetic origin of both (9Z)-violaxanthin and β-citraurin remains unresolved. It is assumed that (9Z)-violaxanthin arises due to isomerization of all-*E*-violaxanthin in vivo. By comparison, β-citraurin (a C_{30} apocarotenoid) is believed to be a degradation product of either zeaxanthin (Yokoyama and White, 1966) or β-cryptoxanthin (Gross, 1981). A similar mechanism is thought to be responsible for the exocentric cleavage of β-carotene in the formation of retinal (van Vliet et al., 1996).

Citrus flavedo is considered an ideal system in which to study the expression of genes/proteins involved in plant stress responses (Sanchez-Ballesta et al., 1999). In earlier work, we showed that levels of the plant stress hormone abscisic acid (ABA), in navel and Valencia flavedo reached a maximum coincident with the onset of color-break and that a subsequent decline in the levels of ABA correlated with expression of full color development (Richardson and Cowan, 1995). We later showed that an enzyme system prepared from orange fruit flavedo converted (9Z)-neoxanthin to xanthoxal (the immediate aldehydic product of xanthophyll cleavage) and ABA (Cowan and Richardson, 1997). Similar findings on the biosynthetic origin of ABA in other plant tissues in response to stress, via dioxygenase-mediated cleavage of (9Z)-xanthophylls, have been reported (Schwartz et al., 1997). The pathway for formation of xanthoxal from (9Z)-xanthophylls is now well-established, and the enzyme responsible for xanthophyll cleavage has been cloned (Cutler and Krochko, 1999). Furthermore, expression of the mRNA for the xanthophyll cleavage enzyme (which is inhibited by low temperature) increases in response to water deficit stress, a stimulus known to induce ABA accumulation (Qin and Zeevaart, 1999). Since the development of color in *C. sinensis* is exacerbated by low temperature, a cold stress may be the stimulus required for synthesis and accumulation of β-citraurin via dioxygenase-mediated cleavage of the parent xanthophyll.

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