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

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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.

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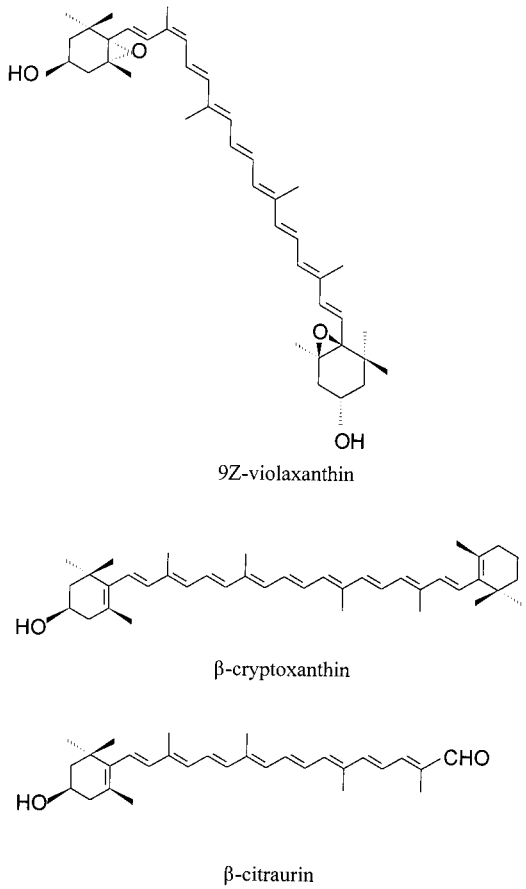

Figure 1. Structures of (9*Z*)-violaxanthin, *β*-cryptoxanthin, and *β*-citraurin, the major color-imparting carotenoids in citrus flavedo.

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). (9*Z*)-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 (9*Z*)-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 (9*Z*)-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 (9*Z*)-violaxanthin and *β*-citraurin was achieved by saponification of zones A and B from TLC, prior to reversed-phase HPLC analy-

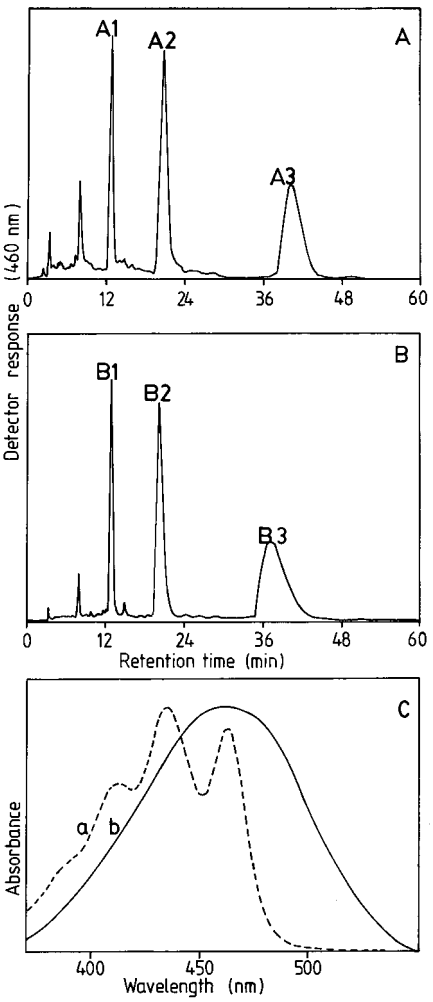

Figure 2. Reversed-phase HPLC chromatograms of unsaponified zone A (A) and zone B (B) from TLC-separated crude pigment extracts of flavedo of color-grade 1 Valencia orange. (C) Absorption spectra of A1, A2, and A3 (a) and B1, B2, and B3 (b).

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,

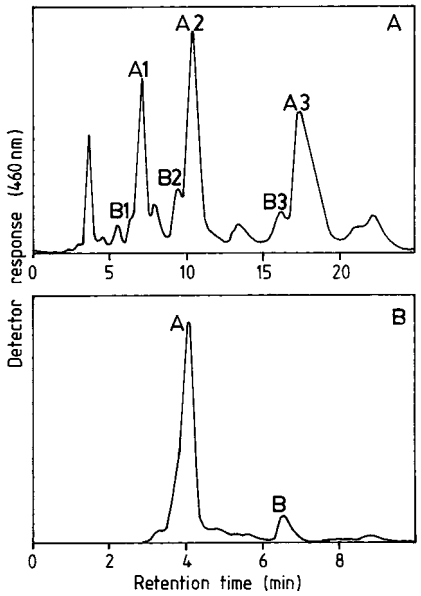

Figure 3. Reversed-phase HPLC chromatograms of unsaponified (A) and saponified (B) pigment extracts of flavedo of mature color-grade 1 Valencia orange fruit. Peaks: A, (9*Z*)-violaxanthin; B, *β*-citraurin.

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

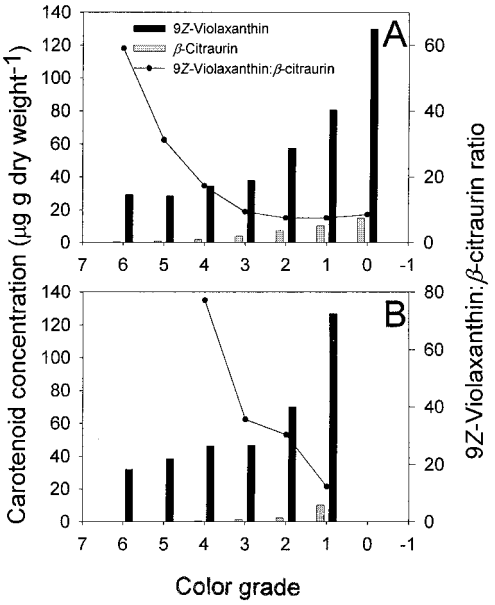

Figure 4. Quantification of (9*Z*)-violaxanthin and *β*-citraurin, and the (9*Z*)-violxanthin:*β*-citraurin ratio in flavedo of mature navel (A) and Valencia (B) fruit color-graded using the Outspan blemish standards chart (no. 19; color). Color grades: 6, poor; to 1, fully/brightly colored.

suggest, therefore, that the amount of (9*Z*)-violaxanthin and *β*-citraurin specify chroma (color intensity), whereas the (9*Z*)-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 (9*Z*)-violaxanthin and *β*-citraurin remains unresolved. It is assumed that (9*Z*)-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 (9*Z*)-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 (9*Z*)-xanthophylls, have been reported (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 (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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