Carotenoids in the Flavedo of Marsh Seedless Grapefruit

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The flavedo of the Marsh seedless grapefruit contains a highly complex carotenoid mixture. At midseason the major colored carotenoids are the epoxides, violaxanthins, and luteoxanthins. The main carotenoid is the colorless polyene phytoene. Phytoene is present in only trace amounts in the early season mature fruit. However, on ripening, phytoene accumulates as the major carotenoid constituent. During this period of maturation the total colored carotenoid content in the flavedo is decreasing. The unique methyl ketone carotenoid reticulataxanthin is present also.

The carotenoids of the Marsh seedless grapefruit have not been studied extensively. Khan and Mackinney (*12*) investigated the major differences in the lycopene, β-carotene, ζ-carotene, and phytofluene content in the pulp of the Marsh white, Marsh pink, and Ruby Red varieties of grapefruit. In the pulp of the mature Marsh white grapefruit, they reported trace amounts of β-carotene and small amounts of phytofluene, ζ-carotene, and a yellow unidentified pigment. Purcell (*21*) reported lycopene in the immature fruit. Preliminary tests on the Marsh seedless grapefruit showed a complex mixture of carotenoids including a number which had not been reported previously. The investigation therefore was continued in greater detail. The authors report herein the carotenoids in the flavedo of the Marsh seedless grapefruit.

Experimental

The grapefuit (*Citrus paradisi* Macf., var. Marsh seedless) used in these experiments were collected from a commercial grove in Indio, Calif., and consisted of two lots of fruit, both collected from the same set of trees. The first lot was collected in December 1965. The fruit was fully matured and deep yellow; the chlorophyll had just disappeared from the flavedo. Estimates were made of total carotenoids and only the hydrocarbon fraction was examined in detail (Table I). The second lot, collected in February 1966, was selected for more detailed study (Table II). This lot corresponded to the midseason stage and the flavedo still retained the yellow color but in addition exhibited a slight golden blush.

Pigment Extraction. The peel was separated from the endocarp by hand, the albedo gently scraped off, and the flavedo (10 kg.) cut up into small pieces. A small amount of magnesium carbonate was then added. The carotenoid pigments were extracted from the flavedo with acetone and methanol in the manner described previously (*29*).

Saponification. The acetone–methanol extract was concentrated in vacuo until all acetone was removed. The extract was then diluted with an equal volume of diethyl ether [previously passed through a column of alumina to remove the peroxides (*8*)] and sufficient saturated NaCl solution added to form two layers. The ether layer was combined with an equal volume of 10% KOH–ethanol, covered with N_2, and kept for 2 hours at room temperature with occasional shaking. The mixture was washed free of alkali with aqueous NaCl solution (the hypophase was re-extracted with ether until no further pigment transfer was observed), dried over anhydrous magnesium sulfate, and filtered. The total carotenoid content was estimated from the spectrophotometrically determined amount in the ether extract using $E_{1\ cm.}^{1\%} = 2700$ at the main λ_{max} in ether. The total carotenoid content is given relative to the wet weight of the flavedo. The ether extract was evaporated in vacuo. The residues were taken up in petroleum ether and methanol in the usual manner for later phase-partition separation.

Chromatography. The complex pigment mixtures were initially submitted to countercurrent distribution with 100 transfers in an all-glass Craig apparatus (the tubes were initially flushed with N_2 and kept under nearly constant N_2 atmosphere). By means of the solvent system (petroleum ether–99% methanol) employed by Curl (*3*, *4*) the carotenoids were fractionated into three groups—hydrocarbons, monols, and diols.

The carotenoids in the hydrocarbon and monol fractions were separated employing column chromatography on magnesium oxide: Hyflo Super-Cel (1 to 1, w./w.). The column was developed stepwise with increasing amounts of acetone or ethanol in petroleum ether. The separation of β-carotene, ζ-carotene, and β-zeacarotene was difficult, but good separation was obtained on rechromatography on a column of alumina (activity grade 1). The column of alumina was developed and eluted (stepwise gradient elution) with petroleum ether containing increasing amounts of ether.

The carotenoids in the diol fraction were separated on a column of Microcel C using petroleum ether containing increasing amounts of acetone as the solvent system.

The column fractions were examined spectrophotometrically on a Cary Model 14 recording spectrophotometer, and the individual pigments were determined quantitatively according to the method described by Davies (*9*). The minor carotenoids were present in amounts permitting some degree of accuracy in estimation.

Identification of Pigments. The individual pigments were identified on the bases of comparison of chromatographic movements and visible spectra with those of authentic samples. Thin-layer chromatography was employed in the comparison of chromatographic properties (*29*). Iodine catalysis was carried out and treat-

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Table I. Chromatographic Separation of the Carotenoids in the Hydrocarbon Fraction from the Flavedo of Early Season Mature Fruit

Fraction[a]	Color	λ_{max} in *n*-Hexane	Required Eluent[b] in Petroleum Ether[c]	Identity	Total Carotenoids, %[d]
1	...	273, 285, 296 (sh)	...	Phytoene	Trace
2	...	333, 349, 368	0.5% *A*	Phytofluene	30.8 (708)
3	Yellow	378, 400, 424	0.5% *A*	η-Carotene[e]	4.7 (108)
4	Yellow-orange	422, 447, 473	0.5% *A*	α-Carotene	0 8 (18)
5a	Yellow-orange	425, 450, 476	1.0% *A* (10% *E*)	β-Carotene	2.7 (62)
5b	Yellow	404 (sh), 425, 452	... (20–30% *E*)	β-Zeacarotene	3.5 (81)
5c	Yellow	378, 400, 424	... (40% *E*)	δ-Carotene	0.7 (16)
6	Orange	414, 438, 468	5% *A*	Neurosporene	0.5 (12)
7	Yellow	408, 420, 447	5% *A*	Mutatochrome	2.8 (64)

[a] Adsorbent MgO–Hyflo Super-Cel (1 to 1, w./w.); pigments in order of increasing adsorption; fraction 5 was rechromatographed on alumina (activity grade 1).
[b] *A* = acetone; *E* = ether; solvents used in elution of fraction on rechromatography are in parentheses.
[c] B.p. 30° to 60° C.
[d] Values in parentheses are in μg./kg. wet weight.
[e] Provisional identification.

Table II. Chromatographic Separation of the Carotenoids in the Flavedo of Midseason Fruit

Fraction[a]	Color	λ_{max} in *n*-Hexane	Required Eluent in Petroleum Ether[b]	Epoxide Test	Identity	Total Carotenoids,[c] %
Hydrocarbon						
1	...	273, 285, 296 (sh)	0.5% acetone		Phytoene	50.6 (759)
2	...	333, 349, 368	0.5% acetone		Phytofluene	24.0 (360)
3	Light orange	425, 450, 476	1.0% acetone		β-Carotene	0.6 (9)
4	Yellow	378, 400, 424	1.5% acetone		ζ-Carotene	2 3 (35)
5	Orange	414, 438, 468	5.0% acetone		Neurosporene	0.3 (5)
Monol						
1	Yellow	422 (sh), 444, 473	0.5% ethanol	+	Cryptoxanthin-5,6-epoxide	0.5
2	Light orange	425 (sh), 450, 479	0.5% ethanol	−	Cryptoxanthin	2.4
3	Yellow	402, 424, 451	1.0% ethanol	−	Unknown	0.7
4	Yellow	403 (sh), 425, 452	1.0% ethanol	+	Cryptoflavin[d]	0.6
Diol						
1	Light orange	420, 442, 470	7% acetone	−	Lutein	1.5
2	Light orange	425 (sh), 447, 473	8% acetone	−	Zeaxanthin	0.7
3	Light orange	422 (sh), 441, 467	8% acetone	−	*cis*-Zeaxanthin	0.4
4	Yellow	420 (sh), 442, 469	9% acetone	+	Antheraxanthin	1.3
5	Pink	463, 490	9% acetone	−	Reticulataxanthin	2.4
6	Yellow	405 (sh), 427, 450	10% acetone	+	Mutatoxanthin	1.1
7	Yellow	415, 438, 468	10% acetone	+	Violaxanthin	5.6
8	Yellow	410 (sh), 433, 460	10% acetone	+	*cis*-Violaxanthin	0.8
9	Yellow	399, 422, 448	12% acetone	+	Luteoxanthin	2.4
10	Yellow	398, 419, 443	12% acetone	+	*cis*-Luteoxanthin	2.1

[a] Hydrocarbon and monol fractions chromatographed on MgO–Hyflo Super-Cel (1 to 1, w./w.); diol fraction chromatographed on Microcel C; carotenoids in order of increasing adsorption.
[b] B.p. 30° to 60° C.
[c] Values in parentheses are in μg./kg. wet weight.
[d] Provisional identification.

ment with acid chloroform was conducted according to the methods described previously (*25*, *28*). The criteria mentioned above were considered as proof of identity of a given carotenoid. Cases where ambiguity exists because authentic samples were not available for direct comparison or because the small amount isolated precluded detailed structural studies are so noted in the results.

The following authentic samples were available for direct comparison: Phytoene (*20*), phytofluene (*32*), ζ-carotene (*20*), and neurosporene (*24*) were isolated from ripe tomatoes; α-carotene from carrots (*15*); cryptoxanthin-5,6-epoxide from Meyer lemons (*5*); cryptoxanthin from egg yolk (*10*); lutein (*26*), zeaxanthin (*26*), and β-zeacarotene (*17*) from corn grain; antheraxanthin from *Euglena gracilus* (*13*); reticulataxanthin from Minneola tangor (*31*); violaxanthin from green leaves (*23*); luteoxanthin was prepared from violaxanthin (*23*) and mutatoxanthin from antheraxanthin (*13*); synthetic β-carotene was obtained from Hoffmann-LaRoche.

Results

The carotenoid mixture in the flavedo of the Marsh seedless grapefruit was highly complex (Table II). As the fruit matured from the early season to the midseason the total carotenoid content in the flavedo decreased from 2.3 to 1.5 mg. per kg. These results confirmed the observations of Miller and Winston (*16*) who had shown that in the white grapefruit the peel carotenoids decreased in amount as the chlorophyll disappeared.

Hydrocarbons. In the early fruit at full maturity (shortly after the disappearance of chlorophyll) phytoene was present only in trace amounts in the flavedo (Table I). In contrast in the midseason fruit phytoene constituted the main constituent in the flavedo (Table II). The colorless component accounted for approximately 51% of the total carotenoids. The two colorless components in the hydrocarbon fraction phytoene and phytofluene accounted for nearly 75% of the total carotenoid content in the flavedo.

η-Carotene-like compound was detected in the flavedo of the early fruit. The unknown carotene was adsorbed just above phytofluene and ahead of α-carotene on a column of magnesium oxide and possessed an absorption spectrum similar to that of ζ-carotene. An authentic sample of ζ-carotene isolated from tomatoes was, however, easily separable from the unknown carotene on TLC. This compound exhibited weaker adsorptive properties. Moreover, both the unknown and ζ-carotene occurred together in the flavedo. The unknown carotene is probably similar to the η-carotene first isolated from *Lonicera* berries by Goodwin (*11*). The structure of η-carotene as a bicyclic ζ-carotene was tentatively suggested by Rabourn (*22*). No direct comparison with authentic η-carotene was attempted. The unknown carotene was identical by chromatographic (TLC) and visible spectral criteria with η-carotene-like compound detected in two other citrus fruits, Sinton citrangequat (*22*) and lemon (*27*).

β-Zeacarotene and α-carotene were present also in the flavedo in the early stage but could not be detected in the midseason stage. An epoxide mutatochrome was detected in the early season fruit but could not be observed in the midseason stage. Neurosporene was present in both the early and midseason fruit.

Monols. Cryptoxanthin was the major component in the monohydroxy fraction in the midseason stage. Smaller amounts of the epoxides cryptoxanthin-5,6-epoxide and a cryptoflavin-like compound were present also.

An unknown pigment with a chromophore (λ_{max} in *n*-hexane 403, 424, and 451 mμ) similar to that of β-zeacarotene was isolated from the monohydroxy fraction. Its polarity is similar to that of cryptoxanthin. On acid chloroform treatment of the unknown pigment, no bathochromic shift in the absorption maxima could be demonstrated, indicating the absence of an allylic hydroxy group. The pigment gave a negative epoxide test. A similar unknown carotenoid was detected previously in larger amounts in the flavedo of the Sinton citrangequat (*29*). Structural studies on the unknown pigment isolated from this source are currently being conducted.

Diols. In the diol fraction the epoxides violaxanthin and luteoxanthin and their cis-isomers constituted the major pigments. Two other epoxides were identified also: antheraxanthin and mutatoxanthin. Lutein and zeaxanthin and its cis-isomer were also in this fraction.

A methyl ketone carotenoid reticulataxanthin was isolated. It was first isolated as a minor constituent from the peel of *Citrus sinensis* (*7*) and was also a minor constituent in the peel of *Citrus reticulata* (*6*). This unique pigment appears as a major constituent in the peel of the *Citrus* hybrids, Minneola tangor (*31*) and Sinton citrangequat (*29*). Although the xanthophyll fraction was not examined in detail, reticulataxanthin could not be detected in the early season fruit.

Discussion

In the ripening fruit the development of the ripe color (carotenoid pigments) begins prior to decrease in chlorophyll (*16*, *21*, *30*). This is the active period of carotenoid pigment formation (*21*). With the disappearance of chlorophyll and further maturation of the fruit, there was a decrease in the total colored carotenoid content in the flavedo; no net synthesis of the total colored carotenoids occurred. However, during this period phytoene accumulated in the flavedo (compare Tables I and II). Thus, a net synthesis of the colorless polyene was observed. The sequential desaturation of phytoene as the pathway for the biosynthesis of lycopene was first proposed by Porter and Lincoln (*19*). And considerable evidence (*1*, *14*, *18*) is available to support the view that the more saturated carotenoids of the phytoene, phytofluene, ζ-carotene, and neurosporene type are normal biosynthetic intermediates in the synthesis of carotenoids. The accumulation of the colorless polyene, phytoene, as the main constituent in the midseason fruit suggests that a selective inhibitory action on carotenoid synthesis takes place. Thus the subsequent steps of the biosynthetic sequence are in-

hibited. As a result of the inhibition, no net synthesis of the total colored carotenoids occurred in the mature fruit; instead, a decrease was observed. The nature of the inhibition or blockage remains to be investigated.

The distribution pattern of the η-carotene-like compound in the ripening fruit is observed also in a number of other citrus fruits (*25*). The unknown carotene is usually present in the fruit at the stage of maturity coincident with the disappearance of the chlorophyll from the flavedo. On further ripening the pigment disappeared. The relationship of this pigment to ζ-carotene remains to be clarified.

A similar pattern was observed in the case of reticulataxanthin. In the early or late season fruit (*25*) reticulataxanthin was not present in detectable amounts; reticulataxanthin could be detected only in midseason fruit. In the other citrus fruits such as Sinton citrangequat and Minneola tangor in which reticulataxanthin is the major carotenoid, seasonal variation in the ketone content was observed (*30*).

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