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THE CAROTENOID PIGMENTS IN THE JUICE AND FLAVEDO OF A MANDARIN HYBRID (*CITRUS RETICULATA*) CV MICHAL DURING RIPENING

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Key Word Index—*Citrus reticulata*; Rutaceae; mandarin hybrid; carotenoids; apocarotenoids; citraurinene; citraurin biosynthesis.

Abstract—The carotenoid pigments of the mandarin hybrid (*Citrus reticulata*) cv Michal, in the juice and flavedo were characterized at three ripening stages, before, during and after colour break. During ripening the characteristic mandarin pattern was formed in the juice, which contained cryptoxanthin as the principal pigment. In the flavedo the chloroplast carotenoid pattern of the green fruit changed into the characteristic pattern of *C. reticulata* with a high level of citraurin which, together with cryptoxanthin, imparts an intensive reddish tint to the hybrid. The flavedo contained an unusual C_{30} apocarotenoid, β-citraurinene (8'-apo-β-caroten-3-ol). The flavedo carotenoids of this accidental hybrid were compared with the carotenoids of the presumed parents Dancy tangerine and Clementine. The hybrid resembles more the second parent, from which it inherited the ability to biosynthesize a higher amount of citraurin as well as citraurinene. Citraurinene, considered a *Citrus* hybrid-specific pigment, was found for the first time in a *Citrus* variety. A possible biosynthetic pathway of the *Citrus* C_{30}-apocarotenoids is proposed.

INTRODUCTION

The Israeli mandarin Michal is a hybrid characterized by a bright reddish colour which is outstanding even for mandarins, the most highly coloured citrus fruit. In this study the carotenoids of the Michal mandarin were characterized at three ripening stages, before, during and after colour break. This would provide a better understanding of carotenogenesis, especially of the formation of C_{30}-apocarotenoids for which the metabolic pathway is not yet fully understood. The Michal mandarin is an accidental hybrid, presumably a cross between the Dancy tangerine and Clementine. To confirm this assumption would be of genetic and taxonomic interest. Therefore, the carotenoid pattern in the developing fruit of the hybrid was compared with those of the two putative parents. The carotenoids in the flavedo of Dancy tangerine were previously characterized [1]. The flavedo carotenoids of maturing Clementine were analysed in this study.

RESULTS

Table 1 lists the carotenoids of the juice and the peel of the ripe Michal mandarin, characterized by their chromatographic and spectrophotometric properties and chemical tests. The pigments are in order of increasing adsorption affinity on the two adsorbents used in TLC. This complex pattern is usually found in *Citrus reticulata* [1–3]. Unusual, however, was the pigment β-citraurinene, a C_{30}-apocarotenoid, isolated and identified for the first time from a *Citrus* hybrid, Robinson (Orlando tangelo × Clementine) [4]. It was detected earlier in the peel of several *Citrus* cultivars as an unknown pigment with a chromophore similar to β-zeacarotene [5]. In this study it was detected in the fraction that follows cryptoxanthin on silica gel plates and that usually contains a mixture of cryptoxanthin epoxides in which the level of cryptoflavin, the 5,8-monoepoxide of cryptoxanthin, varies between 1 and 2% of the total carotenoids. The unexpected high amount of a pigment with the same chromophore (nine conjugated double bonds of which one is in the ring) and the same spectrum (λ_{max}^{EtOH} nm: 402, 424, 450) typical of cryptoflavin, was brought to our attention. However, it gave a negative colour reaction in the epoxide test. Its mass spectrum was as follows: m/z 418 ($C_{30}H_{42}O$) $[M]^{+\cdot}$ (100%), 416 $[M-2H]^{+\cdot}$ (1%), 326 $[M-C_7H_8]^{+\cdot}$ (22%), 312 $[M-C_8H_{10}]^{+\cdot}$ (1%), 265 $[M-C_{10}H_{15}O]^{+}$ (3%). Its formula is $C_{30}H_{42}O$ and its semisystematic name is 8'-apo-β-caroten-3-ol. Consequently it is the same pigment as that isolated from the hybrid Robinson [4]. Some analytical data differed from those of the Robinson citraurinene since the free alcohol was stable and easily separable from cryptoxanthin on silica gel according to the method of Gross [6].

In Table 2 the pigment changes of the juice and the flavedo of the mandarin hybrid (*Citrus reticulata*) cv Michal, at three ripening stages are given. In the juice of the unripe fruit no chlorophyll was detected because generally in *Citrus* the maturation of the endocarp occurs earlier than in the peel. The total carotenoid content increased continuously doubling its content from 7.5 to 13.7 μg/ml. The carotenoid distribution in the earliest stage was already similar in its complex pattern to that in the ripe fruit. During ripening only cryptoxanthin was

Table 1. Characterization of the carotenoids of the mandarin hybrid (*Citrus reticulata*) cv Michal in juice and flavedo

Carotenoid	Semi-systematic name	UV λ_{max}^{EtOH}(nm)	TLC: (1) Si gel (2) MgO–Kieselguhr. Solvent system: Me_2CO–petrol	Epoxide test: hypsochromic shift (nm)	Carbonyl test: UV λ_{max} after reduction with $NaBH_4$
Phytofluene	7,8,11,12,7′,8′-Hexahydro-ψ, ψ-carotene	330, 348, 367*		—	—
α-Carotene	β,ε-Carotene	420, 445, 472*		—	—
β-Carotene	β,β-Carotene	425, 450, 478*		—	—
ζ-Carotene	7,8,7′,8′,-Tetrahydro-ψ, ψ-carotene	378, 400, 422*	1:49	—	—
δ-Carotene	ε, ψ-Carotene	431, 456, 487*		—	—
Mutatochrome	5,8-Epoxy-5,8-dihydro-β,β-carotene	397, 420, 445*		—	—
Lycopene	ψ, ψ-Carotene	444, 470, 503*		—	—
β-Apo-8′-carotenal	8′-Apo-β-caroten-8′-al	456		—	400, 423, 449
α-Cryptoxanthin	β,ε-Caroten-3-ol	420, 444, 472		—	—
β-Cryptoxanthin	β,β-Caroten-3-ol	427, 450, 478		—	—
Cryptoxanthin-5, 6-epoxide	5,6-Epoxy-5,6-dihydro-β,β-caroten-3-ol	420, 444, 473	10:40	20	—
Cryptoxanthin-5′,6′-epoxide	5′,6′-Epoxy-5′,6′-dihydro-β,β-caroten-3-ol	420, 444, 473		18	—
β-Citraurinene	8′-Apo-β-carotene-3-ol	402,424,450		—	—
β-Citraurin	3-Hydroxy-8′-apo-β-caroten-8′-al	456		—	400, 423, 449
Lutein	β,ε-Carotene-3,3′-diol	420,444,473		—	—
Zeaxanthin	β,β-Carotene-3,3′-diol	427, 452, 480		—	—
Mutatoxanthin	5,8-Expoxy-5,8-dihydro-β,β-carotene-3,3′-diol	404, 427, 452		—	—
trans-Antheraxanthin	5,6-Epoxy-5,6-dihydro-β, β-carotene-3,3′-diol	420, 444, 472		18	—
cis-Antheraxanthin	5,6-Epoxy-5,6-dihydro-β,β-carotene-3,3′-diol	418, 442, 470	15:35	18	—
Unknown	—	422, 447, 470		—	—
Luteoxanthin	5,6,5′,8′,-Diepoxy-5,6,5′,8′-tetrahydro-β, β-carotene-3,3′,-diol	398, 420, 444		18	—
trans-Violaxanthin	5,6,5′,6′-Diepoxy-5,6,5′, 6′-tetrahydro-β, β-carotene-3,3′-diol	418, 440, 470		38	—
cis-Violaxanthin	5,6,5′,6′-Diepoxy-5,6,5′,6′-tetrahydro-β, β-carotene-3, 3′-diol	412, 437, 465		35	—
Neoxanthin	5′,6′-Epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol	418, 438, 465		18	—
Neochrome	5′,8′-Epoxy-6,7-didehydro-5,6,5′,8′-tetrahydro-β,β-carotene-3,5,3′-triol	397, 420, 444	20:30	—	—
Trollixanthin	5,6-Dihydro-β,β-carotene-3,3′,5,6-tetrol	420, 442, 470			—
Trollichrome	5,8-Epoxy-5,8,5′,6′-tetrahydro-β, β-carotene-3,3′,5′,6′-tetrol	396, 422, 448		—	—

*Measured in petrol as solvent.

Table 2. Pigment distribution in the juice and flavedo of a mandarin hybrid (*Citrus reticulata*) cv Michal during ripening

	Juice			Peel		
	Green	Colour break	Ripe	Green	Colour break	Ripe
Fruit diameter (cm)	4.70	4.85	5.10	4.70	4.85	5.10
Chlorophyll *a* (μg/g fr. wt)	—	—	—	240.0	52.6	—
Chlorophyll *b* (μg/g fr. wt)	—	—	—	86.0	15.8	—
Total carotenoids (μg/g fr. wt)	7.5	9.5	13.7	143.4	51.0	174.1
Carotenoid pattern (% of total carotenoids)						
Phytofluene	2.4	2.9	5.9	—	5.2	3.1
α-Carotene	0.8	0.5	0.5	9.7	2.4	0.2
β-Carotene	1.3	1.3	1.9	6.9	2.5	0.3
ζ-Carotene	4.1	4.2	3.3	—	—	0.4
δ-Carotene	—	—	—	—	—	0.1
Mutatochrome	—	—	—	—	—	0.5
Lycopene	—	—	0.6	—	—	—
β-Apo-8′-carotenal	—	—	—	0.7	0.7	1.3
α-Cryptoxanthin	1.2	1.9	0.9	1.9	—	—
β-Cryptoxanthin	35.0	35.6	41.0	—	3.1	6.4
Cryptoxanthin 5,6-epoxide	—	—	—	—	—	0.4
Cryptoxanthin 5′,6′-epoxide	—	—	—	—	—	0.3
β-Citraurinene	—	—	—	—	9.5	9.9
β-Citraurin	—	—	—	—	12.3	26.1
Lutein	12.7	10.3	6.5	23.5	12.8	2.6
Zeaxanthin	7.2	8.2	9.1	3.9	1.6	1.0
Mutatoxanthin	1.9	1.8	5.7	1.5	0.2	0.2
trans-Antheraxanthin	10.4	6.4	5.6	3.6	4.3	1.8
cis-Antheraxanthin	6.3	9.5	6.8	—	—	2.5
Luteoxanthin	—	3.5	4.8	5.8	5.6	9.1
trans-Violaxanthin	1.7	2.2	0.5	14.0	11.7	9.8
cis-Violaxanthin	7.3	5.8	3.1	11.0	18.2	19.8
trans-Neoxanthin	2.4	2.8	—	11.7	6.6	4.2
cis-Neoxanthin	0.9	0.5	—	3.6	3.3	—
Neochrome	—	—	0.2	—	—	—
trans-Trollixanthin	3.0	1.9	2.6	—	—	—
Trollichrome	—	—	0.3	—	—	—
Unknown	1.4	0.7	0.7	2.2	—	—

biosynthesized and became the principal pigment reaching 41% of the total carotenoids. An increase in the content of zeaxanthin and mutatoxanthin was observed at lower levels. All other pigments decreased including both isomers of antheraxanthin which is the second main pigment in the juice. Two other pigments, although in minor amounts, were detected, ζ-carotene and trollixanthin (5,6-dihydro-β,β-carotene-3,3′,5,6-tetrol), the structure of which was elucidated by Gross *et al.* [7].

In the flavedo, whereas the chlorophylls disappeared abruptly the total carotenoid level reached a minimum at colour break, from 143 to 51 μg/g, increasing again to 174 μg/g in the ripe fruit. In the green fruit the main carotenoid pattern was that found in photosynthetic tissue containing the four basic chloroplast carotenoids, β-carotene, lutein, violaxanthin and neoxanthin but at already modified levels, violaxanthin (25%) exceeding the level of lutein. Earlier in the season the level of violaxanthin is only one-third of that of lutein found in Dancy tangerine [1]. Other carotenoids which conferred additional complexity to the main pattern were mostly of the α-ionone series, such as α-carotene and α-cryptoxanthin. Minor amounts of the citrus specific C_{30}-apocarotenal, β-apo-8′-carotenal were also detectable. During ripening the principal changes connected with chloroplast–chromoplast transformation occurred, that is the decrease of chloroplast carotenoids especially of the α-ionone series and the synthesis of new specific fruit carotenoids mainly of the β-ionone series. At colour break cryptoxanthin and two C_{30}-apocarotenoids, β-citraurin and β-citraurinene, were present at relatively high levels. Phytofluene was also detectable. In the ripe fruit the pattern became even more complex because besides phytofluene, other precursors of the earlier biosynthetic steps such as ζ-carotene and δ-carotene were also detectable in minor amounts. β-Cryptoxanthin, accompanied by two of its epoxides, doubled its concentration. In the apocarotenoid group β-apo-8′-carotenal and citraurinene changed their level only slightly whereas β-citraurin increased markedly from 12.3 to 26.1%, becoming the second principal flavedo pigment after violaxanthin. During ripening violaxanthin underwent changes observed also in other developing fruits, that is an increasing biosynthesis of the *cis*-isomers [1, 8–10].

In Table 3 the quantitative changes of the flavedo pigments and the changes of the carotenoid pattern of Dancy tangerine and Clementine at three ripening stages are given. In both varieties, whereas chlorophyll disappeared abruptly, the level of total carotenoids showed a pronounced minimum at colour break. The essential change during fruit ripening was the drastic decrease of the chloroplast pigments, except for violaxanthin, which increased substantially reaching in Dancy tangerine a level of 50 %. The pigments from which biosynthesis begins at colour break were the chromoplast pigments cryptoxanthin and the C_{30}-apocarotenoids. The level of cryptoxanthin reached *ca* 14 % in the Dancy tangerine and only 6 % in the Clementine. β-Apo-8′-carotenal was detectable in both varieties in small amounts, but citraurin increased to 8.2 % in Dancy and 32.5 % in Clementine becoming the principal pigment of this variety. The unusual pigment β-citraurinene was totally absent in Dancy tangerine but in Clementine it reached a level as high as 15.7 %.

DISCUSSION

The carotenoid pattern of the developing Michal fruit juice was not different from other mandarin varieties or hybrids [3, 9]. The changes of the juice carotenoid pattern during ripening were very similar to those found in Dancy tangerine. The juice of Michal contained the same high content of cryptoxanthin, but more zeaxanthin 9.1 % vs 5.8 % in Dancy and half the level of violaxanthin of Dancy. The deep colour of the juice is due to the high amount of total carotenoids (13.7 μg/ml) compared to Dancy (12 μg/ml) and to orange juice (*Citrus sinensis*) Shamouti, Valencia and Washington which contain 8, 12.5 and 5.5 μg/ml, respectively [11], and to the high percentage (50 %) of the orange pigments cryptoxanthin and zeaxanthin.

The flavedo colour of the Michal hybrid has a bright reddish tint although its total carotenoid content of 174 μg/g is the average value between the two parent varieties, the orange-red Dancy with 295 μg/g and the orange Clementine with only 75 μg/g. This observation is in accordance with the fact that there is not always a direct correlation between total carotenoid and external colour, the colour depending on internal factors and the localization of the plastids in the tissue [3]. The reddish tint of some citrus fruits is due to the pigments absorbing at longer wavelengths, e.g. cryptoxanthin and especially β-citraurin, even if they do not exceed the level of pre-

Table 3. Quantitative changes of the flavedo pigments in Dancy tangerine and Clementine during ripening

	Dancy tangerine*			Clementine		
	Green	Colour break	Ripe	Green	Colour break	Ripe
Fruit diameter (cm)	4.9	5.3	6.5	4.5	4.7	5.1
Chlorophyll *a* (μg/g fr. wt)	249.2	86.0	—	73.7	39.9	—
Chlorophyll *b* (μg/g fr. wt)	80.0	27.0	—	24.4	18.6	—
Total carotenoids (μg/g fr. wt)	121.5	93.5	295.0	30.8	24.9	74.6
Carotenoid pattern (% of total carotenoids)						
Phytofluene	—	3.9	2.7	—	7.7	3.8
α-Carotene	11.7	4.2	0.2	10.5	3.7	0.1
β-Carotene	8.7	4.0	0.4	11.6	7.2	0.4
ζ-Carotene	—	tr.	0.3		—	0.8
Mutatochrome	—	—	—	—	—	1.2
γ-Carotene	—	—	tr.	—	—	0.2
β-Apo-8′-carotenal	0.1	0.2	0.7	—	—	1.8
α-Cryptoxanthin	1.6	—	—	1.2	1.5	—
β-Cryptoxanthin	1.8	4.5	13.6	5.3	9.7	6.2
Cryptoxanthin 5,6-epoxide	—	—	0.7	—	—	0.2
Cryptoflavin	—	0.8	1.3	—	—	—
Cryptoxanthin 5′,6′-epoxide	—	0.4	2.1	—	—	—
β-Citraurinene	—	—	—	4.1	6.8	15.7
β-Citraurin	—	3.0	8.2	2.3	8.3	32.5
Lutein	25.7	15.5	2.7	34.7	20.6	2.0
Zeaxanthin	1.1	0.8	1.5	1.1	1.3	1.2
Mutatoxanthin	0.2	—	—	0.4	0.7	1.1
trans-Antheraxanthin	1.7	2.3	2.3	1.8	1.9	2.1
cis-Anteraxanthin	—	—	6.2	—	—	
Luteoxanthin	2.1	3.6	2.3	4.0	7.7	10.6
trans-Violaxanthin	20.7	19.6	12.5	4.3	3.8	5.5
cis-Violaxanthin	12.0	29.1	39.3	5.3	8.4	9.3
trans-Neoxanthin	11.1	5.4	1.8	12.8	10.4	5.3
cis-Neoxanthin	1.5	2.7	1.2	—	—	—
Neochrome	—	—	—	0.6	0.3	—

*Reproduced from Gross, 1981 [1].

dominating yellow pigments.

The ripening diagram, the changes in the total level during development, showed a minimum which occurred in midseason in all three cultivars analysed. As revealed in recent systematic studies of pigment changes in ripening fruit, the carotenoid content does not continually increase during maturation but follows different ripening curves [12]. A diagram with a minimum at mid-season was found in tomatoes, apples and some *Citrus* [12–15]. According to Laval-Martin [16] the minimum corresponds to the disorganization of the granal system during the chloroplast–chromoplast transformation.

Comparing the carotenoid pattern of the hybrid with that of its two putative parents, all three revealed an equally complex pattern characteristic of *Citrus reticulata*. The hybrid resembles both its parents but even more the Clementine from which it inherited the ability to synthesize the unusual C_{30}-apocarotenoid, which is absent in the Dancy tangerine. Another similarity with Clementine is the high content of β-citraurin and the lower content of cryptoxanthin. Concerning the second principal pigment violaxanthin, its content (both isomers and luteoxanthin) resembles more the first parent.

The hybrid does not synthesize any carotenoids not found in the parents. Some new hybrid pigments found in Sinton citrangequat, a trigeneric hybrid, namely citranaxanthin and reticulataxanthin [17], were found to be artifacts [18]. Furthermore, β-citraurinene is not necessarily a pigment produced only in hybrids. It seems that the biosynthetic potential of Clementine, which is a variety, to synthesize β-citraurin is inherited by its hybrids. Citraurinene was first isolated from the *Citrus* hybrid Robinson, a cross between Orlando tangelo and Clementine [4]. The other hybrids in which these authors found citraurinene are not specified [5]. The Clementine was considered at the beginning of the century as an accidental hybrid. Only later, evidence was brought to show that Clementine is a variety [19].

The comparison of the carotenoid pattern in all three cultivars, all producing β-citraurin, may permit some speculation concerning its biosynthesis. β-Citraurin was thought to be a degradation product of zeaxanthin [20]. As recently the number of C_{30}-apocarotenoids found in *Citrus* increased to eight, it has been suggested that the biosynthesis of these C_{30}-compounds may follow a new pathway [4, 21, 22]. One possible way suggested, through assymetric degradation of a C_{10}-fragment from one side of a C_{40}-carotenoid is confirmed by this study, the C_{40}-carotenoid being cryptoxanthin.

There is a straightforward precursor–product relationship between cryptoxanthin and citraurin, as indicated in Table 4. Citraurin may further undergo an enzymatic reduction yielding as an intermediate, citraurinol (8′-apo-β-carotene-3,8′-diol), and finally citraurinene. Citraurinol was isolated from the hybrid Robinson [21] but was not found either the Clementine or in Michal. The enzyme system which oxidizes cryptoxanthin to citraurin and, subsequently, reduces the aldehyde to citraurinene is different from the Dancy tangerine which is only able, to a lesser extent, to oxidize cryptoxanthin. Treatment with ethylene induced the accumulation of cryptoxanthin, β-citraurin and citraurinene in the flavedo of Robinson [23], this being additional proof for our proposed pathway. The possibility of a different C_{30}-apocarotenoid metabolic pathway cannot, however, be excluded.

Table 4. Comparison of carotenoid levels as a percentage of total carotenoids in Michal and its parental forms

Carotenoid	Dancy	Clementine % of total carotenoids in	Michal
Cryptoxanthin	14	6	6
Citraurin	8	32	26
Citraurinene	—	16	10

EXPERIMENTAL

Fruits were obtained from a citrus grove on the coastal area near Ness Ziona, Israel. The analytical methods were as previously reported [24]. Certain steps were modified. The saponification time was reduced to 3 hr. CC was omitted, the pigments being separated directly by successive TLC on two adsorbents according to the method of Gross [6]. The first chromatogram on Si gel developed with Me_2CO–petrol (3:7) gives a preliminary fractionation into groups of different polarity. A further separation of each group (excepting the apocarotenals) into individual carotenoids is obtained by rechromatography on MgO–Kieselguhr (1:1). The same solvent system is used, the Me_2CO percentage being increased according to the polarity of each group (from 4 to 30%). The MS is a high resolution spectrum run on a Kratos MS-50 mass spectrometer linked with a Kratos DS-50 data system at a dynamic resolution of 15 000.

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