Carotenoid Composition of Yellow Pepper during Ripening: Isolation of β-Cryptoxanthin 5,6-Epoxide

Zoltán Matus, József Deli, and József Szabolcs*

Institute of Chemistry, Pécs University Medical School, Szigeti út 12, H-7643 Pécs, Hungary

Using a HPLC technique, 54 peaks have been detected in the fruits of yellow pepper; 32 carotenoids (90–94% of the total carotenoid content) have been completely or tentatively identified. The study was carried out quantitatively with fruits in six different stages of maturation. Violaxanthin, antheraxanthin, zeaxanthin, lutein, β-cryptoxanthin, β-carotene, α-cryptoxanthin, α-carotene, and probably "post-mortem artifacts" (furanoid oxides and cis isomers) were found. β-Cryptoxanthin 5,6-epoxide, a precursor of cryptocapsin present in red pepper, was also identified. But carotenoids with κ-, 5,6-dihydro-3,5,6,-trihydroxy-β, and 5,6-dihydro-3,6-epoxy-5-hydroxy-β (oxabicyclo[2.2.1]) end groups usually found in red pepper were not detected. In the massive biosynthesis of violaxanthin during maturation, the rates of hydroxylation exceeded those of epoxidation.

INTRODUCTION

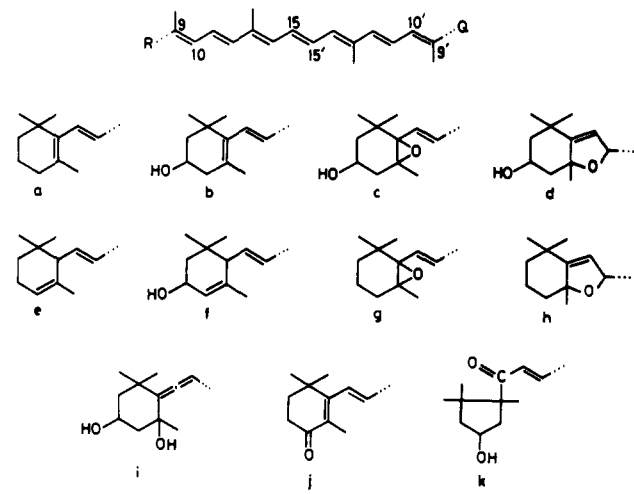
Although paprika (red pepper), as one of the most important and oldest natural food colors, has been investigated for a long time, its yellow variety, whose color never turns red during ripening, has received little attention (Klaüi and Bauernfeind, 1981). The importance of yellow pepper, however, is reflected in its flavoring property and certain vitamin A and C activities. Research on the distribution of carotenoids present in the yellow fruits is also of great importance, as it might provide some clue to the understanding of the biosynthetic pathways of formation not only of yellow carotenoids in yellow paprika but also of both yellow and red carotenoids present in red paprika.

A detailed pioneer work (Cholnoky et al., 1958) on the qualitative and quantitative distribution of carotenoids in yellow paprika (*Capsicum annuum lycopersiciforme flavum*) revealed that the yellow fruits, although containing a large amount of 5,6-epoxy carotenoids (antheraxanthin, violaxanthin) from which the red carotenoids are formed via pinacolone rearrangement (Weedon, 1971) in red pepper (*C. annuum lycopersiciforme rubrum*), were lacking the red carotenoids (capsanthin, capsorubin, cryptocapsin).

The objectives of this work were to study the quantitative changes of carotenoids in a particular variety of yellow paprika (Szentesi sárga paradicsom paprika) during ripening and to correlate the carotenoid biosynthesis of *C. annuum lycopersiciforme flavum* to that of *C. annuum lycopersiciforme rubrum*, first of all, with respect to new minor carotenoids with 5,6-dihydro-3,5,6-trihydroxy-β and 5,6-dihydro-3,6-epoxy-5-hydroxy-β (oxabicyclo[2.2.1]) end groups present in red pepper (Parkes et al., 1986). It was also considered whether cis isomers and furanoid oxides present in pepper were natural or artificial products.

MATERIALS AND METHODS

The yellow peppers used (*C. annuum lycopersiciforme flavum*, cv. Szentesi sárga paradicsom paprika) were obtained commercially in Pécs (southern Hungary) in August 1988. General methods of handling including routine tests (partition coefficients, epoxide–furanoid oxide rearrangement, reduction of carbonyl functions with metal hydrides, etc.) and column chromatography have been described elsewhere (Molnár and Szabolcs, 1979). The quantitative determination of the total carotenoid content of fruits was performed in the absence of chlorophylls



Antheraxanthin, R = c, Q = b; auroxanthin, R = Q = d; canthaxanthin, R = Q = j; α-carotene, R = a, Q = e; β-carotene, R = Q = a; β-carotene monoepoxide, R = g, Q = a; cryptocapsin, R = a, Q = k; α-cryptoxanthin, R = b, Q = e; β-cryptoxanthin, R = b, Q = a; β-cryptoxanthin 5,6-epoxide, R = c, Q = a; lutein, R = b, Q = f; lutein epoxide, R = c, Q = f; luteoxanthin, R = c, Q = d; mutatochrome, R = h, Q = a; mutatoxanthin, R = d, Q = b; neochrome, R = d, Q = i; neoxanthin X, R = c, Q = i; violaxanthin, R = Q = c; zeaxanthin, R = Q = b.

(Davies, 1976). The quantitative determination of chlorophylls was carried out in an ethereal extract (Comar and Zscheile, 1942). Analytical grade chemicals were used, and authentic samples were taken from our collection. The sample solutions were stored under nitrogen, away from light at −20 °C.

Instruments. Melting points were determined with a Boetius hot-stage apparatus and were not corrected. UV–vis spectra were recorded with a Perkin-Elmer 402 instrument. CD spectra were taken in MeOH solutions at room temperature on a Rousset-Juan Dichrographe III (Jobin Ivon), in quartz cells.

High-Performance Liquid Chromatography. *Selection of a Chromatographic Approach.* Considering the complexity of carotenoid extracts, the diversities in polarity of carotenoids, and the need for a practical use of monitoring the process of ripening, a reverse phase and gradient elution were used. This technique enabled chromatographic separation of the polar and nonpolar carotenoids.

Apparatus and Chromatographic Conditions. The chromatographic system consisted of Models 250B and 300B HPLC pumps (Gynkotek), a Glenco injector and a Beckman UV–vis variable-wavelength detector (Ohmacht, 1979). Columns were 250 × 4.6 mm i.d. (Labor MIM) packed with Chromsil C_{18} 6 μm end-

0021-8561/91/1439-1907$02.50/0

Table I. Miscellaneous

properties	stage of maturation 1	2	3	4	5	6
fresh weight, g	596.36	508.19	350.65	311.08	202.50	239.17
dry weight (dw), g	12.92	10.42	6.60	5.86	4.14	4.83
dry weight/fresh weight, %	2.17	2.05	1.88	1.88	2.02	2.02
chlorophyll content, mg/100 g of dw	63.14	39.21	28.87	2.95	0	0
total carotenoid,[a] mg/100 g of dw	13.19	31.40	105.30	186.81	317.03	488.64
total carotenoid,[b] mg/100 g of dw	16.04	22.60	88.67	200.92	263.72	448.78
total carotenoid,[c] mg/100 g of dw	13.00	25.34	98.36	212.10	286.64	476.32
partition coefficient						
before saponification	2.03					0.28
after saponification	1.95					2.29
phytoxanthin, %	76.22					89.75
esterified phytoxanthin, %	12.03					75.63

[a] Calculated by HPLC. [b] Calculated by $E^{1\%}_{1cm}$ = 2300 (Davies, 1976). [c] Calculated by individual $E^{1\%}_{1cm}$.

capped (Labor MIM) and Chromsil C_{18} 6 μm not endcapped (Labor MIM). The eluent was 12% (v/v) H_2O in methanol (A), methanol (B), and 50% (v/v) acetone in methanol (C). The gradient program was 100% A, 8 min, to 80% A/20% B in 8 min, to 50% A/50% B in 8 min, to 100% B in 7 min, 100% B 2 min, to 100% C in 6 min, 100% C 5 min (linear steps). The flow rate was 1.5 mL/min, and detection was at 430, 450, 480, 400, and 340 nm.

Preparation of Samples. After evaporation of the saponified carotenoid extracts (see Carotenoid Extraction), the residues were dissolved in a mixture (3:7) of acetone and methanol. The concentration of the samples was approximately 2–8 × 10^{-4} M (0.1–0.4 mg/mL); the injection volume was 10–50 μL. For standard solutions authentic samples were taken directly from our collection or derivatives were freshly prepared from the authentic samples by means of well-known reactions, i.e., epoxide–furanoid oxide rearrangement (Karrer and Jucker, 1950) and trans–cis stereomutation (Zechmeister, 1962). The derivatives were separated on $CaCO_3$ (Biogal) columns with benzene or a mixture of benzene and petroleum ether (40–60 °C). The furanoid oxide epimers were designated in decreasing order of their adsorption on the $CaCO_3$ columns. The standard solutions were prepared as above, and the concentrations were about 2–4 × 10^{-5} M (0.01–0.02 mg/mL); the injection volume was 5–20 μL. As an internal standard, a 2–4 × 10^{-5} M benzene solution of canthaxanthin [Hoffmann-La Roche, mp 205 °C, recrystallized from benzene–petroleum ether (30–40 °C)] was used. At all times except that of the experiments, the solutions were stored at −20 °C under nitrogen away from light. The total sample handling time was 20 min or less.

Identification of Peaks. To increase the differences in polarity between carotenoids, the carotenoid esters were saponified (see Carotenoid Extraction) before analysis. The peaks in a chromatogram were identified by means of authentic carotenoid samples, different chemical tests, and appropriate variation of the wavelength of detection (Baranyai et al., 1982). For example, the 5,6-epoxides were converted into furanoid oxides by acid treatment and oxocarotenoids into alcohols by $NaBH_4$ reduction; all derivatives showed different retention times and absorption maxima. Similarly, comparison of the corresponding peak heights at different wavelengths of detection allows differentiation between chromophore systems. To achieve complete conversion and avoid trans–cis isomerization, the chemical tests were performed under strictly controlled conditions.

Quantification. The chromatograms were evaluated quantitatively by relating the heights of the individual carotenoids to that of canthaxanthin used as an internal standard (Baranyai et al., 1982). The ratios of the 430-nm mole extinctions of the authentic samples in a chromatogram to that of canthaxanthin were used as detector signals to the amount of identified carotenoids in the sample introduced. For the quantitation of unidentified pigments a mean value of mole extinction was employed. The minor peaks were evaluated in repeated chromatograms by using optimized detection limits for them. The coefficient of variation (CV) varied from major (30–10%) to minor (9–1%) and trace carotenoids (0.9–0.05%), amounting to CV values 0.47–0.79, 1.29–4.84, and 7–30, respectively.

Carotenoid Extraction. The fruits in different stages of ripening were divided into six batches according to their colors from green to orange. Ripe fruits were collected from an open field in August and September 1988. To obtain reliable samples, 200–600 g (fresh weight) of pods, freed from their shells and seeds, were used for extraction. Each of the batches was blended with MeOH and about 1% calcium carbonate. The blendate was allowed to stand in MeOH (100 g of pods requires 300 mL of MeOH) for dehydration. After 18 h, the mixture was filtered and the filter cake extracted with 200 mL of MeOH for 24 h. The extraction was repeated twice with 200 mL of MeOH and finally with Et_2O. After suction, the two MeOH and ethereal extracts were combined, transferred to a separatory funnel, diluted with Et_2O, washed free from methanol with water, dried over anhydrous Na_2SO_4, evaporated in vacuo to about half-volume, and saponified with 30% KOH–MeOH at room temperature for 18 h. After saponification, monitored by liquid–liquid partition tests, the ethereal solution was washed free from alkali and evaporated to dryness in vacuo, and the residue was dissolved in 100 mL of benzene. Since the first methanolic solution obtained in the process of dehydration also contains polar carotenoids, it was worked up separately. Aliquots of the two solutions containing the total carotenoid content were mixed immediately before HPLC analysis.

Preparation of Violaxanthin, Antheraxanthin, Lutein, and Zeaxanthin in Crystalline Form. Ripe fruits, freed from their shells and seeds (3.4 kg of fresh weight), were used for extraction. Further operations were similar to those used for HPLC analysis; however, only the ethereal extract was worked up. After saponification, etc., the residue was dissolved in benzene and the hypophasic pigments were precipitated with hexane (197 mg). The mother liquor was evaporated in vacuo to dryness and dissolved in benzene, and the epiphasic pigments were precipitated with MeOH (63 mg). Separation of the hypophasic pigments was achieved by column chromatography on calcium carbonate (Biogal) with a 3:2 mixture of benzene and hexane. The following zones were obtained: band 1 (unidentified), band 2 (violaxanthin), band 3 (antheraxanthin), band 4 [a mixture of lutein (upper part of the zone) and zeaxanthin], and band 5 (unidentified). Rechromatography of the individual pigments on calcium carbonate (Biogal) with benzene gave violaxanthin [crystallized from MeOH; 26 mg; mp 176 °C; λ_{max} (in benzene) 483, 452, and 426 nm] with 15% hexane in benzene gave antheraxanthin [crystallized from MeOH; 9.8 mg; mp 181 °C; λ_{max} nm (in benzene) 487, 458 and 434 nm], and with 30% benzene in hexane gave lutein [crystallized from benzene–hexane; 4.0 mg; mp 165 °C; λ_{max} nm (in benzene) 488 and 457 nm] and zeaxanthin [crystallized from MeOH; 6.8 mg; mp 176 °C; λ_{max} (in benzene) 493 and 463 nm].

RESULTS AND DISCUSSION

To avoid trans–cis isomerism and epoxide–furanoid oxide rearrangement, great care was taken during the isolation procedures. The individual carotenoid content and total carotenoid and chlorophyll contents were expressed on the basis of the weight and dry weight of fruits. After separation of the carotenoid pigments by HPLC, an

Table II. Relative Carotenoid Content (%) of *C. annum* Fruit at Six Stages of Maturation

peak no.	pigment	stage of maturation 1	2	3	4	5	6
1[a]		0.76	0.44	0.18	0.22	0.16	0.05
2[a]		0.45	0.39	0.25	0.21	0.19	0.21
3[a]		0.14	0.18	0.07	0.13	0.07	0.06
4[a]		0.32	0.33	0.18	0.15	0.10	0.13
5[a]		0.13	0.15	0.12	0.18	0.08	0.06
6[a]		0.45	0.79	0.63	0.69	0.49	0.50
7[a]		0.11	0.14	0.10	0.09	0.08	0.07
8[a]		0.24	0.14	0.13	0.12	0.07	0.08
9[a]		0.16	0.22	0.19	0.15	0.07	0.08
10[a]		0.76	0.71	0.63	0.78	0.36	0.53
11[a]		0.85	0.79	0.62	0.53	0.35	0.20
12	neoxanthin X + neochrome epimer 3[b,c]	0.89	1.11	0.83	1.06	0.46	0.49
13[a]		1.47	1.55	0.74	0.87	0.36	0.34
14	neochrome epimer 1[b,c] + neochrome epimer 4[b,c]	0.32	0.23	0.23	0.19	0.14	0.07
15	neoxanthin + neochrome epimer 2[b,c]	5.53	6.06	2.70	2.46	1.04	0.81
16	violaxanthin + *cis*-neochromes[b]	3.03	8.87	15.97	20.55	26.53	34.07
17	luteoxanthin epimer 2[c]	3.33	7.33	8.21	8.29	7.44	5.84
18	luteoxanthin epimer 1[c]						
19	auroxanthin epimer 2[c]	1.35	2.41	2.30	1.98	1.86	0.73
20	auroxanthin epimer 3[c]	0.53	0.96	0.83	1.68	0.64	0.20
21	auroxanthin epimer 1[c]	1.89	2.60	2.15	1.94	1.80	0.53
22	9-*cis*-violaxanthin	1.71	2.41	1.36	0.92	0.59	0.00
23	lutein epoxide	0.38	0.27	0.46	0.43	0.77	0.53
24	antheraxanthin	0.38	1.64	4.58	6.51	7.79	10.55
25	13-*cis*-violaxanthin	0.45	1.09	1.52	1.50	1.28	1.55
26	mutatoxanthin epimer 2[c]	0.38	0.77	1.36	1.40	1.85	1.17
27	mutatoxanthin epimer 1[c]	0.38	0.90	1.73	1.58	2.18	1.28
28[a]		0.09	0.09	0.08	0.12	0.07	0.09
29[a]		0.25	0.20	0.14	0.16	0.14	0.13
30	lutein	37.83	34.17	19.79	13.43	11.52	9.26
31	zeaxanthin	2.55	3.81	8.04	7.46	7.73	8.50
32[a]		0.07	0.00	0.37	0.42	0.51	0.50
33[a]		0.07	0.22	0.27	0.28	0.38	0.30
34[a]		0.11	0.15	0.08	0.07	0.11	0.40
35	9(9')-*cis*-lutein	1.58	1.25	0.81	0.29	0.52	0.43
36	13(13')-*cis*-lutein + 9-*cis*-zeaxanthin	3.74	1.98	1.66	1.50	1.20	0.94
37	13-*cis*-zeaxanthin	0.32	0.28	0.69	0.66	0.63	0.65
38[a]		0.07	0.07	0.12	0.09	0.09	0.08
39[a]		0.35	0.16	0.09	0.11	0.12	0.08
40	canthaxanthin						
41[a]		0.12	0.14	0.10	0.13	0.12	0.08
42	β-cryptoxanthin 5,6-epoxide	0.00	0.02	0.06	0.10	0.09	0.11
43[a]		0.12	0.12	0.09	0.13	0.13	0.05
44	α-cryptoxanthin	0.76	1.41	3.57	3.82	5.39	4.39
45	β-cryptoxanthin	0.38	0.60	2.01	2.72	2.50	2.20
46[a]		0.10	0.16	0.31	0.43	0.28	0.18
47[a]		0.04	0.09	0.06	0.06	0.05	0.05
48[a]		0.25	0.23	0.10	0.21	0.15	0.11
49[a]		0.12	0.13	0.15	0.34	0.23	0.15
50	mutatochrome	0.45	0.20	0.26	0.40	0.31	0.31
51[a]		0.21	0.16	0.15	0.16	0.15	0.12
52[a]		0.10	0.07	0.13	0.13	0.12	0.05
53	α-carotene	1.17	2.11	5.77	6.50	6.22	5.88
54	β-carotene	19.86	8.32	6.00	4.63	3.59	4.24
55	*cis*-β-carotene	2.75	1.01	0.95	0.97	0.71	0.63
	total carotenoid, mg/100 g of dw	13.19	31.40	105.03	186.81	317.03	488.64

[a] Unidentified. [b] Tentatively identified. [c] The numbers indicate the adsorption affinities, in decreasing order, on a calcium carbonate column.

additional calculation of total carotenoid content was carried out by using the individual molar extinction values for the identified and the average values ($E^{1\%}_{1cm}$ = 2300) for the unidentified components (Davies, 1976). The partition coefficients measured before and after saponification made it possible to determine the percentages of hydrocarbons, phytoxanthins (carotenoid alcohols), and esterified phytoxanthins present in the fruits (Table I).

The changes of the carotenoids present in the fruits of yellow pepper at six different stages of maturation are shown in Table II. We assume that the different stages of maturation can be characterized more exactly by the total carotenoid content of the fruit as a function of temperature, radiation of the sun, etc., than by the duration of maturation in days, chlorophyll content, or color. (Chlorophylls disappear in an early stage of maturation of *C. annuum lycopersiciforme flavum*.) In all the different stages of maturation, the same 54 peaks were found (Figure 1), of which 27 were identified by means of cochromatography using authentic samples, UV–vis spectra, different chemical tests, etc. (Matus et al., 1981). As a result of mixed peaks, 32 carotenoids were identified in the 27 peaks (Figures 1–3). In the course of ripening the amount of identified carotenoids increased from 90 to 94%. In the ripened fruits, violaxanthin (3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*S*) and antheraxanthin (3*S*,5*R*,6*S*,3′*R*) accounted for about 34 and 11% of the total, respectively, zeaxanthin and lutein for about 9% each, β-cryptoxanthin and β-carotene for about

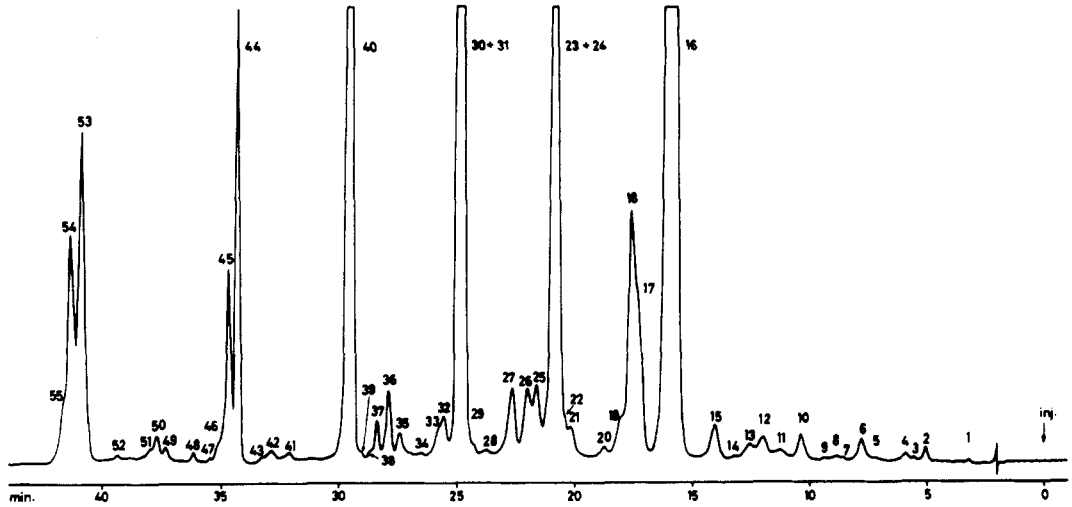


Figure 1. HPLC separation of carotenoids in yellow pepper: Chromsil C_{18} 6 μm endcapped; detection at 430 nm; other conditions as in text. For peak number, see Table II.

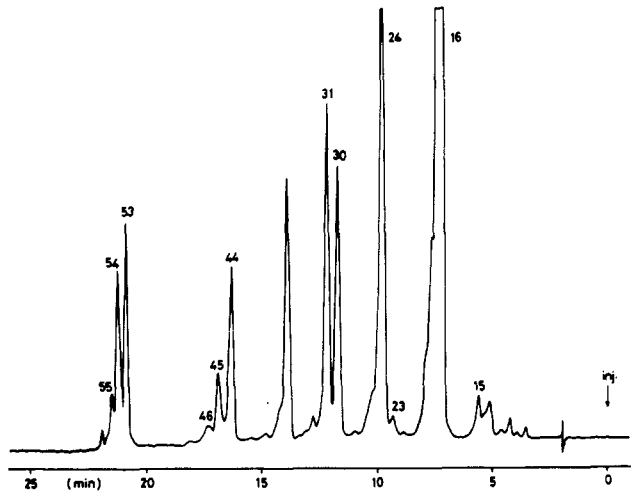


Figure 2. HPLC separation of carotenoids in yellow pepper: Chromsil C_{18} 6 μm not endcapped; detection at 430 nm; other conditions as in text. For peak number, see Table II.

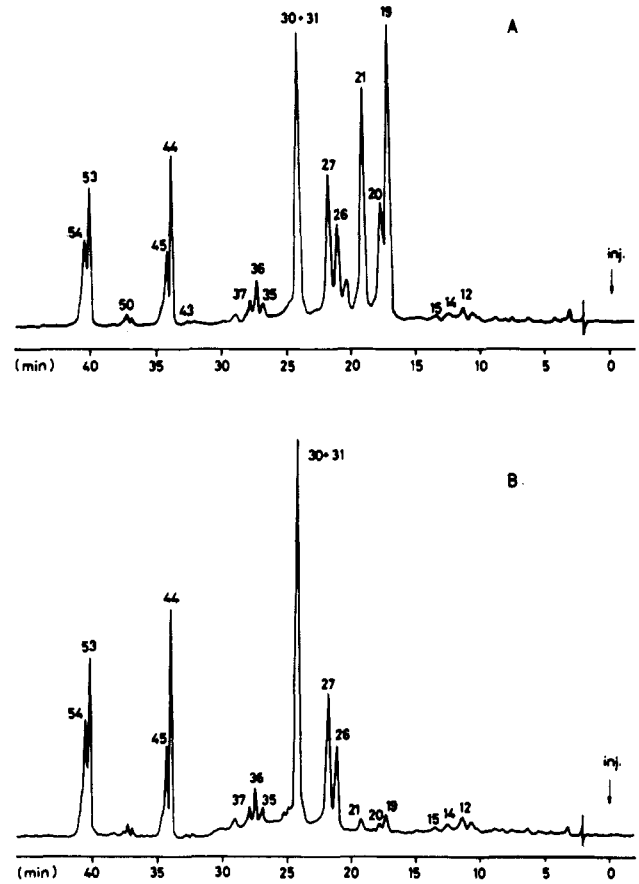


Figure 3. HPLC chromatograms after acid treatment: Chromsil C_{18} 6 μm endcapped; other conditions as in text. For peak number, see Table II. (A) Detection at 430 nm; (B) detection at 450 nm.

4% each, α-cryptoxanthin for about 5%, and α-carotene for about 6%. Numerous furanoid oxides (epimers of auroxanthin, of luteoxanthin, of mutatoxanthin, and of neochrom) and cis isomers, probably as "post-mortem artifacts", were present in an amount of 10% or less.

The changes of total carotenoid content, total epoxide content including furanoid oxides, and the amount of chlorophylls during maturation are presented in Figure 4. By the stage of ripening, characterized by complete disappearance of the chlorophylls from the fruits, their total carotenoid content had already reached two-fifths of the total carotenoid and one-third of the total epoxide contents of the ripest fruits. The total carotenoid and total epoxide contents of the fruits are increased 40-fold and 105-fold, respectively. It should be noted that a 13-fold increase during maturation had been reported earlier (Cholnoky et al., 1958), which was certainly due to the difficulty of defining maturity and, first of all, to the different varieties of *C. annuum lycopersiciforme flavum* investigated. At the beginning of maturation phytoxanthins make up 76% and at its end 90% of the total carotenoid content. During maturation the percentage of esterified phytoxanthins increases from 12 to 76% of the total carotenoid content (Table I).

Figure 5 shows the changes in milligrams per 100 g of dry weight as a function of ripening for various groups of carotenoids. We obtained practically straight lines with different slopes, indicating the rates of formation in decreasing order as follows: epoxides, alcohols, and hydrocarbons. This finding is in agreement with the sequence of carotenoids in the biosynthetic pathway (Scheme I); i.e., the principal epoxide, violaxanthin, is the end product. The same representation for individual carotenoids is shown in Figure 6. During maturation, the concentration of violaxanthin increases 400-fold and even that of β-carotene 8–9-fold in spite of the fact that β-carotene shows a decrease in percentage concentration (Figure 7). The ratio of lutein to zeaxanthin drops from 14.8 to 1.1.

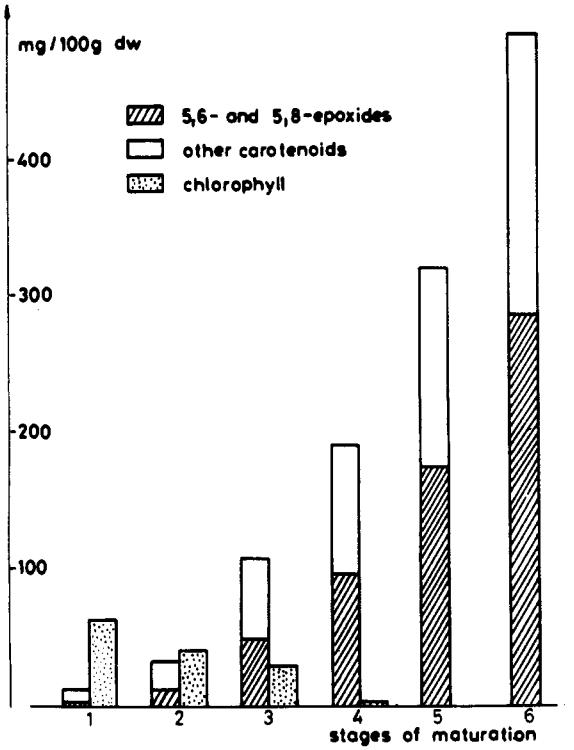


Figure 4. Changes in total carotenoid and chlorophyll content of *C. annuum* fruit.

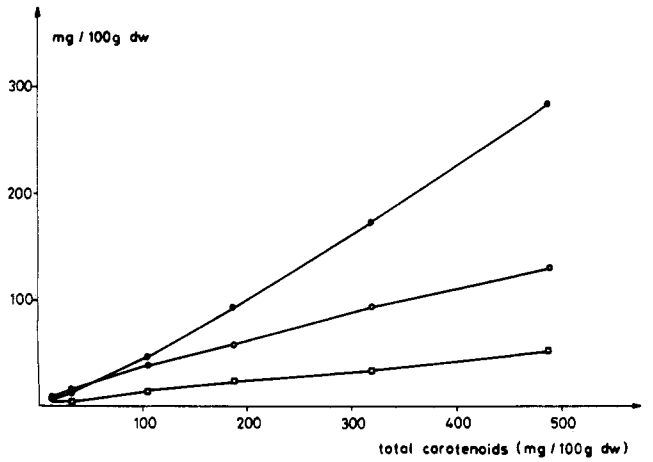
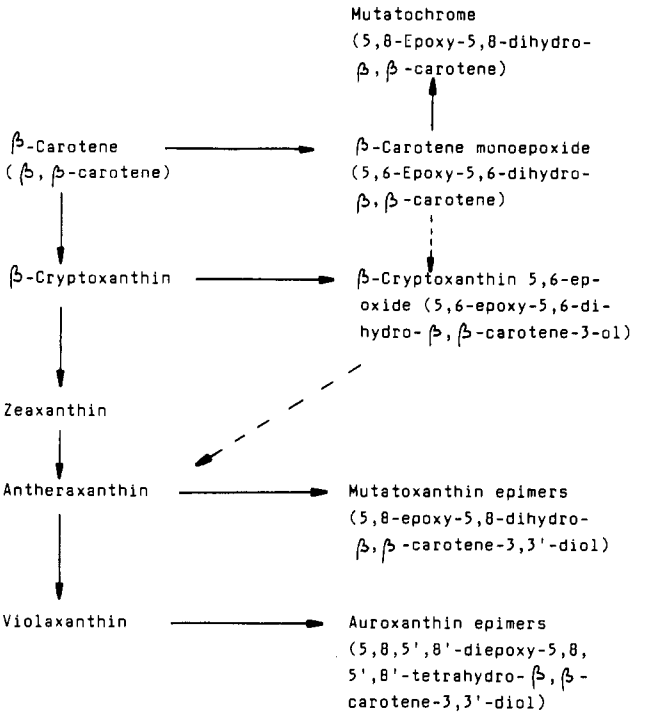


Figure 5. Pigment changes during ripening. (●) Epoxides; (○) monools + diols; (□) carotenes.

Scheme I. Pathway from β-Carotene to Violaxanthin in the Fruits of Yellow Pepper



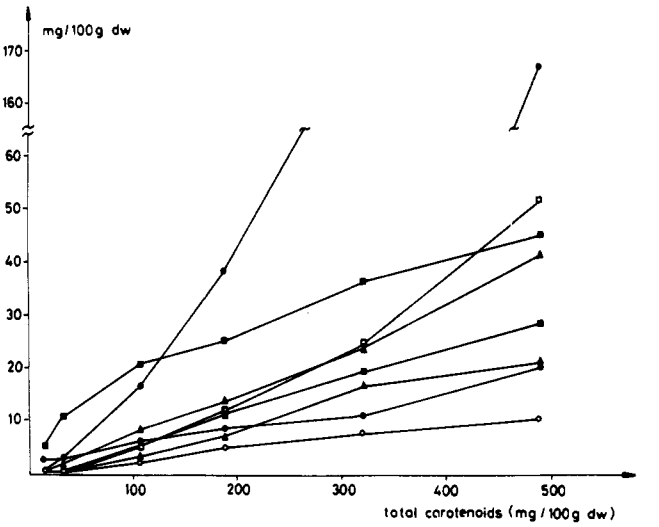


Figure 6. Carotenoid changes during ripening. (●) Violaxanthin; (□) antheraxanthin; (△) zeaxanthin; (○) β-cryptoxanthin; (◐) β-carotene; (■) lutein; (▲) α-cryptoxanthin; (◧) α-carotene.

In Figure 7, the percentage distribution of carotenoids is plotted against the total carotenoid content, i.e., as a function of maturity. Antheraxanthin, zeaxanthin, β-cryptoxanthin, α-cryptoxanthin, and α-carotene increase and β-carotene decreases up to the stage disappearance of chlorophyll when they reach a constant equilibrium concentration. A sharp increase of violaxanthin (end product of the biosynthesis of carotenoids with β end groups) and a sharp decrease of lutein (end product of the biosynthesis of carotenoids with an ε end group) are observed during the whole process of ripening.

In Table II it is demonstrated that furanoid oxides (auroxanthin, luteoxanthin, mutatoxanthin, mutatochrome) are always present during the process of ripening. The percentage concentration of each of them increases in the early stages of maturation, but after the disappearance of chlorophyll there is a steady decrease. The decreasing ratios of the different furanoid oxides and the corresponding 5,6-epoxides are shown in detail in Figure 8. These decreasing ratios point to a situation in which the smoothly rising curves of luteoxanthin plus auroxanthin and those of luteoxanthin plus auroxanthin plus neoxanthin (formed indirectly from zeaxanthin) are converted into nearly horizontal straight lines by plotting milligrams of pigment per 100 g of dry weight vs total carotenoid content, while *trans*-violaxanthin gives a steeply rising curve (Figure 9). It is also illustrated how the curve of violaxanthin vs maturation is converted to a linear line by adding violaxanthin, the parent compound, to its derivatives step by step.

The isolation of furanoid oxides (=5,8-epoxides), however, always raises the question of whether they represent true natural products or are merely artifacts formed via 5,6-epoxide–furanoid oxide rearrangement during isolation. According to data in the literature, the amount of furanoid oxides and their ratio to 5,6-epoxides in plants vary very widely (Szabolcs, 1990; Tóth and Szabolcs, 1970). Since we detected furanoid oxides in decreasing proportion to the corresponding 5,6-epoxides at every stage of ripening

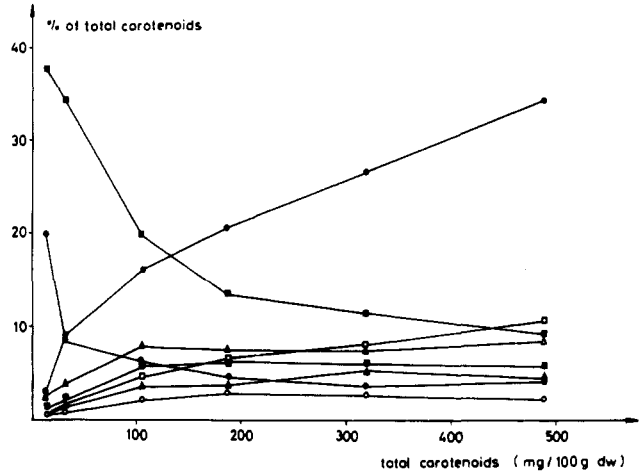


Figure 7. Changes in relative carotenoid content during ripening. (●) Violaxanthin; (□) antheraxanthin; (△) zeaxanthin; (○) β-cryptoxanthin; (◐) β-carotene; (■) lutein; (▲) α-cryptoxanthin; (◧) α-carotene.

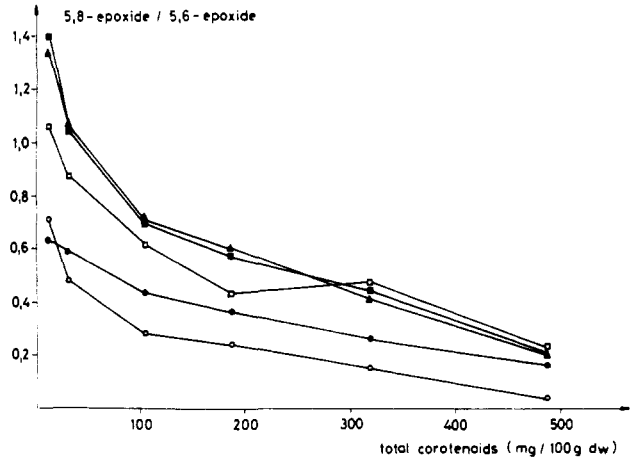


Figure 8. Changes in the ratios of different furanoid oxides and the corresponding 5,6-epoxides during ripening. (●) Luteoxanthins/violaxanthin; (○) auroxanthins/violaxanthin; (▲) luteoxanthins + auroxanthins/violaxanthin; (□) mutatoxanthins/auroxanthin; (■) total furanoid oxide/total 5,6-epoxide.

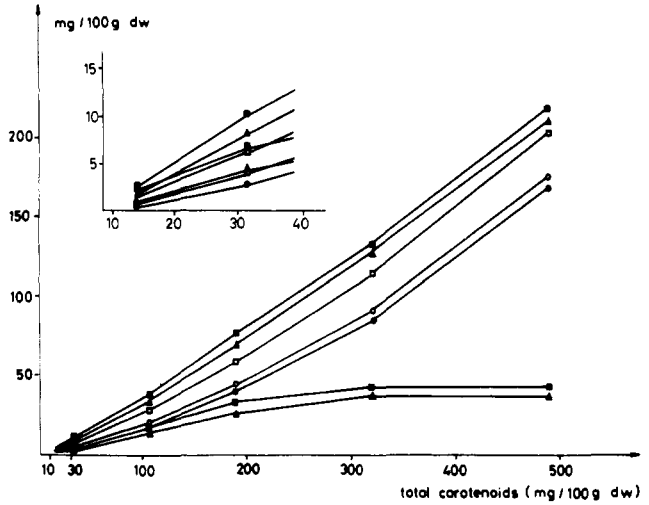


Figure 9. Changes in the content of violaxanthin and its derivatives during ripening. (●) *trans*-Violaxanthin; (○) *trans*- + *cis*-violaxanthin; (□) *trans*- + *cis*-violaxanthin + luteoxanthins; (△) *trans*- + *cis*-violaxanthin + luteoxanthins + auroxanthins; (■) *trans*- + *cis*-violaxanthin + luteoxanthins + auroxanthins + neoxanthins; (▲) luteoxanthins + auroxanthins; (◧) luteoxanthins + auroxanthins + neoxanthins.

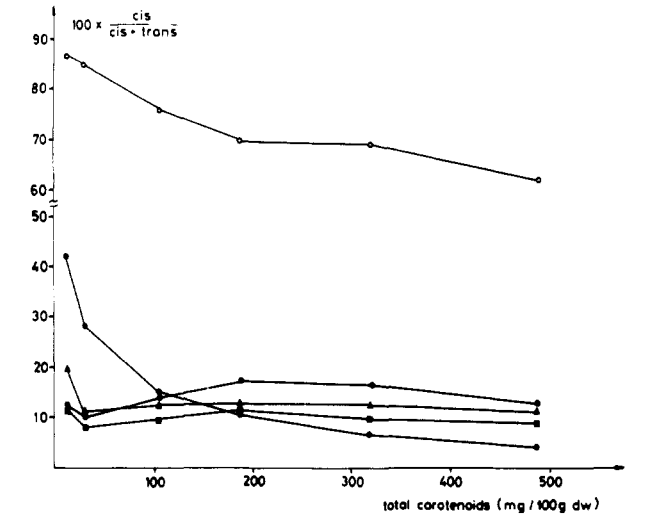


Figure 10. Changes in percentage concentration of cis isomers during ripening. (●) Violaxanthin; (△) zeaxanthin; (◐) β-carotene; (■) lutein; (○) neoxanthin.

and the furanoid oxides with their different epimers were present, we conclude, according to Liaaen-Jensen's definition (Liaaen-Jensen, 1990), that the furanoid oxides are post-mortem artifacts. In our experiment not a single furanoid oxide epimer was found without its epimer(s), which was indicative of the lack of stereospecific enzymatic action.

As we have seen so far, cis isomers were always remotely present in the fruits of yellow pepper in the course of maturation, but their percentage concentration (cis form/cis form plus trans form) decreased with the time of ripening (Figure 10). This tendency is very specific to neoxanthin (the 9-cis form) because the percentage concentration decreases with ripening, as is to be expected, but at the end of ripening the concentration of the 9-cis form reaches even double that of the all-trans form. The curve of *cis*-violaxanthins also starts from a high value of 40% but falls to 5%. *cis*-Zeaxanthins, *cis*-luteins, and *cis*-β-carotenes increase slightly with time until the decomposition of chlorophyll, after which they remain at a more or less constant value. Since the 9-cis and 13-cis isomers occur together and their average total concentration is only about 5–6%, we conclude that the mono-cis isomers are likely to be post-mortem artifacts. However, special attention should be paid to the high ratio of 9-*cis*-neoxanthin to *all-trans*-neoxanthin.

For assessment of the rate of pigment biosynthesis we used what we describe as "integrated carotenoid content", in which the concentration of a carotenoid and those of the others synthesized from it via the biosynthetic pathway are added together. This expression of concentration includes both the actual carotenoid content and the transformed carotenoid content. Thus, the following conversions were considered: β-carotene → β-cryptoxanthin → zeaxanthin → antheraxanthin → violaxanthin, α-carotene → α-cryptoxanthin → lutein → 5,6-lutein epoxide and 5,6-epoxides → 5,8-epoxides (=furanoid oxides). For example, the integrated carotenoid content of zeaxanthin is calculated as follows: actual zeaxanthin content (present in the sample) + actual antheraxanthin content (present in the sample) + actual violaxanthin content (present in the sample).

Figure 11 shows the changes of the integrated carotenoid contents of 5,6- and 5,8-epoxides, mono- and diols, and carotenes as a function of maturation. The values for the slopes of the straight lines were compared (tgα

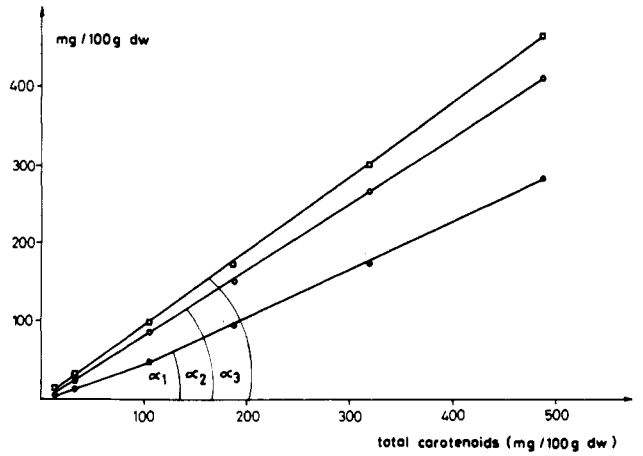


Figure 11. Changes of integrated carotenoid content during ripening. (●) Epoxides; (○) monools + diols; (□) carotenes.

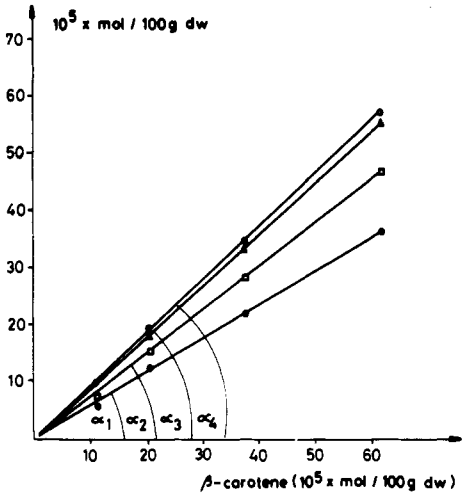


Figure 12. Changes in integrated carotenoid content during ripening (carotenoids with β,β-end groups). (●) Violaxanthin; (□) antheraxanthin; (△) zeaxanthin; (○) β-cryptoxanthin.

(carotenes) > tgα(mono- and diols) > tgα(5,6- and 5,8-epoxides), which led to the conclusion that

$$\frac{v(\text{hydroxylation})}{v(\text{epoxidation})} = \frac{\text{tg}\alpha_2}{\text{tg}\alpha_1} = 1.44\ (\pm 0.04)$$

Thus, disregarding the distinction between β and ϵ end groups, between first and second hydroxylation, and between first and second epoxidation, the overall rate of hydroxylation is 1.5 times as great as that of epoxidation.

Furthermore, for comparison of the rates of the first and second steps of hydroxylation of the β,β structures and those of the first and second steps of epoxidation of the β,β structures (Scheme I), the integrated carotenoid contents were plotted as a function of total carotenoid content (Figure 12). Similar reasoning applied to the evaluation of Figure 11 led to the following proportions:

$$\frac{v(\text{1st hydroxylation})}{v(\text{2nd hydroxylation})} = 1.04\ (\pm 0.01)$$

$$\frac{v(\text{1st epoxidation})}{v(\text{2nd epoxidation})} = 1.31\ (\pm 0.01)$$

$$\frac{v(\text{2nd hydroxylation})}{v(\text{1st epoxidation})} = 1.17\ (\pm 0.01)$$

It should be apparent that the relationship between the slopes is inevitably as follows:

tgα(β-carotene) > tgα(β-cryptoxanthin) > ... tgα(violaxanthin)

Similar graphing of the data for carotenoids with β,β and β,ϵ-structures led to the conclusion that while the rate of the first hydroxylation step slightly exceeds that of the second for β,β structures, the effects is marked for β,ϵ structures.

$$\frac{v(\text{hydroxylation at the } \beta \text{ ring})}{v(\text{hydroxylation at the } \epsilon \text{ ring})} = 1.52\ (\pm 0.08)$$

Furthermore, the rate of epoxidation of the β end group is much greater for β,β structures than for β,ϵ structures.

We succeeded in isolating β-cryptoxanthin 5,6-epoxide [(3*S*,5*R*,6*S*)-5,6-epoxy-5,6-dihydro-β,β-caroten-3-ol] from nature for the first time: peak 42, 0.1%, λ_{max} nm (in methanol) 472, 442 nm; λ_{max} (in methanol) after acid treatment 450, 426 nm. The 5,6-epoxy structure is in complete agreement with the structure of cryptocapsin [(3′*S*,5′*R*)-3′-hydroxy-β,κ-caroten-6′-one), which is formed from β-cryptoxanthin 5,6-epoxide via a pinacolone rearrangement in red pepper. Full details of the determination of the structure of β-cryptoxanthin 5,6-epoxide will be published elsewhere.

We also found β-carotene 5,8-epoxide (originating from the corresponding 5,6-epoxide) and lutein 5,6-epoxide in traces. This finding agrees with our conclusion that the rate of hydroxylation is greater than that of epoxidation. So it is asumed that as soon as a little β-carotene 5,6-epoxide has been formed it begins to transform rapidly into β-cryptoxanthin 5,6-epoxide and antheraxanthin. In contrast, lutein with its β,ϵ structure undergoes a very slow process of epoxidation. It should be mentioned that as post-mortem artifacts of lutein 5,6-epoxide neither flavoxanthin nor chrysanthemaxanthin was found.

Identification of the most important 27 peaks (Table II), totaling 90–94% during ripening, was performed in the HPLC chromatogram, but 27 peaks remained unidentified. Half of the unidentified peaks exceeded the polarity of violaxanthin in the HPLC chromatogram, and they amounted to about 50% of the total of unidentified carotenoids.

Using authentic samples an attempt was made to identify in yellow pepper karpoxanthin and carotenoids with a 5,6-dihydro-3,6-epoxy-β (oxabicyclo [2.2.1]) end group present in red pepper (Parkes et al., 1986), but it was not successful. Therefore, we believe that carotenoids with the oxabicyclo[2.2.1] end group are only formed in red pepper, which finding might be utilized in taxonomic studies in the future.

It should be noted that according to our CD measurements the antheraxanthin and violaxanthin occurring in red pepper do not differ in absolute configuration from the antheraxanthin and violaxanthin occurring in yellow pepper; i.e., the 3-hydroxy-5,6-epoxy-5,6-dihydro-β end groups have the same 3*S*,5*R*,6*S* absolute configuration. Thus, the assumption that the lack of formation of capsanthin and capsorubin in yellow pepper might be due to a difference in absolute configuration between the precursors (antheraxanthin, violaxanthin) must be ruled out.

ACKNOWLEDGMENT

The gifts of yellow paprika from Dr. G. Horváth (Research Institute of Seed Production and Trading Co., Szentes, Hungary) are gratefully acknowledged. We thank Mrs. E. Nyers and Mrs. M. Steiler for skillful assistance.

LITERATURE CITED

Baranyai, M.; Matus, Z.; Szabolcs, J. Determination, by HPLC, of carotenoids in paprika products. *Acta Aliment.* **1982**, *11*, 309.

Cholnoky, L.; Györgyfy, K.; Nagy, E.; Páncél, M. *Acta Chim. Acad. Sci. Hung.* **1958**, *16*, 227.

Comar, C. L.; Zscheile, F. P. Analysis of plant extracts for chlorophylls a and b by a photoelectric spectrophotometric method. *Plant Physiol. (Lancaster)* **1942**, *17*, 198.

Davies, B. H. Carotenoids. In *Chemistry and Biochemistry of Plant Pigments*, 2nd ed.; Goodwin, T. W., Ed.; Academic Press: London, 1976; Vol. 2, Chapter 19, p 149.

Karrer, P.; Jucker, E. *Carotenoids*; Elsevier: Amsterdam, 1950 (English translation by E. A. Braude).

Klaüi, H.; Bauernfeind, J. C. Carotenoids as Food Color. In *Carotenoids as Colorants and Vitamin A Precursors*; Bauernfeind, J. C., Ed.; Academic Press: New York, 1981; Chapter 2, pp 66–70, 141.

Liaaen-Jensen, S. Artifacts of natural carotenoids—Unintended carotenoid synthesis. In *Carotenoids: Chemistry and Biology*; Krinsky, N. I., Matthews-Roth, H. M., Taylor, R. F., Eds.; Plenum Press: New York, 1990; p 149.

Matus, Z.; Baranyai, M.; Tóth, Gy.; Szabolcs, J. Identification of oxo, epoxy and some *cis*-carotenoids in High Performance Liquid Chromatography. *Chromatographia* **1981**, *14*, 337.

Molnár, P.; Szabolcs, J. Alkaline permanganate oxidation of carotenoid epoxides and furanoids. *Acta Chim. Acad. Sci. Hung.* **1979**, *99*, 155.

Ohmahct, R. *Chromatographia* **1979**, *12*, 565.

Parkes, K. E. B.; Pattenden, G.; Baranyai, M.; Molnár, P.; Szabolcs, J.; Tóth, Gy. Novel carotenoid 3,6-epoxides from red paprika, *Capsicum annuum*. *Tetrahedron Lett.* **1986**, *27*, 2535.

Szabolcs, J. Plant Carotenoids. In *Carotenoids: Chemistry and Biology*; Krinsky, N. I., Matthews-Roth, H. M., Taylor, R. F., Eds.; Plenum Press: New York, 1990; p 39.

Tóth, Gy.; Szabolcs, J. Distribution of carotenoids in flowers of Helianthus Annuus, Impatiens Noli Tangere, Ranunculus Acer, Taraxacum Officinale, and in ripe hips of Rosa Canina and Rosa Rubiginosa. *Acta Chim. Acad. Sci. Hung.* **1970**, *64*, 393.

Weedon, B. C. L. Occurrence. In *Carotenoids*; Isler, O., Ed.; Birkhäuser Verlag: Basel, 1971; Chapter 2, p 42.

Zechmeister, L. *cis-trans Isomeric Carotenoids, Vitamin A and Arylpolyenes*; Academic Press: New York, 1962.

Received for review January 2, 1991. Revised manuscript received June 26, 1991. Accepted July 22, 1991.

Registry No. Neoxanthin X, 30743-41-0; neoxanthin, 14660-91-4; violaxanthin, 126-29-4; 9-*cis*-violaxanthin, 26927-07-1; antheraxanthin, 640-03-9; 13-*cis*-violaxanthin, 75715-58-1; lutein, 127-40-2; zeaxanthin, 144-68-3; 9′-*cis*-lutein, 79516-56-6; 9-*cis*-lutein, 29414-89-9; 13′-*cis*-lutein, 79464-33-8; 13-*cis*-lutein, 32499-88-0; 9-*cis*-zeaxanthin, 60497-64-5; 13-*cis*-zeaxanthin, 60497-65-6; canthaxanthin, 514-78-3; β-cryptoxanthin 5,6-epoxide, 17430-14-7; α-cryptoxanthin, 24480-38-4; β-cryptoxanthin, 472-70-8; mutatochrome, 515-06-0; α-carotene, 432-70-2; β-carotene, 7235-40-7; *cis*-β-carotene, 30430-49-0; β-carotene 5,8-epoxide, 15678-54-3; lutein 5,6-epoxide, 28368-08-3.