Formation and Transformation of Pigments during the Fruit Ripening of *Capsicum annuum* Cv. *Bola* and *Agridulce*

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The photosynthetic pigments of the *Capsicum annuum* cv. *Bola* and *Agridulce* were monitored during ripening. Whereas the chlorophyllic pigments, as well as lutein and neoxanthin, disappeared, β-carotene and violaxanthin increased in concentration and other carotenoid pigments were formed *de novo*: zeaxanthin, capsanthin, capsorubin, β-cryptoxanthin, and capsolutein. The biosynthesis of cryptocapsin was not detected. The concentration increase of the carotenoids was always greater in the *Agridulce* variety at the totally ripe stage, but the concentration decreases in both varieties with successive harvests. Those pigments that are the final products of the biosynthetic pathway (red pigments) represent a lower percentage of the total pigments in the last harvest, compared with other pigments such as β-carotene, violaxanthin, and β-cryptoxanthin, which, as intermediates in the synthesis of the red pigments, are present in somewhat higher percentages, and for this reason the provitamin A value increased with successive harvests in each variety.

INTRODUCTION

The pepper (*Capsicum annuum* L.) is an annual herbaceous plant of the Solanaceae family. Its fruit is a rounded or elongated berry, depending on the variety. The different varieties of peppers have proved to be very interesting for researchers working on carotenoids (Cholnoky et al., 1955; Curl, 1962, 1964; Cámara and Monéger, 1978; Davies et al., 1970) for two main reasons: extraordinary changes occur in their carotenoid composition and content during fruit ripening, and pigments appear that are exclusive to this genus, e.g., capsanthin (3,3′-dihydroxy-β,κ-caroten-6′-one), capsorubin (3,3′-dihydroxy-κ,κ-carotene-6,6′-dione), and cryptocapsin (3′-hydroxy-β,κ-caroten-6′-one) (xanthophylls of the pepper) (Curl, 1962; Davies et al., 1970). A profound transformation thus occurs both in the photosynthetic pigment composition and in the pigment content during the various stages of pepper ripening and senescence (Cámara and Monéger, 1978; Davies et al., 1970). As a result, as ripening proceeds, the green color of the fruit disappears and the pepper adopts various tones of orange, finally turning an intense red (Curl, 1962, 1964). This color is chiefly due to the presence of newly formed oxygenated carotenoids (with acylcyclopentanol end groups), principally capsanthin and capsorubin, pigments which are exclusive to the pepper. During this process two simultaneous metabolic processes almost certainly occur. One of these gives rise to the transformation of existing pigments and one includes *de novo* synthesis of these carotenoids. The existence of the latter process is evident from the fact that the overall carotenoid content increases by an order of magnitude of 1 or 2 and, in some cases, there is an increase of as much as 100-fold (Davies et al., 1970).

If, furthermore, one considers the fact that the pepper has a high content of carotenoids with provitamin A activity, principally β-carotene (β,β-carotene) and β-cryptoxanthin (β,β-caroten-3-ol), it is understandable that the changes that occur in these pigments during the ripening process are of interest from a dietary and nutritional point of view.

In the present work, both biosynthesis and transformation of carotenoids pigments are studied during ripening of fruits from two Spanish varieties, *Bola* and *Agridulce*, used exclusively for obtaining of paprika. The changes in provitamin A content are also studied.

EXPERIMENTAL PROCEDURES

Materials. Fruits of *C. annuum* cv. *Bola* (*grossum*) and *Agridulce* (*longum*), grown in the La Vera region (Cáceres, Spain) were used. The study was performed during 2 consecutive years. The fruit of the *Bola* variety has a round shape, its length 4–6 cm and its weight 15–30 g. The fruits of the *Agridulce* variety has an elongated shape, its length 15–25 cm and its weight 15–25 g. Fruits were harvested at different stages of ripeness: in the first year fruits in the green, green-orange (color break), and red stages were selected. In the second year five successive stages of ripeness were selected, again on the basis of their color: green, green with orange zones (color break I), predominantly orange but with green zones (color break II), dark orange (red I), and dark red (red II).

Pigment Extraction. Pigments were extracted from a sample weighing 10 g using Me_2CO, until the complete exhaustion of all color. All extracts were pooled in a separator and shaken with Et_2O. A sufficient quantity of 10% NaCl was added at the end to aid in the separation of the phases. Subsequently, the organic phase was dried over anhydrous Na_2SO_4. This phase contains the pigments, in various stages of esterification with fatty acids, and can be used for chromatographic purposes once the volume has been reduced by drying in a rotary evaporator, if the chlorophyllic pigments are to be studied. If the choice is made to saponify the extract, 100 mL of 20% KOH–MeOH is added and left to act during 1 h (stirring is desirable). The nonsaponifiable pigments are subsequently extracted with Et_2O. The pigments are taken up in a maximum of 25 mL of Me_2CO, and a 1-mL aliquot of this is filtered through a 0.45-μm nylon membrane. The aliquot is frozen at −30 °C until it is to be injected into the HPLC. Losses occurring during the process were monitored using a β-apo-8′-carotenal internal standard, a known quantity of which was added to the sample at the start of the extraction process.

High-Performance Liquid Chromatography. For HPLC analysis, a computerized Perkin-Elmer system with a Series 4 quaternary pump was used. The system was equipped with a Perkin-Elmer Model LC-85B UV–vis detector and a Hewlett-Packard Model 3396-A integrator. The injection valve was a Rheodyne Model 7125. The HPLC system was equipped with a Hewlett-Packard reversed-phase C_{18} Spherisorb ODS-2 (5 μm, 0.4 cm × 25 cm) column. A precolumn (5 cm × 4 mm i.d.) of the same material was fitted to protect the main column.

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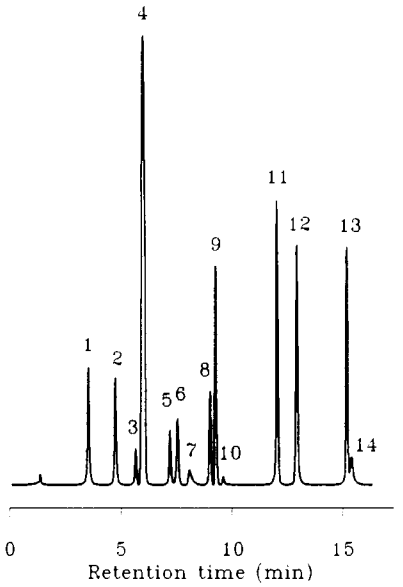

Figure 1. Reversed-phase HPLC chromatogram of a saponified extract of carotenoid pigments from ripe fruit peppers of *Bola* variety. Peak identity: 1, capsorubin; 2, violaxanthin; 3, capsanthin 5,6-epoxide; 4, capsanthin; 5, antheraxanthin; 6, *cis*-capsanthin; 7, mutatoxanthin; 8, capsolutein; 9, zeaxanthin; 10, *cis*-zeaxanthin; 11, β-apo-8′-carotenal (internal standard); 12, β-cryptoxanthin; 13, β-carotene; 14, *cis*-β-carotene.

HPLC Separation and Quantification of Carotenoids. Monitoring and quantification of the carotenoid pigments were carried out using a method previously developed by the authors (Mínguez-Mosquera and Hornero-Méndez, 1993). This method uses a C_{18} reversed-phase column and a binary gradient elution system of H_2O–Me_2CO at a flow rate of 1.5 mL/min, a sample injection volume of 5 μL (loop), and detection at 450 nm. Quantification was carried out using β-apo-8′-carotenal as internal standard for calibration. This pigment has the advantage of being absent from the pepper and of being easily separated from the rest of the carotenoids. In Figure 1, an example HPLC chromatogram of carotenoids from ripe fruit is shown. For the separation and quantification of zeaxanthin and lutein, the method of Juhler and Cox (1990) was used. This method employs an isocratic elution system of THF and H_2O (52:48 v/v) at a flow rate of 1 mL/min and detection at 450 nm.

HPLC Separation and Quantification of Chlorophylls. The procedure followed was that of Mínguez-Mosquera et al. (1991), which uses a reversed-phase and ionic pairs, thus improving the separation of chlorophyllides and pheophorbides. The eluents used were (A) H_2O–ion pair reagent–MeOH (1:1:8 v/v/v) and (B) Me_2CO–MeOH (1:1 v/v). The ion pair reagent consisted of a solution of 0.05 M tetrabutylammonium acetate and 1 M ammonium acetate. The flow rate of the eluents was 2 mL/min, and detection was performed simultaneously at 410 and at 430 nm. Quantification was performed using the detector factors by these authors.

Pigment Identification. These procedures have been described in detail in a previous publication (Mínguez-Mosquera and Hornero-Méndez, 1993) and consist of the following: separation of pigment by TLC and cochromatography with purified pigments; observation of the pigment color on TLC plates under white, UV_{254nm} and UV_{360nm} lights with a Desaga UV–vis lamp; recording of UV–visible spectra in different solvents with a Hewlett-Packard UV–vis diode array spectrophotometer Model 8452A and comparison with the values reported in the literature (Davies, 1976; Davies and Köst, 1988; Foppen, 1971); examination of 5,6-epoxide groups investigated by addition of 2% HCl in EtOH; investigation of carbonyl and hydroxyl groups by FT-IR spectroscopy using a Bio-Rad FTS-7 IR spectrophotometer and also by acetylation with Ac_2O/Py to test for hydroxyl groups and by reduction with $NaBH_4$ in EtOH to test for carbonyl groups.

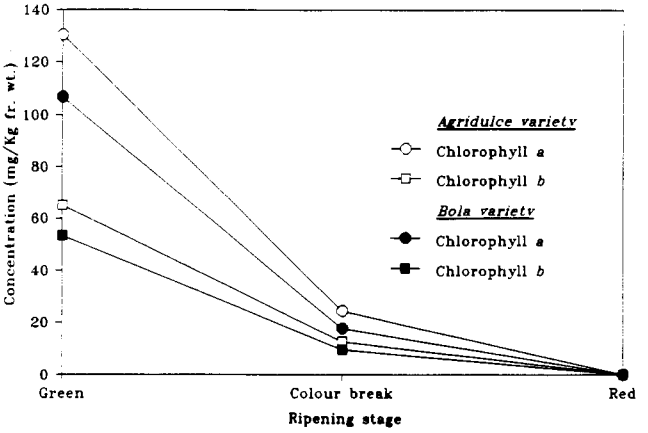

Figure 2. Evolution of the chlorophyllic pigment content during the ripening of fruits of *C. annuum* cv. *Bola* and *Agridulce*.

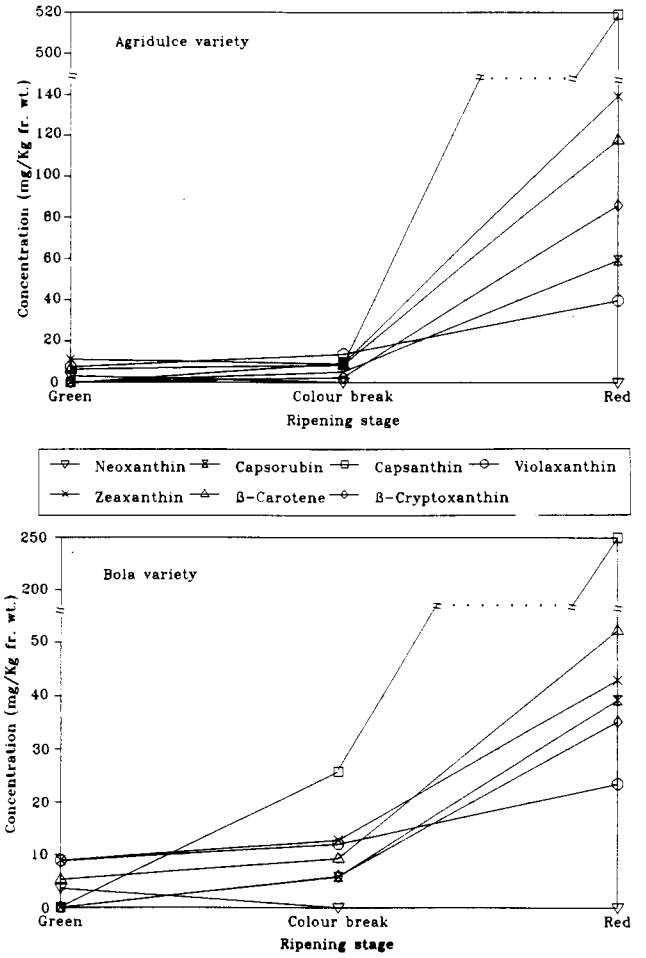

Figure 3. Evolution of the carotenoid pigment content during the ripening of fruits of *C. annuum* cv. *Bola* and *Agridulce*.

RESULTS AND DISCUSSION

Changes in the Pigments during Fruit Ripening. The concentrations of the principal chlorophyllic and carotenoid pigments in fruits from the *Bola* and *Agridulce* varieties were analyzed in three consecutive stages of ripeness: green, green-orange, and red. A very abrupt change was noted in the pigment content, both in qualitative and in quantitative terms. While the chlorophylls disappeared (Figure 2), the increase in the total carotenoid content as a result of ripening indicated that there was a net synthesis of these pigments (Figure 3). The extent of this synthesis depended on the variety of pepper. In the *Agridulce* variety the pigmentation in-

Table 1. Changes in the Chlorophyllic Pigments during the Ripening of Peppers of the *Agridulce* and *Bola* Varieties from the First and Third Harvests

	green		color break I		color break II	
pigment	1st harv	3rd harv	1st harv	3rd harv	1st harv	3rd harv
Agridulce variety						
chlorophyll *a*	137.62	121.30	97.07	61.04	54.72	11.07
chlorophyll *b*	57.52	50.98	41.43	25.83	23.27	5.91
chlorophyllide *a*	tr[a]	tr	tr	nd[b]	nd	nd
chlorophyllide *b*	tr	tr	nd	nd	nd	nd
Chl *a*/Chl *b* ratio	2.39	2.38	2.34	2.36	2.35	1.89
Bola variety						
chlorophyll *a*	69.97	89.88	61.00	86.95	25.76	35.91
chlorophyll *b*	28.28	35.68	27.82	33.77	11.66	14.14
chlorophyllide *a*	3.30	tr	0.86	tr	nd	nd
chlorophyllide *b*	tr	nd	nd	nd	nd	nd
Chl *a*/Chl *b* ratio	2.59	2.52	2.22	2.57	2.20	2.54

[a] tr, traces. [b] nd, not detected.

creased by *ca.* 35-fold, while in the *Bola* variety it increased *ca.* 16-fold. Similar increases in pigment concentration were found by Cholnoky et al. (1955), and Davies et al. (1970) reported increases in pigmentation of up to 100-fold in ripe fruits of red varieties. Since, in the stages of ripeness selected, the synthesis of carotenoids was so pronounced that it was impossible to monitor the individual biosynthetic transformations that occurred during ripening, the following year the study was planned so as to include five perfectly distinguishable and successive stages. At the same time, as the pepper bears fruit at various times during the harvesting period and, as Lease and Lease (1956) have reported, the pigment concentration in the fruits decreases in successive harvests, the fruits monitored were taken at the start and at the end of the harvesting period. A period of about 1 month separated the two samplings.

The changes in the chlorophyll pigments in the fruits during the stages examined are shown in Table 1, chlorophylls *a* and *b* and, in some cases, chlorophyllide *a* appearing from the very start. In none of the fruits was the formation of chlorophyllide *b* detected. The rapid disappearance of the chlorophyllic pigments as ripening advanced coincided with the transformation of the chloroplasts into chromoplasts and, consequently, in neither red stage I nor II were any chlorophyllic compounds detected. The formation of chlorophyllic derivatives lacking the phytol component, although present only in trace amounts, demonstrated the presence of the enzyme chlorophyllase in the fruits. On the other hand, the fact that the ratio of chlorophyll *a* to chlorophyll *b* tended to decrease, chlorophyll *a* disappearing more rapidly than chlorophyll *b*, would seem to indicate that the chlorophyllase enzyme has a higher affinity for the substrate from the *a* series.

The results of the parallel studies carried out on the carotenoid fraction are shown in Tables 2 and 3, where the concentrations are expressed as milligrams of pigment per kilogram of fresh fruit. Overall, while some pigments, lutein (β,ε-carotene-3,3′-diol) and neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol), disappeared, others increased in concentration, β-carotene and violaxanthin (5,6:5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol) and other pigments were formed *de novo*: zeaxanthin (β,β-carotene-3,3′-diol), capsanthin, capsorubin, β-cryptoxanthin, and capsolutein. The absence of cryptocapsin, or its detection at trace levels in both varieties, is in accordance with the observations of Cámara and Monéger (1978) and Simpson et al. (1974). Some authors (Cámara and Monéger, 1978; Ramírez and Tomes, 1964) have reported the possible participation of phytol, liberated by the action of chlorophyllase, on the biosynthesis of the carotenoids. However, as will be shown, the degree of *de novo* synthesis considerably exceeds that which could take place with the aid of phytol alone, although the participation of the latter is not ruled out.

To interpret the increase in the total carotenoid content observed, as well as the transformations that occur in the carotenoids during ripening, the following hypotheses discussed in the literature have been taken into account (Porter and Lincoln, 1950; Weedon, 1971). The possible carotenogenic pathways from common precursors give rise to three main families of carotenoid (Figure 4): those that have two β-ionone rings (**3**), which give rise to the β,β series of carotenes (**5**); those that possess one β-ionone and one ε-ionone ring (**4**), which give rise to the β,ε series of carotenes (**6**); and finally those that possess two ε-ionone rings, which give rise to the ε,ε series of carotenes (**7**). The existence of the ionone rings, of one type or the other, originates from the fact that when cyclization occurs at one extreme of lycopene [ψ,ψ-carotene (**1**)], by the action of the enzyme lycopene cyclase, a carbocation results (**2**). This, through the liberation of a proton, gives rise to a

Table 2. Changes in the Concentration (Milligrams per Kilogram of Fresh Weight) of Carotenoid Pigments in the *Agridulce* Variety with the Stage of Ripening and the Time of Harvesting (First and Third Harvests)

	green		color break I		color break II		red I		red II	
pigment	1st harv	3rd harv	1st harv	3rd harv	1st harv	3rd harv	1st harv	3rd harv	1st harv	3rd harv
neoxanthin	8.48	10.10	3.37	1.08	–[a]	–	–	–	–	–
capsorubin	–	–	8.87	5.06	11.14	7.11	12.92	11.50	76.74	51.84
violaxanthin	9.31	9.76	12.82	14.99	14.72	18.27	20.10	31.08	80.21	71.03
capsanthin 5,6-epoxide	–	–	5.43	5.69	8.82	6.45	5.71	11.24	51.20	35.26
capsanthin	–	–	26.49	18.53	49.73	34.18	140.69	59.16	635.78	360.44
antheraxanthin	0.49	nd[b]	10.12	5.87	10.17	7.03	14.57	8.48	47.48	24.38
cis-capsanthin	–	–	2.65	2.82	7.64	3.68	19.94	7.23	69.83	30.04
capsolutein	–	–	3.46	2.81	8.34	5.47	23.97	8.83	86.11	45.44
zeaxanthin	–	–	6.51	4.54	29.18	11.94	87.71	15.59	98.54	86.53
cis-zeaxanthin	–	–	3.79	3.21	5.48	5.32	9.71	5.57	7.41	4.48
lutein	14.13	9.50	7.53	5.55	0.91	0.37	–	–	–	–
cis-lutein	1.61	2.57	nd	nd	nd	nd	–	–	–	–
β-cryptoxanthin	–	–	7.74	4.33	12.25	9.35	45.73	23.45	78.34	75.77
β-carotene	8.03	4.72	11.26	10.70	17.74	14.32	45.47	30.49	98.60	105.90
total carotenoids	41.56	36.65	110.74	85.18	176.12	123.49	426.52	212.62	1329.74	891.47
% yellow pigments	100.00	100.00	60.77	62.32	56.09	58.36	57.97	58.08	37.35	46.43
% red pigments	0.00	0.00	39.23	37.68	43.91	41.64	42.03	41.92	62.65	53.57

[a] –, absent. [b] nd, not detected.

Table 3. Changes in the Concentration (Milligrams per Kilogram of Fresh Weight) of Carotenoid Pigments in the *Bola* Variety with the Stage of Ripening and the Time of Harvesting (First and Third Harvests)

	green		color break I		color break II		red I		red II	
pigment	1st harv	3rd harv	1st harv	3rd harv	1st harv	3rd harv	1st harv	3rd harv	1st harv	3rd harv
neoxanthin	7.51	6.25	nd[a]	0.26	–[b]	–	–	–	–	–
capsorubin	–	–	4.56	2.81	11.81	10.90	25.46	14.22	56.74	74.19
violaxanthin	9.82	8.29	10.34	24.92	12.22	24.13	23.49	37.02	54.98	68.28
capsanthin 5,6-epoxide	–	–	3.24	2.48	7.36	4.67	17.98	13.13	42.25	41.57
capsanthin	–	–	18.63	27.90	49.27	41.23	188.22	53.07	508.26	253.85
antheraxanthin	0.49	nd	1.78	7.82	4.44	4.25	15.42	14.03	35.43	16.38
cis-capsanthin	–	–	nd	2.31	5.02	3.89	23.63	2.29	61.21	26.17
capsolutein	–	–	2.31	3.57	7.68	5.62	29.51	4.00	68.09	33.25
zeaxanthin	–	–	6.21	7.73	14.65	9.17	37.83	10.81	41.05	32.50
cis-zeaxanthin	–	–	1.53	1.83	2.72	3.07	3.62	6.00	5.53	3.35
lutein	9.09	6.79	2.66	2.58	1.10	0.69	–	–	–	–
cis-lutein	1.49	1.76	nd	nd	nd	nd	–	–	–	–
β-cryptoxanthin	–	–	3.21	5.94	7.69	9.07	27.62	19.00	37.29	26.06
β-carotene	6.36	6.03	7.02	11.58	8.29	16.67	35.26	28.14	51.97	70.59
total carotenoids	34.76	29.12	61.49	101.73	132.25	133.36	428.04	201.71	962.50	646.19
% yellow pigments	100.00	100.00	57.02	65.10	44.45	54.49	40.36	59.00	30.55	38.75
% red pigments	0.00	0.00	42.98	34.90	55.55	45.51	59.64	41.00	69.45	61.25

[a] nd, not detected. [b] –, absent.

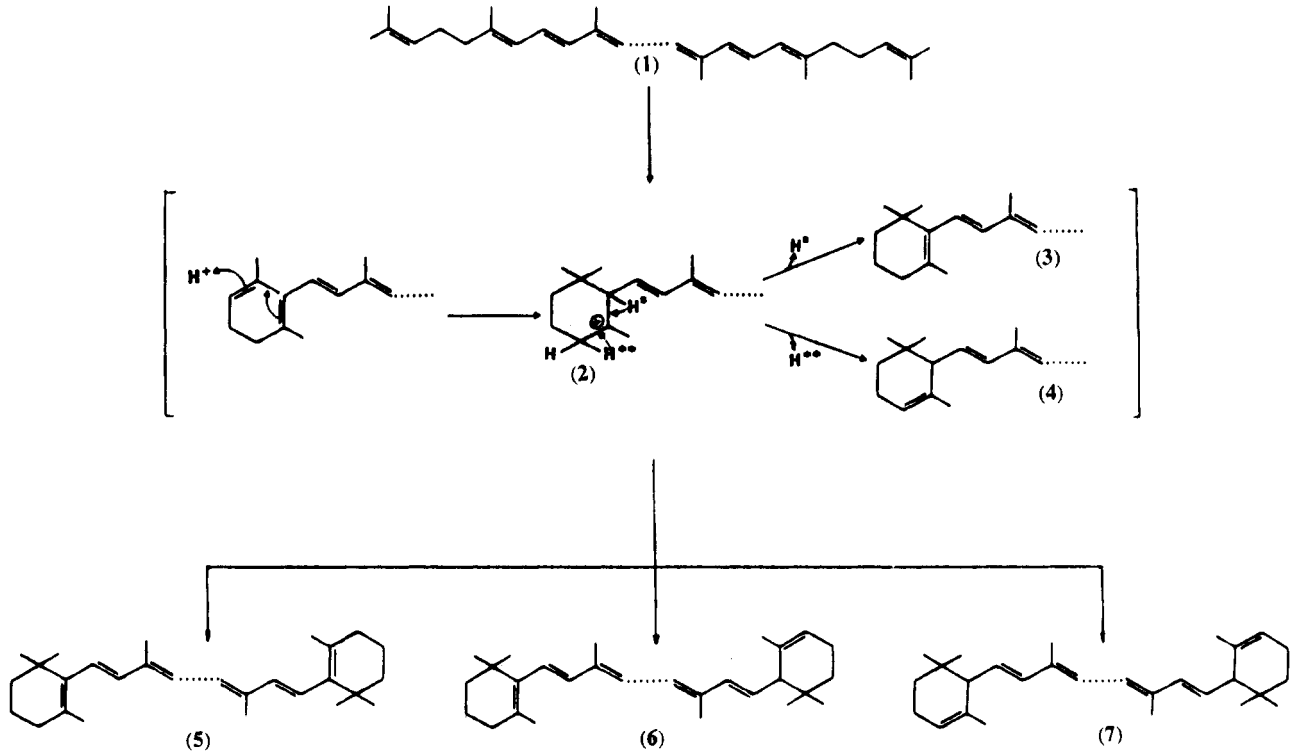

Figure 4. Biosynthetic scheme of formation of β- and ε-ionone rings, giving rise to the β,β-, β,ε-, and ε,ε-carotene series. (Identities of numbers between parentheses are explained in the text.)

double bond, the position of which depends on the location of the hydrogen atom liberated, thus giving rise to β or ε rings.

On the basis of these hypotheses, the main results of the study on the changes in the carotenoids in the two varieties of peppers during ripening can be interpreted. Lutein, the only β,ε series carotenoid present in the green fruits, showed a decrease in concentration in the peppers in the green-orange stage and disappeared altogether from the ripe fruits. This finding coincides with those of other authors (Cholnoky et al., 1955; Cámara and Monéger, 1978; Davies et al., 1970). It would seem that at some point during ripening of these red pepper varieties, the pathway of formation of this pigment is interrupted. It is worthy of mention that in these varieties of pepper, when ripening begins, the pigment capsolutein is synthesized. The name of this latter pigment almost certainly arises from the fact that its absorption spectrum is similar to that of lutein and, since it appears in the ripe pepper, it is considered to be the "lutein of peppers". Given that the most likely structure of this pigment would seem to be 3,3′-dihydroxy-5,8-dihydro-5,8-epoxy-β,κ-carotene, it is unlikely that lutein acts as a direct precursor of it. Another possibility for the structure of this pigment is to correspond to cucurbitaxanthin A (3,6-epoxy-5,6-dihydro-β,β-carotene-5,3′-diol) with properties, both chemical and spectral, similar to those the pigment reported in this paper. Cucurbitaxanthin A was isolated as a new carotenid from pumpkin (*Cucurbita maxima*) (Matsuno et al., 1986) and more recently from red pepper and paprika (Parkes et al., 1986; Deli et al., 1992). It should be noted that the content in capsolutein of the *Bola* variety reaches levels greater than that of zeaxanthin, while in the *Agridulce* variety its levels are similar to that of zeaxanthin. This fact could

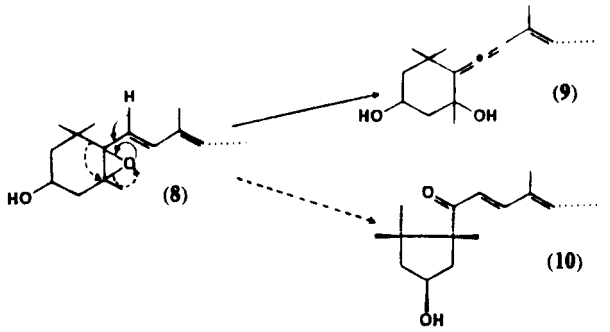

Figure 5. Scheme of the rearrangements of 3-hydroxy-5,6-epoxy-β-ionone end group to 3,5-dihydroxy allene end group (—) and to acylcyclopentanol end group (- - -). (Identities of numbers between parentheses are explained in the text.)

be of use for chemotaxonomic purposes. It is possible that the disappearance of lutein indicates that it has an important role in the photosynthetic process, detained during ripening. This, furthermore, would accord with the frequency with which this pigment is found in the majority of green vegetables. In this regard it is significant that in the fruits of pepper varieties whose final color is yellow (*C. annuum lycopersiciforme flavum*), the biosynthetic process is altered (Davies et al., 1970; Cholnoky et al., 1958; Matus et al., 1991) in such a way that lutein, together with other β,ε series carotenoids is present in high concentrations when the fruit is ripe.

At the same time, in both varieties studied, there was a greater number of β,β series carotenoids than of β,ε series. This probably reflects the fact that during the formation of the ionone rings the reaction tends toward the formation of more β rings than ε rings, which would suggest that the enzyme responsible (lycopene cyclase) is apparently more active for the formation of the β rings than for ε rings (Figure 4). During the ripening process the β,ε series carotenoids disappear, a fact already reported by Cámara and Monéger (1978) and Davies et al. (1970).

In the β,β series there is a carotenoid, neoxanthin, that also disappears when the fruit is ripe, in accordance with the observations of Davies et al. (1970). Neoxanthin is synthesized from a 3-hydroxy-5,6-epoxide end group (8) (violaxanthin) by rearragement to give a 3,5-dihydroxy allene end group (Davies et al., 1970) (Figure 5). This process would seem to be usual in green fruits.

During ripening of the red varieties, there is a synthesis of ketonic pigments with an acylcyclopentanol end group (10 in Figure 5). These are characteristic of the *Capsicum* genus and are formed by a pinacolic rearrangement of the 3-hydroxy-5,6-epoxide end group (Entschel and Karrer, 1960) (Figure 5), such that capsanthin is formed from zeaxanthin via the 5,6-epoxide derivative of the latter, antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol). In the same way violaxanthin (the 5,6:5′,6′-diepoxide derivative of zeaxanthin) gives rise to capsorubin and β-cryptoxanthin via the intermediary 5,6-monoepoxide derivative (5,6-epoxy-5,6-dihydro-β,β-caroten-3-ol) gives rise to cryptocapsin. This double pathway, which via a 3-hydroxy-5,6-epoxide end group gives rise to a 3,5-dihydroxy allene end group (9 in Figure 5) or an acylcyclopentanol end group, seems not to occur at the same time as that leading to the formation of neoxanthin, the latter being interrupted so as to allow the former to proceed. This is confirmed by the absence of this pigment in fruits that have reached an advanced stage of color change. In green fruits, the active enzyme is that which controls the step leading to neoxanthin. During ripening the enzyme involved is that which catalyzes the formation of the acylcyclopentanol end group, giving rise to capsorubin when the substrate is violaxanthin, and consequently no further synthesis of neoxanthin occurs. In yellow pepper varieties, when the fruit ripens, large quantities of neoxanthin are found together with other β,ε and β,β series carotenoids. In these fruits the second enzyme is probably absent or inhibited (Davies et al., 1970) and thus neoxanthin synthesis continues, as does the synthesis in large amounts of other compounds with the 3-hydroxy-5,6-epoxide end group.

Table 4. Percentage Composition of Carotenoid Pigments in Fruits in the Totally Mature Stage (Red II) in the Two Varieties, *Agridulce* and *Bola* (First and Third Harvests)

	Agridulce		*Bola*	
pigment	1st harv	3rd harv	1st harv	3rd harv
neoxanthin	0.00	0.00	0.00	0.00
capsorubin	5.73	5.82	5.90	11.48
violaxanthin	6.03	7.97	5.70	10.57
capsanthin 5,6-epoxide	3.85	3.95	4.39	6.43
capsanthin	47.81	40.43	52.81	39.29
antheraxanthin	3.57	2.73	3.68	2.53
cis-capsanthin	5.25	3.37	6.36	4.05
capsolutein	6.48	5.10	7.07	5.14
zeaxanthin	7.41	9.71	4.26	5.03
cis-zeaxanthin	0.56	0.54	0.57	0.52
lutein	0.00	0.00	0.00	0.00
cis-lutein	0.00	0.00	0.00	0.00
β-cryptoxanthin	5.89	8.50	3.86	4.04
β-carotene	7.42	11.88	5.40	10.92
% yellow pigments	37.35	46.43	57.02	65.10
% red pigments	62.65	53.57	42.98	34.90

The synthesis then of carotenoids throughout ripening is governed by two processes: one that includes the transformation of the pre-existing yellow pigments into the red pigments by the pathway described and one that controls net synthesis of carotenoids. The increase in the concentration of these compounds must represent *de novo* synthesis, which implies new synthesis of carotenoid precursors, such as phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene), phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), and ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene), as well as that of intermediate compounds (violaxanthin, zeaxanthin, β-cryptoxanthin, antheraxanthin, etc). This explains why there is an increase in synthesis of all of these pigments during ripening. To check, qualitatively, this last point (*de novo* synthesis), a study was carried out on the extent of the presence of the initial precursors for the biosynthesis of carotenoids. For this purpose, the presence of phytofluene was monitored in fruits of the *Bola* variety with degrees of ripeness corresponding to the five stages already mentioned, using TLC with plates of silica gel GF_{254}. These were developed using petroleum ether (40–60 °C), and the extent of the presence of the phytofluene was detected by the intensity of the yellow-green fluorescence under ultraviolet light. In the green fruit the precursor pigments were not detected, possibly because the rate of synthesis was low (maintenance) and because as soon as they were formed they were transformed. However, in the fruits that had begun to ripen, their presence was detected and the amount present increased as the fruits ripened, reaching maximum values in the red fruit, these having the highest rate of synthesis.

Comparison between Cultivars. *Carotenoid Content.* Since the pepper plant does not yield all of its fruit simultaneously, harvesting takes place in distinct stages, the fruits from the final stages normally showing a poorer quality than the fruits that ripened earlier. For this reason the changes in the pigments have been studied in fruits of the *Bola* and *Agridulce* varieties during the first and

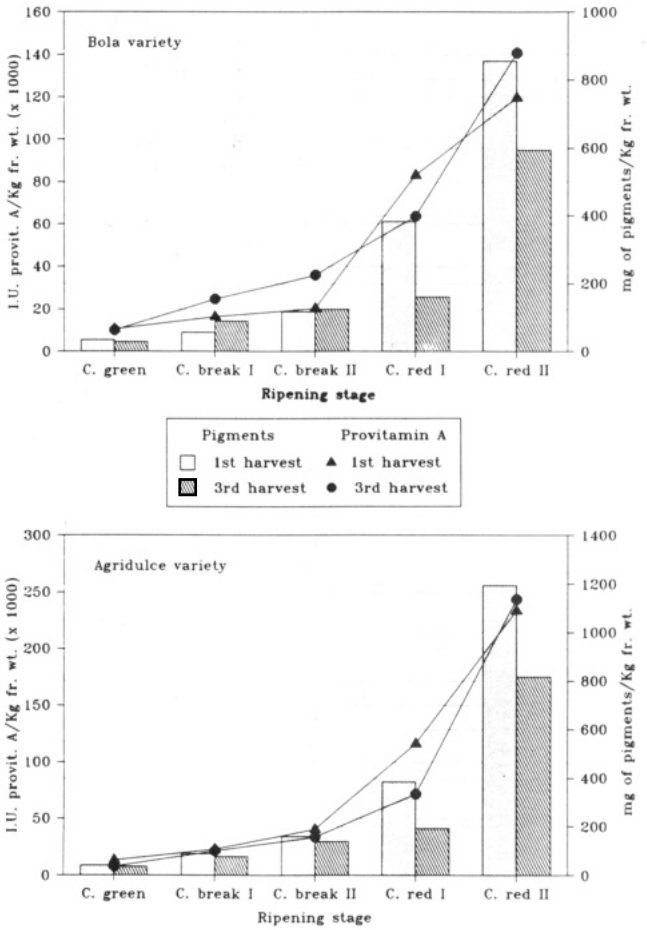

Figure 6. Changes in the provitamin A value in the totally mature stage (red II) of *C. annuum* cv. *Agridulce* and *Bola* fruits with the time of harvesting.

last harvests. In fruits from the first harvest, the changes that occurred in the overall carotenoid content as the two varieties ripened were similar. Both varieties showed a sharp increase in their carotenoid contents in the red I stage. The same occurred in both varieties harvested during the third (or last) harvest. However, when the fruits from the two different harvests were compared, although the concentrations of carotenoids in the initial stages of ripening (green and color break) were similar, in the red stage, those fruits harvested during the first harvest always showed higher concentrations than those from the third harvest. This was true for both varieties. This is almost certainly due to the fact that after a month of continuous fruit-bearing, the plant is in a state of nutritional stress and the stages which correspond to the highest rate of carotenoid synthesis (red I and II) are those most influenced by the physiological state of the plant. This observation is in complete accordance with that of Lease and Lease (1956). These authors pointed out the great effect that the number of harvests borne by a plant has on the carotenoid content of the fruit, the concentration diminishing progressively in successive harvests. The fact that while the fruits are green or color-breaking the plant behaves similarly, whether it is bearing the first or the last harvest of fruit, may be due to the fact that all of the enzymatic systems at that time are active and, consequently, that the synthesis of pigments does not represent a great stress for the plant. All this means that the percentage of each pigment changes somewhat during the final ripening stage, from one harvest to another. In this way, it is seen that the pigments which are the final products of the biosynthetic pathway (red pigments) represent a lower percentage of the total pigments in the third harvest, compared with other pigments such as β-carotene and violaxanthin, which, as intermediates in the synthesis of the red pigments, are present in somewhat higher percentages. It would appear that these accumulate since, due to the physiological state of the plant, they are not used at the same rate as in the first harvest.

Thus, in the *Bola* variety there is 13.52% more capsanthin in the first than in the third harvest, and in *Agridulce* there is 7.38% more in the first than in the third harvest. In the *Bola* variety there is 5.52% more β-carotene in the first than in the third harvest, compared with the *Agridulce* variety, which, in the first harvest has 4.48% more β-carotene. Violaxanthin represents 5.71% of the total carotenoid content of the first harvest from the *Bola* variety and 6.43% of that of the third harvest, while in *Agridulce* the corresponding figures are 6.03% and 7.97%. If we compare the yellow (Y) fractions with the red (R) fractions in the totally ripe state, in *Bola* the percentage of red pigments is 69.45% (R/Y = 2.27) in the first harvest, compared with 61.25% (R/Y = 1.58) in the third harvest. In the *Agridulce* variety the percentage of red pigments is 62.65% (R/Y = 1.67) in the first harvest and in the third 53.57% (R/Y = 1.15) (Table 4).

Provitamin A Value. The provitamin A value varies during ripening, reflecting the changes that occur in those pigments that are capable of giving rise to this vitamin, as can be seen in Figure 6. In the *Agridulce* variety the provitamin A value is always higher than in the *Bola* variety, and the highest levels are found in the totally ripe fruits. In addition to this difference between the two varieties, it is noteworthy that, when fruits of the same variety harvested at two different times 1 month apart are compared, the fruits with the greatest values for provitamin A are those from the third harvest, despite the fact that in both varieties the fruits from the third harvest have a lower total content in carotenoids than those from the first harvest. This fact is true for both varieties but is most evident in the *Bola* variety and is attributable to the fact that those carotenoids with provitamin A activity (β-carotene and β-cryptoxanthin) accumulate, as previously described.

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LITERATURE CITED

Cámara, B.; Monéger, R. Free and Esterified Carotenoids in Green and Red Fruits of *Capsicum annuum*. *Phytochemistry* **1978**, *17*, 91–93.

Cholnoky, L.; Györgyfy, K.; Nagy, E.; Pánczél, M. Investigations of Carotenoid Pigments. I. Pigments of Red Paprika. *Acta Chim. Hung.* **1955**, *6*, 143–171.

Cholnoky, L.; Györgyfy, K.; Nagy, E.; Pánczél, M. Investigations of Carotenoid Pigments. III. Pigments of the Yellow Paprika. *Acta Chim. Acad. Sci. Hung.* **1958**, *16*, 227–246.

Curl, A. L. The Carotenoids of Red Bell Pepper. *J. Agric. Food Chem.* **1962**, *10*, 504–509.

Curl, A. L. The Carotenoids of Green Bell Pepper. *J. Agric. Food Chem.* **1964**, *12*, 522–524.

Davies, B. H. Carotenoids. In *Chemistry and Biochemistry of Plant Pigments;* Goodwin, T. W., Ed.; Academic Press: London, 1976; Vol. 2, p 38.

Davies, B. H.; Köst, H. P. Carotenoids. In *Handbook of Chromatography;* Köst, H. P., Ed.; CRC Press: Boca Raton, FL, 1988; Vol. 1.

Davies, B. H.; Matthews, S.; Kirk, J. T. O. The Nature and Biosynthesis of the Carotenoids of Different Color Varieties of *Capsicum annuum*. *Phytochemistry* **1970**, *9*, 797–805.

Deli, J.; Matus, Z.; Szabolcs, J. Carotenoid composition in the fruits of black paprika (*Capsicum annuum* variety *longum nigrum*) during ripening. *J. Agric. Food Chem.* **1992**, *40*, 2072–2076.

Entschel, R.; Karrer, P. The constitution of capsanthin and capsorubin. *Helv. Chim. Acta* **1960**, *43*, 89–94.

Foppen, F. H. Tables for Identification of Carotenoid Pigments. *Chromatogr. Rev.* **1971**, *14*, 133–298.

Juhler, R. K.; Cox, R. P. High-performance Liquid Chromatographic Determination of Chloroplast Pigments with Optimized Separation of Lutein and Zeaxanthin. *J. Chromatogr.* **1990**, *508*, 232–235.

Lease, J. G.; Lease, E. J. Factors Affecting the Retention of Red Color in Peppers. *Food Technol.* **1956**, *10*, 368–373.

Matsuno, T.; Tani, Y.; Maoka, T.; Matsuo, K.; Komori, T. Isolation and structural elucidation of cucurbitaxanthin A and B from pumpkin *Cucurbita maxima*. *Phytochemistry* **1986**, *25*, 2837–2840.

Matus, Z.; Deli, J.; Szabolcs, J. Carotenoid Composition of Yellow Pepper during Ripening: Isolation of β-Cryptoxanthin 5,6-Epoxide. *J. Agric. Food Chem.* **1991**, *39*, 1907–1914.

Mínguez-Mosquera, M. I.; Hornero-Méndez D. Separation and Quantification of the Carotenoid Pigments in Red Peppers (*Capsicum annuum* L.), Paprika, and Oleoresin by Reversed-Phase HPLC. *J. Agric. Food Chem.* **1993**, *41*, 1616–1620.

Mínguez-Mosquera, M. I.; Gandul-Rojas, B.; Montaño-Asquerino, A.; Garrido-Fernández, J. Determination of Chlorophylls and Carotenoids by High-performance Liquid Chromatography During Olive Lactic Fermentation. *J. Chromatogr.* **1991**, *585*, 259–266.

Parkes, K. E. B.; Pattenden, G.; Baranyai, M.; Molnar, P.; Szabolcs, J.; Toth, G. Novel carotenoid 3,6-epoxides from red paprika (*Capsicum annuum*). *Tetrahedron Lett.* **1986**, *27*, 2535–2538.

Porter, J. W.; Lincoln, R. E. I. Lycopersicon Selections Containing a High Content of Carotenes and Colorless Polyenes. II. The Mechanism of Carotene Biosynthesis. *Arch. Biochem.* **1950**, *27*, 390–403.

Ramírez, D. A.; Tomes, M. L. Relation between Chlorophyll and Carotenoid Biosynthesis in Dirty-red (green-flesh) Mutant in Tomato. *Bot. Gaz.* **1964**, *125*, 221–226.

Simpson, D. J.; Rahman, F. M. M.; Buckle, K. A.; Lee, T. H. Chemical Regulation of Plastid Development. II. Effect of CPTA [2-4(chlorophenylthio) ethyldiethylammonium chloride] on the Ultrastructure and Carotenoid Composition of Chromoplast of *Capsicum annuum* cultivars. *Aust. J. Plant Physiol.* **1974**, *1*, 135–147.

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

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