Carotenoid Biosynthesis Changes in Five Red Pepper (*Capsicum annuum* L.) Cultivars during Ripening. Cultivar Selection for Breeding

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Changes in the biosynthesis of individual carotenoid pigments have been investigated during fruit ripening of five cultivars of red pepper (*Capsicum annuum* L.): Mana, Numex, Belrubi, Delfin, and Negral (a chlorophyll-retaining mutant when ripe). The study was carried out throughout the ripening process, and with special emphasis on the ripe stage, to discover possible differences between cultivars and to characterize these by their carotenoid pattern and content for selecting the best varieties for breeding programs. Ripening fruit of the five cultivars showed the typical and characteristic pattern of carotenoid biosynthesis for the *Capsicum* genus. In the five cultivars, lutein and neoxanthin, both characteristic chloroplast pigments, decreased in concentration with ripening and eventually disappeared. β-Carotene, antheraxanthin, and violaxanthin increased in concentration, and other pigments were biosynthesized *de novo*: zeaxanthin, β-cryptoxanthin, capsanthin, capsorubin, capsanthin-5,6-epoxide, and cucurbitaxanthin A. A pool of zeaxanthin stands out of the rest of pigment during ripening, which reveals the importance of this pigment as a branching point in the carotenoid biosynthesis in *Capsicum*. Quantitatively, Negral cultivar showed the highest increase in total carotenoid content (48.39-fold), followed by Mana and Delfin with 38.03- and 36.8-fold, respectively, and by Belrubi and Numex with 28.03- and 23.48-fold, respectively. In all the red varieties, there was an inverse relationship between total carotenoid content and the red to yellow isochromic pigment fraction ratio (*R*/*Y*) and the capsanthin-to-zeaxanthin ratio (Caps/Zeax). This seems to be related to the carotenogenic capacity of the cultivar, and thus selection and breeding should not only seek a higher total carotenoid content but also attempt to increase these ratios. In the present study, the cultivar Mana had the highest total carotenoid content (13 208 mg/kg dwt), but the lowest *R*/*Y* (1.25) and Caps/Zeax (3.38) ratios, which are therefore the parameters to improve. The cultivar Negral had a high carotenoid content (8797 mg/kg dwt) and high *R*/*Y* and Caps/Zeax ratios and could be used for transfer of these characters in direct crosses with the cultivar Mana. The cultivar Numex had the highest Caps/Zeax ratio (7.17) and is thus an ideal progenitor for this character.

Keywords: *Capsicum annuum; carotenoid; breeding cultivars; paprika; ripening*

INTRODUCTION

The red pepper fruit (*Capsicum annuum*, L.) has been used since ancient times as a source of pigments to add to or change the color of foodstuffs, making them more attractive and acceptable for the consumer. Pepper used as food colorant has traditionally been in the form of paprika (ground powder), although today oleoresins are widely used. The fruits of *C. annuum* owe their intense red color to carotenoid pigments that are synthesized massively during fruit ripening. Among these, the carotenoid pigments mainly responsible for the final red color of the fruits are capsanthin, capsorubin, and capsanthin 5,6-epoxide, which are almost exclusive to the genus *Capsicum* (Davies et al., 1970; Mínguez-Mosquera and Hornero-Méndez, 1994a).

Carotenoids are important natural pigments found in all plants, algae, and many bacteria and fungi, as well as in some animals. In the photosynthetic organisms, carotenoids are always present in the pigment−protein complexes of the photosystems where they harvest light and transfer the energy to the chlorophylls, in addition to playing an important function as photoprotection of the chlorophylls molecules (Frank and Cogdell, 1993). Carotenoid pigments are responsible for the attractive colors of fruits and flowers, having an important role in attracting animals to act as pollinators and seed dispersion vehicles, including in this process the consumption of food by humans. When carotenoids are ingested, they show important biological actions such as being antioxidants and free-radical scavengers and reducing the risk of cancer and having a positive effect on the immune response; in addition, some of them (β-carotene, β-cryptoxanthin, etc.) have provitamin A activity (Edge et al., 1997; Olson, 1989; Ziegler, 1989). Carotenoids are essentially C40 terpenoid compounds formed by the condensation of eight isoprene units, as an important branch of the general and important isoprenoid pathway. The basic carotene structure (i.e.,

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lycopene) can undergo several structural modifications, namely, cyclization, hydroxylation, and epoxidation, yielding the great variety of carotenoids in nature (more than 600) (Britton, 1998). During ripening of the pepper, there is a spectacular synthesis of carotenoid pigments. All the carotenoid pigments present in the pepper are C_{40} isoprenoids containing nine conjugated double bonds in the central polyenic chain, although with different end groups (β, ϵ, κ, 3-hydroxy-5,6-epoxide), which change the chromophore properties of each pigment, allowing them to be classified in two isochromic families: red (R) and yellow (Y). The red fraction contains the pigments exclusive to the *Capsicum* genus (capsanthin, capsanthin-5,6-epoxide, and capsorubin), and the yellow fraction comprises the rest of the pigments (zeaxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, β-carotene, and cucurbitaxanthin A), which act as precursors of the former. Clonation of genes that codify for carotenogenic enzymes, such as lycopene cyclase, zeaxanthin epoxidase, and capsanthin−capsorubin synthase, has proven to be essential for the study of the mechanisms and regulation of carotenoid accumulation in chromoplasts of fruits and flowers, which is a highly regulated process (Bouvier et al., 1996; Bouvier et al., 1997; Cunningham et al., 1996; Kuntz et al., 1998; Ronen et al., 1999; for a detailed revision on carotenoid biosynthesis, see Britton, 1998). Therefore traditional plant-breeding for carotenoid production can now be carried out with a more solid understanding of the process, which is of particular interest in fruit crops such tomato and red pepper.

After red pepper was brought to Spain following the discovery of America, growers have selected many pepper cultivars for the properties and characteristics that were most popular or most profitable agriculturally. The result is a great number of very different cultivars showing a wide range of morphological and organoleptic characteristics, including color, which determine their use. Not all cultivars can be used to produce paprika: they must first meet a series of appropriate agronomic and industrial requirements (Costa, 1980). The most highly valued of these is a high content in carotenoids, as ultimately the commercial value of paprika depends on its coloring capacity, which depends directly on relative pigment richness. Other variety characters of interest are low content in capsaicinoids (that is, reduced hotness); low moisture content and a relatively thinpericarp when ripe (to shorten the drying step of paprika processing, thereby reducing the cost); simultaneous and grouped ripening (to help in mechanical harvesting); and, of course, high agronomic production and yield, together with resistance to factors such as disease, high/low temperatures, and salinity of soil and irrigation water.

In Spain, traditional methods of paprika production have used few cultivars, the main ones being Agridulce (*C. annuum* var. *longum*) and Bola (*C. annuum* var. *grossum*) (Mínguez-Mosquera and Hornero-Méndez, 1994a, 1994b). As these cultivars have been subjected to a long and slow process of selection by growers, the local cultivars that have appeared are well adapted to the climate and very similar to each other genetically, and thus they are unlikely to be improved substantially. The industrialization of paprika production, the opening of new markets, and the introduction of the crop into countries with cheap labor have stimulated a search for more competitive cultivars, such as the recent example of new Jaranda and Jariza cultivars (Pérez-Gálvez et al., 1999), and some other interesting cultivar crosses in Spain and other countries (Almela et al., 1991; Levy et al., 1995). In the present work, five cultivars of pepper (Belrubi, Delfin, Mana, Negral, and Numex) have been characterized by their carotenoid pigment content and composition to enable selection of the best cultivar (or cultivars) for later experiments of variety breeding, or to be used as a vector in the improvement of well-established traditional cultivars.

MATERIALS AND METHODS.

Plant Material. Fruit of peppers (*Capsicum annuum* L.), cultivars Mana, Numex, Negral, Belrubi and Delfin, were used for the present study. Plants were grown at the Escuela Técnica Superior de Ingenieros Agrónomos (Universidad de Castilla-La Mancha, Albacete, Spain). Negral cultivar is a chlorophyll-retaining cultivar, so that the ripe fruit is "chocolate" (green + red), and the rest of cultivars are red when ripe. Belrubi, Numex, and Delfin have long fruits (12−20 cm), Mana has small fruits (4−6 cm long), and Negral has round fruits (4−7 cm diameter).

Six ripening stages were selected as follows: NDG (nondeveloped green fruit), which is a growing fruit and therefore not fully mature; DG (developed green fruit), which is fully developed fruit just before the onset of maturation; CI (changing color fruit), which is a ripening fruit where green areas are more prevalent than red ones; CII (changing color fruit), which is a ripening fruit were red areas are more prevalent thangreen ones; RI and RII (red fruit) which are red fruit with an increasing maturation degree, respectively.

Pigment Extraction. Twenty-five fruits of every ripening stage were devoid of seeds and cutinto small pieces. Samples (10 g) were extracted with acetone, by using a homogenizer Ultraturrax Y25 (Janke Kunkel Ika-LabortechniK), until the complete exhaustion of color (usually 4−5 extractions were enough). All extracts were pooled in a separator and shaken with diethyl ether. 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 containing the pigments, in various stages of esterification with fatty acids, was saponified with 100 mL of 20% KOH−methanol for 1 h at room temperature. The pigments were subsequently extracted with diethyl ether, evaporated in a rotary evaporator, and taken up in a maximum of 25 mL of acetone. Aliquots (1 mL) of this was centrifuged at 12 000 rpm and stored at −30 °C until analyzed. Losses occurring during the process were monitored using a β-apo-8′-carotenal as internal standard; 1 mL of 100 μg/mL internal standard stock solution was added to the sample at the start of the extraction process. All analysis were carried out in quadruplicate.

High-Performance Liquid Chromatography. For HPLC analysis, a Waters 600E quaternary pump equipped with a diode array detector (PDA 996, Waters) and controlled with a Millennium data acquisition station was used. The injection valve was a Rheodyne model 7125. The HPLC system was equipped with a reverse-phase C-18 Spherisorb ODS-2 (5 μm, 0.46 cm × 25 cm) column (Teknokroma, Barcelona, Spain). A precolumn (0.5 cm × 4 mm I. D.) of the same material was fitted in order to protect the main column. Samples were cleaned previous to injection by using a benchtop centrifuge model Micro-Centaur (MSE Scientific Instruments, Sussex, England).

HPLC Separation and Quantification of Carotenoids. Separation and quantification of the carotenoid pigments was carried out using a method previously developed by the authors (Mínguez-Mosquera and Hornero-Méndez, 1993). The method uses a C-18 reverse-phase column and a binary gradient elution system of acetone−H_2O as follows: initially, 75% acetone is maintained for 5 min, changing linearly to 95% in 5 min, and maintained for 7 min. Flow rate was 1.5 mL/min, sample injection volume was 5 μL, and spectrophotomet-

Table 1. Evolution of the Total Carotenoid Content[a] and Isochromic Fraction Ratio (*R*/*Y*) during Fruit Ripening of Five *C. annuum* Cultivars

		ripening stage					
cultivar	pigment fraction	NDG	DG	CI	CII	RI	RII
Delfin	total carotenoid content	450.00	187.56	272.00	1010.48	3138.11	6899.96
	R/*Y* ratio			0.24	1.09	1.55	1.58
Belrubi	total carotenoid content	357.75	281.38	518.27	1051.31	3631.61	7886.00
	R/*Y* ratio			1.17	0.84	1.01	1.42
Mana	total carotenoid content	350.00	347.27	448.16	737.47	5665.64	13207.56
	R/*Y* ratio			0.64	0.35	1.81	1.25
Numex	total carotenoid content	364.08	290.43	275.77	1208.52	3035.57	6818.76
	R/*Y* ratio			0.33	1.30	1.46	1.59
Negral	total carotenoid content	272.22	181.80	513.21	1381.53	3781.53	8797.23
	R/*Y* ratio			0.67	0.99	1.79	1.80

[a] In milligram per kilogram dwt.

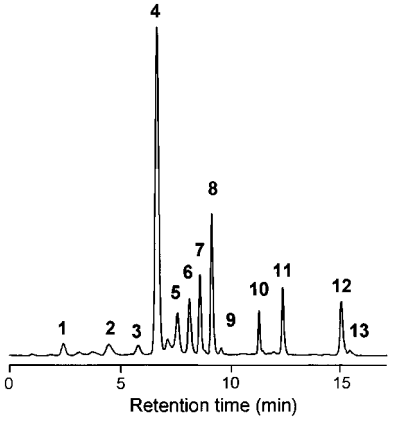

Figure 1. Reverse-phase HPLC chromatogram of a saponified extract of carotenoids pigments from ripe fruit of *Capsicum annuum*. Peak identities: 1, capsorubin; 2, violaxanthin; 3, capsanthin-5,6-epoxide; 4, capsanthin; 5, *cis*-capsanthin; 6, antheraxanthin; 7, cucurbitaxanthin A; 8, zeaxanthin; 9, *cis*-zeaxanthin; 10, all-*trans*-*β*-apo-8′-carotenal (internal standard); 11, *β*-cryptoxanthin; 12, *β*-carotene; 13, *cis*-*β*-carotene.

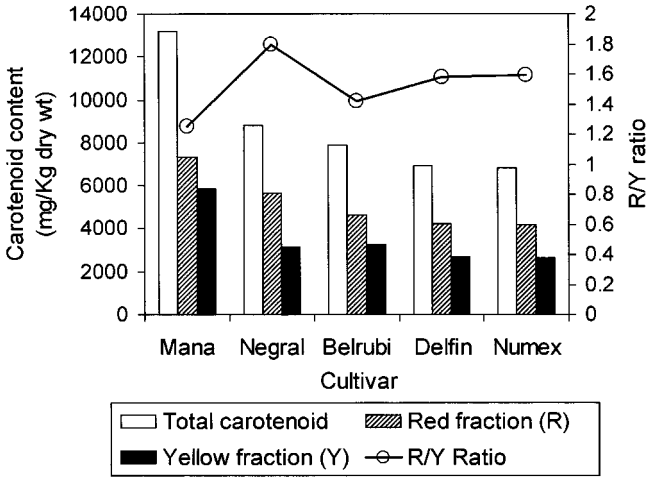

Figure 2. Comparison of the total carotenoid content (mg/kg dwt), red (R) and yellow (Y) isochromic fractions, and *R*/*Y* ratio in fully mature fruits (RII stage of ripeness) of five *Capsicum annuum* cultivars.

ric detection was performed at 450 nm. All-*trans*-*β*-apo-8′-carotenal as internal standard for calibration and quantification. In Figure 1, a sample 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 tetrahydrofurane and H_2O (52:48 v/v) at a flow rate of 1 mL/min and spectrophotometric detection at 450 nm.

Pigment Identification. These have been described in detail in previous publications (Mínguez-Mosquera and Hornero-Méndez, 1993) and consist of the following: separation of pigment by TLC and co-chromatography with purified pigments; observation of the pigment color on TLC plates under white, $UV_{254\ nm}$ and $UV_{360\ nm}$ lights; acquisition of UV−vis spectra in different solvents and comparison with the values reported in the literature; and chemical derivatization microscale tests for the examination of 5,6-epoxide, hydroxyl, and carbonyl groups. Carbonyl and hydroxyl groups were also investigated by FT-IR spectroscopy.

RESULTS AND DISCUSSION

Changes in the Total Carotenoid Content and Isochromic Fractions during Fruit Ripening. Since the commercial value of pepper for paprika is determined by the intensity of its red coloration, the selection and breeding of pepper cultivars must not only increase the total carotenoid content but also (and at the same time) increase the *R*/*Y* ratio, or at least maintain it at the same level as in the progenitors.

Table 1 shows the changes in total carotenoid content and isochromic fractions ratio (*R*/*Y*) during fruit ripening. Figure 2 compares these values for the totally ripe stage (RII) in the five cultivars studied. In the ripe fruit, the cultivar Mana has the highest carotenoid content (13 208 mg/kg dwt), followed by Negral (a cultivar retaining chlorophylls in the totally ripe stage) and Belrubi (8797 and 7886 mg/kg dwt, respectively), and last by Delfin and Numex (6900 and 6818 mg/kg dwt, respectively). In all of the red cultivars, the ratio between the isochromic fractions (*R*/*Y*) tends to increase with the total carotenoid content throughout the ripening, so that a greater content in pigments seems to be mediated by a higher final proportion of red to yellow pigments. In the cultivar Mana, there is a decrease in *R*/*Y* ratio at the end of ripening, which could indicate that the biosynthesis of red carotenoid pigments (capsanthin, capsorubin, etc.) reaches a production maximum, resulting in accumulation of the yellow fraction, containing pigments (violaxanthin, antheraxanthin, zeaxanthin, etc.) that are precursors of the former. The extent of such accumulation depends on the cultivar. In the other cultivars, this phenomenon is shown by a lower rate of increase in *R*/*Y* between states RII and RI than in earlier ripening stages. In terms of the increase in total carotenoid content at the end of ripening with respect to the green stage (DG), Negral has the greatest rise (48.39-fold), followed by Mana (38.03-fold), Delfin (36.8-fold), Belrubi (28.03-fold), and Numex (with the smallest increase, 23.48-fold).

In Negral, there are certain differences with the foregoing. Its *R*/*Y* ratio is the highest (1.80) with a high

carotenoid content as well (8797 mg/kg dwt). An explanation to this observation could be found in the origin of the cultivar, which was a selection of an spontaneous chlorophyll-retaining mutant from a field population of Bola cultivar (Costa, 1980). As shown in a previous paper (Mínguez-Mosquera and Hornero-Méndez, 1994), *R*/*Y* ratio for ripe fruits of Bola cultivar is 2.25 with a total carotenoid content of about 8000 mg/kg dwt, which is very close to the carotenoid content of Negral fruits. Therefore, retention of chlorophylls appears to cause retention of associated pigments (such as β-carotene) in the thylakoids, contributing to an increase in the yellow fraction and a decrease in the *R*/*Y* ratio. This observation could also reflect physiological differences in the transformation of chloroplasts into chromoplasts during ripening of the Negral variety fruits.

Modifications of the Carotenogenesis during Fruit Ripening. Figure 3 shows the changes in individual carotenoid pigments during fruit ripening, for which interpretation the typical scheme for carotenoid pigment biosynthesis of the genus *Capsicum* (shown diagrammatically in Figure 4) must be taken in account.

From the beginning of ripening (stages CI and CII), typical chloroplast pigments, such as lutein and neoxanthin, gradually disappear and are replaced by typical chromoplast pigments such as zeaxanthin and β-cryptoxanthin. This disappearance is very sharp in all varieties (with a decrease of 60−80% at the CI ripening stage) except in Mana where it is somehow delayed (only 5% decrease at the same ripening stage), reflecting some physiological differences during ripening for this cultivar. In general, the role of lutein in green plants appears to be intimately linked with the photosynthetic process as part of the light-harvesting system, so that its gradual disappearance together with chlorophylls seems to be the result of the loss of functionality once photosynthesis is blocked. Lutein and zeaxanthin are synthesized at the same level by action of cyclase enzymes (β- and ϵ-cyclase) (Cunningham et al., 1996), which gives the formation of one end toward the ϵ-ring and another end to β-ring in the case of lutein, and two β-rings in the case of zeaxanthin (see Figure 4). Once ripening begins, only carotenoids having two β-rings are synthesized, and therefore the disappearance in all cultivars of the only carotenoid-containing ϵ-ring, lutein, reveals that cyclase activity is now involved only in the biosynthesis of β,β-series carotenoids (β-carotene, antheraxanthin, violaxanthin, zeaxanthin, β-cryptoxanthin, capsanthin, capsorubin, capsanthin-5,6-epoxide, and cucurbitaxanthin A) as observed in all of the studied varieties (Figure 3). Although the cultivar Negral retains a large part of the initial chlorophylls, it does not retain lutein, which is contrary to what might be expected. This again reveals that ripening blocks the synthesis of lutein.

In a similar way, it has been reported that during ripening of the tomato (*Lycopersicon sculetum* Mill.) the activity of β- and ϵ-cyclase decrease and eventually disappear, resulting in the accumulation of lycopene as the major carotenoid (Ronen et al., 1999).

In the case of neoxanthin biosynthesis, blocking takes place at another level (Figure 4). In all the red cultivars, neoxanthin disappears during ripening, and only in the cultivar Negral, a chlorophyll retainer, are certain levels of neoxanthin maintained in the ripe fruit. The partial preservation of intact thylakoid structures, which retain chlorophylls, might help to retain neoxanthin; however, with no turnover, it eventually disappears. Neoxanthin is formed from its precursor violaxanthin, which is biosynthesized in large amounts during red pepper ripening but is later transformed into capsorubin and capsanthin 5,6-epoxide, so that the step from violaxanthin to neoxanthin is restrained and possibly blocked. Moreover, because much of the antheraxanthin, the precursor of violaxanthin, is used in the synthesis of capsanthin (the major pigment in the ripe fruit), this must also have a negative effect on the turnover of neoxanthin at the beginning of ripening.

In all of the red cultivars (Delfin, Mana, Numex and Belrubi), zeaxanthin is always the major pigment of the yellow fraction throughout ripening (about 60% at the early ripening stages and 30% at the fully ripe fruit), which denotes the central role of this pigment as the pool of the rest of the later intermediaries in the biosynthetic pathway. Zeaxanthin undergoes epoxidation to give antheraxanthin, which in turn is epoxidized to violaxanthin. Both pigments are essential in the synthesis of intrinsic pigments of the red pepper (capsanthin and capsorubin) via pinacolic reorganization of the 3-hydroxy-5,6-epoxide group to acylcyclopentanol or κ-ring (Figure 5). The enzyme responsible for the reorganization of acylcyclopentanol ring is denominated capsanthin-capsorubin synthase (Ccs), or κ-cyclase (Cunningham et al., 1996), which after characterization has shown a high homology with lycopene β-cyclase, leading some authors to suggest that it might evolve from the latter (Pecker et al., 1996; Ronen et al., 1999). Ripening upregulates the activity Ccs over antheraxanthin and violaxanthin to afford capsanthin and capsorubin, respectively. A deficiency of the Ccs enzyme or mutation in the gene that codifies it results in the incapacity of the fruit to form pigments having κ-rings, that is, those of the red fraction, and the final coloration of the ripe fruit is yellow. This fact has been observed in *C. annuum lycopersiciforme flavum* fruits, where lutein and other β,ϵ-series carotenoids are present in high concentrations in the ripe fruit, with an accumulation of pigments having a 3-hydroxy-5,6-epoxide group (Matus et al., 1991). Similarly, although to a lesser degree, the 3-hydroxy-5,6-epoxide group can undergo transformation to the 5-hydroxy-3,6-epoxy-β (or 5-hydroxy-3,6-oxabicycloheptane) end group (Figure 5), so that cucurbitaxanthin A is formed from antheraxanthin. The absence of this pigment in peppers with yellow final coloration suggests that its formation could be mediated by an enzyme similar to Ccs.

In the cultivar Negral, violaxanthin is initially the major pigment, possibly due to its initial presence in the thylakoid structures that do not degenerate completely on chlorophyll retention. However, it is subsequently exceeded in concentration by zeaxanthin, as in the other cultivars. The total loss of lutein, and the great loss of neoxanthin, in this chlorophyll-retaining cultivar indicate that the chlorophyll-retaining chromoplasts are transformed at the carotenogenic level exactly as they are in the red fruit. Thus, chlorophyll retention should be considered a phenomenon separate from carotenoid synthesis, and, being a characteristic of the cultivar, must be controlled at genetic level. Early literature (Smith, 1948, 1950) reports that chlorophyll-retaining cultivars owe this characteristic to homozygosis of the recessive allele of a gene (*cl*), which must ultimately be responsible for the deficiency at a functional or structural level of some enzyme essential in chlorophyll

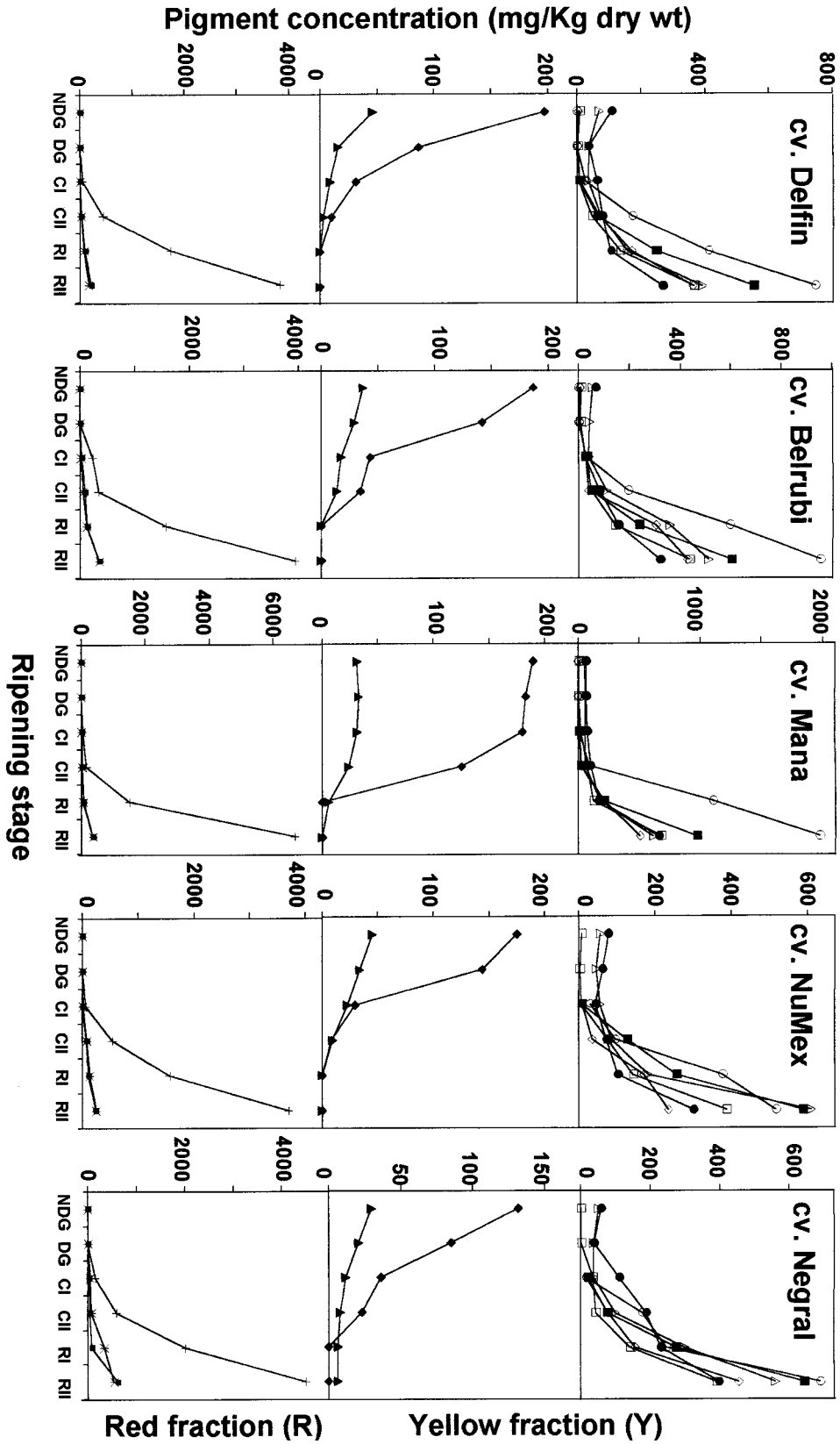

Figure 3. Changes in individual carotenoid composition during fruit ripening of five *C. annuum* cultivars. ▲, neoxanthin; ◆, lutein; +, capsanthin; ×, capsorubin; small filled square, capsanthin-5,6-epoxide; ●, violaxanthin; □, antheraxanthin; ◇, β-cryptoxanthin; △, β-carotene; ■, cucurbitaxanthin A; ○, zeaxanthin.

degradation. Recently, it has been established recently that a deficiency in the enzyme pheophorbide *a* oxygenase is the responsible for such phenotype, so that the chlorophyll-retaining mutants present very low levels of activity for this enzyme (Vicentini et al., 1995).

Comparison and Selection of Cultivars. The higher or lower carotenoid content for a given cultivar depends on various factors: greater or lesser expression of the genes governing carotenogenesis, physiological and morphological characteristics intrinsic to the cul-

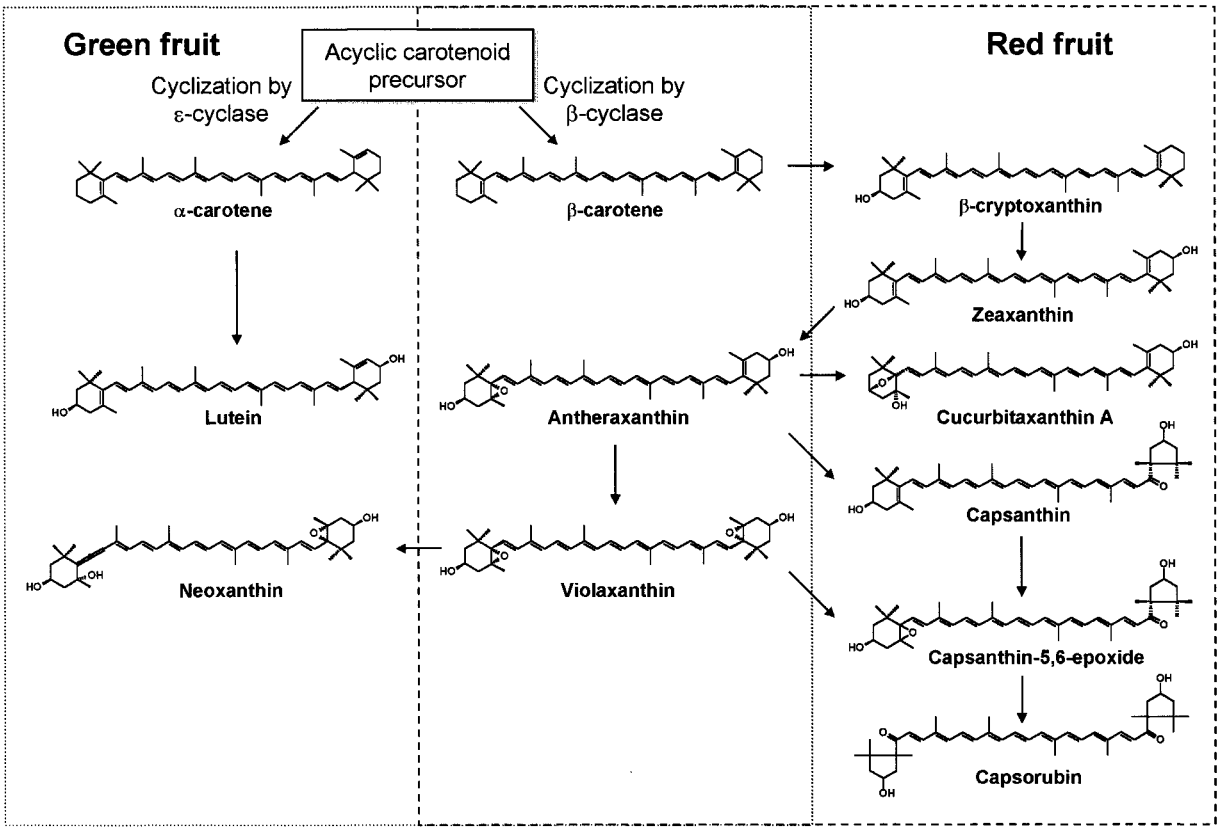

Figure 4. Pathway of carotenoid biosynthesis in *Capsicum annuum*. Dotted line square includes branch pathway for green fruit. Dashed line square includes branch pathway for red fruit.

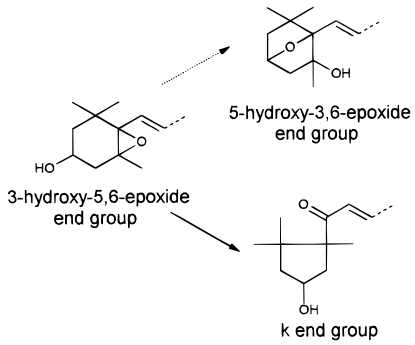

Figure 5. Rearrangement of 3-hydroxy-5,6-epoxy-β end group to give 5-hydroxy-3,6-epoxide end group (·····) and 6-oxo-κ end group (−).

tivar, and growth conditions. Although the last factor is very important in field trials because of its effect on the agronomic yield of the plant, in the present work it can be ignored, because in the greenhouse conditions are the same for all cultivars. Carotenogenesis is also an important branch, but not the only one, of the extensive isoprenoid metabolic pathway, which includes the synthesis of diterpenes, triterpenes, tocopherols, ubiquinone, etc. Depending on the characteristics inherent to each cultivar, certain biosynthetic pathways will be more important than others, and the fruit composition will differ.

As shown in Figure 3, all cultivars experience a decrease in individual pigment contents in the step from NDG to DG ripening stage, that is, as a consequence of a marked growth in size of the fruit. This is mediated by a high rate of synthesis of other metabolites, mainly structural ones. The phenomenon is less marked in the cultivar Mana, whose growth is slow and gradual throughout its vegetative period, rather than concentrated in the first stages as in the other cultivars (Table 2).Zeaxanthin is the major pigment throughout ripening in three of the four red cultivars (Delfin, Mana, and Belrubi), confirming its central role as pigment pool in carotenogenesis. In the cultivar Numex, it is also a major pigment, although jointly with other pigments such as β-carotene and cucurbitaxanthin A. The fact that this cultivar has the lowest total carotenoid content (6818.76 mg/kg dwt) in the ripe fruit and the lowest capsanthin content (3705 mg/kg dwt) indicates that carotenogenesis is less expressed for the formation pathways of intrinsic pepper pigments than in the other cultivars. In Negral, the case is similar regarding zeaxanthin, but opposite in its high capsanthin content, exceeded only in the cultivar Mana. Negral has the highest violaxanthin content throughout ripening. Chlorophyll retention in this cultivar may be accompanied by a persistence of the xanthophyll cycle that helps to maintain a large violaxanthin−antheraxanthin−zeaxanthin pool. This would explain why Negral presents the highest levels of capsorubin (536 mg/kg dwt) and capsanthin-5,6-epoxide (614 mg/kg dwt), 2- to 3-fold those in the other cultivars, as a result of the high content of their precursor, violaxanthin.

In all of the red cultivars, Mana, Belrubi, Delfin, and Numex, there is a direct relationship between total carotenoid content and capsanthin and zeaxanthin content. Mana presents the highest total carotenoid content (13 208 mg/kg dwt), and the highest levels of capsanthin and zeaxanthin (6687 and 1978 mg/kg dwt, respectively). It also presents a low R/Y ratio (1.25), interpreted previously as a maximal synthesis of red pigments (capsanthin, capsorubin, and capsanthin-5,6-

Table 2. Changes in Fruit Weight[a] during Development and Maturation

	ripening stage					
cultivar	NDG	DG	CI	CII	RI	RII
Delfin	1.96 ± 0.3[b]	7.21 ± 0.6	8.14 ± 0.9	8.72 ± 0.9	9.43 ± 1.3	9.26 ± 1.2
Belrubi	7.1 ± 2.0	29.4 ± 4.0	37.6 ± 3.0	43.2 ± 3.0	47.3 ± 4.0	41.2 ± 6.0
Numex	5.3 ± 1.1	16.2 ± 1.8	28.7 ± 3.2	35.3 ± 4.3	38.1 ± 5.1	36.4 ± 4.5
Negral	6.2 ± 2.3	16.3 ± 3.2	27.3 ± 4.6	29.6 ± 7.1	34.2 ± 8.9	31.1 ± 7.6
Mana	1.96 ± 0.2	2.8 ± 0.2	3.4 ± 0.4	4.3 ± 0.3	5.01 ± 0.4	4.62 ± 0.4

[a] In grams. [b] Mean ± SD for 40 fruits.

Table 3. Comparison of the Total Carotenoid Content,[a] Red to Yellow Isochromic Fractions Ratio (*R*/*Y*), and Capsanthin to Zeaxanthin Ratio in Fully Mature Fruits[b] of Five *Capsicum annuum* Cultivars

	cultivar				
	Mana	Belrubi	Delfin	Numex	Negral
total carotenoid[a]	13208	7886	6900	6818	8797
Caps/Zeax ratio	3.38	4.11	5.14	7.17	5.76
R/*Y* ratio	1.25	1.42	1.58	1.59	1.80

[a] In milligram per kilogram dwt. [b] RII stage of ripeness.

epoxide) in the fruit and the accumulation of precursors in their place (yellow fraction).

Cucurbitaxanthin A is, after zeaxanthin, the major pigment in all the cultivars once the fruits ripen (RII stage). As discussed above, this pigment is formed from antheraxanthin by reorganization of the 3-hydroxy-5,6-epoxide end group to 5-hydroxy-3,6-epoxide. Thus, like capsanthin, its precursor is antheraxanthin. The highest levels of this pigment are again seen in the cultivar Mana (972 mg/kg dwt). For the series of cultivars studied, there is a direct relationship between total carotenoid content and cucurbitaxanthin A content.

The first factor for comparing and selecting cultivars is a high total carotenoid content. If this yields more than one cultivar, that with the highest *R*/*Y* ratio will be selected. The *R*/*Y* ratio is a positive variety (i.e., genetic) characteristic and is available for selection and breeding, as well as for transfer from one cultivar to another by crosses between pure lines to form hybrids. The relationship between total carotenoid content and content in capsanthin and zeaxanthin can also be used as a parameter of breeding, with seeds being selected from fruits having high values of both. The first choice is always those with the highest capsanthin/zeaxanthin ratio (Caps/Zeax ratio).

Using these criteria (Table 3), in the present study, the cultivar selected as highest producer of carotenoids would be Mana. However, as already stated, in that cultivar, the *R*/*Y* and Caps/Zeax ratios are low (the lowest of all the cultivars studied), so that these two parameters could be used as indices in a breeding program. The cultivar Negral is also a good carotenoid producer, with around 9000 mg/kg dwt and presents high values of *R*/*Y* and Caps/Zeax. This cultivar could be bred for its total carotenoid content, but its retained chlorophylls in the ripe state darken the paprika, giving erroneously high values of carotenoid content in measurements of extractable color. This cultivar could also be used in crosses with the cultivar Mana, in an attempt to transfer its high *R*/*Y* ratio. The other cultivars (Belrubi, Delfin, and Numex) present total carotenoid contents around 7000−8000 mg/kg dwt, which, although not low, are the lowest of the cultivars studied. Of them, Numex has the highest Caps/Zeax ratio (7.17), and therefore it should be improved for total carotenoid content.

Nevertheless, it must be noted that the complex process of breeding cultivars of pepper for paprika has to take into account not only a high production of carotenoid pigments but also the selection and improvement of other characters (simultaneous ripening, resistance to disease, and agronomic yield), conditioners of viability, and commercial value of the product.

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