Xanthophyll Esterification Accompanying Carotenoid Overaccumulation in Chromoplast of *Capsicum annuum* Ripening Fruits Is a Constitutive Process and Useful for Ripeness Index

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Changes in xanthophyll esterification degree during pepper fruit ripening have been studied in five cultivars (Numex, Mana, Belrubi, Delfin, and Negral). Esterification of xanthophylls with fatty acids is seen to be a process that is contemporary with and directly linked to the transformation of chloroplast (present in the green fruit) into chromoplast (present in the red fruit). Changes in the fractions of free and partially and totally esterified carotenoids are similar between varieties, reflecting the constitutive nature of esterification as part of the ripening process and being controlled by it. From the first stages of ripening, the fraction of totally esterified pigments (zeaxanthin diester, β-cryptoxanthin diester, capsanthin diester, and capsorubin diester) makes up almost 50% of the total carotenoid content. The proportion of the partially esterified pigment fraction (zeaxanthin monoester, capsanthin monoester, and capsorubin monoester) in the total carotenoid content increases, with a gradual decrease in the fraction of free pigments (β-cryptoxanthin, β-carotene, zeaxanthin, capsanthin, and capsorubin). In the fully ripe stage, a balance is reached between the three esterification fractions (free, partially esterified, and totally esterified), with mean values of 24.17 ± 4.06, 31.48 ± 4.61, and 44.36 ± 5.05, respectively, which seems to be largely independent of variety. This suggests a marked control of the carotenoid composition of the totally developed chromoplast, indicating its use as an index of ripeness. The inclusion in the present study of a variety (Negral) that retains chlorophylls when ripening, and which shows the same esterification behavior, supports the idea that carotenogenesis is normal and independent of chlorophyll catabolism.

Keywords: *Carotenoid; Capsicum annuum; cultivars; paprika; xanthophyll esters; provitamin A; ripening*

INTRODUCTION

There is currently a growing demand for the use of natural coloring matters in the food industry. The carotenoids comprise an extended group of natural pigments−around 650−present in vegetables, fruits, animals, fungi, algae, and bacteria. They are mostly tetraterpenoid C40 compounds with an extensive system of conjugated double bonds. Traditionally, they are classified into two large structural groups: carotenes, which are basically hydrocarbons (β-carotene and lycopene), and xanthophylls, which include different oxygenated functions in the molecule (lutein, zeaxanthin, β-cryptoxanthin, etc.). The chromophore and thus the spectral characteristics of the compound differ depending on the extent of the system of conjugated double bonds and the various functional groups contained in the molecule. As a result, the color ranges from yellow to red through different hues of orange (Britton, 1983; Goodwin, 1976).

In nature, the carotenoid pigments have various functions. One of the most noteworthy is their role in trapping light energy at wavelengths scarcely accessible to chlorophyll, to which the energy is subsequently transferred for use in the photosynthetic process. They also have protective functions regarding the photosynthetic apparatus, dissipating excess energy and "quenching" reactive, harmful species such as singlet oxygen and excited chlorophyll (Frank and Cogdell, 1993). Carotenoids present in fruits, seeds, and flowers provide them with vital signals to attract pollen- and seed-dispersion animals, as well as to dissuade potential consumers (Goodwin, 1952).

Carotenoids ingested in the diet have important nutritional and physiological functions, through their general role as antioxidant and as provitamin A in certain cases (mainly β-carotene). Thus, they have been related with a decreased risk for certain types of cancer, an anti-ulcer effect, activation of the immunological system, etc. (Olson, 1989; Swanson and Parker, 1996; Ziegler, 1989).

The red pepper, and traditionally the paprika obtained from certain varieties and more recently the oleoresins, are a good source of carotenoid pigments for use in foodstuffs. Red pepper owes its color to a large family of carotenoid pigments, above all capsanthin and capsorubin, reddish carotenoids which are exclusive to this genus (Davies et al., 1970).

Coloring power is the main characteristic determining the commercial value of paprika and is directly related with the total content in carotenoid pigments. Apart from total carotenoid content, a higher proportion of red (R) pigments than yellow (Y) ones is considered positive, making the R/Y ratio a direct criterion of quality (Mínguez-Mosquera et al., 1984).

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Fruit ripening on the plant takes place gradually, so that depending on the variety, up to three harvests at 7−10-day intervals are necessary. From the agronomic point of view, this means certain disadvantages: lower end-of-harvest fruit quality (Mínguez-Mosquera and Hornero-Méndez, 1994a), greater labor cost, increased risk of disease, etc. Consequently, the grower tends to carry out a single harvest, so that some fruits are left to over-ripen, risking rot, while others do not attain an ideal degree of ripeness. The inevitable result is nonuniformity in the state of ripeness, and thus in the pigment composition, of the fruit harvested. Current work aimed at overcoming this problem is focused on the selection and breeding of varieties having both a clustered flowering and a short ripening cycle that concentrates fruit ripening, making mechanical harvest feasible. These selection characteristics directly affect uniformity of ripeness of the harvested fruit and thus the quality of the end product (Costa, 1980).

Metabolic processes have been reported to take place after fruit harvesting and during the first moments of the drying process to produce paprika. They include transformation of some pigments into others via metabolic pathways operating in the plant but without apparent turnover (Mínguez-Mosquera and Hornero-Méndez, 1994b). Thus, pigments such as capsanthin and capsorubin are synthesized from their precursors antheraxanthin and violaxanthin, respectively, which in turn have been formed sequentially from β-carotene, β-cryptoxanthin, and zeaxanthin. These processes lead to an increase in the red (R) isochromic pigment fraction (the one mainly giving color to the paprika) but at the cost of the other pigments (the yellow isochromic pigment fraction, Y), including carotenoids with provitamin A value (β-carotene and β-cryptoxanthin).

Therefore, it is essential that fruit arrives at the paprika transformation process in an ideal, uniform state of ripeness to ensure product quality and to take full advantage of the biosynthetic capacity of the fruit. It has long been known that processes such as senescence and (above all) fruit ripening in higher plants (e.g., pepper, tomato, etc.) lead in many cases to carotenogenesis accompanied by formation of esters of xanthophylls with various fatty acids. This process seems to be intimately linked with and controlled by the ripening process. Consequently, the present work studies and proposes the degree of xanthophyll esterification as a useful index of ripeness for pepper fruit.

MATERIALS AND METHODS

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

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

Apparatus. HPLC analyses were carried out using a Waters 600E quaternary pump equipped with a diode array detector (PDA 996, Waters) and controlled with a Millennium data acquisition station. The equipment was fitted with a Rheodyne model 7125 injection valve with an injection loop of 10 μL. Samples were cleaned previous to injection by using a benchtop centrifuge model Micro-Centaur (MSE Scientific Instruments, Sussex, England). Other apparatus used were a UV−vis spectrophotometer 8452A (Hewlett-Packard) and a homogenizer Ultraturrax T25 (Janke Kunkel Ika-LabortechniK).

Pigment Extraction. Twenty-five fruits of each ripening stage were devoided of seeds and cut up in small pieces. A 10 g subsample was extracted four times with 40 mL of acetone, until the complete exhaustion of all color. All extracts were pooled in a separator and extracted with 100 mL of 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 and filtered over an anhydrous Na_2SO_4 bed. This phase contains the pigments in various stages of esterification with fatty acids (acyl-carotenol esters) and was dried and taken up to 25−50 mL of acetone. One milliliter was stored for chromatography. The rest was transferred to diethyl ether and saponified with 100 mL of 20% KOH−MeOH for 1 h. The pigments were subsequently extracted with diethyl ether, evaporated in a rotary evaporator, and taken up in a maximum of 25 mL of acetone. A 1 mL aliquot of this was centrifuged at 12 000 rpm and stored at −30 °C until analyzed for individual carotenoid content. 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. All the analyses were carried out in quadruplicate.

HPLC Separation and Quantification of Carotenoids. Monitoring and quantification of the carotenoid pigments was carried out using a method previously developed by the authors (Mínguez-Mosquera and Hornero-Méndez, 1993). This method uses a C18 reverse-phase column (Spherisorb ODS-2, 5 μm, 0.46 cm × 25 cm) and a binary gradient elution system of H_2O−acetone at a flow rate of 1.5 mL/min, a sample injection volume of 5 μL, and detection at 450 nm. Quantification was carried out using all-*trans*-β-apo-8′-carotenal as the internal standard.

Pigment Identification. These have been described in detail in previous publications (Mínguez-Mosquera and Hornero-Méndez, 1993; Davies, 1976; Davies and Köst, 1988, Britton, 1995).

HPLC Separation of Acyl−Carotenol Esters. Chromatographic separation of xanthophyll acyl esters and determination of the degree of esterification was calculated as described by Mínguez-Mosquera and Hornero-Méndez (1994). This is based on chromatography of a direct extract (omitting saponification) and detection at 450 nm. Three fractions, free (F) and partially (PE) and totally esterified (TE) pigments, were separated according to retention time (R_t) elution and expressed as a percentage on the basis of the total integrated area.

RESULTS AND DISCUSSION

During pepper fruit ripening, there are profound transformations associated with and regulated by this phenological state of the plant. One of the most surprising and spectacular changes is the transformation of chloroplast into chromoplast. This process leads to the disappearance of chlorophylls and a de novo biosynthesis of carotenoids that are not present in the green fruit, which are in some cases exclusive to the genus *Capsicum*−mainly capsanthin and capsorubin (Figure 1).

Figure 2 shows the dramatic increase in the total carotenoid content (mg/kg dwt) with fruit ripening in

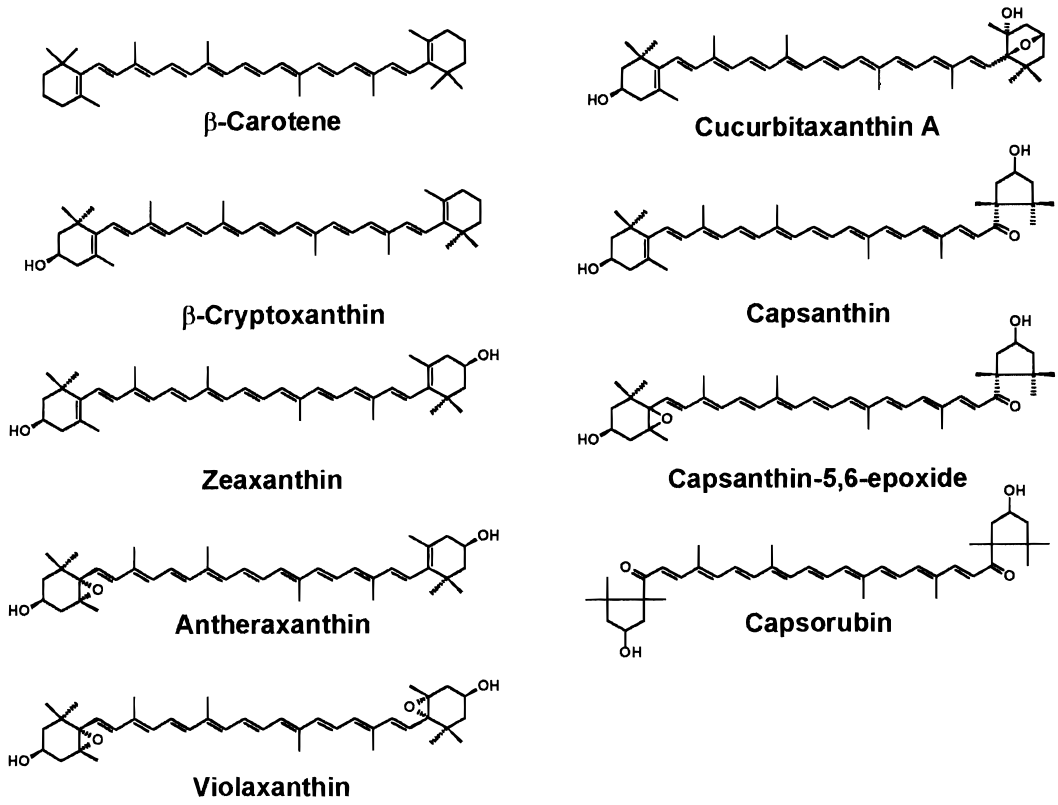

Figure 1. Structures for the carotenoid pigments responsible of the color of *Capsicum annuum* ripe fruit.

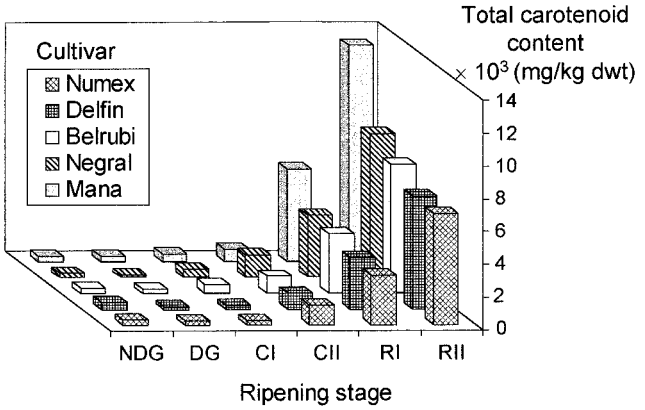

Figure 2. Evolution of the total carotenoid content (mg/kg dwt) during fruit ripening of five selected *Capsicum annuum* cultivars.

five pepper varieties (Mana, Numex, Belrubi, Delfin, and Negral). This increase ranges from 23.5-fold in the case of the variety Numex to 38.0-fold in the case of Mana. In the present study, the variety Negral is characterized by its retaining into the totally ripe stage (RII) a large part of the chlorophylls initially present in the green fruit. This variety belongs to the group of cultivars denominated "chlorophyll retainers", and this phenotype seems to be related with a defect in some key enzyme in the chlorophyll degradation pathway, which causes a retention and lower rate of chlorophyll degradation, resulting in the coexistence of thylakoid structures in the chromoplast of the ripe fruit (chlorochromoplast). Such a process does not appear to affect the carotenoid biosynthesis associated with ripening, which behaves as a parallel but independent phenomenon of chlorophyll catabolism. This variety stands out as having the greatest increase in the total carotenoid content (48.4-fold).

As occurs in senescent leaves, the synthesis and/or transformation of carotenoid pigments associated with ripening in pepper fruit leads to their esterification and their accumulation in chromoplast organelles, the plastoglobules. Esterification takes place mainly at the level of pigments synthesized de novo during ripening (mainly capsanthin, capsorubin, zeaxanthin, and β-cryptoxanthin), although it also occurs at the level of pigments previously present in the green fruit, such as violaxanthin. The mechanism and biosynthetic pathways by which xanthophyll esterification takes place have not been studied much and little is known. The process is not exclusive to plants but also takes place in organisms such as bacteria and algae, where the esterification may even involve substances other than fatty acids (butanoic and hexanoic acid in siphonaxanthin esters). It is generally assumed that these compounds are formed by conventional esterification of the carotenoid hydroxyl group via acyl-CoA, but no biochemical studies have been performed in detail (Britton, 1998). This transformation does not affect the chromophore properties of the pigment. Its function seems to be related—in the case of fruits and flowers—with the aim of attracting animals that are the vehicle for the dissemination of seeds and pollen. Esterification helps to increase attracting power by the overaccumulation of xanthophylls, making them more lipophilic and more readily integrated into the lipid-rich plastoglobules. In senescent leaves, and especially in deciduous species, it has long been observed that chlorophyll disappearance is accompanied by xanthophyll esterification. In this case, the prime function of xanthophyll esterification is obviously not animal attraction, but rather it indicates a phenomenon intimately linked with and inherent to the degeneration of chloroplasts and the formation of chromoplasts. That is, chloroplast degeneration leads to disorganization of the thylakoid membranes, where

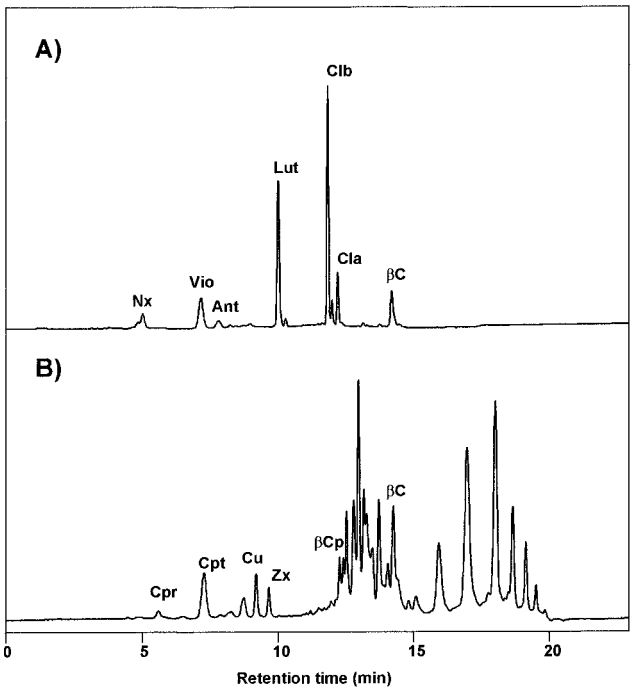

Figure 3. HPLC chromatograms of direct (nonsaponified) pigment extracts from (A) unripe fruit and (B) ripe fruit. Pigment identity: Nx, neoxanthin; Vio, violaxanthin; Antx, antheraxanthin; Lut, lutein; Clb, chlorophyll *b*; Cla, chlorophyll *a*; *β*C, *β*-carotene; Cpr, capsorubin; Cpt, capsanthin; Cu, cucurbitaxanthin; Zx, zeaxanthin; *β*Cp, *β*-cryptoxanthin.

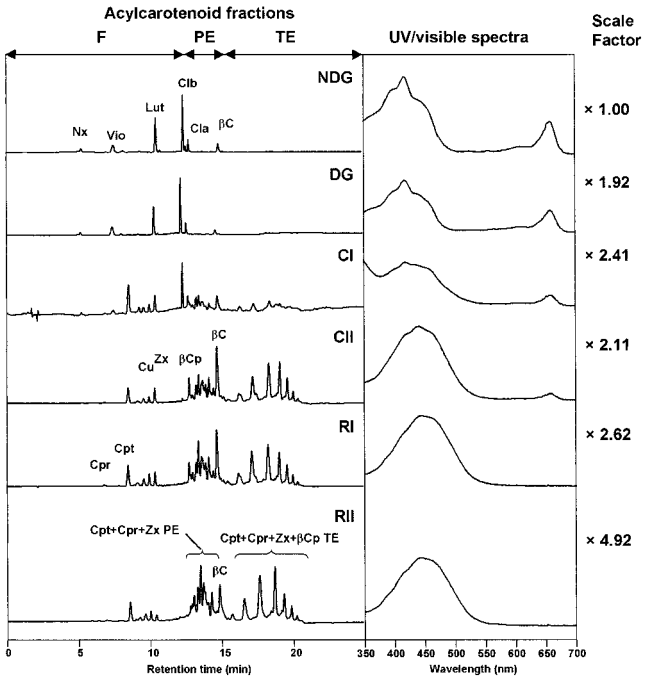

Figure 4. Ripening associated changes in the carotenoid fatty acid esters fractions— free (F), partially esterified (PE), and totally esterified (TE)—and the UV−visible spectra of the pigment extract (cv Mana). Pigment identity: Nx, neoxanthin; Vio, violaxanthin; Lut, lutein; Clb,: chlorophyll *b*; Cla, chlorophyll *a*; *β*C, *β*-carotene; Cpr, capsorubin; Cpt, capsanthin; Cu, cucurbitaxanthin; Zx, zeaxanthin; *β*Cp, *β*-cryptoxanthin.

the xanthophylls, together with the chlorophylls, are found associated to membrane proteins and lipids. The carotenoids are consequently liberated to the stroma, where they are not very soluble. Esterification thus helps in their integration into the membranes and plastoglobules.

Figure 3 shows a chromatogram of a pigment extract from green (unripe) pepper fruit and another of an extract from red (ripe) fruit. While the former shows the presence of the typical chloroplast pigments (chlorophyll *a*, chlorophyll *b*, lutein, violaxanthin, neoxanthin, and *β*-carotene), that of the red fruit shows a striking change in the pigment pattern, with the appearance of typical chromoplast xanthophylls (zeaxanthin and *β*-cryptoxanthin) that include the carotenoids exclusive to the genus *Capsicum*, capsanthin and capsorubin. The chromatogram is complicated by the esterification of these pigments with different fatty acids, resulting in the appearance of a whole family of esterification forms for the same pigment, depending on the number of −OH groups susceptible to esterification, the degree of esterification of these −OH groups, and the nature of the fatty acid involved in the esterification (Biacs and Daood, 1994; Mínguez-Mosquera and Hornero-Méndez, 1994c). Xanthophylls possessing two −OH groups, such as zeaxanthin, capsanthin, and capsorubin, give rise to three types of esterification products: totally esterified (zeaxanthin diester, capsanthin diester, and capsorubin diester), partially esterified (zeaxanthin monoester, capsanthin monoester, and capsorubin monoester), and not esterified (free or non-esterification). Xanthophylls with a single −OH group, such as *β*-cryptoxanthin, give rise to only two types of esterification product: free and totally esterified (*β*-cryptoxanthin monoester).

Esterification drastically changes the polarity of the pigment and thus its chromatographic behavior. Previous studies (Khachik and Beecher, 1988; Mínguez-Mosquera and Hornero-Méndez, 1994c) have shown that under reverse-phase chromatographic conditions, there is a distinct separation between families of compounds, with an elution order clearly correlated with a decreasing polarity of the pigment: free xanthophylls > partially esterified > *β*-carotene > totally esterified. Figure 4 shows the changes in the chromatogram and UV−visible absorption spectrum for the direct extract of chloro-chromoplast pigments with ripening. It can be seen that as the total carotenoid content increases with ripening, so does the complexity of the chromatogram, as a result of xanthophyll esterification, and the pigment profile changes from typically chloroplast to typically chromoplast.

Figure 5 shows the changes in the three fractions—free, partially esterified, and totally esterified—of the carotenoid pigments with the stage of fruit ripeness for the five varieties studied. Initially, and as expected for photosynthetic tissues, the xanthophylls present (mainly lutein, violaxanthin, neoxanthin, and antheraxanthin) are in the nonesterified or free (F) form (NDG and DG stages). During the first stages of ripening (CI and CII stages), the fractions of partial and total esterification (PE and TE) begin to be produced, with totally esterified (TE) xanthophylls being formed more markedly than partially esterified (PE) xanthophylls. Such phenomenon might be the result of a hydrophobicity requirement on the part of the carotenoid, so that with all its −OH groups esterified it will be included more readily in the lipid matrix of chromoplast membranes and organelles (plastoglobules). Moreover, it could be assumed that, as occurs with the triglycerides, the triacylester forms (totally esterified) predominate over those of intermediate esterification. The mechanisms leading to this, and the preferential insertion of determinate

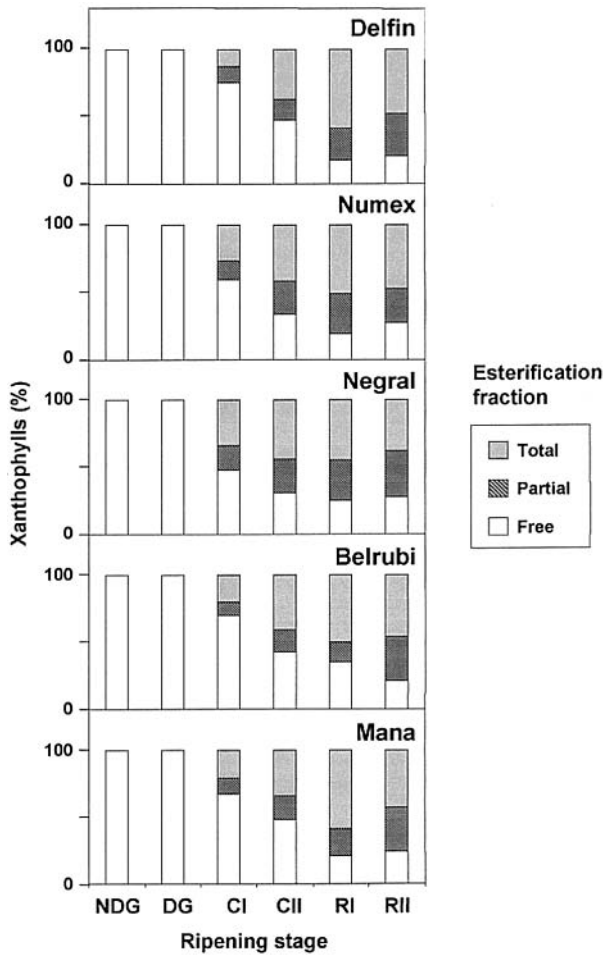

Figure 5. Changes in the xanthophyll esterification degree, defined as percent ratio between free (F), partially esterified (PE), and totally esterified (TE) fractions, during fruit ripening of five selected *Capsicum annuum* cultivars.

fatty acids, are not well-known, but the process must be complex and highly controlled at the biochemical and genetic level. Previous studies (Mínguez-Mosquera and Hornero-Méndez, 1994c) have shown that esterification of the exclusive pepper xanthophylls (capsanthin and capsorubin) is preferentially performed with saturated fatty acids (mainly myristic, lauric, and palmitic). In contrast, the remaining xanthophylls (mainly zeaxanthin and β-cryptoxanthin) are esterified 50−60% with linoleic acid, having a double unsaturation, and with myristic, lauric, and palmitic acids.

In the ripeness stage RI, when the coloration of the fruit is clearly due to carotenoid pigments and the fruit has a high carotenoid content and almost reaches physiological ripeness, the same trend is seen again, with the totally esterified (TE) fraction being the major one (approximately 50%). Later, in the totally ripe stage (RII), there is an increase in the partially esterified (PE) fraction and a consequent decrease in the TE fraction. This seems to be related with the establishment of a balance between the esterification fractions and may be determined by, among other factors, a limitation of the natural reservoirs (membranes) for accommodating these molecules without compromising their integrity.

Recently, Kumagai et al. (1998) have achieved the integration of typical chromoplast carotenoids (capsanthin and capsorubin) in chloroplasts of *Nicotiana benthamiana* via the in vivo expression of the capsanthin-capsorubin synthetase (Ccs) gene of *Capsicum*. The results obtained suggest the existence of a self-regulatory control of the chloroplast carotenoid composition, and corroborate that xanthophyll esterification is a transformation of the chromoplasts, as the integrated chromoplast carotenoids were not transformed. The balance found between the xanthophyll fractions with differing degrees of esterification might also suggest a self-regulation mechanism of the carotenoid composition in the chromoplast. This is in accord with the results of Deruère et al. (1994), who demonstrated the greater efficiency of esterified xanthophylls in the differentiation of the chromoplasts of *Capsicum* and their participation in the organization of the fibrillar structures responsible for overaccumulation of carotenoids in the ripe fruit. The balance reached between the esterification fractions (F, PE, and TE), with mean values of 24.17 ± 4.06, 31.48 ± 4.61, and 44.36 ± 5.05, respectively, seems to be well conserved between varieties, indicating a strong genotype component, and thus could be used as a "ripeness index". The use of this index would ensure uniformity in the state of fruit ripeness and take maximum advantage of the carotenogenic capacity of the fruits, with a direct impact on the quality of the processed products: paprika and oleoresins.

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