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# Studies on the Carotenoid Pigments of Paprika (*Capsicum annuum* L.var Sz-20)

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Fatty acid carotenoid esters and unesterified hypophasic and epiphasic carotenoids were extracted from paprika fruit at different stages of ripening and processing. The pigments were separated by high-performance liquid chromatography (HPLC) on Chromsil $C_{18}$ reversed-phase column with 59:57:4 (v/v/v) isocratic conditions and without prior saponificiation of the samples. Monoesters of capsanthin were found to contain mostly unsaturated fatty acids ($C_{18:2}$) while diesters of both capsanthin and capsorubin contained saturated fatty acids such as $C_{12}$, $C_{14}$, and $C_{16}$. The carotenoid esters were more stable, toward lipoxygenase (LOX) catalyzed linoleic acid oxidation, than free pigments. Furthermore, capsanthin esters containing saturated fatty acids resisted the enzymatic oxidation better than the others did.

Paprika (*Capsicum annuum*) is one of the oldest and most important food colors. Its products are the sources of natural carotenoid concentrates. The total red or yellow pigment content of paprika was determined by measuring the extinction of the benzene extract (Benedek, 1958; Fekete et al., 1976). TLC and open-column chromatography (OCC) were used for the separation of carotenoid pigments from paprika products (Vinkler and Richter, 1972; Buckle and Rahman 1979). Recently, a system of HPLC and supercritical fluid chromatography (SFC) were elaborated and developed for the separation, identification, and determination of paprika oleoresins and associated carotenoids (Baranyai et al., 1982; Gere, 1983). However, the analyses done by these methods are still carried out by gradient systems with or without resorting to saponification of the pigment samples.

Spectrophotometric methods, based on determining the decrease in the absorbance at 460 nm, were used for the measurement of carotenoid destruction through a coupled oxidation with LOX and linoleic acid (Ben Aziz et al., 1971; Nicolas et al., 1982; Hsieh and McDonald, 1984; Edwards and Lee, 1986). These methods are not suitable for the simultaneous determination of several pigments. The HPLC method was first applied by Hoschke et al. (1984) to study the changes occurring in the cartenoids of paprika pigment incubated with LOX and linoleic acid. In the method, ethanol was used in up to 5% of the reaction mixture to solubilize the pigments before the addition of the enzyme.

The purpose of this investigation was the separation and

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identification of paprika pigments and the study of their formation and stability by developed HPLC methods.

## EXPERIMENTAL SECTION

**HPLC Analysis.** A Beckman liquid chromatograph equipped with a Model 114 M pump, a Model 420 controller, and a Model 165 variable-wavelength UV detector was used. The signal was electronically integrated by a Shimadzu Model C-R3A integrator.

The pigments were separated on a 250 × 4.6 mm (i.d.) column packed with Chromsil $C_{18}$ 10-μm phase and eluted with 39:57:4 (v/v/v) acetonitrile–2-propanol–water. Flow rate was 1 mL/min, and detection was carried out at 438 nm.

To determine the cooxidation of paprika pigment by LOX, the mobile phase consisted of 85:5:10 (v/v/v) acetone–hexane–water.

**Pigment Extraction.** Ten grams (random sample) of paprika fruit was taken and disintegrated with quartz sand in a mortar with pestle. The sample was then mechanically shaken for 30 min with 50 mL of acetone. The homogenate was filtered, and the filtrate (fraction 1) was evaporated to dryness under vacuum at 40 °C. The remains (on the filter paper) were shaken with 50 mL of the solvent mixture consisting of 2:1:1 chloroform–2-propanol–acetone. The homogeneous solvent layer was dried on $Na_2SO_4$ and mixed with the solid materials of fraction 1. The solvent was evaporated to dryness under vacuum with a rotary evaporator.

Pigments of paprika powders were extracted by shaking 0.5 g of powder with 100 mL of acetone for 30 min. A 20-mL aliquot of the clear extract was evaporated to dryness under vacuum at room temperature. The dry material was kept at 2–4 °C under $N_2$ gas when not in use.

**Preinjection Preparation.** Solid material of extracted pigments was dissolved in 1 mL of chloroform, and the volume was then completed to 5 mL with the HPLC eluant.

For studying the stability of different pigments towards LOX-catalyzed linoleic acid oxidation, 50-μL portions of both Tween-20 and linoleic acid were added to the solid material of extracted pigments. The mixture was dissolved in 3 mL of chloroform, which was then evaporated under vacuum. The residues were dissolved in 5 mL of 0.1 M phosphate buffer at pH 7.5 with the help of ultrasonication. Cooxidation of paprika pigments was induced by adding 0.1–0.3 mL of partially purified paprika seed LOX [prepared according to Daood and Biacs (1986)]. After gentle shaking, 20 μL was injected into the HPLC column at time intervals of 0, 40, 80, and 120 min. The decrease in the peak area was used as index of pigment oxidation.

**Peak Identification.** The extracted pigments were saponified by refluxing at 60 °C with 5 mL of 20% methanolic KOH, 30 mL of MeOH, and 0.1 g of ascorbic acid for 40 min. Fatty acid methyl esters and saponified pigments were recovered by shaking the mixture twice with 30 mL of petroleum ether. The solvent layer was then separated by separatory funnel, washed with distilled water, and dried on $Na_2SO_4$.

Both carotenoid pigments of saponified and unsaponified samples were separated and identified by TLC method on preparative Kieselgel 60 plates (Merck) using petroleum ether–benzene–acetone–acetic acid (90:10:15:5) as the developing mixture (Vinkler and Richter, 1972; Baranyai et al., 1979). $R_f$ values and absorbance maxima of each band were measured and compared with those reported earlier (Vinkler and Richter, 1972; Buckle and Rahman, 1979). After the solvent was evaporated, the pigments of each band were dissolved in a minimum volume of the HPLC eluent and injected onto the column for measuring the retention time and to scan the maximum wavelengths of absorption between 300 and 700 nm (Table I). Some available standard pigments, such as capsathin, capsorubin, β-carotene, and zeaxanthin (Sigma), were also run by the same TLC and HPLC methods.

**Table I. Data Used for Identification of the Major Carotenoid Pigments of Paprika Extract**

| component | TLC $R_f$ | TLC abs max,[a] nm | HPLC $R_t$, min | HPLC abs max,[b] nm |
|---|---|---|---|---|
| capsorubin | 0.04 | 448, 481, 509 | 3.5 | 476, 517 |
| capsanthin | 0.07 | 478, 507 | 3.9 | 475, 517 |
| zeaxanthin | 0.11 | 422, 454, 480 | 4.2 | 425, 450, 480 |
| lutein | 0.14 | 420, 447, 471 | 4.6 | 422, 450, 478 |
| capsanthin ester I | 0.26 | 476, 494 | 6.1 | 486, 519 |
| capsanthin ester II | 0.27 | 476, 491 | 7.05 | 486, 518 |
| zeaxanthin ester | 0.31 | 429, 457, 482 | 8.3 | 426, 455, 488 |
| β-carotene | 0.97 | 454, 472 | 10.0 | 456, 477 |
| capsorubin ester I | 0.44 | 450, 480, 508 | 11.0 | 492, 517 |
| capsorubin ester II | 0.44 | 450, 480, 508 | 12.6 | 490, 518 |
| capsanthin ester III | 0.57 | 476, 485 | 15.2 | 488, 519 |
| capsanthin ester IV | 0.59 | 479, 489 | 18.6 | 489, 519 |

[a] In acetone. [b] Scanned during elution with 39:57:4 acetonitrile–2-propanol–water.

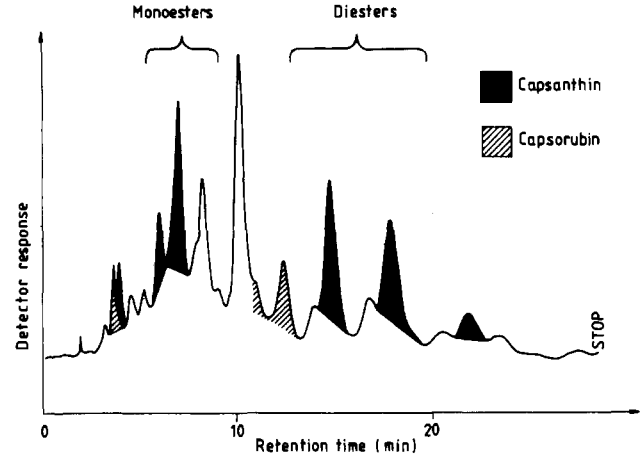


**Figure 1.** HPLC separation of paprika extract on Chromsil $C_{18}$ column, at 438 nm, using 39:57:4 acetonitrile–2-propanol–water as the mobile phase.

**GLC Analysis.** Fatty acid methyl esters obtained after saponification of each carotenoid ester band as well as the methyl esters of fatty acids of the whole paprika fruit were analyzed by gas–liquid chromatography (GLC) according to Gruiz and Biacs (1982) under the following conditions: instrument, Chrom-5; column, 10% poly(ethylene glycol succinate) (PEGS) on Chrom W-80; detector, flame ionization; carrier gaz, nitrogen, 30 mL/min; temperature, 180, 230, and 240 °C for column, injector, and detector respectively.

Standard fatty acid methyl esters (Polyscience Corp.) were used for identification purpose.

## RESULTS AND DISCUSSION

The isocratic separation of the pigments from unsaponified extract of paprika fruit is shown in Figure 1. The hypophasic compounds capsorubin, capsanthin, zeaxanthin, and lutein eluted first followed by the epiphastic pigments such as mono- and diesters of carotenoids and β-carotene. This agrees with the general pattern of reversed-phase chromatographic separation (Simpson, 1976). The carotenoid esters eluted before the β-carotene are due to the mono fatty acid cartoenoid esters, whereas the esters

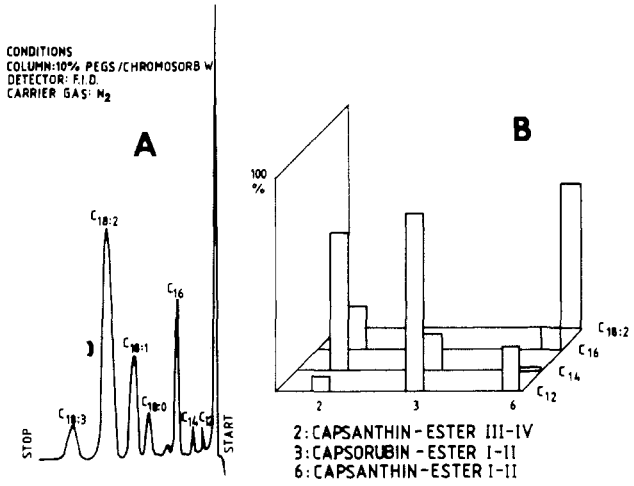


**Figure 2.** (A) GLC separation of fatty acids extracted from paprika powder. (B) Fatty acid composition of carotenoid esters isolated from paprika pigment. See text.

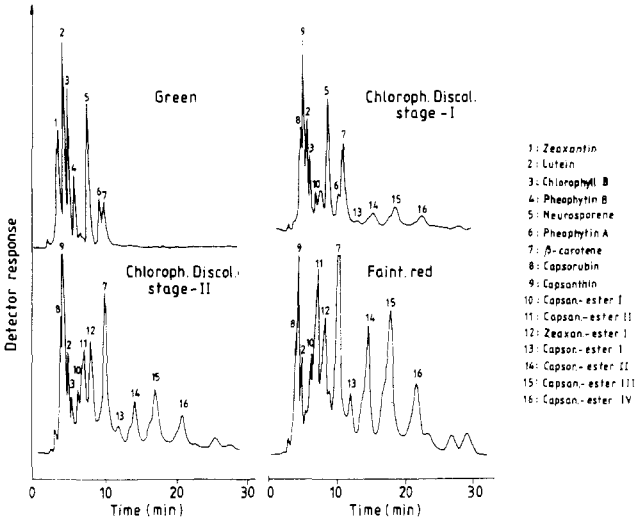


**Figure 3.** HPLC separation of pigments extracted from paprika fruit at the various stages of ripening.

eluted after the β-carotene are due to the bis fatty acid carotenoid esters (Fisher and Kocis, 1987). Also, it was evident that capsorubin was capable of forming bis fatty acid esters more than monoesters since the latter could not be found in paprika extracts.

The fatty acid composition of the carotenoid esters separated by TLC and saponified with alcoholic base is shown in Figure 2. The results indicated that paprika carotenoids esterify mostly with saturated fatty acids such $C_{12}$, $C_{14}$, and $C_{16}$, increasing the stability of the carotenoids toward oxidative damage. On the other hand, monoesters of capsanthin contained unsaturated fatty acids (mostly $C_{18:2}$) in addition to small amounts of $C_{12}$ and $C_{16}$.

**Carotenoid Formation in Paprika Fruit.** Figure 3 illustrates typical chromatograms of pigments extracted from paprika fruit at the various stages of ripening. The mature green fruit contained zeaxanthin, lutein, neurosporene, and β-carotene in addition to the chlorophyll derivates. At this stage neither capsanthin and capsorubin nor their fatty acid esters were detected. The appearance of these xanthophyls was noticed in accord with a decrease in the respiration rate when the chlorophylls started to decompose. After the chlorophylls had been degraded, the accumulation of the red carotenoid pigments was induced to high extent, whereas the concentrations of some carotenoids such as zeaxanthin and lutein decreased, revealing

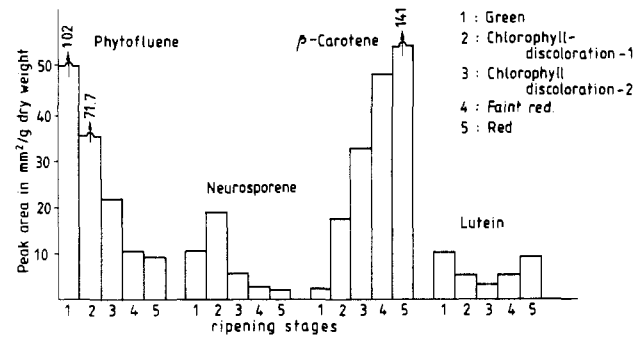


**Figure 4.** Changes in the concentration of phytofluene, neurosporene, β-carotene, and lutein during ripening of paprika fruits.

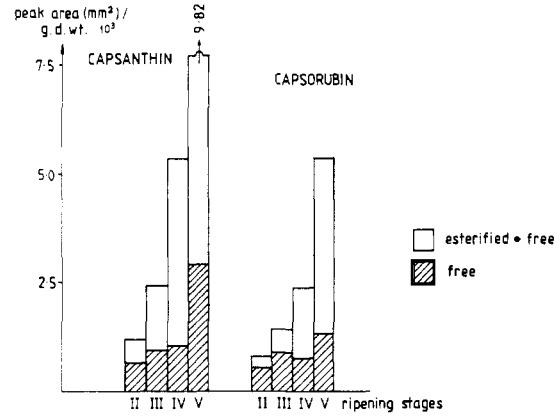


**Figure 5.** Esterification of capsanthin and capsorubin with fatty acids during ripening of paprika fruit.

their important role in the photosynthesis processes (Figure 4).

Capsanthin and capsorubin content of paprika fruit increase proportionally to the advanced ripeness. Figure 5 shows that the esterification of capsanthin and capsorubin with fatty acids was also induced at the same time when these components were being produced. Therefore, the ratio of esterified/free pigment was increasing during ripeness in coincidence with the increase in the total lipid content of the fruit. Concentration of the total capsanthin in paprika fruit and its products was higher than that of the total capsorubin; moreover, capsanthin esters III and IV, which contain saturated fatty acids, were the predominant pigments in paprika.

**Enzymatic Cooxidation of Paprika Pigments.** In the processing of paprika, the seeds are ground with the products (up to 15%) to increase their stability against the oxidation processes. However, a remarkable decrease in color intensity can be noticed during storage of these products. Figure 6 illustrates pigment composition of paprika powders with and without seeds. It is clear that addition of the seeds decreases the total pigment content of the product and damages the most important pigments.

Evidence has been presented for the presence of lipoxygenase in the seeds of paprika (Daood and Biacs, 1986). The enzyme catalyzes the oxidation of fatty acids containing a *cis,cis*-1,4-pentadiene structure, producing an active free radical in the first step of the reaction. The enzyme was found to have carotenoid bleaching activity through its aerobic and anaerobic pathways (Eskin et al., 1977).

Lipoxygenase was extracted from paprika seeds and incubated with the pigments solubilized by Tween-20 in the presence of linoleic acid. The changes in the concentrations of the individual pigments are shown in Figure 7. In general, the free pigments were less stable than the

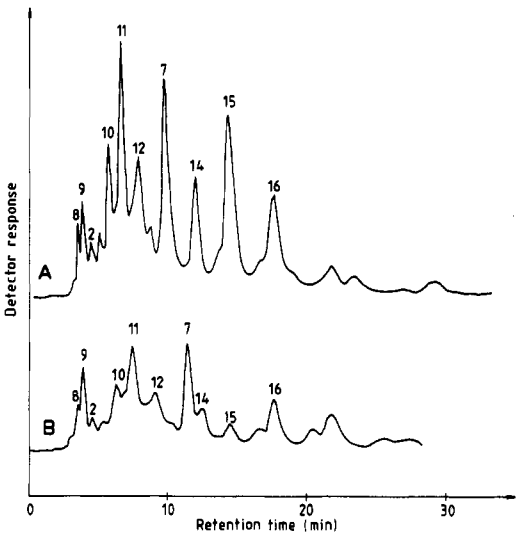


**Figure 6.** HPLC profile of (A) paprika powder and (B) paprika powder containing 10–15% seeds on a Chromsil $C_{18}$ column using 39:57:4 acetonitrile–2-propanol–water as the mobile phase. Peak identification is described on Figure 3.

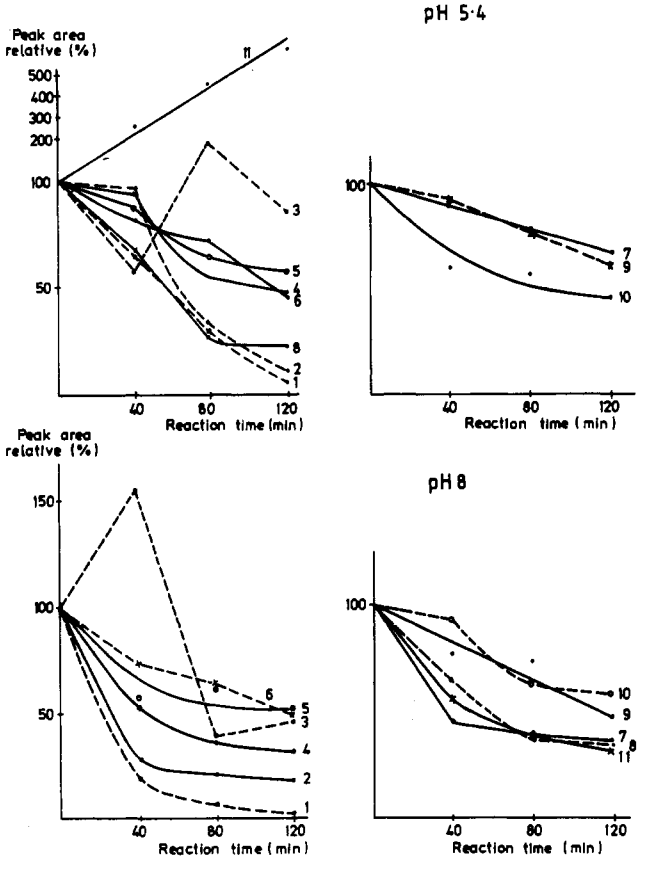


**Figure 7.** Degradation of paprika pigments through paprika seed LOX-catalyzed linoleic acid oxidation: 1, capsorubin; 2, capsanthin; 3, zeaxanthin; 4, capsanthin ester I; 5, capsanthin ester II; 6, zeaxanthin ester; 7, β-carotene; 8, capsorubin ester II; 9, capsanthin ester III; 10, capsanthin ester IV; 11, oxidation product.

esterified ones. Also it was found that capsanthin and their esters were less sensitive to the presence of LOX and linoleic acid than capsorubin and their esters. The most stable pigments were capsanthin esters III and IV, which contain saturated fatty acids.

At pH 8, when both LOX- and hydroperoxide-decomposing enzyme are active, degradation of the pigments was greater than at pH 5, when only acidic lipoxygenase is active. These results revealed the role of hydroperoxide-decomposing enzyme is carotenoid bleaching activity of lipoxygenase reported earlier (Eskin et al., 1977).

The increase in the concentration of some pigments such as zeaxanthin may be due to the presence of fatty acid releasing enzymes that are able to hydrolyze the esters of some carotenoids.

**Registry No.** LOX, 9029-60-1; capsorubin, 470-38-2; capsanthin monolinoleate, 118355-53-6; capsanthin monododecanoate, 34032-39-8; capsanthin monohexadecanoate, 113338-14-0; zeaxanthin, 144-68-3; lutein, 127-40-2; neurosporene, 502-64-7; β-carotene, 7235-40-7; capsanthin, 465-42-9; chlorophyll *b*, 519-62-0; pheophytin B, 3147-18-0; pheophytin A, 603-17-8; phytofluene, 27664-65-9.

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