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Separation and determination of individual carotenoids in a *Capsicum* cultivar by normal-phase high-performance liquid chromatography

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SUMMARY

A normal-phase high-performance liquid chromatographic system was developed for the separation of individual carotenoid pigments in a saponified extract of a *Capsicum* cultivar. Eighteen major components were separated, of which sixteen pigments were identified. The chromatographic system described permits a good separation and quantification of pigments. It can be used in physiological studies for the characterization of different varieties and in the food industry.

INTRODUCTION

Carotenoids constitute one of the most important groups of natural pigments and are widely distributed in the plant, animal and protista kingdoms. The pepper, the fruit of *Capsicum annuum* L., is an ideal plant material for the study of carotenoids as they are present in a great quantity and diversity.

Carotenoids are very important and some are useful for man: β-carotene and β-cryptoxanthin are provitamin A[1,2] and capsanthin, capsorubin and cryptocapsin, which are found nearly exclusively in fruits of *Capsicum*, are used as natural colorants. Finally, their metabolism is interesting owing to the spectacular changes that are produced during the ripening process[3,4].

Many methods have been proposed for the isolation and identification of carotenoids in plant material, but their structural similarities make their resolution difficult. In the particular case of *Capsicum* spp. pigments, paper chromatography[5] and thin-layer chromatography (TLC) are useful methods of separation[6–8]. However, the main inconvenience of these chromatographic systems is that they increase the inherent instability of the carotenoids in light, acids or oxygen[9]. Other workers have used a combination of counter-current chromatography and open-column[10,11] or gas

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chromatography[12]. Of these methods, open-column chromatography and TLC fail from the point of view of reproducibility and accurate quantification, whereas gas chromatography provides this last advantage, but cannot be used with thermolabile carotenoids.

Until now, no method has been reported that separates all *Capsicum* spp. pigments with good resolution using any one chromatographic system. Often it has been necessary to rechromatograph fractions, a method that takes several hours, during which time non-natural products are formed, owing to prolonged exposure to adsorbents, eluents, light and oxygen[1].

High-performance liquid chromatography (HPLC) has recently been applied to the qualitative analysis and determination of carotenoids from different plant materials[13–19]. It offers significant advantages over the techniques previously mentioned, namely rapidity, absence of alterations, on-line detection and sensitivity. When this technique is applied to the carotenoids of *Capsicum* spp. it is necessary to take into account that they are mainly esterified. Studies on the pepper fruit reported so far have used reversed-phase HPLC[20,21] and unsaponified extracts. Different peaks were obtained, some of them corresponding to the same carotenoid with different degrees of esterification[22,23].

In this paper, we describe the separation and identification of individual unesterified carotenoids of *Capsicum annuum* using a normal-phase HPLC system in one step. The method is rapid and reproducible. Also, it can be used in physiological studies, for the characterization of different varieties and in the food industry.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Shimadzu Model LC-6A ternary solvent-delivery system equipped with a Shimadzu Model SLC-6A controller, connected to a Perkin-Elmer Model LC-55 UV–VIS detector. The data were processed by means of a Shimadzu Model CR-6A integrator to evaluate the peak area and peak height.

Absorption spectra of isolated components in various solvents were recorded on a Hitachi Model U-3200 UV–VIS spectrophotometer with a double "monochromator". The absorption spectra of the carotenoids were recorded between 300 and 500 nm.

The purity of the chromatographic peaks was evaluated using a Shimadzu Model SPD-M6A photodiode detector. The spectrophotometric data were processed in a PC with hard disk using a specific chromatographic program. This program provides, among other data, the spectrum, the absorbance ratio and purity of a peak.

Columns

Analytical separation was performed on a stainless-steel column (25 cm × 4.6 mm I.D.) of Spherisorb (5-μm spherical particles) (Phase Separations).

Semipreparative separation was carried out on a Waters Assoc. stainless-steel column (30 cm × 7.8 mm I.D.) of μPorasil (10 μm irregular particles). All columns were equilibrated with the initial elution solvent for 15 min prior to analysis.

Materials and reagents

Detection in the visible zone of the spectrum (400–500 nm) allowed the use of ACS-quality reagents; this provides important savings in solvent costs, without a notable decrease in the operational lifetime of column. The solvents used for liquid chromatography were dehydrated (0.3 nm molecular sieve for acetone; sodium for light petroleum), filtered through 0.45-μm filters and degassed in an ultrasonic bath at reduced pressure.

The fruits of *Capsicum annuum* cv. Belrubí were kindly supplied by the Instituto Nacional de Investigaciones Agrarias (experimental field in Torreblanca, Murcia, Spain). This cultivar was developed from "Cornicabra" (Mexican pepper) for greater productivity. The fruit has a bright red colour, is long and contains capsaicin, which makes its hot. The fruits were harvested fully ripened. Pepper samples (3 kg) of uniform size were collected and taken immediately to the laboratory. The peppers were cut open and the seeds removed and discarded. The remainder of the fruits were chopped into small pieces and 25-g subsamples were removed from each of the samples and stored at $-28°C$ under nitrogen until analysed.

Pigment extraction and saponification

Three 25-g samples of peppers were extracted with methanol in a Sorvall Omni-Mixer blender at high speed for about 20 min. The stainless-steel chamber was immersed in an ice-bath. The mixture was filtered with a sintered-glass funnel and the residue repeatedly extracted with methanol–diethyl ether (90:10) until the triturate was colourless. The filtrates were combined and brought to 500 ml with the same extraction solvent.

A 50-ml aliquot of each sample was saponified with aqueous potassium hydroxide (60%) and the non-saponified fraction of carotenoids was re-extracted with diethyl ether employing the method of Candela *et al.*[8]. The ether solution was brought to a final volume of 50 ml.

For HPLC analysis 3-ml aliquots were dried in a nitrogen stream and the residue was dissolved in 300 μl of acetone and filtered through a 0.45-μm filter (Swinnex, Millipore) prior to injection. Acetone was used because it is a very good solvent for all the pigments.

Other plant materials used as sources of some of the carotenoids present in peppers were anthers from *Asphodelus fistulosus* L., maize seeds, citrus (lemon) leaves and spinach leaves. We also used synthetic β-carotene from Hoffmann-La Roche. Extraction of the carotenoids and the saponification conditions were the same as employed with the pepper fruits. All the manipulations were carried out under red light.

Chromatographic procedures

The separations of carotenoids were carried out under two sets of HPLC conditions. In both, two solvents were used: light petroleum (b.p. 40–60°C) (LP) and acetone (AC). The analytical separations were carried out with HPLC system A and the semipreparative separations with system B.

System A. The initial solvent mixture was LP–AC (95:5, v/v). After injection, a stepwise gradient was introduced as follows: (1) the proportion of LP was decreased to 75% in 30 min with a linear gradient (curve 0 of the system controller); (2) LP was

maintained at 75% for 5 min; (3) LP was increased to 95% in 5 min with a convex gradient (curve −10 of the system controller). Other chromatographic conditions were as follows: flow-rate, 1 ml/min; chart speed, 3 mm/min; wavelength, 460 nm; detection limit, 0.02 a.u.f.s.; and injection volume, 4 μl. The injection of this small, highly concentrated volume prevents distortions of the peak and the formation of artifacts in HPLC[24].

System B. This was identical with A except that in the second step LP was maintained at 75% for 15 min and the flow-rate was 4 ml/min. The detection limit was 0.5 a.u.f.s. and the injection volume was 175 μl.

TLC analyses were made on silica gel 60 plates (10 × 20 cm, layer thickness 0.25 mm; Merck) with a concentration zone (2.5 cm). Development was carried out for 75 min in a rectangular TLC tank previously equilibrated with the elution solvent of LP–AC (77:23) for 5 min.

Pigment quantification

The pigments were quantified by evaluating as capsanthin the red carotenoids: capsanthin, capsanthin epoxide, capsorubin, capsorubin isomer and cryptocapsin. The remaining yellow pigments were quantified as β-carotene. Known amounts between 30 and 600 ng and 100 and 2500 ng of β-carotene and capsanthin, respectively, were analysed with system A and their corresponding areas evaluated. The concentration of these standards in benzene was determined by spectrophotometry, using specific extinction coefficients ($E_{1\,cm}^{1\%}$) of 2337 for β-carotene and 2072 for capsanthin. Six to eight determinations were made for each carotenoid, resulting in a linear calibration graph (area *vs.* concentration). The standard deviation was less than 1.5%.

For the quantification of the problem extracts, a first injection of the standard into the HPLC system was followed by four injections of the corresponding extracts and then the standard.

RESULTS

Separation and identification of carotenoids by semipreparative TLC and HPLC

Concentrated solutions of anthers, maize seeds, citrus (lemon) leaves, spinach leaves and pepper fruit extracts were chromatographed on silica gel thin-layer plates. Of the bands obtained, the following were subsequently analysed by HPLC.

Anthers. Five bands were separated, of which the third in order of chromatographic elution was the largest with $R_F = 0.67$. This band was subsequently purified by semipreparative HPLC (system B), resulting in a retention time (t_R) of 12.31 min and was identified as anteraxanthin. The visible absorption maxima (nm) were hexane, $\lambda_{max} = 421, 443, 473$; benzene, $\lambda_{max} = 433, 457, 486$. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 19.5 nm corresponding to a 5,6-epoxy group.

Maize seeds. Three bands were separated, of which the second ($R_F = 0.84$) and the third ($R_F = 0.38$) in order of elution were scraped off. These bands were subsequently purified by semipreparative HPLC (system B) resulting, in t_R of 10.80 and 23.41 min, respectively.

Band 2 was identified as β-cryptoxanthin. The visible absorption maxima (nm; values in parentheses are shoulders) were hexane, $\lambda_{max} = (425), 450, 477$; benzene, $\lambda_{max} = (435), 463, 490$.

Band 3 was identified as zeaxanthin. The visible absorption maxima (nm) were hexane, λ_{max} = (423), 450, 477; benzene, λ_{max} = (435), 462, 490.

Citrus (lemon) leaves. Eleven bands were separated, of which the ninth in order of chromatographic elution with R_F = 0.20 was scraped off. This band was subsequently purified by semipreparative HPLC (system B), resulting in a t_R 31.34 min. It was identified as violaxanthin. The visible absorption maxima (nm) were hexane, λ_{max} = 420, 444, 473; benzene, λ_{max} = 434, 457, 486. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 40 nm, converting the violaxanthin to auroxanthin (λ_{max} = 380, 400, 425 nm in hexane) within a few minutes.

Spinach leaves. Four bands were separated, scraped off and collected.

Band 1. This band gave R_F = 0.96 on TLC and was subsequently purified by semipreparative HPLC (system B), where it gave a t_R of 2.90 min. The visible absorption maxima (nm) were hexane, λ_{max} = (425), 449, 476; benzene, λ_{max} = (437), 460, 489. This band was identified as β-carotene.

Band 2. This band gave R_F = 0.57 on TLC and was purified by HPLC (system B), resulting in a t_R of 17.80 min. The visible absorption maxima (nm) were hexane, λ_{max} = 420, 447, 472; benzene, λ_{max} = 433, 456, 486. This band was identified as lutein.

Band 3 was identified as violaxanthin.

Band 4. This band gave R_F = 0.13 on TLC. This band was subsequently purified by semipreparative HPLC (system B), resulting in a t_R of 35.74 min. The visible absorption maxima (nm) were hexane, λ_{max} = 412, 435, 465; benzene, λ_{max} = 422, 447, 477. This band was identified as neoxanthin and was converted to neochrome by the addition of a few drops of ethanolic hydrogen chloride, with a hypsochromic shift of 16–17 nm. The visible absorption maxima (nm) of neochrome were hexane, λ_{max} = 400, 423, 450; benzene, λ_{max} = 407, 431, (460).

Pepper fruits. The TLC of a Belrubí (half ripening) pepper extract gave twelve bands, which were recovered and chromatographed by HPLC with the following results.

Band 1 was identified as β-carotene.

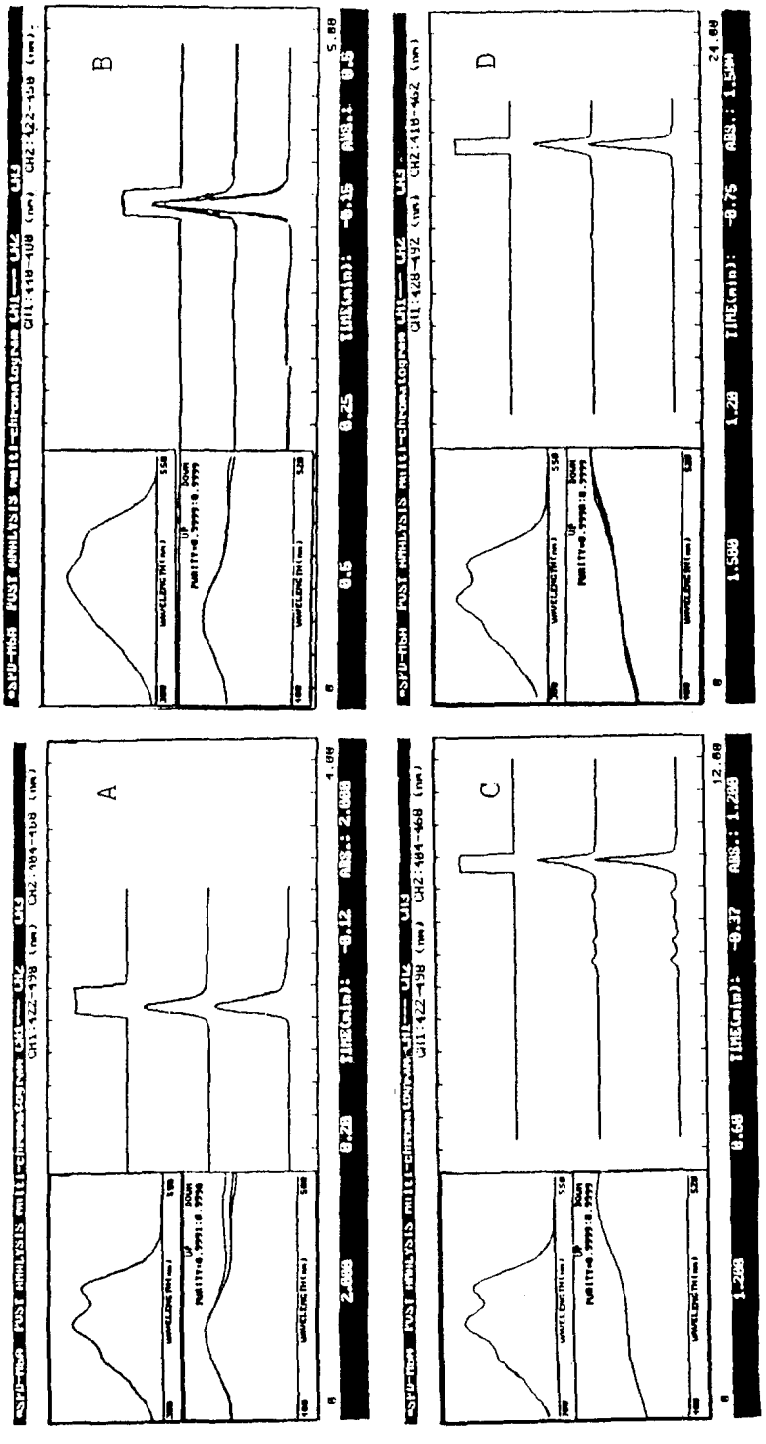
Band 2 had R_F = 0.93 on TLC. When purified by HPLC (system B) it resulted in two peaks with t_R of 3.46 and 4.65 min. The visible absorption maxima (nm) corresponding to the peak of t_R 3.46 min were hexane λ_{max} = (445), 469, 498; benzene, λ_{max} = 483, (518). This band was identified as cryptocapsin[10]. The visible absorption maxima (nm) corresponding to the peak of t_R 4.65 min were hexane λ_{max} = (402), 427, 451; benzene, λ_{max} = (408), 437, 464. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 19 nm. This band was identified as criptoflavin[10].

Band 3 was identified as β-cryptoxanthin.

Band 4 was identified as anteraxanthin.

Band 5 was identified as lutein.

Band 6 had R_F = 0.50 on TLC. Analysis by HPLC (system B) resulted in two peaks with t_R of 20.16 and 22.35 min. The visible absorption maxima corresponding to the peak of t_R 20.16 min were hexane, λ_{max} = 422, 444, 473; benzene, λ_{max} = 433, 458, 484; this band was identified as capsolutein. For the peak of t_R 22.35 min the visible absorption maxima (nm) were hexane, λ_{max} = 399, 422, 449; benzene, λ_{max} = 409, 432, 460. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 20 nm. This band was identified as luteoxanthin.


A
B
C
D

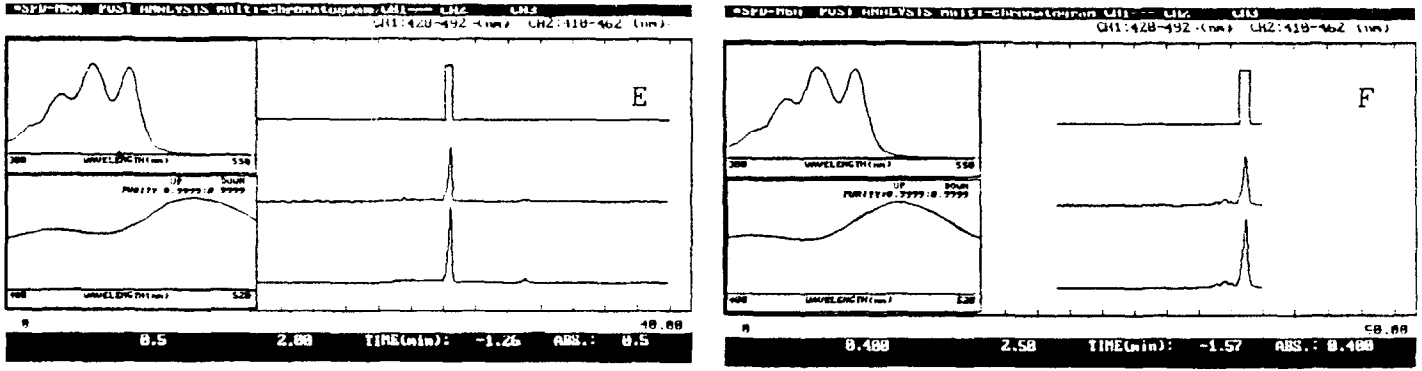


Fig. 1. Absorption spectra, absorbance ratios and purity of some carotenoids. (A) β-Carotene; (B) cryptocapsin; (C) antheraxanthin; (D) zeaxanthin; (E) violaxanthin; (F) neoxanthin.

Band 7 was identified as zeaxanthin.

Band 8 had $R_F = 0.28$ on TLC. Purification by HPLC (system B) resulted in a t_R of 24.82 min and the band was identified as mutatoxanthin. The visible absorption maxima (nm) were hexane, $\lambda_{max} = (406), 427, 453$; benzene, $\lambda_{max} = (415), 439, 466$. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift corresponding to 5,8-epoxy group.

Band 9 had $R_F = 0.24$ on TLC. Purification by HPLC (system B) resulted in two bands with t_R of 29.89 and 30.85 min. The band with $t_R = 29.89$ min was identified as capsanthin. The visible absorption maxima (nm) were hexane, $\lambda_{max} = 470, 497$; benzene, $\lambda_{max} = 483, 511$. The band with $t_R = 30.85$ min was identified as 5,6-epoxy-capsanthin. The visible absorption maxima (nm) were benzene, $\lambda_{max} = 479, (508)$. The addition of a few drops of ethanolic hydrogen chloride gave a hypsochromic shift of 20 nm corresponding to 5,6-epoxy group.

Band 10 was identified as violaxanthin.

Band 11 had $R_F = 0.18$ on TLC. Purification by HPLC (system B) resulted in two peaks with t_R of 33.30 and 33.98 min. The band with $t_R = 33.30$ min was identified as capsorubin. The visible absorption maxima (nm) were hexane, $\lambda_{max} = 440, 467, 501$; benzene, $\lambda_{max} = 460, 488, 523$. The peak with $t_R = 33.98$ min had the same spectrum as capsorubin; it was identified as a capsorubin isomer.

Band 12 was identified as neoxanthin.

DISCUSSION

Once separated and purified by TLC and HPLC, the principal carotenoids responsible for the colour of the *Capsicum annuum* cv. Belrubí fruits were used as standards for normal-phase HPLC separation.

When the photodiode detector was used, the chromatographic software provided, among other data, the absorbance spectrum, the absorbance ratio and purity of each peak. Spectra were obtained using solvent system A. Fig. 1 shows the purity criteria for some of the pigments obtained.

To optimize the HPLC conditions, we tried different gradients with the solvents light petroleum (b.p. 40–60°C) and acetone in order to find the most suitable polarity for the separation of the different pigments. We finally adopted the elution gradient described previously (system A). Of all the bands appearing in the chromatogram, only capsanthin with its epoxide and capsorubin with its isomer are insufficiently separated. Nevertheless, the chromatographic integrator quantified them separately. Modifications of the chromatographic gradient in order to reduce the overlapping of these bands leads to very long analysis times with no significant improvement in resolution. Fig. 2 shows the chromatogram corresponding to this separation.

In Table I, the main chromatographic parameters corresponding to the separation defined by Kirkland[25] are summarized.

For products derived from *Capsicum annuum* the elution order of the individual carotenoids in the normal-phase column is almost the reverse of that obtained by other workers using a reversed-phase column[14].

Fig. 3 shows a chromatogram corresponding to the separation of carotenoids of a saponified extract of the fruits of *Capsicum annuum* cv. Belrubí, harvested completely ripened.

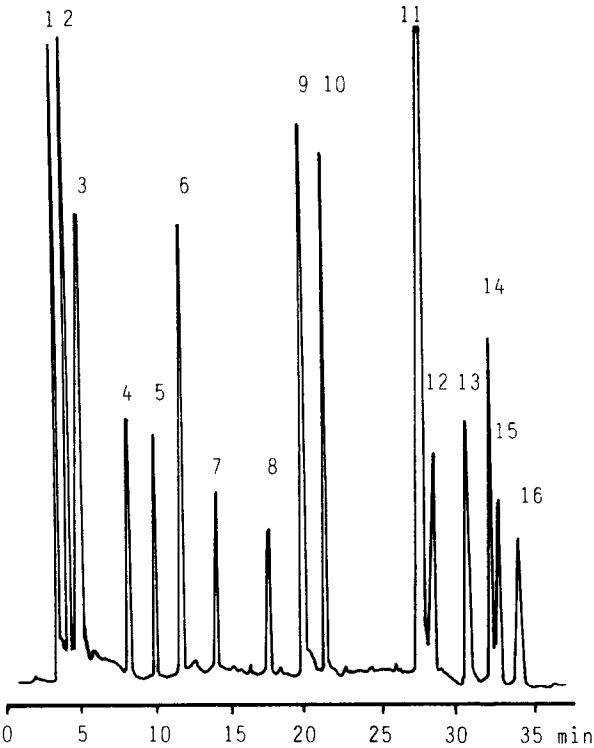


Fig. 2. Separation of carotenoids in a standard mixture (system A). Peak identification: 1 = β-carotene; 2 = cryptocapsin; 3 = cryptoflavin; 4 = β-cryptoxanthin; 5 = antheraxanthin; 6 = lutein; 7 = capsolutein; 8 = luteoxanthin; 9 = zeaxanthin; 10 = mutatoxanthin; 11 = capsanthin; 12 = capsanthin 5,6-epoxide; 13 = violaxanthin; 14 = capsorubin; 15 = capsorubin isomer; 16 = neoxanthin.

TABLE I

MAIN CHROMATOGRAPHIC PARAMETERS CORRESPONDING TO THE SEPARATION OF CAROTENOID PIGMENTS

Retention times were taken from the chromatographic integrator. Symbols: t = retention time (min), t' = reduced retention time ($t' = t - t_0$; dead time t_0 = 1.10 min), ω = band width (min), k' = capacity factor, α = separation factor, R_s = resolution.

Carotenoid	t	t'	ω	k'	α	R_s
β-Carotene	2.75	1.65	0.37	1.51	–	–
Cryptocapsin	3.36	2.26	0.40	2.05	1.36	1.58
Cryptoflavin	4.40	3.30	0.40	3.00	1.46	2.60
β-Cryptoxanthin	7.74	6.64	0.60	6.03	2.01	6.68
Antheraxanthin	9.56	8.46	0.83	7.70	1.27	2.54
Lutein	11.13	10.03	0.73	9.12	1.18	2.01
Capsolutein	13.73	12.63	0.67	11.50	1.26	3.71
Luteoxanthin	17.03	15.93	0.67	14.50	1.26	4.92
Zeaxanthin	19.58	18.48	0.67	16.80	1.16	3.80
Mutatoxanthin	21.39	20.29	0.67	18.45	1.10	2.70
Capsanthin	27.60	26.50	0.67	24.10	1.30	9.27
Capsanthin 5,6-epoxide	28.29	27.19	0.40	24.72	1.03	1.29
Violaxanthin	30.58	29.48	0.73	26.80	1.08	4.05
Capsorubin	32.12	31.02	0.67	28.20	1.05	2.20
Capsorubin isomer	32.48	31.38	0.50	28.53	1.01	0.62
Neoxanthin	33.81	32.71	0.60	29.74	1.04	4.93

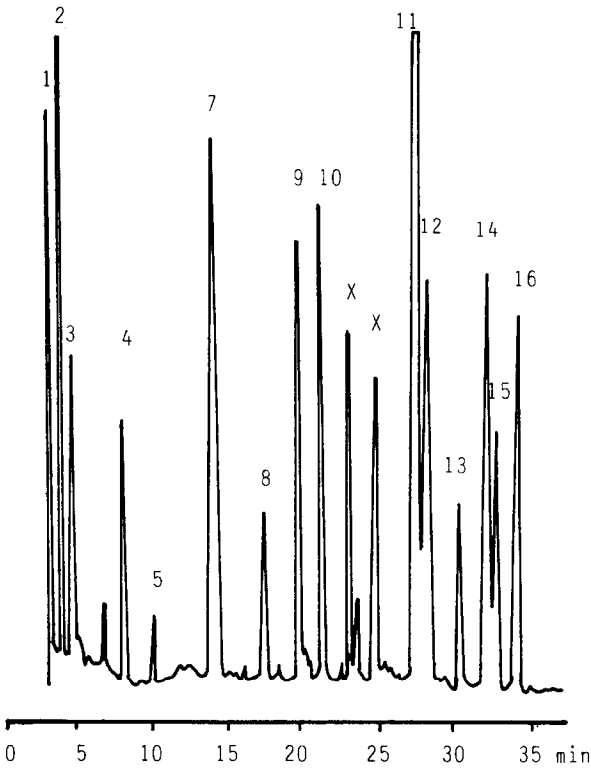


Fig. 3. Normal-phase HPLC of *Capsicum annuum* cv. Belrubí carotenoids. Peak numbers as in Fig. 2; × = unknowns.

Table II lists the distribution of the individual carotenoids, in the manner described in the text. The results obtained demonstrate the high content of carotenoid pigments in the *Capsicum* cultivar studied. It is therefore not surprising that the Belrubí cultivar has been chosen for its intense colour, which in its fresh fruit can be double that of other cultivars now used for the manufacture of paprika.

The proposed chromatographic system permits the identification of capsanthin epoxide, whose presence was questioned by Baranyai and Szabolcs[26], although recognized later when using reversed-phase HPLC[21]. In the pepper cultivars they used, the percentages of carotenoids did not differ essentially from our calculations, except that β-carotene increased at the expense of cryptocapsin, whereas capsorubin corresponded to the total capsorubin and capsorubin isomer calculated with our chromatographic system.

We did not find α-carotene in any of the ripened fruits of *Capsicum annuum*, which is in accordance with the results obtained previously with other varieties[27] and by other workers[28–30]. The previous work confirmed that the essential phenomenon taking place during ripening of pepper fruits is the disappearance of the β,ε-carotene series. The chromatographic system proposed now allows an adequate separation of α-carotene and β-carotene when both isomers are present.

The results obtained by Gregory *et al.*[22], who separated carotenoids acylated

TABLE II

QUANTITATIVE DISTRIBUTION OF *CAPSICUM ANNUUM* cv. BELRUBÍ CAROTENOIDS DETERMINED BY NORMAL-PHASE HPLC

Peak No.	*Carotenoid*	*Concentration (g/g fresh weight × 10^{-4})*	*Proportion (%)*
1	β-Carotene	2.75	6.72
2	Cryptocapsin	8.14	19.90
3	Cryptoflavin	1.09	2.66
4	β-Cryptoxanthin	0.79	1.93
5	Antheraxanthin	0.38	0.93
6	Lutein	—	—
7	Capsolutein	2.13	5.21
8	Luteoxanthin	0.85	2.07
9	Zeaxanthin	1.25	3.06
10	Mutatoxanthin	1.64	4.01
11	Capsanthin	12.07	29.51
12	Capsanthin 5,6-epoxide	2.16	5.28
13	Violaxanthin	0.98	2.40
14	Capsorubin	1.79	4.38
15	Capsorubin isomer	1.38	3.37
16	Neoxanthin	1.74	4.25
Unidentified pigments (as β-carotene)		1.77	4.32
Total		40.91	100.00
Red pigments		25.54	62.43
Yellow pigments		15.37	37.57

with fatty acids, demonstrated a concentration of pigments in the form of capsanthin, capsorubin and β-carotene ester. This suggests that in non-saponified extracts, the lipid part of the molecule predominates in the separation and when the esters of different carotenoids are eluted together, the components present in smaller amount are masked by the larger amounts in such a way that the proportion of the latter appears even greater.

To summarize, the chromatographic system described permits a good separation and quantification of individual carotenoid pigments. The method is applicable to the study of the contents and evolution of these pigments in the fresh fruit of *Capsicum annuum* cv. and their commercial products.

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