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Separation and Quantification of the Carotenoid Pigments in Red Peppers (*Capsicum annuum* L.), Paprika, and Oleoresin by Reversed-Phase HPLC

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Fourteen carotenoids were separated and identified by reversed-phase HPLC in the saponified extract obtained from red pepper fruit, paprika, and oleoresin using gradient elution with acetone and water and UV-visible detection at 450 nm. Quantification was achieved by HPLC with β-apo-8′-carotenal as internal standard. The proposed method minimized artifact formation and permitted easy verification of possible natural or induced transformations as well as any adulteration of the pigments in the commercial presentations: paprika and oleoresin. This method has been used to monitor the changes in the pigments of peppers and in the value of provitamin A at two extreme stages of ripening (green and red) in the *Agridulce* and *Bola* varieties.

INTRODUCTION

The carotenoid pigments are widely distributed among living organisms, both animal and vegetable. As the carotenoids are synthesized only in plants (apart from certain bacteria), their levels in vegetable matter are much higher than in animal matter (Isler, 1971).

The ripe fruits of the different varieties of peppers (*Capsicum annuum* L.) are a good source of carotenoid pigments due to their high concentration in these compounds (Mínguez-Mosquera and Fernández-Díez, 1981). They are widely used as natural colorants of foods, as either concentrated extracts (oleoresins) or a powder (paprika). However, these products lose color easily due to the low stability of the pigments. At the same time, the importance of the carotenoid compounds in the diet has been recognized in recent years. Some of them have provitamin A activity. Red pepper is one of vegetable that has a high content of provitamin A, due to the high concentration of β-carotene and β-cryptoxanthin (Isler, 1971; Simpson, 1983). The appearance of some types of cancer has been linked with the lack of certain oxygenated carotenoids (xanthophylls) in the diet, which are thus considered to be anticancer compounds (Shekelle et al., 1981; Moon and Itri, 1984). Other studies report antiulcer properties, manifested by the compounds' acting as protectors of the gastric mucosa (Javor et al., 1983).

In the ripe peppers most of the pigments are esterified with fatty acids, making them liposoluble (Baranyai et al., 1982; Curl, 1962). Capsanthin is the major pigment among them (Goodwin, 1980; Philip et al., 1971). Classical studies of pigments of peppers used column chromatography (Buckle and Rahman, 1979), paper chromatography (Cholnoky et al., 1956), and TLC (mainly with silica gel as stationary phase) (Vinkler and Richter, 1972; Camara and Moneger, 1978; Mínguez-Mosquera and Garrido-Fernández, 1983). Such polar phases have a big disadvantage: they potentiate isomerization and transformations during the separation. This happens, for example, with violaxanthin, producing 5,8-epoxide derivatives (Philip and Chen, 1988). Recently, an overpressure layer chromatography (OPLC) was applied to the study of pigments in the peppers (Aczel, 1988). Today, the literature includes numerous methods of HPLC analysis for the separation and quantification of carotenoid pigments (Bushway and Wilson, 1982; Davies and Holdsworth, 1980; Schwartz and von Elbe, 1982). Most of these methods have been developed with "reversed-phase" (Bushway and Wilson, 1982; Biacs et al., 1989; Noga and Lenz, 1983; Mínguez-Mosquera et al., 1991a), although those using "normal phase" are also included (Rahmani and Saari-Csallany, 1985; Iriyama et al., 1978). The former are preferred as they require lower equilibrium times and minimize pigment transformations during analysis. The present work proposes a method of gradient HPLC for quantification of the main pigments of peppers, in both direct and saponified extracts. In the case of peppers, authors disagree on the preparation of the extract: some argue that saponification is an aggressive method that produces marked transformations (Fisher and Kocis, 1987; Khachik et al., 1986; Gregory et al., 1987). However, it has been indicated by other authors that saponification under mild, controlled conditions does not give rise to measurable transformations (Javor et al., 1983).

EXPERIMENTAL PROCEDURES

Samples. The study was carried out on pepper fruits (*C. annuum* L.) of *Agridulce* and *Bola* varieties, from the La Vera region (Cáceres, Spain). Paprikas and oleoresins were also studied.

Apparatus used included the following: A Büchi rotavapor, Model R 110; a Desaga UV-vis lamp, provided with white light and ultraviolet $UV_{254,336}$; a Hewlett-Packard UV-vis diode array spectrophotometer, Model 8452A; a Bio-Rad FTS-7 IR spectrophotometer; a computerized Perkin-Elmer system with quaternary pump, Series 4, fitted with a UV-vis detector (Perkin-Elmer Model LC-85B) and an integrator (Hewlett-Packard Model 3396-A); a quaternary pump (Waters Associates Model 600E) with programmable UV-vis photodiode array detector (DAD) (Waters Associates Model 994). Both chromatographs were fitted with an injection valve (Rheodyne Model 7125).

Reagents. All of the reagents used in the development and application of the chromatographic method were of HPLC quality, and the rest analysis quality (ACS). The solvents used in the HPLC were acetone and deionized water, both being vacuum filtered through nylon filtration membranes (0.45 μm; Micron Separations, Westboro, MA) and degassed before analysis. The samples were also filtered before being chromatographed.

Extraction of Pigments. A sample of peppers was sliced up and 10 g extracted three or four times with 50 mL of acetone, using a homogenizer (Polytron homogenizer Ultra-Turrax Model

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T-25), until no more color was extracted. The extracts were combined in a decanting funnel and treated with 100 mL of ethyl ether, shaken, and left to settle. Enough NaCl solution (10%) was added to separate the phases and to transfer the pigments to the ether. This solution was treated several times with anhydrous Na_2SO_4 (2%) to remove all of the water. The ether phase, containing the pigments in different states of esterification with fatty acids, was used for chromatography once its volume had been reduced in the rotary evaporator. If saponification of the extract is opted for, 100 mL of KOH–MeOH (20%) is added and left for 1 h with periodic shaking. The aqueous phase is removed, and the organic phase is washed several times with distilled water until the washings are neutral, filtered through a bed of anhydrous Na_2SO_4, and evaporated to dryness in the rotary evaporator at a temperature lower than 35 °C. The pigments are collected with acetone to a volume of 25 mL and kept refrigerated until their analysis by HPLC.

For paprika, 1 or 2 g of product was extracted with acetone–water (9:1) as described above. For oleoresin, 0.2 g of product was dissolved in acetone to a final volume of 100 mL. The saponification was the same as that for peppers.

An internal standard (β-apo-8′-carotenal) was added at the beginning of the extraction process in a known quantity (around 300–400 μg/10 g of peppers).

Standards. Identification Tests. β-Carotene, canthaxanthin, lycopene, and β-apo-8′-carotenal were purchased, the first two from Hoffmann-La Roche (Nutley, NJ) and the last two from Sigma Chemical Co. (St. Louis, MO). Neoxanthin, violaxanthin, lutein, and antheraxanthin were obtained from a saponified extract of mint (*Menta arvensis* L.), by TLC (Mínguez-Mosquera et al., 1991b, 1992). Mutatoxanthin was obtained by treating an ethanolic solution of antheraxanthin with a few drops of diluted hydrochloric acid to convert the 5,6-epoxide to the 5,8-epoxide. Standards of capsanthin, capsorubin, zeaxanthin, and β-cryptoxanthin were obtained using the method of Mínguez and Garrido (1983), from a direct (not saponified) extract of ripe peppers. For this purpose plates of silica gel 60 GF_{254} (20 × 20 cm plates, thickness 0.7 mm) (Merck, Darmstadt, Germany) were used.

Each compound was identified on the basis of its TLC behavior, its absorption spectrum in different solvents, its IR spectrum, and chemical tests on functional groups described in the bibliography (Davies, 1976; Foppen, 1971) such as reduction with $NaBH_4$ for carbonyl groups and acetylation for hydroxyl groups (both groups were confirmed by bands in IR).

The characteristics of the chromatograms in TLC of the carotenoids from extracts of mint and peppers (direct and saponified), are shown in Table I. To purify the pigments, each compound was scraped from the plate, eluted, and rechromatographed under the same conditions in which they had been obtained. In the case of zeaxanthin and β-cryptoxanthin, the mixture benzene–light petroleum ether (1:1) was used for a better development. Each pigment purified was saponified and rechromatographed in TLC, using light petroleum ether (bp 65–95 °C)–acetone–diethylamine (10:4:1) as solvent system, to check its purity and control its R_f. The use of this new more polar solvent system is necessary due to the changes in the polarity of the de-esterified pigments. Capsorubin required this last step of purification after saponification, being accompanied in its esterified form by considerable amounts of esterification forms of zeaxanthin and β-cryptoxanthin.

Pigments from a saponified extract of peppers are in their de-esterified forms. As a result of this, new bands appear on TLC which were not separated from a direct extract. Among these new bands, two stand out (one red and the other yellow) found between β-cryptoxanthin and zeaxanthin. The red band was found to be cryptocapsin and the yellow one capsolutein. The result of identification tests of the studied pigments are shown in Table II.

High-Performance Liquid Chromatography. A reversed-phase C_{18} column packed with Spherisorb ODS 2, 250 × 4 mm i. d. (Hewlett-Packard), was used. Particle size was 5 μm. To protect the column, a precolumn of the same material was used (5 cm × 4 mm i. d.).

The following HPLC conditions were chosen: flow rate 1.5 mL/min; injection volume, 5 μL (loop), and detection, 450 nm. The gradient program was as follows:

time, min	% acetone	% water	curve
10 (EQ)	75	25	
5	75	25	
5	95	5	linear
7	95	5	
5	100	0	convex
5	75	25	linear

There is thus a 10-min conditioning stage (EQ or equilibrium) at the initial conditions before each injection and, after separation, a stage of cleaning and return to the initial conditions. Thus, the separation itself lasts 17 min for a saponified extract.

Quantification. To quantify, calibration with internal standard was chosen using β-apo-8′-carotenal. This is a pigment absent in the peppers and which under the proposed conditions separates well from the other carotenoids.

Multicomponent mixtures were used for calibration. Once the pigments had been purified, solutions of each were prepared and the concentration was determined spectrophotometrically, using the corresponding values of ϵ_0 (Davies and Köst, 1988). Different mixtures were prepared, taking successively greater aliquots of the "stock" solution of each pigment. The same amount of standard solution was added in each case. These solutions were evaporated in a rotary evaporator at 30 °C, dissolved in 2 mL of acetone, and stored in vials at −30 °C.

Table I. Characteristics of Thin-Layer Chromatograms on Silica Gel 60 GF_{254} Used for Obtaining the Standard Carotenoids

source of pigment	pigment	solvent[a]	color in the chromatogram	R_f
from red peppers direct extract				
	β-carotene + phytofluene and ζ-carotene	A	yellow-orange	0.96
	β-cryptoxanthin	A	yellow	0.85
	zeaxanthin	A	yellow	0.69
	capsanthin	A	intense red	0.37
	capsorubin	A	red-brown	0.21
	rest of pigments (base)	A	yellowish red	0.18–0.00
saponified extract				
	β-carotene	B	yellow-orange	0.96
	β-cryptoxanthin	B	yellow	0.57
	cryptocapsin	B	pale red	0.51
	capsolutein	B	yellow	0.47
	zeaxanthin	B	yellow-orange	0.44
	anteraxanthin	B	yellow	0.42
	capsanthin	B	intense red	0.39
	violaxanthin	B	yellow	0.34
	capsorubin	B	red-brown	0.31
from mint				
	lutein	B	yellow	0.41
	antheraxanthin	C	yellow	0.32
	violaxanthin	B	yellow	0.34
	neoxanthin	B	yellow	0.23

[a] Solvent systems: (A) hexane–ethyl acetate–ethanol–acetone (95:3:2:2); (B) light petroleum ether (bp 65–95 °C)–acetone–diethylamine (10:4:1); (C) methylene chloride–ethyl acetate (4:1).

RESULTS AND DISCUSSION

Separation and Quantification of Saponified Pigments by HPLC. Saponification of extracts containing carotenol fatty acid esters results in the regeneration of parent hydroxycarotenoids with a much simpler HPLC profile.

To optimize the separation of pigments by HPLC and control their t_R, the technique of "successive additions of

Table II. Chromatographic and Spectroscopic Characteristics Used for Pigment Identification in Red Peppers, Paprika, and Oleoresin

pigment identified	R_f value[a]	spectral data, λ_{max}, nm: light petr ether	spectral data, λ_{max}, nm: benzene	bands in IR: OH	bands in IR: C=O	epoxide test (HCl treatment): hypsocromic shift, nm	epoxide test (HCl treatment): color on TLC[b]	acetylation	reduction
neoxanthin	0.23 A	412, 436, 466	422, 442, 478	+	−	14	blue-green	+	−
capsorubin	0.21 B	442, 468, 502	460, 486, 522	+	+	0		+	+
violaxanthin	0.34 A	416, 468, 502	428, 452, 484	+	−	40	blue	+	−
capsanthin	0.37 B	466, 496	486, (520)	+	+	0		+	+
zeaxanthin	0.69 B	(422), 448, 472	(436), 462, 488	+	−	0	brown and green	+	−
lutein	0.41 A	418, 442, 470	429, 456, 485	+	−	0	brown and green	+	−
β-cryptoxanthin	0.85 B	(424), 448, 474	(438), 458, 486	+	−	0	yellow	+	−
β-carotene	1.00 A 0.24 C	(426), 444, 470	463, 492	−	−	0	yellow-orange	−	−
phytofluene	0.12 C	330, 348, 367	338, 353, 374	−	−	0	yellow	−	−
ζ-carotene	0.01 C	378, 400, 422	387, 406, 432	−	−	0	yellow	−	−
antheraxanthin	0.42 A	424, 442, 470	433, 457, 488	+	−	18	blue-green	+	−
mutatoxanthin	0.25 D	(404), 424, 454	(410), 439, 468	+	−	0	blue	+	−
cryptocapsin	0.51 A	(445), 470, 497	486, 518	+	+	0	yellow	+	+
capsolutein	0.47 A	420, 444, 472	434, 458, 486	+	−	0	blue	+	−

[a] Solvent system: (A) light petroleum ether–acetone–diethylamine (10:4:1); (B) hexane–ethyl acetate–ethanol–acetone (95:3:2:2); (C) light petroleum ether (40–60 °C); (D) benzene–acetone (4:1). [b] Chromatographic support for TLC: silica gel 60 GF_{254}.

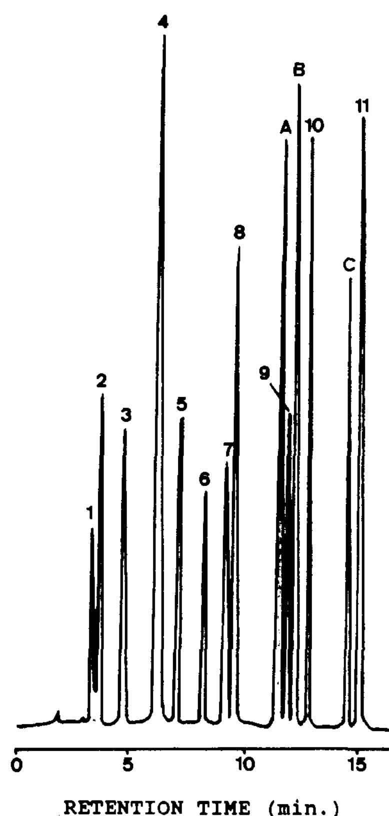

Figure 1. Reversed-phase HPLC of standard mixture. Peaks: (1) neoxanthin, (2) capsorubin; (3) violaxanthin; (4) capsanthin; (5) antheraxanthin; (6) mutatoxanthin; (7) capsolutein; (8) zeaxanthin; (9) cryptocapsin; (10) β-cryptoxanthin; (11) β-carotene; (A) canthaxanthin; (B) β-apo-8′-carotenal; (C) lycopene.

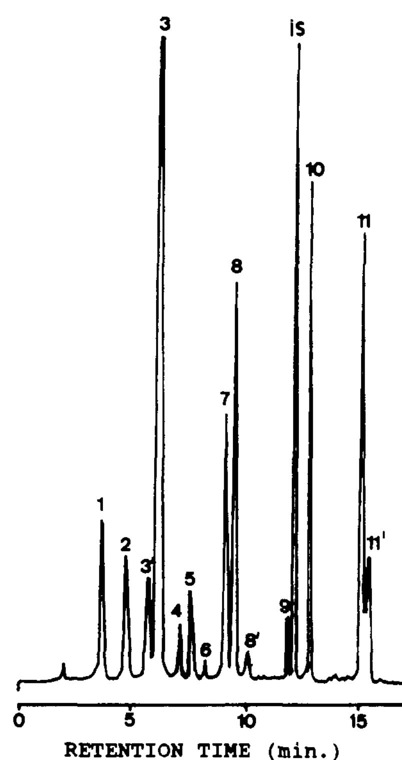

Figure 2. Reversed-phase HPLC of saponified extract from red peppers. Peaks: (1) capsorubin; (2) violaxanthin; (3) capsanthin; (3′) capsanthin 5,6-epoxide; (4) antheraxanthin; (5) *cis*-capsanthin; (6) mutatoxanthin; (7) capsolutein; (8) zeaxanthin; (8′) *cis*-zeaxanthin; (9) cryptocapsin; (10) β-cryptoxanthin; (11) β-carotene; (11′) *cis*-β-carotene; (IS) β-apo-8′-carotenal.

standards" was used. A solution of one of the pigments was injected. Successive additions of each of the remaining pigments were made to this solution, injecting after each addition. In the case of good separation, a new peak should appear after each addition. The standard chromatogram is shown in Figure 1, and the chromatogram obtained after saponification of extract from peppers is shown in Figure 2. Similar chromatograms were obtained for paprika and oleoresin saponified extracts. Besides the coincidence in the t_R of the peaks in both chromatograms, there is also coincidence in the spectra in the mobile phase of the standards and in the peaks in the real sample. Aliquots of each isolated pigment were added individually to a real sample. No new peak appeared—only an increase in the area of the peak corresponding to the pigment added.

Although under the proposed conditions lutein and zeaxanthin (peak 8 in Figures 1 and 2) were not separated, we have not found lutein in the ripe red peppers. Neither was neoxanthin detected. These facts have been verified by absorption spectrum in the mobile phase and identification tests in TLC.

At the same time, the following pigments were tentatively identified by absorption spectrum in the mobile phase: capsanthin 5,6-epoxide and *cis* forms of capsanthin, zeaxanthin, and β-carotene. This was done by comparing their spectra in the mobile phase with those in acetone from the bibliography or in solvents with a similar spectral

Table III. Pigments Separated by HPLC Proposed Method from Extracts of Red Peppers, Paprika, and Oleoresin: Identities, Retention Times (t_R), and Spectral Data

pigment	t_R	spectral data in the mobile phase (λ_{max})			
		cis	I	II	III
non ret peak	1.45				
neoxanthin	3.44		416	438	470
capsorubin	3.49		(452)	482	512
violaxanthin	4.71		418	442	472
capsanthin 5,6-epoxide	5.92			470	(496)
capsanthin	6.35			472	(498)
antheraxanthin	7.43		422	448	478
isomeric *cis*-capsanthin	7.84	360		468	(492)
mutatoxanthin	8.22		(407)	430	458
capsolutein	9.10		424	448	476
zeaxanthin	9.35		426	452	482
isomeric *cis*-zeaxanthin	9.44	340	424	452	480
canthaxanthin	11.43			465	
cryptocapsin	11.85			482	(518)
β-apo-8′-carotenal	11.92			458	
β-cryptoxanthin	12.63		428	454	482
lycopene	14.46		446	472	504
phytofluene	14.82		330	348	367
ζ-carotene	15.08		378	400	422
β-carotene	15.08		(428)	452	480
13,*cis*-β-carotene	15.28	334	(428)	452	480

Table IV. Range of Amount of Pigment Used for the Calibration and Individual Response Factors

pigment	range of wt injected, μg	range of wt ratios[a]	response factor	coeff of correln
neoxanthin	1.62–30.85	0.056–1.056	1.988	0.9987
capsorubin	1.15–17.22	0.039–0.590	1.755	0.9975
violaxanthin	2.36–47.24	0.081–1.617	1.177	0.9986
capsanthin	3.21–83.57	0.110–2.861	1.230	0.9991
zeaxanthin	2.37–45.02	0.081–1.541	1.069	0.9988
β-cryptoxanthin	1.93–50.33	0.066–1.723	1.057	0.9984
β-carotene	1.94–50.58	0.067–1.732	1.047	0.9998

[a] Weight ratio = weight of pigment/weight of internal standard.

effect, such as light petroleum ether, hexane, or ethanol (Foppen, 1971; Davies, 1988). The retention times and the maxima of the spectra of absorption in the mobile phase of these compounds are shown in Table III.

To calibrate, each standard mixture was injected three times to calculate as reproducibly as possible the calibrations in function of the internal standard, taking as response the "peak area". The following formula was used:

$$(\text{weight } X)/(\text{weight IS}) = f_X(\text{area } X)/(\text{area IS})$$

where f_x is the response factor of each pigment, X is each pigment, and IS is the internal standard.

In this way, the response factor of each pigment relative to the internal standard was calculated. The response factors obtained from the calibration straight lines and their coefficients of correlation, as well as range of absolute amounts used for the calibration of each pigment, expressed as micrograms injected, are shown in Table IV. These coefficients can be used during some time without need of recalibration. A new recalibration involves further work of isolation and purification of the pigments studied, as only some of them are commercially available. To avoid this bother, we propose the calculation of "response factors relative to β-carotene", as this and the internal standard are commercially available. These factors are calculated according to

$$F_{i/\beta\text{-car}} = f_i/f_{\beta\text{-car}}$$

where i represents each pigment, $F_{i/\beta\text{-car}}$ is the factor of each pigment i relative to β-carotene, and f_i is the response factor of each pigment.

Table V. Carotenoid Pigment Composition (Milligrams per Kilogram of Fresh Fruit) of Two Varieties of Peppers (*C. annuum* L.), *Bola* and *Agridulce*, Used Industrially for Obtaining Paprika and Oleoresins

pigment	concentration[a]			
	Agridulce		*Bola*	
	green	*red*	*green*	*red*
neoxanthin	8.85		8.12	
capsorubin		78.98		53.44
violaxanthin	7.93	84.17	10.40	52.68
capsanthin 5,6-epoxide		51.56		40.31
capsanthin		656.47		523.21
antheraxanthin		44.08		33.18
cis-capsanthin		72.24		59.38
capsolutein		88.77		68.96
zeaxanthin		99.96		40.30
cis-zeaxanthin		7.52		3.47
lutein	14.09		7.95	
cis-lutein	0.37		0.71	
β-cryptoxanthin		76.72		35.59
β-carotene	7.98	99.51	6.23	51.28
total pigments	39.22	1359.98	33.41	961.80
provitamin A value (IU/kg of fresh fruit)[b]	13302	233626	10385	116909

[a] Mean of two determations. [b] IU of provitamin A/kg fresh fruit = (1667 * mg of β-carotene + 833 * mg of β-cryptoxanthin)/kg fresh fruit.

Thereby, in future calibrations we only have to make one calibration using β-carotene and β-apo-8′-carotenal as internal standard. From the new response factor obtained for β-carotene we can obtain those for the other pigments, using the above expression.

The coefficient of variation of the chromatographic determination was calculated using a standard solution of β-carotene. This was lower than 4%.

The proposed method enables the rapid and reliable separation and determination of the major pigments present in the ripe pepper fruit. The amount of epoxide-type artifacts found is low, in contrast to other methods whose development is sometimes "rich" in these isomers, very possibly formed during the preparation and analysis of the sample. The methodology is useful to follow and control possible natural and/or artificial alterations in the commercial forms: dry peppers, ground peppers, and oleoresins. Moreover, the addition of pigments such as lycopene, canthaxanthin, or β-apo-8′-carotenal, used to increase the color of these products, has also been controlled. The method can also be used to monitor the changes of the pigments of peppers during ripening and the provitamin A value.

Pigments Present in the Red Pepper Varieties *Bola* and *Agridulce*. Using HPLC, the concentration and type of pigments present in two varieties of peppers traditionally used in Spain for the manufacture of paprika and oleoresin have been determined. In Table V the results obtained are shown. The pigments lutein and neoxanthin, peculiar to the green fruits, are absent in the ripe fruits. Other pigments appear when the fruits reach the ripe red state, among which those xanthophylls such as capsanthin, capsorubin, and capsolutein, exclusive to peppers, are prominent. It can be seen that, apart from the overall similarity between the two varieties with respect to the types of carotenoids present, the pigment compositons of their respective unripe fruits are also similar. However, considering the mature fruits, it can be seen that the levels of all carotenoids in the *Agridulce* variety are higher than in the *Bola* variety. Consequently, the total amount of pigments given by the *Agridulce* variety is of the order of 41% more than in the *Bola* variety. Since the coloring

capacity of paprika or oleoresin, and therefore the commercial value of these, is directly related to red pigment content, the fruits with greater value for the manufacture of these products will be those which contain the highest content of, principally, capsanthin and capsorubin. Thus, the *Agridulce* variety, with a red carotenoid content of 859.25 mg/kg, give products with a better coloration than those obtained from the *Bola* variety, with a concentration of 676.34 mg/kg. The *Agridulce* variety also has the advantage over the *Bola* variety of having more provitamin A value, the two varieties showing values of 233 636 and 116 909 IU/kg, respectively. This difference arises from the fact that the former variety has approximately twice as much β-carotene and β-cryptoxanthin as the *Bola* variety.

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