Effects of Enzymatic Treatments of Marigold Flowers on Lutein Isomeric Profiles

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Reversed-phase C_{30} HPLC was applied to study the identity of lutein isomers and to monitor the effects of solids content and elimination of water-soluble substances on the isomeric carotenoid profiles of marigold (*Tagetes erecta*) samples treated with enzymes. The tentative identity of four lutein isomers present in saponified marigold extracts was confirmed. Enzymatic treatment on a 5% solids slurry produced the marigold meal with the highest *all-trans*-lutein content [25.1 g/kg dry weight (dw)]. We did not observe variations in the distribution in percentage of lutein isomers due to enzymatic treatment; the elimination of water solubles had a significant but small effect on such variations. The solids content was the principal factor that affected the carotenoid profiles. An analysis of the distribution showed that 15% solids gave the highest *all-trans*-lutein percentage in treated meals. Interestingly, with 20% solids both the degradation of lutein and the percentage of *all-trans*-zeaxanthin were the highest.

Keywords: *Lutein; carotenoids; pigments; marigold; Tagetes*

INTRODUCTION

Marigold (*Tagetes erecta*) is an annual herbaceous plant native to Mexico, where it is used in folk medicine (Neher, 1968; Tosco, 1970; Mendieta and Del-Amos, 1981). Marigold is commercially cultivated and flower extracts are used as poultry feed supplements for the direct coloring of shanks and yolks (Avila et al., 1990; Hencken, 1992). Lutein is a common carotenoid in nature and is the principal pigmenting agent of marigold flower (see Figure 1 for structure) (Quackenbush and Miller, 1972; Gau et al., 1983; Rivas, 1989).

Carotenoids are widely distributed among plants and are, after chlorophyll, the most abundant kind of pigments. These compounds have received special attention because of their vitamin A activity and also have been involved in other health benefits such as prevention of cancer and cardiovascular and eye diseases (Canfield et al., 1993; Francis, 1995; Peterson et al., 1995). The importance of the carotenoid isomeric form on its biological function has been clearly shown (Davies et al., 1995; Peterson et al., 1995). Hencken (1992) reported that *trans*-carotenoids are more effective pigments because of their redder hue and greater stability. In Nature, they occur as a mixture of *trans*- (60−90%) and *cis*-carotenoids (10−40%). *trans*-Carotenoids change to cis-isomers under processing conditions by factors such as light, temperature, water activity, and oxygen.

On the other hand, enzymes have been used to increase oil extraction from different raw materials with interesting results (Dominguez et al., 1994). In preliminary experiments, we treated marigold flowers (fresh flowers and dehydrated meals) with a commercial enzymatic preparation followed by the elimination of water-soluble substances to obtain meals with a higher content of carotenoids.

Much information is now available about the modifications of the carotenoid content and/or profiles (degradation, synthesis, and isomerization) for different fruits and vegetables by processing (Khachik et al., 1992; Chen et al., 1995; Mínguez-Mosquera and Jaren-Galan, 1995; Wilberg and Rodriguez-Amaya, 1995), but the information for carotenoid profile variations in marigold is scarce. Recently, the development of better liquid chromatographic systems has made it possible to resolve carotenoid isomers (Sander et al., 1994; Schmitz et al., 1995).

One of the objectives was to confirm the tentative identity of lutein isomers present in saponified marigold extracts, and the main one was to determine the effects of solids content and the elimination of water-soluble substances on the carotenoid profiles of marigold samples treated with enzymes.

MATERIALS AND METHODS

Fresh Materials. Fresh marigold (*T. erecta*) flowers were kindly supplied by Industrial Orgánica, S.A., Monterrey, Mexico. The flowers were separated from the receptacles and processed fresh; another portion was dehydrated in a vacuum oven (Forma Scientific, Marietta, OH) to 10% moisture content at 60 °C. The biological material was perfectly mixed before samples were taken for analyses; afterward, dehydrated samples were milled through a 0.5-mm sieve by using a Brinkmann mill (Brinkmann, Wesbury, NY). Moisture content was determined in triplicate according to AOAC methodology (AOAC, 1984).

Oleoresins. Commercial oleoresin obtained from marigold flowers was also provided by Industrial Orgánica, S.A., Monterrey, Mexico.

Standards. *all-trans*-Lutein (75% purity) and *all-trans*-canthaxanthin standards were purchased from Sigma (St. Louis, MO). The purity of standards, as verified by HPLC, was good, and the standards were used without further purification. *all-trans*-Zeaxanthin was separated by HPLC from a sample of yellow maize purchased in the local market (see Figure 1 for structures).

Analytical- and HPLC-grade solvents were from Merck (Darmstadt, Germany).

Lutein Crystallization. Lutein was crystallized from marigold oleoresin according to the procedure reported by Tyczkowski and Hamilton (1991).

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S0021-8561(96)00474-8 CCC: $14.00

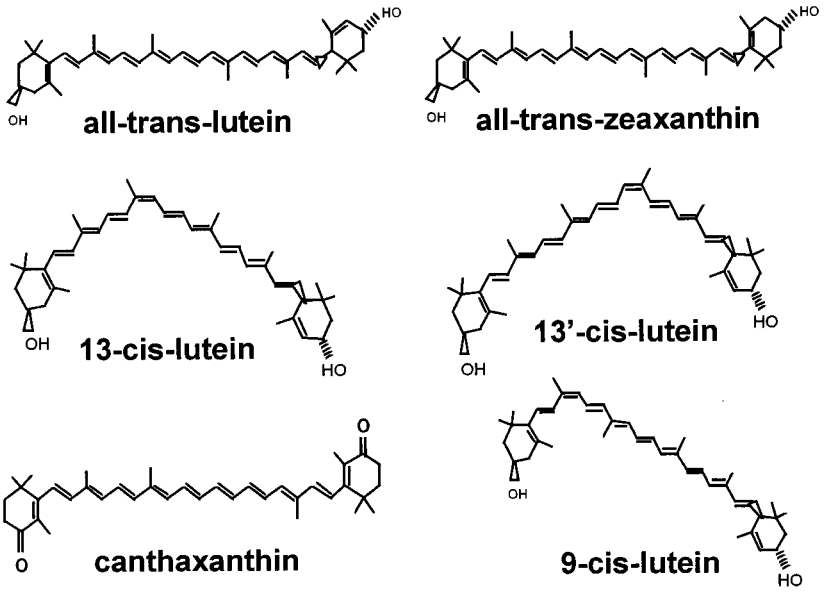

Figure 1. Structures for some lutein isomers.

Photoisomerization. A saturated solution of crystallized lutein in methanol was prepared. An aliquot from this solution was analyzed immediately by HPLC, and another one was illuminated under direct sunlight for 10 min, then covered with aluminum foil, and stored at −20 °C until analysis. Treatment and analysis were carried out twice.

Enzymes and Enzymatic Treatments. The commercial enzyme Econase-cep was kindly supplied by Enzyme Development Corporation (New York, NY). Econase-cep is a cellulase preparation with *endo*-1,4-β-D-glucanase, cellobiohydrolase, and *exo*-1,4-β-D-glucosidase as its principal activities. It also contains other activities such as hemicellulase, β-glucanase, protease, and amyloglucosidase. The same batch of enzyme was used for all treatments. In order to evaluate the effect of the solids-to-water ratio of the enzymatic treatment mixtures on the carotenoid content and isomeric profiles, slurries were prepared to contain 15% solids from fresh marigold flower petals, or 5, 10, 15, or 20% solids from dehydrated marigold meal, each final slurry contained 200 g, and the solids content was determined by taking into account the moisture of the biological material that was used. A series of slurries contained no enzyme (control), while others contained 0.1% (w/w) enzyme. The enzyme was added as a solution containing 0.01% (v/v) Tween 80 and 0.01% (w/v) sodium azide; the control samples were supplemented with the same solution but without enzyme. The pH of all slurries was adjusted to 5.0 (recommended by enzyme manufacturer) with diluted hydrochloric acid, and the pH was evaluated with a Beckman Φ41 pH meter (Beckman Instruments, Inc., Irvine, CA). All slurries were thoroughly mixed, covered with aluminum foil, stored at room temperature, and mixed by hand stirring with a spatula every 12 h during 120 h.

Elimination of Water-Soluble Substances. After the enzymatic treatment, water-soluble substances of 20-g samples (in triplicate) were eliminated by adding deionized water to dilute samples to 1% solids. For the treatment of fresh marigold petals, the samples were first milled in a Waring blender. Each slurry was agitated with a magnetic stirrer for 2 h at room temperature and centrifuged (16274*g*, 4 °C, 15 min); the pellet was subjected to this procedure twice. The water volume used in each elimination was 180 mL. The pellet was recovered, vacuum-dried as described under Fresh Materials, and analyzed immediately or stored in black plastic bags at 4 °C under nitrogen until analysis. Samples treated with enzymes and subsequently subjected to water extraction are heretofore designated as enzymatic-H_2O. The same water extraction procedure was used with control samples (slurries without enzyme). Control samples which were subjected to water extraction are heretofore designated as control-H_2O.

Extraction of Carotenoids. A modified AOAC method was used (AOAC, 1984). Fifty milligrams of sample was mixed with 30 mL of HEAT (hexane−absolute ethanol−acetone−toluene, 10:6:7:7 v/v/v/v), 2 drops of deionized water, and 2 mL of 40% methanolic KOH solution and then swirled (1 min) in a 100-mL boiling flask. The mixture was stirred at 200 rpm for 16 h at 20 °C in sealed flasks covered with aluminum foil, in a reciprocal water shaking bath (New Brunswick Scientific, Edison, NJ) maintained in a dark room. The suspension was transferred to a 100-mL volumetric flask. Then, 30 mL of hexane (enough to obtain a water-immiscible organic layer of 50 mL) was added, and the solution was swirled for 1 min and diluted to volume with a 10% (w/v) Na_2SO_4 solution. The flask was swirled (1 min) and let stand in the dark for 1 h until the two phases were separated. One aliquot (5 mL) was taken from the upper (organic) layer and mixed with 1 mL of canthaxanthin solution (3 mg/50 mL of methanol). The solution was passed through a 0.45 μm PTFE filter membrane (Millipore, Bedford, MA), concentrated to dryness under a nitrogen stream, dissolved in 2 mL of previously filtered methanol−2-propanol (1:1 v/v), and analyzed immediately or stored (less than 1 week) in the dark at −20 °C until the HPLC analysis. A sample of dehydrated marigold meal without any additional treatment and a maize sample (4 g) were extracted as described above.

In order to avoid possible isomerizations, the extraction and saponification procedures were developed at 20 °C; all operations were carried out under dim light conditions (1.5 lx). Samples were analyzed immediately or evacuated with a stream of nitrogen and stored at −20 °C.

Thin Layer Chromatography (TLC). Crystallized lutein and the maize extract were separated by analytical TLC (1 × 6 cm; 0.2-mm layer thickness) with silica gel 60 F_{254} support (Alltech, Deerfield, IL) using three different solvent systems: (A) light petroleum ether (boiling point = 40−60 °C)−acetone, 8:2 v/v; (B) light petroleum ether−acetone, 7:3 v/v; and (C) light petroleum ether−acetone−triethylamine, 20:4:2 v/v/v. After the development of the chromatogram, the plate was removed and dried in a stream of nitrogen and then wetted by spraying with a saturated methanolic silver nitrate solution, as described by Isaksen and Francis (1990). TLC analyses were done three times for each sample and solvent system.

High-Performance Liquid Chromatography (HPLC). The equipment used for HPLC was a Hewlett-Packard (HP) (Palo Alto, CA) Model 1050 equipped with a quaternary pump solvent delivery and degassification system, autosampler, and diode array detector with a built-in Chemstation program. One hundred microliters of each pigment sample was injected. The analysis of carotenoids was performed using a YMC PACK C_{30} (YMC Inc., Morris Plains, NJ) reversed-phase column (4.6 × 250 mm, 5-μm particle size). Solvents were prefiltered through a 0.45-μm PTFE membrane and degassed by bubbling helium (15 min). The solvent-gradient elution was as follows: solvent

A (methanol) was delivered isocratically from 0 to 6 min, 4-min linear gradient to 5% solvent B (2-propanol), 5 min isocratically with 5% solvent B, 10-min linear gradient to 25% solvent B, 5-min linear gradient to 50% solvent B, 10-min linear gradient to 75% solvent B−25% solvent C (hexane), and 10 min isocratically with this mixture. The column was re-equilibrated between samples for a minimum of 17 min with solvent A. Separation was performed at room temperature, approximately 28 °C. Pigments were monitored at 450 nm. The absorption spectra of the pigments were scanned from 260 to 550 nm. The time and wavelength resolution were 1 s and 2 nm, respectively. Data were collected and processed in an HP-vectra 486/66 XM computer.

Peaks on chromatograms were identified by comparing their retention times and spectra with those of authentic standards (*all-trans*-canthaxanthin, *all-trans*-lutein and its photoisomerization products), and also by comparing absorption spectra and *Q* ratios with reference values reported in the literature (Khachick et al., 1986; Goodwin and Britton, 1988; Rivas, 1989; Britton, 1991; Saleh and Tan, 1991; Khachick et al., 1992; O'Neil and Schwartz, 1995), as reported previously (Delgado-Vargas and Paredes-López, 1996).

Quantification of Carotenoids. *all-trans*-Lutein was quantified using an absolute calibration curve. The calibration curve was prepared by measuring the reference compound at concentrations ranging from 2.3 to 18.7 μg/mL. The calibration curves showed good linearity ($r^2 = 0.992$). In the quantitation of total pigments, other lutein isomers were determined as *all-trans*-lutein equivalents. In order to compare the profiles of lutein isomers, the relative percentages of HPLC chromatogram areas were used.

Statistical Analyses. We used ANOVA procedures with Fisher's PLSD multiple comparison tests in the analysis of data (Statview software, Abacus Concepts, 1991). The effect of several processing factors on the carotenoid isomeric profiles of resulting marigold meals was analyzed. The enzyme factor included two levels: without and with enzyme. The elimination factor (elimination of water-soluble substances) had two levels: without and with elimination. The solids factor (solids content in the enzymatic mixtures) considered five levels: fresh marigold flowers (15% solids), and 5, 10, 15, and 20% of solids from dehydrated marigold meal.

RESULTS AND DISCUSSION

Tentative Identification of Lutein Isomers in Saponified Marigold Extracts. TLC analysis of the lutein crystallized from marigold oleoresin showed only one spot with the following R_fs: 0.14 in solvent system A, 0.49 in B, and 0.19 in C. When the maize extract was analyzed by TLC, the principal spot showed a mobility exactly like that of crystallized lutein, although with system C the R_f was slightly higher (0.23). The colors of these spots when developed with the $AgNO_3$ solution were orange for the crystallized lutein and red for the maize extract. These results suggested that the principal spot, with a high probability, corresponded to a pure compound or a mixture of isomeric forms (lutein and zeaxanthin). The red color (bathochromic shift) for the maize spot indicated the presence of a high quantity of zeaxanthin (the compound has two β-ring double bonds), contrasting with the orange color observed for the crystallized lutein (one β-ring double bond) (Isaksen and Francis, 1990).

Figure 2a shows a typical chromatogram for saponified extracts from dehydrated marigold meals. Sample preparation for HPLC did not produce modifications in the quality or quantity of carotenoids, as determined by using canthaxanthin as an internal standard. Most peaks with assigned names presented throughout Figure 2 were tentatively identified in agreement with previously reported spectroscopic data (Delgado-Vargas and Paredes-López, 1996). Peak 8 was tentatively identified in the present study as *all-trans*-zeaxanthin by using the spectroscopic and HPLC chromatographic data obtained from the saponified extract from yellow maize (not shown here). This tentative identity assignment was carried out despite the spectral maxima observed for that peak (Table 1). Previous reports have shown that some spectral maxima obtained for *all-trans*-zeaxanthin are higher than *all-trans*-lutein maxima, when both compounds are dissolved in the same solvent (Britton, 1991; Emenhiser et al., 1995). Our maxima values (Table 1) showed the opposite behavior, which

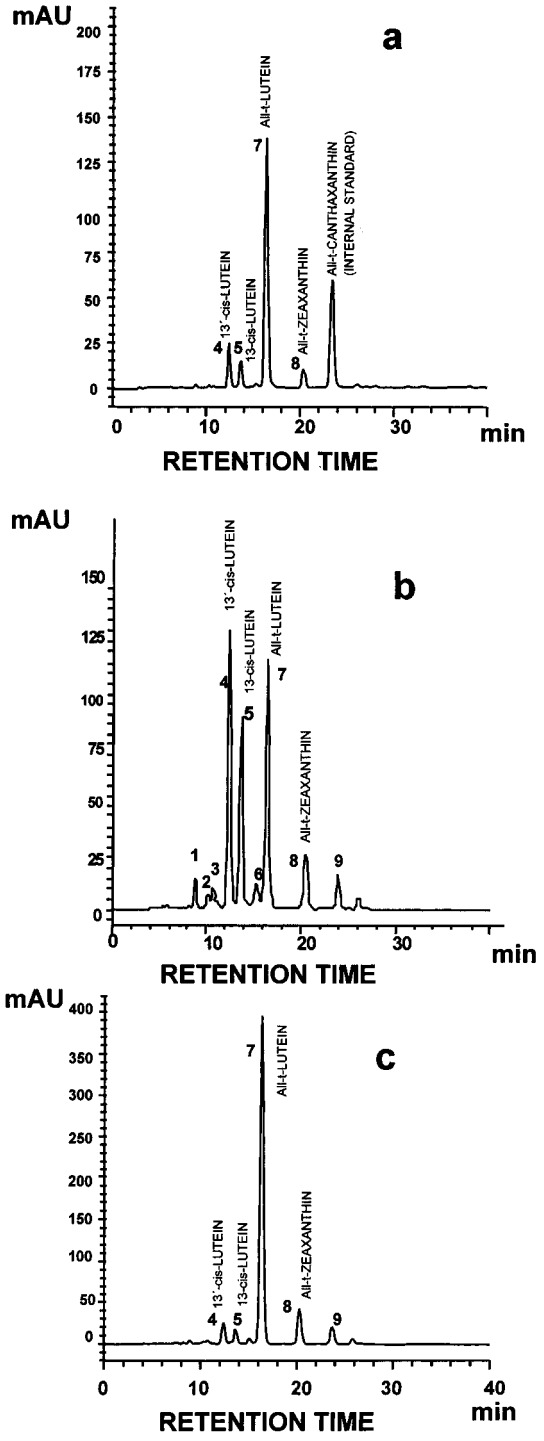

Figure 2. Reversed-phase C_{30} HPLC of saponified marigold pigments: (a) typical chromatogram of pigments from marigold samples extracted under laboratory conditions; (b) chromatogram of a methanolic solution of crystallized lutein obtained from commercial marigold oleoresin in dark conditions; (c) chromatogram of the illuminated solution of crystallized lutein (see Materials and Methods). The identity of the peaks is discussed in the text.

Table 1. Chromatographic Characteristics of Peaks from Crystallized Lutein Separated by HPLC and Detected at 450 nm in the Analyzed Samples

		soln of crystallized lutein							
		kept in the dark				illuminated			
peak[a] no.	t_R[b] (min)	area (mAU·s)	λ[c] (nm)	purity	Rs[d]	area (mAU·s)	λ[c] (nm)	purity	Rs[d]
1	8.9	264.7	275, 389 (sh),[e] 409, 431, 461	999.22	−2.7				
2	10.3	134.6	279, 393 (sh), 411, 433, 465	979.67	2.7−0.7				
3	10.8	275.9	273, 333, 390 (sh), 409, 437, 465	989.53	0.7−2.3				
4	12.5	3142.9	273, 333, 419 (sh), 439, 465	999.88	2.3−2.0	610.5	273, 331, 419 (sh), 441, 467	998.60	−1.9
5	13.7	2235.7	271, 333, 417 (sh), 439, 465	999.97	2.0−2.2	418.8	273, 331, 417 (sh),439,465	999.02	1.9−3.8
6	15.4	419.3	339, 423 (sh), 445, 465	996.38	2.2−1.3				
7	16.5	3218.1	269, 423 (sh), 444, 473	999.91	1.3−5.5	10051.6	269, 423 (sh), 445, 473	999.99	3.8−5.5
8	20.5	766.4	269, 420 (sh), 440, 467	999.69	5.5−4.4	1240.9	269, 333, 421 (sh), 441, 467	999.48	5.5−4.4
9	23.9	475.4	269, 423 (sh), 442, 468	999.24	4.4−	607.3	269, 331, 419 (sh), 441, 469	999.52	4.4−
		$\sum$ area = 10933.0				$\sum$ area = 12929.1			

[a] See Figure 2 for identification. [b] Retention time. [c] Determined in HPLC eluent employing the photodiode array detector (described in the text). [d] Resolution. [e] Shoulder.

could be an effect of using a gradient solvent system on the spectroscopic characteristics (Britton, 1991).

The chromatogram in Figure 2b corresponds to the methanolic solution of crystallized lutein from commercial oleoresin kept in the dark. More isomers were observed in this sample than in the marigold meal extracted under our laboratory conditions (Figure 2a). Tyczkowski and Hamilton (1991) reported only one HPLC peak (silica gel normal phase) after the crystallization of lutein from marigold oleoresin, although Philip and Chen (1988) observed previously that silica gel induced the formation of cis-isomers. From this, it was clear that their HPLC chromatographic system did not show a good resolution. This problem was solved here by using the C_{30} HPLC column introduced by Sander et al. (1994), as can be seen in our chromatograms (Figure 2). After illumination, several peaks disappeared (Figure 2c), which suggested that the additional peaks observed in Figure 2b corresponded to lutein or zeaxanthin isomers. Most of the *cis*-lutein isomers were transformed into the all-trans-isomer. We did not observe degradation as corroborated by the total peak area before and after illumination (Table 1). This interesting change in the isomeric profile due to illumination could be important in view of the observation that *all-trans*-lutein has shown a higher pigmenting activity in broilers and laying hens (Hencken, 1992).

Data in Table 1 suggest that some of the *cis*-lutein isomers (peaks 1 and 2) could be double *cis*-carotenoids by comparing their spectral maxima to *all-trans*-lutein (peak 7) maxima. Zechmeister (1944) reported variations in carotenoid isomeric profiles due to illumination and also observed that *cis*-carotenoid spectra showed a hypsochromic change in relation to the all-trans-isomer (1−5 nm for the mono-cis and higher for double-cis carotenoids). Similar results were observed by Chen et al. (1994). Peaks 3 and 6 might be isomers of zeaxanthin if we consider the maxima for the *all-trans*-zeaxanthin (peak 8) in the elution solvent system. Peak 9 could be the 9- or 9′-cis-isomer of lutein, in agreement with the spectral data in Table 1 (we did not observe a peak around 300 nm). The isomerization of carotenoids produces the appearance of a peak in the ultraviolet region (320−380 nm); additionally, the extinction coefficient is higher for central isomers (e.g., 13-cis, 13′-cis) than for terminal isomers (e.g., 9-cis, 9′-cis). This is because the extinction coefficient depends on the dipolar moment in the molecule, which is in turn related to the perpendicular distance between the cis bond and an imaginary line joining the ends of the carotenoid molecule (Zechmeister, 1944). Also, it was clear from Table 1 that our system gave a good resolution for most of the analyzed compounds (Rs > 1.5) (Kirkland, 1971). These values were similar to those reported by Sander et al. (1994) and by Emenhiser et al. (1995). Moreover, the peak purity was excellent in all cases.

Table 2. Effect of Solids Content in Enzymatic Mixtures on the *all-trans*-Lutein Level of Marigold Samples after Treatment with Econase-cep at 0.1% w/w for 120 h[a]

		enzymatic	
solids content (% w/w)	control *all-trans*-lutein	*all-trans*-lutein	total pigments[b]
	Fresh Flowers		
15	14.1 ± 0.0 a	13.7 ± 0.7 a	18.5 ± 1.13
	Dehydrated Meal		
5	11.8 ± 0.6 a	25.1 ± 0.3 b	34.0 ± 0.5
10	11.3 ± 0.1 a	21.9 ± 0.3 b	29.4 ± 0.2
15	15.5 ± 1.4 a	21.9 ± 0.8 b	28.5 ± 0.8
20	12.3 ± 0.2 a	17.0 ± 0.1 b	24.9 ± 0.2

[a] Evaluated by HPLC (see Materials and Methods). Values (g/kg dw) are the mean of three determinations ± standard error. Different letters on the same row indicate significant differences ($\alpha = 0.05$). [b] Estimated as *all-trans*-lutein equivalents (g/kg dw).

Effect of the Enzymatic Treatments on the *all-trans*-Lutein Levels of Marigold Meals. Table 2 shows that *all-trans*-lutein levels in enzymatically treated dehydrated marigold samples were significantly higher than in the controls, whereas the enzymatic treatment did not affect significantly the *all-trans*-lutein content of fresh marigold flowers. The same trends were observed when total xanthophylls were quantified spectroscopically by the AOAC modified method (data not shown) or by HPLC (Figure 2a) as *all-trans*-lutein equivalents (Table 2). The coefficient of variation for the *all-trans*-lutein determination was 5%, and the results were highly significant ($P < 0.005$). Currently we do not have an explanation for these results, but it seemed logical to expect that the cell walls had different structures in fresh and dehydrated marigold. Cell wall modifications during processing have been demonstrated with other materials (Massiot et al., 1992; Vardar-Sukan et al., 1993). Marigold fresh flowers were treated with enzymes before the drying processes. Thus, sugars and acids, among other substances, liberated by the enzymatic treatment could form new compounds, as a result of exposure to heat during the drying, which might counteract the effect of enzymes. However, this effect was not observed in dehydrated marigold meals treated with enzymes, and further studies will be necessary to fully explain the effect of drying on cell wall structure and its availability to

Table 3. Distribution in Percentage of Lutein Isomers from Extracts of Dehydrated Marigold Meal Produced by Different Treatments[a]

	factors								
	enzyme[b]		elimination[c]		solids[d]				
compd	without	with	without	with	FF	5%	10%	15%	20%
13′-*cis*-lutein	11.20 a	11.09 a	11.48 b	10.81 a	11.48 b	11.64 b	11.34 b	9.82 a	11.44 b
13-*cis*-lutein	7.81 a	7.77 a	8.05 b	7.53 a	7.18 b	8.12 c	7.93 c	6.45 a	9.28 d
all-trans-lutein	74.90 a	74.73 a	74.32 a	75.31 b	75.25 b	74.78 b	74.90 b	78.12 c	71.04 a
all-trans-zeaxanthin	6.12 a	6.41 b	6.16 a	6.37 a	6.10 c	5.47 a	5.90 bc	5.61 ab	8.25 d

[a] Values are the average of 15 determinations per treatment. Different letters on the same row within each factor indicate significant differences ($\alpha = 0.05$). [b] Enzyme: without and with Econase-cep treatment, respectively. [c] Elimination: Without and with the elimination of substances soluble in water, respectively. [d] Solids. The values corresponded to the percentage of solids content in the enzymatic mixture; FF = fresh flower (15% solids) (see Materials and Methods).

enzymatic attack. Five percent of solids content in the enzymatic mixture gave the dehydrated marigold meal with the highest *all-trans*-lutein level (25.1 g/kg dw).

Effect of Some of the Enzymatic Treatment Factors on the Carotenoid Isomeric Profiles of Marigold Meals. Several reports have shown that processing conditions (e.g., heating, illumination) of different plant materials cause modifications in carotenoid isomeric profiles (Chen et al., 1994, 1995), although in other studies no variations have been observed (Khachik et al., 1986; Chen and Chen, 1993). Thus, it was important for us to find the adequate processing conditions that could result in marigold meals containing carotenoids in the most suitable isomeric form, together with a higher carotenoid content. The distribution of lutein isomers of marigold meals resulting from the enzymatic treatments is shown in Table 3. The enzyme factor did not have a significant effect on the isomeric profiles; the variation for *all-trans*-zeaxanthin, although significant ($P = 0.024$), was not accompanied by variation in the other isomers. For the elimination factor, the elimination of water-soluble substances had a slightly positive effect resulting in a significantly higher *all-trans*-lutein percentage ($P = 0.005$). *all-trans*-Zeaxanthin did not show significant variation with this factor ($P = 0.0878$). Regarding the solids factor, the highest percentage of *all-trans*-lutein in a treated marigold meal was obtained when 15% solids from dehydrated marigold meal was used in the enzymatic mixture; corresponding lower values for the *cis*-lutein isomers were observed ($P < 0.001$). Again, at 15% *all-trans*-zeaxanthin did not vary. Interestingly, in Table 2 we have shown that meals with the lowest pigment contents resulted from enzymatic mixtures containing 20% solids, and in Table 3 these samples exhibited a lower *all-trans*-lutein percentage and unexpectedly the highest *all-trans*-zeaxanthin percentage. All experiments showed coefficients of variation lower than 6%.

Figure 3A shows the results for individual experiments, as compared with a dehydrated marigold meal without any further treatment. It can be noticed that additional processing of a dehydrated sample caused a reduction in *all-trans*-lutein levels; this was accompanied by corresponding increments of the cis-isomer levels (graphs not shown). Additionally, all treatments with 15% solids resulted in the highest values for *all-trans*-lutein, supporting the general data reported in Table 3. After the elimination of water-soluble substances, the *all-trans*-lutein contents were significantly higher (control vs control-H_2O and enzymatic vs enzymatic-H_2O), although differences are not clearly visible. In general, *all-trans*-zeaxanthin levels (Figure 3B) showed no variations as a result of most of the treatments; however, it was clear that the 20%-solids mixture yielded significantly higher values. This result,

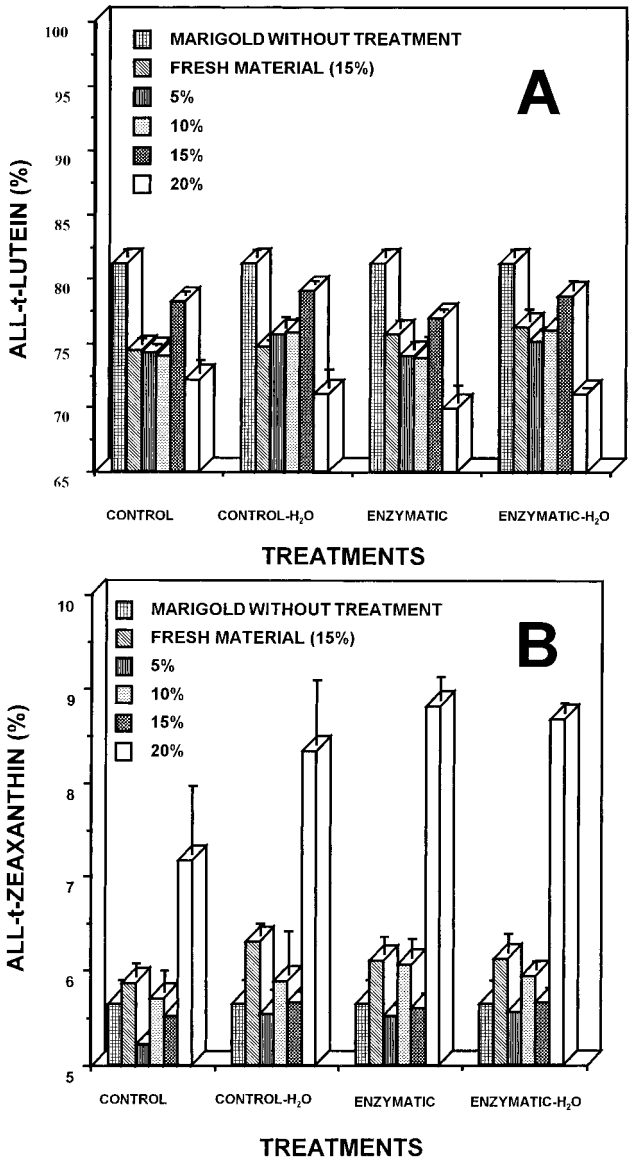

Figure 3. *all-trans*-Lutein (A) and *all-trans*-zeaxanthin (B) composition (percentage of total area) of dehydrated marigold meals obtained after different enzymatic treatments. The elimination of water-soluble substances is indicated by control-H_2O and enzymatic-H_2O. Vertical lines on top of the columns represent the standard error of three determinations.

together with the lower pigment content reported in Table 2, suggests that, with 20% solids, a degradation of lutein occurred. The higher percentage of *all-trans*-zeaxanthin could be explained by a superior stability of this compound under the experimental conditions. In summary, our study showed that solids content in the enzymatic mixture was the principal factor which

induced variations both in the carotenoid content and in the carotenoid isomeric profiles of treated marigold meals.

ACKNOWLEDGMENT

Marigold and enzyme samples were kindly provided by Industrial Orgánica, S.A. (Monterrey, Mexico), and Enzyme Development Corporation (New York, NY), respectively. Thanks are also due to Fidel Guevara-Lara (CINVESTAV-Irapuato) for reviewing the manuscript, and to Roman De La Vara (CIMAT-Guanajuato) for the statistical analyses.

LITERATURE CITED

AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Arlington, VA, 1984.

Avila, E.; Shimada, A. S.; LLamas, G. Otros aditivos. In *Anabólicos y Aditivos en la Producción Pecuaria*; Colegio de Posgraduados: Chapingo, Mexico, 1990; pp 239−250.

Britton, G. Carotenoids. In *Methods in Plant Biochemistry*; Dei, Y., Ed.; Academic Press: New York, 1991, pp 473−518.

Canfield, L. M., Krinski, N. I., James, A. O., Eds. *Carotenoids in Human Health*; New York Academy of Sciences: New York, 1993.

Chen, B. H.; Chen, Y. Y. Stability of chlorophylls and carotenoids in sweet potato leaves during microwave cooking. *J. Agric. Food Chem.* **1993**, *41*, 1315−1320.

Chen, B. H.; Chen, T. M.; Chien, J. T. Kinetic model for studying the isomerization of α and β-carotene during heating and illumination. *J. Agric. Food Chem.* **1994**, *42*, 2391−2397.

Chen, B. H.; Peng, H. Y.; Chen, H. E. Changes of carotenoids, color, and vitamin A contents during processing of carrot juice. *J. Agric. Food Chem.* **1995**, *43*, 1912−1918.

Davies, C. M.; Bustamante, P. L.; Loach, P. A. Reconstitution of the bacterial core light-harvesting complexes of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* with isolated α and β-polypeptides, bacteriochlorophyll a, and carotenoid. *J. Biol. Chem.* **1995**, *270*, 5793−5804.

Delgado-Vargas, F.; Paredes-López, O. Correlation of HPLC and AOAC methods to assess the all-t-lutein content in marigold flowers. *J. Sci. Food Agric.* **1996**, *72*, 283−290.

Domínguez, H.; Nuñez, M. J.; Lema, J. M. Enzymatic pretreatment to enhance oil extraction from fruits and oilseeds: a review. *Food Chem.* **1994**, *49*, 271−286.

Emenhiser, C.; Sander, L. C.; Schwartz, S. J. Capability of a polymeric C_{30} stationary phase to resolve cis-trans carotenoid isomers in reversed-phase liquid chromatography. *J. Chromatogr. A* **1995**, *707*, 205−216.

Francis, F. J. Carotenoids as colorants. *World Ingred.* **1995**, September−October, 34−38.

Gau, W.; Ploscke, H. J.; Wünsche, Ch. Mass spectrometric identification of xanthophyll fatty acid esters from marigold flowers (*Tagetes erecta*) obtained by high performance liquid chromatography and Craig counter-current distribution. *J. Chromatogr.* **1983**, *262*, 277−284.

Goodwin, T. W.; Britton, G. Distribution and analysis of carotenoids. In *Plant Pigments*; Goodwin, T. W., Ed.; Academic Press: London, 1988; pp 62−132.

Hencken, H. Chemical and physiological behavior of feed carotenoids and their effects on pigmentation. *Poultry Sci.* **1992**, *71*, 711−717.

Isaksen, M.; Francis, G. W. Silver ion spray reagent for the discrimination of β- and ϵ-end groups in carotenoids on thin-layer chromatograms. *Chromatographia* **1990**, *29*, 363−365.

Khachik, F.; Beecher, G. R.; Whittaker, N. F. Separation, identification and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *J. Agric. Food Chem.* **1986**, *34*, 603−616.

Khachik, F.; Goli, M. B.; Beecher, G. R.; Holden, J.; Lusby, W. R.; Tenorio, M. D.; Barrera, M. R. Effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables. *J. Agric. Food Chem.* **1992**, *40*, 390−398.

Kirkland, J. J. *Modern Practice at Liquid Chromatography*; Kirkland, J. J., Ed.; Wiley: New York, 1971.

Massiot, P.; Guiller, I.; Baron, A.; Drilleau, J. F. Cell wall polysaccharides modifications during heat treatment and enzymatic degradation of carrot tissues. *Food Sci. Technol. (London)* **1992**, *25*, 559−563.

Mendieta, R. M.; Del-Amos, S. R. *Plantas Medicinales del Estado de Yucatán*; Instituto Nacional de Investigaciones sobre Recursos Bióticos; CECSA: Yucatán, Mexico, 1981.

Mínguez-Mosquera, M. I.; Jaren-Galan, M. Kinetics of the decolouring of carotenoid pigments. *J. Sci. Food Agric.* **1995**, *67*, 153−161.

Neher, R. T. The Ethnobotany of *Tagetes*. *Econ. Bot.* **1968**, *22*, 317−324.

O'Neil, C. A.; Schwartz, S. J. Photoisomerization of β-carotene by photosensitization with chlorophyll derivatives as sensitizers. *J. Agric. Food Chem.* **1995**, *43*, 631−635.

Peterson, T.; Ernström, U.; Griffiths, W.; Sjövall, J.; Bergman, T.; Jörnvall, H. Lutein associated with a transthyretin indicates carotenoid derivation and novel multiplicity of transthyretin ligands. *FEBS Lett.* **1995**, *365*, 23−26.

Philip, T.; Chen, T. S. Separation and quantitative analysis of some carotenoid fatty acid esters of fruits by liquid chromatography. *J. Chromatogr.* **1988**, *435*, 113−126.

Quackenbush, F. W.; Miller, S. L. Composition and analysis of the carotenoids in marigold petals. *J. Assoc. Off. Agric. Chem.* **1972**, *55*, 617−622.

Rivas, J. D. L. Reversed-phase high performance liquid chromatographic separation of lutein and lutein fatty acid esters from marigold flower petal powder. *J. Chromatogr.* **1989**, *464*, 442−447.

Saleh, M. H.; Tan, B. Separation and identification of cis/trans carotenoid isomers. *J. Agric. Food Chem.* **1991**, *39*, 1438−1443.

Sander, L. C.; Sharpless, K. E.; Craft, N. E.; Wise, S. A. Development of engineered stationary phases for the separation of carotenoid isomers. *Anal. Chem.* **1994**, *66*, 1667−1674.

Schmitz, H. H.; Emenhiser, C.; Schwartz, S. J. HPLC separation of geometric carotene isomers using a calcium hydroxide stationary phase. *J. Agric. Food Chem.* **1995**, *43*, 1212−1218.

Tosco, U. *Diccionario de Botánica*; Instituto Geográfico de Agostini; Teide: Barcelona, 1970.

Tyczkowski, J. K.; Hamilton, P. B. Research note: preparation of purified lutein and its diesters from extracts of marigold (*Tagetes erecta*). *Poultry Sci.* **1991**, *70*, 651−654.

Vardar-Sukan, F.; Suha-Sukan, S.; Öngen-Baysal, G. Enzymatic hydrolysis of complex substrates following thermal pretreatment operations. *Biotechnol. Lett.* **1993**, *15*, 657−660.

Wilberg, V. C.; Rodriguez-Amaya, D. B. HPLC quantitation of major carotenoids of fresh and processed guava, mango and papaya. *Food Sci. Technol. (London)* **1995**, *28*, 474−480.

Zechmeister, L. Cis-trans isomerization and stereochemistry of carotenoids and diphenyl polyenes. *Chem. Rev.* **1944**, *34*, 267−344.

Received for review June 28, 1996. Revised manuscript received December 27, 1996. Accepted January 10, 1997.[⊗] We gratefully acknowledge financial support by the Consejo Nacional de Ciencia y Tecnología—México.

JF960474A

[⊗] Abstract published in *Advance ACS Abstracts*, February 15, 1997.