Characterization and Stability of Pigments Extracted from *Terminalia Catappa* Leaves

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ABSTRACT: ***Terminalia catappa*** **is a tree growing in Southeast Mexico; its leaf color ranges from red to yellow and it is a potential source of natural pigments. Petroleum ether:acetone and hexane:acetone were used for pigment extraction. The maximum extraction was 24 mg/100 g of leaves with 70:30 petroleum ether:acetone. Seven pigments were identified in the extracts (violaxanthin, violeoxanthin, lutein epoxide, lutein, two lutein isomers and β-cryptoxanthin). A lower pigment concentration was obtained in saponified than in unsaponified extracts. Antioxidant-free extracts underwent deterioration when stored under light. Conversely, pigments remained stable after 10 days at 40 °C, provided they were preserved with 0.1% BHT and stored in the dark.**

Keywords: carotenoids, ***Terminalia catappa*****, solvent extraction, stability**

Introduction

THERE IS A WORLDWIDE AND GROWING INTEREST IN NATURAL colorants, as they are considered suitable for various applications in foods, pharmaceuticals and cosmetics (López-Hernández and others 1993). Studies reported in the literature demonstrate that a number of synthetic colorants are toxic, causing hepatic alterations, excessive sensitivity and behavioral disturbances (Baublis and others 1994; Giusti and Wrolstad 1996; Gao and Mazza 1996).

Among natural colorants, carotenoids have been widely accepted due to their coloring properties as well as for their nutraceutical properties such as ulcer and cancer prevention (Adewussi and Bradbury 1993; Yen and Chen 1995); protection of the gastric mucosa (Mínguez-Mosquera and Hornero-Méndez 1993); and aging prevention (Willis and Ranga 1996).

Our study focused on the extraction of carotenoids from *Terminalia catappa*, a widely distributed tree growing in Southeast Mexico, mainly used for ornamental purposes, although of no commercial use. Its common name is "almedro tropical" (tropical almond), a *Combretaceae,* native of Malaysia and widely distributed in the tropics. Its leaves are approximately 25 cm long and purplish-red when fully ripened turning yellow during senescence; it has small white flowers and 5 cm long ovoid, reddish drupe fruit with an edible almond (Cano and Marroquín 1994). The objective of this work was to define the best extraction method using organic solvents, as well as to identify the pigments in saponified and unsaponified extracts. Pigment stability was also followed throughout the study in extracts with and without BHT, at 2 illumination conditions (light and darkness) and 4 temperatures.

Materials and Methods

Raw material

Leaves of *Terminalia catappa* were collected when the predominant color was red, from January through March in Villahermosa, Southern Mexico. Due to the fact that there was abundant supply of *T. catappa* leaves, samples were collected early in the morning and immediately processed, without the need of storage before analysis.

Protein and lipid analysis

Protein content was determined by the Lowry method using bovine serum albumin as standard (Robyt and White, 1990) using a standard curve ($Y = 0.052 + 0.004153x$; $r = 0.98$) reading at 750 nm. Protein content was important as protein-pigment complex could be formed making extraction difficult. Lipid content was determined according to the method reported in AOAC (1980).

Carotenoid extraction

To fresh leaves (30 g, 59.7 % moisture) were added 0.1% BHT, 3 g $CaCO_3$, 10 g anhydrous Na_2SO_3 and 400 mL of a solvent system, according to the method reported by Cano (1991). Samples were then homogenized in a Waring blender for 15 s. Different mixtures of petroleum ether-acetone and of hexane-acetone were used as extraction systems (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100). Each extraction was repeated 10 times. The extracted pigments were purified using low-pressure chromatography with a silica gel column (40 to 140 mesh, Baker, Mexico City) and hexane-ethyl acetate (50:50) in a first elution and methanol-hexane (80:20) in a second elution. After the extraction, the samples were left in the dark for 2.5 h in order to achieve complete pigment extraction while preventing oxidation due to light incidence.

Total carotenoid content was analyzed using a Genesys 2PC spectrophotometer (Genesys Co., Rochester, NY) according to the method reported by Hart and Scott (1995). A standard curve ($Y = 0.0283 + 212.343x$; $r = 0.9996$) was used. Maximum absorbance was fixed at 450 nm after wavelength scanning in the visible range of the 60:40 hexane-acetone extracts and expressed as total xanthophylls. Absorbance at the various solvent proportions was obtained for the 2 solvent systems. The maximum absorbance was considered as the best extraction.

Pigment saponification

Half of each concentrated sample was saponified with KOH-20% methanol in aqueous solution at room temperature followed by 1 hour standing in the dark. The methanol phase was extracted with n-hexane, and the organic phase was

Table 1—Conditions used in HPLC analysis of carotenoids

Time (min)	% water	% acetone
0	25	75
10	25	75
15	25	75
20	5	95
27	5	95
32	25	75
37	25	75

washed with water until alkaline-free at pH = 7 (Lasellier and others 1993; Hart and Scott 1994). The maximum absorbance of saponified and unsaponified extracts was then obtained.

HPLC analysis

The pigments present were identified in the extracts after filtration and concentration at under vacuum (Rivas 1989). The extracts were then filtered through 0.45-μ membranes before injection. R_f values obtained from the analysis were compared to R_f values reported in the literature (Table 2) in analysis carried out under similar conditions. Pigment concentration was calculated from a standard curve (Y = 0.08347 + 75.283x; r = 0.9763) correlating peak areas and a-carotene concentration in the same solvent system as standard. A Waters HPLC, model 2487, fitted with a diode arrangement detector and Millennium software, was used; the column was a reverse-phase Waters Symmetry C18, 5 mm particle size, 4.6 mm internal diameter, 250 mm length fitted with a pre-column of the same material (5 cm length × 4 mm I.D.). The analytical conditions were: 1.5 mL/min flow rate, 20 μL injection volume and detection at 450 nm, using the acetone-water gradient shown in Table 1, and a total time of analysis of 40 min. A 25/75 water/acetone gradient was used to re-equilibrate the column for the next run.

Pigment stability

Pigment stability was tested in extracts randomly allocated to the following conditions and levels: antioxidant (0 and 0.1% BHT) (Tsimidou and Tsatsaroni 1993); storage in the dark or under light; temperature (4, 25 and 40 °C) (Giusti and Wrolstad 1996). The lamp used was a daylight-21 cm diameter, 127 V, 60 Hz, 1105 lumen Phillips model TLE 22W (Bangkok, Thailand). An extra storage condition at 60 °C was only applied to samples stored in the dark. Illumination was achieved by using a circular 22-Watt incandescent lamp at 300 mm from the samples (Baublis and others 1994). All samples were stored in the air. Total carotenoid content, expressed as total xanthophylls, was analyzed using a Genesys (Rochester, NY) 2PC spectrophotometer, as described above. The experiment was replicated 4 times. The experimental design was a $2^2 \times 3 \times 4$ complete factorial in a random design for illuminated samples, and a $2^2 \times 4^2$ complete factorial for samples stored in the dark. Data were analyzed using a SAS package (SAS Institute 1989).

Results and Discussion

THE PROXIMATE COMPOSITION OF *TERMINALIA CATAPPA* LEAVES was: water 59.7%; ether extract 3.72%; nitrogen-free extract 31.37%. Total carotenoid content was 36.36 mg/100 g, expressed as total xanthophylls. The maximum extraction yield was obtained using petroleum ether-acetone (70:30) and hexane-acetone (60:40) as shown in Figure 1 measured at 450 nm.

The mean concentration of extracted pigments with these 2 systems was 24 mg/100 g of leaves obtained with 70:30 petroleum ether:acetone, as shown in Figure 2. This concentration is much higher than the one found in other plants such as *Amaranthus udinis* (12.6 mg/100g), *Mangifera indica* (2.21 mg/100 g), *Ashirantes aspera* (2.67 mg/100 g) and *Carica papaya* (2.7mg/ 100 g) (Bhaskarachary and others 1995) making *Terminalia catappa* a better carotenoid source than the other plants.

Extraction yields of saponified samples were considerably lower than in unsaponified samples (Figure 2) and higher when petroleum ether:acetone was used. Lasellier and others (1993) concluded that if the sample does not have a high

Table 2—R_f values of carotenoids found in *T. catappa* and in the literature (López-Hernández and others 1993; Fisher and Kocis 1987; Adewusi and Bradbury 1993)

Identified pigments	Rf literature	Rf experimental	λmax literature	λmax experimental
Violaxanthin	0.34	—	420, 443, 462	420, 445, 472
Violeoxanthin	0.39	0.41	419, 444, 469	419, 444, 469
Lutein	0.6	0.63	424, 445, 475	424, 448, 475
Lutein epoxide	0.5	0.49	421, 443, 478	424, 442, 474
Lutein isomer	0.55	0.55	—	—
Lutein isomer	0.56	0.56	—	—
β-Cryptoxanthin	0.85	0.85	422, 450, 476	421, 450, 476

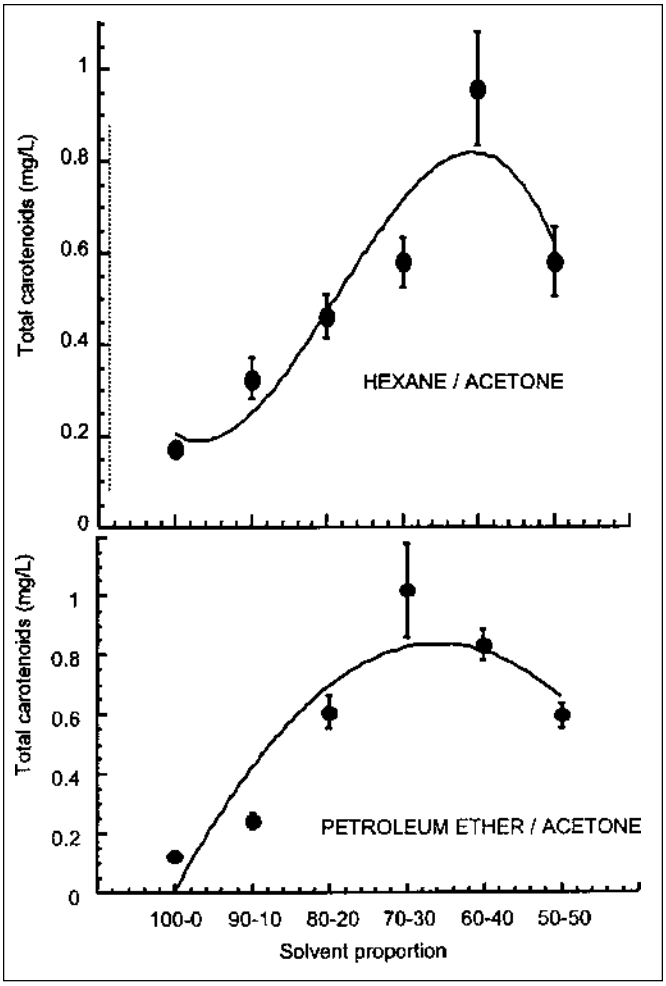

Figure 1—Yields of pigments extracted with organic solvents

chlorophyll concentration, it is advisable not to saponify. This is because during saponification an aldol condensation to β-8′ apocarotenal may occur.

Figure 3 shows the results of HPLC; 7 compounds were identified in unsaponified extracts of *T. catappa:* violaxanthin (1), violeoxanthin (2), lutein epoxide (3), lutein (4), lutein isomers (5 and 6) and β-cryptoxanthin (7). Besides comparing retention times of peaks versus standards, values reported in the literature (Table 2) were used to identified the pigments (Fisher and Kocis 1987; López-Hernández and others 1993; Adewusi and Bradbury 1993; Mínguez-Mosquera and Hornero-Méndez 1993; Britton and others 1995). Lycopene, β-carotene, canthaxanthin, γ-carotene and 8′-apo-β-carotenal were absent in saponified or unsaponified extracts.

Figure 3 also shows the effect of saponification on the extracts. There was a decrease in concentration of lutein, violeoxanthin, lutein epoxide and β-cryptoxanthin in saponified extracts. However, no stereochemical changes were observed, in agreement with Chen and Chen (1983). Violaxanthin was not present in saponified samples; lutein isomers were not affected.

Carotenoid degradation occurs at a high rate due to reaction with free radicals originated by lipid oxidation. The oxidation reaction occurs even faster due to the presence of lipoxygenases (Gordon and An 1995). Because of the presence of double bonds, these compounds are highly reactive to light and oxygen as well as to acid degradation (Chen and Chen 1993).

Table 3—Pigment stability. Duncan multiple range tests

Source of variation	Mean values*			
Antioxidant addition	yes	no		
	5.113[a]	4.010[b]		
Temperature (ºC)	40	4	62	25
	4.945[a]	4.707[b]	4.612[b]	4.201[c]
Illumination	darkness	light		
	4.948[a]	4.124[b]		
Storage time (days)	0	10	20	30
	5.737[a]	5.260[b]	4.024[c]	3.259[d]

*means with different letters are significantly different

Pigment stability, expressed as total carotenoid concentration, depends on oxidation and subsequent disintegration of carotenoids. This process always starts in the open end of the molecule before taking place in the β-ionone ring. Double bonds are saturated and the characteristic color disappears. On the other hand, the pigment undergoes autoxidation after reacting with oxygen from the air. The rate at which this reaction takes place depends on the presence of light, as well as pro- and antioxidants. These reactions occur due to coupled lipid-pigment oxidation (Britton and others 1995).

Under these circumstances, all sources of variation (antioxidant addition, temperature, illumination and storage time) as well as interactions were significant ($P > 0.0001$). Table 3 shows Duncan multiple range tests for pigment concentrations. Storage conditions (antioxidant-light, antioxidant-darkness, without antioxidant-light, without antioxi-

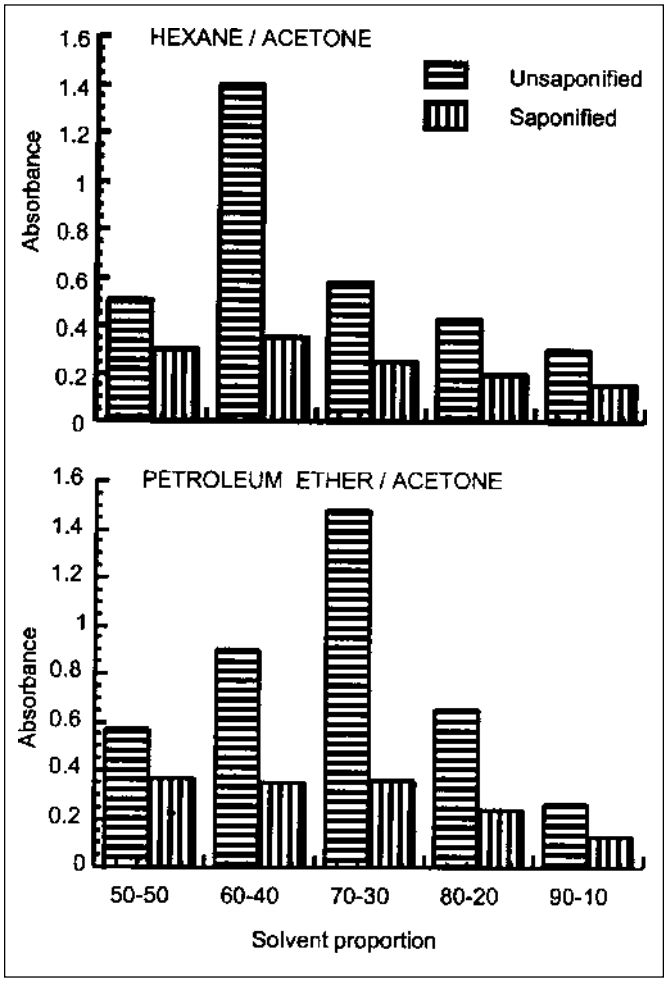

Figure 2—Mean concentration in unsaponified and saponified samples of pigments extracted with organic solvents

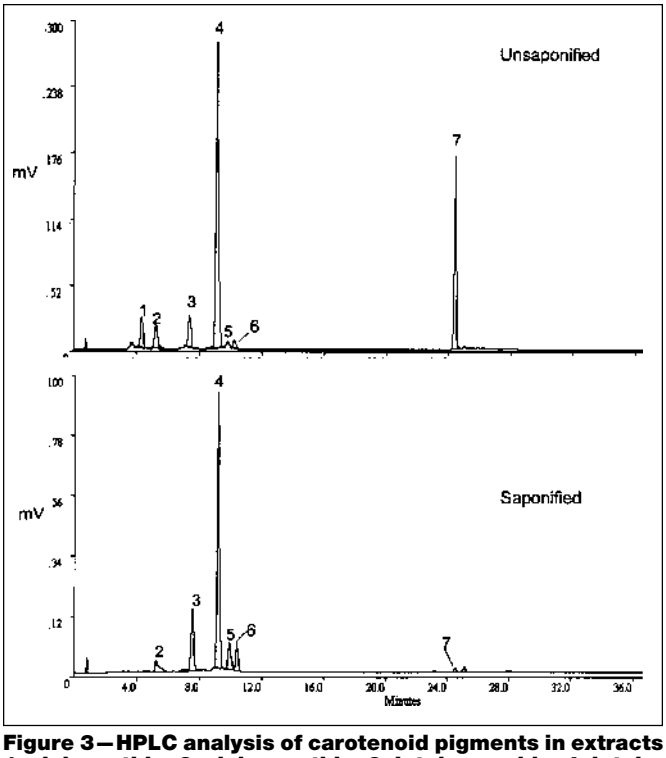

Figure 3—HPLC analysis of carotenoid pigments in extracts 1. violaxanthin; 2. violeoxanthin; 3. lutein epoxide; 4. lutein; 5. lutein isomer; 6. lutein isomer; 7. β-cryptoxanthin

dant-darkness) produced significantly different pigment stability. Table 4 shows Duncan multiple range tests for the 4 storage conditions.

It was assumed that these differences are due to the type of pigment present. Xanthophylls are very susceptible to oxidative degradation via linoleic acid oxidation, catalyzed by a lipoxygenase (Biacs and others 1992). Degradation started by light incidence occurs via stereomutarotation due to the fact that double bonds are in trans configuration in nature and change to cis.

Storage under light affected pigment stability in all samples (Figures 4 and 5). When light was combined with high temperature (40 and 60 °C) carotenoid concentration was reduced to values as low as 0.15 and 0.7 mg/L in samples with 0 and 0.1% BHT, respectively. Samples stored in the dark showed higher xanthophyll concentration, although significantly different when stored at 4 and 25 °C. No significant differences were found in samples stored at 0 or 10 days (Table 4).

Antioxidant addition prevented xanthophyll deterioration to a certain extent in all storage conditions. No significant differences were observed in antioxidant-free samples stored at 4 and 25 °C after 20 days of storage, meaning fast deterioration even when stored under refrigeration. Significantly lower values were obtained in samples stored at 40 °C. Samples with BHT stored in the dark at 4, 25 and 40 °C had similar values. Conversely, rapid deterioration took place after day 10 in all samples with BHT when stored under light (Figure 5).

It can be concluded that illumination was a more determinant factor of stability than temperature. Stability of pigments stored under light has been reported by several authors, although stability at high temperatures is not documented in the literature. Extracts with BHT can be stored up to 40 °C for 30 days, undergoing similar deterioration as BHT-free extracts. In both cases, pigments must be stored in the dark. In our case, temperature stable pigments are an important fact due to high ambient temperatures in the humid tropics (30 to 45 °C). All samples remained stable for the first 10 days of storage, apparently the initiation period before autoxidation takes place.

In general, concentration was lower than in antioxidant-added samples. However, when stored in the dark, concentrations were significantly higher for all samples (Figures 4 and 5) as compared to samples stored in under light. Antioxidant addition and storage in the dark are the 2 major factors necessary for pigment stability.

Table 4—Duncan multiple range tests. Four storage conditions

Condition**	Source of variation	Mean values*			
I	Temperature (ºC)	4	25	40	
		5.360[a]	4.898[b]	3.896[c]	
	Storage time (days)	0	10	20	30
		6.890[a]	5.650[a]	3.627[b]	2.268[c]
II	Temperature (ºC)	4	25	40	60
		5.797[a]	5.570[b]	5.444[c]	4.856[d]
	Storage time (days)	0	10	20	30
		6.590[a]	5.635[a]	4.914[b]	4.447[c]
III	Temperature (ºC)	40	25	4	
		4.025[a]	3.405[b]	2.846[c]	
	Storage time (days)	0	10	20	30
		4.720[a]	4.698[a]	2.835[b]	1.174[c]
IV	Temperature (ºC)	4	25	40	62
		4.550[a]	4.387[b]	4.439[b]	4.368[b]
	Storage time (days)	0	10	20	30
		4.720[a]	4.698[a]	4.212[b]	4.065[b]

*means with different letters are significantly different
*I antioxidant-light
II antioxidant-darkness
IIIwithout antioxidant-light
IV without antioxidant-darkness

Conclusion

TERMINALIA CATAPPA COULD BE CONSIDERED TO BE A BETTER carotenoid source as good as other plants, although it need further confirmation. It has a maximum extraction yield of 24 mg/100 g leaves. Seven compounds were identi-

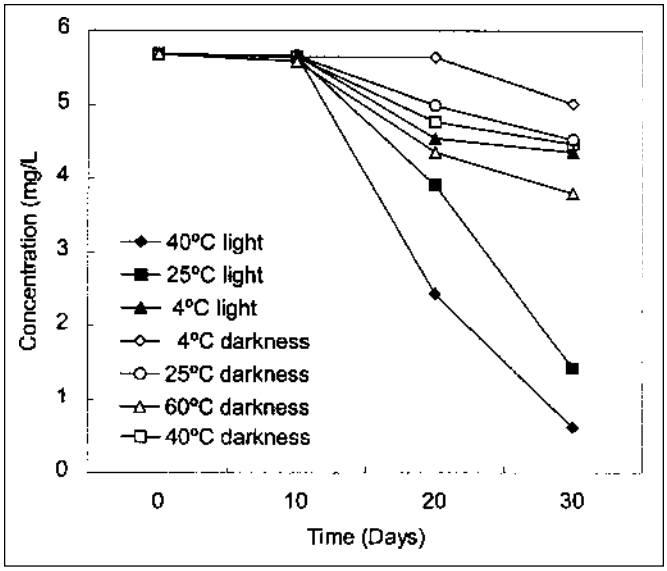

Figure 4—Carotenoid concentration in extracts stored with antioxidant under two illumination conditions

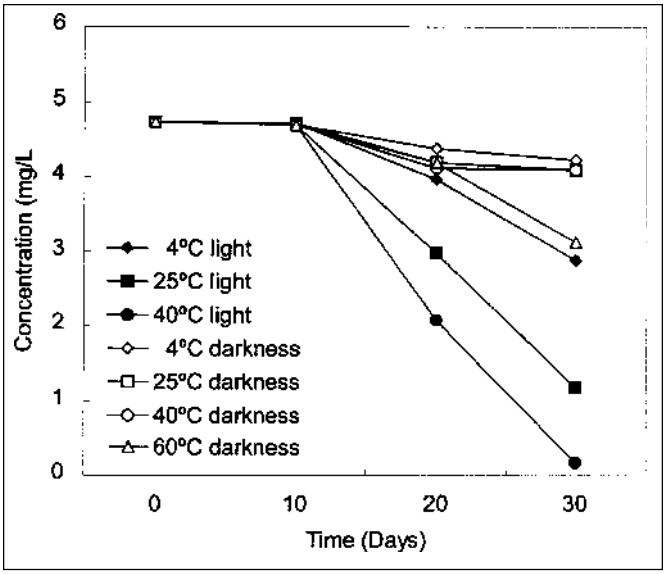

Figure 5—Carotenoid concentration in extracts stored without antioxidant under two illumination conditions

fied by HPLC in the extracts: violaxanthin, violeoxanthin, lutein epoxide, lutein, two lutein isomers and b-cryptoxanthin. Although storage at 40 and 60 °C resulted in faster deterioration, more significant factors were illumination and antioxidant addition. This fact is important, due that ambient temperature in the humid tropical, where *Terminalia catappa* can be industrialized by pigment production are generally around 40 °C. This pigment could be used in several food products in Southeast Mexico, such as butter as this is an important dairy producing area. However, it is necessary to carry out toxicology tests.

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