Carotenoid Composition of Marigold (*Tagetes erecta*) Flower Extract Used as Nutritional Supplement

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Commercially prepared marigold flower (*Tagetes erecta*) extract was saponified and analyzed for carotenoid composition. HPLC analyses were performed on two normal-phase columns (*β*-Cyclobond and silica) and on a C_{30} reversed-phase column. The extract contained 93% utilizable pigments (detected at 450 nm), consisting of all-trans and cis isomers of zeaxanthin (5%), all-trans and cis isomers of lutein, and lutein esters (88%). All were identified by chromatographic retention, UV–visible spectra, and positive ion electrospray mass spectrometry in comparison to authentic standards. Contrary to previous findings, insignificant levels (<0.3%) of lutein oxidation products were detected in the saponified extract. This compositional determination is important for the application of marigold extract in nutritional supplements and increases its value as a poultry feed colorant because it contains more biologically useful lutein compounds than previously believed.

Keywords: *Lutein; marigold (Tagetes erecta); poultry; feed colorant*

INTRODUCTION

The natural pigmentation of poultry (broilers, in particular) is affected by the composition, especially the carotenoid content, of their diet. Extract of marigold flowers is used commercially as an additive to poultry feed to improve bird (fat and skin) and egg yolk pigmentation (Bailey and Chen, 1981; Tyczkowski and Hamilton, 1987). Carotenoids, in particular the dihydroxycarotenoids (also referred to as xanthophylls) lutein and zeaxanthin, are the compounds of interest in the marigold flower extract for poultry pigmentation (Marusich, 1971). For uptake and distribution, free (Hencken, 1992) or esterified (Tyczkowski and Hamilton, 1986b) lutein is absorbed into the bloodstream. Partial cleavage (40–60%) of esterified pigments may also occur within the gut of the broiler (Hencken, 1992; Tyczkowski and Hamilton, 1986a). Saponification is generally performed during the manufacture of commercial preparations to enhance the pigmentation value of the extract (Fletcher and Papa, 1986). Once consumed, free (unesterified) lutein is transported and stored in the liver or integumentary sites as diesterified lutein (Tyczkowski and Hamilton, 1986a,b). A bright yellow color in egg yolks, skin, and fatty tissues is often associated with good health and premium quality by the consumer.

It has been shown that the lutein esters of marigold extract are efficiently absorbed into the human blood stream (Bowen et al., 1997). Lutein from marigold extract suppresses mammary tumor growth and enhances lymphocyte proliferation (Chew et al., 1996; Park et al., 1997). High plasma levels of lutein from ingested lutein from marigold extract increased human macular pigment optical density, which reduces the risk of age-related degeneration of the human macula (Landrum et al., 1997). It thus appears that lutein has important biological functions within those predicted for the carotenoids in general (Krinsky, 1994).

Previous papers indicated that marigold extracts contain epoxides (for example, lutein 5,6-epoxide) and other oxidation products of lutein (Alam et al., 1968; Goodwin, 1980). Early work by Valadon and Mummery (1967) concluded that ≤10% of the carotenoids within *Tagetes erecta* was intact lutein and zeaxanthin; epoxycarotenoids were found to be the main pigments. Later, Quackenbush and Miller (1972) concluded that, of 17 different separated pigments, 88–92% were lutein (predominantly) and zeaxanthin and <3% were epoxy pigments. It was hypothesized that these epoxides and oxidation products may be formed during the industrial processing of marigold flowers. However, Quackenbush and Miller (1972) found no significant compositional difference between extracts of processed or unprocessed marigold flowers. McGeachin and Bailey (1995) claim to have found epoxides in a marigold extract product, assigning to violaxanthin and neoxanthin the peaks appearing after *trans*-lutein and *trans*-zeaxanthin in the HPLC on silica.

Due to the fact that free and esterified carotenoids (with various fatty acids) have different stabilities and

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biological actions, as well as different color effects, previous reversed-phase HPLC-based research has focused on the presence of lutein and lutein esters in marigold extract (Gau et al., 1983; Gregory et al., 1986; Rivas, 1989). However, further analysis of the marigold preparation for human nutrition and for poultry supplementation is needed to determine the isomeric forms of lutein in the extract, regardless of fatty acid composition. Free, unesterified, lutein occurs naturally and is chemically most stable in the all-trans conformation. However, heat, light, oxygen, acid, and certain active surfaces have been shown to catalyze isomerization from the all-trans to various mono- and di-cis isomeric forms (Zechmeister and Polgar, 1944b). Due to the difficult nature of carotenoid geometric isomer separation using HPLC, investigators have not focused on elucidating the isomeric species of lutein that result from extraction and subsequent saponification of dried marigold flowers for poultry feed. Lutein geometric isomers have been identified in a paper on the detailed compositional analysis of human plasma (Khachik et al., 1992) and in a study of reversed-phase C_{30} chromatography (Emenhiser et al., 1995). Recently Khachik et al. (1999) reported on the relative distribution of carotenoids in extracts from marigold flowers and the isolation and characterization of (13*Z*,13′*Z*)-lutein. All isomeric forms of lutein and zeaxanthin contribute to the biological value of the marigold product to various degrees. The goal of this research was to (1) separate the carotenoid pigments present in saponified commercially representative marigold extract using normal and reversed-phase HPLC and (2) identify these pigments using UV−visible spectrophotometric analysis, retention time comparison, and positive ion electrospray mass spectrometry.

EXPERIMENTAL PROCEDURES

Materials. For the raw material, a marigold oil containing 4.5% lutein in the form of lutein esters was obtained from INEXA C.A. (Quito, Ecuador). Lutein, grade 1 all-trans, was purchased from Kemin Industries (Des Moines, IA). Zeaxanthin was obtained from Indofine Chemical Co., Inc. (Belle Meade, NJ). Authentic violaxanthin was obtained as described by Karrer and Jucker (1950).

Preparation of Standards and Samples. Heat isomerization of the *all-trans*-lutein standard to produce cis isomers was performed in hexane under a nitrogen headspace in a 70 °C water bath in the absence of light for 1 h. Marigold extract saponification was accomplished by dissolving the extract in ethyl ether and reacting it with 15% potassium hydroxide in methanol for 1 h in the absence of light. This mixture was then washed with deionized water until a neutral pH was reached as indicated by pH paper. The lower aqueous phase was washed with ethyl ether for re-extraction until the aqueous phase was colorless in white light (i.e., no carotenoids present). The re-extracted ethyl ether was then combined with the original upper solvent phase, which contained the lipid and carotenoid compounds, to be passed through sodium sulfate to dry. The sample was, finally, evaporated to dryness under nitrogen and stored frozen with nitrogen headspace at −20 °C. For analysis, dried samples were brought to the desired volume with mobile phase.

Instrumentation. The chromatography system consisted of a Waters (Milford, MA) Model 510 solvent delivery system fitted with a U6K injector, a Model 996 photodiode array detector collecting data in a range from 250 to 550 nm, and a Millennium 2010 Chromatography Manager v. 2.10 data processor.

Chromatography/UV−Visible Spectroscopy. The first normal-phase system consisted of a Zorbax SIL 5 μm column (4.6 mm i.d. × 250 mm) (MacMod Analytical, Inc., Chadds
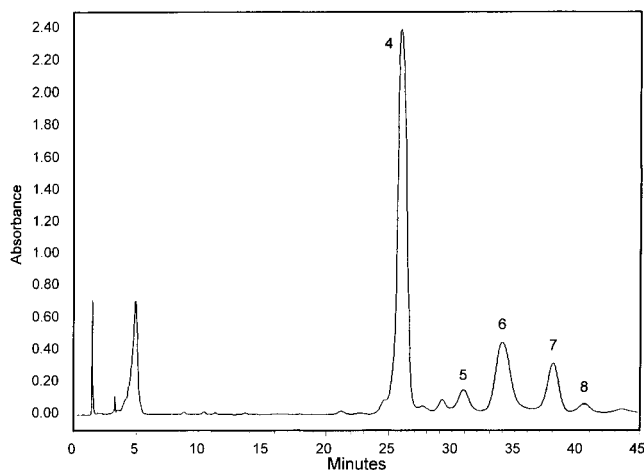

Figure 1. Marigold extract chromatographed on silica column, using hexanes/ethyl acetate (75:25) mobile phase at a flow rate of 2.0 mL/min. Peak identifications: 4 = *all-trans*-lutein; 5 = *all-trans*-zeaxanthin; 6 = 9- or 9′-*cis*-lutein isomers; 7 = 13- or 13′-*cis*-lutein isomers, 8 = 15-*cis*-lutein isomer.

Ford, PA) with hexanes/ethyl acetate (75:25) mobile phase, flowing at 2.0 mL/min. The second normal-phase system included a β-Cyclobond 5 μm column (4.6 mm i.d. × 250 mm) (Advanced Separations Technologies, Inc., ASTEC, Whippany, NJ) with *n*-hexane/ethyl acetate (87:13) mobile phase at a flow rate of 2.0 mL/min.

Reversed-phase chromatography was performed using a YMC S3-SIL200 3 μm silica C_{30} column (YMC, Wilmington, NC) with an isocratic mobile phase consisting of 3% methyl *tert*-butyl ether (MTBE) in methanol, flowing at 1.0 mL/min.

Hexanes, ethyl acetate, and methanol were of certified ACS grade (Fisher Scientific, Fair Lawn, NJ), *n*-hexane (Sigma Chemical Co., St. Louis, MO) and methyl *tert*-butyl ether (Fisher Scientific) were of HPLC grade. All mobile phases and samples were filtered before use. Separations were carried out at room temperature. All injection volumes were 10 μL.

Mass Spectrometry. Positive ion electrospray mass spectra were obtained using a Hewlett-Packard (Palo Alto, CA) 5989B mass spectrometer as described previously (van Breemen, 1995). Peaks were collected manually as they eluted from the HPLC system using absorbance detection. Each solution was evaporated to dryness and shipped on dry ice to the Department of Medicinal Chemistry and Pharmacognosy at the University of Illinois at Chicago. Samples were redissolved in methanol/methyl *tert*-butyl ether (50:50 v/v) and infused into the electrospray ion source at a rate of 10 μL/min.

RESULTS AND DISCUSSION

Zorbax SIL. Representative samples of saponified marigold extract were chromatographed on the Zorbax SIL silica column (Figure 1). With the knowledge that lutein is the most abundant carotenoid in marigold flowers (McGeachin and Bailey, 1995; Tyczkowski and Hamilton, 1991), peak 4, being the largest peak detected at 450 nm, was tentatively identified as *all-trans*-lutein and, later, confirmed by cochromatography with an authentic standard. The relative retention times and UV−visible spectra of peaks 6−8, which elute after *all-trans*-lutein, correspond to those obtained for the cis isomer peaks (Figure 2; Table 1) produced by heat isomerization of the lutein standard. Identification of these peaks was also supported by electrospray mass spectrometry with both the *all-trans*- and the *cis*-lutein isomers exhibiting a molecular ion of *m/z* 568 (Figure 3). Therefore, peak 4 is identified as *all-trans*-lutein and peaks 6−8 are identified as *cis*-lutein isomers.

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Table 1. Comparison of Maximum Peak Absorbances of Isomerized Lutein Standard and Marigold Extract Components Chromatographed under Conditions As Listed in Figure 4

	marigold extract				isomerized lutein standard			
peak	$\lambda_{Bmax}{}^a$ (nm)	$\lambda_{IImax}{}^a$ (nm)	$\lambda_{IIImax}{}^a$ (nm)	Q ratio A_B/A_{II}	$\lambda_{Bmax}{}^a$ (nm)	$\lambda_{IImax}{}^a$ (nm)	$\lambda_{IIImax}{}^a$ (nm)	Q ratio A_B/A_{II}
1	329.2	433.5	461.3	0.17	329.2	433.5	461.3	0.19
2	329.2	435.9	463.7	0.06	329.2	435.9	463.7	0.08
3	330.4	433.5	460.1	0.13	330.4	433.5	460.1	0.22
4	332.8	444.4	473.4	0.06	331.6	445.6	473.4	0.06
6	331.6	440.7	468.6	0.08	330.4	440.7	468.6	0.08
7	331.6	439.5	467.4	0.37	331.6	439.5	467.4	0.36

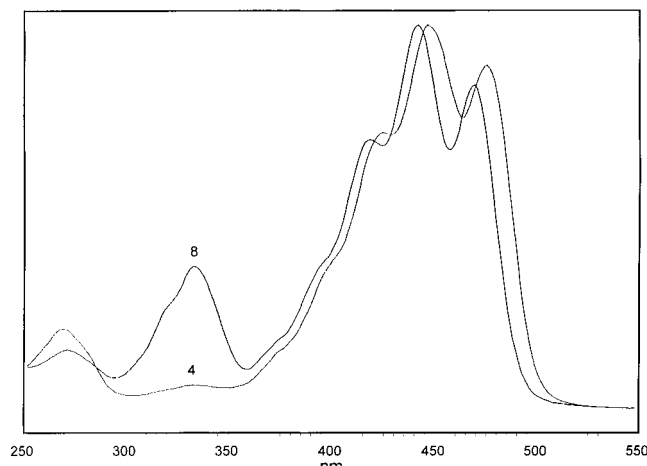[a] Notation as in Britton (1995).

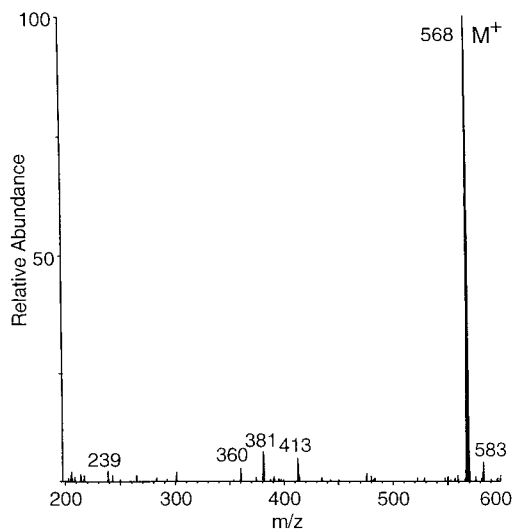**Figure 2.** UV−visible absorption spectra for *all-trans*-lutein (peak 4) and 15-*cis*-lutein isomer (peak 8).

Figure 3. Positive ion electrospray mass spectrum of lutein, representative of compounds **1−8**, chromatographed under both silica and β-Cyclobond column conditions.

Figure 4. Marigold extract chromatographed on β-Cyclobond column, using *n*-hexane/ethyl acetate (87:13) mobile phase at a flow rate of 2.0 mL/min. Peak identifications: 1−3 = *cis*-lutein isomers; 4 = *all-trans*-lutein; 5 = *all-trans*-zeaxanthin; 6 = 9- or 9′-*cis*-lutein isomers; 7 = 13- or 13′-*cis*-lutein isomers.

Figure 5. Isomerized lutein standard chromatographed on β-Cyclobond column, using *n*-hexane/ethyl acetate (87:13) mobile phase at a flow rate of 2.0 mL/min, showing *cis*-lutein isomers.

Similarly, *all-trans*-zeaxanthin, peak 5, was also identified by UV−visible absorption spectra, cochromatography of an all-trans standard with saponified marigold extract, and molecular weight determination (M$^+$ of *m/z* 568) by mass spectrometry (Figure 3).

β-Cyclobond. Analysis of the saponified extract was also carried out on a normal phase β-Cyclobond column (Figure 4). For comparison, a heat-isomerized lutein standard was also analyzed using this column (Figure 5). On the basis of the coelution with authentic standard, peak 4 at 54 min was identified as *all-trans*-lutein. Peaks 1−3, 6, and 7 were identified as *cis*-lutein isomers on the basis of relative retention times (Figures 4 and
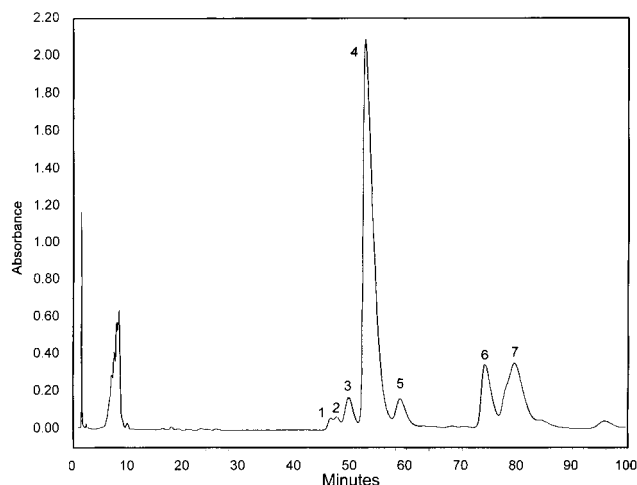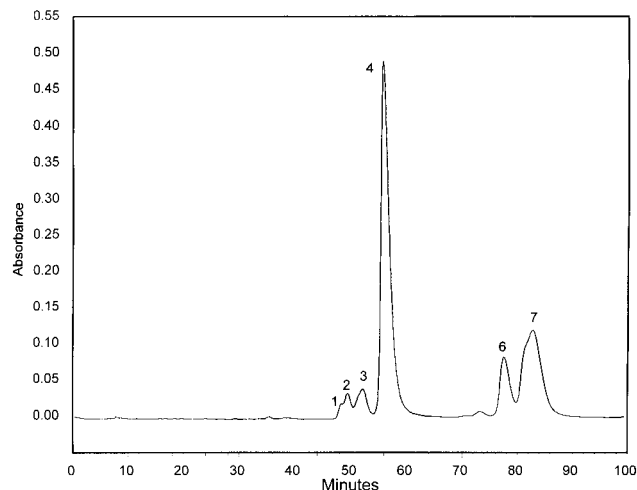
5), UV−visible absorption spectral properties (Table 1), and mass spectrometric analysis.

Approximately 13% of identified peaks detected at 450 nm eluted before 10 min (Figure 4). Some of these compounds are incomplete saponification products (having UV−visible spectra of lutein), which are less polar and would elute before lutein in a normal phase system (Tyczkowski and Hamilton, 1986a). Spectral characteristics of additional components found in this region also indicated the presence of β-cryptoxanthin and some nonpolar carotenoids such as α- and/or β-carotene and

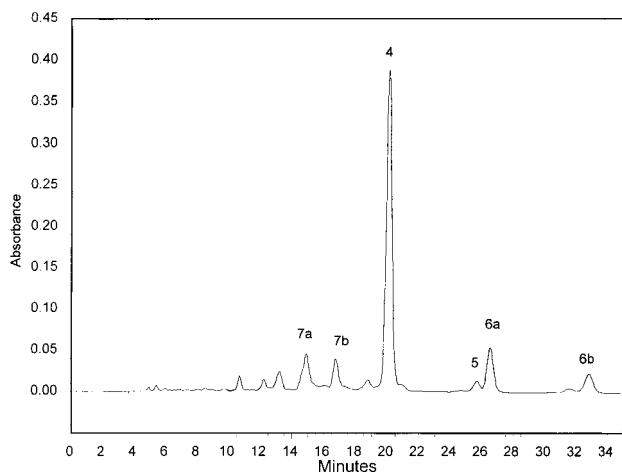**4192** *J. Agric. Food Chem.,* Vol. 47, No. 10, 1999

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Figure 6. Marigold extract chromatographed on a YMC 3 μm silica C_{30} column, using 3% MTBE/methanol mobile phase at a flow rate of 1.0 mL/min. Peak identifications: 4 = *all-trans*-lutein; 6a = 9-*cis*-lutein isomer; 6b = 9′-*cis*-lutein isomer; 5 = *all-trans*-zeaxanthin; 7a = 13-*cis*-lutein isomer; 7b = 13′-*cis*-lutein isomer.

phytofluene, which agrees with previous work done by Bodwell and Nelson (1991).

Epoxides and other oxidation products are more polar than *all-trans*-lutein and, therefore, if present, would be expected to elute after *all-trans*-lutein (Buchecker et al., 1976) during normal phase chromatography. The fine structure of the epoxide absorbance spectra is readily distinguishable from lutein due to the high Q ratios (A_B/A_{II}) close to or equal to 1. For example, lutein epoxide exhibits a Q ratio of 1.07 as detected in olive oil (Minguez-Mosquera et al., 1992) compared to 0.05–0.4 for lutein cis isomers in this sample. On the basis of UV–visible spectra and retention times relative to those of *all-trans*-lutein, two small peaks eluting at ~68 min (<0.3% of detected compounds) are tentatively identified as epoxides. Because of their low concentrations, these epoxides are not apparent within the chromatogram shown and are detectable upon analysis of highly concentrated samples. A hypsochromic shift of ~16 nm compared to *all-trans*-lutein indicates a diepoxide, probably violaxanthin, eluting at 70.5 min in the β-Cyclobond chromatography. Tentatively, the compound that elutes at 68.3 min is identified as a monoepoxide of lutein, antheraxanthin (Matus et al., 1981).

C_{30}. Analysis using C_{30} reversed-phase chromatography showed more complete separation of cis isomers of lutein (Figure 6), which confirms recent work by others (Delgado-Vargas and Paredes-López, 1997). In addition, the chromatogram shows *all-trans*-lutein (peak 4), 9- and 9′-*cis*-lutein isomers (peaks 6a and 6b), 13- and 13′-*cis*-lutein isomers (peaks 7a and 7b), and *all-trans*-zeaxanthin (peak 5) were identified. The two all-trans compounds were confirmed by cochromatography with authentic standards. The cis isomers of lutein were identified by comparison of their respective spectra with isomerized lutein standard spectra and with published data (Emenhiser et al., 1995).

Identification of Peaks. *all-trans*-Lutein and *cis*-lutein isomers identified by UV–visible spectral characteristics, chromatographic retention time, and electrospray mass spectrometry accounted for 88% of the signal at 450 nm. At least 5% of the compounds detected at 450 nm are *all-trans*-zeaxanthin and isomers. The

remaining 7% of compounds at 450 nm eluted early during normal phase chromatography and are tentatively attributed to other carotenoids. Possible epoxides or other oxidation products of lutein were identified as <0.3% of the saponified extract. Contrary to previous findings (Valadon and Mummery, 1967), lutein oxidation products were determined to be an insignificant fraction of the extracts of processed marigold flowers used in poultry feed. These results agree with those of Quackenbush and Miller (1972).

Negative Acid-Shift Test for Epoxides. The carotenoids of peaks 6–8 (Figure 1) were isolated by semipreparative chromatography on an open silica/Celite (1:1) column. The fractions eluting from the chromatographic column were collected manually, on the basis of the visually observable bands that descended through the column. The visible spectrum of the effluent solution (3 mg of carotenoids/L in methanol) was examined before and after addition of a trace of 0.1 M hydrochloric acid (Livingston et al., 1969). None of the hypsochromic shifts of 20 or 40 nm that are characteristic for the 5,6-epoxides were found. The procedure was validated by a positive test obtained with authentic violaxanthin. This shows that peaks 6–8 are not epoxides.

Iodine-Catalyzed Trans-to-Cis Isomerization. Pure *trans*-lutein (5 mg) was dissolved in cyclohexane (50 mL). After addition of a trace of iodine, the solution was irradiated with a fluorescent lamp (Zechmeister and Lemmon, 1944a) and examined in the spectrophotometer. The "B-band" absorption around 330 nm appeared immediately concurrent with a hypsochromic shift of ~3 nm in the absorption maximum and an overall decrease of absorbance at 445 nm. HPLC of the resulting solution showed the formation of the same peaks as the heat-isomerized *trans*-lutein (Figure 5), thus providing a further indication that peaks 6–8 are cis isomers of lutein and not epoxides as formerly considered.

Absence of Cis Isomers in Normal, Freshly Harvested Marigold Flowers. A Soxhlet extract of normal fresh marigold flowers after drying without heat by lyophilization showed the *trans*-lutein and *trans*-zeaxanthin peaks with only traces of other peaks. A similar extract from commercially heat-dried marigold flowers showed the cis peaks (as in Figure 4). This demonstrates that the cis isomers are artifacts of the normal drying process of the marigold flowers.

Reversible Isomerization. Upon exposure to light, dilute hexane solutions of marigold extract show a progressive disappearance of the *cis*-lutein isomers with concurrent increase of *trans*-lutein. Marigold extract (30 mg/100 mL in hexane) with an initial isomeric composition of 70% *trans*-lutein and 30% *cis*-lutein isomers was exposed in a transparent volumetric flask to the normal fluorescent lighting of the laboratory (800 lx).

During the first 3 h a progressive decrease of the cis peaks occurred with a concurrent increase of *trans*-lutein leading to a composition of 95% *trans*-lutein and 5% cis isomers. This cis-to-trans change causes an increase of optical absorbance at 445 nm of the solution by ~8%, consistent with the higher extinction coefficient of *trans*-lutein as compared to the *cis*-lutein isomers. Photodecomposition sets in at longer exposure times to light, and the optical absorbance of the solution begins to decline. This change in isomeric composition of lutein in dilute hexane solution by exposure to light underscores the importance of working under subdued light-

ing conditions when analytical lutein determinations of marigold extracts are performed.

The reversibility of the trans/cis isomerization is another argument against the earlier assignment of these peaks as epoxides. It is well-known that a de-epoxidation involves hydrogenolytic opening of the 5,6-epoxides or the furanoid oxides, which does not occur without strong chemical treatment (Liaaen-Jensen, 1971). A cis-to-trans isomerization of carotenoids has been reported to occur when isomeric mixtures are heated in polar solvents (Surmatis, 1976).

Possible Biological Significance of the Trans/Cis Equilibrium of Lutein. The trans-to-cis isomerization of lutein by heat, and the reverse cis-to-trans isomerization by light in the absence of water, may also occur in nature, that is, in the living marigold flower. It could be a chemical signaling mechanism of basic importance to the marigold plant. Upon heat stress of the plant with excessive evaporation of water (drought conditions), the formation of *cis*-lutein may signal heat damage and trigger repair mechanisms. As the repair proceeds under the action of sunlight the reverse cis-to-trans isomerization may turn off the repair mechanisms when the action is completed.

Summary. We present evidence that commercial marigold flower extracts contain *trans*-lutein as the main carotenoid component with several *cis*-lutein isomers as minor components. Carotenoid epoxides could not be identified in marigold extract. The *cis*-lutein isomers are formed in the harvested marigold flower by the action of heat used during commercial dehydration prior to industrial extraction. This trans-to-cis isomerization is reversed by the action of light on dilute hexane solutions of the marigold extract. A theory is presented about a possible biological significance of shifts in the trans/cis equilibrium.

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