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Lutein and Zeaxanthin in Leafy Greens and Their Bioavailability: Olive Oil Influences the Absorption of Dietary Lutein and Its Accumulation in Adult Rats

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This study determined the lutein level in various green leafy vegetables (GLVs) and the influence of olive and sunflower oils on the postprandial plasma and eye response of dietary lutein in adult rats, previously induced with lutein depletion (LD). Fresh GLVs ($n = 35$) were assessed for lutein (L) and its isomer zeaxanthin (Z) levels by high-performance liquid chromatography and liquid chromatography−mass spectrometry. Among GLVs analyzed, *Commelina benghalensis* L. contained a higher level of L + Z (183 mg/100 g dry wt) and was used as a lutein source for feeding studies. Rats with LD were fed a diet containing powdered *C. benghalensis* (2.69 mg lutein/kg diet) with either olive oil (OO group), sunflower oil (SFO group), or groundnut oil (GNO group) for 16 days. The L + Z levels of the OO group were markedly ($p > 0.05$) higher than those of SFO and GNO groups, in plasma (37.6 and 40.9%) and eyes (22.7 and 30.8%), respectively. These results suggest that oleic acid or OO can be used as a suitable fat source to modulate the absorption of dietary lutein to manage age-related macular degeneration.

KEYWORDS: Lutein; leafy greens; bioavailability; carotenoids

INTRODUCTION

The two major xanthophyll carotenoids found in the human eyes are lutein (β,ϵ-carotene-3,3′-diol) and zeaxanthin (β,β-carotene-3,3′-diol) and are often referred to as macular pigments. Their level in the macula is five-fold higher than the peripheral retina (*1*). They protect the eyes from phototoxic damage (*2*), and an association between the intake of carotenoid-rich plant foods and the macular pigment density in human subjects has been suggested (*3*). Studies have reported that the consumption of green leafy vegetables (GLVs) is associated with a lower risk of cataract and age-related macular degeneration (AMD) (*4*, *5*).

GLVs are rich sources of lutein (L) and zeaxanthin (Z). Data on the level of these pigments in various agri/horticultural produce are vital to choose the right dietary source. Various studies have reported the carotenoids content of foods with reference to developing countries (*6*−*8*). Except for a few studies on provitamin A carotenoids (*9*−*11*), comprehensive data on the levels of L and Z in Indian GLVs are limited. Hence, generating data on these pigments in wild and agri/horticulture produce may be useful from an ethnopharmacological point of view (*12*, *13*). Data on L and Z contents of GLVs will aid in assessing the dietary intake of these pigments and their relationship with AMD. The present work emphasizes the importance of suitable dietary fat to improve lutein bioavailability from leafy greens to manage AMD. Dietary intake is the only source of lutein, as the human body cannot synthesize it. Various dietary factors influence the bioavailability of lutein, among which dietary fat plays a major role (*14*, *15*). Earlier, we reported that micellar phospholipids, oleic and eicosapentaenoic acids, greatly enhanced the bioavailability of lutein and β-carotene in rats and mice (*16*−*18*).

The present study was conducted to evaluate lutein and zeaxanthin levels in various GLVs and to determine the influence of vegetable oils on the plasma and eye response to dietary lutein in lutein-depleted adult rats. This study provides scientific data on superior food sources of lutein and the importance of a suitable dietary lipid to enhance lutein bioavailability under conditions such as AMD.

MATERIALS AND METHODS

Leafy Vegetables. GLVs ($n = 30$) were obtained fresh from either local horticultural farms (familiar, $n = 16$) or collected from agricultural fields (less familiar, $n = 19$). The botanical and common names of these GLVs are listed in **Tables 1** and **2**. The leafy portions were used for the extraction of carotenoids.

Chemicals. Lutein (98%) and DL-α-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO). Dr. A. Nagao (NFRI, Tsukuba, Japan) kindly donated the zeaxanthin (98%). High-performance liquid chromatography (HPLC) grade acetonitrile, hexane, methanol, and dichloromethane were purchased from Sisco Research Laboratories (Mumbai,

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Table 1. Lutein and Zeaxanthin Levels in Less Familiar Leafy Greens[a]

botanical name	family	common name	lutein	zeaxanthin
Allmania nodiflora (L.) R. Br.	Liliaceae	onion stalks	19.71	0.12
Alternanthera pungens Kunth	Amaranthaceae	celosia	71.86	0.67
Alternanthera sessilis (L.) DC.	Amaranthaceae	khaki weed	62.20	0.50
Basella rubra L.	Bassellaceae	red spinach	67.94	2.25
Boerhavia diffusa L.	Nyctaginaceae	hog weed	26.83	0.19
Chenopodium album L.	Chenopodiaceae	lamb's quarters	19.51	0.52
Commelina benghalensis L.	Commelinaceae	jio	181.30	2.10
Daucus carota L.	Apiaceae	carrot greens	40.17	0.59
Gynandropsis pentaphylla L.	Capparidaceae	spider wisp	42.65	1.28
Hibiscus cannabinus L.	Malvaceae	kenaf	33.97	0.17
Hydrocotyle asiatica L.	Apiaceae	Indian pennywort	15.93	ND
Phyllanthus niruri L.	Euphorbiaceae	chanca piedre	77.55	1.63
Piper betle L.	Piperaceae	betel leaf	36.43	0.47
Raphanus sativus L.	Cruciferae	radish	22.30	0.75
Sesbania grandiflora (L.) Poir.	Papilionacae	agase	16.90	0.59
Solanum nigrum L.	Solanaceae	black night shade	84.38	ND
Talinum cuniefolium Willd.	Portulacaceae	ceylon spinach	89.79	1.22
Trianthema portulacastrum L.	Aizoaceae	desert horse	41.51	0.44
Tribulus terrestris L.	Zygophyllaceae	puncture vine	56.39	0.01

[a] Data presented as mg/100 g dry weight (values are means of three analyses).

Table 2. Lutein and Zeaxanthin Levels in Familiar Leafy Greens[a]

botanical name	family	common name	lutein	zeaxanthin
Amaranthus gangeticus L.	Amaranthaceae	amaranthus	20.26	0.21
Amaranthus tristis L.	Amaranthaceae	arai keerai	25.41	0.19
Amaranthus viridis L.	Amaranthaceae	slender	90.43	0.16
Basella alba L.	Bassellaceae	Indian spinach	113.82	1.76
Beta vulgaris L.	Chenopodiaceae	beat greens	26.76	0.14
Brassica oleracea var. *botrytis* L.	Brassicaceae	broccoli	54.5	ND
Coriandrum sativum L.	Apiaceae	coriander leaves	99.27	ND
Lactuca sativa L.	Asteraceae	lettuce	87.12	ND
Mentha spicata L.	Lamiaceae	spearmint	17.74	0.28
Moringa oleifera Lam.	Moringacae	drumstick leaves	50.40	4.13
Murraya koenigii L.	Rutaceae	curry leaf	26.96	0.16
Peucedanum sowa Roxb.	Umbelliferae	Indian dill	92.99	2.25
Portulaca oleracea L.	Portulacacae	purslane	50.84	0.99
Rumex acetocella L.	Polygonaceae	sheap sorrel	53.00	ND
Spinacia oleracea L.	Chenopodiaceae	spinach	77.58	1.51
Trigonella foenum graecum L.	Fabaceae	fenugreek leaves	59.60	0.95

[a] Data presented as mg/100 g dry weight (values are mean of three analyses).

India). Casein was purchased from Nimesh Corp. (Mumbai, India). Food grade vitamins and minerals were purchased from Hi-Media Laboratories (Mumbai, India). Olive oil (OO), sunflower oil (SFO), and groundnut oil (GNO) were obtained from the local market.

Extraction of Carotenoids from GLVs. Fresh GLVs (10 g each) were ground well in a pestle and mortar along with sodium sulfate (5 g) and 2 mM α-tocopherol in methanol (100 μL/g). Carotenoids were extracted thrice in ice-cold acetone (total volume, 100 mL). The pooled extract was dried over anhydrous sodium sulfate (5 g) and filtered through Whatman #1 filter paper. An aliquot (100 μL) of the extract was dried under a stream of nitrogen, and the residue was redissolved in acetonitrile:methanol:dichloromethane (60:20:20, v/v/v) for HPLC analysis (*19*).

Animals and Diet. Animal experiments were performed after due clearance from the institutional animal ethics committee. Male albino rats [OUTB− Wistar, IND-cft (2c)] weighing 250 ± 5 g were housed in steel cages at room temperature (28 ± 2 °C) with 12 h light/dark cycles in the institute animal house facility. Rats received fresh diet daily and had free access to tap water. *Commelina benghalensis* leaves (lutein source) were washed with deionized water, dried under shade, and powdered by a mixer (Mitaso, India). A standard diet (*20*) supplemented with this powdered GLV was used (4.21 g/kg diet) for feeding trials. The amount of L and Z together was 2.69 mg/kg diet, and their content in dried leaf powder and diet was analyzed by HPLC (*19*). The diet contained 10% fat derived from either OO (oleic acid source) or SFO (linoleic acid source) or GNO (control).

Induction of Lutein Depletion (LD) and Refeeding. To induce LD, rats ($n = 60$) were fed with lutein-depleted diet for 2 weeks. Initially (0 day, baseline) and after 2 weeks of feeding a LD diet, blood was drawn from the orbital plexus and analyzed for L and Z to ensure the depletion status (<0.1 pmol/mL). After induction of LD, rats were divided into three groups ($n = 20$/group) and were fed lutein-supplemented diet (powdered *C. benghalensis*) with either OO group, SFO group, or GNO group. To study the time−course response of L and Z, each group was further divided into four subgroups ($n = 5$/group) to measure their levels after 2, 4, 8, and 16 days of refeeding of a lutein-supplemented diet. At the termination of each experiment, blood and eyes were sampled and processed immediately for L and Z analysis by HPLC and liquid chromatography−mass spectrometry (LC-MS).

Extraction of Lutein from Plasma and Eye Samples. Plasma L and Z were extracted according to the procedure of Lakshminarayana et al. (*17*). In brief, to the plasma (0.8 mL), 3 mL of dichloromethane: methanol (2:1,v/v) containing α-tocopherol (2 mM) was added and vortexed. To the mixture, hexane (1.5 mL) was added, mixed well, and centrifuged at 1000*g* for 5 min, and the resulting upper hexane/dichloromethane phase was collected. The extraction procedure was repeated twice with 1 mL of dichloromethane and 1.5 mL of hexane. The extracts were pooled, evaporated to dryness using nitrogen, redissolved in 100 μL of methanol, and used for HPLC analysis.

Eye samples were homogenized (Potter−Elvehjem homogenizer) separately with 9 parts of ice-cold isotonic saline. The homogenate (0.8 mL) was used for L and Z extraction according to the procedure

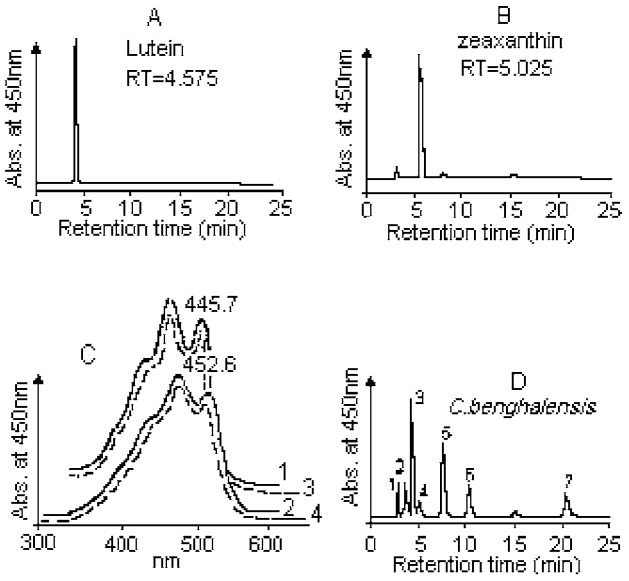

Figure 1. HPLC profiles of standard lutein (**A**) and zeaxanthin (**B**) and their absorption spectra (**1** and **2**) along with spectra of lutein (**3**) and zeaxanthin (**4**) eluted from *C. benghalensis* (**C**) and its typical chromatogram of carotenoids (**D**). Peaks: 1, neoxanthin; 2, violaxanthin; 3, lutein; 4, zeaxanthin; 5 and 6, chlorophyll b and a; and 7, β-carotene.

described above. These operations were done in dim yellow light at 4 °C to minimize isomerization and oxidation of carotenoids by light.

HPLC Analysis of Lutein and Zeaxanthin. L and Z levels in the extracts of GLVs, plasma, and eyes were analyzed by HPLC (*19*, *17*). In brief, L and Z were separated under isocratic conditions at a flow rate of 1 mL/min on a SGE C-18 (ODS) column, 25 cm × 4.6 mm id, 5 μm, 120A0 (SGE Co., India) using acetonitrile:methanol:dichloromethane (60:20:20, v/v/v) containing 0.1% ammonium acetate, as a mobile phase at 450 nm with UV−visible detector (Shimadzu, Japan). Peak identities, λ_{max}, and levels of L and Z were confirmed and quantified by their retention times, characteristic spectra, and peak areas of reference standards and recorded with a Shimadzu model LC-10Avp series equipped with SPD-10AVP detector (**Figure 1**). In this study, we have not included quantitative data on hydrocarbon carotenoids and xanthophylls other than L and Z since the objective was to determine their level. In the case of plasma and eye samples, L and Z levels are presented together as L + Z, due to their unsatisfactory resolution (**Figure 3A**,**C**).

LC-MS for Lutein and Zeaxanthin. LC-MS for L and Z was performed according to the procedure of Breithaupt et al. (*21*). In brief, LC-MS was performed on a Waters 2996 modular HPLC system [autosampler, gradient pump, thermoregulator, and diode array detector (DAD)], coupled to a Q-TOF Ultima (UK) mass spectrometer. The atmospheric pressure chemical ionization (APCI) source was heated at 130 °C, and the probe was kept at 500 °C. The corona voltage was optimized to 5 kV, the HV lens to 0.5 kV, and the cone voltage to 30 V. Nitrogen was used as a sheath and drying gas at 100 and 300 L/h, respectively. The spectrometer was calibrated in the positive ion mode (scan range *m*/*z* 80−1200), and the resulting $(M + H)^+$ signals as well as the $(M + NH_4)^+$ ions were evaluated. Mass spectra of L and Z were acquired with an *m*/*z* 200−1200 scan range, and the UV absorption was recorded at 450 nm by using a DAD. The MS identities of L and Z in samples were confirmed using respective reference standards (**Figures 2** and **3**). Data were processed with Mass Lynx 3.2 software.

Fatty Acid Analysis. Fatty acids in the vegetable oils used were analyzed by gas chromatography (Shimadzu 14B, fitted with flame ionization detection) using a fused silica capillary column 25 m × 0.25 mm (Parma bond FFAP-DF-0.25; Machery-Nagel Gm BH Co, Duren, Germany) as methyl esters (*22*).

Statistical Analysis. To quantify the postprandial L + Z levels in plasma and eye samples over 16 days of refeeding of lutein-supplemented diet, the area under the curve (AUC) was calculated by trapezoidal approximation. Data were tested for homogeneity of variances by the Bartlett test. When homogeneous variances were confirmed, the data were tested by analysis of variance (ANOVA) and significant differences in means among groups and at different days of intervals were evaluated by Tukey's test. Differences in means were considered significant at a level of $p < 0.05$.

RESULTS AND DISCUSSION

This study reports the levels of L and Z in GLVs (**Tables 1** and **2**). The typical HPLC profile of L and Z in *C. benghalensis* is shown in **Figure 1D**. The LC-MS spectra of L and Z, separated from *C. benghalensis* extract, further confirmed their identity (**Figure 2A**). L and Z are composed of two different ionone rings, one with the β-configuration and the other with the ϵ-configuration. The mass spectra signals are characterized by loss of the water (L) or no loss of water (Z) moiety from the respective quasimolecular ions, which is found in each MS, representing the lutein backbone ($[M + H - H_2O]^+$; *m*/*z* 551). Wingerath et al. (*23*) and Breithaupt et al. (*21*) also reported a similar pattern of mass spectra for lutein and its isomers using matrix-assisted laser desorption/ionization mass spectroemtry and APCI-MS. Furthermore, the mass spectra of L and Z eluted from the samples were comparable with the mass spectra of the respective reference standards (**Figure 2B**).

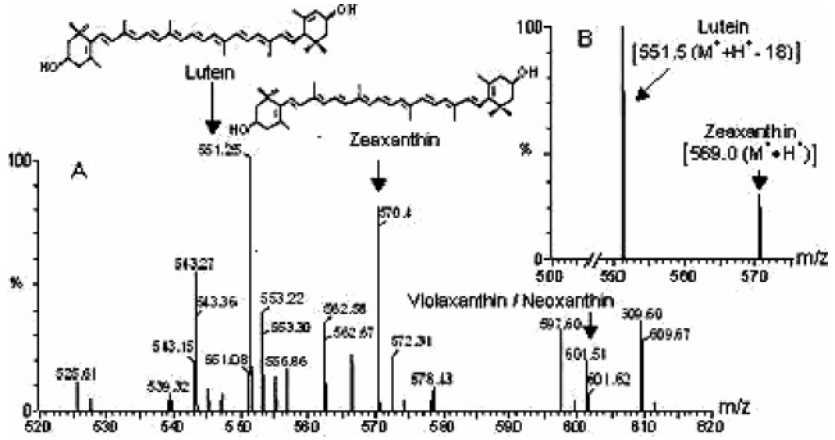

Figure 2. Mass spectrum of carotenoids extracted from *C. benghalensis* (**A**) and standard lutein and zeaxanthin (**B**). LC-MS conditions: Waters 2996 modular HPLC system coupled with mass spectrometer. The APCI source was heated at 130 °C, and the probe was kept at 500 °C. The corona voltage was optimized to 5 kV, the HV lens to 0.5 kV, and the cone voltage to 30 V. Nitrogen was used as the sheath and drying gas at 100 and 300 L/h, and the spectrometer was calibrated in the positive ion mode (scan range *m*/*z* 80–1200). Mass spectra of lutein and zeaxanthin were acquired with an *m*/*z* 200–1200 scan range.

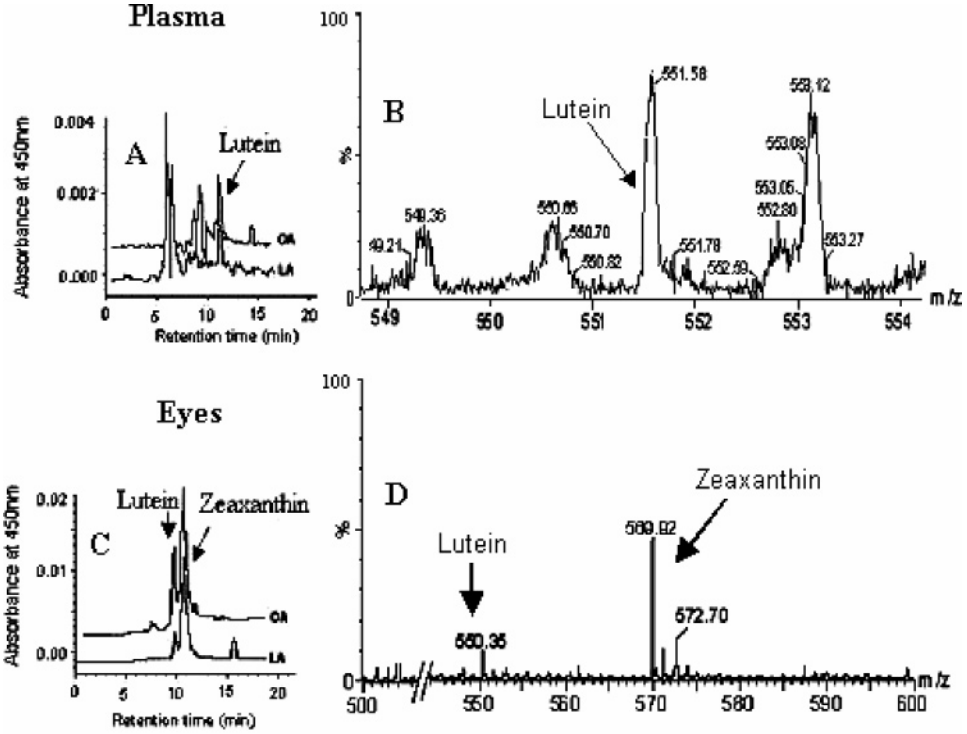

Figure 3. HPLC and MS profiles of lutein and zeaxanthin extracted from plasma (**A** and **B**) and eyes (**C** and **D**) of aged rats after refeeding of the lutein-supplemented diet. LC-MS conditions are the same as given for **Figure 2**.

Although the HPLC profile of L and Z in GLVs appears analogous, their concentrations are different (**Tables 1** and **2**). For example, less familiar GLVs such as *C. benghalensis* (183.40), *Alternanthera pungens* (72.53), *Phyllanthus niruni* (77.6), *Basella rubra* (67.94), *Talinum cuniefolium* (91.01), and *Solanum nigrum* (84.38) contained comparatively higher levels of L + Z (mg/100 g dry weight) than the other leafy vegetables. Among familiar GLVs, *Basella alba* (115.58), *Coriandrum sativum* (99.27), *Peucedanum sowa* (92.24), *Amaranthus viridis* (90.59), *Lactuva sativa* (87.12), and *Spinacia oleracea* (77.58) contained higher levels of L + Z. Results revealed that less familiar GLVs possess equally or even higher levels of L + Z as compared to those in familiar ones. These differences may be attributed to species variations (*24*). Chen and Chen (*25*) reported that factors such as species, part of the plant, degree of maturity at harvest, and postharvest handling practices may also influence the carotenoid levels. Hart and Scott (*26*) also reported lutein levels in *S. oleracea*, *S. pumila*, and *A. viridis* as 3.39, 3.17, and 6.71 mg/100 g edible portions, respectively, which are different from the values obtained in this study for the same species. The difference in the level of L + Z between cultivated and wild GLVs might be attributed to the impact of cultivation practices (*27*, *28*). This study facilitated *C. benghalensis* as the choice for supplementation as it was found to be the richest dietary source of lutein among the GLVs analyzed and hence was utilized for animal feeding experiments. Furthermore, less familiar GLVs that contained higher levels of lutein could be exploited as good source of L + Z to derive maximum health benefits.

Dietary Study. No difference ($p > 0.05$) was found between groups in food intake and gain in body weight. Previously, we have shown that enhanced intestinal uptake of β-carotene and lutein solubilized with specific phospholipids and fatty acids in normal rats (*17*, *29*). The fatty acid composition of vegetable oils used in this study is given in **Table 3**. This study determined the influence of specific fatty acid-rich vegetable oils on the uptake of dietary lutein and its accumulation in lutein-depleted rats. The efficiency of OO and SFO (source of oleic and linoleic acids) on the bioavailability of lutein from GLV (*C. benghalensis*) was determined in the present study. The postprandial plasma and eye responses of L + Z during the 16 day dietary feeding of *C. benghalensis* with either OO, SFO, or GNO (control) are shown in **Figures 4** and **5**. The baseline data (before feeding lutein depleted diet) on L and Z in plasma and eyes of rats were found to be 4.7 pmol/mL and 32.7 pmol/g, respectively. Plasma L + Z was detected after 2 weeks feeding of the lutein-depleted diet, whereas their level in eye samples was 19 ± 2 pmol/g. However, after 16 days of feeding, plasma L + Z levels (AUC) of the OO group were significantly higher ($p < 0.05$) by 37.6 and 40.9% than that of SFO and GNO groups, respectively. Similarly, in eyes, the level was higher by 22.7 and 30.8% in the OO group as compared to SFO and GNO groups, respectively.

Table 3. Fatty Acid Composition of Vegetable Oils Used in the Animal Diet[a]

fatty acid (%)	OO	SFO	GNO
16:0	11.76 ± 1.6	6.44 ± 1.2	13.09 ± 2.0
16.1	0.34 ± 0.0	1.52 ± 0.6	ND[b]
18:0	2.31 ± 0.5	5.14 ± 1.5	1.77 ± 0.3
18:1	75.77 ± 6.8	29.41 ± 4.3	46.07 ± 7.5
18:2 (n-6)	9.84 ± 1.2	57.46 ± 5.2	33.71 ± 3.7
18:3 (n-3)	ND	ND	1.46 ± 0.4
20:0	ND	ND	ND
20:4 (n-6)	ND	ND	0.9 ± 0.1
20:5 (n-3)	ND	ND	ND
22:0	ND	ND	2.96 ± 0.2
22:6 (n-3)	ND	ND	ND

[a] Values are means ± SD, $n = 3$. [b] ND, not detected.

In this study, we analyzed the postprandial plasma and eye levels of L and Z as a measure of intestinal absorption (*17*). However, Gartner et al. (*30*) measured the level of L and Z in chylomicrons fractions. Ahuja et al. (*31*) and Clark et al. (*32*)

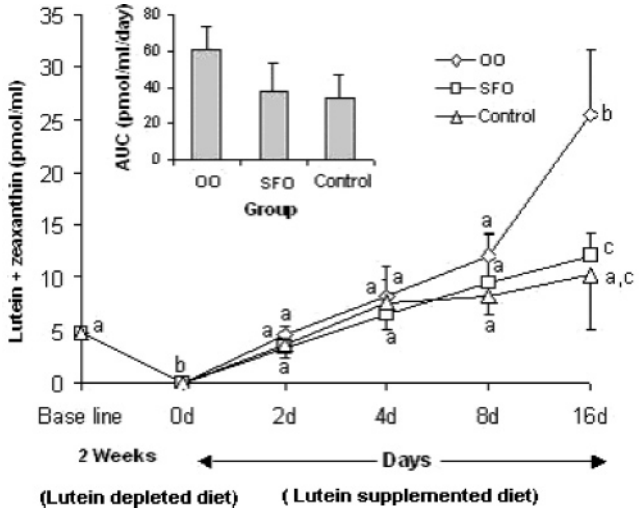

Figure 4. Lutein + zeaxanthin level in plasma of rats fed a lutein-supplemented diet with either OO or SFO or GNO (control) after LD. Data represent the means ± SD, $n = 5$. The values at each time point not sharing a common letter are significantly different ($p < 0.05$) between groups as determined by repeated measures ANOVA and Tukey's test.

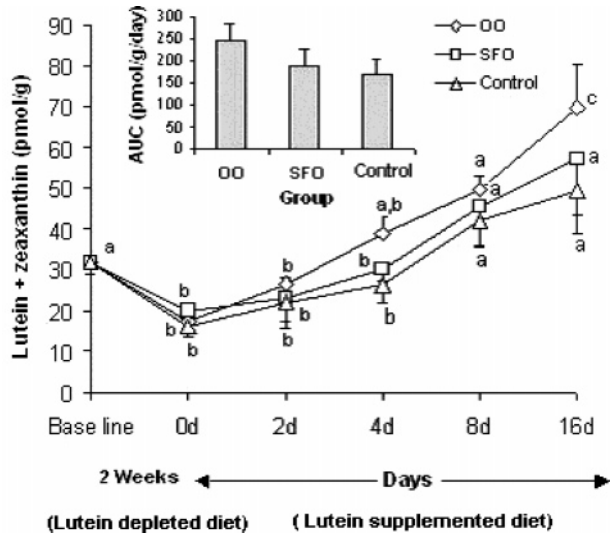

Figure 5. Lutein + zeaxanthin level in eye samples of rats fed a lutein-supplemented diet with either OO or SFO or GNO (control) after LD. Data represent the means ± SD, $n = 5$. The values at each time point not sharing a common letter are significantly different ($p < 0.05$) between groups as determined by repeated measures ANOVA and Tukey's test.

found that the rate of lycopene and astaxanthin absorption from diet in human subjects and rats. Their absorption was higher after the addition of OO to the diet as compared with corn and SFO. They also affirmed that the rate of bioavailability of those pigments depends upon the hydrophobicity of fatty acid in which carotenoids were solubilized and delivered. Unlu et al. (*33*) also reported that dietary lipids are an important factor for carotenoid bioavailability and reported that adding avocado oil as a source of oleic acid with salad significantly enhanced the lutein (3.3-fold) and β-carotene (15.3-fold) when compared to salad without added avocado oil. Van het Hof et al. (*34*) reported that the particle size of the emulsion is one of the factors that influences the bioavailability of carotenoids. It has been suggested that micelles containing linoleic or eicosapentaenoic acid are larger in size, and hence slower in diffusion through enterocytes, thereby decreasing the rate of β-carotene absorption in contrast to micelles containing oleic acid (*18*, *35*). This result suggests that OO may influence the intestinal uptake of lutein by positively favoring mixed micelles formation at the intestinal level as reported by Raju et al. (*18*).

The results of the present study and the previous in vitro and in vivo studies (*36*, *37*) suggest that the lipolysis or hydrolysis of triglycerides and release of fatty acids in the intestinal tract by lipase are vital for the efficient uptake of carotenoids by the enterocytes. Moreover, these fatty acids play a significant role in the solubilization of carotenoids in lipid micelles (*38*). Whereas OO and SFO have an almost similar fatty acid profile except for oleic acid, which is higher in OO, and linoleic acid, which is higher in SFO, they differ in the level of unsaturation (**Table 3**). Because of differences in chemical properties, vegetable oils may act differently at various stages of absorption and metabolism of carotenoids. These properties of fatty acids in oils would make it possible to modify the bioavailability of lipophilic substances such as lutein from the diet.

In conclusion, less familiar GLVs were also found to contain higher levels of L + Z as compared with familiar ones and these could be exploited as a good source of L + Z to derive maximum health benefits for combating AMD. The dietary study revealed that OO might enhance the intestinal accessibility of lutein, which is reflected in higher levels of L + Z in plasma and eyes of aged lutein deficient rats. The mechanism by which dietary OO influences the intestinal uptake of lutein may be due to its fatty acid (oleic acid) content, which facilitates the formation of micelles and incorporation of lutein into the micelles. Furthermore, L + Z rich GLVs and OO or oleic acid combinations could be suitable recommendations to raise macular pigment densities in patients with AMD so as to prevent further progression of the AMD.

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