

Identification of Synthetic Regioisomeric Lutein Esters and Their Quantification in a Commercial Lutein Supplement

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Synthetic mixtures of 24 mono- and diesters of the asymmetric hydroxylated carotenoid lutein with lauric, myristic, palmitic, and stearic acids were analyzed by liquid chromatography–ultraviolet/visible spectroscopy (LC-UV–vis) and characterized by LC–mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). These compounds were then used for identifying the composition of a commercial lutein supplement. This is the first report of chromatographic separation of mixed fatty acid lutein diesters. Preferential MS loss of fatty acids or water occurred initially at the 3'-hydroxy position in the ϵ -ionone ring and subsequently at the 3-hydroxy position in the β -ionone ring. This selective fragmentation leads to facile assignment of the specific fatty acids to the appropriate regioisomeric ionone ring. A commercial lutein supplement contained low levels of two pairs of regioisomeric monoesters and nearly equal levels of three homogeneous diesters and five pairs of mixed diesters. Palmitic acid was the predominant fatty acid, with lower amounts of myristic, stearic, and lauric acids.

KEYWORDS: Lutein; lutein esters; regioisomers; marigold; LC-UV–vis; LC-MS; lutein supplement

INTRODUCTION

Lutein is an asymmetric dihydroxylated carotenoid that has a wide range of applications in the food industry and medicine. The occurrence of lutein in nature is quite widely spread, although the highest levels are found in marigold (*Tagetes erecta* L.) flowers, generally as C14 to C18 even-numbered straight-chain fatty acid esters (1). Lutein is readily absorbed from foods and dietary supplements, whereas to enter the bloodstream, the esters require prior de-esterification by intestinal enzymes (2). Applications include as food colorant additives to poultry feed (3) and as human dietary supplements (4) because lutein may show anticancer (5) and antioxidant activity (6, 7), inhibit age-related macular degeneration (8, 9), which causes irreversible blindness in the elderly, and protect the skin from UV-induced damage and reduce the risk of cardiovascular disease (2).

With the advent and application of more powerful chromatographic and detection technologies, the isolation and characterization of even more minor carotenoid constituents in marigold oleoresins and other plant extracts has become possible. In the case of lutein, many of the extracts are rich in fatty acid diesters and some monoesters. Because lutein is asymmetrical and has β - and ϵ -ionone rings, monoacylation and diacylation by two different fatty acids lead to regioisomers.

Until recently, the regioisomers were not suspected or observed. In an early paper on marigold extracts, Gau et al. (10) noted the presence of the xanthophyll homogeneous diesters of myristic, palmitic, and stearic acids, which had been reported before. However, they were the first to report the mixed lutein myristate–palmitate and lutein palmitate–stearate diesters. The possibility of regioisomers was not recognized because the structure shown for lutein (called xanthophyll in this paper) was incorrectly published as that of zeaxanthin, which does not form regioisomers. The presence of the monoesters of myristic, palmitic, and stearic acids was then observed by Rivas (11), and the chromatograms showed only single peaks for these esters; there was no reference to regioisomers. Piccaglia et al. (12) made a similar observation, and their chromatograms showed no resolution of regioisomers. Among the diesters, Tsao et al. (1) also reported finding lutein laurate–myristate, lutein laurate–palmitate, and lutein myristate–stearate and, for the first time, many *cis* isomeric compounds, which correspond to the known *trans* compounds. Here, too, no resolution of regioisomers was observed. This lack of resolution in all of these studies may be due to the insufficient resolving power of the C18 reversed phase columns used.

The first reported resolution of regioisomeric lutein mono-myristate esters, albeit not from natural sources, was made by Khachik et al. (13), interestingly, on a C18 reversed phase column. Reasonable resolutions of the four regioisomeric pairs of mono lutein laurate, myristate, palmitate, and stearate esters,

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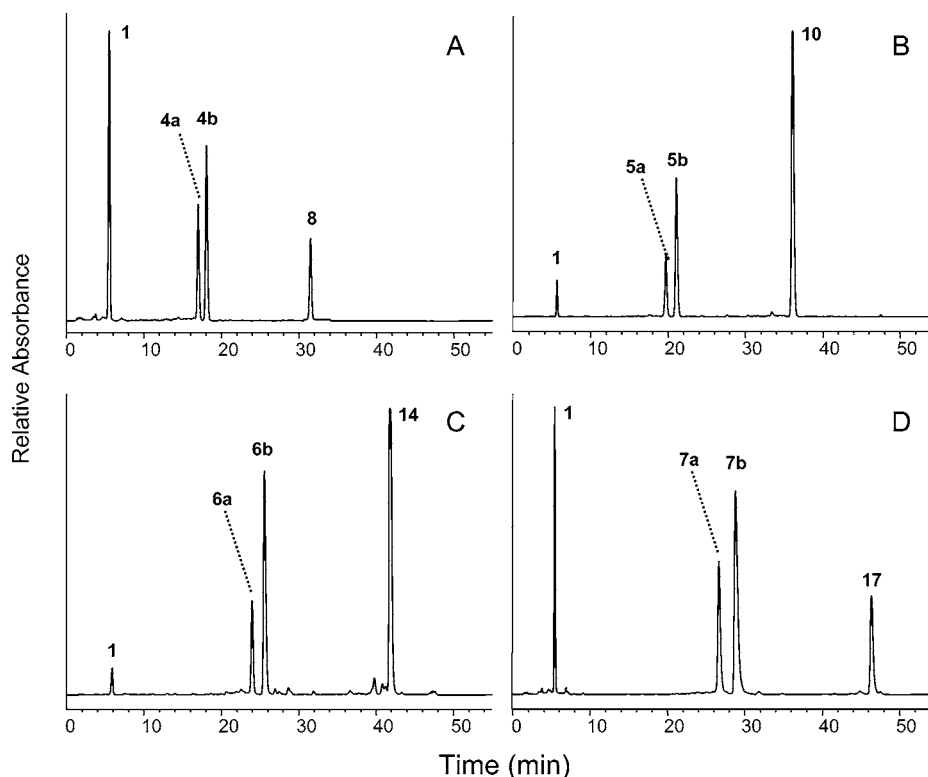


Figure 1. LC-UV-vis chromatograms of synthetic laurate, myristate, palmitate, and stearate monoesters of lutein: (A) reaction mixture from lauric acid plus lutein; (B) reaction mixture from myristic acid plus lutein; (C) reaction mixture from palmitic acid plus lutein; (D) reaction mixture from stearic acid plus lutein. See Table 1 for identity of numbered peaks.

obtained by incomplete enzymatic saponification of marigold oleoresin, were achieved by Breithaupt et al. (14) on a more powerful C30 reversed phase column. The problem now was to distinguish between each pair of isomers. By use of mass spectrometry, nuclear magnetic resonance (NMR), and relative retention times, Khachik et al. (13) determined that of the pair of synthesized lutein monomyristate esters, lutein 3-*O*-myristate (labeled in their publication as β,ϵ -carotene-3-monol monomyristate-3'-monol) was the first to elute. However, by the use of mass spectrometry and some *O*-methylation chemistry on a pair of synthesized lutein monomyristate esters, Breithaupt et al. (14) concluded that monoesters acylated at the 3'-hydroxyl group show shorter retention times. Mixed diester regioisomers were not resolved in either of these two studies.

In the course of our studies to develop high-lutein functional foods, our previous study (15) showed the potential of einkorn, kamut, khorasan, and durum wheats and yellow corn as promising raw materials; however, they still need to be fortified with lutein to deliver the recommended physiological dose. Because lutein in an esterified form isolated from marigold is the only commercially available source, there is a need for analytical procedures to completely characterize lutein esters and also resolve the above-mentioned contradiction regarding the structural identity of lutein ester regioisomers. To this end, we synthesized 24 mono- and diesters of lutein plus two esters of zeaxanthin. The reaction mixtures and a lutein supplement were analyzed by use of LC-UV-vis, LC-MS-atmospheric pressure chemical ionization positive ion mode (APCI+ve), and NMR.

MATERIALS AND METHODS

Chemicals. *all-trans*-Lutein (95% purity) and zeaxanthin (95% purity) standards were purchased from ChromaDex (Santa Ana, CA); lauric acid was from Nu-Check Prep, Inc. (Elysian, MN); myristic acid,

palmitic acid, and stearic acid were from The Hormel Institute (Austin, MN); 1,3-dicyclocarbodiimide and 4-(dimethylamino)pyridine were from Sigma-Aldrich (Oakville, ON); acetonitrile, 1-butanol, dichloromethane, methanol, and methyl *tert*-butyl ether were from Fisher Scientific (Ottawa, ON); and lutein supplement (an extract from marigold containing 90% lutein esters) was kindly provided by Blue California (Rancho Santa Margarita, CA).

Syntheses of Lutein and Zeaxanthin Esters. Esters were prepared according to the method of Tsao et al. (16), which typically consisted of starting with about 1 μ mol of lutein and 3 μ mol each of one or two fatty acids and mixing with about 5 μ mol of 1,3-dicyclocarbodiimide and a crystal (\sim 30 μ mol) of 4-(dimethylamino)pyridine dissolved in 300 μ L of dichloromethane. Nominally, the ratio of lutein and 1,3-dicyclocarbodiimide would be closer to 1:1; however, given the small scale of these reactions, it was not possible to accurately add tiny amounts of powdered material. This mixture was stirred at room temperature for 4 h and then taken to dryness under a stream of nitrogen and redissolved in 400 μ L of a 1:1 (v/v) mixture of acetonitrile and methyl *tert*-butyl ether. Undissolved material was separated by centrifugation at 14000 rpm for 5 min. In a separate experiment, zeaxanthin was reacted with stearic acid and the other reagents.

Liquid Chromatography-Ultraviolet Spectroscopy. Carotenoids were separated and quantified by LC 1100 series Agilent Technologies Canada Inc. (Mississauga, ON) equipped with a quaternary pump G1311A, temperature-controlled injector G1329A by G1330 thermostat, temperature-controlled column thermostat G13161A, degasser G1322A, DAD detector G1315B, and data acquisition system ChemStation for LC 3D Rev. A v. 8.0-1. Reaction mixtures were analyzed by taking a 10 μ L aliquot and injecting it onto a 100 \times 4.6 i.d. mm, 3 μ m LC column packed with reversed phase C30 YMC Carotenoid (Waters, Mississauga, ON) (17). The column, at 35 $^{\circ}$ C, was eluted at 1.0 mL/min with a gradient program consisting of solvent A [methanol/methyl *tert*-butyl ether/water (81:15:4, v/v/v)] and solvent B [methanol/methyl *tert*-butyl ether (90:10, v/v)] (15). The mixture was changed linearly from 100 to 50% A over 44 min, to 0% A over 2 min, then held for 3 min, and returned to 100% A over 5 min. Eluting compounds were

Table 1. Selected Free Carotenoids and Laurate, Myristate, Palmitate, and Stearate Mono- and Diesters of Lutein and Zeaxanthin

compound no. ^a	compound	retention time ^b (min)
1	<i>all-trans</i> -lutein	5.37
2	<i>all-trans</i> -zeaxanthin	6.43
3	β -cryptoxanthin	11.73
4a	lutein 3'- <i>O</i> -laurate	16.98
4b	lutein 3- <i>O</i> -laurate	18.10
5a	lutein 3'- <i>O</i> -myristate	19.97
5b	lutein 3- <i>O</i> -myristate	21.37
6a	lutein 3'- <i>O</i> -palmitate	23.58
6b	lutein 3- <i>O</i> -palmitate	25.38
7a	lutein 3'- <i>O</i> -stearate	28.12
7b	lutein 3- <i>O</i> -stearate	30.48
8	lutein dilaurate	31.60
9a	lutein 3'- <i>O</i> -laurate-3- <i>O</i> -myristate	34.15
9b	lutein 3'- <i>O</i> -myristate-3- <i>O</i> -laurate	34.38
10	lutein dimyristate	36.66
11a	lutein 3'- <i>O</i> -laurate-3- <i>O</i> -palmitate	36.95
11b	lutein 3'- <i>O</i> -palmitate-3- <i>O</i> -laurate	37.43
12a	lutein 3'- <i>O</i> -myristate-3- <i>O</i> -palmitate	39.40
12b	lutein 3'- <i>O</i> -palmitate-3- <i>O</i> -myristate	39.70
13a	lutein 3'- <i>O</i> -laurate-3- <i>O</i> -stearate	40.53
13b	lutein 3'- <i>O</i> -stearate-3- <i>O</i> -laurate	41.35
14	lutein dipalmitate	42.07
15a	lutein 3'- <i>O</i> -myristate-3- <i>O</i> -stearate	42.52
15b	lutein 3'- <i>O</i> -stearate-3- <i>O</i> -myristate	43.27
16a	lutein 3'- <i>O</i> -palmitate-3- <i>O</i> -stearate	45.39
16b	lutein 3'- <i>O</i> -stearate-3- <i>O</i> -palmitate	45.53
17	lutein distearate	46.87
18	zeaxanthin monostearate	32.05
19	zeaxanthin distearate	47.22

^a Numbers correspond to peaks shown in **Figures 1** and **3–5**. ^b Retention times for UV liquid chromatogram of lutein and zeaxanthin esters on a C30 column under conditions described in the text.

detected at 450 nm. The lutein supplement was analyzed in triplicate as described above.

Liquid Chromatography–Mass Spectrometry (LC-MS). Starting materials (20 μ L aliquots) and products separated under identical chromatographic conditions were also detected with a Finnigan (Thermo-Finnigan, San Jose, CA) SpectraSystem UV6000LP ultraviolet detector and a Finnigan LCQ Deca ion trap MS operating in the APCI (positive) mode. Machine operating conditions were as follows: shear gas and auxiliary flow rates, 58 and 3 (arbitrary units); voltages, (source) 6.0 kV, (capillary) 45.5 V, (tube lens offset) 3.0 V, (multipole RF amplifier) 290.0 V (p–p), (multipole 1 offset) –6.1 V, (multipole 2 offset) –9.5 V, (intermultipole lens) –59.0 V, (entrance lens) –56.0 V, and (trap DC offset) –10.0 V; capillary and vaporizer temperatures, 150 and 450 °C, respectively; source current, 5 μ A.

Nuclear Magnetic Resonance (NMR). ¹H NMR spectra were acquired at 500.13 MHz on an ARX500 spectrometer (Bruker Biospin Corp., Billerica, MA) equipped with a XWINNMR data system. Samples of lutein standard and the isolated LC peaks of the mono- and diesters were run in acetonitrile-*d*₃ in a 5 mm probe. ¹H/¹H correlation spectra (COSY-45) were run to confirm resonance assignments, particularly to define the 3 and 3' protons.

RESULTS AND DISCUSSION

Synthesis of Lutein Esters. Other groups (13, 14) have synthesized lutein esters by reaction between acyl chlorides and lutein in pyridine. Because of its simplicity, we chose the method of Tsao et al. (16), which involves reaction between fatty acids and lutein in the presence of 1,3-dicyclocarbodiimide and 4-(dimethylamino)pyridine.

Figure 1 shows the results of reaction between the saturated straight-chain lauric, myristic, palmitic, and stearic fatty acids and lutein. **Table 1** lists the various esters encountered in this study and refers to the chromatograms shown in **Figures 1** and

3–5. Four products in various yields were observed in each reaction mixture: unreacted lutein, two closely eluting compounds, and a late eluting peak. On the basis of LC-MS data, the two closely eluting products were identified as being the two regioisomers of the monoesters and the last peak as being the corresponding diester of lutein. Under our LC conditions, each pair of regioisomers was completely resolved. Structures of typical lutein regioisomers are shown in **Figure 2**. Of interest was the observation that in each reaction, the ratios of area response for the **4–7 a** and **b** products were very close to 1:2. Khachik et al. (13) observed about a 1:3 ratio for the regioisomeric lutein monomyristate esters under different synthetic conditions. They attributed the lower yield of the 3'-*O*-isomer to the electron-withdrawing effect of the allylic double bond in the ϵ -ionone ring. Because the product ratios Breithaupt et al. (14) report were about 1:1, such deactivation does not appear to operate under their incomplete enzymatic saponification conditions.

The product mixtures of reactions between the six possible combinations of two acids and lutein were more complex on each chromatogram. Nine products were observed in various yields (**Figure 3**). These included unreacted lutein, and from LC-MS data, two pairs of regioisomeric monoesters, two homogeneous diesters, and a pair of mixed diesters. The mixed diesters were partially or completely resolved under our LC conditions. Again, the ratio of the two monoesters was close to 1:2, although the ratio of the mixed diesters was close to 1:1. In their studies, Breithaupt et al. (14) and Tsao et al. (1) were not able to resolve chromatographically regioisomeric mixed diesters.

The separations achieved of a mixture of synthetic lutein esters plus added standard zeaxanthin and β -cryptoxanthin by use of a C30 reversed phase column are shown in **Figure 4**.

To ensure that the regioisomeric pairs were not artifacts of the esterification procedure, symmetrical zeaxanthin was reacted with stearic acid. The only products, as expected, were a single zeaxanthin monostearate and zeaxanthin distearate (data not shown).

LC-MS Fragmentation Patterns of Lutein Monoesters. The ion trap APCI+ve MS fragmentation patterns of the four pairs of monoester regioisomers are shown in **Table 2**. For each pair of monoesters, one might anticipate that there could be a more or less similar probability of neutral loss of a fatty acid or water molecule from either end of the lutein molecule. Clearly, the resulting fragmentation intensity data in the table show otherwise. The preferred fatty acid or water losses occur from the 3'-position due to allylic stabilization of the cation generated by cleavage in the ϵ -ionone ring for the **a** and **b** isomers, respectively. Subsequent losses of water and fatty acid then occur at the 3-position in the β -ionone ring. Clear differences in MS fragment patterns enable facile discrimination between the 3'-*O*- and 3-*O*-monoester regioisomers of lutein. This difference in fragmentation pattern was also observed between free lutein, which has a β -ionone ring and an ϵ -ionone ring and free zeaxanthin, which has two β -rings. Because water is readily lost only from the ϵ -ionone ring (15), the lutein base peak is the M + H – 18 fragment at *m/z* 551, whereas that of zeaxanthin is the protonated molecular ion M + H at *m/z* 569.

Lutein esters exhibit two types of MS fragmentations under ion trap MS conditions. The first involves neutral loss of a fatty acid or water from the ϵ -ionone ring followed by a neutral loss of water and a fatty acid to give the characteristic lutein backbone ion at *m/z* 533. The other involves loss of *m/z* 92

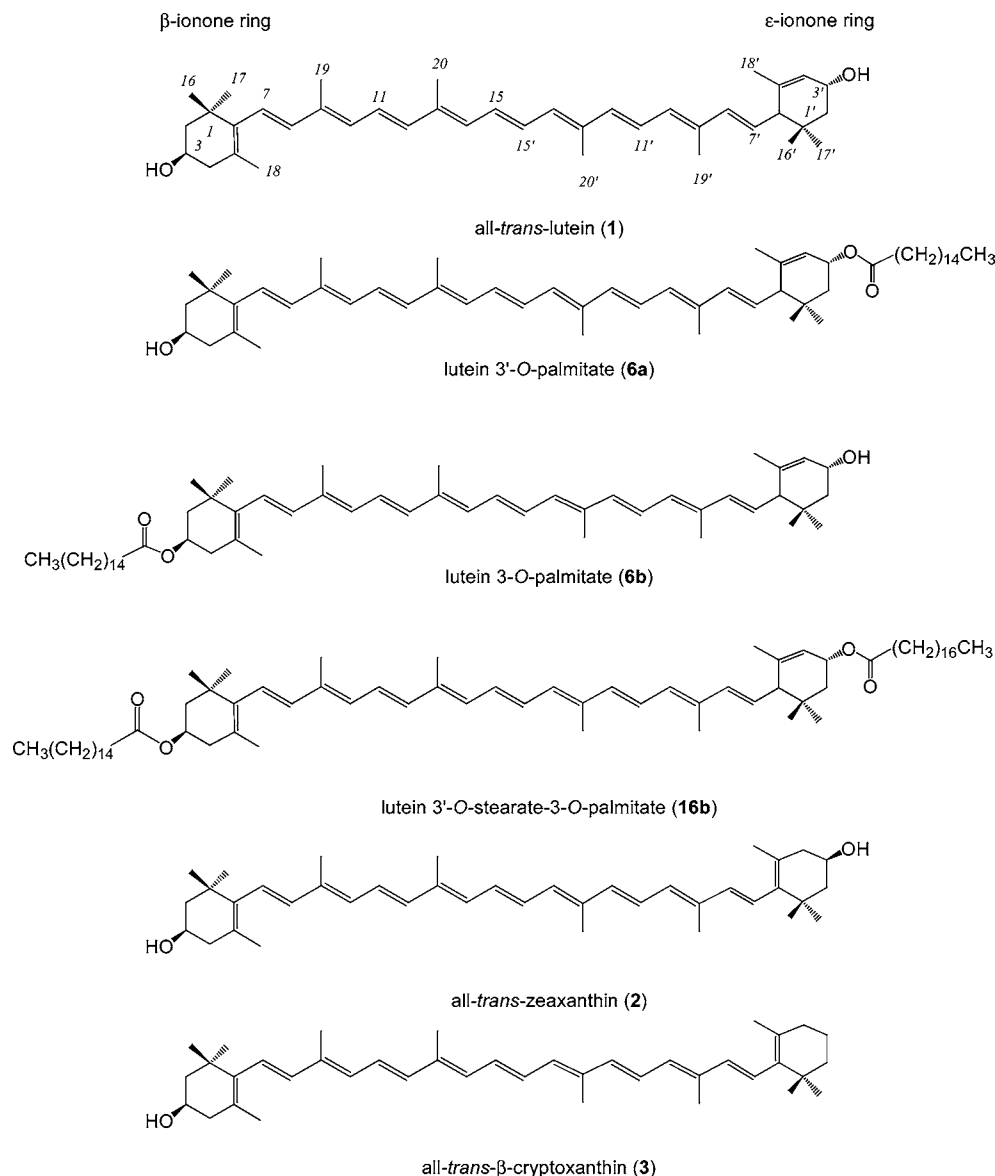


Figure 2. Structures of all-*trans*-lutein, zeaxanthin, β -cryptoxanthin, and selected lutein esters.

from the protonated molecular ion, from the first fragment ion, and from the backbone ion. This has been attributed to loss of toluene (18). Under quadrupole APCI+ve MS conditions, Breithaupt et al. (14) did not report the protonated molecular ion or the ions from loss of toluene.

LC-MS Fragmentation Patterns of Lutein Diesters. The ion trap APCI+ve MS fragmentation patterns of the various lutein diesters are shown in Table 3. As with the monoesters, the preferred initial neutral loss of a fatty acid was from the 3'-*O*-position in the ϵ -ionone ring. Subsequent fatty acid neutral loss occurred at the 3-*O*-position in the β -ionone ring to afford a lutein backbone ion at m/z 533. Fragment ions from loss of toluene from the protonated molecular ion, from the first fragment ion, and from the backbone ion were also observed. These ions were not reported by Breithaupt et al. (14) or Tsao et al. (1). Similar fragmentations have been reported under electron impact (19), matrix-assisted laser desorption ionization (20), and desorption chemical ionization (13) conditions.

Regioisomer Structure Assignments. There are conflicting assignments of structures for the regioisomers of lutein monoesters. On the basis of physical (NMR, MS), chemical, and chromatographic properties, Khachik et al. (13) proposed that the earlier eluting monoesters are those acylated at the 3-hy-

droxyl group, whereas Breithaupt et al. (14) tentatively concluded that it is those monoesters acylated at the 3'-hydroxyl group. To test these hypotheses, the product mixture from reaction between palmitic acid and lutein (see Figure 1C) was separated by preparative LC, and the purified components were subjected to ^1H NMR.

Assignments of the lutein standard were in agreement with published values (21). In particular, the ^1H NMR spectrum of pure lutein showed chemical shifts of 3.85 and 4.12 ppm for the C-3 and C-3' protons on the β - and ϵ -ionone rings, respectively, which are in reasonable agreement with the reported shifts of 3.98 and 4.23 ppm, respectively. Table 4 shows the chemical shifts of the C-3 and C-3' protons in lutein, its two regioisomeric monopalmitate esters, and lutein dipalmitate. One can clearly distinguish between the functionalities at C-3 and C-3'. The C-3' proton chemical shift values for the 3'-*O*-monopalmitate ester (6a) (3.85 and 5.25 ppm, respectively) are also in reasonable agreement with the values reported for the 3'-*O*-monomyristate ester (5a) [4.09 and 5.35 ppm, respectively (13)]. Similarly, the C-3 and C-3' proton chemical shift values for the 3-*O*-monopalmitate ester (6b) (5.01 and 4.12 ppm, respectively) are in reasonable agreement with the values reported for the 3-*O*-monomyristate ester (5b) [5.06 and 4.30

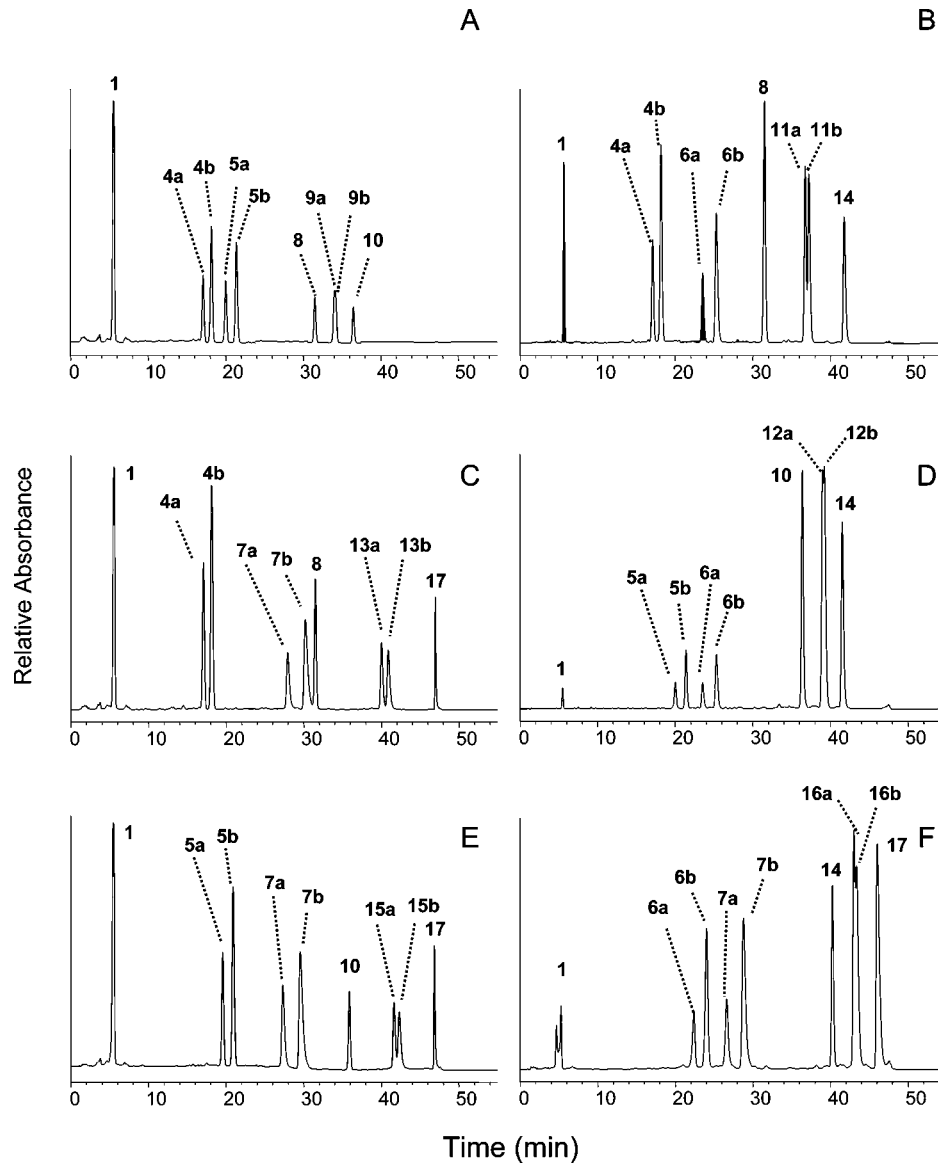


Figure 3. LC-UV-vis chromatograms of synthetic laurate, myristate, palmitate, and stearate diesters of lutein: (A) reaction mixture from lauric and myristic acids plus lutein; (B) reaction mixture from lauric and palmitic acids plus lutein; (C) reaction mixture from lauric and stearic acids plus lutein; (D) reaction mixture from myristic and palmitic acids plus lutein; (E) reaction mixture from myristic and stearic acids plus lutein; (F) reaction mixture from palmitic and stearic acids plus lutein. See **Table 1** for identity of numbered peaks.

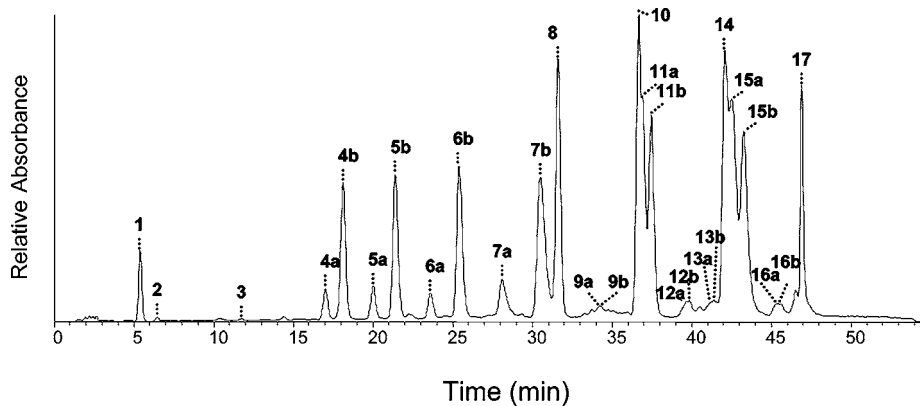


Figure 4. LC-UV-vis chromatogram of synthetic laurate, myristate, palmitate, and stearate mono- and diesters of lutein plus added zeaxanthin and β -cryptoxanthin. See **Table 1** for identity of numbered peaks.

ppm, respectively (13)]. Our combined LC, MS, and NMR results show that the monoester acylated at the 3'-hydroxyl group elutes first, in agreement with the conclusions drawn by Breithaupt et al. (14).

Analysis of a Commercial Lutein Supplement. A commercial food grade sample of lutein supplement was analyzed for its content of mono- and diesters. **Figure 5** shows the separation of the 17 various esters detected in the supplement.

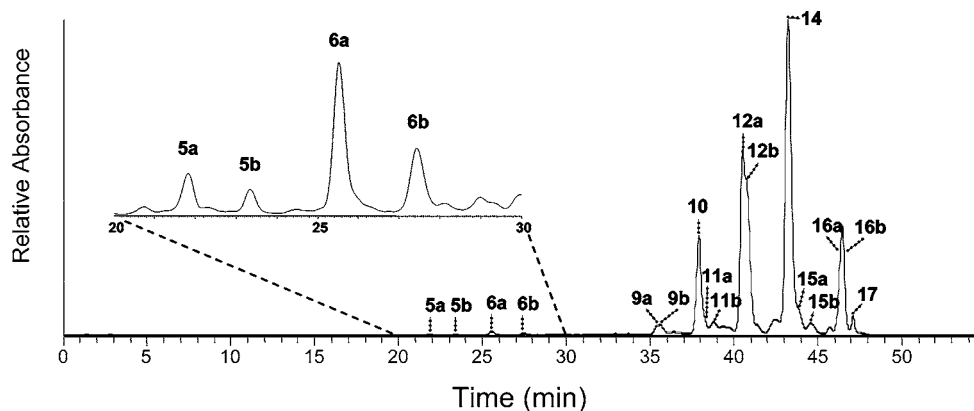


Figure 5. LC-UV-vis chromatogram of a lutein supplement extract. See Table 1 for identity of numbered peaks.

Table 2. Liquid Chromatographic–Mass Spectra Fragment Ions (m/z) of Synthetic Laurate, Myristate, Palmitate, and Stearate Mono Esters of Lutein

compound	$[M+H]^+$	$[M+H-W_\epsilon]^+$ ^a	$[M+H-W_\beta]^+$ ^b	$[M+H-92]^+$	$[M+H-W\epsilon-92]^+$	$[M+H-A_\epsilon]^+$ ^c	$[M+H-A_\epsilon-W_\beta]^+$	$[M+H-A_\beta]^+$ ^d	$[M+H-A_\beta-W_\epsilon]^+$	$[M+H-A_\epsilon-92]^+$	$[M+H-A_\epsilon-W_\beta-92]^+$	$[M+H-A_\beta-92]^+$	$[M+H-W\epsilon-92]^+$
lutein 3'-O-laurate (4a) ^e	751 ^f (20) ^g		733 (2)	658 (8)	551 (100)	533 (9)				459 (18)	441(2)		
lutein 3-O-laurate (4b)	751 (42)	733 (100)		658 (5)	641 (13)			551 (17)	533 (69)			459 (7)	441(16)
lutein 3'-O-myristate (5a)	779 (23)		761 (2)	686 (8)	551 (100)	533 (10)				459 (19)	441(2)		
lutein 3-O-myristate (5b)	779 (43)	761 (100)		686 (5)	669 (12)			551 (15)	533 (64)			459 (9)	441(15)
lutein 3'-O-palmitate (6a)	807 (25)		789 (2)	714 (9)	551 (100)	533 (10)				459 (19)	441(2)		
lutein 3-O-palmitate (6b)	807 (39)	789 (100)		714 (5)	697 (11)			551 (14)	533 (69)			459 (8)	441(14)
lutein 3'-O-stearate (7a)	835 (27)		817 (3)	742 (9)	551 (100)	533 (13)				459 (19)	441(2)		
lutein 3-O-stearate (7b)	835 (39)	817 (100)		742 (5)	725 (10)			551 (13)	533 (62)			459 (6)	441(14)
zeaxanthin 3-O-stearate (19)	835 (100)	817 (33)		743 (18)	725 (7)			551 (67)	533 (20)				

^a W_ϵ = loss of water from 3'-hydroxyl in ϵ -ionone ring. ^b W_β = loss of water from 3-hydroxyl in β -ionone ring. ^c A_ϵ = loss of fatty acid from acylated 3'-hydroxyl in ϵ -ionone ring. ^d A_β = loss of fatty acid from acylated 3-hydroxyl in β -ionone ring. ^e Numbers of the lutein monoesters correspond to the numbers in Table 1 and peak numbering in Figure 4. ^f The fragment ion in m/z . ^g The percent signal intensity (in parentheses).

Table 3. Liquid Chromatographic–Mass Spectra Fragment Ions (m/z) of Synthetic Laurate, Myristate, Palmitate, and Stearate Diesters of Lutein

compound	$[M+H]^+$	$[M+H-92]^+$	$[M+H-A_\epsilon]^+$ ^a	$[M+H-A_\beta]^+$ ^b	$[M+H-A_\epsilon-92]^+$	$[M+H-A_\epsilon-A_\beta]^+$	$[M+H-A_\epsilon-A_\beta-92]^+$
lutein dilaurate (8) ^c	933 (31) ^d	840 (5)	733 (100)		641 (18)	533 (67)	441 (15)
lutein 3'-O-laurate-3-O-myristate (9a)	961 (7)		733 (100)	761 (2)	641 (18)	533 (82)	
lutein 3'-O-myristate-3-O-laurate (9b)	961 (6)		761 (100)	733 (10)	669 (17)	533 (88)	
lutein dimyristate (10)	989 (34)	896 (5)	761 (100)		669 (15)	533 (57)	441 (11)
lutein 3'-O-laurate-3-O-palmitate (11a)	989 (8)		733 (100)		641 (19)	533 (62)	441 (13)
lutein 3'-O-palmitate-3-O-laurate (11b)	989 (6)		789 (100)		697 (16)	533 (80)	441 (15)
lutein 3'-O-myristate-3-O-palmitate (12a)	1017 (3)		761 (100)		669 (14)	533 (47)	441 (6)
lutein 3'-O-palmitate-3-O-myristate (12b)	1017 (3)		789 (100)		697 (10)	533 (31)	441 (6)
lutein 3'-O-laurate-3-O-stearate (13a)	1017 (8)		733 (100)		641 (19)	533 (96)	441 (25)
lutein 3'-O-stearate-3-O-laurate (13b)	1017 (6)		817 (100)		725 (13)	533 (96)	441 (26)
lutein dipalmitate (14)	1045 (30)	953 (5)	789 (100)		697 (18)	533 (63)	441 (13)
lutein 3'-O-myristate-3-O-stearate (15a)	1045 (33)	953 (9)	761 (100)		669 (12)	533 (100)	441 (23)
lutein 3'-O-stearate-3-O-myristate (15b)	1045 (18)	953 (3)	817 (100)		725 (10)	533 (53)	441 (12)
lutein 3'-O-palmitate-3-O-stearate (16a)	1073 (3)		789 (100)		697 (16)	533 (43)	441 (8)
lutein 3'-O-stearate-3-O-palmitate (16b)	1073 (4)		817 (100)			533 (54)	441 (8)
lutein distearate (17)	1101 (25)	1009 (6)	817 (100)		725 (15)	533 (69)	441 (14)
zeaxanthin distearate (19)	1101 (76)	1009 (10)	817 (100)		725 (15)	533 (48)	441 (12)

^a A_ϵ = loss of fatty acid from acylated 3'-hydroxyl in ϵ -ionone ring. ^b A_β = loss of fatty acid from acylated 3-hydroxyl in β -ionone ring. ^c Numbers of the lutein monoesters correspond to the numbers in Table 1 and peak numbering in Figure 4. ^d The fragment ion in m/z and the percent signal intensity (in parentheses).

The identity of each ester was confirmed by congruence of LC retention times and LC-MS-APCI+ve spectra with those of the synthetic lutein esters. The percentage composition of the esters is given in Table 5. Several compositional trends were readily noticeable: the level of monoesters was very low (1.5%), mixed diesters were present at about the same levels as the homogeneous diesters (50.2 vs 48.0%, respectively), and palmitic acid

was the predominant fatty acid (57.2%) followed by myristic acid (30.2%), stearic acid (10.2%), and lauric acid (2.4%). There was also a trend with the regioisomers: the first eluting isomer was present in higher amounts, which was opposite to the proportions observed after chemical synthesis. This variance may result from differences between the enzymatic mechanism of synthesis for the in vivo isomers and a chemical mechanism

Table 4. NMR Chemical Shifts of C-3 and C-3' Protons in Lutein and Its Palmitate Esters

compound	chemical shift (ppm)	
	C-3	C-3'
lutein	3.85	4.12
lutein 3'-O-palmitate	3.85	5.26
lutein 3-O-palmitate	5.01	4.12
lutein dipalmitate	5.01	5.26

Table 5. Ester Composition of a Commercial Lutein Supplement

compound relative	composition %
monoesters	
lutein 3'-O-myristate (5a)	0.2 ± 0.02 ^a
lutein 3-O-myristate (5b)	0.1 ± 0.01
lutein 3'-O-palmitate (6a)	0.8 ± 0.02
lutein 3-O-palmitate (6b)	0.3 ± 0.03
homogeneous diesters	
lutein dimyristate (10)	9.2 ± 0.93
lutein dipalmitate (14)	39.9 ± 0.78
lutein distearate (17)	1.1 ± 0.07
mixed diesters	
lutein 3'-O-laurate-3-O-myristate (9a)	0.7 ± 0.04
lutein 3'-O-myristate-3-O-laurate (9b)	0.7 ± 0.04
lutein 3'-O-laurate-3-O-palmitate (11a)	1.3 ± 0.12
lutein 3'-O-palmitate-3-O-laurate (11b)	1.0 ± 0.06
lutein 3'-O-myristate-3-O-palmitate (12a)	17.8 ± 0.25
lutein 3'-O-palmitate-3-O-myristate (12b)	12.5 ± 0.58
lutein 3'-O-myristate-3-O-stearate (15a)	2.0 ± 0.24
lutein 3'-O-stearate-3-O-myristate (15b)	1.4 ± 0.11
lutein 3'-O-palmitate-3-O-stearate (16a)	4.4 ± 0.29
lutein 3'-O-stearate-3-O-palmitate (16b)	6.2 ± 0.43

^a Average of triplicate determinations ± standard deviation.

for the in vitro isomers. General agreement was observed between the values of diesters taken from the lutein supplement and averages of literature values for native marigold esters (10–12). In the present and previous studies (10–12), lutein dipalmitate is present at the highest level (39.9 vs 36.5%, respectively), followed by lutein 3'-O-myristate-3-O-palmitate (17.9%) and lutein 3'-O-palmitate-3-O-myristate (13.3%). These two mixed diesters were observed as one compound in the previous studies (10–12) averaging 24.7%, which is close to the sum of the two mixed regioisomeric diesters (31.2%). The fourth highest compound in the present and previous studies is lutein dimyristate (9.2 vs 12.8%, respectively).

There is growing evidence for the role of lutein in the health of eyes and skin, which has led to increasing demands for lutein supplements and product. This study provides analytical techniques for the identification and quantification of lutein esters in lutein supplements and lutein-containing products using synthetic lutein esters. From mass spectrometric and NMR properties of 20 synthetic lutein esters, it was possible to assign unequivocal structures to the regioisomers in order of LC elution. These compounds were then used to identify 17 lutein esters found in a commercial lutein supplement. The present method would help to improve quality control of lutein supplements and other lutein-based products.

ABBREVIATIONS USED

APCI+ve, atmospheric pressure chemical ionization positive ion mode; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; UV–vis, ultraviolet–visible.

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