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Separation of geometric isomers of native lutein diesters in marigold (*Tagetes erecta* L.) by high-performance liquid chromatography–mass spectrometry

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Abstract

Lutein is found in many foods; the richest and purest plant source is marigold flower (*Tagetes erecta* L.). In this plant, lutein is in the form of saturated fatty acid diesters. By using a binary mobile phase consisting of ethyl acetate and acetonitrile–methanol (9:1), improved separation was achieved on a C_{18}-bonded phase. The unique absorption of lutein *cis* isomers at 330 nm was used in combination with MS to identify the novel *cis*-lutein isomeric dimyristate, myristate-palmitate, dipalmitate, and palmitate-stearate diesters, as well as the rare combinations of both *trans*- and *cis*-lutein laurate-palmitate and *trans*- and *cis*-lutein myristate-stearate. The presence of the all-*trans*-lutein laurate-myristate, dimyristate, myristate-palmitate, palmitate-stearate, and distearate diesters, reported by others, was also confirmed.

Keywords: Geometric isomers; *Tagetes erecta*; Lutein; Fatty acids

1. Introduction

Lutein is a dihydroxy carotenoid or xanthophyll (Fig. 1) that has recently received the attention of many researchers for its anticancer activity and effect on degenerative diseases of the eyes [1,2]. It has in the past several years been the subject of many studies associating it with risk reduction for failing eyesight due to diseases such as age-related macular degeneration, the leading cause of irreversible blindness amongst senior populations. Lutein is also a potent antioxidant [3] and is found to enhance immune function as well [2].

Although many fruits and vegetables contain lutein, the best commercial source of pure lutein is marigold (*Tagetes erecta* L.). Marigold is a Compositeae family plant, and its flowers are the richest common source of xanthophylls, ranging from 0.6 to 2.5% on a dry basis; 88–92% of the total xanthophylls in marigold flowers are lutein and zeaxanthin with lutein as the predominant xanthophyll [4–6]. Although esters of lutein can be partially hydrolyzed in the body [7,8], they are usually hydrolyzed by saponification during the manufacture of lutein-containing extracts. The majority of lutein esters in marigold are diesters (Fig. 1) due to the two hydroxy groups, one at each ionone ring. The fatty acids of the diesters have been found to be saturated with a carbon length between C_{12}–C_{18}. Dimyristate and dipalmitate were reported as the major diesters of lutein (83–92% of total esters) in marigold flower [9,10]. Separation of lutein and its esters has been a difficult task because of the similarity in polarity of these compounds. However, using reversed-phase LC (C_{18} column), up to five diesters in marigold extract were separated, and eluted in the following order: dimyristate (C_{14}/C_{14}), myristate-palmitate (C_{14}/C_{16}), dipalmitate (C_{16}/C_{16}), palmitate-stearate (C_{16}/C_{18}), and distearate (C_{18}/C_{18}) [11,12]. Similar methods have also been developed for simultaneous detection of free lutein, three monoesters (C_{14}, C_{16} and C_{18}), and five of the above diesters [5,13]. C_{30} columns have been used for the separation of free lutein and its geometric isomers [6,14–18], and most recently in an LC–MS method for eight regioisomeric

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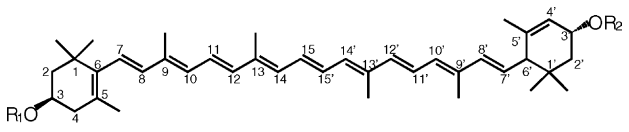

	R1	R2
Lutein (all-*trans*)	H	H
laurate-myristate (C12/C14)	Lauroyl [a]	myristoyl [a]
dimyristate (C14/C14)	myristoyl	myristoyl
myristate-palmitate (C14/C16)	myristoyl [a]	palmitoyl [a]
dipalmitate (C16/C16)	palmitoyl	palmitoyl
palmitate-stearate (C16/C18)	palmitoyl [a]	stearoyl [a]
distearate (C18/C18)	stearoyl	stearoyl

a: regioisomers were arbitrarily assigned.

Fig. 1. Lutein and its commonly found diesters in marigold flowers.

monoesters and six lutein diesters, with the additional diester being lutein laurate-myristate (C_{12}/C_{14}) [19]. Four fatty acids (C_{12}, C_{14}, C_{16} and C_{18}) were involved in the eight monoester regioisomers at the 3-O- and 3′-O positions, and they were identified by selective 3′-O methylation of the isomer (Fig. 1). It was not possible to separate regioisomers of mixed lutein diesters with the method [19].

Separation of geometric isomers of lutein diesters is highly difficult as they may be co-eluting, and virtually impossible to be identified using photodiode array detection (DAD) because the UV absorption patterns of these diesters are nearly the same. There is no clear indication whether or not these diesters are all-*trans* or *cis* isomers in marigold flowers, because no strong absorption near 330 nm, a characteristic UV maximum for *cis*-isomers of lutein, was found [15,17]. Nonetheless, the predominant geometric isomer of lutein is considered all-*trans* because it is chemically most stable and the predominating hydrolysis product of lutein esters [6,15–17,19].

Mass spectrometry (MS) has been used as a detector in the identification of lutein and its esters [19,20]. The matrix-assisted laser desorption ionization MS method of Wingerath et al. [20] did not distinguish geometric isomers, and the method by Breithaupt et al. [19] only identified regioisomers of the monoesters of lutein. UV detection can be useful for distinguishing the geometric isomers because the *cis* and *trans* isomers have different UV spectra, particularly near 330 nm [15]. MS detection, on the other hand, can usually tell if a peak contains more than one compound. Using selected ions, different components under the same peak can be identified as long as there is partial resolution between them. For example, lutein dipalmitate (C_{16}/C_{16}) has two major fragments, *m/z* 533 and 789 [19]. However, if the peak known as C_{16}/C_{16} also had other typical diester ions, e.g. C_{14} and C_{18}, it is highly likely that the peak also contains other diesters, in this case a lutein diester of myristic acid (C_{14}) and stearic acid (C_{18}).

In this paper, we report on identification of lutein diesters and their geometric isomers in native marigold flower extract. Lutein diesters are carefully examined against reported combinations of fatty acids. Possibilities of new combinations of saturated fatty acids in the diesters are discussed using data collected from selected ion chromatograms.

2. Experimental

2.1. Chemicals and solvents

Lutein was purchased from Sigma–Aldrich (Oakville, Canada). HPLC-grade solvents (hexane, ethyl acetate, methanol and acetonitrile) were purchased from Caledon Labs. (Georgetown, Canada).

2.2. Sample preparation

All procedures for the sample preparation were performed in dim light in order to minimize the photodegradation of lutein esters. Dried marigold flower powder was provided by the Healing Arc (Kitchener, Canada). A 10 g amount of the powder was extracted four times with 100 mL hexane (1:10, w/v). Each time the mixture was filtered through a Whatman No. 1 filter paper (Whatman, Maidstone, UK), and all the filtrates were combined and concentrated to dryness in vacuo at $\leq$40 °C in dark. The crude extract (oleoresin) was then reconstituted in ethyl acetate at a concentration of 10 mg/mL and filtered through a 0.45 μm Gelman GHP Acrodisc GF syringe filter (Pall Canada, Mississauga, Canada) before LC analysis.

2.3. LC conditions

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler and a DAD system was used for identification of lutein and its esters in the extract. Separation of lutein esters was carried out in a Waters Spherisorb ODS(2) column (150 mm × 4.6 mm i.d.; particle size, 5 μm) with a C_{18} guard column. The binary mobile phase consisted of ethyl acetate (solvent A) and acetonitrile–methanol (9:1, v/v; solvent B). All solvents were filtered through a 0.45 μm aforementioned syringe filter prior to analysis. The flow rate was kept constant at 1.0 mL/min for a total run time of 30 min. The system was run with a gradient program: 20% B to 60% B in 15 min, 60% B isocratically for 10 min, 60% B to 80% B in 2 min, and 80% B to 20% B in 3 min. The injection volume was 20 μL. The detector was set at 450 nm. Tentative identification of lutein esters was achieved by comparing their elution pattern with literature data, and by congruent UV–vis spectra with that of authentic lutein standard.

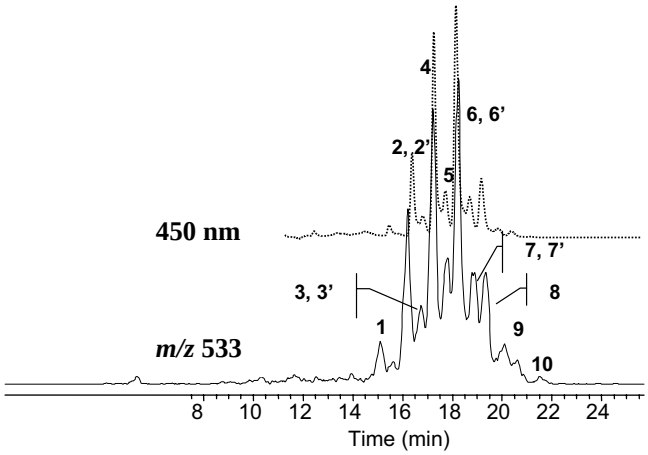

Fig. 2. LC–UV_{450} (top), and selected-ion LC–MS using *m/z* 533 (bottom). See Table 2 for identities of numbered peaks.

2.4. LC–MS for identification

LC–MS was performed using LC coupled to a photodiode array UV detector (Finnigan MAT Spectra System UV6000LP, San Jose, CA, USA) equipped with a Finnigan ion trap LCQ Deca atmospheric pressure chemical ionization mass spectrometry (APCI-MS) system operated in the positive ion mode. Conditions of the APCI source: vaporization temperature: 450 °C; sheath gas flow rate: 80 (arbitrary units); auxiliary gas flow rate: 0 (arbitrary units); discharge voltage of the needle was set at 4.5 kV resulting a current at 10 μA; capillary temperature: 200 °C. The instrument performance parameters were optimized for lutein prior to sample analysis. Total ion chromatography was obtained between *m/z* 50 and 2000, however, since the molecular ion or protonated molecule cannot be detected [19,20], we used selected ions $[M\text{-}FA_1 + H]^+$ and $[M\text{-}FA_2 + H]^+$ (protonated mono-ester ions), and $[M\text{-}FA_1\text{-}FA_2 + H]^+$ (*m/z* 533, protonated lutein backbone ion) for peak identification. FA stands for fatty acid. The same separation conditions were used as in the LC–UV experiment.

3. Results and discussion

3.1. LC separation of lutein diesters

The analysis time of the method described here is shorter than most of the reported methods. By using a C_{18}-bonded phase, good separations were achieved within 25 min (Fig. 2).

The oleoresin of dried marigold flower petals showed a diester pattern (Fig. 2) in major peaks similar to those in previous reports [11,19]. Peaks 1, 2, 4, 6, 8, and 10 (Fig. 2) were the six lutein diesters with C_{12}/C_{14}, C_{14}/C_{14}, C_{14}/C_{16}, C_{16}/C_{16}, C_{16}/C_{18} and C_{18}/C_{18} fatty acids, respectively that have been reported by others. The identification of these compounds was based on UV and MS data, and on literature reports [11,19]. In addition, some minor peaks (peaks 3, 5, 7, and 9 in Fig. 2) were also separated from the major diester peaks in the oleoresin. These peaks are new and not previously reported.

Peaks 1, 2, 4, 6, 8, and 10 showed an UV absorption at 270 nm, and three additional absorption maxima at 424, 448 and 476 nm (Table 1), typical of the previously reported *trans* isomers of free lutein [15,16]. However, the smaller new peaks 3, 5, 7, and 9, had an extra UV absorption at 332 nm (Fig. 3 and Table 1), which is known as the *cis* peak for the *cis* isomers of free lutein [15,16]. We also noticed that the shoulder at 270 nm for the *trans* isomers of these new peaks shifted to a slightly longer wavelength at 272 nm, and the three maxima at 424, 448 and 476 nm shifted to slightly shorter wavelengths at 418, 440, and 468 nm, respectively (Table 1). These shifts have only been reported with free lutein isomers [16]. The position of the *cis* double bond was not determined in this study, although it was suggested that it might be at 13-, 13′- or 15-position (Fig. 1), because only the central ones (13, 13′ and 15) have a dipole

Table 1
Peak identification of native lutein diesters in marigold flower oleoresin separated by HPLC and detected by DAD

Peak	t_R (min)	Isomer[a]	Absorption maxima (nm)[b] (% mAU of the maxinum)[c]				
1	15.82	All-*trans*	270 (23)		424 (76)	448 (100)	476 (88)
2	16.86	All-*trans*	270 (19)		424 (69)	448 (100)	476 (91)
3	17.34	*Cis*	272 (18)	332 (60)	418 (68)	440 (100)	468 (87)
4	17.86	All-*trans*	270 (19)		424 (69)	448 (100)	476 (91)
5	18.37	*Cis*	272 (16)	332 (46)	420 (71)	442 (100)	468 (84)
6	18.80	All-*trans*	270 (19)		424 (69)	448 (100)	476 (91)
7	19.47	*Cis*	272 (16)	332 (51)	418 (72)	440 (100)	468 (83)
8	19.99	All-*trans*	270 (19)		424 (68)	448 (100)	476 (91)
9	20.70	*Cis*	272 (16)	332 (51)	418 (73)	440 (100)	468 (83)
10	21.38	All-*trans*	270 (19)		424 (67)	448 (100)	476 (92)

[a] Geometric isomer confirmation determined based on the absorption maximum at near 332 nm (see text).
[b] Absorption maxima of the peaks presented in Fig. 2. Data were collected using the DAD in the mobile phase.
[c] Percentage of the milli-absorbance units of each shoulder to the maximum absorption. The latter is the absorbance at 448 nm for the all-*trans*, and 440 or 442 nm for the *cis* isomers.

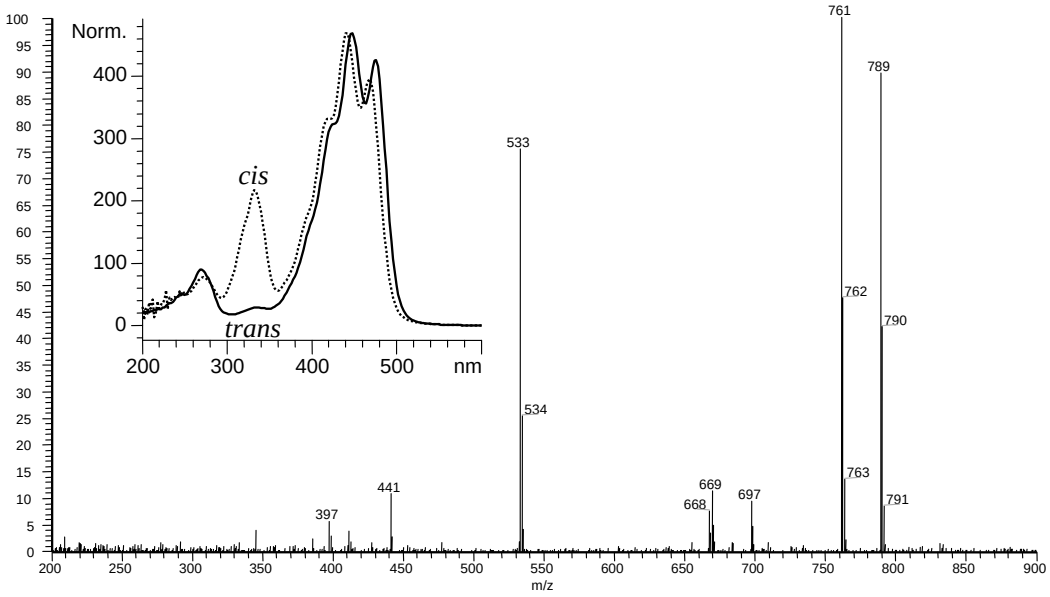

Fig. 3. UV spectra of all-*trans* and *cis* isomers of lutein myristate-palmitate (peaks 4 and 5, respectively) and mass spectrum of peak 4.

moment big enough to produce a considerable *cis* peak [15]. These minor peaks were well separated in this method, and all spectral data were collected using in-line DAD in the LC (Fig. 2, Table 1), making it possible for direct detection and identification of native geometric isomers of lutein diesters. UV spectral data was critical in the identification of the geometric conformation of *cis* isomers; however, MS data were also important because they confirmed that these *cis* isomers had the same molecular weight and similar fragmentation pattern as their respective *trans* isomers. Peaks 3, 5, 7, and 9 were therefore identified as *cis* isomers of lutein diesters with C_{14}/C_{14}, C_{14}/C_{16}, C_{16}/C_{16} and C_{16}/C_{18} (Table 2), geometric isomers of known all-*trans* diesters, peaks 2, 4, 6 and 8, respectively.

3.2. Fragmentation pattern of lutein diesters in LC–MS and structure identification

The positive ion APCI-MS data of lutein diesters generally show three major fragments, $[M\text{-}FA_1 + H]^+$, $[M\text{-}FA_2 + H]^+$, and $[M\text{-}FA_1\text{-}FA_2 + H]^+$ (Table 2). The first two were from the neutral loss of a single fatty acid from the protonated molecule or quasimolecular ion $[M + H]^+$ and the last one is the residual lutein backbone after loss of both fatty acid moieties. Diesters with identical fatty acids will only have two ions [19] (Table 2). The quasimolecular ion could not be detected and structural assignments must be made according to the fragmentation patterns. This is in agreement with the report of Breithaupt et al. [19], which

Table 2
APCI-MS data of native lutein diesters from marigold oleoresin separated by LC

Peak	Identity[a]	M_W	$[M + H - FA_1]^{+b}$ *m/z* (%)	$[M + H - FA_2]^{+b}$ *m/z* (%)	$[M + H - FA_1 - FA_2]^{+b}$ *m/z* (%)
1	All-*trans*-lutein laurate-myristate	960	733 (30)	761 (80)	533 (100)
2	All-*trans*-lutein dimyristate	988	761 (100)		533 (50)
2′	All-*trans*-lutein laurate-palmitate	988	733 (15)	789 (10)	533[c]
3	*Cis*-lutein dimyristate	988	761 (100)		533 (50)
3′	*Cis*-lutein laurate-palmitate	988	733 (10)	789 (10)	533[c]
4	All-*trans*-lutein myristate-palmitate	1016	761 (100)	789 (70)	533 (75)
5	*Cis*-lutein myristate-palmitate	1016	761 (100)	789 (30)	533 (35)
6	All-*trans*-lutein dipalmitate	1044	789 (100)		533 (50)
6′	All-*trans*-lutein myristate-stearate	1044	761 (10)	817 (10)	533[c]
7	*Cis*-lutein dipalmitate	1044	789 (100)		533 (45)
7′	*Cis*-lutein myristate-stearate	1044	761 (15)	817 (15)	533[c]
8	All-*trans*-lutein palmitate-stearate	1072	789 (70)	817 (100)	533 (80)
9	*Cis*-lutein palmitate-stearate	1072	789 (100)	817 (55)	533 (90)
10	All-*trans*-lutein distearate	1100	817 (100)		533 (30)

[a] Determination of geometric isomers was based on the UV spectra (see Table 1and text). Allocation of the *cis*- bond was not attempted.
[b] MS ions (signal intensity in percentage).
[c] Signal intensity was not determined due to overlaps with the same ion from the other co-eluting compound.

supported the lutein diester pattern of native marigold oleoresin as proposed by Rivas [5] and Gregory et al. [12]. Using the same technique, and aided by the good separation of the geometric isomers of lutein diesters, we were able to confirm that the *cis* isomers also followed the same LC–MS fragmentation pattern (Table 2).

Furthermore, by using selected ions to obtain simplified chromatograms, we found that it is much more complicated than what has heretofore been reported in terms of the lutein diester pattern in marigold flowers. The *cis*- and all-*trans*-lutein diesters discussed insofar have not gone beyond those with known fatty acid patterns, i.e. C_{12}/C_{14}, C_{14}/C_{14}, C_{14}/C_{16}, C_{16}/C_{16}, C_{16}/C_{18}, and C_{18}/C_{18}. However, we found that although not separated chromatographically using UV detector, certain peaks were not pure of known lutein diesters. From the UV chromatogram (Fig. 2), peak 2 appeared to be a single compound: all-*trans*-lutein dimyristate, and in the MS, this peak showed the expected ions (*m/z* 533 and 761, or $[M\text{-}2C_{14} + H]^+$ and $[M\text{-}C_{14} + H]^+$). However, this peak also showed *m/z* 733 (Fig. 4B) and *m/z* 789 (Fig. 4D), ions consistent with loss of palmitate ($[M\text{-}C_{16} + H]^+$) and laurate ($[M\text{-}C_{12} + H]^+$) moieties. A very close examination of the single ion chromatograms for *m/z* 761 (Fig. 4C), *m/z* 789 (Fig. 4D) and *m/z* 733 (Fig. 4B) revealed that the compound giving rise to the *m/z* 761 peak eluted slightly ahead of the compound giving rise to the two other ions. Thus, these nearly overlapped peaks are labelled 2 and 2′, respectively. The absence of an absorption band at 332 nm in the UV suggests an all-*trans* orientation in the polyene section of both molecules. Identification of peak 2′ as all-*trans*-lutein lauryl palmitate is consistent with the data. A similar examination of peak 3 revealed the presence of two nearly co-eluting compounds that are isomeric with those in peak 2; the presence of a UV absorption band at 332 nm is suggestive that 3 and 3′ are *cis*-lutein dimyristate and *cis*-lutein lauryl palmitate, respectively. Compounds 2′ and 3′ are a new type of isomeric lutein diesters that have not been reported in the literature as native in marigold.

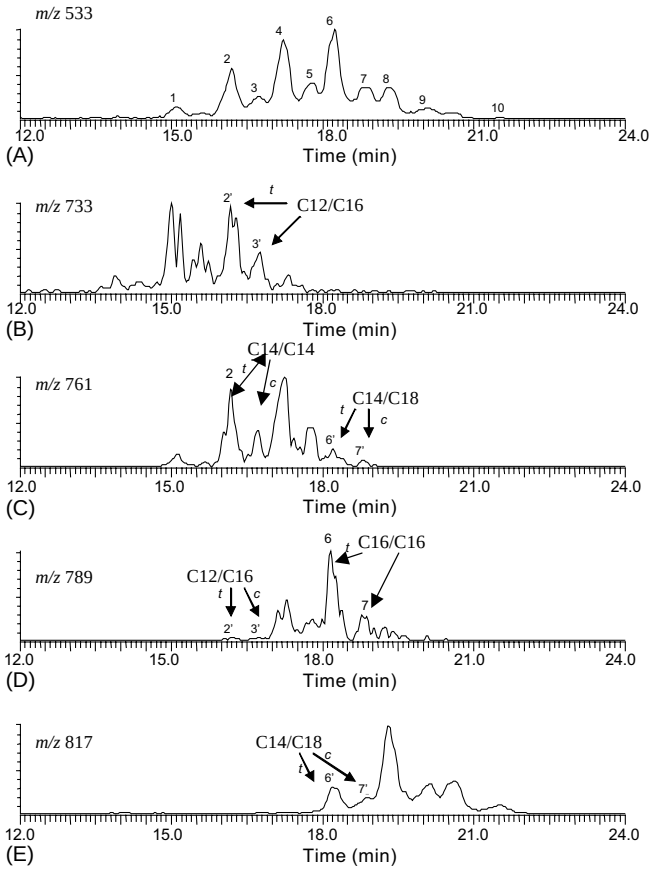

Fig. 4. Selected ion chromatograms of lutein diester isomers in marigold oleoresin. *t: trans; c: cis.* (A) Single-ion trace chromatogram at *m/z* 533. (B) Single-ion trace chromatogram at *m/z* 733. Peaks 2′ and 3′ are all-*trans*- and *cis*-lutein laurate-palmitate (C_{12}/C_{16}) having the same molecular mass as peaks 2 and 3, all-*trans*- and *cis*-lutein dimyristate (C_{14}/C_{14}), respectively (also see C and D). (C) Single-ion trace chromatogram at *m/z* 761. Peaks 6′ and 7′ are all-*trans*- and *cis*-lutein myristate-stearate (C_{14}/C_{18}) having the same molecular mass as peaks 6 and 7, all-*trans*- and *cis*-lutein dipalmitate (C_{16}/C_{16}), respectively (also see D). (D) Single-ion trace chromatogram at *m/z* 789 (also see B and C). (E) Single-ion trace chromatogram at *m/z* 817 (also see C and D).

Peaks 4 and 5 each appear to be single components and on the basis of the spectral data have been assigned as all-*trans*-lutein myristate-palmitate and the isomeric *cis*-lutein myristate-palmitate, respectively (Tables 1 and 2).

Following the same analysis as for peaks 2 and 2′, and 3 and 3′, we found that there was a peak 6′ eluting very slightly after peak 6, and peak 7′ slightly after 7 (Fig. 4C and D). These two compounds, peaks 6′ and 7′, were similarly assigned the identities of *trans*- and *cis*-lutein myristate-stearate and appear to be novel in this source as well (Tables 1 and 2).

4. Conclusion

The LC method reported here not only confirmed the lutein diester profile of marigold flower oleoresin reported by others, but through improved separation also indicated that geometric isomers of these known diesters exist in the oleoresin. This is the first report that native geometric isomers of lutein diesters have been separated and identified. So far, only those of free lutein, a saponification product of marigold oleoresin, have been separated and identified. This study also showed that some novel combinations of fatty acids may have resulted in the formation of diesters as well. The determination of geometric isomers and the fatty acid combinations were based on the LC–UV and LC–MS data as well as on literature reports. Although the compounds were not isolated and subjected to full structural identification, the UV and MS data provide evidence of the existence of *cis* isomers and novel lutein diesters such as lutein laurate-palmitate, and lutein myristate-stearate. The findings of this study will help further understand the chemistry and biosynthesis of native lutein diesters in marigold flower, and the role of these diesters in human health maintenance.

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