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Differentiation between Lutein Monoester Regioisomers and Detection of Lutein Diesters from Marigold Flowers (*Tagetes erecta* L.) and Several Fruits by Liquid Chromatography–Mass Spectrometry

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Liquid chromatography−atmospheric pressure chemical ionization mass spectrometry (LC-APCIMS) was employed for the identification of eight lutein monoesters, formed by incomplete enzymatic saponification of lutein diesters of marigold (*Tagetes erecta* L.) by *Candida rugosa* lipase. Additionally, the main lutein diesters naturally occurring in marigold oleoresin were chromatographically separated and identified. The LC-MS method allows for characterization of lutein diesters occurring as minor components in several fruits; this was demonstrated by analysis of extracts of cape gooseberry (*Physalis peruviana* L.), kiwano (*Cucumis metuliferus* E. Mey. ex Naud.), and pumpkin (*Cucurbita pepo* L.). The assignment of the regioisomers of lutein monoesters is based on the characteristic fragmentation pattern: the most intense daughter ion generally results from the loss of the substituent (fatty acid or hydroxyl group) bound to the ϵ-ionone ring, yielding an allylic cation. The limit of detection was estimated at 0.5 μg/mL with lutein dimyristate as reference compound. This method provides a useful tool to obtain further insight into the biochemical reactions leading to lutein ester formation in plants.

KEYWORDS: LC-MS; carotenoids; regioisomers; lutein monoester

INTRODUCTION

Carotenoids represent an important class of widely distributed natural pigments, responsible for the coloration of many fruits and vegetables. One of the main industrial sources is flower petals of marigold (*Tagetes erecta* L.), being intensely orange. In Central America several varieties of marigold are cultivated for the purpose of carotenoid extraction. Extracts of marigold petals have been used in traditional folk medicine for a long time (*1*) and are nowadays of growing interest because they are employed commercially as additives to poultry feed to improve chicken skin and egg yolk coloration (*2*). The use of lutein as a low-priced basic material for synthesis of rare carotenoids is still under investigation.

The coloring component of marigold is *all-trans*-lutein (3*R*,3′*R*,6′*R*-β,ϵ-carotene-3,3′-diol), an asymmetric dihydroxy-carotenoid (xanthophyll). Because lutein bears one hydroxyl group at each ionone ring, it can be esterified with fatty acids in plant cells, resulting in mono- and diacylated derivatives. Alkaline saponification is preferentially performed before carotenoid extract analysis by HPLC to remove triacylglycerides and other interfering compounds (*3*). Thus, no information concerning carotenoid esters in natural samples can be obtained. As a consequence, the results of different workers are confusing and often contradictory: Gregory et al. (*4*) and Rivas (*5*) showed that a freshly prepared marigold flower extract contains no free lutein but is rich in several lutein diesters with lutein dipalmitate and myristate palmitate being the major esters in dark orange flowers. Zonta et al. (*6*) isolated the ester fraction by column chromatography on aluminia; the extract was saponified, and the fatty acids were analyzed after methylation via gas chromatography. The results of this time-consuming procedure showed C14:0, C16:0, C18:0, C18:1, and C18:2 to be the main fatty acids esterifying lutein in marigold. Scalia and Francis (*7*) described a method for separating five lutein diesters by preparative reversed-phase HPLC. GC-MS analyses of the transesterified carotenoid esters proved only saturated fatty acids to be responsible for lutein diester formation in marigold (C12/C14; C14/C14; C14/C16; C16/C16; C16/C18), whereas unsaturated fatty acids were absent. More recently, the isomeric profile of lutein was studied by Hadden et al. and Delgado-Vargas et al. (*8*−*10*), who used C30 reversed-phase material for separation and found 9-, 9′-, 13-, and 13′-*cis*-lutein to be native minor components present in saponified marigold extracts; additionally, small amounts of *all-trans*-zeaxanthin were detected.

Lutein has β- and ϵ-ionone rings, so monoacylation as well as diacylation by two different fatty acids leads to regioisomers. The objective of this study was to analyze all lutein monoester

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regioisomers, which were obtained by incomplete enzymatic saponification of lutein diesters derived from marigold oleoresin. Additionally, we determined for the first time the main native lutein diesters present in marigold extracts by LC-MS without using time-consuming procedures for fatty acid analysis. To investigate whether the LC-MS method is applicable for characterization of lutein diesters occurring as minor components in several fruits, extracts of cape gooseberry (*Physalis peruviana* L.), kiwano (*Cucumis metuliferus* E. Mey. ex Naud.), and pumpkin (*Cucurbita pepo* L.) were analyzed.

MATERIALS AND METHODS

Chemicals. Light petroleum (boiling fraction 40−60 °C), methanol, ethyl acetate, and acetone were purchased from Merck (Darmstadt, Germany); *tert*-butyl methyl ether, *n*-hexane, pyridine (99.8%, over molecular sieve), myristoyl chloride (99%), palmitoyl chloride (99%), *Candida rugosa* lipase type VII (EC 3.1.1.3), and polyethylene glycol (PEG) 200, 400, 600, and 1000 were purchased from Sigma-Aldrich (Taufkirchen, Germany), and silica gel (0.063−0.2 mm) was purchased from J. T. Baker (Griesheim, Germany). All solvents were distilled before use. High-purity water was prepared with a Milli-Q 185 Plus water purification system (Millipore, Eschborn, Germany). Soy lecithin (Epikuron 200; phosphatidylcholine content > 92%) was a gift from Lucas Meyer GmbH (Hamburg, Germany). Lutein was provided by Hoffmann-La Roche (Basel, Switzerland). Marigold oleoresin (oil from *T. erecta*) and marigold flowers (supplied as frozen goods) were kindly supplied by Euram Food GmbH (Stuttgart, Germany). The oil was manufactured in the country of origin from minced petals by solvent extraction with *n*-hexane.

Fruits. Fully ripe fruits of cape gooseberry (*P. peruviana*), kiwano (*C. metuliferus*), and pumpkin (*C. pepo*) were obtained from retail shops. Cape gooseberries, native to Peru and Chile, are cherry-like fruits with a golden-orange skin, enclosed in a straw-colored inedible husk. Kiwano (horned melon), originating from southern Africa, is an ellipsoid fruit that is bright yellow-orange in color when mature and shaped like a short cucumber with many thorns on its surface. Both kiwano and cape gooseberry are currently promoted as speciality fruits for export to the European and U.S. markets. The genus *Cucurbita* includes numerous popular species. We used yellow round giant pumpkins with a fruit weight of ∼1 kg.

Preparation of Samples. *1. Cleanup Procedure.* Because high contents of triacylglycerides complicate LC-MS measurements, purification by open column chromatography was applied. A marigold oleoresin solution (3 mL; 140 mg/100 mL of light petroleum) was subjected to column chromatography (200 × 10 mm) on silica gel (3 g) suspended in light petroleum. For removal of triacylglycerides, the column was washed twice with light petroleum (20 mL each). The more polar carotenoid esters were eluted with mixtures of acetone in light petroleum [10, 20, and 40% (v/v); 20 mL each]. Colored fractions were combined, the solvents were evaporated under reduced pressure (50 mbar, 30 °C), and the residue was redissolved in 2 mL of *tert*-butyl methyl ether/methanol (1:1 v/v). After membrane filtration (0.45 μm), the final solution was subjected to LC-MS analysis. To avoid *cis*−*trans* isomerization, all procedures were performed under dim light and all samples were analyzed immediately after cleanup.

2. Enzymatic Cleavage of Lutein Diesters. A marigold oleoresin solution (3 mL; 140 mg/100 mL of light petroleum) was transferred to a 50 mL sealed tube and evaporated to dryness under a gentle stream of nitrogen. The residue was immediately dissolved in 0.1 M phosphate buffer (pH 7.4; 10 mL). For emulsification, 10 μL of a methanolic solution of soy lecithin (25 mg/mL), and 250 μL of a solution containing sodium chloride (175 mg/mL) and calcium chloride (8.3 mg/mL) were added. After sonication (1 min), 1 mL of an aqueous suspension containing 5 mg of *C. rugosa* lipase was added and the mixture incubated on a horizontal shaker (37 °C; 1 h). For extraction of lutein derivatives, the solution was shaken three times with a mixture of methanol/ethyl acetate/light petroleum (1:1:1 v/v/v; 20 mL each). The upper layers were collected, dried with anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The residue was dissolved in 3 mL of light petroleum and purified (see Cleanup Procedure; final volume = 2 mL).

3. Extraction of Marigold Flowers. Marigold flowers (25 g) were minced with an Ultra Turrax T 25 (Janke and Kunkel, Staufen, Germany) for 1 min and extracted twice with a mixture of methanol/ethyl acetate/light petroleum (1:1:1 v/v/v; 150 mL each) on a horizontal shaker (30 min, 150 rpm). The supernatants were collected and filtered, dried with anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The residue was dissolved in 25 mL of light petroleum. An aliquot (5 mL) was used for purification (see Cleanup Procedure; final volume = 2 mL).

4. Extraction of Fruits. Edible portions were cut into small pieces and homogenized with an Ultra Turrax T 25 for 1 min. Samples of 50 g (cape gooseberry and pumpkin) or 10 g (kiwano), respectively, were extracted three times with a mixture of methanol/ethyl acetate/light petroleum (1:1:1 v/v/v; 50 mL each). The supernatants were collected and filtered, dried with anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The residues were dissolved in light petroleum (5 mL each), quantitatively transferred to chromatography columns, and purified (see Cleanup Procedure; final volume = 2 mL each).

Structural Assignment of Lutein Myristoyl Monoester Regioisomers by O-Methylation. *1. Synthesis.* Lutein (10 mg) was dissolved in dry pyridine (3 mL), myristoyl chloride (30 mg) added dropwise, the solution stirred at room temperature for 2 h, and water (20 mL) added. The reaction products were extracted three times with a mixture of methanol/ethyl acetate/light petroleum (1:1:1 v/v/v; 20 mL each). The organic layer was washed four times with water (50 mL each), and the upper phase was dried with anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in *tert*-butyl methyl ether/methanol (1:1 v/v; 2 mL) and subjected to LC-MS analysis. Besides two lutein monoesters [**A**, t_R = 26.1 min; 11%; MS *m/z* 551 (100) and 533 (5); **B**, t_R = 27.2 min; 13%; MS *m/z* 761 (100), 551 (34), and 533 (43); λ_{max} (identical for **A** and **B**) 422, 444, and 472 nm, monitored in the HPLC solvents], lutein dimyristate (11%) and unreacted lutein (44%) were present; 21% corresponded to several *cis*-isomers. For structural identification of lutein monoesters, **A** and **B** were isolated by semipreparative HPLC as described previously (*11*). The concentrations were calculated on the basis of an ϵ value of 1.451×10^5 [L/(mol·cm)] in ethanol at 445 nm (*12*).

2. O-Methylation. Methanolic solutions containing **A** or **B** (10 nmol/mL; 10 mL each), respectively, were treated with methanolic hydrochloric acid (1%; 150 μL) for 10 min at room temperature, and sodium hydrogen carbonate solution (10% w/v, 10 mL each) was added. The reaction products were extracted three times with a mixture of methanol/ethyl acetate/light petroleum (1:1:1 v/v/v; 20 mL each), and the organic layers were dried with anhydrous sodium sulfate and evaporated to dryness. The residues were dissolved in *tert*-butyl methyl ether/methanol (1:1 v/v; 2 mL) and subjected to LC-MS analysis. The chromatogram for lutein monoester fraction **A** showed only unreacted **A**, whereas the chromatogram of lutein monoester fraction **B** showed one additional peak [t_R = 32.6 min; 15%; MS *m/z* 793 (13), 761 (100), 565 (14), and 533 (28); λ_{max} 422, 444, and 472 nm, monitored in the HPLC solvents].

Liquid Chromatography—Mass Spectrometry (LC-MS). *1. Apparatus and Conditions.* LC-MS was performed on an HP1100 modular HPLC system (autosampler, gradient pump, thermoregulator, and DAD) from Hewlett-Packard (Waldbronn, Germany), coupled to a Micromass (Manchester, U.K.) VG platform II quadrupole mass spectrometer. The APCI source was heated at 150 °C, and the APCI probe was kept at 400 °C. The corona voltage was optimized to 3.7 kV, the HV lens to 0.5 kV, and the cone voltage to 30 V. Nitrogen was used as sheath and drying gas at 75 and 300 L/h, respectively. The mass spectrometer was calibrated in the positive ion mode (scan range *m/z* 80−1200) using a mixture of four PEG fractions [PEG 200 (25 mg/L), 400 (50 mg/L), 600 (75 mg/L), and 1000 (250 mg/L) in acetonitrile/0.002 M ammonium acetate, 1:1 v/v]. For calibration, the resulting $[M + H]^+$ signals as well as the $[M + NH_4]^+$ ions were evaluated. Mass spectra of all lutein esters were acquired with an *m/z* 200−1200 scan range, and the UV absorbance was recorded at 450 nm by using a diode array detector (DAD). Data were acquired and processed with MassLynx 3.2 software. For HPLC separation, a YMC analytical column (YMC Europe,

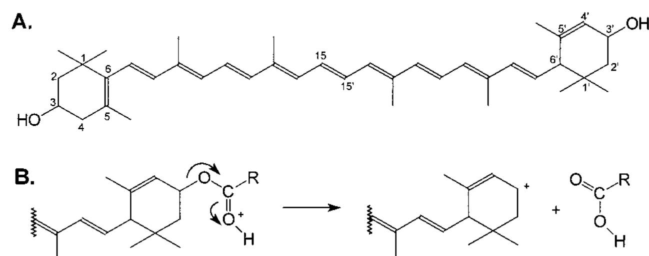

Figure 1. (A) Structure and numbering of lutein (3*R*,3′*R*,6′*R*-β,ϵ-carotene-3,3′-diol). (B) Major fragmentation pathway of a lutein monoester, acylated at the ϵ-ionone ring.

Schermbeck, Germany) with 5 μm C30 reversed-phase material (250 × 4.6 mm i.d.) including a precolumn (Nucleosil 5 μm C18, 10 × 4.6 mm i.d., Bischoff, Leonberg, Germany) was used and kept at 35 °C. The mobile phase consisted of mixtures of methanol/*tert*-butyl methyl ether/water [81:15:4 v/v/v (A) and 6:90:4 v/v/v (B)], starting with 10 min isocratic 100% A, followed by a gradient to obtain 50% B at 40 min, 100% B at 50 min, 100% A at 55 min, and isocratic 100% A from 55 to 60 min at a flow rate of 1 mL/min. The injection volume was 20 μL.

2. Detection Limit. The detection limit for lutein esters was determined using a solution of lutein dimyristate (2.5 μg/mL) in *tert*-butyl methyl ether/methanol (1:1 v/v) based on a signal-to-noise ratio of 3:1. Lutein dimyristate was synthesized according to a procedure described by Khachik et al. (*13*). The concentration was calculated on the basis of an ϵ value of 145.1 × 10^3 [L/(mol·cm)] in ethanol at 445 nm (*12*) and a molecular mass of 989.6 g/mol.

RESULTS AND DISCUSSION

Fragmentation Pattern of Lutein Monoesters in LC-MS Analysis. Lutein bears two different ionone rings, one with the β-configuration and the other with the ϵ-configuration (Figure 1A). The double bond of the ϵ-ionone ring is isolated. Monoacylation—as well as diacylation with two different fatty acids—leads to the formation of regioisomers. As a consequence, in the case of monoesters protonation with subsequent elimination of the fatty acid or water in the mass spectrometer reveals different positive fragment ions. Because the cation formed by cleavage at the ϵ-ionone side is allylic, it is more stable than the secondary cation generated from the respective cleavage at the β-ionone side (see the fragmentation scheme in Figure 1B). Thus, the substitution pattern of lutein monoesters can be determined by comparison of the respective daughter ion signal intensities. For confirmation of this theory it is necessary to subject lutein monoesters with established structure to LC-MS analysis. To identify which regioisomer was generated we used the fact that the 3′-hydroxy function of lutein can easily be O-methylated by adding methanolic hydrochloric acid to a methanolic solution of lutein; the respective 3-hydroxyl group does not react (*14*, *15*). The allylic hydroxyl group of lutein monoesters that is *not* esterified therefore is supposed to undergo O-methylation when methanolic hydrochloric acid is added; the one that is esterified does not react.

An LC-MS analysis of a sample containing synthesized lutein myristoyl esters proved two lutein monoesters, **A** (11%) and **B** (13%), as well as lutein dimyristate, to be present. **A** and **B** were isolated by semipreparative HPLC and O-methylated with methanolic hydrochloric acid in methanol. LC-MS analysis showed that only in the case of **B** was a reaction product formed, giving the molecular mass and fragmentation pattern of *O*-methoxy-*O*-myristoyl-lutein (*m*/*z* 793 [M + H]$^+$). This result proved unambiguously that **B** consisted of 3-*O*-myristoyl-lutein, whereas **A** is 3′-*O*-myristoyl-lutein.

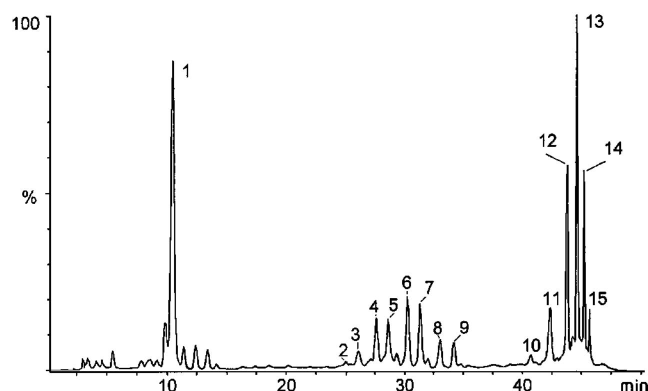

Figure 2. Chromatogram (DAD, 450 nm) of a sample obtained by incomplete enzymatic saponification of marigold lutein diesters using *C. rugosa* lipase. Peak 1 represents free *all-trans*-lutein, peaks 2–9 correspond to lutein monoesters, and peaks 10–15 are lutein diesters. For precise peak assignment see Tables 1 and 2.

Analysis of Lutein Mono- and Diesters. LC-MS analyses were performed using an atmospheric pressure chemical ionization interface, operating in the positive-ion mode (APCI$^+$). With C30 reversed-phase material carotenoid esters from peppers were separated properly (*16*); this also holds for lutein esters. Figure 2 shows the chromatogram of a sample obtained by incomplete enzymatic saponification of marigold lutein diesters (DAD, 450 nm). Both lutein diesters, the native compounds of marigold oleoresin (retention time window = 40−46 min), and lutein monoesters (retention time = 24−34 min), representing minor constituents of marigold oleoresin, are present in comparable concentrations. Free *all-trans*-lutein (retention time = 10.5 min) occurs in native marigold extracts only in trace amounts. The peak detected in this sample is due to enzymatic lutein diester cleavage. Several low-concentration compounds near the *all-trans*-lutein peak may be ascribed to lutein *cis*-isomers, as reported by Hadden et al. (*8*).

The LC-MS data of eight lutein monoesters, obtained by enzymatic cleavage of native marigold lutein diesters, are given in Table 1. The mass spectra are generally characterized by three signals. Two of them are generated by the neutral loss of the fatty acid or water from the respective quasimolecular ions, whereas the third signal, which is found in each mass spectrum, represents the lutein backbone ([M + H − H_2O − FA]$^+$; *m*/*z* 533). Such a pattern was also observed by Wingerath et al. (*17*), who used MALDI-MS for analysis of several carotenol fatty acid esters; however, they disregarded the problem of regioisomers. Due to low signal intensity, it was difficult to distinguish between 3′-*O*-lauroyl- and 3-*O*-lauroyl-lutein (peaks 2 and 3 in Figure 2). Because monoesters acylated at the 3′-hydroxyl group show shorter retention times than their corresponding 3-*O*-regioisomers, peak 2 is tentatively assigned to 3′-*O*-lauryol-lutein and peak 3 to 3-*O*-lauryol-lutein.

Myristoylpalmitoyl-lutein was synthesized following the procedure outlined for the synthesis of lutein dimyristate to test whether regioisomers of mixed lutein diesters can be separated (data not given). An LC-MS analysis of the resulting mixture showed only three signals for the four possible lutein diester isomers. The products were identified on the basis of their MS data (see below) as dimyristoyl-lutein, myristoylpalmitoyl-lutein, and dipalmitoyl-lutein. Obviously it is not possible to separate regioisomers of mixed lutein diesters with this chromatographic method. In the case of mixed lutein diesters the mass spectra show three specific signals (Table 2): Two of them result from the neutral loss of the corresponding fatty acid from the

Table 1. LC-MS Data of Lutein Monoesters (FAϵ/β Indicates the Position of Fatty Acids at the ϵ- or β-Ionone Ring)[a]

	$[M+H-FA\epsilon]^+$	$[M+H-H_2O\epsilon/\beta]^+$	$[M+H-FA\beta]^+$	$[M+H-H_2O-FA]^+$
3′-*O*-lauroyl-lutein (**2**)	*m*/*z* 551[b] C12:0	*m*/*z* 733[b]		*m*/*z* 533[b]
3-*O*-lauroyl-lutein (**3**)		*m*/*z* 733 (100%)	*m*/*z* 551[b] C12:0	*m*/*z* 533 (12%)
3′-*O*-myristoyl-lutein (**4**)	*m*/*z* 551 (100%) C14:0	*m*/*z* 761[b]		*m*/*z* 533 (5%)
3-*O*-myristoyl-lutein (**5**)		*m*/*z* 761 (100%)	*m*/*z* 551 (44%) C14:0	*m*/*z* 533 (48%)
3′-*O*-palmitoyl-lutein (**6**)	*m*/*z* 551 (100%) C16:0	*m*/*z* 789[b]		*m*/*z* 533 (6%)
3-*O*-palmitoyl-lutein (**7**)		*m*/*z* 789 (100%)	*m*/*z* 551 (26%) C16:0	*m*/*z* 533 (56%)
3′-*O*-stearoyl-lutein (**8**)	*m*/*z* 551 (100%) C18:0	*m*/*z* 817[b]		*m*/*z* 533 (8%)
3-*O*-stearoyl-lutein (**9**)		*m*/*z* 817 (100%)	*m*/*z* 551 (13%) C18:0	*m*/*z* 533 (81%)

[a] In the first line, the fragment ion and the signal intensity (in parentheses) are given; the second line specifies the fatty acid, which is lost. The numbers of the lutein monoesters **2**–**9** correspond to the peak numbering in Figure 2. [b] The fragment ion is detectable, but due to low intensity no percentage is given.

Table 2. LC-MS Data of Native Lutein Diesters from Marigold Oleoresin (FA = Fatty Acid)[a]

	$[M+H-FA_1]^+$	$[M+H-FA_2]^+$	$[M+H-FA_1-FA_2]^+$
lauroylmyristoyl-lutein (**10**)	*m*/*z* 733 (100%) C14:0	*m*/*z* 761 (77%) C12:0	*m*/*z* 533 (83%)
dimyristoyl-lutein (**11**)	*m*/*z* 761 (100%) C14:0		*m*/*z* 533 (61%)
myristoylpalmitoyl-lutein (**12**)	*m*/*z* 761 (100%) C16:0	*m*/*z* 789 (71%) C14:0	*m*/*z* 533 (86%)
dipalmitoyl-lutein (**13**)	*m*/*z* 789 (100%) C16:0		*m*/*z* 533 (53%)
palmitoylstearoyl-lutein (**14**)	*m*/*z* 789 (100%) C18:0	*m*/*z* 817 (77%) C16:0	*m*/*z* 533 (53%)
distearoyl-lutein (**15**)	*m*/*z* 817 (100%) C18:0		*m*/*z* 533 (43%)

[a] In the first line, the fragment ion and the signal intensity (in parentheses) are given; the second line specifies the fatty acid, which is lost. The numbers of the lutein diesters **10**–**15** correspond to the peak numbering in Figure 2.

quasimolecular ion, whereas the third signal represents the lutein backbone ($[M + H - FA_1 - FA_2]^+$; *m*/*z* 533). In contrast, MS spectra of lutein acylated with one type of fatty acid display only two signals: one represents the loss of a single fatty acid, the other corresponds to the lutein backbone. The quasimolecular ion $[M + H]^+$ generally could not be detected. Peak assignment of the main lutein diesters present in native marigold oleoresin (peaks 10−15 in Figure 2) are given in Table 2. To ensure that the lutein diester pattern is not affected by the manufacturing process, which is not exactly known, we analyzed a self-made extract of marigold flowers. The chromatogram showed the same lutein diesters as extracts from commercial marigold oleoresin, so we concluded that the carotenoid ester pattern is not noticably influenced by the manufacturing method. Thus, the lutein diester pattern of native marigold as proposed by Gregory et al. (*4*) and Rivas (*5*) is supported by this LC-APCI procedure.

Detection Limit of Lutein Diesters (LC-MS). For estimating the detection limit of lutein diesters, lutein dimyristate as model compound was employed. On the basis of the intensity of the fragment ion at *m*/*z* 761 (loss of myristic acid from the quasimolecular ion), a signal-to-noise ratio of 3:1, and an injection volume of 20 μL, we determined the detection limit to be ∼0.5 μg/mL. By using single ion monitoring (SIM) of characteristic daughter ions of lutein esters this value can be lowered by a factor of 10.

Lutein Diesters in Some Other Fruits. Although not common in every food plant, small amounts of lutein diesters occur in the carotenoid fraction of several fruits. Thus, we investigated whether this LC-MS method is helpful for the detection of trace amounts of lutein diester regioisomers of cape gooseberry (*P. peruviana*), kiwano (*C. metuliferus*), and pumpkin (*C. pepo*). In each fruit extract we were able to identify several lutein diesters (Table 3). In the case of pumpkin and cape gooseberry, the main carotenoid is β,β-carotene. Lutein diesters form about 2 and 5%, respectively, of the total carotenoid content, from a comparison of the peak areas. Kiwanoshows a complex carotenoid pattern including different free and mono- and diesterified carotenoids, whereas lutein diesters form ∼10% of the total carotenoid content.

Table 3. Lutein Diesters, Found in Cape Gooseberry (*P. peruviana*), Kiwano (*C. metuliferus*), and Pumpkin (*C. pepo*)

fruit	lutein diester
cape gooseberry	dimyristoyl-lutein myristoylpalmitoyl-lutein dipalmitoyl-lutein
kiwano	dilauroyl-lutein lauroylmyristoyl-lutein dimyristoyl-lutein myristoylpalmitoyl-lutein
pumpkin	dimyristoyl-lutein myristoylpalmitoyl-lutein dipalmitoyl-lutein

In contrast to previously published results (*6*), we detected in marigold extracts only lutein acylated with saturated fatty acids. Thise finding may be due to variations in the genetic

background, the age of plants at time of harvest, and the provenance of the material used in the respective studies. The analyses of a self-made marigold flower extract proved that the manufacturing process did not affect the lutein diester variations. Zonta et al. (*6*) analyzed the fatty acid pattern of an extract of marigold petals by means of gas chromatography and found palmitic acid to be the main component, followed by linoleic, oleic, myristic, and stearic acid. This distribution is not reflected by the lutein diester pattern of native marigold oleoresin, where lutein is diacylated mainly with palmitic, myristic, and stearic acid (see Table 2 and Figure 2). These findings are in agreement with our results, obtained by analyses of red pepper (*Capsicum annuum* L.) carotenoid esters (*15*), which established significant differences between the fatty acid pattern of triacylglycerides on the one hand (the main fatty acid is linoleic acid) and the carotenoid ester composition on the other (capsanthin is acylated mainly with lauric, myristic, and palmitic acid). Furthermore, in mixed carotenoid esters, derived from either red pepper or marigold, only fatty acids that differ in two methylene groups are present. These findings are in accordance with the fatty acid pattern of lutein diesters found as minor components in cape gooseberry (*P. peruviana*), kiwano (*C. metuliferus*), and pumpkin (*C. pepo*). Taken together, these results indicate a general enzymatic reaction step, responsible for selective acylation in the biochemical pathway of cellular carotenoid ester formation. The enzymes responsible for acylation as well as the consequences for the plant cell are as yet unknown or not fully understood. Further studies have to be carried out to gain a better insight into the biochemical reactions leading to carotenoid ester formation in plants. For this purpose this LC-APCIMS method is an essential technique to resolve analytical questions.

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