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Article

Carotenoid Composition of *Calendula officinalis* Flowers with Identification of the Configuration of 5,8-Epoxy-carotenoids

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ABSTRACT: The carotenoid composition of petals, florets, and full inflorescences of *Calendula officinalis* grown in sun and shade was investigated by the HPLC-DAD-MS method. The total carotenoid content of flowers grown in the shade was higher than those grown in the sun (1.154 and 0.872 mg/g in petals), while no differences were found in the proportion of individual carotenoids. In all samples, 29 components were identified, from which the main carotenoids, besides lutein, were 5,8-epoxy-carotenoids with 8*S* and 8′*R* configurations. The main 5,8-epoxy-carotenoid was chrysanthemaxanthin with the 8*S* configuration and not flavoxanthin with the 8*R* configuration, as published earlier. In addition, (all-*E*,8′*R*)- and (9*Z*,8′*R*)-luteoxanthin were detected in larger amounts. The 5,8-epoxy-carotenoids (flavoxanthin, chrysanthemaxanthin, and luteoxanthin epimers) were also prepared via an acid-catalyzed reaction of the parent carotenoid 5,6-epoxides. The structures of the epimers were elucidated by NMR measurements.

KEYWORDS: *Calendula officinalis, HPLC-DAD-MS analysis, carotenoids, NMR, furanoid oxide rearrangement*

INTRODUCTION

Carotenoids are synthesized in plants and microorganisms, but the animal and human body can only provide them from external sources.[1,2] By 2013, more than 750 different carotenoid molecules had been isolated, of which usually six have been shown to be adsorbed and metabolized in the human body: α- and β-carotene, lycopene, β-cryptoxanthin, zeaxanthin, and lutein.[3,4] Carotenoids play a crucial role in the life of a plant in photosynthesis,[5] but the exact mechanisms controlling carotenoid accumulation are not fully understood. Currently, the use of carotenoids in the food and feed industry is becoming increasingly significant due to their natural coloring, antioxidant, and favorable physiological effects.[6]

Carotenoids have a range of functions in human health. They primarily exert antioxidant effects, but individual carotenoids may also act through other mechanisms; for example, β-carotene has a pro-vitamin A function, while lutein/zeaxanthin constitutes macular pigment in the eye. There is evidence that carotenoids, in addition to beneficial effects on eye health, also produce improvements in cognitive function and cardiovascular health, and may help to prevent some types of cancer.[7] This brings up the important aspect of the bioavailability of dietary carotenoids, which depends on other dietary factors, especially the contribution of dietary fats.[8] It seems that the epoxyxanthophylls, such as violaxanthin and neoxanthin, which are abundant in plants, particularly in green tissues, cannot be incorporated into and used by the human body. Despite the evidence of the health benefits of carotenoids, large population-based supplementation studies have produced mixed results for some of the carotenoids. To establish and confirm the health benefits of the different carotenoids, more research, including clinical studies, is needed.

Garden marigold (*Calendula officinalis*) belongs to the order *Asterales*, the family *Asteraceae*, and the subfamily *Asteroidae*. The head inflorescence (capitulum) has a diameter of 3−7 cm, comprising both ray (ligulate) and disc (tubular) florets, whose color varies from pale yellow to dark orange. It is an annual, herbaceous plant that originated from the Mediterranean and can be grown almost all over the temperate zone. Its cultivation is typical in home gardens but it is also known in large-scale cultivation technology.[9,10] The marigold drug and its various extracts, as well as the preparations made from them, are an integral part of both folk medicine and modern phytotherapy.[11] The dried flower (*Calendulae flos*) is an official drug recognized by numerous monographs and accepted in the current European Pharmacopoeia, and the more valuable flower drug picked from the disk (*Calendula flos sine calycibus*) is also available in trade.[9]

The most important hydrophilic active ingredients of marigold are flavonoids (isorhamnetin, quercetin and quercetin glucosides, narcissin, calendoflavoids).[12,13] Lipophilic biomolecules of particular value to the cosmetic industry are the anti-inflammatory, antioxidant, and skin-regenerating carotenoids (β-carotene (**1**), (all-*E*)-lutein (**2**), (9*Z*)-lutein, luteoxanthin (**3**), flavoxanthin (**4**), violaxanthin (**5**), rubixanthin (**6**), γ-carotene (**7**), and lycopene (**8**), Figure S1).[14−16] In addition, the determination of *Calendula* genus metabolic products has a

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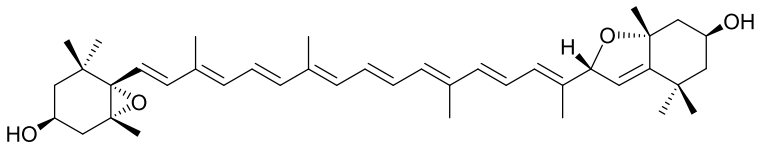
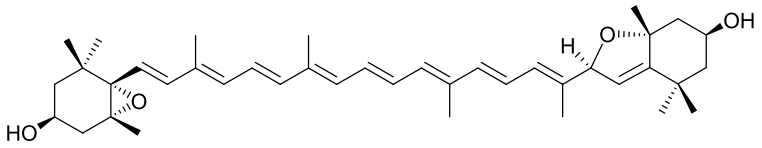
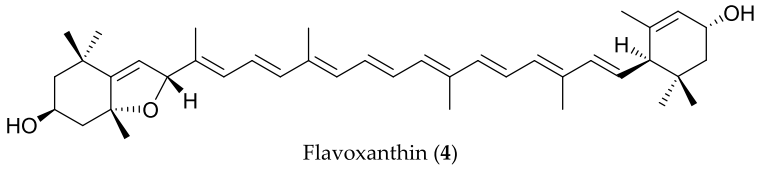


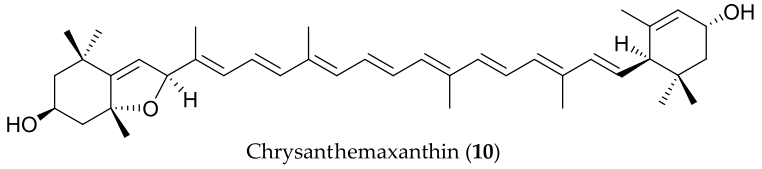
(8′*R*)-Luteoxanthin ((8′*R*)-**3**)
(all-*E*,3*S*,5*R*,6*S*,3′*S*,5′*R*,8′*R*)-5,6:5′,8′-Diepoxy-5,6,5′,8′-tetrahydro-β,β-carotene-3,3′-diol)



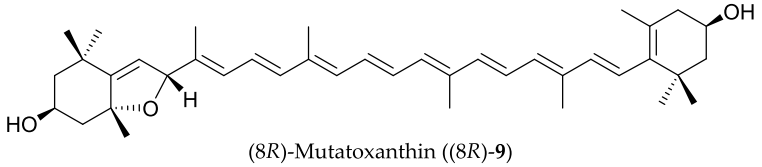
(8′*S*)-Luteoxanthin ((8′*S*)-**3**)
(all-*E*,3*S*,5*R*,6*S*,3′*S*,5′*R*,8′*S*)-5,6:5′,8′-Diepoxy-5,6,5′,8′-tetrahydro-β,β-carotene-3,3′-diol)



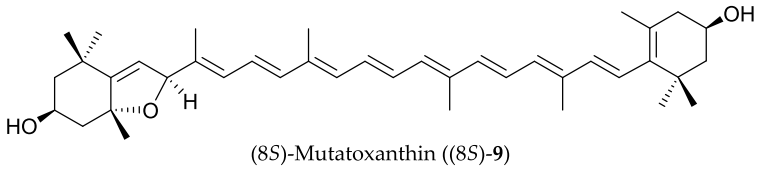
Flavoxanthin (**4**)
(all-*E*,3*S*,5*R*,8*R*,3′*R*,6′*R*)-5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3′-diol



Chrysanthemaxanthin (**10**)
(all-*E*,3*S*,5*R*,8*S*,3′*R*,6′*R*)-5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3′-diol



(8*R*)-Mutatoxanthin ((8*R*)-**9**)
(all-*E*,3*S*,5*R*,8*R*,3′*R*)-5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3′-diol



(8*S*)-Mutatoxanthin ((8*S*)-**9**)
(all-*E*,3*S*,5*R*,8*S*,3′*R*)-5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3′-diol

Figure 1. Structures of 5,8-epoxy-carotenoids.

tradition of more than a century, during which 656 characteristic molecules have been identified for taxa.[17]

According to the literature, the carotenoid content of calendula drug is influenced by various factors, such as variety, growing location, harvest time, soil nutrient composition, or temperature.[6] Based on the results of Andreeva,[18] dark orange calendulas can have up to 1.5% carotenoid content. As reported by Raal et al.,[19] the total carotenoid content of different calendula genotypes grown in Estonia was between 0.2 and 3.5%. According to the conclusions of Pintea et al.[14] the carotenoid content of fresh flowers of orange-colored varieties is higher than that of yellow-colored varieties, which can be explained by a change in the ratio of hydrocarbon and oxygenated compounds.

The main carotenoids of calendula petals are lutein (**2**) and its derivatives.[20] Kishimoto et al.[21] identified 19 carotenoids in extracts of petals of orange- and yellow-flowered calendulas, ten of which were peculiar to orange-flowered cultivars. Besides α-carotene, all of these characteristic components of orange varieties contained an Ψ-end group, and six of them had (5*Z*) or (5′*Z*) configuration. The (5*Z*)-carotenoids were proposed to be the result of an unusual enzymatic isomerization.

More than 20 years ago, we investigated the carotenoid composition of petals, pollens, and industrial products of calendula (herbal teas, tinctures, creams).[22] At that time, we carried out the separation of carotenoids on a C_{18} stationary phase, which could not separate well the apolar carotene hydrocarbons. Among the 5,8-epoxy-carotenoids, we identified

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flavoxanthin (**4**), luteoxanthin (**3**), and mutatoxanthin (**9**) (Figure 1).

Zubay et al.[23] evaluated the shade effects on seven different species (including *Calendula*) for plant height and width, fresh yield, drug mass, essential oil content, and content of other biologically active compounds. In that work, only the carotenoid content of the dried petals was used in industry.

Here, we report the results of a detailed reinvestigation of petals and florets of *C. officinalis* grown in sun and shade. In addition, we describe the preparation and characterization of flavoxanthin (**4**), chrysanthemaxanthin (**10**), and luteoxanthin (**3**) epimers.

MATERIALS AND METHODS

Plant Materials. Plants sampled for this study were grown in a 3 year in vivo agroforestry model experimental set up in the Experimental and Research Farm of the Hungarian University of Agriculture and Life Sciences in Budapest, Hungary (47°24′09.5″N 19°08′60.0″E). In this experiment, artificial (by commercially available agro green shade nets) shade (30% light intensity decrease all day long) was applied as treatment, and no shade was applied (plants grown in sun all day long) as control. The area of both experimental plots was 30 m^2. The propagation material (species: *C. officinalis* L.; variety: Claudia; characteristics: high amount of flavonoids; registration code of the variety: 239987) originated from the gene bank of the Research Institute for Medicinal Plants and Herbs Ltd. The cultivation technology consisted of the following main steps: direct sowing (50 cm between rows), thinning, mechanical weed control, regular irrigation in dry periods, and harvesting of the inflorescences by hand. Sampling for this study took place on July 19, 2021, which was the 15th day after the appearance of the first flowering individuals of the experimental population, during which a significant part of the population had already been in bloom. Inflorescences from 10 treated and 10 control plant individuals were collected fresh and then stored in a refrigerator until use.

Pigment Extraction and Determination of Carotenoid Content. Analytical-grade chemicals were used for the extractions. Extraction: Flower samples were extracted twice with acetone and once with Et_2O. After evaporation, the residue of the acetonic extracts was dissolved in Et_2O. The ethereal solutions were combined, and this total extract was saponified in the heterogeneous phase (30% KOH/MeOH) overnight. The reaction mixture was washed with water ten times. The saponified pigments were stored in benzene at −20 °C under nitrogen.

The total carotenoid content of the plant materials was determined photometrically.[24] UV−vis spectra were recorded with a Jasco V-530 spectrophotometer (Jasco Corporation, Tokyo, Japan). NMR spectra were recorded with a Bruker Avance III Ascend 500 spectrometer (500/125 MHz for $^1H/^{13}C$) in $CDCl_3$. Chemical shifts are referenced to Me_4Si (^1H) or to the residual solvent signals (^{13}C).

High-Performance Liquid Chromatography on C_{30}. Solvents for the analysis (MeOH: methanol; MTBE: *tert*-butylmethyl ether, acetone, water) were of HPLC grade. The HPLC-DAD separations on a C_{30} stationary phase were performed by a Dionex 3000 HPLC system (Thermo Fisher Scientific Inc., Waltham, Massachusetts). Chromatograms were detected at 450 nm wavelength, and the data acquisition was performed by Chromeleon 7.20 software. The separation was carried out on an end-capped C_{30} column (250 mm × 4.6 mm i.d.; YMC C_{30}, 3 μm, YMC Europe GmbH, Dinslaken, Germany). Eluents: (A) MeOH/MTBE/H_2O = 81:15:4 v/v% and (B) MeOH/MTBE/H_2O = 6:90:4 v/v%. Chromatography was performed in a linear gradient from 100% A eluent to 75% B mixture in 75 min, with 1.00 mL/min flow rate at 22 °C.

HPLC-DAD-MS chromatograms were recorded by a 6530 Accurate-Mass Q-TOF LC/MS apparatus (Agilent Technologies, Singapore); the data acquisition was performed by MassHunter B.04.00 software. For LC−(APCI)MS, the positive ion mode was used, with TIC, scanning range 200−1500 *m*/*z*, corona voltage 2.6 kV, fragmentor voltage 150 V, skimmer 60 V, and Oct 1RF Vpp 750 V. The flow rate of the dried nitrogen as nebulizer gas was 240 L/h, and the vaporizer temperature was 400 °C. The HPLC conditions were the same as described above.

High-Performance Liquid Chromatography on C_{18}. The HPLC analysis was performed with a Dionex P680 quaternary analytical pump, a Dionex PDA 100 UV/vis detector (Thermo Fisher Scientific, Inc., Waltham, Massachusetts) with Chromeleon 6.8 software, and a column temperature control module. Chromatograms were developed on a 250 mm × 4.6 mm stainless steel LiChrospher 100 RP 18e, 5 mm (Merck KGaA, Darmstadt, Germany) column, with 1.25 mL/min flow rate, at 22 °C. Eluents were (A) H_2O/MeOH = 12:88 v/v%; (B) MeOH; and (C) acetone/MeOH = 50:50 v/v%. The gradient program was the following: 0−2 min 100% A, 2−10 min to 80% A/20%B, 10−18 min to 50% A/50% B,18−25 min to 100% B, 25−27 min 100% B, 27−33 min to 100% C, 33−38 min 100% C, and 38−40 min to 100% B (in linear steps).

Identification of the Peaks. The carotenoids were identified using the following data: elution order on the C_{30} HPLC column,[25] spiking with authentic standards, UV−visible spectrum (λ_{max}), spectral fine structure (%III/II), *cis* peak intensity (%A_B/A_{II}),[26] and mass spectrum (molecular ion and fragments) compared to standards and data available in the literature.[27] Authentic standards were taken from our collection.

Isomerization of Lycopene, γ-, and δ-Carotene. Lycopene (**8**), γ-(**7**), and δ-carotene (**17**) were taken from our collection. A solution of 2 mg of carotenoid in 50 mL of toluene was isomerized in the presence of 0.02 mg of I_2 (∼2% to the carotenoid) under N_2 in scattered daylight.[28,29] The isomerization was monitored by UV−vis spectrometry, and when the thermodynamic equilibrium was reached (ca. 40 min), the mixture was washed free of I_2 with 5% $Na_2S_2O_3$ solution and, after the usual workup, submitted to HPLC.

Preparation of Flavoxanthin and Chrysanthemaxanthin. To a solution of 10 mg of lutein 5,6-epoxide (**11**) (extracted from *Chelidonium majus*) in 100 mL of Et_2O was added 0.1 mL of cc. HOAc/HCl (9:1) solution at room temperature. The mixture was kept under N_2 in the dark. The reaction was monitored by UV−vis spectrometry. After 0.5 h, the mixture was washed with 5% aqueous $NaHCO_3$ solution, and the organic phase was dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in toluene and submitted to open column chromatography ($CaCO_3$, Merck, Germany, 6 cm × 30 cm, toluene). After development, two fractions were visible: fraction 1:20 mm yellow band, flavoxanthin (**4**) and fraction 2:30 mm yellow band, chrysanthemaxanthin (**10**). After processing, which consisted of cutting the column packing into sections and extracting each section, fractions 1 and 2 were obtained, which were crystallized from benzene with *n*-hexane, yielding 5 mg of flavoxanthin (**4**) and 3 mg of chrysanthemaxanthin (**10**).

Preparation of (8′*R*)- and (8′*S*)-Luteoxanthins. To a solution of 60 mg of violaxanthin (**5**) (extracted from spinach) in 50 mL of Et_2O, 0.1 mL of cc. HOAc/HCl (9:1) solution was added at room temperature. The mixture was kept under N_2 in the dark. The reaction was monitored by UV−vis spectrometry. After 20 min, the mixture was washed with a 5% aqueous $NaHCO_3$ solution, and the Et_2O phase was dried with Na_2SO_4 and evaporated to dryness. The residue was dissolved in toluene and submitted to open column chromatography ($CaCO_3$, Merck, Germany, 6 cm × 30 cm, toluene). After development, three fractions were visible: fraction 1:10 mm pale yellow band, mixture of auroxanthin epimers; fraction 2:30 mm yellow band, (5′*R*,8′*R*)-luteoxanthin; and fraction 3:30 mm yellow band, (5′*R*,8′*S*)-luteoxanthin. After processing, which consisted of cutting the column packing into sections and extracting each section, fractions 2 and 3 were obtained, which were crystallized from benzene with *n*-hexane, yielding 15 mg of (5′*R*,8′*R*)-luteoxanthin ((8′*R*)-**3**) and 8 mg of (5′*R*,8′*S*)-luteoxanthin ((8′*S*)-**3**).

Preparation of (9*Z*,8′*R*)-Luteoxanthin. A solution of 10 mg of (all-E,5′*R*,8′*R*)-luteoxanthin ((8′*R*)-**3**) in 100 mL of toluene was isomerized in the presence of 0.01 mg of I_2 (∼2% to the carotenoid) under N_2 in scattered daylight.[28,29] The isomerization was monitored by UV−vis spectrometry, and when the thermodynamic equilibrium

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Table 1. ^{1}H and ^{13}C NMR Data of Flavoxanthin and Chrysanthemaxanthin at 500/125 MHz, resp., in $CDCl_3$ at Room Temperature[a]

	flavoxanthin (8*R*) (**4**)				chrysanthemaxanthin (8*S*) (**10**)		
position	δ(H) [ppm]	^{3}J [Hz]	δ(C) [ppm]	position	δ(H) [ppm]	^{3}J [Hz]	δ(C) [ppm]
1			33.7	1			34.2
2	α: 1.51 (dd)	14.3, 3.4	46.8	2	α: 1.47 (dd)	14.1, 3.7	47.41[b]
	β: 1.77 (dd)	14.3, 4.3			β: 1.77−1.82 (m)		
3	4.20−4.30 (m)		67.8	3	4.24 (brs)		67.9
4	α: 2.00 (dd)	13.9, 4.3	47.5	4	α: 1.88−1.92 (m)		47.45[b]
	β: 2.13 (pdd)	14.0, 3.4			β: 2.09−2.13 (m)		
5			86.9	5			87.2
6			154.1	6			153.2
7	5.25 (brs)		119.9	7	5.30 (d)	1.6	118.8
8	5.16 (brs)		87.8	8	5.07 (s)		88.4
9			138.0[b]	9			138.7
10	6.19 (d)	11.1	127.3	10	6.19 (d)	10.9	126.2
11	6.49 (dd)	15.1, 11.1	124.4	11	6.50 (dd)	15.0, 11.0	124.51[b]
12	6.32 (d)[b]	15.0	137.6	12	6.32 (d)	15.0	137.6
13			136.3[b]	13			136.3
14	6.21−6.25 (m)		132.3[b]	14	6.21−6.25 (m)		132.1[b]
15	6.57−6.64 (m)		130.0[b]	15	6.57−6.65 (m)		129.8[b]
16	1.17 (s)		31.4	16	1.19 (s)		31.3
17	1.33 (s)		29.0	17	1.34 (s)		28.2
18	1.62 (brs)		29.1	18	1.68 (s)		30.6
19	1.71 (s)		12.7	19	1.80 (s)		13.4
20	1.94 (s)[g]		12.9	20	1.95 (s)[b]		12.8
1′			34.1	1′			34.0
2′	α: 1.83 (dd)	13.2, 5.9	44.7	2′	α: 1.84 (dd)	13.2, 5.9	44.6
	β: 1.36 (dd)	13.1, 6.9			β: 1.36 (dd)	13.0, 6.9	
3′	4.20−4.30 (m)		66.0	3′	4.24 (brs)		65.9
4′	5.54 (brs)		124.5	4′	5.54 (brs)		124.50[b]
5′			138.0[b]	5′			138.0
6′	2.40 (d)	9.8	55.0	6′	2.40 (d)	9.9	55.0
7′	5.43 (dd)	15.4, 9.9	128.7	7′	5.42 (dd)	15.5, 9.9	128.7
8′	6.11−6.15 (m)		137.8	8′	6.11−6.15 (m)		137.7
9′			135.0	9′			135.0
10′	6.11−6.15 (m)		130.8	10′	6.11−6.15 (m)		130.8
11′	6.57−6.64 (m)		124.8	11′	6.57−6.65 (m)		124.7
12′	6.35 (d)[b]	14.9	137.6	12′	6.35 (d)	14.9	137.3
13′			136.4[b]	13′			136.2
14′	6.21−6.25 (m)		132.5[b]	14′	6.21−6.25 (m)		132.5[b]
15′	6.57−6.64 (m)		130.04[b]	15′	6.57−6.65 (m)		130.0[b]
16′	0.85 (s)		24.4	16′	0.85 (s)		24.3
17′	1.00 (s)		29.5	17′	1.00 (s)		29.5
18′	1.62 (brs)		22.8	18′	1.62 (s)		22.8
19′	1.91 (s)		13.1	19′	1.91 (s)		13.1
20′	1.97 (s)		12.9	20′	1.96 (s)[b]		12.8

[a]All ^{1}H and ^{13}C spectra were assigned with the help of $^{1}H-^{1}H$-COSY, $^{13}C-^{1}H$-HSQC, and $^{13}C-^{1}H$-HMBC experiments. [b]Assignment may be exchanged.

was reached (ca. 40 min), the mixtures were washed free of I_2 with 5% $Na_2S_2O_3$ soln., and, after the usual workup, were submitted to OCC (6 cm × 30 cm column, $CaCO_3$). The separation of the thermal isomerization mixture (toluene/acetone 95:5) resulted in the following picture after development: fraction 1:4 mm pale yellow (unidentified), fraction 2:15 mm yellow ((13*Z*)-isomer of **3**), fraction 3:20 mm pale yellow ((9*Z*)-isomer of **3**), and fraction 4:30 mm yellow ((all-E,8′*R*)-**3**).

RESULTS AND DISCUSSION

Semisynthesis and Identification of 5,8-Epoxy-carotenoids (Furanoids). 5,8-Epoxycarotenoids are easily prepared by treating 5,6-epoxides with acid. Naturally occurring (3*S*,5*R*,6*S*)-3-hydroxy-5,6-epoxycarotenoids produce two 5,8-epoxy epimers, namely, the (3*S*,5*R*,8*R*) and (3*S*,5*R*,8*S*) epimers in almost the same proportion. In the case of 3-hydroxy compounds, the epimers can be easily separated by HPLC or open column chromatography (OCC) on $CaCO_3$,[30,31] while in the absence of 3-hydroxyl group, they can be separated only on a chiral phase.[32,33] The differentiation of (8*S*) and (8*R*) isomers of the 3-hydroxy derivatives is based on their ^{1}H NMR spectra, according to Eugster's criteria: for the (8*S*) compound, $\delta_{(H\text{-}7)} - \delta_{(H\text{-}8)} = 0.15-0.22$ ppm and $^{3}J_{H\text{-}7,8} > 1.4$ Hz, while in the (8*R*) epimer, $\delta_{(H\text{-}7)} - \delta_{(H\text{-}8)} \leq 0.09$ ppm, and H-7 gives a broad singlet.[34]

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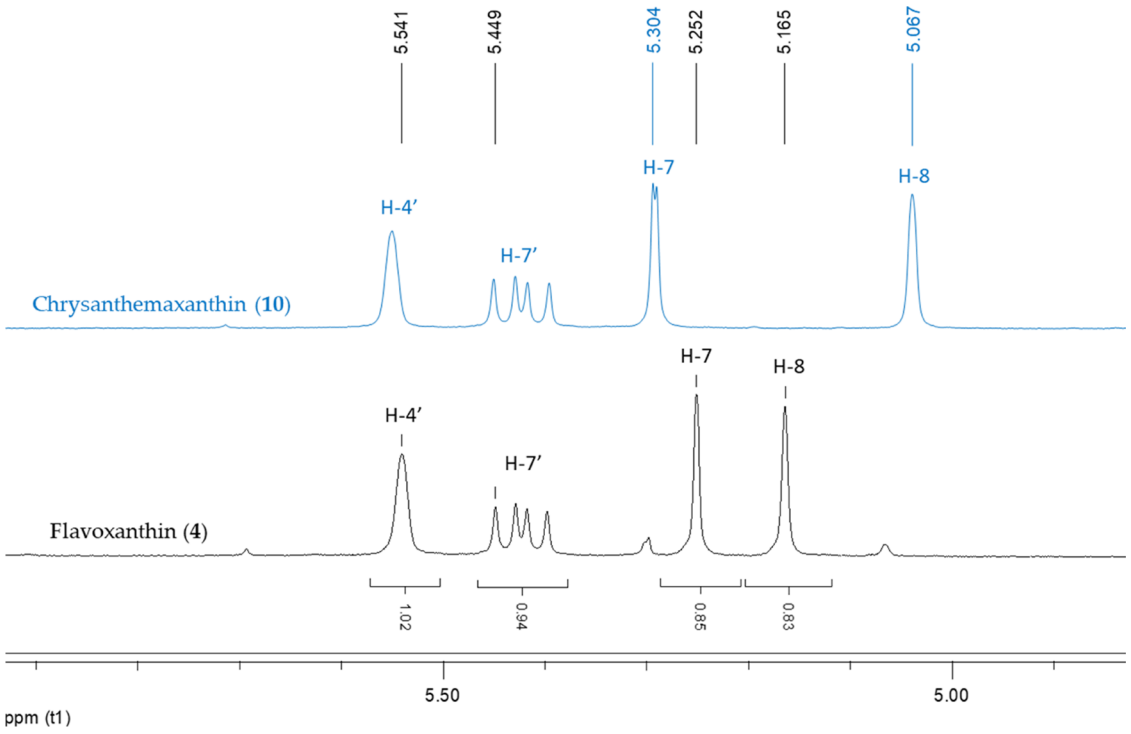

Figure 2. Comparison of the ^{1}H NMR spectrum of flavoxanthin (**4**) and that of chrysanthemaxanthin (**10**).

Preparation and Structure Elucidation of Flavoxanthin (4) and Chrysanthemaxathin (10). The treatment of lutein 5,6-epoxide (**11**) with cc. HOAc/HCl (9:1) produced a mixture of flavoxanthin (**4**) (5*R*,8*R*) and chrysanthemaxanthin (**10**) (5*R*,8*S*). These isomers could be separated on both C_{30} and C_{18} stationary phases by HPLC and classical column chromatography. The configuration of the separated epimers was evaluated by ^{1}H NMR and ^{13}C NMR spectroscopy in $CDCl_3$ (Table 1, Figures 2 and S2−S5). The comprehensive work by Cadosch et al.[35] helps to identify the isomers: the characteristic difference in the ^{1}H NMR spectra of **4** and **10** is the chemical shift of H-8 (5.16 ppm for **4**, 5.07 ppm for **10**), which can unambiguously distinguish the two molecules.[35,36] A similar tiny divergence can be observed in the chemical shifts of H-19 (1.71 ppm for **4**, 1.80 ppm for **10**).[35] In $CDCl_3$, the ^{13}C shifts of C-6 and C-7 of flavoxanthin differ by ∼1 ppm from the corresponding signals of chrysanthemaxanthin.

Preparation and Structure Elucidation of Luteoxanthin (3) C-8′ Epimers. Since violaxanthin (**5**) is a 5,6,5′,6′-diepoxide, the acidification had to be done carefully so that only one of the 5,6-epoxide groups was converted to the 5,8-epoxide. This can be achieved by frequent monitoring of the reaction mixture; thus, the main products became (8′*R*)- and (8′*S*)-luteoxanthin (**3**).[37] The resulting luteoxanthin (**3**) epimers can be easily separated from each other and from the starting violaxanthin by column chromatography on $CaCO_3$. The configuration of the epimers obtained by the OCC was established using ^{1}H NMR and ^{13}C NMR spectroscopy. The measured chemical shifts (Table 2) are in accordance with the literature data.[34,37] Similar to the distinction of flavoxanthin (**4**) and chrysanthemaxanthin (**10**), the chemical shifts of H-8′ and H-19′, as well as those of C-6′ and C-7′, can be used to differentiate the C-8′ epimers. The prepared compounds were used as standards on the C_{18} stationary phase to establish which epimer is present in *Calendula*. On the C_{30} stationary phase, the two luteoxanthin (**3**) epimers give one peak.[25]

Preparation and Structure Elucidation of (9*Z*,8′*R*)-Luteoxanthin. To prepare a standard, iodine-catalyzed isomerization of (all-*E*,8*R*)-luteoxanthin (**3**) was performed in a toluene solution. The configuration of the (9*Z*)-isomer obtained by open column chromatography was identified by ^{1}H NMR and ^{13}C NMR spectroscopy (Table 2).[37] The position of the *Z* double bond was unambigously determined by the characteristic ^{1}H chemical shift of H-8 (6.29 ppm for the all-*E* compound and 6.83 ppm for the 9*Z* isomer) and that of H-11 (6.57−6.63 ppm *m* for the all-*E* compound and 6.76 ppm *dd* for the 9*Z* isomer). ^{13}C chemical shifts of C-8, C-9, and C-10 also reveal the existence of a 9*Z* compound ($\Delta\delta$ = $\delta(E) - \delta(Z)$: $\Delta\delta$ = 7.9 ppm for C-8, $\Delta\delta$ = 1.5 ppm for C-9, and $\Delta\delta$ = 1.4 ppm for C-10).[38]

Analysis of Flowers. The investigated head inflorescence (capitulum) had a diameter of 3−7 cm, comprising both ray (ligulate) and disc (tubular) florets, whose color was orange. In our analysis, we used both types of flowers and the full inflorescences. The green parts of the inflorescence (bracts and receptacles) were excluded. The total carotenoid contents were determined by UV−vis spectrophotometry.[24]

The HPLC-DAD and HPLC-DAD-MS analyses were performed on a C_{30} phase. Our previous results showed that this stationary phase separates the isomers of interest well, while a C_{18} phase is less suitable.[25] The carotenoids were identified by their elution order on the C_{30} HPLC column, spiking with authentic standards, UV−visible spectra (λ_{max}, spectral fine structure (%III/II)), *cis* peak intensity (%A_B/A_{II}), and mass spectrum compared to standards and data available in the literature.[27]

In the HPLC chromatograms of all of the investigated total extract samples on a C_{30} column at 450 nm wavelength, 31 peaks were characterized (Table 3). The identification of the

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Table 2. ^{1}H NMR and ^{13}C NMR Data of Luteoxanthin Isomers at 500/125 MHz, Respectively, in $CDCl_3$ at Room Temperature

	(8′*R*) luteoxanthin (8′*R*-**3**)			(8′*S*) luteoxanthin (8′*S*-**3**)			(9*Z*,8′*R*) luteoxanthin (9*Z*,8′*R*-**3**)			
position	δ(H) [ppm]	^{3}J [Hz]	δ(C) [ppm]	δ(H) [ppm]	^{3}J [Hz]	δ(C) [ppm]	δ(H) [ppm]	^{3}J [Hz]	δ(C) [ppm]	position
1			35.3			35.4			35.3	1
2	α: 1.60−1.65 (m)		47.2	α: 1.60−1.65 (m)		47.2	α: 1.63−1.68 (m)		47.2	2
	β: ∼1.25 (m)			β: ∼1.25 (m)			β: ∼1.26 (m)			
3	3.91 (ddd)	13.8, 8.8, 4.7	64.3	3.91 (ddd)	13.9, 8.9, 4.9	64.3	3.92 (ddd)	13.9, 8.9, 5.0	64.3	3
4	α: 1.60−1.65 (m)		41.0	α: 1.60−1.65 (m)		41.0	α: 1.63−1.68 (m)		41.0	4
	β: 2.38 (dd)	14.2, 3.7		β: 2.40 (dd)	14.0, 3.6		β: 2.40 (dd)	14.2, 4.9		
5			66.9			66.9			66.8	5
6			70.3			70.3			70.6	6
7	5.88 (d)	15.5	123.8	5.87 (d)	15.5	123.8	5.93 (d)	15.4	125.9	7
8	6.29 (d)	15.6	137.4	6.28 (d)	15.5	137.4	6.83 (d)	15.4	129.5	8
9			134.2			134.2			132.7	9
10	6.19 (d)	11.1	132.2	6.19 (d)	11.6	132.3	6.07 (d)	10.3	130.8	10
11	6.57−6.63 (m)		124.6	6.59−6.63 (m)		124.6[a]	6.76 (dd)	14.7, 11.7	123.5	11
12	6.37 (d)	15.0	138.2	6.37 (d)	14.9	138.2	6.29 (d)	15.7	137.6[a]	12
13			136.2			136.1			136.4	13
14	6.22−6.26 (m)		132.2[a]	6.23−6.27 (m)		132.9	6.20−6.24 (m)		132.5[a]	14
15	6.57−6.63 (m)		129.9[a]	6.57−6.63 (m)		129.8[a]	6.57−6.64 (m)		128.98[a]	15
16	0.97 (s)		24.9	0.98 (s)		24.9	1.00 (s)		25.0	16
17	1.15 (s)		29.6	1.15 (s)		29.6	1.16 (s)		29.6	17
18	1.19 (s)		20.0	1.18 (s)		20.0	1.21 (s)		20.0	18
19	1.92 (s)		13.0	1.92 (s)		13.0	1.93 (s)		21.0	19
20	1.94 (s)[a]		12.80[a]	1.95 (s)		12.8	1.94 (s)[a]		12.8[a]	20
1′			33.7			34.2			33.7	1′
2′	α: 1.51 (dd)	14.1, 3.3	46.7	α: 1.47 (dd)	14.1, 3.7	47.4	α: 1.52 (dd)	14.1, 3.2	46.7	2′
	β: 1.76 (dd)	14.2, 3.5		β: 1.78−1.81 (m)			β: 1.76 (dd)	15.1, 4.0		
3′	4.22−4.26 (m)		67.7	4.24 (pt)	3.8	67.9	4.24 (br m)		67.7	3′
4′	α: 1.99 (dd)	13.7, 3.9	47.4	α: 1.90 (dd)	14.0, 4.1	47.5	α: 1.98 (dd)	14.4, 4.1	47.4	4′
	β: 2.12 (dd)	13.6, 3.4		β: 2.11 (pd)	13.8		β: 2.12 (dd)	13.8, 3.2		
5′			86.8			87.2			86.9	5′
6′			154.1			153.2			154.1	6′
7′	5.25 (brs)		119.9	5.30 (d)	1.5	118.7	5.25 (s)		119.9	7′
8′	5.17 (brs)		87.7	5.07 (s)		88.4	5.16 (s)		87.7	8′
9′			138.0			138.7			137.9	9′
10′	6.19 (d)	11.1	127.2	6.18 (d)	10.7	126.2	6.19 (d)	10.8	127.2	10′
11′	6.49 (dd)	15.0, 11.3	124.4	6.50 (dd)	15.0, 11.1	124.5[a]	6.48 (dd)	15.0, 11.1	124.3	11′
12′	6.32 (d)	15.2	137.6	6.32 (d)	15.1	137.3	6.32 (d)	16.2	137.5[a]	12′
13′			136.4			136.4			136.2	13′
14′	6.22−6.26 (m)		132.8[a]	6.23−6.27 (m)		132.1	6.20−6.24 (m)		132.3[a]	14′
15′	6.57−6.63 (m)		130.2[a]	6.57−6.63 (m)		130.2[a]	6.57−6.64 (m)		128.96[a]	15′
16′	1.17 (s)		31.4	1.19 (s)		31.4	1.17 (s)		31.4	16′
17′	1.33 (s)		28.9[a]	1.34 (s)		28.2	1.33 (s)		28.9	17′
18′	1.62 (s)		29.0[a]	1.68 (s)		30.6	1.61 (s)		29.0	18′
19′	1.71 (s)		12.6	1.80 (s)		13.4	1.71 (s)		12.6	19′
20′	1.96 (s)[a]		12.78[a]	1.95 (s)		12.8	1.96 (s)[a]		13.0[a]	20′

[a]Assignment may be exchanged.

components is shown in the example of *C. officinalis* petals (Figure 3).

The main component, peak 4, was identified as chrysanthemaxanthin (**10**) with (5*R*,8*S*) configuration by its UV−vis (λ_{max}: 398, 421, 448 nm, %III/II: 92) and MS spectra (m/z 567, $[M - H_2O + H]^+$) and cochromatography with an authentic standard. Peak 8 was tentatively identified as the (9*Z*)-isomer of chrysanthemaxanthin ((9*Z*)-**10**). This peak showed characteristic UV−visible spectra (λ_{max}: 395, 417, 443, %III/II: 101, $\%A_B/A_{II}$: 6), with a slightly decreased spectral fine structure and a hypsochromic shift of 4 nm compared to the all-*trans* isomer (peak 4). The identification of this compound was also supported by its m/z value (567, $[M - H_2O + H]^+$).

Peak 3 exhibited UV−visible spectra similar to those of peak 4 (λ_{max}: 399, 421, 448 nm (%III/II: 91)). The molecular mass ($[M + H]^+$), detected at m/z 601 for the compound, seemed to correspond to luteoxanthin (**3**). As mentioned above, the

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Table 3. Carotenoid Composition of *C. officinalis*

					%(of total carotenoids)[a]					
					petals sun	petals shade	floret sun	floret shade	inflorensce sun	inflorensce shade
no.	carotenoid	retention time (min)	UV−vis λ_{max} (nm)	MS (m/z)						
1	neoxanthin	7.71	412, 438, 467	601 $[M + H]^+$	0.72	0.55	0.29	1.82	0.49	0.58
2	(9*Z*)-neoxanthin	8.24	413, 436, 463	601 $[M + H]^+$	0.42	0.51	0.22	0.93	0.39	0.30
3	(8′*R*)-luteoxanthin	9.18	399, 421, 448	601 $[M + H]^+$	10.41	10.15	tr	tr	8.21	10.05
4	chrysanthemaxanthin	11.44	398, 421, 448	567 $[M - H_2O + H]^+$	14.99	16.75	12.85	12.23	11.16	15.53
5	(9*Z*,8′*R*)-luteoxanthin	12.44	397, 417, 442	601 $[M + H]^+$	7.53	7.90	2.90	3.59	8.36	8.26
6	lutein	13.55	444, 471	551 $[M - H_2O + H]^+$	11.03	10.76	14.77	14.96	7.90	7.53
7	(8*S*)-mutatoxanthin	13.78	427, 452	585 $[M + H]^+$	4.42	3.30			4.64	4.00
8	(9*Z*)-chrysanthemaxanthin	14.43	395, 417, 443	567 $[M - H_2O + H]^+$	0.54	0.44	1.76	1.57	0.61	0.71
9	mixture	15.13	321, 422, 446		0.56	0.74	tr	tr	0.94	0.44
10	zeaxanthin	15.66	450, 476	569 $[M + H]^+$	1.31	0.83	5.28	5.25	1.41	1.33
11	(9*Z*)-lutein	16.83	331, 439, 467	551 $[M - H_2O + H]^+$	6.26	6.53	8.99	11.15	5.11	5.01
12	(9′*Z*)-lutein	19.40	330, 440, 467	551 $[M - H_2O + H]^+$	2.64	2.45	7.58	8.03	2.00	2.22
13	(9*Z*)-zeaxanthin	20.59	340, 445, 471	569 $[M+ H]^+$	3.27	3.41	4.01	3.53	2.98	2.78
14	*β*-cryptoxanthin	24.70	451, 476	553 $[M+ H]^+$	0.59	0.37	4.60	3.91	0.66	0.94
15	(13*Z*)-*β*-Carotene	27.60	348, 450, 474	537 $[M+ H]^+$	0.54	0.62	0.70	0.78	2.92	0.86
16	mixture	29.93	339, 442		0.58	0.21	0.73	0.58	2.52	0.28
17	*α*-carotene	31.91	445, 472	537 $[M + H]^+$	0.58	0.81	0.73	0.41	3.81	0.76
18	(5′*Z*,9′*Z*)-rubixanthin	34.82	350, 455, 484	553 $[M + H]^+$	3.09	3.24	1.54	1.46	0.92	4.91
19	*β*-carotene	35.52	452, 478	537 $[M + H]^+$	5.10	6.32	13.08	13.49	10.00	5.80
20	(9*Z*)-*β*-carotene	37.81	347, 447, 467	537 $[M + H]^+$	1.06	0.97	4.00	3.90	2.86	1.27
21	rubixanthin	41.94	459, 489	553 $[M + H]^+$	1.11	0.83	0.14	0.44	1.71	1.30
22	(5′*Z*)-rubixanthin	43.03	460, 490	553 $[M + H]^+$	4.74	4.23	1.61	1.78	3.51	5.01
23	(di-*Z*)-*γ*-carotene	45.49	350, 455, 484	537 $[M + H]^+$	0.70	0.65	0.55	0.56	1.27	1.33
24	*δ*-carotene	47.95	432, 457, 487	537 $[M + H]^+$	0.69	0.73	1.80	1.90	1.22	0.74
25	(5*Z*)-*δ*-carotene	48.65	431, 455, 486	537 $[M + H]^+$	0.38	0.43	tr	tr	0.32	0.51
26	*γ*-carotene	52.20	460, 490	537 $[M + H]^+$	2.14	2.36	1.47	1.21	2.68	2.14
27	(5*Z*)-*γ*-carotene	53.15	360, 461, 491	537 $[M + H]^+$	5.45	5.60	3.56	2.74	5.03	5.89
28	(5*Z*,9*Z*,5′*Z*)-lycopene	59.92	440, 465, 496	537 $[M + H]^+$	0.47	0.27	0.18	0.10	0.63	0.44
29	(5*Z*,9*Z*)-lycopene	61.18	440, 465, 496	537 $[M + H]^+$	2.61	2.71	1.16	1.25	2.13	3.92
30	lycopene	68.95	446, 471, 502	537 $[M + H]^+$	0.89	0.83	0.26	0.16	0.49	0.69
31	(5*Z*)-lycopene	69.80	445, 471, 502	537 $[M + H]^+$	5.12	4.40	1.55	1.15	3.06	4.39
	total carotenoid	mg/g			0.872	1.154	0.0986	0.123	0.406	0.806

[a]Percentage of peak area in the HPLC chromatogram at 450 nm.

two C-8 stereoisomers of luteoxanthin (**3**) are inseparable in the C_{30} stationary phase. After separation of the calendula extract on the C_{18} stationary phase (Figure S6), only one stereoisomer was detected. Cochromatography with the authentic standard obtained by acid-catalyzed rearrangement of violaxanthin confirmed the identity of (5′*R*,8′*R*)-luteoxanthin ((8′*R*)-**3**) (Figure S7).

(9*Z*,5′*R*,8′*R*)-Luteoxanthin ((9*Z*,8′*R*)-**3**) (peak 5) showed characteristic UV−visible spectra (λ_{max}: 397, 417, 442, %III/II: 69, $\%A_B/A_{II}$: 6), with a *cis* peak, slightly decreased spectral fine structure, and a hypsochromic shift of 6 nm compared to the all-*trans* isomer (peak 3). The identification of this compound was supported by its m/z value (601, $[M + H]^+$) and spiking

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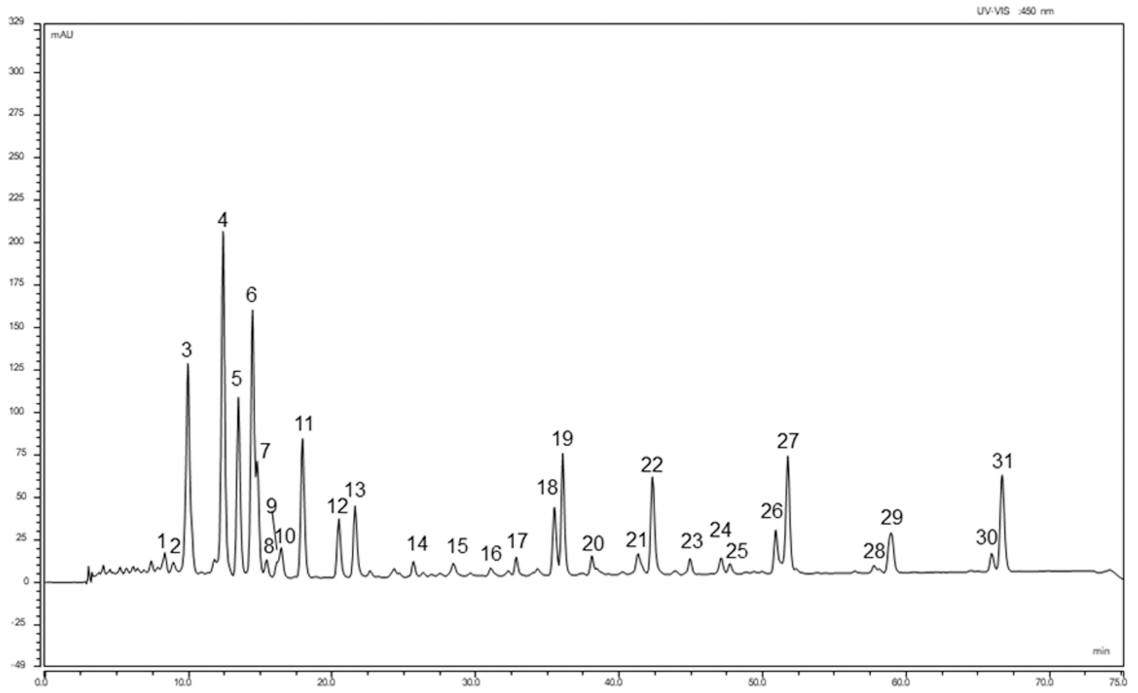

Figure 3. HPLC-DAD full chromatogram of *C. officinalis* petals. Peak numbering is given in Table 3.

with an authentic sample obtained by stereomutation of the appropriate all-*E* compounds.

Peak 6 was identified as (all-*trans*)-lutein (**2**) by its UV−vis (λ_{max}: 444, 472 nm, %III/II: 51) and MS spectra (*m*/*z* 551, [M − H_2O + H]$^+$) and cochromatography with authentic standards. Peaks 11 and 12 were attributed to the 9-*cis* and 9′-*cis* isomers of lutein, respectively, according to the characteristic hypsochromic shift and the low intensity of the *cis* peak in the UV−visible spectra (λ_{max}: 330, 440, 467 nm, %III/II: 55, %A_B/A_{II}: 9), MS ([M − H_2O + H]$^+$ at *m*/*z* 551), and spiking with authentic standards obtained from the iodine-catalyzed isomerization of lutein.[28,29]

Peak 7, the shoulder of peak 6, was identified as (5*R*,8*S*)-mutatoxanthin ((8*S*)-**9**). The molecular mass ([M + H]$^+$) detected at *m*/*z* 585 and UV−vis spectrum (λ_{max}: 427, 452 nm, %III/II: 69) seemed to correspond to mutatoxanthin (**9**). Our earlier result showed that lutein and (5*R*,8*S*)-mutatoxanthin eluted very closely on a C_{30} stationary phase.[25] This assumption was confirmed by coelution with the authentic standard prepared earlier in our laboratory.[31,39]

In plants, lutein (**2**) is usually accompanied by more or less zeaxanthin (**14**) (Figure S1). Peaks 10 and 13 were identified as zeaxanthin (**14**) and (9*Z*)-zeaxanthin ((9*Z*)-**14**), respectively, by their UV−vis (peak 10 λ_{max}: 450, 476 nm, %III/II: 22, peak 13 λ_{max}: 341, 445, 471 nm, %III/II: 23, %A_B/A_{II}: 10) and MS spectra (*m*/*z* 569, ([M + H]$^+$)). Peak 14 exhibited UV−visible spectra similar to those of peak 10. The molecular mass ([M + H]$^+$), detected at *m*/*z* 553, seemed to correspond to *β*-cryptoxanthin (**15**).

From the polar carotenoids, (all-*trans*)-neoxanthin (**12**) (peak 1) showed characteristic UV−visible spectra with fine structure (λ_{max}: 414, 438, 468 nm, %III/II: 56). The identity of peak 1 was supported by coelution with an authentic standard and by its *m*/*z* value (600). Peak 2 was identified similarly to peak 5 as (9′*Z*)-neoxanthin ((9′*Z*)-**12**). Both compounds appeared in very small amounts. It should be noted here that while Kishimoto[21] found a larger amount (7%) of auroxanthin (**13**, Figure S1) in *Calendula* petals, we were able to detect it only in trace amounts.

Peak 21 was identified as all-*trans*-rubixanthin (**6**) (Figure S1). It had a UV−vis spectrum with 459 and 489 nm absorption maxima (%III/II: 43), and the *m*/*z* value of 553 ([M + H]$^+$) confirmed the occurrence of one hydroxyl group in the molecule. The identity of peak 21 was supported by spiking with an authentic standard. Peak 22 was identified as the 5*Z*-isomer of rubixanthin (**6**) (λ_{max}: 460, 490 nm, %III/II: 47). The spectrum of peak 18 showed a hypsochromic shift of 5 nm compared to peak 21. The *m*/*z* value of 553 ([M + H]$^+$) indicates the presence of a hydroxyl group. In the absence of an authentic standard, the component was tentatively identified as (5′*Z*,9′*Z*)-rubixanthin based on the UV−vis spectrum (λ_{max}: 350, 455, 484 nm, %III/II: 45, %A_B/A_{II}: 12) and retention time, which was in accordance with data published by Kishimoto.[21]

Peak 19 had UV−visible spectra similar to those of zeaxanthin (**14**). The molecular mass detected at 536 seemed to correspond to (all-*trans*)-*β*-carotene (**1**). Peak 20 corresponded to 9*Z*-, and peak 15 to 13*Z*-isomers of *β*-carotene (**1**) based on their UV−vis (peak 20 λ_{max}: 347, 447, 467 nm, %A_B/A_{II}: 15, peak 15 λ_{max}: 348, 450, 474 nm, %A_B/A_{II}: 40) and MS spectra. Peak 17 with a spectrum similar to that of lutein (**2**) and a [M + H]$^+$ value of 537 was identified as *α*-carotene (**16**).

In addition to *α*- and *β*-carotene, the extract also contained carotene hydrocarbons with a Ψ-end group. Peak 30 was identified as (all-*E*)-lycopene (**8**) by its UV−vis spectrum (λ_{max}: 446, 471, 502 nm, %A_B/A_{II}: 68) and molecular mass (*m*/*z* 537, [M + H]$^+$).

In the lack of standard materials, the identification of peaks 28, 29, and 31 was based on the cochromatography with the iodine-catalyzed isomerization mixture of lycopene and the data published earlier by Kishimoto et al.[21] The similar UV−vis spectra of peaks 30 and 31 (λ_{max}: 445, 471, 502 nm, %A_B/A_{II}: 69) showed the isomerization in position C-5; thus, peak

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31 was tentatively identified as (5*Z*)-lycopene. Peaks 29 and 28 were attributed to the 5-*cis* and 5,5′-di-*cis* isomers of (9*Z*)-lycopene, respectively, according to the characteristic hypsochromic shift and the low intensity of the *cis* peak in the UV−visible spectra (λ_{max}: 352, 440, 465, 496 nm, %III/II: 64, $\%A_B/A_{II}$: 13).

The peaks 24 and 26 were identified as δ-carotene (**17**) and γ-carotene (**7**) by their UV−vis and mass spectra and cochromatography with an authentic sample. Based on spiking with the iodine-catalyzed isomerization mixture of δ- and γ-carotene and the data published by Kishimoto et al.,[21] peak 25 and peak 27 were identified as 5′*Z*-isomers of δ- and γ-carotene, respectively. Peak 23 was tentatively identified as a *Z*-isomer of γ-carotene (**7**) from its UV−vis and mass spectra.

Only two peaks, peaks 9 and 16, remained unidentified, which are presumably mixed components.

DISCUSSION

We found a slight difference in the total carotenoid content between plants grown in the sun and in the shade. Flowers grown in the shade had higher carotenoid content than those grown in the sun (Table 3). This was consistent with our previous study,[23] however, our one-year investigation cannot be considered decisive. The examined petals and florets showed a similar carotenoid composition except for (all-*E*)-luteoxanthin (**3**). While the petals contained a large amount of (all-*E*)-luteoxanthin (**3**), it was found only in traces in the florets, while (9*Z*)-luteoxanthin ((9*Z*)-**3**) occurred in both. With a few exceptions, we found the same carotenoids as previously described.[14,21] We detected the open-chain lycopene (**8**), γ-carotene (**7**), δ-carotene (**17**), as well as their *cis*- and di-*cis*-isomers, which were previously isolated and identified by Kishimoto et al.[21] Among the carotene hydrocarbons, we also found α-carotene (**16**) and β-carotene (**1**) in small amounts. Monohydroxy carotenoids are represented by β-cryptoxanthin (**15**) and rubixanthin (**6**) and their *cis*-isomer. Lutein (**2**) occurred as one of the main components, while (9*Z*)-, (9′*Z*)-lutein, and (all-*E*)-zeaxanthin (**14**) were found only in small amounts in petals and florets.

Contrary to the results of Pintea[14] and Kishimoto,[21] we could not detect carotenoid 5,6-epoxides (antheraxanthin (**18**), lutein 5,6-epoxide (**11**); Figures S1 and S7) or their isomers, and we only found trace amounts of neoxanthin (**12**) and (9′*Z*)-neoxanthin ((9′*Z*)-**12**) (Figure S1). A significant difference was found in the case of 5,8-epoxy-carotenoids. Previously, the main component of *Calendula* was identified as flavoxanthin (**4**) ((5*R*,8*R*)-epimer).[14,21,22] Our current detailed NMR studies show that the configuration of the main component is (5*R*,8*S*), i.e., it is chrysanthemaxanthin (**10**) (Figure S9). Surprisingly, however, the configuration of luteoxanthin (**3**) and (9*Z*)-luteoxanthin ((9*Z*)-**3**) was (5′*R*,8′*R*) and that of mutatoxanthin (**9**) was (5*R*,8*S*).

The naturally occurring (5*R*,6*S*)-carotenoid 5,6-epoxides produce two 5,8-epoxy isomers (also called furanoids) in almost equal amounts, namely, the (5*R*,8*R*) and the (5*R*,8*S*)-epimers upon the influence of plant acids.[34] The general assumption that furanoids arise from the rearrangement of 5,6-epoxides in acidic media during isolation and purification protocols has led to the consideration of these natural products as artifacts. The fact that only one furanoid epimer was found in *Calendula* suggests that here the epoxide-furanoid rearrangement is not acid-catalyzed but enzyme-catalyzed. It also seems to be confirmed by the absence of 3,5,6-trihydroxy derivatives of violaxanthin (**5**) (latoxanthin, 6-epilatoxanthin) in the extracts, since, in addition to 5,8-epoxy carotenoids, a small amount of 3,5,6-trihydroxy component is always formed by ring opening under acidic conditions.[34] Previously, we obtained karpoxanthin and 6-epikarpoxanthin by acidic ring opening of antheraxanthin (**18**) in addition to mutatoxanthin epimers (**9**) formed in large quantities.[31] In red peppers, 3,5,6-trihydroxy-carotenoids are formed enzymatically, catalyzed by the capsanthin−capsorubin synthase (CCS) enzyme.[40,41]

The (8*S*) configuration of chrysanthemaxanthin (**10**) and mutatoxanthin (**9**), as well as the (8′*R*) configuration of (all-*E*)- and (9*Z*)-luteoxanthin (**3**), suggests that different enzymes catalyze the epoxide-furanoid rearrangement of monoepoxides (lutein 5,6-epoxide (**11**), antheraxanthin (**18**)) and that of the diepoxide violaxanthin (**5**). In contrast to the results of Kishimoto[21] and Pintea,[14] we did not find auroxanthin (**13**) (Figure S1) even with detection at 420 nm. The molar mass of auroxanthin (**13**) *m*/*z* 601 was not found in the MS spectra either, even though auroxanthin (**13**) and luteoxanthin (**3**) are well separated on the C_{30} phase from each other and from the parent violaxanthin (**5**).

In order to clearly identify the structure, carotenoid 5,8-epoxides were prepared from the corresponding carotenoid 5,6-epoxides and their configuration was confirmed by NMR studies. This is the first time reporting the complete ^{1}H NMR and ^{13}C NMR data of chrysanthemaxanthin (**10**), flavoxanthin (**4**), and luteoxanthin (**3**) epimers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.3c00367.

Structure of carotenoids (Figure S1); ^{1}H- and ^{13}C NMR spectra of flavoxanthin (**4**) in $CDCl_3$ (Figure S2−S5); HPLC separation of *Calendula* extract on a C_{18} stationary phase (Figure S6); comparison of *Calendula* extract with the standard mixtures of luteoxanthins, mutatoxanthin epimers, antheraxanthin, flavoxanthin, and chrysanthemaxanthin on a C_{18} stationary phase (Figures S7−S9); and UV−vis and EIC chromatogram of *Calendula* petals extract (Figure S10) (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Britton, G. Structure and properties of carotenoids in relation to functions. *FASEB J.* **1995**, *9*, 1551−1558.
(2) Olson, J. A.; Krinsky, N. I. Introduction: the colourful fascinating world of the carotenoids: important physiologic modulators. *FASEB J.* **1995**, *9*, 1547−1550.
(3) Takaichi, S. Tetraterpenes: Carotenoids. In *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics, and Terpenes*; Ramawat, K. G.; Merillon, J. M., Eds.; Springer: Berlin, Heidelberg, Germany, 2013; Vol. *1*, pp 3251−3283.
(4) Britton, G.; Khachik, F. Carotenoids in Food. In *Carotenoids*; Britton, G.; Liaaen-Jensen, S.; Pfander, H., Eds.; Birkhäuser Verlag: Basel, Switzerland, Boston, MA, USA; Berlin, Germany, 2009; Vol. *5: Nutrition and Health*, pp 45−66.
(5) Domonkos, I.; Kis, M.; Gombos, Z.; Ughy, B. Carotenoids, versatile components of oxygenic photosynthesis. *Prog. Lipid Res.* **2013**, *52*, 539−561.
(6) Sausserde, R.; Kampuss, K. In *Composition of Carotenoids in Calendula (Calendula officinalis L.) Flowers*, 9th Baltic Conference on Food Science and Technology "Food for Consumer Well-Being" FOODBALT 2014 Conference Proceedings; Straumite, E., Ed.; LLU: Jelgava, Latvia, 2014; pp 13−18. ISSN 2255−9817.
(7) Eggersdorfer, M.; Wyss, A. Carotenoids in human nutrition and health. *Arch. Biochem. Biophys.* **2018**, *652*, 18−26.
(8) Perera, C. O.; Yen, G. M. Functional properties of carotenoids in human healths. *Int. J. Food Prop.* **2007**, *10*, 201−230.
(9) Szabó, K.; Lenchés, O. *Calendula officinalis* L.−Kerti körömvirág. In *Vadon termő és termesztett gyógynövények*; Bernáth, J., Ed.; Mezőgazda Kiadó: Budapest, Hungary, 2013; pp 200−203.
(10) Veselinov, B.; Adamovic, D.; Martinov, M.; Viskovic, M.; Golub, M.; Bojic, S. Mechanized harvesting and primary processing of *Calendula officinalis* L. Inflorescences. *Span. J. Agric. Res.* **2014**, *12*, 329−337, DOI: 10.5424/sjar/2014122-4876.
(11) *European Medicines Agency Committee on Herbal Medicinal Products. Assessment report on Calendula officinalis L. flos.; London, UK* 2018.
(12) Arora, D.; Rani, A.; Sharma, A. A review on phytochemistry and ethnopharmacological aspects of genus Calendula. *Pharmacogn. Rev.* **2013**, *7*, 179−187.
(13) Engel, R.; Szabó, K.; Abrankó, L.; Rendes, K.; Füzy, A.; Takács, T. Effect of Arbuscular Mycorrhizal Fungi on the Growth and Polyphenol Profile of Marjoram, Lemon Balm, and Marigold. *J. Agric. Food Chem.* **2016**, *64* (19), 3733−3742.
(14) Pintea, A. HPLC analysis of carotenoids in four varieties of *Calendula officinalis* L. flowers. *Acta Biol. Szegediensis* **2003**, *47*, 37−40.
(15) Szopa, A.; Klimek-Szczykutowicz, M.; Jafernik, K.; Koc, K.; Ekiert, H. Pot marigold (*Calendula officinalis* L.) − A position in classical phytotherapy and newly documented activities. *Acta Sci. Pol. Hortorum Cultus* **2020**, *19* (3), 47−61, DOI: 10.24326/asphc.2020.3.5.
(16) Verma, P. K.; Raina, R.; Agarwal, S.; Kaur, H. Phytochemical ingredients and Pharmacological potential of *Calendula officinalis* Linn. *Pharm. Biomed. Res.* **2018**, *4*, 1−17.
(17) Olennikov, D. N.; Kashchenko, N. I. Marigold Metabolites: Diversity and Separation Methods of *Calendula* Genus Phytochemicals from 1891 to 2022. *Molecules* **2022**, *27*, 8626.
(18) Andreeva, L. G. Localization and content of carotenoids in highly productive forms of *Calendula officinalis*. *Aptechnoe. Delo* **1961**, *10*, 46.
(19) Raal, A.; Orav, A.; Nesterovitsch, J.; Maidla, K. Analysis of Carotenoids, Flavonoids and Essential Oil of *Calendula officinalis* Cultivars Growing in Estonia. *Nat. Prod. Commun.* **2016**, *11*, No. 1934578X1601100831, DOI: 10.1177/1934578X1601100831.
(20) Tanaka, Y.; Sasaki, N.; Ohmiya, A. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* **2008**, *54*, 733−749.
(21) Kishimoto, S.; Maoka, T.; Sumimoto, K.; Ohmiya, A. Analysis of carotenoid composition in petals of calendula (*Calendula officinalis* L.). *Biosci. Biotechnol., Biochem.* **2005**, *69*, 2122−2128.
(22) Bakó, E.; Deli, J.; Tóth, G. HPLC study on the carotenoid composition of *Calendula* products. *J. Biochem. Biophys. Methods* **2002**, *53*, 241−250.
(23) Zubay, P.; Ruttner, K.; Ladányi, M.; Deli, J.; Zámboriné, É. N.; Szabó, K. In the shade - Screening of medicinal and aromatic plants for temperate zone agroforestry cultivation. *Ind. Crops Prod.* **2021**, *170*, No. 113764, DOI: 10.1016/j.indcrop.2021.113764.
(24) Schiedt, K.; Liaaen-Jensen, S. Isolation and Analysis. In *Carotenoids*; Britton, G.; Liaaen-Jensen, S.; Pfander, H., Eds.; Birkhäuser Verlag: Basel, Switzerland, Boston, MA, USA; Berlin, Germany, 1995; Vol. *1A: Isolation and Analysis*, pp 81−108.
(25) Turcsi, E.; Nagy, V.; Deli, J. Study on the elution order of carotenoids on endcapped C_{18} and C_{30} reverse silica stationary phase. A rewiev of the database. *J. Food Compos. Anal.* **2016**, *47*, 101−112, DOI: 10.1016/j.jfca.2016.01.005.
(26) Britton, G. UV/Visible Spectoscopy. In *Carotenoids*; Britton, G.; Liaaen-Jensen, S.; Pfander, H., Eds.; Birkhäuser Verlag: Basel, Switzerland, Boston, MA, USA; Berlin, Germany, 1995; Vol. *1B: Spectroscopy*, pp 16−22.
(27) Enzell, C. R.; Back, S. Mass Spectometry. In *Carotenoids*; Britton, G.; Liaaen-Jensen, S.; Pfander, H., Eds.; Birkhäuser Verlag: Basel, Switzerland, Boston, MA, USA; Berlin, Germany, 1995; Vol. *1B: Spectroscopy*, pp 261−320.
(28) Zechmeister, L. *Cis-Trans Isomeric Carotenoids, Vitamins A and Arylpolyenes*; Springer Verlag: Wien, Austria, 1962.

(29) Molnár, P.; Szabolcs, J. (*Z*/*E*)-Photoisomerization of C_{40}-carotenoids by iodine. *J. Chem. Soc., Perkin Trans. 2* **1993**, 261−266.
(30) Deli, J.; Molnár, P.; Matus, Z.; Tóth, G.; Steck, A.; Pfander, H. Partial Synthesis and Characterization of Capsokarpoxanthins and 3,6-Epoxycapsanthins. *Helv. Chim. Acta* **1998**, *81*, 1242−1253.
(31) Molnár, P.; Deli, J.; Matus, Z.; Tóth, G.; Steck, A.; Pfander, H. Partial Synthesis and Characterization of Karpoxanthins and Cucurbitaxanthin A Epimers. *Helv. Chim. Acta* **1999**, *82*, 1994−2002.
(32) Gulyás-Fekete, G.; Murillo, E.; Kurtán, T.; Papp, T.; Illyés, T. Z.; Drahos, L.; Visy, J.; Agócs, A.; Turcsi, E.; Deli, J. Cryptocapsinepoxide-Type Carotenoids from Red Mamey, *Pouteria sapota*. *J. Nat. Prod.* **2013**, *76*, 607−614.
(33) Murillo, E.; Agócs, A.; Nagy, V.; Király, S. B.; Kurtán, T.; Toribio, E. M.; Lakey-Beitia, J.; Deli, J. Isolation and identification of sapotexanthin 5,6- and 5,8-epoxide from red mamey (*Pouteria sapota*). *Chirality* **2020**, *32*, 579−587.
(34) Eugster, C. H. Carotenoid structures, old and new problems. *Pure Appl. Chem.* **1985**, *57*, 639−647.
(35) Cadosch, H.; Vögeli, U.; Rüedi, P.; Eugster, C. H. Über die Carotinoide Flavoxanthin und Chrysanthemaxanthin: 1H-NMR.-, 13C-NMR.-und Massen-Spektren und absolute Konfiguration.(Kritische Überprüfung veröffentlichter chemischer und physikalischer Daten). *Helv. Chim. Acta* **1978**, *61*, 783−794, DOI: 10.1002/hlca.19780610226.
(36) Englert, G. NMR Spectroscopy. In *Carotenoids*; Britton, G.; Liaaen-Jensen, S.; Pfander, H., Eds.; Birkhäuser Verlag: Basel, Switzerland; Boston, MA, USA; Berlin, Germany, 1995; Vol. *1B*, pp 147−260.
(37) Märki-Fischer, E.; Buchecker, R.; Eugster, C. H. Eine Neuuntersuchung der Carotinoide aus Rosa foetida: Struktur von 12 neuen Carotinoiden; stereoisomere Luteoxanthine, Auroxanthine, Latoxanthine und Latochrome. *Helv. Chim. Acta* **1984**, *67* (8), 2143−2154, DOI: 10.1002/hlca.19840670814.
(38) Nagy, V.; Agócs, A.; Balázs, V. L.; Purger, D.; Filep, R.; Sándor, V.; Turcsi, E.; Gulyás-Fekete, G.; Deli, J. Lutein isomers: preparation, separation, structure elucidation and occurrence in twenty medicinal plants. *Molecules* **2023**, *28*, 1187.
(39) Molnár, P.; Deli, J.; Matus, Z.; Tóth, G.; Steck, A.; Pfander, H. Isolation and Characterization of Mutatoxanthin-epimers from Red Paprika (*Capsicum annuum*). *Eur. Food Res. Techn.* **2000**, *211*, 396−399.
(40) Deli, J.; Molnár, P.; Matus, Z.; Tóth, G.; Steck, A.; Pfander, H. Isolation of carotenoids with 3,5,6-trihydroxy-5,6-dihydro-*β*-end groups from red paprika (*Capsicum annuum*). *Helv. Chim. Acta* **1998**, *81*, 1233−1241.
(41) Mialoundama, A. S.; Heintz, D.; Jadid, N.; Nkeng, P.; Rahier, A.; Deli, J.; Camara, B.; Bouvier, F. Characterization of plant carotenoid cyclases as members of the favoprotein family functioning with no net redox change. *Plant Physiol.* **2010**, *153*, 970−979, DOI: 10.1104/pp.110.155440.

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