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Cryptoxanthin Structural Isomers in Oranges, Orange Juice, and Other Fruits

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Citrus fruits contain a wide range of bioactive compounds. Their carotenoid fraction is *inter alia* dominated by structural cryptoxanthin isomers as β-cryptoxanthin and zeinoxanthin. Both xanthophylls were identified in saponified citrus fruit extracts by comparison to reference compounds extracted from corn and by their typical fragmentation pattern in LC-(APCI)MS analyses. α-Cryptoxanthin, another structural cryptoxanthin isomer usually found in carrot leaves, was not identified in the citrus fruits studied. Cryptoxanthin concentrations of direct orange juices (D) and reconstituted juices (C) were compared. Although the respective mean values [β-cryptoxanthin, 62 (C) versus 110 μg/100 g (D); zeinoxanthin, 22 (C) versus 37 μg/100 g (D)] were statistically distinguishable ($P < 0.05\%$), a doubtless classification is not possible because the concentration ranges overlap. To identify esters of structural cryptoxanthin isomers in native orange juice extracts, four saturated acyl esters were synthesized. LC-(APCI)MS studies revealed for the first time that the dominant acylation partners of both xanthophylls were C12:0, C14:0, and C16:0 in nearly equal amounts of roughly one-third, whereas C10:0 and C18:1 were present at lower extents of 5−14%; other acylation partners were not identified. The presented method is appropriate to gain deeper insight into the pattern of structural cryptoxanthin isomers of citrus fruits. Knowledge of acylated cryptoxanthin isomers may be important in the evaluation of the bioavailability of individual esters in future human digestion studies.

KEYWORDS: **β-Cryptoxanthin; α-cryptoxanthin; zeinoxanthin; orange juice; LC-(APCI)MS**

INTRODUCTION

The carotenoid family can be divided into two subclasses by the presence or absence of oxygen in their structure. If at least one oxygen atom is present in the molecule, the substances are called xanthophylls; otherwise, they are termed carotenes. Hydroxylation of β-carotene gives rise to a prominent monohydroxylated xanthophyll known as β-cryptoxanthin [(*3R*)-β,β-caroten-3-ol]. Although the β,β-skeleton is symmetric and metabolic hydroxylation generates only one (*R*)-isomer, hydroxylation of the asymmetric β,ε-backbone results in two structural (*R*)-configured cryptoxanthin isomers, called α-cryptoxanthin [(*3′R,6′R*)-β,ε-caroten-3′-ol] and zeinoxanthin [(*3R,6′R*)-β,ε-caroten-3-ol] (**Figure 1**). This designation is in accordance with nomenclature given in the *Carotenoids Handbook* (*1*). Due to the presence of one unsubstituted β-ionone ring, only α- and β-cryptoxanthin act as provitamin A precursors, whereas zeinoxanthin is vitamin-inactive. Up to now, only β-cryptoxanthin is commercially available as reference compound. However, Khachik provided a method for the production of α- and β-cryptoxanthin from commercially available lutein (*2*).

The most popular compound, β-cryptoxanthin, is found in red paprika, citrus fruits, squash, persimmon, and tropical fruits such as papaya and mango (*3*−*5*). Citrus fruits accounted for 68% of the total β-cryptoxanthin present in the common Spanish diet (*6*). Analytical methods to quantify free β-cryptoxanthin as well as several esterified derivatives have been established (*7*, *8*). In a recent investigation, β-cryptoxanthin was actually used as a marker to quantify the orange color in food (*9*). Several studies concerning the biological activity have been undertaken. Derived from the results of an epidemiological study, ingestion

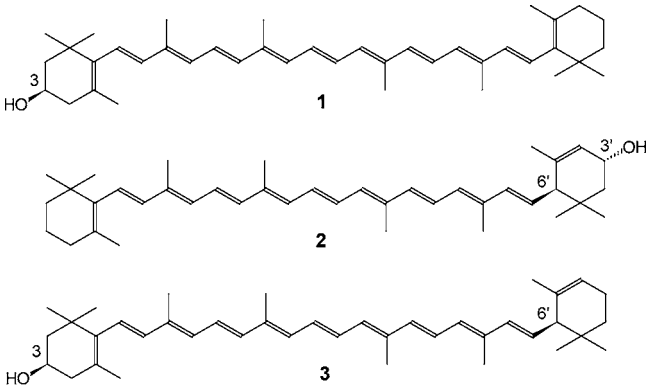

Figure 1. Chemical structures of cryptoxanthin isomers: β-cryptoxanthin [**1**; (*3R*)-β,β-carotene-3-ol]; α-cryptoxanthin [**2**; (*3′R,6′R*)-β,ε-carotene-3′-ol]; zeinoxanthin [**3**; (*3R,6′R*)-β,ε-carotene-3-ol].

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of β-cryptoxanthin together with zinc may reduce the risk of rheumatoid arthritis (*10*). Takuji et al. found that special fractions of Satsuma mandarins (*Citrus unshiu*) inhibit chemically induced rat colon and mouse lung tumorigenesis (*11*). Uchiyama et al. demonstrated that β-cryptoxanthin from Satsuma mandarins has an anabolic effect on bone components in aged female rats *in vivo* and *in vitro* (*12*), and Yamaguchi proposed that β-cryptoxanthin may be a preventive remedy for bone diseases such as osteoporosis (*13*). Yuan et al. proposed that dietary β-cryptoxanthin acts as a chemopreventive agent for lung cancer in humans (*14*). Little is known about physiological roles of α-cryptoxanthin and zeinoxanthin. Recently, Craft et al. found that α- and β-cryptoxanthin belong to the major xanthophylls found in the human brain. In particular, the frontal cortex, which is generally susceptible to Alzheimer's disease, had higher concentrations of antioxidants than other parts (*15*). Fundamental investigations of Wingerath et al. straightened out the bioavailability of native β-cryptoxanthin esters from tangerines and the absence of related esters in human chylomicrons (*16*). A comparison of the bioavailability of β-cryptoxanthin esters and the free form was presented by Breithaupt et al. (*17*). A review on the role of carotenoids, among others, β-cryptoxanthin, in human physiology was given by Landrum et al. (*18*).

The structural cryptoxanthin isomers zeinoxanthin and α-cryptoxanthin were often confused in the literature, causing misinterpretation of the carotenoid content of foods. For example, Meléndez-Martínez et al. described an HPLC method for the separation of carotenoids in orange juice. β-Cryptoxanthin and zeinoxanthin were separated well, but they designated the—however correct—structure of the latter as α-cryptoxanthin (*5*). The same incorrect nomenclature was used in other papers (*19*−*23*). Thus, readers may conclude that especially orange juice contains α-cryptoxanthin. The problem of structural cryptoxanthin isomer confusion was recognized three years ago by Mercadante and Rodriguez-Amaya (*24*). They pointed out that a lot of Brazilian green leafy vegetables contained α-cryptoxanthin, not β-cryptoxanthin as reported in the literature; the α-cryptoxanthin standard used by this group was isolated from *Amaranthus viridis*. Sometimes, the clearly visible zeinoxanthin peak eluting in front of β-cryptoxanthin was ignored (e.g., ref *25*). On the other hand, the presence of a structural cryptoxanthin isomer in corn grain has been known for decades (*26*, *27*). Carrot leaves are known as good sources of α-cryptoxanthin (28). Recently, zeinoxanthin was determined in the hips of *Rosa rubiginosa* (5.9 μg/g; ref *29*). A unique occurrence of zeinoxanthin was not established.

Because the xanthophyll pattern of oranges (*Citrus sinensis*) is complex and is subject to considerable variations (*25*), quantitative data on structural cryptoxanthin isomers are inconsistent. Furthermore, most databases (e.g., the USDA-NCC carotenoid databases; ref *30*) do not list structural cryptoxanthin isomers apart from β-cryptoxanthin. Knowledge about the native occurrence of cryptoxanthin isomer esters in plants is satisfying only for β-cryptoxanthin. Several characteristic β-cryptoxanthin esters (mainly C10:0 −C16:0 esters) have been identified in orange juice concentrate (e.g., refs *31* and *32*). Zeinoxanthin esters (even though designated α-cryptoxanthin esters) were tentatively identified by Philip et al. (*33*). However, reference standards were not available, and MS data are lacking.

Taken together, the main purpose of the present study was to clarify the confusing situation concerning the correct structures of structural cryptoxanthin isomers, especially in orange juice. Thus, standards were isolated from food plants and were characterized by HPLC(DAD) and by liquid chromatography atmospheric pressure chemical ionization mass spectrometry [LC-(APCI)MS]. Special attention was paid to the possibility to differentiate direct orange juice and juice made from concentrate by taking advantage of the relationship of structural cryptoxanthin isomer concentrations. Furthermore, LC-(APCI)-MS experiments were carried out to identify esters of structural cryptoxanthin isomers in concentrated native orange juice extracts.

MATERIALS AND METHODS

Chemicals. Light petroleum ether (boiling fraction 40−60 °C), methanol, ethyl acetate, ethanol, diethyl ether, and silica gel 60 (0.063−0.200 mm) were purchased from Merck (Darmstadt, Germany); *tert*-butyl methyl ether (TBME), magnesium hydroxycarbonate, pyridine, and acyl chlorides (C10:0, C12:0, C14:0, C16:0; purity = 99% each) were from Sigma-Aldrich (Taufkirchen, Germany). All solvents were distilled before use.

Reference Compounds. The reference substance β-cryptoxanthin was generously provided by DSM (Kaiseraugst, Switzerland).

Samples. Oranges, orange juice, minneola, tangerine, kumquat, tangelo, nectarine, avocado, canned corn (sweet yellow whole kernels, drained), and fresh carrot leaves were obtained from local supermarkets. Four direct orange juices, obtained at the place of manufacture in the country of origin (see **Table 2**), were kindly provided by SGF International (Schutzgemeinschaft der Fruchtsaftindustrie e.V, Nieder-Olm, Germany).

Preparation of Samples. *Extraction.* Edible portions were cut into small pieces and homogenized for ∼1 min. Orange juices were shaken well before sampling. Samples of citrus fruits, orange juices, and corn (10.0 g each) were extracted four times with a mixture of methanol/ethyl acetate/light petroleum (1:1:1, v/v/v; 15 mL each). The supernatants were collected; 2 mL of ethanol was added to remove traces of water, and then they were evaporated to dryness. The residues was saponified or dissolved in TBME/methanol (1:1, v/v; 2 mL) and subjected to HPLC-DAD analyses.

Saponification. For saponification, the residue was dissolved in diethyl ether (50 mL). After the addition of methanolic KOH (30%, w/v; 5 mL), the flask was stored overnight in the dark at room temperature. The reaction mixture was transferred into a separator funnel, washed three times with water, mixed with 2 mL of ethanol to remove traces of water, evaporated to dryness under reduced pressure, dissolved in 2 mL of TBME/methanol (1:1, v/v) and subjected to HPLC-DAD analyses.

Influence of Magnesium Hydroxycarbonate (MHC) on Extraction Yield. To test whether there is an influence of the pH on extraction yields, MHC (20 mg) was added to orange juice samples (10 mL) as suggested by Cortés et al. (*19*), causing a pH shift from 3.6 to 4.1 in one test sample. Workup was done as described, and the resulting β-cryptoxanthin and zeinoxanthin concentrations were compared to those obtained without the addition of MHC. The following results were obtained ($n = 3$): β-cryptoxanthin, 78.1 mg (with MHC), 76.0 mg (without MHC); zeinoxanthin, 26.9 mg (with MHC), 27.7 mg (without MHC). Using F and t tests, results were statistically not distinguishable ($P < 0.05$).

Isolation of α-Cryptoxanthin from Carrot Leaves. Fresh carrot leaves (100 g, without thick stems) were minced with a knife, homogenized using an ESGE M100-mixer (ESGE AG, Mettlen, Switzerland; 1 min), and extracted three times with a mixture of methanol/ethyl acetate/light petroleum ether (1:1:1 v/v/v; 100 mL each). For removal of chlorophylls, the organic extract was saponified as described above (10 mL of methanolic KOH/100 mL of diethyl ether), washed to remove alkali, and evaporated to dryness. The residue was dissolved in light petroleum ether (20 mL) and subjected to preparative open column chromatography (glass column, 400 × 20 mm) on silica gel (10 g) suspended in light petroleum ether. Aliquots of 5 mL were poured onto the column head. The first band, obtained by elution with pure light petroleum ether, consisted mainly of β-carotene and was discarded. The second band (light petroleum ether/acetone 95:5, v/v) was shown to consist of α-cryptoxanthin, which was identified by LC-

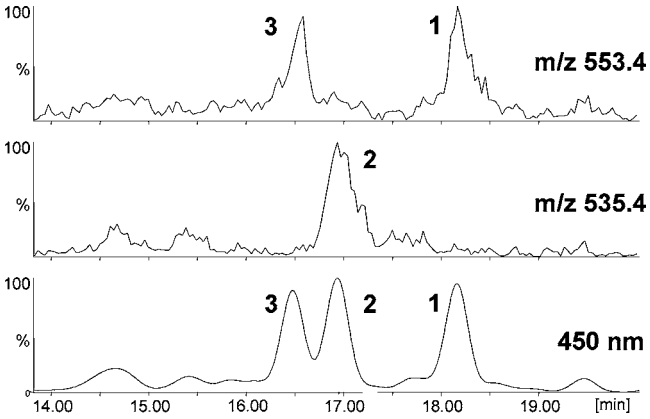

Figure 2. LC-(APCI)MS analysis (extended section) of a mixture of structural cryptoxanthin isomers. The lower trace corresponds to the UV–vis detection at 450 nm (DAD); other traces show selected molecular masses, suitable for detection of α-cryptoxanthin (**2**; *m*/*z* 535.4, $[M + H - H_2O]^+$) or β-cryptoxanthin (**1**) and zeinoxanthin (**3**) (*m*/*z* 553.4, $[M + H]^+$).

(APCI)MS analyses, the UV−vis spectrum, and the retention time (**Figure 2**). Further polar carotenoids remaining on the column were discarded with the silica gel.

Synthesis of β-Cryptoxanthin and Zeinoxanthin Acyl Esters. Canned corn (500 g) was homogenized using an ESGE M100-mixer (ESGE AG; 1 min). Aliquots (100 g) of the resulting mush were repeatedly extracted with a mixture of methanol/ethyl acetate/light petroleum ether (1:1:1 v/v/v; volume of combined organic phases = 1 L). The extract was saponified as described for carrot leaves. The final carotenoid extract was dissolved in TBME/methanol (1:1, v/v; 20 mL) and used for xanthophyll ester synthesis as described earlier (*7*). In brief, aliquots (1 mL) were dried in a stream of nitrogen, and the residue was dissolved in dry pyridine (2 mL) and reacted dropwise with four acyl chlorides (C10:0, C12:0, C14:0, or C16:0, respectively; 80 μL each) over a period of 1 h. The products were extracted with methanol/ethyl acetate/light petroleum ether (1:1:1 v/v/v; 5 mL), and the extract was washed three times with sodium hydrogen carbonate solution (1% w/v; 2 mL each). The organic phase was mixed with ethanol (1 mL) to remove traces of water and evaporated to dryness using a rotary evaporator. The carotenoid ester containing solution was redissolved in TBME/methanol (1:1, v/v; 2 mL) and stored at −20 °C. Thus, the respective esters of both xanthophylls were obtained in one step. Aliquots (1 mL) of all obtained solutions were mixed in a final step, concentrated to 0.5 mL in a stream of nitrogen, and subjected to LC-(APCI)MS analyses.

Fractionation of Orange Juice Samples for LC-(APCI)MS Measurements. To obtain samples with high concentrations of cryptoxanthin isomers and low concentrations of interfering compounds (triacylglycerides), a cleanup step on silica gel was performed. One hundred milliliters of orange juice was extracted in aliquots as described above. The final carotenoid extract was dissolved in light petroleum ether (10 mL) and subjected to preparative open-column chromatography (glass column, 400 × 20 mm) on silica gel (10 g) suspended in light petroleum ether. The first band (mainly β-carotene), obtained by elution with pure light petroleum ether, was discarded. Bands obtained by elution with light petroleum ether/acetone 95:5 (v/v; elution of cryptoxanthin esters and free cryptoxanthin structural isomers) and 90:10 (v/v; to ensure complete elution) were collected together. Polar free xanthophylls, remaining on the column, were discarded together with the silica gel. The solvent was removed under vacuum, and the residue was dissolved in TBME/methanol (1:1, v/v; 1.5 mL) and directly subjected to LC-(APCI)MS analysis. In additional experiments, it was assured that the respective peak patterns found in HPLC chromatograms of orange juice extracts and of cleaned samples were identical. Thus, elution with the solvent systems given allows for isolation of free and esterified cryptoxanthin structural isomers.

High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography−Mass Spectrometry Using an Atmospheric Pressure Chemical Ionization Interface [LC-(APCI)MS]. *Apparatus and Conditions.* The HPLC consisted of a modular system HP1100 (Hewlett-Packard GmbH, Waldbronn, Germany) with a diode array detector (DAD, 450 nm). For separation, a YMC analytical column (YMC Europe, Schermbeck, Germany) with 5 μm C30 reversed phase material (250 × 4.6 mm i.d.) including a precolumn (10 × 4.0 mm i.d.) was used and kept at 35 °C. LC-(APCI)MS was performed on an HP1100 modular HPLC system, coupled to a Micromass (Manchester, U.K.) VG platform II quadrupole mass spectrometer. The MS parameters and the mobile phases have been detailed earlier (*34*). Mass spectra were acquired with a scan range *of m*/*z* 300−1200, and the data were processed with MassLynx 3.2 software.

Quantification. β-Cryptoxanthin was quantified in the range of 0.5−21.5 mg/L using a reference standard. A calibration curve was recorded by plotting the peak area (DAD, 450 nm) *versus* the concentration. Due to similar molar extinction coefficients, the same graph was applied for the quantification of zeinoxanthin.

Evaluation of the Workup Procedure. To determine possible losses of structural crpytoxanthin isomers during workup, recovery experiments using zeaxanthin as reference compound were performed. Aliquots of an orange juice (10 mL) were spiked with 1 mL of ethanolic zeaxanthin solution (c = 4.5 μg/mL; based on ϵ = 144 500 L mol^{-1} cm^{-1} at 451 nm in ethanol; isolated from saponified orange pepper extract) and were worked up as described above (final volume = 2 mL). The amount of zeaxanthin added mimicked the β-cryptoxanthin concentration usually found in orange juice. The zeaxanthin concentration of this sample is referred to as c_2; the native zeaxanthin concentration is designated c_0 (n = 4). For preparation of a reference solution, another aliquot (1 mL) was evaporated under vacuum and redissolved in TBME/methanol (1:1 v/v; 1 mL; c_1). Recoveries of zeaxanthin were calculated as follows: % recovery = $(c_2 - c_0) \times 100/c_1$. The following recovery was obtained (n = 3): 94.8 ± 8.7%. This value refers to the entire workup process and includes extraction as well as saponification.

Detection Limit of Cryptoxanthin Isomers. On the basis of the use of a 10 mL sample, a final volume of 2 mL for HPLC, and an injection volume of 20 μL, the limit of detection (LOD) was estimated from the β-cryptoxanthin calibration graph on the basis of a signal-to-noise (S/N) ratio of 3:1 to be 6 μg/100 g.

RESULTS AND DISCUSSION

Identification of Cryptoxanthin Isomers. To identify α-cryptoxanthin and zeinoxanthin, two plant sources were chosen for extraction of reference compounds: α-cryptoxanthin was isolated from a saponified extract of carrot leaves by open-column chromatography on silica gel, whereas zeinoxanthin and β-cryptoxanthin were obtained from saponified corn extract. To evaluate the separation efficiency of the C30 phase applied and to identify structural cryptoxanthin isomers unequivocally by LC-(APCI)MS, aliquots of the prepared α-cryptoxanthin solution and of the mix of zeinoxanthin and β-cryptoxanthin (solutions in TBME/methanol, 1:1, v/v) were combined and analyzed using an APCI interface, operated in the positive mode (**Figure 2**). All cryptoxanthin isomers were well separated. For identification, two mass traces were utilized: β-cryptoxanthin (**1**) and zeinoxanthin (**3**) were identified on the basis of their quasimolecular ion at *m*/*z* 553.4 ($[M + H]^+$, 100%). The tendency for fragmentation was low in both cases ($[M + H - H_2O]^+$, 3%). This behavior is different from that of α-cryptoxanthin (**2**), which showed a quasimolecular ion with low relative abundance (9%) and an intense fragment ion of 535.4 Da (100%). This pattern is typical for xanthophylls comprising at least one hydroxylated α-ionone ring in their structure (e.g., lutein; ref *34*) and is in accordance with the findings of Mercadante and Rodriguez-Amaya (70 eV-EI-MS; ref *24*), supporting the assignment of α-cryptoxanthin. The isotopic ratio pattern of the $[M + H]^+$ ions of **1** and **3** clearly indicated the

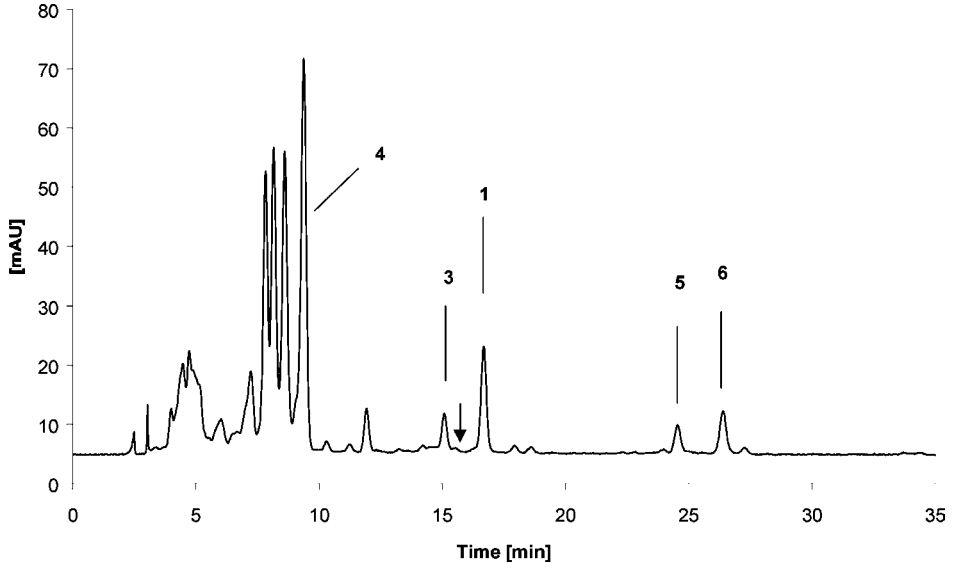

Figure 3. Typical HPLC chromatogram (DAD, 450 nm; extended section) of a saponified orange juice extract (according to sample D8, **Table 2**). Peak assignment: **1**, β-cryptoxanthin; **3**, zeinoxanthin; **4**, lutein; **5**, α-carotene; **6**, β-carotene. The expected retention time of α-cryptoxanthin (**2**) is marked by an arrow.

presence of xanthophylls with C40 skeletons (e.g., **1**, $[M + H]^+$ 100%; $[M + H + 1]^+$ 44%; $[M + H + 2]^+$ 11%) and is in accordance with that theoretically calculated for $C_{40}H_{57}O$ (MS software MassLynx 3.2). Additionally, UV−vis maxima obtained by DAD were used to characterize cryptoxanthin isomers. The following maxima were obtained [data (nm) given at time of elution; "s" designates a shoulder]: β-cryptoxanthin (**1**), 426-(s)/452/480; α-cryptoxanthin (**2**), 424(s)/446/474; zeinoxanthin (**3**), 424(s)/446/474. The similarity of the UV−vis maxima of **2** and **3** and a 4−6 nm bathochromic shift of the central maximum of **1** is in accordance with literature data (*1*). Furthermore, β-cryptoxanthin identification was ascertained by spiking the mixture with a β-cryptoxanthin reference standard, resulting in coelution at the retention time anticipated.

Structural Cryptoxanthin Isomers in Fruits. Extracts of citrus fruits were screened for the presence of structural cryptoxanthin isomers by HPLC-DAD. α-Cryptoxanthin was identified in no case unequivocally, although a small peak appeared at the respective retention time (see **Figure 3**, peak marked with an arrow). However, the concentration was too low to state quantitative data (LOD = 6 μg/100 g). As shown in **Table 1**, β-cryptoxanthin was present in each fruit extract, and zeinoxanthin was found in most of them in variable amounts. Highest β-cryptoxanthin concentrations were determined in minneola (761 μg/100 g) and tangerine samples (755 μg/100 g). In citrus fruits, β-cryptoxanthin formed the predominant structural cryptoxanthin isomer. Consequently, the ratio of the β-cryptoxanthin and zeinoxanthin concentrations was >1.0. The opposite occurred for the same ratio calculated for corn samples because zeinoxanthin formed the major structural isomer (**Table 1**). In extracts of minneola and tangerine—which comprised exceptionally high β-cryptoxanthin concentrations—it was not possible to unequivocally identify minute amounts of zeinoxanthin because *cis*-configured carotenoids (with typical *cis*-bands in their UV−vis spectra) coeluted at the respective retention time. These additional peaks may represent *cis*-β-cryptoxanthin isomers. Thus, in those cases the zeinoxanthin concentration is not given. Further on, the only green fruit investigated comprising β-cryptoxanthin and zeinoxanthin in substantial concentrations was avocado. The following amounts were found in two avocado samples originating from Israel (cv. Pinkerton) and Spain (cv. Hass), respectively: β-cryptoxanthin, 41.0/88.5 μg/100 g; zeinoxanthin, 28.7/80.8 μg/100 g.

Table 1. Concentrations of β-Cryptoxanthin and Zeinoxanthin Determined after Saponification in Citrus Fruits and Corn[a]

sample	β-cryptoxanthin (μg/100 g)	zeinoxanthin (μg/100 g)	β-C/Z
orange 1 (Spain)	183.9 ± 12.6	68.7 ± 4.9	2.7
orange 2 (Spain)	163.1 ± 12.2	44.1 ± 3.2	3.7
orange 3 (South Africa)	117.8 ± 5.2	49.7 ± 1.6	2.4
orange 4 (South Africa)	87.8 ± 6.1	42.2 ± 2.8	2.1
orange 5 (Spain)	73.0 ± 0.5	17.2 ± 0.5	4.2
orange 6 (Spain)	237.7 ± 17.9	42.8 ± 1.6	5.6
blood orange (Italy)	162.7 ± 6.3	50.6 ± 1.1	3.2
tangelo (Jamaica)	114.7 ± 6.2	30.4 ± 1.5	3.8
minneola (Greece)	760.8 ± 76.0	*b*	
minneola (Israel)	264.4 ± 27.6	*b*	
tangerine (Spain)	754.6 ± 19.0	*b*	
nectarine	100.0 ± 5.7		
kumquat	79.5 ± 17.2		
canned corn 1	21.2 ± 2.5	76.4 ± 5.3	0.3
canned corn 2	22.4 ± 5.1	89.7 ± 1.7	0.2
canned corn 3	36.2 ± 3.4	223.9 ± 14.9	0.2
canned corn 4	39.0 ± 0.1	78.5 ± 8.7	0.5
sweet corn[c]	47.9 ± 4.7	73.6 ± 6.3	0.7

[a] Quantitative amounts (n = 3) were calculated from the β-cryptoxanthin calibration curve. The ratio of the β-cryptoxanthin and zeinoxanthin concentrations is given. Outliers were eliminated according to the method of Nalimov (P < 0.05). [b] Due to the coelution with *cis*-β-cryptoxanthin isomers, unequivocal identification is not possible. [c] Ready-cooked, on the cob.

Cryptoxanthin Isomers in Saponified Orange Juice Extracts. Special attention was paid to cryptoxanthin analysis in orange juices. Again, α-cryptoxanthin was not unequivocally identified. Zeinoxanthin formed one of the monohydroxylated xanthophylls in orange juice (**Figure 3**). On the basis of their typical UV−vis spectra and in accordance with literature data (*5*, *25*), lutein was identified in the polar region, and α- and β-carotene were identified in the more apolar region of the chromatogram. Assignment of further xanthophylls was not accomplished. According to other publications (*5*, *19*, *20*), it is assumed that neoxanthin, neochrome, violaxanthin, antheraxanthin, mutatoxanthin, lutein-5,6-epoxide, and various *cis*-

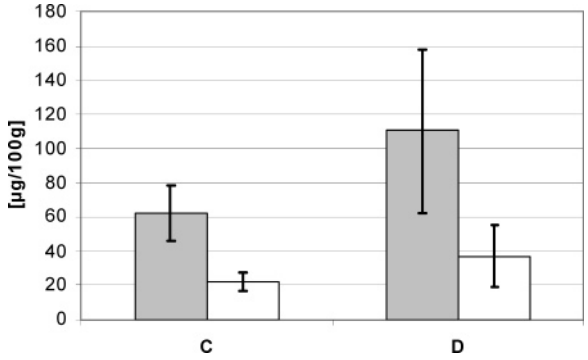

Figure 4. Statistical spread of β-cryptoxanthin and zeinoxanthin concentrations (μg/100 g) in direct orange juices (D) and in orange juices made from concentrate (C; $n = 15$, each). Gray bars represent β-cryptoxanthin and white bars, zeinoxanthin. The complete data set is given in **Table 2**.

derivatives thereof form the major part of the total xanthophyll fraction.

For comparison of quantitative data it is anticipated that results given in the literature are referred to zeinoxanthin and not to α-cryptoxanthin as often indicated. Average concentrations of β-cryptoxanthin and zeinoxanthin in juices made from concentrate by reconstitution (C) or direct juice (D) were calculated as follows: for β-cryptoxanthin, 61.8 ± 16.1 μg/100 g (C) *versus* 110.3 ± 47.9 μg/100 g (D); and for zeinoxanthin, 21.8 ± 5.2 μg/100 g (C) *versus* 37.1 ± 18.3 μg/100 g (D) (**Figure 4**). Extracts made from minced fresh fruits lay—with the exception of orange sample 6 (**Table 1**)—in the overall ranges found for both xanthophylls in direct juices (not for reconstituted juices). For raw orange juice, the USDA-NCC carotenoid database states mean β-cryptoxanthin concentrations varying from 15 to 324 μg/100 g for hybrid varieties (*30*). Cortés et al. (*19*) determined the following mean concentrations in orange juice (β-cryptoxanthin *versus* zeinoxanthin): 118.31/42.29 μg/100 g. Sánchez-Moreno et al. (*21*) gave for fresh Valencia orange juice 314.09/161.82 μg/100 mL. Nam et al. quantified only β-cryptoxanthin in the flesh of citrus fruits cultivated in Korea and America (70 μg/100 g in Valencia orange flesh) (*35*). Pupin and co-workers gave a mean β-cryptoxanthin level in hand-squeezed Brazilian orange juice from seven varieties of only 5 μg/100 mL (*25*). Apart from the latter results, the results reported here lie within the expected range.

Special attention was paid to the question of whether the cryptoxanthin isomer pattern allows for distinguishing direct orange juice from reconstituted juice. Remarkably, the β-cryptoxanthin concentration of reconstituted juice was found to be in a narrow range (41−97 μg/100 g, $n = 15$), whereas that of direct juice varied widely (43−183 μg/100 g; $n = 15$) (**Figure 4**). The same trend—however, less explicit—was observed with the corresponding zeinoxanthin concentrations. The respective mean values [β-cryptoxanthin, 62 μg (C) *versus* 110 μg/100 g (D); zeinoxanthin, 22 μg (C) *versus* 33 μg/100 g (D)] were statistically distinguishable ($P < 0.05\%$). Because the respective ratios of β-cryptoxanthin and zeinoxanthin (β-C/Z values; **Table 2**) were statistically not distinguishable ($P < 0.05$), they offer no possibility to differentiate between both juice types. Because the concentration ranges of β-cryptoxanthin and zeinoxanthin overlap, assignment of unknown juices to one fruit juice type on the basis of only the cryptoxanthin isomer concentration is problematic. However, typical results may contribute together with other analytical parameters to a correct classification. One reason for the overall lesser cryptoxanthin isomer concentration in processed juice may be found in degradation reactions during juice production (e.g., multiple heating and cooling cycles). Further studies will have to clarify this assumption.

Table 2. Concentrations of β-Cryptoxanthin and Zeinoxanthin Determined after Saponification in Orange Juices [Direct Juice (D) or Juice Made from Concentrate (C)][a]

orange juice	β-cryptoxanthin (μg/100 g)	zeinoxanthin (μg/100 g)	β-C/Z
C 1	51.5 ± 2.9	17.3 ± 1.3	3.0
C 2	48.6 ± 2.4	15.8 ± 0.2	3.1
C 3	61.6 ± 2.0	19.9 ± 0.4	3.1
C 4	40.8 ± 1.3	19.6 ± 0.4	2.1
C 5	40.9 ± 2.5	15.2 ± 0.3	2.7
C 6	51.9 ± 2.6	16.3 ± 0.6	3.2
C 7	71.0 ± 3.4	21.1 ± 0.7	3.4
C 8	48.4 ± 4.3	18.0 ± 0.9	2.7
C 9	65.8 ± 4.8	27.4 ± 0.6	2.4
C 10	64.2 ± 0.1	23.6 ± 0.1	2.7
C 11	88.8 ± 9.1	28.6 ± 0.5	3.1
C 12	59.9 ± 2.0	21.3 ± 0.6	2.8
C 13	97.0 ± 6.9	32.3 ± 0.6	3.0
C 14	64.9 ± 1.0	25.1 ± 0.1	2.6
C 15	71.5 ± 3.1	25.8 ± 0.3	2.8
D 1*	130.6 ± 0.3	40.5 ± 1.3	3.2
D 2*	65.2 ± 7.0	40.6 ± 1.9	1.6
D 3*	183.2 ± 0.8	82.2 ± 2.8	2.2
D 4*	61.4 ± 1.5	29.5 ± 2.0	2.1
D 5	80.2 ± 2.4	19.9 ± 0.4	4.0
D 6	42.9 ± 1.9	17.4 ± 1.6	2.5
D 7	80.3 ± 0.7	22.5 ± 1.0	3.6
D 8	61.2 ± 0.4	23.1 ± 0.4	2.6
D 9	115.8 ± 8.1	31.2 ± 1.6	3.7
D 10	130.5 ± 0.1	31.8 ± 2.0	4.1
D 11	152.6 ± 9.2	51.7 ± 0.9	3.0
D 12	169.5 ± 4.0	36.5 ± 1.5	4.6
D 13	64.2 ± 1.5	24.5 ± 1.0	2.6
D 14	139.5 ± 1.8	36.5 ± 0.8	3.8
D 15	177.0 ± 13.2	69.1 ± 0.7	2.6

[a] Quantitative amounts ($n = 3$) were calculated from a β-cryptoxanthin calibration curve. The ratio of the β-cryptoxanthin and zeinoxanthin concentrations is given. Outliers were eliminated according to the test of Nalimov ($P < 0.05$). Original juice samples marked with an asterisk (*) were directly obtained at the place of manufacture by SGF International (Schutzgemeinschaft der Fruchtsaftindustrie e.V., Nieder-Olm, Germany). Country of origin: D1, Cyprus; D2 and D4, Brazil; D3, Spain.

Native Cryptoxanthin Isomer Esters in Orange Juice Extracts. To identify native zeinoxanthin and β-cryptoxanthin esters, saturated acyl esters (C10, C12, C14, and C16) were synthesized as reference compounds. As a readily available basic material, crude saponified corn extract, containing both xanthophylls in roughly equal amounts, was applied. Thus, synthesis of two acyl derivatives was achieved in one step. The resulting xanthophyll ester solutions were combined and analyzed by LC-(APCI)MS in the positive mode without further cleanup. The resulting chromatogram (**Figure 5**, bottom trace) documents that it is possible to separate zeinoxanthin and β-cryptoxanthin acyl derivatives by C30 HPLC, although the structures differ only in the position of one double bond, which is either allylic or conjugated. Small peaks belong to *cis*-isomers, formed as reaction byproducts. Each MS spectrum was dominated by loss of the respective fatty acid from the quasimolecular ion, resulting in a common backbone ion at m/z 535.4 $[M + H - \text{fatty acid}]^+$. Because the fragmentation and the intensity of the backbone ions were similar, the bathochromic shift of the central β-cryptoxanthin maximum offers the only possibility to distinguish zeinoxanthin and β-cryptoxanthin esters. Unfortunately, baseline separation of native xanthophyll esters was not achieved, hampering identification of individual esters on the basis of their UV−vis spectra (**Figure 5**, sample trace). If the masses of the

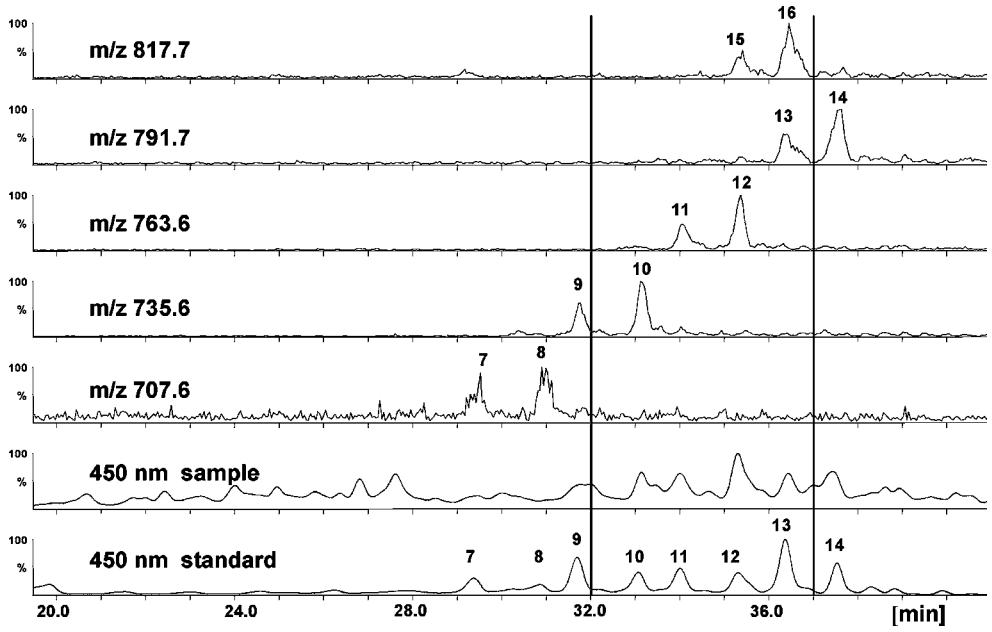

Figure 5. LC-(APCI)MS chromatogram (extended section; DAD and selected mass traces) of a mixture of synthesized β-cryptoxanthin and zeinoxanthin esters (C10:0, C12:0, C14:0, and C16:0) and one direct orange juice extract (cleaned and concentrated by open-column chromatography on silica gel). Peak assignment (β-cryptoxanthin, Cr; zeinoxanthin, Ze): **7**, Ze-C10:0; **8**, Cr-C10:0; **9**, Ze-C12:0; **10**, Cr-C12:0; **11**, Ze-C14:0; **12**, Cr-C14:0; **13**, Ze-C16:0; **14**, Cr-C16:0; **15**, Ze-C18:1; **16**, Cr-C18:1.

respective β-cryptoxanthin and zeinoxanthin esters were used to scan the total ion current trace, a unique peak pattern was received (**Figure 5**). Comparison of the respective retention times of the standard compounds with the mass signals found in native orange juice extracts supports the assumption that the masses de facto belong to β-cryptoxanthin and zeaxanthin esters, the latter generally eluting in front of the respective β-cryptoxanthin esters. However, coelution forestalled read-out of undisturbed UV−vis spectra to affirm peak assignment. Identification is assisted by the intensity of the mass signals, which correspond to the concentration ratio found after saponification. Remarkably, only signals originating from the respective C10:0 (*m*/*z* 707.6), C12:0 (*m*/*z* 735.6), C14:0 (*m*/*z* 763.6), C16:0 (*m*/*z* 791.7), and C18:1 (*m*/*z* 817.7) esters were detected. Although the respective C18:1 esters were not synthesized as reference compounds, the mass signals, the retention times, and the UV−vis spectra assist doubtless identification. Screening for masses corresponding to other esters resulted in no signals. However, they may be present in minute amounts.

To get an idea about the ratio of the main fatty acids found in the cryptoxanthin isomer ester fraction, calculated mass signals belonging to the acyl derivatives were integrated and summed. The relative percentages calculated from LC-(APCI)-MS analyses of six direct juices (D3, D8, D9, D10, D11, D15; **Table 2**) are illustrated in **Figure 6**. If the mean concentrations (marked with a cross) were considered, the dominant acylation partners of both xanthophylls were C12:0, C14:0, and C16:0 in nearly equal amounts of roughly one-third (25, 28, and 29 area %), whereas C10:0 and C18:1 (5 and 14 area %) were present to a lower extent. Remarkably, oleic acid was the only unsaturated fatty acid found in this fraction. This is in clear contrast to the fatty acid pattern of the triacylglycerol fraction, where, besides oleic acid (29%), linoleic (32%) and linolenic acid (16%) form the principal components (*36*).

With respect to the selection of orange varieties high in carotenoids of the cryptoxanthin family or for production of new varieties by genetic engineering, it is indispensable to correctly identify individual compounds. The presented method

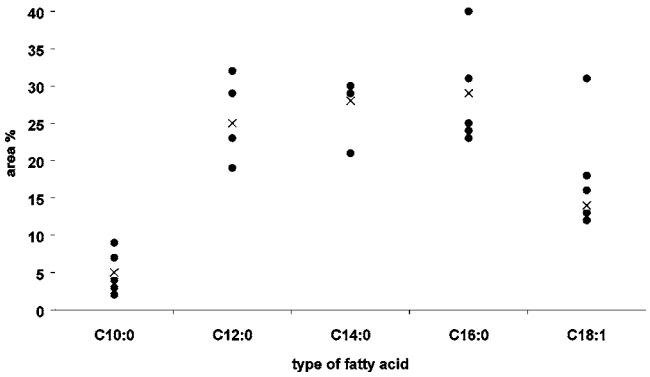

Figure 6. Relative area percent of β-cryptoxanthin and zeinoxanthin esters (C10:0, C12:0, C14:0, C16:0, and C18:1) in six direct orange juices (D3, D8, D9, D10, D11, and D15; **Table 2**), received by integration of the respective mass signals (summed) belonging to individual acyl derivatives. The cross marks mean values ($n = 6$).

is appropriate to gain deeper insight into the structural cryptoxanthin isomer pattern of citrus fruits and may contribute to a better understanding of vitamin A deficiency in human diets. Furthermore, the correct identification of acylated cryptoxanthin isomers is important to evaluate the bioavailability of individual esters in future human digestion studies.

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