Genuine Carotenoid Profiles in Sweet Orange (*Citrus sinensis* (L.) Osbeck cv. Navel) peel and pulp at Different Maturity Stages

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Abstract

The carotenogenesis in the endocarp and flavedo of Navel oranges over four consecutive maturity stages was assessed by HPLC-DAD-APCI-MSⁿ. After optimizing the extraction method, a total of 77 carotenoids including 26 mono- and 33 diesters of violaxanthin, β -citraurin, and antheraxanthin was characterized. Whereas chloroplast-specific pigments such as (all-*E*)-lutein and (all-*E*)- β -carotene predominated in the flavedo of green-ripe fruit, a highly complex pattern of xanthophyll esters was found in the mature oranges. Total carotenoid contents of flavedo were approximately nine-fold higher (12,605 $\mu\text{g}/100\text{ g}$ of FW) than those in the endocarp (1,354 $\mu\text{g}/100\text{ g}$ of FW) at fully mature stage. The mature endocarp abundantly contained violaxanthin mono- and diesters, in addition to diverse antheraxanthin esters, which were exclusively detected in this fruit fraction. Likewise, β -citraurin esters were found to be unique flavedo constituents of mature fruit. Therefore, they may support the detection of fraudulent use of peel fractions during orange juice production.

Keywords: genuine carotenoid profiles; citraurin; antheraxanthin; violaxanthin; xanthophyll esters; Citrus fruit; ripening;

Introduction

Oranges (*Citrus sinensis* (L.) Osbeck) and juices derived thereof are among the most popular fruits worldwide. Their world production accounted for 73.3 million metric tons in 2017.¹ As a rich source of minerals, vitamin C, flavanones, and carotenoids, oranges and orange juice contribute to a healthy human diet.^{2–4} For instance, vascular health benefits such as a decrease of diastolic blood pressure have been proposed to be related with the dietary intake of orange juice.⁵ Furthermore, the regular consumption of orange juice has been shown to lower uric acid levels in human blood plasma, potentially contributing to the prevention of gout.⁶ While the mechanisms involved in the aforementioned health-related benefits are not fully elucidated, the provision of vitamin A by dietary consumption of *Citrus* fruits rich in provitamin A-carotenoids (β -cryptoxanthin, α -carotene, and β -carotene) is well established. In addition, xanthophylls such as antheraxanthin and violaxanthin have been reported to be prevailing pigments in orange pulp and derived juices.^{7,8}

The hydroxyl groups of xanthophylls may be esterified with diverse fatty acids, resulting in a highly complex carotenoid profile in many fruit and vegetable tissues.^{9,10} Approximately 40–80% of the xanthophylls in various *Citrus* fruits were found to be esterified.^{10,11} The acyl moieties reported in the literature comprise saturated (C12, C14, C16, and C18) and monounsaturated (C16:1 and C18:1) fatty acids.¹⁰ Up to 93% of the total carotenoid content presented in orange juice are xanthophyll esters.¹² A recent comparative analysis of the esterified xanthophylls in juices of 21 orange and seven mandarin genotypes revealed a complex pattern highly depending on the varieties and the processing technology.¹³ For simplification of the analytical procedure, a saponification step is frequently included in the analytical protocol for carotenoid determination in orange juice.^{14,15} The most abundant xanthophylls in the saponified pulp extract of Navel oranges were (9Z)-violaxanthin and antheraxanthin, contributing to 67 and 11% of the total carotenoid contents, respectively.¹⁶ After alkaline hydrolysis, (all-*E*)- and (9Z)-violaxanthin at a ratio of approximately 1:4 were found to be the prevailing xanthophylls in the flavedo of mature Navelate oranges.¹⁷ Whereas several studies have reported the xanthophyll ester composition in the edible fraction of sweet oranges at full maturity,^{18,19,13} their ripening-dependent genesis in the flavedo has not been previously considered. Moreover, xanthophyll esters have been proposed to be suitable authentication markers for orange juice.¹² They might be used for the detection of mutual

adulteration of juices derived from different *Citrus* species, and also to unravel the fraudulent admixture of peel extracts or comminuted products to orange juices. In addition, carotenoid profiling may permit to assess the maturity stage of the fruits used for juice extraction. Therefore, the goal of the current study was to characterize esterified and free carotenoids in the endocarp and flavedo of Navel sweet oranges during fruit maturation from mature green to fully ripe fruit by HPLC-DAD-APCI-MSⁿ analyses.

Materials and methods

Reagents

Acetone, calcium carbonate, diethyl ether, methyl *tert*-butyl ether (MTBE), *n*-hexane, ethyl acetate, ethanol, 2-propanol, and sodium hydrogen carbonate were obtained from Merck (Darmstadt, Germany). Methanol and petroleum ether (b.p. 40–60°C) were from VWR International (Fontenay-sous-Bois, France). Butylated hydroxytoluene (BHT) was purchased from Merck-Schuchardt (Hohenbrunn, Germany). (all-*E*)-Violaxanthin was obtained from Sigma-Aldrich (Buchs, Switzerland). Marigold extract beadlets containing 10% lutein were purchased from BioActives Europe (Frankfurt, Germany). (all-*E*)-Antheraxanthin and β -citraurin were supplied by CaroteNature (Ostermündingen, Switzerland). (all-*E*)-Zeaxanthin was from Cayman Chemical (Ann Arbor, MI). (all-*E*)- β -Apo-8'-carotenal, (all-*E*)- β -carotene, (all-*E*)- α -carotene, (all-*E*)-neoxanthin, and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water from an arium[®] 611 (Sartorius, Göttingen, Germany) water treatment system was used throughout. All chemicals were at least of HPLC grade.

Orange fruit samples

Fully ripe Navelina fruit (*Citrus sinensis* (L.) Osbeck) harvested in Valencia (Spain) were purchased from a local supermarket and used for method development. For our investigation into the ripening-dependent changes in carotenoid profile, Navel oranges were cultivated under standard agronomical practices in an experimental orchard in Museros (Valencia, Spain). For each replication and maturity stage, at least 15 fruit were harvested from three adult trees. Fruit were selected for color and size uniformity, and absence of any lesion or injury. The different maturation stages were collected in August (green fruit, G), October (mature-green breaker fruit, GB), December, 2017 (mature fruit, M), and in

January, 2018 (fully mature fruit, FM), corresponding approximately to 120, 180, 240, and 270 days after anthesis.

Carotenoid analyses

Sample preparation

For the method optimization and validation, four oranges were manually peeled, and the endocarp was homogenized under liquid nitrogen in a laboratory blender (Model 8011ES, Waring Products, Torrington, CT).

Oranges harvested at the above-mentioned four maturity stages were processed as follows. Two replicate samples of flavedo of at least 15 fruit per maturity stage were separated with a scalpel. Flavedo and endocarp were immediately frozen in liquid nitrogen and homogenized for 30 s with an electric coffee grinder (Taurus, Barcelona, Spain). Samples were stored in 50-mL tubes under light exclusion and nitrogen atmosphere at $-80\text{ }^{\circ}\text{C}$ until extraction.

Method development and optimization

For method optimization, extraction yields using four different solvent mixtures were compared. The tested solvents comprised two ternary and two binary mixtures, i.e., ethanol/ethyl acetate/*n*-hexane (1/1/1, v/v/v),¹⁸ methanol/ethyl acetate/petroleum ether (1/1/1, v/v/v),²⁰ petroleum ether/2-propanol (1/1, v/v),⁴ and a mixture of acetone/diethyl ether/*n*-hexane (1/1/1, v/v/v). Each 0.1 g/L BHA and BHT were added as antioxidants.

An aliquot of 2.00 ± 0.05 g endocarp, 40 mg of calcium carbonate, and 20 mg of sodium hydrogen carbonate were combined with 3 mL of the respective extraction solvent. After mixing using a test tube shaker (model REAX 1 R, Heidolph Instruments, Schwabach, Germany) and centrifugation, the organic phase was recovered and the sample was re-extracted four times with each 2 mL. After the admixture of 1 mL of methanol to precipitate pectins, the combined organic phase was washed twice with each 3 mL of water. The organic layer was evaporated to dryness under a nitrogen stream and dissolved in acetone. The total carotenoid content expressed as violaxanthin equivalents was determined at 442 nm using a molar extinction coefficient of $144,000\text{ L}/(\text{cm} \cdot \text{mol})$ ²¹ and a UV/Vis spectrometer Lambda 35

(Perkin Elmer, Singapore, Republic of Singapore). The solvent with the highest yield after four extraction steps was selected and the minimum number of extraction steps determined.

Final extraction method

A representative sample of 2.00 ± 0.05 g endocarp or 0.50 ± 0.02 g of flavedo tissue was mixed with appropriate amounts of calcium carbonate and sodium hydrogen carbonate for the neutralization of genuine fruit acids. The required quantities were determined in preliminary experiments to adjust the pH to 6.8–7.4. The flavedo samples were additionally homogenized after adding 1 mL of water using an Ultra Turrax® T25 (IKA, Staufen, Germany). Subsequently, samples were extracted with 3 mL methanol/ethyl acetate/petroleum ether (1/1/1, v/v/v) containing each 0.1 g/L BHA and BHT. The extraction was thrice repeated with each 2 mL of the aforementioned solvent. Following the admixture of 1 mL of methanol, the combined organic layer was washed twice with 3 mL of water. After evaporation of the solvent with a gentle nitrogen stream, the carotenoids were dissolved in 300 μ L MTBE/methanol (50/50, v/v), membrane-filtered, and analyzed by LC-MS as detailed below.

Recovery experiments

Since commercial standards of orange xanthophyll esters were not available, recovery experiments ($n = 3$) were performed by spiking orange endocarp with a genuine orange extract.²² Briefly, an endocarp sample was extracted as described above and 100 μ L of the resulting organic phase was filled into an extraction tube to be evaporated to dryness under a gentle nitrogen stream. An aliquot of 2.00 ± 0.05 g of an endocarp sample was weighed into the same tube and sample workup was carried out as detailed above. Recoveries were estimated by comparing the peak area of the spiked sample to those of the control (no endocarp sample added) and the admixed extract alone. The detection wavelengths were 286 nm for phytoene (recovery 100%), 348 nm for phytofluene (106%), and 439 nm for (all-*E*)-violaxanthin (111%), (all-*E*)-zeaxanthin (101%), (9*Z*)-violaxanthin myristate (108%), (9*Z*)-violaxanthin palmitate (109%), (9*Z*)-violaxanthin laurate-palmitate (106%), (9*Z*)-violaxanthin myristate-palmitate, and (all-*E*)-violaxanthin palmitate-oleate (110%).

128 *HPLC-DAD-APCI-MSⁿ analyses*

129 HPLC-DAD-APCI-MSⁿ analyses were performed using a series 1100 HPLC system with a G1315B
130 diode array detector (both from Agilent, Waldbronn, Germany) coupled on-line to an Esquire 3000+ ion
131 trap mass spectrometer (Bruker Daltonik, Bremen, Germany). The latter was equipped with an
132 atmospheric pressure chemical ionization (APCI) source. HPLC conditions were as detailed by Petry
133 and Mercadante,¹⁹ except for the particle size of the used column (C30 stationary phase, 250 × 4.6 mm
134 i.d., particle size $d_p = 3 \mu\text{m}$ YMC Europe, Dienslaken, Germany). The latter was protected by a C30
135 guard column (10 × 4.6 mm i.d., $d_p = 3 \mu\text{m}$; YMC Europe). UV/Vis spectra were recorded between 200–
136 800 nm, detection wavelengths were 286, 348, and 439 nm. Mass spectra were acquired in the positive
137 ion mode at a capillary potential of +2779 V and a scan range of m/z 100–1,200. Vaporizer and drying
138 temperatures were 400 and 350 °C, respectively. Nitrogen was used as drying (3.5 L/min) and
139 nebulizing gas (65 psi). Fragmentation experiments were performed with helium as collision gas at an
140 amplitude of 1.0 V and an isolation width of 4.0 Th. Control of the LC-MS system and data evaluation
141 were achieved with ChemStation for LC version A.00.03 (Agilent) and Esquire version 5.1 software
142 (Bruker), respectively. Individual compounds were assigned based on retention times (t_R), UV/Vis, and
143 mass spectrometry as well as the comparison with published data^{23,19,24–26} and authentic reference
144 standards.

145

146 *Quantitation*

147 Linear calibration curves were prepared for (all-*E*)-violaxanthin (0.03 to 96.93 mg/L), (all-*E*)-lutein
148 (0.03 to 96.93 mg/L), β -apo-8'-carotenal (0.33 to 21.25 mg/L), and (all-*E*)- β -carotene (0.18 to 20.78
149 mg/L). Violaxanthin, neoxanthin, antheraxanthin, unspecified compounds, and their esters were
150 quantitated using (all-*E*)-violaxanthin. The concentration of (all-*E*)-zeaxanthin was estimated using the
151 calibration curve of (all-*E*)-lutein. (all-*E*)- β -Carotene was used for the quantitation of carotenes as well
152 as phytoene and phytofluene considering their molar extinction coefficients.²³ β -Citraurin was calibrated
153 with β -apo-8'-carotenal. The concentrations of (9*Z*)-isomers were determined using the corresponding
154 (all-*E*)-xanthophylls. All aforementioned carotenoids were quantitated using the HPLC method detailed

above. Lutein was not baseline resolved from an interfering pigment in the early maturity stages and thus, quantitated using the HPLC method established by Hempel et al.²⁷

The concentrations of the individual carotenoids were used to calculate the total colorless carotene (phytoene and phytofluene), total colored carotene (α - and β -carotene), and total free xanthophyll (violaxanthin, antheraxanthin, neoxanthin, zeaxanthin, and lutein) contents. The individual xanthophyll esters were classified as mono- and diesters. Total carotenoid levels represented the sum of colorless and colored carotenes, free and esterified xanthophylls, and unspecified carotenoid compounds (as violaxanthin).

Statistical analyses

Results were evaluated using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) with Tukey's multiple comparison test were applied to determine significant differences of means.

Results and discussion

Comparison of extraction solvents for the extraction of *Citrus* carotenoids

As shown in **Figure 1a**, the yields in total carotenoid contents achieved after four extractions with solvents B (methanol/ethyl acetate/petroleum ether, 1/1/1, v/v/v), C (petroleum ether/2-propanol, 1/1, v/v), and D (acetone/diethyl ether/*n*-hexane, 1/1/1, v/v/v) were significantly higher than that obtained with solvent mixture A (ethanol/ethyl acetate/*n*-hexane, 1/1/1, v/v/v). Although extraction mixture C was shown to be suitable for the extraction of carotenoids from pasteurized and fresh orange juice,⁴ the highest yield of violaxanthin equivalents, amounting to 1.02 mg/100 g of FW, was obtained using mixture B. This ternary mixture of methanol/ethyl acetate/petroleum ether has been previously used for the extraction of diverse xanthophyll esters, e.g., violaxanthin esters from pineapple (*Ananas comosus* (L.) Merr.) flesh²⁸, and β -cryptoxanthin esters from papaya (*Carica papaya* L.) fruits.²⁹ As illustrated by **Figure 1b**, four consecutive steps sufficed for exhaustive extraction of carotenoids from the endocarp of fully ripe oranges. The recoveries achieved for selected compounds compiled above ranged between 100 and 111%, thus being slightly higher than the 96–97% previously reported for diverse xanthophyll

esters extracted from red pepper.²² In brief, the application of four extraction cycles with solvent mixture B (methanol/ethyl acetate/petroleum ether, 1/1/1, v/v/v) was chosen for the quantitative isolation of carotenoids from oranges, allowing the simultaneous extraction of carotenes, xanthophylls as well as their mono- and diesters.

HPLC-DAD-APCI-MSⁿ analysis of genuine carotenoid pattern

Endocarp and flavedo of Navel orange fruit at four different maturity stages were analyzed by HPLC-DAD-APCI-MSⁿ (**Table 1**). Representative chromatograms of flavedo and endocarp extracts from green and fully mature oranges are illustrated in **Figure 2**.

The endocarp of green fruit merely contained the xanthophylls (all-*E*)-violaxanthin (1), (all-*E*)-neoxanthin (2), (9*Z*)-violaxanthin (3a), and (all-*E*)-lutein (7). Most abundant carotenoids in the flavedo of green-ripe fruit were (all-*E*)-violaxanthin (1), (all-*E*)-neoxanthin (2), (9*Z*)-violaxanthin (3a), (all-*E*)-antheraxanthin (3b), (all-*E*)-lutein (7), and (all-*E*)- β -carotene (35). These compounds resembled typical chloroplast-specific accessory pigments as previously reported in *inter alia* immature Cara Cara oranges³⁰ and green goji berries.²⁷ Moreover, (all-*E*)-zeaxanthin (8), (13'*Z*)- and (13*Z*)-lutein (4 and 5) were detected at low abundance, in addition to (all-*E*)- α -carotene (29).

The MS¹ spectrum of (all-*E*)-lutein displayed abundant precursor ions at m/z 551 ([M + H – H₂O]⁺) from the in-source elimination of water as previously reported.³¹ This in-source elimination was also observed in the case of (all-*E*)-neoxanthin ([M + H – H₂O]⁺ at m/z 583.4), whereas the remaining xanthophylls displayed protonated molecules ([M + H]⁺). Noteworthy, (9*Z*)-violaxanthin and (all-*E*)-antheraxanthin (3ab) were not base-line resolved. However, both xanthophylls can be distinguished based on their [M + H]⁺ at m/z 601.5 and 585.5, respectively, and their characteristic mass fragmentations (**Table 1**).

The elution order of (all-*E*)-violaxanthin prior to (9*Z*)-violaxanthin on a C30 stationary phase was in agreement with the literature.⁸ In addition to carotenoids, chlorophylls and their putative degradation products were present in the flavedo and endocarp of green fruit, however, without further characterization in the present work (compounds labelled with asterisks in **Figure 2**).

In the green-breaker stage, diverse xanthophyll mono- and diesters simultaneously emerged in the endocarp and their diversity and quantity increased with progressing maturation. In the flavedo, a few

xanthophyll monoesters were observed in the green-breaker samples and additional mono- and diesters were found in progressed maturity stages.

In the flavedo of fully mature fruit, 22 xanthophyll mono- and 28 diesters were identified. The endocarp contained 21 and 32 mono- and diesters, respectively. The most abundant carotenoids in endocarp and flavedo of fully mature oranges were assigned to (all-*E*)- and (9*Z*)-violaxanthin esters. The latter carotenoids carried both saturated (caprate, C10; laurate, C12; myristate, C14; palmitate, C16; stearate, C18) and unsaturated (palmitoleate, C16:1; oleate, C18:1) acyl moieties. Violaxanthin monoesters displayed protonated molecules $[M + H]^+$ in the APCI(+)-MS¹ spectra, in addition to precursors from the in-source elimination of water ($[M + H - H_2O]^+$, not shown in **Table 1**). In the MS² experiment, collision-induced dissociation (CID) of the protonated molecules resulted in product ions from the elimination of water and the neutral loss of the fatty acyl moiety, i.e., $[M + H - H_2O]^+$, $[M + H - FA]^+$, and $[M + H - H_2O - FA]^+$.

Violaxanthin homodiesters such as (all-*E*)-violaxanthin dilaurate (37b) displayed fragment ions resulting from the eliminations of water and one of two fatty acids ($[M + H - H_2O]^+$, $[M + H - FA_{1/2}]^+$, and $[M + H - H_2O - FA_{1/2}]^+$). The common product ion at m/z 565 was generated by the loss of both fatty acyl moieties ($[M + H - FA_1 - FA_2]^+$). In addition, CID of the $[M + H - H_2O]^+$ precursors resulted in product ions generated by $[M + H - H_2O - FA_1 - FA_2]^+$ and typical in-chain eliminations of toluene from the polyene chain ($[M + H - H_2O - \text{toluene}]^+$) as, e.g., observed for compound 52c assigned to (all-*E*)-violaxanthin palmitate oleate. The losses of toluene and water were also observed for violaxanthin homodiesters (not shown in **Table 1**).

Violaxanthin heterodiesters were distinguished from violaxanthin homodiesters by non-identical fragment ions generated by the eliminations of one of the two fatty acids without ($[M + H - FA_1]^+$ and $[M + H - FA_2]^+$) or with concomitant water loss ($[M + H - H_2O - FA_1]^+$ and $[M + H - H_2O - FA_2]^+$). For instance, CID of (9*Z*)-violaxanthin myristate-oleate (50) that displayed protonated molecules $[M + H]^+$ at m/z 1076.1 resulted in fragment ions at m/z 847.8 ($[M + H - FA_1]^+$) and 793.5 ($[M + H - FA_2]^+$) from the neutral loss of myristic and oleic acid, respectively. The loss of both fatty acid moieties resulted in the fragment m/z 565.4 ($[M + H - FA_1 - FA_2]^+$). These fragmentation patterns of violaxanthin homo- and diesters were in agreement with previous findings.^{19,32}

(all-*E*)- and (9*Z*)-isomers were distinguished based on their UV/Vis absorption maxima (λ_{max}). For instance, (all-*E*)-violaxanthin oleate (22) and (9*Z*)-violaxanthin oleate (27) displayed identical protonated molecules and fragment ions. The Vis λ_{max} of (9*Z*)-violaxanthin oleate at 413, 436, and 465 nm displayed a slight hypsochromic shift of approximately 3 nm compared to those of (all-*E*)-violaxanthin oleate at 416, 439, and 468 nm, as expected for (*Z*)-configured carotenoids²¹. The Vis λ_{max} were in agreement with the literature.^{18,23} Slight deviations of approximately ± 1 nm may be attributed to the different compositions of the eluents or, in some instances, individual esters that were not baseline resolved resulting in superimposed absorption spectra.

By analogy to their non-esterified counterparts, (all-*E*)-violaxanthin esters eluted prior to their corresponding (9*Z*)-isomers. Interestingly, for mono- and diesters of a given xanthophyll, e.g., (9*Z*)-violaxanthin, retention times consistently increased by approximately 4 min upon elongation of the fatty acid moiety by C₂H₄ units (**Figure 3**). In agreement with the literature, unsaturated mono- and heterodiesters eluted prior to their counterparts carrying the corresponding saturated fatty acids with the same carbon number (CN).¹⁹ Noteworthy, the esters of monounsaturated fatty acids eluted prior to their saturated counterparts with acyl moieties of equivalent carbon number (ECN = CN – 2 × DB, here with DB representing the number of double bonds in the acyl moiety).³³ For instance, (9*Z*)-violaxanthin palmitoleate (C16:1, 23a) eluted prior to (9*Z*)-violaxanthin myristate (C14, 25b) as illustrated by **Figure 3**. In conclusion, the detection and assignment of violaxanthin esters was undergirded using their elution order as exemplarily displayed for (9*Z*)-violaxanthin esters in **Figure 3**. By this approach, esters carrying unsaturated fatty acyl moieties other than C16:1 or C18:1 were not detected.

In addition to violaxanthin esters, (all-*E*)-antheraxanthin myristate (32), (9*Z*)-antheraxanthin laurate (34), myristate (39), oleate (41), and several antheraxanthin diesters (56, 62, and 64ab) were tentatively assigned in the endocarp at full maturity. The presence of antheraxanthin laurate, myristate, and palmitate has been previously reported in orange juice and pumpkins.^{12,25,34}

Esters of β -citraurin (26a, 31, 33, and 38), a C30 *apo*-carotenal, were exclusively detected in the flavedo of fully mature oranges. The characteristic orange-reddish tint color of mature Navel and Valencia orange peel has been attributed to esterified β -citraurin and (9*Z*)-violaxanthin, however, without complete elucidation the nature of their acyl moieties.³⁵ The pronounced Vis absorption maximum of β -

citraurin esters at 460 nm matched that of an authentic β -citraurin standard, thus being in accordance with the literature.³⁶ Occasionally observed slight shifts of up to 2 nm may be attributed to the different eluent system applied herein and the later elution of esterified β -citraurin. The mass fragmentation of β -citraurin esters resembled those of violaxanthin monoesters described above. **Figure 4** illustrates the MS² spectrum of β -citraurin laurate (26a) that displayed protonated molecules at m/z 615.5. Characteristic fragment ions were detected at m/z 597.5 ($[M + H - H_2O]^+$), 523.5 ($[M + H - \text{toluene}]^+$), and 415.3 ($[M + H - FA]^+$), resulting from the elimination of water (18 amu), toluene (92 amu), and the fatty acyl moiety (here 200 amu). The fragment ion at m/z 397.1 ($[M + H - H_2O - FA]^+$), resembling dehydrated β -citraurin, may be generated by the neutral loss of both the fatty acid moiety (200 amu) and water (18 amu). β -Citraurin laurate (26a), myristate (31), oleate (33), and palmitate (38) were exclusively found in the flavedo of mature and fully mature orange fruit. Their elution order matched that of the violaxanthin monoesters as discussed above. Even though β -citraurin laurate, myristate, and palmitate have been previously reported in the flavedo of mature Satsuma mandarins (*Citrus unshui* Marc.),³⁷ to the best of our knowledge, their biosynthetic origins have not been explicitly described in the literature. Carotenoid cleavage dioxygenase 4 (CCD4/CCD4b) has been identified as the enzyme responsible for the biosynthesis of β -citraurin from its precursors zeaxanthin and β -cryptoxanthin^{38–40}, but the esterification is currently unknown.

In endocarp and flavedo samples, two phytoene (11 and 17) and three phytofluene isomers (15, 21, and 24) were detected at 286 and 348 nm, respectively. Phytoene and phytofluene recently gained increasing attention as their bioaccessibility from carrot, tomato, and blood orange juices was superior to those of β -cryptoxanthin, α -carotene, and β -carotene.³⁶

Quantitation of carotenoids in endocarp and flavedo

Total carotenoid contents in the endocarp of orange fruit increased during ripening from 50 ± 2 to $1,354 \pm 23$ $\mu\text{g}/100$ g of fresh weight (FW) in unripe (G) and fully mature fruit (FM), respectively (**Figure 5a**). This may be attributed to the *de novo* biosynthesis of xanthophyll esters, reaching their maximum concentrations at full maturity. Whereas the endocarp of green-breaker (GB) and mature (M) oranges contained mono- and diesters at almost equal proportions, that of the fully mature (FM) sample mostly

295 contained diesters. Total carotenoid levels commonly increase during fruit maturation of many *Citrus*
296 species and varieties, including tangor (*Citrus reticulata* × *C. sinensis*) Murcott, Pêra oranges, and their
297 interspecific hybrids,⁴¹ grapefruits, and oranges^{42,43}. In general, concentrations of xanthophyll esters in
298 the flavedo ($10,976 \pm 288$ µg/100 g of FW) of Navel oranges exceeded those in the endocarp ($1,223 \pm$
299 19 µg/100 g of FW) at fully mature stage. Total diester contents in the flavedo of $5,963 \pm 141$ µg/100 g
300 of FW were approximately eight-fold higher than those in the endocarp of 772 ± 3 µg/100 g of FW.
301 Even more pronounced differences have been reported for some pomelo (*Citrus grandis*) varieties, an
302 ancestral *Citrus* species, where a 250-fold higher carotenoid level in the peel than in the juice sacs has
303 been found, indicating a differently regulated carotenogenesis in both fruit tissues.⁴⁴

304 Total carotenoid levels in flavedo samples significantly dropped from $9,692 \pm 55$ in immature green (G)
305 to $4,178 \pm 329$ µg/100 g of FW in green-breaker (GB) fruit (**Figure 5b**). Hereby, in particular, the
306 concentrations of carotenes (α - and β -carotene) and the free xanthophylls neoxanthin, zeaxanthin, and
307 lutein declined. A minimum carotenoid content at the breaker stage has been previously reported in the
308 flavedo of other orange varieties.¹⁷ Chloroplasts are converted into chromoplast at this stage.
309 Simultaneously, thylakoid membranes and the contained carotenoids degrade, but biosynthesis of
310 chromoplast-specific carotenoids has not yet commenced, ultimately resulting in the aforementioned
311 declining carotenoid levels. Whereas the flavedo at green breaker-stage contained xanthophyll
312 monoesters only as minor constituents, the concentrations of mono- and diesters increased tremendously
313 with progressing maturation. The esterified xanthophylls reached maximum concentrations of
314 $5,013 \pm 146$ and $5,963 \pm 141$ µg/100 g of FW as determined for mono- and diesters, respectively, in the
315 fully mature fruit. Total carotenoid levels thereby amounted to $12,605 \pm 351$ µg/100 g of FW.

316 Noteworthy, diverse violaxanthin esters prevailed in the flavedo of mature and fully mature fruit,
317 whereas (all-*E*)-neoxanthin, (all-*E*)- α -, and (all-*E*)- β -carotene were not detected. The accumulation of
318 (9*Z*)-violaxanthin concomitantly with decreasing concentrations of the aforementioned carotenoids has
319 been attributed to the interconversion of chloroplasts into chromoplasts in the flavedo of maturing
320 oranges. The alteration of the pigment profile has been associated with the up-regulation of genes
321 encoding for ζ -carotene desaturase (ZDS) and phytoene synthase (PSY). Moreover, the observed shift
322 from carotenoids of the β,ϵ -branched type (i.e. α -carotene and lutein) to those of the β,β -path (here

violaxanthin) may be attributed to the downregulation of ϵ -lycopene cyclase (ϵ -LCY) and the boosted expression of β -LCY isoform chromoplasts-specific (LCY2) and β -carotene hydroxylase (β -CHX) in the flavedo of maturing oranges.^{17,45,46} The metabolic pathways and their regulation as involved in the biosynthesis of esterified xanthophylls is not fully understood to date and merits further investigation. In conclusion, carotenoid profiles in endocarp and flavedo samples of Navel orange fruit harvested at four consecutive maturity stages were elucidated. After optimizing and validating our extraction procedure, detailed HPLC-DAD-APCI-MSⁿ experiments revealed a highly complex pattern of at least 77 different carotenoids, including 26 xanthophyll mono- and 33 diesters of violaxanthin, antheraxanthin, and β -citraurin. Compound assignments were substantiated considering a conserved and consistent elution order of the individual esters. Thereby, we revealed that ECNs supporting the identification of triacylglycerols^{33,47} may also be highly instrumental for the designation of xanthophyll esters, in particular, those carrying monounsaturated acyl moieties. During maturation, the shift of the carotenoid pattern from derivatives mainly of β,ϵ -type to those of the β,β -branched types indicated the conversion of chloroplasts to chromoplasts in the flavedo. Concomitantly, carotenoid concentrations in the endocarp increased exponentially with progressing maturation, indicating their *de novo* synthesis during chromoplast development from colorless plastids, such as, e.g., amyloplasts.⁴⁸ In particular, pathways involved in the biosynthesis of diverse xanthophyll esters, including those of the *apo*-carotenal β -citraurin, in maturing orange fruit have not been assessed. Detailed reports on acyltransferases and/or GDSL esterases/lipases being involved in the esterification of carotenoids are widely lacking.^{49,50} Therefore, this particular biosynthetic step in orange fruit merits further investigation. Future studies may additionally assess the authentication of orange juices based on their genuine carotenoid patterns as, e.g., β -citraurin esters were exclusively found in the flavedo of Navel oranges.

Abbreviations

APCI, atmospheric pressure chemical ionization; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CID, collision induced dissociation; CN, carbon number; ECN, equivalent carbon

351 number; *FA*, fatty acid; *HPLC-DAD*, high performance liquid chromatography-diode array detection;
352 *MSⁿ*, multiple-stage mass spectrometry; *MTBE*, methyl *tert*-butyl ether

353

354 *Acknowledgements*

355 This work was partially supported by research grant RTI2018-095131-B-I00 (Ministerio Ciencia,
356 Innovación y Universidades, Spain). MJR and LZ are members of Eurocaroten (COST_Action
357 CA15136) and CaRed (Spanish Carotenoid Network BIO2017-90877-REDT, Ministerio de Ciencia,
358 Innovación y Universidades, Spain).

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494

Figure Captions

Figure 1. Carotenoid concentrations achieved after four extraction cycles of orange endocarp (a) using ethanol/ethyl acetate/*n*-hexane (A), methanol/ethyl acetate/petroleum ether (B), petroleum ether/2-propanol (C), and acetone/diethyl ether/*n*-hexane (D). Extraction yields after repeated extractions with solvent B (b). Values represent means $\pm$ standard deviations ($n = 2$). Small letters indicate significant ($P < 0.05$) differences of means.

Figure 2. HPLC-DAD chromatograms of Navel orange flavedo and endocarp extracts from green (a, b) and fully mature fruit (c, d). The insert (b') displays the chromatographic separation of (all-*E*)-lutein (7) from chlorophyll derivatives using the method of Hempel et al. 2017.²⁷

Figure 3. Relation between carbon number of the fatty acid moiety and the elution order of (9*Z*)-violaxanthin mono- and diesters. Esters carrying monounsaturated or exclusively saturated fatty acids are indicated by *circles* or *filled circles*, respectively. (9*Z*)-violaxanthin Monoesters (Cx) are shown in *blue*. Diesters with at least one lauric (C12-Cx), myristic (C14-Cx), and palmitic (C16-Cx) acid are displayed in *red*, *green*, and *purple*, respectively, (9*Z*)-violaxanthin dioleate (52a) in *cyan*. The labels indicate the carbon number of the fatty acid moiety of the monoesters and the carbon number of the second fatty acid of diesters, respectively.

Figure 4. APCI(+)-MS² spectrum of compound 26a in the flavedo of fully mature Navel oranges assigned to β -citraurin laurate.

Figure 5. Composition of carotenoids in the endocarp (a) and flavedo (b) of four progressing maturity stages of Navel oranges. *G*, green unripe fruit. *GB*, green-breaker fruit. *M*, mature fruit. *FM*, fully mature fruit. Values represent means $\pm$ standard deviations ($n = 2$). Lowercase letters indicate significant ($P < 0.05$) differences of the total carotenoid concentrations.

Tables

Table 1. HPLC-DAD-APCI-MSⁿ Data of Carotenoids from Navel Orange (*Citrus sinensis* (L.) Osbeck) Fruit

No.	t _R (min)	λ _{max} (nm)	D _B /D _{II} ^a (%)	D _{III} /D _{II} ^b (%)	[M + H] ⁺ (m/z)	APCI(+)-MS ⁿ experiment (m/z)	Proposed structure
1	8.1	266, 327 416/439/469	-	91	601.5	[601.5]: 583.4, 565.4, 509.4, 491.4, 445.3, 429.3, 221.1, 181.0	(all- <i>E</i>)-violaxanthin ^c
2	8.4	266, 327 412/436/464	-	93	583.4*	[583.4*]: 565.4, 547.4, 509.4, 491.3, 445.2, 393.2, 221.0, 180.8	(all- <i>E</i>)-neoxanthin ^c
3ab	11.0	267, 327 413, 436, 464	8	85	601.5	[601.5]: 583.4, 565.4, 509.4, 491.3, 445.3, 429.3, 221.1, 181.0	(9 <i>Z</i>)-violaxanthin ^{c,f}
		267, 334 sh423/446/473	6	54	585.5	[585.5]: 567.5, 549.3, 493.3, 221.0, 181.0	(all- <i>E</i>)-antheraxanthin ^{c,f}
4	11.8	270, 331 sh415/437/465	46	48	551.5*	[551.5*]: 533.4, 495.3, 477.3, 459.4, 429.3	(13 <i>Z</i>)-lutein
5	12.7	270, 331 sh415/439/465	41	44	551.5*	n.d.	(13' <i>Z</i>)-lutein
6	13.3	442	-	-	n.d.	n.d.	n.i.
7	13.7	267, 332 sh423/445/473	8	65	551.5*	[551.5*]: 533.4, 495.3, 477.4, 459.3, 429.6	(all- <i>E</i>)-lutein ^c
8	15.8	276, 332 422/451/476	9	27	569.5	[569.5]: 551.4, 549.4, 533.3, 477.3, 463.3, 459.3, 411.2, 393.3	(all- <i>E</i>)-zeaxanthin ^c
9	16.4	442	-	-	n.d.	n.d.	n.i.
10	16.8	433	-	-	n.d.	n.d.	n.i.
11	17.7	sh275/286/300	-	-	545.5	[545.5]: 503.5, 489.5, 475.5, 463.5, 435.4, 393.3	Phytoene (isomer 1)
12	18.1	430/452	-	-	n.d.	n.d.	n.i.
13	19.0	270, 315 sh420/445/472	-	-	569.5	[569.5]: 551.4, 549.4, 533.3, 477.3, 463.3, 459.3, 411.3, 393.3	(9 <i>Z</i>)-zeaxanthin
14	19.8	-	-	-	783.7	[783.7]: 765.7, 583.4, 565.4	n.i. xanthophyll laurate (C12)
15	20.0	332/348/367	-	70	543.5	[543.5]: 501.4, 487.3, 473.3, 461.3, 433.3, 405.2, 323.1	Phytofluene (isomer 1)
16	21.3	267, 327 416/440/470	-	89	783.7	[783.7]: 765.6, 583.4, 565.4	(all- <i>E</i>)-violaxanthin laurate (C12)
17	23.1	sh275/286/300	-	-	545.5	[545.5]: 503.4, 489.4, 475.4, 463.4, 435.4, 393.3	Phytoene (isomer 2)
18	23.3	265, 327 419/441/470	-	90	837.7	[837.7]: 819.7, 583.5, 565.5	(all- <i>E</i>)-violaxanthin palmitoleate (C16:1)
19ab	24.1	266, 328 413/436/465	14	89	783.7 811.7	[783.7]: 765.6, 583.4, 565.4 [811.7]: 793.7, 583.5, 565.4	n.i. xanthophyll laurate (C12) n.i. xanthophyll myristate (C14)
20abc	25.5	266, 328 413/436/465	15	86	569.5 811.7 865.8	[783.7]: 765.6, 583.4, 565.4 [811.7]: 793.7, 583.5, 565.4 [865.8]: 847.7, 583.5, 565.3	(9 <i>Z</i>)-violaxanthin laurate (C12) (all- <i>E</i>)-violaxanthin myristate (C14) ^c n.i. xanthophyll oleate (C18:1)
21	25.9	332/348/367	-	69	543.5	[543.5]: 501.4, 487.4, 473.3, 461.4, 433.3, 405.1, 323.1	Phytofluene (isomer 2)
22	27.3	267, 327 416/439/468	-	88	865.8	[865.8]: 847.8, 583.5, 565.5	(all- <i>E</i>)-violaxanthin oleate (18:1)
23ab	28.3	286, 329 413/436/463	12	84	811.7 837.7	[811.7]: 793.7, 583.4, 565.5 [837.7]: 819.8, 583.4, 565.5	n.i. xanthophyll myristate (C14) (9 <i>Z</i>)-violaxanthin palmitoleate (C16:1)
24	29.3	330/348/367	-	65	543.5	[543.5]: 501.3, 487.3, 473.3, 461.4, 433.3, 405.2, 323.2	Phytofluene (isomer 3)
25ab	29.5	266, 327 413/436/465	13	93	839.8 811.7	[839.8]: 821.8, 583.4, 565.3 [811.7]: 793.7, 583.4, 565.5	n.i. xanthophyll palmitate (C16) (9 <i>Z</i>)-violaxanthin myristate (C14)

26ab	30.4	460 268, 329 417/440/470	- - -	- - -	615.5 839.8	[615.5]: 597.5, 523.5, 415.3, 397.1 [839.8]: 821.7, 583.5, 565.4	β -citaurin laurate (C12) ^d (all- <i>E</i>)-violaxanthin palmitate (C16)
27	31.7	267, 328 413/436/465	12	90	865.8	[865.8]: 847.8, 583.6, 565.5	(9 <i>Z</i>)-violaxanthin oleate (C18:1)
28	32.9	267, 327 416/436/465	7	86	839.8	[839.7]: 821.8, 583.4, 565.4	n.i. xanthophyll palmitate (C16)
29	34.1	266 422/446/474	17	55	537.5	[537.5]: 481.4, 457.5, 445.4, 444.5, 413.2, 401.3, 399.1, 387.3, 347.4, 321.3, 281.2	(all- <i>E</i>)- α -carotene ^c
30	34.1	267, 327 413/436/465	14	88	839.7	[839.7]: 821.8, 583.5, 565.4	(9 <i>Z</i>)-violaxanthin palmitate (C16)
31	35.1	460	-	-	643.5	[643.5]: 625.5, 551.1, 415.3, 397.1	β -citaurin myristate (C14) ^d
32	35.0	269, 329 sh428/446/473	-	65	795.7	[795.7]: 777.7, 567.4, 549.4	(all- <i>E</i>)-antheraxanthin myristate (C14)
33	36.6	460	-	-	697.5	[697.5]: 679.5, 605.5, 415.3, 397.1	β -citaurin oleate (C18:1) ^d
34	37.3	269, 329 sh415/442/469	8	54	767.6	[767.6]: 749.6, 567.4, 549.4	(9 <i>Z</i>)-antheraxanthin laurate (C12)
35	38.8	266, 340 sh428/452/477	4	18	537.5	[537.5]: 481.3, 457.3, 445.3, 444.3, 413.2, 401.3, 399.3, 387.2, 347.3, 321.3, 281.2	(all- <i>E</i>)- β -carotene ^c
36	40.1	266, 328 413/435/465	7	83	937.8	[937.8]: 919.8, 765.7, 747.6, 737.6, 719.5, 565.4	(9 <i>Z</i>)-violaxanthin caprate-laurate (C10-C12)
37ab	40.4	n.d.	-	-	867.8 965.9	[867.8]: 849.8, 583.4, 565.5 [965.9]: 947.9, 765.7, 747.7, 565.4	(9 <i>Z</i>)-violaxanthin stearate (C18) ^c (all- <i>E</i>)-violaxanthin dilaurate (C12-C12) ^c
38	41.4	460	-	-	671.6	[671.6]: 653.6, 579.2, 415.3, 397.1	β -citaurin palmitate (C16) ^d
39	42.2	268, 333 418/441/468	8	67	795.7	[795.7]: 777.7, 759.7, 567.4, 549.4	(9 <i>Z</i>)-antheraxanthin myristate (C14)
40	43.2	267, 328 413/435/464	14	89	965.9	[937.8]: 919.8, 765.7, 747.6, 737.6, 719.5	(9 <i>Z</i>)-violaxanthin dilaurate (C12-C12)
41	44.3	268, 330 sh418/440/467	8	69	849.8	[849.8]: 831.7, 813.7, 567.4, 549.4	(9 <i>Z</i>)-antheraxanthin oleate (C18:1)
42	45.2	267, 328 418/439/470	-	-	993.9	[993.9]: 975.9, 793.8, 775.7, 765.6, 747.7, 565.5	(all- <i>E</i>)-violaxanthin laurate-myristate (C12-C14)
43	46.0	267, 328 413/437/465	15	88	1019.9	[1019.9]: 1002.0, 819.7, 801.7, 765.7, 747.7, 565.4	(9 <i>Z</i>)-violaxanthin laurate-palmitoleate (C12-C16:1)
44	47.2	267, 328 413/436/465	14	86	993.9	[993.9]: 975.9, 793.7, 775.8, 765.6, 747.7, 565.4	(9 <i>Z</i>)-violaxanthin laurate-myristate (C12-C14)
45	48.2	221, 329 406/430/457	25	51	1021.9	[1021.9]: 1003.9, 821.7, 803.8, 765.7, 747.6, 565.4	n.i. xanthophyll laurate-palmitate (C12-C16)
46abc	49.1	267, 328 416/438/467	10	62	1076.1 1047.9 1022.0	[1076.1]: 1058.0, 847.7, 829.7, 793.7, 775.7, 565.4 [1047.9]: 1029.9, 847.8, 829.6, 765.7, 747.7, 565.4 [1022.0]: 1004.0, 793.7, 775.6, 565.5	n.i. xanthophyll myristate-palmitate (C14-C16) (9 <i>Z</i>)-violaxanthin laurate-oleate (C12-C18:1) ^c (all- <i>E</i>)-violaxanthin dimyristate (C14-C14) ^c
47abc	49.7	266, 327 418/440/470	-	90	1076.1 1021.9	[1076.1]: 1058.0, 847.8, 829.7, 821.7, 819.7, 803.7, 801.6, 793.6, 775.7, 565.3 [1021.9]: 1003.8, 821.7, 803.8, 765.6, 747.6, 565.4	n.i. xanthophyll myristate-oleate / palmitate-palmitoleate (C14-C18:1/ C16-C16:1) (all- <i>E</i>)-violaxanthin laurate-palmitate (C12-C16)
48ab	50.6	267, 328 413/436/465	14	82	1076.1 1022.0	[1076.1]: 1057.9, 847.7, 829.7, 793.5, 775.5, 565.1 [1022.0]: 1004.0, 793.7, 775.5, 565.6, 547.4	(all- <i>E</i>)-violaxanthin myristate-oleate (C14-C18:1) ^c (9 <i>Z</i>)-violaxanthin dimyristate (C14-C14)
49ab	51.2	267, 328 413/437/465	13	93	1050.0 1022.0	[1050.0]: 1031.8, 821.6, 803.5, 793.6, 775.7, 565.3 [1022.0]: 1004.0, 821.8, 803.8, 765.7, 747.7, 565.4	n.i. xanthophyll myristate-palmitate (C14-C16) (9 <i>Z</i>)-violaxanthin laurate-palmitate (C12-C16)
50	52.2	267, 327 412/436/464	13	82	1076.1	[1076.1]: 1058.1, 847.8, 829.8, 793.5, 775.7, 565.4	(9 <i>Z</i>)-violaxanthin myristate-oleate (C14-C18:1)

51	52.8	265, 328 418/440/470	-	93	1050.0	[1050.0]: 1032.0, 821.7, 803.7, 793.8, 775.7, 565.45	(all- <i>E</i>)-violaxanthin myristate-palmitate (C14-C16)
52abc	54.0	266, 328 414/437/466	16	84	1130.0	[1130.0]: 1111.8, 847.6, 829.5, 565.4	(9 <i>Z</i>)-violaxanthin dioleate (C18:1-C18:1) ^e
					1050.0	[1050.0]: 1032.0, 821.7, 803.7, 793.8, 775.7, 565.4, 547.5	(9 <i>Z</i>)-violaxanthin myristate-palmitate (C14-C16) ^e
					1104.1	[1104.1]: n.d.	(all- <i>E</i>)-violaxanthin palmitate-oleate (C16-C18:1) ^e
					1086.1*	[1086.1*]: 994.0, 829.7, 803.7, 547.2	
53	54.9	223, 329 409/432/457	31	53	1078.1	[1078.1]: 1059.9, 821.8, 803.8, 565.4	n.i. xanthophyll dipalmitate (C16-C16)
54	55.3	267, 327 413/436/465	6	93	1104.1	[1104.1]: 1086.0, 847.8, 829.7, 821.7, 803.8, 565.4	n.i. xanthophyll palmitate-oleate (C16-C18:1)
55	55.5	267, 327 413/436/465	8	89	1104.1	[1104.1]: 1086.0, 847.8, 829.8, 821.8, 803.7, 565.4	(9 <i>Z</i>)-violaxanthin palmitate-oleate (C16-C18:1)
56	55.7	269, 333 419/444/471	7	59	977.9	[977.9]: 959.9, 777.7, 759.7, 749.6, 731.6, 549.4	antheraxanthin laurate-myristate (C12-C14)
57	56.0	266, 329 418/440/470	-	93	1078.1	[1078.1]: 1060.0, 821.7, 803.8, 565.5	(all- <i>E</i>)-violaxanthin dipalmitate (C16-C16)
58	57.2	267, 328 413/436/465	14	89	1078.1	[1078.1]: 1060.1, 821.7, 803.8, 565.3	(9 <i>Z</i>)-violaxanthin dipalmitate (C16-C16)
59	58.1	415/440/468	-	89	1078.1	[1078.1]: n.d.	n.i.
60	58.7	455/481	-	36	1078.0	[1078.1]: n.d.	n.i.
61	59.3	268, 333 413/435/465	15	78	n.d.	n.d.	n.i.
62	59.9	268, 333 sh420/442/470	7	55	1005.9	[1005.9]: 987.9, 777.7, 759.7, 549.4	(9 <i>Z</i>)-antheraxanthin dimyristate (C14-C14)
63	60.7	266, 329 418/440/470	-	84	1106.1	[1106.1]: n.d.	(all- <i>E</i>)-violaxanthin palmitate-stearate (C16-C18)
					1088.1*	[1088.1*]: 996.0, 831.7, 803.7, 547.5	
64ab	61.4	269, 333 sh421/442/470	7	66	1060.0	[1060.0]: 1041.9, 831.8, 813.8, 777.7, 759.7, 549.4, 531.4	(9 <i>Z</i>)-antheraxanthin myristate oleate (C14-C18:1)
					1060.0	[1060.0]: 1041.9, 805.7, 803.8, 787.7, 785.8, 549.4, 531.4	(9 <i>Z</i>)-antheraxanthin palmitoleate palmitate (C16:1-C16)
65	61.7	266, 328 413/436/465	13	69	1106.1	[1106.1]: n.d.	(9 <i>Z</i>)-violaxanthin palmitate-stearate (C16-C18)
					1088.1*	[1088.1*]: 996.1, 831.8, 803.7, 547.5	

sh: shoulder.

n.d. not detected. n.i. not identified.

^a D_b/D_{II}: ratio between the absorbance of the cis-peak (D_b) and that of the middle main absorption band (D_{II}).

^b D_{III}/D_{II}: Ratio of the absorbance at the longest wavelength absorption band (D_{III}) to that at the middle main absorption band (D_{II}).

^c Verified by an authentic reference standard.

^d UV/Vis absorption spectrum verified by β-citraurin standard.

^e UV/Vis absorption spectrum not clearly defined (co-elution).

^f UV/Vis data taken from alkaline saponified extracts.

* In-source elimination of water ([M +H – H₂O]⁺).

Figure 1. Carotenoid concentrations achieved after four extraction cycles of orange endocarp (a) using ethanol/ethyl acetate/*n*-hexane (A), methanol/ethyl acetate/petroleum ether (B), petroleum ether/2-propanol (C), and acetone/diethyl ether/*n*-hexane (D). Extraction yields after repeated extractions with solvent B (b). Values represent means $\pm$ standard deviations ($n = 2$). Small letters indicate significant ($P < 0.05$) differences of means.

83x57mm (300 x 300 DPI)

Figure 2. HPLC-DAD chromatograms of Navel orange flavedo and endocarp extracts from green (a, b) and fully mature fruit (c, d). The insert (b') displays the chromatographic separation of (all-*E*)-lutein (7) from chlorophyll derivatives using the method of Hempel et al. 2017.²⁷

141x216mm (300 x 300 DPI)

Figure 3. Relation between carbon number of the fatty acid moiety and the elution order of (9Z)-violaxanthin mono- and diesters. Esters carrying monounsaturated or exclusively saturated fatty acids are indicated by *circles* or *filled circles*, respectively. (9Z)-violaxanthin Monoesters (Cx) are shown in *blue*. Diesters with at least one lauric (C12-Cx), myristic (C14-Cx), and palmitic (C16-Cx) acid are displayed in *red*, *green*, and *purple*, respectively, (9Z)-violaxanthin dioleate (52a) in *cyan*. The labels indicate the carbon number of the fatty acid moiety of the monoesters and the carbon number of the second fatty acid of diesters, respectively.

155x65mm (300 x 300 DPI)

Figure 4. APCI(+)-MS² spectrum of compound 26a in the flavedo of fully mature Navel oranges assigned to β -citaurin laurate.

176x56mm (300 x 300 DPI)

Figure 5. Composition of carotenoids in the endocarp (a) and flavedo (b) of four progressing maturity stages of Navel oranges. *G*, green unripe fruit. *GB*, green-breaker fruit. *M*, mature fruit. *FM*, fully mature fruit. Values represent means $\pm$ standard deviations ($n = 2$). Lowercase letters indicate significant ($P < 0.05$) differences of the total carotenoid concentrations.

173x64mm (300 x 300 DPI)

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73x37mm (300 x 300 DPI)