

In Vitro Bioaccessibility of Carotenoids, Flavonoids, and Vitamin C from Differently Processed Oranges and Orange Juices [*Citrus sinensis* (L.) Osbeck]

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S Supporting Information

ABSTRACT: Carotenoid, flavonoid, and vitamin C concentrations were determined in fresh orange segments and a puree-like homogenate derived thereof, as well as freshly squeezed, flash-pasteurized, and pasteurized juices. Lutein and β -cryptoxanthin were slightly degraded during dejuicing, whereas β -carotene levels were retained. Vitamin C levels remained unaffected, whereas flavonoid levels decreased 8-fold upon juice extraction, most likely due to the removal of flavonoid-rich albedo and juice vesicles. Likewise, the presence of such fibrous matrix compounds during in vitro digestion was assumed to significantly lower the total bioaccessibility (BA) of all carotenoids from fresh fruit segments (12%) as compared to juices (29–30%). Mechanical disruption of orange segments prior to digestion did not alter carotenoid BA, whereas pasteurization of the freshly squeezed juice slightly increased BA by 9–11%. In addition to carotenoid BA, the stabilities of hesperidin, narirutin, and vitamin C including dehydroascorbic acid during in vitro digestion were monitored, and applied analytical methods were briefly validated.

KEYWORDS: fruit matrix, bioavailability, dietary fiber, bioactive compounds, health and nutrition

INTRODUCTION

Orange juice is the most popular fruit juice in the European Union and the United States.¹ Consumers perceive orange juice as a healthy and natural source of vitamins and other health-promoting nutrients, resulting in an increasing worldwide demand and production.² Additionally, the convenient packaging and long shelf life of juices are advantageous compared to fresh fruit. Recent intervention studies demonstrated the health benefits of long-term orange juice consumption, such as an increased total antioxidant status, lower total cholesterol levels, and the prevention of endotoxin increases after meals high in fat and carbohydrate.^{3–6} However, greater consumption of orange juice has also been criticized because of its high intrinsic sugar level, being associated with a higher risk of type 2 diabetes and cardiovascular diseases.⁷

Oranges [*C. sinensis* (L.) Osbeck] and products thereof are rich sources of carotenoids, flavonoids, and vitamin C.^{8–11} The carotenoid profile of oranges has been reported to be one of the most complex in fruits,¹² making its determination a popular research objective over the past few decades.⁹ In addition to the provitamin A carotenoids β -cryptoxanthin, α -carotene, and β -carotene, oranges contain a large diversity of free and esterified xanthophylls, among them lutein and zeaxanthin. The latter compounds are believed to play an important role in preventing age-related macular degeneration and, possibly, cognitive impairment in elderly people.¹³ Sufficient bioavailability is a prerequisite for their bioactivity in humans, that is, their liberation from the food matrix, incorporation into mixed micelles, and subsequent absorption by the enterocyte.¹⁴ The term “bioaccessibility” refers to “the fraction of a compound

that is released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption”. Because only micellarized carotenoids are potentially bioavailable, common in vitro digestion assays include analyses of the intestinal micellar fraction.¹⁵ In vitro digestion models measuring the bioaccessibility of micro- and macronutrients in food by mimicking the physiological conditions in the intestinal tract have become a popular low-cost–high-throughput research tool.^{15,16} Although presenting major drawbacks compared to in vivo studies, in vitro models allow the fast and inexpensive screening of a larger sample set, serving as a scientific basis for further in vivo studies.

Numerous reports have described the bioavailability and bioaccessibility of carotenoids from different food matrices.^{14,17–19} Simultaneous intake of fat^{20,21} as well as thermal and mechanical processing^{22,23} were shown to favor carotenoid liberation and micellarization from tomatoes, carrots, and spinach, thus resulting in higher carotenoid bioavailability. Although some studies evaluated the effects of different orange juice production on carotenoid bioaccessibility,^{24–26} comparative data regarding the bioaccessibility of unprocessed orange fruits and products derived thereof are lacking.

In addition to carotenoids, the flavanone glycosides hesperidin and narirutin represent potentially bioactive phytochemicals in oranges. In numerous epidemiological

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studies, an inverse relationship between dietary flavonoid intake and cardiovascular diseases has been demonstrated, mainly due to the antioxidant properties of the flavanones.¹¹ Various other health-beneficial mechanisms have been reported, such as anti-inflammatory and chemopreventive activities.¹¹ The metabolism of hesperidin and narirutin involves the deglycosylation of the rhamnose moiety by the colon microbiota, their absorption by colonocytes, and their subsequent conjugation with hydrophilic compounds such as glucuronic acid.¹⁰ The solubilization of flavonoids during processing and digestion is a key factor in this metabolism, thus strongly affecting their bioavailability.²⁷ In vitro models commonly used for the evaluation of carotenoid bioaccessibility include ultracentrifugation and microfiltration of the digesta,¹⁸ thus also allowing the measurement of soluble flavonoids in the intestinal fluid.

Orange fruits and products thereof contain high amounts of vitamin C, with concentrations averaging 500 mg/L.^{8,28} Although the bioavailability of vitamin C (comprising ascorbic acid and dehydroascorbic acid) ranges between 80% and 100% at doses normally ingested,⁸ its oxidation and further degradation during processing, storage, and digestion might limit the nutritional value of a dietary source.

The first aim of this work was to evaluate the impacts of different steps of orange fruit processing on levels of carotenoids, flavonoids, and vitamin C. All test foods were produced from the same batch of navel oranges to ensure uniformity of analyte levels. The main objective was then to evaluate the in vitro bioaccessibility of nutritionally relevant carotenoids and flavonoids in these test foods. In addition, levels of vitamin C were monitored before and after in vitro digestion.

MATERIALS AND METHODS

Materials and Reagents. Fresh, untreated oranges [*Citrus sinensis* (L.) Osbeck cv. "Navel Lane Late"] were obtained from a wholesale market (Stuttgart, Germany). Ammonium oxalate ($\geq 99.5\%$), calcium carbonate (98.5–100.5%), calcium chloride dihydrate (p.a.), dipotassium hydrogen phosphate ($\geq 99\%$), ethanol ($\geq 99.9\%$), aqueous hydrochloric acid (37%), potassium dihydrogen phosphate (p.a.), potassium chloride (p.a.), *N,N*-dimethylformamide (DMFA, $\geq 99.8\%$), sodium hydrogen carbonate (p.a.), sodium hydroxide (1 mol/L, Titrisol), 2-propanol ($\geq 99.8\%$), methyl *tert*-butyl ether [MTBE, high-performance liquid chromatography (HPLC) gradient grade], and BIOQUANT total dietary fiber kits (1.12979.0001) were purchased from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT) was from Merck Schuchardt (Hohenbrunn, Germany). Ascorbic acid (Ph. Eur.), acetic acid (100%), sodium chloride (100%), potassium hydroxide (85.9%), magnesium chloride hexahydrate (Ph. Eur.), methanol (HPLC grade), metaphosphoric acid (GPR), and petroleum benzene (bp 40–60 °C, GPR rectapur) were purchased from VWR International (Leuven, Belgium). Porcine pancreatic α -amylase (46.4 U/mg), porcine bile extract, cholesterol esterase from porcine pancreas (42.9 U/mg), dehydroascorbic acid, disodium hydrogen phosphate, pancreatin from porcine pancreas, and pepsin from porcine gastric mucosa were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). β -Carotene ($\geq 97\%$), β -cryptoxanthin ($\geq 97\%$), 3-*tert*-butylhydroxyanisole (BHA), and hesperidin ($\sim 90\%$) were from Fluka Chemie (Buchs, Switzerland), whereas antheraxanthin (95.0%), zeinoxanthin (97.7%), α -carotene (98.9%), lutein (99.9%), mutatoxanthin (95.5%), violaxanthin (99.3%), and zeaxanthin (99.8%) were purchased from CaroteNature (Ostermündingen, Switzerland). Narirutin (96%) was obtained from ChromaDex (Irvine, CA), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was from Alfa Aesar (Karlsruhe, Germany). Ultrapure water (Milli-Q system, Millipore, Bedford, MA) was used for all analyses.

Production of Orange Test Foods. Figure 1 illustrates the processing steps used to obtain five different test products (fresh

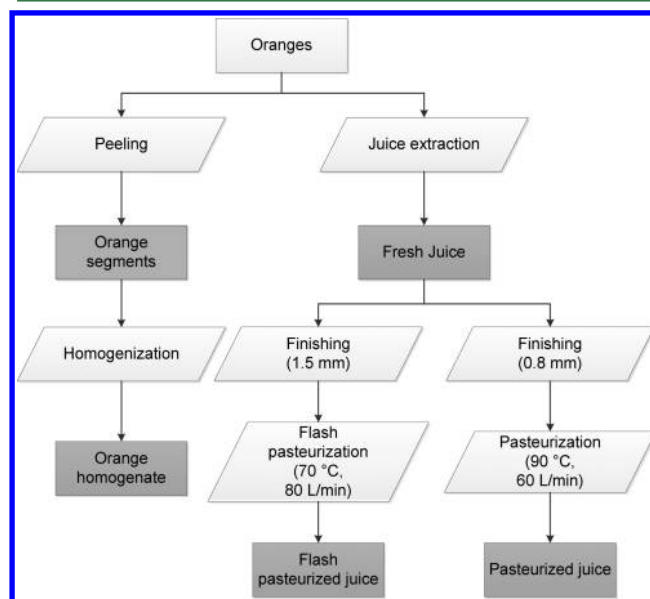


Figure 1. Production of the test foods (gray boxes) for in vitro digestion from the same batch of fresh "Navel Lane Late" orange fruits.

unprocessed orange segments, homogenized segments, freshly squeezed juice, pasteurized juice, flash-pasteurized juice). A minimum of 8 kg of oranges was used for the production of each test food to minimize the influence of raw-material heterogeneity. Unprocessed orange segments were manually peeled, and the adhering albedo and the columella were almost completely removed. A portion of the obtained segments was immediately frozen in liquid nitrogen, vacuum-sealed in plastic bags, and stored at -80 °C until further analyses. Residual segments were homogenized with a PUC colloid mill (Probst and Class, Rastatt, Germany) at 5050 rpm and a gap width of 1 mm. Subsequently, homogenization was repeated thrice with decreasing gap widths (350, 50, and 50 μ m, respectively). The homogenized segments were filled into 20 mL plastic flasks, and the headspace was flushed with nitrogen prior to sealing. The plastic flasks were then immediately frozen in liquid nitrogen and stored at -80 °C until further analyses.

For orange juice production, oranges were washed with cold water and dejuiced using a semiautomatic Orange X-Press extractor (Brimato, Hilter, Germany). An aliquot of the freshly squeezed juice was filled into plastic flasks, frozen with liquid nitrogen, and stored at -80 °C until further use. The remaining freshly squeezed juice was finished using a commercial paddle pulper (PAP 0533, Bertuzzi, Brughiero, Italy) with mesh sizes of 1.5 and 0.8 mm for the production of the flash-pasteurized and pasteurized orange juice, respectively. As shown in Figure 1, the flash-pasteurized juice was continuously heated to 70 °C at a flow rate of 80 L/h using a tubular heater (Ruland Engineering & Consulting, Neustadt/Weinstraße, Germany), whereas the pasteurized juice was heated to 90 °C at 60 L/h. Subsequently, the juices were hot-filled into 500 mL glass bottles and cooled to room temperature in a water bath. The juices were filled into plastic flasks, and the headspace was flushed with nitrogen before sealing and freezing with liquid nitrogen. All samples were stored at -80 °C until further analyses. Product temperature was recorded throughout the process, and pasteurization values were calculated using *Bacillus coagulans* as the reference germ ($T_{ref} = 93.3$ °C, $z = 8.9$ K) according to the method of Hirsch et al.²⁹ Pasteurization values were 0.002 and 0.504 min for the flash- and conventionally pasteurized juices, respectively.

In Vitro Digestion Model. After being thawed at ambient temperature for less than 1 h, unprocessed orange segments were cut into cubic pieces of 5-mm length to mimic coarse comminution obtained by chewing. All other test foods were thawed and subjected

to the digestion model without further pretreatment. The *in vitro* digestion protocol was based on the method reported by Schweiggert et al.,¹⁸ with the following modifications. During the oral phase, 10 mL (instead of 5 mL) of artificial saliva solution was used, and 7 mL (instead of 9 mL) of porcine bile extract/pancreatin solution was added at the beginning of the intestinal phase. After completion of the intestinal phase, 35 mL of the artificial intestinal fluid was centrifuged at 7500g for 60 min at 10 °C in a JA-25.50 rotor (Avanti J-26 XP/XPI, Beckman Coulter, Krefeld, Germany) to separate solids from the aqueous phase. The thus-obtained supernatant (~35 mL) was considered to contain the nutrients liberated from the solid food matrix. A volume of 5 mL of the supernatant was then filtered through a cellulose acetate-based 0.2- μ m syringe filter (Klaus Ziemer, Mannheim, Germany) to yield the micellar fraction. Only micellized lipophilic nutrients such as carotenoids are considered to be potentially available for absorption, thus representing the *bioaccessible* fraction. *In vitro* liberation and bioaccessibility were calculated as the percentages of the respective nutrient transferred from the test food to the supernatant obtained after centrifugation and to the micellar phase obtained by microfiltration of the supernatant, respectively, as described by Schweiggert et al.¹⁸ The *in vitro* digestion was performed in triplicate.

Carotenoid, Flavonoid, and Vitamin C Analyses. *Sample Preparation.* Frozen orange segments and homogenized segments were milled under liquid nitrogen using a model 38BL41 blender (Waring, Torrington, CT) prior to analyses, whereas juices and digesta were analyzed without further pretreatment.

Carotenoids. An aliquot of 2.0 ± 0.1 g and 2.0 mL of test food and digesta, respectively, were combined with 50 mg of CaCO_3 and 3 mL of petroleum benzene/2-propanol (1:1, v/v), containing 50 mg of BHA and 50 mg of BHT/100 mL solvent mixture. Extraction was supported by a probe sonicator (Sonoplus HD 3080 with MS 72 sonotrode, Bandelin, Berlin, Germany) at 70% amplitude for 30 s. After centrifugation (3000 rpm, 3 min, Labofuge 200, Heraeus, Hanau, Germany), the upper organic layer was collected, and the sample was re-extracted twice with 2 mL of the above-mentioned extraction solvent. The combined organic phases were washed with 2 mL of aqueous NaCl (0.1 M) and dried under a stream of nitrogen at ≤ 25 °C. Subsequently, the dried extracts were redissolved in 3 mL of petroleum benzene, and saponification was started by adding 3 mL of ethanolic KOH (10%, w/v). After incubation for 3 h under continuous shaking using an orbital shaker at 130 rpm (Type A2, Edmund Bühler, Tübingen, Germany), the samples were washed with 2 mL of aqueous NaCl (0.1 M), centrifuged, and the upper organic layer was separated. The lower ethanolic phase was re-extracted with 1 mL of MTBE, and the combined organic phases were evaporated to dryness under a stream of nitrogen at ≤ 25 °C. Samples were redissolved in 400 μ L of 2-propanol and filtered (0.45 μ m) into amber vials prior to HPLC analysis. The extraction procedure was conducted in duplicate under subdued light.

Carotenoid identification and quantification were performed on a series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a G1379A degasser, a G1312A binary gradient pump, a G1313A autosampler, a G1316A column oven, and a G1315B diode array detector (DAD) coupled to a Bruker 3000+ ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Carotenoids were separated on a YMC C30 reverse-phase column (150 \times 3.0 mm i.d., 3- μ m particle size, YMC Europe GmbH, Dinslaken, Germany) protected by a YMC C30 guard column (10 \times 3.0 mm i.d., 3- μ m particle size) and operated at 40 °C. The mobile phase consisted of methanol/water (90:10 v/v, eluent A) and MTBE/methanol/water (78:20:2 v/v/v, eluent B), both containing 1.5 g/L ammonium acetate. The following gradient was used (min/percentage of A): 0/98, 24/83, 28/54, 35/22.5, 35.1/0, 38/0, 41/98, 43/98. The total run time was 43 min at a flow rate of 1 mL/min. The injection volume was 20 and 80 μ L for the analyses of test foods and digested samples, respectively. Carotenoids were monitored at 450 nm, and additional UV–vis spectra were recorded between 200 and 700 nm. Carotenoid identification was carried out by comparing the retention times, UV–vis spectra, and mass spectra of detected compounds with those

of external standards and literature references.³⁰ To support the identification of carotenoid (Z) isomers, $D_{\text{g}}/D_{\text{II}}$ ratios were determined according to the method of Britton.³⁰ Carotenoids were quantified by HPLC-DAD using linear calibration curves of external standards.

Vitamin C Analyses. Vitamin C analyses were conducted by HPLC-DAD based on the findings of Wechtersbach and Cigić.³¹ A sample aliquot of 2.0 ± 0.1 g was transferred to a 20 mL volumetric flask containing 15 mL of metaphosphoric acid (1% w/v) adjusted to pH 3.8 with dipotassium hydrogen phosphate. A second aliquot of 2.0 ± 0.1 g was transferred to an identical buffer additionally containing 10 mM TCEP. The samples were made up to 20 mL with the respective buffer, vortexed, and allowed to stand for 30 min for the reduction of dehydroascorbic acid (DHAA) to ascorbic acid (AA) in the TCEP experiment. Subsequently, samples were membrane-filtered (0.45 μ m) and analyzed by HPLC. For quantification of ascorbic acid by HPLC-DAD, the above-described HPLC system (series 1100 HPLC) was equipped with a reverse-phase C18 Sunfire column (250 mm \times 4.6 mm i.d., 5- μ m particle size, Waters, Eschborn, Germany) operated at 25 °C. The mobile phase consisted of aqueous metaphosphoric acid (1% w/v) adjusted to pH 2.6 with dipotassium hydrogen phosphate (eluent A) and methanol (eluent B). The following gradient was used (min/percentage of A): 0/100, 17/100, 20/0, 30/0, 33/100, 43/100. The total run time was 43 min at a flow rate of 0.8 mL/min. The injection volume was 20 μ L. AA was monitored at 254 nm and quantitated by external linear calibration. DHAA concentrations were determined by subtracting the initial AA concentration from that obtained by the above-described TCEP experiment. The reported vitamin C levels represent the sum of AA and DHAA.

Flavonoids. Flavonoid extraction was performed according to method no. 58 of the International Federation of Fruit Juice Producers³² with some modifications. Exactly weighed samples (2.0 ± 0.1 g of test foods and supernatant, 1.0 ± 0.1 g of micellar phase) were transferred into 50, 10, and 5 mL volumetric flasks for test foods, supernatant, and micellar phase, respectively. The following description refers to the experiment using the 50 mL volumetric flask. Experiments using 10 and 5 mL flasks were carried out identically using proportional amounts of reagents. After addition of 10 mL of ammonium oxalate solution (0.025 M) and 10 mL of DMFA, the samples were made up to 50 mL with deionized water and incubated in a type 461 water bath (Büchi Labortechnik, Flawil, Switzerland) at 90 °C for 10 min. Subsequently, samples were membrane-filtered (0.45 μ m, Chromafil RC-45/15MS, Macherey-Nagel, Düren, Germany) into amber HPLC vials. Flavonoid separation was performed on the above-described series 1100 HPLC system, using a reverse-phase C18 Sunfire column (250 \times 4.6 mm i.d., 5- μ m particle size, Waters, Eschborn, Germany) at 25 °C. The mobile phase consisted of acetic acid (2%, eluent A) and methanol (eluent B) using the following gradient (min/percentage of A): 0/100, 10/100, 20/55, 30/55, 33/0, 40/0, 43/100, 50/100. The total run time was 50 min at a flow rate of 0.8 mL/min. The injection volume was 10 μ L, except for supernatant and micellar phases of all juices (20 μ L). Flavonoids were monitored at 287 nm and quantitated by external linear calibration curves.

Method Validation of Carotenoid, Flavonoid, and Vitamin C Determinations. *Repeatability.* The intra-assay precision and the time-dependent intermediate precision³³ of the methods described for analysis of the bioactive compounds were determined by analyzing a commercial orange juice in quadruplicate by the same person and with the same equipment on three different days within 1 month.³⁴ The orange juice was aliquoted and stored at -20 °C prior to the performance of the above-described determinations. Relative standard deviations (RSDs) for quantification of flavonoids and vitamin C were <0.7%. RSDs for quantification of carotenoids were <4.4%, except for the interday RSD of lutein (6.1%). The results illustrate the high precision of the methods applied. Further details are provided in Table S1 (Supporting Information).

Recovery. The accuracy of the methods described for analysis of bioactive compounds was determined by spiking different levels of the

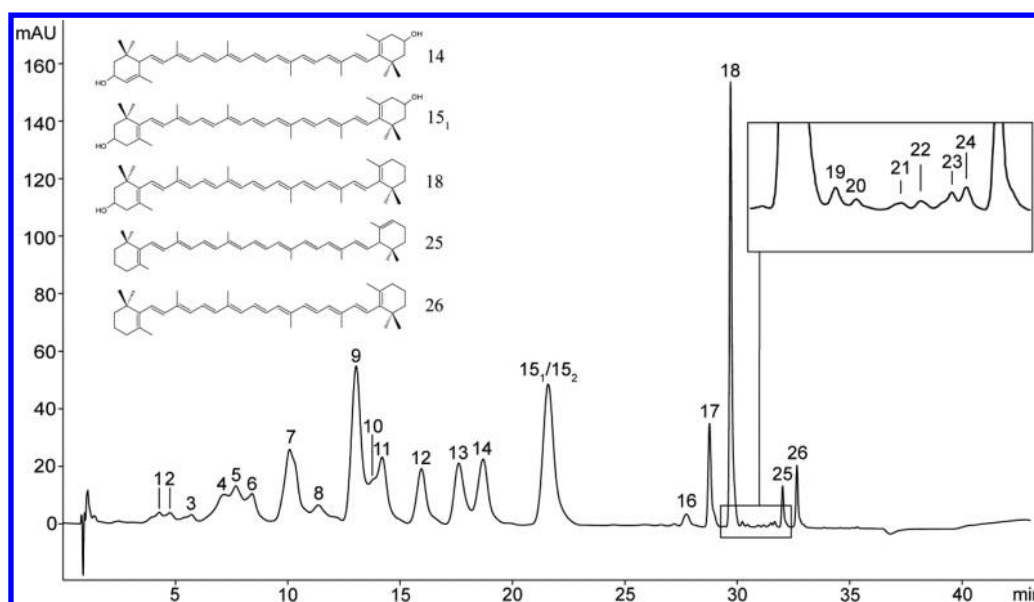


Figure 2. Chromatogram at 450 nm showing carotenoids in the pasteurized orange juice sample. Peak assignments in Table 1.

Table 1. Identification of Carotenoids Found in Orange Fruit and Derived Products

compound no.	retention time (min)	identity	UV-vis absorption maxima (nm)	[M + H] ⁺ m/z
1	4.2	NI ^a	416/440/468	583
2	4.7	NI	418/442/468	601
3	5.7	NI	400/422/448	601
4	7.1	NI	408/432/458	601
5	7.6	NI	418/442/468	601
6	8.3	violaxanthin ^b	418/440/468	601
7	10.0	NI	400/422/448	601
8	11.3	NI	404/426/446	601
9	13.0	(9Z)-violaxanthin ^c	328/416/438/464	601
10	13.7	NI	420/444/470	601
11	14.1	NI	396/418/444	601
12	15.9	mutatoxanthin (8R/8S) ^b	406/428/452	585
13	17.5	mutatoxanthin (8R/8S) ^b	406/428/452	585
14	18.6	lutein ^b	422/446/472	551 ^d
15 ₁	21.5	zeaxanthin ^b	428/450/476	551 ^d
15 ₂	21.5	(9Z)-antheraxanthin ^c	332/420/442/468	585
16	27.7	(13Z)-β-cryptoxanthin ^c	340/420/444/468	553
17	28.7	zeinoxanthin ^b	422/446/474	553
18	29.6	β-cryptoxanthin ^b	430/452/478	553
19	30.2	NI	ND ^e	ND
20	30.4	NI	332/348/368	ND
21	30.9	NI	ND	ND
22	31.1	NI	334/348/368	ND
23	31.4	NI	378/400/424	ND
24	31.6	NI	378/398/422	ND
25	31.9	α-carotene ^b	422/446/474	537
26	32.6	β-carotene ^b	428/452/478	537

^aNI = not identified ^bIdentified using authentic standards. ^cTentative identification by UV-vis absorption spectrum and [M + H]⁺. ^dD_B/D_{II} (compound): 0.078 (9), 0.075 (15₂), 0.459 (16). ^e[M + H - H₂O]⁺. ^eND = not detectable (trace amounts).

analytes into orange juice.³³ Relative recovery was calculated based on the predicted and measured concentrations. Recovery rates were generally 98–100%, except for the addition of intermediate levels of β-carotene (95.3%) and high levels of β-cryptoxanthin (94.8%). Further details are provided in Table S2 (Supporting Information).

Limit of Detection (LOD) and Limit of Quantification (LOQ). LOD and LOQ were determined according to DIN 32645 from the respective calibration curves.³⁵ All measured concentrations of

secondary plant metabolites were above the LOQ. Detailed information can be found in the Supporting Information (Table S2).

Quantitation of Pectin and Total Dietary Fiber. Pectin levels were quantitated spectrophotometrically according to IFU methods.³⁶ Pectin levels are expressed as milligrams of galacturonic acid (GA) per 100 g of test product. Dietary fiber levels were determined gravimetrically using a BIOQUANT total dietary fiber kit according to the supplier's instructions. Samples were analyzed in quadruplicate.

Table 2. Carotenoid Levels^a in Test Foods, Supernatants, and Micelles (Based on Fresh Weight of Test Foods)

	orange segments	orange homogenate	juice _{fresh}	juice _{flash-pasteurized}	juice _{pasteurized}
In Test Foods					
sum (C _p) ^b	224.3 ± 5.0 b	271.5 ± 9.5 a	204.5 ± 8.5 bc	178.3 ± 3.5 c	183.5 ± 9.5 c
lutein	96.2 ± 3.7 a	89.2 ± 6.6 a	82.3 ± 0.3 ab	66.3 ± 2.0 c	68.3 ± 3.0 bc
β-cryptoxanthin	104.9 ± 0.9 b	151.4 ± 2.7 a	101.5 ± 6.9 b	91.8 ± 1.3 b	94.8 ± 5.5 b
α-carotene	10.0 ± 0.1 a	9.0 ± 0.2 a	7.8 ± 0.3 b	7.1 ± 0.2 b	7.7 ± 0.4 b
β-carotene	13.2 ± 0.3 b	21.9 ± 0.0 a	12.9 ± 1.0 b	13.1 ± 0.1 b	12.8 ± 0.6 b
ZEA + (9Z)-ANT ^c	61.7 ± 1.6 bc	82.0 ± 2.1 b	51.1 ± 1.5 c	115.1 ± 12.2 a	116.7 ± 4.4 a
In Supernatant ^d					
sum (C _L)	51.8 ± 2.8 b	61.2 ± 5.5 b	91.9 ± 9.9 a	90.1 ± 3.5 a	99.0 ± 3.7 a
lutein	12.1 ± 0.3 c	14.5 ± 0.9 c	23.9 ± 2.7 b	23.9 ± 1.6 ab	27.3 ± 1.4 a
β-cryptoxanthin	34.5 ± 2.8 b	37.3 ± 3.8 b	55.9 ± 6.0 a	54.2 ± 2.2 a	58.9 ± 2.4 a
α-carotene	1.5 ± 0.1 c	2.9 ± 0.3 b	4.4 ± 0.5 a	4.3 ± 0.1 a	4.9 ± 0.2 a
β-carotene	3.6 ± 0.4 c	6.6 ± 0.6 b	7.7 ± 0.7 a	7.6 ± 0.2 a	7.9 ± 0.2 a
ZEA + (9Z)-ANT ^c	22.7 ± 1.1 c	25.0 ± 1.8 cb	30.0 ± 4.2 a	30.5 ± 2.1 a	27.6 ± 0.6 ab
In Micelles ^e					
sum (C _M)	24.2 ± 0.4 d	32.4 ± 1.1 c	57.9 ± 5.1 b	67.1 ± 1.1 a	72.7 ± 2.4 a
lutein	6.4 ± 0.3 c	8.2 ± 0.6 c	15.8 ± 0.9 b	17.2 ± 0.3 b	19.4 ± 1.1 a
β-cryptoxanthin	15.2 ± 0.5 c	19.0 ± 0.5 c	34.2 ± 4.1 b	40.6 ± 1.0 a	43.4 ± 1.3 a
α-carotene	0.8 ± 0.1 d	1.4 ± 0.1 c	2.8 ± 0.3 b	3.4 ± 0.2 a	3.7 ± 0.1 a
β-carotene	1.9 ± 0.1 d	3.7 ± 0.2 c	5.0 ± 0.6 b	5.9 ± 0.2 a	6.1 ± 0.1 a
ZEA + (9Z)-ANT ^c	9.7 ± 0.6 b	10.6 ± 0.4 b	17.7 ± 2.5 a	19.0 ± 0.5 a	18.4 ± 1.4 a

^aMean ± standard deviation, $n = 2$ for quantification of carotenoids in the test foods, $n = 3$ for in vitro digestion. Units of $\mu\text{g}/100 \text{ g}$ of FW. Different letters within rows indicate significant differences ($P \leq 0.05$). ^bSum (C_p) = sum of lutein, β-cryptoxanthin, α- and β-carotene levels. ^cZEA, zeaxanthin; (9Z)-ANT, (9Z)-antheraxanthin. ^dObtained after centrifugation of the digesta. ^eObtained after centrifugation and microfiltration (0.2 μm).

Statistics. Statistically significant differences of means were identified by Tukey's and Duncan's test, using SAS 9.1.3 (SAS Institute, Cary, NC). Differences were considered significant at $p < 0.05$. Normal distribution of the results was verified by the Shapiro–Wilk test. All reported data represent mean ± standard deviation. The standard deviation of the bioaccessibility was calculated by the Gaussian law of error propagation from the standard deviations of the levels in the test foods and of the total bioaccessible amounts of the respective analyte.

RESULTS AND DISCUSSION

Carotenoid Identification in the Test Foods. Figure 2 presents a chromatogram of a carotenoid extract from pasteurized orange juice, showing 26 detected carotenoids. Unambiguous identification of 9 carotenoids (peaks 6, 12–15, 17, 18, 25, 26) was achieved by comparing their retention times, UV–vis spectra, and molecular masses with those of authentic standards. Among them, lutein (14), zeaxanthin (15₁), zeinoxanthin (17), and β-cryptoxanthin (18) were the major ones, in accordance with previous studies.^{9,28,37–39} The two enantiomeric forms (8R)- and (8S)-mutatoxanthin were also identified using authentic standards, although the assignment of the enantiomers to the respective peaks (12 and 13) was impossible. Using authentic standards (compounds 6, 25, and 26, respectively), violaxanthin and α- and β-carotene were unambiguously assigned.

Compound 9 was tentatively identified as (9Z)-violaxanthin. Its UV–vis spectrum was highly similar to that of all-*E*-violaxanthin (6), except for a characteristic hypsochromic shift of 2–4 nm (Table 1). In addition, the UV spectrum exhibited a *cis* peak at 328 nm with a D_B/D_{II} ratio of 0.078, and the compound eluted after its all-*trans* isomer, both being typical of (9Z)-carotenoid isomers. In agreement with the previous report of Dugo et al.,⁴⁰ a predominant quasimolecular ion was

detected at m/z 601, thus supporting the identity of compound 9 as (9Z)-violaxanthin.

By analogy, compound 15₂ was tentatively assigned to (9Z)-antheraxanthin. It is noteworthy that this compound coeluted with zeaxanthin (15₁) and was detectable only in the flash-pasteurized and pasteurized orange juices, thus indicating its thermally induced formation. Meléndez-Martínez et al.⁴¹ reported the presence of high amounts of *cis*-antheraxanthin in orange juice. Therefore, the zeaxanthin contents determined for pasteurized juices can be partly attributed to (9Z)- or (9'Z)-antheraxanthin. Because of the observed coelution, the fate of zeaxanthin during processing and digestion remains unclear.

Compound 16 revealed a slightly hypsochromic shift of the UV–vis spectrum similar to β-cryptoxanthin. In contrast to compounds 9 and 15₂, compound 16 eluted before its all-*E* isomer and had a D_B/D_{II} ratio of 0.459, which is characteristic of (13Z) isomers.³⁹ Therefore, compound 16 was tentatively assigned to (13Z)-β-cryptoxanthin.

Absolute Carotenoid Levels in Test Foods. Table 2 summarizes the carotenoid levels in the test foods, as well as the liberated and micellarized carotenoid fractions after in vitro digestion. Carotenoid epoxides such as violaxanthin and mutatoxanthin are not found in human plasma and thus are considered not to be bioavailable in humans.⁴² Accordingly, we limited our bioaccessibility study to bioavailable carotenoids, because only such carotenoids would be relevant for the evaluation of assumed health-beneficial effects. Because of the coelution of the tentative (9Z)-antheraxanthin and zeaxanthin in both flash- and conventionally pasteurized juices, their carotenoid concentrations were apparently higher than those in the freshly squeezed juice. We therefore did not include these carotenoids in the sum of total (bioaccessible) carotenoids reported in Table 2 and Figure 3.

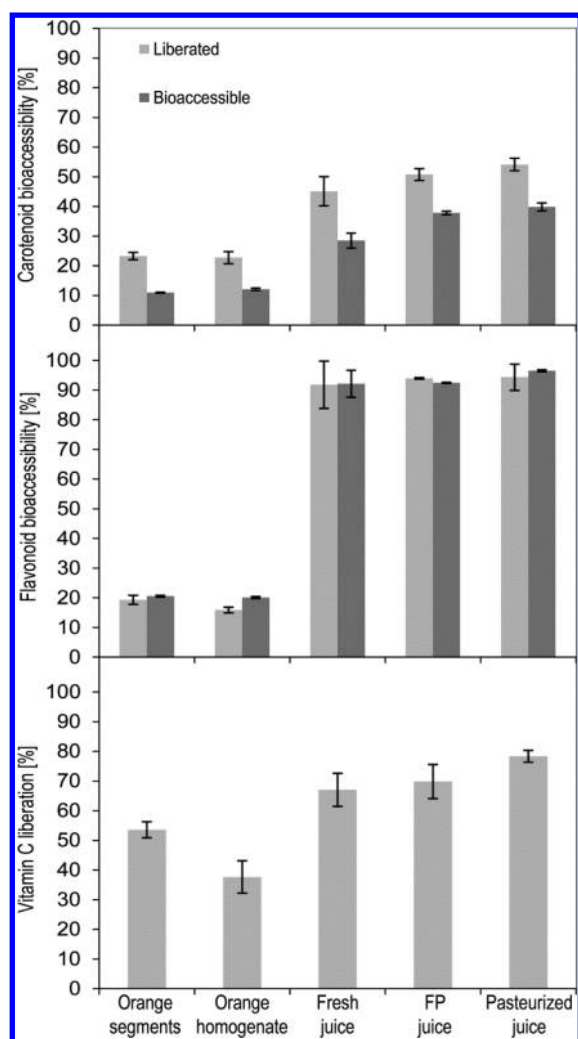


Figure 3. Bioaccessibility of potentially bioavailable carotenoids (lutein, β -cryptoxanthin, α - and β -carotene), flavonoids, and vitamin C from differently processed oranges (FP = flash-pasteurized). Liberated and bioaccessible fractions were calculated as the percentages of the respective nutrients transferred from the test food to the supernatant obtained after centrifugation and to the micellar phase obtained by microfiltration of the supernatant, respectively.

A decrease in total carotenoid levels (C_p) was noted during the processing of fresh oranges ($224 \mu\text{g}/100 \text{ g}$ of FW) into pasteurized orange juices ($178\text{--}184 \mu\text{g}/100 \text{ g}$ of FW, Table 2). However, the homogenized orange segments showed higher carotenoid levels ($272 \mu\text{g}/100 \text{ g}$ of FW), possibly due to the inevitable heterogeneity of the fruit or a slightly better carotenoid extractability during analyses. Among the nutrition-

ally relevant carotenoids (Table 2), β -cryptoxanthin was predominant in fresh orange segments, homogenate, and freshly squeezed juice ($102\text{--}151 \mu\text{g}/100 \text{ g}$ of FW), being slightly lowered by 7–10% after pasteurization ($92\text{--}95 \mu\text{g}/100 \text{ g}$ of FW). According to the classification of Britton and Khachik,⁴³ β -cryptoxanthin levels of orange can be considered as being low ($<100 \mu\text{g}/100 \text{ g}$ of FW) to moderate ($100\text{--}500 \mu\text{g}/100 \text{ g}$ of FW), whereas papaya fruits (up to ca. $2500 \mu\text{g}/100 \text{ g}$ of FW⁴⁴) and red chili peppers ($1834 \mu\text{g}/100 \text{ g}$ of FW⁴⁵) are among fruits and vegetables rich in β -cryptoxanthin. Lutein had the second highest concentrations in segments, homogenate, and freshly squeezed juice, reaching $82\text{--}96 \mu\text{g}/100 \text{ g}$ of FW. Its contents were reduced by 17–19% after pasteurization ($66\text{--}68 \mu\text{g}/100 \text{ g}$ of FW, Table 2). Regarding the above-mentioned classification, lutein levels were considered as being low, particularly when compared to those of green leafy vegetables ($>2000 \text{ mg}/100 \text{ g}$ of FW⁴³). As mentioned above, the exact quantitation of zeaxanthin was hampered by the presence of (9Z)-antheraxanthin. Nevertheless, the sum of the aforementioned carotenoids more than doubled after processing of the freshly squeezed juice ($51 \mu\text{g}/100 \text{ g}$ of FW) to the pasteurized juices ($115\text{--}117 \mu\text{g}/100 \text{ g}$ of FW). This increase was ascribed to the heat-induced formation of the tentative (9Z)-antheraxanthin from yet-unknown precursors, because the practical absence of all-*E*-antheraxanthin in the fresh juice was verified using an authentic standard. For α - and β -carotene, degradation and isomerization were insignificant (Table 2), confirming their high stability at temperatures below 120°C .⁴⁶

Absolute Carotenoid Levels in Micellar Fractions and Bioaccessibility. After *in vitro* digestion, the so-called bioaccessible carotenoids are contained in the micellarized fraction resulting from microfiltration of the supernatant.⁴⁷ The lowest levels of bioaccessible carotenoids were found for orange segments ($24.2 \pm 0.4 \mu\text{g}$ of micellarized total carotenoids/100 g of test food). Hence, only 10.8% of the carotenoids contained in fresh orange segments was micellarized and thus bioaccessible (Figure 3). Although the micellarized carotenoid concentration after digestion of homogenized segments was higher ($32.4 \pm 1.1 \mu\text{g}/100 \text{ g}$), the bioaccessibility was similar (11.9%) to that of intact segments (Figure 3), thus indicating only a minor contribution of mechanical cell disruption to carotenoid BA in oranges. The previously reported supportive effect of comminution on carotenoid bioavailability from tomatoes by disrupting the cell matrix²² might be less decisive for oranges, because of either the absence of large carotenoid crystals as reported for tomatoes¹⁸ or the high intrinsic fiber levels of oranges ($13.9 \text{ g}/100 \text{ g}$, Table 3) as compared to tomatoes ($1.2 \text{ g}/100 \text{ g}$ ⁴⁸). The amount of soluble fiber such as pectin can strongly affect micellarization of carotenoids in the duodenum, because previous studies demonstrated hindering of

Table 3. Pectin and Dietary Fiber Contents in the Test Foods

	orange segments	orange homogenate	juice _{fresh}	juice _{flash-pasteurized}	juice _{pasteurized}
pectin ^a	$287.8 \pm 7.2 \text{ b}$	$425.5 \pm 1.9 \text{ a}$	$44.2 \pm 1.2 \text{ c}$	$30.8 \pm 0.7 \text{ c}$	$27.5 \pm 0.4 \text{ c}$
dietary fiber ^c					
soluble	$5.7 \pm 0.2 \text{ a}$	$5.4 \pm 0.4 \text{ a}$	$0.7 \pm 0.0 \text{ b}$	$0.6 \pm 0.1 \text{ b}$	$0.5 \pm 0.0 \text{ b}$
insoluble	$8.2 \pm 0.3 \text{ a}$	$8.2 \pm 0.3 \text{ a}$	$0.7 \pm 0.0 \text{ b}$	$0.6 \pm 0.1 \text{ b}$	$0.2 \pm 0.0 \text{ b}$
total	$13.9 \pm 0.5 \text{ a}$	$13.6 \pm 0.7 \text{ a}$	$1.4 \pm 0.0 \text{ b}$	$1.2 \pm 0.1 \text{ b}$	$0.7 \pm 0.0 \text{ b}$

^aMean \pm standard deviation, $n = 2$. Units of mg of GA/100 g of FW, where GA = galacturonic acid. Different letters within rows indicate significant difference ($P \leq 0.05$). ^cMean \pm standard deviation, $n = 4$. Units of g/100 g of FW. Different letters within rows indicate significant difference ($P \leq 0.05$).

Table 4. Flavonoid, Ascorbic Acid (AA), and Vitamin C^a Levels in Test Foods, Supernatants, and Micelles (Based on Fresh Weight of Test Product)

	orange segments	orange homogenate	juice _{fresh}	juice _{flash-pasteurized}	juice _{pasteurized}
Flavonoid Levels ^a (mg/100 g of FW)					
In Test Foods					
hesperidin	241.7 ± 7.9 a	238.8 ± 3.3 a	29.3 ± 0.5 b	28.9 ± 0.0 b	29.7 ± 0.0 b
narirutin	46.6 ± 0.4 a	46.1 ± 1.0 a	5.3 ± 0.0 b	5.5 ± 0.0 b	5.1 ± 0.0 b
In the Supernatant					
hesperidin	32.1 ± 3.9 a	25.6 ± 1.6 a	26.8 ± 2.3 a	27.3 ± 0.0 a	28.2 ± 1.4 a
narirutin	23.6 ± 0.8 a	19.7 ± 1.1 b	5.0 ± 0.5 c	5.1 ± 0.0 c	4.7 ± 0.2 c
In Micelles					
hesperidin	33.9 ± 1.4 a	32.3 ± 0.1 a	26.7 ± 1.3 b	26.8 ± 0.0 b	28.8 ± 0.0 b
narirutin	25.4 ± 1.9 a	24.9 ± 0.1 a	5.1 ± 0.2 b	5.0 ± 0.0 b	4.7 ± 0.0 b
Levels of Ascorbic Acid (AA) and Vitamin C ^b (mg/100 g of FW)					
In Test Foods					
AA	43.1 ± 0.6 c	32.6 ± 0.2 d	48.6 ± 0.5 a	45.5 ± 0.0 b	44.6 ± 0.1 bc
vitamin C	51.1 ± 0.6 a	47.9 ± 0.2 c	49.4 ± 0.1 b	47.1 ± 0.1 d	46.5 ± 0.1 d
In the Supernatant					
AA	22.9 ± 0.4 b	12.4 ± 2.5 c	26.1 ± 2.0 b	26.1 ± 2.0 b	33.6 ± 1.1 a
vitamin C	27.4 ± 1.4 b	18.0 ± 2.6 c	33.1 ± 2.8 ab	32.9 ± 2.7 ab	36.5 ± 0.9 a

^aCalculated as the sum of ascorbic acid and dehydroascorbic acid. ^aMean ± standard deviation, *n* = 3. Different letters within rows indicate significant differences (*P* ≤ 0.05). ^bMean ± standard deviation, *n* = 2 for quantification of AA and vitamin C in the test foods, *n* = 3 for quantification of AA and vitamin C in the supernatant. Different letters within rows indicate significant differences (*P* ≤ 0.05).

β-carotene bioavailability in humans by dietary fibers and, particularly, citrus pectin.⁴⁹ In our study, pectin and soluble-fiber levels confirmed their inverse correlation with carotenoid BA. Whereas dietary fiber and bioaccessibility in fresh and homogenated oranges were 13.6–13.9 g/100 g and 10.8–11.9%, respectively, juices containing only 0.7–1.4 g of dietary fiber/100 g had a far higher BA of 28.3–39.5% (Tables 2 and 3).

Because of the diminished content of dietary fiber, micellar carotenoid levels increased to 57.9 ± 5.1 μg/100 g after the digestion of freshly squeezed orange juice, resulting in a 2.6-fold higher bioaccessibility (28.3%) when compared to carotenoids from orange segments. Highest concentrations of bioaccessible carotenoids were observed in the flash-pasteurized (67.1 ± 1.1 μg/100 g) and pasteurized (72.7 ± 2.4 μg/100 g) juices. The bioaccessibility of total carotenoids was significantly higher in the pasteurized juices (37.6–39.5%) than in the freshly squeezed juice (28.3%). Thermal processing and finishing of the fresh juice increased the BA of lutein, β-cryptoxanthin, α-carotene, and β-carotene, whereas the (9Z)-antheraxanthin/zeaxanthin BA decreased from 35% to 17% and 16% in flash-pasteurized and pasteurized juices, respectively. Note that this decrease might be attributable to the instability of epoxy carotenoids such as antheraxanthin during digestion. Higher pasteurization temperatures and intensified finishing with smaller mesh size did not result in significant changes of the carotenoid BA. Consequently, a moderate thermal treatment and finishing appear to be key factors in increasing carotenoid BA. The insignificant change in carotenoid BA from the flash-pasteurized to the pasteurized juice indicates a “saturation” of these processing effects, meaning that further heating or finishing beyond such an “optimum processing” does not further improve carotenoid BA. If this hypothesis is valid, the heat exposure during the production of the flash- and conventionally pasteurized juices (*P* values of 0.002 and 0.504 min, respectively) exceeded this optimum heating threshold. However, the definition of an “optimum thermal treatment” regarding carotenoid bioaccessibility is expected to

be highly intricate because of the numerous interactions with other dietary factors, such as the amount of ingested dietary lipids. Unfortunately, we cannot differentiate whether the thermal treatment or the finishing step had the greater impact on carotenoid BA. Both treatments are assumed to contribute to the enhanced carotenoid bioaccessibility.

In comparison to this study, Stinco et al.²⁴ reported higher relative carotenoid absorption from simulated digesta, ranging from 34% in fresh hand-squeezed juice to 40% in industrially pasteurized juice and up to 52% in industrially squeezed and finished juice. According to their work, finishing resulted in a particle size reduction, thus improving carotenoid liberation, whereas thermal pasteurization apparently diminished carotenoid liberation during digestion. Note that Stinco et al.²⁴ did not separate the micellar fraction by microfiltration of the supernatant, which is rather more comparable to the liberated carotenoids displayed in Figure 3 than to the bioaccessible fraction. In agreement with our study, carotenoid liberation was 44.9–53.9% in freshly squeezed and pasteurized juices, thus being significantly higher than the respective bioaccessibility (28.3–39.5%). The observation of higher liberation as compared to bioaccessibility is in accordance with previous studies, which reported significantly lowered carotenoid levels after microfiltration of the aqueous supernatant.^{18,47,50}

Flavonoid Levels in Test Foods and Digesta. Flavonoid contents in the orange juices were 8-fold lower than those of orange segments and homogenated segments (Table 4). According to previous studies, hesperidin levels in membranes and albedo of *Citrus sinensis* are between 8.5- and 16.0-fold higher than those in juice vesicles.⁵¹ These findings correlate well with the hesperidin and narirutin levels determined in our test foods, because juice extraction resulted in the extensive separation of membranes and albedo. For instance, the hesperidin content decreased from approximately 240 mg/100 g in fresh orange segments to 30 mg/100 g of juice (Table 4). Despite the large differences in the test foods, the absolute hesperidin and narirutin levels in the supernatant and micellar fractions were similar after their *in vitro* digestion. Apparently,

their solubilization was dose-independent in our experiments, indicating a saturation of the intestinal fluid with soluble hesperidin and narirutin at 26–34 mg/100 g and 20–25 mg/100 g, respectively. Consequently, the bioaccessibility of hesperidin and narirutin increased significantly upon juice extraction (Figure 3). Whereas only 20.5% of the flavonoids were bioaccessible from orange segments, a total of 92.1–96.5% of the flavonoids occurring in the juices was bioaccessible, equaling a gain of almost 500% as compared to the orange segments. Comparing the digestion of intact and homogenized segments, differences in flavonoid BA were insignificant. Thus, homogenization of the orange segments turned out to be ineffective regarding flavonoid BA (Figure 3).

Our findings are in good agreement with those of a previous study by Vallejo et al., who found only the soluble fraction of flavonoids in orange juice to be bioavailable.²⁷ Supported by these findings, it is assumed that the higher hesperidin level in orange fruits compared to orange juice offers only a limited nutritional benefit, because differences in the hesperidin contents in the supernatant and micellar phase from the digested test foods were found to be insignificant. However, it is worth mentioning that our in vitro model did not include the simulation of colonic digestion, where hesperidin is deglycosylated by the microbiota to hesperetin 7-O-glucosid and hesperetin.¹⁰ Insoluble hesperidin can thus be further solubilized, metabolized, and absorbed in the colon. Nevertheless, in an in vivo study, Brett et al.⁵² found differences in flavonoid bioavailability to be insignificant based on total urinary hesperetin excretion of human subjects after consumption of orange fruit and juice.

Ascorbic Acid and Vitamin C in Test Foods and Digesta (Supernatant). When exposed to oxygen, ascorbic acid (AA) is reversibly oxidized to dehydroascorbic acid (DHAA), which also exerts vitamin efficacy. According to most reports, vitamin C efficacy is lost upon the irreversible degradation of DHAA to 2,3-diketogulonic acid.³¹ Consequently, both AA and DHAA levels were monitored in our study (Table 4). AA levels in the fresh and flash-pasteurized juices (48.6 ± 0.5 and 45.5 ± 0.0 mg/100 g, respectively) were slightly higher compared to those in the orange segments (43.1 ± 0.6 mg/100 g) and the pasteurized juice (44.6 ± 0.1 mg/100 g). Although these slight differences might be due to unavoidable heterogeneities within the used batch of oranges, homogenization of the orange segments led to a significant loss of AA from 43.1 to 32.6 mg/100 g and, at the same time, to an increase in DHAA from 8.0 to 15.3 mg/100 g, possibly due to oxygen exposure during homogenization. However, the vitamin C values of all test foods ranged between 46.5 and 51.1 mg/100 g, confirming the stability of AA and DHAA at low pH.

The bioavailability of vitamin C ranges from 80% to 100% at doses normally ingested (≤ 180 mg).⁸ Thus, the concentration in the intestinal digesta is rather a demonstration of vitamin C stability during digestion than of bioaccessibility. Whereas digestion of the orange segments resulted in residual vitamin C levels of 54% as compared to the test food, a level of only 38% was found after digestion of homogenized orange segments. The high input of air during homogenization probably favored further degradation of vitamin C during in vitro digestion. In contrast, the production of the pasteurized orange juice significantly improved vitamin C stability during digestion, resulting in a residual level of 78% as compared to the test food. Vitamin C levels in the digesta of the different juices did not differ significantly (Figure 3).

In conclusion, processing of the oranges into fresh and pasteurized juices slightly diminished carotenoid and vitamin C levels by 3–18%. However, flavonoid and dietary fiber levels were decreased to approximately one-tenth upon dejuicing. At the same time, carotenoid BA from orange segments was enhanced from 10.8% to 28.3% for freshly squeezed orange juice, whereas flavonoid BA was boosted by almost 5-fold to 96.5%. However, the total hesperidin levels in the digesta of all test foods were quite similar. The lower flavonoid levels in orange juices as compared to orange segments might be less relevant regarding their intestinal absorption, because low flavonoid solubility in the digestive fluids is considered to be the limiting factor. In contrast to dejuicing, homogenization of orange segments to a puree did not enhance carotenoid BA, thus indicating a minor role of cell disruption and comminution compared to the removal of fibers in orange products. To substantiate our findings, performing an in vivo bioavailability study is the next aim of our ongoing investigations.

■ ASSOCIATED CONTENT

● Supporting Information

Data on reproducibility, recovery, and limit of detection/limit of quantification for carotenoid, flavonoid, and vitamin C quantification. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AA, ascorbic acid; BA, bioaccessibility; BHA, 3-*tert*-butylhydroxyanisole; BHT, butylated hydroxytoluene; DHAA, dehydroascorbic acid; DMFA, *N,N*-dimethylformamide; FP, flash-pasteurized; MTBE, methyl *tert*-butyl ether; TCEP, tris(2-carboxyethyl)phosphine hydrochloride

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