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Carbon and Hydrogen Stable Isotope Ratios of Carotenoids and β-Carotene-Based Dietary Supplements†

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Considering the increasing nutritional and commercial importance of carotenoids, there is an interest in developing a reliable method for authenticity assessment of these compounds. Applying isotope ratio mass spectrometry using elemental analysis in the "combustion" (C) and "pyrolysis" (P) modes (EA−C/P−IRMS), the $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ values of selected carotenoids and α/β-carotene-based commercial dietary supplements were determined in comparison to those of synthetic and "natural" references. The $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ values of synthetic β-carotene samples ($n = 4$), ranging from −25.3‰ to −26.4‰ and from −144‰ to −155‰, respectively, differed clearly from the data determined for carotenoids from various natural sources, including C_3 plant material ($n = 9$; $\delta^{13}C_{V\text{-}PDB}$ ranging from −28.5‰ to −32.8‰ and $\delta^{2}H_{V\text{-}SMOW}$ from −180‰ to −275‰) and microalgae *Dunaliella salina* ($n = 1$; $\delta^{13}C_{V\text{-}PDB}$ value of −15.6‰ and $\delta^{2}H_{V\text{-}SMOW}$ value of −191‰). From five commercial dietary supplements under study, two revealed $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ values in areas as found for synthetic references, and the other three had values near those of biotechnological β-carotene produced by *D. salina*. The $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ values recorded for natural lycopene ($n = 4$) and lutein ($n = 5$) ranged from −31.1‰ to −31.8‰ and from −180 to −201‰, as well as from −28.8‰ to −32.2‰ and from −186‰ to −245‰, respectively. Synthetic canthaxanthin ($n = 3$) exhibited $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ values ranging from −25.0‰ to −28.6‰ and from −133‰ to −153‰, respectively. The EA−C/P−IRMS application of this study showed that the natural stable isotopic composition of carotenoids is a powerful tool for determining their origin.

KEYWORDS: α-Carotene; β-carotene; lycopene; lutein; canthaxanthin; $^{13}C/^{12}C$ ratio; $^{2}H/^{1}H$ ratio; EA−C/P−IRMS; authenticity; dietary supplements; *Dunaliella salina*

INTRODUCTION

Carotenoids are one of the most important groups of natural pigments. As they can only be biosynthesized by plants and microorganisms, their presence in animals is attributed to ingestion via food and accumulation in certain tissues (pink feathers of flamingo, egg yolk). Apart from the function of color, carotenoids act as precursors of diverse apocarotenoids widely spread in nature. Biologically and commercially important apocarotenoids include the phytohormone abscisic acid, the visual and signaling molecules retinal and retinoic acid, and the flavor compounds α- and β-ionone (*1, 2*).

Although more than 600 carotenoids have been identified from various natural sources, only about 40 are ingested by humans; among them, α- and β-carotene, lycopene, β-cryptoxanthin, lutein, and zeaxanthin are most important (**Figure 1**).

Due to their role as efficient antioxidants, carotenoids have a growing nutritional relevance (*3–5*). As a consequence, the growing interest in carotenoids as part of the human diet has made great demands on the carotenoid industry. Primarily, carotenoids are used industrially for coloring purposes, in animal feeding, in particular, in poultry farming and in aquaculture, and in pharmaceuticals and cosmetics. In addition, the application in functional foods and dietary supplements (*6*) has led to an expanding international carotenoid market in recent years. Most of the carotenoids available on the market are produced by total chemical synthesis. For instance, synthetic β-carotene has been produced commercially since 1954; the annual output is thought to exceed 500 t, which corresponds to around 17% of the global and 40% of the European colorant market (*7*). An overview about the technical synthesis of some commercially important carotenoids is shown in **Figure 2**.

Apart from technical synthesis of carotenoids, the (more expensive) extraction from plant material is still in use, in addition to the biotechnological processes which have become an attractive alternative to "naturally grown" carotenoids; for

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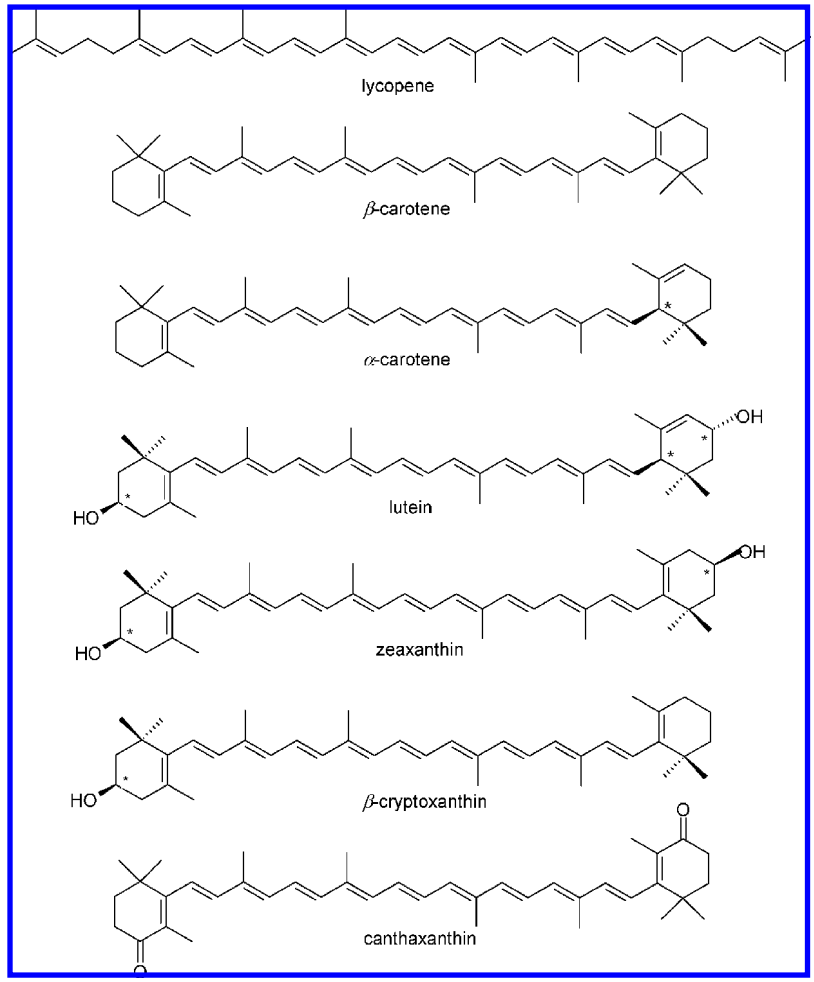

Figure 1. Chemical structures of some biologically important carotenoids.

instance, the production of β-carotene from the microalgae *Dunaliella salina* has been well established since 1986. The cultivation of *D. salina* has the advantage of extremely high β-and α-carotene contents that can reach under certain extreme conditions (hypersaline, low availability of nitrogen, and high levels of solar radiation) up to 14% of its dry weight. The *D. salina* biomass market comprises about 1200 t of dry matter/year employed for human nutrition, aquaculture, and cosmetics (*8*).

The expansion to use "natural" production ways is caused by the increasing awareness of modern consumers and their demand for natural products. In the view of the still increasing trend to natural products on one hand and the significant price differences between synthetic and natural carotenoid compounds on the other hand, there is an obvious risk for fraud by false origin declaration. Thus, there is sufficient interest to develop a method that is suitable to determine the authenticity of carotenoids. For authenticity assessment, the determination of the stable isotope ratios of the bioelements in foods has been established as an important approach. The application of this technique for pigment authentification was recently reported for betanin and isobetanin (*9*); the information on carotenoids, however, is rather scarce in the literature and limited to $^{13}C/^{12}C$ values (**Table 1**).

In this study, we extend the scope of isotope analysis of carotenoids from various sources achieved by assessment of $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios using elemental analysis−combustion/pyrolysis−isotope ratio mass spectrometry (EA−C/P−IRMS). In addition, β-carotene isolated from commercially available dietary supplements was analyzed by this method.

MATERIALS AND METHODS

Samples and Chemicals. Rose hip pulp and tomato puree were purchased at the local market and in local supermarkets (Würzburg, Germany), respectively. Raw palm oil was from Care Naturkost GmbH & Co. (Sittensen, Germany). Betatene (10% N) as a microencapsulated tablet grade powder containing approximately 10% mixed carotenes from *D. salina* was a gift from Cognis Australia Pty Ltd. (Cheltenham, Victoria, Australia). *Herba urticae* (dry leaves) and *Tagetes erecta* (dry marigold flower) were obtained from a local drugstore and from an Internet shop, respectively. Three samples of commercial dietary supplements were purchased from Internet shops, one sample was from a local drugstore, and one sample was from a local supermarket. Their origin is detailed in **Table 3**, in which they are numbered from Su1 to Su5. References of β-carotene, α/β-carotene (carotenoid mixture from carrots; the ratio of β- to α-carotene is approximately 2:1), lycopene, lutein, and canthaxanthin were supplied by Sigma-Aldrich (Schnelldorf, Germany), Fluka (Deisenhofen, Germany), Extrasynthèse (Genay, France), DSM Nutritional Products (Saint-Louis, France), and Hoffmann-La Roche AG (Grenzach, Germany). All commercial carotenoid references contained only *all-trans* isomers. All samples were stored at −20 °C until use. All other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). Solvents were redistilled before use. High-performance liquid chromatography (HPLC) solvents were of HPLC gradient grade.

Isolation of Carotenoids from Plant Materials. *Extraction and Saponification.* To avoid degradation and isomerization of carotenoids, dark-colored glassware was used and extraction was performed under

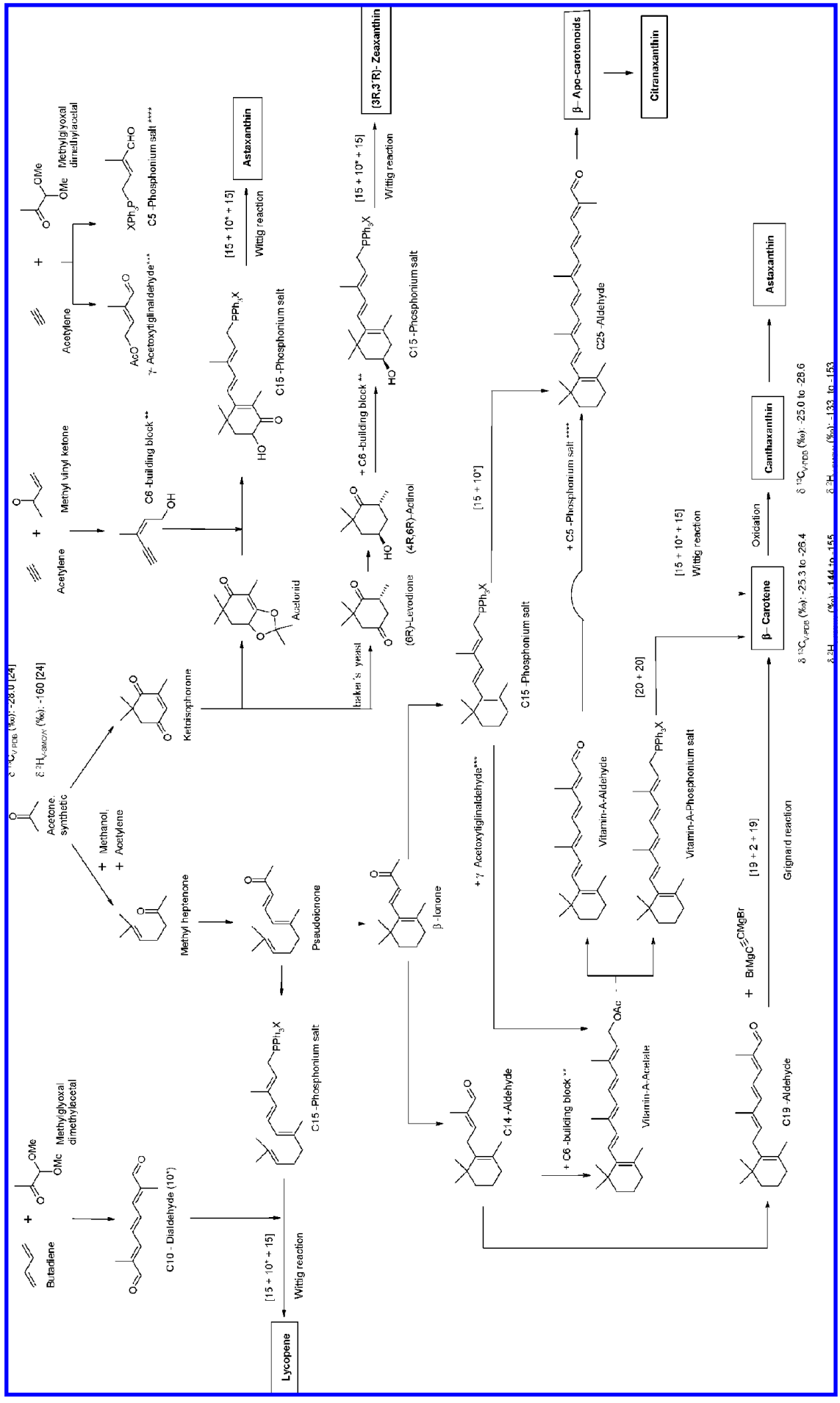

Figure 2. Scheme of industrial carotenoid synthesis (modified from refs 25 and 26) including data of carbon and hydrogen isotope values of synthetic acetone (24), synthetic β-carotene, and canthaxanthin (self-determined). (Re)used intermediates are marked with one or more asterisks.

Table 1. $\delta^{13}C_{V\text{-}PDB}$ Data Published in the Literature (*16, 17*) for Carotenoids

substance	origin/source	photosynthesis type	$\delta^{13}C_{V\text{-}PDB}$ (‰)	ref
lutein	*Tagetes erecta*	C_3	−29.9	*17*
	Lupinus sp.	C_3	−31.1	*16*
	corn gluten meal (*Zea mays*)	C_4	−19.8	*17*
carotenoids	*Sorghum*	C_4	−14.9 to −20.7	*16*
	cotton (*Gossypium*)	C_3	−29.2	*16*

Table 2. $\delta^{2}H_{V\text{-}SMOW}$ and $\delta^{13}C_{V\text{-}PDB}$ Isotope Values [a] (‰) of Carotenoids from Various Sources

substance	purity[b] (%)	status[c]	origin/source	phot type[d]	$\delta^{2}H_{V\text{-}SMOW}$	$\delta^{13}C_{V\text{-}PDB}$
β-carotene	≥97	syn/comm			−144	−26.4
	UV	syn/comm			−149	−25.8
	≥95	syn/comm			−155	−25.3
	≥96	syn/comm			−153	−26.1
	∼99	n/ex	tomato puree (*Lycopersicon esculentum*)	C_3	nd	−32.8
β/α-carotene (2:1)	≥95	n/comm	*Daucus carota*	C_3	−275	−28.5
β/α-carotene/lutein (30:2:1)	∼99	n/ex	*Dunaliella salina* (marine microalgae)		−191	−15.6
β/α-carotene (3:1)	∼99	n/ex	palm oil (*Elaeis guineensis*)	C_3	−197	−30.0
lycopene	≥90	n/comm	*L. esculentum*	C_3	−180	−31.8
	∼97	n/ex	tomato puree (*L. esculentum*)	C_3	−188	−31.1
	∼96	n/ex	rose hip pulp (*Rosa rubiginosa*)	C_3	−201	−31.1
	UV	n/comm	?		−189	−31.4
	≥96	comm	?		−181	−26.5
lutein	∼90	n/comm	alfalfa (*Medicago sativa*)	C_3	−229	−30.1
	UV	n/comm	?		−245	−28.8
	∼90	n/comm	?		−204	−29.6
	∼98	n/ex	*Tagetes erecta*	C_3	−186	−29.5
	∼99	n/ex	*Herba urticae*	C_3	−232	−32.2
canthaxanthin	≥90	syn/comm			−153	−28.6
	≥97	syn/comm			−145	−25.0
	≥96	syn/comm			−133	−27.6

[a] Standard deviations of ±5‰ and ±0.1‰ for $\delta^{2}H_{V\text{-}SMOW}$ and $\delta^{13}C_{V\text{-}PDB}$ determinations, respectively. [b] By HPLC (UV/vis), or UV = UV/vis absorption data are consistent with those of the reference. [c] n = natural, syn = synthetic, comm = commercial, and ex = self-extracted. [d] phot type = photosynthesis type, and nd = not determined.

Table 3. $\delta^{2}H_{V\text{-}SMOW}$ and $\delta^{13}C_{V\text{-}PDB}$ Isotope Values[a](‰) of Carotenoids Isolated from Dietary Supplements (Su's)

sample	declaration for β-carotene[b]	source	isolated carotenoid	purity total[c] (%)	$\delta^{2}H_{V\text{-}SMOW}$	$\delta^{13}C_{V\text{-}PDB}$
Su1	n/from *Dunaliella salina*	local drugstore	β/α-carotene/lutein (27:3:1)	∼97	−201	−16.4
Su2	n/from *D. salina*	local supermarket	β/α-carotene (9:1)	∼99	−218	−20.3
Su3	n/from *D. salina*	Internet shop	β/α-carotene (15:1)	∼98	−192	−16.5
Su4	n	Internet shop	β-carotene	∼99	−148	−28.5
Su5	n	Internet shop	β-carotene	∼99	−134	−28.2

[a] Standard deviations of ±5‰ and ±0.1‰ for $\delta^{2}H_{V\text{-}SMOW}$ and $\delta^{13}C_{V\text{-}PDB}$ determinations, respectively. [b] n = natural. [c] Purity determination by HPLC as described in the text.

dim light. Rose hip pulp (approximately 800 g), tomato puree (approximately 800 g), palm oil (approximately 100 g), and Betatene (10% N) (approximately 10 g) were used directly; *H. urticae* leaves (approximately 250 g) and *Tagetes* flowers (approximately 100 g) were powdered before extraction. The plant materials were transferred into Erlenmeyer flasks and homogenized in diethyl ether (approximately 300−400 mL, for extraction of lutein and α/β-carotenes) or in heptane (approximately 250 mL/100 g of plant material, for extraction of lycopene) by mechanical blending. Then an equal volume of methanolic KOH solution (10%, w/v) was added, the Erlenmeyer flasks were flushed with nitrogen or argon and then closed, and the saponification proceeded for 10−14 h using a magnetic stirrer at room temperature in the dark. Afterward the solid plant debris was removed by filtration under suction and re-extracted with a fresh appropriate organic solvent. The extracts were combined, and the volume of organic solvents was reduced in a rotary evaporator (water bath temperature <30 °C). The resulting two-phase reaction mixture was transferred into a separating funnel, and the organic phase was washed several times with water (each 200−300 mL) to remove alkali. Subsequently, the organic layer was evaporated to dryness using a rotary evaporator. The dried residues were used immediately for further purification steps or stored under nitrogen at −80 °C until use.

Isolation and Purification. The carotenoids were separated and purified from the dried plant extracts after simultaneous extraction and saponification steps (see above) via crystallization and recrystallization (used for *H. urticae*, *T. erecta*, palm oil, Betatene (10% N) samples) or by use of a combination of column chromatography and crystallization (used for rose hip pulp and tomato puree samples).

Crystallization. For the crystallization of lutein from the dried plant extracts, the solvent mixture pentane + methanol (80:20) was applied. A suitable solvent for the crystallization of lycopene and α- and β-carotene was diethyl ether. The resulting crystalline or powdery substances including the carotenoids were filtered sharply and dried under vacuum. The purity of the carotenoids was checked by HPLC via simultaneous UV−vis and evaporative light scattering detection. Prior to HPLC analysis, an aliquot of purified carotenoids was dissolved in a small volume of corresponding HPLC solvent and membrane-filtered. The pure dried crystals (with purity ≥96%, UV−vis) were stored under nitrogen at −80 °C until IRMS analysis.

Column Chromatography. Column chromatography was used to separate the carotenoid mixture from rose hip pulp and tomato puree. The residual total extract after the saponification procedure was dissolved in a small volume of hexane and applied to a dried Celite/MgO (1:1) column (100 cm × 4 cm i.d.) by use of hexane as the starting solvent. The elution was continued with each of 500 mL of hexane + diethyl ether (95:5, 90:10, 80:20, 60:40, 20:80) and then with hexane + diethyl ether + acetone (20:40:40). The column was covered by an aluminum foil coating and was cooled at 3 °C using a refrigerated

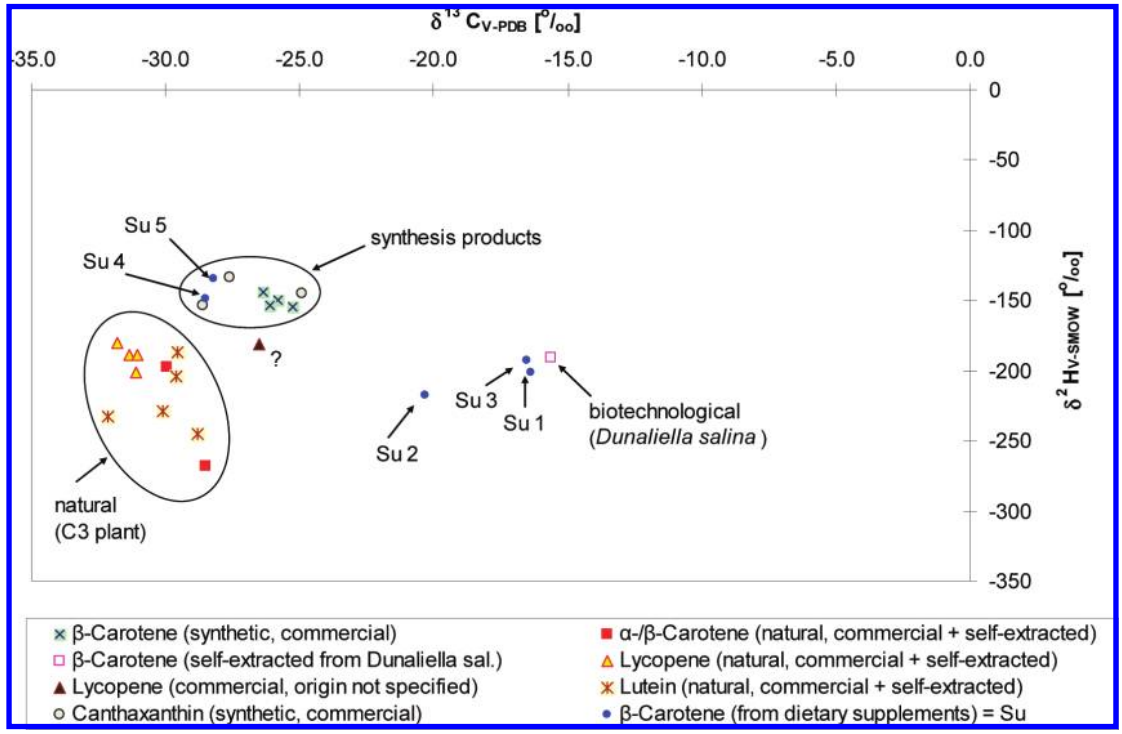

Figure 3. Correlation of $\delta^2H_{V\text{-}SMOW}$ and $\delta^{13}C_{V\text{-}PDB}$ values (‰) of carotenoids isolated from various natural sources and dietary supplements (Su's) in addition to those of synthetic and natural commercial references.

recirculator. The purity of an individual elute was analyzed by HPLC on the C_{30} phase against respective reference standards. The fractions containing lycopene were combined and evaporated; for the tomato puree also the β-carotene fraction was collected. Final purification was achieved by crystallization as described above.

Extraction of β-Carotene from Dietary Supplements. A total of 20−100 capsules or tablets (corresponding to a dose of about 200 mg of β-carotene) of commercial source were used for β-carotene extraction. Oil-filled capsules were opened with a scalpel, and after being washed with diethyl ether in a beaker, the capsules were separated. The capsules with a powdery content were opened, and the powdery substances were transferred into an Erlenmeyer flask. Tablets were ground using a mortar and pestle. Extraction and saponification were carried out simultaneously as described above. The volume of diethyl ether used for extraction was applied according to the literature data of the relative solubility of β-carotene in diethyl ether (1 mg of β-carotene/mL of diethyl ether) (*10*). After the extraction and saponification procedure the volume of diethyl ether was reduced in a rotary evaporator (water bath temperature <30 °C). The residue of organic solvent was completely removed under a nitrogen stream. The following isolation and purification of β-carotene were performed by crystallization from diethyl ether or 2-propanol, and if necessary, subsequent recrystallization was applied. After separation from the solvent and careful drying under a vacuum crystalline or powdery substances were obtained. Carotenoid components and their purity were analyzed by HPLC (total purity ≥97%, at 450 nm) on a C_{30} column. Details are given in **Table 3**.

High-Performance Liquid Chromatography. HPLC analysis was performed on a Knauer (Berlin, Germany) HPLC WellChrom including Eurochrom 2000 software, coupled with a UV−vis detector (Knauer) and an evaporative light scattering detector (ELSD) (30 °C), SEDEX 55 (SEDERE, France), using an analytical scale C_{30} reversed-phase column (Pronto Sil 200-3, 250 mm × 4.6 mm i.d.) with a particle size of 5 μm (Knauer, Berlin, Germany). The column was covered in a steel coating, which was cooled with tap water (22 °C). Peak identification of (*all-E*)-carotenoid isomers was made by retention time versus commercial standards. The *Z*-isomers (*cis*-peaks) of β-carotene and lycopene were identified on the basis of day-to-day tests and by comparison with the literature data (*11*). The tests for *cis*-isomer identification were carried out with commercial standards of (*all-E*)-β-carotene and (*all-E*)-lycopene, which were dissolved in a methanol/*tert*-butyl methyl ether (MTBE)/ethyl acetate mixture (50:40:10), stored at room temperature in the dark, and analyzed via HPLC (solvent systems 1 and 2, respectively; cf. below) on six different days within two weeks. The appearance of new peaks at 450 nm (for β-carotene) and 470 nm (for lycopene) was noticed as *cis*-peaks. Monitoring was performed at the visible area and by ELSD for simultaneous detection of carotenoids and other cosubstances. Purity results and quantity ratios from carotenoids were determined from intergraded peak areas at 450 nm for α- and β-carotenes, at 470 nm for lycopene, and at 448 nm for lutein and canthaxanthin. The *E*/*Z* isomers were received as total. For α- and β-carotene, lycopene, lutein, and canthaxanthin different solvent systems were needed for HPLC analysis. Solvent system 1: α- and β-carotenes were eluted isocratically with a methanol/MTBE mixture (60:40) and detected by ELSD and vis (450 nm). The flow rate was 0.5 mL/min. Solvent system 2: Lycopene was eluted with a methanol/MTBE/ethyl acetate mixture (50:40:10) with a flow rate gradient (0−30 min, 0.75 mL/min; 30−50 min, from 0.75 to 1.0 mL/min; 50−80 min, 1.0 mL/min). Visible detection was performed at 470 nm in addition to ELSD detection. Solvent system 3: Lutein and canthaxanthin were eluted isocratically with a methanol/MTBE mixture (80:20) and detected by ELSD and vis (448 nm). The flow rate was 0.5 mL/min.

Elemental Analysis−Combustion/Pyrolysis−Isotope Ratio Mass Spectrometry. The isotope ratio analysis was performed by 5-fold measurements using a Delta plus XL (Finnigan MAT, Bremen, Germany) isotope ratio mass spectrometer coupled to elemental analyzers operating in the "combustion" (C) and "pyrolysis" (P) modes.

Combustion (EA−C) Mode. Determination of $^{13}C/^{12}C$ ratios was performed in the combustion mode with an oxidative reactor (quartz tube filled with tungsten oxide, quartz wool, and copper filling; Euro EA 3000 series, HEKAtech, Wegberg, Germany). The operating reactor temperature was 1000 °C, and the carrier gas (helium 5.3) flow rate was 80 mL/min. A 2 m × 5 mm i.d. Porapak QS column (Euro Vector, Milano, Italy) and an oven temperature of 60 °C were used.

Pyrolysis (EA−P) Mode. The reactor used for $^2H/^1H$ ratio determination consisted of an outer ceramic tube and an inner glass carbon tube filled with nickelized carbon and glass carbon pieces (HT Sauerstoff Analysator, HEKAtech). The operating reactor temperature was 1460 °C, and the carrier gas (helium 5.3) flow rate was 70 mL/

min. A 1 m × 5 mm i.d. 5 Å molecular sieve column (Euro Vector) and an oven temperature of 85 °C were used.

Procedure. In general, 6-fold determinations were carried out with about 0.25 and 1 mg sample for carbon and hydrogen measurements, respectively. Dry samples were weighed into tin cups (3.5 mm × 5 mm, HEKAtech) for the EA−C mode and into silver cups (3.5 mm × 5 mm, HEKAtech) for the EA−P mode and submitted to EA measurement as described above. The ion source of the mass spectrometer was heated at 70 °C. Electron impact ionization was performed at an electron energy of 70 eV. The cathode voltage was 3 kV, and transfer capillaries were not heated.

Daily system checks were carried out by measuring reference samples with known $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios. Stability checks of the used reference gases were routinely performed by measuring certified International Atomic Energy Agency (IAEA; Vienna, Austria) standards with defined $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios, namely, for $^{13}C/^{12}C$ IAEA CH-7 and for $^{2}H/^{1}H$ IAEA CH-7 as well as NBS 22 (oil, IAEA).

The $^{13}C/^{12}C$ and $^{2}H/^{1}H$ isotope ratios were expressed in per mil deviation relative to the values for the V-PDB (Vienna Pee Dee belemnite) and V-SMOW (Vienna standard mean ocean water) international standards, respectively. The results were calculated as follows:

$$\delta^{13}C_{V\text{-}PDB}\ (‰) = \left(\frac{R_{sample} - R_{V\text{-}PDB}}{R_{V\text{-}PDB}}\right) \times 1000$$

where R is the isotope ratio $^{13}C/^{12}C$. For $\delta^{2}H_{V\text{-}SMOW}$ the corresponding formula is valid, where $R = {}^{2}H/{}^{1}H$, respectively.

For $^{13}C/^{12}C$ measurements the mass spectrometer was calibrated with certified CO_2 gas ($\delta^{13}C_{V\text{-}PDB} = -24.9 \pm 0.1‰$; Messer Griesheim, Frankfurt, Germany). For $^{2}H/^{1}H$ measurements calibration was performed with certified H_2 gas ($\delta^{2}H_{V\text{-}SMOW} = -207 \pm 5‰$; Messer Griesheim). Standard deviations were ±0.1‰ and ±5‰ for $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ determinations, respectively. The influence of sample preparation on the isotope ratios checked by model Celite/MgO (1:1) column separation and crystallization were found to be within the range of standard deviation and thus negligible (data not shown).

RESULTS AND DISCUSSION

Carotenoids such as α- and β-carotenes, lutein, and lycopene were isolated from six different natural sources by simultaneously performed extraction and saponification steps as well as subsequent crystallization using suitable solvents, according to methods previously described by various authors (*12–14*). Since lycopene in the samples rose hip pulp and tomato puree could not be satisfactorily separated from other cocarotenoids by crystallization, column chromatography on Celite/MgO was required. Applying this method, not only was lycopene separated, but also isolation of β-carotene (5 mg) from tomato puree was achieved. The yields of other carotenoids from natural sources ranged from 43 to 240 mg, depending on the sample. Purification procedures such as crystallization and column chromatography yielded purities >96%. Finally, β-carotene was isolated analogously from five commercial dietary supplements. The subsequently applied crystallization procedure yielded purities >97%.

IRMS Analysis of Authentic Carotenoid Samples. The $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios of authentic carotenoid samples, i.e., β-carotene, the mixture of α- and β-carotene, lycopene, lutein, and canthaxanthin, from various sources were determined. The results of both $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ determinations are compiled in **Table 2**. The $\delta^{13}C_{V\text{-}PDB}$ values of some carotenoids reported previously by other authors are shown in **Table 1**. Four different categories of sample sources were taken into account: C_3 and C_4 terrestrial plants, marine microalgae, and synthetic samples.

The isotopic analysis of carotenoids from C_3 plants (β-carotene and lycopene from *Lycopersicon esculentum*, β/α-carotene mixture from *Daucus carota* and *Elaeis guineensis*, lycopene from *Rosa rubiginosa*, and lutein from *Medicago sativa*, *T. erecta*, and *H. urticae*) led to $\delta^{13}C_{V\text{-}PDB}$ values of −28.5‰ to −32.8‰. This finding is in good agreement with earlier published literature data of $\delta^{13}C_{V\text{-}PDB}$ values ranging from −29.2‰ to −31.1‰ for carotenoids from C_3 plants (lutein from *T. erecta* and *Lupinus* sp., as well as a carotenoid mixture from *Gossypium*). Hence, these results are consistent with carbon isotope ratios of −24‰ to −32‰ generally reported for products of C_3 plants (*15*). In addition, some commercial samples declared to be natural (without indication of origin) showed $^{13}C_{V\text{-}PDB}$ values (lycopene, −31.4‰; lutein, −28.8‰ and −29.6‰) quite similar to those of the references originating from the C_3 plants discussed above.

In contrast to those from C_3 plants the carotenoids from C_4 plants (lutein from *Zea mays*, a carotenoid mixture from *Sorghum*) have shown in previous studies more positive $\delta^{13}C_{V\text{-}PDB}$ values ranging from −14.9‰ to −20.7‰ (*16, 17*) (cf. **Table 1**). These data ranges fit well to the well-known carbon isotope values from C_4 plant products generally ranging between −10‰ and −16‰ (*15*).

Aquatic plants have to be considered separately due to some differences of environmental influences, in particular, in relation to the primary source of the carbon. Marine plants take up preferably HCO_3^- ($\delta^{13}C_{V\text{-}PDB} = \pm 0‰$) dissolved in seawater as a more available carbon source than atmospheric CO_2 ($\delta^{13}C_{V\text{-}PDB} = -7‰$). Hence, marine plants and phytoplankton tend to be enriched in ^{13}C compared to terrestrial plants with a corresponding photosynthetic pathway (*15, 18, 19*). Previous studies reviewed by Descolas-Gros and Fontugne (*19*) reported that some marine algae may show different pathways of carbon fixation catalyzed by RUBISCO (ribulose 1,5-bisphosphate carboxylase) and/or by β-carboxylases (phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, and pyruvate carboxylase). The rate of β-carboxylation relative to RUBISCO activity in the same algae species can vary with environmental factors (temperature, photoperiod) and the physiological state of the cells (aging) that cause a variation in the carbon isotope discrimination rate. Different species of marine phytoplankton showed in earlier studies a variation in $\delta^{13}C_{V\text{-}PDB}$ from −14‰ to about −35‰ (*16, 19–22*). In our investigations, one sample of a carotenoid mixture with β-carotene as the major component isolated from the marine microalgae *D. salina* exhibited a relatively positive $\delta^{13}C_{V\text{-}PDB}$ value of −15.6‰ (**Table 2**). A similar carbon isotopic composition has been reported previously for cell materials of *Dunaliella tertiolecta* ($\delta^{13}C_{V\text{-}PDB} = -16.2‰$) (*23*) and *Dunaliella marina* ($\delta^{13}C_{V\text{-}PDB} = -18‰$) (*19*).

Commercial synthetic β-carotene references ($n = 4$) from different suppliers revealed $\delta^{13}C_{V\text{-}PDB}$ values from −25.3‰ to −26.4‰ that clearly differed from those determined for natural β-carotene and other natural carotenoid samples described above. In addition, it was of interest to investigate synthetic canthaxanthin ($n = 3$), usually derived in the industrial large-scale production from β-carotene by way of oxidation (**Figure 2**). The $\delta^{13}C_{V\text{-}PDB}$ data of canthaxanthin showed with values ranging from −24.9‰ to −28.6‰ a broader variation than those of the corresponding synthetic β-carotene. Canthaxanthin of natural source was not analyzed in this work.

To our best knowledge, there are no isotope data for synthetic carotenoids available in the literature to date. Because β-carotene, canthaxanthin, and other carotenoids are synthetically produced from acetone as the starting material in the range of large-scale manufacturing, their well-known isotopic data were taken into account and are presented in **Figure 2**. The slight

enrichment and partial similarity in $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ values of the investigated synthetic products β-carotene ($\delta^{13}C_{V\text{-}PDB}$ values from $-25.3‰$ to $-26.4‰$; $\delta^{2}H_{V\text{-}SMOW}$ values from $-144‰$ to $-155‰$) and canthaxanthin ($\delta^{13}C_{V\text{-}PDB}$ values from $-25.0‰$ to $-28.6‰$; $\delta^{2}H_{V\text{-}SMOW}$ values from $-133‰$ to $-153‰$) were obvious in comparison to isotope data of synthetic acetone ($\delta^{13}C_{V\text{-}PDB} = -28.0‰$; $\delta^{2}H_{V\text{-}SMOW} = -160‰$) previously reported by our group (*24*).

Not only did $\delta^{13}C_{V\text{-}PDB}$ results show clear differences between natural and synthetic carotenoid references, but also $\delta^{2}H_{V\text{-}SMOW}$ measurements confirmed this tendency: $\delta^{2}H_{V\text{-}SMOW}$ values determined for all natural carotenoid samples (ranging from $-180‰$ to $-275‰$) were more negative than those of synthetic β-carotene and canthaxanthin (ranging from $-133‰$ to $-155‰$). To our best knowledge, there are no hydrogen isotope data ($\delta^{2}H_{V\text{-}SMOW}$ values) for any carotenoid available in the literature to date.

To illustrate the differences in the isotope ratio composition of carotenoids from various sources, the resulting $\delta^{13}C_{V\text{-}PDB}$ values were plotted versus the $\delta^{2}H_{V\text{-}SMOW}$ values in **Figure 3**: three clusters of data points representing synthesis products, C_3 plant origin, and biotechnological source from marine microalgae can be distinguished. One sample of commercial lycopene for which the origin was not specified could not be assigned to these fields. The resulting tendency of origin differentiation by carotenoids on the basis of stable isotope correlations allowed further analysis of dietary supplements containing carotenoids.

IRMS Analysis of Dietary Supplements. Five commercial dietary supplements containing β-carotene, in all cases declared to be natural, were analyzed by EA−C/P−IRMS. The recorded $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ values of β-carotene as the major compound are shown in **Table 3**. The relationship of these results is additionally illustrated in **Figure 3** in comparison to the current collection of isotope data for synthetic and natural carotenoid references. Among the dietary supplements under study, with $\delta^{13}C_{V\text{-}PDB}$ values of β-carotene ranging from $-16.4‰$ to $-28.5‰$ and $\delta^{2}H_{V\text{-}SMOW}$ data ranging from $-134‰$ to $-218‰$, two samples exhibited $\delta^{13}C_{V\text{-}PDB}$ and $\delta^{2}H_{V\text{-}SMOW}$ data as found for synthetic β-carotene and the other three were positioned near biotechnological β-carotene.

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