JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Heating and Illumination on *Trans*−*Cis* Isomerization and Degradation of β-Carotene and Lutein in Isolated Spinach Chloroplasts

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The influence of thermal treatment and light exposure on degradation and isomerization of the predominant carotenoids (lutein and β-carotene) occurring in green leafy vegetables was assessed. The effect of lipid addition on carotenoid stability was also evaluated. For the first time, the stabilities of pure carotenoids and chloroplast-bound carotenoids were compared. Besides degradation, heating caused carotenoid isomerization in all samples. Whereas pure carotenoids favor 13-*cis* isomers, in native chloroplasts and heated chloroplasts 9-*cis* isomers were predominant. Illumination of freshly prepared chloroplast isolates caused an initial increase in the level of lutein (9.6%) and β-carotene (29.8%), while pure carotenoids exhibited time-dependent degradation. The addition of lipids to chloroplast preparations had the reverse effects on the retention of both carotenoids after heating; isomerization was not significantly affected. It was demonstrated that carotenoid stability has to be evaluated for every individual pigment in its genuine environment. Stability data based on model systems (e.g., pure carotenoids) may not be transferred to complex food matrices without intensive investigation.

KEYWORDS: Spinach; chloroplast; β-carotene; lutein; *trans*−*cis* isomers; heat; illumination

INTRODUCTION

In plants, beside their role as color, carotenoids represent essential accessory light-harvesting pigments in the photosynthetic apparatus (*1*). Furthermore, carotenoids have been reported to be excellent scavengers of reactive oxygen species (*2*). Numerous epidemiological studies have suggested an inverse correlation of carotenoid ingestion and the risk of certain types of cancer (*3*−*5*). In addition, evidence for protection against age-related macular degeneration has been provided, particularly for lutein (*6*).

Whereas natural carotenoids occur mainly in their thermodynamically more stable all-*trans* configuration, the *cis* isomers are only present in minor amounts (*7*) and have been demonstrated to be formed as a consequence of food processing such as heating and illumination (*8*−*11*). These conversions have nutritional consequences because of changes in bioavailability and physiological activity (*12*−*15*). Literature data suggest that each isomer displays individual characteristics of absorption, plasma transport, and metabolism.

In previous studies, *cis* stereoisomers were shown to be concomitant with all-*trans* carotenoids in raw vegetables and fruits (*16*). In yellow and red vegetables, thermal processing of food facilitated the formation of 13-*cis* isomers, while illumination induced 9-*cis* isomerization (*9*, *10*, *17*, *18*). These results comply with studies with pure carotenoids yielding similar isomeric composition (*16*, *19*). In contrast, in green-colored vegetables and fruits, 9-*cis*-β-carotene was found to be the major *cis* isomer (*16*). Mixtures of pure all-*trans*-β-carotene and chlorophyll fractions showed an increase in 9-*cis* stereoisomers after illumination, explaining the elevated content of 9-*cis* isomers in raw green vegetables (*20*). Unexpectedly, the level of 9-*cis* stereoisomers was also increased after thermal processing (*17*, *18*, *21*). After light exposure, pure carotenoids exhibited a much better light stability than in the presence of chlorophyll (*22*). Obviously, pigments such as chlorophylls act as sensitizers of carotenoid degradation and isomerization. Similarly, chlorogenic acid enhances the isomerization rate of all-*trans*-β-carotene (*23*).

In plant cells, carotenoids are accumulated and sequestered in chromoplasts and chloroplasts. Carotenoids are deposited as the carotenoid−protein complex, in crystalline form, or as oily droplets, depending on the chromoplast type (*24*). In chloroplasts, carotenoids form chlorophyll−carotenoid−protein complexes which are located either in hydrophobic domains of membrane proteins or in the lipid membrane matrix (*1*). In many studies, carotenoid stability was investigated by the use of pure pigments (*11*, *20*). However, in such model systems, interactions with the matrix, e.g., in cell organelles, are not considered. It was demonstrated that dissolved mixtures of chlorophylls and carotenoids were generally highly unstable and underwent rapid

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10.1021/jf050926w CCC: $30.25
Published on Web 10/29/2005

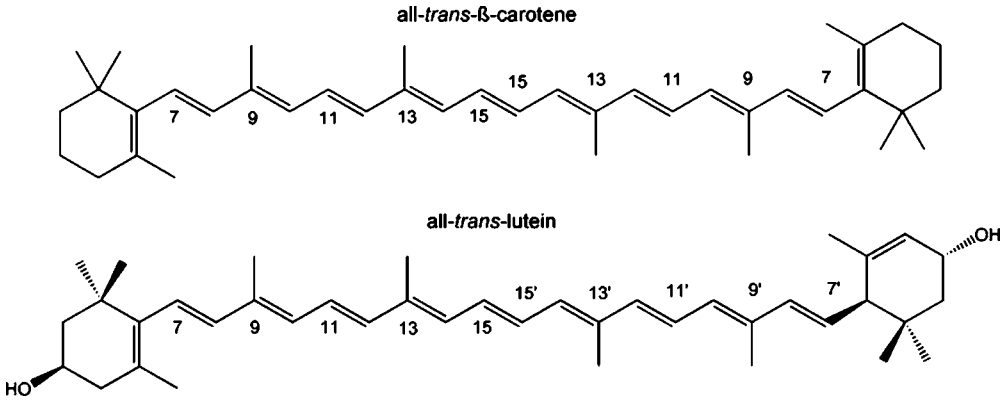

Figure 1. Structures of all-*trans*-β-carotene and all-*trans*-lutein.

bleaching in the presence of light and oxygen, while the stability of these pigments was significantly improved when they were bound to apoproteins of pigment−protein complexes (*25*).

The objective of this study was to compare the degradation and isomerization of pure lutein and β-carotene and of a green leafy vegetable that contains both carotenoids as the main carotenoid species (**Figure 1**). Spinach, which represents an important dietary source of both pigments (*26*), was used. Intact chloroplasts were isolated by gradient centrifugation, discriminating between the complex interactions between the food matrix and carotenoids. Because dietary fat has been shown both to promote carotenoid absorption (*27*) and to facilitate isomerization (*10*), the effect of lipid addition on carotenoid stability was also evaluated.

MATERIALS AND METHODS

Materials. All chemicals and HPLC solvents were purchased from VWR (Darmstadt, Germany) and were reagent-grade and gradient-grade, respectively. Ultrapure water was used for HPLC analyses and sample preparation. Miracloth and Ficoll 400 were supplied by Calbiochem-Merck (Darmstadt, Germany). Percoll separating solution (density of 1.077 g/mL) and bovine serum albumin were purchased from Biochrom (Berlin, Germany) and Serva (Heidelberg, Germany), respectively. Sorbitol and polyethylene (MW 6000) were obtained from Mallinckrodt Baker (Deventer, The Netherlands) and Schuchardt (Hohenbrunn, Germany), respectively. All-*trans*-β-carotene was from Sigma-Aldrich (Taufkirchen, Germany). All-*trans*-lutein [(*all-trans*,3*R*,3′*R*,6′*R*)-β,ϵ-carotene-3,3′-diol] was provided by Hoffmann-La Roche (Basel, Switzerland). Spinach (*Spinacia oleracea* L.) and coconut fat (100%, hardened) (Biskin, Elmshorn, Germany) were purchased from the local market.

Preparation of Solutions for Gradient Centrifugation. For GM (grinding mix), 2.225 g of sodium pyrophosphate was dissolved in 40 mL of boiling water. After the mixture had cooled at 20 °C, 59.58 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 300.6 g of sorbitol, 3.72 g of EDTA, 1.016 g of $MgCl_2$, and 0.99 g of $MnCl_2$ were added. The solution was dissolved in 700 mL of water. After the pH was adjusted to 6.8 with 6 N NaOH, the volume was increased to 1000 mL. Portions of 200 mL were stored at −20 °C.

For GM dilution, immediately after thawing, 200 mL of GM and 308 mg of dithiothreitol were mixed with water to a volume of 1000 mL.

For PCBF dilution, 3 g of polyethylene (MW 6000), 1 g of bovine serum albumin, and 1 g of Ficoll were dissolved in 100 mL of Percoll separating solution.

For the 10% Percoll gradient, 6 mg of glutathione and 10 mL of PCBF dilution were added to 20 mL of GM. The volume was increased to 100 mL with water.

For the 80% Percoll gradient, 6 mg of glutathione was added to 20 mL of GM and the volume was increased to 100 mL with PCBF dilution.

Isolation of Chloroplasts from Spinach. The isolation of chloroplasts was performed according to the method of Gruissem et al. (*28*). Approximately 100 g of fresh spinach leaves was washed with cold water and drained. All following preparation steps were carried out at 0−4 °C. Spinach portions of 50 g were homogenized using a model 38BL41 Waring blender (Waring Products, Torrington, CT) in 150 mL of GM dilution for 20 s at high speed (level II).

The homogenate of one portion was filtered through four layers of miracloth, and the filtrate was centrifuged (Suprafuge 22, Heraeus, Hanau, Germany) for 1 min (4300*g* at 2 °C). The slightly green supernatant and the upper layer of the chloroplast pellets, mostly consisting of broken chloroplasts, were discarded. The pellets retained in the tubes and the chloroplasts from the subsequent homogenate were collected with the former chloroplast pellets. The resulting pellets were suspended with GM dilution and transferred to a brown flask, and the volume was increased to 25 mL with GM dilution.

For gradient centrifugation, 4 mL each was layered onto six Percoll gradients (each 24 mL). For this purpose, 12 mL of the 10% Percoll gradient was put into a centrifuge tube and 12 mL of the 80% Percoll gradient was underlaid with a syringe. The gradients were centrifuged for 20 min (8050*g* at 2 °C). Intact chloroplasts were recovered from the lower band, while fragments were located in the upper band. For removal of Percoll, each chloroplast suspension was diluted with 10 mL of GM dilution and centrifuged for 1 min (8050*g* at 2 °C). The chloroplast pellets were transferred to a brown flask, and the volume was increased to 50 mL with GM dilution and the mixture stored at 4 °C.

Preparation of Carotenoid Solutions. Approximately 1 mg of carotenoid was dissolved in toluene and the volume increased to 50 mL.

Heating Experiments. For heating experiments, 2 mL of chloroplast suspension and 3 mL of GM dilution were placed in a test tube. To investigate the effect of lipophilic solvents, 0.5 g of coconut fat was added. Experiments with pure carotenoids were carried out using tubes containing 2 mL of carotenoid solution and 3 mL of toluene. To avoid pigment degradation in the presence of oxygen, samples were flushed with nitrogen. The vessels were sealed tightly and shaken vigorously before being heated. Solutions were heated in a boiling water bath at 98 °C for 0, 0.5, 1, 2, 5, 10, 20, and 60 min under dim light conditions and agitation with a magnetic stir bar. Experiments were carried out in duplicate.

Illumination Experiments. Chloroplast isolates and solutions of pure carotenoids were prepared as described above. The illumination experiments were performed by exposing the samples to fluorescent light [polychrome; one fluorescent lamp (L58W/25 universal white) and four fluorescent lamps (L36W/76 nature de luxe) (Osram, Munich, Germany)] for 0, 0.5, 1, 2, 5, 10, 20, and 60 min at 20 °C. All samples were agitated with a magnetic stir bar. The illumination intensity (3750 lux) was determined with a luxmeter (Mavolux-digital, Gossen, Nuernberg, Germany). All experiments were performed in duplicate.

Carotenoid Analysis. *Sample Preparation.* To avoid degradation and isomerization of carotenoids, amber glassware was used, and extraction was performed under dim light. For the assessment of

carotenoids, chloroplast preparations were subjected to saponification to remove chlorophylls, which affect chromatographic performance. For this purpose, 30 mL of a potassium hydroxide solution [10 g in 100 mL of a 50:50 (v/v) water/methanol mixture] and 60 mL of light petroleum (40−60 °C) containing butylated hydroxytoluene (BHT) (100 mg/L) and butylated hydroxyanisole (BHA) (100 mg/L) as antioxidants were added to a 100 mL flask. After being flushed with nitrogen, the flask was sealed tightly, and the contents were agitated with a magnetic stir bar for 1 h. The solution was transferred to an amber glass separatory funnel and washed once with 50 mL of a sodium chloride solution (10 g/100 mL) and twice with 50 mL of water to remove alkali. The aqueous layer was reextracted with ethyl acetate until it was colorless, and the pooled organic phases were dried over sodium sulfate. Glassware and sodium sulfate were rinsed with toluene, and the combined organic phases were evaporated to dryness in vacuo (T < 30 °C). The residue was dissolved in 2-propanol, and the volume was increased to 25 mL. Standard solutions after heating and illumination were evaporated in vacuo (T < 30 °C), and the residue was dissolved in 2-propanol. The volume was increased to 5 mL.

Separation of Carotenoid Stereoisomers by HPLC. HPLC analyses were performed on a model 2690 Waters separation module equipped with an autosampler injector, a model Jetstream 2 plus Waters column oven, and a model 2996 Waters UV−visible photodiode array detector controlled by a Millennium 32 (version 3.20) workstation (Waters, Milford, MA). The separation of the carotenoid stereoisomers was carried out using a 3 μm analytical scale C_{30} reversed phase column (150 mm × 3.0 mm inside diameter) protected by a 3 μm C_{30} reversed phase guard column (10 mm × 4.0 mm inside diameter) (YMC, Wilmington, MA) at 20 °C and a flow rate of 0.42 mL/min.

β-Carotene isomers were separated within 34 min using a linear gradient from 100% A [81:15:4 (v/v) methanol/*tert*-butyl methyl ether (MTBE)/water mixture] to 51% eluent B [90:6:4 (v/v) MTBE/methanol/water mixture]. The separation of lutein isomers was performed as previously reported (*29*). The injection volume was 10 μL. The carotenoids were detected at the wavelengths of maximum absorption in the corresponding HPLC solvent: all-*trans*-β-carotene, 452 nm; 9-*cis*-β-carotene, 445 nm; 13-*cis*-β-carotene, 445 nm; all-*trans*-lutein, 445 nm; 9-*cis*-lutein, 438 nm; 9′-*cis*-lutein, 438 nm; 13-*cis*-lutein, 438 nm; and 13′-*cis*-lutein, 438 nm. HPLC analyses were carried out in duplicate.

Identification and Quantification of Carotenoids. Standards of *cis* isomers were obtained by iodine-catalyzed photoisomerization of all-*trans*-β-carotene and all-*trans*-lutein (*30*), and recovered by preparative HPLC. Isolation of the major stereoisomers of β-carotene and lutein was performed as described previously (*31*). The predominant chromatographic peaks were identified by LC−MS (APCI$^+$ mode) and HPLC−NMR spectroscopy (*29*, *33*). One- and two-dimensional NMR spectra were recorded in the stopped-flow mode. Carotenoids extracted from spinach samples were identified by comparison of their retention times and absorption spectra with those of authentic standards. Concentrations were based on linear calibration curves. Concentrations of stock solutions were determined spectrophotometrically on the basis of published data (*32*). Specific absorption coefficients ($A^{1\%}_{1cm}$) and wavelength maxima that were used were as follows: all-*trans*-β-carotene, 2592 at 450 nm (hexane); all-*trans*-lutein, 2550 at 445 nm (ethanol). Concentrations of *cis* isomers were calculated using the calibration curves of the corresponding all-*trans* carotenoids.

RESULTS

All experiments were carried out in duplicate. Each point of the figures represents the mean of duplicate determinations. Relative standard deviations observed for total carotenoids and *cis* carotenoids were 1.54% (from 0.07 to 4.94%) and 7.29% (from 4.24 to 13.93%), respectively.

Effects of Heating. *Stability of β-Carotene*. Heat treatment (T = 98 °C) entailed loss of total amounts of β-carotene in all samples that were investigated (**Figure 2**). In the standard solution, 84.7% of the initial content was retained after heating for 60 min. In chloroplasts, a similar degradation rate of β-carotene, amounting to 83.2%, was observed. The addition of fat to chloroplasts resulted in a decrease to 82.0%. Pure β-carotene and chloroplasts showed a similar pattern of degradation throughout heating. However, β-carotene localized in the chloroplast appeared to be slightly more stable during the first 10 min, whereas the addition of fat enhanced β-carotene degradation during this period.

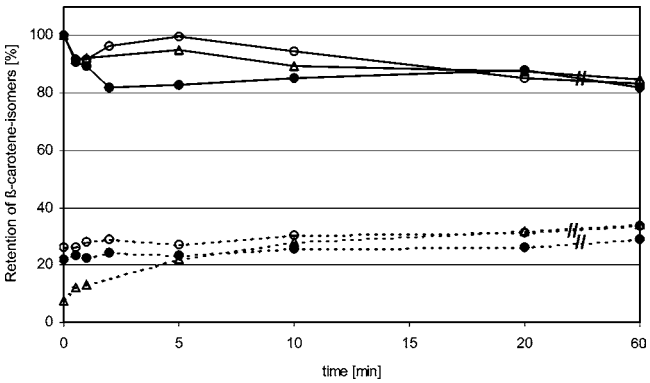

Figure 2. Retention of β-carotene isomers (%) during heating at 98 °C of pure carotenoid (△), chloroplast isolate (○), and chloroplast isolate with added lipids (●), for total β-carotene (—) and *cis*-β-carotene isomers (•••). Each point represents the mean of duplicate determinations.

Isomerization of β-Carotene. Apart from degradation, all-*trans*-β-carotene was partially converted into its *cis* isomers. The unheated standard consisted of 92.5% all-*trans*-, 5.5% 13-*cis*-, and 2.0% 9-*cis*-β-carotene. Heat treatment increased the amount of 13-*cis*-β-carotene, whereas the formation of 9-*cis*-β-carotene was negligible. After the sample had been heated for 60 min, the proportion of 13-*cis*-β-carotene and 9-*cis*-β-carotene increased to 28.8 and 5.1%, respectively. In contrast, unheated chloroplasts were characterized by an elevated proportion of *cis* isomers (26.0%), with 9-*cis*-β-carotene being predominant. After heating, the level of 13-*cis*- and 9-*cis*-β-carotene increased from 11.9 to 15.3% and from 14.2 to 22.1%, respectively. Analogously, the addition of fat to the chloroplasts caused an increase in the level of 13-*cis*- and 9-*cis*-β-carotene from 9.2 to 10.2% and from 14.7 to 21.5%, respectively.

Isomeric Composition of β-Carotene. The retention of total to *cis*-β-carotene isomers (9- and 13-*cis*) during heating of the pure carotenoid and of the chloroplasts with and without added fat is shown in **Figure 2**. After 60 min, 34.0% *cis* isomers and 66.0% all-*trans*-β-carotene were reached in the standard, thus representing a pseudoequilibrium. In heated chloroplasts, a similar *cis* isomerization rate (33.6%) was determined. In contrast, after addition of lipid to the chloroplast suspension, the *cis* isomer proportion slightly decreased to 29.0% at the end of thermal processing.

Stability of Lutein. In almost all samples, the total lutein content was lowered upon heating compared to that of the unheated control (**Figure 3**). In the standard solution, 83.8% of the initial lutein content was retained after heat exposure for 60 min, resulting in degradation rates similar to those found for β-carotene (84.7%). In the heated chloroplast isolate, a more pronounced degradation was observed with only 72.0% lutein retention after 60 min. Obviously, lutein localized in the chloroplast was more prone to degradation than β-carotene (83.2%). After the addition of lipids to the chloroplast preparation, enhanced lutein stability was found, with the major part (93.0%) still being intact after heat exposure for 60 min. In contrast to β-carotene degradation (82.0%), lutein appeared to be stabilized by addition of lipids. As indicated by a time lag during the first 2 min, all samples initially exhibited good stability of total lutein (>95.0%). However, upon continuous

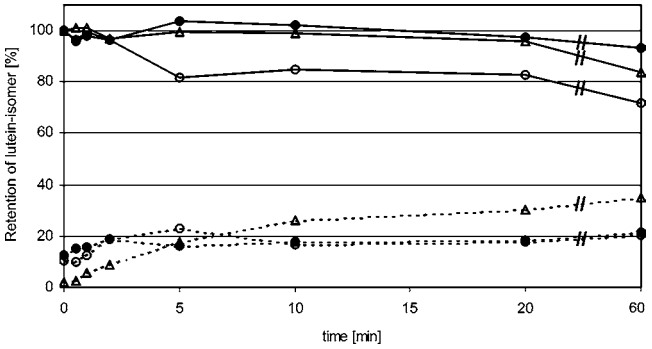

Figure 3. Retention of lutein isomers (%) during heating at 98 °C of pure carotenoid (△), chloroplast isolate (○), and chloroplast isolate with added lipids (●), for total lutein (—) and *cis*-lutein isomers (•••). Each point represents the mean of duplicate determinations.

heating the lutein content dropped significantly in chloroplasts, while the addition of fat resulted in marginal lutein decomposition.

Isomerization of Lutein. Furthermore, thermal processing caused *trans*−*cis* isomerization of lutein. Whereas the all-*trans*-lutein standard was found to contain 1.1% 13-*cis*- and 0.9% 13′-*cis*-lutein, heating at 98 °C for 60 min increased the relative content of both isomers to 18.3 and 16.8%, respectively, whereas 9-*cis* isomers were not detectable. In the unheated chloroplast isolate, besides all-*trans*-lutein, 2.0% 13-*cis*, 1.4% 13′-*cis*, 3.4% 9-*cis*, and 3.7% 9′-*cis* isomers were present. After heating for 60 min, 13-*cis*- and 13′-*cis*-lutein contents were only slightly lowered (3.1 and 2.2%, respectively), whereas 9-*cis*- and 9′-*cis*-lutein contents considerably increased to 8.5 and 6.5%, respectively. With the addition of lipids to the chloroplasts, a similar isomerization pattern was obtained. Whereas the levels of 9-*cis*- and 9′-*cis*-lutein increased from 3.9 to 8.5% and from 2.9 to 7.4%, respectively, levels of 13-*cis*- and 13′-*cis*-lutein were hardly affected (from 3.0 to 2.1% and from 2.7 to 3.4%, respectively). Generally, a similar trend toward the formation of 9-*cis* isomers was found for lutein and β-carotene in thermally treated chloroplasts.

Isomeric Composition of Lutein. The isomeric composition of lutein during heating is shown in **Figure 3**. In the standard, the total *cis* isomer content (9- and 13-*cis*) reached 35.0% within 60 min, which is in good agreement with β-carotene isomerization upon heating. In contrast, lutein in the chloroplasts, where ∼80% of the all-*trans* configuration was observed, exhibited a lower extent of conversion to the *cis* isomers. The addition of lipids to the chloroplast isolate caused a similar isomerization rate (21.7%). After a prolonged heating over 180 min, a pseudoequilibrium of the isomeric composition as observed in the standard solution was found (data not shown).

Effects of Illumination. *Stability of β-Carotene*. Light exposure (3750 lux) of the standard solution caused degradation of total β-carotene (**Figure 4**). After an induction time of 20 min, the total level of β-carotene decreased from 98.4 to 80.6% of the initial content. Astonishingly, chloroplasts after illumination were found to behave completely differently: the total level of β-carotene considerably increased to 126.7% within 0.5 min followed by a drop to a minimum after 5 min (120.4%) and reaching a maximum after 10 min (129.8%). Subsequently, the β-carotene level declined linearly to 121.4% within 60 min. Continued illumination over 180 min caused a further decrease to 120.3% (data not shown).

Isomerization of β-Carotene. The all-*trans*-β-carotene standard used in this study was characterized by isomeric impurities consisting of 8.1% 13-*cis*- and 3.7% 9-*cis*-β-carotene. Illumination did not affect its initial isomeric composition, since 13-*cis*- and 9-*cis*-β-carotene contents only marginally increased to 9.9 and 4.4%, respectively. Also, the native chloroplast isolates already contained notable proportions of 13-*cis*- (11.9%) and 9-*cis*-β-carotene (10.4%). Whereas the level of 13-*cis*-β-carotene decreased to 9.5% during light exposure, the level of 9-*cis*-β-carotene, as the predominant photoinduced *cis* isomer, expectedly increased to 12.3% after illumination for 60 min.

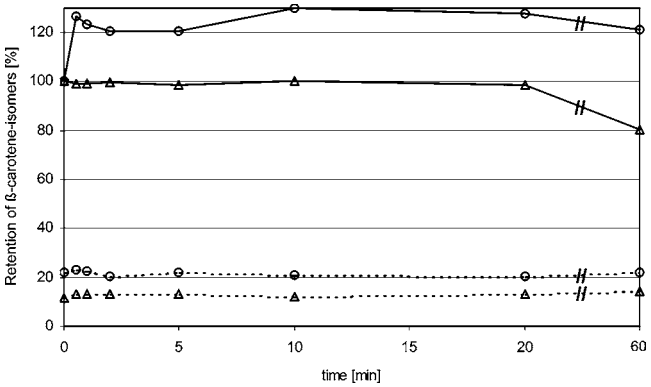

Figure 4. Retention of β-carotene isomers (%) during illumination (3750 lux) at 20 °C of pure carotenoid (△) and chloroplast isolate (○), for total β-carotene (—) and *cis*-β-carotene isomers (•••). Each point represents the mean of duplicate determinations.

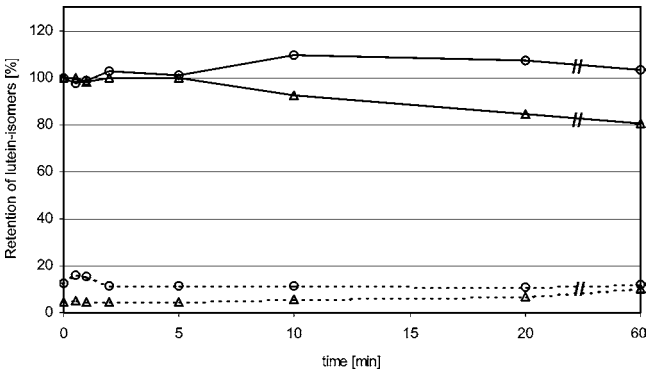

Figure 5. Retention of lutein isomers (%) during illumination (3750 lux) at 20 °C of pure carotenoid (△) and chloroplast isolate (○), for total lutein (—) and *cis*-lutein isomers (•••). Each point represents the mean of duplicate determinations.

Isomeric Composition of β-Carotene. The distribution of *cis* and *trans* isomers did not vary significantly in the standard solution (**Figure 4**) since the initial isomeric ratio of 11.8:88.2 was only slightly changed to 14.3:85.7. Likewise, the isomeric composition of chlorophyll-bound β-carotene showed minimal differences ranging from a 22.3:77.7 ratio to a 21.8:78.2 ratio after exposure to light for 60 min.

Stability of Lutein. The isomeric composition of lutein exhibited a time-dependent degradation following light exposure (**Figure 5**). The lutein level continuously decreased to 80.4% of the initial content after 60 min, showing retention rates nearly identical to that observed for β-carotene (80.6%). The initial lutein content was retained (99.7%) for only the first 5 min, whereas β-carotene persisted for more than 20 min (98.4%), showing a superior light stability during initial illumination. These investigations of chloroplasts revealed increased lutein contents during the first 10 min (109.6%) and a subsequent decrease afterward (103.3%). Thus, a similar but less pronounced trend as noticed for β-carotene was confirmed.

Isomerization of Lutein. The lutein standard consisted of 1.0% 13-*cis*-lutein, 1.1% 13′-*cis*-lutein, 0.8% 9-*cis*-lutein, and 1.5%

9′-*cis*-lutein as minor compounds. As shown for β-carotene, the composition of *cis* isomers varied only marginally following illumination. Except for 13-*cis*- and 13′-*cis*-lutein isomerization (3.2 and 4.2%, respectively), 9-*cis*- and 9′-*cis*-lutein levels remained unchanged (1.3 and 1.7%, respectively). Chlorophyll-bound lutein contained 2.5% 13-*cis*, 3.1% 13′-*cis*, 4.2% 9-*cis*, and 2.8% 9′-*cis* isomers. After 60 min, 2.6% 13-*cis*, 3.9% 13′-*cis*, 3.3% 9-*cis*, and 3.0% 9′-*cis* isomers were detected, suggesting a negligible change in the isomeric distribution.

Isomeric Composition of Lutein. The isomeric composition was hardly changed after light exposure (**Figure 5**). In the standard, the initial ratio of *cis* to *trans* isomers (4.4:95.6) of the control was increased slightly to 10.5:89.5. Continued illumination over 180 min did not further affect the isomeric composition (data not shown). Likewise, in isolated chloroplasts, the *cis*- and *trans*-lutein pattern was only slightly changed from the initial 12.6:87.4 ratio to 11.9:88.1 after illumination over 60 min.

DISCUSSION

Vegetables are thermally processed for product preservation, most often by blanching, pasteurization, and sterilization. However, thermal inactivation of microorganisms and degradative enzymes is generally accompanied by losses in quality attributes such as color, flavor, texture, and degradation of valuable constituents. In addition, domestic food preparation such as baking or frying further contributes to thermal decomposition of heat sensitive compounds. These treatments, often carried out with the addition of fat, are characterized by long heating periods. The amount of fat added to the chloroplast isolates was allocated according to common household preparation of spinach (approximately 5−10 g per 100 g of spinach). Besides thermal treatment, vegetables are exposed to illumination from cultivation until consumption. In this study, 9-*cis* and 13-*cis* isomers were evaluated, because these isomers were predominantly detected in processed vegetables (*10*, *33*).

Effect of Heating. *Total Carotenoid Content*. Thermal treatment applied to pure carotenoids caused a continuous decrease in their total contents. After heating at 98 °C was carried out for 60 min, retention values of 84.7% for β-carotene and of 83.4% for lutein were found.

Within the first 20 min, total lutein content declined only slightly, indicating a higher thermal stability compared to that of β-carotene. Contrasting results were observed for carotenoid levels of chloroplast isolates, suggesting improved β-carotene stability. Whereas the β-carotene level was reduced to less than 90% of the initial content within 20 min, that of lutein reached 90% within less than 5 min. The assumption of improved β-carotene stability during the first period of heating was supported by its higher level of retention at the end of the thermal process. In chloroplast preparations, 83.2% of initial β-carotene and 72.0% of its lutein content were still detectable. These results are in contrast to those of Choe et al. (*34*), attributing a greater thermal stability to lutein after blanching of spinach for 20 min in boiling water.

The addition of lipids did not affect the retention of total β-carotene (82.0%), although its degradation was enhanced during the first 10 min. In contrast, added fat abated lutein degradation. Within the first 20 min of heating, no significant loss was noticed, and after 60 min, 93.0% of the initial content remained. In contrast, in a previous study a similar degradation of carotenoids present in paprika oleoresins was observed after thermal treatment. Furthermore, a significant effect of variable fatty acid composition of various used oleoresins on carotenoid stability could not be found. It was assumed that lipid substrates with different degrees of unsaturation supply the same stability of carotenoids (*35*). In oleoresins, carotenoids are mainly dissolved in the lipophilic phase, and therefore, other factors responsible for different retention levels of lutein and β-carotene after heating of chloroplasts have to be considered in this study. Because in chloroplasts carotenoids are associated with proteins, it is assumed that in their presence the stability of chloroplast-bound carotenoids is strongly influenced.

Effect on Predominant Carotenoid Isomers. The amount of all-*trans* isomers was smaller in all heated samples compared to the controls, caused by degradation and *trans*−*cis* isomerization. In the unheated standard solutions, *cis* isomers were detected in trace amounts only, whereas a significant proportion of *cis* isomers was already present in the native chloroplasts. The levels are in agreement with earlier investigations of spinach leaves (*17*, *18*, *36*), where chloroplasts contained 26.0% *cis*-β-carotene and 10.6% *cis*-lutein. The predominant *cis* isomers were the 9-*cis* isomers followed by smaller quantities of 13-*cis* isomers as shown by others (*17*, *18*, *36*). Because commonly all-*trans* isomers are predominantly found in membranes and protein−pigment complexes of chloroplasts, large amounts of 9-*cis*-β-carotene in the cytochrome b_6f complex support a unique structural and functional role (*37*). It has been suggested that it may act as an efficient antioxidant to protect chlorophyll *a* or other components from oxidation by singlet oxygen. The increased proportion of *cis* isomers prior to processing may be ascribed to the chlorophylls acting as a sensitizer to isomerization as previously described (*20*). Additionally, heating of spinach chloroplasts significantly increased 9-*cis*-β-carotene levels from 14.2 to 18.8% and those of 9-*cis*- and 9′-*cis*-lutein from 7.1 to 15.0%, whereas the levels of 13-*cis* isomers remained virtually unchanged (2.9 and 1.9%, respectively). This trend disagrees with results from investigations on pure carotenoids, which mainly suffer from 13-*cis* isomerization after heat treatment (*11*, *16*). The different isomeric profile after heating may result from interactions of chlorophylls in the chloroplast enhancing the formation of the 9-*cis* isomers. It is remarkable that these effects, occurring in well-organized chlorophyll−protein complexes, were still observed after heat treatment at 98 °C for 60 min, resulting in the denaturation of the chlorophyll−carotenoid complexes.

Isomeric Ratio of Carotenoids. Heating of β-carotene and lutein solutions yielded similar *trans*:*cis* ratios of approximately 65:35. Further thermal processing for 120 min did not affect the final isomeric proportion (data not shown), indicating that a pseudoequilibrium had been reached. Heat exposure of chloroplasts brought about a similar isomeric ratio for β-carotene, while the ratio of *trans*- to *cis*-lutein was lowered (80:20). These results implied that lutein was less prone to isomerization than β-carotene. The addition of fat to the chloroplasts had a negligible effect on the isomerization rate of both carotenoids. In a previous study, the physical state of carotenoids was shown to strongly influence pigment stability. Whereas crystalline all-*trans*-β-carotene of carrot chromoplasts was only minimally isomerized after heat treatment, the addition of lipids enhanced the formation of *cis* isomers, demonstrating that dissolution was a prerequisite for carotene isomerization (*10*). According to these results, the presence of crystalline carotenoids can therefore be ruled out.

Effect of Illumination. *Total Carotenoid Content*. Illumination correlated with the degradation of β-carotene and lutein dissolved in toluene. After they had been exposed to light for 60 min, 80.6% of β-carotene and 80.4% of lutein were retained,

demonstrating that the impact of photodegradation was in the range of thermal treatment, resulting in 84.7 and 83.5% after heating for 60 min at 98 °C, respectively. Particularly with respect to short-time illumination for 20 min, β-carotene turned out to be more stable (98.4%) than lutein, since the latter exhibited a significantly lower level of retention (92.3%) after light exposure for 10 min. Compared to heat treatment, which resulted in an enhanced degradation of β-carotene, light stability of both carotenoids was the reverse. Remarkably, the illumination of chloroplasts was associated with a considerable increase in carotenoid levels during the first 10 min when 129.8 and 109.6% of the initial contents of β-carotene and lutein were observed, respectively. Subsequently, both levels continuously fell to 121.4% β-carotene and 100.3% lutein within 60 min. The initial increase in the level of carotenoids was in agreement with early reports revealing changes in the levels of chloroplast carotenoids in response to light intensity (*38*). Also, a slight increase in the lutein level was found in intense light environment (*39*), and β-carotene synthesis was specifically stimulated (*40*). Carotenoids of chloroplasts are located in photosystems PSI and PSII, each consisting of a core complex (CCI and CCII) together with an antenna complex (LHCI and LHCII). In general, β-carotene is abundant in reaction center proteins of CCI and CCII, in which protection against radicals by quenching the excitation energy of $^1O_2$ produced by ^{3}Chl probably occurred (*41*). Xanthophylls are the major carotenoids in light-harvesting proteins, whereas lutein is considered to be predominantly associated with the LHC pigment−protein complex, acting as a concomitant pigment for light harvesting (*42*). The increase in the level of β-carotene ascertained in this study may be due to reinforced protection of chlorophylls in response to light exposure. The disparate increase in levels of lutein and β-carotene found in this study concurs with the observation of a decreased ratio of xanthophylls to carotenes in plants grown under intensive light (*25*). The localization of each carotenoid with its different functions in the photosynthetic apparatus was suggested to be responsible for the observed different responses after light sensitization. Beyond synthesis, the elevated level of both pigments may also be facilitated by an increased stability, as shown in previous studies for carotenoids when bound to apoproteins in the pigment−protein complexes (*25*).

Effect on Predominant Carotenoid Isomers. The isomeric composition did not vary significantly after light exposure. Levels of *cis*-β-carotene and *cis*-lutein increased only marginally in the standard solutions. These results comply well with previous reports (*16*, *20*, *43*) showing insignificant isomerization after light exposure of dissolved all-*trans*-β-carotene. Because *cis* isomers were detected in the photosynthetic apparatus, carotenoid isomerization in the presence of chlorophylls was concluded (*43*). A variety of chlorophyll derivatives were reported to act as photosensitizers inducing the formation of *cis* isomers.

Isomeric Ratio of Carotenoids. Significant differences in their isomeric pattern were ascertained after illumination of mixtures of all-*trans*-β-carotene and chlorophylls. It was reported that the level of *cis* isomers increased up to 17% until an equilibrium was reached (*20*). Further illumination of chloroplasts already containing elevated levels of *cis* isomers only minimally affected their isomeric composition. The percentage of *cis* isomers ranged from 22.3 to 21.8% and from 12.6 to 11.9% for β-carotene and lutein, respectively. This pattern remained unchanged even after prolonged illumination for 180 min (data not shown). These findings suggested a quasi-stationary equilibrium of several stereoisomers in the native chloroplast independent of further illumination.

In conclusion, stability data derived from pure lutein and β-carotene cannot be applied to chloroplast-bound carotenoids. In chloroplasts, thermal treatment turned out to be the major cause of degradation and isomerization of carotenoids. The results revealed an enhanced stability of β-carotene compared to that of lutein. Unexpectedly, the addition of lipids to chloroplast preparation had a reverse effect on both carotenoids, inverting the sensitivity of β-carotene and lutein, the latter being improved. In the native as well as in the heated chloroplast isolates, 9-*cis* isomers were identified as the predominant stereoisomers. These results were inconsistent with data from heated pure carotenoids favoring 13-*cis* isomers. After heating of chloroplasts, β-carotene, in contrast to lutein, reached an equilibrium comparable to that of the standard. Illumination of freshly prepared chloroplast isolates caused an initial increase in the levels of lutein and predominantly β-carotene. The isomeric composition was not significantly changed. Compared to the thermal impact on carotenoid stability, illumination was insignificant. Therefore, light exposure may be neglected with respect to the carotenoid integrity of raw and processed vegetables. It was demonstrated that carotenoid stability has to be evaluated for every individual pigment in its genuine environment. Degradation and isomerization of β-carotene and lutein even in the same organelle behaved differently after heating and light exposure. Stability data based on model systems (e.g., pure carotenoids) may not be portable to complex food without intensive investigation.

ACKNOWLEDGMENT

We thank Sandra Bayha for her excellent technical assistance, and we acknowledge the valuable discussion with Prof. Dr. Nicolaus von Wirén (Institute of Plant Physiology, Hohenheim University). All-*trans*-lutein was kindly donated by Hoffmann-La Roche.

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Received for review April 21, 2005. Revised manuscript received September 15, 2005. Accepted September 26, 2005.

JF050926W