Detection of Cis–Trans Carotene Isomers by Two-Dimensional Thin-Layer and High-Performance Liquid Chromatography

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High-performance liquid chromatography (HPLC) was used to detect cis–trans isomers of α- and β-carotene. Monitoring HPLC eluants at both 436 and 340 nm and calculating the peak height ratio, 340 nm/436 nm, permit detection of isomers in carotene–petroleum ether solutions and plant extracts. Separation of individual isomers was achieved on thin-layer chromatography (TLC) plates prepared with calcium hydroxide layers. Equilibration of the plates to 44% relative humidity prior to development with 1.5% p-methylanisole in petroleum ether was necessary to achieve optimal resolution. No isomeric carotenoids were found in the fresh yellow-orange vegetables, but analyses of chlorophyll-containing vegetables indicated that isomers were present. Heat-processed products contained a variety of cis isomers. Sample handling, saponification methods, and sample pH did not influence the formation of isomers.

INTRODUCTION

α- and β-carotene are predominant vitamin A precursors found in many fruits and vegetables (Bauernfeind, 1972). The all-trans forms of these carotenoids, relative to the cis isomers, contain the highest vitamin A activity (Simpson, 1983). Cis–trans isomerization reactions, which may occur during food processing, reduce the bioavailability of the provitamins in rats (Sweeney and Marsh, 1971, 1973). Because of the lowered biological activity of the cis isomers, characterization of the isomeric forms in cooked and processed products would be desirable (National Research Council, 1980). However, a rapid and convenient method for this analysis is not currently available. Beecher and Khachik (1984) are developing an HPLC method that does, at this time, resolve some of the cis isomers from the main α- and β-carotene peaks. They are working on positive identification of these isomers and also on improved chromatography.

Recent review articles have been published covering the literature related to the analysis of carotenoids by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (Schwartz and von Elbe, 1982; Taylor, 1983; Krinsky and Welankiwar, 1984). Relatively little research has been aimed at the analysis of isomeric carotenoids. Isomers of α- and β-carotene have been analyzed by chromatography on supports of calcium hydroxide, magnesium oxide, or calcium hydroxide–magnesium hydroxide mixtures (Bickoff et al., 1949; Panalaks and Murray, 1970; Sweeney and Marsh, 1970; Sadowski and Wojcik, 1983). We have observed that the published techniques required for separation of isomers are cumbersome and results are difficult to reproduce. A more reproducible method requiring less expertise to perform is needed to obtain information on the presence and formation of carotenoid isomers in fresh and processed foods.

The purpose of this study is to (1) develop a TLC and/or HPLC method to detect cis–trans carotene isomers and (2) determine the utility of the method to analyze carotenoid isomers in fresh and processed foods.

MATERIALS AND METHODS

α- and β-carotene were purchased from Sigma Chemical Co. (St. Louis, MO). Vegetable products were obtained from local sources. All other chemicals and solvents were reagent grade.

Extraction of Tissue. Plant tissue (20 g) was homogenized in a Tissumizer (Tekmar, Cincinnati, OH) in 75 mL of acetone–hexanes (9:1 v/v). Acetone was removed by washing five times with an equal volume of water. If needed, 10% NaCl was used for the initial wash to disrupt emulsions. The extract diluted to 50 mL was saponified at room temperature for 30 min with 12.5 mL of potassium hydroxide saturated methanol and then washed (5×) free of base with water or saturated NaCl. The extract was dried over anhydrous sodium sulfate, evaported to near dryness under reduced pressure, and redissolved in petroleum ether (10 mL). The entire procedure was performed in dim light. Unsaponified extracts were also analyzed. All extracts were stored refrigerated (4 °C) under nitrogen.

Isomerization. Isomerization was catalyzed by iodine according to the method of Zechmeister (1944). β-Carotene and iodine, both in petroleum ether, were mixed so that the final concentration of β-carotene was 0.1 mg/mL and iodine was 2% of β-carotene by weight. The reaction proceeded for 1 h under fluorescent light. UV–visible spectra were obtained on a Shimadzu (Columbia, MD) Model 240 UV recording spectrophotometer.

HPLC Conditions. All chromatograms were obtained at room temperature on a Model 510 solvent delivery system, U6K injector, μ-Bondapak C18 column (10-μm particle size, 3.9 mm × 30 cm column dimensions), and 440 dual wavelength absorbance detector (Waters Associates, Milford, MA). The column was protected with a guard column (Waters Associates) packed with 30–38 μm pellicular ODS media (Whatman, Clifton, NJ). Column eluants were monitored at both 436 and 340 nm.

A volume of sample (10–25 μL) was injected in each analysis so that the peak heights at 436 nm for every injection were approximately equal. The solvent system employed (Broich et al., 1983) was methanol–acetonitrile–chloroform (47:47:6). The flow rate was maintained at 2.0 mL/min. Detector sensitivity was set at 0.05 (436 nm) and 0.01 (340 nm) absorbance unit full scale. The analog signal was displayed on a dual-pen Series 5000 Fisher recorder (Fisher Scientific, Raleigh, NC). A ratio of cis peak absorbance at 340/436 nm absorbance was derived by measuring the two peak heights. A correction was made for the difference in detector sensitivity settings.

TLC. Calcium hydroxide powder (Aldrich Chemical Co., Milwaukee, WI) was stored in an enclosed chamber equilibrated to 44% relative humidity prior to slurry preparation. The slurry composition was 50 g of powder to 120 mL of water. Glass plates (20 × 20 cm) were poured to an adsorbent thickness of 0.25 μm with a Dasaga-Brinkmann spreader (Brinkmann, Westbury, NY). All plates were stored, after air-drying for 3 h, in 44% relative humidity for at least 1 day or until use. The atmosphere

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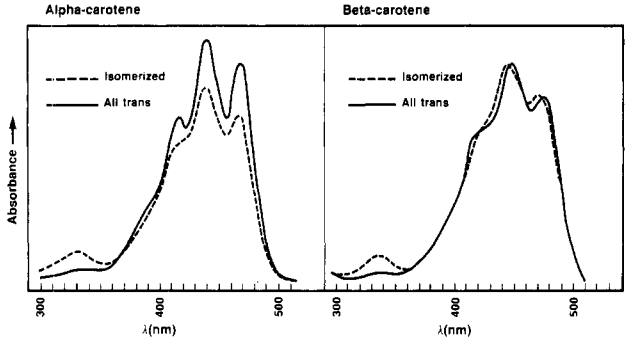

Figure 1. Absorption spectra of α- and β-carotene in petroleum ether before and after iodine isomerization.

of the developing tanks was saturated with the solvent 1.5% *p*-methylanisole (Eastman Kodak Co., Rochester, NY) in petroleum ether before development. In a few cases, to obtain spectrophotometric scans without interference from *p*-methylanisole, the solvent system was 1.2% acetone in petroleum ether.

Plates were spotted with a microcapillary tube and allowed to develop between 1 and 1.5 h in each direction. Plates were dried rapidly (<1 min), under a stream of nitrogen, when the direction was changed. The tank cover was opened slightly when the solvent front was at the plate edge to allow further development (~20 min) of the plate in the second direction. Loading and development were carried out under dim light, and the tank was covered with aluminum foil. Separated compounds, after removal from TLC plates, were identified by comparison to their absorption spectra (Zechmeister, 1944).

Effect of pH on Isomerization. All-trans β-carotene (0.1–0.2 mg) dissolved in 10.0 mL of two solvent systems (petroleum ether–, acetone–petroleum ether (50:50)) was used to check the effect of pH on isomerization. An equal volume of each buffer solution (pH 1, 2, 3, 4, and 7; 0.05 M KCl, 0.05 M KCl, 0.05 M potassium biphthalate, 0.25 M potassium acid phthalate, and 0.10 M sodium phosphate, respectively, and 10 M HCl) was added and the mixture stirred vigorously at room temperature for various time intervals (30 min; 2, 6, 15 h) in the dark. Acetone was removed as previously described, and each extract was filtered through a 0.45-μm prep-Disc (Bio-Rad, Richmond, CA) prior to HPLC analysis.

RESULTS AND DISCUSSION

Figure 1 shows the absorption spectra of α- and β-carotene in petroleum ether before and after iodine-catalyzed isomerization. Irradiation of carotene solutions in the presence of iodine forms an equilibrium mixture of cis–trans isomers. Characteristically, the spectra of cis–trans mixtures exhibit a shift toward shorter wavelengths of a few nanometers and the formation of a "cis peak" that appears at 300–360 nm. The presence of the "cis peak" is useful to selectively detect cis carotenoid isomers (Zechmeister, 1944).

Monitoring HPLC eluants at both 436 and 340 nm results in the sample chromatograms shown in Figure 2. The chromatograms illustrate the results obtained for all-trans β-carotene and isomerized β-carotene. Similar chromatograms were also found for the analysis of carotenoids in vegetable extracts. The 340-nm wavelength detector sensitivity was set at 0.01 absorbance unit full scale. This is a 5 times increase in sensitivity relative to the 436-nm measurement, thereby enhancing the detection of the "cis peak" signal. The peak height ratio, 340 nm/436 nm, increased in mangitude when cis isomers were present relative to the all-trans form because of the increase in

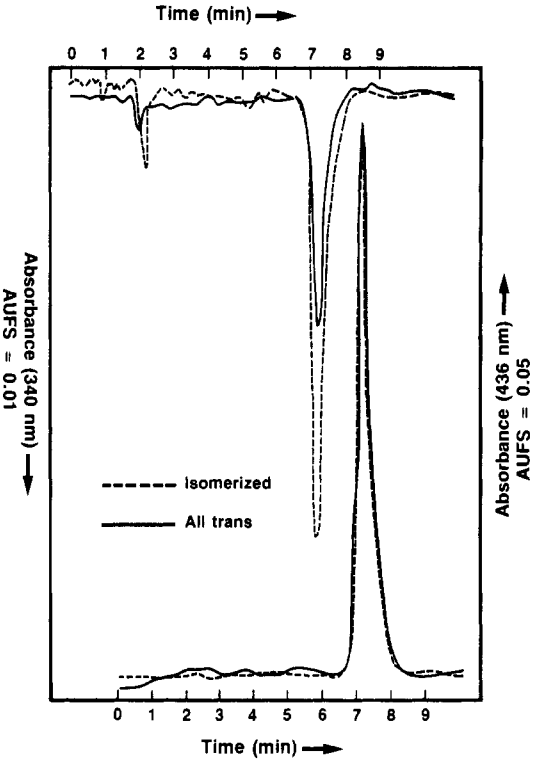

Figure 2. HPLC chromatograms of all-trans and isomerized β-carotene.

Table I. Cis–Trans Carotenoids Found in Selected Vegetables by HPLC and TLC

product	HPLC abs ratio[a] (±S.E.)	carotene isomers found by TLC[c] β	α
controls			
trans β-carotene	0.079 ± 0.004	trans	
isomerized β-carotene	0.160 ± 0.003	trans, neo B, U	
trans α-carotene	0.077 ± 0.002		trans
isomerized α-carotene	0.138 ± 0.003		trans, neo B, W, U
spinach (fresh)	0.099 ± 0.006	trans, neo B, U	
spinach (canned)	0.102 ± 0.002	trans, neo B, U	
cucumber	0.103 ± 0.002	trans, neo B, U	
pickle	0.110 ± 0.008	trans, neo B, U	
sweet potato (fresh)	0.075 ± 0.002	trans	
sweet potato (blanched)	0.103 ± 0.006	trans, neo B, U	
carrot (fresh)	*b*	trans	trans
carrot (processed)	*b*	trans, neo B, U	trans, neo B

[a] Measured from peak heights at 340 and 436 nm; deviations calculated from triplicate measurements. [b] Peak height measurements inadequate due to incomplete resolution of α- and β-carotene. [c] Refer to Figure 3.

absorbance of the cis peak in the 340-nm region. With use of the described HPLC system, a mixture of isomers appeared as a single peak and coeluted with all-trans α- or β-carotene. In addition, no peak broadening or shoulders were observed when cis isomers were present.

Table I lists the ratios, 340 nm/436 nm, corrected for detector sensitivity settings, for all-trans α- and β-carotene and their respective equilibrium mixtures of isomers formed by irradiation of carotene solutions catalyzed by iodine. The differences in ratios and standard deviations found between all-trans β- and α-carotene and equilibrium mixtures of isomers indicate that this measurement is useful to detect the presence of isomers in plant extracts. A ratio measurement greater than 0.079 for β-carotene and

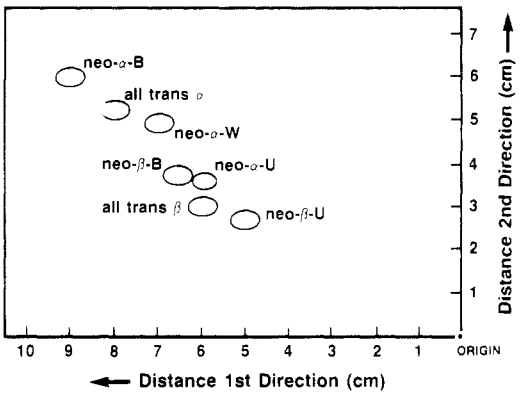

Figure 3. Two-dimensional TLC chromatogram of cis–trans α- and β-carotene isomers.

0.077 for α-carotene suggests the presence of cis isomers. In addition, the ratios found for vegetable extracts (spinach, cucumber, pickle, sweet potato) are also listed. The data in Table I show that cis isomers should be found in spinach (fresh and canned), cucumber, pickle, and blanched sweet potato. Because of interferences from other compounds found in these plant extracts, this information cannot be obtained from absorption spectral scans and a method such as HPLC is required to separate individual carotenoids from interfering constituents.

For those extracts that contain both α- and β-carotene, complete resolution is required ($R \geq 1.0$) in order for the ratio measurements to be applied. Such separations might be achieved on columns containing a greater number of theoretical plates than the one used in this study.

The molar absorptivity values vary for each isomer in the cis peak region. Therefore, this technique cannot be used to accurately quantitate the different isomeric forms present in sample mixtures. Separation of each isomer followed by a quantitative method of analysis would be required.

Separation of the isomers can be achieved on TLC plates prepared with calcium hydroxide layers (0.25 μm). It has been reported that the source (suppliers or manufactures) of calcium hydroxide can significantly affect resolution (Bickoff et al., 1949). Similar observations were made during this study. Equilibration of the plates to 44% relative humidity is critical to achieve optimal resolution. In addition, plates must be run in two dimensions to obtain the required number of theoretical plates for separation. In most cases, continuous development in the second dimension is necessary to achieve the desired resolution. *p*-Methylanisole is employed to minimize diffusion of spots (Bickoff, 1948). No isomeric carotenoids were formed during the TLC development of all-trans α and β standards. Therefore, it was assumed that the cis forms did not further isomerize during separation.

Figure 3 illustrates a typical developed TLC plate for a mixture of α- and β-carotene isomers. Specific configurations of individual isomers are still unknown; therefore, the "neoisomer" nomenclature proposed by Zechmeister (1944) is used. Identification of the isomers was performed on the basis of their chromatographic properties and absorption spectra. Comparisons were made of wavelength maxima and absorption coefficients of cis peaks in order to identify each compound. The compounds were isolated by removing the appropriate spots from the TLC plate and dissolving the carotenoid compound in petroleum ether prior to spectral analysis. Expected isomers from the iodine-catalyzed reaction are neoisomers U and B for β-carotene and neoisomers U, W, and B for α-carotene (Zechmeister, 1944).

Table II. Influence of pH on Cis–Trans Isomerization[a]

treatment	HPLC abs ratio[b] (±S.E.)
trans, β-carotene (untreated)	0.079 ± 0.004
10.0 M HCl	0.123 ± 0.014
pH 7	0.077
pH 4	0.075 ± 0.005
pH 3	0.079
pH 2	0.074
pH 1	0.078 ± 0.003

[a] Petroleum ether solutions of trans β-carotene incubated with buffer solution at 25 °C for 30 min prior to HPLC analysis. [b] Measured from peak heights at 340 and 436 nm; deviations calculated from triplicate measurements

Table I lists the isomers found by TLC in selected fresh and processed vegetables. Neoisomers in vegetable extracts were identified by their relative position to all-trans isomers obtained from plates of pure isomerized solutions. R_f values were not used because of their variability. This variability may be due to differences in composition of plant material, plate moisture, amount of pigment loaded on the plate, or drying time while changing directions.

No differences were found in the number of isomers for saponified and unsaponified tissue extracts. Control solutions of carotenes subjected to extraction and saponification methods were checked to ensure that isomerization did not take place during sample extraction or handling. Saponification was performed to remove chlorophyll and fat-soluble substances; the latter cause diffusion of the pigment spots (Strain and Svec, 1969).

Extracts of both raw carrots and sweet potatoes did not exhibit any cis isomers (Table I) even after the petroleum ether extracts were stored refrigerated for 3 days. However, iodine isomerization of these samples resulted in the formation of the expected isomers. Heat-processed products (pureed carrot and water-blanched sweet potato) contained a variety of cis isomers. The reason only five isomers were detected in the pureed carrot rather than the expected seven (four α and three β) is unknown. Presumably, the reaction products (equilibrium mixture) for heat-induced isomerization differ from those of iodine catalysis; or possibly, our present system does not separate and/or detect a small quantity of isomer present.

Fresh cucumber and pickle extracts both had three β-carotene isomers as did fresh and canned spinach. α-Carotene was not detected in these three vegetables. Xanthophylls were present in most extracts, particularly spinach, but because of their greater polarity, these compounds did not migrate from the point of sample application. No attempts were made to resolve or identify these components.

Comparing the absorbance ratio data obtained by HPLC to the compounds identified by TLC analysis indicates that the HPLC method is useful to monitor the presence of isomers found in carotenoid extracts. The developed TLC method can then be employed to separate and identify the individual isomers. New developments in HPLC methodology are needed to rapidly separate and quantify cis–trans carotenoid compounds.

The effect of pH on isomerization was investigated to test the ability of sample pH to cause isomerization. High concentrations of acid have been shown to cause isomerization in petroleum ether solutions (Zechmeister, 1944). Petroleum ether solutions of all-trans carotenes, when incubated with buffers ranging from pH 1 to 7, showed no detectable isomers (Table II). In addition, no differences were observed in solutions of 50:50 petroleum ether–acetone. HCl (10 M) was needed to form the expected isomeric equilibrium. These results indicate that the pH of the

tested vegetable products did not affect isomerization during the 30-min incubation period. Furthermore, pH 1 solutions showed no change after 15 h of incubation.

It is interesting to note that no isomeric carotenoids were detected in the fresh yellow-orange vegetables, but analysis of all the fresh green vegetables selected indicated that isomers were present. A number of factors such as heat, light, iodine, and acid are well-known catalysts for isomerization (Klaui and Bauernfeind, 1981). Also, chlorophyll has been reported to influence photoisomerization of certain carotenoids through a direct energy-transfer mechanism (Claes, 1961). If chlorophyll can cause carotenoid photoisomerizations, then this reaction may be an important factor during sample workup, thus accounting for the presence of the cis forms found in chlorophyll-containing vegetable extracts. Furthermore, the possible function of carotenoids as accessory pigments in photosynthesis (Krinsky, 1968) may also influence the formation of these isomers in chloroplasts due to their sensitivity to light-induced isomerization. Additional work is needed to determine the factors that influence the formation of cis carotenoid isomers in food products.

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Registry No. *all-trans*-β-Carotene, 7235-40-7; neo-α-carotene B, 83058-23-5; neo-α-carotene W, 86851-59-4; neo-β-carotene B, 6811-73-0; neo-α-carotene U, 29907-61-7; neo-β-carotene U, 13312-52-2; *all-trans*-α-carotene, 432-70-2.

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Identification of Caffeine in Citrus Flowers and Leaves

Ivan Stewart

Caffeine was isolated and identified in extracts from flower buds of several citrus cultivars and from leaves of Valencia oranges (*Citrus sinensis* L. Osbeck). No caffeine was detected in orange juice. Identification was by high-performance liquid chromatography (HPLC), gas chromatography (GC), ultraviolet spectroscopy (UV), and mass spectra (MS).

Our laboratory has been isolating compounds from citrus tissue and assaying them for cytokinin activity using soybean callus growth as a criteria (Miller, 1963). The extraction procedure used not only removed cytokinins from the tissue but various kinds of bases and other compounds. During the separation of a citrus flower extract, one of the fractions was found to induce growth of soybean callus, suggesting the presence of cytokinins. However, upon further fractionation of the extract, the main constituent, as indicated by HPLC, no longer induced callus growth. Obviously, during the purification procedure, the growth-active compound had either been removed or deactivated. The inactive fraction was then subjected to GC–MS analysis and was found to contain virtually pure caffeine.

Caffeine is said to occur in some 60 species of plants (Roberts and Barone, 1983); the more common are from the genera *Caffea*, *Thea*, *Theobroma*, *Cola*, *Paullinia*, *Ilex*, and *Copernecia*. However, this is believed to be the first report of the occurrence of caffeine in citrus. This paper reports on the isolation and identification of caffeine in citrus flowers and leaves, on the amounts of caffeine in these tissues, and a discussion on some of the biological properties of caffeine in plants.

EXPERIMENTAL SECTION

Isolation. Flower buds and open flowers from trees of the Valencia orange (40-kg fresh weight) were collected and extracted in a Waring blender with 80% ethanol and then filtered through a fritted glass funnel. The extract was adjusted to pH 4.0 with HCl, and a portion equivalent to

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