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Cis/Trans Isomerization of Retinyl Palmitate in Foods

Steven J. Schwartz

Heat treatment (120–170 °C) of *all-trans*-retinyl palmitate for various time intervals showed an increase in the ratio of 13-cis/all-trans. The increase followed first-order kinetics, and an apparent Arrhenius activation energy was calculated as 12 kcal/mol. Analysis of selected food products showed 13-cis/trans ratios ranging from 0.15 to 0.63. Pasteurization and ultrahigh-temperature processing of skim milk supplemented with retinyl palmitate produced no changes in isomeric composition. Commercial retinyl palmitate samples used to prepare supplementation and fortification mixes had variable amounts of 13-cis isomer. Exposure of samples to indirect sunlight induced primarily the formation of 9-*cis*-retinyl palmitate. Retinyl palmitate isomers found in food products may in part be attributed to the composition of the added supplemental vitamin as well as effects of food-processing treatments, particularly heating.

Synthetic retinyl palmitate is a major source of vitamin A in the diet of many individuals (Parrish, 1977). Retinyl palmitate is commonly added to foods supplemented or fortified with vitamin A. Ester hydrolysis to retinol occurs readily during digestion (Goodman and Blaner, 1984).

The all-trans form of retinol and retinyl palmitate have the greatest biological activity relative to other isomeric forms. The 13-cis and 9-cis isomers of retinyl palmitate have biological activities of 75% and 26%, respectively (Ames et al., 1960). Prompted by these findings, a number of laboratories have reported analysis of cis–trans retinoid isomers in foods and feeds (Steuerle, 1985; Sivell et al., 1984; Wiggins et al., 1982; Stancher and Zonta, 1982; Egberg et al., 1977). These studies have found that 13-*cis*-retinol was the most common of the cis isomers in foods and detected in varying amounts. If the isomeric composition of retinoids is not accounted for, inaccurate nutritional estimates may result. Generally, for determination of vitamin A in foods with added retinyl palmitate, interfering lipids must be removed by saponification. Hydrolysis of palmitate ester occurs and analyses are quantified as retinol.

In two recent studied (Landers and Olson, 1986; Mulry et al., 1983), it was reported that upon photolysis of *all-trans*-retinyl palmitate, the 13-cis isomer was formed as a minor component. The 9-cis isomer predominated and varied in content depending upon the solvent used during light exposure. There are no reports that have detected measurable levels of 9-*cis*-retinyl palmitate in foods. Furthermore, reasons for the presence of appreciable amounts of 13-*cis*-retinyl palmitate in food samples remains unclear.

The objectives of this study were to investigate the mechanism for the formation of 13-*cis*-retinyl palmitate in foods and to determine whether isomerization of *all-trans*-retinyl palmitate can be induced by heat treatments, such as those used in commercial food-processing facilities.

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MATERIALS AND METHODS

Source of Materials. *all-trans*-Retinal, retinol, retinyl palmitate, and 9-*cis*-retinal were purchased from Sigma Chemical Co. (St. Louis, MO), and 13-*cis*-retinal was from Eastman Kodak Co. (Rochester, NY). Retinyl palmitate fortification samples (corn oil as carrier) and skim milk (0.2% fat) for processing were provided by North Carolina State University Dairy Processing Plant. Food products were purchased from local sources. Infant formula powders were reconstituted following package directions. All other chemicals and solvents were reagent grade.

HPLC Conditions. The HPLC system consisted of a Model 510 solvent delivery system with U6K injector, both from Waters Associates (Milford, MA), a Du Pont guard column packed with μ-Porasil (10 μm) a Du Pont Zorbax Sil normal-phase column (4.6 mm × 25 cm), 5-μm particle size (Wilmington, DE), a Chira Tech UV-106 HPLC detector (Fort Collins, CO) equipped with a 340-nm filter, and a Fisher dual-pen Series 5000 recorder (Fisher Scientific, Raleigh, NC). Spectral scans were obtained with a linear UV-203 HPLC detector (Reno, NV) in the stopped-flow mode.

Solvent systems employed were ethyl acetate/methylene chloride/hexane (0.4/1.0/98.6, v/v/v) to separate retinyl palmitate isomers and ethyl acetate/methylene chloride/hexane (6.2/7.7/86.1) to resolve retinol isomers (Tsukida et al., 1977b). Injections of 25–50 μL were made, and all chromatograms were run at ambient temperature at a flow rate of 2 mL/min. Absorbance units full scale varied from 0.01 to 1.0. All ratios reported were determined from peak heights and calculated from duplicate experiments.

Assignment of Peaks. Retinoid isomers were assigned by comparison to standards when available. The 9-*cis*- and 13-*cis*-retinol standards were prepared by reduction of 9-*cis*- and 13-*cis*-retinals following the method described by Egberg et al. (1977). Mixtures of retinol isomers were obtained by photochemical catalysis of *all-trans*-retinol and by iodine-catalyzed isomerization followed by $NaBH_4$

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reduction of *all-trans*-retinal (Tsukida et al., 1977a,b).

Determination of Rate Constants and Activation Energy of the Heat Isomerization of Retinyl Palmitate. Retinyl palmitate (250 mg) was placed in a screw-cap vial (5.0 mL) with air-tight seal in a Thelco GCA/Precision oven (Precision Scientific Co., Chicago, IL) previously equilibrated to the desired temperature (120, 130, 140, 150, 160, 170 °C) and held for the specific time interval (0, 30, 45, 60, 90 min). Considering the length of the heating period, heat up and cooldown times were neglected. Sample aliquots were dissolved in petroleum ether (25 mL) and analyzed by HPLC.

Rate constants (s^{-1}) were determined from regression lines of ln (13-cis/all-trans) vs. heating time. The activation energy was calculated from the regression line of ln k vs. $1/T$ (K^{-1}).

Saponification Conditions. Solutions of retinyl palmitate (20 g/mL) in ethanol were saponified by a similar procedure described by Stancher and Zonta (1982). To 25-mL sample aliquots, in a foil-covered flask, were added 33 mL of absolute ethanol, 32 mL of 14 N KOH, and 25 mL of deionized water. The mixture was stirred at room temperature for 16 h. Control solutions were prepared without KOH, and retinol control solutions were also analyzed. Skim milk and commercial food products (25 mL) were saponified as described; however, ethanol rather than deionized water was used to ensure that the water to ethanol ratio (50/50) was constant.

Extraction of Foods. Following saponification, samples were extracted with 250 mL of hexane/methylene chloride (3/1, v/v). The organic layer was washed with 150 mL of 3.5 M NaCl followed by three 200-mL water washings. The extract was dried over anhydrous Na_2SO_4 (35 g) and the solvent evaporated under reduced pressure. The residue was dissolved in petroleum ether (5.0 mL) and filtered (0.45 μm) for HPLC analysis. All sample and workup procedures were performed in subdued lighting.

Processing Conditions. Skim milk, fortified with 2.2 IU of retinyl palmitate/mL, was pasteurized at 77 °C for 17 s in the North Carolina State Dairy University Processing Plant. A Cherry-Burrell Unitherm (Model XLV No-Bac Unitherm, Cherry-Burrell Inc., Cedar Rapids, IA) system was used for ultrahigh-temperature (UHT) processing. This system has been described by Biziak et al. (1982). Skim milk was processed at 100, 125, and 150 °C for both 18 and 31 s. Skim milk was also canned in 303 × 406 containers, sealed, and retorted at 117 °C for 20 min. Samples were saponified for extraction immediately after processing.

Illumination. Fluorescent lighting was obtained with standard General Electric cool white fluorescent bulbs at a distance of 3 m. Samples exposed to intermittent indirect sunlight were placed 5 ft from a laboratory window. Dark control samples were wrapped in aluminum foil. All samples were held at room temperature for a 3-day illumination period.

RESULTS AND DISCUSSION

Figure 1 (sample A) illustrates a typical chromatogram from an HPLC analysis of an *all-trans*-retinyl palmitate standard. Two peaks predominate: *all-trans*- (peak 3) and 13-*cis*-retinyl palmitate (peak 1). The 13-cis isomer was assigned by its conversion via base hydrolysis to 13-*cis*-retinol. The relative retention times of retinyl palmitate isomers on silica columns and UV absorption maxima reported by others (Landers and Olson, 1986) are very similar to that observed in this study. This provides additional evidence for their identity. These values are listed in Table I.

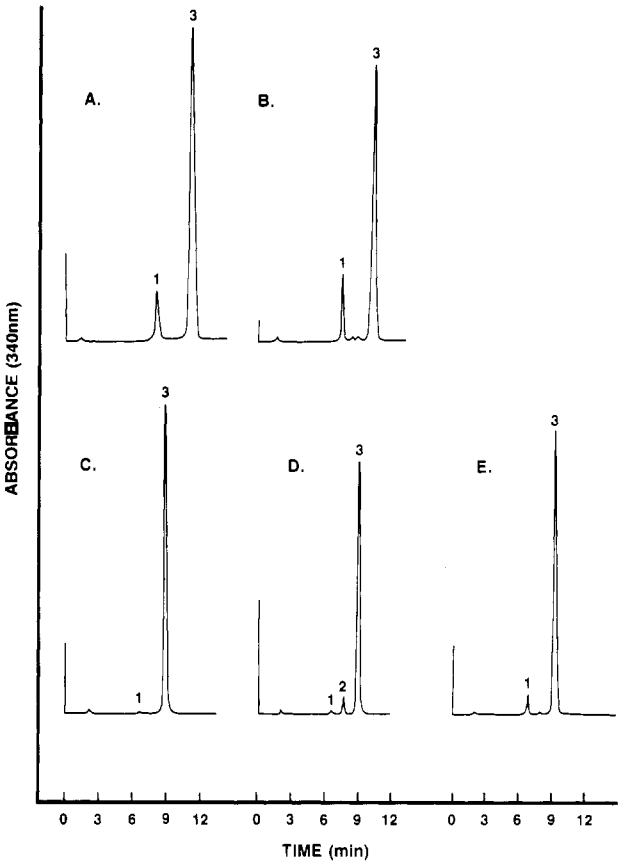

Figure 1. Chromatograms of retinyl palmitate subjected to various treatments: peak 1, 13-*cis*-retinyl palmitate; peak 2, 9-*cis*-retinyl palmitate; peak 3, *all-trans*-retinyl palmitate; A, *all-trans*-retinyl palmitate standard; B = after heat treatment of sample A (140 °C, 60 min); C = purified *all-trans*-retinyl palmitate; D = after indirect sunlight exposure of C; E = after heat treatment of C.

Table I. Relative Retention Times and UV Absorbance Maxima of Retinyl Palmitate Isomers

peak no.	found RRT[a]	found λ_{max}[b]	lit.[c] RRT	lit.[c] λ_{max}	assignment
1	0.75	329	0.76	328	13-*cis*-retinyl palmitate
2	0.83		0.81	324	9,13-di-*cis*-retinyl palmitate
3	0.88	322	0.87	322	9-*cis*-retinyl palmitate
4	1.00	327	1.00	325	*all-trans*-retinyl palmitate

[a] RRT = relative retention time based on elution of *all-trans*-retinyl palmitate in 8.6 min (RRT = 1.0). [b] λ_{max} reported obtained from HPLC stopped-flow scans (solvent ethyl acetate/methylene chloride/hexane, 0.4/1.0/98.6, v/v/v). [c] Landers and Olson, 1986. λ_{max} determined in hexane.

Heat treatment of *all-trans*-retinyl palmitate (Figure 1, sample A) to various temperatures produced an increase in the ratio of 13-*cis*- to *all-trans*-retinyl palmitate. Figure 1 (sample B) illustrates a typical chromatogram of a sample heated at 140 °C for 60 min. Ratio and heating time data revealed that first-order kinetic equations could be used to predict a change in isomeric composition. A plot of ln k vs. 1/K (Figure 2) resulted in an apparent activation energy of 12 kcal/mol. Thus, heat treatment induced trans to cis isomerization of retinyl palmitate, the extent being temperature dependent.

Measurements of the 13-cis/trans ratio in selected food products were obtained following saponification treatment. This alleviated the problem of interferences from lipids and caused ester hydrolysis of retinyl palmitate to retinol. No change in the amounts of *cis*/*trans*-retinol were detected after saponification of a cis/trans mixture of retinyl

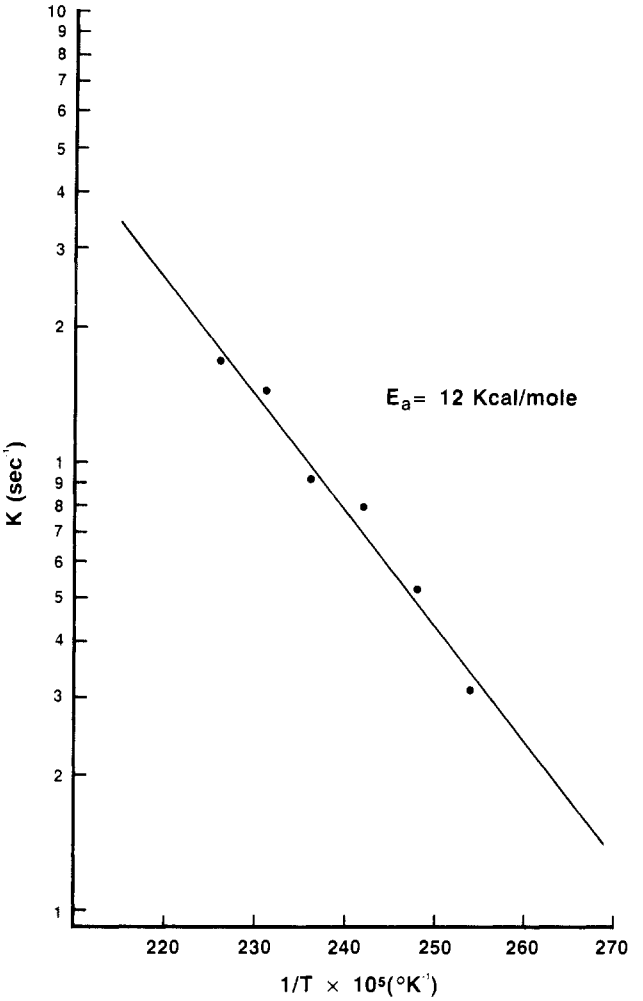

Figure 2. Logarithmic plot of rate constants for retinyl palmitate (13-cis/trans) isomerization vs. reciprocal temperature (1/K).

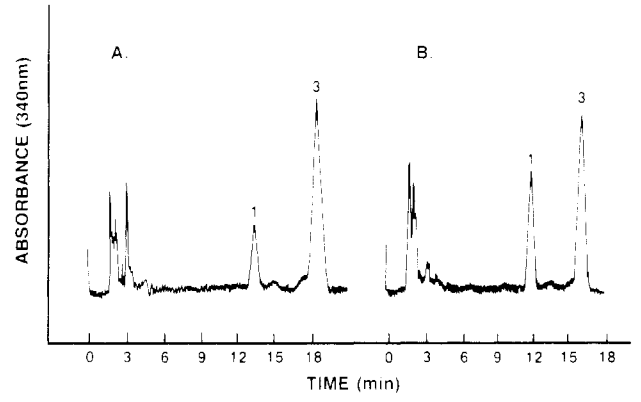

Figure 3. Chromatograms of retinol obtained from analyses of evaporated milk (A) and infant formula (B): peak 1, 13-*cis*-retinol; peak 3, *all-trans*-retinol.

palmitate. This calculation is valid since molar absorbtivity values of 13-cis isomers are similar to their all-trans parent (Groenendijk et al., 1980). Therefore, cis/trans ratio measurements of retinol, obtained following saponification at room temperature, accurately reflect the ratio of retinyl palmitate prior to ester hydrolysis.

Analyses obtained from selected commercial food products and processed skim milk are listed in Table II. The ratio of 13-*cis*/*trans*-retinol ranged from 0.15 ± 0.01 to 0.63 ± 0.04. Typical chromatograms for saponified extracts of evaporated milk and infant formula are shown in Figure 3. Supplemented products that have been

Table II. Ratio of 13-*cis*/*trans*-Retinol Found in Analyses of Selected Products

product	ratio ± SE[a]
Commercial Samples	
milk	
skim pasteurized	0.12 ± 0.01
skim, powder	0.15 ± 0.01
UHT, whole (3.25% fat)	0.27 ± 0.02
UHT, 2% fat	0.36 ± 0.03
evaporated	0.36 ± 0.02
infant formula	
soy based	
powder	0.17 ± 0.01
liquid	0.40 ± 0.02
milk based	
powder	0.14 ± 0.02
liquid	0.63 ± 0.04
Experimental Samples[b]	
fortification samples	0.13 ± 0.01
skim milk	
fortified unprocessed	0.13 ± 0.01
pasteurized	0.13 ± 0.01
UHT processed	0.12 ± 0.01
canned	0.14 ± 0.01

[a] SE = standard error. [b] Milk samples processed at North Carolina State University Dairy Processing Plant. Refer to Materials and Methods for processing conditions.

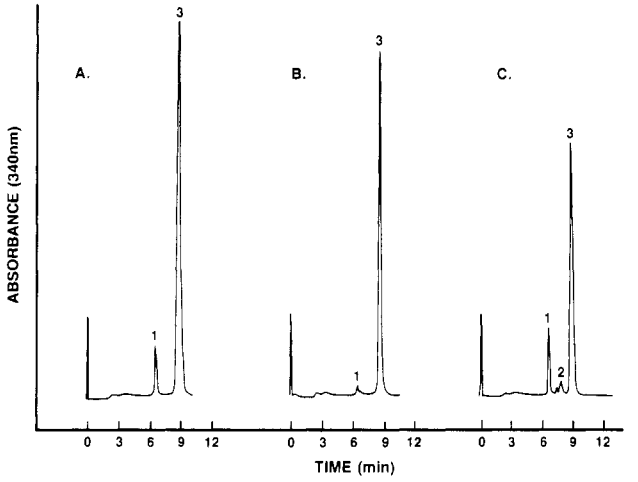

Figure 4. Chromatograms of retinyl palmitate samples used in supplementation and fortification mixes: peak 1, 13-*cis*-retinyl palmitate; peak 2, 9-*cis*-retinyl palmitate; peak 3, *all-trans*-retinyl palmitate.

sterilized (infant formulas, UHT—2% fat) appeared to have greater cis/trans ratios (Table II). These results suggest that relatively severe heat treatments, such as those used in canning facilities, are required to form appreciable levels of cis isomers. UHT (3.2% fat) and evaporated milk samples were not commercially fortified, and the isomeric composition measured represents the native retinoid compounds.

Changes in isomeric composition were not observed during processing of skim milk (experimental samples, Table II). Raw skim milk, after supplementation with retinyl palmitate, was subjected to various processing conditions. The fortification mix and unprocessed and pasteurized milk all yielded ratios at control levels. Canning at 117 °C for 20 min did not cause any further significant isomeriation. Since these heat treatments did not cause isomerization, the ratios shown in Table II may be attributed to the isomeric composition of the fortification mixture used for each product.

Analyses of retinyl palmitate samples prepared by three different manufacturers, for the purpose of blending into

fortification mixes, revealed that various levels of isomers were present. Figure 4 illustrates typical chromatograms for these fortification samples. The ratios of 13-cis/trans were measured as 0.06, 0.02, and 0.27 for samples A–C, respectively. On the basis of relative retention time data (Landers and Olson, 1986), the smallest peak eluting after the 13-cis isomer (Figure 4, sample C), may be 9,13-di-*cis*-retinyl palmitate. Presumably, the differences in isomeric composition found in these samples result as end products when synthesizing retinyl palmitate and may depend upon the temperatures or conditions used during synthesis. It follows that the isomers found in food products are in part attributable to the composition of the fortification samples.

Exposure of fortification mix to fluorescent laboratory lighting, darkness, and intermittent indirect sunlight for 3 days revealed a change in isomeric composition for samples only exposed to sunlight. Upon exposure to sunlight, no change in the amount of 13-cis isomer was observed. However, a decrease in the level of all-trans isomer occurred corresponding to the formation of 9-*cis*-retinyl palmitate. Exposure of purified *all-trans*-retinyl palmitate (Figure 1, sample C), obtained by repeated HPLC injections of sample A, to indirect sunlight resulted in the formation of the 9-cis isomer (peak 2, Figure 1, sample D). These results confirm those mentioned earlier (Mulry et al., 1983; Landers and Olson, 1986). Although *all-trans*-retinyl palmitate is susceptible to light-induced degradation (Zahar et al., 1986), these observations suggest that relatively harsh illumination conditions are required for photoisomerization to occur. Heat treatment of the all-trans compound (sample C) to 120 °C for 30 min showed the formation of primarily the 13-cis isomer (Figure 1, sample E). The results of this study indicate that 9-*cis*-retinyl palmitate is formed predominately via a photocatalytic mechanism while formation of 13-*cis*-retinyl palmitate is predominately heat induced. *all-trans*-Retinol has been reported to react similarly (Woollard and Indyk, 1986). No change in isomeric composition was detected when samples and extracts were allowed to set at room temperature or during refrigerated storage. Heating dilute aqueous solution of *all-trans*-retinol also produced the 13-cis isomer, however, to a much lesser extent than that observed for retinyl palmitate. The formation of this isomer, relative to other cis geometries, implies that the 13-cis form is the most thermodynmically stable configuration.

The presence of appreciable levels of *cis*-retinyl palmitate in foods suggests that this form of vitamin A should be accounted for in nutritional content determinations. Many HPLC methods, for quantitative vitamin A analyses, elute all vitamin A forms as a single peak (Landen et al., 1985; Mulry et al., 1983; Head and Gibbs, 1977; Thompson and Maxwell, 1977). Considering that 13-*cis*-retinol has 75% of the activity of *all-trans*-retinol, these methods may result in inaccurate nutritional estimates. This conclusion has been recently emphasized by Woollard and Indyk (1986). Further research is needed to develop techniques to account for the bioavailability of isomeric vitamin A forms and to determine reasons for their presence and formation in food products.

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Registry No. Retinyl palmitate, 79-81-2; 13-*cis*-retinyl palmitate, 26771-20-0; 9-*cis*-retinyl palmitate, 34356-29-1; 9-*cis*,13-*cis*-retinyl palmitate, 34356-30-4.

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