Isomerization of Retinyl Palmitate in Fortified Skim Milk under Retail Fluorescent Lighting

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The rate of loss of *all-trans*-retinyl palmitate (RP) and 13-*cis*-RP was monitored under fluorescent lighting (1076 lx) in fortified skim milk in paperboard, polyethylene, and glass milk containers to simulate commercial display cases. Commercially fortified milk contained 5–10% 13-*cis*-RP, initially. The rate of *all-trans*-RP loss was apparent first order, with rate constants of 0.016 90 ± 0.001 22 and 0.011 43 ± 0.0017/h for glass and plastic containers, respectively. 13-*cis*-RP was lost at 0.0116 ± 0.003 51 and 0.007 84 ± 0.002 65/h in glass and plastic containers, respectively. No significant loss of either isomer occurred in paperboard containers. 9-*cis*-RP was observed only in glass and plastic containers after 3 days of illumination. The mechanism to explain the loss of RP is more complex than first order and probably includes isomerization and oxidation.

Vitamin A degradation due to photoisomerization has been observed for some time, but the extent of this process has not been investigated in great detail in very many foods. The presence of *cis*-retinyl isomers in food has been observed in many types of food, including cheeses (Stancher and Zonta, 1982a,b), UHT milk, margarine, ready-to-eat cereal (Mulry, 1983), sausage, dry cat food, margarine, and butter (Egberg et al., 1977). The rate loss of vitamin A from isomerization and degradation has been investigated in milk in glass (Gaylord et al., 1986; Zahar et al., 1987) and in various solvent systems (Mulry et al., 1983). The effect of fluorescent light on *all-trans*-retinyl palmitate (RP) results in the formation of 13-cis and 9-cis isomers of RP initially, followed by formation of 11-cis, 9,11-cis,cis, 11,13-cis,cis, and 9,13-cis,cis isomers of RP, as well as degradation of all the RP isomers. The 9- and 13-cis isomers are the predominant forms produced in food from isomerization of *all-trans*-RP. These two isomers have reduced vitamin A activity when compared with the all-trans isomer. Relative biological activities for the RP moieties are 100% for *all-trans*-RP, 75% for 13-*cis*-RP, and 25% for 9-*cis*-RP (Ames, 1966). Thus, prolonged exposure to light results in loss of total vitamin A potency.

The kinetics of vitamin A degradation and isomerization have been reported in beef liver puree (Wilkinson et al., 1981, 1982), in pharmaceutical preparations (Slater et al., 1979), and in coconut oil model systems with and without methyl linoleate (Mulry, 1983). The rate of vitamin A loss in milk and the extent of the loss have only been investigated in glass and only for *all-trans*-RP (Gaylord et al., 1986; Zahar et al., 1987). Most of the milk sold at the retail level is packaged in coated paperboard and plastic. Before purchase, this product is exposed to fluorescent light in the display case and the room lights. The extent of vitamin A loss under these conditions has not been previously investigated.

The purpose of this study was to evaluate the extent of isomerization and degradation of RP-fortified skim milk in three container types under commercial lighting. Because 9- and 13-*cis*-RP are not available commercially, they were synthesized from 9- and 13-*cis*-retinal. The isomers of RP were quantified by using normal-phase high-performance liquid chromatography (HPLC).

METHODS AND MATERIALS

Vitamin A fortified skim milk in paperboard (Pure-Pak) was purchased locally as soon as delivered from the commercial dairy. The milk was equally distributed into glass, polyethylene, and paperboard milk containers. The containers were placed in a commercial display case held between 7 and 10 °C. The display case fluorescent lights yielded an average of 1076 lx as measured by a light meter. The milk samples were subjected to continuous illumination and sampled over a 4-day period.

HPLC analysis of the RP isomers was a modification of Mulry et al. (1983). The mobile phase was 2% isopropyl ether (Fisher) in hexane (HPLC grade, Fisher) with 0.1% BHT to retard oxidation and filtered through a 0.45-μm filter (Alpha-450 Metricel, Fisher). Two different analytical systems were used in this study. The first analytical system consisted of a Beckman 110A pump, an Altex Model 210 injector with a 250-μL loop, a silica guard column (Brownlee), a Zorbax-SIL (DuPont) 4.6 × 250 mm column, an Aminco Fluoro-Monitor (American Instruments, Inc.) equipped with a Corning 7-51 primary filter, and Wratten No. 8 secondary filter, and a recorder. The other analytical system consisted of two Beckman 110B pumps, a Beckman Model 420 controller, a Spectra-Physics Model 8780 autosampler equipped with a 250-μL loop, a silica guard column, a Zorbax-SIL (DuPont) or a Beckman Ultrasphere-Si column (4.6 × 250 mm), a Beckman Model 163 variable-wavelength UV detector at 323 nm, and a Beckman Model 427 integrator. The mobile phase was metered at 1.2 mL/min. Peak height was correlated with RP concentration in the former HPLC system, and peak area was integrated in the latter. Duplicate injections of all samples were routinely performed. All steps in synthesis and analysis were conducted under gold fluorescent light (Phillips/Westinghouse F4OGO).

The 9- and 13-*cis*-RP were synthesized by a modification of the procedure reported by Mulry et al. (1983) and Mulry (1983). 9- and 13-*cis*-retinal were used as obtained (Sigma). The aldehydes were reduced to their respective alcohols with $NaBH_4$ in ethanol. The alcohols were esterified with palmitoyl chloride (Sigma) in methylene chloride by using triethylamine as a catalyst. The reaction mixture was cleaned up by column chromatography on silica gel deactivated with 5% (w/w) H_2O. The crude RP isomers were eluted with a 5% isopropyl ether in hexane solution. The crude cis isomers and commercially available *all-trans*-RP were further purified by semipreparative HPLC.

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Table I. Standard Curves

RP isomer	equation	r
	Fluorescence[a]	
all-trans	$y = 0.236x + 2.217$	0.9987
13-cis	$y = 0.962x - 2.702$	0.9993
	Absorbance[b]	
all-trans	$y = 0.091x - 5.799$	0.9991
13-cis	$y = 0.103x + 10.735$	0.9993

[a] y = peak height; x = nanograms/milliliter. [b] y = integrator units; x = nanograms/milliliter.

Semipreparative HPLC of the crude retinyl palmitate isomers was performed with a Beckman Ultrasphere-Si, 100 × 250 mm column. Samples were introduced to the chromatographic system through a 2000-μL loop. The mobile phase consisted of 5% isopropyl ether in hexane and was pumped at a flow rate of 2–3 mL/min. Peaks were detected in a preparative flow cell for the Beckman Model 153 UV detector at 254 nm. The peaks of interest were collected, pooled, and rechromatographed on the analytical HPLC system to confirm their respective purity. Purified isomers were stored in the dark under nitrogen at −20 °C.

Skim milk was extracted for the RP isomers according to Thompson et al. (1980) by using a hexane/ethanol mixture. All samples were filtered through a 0.45-μm Alpha-450 (Fisher) before chromatography. The HPLC column was routinely reactivated according to the procedure of Bredeweg et al. (1979) to yield reproducible retention times.

Standard curves for *all-trans*-RP, 13-*cis*-RP, and 9-*cis*-RP were prepared and plotted as linear regression equations. The concentrations of the RP isomers were determined from these lines. The loss of the individual isomers was plotted versus time by using the SAS statistical package (SAS, 1985). Rate constants were generated from plots of ln (percent remaining) versus time as a linear regression equation (SAS, 1985). Recovery of RP from skim milk ranged from 95 to 105%. Shelf life predictions were expressed as the half-life in hours for each container–RP isomer pair. The rate loss experiment was repeated eight times. Mean rate constants were compared by using Student's t-test.

RESULTS

Resolution of the isomers of RP was achieved by using the silica-based HPLC column and an isopropyl ether/hexane mobile phase. The retention order of the RP isomers was not different from that observed by Landers and Olson (1986) using the same mobile phase, by Mulry et al. (1983) with methyl *tert*-butyl ether/hexane mobile phase, or by Gaylord et al. (1986) using diethyl ether/hexane mobile phase. Commercially available *all-trans*-RP always contains 5–10% 13-*cis*-RP. This observation has been reported by others (Gaylord et al., 1986; Mulry, 1983). Milk not exposed to light after purchase routinely contained 5–10% 13-*cis*-RP that probably was present in the fortification mixture. In milk stored in paperboard cartons for 30 days, no change in the ratio of *all-trans*-RP to 13-*cis*-RP was observed (data not shown).

Preparation of the 13-*cis*- and 9-*cis*-RP was relatively easy, although yields of the esterified forms were relatively low (25–30%). The amounts were more than adequate to prepare standard curves and identify the isomers. The purified isomers were quite stable in solution when stored under nitrogen at −20 °C for several months, as long as they were protected from light.

A comparison of the standard curves generated from absorbance detection versus fluorescent detection yields an interesting comparison (Table I). The extinction coefficients for absorbance of *all-trans*-RP and 13-*cis*-RP at 323 nm are not significantly different. The extinction coefficient for the fluorescent emission was 4 times greater for the 13-cis isomer than for the all-trans one. Thus, it seems that fluorescence detection is much more sensitive for the cis isomers (data not shown for 9-*cis*-RP) than for *all-trans*-RP.

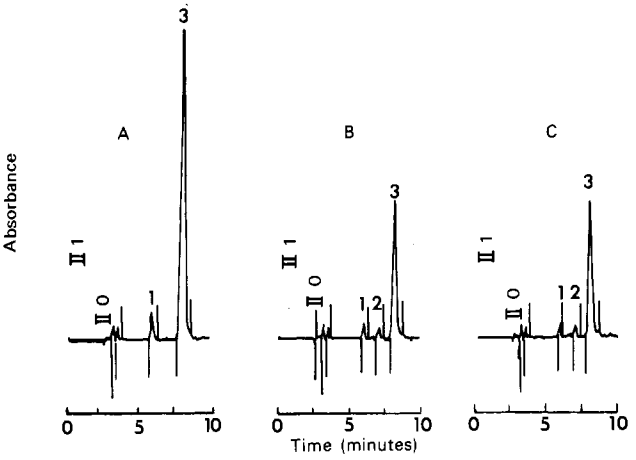

Figure 1. HPLC of RP isomers in skim milk: (a) in carton at time zero; (b) in plastic after 3 days of illumination; (c) in glass after 3 days of illumination. Key: peak 1 = 13-*cis*-RP; peak 2 = 9-*cis*-RP; peak 3 = *all-trans*-RP. Absorbance detection at 343 nm; AUFS = 0.1.

Table II. Vitamin A Loss in Skim Milk[a]

container	k of RP vitamer, h^{-1}	
	all-trans[b]	13-cis[c]
glass	0.016 90 ± 0.001 22	0.011 60 ± 0.003 51
plastic	0.011 43 ± 0.001 77	0.007 84 ± 0.002 65
carton	0.000 69 ± 0.002 78	0.001 20 ± 0.003 37

[a] n = 8. [b] Rate constants for *all-trans*-RP loss are significantly different at 0.001. [c] Rate constants for 13-*cis*-RP loss are significantly different at 0.025.

Both 13-*cis*-RP and *all-trans*-RP were lost in skim milk as illumination time increased in light-permeable containers. No 9-*cis*-RP was observed in the milk until 3 days of illumination had occurred. No 9-*cis*-RP was observed in the paperboard containers. The amount of 9-*cis*-RP present on the fourth day was extremely small (Figure 1). The 9-*cis*-RP concentration averaged 150 ng/mL of milk on day 3 or about 5% of the RP content.

Vitamin A was lost from the glass and plastic containers in an apparent first-order rate process (Table II; Figures 2 and 3). No significant loss of *all-trans*- or 13-*cis*-RP was observed in paperboard containers (slopes were not significantly different from zero). The rate of loss was fastest in glass containers. The rate constants for *all-trans*-RP loss were significantly greater than for 13-*cis*-RP in glass. This suggests that the rate constant observed for 13-*cis*-RP may reflect formation of 13-*cis*- from *all-trans*-RP and degradation of 13-*cis*-RP, concomitantly. The rate of loss of *all-trans*-RP from plastic containers was significantly less than in glass but still at an appreciable rate compared with glass (67%). The rate of loss of 13-*cis*-RP in plastic was also reduced by 34% as compared with glass.

These rate constants allow us to predict the half-life of the two vitamers in the different container types. We can expect a half-life for *all-trans*-RP of 1004, 60, and 41 h in paperboard, plastic, and glass, respectively. The half-lives predicted for 13-*cis*-RP were 580, 88, and 60 h in paperboard, plastic, and glass, respectively. When the vitamin A content is expressed as retinol equivalents, the loss is

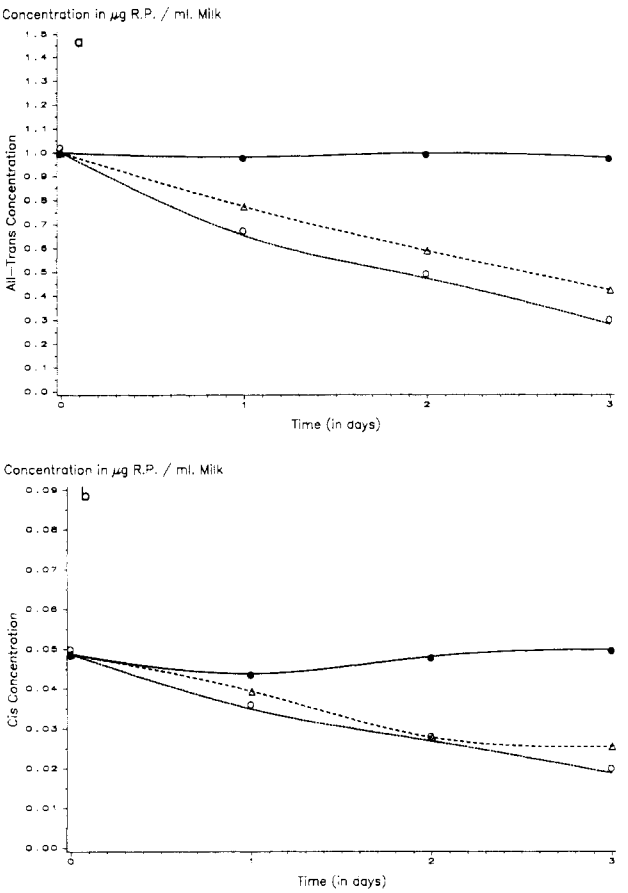

Figure 2. Loss of (a) *all-trans*-retinyl palmitate and (b) 13-*cis*-retinyl palmitate in paperboard (●), plastic (△), and glass (○) containers of skim milk as a function of time.

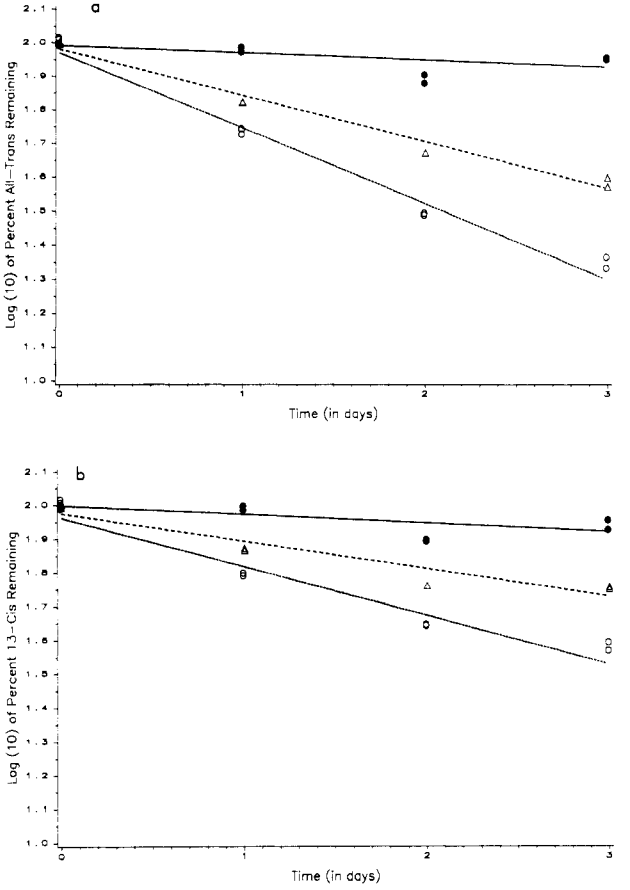

Figure 3. First-order rate plot for the loss of (a) *all-trans*-retinyl palmitate and (b) 13-*cis*-retinyl palmitate in glass (○), plastic (△) and paperboard (●) carton containers versus time.

approximately the same. This is due to the relative high potency of the 13-cis isomer compared with *all-trans*-RP and because very little 9-cis isomer was formed in milk. These data indicate that significant loss of vitamin A occurs in plastic and glass containers even in relatively short time under fluorescent lights. The data indicate that although loss of vitamin A is significantly slower in polyethylene plastic containers, the loss is such that milk at retail outlets undergoes a significant loss of vitamin A before purchase by the consumer.

DISCUSSION

The data for these experiments indicate that vitamin A is lost rapidly from milk under fluorescent illumination as both *all-trans*-RP and 13-*cis*-RP degrade. Little 9-*cis*-RP formed under the conditions of our experimental design. This observation is different from that reported by Gaylord et al. (1986). This probably is due to the different sizes of containers used to illuminate the milk. We tried to mimic retail conditions with standard-size milk containers, whereas Gaylord et al. (1986) used test tubes for their containers. With greater surface area exposed to light per milliliter of milk in the Gaylord et al. (1986) experiments, more isomerization to the 9-cis isomer should be expected. We both observed the same reaction(s) as indicated by our calculated rate constants that were not statistically different for the loss of *all-trans*-RP in glass (0.0198/h and 0.01696/h).

RP in milk evidently does not isomerize in the same manner as observed in organic solvents (Mulry et al., 1983; Mulry, 1983; Landers and Olson, 1986). In organic solvents, significant amounts of 9-*cis*-RP formed under illumination as *all-trans*-RP decreases, and 13-*cis* seems to remain relatively constant (Mulry et al., 1983). Mulry (1983) reported that, in model systems (in amber vials of coconut oil without methyl linoleate incubated at 121 °C), *all-trans*-RP was lost as 9- and 13-cis isomers increased in concentration. With methyl linoleate present in the model system, degradation of vitamin A did not occur (Mulry, 1983). Zahar et al. (1987) also reported that when the carrier oil containing vitamin A was illuminated, the rate of loss was fastest in coconut oil and slowest in peanut and corn oils. In contrast, Zahar et al. (1987) reported the opposite results for vitamin A carriers when incorporated into dairy products. Vitamin A in corn oil in fortified skim milk was lost to a greater extent than vitamin A in coconut oil under illumination at 4 and 35 °C. Wilkinson et al. (1982) reported apparent first-order rate constants for the degradation of vitamin A in beef liver puree. They reported increasing E_a with increasing moisture content. These data suggest that interpretation of our data in milk could be complex, with moisture providing a protective effect, and the small amounts of remaining lipid may yield a negative effect on vitamin A stability. Because the rate constants for *cis*- and *trans*-RP loss were not the same within a container type, the mechanism probably is more complex than first order. A suggested mechanism in order to interpret our results awaits further experimentation.

In conclusion, vitamin A was lost from skim milk in an apparent first-order rate process, but the reaction mechanism probably is more complex. The loss of vitamin A from plastic milk containers is significant under fluorescent lighting but less than that observed in glass. For milk processors to meet FDA labeling claims for vitamin A content, milk in glass and plastic will be less than the allowed 20% error after 13.2 and 19.5 h of exposure to light, respectively. Because the majority of milk purchased in the United States is in plastic containers, some care in

retail handling of milk is recommended.

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Changes in Free Amino Acids and Adenine Nucleotides in Boiled Muscle Extracts of Yellowtail (*Seriola quinqueradiata*) Stored in Ice

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Free amino acids (FAA), adenine nucleotides, and their related compounds in boiled muscle extracts of yellowtail (*Seriola quinqueradiata*) were determined during ice storage. Little change in the amount of major FAA except alanine was detected in the white muscle during over 40 days of storage, while in the dark muscle almost all FAA, except taurine and histidine, increased significantly in the early stage of storage. Inosine 5′-monophosphate (IMP) content was abundant in the very fresh white muscle and then decreased gradually during storage. In the dark muscle, inosine (HxR) and IMP were predominant initially, followed by an extremely rapid decrease in IMP and a concomitant increase in HxR level within 1 day.

Fish muscle contains a variety of nonprotein nitrogenous compounds, some of which are important from a food chemical point of view (Konosu and Yamaguchi, 1982). The changes in content of adenine nucleotides and their related compounds during storage are closely associated with the quality of the fish (Uchiyama and Ehira, 1970; Connell and Shewan, 1980). Also the changes in amount of free amino acids (FAA) relate to autolysis and subsequent bacterial action (Shewan, 1962; Liston, 1980). FAA are known to evoke a meaty taste sensation together with adenine nucleotides (Maga, 1983). In our previous papers, we reported the changes in content of major FAA, adenine nucleotides, and their related compounds in yellowtail (*Seriola quinqueradiata*) muscle (Sakaguchi et al., 1982; Murata and Sakaguchi, 1986). Yellowtail is a dark-flesh fish and one of the most important species of cultured fish in Japan. Dark-fleshed fish have dark muscle along the lateral line in an amount of 10–20% of the whole musculature (Obatake and Heya, 1985). The rates and patterns of the changes in content of the above compounds during ice storage differ greatly between white and dark muscles. In the white muscle most FAA showed little change in content; in the dark muscle they increased markedly (Sakaguchi et al., 1982, 1984). Inosine 5′-monophosphate (IMP), among adenine nucleotides, accumulated in the former tissue in a certain early stage of storage, followed by the gradual decrease in level. IMP levels in the latter fall very rapidly, and inosine (HxR) rises concomitantly (Dyer et al., 1966; Murata and Sakaguchi, 1986). For extraction of nonprotein nitrogenous compounds from the muscle tissue, several deproteinizing reagents are commonly used. Such extracts obtained, however, are not applicable to sensory evaluation. In the present paper, we describe the changes in content of FAA, adenine nucleo-

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