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The Stability of All-*trans* Retinol and Reactivity Towards Transition Metals

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ABSTRACT

*The stability of all-*trans *retinol in solution and in a simple model system was studied by high performance liquid chromatography. Approximately 50% all-*trans *retinol was decomposed in chlorinated solvents exposed to sunlight (20 min) and in methanol under ultra violet light (30 min) at laboratory temperature. All the degradation products (9-*cis, *9, 13 di-*cis, *11-*cis, *11, 13 di-*cis*) were higher in chlorinated solvents than non-chlorinated solvents and 9-*cis *and 13-*cis *retinol were the major isomers in all the solvents during sunlight induced photolysis. Assay of all-*trans *retinol dispersed on a microcrystalline cellulose model system showed that the preparation technique affected the % recovery and the precision of the method. About 93% of the all-*trans *retinol was recovered from well-homogenised model systems (MS1, MS2) and the recovery was not affected by the level of addition. Mineral fortification had a significant effect on the retinol stability and the half-life dropped to approximately* $\frac{1}{3}$ *and* $\frac{1}{4}$ *of the unsupplemented value due to iron and copper supplementation, respectively. The loss of all-*trans *retinol followed first order kinetics.*

INTRODUCTION

All-*trans* retinol is an unsaturated monohydric alcohol with 20 carbon atoms, consisting of a cyclohexane ring linked to a polyunsaturated chain which terminates in an alcohol group. The five conjugated double bonds

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(Lundberg, 1961) in the configuration of vitamin A are easy points of attack for oxygen; this theoretically permits sixteen *cis–trans* isomers of reduced biological activity (Stancher & Zonta, 1984). Vitamin A alcohol (retinol) exhibits full biological activity, the 2-mono *cis* (neo) or 13-*cis* $\frac{3}{4}$ activity and the 6-mono *cis* or 9-*cis* and 2, 6 di-*cis* or 9, 13 di-*cis* $\frac{1}{4}$ activity (Bauernfeind & Cort, 1973).

A number of investigators have studied the stability of retinyl palmitate and retinyl acetate to oxidation by atmospheric oxygen, light, heat and other oxidizing agents (Stancher & Zonta, 1982*a*,*b*; Lambert *et al.*, 1985; Murphy *et al.*, 1988). The oxidation kinetics of retinyl acetate and retinol in solutions (Landers & Olson, 1986) and in the solid state (Finkel'shtein *et al.*, 1970) were investigated and it was found that there is a radical chain process during the autoxidation. deMan (1981) found that the vitamin A content of whole milk dropped by 67·7%, when milk samples were exposed to fluorescent light for 48 h at refrigerated temperature.

Similarly, the effect of acids (Schwieter & Isler, 1967), heat (Sweeney & Marsh, 1971; Villota *et al.*, 1980; Parrish *et al.*, 1980*a*; Wilkinson *et al.*, 1981; 1982; Parrish & Patterson, 1983; Le Maguer & Jackson, 1983; Lambert *et al.*, 1985; Woollard & Fairweather, 1985), water (Cartensen, 1964; Kirk, 1981) and enzymes (Chou & Breene, 1972; Gardner, 1980) on vitamin A content have been extensively studied. Very little information is available regarding the stability of all-*trans* retinol. This project was therefore initiated to study the stability of all-*trans* retinol under normal atmospheric conditions in solutions and in a simple model system fortified with some transition metals, simulating dehydrated foods.

MATERIALS AND METHODS

Pure standards of vitamin A alcohol (all-*trans* retinol) were obtained from Fluka AG (Buchs, Switzerland); 13-*cis* retinal, 13-*cis* retinol (85% pure) and 9-*cis* retinal were supplied by Sigma Chemical Co. Limited. As neither 13-*cis* nor 9-*cis* retinol (pure) standards could be obtained, reduction of 13-*cis* and 9-*cis* retinals using a procedure of Egberg *et al.* (1977), to 13-*cis* and 9-*cis* retinols was used. HPLC grade methanol, hexane, water and isopropanol were purchased from Rathburn Chemical Co. Limited. Microcrystalline cellulose (MCC) (Avicel PH101) was supplied by Honeywill and Stein Ltd. All other chemicals used were of AnalaR grade. They were used without further purification.

High performance liquid chromatography (HPLC)

An Applied Chromatography System (ACS) Model LC 750, with a 20 μl loop injector Model 7010 Rheodyne valve (Jones Chromatography Ltd.),

equipped with a continuously variable wavelength detector Varian Model UV 50 and Model 600/601 Tarkan recorder (W + W Bedienungsanleitung) was used.

HPLC column

HPLC column Lichrosorb Si-60 (E. Merck. Durmstadt, FR Germany), column length 250 mm and internal diameter 4 mm.

Photolysis

Retinol stability was studied in solvents most frequently used in our laboratory to assess their effect on retinol assays. Pure all-*trans* retinol solutions (20 μg/ml) in different solvents (chloroform, dichloromethane, hexane, methanol, ethanol) were placed in direct sunlight and under ultra violet light (Universal UV Lampe Camag, Wavelength 350 nm) at room temperature for different time intervals. The isomerisation of retinol which occurred as a result of photolysis (Tsukida *et al.*, 1977; Stancher & Zonta, 1984) was studied by HPLC chromatography according to the method of Stancher and Zonta (1984) with the chromatographic conditions:

Stationary Phase:	Lichrosorb Si-60
Eluent Composition:	Hexane:Propan-2-ol (99·6:0·4) % v/v
Loop Capacity:	20 μl
Detector:	UV 50 using 326 nm
Column and Detector Temperature:	45°C
Peak Evaluation:	Peak Height
Flow Rate:	1·5 ml/min
Absorbance Range:	0·005–0·1
Recorder Sensitivity:	20 mv
Band Width:	8

Preparation of model system

Microcrystalline cellulose (MCC) as an inactive organic compound was used as a solid support medium for retinol study. A number of previous workers (Chou & Breene, 1972; Chou & Labuza, 1974; Kanner *et al.*, 1978; Ramakrishman & Francis, 1979*a*) also used MCC for vitamin assays. MCC was exhaustively washed with 0·05M ethylene diamine tetra acetic acid (EDTA) solution in order to remove the metals (Tjhio *et al.*, 1969; Labuza *et al.*, 1971*b*) from the model system. The MCC was dried after thoroughly washing with distilled water and passed through a (125 μm aperture) plastic mesh sieve.

Approximately $5{\cdot}2363 \times 10^{-4}$ mol retinol was added in ethanolic solution to each gram of MCC and dried in a rotary evaporator under vacuum at 40°C. The mixture was transferred to a food processor with a plastic blade and bowl and homogenized for 3–5 min in a nitrogen atmosphere. The transition mineral elements such as iron, copper, zinc and calcium in the form of ferrous sulphate (1·3 μg), copper sulphate (0·17 μg), zinc oxide (2·81 μg) and calcium carbonate (144 μg) were added to the model systems in proportion to retinol as described in the Present Day Practice in Infant Feeding (1980).

Recovery of retinol from MS

The aim of this investigation was to establish the per cent recovery of all-*trans* retinol in MS and its analysis, whether or not losses occurred during preparation of MS and the precision and efficiency of the analytical method of extraction. The percentage of retinol recovered from the MS was calculated using the equation:

$$\% \text{ Retinol Recovery} = \frac{\text{Amount of all-}trans\text{ retinol measured}}{\text{Amount of all-}trans\text{ retinol added}} \times 100$$

Storage of the MS

The water activity (a_w) level in the MS was adjusted over saturated salt solution prepared according to the method described by Rockland (1960). Equilibration to the desired temperature and a_w was allowed to proceed for about 48 h, under nitrogen. Model systems were then exposed to air and samples (1–2 g) were taken from the storage cabinet randomly at different time intervals for retinol assay. All operations were done under subdued light and in the presence of nitrogen gas, wherever necessary.

Extraction of sorbed retinol

About 20 ml of MEA reagent (methanol:ethanol:acetone, 6:3:1) % v/v, containing an antioxidant (BHT, 20 mg/100 ml) was added to the MS (1–2 g). The flasks were stoppered and shaken for 1 h by means of a mechanical shaker (Gallenkamp). The slurry was filtered through a 1A sintered glass filter, with the aid of a water suction pump and solvent was removed by evaporation under vacuum at 40°C with a rotary vacuum evaporator. The residues in the flasks were dissolved in HPLC-grade methanol containing antioxidant (BHT, 20 mg/100 ml).

Retinol analysis

A series of standards of all-*trans* retinol, 13-*cis* retinol and 9-*cis* retinol were prepared in methanolic solution (BHT, 20 mg/100 ml). The standard solutions and the extracts from model systems were analysed by the same chromatographic conditions as described earlier. The concentrations of all-*trans*, 13-*cis* and 9-*cis* retinol were obtained by comparing the height of the peak of extracted retinol with the peak height of a standard solution of a known concentration. Tentative identification of 9, 13 di-*cis*, 11, 13 di-*cis* and 11-*cis* was based on the work of Stancher and Zonta (1984).

Kinetics study

The kinetics of all-*trans* retinol loss were computed using the method of Benson (1960). The integrated equation for rate constant (k) used was $k_1 = \ln(a/a-x)$ and for half life ($t_{1/2}$) was $\ln 2/k_1$. Where 'a' is the concentration of reactant at time $t=0$ and 'x' is the concentration of reactant at time $t=t$.

RESULTS AND DISCUSSION

Table 1 shows the results for all-*trans* retinol isomers during sunlight and UV-induced photolysis and Fig. 1 shows a typical chromatogram of photolysis of all-*trans* retinol. The results indicated that all-*trans* retinol in

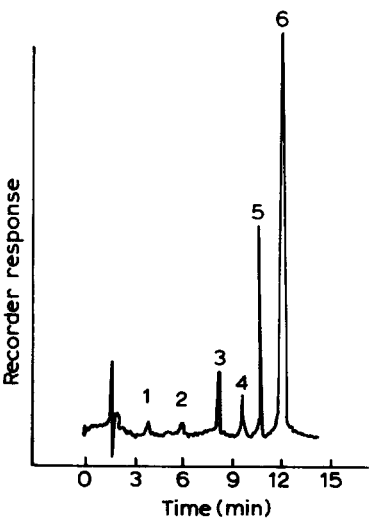

Fig. 1. Chromatogram of sunlight-induced photolysis of retinol. For chromatographic conditions see text. Peaks: 1. 11,13 di-*cis* 2. 11-*cis* 3. 13-*cis* 4. 9,13-*cis* 5. 9-*cis* 6. all-*trans* retinol.

TABLE 1
Effect of Sunlight and UV-Induced Photolysis on All-*Trans* Retinol in Different Solvents[a]

Time (*min*)	9-cis (%)	*9, 13* *di*-cis[b]	*13*-cis (%)	*11*-cis[b]	*11, 13* *di*-cis[b]	*All*-trans (%)
Photolysis in chloroform[c]						
13	20·4	1·71	2·13	1·70	0·85	46·8
20	23·4	2·12	3·83	2·13	0·85	42·6
30	27·7	5·96	8·08	2·55	1·06	37·0
Photolysis in dichloromethane[b]						
13	18·2	0·86	1·81	0·96	0·35	55·6
20	22·1	1·10	2·05	1·10	0·61	48·6
30	28·3	3·21	5·98	2·00	0·98	40·9
Photolysis in ethanol[c]						
60	0·45	0·61	13·3	—	0·31	81·1
120	0·49	0·75	18·4	—	0·53	78·4
Photolysis in hexane[c]						
20	1·11	0·25	13·3	—	—	85·3
30	2·21	0·29	22·3	—	—	74·2
40	3·30	0·34	25·6	—	—	75·4
45	4·44	0·34	31·1	—	—	63·3
Photolysis in methanol[c]						
30	—	—	0·34	—	—	98·8
60	—	—	0·41	—	—	96·9
Photolysis in methanol[d]						
8	—	—	0·57	—	—	90·0
20	—	—	2·30	—	—	60·9
30	—	—	3·45	—	—	51·3

[a] Average of two determinations.
[b] Values are expressed as peak height count percentage.
[c] Sunlight-induced photolysis.
[d] UV-induced photolysis.

chloroform and dichloromethane solutions was decomposed more rapidly than in non-chlorinated solvents. Rather more than 50% of all-*trans* retinol in chlorinated solvents was decomposed when exposed to sunlight for 20 min at laboratory temperature. All the degradation products (9-*cis*, 9, 13 di-*cis*, 11-*cis*, 11,13 di-*cis*) were higher in chlorinated solvents compared with non-chlorinated solvents. 9-*cis* and 13-*cis* retinols were the major isomers in all the solvents during sunlight-induced photolysis. The fast isomerisation in chlorinated solvents may possibly be due to a photochemically induced free radical mechanism. Chlorinated solvents are unstable towards light and

oxygen, forming phosgene and HCl (Mulry *et al.*, 1983) and may have initiated the acid-catalysed isomerisation of the conjugated double bonds system (Seltzer, 1972).

Isomerisation of all-*trans* retinol was greater in hexane than in other non-chlorinated solvents. The major break-down product was 13-*cis* retinol during sunlight-induced photolysis. The peak presumed to be 11-*cis* retinol was totally absent during sunlight and UV-induced photolysis in hexane, ethanol and methanol contrasting with the results obtained by Stancher and Zonta (1984) who found 11-*cis* retinol as the main product in fish liver oil, when subjected to fluorescent light. All-*trans* retinol was very stable in methanol and less than 5% was decomposed in 1 h when exposed to sunlight at laboratory temperature, whereas in ultra violet about 50% was destroyed in 40 min, possibly due to secondary photoxidation during irradiation. Irradiation causes a series of changes in the absorption spectrum including a shift in the UV absorption maximum from 326–328 nm to 274–275 nm (Bolomey *et al.*, 1947) during the autoxidation of vitamin A in fish liver oil.

The rate of loss of vitamin A during isomerisation and degradation has been investigated in various solvents (Mulry *et al.*, 1983) and in milk in glass (Gaylord *et al.*, 1986; Zahar *et al.*, 1987). They found 13-*cis*, 9-*cis*, 9, 13 di-*cis*, and 11, 13 di-*cis* isomers of retinyl palmitate produced by fluorescent light and observed 9-*cis* and 13-*cis* isomers predominantly in foods. The results obtained in our investigations follow a similar pattern.

Table 2 shows the mean all-*trans* retinol, standard deviation, coefficient of variation and % recovery of all-*trans* retinol from the model systems. Comparison of the % recovery of the analysis of MS showed that the preparation technique affected the precision. The large CV (5·79) between samples and the high recovery of retinol in the MS3 showed that the model system was not homogeneous. When a homogenised MS (MS1, MS2) was used the precision of the analysis was improved. The effect of concentration of all-*trans* retinol on recovery was examined (MS4) and it was found that increasing the retinol level in MS did not affect the recovery obtained. The recovery of retinol (MS5) was significantly affected when MCC and other equipment used were not properly washed with EDTA solution. Previous workers (Tjhio *et al.*, 1969; Labuza *et al.*, 1971*b*) used EDTA solution for the removal of metallic ions from the model system. No literature data have been found to compare with our results. The amount of retinol added in the preparation of MS was only a guide to the level of retinol in the MS at the beginning of the storage test.

The influence of mineral fortification on the stability of all-*trans* retinol in a model system stored at a a_w 0·42 and temperature 30°C was studied. The results (Table 3) obtained indicated that mineral fortification reduced the retinol stability. Copper and iron have the most significant effect on the rate

TABLE 2
Recovery of All-*Trans* Retinol from Different Model Systems[a]

Model system	*Retinol concentration*		*SD*	*CV*	*Recovery* (%)
	Added	*Measured*			
	(μg/g)				
	17·0	15·8			93·2
1.	17·0	15·8	0·05	0·32	93·0
	17·0	15·8			92·7
Mean		15·8			93·0
	17·0	15·7			92·1
2.	17·0	15·9	0·13	0·86	93·6
	17·0	15·8			92·9
Mean		15·8			92·9
	17·0	15·1			88·8
3.	17·0	13·1	0·82	5·79	78·0
	17·0	14·3			84·1
		14·1			83·3
	25·0	23·1			92·5
4.	25·0	22·8	0·12	0·55	91·3
	25·0	23·0			92·0
Mean		23·0			92·0
	17·0	14·1			70·5
5.	17·0	12·3	0·74	5·61	61·8
	17·0	13·0			65·8
Average		13·1			66·0

[a] Mean of three determinations. 1, 2. A homogeneous model system of all-*trans* retinol and microcrystalline cellulose. 3. Non-homogeneous model system of all-*trans* retinol and microcrystalline cellulose. 4. The initial concentration of all-*trans* retinol in the model system was higher than all other model systems and was homogeneous. 5. The materials used in the model system were not washed with EDTA solution in order to see the effect of trace mineral elements on retinol recovery.

constant and half life of retinol. The smaller catalytic effect of zinc and calcium may be due to their lower solubility characteristics. The half life of retinol dropped to approximately $\frac{1}{3}$ and $\frac{1}{4}$ of the unfortified value due to iron and copper supplementation, respectively. This effect can be attributed to a greater ionic mobility relative to other mineral cations. The results were

TABLE 3
Rate Constant (k) and Half Lives ($t_{1/2}$) for Retinol at a_w 0·42, Temp., 30°C and Mineral Fortification

Temp./a_w/Mineral (°C)	k	$t^{1/2}$
30/0·42/Control	0·50	138
30/0·42/$FeSO_4$	1·39	49
30/0·42/$CuSO_4$	2·03	34
30/0·42/ZnO	0·73	94
30/0·42/$CaCO_3$	0·78	89

Note: k, first order rate constant $\times 10^{-2}\,h^{-1}$.
$t_{1/2}$ half life, h.

somewhat similar to the findings of Dennison (1978) and Kirk (1981). They studied the effect of mineral supplementation on ascorbic acid content and retinyl acetate in a dehydrated model food system stored in enamelled metal cans at different a_w and temperatures. They noted that the level of mineral fortification had no significant effect on the degradation half-lives of ascorbic acid and retinyl acetate when 10% or 25% mineral supplement was added to the model system.

The plot of $\ln(a/a-x)$ against storage time in Fig. 2 shows that first-order character was observed in all the model systems. A first-order reaction was found by Kirk (1981) for retinyl acetate in a dry model food system composed of 10% soya protein, 1% fat, 76·6% powdered starch, 5·1%

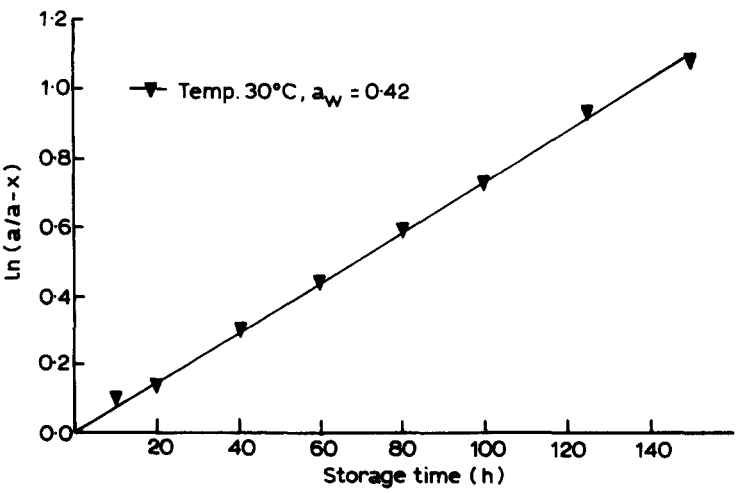

Fig. 2.

reducing sugar, 5·1% sucrose and 2% salt, stored in enamelled metal cans at 30, 37 and 45°C after equilibrating to a_w of 0·24, 0·40 and 0·65. First-order kinetics have been reported for β-carotene adsorbed on solid supports or model systems by Ramakrishnan and Francis (1979*a*) and Chou and Breene (1972). However, Chen and Gutmanis (1968) found second order kinetics for ground chilli pepper extracts. Carstensen (1964) found a pseudo first-order character for vitamin A palmitate oil in water:micelle systems and also for vitamin A acetate and palmitate beadlets in sugar coated tablets.

The differences in the rate order may be attributed to differences in the nature of vitamin A compounds in the MS, the degradation mechanism and the composition of MS as well as reaction mechanisms and reaction rates which are frequently sensitive to metallic ions (Kirk, 1981) and to acids or bases or to electrolytes that may be present (Bunnet, 1974).

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