Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 921–931
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Model lipophilic formulations of retinyl palmitate: influence of conservative agents on light-induced degradation

M. Scalzo [a,*], E. Santucci [a], F. Cerreto [a], M. Carafa [b]

[a] *Dip. Studi Chim. Tecnol. Sost. Biol. Attive, University of Rome "La Sapienza", P.le A. Moro 5, Rome 00185, Italy*
[b] *Dip. Scienze del Farmaco, University "G. D'Annunzio", Chieti, Italy*

Received 29 July 2003; received in revised form 13 November 2003; accepted 13 November 2003

**Abstract**

Vitamin A is widely employed in pharmaceuticals and cosmetics. The all-trans (AT) isomer (100% of biological potency) is sensible to different factors, such as light, heat and formulation components, leading to its degradation or isomerization. The main objective of this work was to study, in model cosmetic lipophilic vehicles, the degradation of retinyl palmitate (RetP) to the less active cis-isomers in presence of widely used conservative agents (propyl gallate and Vitamin E). Two lipophilic phases were used (liquid paraffin and almond oil) because liquid paraffin, almost composed of satured hydrocarbons, is not degraded by light exposure, while almond oil, containing several double bonds, could interfere with light-induced degradative process of RetP. In the first phase, the more suitable analytical method was chosen between normal and reverse phase HPLC to follow the degradation of RetP. In the second phase, RetP light-induced degradation was studied to simulate storage condition effect on cosmetic products ageing. The results showed that: (a) the reverse phase HPLC technique, unable to separate the all-trans from the 13-cis and 9-cis isomers, derived by Vitamin A isomerization, leads to an incorrect quantitation of RetP; (b) the lipophilic vehicle influences the isomerization–degradation process; (c) the conservative agents do not protect from degradation.


*Keywords:* Retinyl palmitate; Degradation; Isomerization; Lipophilic vehicles; Conservative agents; Normal phase HPLC

## 1. Introduction

The sensitivity of Vitamin A compounds in foods, pharmaceutical formulations and cosmetics to moisture, oxygen, acids, metals and light has been reviewed in [1]. These agents affect chemical stability of the isoprenic and β-ionic parts of vitamin molecule, leading to degradation and isomerization of all-trans (AT) retinol. On the other hand, Vitamin A esters are rapidly inactivated in aqueous solution during exposure to daylight [2,3].

The great interest in evaluating the degradation–isomerization kinetics [4–8] of Vitamin A and its esters is due to the loss of potency occurring after degradative processes.

The biopotency of the isomers 9-cis and 13-cis, the principal products of isomerization of all-trans retinol, are reported to be 24 and 75% of the all-trans value, respectively [9,10].

* Corresponding author. Tel.: +39-06-499-13669; fax: +39-06-499-13133.
*E-mail address:* marcello.scalzo@uniroma1.it (M. Scalzo).

0731-7085/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00653-8

In the past, most studies on the degradation rate of retinol and retinyl esters have been monitored by UV absorbance modification at wavelengths at which also degradative products absorb [11,12].

Although UV spectrophotometry is the official method for RetP pharmaceutical formulations [13,14], HPLC methods are actually used for better resolution of retinol and retinyl esters from degradative compounds (reverse phase HPLC) and to differentiate among geometric isomers (normal phase HPLC) in food and cosmetic analyses [15].

Nevertheless, more complex procedure related to sample preparation for normal phase HPLC analyses lead to a wide use of reverse phase HPLC to study degradation of Vitamin A and its derivatives in pharmaceutical, biological and cosmetic field [16–24].

The main objective of this work was to study the degradation of retinyl palmitate (RetP, Fig. 1) to the less active cis-isomers, in model cosmetic lipophilic vehicles.

Two lipophilic phases were used: liquid paraffin and almond oil, both widely used in the cosmetic field, in presence of conservative agents (propyl gallate and Vitamin E).

Liquid paraffin, almost composed of satured hydrocarbons, is not degraded by light exposure, while almond oil, containing several double bonds, could interfere with light-induced degradative process of Vitamin A palmitate.

In the first phase, to follow the degradation of RetP, the more suitable analytical method was chosen (between normal and reverse phase HPLC). In the second phase, RetP light-induced degradation was studied at different Vitamin A ester content, to simulate storage condition effect on cosmetic products ageing.

Irradiation experiments were performed using a fluorescent lamp, as reported by several authors to investigate the stability of Vitamin A in foods [25–29]. Since the light emitted by fluorescent sources contains a small intensity of UV radiation (Technical Report from OSRAM), a filament lamp, lacking in UV emissions, was used to perform alternative experiments on vitamin light-induced isomerization–degradation process.

## 2. Materials and methods

### 2.1. Materials

Analytical quality retinyl palmitate (RetP), propyl gallate and Vitamin E were Fluka Chemie AG products.

Pharmacopeia quality almond oil and liquid paraffin were Agrar products.

*n*-Hexane, methanol and acetonitrile were all HPLC grade as supplied by Riedel-deHaën GMBH.

For irradiation experiments a fluorescent lamp (Circolux EL 32 W Osram) and a filament lamp (60 W Osram) were used.

### 2.2. Sample preparation and irradiation experiments

Samples at different RetP concentrations, according to del Pozo and Alemany [30], were prepared (Table 1). Samples prepared in hexane were used as references, because this solvent is unable to assist the free radical formation needed for the vitamin photoisomerization [26].

Samples at same RetP content were then prepared in liquid paraffin and almond oil, respectively, in presence and in absence of 0.1% w/v of conservative agents (Table 1).

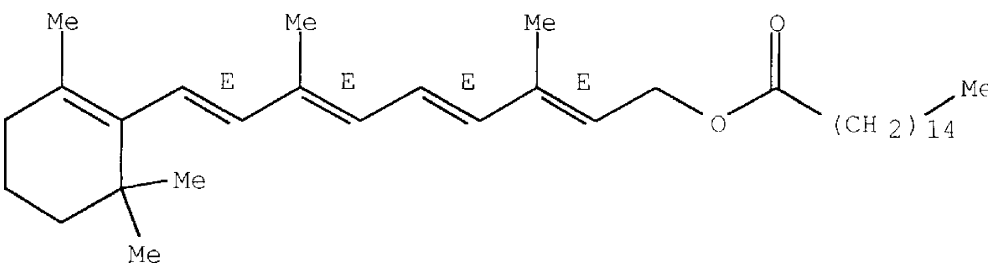



Fig. 1. Retinyl palmitate.

Table 1
Sample compositions (mg/ml)

| Sample | RetP | Hexane | Liquid paraffin | Almond oil | Almond oil (peroxidation) | Propyl gallate | Vitamin E |
|---|---|---|---|---|---|---|---|
| 1 | 6 | * | | | | | |
| 2 | 4.5 | | | | | | |
| 3 | 3 | * | | | | | |
| 4 | 1.5 | * | | | | | |
| 1LP | 6 | | * | | | | |
| 2LP | 4.5 | | * | | | | |
| 3LP | 3 | | * | | | | |
| 4LP | 1.5 | | * | | | | |
| 1AO | 6 | | | * | | | |
| 2AO | 4.5 | | | * | | | |
| 3AO | 3 | | | * | | | |
| 4AO | 1.5 | | | * | | | |
| 1LPG | 6 | | * | | | * | |
| 2LPG | 4.5 | | * | | | * | |
| 3LPG | 3 | | * | | | * | |
| 4LPG | 1.5 | | * | | | * | |
| 1AOG | 6 | | | * | | * | |
| 2AOG | 4.5 | | | * | | * | |
| 3AOG | 3 | | | * | | * | |
| 4AOG | 1.5 | | | * | | * | |
| 1AOP | 6 | | | | * | | |
| 2AOP | 4.5 | | | | * | | |
| 3AOP | 3 | | | | * | | |
| 4AOP | 1.5 | | | | * | | |
| 1AOE | 6 | | | * | | | * |
| 2AOE | 4.5 | | | * | | | * |
| 3AOE | 3 | | | * | | | * |
| 4AOE | 1.5 | | | * | | | * |

Samples (50 ml volumetric flask) were exposed continuously, in a darkened room, to the light of a fluorescent lamp; duplicate samples were exposed, in the same conditions, to the light of a filament lamp. The light intensity was set at 1200 lx for each sample (measured with Light Meter LX-103 by LUTRON) by adjusting the distance between the sample and the light source.

### 2.3. HPLC determination of retinyl palmitate content

The residual concentration of Vitamin A palmitate in each sample was determined weekly by normal and reverse phase HPLC. The normal phase analyses were carried-out using normal silica phase [15,27–29,31], and replied on silica gel derivatized with an aminopropyl function, according to the 24 US Pharmacopeia.

The reverse phase HPLC was carried out on an octyl-bonded silica [21,23].

The analytical methods were developed and then validated, using the equipment, mobile phases, chromatographic conditions and protocols described below.

#### 2.3.1. Equipment

The HPLC system consisted of Model 600 solvent delivery system, of a Photodiode Array Detector 991 (DAD) from Water Associates (Milford, MA) and a model 7125 syringe loading sample injector from Rheodyne (California, USA) equipped with a 50 μl loop.

#### 2.3.2. Chromatographic conditions

- Normal phase
  - stationary phase: 250 mm × 4.6 mm LiChrosorb™ Si 60 (5 μm);
  - temperature: ambient;

- mobile phase: *n*-hexane/ethyl ether 99/1;
- isocratic flow: 1 ml/min;
- detector: 325 nm;
- volume injected: 50 μl.

- Normal phase
  - stationary phase: 250 mm × 4.6 mm LiChrosorb$^{TM}$ –$NH_2$ (10 μm);
  - temperature: ambient;
  - mobile phase: *n*-hexane;
  - isocratic flow: 1 ml/min;
  - detector: 325 nm;
  - volume injected: 50 μl.
- Reverse phase
  - stationary phase: 250 mm × 4 mm LiChrosorb$^{TM}$ RP 8 (7 μm);
  - temperature: ambient;
  - mobile phase: acetonitrile;
  - isocratic flow: 1 ml/min;
  - detector: 325 nm;
  - volume injected: 50 μl.

### 2.3.3. Experimental protocol

Weekly, 1 ml of each vitamin sample was drawn from the volumetric flask and opportunely diluted with *n*-hexane (normal phase analysis) or methanol (reverse phase analysis) to obtain area values ranging from 0.5 to 0.1 AU min. Sample analyses were performed in triplicate. Typical chromatograms are shown in Fig. 2. In normal phase HPLC (A, –$SiO_2$) three different peaks were obtained (retention time: all-trans = 11 min; 13-cis = 8 min; 9-cis = 9.2 min); in reverse phase (B) the RetP unresolved peak was eluted at 7 min.

Before exposure to light, for samples analyzed by normal phase HPLC, only two peaks were observed and easily referred to all-trans (Fig. 3a, peak a) and to 13-cis (Fig. 3a, peak b) isomers, according to literature data [29]. The 13-cis isomer (5–10%) is always present in commercially available RetP. After exposure to light, a decrease of peaks (a) and (b) and an increase of peak (c) was observed (Fig. 3b). The UV spectra (maximum 322 nm) and NMR spectra (Bruker 200 MHz) of collected fractions of peak (c), in agreement with the data reported from Landers and Olson [26], confirmed that peak (c) can be referred to the 9-cis isomer. The normal phase separation, carried-out using a "–$NH_2$" stationary phase, gave similar results (Fig. 4). On the other hand, in reverse phase analyses, we observed only one significative peak (Fig. 2B, Ua), that was related to unresolved RetP isomers mixture (AT, 9-cis, 13-cis). Analyzing, by means of reverse phase HPLC, the three different isomer fractions,

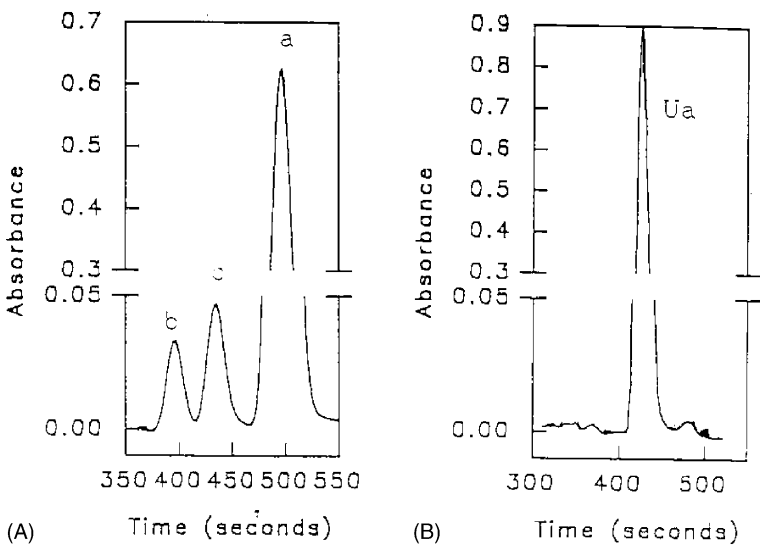



Fig. 2. Typical chromatograms of RetP samples, in hexane, analyzed by means of "–$SiO_2$" normal phase HPLC (A) and "–$C_8$"reverse phase HPLC (B). (a) AT; (b) 13-cis; (c) 9-cis; Ua, unresolved peak.

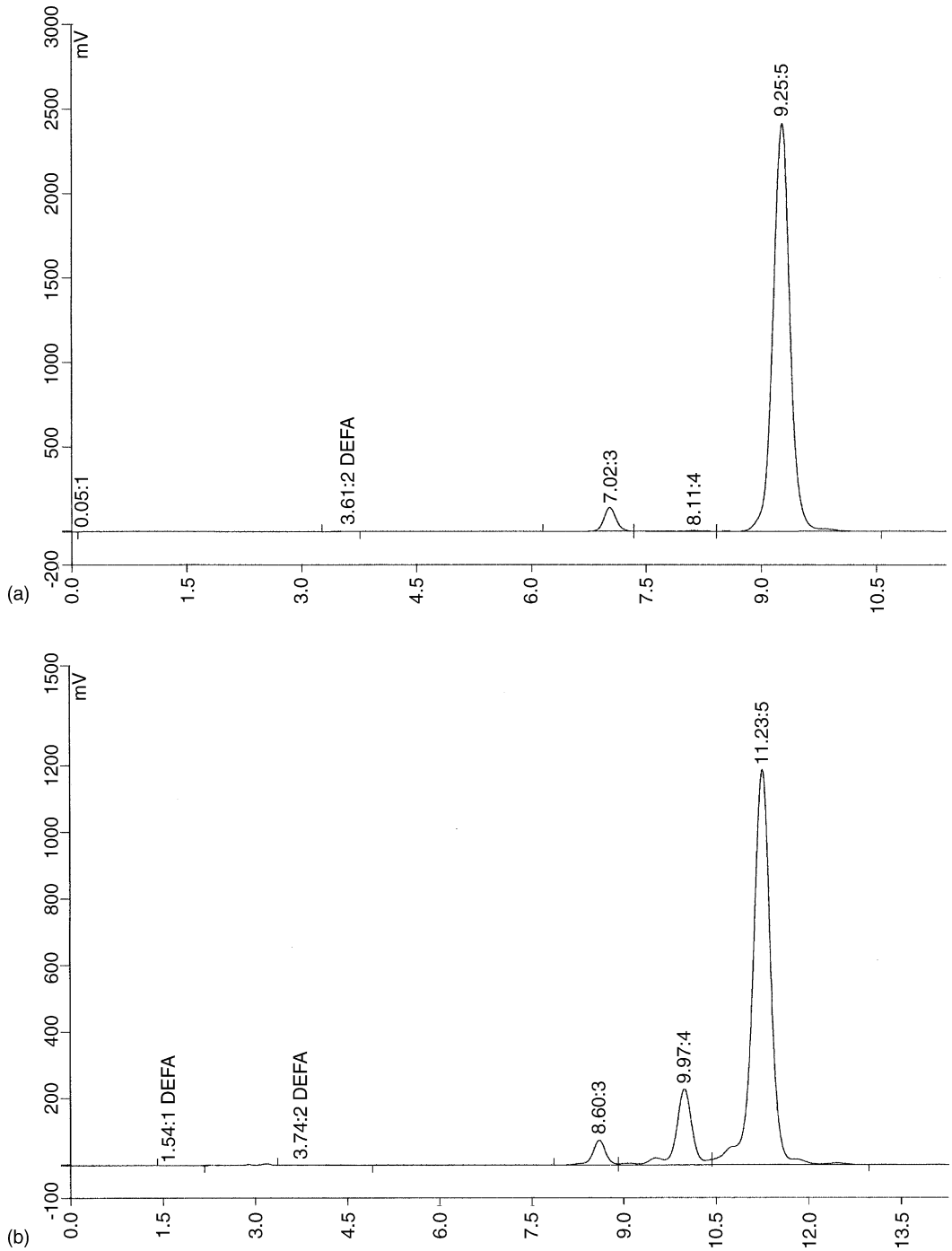



Fig. 3. Typical chromatograms of RetP samples, in hexane, analyzed by means of "–$SiO_2$" normal phase HPLC at time = 0 (a) and after exposure to light (b).

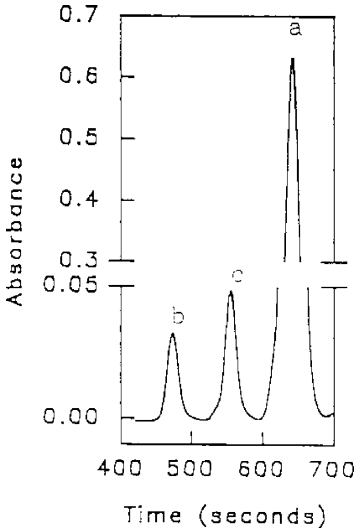



Fig. 4. Typical chromatograms of a RetP sample, in hexane, analyzed by means of "–$NH_2$" normal phase HPLC.

collected by normal phase HPLC, we obtain, for each sample, similar retention time.

*2.3.4. Validation of analytical method*

In the validation of the analytical method the following points were determined: linearity and replicability.

*2.3.4.1. Linearity assay.* The linearity of an analytical method is the ability to obtain linear results proportionally to the concentration of analyte in the sample, in a determined time. Seven hexane solutions of Vitamin A palmitate ranging from 0.1115 to 1.115 mg/ml were prepared and analyzed, in triplicate, by means of the three different analytical method. The least-squares fitting technique applied on the peak area values gave satisfactory results.

*2.3.4.2. Replicability assay.* The replicability is defined as the measure of accuracy of a method applied

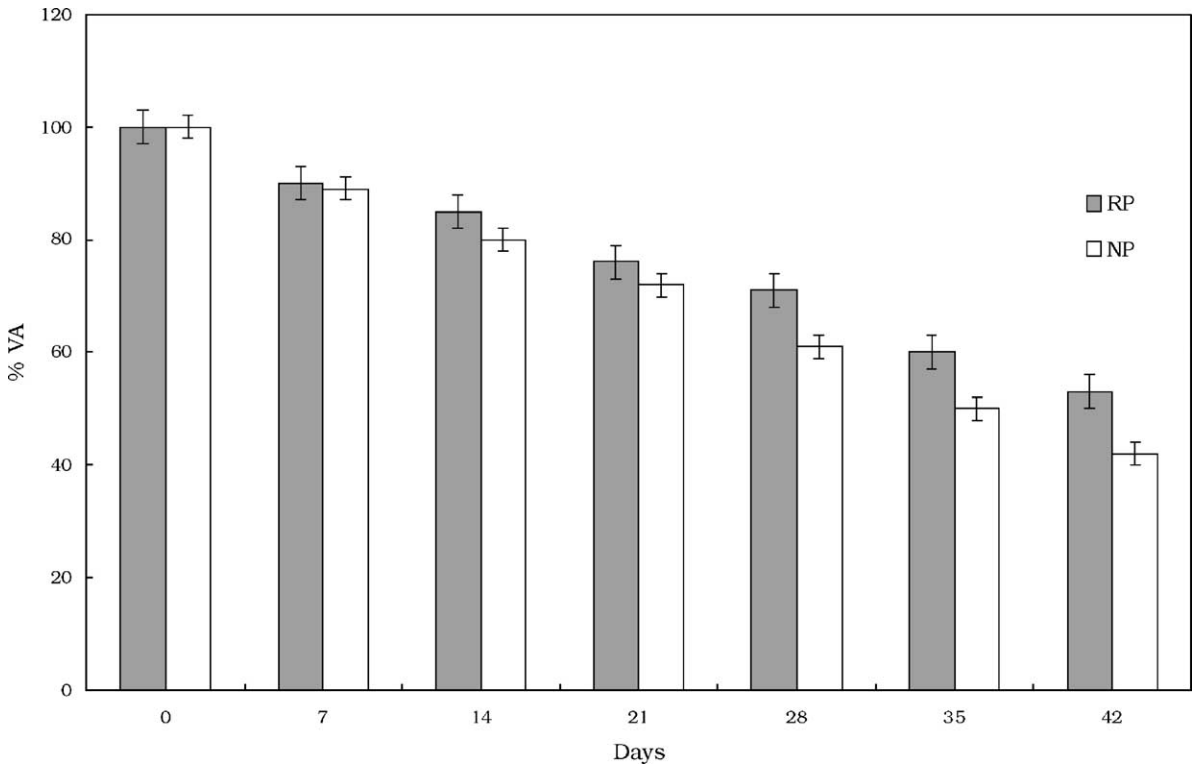



Fig. 5. Comparison of vitaminic activity evaluated using the two different HPLC techniques, in function of time.

to the same sample under the same conditions and after a short period of time.

The replicability of the analytical methods was determined from the values obtained in the linearity assay. It was calculated using the following equation:

$$R\,(\%) = \frac{\text{S.D.}}{C} \times 100 \quad (1)$$

where S.D. is the standard deviation of the sample at each concentration and $C$ the mean experimental concentration. In the analyses of collected samples a variation coefficient lower than 3% is considered acceptable.

*2.3.5. Statistical treatment*

Statistical analyses were used to compare the influence of experimental parameters on kinetics and rates of RetP degradation processes. The *t*-difference test, applied to degradation curve slopes, was used, comparing curves two by two.

## 3. Results and discussion

### *3.1. Choice of analytical method*

Comparing different HPLC methods, the normal phase HPLC appears the most useful in evaluating the influence of light exposure on model cosmetic formulations, offering the possibility of separating the main RetP isomers with different vitaminic activity.

On the other hand, the normal phase "–$NH_2$" shows always higher area values for AT isomer, compared to "Si-60", this probably related to the presence of unknown substances, strongly retained by silica phase, coeluted with AT in "–$NH_2$" phase . In fact, using a

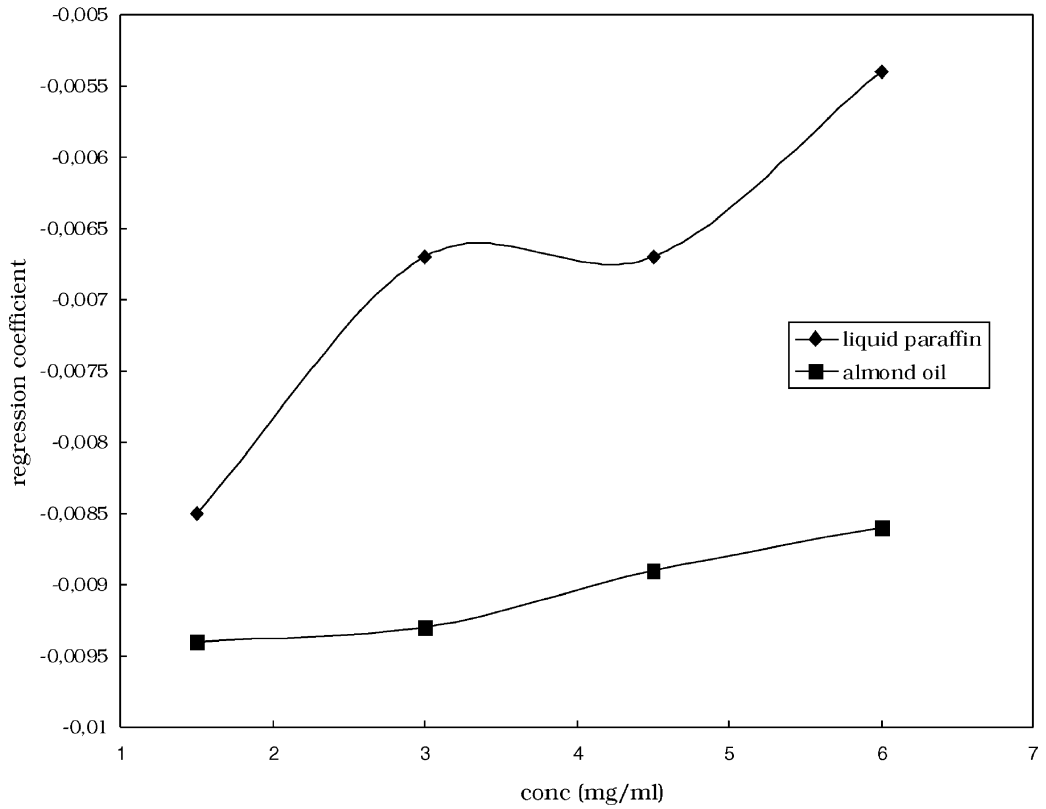



Fig. 6. Influence of initial RetP content on vitamin degradation rate, expressed as regression coefficient of degradation curves, in the dark.

"Si-60" precolumn in "$NH_2$" analyses, the differences in area values are negligible.

Manan et al. [32] reported that the assay method affects the kinetic interpretation of the degradation of retinol.

In agreement with this article, we obtained, using normal phase HPLC, degradative processes described by first-order kinetics.

Moreover, we found that the use of reverse phase HPLC leads to the possibility of incorrect evaluation of vitaminic potency. The vitaminic activity, for samples analyzed by normal phase HPLC, was evaluated as follows:

$$\text{VA}\,(\%) = [\text{AT}] \times 100\%\text{VP} + [\text{13-cis}] \times 75\%\text{VP} + [\text{9-cis}] \times 24\%\text{VP} \quad (2)$$

where VA, vitaminic activity and VP, vitaminic potency.

In reverse phase HPLC the vitaminic activity, related to the unresolved peak, was always higher than that obtained from Eq. (2) and the difference increases with time (Fig. 5).

### 3.2. Influence of lamp emission on degradation–isomerization pathway

Samples were exposed continuously, in a darkened room, to the light of a fluorescent lamp; duplicate samples were exposed, in the same conditions (1200 lx), to the light of a filament lamp.

This to compare the influence of residual UV radiations, present in light emission spectrum of fluorescent lamp (Osram Circolux 32 W). In this case the UV radiations emitted by the fluorescent lamp speeds up the AT degradation and the 9-cis formation, increasing the 9-cis/AT ratio and strongly shortening the time needed to observe a significant difference

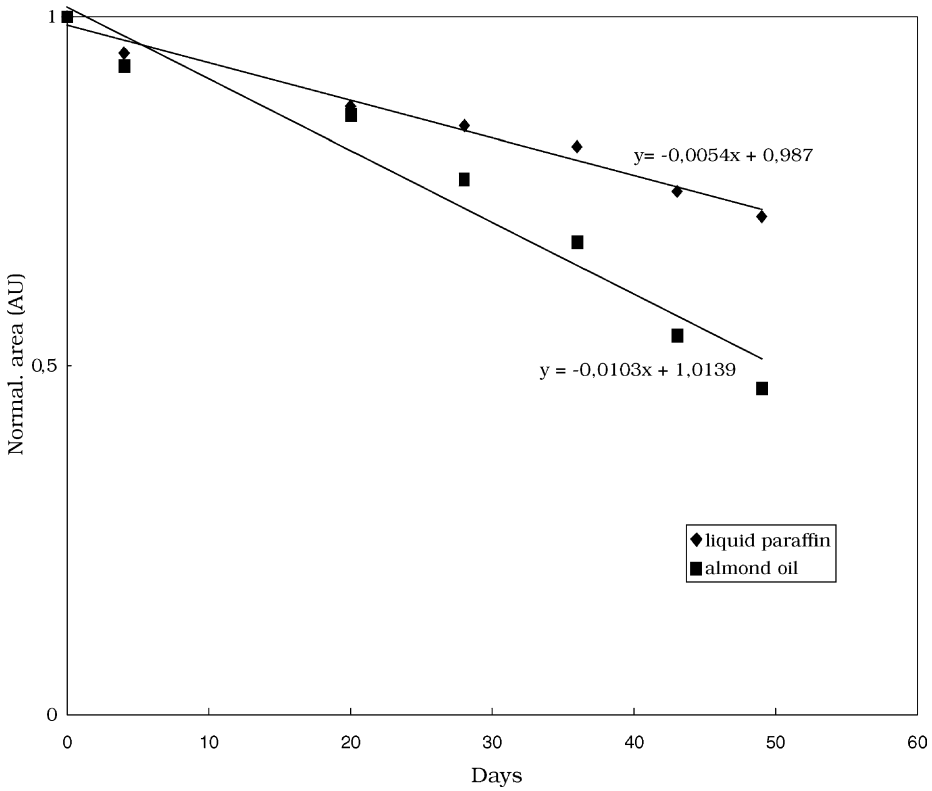



Fig. 7. Influence of lipophilic vehicles on vitamin degradation rate.

between the normal and the reverse phase analysis data.

### 3.3. Influence of initial RetP content on degradation

Comparing the regression coefficients of four samples, at different initial RetP concentration, in the same model vehicle, it can be evidenced that only in liquid paraffin, vitamin degradation rate is influenced by vitamin content (Fig. 6).

It can be assumed that liquid paraffin, almost composed of satured hydrocarbons, not degraded by light exposure, and so not involved in RetP degradative pathway, does not mask the slight effect of different vitamin concentrations.

On the other hand, almond oil, containing several double bonds, could interfere with light-induced degradative process of Vitamin A palmitate and "cover" the initial RetP content effect.

### 3.4. Influence of model lipophilic vehicle

Comparing the degradation curve of samples at the same concentration in different lipophilic vehicles it can be assumed, as predictable, that the presence of double bonds (almond oil) facilitates the degradation of retinyl palmitate (Fig. 7).

On the other hand, the vehicle "age", expressed as peroxidation number of almond oil (PN = 76), strongly affects the degradative processes (rate and kinetic), as shown in Fig. 8.

The addition of conservative agents, as propyl gallate and Vitamin E, does not modify the degradation kinetics in both model vehicles (Fig. 9a and b).

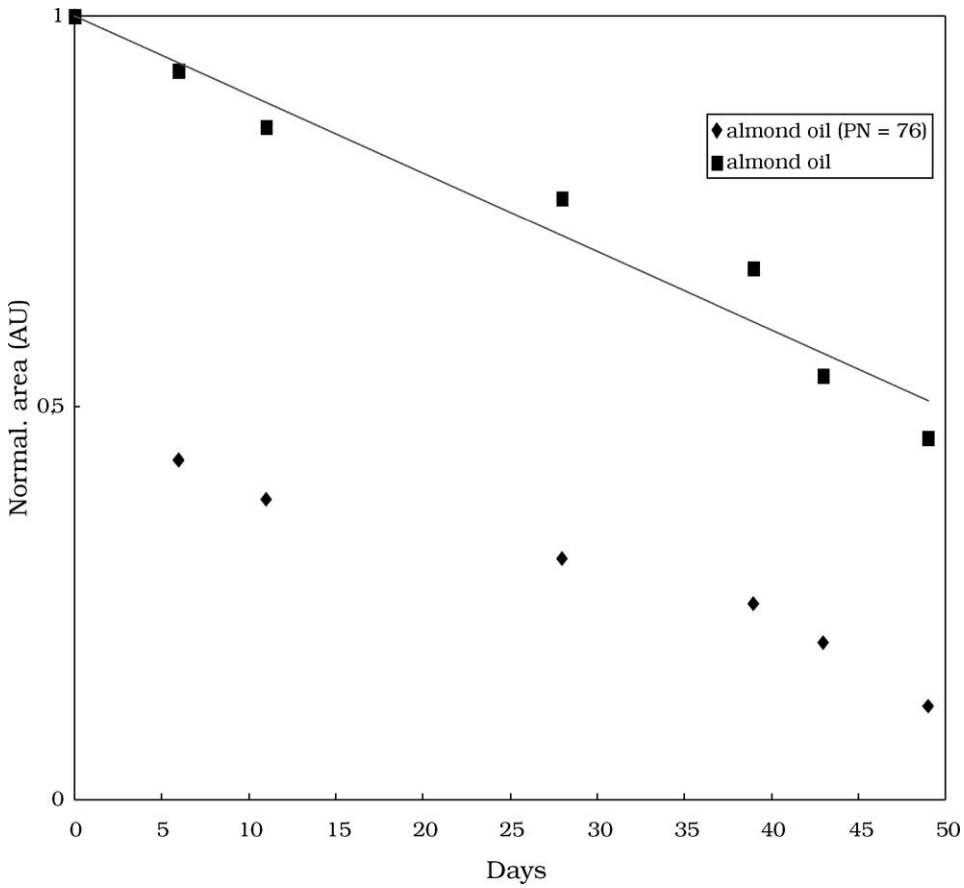
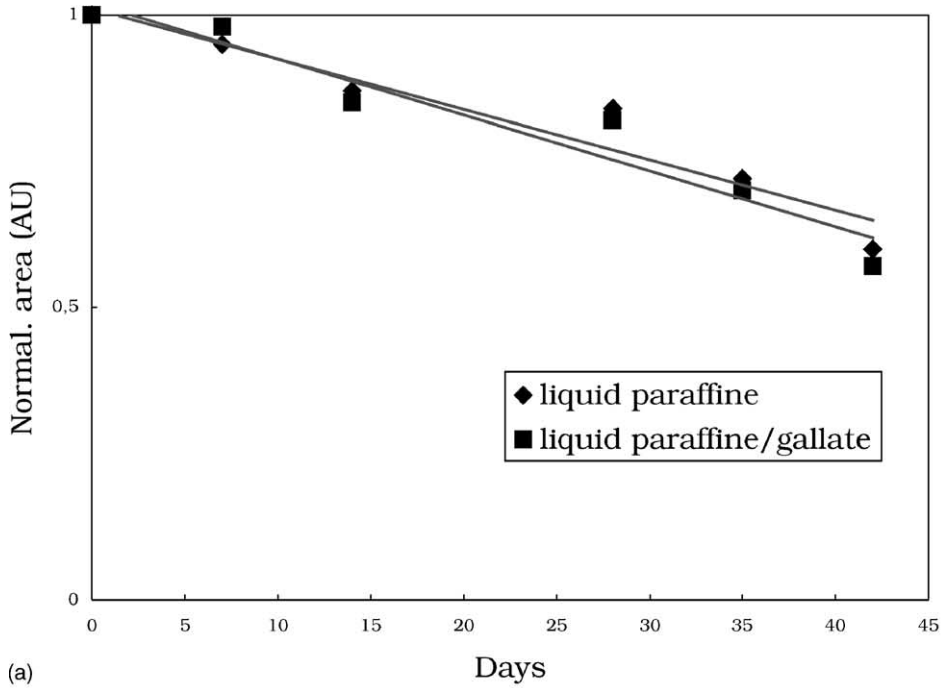



Fig. 8. Influence of age of lipophilic vehicle (expressed as peroxidation number of almond oil) on vitamin degradation rate.



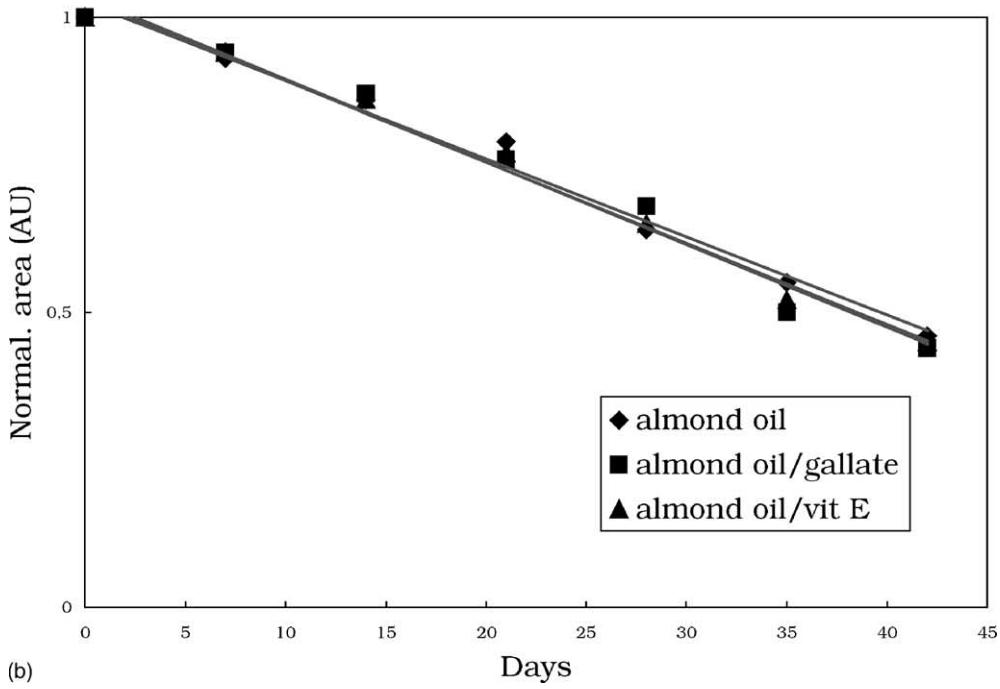




Fig. 9. Influence of the addition of conservative agents to lipophilic vehicles (a) liquid paraffin and (b) almond oil, on vitamin degradation rate.

## 4. Conclusions

The collected results showed that the reverse phase HPLC, unable to separate the all-trans from the 13-cis and 9-cis isomers, derived by Vitamin A isomerization, leads to overstimating the true retinyl palmitate content, in stability studies. From the reported data it can also be deduced that the lipophilic vehicle influences the isomerization–degradation process and that the chosen conservative agents do not protect, in the reported experimental conditions, from isomerization.

Thus, a determining factor in the preformulation of liquid mixture of retinyl palmitate with lipophilic vehicles is the necessary control of the system stability against light-induced isomerization, in addition to tests of oxidative stability.

## References

[1] E. De Ritter, J. Pharm. Sci. 71 (1982) 1073–1096.
[2] M.C. Allwood, J.H. Plane, Int. J. Pharm. 19 (1984) 207–213.
[3] G. Panquette, M.A. Kanaan, Food Chem. 18 (1985) 211–231.
[4] E.R. Garret, J. Pharm. Sci. 51 (1962) 811–833.
[5] J.T. Cartensen, J. Pharm. Sci. 53 (1964) 839–840.
[6] A.H. El-Tinay, C.O. Chichester, J. Org. Chem. 35 (1970) 2290–2293.
[7] A.K. Baloch, K.A. Buckle, R.A. Edwards, J. Food Technol. 12 (1977) 309–316.
[8] F. Manan, A. Baines, J. Stone, J. Ryley, Food Chem. 52 (1995) 267–273.
[9] H. Weiser, G. Somorjai, Int. J. Vitamin Nutr. Res. 62 (1992) 201–208.
[10] S.R. Ames, J. Assoc. Off. Anal. Chem. 49 (1966) 1071–1078.
[11] T. Anmo, M. Washitake, Y. Takashima, M. Isohata, K. Koike, Vitamins 46 (1972) 193–203.
[12] M. Goldman, B. Horev, I. Saguy, J. Food 48 (1983) 751–754.
[13] US Pharmacopeia, vol. 24, 2000, pp. 1890–1891.
[14] E. Pharmacopeia, Fourth ed., 2002, pp. 2122–2123.
[15] G.M. Landers, Methods Enzymol. 189 (1990) 70–79.
[16] L.R. Chaudhary, E.C. Nelson, J. Chromatogr. 294 (1984) 465–470.
[17] M.I. Santoro, J. Magalhaes, E. Hackmann, J. Assoc. Off. Anal. Chem. 65 (1988) 619–622.
[18] K.E. Savolainen, K.M. Pynnonen, S.P. Lapinjoki, M.T. Vidgren, J. Pharm. Sci. 77 (1988) 802–803.
[19] F. Billion-Rey, M. Guillaumont, A. Frederich, G. Aulagner, J. Pharm. Clin. 10 (1991) 99–105.
[20] T. Van Vliet, F. Van Schaik, J. Van Schoonhoven, J. Schrijver, J. Chromatogr. 553 (1991) 179–186.
[21] A. Semenzato, M. Secchieri, A. Bau, A. Nicolato, A. Bettero, Farmaco 47 (1992) 1407–1417.
[22] S. Scalia, A. Renda, G. Ruberto, F. Bonina, E. Menegatti, J. Pharm. Biomed. Anal. 3 (1995) 273–277.
[23] S. Scalia, G. Ruberto, F. Bonina, J. Pharm. Sci. 84 (1995) 433–436.
[24] L. Halbaut, C. Barb, M. Aroztegui, C. de la Torre, Int. J. Pharm. 147 (1997) 31–40.
[25] A.M. Gaylord, J.J. Warthesen, D.E. Smith, J. Food Sci. 51 (1986) 1456–1458.
[26] G.M. Landers, J.A. Olson, J. Assoc. Anal. Chem. 69 (1986) 50–55.
[27] S.J. Schwartz, J. Agric. Food Chem. 35 (1987) 748–751.
[28] M. Zahar, D.E. Smith, J. Dairy Sci. 70 (1987) 13–19.
[29] P.A. Murphy, R. Engelhardt, S. Smith, J. Food Chem 36 (1988) 592–595.
[30] A. del Pozo, P. Alemany, Pharm. Acta Helv. 38 (1963) 508–515.
[31] J.N. Thompson, G. Hatina, W.B. Maxwell, J. Assoc. Off. Anal. Chem. 63 (1980) 894.
[32] F. Manan, L.V.J. Guevara, J. Ryley, J. Micronutr. Anal. 7 (1991) 349–355.