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The kinetics of the loss of *all-trans* retinol at low and intermediate water activity in air in the dark

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All-trans retinol was suspended on a support of microcrystalline cellulose and stored in air in the dark after equilibration at water activities from 0·11 to 0·75. The loss of *all-trans* retinol was monitored by normal phase HPLC. The rate of loss could be satisfactorily described by first-order kinetics with respect to *all-trans* retinol (up to more than 50% loss). The rate increased with increasing water activity in the range 0·11–0·75 a_w. Arrhenius activation energies were found to be in the range of 28–48 × 10^3 J mol^{-1}. A correlation between enthalpy change and Gibbs free energy change indicated a chemical compensation effect in the range 0·11–0·66 water activity.

INTRODUCTION

The sensitivity of vitamin A compounds in foods and pharmaceutical products to moisture, oxygen, acid, metals and light has been reviewed by De Ritter (1982). Loss of vitamin A potency due to isomerisation has been recognised for many years from the discrepancy between Carr–Price values (which are not influenced by isomerisation) and bio-assay values (De Ritter, 1961). The biopotency of *13-cis* and *9-cis* forms are reported to be 75% and 24% of the *all-trans* value (Ames, 1966). Most studies of the degradation rate of retinyl acetate, retinol and carotenes have been monitored by the change in absorbance at wavelengths at which degradation products also absorb (Anmo *et al.*, 1972; Baloch *et al.*, 1977; Arya *et al.*, 1979; Goldman *et al.*, 1983; Haralampu & Karel, 1983; Paquette & Kanaan, 1985). However, developments in HPLC have enabled both better resolution of vitamin A compounds from degradation products (Takashima *et al.*, 1979) and differentiation between geometric isomers (Paanakker & Groenendjik, 1979; Stancher & Zonta, 1982) to be achieved. Assays using normal and reversed phase columns have been applied to foods in recent years to study the isomer composition (Egberg *et al.*, 1977; Woollard & Indyk, 1986). Activation energies for β-carotene loss have been reported in toluene (El-Tinay & Chichester, 1970), and on microcrystalline cellulose (Chou & Breene, 1972; Baloch *et al.*, 1977). Activation energies for the degradation of retinol have not been reported.

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✠ It is with great regret that the co-authors report that Dr Alan Baines died before this paper was finalised.
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In this study, the loss of *all-trans* retinol has been monitored in the range of temperature and water activity relevant to the storage of dehydrated and intermediate moisture content foods. Although the relationship between β-carotene stability and water activity has been studied by many workers in relation to loss of colour because of the importance of colour to the sensory quality of food, comparable studies have not been carried out for vitamin A compounds.

Model systems simulating dehydrated foods in which the reactant has been supported on an adsorbent have previously been used by many workers. Labuza *et al.* (1966) and Maloney *et al.* (1966) selected microcrystalline cellulose after reviewing numerous solid supports used by others. Arya *et al.* (1979), Baloch *et al.* (1977), Chou and Breene (1972), Kanner *et al.* (1978), Ramakrishnan and Francis (1979), Teixeira-Neto *et al.* (1981), Goldman *et al.* (1983) and Premavalli and Arya (1985) used a microcrystalline cellulose support for studying the degradation of β-carotene or pigments extracted from foods.

MATERIALS AND METHODS

The materials and methods have been previously described by Manan *et al.* (1991). All manipulations involving retinol were carried out in subdued light in glassware protected by aluminium foil.

Preparation of microcrystalline cellulose/retinol system

Microcrystalline cellulose (Honeywill and Stein, UK) was exhaustively washed with 0·05 M ethylene diamine tetra-acetic acid to remove metals, washed with distilled

water, dried at 105°C and passed through a plastic 125 μm aperture sieve. An ethanolic solution of *all-trans* retinol (Fluka) was added to give a final level of 5×10^{-4} mol g^{-1} of cellulose and the ethanol removed on a rotary evaporator at 40°C under vacuum. The powder was transferred to a purpose-made all-plastic homogeniser (including the blade) and mixed under nitrogen.

Storage studies

The samples were equilibrated to the desired water activity by storage in desiccators containing dishes of the appropriate saturated salt solutions under nitrogen in the dark for 48 h (Rockland, 1960). At the end of the equilibration period the atmosphere in the desiccators was replaced by air bubbled through a saturated solution of the same salt. Temperature control was achieved by placing the desiccators containing the samples suspended over salt solutions in water baths controlled to ±0·1°C throughout the equilibration and storage periods. Samples were removed at intervals for retinol assay until the loss of *all-trans* retinol had exceeded 50% of the initial concentration.

Extraction and assay

A 1–2 g sample was shaken with 20 ml of methanol/ethanol/acetone (6:3:1) containing BHT (20 mg/100 ml). The slurry was filtered and washed through a sintered glass filter and the solvent removed by rotary evaporation. The residue was dissolved in HPLC grade methanol containing BHT (20 mg/100 ml) and made up to volume to give a concentration in the range 2–20 μg ml^{-1}. Aliquots (20 μl) were injected onto a Lichrosorb Si-60 column (250 × 4 mm) and eluted at 45°C with hexane-propan-2-ol (99·6:0·4 v/v) at a flow rate of 1·5 ml min^{-1} and monitored at 326 nm. Peak heights were compared with standard *all-trans* retinol. *13-cis* retinol was identified by comparison of retention time with that of a known sample (Sigma Chemical Co Ltd) and by spiking the extract.

Data processing

The concentration of residual *all-trans* retinol was calculated by comparison of peak heights of the unknowns with standards. For each water activity level, first-order rate constants were determined from the relationship

$$C_{ij} = C_{oj} e^{-k_j t_{ij}}$$

where t_{ij} is the ith time point at temperature T_j, C_{ij} is the concentration at time t_{ij}, k_j is the rate constant at the given temperature and c_{oj} is the initial concentration, by transformation to

$$\ln (c_{ij} / c_{oj}) = -k_j t_{ij} \quad (1)$$

and determining the slope of the linear regression of $\ln c_{ij}$ on t_{ij}.

The dependence of k_j on temperature is modelled by the Arrhenius equation:

$$k_j = k_o e^{-E/RT_j} \quad (2)$$

where k_o is a constant (the frequency factor) for the given water activity level, E is the Arrhenius activation energy and R is the gas constant.

After transformation, eqns (1) and (2) constitute the usual two-step method for estimating values of E. By combining eqns (1) and (2), the relationship of c to t and T can be expressed as:

$$\ln\ln(c_{oj}/c_{ij}) - \ln t_{ij} = \ln k_o - E/RT_j \qquad (c_{ij} \neq c_{oj}) \quad (3)$$

The fitting of such a model by regression analysis constitutes a one-step approach which, if valid, will provide better estimates and confidence limits for E at a given water activity level.

Regression analysis for the one-step model and validity of the model

In order to fit the one-step model equation (3), the best estimates of c_{oj} must be used. These are obtained by transforming eqn (1) to :

$$\ln c_{ij} = \ln c_{oj} - k_j t_{ij}$$

and regressing $\ln c_{ij}$ on t_{ij} for each temperature ($\ln \hat{c}_{oj}$ is the intercept of the fitted line at each temperature). Then

$$y_{ij} = \ln\ln(\hat{c}_{oj}/c_{ij}) - \ln t_{ij} = \ln k_o - E/RT_j \quad (4)$$

The validity of generating y_{ij} was tested for each temperature by fitting the regression of $\ln\ln \hat{c}_{oj}/c_{ij}$ on t_{ij} to the data, excluding the initial observation of c_{ij}, and performing a t-test (1% significance level) to show that the slope of the relationship was not significantly different from unity. If this test was satisfactory, the sum of squares $\sum_{i=1}^{n_j} (y_{ij} - \bar{y}_j)^2$ with $n_j - 2$ degrees of freedom was calculated, where n_j is the number of observations of concentration at temperature T_j.

A total residual sum of squares, SS_2, was then calculated as

$$SS_2 = \sum_{j=1}^{3} \sum_{i=1}^{n_j} (y_{ij} - \bar{y}_j)^2$$

with $\sum_{j=1}^{3} n_j - 6$ degrees of freedom.

The regression of y_{ij} on $1/T_j$ and its residual sum of squares, SS_1, were determined with $\sum_{j=1}^{3} n_j - 5$ degrees of freedom.

The sums of squares SS_1 and SS_2 and their associated degrees of freedom were used to construct the ANOVA table shown as Table 1.

The F ratio $[\sum_{j=1}^{3} n_j - 6] (SS_1 - SS_2)/SS_2$ is a test statistic for the null hypothesis that the fitted one-step model is a valid procedure for combining the data for different temperatures within one water activity level. If the F

Table 1. ANOVA table

Source	Degrees of freedom	Sums of squares	Mean square
Residuals about separate temperature model	$\sum_{j=1}^{3} n_j-6$	SS_2	$SS_2/\sum_{j=1}^{3} n_j-6$
Difference between residuals	1	(SS_1-SS_2)	(SS_1-SS_2)
Residuals about one-step model	$\sum_{j=1}^{3} n_j-5$	SS_1	

ratio is not significantly high then it is reasonable to use the fitted one-step model as a valid procedure for estimating the Arrhenius activation energy, i.e. the slope of the fitted regression estimates the quantity $(-E/R)$.

The estimates of E determined by the above one-step model were then used to calculate the frequency factor (k_o) at each water activity level and the rate constant at the harmonic mean temperature using eqn (2). The thermodynamic parameters ΔH, ΔG and ΔS at the harmonic mean temperature were calculated from the following thermodynamic relationships:

$$\Delta H = E-RT_j$$

$$\Delta G = RT_j \ln \frac{k_b T_j}{h\,k_j} \quad (k_b \text{ is Boltzmann constant})$$

$$\Delta S = \frac{\Delta H-\Delta G}{T_j} \quad (\text{using } T_j = \text{harmonic mean temperature})$$

RESULTS AND DISCUSSION

The loss of *all-trans* retinol could be adequately described by a pseudo first-order plot up to more than 50% loss under all the conditions studied. Figure 1 shows one data set. Figure 2 shows a plot of rate constant against water activity for the loss of *all-trans* retinol showing the expected increase in the rate above the BET monolayer after which capillary water increases the mobility of reactants.

An increased rate of reaction with water activity in the region of 0·4–0·75, as shown in Fig. 2, has also been reported for ascorbic acid in sweet potato (Haralampu & Karel, 1983). However, the degradation rate of β-carotene in sweet potato (Haralampu & Karel, 1983) and β-carotene on microcrystalline cellulose (Ramakrishnan & Francis, 1979) has been reported to be insensitive to water activity changes in this region. This difference in behaviour between retinol and β-carotene can possibly be attributed to the poorer solubility of β-carotene in water.

The values obtained for the first-order rate constants for the loss of *all-trans* retinol in the range 0·3–0·4 a_w (Fig. 2) are very similar to values reported by Chou

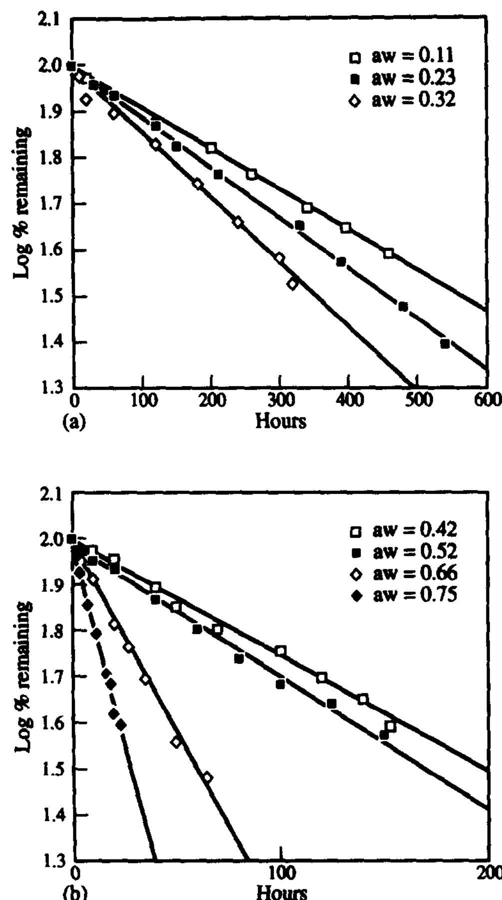

Fig. 1. First-order plots for the degradation of *all-trans* retinol at 30 °C.

and Breene (1972) and Baloch *et al.* (1977) for β-carotene on microcrystalline cellulose and to the rate of decolorisation reported for β-carotene in sweet potato powder (Haralampu & Karel, 1983). Since the spectrophotometric assay in these studies did not differentiate between initial reactant and degradation products which absorbed at the same wavelength, it is likely that the degradation rate of *all-trans* β-carotene, if monitored by a single species assay, would be greater than that of retinol stored under the same conditions. This is compatible with the greater reactivity conferred on

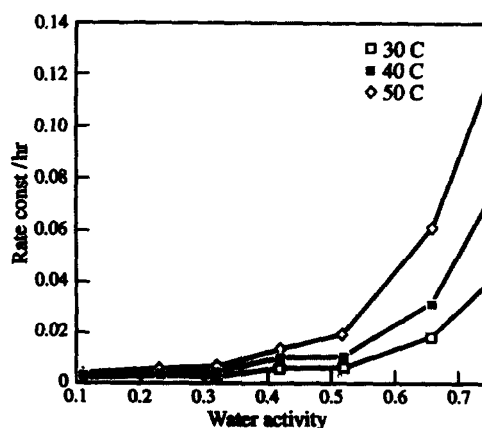

Fig. 2. Relationship between water activity and degradation rate for *all-trans* retinol.

Table 2. Arrhenius activation energies (E) calculated by the one-step method

a_w	E(kJ mol^{-1})[a]
0·11	28·6 ± 6·1
0·23	32·1 ± 2·3
0·32	32·2 ± 10·3
0·42	38·8 ± 5·1
0·52	44·0 ± 3·6
0·66	48·2 ± 3·3
0·75	42·9 ± 3·6

[a]95% confidence intervals.

β-carotene as a result of its additional conjugation relative to retinol (El-Tinay & Chichester, 1970).

The induction period shown by Chou and Breene (1972) for β-carotene decolorisation on microcrystalline cellulose was not apparent for any of the first-order plots in this study. Induction periods are characteristic of free radical chain reactions—the mechanism by which the oxidation of retinyl polyenes has been shown to occur (Koslov *et al.*, 1969). However, many free radical processes do not show an induction period, possibly due to the reactivity of the molecules involved (Guillory & Higuchi, 1962). Chou and Breene (1972) commented that the induction period was shorter at 35°C than at 5°C. Very short induction periods of 1–2 min were observed by Kozlov *et al.* (1969) in the oxidation of retinyl acetate and β-carotene in the solid state in a thin film in the range 10–25°C. El-Tinay and Chichester (1970) noted the absence of a lag period in the aerial oxidation of β-carotene in toluene, except when free radical initiators were added.

Two sets of data (a_w = 0·57 and a_w = 0·69) failed the test of validity for the one-step method for the calculation of activation energy and were discarded. Using the one-step method rather than the conventional two-step method increased the number of degrees of freedom available for estimating the value of E at each water activity level from j–2 (=1 for three temperature values) to n_j–5. For example, in the estimation of E for a_w = 0·11, the confidence limits for E are given by mean ± t × SD. At the 5% significance level using the two-step model there is just one degree of freedom available for t and its value is 12·7; using the one-step method gives 22 degrees of freedom for t and its value is 2·08.

The values of E for the seven data sets which passed the test of validity are listed in Table 2 and shown graphically in Fig. 3 as a function of water activity.

The values obtained for E are of the same order as those obtained for lipid oxidation in the absence of antioxidants (Ragnarsson *et al.*, 1977) and ascorbic acid oxidation in seaweed (Jensen, 1969).

Activation energies of the order of 40 kJ mol^{-1} have also been obtained for the oxidation of β-carotene on microcrystalline cellulose at 0·3 a_w (Baloch *et al.*, 1977), in the 'dry' state (Chou & Breene, 1972) and in toluene (El-Tinay & Chichester, 1970) and for retinyl acetate in crystal form (Guillory & Higuchi, 1962). Activation

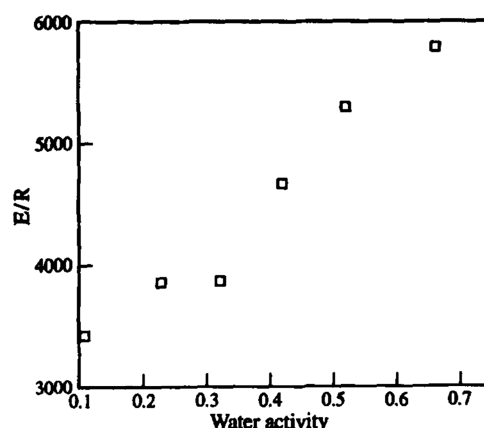

Fig. 3. Relationship between Arrhenius activation energy E and water activity for the degradation of *all-trans* retinol.

energies in the range 36–54 kJ mol^{-1} were obtained by Widicus *et al.* (1980) for a system of α-tocopherol in the water activity range 0·1–0·65 at 20–37 °C.

The Arrhenius activation energy increased with increasing water activity in the range 0·32–0·66 but then decreased at the water activity level usually associated with the appearance of free water in the system. Studies of the relationship between water activity and activation energy for a range of systems have been reviewed by Labuza (1980). Most studies have used a more limited range of water activity but both increases and decreases have been found.

Table 3 shows that the frequency factor, k_o, also varied with water activity in a similar manner. The relationship between a_w and k_o is shown graphically in Fig. 4.

The enthalpy, ΔH, and the entropy, ΔS, changes also increase in the a_w range 0·11–0·66 and then decrease. In contrast, the Gibbs free energy change, ΔG, decreases progressively as water activity increases.

It is usual to account for such variations by examining the data for enthalpy–entropy compensation (Leffler, 1966; Labuza, 1980) but there is considerable controversy concerning appropriate statistical methodology (Exner, 1964; Krug *et al.*, 1976; Good & Stone, 1972).

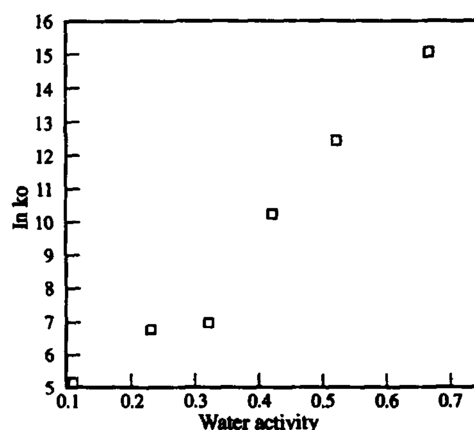

Fig. 4. Relationship between the frequency factor, k_o, and water activity for the degradation of *all-trans* retinol.

Table 3. Thermodynamic parameters (with 95% confidence intervals)

a_w	E/R	$\ln k_O$	$\ln k_j$ (at harmonic mean temperature) (h^{-1})	ΔH ($J\ mol^{-1}$)	ΔG ($J\ mol^{-1}$)	ΔS ($J\ mol^{-1}K^{-1}$)
0·11	3431 (±731)	5·14	−5·82 (±0·31)	$25·92 \times 10^3$ ($\pm 6·08 \times 10^3$)	$92·92 \times 10^3$ (±117)	−210·9 (±19·4)
0·23	3859 (±280)	6·77	−5·56 (±0·12)	$29·48 \times 10^3$ ($\pm 2·33 \times 10^3$)	$91·24 \times 10^3$ (±17)	−197·3 (±7·4)
0·32	3868 (±1243)	6·97	−5·39 (±0·52)	$29·56 \times 10^3$ ($\pm 10·3 \times 10^3$)	$90·79 \times 10^3$ (±336)	−195·7 (±33·0)
0·42	4665 (±617)	10·24	−4·67 (±0·25)	$36·18 \times 10^3$ ($\pm 5·13 \times 10^3$)	$88·91 \times 10^3$ (±78)	−168·5 (±16·4)
0·52	5297 (±433)	12·44	−4·49 (±0·18)	$41·44 \times 10^3$ ($\pm 3·60 \times 10^3$)	$88·44 \times 10^3$ (±38)	−150·2 (±11·5)
0·66	5795 (±411)	15·13	−3·39 (±0·17)	$45·58 \times 10^3$ ($\pm 3·42 \times 10^3$)	$85·58 \times 10^3$ (±34)	−127·8 (±10·9)
0·75	5157 (±394)	13·83	−2·65 (±0·15)	$40·27 \times 10^3$ ($\pm 3·28 \times 10^3$)	$83·66 \times 10^3$ (±29)	−138·6 (±10·5)

T is harmonic mean temperature 313 K.
$\ln k_T$ is ln of rate constant at harmonic mean temperature.
$\Delta H = E - RT$, where R is 8·314 J mol^{-1}.
$\Delta G = RT \ln \frac{k_b T}{h k_T}$, where k_b is Boltzmann constant $1·38 \times 10^{23}$ and h is Planck constant $6·626 \times 10^{-34}$.
$\Delta S = \frac{\Delta H - \Delta G}{T}$.

According to Exner (1964), linear relationships such as that shown between enthalpy and entropy changes (Fig. 5) are suspect because the method of calculation can lead to linear correlations which are statistically significant but physically meaningless and are likely to give slope values insignificantly different from the experimental temperature. However, the isokinetic temperature (slope) is 225 for all seven points and 249 for six points (excluding the point at 0·75 water activity) compared with the harmonic mean temperature of 313 K. Krug *et al.* (1976) concluded that correlations between enthalpy and free energy estimates, evaluated at the harmonic mean temperature, in data sets which are thermally consistent and allow the calculation of a unique harmonic mean temperature, are a better indicator of chemical causality. The use of the harmonic mean temperature minimises estimated error in ΔG as shown in Table 3. Figure 6 shows that a linear relationship can be fitted (R^2 = 0·945) between enthalpy and Gibbs free energy changes within the water activity range 0·11–0·66. Good and Stone (1972) also pointed out that any relationship in the enthalpy versus entropy plane is a consequence of a relationship in the E versus $\ln k_o$ plane and is not necessarily linear. Although, in Figs 5–7, a straight line can be fitted to the data, when the order of the points in relation to increasing values of water activity is taken into account, the points may lie on curves which have a turning point at

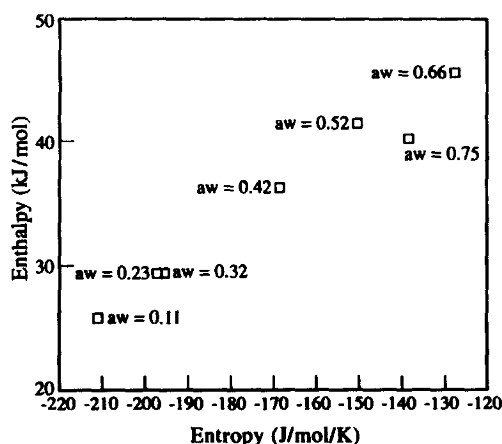

Fig. 5. Relationship between enthalpy and entropy for the degradation of *all-trans* retinol.

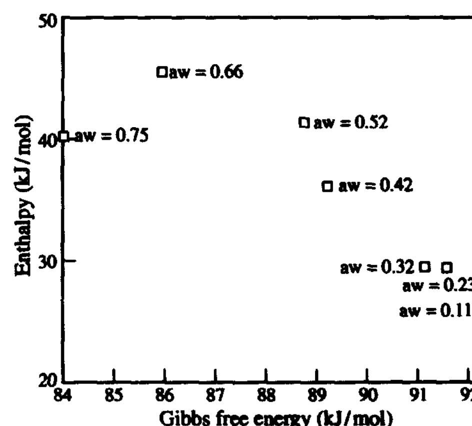

Fig. 6. Relationship between enthalpy and Gibbs free energy for the degradation of *all-trans* retinol.

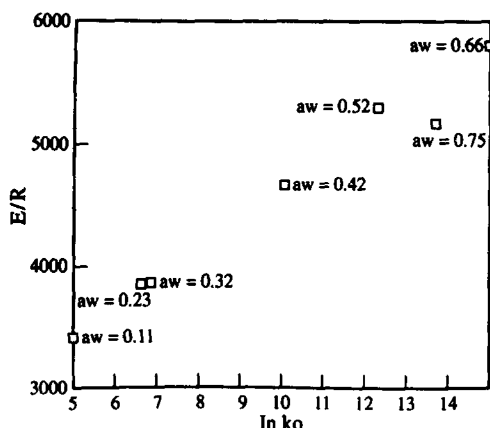

Fig. 7. Relationship between the Arrhenius activation energy, E, and the frequency factor, k_0, for the degradation of *all-trans* retinol.

water activity = 0·75. Further studies to enable testing for departure from linearity in the E versus k_0 plane above 0·66 water activity might be worthwhile.

Thus the variation with water activity may be interpreted in terms of a compensation effect related to the role of water in the range associated with bound and capillary water.

CONCLUSION

The loss of *all-trans* retinol suspended on microcrystalline cellulose in the dark, in air, can be adequately described by a first-order relationship in the water activity range 0·11–0·75. The activation energy values obtained indicate that, at low water activity levels, 0·11–0·32, the degradation of *all-trans* retinol has similarities to that of β-carotene under the same conditions. Examination of the thermodynamic parameters indicate a chemical compensation effect in the water activity range associated with bound and capillary water.

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