Investigation of γ-irradiation of α-tocopherol and its related derivatives by high-performance liquid chromatography using a rapid scanning spectrophotometer[*]

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ABSTRACT

The behaviour of α-tocopherol in differently reactive model systems was investigated immediately after irradiation or chemical reaction by reversed-phase high-performance liquid chromatography with rapid-scanning UV detection. The main advantage of this technique is the generation of the complete spectral and chromatographic information in one experiment. The other advantage of the method is the ability to study α-tocopherol in different environments (solvents) without tedious sample preparation and the characterization of the main primary products. The method is especially well suited for the investigation of α-tocopherol and related substances, which are sensitive to oxidation, which could not be studied with other conventional techniques.

INTRODUCTION

Tocopherols, a group of lipid-soluble compounds, are used as food ingredients and are biochemically interesting compounds. They are achieving increasing attention, due to α-tocopherol revealing among its properties as vitamin E, to be the most important natural antioxidant. It is able to protect lipids in the lipid phase of foods and in the membrane of living cells from autoxidation [1,2]. As irradiation is increasingly used to conserve foods, especially to protect poultry meat, vegetables, fruits
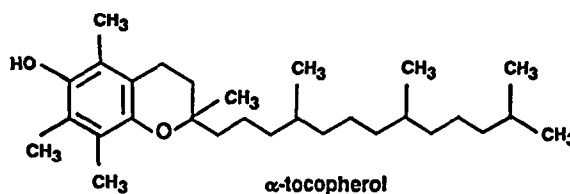
α-tocopherol

and spices from decomposition by various mechanisms and means [3], we wanted to study the behaviour of α-tocopherol during irradiation processes in different environments.

γ-Irradiation leads to changes in food composition, *e.g.*, owing to oxidation in the lipid phase [4]. Several workers have stated that substantial destruction of α-tocopherol occurs during the irradiation processes [5–9]. Therefore, the effect of irradiation on tocopherols and their capacity to act as antioxidants should be investigated.

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There have been several reports of the determination of tocopherols by high-performance liquid chromatography (HPLC) in different systems, such as foods, pharmaceutical preparations and mixtures containing tocopherols and other components, e.g., vitamin mixtures [10]. However, no methods for dealing with products of α-tocopherol immediately after irradiation have been reported, so we wanted to develop an HPLC technique for this purpose.

Normal- and reversed-phase (RP) HPLC have been applied after the reaction of α-tocopherol with various oxidants [11–14]. Cillard et al. [11] separated three different tocopherol dimers by normal-phase HPLC after reaction of α-tocopherol with alkaline hexacyanoferrate(II). Yamauchi and co-workers [12–14] investigated the mixture resulting from the reaction of α-tocopherol with 2,2′-azobis (2,4-dimethylvaleronitrile) on μBondasphere C_{18} or Wakosil C_{18} with methanol, with gradient elution from methanol to methanol–ethyl acetate (3:7, v/v) and with methanol–ethyl acetate (7:3, v/v), respectively.

Yamauchi et al. [15] separated trimers and other tocopherol oxidation products in autoxidizing methyl lineolate by RP-HPLC on Wakosil C_{18} with a linear gradient from methanol to methanol–diisopropyl ether (1:1, v/v). Gottstein and Grosch [16] studied the products of autoxidation of linoleic acid in the presence of tocopherol model compounds using normal-phase HPLC.

Ha and Csallany [17] separated a mixture of α-tocopherol and five oxidation products by normal-phase HPLC with hexane–chloroform–2-propanol (95:4.5:0.5) (v/v/v) as the mobile phase. Koskas et al. [18] separated tocopherol, tocopherylquinone and a tocopheryl dimer by RP-HPLC on Spherisorb ODS with gradient elution from methanol–water (85:15, v/v) to methanol.

Howell and Wang [19] separated α-tocopherol, α-tocopherol acetate, α-tocopherylquinone, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid and γ-tocopherol by RP-HPLC on Partisil ODS and on Spherisorb ODS in an isocratic mode using methanol–water (87:13, v/v) and gradient elu-

TABLE I

FURTHER HPLC METHODS FOR SEPARATION OF TOCOPHEROL AND TOCOPHERYLQUINONE

Column	Eluent (v/v)	Detection[a]	Compounds[b]	Ref.
Zorbax C_{18}	Methanol–water (98:2)	UV, 265 nm	T-quinone	20
C_{18}	Methanol–water (97.5:2.5)	UV, 280 nm	T-quinone	21
Spherisorb ODS	(a) Methanol–water (85:15) (b) Gradient from methanol–water (85:15) to methanol	UV, 290 nm	α-T, γ-T, T-dimer	22
μBondapak C_{18}	Acetonitrile	UV, 254 nm	Vitamin E Vitamin E quinone	23
C_{18}	Methanol–water (96:4)	UV, 265, 292, 215 nm	α-T, α-Tqu, γ-T, α-Tac, cholesterol	24
Ultrasphere ODS	2-Propanol–acetonitrile–water–triethylamine–acetic acid (60:20:19.4:0.5:0.1)	ED	T, T-quinone	25
Spherisorb ODS Adsorbosphere C_{18} guard column	96% methanol with sodium perchlorate (50 mM)	ED	T, T-quinone	26
C_{18}	Mixtures of ethanol and methanol containing 50 mM $NaClO_4$ and 2 mM $HClO_4$	ED	Prenyl quinones	27

[a] ED = Electrochemical detection.
[b] α-T = α-Tocopherol; γ-T = γ-tocopherol; α-Tac = α-tocopheryl acetate; Tqu = tocopheryl quinone; T-dimer = tocopheryl dimer.

tion from methanol–water (88:12, v/v) to methanol, respectively.

Some workers have observed α-tocopherylquinone using RP-HPLC in the presence of α-tocopherol (Table I).

In most HPLC analyses of tocopherols, UV, fluorescence or electrochemical detection techniques were used. The application of diode-array detection was described by Greenspan et al. [28] for the analysis of α-tocopherol, retinol, cholesterol and other lipid-soluble substances and by Miller and Yang [29] for a mixture of α-tocopherol with carotenoids.

The application of rapid scanning spectrophotometers has the advantage of protecting unstable compounds, which could be formed after gamma irradiation of α-tocopherol, owing to the use of linear optics, which means the application of less light energy to the sample in the detector cell. The use of such a detector for the study of tocopherol oxidation products has not been described previously, however. In our work we used a BarSpec Chrom-A-Scope rapid-scanning UV spectrophotometer as a detector.

EXPERIMENTAL

Instrumentation

A modular HPLC system was constructed, with a Knauer (Berlin, Germany) Type 64.00 pump, a Rheodyne (Cotati, CA, USA) model 7125 injector, a Nucleosil C_{18} (5 μm) column (250 × 4.6 mm I.D.) (Molnar, Berlin, Germany), a Victor (Frankfurt, Germany) PC 386A computer with Model 387 mathematical coprocessor, an NEC P6 (Berlin, Germany) printer and Chrom-A-Scope rapid-scanning spectrophotometer with a wavelength range from 190 to 370 nm (BarSpec, Rehovot, Israel). The eluent was acetonitrile (HPLC grade, Merck, Darmstadt, Germany) at a flow-rate of 2.0 ml/min.

For the investigation of venritidin HCl we used the following conditions: Column, EnCaPharm 100 RP-18 (5 μm) (120 × 4.6 mm I.D.) (Molnar); eluent, acetonitrile–0.4 M phosphate buffer (pH 2.1) (10.5:89.5, v/v); temperature, 60°C; injection volume, 40 μl (1 mg/ml); flow-rate 2.0 ml/min.

γ-Irradiation, ^{60}Co MRCH-τ-100 (Humboldt University, Berlin, Germany), irradiation dose 1.3 kGy, dose rate 64 Gy/min, temperature 293 K. To trap the irradiated state, the samples were frozen by using liquid nitrogen.

Reference substances and chemicals

The reference substances used were α-tocopherol (Merck) and tocopherylquinone (Serva, Heidelberg, Germany).

Formyltocopherol, ethoxymethyltocopherol and tocopherone were isolated with the chromatographic system described above. Fractions were evaporated and characterized by UV, IR and ^{1}H NMR spectroscopy [30] and identified by comparing the results with literature data [9,31,32]. The spirodimer of α-tocopherol was synthetised by the method of Boguth and Hackel [33], characterized by ^{1}H NMR, UV and IR spectroscopy [30] and identified by comparison with data from the literature [33].

Acetonitrile and methanol as eluents were of HPLC grade, other chemicals were of analytical-reagent grade (Merck).

RESULTS AND DISCUSSION

Method development

The synthesized spirodimer could not be eluted with methanol in the capacity factor (k') range 1–20. Using propionitrile as an eluent of higher elution strength, however, we could elute tocopherol dimers, but had problems with the reproducibility of the results owing to the high viscosity of this eluent. Mixtures of propionitrile with acetonitrile were also still fairly viscous. Methanol did separate well the polar oxidation products of α-tocopherol, but had a high absorbance below 220 nm. With acetonitrile, however, the irradiation products could be

TABLE II

α-TOCOPHEROL CONTENT (%, ±2.0%) AFTER γ-IRRADIATION IN DIFFERENT SOLVENTS [30,34]

Irradiation dose 1.3 kGy in the presence of air.

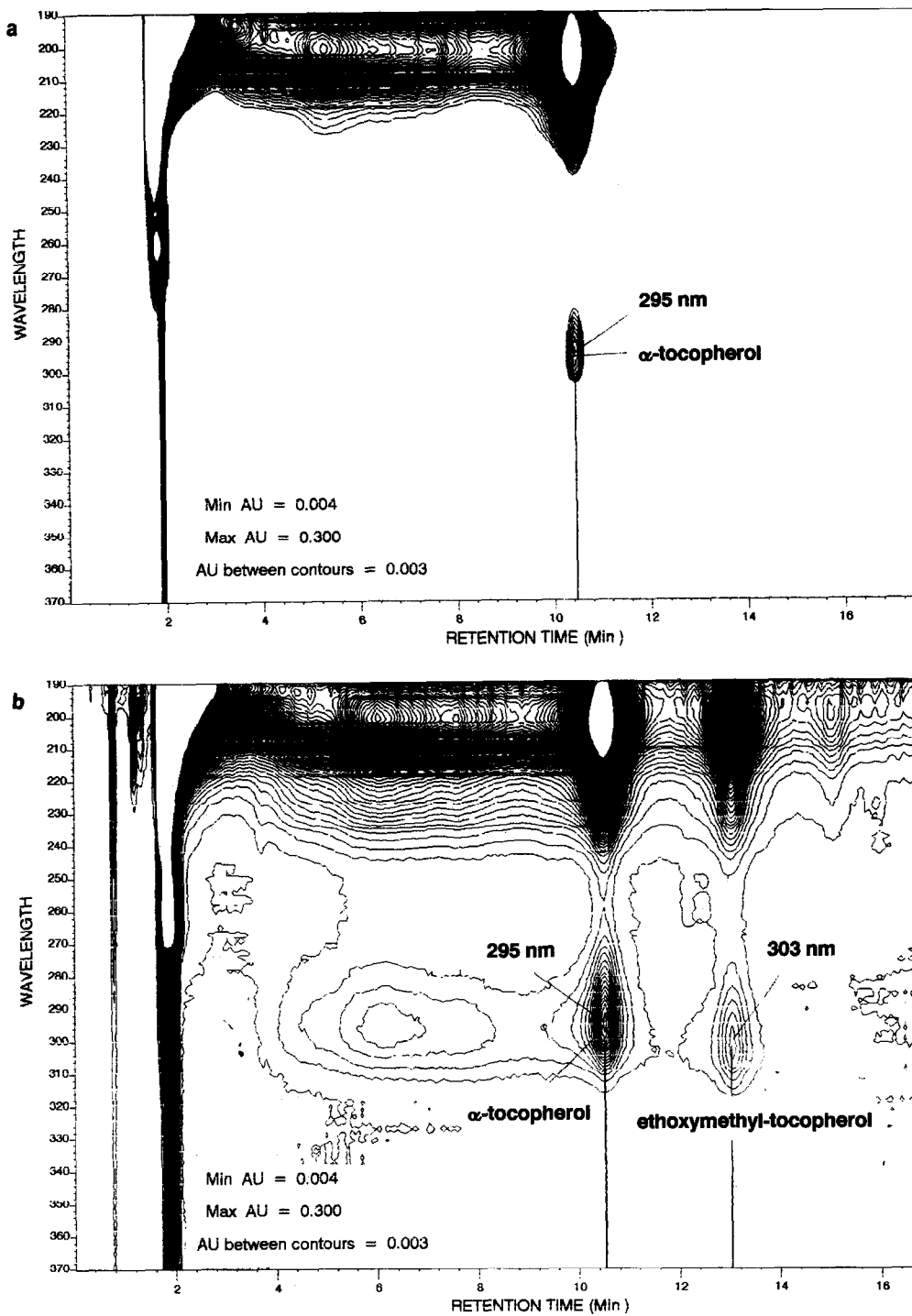| Solvent	Initial α-tocopherol concentration (g per 100 ml)		
	0.03–0.05	0.07–0.1	0.9–1.0
Benzene	100.0	100.0	100.0
Acetonitrile	93.5	96.9	100.0
Ethanol	68.0	87.7	–
Hexane	52.1	78.1	97.1
Chloroform	0.0	50.7	96.0[a]

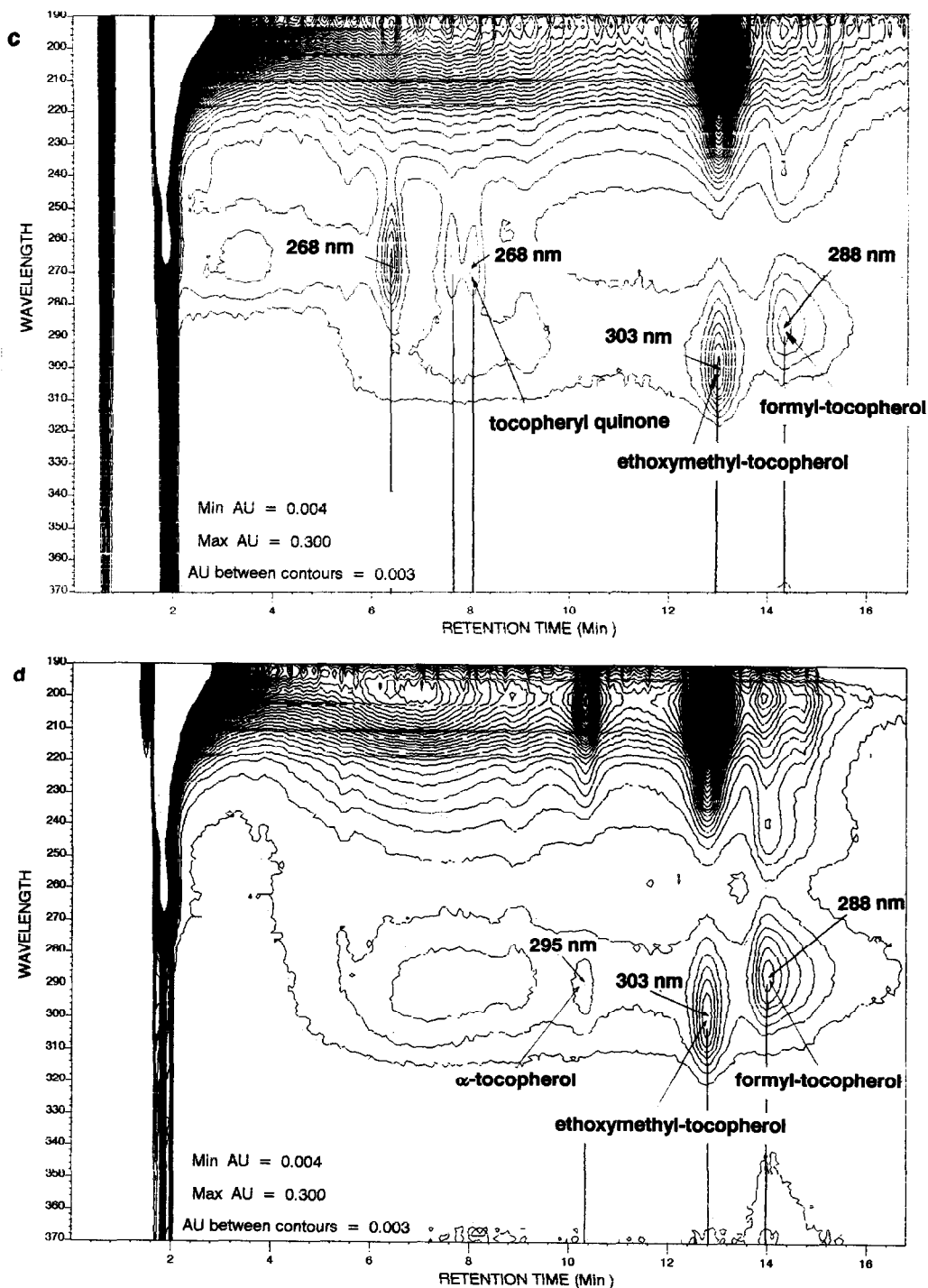[a] Initially 0.7 g of α-tocopherol per 100 ml.

Fig. 1.

Fig. 1. HPLC contour map of γ-irradiated α-tocopherol in chloroform at low concentration (0.04 g per 100 ml). Injection volume, 100 μl. (a) Without irradiation; (b) *in situ* state after irradiation in the absence of oxygen, under nitrogen; (c) immediately after irradiation in the presence of oxygen; (d) as (c), but after storage for 1 day at 255 K.
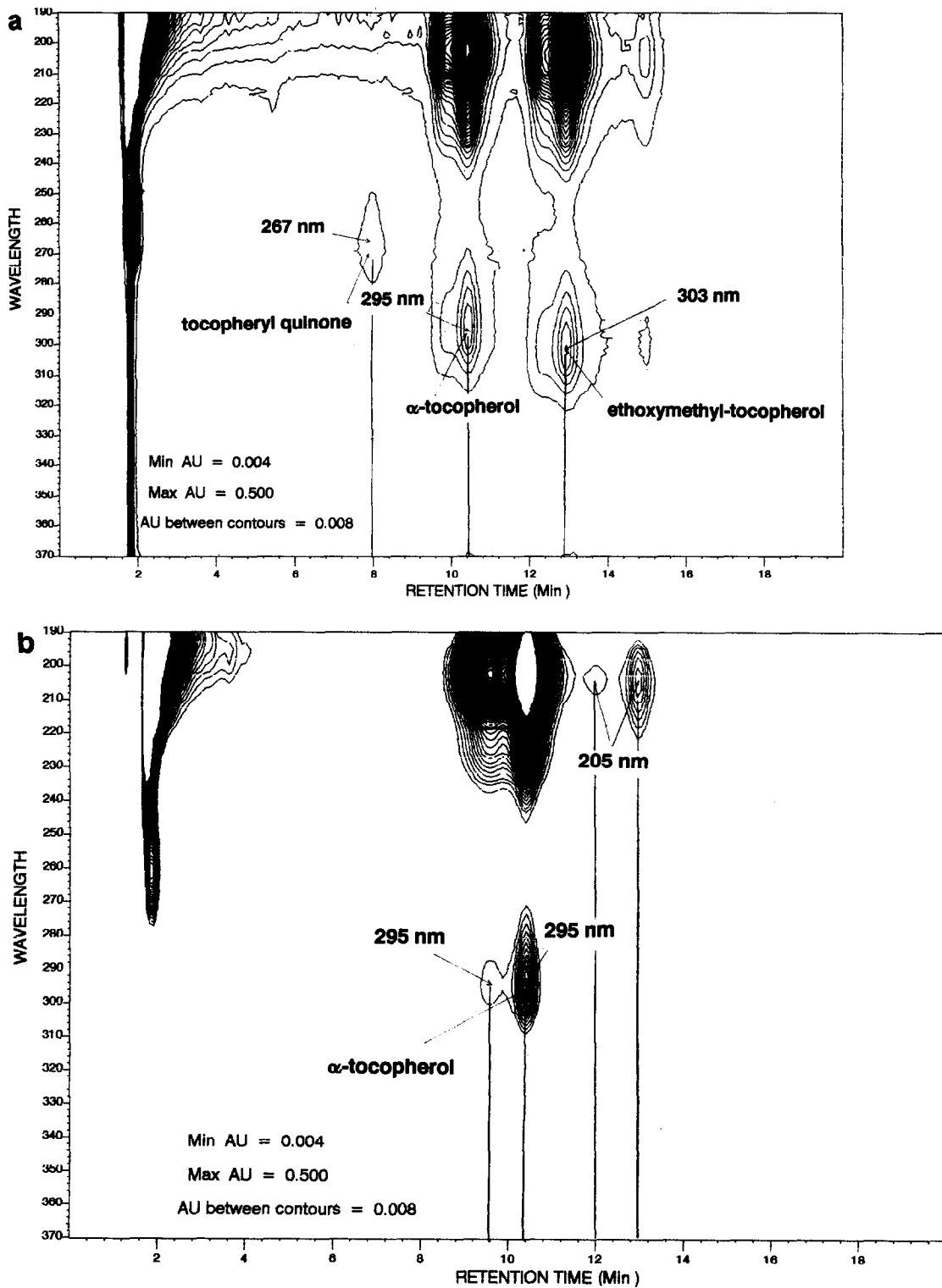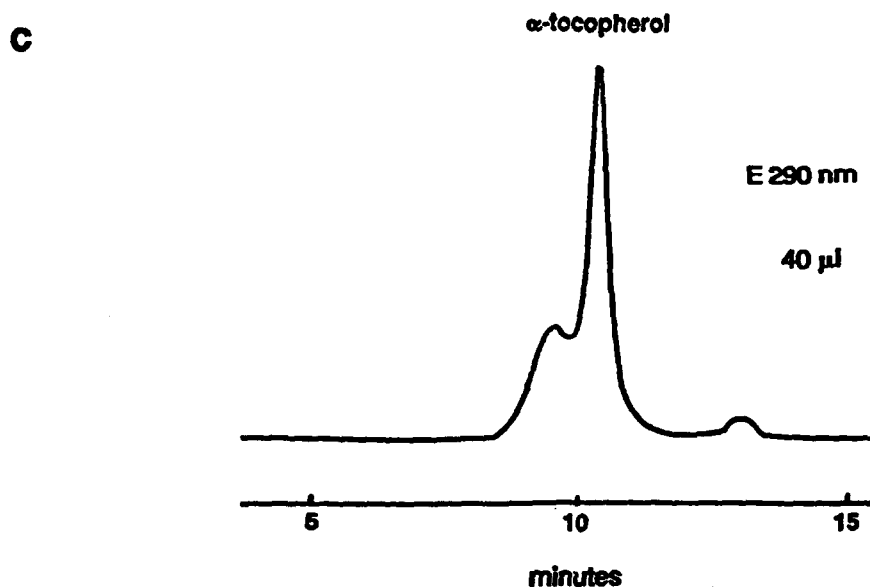
Fig. 2.

C

α-tocopherol

E 290 nm

40 µl

5   10   15

minutes

Fig. 2. HPLC and contour map of γ-irradiated α-tocopherol in chloroform at 0.1 g per 100 ml (immediately after irradiation). Injection volume, 40 µl. (a) Irradiation in the presence of oxygen; (b) irradiation in the absence of oxygen, under nitrogen; (c) chromatogram after irradiation under nitrogen (295 nm), from (b).

well separated and detected below 200 nm, making a correlation with the irradiation process possible. This and other investigations in hexane demonstrated further that significant amounts of tocopherol dimers are formed only in irradiated hexane.

Solvent-dependent results of γ-irradiaton

The investigations in different solvent systems revealed that the mechanism (how the reaction prod-

ucts are generated from α-tocopherol) and the degree of α-tocopherol destruction are variable and depend on the reactivity of the radical solvent species formed during the irradiation process (see Table II) [30,34].

Irradiation of the tocopherol molecule in highly reactive media (chloroform, ethanol, hexane) is terminated in oxidation processes and different reaction products are formed: (a) in chloroform, for-
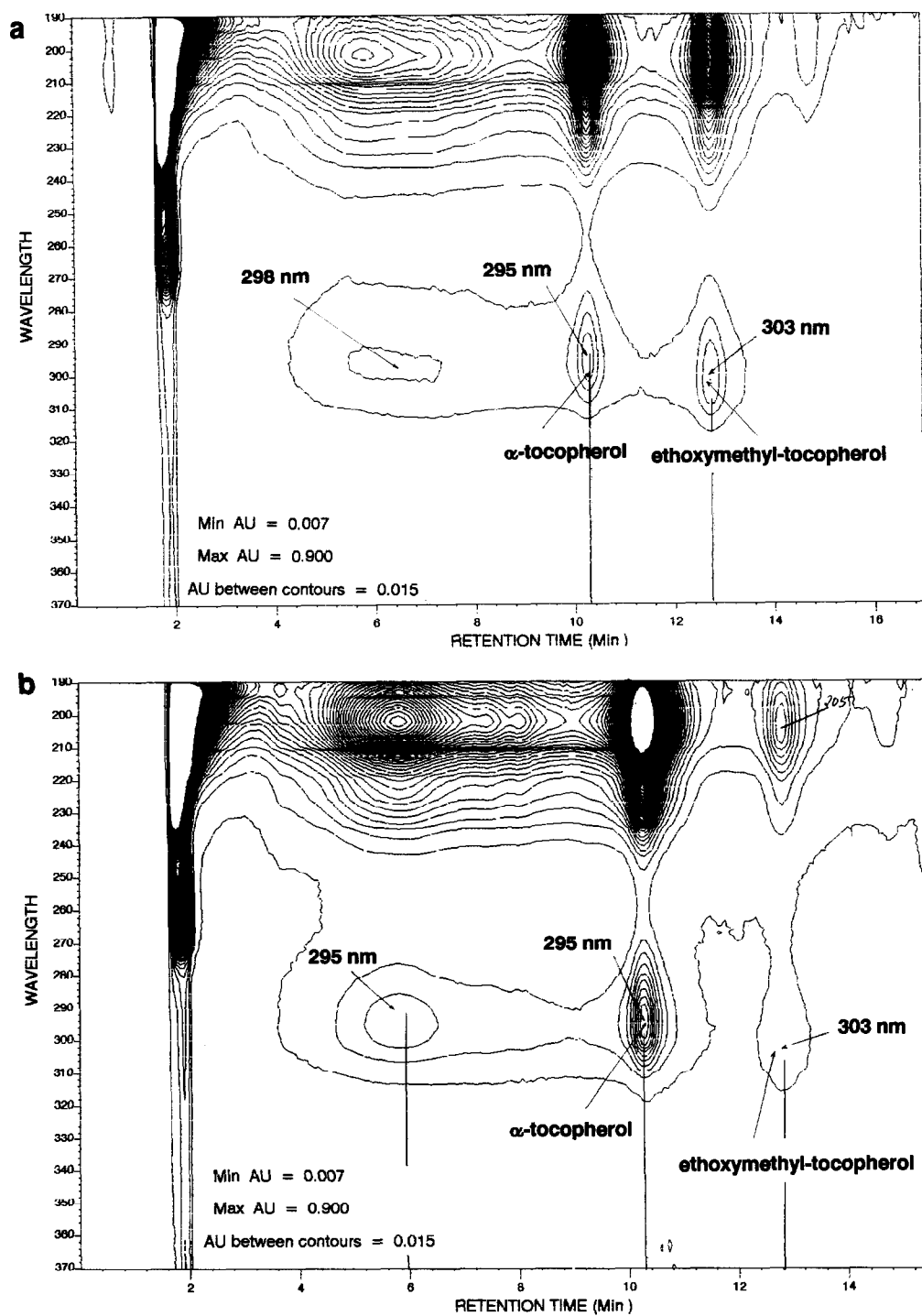

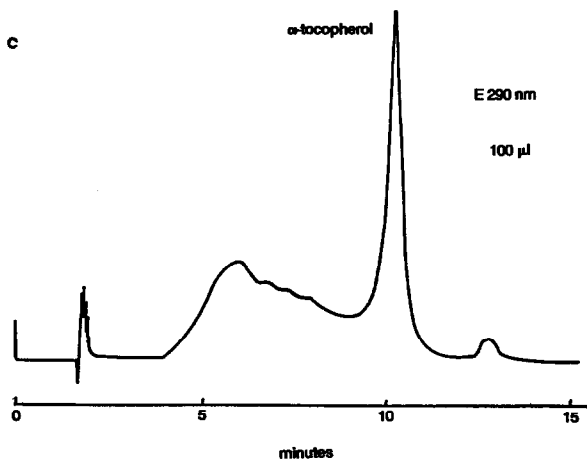Tocopherone

R = H, OH

Tocopheryl quinone

5-Formyl tocopherol

5-Ethoxymethyl tocopherol
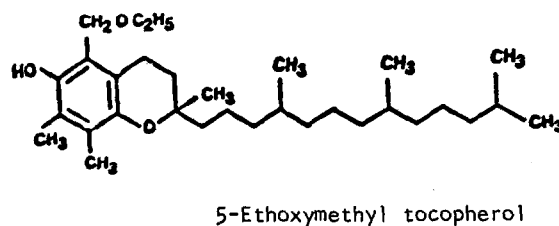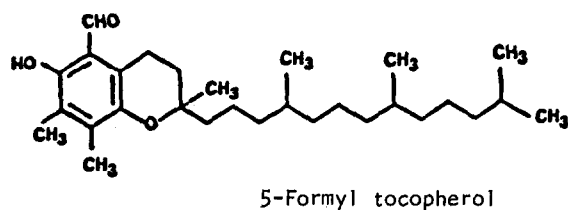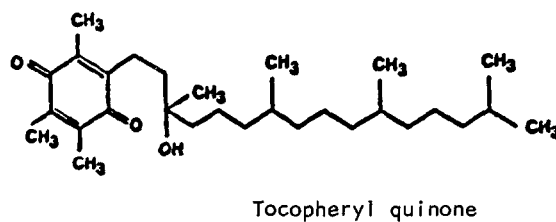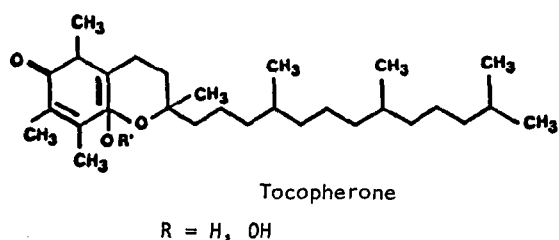
Fig. 3.

Fig. 3. HPLC and contour map of γ-irradiated α-tocopherol in chloroform at 0.1 g per 100 ml after storage for 1 day at 255 K. Injection volume 100 μl. (a) Irradiation in the presence of oxygen; (b) irradiation in the absence of oxygen, under nitrogen; (c) chromatogram of (b).

myl- and ethoxymethyltocopherol and quinones (Figs. 1–3); (b) in ethanol, tocopherone and tocopherylquinone (Fig. 4); and (c) in hexane, also to-

copherylquinone (Fig. 5) and spirodimer (not eluted in Fig. 5), but measured by thin-layer chromatography elsewhere). Formyl-, and ethoxymethyltocopherol are also products of oxidation of α-tocopherol with *tert.*-butyl hydroperoxide in chloroform [31].

Dependence of the irradiation reaction on the presence of oxygen

The investigation showed that the products formed depend on the reactivity of the medium. The solubility of oxygen in the solvent plays an important role in the irradiation process. It is greater in hexane than in chloroform or in ethanol.

Following γ-irradiation in the absence of oxygen, under nitrogen, (a) in chloroform, formyltocopherol and quinones are not formed (Figs. 1–3); (b) in ethanol no products are formed (Fig. 4); and (c) in hexane the same products are formed with or without the presence of dissolved oxygen.

After irradiation in chloroform, an unknown species could be detected which had a shorter retention time and an extremely broad peak form (Figs. 2 and
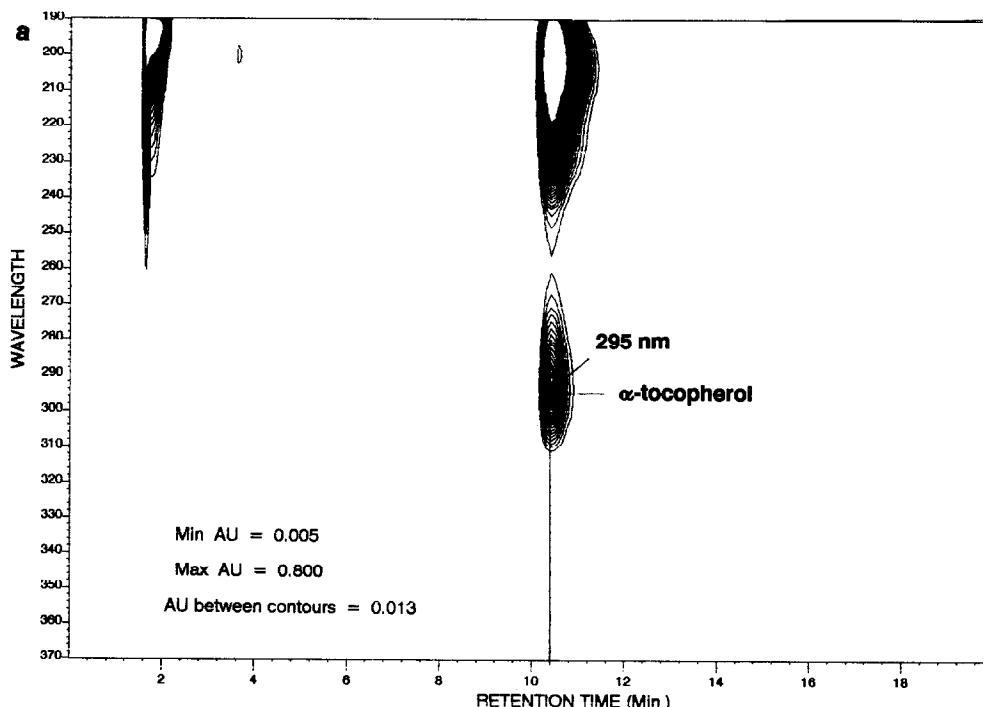

Fig. 4.

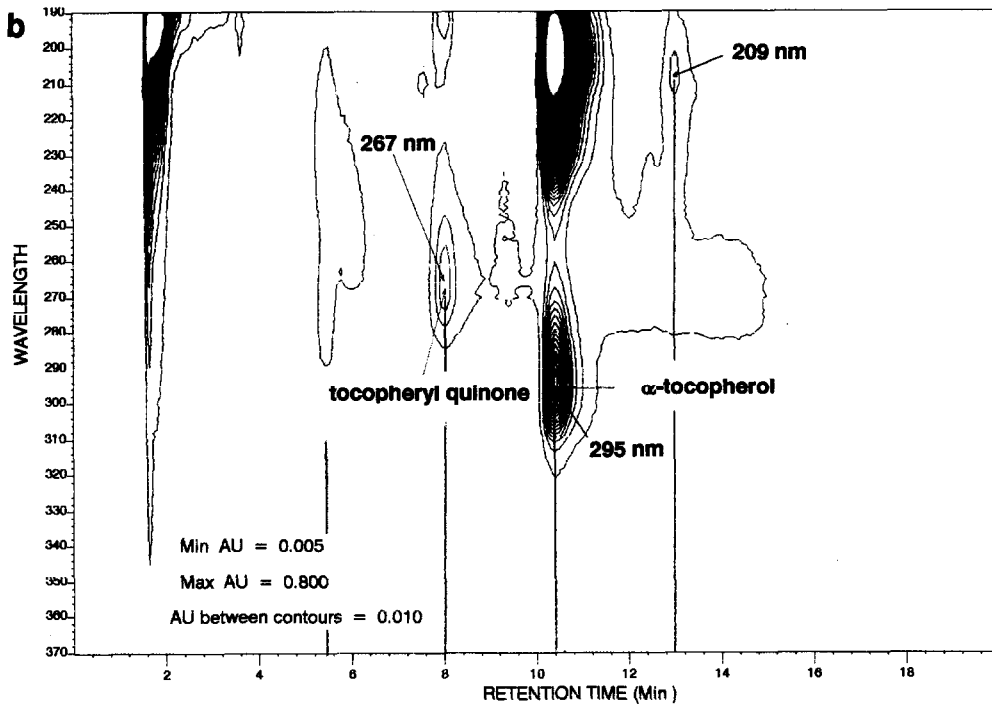Fig. 4. HPLC contour map of γ-irradiated α-tocopherol in ethanol at 0.05 g per 100 ml (immediately after irradiation). Injection volume, 100 μl. (a) Irradiation in the absence of oxygen, under nitrogen; (b) irradiation in the presence of oxygen.
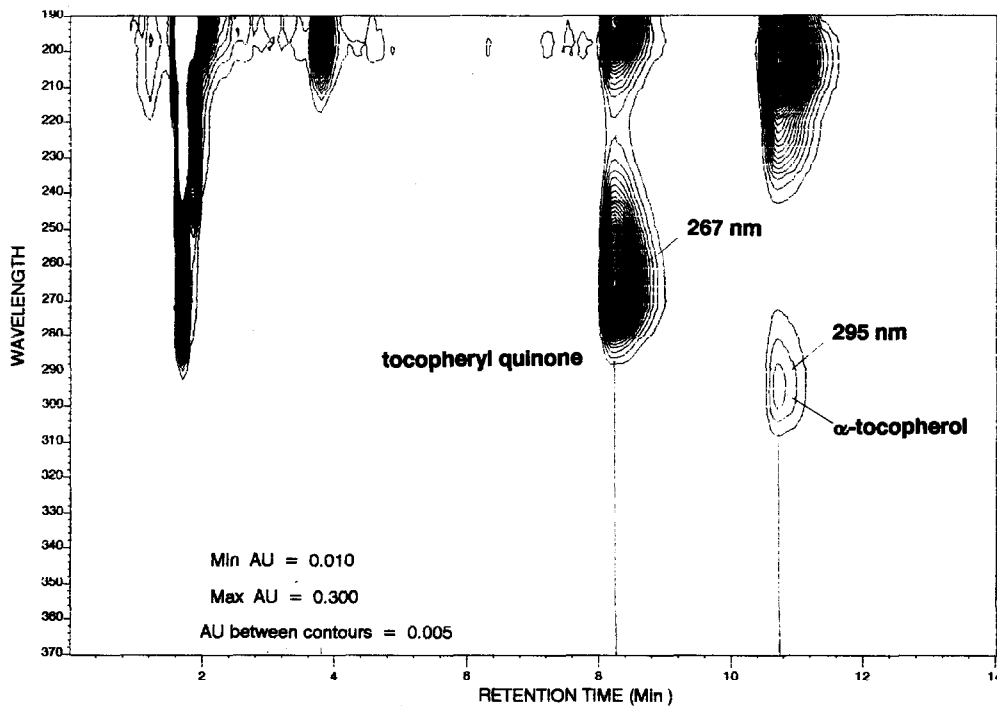

Fig. 5. HPLC contour map of γ-irradiated α-tocopherol in hexane at 0.08 g per 100 ml (*in situ* state). Injection volume, 40 μl.
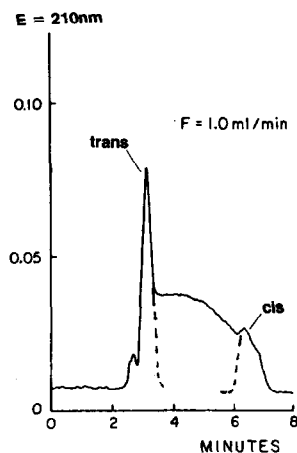
Fig. 6. Chromatogram of the *cis–trans* isomeric mixture of L-alanyl-L-proline as described by Melander *et al.* [36]. Column, LiChrosorb RP-18, 10 μm (250 × 4.6 mm I.D.); eluent, 0.05 *M* phosphate buffer (pH 6.0); flow-rate, 1 ml/min; temperature, 25°C.

3). This type of peak form is known for acid–base equilibria as described in the solvophobic theory of Horváth *et al.* [35]. They found that the ratio of the k' values of benzoic, salicylic and homovanillic acids to anions were between 4 and 6, which leads in the absence of buffer to broad peaks, besides protic equilibria. Also *cis–trans* isomers give in RPC in some instances this type of broad peak form, as is the case with L-alanyl-L-proline. Whereas polar and non-polar residues in the *cis* conformation can be placed on opposite sites of a hypothetical plane, no such plane exists for the *trans* conformation, so the *trans* isomer is less retarded (Fig. 6) [36].

Broad peaks are usually observed in RPC for strong bases having a positive charge and also a large solvophobic contact surface area. Similar observations of broad multiple peak formation were found with venritidin, a furane derivative used in ulcer therapy as an H_2-antagonist. At low ionic strength we observed here also instead of one peak, three peaks with an unusual broad peak in the middle. All three peaks have identical UV spectra, which can be seen in the contour map using the rapid scanning spectrophotometer. The three bands probably result from an equilibrium of a *cis–trans* isomer pair similar to L-alanyl-L-proline (Fig. 7) [37].
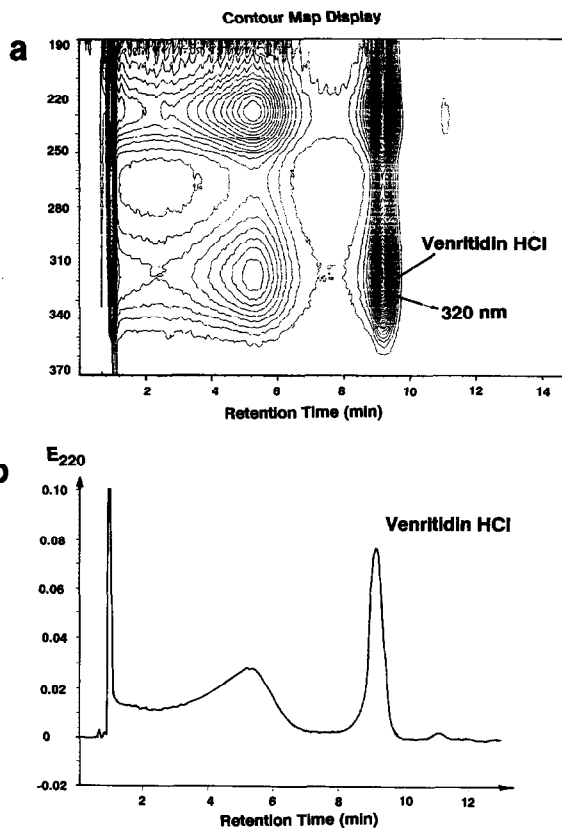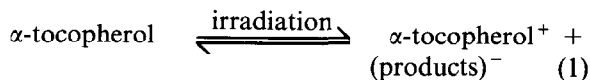

Fig. 7. (a) HPLC contour map of venritidine. (b) The corresponding chromatogram at 205 nm showing three bands: one very polar at t_0, a broad one in the middle and a very sharp third one, all having identical spectral properties. For chromatographic conditions, see Experimental.

The UV spectrum of the broad peak was likewise identical with that of α-tocopherol. After fraction collection of the broad peak in Fig. 3b, concentration by evaporation and re-injection, we found α-tocopherol and some more polar components (Fig. 8). Further work on this aspect is planned.

We assumed that the broad band could represent an equilibrium of the following type:

$$\alpha\text{-tocopherol} \; \underset{}{\overset{\text{irradiation}}{\rightleftharpoons}} \; \alpha\text{-tocopherol}^+ + (\text{products})^- \qquad (1)$$

Of all the irradiated model solvents that were studied, air-saturated chloroform contained the most reactive short-living radicals, such as the trichloromethaneperoxy radical, $CCl_3OO^•$ (see Table III).
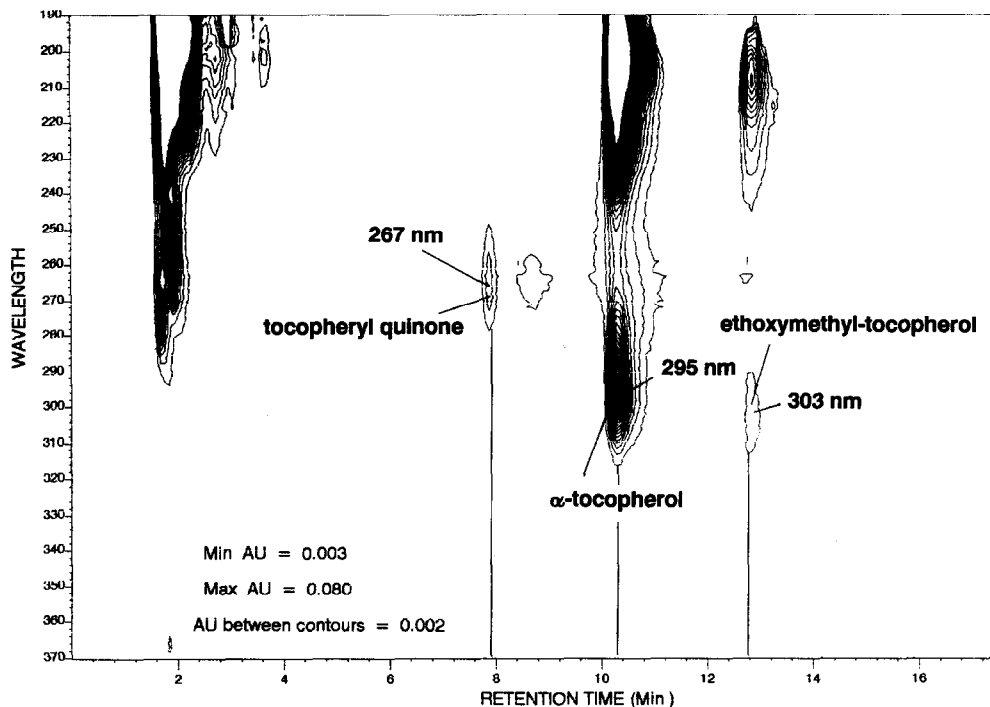
Fig. 8. HPLC contour map of isolated bands with retention times from 5 to 10 min as in Fig. 3b. Concentration of α-tocopherol, 0.1 g per 100 ml; injection volume, 100 μl.

Dependence of the irradiation reaction on the concentration of α-tocopherol

It could be shown that the effect of irradiation on α-tocopherol decreases with increasing concentration in the same solution. Concentration-dependent irradiation in chloroform has shown that formyltocopherol could be formed only at low concentrations, *i.e.*, below 300 ppm α-tocopherol, whereas ethoxymethyltocopherol could be observed at all investigated concentrations (Figs. 1–3).

TABLE III

RATE CONSTANTS FOR THE REACTION ROO· + α-to-co–OH → ROOH + α-toco–O·

ROO·	k (l mol^{-1})	Ref.
C_6H_{11}–OO·	$2.3 \cdot 10^7$	38
CCl_3–OO·	$5 \cdot 10^8$	39
CH_3CH–OO·–OH	$9.1 \cdot 10^4$	9
R–OO· from oleic acid	$2.5 \cdot 10^6$	38

To explain the mechanism of tocopherol destruction via γ-irradiation, the dependence on different solvent properties and on the oxygen solubility, α-tocopherol was also chemically oxidized, especially with superoxide anion radical, $O_2^{-\cdot}$, and Fe^{3+} [30]. The products of the reaction of α-tocopherol with a tenfold excess of $O_2^{-\cdot}$ (Fig. 9) in acetonitrile are similar to the products formed following irradiation of α-tocopherol in ethanol (Fig. 4), so that a correlation between the two mechanisms is possible. The method also allows the investigation of the time-dependent reaction of α-tocopherol with superoxide anion radicals.

Products arising from the reaction of α-tocopherol with Fe^{3+} ions in acetonitrile, *viz.* tocopherylquinone and tocopherone (Fig. 10), are similar to the products of the reaction with superoxide and to the products of the irradiation in ethanol. Both reactions are well documented [40–44].

Concerning electron spin resonance (ESR) studies on different derivatives of α-tocopherol and several tocopherol isomers (α-, β- and γ-tocopherol, to-
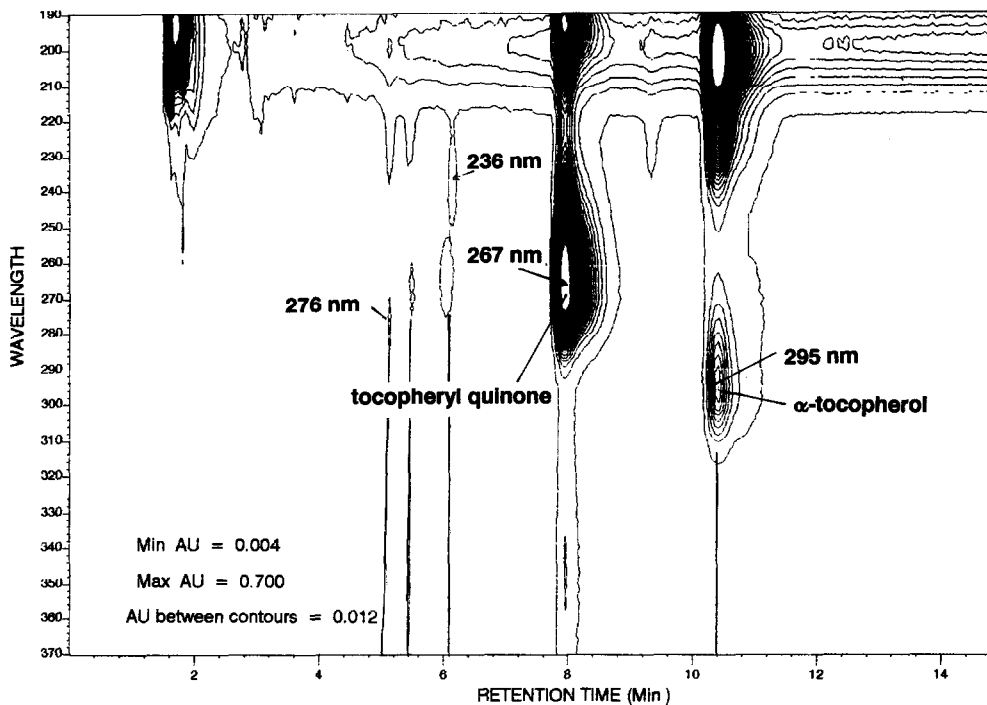
Fig. 9. HPLC contour map of α-tocopherol after oxidation in acetonitrile: $O_2^{-\cdot}$ = 1:8 (mol/mol). Injection volume, 100 μl of a 400 ppm solution of α-tocopherol in acetonitrile.
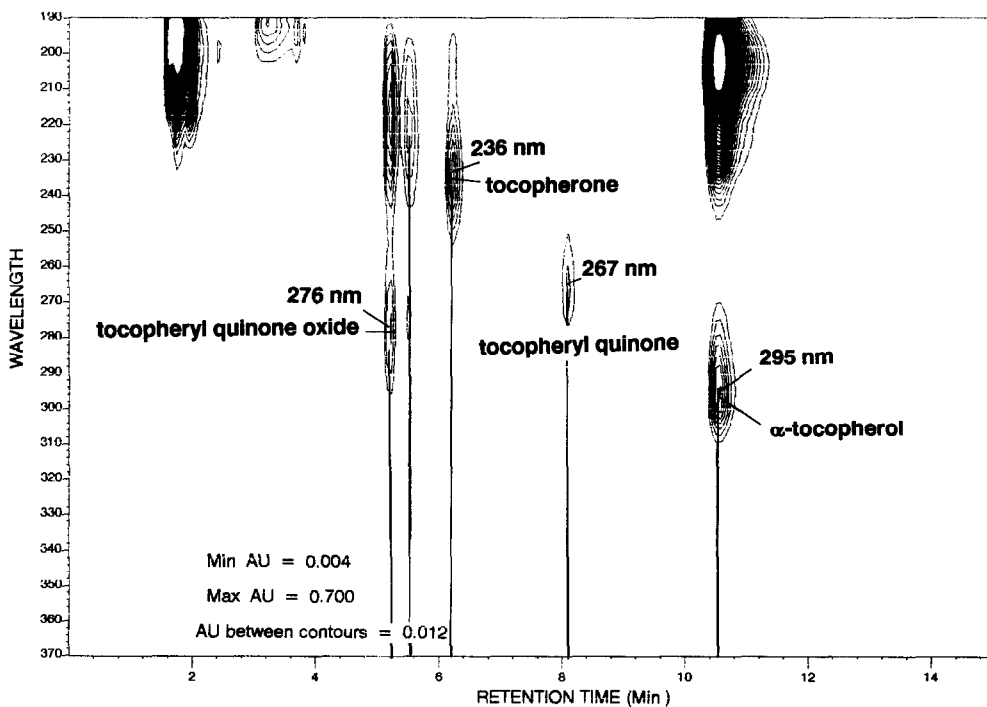

Fig. 10. HPLC contour map of α-tocopherol after oxidation with $FeCl_3$ in acetonitrile at a concentration of 1500 ppm. Injection volume, 40 μl.

col and 2,2,5,7,8-pentamethyl-6-chromanol) [30,45], we concluded that the irradiation process leads in the first step to the formation of a cation radical of α-tocopherol. It could be also observed that the reaction of α-tocopherol with $FeCl_3$ and $O_2^-{}^\cdot$ includes in the first step an electron transfer to form Fe^{2+} and O_2^{2-} and an α-tocopherol cation radical, respectively [30,45]. The primary formation of an α-tocopherol cation radical is a possible interpretation to explain the influence of different solvent properties (pH, solvation power, etc.) on tocopherol oxidation and product formation. This cation radical is stabilized to form an α-tocopheryl-radical by releasing a proton.

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