Journal of Chromatography, 130 (1977) 378–383
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CHROM. 9578

Note

Fractionation of the oxidation products of α-tocopherol and their condensation products with L-lysine by combined thin-layer and gel chromatography

NGUYEN-THIEN LUAN and J. POKORNÝ
Department of Food Chemistry, Institute of Chemical Technology, 166 28 Prague 6 (Czechoslovakia)
J. ČOUPEK
Chemical Research Department, Laboratory Instruments Works, 162 00 Prague 6 (Czechoslovakia)
and
S. POKORNÝ
Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, 162 06 Prague 6 (Czechoslovakia)
(Received July 20th, 1976)

Tocopherols present in vegetable oils can be oxidized directly by air oxygen, by lipidic hydroperoxides and by iron(III) salts present as contaminants. In the first two instances, hydroperoxides are the primary oxidation products[1]. Organic peroxides[2] and hydroperoxides[3] catalyze the decomposition of tocopherols in vegetable oils. The final oxidation products of tocopherols are predominantly various types of oligomers[4], so that in oils stored for a long time the tocopherols are converted into various polymeric derivatives[3,5]. Polymerization reactions remove the antioxidative activity of tocopherol derivatives[6]. Tocopherols also lose their antioxidative activity in the presence of iron(III) salts[7] because of their conversion into a mixture of quinoid oxidation products[8].

This paper reports the separation of the oxidation products of α-tocopherol by combined chromatographic techniques.

EXPERIMENTAL AND RESULTS

DL-α-Tocopherol was prepared by the alcoholysis of DL-α-tocopherol acetate (Nisshin Chemicals, Tokyo, Japan) with methanol. The α-tocopherol thus obtained was oxidized[9] in methanol by treatment with iron(III) chloride (reagent grade, Merck, Darmstadt, G.F.R.). L-Lysine hydrochloride dihydrate was produced in the Research Institute of Antibiotics and Biotransformations (Roztoky, near Prague, Czechoslovakia) and re-purified by ion-exchange chromatography.

The oxidation products of DL-α-tocopherol were separated on a thin layer of Silpearl macroporous silica gel (Kavalier Glassworks, Votice, Czechoslovakia) by using the following development procedures: A, with benzene; B, with a mixture of light petroleum (b.p. 40–60°), diethyl ether and acetone (94:5:1) for preparation

purposes and a mixture of light petroleum (b.p. 40–60°), diethyl ether and acetic acid (94:5:1) for analytical purposes.

The fractions were eluted with methanol and their spectra determined (CF 4 spectrophotometer, Optica, Milan, Italy). The fractions were analyzed by gel chromatography[5,10] on an apparatus constructed at the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences (Prague, Czechoslovakia) by using a series of five columns, 1.2 m × 8 mm, packed with the styrene–divinylbenzene copolymer S-GEL-832 (Institute of Macromolecular Chemistry), exclusion limit 1000 units of molecular weight. The elution was carried out at 25° with tetrahydrofuran at a flow-rate of *ca.* 35 ml/h. A Waters R-4 differential refractometer and a flow spectrometer (Development Workshop, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) were used as detectors at a wavelength of 254 nm. Both detectors were connected in series.

The reaction mixture obtained by the oxidation of DL-α-tocopherol with iron(III) chloride was fractionated by a triple development with a mixture of light petroleum (b.p. 40–60°), diethyl ether and acetone (Fig. 1). In this way some fractions which had not been separated by the elution with benzene[8] could be separated (Table I), as shown by repeated chromatography of fractions eluted with ethanol, this time with benzene as the developing solvent.

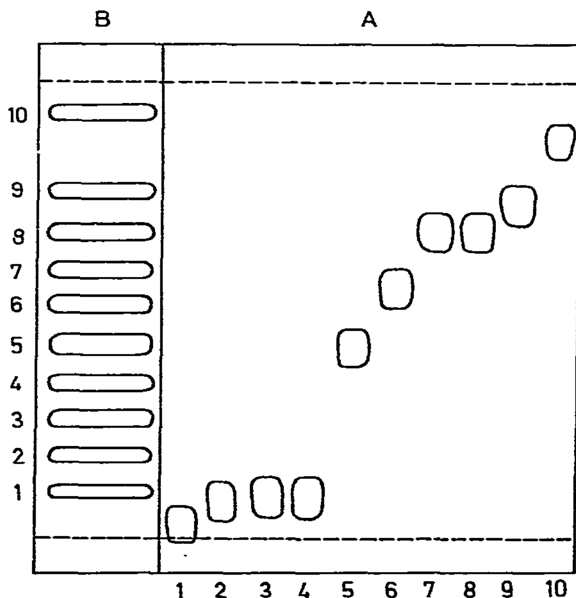

Fig. 1. Preparative thin-layer chromatogram of oxidation products of α-tocopherol. Sample: 1 g of α-tocopherol and 10 g of iron(III) chloride in 100 ml of methanol were heated at 50° for 3 h, then 100 ml of water added, the mixture was extracted with diethyl ether, the solvent distilled off at room temperature and the volume of the extract adjusted to 2 ml. Conditions of separation: Silpearl wide-pore silica gel (particle size 30–70 μm) plus 15% of plaster of Paris; layer thickness 0.9 mm; activation for 1 h at 105°. Chromatogram A: 50 mg of extract spread on a 40-mm band; developing mixture, light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (94:5:1); front distance, 145 mm. Chromatogram B: fractions from chromatogram A eluted with ethanol, spread on a Silpearl layer and developed with benzene to a front distance of 120 mm. 1–10 = Numbers of fractions.

TABLE I

CHROMATOGRAPHY OF OXIDATION PRODUCTS OF α-TOCOPHEROL ON A THIN LAYER OF SILICA GEL

*Number of fraction**	R_F**			*Colour of spot*	*Absorption maximum of extract (nm)*
	B	*A*	*according to ref. 8*		
1[a]	0.10	0.02	0.01 -0.02	Red	480
2[b]	0.18	0.08	0.001-0.07	Red	474
3	0.26	0.09	—	Orange	442
4[c]	0.33	0.08	0.01 -0.06	Red	463, 513
5[d]	0.43	0.41	0.37 -0.43	Yellowish	—
6	0.51	0.54	0.41 -0.54	Slightly violet	465
7	0.58	0.66	—	Yellow	425
8	0.67	0.66	—	Yellow	440
9	0.76	0.72	—	Yellow	435
10[e]	0.93	0.88	0.59 -0.63	Yellow	420

* The superscripts indicate identified components: a = tocored; b = α-tocopherylquinone; c = tocopurple; d = α-tocopherol; e = tocopherol ketoether dimer.

** Procedure A, developed with benzene; procedure B, developed with a mixture of light petroleum (b.p. 40–60°), diethyl ether and acetic acid (94:5:1); other conditions as in Fig. 1.

Fractions obtained by thin-layer chromatography were eluted with ethanol; on distilling off the solvent under reduced pressure and at room temperature they were separated by gel chromatography. The UV detection was five to six times more sensitive than detection with a differential refractometer. Fractions 1 (tocored), 2 (tocopherylquinone) and 3 and 4 (tocopurple) consisted of monomers and of a small amount of dimers (Fig. 2). These dimers were obviously formed only during the elution from the layer and the concentration of the extract, because they were readily detected also by thin-layer chromatography, where different R_F values were observed. With fractions 1 and 2, the main component was preceded by a small, incompletely separated side-component (Fig. 2), probably formed by solvation of the original molecule with tetrahydrofuran. Four less polar fractions (Nos. 7–10) consisted of a mixture of monomeric derivatives and of a minor amount of oligomers: only dimer for fraction 7, and dimer and trace amounts of trimers and higher oligomers for fractions 8–10 (Fig. 2). The elution volumes of the fractions are given in Table II. Fraction 10, containing spiroketo dimer, also contained a predominant monomeric fraction.

Monomeric and oligomeric fractions obtained by gel chromatography were again analyzed on a thin layer; an example is given in Fig. 3. It can be seen that the R_F values of both fractions are identical and therefore they could not be separated only on a thin layer of silica gel. The monomeric quinoid oxidation products are readily converted into dimeric and further into trimeric compounds[11–15]; the conversion could take place under the reaction conditions used.

The reaction of quinoid oxidation products of tocopherol with amino acids (which probably occurs as the main reaction in natural materials containing a large amount of free amino acids and proteins) gave rise to a number of coloured compounds; for instance, the reaction of tocopherylquinone (fraction 2, Fig. 1) with L-lysine [a mixture of 0.05 M L-lysine hydrochloride dihydrate in 1 M acetate buffer,

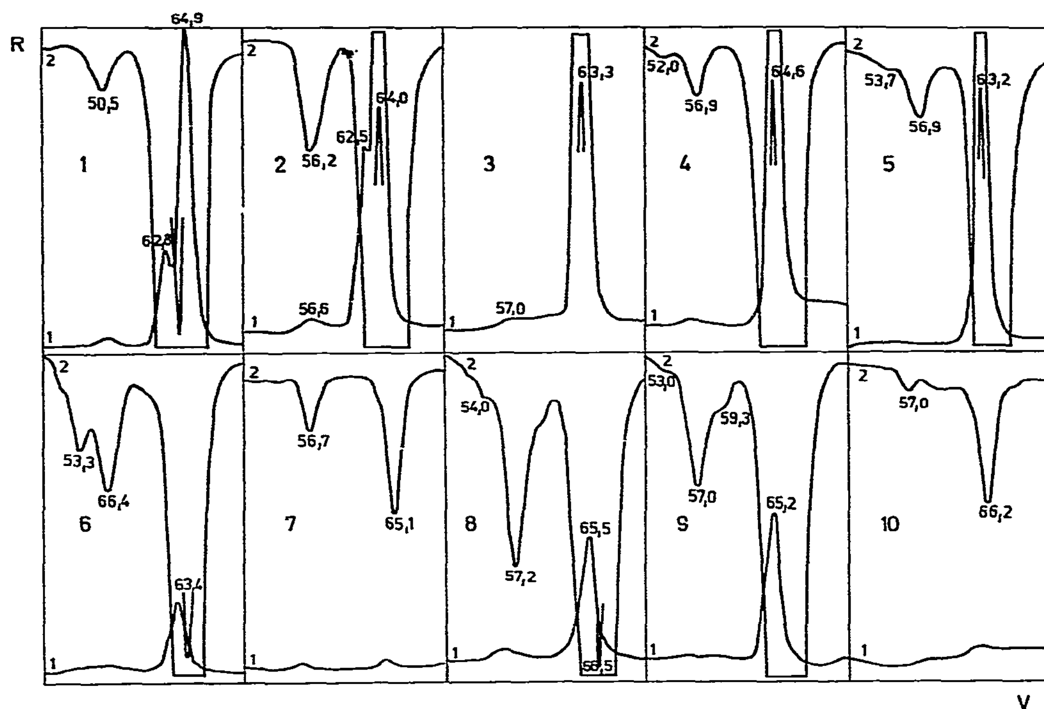

Fig. 2. Separation of oxidation products of α-tocopherol by gel chromatography. Conditions: 6 m $\times$ 8 mm columns; S-GEL-832; solvent, tetrahydrofuran; flow-rate, 35 ml/h; detector, Waters R-4 differential refractometer and flow UV detector (254 nm) connected in series. R = detector response; 1 = refractometer; 2 = spectrophotometer; V = elution volume (counts); 1–10 = numbers of fractions (*cf.*, Table I).

TABLE II

CHROMATOGRAPHY OF OXIDATION PRODUCTS OF α-TOCOPHEROL ON S-GEL-832

Conditions as in Fig. 2. Samples: fractions isolated by preparative thin-layer chromatography (*cf.*, Fig. 1).

Number of fraction	*V_e (counts)**				*Identified*
	M		*D*	*T*	
1	**64.3**	62.8[a]	57.0[b]	50.5[b]	Tocored
2	**64.0**	62.5[a]	56.2[b]		α-Tocopherylquinone
3	**63.3**		57.0		—
4	**64.6**		56.9[b]	52.0[b]	Tocopurple
5	**63.2**		56.9[b]	53.7[b]	α-Tocopherol
6	**64.3**[b]		56.4[b]	53.3[b]	—
7	**65.1**[b]		**56.7**[b]		—
8	**66.5**		57.2[b]	54.0[b]	—
9	**65.2**	59.3[b]	57.0[b]	53.0[b]	—
10	**66.2**[b]	60.5[b]	57.0[b]		Ketoether dimer

* Main fractions in bold. Superscripts: a, side component (see text); b, spectrophotometric detection; no superscript, refractometric detection. Subfractions: M (monomeric), D (dimeric), T (trimeric).

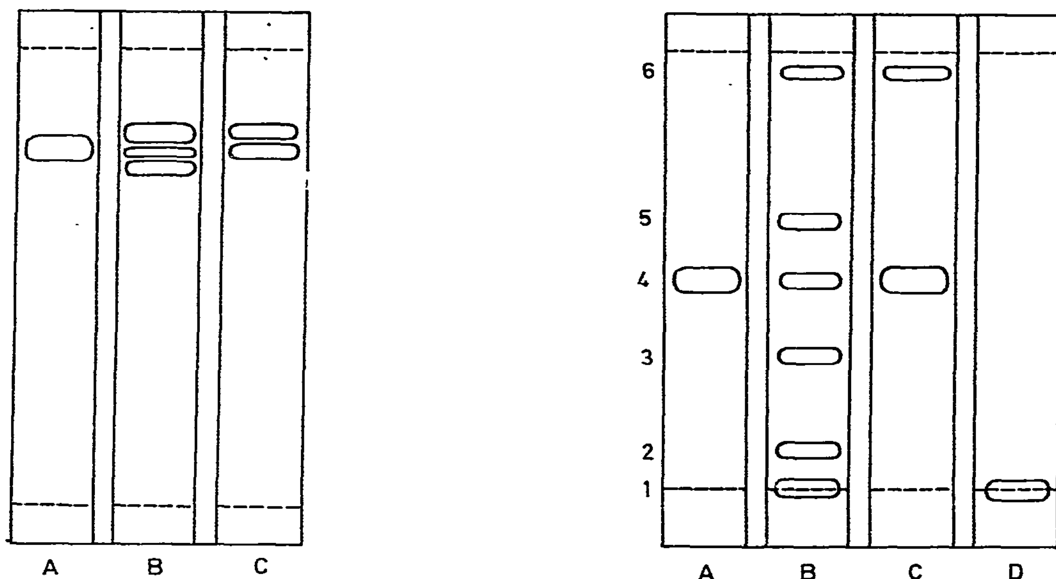

Fig. 3. Thin-layer chromatogram of sub-fractions obtained by gel chromatography. Sample: fraction No. 9 from preparative thin-layer chromatography (Fig. 1) after separation by gel chromatography (Fig. 2). Separation conditions: layer of Silufol UV_{254} wide-pore silica gel connected with starch (Kavalier Glassworks); activation, 30 min at 105°; developing mixture, light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (94:5:1); front distance, 120 mm; detection, 5% methanolic solution of molybdophosphoric acid. A = Original fraction No. 9; B = dimeric sub-fraction (57.0 counts in Fig. 2); C = monomeric sub-fraction (65.2 counts in Fig. 2).

Fig. 4. Thin-layer chromatogram of condensation products of α-tocopherylquinone with L-lysine. Conditions of separation: layer of Silufol UV_{254} wide-pore silica gel; activation, 30 min at 105°; amount of sample spread, 40 μg; developing system; light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (90:19:1); front distance, 115 mm. Samples: A = original α-tocopherylquinone (fraction No. 2, Fig. 1); B = reaction mixture (0.005 *M* tocopherylquinone and 0.025 *M* L-lysine in a mixture of 50% methanol and 50% 1 *M* acetate buffer, pH 5.6, heated at 60° for 9 h); C = control (similar to B, but without addition of L-lysine); D = control (similar to B, but without addition of α-tocopherylquinone). Colours of fractions: 1, brown (B) or almost colourless (D); 2, yellow-brown; 3, violet; 4, red; 5, yellow; 6, yellow.

pH 5.6, and a 0.01 *M* methanolic solution of tocopherylquinone in a ratio of 1:1 (v/v) was heated at 60° for 9 h] gave rise to a reaction mixture, the chromatography of which on a thin layer of silica gel yielded five coloured fractions (Fig. 4), which were eluted with ethanol and separated by gel chromatography. The chromatograms show (Fig. 5) that all fractions consisted of a monomeric sub-fraction and several oligomeric sub-fractions. The mechanism of the condensation reactions was probably similar to that of the reactions of the quinoid oxidation products of tocopherol with proteins[15].

The results in Tables I and II indicate that neither thin-layer chromatography on silica gel with solvent systems, when compounds are separated according to their polarity, nor chromatography on S-GEL-832 with tetrahydrofuran as solvent, when compounds are separated according to the size of the molecule, is adequate for the separation of the oxidation products of tocopherol and their condensation

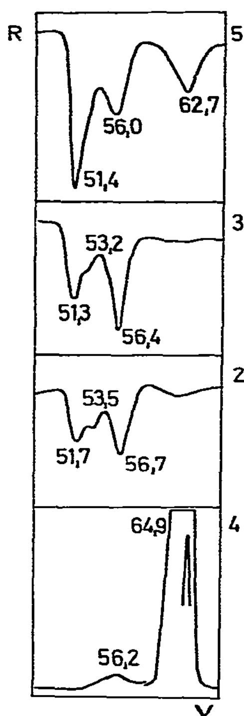

Fig. 5. Separation of condensation products of α-tocopherylquinone and L-lysine by gel chromatography. Conditions of separation as in Fig. 2. Samples: 5, 3, 2, 4 = fractions from Fig. 4. R = detector response (refractometer for fraction No. 4, spectrometer for fractions Nos. 2, 3 and 5); V = elution volume (for individual peaks, elution volumes in counts).

products with lysine. Only by combining both chromatographic techniques is it possible to separate the monomeric, dimeric and trimeric fractions of the oxidation products, which have different polarities.

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