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Note

Separation of α-tocopherol, α-tocopherolquinone and α-tocopherol dimer by reversed-phase high-performance liquid chromatography

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α-Tocopherol (Vitamin E, α-T) is important in physiology and biochemistry: it protects against various animal and human diseases (especially in childhood)[1]. Furthermore, α-T is a free radical scavenger and a singlet oxygen quencher[2]. *In vitro*, depending on its concentration, α-T acts as an antioxidant or a prooxidant towards polyunsaturated fatty acids[3]. It is oxidized simultaneously into α-tocopherolquinone (α-TQ) and α-tocopherol dimer (DT)[4]. The last two compounds are also found as metabolites of α-T *in vivo*.

α-T and related compounds have often been analyzed by high-performance liquid chromatography (HPLC) in plasma and blood[5–9], brain[10], fish liver[11] and food[12], or to obtain purified α-TQ[13] or to separate α-T, α-tocopheryl acetate (α-TA) and α-TQ[14]. This study describes an HPLC separation of α-T, α-TQ, DT and γ-tocopherol (γ-T). The last compound is used as an internal standard to allow a quantitative analysis of α-T, α-TQ and DT.

EXPERIMENTAL

Reagents and chemicals

α-T and γ-T were purchased from Hoffmann-Laroche (France) and methanol from Prolabo (France).

α-TQ was prepared according to the procedure of Schudel *et al.*[15] and then purified by HPLC. DT was obtained by the method of Skinner and Alaupovic[16] and then purified by HPLC.

Instrumentation

An LDC high-performance liquid chromatograph was purchased from Sopares (France) and equipped with two Constametric III pumps, a Gradient Master programmer, a Valco 7000-p.s.i. injector and a Spectromonitor III UV detector set at 290 nm and 0.16 a.u.f.s. A Linear recorder was used at a speed of 2 mm/min.

For gradient elution the entire system was employed. On the other hand, isocratic elutions were obtained by cutting out one pump and the gradient programmer.

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Chromatography

Chromatography was carried out on a stainless-steel column (20 × 0.47 cm I.D.) of Spherisorb ODS (particle size 5 μm) at room temperature.

In isocratic elutions, two solvent systems were used: I, methanol–water (85:15, v/v), to separate α-TQ, γ-T and α-T; II, pure methanol, to chromatograph DT. Gradient elutions of the four compounds were performed using a convex gradient with an initial mobile phase composed of 100% solvent I increasing over 8, 15 or 18 min to 100% solvent II. The flow-rate was 2 ml/min in both cases.

Sample preparations

The standard solutions were 0.5, 0.5, 2 and 2 μg per μl of methanol respectively for α-T, γ-T, α-TQ and DT. The samples injected into the chromatograph contained respectively 7.5, 7.5, 30 and 30 μg.

RESULTS AND DISCUSSION

Isocratic elution

Fig. 1A shows a chromatogram of α-TQ, γ-T and α-T separated by isocratic elution with solvent I (methanol–water, 85:15, v/v). The respective retention times were 11, 15 and 17.5 min; DT was not eluted. Fig. 1B shows a chromatogram of the four compounds obtained by isocratic elution with solvent II (pure methanol). α-TQ, γ-T and α-T were rapidly eluted and not separated; DT was eluted at 7.5 min.

Solvents containing between 85 and 100% methanol did not allow the complete

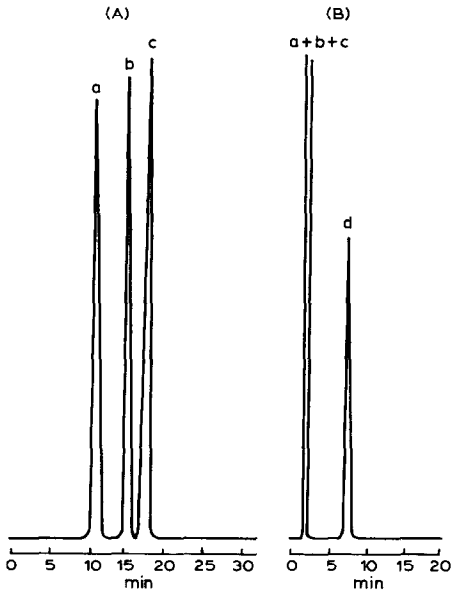

Fig. 1. HPLC separation of α-tocopherol and related compounds by isocratic elutions using: A, methanol–water (85:15, v/v) as solvent I, peak d is not eluted; B, pure methanol as solvent II, peaks a, b and c are not separated. Peaks: a = α-tocopherolquinone; b = γ-tocopherol; c = α-tocopherol; d = dimer of α-tocopherol.

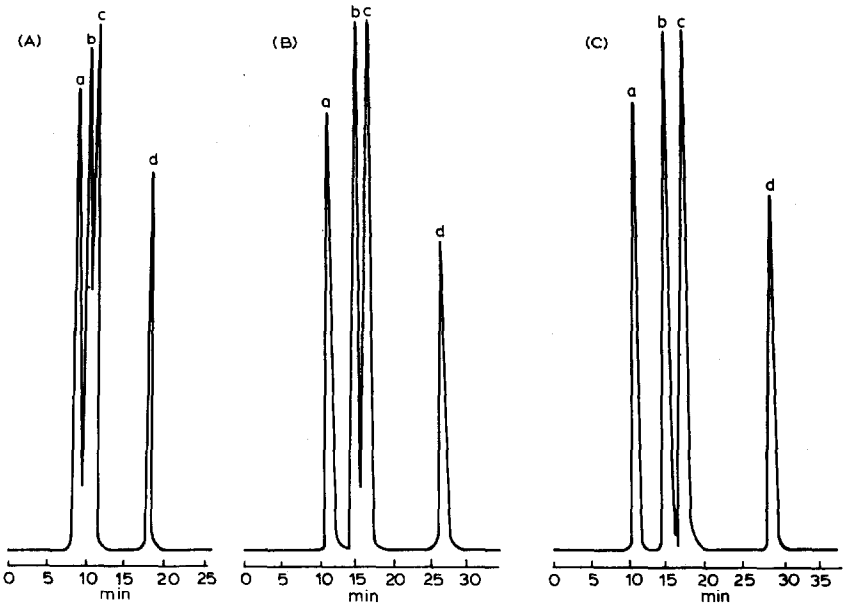

Fig. 2. HPLC separations of α-tocopherol and related compounds by convex gradient elutions with an initial mobile phase of 100% solvent I, methanol–water (85:15, v/v), increasing to 100% solvent II, pure methanol: A, gradient time 8 min, peaks a, b and c are not well separated; B, gradient time 15 min, b and c are not completely separated; C, gradient time 18 min, a, b, c and d are completely separated. Identity of peaks as in Fig. 1.

separation of α-TQ, γ-T and α-T. Furthermore, the retention time of DT was too large.

Gradient elution

The above results led us to attempt a convex gradient elution with an initial mobile phase of solvent I and a final phase of solvent II. Different rates of increase to 100% solvent II were tested. The retention times of the four compounds are reported in Table I and chromatograms in Fig. 2.

The retention times varied with the gradient. A time of 8 min did not allow complete separation of γ-T and α-T; neither did a time of 15 min although the separation of γ-T and α-T was almost complete. The best result was obtained with a

TABLE I

RETENTION TIMES (min) OF α-TOCOPHEROLQUINONE (α-TQ), γ-TOCOPHEROL (γ-T), α-TOCOPHEROL (α-T) AND α-TOCOPHEROL DIMER (DT)

Gradient time (min)	*α-TQ*	*γ-T*	*α-T*	*DT*
8	8	10	10.5	18
15	11.5	15	17	27
18	11.5	15.5	18	29.5

TABLE II

MEAN CONCENTRATIONS (M.C.) ± STANDARD DEVIATION (S.D.), COEFFICIENTS OF VARIATION (C.V.) AND EFFICIENCIES OF RECOVERY (E.R.)

Values found after six injections of 1 ml of solvent containing 4, 8 and 10 μg of α-T, α-TQ and DT, respectively.

	α-T	*α-TQ*	*D-T*
M.C. (μg/ml)	3.97	7.76	9.91
S.D. (μg/ml)	0.14	0.18	0.24
C.V. (%)	3.49	2.30	2.47
E.R. (%)	99.25	97.04	99.12

time of 18 min which allowed complete separation of all compounds at the maximum rate.

UV monitoring at 290 nm (maximum absorptions of α-T, γ-T, α-TQ and DT occur respectively at 294, 294, 270 and 300 nm) allowed us to work with higher sensitivity: 0.01 a.u.f.s. The change in the baseline due to the change in the absorption of the modified solvent did not affect the analysis of the compounds.

The limits of detection were 0.10, 0.40 and 0.60 μg/ml for α-T, α-TQ and DT, respectively. Calibration curves were obtained as follows. One millilitre of solvent (phosphate-buffered aqueous solution was added to 4 μg of γ-T (internal standard) and different concentrations of α-T (0.5–8 μg), α-TQ (1–18 μg) and DT (1–20 μg). After direct injection, the peak height (p.h.) ratios (p.h. α-T, α-TQ or DT/p.h. γ-T) were calculated. A linear relationship was found between these p.h. ratios and the concentrations, c (μg/ml), of α-T, α-TQ or DT (regression coefficients: p.h. α-T/p.h. γ-T and $c_{\alpha\text{-T}}$, $r = 0.999$; p.h. α-TQ/p.h. γ-T and $c_{\alpha\text{-TQ}}$, $r = 0.995$; p.h. DT/p.h. γ-T and c_{DT}, $r = 0.997$).

Ten millilitres of the solvent were then added to 40, 40, 80 and 100 μg of α-T, γ-T (internal standard), α-TQ and DT respectively, injected six times (one 1-ml injection per day during six consecutive days) (using a 1-ml loop) and the p.h. ratios (p.h. α-T/p.h. γ-T, p.h. α-TQ/p.h. γ-T and p.h. DT/p.h. γ-T) and the corresponding concentrations of α-T, α-TQ and DT calculated using the calibration curves. Table II gives the resulting mean concentrations (M.C.)± standard deviation (S.D.), the coefficients of variation (C.V.) and the efficiencies of recovery (E.R.).

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