Separation of α-Tocopherol and Its Oxidation Products by High Performance Liquid Chromatography

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A very sensitive high performance liquid chromatographic (HPLC) method was developed for the separation of α-tocopherol (α-T) and its five oxidation products: α-tocopheryl quinone (TQ), dimer (D), dihydroxy dimer (DHD), trimer (T) and 9-methoxy-α-tocopherone commonly called α-tocopheroxide (TO). The separation was achieved on a normal-phase silica-based column (Ultrasphere-Si), using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) at a flow rate of 0.4 ml/min, and the eluants were monitored simultaneously at their maximum absorptions using a variable-wavelength UV detector. The minimum detection limit is 0.01 μg for α-T, TQ and TO, 0.05 μg for DHD and D, and 0.1 μg for T/injection. This normal-phase method has the combined advantages of being very sensitive, fast and capable of separating all six compounds at the same time.
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α-Tocopherol (α-T), a naturally occurring antioxidant, reacts with free radicals or oxidizing agents to form several oxidation products. Some of these oxidation products include α-tocopheryl quinone (TQ), and dihydroxy dimer (DHD), dimer (D) and trimer (T) of α-tocopherol and 9-methoxy-α-tocopherone commonly called α-tocopheroxide (TO) (1-6). The first four of these oxidation products have been isolated as metabolites, and TQ was found to be the major metabolite (1,3,7).

Quantitative determination of α-T and its oxidation products (TOP) can be used to measure the degree of cell membrane peroxidation and the metabolic pathways of α-T, and useful in the prediction of the stability of oils (7,8). Gas chromatography, column chromatography and thin layer chromatography were methods used commonly for the separation of α-T and TOP (9-12). These methods have serious limitations because of their comparatively low sensitivity, long analysis time and possibility of increased oxidation. Lately, various high performance liquid chromatographic (HPLC) methods have been applied successfully to determine α-T in foods and feeds (13,14), and in tissues (15,16). HPLC methods also were reported for the simultaneous separation of α-T, TQ or TO (17-21) and for the separation of dimers of α-T (22).

Currently, a HPLC method is not available in the literature for the simultaneous separation of α-T and its five oxidation products (TQ, DHD, D, T and TO) with a very high sensitivity.

The present method is not only capable of simultaneously separating α-T and its most important five oxidation products in a single injection, but it has higher sensitivity than the previously published methods.

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Abbreviations: α-T, α-tocopherol; AUFS, absorption unit full scale; D, dimer; DHD, dihydroxy dimer; HPLC, high performance liquid chromatography; T, trimer; TO, α-tocopheroxide; TOP, α-T oxidation products; TQ, α-tocopheryl quinone.

MATERIALS AND METHODS

Reagents. α-T was purchased from Eastman-Kodak Company (Rochester, NY). Hexane (HPLC grade), methylene chloride (HPLC grade), methanol (ACS grade), ethanol (ACS grade) and diethyl ether (ACS grade) were purchased from Fisher Scientific Co. (Itasca, IL). Aluminum oxide was purchased from Bio-Rad Laboratory (Richmond, CA). All other chemicals used were reagent grade.

Synthesis and purification of standard TOP. The TQ was synthesized by the method of Eggitt and Norris (23), DHD, D and T according to Csallany et al. (3) and TO by the modified method of Boyer (24). Modification consisted of a change from ethanol to methanol in the synthesis of the compound. The TQ, DHD, D and T were purified by column chromatography on aluminum oxide, deactivated with 6% water, followed by HPLC on a 250 mm × 10 mm (i.d.) C_{18} reversed-phase semipreparatory column packed with Ultrasil-ODS (10 μm) (Beckman Instruments, Inc., Berkeley, CA). Purification of TO was carried out only on the above-mentioned reversed-phase C_{18} column. Standard stock solutions were made weight by volume. The HPLC was performed with a mobile phase of methanol/hexane/methylene chloride (95:4:1, v/v/v) at a flow rate of 0.4 ml/min for the purification of TQ and TO, and a mobile phase of methanol /hexane/methylene chloride (30:21:1, v/v/v) at a flow rate of 1.5 ml/min for the purification of D, DHD and T. The absorption spectrum of each purified compound was monitored by a DU-8 Spectrophotometer (Beckman Instruments, Inc.), using a quartz cuvette (1 × 1 cm).

HPLC equipment and conditions. The HPLC equipment for the separation of α-T and TOP consisted of a model 110A solvent metering pump (Beckman Instruments, Inc.) and an Altex Model 210 solvent injector equipped with a 100-μl loop (Beckman Instruments, Inc.). A normal-phase HPLC chromatography was performed on a 6-μm Ultrasphere-Si, 250 mm × 4.6 mm (i.d.) column, using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v), at a flow rate of 0.4 ml/min to separate α-T, TQ, DHD, D, T and TO. The UV absorptions were monitored at 292, 268, 292, 294, 298 and 240 nm for α-T, TQ, DHD, T, D and TO, respectively, using a Beckman model 165 variable-wavelength detector (with two channels) equipped with a 20-μl quartz flow cell (Beckman Instruments, Inc.). The wavelength was changed from 295 nm to 292 nm in the first channel after eluting T, D and DHD in order to monitor α-T at their maximum absorptions. Similar changes were made in the second channel which monitored TO at 240 nm, and ollowed by TQ at 268 nm. The detector was operated at a sensitivity of 0.05 absorption unit full scale (AUFS).

For preparation of the mobile phase, methanol was glass distilled, filtered through a 0.45-μm α-metricel filter (Gelman Sciences, Ann Arbor, MI), and degassed under vacuum. Hexane and methylene chloride also were degassed under vacuum.

TABLE 1

Preparation of Stock Solutions and Working Solutions of α-T and TOP

Compounds	Dilutants	Stock solutions (μg/ml)	Working solutions (μg/ml)
α-Tocopherol	a	310	30.8 – 6.2
α-Tocopheryl quinone	a	450	45.0 – 0.9
Dihydroxy dimer	a	370	37.4 – 0.6
Dimer	a	580	58.0 – 2.4
Trimer	a	460	230.0 – 8.7
9-Methoxy-α-tocopherone	a	230	22.7 – 3.8

[a]Hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v).

Preparation of calibration curves of α-T and TOP. The stock solutions containing 310 μg α-T, 450 μg TQ, 370 μg DHD, 590 μg T and 230 μg TO/ml were diluted with the mobile phase to the desired concentrations for the preparation of standard curves (Table 1). A 60-μl of diluted standard solution was injected with a 100-μl blunt-needle Hamilton syringe (Rainin Instrument Co., Inc., Woburn, MA) onto the column. Peak heights and areas were recorded by a Spectra Physics 4270 computing integrator (Arlington, IL).

RESULTS AND DISCUSSION

Purification of TOP. Five TOP (TQ, D, DHD, T and TO) were synthesized and purified. After purification, each peak of the TOP was collected from a separate chromatographic run of a given sample on a reversed-phase C_{18} semipreparatory column and scanned from 400 nm to 200 nm. The absorbance maxima were found to be for α-T, TQ, DHD, D, T and TO at 292, 268, 292, 298, 294 and 240 nm, respectively, in the appropriate mobile phase. No changes were observed in absorbance maxima of α-T and the five TOP, when solvents were changed from the mobile phase of the C_{18} column to hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v), which is used for the normal-phase HPLC. The absorbance maxima were slightly different in the mobile phase for α-T and TOP when compared to the published data (5,24) because absorbances of α-T, DHD, D and T were measured in isooctane, and TO was measured in ethanol.

Separation of α-T and TOP by HPLC. A typical separation of α-T and the five TOP on a normal-phase column, using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) at a flow rate of 0.4 ml/min is demonstrated in Figure 1. Elution times for T, D, DHD and α-T recorded by the first channel were 5.3, 6.9, 7.5 and 14.0 min, respectively. Elution times for TO and TQ recorded by the second channel were eluted at 10.5 and 52.0 min, respectively. Widicus and Kirk (20) have reported the separation of TO and TQ by normal-phase HPLC, however, these compounds were closely eluted. The rest of the TOP, therefore, do not separate well in this system. In the present method, the elution time is about 40 min between TO and TQ, thus the TOP can separate very well.

Calibration curves. Calibration curves, prepared by injection of 50 μl working solutions, were obtained by measuring the peak height response (Figs. 2 and 3). The relationships of peak heights to the amounts injected under these conditions were linear in the tested range. The following linear regression equations were obtained: Y = 4438X + 125, α-T; Y = 8831X + 105, TO; Y = 33878X + 0.99, TQ; Y = 6312X − 251, DHD; Y = 4768X + 570, D; and Y = 500X + 20, T. Correlation coefficients were found to be greater than 0.999 for each compound. Correlations of peak areas to the amounts injected also were calculated but no differences were found in comparison

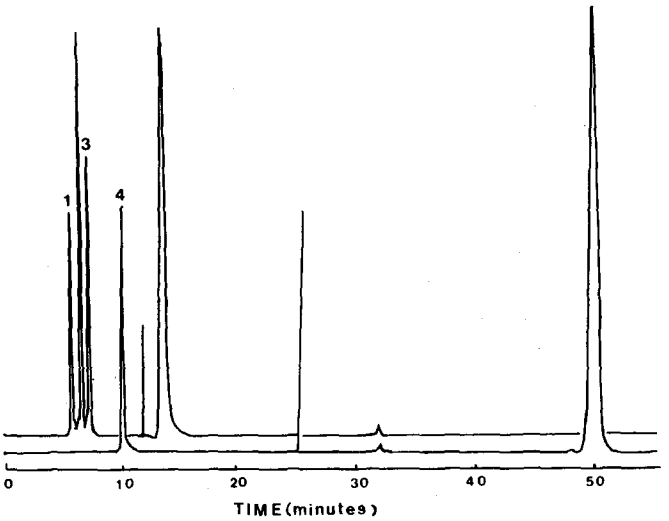

FIG. 1. Elution profile of α-tocopherol and its oxidation products on a 5-μm Ultrasphere-Si, 250 mm × 4.6 mm (i.d.). Peaks identification: trimer (1); dimer (2), dihydroxy dimer (3); 9-methoxy-α-tocopherone (4); α-tocopherol (5); α-tocopheryl quinone (6). Conditions: mobile phase, hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v); flow rate, 0.4 ml/min; detector with 295 nm for trimer, dimer and dihydroxy dimer, 292 nm for α-tocopherol, 240 nm for tocopherone and 268 nm for α-tocopheryl quinone; and attenuation at 0.05 AUFS.

FIG. 2. Calibration curves for high performance liquid chromatography of α-tocopherol (■—■), α-tocopheryl quinone (●—●) and 9-methoxy-α-tocopherone (O—O) on a 5-μm Ultrasphere-Si, 250 × 4.6 mm (i.d.). Data points represent the means of triplicate sample measurements; SEM was less than data point.

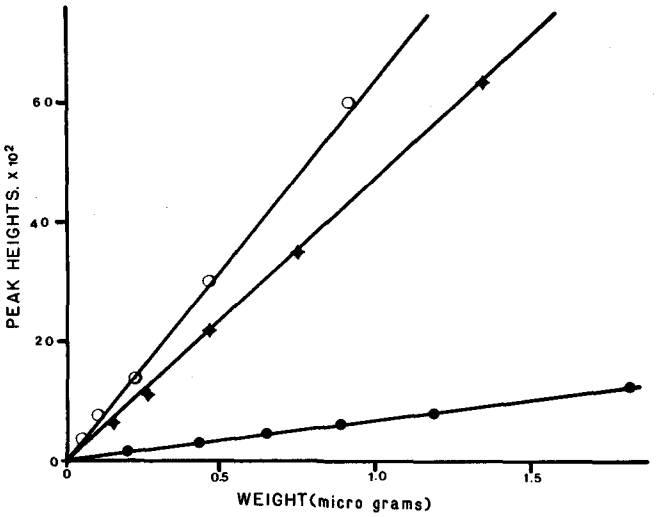

FIG. 3. Calibration curves for high-performance liquid chromatography of dihydroxy dimer (■—■), dimer (●—●), and trimer (○—○) on a 5-μm Ultrasphere-Si, 250 × 4.6 mm (i.d.). Data points represent the means of triplicate sample measurements; SEM was less than data point.

to peak heights. The lowest amounts detected by these newly developed normal-phase HPLC method were 0.01 μg for α-T, TQ and TO; 0.05 μg for DHD and D; and 0.1 μg for T/injection.

In summary, α-T, TQ, DHD, D, T and TO simultaneously were separated on a Ultrasphere-Si column using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v). This new normal-phase HPLC method has the following advantages compared to the previously published methods: the simultaneous separation of α-T and five TOP by a single chromatographic run, much higher sensitivity, and relatively longer retention time between TO and TQ ensuring good separation of TOP.

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