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Reversed-phase high-performance liquid chromatographic method using a pentafluorophenyl bonded phase for analysis of tocopherols

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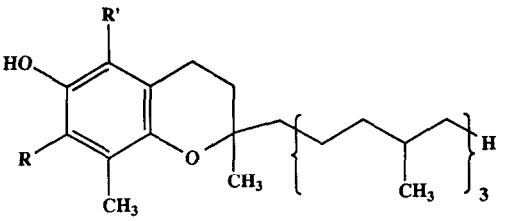
Abstract

A high-performance liquid chromatographic (HPLC) method to determine four tocopherols (α-, β-, γ-, and δ-) simultaneously was developed using a pentafluorophenyl reversed-phase column. A mobile phase of 92% aqueous methanol was used. Tocopherols were measured in vegetable oil, soybean oil deodorizer distillate, mixed tocopherol concentrate, and vitamin E pharmaceutical preparations.

1. Introduction

Several reports have appeared on the simultaneous measurement of tocopherols by high-performance liquid chromatography (HPLC) using normal-phase columns [1–4]. These methods appear to adequately separate the four major tocopherols (Fig. 1) but utilize silica columns and normal-phase solvents such as chloroform, tetrahydrofuran (THF), isopropanol (IPA), and hexane. On the other hand, to our knowledge there have been no methods reported that give satisfactory separation of α-, β-, γ-, and δ-tocopherols by reversed-phase HPLC. The difficulty appears to be in the separation of the isomeric β- and γ-tocopherols. Several groups have reported the separation of α-, β- and γ-, and δ-tocopherols [5–9], using reversed-phase columns. Satomura et al. [10] succeeded in partially separating these tocopherol isomers on a 120-Å C_{18} column, but the β- and γ-tocopherols were not baseline separated. Moreover, the analysis required elevated temperature and a 50-min run time. Because there are definite advantages in using reversed-phase columns and solvents, we investigated this separation



	R	R'	
1	H	H	δ - tocopherol
2	H	CH_3	β - tocopherol
3	CH_3	H	γ - tocopherol
4	CH_3	CH_3	α - tocopherol

Fig. 1. Chemical structures of the tocopherols.

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problem on a variety of reversed-phase chemistries and report in this paper on the separation of all four tocopherols in less than 20 min using a commercially available pentafluorophenyl (PFP) column.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Model L-6200 pump, a Model AS-4000 autosampler equipped with a 100-μl loop, and a Model L-4500A diode array detector (Hitachi Instruments, Fremont, CA, USA). The system was equipped with a Dynova 486 computer with a 320-megabyte hard drive (Foxborough, MA, USA) and DAD System Manager HPLC software (Hitachi Instruments). Chromatographic reports were printed on a Hewlett-Packard LaserJet 4 printer.

The preferred column was a 25 cm × 4.6 mm Taxsil, PFP reversed-phase column with 5-μm spherical particles (MetaChem Technologies, Torrance, CA, USA). Other PFP columns evaluated were a 25 cm × 4.6 mm Chromegabond PFP column (ES Industries, Marlton, NJ, USA), and a 25 cm × 4.6 mm TAC1 PFP column (Whatman, Clifton, NJ, USA).

2.2. Reagents

The tocopherols *d*-α, *d*-γ, and *d*-δ were obtained from Sigma (St. Louis, MO, USA). Rac-β-tocopherol was purchased from Matreya (Pleasant Gap, PA, USA). Methanol (S/P grade) was purchased from Baxter Diagnostics (Deerfield, IL, USA) and water was from the output of a Barnsted Model D4754 NANOpure water system (Dubuque, IA, USA). IPA, reagent alcohol, acetonitrile, and THF (UV grade) were Burdick and Jackson brand from Baxter Scientific Products (Bedford, MA, USA).

2.3. Chromatographic conditions

All columns were used without a guard column. The mobile phase was a 92:8 mixture of methanol and water made by placing 80 ml of water in a 1-l mixing cylinder and adding methanol to volume and mixing. The flow-rate was 1.0 ml/min and the temperature was ambient. An aliquot of 10 μl of sample was injected in isopropanol or tetrahydrofuran and detection was at 290 nm.

2.4. Procedure

A standard solution of the four tocopherols was prepared in IPA. Approximately 1 g of vegetable oil, accurately weighed, was placed in a 10-ml volumetric flask and diluted to volume with IPA. Similarly, 0.5 g of soybean oil deodorizer distillate and 10–20 mg of vitamin E preparations or mixed tocopherol concentrates were each diluted to 10.0 ml with IPA. In the event the sample did not give a clear solution in IPA, UV-grade THF was used instead. The sample and standard solutions (10 μl) were injected directly into the column without any prior filtration. Under these conditions, triglycerides present in vegetable oils did not elute from the column but the accumulated triglycerides could be washed from the column using IPA.

3. Results

3.1. Investigation of different columns and mobile phases

Satomura et al. [10] reported poor separation of β- and γ-tocopherols on a C_{18} column using mobile phases containing methanol–water and ethanol–water. They achieved partial separation on this column using a 65:35 mixture of IPA and water at elevated temperature. A mixture of β- and γ-tocopherols in methanol was prepared and injected into a variety of different reversed-phase columns using a mobile phase of methanol–water (95:5). We observed poor to no separation of β- and γ-tocopherols on a variety of different C_{18}, proprietary media, phenyl, diphenyl, and cyano columns. However, there was nearly baseline separation of these two isomers using a PFP column. Two other commercially available 25 cm × 4.6 mm PFP columns

Table 1
Peak resolution (R_s) of β- and γ-tocopherols utilizing the MetaChem PFP column under different HPLC conditions

Mobile phase Methanol–water	Flow-rate (ml/min)	Retention time of γ-tocopherol (min)	R_s
95:5	1.0	6.9	0.83
92:8	1.0	16.5	1.53
92:8	1.5	10.5	1.36
92:8[a]	1.5	9.2	1.26
92:8[b]	1.5	6.6	1.31
90:10	1.0	26.4	1.80
90:10	1.5	16.3	1.61
85:15	1.0	74.7	2.78
85:15	1.5	51.3	2.28

[a]ES Industries Chromegabond PFP column (25 cm × 4.6 mm).
[b]Whatman TAC1 PFP column (25 cm × 4.6 mm).

were also investigated. Both the ES Industries PFP and the Whatman TAC1 PFP showed good separation of β- and γ-tocopherols, but the separation was best with the MetaChem PFP column (see also Table 1). Aqueous mobile phase mixtures employing ethanol, IPA, and acetonitrile were also investigated, but these did not improve the separation of β- and γ-tocopherols.

3.2. Selectivity of the PFP column

The chromatogram illustrated in Fig. 2 shows the results obtained with isocratic elution on a PFP column at ambient temperature using a 92:8 mixture of methanol and water on a standard mixture of α-, β-, γ-, and δ-tocopherols. Because of the unique selectivity of the PFP reversed-phase chemistry, baseline separation of these four tocopherol isomers could be easily obtained using a variety of methanol, water, flow-rate, and temperature conditions. Improved separation of β- and γ-tocopherols was obtained by increasing the water content of the mobile phase. This caused a corresponding increase in retention time of the tocopherols that could be offset partially by increasing either the flow rate or the temperature (see Table 1). However, our goal was to develop a rapid, isocratic method for the separation of tocopherols, and a mobile

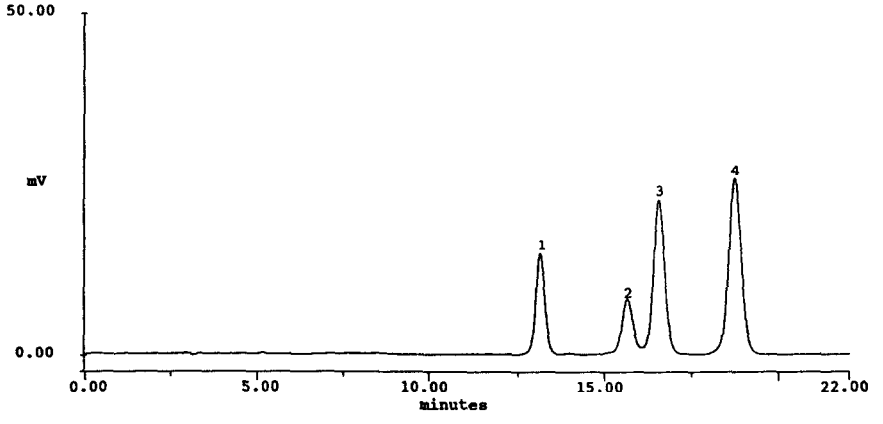

Fig. 2. Chromatogram of a standard mixture of tocopherols. Column: MetaChem PFP, 5 μm; eluent: methanol–water (92:8, v/v); flow-rate: 1 ml/min; UV detection: 290 nm. Peaks: 1 = δ-tocopherol; 2 = β-tocopherol; 3 = γ-tocopherol; 4 = α-tocopherol.

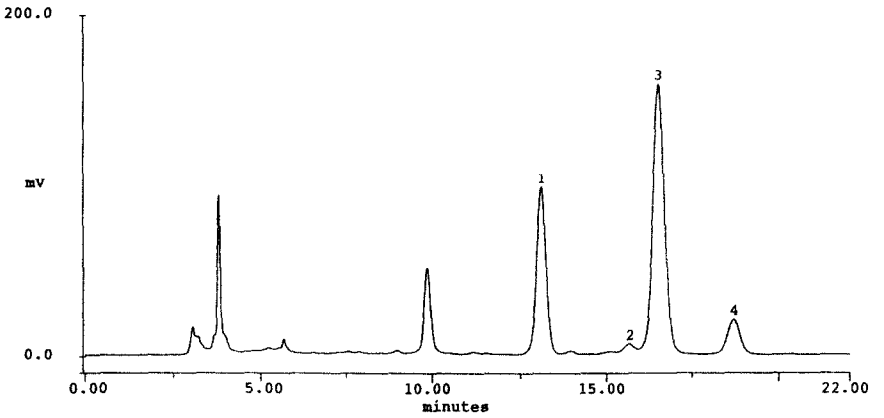

Fig. 3. Chromatogram of soybean oil deodorizer distillate, 0.5 g diluted to 10.0 ml with IPA. Chromatographic conditions and peaks are the same as in Fig. 2.

phase mixture of 92% aqueous methanol flowing at 1.0 ml/min gave baseline resolution of β- and γ-tocopherols in minimum time.

3.3. Analysis of samples

Fig. 3 shows the chromatogram obtained on soybean oil deodorizer distillate. This distillate is the starting material for most commercial production of naturally derived mixed tocopherols and vitamin E [11]. There are numerous mixed tocopherol antioxidant and vitamin E preparations on the market that could be analyzed by this method. Fig. 4 illustrates a typical chromatogram obtained on a liquid-filled capsule of naturally derived vitamin E.

Fig. 5 shows the chromatogram obtained on soybean oil. The method permits the identification and estimation of the four individual tocopherols in vegetable oils where they are present in low concentration. One advantage of the method when analyzing such samples is that the small β-tocopherol peak elutes before the much larger γ-tocopherol peak.

Using this method, d-α-tocopherol acetate eluted at a retention time of 1.3 relative to α-tocopherol, and hence, this semisynthetic tocopherol could serve as an internal standard

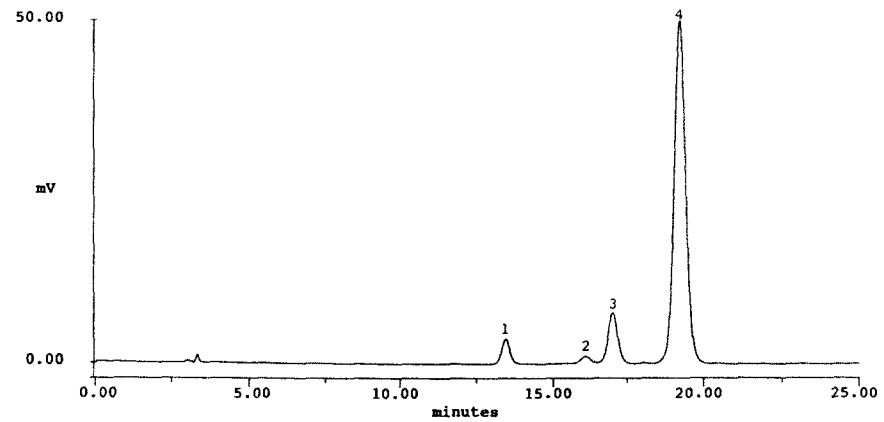

Fig. 4. Chromatogram of a sample of a 400-mg, natural-source, soft-gelatin, vitamin E capsule, 10 mg fill-diluted to 10.0 ml with IPA. Chromatographic conditions and peaks are the same as in Fig. 2.

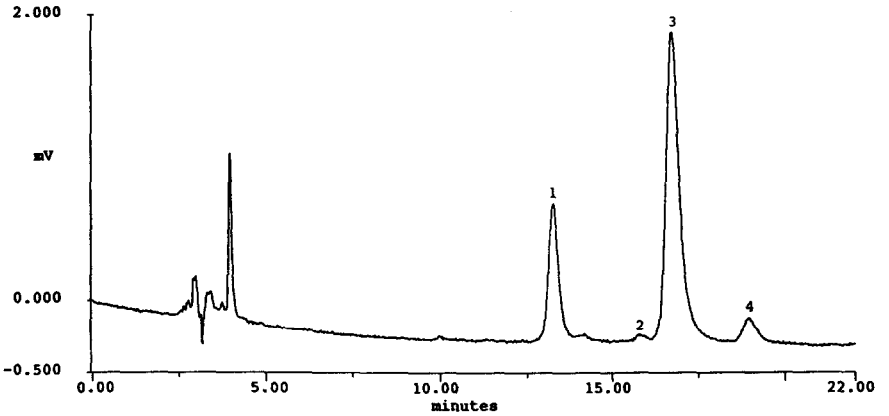

Fig. 5. Chromatogram of refined soybean oil, 1.0 g diluted to 10.0 ml with IPA. Chromatographic conditions and peaks the same as in Fig. 2.

for the analysis of naturally occurring tocopherols. In addition *trans*-retinol (vitamin A) and *trans*-β-carotene eluted at relative retention times of 0.4 and 4.7, respectively, using this method.

4. Discussion

The pentafluorophenyl bonded phase appears to provide unique selectivity for the separation of a variety of aromatic compounds [12,13]. A recent example of this unique selectivity is the report from our laboratories on the use of the PFP column for the difficult separation of taxol from other related taxanes [14]. Unlike more traditional reversed-phase media (C_{18}, C_8, phenyl), the order of elution of β- and γ-tocopherols was reversed using the PFP column, and β-tocopherol eluted before γ-tocopherol (in the same order as normal-phase chromatography). However, the elution of the δ- and α-tocopherol was the same as with all other reversed-phase columns tested. This reversal of elution of β- and γ-tocopherols is an advantage because the natural abundance of γ-tocopherol is normally many times greater than β-tocopherol, and it is easier to separate and quantify a small fronting peak than a small tailing peak.

It is important to be able to individually quantify β- and γ-tocopherol because β-tocopherol has five times the vitamin E activity of γ-tocopherol [15]. However, many of the published methods (including the official methods of the USP/NF [16], FCC [17], and AOAC [18], which use GC separation of the propionate esters of tocopherols) do not separate β- and γ-tocopherols. Reversed-phase HPLC has become a common method for assaying vitamins and other pharmaceutical components because of its high sensitivity, selectivity, accuracy, ease of use, and ruggedness. The method described here is much simpler to perform and more rapid than the GC methods of the official compendia and the normal-phase methods of various authors. In addition, the method determines the content of the four major tocopherols simultaneously in samples of vegetable oil, pharmaceutical vitamin E preparations, antioxidant preparations, mixed tocopherol concentrates, and crude vegetable oil distillates.

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