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Separation of tocopherols on various chemically bonded phases in microcolumn liquid chromatography

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Vitamin E, as it occurs naturally, consists of eight compounds which belong to two series of methyl-substituted chromanols with either a saturated (the tocopherols) or unsaturated (the tocotrienols) side chain in the 2-position. The four naturally occurring tocopherols, *i.e.*, *d*-α, *d*-β, *d*-γ and *d*-δ tocopherols, differ in the number and position of the methyl groups on the chromanol ring. They show pronounced quantitative differences in biological activity and, hence, contribute to different degrees to the overall vitamin E supply. They also differ in their ability to protect natural products, *e.g.*, vegetable oils, from oxidative degradation (antioxidant properties)[1].

Tsen[2] reported an improved spectrometric method for the determination of tocopherols, using 4,7-diphenyl-1,10-phenanthroline. A simultaneous determination of α-tocopherol and retinol (vitamin A) is meaningful in view of the recently suggested biological interaction between the two vitamins, and several have been established for their simultaneous in biological materials, especially in human serum using high-performance liquid chromatography (HPLC)[3–5]. Cort *et al.*[6] reported the separation and identification of vitamin E isomers using a normal-phase Chromegasphere SI 60 column with 2.5% tetrahydrofuran (THF) in isooctane as the mobile phase and fluorescence detection.

Normal-phase chromatography on a polar stationary phase allows rapid and easy differentiation of positional vitamin E isomers, in agreement with the well known stereochemical selectivity of silicic acid for isomers[1]. Generally, the separation of tocopherols is achieved using normal-phase silica columns[7–10] and also amino bonded columns[11]. Separation of the isomers using reversed-phase LC was tried[10,11], and the tocopherols were easily separated into α-, β- plus γ- and δ-forms. Separation of β- and γ-tocopherols could not be achieved under a variety of experimental conditions, however.

Developments in microcolumn LC have recently led to high resolution and higher sensitivity[12]. In this investigation, separations of tocopherols using microcolumn LC have been performed on various chemically bonded phases in order to find a good system to separate β- and γ-tocopherols.

EXPERIMENTAL

The *d*-α-, *d*-β-, *d*-γ- and *d*-δ-tocopherols (Ezai, Tokyo, Japan) were dissolved in *n*-hexane (Wako, Osaka, Japan) and injected directly. Hexafluoroisopropanol (FIPA)

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TABLE I

PACKING MATERIALS USED

No.	*Stationary phase*	*Particle diameter (μm)*	*Pore size (Å)*
1	Naphthylethyl (N. Tanaka, Kyoto Institute of Technology, Kyoto, Japan)	5	147
2	Phenyl (M. Okamoto, Tajimi Hospital, Tajimi, Japan)	5	150
3	Diphenyl (M. Okamoto)	5	150
4	Triphenyl (M. Okamoto)	5	180
5	Pyrenylethyl (N. Tanaka)	5	147
6	Polymer-based C_{18} (I) (TSK Gel octadecyl PW4; Tosoh, Nanyo, Japan)	7	110–120
7	Polymer-based C_{18} (II) (Asahi pak ODP; Asahi Kasei, Kawasaki, Japan)	5	*
8	Polymeric C_{18} (Vydac 201TPB5; Separation Group, Hesperia, CA, U.S.A.)	5	300
9	Monomeric C_{18} (N. Tanaka)	5	110
10	Polymer-coated C_{18} (Capcell pak; Shisheido, Tokyo, Japan)	5	120

* Informations not available.

(Showa Denko, Tokyo, Japan) was used as a mobile phase modifier for the normal-phase system. All other chemicals such as methanol, acetonitrile and isopropanol (IPA) were commercially available.

The microcolumn LC system was comprised of a MF-2 microfeeder (Azuma Electric, Tokyo, Japan) and an Uvidec 100 detector (Jasco, Tokyo, Japan) set at 295 nm, the cell volume being 0.65 μl. A microloop injector Jasco ML-422 (0.1 μl) was used for sample introduction. The columns used were fused-silica capillaries (Quadrex; Kasei Kogyo, Tokyo, Japan), 500–1000 mm × 0.53 mm I.D., packed with various bonded phases by the conventional slurry technique. Details of the packing materials utilized are summarized in Table I.

RESULTS AND DISCUSSION

LC is very suitable for the analysis of tocopherol isomers, especially since specific detection methods can be employed and exposure of the tocopherols to air can be minimized. LC analysis of tocopherols has mainly been performed with silica columns, *e.g.*, Corasil[10]. Although chemically bonded polar phases have been little used, Westerberg *et al.*[11] resolved all tocopherols using a μBondapak-NH_2 column with *n*-hexane–ethanol (99.2:0.8) as the mobile phase. In this work, microcolumns packed with various chemically bonded phases were tried for tocopherol separation in both normal- and reversed-phase separation systems, in order to find a better separation system for vitamin E isomers.

For the normal-phase system, bonded phases such as naphthylethyl, pyrenylethyl, phenyl, diphenyl and triphenyl were evaluated. First, 100% *n*-hexane was used as the mobile phase and the relative retention times for all tocopherols are listed in Table II. It is apparent that the pyrenylethyl, phenyl, diphenyl and triphenyl bonded phases did not give sufficient retention to enable separation of the four tocopherols, especially β- and γ-tocopherols. However, with the naphthylethyl bonded phase column, the tocopherols seem to be completely separated, and an unique elution order was observed. Generally, the elution order to tocopherols in a normal-phase system is

TABLE II

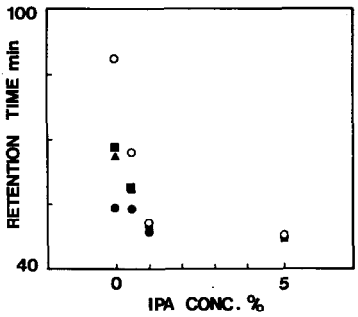
RELATIVE RETENTION TIMES OF TOCOPHEROLS IN THE NORMAL-PHASE SYSTEM USING 100% HEXANE AS THE MOBILE PHASE

Stationary phases are numbered as in Table I.

Stationary phase	*Relative retention time*		
	β/α	γ/α	δ/α
1	1.258	1.218	1.635
2	1.145	1.156	1.447
3	1.212	1.215	1.433
4	1.112	1.125	1.224
5	1.008	1.012	1.020

α-, β-, γ- and δ-tocopherols, respectively[6–11]. However, on the naphthylethyl bonded phase column the order is α-, γ-, β- and δ-tocopherols. This indicates the existance of some effect of the naphthylethyl surface bonded phase on the retention, because the shape of the aromatic moieties on the stationary phase surface is planar and they are readily polarizable; thus some π–π interactions are expected. Consequently, the naphthylethyl bonded phase should be more retentive for aromatic substances, particularly in certain biochemical separations and for molecules having rigid three-dimensional structures, *e.g.*, tocopherols. Finally, it appears that, on the naphthylethyl bonded phase column, γ-tocopherol seems to be more bulky or planar than β-tocopherol, although in the normal-phase system generally the elution order is that of decreasing sample size. However, the γ- and β- order as seen on the naphthylethyl phase is the same as that found by Lin and Horning[13] in gas chromatography using an open-tubular glass capillary column, coated with a polar phase PZ-176, on which the tocopherols were separated as their trimethylsilyl ether derivatives.

Further evaluation of the separation of tocopherols in the normal-phase system was carried out by using a naphthylethyl bonded phase column (750 mm × 0.53 mm I.D.), because this is very promising for the separation. The effect of the mobile phase composition on the resolution was evaluated with various concentration rates of isopropanol in *n*-hexane. The results are shown in Fig. 1, where the relationship between the retention times of tocopherols and the isopropanol concentration in the mobile phase is illustrated. These results indicated that isopropanol is not suitable as a modifier for tocopherol separation on the naphthylethyl bonded phase column. Therefore, the separation of tocopherols was then performed using hexafluoroisopropanol in *n*-hexane as the mobile phase. The relationship between the retention times of the tocopherols and the hexafluoroisopropanol concentration in the mobile phase is shown in Fig. 2. It is seen that good separation should be achieved at 0, 0.05 and 0.1% hexafluoroisopropanol in the mobile phase. The time required for the analysis at 0.1% hexafluoroisopropanol is shorter than that at 0 or 0.05% hexafluoroisopropanol. In addition, at 0 and 0.05% hexafluoroisopropanol the tocopherols peaks show some tailing. It is concluded that 0.1% hexafluoroisopropanol in *n*-hexane as the mobile phase gives the best separation of tocopherols on the naphthylethyl bonded phase column.



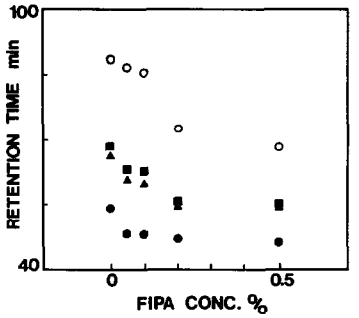


Fig. 1. Relationship between the retention times of tocopherols and the isopropanol (IPA) concentration in *n*-hexane as the mobile phase on a naphthylethyl bonded phase column (750 mm × 0.53 mm I.D.). ●, α-; ■, β-; ▲, γ- and ○, δ-tocopherol.

Fig. 2. Relationship between the retention times of tocopherols and the hexafluoroisopropanol (FIPA) concentration in *n*-hexane on a naphthylethyl bonded phase column. Details as in Fig. 1.

A longer column of the naphthylethyl bonded phase (1000 mm × 0.53 mm I.D.) was then tested using 0.1% hexafluoroisopropanol in *n*-hexane as the mobile phase. Fig. 3 shows the resulting chromatogram of the separation of tocopherols.

Although the reversed-phase system offers some practical advantages, *e.g.*, column stability, reproducibility of retention times and rapid equilibration, the retention on this type of support is essentially governed by the number of carbon atoms in a molecule. Therefore, this system generally fails to resolve some positional isomers[1]. Nevertheless, the separation of tocopherols using reversed-phase systems has been attempted[10,11] and the tocopherols were easily separated into α-, β- plus γ- and δ-forms.

In this work, the separation of tocopherols using a reversed-phase system has been atempted using various stationary phases. We sought the optimum separation system for β- and γ-tocopherols. No separations have been observed on bonded phases such as phenyl, diphenyl, triphenyl, naphthylethyl and pyrenylethyl with various mobile phase systems. Therefore, C_{18} phases were evaluated, namely two polymer-based C_{18}, polymeric C_{18}, monomeric C_{18} and polymer-coated C_{18} phases. First, 100% methanol as the mobile phase was tried and the relative retention times of the tocopherols are summarized in Table III. From these results it appears that β- and γ-tocopherols do not have sufficient difference in retention for their good separation. 100% Acetonitrile was then tried as the mobile phase and the relative retention times of the tocopherols are listed in Table IV. It is apparent that only the polymeric C_{18} column gave a reasonable separation, although the tocopherols peaks showed tailing.

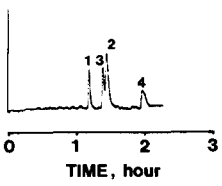


Fig. 3. Chromatogram of tocopherols on a naphthylethyl bonded phase column with 0.1% hexafluoroisopropanol in *n*-hexane as the mobile phase. Peak assignment: 1 = α-; 2 = β-; 3 = γ- and 4 = δ-tocopherol.

TABLE III

RELATIVE RETENTION TIMES OF TOCOPHEROLS IN THE REVERSED-PHASE SYSTEM USING 100% METHANOL AS THE MOBILE PHASE

Stationary phases numbered as in Table I.

Stationary phase	*Relative retention time*		
	β/α	γ/α	δ/α
6	0.926	0.932	0.840
7	0.878	0.872	0.760
8	0.856	0.875	0.815
9	0.860	0.850	0.732
10	0.878	0.871	0.760

TABLE IV

RELATIVE RETENTION TIMES OF TOCOPHEROLS IN THE REVERSED-PHASE SYSTEM WITH 100% ACETONITRILE AS THE MOBILE PHASE

Stationary phases numbered as in Table I.

Stationary phase	*Relative retention time*		
	β/α	γ/α	δ/α
6	0.980	0.986	0.978
7	0.969	0.964	0.890
8	0.773	0.790	0.708
9	0.946	0.943	0.774
10	0.844	0.842	0.710

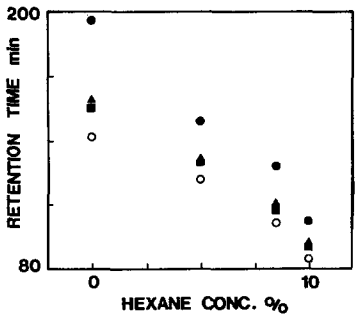


Fig. 4. Relationship between the retention times of tocopherols and the *n*-hexane concentration in acetonitrile as the mobile phase on a polymeric C_{18} column (750 mm × 0.53 mm I.D.). Other details as in Fig. 1.

Fig. 5. Chromatogram of tocopherols on the polymeric C_{18} column with 8.5% *n*-hexane in acetonitrile as the mobile phase. Peaks numbered as in Fig. 3.

From these results, the separation of four tocopherols on the polymeric C_{18} column was investigated using various mobile phase compositions, *e.g.*, acetonitrile–methanol, acetonitrile–THF and acetonitrile–hexane. Acetonitrile–methanol and acetonitrile–THF could not separate β- and γ-tocopherols, although there was no peak tailing. Acetonitrile–hexane gave a good separation of tocopherols.

The longer polymeric C_{18} column (length 750 mm) was employed to improve the resolution, using various concentration ratios of *n*-hexane in acetonitrile as the mobile phase. The result is shown in Fig. 4, where the relationship between the retention of tocopherols and the *n*-hexane concentration in the mobile phase is demonstrated. These results indicate that the best separation should be achieved with a mobile phase of 8.5% *n*-hexane in acetonitrile. A chromatogram of the separation of tocopherols in the reversed-phase system using the polymeric C_{18} phase is presented in Fig. 5.

CONCLUSION

The separation of tocopherols on various chemically bonded phases in microcolumn liquid chromatography has been investigated. For the normal-phase system, a naphthylethyl bonded phase column with 0.1% hexafluoroisopropanol in *n*-hexane as the mobile phase gave the best separation. The elution order of the tocopherols was unique, namely α-, γ-, β- and δ-tocopherol. A polymeric C_{18} column with 8.5% *n*-hexane in acetonitrile as the mobile phase gave the best results in various reversed-phase systems, the four tocopherols being well separated. Based on the results, a reversed-phase separation of tocopherol isomers is proposed.

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