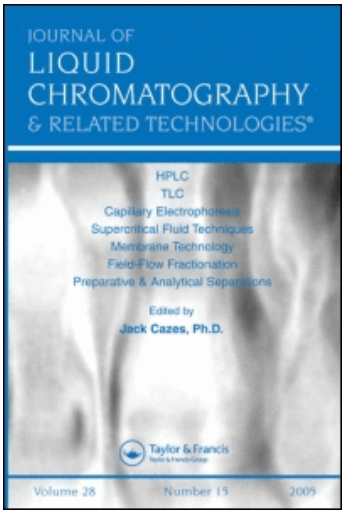

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SEPARATION OF TOCOLS BY HPLC ON AN AMINO-CYANO POLAR PHASE COLUMN

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ABSTRACT

A method is described for separating α-, β-, γ- and δ-tocopherols, their corresponding tocomonoenols and tocotrienols, and plastochromanol-8 on a 5 μm amino-cyano HPLC column using hexane:tetrahydrofuran (94:6) as eluant. The detection limit for α-tocopherol using fluorescence excitation at 210 nm was 1 ng. Results of analysis on six seed oils are presented.

INTRODUCTION

The tocols are a group of structurally related compounds that are individually named according to whether the phytyl side chain to the chroman ring has no double bonds (tocopherols : T), one double bond (tocomonoenols : T-1), or three double bonds (tocotrienols : T-3). Further classification is by the methyl substitution on the phenolic ring : 5,7,8-trimethyltocol (α-T); 5,8-dimethyltocol (β-T); 7,8-dimethyltocol (γ-T), and 8-methyltocol (δ-T). Interest in these tocols centres on their

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varying vitamin E activity (1) which is directly related to their antioxidant activity (2).

The first tocol identified, and having the highest vitamin E activity, was α-T. The other naturally occurring tocols were identified as analytical methods improved. Initially, the tocopherols were separated by column chromatography (3) and then by paper chromatography (4), thin-layer chromatography (5), and high-performance liquid chromatography (HPLC). The first HPLC systems for the separation of the four tocopherols comprised a silica column with hexane eluant containing a polar modifier such as diisopropyl ether (6). Because of the inherent instability and consequent poor reproducibility with silica columns, the more stable reversed phase system came to be more commonly used. However, analytical problems associated with the high polarity of the typical methanol : water eluant often arise with this system. To overcome these problems we developed a method for the separation and determination of α-T based on a reversed phase octadecylsilane (ODS) column with hexane:propan-2-ol (99:1) as eluant (7). We now report modifications of this method that improve sensitivity and reproducibility, and that enable the separation of the above 12 tocols and plastochromanol-8 (P-8), a tocol structurally related to γ-T-3 (8), to be achieved.

EXPERIMENTAL

Chemicals

Hexane was distilled over potassium hydroxide from technical hexane (Mobil Pegasol 1516). L-ascorbic acid was Reagent grade and ethanol, potassium hydroxide, sulphuric acid, propan-2-ol, and 1,4-dioxan were Analar grade, British Drug Houses (Poole, U.K.). Tetrahydrofuran (THF) was Unichrom grade, Ajax Chemicals (Sydney, Australia). Palm, castor, wheat germ, soybean, maize, and linseed oils were obtained from retail outlets.

α-T was obtained from Sigma (St Louis, U.S.A.), β-T from Supelco (Bellefonte, U.S.A.), and γ-T from Eastman Kodak

(Rochester, U.S.A.). δ-T was isolated from soybean oil by separation with chloroform on Sephadex LH20 (Hoogenboom, unpublished). The other tocols were not available commercially and so were identified in the various oils by reference to published data on the natural occurrence of these tocols (1,9) and their retention times relative to the three commercially available tocopherols and δ-T using various HPLC column/eluant systems (9).

Instrumentation

The HPLC system comprised a Tracor (Austin, U.S.A.) 995 pump; Rheodyne (Berkeley, U.S.A.) 7120 injector with 40 µl loop; Rheodyne 7302 column inlet filter; Whatman Partisil PXS 5/25 PAC or PXS 10/25 PAC column; and a Varian (Palo Alto, U.S.A.) Fluorichrom fluorescence detector with deuterium lamp fitted with forced air convection cooling. A Corion (Holliston, U.S.A.) 210 nm interference filter was used for excitation and a Varian 325 nm band filter for emission. Detector responses were monitored on a Spectra-Physics 4270 integrator. Eluant mixtures were de-gassed and pumped isocratically at the stated flow rates.

A Shimadzu RF-540 spectrofluorimeter was used to measure the relative fluorescence of α-T in various eluants.

RESULTS AND DISCUSSION

Most experimental work was carried out on the 5 µm PAC column using hexane modified by the addition of propan-2-ol, 1,4-dioxan, or THF. The effect of these polar modifiers on the retention times for the four tocopherols is shown in Fig. 1. Propan-2-ol at concentrations of 1-2% gave good separation of α-T and δ-T with comparatively short retention times of 2-11 min, but β-T and γ-T were less well resolved. THF and 1,4-dioxan readily separated all four tocopherols but higher concentrations (5-8%) of these polar modifiers were required to achieve similar short retention times.

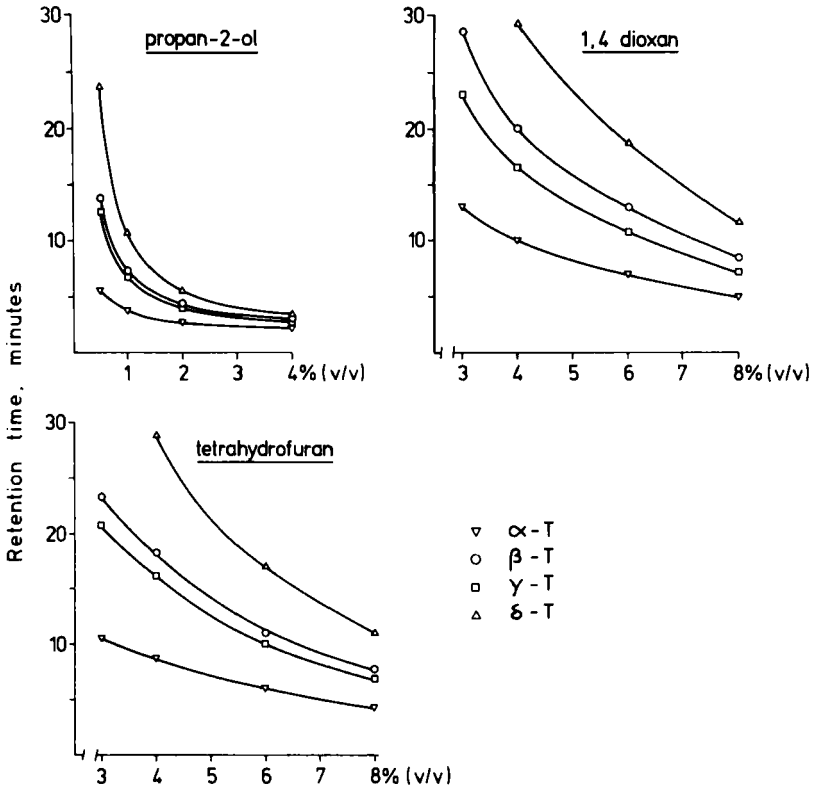

FIGURE 1. Effect of polar modifiers in hexane eluant on the retention of α-, β-, γ-, and δ-tocopherols on a 5 μm PAC column

Table 1 shows the relative retention times for the 13 tocols with four eluant systems formulated on the basis of Fig. 1. Eluant 1 (hexane:propan-2-ol, 99.5:0.5) did not resolve any of the T-1 peaks. Eluant 2 (hexane:1,4-dioxan, 94:6) did not resolve β-T-1 from δ-T or β-T-3 from P-8. Eluant 3 (hexane:THF, 94:6) resolved all peaks except those for β-T-3 and γ-T-1. Eluant 4 (hexane:THF:1,4-dioxan, 94:3:3) gave a slightly better resolution. However, the best eluant system in practice will depend upon the relative amounts of those tocols of interest to the analyst.

Table 1. HPLC Retention Times for Tocols, Relative to α-T, with Various Eluants on a Partisil PXS 5/25 PAC Column.

	*Eluant			
Tocol	1	2	3	4
α-T	1.00	1.00	1.00	1.00
α-T-1	1.00	1.09	1.09	1.08
α-T-3	1.14	1.27	1.29	1.24
β-T	2.37	1.71	1.55	1.60
β-T-1	2.37	1.91	1.70	1.74
β-T-3	2.80	2.25	2.05	2.08
γ-T	2.59	1.91	1.88	1.84
γ-T-1	2.59	2.07	2.05	2.00
γ-T-3	3.06	2.53	2.51	2.42
δ-T	4.45	2.92	2.69	2.68
δ-T-1	4.45	3.23	2.93	2.95
δ-T-3	5.27	3.92	3.67	3.60
P-8	2.19	2.25	2.19	2.13

* Eluant 1 hexane:propan-2-ol (99.5:0.5)
Eluant 2 hexane:1,4-dioxan (94:6)
Eluant 3 hexane:THF (94:6)
Eluant 4 hexane:THF:1,4-dioxan (94:3:3)

The retention times of the tocomonoenols and tocotrienols relative to the corresponding tocopherols are a useful aid in the identification of individual tocols (Table 2). These relative retention times are similar to those found by Müller-Mulot et al (9) using a 5 μm Lichrosorb Si 60 (silica) column with hexane:ethyl acetate (96.5:3.5) and hexane:dioxan (96:4) as eluants. The relative positions of the β-T and P-8 peaks using propan-2-ol as polar modifier were also similar to those reported by Müller-Mulot.

The 5 μm PAC column showed a minimum of 9100 theoretical plates (with eluant 1) to a maximum of 11900 plates (with eluants 3 and 4) for γ-tocopherol. In contrast, the 10 μm PAC column showed 3000 theoretical plates (with eluant 1) and correspondingly poorer peak resolution : the tocotrienols, but not the tocomonoenols, were resolved from the corresponding tocopherols.

Table 2. HPLC Retention Times for Tocomonoenols and Tocotrienols, Relative to the Corresponding Tocopherol, with Various Eluants.

Tocol	*Eluant 1	2	3	4
α-T-1	**	1.09	1.09	1.08
β-T-1	**	1.12	1.10	1.09
γ-T-1	**	1.08	1.09	1.08
δ-T-1	**	1.09	1.10	1.10
α-T-3	1.14	1.27	1.29	1.23
β-T-3	1.18	1.32	1.33	1.28
γ-T-3	1.19	1.33	1.34	1.30
δ-T-3	1.19	1.36	1.37	1.30

* See Table 1
** Not resolved from corresponding tocopherol on chromatogram

The 10 μm PAC column has been routinely used with hexane:propan-2-ol (99:1) as eluant for the determination of α-T in 1500 biological samples and animal feedingstuffs over an 18 month period. During this time the column efficiency fell from 3000 to 2300 theoretical plates and the α-T peak gradually became positively skewed, presumably as a result of inlet bed problems (10). Column deterioration was delayed by washing with propan-2-ol after each batch of analyses. The 5 μm PAC column, used for detailed analysis of oils for the various tocols, was washed with the same solvent as that used as polar modifier in the eluant. When not in use, the PAC column should preferably be stored in hexane and tightly capped.

The use of polar-modified hexane as eluant facilitated the direct HPLC analysis of seed oils as a 1% solution in hexane (Fig. 2). This direct analysis of unsaponified oils avoids the destruction of tocotrienols that has been reported by other workers (11). Using 20 μl injections, peak symmetry was maintained with no detectable peak broadening when compared with

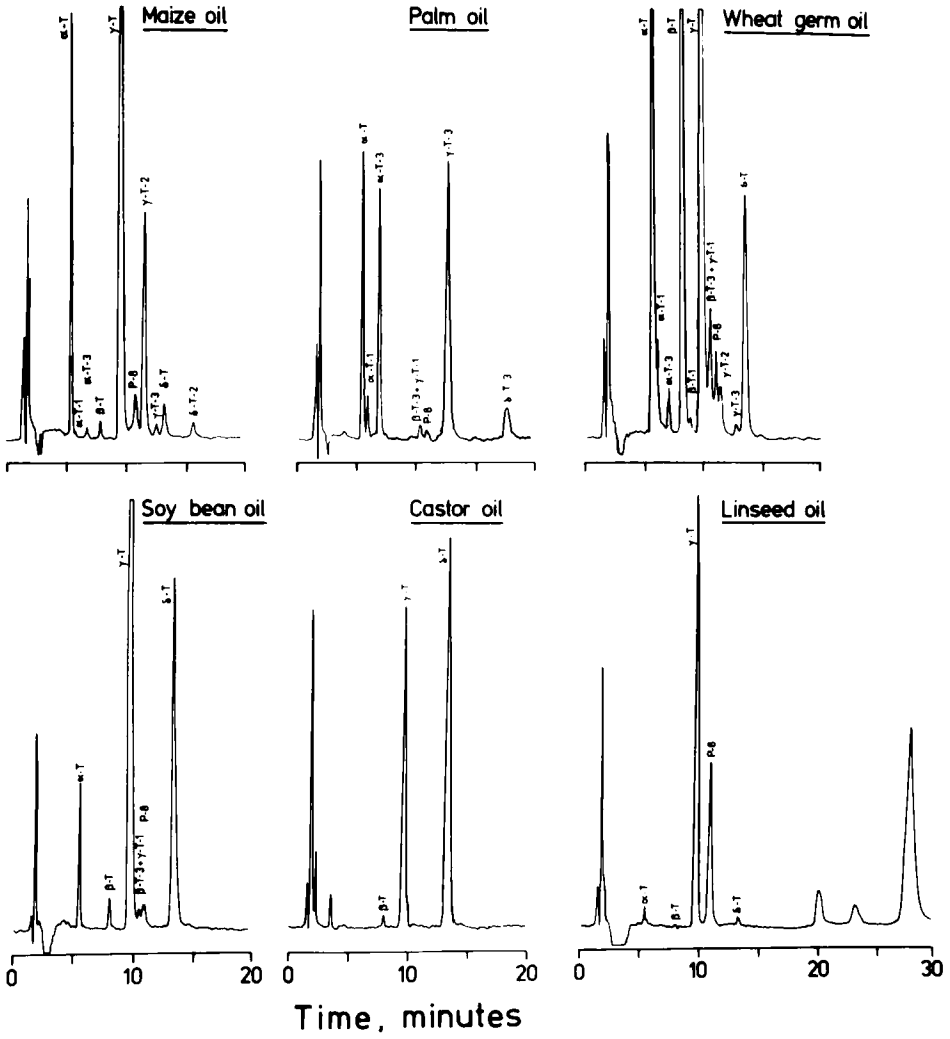

FIGURE 2. HPLC chromatograms of seed oils, as 1% solutions in hexane, on 5 µm PAC column using hexane:THF (94:6) as eluant.

Table 3. Precision Data for 6 Replicate Injections of Seed Oil in Hexane.

Tocol	Retention time $\bar{X}$ min	± S.D.	%C.V.	Peak Area $\bar{X}$ units	± S.D.	%C.V.
α-T	5.77	0.04	0.7	14228	247	1.7
α-T-1	6.00	0.04	0.8	495	20	4.0
α-T-3	6.98	0.05	0.7	1052	60	5.7
β-T	8.13	0.06	0.7	5817	121	2.1
β-T-3	10.47	0.08	0.8	847	35	4.2
γ-T	9.78	0.07	0.7	9645	101	1.1
γ-T-3	12.78	0.09	0.7	1908	45	2.3
δ-T	13.53	0.08	0.6	3092	52	1.7
δ-T-3	17.86	0.11	0.6	331	92	27.9

injections of hexane extracts of saponified oils. The reproducibility with replicate injections of a 1% solution of wheat germ:palm:castor oils (2:2:1) in hexane is shown in Table 3.

Table 4 shows the approximate tocol contents of the six seed oils analysed using hexane:THF (94:6) as eluant at a flow rate of 2 ml/min. The tocol values were calculated using the α-T fluorescence yield for all tocols : accurate determinations were impossible without the required tocol standards. The large differences between oils in both the proportions of the tocols and the total tocol contents have been noted, to various extents, by previous workers. With the maize oil (Fig. 2) the peaks with retention times intermediate between those for γ-T and γ-T-3, and between those for δ-T and δ-T-3 were tentatively identified as the tocodienols γ-T-2 and δ-T-2. Their retention times relative to γ-T and δ-T were 1.20 and 1.18, respectively. Apart from a personal communication cited in a paper by Threlfall and Whistance (12) there appear to be no published reports on the occurrence of tocodienols in oils. The peaks with retention times longer than δ-T-3 in the maize and linseed oils (Fig. 2) could not be identified.

Table 4. Approximate Tocol Content* of six Seed Oils.

Tocol	Tocol, as % of total tocol in oil					
	Maize	Palm	Wheat Germ	Soybean	Castor	Linseed
α-T	13	18	44	3.8	-	2.2
α-T-1	0.1	3.5	0.3	-	-	-
α-T-3	0.6	21	0.5	-	-	-
β-T	0.9	-	18	1.1	2.0	0.7
β-T-1	-	-	0.2	-	-	-
β-T-3/γ-T-1	-	2.2	2.2	0.4	trace	
γ-T	66	-	27	72	36	64
γ-T-2(?)**	13	-	0.8	-	-	-
γ-T-3	0.8	47	0.3	-	-	-
δ-T	2.3	-	5.0	22	62	2.2
δ-T-1	-	-	trace	-	trace	-
δ-T-2(?)**	1.4	-	-	-	-	-
δ-T-3	-	6.7	-	-	-	-
P-8	2.3	2.0	1.5	0.7	-	31
Total tocol (mg/kg)	1600	730	5600	1800	810	830

* Calculated using the same fluorescence yield as α-T for all other tocols.
** Tentatively identified as tocodienols.

Partisil PAC is a moderately polar material in which cyano and amino groups, in a ratio of 1:2, are bonded to the surface hydroxyls of silica. The cyano groups selectively react with compounds having double bonds while the amino groups are quaternised when used with a polar organic eluant so imparting weak anion exchange properties to the column (13,14). With the polar modified eluant systems used in the present study, the amino groups appeared to be in the quaternised form since a 0.1M NH_4OH wash increased retention times and markedly broadened peaks while a wash with THF:formic acid (99:1) had no effect on the tocol separation characteristics. Incorporation of 0.01% formic acid in the eluant gave slightly sharper peaks and slightly shorter retention times but this system was not adopted as a routine because of uncertainty about its effect on long term column stability.

On a polar stationary phase, where separation is principally by adsorption processes, solute retention increases with the number and polarity of the functional groups, and also with the molecular size (15). The main factor affecting the retention of individual tocols on the PAC column is presumably steric hindrance of the phenolic OH group by the methyl substituents on the phenolic ring. The increased retention times for the tocomonoenols and tocotrienols relative to the corresponding tocopherols are attributed to selective reaction with the cyano groups on the column. The combined effect of the amino and cyano groups appears to make the PAC column ideal for the separation of tocols.

The low naturally occurring levels of some tocols necessitates the use of fluorescence for detection. The fluorescence intensity was maximised by using excitation at 210 nm rather than 292 nm, the absorbance maximum for α-T (7). The relative quantum yields for α-T at 210 nm in eluants 1, 2, 3 and 4 were 1.0, 2.7, 3.4 and 2.7, respectively. Overall, hexane:THF (94:6) was therefore the best eluant since it gave good separation of tocols and the highest sensitivity. The detection limit (S/N=3) for α-T using this eluant was 1 ng.

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