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Normal-phase high-performance liquid chromatography of tocopherols and tocotrienols Comparison of different chromatographic columns

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Abstract

Natural vitamin E is composed of eight different vitamers (α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols). As these eight vitamers have different antioxidant and biological activities, it is necessary to have quantitative data on each substance separately. The aim of this study was to find universal HPLC columns for the separation of all eight components and to test if a few columns of the same material (different batches) will give reproducible results. Normal-phase HPLC separations of vitamin E compounds in a prepared mixture (containing oat extracts, palm oil and tocopherol standards) were tried on six silica, three amino and one diol columns. As shown by calculations of retention factors (k), separation factors (α), numbers of theoretical plates (N) and resolutions (R_s), the best separations were obtained on three silica columns and two amino columns using 4 or 5% dioxane in hexane as the mobile phase as well as on a diol column using 4% *tert*.-butyl methyl ether in hexane as the mobile phase.

Keywords: Food analysis; Tocopherols; Tocotrienols; Vitamins

1. Introduction

Vitamin E is an important natural antioxidant in foods, especially those rich in polyunsaturated fatty acids [1]. Due to its role as a scavenger of free radicals, vitamin E is also believed to protect our bodies against degenerative malfunctions, mainly cancer and cardiovascular diseases [2]. Natural vitamin E is composed of eight chemical compounds: α-, β-, γ- and δ-tocopherols and four corresponding tocotrienols (Fig. 1). All four members of each series are neither isomers nor homologues, they differ in both the number of methyl substituents and their positions on the phenolic ring. To facilitate their description, the term ''E-vitamers'' will be used in this report as a collective name for the eight compounds.

As the eight vitamin E compounds have different antioxidant and biological activities [1,3], it is necessary to be able to have quantitative data on the biological levels of each substance separately. Different methods are described in the literature for the analysis of vitamin E by gas chromatography (GC) as well as by high-performance liquid chromatography (HPLC) [4] and references cited below. For GC analysis, a pre-treatment for the elimination of

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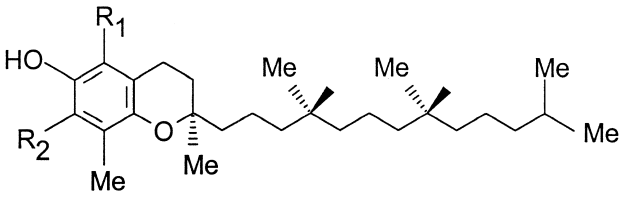
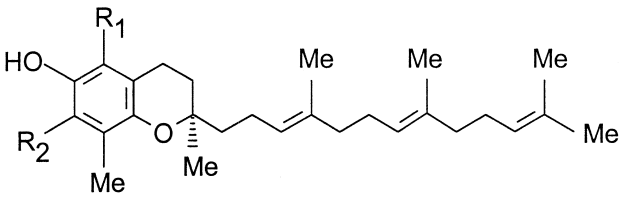


Tocopherols



Tocotrienols

Tocol Structure	Trienol Structure	R_1	R_2
α-Tocopherol (α-T) *(5,7,8-Trimethyl tocol)*	α-Tocotrienol (α-3) *(5,7,8-Trimethyl tocotrienol)*	Me	Me
β-Tocopherol (β-T) *(5,8-Dimethyl tocol)*	β-Tocotrienol (β-3) *(5,8-Dimethyl tocotrienol)*	Me	H
γ-Tocopherol (γ-T) *(7,8-Dimethyl tocol)*	γ-Tocotrienol (γ-3) *(7,8-Dimethyl tocotrienol)*	H	Me
δ-Tocopherol (δ-T) *(8-Monomethyl tocol)*	δ-Tocotrienol (δ-3) *(8-Monomethyl tocotrienol)*	H	H

Fig. 1. Structures and methyl positions of the eight natural E vitamins. The Chemical Abstracts name for tocol is 3,4-dihydro-2-methyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol and for tocotrienol is 2-methyl-2-(4′,8′,12′-trimethyltrideca-3′,7′,11′-trienyl)-6-chromanol. The Chemical Abstracts registry numbers for the tocopherols are α-T [59-02-9]; β-T [16698-35-4]; γ-T [54-28-4] and δ-T [119-13-1] and for the tocotrienols are α-T3 [1721-51-3; 493-35-6]; β-T3 [490-23-3] and γ-T3 [91-86-1].

interfering substances (mainly saponification) is necessary to separate the tocopherols mainly from the acyl lipids. Not necessarily needed in HPLC analysis, this preparation step may cause losses of vitamin E which have to be taken into account during quantification [5]. Furthermore, HPLC analysis needs less sample preparation and no derivatisation. Hence, it provides a fast, simple, sensitive, selective and more robust technique than GC [6,7].

HPLC separations of tocopherols are performed on both normal- and reversed-phase columns. Although reversed-phase columns are generally known to have the advantages of better stability and longer durability than normal-phase columns, most reversed-phase columns are not able to separate the β- and γ-isomers of tocopherols and tocotrienols [7–10]. It was, however, possible to separate β- and γ-tocopherol isomers on pentaflurophenyl [11] and octadecylpolyvinyl alcohol [12] reversed-phase columns. Besides their better selectivity for fat-soluble vitamins and their ability to separate the β- and γ-isomers, the advantages of normal- over reversed-phase columns in the separation of E-vitamers include: (i) the ability to operate with organic solvents, allowing a high solubility for lipids to be possible; (ii) the ability to tolerate high loads of lipids which are easy to wash-out by non-polar solvents and (iii) the ability to provide a wide range

of selectivity through the inclusion of different polar modifiers in the mobile phase [8,13].

Since human and animal tissues and most foods do not contain all eight vitamers [14], it is not always necessary to separate all tocopherols and tocotrienols. Animal products and vegetables, containing only α- and γ-tocopherols, can easily be analysed on several types of columns [4]. However, the use of normal-phase columns is needed for the analysis of food samples having a more complicated vitamin E composition, mainly cereal foods and mixed diets containing the β- and γ-isomers of tocopherols and/or tocotrienols [14]. Normal-phase HPLC separations of E-vitamers were previously achieved using silica, aminopropyl- or diol-bonded column packings [4]. According to our experience with cereal E-vitamers, silica columns from different manufacturers generally provide good separations for the four tocopherols and α- and δ-tocotrienols. Nevertheless, not all silica columns are able to provide satisfactory results as regards to the separations of β- and γ-tocotrienols from their β- and γ-tocopherol relatives.

The most generally used mobile phases employ a binary solvent system based on hexane [15] together with a variety of organic modifiers including diethyl ether [16], diisopropyl ether [17], *tert.*-butyl methyl ether [8], methanol [18], isopropanol [9,10] and 1,4-dioxane [5]. The use of ethers includes a risk of peroxide formation and the use of alcohols is accompanied by difficulties in achieving accurate mobile phase proportions since relatively very small volumes of these alcohols are needed because of their very high polarities [19]. Compared to other ethers with similar polarity, *tert.*-butyl methyl ether was reported to show a lower risk of peroxide build-up [19].

For the determination of vitamin E, fluorescence detectors are generally used [6,15,17] because of their higher sensitivity and specificity compared with ultraviolet [20] and evaporative light scattering detectors [21,22]. However, these other detectors may sometimes be preferable because they allow the simultaneous analysis of other lipophilic compounds such as triacylglycerols, phenolic antioxidants, ubiquinones, carotenoids, sterols and vitamins A, D and K, etc. [22–29]. Vitamin E compounds can also be quantified after sensitive and specific coulometric detection [28] but since this detector is still not able to differentiate between the β- and γ-isomers {redox potentials are +0.343 (β-T) and +0.348 (γ-T) [30]}, separation of these isomers still needs to be performed on the column.

According to our experience, old types of silica columns gave irreproducible results and had very short life-times for the separation of the β- and γ-tocopherol and tocotrienol vitamers. The aim of this study was to find universal HPLC columns for the separation of all eight natural vitamin E components by comparing several columns with new silica types for the HPLC separation of vitamin E using different mobile phases and to test if a few columns of the same material (different batches) would give reproducible separations.

2. Experimental

Tocopherols (article no. 15496) and dl-tocotrienols (article no. 8524) were purchased as isomer kits from Merck (Darmstadt, Germany). HPLC-grade solvents (*n*-hexane, *tert.*-butyl methyl ether, tetrahydrofuran, diisopropyl ether, 2-propanol, methanol and 1,4-dioxane) were also purchased from Merck and were used without further purification. Tocopherol/tocotrienol extracts were obtained from oat flour by methanol extraction according to Peterson and Qureshi [31]. Oat extracts, palm oil (a commercial red oil) and standards of β-, γ- and δ-tocopherols were mixed in hexane to provide a balanced mixture of tocopherols and tocotrienols to be used for the evaluation of the chromatographic performance of the different columns.

The HPLC equipment consisted of an 2248 HPLC pump (Pharmacia LKB Biotechnology, Uppsala, Sweden), an LKB 2157 autoinjector and a Fluor LC 304 fluorescence detector (Linear Instruments, Reno, NV, USA). Peaks were recorded using the chromatography software JCL 6000 (Jones Chromatography, Mid-Glamorgan, UK). Fluorimetric detection of all peaks was performed at an excitation wavelength of 294 nm and an emisson wavelength of 326 nm. HPLC separations on the prepared mixture were tried on the following columns: (i) six different silica columns from Alltech (Deerfield, IL, USA), Chrompack International (Middelburg, Netherlands), Jones Chromatography and Merck; (ii) three amino col-

Table 1
Survey of the columns used in this study

No.	Name	Dimensions (mm)	Packing material	Retention principle
A	Alltima SI 5U (Alltech) (3x)[a]	250×4.6	d_p=5 μm	Normal-phase
B	LiChroCART Superspher Si 60 (Merck)	250×4	Porous silica d_p=4 μm	Normal-phase
C	Inertsil SI (Chrompack)	250×4.6	d_p=5 μm	Normal-phase
D	Chromspher SI (Chrompack)	250×4.6	d_p=5 μm	Normal-phase
E	Apex silica (Jones)	250×4.6	d_p=5 μm	Normal-phase
F	Genesis silica (Jones) (3x)[b]	250×4.6	d_p=4 μm	Normal-phase
G	Hypersil APS-2[c] (Chrompack)	250×4.6	d_p=5 μm	Normal-phase
H	LiChrosorb NH_2 (Merck)	250×4	d_p=5 μm	Normal-phase
I	LiChroCART Purospher NH_2(Merck)	250×4	Porous silica deactivated d_p=4 μm	Normal-phase
J	LiChrosorb Diol Hibar (Merck) (2x)[d]	250×4	Porous silica d_p=5 μm	Normal-phase

[a] Alltima columns were from packing lot AT0121, AT0124 and AT0127.
[b] Genesis columns were from batches 9619601, 9619701 and 9619801.
[c] APS=Aminopropyl silica.
[d] Diol columns were from cartridge no. 628697 and 742586.

umns from Alltech, Chrompack and Merck and (iii) one diol column from Merck. The characteristics of the different columns used are shown in Table 1. The performance of the normal-phase columns was tested using the mobile phases listed in Table 2.

Columns were equilibrated for at least 0.5 h, with a minimum of 30 column volumes, before chromatographic data were collected. Chromatographic separations were evaluated based on three replicate determinations using the following formulas [32,33]:

$$k = \frac{t_E - t_0}{t_0} \quad (1)$$

retention factor (formerly called capacity factor, k')

$$\alpha = \frac{k_b}{k_a} \quad (2)$$

separation factor (formerly called selectivity)

$$N = 5.54 \cdot \left(\frac{t_E}{W_E}\right)^2 \quad \text{number of theoretical plates} \quad (3)$$

$$R_S = 1.18 \cdot \frac{t_b - t_a}{w_b + w_a} \quad \text{resolution} \quad (4)$$

where,

t_0=retention time of unretained solute
t_E=retention time of vitamin E components

Table 2
The different mobile phases (solvents) used for normal-phase HPLC

No.	Components	Ratio of components
1	Hexane–*tert*.-butyl methyl ether–tetrahydrofuran–methanol	79:20:1:0.1
2	Hexane–*tert*.-butyl methyl ether–tetrahydrofuran	79:20:1
3	Hexane–*tert*.-butyl methyl ether	90:10
4	Hexane–*tert*.-butyl methyl ether	96:4
5	Hexane–*tert*.-butyl methyl ether	97:3
6	Hexane–diisopropyl ether	90:10
7	Hexane–diisopropyl ether	97:3
8	Hexane–1,4-dioxane	97:3
9	Hexane–1,4-dioxane	96:4
10	Hexane–1,4-dioxane	95:5
11	Hexane–2-propanol	99:1

a, b = adjacent components ($t_a < t_b$)
w_E = bandwidth at half-height (w_a and w_b are and widths at half-height for peaks a and b, respectively).

3. Results and Discussion

Tocopherol and tocotrienol standards are available in isomer kits from Merck (see Section 2) but on chromatography, the β-, γ-, δ-tocotrienol standards showed double peaks (Fig. 2), most probably due to the formation of a racemic mixture of 2*R* and 2*S* stereoisomers during synthesis [34,35]. Since our aim was to separate only the natural E-vitamers, a mixture containing oat extracts rich in α-tocopherol (α-T), β-tocopherol (β-T), α-tocotrienol (α-T3) and β-tocotrienol (β-T3) [32], palm oil as a source of α-T, α-T3, γ-tocotrienol (γ-T3) and δ-tocotrienol (γ-T3) [36,37] and Merck β-T, γ-tocopherol (γ-T) and δ-tocopherol (δ-T) standards was prepared and used for the testing of the chromatographic methods studied (Fig. 3–5). No attempt has been made to study parameters important for the quantitative determination of the E-vitamers. However, it was reported that using the fluorescence detector, each tocotrienol exhibits quantitatively the same response as its corresponding tocopherol [16].

In normal-phase HPLC, natural E-vitamers are basically separated by adsorption according to the number of methyl groups on their chromanol rings which is the main factor affecting their polarity. In addition, these methyl substituents induce different steric effects on the phenolic group influencing its interaction with the silanol groups of the silica [38]. Moreover, the unsaturation in the side chain also influences the polarity of these compounds making the tocotrienols slightly more polar than their corresponding tocopherols. Generally, normal-phase columns are more retentive towards the more polar vitamers having less methyl substituents in their chromanol rings (retention: $\alpha < \beta \leq \gamma < \delta$) [9]. Although both β- and γ-isomers have two such methyl substituents, the position of these in the γ-isomers (7,8-dimethyls) appear to increase their degree of asymmetry and their polarity compared with the β-isomers (5,8-dimethyls) [39]. Because of these structural properties, it can be difficult to separate

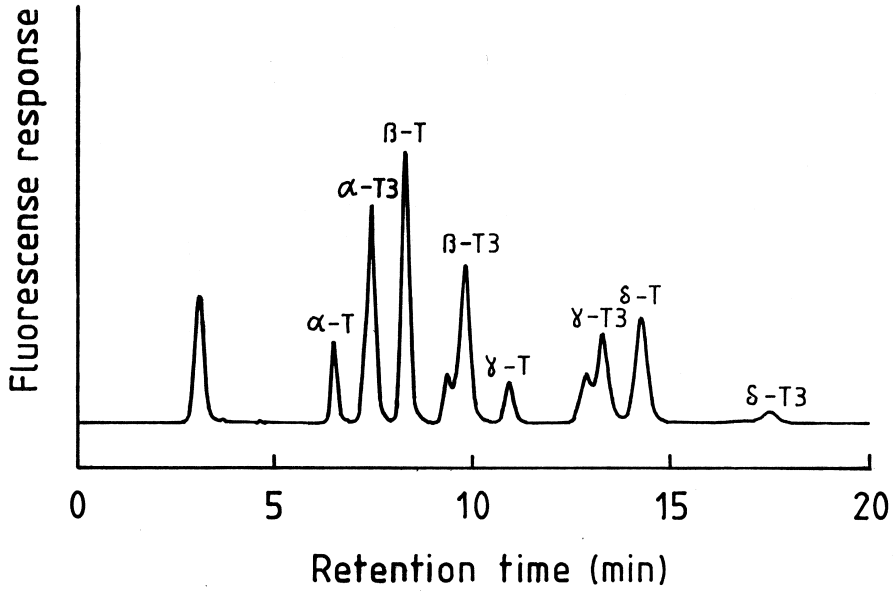


Fig. 2. Separation of Merck tocopherol and tocotrienol vitamers on an amino-bonded column (H, Table 1) using hexane–*tert.*-butyl methyl ether–tetrahydrofuran–methanol (79:20:1:0.1, v/v/v/v, solvent 1; Table 2) at a flow-rate of 1.0 ml min^{-1}. Peaks: α-T (α-tocopherol), α-T3 (α-tocotrienol), β-T (β-tocopherol), β-T3 (β-tocotrienol), γ-T (γ-tocopherol), γ-T3 (γ-tocotrienol), δ-T (δ-tocopherol) and δ-T3 (δ-tocotrienol).

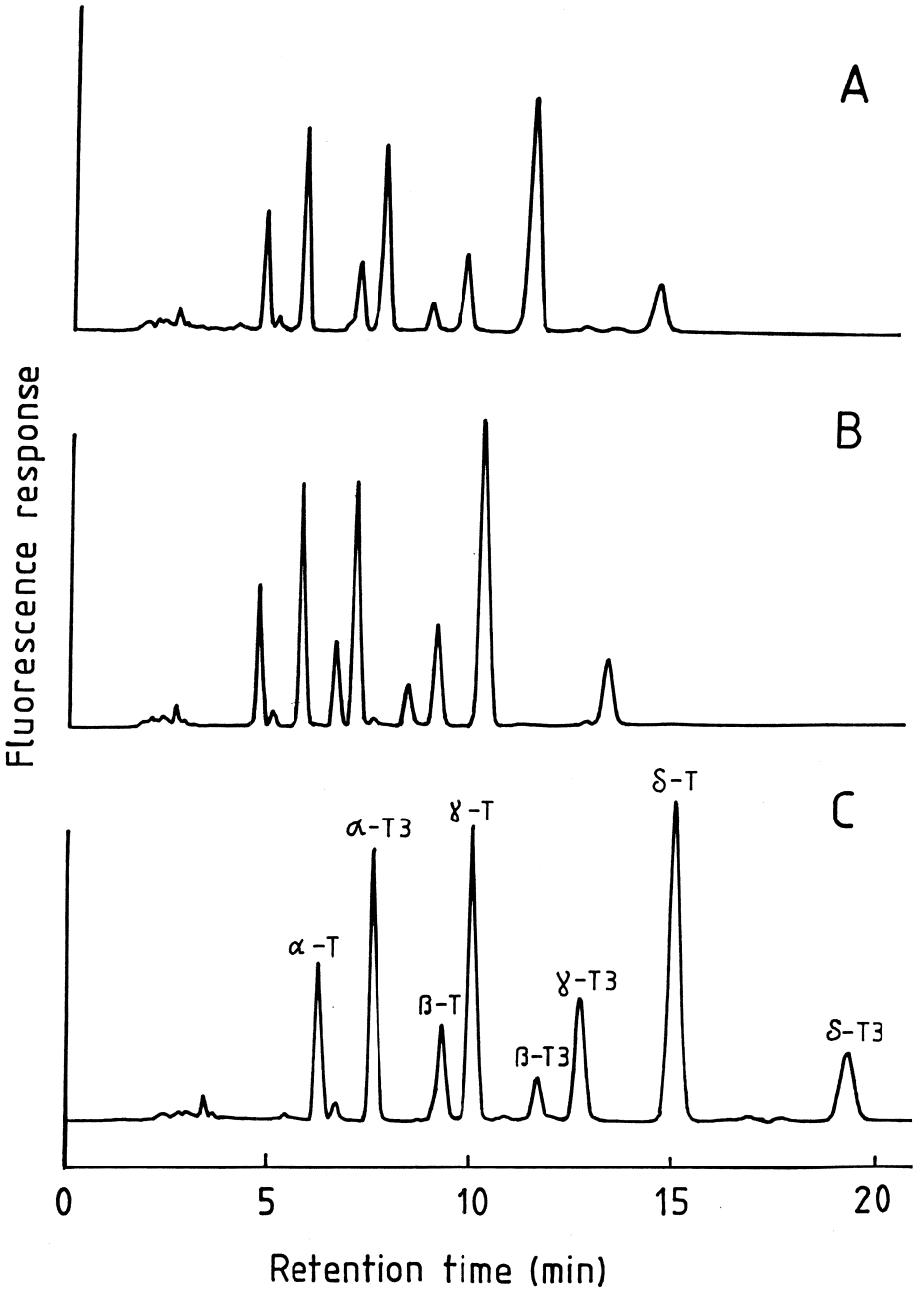


Fig. 3. Separation of a balanced mixture of tocopherols and tocotrienols on three silica columns (A, C, F; Table 1) using mixtures of hexane and 1,4-dioxane (solvents 9 and 10; Table 2) as mobile phases: (A) column A + solvent 9 at 2.0 ml min^{-1}; (B) column C + solvent 10 at 2.0 ml min^{-1} and (C) column F + solvent 9 at 1.5 ml min^{-1}. For solvents, see Table 2 and for identification of peaks, see legend to Fig. 2.

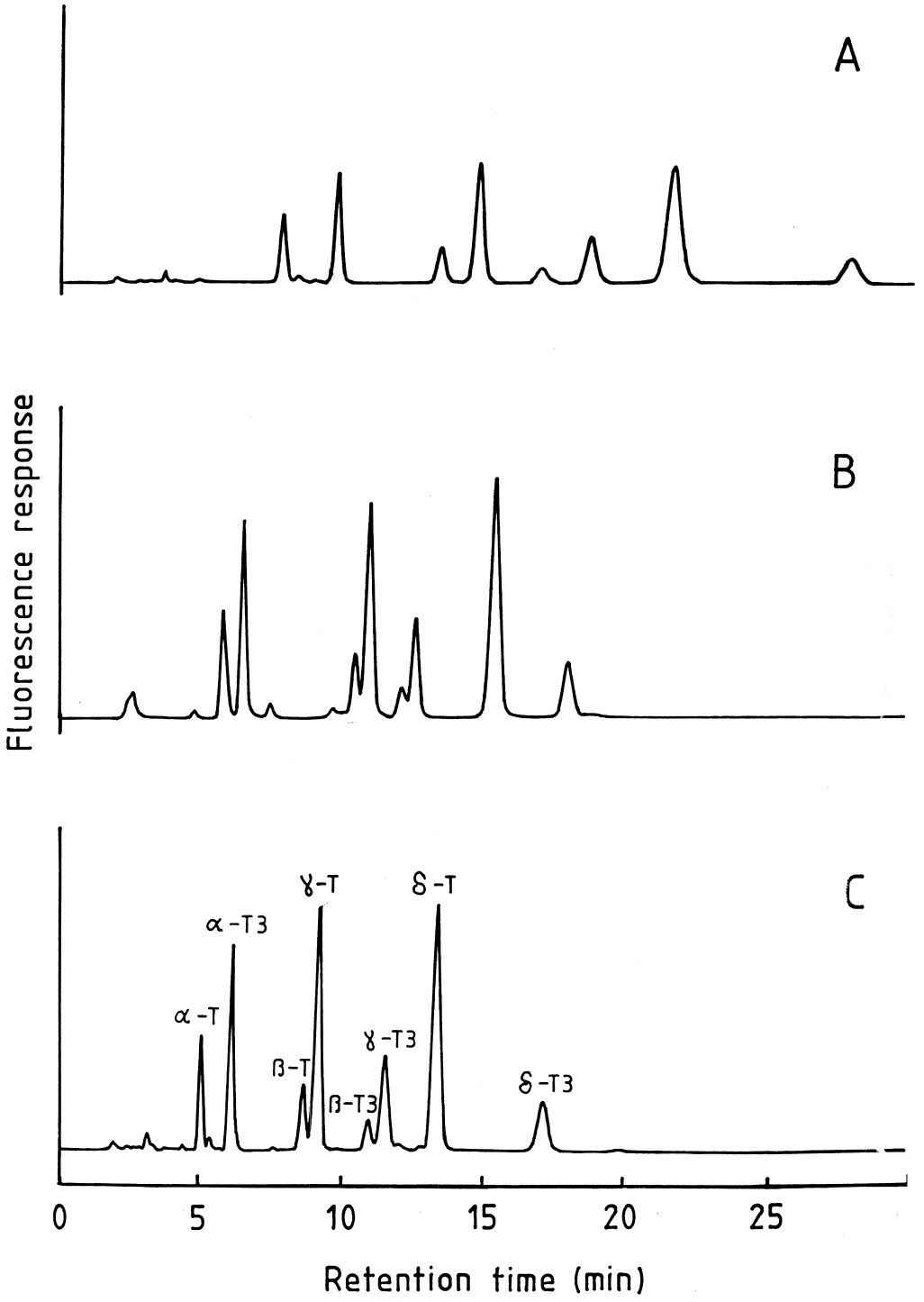


Fig. 4. Separation of a balanced mixture of tocopherols and tocotrienols on diol-bonded silica column (J; Table 1) using different mobile phases: (A) solvent 4 at 2.0 ml min^{-1}; (B) solvent 11 at 1.0 ml min^{-1}; and (C) solvent 9 at 1.5 ml min^{-1}. For solvents, see Table 2 and for identification of peaks, see legend to Fig. 2.

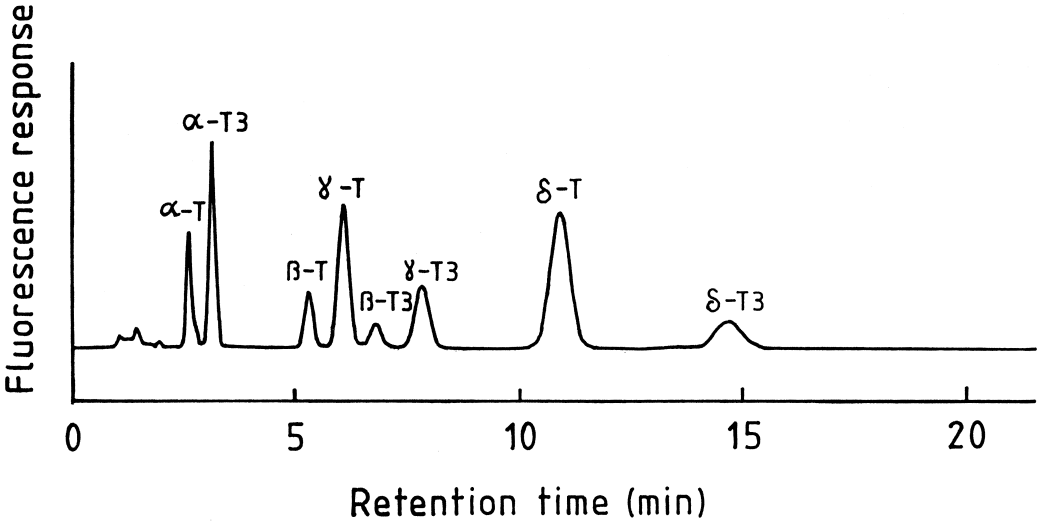


Fig. 5. Separation of a balanced mixture of tocopherols and tocotrienols on an amino-bonded silica column (G; Table 1) using solvent 10 (Table 2) at 2.5 ml min^{-1}. For solvent, see Table 2 and for identification of peaks, see legend to Fig. 2.

α-T3 and β-T, β-T and γ-T, β-T3 and γ-T, γ-T and γ-T3, and/or γ-T3 and δ-T under some normal-phase conditions.

Previously, it was believed that unmodified silica phases were inferior to polar-bonded phases in terms of reproducibility and retention of some polar compounds [32,39,40]. It is, however, our experience that the newer types of unmodified silica columns provide better separations for the slightly polar E-vitamers. The problem of poor batch-to-batch reproducibility of the old generations of silica due to inhomogeneous surfaces and uncontrolled levels of trace metal ions [41] has been eliminated in many of the new silica types. Of the different silica columns tried in this study, three columns (A, C and F) led to satisfactory separations of all eight E-vitamers with the use of 1,4-dioxane at the 4 or 5% level in hexane (solvents 9 and 10, Fig. 3). The order of elution of the tocopherols/tocotrienols in all silica columns was α-T<α-T3<β-T<γ-T<β-T3<γ-T3<δ-T<δ-T3. Table 3 lists results on the calculations of the retention factors (k) and number of theoretical plates (N) for each peak and the separation factor (α) and the resolution (R_s) for adjacent peak pairs according to Eqs. (1)–(4) shown in Section 2. Using these column–solvent combinations, all eight E-vitamers were baseline separated ($R_s \geq 1.5$) by the three columns within 15–22 min. In agreement with structural similarities, the poorest separations were obtained for the β- and γ-tocopherol and tocotrienol pairs. Using 4% dioxane in hexane, baseline separations were obtained on column B for the E-vitamers except γ-T3 and δ-T (results not shown).

Columns from three different batches from two silica columns, viz. A and F, were used to study the reproducibility of the previously obtained separations. The separation factors (α) (Table 3) were very close for the same type of column. Furthermore the α values were close for the two silica columns, although the retention factors (k) were more than 50% higher on column F which had the highest number of theoretical plates for all eight E-vitamers. The silica columns showed good reproducibility in contrast to Kramer et al. [10]. It may be possible that these authors tested a silica column type-A while we have tested the new, rugged, and reproducible type-B silicas characterised by a homogeneous, highly hydroxylated silica surface and by low trace metal content [41].

Some separations were obtained using the silica columns A, C, F and B with *tert.*-butyl methyl ether in hexane as the mobile phase (results not shown).

Table 3
Performance characteristics of the columns that provided the best separation of the eight natural E vitamers[a]

Column + mobile phase	Total run time (min)	α-T				α-T3				β-T				γ-T				β-T3				γ-T3				δ-T				δ-T3	
		k	*α*	*N*	R_s	*k*	*α*	*N*	R_s	*k*	*α*	*N*	R_s	*k*	*α*	*N*	R_s	*k*	*α*	*N*	R_s	*k*	*α*	*N*	R_s	*k*	*α*	*N*	R_s	*k*	*N*
Silica columns																															
A1+ 9	15	0.85	1.46	5602	3.80	1.24	1.38	6881	4.03	1.71	1.14	7288	1.83	1.95	1.23	8591	3.19	2.40	1.14	7633	2.13	2.72	1.25	9186	4.16	3.40	1.35	10 805	6.24	4.60	10 687
A2+9	15	0.83	1.47	5028	3.44	1.22	1.39	5126	3.54	1.70	1.13	5281	1.49	1.92	1.23	6182	2.89	2.36	1.13	7164	1.95	2.68	1.26	7795	3.88	3.39	1.35	7673	5.31	4.58	7958
A3+9	15	0.81	1.47	4986	3.65	1.20	1.39	6555	3.87	1.66	1.13	6613	1.60	1.87	1.23	7336	3.08	2.30	1.14	8128	2.11	2.62	1.26	8940	3.99	3.28	1.35	8707	5.78	4.44	10 003
C+10	15	1.27	1.42	6372	4.59	1.81	1.24	8803	3.35	2.25	1.11	8005	1.74	2.50	1.25	9297	4.13	3.14	1.11	10 258	2.12	3.49	1.15	10 852	2.96	4.02	1.37	11 597	7.12	5.52	12 216
F1+9	20	1.64	1.34	7472	4.55	2.19	1.32	10943	4.98	2.89	1.11	9403	1.99	3.21	1.21	11 009	3.97	3.89	1.11	11 384	2.31	4.34	1.23	11 479	4.70	5.32	1.33	12 974	7.11	7.09	13 778
F2+9	20	1.60	1.35	7980	4.56	2.15	1.34	9918	5.28	2.88	1.11	10 651	2.15	3.20	1.20	11 669	3.92	3.84	1.12	12 978	2.62	4.30	1.21	13 166	4.60	5.23	1.33	13 034	6.89	6.94	12 749
F3+9	20	1.74	1.35	7318	4.62	2.36	1.35	9472	5.35	3.18	1.11	9915	2.02	3.52	1.21	11 580	4.18	4.26	1.12	12 612	2.57	4.76	1.24	12939	5.16	5.89	1.34	13 513	7.66	7.87	15 909
Diol columns																															
J1+4	23	2.93	1.31	4756	3.74	3.85	1.54	5507	6.71	5.92	1.12	5973	1.96	6.65	1.17	6120	2.70	7.79	1.12	5942	1.89	8.69	1.17	6077	2.94	10.21	1.31	6965	5.01	13.32	6542
J2+4	22	2.94	1.31	5195	3.97	3.85	1.48	6411	6.31	5.69	1.12	6193	1.99	6.36	1.18	7502	3.10	7.51	1.11	7198	1.96	8.33	1.17	7277	2.94	9.73	1.31	6757	5.37	12.79	7922
J2+9	18	1.60	1.36	6226	3.96	2.17	1.59	6224	7.53	3.46	1.07	7902	1.29	3.69	1.26	8248	4.40	4.64	1.06	9819	1.21	4.92	1.19	8121	3.49	5.87	1.34	8204	6.05	7.84	9413
Amino column																															
G+10	16	1.46	1.34	2119	3.75	1.96	2.06	2455	11.70	4.04	1.18	3072	3.02	4.76	1.15	2620	2.64	5.47	1.18	3048	3.32	6.44	1.45	3300	7.66	9.32	1.37	2916	6.70	12.77	3146

[a] For codes of columns and solvents, see Tables 1 and 2, and for calculations of N and R_S, see Section 2. Flow-rates: A+9, C+10=2.0 ml min^{-1}; F+9=1.5 ml min^{-1}; J+4=2.0 ml min^{-1}; J+9=2.0 ml min^{-1} and G+10=2.5 ml min^{-1}.

Different percentages of *tert.*-butyl methyl ether were needed to provide separations on these columns but we were only able to separate seven peaks with this modifier. The non-separable peaks were β-T3 and γ-T except for column A where they were α-T3 and β-T. These results showed that the chromatographic separations of E-vitamers were affected by both the column material and solvent and that the stronger polar modifier, 1,4-dioxane, provided better selectivity (α) compared with the weaker modifier, *tert.*-butyl methyl ether in agreement with previous reports [39]. The lack of separation of β-T3 and γ-T was obtained with the same columns using 10% diisopropyl ether or 1% isopropanol in hexane (results not shown). The other two columns (D and E) were not able to provide acceptable separations in combination with any of the solvents used.

Previous work showed that all eight E-vitamers could be separated on diol-bonded silica columns using *tert.*-butyl methyl ether [8] or 2-propanol [10] in hexane. We tried these two polar modifiers (solvents 4 and 11) as well as 4% dioxane in hexane (solvent 9) on one diol column and obtained a very good separation using 4% *tert.*-butyl methyl ether (Fig. 4, Table 3). Mobile phases 9 and 11 could not completely separate β-T and γ-T and β-T3 and γ-T3, respectively, thus, a more weakly polar modifier was necessary. On the diol column (J), the retention factor (k) for α-T and α-T3 was in the desired range, between 1 and 5 [42]. Combined with 4% *tert.*-butyl methyl ether in hexane (solvent 4), all other retention factors were higher, ranging from 5.69 to almost 13, and the resolution was very good. Diol columns were reported to give consistent and reproducible results [10] but the results for two different batches of the diol column in this study (Table 3) were less reproducible compared to those obtained on the silica columns. Moreover, silica columns are more stable than diol columns.

Poor reproducibility of silica column separations was discussed by Rammel and Hoogenboom [38] who proposed the use of an amino-cyano column which offers good separation of all tocopherols and tocotrienols. It was also mentioned by Abidi and Mounts [39] that amino columns offer better selectivity than diol-bonded columns. Separation of the four tocopherols (but not the tocotrienols) using a Bondapak amino-bonded silica (10 μm) and 10% dioxane in hexane as the mobile phase has been previously suggested [39]. In this study, three amino columns were tested (viz. G, H and I). Column G provided a very good separation for all tocopherols and tocotrienols (Fig. 5, Table 3) when used with 5% 1,4-dioxane in hexane (solvent 10) and the E-vitamers eluted in the same order as they did on the three silica columns (Fig. 3). A good separation of all eight tocopherols and tocotrienols was also obtained once using column H together with hexane–*tert.*-butyl methyl ether–tetrahydrofuran–methanol (79:20:1:0.1, solvent 1) as a mobile phase (Fig. 2). The order of elution for the eight peaks was α-T<α-T3<β-T<β-T3<γ-T<γ-T3<δ-T<δ-T3, different from that obtained on column G (Fig. 5). The performance of column I was tested with hexane–2-propanol (99:1 v/v, solvent 11, 2.5 ml min^{-1}) and with hexane–*tert.*-butyl methyl ether–tetrahydrofuran–methanol (79:20:1:0.1, solvent 1) as the mobile phases. In both cases, six peaks were separated in the following order of elution: α-T<α-T3<β-T<β-T3=γ-T<γ-T3=δ-T<δ-T3. This amino column was also different from amino column G in the order of elution of β-T, γ-T, β-T3, γ-T3 and in that it is the β-T3 and γ-T pair (on one hand) and the γ-T3 and δ-T pair (on the other hand) which are difficult to separate. Although column G provided a very good separation (Fig. 5, Table 3) comparable to those obtained on the silica columns (Fig. 3), it is generally believed that amino columns are less stable than silica columns due to ionisation and wash-out of the amino groups which causes increased retention, decreased selectivity and peak broadening [38].

This work presents our first trials towards the understanding of the resolution of all eight E-vitamers on silica-based columns. The results show that some of the new silica materials are very promising for E-vitamer separations with no significant, between-batch variations, making HPLC silica columns suitable for this application. Relevant to this investigation is a study by Abidi and Mounts [43] on the separation of tocopherols on β- and γ-cyclodextrin-bonded silica columns. Trials to separate all eight E-vitamers on cyclodextrin-silica as well as on various reversed-phase columns are warranted. The ability to obtain E-vitamer separations on normal-phase as well as on reversed-phase silica will enable easy analysis of a wide range of biological samples.

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