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SEPARATION OF TOCOL-DERIVATIVES BY ELEVATED TEMPERATURE NORMAL-PHASE LIQUID CHROMATOGRAPHY

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Abstract. A novel method for the separation of eight vitamin E isomers (α-, β-, γ-, δ-tocopherol, and α-, β-, γ-, δ-tocotrienol) and α-tocopherol acetate on normal phase high performance liquid chromatography (NP-HPLC) at elevated column temperature has been developed. The separation was optimized by varying the eluent composition and column temperature. All the isomers were successfully separated using NP-HPLC on amino and silica columns. By simply increasing the temperature for silica column, excellent separation efficiencies and shorter analysis times were achieved without significant loss in resolution. The developed separation method is rapid, shows excellent reproducibility, and suitable to be used as a quantitative method in analyzing tocopherols and tocotrienols.

Keywords: Elevated temperature HPLC, tocopherols, tocotrienols, vitamin E

Abstrak. Kaedah baru bagi pemisahan lapan isomer vitamin E (α-, β-, γ-, δ-tokoferol, dan α-, β-, γ-, d-tokotrienol) dan α-tokoferol asetat menggunakan kromatografi cecair prestasi tinggi fasa normal (NP-HPLC) pada suhu turus yang tinggi telah dibangunkan. Pemisahan itu telah dioptimumkan dengan mengubah komposisi pengelusi dan suhu turus. Kesemua isomer itu berjaya dipisahkan menggunakan NP-HPLC di atas turus amino dan silika. Dengan hanya meningkatkan suhu turus silika, pemisahan berkecekapan tinggi dan masa analisis yang singkat telah diperoleh tanpa kehilangan resolusi yang signifikan. Kaedah pemisahan yang dibangunkan ini adalah cepat, menunjukkan kebolehulangan yang baik, dan sesuai sebagai kaedah kuantitatif dalam analisis tokoferol dan tokotrienol.

Kata kunci: HPLC suhu tinggi, tokoferol, tokotrienol, vitamin E

1.0 INTRODUCTION

In 1937, Emerson *et al.* [1] described the existence of various vitamin E homologues having different abilities to prevent vitamin E deficiency. The naturally occurring tocopherols and tocotrienols constitute the majority of the vitamin E group of compounds and they are composed of eight vitamers: α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), and δ-tocopherol (δ-T) and their four

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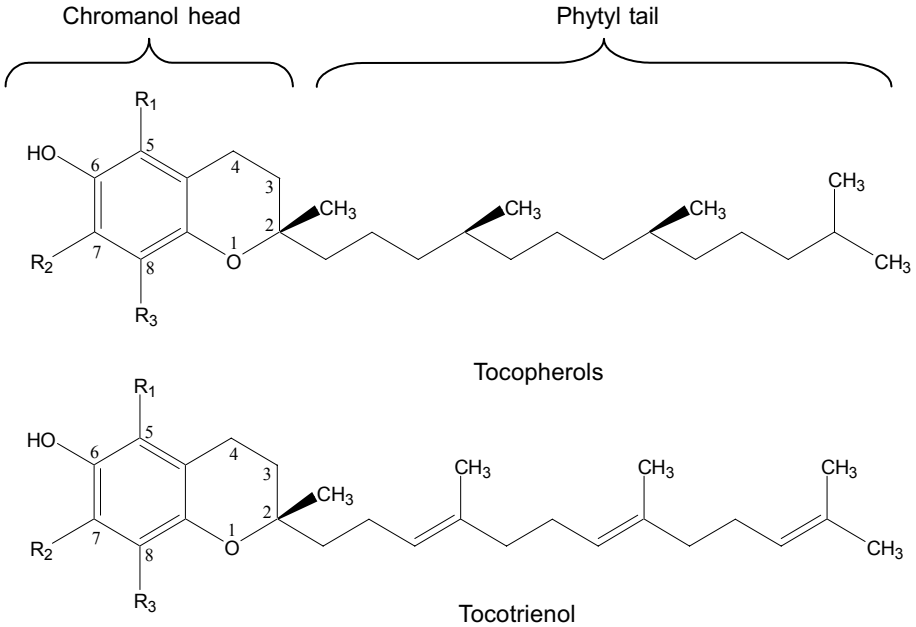
corresponding unsaturated congeners, namely α-tocotrienol (α-T_3), β-tocotrienol (β-T_3), γ-tocotrienol (γ-T_3), and δ-tocotrienol (δ-T_3). The basic structures of tocopherols and tocotrienols are shown in Figure 1. Tocopherols are methyl-substituted hydroxychromans with a saturated phytyl side chain and the tocotrienols with an unsaturated side chain. The main interest for studying vitamin E is its natural antioxidant capabilities. α-tocopherol has been known as the most efficient antioxidant for breaking free radical driven chain reactions. However, recent results indicate that α-tocotrienol is at least 3-fold more efficient as a scavenger of peroxyl radicals than α-tocopherol [2]. The number and localization of the methyl groups of their chromanol rings play an important role in influencing their biological activities, δ-tocotrienol being the most potent cholesterol inhibitor, followed by γ-tocotrienol and α-tocotrienol [2].

Normal phase HPLC techniques have been widely used for the analysis of the antioxidant mixtures in various sample matrices. The most significant advantage of using normal phase columns is the ability to separate β and γ isomers of both



Isomers	R_1	R_2	R_3	Position of methyl group
α	CH_3	CH_3	CH_3	5,7,8-trimethyl
β	CH_3	H	CH_3	5,8-dimethyl
γ	H	CH_3	CH_3	7,8-dimethyl
δ	H	H	CH_3	8-monomethyl

Figure 1 Chemical structures of tocopherols and tocotrienols

tocopherols and tocotrienols. Normal phase systems show elution of homologues in order of increasing polarity with separation based on methyl substituents on the chromanol moiety [3]. Normal phase separations also offer the advantages of operating with organic solvents, allowing a high solubility for lipids and very suitable for the direct analysis of oils and fats. Various types of silica-based column have been utilized to separate all the isomers. Recent review by Abidi [4] clearly specified the ability of pure silica-based phases and polar silica-based column such as amino-, cyano-, cyclodextrin-, diol-, and nitro-bonded silica in separating all eight vitamin E isomers.

Reversed-phase HPLC techniques have been widely used in the analysis of lipid antioxidants in cases where one component of an isomer pair is absent in the sample and the isomer separation is unimportant in specific research work. The advantages offered by the reversed-phase techniques are easy equilibration of mobile phases, reproducible chromatographic peak characteristics, compatible with highly sensitive electrochemical detection, and low volatility of mobile phase solvents. Several researchers have reported the separation of β and γ isomers of vitamin E on new stationary phases encompassing pentafluorophenylsilica (PFPS) [5], long-chain alkylsilica [6], and nonsilica-based octadecanoyl polyvinyl alcohol (ODPVA) [7]. Among the three alternative unconventional phases, PFPS offers the best baseline resolution and appears to be best suited for the routine reversed-phase analysis of the vitamin E isomers [4]. Although reversed-phase separations are generally known to have the advantages of better stability and longer column durability than normal phase separations, the latter are more efficient in separating β and γ isomers of tocopherols and tocotrienols.

In this work, we report the results of our comprehensive study on elevated temperature separation of the tocopherol and tocotrienol isomers using NP-HPLC. Aside from our work, no reports have described the use of elevated temperature in separating the tocol-derivatives using NP-HPLC.

2.0 MATERIALS AND METHODS

2.1 Reagents

Tocopherols and tocotrienols were purchased as isomer kits from Merck (Damstadt, Germany). α-tocopherol acetate (α-TAc) was obtained from Sigma Aldrich (USA). Hexane, 2-propanol, diethyl ether, ethyl acetate, absolute ethyl alcohol, and 1,4-dioxane were analytical grade obtained from various suppliers.

2.2 Chromatographic Conditions

The HPLC systems consisted of a conventional HPLC system coupled with a column oven of a Perkin Elmer Autosystem Gas Chromatography (USA). HPLC separations were carried out using a Waters 515 HPLC pump (Milford, USA) for mobile phase

delivery. A Rheodyne 7125 injection valve (Cotati, USA) fitted with a 5 μL loop was used for sample introduction. Analyte peaks were detected using a Shimadzu SPD-6A UV detector (Kyoto, Japan) and were recorded on a Hewlett Packard HP 3396 Series II integrator (USA). The analytical columns used were: (i) 5 μm Hypersil silica (200 mm × 4.6 mm I.D.) (Sigma-Aldrich, Milwaukee, USA); and (ii) 5 μm Hypersil APS amino column (100 mm × 2.1 mm I.D.) (Hewlett Packard, USA).

2.3 Separation of Tocopherols and Tocotrienols by NP-HPLC on Amino and Silica Columns at Ambient Temperature

Samples of eight vitamin E isomers (α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol, and δ-tocotrienol) and internal standard α-tocopherol acetate were dissolved in ethanol and stored at –18°C in darkness. HPLC separations of the prepared mixture were performed on two columns: (i) silica column from Hypersil and (ii) amino column from Hypersil APS. The column performance was tested at different separation conditions (Table 1). The prepared mixture was injected in triplicate onto the column and the solute concentrations were 0.1– 0.3 mg/mL. UV detection of analytes for the comparison study was at 295 nm. All the separations were performed at ambient temperature (~23°C).

Table 1 Separation conditions used for NP-HPLC at ambient temperature

Column	**Mobile phase**		
	Flow rate	**Components**	**Ratio of components**
Hypersil APS amino column 100 × 2.1 mm I.D.	0.2 mL/min	Hexane - diethyl-ether	97.0 : 3.0
		Hexane - ethyl acetate	98.5 : 1.5
		Hexane - isopropanol	99.8 : 0.2
		Hexane - 1,4-dioxane - ethyl acetate	98.9 : 0.1 : 1.0
Hypersil silica column 200 × 4.6 mm I.D.	1.0 mL/min	Hexane - diethyl-ether	95.0 : 5.0
		Hexane - isopropanol	99.5 : 0.5
		Hexane - 1,4-dioxane	96.4 : 4.0
		Hexane - 1,4-dioxane - isopropanol	96.5 : 2.5 : 1.0

2.4 Separation of Tocopherols and Tocotrienols by NP-HPLC on Silica Columns at Elevated Temperature

Separation of prepared mixture at elevated temperature was carried on a Hypersil silica column using mobile phase hexane-1,4-dioxane 96.0:4.0 (v/v) at elevated temperatures (30°C – 60°C). The prepared mixture was injected in triplicate onto

the column and the solute concentrations were 0.1– 0.3 mg/mL. UV detection of analytes for the comparison study was at 295 nm.

3.0 RESULTS AND DISCUSSION

3.1 Separation of Tocopherols and Tocotrienols by NP-HPLC on Amino and Silica Columns at Ambient Temperature

All eight main isomers of tocopherols and tocotrienols were successfully separated by NP-HPLC. Based on the chromatograms obtained in this study (Figures 2 - 3), it was noted that the elution order for the nine vitamin E isomers on both columns were strongly in order of increasing analyte polarity: α-T-Ac→α-T→α-T_3→β-T→ γ-T→β-T_3→γ-T_3→δ-T→δ-T_3. The isomers with the lowest polarity were eluted first followed by the isomers with higher polarity. Thus, the normal-phase column provided separation based on the number as well as the position of methyl substituents on the chromanol moiety. Hence, the NP-HPLC separations confirm that the polarity of the tocopherol and tocotrienol isomers decrease with increased number of methyl groups [8].

The resolution of adjacent peaks and efficiencies for both columns were investigated and the results were summarized (Table 2). It was observed that in general, the amino-column showed greater retention for all the isomers studied. Approximately 42 minutes was required to separate all the isomers (Figure 2). The column efficiency for the amino-column was generally unsatisfactory relative to the silica column. Based on the resolution data, it was noted that elution with hexane-diethyl ether resulted in better separations with all R_s values greater than 1.25.

Because of its high efficiency, the silica column was then used for the optimization study for the isomers separation using NP-HPLC with different mobile phase compositions. The overall results obtained were better than the results generated by the amino-column. Complete separation of all nine vitamin E isomers was achieved within 20 minutes (Figure 3). The overall column efficiency (N/m) observed for the silica column was reasonable (>20 000 plates/m). Based on the resolution data gathered in this study, the best separation was obtained using a mixture of hexane with 1,4-dioxane as the eluent. All the resolution values obtained was higher than 1.25, which means the set of separation conditions was suitable for quantification purposes. However, we observed that γ-T~β-T_3 isomer pair was not successfully separated when a more polar modifier, such as diethyl ether or 2-propanol, was used as the modifier in mixture with hexane. Although good result could be obtained using mobile phase with tertiary solvent system such as hexane-1,4-dioxane-isopropanol, it was difficult to accurately reproduce this conditions with such small modifier composition, and a fresh eluent was needed each day.

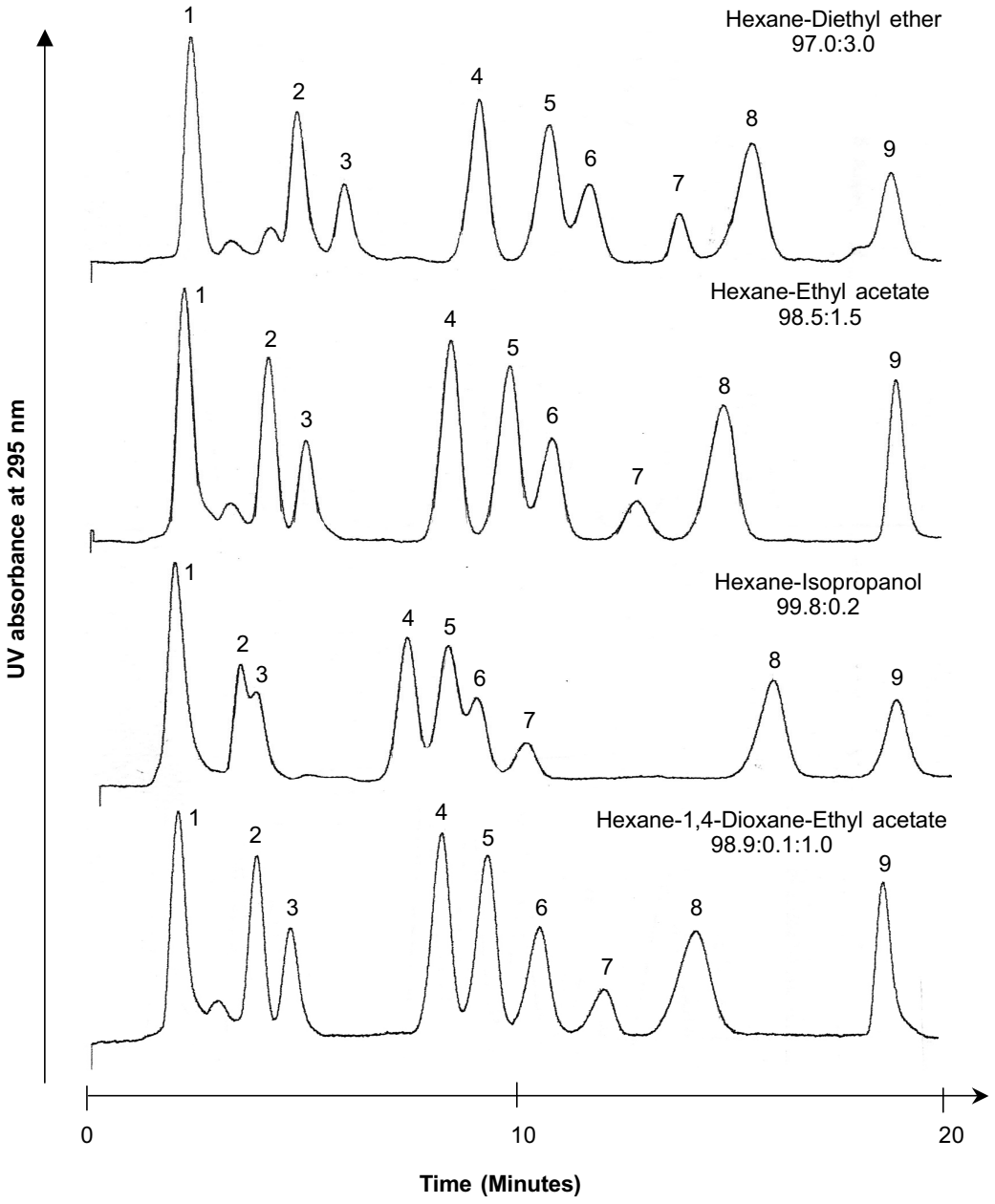


Figure 2 NP-HPLC separations of a balanced mixture of tocopherols and tocotrienols on amino-bonded silica column at ambient temperature with different mobile phases. Chromatographic conditions: flow rate: 0.2 mL/min. Peaks: 1 – α-tocopherol acetate, 2 – α-tocopherol, 3 – α-tocotrienol, 4 – β-tocopherol, 5 – γ-tocopherol, 6 – β-tocotrienol, 7 – γ-tocotrienol, 8 – δ-tocopherol, 9 – δ-tocotrienol

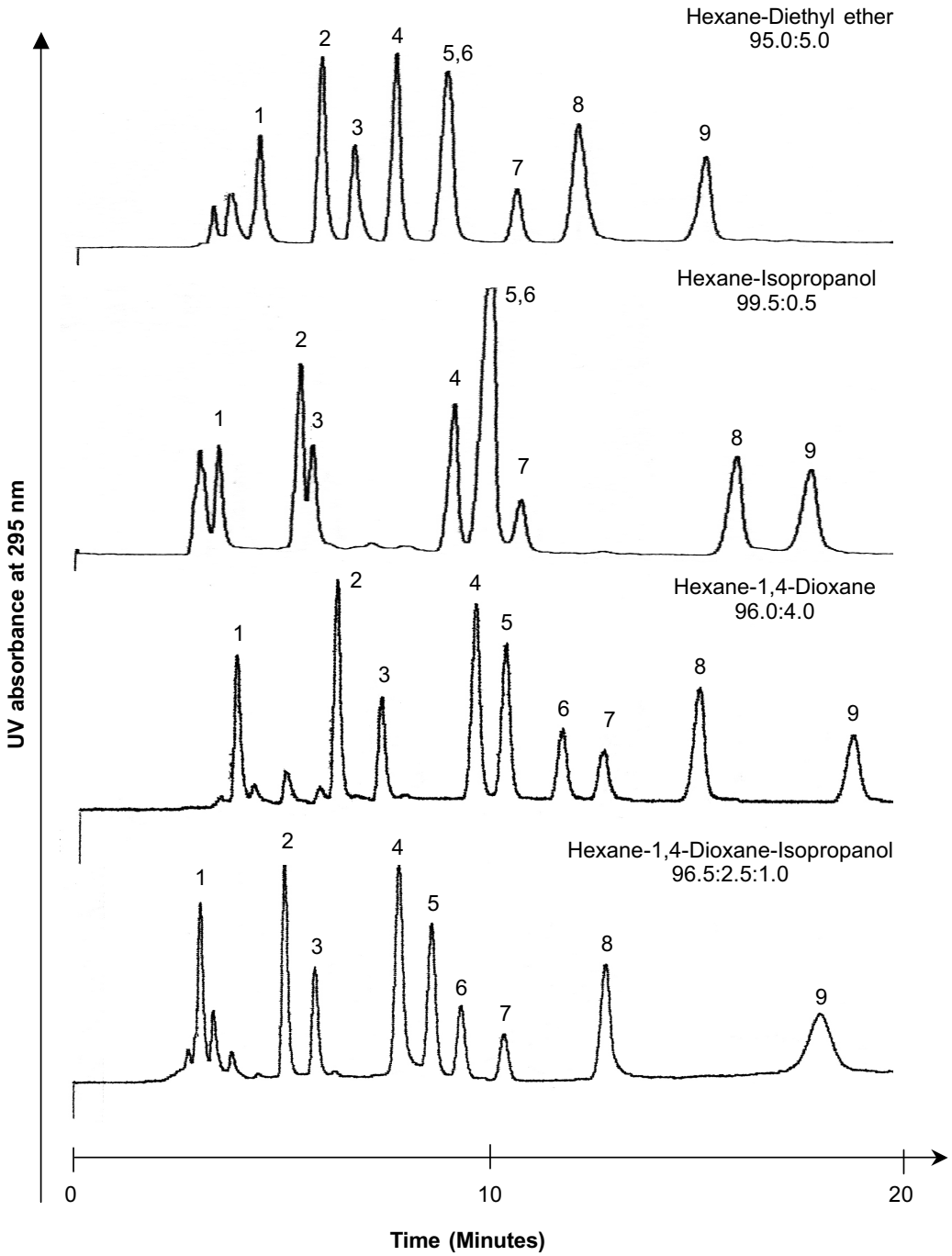


Figure 3 NP-HPLC separations of a balanced mixture of tocopherols and tocotrienols on Hypersil silica column at ambient temperature with different mobile phases. Chromatographic conditions: flow rate: 1 mL/min. Peaks: 1 - α-tocopherol acetate; 2 - α-tocopherol, 3 - α-tocotrienol, 4 - β-tocopherol, 5 -γ-tocopherol, 6 - β-tocotrienol, 7 - γ-tocotrienol, 8 - δ-tocopherol, 9 - δ-tocotrienol

Table 2 Resolution (R_s) of adjacent peaks and efficiencies (N/m) of the amino and silica columns in separating eight natural vitamin E isomers at ambient temperature

Mobile phase*	Total run time (min)	α-T		α-T_3		β-T		γ-T		β-T_3		γ-T_3		δ-T		δ-T_3
		N/m	R_s	*N/m*	R_s	*N/m*	R_s	*N/m*	R_s	*N/m*	R_s	*N/m*	R_s	*N/m*	R_s	*N/m*
Amino column																
Hx-diethyl ether 97.0:3.0 (v/v)	42	6000 (4.1)	2.08 (0.6)	79600 (3.1)	8.71 (2.1)	60500 (2.0)	3.18 (1.8)	55600 (4.2)	2.02 (5.0)	177600 (5.5)	7.15 (3.7)	552000 (4.3)	4.08 (3.4)	102400 (4.5)	5.72 (2.0)	221700 (2.0)
Hx-ethyl acetate 98.5:1.5 (v/v)	41	2200 (0.7)	1.14 (2.8)	3100 (3.8)	3.66 (1.9)	5300 (3.6)	1.73 (2.4)	23100 (4.4)	1.59 (2.3)	20300 (3.1)	3.02 (2.5)	30300 (4.1)	2.77 (4.4)	26400 (1.9)	4.92 (0.9)	41000 (2.5)
Hx-ISP 99.5-0.5 (v/v)	43	2600 (5.2)	0.80 (0.9)	10700 (3.0)	9.37 (1.1)	19600 (2.0)	2.17 (3.5)	32900 (2.5)	1.67 (5.0)	52000 (4.7)	3.90 (3.3)	166800 (3.7)	13.05 (3.2)	40300 (3.5)	5.04 (5.3)	106800 (3.8)
Hx-1,4-dx-e-ace 98.9:0.1:1.0 (v/v)	40	2000 (2.2)	1.00 (2.0)	2500 (3.5)	3.87 (91.3)	5200 (1.6)	1.03 (1.7)	6100 (3.4)	1.49 (2.2)	25300 (3.9)	2.48 (1.2)	31300 (0.4)	2.84 (1.3)	21000 (4.5)	5.18 (2.2)	41800 (4.4)
Silica column																
Hx-diethyl ether 95.0:5.0 (v/v)	15	15100 (5.0)	1.79 (1.4)	14700 (2.2)	2.03 (1.2)	20300 (4.5)	2.16 (2.6)	14100 (3.6)	-	14100 (3.6)	2.91 (4.4)	29600 (1.5)	2.49 (2.6)	29700 (3.5)	3.96 (0.7)	36700 (5.4)
Hx-ISP 99.5:0.5 (v/v)	18	12400 (4.1)	0.71 (2.1)	10300 (1.1)	6.51 (1.9)	22000 (2.3)	1.19 (1.3)	12500 (2.2)	-	12500 (2.2)	1.21 (1.3)	22600 (5.0)	7.18 (1.0)	32500 (1.2)	2.17 (0.9)	33600 (2.5)
Hx-1,4-Dioxane 96.0:4.0 (v/v)	21	24400 (3.9)	3.84 (2.1)	83300 (2.9)	6.37 (1.1)	30600 (2.7)	1.52 (0.8)	34000 (2.5)	2.73 (1.2)	40000 (2.4)	1.89 (0.9)	44100 (2.2)	3.71 (2.1)	40700 (2.5)	5.26 (1.9)	44600 (2.4)
Hx-1,4-dxe-ISP 96.5:2.5:1.0 (v/v)	23	19600 (3.2)	2.31 (0.3)	26800 (6.2)	5.55 (0.8)	27200 (2.1)	1.80 (0.1)	28800 (0.5)	1.60 (1.8)	32700 (0.5)	2.14 (0.7)					20700 (1.0)

- Not separated (R.S.D. %) based on triplicate injections N/m = Theoretical plates/column length

*Solvent: Hx = Hexane, ISP = Isopropanol. 1,4-dx = 1,4-diaxane, e-ace = ethyl acetate

3.2 Separation of Tocopherols and Tocotrienols by NP-HPLC on Silica Columns at Elevated Temperature

Based on the results obtained for the vitamin E isomers separation using NP-HPLC, the following separation conditions were chosen: hexane-1,4-dioxane (96.0:4.0 (v/v) as the mobile phase, Hypersil silica column: (200 × 4.6 mm I.D.), flow rate of 1.0 mL/min and UV wavelength at 295 nm. Although good separations could be obtained using the optimum conditions mentioned above, better column efficiencies and reduced analysis times could be achieved by simply increasing separation temperature. Therefore, the optimum condition was investigated at different column temperatures from 30°C to 60°C with 10°C increments. From the chromatograms obtained (Figure 4), it was found that the elution times were somewhat inversely proportional to the column temperature. Based on the retention factors obtained in our study (Table 3), it was observed that for each isomer, the retention factor decreased by a factor of 1.08 when the column temperature was increased by 10°C. This observed trend was found consistently for the vitamin E isomers at all column temperatures studied.

It was also noted from Table 3 that the column efficiency was directly proportional to column temperature. This was probably due to the increase in the diffusion coefficients of the mobile phase and the analytes with the increase in column temperature. The resolution data gathered in our study demonstrated that the best separation was achieved at a column temperature of 40°C. In order to determine the repeatability and reproducibity of the separation, a study was conducted under the optimum separation conditions. In the study, the retention factors and the peak area response factor were observed on the same day and on different days. It was found that the results achieved on the same working day were generally acceptable with RSD value of <5%. Based on the results obtained on three consecutive days, slightly increased RSD % values were observed. Nevertheless, all the RSD % values were lower than 7%. Therefore, the elevated temperature NP-HPLC system for the separation of vitamin E isomers developed in this study is suitable to be used as a separation and quantification technique. The detection limit values, expressed as amount injected, were 5 ng (δ-tocopherol and δ-tocotrienol), 10 ng (γ-tocopherol, β-tocopherol and β-tocotrienol), 15 ng (α-tocopherol), and 20 ng (α-tocotrienol and γ-tocotrienol).

3.3 Determination of Tocol Derivatives in Crude Palm Oil (CPO) Using Elevated Temperature NP-HPLC

Figure 5 shows a HPLC chromatogram of crude palm oil (CPO) using optimized separation conditions developed in this study. The major forms of vitamin E isomers that exist in the CPO are γ-tocotrienol (55%) followed by α-tocotrienol (20%), α-tocopherol (15%), and δ-tocotrienol (10%). These results were comparable with those obtained by Maclellan [9]. In his report, NP-HPLC at ambient operating

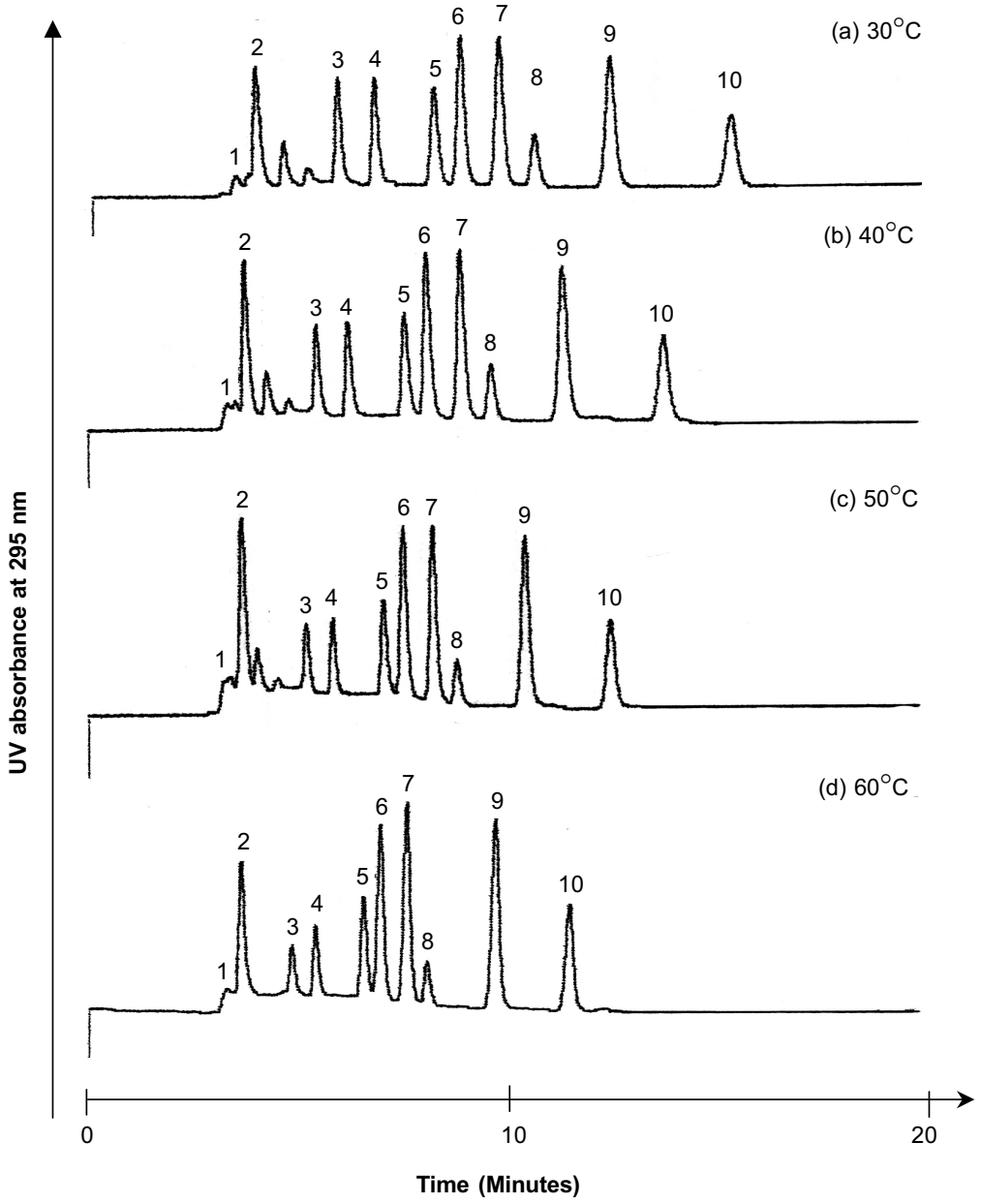


Figure 4 NP-HPLC separations of a balanced mixture of tocopherols and tocotrienols on Hypersil silica column at different column temperature. Chromatographic conditions: mobile phase: hexane-1, 4-dioxane (96.0:4.0 v/v); flow rate: 1 mL/min. Peaks: 1 - solvent, 2 - α-tocopherol acetate; 3 - α-tocopherol, 4 - α-tocotrienol, 5 - β-tocopherol, 6 -γ-tocopherol, 7 - β-tocotrienol, 8 - γ-tocotrienol, 9 - δ-tocopherol, 10 - δ-tocotrienol

Table 3 Retention factors (k') and resolution (R_s) of adjacent peaks for eight vitamin E isomers as a function of column temperature using hexane-1,4-dioxane 96.0:4.0 (v/v) as the eluent on silica column

Vitamin E isomers	Column temperature											
	30°C			40°C			50°C			60°C		
	k'	N/m	R_s	k'	N/m	R_s	k'	N/m	R_s	k'	N/m	R_s
α-tocopherol	0.60	21100	2.36	0.56	21400	2.23	0.52	22900	2.09	0.47	29200	2.00
	(0.0)	(0.8)	(0.1)	(1.0)	(3.4)	(1.7)	(0.1)	(3.8)	(0.8)	(1.2)	(1.2)	(0.9)
α-tocotrienol	0.85	23000	3.33	0.78	25200	3.59	0.71	26100	3.74	0.65	23800	3.60
	(0.7)	(0.5)	(0.5)	(0.2)	(5.0)	(1.9)	(0.1)	(2.6)	(1.2)	(0.9)	(5.1)	(1.1)
β-tocopherol	1.23	27000	1.39	1.16	29100	1.33	1.08	32900	1.28	1.00	31500	1.17
	(0.5)	(1.2)	(0.8)	(0.9)	(7.0)	(2.7)	(0.5)	(1.4)	(1.2)	(1.0)	(1.4)	(1.3)
γ-tocopherol	1.40	29000	1.99	1.31	30700	1.94	1.22	32800	1.88	1.12	31600	1.75
	(0.4)	(0.4)	(0.6)	(0.9)	(4.7)	(2.1)	(0.8)	(1.9)	(0.5)	(0.9)	(3.0)	(2.0)
β-tocotrienol	1.66	30900	1.72	1.55	34300	1.67	1.43	37100	1.59	1.31	35800	1.41
	(0.3)	(1.2)	(0.6)	(1.0)	(5.3)	(2.4)	(0.7)	(1.2)	(1.3)	(0.8)	(5.3)	(1.5)
γ-tocotrienol	1.90	37100	3.47	1.75	41300	3.72	1.60	47000	4.16	1.46	46600	4.30
	(0.6)	(2.5)	(1.2)	(0.7)	(5.6)	(2.7)	(0.4)	(1.1)	(2.0)	(0.7)	(6.1)	(2.2)
δ-tocopherol	2.40	38000	4.60	2.25	39600	4.41	2.11	43300	4.35	1.95	42900	4.12
	(0.6)	(1.4)	(0.4)	(0.4)	(3.7)	(1.3)	(0.1)	(1.4)	(0.5)	(0.6)	(3.2)	(0.4)
δ-tocotrienol	3.17	44800		2.93	46800		2.71	53500		2.49	56000	
	(0.3)	(2.3)		(0.7)	(3.4)		(0.1)	(0.6)		(0.6)	(0.6)	
Total run time	16 min			14 min			13 min			12 min		

(R.S.D. %) based on triplicate injections

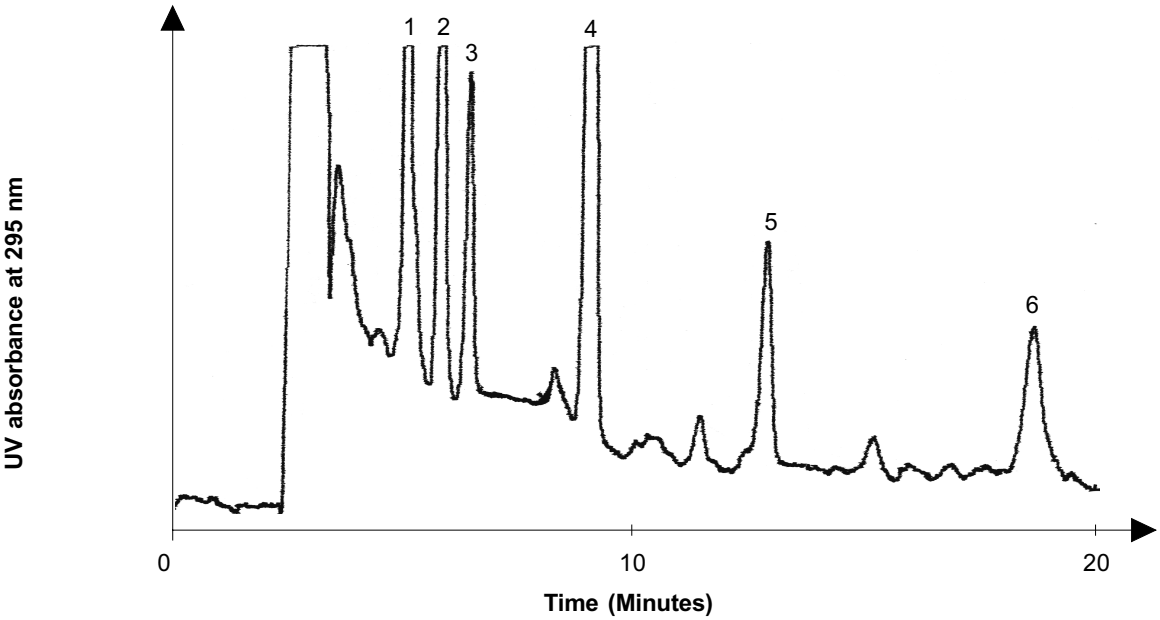


Figure 5 NP-HPLC separations of crude palm oil on a Hypersil silica column. Chromatographic conditions: mobile phase: *n*-hexane-1,4-dioxane (96.0:4.0 v/v); flow rate: 1 mL/min; temperature: 40°C. Peaks: 1 - α-tocopherol, 2 - α-tocotrienol, 4 - γ-tocotrienol, 5 - δ-tocotrienol, 3 & 6 - unknown

temperature was utilized as the separation method. The total analysis time required in Maclellan study was generally longer that the analysis time required in our study.

4.0 CONCLUSIONS

Separation of eight natural vitamin E isomers and α-tocopherol acetate using NP-HPLC on both amino and silica column was developed successfully in our research work. Silica column generally resulted in better separation efficiency and resolution relative to amino column. It was found that by simply increasing the column temperature, excellent separation efficiencies and shorter analysis times were achieved without significant loss in resolution. The successfully developed method was applied to the analysis of tocol derivatives in CPO and the separations were comparable with the previous report in terms of resolution. However, the developed method generally resulted in shorter analysis times, and thus advantageous for separation of the vitamin E isomers. Current work provides a great interest to further investigate on the applicability of the separation method to the analysis of vitamin E isomers content in other sample matrices such as food, medicines, etc.

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