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ANALYSIS OF TOCOPHEROLS AND TOCOTRIENOLS IN PALM OIL AND PALM OLEIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) : A PRELIMINARY STUDY

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Keywords: Palm oil, Palm olein, Tocopherols, Tocotrienols, HPLC and Synthetic antioxidants.

RINGKASAN

Pengasingan dan penjumlahan 'tocopherol' dan 'tocotrienol' yang berada dalam minyak kelapa sawit telah dijayakan dengan kaedah 'High Performance Liquid Chromatography, (HPLC)'. Kaedah ini memerlukan saponifikasi contoh, diikuti dengan suntikan bahan tidak tersaponifikasi ke dalam sistem HPLC yang mempunyai 'adsorption column', 7% etil asitat dalam n-heptane sebagai fasa bergerak dan pengesan 'ultra violet'.

INTRODUCTION

Tocopherols and their related compounds, tocotrienols are naturally occurring substances which show antioxidative and vitamin E properties in most vegetable oils. At present eight forms of tocopherols (T) and tocotrienols (T_3) are known to exist in nature *(Appendix I)* and it is found that palm oil contains a variety of the homologues. The role of antioxidant such as tocopherol in inhibiting the process of auto-oxidation has been discussed by a number of authors (LUNDBERG, 1962; WITTING, 1975; JACOBSBERG *et al.*, 1978). In view of the fact that each individual form of tocopherols has its own degree of anti-oxidative and vitamin E activities, it is of interest to be able to quantitate the amount of various forms individually.

BUNNEL, (1971) reviewed the various methods of tocopherols determination in oils and fats. In the case of palm oil, DPPH method was the most widely used. This method is unspecific and measures the total amount of reducing material in the oil, and therefore it is unsuitable for refined palm oil (JACOBSBERG *et al.*, 1973). Determination of individual tocopherols and tocotrienols normally requires pretreatment of sample such as removal of triglycerides and interfering substances and therefore the method becomes tedious and time-consuming.

Triglycerides are normally removed by saponification, a simple and fast method compared to other possible methods such as crystallization or molecular distillation. Quantitative determination of individual tocopherols and tocotrienols is then achieved either through TLC separation followed by spectrophotometric Emmerie-Engel reaction or by GLC with or without derivatization. Most of the GLC methods however, including the method developed by MEIJBOOM (1979) (TLC+ GLC technique), fail to separate the β/γ tocopherols except the method developed by MORDRET, 1978 (Capillary GLC). A fast technique, Differential Pulse Voltammetry (DELDRIME *et al.*, 1977; HENDRIKSE *et al.*, 1978; PODLAHA *et al.*, 1978), requires no pretreatment of sample but still fails to separate β/γ tocopherols.

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By a more recent technique, High Performance Liquid Chromatography (HPLC), the separation of $\alpha-$, $\beta-$, $\gamma-$ and $\delta-$ tocopherols were effected (NIEKERK, 1973; ERIKSON, 1977; TANGNEY, 1979 and CARPENTER, 1979). An attempt to separate tocotrienols from its tocopherols was first recorded by ERIKSON *et al.*, (1977). Using a Corasil II column (1.50 m x 0.64 cm), with 0.7 ml/min flow rate of 0.5% tetrahydrofuran in n-hexane and spectrofluorimeter detector they managed to get fairly good separation of $\alpha-$tocopherol from $\alpha-$tocotrienol but both $\gamma-$tocotrienol and $\delta-$tocotrienol were shown as broad peaks.

Our previous study (ABD. GAPOR *et al.*, 1979) on the application of HPLC for the separation and quantitative determination of tocopherols and tocotrienols in palm oil involved isocratic elution using hexane – isopropyl alcohol, hexane – ethyl acetate, heptane – isopropyl alcohol and n–heptane – ethyl acetate systems at various solvent proportion on adsorption mode column with ultraviolet detection and the results indicated that 7% ethyl acetate in n–heptane was the best mobile phase. The objective of this paper is to report the progress we have made using the best mobile phase we have found.

MATERIALS AND METHODS

Chemicals:

(1) DL – alpha – Tocopherol for biochemistry; Content 99%; $C_{29}H_{50}O_2$; Molecular weight 430.72; Merck.

(2) d–Beta–Tocopherol: $C_{28}H_{48}O_2$; Molecular weight 416.69; Eastman Kodak Co.

(3) d–Gamma–Tocopherol; $C_{28}H_{48}O_2$; Molecular weight 416.69; Eastman Kodak Co.

(4) Tocomix D Liquid : Mixture of natural tocopherols, mainly $\alpha-$T and $\gamma-$T in natural glycerides. Jandekkerby, Naarden International Chemical Division.

(5) Ethyl acetate : Not less than 99% esters as $C_4H_8O_2$; May and Baker.

(6) n–Heptane : Content (G.C.) 99%; Merck.

Equipment:

HPLC: Hewlett-Packard Liquid Chromatograph 1084 B
- Double head (gradient); reciprocating diaphragm pump.
- Temperature controlled column compartment.
- Variable volume injector.
- Microprocessor control.
- User terminal with thermal printer/plotter.

Column: HP 79920 A for adsorption;
250 mm long; 4.6 mm int. diameter; 2μm frit pore;
Stationary phase : Lichrosorb Si 100 particles; size 5μm.

Detector: Variable UV – detector.

Samples:

RBD palm olein A; samples on 19.9.78 (30 – 50 ppm BHT added).
Refined palm oil B; samples on 8.6.79.
Crude palm oil C; samples on 21.6.79.

Sample treatment and HPLC injection

Sample (5g) was saponified according to the method by MEIJBOOM (1979) and the unsaponifiable matter was then separated out and dissolved in 5 ml of the mobile phase solvent. The tocopherols/tocotrienols analyses were done by injecting 20 μl of sample solution into the High Performance Liquid Chromatography system with the following working conditions:

Working conditions:

Mobile phase: 7% ethyl acetate in n-heptane

Flow: 2.98 ml/min

Column Pressure: 100 bar (Maximum pressure of the system 400 bar and minimum pressure 0 bar).

Solvent Temp. Reservoir A and B: 30°C

Oven Temperature: 30°C

Variable Wavelength: detection wavelength 300 nm and reference wavelength 430 nm.

Chart Speed: 1.00 cm/min.

Attenuation 2^{+}: 6 (2^{6})

Area Rejection: 300

Slope Sensitivity: 1.00

(Three minutes after injection, the slope sensitivity was changed to 0.50).

Calibration and quantitation

5, 10, 15, 20, 25 μg in 20 μl solution of α–T, β–T and γ–T standards were injected and the areas produced were plotted against the concentration.

Tocopherols and tocotrienols in palm oil samples were quantitated by the external standard method, based on α–tocopherol standard.

Influence of added antioxidants

In order to prevent oxidation, some oils are added with synthetic antioxidants such as Butylated Hydroxyanisole (BHA; 2, (3) – Terbutyl – 4 – Hydroxyanisole), Butylated Hydroxytoluene (BHT), Tertiarybutylhydroquinone (TBHQ), Propylgallate (PG), and Octylgallate (OG). In view of this, we decide to check if these compounds will show up under the working conditions for tocopherols. 10 μl of a 1% solution of the above synthetic antioxidants were injected into the HPLC system.

RESULTS AND DISCUSSION

Detector responses displayed in terms of area of α–, β– and γ– tocopherol standards within the likely range of tocopherols/tocotrienols concentration in palm oil samples were found to be linear as shown in *Figure 1*.

A typical chromatogram obtained from the oil samples in examplified by the chromatogram of Refined Palm Oil, as shown in *Figure 2*. For identification and quantitation purposes, chromatograms of laboratory-prepared mixture of standards (α–, β–, γ–tocopherols), commercially–prepared mixture of standards (α–, β–, γ– and δ– Tocopherols) and Refined Palm Oil with added β– tocopherol are shown in *Figures 3, 4* and *5* respectively.

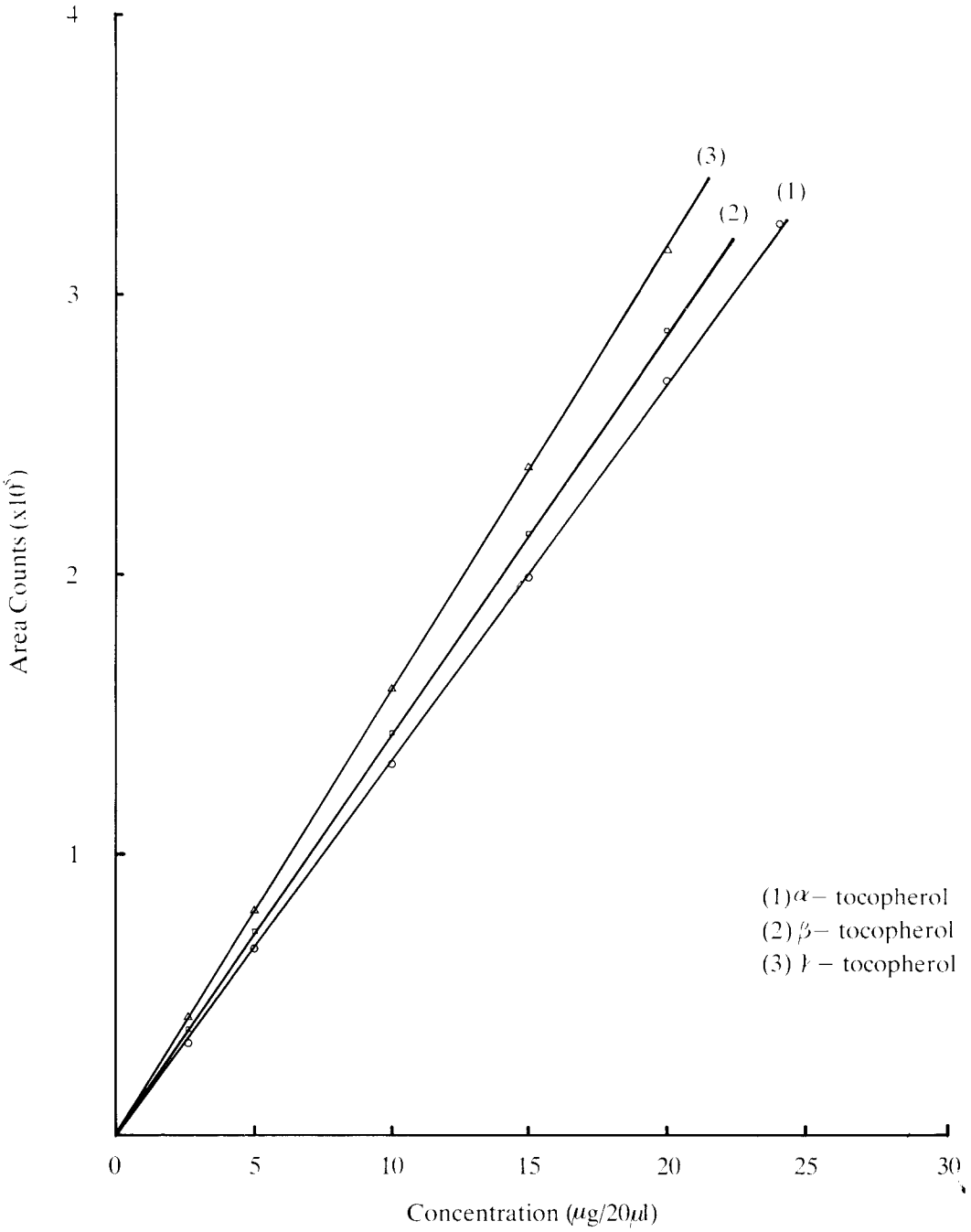

Figure 1. Calibration graph of α−, β− and γ−tocopherols

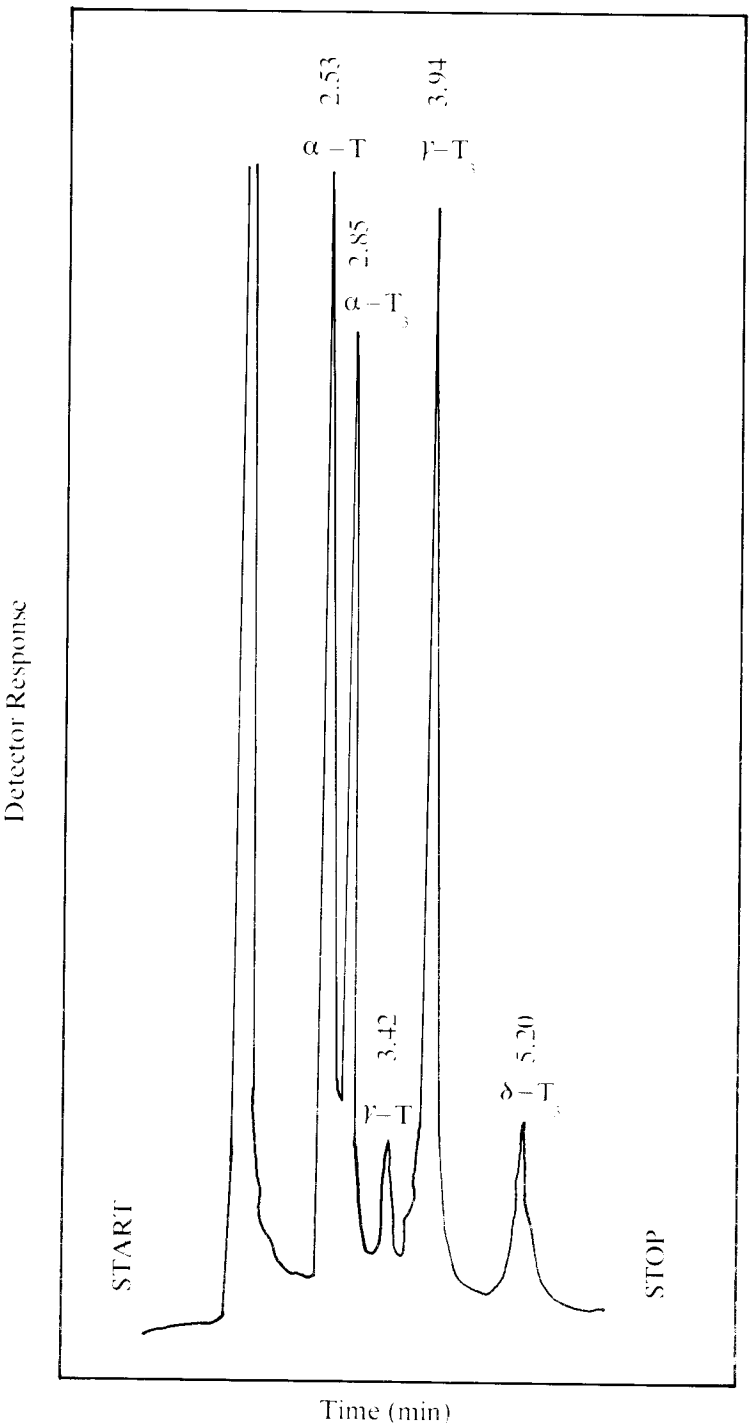

Figure 2. Chromatogram of Refined Palm Oil

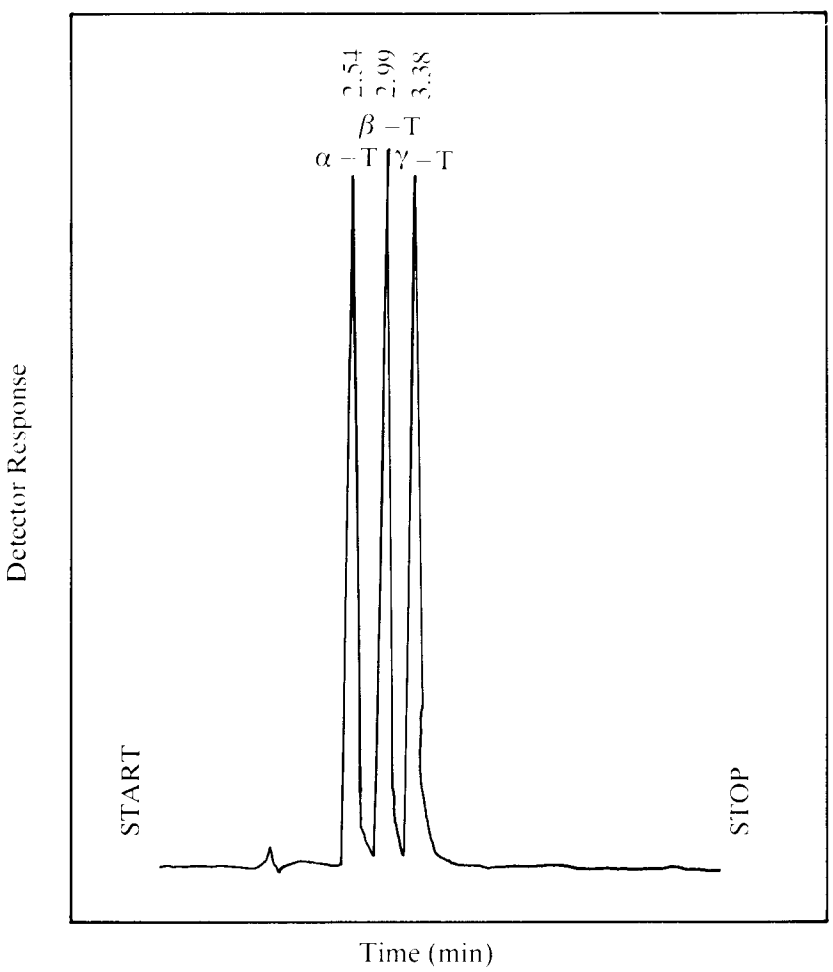

Figure 3. Mixture of α–T, β–T and γ–T standards.

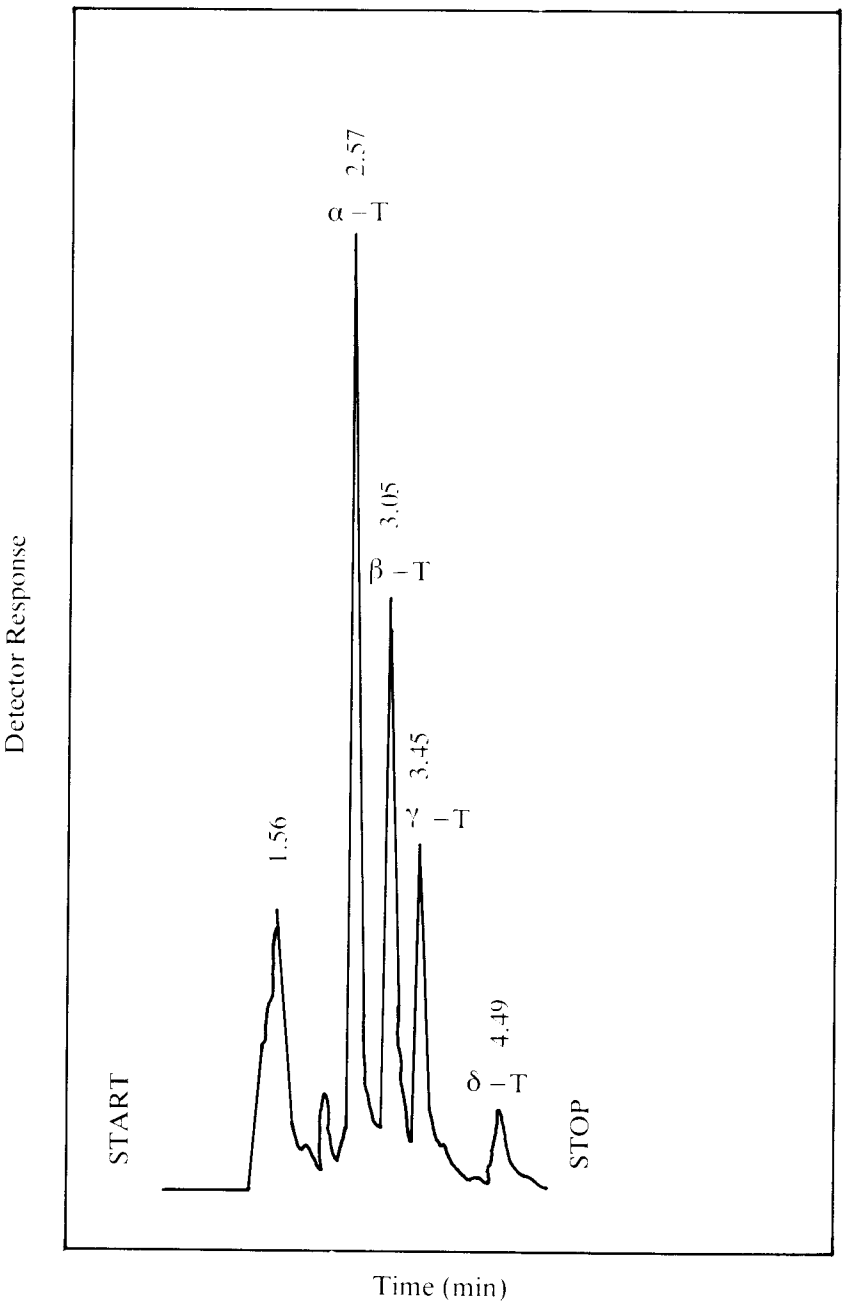

Figure 4. Tocomix D, mixture of commercial mixture of α–, β–, γ – and δ– tocopherols.

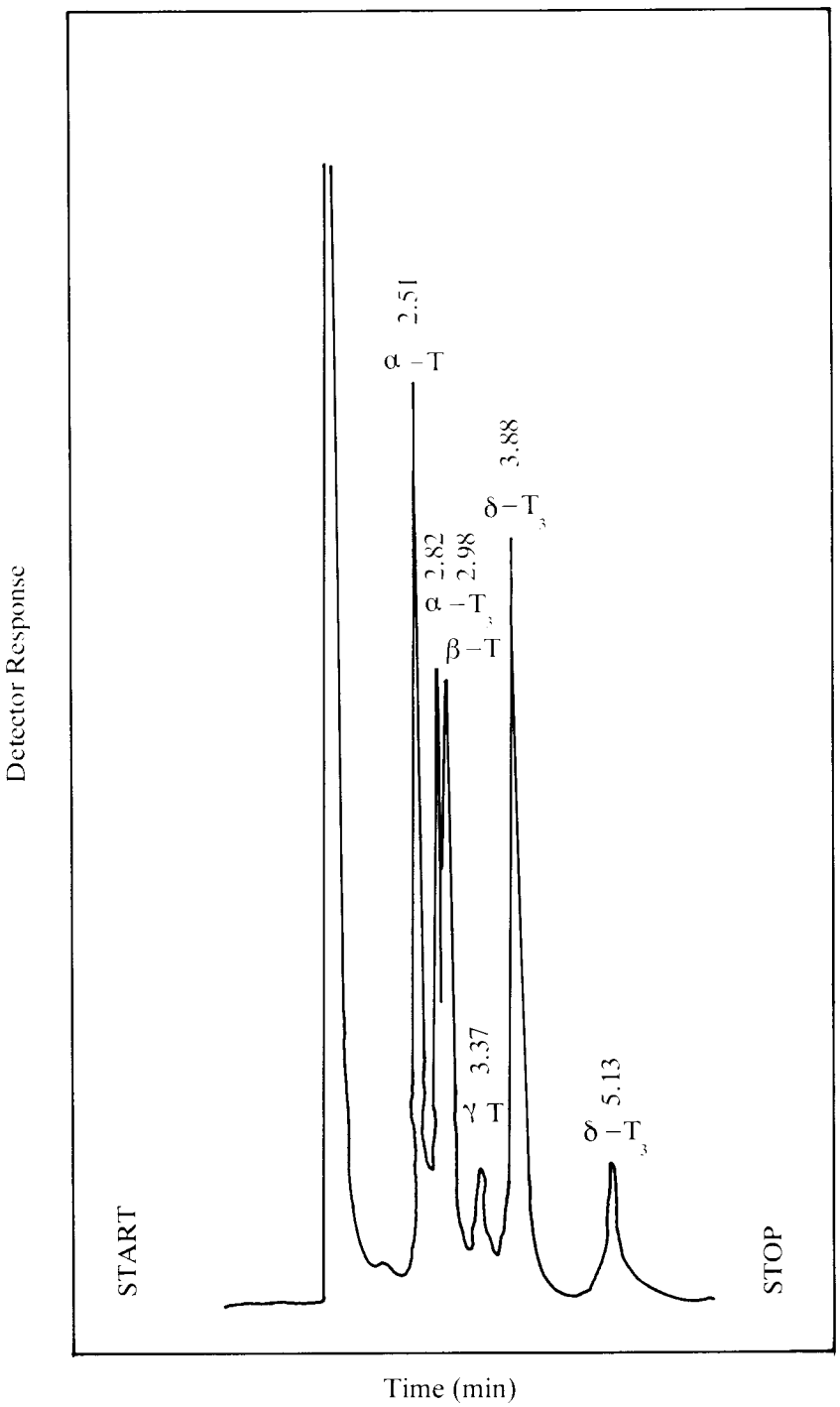

Figure 5. Refined Palm Oil with added β−T

Identification of Tocopherols and Tocotrienols in Palm Oil

WHITTLE *et al.*, (1967) established the existence of α-T, α-T_3, β-T_3, γ-T and δ-T_3 by two-dimensional thin-layer chromatography and subsequent colorimetric determination. JACOBSBERG *et al.*, (1978) confirmed the presence of δ-T_3 by mass-spectrometer and found no δ-T. The presence of β-T_3 was also reported by SEHER (1973).

Our work involved spiking palm oil with α-T, β-T, and γ-T, the results confirmed that there are α, γ – tocopherols and no β – tocopherol. However, by comparing the results with established reports the increase of γ – tocopherol peak could also be assigned to β-T_3 which could possibly be having about the same retention time as the γ – tocopherol. Due to unavailability of tocotrienol standards, we propose the other peaks be assigned to α-T_3, γ-T_3 and δ-T_3 components. Furthermore, the addition of Tocomix D to RBD palm oil showed an in α-T and other peaks remained the same.

Tocopherols and Tocotrienols contents

The contents of tocopherols and tocotrienols of the samples are presented in *Table 1*. The concentration was calculated with respect to α-T standard.

It is of interest to note that the commercially prepared tocopherols concentrate, Tocomix D was found to have a concentration of about 10% of tocopherols consisting of 46.0% α-T, 29.7% β-T, 20.2% γ-T and 4.2% δ-T.

TABLE 1. TOCOPHEROLS/TOCOTRIENOLS CONTENT
(EXPRESSED AS P.P.M. -TOCOPHEROL)

Components	α-T	α-T_3	β-T	β-T_3	γ-T	γ-T_3	δ-T	δ-T_3	Total	Note
Rt (from standards)	2.54		3.01		3.40		4.44			a
Rt (from samples)	2.53	2.83			3.39	3.90		5.14		a
Crude Palm Oil	279	274			61	398		69	1081	b
	(25.8%)	(25.3%)			(5.6%)	(36.8%)		(6.4%)		
Refined Palm Oil	236	191			41	290		44	802	b
	(29.4%)	(23.8%)			(5.1%)	(36.2%)		(5.5%)		
RBD Palm Olein	237	190			49	269		46	791	b
	(30.0%)	(24.0%)			(6.2%)	(34.0%)		(5.8%)		

a – Average of six readings
b – Average of two determinations

Possible interference by added synthetic antioxidants in tocopherols/tocotrienols determination

Figure 6 shows the chromatograms of BHA, BHT, TBHQ, PG and OG obtained by injecting 10μl of 10,000 ppm of the respective antioxidant solution. By comparing the retention times of the synthetic antioxidants *(Table 2)* with those of tocopherols and tocotrienols from *Table 1*, it seemed that BHA, PG and TBHQ could interfere in the tocopherols and tocotrienols determination.

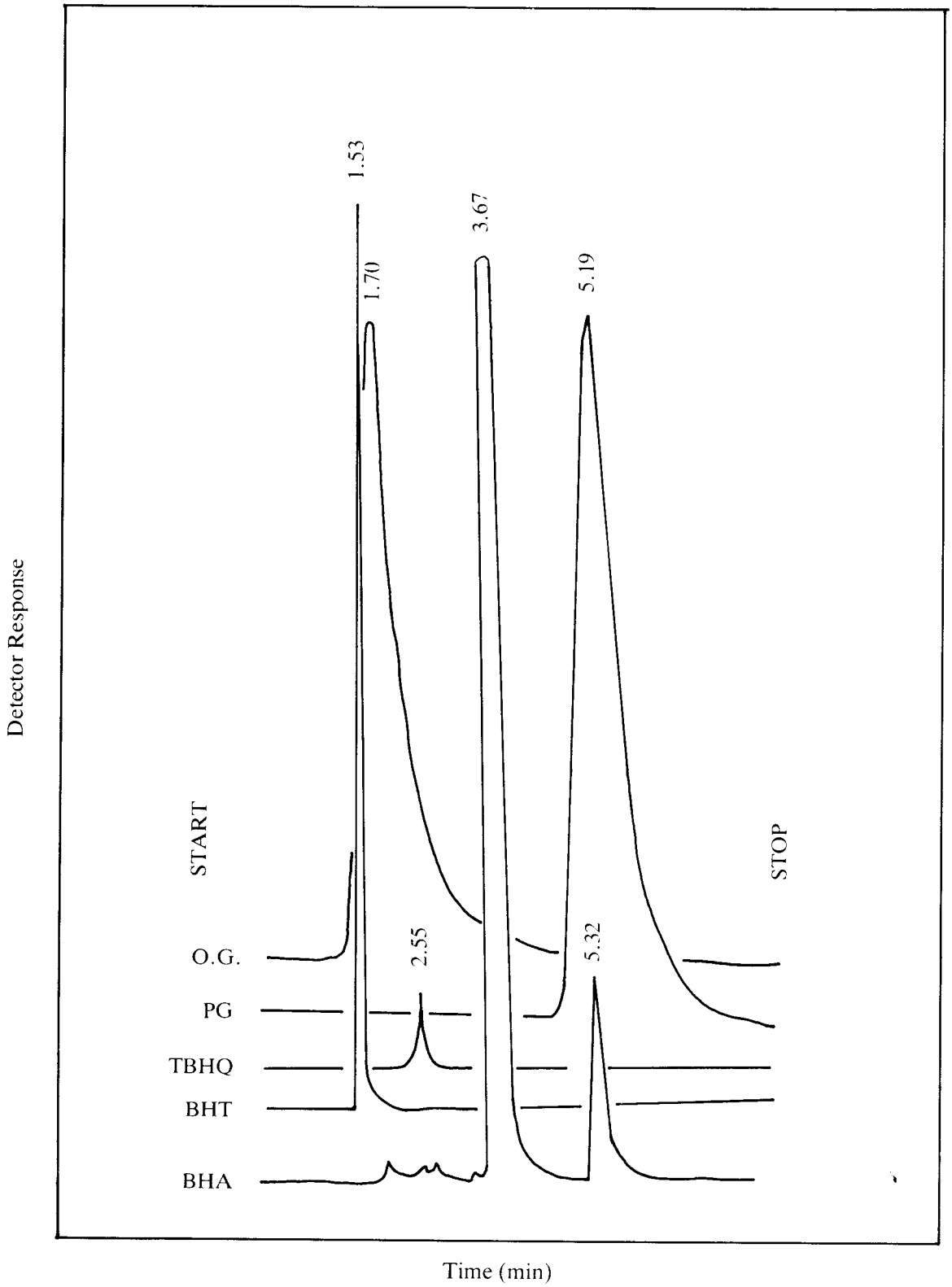

Figure 6. Chromatograms of synthetic antioxidants (10,000 p.p.m. 10μℓ injection)

TABLE 2. RETENTION TIMES (Rt) OF SYNTHETIC ANTIOXIDANTS

Synthetic antioxidants	BHA	BHT	TBHQ	PG	OG
Rt	3.67 (96%) 5.32 (4%)	1.53	2.55	5.19	1.70

However, should there be any antioxidant added, the level normally is less than 100 ppm. Further work on this aspect is to be carried out.

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SUMMARY

Separation and quantitation of tocopherols and tocotrienols present in palm oil were effected by High Performance Liquid Chromatography (HPLC) technique. The method requires saponification of sample followed by isolation and injection of the unsaponifiable matter into the HPLC system having adsorption column, 7% ethyl acetate in n–heptane as the mobile phase and ultraviolet detector.

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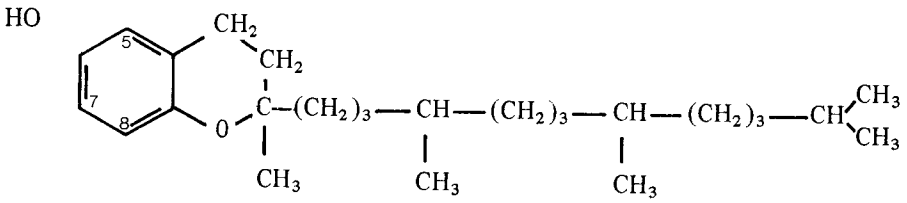
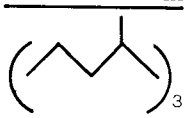
APPENDIX 1 : TOCOPHEROLS FOUND IN VEGETABLE MATTER
(JACOBSBERG *et al.*, 1978)



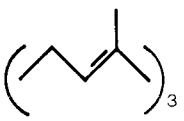
Tocol

α	5,7,8	trimethyltocol
β	5,8	dimethyltocol
γ	7,8	dimethyltocol
∫	8	methyltocol

Tocol side chain



Tocotrienol side chain



These homologues exist as tocopherols and tocotrienols.

NOTE: The ϵ, η and ζ tocopherols of older literature have been more recently identified respectively as β, γ and α tocotrienols.