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SEPARATION OF INDIVIDUAL TOCOPHEROLS FROM HUMAN PLASMA AND RED BLOOD CELLS BY THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

A thin-layer chromatography–gas–liquid chromatography system has made it possible to separate the individual tocopherols for qualitative and quantitative determination in blood plasma and red blood cells. The peak of the individual tocopherols found in plasma and red blood cells were identified by their R_F values and comparing their relative retention times with that of 5,7-dimethyltocol which served as an internal reference standard.

The detection limits for the tocopherol with the hydrogen flame detector was found to be in the range of 0.03 μg.

Fat-soluble reducing substances which constitute the major source of interference in the commonly used methods do not interfere with the separation of the tocopherols.

INTRODUCTION

Within the last decade the group of naturally occurring compounds possessing vitamin E activity has been shown to be comprised of α-tocopherol (5,7,8-trimethyltocol), β-tocopherol (5,8-dimethyltocol), γ-tocopherol (7,8-dimethyltocol), and δ-tocopherol (8-methyltocol), and their analogous tocotrienols containing three unsaturated bonds in the side chain[1].

American astronauts in some space flights have experienced the loss of significant amounts of red cell mass in the absence of nitrogen[2]. Other related changes reported in Apollo 9 astronauts included decreases in plasma levels of vitamins E and A, in red blood cell lipid, and in glutathione of red cells. Tappel[3] stated that vitamin E serves as the biologic lipid antioxidant. Roehm[4] suggested that it is entirely possible that vitamin E provides an important measure of protection to vitamin A against destructive oxidation.

To understand better the cellular mechanisms of vitamin E actions on biologic systems, the complete separation of the individual tocopherols is necessary. The

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development of analytical methods employing paper, column, thin-layer chromatography (TLC) or gas–liquid chromatography (GLC) has afforded much more sensitive and specific means for determining the contents of tocopherols. The use of TLC for the analysis of tocopherols has made rapid advances in the last 10 years so that it is now one of the most important analytical tools in this field. The choice of a mobile phase is usually dictated by the pattern of tocopherols present, the impurities which must be removed, and whether the investigator wishes to quantitatively estimate all the individual tocopherols which are present in the sample.

Seher[5] reported useful separation of tocopherols on thin layers of alumina with benzene as the developing solvent, and on silica gel with chloroform as the solvent. Bolliger[6] and Dilley and Crane[7] reported better separation on silica gel G with benzene–methanol (98:2) than with cyclohexane–diethyl ether (80:20), but with either of these solvent systems the separation of β- and γ-tocopherols was not accomplished.

Bro-Rasmussen and Hjarde[8,9] reported that secondary magnesium phosphate gave a useful separation of α- and β-tocopherols by column chromatography, but it proved slightly inferior to silica gel as a thin layer. Schmandke[10] reported the separation of α- from γ-tocopherol, but poor resolution was obtained. Marcinkiewicz and Green[11] reported a nitroso-derivative separation of β- and γ-tocopherols with two-dimensional paper chromatography. Others have reported TLC methods for vitamin E[12–15]. Stowe[16] was the first to report on a qualitative TLC separation of β- and γ-tocopherols in the presence of α- and δ-tocopherols using silica gel G, but because of the complexity of the tocopherols applied, the R_F values were not reported.

Pennock and his co-workers[17,18] introduced further refinements of silica gel TLC of the tocopherols by using two-dimensional systems to separate the tocopherols and tocotrienols. During the course of the development of their methods, it was found that saponification destroyed a significant amount of tocotrienols. Whittle and Pennock[18] stated that in order to handle an unknown compound it is necessary to run a qualitative two-dimensional thin-layer chromatogram with tocopherol standards to learn the pattern of the tocopherols and interfering substances.

Nair *et al.*[19] separated the isomeric dimethyltocols by GLC on a binary mixture of rubber SE-92 and XE-60 after oxidizing the tocopherols to their *p*-quinones.

The technique of Duggan[20], the GLC procedure of Wilson *et al.*[21], and the partition chromatography method proposed by the Analytical Methods Committee[22] do not separate β- and γ-tocopherols.

A combination of radiolabel techniques, TLC, and GLC was used by Bieri *et al.*[23] in solving the problem of the determination of α-tocopherol in erythrocytes. Preliminary experiments had indicated erratic results and only 30% to 60% recovery of [α-^{14}C]tocopherol added to erythrocytes. Bieri and Prival[24] reported the determination of α- and ($\beta+\gamma$)-tocopherol by TLC and GLC, but separation of β- and γ-tocopherols and the detection of δ-tocopherol were not achieved. It was suggested by these authors that it would be desirable to know what proportions of the total serum tocopherols are contributed by the individual tocopherols. Lehmann and Slover[25] reported a method for the determination of plasma α- and γ-tocopherol-trimethylsilyl ethers on 0.5% Apiezon L by GLC. Krishnamurthy and Bieri[26] and Sternberg and Pascoe-Dawson[27] measured red cell radioactivity after administering

[α-^{14}C]tocopherol to rats. Bratzler *et al.*[28] indirectly determined the tocopherol content of swine erythrocytes.

The purpose of this investigation is to report the separation of the individual tocopherols by TLC and quantitative measurement of these individual vitamins in blood plasma and red blood cells by GLC.

MATERIALS AND METHODS

The TLC equipment was obtained from the Research Specialties Co., Richmond, Calif. Thin-layer plates (20 × 20 cm) were coated with silica gel G (250 μm thick). These plates must be thoroughly cleaned with ethanol and air-dried before the silica gel G is applied. After the plates are prepared with the silica gel G, they are placed in a rack and heated at 105° for 2–3 h and stored in a desiccator over silica gel until needed. All fat-soluble vitamins and standards were applied to the thin-layer plates with an applicator developed by Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif. The stationary phase was silica gel G with gypsum binder (Warner-Chilcott Laboratories, Instruments Division, Richmond, Calif.). Mobile phase components include cyclohexane (Fisher Certified Reagent), *n*-hexane (99 moles% pure certified), isopropyl ether (Fisher Certified Reagent), and ammonium hydroxide (Mallinckrodt, analytical reagent). Bis-(trimethylsilyl)ether acetamide (BSA)–trimethylsilylchlorosilane (TMCS), and bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Supelco, Inc., Bellefonte, Pa.

Standard reference tocopherols (Distillation Products) were prepared by the method originally described previouvsly[29].

Internal reference standard 5,7-dimethyltocol (Koch-Light Laboratories, Colnbrook-Bucks, Great Britain) was prepared by weighing exactly 15.0 mg of the internal standard. This standard was transferred to a 10-ml volumetric flask and dissolved in hexane and made up to volume with the same solvent. The internal reference solution contains 1.5 μg/μl.

Solvent system and combination color reagent were prepared by the method described by Lovelady[29].

PROCEDURE

Preparation of blood plasma and red blood cells

On five different occasions 10 ml of whole blood were withdrawn from a human male into 10.0-ml syringes containing heparin. The blood plasma and red blood cells were separated by centrifugation.

Extractions

Volumes of 3.0 ml of plasma and 3.0 ml of red blood cells were pipetted into two separate (15 × 150 mm) glass-stoppered test tubes; 6.0 ml of 95% ethyl alcohol were added to each, and the tubes were placed in a heating block for 25 min at a temperature of 50°. The tubes were removed from the heating block to cool, and 1.5 ml of 10 *N* NaOH were added to each tube. The tubes were returned to the heating block for 40 min. The tubes were then removed from the heating block, and 3.0 ml of deionized distilled water were added to each tube. The tubes were mixed thor-

oughly, and the samples were extracted twice with 12.0 ml of *n*-hexane (spectro-analyzed grade). The top (hexane) layers of the two-phase extracts were removed and transferred to separate glass-stoppered centrifuge tubes and evaporated to dryness with a stream of nitrogen gas. The residues were dissolved in 1.0 ml of *n*-hexane for use in TLC analysis.

Thin-layer chromatography

Aliquots of the plasma and red blood cell extracts were applied to the thin-layer plates for separation. All separations were carried out using the following solvent system: cyclohexane–*n*-hexane–isopropyl ether–ammonium hydroxide (conc.) (40:40:20:2). The development was carried by the ascending method in closed rectangular tanks (24×23×8 cm), saturated 30 min with the appropriate solvent system. The tanks were lined with 3-mm filter paper for good equilibration. The time required for development (the solvent front moved 17 cm) was approximately 90 min. After development the chromatograms were removed from the tanks, dried and sprayed with the detecting reagent until uniformly light yellow. The chromatograms were dried with air from an airjet dryer for 3–4 min during which time the color intensity of the sample's bands increased. The vitamin samples, separated on the chromatograms, were detected by inspection in ordinary light or by observing the fluorescence produced by ultraviolet radiation lamp (2540 Å).

The vitamin samples were marked after identification on the thin-layer plates. The sample bands were removed from the plates by scraping with a spatula and were transferred to 15-ml glass-stoppered centrifuge test tubes; 1.0 ml of methanol was added to each tube, mixed and centrifuged for 15 min to remove the silica gel G. The methanol extracts from each tube were transferred to 15-ml glass-stoppered centrifuge tubes and evaporated to dryness with a stream of nitrogen gas.

Preparation of tocopherol derivatives

The residues containing tocopherols are dissolved in 0.2 ml of *n*-hexane with the addition of 0.25 ml of BSTFA and 0.05 ml of BSA–TMCS (5:1). Mix thoroughly before proceeding. The mixtures are placed in a heating block for 45 min at 50°. The mixtures are evaporated to dryness under a stream of nitrogen. The residues are dissolved in 0.25–1.0 ml of *n*-hexane for use in GLC analysis.

Gas–liquid chromatography

A Beckman GC-5 chromatograph with a dual hydrogen flame detector was used. The stainless-steel columns (6 ft.×1/8 in. O.D.) were packed in our laboratory with 3% OV-1 on 80–100 mesh Supelcoport (Perco Supplies, P.O. Box 201, San Gabriel, Calif.) and conditioned for 24 h at 250°. Operating conditions: column temperature isothermal at 240°; detector at 290°. Gas flow: carrier gas helium, 80 ml/min at 52 p.s.i.; hydrogen pressure at 40 p.s.i., air pressure at 50 p.s.i., respectively. Beckman Ten-Inch Recorder, pen response at 0.5 sec full scale, a high accuracy of ±0.25%, and a sensitive 1 mV range. The sensitivity 2.5×10^{-10} a.f.s. for most analyses.

Calibration curves

The standards were prepared in hexane to contain 0.4–1.8 μg of δ-tocopherol; 0.6–2.6 μg of γ-tocopherol; 0.5–1.8 μg of β-tocopherol and 0.6–2.4 μg of α-tocopherol.

2.5-μl volumes of each standard were injected into the column. Before injection, a flush volume of 1 μl of hexane was taken into the syringe before each sample. The standards were injected into the gas chromatograph. Calibration curves of each individual tocopherol were prepared from data obtained from the gas chromatograph. The average area of each reference standard under the GC peak, as determined by height $\times$ width at half-height were plotted against the tocopherol concentrations. The identities of the standard and/or unknown tocopherol peaks were ascertained by comparing the relative retention times with that of 5,7-dimethyltocol, the internal reference.

Quantitative data were determined by calculating the correction factors relative to α-tocopherol. The weights "W" injected are known. The areas "A" are measured. The ratio A/W is calculated for each peak. The correction factor "F" is calculated by dividing the A/W of each peak by the α-tocopherol A/W. These factors are relative to α-tocopherol. The α-tocopherol factor is arbitrarily set to equal to 1.00.

Under the same detector conditions, these factors can be used time and time again to calculate the weight percent of β-, γ-, and δ- relative to α-tocopherol.

From these results the weight of an unknown "b" can be calculated:

$$W_b = \frac{W_a \cdot A_b}{F_b \cdot A_a}$$

where:

W_b = weight of α-, β-, γ-, or δ-tocopherol

W_a = weight of standard α-tocopherol

A_a = measured area of standard α-tocopherol

A_b = measured area of component β-, γ-, or δ-tocopherol

F_b = correction factor of compound β-, γ- or δ-tocopherol relative to α-tocopherol at equal weights. The response of flame ionization detectors (FID) is independent of temperature, carrier gas, and flow-rate. This makes it well suited, possibly the best detector, for quantitative analysis.

RESULTS AND DISCUSSION

Many biologically important compounds *e.g.* cholesterol, vitamin A, vitamin K and β-carotene do not interfere in the TLC or GLC analysis of the tocopherols in this study. The greatest separation between the individual tocopherols was obtained with the solvent system described previously[29]. When the unknown samples are chromatographed on the TLC, the compounds are separated in ascending order, cholesterol >, δ->, γ->, β->, α->, vitamin A >, vitamin K >, and β-carotene. Vitamin A in the acetate form after hydrolysis yields the free alcohol and the ester.

In contrast to the results of Stowe[16], in this study the movement of individual tocopherols in relation to the solvent front was not altered by the complexity of the tocopherols applied to the thin-layer plates.

In silica gel TLC of hypnotics, R_F values are affected by variations in humidity; constant humidity is essential for reproducible TLC. R_F values in TLC systems are very sensitive to changes in organic ligand.

Identification of the two isomeric dimethyltocols was facilitated by their visible separation and R_F values when sprayed with the combination color reagent. The

color reagent stabilizes the tocopherols on the thin-layer plates. The solvent system, color reagent and R_F values found by TLC permit identification of the individual tocopherols for qualitative determination in blood plasma and red blood cells.

Preliminary investigation of the individual tocopherols analyzed by GLC on 3% OV-1 (methyl-substituted silicones) non-polar stationary phase produced chromatograms with no tailing. The tailing will occur with aging of the column. The column has a lifetime of at least 4 months. When the tocopherols are not derivatized, a small amount of tailing occurs in the GLC analysis. The amount of tailing was so small that no attempt was made to calculate the percent of the tailing factor. In this GLC analysis the tocopherols are derivatized with a combination of BSTFA and BSA–TMCS.

The column was cured at 250° for a minimum of 24 h with carrier gas flow and the temperature reduced to 240° for all analysis.

Efficiency of the 3% OV-1 column for the tocopherols was obtained from the number of theoretical plates in *n*-hexane. The column demonstrated an apparent efficiency of theoretical plates for δ- (807); γ- (389); β- (424); α- (768) and 5,7-dimethyltocol (internal standard, IS) (553).

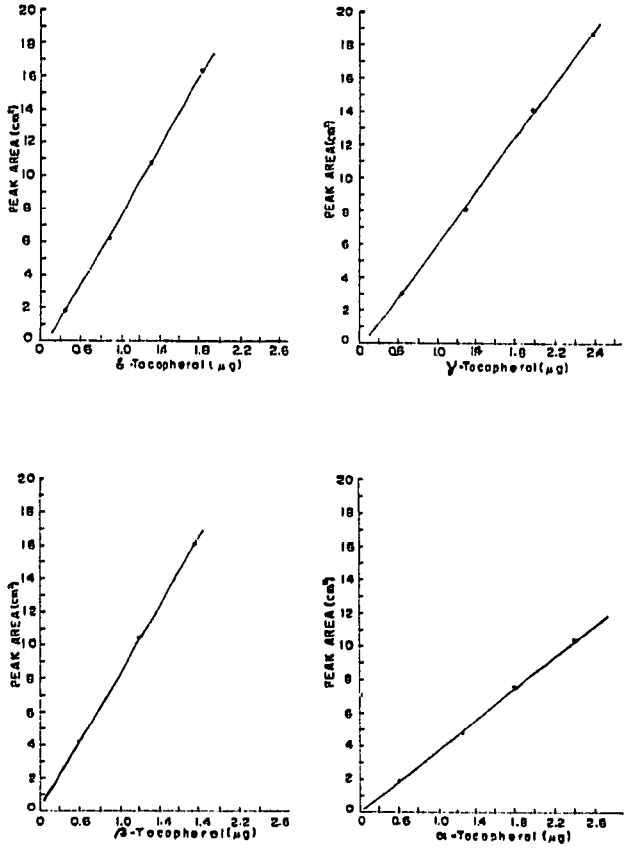

Fig. 1. Calibration curves of δ-, γ-, β- and α-tocopherol. GLC conditions as described in legend of Fig. 2.

Linear detector response was obtained by establishing an absolute calibration curve for each individual tocopherol (see Fig. 1). Standard solutions of tocopherol were prepared over the concentration range of approx. 0.4–2.6 μg. In each case 2.5-μl samples were injected into the gas chromatograph.

The linear correlation coefficient for α-tocopherol was 0.999 with a standard error estimate of (1.11%); β-tocopherol, 0.985 with a standard error estimate of (9.31%); γ-tocopherol, 0.998 with a standard error of (3.82%); and δ-tocopherol, 0.998 with a standard error estimate of (3.32%).

In comparing the absolute calibration with the FID correction factor, the absolute calibration has disadvantages: one has to inject the exact amount at all times, and the detector's injection temperature has to be kept at a constant temperature at all times. The correction factor is independent of these factors.

In order to verify the capability of the GLC system to resolve complex mixtures, a qualitative mixture of δ-, internal standard and α-tocopherol was injected into the gas–liquid chromatograph in hexane. The second mixture consisting of δ-, β-tocopherol, and IS was injected into the gas–liquid chromatograph in hexane. The resulting chromatograms were similar to those shown in Fig. 3. The suitability test for inertness of support shows single symmetrical peaks for injected tocopherol standards with no evidence of decomposition.

Data in Table I show the relative retention times from the GLC analysis of the individual tocopherol standards and those found in blood plasma and red blood cells.

Experiments were performed to measure the recovery of tocopherols. Standards were added to vitamin E deficient rat plasma and red blood cells. The extractions were carried out as stated in the text and separated by TLC and analyzed by GLC. The percent recoveries of the tocopherols are shown in Table II.

Table III shows the individual concentration of the tocopherols found in

TABLE I

RETENTION TIMES OF COMPONENTS IN STANDARD MIXTURES AND RED BLOOD CELL (RBC) AND BLOOD PLASMA EXTRACTS

Column temperature: 240°; carrier gas flow-rate: 80 ml/min.

Compound	*Relative retention time (min)*		
	*Standard**	*RBC*	*Plasma*
8-Methyltocol (δ-tocopherol)	0.64	0.64	0.64
7,8-Dimethyltocol (γ-tocopherol)	0.82	0.83	0.83
5,8-Dimethyltocol (β-tocopherol)	0.81	0.81	0.82
5,7,8-Trimethyltocol (α-tocopherol)	1.18	1.18	1.18
5,7-Dimethyltocol (internal reference standard)	1.00	1.00	1.00

* Retention time relative to 5,7-dimethyltocol.

TABLE II

RECOVERY OF TOCOPHEROLS ADDED TO BLOOD PLASMA AND RED BLOOD CELLS OF VITAMIN E DEFICIENT RATS

Male rats were depleted of vitamin E for 3 weeks. Blood from these rats was used in the recovery analysis. The indicated amount of the tocopherols was added to 1.0 ml of plasma and 1.0 ml of red blood cells which were then carried through the extraction and separation procedure. All determinations were carried out in triplicate.

Compound	*Amount added to plasma (μg)*	*Found by GLC (μg)*	*% recovered*	*Amount added to RBC (μg)*	*Found by GLC (μg)*	*% recovered*
8-Methyltocol (δ-tocopherol)	35.9	30.0 ± 5.01	84	35.9	37.7 ± 0.96	105
7,8-Dimethyltocol (γ-tocopherol)	51.7	52.3 ± 0.00	101	51.7	55.2 ± 0.06	107
5,8-Dimethyltocol (β-tocopherol)	47.6	50.7 ± 3.99	107	47.6	47.9 ± 0.42	100
5,7,8-Trimethyltocol (α-tocopherol)	48.2	47.6 ± 4.34	99	48.2	48.3 ± 0.12	100

TABLE III

INDIVIDUAL TOCOPHEROL DETERMINATIONS IN NORMAL HUMAN PLASMA AND RED BLOOD CELLS

Column temperature: 240°; carrier gas flow-rate: 80 ml/min.

Compound	*Number of determinations*	*Plasma (μg/100 ml)*	*Red cells (μg/100 ml)*	*RBC/plasma*
8-Methyltocol (δ-tocopherol)	5	239.0 ± 10.0*	63.1 ± 13.11*	0.264
7,8-Dimethyltocol (γ-tocopherol)	5	324.7 ± 13.65	132.2 ± 11.8	0.407
5,8-Dimethyltocol (β-tocopherol)	12	27.0 ± 0.55	18.6 ± 2.14	0.689
5,7,8-Trimethyltocol (α-tocopherol)	9	591.2 ± 38.5	176.6 ± 39.6	0.299

* Standard deviation of the mean.

plasma and red blood cells with their red blood cell:plasma ratios. These values for α- and γ-tocopherols are somewhat lower than those reported in refs. 23–25. The lower values in this study may be the result of elimination of trace impurities and their interferences (*e.g.* cholesterol and vitamin A) in the subsequent quantitative determination.

The values of β- and δ-tocopherols are the concentrations found in this study. To date, no values have been reported for β- or δ-tocopherols in biologic materials.

Fig. 2 shows the gas chromatogram peaks of δ-, γ-, and α-tocopherol obtained from plasma–hexane extract. The β-tocopherol zone was removed from the TLC plate before GC analyses were performed. In the bottom chromatogram, the γ-tocopherol zone was removed from the TLC plate before GC analysis was performed.

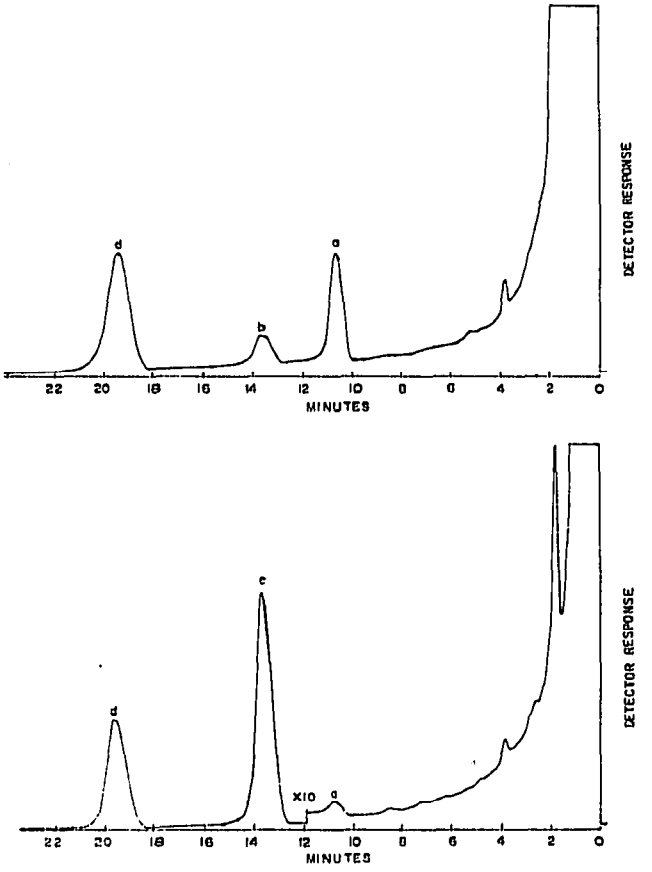

Fig. 2. Gas chromatogram at top represents a fraction of plasma–hexane extract of a, δ-; b, γ-; and d, α-tocopherol. Gas chromatogram on bottom represents a fraction of plasma–hexane extract of a, δ- (trace); c, β-; and d, α-tocopherol. Operating conditions: Column: 3% OV-1 on 80–100 mesh Supelcoport. Temperatures: column, isothermal at 240°; injector, 260°; hydrogen flame detector, 290°. Sample size: 1.5 ml.

All the gas chromatograms in this investigation were carried out in the same manner as above. The reason, that the β- or γ-tocopherol has to be removed from each zone before GC analysis is performed, is because they do not separate on the gas chromatograph. The relative retention times of the two tocopherols are about the same. Therefore one has to remove one or the other in order to get the separate chromatograms.

Fig. 3 shows gas chromatograms of standard tocopherols, with the internal reference standard. The peaks for the tocopherols are represented as follows: at the top β-, IS, and α-; at the bottom δ-, γ- and IS.

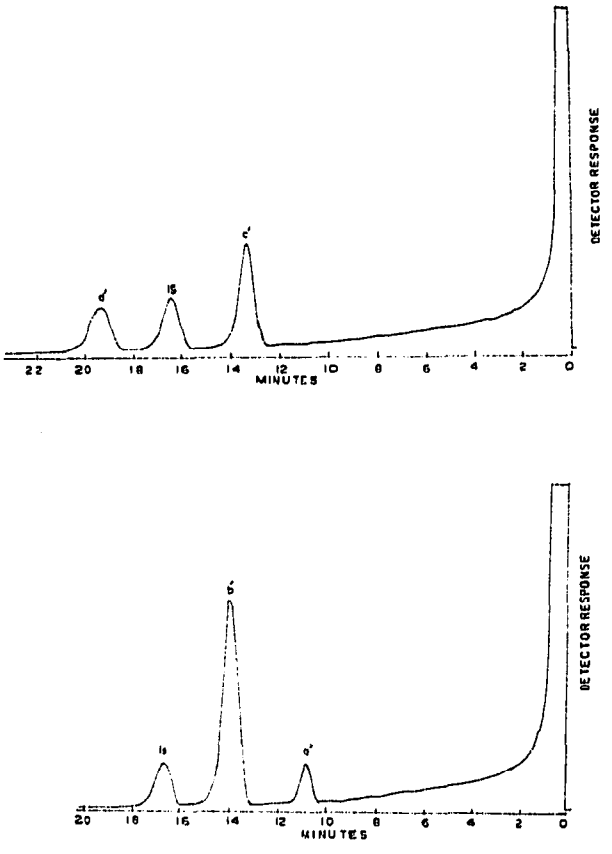

Fig. 3. Gas chromatogram at top represents standard mixture of c', β-; IS, internal reference standard 5,7-dimethyltocol; and d', α-tocopherol in hexane. Gas chromatogram at bottom represents standard mixture of a', δ-; b', γ-; and IS, internal reference standard in hexane. GLC conditions as described in legend of Fig. 2.

Fig. 4 shows the gas chromatogram obtained from the red blood cell–hexane extract. The peaks for the tocopherols are represented as follows: at the top β- and α-; at the bottom δ-, γ- and α-. The relative retention times for all tocopherols found in plasma and the red blood cells are the same as those of the standards shown in Table I.

The red blood cell tocopherols showed clean peaks free of interference from other fat-soluble vitamins. The results showed that the relative retention time ratios were fairly constant for both the pure and the unknown mixtures. With the above conditions the detection limits for the tocopherols with the hydrogen flame detector were found to be approximately 3×10^{-8}g.

In contrast to the commonly employed spectrophotometric methods, vitamin A, and other fat-soluble reducing substances do not interfere with the TLC and GLC techniques described in this paper.

In conclusion, the TLC system, the combination color reagent and GLC

method presented in this communication have made an important contribution to the study of the plasma and red blood cells containing vitamin E (antioxidant).

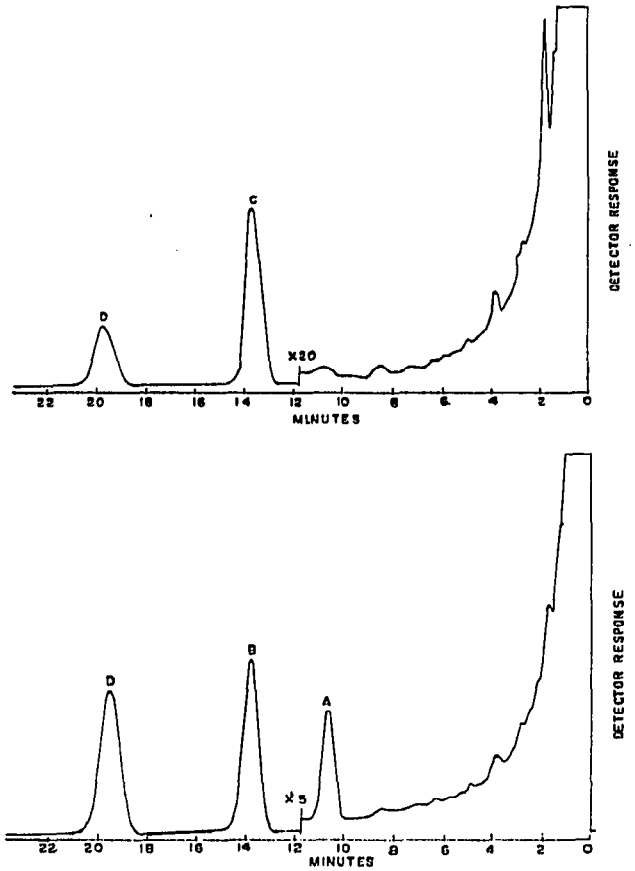

Fig. 4. Gas chromatogram at top represents a fraction of red blood cell–hexane extract of C, β-; and D, α-tocopherol. Gas chromatogram on bottom represents a fraction of red blood cell–hexane extract of A, δ-; B, γ-; and D, α-tocopherol. GLC conditions as described in legend of Fig. 2.

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