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Separation of Tocopherols by Gas-Liquid Chromatography

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Two distinct series of tocopherol compounds found in Nature are recognized. The isoprenoid side chain of the tocol series is fully saturated, whereas that of the other series appears to have three double bonds (Fig. 1). For the latter a trivial name tocotrienol has been proposed (Bunyan, McHale, Green & Marcinkiewicz, 1961). The tocol series comprises eight compounds, and all but two have been found in Nature. Of the tocotrienols, only the 5,8-dimethyl and the 5,7,8-trimethyl derivatives (ϵ - and ζ -tocopherols) are known, but others are possible, corresponding to the remaining members of the tocol series.

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The large number of tocopherols requires two-dimensional paper chromatography for their separation as described by Green, Marcinkiewicz & Watt (1955) and by the Analytical Methods Committee (1959), but even then certain pairs are difficult to resolve.

The versatility and high resolving power of gas-liquid chromatography suggested itself as a useful technique in the analysis of mixtures of tocopherols. As far as we are aware the only reference to the gas chromatography of tocopherols is the report of Nicolaides (1960) that α -tocopherol can be separated nearly completely from γ -tocopherol on columns coated with silicone-rubber gums. In this paper we describe procedures for the complete

Fig. 1. Formulae of (a) tocot and (b) tocotrienol.

separation of a number of known tocopherols. Group separation factors have been calculated which permit the prediction of retention times of the so far unknown tocotrienol derivatives, and also confirm the presence of three double bonds in the side chain of the tocotrienols as referred to by Bunyan *et al.* (1961).

METHODS

Gas-chromatographic columns

Glass columns (4 ft. long, 4 mm. bore) were used in the Pye Argon Gas Chromatograph with a strontium ionization detector. The glass columns were packed up to 3 ft. 3 in. with coated, silicone-treated Celite.

Celite (mesh 100–120) supplied by W. G. Pye and Co. Ltd. was treated as follows. The Celite (100 g.) was suspended for 1 hr. in 500 ml. of 5% (v/v) dimethyldichlorosilane in toluene, and stirred occasionally to ensure complete 'wetting'. The silicone-treated Celite was collected on a filter, washed with toluene and then with methanol, and dried at 90°. Three different stationary phases were used.

Silicone polymer SE-30, 4% (*General Electric Co.*). To 9.6 g. of silicone-treated Celite was added 0.4 g. of SE-30 in 40 ml. of toluene. The solvent was evaporated carefully *in vacuo* with warming, and dried at 80° for 1 hr. The packed column was conditioned by preheating at 300° for 24 hr.

Fluoroalkyl silicone polymer QF-1, 5% (*DC-QF-1-0065/10 000, Dow Corning Corp. Inc.*). To 9.5 g. of silicone-treated Celite was added 0.5 g. of QF-1 in 40 ml. of acetone, and the further procedure was as described above. The packed column was conditioned for 24 hr. at 250°, since QF-1 is unstable above this temperature.

Poly(ethylene glycol adipate), 4% (*W. G. Pye and Co. Ltd.*). To 9.6 g. of silicone-treated Celite was added 0.4 g. of poly(ethylene glycol adipate) in 40 ml. of methylene dichloride, and the mixture was treated as above. The packed column was conditioned very slowly, the temperature being increased daily in steps of 10° from 200° to 250°.

Application of samples

The substance (1–10 µg.) dissolved in benzene or acetone was applied, as suggested by Dr B. A. Knights (personal communication), to pieces of 50-mesh stainless-steel

gauze (2 mm. × 15 mm.) that had been doubled over twice at one end. The solvent was evaporated at room temperature before the gauze strip was dropped into the column. This procedure allowed quantitative application of several tocopherols to one gauze without producing a large solvent peak. An alternative quantitative application for a single solution consisted of dropping into the column Drummond Microcaps pipettes (Shandon Scientific Co. Ltd.) that hold 1–10 µl. of solution.

Samples of tocopherols

DL- α -Tocopherol and DL- α -tocopheryl acetate were obtained from Roche Products Ltd. The natural β -, γ - and δ -tocopherol samples from Distillation Products Ind. were kindly supplied by Dr R. J. Ward. Synthetic tocot, 5-methyltocot, 7-methyltocot (η -tocopherol), 5,7-dimethyltocot (ζ_2 -tocopherol), the natural 5,8-dimethyltocotrienol (ϵ -tocopherol) and an impure concentrate from wheat containing 5,7,8-trimethyltocotrienol (ζ_1 -tocopherol) were gifts from Dr J. Green and Dr D. McHale.

Esterification of tocopherols

The acetylation procedure as used for steroids by Dr B. A. Knights (personal communication) was employed. The tocopherol (1 mg.) was dissolved in 1 ml. of pyridine-acetic anhydride (2:1, v/v), and left overnight at room temperature. The solvent was then evaporated with a stream of nitrogen and the residue desiccated *in vacuo*, leaving the sample ready for gas chromatography. An authentic sample of DL- α -tocopheryl acetate was compared with a sample of α -tocopherol acetylated by the procedure described above. There was no difference in their chromatographic behaviour, and no non-esterified tocopherol could be detected, so that the esterification was evidently complete.

Paper chromatography

Tocopherols from natural sources were separated by two-dimensional paper chromatography according to Green *et al.* (1955) but with Whatman no. 4 filter paper and with the modifications proposed by Booth (1962). To obtain the separated tocopherols for gas chromatography, the spots were eluted into light petroleum (b.p. 30–40°), re-run in the first dimension on ZnCO₃-impregnated paper (Fig. 2) to remove paraffin, and then eluted into acetone. Parallel

determinations of the amount of tocopherol in eluates were done by the FeCl_3 -dipyridyl method (Analytical Methods Committee, 1959).

RESULTS

Fig. 2 shows the separation of tocopherols by two-dimensional paper chromatography. In addition to the reported positions of methylated derivatives, the spots for tocol and for α -, γ - and

Fig. 2. Schematic two-dimensional paper chromatogram of tocopherols. Whatman no. 4 paper (22 cm. $\times$ 32 cm.) impregnated with ZnCO_3 was used. The solvents were: first dimension, 1.5% (v/v) acetone in light petroleum (b.p. 40–60°); second dimension, 92% (v/v) methanol after impregnation with medicinal paraffin. The positions of individual tocopherols are denoted by their Greek symbols; 5-t, 5-methyltol; t, tocol; Ac, acetate.

δ -tocopheryl acetates are indicated. The monomethyltolcols, namely 5-methyltolcol and η -tocopherol, cannot be readily separated from the 5,8-dimethyltocotrienol (ϵ -tocopherol). The same difficulty applies to the separation of the pair of dimethyltolcols, β - and γ -tocopherol, which are distinguishable only by their reaction with diazotized *o*-dianisidine. The spot of 5,7-dimethyltolcol (ζ_2 -tocopherol) merges with that of the 5,7,8-trimethyltocotrienol (ζ_1 -tocopherol).

Gas chromatography of free tocopherols. The retention volumes relative to tocol obtained with authentic samples of tocopherols are shown in Table 1. They represent the mean of 3–6 replicates. The gas-chromatographic behaviour of α -, γ - and δ -tocopherol was identical with that of corresponding tocopherols from natural sources after elution from paper chromatograms. Retention volumes of samples of authentic η -, ϵ - and ζ_1 -tocopherols were also checked after purification of the individual substances by paper chromatography.

With the silicone polymer SE-30 as the stationary phase, the 5-methyltolcol was well separated from the other two monomethyl derivatives, which, however, were not resolved from one another. The dimethyltolcols did not separate from each other, but the trimethylated derivative (α -tocopherol) was completely separated from all the other members of the tocol series. Of the tocotrienol derivatives, the 5,8-dimethyl compound (ϵ -tocopherol) had a significantly lower retention volume than the trimethyltocotrienol, but was not separated from α -tocopherol.

With the fluoroalkyl silicone polymer QF-1, it was again the 5-methyltolcol that separated from the other monomethyl derivatives, but under these conditions it merged with the peaks of the dimethyltolcols. Of these, 5,7-dimethyltolcol was partially resolved from 5,8-dimethyltolcol. The main advantage of this stationary phase is the good separation of the two tocotrienols from each other and from all the methylated tocols.

Table 1. *Relative retention volumes of tocopherols on coated, silicone-treated Celite columns*

The conditions used were: for SE-30, a temperature of 250° and a flow rate of 40 ml./min.; for QF-1, a temperature of 240° and a flow rate of 50 ml./min.; for poly(ethylene glycol adipate), a temperature of 250° and a flow rate of 50 ml./min. The retention times of tocol on SE-30-, QF-1- and poly(ethylene glycol adipate)-coated columns were 17, 10 and 18 min. respectively. PEGA, Poly(ethylene glycol adipate).

Substance	SE-30	QF-1	PEGA
Tocol	1.00	1.00	1.00
8-Methyltolcol (δ -tocopherol)	1.13	1.05	0.96
7-Methyltolcol (η -tocopherol)	1.15	1.00	0.92
5-Methyltolcol	1.27	1.17	1.03
5,7-Dimethyltolcol (ζ_2 -tocopherol)	1.44	1.11	0.89
5,8-Dimethyltolcol (β -tocopherol)	1.42	1.20	0.94
7,8-Dimethyltolcol (γ -tocopherol)	1.45	1.16	0.96
5,7,8-Trimethyltolcol (α -tocopherol)	1.79	1.31	0.89
5,8-Dimethyltocotrienol (ϵ -tocopherol')	1.81	1.53	1.98
5,7,8-Trimethyltocotrienol (ζ_1 -tocopherol')	2.33	1.73	2.05

Table 2. *Relative retention volumes of tocopheryl acetates*

The conditions used were as described in Table 1. The method of calculation of the retention volumes of the esterified tocopherols is given in the text.

Substance	SE-30		QF-1 Found
	Found	Calc.	
Tocol	1.00	—	1.00
Acetate esters of tocol	1.25	[1.25]	1.55
8-methyltol (δ-tocopherol)	1.39	1.39	1.61
7-methyltol (ζ-tocopherol)	1.38	1.39	1.61
5-methyltol	1.54	1.55	2.00
5,7-dimethyltol (ζ ₂ -tocopherol)	1.72	1.72	1.96
5,8-dimethyltol (β-tocopherol)	1.69	1.72	2.06
7,8-dimethyltol (γ-tocopherol)	1.74	1.72	1.94
5,7,8-trimethyltol (α-tocopherol)	2.13	2.13	2.31
5,8-dimethyltocotrienol ('ε-tocopherol')	2.19	2.22	2.56
5,7,8-trimethyltocotrienol ('ζ ₁ -tocopherol')	2.74	2.74	2.93

When poly(ethylene glycol adipate) was used as the stationary phase, most of the peaks of the methylated tocols overlapped except that of the 5-methyltol. The tocotrienols were well separated from the tocols, but not from each other.

Gas chromatography of esters. The acetate esters ran more slowly than the non-esterified compounds on the SE-30 and QF-1 columns, but the individual separation was the same (Table 2). However, the retention volumes were more reproducible, and the peaks tailed less because the hydroxyl groups were covered. A characteristic gas-liquid chromatogram of a mixture of tocopheryl esters is shown in Fig. 3. The esters of ζ₁-, ζ₂-, ε- and η-tocopherols and of 5-methyltol were well separated.

Effect of methyl groups. The relationship between log (relative retention volume) and number of methyl groups on the chromanol moiety for the SE-30 column is shown in Fig. 4. The full lines represent acetate esters, the broken lines free tocopherols. The acetate esters were displaced by a factor that, for all the tocopherols, averaged 1.21 ± 0.002 .

Not all methyl groups had the same effect on the retention by the column. From the results for all the tocopheryl esters, the separation factors for the methyl groups and the influence of configuration were calculated (Table 3). The separation factor corresponded to the general formula x^n , where x is the separation factor for one methyl group and n the influence of the position of such a group. When position 5 (see Fig. 1) was occupied by a methyl group or when, in the presence of a methyl group in position 8, position 7 had a methyl group attached, the separation factor was the square of that for a single methyl group in positions 7 or 8. The corrected separation factor for one methyl group, irrespective of the positional influence, was 1.115 and 1.108 respectively. Since there was no significant difference between these two values ($0.1 < P < 0.2$), the mean, 1.112, was used for

Fig. 3. Gas-liquid chromatogram of a mixture of tocopheryl esters on 4% SE-30 stationary phase. The temperature was 250° and the flow rate 40 ml./min. The sensitivity was 10% and the detector voltage 1500 v. The symbols of individual tocopheryl esters are as in Fig. 2.

calculation of predicted retention volumes. Thence a linear relationship could be obtained between log (relative retention volume) and log (separation factor) (i.e. $\log x^n$) (Fig. 5). It is evident that the

latter will be a multiple of the function n , since x is constant. Thus n is 1 for 7-methyl- and 8-methyl-tocol, 2 for 5-methyltocol, 3 for the dimethyl-tocols, and 5 for the trimethyltocol, each in its ester form. Although the retention volumes of the dimethyltocyl acetates did not differ significantly, the 5,8-dimethyl derivative had consistently the smallest retention volume, indicating that even among the dimethyl derivatives there was a slight secondary differential effect of the two methyl groups.

Fig. 4. Relationship between log (retention volume relative to tocol) and the number of additional methyl groups on the chromanol moiety of tocopherols. The stationary phase was 4% SE-30. —, Acetate esters; - - -, free tocopherols. The symbols are as in Fig. 2.

Effect of double bonds. The greater retention volume of the two tocotrienols was due to the effect of the double bonds in the side chain, with a separation factor for 1 double bond of 1.089 on the SE-30 column and of 1.079 for the QF-1 column. This factor is of the same order of magnitude as that for each double bond in squalene, namely 1.071 and 1.069, when the retention volumes of squalene and squalane were compared on the two columns (Table 4). The small difference is most likely caused by the fact that the tocotrienols have a ring structure attached to the isoprenoid chain. A line parallel to that of the tocyl esters was constructed that passed through the log

Fig. 5. Relationship between log (retention volume relative to tocol) and function n of log (separation factor) (i.e. $\log x^n$) for additional methyl groups on the chromanol moiety of tocopheryl acetates (x , constant separation factor for CH_3 group irrespective of position). The stationary phase was 4% SE-30. The symbols are as in Fig. 2.

Table 3. Separation factors for additional methyl groups on chromanol ring of tocopheryl acetates, and the influence of the methyl position, in gas-liquid chromatography on silicone polymer SE-30

The separation factor is defined as the ratio of the retention volumes of two substances differing with respect to one specific chemical grouping. The values in parentheses denote the number of pairs from which the s.e.m. values were calculated.

Chemical grouping	Separation factor on SE-30 (x^n)	Influence of position of CH_3 (n)	Influence of one CH_3 group (x)
CH_3 in position 5; or in position 7 in the presence of CH_3 in position 8	1.242	2	1.115 ± 0.002 (9)
CH_3 in position 7 or 8	1.108	1	1.108 ± 0.004 (4)
		Mean	1.112 ± 0.005

(relative retention volume of the 5,7,8-trimethyl-tocotrienol). The 5,8-dimethyltocotrienol was displaced below this line to the same extent as the corresponding saturated tocol, namely the 5,8-dimethyl derivative, was displaced below the line of the saturated series (Fig. 5).

Calculation of relative retention volumes. With the above-mentioned separation factors and the found relative retention volume for tocyol acetate (1.25) on SE-30 coating, the retention volumes of the acetate esters of tocopherols were calculated (Table 2). The calculated relative retention volumes agree well with those obtained by direct measure-

The influence of methyl groups on the other two coatings, for both the free and esterified tocopherols, was more complex, and no clear derivation of group-separation factors could at present be computed.

Sensitivity and application to a natural source. As little as 0.2 µg. of tocopherol could be estimated, giving 9 % of full-scale deflexion at 33 % sensitivity and 1750 v on the detector.

The separation technique was applied to a natural soya-bean concentrated extract (Sotoco, Joseph Stanley Co.). Fig. 6 (a) shows a gas chromatogram of Sotoco, 20 µg., applied on the SE-30 column. The α-, γ- and δ-tocopherols were well separated from the other constituents.

Table 4. Separation factors for double bonds in the isoprenoid chain of tocopheryl acetates and of squalene in gas-liquid chromatography on silicone polymers SE-30 and QF-1

The definition of the separation factor is given in Table 3.

Substance	Double bonds in isoprenoid chain	Separation factor for double bonds			
		SE-30		QF-1	
		Found	Calc. for 1 double bond	Found	Calc. for 1 double bond
5,8-Dimethyltocotrienyl acetate	3	1.296	1.089	1.243	1.079
5,7,8-Trimethyltocotrienyl acetate	3	1.286		1.268	
Squalene	6	1.510	1.071	1.490	1.069

Fig. 6. Gas-liquid chromatograms of soya-bean concentrated extract Sotoco, (a) before and (b) after acetylation. The stationary phase was 4% SE-30, the temperature 250° and the flow rate 40 ml./min. The sample weight was 20 µg. The sensitivity was 33% and detector voltage 1750 v. Tocopherols are denoted by their Greek symbols; X, unknown compound with a retention volume of 0.85 relative to tocol.

Table 5. Comparison of tocopherol assays on soya-bean concentrated extract by two methods

Experimental details are given in the text.

Method	Total tocopherols (mg./g.)	Percentage composition of tocopherols		
		α	γ	δ
Gas-liquid chromatography	80	16	63	21
Paper chromatography	73	16	65	19

Acetylation of Sotoco yielded a mixture that produced the gas chromatogram shown in Fig. 6 (b). The esters of the three tocopherols were each displaced by a separation factor for the acetate grouping. In the range where known acetylated tocopherols can be encountered, there were no other peaks. The three slowest unknown substances were each displaced by a factor that was the square of the acetate retention factor. The latter finding suggests that they are substances with two esterifiable hydroxyl groupings. Other, fast-running, substances were not affected by acetylation, and one of them, X, had a retention volume of 0.85 relative to tocol, the same as that of squalene.

Quantitative estimation of the tocopherols by measurement of the area under their peaks, and comparison with tocopherol standards, gave values that agreed with those obtained by quantitative assay with two-dimensional paper chromatography (Table 5).

DISCUSSION

The technique of introducing the sample into the column either solid on steel gauze or in solution in Microcaps pipettes ensured quantitative application of small quantities of substance and gave reproducible peak areas. Thus the disadvantages of measuring the length of the liquid in the capillary, as suggested by Gerson (1961), are obviated. Acetate esters gave more reproducible peaks, without tailing, and were therefore preferred to the non-esterified tocopherols.

The gas-chromatographic separation of tocopherols is complementary to two-dimensional paper chromatography. The latter technique separates compounds in the second dimension by partition chromatography, in which migration rates are decreased according to the number of methyl groups, but increased by double bonds in the side chain. Thus 5,7,8-trimethyltocotrienol (ζ_1 -tocopherol) merges with 5,7-dimethyltocol (ζ_2 -tocopherol) and can be separated only with great difficulty (Green, McHale, Marcinkiewicz, Mamalis & Watt, 1959). Similarly 5,8-dimethyltocotrienol (ϵ -tocopherol), 5-methyltocol and 7-methyltocol (η -tocopherol) are difficult to separate from each other by paper chromatography. In gas-liquid chromatography on the silicone polymers the

influence of double bonds and number of methyl groups is additive. The SE-30 stationary phase, therefore, separates ζ_1 -tocopherol from ζ_2 -tocopherol and distinguishes ϵ -tocopherol from 5-methyltocol and from η -tocopherol. However, it does not separate the monomethyl compounds, δ - and η -tocopherol, that are distinguishable by paper chromatography. With the QF-1 stationary phase it is possible to distinguish trimethyltocol (α -tocopherol) from 5,8-dimethyltocotrienol (ϵ -tocopherol).

5-Methyltocol separates well from the other monomethyltocols on all three columns, since apparently the methyl group in position 5 (vicinal to the hydroxyl group) exerts a particularly strong retarding influence that amounts, with the SE-30 stationary phase, to the square of that exerted by other single methyl groups. The same retarding effect is exerted by a methyl group in position 7 (vicinal to the hydroxyl group) if this group is accompanied by a methyl group in position 8. The special behaviour of the 5-methyltocol on gas-liquid columns appears to be paralleled by the somewhat anomalous behaviour of the nitroso derivative compared with that of the other monomethyltocols on paper impregnated with zinc carbonate (Green *et al.* 1959; Marcinkiewicz & Green, 1959).

The outstanding advantage of the gas-chromatographic technique is the separation of the tocotrienols from tocols. Green, Mamalis, Marcinkiewicz & McHale (1960) and McHale, Green & Marcinkiewicz (1961) have proposed, on the basis of their chemical studies, that ϵ -tocopherol is a 5,8-dimethyl derivative of 2-methyl-2-(4',8',12'-trimethyltrideca-3,7,11-trienyl)chroman-6-ol, being thus a member of a new series containing a trienyl isoprenoid side chain. Since ϵ -tocopherol could be converted by chloromethylation into ζ_1 -tocopherol, Green *et al.* (1960) suggested that the latter is a homologous tocotrienol. We are able to confirm their conclusions from the evidence of the behaviour of tocotrienols on gas-liquid chromatography. These compounds having a trienyl side chain are separated from corresponding saturated compounds by a separation factor that is consistent with the presence of three double bonds. This evidence is derived from the observed effect of double bonds in squalene.

Further, the ζ_1 -tocopherol behaves on gas-chromatographic columns as the higher homologue of ϵ -tocopherol; therefore both compounds must have a similar structure. The relationship between log (relative retention volumes) and the influence of the group separation factor, as shown in Fig. 5, permits us to predict the relative retention volumes of lower homologues of tocotrienols, should they be found in Nature.

SUMMARY

1. The separation of tocol, methylated tocols and tocotrienols by gas-liquid chromatography has been investigated.

2. A number of tocopherols could be resolved from one another that were separable only with great difficulty by two-dimensional paper chromatography. Thus 5-methyltocol, ϵ -tocopherol and η -tocopherol were separated, as was ζ_1 -tocopherol from ζ_2 -tocopherol.

3. Acetylation of tocopherols yielded esters that gave more reproducible results than non-esterified compounds.

4. On the silicone polymer SE-30, separation factors, calculated for methyl groups and for double bonds, permitted the prediction of relative retention volumes for tocopherols.

5. These separation factors, with silicone polymers as stationary phases, were additive, whereas in paper chromatography the effects of a

methyl group and the trienyl grouping cancel one another.

6. The gas-chromatographic behaviour of ϵ - and ζ_1 -tocopherols is in keeping with their trienyl structure.

7. As little as 0.2 μ g. of tocopherol could be assayed either in mixtures or in a natural source. The assay of the latter gave results that agreed with those obtained by paper chromatography.

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Utilization of Serum-Protein-Bound Glycine in the Formation of Globin and Haem

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The nature, significance and extent of the re-utilization of protein-bound amino acids in the formation of new protein (and other metabolites) have been a subject of interest for many years (Whipple, 1956; Loftfield & Harris, 1956; Walter *et al.* 1957; Walter, 1960). Thus estimates of the contribution by proteins of amino acid residues to the formation of other specific protein have ranged from minor (Cohen, Holloway, Matthews & McFarlane, 1956) to major (Loftfield & Harris, 1956), and the suggested intermediates have in-

cluded amino acids, peptides and activated amino acids (Loftfield & Harris, 1956; Whipple, 1956; Walter & Mahler, 1958).

In the present work we attempted to ascertain whether free amino acids were intermediates in the transfer of serum-protein-bound glycine to globin and haem. We also obtained results on the relative utilization of free and protein-bound glycine in the biosynthesis of globin and haem and investigated the percentage of the total glycine required in globin synthesis that is supplied by the serum proteins.

Haemoglobin was chosen for these experiments because it is a protein which, contained in the red

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