Journal of Chromatography, 103 (1975) 327–340

CHROM. 7876

GAS–LIQUID CHROMATOGRAPHY OF CAROTENOIDS AND OTHER TERPENOIDS

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(Received July 15th, 1974)

SUMMARY

The retention behaviour of over seventy terpenoids on three silicone polymer liquid phases under both isothermal and temperature-programmed conditions is reported. Terpenoids with conjugated unsaturation (*e.g.*, carotenoids) were hydrogenated prior to analysis in order to prevent thermal decomposition. Analyses of the acetates and TMS ethers of both the natural hydroxycarotenoids and their perhydro-derivatives are also reported. In addition, a system is described for the routine analysis of terpenols, including those whose pyrophosphates are intermediates in sterol and carotenoid biosynthesis.

INTRODUCTION

The potential of gas–liquid chromatography (GLC) as a means of analyzing carotenoids and related terpenoids is limited by the thermal instability of conjugated polyene chains at the temperatures which would be required. Because of their inherent lability, carotenoids must be hydrogenated prior to GLC analysis. This approach was first used to separate perhydro-β-carotene and perhydrolycopene on a liquid phase of high-vacuum grease (HVG)[1] and was later applied to the separation of the hydrogenation products of enzymically synthesized carotenoids such as phytoene, phytofluene, ζ-carotene, neurosporene, lycopene, γ-carotene and β-carotene[2,3]. Recently, the examination of the hydrogenation products of the carotenes from a pigmented Streptococcus, *S. faecium* UNH 564P, by GLC was instrumental in the discovery of the first triterpenoid carotene series known to occur *in vivo*[4].

In a previous report[5], the separation of a number of hydrogenated carotenoids on a stationary phase of HVG was described. This phase, like the SE-30 liquid phase used by other workers[3,6], is relatively non-selective so other more selective stationary phases have been tested as alternatives or complements to HVG for the separation of terpenoids. A further object of the present study was to determine whether the behaviour of a terpenoid on different GLC systems is a useful criterion in structural determination. In addition, we report the effects of acetylation and silylation on

the GLC behaviour of hydrogenated xanthophylls and we have extended the use of our GLC systems to the analysis of terpenoid precursors.

EXPERIMENTAL

Materials

Phytoene, phytofluene, ζ-carotene and β-zeacarotene were isolated from mutants of *Phycomyces blakesleeanus* by standard methods used in this laboratory[7,8]. 4,4'-Diapocarotenes were isolated from *S. faecium* UNH 564P as described elsewhere[4]. Neurosporene was isolated from a nicotine-inhibited culture of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* and was provided by Dr. Aung Than (of this laboratory). Squalene was purchased from Eastman-Kodak (Kirkby, Great Britain) and phytol, cholestane, cholesterol, lanosterol, stigmasterol, lycopene and β-carotene were obtained from Sigma London Chemical (Kingston-upon-Thames, Great Britain). Ergosterol was from BDH (Poole, Great Britain) and linalool, terpineol, geraniol, nerolidol and farnesol were from Koch-Light (Colnbrook, Great Britain). All the remaining carotenoids as well as geranyllinalool, geranylgeraniol and lycopersene (7,8,11,12,15,7',8',11',12',15'-decahydro-ψ,ψ-carotene) were generous gifts from Hoffmann-La Roche (Basel, Switzerland) through Dr. F. Leuenberger.

It is a recommendation of the Commission on Biochemical Nomenclature[9] that trivial names for carotenoids should always be accompanied by their corresponding semi-systematic names. These are recorded in Table I and structures of the relevant parent carotenes are shown in Fig. 1.

All solvents used were of analytical reagent grade (AnalaR) and were purchased from BDH. Diethyl ether was sodium-dried and then glass-redistilled from reduced iron powder; it was peroxide free. Chloroform was glass-redistilled twice, stored in the dark and used within a week of purification. Pyridine was refluxed over solid KOH for 1 h and then redistilled, the distilling condenser being fitted with an anhydrous $CaCl_2$ moisture trap. The dry pyridine was stored over Union Carbide molecular sieve 13X (BDH).

Gas–liquid chromatography

All analyses were carried out on a Pye-Unicam (Cambridge, Great Britain) Series 104 gas chromatograph equipped with a dual flame ionization detector system. Glass columns, 5 ft. × 0.25 in. O.D., were silylated by washing them with 10% (v/v) hexamethyldisilazane (Sigma London Chemical) in glass-redistilled toluene and were then dried at 125° for 4–8 h prior to packing. Column packings were either purchased *per se* or prepared by methods described previously[5]. Three packings were used: (1) 2% SE-52 (silicone gum rubber SE-52) on Gas-Chrom Q (80–100 mesh), (2) 2% Dow-Corning High-Vacuum Grease (HVG) on Chromosorb W AW DMCS (85–100 mesh) and (3) 3% OV-17 on Universal B (85–100 mesh). Packed columns were conditioned for at least 72 h at 325° with a nitrogen carrier gas flow-rate of 40 ml/min prior to use.

Samples (1–3 μl of 1–5 mg/ml solutions in chloroform) were injected directly onto silylated glass wool plugs in the columns. Blocking of any remaining or newly appearing active sites on the columns was accomplished by periodic injections of Silyl-8 (Pierce, Rockford, Ill., U.S.A.).

TABLE I

STRUCTURES OF CAROTENOIDS*

Trivial name	*Semi-systematic name*
β-Apo-4′-carotenal	4′-Apo-β-caroten-4′-al
β-Apo-8′-carotenal	8′-Apo-β-caroten-8′-al
β-Apo-10′-carotenal	10′-Apo-β-caroten-10′-al
β-Apo-8′-carotenoic acid	8′-Apo-β-caroten-8′-oic acid
β-Apo-8′-carotenoic acid ethyl ester	Ethyl 8′-apo-β-caroten-8′-oate
β-Apo-8′-carotenoic acid methyl ester	Methyl 8′-apo-β-caroten-8′-oate
Astacene	3,3′-Dihydroxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione or β,β-carotene-3,4,3′,4′-tetrone
Azafrin	5,6-Dihydroxy-5,6-dihydro-10′-apo-β-caroten-10′-oic acid
Bixin	Methyl hydrogen 9′-*cis*-6,6′-diapocarotene-6,6′-dioate
Canthaxanthin	β,β-Carotene-4,4′-dione
α-Carotene	β,ε-Carotene
β-Carotene	β,β-Carotene
γ-Carotene	β,ψ-Carotene
ζ-Carotene	7,8,7′,8′-Tetrahydro-ψ,ψ-carotene
β-Carotenone	5,6,5′,6′-Diseco-β,β-carotene-5,6,5′,6′-tetrone
Carotinin	15,15′-Didehydro-β,β-carotene
Capsanthin	3,3′-Dihydroxy-β,κ-caroten-6′-one
Crocetin	8,8′-Diapocarotene-8,8′-dioic acid
Cryptoxanthin	β,β-Caroten-3-ol
Dehydro-β-apo-8′-carotenal	3,4-Didehydro-8′-apo-β-caroten-8′-al
Dehydro-β-carotene	3,4-Didehydro-β,β-carotene
4,4′-Diapo-ζ-carotene	7,8,7′,8′-Tetrahydro-4,4′-diapo-ψ,ψ-carotene
4,4′-Diaponeurosporene	7,8-Dihydro-4,4′-diapo-ψ,ψ-carotene
4,4′-Diapophytoene	7,8,11,12,7′,8′,11′,12′-Octahydro-4,4′-diapo-ψ,ψ-carotene
4,4′-Diapophytofluene	7,8,11,12,7′,8′-Hexahydro-4,4′-diapo-ψ,ψ-carotene
Diethylcrocetin	Diethyl 8,8′-diapocarotene-8,8′-dioate
Dimethylcrocetin	Dimethyl 8,8′-diapocarotene-8,8′-dioate
Echinenone	β,β-Caroten-4-one
Fucoxanthin	5,6-Epoxy-3,3′,5′-trihydroxy-6′,7′-didehydro-5,6,7,8,5′,6′-hexahydro-β,β-caroten-8-one 3′-acetate
Isocryptoxanthin	β,β-Caroten-4-ol
Isozeaxanthin	β,β-Carotene-4,4′-diol
Lycopene	ψ,ψ-Carotene
Methylazafrin	Methyl 5,6-dihydroxy-5,6-dihydro-10′-apo-β-caroten-10′-oate
Methylbixin	Dimethyl 9′-*cis*-6,6′-diapocarotene-6,6′-dioate
Neurosporene	7,8-Dihydro-ψ,ψ-carotene
Physalien	β,β-Carotene-3,3′-diol dipalmitate
Phytoene	7,8,11,12,7′,8′,11′,12′-Octahydro-ψ,ψ-carotene
Phytofluene	7,8,11,12,7′,8′-Hexahydro-ψ,ψ-carotene
Torularhodin	3′,4′-Didehydro-β,ψ-caroten-16′-oic acid
β-Zeacarotene	7′,8′-Dihydro-β,ψ-carotene
Zeaxanthin	β,β-Carotene-3,3′-diol

* Structures of the relevant parent carotenes are shown in Fig. 1.

Both isothermal and temperature-programmed analyses were made. All columns were run isothermally at 240°, 275° and 300° with a nitrogen carrier gas flow-rate of 60 ml/min. The injection port and detector temperatures were maintained at 25–50° above the isothermal temperature used. In addition, C_{10}–C_{20} terpe-

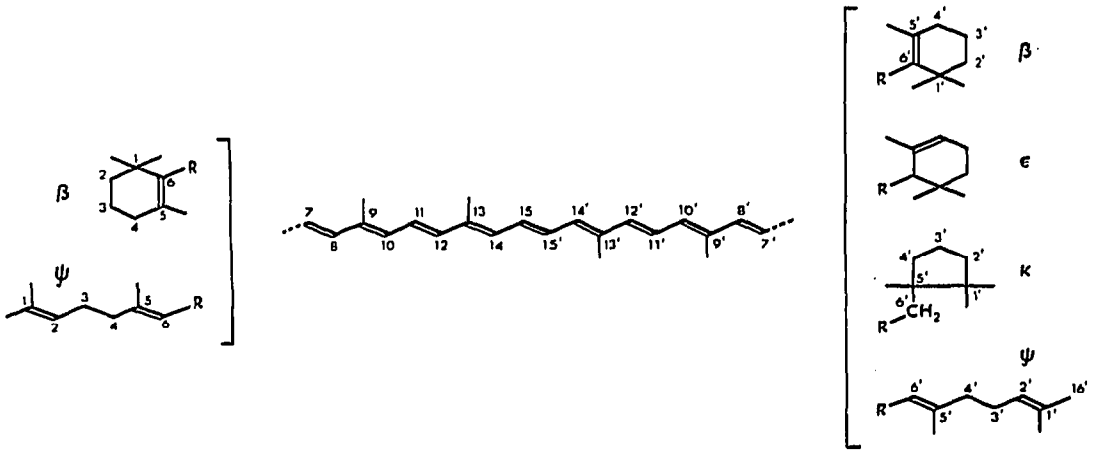

Fig. 1. Structures of the parent carotene hydrocarbons. The appropriate combinations of end groups with the polyene chain show the structures of β,β-carotene (*i.e.*, β-carotene), β,ε-carotene (*i.e.*, α-carotene), β,κ-carotene, β,ψ-carotene (*i.e.*, γ-carotene), and ψ,ψ-carotene (*i.e.*, lycopene).

nols were analyzed on the SE-52 columns at temperatures of 125°, 150°, and 175°.

Programmed analyses of carotenoids and other terpenoids were run on all three columns from 225 to 300° with a nitrogen carrier gas flow-rate of 60 ml/min. After an initial isothermal period of 3 min, the temperature was programmed to rise at a rate of 3°/min. During such runs, the injection port and detector oven temperatures were maintained at 325 and 350°, respectively. The C_{10}–C_{20} terpenols were also analyzed by programmed runs on the SE-52 and HVG columns; the SE-52 programme ran from 100 to 225° while that for HVG was from 125 to 250°. In both cases, the temperature was programmed to rise at a rate of 6°/min after an initial isothermal period of 3 min.

Hydrogenation

Hydrogenations were carried out in a Gallenkamp micro-hydrogenation apparatus (A. Gallenkamp, London, Great Britain). Samples (50–500 μg) were dissolved in 25 ml chloroform containing 50 mg platinum oxide (Hopkin and Williams, Romford, Great Britain) and were maintained at room temperature with shaking for 2–4 h under a positive hydrogen gas pressure of 100 mmHg. After this time, the catalyst was removed from the reaction mixture by filtering the mixture through a sintered glass funnel and the resulting colourless filtrate was concentrated *in vacuo* to yield a sample concentration of approx. 10 μg/μl chloroform. From 1 to 3 μl of this final solution was used for GLC analysis.

Acetylation and silylation

Acetylation of xanthophylls[10] was carried out by dissolving 50–200 μg of a carotenoid or its hydrogenation product in 1 ml dry pyridine, adding 0.01 ml acetic anhydride (Hopkin and Williams) and maintaining the mixture under nitrogen in the dark at room temperature for 12–24 h. After this time, 2 ml distilled water was added to the reaction mixture and the acetylated product was extracted with 3 ml diethyl

ether. Extraction was repeated three times, each time with a fresh volume of solvent, and the ether extracts were combined, washed with water to remove pyridine and concentrated *in vacuo* to dryness. The residue was dissolved in the minimum volume of chloroform for GLC analysis.

Silylation of xanthophylls[11] with hexamethyldisilazane and trimethylchlorosilane (both from Koch-Light) was carried out in pyridine by the standard method used in this laboratory[12].

Calculation of relative retention times

Because of the large differences in the retention times of the C_{10}–C_{50} terpenoids studied, all GLC data are reported in terms of relative retention times, using as standards those compounds best suited to the different temperature ranges. These were as follows: (1) squalene for temperature-programmed analyses of the higher terpenoids and for isothermal analyses at 240°, (2) perhydro-β-carotene for isothermal analyses at 275 and 300°, and (3) all-*trans*-farnesol for programmed and isothermal analyses of C_{10}–C_{20} terpenols.

RESULTS AND DISCUSSION

The relative retention times of the carotenoids and related terpenoids analyzed by the various systems are recorded in Tables II and III. A number of both general and specific observations may be made on the basis of these results.

In general, only unsaturated compounds containing non-conjugated carbon–carbon double bonds could be analyzed *per se* by the GLC systems used. Any conjugated unsaturation in a terpenoid acyclic chain (as in a carotenoid polyene system) resulted in thermal destruction, so all the carotenoids had to be hydrogenated prior to analysis. In an earlier report[5], it was claimed that phytoene, lycopene and echinenone could be analyzed *per se* by GLC on 5% HVG on Chromosorb W. It is possible that these samples were contaminated with a thermostable component with a molecular weight similar to that of squalene. It was found in the present study that pure samples of these three carotenoids all required prior hydrogenation for successful analysis.

Acyclic C_{30} and C_{40} carotenes yielded squalane and lycopersane, respectively, on hydrogenation. Acetylation or silylation of xanthophylls did not produce thermally stable derivatives, presumably because the long conjugated polyene chains of the acetates and trimethylsilyl (TMS) ethers remained unprotected. Acetylation or silylation carried out after hydrogenation of the xanthophylls, however, produced derivatives with retention characteristics distinct from those of the parent perhydroxanthophylls. These reactions must be carried out after and not before hydrogenation; otherwise losses of the acetyl or TMS groups occur, possibly by hydrogenolysis. Even here, the processes of acetylation and silylation of hydrogenated xanthophylls (cryptoxanthin, zeaxanthin, isocryptoxanthin and isozeaxanthin) were found in many trials to lead to a 25–40% production of perhydro-β-carotene, perhaps because of an interfering dehydration side reaction occurring during derivatization.

In many cases, pairs of compounds with very similar retention times were found to influence each other in their elution characteristics. This was especially true in the case of perhydro-β-carotene and compounds such as perhydro-torularhodin

TABLE II

GLC RETENTION TIMES RELATIVE TO SQUALENE OR PERHYDRO-β-CAROTENE OF HYDROGENATED CAROTENOIDS, THEIR ACETATES AND TMS ETHERS AND RELATED TERPENOIDS UNDER ISOTHERMAL AND TEMPERATURE-PROGRAMMED CONDITIONS

Compound and molecular size	*Relative retention times on systems 1, 2 and 3* isothermal or programmed*											
	*240°***			*275°****			*300°****			*Programmed**,§*		
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
Geranyllinalool (C_{20})	0.12	§§	0.07	§§	§§	§§	§§	§§	§§	0.15	0.16	0.13
Phytol (C_{20})	0.14	§§	0.05	§§	§§	§§	§§	§§	§§	0.18	0.17	0.10
Geranylgeraniol (C_{20})	0.18	§§	0.09	§§	§§	§§	§§	§§	§§	0.21	0.22	0.15
Squalene (C_{30})	1.00	1.00	1.00	0.14	0.14	0.15	0.16	0.17	0.18	1.00	1.00	1.00
Cholestane (C_{27})	1.00	1.00	1.00	0.16	0.16	0.18	0.22	0.23	0.25	1.00	1.00	1.00
Cholesterol (C_{27})	2.04	2.01	§§§	0.28	0.28	0.49	0.36	0.35	†	1.69	1.46	1.67
Ergosterol (C_{28})	2.59	2.44	§§§	0.35	0.34	0.59	0.43	0.42	†	1.98	1.65	1.80
Stigmasterol (C_{29})	2.92	2.78	§§§	0.38	0.37	0.61	0.45	0.44	†	2.14	1.82	1.95
Lanosterol (C_{30})	3.49	3.18	§§§	0.44	0.41	0.72	0.51	0.48	†	2.31	1.91	2.11
Lycopersene (C_{40})	§§§	§§§	§§§	1.03	1.02	1.38	1.06	1.00	1.02	3.95	3.15	2.76
Hydrogenation products of:												
Retinol (C_{20})	0.15	§§	0.04	§§	§§	§§	§§	§§	§§	0.11	0.13	0.07
Retinaldehyde (C_{20})	0.15	§§	0.04	§§	§§	§§	§§	§§	§§	0.10	0.10	0.06
Crocetin (C_{20})	0.07	0.05	0.28	§§	§§	0.02	§§	§§	§§	0.13	0.11	0.13
Dimethylcrocetin (C_{22})	0.29	0.21	0.32	0.03	0.03	0.05	§§	§§	§§	0.34	0.33	0.40
Diethylcrocetin (C_{24})	0.39	0.39	0.43	0.06	0.05	0.07	§§	§§	§§	0.45	0.45	0.50
Bixin (C_{25})	1.13	1.12	1.33	0.15	0.15	0.20	0.18	0.18	0.21	1.14	1.09	1.20
Methylbixin (C_{26})	1.11	1.11	1.22	0.14	0.14	0.19	0.17	0.17	0.18	1.08	1.08	1.18
β-Apo-10′-carotenal (C_{27})	0.41	0.42	0.27	0.06	0.07	0.05	§§	0.09	0.05	0.42	0.52	0.41
Azafrin (C_{27})	2.61	1.97	1.63	0.27	0.27	0.45	0.32	0.30	0.49	2.08	1.75	1.80
Methylazafrin (C_{28})	1.99	1.74	1.35	0.21	0.23	0.37	0.30	0.27	0.28	1.70	1.49	1.73
Squalene (*i.e.*, squalane, C_{30})	0.61	0.67	0.37	0.09	0.09	0.07	§§	0.12	0.09	0.66	0.73	0.54
4,4′-Diapophytoene (C_{30})	0.61	0.67	0.37	0.09	0.09	0.07	§§	0.12	0.09	0.67	0.73	0.53
4,4′-Diapophytofluene (C_{30})	0.60	0.67	0.37	0.09	0.09	0.06	§§	0.11	0.09	0.67	0.74	0.54
4,4′-Diapo-ζ-carotene (C_{30})	0.61	0.66	0.35	0.09	0.09	0.07	§§	0.12	0.08	0.67	0.73	0.53
4,4′-Diaponeurosporene (C_{30})	0.61	0.67	0.36	0.09	0.09	0.07	§§	0.12	0.09	0.67	0.73	0.53
β-Apo-8′-carotenal (C_{30})	0.79	0.83	0.54	0.11	0.10	0.10	§§	0.15	0.12	0.82	0.85	0.70
3,4-Dehydro-β-apo-8′-carotenal (C_{30})	0.79	0.83	0.54	0.11	0.10	0.10	§§	0.15	0.12	0.82	0.85	0.69
β-Apo-8′-carotenoic acid (C_{30})	1.65	1.80	1.77	0.23	0.23	0.24	0.27	0.27	0.27	1.57	1.48	1.40
β-Apo-8′-carotenoic acid methyl ester (C_{31})	1.80	1.88	1.85	0.24	0.24	0.26	0.26	0.26	0.28	1.61	1.54	1.46
β-Apo-8′-carotenoic acid ethyl ester (C_{32})	2.02	2.05	1.96	0.26	0.26	0.28	0.30	0.30	0.30	1.77	1.59	1.50
β-Apo-4′-carotenal (C_{35})	2.55	2.55	§§§	0.30	0.33	0.26	0.35	0.35	0.29	2.10	1.84	1.44
Lycopersene (*i.e.*, lycopersane, C_{40})	6.30	6.80	§§§	0.59	0.64	0.46	0.62	0.64	0.46	3.15	2.67	1.76
Phytoene (C_{40})	6.32	6.85	§§§	0.60	0.65	0.45	0.62	0.63	0.46	3.14	2.64	1.77
Phytofluene (C_{40})	6.32	6.85	§§§	0.60	0.65	0.46	0.61	0.64	0.44	3.15	2.66	1.75
ζ-Carotene (C_{40})	6.33	6.83	§§§	0.61	0.63	0.46	0.62	0.64	0.46	3.13	2.66	1.78
Neurosporene (C_{40})	6.32	6.79	§§§	0.60	0.65	0.46	0.62	0.62	0.45	3.14	2.68	1.77
Lycopene (C_{40})	6.32	6.85	§§§	0.60	0.65	0.46	0.62	0.64	0.46	3.15	2.66	1.75
γ-Carotene (C_{40})	§§§	§§§	§§§	0.78	0.81	0.67	0.79	0.81	0.68	3.66	2.83	2.24
β-Zeacarotene (C_{40})	§§§	§§§	§§§	0.79	0.81	0.67	0.80	0.82	0.68	3.66	2.83	2.24
β-Carotene (C_{40})	§§§	§§§	§§§	1.00	1.00	1.00	1.00	1.00	1.00	3.81	2.88	2.43
α-Carotene (C_{40})	§§§	§§§	§§§	1.00	1.00	1.00	1.00	1.00	1.00	3.81	2.87	2.43

TABLE II *(continued)*

Compound and molecular size	*Relative retention times on systems 1, 2 and 3*, isothermal or programmed*											
	*240°***			*275°****			*300°****			*Programmed**,§*		
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
Dehydro-β-carotene (C_{40})	§§§	§§§	§§§	1.00	1.00	1.00	1.00	1.00	1.00	3.81	2.88	2.43
Carotinin (C_{40})	§§§	§§§	§§§	1.00	1.00	1.00	1.00	1.00	1.00	3.81	2.88	2.42
Echinenone (C_{40})	§§§	§§§	§§§	1.71	1.64	2.25	1.61	1.54	1.38	4.65	3.59	3.19
Canthaxanthin (C_{40})	§§§	§§§	§§§	2.85	2.73	3.01	2.58	2.47	2.06	5.90	4.20	3.66
β-Carotenone (C_{40})	§§§	§§§	§§§	2.69	2.64	2.55	2.53	2.25	1.74	5.33	4.12	3.09
Torularhodin (C_{40})	§§§	§§§	§§§	1.07	1.00	1.26	1.05	1.00	1.13	4.23	2.99	2.58
Capsanthin (C_{40})	§§§	§§§	§§§	1.87	1.17	1.49	2.03	1.11	1.40	5.13	3.22	2.81
Astacene (C_{40})	§§§	§§§	§§§	1.70	1.64	1.46	1.63	1.55	1.34	5.79	3.58	3.00
Physalien (C_{40} + $2C_{16}$): C_{40} fragment	§§§	§§§	§§§	1.46	1.42	1.88	1.43	1.35	1.73	3.90	2.92	2.42
acyl fragment?	§§	§§	§§	§§	§§	§§	§§	§§	§§	0.17	0.14	0.13
Cryptoxanthin (C_{40})	§§§	§§§	§§§	1.27	1.25	1.43	1.25	1.34	1.38	4.14	3.33	2.55
Cryptoxanthin, Ac††	§§§	§§§	§§§	1.23	1.25	1.76	1.21	1.33	1.76	4.10	3.28	2.53
Cryptoxanthin, TMS††	§§§	§§§	§§§	1.47	1.45	1.82	1.41	1.40	1.76	4.12	3.31	2.54
Isocryptoxanthin (C_{40})	§§§	§§§	§§§	1.20	1.22	1.40	1.18	1.17	1.37	3.92	2.91	2.53
Isocryptoxanthin, Ac††	§§§	§§§	§§§	1.21	1.23	1.74	1.21	1.33	1.74	3.78	2.81	2.44
Isocryptoxanthin, TMS††	§§§	§§§	§§§	1.43	1.44	1.80	1.36	1.38	1.76	4.07	3.02	2.51
Zeaxanthin (C_{40})	§§§	§§§	§§§	1.68	1.50	2.21	1.41	1.39	1.79	4.68	3.45	3.30
Zeaxanthin, diAc††	§§§	§§§	§§§	1.63	1.40	1.93	1.39	1.33	1.74	5.04	3.50	2.98
Zeaxanthin, diTMS††	§§§	§§§	§§§	1.66	1.44	1.91	1.39	1.38	1.71	5.07	3.55	3.05
Isozeaxanthin (C_{40})	§§§	§§§	§§§	1.48	1.32	1.90	1.24	1.[illegible]	1.74	4.16	3.00	2.59
Isozeaxanthin, diAc††	§§§	§§§	§§§	1.54	1.37	1.64	1.25	1.31	1.57	4.31	3.14	2.47
Isozeaxanthin, diTMS††	§§§	§§§	§§§	1.58	1.41	1.58	1.25	1.33	1.55	4.35	3.17	2.53
Dimethoxyzeaxanthin (C_{42})	§§§	§§§	§§§	2.02	1.96	2.68	1.84	1.78	2.22	5.15	3.67	3.44
Dimethoxyisozeaxanthin (C_{42})	§§§	§§§	§§§	1.23	1.10	1.46	1.05	1.03	1.34	4.02	2.88	2.37
Fucoxanthin (C_{42})	§§§	§§§	§§§	2.24	1.70	2.56	2.22	1.62	2.35	6.24	3.49	3.42
Decapreno-β-carotene (C_{50})	§§§	§§§	§§§	6.35	6.12	6.87	5.04	4.86	5.45	7.54	5.58	5.32
Retention time (min) of:												
Squalene	2.95	5.96	11.23	0.70	1.44	2.48	0.29	0.68	1.06	5.50	8.65	11.15
Perhydro-β-carotene	§§§	§§§	§§§	5.13	10.45	16.65	1.82	3.92	6.01	20.95	24.91	27.09

* Column systems: 1 = SE-52, 2 = HVG, 3 = OV-17. See text for details.
** Retention times relative to squalene.
*** Retention times relative to perhydro-β-carotene.
§ Temperature programme proceeds from 225 to 300° with a 3°/min rate of increase after an initial isothermal period of 3 min.
§§ Compound is eluted with solvent.
§§§ Compound spreads and tails; accurate retention time cannot be determined.
† Compound shows an irregular elution peak indicative of thermal decomposition on the column.
†† Ac = Acetate of perhydro-monohydroxycarotenoid; diAc = diacetate of perhydro-dihydroxycarotenoid; TMS = TMS ether of perhydro-monohydroxycarotenoid; diTMS = TMS diether of perhydro-dihydroxycarotenoid.

and perhydro-dimethoxyisozeaxanthin. For example, at 300° and particularly on the HVG column, the latter two compounds not only failed to resolve completely from perhydro-β-carotene but also tended to increase its normal retention time. Thus compounds with similar retention times need to be examined at a number of temperatures on different liquid phases.

Temperature programming provided the only means by which a range of terpenoids containing from twenty to fifty carbon atoms could be analyzed together. In general, such analyses showed retention times for specific compounds which paralleled those found in isothermal runs. Exceptions to the isothermal elution order did occur, however, on temperature-programmed analysis, notably in the case of the hydrogenated mono- and dihydroxycarotenoids, their acetates and their TMS ethers. The reasons for these discrepancies are not fully understood but may involve the gradually increasing temperature in programmed analysis having a greater influence on separation than such factors as molecular weight and shape and functional group interactions, all of which appear to be important in isothermal separations.

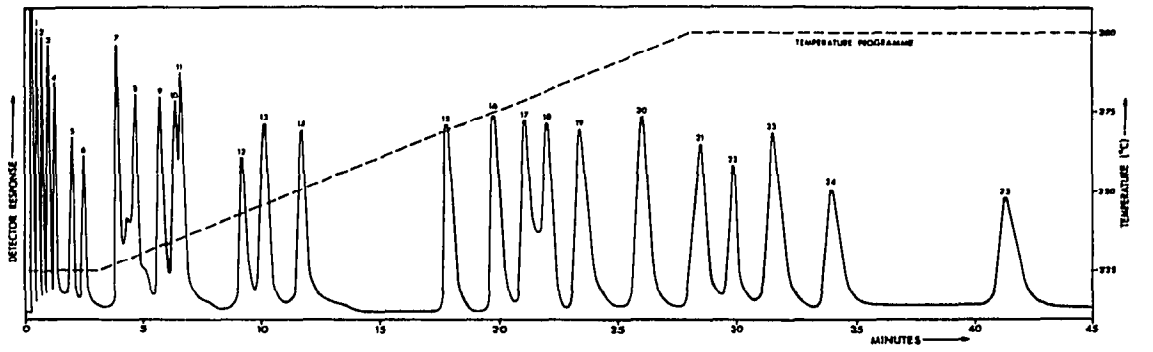

Fig. 2. GLC separation of a mixture of perhydro-carotenoids and other terpenoids on a column of 2% SE-52 on Gas-Chrom Q programmed from 225–300° at 3°/min increase after an initial isothermal period of 3 min. 1 = Perhydro-retinol; 2 = geranyl-linalool; 3 = phytol; 4 = geranylgeraniol; 5 = perhydro-dimethylcrocetin; 6 = perhydro-diethylcrocetin; 7 = squalane; 8 = perhydro-β-apo-8′-carotenal; 9 = squalene; 10 = perhydro-methylbixin; 11 = perhydro-bixin; 12 = methyl ester of perhydro-β-apo-8′-carotenoic acid; 13 = ethyl ester of perhydro-β-apo-8′-carotenoic acid; 14 = perhydro-β-apo-4′-carotenal; 15 = perhydro-lycopene; 16 = perhydro-γ-carotene; 17 = perhydro-β-carotene; 18 = lycopersene; 19 = perhydro-torularhodin; 20 = perhydro-echinenone; 21 = perhydro-capsanthin; 22 = perhydro-β-carotenone; 23 = perhydro-canthaxanthin; 24 = perhydro-fucoxanthin; 25 = perhydro-decapreno-β-carotene.

Each of the three columns used has both general advantages and disadvantages for the separation of terpenoids. The SE-52 column produced the lowest retention times and the sharpest peaks, especially when used with a temperature programme (see Fig. 2) and was best suited for the general separation of not only perhydrocarotenes but also of the hydrogenation products of hydroxy-, mixed-function (*e.g.*, fucoxanthin) and long-retention-time (*e.g.* decapreno-β-carotene, a synthetic C_{50} analogue of β-carotene) carotenoids. The SE-52 column proved to be the most versatile and dependable of the systems used but it suffered from the disadvantage that C_{20} and C_{30} compounds tended to run on it with very similar, and sometimes overlapping, retention volumes.

The HVG column was found to complement the SE-52 column in that although compounds were eluted in the same order from the two columns, retention times on HVG were approximately double those of the same compounds on SE-52. Thus the HVG column gave a better separation of those compounds (in the C_{20}–C_{30} range) which tended to overlap on SE-52. Because of their long retention times on

HVG, the perhydro-xanthophylls tended to be eluted as rather broad and sometimes tailing peaks. HVG is therefore most useful for the analysis of terpenoids of molecular size up to and including the C_{40} perhydrocarotenes.

The terpenoids were retained longer on OV-17 columns than on either HVG or SE-52. In spite of the long retention times, most compounds were eluted from OV-17 as sharp peaks, the only exceptions being the mixed-function perhydro-carotenoids, which tended to be eluted as broad and tailing peaks. Because of the selectivity of this liquid phase, the order in which many compounds were eluted from OV-17 differed from that observed in analyses on HVG or SE-52.

When the behaviour of the terpenoids on the three columns used is considered in more detail, more specific conclusions may be reached; these are that separation is dependent on the molecular weights and polarities of the compounds and the nature and position of their functional groups as well as on the selectivity of the stationary phase. HVG is a methyl silicone polymer and may be classified as a low-polarity, non-selective liquid phase. SE-52 is a phenylmethyl silicone polymer containing 10 mol% of phenyl groups and is thus more selective than HVG. OV-17 is also a phenylmethyl silicone polymer but contains 50–55 mol% phenyl groups; it is thus an intermediate-polarity liquid phase and is the most selective of the three liquid phases tested.

Table II shows that the more selective the liquid phase, the less dependent terpenoid separation is on molecular weight alone. Thus HVG and, for the most part, SE-52 tended to separate compounds firstly by molecular weight and then by molecular shape and symmetry, type and number of functional groups etc., while OV-17 was more selective in separating the terpenoids according to factors other than molecular weight. For example, OV-17 gave the best separations of compounds containing carbon–carbon unsaturation from their hydrogenated derivatives (*cf.* squalene and squalane) and of hydroxylated compounds from their acetates or TMS ethers. This specific selectivity of OV-17 has been used for the separation of TMS ethers of sterols; these are eluted from OV-17 columns in advance of their parent compounds[13]. The increased separation of terpenoids on OV-17 is also exemplified by the behaviour of lycopersene and perhydro-β-carotene; while these show considerable overlap of their retention volumes on SE-52 and HVG, their resolution on OV-17 at 275° is complete.

Compounds in the C_{20}–C_{30} molecular-weight range all had similar retention behaviour on the three columns. Not all the compounds in this range appear to be separated primarily by molecular weight. For example, the methylation of azafrin results in the volatility of the perhydro-methylazafrin being higher, because of the lower polarity of the methyl ester, than that of the corresponding free acid, perhydro-azafrin. Above the C_{30} molecular-weight range, however, molecular weight is the dominant factor in separation; the methyl and ethyl esters of perhydro-β-apo-8′-carotenoic acid are increasingly less volatile than the free acid.

Unsaturated compounds (*e.g.* squalene and lycopersene) are less volatile than their saturated counterparts (squalane and lycopersane, respectively), the greatest separation of such pairs being on OV-17. All three columns gave the same pattern of molecular shape-dependent separation of the perhydro-C_{40}-carotenes. The bicyclic compounds (*e.g.* perhydro-β-carotene) were less volatile than the corresponding monocyclic compounds (*e.g.* perhydro-γ-carotene) and these, in turn, were eluted after the acyclic perhydrolycopene (lycopersane). Again, the greatest separation was

on OV-17. The retention times in isothermal analyses at 300° of the perhydro-derivatives of the isomeric carotenes, β-carotene, γ-carotene and lycopene, were in the ratio 100:79:62 on SE-52 and 100:81:64 on HVG but in the ratio 100:68:46 on OV-17. Perhydrolycopene is eluted from OV-17 columns before most of the sterols analyzed in both the isothermal and temperature-programmed runs.

The retention times of perhydro-β-apo-8'-carotenal and perhydro-3,4-dehydro-β-apo-8'-carotenal were identical on each of the three columns as were those of the perhydro-derivatives of α-carotene, β-carotene, 3,4-dehydro-β-carotene and carotinin. Although catalytic hydrogenation appears to saturate all the carbon–carbon double bonds (and acetylenic bonds, as in carotinin) in the terpenoids tested, it does not reduce carbonyl double bonds. The perhydro-ketocarotenoids are, in most cases, less volatile than their corresponding perhydro-hydroxycarotenoid analogues; the retention times of perhydro-echinenone and perhydro-canthaxanthin are greater, respectively, than those of perhydro-isocryptoxanthin and perhydro-isozeaxanthin, although the retention times of each member of an analogue pair are similar at 300°. The increased retention of the keto-containing perhydro-carotenoids compared with their hydroxyl-containing analogues may be a function of a capacity to enolize, in which case the difference between the molecules would effectively be an olefinic bond at each end group. It is noteworthy that perhydro-canthaxanthin (with both cyclic end groups carrying 4-keto substituents) has a lower volatility even than perhydro-β-carotenone (an acyclic tetraketone). The linearity of the latter molecule may permit a degree of intramolecular hydrogen bonding sufficient to increase its volatility above that of perhydro-canthaxanthin. Intramolecular hydrogen bonding between hydroxyl and keto groups might also account for the high volatility of perhydro-astacene, a compound which may be visualized in its enol form as a dihydroxydiketone.

The influence of substituent position on GLC behaviour is illustrated by the separation of the perhydro-derivatives of hydroxylated carotenoids. Perhydro-isocryptoxanthin and perhydro-isozeaxanthin (one and two 4-hydroxyl substituents, respectively) are each more volatile than their corresponding 3-hydroxy-substituted isomers, perhydro-cryptoxanthin and perhydro-zeaxanthin. It is noteworthy in this context that an oxygen substituent at C_4 of a cyclic end group can be concealed much more effectively by intramolecular folding than a similar function at C_3. This may also be relevant in explaining the otherwise anomalous effect of methylating the dihydroxy-isomers. The volatility of perhydro-isozeaxanthin is increased by methylation whereas that of perhydro-zeaxanthin is decreased; perhaps the effects of depolarizing the hydroxyl groups in the latter compound are outweighed by the increase in overall molecular size (rather than in molecular weight alone).

Acetylation or silylation of perhydro-cryptoxanthin and perhydro-isocryptoxanthin usually decreased their volatilities, particularly on OV-17, although the acetates were sometimes more volatile than the parent compounds (*e.g.* cryptoxanthin on SE-52). The corresponding derivatives of perhydro-isozeaxanthin were less volatile than perhydro-isozeaxanthin on HVG and SE-52 but more volatile on OV-17. The diacetate and diTMS ether of perhydro-zeaxanthin were both eluted before the parent compound in all isothermal analyses, but this was not the case in temperature-programmed analyses on SE-52 or HVG. On these two columns, acetates were more volatile than the corresponding TMS ethers; the situation was reversed, however, on OV-17 for the derivatives of perhydro-dihydroxycarotenoids.

The behaviour of the mixed-function compounds, perhydro-fucoxanthin and perhydro-capsanthin, showed that there was an increase in retention time on each of the three columns as the number of functional groups in the molecule (and hence molecular weight) increased. Even though perhydro-fucoxanthin has a number of functional groups, it still showed a lower retention time in isothermal analyses on HVG and SE-52 than either perhydro-canthaxanthin or perhydro-β-carotenone.

Perhydro-physalien, the dipalmitate of perhydro-zeaxanthin, appeared to lose its fatty acyl groups during hydrogenation and/or on the GLC columns. For this reason, the major peak for perhydro-physalien on each of the three columns was in the perhydro-hydroxycarotenoid region. An additional peak, close to the solvent peak and probably corresponding to the acyl fragment(s), was found in programmed analyses. Although the major peak for perhydro-physalien had a constant retention time in a number of analyses, its elution characteristics did not correspond to those of perhydro-zeaxanthin. It must therefore be due to another cleavage product formed from the original compound.

TABLE III

GLC RETENTION TIMES RELATIVE TO ALL-*trans*-FARNESOL OF C_{10}–C_{20} TERPENOLS UNDER ISOTHERMAL AND TEMPERATURE-PROGRAMMED CONDITIONS

Systems: (1) 2% SE-52 on Gas-Chrom Q; (2) 2% HVG on Chromosorb W.

Compound	*System 1*				*System 2*
	125°	*150°*	*175°*	*Programmed**	*Programmed**
Linalool (C_{10})	0.03	**	**	0.11	0.10
Terpineol (C_{10})	0.05	**	**	0.14	0.14
Geraniol (C_{10})	0.09	0.11	**	0.19	0.26
Cis-Nerolidol (C_{15})	0.33	0.39	0.42	0.61	0.46
All-*trans*-nerolidol (C_{15})	0.40	0.45	0.51	0.68	0.53
cis-Farnesol (C_{15})	0.87	0.89	0.91	0.96	0.93
All-*trans*-farnesol (C_{15})	1.00	1.00	1.00	1.00	1.00
Perhydro-retinol (C_{20})	***	1.65	1.59	1.04	1.11
Geranyllinalool (C_{20})	***	3.91	3.31	1.47	1.36
Phytol (C_{20})	***	6.09	4.69	1.60	1.59
cis-Geranylgeraniol (C_{20})	***	7.58	5.58	1.65	2.01
All-*trans*-geranylgeraniol (C_{20})	***	8.47	6.16	1.69	2.01
Retention time (min) of all-*trans*-farnesol	10.08	2.96	0.99	11.80	11.62

* See text for details.
** Compound is eluted with solvent.
*** Compound spreads and tails; accurate retention times cannot be determined.

In addition to the separations of terpenoids recorded in Table II, a number of C_{10}–C_{20} terpenols were analyzed at lower temperatures on SE-52 and HVG columns (see Table III). The SE-52 column has been selected as our standard routine system for the separation of these compounds because of a number of features. These include its low bleed rate, its excellent resolution (especially in temperature-programmed analyses; see Fig. 3) and its extreme sensitivity (1 ng farnesol, 500 pg geraniol).

In addition, farnesol, nerolidol and geranylgeraniol each separated into two distinct isomers on the SE-52 column. Of these, the major and assumed all-*trans* isomer[3,6,14,15] had the longer retention time in each case. HVG and OV-17 columns were less satisfactory; detection sensitivities were only half of those on SE-52, isomer separations were not complete and peaks tended to spread and tail. Allylic alcohols such as geraniol, farnesol and geranylgeraniol were particularly prone to spreading and tailing on both HVG and OV-17; they had very broad peaks on HVG and thermal destruction of the samples was a feature of analyses on OV-17.

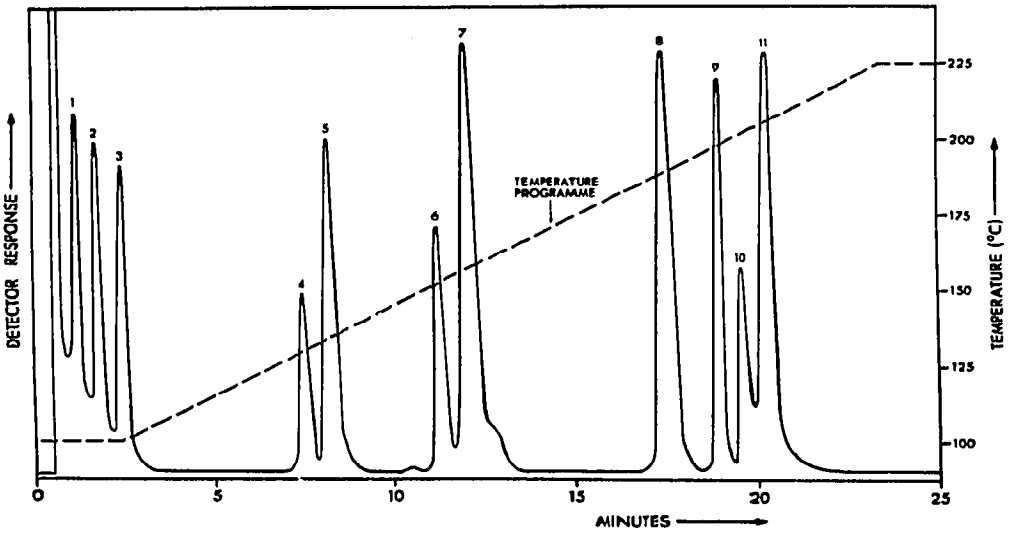

Fig. 3. GLC separation of a mixture of terpenols on a column of 2% SE-52 on Gas-Chrom Q programmed from 100–225° at 6°/min increase after an initial isothermal period of 3 min. 1 = Linalool; 2 = terpineol; 3 = geraniol; 4 = *cis*-nerolidol; 5 = all-*trans*-nerolidol; 6 = *cis*-farnesol; 7 = all-*trans*-farnesol; 8 = geranyl-linalool; 9 = phytol; 10 = *cis*-geranylgeraniol; 11 = all-*trans*-geranylgeraniol.

The separation of C_{10}–C_{20} terpenols has been achieved with varying degrees of success by other workers. The systems used range from 5–20% loadings of polar phases such as ethylene glycol adipate[14], diethylene glycol succinate[16], ethylene glycol succinate or Carbowax 20M[15,17], and butanediol succinate[6,18], to non-polar or low-polarity liquid phases such as Apiezon L[14] and SE-30[19]; more recently, low-percentage loadings of SE-30 and QF-1 have been used[20]. The low-polarity phases give much faster separations of terpenols, with equal if not superior resolution, than those of higher polarity. All-*trans*-farnesol and geranylgeraniol are eluted from 20% butanediol succinate–Chromosorb W (isothermal 190°) in approx. 9 and 43 min, respectively[6], while all-*trans*-farnesol requires some 30 min for elution from 10% Carbowax 20M–Celite (isothermal 180°)[15]. In comparison, all-*trans*-farnesol and geranylgeraniol are eluted, respectively, in approx. 3 and 18 min from a column of 3% SE-30–Gas-Chrom Q (isothermal 195°)[20]. The results in Table III show that these two terpenols are eluted in approx. 1 and 6 min, respectively, from 2% SE-52–Gas-Chrom Q (isothermal 175°). This rapid elution of terpenols from the silicone polymer liquid phase

columns ensures that thermal destruction and quantitative losses of the thermolabile allylic terpenols are minimal. Of the many systems tested, the SE-52 phase is apparently the best for the routine analysis of C_{10}–C_{20} terpenols.

CONCLUSION

The use of gas chromatography for the analysis of carotenoids is obviously limited by the necessity for prior hydrogenation of the samples. GLC analysis can, however, provide a rapid and reliable method for the determination of the molecular size of an unknown carotenoid and is capable of defining the number of cyclic end groups and the type, position and number of functional groups.

The analysis of perhydro-xanthophylls both before and after acetylation and/or silylation provides valuable information on the nature of hydroxyl groups in the molecule. It is clear that GLC analysis, especially if different column systems are used, will be a valuable addition to the chromatographic and spectroscopic methods already in use for structural analysis and identification in the carotenoid field. Indeed, details of its use in this context have recently been reported[4].

The systems reported here are also useful in the analysis of terpenoids which do not have conjugated unsaturation and which therefore do not require prior hydrogenation. The SE-52 system is of particular value in the separation of C_{10}–C_{20} terpenols. It is now in routine use in this laboratory for the analysis of radioactively labelled terpenols released by the standard alkaline phosphatase method[21] from their pyrophosphates which are key intermediates in sterol and carotenoid biosynthesis.

ACKNOWLEDGEMENTS

We thank F. Hoffmann-La Roche and Co. Ltd., Basel, Switzerland, for a generous gift of a number of samples used in this study and Dr. Aung Than for providing a sample of neurosporene. R.F.T. was in receipt of a NATO Postdoctoral Fellowship in Science.

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