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AN EVALUATION OF RECENT GAS–LIQUID CHROMATOGRAPHIC LIQUID PHASES FOR RESOLUTION OF ACETYLATED PLANT STEROLS

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SUMMARY

The relative retentions of twelve acetylated plant sterols were determined on fourteen gas–liquid chromatographic liquid phases. These phases were rated according to their ability to resolve three "critical sterol pairs". The modified Carbowax, SP-1000**, adequately resolved all three pairs in thirty minutes at 240°.

INTRODUCTION

In recent years considerable interest has developed in tentatively identifying plant sterols by their gas–liquid chromatographic (GLC) retention times on various liquid phases[1–3]. This interest appears to have evolved from the discoveries of COPIUS PEEREBOOM[4], KNIGHTS[5], KNIGHTS AND LAURIE[6], and others that the composition of sterols in plants are more complex than first reported. Liquid phases employed for the analyses of phytosterols were essentially limited to SE-30, QF-1 and NGS. Three sets of "critical sterol pairs" are present in plants which challenge the GLC investigator. Examples of these critical pairs are stigmastanol–sitosterol, stigmasterol–campesterol and sitosterol–fucosterol and/or isofucosterol. Resolution of one or two of these critical pairs is achieved on any one of the three major sterol liquid phases, however, a single phase will not resolve all three critical pairs.

In our study of phytosterols in orange juice sac lipids[7] we became aware of the necessity of further elucidating by GLC the minor sterol components which were obscured by major sterol peaks, *viz.*, cholesterol, campesterol, stigmasterol and sitosterol[8]. A single liquid phase which would resolve in a reasonable time the sterols in all three of the "critical pair" regions was needed. To achieve this goal fifteen new or improved phases were studied with the most prominent desmethyl phytosterols.

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** References to brand names are for identification and do not imply endorsement.

These phases were chosen because of their reported increased thermostability, increased polarity and their ability to resolve specific isomeric structures. The acetate form of sterols was investigated since it has been shown[4,5,8] that they are resolved by preparative column chromatography to the greatest extent.

EXPERIMENTAL

GLC conditions

Dexsil-300 GC and AN-600 were obtained from Analabs, Inc., North Haven, Conn. OV-101, OV-210 and OV-225 were obtained from Western Analytical Service, Grinda, Calif. SP-525, SP-1000 and SP-2401 were obtained from Supelco, Inc., Bellefonte, Pa. PMPE (polymetaphenoxylene), Carbowax 20M–Terephthalic Acid (CTpA), HI-EFF-8BP (cyclohexanedimethanol succinate), GE-F-50, OV-17, OV-25 and Poly-I-110 were obtained from Applied Science Laboratories, Inc., State College, Pa. All liquid phases were applied as 1% coatings on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories). The phases were dissolved in their recommended solvents and poured over the solid support in an evaporation dish. The mixture was gently folded with a spoon while the solvent evaporated. The packings were fluidized for 30 min at 70° under nitrogen. All packings were poured into 6 ft. × 4 mm coiled glass columns using vibration and nitrogen pressure and cured overnight at 230° with a helium flow-rate of 20 ml/min. All phases were studied on an F & M Model 5750 gas chromatograph equipped with a flame ionization detector. The detector temperature was 270° and the helium flow-rate was 80 ml/min. For each liquid phase the oven temperature was raised until both internal standards, cholestane and cholesterol acetate, eluted within 7 and 19 min, respectively. On-column injection of the sterol acetate standards was utilized at these "minimum" oven temperatures ranging from 200 to 250°. The six most polar phases were also analyzed on an F & M Model 7610A research gas chromatograph utilizing 6 ft. × 4 mm U-tube glass columns and flame ionization detectors under the same conditions as employed with the Model 5750. The values obtained on both instruments were quite comparable and are presented in composite form.

Sterol standards

Cholestane, cholestan-3-β-ol, campesterol, stigmasterol, desmosterol and sitosterol were obtained from Applied Science Laboratories. Cholesterol acetate was obtained from Steraloids, Inc., Pawling, N.Y., and ergosterol from Eastman Organic Chemicals, Rochester, N.Y. Fucosterol was isolated from Fucus and Ascophyllum[9] obtained from Carolina Biological Supply Company, Burlington, N.C. A concentrate of brassicasterol was isolated from cabbage seeds[10]. The acetates of all sterols were prepared by heating the sterols for 1 h at 75° with a pyridine–acetic anhydride acetylation reagent (Applied Science Laboratories) in sealed acetylation tubes. The acetates were purified by TLC on Silica Gel G plates with hexane–ethyl acetate (160:40). Standards were injected as 1% solutions of the acetates in heptane with cholestane and cholesterol acetate as internal references. Retention times were recorded relative to cholesterol acetate[3]. For standards having retention times very near cholesterol acetate (cholestanol acetate), a sample free of the cholesterol acetate was also injected.

TABLE I

LIQUID PHASES FOR STEROL ACETATE ANALYSES

Liquid phase	*Phase structure*	*Similar phase (s)*	*McReynolds constant x'*
OV-101	Dimethyl silicone	SE-30, UC-W-98	17
GE-F-50	Methyl, chlorophenyl silicone	(Versilube)	19
Dexsil-300	Polycarboranesiloxane		47
OV-17	50% Phenyl, methyl silicone		119
OV-210	50% Trifluoropropyl, methyl silicone	QF-1, FS-1265	148
SP 2401	50% Trifluoropropyl, methyl silicone	QF-1, OV-210	148
OV-25	75% Phenyl, methyl silicone		178
AN-600	50% Cyanoethyl, methyl silicone	XE-60, OV-225	204[a]
SP-525	"Aromatic hydrocarbon"		225
OV-225	25% Cyanopropyl, 25% phenylmethyl silicone	XE-60	228
PMPE	Polymetaphenoxylene	PPE-20	257[b]
HI-EFF-8BP	Cyclohexanedimethanol succinate	CHDMS, NGS	271
CTpA	Carbowax 20M–Terephthalic acid		321
SP-1000	"Modified Carbowax 20M"		332
Poly-I-110	Polyamide		[c]

[a] Reported for XE-60.
[b] Reported for PPE-20.
[c] McREYNOLDS constant x' not available.

RESULTS AND DISCUSSION

Table I lists the fifteen liquid phases in increasing polarity as determined by reported McREYNOLDS constant x' values[11,12]. OV-101 has nearly the same McREYNOLDS constants as SE-30, however, it has been reported that OV-101 is superior in resolution and offers more symmetrical peaks than SE-30 for free sterols[13]. GE-F-50, also known as Versilube, is included in the list as being an example of a chlorophenylmethylsilicone of high stability. Dexsil-300 GC is a new type of phase having very low bleed at high temperatures. Although no data have been previously recorded on the use of this phase for sterol acetates, various steroid derivatives have been analyzed on this phase[14,15]. OV-17 has been reported to give good resolution of campesterol–stigmasterol[16,17] as well as being a good phase for GLC–mass spectrometry analyses of fucosterol[9,18]. No reports on the use of OV-25 for sterol acetates appear in the literature. Its increased percentage of the phenyl moiety over OV-17 makes it a promising candidate for even further improvement in resolution of the campesterol–stigmasterol and sitosterol–fucosterol pairs. AN-600, SP-525 and OV-225 are all three improved versions or replacements for the frequently used cyanosilicone XE-60[2,10]. Polymetaphenoxylene was presented as being the most stable polar phase available in 1969 (ref. 19). Excellent resolution of sterol acetates was reported by PATTERSON[3] for this phase. OV-210 and SP-2401 are both improved versions of QF-1. The supplier (Supelco Inc.) reports that SP-2401 has a lower viscosity than either QF-1 or OV-210 and thus produces columns with increased efficiency. Very good resolution of the cholesterol–cholestanol pair was reported for this phase[20]. HI-EFF-8BP is presently the most stable version of the frequently used sterol phases, CHDMS or NGS. It is, as its predecessors, limited in thermostability. Both CTpA and SP-1000 have not been studied previously for their sterol resolving capabilities

TABLE II

COLUMN CONDITIONS FOR STEROL ACETATE ANALYSES

The net retention time is expressed relative to solvent peak heptane.

Liquid phase	*Oven temp. (°C)*	*Net retention time (min)*		*Theoretical plates/ft.*
		Cholestane	*Cholesterol acetate*	
OV-101	230	6.16	16.45	326
GE-F-50	230	5.22	14.46	458
Dexsil-300	240	4.40	12.63	551
OV-17	240	4.50	15.62	338
OV-210	200	2.64	13.99	362
SP 2401	220	2.35	9.92	511
OV-25	230	3.24	12.41	186
AN-600	210	2.40	11.71	170
SP-525	250	3.45	12.48	603
OV-225	235	2.94	13.61	267
PMPE	230	2.56	12.21	473
HI-EFF-8BP	230	3.68	18.69	555
CTpA	230	2.44	12.96	267
SP-1000	240	2.70	11.48	499
Poly-I-110	240		28.3	

presumably because Carbowax 20M, their major component, is limited to a maximum operating temperature of 200°. Poly-I-110 with a reported upper limit of 300° for packed columns can easily resolve cholesterol from cholestanol on capillary columns[21].

Table II lists the GLC conditions under which each of the fifteen phases were analysed. It is clear that these phases differ markedly in their viscosity and ability to retain the cholestane and cholesterol acetate standards.

Although Poly-I-110 is reported to resolve saturated from mono-unsaturated sterols on capillary columns, the extended time of 28.3 min for elution of cholesterol

TABLE III

RELATIVE RETENTION TIMES OF PLANT DESMETHYL STEROL ACETATES TO CHOLESTEROL ACETATE

Sterol acetate	*Abbreviation*	*Liquid phase*				
		OV-101	*GE-F-50*	*Dexsil 300*	*OV-17*	*OV-210*
Cholesterol	FC_{27}	1.00	1.00	1.00	1.00	1.00
Cholestanol	C_{27}	1.02	1.01	1.05	1.00	1.04
Desmosterol	$FC_{27}F$	1.10	1.11	1.12	1.22	1.12
25-Dehydrocholesterol	$FC_{27}F$			1.15		
Campesterol	FC_{28}	1.31	1.31	1.33	1.31	1.34
Ergostanol	C_{28}	1.33	1.33	1.39	1.31	1.38
Brassicasterol	$FC_{28}F$	1.12	1.11	1.11	1.14	1.09
Ergosterol	$2FC_{28}F$	1.09	1.10	1.10	1.29	1.21
Sitosterol	FC_{29}	1.65	1.65	1.71	1.65	1.64
Stigmastanol	C_{29}	1.68	1.69	1.78	1.65	1.72
Stigmasterol	$FC_{29}F$	1.42	1.42	1.41	1.45	1.37
Fucosterol	$FC_{29}F$					
Isofucosterol	$FC_{29}F$	1.66	1.71	1.76	1.84	1.65

acetate at 240° shows that Poly-I-110 was not practical and, therefore, further studies with this phase were discontinued.

Table III lists the relative retention times of twelve sterol standards on fourteen liquid phases. In general, the fourteen phases show relative retention times quite comparable to those reported by CLAYTON[1] for methyl ethers, IKEKAWA *et al.*[2] for free sterols and PATTERSON[3] for sterol acetates. The presence of a C-24 double bond increases the relative retention time of desmosterol acetate from that of cholesterol acetate by 0.1 units for non-selective phases (*e.g.* OV-101 and GE-F-50); to 0.2 units for phenyl silicones (*e.g.* OV-17 and OV-25) and to 0.3 or higher for polar polyesters. The presence of two isomers of standard desmosterol, presumably Δ^{24} and Δ^{25} (ref. 22), are observed on both Dexsil-300 and SP-2401. These two isomers were previously observed on SE-30, QF-1 and NGS (ref. 22) with QF-1 showing the greatest separation factor of 1.04. This compares favorably with a value of 1.03 calculated for SP-2401. Desmosterol can readily be resolved from campesterol on the three most non-polar phases (OV-101, GE-F-50, Dexsil-300), however, on several polar phases (SP-2401, SP-525, OV-225, PMPE) resolution is poor. Ergosterol with two double bonds in the ring and one in the side-chain has nearly identical relative retention times as desmosterol for the three most non-polar phases. This is in contradistinction to 1.09 and 1.22 reported for desmosterol and ergosterol by PATTERSON[3] on an SE-30 column. The additional double bond and a methyl group at C-24 of ergosterol adds 0.1 to 0.2 relative retention units to that observed for desmosterol on OV-17, OV-210, SP-2401, HI-EFF-8BP and SP-1000 phases. Ergosterol can readily be resolved from campesterol on the three most non-selective phases and on OV-210, SP-2401, AN-600, SP-525 and SP-1000, however, on the remaining six phases these two sterols elute together. Brassicasterol with two double bonds and one C-24 methyl group should possess retention characteristics somewhat between campesterol and ergosterol. The relative retention values for brassicasterol, however, on all fourteen phases are 1.11 ± 0.03 or 0.2 units lower than 1.32 ± 0.02, the values

SP-2401	*OV-25*	*AN-600*	*SP-525*	*OV-225*	*PMPE*	*HI-EFF-8BP*	*CTpA*	*SP-1000*
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.05	0.99	1.00	1.03	1.03	1.03	1.00	0.96	0.95
1.24	1.25	1.18	1.28	1.28	1.32	1.27	1.35	1.37
1.28								
1.33	1.33	1.32	1.31	1.31	1.32	1.33	1.30	1.30
1.35	1.31	1.32	1.34	1.34	1.35	1.33	1.24	1.23
1.10	1.14	1.08	1.11	1.12	1.08	1.10	1.13	1.12
1.44	1.28	1.17	1.19	1.27	1.36	1.35	1.34	1.48
1.64	1.65	1.65	1.60	1.59	1.57	1.63	1.57	1.60
1.66	1.63	1.65	1.63	1.62	1.60	1.63	1.52	1.53
1.38	1.44	1.36	1.36	1.41	1.35	1.37	1.36	1.39
			1.72	1.79	1.72	1.77	1.77	1.74
1.59	1.75	1.75	1.86	1.80	1.84	1.87	1.86	1.92

TABLE IV

SEPARATION FACTORS FOR FIVE STEROL ACETATE PAIRS

1 = sitosterol/stigmastanol; 2 = campesterol/stigmasterol; 3 = sitosterol/fucosterol; 4 = sitosterol/isofucosterol; 5 = fucosterol/isofucosterol.

Liquid phase	*Sterol acetate pairs*				
	1	*2*	*3*	*4*	*5*
OV-101	0.98	0.92		0.99	
GE-F-50	0.98	0.92		0.96	
Dexsil 300	0.96	0.94		0.97	
OV-17	1.00	0.90		0.90	
OV-210	0.95	0.98		0.99	
SP-2401	0.99	0.96		1.03	
OV-25	1.01	0.92		0.94	
AN-600	1.00	0.97		0.94	
SP-525	0.98	0.96	0.93	0.86	0.92
OV-225	0.98	0.93	0.89	0.88	0.99
PMPE	0.98	0.98	0.91	0.85	0.93
HI-EFF-8BP	1.00	0.97	0.92	0.87	0.95
CTpA	1.03	0.96	0.89	0.84	0.95
SP-1000	1.05	0.94	0.92	0.83	0.91

recorded for campesterol. It therefore appears as if no phase possesses any significant selectivity for the C-22 double bond of brassicasterol.

Table IV shows the separation factors for five critical pairs of sterol acetates. Table IV reveals that only four of the fourteen phases show possibilities for resolving sitosterol from stigmastanol (column 1). OV-210 has a 0.03 lower value than that reported for its predecessor, QF-1, while Dexsil-300 is 0.02 units lower than SE-30[8]. CTpA and SP-1000 show stigmastanol acetate eluting before sitosterol acetate by 0.03 and 0.05 units, respectively. Examination of column 2 (campesterol–stigmasterol) shows that the three non-polar phases (OV-101, GE-F-50 and Dexsil-300) exhibit "non-selectivity" in resolving stigmasterol acetate from campesterol acetate. Phenyl- and cyanosilicones also resolve these two standards very well with OV-17 having a value of 0.90. On highly polar phases, however, these two sterol acetates tend to elute together. An exception appears to be SP-1000 in which the two sterols are as resolved as observed on the non-polar Dexsil-300. For the sitosterol–fucosterol pair (column 3) all polar phases show excellent resolution with separation factors 0.91 $\pm$ 0.02. These polar phases show even better resolution for the sitosterol–isofucosterol pair (column 4) with separation factors of 0.85 $\pm$ 0.03. Less resolution (0.92 $\pm$ 0.02) is encountered for the phenyl- and cyanomethyl silicones. The non-selective and fluorosilicone phases fail to separate these two sterol acetates. Column 5 lists the separation factors for the two $\Delta^{5,24(28)}$ sterol acetate isomers on the six most polar phases. Excellent resolution is obtained on SP-525, PMPE and SP-1000 (0.92 $\pm$ 0.01). These isomeric sterols are less resolved on HI-EFF-8BP and CTpA, while OV-225 fails to resolve the pair.

The data presented clearly distinguish SP-1000 as being superior to all other phases tested in its ability to resolve all five critical pairs. In addition this phase separates other major phytosterols including desmosterol. This phase is stable and

offers a reasonably short time of 25 min at 240° for eluting all Δ^5 desmethyl sterol acetates. An additional 8 to 10 min is needed to elute the Δ^7 desmethyl phytosterol acetates[8]. The phase is excellent for monitoring sterol acetates separated by argentation column chromatography[8]. It is recommended that this phase be used in conjunction with Dexsil-300 and OV-17 for a complete GLC analysis of desmethyl phytosterol acetates.

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