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STEROLS OF THE COCKLE *CERASTODERMA EDULE*

EVALUATION OF THERMOSTABLE LIQUID PHASES FOR THE GAS–LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF THE TRIMETHYLSILYL ETHERS OF MARINE STEROLS

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SUMMARY

Some recent liquid phases for gas–liquid chromatography with improved thermostability have been evaluated for their use in the gas–liquid chromatographic–mass spectrometric (GC–MS) analysis of the complex mixtures of sterol trimethylsilyl ethers obtained, often in small amounts, from marine organisms. A combination of Dexsil 300 GC, SE-30 "ultraphase" and Silar 5CP is recommended for use in this technique because of the excellent separating efficiency and the very low substrate bleed. GC–MS analysis of the sterol trimethylsilyl ethers obtained from the cockle *Cerastoderma edule* has established the identity and relative proportions of the eleven sterols present.

INTRODUCTION

Over the last few years there has been an upsurge of interest in the role played by sterols (see refs. 1–7 and references therein) in the marine environment. This increased interest is due in part to the introduction of gas–liquid chromatography (GLC) as an analytical technique, as the sterols could be identified by comparison of the GLC retention times on various liquid phases[8–10]. However, marine sterol mixtures have proved to be very complex indeed and some of the reported identifications must be considered as tentative, due to inadequate separations on the GLC columns.

Some improvement was achieved by the introduction of argentated preparative thin-layer chromatography (AgPLC) as a preliminary step to GLC analysis[11,12], as mixtures of sterol acetates could be separated into various zones depending on their complexing properties with silver ion. GLC analysis of the sterol acetates within each zone resulted in a considerable simplification of the chromatograms and some overlapping peaks have been identified in this way[12].

Mass spectrometry has often been employed to identify components which have been separated on preparative GLC columns and various derivatives have been employed to improve the volatility, GLC resolution, or mass-spectrometric response of the free sterols. In particular acetates[8], methyl ethers[10,13] and trimethylsilyl ethers[14] have been examined and their GLC and mass-spectrometric performance tabulated[15–17].

Coupled gas–liquid chromatography–mass spectrometry (GC–MS) has recently been employed for the investigation of sterols[7,15–17] and has considerable potential for the rapid identification of the complex mixtures from marine sources. However, the technique suffers from the general problems[18] of (a) inadequate resolution on the GC column, (b) column bleed at the high temperatures necessary for the investigation of sterols, (c) condensation or decomposition in the transfer lines or separator, and (d) determination of the steroid structure from the mass spectral fragmentations. In our study of the sterols present in the cockle we decided to investigate the utility of the GC–MS technique for this type of analysis.

Trimethylsilyl (TMS) ethers were selected by us as the most suitable type of derivative for this investigation[16], for although methyl ethers and acetates had been recommended because of their reported superior resolution characteristics on GC columns[8,19,20], the methyl ethers and acetates were found to be much more troublesome to prepare quantitatively from biological samples and to have less well defined and less well documented mass spectra[13,15,17]. The TMS derivatives were prepared quantitatively in seconds[14] and were found to have adequate resolution on the GLC columns employed in this investigation as well as much shorter retention times than those reported for the methyl ethers[20] or free sterols. They were also found to be extremely stable in solution, to have excellent transfer characteristics through the silicone membrane separator and transfer lines used in our apparatus and had well defined reproducible[16] mass spectra.

Substrate bleed from the liquid phases commonly used for the GLC analysis of sterols and their derivatives was a serious problem[18] in GC–MS work, as many of the columns were used at temperatures close to their maximum recommended temperature and even a small quantity of bleed could swamp the sterol mass spectrum with contamination ions. It was decided to try to minimise substrate bleed by investigating the performance of a series of new or "improved" liquid phases for their separating efficiency for sterol TMS derivatives and their thermostability in GC–MS operations.

A series of marine sterol standards was used in this study as well as the sterol mixture obtained from the cockle *Cerastoderma edule*.

The sterol compositions of the Mollusc family have been investigated extensively for several years and the findings have been reviewed[21–23]. However, no detailed investigation has been reported on the sterols of the cockle, although a Canadian variety was the subject of a preliminary study by Fagerlund and Idler in 1956[24]. The cockle was selected for GC–MS investigation because of the expected complexity[23] of its sterol profile and its local abundance.

EXPERIMENTAL

GLC columns and conditions

SE-30 "ultraphase", SP-1000 and STAP were obtained from Phase Separations

(Queensferry, Great Britain); HI-EFF-8BP was obtained from Applied Science Labs. (State College, Pa., U.S.A.); OV-1 was obtained from Pye Unicam (Cambridge, Great Britain); Dexsil 300 GC was obtained from Analabs (North Haven, Conn., U.S.A.); and Silar-5CP was obtained from Field (Richmond, Great Britain).

The liquid phases were dissolved in their recommended solvents and applied as 1% coatings to 100–120 mesh Diatomite CQ (Pye Unicam). The packings were inserted into coiled glass columns (2.5 m × 4 mm I.D.) and conditioned overnight near to their maximum recommended temperatures (Dexsil 300 GC at 350°) with a helium flow-rate of 50 ml/min.

The performance of the columns was compared by on-column injection in a Pye Model 104 gas chromatograph fitted with dual flame ionisation detectors (FIDs), one of which was connected to a mass spectrometer interface. Helium flow-rates were 50 ml/min and detector temperatures were 300° with oven temperatures between 220–260° depending upon the thermostability of the liquid phase (see Table II).

GC–MS interface

The Pye Model 104 gas chromatograph was fitted with a 1:1 effluent splitter, one branch of which was connected to an FID and the other to a mass spectrometer interface*. The interface consisted of a glass solvent dump valve and a Llewellyn–Littlejohn[25] type of silicone membrane separator mounted on a glass sinter**. The reverse side of the membrane was connected to an AEI Model MS9 mass spectrometer, modified with fast scanning capability, via a heated glass transfer line and glass source re-entrant tube. The mass spectrometer was fitted with a total ion monitor (TIM) recorder and was operated at 20 eV, which was below the ionisation potential of the helium carrier gas, to record the TIM chromatogram and was automatically switched to 70 eV when a mass-spectral magnetic scan was initiated.

Mass spectra were recorded at a source temperature of 220° and a trap current of 100 μA, using scan times of 5 or 10 sec per decade with UV galvanometer recording. The temperature of the membrane separator was maintained at 240° and the transfer lines at 250° for each column system investigated. Helium pressure in the MS source was of the order of 1×10^{-5} torr during GC–MS runs. The solvent dump valve was used to bypass the solvent effluent, N,O-bis-(trimethylsilyl)-acetamide (BSA), to air to prevent contamination of the MS source. The vent valve was operated manually when required, as indicated by the FID recorder trace.

The transfer efficiency of the membrane separator and transfer lines was monitored continuously by direct comparison of FID and TIM traces. There was a delay of about 10 sec between a peak appearing on the FID and on the TIM recorders. MS scans were initiated on the appropriate portions of each GC peak as it appeared on the TIM chromatogram. In this way several MS scans could be performed on each GC peak to determine its homogeneity.

Sterol standards

Sterols (*ca.* 1 mg) were converted to their TMS ethers by dissolving in BSA

* A.E.I. Membrane Separator Interface Type WM-075.
** Designed by Hirst Research Centre, Wembley, Great Britain.

(1 ml) at room temperature or by gently warming[14]. In some cases a small quantity of pyridine was necessary to aid dissolution. GLC analysis of the solutions after 5 min indicated that quantitative conversion to the TMS ethers had taken place.

Isolation of cockle sterols

Fresh cockles, *Cerastoderma edule*, were obtained from the cockle beds* off Penclawdd, South Wales, and the flesh (22.0 g) was extracted into 20 vol. of chloroform–methanol (2:1). All manipulations were performed under nitrogen atmosphere and the extracts were stored under nitrogen in the dark at −22°. The resultant lipid fraction (154 mg) was saponified with 20 vol. of 10% KOH–ethanol (1:1) at 20° for 16 h. Sterols and other non-saponifiable lipids (45 mg) were extracted into diethyl ether and separated by PLC on Kieselgel G (500 μm layers) using toluene–diethyl ether–ethanol–acetic acid (50:40:4:2)[26] as eluent. The sterol zone, which was detected by spraying with either Rhodamine 6G solution or concentrated sulphuric acid–ethanol (1:3) solution, was removed and further purified by rapid column chromatography to give a pure sterol mixture (22 mg). The TMS ethers were prepared as described and injections of 1 μl of BSA containing approx. 1–10 μg of TMS ethers were used for the GC–MS analyses.

TABLE I

POTENTIAL THERMOSTABLE LIQUID PHASES FOR THE ANALYSIS OF STEROL TMS ETHER MIXTURES

Liquid phase	*Phase description*	*McReynolds' constant, x'*	*Maximum temperature (°C)*	*Number of theoretical plates**
SE-30 "ultraphase"	"improved" methylsilicone gum	15	350	4224
OV-1	dimethylsilicone gum	16	350	2557
Dexsil 300 GC	polycarboranesiloxane	47	500	3708
SP1000	"Carbowax 20M + substituted terephthalic acid"	332	275	2745
STAP	"Steroid Analysis Phase"	345	255	3410
HI-EFF-8BP	cyclohexane dimethanol succinate	271	250	1722
Silar 5CP	50% cyanopropyl, 50% phenyl silicone	319	275	2291

* As measured on the cholesteryl TMS ether peak.

RESULTS AND DISCUSSION

The seven new or "improved" GLC liquid phases which were studied in this investigation are described in Table I. The different polarities are described in terms of the McReynolds constant x' (ref. 27). The non-polar methyl silicone phases have been widely used for sterol analysis[16,17] and the improved phase, SE-30 "ultraphase", was selected for comparison with the commonly used OV-1 phase. These phases would have the advantage of a very high maximum working temperature and were reported to have little substrate bleed at normal temperatures[28].

* We are indebted to Mrs. S. Littlejohns, Swansea University College, for the gathering of fresh samples.

Dexsil 300 GC, a remarkably thermostable new phase of low polarity, had been recommended for the GLC analysis of sterol acetates[8]. The polar phases SP1000 and HI-EFF-8BP had also been found useful in the GLC analysis of sterol acetates[8] but had a much lower thermal stability than Dexsil 300 GC although HI-EFF-8BP is the most thermostable of all of the polyester phases. The polar "steroid analysis phase", STAP, was included in the survey although it had been reported as having bleed problems in GC–MS operations[18].

Silar 5CP is a new, fairly stable, silicone phase of high polarity and was reported to have potential in the resolution of double bond isomers[29].

Table II lists the GLC relative retention time (*RRT*) of fourteen typical marine sterol TMS ethers on each of the liquid phases under investigation. The general features of the separations of the sterol TMS ethers are displayed graphically in Fig. 1 and have similarities to those reported previously for free sterols[30], sterol methyl ethers[10] and sterol acetates[8,9] as well as in previous work on TMS ethers[16,17].

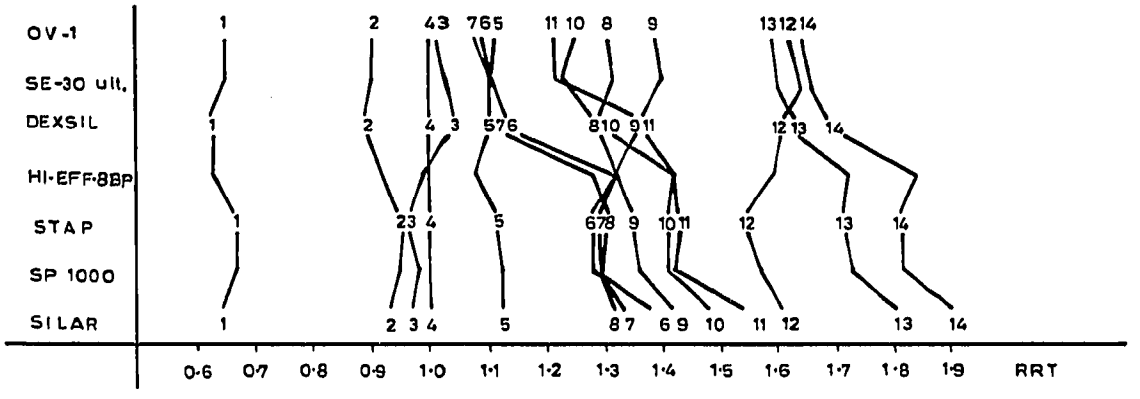

Fig. 1. Comparative retention times of marine sterol TMS ether standards on thermostable phases relative to cholesteryl TMS ether. The numbers with the curves correspond to those given in Table II.

On all the column systems the $\Delta^{5,22}$-sterol TMS ethers precede the corresponding Δ^{5}-sterol TMS ethers but none of the columns have any particular selectivity for this feature. The $\Delta^{5,7}$ homoanular diene had a longer retention time than the corresponding Δ^{5} compound on each column but the diene was much more strongly adsorbed on the polar columns (Silar 5CP > STAP > HI-EFF-8BP > SP1000) than on the non-polar columns.

A similar feature was observed with the C_{24-25} double-bonded compound desmosterol TMS ether (compound 7) which had a longer retention time than cholesterol TMS ether (compound 4) on each column but was much more strongly adsorbed on the polar columns (in the same order) than on the non-polar columns. The C_{24-28} double bond in fucosterol TMS ether and isofucosterol TMS ether was again strongly adsorbed by particular column systems. In all systems the Z compound had a longer retention time than either the E compound or β-sitosterol TMS ether and was much more strongly adsorbed by the polar columns than by the non-polar columns. However, it was found that the E compound was less strongly adsorbed

TABLE II

RELATIVE RETENTION TIMES OF STANDARD STEROL TMS DERIVATIVES

No.	*Sterol TMS derivatives*	*Notation*	*SE-30 "ultra"*	*OV-1*	*Dexsil 300 GC*	*SP1000*	*STAP*	*HI-EFF-8BP*	*Silar 5CP*
1	22-*trans*-24-Norcholesta-5,22-dien-3β-ol	C26:5,22	0.65	0.65	0.63	0.67	0.67	0.63	0.64
2	22-*trans*-Cholesta-5,22-dien-3β-ol	C27:5,22	0.90	0.90	0.89	0.95	0.95	0.92	0.93
3	Cholestan-3β-ol	C27:0	1.03	1.01	1.04	0.98	0.96	0.99	0.97
4	Cholesterol	C27:5	(15)*	(9)*	(19)*	(10)*	(10)*	(14)*	(16)*
5	Brassicasterol	C28:5,22	1.10	1.11	1.10	1.12	1.11	1.08	1.12
6	Cholesta-5,7-dien-3β-ol	C27:5,7	1.10	1.09	1.13	1.28	1.28	1.32	1.40
7	Desmosterol	C27:5,24	1.10	1.08	1.10	1.28	1.30	1.28	1.34
8	Campesterol	C28:5	1.31	1.30	1.29	1.29	1.29	1.32	1.32
9	Stigmasterol	C29:5,22	1.40	1.39	1.36	1.36	1.35	1.32	1.43
10	24-Methylenecholesterol	C28:5,24(28)	1.23	1.25	1.29	1.41	1.41	1.42	1.49
11	Ergosterol	C29:5,7,22	1.22	1.21	1.36	1.42	1.43	1.42	1.57
12	β-Sitosterol	C29:5	1.64	1.62	1.61	1.57	1.54	1.59	1.61
13	Fucosterol	C29:5,24(28)E	1.60	1.59	1.62	1.73	1:71	1.72	1.82
14	28-Isofucosterol	C29:5,24(28)Z	1.66	1.64	1.69	1.82	1.81	1.84	1.92
	GLC oven temperatures, °C		250	250	260	250	250	245	220

* Retention time in minutes for cholesteryl TMS ether.

than β-sitosterol TMS ether on the non-polar columns, but on the polar columns the reverse situation was observed.

The separation efficiency of the column systems for particular pairs of sterol TMS ethers can be assessed by calculating either the separation factors as employed by both Patterson[9] and Nordby and Nagy[8] or by calculating the resolution between the pairs[31]. Table III contains the results of these calculations for sterol pairs which commonly occur in marine sterol samples.

The results indicate that in several cases the resolution of the TMS ethers on the best column systems available is almost as good as the best separations achieved with the sterol acetates[8,9]. It should be appreciated that the resolution figures, which take into account the peak widths[31], are of much more value than the separation factors in assessing the most suitable column system to be used for a particular sterol separation. For example, the separation factors between 22-*trans*-cholesta-5,22-dien-3β-ol TMS ether and cholesteryl TMS ether would suggest no difference in performance between SE-30 and OV-1 for this separation, but in fact the chromatograms indicate that there is a much better resolution between these two sterol derivatives on SE-30, as indicated by the calculated resolution figure (R).

By the use of Table III together with Fig. 1, it is possible to deduce which GLC column system may be best employed for the separation of any pair of these marine sterol TMS ethers. For example, to achieve the best separation between brassicasterol TMS ether (5) and desmosterol TMS ether (7), it is advisable to use a Silar 5CP column ($R = 1.81$); to achieve the best separation between desmosterol TMS ether (7) and campesterol TMS ether (8) it is advisable to use an OV-1 column ($R = 1.90$); to achieve the best separation between 22-*trans*-cholesta-5,22-diene-3β-ol TMS ether (2) and cholestanol TMS ether (3), it is advisable to use a Dexsil 300 GC column ($R = 1.94$); and to achieve the best separation between ergosterol TMS ether (11) and β-sitosterol TMS ether (12) it is advisable to use an SE-30 column ($R = 4.04$).

Clearly, no one GLC column system will resolve satisfactorily the common mixtures of marine sterol TMS ethers but two or three carefully chosen column systems, used in conjunction, could resolve most problems of overlapping peaks. Also the use of GC–MS multiple scanning over any incompletely resolved doublet should make the problem of the identification of the components much easier than with GLC alone and thus the complete resolution of two components on the GLC column, although highly desirable, would no longer be essential for the identification of known materials.

It is apparent from this survey that, with regard to the partition of sterol TMS ethers, there are essentially three different types of GLC columns, (i) OV-1 and SE-30, (ii) Dexsil 300 GC, and (iii) Silar 5CP, SP1000, STAP and HI-EFF-8BP. For any GLC analysis of marine sterols it would therefore be desirable to have one of each of these types available and clearly for GC–MS analysis the thermostability of each of the liquid phases would be of paramount importance. To obtain information on this feature each of the seven columns was fitted into the GC–MS instrument and a combined background spectrum of the column and membrane separator bleed was recorded under the normal operating conditions of the GC column and MS interface with helium flowing. The background bleed of the silicone membrane itself was determined by flowing helium through an empty column in the GC oven at 250° and recording the mass spectrum. The results of this investigation are shown in Table IV.

TABLE III

RESOLUTION CHARACTERISTICS OF THE GLC LIQUID PHASES FOR DIFFICULT PAIRS OF STEROL TMS ETHERS

	Sterol pairs		*Resolution of pairs*							*Separation factor*						
			SE-30	*OV-1*	*Dexsil*	*SP1000*	*STAP*	*HI-EFF-8BP*	*Silar 5CP*	*SE-30*	*OV-1*	*Dexsil*	*SP1000*	*STAP*	*HI-EFF-8BP*	*Silar 5CP*
2	22-*trans*-Cholesta-5,22-dien-3β-ol	C27:5,22	1.40	1.11	1.36	0.52	0.47	0.73	0.70	0.90	0.90	0.89	0.95	0.95	0.92	0.93
4	Cholesterol	C27:5														
5	Brassicasterol	C28:5,22		0.21	0	1.45	1.73	1.56	1.81	1.00	1.03	1.00	0.88	0.85	0.84	0.83
7	Desmosterol	C27:5,24														
8	Campesterol	C28:5	0.86	0.70	0.70	0.54	0.53	0	0.83	0.94	0.94	0.95	0.94	0.96	1.00	0.92
9	Stigmasterol	C29:5,22														
8	Campesterol	C28:5	0.86	0.41	0.12	0.86	1.04	0.69	1.26	1.07	1.04	1.00	0.91	0.91	0.92	0.88
10	24-Methylene-cholesterol	C28:5,24(28)														
12	β-Sitosterol	C29:5	0.33	0.20	0.06	1.01	1.24	0.77	1.33	1.03	1.02	0.99	0.91	0.90	0.92	0.88
13	Fucosterol	C29:5,24(28)E														
12	β-Sitosterol	C29:5	0.13	0.13	0.67	1.40	1.91	1.45	1.91	0.99	0.99	0.95	0.86	0.85	0.86	0.83
14	28-Isofucosterol	C29:5,24(28)Z														
13	Fucosterol	C29:5,24(28)E	0.51	0.32	0.55	0.54	0.67	0.67	0.58	0.96	0.97	0.96	0.95	0.94	0.93	0.94
14	28-Isofucosterol	C29:5,24(28)Z														

TABLE IV

COLUMN BLEED CHARACTERISTICS

Liquid phase	*Total ion abundance* Σ_{44}^{510}	*Relative column bleed*	*Eight most prominent ions in the mass spectrum of membrane and column bleed, m/e (ion current)*
Emtpy column	138	–	207 (65), 281 (12), 73 (12), 96 (11), 208 (10), 209 (8), 57 (7), 133 (6)
Dexsil 300 GC	141	1	207 (65), 281 (16), 73 (15), 96 (12), 208 (14), 209 (9), 133 (4), 355 (4)
SE-30 "ultraphase"	170	11	207 (65), 281 (17), 73 (16), 96 (13), 208 (13), 209 (10), 133 (6), 355 (6)
Silar 5CP	175	12	207 (65), 73 (19), 281 (14), 503 (14), 96 (11), 208 (14), 133 (8), 355 (4)
OV-1	176	13	207 (65), 281 (17), 73 (15), 208 (14), 96 (12), 209 (11), 133 (6), 191 (6)
HI-EFF-8BP	770	210	108 (120), 109 (10), 207 (65), 67 (58), 93 (38), 79 (33), 81 (30), 55 (29)
SP1000	801	220	45 (130), 73 (130), 117 (77), 207 (65), 59 (65), 89 (36), 103 (32), 87 (30)
STAP	943	268	45 (215), 73 (125), 59 (75), 207 (65), 89 (61), 87 (50), 117 (31), 103 (29)

A quantitative estimation of the column substrate bleed was obtained in each case by measuring the sum of the MS ion abundances. This showed that the silicone membrane in the molecular separator had a measurable quantity of bleed at 240° and that Dexsil 300 GC and the three silicone columns had very little substrate bleed under these conditions. However, the polyester and modified polyethylene glycol columns were not so thermostable and considerable substrate bleed took place with these columns. In fact, the STAP column had 268 times more bleed than the Dexsil 300 GC column.

The major ion in the bleed from the silicone membrane, *m/e* 207, did not correspond to any common ion in the mass spectra of any of the sterol TMS ethers and was usually found to be a small contaminant during GC–MS runs. For example, an injection of 0.5 μg of cholesterol TMS ether produced a spectrum in which the abundance of the *m/e* 207 ion was less than 1 % of the abundance of the base peak of the spectrum.

From these results it would seem that the best of these columns to use for the analysis of marine sterol TMS ethers by the GC–MS technique would be a combination of Dexsil 300 GC, SE-30 "ultraphase" and Silar 5CP, which would have good resolution characteristics coupled with very low substrate bleed.

GC–MS analysis of the sterols present in the cockle Cerastoderma edule

For the GC–MS investigation of the TMS ethers of the cockle sterols a combination of three column systems was used, Dexsil 300 GC, SE-30 "ultraphase" and SP1000. (At the time when this investigation was in hand, Silar 5CP was not available and it was not until a later stage that the particular advantages of this phase became apparent.) The FID chromatograms on the Dexsil 300 GC column (Fig. 2) and the SP1000 column (Fig. 3) were each found to contain seven peaks, some of which contained more than one sterol. The TIM chromatograms from these columns indicated that excellent transfer efficiencies were involved with little peak widening or tailing visible when compared with the FID chromatograms. By multiple MS scanning of each TIM peak, it was possible to identify the sterols present in each peak, after careful inspection of the mass spectra. Table V summarises the results of this investigation and gives the quantitative estimation of the eleven sterols found to be present in these cockle samples.

Sterol C1. Sterol C1 was separated on both Dexsil (peak A) and SP1000 (peak A) and was identified as 22-*trans*-24-norcholesta-5,22-dien-3β-ol[32] [TMS *m/e* (%): 442 (17), 427 (6), 352 (21), 337 (10), 313 (23), 255 (22), 215 (7), 213 (6), 129 (48), 97 (100)] by comparison of GLC retention times and mass spectra with those of an authentic sample* run under identical GC–MS conditions.

Sterol C2. Sterol C2 was separated by Dexsil (peak B) but occurred as a shoulder on SP1000 (peak B). It was identified as 22-*trans*-cholesta-5,22-dien-3β-ol[20] [TMS *m/e* (%): 456 (19), 441 (6), 366 (25), 351 (23), 327 (29), 255 (29), 215 (10), 213 (6), 129 (62), 111 (100)] by comparison of GLC retention times and mass spectra with those of an authentic sample** run under identical GC–MS conditions. Although the mass

* We are indebted to Dr. D. R. Idler, Marine Sciences Research Laboratory, Nova Scotia, Canada, for this sample.

** We are indebted to Dr. L. J. Goad, Biochemistry Department, University of Liverpool, for this sample.

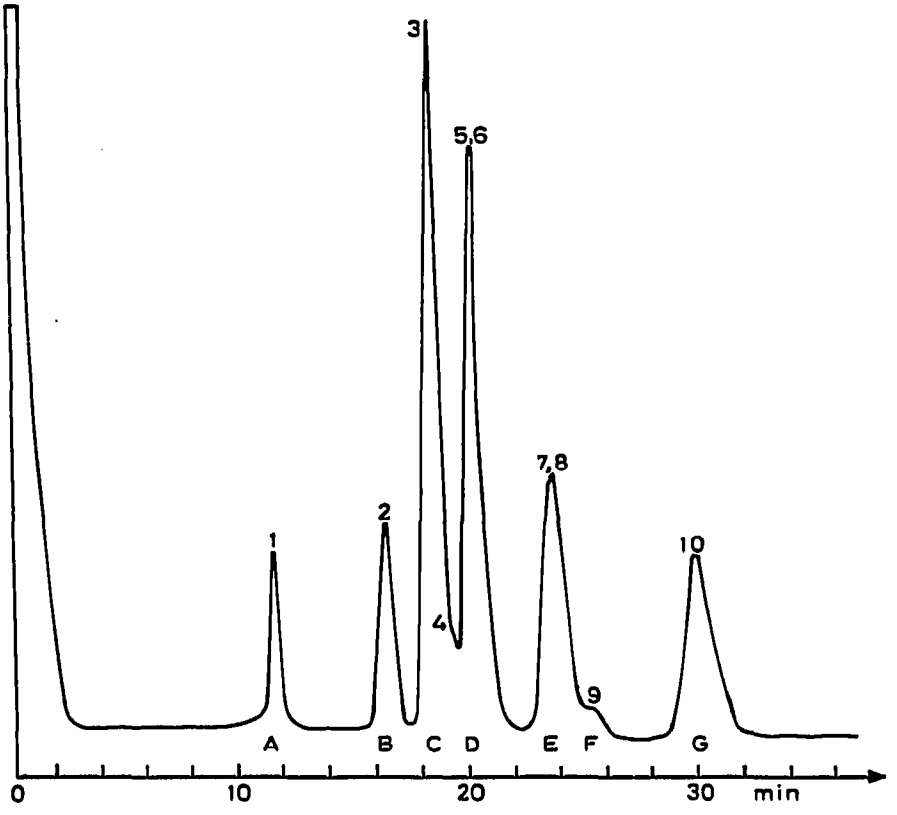

Fig. 2. FID chromatogram of cockle sterol TMS ethers on a Dexsil 300 GC column. For identification of peak numbers, see Table II.

spectra of the *cis* and *trans* isomers of this sterol are identical[20], the non-identity of sterol C2 with the *cis* isomer was established as these two isomers were easily separated on the Dexsil column.

Sterol C3. Sterol C3 was the major component of the sterol mixture. It was not completely separated from minor components on either Dexsil (peak C) or SP1000 (peak B) but was easily identified as cholesterol [TMS *m/e* (%): 458 (33), 443 (14), 368 (62), 353 (33), 329 (100), 275 (10), 255 (14), 247 (19), 213 (10), 129 (100)] by comparison of GLC retention times and the mass spectra obtained by scanning the top of peak C in the Dexsil chromatogram with those of an authentic sample* run under identical GC–MS conditions.

Sterol C4. Sterol C4 was a very minor component which formed a shoulder prior to cholesterol on SP1000 and a shoulder after cholesterol on Dexsil. Because of these characteristic retention times and by examination of the mass spectra taken at the point of each shoulder, this sterol was identified as cholestanol [TMS *m/e* (%): 460 (27), 455 (38), 370 (11), 355 (18), 306 (15), 305 (13), 262 (8), 237 (8), 230 (13), 217

* Supplied by R. N. Emmanuel Ltd., Alperton, Great Britain.

TABLE V

STEROLS OF THE COCKLE *CERASTODERMA EDULE*

Standard sterols (TMS)	*Dexsil RRT*	*SP1000 RRT*	*Cockle sterols (TMS)*	*Dexsil RRT*	*Peak (Fig. 2)*	*SP1000 RRT*	*Peak (Fig. 3)*	*Cockle sterol identity*	*Sterol in cockle (%)*
22-*trans*-24-Norcholesta-5,22-dien-3β-ol	0.63	0.67	C 1	0.63	A	0.67	A	22-*trans*-24-norcholesta-5,22-dien-3β-ol	4.2
22-*trans*-Cholesta-5,22-dien-3β-ol	0.90	0.95	C 2	0.90	B	0.95		22-*trans*-cholesta-5,22-dien-3β-ol	7
Cholesterol	1.00	1.00	C 3	1.00	C	1.00	B	cholesterol	30
Cholestanol	1.04	0.98	C 4	1.03		0.98		cholestan-3β-ol	1
Brassicasterol	1.10	1.12	C 5	1.09	D	1.11	C	24-methylcholesta-5,22-dien-3β-ol	17
Desmosterol	1.10	1.28	C 6	1.09		1.28	D	cholesta-5,24-dien-3β-ol	10
Campesterol	1.29	1.29	C 7	1.29	E	1.28		24-methylcholest-5-en-3β-ol	4
24-Methylenecholesterol	1.29	1.40	C 8	1.29		1.40	E	24-methylcholesta-5,24(28)-dien-3β-ol	11
Stigmasterol	1.36	1.36	C 9	1.37	F	1.36		24-methylcholesta-5,22-dien-3β-ol	1.5
β-Sitosterol	1.60	1.57	C10	1.61	G	1.57	F	24-ethylcholest-5-en-3β-ol	13.5
28-Isofucosterol	1.69	1.82	C11	1.67		1.83	G	24-ethylcholesta-5,24(28)Z-dien-3β-ol	2.4

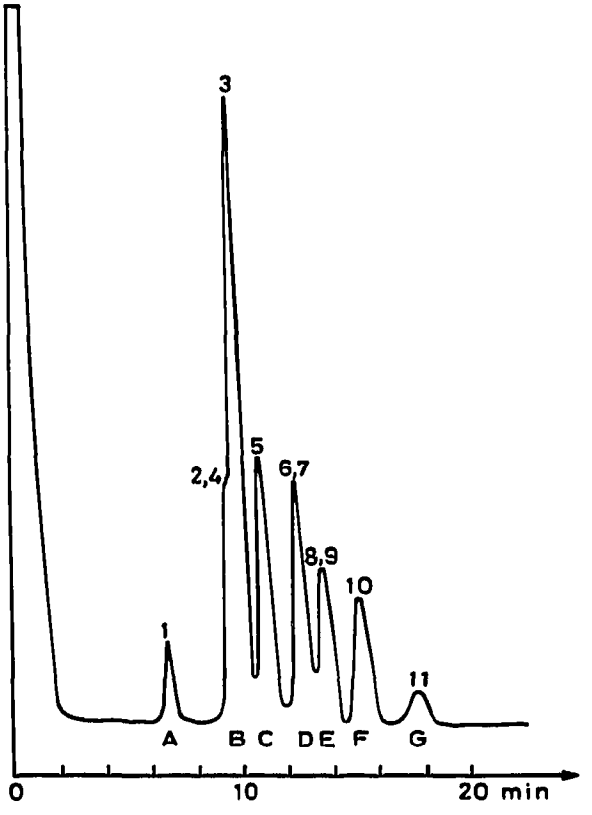

Fig. 3. FID chromatogram of cockle sterol TMS ethers on an SP1000 column. For identification of peak numbers, see Table II.

(22), 216 (28), 215 (45), 75 (100)] by comparison with an authentic sample* run under identical GC–MS conditions.

Sterol C5. Sterol C5 was separated by SP1000 (peak C) but co-occurred with sterol C6 in peak D in the Dexsil chromatogram. It was identified as 24 (R or S)-methylcholesta-5,22-dien-3β-ol [TMS *m/e* (%): 470 (35), 380 (39), 365 (13), 341 (28), 340 (11), 337 (11), 255 (50), 215 (13), 213 (13), 129 (95), 125 (100)] by comparison of GLC retention times and mass spectra with those of an authentic sample** run under identical GC–MS conditions. However, neither GLC nor MS techniques can distinguish between the C_{24} epimers of this sterol and this compound could be either the 24R isomer (brassicasterol) or the 24S isomer (crinosterol).

Sterol C6. Sterol C6 could not be separated from other sterols on either GLC system as in the Dexsil chromatogram it was mixed with sterol C5 in peak D and in the SP1000 chromatogram it was mixed with sterol C7 in peak D. By examination of these several mass spectra obtained by multiple scans of peak D in each TIM chro-

* Supplied by Applied Science Labs., State College, Pa., U.S.A.
** We are indebted to Dr. L. J. Goad for this sample.

matogram, the composite spectrum of sterol C6 could be determined by difference. Also the retention times of sterol C6 were established in each GLC system and in this way it was identified as desmosterol [TMS *m/e* (%): 456 (24), 441 (16), 366 (31), 351 (20), 343 (23), 327 (40), 255 (8), 253 (11), 245 (11), 217 (9), 213 (8), 129 (100)] when compared with an authentic sample* run under identical GC–MS conditions. In particular the prominent MS ions at *m/e* 456 (M), 366 (M — 90) and 253 (M — 90 — side chain — 2H) served to distinguish[16] desmosterol from its near neighbours.

Sterol C7. Sterol C7 could not be separated from other sterols on either GLC system as in the Dexsil chromatogram it was mixed with sterol C8 in peak E and in the SP1000 chromatogram it was mixed with sterol C6 in peak D. By multiple MS scanning of each of these TIM peaks and by comparison of GLC retention times with those of an authentic sample of campesterol* run under identical GC–MS conditions, sterol C7 was identified as 24 (R or S)-methylcholest-5-en-3β-ol [TMS *m/e* (%): 472 (28), 457 (12), 382 (54), 367 (28), 343 (83), 261 (8), 255 (12), 213 (8), 129 (100)]. In particular the prominent MS ions at *m/e* 472 (M), 382 (M — 90), and 367 (M — 105) served to distinguish it from its near neighbours.

Sterol C8. Sterol C8 could not be separated from other sterols on either GLC system as in the Dexsil chromatogram it was mixed with sterol C7 in peak E and in the SP1000 chromatogram it was mixed with sterol C9 in peak E. By multiple MS scanning of each of these TIM peaks and comparison with standard spectra[20], sterol C8 was identified as 24-methylcholesta-5,24(28)-dien-3β-ol [TMS *m/e* (%): 470 (9), 455 (8), 386 (20), 380 (19), 365 (10), 343 (10), 341 (25), 296 (13), 257 (16), 255 (6), 253 (7), 213 (8), 129 (100)]. In particular the prominent ions at *m/e* 470 (M), 386 (M — 84), 380 (M — 90), 341 (M — 129) and 296 (M — 90 — 84) served to distinguish it from its near neighbours and to identify this sterol[22,33]. Sterol C8 could be isolated in a pure form by AgPLC of cockle sterol acetates in an identical manner to that described by Idler[12] for the isolation of 24-methylcholesta-5,24(29)-dien-3β-ol from the scallop.

Sterol C9. Sterol C9 co-occurred with sterol C8 in peak E of the SP1000 chromatogram but was separated in the Dexsil chromatogram in peak F. It was identified as 24(R or S)-ethylcholesta-5,22-dien-3β-ol [TMS *m/e* (%): 484 (22), 469 (5), 394 (24), 379 (6), 355 (10), 351 (12), 255 (29), 213 (8), 139 (18), 129 (50), 83 (100)] by comparison of GLC retention times and mass spectra with those of an authentic sample of stigmasterol** run under identical GC–MS conditions.

Sterol C10. Sterol C10 co-occurred with sterol C11 in peak G of the Dexsil chromatogram but was separated in the SP1000 chromatogram in peak F. It was identified as 24(R or S)-ethylcholest-5-en-3β-ol [TMS *m/e* (%): 486 (36), 471 (9), 396 (50), 381 (23), 357 (71), 303 (6), 275 (9), 255 (11), 213 (9), 129 (100)] by comparison of GLC retention times and mass spectra with an authentic sample of β-sitosterol** run under identical GC–MS conditions.

Sterol C11. Sterol C11 co-occurred with sterol C10 as a shoulder on peak G of the Dexsil chromatogram but was separated in the SP1000 chromatogram in peak G. It was identified as 24-ethylcholesta-5,24(28)Z-dien-3β-ol [TMS *m/e* (%): 484 (8), 386 (84), 371 (11), 355 (10), 296 (65), 281 (45), 257 (35), 255 (11), 253 (10), 213 (13),

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** Supplied by R. N. Emmanuel Ltd.

211 (13), 129 (100), 73 (90)] by comparison of GLC retention times and mass spectra with those of an authentic sample of 28-isofucosterol* run under identical GC–MS conditions. In particular the prominent ion at *m/e* 386 (M — 98) was characteristic[34] of a 24-ethylidene sterol. The non identity of sterol C11 with the isomeric fucosterol was easily established as sterol C11 was separated from an authentic sample of fucosterol* on the SP1000 column.

The relative amounts of each of the eleven cockle sterols were calculated from the GLC traces and are included in Table V. The predominance of cholesterol and 24-methylenecholesterol is a common feature amongst other members of the pelecypod family of molluscs[23]; however, the level of 24-methylenecholesterol which was found to be present in our cockle sample was much lower than that observed by Fagerlund and Idler in the earlier study of the Canadian cockle[24]. It was also lower than the levels of 24-methylenecholesterol reported in scallops and oysters[22,23,35] but is similar to the levels found previously in some species of clam[22]. There is some evidence that the level of 24-methylenecholesterol in the cockle may be affected by seasonal changes since later batches were found to have considerably higher proportions of this sterol.

The quantities of desmosterol and 24-ethylcholest-5-en-3β-ol in the cockle were found to be unusually high when compared to other pelecypods, indeed clams, scallops and oysters were reported to have no desmosterol at all[35] and only very low levels of β-sitosterol have been observed previously[22,23].

CONCLUSIONS

The determination of marine sterols in the cockle by GC–MS analysis of the TMS ethers has proven to be a very sensitive, rapid and effective technique. It is probable that the use of Silar 5CP in conjunction with Dexsil 300 GC and SE-30 "ultraphase" would have made the separations more effective, and the identification of the subsequent mass spectra more easy than with the SP1000 phase, due to a much reduced level of substrate bleed, making this procedure recommendable. The one disadvantage of this very sensitive GC–MS technique is its inability to establish the configuration at C_{24} in the methyl- and ethyl-substituted compounds. At the present time it would require sufficient quantities of the sterols to be separated by either preparative GLC or AgPLC for the configuration at C_{24} to be established by optical rotary dispersion, nuclear magnetic resonance[36] or by comparison of derivative melting points. In many cases such large quantities of marine organisms are simply not available.

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REFERENCES

1 D. R. Idler, L. M. Safe and E. F. MacDonald, *Steroids*, 18 (1971) 545.
2 A. Alcaide, J. Viala, F. Pinte, M. Itoh, T. Nomura and M. Barbier, *C.R. Acad. Sci., Ser. C*, 273 (1971) 1386.
3 P. A. Voogt, *Arch. Int. Physiol. Biochim.*, 81 (1973) 401.
4 T. R. Edrman and R. H. Thomson, *Tetrahedron*, 28 (1972) 5163.
5 S. Yasuda, *Comp. Biochem. Physiol.*, 48B (1973) 225.
6 S. Teshima, A. Kanazawa and T. Ando, *Comp. Biochem. Physiol.*, 41B (1972) 121.
7 A. G. Smith, I. Rubinstein and L. J. Goad, *Biochem. J.*, 135 (1973) 443.
8 H. E. Nordby and S. Nagy, *J. Chromatogr.*, 75 (1973) 187.
9 G. W. Patterson, *Anal. Chem.*, 43 (1971) 1165.
10 R. B. Clayton, *Biochemistry*, 1 (1962) 357.
11 H. E. Vroman and C. F. Cohen, *J. Lipid Res.*, 8 (1967) 150.
12 D. R. Idler and L. M. Safe, *Steroids*, 19 (1972) 315.
13 D. R. Idler, L. M. Safe and S. H. Safe, *Steroids*, 16 (1970) 251.
14 E. M. Chambaz and E. C. Horning, *Anal. Biochem.*, 30 (1969) 7.
15 B. A. Knights, *J. Gas Chromatogr.*, 5 (1967) 273.
16 C. J. W. Brooks, E. C. Horning and J. S. Young, *Lipids*, 3 (1968) 391.
17 C. J. W. Brooks, W. Henderson and G. Steel, *Biochim. Biophys. Acta*, 296 (1973) 431.
18 W. McFadden, *Techniques of Combined Gas Chromatography/Mass Spectrometry*, Wiley, New York, 1973, Ch. 3.
19 D. R. Idler and P. Wiseman, *Int. J. Biochem.*, 2 (1971) 91.
20 D. R. Idler and P. Wiseman, *Comp. Biochem. Physiol.*, 38A (1971) 581.
21 J. Austin, *Advan. Steroid Biochem. Pharmacol.*, 1 (1970) 73.
22 D. R. Idler and P. Wiseman, *Int. J. Biochem.*, 2 (1971) 516.
23 D. R. Idler and P. Wiseman, *J. Fish Res. Board Can.*, 29 (1972) 385.
24 U. H. M. Fagerlund and D. R. Idler, *J. Org. Chem.*, 21 (1956) 372.
25 P. M. Llewellyn and D. P. Littlejohn, *Pittsburgh Conf. Anal. Chem. Appl. Spectrosc., February 1966.*
26 C. P. Freeman and D. West, *J. Lipid Res.*, 7 (1966) 324.
27 W. O. McReynolds, *J. Chromatogr. Sci.*, 8 (1970) 685.
28 W. H. Elliot, L. B. Walsh, M. M. Mui, M. A. Thorne and C. M. Siegfried, *J. Chromatogr.*, 44 (1969) 452.
29 R. G. Ackman and S. N. Hooper, *J. Chromatogr. Sci.*, 12 (1974) 131.
30 N. Ikekawa, R. Walanuki, K. Tsuda and K. Sakai, *Anal. Chem.*, 40 (1968) 1139.
31 J. H. Purnell, *Gas Chromatography*, Wiley, New York, 1962, p. 115.
32 D. R. Idler, P. M. Wiseman and L. M. Safe, *Steroids*, 16 (1970) 451.
33 S. G. Wyllie and C. Djerassi, *J. Org. Chem.*, 33 (1968) 305.
34 C. J. W. Brooks, B. A. Knights, W. Sucrow and B. Raduchel, *Steroids*, 20 (1972) 487.
35 D. Kritchevsky, S. A. Tepper, N. W. DiTullo and W. L. Holmes, *J. Food Sci.*, 32 (1967) 64.
36 I. Rubinstein and L. J. Goad, *Phytochemistry*, 13 (1974) 485.