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MARINE STEROLS

XIII. EVALUATION OF GLASS SUPPORT-COATED OPEN TUBULAR COLUMNS, PREPARED BY THE MERCURY-PLUG DYNAMIC TECHNIQUE, FOR MARINE STEROL ANALYSIS

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SUMMARY

Modifications of the mercury plug double dynamic coating procedure are described for the manufacture of high-phase-ratio support-coated open tubular (SCOT) columns with Silanox 101 as the solid support for non-polar stationary phases, such as Dexsil 300 GC and SE-30 Ultraphase, and Hypersil as a support for the preparation of polar POLY-S 176 SCOT columns.

These columns have been used in conjunction with a falling-needle injection system, for an improved analysis of complex sterol mixtures from marine sources.

INTRODUCTION

Sterols found in the marine environment are usually complex mixtures, containing constituents which are unknown in terrestrial plants and animals. Recent reviews by Goad[1,2], Morris and Culkin[3], and Schmitz[4] have highlighted the wide range of unusual sterols which have been identified, and the recent work of Djerassi and co-workers[5,6] has indicated the large number of sterols which can be found in a single marine invertebrate specimen.

As part of our investigations into the complex mixtures of sterols from marine invertebrate samples, we have established the value of packed-column gas chromatographic–mass spectrometric (GC–MS) techniques for the identification of marine sterols[7]. However the technique has been limited by the lack of resolution of the gas–liquid chromatographic (GLC) packed columns for this very complex mixture of homologues, double-bond isomers and position isomers, all of which are based upon an identical carbon skeleton.

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Although these limitations have been known for some time, relatively few research groups[8–14] have utilised the high resolution of open tubular GC techniques for sterol analysis. This report is concerned with the laboratory preparation and evaluation of thermostable support-coated open tubular (SCOT) columns, manufactured by a double dynamic mercury plug coating technique in which modifications of the methods developed by Schomburg and co-workers[15,16], were used, and their evaluation for marine sterol analysis.

The thermostable stationary phases, Dexsil 300 GC, SE-30 Ultraphase and POLY-S 176, which were selected for this investigation were those that had been used previously with success in this laboratory for the analysis of marine sterol mixtures for packed-column GLC.

The polycarborane siloxane phase, Dexsil 300 GC, is one of the most thermostable GC stationary phases known. It has a low polarity and has proved to be particularly useful in packed columns for the separation of Δ^5-sterol–5α-stanol pairs[7,17] in marine sterol mixtures. Although several groups have reported the use of Dexsil as a stationary phase in wall-coated open tubular (WCOT) columns[15,18–20], it has not been commonly used in SCOT columns.

The methyl silicone gum phase, SE-30, was one of the most popular stationary phases for the preparation of early open tubular columns because of the superior wetting characteristics of this gum phase[21]. It has been used previously in conjunction with Silanox 101 by German and co-workers[22,23] to produce efficient SCOT columns with higher stationary phase loadings (lower phase ratio).

The polyphenylether sulphone phase, POLY-S 176 (formerly PZ-176), has high thermal stability and was developed by Mathews and co-workers[24,25]. It had proved in our previous work to be particularly useful in the separation of geometrical isomers of marine sterols in packed columns[26].

EXPERIMENTAL

Laboratory preparation of glass SCOT columns

Coating Procedure. A coating reservoir [Scientific Glass Engineering (SGE) London, Great Britain] was modified as in Fig. 1 to facilitate the introduction of a mercury plug by the incorporation of a sliding seal at the top of the reservoir in place of the septum seal supplied. Approximately 5 cm of PTFE tape was wrapped around the SGE bolt and the stainless-steel delivery tube. When the connecting nut was partially tightened, a position could be found where, although the seal was gas-tight, the stainless-steel delivery tube could easily be slipped up and down. After removal of the delivery tube, approximately 10% of the reservoir was filled with clean mercury followed by the coating solution (or suspension *ca.* 2 cm^3).

The Pyrex open tubular column was placed with the coils flat on a bench and spread out so that all of the coils could be observed from above. The column entrance was manoeuvred to a suitable position by careful bending of the end with a microburner, and connected via a small piece of glass tubing to the stainless steel delivery tube with heat-shrink PTFE linkages. The column exit was connected with heat-shrink PTFE to a dummy column of about fifteen coils of similar dimensions so as to prevent an increase in plug velocity when the plug left the column proper.

The side arm of the reservoir was connected via a needle valve to a regulated

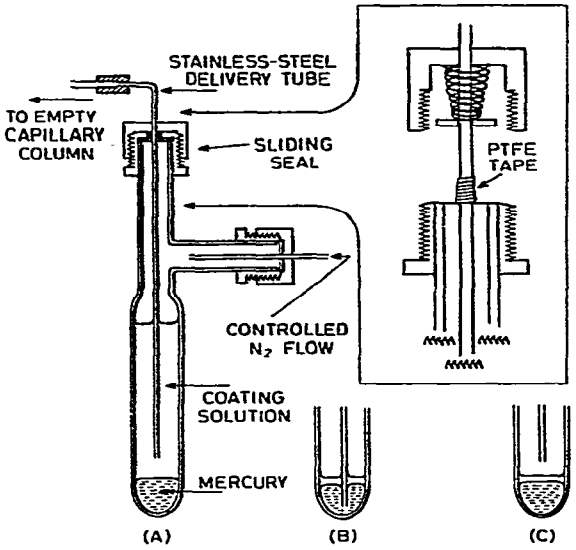

Fig. 1. Coating reservoir and sliding seal.

30 p.s.i. nitrogen supply, and the needle valve was opened so as to purge the gas line and reservoir head space with inert gas. After closing the needle valve, the delivery tube was pushed, via the sliding seal, into the coating solution as shown in Fig. 1A and the needle valve was opened slightly so that the coating solution passed into the capillary. After a short period of time to allow equilibrium to be achieved, the velocity of the plug was measured by timing the front of the coating plug as it passes through several coils of the tube, and adjustments were made with the needle valve so as to maintain the desired flow-rate. Just before the level of the coating solution (or suspension) fell below the entrance of the delivery tube, use was made of the sliding seal to push the tube into the mercury layer as in Fig. 1B. A mercury plug entered the capillary, and after 3–5 sec, the tube was withdrawn quickly above the coating solution as in Fig. 1C.

In this way, a mercury plug of 10–30 cm was forced into the capillary, without a gas pocket between the coating solution and the mercury and without any coating solution following the mercury plug. The velocity of the coating plug was measured several times during the coating step and was found to decrease only very slightly as coating continued. As soon as the coating solution and mercury plug had entered the dummy column, the needle valve was opened fully, and the solvent was allowed to evaporate from the column for several hours.

When the column was dry, the PTFE connection to the coating reservoir was severed carefully with a razor blade, held parallel to the coils, and if a second coating step was required, the reservoir was cleaned and refilled, and the procedure was repeated.

Preparation of a Dexsil 300 GC–Silanox 101 SCOT column. A Pyrex glass open tubular column (Phase Separations, Queensferry, Great Britain (57 m × 0.4 mm I.D.) was silanised prior to coating, according to the method described by German *et al.*[22] and attached to the coating reservoir. A suspension of 0.20 g Silanox 101 (Cabot Cor-

poration, Boston, MA, U.S.A.) and 0.05 g Dexsil 300 GC in 10 cm^3 tetrachloromethane was mixed for 3 h in an ultrasonic bath, before a 2.0-cm^3 plug of this suspension was forced through the column under nitrogen pressure in front of a 30-cm mercury column at a plug velocity of 3 cm sec^{-1}.

The remaining solvent was removed by nitrogen flow for 3 h and the mercury-plug coating technique was repeated with a 2-cm^3 plug of Dexsil 300 GC in isooctane (0.4 g in 10 cm^3) at a plug velocity of 3 cm sec^{-1}, removing the residual solvent after the coating step. The column was installed in the chromatograph oven and conditioned for 15 h at 250°C before being silanised by several injections of bis (trimethylsilyl)acetamide (BSA) at 250°C.

Preparation of an SE-30 Ultraphase–Silanox 101 SCOT column. A suspension of 0.05 g SE-30 Ultraphase (Phase Separations) and 0.2 g Silanox 101 in 10 cm^3 tetrachloromethane was sonicated for 3 h and pushed through a silanised Pyrex column (57 m × 0.4 mm I.D.) behind a 15-cm mercury plug at a plug velocity of 2.7 cm sec^{-1}. As soon as the coating plug had left the column, the nitrogen pressure was increased and the solvent vapour was removed for 30 min. A second coating plug of SE-30 Ultraphase in isooctane (0.2 g in 10 cm^3), prepared by mechanical stirring overnight at 20°C, was pushed through the column behind a mercury plug at a velocity of 3.3 cm sec^{-1}. Since this coating plug was rather viscous, the nitrogen pressure had to be increased during the second coating step to maintain a constant plug velocity. The column was then conditioned and silanised as previously described.

A commercial WCOT column with SE-30, coated on an etched inside surface (25 m × 0.35 mm I.D., LKB Instruments, Stockholm, Sweden) was used for comparison with the SCOT columns coated by the dynamic mercury plug methods.

Preparation of POLY-S 176 SCOT columns with Silanox 101 and Hypersil as substrates

(a) POLY-S 176–Silanox 101 column. An unsilanised Pyrex capillary (54 m × 0.4 mm I.D.) was coated with a suspension of 0.05 g POLY-S 176 (Applied Science Labs. and 0.25 g Silanox 101 in 10% (v/v) tetrahydrofuran in tetrachloromethane (11 cm^3). A plug of approximately 2 cm^3 of this suspension was forced through the column by 25 cm of mercury at a velocity of 3 cm sec^{-1} during the first coating step. After evaporation of the solvents the procedure was repeated with a coating solution of POLY-S 176 in tetrahydrofuran (10%, v/v). The remaining solvent was removed and the column conditioned overnight at 290°C.

(b) POLY-S 176–Silanox 101-Carbowax 20M column. Carbowax 20M (0.21 g) was dissolved in 35 cm^3 warm methanol. Silanox 101 (0.49 g) was added and the resultant suspension was shaken vigorously before removal of the methanol by rotary evaporation at 45°C. Tetrachloromethane (50 cm^3) was added and the resultant suspension sonicated for 1 h. An un-silanised Pyrex capillary (30 m × 0.4 mm I.D.) was coated by the mercury-plug dynamic technique with 2 cm^3 of the above suspension at a plug velocity of 3 cm sec^{-1}. After removal of residual solvent with nitrogen, the column ends were sealed with a microburner, and the column was placed in a GC oven at 280°C for 5 h. The column ends were opened and the column was subjected to two further coating steps with the Carbowax 20M–Silanox suspension at plug velocities of 15–20 cm sec^{-1}. Following the removal of the solvent with nitrogen, the column was connected in the GC oven, and excess Carbowax 20M was removed by flushing the column with nitrogen carrier gas at 280°C for 14 h. Finally, the column

was coated with a solution of 0.04 g POLY-S 176 in 2 cm^3 dichloromethane with a mercury plug velocity of 3 cm sec^{-1}. After evaporation of residual solvent by nitrogen, the column was conditioned overnight at 280°C.

(c) POLY-S 176–Hypersil (BTPPC) column. A solution of 0.05 g benzyltriphenylphosphonium chloride (BTPPC) (Aldrich, Milwaukee, WI, U.S.A.) in 5 cm^3 dichloromethane was carefully added to 0.34 g Hypersil [5.5 μm microparticulate silica for high-performance liquid chromatography (HPLC); Shandon, London, Great Britain] in a centrifuge tube by carefully running the solution down the walls. The resultant suspension was shaken to ensure complete mixing and left to stand for 10 min. The suspension was then centrifuged for 10 min to 2000 rpm, after which time the supernatant BTPPC solution was discarded. The remaining solid was washed twice with 4 cm^3 dichloromethane, and the solvent was removed by centrifugation. The BTPPC-treated Hypersil was suspended in 10 cm^3 dichloromethane and added to a solution of 0.3 g POLY-S 176 in 40 cm^3 dichloromethane before sonication for 1 h.

A 2-cm^3 plug of this suspension was introduced into an unsilanised Pyrex capillary (30.5 m × 0.4 mm I.D.) behind a wetting plug of tetrachloromethane (120 cm) and propelled through the column at a velocity of 2.5 cm sec^{-1} by a 15-cm mercury plug. The excess solvent was removed by nitrogen flow and in a second coating step, a 0.25-g plug of POLY-S 176 in 2 cm^3 dichloromethane was pushed through at a velocity of 2.5 cm sec^{-1} in front of a 10-cm mercury plug. The solvent was removed with nitrogen, and the column was conditioned at 270°C overnight.

Installation of the SCOT columns into the gas chromatograph oven

The columns were suspended in the oven of a Pye 104 gas chromatograph from a horizontal nickel spatula, one end of which was bent into a hook and fixed to the detector supporting bridge so as to give a horizontal support. The inlet and exit ends of the column were bent into shape and connected with heat-shrink PTFE to more permanent glass capillary connecting pieces which had been inserted into both the injector and detector. The detector connection was modified with a drilled-out T piece to accommodate make-up gas and was fitted with a glass insert, to which the column exit was attached.

Two different types of injector systems were tested with the SCOT columns; a conventional side-arm variable-split injector in glass-lined stainless steel and an inexpensive home-made all-glass falling-needle injector for the solventless injection of solids.

The variable-split injector had Pye fittings which were drilled out to accept a glass insert, connected by polyimide ferrules, and the SCOT columns were attached to this glass insert by heat-shrink PTFE links.

The falling-needle injector was similar in principle to that described by Van den Berg and Cox[27] and was fabricated in glass from two Pasteur pipettes and two commercial glass connectors (Scientific Glass Engineering) and odd lengths of glass tubing of suitable dimensions as shown in Fig. 2.

The falling needle (1) which was sealed at the fine end, was manipulated via a cylindrical magnet (8) acting upon a soft-iron core (9), which was glued into the top end of the pipette with Araldite. The falling needle was of suitable dimensions to allow free movement up and down the main injection chamber (7) and venting of the carrier gas to the atmosphere via the drawn-out Pasteur pipette leak (2). This was attached to

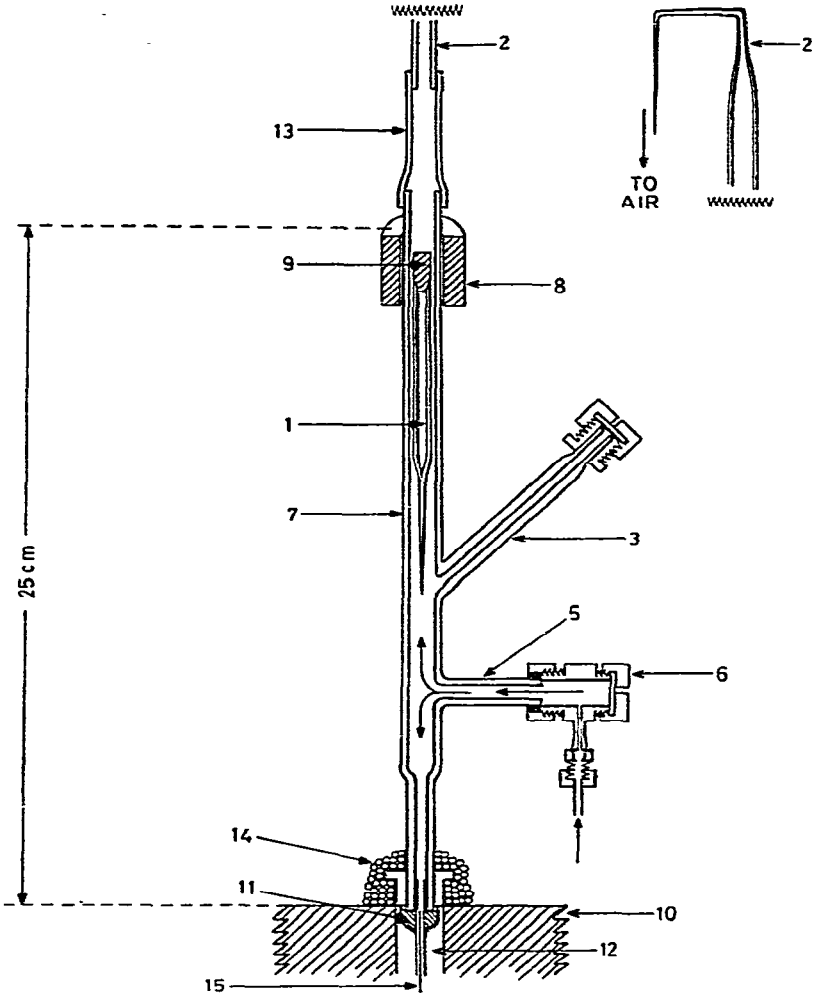

Fig. 2. Detailed drawing of falling-needle injector. 1 = Pasteur pipette falling needle; 2 = Pasteur pipette variable jet; 3 = SGE connector-septum injector; 4 = SGE connector-heated vapourisation zone; 5 = side-arm; 6 = Pye 104 injector heater block; 7 = main injector chamber; 8 = ring magnet; 9 = soft-iron core; 10 = Pye 104 injector heater block; 11 = Araldite connection; 12 = melting-point capillary down-tube; 13 = rubber tubing; 14 = asbestos wool insulation; 15 = SCOT column.

the main injection chamber by a short length of thick-walled rubber tubing (13), which was found to hold in position at gas pressures up to 50 p.s.i. but was easily removed in order to replace the venting pipette, or to gain access to the falling-needle for cleaning or replacement purposes.

A Pyrex melting-point capillary (12) was inserted into the SGE connector (4) and glued into place with Araldite to make as gas-tight seal. The straightened end of the SCOT column was attached within this down-tube by heat-shrink PTFE tubing.

In operation, the glass needle was raised as shown in Fig. 2, the magnet held in position by an elastic band, and the sample solution was applied to the tip of the glass

needle through the septum seal (3), using a long-needle 10-ml syringe. The solution volumes which could be deposited on to the needle in one operation varied from 0.2–2 μl, and the solvent was evaporated by the passage of carrier gas through the venting capillary. It was possible to repeat injections onto the glass needle when very dilute solutions were encountered. The helium carrier gas-split ratio between the column and the vent could easily be varied by drawing out the Pasteur pipette to suitable dimensions. In general, for volatile solvents, approximately 30–60 sec were sufficient for solvent removal, but with the more involatile BSA–pyridine solutions, which were commonly used in this work, it was sometimes necessary to vent for several minutes. Injection was carried out by removing the elastic band and carefully but quickly lowering the magnet until the glass needle penetrated slightly beyond the Araldite seal into the heated zone (290–300°C). The needle was left in position for 2–3 sec and quickly returned to its original position. Tests indicated that these injection conditions removed all of the sample. On repeated use, the tip of the glass needle tended to blacken slightly, but this was easily overcome by removal of the falling needle, followed by cleaning with a tissue, soaked in ether.

The falling needle required replacement just once during one year's almost continual operation when it was broken during cleaning of the needle tip. The Araldite seal in the heated injector zone was renewed on about three occasions during this period, but this was a straightforward operation, involving removal of the brittle pyrolysed resin and replacing the melting-point tube and capillary insert.

Chromatographic conditions

All of the chromatograms were obtained under the following standard conditions: column temperature 250°C isothermal; injection port temperature 290–300°C; detector temperature 280°C; helium carrier gas flow-rate 4 $cm^3\ min^{-1}$; nitrogen make-up gas flow-rate 15 $cm^3\ min^{-1}$.

RESULTS

Separation of marine sterols trimethylsilyl (TMS) ethers on Dexsil 300 GC–Silanox 101 SCOT columns

Cerastoderma edule. The GC profile for the sterol TMS ethers obtained after extraction of the cockle, *Cerastoderma edule*, on the 57-m SCOT column is shown in Fig. 3a. This chromatogram is compared with the result obtained for a 1% Dexsil 300 GC packed column on the same sample (Fig. 3b)[28]. An analysis of the sterol content of the sample with the Dexsil 300 GC SCOT capillary column in terms of Kováts[29] retention index values, obtained by the co-injection of *n*-C_{28} and *n*-C_{36}, is given in Table I.

Ascidia mentula. The GC profile for the sterol TMS ethers obtained from extracts of the coastal tunicate, *Ascidia mentula*[17], on the 57-m Dexsil 300 GC SCOT column is shown in Fig. 4a and is compared with the performance of a packed column of 1% Dexsil 300 GC for the same sample (Fig. 4b). Kováts retention index values for the sterol TMS ethers are given in Table II.

The column efficiency was determined with respect to theoretical plate numbers for cholesterol TMS ether, measured at 92,000 (retention time 11.9 min, and capacity factor 2.19) or 1614 plates m^{-1}.

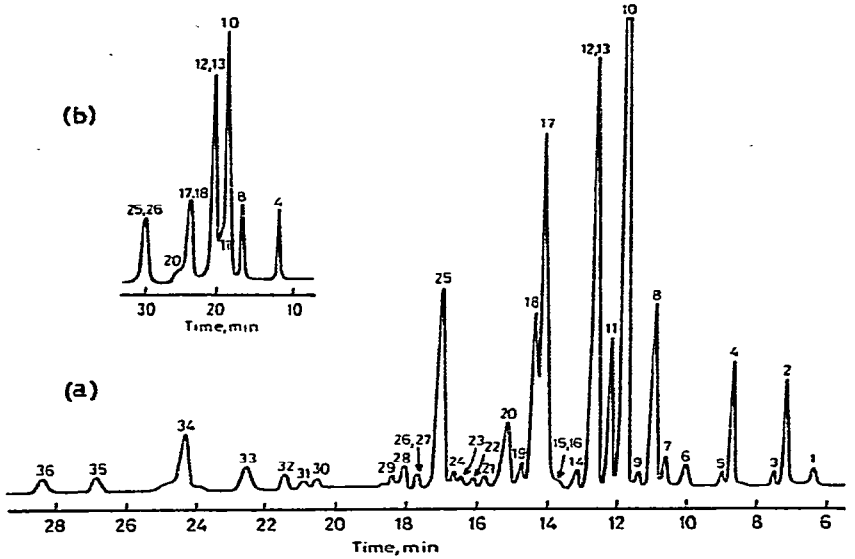

Fig. 3. (a) Chromatogram of cockle sterol TMS ethers on Dexsil 300 GC–Silanox 101 SCOT column. (b) Identical sample on a Dexsil 300 GC packed column (2.5 m × 4 mm I.D.).

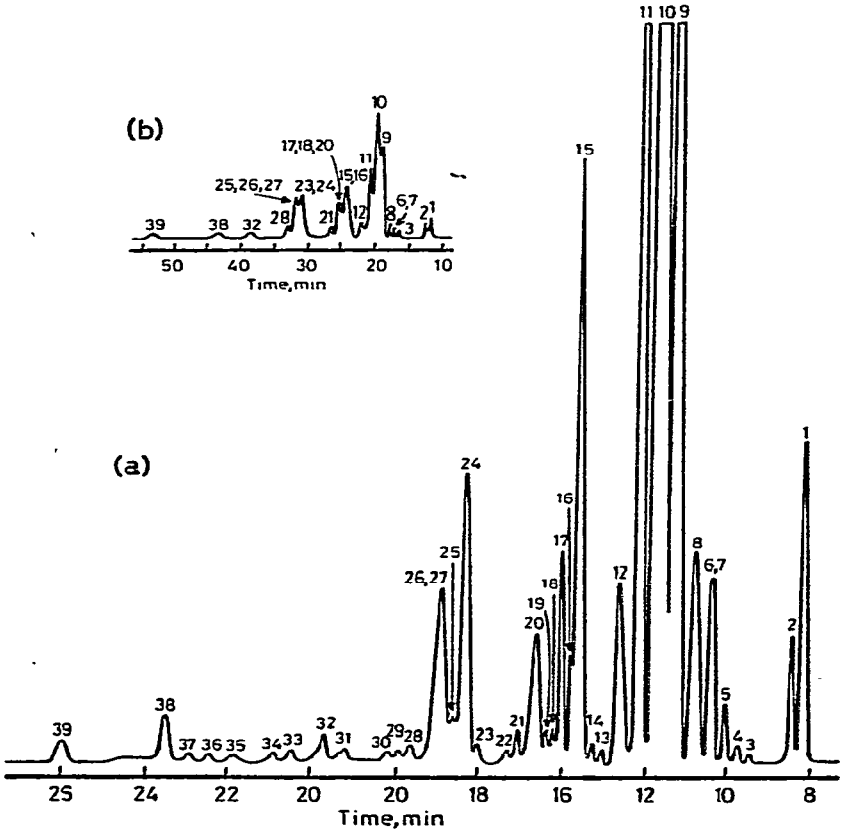

Fig. 4. (a) Chromatogram of tunicate sterol TMS ethers on Dexsil 300 GC–Silanox 101 SCOT column. (b) Identical sample on a Dexsil 300 GC packed column (2.5 m × 4 mm I.D.).

TABLE I

PERFORMANCE OF COCKLE STEROL TMS DERIVATIVES ON DEXSIL SCOT COLUMN

— Not identified.

Cockle sterols (Fig. 3)	*Sterol identity**	*Kováts retention index[29], I_{250}*
C-1	—	2707
C-2	—	2801
C-3	—	2826
C-4	26C 5, 22*E*	2944
C-5	26C 22*E***	2963
C-6	26C**	3041
C-7	Occelasterol	3078
C-8	27C 5, 22*E*	3096
C-9	27C 22*E***	3117
C-10	27C 5	3142
C-11	27C	3160
C-12	27C 5. 24(25)	3181
C-13	28C 5, 22*E*	3181
C-14	28C 22*E***	3200
C-15	—	3230
C-16	—	3235
C-17	28C 5, 24(28)	3243
C-18	28C 5	3251
C-19	28C 24(28)**	3263
C-20	29C 5, 22*E*	3280
C-21	29C 22*E***	3300
C-22	—	3315
C-23	28C 7**	3324
C-24	29C 5, 24(28) *E***	3331
C-25	29C 5	3341
C-26	29C 5, 24(28) *Z*	3358
C-27	29C**	3358
C-28	29C 24(28) *Z***	3374
C-29	—	3380
C-30	27C 5(24 oxo)**	3435
C-31	—	3443
C-32	—	3457
C-33	30C**	3481
C-34	31C?	3516
C-35	32C?	3563
C-36	32C?	3589

* The shorthand notation for the sterols refers to the number of carbon atoms followed by C, followed by the position of any double bonds and an indication of their geometrical isomerism. All of the compounds are 3β-sterols and the additional carbon atoms are attached to C-24. Note that it is not possible to distinguish between C-24 epimers by GC[25]; *e.g.*, 27C is 5α-cholestan-3β-ol; 28C 5, 22 *E* is (22*E*, 24ξ)-24-methylcholesta-5,22-dien-3β-ol, *i.e.*, either brassicasterol (24*R*) or crinosterol (24*S*); and 29C 5, 24(28) *Z* is (24*Z*)-24-ethylcholesta-5, 24(28)-dien-3β-ol, *i.e.*, 28-isofucosterol.

** Tentative identification only.

Separation of marine sterols TMS ethers on SE-30–Silanox 101 SCOT columns

Synops sp. The GC profile of the sterol TMS ethers obtained from the extraction of the oceanic siliceous sponge, *Synops* sp.[30], on the 57-m SCOT column is shown in Fig. 5a) and compared with the result from an 1% SE-30 packed column (Fig. 5b). Kováts retention index values are given in Table III.

TABLE II

PERFORMANCE OF TUNICATE STEROL TMS DERIVATIVES ON DEXSIL SCOT COLUMN

— Not identified.

Tunicate sterols (Fig. 4)	*Sterol identity**	*Kováts retention index[29], $I_{250°}$*
AM-1	26C 5,22*E*	2944
AM-2	26C 22*E*	2964
AM-3	26C**	3040
AM-4	—	3060
AM-5	Occelasterol	3077
AM-6	Patinosterol	3094
AM-7	27C 5,22*E*	3094
AM-8	27C 22*E*	3113
AM-9	27C 5	3141
AM-10	27C	3159
AM-11	28C 5,22*E*	3181
AM-12	28C 22*E*	3200
AM-13	27C 7	3206
AM-14	—	3220
AM-15	28C 5,24(28)	3242
AM-16	28C 5	3250
AM-17	28C 24(28)	3260
AM-18	28C	3269
AM-19	—	3275
AM-20	29C 5,22*E*	3281
AM-21	—	3288
AM-22	29C 22*E*	3300
AM-23	29C 5,24(28)*E*	3331
AM-24	29C 5	3341
AM-25	29C 24(28)*E*	3350
AM-26	29C	3357
AM-27	29C 5,24(28)*Z*	3357
AM-28	29C 24(28)*Z*	3378
AM-29	—	3386
AM-30	—	3393
AM-31	—	3423
AM-32	27C 5,(24 oxo)	3435
AM-33	—	3454
AM-34	30C 5**	3462
AM-35	30C**	3484
AM-36	—	3497
AM-37	—	3507
AM-38	31C?	3520
AM-39	32C?	3566

* See Table I.

** Tentative identification only.

Periphylla periphylla. The GC profile of the sterol TMS ethers obtained by extraction of the oceanic jellyfish, *Periphylla periphylla*[7], on the 57-m SCOT column is shown in Fig. 6a and compared with the results from a Dexsil packed column (Fig. 6b). Kováts retention index values are given in Table IV.

The column efficiency was determined with respect to theoretical plate num-

TABLE III

PERFORMANCE OF SYNOPS STEROL TMS DERIVATIVES OF SE-30 ULTRAPHASE SCOT COLUMN

— Not identified.

Synops sterols (Fig. 5)	*Sterol identity**	*Kováts retention index[29], I_{250}**
S-1	26C 5,22*E*	2974
S-2	26C 22*E*	2990
S-3	26C 5	3032
S-4	26C	3041
S-5	Occelasterol**	3087
S-6	Patinosterol**	3100
S-7	27C 5,22*E*	3100
S-8	27C 22*E*	3113
S-9	27C 5	3141
S-10	27C	3152
S-11	28C 5,22*E*	3180
S-12	28C 22*E*	3189
S-13	27C 7	3205
S-14	—	3216
S-15	28C 5,24(28)	3227
S-16	28C 5	3239
S-17	28C 24(28)	3239
S-18	28C	3254
S-19	29C 5,22*E*	3262
S-20	28C 7,24(28)**	3281
S-21	28C 7**	3291
S-22	29C 5	3321
S-23	29C	3333
S-24	29C 5,24(28)*Z*	3333
S-25	29C 24(28)*Z*	3345
S-26	29C 7,22*E*	3358
S-27	29C 7,24(28)*E***	3375
S-28	29C 7	3390
S-29	29C 7,24(28)*Z***	3401
S-30	30C 5	3421
S-31	30C	3434
S-32	—	3458
S-33	31C 5?	3481
S-34	31C ?	3492
S-35	32C?	3524
S-36	—	3572

* See Table I.

** Tentative identification.

bers with respect to cholesterol TMS ether, measured at 80,000 plates (retention time 12.0 min, capacity factor 1.04) or 1403 plates m^{-1}. (Cholestane was measured at 125,000 plates or 2192 plates m^{-1}).

Separation of marine sterol TMS ethers on the commercial SE-30 WCOT column

Ascidia mentula. The performance of the commercial 25-m SE-30 WCOT column for the separation of marine sterol TMS ethers from the extracts of the tunicate, *Ascidia mentula*,[17] (Fig. 7a) was compared with an SE-30 packed column (Fig. 7b).

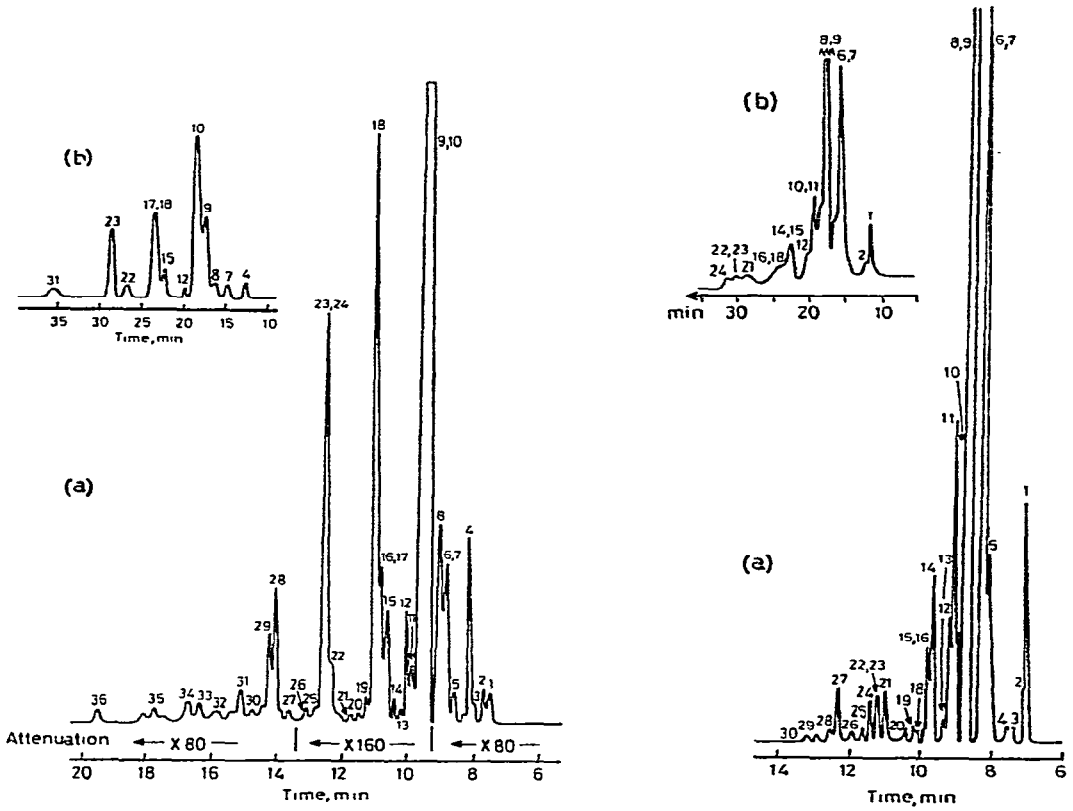

Fig. 5. (a) Chromatogram of *Synops* sterol TMS ethers on SE-30 Ultraphase–Silanox 101 SCOT column. (b) Identical sample on SE-30 Ultraphase packed column (2.5 m × 4 mm I.D.).

Fig. 6. (a) Chromatogram of *Periphylla* sterol TMS ethers on SE-30 Ultraphase–Silanox 101 SCOT column. (b) Identical sample on a Dexsil 300 GC packed column (2.5 m × 4 mm I.D.).

The column efficiency was high, 55,000 theoretical plates for cholesterol TMS ether (2200 plates m^{-1}), but the retention time was very long (approx. 60 min) (capacity factor 22.0). This column operated efficiently with both injector systems, but it took 170 min to complete the analysis.

Separation of marine sterols TMS ethers on POLY-S 176 SCOT columns

POLY-S 176–Silanox 101. Fig. 8a illustrates the performance of a 53-m POLY-S 176–Silanox 101 column for the separation of a mixture of cholesterol, brassicasterol, and desmosterol TMS ethers. The column efficiency was 25,000 theoretical plates (463 plates m^{-1}) for cholesterol TMS ether (retention time 19 min, capacity factor 1.70).

POLY-S 176–Silanox 101, deactivated with Carbowax 20M. Fig. 8b illustrates the performance of the 25-m column of POLY-S 176 on Carbowax 20M–deactivated Silanox 101 for the separation of a mixture of cholesterol, brassicasterol, and desmosterol TMS ethers. The column efficiency was 23,000 theoretical plates (920 plates m^{-1}) for cholesterol TMS ether (retention time 3.85 min, capacity factor 0.7).

TABLE IV

PERFORMANCE OF JELLYFISH STEROL TMS DERIVATIVES ON SE-30 ULTRAPHASE SCOT COLUMN

— Not identified.

Periphylla sterols (Fig. 6)	*Sterol identity**	*Kováts retention index[29], I_{250}**
PP-1	26C 5,22*E*	2970
PP-2	26C 22*E*	2987
PP-3	26C 5**	3033
PP-4	26C**	3044
PP-5	Occelasterol**	3088
PP-6	27C 5,22*E*	3099
PP-7	27C 22*E*	3106
PP-8	27C 5	3138
PP-9	27C	3150
PP-10	27C 5,24(25)	3170
PP-11	28C 5,22*E*	3178
PP-12	28C 22*E*	3188
PP-13	27C 7**	3208
PP-14	28C 5,24(28)	3227
PP-15	28C 5	3236
PP-16	28C 24(28)	3236
PP-17	28C**	3246
PP-18	29C 5,22*E*	3260
PP-19	29C 22*E*	3268
PP-20	28C 7,24(28)**	3279
PP-21	29C 5	3320
PP-22	29C	3333
PP-23	29C 5,24(28)*Z*	3333
PP-24	29C 24(28)*Z*	3344
PP-25	29C 7,22*E***	3357
PP-26	—	3375
PP-27	29C 7**	3391
PP-28	29C 7,24(28)*Z***	3402
PP-29	—	3415
PP-30	—	3423

* See Table I.

** Tentative identification only.

The lack of resolution for brassicasterol and desmosterol TMS ethers on this column indicates that it is essentially a Carbowax 20M–Silanox 101 SCOT column of reasonable efficiency but low loading! The separating characteristics are those of Carbowax 20M rather than POLY-S 176.

POLY-S 176-Hypersil deactivated with BTPPC

(a) Standard sterol TMS ethers. Fig. 8c illustrates the performance of a 30-m column of POLY-S 176 on Hypersil, deactivated with BTPPC for the separation of a mixture of cholesterol, brassicasterol, and desmosterol TMS ethers. The column efficiency was 25,500 theoretical plates (850 plates m^{-1}) for cholesterol TMS ether was 25,500 theoretical plates (850 plates m^{-1}) for cholesterol TMS ether (rentention time 10.8 min, capacity factor 1.94).

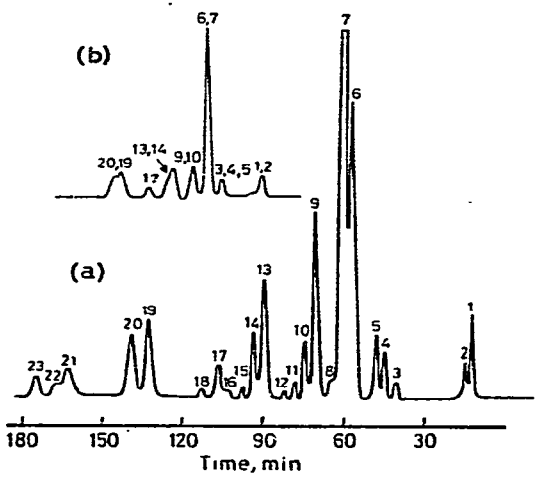

Fig. 7. (a) Chromatogram of tunicate sterol TMS ethers on a commercial WCOT SE-30 column (LKB). (b) Identical sample on SE-30 Ultraphase packed column (2.5 m × 4 mm I.D.).

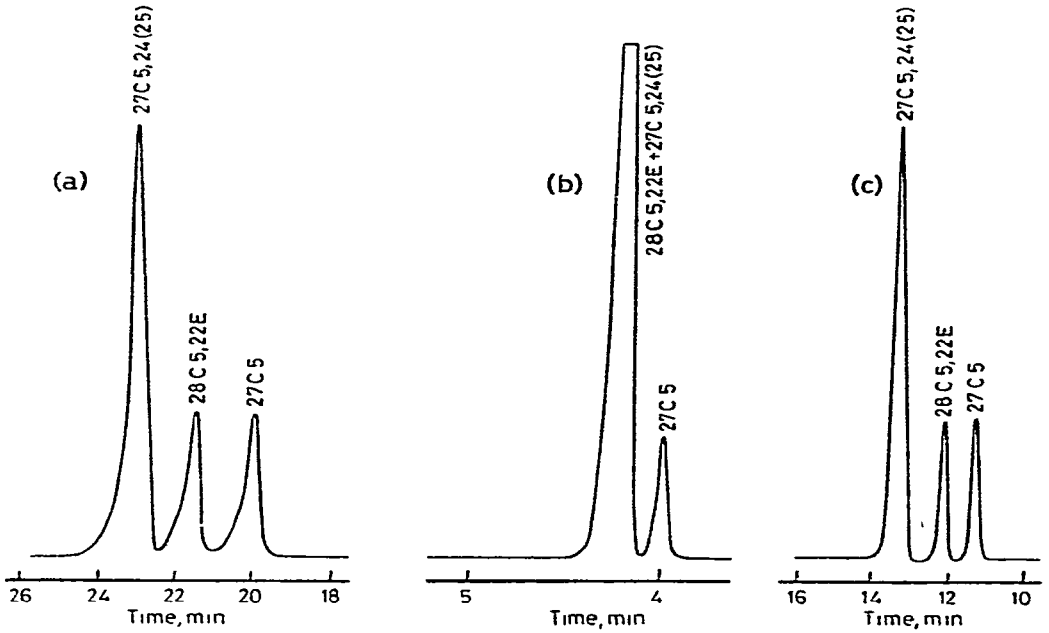

Fig. 8. Comparison of the separations achieved with a mixture of TMS ethers of cholesterol, brassicasterol and desmosterol on (a) POLY-S 176–Silanox 101 SCOT column (53 m), (b) POLY-S 176–Silanox 101 (deactivated with Carbowax 20M) SCOT column (30 m) and (c) POLY-S 176–Hypersil (deactivated with BTPPC) SCOT column (30 m).

(b) Cerastoderma edule. The GC profile for the cockle sterol TMS ethers on the 30-m SCOT column of POLY-S 176–Hypersil (Fig. 9a) is compared with the result obtained from a 1% POLY-S 176 packed column[23]. The sterol identities and Kováts retention indices are given in Table V.

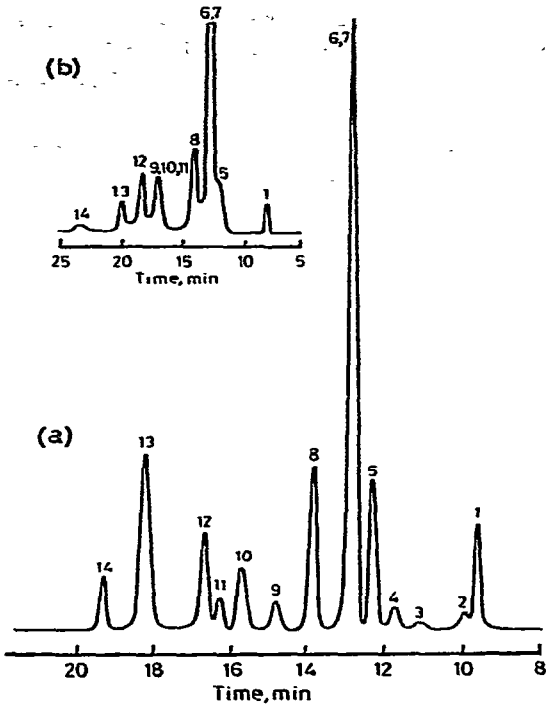

Fig. 9. (a) Chromatogram of cockle sterol TMS ethers on POLY-S 176–Hypersil (BTPPC) SCOT column. (b) Identical sample on a POLY-S 176 packed column (2.5 m × 4 mm I.D.).

TABLE V

PERFORMANCE OF COCKLE STEROL TMS DERIVATIVES ON POLY-S 176 SCOT COLUMN

— Not identified.

Cockle sterols (Fig. 9)	*Sterol identity*[a]	*Kováts retention index*[29], I_{250}
C-1	26C 5,22*E*	3152
C-2	—	3177
C-3	—	3250
C-4	Occelasterol	3284
C-5	27C 5,22*E*	3314
C-6	27C 5	3341
C-7	27C	3341
C-8	28C 5,22*E*	3387
C-9	27C 5,24(25)	3431
C-10	28C 5	3456
C-11	29C 5,22*E*	3477
C-12	28C 5,24(28)	3491
C-13	29C 5	3537
C-14	29C 5,24(28)*Z*	3565

[a] See Table I.

(c) Purple holothurian. The sterols present in a deep-ocean purple holothurian specimen[31] were analysed using the 30-m SCOT column of POLY-S 176–Hypersil (Fig. 10a) and compared with the results from a 1% POLY-S 176 packed column (Fig. 10b).

These extracts contain a complex mixture of Δ^5- and Δ^7-sterol and stanol compounds and will be the subject of further investigation.

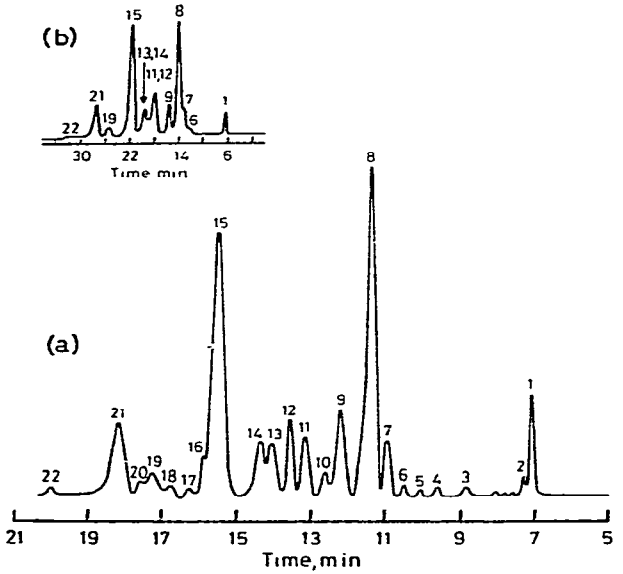

Fig. 10. (a) Chromatogram of holothurian sterol TMS ethers on POLY-S 176–Hypersil (BTPPC) SCOT column. (b) Identical sample on a POLY-S 176 packed column (2.5 m × 4 mm I.D.).

Comparison of the phase ratio for laboratory-prepared SCOT columns vs. WCOT column

It was possible to make rough comparisons of the levels of liquid loading of the commercial SCOT column with the columns prepared by the double dynamic mercury plug technique by comparing the phase ratio, β.

The commercial column had a quoted stationary phase thickness of 0.5 μm, and hence the phase ratio, β, could be calculated using $\beta = r/2d_f$, where r = the internal radius of the column (quoted as 0.17 mm) and d_f = the average film thickness.

The phase ratio for the commercial WCOT column was therefore calculated as 175. Now, for a particular stationary phase at constant temperature the partition coefficient, K, of a solute depends upon the phase ratio and the capacity factor so that $K = \beta_1 k_1 = \beta_2 k_2$, where β_1 is the phase ratio of the commercial column; k_1 is the capacity factor of cholesterol TMS ether on commercial column; β_2 is the phase ratio of a laboratory-prepared column; k_2 is the capacity factor of cholesterol TMS ether on a laboratorium column.

Now, although some gross assumptions have been used in this relationship, it is possible to use it to obtain a rough comparison of the phase ratio and liquid loading of these mercury plug double dynamic coated columns with a commercial column. These comparisons are in Table VI.

TABLE VI

PHASE RATIO COMPARISONS FOR SCOT *VS.* WCOT COLUMNS

Column stationary phase	*Capacity factor, k, for cholesterol TMSE*	*Phase ratio, β*
SE-30 (commercial) WCOT	22.0	175
Dexsil 300 GC–Silanox 101	2.19	1760
SE-30–Silanox 101	1.04	3700
POLY-S 176–Silanox 101	1.70	2260
Carbowax 20M–Silanox 101	0.7	5500
POLY-S 176–Hypersil BTPPC	1.94	1980

From Table VI it can be seen that the phase ratio of the mercury-plug dynamic coated columns is much higher than that of the commercial etched-wall WCOT column and, hence, they have a much lower loading of stationary phase and have correspondingly shorter retention times for high-boiling solutes.

DISCUSSION

The present study has demonstrated that the double dynamic column coating procedure using the mercury-plug technique, initially used to deposit stationary phase layers in WCOT columns[15], can be extended to prepare high-phase-ratio SCOT columns from both non-polar and polar phases.

Supplementing our previous report concerning Apolane-87 (ref. 32), the non-polar phases of Dexsil 300 GC and SE-30 Ultraphase have been successfully coated on to a Silanox 101 support to give low-loaded SCOT columns with efficiencies measured as the number of theoretical plates for cholesterol TMS ether, of 1428 (ref. 32), 1614 and 1403 plates m^{-1}, respectively.

Horning and co-workers[8,33] had already showed that POLY-S 176 could be coated onto Silanox 101 by a static evaporation technique to produce SCOT columns which could be used for steroid analysis. Some difficulties had been reported by several research groups[34–36] in the use of Silanox 101 with polar phases, and this was our experience also with columns coated by the mercury plug dynamic technique. Poor resolution and unacceptable tailing of peaks were observed, and attempted deactivation of the support with Carbowax 20M resulted only in the production of a SCOT column which had the properties of a lightly loaded Carbowax column. A highly satisfactory POLY-S 176 column was obtained by using a 5-μm microparticulate silica, developed for HPLC, as the solid support to produce a lightly loaded POLY-S 176 column by the double dynamic mercury plug technique having efficiency of 850 theoretical plates m^{-1} for cholesterol TMSE. In addition, the dynamic nature of the coating steps resulted in a drastic reduction of the time previously required to prepare such columns[8,33].

The low loading of stationary phase allows these SCOT columns to perform fast analyses of high-boiling compounds, such as sterol TMS ethers. The design of injection system is critical to the good performance of these low-loaded columns. It was found that although both the falling-needle injector and the variable-splitter injector worked well with the commercial lower-phase-ratio WCOT column, the variable-splitter injector was unsatisfactory with laboratory-prepared SCOT columns

of high phase ratio. Problems were encountered with solvent tailing, which were totally overcome by the use of the all-glass falling needle injector system.

It has already been shown by our earlier studies with packed columns[26,28] that the thermostable phases Dexsil 300 GC and POLY-S 176 were complemenatry in their separation characteristics for marine sterol constituents and hence high-resolution SCOT columns of these two phases would be optimal for the identification of sterols. A comparison of the sterol profiles of the cockle, *Cerastoderma edule*, on these two columns (Figs. 3 and 9) illustrates the increase in resolution which these high-phase-ratio SCOT columns have achieved. Thirty-six sterol constituents have been resolved compared with the eleven observed in the previous packed-column GC–MS analysis[28].

The increased resolving power of the SCOT columns enabled increased numbers of sterols to be detected in all of the marine sterol extracts examined: 39 sterols (compared with 26) in the tunicate, *Ascidia mentula*[17]; 36 sterols (compared with 12) in the oceanic siliceous sponge, *Synops* sp.[30]; 30 sterols (compared with 17) in the pelagic jellyfish, *Periphylla periphylla*[7], and 22 sterols (compared with 13) in an oceanic holothurian[31]. In addition, the greatly increased resolution allowed a greater accuracy of quantitation for each sterol.

From our results it is clear that, as the separation power of analytical columns is improved, the complexity of the natural product sterol mixtures present in many marine animals can be properly appreciated. Most of the organisms analysed in this work are unlikely to have had nearly such a complex dietary mixture of sterols as are found in their tissues. For such animals the overriding conclusion is that their tissue sterols are being specifically synthesised, either *de novo* or by modification of available dietary sterols, for subsequent incorporation into the animal's membranes where, presumably, they have very specialised roles.

We believe that for a better understanding of sterol function in a whole range of marine organisms, there must be a continual improvement in our analytical techniques. Only with highly efficient separation methods is it possible to determine the true spectrum of an organism's component sterols.

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REFERENCES

1 L. J. Goad, in D. C. Malins and J. R. Sargent (Editors), *Biochem. Biophys. Perspectives in Mar. Biol.*, Vol. 3, Academic Press, New York, Lonodn, 1976, p. 213.
2 L. J. Goad, in P. J. Scheuer (Editor), *Marine Natural Products*, Vol. II, Academic Press, New York, London, 1978, p. 75.
3 R. J. Morris and F. Culkin, *Oceanogr. Mar. Biol. Annual Rev.*, 15 (1977) 73.
4 F. J. Schmitz, in P. J. Scheuer (Editor), *Marine Natural Products*, Vol. I, Academic Press, New York, London, 1978, p. 241.
5 S. Popov, R. M. K. Carlson, A. Wegmann and C. Djerassi, *Steroids*, 28 (1976) 695.

6 C. Djerassi, N. Theobald, W. C. M. C. Kokke, C. S. Pak and R. M. K. Carlson, *Pure Appl. Chem.*, 51 (1979) 1815.
7 J. A. Ballantine, J. C. Roberts and R. J. Morris, *Biomed. Mass Spectrom.*, 3 (1976) 14.
8 P. van Hout, J. Szafranek, C. D. Pfaffenberger and E. C. Horning, *J. Chromatogr.*, 99 (1974) 103.
9 D. R. Idler, M. W. Khail, J. D. Gilbert and C. J. W. Brooks, *Steroids*, 27 (1976) 155.
10 M. Novotny, M. L. Lee, C. E. Low and M. P. Maskarinec, *Steroids*, 27 (1976) 665.
11 C. G. Edmonds and C. J. W. Brooks, *J. Chromatogr.*, 116 (1976) 173.
12 C. G. Edmonds, A. G. Smith and C. J. W. Brooks, *J. Chromatogr.*, 133 (1977) 372.
13 M. Basic, Lj. Basic, J. A. Jovanovic and G. Spiteller, *J. Amer. Oil Chem. Soc.*, 54 (1977) 525.
14 D. R. Idler, M. W. Khalil, C. J. W. Brooks, C. G. Edmonds and J. D. Glibert, *Comp. Biochem. Physiol.*, 59B (1978) 163.
15 G. Schomburg, H. Husmann and F. Weeke, *J. Chromatogr.*, 99 (1974) 63.
16 G. Schomburg and H. Husmann, *Chromatographia*, 8 (1975) 517.
17 J. A. Ballantine, A. Lavis, J. C. Roberts and R. J. Morris, *J. Exp. Mar. Biol. Ecol.*, 30 (1977) 29.
18 J. J. Franken, G. A. F. M. Rutten and J. A. Rijks, *J. Chromatogr.*, 126 (1976) 117.
19 G. Schomburg, R. Dielmann, H. Husmann and F. Weeke, *J. Chromatogr.*, 122 (1976) 55.
20 M. Novotny and A. Zlatkis, *J. Chromatogr.*, 56 (1971) 353.
21 K. Grob, *Chromatographia*, 10 (1977) 625.
22 A. L. German, C. O. Pfaffenberger, J.-P. Thenot, M. G. Horning and E. C. Horning, *Anal. Chem.*, 45 (1973) 930.
23 A. L. German and E. C. Horning, *J. Chromatogr. Sci.*, 11 (1973) 6.
24 R. D. Schwartz, R. G. Mathews, S. Ramachandran, R. S. Henly and J. E. Doyle, *J. Chromatogr.*, 112 (1975) 111.
25 R. G. Mathews, R. D. Schwartz, C. D. Pfaffenberger, S.-N. Lin and E. C. Horning, *J. Chromatogr.*, 99 (1974) 51.
26 J. A. Ballantine and K. Williams, *J. Chromatogr.*, 148 (1978) 504.
27 P. M. J. van den Berg and T. P. H. Cox, *Chromatographia*, 5 (1972) 301.
28 J. A. Ballantine, J. C. Roberts and R. J. Morris, *J. Chromatogr.*, 103 (1975) 289.
29 K. Kováts, *Advan. Chromatogr.*, 1 (1965) 229.
30 J. A. Ballantine, A. Lavis and R. J. Morris, *Comp. Biochem. Physiol.*, 63B (1979) 119.
31 A. Lavis, *Ph.D. Thesis*, University College of Swansea, 1978.
32 J. A. Ballantine, K. Williams and R. J. Morris, *J. Chromatogr.*, 166 (1978) 491.
33 S.-N. Lin, C. D. Pfaffenberger and E. C. Horning, *J. Chromatogr.*, 104 (1975) 319.
34 P. Arpino, C. Vidal-Madjar, G. Guiochon and S. Békássy, *J. Chromatogr.*, 138 (1977) 173.
35 R. S. Deelder, J. J. M. Raemakers, J. H. M. van den Berg and M. L. Wetzels, *J. Chromatogr.*, 119 (1976) 99.
36 C. A. Cramers, E. A. Vermeer and J. J. Franken, *Chromatographia*, 10 (1977) 412.