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THE MAJOR STEROLS OF SARGASSUM VULGARE C. AGARDH INVESTIGATED BY MASS CHROMATOGRAPHY

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Increasing interest is being shown in the sterol contents of marine plants (Patterson, 1971 ; Goodwin, 1974) and invertebrates (Patterson *et al.*, 1975) for such reasons as chemo-taxonomic significance, economics and diet. The recent introduction of more effective analytical methods in this area *e.g.* gas chromatography-mass spectrometry and hydrophobic gel column chromatography (Patterson *et al.*, 1975) are helping to reduce many problems concerning sensitivity, specificity and separating powers which beset earlier workers. It is generally recognized that the brown algae, Phaeophyceae, contain fucosterol as the major sterol component (Ikekawa *et al.*, 1968 ; Patterson, 1971 ; Goodwin, 1974) .

MATERIAL AND METHODS

Unless otherwise stated in the text all reagents used were of analytical grade obtained from E. Merck A.G., Darmstadt. Fucosterol (5,24(28) E-cholestadiene-3 $^{\beta}$ - ol) was obtained from Steraloids Inc., Wilton N.H., U.S.A. and 28-iso-fucosterol (5,24(28) Z-cholestadiene-3 $^{\beta}$ - ol) was a gift from Prof. W. Sucrow, Paderborn.

Sargassum vulgare was harvested near Fortaleza, Brazil during the month of July. An ethanolic extract was made by grinding 200 g of clean wet alga with 100 ml of distilled water and 30 ml of ethanol. The resulting mixture was filtered, evaporated to dryness (75°-80°C) , made up to 160 ml with ethanol and stored in a refrigerator. From this solution 40 ml were taken, evaporated to dryness and made up to 200 ml with distilled water.

A 2.1 x 25.5 cm column containing 50 g of Amberlite XAD-2 (Serva, Heidelberg; 300 - 1000 Mesh) was prepared for use by washing with 250 ml methanol, 250 ml acetone and 500 ml water. The 200 ml aqueous solution of algal extract was then passed through at a rate of 20 drops/minute. After washing with 100 ml of an ethanol-water (30-70 v/v) mixture, 100 ml of ethanol was applied yielding fraction 1 and then 200 ml of methanol yielding fraction 2 . The further description relates to this latter fraction as both gave similar analytical results.

A small aliquot of fraction 2 was evaporated to dryness under nitrogen and sonicated with 1 ml phosphate buffer (0.05 N) giving pH 6 . The solution was incubated for 2 hours at 35-40°C with 4 drops of $^{\beta}$-glucuronidase (Institute Pasteur, Paris). 6 ml of distilled water were added and the solution sonicated for a few minutes before extracting with three 20 ml portions of diethyl ether and drying over anhydrous sodium sulphate.

Silylation was carried out by adding 0.6 ml dry pyridine, 0.4 ml hexamethyldisilazane (Eastman-Kodak) and 0.2 ml trimethylchlorosilane and leaving for one hour at room temperature. Pyridine and excess reagents were removed under nitrogen and the residue dispersed in n-hexane by sonication. After centrifugation the supernatant was removed and used for further analyses.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry was carried out with an LKB 9000S instrument equipped with a 2 m x 3.2 mm ID glass column packed with 3% OV-101 on Chromosorb W HP 60/100 .

Operating conditions were: column temperature, 240°C ; injector temperature, 260°C ; ion source temperature, 290°C ; helium separator temperature, 270°C ; ionization potential, 22.5 eV ; ionization current, 60 *u*A ; flow-

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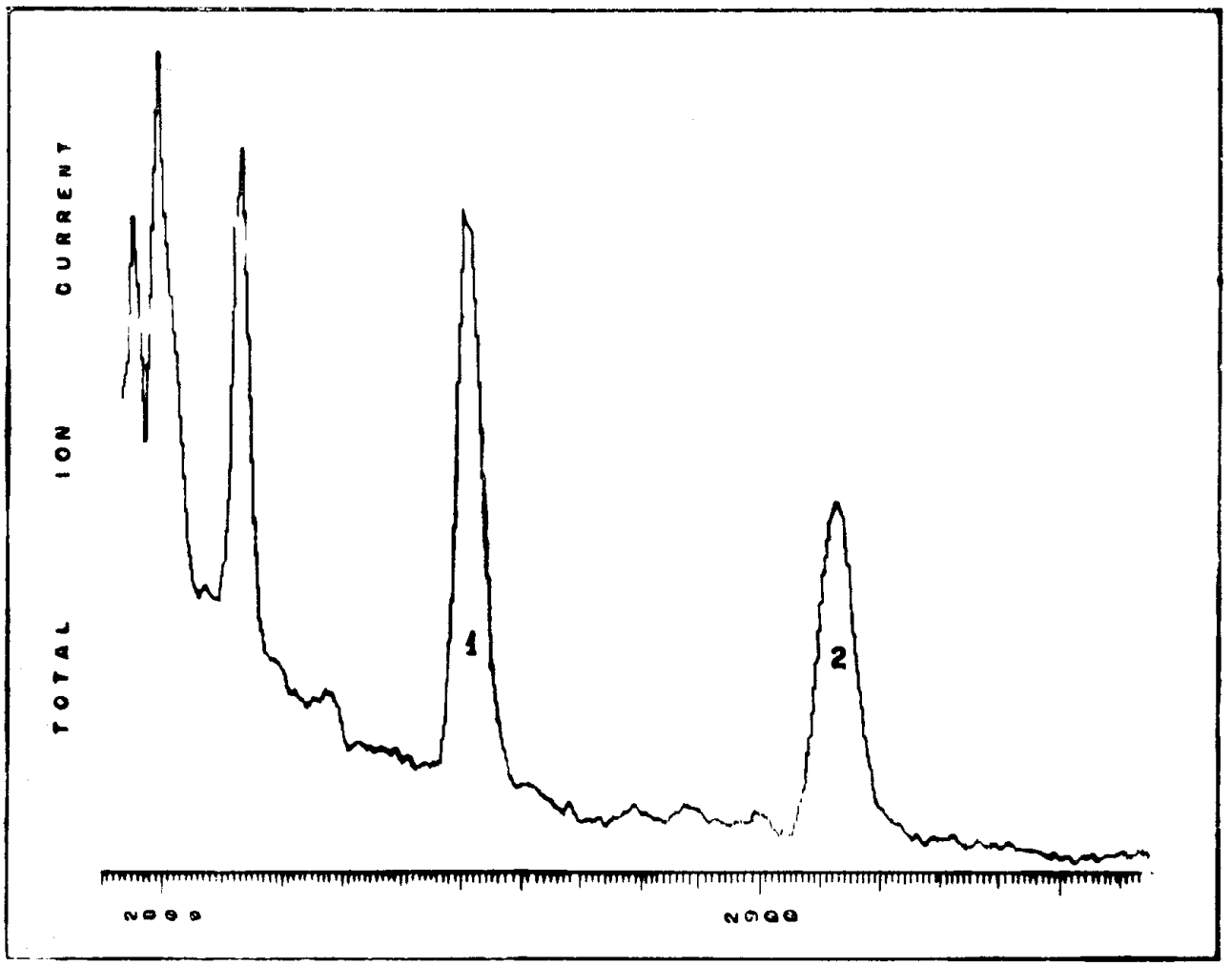

Figure 1 — Total ion-current chromatogram of silylated extract from *Sargassum vulgare* showing peaks 1 and 2 having R_t typical of cholesterol and fucosterol trimethylsilyl ethers, respectively.

-rate, 30 ml/min helium. Successively scanned mass spectra were recorded on magnetic tape via an LKB 2130 data system connected on line to the mass spectrometer.

Gas chromatography

Gas chromatography was performed with a Pye 104 chromatograph: 2.7 m x 4 mm column of OV-101 (phase identical to that used in GC-MS) at 240°C and at 220°C ; flow-rate, 30 ml/min nitrogen; detector temperature, 250°C .

Thin-layer chromatography

Thin-layer chromatography was carried out using a pre-coated 20 x 20 cm Silica Gel G plate, 0.25 mm layer, using cyclohexane: ethyl acetate 50:50 . In this system sterols have an R_F-value of approximately 0.5 . The sterol zone was scraped off, transferred to a Pasteur pipette containing a glass wool plug and the contents eluted with a few ml of ethanol.

RESULTS AND DISCUSSION

The computer constructed gas chromatogram obtained from the *S. vulgare* extract trimethylsilyl ether (TMSi) derivative is shown in Figure 1 . The vertical axis represents the total ion currents of the successively scanned mass spectra and the horizontal axis shows time and spectrum number. Conditions are given under Material and Methods.

Two major peaks show up in the region where sterols normally appear. Peak 1 (retention relative to 5α — cholestane: 2.02) and peak 2 (relative retention: 3.18) could be due to cholesterol TMSi and fucosterol TMSi , respectively (actual values on stationary phase OV-101 of 2.10 and 3.20 at 240°C) .

In order to detect specific sterols mass chromatograms were constructed consisting of ion-current plots of certain preselected ions from each successively recorded mass spectrum instead of the total ion current. Thus, when ions characteristic of particular sterols are chosen the computer will indicate their presence or absence. In Figure 2*a* is

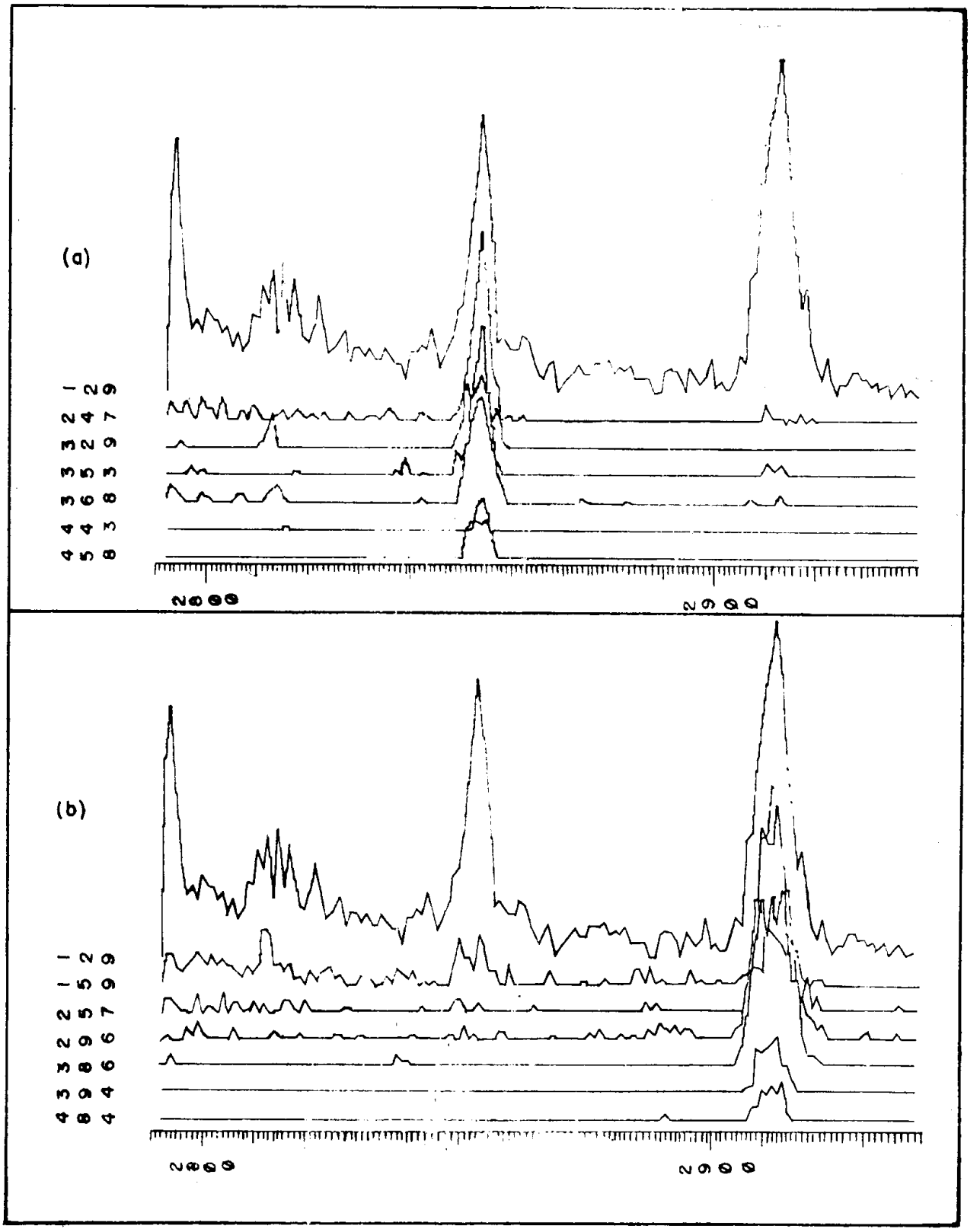

Figure 2 — Mass chromatograms constructed from: (a) ions characteristic of cholesterol trimethylsilyl ether; (b) ions characteristic of fucosterol trimethylsilyl ether.

shown a mass chromatogram having the same spectra as Figure 1 but with only the ions characteristic of cholesterol TMSi (m/e: 458, 443, 368, 353, 329, 247, 129). Similarly, in Figure 2*b* are shown the ions characteristic of fucosterol TMSi (m/e: 484, 394, 386, 296, 257, 159, 129).

The mass chromatograms therefore strongly indicate the presence of both sterols as suggested by relative retention values. Ho-

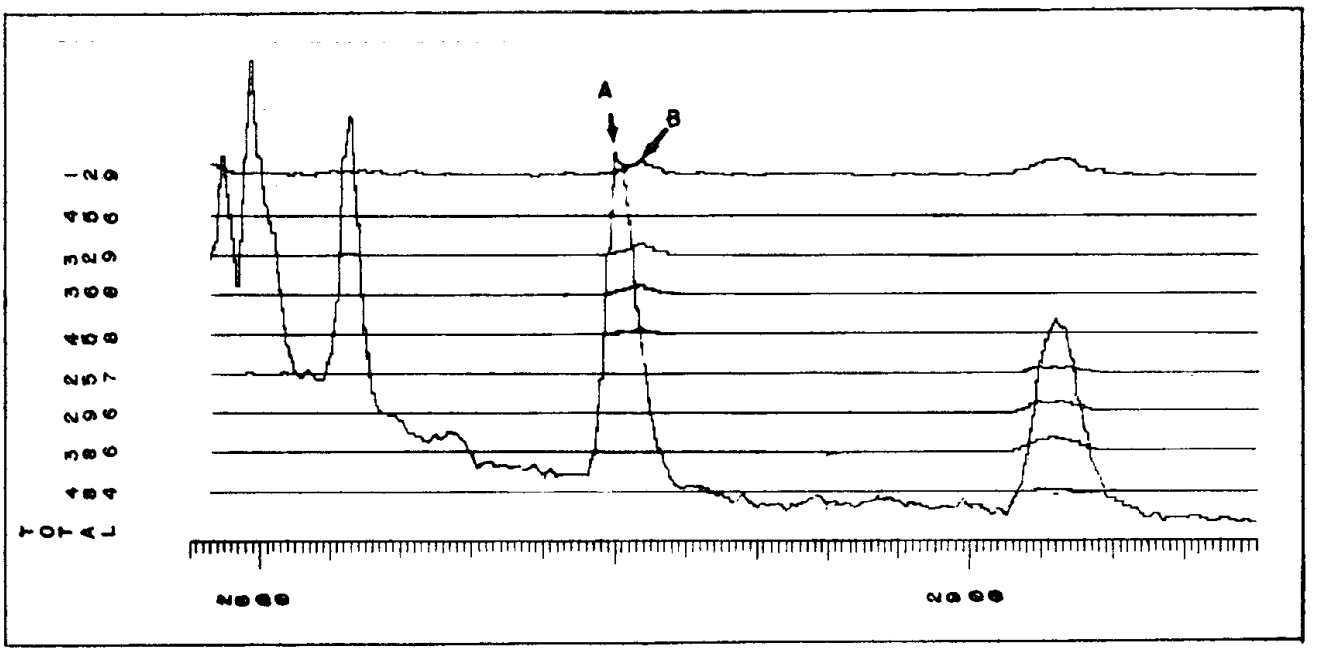

Figure 3 — Mass chromatogram constructed from ions typical of both cholesterol and fucosterol trimethylsilyl ethers together with a total ion-current plot revealing the occurrence of the former compound in the latter part of peak 1, *i.e.* centred on spectrum B.

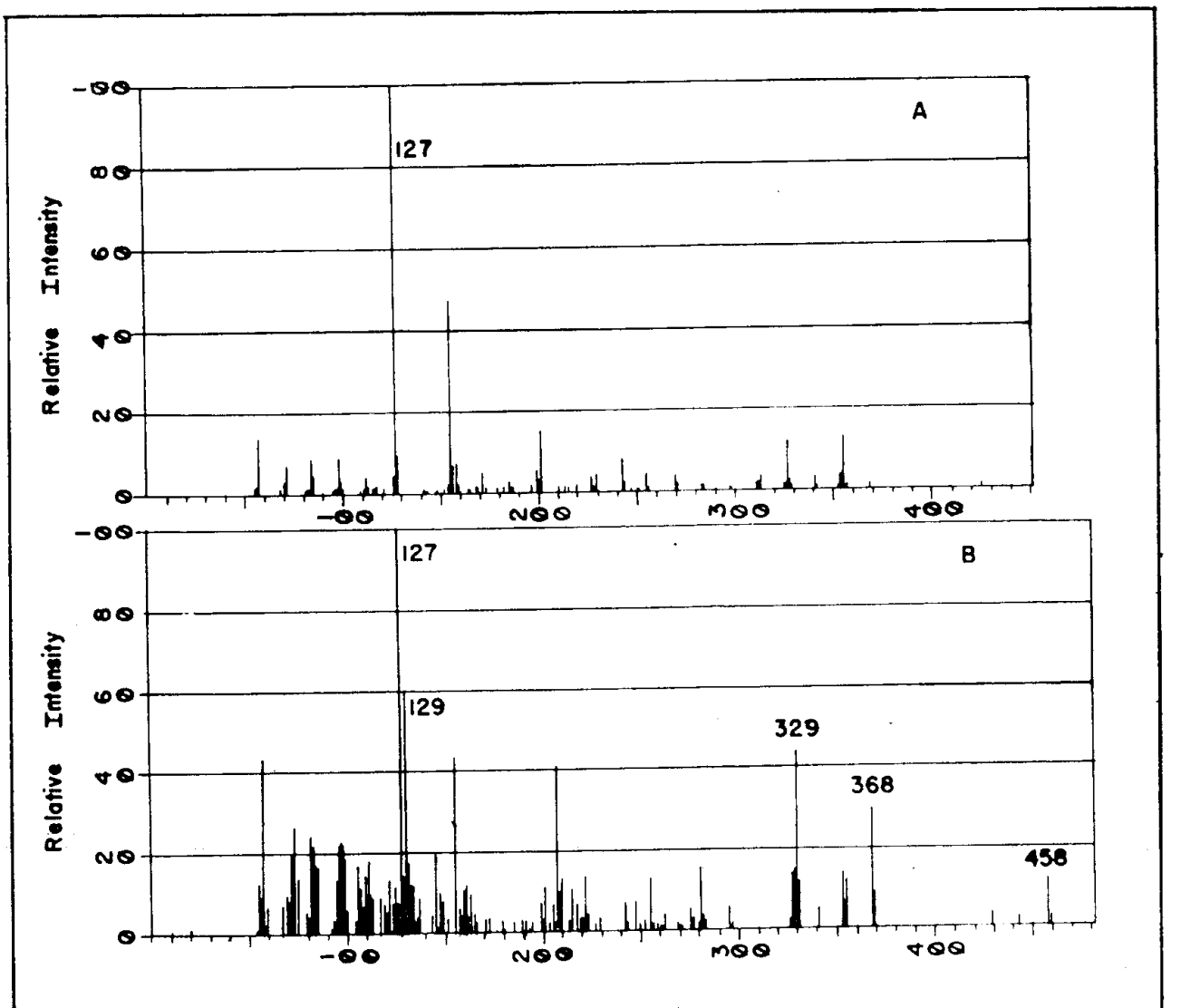

Figure 4 — The mass spectra A and B showing the appearance of cholesterol trimethylsilyl ether peaks.

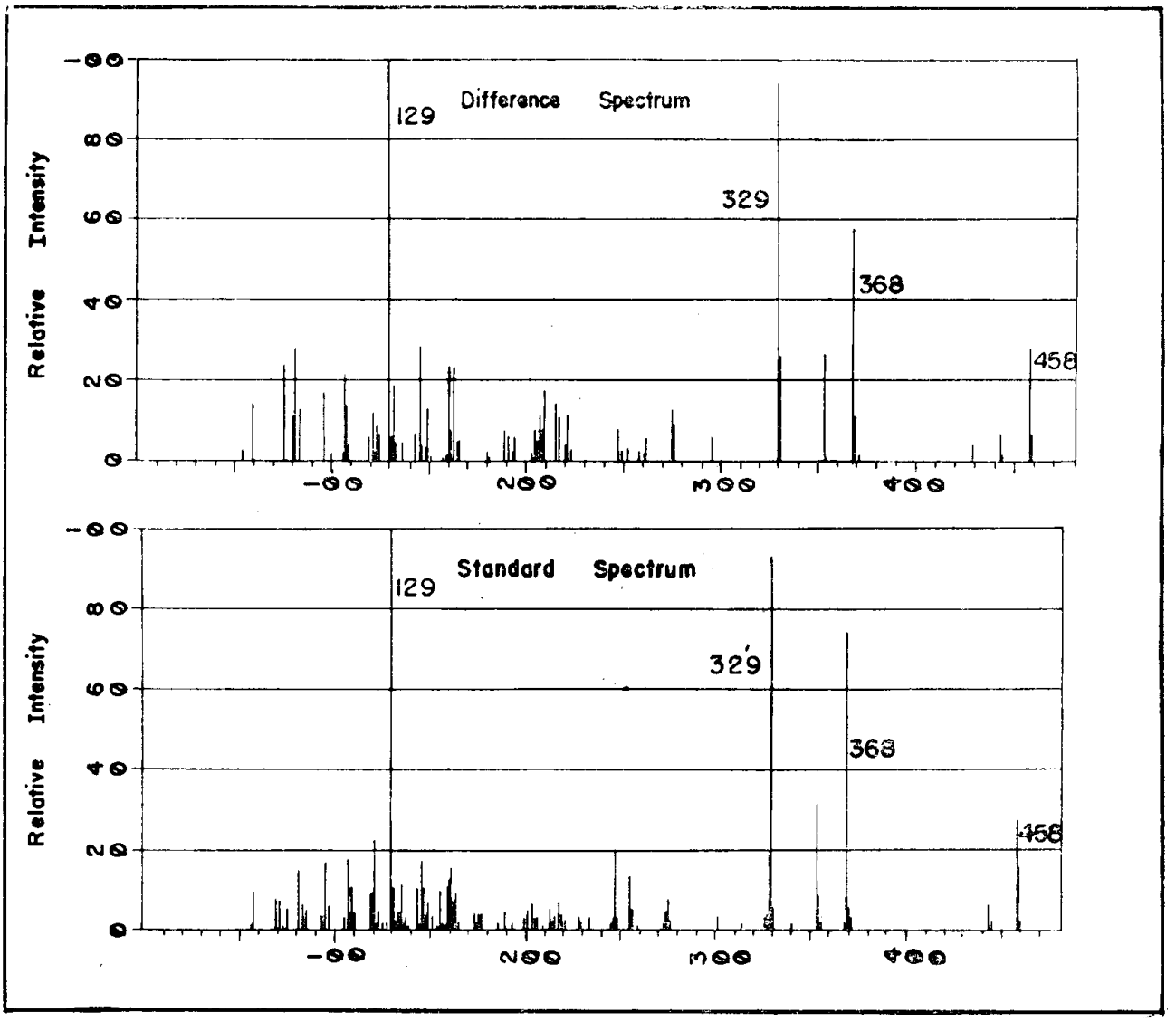

Figure 5 — The difference spectrum obtained by subtr acting spectrum A from spectrum B (figure 4) together with a standard spectrum of ch olesterol trimethylsilyl ether.

wever, the mass spectra obtained from the centres of the peaks were not characteristic of sterols and this fact in view of the good mass chromatograms suggested contaminating substances present in overwhelming amounts. In Figure 3 is shown a mass chromatogram constructed from the most significant ions in both sterol ethers together with a total ion-current plot.

The position of the cholesterol ether in peak 1 is revealed as lying to the right of centre. The mass spectrum A at the centre is shown in Figure 4 together with the spectrum having a maximum of cholesterol ether ions, B. The fact that these ions are off--centre suggested the possibility of obtaining a cleaner spectrum by subtraction. This was readily accomplished by computerized manipulation and the resulting spectrum is shown in Figure 5 together with a standard cholesterol TMSi spectrum. The main ions are: m/e 458 (molecular ion, M+), 443 (M+ — 15), 368 (M+ — 90), 353 (M+ — 90 — 15), 329 (M+ — 129) and 129. The presence of major peaks having m/e 129 and also (M+ - 129) is characteristic of $\triangle 5$ - 3^{β} - ol trimethylsilyl ethers (Brooks *et al.*, 1968).

It was not possible to obtain a clean spectrum of fucosterol TMSi from peak 2 by this means owing to coincidence of retention times. The distinction between fucosterol and its naturally occuring isomer, 28-iso-fucosterol, was made by comparison of retention times (Knights & Brooks, 1969) on a 2.7 m column.

At 240°C it was not possible to resolve a 50:50 mixture of fucosterol and 28-iso-fucosterol as their trimethylsilyl ethers but one peak was obtained having a relative retention time (R_t) of 3.88, *i.e.* between the R_t - values of the pure components (table I). In spite of the broadness of peak 2 the relative

TABLE I

Stereochemistry at C_{28} : Retention data of standards and algal extract hydrolysate both before and after cleaning with TLC .

Temperature (°C)	Sterol TMSi-ether	R_t (cholestane) (± 0.05)
240	28-iso-fucosterol	3.95
	fucosterol	3.77
	algal hydrolysate peak 2	3.80
	peak 2 after TLC	3.77
220	peak 2 after TLC	4.33
	iso-fucosterol	4.50
	fucosterol	4.38

retention favours fucosterol. In order to obtain more certainty a sample of hydrolysate was subjected to thin-layer chromatography and that band corresponding to sterols was extracted and derivatized. The peak obtained was narrower and a better agreement with fucosterol was drawn. At 220°C a partial separation of the 50:50 mixture of both sterol ethers was obtained and the measurements of R_t confirm the identity as fucosterol. The result does not preclude the presence of a small proportion of the isomer and work is at present being carried out to investigate this possibility. 28-iso-fucosterol is found in many green algae.

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SUMMARY

The application of computerized gas chromatography-mass spectrometry and particularly mass chromatography in the investigation of crude algal extracts is demonstrated. Fucosterol and cholesterol were found to be the major sterol components of the brown alga *Sargassum vulgare* C. Agardh. These findings concur with results obtained for other brown algae.

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