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Rapid Method for Detection and Characterization of Steroids

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IDENTIFICATION OF INDIVIDUAL COMPONENTS from complex mixtures of steroids usually requires rigorous application of chromatographic techniques and mass spectrometry. In many mammalian systems, cholesterol is the predominant steroid and thus utilization of these techniques becomes almost a routine process. However, in the area of comparative phytochemistry, steroid fractions are often extremely complex mixtures and therefore, the investigation of the steroid content of one such sample becomes a major undertaking. While recognizing that unequivocal structure determinations necessitate detailed analysis as reported by many workers (1-3), it is possible to arrive at tentative structural assignments of sterol components by a more rapid method which we have found to be accurate.

Our investigations of the chemistry of a contemporary lake (Mono Lake, Calif.) and the historical record of the lake metabolism as evidenced by the stratigraphic sequence have required analyses of many samples of macro- and micro-organisms, lake water, bottom muds, and consolidated sediments. We have shown that steroids are present in the organisms and sediments (4, 5) of Mono Lake, and it appears that their distributions and identities reflect variations in the biological composition of contemporary and ancient environments (4).

Further investigation of these variations required structural analyses of complex steroid mixtures from more than 100 different samples, first of all to determine the presence or absence of steroids, and second, to obtain tentative structural identifications.

The method developed uses the present knowledge of the polarity and behavior of sterols on liquid-solid chromatography for isolation of a crude sterol fraction which is then treated with a trimethylsilylation reagent and the resulting silyl ethers are separated from extraneous materials by preparative TLC (6, 7). These derivatives are then analyzed by temperature programmed direct inlet probe mass spectrometry at high and low voltage. The recorded ion abundances are correlated with those of the six major ions occurring in the spectra of a reference library of authentic sterol TMS ethers.

EXPERIMENTAL

Isolation of a Sterol Fraction. Organic material was extracted from the finely powdered sediment (~15 grams) by room temperature sonication in benzene:methanol (3:1 v/v); (4 × 150 ml). The combined extracts were taken to dryness on a rotary evaporator ($\leq 40^\circ\text{C}$) and the residue was dissolved

in heptane:ethyl acetate (20:1 v/v) in preparation for silica gel column chromatography (60-200 mesh, J.T. Baker Chemical Co., Phillipsburg, N.J.). The chromatography was accomplished by successive elutions with heptane:ethyl acetate (20:1 v/v) and with pure ethyl acetate. The ethyl acetate fraction was shown by analytical TLC to contain sterols which were trimethylsilylated using hexamethyldisilazane:trimethylchlorosilane (5:1 v/v) in pyridine solution. The reagents were removed under a stream of dry nitrogen and the resulting derivatives were dissolved in ethyl acetate and the solution was centrifuged to remove solid material. The total etherified fraction was chromatographed preparatively on one 20 × 20 cm thin-layer chromatographic plate coated with silica gel G (E. Merck, Darmstadt, Germany; 0.25 mm) using heptane:benzene (1:1 v/v) as mobile phase. An area R_f 0.38- R_f 0.62 was scraped from the chromatogram after location by comparison to the mobilities of reference sterol TMS ethers. TMS ethers were extracted from the silica gel G by sonication in ethyl acetate at 20°C . The solvent was evaporated and the fraction weighed and made up in ethyl acetate to a concentration of approximately 1 mg/ml.

Probe Mass Spectrometry. The mass spectrometer used for the analysis of the sterol TMS ethers was a Du Pont 492-1 instrument (Du Pont Instrument Division, Monrovia, Los Angeles), equipped with linear magnetic scan and an independently heated direct introduction probe. This allowed controlled temperature programming of the probe tip from 50-350 $^\circ\text{C}$, while maintaining a source temperature of 250 $^\circ\text{C}$. Samples, 1-2 μL , of sterol TMS ether mixtures were deposited in clean glass capillaries, and the solvent was evaporated with slight heating. The capillary was then introduced into the source of the mass spectrometer via the direct introduction probe.

The source and analyzer pressures were maintained at approximately 5×10^{-8} Torr by means of differential pumping using a 4-in. diameter source diffusion pump and a 2-in. analyzer diffusion pump. The spectra were recorded, on an Oscillomink E spray ink recorder (Siemens, Karlsruhe, Germany), every 30 seconds during programmed heating. Spectra are recorded at 70 eV and 16 eV during the course of a temperature program. The temperature rise may be controlled manually such that isothermal periods are maintained at significant temperatures. Considerable fractionation of components is observed, but quantitative results would be better achieved using a data handling system.

RESULTS AND DISCUSSION

The mass spectra of the trimethylsilyl ethers of non-epimeric sterols are fairly unique (8). Table I shows the six most abundant high mass ions in the mass spectra of a series of reference sterol TMS ethers. Examination of these data shows that even when only six ions are considered, very distinct patterns emerge. The fragmentation processes leading to many of the ion fragment m/e values shown in this table have been exhaustively described by other groups of workers (8-10), and they will not be discussed in the present communication. Figure 1 shows normalized line diagrams of the high mass

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Table I. Mass Spectra of Reference Sterol TMS Ethers

Parent	Mol wt, TMS	Principal ions* in mass spectrum					
Cholesterol	458	129 (100%)	329 (87%)	368 (52%)	145 (38%)	121 (36%)	353 (32%)
Cholestanol	460	215 (100%)	216 (59%)	217 (41%)	147 (37%)	121 (32%)	355 (25%)
Brassicasterol	470	125 (100%)	129 (98%)	380 (84%)	255 (75%)	145 (58%)	147 (55%)
Ergosta-7,22-diene-3 β -ol	470	255 (100%)	147 (68%)	145 (55%)	159 (52%)	229 (49%)	343 (47%)
24-Methylenecholesterol	470	129 (100%)	119 (38%)	386 (36%)	380 (34%)	341 (30%)	296 (25%)
Campesterol	472	129 (100%)	121 (55%)	119 (44%)	147 (32%)	343 (25%)	382 (19%)
Stigmasterol	484	129 (100%)	255 (63%)	133 (38%)	394 (35%)	145 (32%)	484 (22%)
Fucosterol	484	129 (100%)	386 (73%)	296 (62%)	119 (41%)	145 (33%)	355 (27%)
Stigmasta-5,25-diene-3 β -ol	484	129 (100%)	119 (43%)	121 (37%)	145 (29%)	147 (27%)	394 (26%)
β -Sitosterol	486	129 (100%)	357 (98%)	396 (66%)	121 (58%)	145 (45%)	215 (42%)
Stigmastanol	488	215 (100%)	216 (52%)	121 (40%)	147 (32%)	217 (30%)	305 (22%)

* Only ions over m/e 110 have been considered.

Figure 1. Line diagrams of high-mass ends of probe mass spectra obtained from the trimethylsilyl ethers of:

A. the sterol fraction from Mono Lake algae

B. the sterol fraction from a Mono Lake sediment. Spectra were normalized on the most abundant ion over m/e 200

ends of the probe mass spectra (obtained as described above) of trimethylsilylated sterol fractions of: A, Mono Lake algae; and, B, of a consolidated Mono Lake sediment with an approximate age of 130,000 years. The spectrum derived from the algae gave what looked like distinct molecular ions at m/e 486, 472, and 458. The ion at m/e 458 together with those at m/e 368, 353, 255, 213, and 129 and, more significantly, at m/e 329 ($458 - 129$), clearly pointed to the presence of cholesterol TMS ether in this sample. A number of alternative structures are possible having a molecular ion at m/e 472 (i.e., TMS ethers of campesterol or the ergosterols).

Campesterol (24 α -methylcholest-5-en-3 β -ol) TMS ether seemed a strong possibility since a significant ion found at m/e 343 was thought to be due to the loss of 129 mass units from the molecular ion, and this together with the corresponding ion at m/e 129 suggests a Δ^3 3 β -OTMS containing structure (8, 10) as is found in campesterol TMS ether.

It seemed unlikely that the abundant molecular ion at m/e 486 was due to β -sitosterol (24 α -ethylcholest-5-en-3 β -ol), since this would have produced a major ion at m/e 357 due to loss of 129 mass units from the molecular ion, and m/e 357 is almost completely absent from the present spectrum.

An examination of the spectrum shown in Figure 1B (sediment sample) showed abundant ions at m/e 215, 216, 217, and 257 and these, together with possible molecular ions at m/e 460 and 488 suggested that saturated sterols were present (8) and the structures cholestanol TMS ether and stigmastanol TMS ether were tentatively assigned. It is also clear from molecular ions at m/e 458, 470, 472, and 484 that the unsaturated counterparts of cholestanol and stigmastanol are also present and from an examination of the fragment ions (including those below m/e 200) cholesterol, brassicasterol, campesterol, and stigmasterol were tentatively identified. The tentative identifications made here by probe mass spectra have been shown to be very accurate when a complete GLC and GC-MS study was carried out.

This method is suggested as a technique for screening a large number of samples to decide which fractions contain

sterols which merit a fuller investigation by the more time-consuming conventional approach. As illustrated above, it is also possible to arrive at some preliminary identifications especially where some of the compounds being examined yield fairly unique fragments in their mass spectra. It should be emphasized that the method described here could equally well be applied to many other groups of compounds or their derivatives having appropriate volatility.

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