An updated look at the analysis of unsaturated C_{27} sterols by gas chromatography and mass spectrometry

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Abstract Gas chromatography–mass spectrometry (GC-MS) and GC are commonly used methods for the identification and quantitation of sterols from samples of biological origin. To investigate the utility and limitations of these methods, we have determined gas chromatographic mobilities and mass spectral properties of 5α-cholestan-3β-ol and 26 unsaturated C_{27} sterols as their acetate and trimethylsilyl (TMS) ether derivatives by GC and GC-MS. The GC retention data showed that numerous sterols were essentially coeluted on capillary GC columns coated with either 5% phenyl–95% methyl polysiloxane or polyethylene glycol, although the peaks were more widely dispersed on the latter column. Mass spectra of many groups of sterol isomers were also quite similar. Sterol mixtures of any complexity are likely to contain coeluting components, and attempts to establish structures based on mass spectra that may represent a mixture of sterol isomers could easily lead to errors. Our results demonstrate that GC and GC-MS alone cannot generally be used for rigorous structure determinations of individual components in mixtures of unsaturated sterols. However, all but a few of the 26 sterols could be distinguished by their combined chromatographic mobilities on the two GC columns coupled with critical examination of their mass spectra. GC-MS analysis of appropriate sterol subclasses or preferably individual sterol components obtained by prior purification by other methods may provide valuable supporting evidence for the identification of sterol structures. Reliability of identification is dependent upon careful attention to GC and MS conditions, calibration of GC and MS data with authentic sterol standards, and consideration of possible decomposition under GC conditions and of the effect of overloading on GC retention times.—**Gerst, N., B. Ruan, J. Pang, W. K. Wilson, and G. J. Schroepfer, Jr.** An updated look at the analysis of unsaturated C_{27} sterols by gas chromatography and mass spectrometry. *J. Lipid Res.* 1997. **38:** 1685–1701.

Supplementary key words GC retention times • column overloading • column bleed • lability of sterols • ion abundances • structure elucidation • Smith-Lemli-Opitz syndrome

Determination of the chemical nature and levels of sterol intermediates in cholesterol biosynthesis is of considerable importance in a number of current problems in biomedical research. Some of these problems have been enumerated and discussed elsewhere (1). Among these interesting areas of investigation are studies of the structures and levels of noncholesterol sterols accumulating in individuals with the Smith-Lemi-Opitz syndrome (SLOS), a severe hereditary developmental disorder affecting multiple organ systems (2). Reported sterols accumulating in blood and/or tissues in SLOS (based upon gas chromatography–mass spectrometry (GC-MS) and/or GC) include the following: cholesta-5,7-dien-3β-ol (2, 3), cholesta-5,8-dien-3β-ol (4), 5α-cholesta-6,8-dien-3β-ol (5–7), 5α-cholesta-6,8(14)-dien-3β-ol (8), 5α-cholest-7-en-3β-ol (6, 7), 5α-cholest-8(14)-en-3β-ol (6), cholesta-5,7,9(11)-trien-3β-ol (9), and 19-norcholesta-5,7,9-trien-3β-ol (9–11).

Sterol mixtures from biological sources can be resolved into subclasses by liquid chromatography or into individual components by Ag^+-high performance liquid chromatography (Ag^+-HPLC) (1) and rigorously identified by ^1H NMR at high field (12). However, the relatively high detection limit (~100 ng) for ^1H NMR and other considerations have led many investigators to rely on GC or GC-MS, commonly without any prior fractionation by other methods. Examination of reports on the identification and quantitation of unsaturated C_{27} sterols by GC or GC-MS indicates the critical importance of an extensive collection of authentic standards and an evaluation of the capability of separating and distinguishing the various sterols. We recently reported the preparation of a large number of unsaturated C_{27} sterols

Abbreviations: GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry or mass spectrum; NMR, nuclear magnetic resonance (spectroscopy); RRT, relative retention time (relative to 5α-cholestane); R_s, resolution; SC, side chain; SLOS, Smith-Lemli-Opitz syndrome; TMS, trimethylsilyl; TMSOH, trimethylsilanol.

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in high purity (12 and references therein) and described their ^1H and ^{13}C NMR spectral properties (12). We have now characterized these compounds by GC and GC-MS. Reported herein are GC retention data and mass spectral properties for the acetate and TMS ether derivatives of 26 unsaturated C_{27} sterols. These data have been analyzed with the objective of evaluating the utility and limitations of GC and MS in the identification of sterols from samples of biological origin. Structures of the sterols studied herein are shown in **Fig. 1.**[2]

MATERIALS AND METHODS

Except as noted below, authentic standards of C_{27} sterols were prepared as described previously (12 and references therein) and showed ≥97% purity. Other standards were obtained by chemical synthesis or from commercial sources and analyzed for purity and authenticity by ^1H NMR as described previously (12). The purity of some commercial samples, analyzed shortly after receipt, was surprisingly low. Cholesta-2,4-diene and cholesta-3,5-diene were purchased from Steraloids (Wilton, NH). NMR analysis showed the $\Delta^{2,4}$ diene sample to be a 9:1 mixture of $\Delta^{2,4}$ and $\Delta^{3,5}$ dienes. GC analysis indicated the $\Delta^{3,5}$ diene sample to be of very low purity, and NMR analysis gave an assay of only 7% purity.[3] Filtration of the $\Delta^{3,5}$ diene sample (25 mg) through silica gel (elution with dichloromethane–hexane 1:1) gave a white solid (2 mg) of ~95% purity by ^1H NMR. Cholesta-2,4,6-triene was obtained as a byproduct in the preparation of the $\Delta^{4,6}$ acetate (13) by bromination–dehydrobromination of cholesteryl acetate (1 g); after filtration through silica gel, the crude yellow solid (0.94 g; 84:9:6 mixture of $\Delta^{4,6}$, Δ^5, and $\Delta^{5,7}$ acetates by ^1H NMR) was adsorbed onto silica gel and subjected to chromatography on $AgNO_3$-silica gel (1 m × 10 mm i.d. column; elution with toluene–hexane 1:4; 20-ml fraction volumes); fraction 9 contained the $\Delta^{2,4,6}$ triene (~95% purity). (22*E*)-3β-Acetoxycholesta-5,22-diene and its 22*Z* isomer were purchased from Research Plus (Bayonne, NJ) and showed ~98% purity by NMR. The $\Delta^{5,22E}$ free sterol was purchased from Steraloids (catalog #3140; Δ^{22} configuration not designated); NMR analysis indicated ~70% purity and the presence of 6% of the $\Delta^{5,22Z}$ isomer. (20(22)*E*)-Cholesta-5,20(22)-dien-3β-ol was prepared essentially as described previously (14), and the $\Delta^{5,20(22)Z}$ isomer was obtained as a minor byproduct. 25,26,26,26,27,27,27-Heptafluorocholest-5-en-3β-ol (F_7-cholesterol) was prepared as described previously (15). Acetates of F_7-cholesterol and the $\Delta^{5,20(22)}$ dienes were prepared by treatment of the free sterols with acetic anhydride–pyridine 1:1 overnight at room temperature. 19-Norcholesta-5,7,9-trien-3β-ol and its acetate derivative were prepared as described previously (16). Trimethylsilyl (TMS) ether derivatives were prepared by treatment of the sterols with a 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide and pyridine for 1 h at room temperature, followed by evaporation to dryness at 30°C under nitrogen. 5α-Cholestane (99% purity) and bis(trimethylsilyl)trifluoroacetamide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Mixtures of *n*-alkane standards (C_{28}, C_{30}, C_{32}, and C_{34}) were purchased from Alltech Associates (Deerfield, IL).

All C_{27} sterols were characterized by NMR. In addition to data given in reference 12, the following ^1H NMR signals were observed for H–18 (s), H–19 (s or d, J ~0.5 Hz), and H–21 (d, J ~6.6 Hz): $\Delta^{2,4}$ diene, δ 0.687, 0.927, 0.907; $\Delta^{3,5}$ diene, δ 0.704, 0.953, 0.921; $\Delta^{5,20(22)E}$ free sterol, δ 0.545, 1.010, ~1.628; $\Delta^{5,20(22)E}$ acetate, δ 0.544, 1.020, 1.625; $\Delta^{5,20(22)Z}$ acetate, δ 0.664, 1.027, 1.700; $\Delta^{5,22E}$ free sterol, δ 0.694, 1.010, 1.009; $\Delta^{5,22E}$ acetate, δ 0.692, 1.020, 1.009; $\Delta^{5,22Z}$ acetate, δ 0.720, 1.027, 0.956; $\Delta^{2,4,6}$ triene, δ 0.714, 0.943, 0.916; F_7-cholesteryl acetate, δ 0.685, 1.020, 0.940.

GC retention times for authentic samples of unsaturated C_{27} sterol derivatives were determined using capillary columns coated with DB-1 (60 m × 0.25 mm i.d.; 100% methyl polysiloxane; 0.1 μm film thickness; J&W Scientific; Folsom, CA), DB-5ms (30 m or 60 m × 0.25 mm i.d.; 5% phenyl–95% methyl polysiloxane; 0.1 μm film thickness; J&W Scientific); Stabilwax (30 m or 60 m × 0.25 mm i.d.; polyethylene glycol; 0.1 μm film

[2]Unsaturated C_{27} sterols are all of 5α configuration (or Δ^4 or Δ^5) and are designated by their unsaturation as follows: Δ^0, 5α-cholestan-3β-ol; Δ^4, cholest-4-en-3β-ol; Δ^5, cholest-5-en-3β-ol; Δ^6, 5α-cholest-6-en-3β-ol; Δ^7, 5α-cholest-7-en-3β-ol; Δ^8, 5α-cholest-8-en-3β-ol; $\Delta^{8(14)}$, 5α-cholest-8(14)-en-3β-ol; Δ^{14}, 5α-cholest-14-en-3β-ol; $\Delta^{2,4}$, cholesta-2,4-diene; $\Delta^{3,5}$, cholesta-3,5-diene; $\Delta^{4,6}$, cholesta-4,6-dien-3β-ol; $\Delta^{5,7}$, cholesta-5,7-dien-3β-ol; $\Delta^{5,8}$, cholesta-5,8-dien-3β-ol; $\Delta^{5,8(14)}$, cholesta-5,8(14)-dien-3β-ol; $\Delta^{5,20(22)E}$, (20(22)*E*)-cholesta-5,20(22)-dien-3β-ol; $\Delta^{5,20(22)Z}$, (20(22)*Z*)-cholesta-5,20(22)-dien-3β-ol; $\Delta^{5,22E}$, (22*E*)-cholesta-5,22-dien-3β-ol; $\Delta^{5,22Z}$, (22*Z*)-cholesta-5,22-dien-3β-ol; $\Delta^{5,24}$, cholesta-5,24-dien-3β-ol; $\Delta^{5,25}$, cholesta-5,25-dien-3β-ol; $\Delta^{6,8}$, 5α-cholesta-6,8-dien-3β-ol; $\Delta^{6,8(14)}$, 5α-cholesta-6,8(14)-dien-3β-ol; $\Delta^{7,9(11)}$, 5α-cholesta-7,9(11)-dien-3β-ol; $\Delta^{7,14}$, 5α-cholesta-7,14-dien-3β-ol; $\Delta^{7,24}$, 5α-cholesta-7,24-dien-3β-ol; $\Delta^{8,14}$, 5α-cholesta-8,14-dien-3β-ol; $\Delta^{8,24}$ 5α-cholesta-8,24-dien-3β-ol; $\Delta^{2,4,6}$, cholesta-2,4,6-triene; $\Delta^{5,7,9(11)}$, cholesta-5,7,9(11)-trien-3β-ol; $\Delta^{5,22E,24}$, (22*E*)-cholesta-5,22,24-trien-3β-ol; $\Delta^{6,8,14}$, 5α-cholesta-6,8,14-trien-3β-ol; 19-nor-$\Delta^{5,7,9}$, 19-norcholesta-5,7,9-trien-3β-ol. Other sterols: campesterol, (24*R*)-ergost-5-en-3β-ol; ergosterol, (22*E*,24*R*)-ergosta-5,7,22-trien-3β-ol; sitosterol, (24*R*)-stigmast-5-en-3β-ol; stigmasterol, (22*E*,24*S*)-stigmasta-5,22-dien-3β-ol; lanosterol, lanosta-8,24-dien-3β-ol; dihydrolanosterol, lanost-8-en-3β-ol; F_7-cholesterol, 25,26,26,26,27,27,27-heptafluorocholest-5-en-3β-ol.

[3]A mixture prepared from the $\Delta^{3,5}$ diene sample (4.21 mg) and Δ^7 acetate (0.56 mg; ≥99% purity) was analyzed by ^1H NMR. Comparison of intensities for both olefinic and methyl signals showed a 6:10 molar ratio of $\Delta^{3,5}$ diene to Δ^7 acetate. This assay indicated that the diene sample contained only 0.29 mg of the $\Delta^{3,5}$ diene.

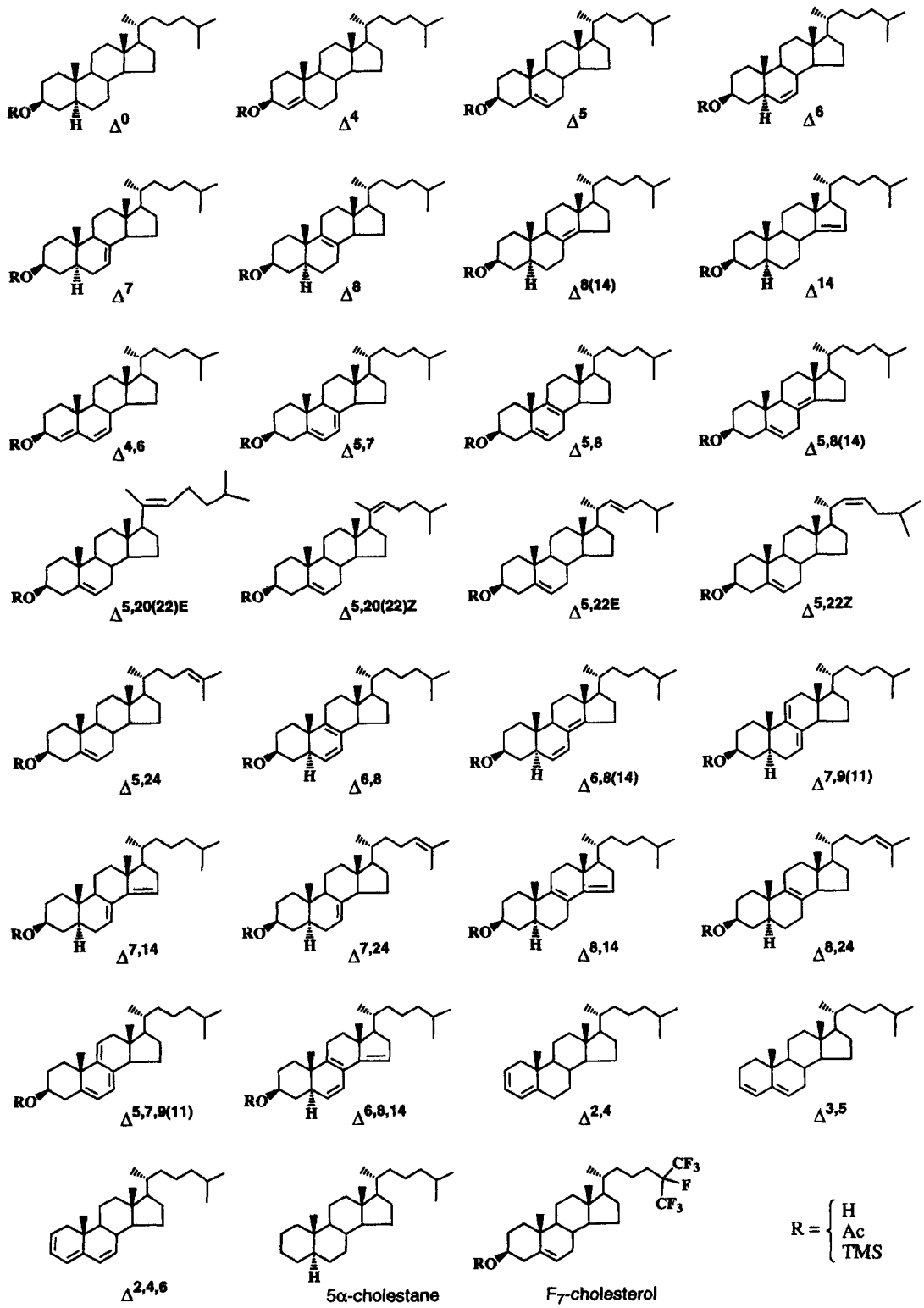

Fig. 1. Structures of unsaturated C_{27} 3β-hydroxysterols described herein and related steroids.

thickness; Restek; Bellefonte, PA), DB-225 (15 m × 0.25 mm i.d.; 50% cyanopropyl-phenyl–50% methyl polysiloxane; 0.1 μm film thickness; J&W Scientific), Rtx-1701 (15 m × 0.25 mm i.d.; 14% cyanopropyl-phenyl–86% methyl polysiloxane; 0.1 μm film thickness; Restek), and CP-Wax 57 CB (25 m × 0.32 mm i.d.; high polarity polyethylene glycol; 0.2 μm film thickness; Chrompack; Raritan, NJ). For brevity, the DB-5ms and CP-Wax 57 CB columns are designated as DB-5 and CP-Wax. Sterol samples were injected in hexane solution (1–2 μl).

The GC analyses for DB-1, DB-5, Stabilwax, and CP-Wax columns were done isothermally on a Shimadzu GC-9A instrument (25-m and 30-m columns) or a Perkin-Elmer Sigma 2000 chromatograph (60-m columns). For these columns, the Shimadzu GC-9A was operated with nitrogen (1.1 kg/cm^2, 26-cm/s linear velocity for the DB-5 column) using split injection with a 50:1 split ratio or, in the case of the CP-Wax column, with helium (1.1 kg/cm^2; 40-cm/s linear velocity) using split injection with a 20:1 split ratio. The Sigma 2000 instrument was operated with nitrogen (1.3 kg/cm^2; 15-cm/s linear velocity) using split injection with a 90:1 split ratio. Injector and flame ionization detector temperatures were held at 250°C and 290°C, respectively. Analyses on the DB-225 and Rtx-1701 columns were done on the Shimadzu GC-9A instrument as described above except for nitrogen gas pressure (1.3 kg/cm^2), splitless injection, and temperature programming (see below). GC results were processed with ChromPerfect software (Justice Innovations; Mountain View, CA). GC-MS analyses were done isothermally[4] at 250°C on a Hewlett-Packard 5890A chromatograph (1.4 kg/cm^2 helium, 30-cm/s linear velocity, splitless injection; 60-m DB-5ms column). The temperatures of the injector and GC-MS interface were 270°C (or 290°C in some cases). Mass spectra (m/z 50 to 700) were measured on a ZAB-HF reverse-geometry double-focusing instrument at 70 eV with an electron-impact ion source (200°C). The accelerating voltage was 8 kV, and the resolution was 1000 (10% valley).

RESULTS

GC retention times of unsaturated C_{27} sterols

A large collection of C_{27} sterols was analyzed on DB-5 (30 m and 60 m) and CP-Wax (25 m) columns as their acetate or TMS derivatives. Although the sterols were eluted over a narrow range on the DB-5 columns, many of the sterols could be separated as their TMS ethers, as illustrated in **Fig. 2.** The sterol peaks were more widely dispersed on the CP-Wax column, which could also resolve many sterols, as shown in **Fig. 3.** However, the standards shown in Figs. 2 and 3 represent only a small fraction of the sterols listed in **Table 1,** which contains relative retention times (RRT) for the DB-5 and CP-Wax columns. Simple inspection of retention data in Table 1 and **Fig. 4** indicates that, with a more comprehensive

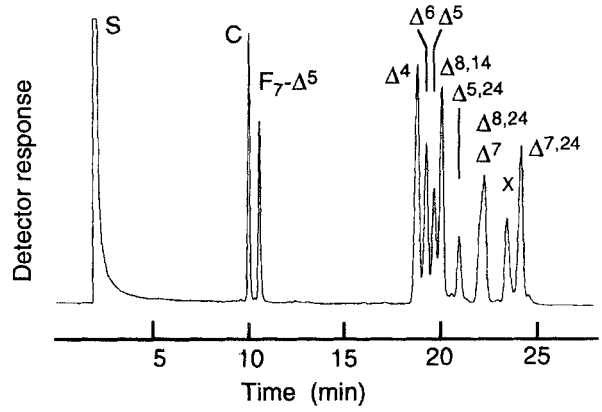

Fig. 2. Gas chromatogram showing separation of a mixture of selected sterol TMS ethers on a 30-m DB-5 column (250°C, isothermal elution with nitrogen). Additional chromatographic peaks correspond to solvent (S), 5α-cholestane (C), F_7-cholesterol TMS ether (F_7-Δ^5), and cholest-4-en-3-one (X). The amount of sterol derivative injected was 12–40 ng per component.

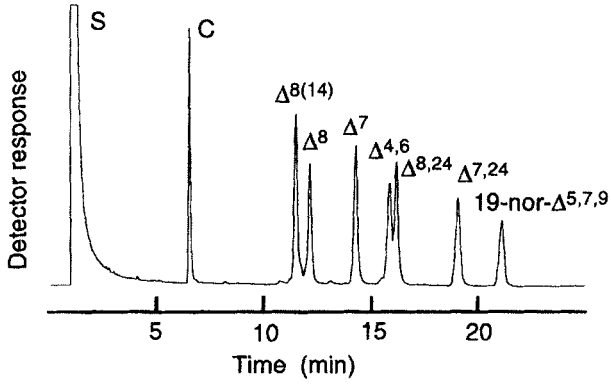

Fig. 3. Gas chromatogram showing separation of a mixture of sterol TMS ethers resolvable on a CP-Wax column (225°C, isothermal elution with helium). Additional chromatographic peaks correspond to solvent (S) and 5α-cholestane (C). The amount of sterol derivative injected was ~30 ng per component.

[4]Analyses were generally carried out isothermally because the sterol derivatives were eluted over a relatively narrow range. Although splitless injections normally start with lower initial column temperatures (as described for the DB-225 and Rtx-1701 columns), GC-MS analyses done isothermally with splitless injection gave acceptable peak shapes, probably owing to the very high boiling points of the sterol derivatives.

TABLE 1. Gas chromatographic retention data for acetate and TMS ether derivatives of unsaturated C_{27} 3β-hydroxysterols and related compounds

	3β-OTMS		3β-OAc			3β-OTMS
	DB-5[a] 30 m	DB-5[b] 60 m	DB-5[b] 30 m	DB-5[b] 60 m		CP-Wax[c] 25 m
	Retention times relative to 5α-cholestane					
$\Delta^{5,20(22)Z}$	1.70	1.62	2.10		$\Delta^{5,20(22)Z}$	1.72
$\Delta^{5,22Z}$			2.11	1.98	$\Delta^{5,22E}$	1.73
$\Delta^{5,22E}$	1.75	1.66	2.19	2.05	Δ^{0}	1.75
Δ^{4}	1.87	1.75	2.33[d]	2.18[d]	$\Delta^{8(14)}$	1.76
$\Delta^{5,8(14)}$	1.90	1.78	2.35	2.21[e]	Δ^{5}	1.82
$\Delta^{5,20(22)E}$	1.92	1.81	2.41	2.24	Δ^{14}	1.82
$\Delta^{8(14)}$	1.92	1.81	2.40	2.24	$\Delta^{5,8(14)}$	1.84
Δ^{5}	1.92	1.81	2.40	2.24	Δ^{4}	1.85
Δ^{14}	1.92	1.81	2.40	2.24	Δ^{8}	1.86
$\Delta^{5,7,9(11)}$	1.93	1.83	2.37	2.22	Δ^{6}	1.89
Δ^{6}	1.96	1.85	2.41	2.24	$\Delta^{5,20(22)E}$	1.99
Δ^{0}	1.96	1.85	2.47	2.30	$\Delta^{5,8}$	2.00
$\Delta^{5,8}$	1.97	1.85	2.45	2.29	$\Delta^{7,14}$	2.04
$\Delta^{6,8,14}$	1.98[f]	1.86[f]	2.38[f]	2.23[f]	$\Delta^{8,14}$	2.05
$\Delta^{7,14}$	1.98	1.87	2.44	2.27	$\Delta^{6,8}$	2.15[f]
$\Delta^{8,14}$	1.99	1.88	2.48	2.30	$\Delta^{5,7,9(11)}$	2.18
Δ^{8}	2.03	1.91	2.53	2.36	$\Delta^{7,9(11)}$	2.18
$\Delta^{4,6}$	2.07	1.94	[g]	[g]	Δ^{7}	2.20
$\Delta^{5,24}$	2.09	1.97	2.64	2.43	$\Delta^{6,8,14}$	2.28
$\Delta^{7,9(11)}$	2.10	1.97	2.59	2.41	$\Delta^{6,8(14)}$	2.32
$\Delta^{6,8(14)}$	2.11	1.98	2.60	2.40	$\Delta^{5,7}$	2.39[f]
$\Delta^{6,8}$	2.12[f]	1.98[f]	2.59[f]	2.42[f]	$\Delta^{5,24}$	2.40
19-nor-$\Delta^{5,7,9}$	2.13	1.99	2.65	2.45	$\Delta^{4,6}$	2.44
$\Delta^{5,7}$	2.14[f]	1.99[f]	2.66[f]	2.44[f]	$\Delta^{8,24}$	2.49
Δ^{7}	2.20	2.07	2.75	2.55	$\Delta^{7,24}$	2.92
$\Delta^{8,24}$	2.23	2.10	2.79	2.57	19-nor-$\Delta^{5,7,9}$	3.24
$\Delta^{5,22E,24}$			2.88	2.66		
$\Delta^{7,24}$	2.41	2.25	3.00	2.76		
$\Delta^{2,4}$	0.96	0.97	0.96	0.97	$\Delta^{2,4}$	1.25
$\Delta^{3,5}$	1.09	1.08	1.09	1.08	$\Delta^{3,5}$	1.52
$\Delta^{2,4,6}$	1.04	1.04	1.04	1.04	$\Delta^{2,4,6}$	1.64
	Actual retention times (min)					
5α-Cholestane	10.1	29.3	10.1	29.3	5α-Cholestane	6.8
F_7-Δ^5	10.7	30.1	13.1	36.6	F_7-Δ^5	5.1
Δ^5	19.4	53.1	24.1	65.6	Δ^5	12.4
Methane	1.92	6.7	1.92	6.7	Methane	1.03

[a]Isothermal elution with nitrogen (1.1 kg/cm^2; 26 cm/s) at 250°C; 30-m DB-5ms column (0.25 mm i.d.; 0.1 μm film thickness); split injection (250°C); flame-ionization detection (280°C). Retention times for alkane standards: C_{28}, 9.6 min; C_{30}, 15.65 min; C_{32}, 26.3 min; C_{34}, 44.95 min.

[b]Isothermal elution with nitrogen (1.3 kg/cm^2; 15 cm/s) at 250°C; 60-m DB-5ms column (0.25 mm i.d.; 0.1 μm film thickness); split injection (250°C); flame-ionization detection (290°C). Retention times for alkane standards: C_{28}, 26.4 min; C_{30}, 41.05 min; C_{32}, 66.15 min; C_{34}, 108.9 min.

[c]Isothermal elution with helium (1.1 kg/cm^2; 40 cm/s) at 225°C; 25-m CP-Wax 57 CB column (0.32 mm i.d.; 0.2 μm film thickness); split injection (250°C); flame-ionization detection (280°C).

[d]GC analysis gave, in addition to the Δ^4 acetate, two peaks in 3:2 mixture with relative retention times corresponding to those of the $\Delta^{2,4}$ and $\Delta^{3,5}$ dienes, respectively.

[e]Approximate RRT for this compound was estimated from chromatograms showing significant overloading.

[f]RRT is approximate because peaks were broad and asymmetrical (retention times were measured at the apex): $\Delta^{5,7}$ diene, 3 × normal width with fronting on DB-5 columns; normal width but with a wide, low hump in front on the CP-Wax column; $\Delta^{6,8}$ diene, 4 × normal width and fronting on DB-5 columns; normal width but with a trailing wide, low hump on the CP-Wax column; $\Delta^{6,8,14}$ triene, 3 × normal width and slightly tailing on DB-5 columns.

[g]GC analysis gave mainly one peak corresponding in retention to the $\Delta^{2,4,6}$ triene.

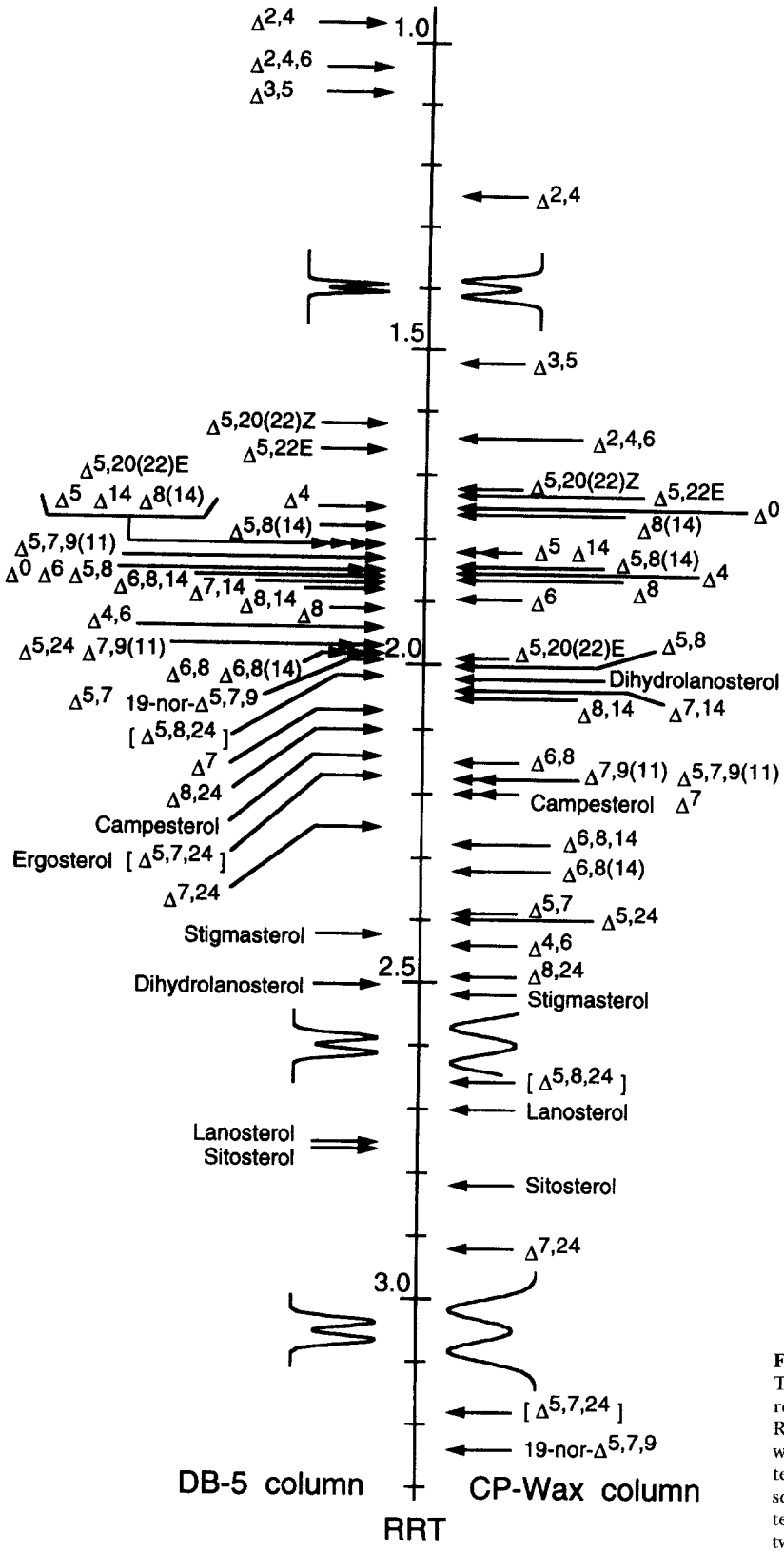

Fig. 4. Retention times relative to 5α-cholestane for TMS ether derivatives of unsaturated C_{27} sterols and related sterols on DB-5 (60-m) and CP-Wax columns. Retention data for the $\Delta^{5,8,24}$ and $\Delta^{5,7,24}$ TMS ethers were predicted according to observed changes in retention time upon introduction of a Δ^{24} bond, as described in the Results. Simulated Gaussian peaks centered at RRT 1.4, 2.6, and 3.05 show the spacing of two *non-overloaded* components at resolution R_s 1.0.

set of standards, both columns would fail to separate numerous sterols, even on a highly efficient capillary column.

Among the unsaturated C_{27} sterols, the $\Delta^{5,20(22)Z}$ derivatives were eluted first and the $\Delta^{7,24}$ derivatives last. Retention times of the TMS ethers ranged from 17.2 min to 24.3 min for the 30-m DB-5 column, from 47.3 min to 65.7 min for the 60-m DB-5 column, and from 11.7 min to 19.9 min for the 25-m CP-Wax column. These data gave sterol peak capacities[5] of 18, 36, and 25 for the three respective columns with the criterion of resolution R_S set at 1.0 (corresponding to a 27% valley between two Gaussian peaks of equal height). Based on data given below, the DB-1 and Stabilwax columns appeared to have sterol peak dispersions paralleling those of the DB-5 and CP-Wax columns, respectively. For the data in Table 1, a pair of sterol TMS derivatives showing a resolution R_S of 1.0 and having a mean RRT of 2.0 would differ in RRT by 0.04 (CP-Wax and 30-m DB-5 columns) or 0.02 (60-m DB-5 column). Similar information for pairs of peaks approximately at RRT 1.4, 2.6, and 3.05 is indicated in Fig. 4. The resolving power of the columns given above is valid only in the absence of overloading.

On both DB-5 and CP-Wax columns, introduction of Δ^7 or Δ^{24} bonds markedly increased retention, whereas $\Delta^{20(22)}$ and Δ^{22} bonds decreased retention. The Δ^5 and Δ^{14} TMS ethers had identical mobilities on both phases. The retention order and peak dispersion of the acetates generally paralleled those of the TMS ethers, but acetate retention times were 20–25% longer. These retention patterns are compatible with previous findings (17–22). In some cases, derivatization had a small effect on relative GC mobilities. Several pairs of acetates, including $\Delta^{7,14}$ and $\Delta^{8,14}$, Δ^5 and $\Delta^{5,7,9(11)}$, and $\Delta^{5,24}$ and $\Delta^{6,8(14)}$, were resolved on the 60-m DB-5 column but were essentially coeluted as TMS ethers on DB-5 and CP-Wax columns.

As has been documented extensively in past work (18–25), double bonds at various positions have a characteristic multiplicative effect on retention times (or additive effect on logarithmic retention indices) for each stationary phase, and these effects can be used to predict chromatographic mobility. Comparison of the retention data for $\Delta^{5,24}$, $\Delta^{7,24}$, and $\Delta^{8,24}$ derivatives in Table 1 with those of the corresponding sterols lacking a Δ^{24} bond indicated that introduction of a Δ^{24} bond leads to a 9% increase in RRT on the DB-5 columns (TMS ethers and acetates) and a 33% increase in RRT on the CP-Wax column. Based on these trends, retention data can be estimated for Δ^{24} analogs not available in the present work. Figure 4 includes predicted retention data for $\Delta^{5,7,24}$ and $\Delta^{5,8,24}$ TMS ethers.

The relative retention times in Table 1 were derived from numerous chromatograms with careful consideration of the effects of column overloading. Over 100 injections of the unsaturated sterols (individually and in defined mixtures) were made for each column. 5α-Cholestane and F_7-cholesterol (as its acetate or TMS derivative) were used as internal standards. The number of theoretical plates, as judged by both peak widths and by valleys between partially resolved peaks for non-overloaded injections of unsaturated sterols, was typically 35000, 45000, and 190000 for the CP-Wax, 30-m DB-5, and 60-m DB-5 columns, respectively. The relative responses (based on mass injected) of the acetates of F_7-cholesterol, Δ^5, Δ^7, $\Delta^{5,7}$, and $\Delta^{5,8}$ sterols and of 5α-cholestane to the flame ionization detectors were 1.0, 1.7, 1.7, 1.7, 1.6, and 1.8, respectively. Except for the $\Delta^{5,7}$ acetate, these sterols showed linear detector responses with 30-m and 60-m DB-5 columns over the range of 3 ng to 150 ng. The $\Delta^{5,7}$ acetate gave a broad peak, and the resulting lower sensitivity hampered reliable quantitation below 5 ng (30-m column) or 20 ng (60-m column).

Retention times were reproducible with a standard deviation of 0.2% for ~10 successive injections of 5α-cholestane or F_7-cholesteryl acetate on DB-5 columns when the mass injected was held constant. However, as shown in **Fig. 5** and **Fig. 6,** column overloading led to significant increases in retention times of unsaturated sterols. The characteristic broadening and fronting was easily observed on heavily overloaded peaks, but moderate overloading resulted in appreciable increases in retention time with only subtle effects on peak shape (Fig. 5). As illustrated in Fig. 6, percentage errors in retention times were greater for later eluting components. Increased retention times were also noted for a minor component if a nearby eluting major component showed overloading. Peaks were monitored for overloading by their width and skew and by their absolute areas. The well-known effects of column overloading have been noted previously in sterols analyses on both packed (22) and capillary (26) columns.

As the columns aged, retention times decreased by 2% per year for the DB-5 columns and by 2% per week for the CP-Wax column.[6] The DB-5 columns were kept

[5]The theoretical peak capacity is defined as $C_p = (\sqrt{N}/4R_S)[\ln(t_{last}/t_{first})]$, where t_{first} and t_{last} are the retention times of the earliest and latest peaks of interest, R_S is the desired resolution, and N is the number of theoretical plates. C_p gives an estimate of the number of peaks that could be resolved within the observed elution range. Of C_{27} sterol samples not available for this work, literature data suggest that only the very late eluting $\Delta^{5,22E,24}$ (17) would fall substantially outside the range of retention times in Table 1.

[6]Polyethylene glycol phases vary considerably in stability depending on their preparation and protection from oxygen and moisture. Our CP-Wax column was protected by indicating oxygen and moisture traps. Apart from preliminary work with the Stabilwax columns, we

at 250°C. The CP-Wax column was operated at 225°C, but the temperature was lowered to 100°C at other times (e.g., night and weekends), as suggested previously for high-bleed columns (23). Column age had little effect on retention times relative to 5α-cholestane. Due to the objectionable bleeding of the CP-Wax column, we selected DB-5 columns for routine work and GC-MS analyses. The modest peak dispersion on DB-5 was partially offset by use of a highly efficient column, i.e., small diameter (0.25 mm i.d.), ample length (60 m), thin film of stationary phase (0.10 μm), and operation near the optimal linear velocity for nitrogen.[7]

Relative retention data in Table 1 are given for injections corresponding to ~20 ng of sterol and have a precision of approximately ±0.01. The data from the 30-m DB-5 column could be related to data of the 60-m DB-5 column operated on a different instrument at a markedly different linear velocity through the formula RRT(60) = 0.879 × RRT(30) + 0.124, where RRT(30) and RRT(60) are the relative retention times on 30-m and 60-m columns. This result, obtained by linear regression,[8] suggests the possibility of adapting the data in Table 1 for use with DB-5 columns in other laboratories. The calibration process would require several authentic sterol standards spanning a wide range of retention times.

Retention times are also given for *n*-alkane standards (for DB-5 columns), methane, and cholesterol so that data in Table 1 can be converted quickly to another format by simple manipulations on a computer spreadsheet.[9] In addition, retention times (uncorrected for effects of overloading) were measured on other columns for a limited set of C_{27} sterols as their TMS derivatives: DB-1 column (60 m; 240°C): Δ^5, 73.4 min; Δ^7, 82.7 min; Δ^8, 77.0 min; $\Delta^{8(14)}$, 72.0 min; $\Delta^{5,7}$, 79.6 min; $\Delta^{5,8}$, 74.6 min; $\Delta^{7,9(11)}$, 78.9 min; $\Delta^{7,14}$, 74.7 min; $\Delta^{8,14}$, 75.9 min; Stabilwax column (60 m; 240°C): Δ^5, 59.0 min; Δ^7, 71.2 min; Δ^8, 61.1 min; $\Delta^{8(14)}$, 59.4 min; $\Delta^{5,7}$, 70.2 min; $\Delta^{5,8}$, 64.9 min; $\Delta^{7,9(11)}$, 70.6 min; $\Delta^{7,14}$, 65.6 min; $\Delta^{8,14}$, 65.7 min; DB-225 column (15 m; 100°C for 3 min, then increased at 20°C/min to 240°C): Δ^5, 12.3 min; Δ^7, 13.0 min; $\Delta^{8(14)}$, 12.4 min; $\Delta^{5,7}$, 12.9 min; $\Delta^{7,14}$, 12.7 min; Rtx-1701 column (15 m; 100°C for 3 min, then increased at 20°C/min to 250°C): Δ^5, 13.9 min, Δ^7, 14.6 min; $\Delta^{8(14)}$, 13.8 min; $\Delta^{5,7}$, 14.5 min; $\Delta^{7,14}$, 14.1 min. Similar data (not shown) were collected for a 30-m DB-1 and a 15-m Stabilwax column.

Several unsaturated C_{27} sterols were labile under the GC conditions. As has been noted previously for methyl ether derivatives (23), the Δ^4 acetate and TMS ethers tended to undergo elimination reactions in the injector. We observed additional peaks at retention times corresponding to $\Delta^{2,4}$ and $\Delta^{3,5}$ dienes, and GC-MS analyses were compatible with these assignments of structure. The $\Delta^{4,6}$ derivatives behaved similarly, undergoing elimination to material virtually identical in GC and MS properties to the $\Delta^{2,4,6}$ triene. The Δ^4 and $\Delta^{4,6}$ acetates were much more labile than the corresponding TMS ethers. In the absence of overloading, narrow, symmetrical peaks were observed for all compounds except the $\Delta^{5,7}$, $\Delta^{6,8}$, and $\Delta^{6,8,14}$ sterols. On DB-5 columns, the triene gave a broad symmetrical or tailing peak, whereas the $\Delta^{5,7}$ and $\Delta^{6,8}$ TMS and acetate derivatives gave broad, fronting peaks. The excessive peak widths suggested decomposition or isomerization on the column, but mass spectra examined across GC-MS peaks for $\Delta^{5,7}$ acetate and TMS ether derivatives on a DB-5 column showed no significant differences. On the CP-Wax column, the $\Delta^{5,7}$ TMS ether gave a narrow peak preceded by a small hump, and the $\Delta^{6,8}$ TMS ether produced a narrow peak followed by a broad, low hump. The $\Delta^{5,8}$ TMS and acetate derivatives were also labile under GC conditions. As reported previously (16), the $\Delta^{5,8}$ derivatives undergo facile thermal decomposition to the corresponding derivatives of 19-norcholesta-5,7,9-trien-3β-ol. The amount of 19-nortriene formed varies directly with sample loading and inversely with injector head pressure. Although substantial amounts (>20%) of the 19-nor-

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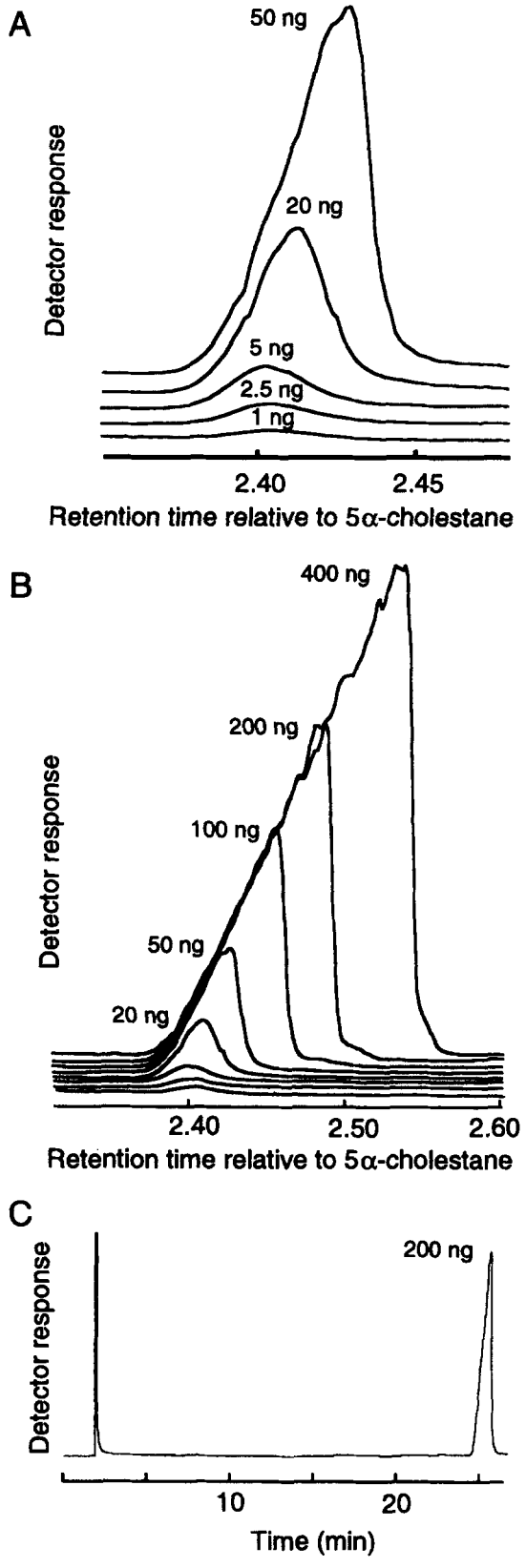
have not explored the stability or selectivity of polyethylene glycol columns from other suppliers. Myher and Kuksis (26) reported high day-to-day reproducibility of retention times for a Supelcowax 10 capillary GC column operated at 250°C.

[7]These parameters, which have not been optimized with respect to column temperature, type of carrier gas, and linear velocity, gave excellent efficiency at the expense of long retention times. Increasing the column temperature to 280°C resulted in much shorter retention times with only modest loss of efficiency, but lability of the sterols and subtle effects on RRT have not been explored. The high efficiency of the 60-m DB-5 column relative to the 30-m column is attributable to differences in linear velocity of the nitrogen carrier gas.

[8]The calculation was performed using acetate and TMS ether data for sterols shown in Fig. 4. Similar results were obtained by considering either set of RRT as the dependent variable. The coefficient of determination (r^2) was >0.999.

[9]Retention times are reported relative to 5α-cholestane because 5α-cholestane was used as an internal standard and because the values given to two decimal places correspond well with the experimental precision. However, the considerable difference between retention times of 5α-cholestane and unsaturated sterols leads to difficulties in comparison of sterol retention data among laboratories (19), and we observed deviations even between our own data collected on 30-m and 60-m DB-5 columns (at different linear velocities on different instruments). Except for the early-eluting hydrocarbon steroids, the discrepancies largely disappeared when the retention data were calculated relative to cholesterol, an expedient scheme known to provide better inter-laboratory comparisons (19). Kovats indices are also widely used for inter-laboratory comparisons, and similar indices have been used to correlate structure with retention times (43, 44). Although actual retention times are used herein, relative retention data and Kovats indices are properly measured using adjusted retention times, which are obtained by subtracting the retention time of a non-retained peak, such as methane.



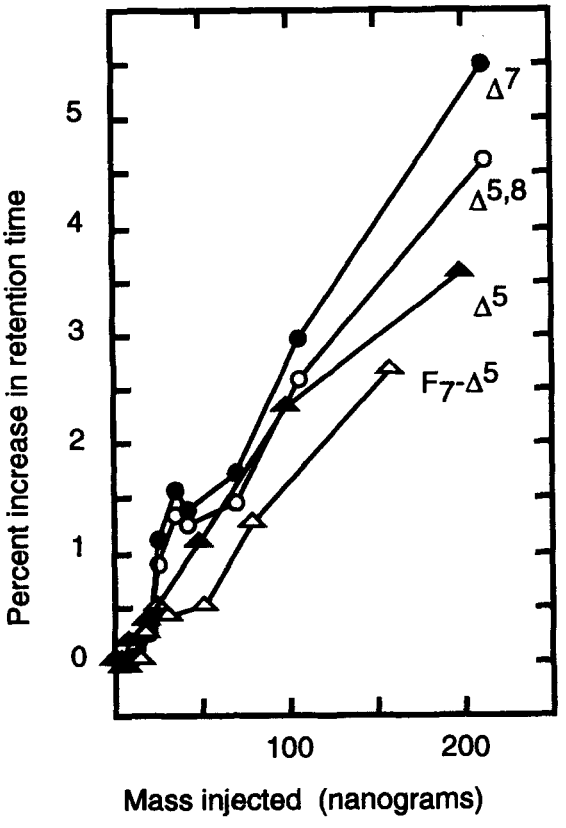


Fig. 6. Increase in retention time as a function of the mass of sterol injected onto the 30-m DB-5 column: Δ^7 acetate, closed circles; $\Delta^{5,8}$ acetate, open circles; Δ^5 acetate, closed triangles; F_7-cholesterol, open triangles.

triene were formed under appropriate conditions (16), only 0–2% was observed under the conditions of the present study. Similar byproduct formation was not observed at significant levels (>2%) among other sterols.

Mass spectra of unsaturated C_{27} sterols

Electron-impact mass spectra for unsaturated C_{27} sterols and their acetate and TMS derivatives were measured by GC-MS, which gave slightly different relative ion abundances from those measured by direct probe. Mass spectral data for saturated and triene derivatives and hydrocarbon steroids are presented as *m/z* (relative

Fig. 5. Effect of mass of sterol injected on peak shapes and retention times of the Δ^5 acetate on the 30-m DB-5 column: (A) Slight to moderate overloading (20–50 ng) causes an increase in relative retention time with only subtle effects on peak width and asymmetry. (B) Severe overloading leads to a major increase in relative retention time and broad, fronting peaks. (C) The prominent fronting caused by severe overloading (200 ng) is less obvious in the normal (unexpanded) chromatogram.

TABLE 2. Relative abundances for major ions and other ions of diagnostic value in electron-impact mass spectra of monounsaturated C_{27} steryl acetates[a–f]

m/z	Δ^5	Δ^6	Δ^7	Δ^8	$\Delta^{8(14)}$	Δ^{14}
428	0	56	100	100	100	5
413*	0	10	17	20	21	4
368*	100	100	5	4	13	12
353*	17	23	8	11	15	10
315*	0	5	8	7	2	56
273	1	4	6	4	1	1
260	10	9	2	4	3	2
255*	11	44	41	15	16	100
247	15	32	2	2	3	2
229	2	10	15	21	20	2
213	10	26	18	30	20	4
120	11	20	5	7	5	5

[a]Abundances relative to the base peak (*m/z* 100–700) were taken from GC-MS data on a sector instrument at 70 eV.

[b]Ions corresponding to loss of CH_3, side chain, and CH_3COOH (or TMSOH or H_2O in other tables) and combinations thereof are marked by an asterisk.

[c]Ions at *m/z* 207, 281, and 429 were frequently observed in low to moderate abundance and were attributed to bleeding of the DB-5ms column. Also not listed are ions two mass units below a major ion; these ions appeared to be dehydrogenation artifacts (present in low abundance in monoene acetate data given here).

[d]Clusters including ions one or two mass units lower or higher than *m/z* 260, 247, and 229 were notable.

[e]GC-MS analysis of the Δ^4 isomer gave two peaks at RRT 0.97 and RRT 1.09 in a 3:2 ratio, with mass spectra very similar to those of $\Delta^{2,4}$ and $\Delta^{3,5}$ dienes, respectively.

[f]Selected ion assignments: 428 (M^+), 413 (M–CH_3), 368 (M–CH_3COOH), 353 (M–CH_3COOH–CH_3), 315 (M–SC), 255 (M–CH_3COOH–SC).

abundance), and other mass spectral data are presented in tabular form: monounsaturated acetates, **Table 2;** monounsaturated TMS ethers, **Table 3;** monounsaturated free sterols, **Table 4;** diene acetates, **Table 5;** diene TMS ethers, **Table 6;** diene free sterols, **Table 7.** Ion abundances are given relative to the base peak (*m/z* 100–700). Ions corresponding to M–CH_3, M–SC, M–CH_3COOH, M–CH_3COOH–CH_3, M–CH_3COOH–SC, M–TMSOH, M–TMSOH–CH_3, M–TMSOH–SC, M–H_2O, M–H_2O–CH_3, and M–H_2O–SC are marked by an asterisk.

For many compounds, repeated GC-MS measurements (typically months apart) were made over the course of 2 years. Considerable variation was observed in the relative abundances of certain ions, and some spectra showed rather large abundances for ions involving loss of TMSOH, CH_3COOH, H_2O, and 2H. These losses may be partially attributable to thermal elimination and dehydrogenation reactions catalyzed by active sites in the ion source (27). The $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{6,8}$, and $\Delta^{6,8(14)}$ acetates, TMS ethers, and free sterols appeared to be more sensitive than other dienes to dehydrogenation and showed notable abundance for ions at *m/z* 364 and 349, even with a clean ion source and highly purified samples.

TABLE 3. Relative abundances for major ions and other ions of diagnostic value in electron-impact mass spectra of TMS derivatives of monounsaturated C_{27} sterols[a–f]

m/z	Δ^4	Δ^5	Δ^6	Δ^7	Δ^8	$\Delta^{8(14)}$	Δ^{14}
458	40	51	80	100	100	100	16
443*	2	13	25	19	21	19	12
368*	100	90	100	7	6	6	4
353*	24	39	40	11	15	9	7
345*	1	1	6	5	5	6	83
329	1	100	49	2	1	1	1
275	1	9	5	1	1	1	0
260	12	3	5	2	3	2	1
255*	26	17	49	52	12	11	100
247	21	20	36	3	3	2	1
229	4	4	13	12	16	16	2
213	17	12	28	17	17	14	4
143	70	14	20	5	5	4	4
142	58	2	4	2	2	1	2
129	13	94	51	8	6	5	7

[a–c]See footnotes *a–c* of Table 2. Levels of dehydrogenation artifacts were low (<5% of major ion intensities).

[d]Clusters including ions one or two mass units lower or higher than *m/z* 329, 275, 260, 247, and 229 were notable.

[e]Selected ion assignments: 458 (M^+), 443 (M–CH_3), 368 (M–TMSOH), 353 (M–TMSOH–CH_3), 345 (M–SC), 255 (M–TMSOH–SC).

Mass spectra of saturated derivatives

Δ^0 acetate, 430 (M^+, 60), 415* (4), 370* (55), 366 (53), 355* (32), 351 (57), 317* (3), 316 (10), 276 (36), 257* (15), 230 (33), 215 (100); Δ^0 TMS ether, 460 (M^+, 94), 445* (99), 403 (18), 370* (33), 355* (43), 347* (0), 331 (11), 306 (25), 305 (28), 262 (11), 257* (10), 215 (100).

TABLE 4. Relative abundances for major ions and other ions of diagnostic value in electron-impact mass spectra of monounsaturated C_{27} free sterols[a–f]

m/z	Δ^5	Δ^6	Δ^7	Δ^8	$\Delta^{8(14)}$	Δ^{14}
386	100	100	100	100	100	7
371*	34	35	27	35	28	5
368*	56	63	22	40	28	5
353*	37	30	12	23	12	4
301	34	15	1	1	0	1
275	58	24	1	1	0	4
273*	17	22	15	16	13	100
255*	25	63	59	21	13	74
247	20	50	8	10	7	1
231	18	23	18	16	8	1
229	7	27	23	32	20	1
213	32	39	25	29	19	3

[a–c]See footnotes *a–c* of Table 2. Spectra of Δ^6, Δ^7, Δ^8, and Δ^{14} sterols showed ions corresponding to M–2H and M–H_2O–2H in moderate abundance.

[d]Clusters including ions one or two mass units lower or higher than *m/z* 275, 247, 231, and 229 were notable.

[e]GC-MS of the Δ^4 isomer gave mainly two peaks in a 1.8:1 ratio, with mass spectra and retention times very similar to those of $\Delta^{2,4}$ and $\Delta^{3,5}$ dienes, respectively.

[f]Selected ion assignments: 386 (M^+), 371 (M–CH_3), 368 (M–H_2O), 353 (M–H_2O–CH_3), 273 (M–SC), 255 (M–H_2O–SC).

TABLE 5. Relative abundances for major ions and other ions of diagnostic value in electron-impact mass spectra of diunsaturated C_{27} steryl acetates[a–k]

m/z	$\Delta^{5,7}$	$\Delta^{5,8}$	$\Delta^{5,8(14)}$	$\Delta^{5,20(22)E}$	$\Delta^{5,22}$	$\Delta^{5,24}$	$\Delta^{6,8}$	$\Delta^{6,8(14)}$	$\Delta^{7,9(11)}$	$\Delta^{7,14}$	$\Delta^{7,24}$	$\Delta^{8,14}$	$\Delta^{8,24}$
426	12	4	1	0	0	0	8	39	100	99	24	99	100
411*	1	3	1	0	0	0	0	20	18	21	27	15	47
366*	100	95	89	100	100	100	100	38	50	38	24	42	38
351*	51	100	39	10	11	27	65	40	46	25	24	100	30
350	15	8	1	0	1	0	8	2	1	1	0	1	1
342	1	0	1	0	0	0	2	0	1	0	11	1	5
325	7	13	1	0	1	1	9	0	2	0	1	2	0
313*	3	3	3	2	1	1	3	85	16	100	100	13	13
312	3	5	1	0	1	2	4	2	4	7	3	13	2
299	1	1	1	0	1	0	0	7	2	30	2	3	3
286	1	1	1	0	0	0	0	3	10	0	1	0	0
259	2	1	2	1	2	3	2	7	16	3	2	4	4
255	3	2	3	2	35	15	4	6	2	4	28	2	12
253*	39	45	100	10	5	44	88	100	28	57	51	19	11
245	1	1	2	2	5	24	3	2	2	0	3	0	2
239	4	5	7	2	2	3	9	13	4	17	4	14	6
238	2	4	5	0	0	1	5	7	2	4	1	24	2
229	2	1	2	5	2	3	3	0	2	2	12	1	25
228	2	2	3	27	2	7	4	9	2	3	8	2	10
227	7	6	13	6	1	4	12	45	5	7	8	2	13
213	6	5	5	16	8	18	5	8	8	3	31	4	45
211	15	27	16	9	2	6	24	21	39	7	4	9	11
158	23	16	23	9	5	17	22	11	8	6	6	3	6
111	2	3	2	1	20	7	3	3	3	0	3	2	5

[a–c]See footnotes *a–c* of Table 2. Spectra of $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{6,8}$, and $\Delta^{6,8(14)}$ isomers showed *m/z* 364, 349, and 251 in moderate abundance.

[d]The abundance of *m/z* 350 was corrected for isotope contributions from *m/z* 349.

[e]Clusters including ions one or two mass units lower or higher than *m/z* 238 and 228 were notable.

[f]The Δ^{24} sterols showed *m/z* 69 in much higher in abundance than did the other sterols.

[g]Mass spectra of $\Delta^{5,22E}$ and $\Delta^{5,22Z}$ acetates were essentially identical.

[h]Additional ions: $\Delta^{5,8}$, *m/z* 362 (11); $\Delta^{7,9(11)}$, *m/z* 311 (6), 272 (6), 271 (7), 258 (7); $\Delta^{8,24}$, *m/z* 315 (5), 288 (7), 273 (8), 241 (6).

[i]As described previously (33), the $\Delta^{5,7}$ acetate showed ions at *m/z* 128, 143, and 158. However, the diagnostic values of these ions was diminished by the presence of clusters of ions centered about *m/z* 128, 144, and 158 in $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$, and $\Delta^{6,8}$ isomers.

[j]GC-MS of the $\Delta^{4,6}$ isomer gave predominantly a peak at RRT 1.05 with a mass spectrum very similar to that of the $\Delta^{2,4,6}$ triene.

[k]Selected ion assignments: 426 (M^+), 411 ($M-CH_3$), 366 ($M-CH_3COOH$), 351 ($M-CH_3COOH-CH_3$), 313 (M–SC), 253 ($M-CH_3COOH$–SC).

Mass spectra of triene derivatives

$\Delta^{5,7,9(11)}$ acetate, 424 (M^+, 3), 409* (0.5), 364* (100), 349* (49), 311* (0), 251* (38), 235 (24), 223 (14), 209 (60), 195 (53); $\Delta^{5,7,9(11)}$ TMS ether, 454 (M^+, 15), 439* (13), 364* (100), 349* (21), 298 (10), 251* (32), 237 (10), 209 (35), 195 (28); $\Delta^{5,22E,24}$ acetate, 424 (2), 364* (15), 349* (4), 313 (11), 282 (49), 267 (9), 255* (20), 253 (42), 213 (8), 109 (100); $\Delta^{5,22E,24}$ free sterol, 382 (5, M^+), 364* (4), 300 (7), 285 (5), 282 (11), 271 (27), 267 (8), 255* (12), 253 (9), 109 (100); $\Delta^{6,8,14}$ acetate, 424 (M^+, 32), 409* (3), 364* (82), 362 (28), 360 (22), 349* (100), 345 (14), 311* (12), 251* (70), 249 (9), 235 (32), 209 (33), 207 (30); $\Delta^{6,8,14}$ TMS ether, 454 (M^+, 49), 439* (5), 364* (28), 349* (100), 341* (4), 323 (13), 251* (20), 209 (11).

Mass spectra of steroid hydrocarbons

$\Delta^{2,4}$ diene, 368 (80, M^+), 353* (13), 261 (4), 260 (4), 255* (24), 250 (7), 247 (8), 213 (13), 211 (22), 118 (34), 106 (85), 105 (100); $\Delta^{3,5}$ diene, 368 (100, M^+), 353* (27), 260 (20), 255* (16), 247 (25), 213 (17), 120 (23), 106 (12), 105 (41); $\Delta^{2,4,6}$ triene, 366 (100, M^+), 351* (5), 253* (8), 247 (28), 211 (6), 158 (20), 142 (25), 118 (15).

DISCUSSION

Gas chromatographic analyses

Gas chromatography has long been recognized as a valuable method for the identification of sterols available in only microgram or nanogram quantities. During the past 35 years, GC retention data have been reported for approximately 40 different unsaturated C_{27} 3β-hydroxysterols, including 11 methyl ethers analyzed by Clayton (23) in 1962 on a diethyleneglycol succinate

TABLE 6. Relative abundances for major ions and other ions of diagnostic value in electron-impact mass spectra of TMS derivatives of diunsaturated C_{27} sterols[a–i]

m/z	$\Delta^{4,6}$	$\Delta^{5,7}$	$\Delta^{5,8}$	$\Delta^{5,8(14)}$	$\Delta^{5,20(22)E}$	$\Delta^{5,22E}$	$\Delta^{5,24}$	$\Delta^{6,8}$	$\Delta^{6,8(14)}$	$\Delta^{7,9(11)}$	$\Delta^{7,14}$	$\Delta^{7,24}$	$\Delta^{8,14}$	$\Delta^{8,24}$
456	100	34	36	88	100	26	35	42	49	100	100	37	100	100
441*	6	4	6	18	12	5	30	5	20	15	17	35	16	38
372	0	0	0	1	0	4	18	1	1	0	0	12	1	4
366*	8	23	9	59	28	58	58	41	40	22	9	6	13	17
351*	1	100	100	100	24	17	36	100	44	27	17	11	98	24
350	1	20	7	0	0	1	0	4	1	0	1	1	1	0
343*	1	2	1	14	5	2	65	4	50	6	48	100	7	9
329	1	1	0	1	0	1	3	1	4	0	17	2	1	1
327	0	5	4	5	8	30	52	5	1	3	1	1	2	1
325	0	72	61	34	4	2	4	62	3	2	2	1	3	2
255	1	1	0	3	3	46	13	2	3	2	2	19	1	9
253*	2	12	7	87	10	8	40	49	100	21	29	9	15	7
247	7	1	1	3	0	1	1	2	1	0	1	1	1	1
245	0	1	0	2	2	7	24	1	1	0	1	2	1	3
239	0	2	2	8	7	4	5	6	8	3	7	2	10	3
238	0	2	1	4	3	1	1	2	3	2	3	1	15	1
229	1	1	1	1	7	3	5	2	1	0	1	9	1	20
228	1	1	1	4	21	4	6	2	5	1	1	5	1	7
227	1	5	3	20	10	3	7	8	27	4	4	6	2	8
213	2	5	3	11	26	11	18	6	8	5	3	20	3	27
211	2	18	15	33	14	3	7	20	19	35	5	3	8	7
182	1	5	2	6	3	1	2	5	4	6	5	2	48	4
129	9	15	9	20	55	55	100	15	13	15	8	11	8	10
111	2	2	2	3	3	100	11	2	2	2	1	2	1	3

[a–f]See footnotes *a–f* of Table 5. Spectra of $\Delta^{5,8(14)}$ and $\Delta^{6,8(14)}$ isomers showed *m/z* 364, 349, and 251 in moderate abundance.

[g]Mass spectral data for the $\Delta^{5,20(22)E}$ and $\Delta^{5,20(22)Z}$ isomers were very similar.

[h]Additional ions: $\Delta^{5,20(22)E}$, 330 (9); $\Delta^{5,24}$, *m/z* 243 (7); $\Delta^{7,9(11)}$, 289 (9), 272 (5); $\Delta^{7,24}$, *m/z* 345 (5); $\Delta^{8,14}$, *m/z* 341 (5); $\Delta^{8,24}$, *m/z* 303 (5).

[i]Selected ion assignments: 456 (M^+), 441 ($M-CH_3$), 366 (M–TMSOH), 351 ($M-TMSOH-CH_3$), 343 (M–SC), 253 (M–TMSOH–SC).

column, 8 free sterols analyzed by Ikekawa et al. (18) in 1968 on six different phases, 13 acetates analyzed by Patterson (19) in 1971 on four different phases, 9 TMS ethers analyzed by Schroepfer et al. (28) in 1972 on a QF-1 column, 8 TMS ethers analyzed by Homberg (21) in 1977 on eight different phases, 9 acetates analyzed by Itoh et al. (20) in 1982 on OV-1 and OV-17 capillary columns, 18 free sterols analyzed by Xu et al. (29) in 1988 on an SE-30 column, 5 diene benzoates analyzed by Wilson and Schroepfer (30) in 1988 on a DB-5 capillary column, 17 free sterols analyzed by Chitwood and Lusby (17) in 1991 on an OV-17 column and a DB-1 capillary column, and 3 diene TMS ethers analyzed by Axelson (31) on a DB-1 capillary column in 1991.

Most of the foregoing results were obtained on packed columns, which, despite their low efficiency, can rival capillary columns in providing precise retention measurements for individual authentic standards. Because the bonded, cross-linked phases now used in capillary columns have close counterparts in the liquid phases used in packed columns, retention data from packed columns are valuable for estimating behavior on capillary columns. Retention times relative to cholesterol for a given stationary phase generally show reasonable consistency between packed and capillary columns, among various types of derivatization, and among different laboratories (17–21, 28, 29). Nevertheless, precise elution orders cannot be determined reliably from retention data collected under diverse conditions. A further problem in using literature data is the lack of reported analyses of unsaturated C_{27} sterols on polar stationary phases comparable to those now available for capillary columns.

In the present work, we have measured capillary GC retention data for unsaturated C_{27} sterols on DB-5 (nonpolar) and CP-Wax (polar) columns. As illustrated in Fig. 4, numerous sterols could not be resolved on either the highly efficient 60-m DB-5 column or the highly selective CP-Wax column. On the DB-5 column, the Δ^5 TMS ether was accompanied by a cluster of 10 other C_{27} sterols, few if any of which could be resolved depending on the level of overloading and the efficiency of the column. Similarly, the $\Delta^{5,7}$ TMS ether was eluted in a cluster of 5 other unsaturated C_{27} sterols (Fig. 4). Despite the better peak dispersion on the CP-Wax column, Δ^5 TMS ether was accompanied by several other sterols, and numerous additional pairs of sterols were essentially inseparable. Although most sterols could be distinguished by their combined behavior on both columns, the Δ^5 and Δ^{14} TMS ethers and the $\Delta^{5,7}$ and $\Delta^{5,24}$ deriva-

TABLE 7. Relative abundances for major ions and other ions of diagnostic value in electron-impact mass spectra of monounsaturated C_{27} free sterols[a–h]

m/z	$\Delta^{5,7}$	$\Delta^{5,8}$	$\Delta^{5,8(14)}$	$\Delta^{5,20(22)E}$	$\Delta^{5,22E}$	$\Delta^{5,24}$	$\Delta^{5,25}$	$\Delta^{6,8}$	$\Delta^{6,8(14)}$	$\Delta^{7,9(11)}$	$\Delta^{7,14}$	$\Delta^{7,24}$	$\Delta^{8,14}$	$\Delta^{8,24}$
384	38	32	96	53	54	29	76	36	39	100	81	19	100	100
369*	5	6	21	5	11	28	26	8	30	32	22	30	82	45
366*	46	24	53	83	53	8	58	52	20	27	15	5	11	11
351*	100	100	99	29	18	14	28	89	34	32	14	7	40	9
350	2	4	4	0	1	0	0	3	0	0	0	0	0	0
325	25	14	10	1	1	1	0	22	1	2	1	0	2	2
300	1	1	1	1	51	22	14	2	1	1	1	13	1	5
299	1	1	4	2	9	10	25	2	6	4	2	6	3	4
273	3	2	2	2	26	11	21	6	4	4	4	5	3	6
271*	10	7	41	6	31	100	45	19	100	30	100	100	25	11
257	3	2	4	2	8	3	6	7	8	4	32	5	12	4
255	4	2	5	4	100	7	27	5	5	4	6	19	13	6
253*	89	18	100	34	14	10	13	100	73	25	33	13	14	5
245	5	5	9	13	13	4	18	11	11	4	3	4	2	7
244	2	2	3	3	2	1	4	5	4	14	2	3	2	3
239	9	4	11	7	8	1	5	12	8	5	8	1	14	4
238	7	3	7	1	0	4	2	7	4	3	3	0	22	2
231	8	7	4	6	7	5	15	16	5	5	2	13	3	17
229	7	6	10	19	11	7	14	13	5	21	3	10	5	17
228	5	3	4	42	8	3	12	10	6	2	2	4	2	5
227	15	5	22	29	7	4	10	19	27	5	6	5	5	5
217	9	16	12	3	5	2	9	21	17	23	4	3	8	6
213	9	7	13	55	29	12	38	12	9	8	4	18	5	22
211	26	23	43	29	7	3	11	34	22	32	7	3	11	6
111	4	4	4	5	93	2	30	5	2	4	2	4	2	5

[a–f]See footnotes *a–f* of Table 5. Spectra of $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$, and $\Delta^{6,8}$ isomers showed *m/z* 364, 349, and 251 in moderate abundance.

[g]GC-MS of the $\Delta^{4,6}$ isomer gave predominantly a peak similar in retention time and mass spectrum to those of the $\Delta^{2,4,6}$ triene.

[h]Selected ion assignments: 384 (M^+), 369 ($M-CH_3$), 366 ($M-H_2O$), 351 ($M-H_2O-CH_3$), 271 (M–SC), 253 ($M-H_2O$–SC).

tives showed very similar chromatographic mobilities on both columns.

Comparison of the nonpolar and polar columns indicated important differences. The DB-5 columns produced minimal bleed, whereas excessive bleeding of the CP-Wax column was a serious impediment to its routine use. On the DB-5 columns, which separate analytes largely by volatility, dienes and monoenes had similar retention times, while most dienes were retained more strongly than monoenes on the CP-Wax column. On the DB-5 columns, hydrocarbon steroids and sterols containing additional methyl groups were eluted either well before or generally after the C_{27} sterols, whereas many of these compounds were eluted within the C_{27} sterol region on the CP-Wax column. These elution patterns, some of which have been observed previously (18, 19, 23), indicate the need for vigilance in the use of polar GC columns for analyzing mixtures that might contain plant sterols, triterpene sterols, or sterols that may undergo thermal decomposition to hydrocarbons. The CP-Wax column dispersed the unsaturated C_{27} sterols over a much wider range than the DB-5 columns, but other polar columns did not necessarily have this desirable feature. Apart from methyl silicone containing 35–50% phenyl (17, 18, 20), other polar stationary phases appeared to give at best modest peak dispersion for unsaturated C_{27} sterols based on our preliminary data for DB-225 and Rtx-1701 columns and results of others using capillary or packed columns containing methyl silicones (17–21, 29), cyanopropyl-phenyl methyl silicones, (10, 21, 32) and trifluoropropyl methyl silicones (18, 19, 21, 28).

Two technical issues warrant discussion. Several unsaturated C_{27} sterols, including Δ^4, $\Delta^{4,6}$, $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{6,8}$, and $\Delta^{6,8,14}$ showed some lability either in the injector or on the column. As documented previously for the $\Delta^{5,8}$ derivatives (16), the extent of lability may depend in a complex manner on various GC operating conditions, including the condition of the inlet, injector temperature, and flow rate. Failure to recognize the potential for decomposition can lead to serious errors in quantitation or identification (16). For example, the 19-nor-$\Delta^{5,7,9}$-triene shows chromatographic behavior similar to that of sterols of potential importance in SLOS on both DB-5 and CP-Wax columns (Fig. 4), and hydrocarbon decomposition products are eluted near some unsaturated C_{27} sterols on CP-Wax columns.

Another technical problem concerns the difficulty of obtaining reproducible retention data. Under favorable conditions, retention times were reproducible to

±0.2% (standard deviation), but overloading led to increases in retention time of 5% or greater (Fig. 6). Severe overloading could cause an early eluting sterol (e.g., Δ^5 acetate, RRT 2.40 on DB-5) to appear at the position (RRT 2.54) of a rather late-eluting sterol (Fig. 5). Such overloading is obvious from excessive peak width and fronting in expanded chromatograms, but symptoms of overloading may be less apparent from the usual chromatogram traces (Fig. 5, panel C), and modest overloading corresponding to a 1% increase in retention time may have only subtle effects on peak shape (Fig. 5, panel A). Overloading is likely to occur in trace analysis when the major sterol (e.g., cholesterol) has not been removed prior to GC or GC-MS.

Mass spectral analyses

Mass spectral data have been reported for a large number of unsaturated C_{27} sterols and their TMS and acetate derivatives, including electron-impact data by Galli and Maroni (33) for Δ^5, $\Delta^{5,7}$, $\Delta^{5,7,24}$, Δ^7, Δ^8, and $\Delta^{8,24}$ acetates, by Knights (34) for Δ^5, Δ^7, and $\Delta^{5,24}$ sterols, by Moiseichuk et al. (35) for $\Delta^{5,7}$, $\Delta^{5,7,22}$, $\Delta^{5,8}$, $\Delta^{5,24}$, $\Delta^{7,9(11)}$, $\Delta^{7,14}$, and $\Delta^{8,14}$ free sterols, by Chitwood and Lusby (17) for $\Delta^{5,7,9(11)}$, $\Delta^{8(14)}$, Δ^5, Δ^0, Δ^8, $\Delta^{7,9(11)}$, $\Delta^{5,7,9(11),24}$, $\Delta^{5,24}$, $\Delta^{5,7}$, Δ^{24}, Δ^7, $\Delta^{8,24}$, $\Delta^{5,7,9(11),22E,24}$, $\Delta^{5,7,24}$, $\Delta^{7,24}$, and $\Delta^{5,7,22E,24}$ acetates, and by Wilson et al. (12) for $\Delta^{5,8}$, $\Delta^{5,24}$, $\Delta^{6,8}$, and $\Delta^{6,8(14)}$ sterols. Also, fragmentation patterns have been elucidated for various Δ^5 derivatives (36–39), Δ^7 and $\Delta^{8(14)}$ species (40), and side-chain unsaturated sterols (41, 42). Despite this wealth of MS data, no single report contains results for a large set of isomeric sterols measured under standardized conditions. Rigorous use of MS to differentiate unsaturated sterol isomers requires uniformity in data collection and reporting, some knowledge about the extent of reproducibility of ion abundances, and the ability to compare the abundances of all ions that might be of diagnostic value in structure determinations. In Tables 2–7, we have presented ion abundances for a large set of sterols in a format allowing ready comparisons among isomers.

Many sterol isomers showed very similar mass spectra, but, with careful consideration of the limited reproducibility of ion abundances, most of the unsaturated C_{27} sterols could be tentatively identified by various diagnostic ions. Among monoenes, Δ^{14} acetate and TMS derivatives were distinctive for their high abundance of ions corresponding to M–SC and M–SC–CH_3COOH or M–SC–TMSOH. The Δ^4 TMS derivative was unique among monoenes in having an abundant ions at *m/z* 368 and 142 but negligible abundance for *m/z* 329. The Δ^5 and Δ^6 TMS ethers showed distinctive ions at *m/z* 329, 275, and 129 and could be distinguished from each other as acetate derivatives by their abundances for M^+. Apart from somewhat higher abundance for *m/z* 255 in Δ^7 spectra (evident also in spectra of others (17)), the remaining monoenes (Δ^7, Δ^8, and $\Delta^{8(14)}$) had highly similar mass spectra as the free sterol and acetate and TMS derivatives. Isomerization after electron impact has been proposed to explain spectral similarities in analogous isomers (40).

Among the 16 diene isomers, various ions in spectra of acetate or TMS ether derivatives are potentially useful in structure elucidation. The ions at *m/z* 329 of the $\Delta^{7,14}$ TMS ether and *m/z* 182 of the $\Delta^{8,14}$ isomer were of low abundance in all other dienes. The Δ^{24} sterols showed an unusually abundant ion at *m/z* 69. Characteristic ions for $\Delta^{5,24}$ and $\Delta^{7,24}$ TMS ethers at *m/z* 129 and 343, respectively, facilitated distinctions among the Δ^{24} sterols. The $\Delta^{5,20(22)}$ and $\Delta^{5,22}$ acetates showed diagnostic ions at *m/z* 228 and 111, respectively. Among the remaining dienes, only the $\Delta^{6,8(14)}$ acetate showed high abundance for *m/z* 313, thus leaving the $\Delta^{7,9(11)}$ diene as the only remaining acetate with M^+ as the base peak. The $\Delta^{4,6}$ TMS ether then became unique in having M^+ as the base peak and negligible intensity for *m/z* 351. Of the remaining dienes, only the $\Delta^{5,8(14)}$ TMS ether showed high abundance for *m/z* 343 and 253 and low abundance for *m/z* 325. The $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{6,8}$ dienes were not easily distinguished as their TMS ethers or acetates, although the $\Delta^{6,8}$ derivatives showed rather high abundance for *m/z* 253. The mass spectra for the $\Delta^{5,20(22)E}$ and $\Delta^{5,20(22)Z}$ isomers were essentially identical, as were those of $\Delta^{5,22E}$ and $\Delta^{5,22Z}$ acetates.

The foregoing structure assignment algorithms depend at various stages on the abundance of only one or two ions and thus presuppose that the MS data are derived from a single component of high purity. The algorithms also depend on the reproducibility of ion abundances. Although the mass spectral data in Tables 2–7 were collected on a single instrument under uniform operating conditions, ions resulting in part from thermal eliminations showed noticeable variation in relative abundance. Variations may also arise from differences in tuning conditions, electron energies, and the type of mass analyzer. Effective use of the above algorithms requires that the pattern of relative abundances measured on another GC-MS system be gauged against those given herein to compensate for variability in thermal elimination. Despite these concerns about reproducibility of MS data, ion abundances reported by others (17, 34, 35), including those using quadrupole instruments, are reasonably similar to those in Tables 2–7.

General remarks on use of GC-MS for identification of sterols

Interest in sterol analysis is quite broad, and sterol samples from a wide range of biological materials have

been subjected to GC or GC-MS analyses. Some investigators have purified the sterol mixtures by liquid chromatography prior to GC analysis to isolate C_{27} sterols as subclasses or individual components derived therefrom. With the advent of capillary columns, GC analyses are now frequently done on crude sterol preparations, often with no prior fractionation of C_{27} sterols from other sterols. Direct GC-MS analysis of complex sterol mixtures may appear to be sound methodology because of the extraordinarily high resolving power of capillary GC combined with mass spectral data containing intensity measurements for hundreds of ions. However, our results indicate that structure identifications based primarily on GC-MS data for mixtures of unsaturated C_{27} sterols simply cannot be considered reliable. Despite the high efficiency of capillary GC, most C_{27} sterols are eluted within a narrow range, and many isomers have essentially identical retention times (Fig. 4). The probability of coelution is even higher with polar columns, on which plant and triterpene sterols appear in the unsaturated C_{27} sterol region. Consequently, a GC peak from analysis of a complex sterol mixture may represent any of several possible sterols or a mixture thereof. If the peak corresponds to a mixture of sterol isomers, then attempting to decipher the MS data is usually futile in the absence of further information. The dilemma of analyzing sterol mixtures by GC-MS is further compounded by the inability of GC-MS data alone to demonstrate the homogeneity of a peak. Even knowledge that a peak corresponds to a single sterol may not be sufficient for identification by GC-MS. For example, $\Delta^{5,7}$ and $\Delta^{6,8}$ TMS ethers cannot be rigorously distinguished from their mass spectra (Table 6) or their GC retention times on a DB-5 column (Table 1), and other work (31) confirms the near impossibility of distinguishing these isomers by GC-MS on a DB-1 column. If isomerization occurs in the ion source (as has been proposed for some monoenes (40)) or in the GC column, then GC-MS-MS and two-dimensional GC may also be incapable of distinguishing these isomers.

GC-MS analysis of mixtures does have some value. The absence of a sterol in a mixture can be demonstrated at an appropriate detection level by GC or GC-MS if no peak is observed at the retention time of the corresponding authentic standard. Also, GC-MS analysis of mixtures can be valuable in confirming analyses by other methods, such as Ag^+-HPLC (1) and NMR (12). Finally, GC-MS of mixtures can provide useful albeit equivocal clues about the structure of individual components. Highly tentative structure proposals, guided by intelligent conjecture, often prove ultimately to be correct. However, our data indicate great potential for error when structure assignments are based primarily upon GC-MS analysis of mixtures. Our data also obviously suggest the potential for error in the uncritical application of selective ion monitoring for the detection or quantitation of unsaturated sterols.

Identification of sterols by GC-MS has much more credibility if the sample has first been fractionated into individual isomers by other chromatographic methods. If this is done, then critical evaluation of combined GC retention data on DB-5 and CP-Wax columns can define an unsaturated C_{27} 3β-hydroxysterol almost uniquely. Mass spectra of the TMS and acetate derivatives would dispel any remaining ambiguities (e.g., Δ^5 vs. Δ^{14} and $\Delta^{5,7}$ vs. $\Delta^{5,24}$). In all but a few cases, identification could be made independently by GC and by MS. Credible use of this methodology requires utmost attention to chromatographic and spectroscopic conditions. Possible decomposition under the GC conditions and effects of overloading must be considered. In the absence of a large collection of authentic standards, calibration of GC retention times and MS ion abundances against literature data is essential. A minimal set of commercially available standards spanning a wide range of retention times might include Δ^0, Δ^5, Δ^7, $\Delta^{5,7}$, $\Delta^{5,22E}$, and $\Delta^{5,24}$ sterols as well as various plant and triterpene sterols. In view of the low purities noted above for some commercial samples, analysis of standards for purity and authenticity is indicated. Critical examination of the MS data is important because many pairs of unsaturated C_{27} sterols show only subtle differences in ion abundances. Even with these measures, structure elucidation based solely on GC-MS must be regarded as only tentative because the data presented herein do not include all possible unsaturated C_{27} 3β-hydroxysterols of 5α configuration (including Δ^4 and Δ^5) or various epimers thereof. Most of these isomers can usually be disregarded as unrealistic possibilities in samples of biological origin on the basis of current knowledge of sterol biosynthesis, but a large number of compounds not described herein remain nevertheless.

Summary

We have presented GC and GC-MS data for a large collection of unsaturated C_{27} 3β-hydroxysterols, including several that have not previously been analyzed by GC or GC-MS. The GC retention times span a narrow range, and numerous sterols are coeluted on both polar and nonpolar columns. Because identification of a GC peak from its mass spectrum requires knowledge that the peak represents a single sterol component, GC-MS alone cannot generally be used for reliable structure determination of mixtures of unsaturated sterols. However, purification of sterol mixtures by HPLC or Ag^+-HPLC, followed by GC-MS analysis of individual sterol components can lead to a credible albeit tentative structure. Careful attention to possible decomposition under

GC conditions, effects of overloading on retention time, and calibration of GC and MS data with authentic sterol standards is essential. If ≥100 ng of material is available, much more reliable structure assignments can be made by supplementing the GC-MS data with analyses by Ag^+-HPLC (1) and ^1H NMR (12).

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