HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, INFRARED AND RAMAN SPECTRA OF STEROIDAL SAPOGENINS

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ABSTRACT.—The six steroidal sapogenins, diosgenin, yamogenin, tigogenin, neotigogenin, smilagenin, and sarsasapogenin, in the form of their acetates, were completely separated by high-performance liquid chromatography, and their infrared and Raman spectra were determined.

The steroidal sapogenins are a group of widely distributed plant products useful as starting materials for the partial synthesis of steroid hormones (1, 2). We have recently described methods for high-performance liquid chromatography (hplc) and spectroscopy of triterpenoid sapogenins (3), but analogous methods for steroidal sapogenins are still needed. So far, only one paper on hplc analysis of the benzoate esters of the sapogenins in *Agave* has been published (4). Although there is ample literature on the infrared (ir) spectra of steroids (5, 6), isolation problems cast some doubt on the authenticity of older spectra. Raman spectra provide valuable information about steroids (7), but, to our knowledge, sapogenins have not yet been investigated by this method. Our paper describes the separation of six common isomeric steroidal sapogenin acetates by hplc and their analysis by ir and Raman spectroscopy.

EXPERIMENTAL

HPLC.—The hplc apparatus was assembled from commercially available components. The pump was of the single-piston reciprocating type[1] with a 5-μm inlet filter.[1] The pump was connected to the column through a sample injection valve.[2] The column consisted of 4 stainless-steel chromatographic tubes,[3] each 250x4.6 mm I.D., which were connected in series. The column outlet was connected to a variable-wavelength detector[4] set at 210 nm. The detector signal was fed into a single-channel recorder.[5]

Two columns were used. One contained Zorbax SIL,[6] a brand of spherical silica gel, and the other one contained Zorbax ODS,[6] a C-18 bonded phase. The columns were packed in our laboratory with the aid of a Haskel hplc slurry packing unit[7]; the packing materials were suspended in a mixture of tetrabromoethane and tetrachloroethane of "balanced" density.

All solvents were highly purified.[8] The Zorbax SIL column was eluted with *n*-hexane-acetone (99:1), and the Zorbax ODS column was eluted with acetonitrile-*n*-hexane-tetrahydrofuran (17:2:1). Eluate fractions containing ca. 10 mg of individual sapogenin acetates were collected, and the solvent was evaporated under a stream of nitrogen.

IR.—Infrared spectra were measured in a Cary spectrophotometer[9] on about 1.5 mg of sample in 250 mg KBr. The KBr sample and reference disks had a diameter of 12.7 mm. The instrumental bandwidth dropped from 3 cm^{-1} at 1880 cm^{-1} to 2.1 cm^{-1} or less between 1700 cm^{-1} and 1200 cm^{-1}, where the grating changes. Between 1200 cm^{-1} and 1100 cm^{-1}, the bandwidth again dropped from 3 cm^{-1} to 2 cm^{-1} and remained at that value down to 700 cm^{-1}.

[1]Model 110A, Altex, Berkeley, California. Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.
[2]Model 7120, Rheodyne, Berkeley, California.
[3]Alltech, Arlington Heights, Illinois.
[4]Model 100-30, Altex-Hitachi, Berkeley, California.
[5]Model 355, Linear, Irvine, California.
[6]Du Pont Instruments, Wilmington, Delaware.
[7]Model 29426, Alltech, Arlington Heights, Illinois.
[8]"Distilled-in-Glass" quality, Burdick & Jackson, Muskegon, Michigan.
[9]Model 90, Varian Instruments, Palo Alto, California.

RAMAN.—The Raman spectra were measured in the photon counting mode essentially as previously described (8–11) with the following exceptions. An argon laser (5145Å)[10] was used for sample excitation, the power being regulated as stated in table 1. The crystallized samples were irradiated as micropelletes, 1 mm in diameter, which were supported in a steel rod with a 6 x 6-mm square cross section having a round hole 1 mm in diameter drilled through the side. A force of 2 kg, easily exerted by hand on a drill rod ram, compacted the sample crystallites against a plate-glass anvil with a pressure of about 250 kg/cm². The 0.3 to 0.5 mg micropellet was placed on a magnetic fixture and located with a micropositioner on the spectrometer entrance axis at the focus of the laser. The converging laser beam and the surface of the pellet formed a 20° angle.

TABLE 1. Raman operating conditions.

Sapogenin	Laser mw	LoLimit x 10³[a]	HiLimit x 10³[b]	Seconds per data point	Watt·Sec
Diosgenin	500	1.4	13.3	2	1.0
Yamogenin	400	1.1	2.3	2	0.8
Tigogenin	400	4.4	44	4	1.6
Neotigogenin	200	535	915	54	10.8
Smilagenin	200	10.0	51	5	1.0
Sarsasapogenin	500	1.3	15.2	4	2.0

[a]The zero ordinate of the spectrum. A background of this number of counts has been subtracted from each data point in the spectrum.

[b]The difference between HiLimit and LoLimit is the ordinate scale of the Raman spectrum for the compound.

RESULTS AND DISCUSSION

HPLC.—We have selected the acetates as derivatives for our work because they have been used for most of the published ir spectra of steroidal sapogenins (12, 13). Also, the ultraviolet (uv) absorption of saturated sapogenins is too

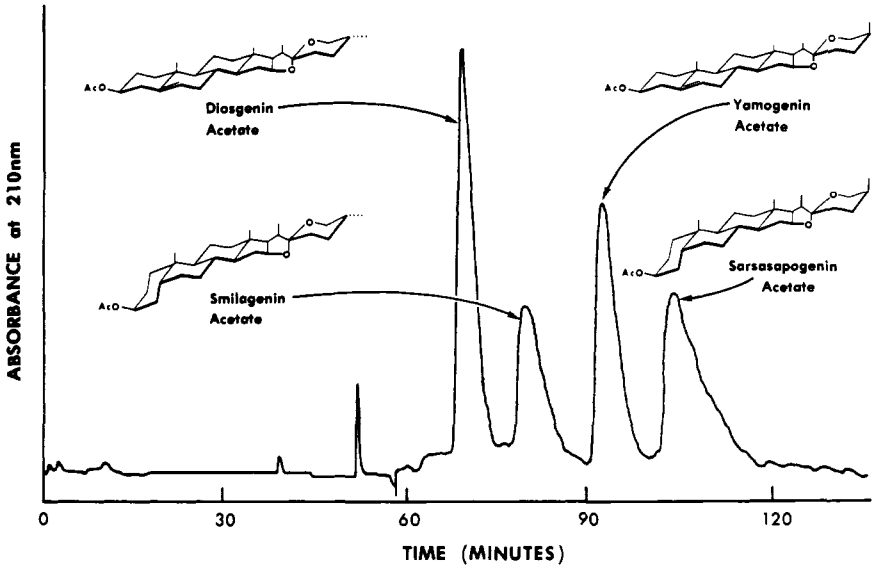

FIG. 1. HPLC of sapogenin acetates epimeric at C-5 and C-25. Sample, 28 μg diosgenin acetate, 620 μg smilagenin acetate, 45 μg yamogenin acetate, 1153 μg sarsasapogenin acetate; column, Zorbax SIL, 1 m x 4.6 m I.D.; eluent, *n*-hexane-acetone (99:1), flow rate, 2.0 ml/min; pressure, 3200 psi. Detector at 210 nm; range, 0.1; recorder speed, 10 min/cm; span, 10 mV.

[10]Model 52C, Coherent, Palo Alto, California.

weak for the hplc detector unless they are suitably derivatized. Our reference compounds, dating back to the 1950's, were in the form of the acetate derivatives and were, at that time, considered to be very pure.

Figure 1 shows the resolution of two pairs of sapogenin acetates, epimeric at C–5 and C–25, by adsorption chromatography. There is a baseline separation of the two Δ^5-steroids from the two more polar 5β-analogs and even greater separation of the sapogenins with an equatorial (α-) methyl group at C–25 from the more polar analogs with an axial (β-) methyl group at C–25.

However, the 5α-sapogenins cannot be separated from the Δ^5-sapogenins by this method. To accomplish this separation, we used the hydrophobic octadecyl-bonded column with a nonaqueous eluent mixture. Complete separation of diosgenin acetate from its 5α-analog, tigogenin acetate, is shown in fig. 2, and a

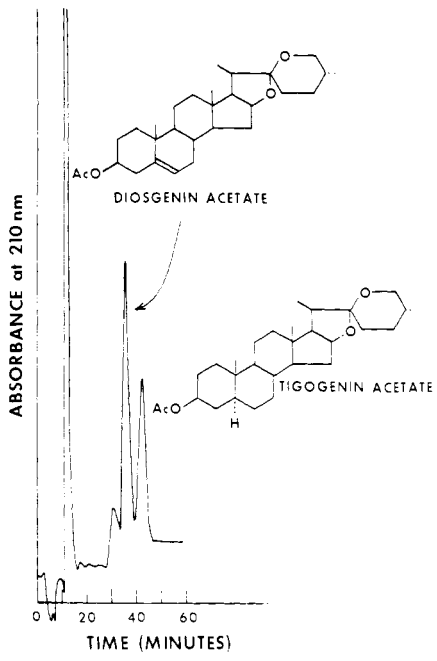

FIG. 2. HPLC of diosgenin acetate and tigogenin acetate. Sample, 16 μg diosgenin acetate, 3350 μg tigogenin acetate; column, Zorbax ODS, 1 m x 4.6 mm I.D.; eluent, acetonitrile-*n*-hexane-tetrahydrofuran (17:2:1); flow rate, 1.0 ml/min; pressure, 1100 psi. Detector at 210 nm; range 0.2. Recorder speed, 10 min/cm; span, 10 mV.

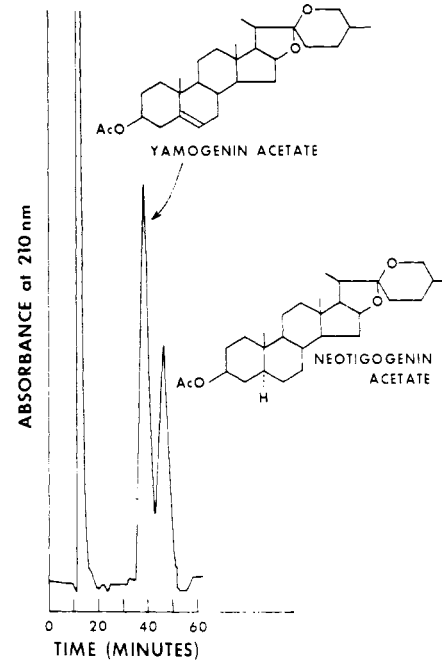

FIG. 3. HPLC of yamogenin acetate and neotigogenin acetate. Sample, 90 μg yamogenin acetate, 1258 μg neotigogenin acetate. For other details see legend for Fig. 2.

similar chromatogram for yamogenin acetate and its 5α-analog, neotigogenin acetate, is shown in fig. 3. The notoriously difficult resolution of Δ^5/5α-pairs of steroid analogs (14) is readily achieved by hydrophobic adsorption chromatography, as demonstrated earlier (15).

It will be noted that milligram quantities of the saturated sapogenins were used in our experiments to furnish adequate elution curves by uv absorption at

210 nm. Ultra-pure sapogenin acetate samples for spectroscopy were prepared by first passing the presumably pure reference substances through the Zorbax ODS column and collecting the unsaturated and saturated steroid acetates separately. Each fraction was then chromatographed on the Zorbax SIL column to resolve the C–5 and C–25 epimers, as indicated in fig. 1.

Thus, a combination of two hplc systems yields ultra-pure sapogenins and eliminates the uncertainties that have beset older chromatographic procedures.

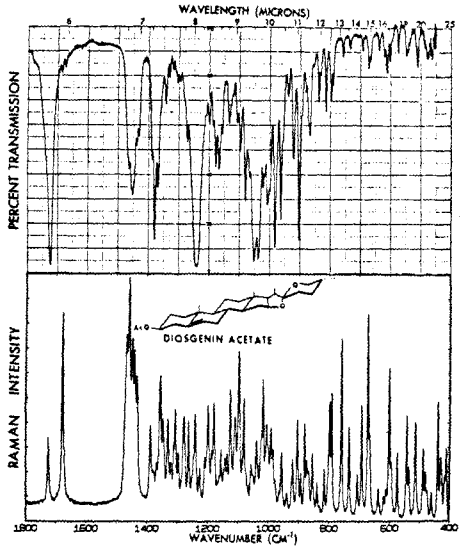

FIG. 4. IR (top) and Raman (bottom) spectra of diosgenin acetate.

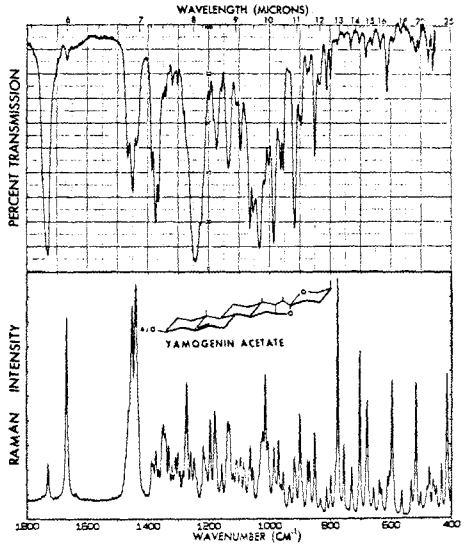

FIG. 5. IR (top) and Raman (bottom) spectra of yamogenin acetate.

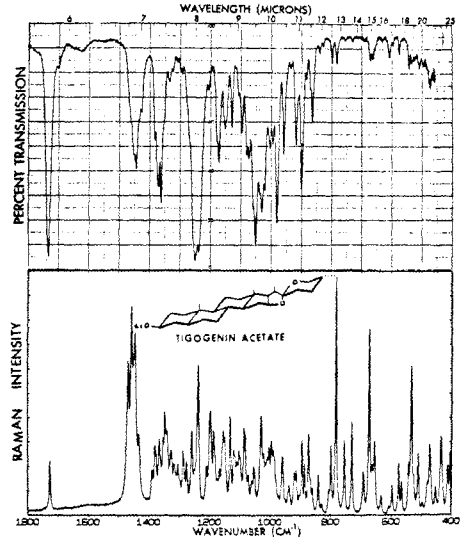

FIG. 6. IR (top) and Raman (bottom) spectra of tigogenin acetate.

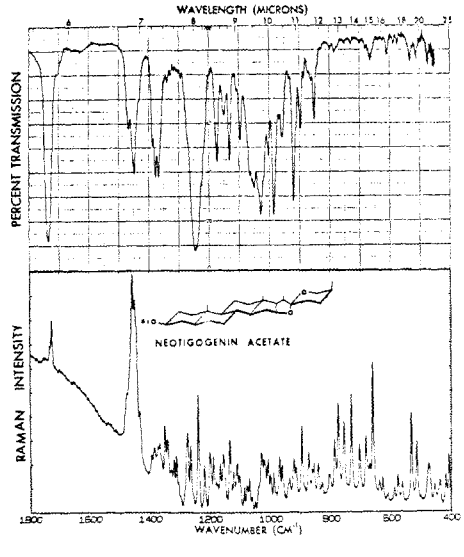

FIG. 7. IR (top) and Raman (bottom) spectra of neotigogenin acetate.

For instance, conventional silica gel column chromatography does not separate the 5α-sapogenins from their Δ^5-analogs, and ir spectroscopy must be used to determine the relative amounts of 25α- and 25β-epimers in the eluate (16).

IR.—Infrared and Raman spectrometry were used to establish the identity of the purified sapogenin acetates. The spectra of the six steroidal sapogenin acetates are shown in figs. 4–9. Compared to published solution spectra, some of which show evidence of impurities, our solid state spectra are characterized by the absence of solvent shifts (17) and extraneous bands, while our instrument resolution is either equal or better. For example, with our preparation of tigogenin acetate

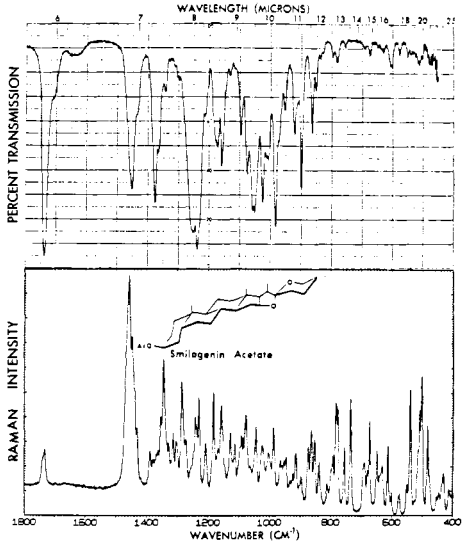

FIG. 8. IR (top) and Raman (bottom) spectra of smilagenin acetate.

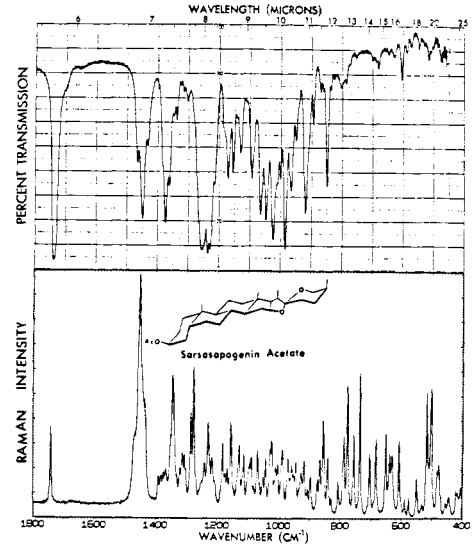

FIG. 9. IR (top) and Raman (bottom) spectra of sarsasapogenin acetate.

in a KBr disk (fig. 6), we do not find the prominent shoulder near 950 cm^{-1} shown in the CS_2 solution spectra (12, 13). However, a decided disadvantage of solid state spectra is that crystal field effects induce splitting of the absorption bands found in solution spectra at 1025, 1074, 1243, and 1298 cm^{-1}. For neotigogenin acetate, our KBr disk spectrum (fig. 7) and one in KCl (18) shows crystal field splitting of the CS_2 solution bands at 1063 and 1358 cm^{-1} (19).

Our diosgenin acetate in KBr (fig. 4) does not show the shoulder near 890 cm^{-1} observed in CS_2 solution (13). We find crystal field splitting of the absorption bands shown in CS_2 solution (12) at 838, 866, 1009, 1159, 1176, 1302 cm^{-1} and the weak C=C stretch band near 1670 cm^{-1} in CCl_4 (13). Our yamogenin acetate (fig. 5) does not show the shoulders near 1135 and 1215 cm^{-1} of the CS_2 solution spectrum (13), but we find crystal field splitting of the absorption band at 900 cm^{-1} in solution. However, unlike diosgenin acetate, yamogenin acetate does not exhibit crystal field splitting of the weak C=C stretch absorption band near 1667 cm^{-1}.

No problems of sample identity should arise from crystal field splitting in comparing any of these solid state spectra with CS_2 solution spectra.

RAMAN.—Since steroids do not possess any of the symmetry elements other than the trivial identity, the selection rules allow all 3n-6 vibrations of an n-atom molecule to appear as absorption or scattering bands, respectively, in the ir and Raman spectra. With molecules of 77 or 79 atoms, as in the present instances, we would expect 225 or 231 spectral features at the same frequencies in both ir and Raman spectra. In fact, we find less than those numbers, even in the sums of the observed ir and Raman spectral features for the individual compounds.

The ir and Raman spectra arise through different mechanisms, namely, the modulation by the molecular motions of, respectively, the molecular dipole moment and the molecular polarizability. It follows that the relative ir and Raman intensities, even for modes allowed in both spectra, can be very different. The probability of very weak as well as overlapping and accidentally coincident bands in either or both, ir and Raman spectra, explains the discrepancy. Thus, finding a coincidence of a prominent band in both ir and Raman in a limited series of molecules of this size does not of itself prove that the ir and Raman features arise from the same molecular vibration in the fingerprint region. Limitations of computer speed and memory have restricted detailed calculations of molecules of this size, but advances in both computer technology and computational methods can be expected to ease these handicaps (20).

The availability of Raman spectra of steroids has lagged behind the comparable ir literature. In fact, we have been unable to find in the literature Raman spectra of any of the steroidal sapogenins. Until considerably more spectra have accumulated, the scheme for structural elucidation developed for steroids (7) may be modified and applied with caution to the steroidal sapogenins.

In the Raman spectra of all our compounds, the CH_2 scissoring mode gives rise to a series of bands near 1450 cm^{-1} of relative intensity comparable to their corresponding ir spectra. The ester C=O stretch near 1730 cm^{-1} is of 0.1 to 0.25 relative intensity in the Raman compared to the ir, when normalized against the 1450 cm^{-1} bands. In contrast, the C=C stretching band from one isolated double bond near 1670 cm^{-1} in the Raman spectra (figs. 4 and 5) is about as intense as the CH_2 scissoring bands, but very weak in the ir for tetrasubstituted C=C in steroids. Thus, the presence of a strong Raman band at about 1620 to 1680 cm^{-1} is a sensitive indicator of the presence of one or more double bonds in the molecule, and in many cases it will provide unequivocal evidence of its position in the molecule (21).

The susceptibility of a few compounds to crystal field splitting of the C=O and C=C stretching bands in ir and Raman spectra of steroids has been noted previously (21). Two unconjugated steroid esters exhibited no splitting of either ir or Raman C=O or C=C bands. One of our two examples of 3-hydroxy-5-enes, diosgenin acetate, showed crystal field splitting of only the trisubstituted C=C stretch in the ir, but no splitting of the C=O or C=C stretch bands in the Raman spectrum (fig. 4). However, yamogenin acetate, which also has a trisubstituted unconjugated C=C, showed no splitting of either C=O or C=C stretch bands in either the ir or Raman spectra (fig. 5).

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