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AN EXAMINATION OF THE HEXANE EXTRACT OF FLUE-CURED TOBACCO INVOLVING GEL PERMEATION CHROMATOGRAPHY[1]

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Abstract—Techniques have been investigated for separating the hexane extract of flue-cured tobacco into classes based on polarity and molecular size. The method developed, which depends upon counter-current distribution for polarity separation and gel permeation chromatography for molecular size separation, is reproducible, non-destructive, and permits quantitative recovery. Its applicability has been demonstrated by the isolation and identification of several compounds and classes of compounds by following it with conventional chromatographic techniques (column, thin-layer, and gas/liquid chromatography). Among the substances isolated were glycerides, solanesol esters, sterol esters (including cholesterol esters), solanochromene (plastochromenol-8), solanesol, α-tocopherol, β-amyrin, and sterols (including cholesterol). Molecular weight determinations indicated that 70 per cent of the non-basic hexane extract was below 800 in molecular weight.

INTRODUCTION

ACCORDING to a recent review[2] over 1200 compounds have been identified in tobacco and smoke. The hexane-extractable fraction has received considerable attention, primarily because of its suggested involvement with leaf quality and the generation of polynuclear aromatic hydrocarbons during leaf pyrolysis. In addition to the numerous studies of individual compounds or classes of compounds (see Ref. 2), Swain *et al.* reported a comprehensive study of this fraction in 1961.[3] In this study, as well as others,[4,5] a large portion (69 per cent) of the hexane extract was reported to consist of resinous material which could not be definitely characterized. Hellier developed a paper chromatographic, differential solubility technique which divided tobacco extracts into "soft" and "hard" resins.[6,7] The latter resembled the resin fraction described by Swain *et al.*

An indication that the resin fraction had a high molecular weight[3] suggested that it might be advantageous to separate the hexane extract by molecular weight differences before

[1] (a) A report of work done under Contract No. 12-14-100-7702(73) with the U.S. Department of Agriculture and authorized by the Research and Marketing Act of 1946. The contract was supervised by the Eastern Utilization Research and Development Division of the Agricultural Research Service. (b) Presented in part at the 21st Annual Tobacco Chemists Conference, 19–20 October 1967, Abstracts, p. 4. (c) For a preliminary report of some of this work see C. E. COOK, MARGARET E. TWINE and M. E. WALL, *Experientia* **23**, 987 (1967).

[2] R. L. STEDMAN, *Chem. Rev.* **68**, 153 (1968).

[3] A. P. SWAIN, W. RUSANIWSKYJ and R. L. STEDMAN, *Chem. Ind.* 435 (1961).

[4] R. A. W. JOHNSTONE and J. R. PLIMMER, *Chem. Rev.* **59**, 885 (1959).

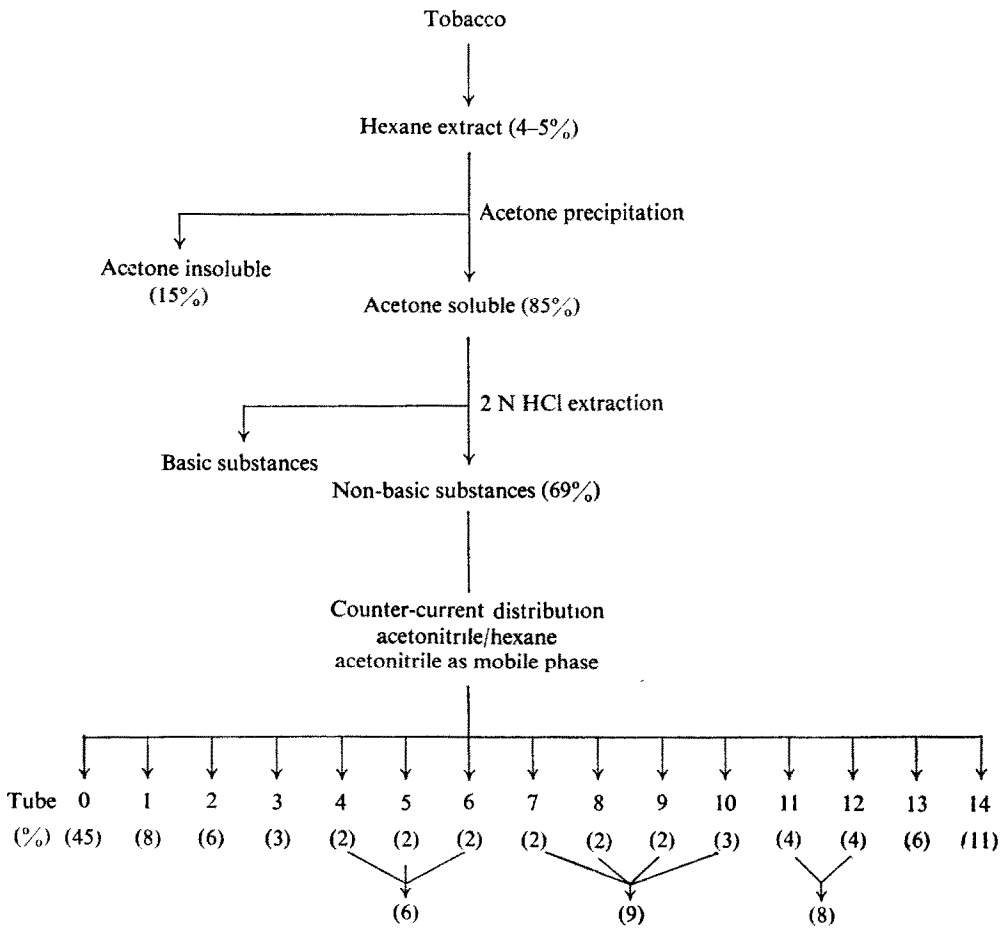
[5] W. G. FRANKENBURG, *Advan. Enzymol.* **6**, 309 (1946), quoted in Ref. 3.

[6] D. N. HELLIER, *Chem. Ind.* 260 (1959).

[7] W. W. REID and D. N. HELLIER, *Chem. Ind.* 1489 (1961).

applying more conventional techniques. Although compounds with different functional groups are expected to be separable by adsorption chromatography because of their differing polarities, these differences may well be blurred by widely disparate molecular weights. Therefore adsorption chromatography should be simplified by a prior molecular weight separation. Gel permeation chromatography using beads formed from polystyrene–divinylbenzene copolymer has found significant use in molecular weight separations among polymer chemists.[8] Less attention has been paid to use of this material in the examination of natural products, although such a use was first demonstrated in 1964.[9] This report deals with application of such a technique to the hexane-extractable fraction from flue-cured tobacco.

TABLE 1.



Percentages are given with respect to each separation. Cumulative percentages are found by multiplication. For example, tube 0 contains 45% of the non-basic substances, 31% (0·45 × 69%) of the acetone-soluble fraction, 26% (0·31 × 85%) of the hexane extract, or 1·0–1·3% (0·26 × 4–5%) of the tobacco.

[8] J. C. Moore, *J. Polymer Sci.* **2A**, 835 (1964).
[9] C. L. Tipton, J. W. Paulis and M D. Pierson, *J. Chromatogr.* **14**, 486 (1964).

RESULTS AND DISCUSSION

Extraction and Preliminary Separation

Table 1 outlines the procedure used for obtaining material. Continuous extraction with hot hexane, precipitation of acetone-insoluble substances, and removal of basic substances were followed by a short counter-current distribution so as to divide the extract into rough classes according to polarity. A relatively non-polar system (acetonitrile-hexane) was chosen on the basis of preliminary studies. If basic substances were not removed before the counter-current distribution step, they were found in the center tubes. All the steps in Table 1 could be carried out on a large scale, so the preliminary separation could be done quite efficiently.

Gel Permeation Chromatography

Initial experiments with polymer beads containing 2, 4, and 10 per cent divinylbenzene showed that best separation was achieved with the 2 per cent cross-linkage. Reproducibility was demonstrated by chromatographing several samples in sequence, when closely similar elution patterns were obtained. Since recovery was essentially quantitative a new sample could be applied before the first was completely eluted. Although sample size had to be limited to *ca.* 7 mg per g of dry polymer, the column size was readily scaled up with no loss in efficiency.

When all of the counter-current distribution fractions were chromatographed, the results shown in Table 2 were obtained. (Percentages in Table 2 are the average of several runs, average deviation ± 1–2 per cent). In spite of the differences in polarity of material from the various CCD tubes, the contents of all the tubes were eluted within approximately the same volume of solvent. However, the more polar tubes contained relatively more low molecular weight material.

TABLE 2. GEL PERMEATION CHROMATOGRAPHY (GPC) OF THE COUNTER-CURRENT DISTRIBUTION (CCD) TUBES

CCD tube No.	GPC Fraction	Ave. M.W.	GPC tube Nos.	% of CCD tube	% of non-basics	% of hexane extract
0	A		14–16	6		
	B	2049	17–18	8	9	5·3
	C		19	3		
	D		20	3		
	E	934	21–23	20	9	5·3
	F		24	6	2·7	1·6
	G	614	25–27	35	15·7	9·2
	H		28–30	11	8·6	5·0
	I	298	31–35	8		
1	A		15–17	2		
	B		18	1	0·6	0·4
	C	1596	19–20	3		
	D		21	2		
	E	1091	22–23	12	1·0	0·6
	F		24	6	0·5	0·3
	G	630	25–28	56	4·5	2·6
	H		29–30	11	1·8	1·1
	I	341	31–36	11		

TABLE 2—*continued*

CCD tube No.	GPC fraction	Ave. M.W.	GPC tube Nos.	% of CCD tube	% of non-basics	% of hexane extract
2	A	2213	16–21	5	0·4	0·2
	B		22	2		
	C	1179	23–24	7	0·7	0·4
	D		25	4		
	E	670	26–29	56	3·4	2·0
	F		30–32	10	1·3	0 8
	G	368	33–38	12		
3	A		13 5–17	7		
	B		18–19	6	0·6	0·4
	C		20–21	8		
	D		22	5	1 6	0·9
	E	581	23–26	50		
	F		27–29	11	0·6	0·4
	G	373	30–33	10		
4–6	A		14–17	4		
	B		18–19	6	0·7	0·4
	C		20	2		
	D		21–22	7	0·7	0·4
	E		23	4		
	F	603	24–27	50	3·0	1·8
	G		28–30	8		
	H		31–32	4	1·5	0·9
	I		33–36	13		
7–10	A		14–15	2		
	B		16–18	4	1·1	0·6
	C		19–20	4		
	D		21	2		
	E	580	22–27	34	3·4	2·0
	F		28–30	4		
	G		31–33	19	4 0	2·3
	H		34–38	25		
11–12	A	1567	14–22	9	0·7	0·4
	B		23–24	7	0·7	0·4
	C		25	2		
	D	625	26–27	7	1·0	0·6
	E		28–29	5		
	F	368	30–33	47	5·3	3·1
	G	291	34–37	19		
13	A		14–23	9	0·5	0·3
	B		24–25	5	0·5	0·3
	C		26	3		
	D	499	27–28	7	0 7	0·4
	E		29	4		
	F	407	30–34	66	4 7	2·8
	G		35–37	12		
14	A		13–16	4	1·1	0·6
	B		17–19	6		
	C	731	20–24	20	2·2	1·3
	D		25–26	7	1·8	1·1
	E		27–28	9		
	F	296	29–32	42	6·4	3·7
	G		33–36	16		

The number-average molecular weights[10] in Table 2 show that the order of elution is always that of descending molecular weight and that the molecular weights for any given set of GPC tubes are closely similar. This is strong evidence that the gel column used fractionates by molecular size over the entire polarity range tested—a hypothesis borne out by the isolation of principal components in several of the fractions (see below). Thus the order of elution for tube zero is glycerides (M.W. 800–900), sterol esters (M.W. 600–700), free sterols (M.W. *ca.* 400).

It should be emphasized that the molecular weights determined for gel column fractions are average values, that they do not in themselves define the upper and lower limits of molecular weights in a mixture, and that the crude nature of the material renders them imprecise. Nevertheless it is instructive to consider their implications concerning the amounts of material in various molecular weight ranges. If we consider only the portion of the hexane extract which is non-basic and soluble in acetone, roughly 14 per cent has M.W. >1200, 16 per cent lies in the range 800–1200, 36 per cent in the range 450–800, and 35 per cent is lower than 450 in molecular weight. The highest range could be said to include "polymeric" materials; the second contains glycerides and solanesol esters plus some "polymers". Molecular weights in the third range are of the order of solanesol, solanochromene, and sterol esters, and the lowest range should include such components as sterols, triterpenes, phenolics, and hydrocarbons.

These percentages would doubtless fluctuate with the type and previous history of the tobacco sample. None the less the results suggest that at least 70 per cent of the non-basic hexane extract from the sample examined consists of material in a size range amenable to structure determination by ordinary chemical and physical methods. The fact that percentages of identified compounds add up to a considerably lower figure (both in our hands and those of others) may be attributed to losses in purification—the difficulty of which is magnified by the multitude of compounds present in tiny amounts—and the presence of many unsaturated compounds which readily undergo autoxidation with a resulting increase in the resin fraction.

Thin-layer chromatograms of all the gel column fractions showed that marked simplification of adsorption chromatography had been achieved by the gel chromatography. Subsequent isolation of components was then carried out by either column or preparative thin-layer chromatography.

Identification of Components

I.r. spectra of 0-E, 1-E, and 2-C showed strong ester bands. The major component was isolated from 0-E by column chromatography and preparative TLC and identified as a mixture of glycerides by a color test for glycerol[11] and gas–liquid chromatography (GLC) of the acid components as the methyl esters.[12] The major acids were palmitic, linoleic, and linolenic, with lesser amounts of stearic and oleic, and minor quantities of myristic, capric, and unidentified acids.[13] The glycerides of tobacco seed oil have received considerable study,[4] with less attention given to leaf glycerides.[6] The isolated glyceride fraction constituted over 1 per cent of the non-basic hexane extract. It seems likely that much of the other ester material

[10] For a background discussion of the method used (which is actually a thermoelectric vapor pressure method) see R. U. BONNAR, M. DIMBAT and F. H. STROSS, *Number-Average Molecular Weights*, pp. 263–268, Interscience Publishers, New York (1958).

[11] R. D. SPENCER and B. H. BEGGS, *J. Chromatogr.* **21**, 52 (1966).

[12] A. RODGMAN, L. C. COOK and P. H. LATIMER, JR., *Tobacco Sci.* **3**, 125 (1959).

[13] Cf. A. P. SWAIN and R. L. STEDMAN, *J. Assoc. Offic. Agr. Chem.* **45**, 536 (1962).

in fractions 0-E, 1-E, and 2-C arises by autoxidation of the glycerides. A minor component was a mixture of solanesol esters, identified by hydrolysis and conversion of solanesol to the *p*-phenylazobenzoyl ester. Solanesol palmitate (M.W. 868) was previously isolated from tobacco.[14]

Chromatography of fraction 0-G (Table 2) (hexane–ether gradient) yielded two major components—a sterol ester mixture (21 per cent) and solanesol (46 per cent). These were less prominent in 1-G (3·5 and 30 per cent, respectively) and almost non-existent in the equivalent fraction 2-E. A third component of 0-G (*ca.* 6 per cent) was identified as solanochromene (plastochromenol-8) by high resolution mass spectrometry, u.v. spectrum,[15,16] NMR spectrum[17] and hydrogenation to yield a 2·5:1 mixture of octadecahydro and hexadecahydro derivative.[15,16]

Solanesol was identified by comparison of its properties with those of a genuine sample. This method of isolation gave a yield of solanesol amounting to *ca.* 0·3 per cent of the tobacco. (Considerable solanesol was removed in the acetone-insoluble fraction.) Analyses have indicated tobacco to contain 1·9–2·5 per cent of solanesol-like substances.[18]

The principal acid components of the sterol ester fraction were shown by GLC to be palmitic, stearic, and oleic, with lesser amounts of lauric, myristic, and linoleic acids, and an unidentified, possibly C_{16} acid. GLC (as trimethylsilyl ethers) indicated the major sterols to be cholesterol, campesterol, stigmasterol, and β-sitosterol. Preparative GLC of the dimethylsilyl ethers cleanly separated cholesterol, and the mass spectrum of the acetate prepared from it was identical with that of a genuine sample.[1b,c] Mass spectrometry also identified the other three sterols, which have previously been isolated from tobacco.[2]

Fraction 1-I (Table 2) was a mixture of crystals and oil containing four major components readily separable by preparative TLC: neophytadiene[19] (*ca.* 40 per cent), α-tocopherol,[15,20] an amyrin fraction, and a sterol fraction (*ca.* 40 per cent). The first two components were identified by spectral methods. It is of interest that the mass spectrum of the α-tocopherol showed no evidence for the presence of homologs.

Preparative GLC of the amyrin fraction (as dimethylsilyl ethers) yielded β-amyrin, identified by retention time, m.p., and mass spectrum comparison with a genuine sample. A minor component had the retention time of α-amyrin and a molecular ion at *m/e* 426, but the characteristic amyrin peak at *m/e* 218[21] was relatively small and the compound could not be identified as α-amyrin. Esters of β-amyrin have been reported recently in tobacco.[22] GLC of the sterols from our ester fraction also indicated the presence of small amounts of β-amyrin.

Gas chromatography and mass spectrometry showed the free sterol fraction to have essentially the same composition as the sterol ester fraction. By preparative GLC, cholesterol was isolated in 5 per cent yield and identified by m.p. (146·5–148·5°) and mass spectrum comparison. Fraction 0-I contained a similar sterol mixture, and the isolated free sterol content of the tobacco was estimated to be *ca.* 0·5 per cent of the non-basic hexane extract.

[14] R. L. Rowland and P. H. Latimer, *Tobacco Sci.* **3**, 1 (1959).
[15] R. L. Rowland, *J. Am. Chem. Soc.* **80**, 6130 (1958).
[16] R. L. Rowland and J. A. Giles, *Tobacco Sci.* **4**, 29 (1960).
[17] H. Mayer, J. Metzger and O. Isler, *Helv. Chim. Acta* **50**, 1376 (1967).
[18] W. R. Bilinsky and R. L. Stedman, *J. Assoc. Offic. Agr. Chem.* **45**, 532 (1962).
[19] R. L. Rowland, *J. Am. Chem. Soc.* **79**, 5007 (1957).
[20] R. L. Stedman, A. P. Swain and W. Rusaniwskyj, *Tobacco Sci.* **6**, 1 (1962).
[21] C. Djerassi, H. Budzikiewicz and J. M. Wilson, *Tetrahedron Letters* 213 (1962).
[22] J. D. Frederickson, 20th Tobacco Chemists Research Conference, Winston-Salem, N.C., 1966, quoted in Ref. 1.

As a check against inadvertent contamination with cholesterol, a 1-kg sample of the tobacco was washed with benzene to remove surface contaminants before being carried through the extraction procedure. Solvents used were freshly opened reagent grade or distilled, and all steps were carried out by an operator wearing rubber gloves. Results were the same as before.

The presence of cholesterol in tobacco is not without precedent in the plant kingdom. Since its isolation from date palm pollen,[23a] its presence has been inferred in other plants by mass spectrometry and/or gas chromatography.[23b–h] The sterol fraction from saponification of tobacco tissue cultures was reported to contain *ca.* 1 per cent of a substance with the retention time and molecular weight of cholesterol, but positive identification was not made.[24] The present work provides rigorous identification. GLC analysis[25] indicated *ca.* 5 per cent of the sterol fraction was cholesterol.[26]

CONCLUSIONS

The combination of counter-current distribution and gel permeation chromatography allows rapid fractionation of the non-basic hexane extract of tobacco into classes based on polarity and molecular weight. Both techniques are mild and permit complete recovery of material. In certain cases they form a convenient means of isolating a given substance. Thus the glyceride fraction is readily obtained almost pure after a simple silica gel chromatography, the sterol ester fraction is rapidly separated by a similar technique, and the main sterol fraction is partially crystalline and pure enough for direct GLC analysis of the sterols. These techniques may thus prove useful in comparison studies of different tobaccos and processing.

EXPERIMENTAL

Tobacco

A 1000-lb lot of Hicks variety flue-cured tobacco, U.S. government grade B4LV, harvested from just above mid-stalk (sixth of ten predicted primings) in late July, 1964, was kept in freezer storage. The crop was grown in Georgia on Tifton series soil. Fertilizer (4-8-12) was used at a rate of 1800 lb/acre, and MH-30 (maleic hydrazide) was used for sucker control.

Solvents

Solvents were carefully checked for non-volatile residue. Hexane for extractions was distilled before use unless it contained less than 10 mg of residue per l. Other solvents were reagent grade, but were also distilled unless obtained in glass bottles.

Extraction and Preliminary Fractionation (Table 1)

Tobacco (9·5 kg, ground in a ball and jewel mill) was soaked in 38 l. of hexane overnight and then extracted continuously with heating for 8 hr. Hexane was drawn off and 38 l. more added. Hot extraction was carried

[23] (a) R. D. BENNETT, S.-T. KO and E. HEFTMANN, *Phytochem.* **5**, 231 (1965). (b) M.-F. HUGEL, W. VETTER, H. AUDIER, M. BARBIER and E. LEDERER, *Phytochem.* **3**, 7 (1964). (c) P. DUPERON, W. VETTER and M. BARBIER, *Phytochem.* **3**, 89 (1964). (d) C. DJERASSI, J. C. KNIGHT and H. BROCKMANN, JR., *Chem. Ber.* **97**, 3118 (1964). (e) J. W. ROWE, *Phytochem.* **4**, 1 (1965). (f) E. HEFTMANN, E. R. LIEBER and R. D. BENNETT, *Phytochem.* **6**, 225 (1967). (g) R. D. BENNETT and E. HEFTMANN, *Archs Biochem. Biophys.* **112**, 616 (1965). (h) B. A. KNIGHTS, in *Gas Liquid Chromatography of Steroids* (edited by J. K. GRANT), p. 217, University Press, Cambridge (1967). Cholesterol percentages range from traces in some plants up to 11–12 per cent [in tomato (*Lycopersicom esculentum*) leaves and lemon (*Citrus limoni*) peel] of total sterols.

[24] P. BENEVENISTE, L. HIRTH and G. OURISSON, *Phytochem.* **5**, 31 (1966).

[25] A. ROZANSKI, *Anal. Chem.* **38**, 36 (1966).

[26] Since our preliminary report[1b,c] appeared, B. RICHARDSON, J. R. BAUR, R. S. HALLIWELL and R. LANGSTON [*Steroids* **11**, 231 (1968)] have reported on the GLC analysis of sterols in tobacco, and suggest that the level of cholesterol may fluctuate.

out overnight, and the hexane was again withdrawn and replaced. A third overnight hot extraction yielded the last fraction. The three extracts were combined, filtered, and concentrated on a cyclone evaporator. Insoluble material was filtered off, and the filtrate was evaporated under reduced pressure at 50°. Traces of solvent were removed by vacuum pump at room temperature. (In one case a Waring blender was used for initial extraction of whole leaves, followed by Soxhlet extraction of the residue. Results were equivalent to those in Table 1.) The residue was dissolved in warm acetone (10 ml/g) and kept in a refrigerator 20–60 hr. The mixture was filtered and the filter cake washed with three portions (0·3 ml/g) of cold acetone, to leave an off-white waxy solid which contained considerable solanesol by TLC. The combined filtrates were evaporated as before and the residue taken up in ether (10 ml/g). The ether was extracted with 2 N HCl (one 25 per cent volume and two 5 per cent volumes), distilled water (two 5 per cent volumes), a saturated solution of $NaHCO_3$ (one 5 per cent volume), distilled water (one 5 per cent volume), and a saturated solution of NaCl (one 5 per cent volume). (Percentages are given with respect to the volume of ether.) The ether was dried over Drierite and evaporated.

Counter-current Distribution (Table 1)

Distilled hexane and technical grade acetonitrile (negligible residue) were used. The material to be distributed was dissolved in a mixture of equal amounts of upper and lower phases (3·2 ml of each per g). The distribution was generally carried out manually, using a train of 15 separatory funnels (lower phase mobile). Emulsion problems were not severe, solvents were readily evaporated, and a 250 g batch of material could generally be distributed in 3 days.

Gel Permeation Chromatography

Beads (1 kg, 200–400 mesh) of polystyrene cross-linked with 2% divinylbenzene were swollen in benzene and poured into a column. Bed dimensions were 8·2 × 103 cm, void volume 1420 ml, gel volume 4020 ml, and exclusion limit *ca.* 2000. Samples (up to 7 g) were dissolved in 20 ml of benzene, placed on the column, and eluted with benzene. Fractions of 100 ml each were collected. Elution curves were prepared by weighing the residue from 2-ml aliquots in aluminium foil cups. Complete elution required 3400–3800 ml. Since the material was completely eluted, a second sample could be applied before the first one was off. Thus about 2 5 l. of benzene was used for each 7 g sample. Since the colored materials of the sample provided visual markers, in repetitive runs fractions in the later runs could be identified by tube number and color. A smaller column (5 × 99 cm) was prepared from 400 g of beads and used for samples up to 3 g. Number-average molecular weights ($M.W. = \sum n_i M_i / \sum n_i$) were obtained on a Mechrolab Vapor Pressure Osmometer.[10] The solvent used was benzene and the instrument was standardized with benzhydrol. Concentrations were in the range of 2·5–5 mg/ml, and determinations were made at two to four different concentrations. In the concentration range used and for the accuracy required it was not found necessary to extrapolate to infinite dilution and the results were merely averaged.

Adsorption Chromatography

Chromatography of various fractions was carried out on columns of Davison silica gel (Grade 923, 100–200 mesh, 100:1 ratio). For example, a 2·96 g sample of fraction 0-G (Table 2) in hexane was placed on a column of 300 g of silica gel packed in hexane. Elution was carried out first with 500 ml of hexane (collected in one fraction, weight 61 mg), then with a gradient elution system using 1500 ml of hexane and 1500 ml of ether (20-ml fractions, total weight 2578 mg), and finally with ethyl acetate (one fraction, weight 318 mg).

Fractions were routinely examined by TLC on silica gel HF_{254}, using *ca.* 0·25 mm layers on microscope slides or lantern slides and hexane–acetone mixtures. Spots were visualized by examination under u.v. light (254 and 365 nm) and by phosphomolybdic acid spray. Preparative TLC was carried out using 1 mm layers. For preparative work plates were pre-washed with acetone and then reactivated for 30 min at 100–110°. In general the desired bands were located by spraying the edges of the plate with phosphomolybdic acid and heating briefly on the edge of a steam bath. The bands were eluted with acetone. Filtration of the acetone eluate through membrane filters (S & S 02, 0·45 μ pore size) was used to remove suspended silica gel.[27]

Gas–Liquid Chromatography

GLC was carried out on a F & M Model 402 gas chromatograph equipped with flame ionization detector. Analytical work on sterols was done on 4 ft columns of 1–3·8 per cent SE-30 or 2 per cent OV-17 on acid-washed, silanized, Gaschrom W coated by the method of Fales.[28] Preparative work was carried out on 5 ft long, 6 mm i.d. columns of 1 per cent OV-17 on acid-washed, silanized Gaschrom G, using an effluent splitter

[27] R. D. Spencer and B. H. Beggs, *J. Chromatogr.* **21**, 52 (1966).

[28] H. M. Fales, in *Biomedical Applications of Gas Chromatography* (edited by H. A. A. Szymanski), pp. 51–52, Plenum Press (1964).

supplied by F & M (split ratio *ca.* 50:1 to 200:1). The sample size ranged from 400–1000 μg in 20–50 μl of solution. Effluent samples were collected in Teflon tubes and rinsed into vials with CH_2Cl_2. Hydroxy compounds (e.g. sterols) were often converted to the corresponding trimethylsilyl (TMS) or dimethylsilyl (DMS) ethers by heating for 0·25 hr on a steam bath with pyridine and either hexamethyldisilazane (for TMS ethers) or *sym*-tetramethyldisilazane (for DMS ethers). Methyl esters were analyzed on a 4 ft, 3 mm i.d. 10% EGSSX column (Applied Science Laboratories), with methyl pentadecanoate as an internal reference. Good separation was obtained at 180° for acids from C_{12}–C_{20} (the separation factor for stearic and oleic esters was 1·21).

Spectra

I.r. spectra were determined in CH_2Cl_2, u.v. spectra in hexane or methanol on a Cary 14 Spectrophotometer, NMR spectra in $CDCl_3$ with internal tetramethylsilane standard on a Varian A-60 and mass spectra on a Perkin-Elmer-Hitachi (for which we thank Dr. Maurice Bursey of UNC-Chapel Hill), or an AEI MS-902 (Dr. David Rosenthal, Research Triangle Institute Regional Center for Mass Spectrometry).

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