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Separation of Steroids by Gas Chromatography

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IT IS NOW generally recognized that compounds of moderately high molecular weight and with comparatively complex structures may be separated or identified by gas chromatographic methods. The key to successful application of these methods lies in the choice and preparation of chromatographic packings containing relatively thin films of highly thermostable liquid phases. These are best used with high-sensitivity ionization detectors. With suitable equipment and suitable columns, it is usually possible to find appropriate separation conditions for use with highly complex mixtures. Further, since the relative retention time of a given substance is dependent upon solute-solvent interaction, gas chromatographic data may be used to gain information about the molecular size and functional groups for compounds of unknown structure.

The development of basic gas chromatographic methodology has resulted in many new applications of these techniques in the biological and medical sciences as well as in the physical sciences. The superb resolving power, the feasibility of using micro samples, and the ability to obtain qualitative and quantitative data at the same time, together with a wide degree of generality of use, suggest that many additional applications will be found in the future. The following summary should be regarded as a discussion of a still rapidly developing subject with particular reference to steroid problems.

The use of very small samples is desirable in order to obtain the highest possible efficiency for thin-film columns. Fortunately this requirement is in line with the circumstances prevailing in many biological experiments; the sample in some cases may contain only microgram or sub-microgram levels of significant components. The development of highly sensitive ionization detectors makes it possible to work with small amounts of material, and much work has been done with the Lovelock argon ionization detector. This very stable and relatively simple detection system uses a radioactive source in the detection cell, and argon is used as the gas phase. Other detection methods that may be used include hydrogen flame ionization devices, the Lovelock photon-activated ionization detector and the radiofrequency detector of Karmen and Bowman. In some instances thermal conductivity detectors may be used. In every case attention must be given to the mass-response relationship if quantitative results are desired.

Liquid phases. Brown (2) has suggested that liquid phases may be classified as non-selective, selective electron-donating, and selective electron-accepting. In practice selective phases are also classified in terms of the effects seen with functional groups of various kinds. The appropriate choice of phase is a matter of

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great importance in achieving satisfactory separations.

For steroid work the best non-selective phases are methyl silicone polymers: SE-30, SF-96, and SE-52 (containing a small amount of phenyl groups)[2] are examples. Of these the most useful is SE-30. This phase was used in the first practical demonstration of the use of gas chromatography for steroid separations under moderate conditions (15) (Fig. 1). Apiezon

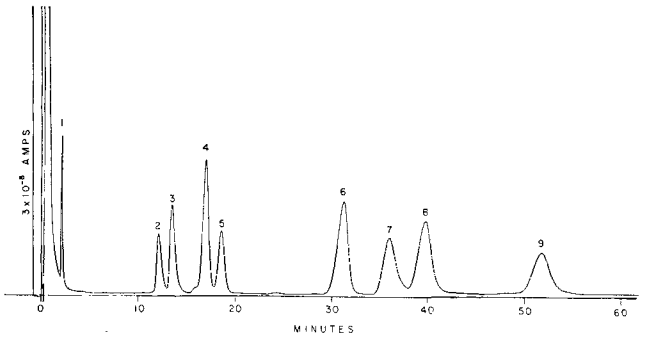

FIG. 1. Separation of (1), androstane; (2), pregnane-3,20-dione; (3), 5α-pregnane-3,20-dione; (4), coprostane; (5), cholestane; (6), stigmastane; (7), cholesterol; (8), cholestan-3-one; (9), stigmasterol. Conditions: 3% SE-30 silicone polymer on Chromosorb W (80–100 mesh); 6 ft. x 4 mm. I.D. column; 222°C; argon inlet pressure 10 psi.

phases, although widely used in other applications, are less satisfactory for steroid separations than silicone polymers.

A number of thermostable selective phases are now known. One of the most useful of these is the fluoroalkyl silicone polymer QF-1-0065[3] (18). This material may be used in exactly the same way as methyl silicone polymers. It has a high degree of stereospecific selectivity, and it is particularly useful with mixtures containing alcohols and ketones. Comparison of functional group effects may be made through comparison of T values as defined by Haahti, Vanden Heuvel and Horning (5, 8); when this is done it is found that QF-1-0065 has a greater selective retaining effect for ketones than for the corresponding alcohols. However with polycyclic systems the effect found for hydroxyl groups depends upon the stereochemical configuration of the group. Axial hydroxyl groups lead to shorter retention times than equatorial hydroxyl groups. It is therefore possible to use this phase to separate epimers; this is usually not possible with a nonselectitve phase. A selective retention effect is not observed for compounds having double bonds with this phase.

Silicone polymers may also be prepared from phenyl-containing precursors; phases made in this way usually contain a relatively high percent of phenyl groups along with some methyl groups. These phases show a different kind of selective action; for example, selective retention effects are observed for alcohol, but epimers are not separated. This suggests that a hydrogen bonding effect, exerted through the H of the OH group, is involved in this instance. Phases of this kind are highly thermostable, and they have been used in specific applications.

[2] These silicone polymers are made by the General Electric Company.
[3] QF-1-0065 (10,000 CS) is a silicone polymer made by the Dow-Corning Corp.

TABLE I

Comparison of Relative Retention Times for Certain Steroids on Three Phases

Compound	Relative retention time		
	SE-30[a] 214°	NGS[b] 212°	QF-1[c] 187°
Cholestane	1.00[d]	1.00[e]	1.00[f]
Coprostane	0.90	0.91	0.90
Epicholestanol	1.97	5.79	3.18
Cholestanol	2.00	6.34	3.70
Cholestanol acetate	3.12	5.75	5.73
Cholestan-3-one	2.14	6.70	7.30
Δ⁵-Cholestene	1.00	1.06	0.98
Cholesterol	1.95	6.85	3.39
Cholesterol acetate	3.12	5.88	5.54
4-Cholesterol acetate	2.64	10.4	11.9
Ergosterol	2.35	10.45	4.35

[a] Column, 6 ft. x 4 mm. I.D., 0.75% SE-30 on 100–140 mesh Gas-Chrom P, 14 psi.
[b] Column, 6 ft. x 4 mm. I.D., 0.75% neopentyl glycol succinate on 100–140 mesh Gas-Chrom P, 15 psi.
[c] Column, 6 ft. x 5 mm. I.D., 1% QF-1 (10,000 CS) on 100–140 mesh Gas-Chrom P, 12 psi.
[d] Time, 8.4 minutes.
[e] Time, 4.6 minutes.
[f] Time, 6.8 minutes.

Several thermostable polyesters are suitable for use in steroid separations (5). Of these neopentyl glycol succinate (NGS) (5) and ethylene glycol isophthalate (EGIP) (12) are perhaps the most useful. Other neopentyl glycol esters that have been recommended include the adipate polymer (5, 9) and the sebacate polymer (9). These polyesters may be used at temperatures up to about 250°C. and they have a marked selective action with regard to keto and hydroxyl groups. A selective retention effect is also shown for compounds with double bonds, although this is less marked than the effect observed with ethylene glycol succinate (EGS). The latter polyester may in fact be used in steroid separations, although this is not recommended because of its limited thermal stability.

Polyester phases are particularly useful for work with mono or difunctional steroids, and with relatively non-polar derivatives of steroids. Polyfunctional compounds generally have unduly long retention times. Epimers may usually be separated with polyester phases.

For steroid work all of these phases should be used in relatively thin-film columns. The amount of phase is usually in the range 0.5–3%; the most useful levels are 0.75%, 1.0% and 1.5% with Gas-Chrom P or Chromosorb W as the support. Columns containing 3–5% or more of liquid phase are not usually used for steroid separations because of the very long retention times or very high temperatures needed for the separations. However, some steroids will withstand extreme conditions without extensive decomposition. The use of high-temperature Apiezon or silicone columns for steroid separations has been reported in a number of instances (1, 3, 10, 11, 22). It was earlier believed that columns containing less than about 5% of liquid phase were not satisfactory for gas chromatographic work, but this is now known to be an erroneous view.

Supports used in steroid work should be inactivated; this may be done through treatment with dimethyldichlorosilane or hexamethyldisilazane. Some polyesters do not require prior inactivation of the support, and by the use of appropriate alkali washing or polyester inactivation it is possible to prepare satisfactory packings without a siliconizing treatment. However the introduction of siliconized supports (7)

permitted the preparation of reproducible and long-lived column packings, and this is now a widely used practice.

Steroid applications. Many naturally occurring and synthetic steroids may be separated by gas chromatography. Animal and plant sterols including cholesterol, cholestanol, coprostanol, the sitosterols, ergosterol, stigmasterol, and the marine sterols may be separated readily. If the carbon content is different the most useful phase is SE-30; separations with this phase are based chiefly on molecular size (15). If stereoisomerism based on the A/B ring system is involved, SE-30 is again a suitable phase; steroid 5α-H and 5β-H isomers may be separated with non-selective phases (15). The cholesterol-cholestanol separation is difficult to achieve, but it may be carried out with QF-1-0065 columns of about 3000-4000 plates. Biosynthetic precursors of cholesterol may be separated readily in mixtures of biological origin; squalene, lanosterol, desmosterol, and cholesterol may be separated with SE-30 or phenyl silicone polymer phases.

Sex hormones and urinary 17-keto-steroids have relatively simple steroid structures. These compounds may be studied with suitable phases. For the sex hormones, SE-30 and QF-1 are suitable phases (13), while for urinary steroids the best phases at present are SE-30 and NGS (4). An example of a urinary steroid separation is shown in Fig. 2.

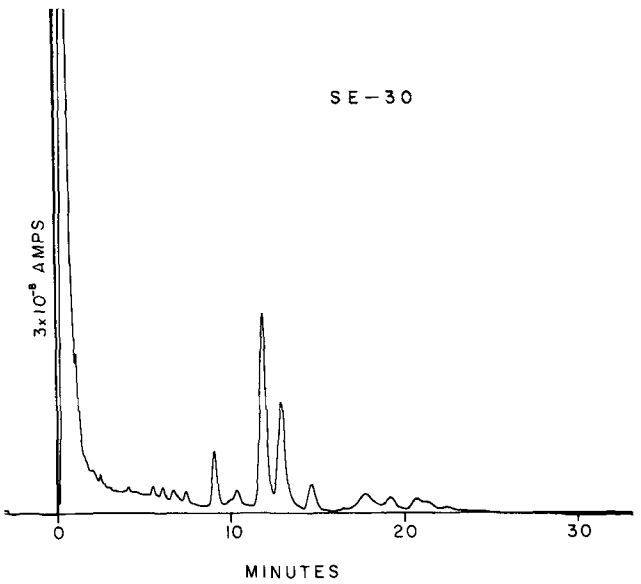

FIG. 2. Gas chromatographic separation of urinary steroids from a normal female. The steroid sample was obtained by an enzymic hydrolysis procedure, and was not subjected to column or paper chromatographic purification. The two major peaks correspond to 5β-androstan-3α-ol-17-one (earlier major peak) and a mixture of androstan-3α-ol-17-one and Δ^5-androsten-3β-ol-17-one (later major peak). The identity of other components of the mixture has not been established. Conditions: 1.5% SE-30 silicone polymer on 100-140 mesh Gas-Chrom P; 12 ft. x 3 mm. I.D. column; 216°C; argon inlet pressure 20 psi.

Bile acids must be converted to methyl esters before chromatography. The most generally useful phase is SE-30 (13), and QF-1 may be used for some of the bile acid esters.

Sapogenins may be separated readily with an SE-30 phase (20) and steroidal amines may be separated in the same way (19).

Adrenal cortical steroid hormones present a special problem. If the structure includes a 20-keto-17α,21-diol side chain system, a thermal elimination occurs during gas chromatography and the product seen in the usual way is the corresponding 17-keto compound (14). The preparation of derivatives may be required for a suitable analytical separation procedure in this instance.

Synthetic steroids present no particular problem as far as is known at the present time. Fluorine-containing steroids, for example, may be separated under ordinary conditions (21).

Thermal alterations. Two specific thermally-induced transformations have been found. One of these involves the side-chain loss mentioned earlier, and the second is the cyclization of compounds of the Vitamin D group to polycyclic compounds of isopyrocalciferol-pyrocalciferol type (23). In this connection it may be mentioned that compounds in the A, E, and K vitamin series may also be separated by gas chromatography (21).

Derivatives. For some purposes it may be desirable to convert the initial compounds into derivatives before gas chromatography. Carboxylic acid groups, for example, are converted to methyl esters. Keto groups are not usually altered before chromatography, but hydroxyl groups may be esterified with acetyl or trifluoracetyl groups. This is usually not necessary unless a relatively large number of hydroxyl groups are present. For example, sugars may be separated as acetates; QF-1-0065 is a particularly good phase for this purpose (17). Trifluoracetates of steroids are eluted far more rapidly than the corresponding hydroxy compounds (16). An example of a separation of trifluoracetyl derivatives is illustrated in Fig. 3.

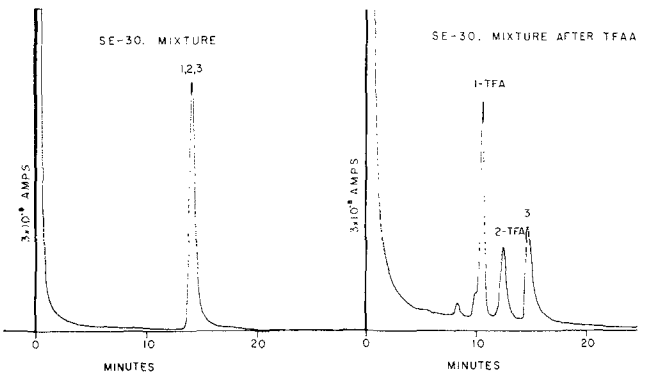

FIG. 3. Gas chromatographic separations of a mixture of (1) 5α-pregnan-3β,20α-diol, (2) 5α-pregnan-30β-ol-3-one and (3) 5α-pregnan-3,20-dione, before and after treatment with trifluoracetic anhydride. No separation occurs for these three steroids on an SE-30 column. After trifluoracetic anhydride treatment, the compounds are (1) 3β,20α-di-(trifluoracetoxy)-5α-pregnane, (2) 20β-trifluoracetoxy-5α-pregnan-3-one and (3) unchanged 5α-pregnan-3,20,dione; the three steroids are now separated readily. Conditions: 0.75% SE-30 silicone polymer on 100–140 mesh Gas-Chrom P; 6 ft. x 4 mm. I.D. Column; 197°C.; argon inlet pressure 16 psi.

High resolution columns. At the present time capillary columns are not very satisfactory for steroid work. However in some applications it is desirable to use columns of higher efficiency than the usual 2000–3000 plates attainable with ordinary packed columns. In these instances a 12 ft. column, 3.4 mm. in diameter, may be used; with appropriate packings 5000–7000 plates may be obtained for steroid separations.

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The Analysis of Glycerides by High Temperature Gas-Liquid Partition Chromatography

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GAS CHROMATOGRAPHY appears to be a powerful tool for the analysis of triglyceride mixtures. Even though higher molecular weight compounds are inherently more difficult to analyze by means of gas chromatography, useful quantitative analyses of triglyceride mixtures can be made if the operating conditions are carefully chosen.

Gas-liquid partition chromatography has been practically universally adopted as a means of determining the fatty acid composition of fats and oils. Analysis of fatty acid mixtures by gas chromatography has been found to be extremely rapid, sensitive, and reliable. Relatively little effort has been expended in using gas chromatography for the analysis of the triglycerides themselves. Presumably this is due to the extremely low volatility of triglycerides. It would be advantageous to analyze the actual glycerides of natural fats rather than their hydrolysis products. Perhaps an even greater need exists for a method capable of analyzing chemically altered fats and synthetic glycerides. It is hoped that this discussion will stimulate more interest in the analysis of glyceride mixtures by means of gas chromatography. Also, since gas chromatography is a highly flexible analytical tool, many other types of high molecular weight compounds could be analyzed by using the same general procedures which are used for glycerides.

Some preliminary investigations have shown that it is entirely feasible to analyze glycerides by means of high temperature gas chromatography. McInnes *et al.* (1) converted monoglycerides to allyl esters of their corresponding fatty acids and found that these volatile derivatives could be separated with an Apiezon M column operated at 240°C. Acetylated mono- and diglyceride mixtures were analyzed on a silicone grease column operated under isothermal conditions (2). Acetylated mono-, di-, triglyceride mixtures were analyzed on a silicone gum rubber column which was temperature programmed (3). Trimyristin was the largest glyceride glyceride eluted. Preliminary experiments by Fryer *et al.* (4) and Martin *et al.* (5) indicated that it may be possible to analyze natural fats containing glycerides as large as tristearin. Martin *et al.* (5) obtained approximately 12 peaks when they chromatographed margarine stock. Fryer *et al.* (4) obtained "fingerprint" chromatograms of various natural fats and oils.

Although the low volatility of triglycerides necessitates higher operating temperatures, these higher temperatures do not, in themselves, create any difficult instrumental problems. Several commercial gas chromatography detectors are now capable of operation at temperatures adequate for the analysis of glycerides. The thermal conductivity detector used in this study was of conventional design. The reference and detector cells used 50-ohm tungsten filaments operated at 12 volts. Both the detector and injection port were maintained at 370°C. for all triglyceride analyses. Helium carrier gas was preheated by passing it through a 12-in. length of stainless steel tubing contained in the column chamber. The rubber septum was cooled by passing air through a tube coiled near the top of the injection port. Operation of this detector at elevated temperatures decreased sensitivity, increased noise level, and increased the tendency for base line drift. Although these high-temperature effects are certainly undesirable, they did not seriously impair the value of high temperature gas chromatography.

Certain inherent characteristics of higher molecular weight compounds such as triglycerides may create serious analytical difficulties. Glyceride mixtures are likely to be highly complicated and contain components having a wide range of volatility. Thus, while the major fatty acids of lard differ by only four carbons (myristate to stearate), commercial monoglycerides prepared from lard are likely to contain components differing by 54 carbons (glycerol to tristearin). Isothermal analysis of this mixture at a temperature high enough to elute tristearin would result in such a rapid elution of the monoglycerides that resolution would be seriously impaired. This difficulty is minimized by temperature programming of the

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